Njavara Rice Bran: Antiproliferative and other Medicinal Properties

A thesis submitted to the University of Hyderabad for the award of a Ph.D. degree in Plant Sciences

By

Akiri S.V. Chalapathi Rao

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad – 500046
India

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Enrollment No. 04LPPH10

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD 500 046

DECLARATION

I, **Akiri S.V. Chalapathi Rao** hereby declare that this thesis entitled **"Njavara rice bran: antiproliferative and other medicinal properties"** submitted by me under the guidance and supervision of Professor Attipalli R Reddy is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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Signature of the Student:

Regd No. 04LPPH10

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UNIVERSITY OF HYDERABAD
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CERTIFICATE

This is to certify that this thesis entitled **"Njavara rice bran: antiproliferative and other medicinal properties"** is a record of bonafide work done by Mr. Akiri S. V. Chalapathi Rao, a research scholar for Ph.D. programme in Plant Sciences Department, School of Life Sciences, University of Hyderabad under my guidance and supervision.

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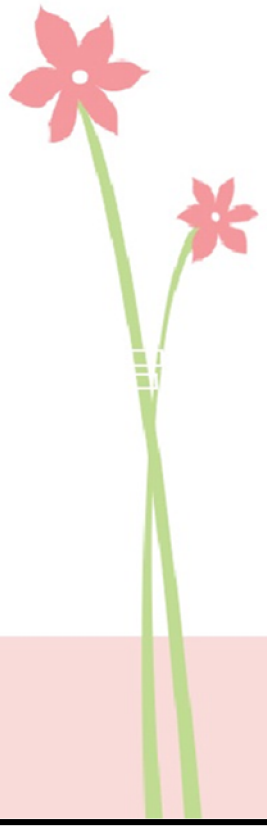

Late, Akiri Appa Rao

DEDICATED TO MY FATHER

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Chalapathi....✍

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Abbreviations

ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
BCIP	5-Bromo-4-chloro-3′-indoylphosphate p- toluidine salt
COX-1	Cyclo-oxygenase-1
COX-2	Cyclo-oxygenase-2
DMSO	Dimethyl Sulfoxide
DNS	Dinitrosalicylic
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetate
ERK 1/2	Extracellular signal regulated kinase ½
GA	Gallic acid
GBM	Glioblastoma multiforme
FTIR	Fourier transformed infrared spectroscopy
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
H_2O_2	Hydrogen peroxide
I κ B	Inhibitor of κ B
JNK	c-Jun NH_2 -terminal kinase
MAPK	Mitogen activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAR	Naringenin
NARN	Naringenin Nanoparticles
NBM	Njavara rice bran methanolic extract
NBT	Nitro-blue tetrazolium chloride
NF- κ B	Nuclear factor κ B
OHG	Oral hypoglycemic drugs
$O_2^-\bullet$	Superoxide

OH•	Hydroxyl radical
PARP	Poly (ADP-ribosyl) polymerase
PBG	Plasma blood glucose
PBS	Phosphate buffered saline
PGG_2	Prostaglandins G_2
PGH_2	Prostaglandins H_2
PMS	Phenazine methosulfate
PMSF	Phenyl methyl sulphonyl fluoride
PPHG	Postprandial hyperglycemia
PUFA	polyunsaturated fatty acids
PVA	Poly vinyl alcohol
QE	Quercetin
ROS	Reactive oxygen species
T2DM	Type II diabetes mellitus
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween 20
TBA	Thiobarbuteric acid
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TFC	Total flavonoid content
TMPD	N,N,N,N-tetra methyl-p-phenylene diamine
TPC	Total phenolic content
TNF- α	Tumor necrosis factor α
WHO	World health organization
XRD	X-ray diffractometry

General Introduction

I. Free radicals

Free radicals are atoms or molecules or ions with one or more unpaired electrons on an open shell configuration. This electron imbalance causes high reactivity creating other free radicals by chain reactions. The majority of free radicals that damage biological systems are oxygen radicals and other reactive oxygen species (ROS), the main byproducts formed in the cells of aerobic organisms and have important roles in cell signaling (Waris and Ahsan, 2006; Valko *et al.*, 2007, Huy *et al.*, 2008) . However, during times of stressful conditions, ROS levels can increase dramatically. This may result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. Examples of oxygen free radicals are singlet oxygen [O_2^1], superoxide [O_2^{-}•], hydroxyl [OH•], lipid peroxyl [LO_2•], lipid alkoxyl [LO•], and hydroperoxyl [HO_2•] radicals (Apel and Hirt, 2004; Lee *et al.*, 2004). Oxygen free radicals can be converted to other non-radical reactive species, such as hydrogen peroxide [H_2O_2], hypochlorous acid [HOCl], hypobromous acid [HOBr].

II. Production of free radicals in cells

With the exception of the unusual circumstances such as ionising radiation, ultraviolet light and other forms of high energy exposure, free radicals are produced in cells, in general, by electron transfer reactions (Cheeseman and Slater, 1993). The production of free radicals in cells can happen both accidentally or deliberately. An example of deliberate reaction is the superoxide generated by activated phagocytes and in catalytic reaction by ribonucleotide reductase (Stubbe, 1990). An example of accidental generation of free radicals would be the leakage of O_2^{-}•, H_2O_2 and other ROS at the interface of the bacterium and the activated phagocyte (Droge, 2002;

Halliwell, 1991). However, the major source of free radicals under normal circumstances is the electron leakage that happens from electron transport chain as seen in the mitochondria, generating O_2^{-}• from O_2 (Matsuzaki, 2009).

III. Source of free radicals

ROS and their products can be produced by both endogenous and exogeneous sources (Figure 1). Potential endogenous sources include oxidative phosphorylation, cytochrome P450 metabolism, peroxisomes and inflammation cell activation. Exogenous sources are exposoure to X-rays, ozone, cigarette smoking, air pollutions and industrial chemicals (Valko *et al.*, 2007; Huy *et al.*, 2008).

i) Endogenous sources

a. Autoxidation:

Autoxidation is a by-product of the aerobic internal milieu. Autoxidation of either catecholamines, haemoglobin, myoglobin, reduced cytochrome C and thiol in a reaction results in the reduction of the oxygen diradical and the formation of ROS. Superoxide is the primary radical formed during this process. Conversion of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) is an example for autooxidation during which O_2^{-}• is formed (Fridovich, 1994).

b. Enzymatic oxidation:

A variety of enzyme systems are capable of generating significant amounts of free radicals, including xanthine oxidase which is activated in ischemia-reperfusion (Fisher, 2004; Bhakuni *et al.*, 2005), prostaglandin synthase (Jeng *et al.*, 2006), lipoxygenase (Huang *et al.*, 2006), aldehyde oxidase (Shaw and Jayatilleke, 1990)

and amino acid oxidase (Rojo *et al.*, 2007). The enzyme myeloperoxidase produced in activated neutrophils utilizes H_2O_2 to oxidize chloride ions into the powerful oxidant HOCl (Winterbourn and Kettle, 2000).

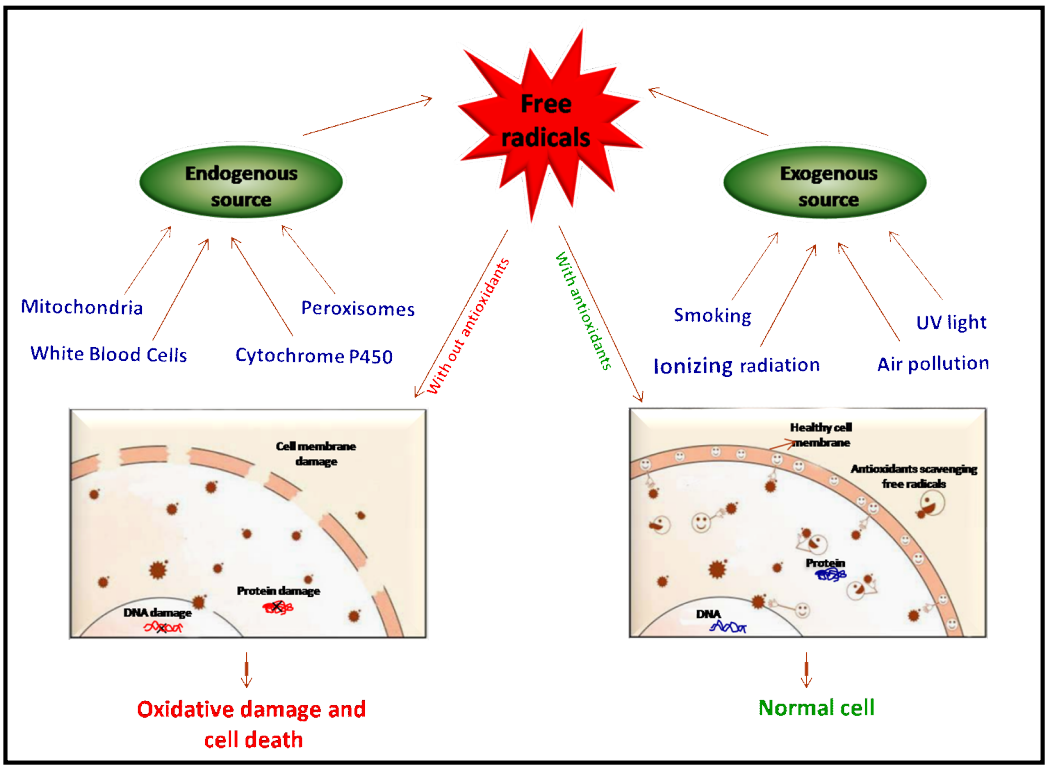


Figure 1: Sources of free radicals and antioxidant protection. Free radicals are produced both exogenously and endogenously. The excess free radicals generated during unfavourable conditions are scavenged by antioxidant defense system.

c. Respiratory burst:

Respiratory burst (sometimes called oxidative burst) is the rapid release of ROS ($O_2^{\bullet -}$ and H_2O_2) from different types of cells and plays an important role in immune system. It is a crucial reaction that occurs in phagocytes to degrade internalized particles and bacteria. During this process, immune cells use NADPH oxidase to reduce O_2 to oxygen free radical and then to H_2O_2. Neutrophils and monocytes utilize myeloperoxidase to further combine H_2O_2 with Cl^- to produce hypochlorite,

which plays a role in destroying bacteria. (Winterbourn and Kettle, 2000; Tung *et al.*, 2009)

d. Subcellular organelles:

Organelles such as mitochondria, microsomes, peroxisomes and nuclei have been shown to generate ROS. Mitochondria are the main cellular organelle for cellular oxidation reactions and the main source of reduced oxygen species in the cell. The leaks in mitochondrial electron transport system allow O_2 to accept a single electron forming $O_2^{\bullet -}$ (Murphy, 2009). It has been shown that $O_2^{\bullet -}$ production by the mitochondria increases under two conditions; either when the oxygen concentration is greatly increased or when the respiratory chain becomes fully reduced (as happens during ischemia). Microsomes are responsible for 80% of the H_2O_2 produced *in vivo* at 100 % hyperoxia sites (Stohs and Bagchi, 1995). Peroxisomes are known to produce H_2O_2 under physiological conditions. Peroxisomal oxidation of fatty acids has recently been recognized as a potentially important source of H_2O_2 production with prolonged starvation. (Valko *et al.*, 2004)

e. Transition metals ions:

Transition metal ions participate in the Haber-Weiss reaction that generates OH• from $O_2^{\bullet -}$ and H_2O_2 (Halliwell, 1995). This reaction can occur in cells and is therefore a possible source for oxidative stress. The reaction is very slow, but is catalyzed by divalent metal. For example, the conversion of Fe^{3+} to Fe^{2+} is the first step in the catalytic cycle as shown below:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \text{ (First step)}$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet} \text{ (Second step)}$$
$$O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2 \text{ (Net reaction)}$$

ii) Exogenous sources

a. Drugs:

A number of drugs can increase the production of free radicals in presence of increased oxygen tensions: for example, drugs that depend on quinoid groups or bound metals for activity (nitrofurantoin). Some other drugs possess pro-oxidant activity which include bleomycin, anthracyclines (adriamycin) and methotrexate (Phillips *et al.,* 2003). In addition, radicals derived from penicillamine, phenylbutazone, some fenamic acids and the aminosalicylate component of sulphasalazine might inactivate protease and deplete ascorbic acid, accelerating lipid peroxidation (Grisham *et al,.* 1992; Halliwel *et al.,* 1992; Evans *et al.,* 1994; Gupte *et al.,* 2008).

b. Radiation:

Radiotherapy may cause tissue injury that is caused by excess free radicals. Electromagnetic radiation (X rays, gamma rays) and particulate radiation (electrons, photons, neutrons, alpha and beta particles) generate primary radicals by transferring their energy to cellular components such as water. These primary radicals can undergo secondary reactions with dissolved O_2 or with cellular solutes and generate radicals, especially OH• (Demir *et al*., 2003; Kasapovic *et al.,* 2009).

c. Tobacco smoking:

Oxidants present in tobacco severely deplete intracellular antioxidants in the living cells and cause severe damage to the respiratory tract (Barreiro *et al.,* 2010). Each puff of smoke has an enormous amount of aldehydes, epoxides, peroxides, nitric oxide, peroxyl radicals, carbon centered radicals and some semiquinone radicals derived from quinines and hydroquinones (Aycicek and Ipek 2008).

Scientific investigates show that smokers have elevated amounts of neutrophils in the lower respiratory tract that could contribute to a further elevation of concentrations of the free radicals (Alberg, 2002; Mahapatra *et al.*, 2008).

d. Inorganic particles:

Inhalation of inorganic particles also known, as mineral dust (e.g. asbestos, quartz, silica), can lead to lung injury mediated by free radical production. Asbestos inhalation has been linked to an increased risk of developing pulmonary fibrosis (asbestosis), mesothelioma and bronchogenic carcinoma. Silica particles as well as asbestos are phagocytosed by pulmonary macrophages. These cells then rupture, releasing proteolytic enzymes and chemotactic mediators causing infiltration by other cells such as neutrophils, thus initiating an inflammatory process (Manning *et al.*, 2002), that leads to increased production of free radicals and other ROS.

e. Gases:

Ozone (O_3) is not a free radical but a very powerful oxidising agent. Ozone contains two unpaired electrons which degrade under physiological conditions to OH•, suggesting that free radicals are formed when ozone reacts with biological substrates. In support of this hypothesis, ozone can generate lipid peroxidation *in vitro*, although similar findings *in vivo* have not been demonstrated (Kontorshchikova and Soloveva, 1996; Chen *et al.*, 2007; Guzman *et al.*, 2009).

f. Others:

Fever, excess glucocorticoid therapy and hyperthyroidism decrease oxygen tolerance in experimental animals. The decrease is attributable to the increased generation of oxygen-derived radicals that accompanies increased metabolism. In

addition, a wide variety of environmental agents including photochemical air pollutants as pesticides, solvents, anaesthetics, exhaust fumes and the general class of aromatic hydrocarbons are also important sources of free radicals (Huy *et al.*, 2008).

IV. Deleterious activities of free radicals

Oxidative stress is defined as the state in which the level of toxic reactive oxygen intermediates (ROI) overcomes the endogenous antioxidant defenses of the host. This state results in an excess of free radicals, which can react with cellular lipids, proteins and nucleic acids, leading to local injury and eventual organ dysfunction. Lipids are probably the most susceptible biomolecules for free radical attack (Huy *et al.*, 2008).

a. Lipid peroxidation:

It refers to the oxidative degradation of lipids. It is the process whereby free radicals steal electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. (Marnett, 1999; Ran *et al.*, 2006). It affects polyunsaturated fatty acids (PUFA), because they contain multiple double bonds in between methylene -CH_2- groups, that possess especially reactive hydrogens.

b. Protein damage:

Proteins and nucleic acids seem to be less susceptible than PUFAs to free radicals. Random attack of radicals on proteins occurs only if radicals are allowed to accumulate (which is not likely in normal cells), or if the damage is focussed on a particular site of the protein. The radical-protein reactions can impair the function of important cellular

and extracellular proteins. It has been estimated that oxidized protein in old animals may constitute 30-50% of the total cellular protein (Stadtman, 1995)

c. DNA damage:

Oxidising radicals readily attack DNA if they are formed in its vicinity and can break its strands or delete a base. This DNA damage can be a lethal event for any organism. The rate of DNA damage inflicted by free radicals, is considerably high; it is estimated that an average of more than 10,000 oxidative hits occur each day in the DNA of a human cell (Ames *et al.,* 1993). Although cellular repair system corrects much of these damages, the radical-induced DNA lesions accumulate with age, which is an important etiology of aging processes (Wickens, 2001).

V. Oxidative stress and human diseases

Oxidative stress, induced by ROS, has been associated with the development of many chronic and degenerative diseases (Figure 2), including cardiovascular disease, cancer, diabetes mellitus, neuronal degeneration such as Parkinson's disease, Alzheimer's disease, as well as involved in the process of aging (Huy *et al.,* 2008). Usually human body has natural defense system for scavenging the free radicals but the capability of natural defense systems of living organism against excess production of these species decreases when influenced with negative environmental factors such as air pollution, cigarette smoking, chemicals, environmental toxins and aging. As a result, different cellular and extra cellular components, and especially lipids, DNA and proteins are damaged, causing or enhancing a number of diseases. Therefore, antioxidants that are believed to play very important role in the body defense system against ROS are of great value in preventing chronic and degenerative diseases.

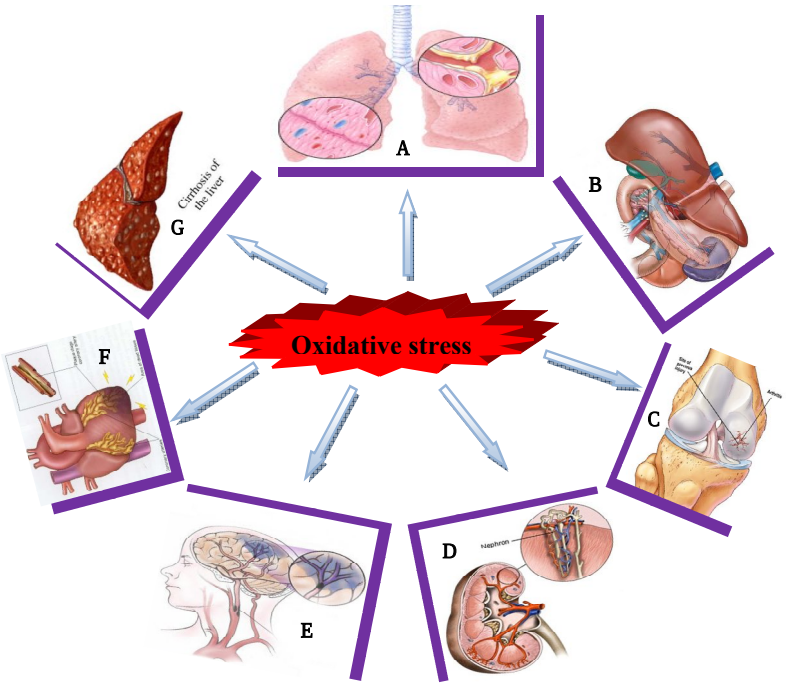


Figure 2: Oxidative stress induced-diseases in humans. Asthma, chronic bronchitis, etc [lungs (A)]; Cancer, Diabetes, Aging, etc [Multiorgan (B)]; Arthritis, Rheumatism [Joints (C)]; Glomerulonephritis, Chronic renal failure [Kidney (D)]; Alzheimer's, Parkinson's, Memory loss, etc [Brain (E)]; Artherosclerosis, Hypertension, cardiac fibrosis, Ischemia, etc [Heart (F)]; Cirrhosis of the liver (G).

VI. Natural defense systems against free radicals

Although free radicals are produced in abundance in all cells, there are numerous natural defenses to either prevent their formation or to neutralize them after they are formed. Both enzymatic and non-enzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS and to contribute to disease prevention. To provide maximum intracellular protection, these scavengers are strategically compartmentalized in the cell. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (GR). SOD, the first line of defense against free radicals, catalyzes the dismutation of $O_2^{\bullet -}$ into H_2O_2 by reduction. The oxidant formed is transformed into water and oxygen by CAT and GPx. During this process, reduced glutathione (GSH) is

converted to oxidized glutathione (GSSG) by GPx. This oxidized glutathione is regenerated back to GSH through GR activity and maintains the redox homeostasis (Willcox *et al.*, 2004; Genestra *et al.*, 2007; Halliwell, 2007; Pacher *et al.*, 2007). The non-enzymatic antioxidants include metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants are endogenous antioxidants which are produced during metabolism including glutathione, L-arginine, coenzyme Q10, melatonin, uric acid, bilurubin, metal chelating proteins, transferrin, etc. (Droge, 2002, Willcox *et al.*, 2004; Genestra *et al.*, 2007; Pacher *et al.*, 2007); while nutrient antioxidants belong to exogenous antioxidants, which must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, flavonoids, etc. Scientific study on these nutrient antioxidants is an important area because of their importance in disease prevention, when the endogenous antioxidant system is unable to scavenge the excess ROS generated during stressful conditions.

VII. Antioxiants

An antioxidant is defined as a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions (Gutteridge and Halliwell, 2000). They do so by being oxidized themselves; so antioxidants act often as reducing agents such as thiols, ascorbic acid or polyphenols. Antioxidants also act as radical scavengers, hydrogen donars, electron donars, peroxide

decomposers, singlet oxygen quenchers, enzyme inhibitors and metal-chelatating agents (Young and Woodside, 2001; Clark, 2002; Scheibmeir *et al.*, 2005).

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex antioxidant defense system against oxidative stress. It is composed of several lines and the antioxidants are classified into four different categories based on function (Halliwell, 1999; Young and Woodside, 2001)

i. First line of defense is the preventive antioxidants, which suppress formation of free radical (SOD, GPx, CAT, selenoprotein, ferritin, lactoferrin, carotenoids, etc).
ii. Second line of defense is the radicals scavenging antioxidants suppressing chain initiation and /or breaking chain propagation reactions: radical scavenging antioxidants.
iii. Third category: repair and *de novo* antioxidant (some proteolytic enzymes, DNA repairing enzymes etc).
iv. Fourth line is an adaptation where the signal for the production and reactions of free radicals induces formation and transport of the appropriate antioxidants to right sites.

VIII. Dietary antioxidants and health

There is now increasing interest in antioxidant activity of phytochemicals present in the diet. Increasing intake of dietary antioxidants may help to maintain an adequate antioxidant status and therefore, the normal physiological function of a living

system. Some functional foods, vegetables and cereal grains are the important sources of exogenous antioxidants. The most common antioxidants present in foods are vitamin C and E, carotenoids, flavonoids.

Clinical trial and epidemiological studies have established an inverse correlation between the intake of whole grain cereals and the occurrence of disease such as inflammation, cardiovascular disease, cancer and type II diabetes. Whole grain cereal antioxidants, including polyphenolic compounds, vitamin E and C and carotenoids are believed to be the effective nutrients in the prevention of these oxidative stress-related diseases. Whole grain cereals have thus become a topic of increasing interest recently.

IX. Whole grain cereals and phytochemicals

Cereal grains have long been thought to be less important sources of antioxidants than fruits and vegetables (Liu, 2007), although they contain many antioxidants and are the major dietary components worldwide. Compared to refined grains, whole grains are much richer in bioactive molecules, such as simple phenols, flavonoids, anthocyanins and phytoestrogens apart from its high insoluble fiber, minerals and vitamins; which are generally recommended as important beneficial part of human diet. (Chatenoud *et al.*, 1998; Okarter and Liu, 2010). Some examples of highest intake of whole grain foods and health benefits are; reduced risk of gastric cancer (Wu-Williams *et al.*, 1990), colorectal cancer (Schatzkin, 2007; Slattery *et al.*, 1997), breast cancer, endometrial cancer risk (Franceschi *et al.*, 1996; Levi *et al.*, 1993), pancreatic cancer (Chan *et al.*, 2007), coronary heart disease (Anderson, 2004) and type II diabetes (Venn and Mann, 2004). Among the whole grain cereals (including maize, wheat, corn, rice, oats, barley, rye, sorghum), rice is one of the most important

staple food source for over half of the world's population (Hu *et al.*, 2003). In Asia alone, more than 2000 million people obtain 60 to 70 % of their calories from rice and its products.

X. Rice

Rice (*Oryza sativa* L.) is a short-lived plant related to the grass family, with a life cycle of 3-7 months. The span of one cycle varies, depending on its type and the growing environment. Recent studies on rice grain have demonstrated that its potential health benefits appear to be related to its pericarp colour. Ling *et al.*, (2001) found that liver reactive oxygen species, aortic malondialdehyde and the area of atherosclerotic plaque were significantly lower in rabbits, fed rice with red or purple bran than in those fed rice with white bran. In another study, rats fed rice with dark coloured bran showed protective effect against renal lipid peroxidation compared to the rats fed rice with light bran colour (Toyokuni *et al.*, 2002).

The higher antioxidative effect of coloured rice as compared with that from white or light bran coloured rice may be explained by differences in the phenolic content or other components in the rice kernel (Goffman and Bergman, 2004). In fact the phenolic concentration in rice appears to be strongly related to bran colour, with red or purple bran showing 20 times higher concentrations compared with those with white or light-brown bran (Goffman and Bergman, 2004). The predominant form of rice found in today's market is polished rice where the bran fraction is removed during milling. The bran fraction of higher nutritive value is used as an animal feed. This implied not only the loss of a nutritionally valuable rice component in human diet, but also a reduction of the quantity of rice available for human nutrition by 10-15 %.

Therefore, studies dealing with rice whole grain and its phytochemicals are of greater importance.

XI. Rice grain structure

Freshly harvested rice is called paddy grain or rough rice. The pearly white starch grain used for cooking is the centre of the rice seed and is covered and protected by the hull. Inside the hull, the familiar white grain is covered by a layer called bran. The embryo, a small structure at the base of the grain, is also contained within the bran layer. Together, the grain, embryo and bran are called brown rice (Figure 3).

During processing hull is removed from the paddy to obtain brown rice. It consists of the outer layers of pericarp, seed coat, nucellus, the germ or embryo and the endosperm. The endosperm consists of the aluerone layer and endosperm proper. Further milling of brown rice produces white rice with the loss of bran fraction which is the most important part of the rice residues.

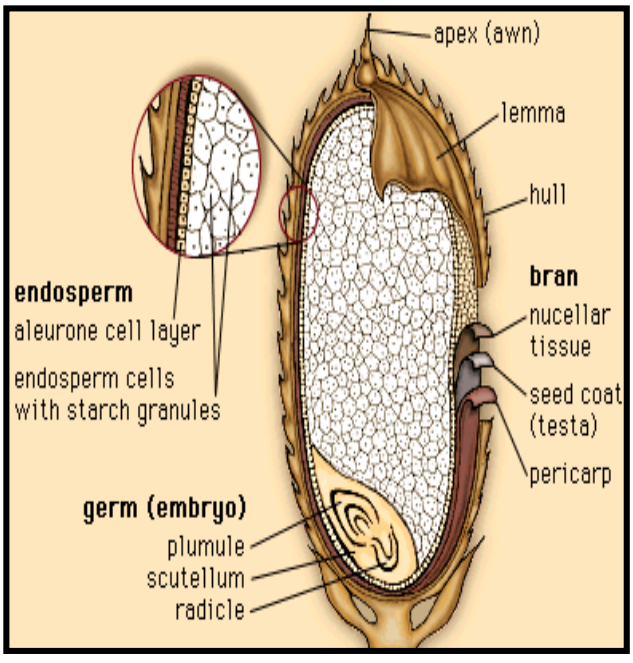


Figure 3: Longitudinal section of rice grain.

XII. Rice bran stabilization and its importance

Freshly milled rice bran has a short shelf life because of the decomposition of the lipids (triglycerols) into free fatty acids, making it unsuitable for human consumption or the economical extraction of edible oil. When the bran layers are removed from the endosperm during the milling process, the individual cells are disrupted and the rice bran lipids come into contact with lipases, an enzyme that rapidly hydrolyzes oil to free fatty acids and glycerol, and results in drastic reduction in quality of the bran. Therefore deactivating the enzyme lipase by heat treatment extends the shelf life of the rice bran. Lakkakula *et al.,* (2004) and Ramezanzadeh *et al.,* (1999) have reported the stabilization of rice bran by ohmic heating and microwave heating respectively and succeeded in inactivating lipase and extending the shelf life of rice bran which could be useful for the potential recovering valuable oil and functional ingredients like bioactive compounds for the human diet (Abdul-Hamid *et al.,* 2007).

XIII. Rice bran phytochemicals

Rice bran is composed of pericarp, seed coat, nucellus, aleurone layers and germ. The lipid content of rice bran is approximetly 15-20 % with especially unsaturated fatty acids (Hu *et al.,* 1996). In addition to good quality protein, high fiber content, ash, vitamins and minerals, rice bran has the potential to promote health because they contain antioxidative compounds that have the ability to inhibit the formation or to reduce the concentrations of reactive cell-damaging free radicals. These compounds include ferulic acid, its esterified derivative (γ-oryzanol) and unsaponifiable components such as tocols (tocopherols + tocotrienols), anthocyanins, flavonoids, simple phenolic compounds such as 4-hydroxycinnamic acid, *p*-coumeric acid,

protocatechuic acid, synergic acid, vanillic acid, sinapinic acid, chlorogenic acid, etc. (Isao *et al.*, 2004; Nam *et al.*, 2006; Devi and Arumugham, 2007; Lai *et al.*, 2009).

a. γ-Oryzanol

Nearly ten components of γ-oryzanols were identified as a mixture of ferulic acid esters of plant sterols and trieterpene alcohols, having powerful antioxidant activity and occurring naturally only in rice bran (Xu and Godber, 1999). Ferulic acid is a derivative of 3,4,-dihydroxy cinnamic acid, which is abundantly found in rice bran. The content of γ-oryzanol present in rice bran is 13-20 times (w/w) greater than the total tocols present in rice bran (Chen and Bergman, 2005). It has also been reported to possess some health-beneficial properties such as reducing cholesterol absorption (Rong *et al.*, 1997), anti-inflammatory activity (Akihisa *et al.*, 2000), inhibition of platelet aggregation, improvement of plasma lipid pattern and can inhibit cholesterol oxidation *in vitro* (Xu *et al.*, 2001; Cicero and Gaddi, 2001). In addition, it is a potent antioxidant in food, pharmaceutical and cosmotic industries (Lilitchan *et al.*, 2008).

b. Tocols

It includes four homologs of α,-β,-γ,-δ- tocopherols (Ts) and four homologs of α,-β,-γ,-δ- tocotrienols (T3s). Among the tocols, especially the naturally occurring d-α-tocopherol comprising of about 90 % of the tocopherols in animal tissues and displays the highest biological activities in most bioassay system (Wolf *et al.*, 1998; Chen and Bergman, 2005). Vitamin E is nature's most effective lipid-soluble antioxidant protecting unsaturated fatty acids in cell membranes that are important for membrane function and structure. It is the major chain-breaking antioxidant in body tissues and is considered to be the first line of defense system against lipid peroxide. It also protects

cell membranes at an early stage of free radical attack that are important for membrane function and strucuture (Mascio *et al.,* 1991; Brigelius-Flohe and Traber, 1999). Many studies have demonstrated the role of vitamin E vitamers in the prevention and treatment of some chronic, age-related diseases such as cardiovascular diseases, atherosclerosis, cancer, arthritis, alzhemer's and Parkinson's disease. It is also known to enhance immune responses (Ozer *et al.,* 1995; Brigelius-Flohe and Traber, 1999; Bramley *et al.,* 2000; Shui and Leong, 2005). In addition; tocotrienols have shown peculiar physiological potential including antitumor properties towards mammary cancer (Nasaretnam, 2005), inhibit cholesterol synthesis, lower serum-cholestrol levels in various animal models, suppress tumor cell proliferation with γ- and δ- homologs demonstrating greater potency than the α-homolog (Qureshi *et al.,* 2000).

Rice bran is a rich natural source of vitamin E, containing upto 300 mg/kg (Shin *et al.,* 1997). The major component of vitamin E in rice bran is α-tocopherol. Moreover, Aguiler-Garcia *et al*., (2007) reported that total tocopherols of rice ranged from 41.0 to 61.3 µg/g dry weight in brown rice whereas total tocotrienols ranged from 155 to 163 µg/g dry weight in rice bran.

c. β-Carotene

It is a type of carotenoid, which is one of the most important classes of plant pigments and an effective antioxidant for quenching singlet oxygen species and can directly scavenge other free radicals (Mascio *et al.,* 1991; Ni, 1993; Sies and Stahl, 1995). In addition, β-carotene is efficient in a chain termination at low partial oxygen pressures. A study on phillipines upland rice varieties found distinguished differences in β-carotene content depending on the grain colour. The highest average content was found in black variety, with values reaching up to 0.13 mg/kg (brown rice). Similar

results were obtained in another survey including varieties from Malaysia, Vietnam and Thailand. The highest concentration was detected in a black variety from Malaysia with 0.22mg/kg in black or purple landrace from Philippines (Frie and Becker, 2004)

d. Polyphenols

The phenolic component of plants constitutes a complex mixture, and only a small number of plants have been examined systematically for their phenolic content. Studies indicated that phenolic compounds have health beneficial properties and are the integral part of the human diet. Even though rice is a staple food for more than half of the world's population; data on rice phenolics are limited but suggest that the predominant phenols are phenolic acids, primarily ferulic acid (FA) and *p*-coumaric acid (Isao *et al.*, 2004). Evidence indicates that polyphenols have potent antioxidant properties and exert various physiological effects in humans such as preventive oxidative damage of lipids and low-density lipoproteins, inhibiting platelet aggregation and reducing the risk of cardiovascular disease, diabetes and cancer (Chotimarkorn *et al.*, 2008; Zhang *et al.*, 2010a).

The total phenolics of black rice bran ranged from 2365 to 7367 mg of gallic acid equiv/100g of dry weight (dw) among the 12 black rice varieties as analyzed by Zhang *et al.*, (2010a). However, in another study, the total phenolic content of five long-grained white rice bran from commercial available cultivars in Thailand was reported in the range of 220-320 mg of gallic acid equiv/100g of dw (Chotimarkorn *et al.*, 2008). Because of the high phenolic content in black rice varieties, China and many other Asian countries use black rice varieties as staple food to replace white rice. The distinct phytochemicals from black rice, especially flavonoids and anthocyanins, have been

shown to have beneficial effects in the prevention of chronic diseases, associated with oxidative stress (Zhang *et al.*, 2010a).

e. Anthocyanins

Anthocyanins occur ubiquitously in the plant kingdom and are a subclass of natural phenolic compounds that are widely spread in food of vegetal origin. There are over 250 natural anthocyanins, and all are *O*-glucosylated with different sugar substitutes (Chen *et al.*, 2006). This group of polyphenolic compounds have been reported to have positive effects in the treatment of various diseases and are prescribed as medicines in many countries (Cao *et al.*, 2001).

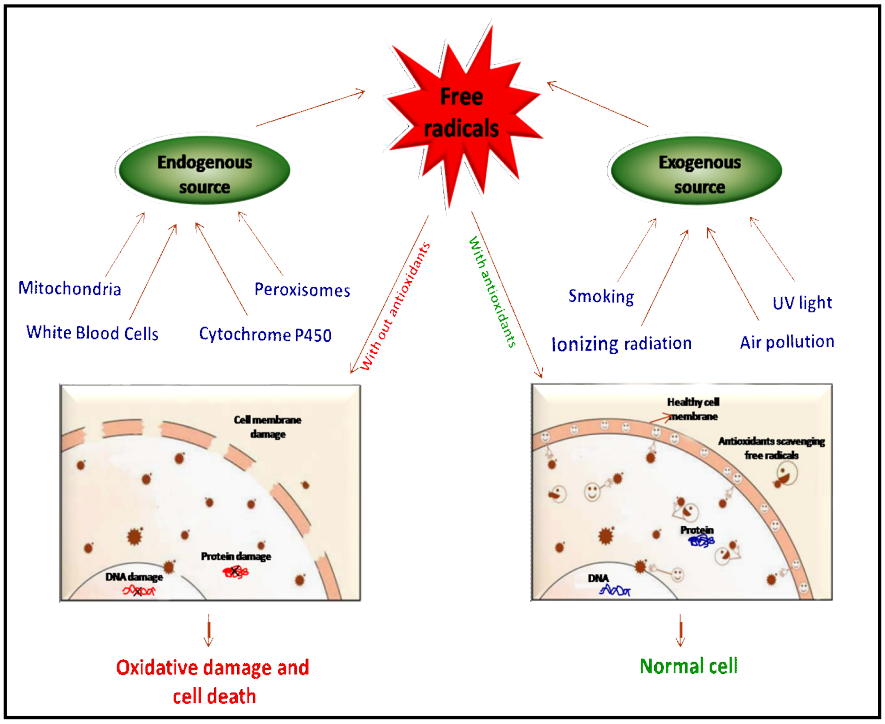
Significant amounts of anthocyanins are found in red rice and black rice varieties. The principal anthocyanin in rice is cyanidin-3-glucoside (0-493 mg/100g), followed, in minor proportion by peodinin-3-glucoside (0-40 mg/100g) (Ryu *et al.*, 1998). Recently, Hu *et al.*, (2003) isolated and determined the content of anthocyanins in black rice. They found that the content of total anthocyanins in the whole grain was 0.16 % and of the bran was 1.36 % which showed the capacity of anthocyanin extract to neutralize reactive oxygen and nitrogen species in model cell culture. These authors also reported that, as consequences of a marked antioxidant activity and capacity to capture free radicals *in vitro*, the pigmented fraction of black rice prevents DNA scission and the deterioration of human LDL, induced by ROS. Xia *et al.*, (2003) showed cardiovascular protection of anthocyanins. Morimitsu *et al.*, (2002) showed the anthocyanins of pigmented rice as potential inhibitors of the formation of cataracts in diabetic. Chen *et al.*, (2006) showed black rice anthocyanins inhibit invasion and motility of the cancer cells (SKHep-1, Huh-7 and HeLa) and also inhibited the growth of SKHep-1 cells in *in vivo* studies.

Increasing intake of whole grain antioxidants may help to maintain an adequate antioxidant status and normal physiological function of a living system. Hence, diet with antioxidant properties has become a topic of increasing interest all over the world. Since the Ayurvedic practice started in India, plant extracts are being used for the treatment of various disorders. The exact mechanism of these plant-derived preparations is not well understood and requires to be scientifically investigated. Although Njavara, a medicinal rice variety, which is extensively used in Ayurveda for the treatment of various disorders, there are no scientific investigations on the medicinal properties of this rice variety. The present work was undertaken with the following objectives:

Objectives of the study

1) Comparison of antioxidant activities of Njavara rice bran with those of Vasumathi, Yamini and Jyothi rice varieties.
2) Antimicrobial, Antidiabetic and Antiproliferative properties of Njavara Rice bran.
3) *In vitro* antiproliferative and proapoptotic effects of Njavara rice bran methanolic extracts on glioma cell lines.
4) Naringenin – induced cytotoxicity in glioma cell lines and preparation of Naringenin nanoparticles.

1
Chapter



Comparison of antioxidant activity of Njavara rice bran with Vasumathi, Yamini and Jyothi rice varieties

1.1. Introduction

It is widely recognized that many of the today's diseases are due to the oxidative stress that results from an imbalance between formation and neutralization of pro-oxidants (Hazra *et al.*, 2008; Huy *et al.*, 2008). Cells have developed antioxidant mechanisms to quench the free radicals but when the generation of free radicals exceeds the scavenging capacity of the cell, the excess free radicals seek stability through electron pairing with biological macromolecules such as lipids, DNA and proteins in healthy human cells leading to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases (Devi and Arumugham, 2007; Aswatha *et al.*, 2008; Chotimarkorn *et al.*, 2008; Hodzic *et al.*, 2009; Lai *et al.*, 2009). The free radicals are known to be scavenged by synthetic antioxidants, but due to their adverse side effects, search for effective and natural antioxidants has become crucial (Choi *et al.*, 2007; Adeolu *et al.*, 2009). Rice bran is a by-product of rice milling which contains a significant amount of natural phytochemicals including sterols, higher alcohols, γ-oryzanol, tocopherols, tocotrienols and phenolic compounds (Isao *et al.*, 2004; Nam *et al.*, 2006; Devi and Arumugham, 2007; Lai *et al.*, 2009).

1.1.1. Njavara Rice

The ayurvedic treatise records show the existence of several medicinal rice varieties in India. Thus, proper documentation and research data on these varieties are of greater importance. Njavara is one of such important Indian medicinal rice variety, grown in Southern India and is used mainly for Ayurvedic treatments (Deepa *et al.*, 2008; Simi and Abraham, 2008). It is considered as gold among the paddy varieties. It is believed to be a progenitor of Asiatic rice with an unadulterated gene pool. The

dehusked rice is dark red in colour. In southern United States, Greece, Latin America, Spain and other temperate regions, red rice is considered a weed, which grows along with cultivated rice. However in Bhutan, China, India, Srilanka, Philippines, red rice is grown as a staple rice cultivar (Itani *et al.,* 2004). Red rice is gaining popularity in Japan as a functional food because of its high polyphenols and anthocyanin content.

Njavara is at the brink of extinction due to its low yield and high cost. It is presently used in limited Ayurvedic preparations / treatments. It is bestowed with many medicinal properties and the medicinal quality of this rice is preserved by using only dehusked rice (Deepa *et al.,* 2008).

1.1.2. Health benefits and nutrient composition of Njavara rice

A. Uses in Ayurveda

- Increases the growth of muscles and stimulates the nerve endings.
- Grains are used mainly in disorders like muscle wasting and cervical spondylitis.
- The roots of this rice are said to be cooling, diuretic and febrifuge and are useful in burning sensation, dyspepsia, bilious fever and diabetes.
- Usually used in external application as *Njavara kizhi*, a type of "warm sweating" treatment and as *Njavara theppu*, covering the body with hot Njavara rice.

Njavara kizhi and *Navara theppu* are the two major treatments in ayurveda for arthritis, paralysis, neurological disorders, degeneration of muscles and tuberculosis. In *Njavara Kizhi*, a poultice is prepared by cooking Njavara rice in

milk, with certain herbs like *Sida rectusa* and *Alpinia galangal*. It is then enclosed in cloth pouches (*kizhis*) and used for topical massage of the entire body. This treatment leads to heat generation, extensive perception of the body, increase in blood circulation and relieves stiffness of the joints and arthritic pain. In *Njavara theppu*, a paste boiled Njavara rice of light warmth is applied on the body. Here again the rice is boiled in *kurunthotti kashayam* (a decoction of sida root and milk). It is recommended for those patients who were unable to bear the *Njavara Kizhi* treatment.

B. Local uses

- Regular consumption of *Paal Kanji* (rice gruel made in cow's milk with sugar added) once a day ensures longevity. It is believed that this was the food of holy leaders in ancient India.
- Consuming this rice boiled in some vessel made of copper prevents rheumatic complaints and gives exceptionally high energy.
- For acute complaints of piles, consume the rice roasted with small onions in cows gee is consumed for 21 days on an otherwise empty stomach. (Cook the rice separately in a clay pot and then roast it with onion.) Another method is consuming the cooked rice mixed with curry leaf, sour buttermilk and pepper.
- Roots are employed in the form of decoction, which is useful in children's urinary complaints.
- Eating Njavara rice flakes, pounded with *Aswagandha* roots and sugar, will increase vitality and body weight and act as an aphrodisiac.
- Consuming *Payasam*, a sweet dish made of this rice in jaggery and gee, increases mother's milk.

- Warm rice paste is applied once or twice for a week for burning sensation of foot.
- Consumption of *Marunnu kanji*, Njavara gruel, along with several other medicinal herbs prevents ailments common in the monsoon season.
- Njavara rice is recommended for diabetic patients.
- Applying rice paste in the pustules formed due to the biting of a viper reduces pain.
- For stomach ulcer, a dish made of Njavara rice flour and squashed banana is consumed.
- For premature falling of hair, cleansing the head regularly with washed-away water of Njavara is useful.
- For cough, Njavara rice is boiled along with *Moringa* leaves, pounded it and has to be taken with the flakes made of Njavara.
- Eating Njavara rice increases semen and fertility in males. Recommended for childless couples.

C. Nutrient composition of Njavara brown rice

Cereals are the major source of carbohydrate, proteins, fats, minerals and vitamins to the vegetarian population worldwide. Rice is one of the most important cereal and hence its grain quality and the nutrition composition plays a vital role in health benefits.

Table 1: Nutrient composition of Njavara brown rice (Deepa *et al.*, 2008).

	Njavara brown rice
Proximate composition	
Total carbohydrate (g per 100g)	73.5 ± 13.21
Protein (g per 100 g)	9.52 ± 0.34
Crude lipid (g per 100 g)	2.48 ± 0.50
Ash (g per 100 g)	1.42 ± 0.06
Fiber (g per 100 g)	8.03 ± 0.03
Energy value (kJ per 100 g)	1630 ± 7.2
Mineral composition (mg per 100 g)	
Iron	1.93 ± 0.01
Calcium	11.6 ± 0.08
Sodium	30.9 ± 0.14
Magnesium	216 ± 0.10
Potassium	304 ± 0.48
Phosphorus	354 ± 15.42
Vitamin content (mg per 100 g)	
Folic acid	0.05 ± 0.001
Thiamine	0.52 ± 0.01
Riboflavin	0.071 ± 0.001
Niacin	7.32 ± 0.81
Dietary fibre (g per 100 g)	
Insoluble fibre	7.56 ± 0.05
Soluble fibre	0.52 ± 0.03
Amylose equivalent (%)	
Insoluble amylose	14.3±1.17
Soluble amylose	8.45±1.35
Fatty acid composition (%)	
Palmitic acid (16:0)	22.1
Stearic acid (18:0)	02.4
Oleic acid (18:1)	42.6
Linoleic acid (18:2)	31.0
Linolenic acid (18:3)	1.54

According to Adu-Kwarteng *et al.,* (2003) intermediate amylose rice (20-25%) varieties does not increase rapid rise in blood sugar levels when they are taken as diet. Therefore, intermediate amylose rice varieties are the most preferred ones for diet, especially with reference to diabetic patients. Deepa *et al.,* (2008) showed the higher protein, total fiber content, intermediate amount of amylose content, significant amounts of vitamins and minerals in Njavara brown rice (Table 1). The high thiamin content of Njavara rice could be useful in treating muscle weakness, neuritis and other symptoms related to deficiency of vitamin B_1. High potassium, calcium and magnesium could be helpful in improving muscle activity in patients suffering from muscle wasting.

Inspite of enormous medicinal properties and high nutritive value, scientific studies on Njavara rice variety are scarce. Recently Deepa *et al.,* (2008 and 2009) showed the high nutritive value, physiochemical and genetic analysis of Njavara rice. While Simi and Abraham, (2008) studied the physiochemical, rheological and thermal properties of Njavara starch. More recently, Kumar *et al.,* (2010) studied Njavara leaf isozyme analysis of alcohol dehydrogenase to establish an index of genetic similarities among different ecotypes. However, no scientific data are available on the phytochemical content and free radical scavenging properties of the Njavara rice.

1.1.3. Objective

Our main objective in this investigation was to evaluate antioxidative and radical scavenging properties of Njavara rice bran methanolic extracts. These properties were compared with commercially available two Indian basmati varieties: Vasumathi, Yamini and a non-medicinal variety, Jyothi.

1. 2. Materials and Methods

1.2.1. Chemicals

Folin-Ciocalteus's phenol reagent, sodium carbonate, gallic acid (GA), quercetin (QE), $FeCl_3$, $NaNO_2$, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), reduced nicotinamide dinucleotide (NADH), xylenol orange, thiobarbuteric acid (TBA), ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium nitro preside (SNP), α-napthyl-ethylenediamine, potassium ferricyanide, trichloroacetic acid (TCA), ammonium molybdate, dimethyl sulphoxide (DMSO), 2-deoxy-2-ribose were purchased from Merck Chemical Supplies (Damstadt, Germany). All the chemicals, including the solvents, were of analytical grade.

1.2.2. Plant material

Seeds of Njavara variety were obtained from Kerala Agricultural University, Kerala, Vasumathi, Yamini and Jyothi varieties were obtained from Directorate of Rice Research, Hyderabad; Central Soil Salinity Research Institute, Karnal and National Seed Corporation, Warangal respectively. Vasumathi and Yamini are the two commercially available basmati varieties which are commonly taken as staple food in India, Jyothi is a pigmented variety and its dehusked seed is red in colour. Njavara is also a pigmented variety, its paddy is black in colour and dehusked seed will be red in colour (Figure 4).

Figure 4: Morphology of paddy, brown rice and polished rice from four rice varieties. 1,2,3, represents paddy, brown rice and polished rice. A, B, C and D represent Vasumathi, Yamini, Jyothi and Njavara rice varieties respectively.

1.2.3. Rice bran preparation and stabilization

Rice bran from the four varieties was obtained by milling rice grain in a local grinding mill, followed by sieving to separate grain from bran. Stabilization of rice bran was done by heating the bran in microwave oven at 850W and with 2450 MHz. The microwave chamber was preheated to 100% power for 3 min. The moisture content of the raw rice bran was adjusted to 21% by adding deionized water (Malekian, 1992). The sample was heated for 3 min at 100% power (Figure 5). The temperature of the sample after heating in the microwave was 107± 2ºC. The sample was allowed to cool to room temperature and stored in an ultralow freezer (-80ºC) until further analysis.

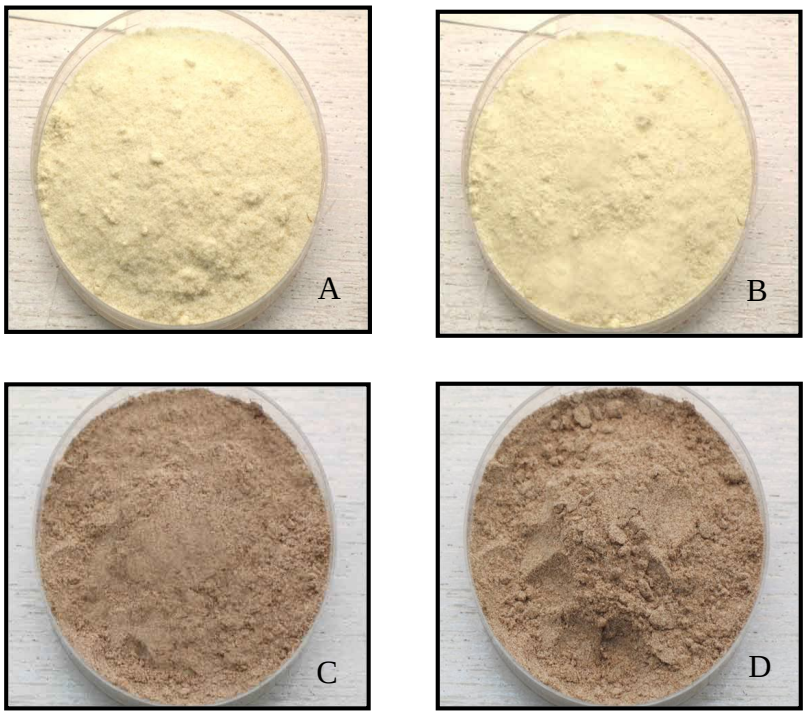


Figure 5: Bran from four rice varieties. A, B, C and D represents Vasumathi, Yamini, Jyothi and Njavara rice bran respectively.

1.2.4. Sample preparation

Rice bran/brown rice flour/polished rice flour extract was prepared according to a modified method of Choi *et al.,* (2007). Each sample (5 g) was extracted thrice with 30 ml methanol/ethyl acetate/hexane for 3 h in an electrical shaker at 40 ºC. The extracts were filtered through Whatman No.2 filter paper and evaporated under vaccum using a rotary evaporator (Heidolph, Germany). The residual crude rice bran extracts were weighed, dissolved in dimethyl sulphoxide (DMSO), filtered through a 0.45µm of Nylon membrane filter and stored at -20 ºC until further analysis.

1.2.5. Determination of total phenolic content

The total phenolic content of the samples were determined using the Folin-Ciocalteu reagent (Gulcin *et al.,* 2007). The reaction mixture contained: 200 µl of diluted sample (bran 1:100; brown rice 1:50; polished rice 1:10), 800 µl of freshly prepared diluted Folin Ciocalteu reagent and 2 ml of 7.5 % sodium carbonate. The final mixture was diluted to 7 ml with deionized water. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction and the absorbance was measured at 765 nm. Gallic acid was used as standard and the results were expressed as mg gallic acid (GAE)/g sample.

1.2.6. Determination of total flavonoid content

Total flavonoid content was determined using aluminium chloride ($AlCl_3$) according to a standard method using quercetin as a standard (Zhishen *et al.,* 1999). 100 µl of sample was added to 300 µl distilled water followed by 30 µl of 5 % $NaNO_2$. After 5 min at 25 ºC, 30 µl of 10 % $AlCl_3$ was added. After further 5 min, the reaction

mixture was treated with 200 µl of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. The results were expressed as mg quercetin (QE)/g sample.

1.2.7. Determination of total antioxidant activity

For total antioxidant assay various concentrations (20, 40, 60, 80 and 100 µg) of rice bran extract were mixed with 1 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated in a water bath at 95 ºC for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm (Preito *et al.*, 1999). Ascorbic acid was used for comparison.

1.2.8. Determination of reducing power

The reducing power of rice bran extract was measured according to the method described by Yen and Duh, (1993) with some modifications. Various concentrations (100, 200, 300, 400 and 500 µg) of rice bran extract were mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6). The dilute sample was then mixed with 5.0 ml of 1 % potassium ferricyanide and the mixture was incubated at 50 °C for 20 min. 5.0 ml of 10 % trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 5.0 ml of the supernatant was mixed with 5.0 ml of distilled water and 1.0 ml of ferric chloride (1 %). The absorbance was measured at 700 nm. Standard ascorbic acid was used for comparison.

1.2.9. Measurement of nitric oxide scavenging ability

Nitric oxide scavenging activity was determined according to Griess Illosvoy reaction (Garratt, 1964). The reaction mixture contained: 10 mM SNP in 0.5 M phosphate buffer (pH 7.4) and various concentrations (50–250 µg/ml) of the bran samples in a final volume of 3 ml. After incubation for 60 min at 37 ^{o}C, Griess reagent (0.1 % α-napthyl-ethylenediamine in water and 1 % sulphanilic acid in 5 % H_3PO_4) was added. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with α-napthyl-ethylenediamine was measured spectrophotometrically at 540 nm. Ascorbic acid was used as a positive control. Nitric oxide scavenging ability (%) was calculated by using the formula:

$$[A_{540\ nm\ of\ control} - A_{540\ nm\ of\ sample} / A_{540\ nm\ of\ control}] \times 100.$$

1.2.10. Determination of DPPH˙ scavenging assay

DPPH radical scavenging activity of rice bran extracts was determined according to the method of Blois (1958) with slight modifications. An aliquot of 0.5 ml of sample solution in methanol was mixed with 2.5 ml of 0.5 mM methanolic solution of DPPH˙. The mixture was shaken vigorously and incubated for 37 min in the dark at room temperature. The absorbance was measured at 517 nm using UV-vis spectrophotometer. Ascorbic acid was used as a positive control. DPPH free radical scavenging ability (%) was calculated by using the formula:

$$[A_{517\ nm\ of\ control} - A_{517\ nm\ of\ sample} / A_{517\ nm\ of\ control}] \times 100.$$

1.2.11. ABTS radical scavenging Assay:

ABTS radical cations were produced by reacting ABTS (7 mM) and potassium persulfate (2.45 mM) on incubating the mixture at room temperature in dark for 16 hours. The solution thus obtained was further diluted with PBS to give an absorbance of 0.8. Different concentrations of the rice bran methanolic extracts in 50 µl were added to 950 µl of ABTS working solution. The absorbance was recorded immediately at 734 nm (Auddy *et al*., 2003). Gallic acid was used as reference standard. ABTS free radical scavenging ability (%) was calculated by using the formula:

$$[A_{734\ nm\ of\ control} - A_{734\ nm\ of\ sample} / A_{734\ nm\ of\ control}] \times 100.$$

1.2.12. Hydrogen peroxide scavenging

This activity was determined according to a previously described method (Hazra *et al*., 2008). An aliquot of 50 mM H_2O_2 and various concentrations (0–2 mg/ml) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 90 µl of the H_2O_2 sample solution was mixed with 10 µl HPLC-grade methanol and 0.9 ml FOX reagent was added (prepared in advance by mixing 9 volumes of 4.4 mM BHT in HPLC grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm.

1.2.13. Superoxide radical scavenging

This activity was measured by the reduction of NBT according to Fontana *et al.*, (2001). The nonenzymatic phenazine methosulfate-nicotinamide adenine dinucleotide

(PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various concentrations (0–20 µg/ml) of sample solution. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. Quercetin was used as positive control.

1.2.14. Hydroxyl radical scavenging

Hydroxyl radical scavenging was assayed as described by Elizabeth and Rao (1990) with slight modification. The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe3+-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction mixture contained (in a final volume of 1 ml): 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and various concentrations (0–200 µg/ml) of the bran samples or reference compound. After incubation for 1 h at 37 °C, 0.5 ml of the reaction mixture was added to 1 ml 2.8 % TCA; then 1 ml 1 % aqueous TBA was added and the mixture was incubated at 90 °C for 15 min. After cooling, the absorbance was measured at 532 nm. Mannitol, a classical $OH^{\cdot}$ scavenger, was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

A dose response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50 % of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

1.2.15. Statistical analysis

The data were subjected to correlation coefficient by using Sigmastat version 3.1 statistical analysis software. The correlation of the data was determined by Pearson's test. $P < 0.05$ was considered as statistically significant.

1.3. Results

1.3.1. Solvent selection for phytochemical extraction

Initially Njavara rice bran was extracted with methanol (MeOH), ethylacetate (EtOAc) and hexane (Hex). MeOH extract showed highest TPC content (12.75 µg/g GAE) followed by EtOAc (7.89 µg/g GAE) and Hex (2.92 µg/g GAE).

1.3.2. Total phenolic content (TPC)

The total phenolic content (TPC) was expressed as gallic acid equivalents. Significant differences were observed for TPC among the four rice varieties. TPC was in the range of 3.27-12.4 mg GAE/g bran. Highest TPC was observed in Njavara followed by Jyothi. Yamini and Vasumathi showed nearly equal TPC content which

Table 2: Total phenolic content of methanolic extract in four rice varieties.

TPC expressed as mg gallic acid equivalents/100 grams

Variety	Polished Rice	Brown Rice	Bran
Vasumathi	0.54 ± 0.11	1.21 ± 0.15	3.31 ± 0.3
Yamini	0.58 ± 0.18	1.84 ± 0.21	4.23 ± 0.4
Jyothi	0.99 ± 0.22	3.56 ± 0.18	9.44 ± 0.2
Njavara	1.94 ± 0.16	4.62 ± 0.24	12.72 ± 0.6

Values are of three experiments ± SE

were in low quantities compared to Njavara bran. TPC of brown rice was higher than the TPC of polished rice in all the varieties studied (Table 2).

1.3.3. Total flavonoid content (TFC)

Total flavonoid content of the methanolic extract was significantly high in Njavara compared to the other three varieties as recorded in quercetin equivalents. TFC of Vasumathi, Yamini, Jyothi, and Njavara were 1.68, 2.57, 5.33 and 8.5 mg QEE/g bran respectively. TFC of brown rice was higher than the TFC of polished rice in all the varieties studied (Table 3).

Table 3: Total flavonoid content of methanolic extract in four varieties.

TFC expressed as mg Quercetin equivalents/100 grams

Variety	Polished Rice	Brown Rice	Bran
Vasumathi	0.05 ± 0.015	0.11 ± 0.012	1.68 ± 0.032
Yamini	0.05 ± 0.018	0.15 ± 0.016	2.57 ± 0.041
Jyothi	0.11 ± 0.026	0.94 ± 0.021	5.33 ± 0.072
Njavara	0.35 ± 0.021	1.72 ± 0.019	8.51 ± 0.053

Values are of three experiments ± SE

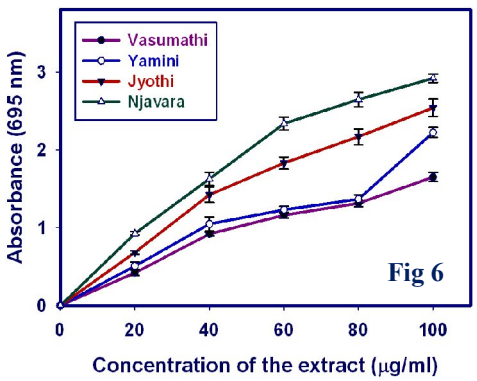
1.3.4. Total antioxidant activity (TAA)

Total antioxidant activity of the rice bran extracts increased with increasing concentration of the extracts and a significant change was observed at 0.02 to 0.1 mg/ml concentration of the extract (Figure 6). At 0.1 mg/ml of the methanolic rice bran extracts, the absorbance values of Vasumathi, Yamini, Jyothi and Njavara were 1.65, 2.21, 2.52 and 2.93 respectively. However, the total antioxidant activities of all

the rice bran extracts were less than that of the positive control ascorbate (2.5-40 µg showed an absorbance 0.117 - 2.0).

1.3.5. Reducing power (RP) assay

The methanolic extracts from all the samples had shown considerable amount of reducing activity. The reducing power of the rice bran extracts increased with the increasing concentration and a significant change was observed at 0.1 to 0.5 mg/ml concentration of the rice extract (Figure 7). 0.5 mg/ml of the methanolic rice bran extracts showed absorbance values of 0.59, 1.04, 1.93 and 2.98 corresponding to Vasumathi, Yamini, Jyothi and Njavara respectively. While 5–25 µg of the positive control ascorbate showed an absorbance 0.06 - 0.28.



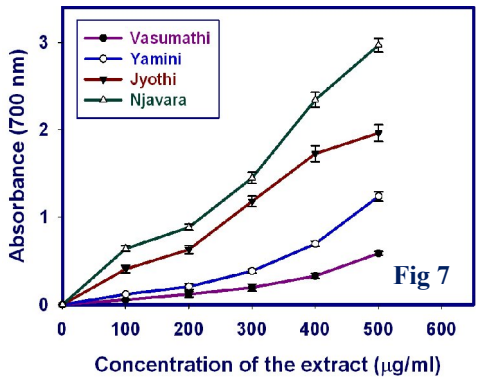


Figure 6: Total antioxidant activity. TAA carried out with different concentrations of methanolic rice bran extracts from four rice varieties. Results represent means ± standard deviation (n = 3).

Figure 7: Reducing power. RP of different concentrations of methanolic rice bran extracts from four rice varieties. Results represent means ± standard deviation (n = 3).

1.3.6. DPPH radical scavenging activity

Free radical scavenging activities of the rice bran methanolic extracts were assessed by the DPPH assay. Figure 8 illustrates a significant decrease in the

concentration of DPPH radical due to scavenging ability of the rice bran. The results showed that Njavara had the highest DPPH• scavenging activity with an IC_{50} value of 30.85 µg/ml. IC_{50} values of the other three varieties were 48.88, 70.58 and 87.72 µg/ml for Jyothi, Yamini and Vasumathi respectively. IC_{50} value of the positive control ascorbic acid was 3.2 µg/ml.

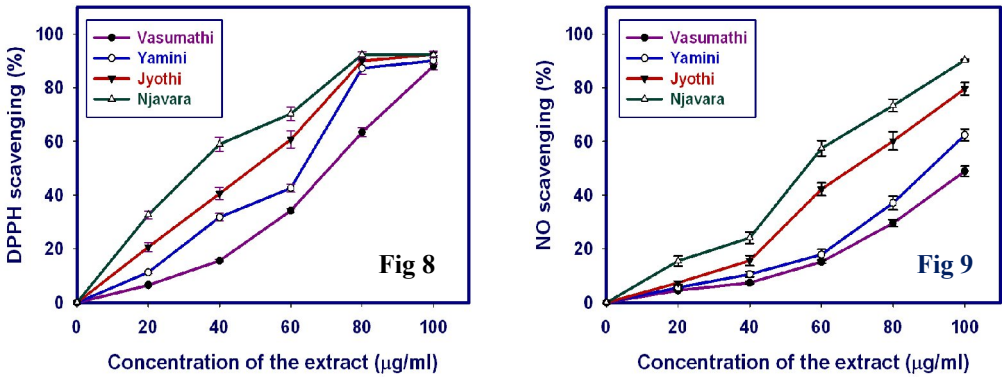


Figure 8: DPPH radical scavenging activity (%). DPPH scavenging activity of different concentrations of methanolic rice bran extracts from four rice varieties. Results represent means ± standard deviation (n = 3).

Figure 9: Nitric oxide scavenging activity (%). NO scavenging activity in the methanolic rice bran extracts from four rice cultivars. Results represent means ± standard deviation (n = 3).

1.3.7. Nitic oxide (NO) scavenging

Njavara extract showed the highest nitric-oxide scavenging activity compared to the other three rice bran methanolic extracts in a moderate dose- dependent inhibition of nitric oxide with an IC_{50} value of 52.25 µg/ml. IC_{50} values of Jyothi, Yamini and Vasumathi were 71.41, 107.18 and 102.48 µg/ml respectively (Figure 9). Ascorbic acid was used as a reference compound and its IC_{50} value is 4.6 µg/ml.

1.3.8. ABTS radical and Hydrogen peroxide scavenging

The methanolic extracts of bran from Njavara variety is very effective scavenger of the ABTS radical (Figure 10) and this activity is comparable with that of gallic acid. Vaumathi and Yamini showed least scavenging activity compared to the Njavara and Jyothi. Figure 11 showed dose-response curve of H_2O_2 scavenging activity of the methanolic extracts of the bran extracts. It was observed that methanolic extract from Njavara showed effective scavenging activity with least IC_{50} value 56 µg/ml.

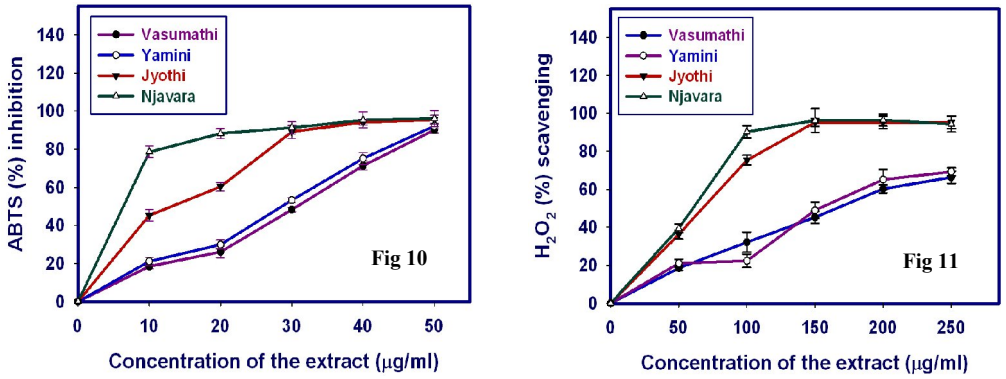


Figure 10: ABTS radical scavenging activity (%). ABTS scavenging activity of different concentrations of methanolic rice bran extracts from four rice varieties. Results represent means ± standard deviation (n = 3).

Figure 11: H_2O_2 scavenging activity (%). H_2O_2 scavenging activity in the methanolic rice bran extracts from four rice cultivars. Results represent means ± standard deviation (n = 3).

1.3.9. Superoxide and Hydroxyl scavenging activity

Although all the extracts showed superoxide scavenging activity, extract from Njavara had more scavenging activity. As shown in Figure 12, the superoxide scavenging activity of the four samples were in the order: Njavara > Jyothi > Yamini > Vasumathi with IC_{50} values 41, 48, 102, 140 µg/ml respectively. The hydroxyl radical scavenging activity of all the samples increased with increasing sample concentrations (Figure 13).

The extracts showed considerable hydroxyl scavenging activity. At concentrations from 50 – 100 µg/ml, all the extracts scavenged more than 70 % of the available free radical.

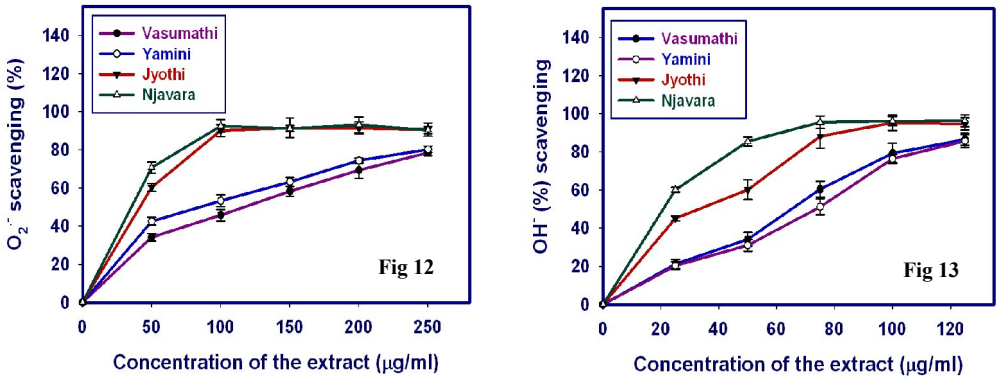


Figure 12: Superoxide scavenging activity (%). superoxide scavenging activity of different concentrations of methanolic rice bran extracts from four rice varieties. Results represent means ± standard deviation (n = 3).

Figure 13: Hydroxyl radical scavenging activity (%). Hydroxyl radical scavenging activity of different concentrations of methanolic rice bran extracts from four rice cultivars. Results represent means ± standard deviation (n = 3).

Table 4: IC_{50} values for scavenging activities of the bran extracts from four rice varieties

Antioxidant activity	**Vasumathi** IC_{50} value (µg/ml)	**Yamini** IC_{50} value (µg/ml)	**Jyothi** IC_{50} value (µg/ml)	**Njavara** IC_{50} value (µg/ml)	**Standard** IC_{50} value (µg/ml)
DPPH	74	64	49	35	3.2 (Asc)
NO	110	95	72	59	4.6 (Asc)
ABTS	33	31	16	8.0	2.8 (Ga)
H_2O_2	186	168	65	56	11.1 (Asc)
$O_2^{\cdot-}$	140	102	48	41	8.2 (Qur)
$OH^{\cdot-}$	79	68	38	22	6.3 (Man)

IC_{50} : The effective concentration at which radicals were scavenged by 50%

Asc-ascorbic acid, **Ga**-gallic acid, **Qur-** quercetin, **Man**-mannitol

1.3.10. Statistical analysis

Table 5 shows highly positive significant correlation coefficient values for total phenolic content with various scavenging activities of rice bran in all the four varieties studied.

Table 5: Correlation of phenolic content from the methanolic bran extracts of different rice varierties with various scavenging activities

Variety	Total Phenolic Content							
	TAA	**RP**	**DPPH**	**NO**	**ABTS**	**H_2O_2**	**$O_2^{\bullet-}$**	**$OH^{\bullet-}$**
Vasumathi								
R value	0.961	0.9555	0.964	0.957	0.984	0.962	0.946	0.9612
P value	0.0031	0.0114	0.0024	0.0105	0.0001	0.0021	0.0015	0.0031
Yamini								
R value	0.953	0.950	0.968	0.952	0.981	0.974	0.964	0.954
P value	0.0123	0.0132	0.0068	0.0125	0.0024	0.0031	0.0012	0.0214
Jyothi								
R value	0.965	0.969	0.961	0.992	0.991	0.958	0.974	0.981
P value	0.0021	0.0014	0.0032	0.0009	0.0015	0.0014	0.0021	0.0014
Njavara								
R value	0.977	0.992	0.964	0.967	0.9888	0.981	0.989	0.975
P value	0.0042	0.0008	0.0062	0.0042	0.0052	0.0014	0.0016	0.0056

In all the experiments number of samples (n) = 5. The pair(s) of variables with positive correlation coefficients and P values below 0.050 tend to increase together. For pairs with P values greater than 0.050, there is no significant relationship between the two variables.

1.4. Discussion

In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biological molecules leading to various diseases.(Hazra *et al.*, 2008; Huy *et al.*, 2008). Many synthetic drugs protect against oxidative damage but, because of their adverse side effects an alternative solution to this problem is to

consume natural antioxidants through food supplements and traditional medicines (Yazdanparast and Ardestani, 2007; Yazdanparast *et al.*, 2008). Recently, many plant extracts have been shown to be potent free radical scavengers. In the present study Njavara rice bran phytochemicals have been extracted with methanol and compared with the commercially available varieties; Vasumathi and Yamini and with a non-medicinal rice variety including Jyothi. Njavara showed highest phyotochemical content compared to the other three varieties.

For taking insight into the effect of solvent properties on the phytochemical content of rice bran extracts, three solvents (MeOH, EtOAc and Hex) with different polarities were evaluated with Njavara rice bran. Highest TPC was observed in methanolic extracts followed by EtOAc and Hex. Previous studies suggested that the total polyphenolic content of the plant extracts will be positively correlated to the scavenging activities. Hence further studies were carried out using MeOH as a solvent for phytochemical extraction from bran, brown rice and polished rice from all the four rice varieties. The results indicated that the TPC and TFC were more in bran compared to brown rice and polished rice in all the four varieties. Njavara variety showed highest TPC and TFC followed by Jyothi, Yamini and Vasumathi (Table 2 and 3). Both these classes of compounds have good antioxidant potential and their effects on human nutrition and health are considerable (Havsteen, 2002; Gumul *et al.*, 2007; Petti and Scully, 2009). Since bran extracts showed highest phytochemical content further radical scavenging activities were evaluated only with bran from all the four rice varieties.

Total antioxidant activity of the four rice bran extracts was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically. Our studies on the total antioxidant activity of the rice bran

methanolic extracts increased with increasing concentration of the extracts indicating the potential of extracts as antioxidants (Figure 6). Relatively high total antioxidant activity in the Njavara rice bran compared to the other samples showed a significant correlation with polyphenolic contents (Table 5) suggesting the importance of polyphenolics as potential antioxidant biomolecules. In reducing power assay ferric–ferricyanide complex was reduced to a ferrous form depending on the concentration of antioxidants in the rice bran extracts. Reducing power has been used to evaluate the ability of natural antioxidants to donate electrons (Chotimarkorn *et al.*, 2008). Njavara had relatively higher reducing power than other samples (Figure 7), indicating a significantly higher correlation with polyphenolic content (Table 5). The results indicate that rice bran methanolic extracts are capable of donating electrons which can react with free radicals to convert them as more stable products and strongly inhibiting radical chain reaction.

DPPH radical scavenging is considered as good *in vitro* model, widely used to assess antioxidant efficacy within a very short time. In its radical form, DPPH$^{\cdot}$ has an absorbance at 515 nm which disappears on reduction by an antioxidant compound or a radical species to become a stable diamagnetic molecule resulting colour change from purple to yellow, which could be taken as an indication of the hydrogen donating ability of the tested samples (Lee *et al.*, 2007; Marxen *et al.*, 2007). DPPH radical scavenging abilities of the extracts were significantly lower than that of ascorbic acid. However, all the rice varieties in our study exhibited appreciable scavenging activity and there was a significant correlation between DPPH radical scavenging activity and polyphenolic content (Table 5). IC_{50} value of Njavara indicates that it has the highest proton donating ability among the four tested rice varieties (Figure 8). These results indicate that the

rice bran extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

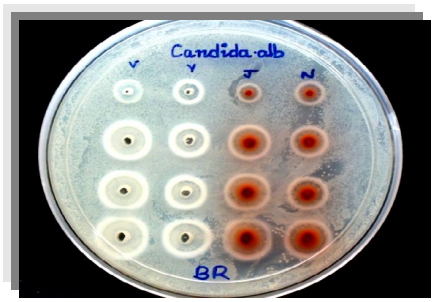
The nitric oxide generated from sodium nitropruside reacts with oxygen to form nitrite. The rice bran extracts inhibited nitrite formation by directly competing with oxygen in the reaction with nitric oxide (Tylor *et al.*, 1997). The present study proved that the rice bran methanolic extracts have potent nitric oxide scavenging activities and Njavara had the highest nitric oxide scavenging activity compared to the other three varieties (Figure 9). These oxy radicals are toxic to the tissues and are responsible for various inflammatory responses and carcinomas. Excess nitric oxide which accumulate is known to react with oxygen to form nitrite ions in the acidic environment and induce mutagenic reactions (Yin *et al.*, 2007). It has also been reported that phenolic compounds have a greater nitrite scavenging activity in environments with low pH (Noh *et al.*, 2002).

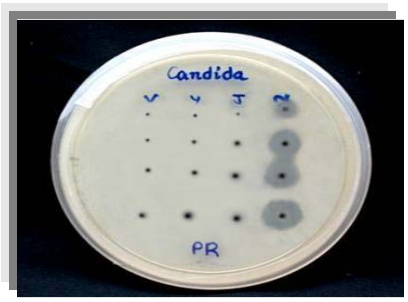
$ABTS^{\cdot+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate (Auddy *et al.*, 2003). Addition of rice bran extract to this pre-formed radical cation reduced it to ABTS in a concentration dependent manner (Figure 10). These results were compared with those obtained with gallic acid which indicates that the extract is a potent antioxidant. Similar results were seen with H_2O_2 scavenging by rice bran extracts (Figure 11). Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage (Hazra *et al.*, 2008). They were produced *in vitro* by Elizabeth and Rao (1990) method and the IC_{50} values indicated that the Njavara bran extract is a better hydroxyl radical scavenger than the other three rice varieties (Table 4). Similar trend of scavenging superoxide anion indicates that Njavara bran extract is a potent superoxide scavenger (Figure 12).

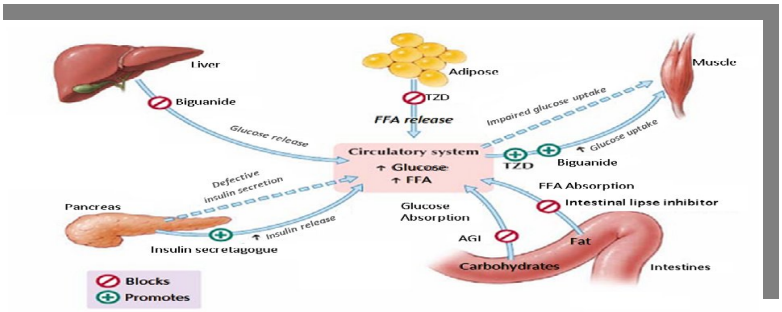
In conclusion, our data in this chapter strongly suggest that Njavara rice bran is a source of potential antioxidant for radical scavenging. The highly positive correlation of antiradical scavenging activity and total polyphenolic content in rice bran indicates that polyphenols are the important components which could be used for the free radical scavenging activity. This is the first scientific study on the antiradical efficiency of the Indian medicinal rice, Njavara. The present results formed the basis for selection of Njavara rice bran for further investigations on its biological activities including antimicrobial, antidiabetic and antiproliferatory properties.

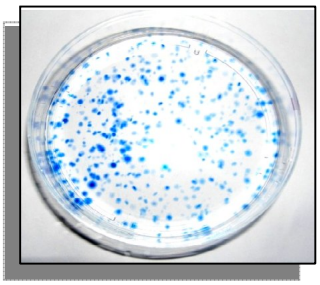
2

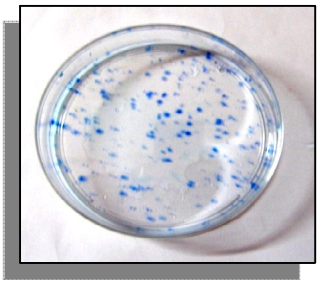
Chapter

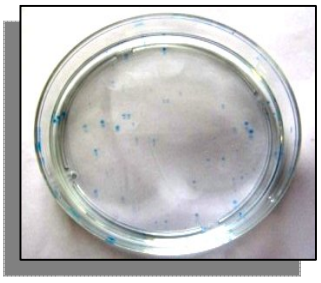












Antimicrobial, Antidiabetic and Antiproliferative properties of Njavara Rice bran.

2.1 Introduction

Numerous plants used in traditional medicine are effective in treating various ailments caused by oxidative stress, bacterial and/or fungal infections (Duffy and Power, 2001; Adeolu *et al.*, 2009). Epidemiological studies have shown that regular consumption of whole grains and whole grain products was associated with reduced risks of various types of chronic diseases such as cardiovascular disease, type II diabetes and some cancers (Anderson, 2004; Venn and Mann, 2004; Chan *et al.*, 2007; Hodzic *et al.*, 2009; Okarter and Liu, 2010). Therefore, increased dietary consumption of a wide variety of fruits, vegetables and whole grains daily is a practical strategy to optimize the health and reduce the risk of chronic diseases. Whole grains are rich in phytochemicals and provide unique bioactive compounds that are complementary to those in fruits and vegetables when consumed together (Liu, 2007). However, an increasing volume of convincing evidence suggests that the benefits of phytochemicals in fruits, vegetables and whole grains may be even greater than is currently thought because the oxidative stress, induced by free radicals, is involved in the etiology of a wide range of chronic diseases (Liu, 2007; Okarter and Liu, 2010). Overall, the available data indicates plants with high radical scavenging activities are able to show protective activities against microbial infections, diabetes and certain cancers.

2.1.1. Antimicrobial property of medicinal plants

Incidences of food borne illnesses are still a major problem, even in developed countries and has been shown that most of the illnesses and annual deaths were attributed to food borne pathogens (Alzoreky and Nakahara, 2003). The antimicrobials of plant origin are effective in the treatment of infectious diseases and also as food

preservatives while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Al-Bakri and Afifi, 2007; Das and Devaraj, 2009).

Leaves of etiponla, ayape, pakoljebu, roots of inabiri and bark of guofa are used for the treatment of diseases caused by microbes including abscess, boils, malaria, mouth infections, skin diseases and scabes (Aladesanmi *et al.*, 2007). Rios and Recio (2005) showed use of Bearbeer and Cranberry juices against urinary tract infections. Extracts of garlic, cinnamon, curry, mustard, basil, ginger and other herbs exhibit antimicrobial properties (Arora and Kaur, 1999; Marino *et al.*, 1999; Ohara *et al.*, 2008). Furthermore, essential oils of many aromatic plants including basil, bay, clove, thyme and rosemary possess antimicrobial activities (Cosentino *et al.*, 1999; Ogara *et al.*, 2000; Elgayyar *et al.*, 2001). Phenolic compounds and their derivatives have been reported either as fungicidal or antibacterial compounds or antiviral; for instances, Cinnamic acid, caffeic acid, Catechol, pyogallol, Eugenol, Hypericin, Chrysin, totarol, berberine, capsaicin, benzoic acid, p-hydroxybenzoic acid, p-coumaric acid, vanillic acid, syringic acid, and procatechuic acid (cowen *et al.*, 1999; Jayaprakasha *et al.*, 2003). Recent findings of Fukuta *et al.*, (2007) demonstrated that phytoalexin from rice, momilactones (M_A and M_B) exert antibacterial, antifungal and antioxidant properties.

2.1.2. Diabetes

2.1.2.A. Glucose homeostasis

Blood glucose levels are naturally tightly regulated by the interaction of many organs. Both insulin-dependent (pancreas, liver, muscle) and insulin-independent (brain and nervous system) processes contribute to fasting and postprandial plasma glucose

regulation. Together, these processes endeavor to maintain plasma glucose levels within a narrow range as a part of metabolic homeostasis in normal individuals (Baynes and Dominiczak, 2004). The brain and nervous system are insulin-independent; they autonomously regulate their use of glucose as a metabolic fuel. Glucose transport is mediated by a protein called glucose transporter 1 (GLUT-1) that actively transports glucose across the cell membrane of nervous tissue, irrespective of insulin or glucose levels (Rang *et al.*, 2003). Muscle and adipose tissues use glucose as primary metabolic fuel when the insulin levels are high and decreases postprandial plasma glucose; but when insulin levels are low, both these tissues use free fatty acids/ ketones and reduce the uptake of glucose (Mentreddy, 2007).

The pancreas plays a primary role in the metabolism of glucose by secreting the hormones including insulin and glucagon (Figure 14). When the blood glucose level is increased, the β-cells of the pancreas secrete insulin directly into the blood and maintain the blood glucose levels by suppressing hepatic production of glucose (glycogenolysis and gluconeogenesis), stimulate hepatic glucose uptake and storage, regulates glucose uptake in muscle and to a lesser extent, adipose tissue. Glucagon is a hormone that opposes the action of insulin. It is secreted when blood glucose level falls. It increases blood glucose concentration partly by breaking down stored glycogen in the liver by a pathway known as glycogenolysis (Baynes and Dominiczak, 2004).

The gastrointestinal tract also participates in glucose homeostasis by permitting glucose entry to the body during digestion. This process occurs episodically, delivering large amounts of glucose into the portal vein with meals. In patients with insulin resistance or impaired glucose tolerance, gastrointestinal absorption of glucose can stress already compromised glucose regulatory systems by overwhelming the other

organ's abilities to dispose of elevated postprandial glucose. This results in postprandial hyperglycemia and it is the major risk factor for the development of type II diabetes (Perfetti *et al.*, 1999)

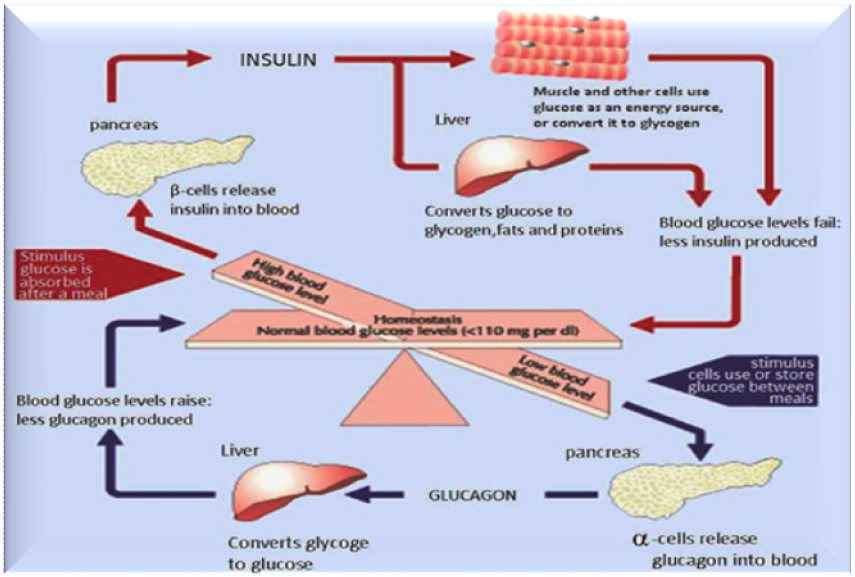


Figure 14: Glucose homeostasis

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2.1.2.B. Diabetes mellitus

Diabetes mellitus is a metabolic disorder initially characterized by a loss of glucose homeostasis resulting from defects in insulin secretion, insulin action or both (Barcelo and Rajpathak, 2001). Without enough insulin, the cells of the body cannot absorb sufficient glucose from the blood; hence blood glucose levels increase, which is termed as hyperglycemia. If the glucose level in the blood remains high over a long period of time, this can result in longterm damage to organs, such as the kidneys, liver, eyes, nerves, heart and blood vessels. Complications in some of these organs can lead to death (Pari and Saravanan, 2004). The two most common types of diabetes are type I and type II diabetes mellitus

2.1.2.B.1. Type I diabetes mellitus

Autoimmune destruction of the insulin secreting β-cells of the pancreas results in an absolute deficiency of insulin leading to type I diabetes mellitus (T1DM). It occurs in children, although disease onset can occur at any age. Patients with T1DM must rely on insulin medication for survival. It may account for 5 -10 % of all diagnosed cases of diabetes. Autoimmune, genetic and environmental factors are the major risk factors for type I diabetes (Abebe *et al*., 2003; Mentreddy, 2007).

2.1.2.B.2. Type II diabetes mellitus

Insulin resistance (decreased ability of insulin to stimulate glucose uptake in peripheral tissues) and pancreatic β-cell failure (inability of the β-cell to secrete insulin adequately) are the two key features in the pathogenesis of type II diabetes mellitus (T2DM). The major sites of insulin resistance in T2DM are the liver, skeletal muscle and adipose tissue (Ostenson, 2001; White *et al*., 2003). Both defects, insulin resistance and β-cell failure, are caused by a combination of genetic and environmental factors. Environmental factors such as lifestyle habits (*i.e.*, physical inactivity and poor dietary intake), obesity and toxins may act as initiating factors or progression factors for T2DM. Type II diabetes is increasingly being diagnosed at any age nowadays and it accounts for 90-95% of all diagnosed cases of diabetes. (Holt, 2004; Mentreddy, 2007).

2.1.2.C. Management of diabetes mellitus

Diet, exercise, modern drugs including insulin and oral administration of hypoglycaemic drugs such as sulfonylureas and biguanides manage the pathogenesis of diabetes mellitus. In individuals with T2DM, a common sequence of therapy starts with diet treatment and exercise followed by oral antihyperglycemic agents. In general,

insulin therapy has been considered to be the last therapeutical option when diet, exercise and oral antihyperglycemic agent therapies have failed. One of the common complications seen in diabetic patients is postprandial hyperglycemia (PPHG). Currently, there are six types of oral hypoglycemic drugs (OHG) against T2DM. Type 1 OHG will stimulate β-cells of the pancreas to secrete insulin (eg. insulin secretagogue, chlorpropamide). Type 2 OHG will block the glucose release from the liver (eg. Metformin). Type 3 OHG will inhibit the free fatty acid release from the adipose tissue (eg. Thiazolidinedione). Type 4 OHG will increase glucose absorption by the muscle tissue (eg. Biguanide). Type 5 OHG will inhibit the intestinal lipase activity (eg. orlistat) and Type 6 OHG will inhibit the α-glucosidase activity (eg. Acarbose) (Figure 15).

Although different types of OHG's are available for the treatment of T2DM, there is a growing interest in natural products, due to several side effects associated

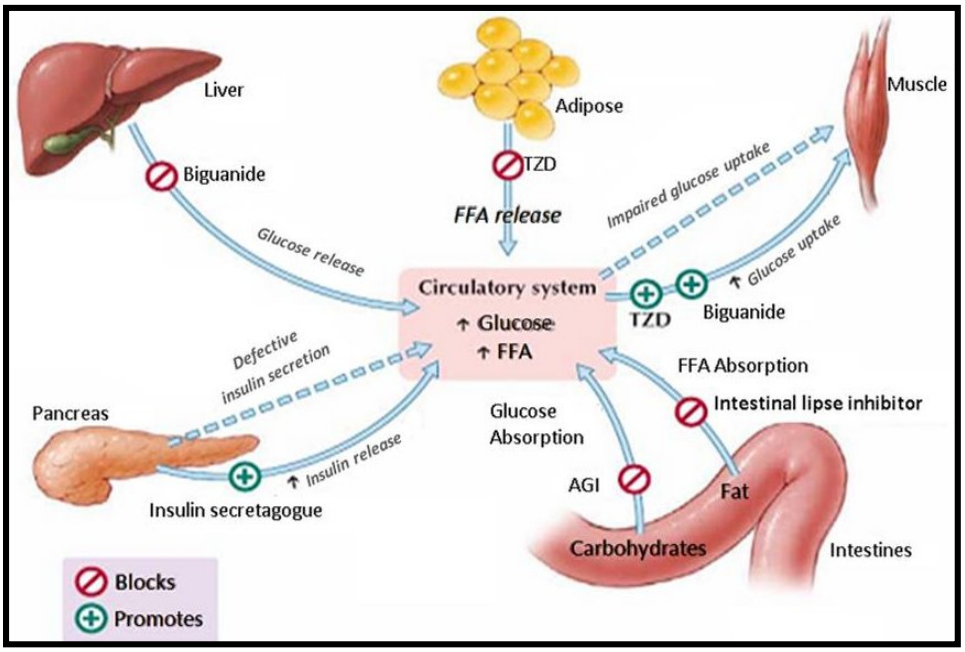


Figure 15: Major target organs and action of orally administered antihyperglycemic agents in type II diabetes mellitus. TZD = thiozolidinedione; FFA = free fatty acid; AGI = α- glucosidase inhibitor (Cheng and Fantus, 2005)

with the above therapeutic agents. Therefore management of diabetes without any side effect is still a challenge for the medical system. This leads to an increasing search for improved antidiabetic drugs.

2.1.2.D. Postprandial hyperglycemia and its complications

Increase in blood glucose levels after the carbohydrate rich diet is termed as postprandial hyperglycemia (PPHG). Starch and sucrose account for 80-90 % of our daily intake of carbohydrates. α-amylases are endoglucanases, which hydrolyze the internal α-1,4 glucosidic linkages of starch and converts starch to oligosaccharides and disaccharides. α-Glucosidase which is located in the brush border surface membrane of intestinal cells converts oligosaccharides and disaccharides to monosacharides and is a key enzyme for carbohydrate digestion (koga *et al.*, 2006). These enzymes have been recognized as one of the therapeutic targets for modulation of PPHG. Postprandial blood glucose levels may be elevated in the presence of normal levels of fasting plasma glucose, constituting an early stage in T2DM, referred as 'postprandial diabetes' (Baron, 1998). This state, not only initiates the development of micro- and macro-vascular complications, but can also contribute to a more rapid progression to symptomatic diabetes by causing glucose toxicity in muscle and pancreatic cells (Mentreddy, 2007). Early identification of PPHG and its effective control will aid in early intervention and prevention of diabetic complications (Ratner, 2001).

2.1.2.E. Medicinal plants as alternatives for diabetic treatment

The incidence of diabetes worldwide affects 230 million people of which 30 million are in India. It has been estimated that by the year 2025, the global incidence of

diabetes would increase to 350 million (Arumugam *et al.*, 2008). World Health Organization (WHO) has recommended the evaluation of traditional plant treatments for diabetes as they are effective and non-toxic with less or no side effects (Bhat *et al.*, 2008). Recently, Mankil *et al.*, (2006) have reviewed many medicinal plants possessing experimental and clinical antidiabetic activity that have been used in traditional systems of medicine. It has been estimated that about 80-85 % of population both in developed and developing countries rely on traditional medicine for their primarily health care needs and it is assumed that a major part of traditional therapy involves the use of plant extracts or their active principles (Kim *et al.*, 2009; Shabeer *et al.*, 2009; Zhang *et al.*, 2010).

Previously, several *in vitro* studies have been performed yielding potential α-glucosidase and amylase inhibitors from various food components and the plant extracts like cranberry (Apostolidis *et al.*, 2006), pepper (Pullela *et al.*, 2006), soy bean extracts, (Georgetti *et al.*, 2006), cheese (Apostolidis *et al.*, 2007), oregano (McCue *et al.*, 2004), fenugreek and balanite (Gad *et al.*, 2006) also showed α-glucosidase and amylase inhibitory nature and reduced PPHG. Therefore, natural α-glucosidase and amylase inhibitors from plant sources offer an attractive strategy for the control of PPHG.

2.1.3. Cancer

Cancer is a class of disease in which a cell, or a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). Cancer affects people at all ages with the risk for most types increasing with age (Vogelstein and Kinzler, 2002). Cancer continues to be a

worldwide killer, despite the enormous amount of research and rapid developments during the past decades. According to the recent statistics, the world population is expected to reach 7.5 billion, by 2020; and approximately 15 million new cancer cases will be diagnosed and 12 million cancer patients will die (Anand *et al.*, 2008). Cancer is primarily an environmental disease with 90-95 % of cases due to lifestyle and environmental factors and 5-10 % due to genetics. Common environmental factors leading to cancer death include: tobacco (25-30 %), diet and obesity (30-35 %), infections (15-20 %), radiation, stress, lack of physical activity, environmental pollutants (Anand *et al.*, 2008).

2.1.3.A. Types of cancer

Cancers are classified by the type of cell that resembles the tumor and therefore, the tissue presumed to be the origin of the tumor. Cancers are grouped into five major categories: carcinoma, sarcoma, myeloma, leukemia and lymphoma. In addition, there are also some cancers of mixed types.

2.1.3.A.I. Carcinoma

Carcinoma refers cancer of epithelial origin or of the internal or external lining of the body. Carcinomas account for 80-90% of all cancer cases.

2.1.3.A.II. Sarcoma

Sarcoma originates in supportive and connective tissues such as bone, tendons, cartilage, muscle and fat. Sarcoma tumors usually resemble the tissue in which they grow.

2.1.3.A.III. Myeloma

Myeloma is cancer that originates in the plasma cells of bone marrow.

2.1.3.A.IV. Leukemia

Leukemias (liquid or blood cancer) are cancers of the bone marrow (the site of blood cell production.

2.1.3.A.V. Lymphoma

Lymphomas develop in the glands or nodes of the lymphatic system, a network of vessels, nodes and organs (specifically the spleen, tonsils and thymus) that purify bodily fluids and produce infection-fighting white blood cells or lymphocytes. Unlike the leukemias lymphomas are solid cancers. Lymphomas may also occur in specific organs such as stomach, breast or brain.

2.1.3.B. Cyclooxygenases and cancer

Cyclooxygenase (COX) is a rate limiting enzyme in the biosynthesis of prostanoids. It has two distinct activities: a cyclooxygenase activity, which catalyzes the formation of prostaglandin G_2 (PGG_2) from arachidonate and peroxidase activity which reduces the hydroperoxide group to PGG_2 to form PGH_2 (Pagels *et al.,* 1983). The two isoforms of COX are COX-1 and COX-2. COX-1 is constitutively expressed in all mammalian tissues and maintains homeostatic functions where as COX-2 is expressed in most tissues and cells at very low levels unless induced by mitogenic or hormonal stimuli. Therefore the inhibition of COX-1 is undesirable whereas the inhibition of COX-2 is desirable (Kubuju *et al.,* 1991). Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in variety of cancer cell lines including intestine, prostate and breast (Cai *et al.,* 2005; Sarkar *et al.,* 2007). High level expression of COX-2 enzyme was seen in proliferation and differentiation of various cancer cell lines and different tumor types (Sheng *et al.,* 2001).

2.1.3.C. Diet and cancer control

Dietary patterns, foods, nutrients and other dietary constituents are closely associated with the risk for several types of cancer. All most all cancers (80-90 %) are caused due to environmental factors and of this 30-40 % of cancers are directly linked to the diet. Epidemiological data suggest that there is a significant relation between lifestyle (including food consumption) and cancer prevention (Sommakerkamp and Khauli 2005; Manson *et al.,* 2007; Steck *et al.,* 2007; Nishino 2009). Nutrients (antioxidants) in our food have been shown to minimize the mutations which causes cancer by neutralizing the free radicals generated during adverse conditions (Slavin, 2000; Nishino, 2009). Several studies have found that when isolating the nutrients by extracting it from the food source, no benefits regarding cancer prevention were found. However, when increased amounts of the whole foods were used, cancer occurrences were limited (Abdulla and Gruber 2000; Slavin, 2000).

Some studies reported that reduced meat consumption is associated with decreased risk of colon cancer (Slattery *et al.,* 1998), consumption of grilled meat causes an increased risk of stomach cancer (Larsson and Wolk, 2007), colon cancer (Sinha *et al.,* 2005), breast cancer (Steck *et al.,* 2007) and intake of diets cooked at high temperatures leads to the development of pancreatic cancer due to the presence of carcinogen benzopyrene (Anderson *et al.,* 2005). Diet rich in high fiber are known to reduced the risk of developing colon cancer, A few studies have also shown a reduced risk of the breast, bowel, prostate, liver, skin and stomach cancer with diets rich in fruits, vegetables, whole grains and their products (Go *et al.,* 2001 & 2004; Kowalczyk *et al.,* 2010). Many reviews and scientific data supports that the phytochemicals

present in the whole grains are one of the major constituents responsible for their positive health benefits against these diseases.

2.1.4. Objective

In our previous chapter we have shown that Njavara rice bran methanolic extract was superior in terms of phytochemical content and free radical scavenging activity, compared to the other three varieties. Therefore our main objective of this study was to evaluate the antimicrobial, antidiabetic and antiproliferatory properties of Njavara rice bran methanolic extract.

2.2. Materials and methods

2.2.1. Chemicals

yeast α-glucosidase, p-nitrophenyl α-glucopyranoside (pNPG), porcine pancreatic α-amylase, dinitrosalisylic acid (DNS), starch, dimethylsulfoxide (DMSO), alloxan monohydrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N,N,N,N-tetra methyl-p-phenylene diamine (TMPD), hematin, EDTA, arachidonic acid were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). All the chemicals used including the solvents, were of analytical grade.

2.2.2. Antimicrobial property of Njavara rice bran methanolic extract (NBM)

2.2.2.A. Sample preparation

Polished rice & bran methanolic extracts were prepared from four rice varieities (Vasumathi, Yamini, Jyothi & Njavara) as described earlier (chapter 1, section 1.2.4)

2.2.2.B. Microbial strains used for the study

Gram negative bacteria: *Escherichia coli (ATCC 25922)*, *Salmonella typhi (CI)*, *Pseudomonas aeruginosa (ATCC 27853)*

Gram positive bacteria: *Staphylococcus aureus (CI)*, *Bacillus subtilis (QST713)*

Fungi: *Candida albicans (3153A)*

2.2.2.C. Test assays for antimicrobial activity

The screening of antimicrobial activities against Gram^{+ve} and Gram $^{-ve}$ bacteria and fungi were achieved by an adaptation of the agar streak dilution method based on radial diffusion (Sousa *et al.*, 2006). Suspensions of the microorganism were prepared to contain approximately 10^8 cfu/mL, and the plates containing agar medium were inoculated (100 µL; spread on the surface). Different concentrations of sample was placed in a hole (3 mm depth, 4 mm diameter) made in the centre of the agar. The MIC was considered to be the lowest concentration of the tested sample to inhibit the growth of microbe after 24 h. The diameters of the inhibition zones were measured using a ruler, with an accuracy of 0.5 mm. Each inhibition zone diameter was measured three times (three different plates) and the average was considered.

2.2.3. Antidiabetic property of NBM

2.2.3.A. Rat intestinal α-glucosidase inhibition assay

Rat intestinal α-glucosidase assay as described by Kwon *et al.*, (2008) was used with slight modifications. One gram of rat-intestinal acetone powder was suspended in

3 ml of 0.9 % saline and the suspension was sonicated twelve times for 30 s at 4 ºC. After centrifugation (10000g, 30 min, 4 ºC), the resulting supernatant was used for the assay. Various concentrations of NBM extract in 50 µl solution were pre-incubated with 100 µl of rat-intestinal α-glucosidase (0.02 U) solution at 37 ºC for 30 min. After pre-incubation, 50 µl of 5 mM p-nitrophenyla-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added. The reaction mixtures were incubated at 37 ºC for 15 min. Absorbance readings were recorded at 405 nm and compared to a control which had 50 µl of solvent in place of the sample solution. The rat α-glucosidase inhibitory activity was expressed as inhibition (%) which was calculated as follows:

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

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample.

2.2.3.B. Porcine pancreatic α-amylase inhibition assay

Porcine pancreatic α-amylase inhibition was followed according to the method of Kwon *et al.,* (2008). Various concentrations of NBM extract in 200 µl solution were pre-incubated with 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.33 U) at 25 ºC for 30 min. After pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer were added. The reaction mixture was then incubated at 25 ºC for 10 min. The reaction was stopped with 1.0 ml of dinitrosalicylic (DNS) acid, colour reagent. The reaction mixture was then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml of water and absorbance was measured at 540 nm.

2.2.3.C. Oral glucose tolerance in normal rats

The oral glucose tolerance test was performed on overnight fasting (16 h) normal rats. Distilled water, aqueous suspension of NBM extract at three doses (100, 300 and 500 mg/kg) or acarbaose (2 mg/kg) were administered to four different groups of rats. Glucose (2g/kg) was fed 90 min after treatment. Blood was taken from the tail at 0, 30, 60 and 120 min from control and experimental animals and the blood glucose levels were determined by using glucometer.

2.2.3.D. Induction of diabetes in rats

Wistar male rats weighing 125–165 g were used for the study. The animals were housed under standard conditions (25 °C, 12 h light and 12 h dark cycle) and fed with rodent diet and water *ad libitum*. Diabetes was induced in rats by a single intraperitoneal injection of alloxan monohydrate (120 mg/kg) after overnight fasting for 12 h. Alloxan was first weighed individually for each animal according to their weight and then solublized with 0.5 ml sodium citrate just prior to injection. Food and water were given to the animals 30 minutes after drug administration. Two days after alloxan injection, plasma blood glucose levels of each animal was determined using glucometer. The rats with effective and permanent elevated plasma glucose levels (3.10 –3.66 g/l) were selected for experimental study.

2.2.3.E. Experimental design

The rats were divided randomly into 5 groups with five animals in each group. Group 1 served as control (took only the vehicle), Groups 2, 3 and 4 were treated with

1 ml aqueous suspension of NBM extract (100, 300 & 500 mg/kg respectively) using a gavage daily for 2 weeks and Group 5 was given standard drug (acarbose, 2 mg/kg per day orally).

2.2.3.F. Antihyperglycemic effect of NBM extract on alloxan induced diabetic rats

Distilled water, aqueous suspension of NBM extract at three doses (100, 300 and 500 mg/kg) or acarbaose (2 mg/kg) were administered to the rats for 14 days. Blood samples were drawn at weekly intervals till the end of the study, 2 weeks. Fasting blood glucose levels were recorded on day 1, 7 and 14 during the experimental period using glucometer.

2.2.3.G. Postprandial antihyperglycemic effect of NBM in diabetic rats

After two weeks of treatment with the plant extracts and acarbose, the animals were made to fast for 12-14 hours and the glucose solution (2 g/kg body weight) was administered orally in a volume of 1 ml. Blood samples were collected at 30, 60 and 120 minutes after administration of glucose in order to evaluate their blood glucose levels (Kumar *et al*., 2006) using glucometer.

2.2.4. Antiproliferatory property of NBM

2.2.4.A. cell lines and cell culture

U373 and C6 glioma cell lines were cultured in RPMI media, supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5 % CO_2 at 37° C.

2.2.4.B. MTT assay

Cytotoxicity of NBM on glioma cell lines was measured by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. U373 and C6 cells were plated ($1x10^4$ cells/well) into 96 well plates. After overnight incubation, the cells were treated with varying concentrations of NBM (25 µg, 50 µg, 75 µg and 100 µg) or vehicle (0.1 % DMSO) alone for 24 h. Then MTT reagent was added to each well and incubated for 4 h at 37° C. The reduced formazan crystals were solubilized in acidic iso-propanol and absorbance was then measured at 540 nm on micro plate ELISA reader. All treatments were performed in triplicate and results expressed as mean ± S.D.

2.2.4.C. Cell viability and clonogenic assays

Effect of NBM on cell proliferation was assessed by trypan blue exclusion assay. Briefly, U373 and C6 cells were seeded ($1 x10^5$/well) in 6-well plates and treated with varying concentrations of NBM or vehicle (0.1 % DMSO) for 24 h, 48h and 72 h. Then, number of trypan blue-excluding viable cells was counted and expressed as % of control. Clonogenic assay was performed by plating 500 U373 and C6 cells in 100-mm culture dishes and treated with different concentrations of NBM or vehicle (0.1 % DMSO) for 24 h. The cells were allowed to grow for 7 days and stained with 0.5 % methylene blue in 50 % methanol. Colonies that contain more than 50 cells were counted (Du *et al.*, 2004). All treatments were performed in triplicate and results were expressed as mean ± S.D.

2.2.4.D. Cyclooxygenase (COX-1 and COX-2) assay

Enzymatic activities of COX-1 and COX-2 was measured according to the method of Copeland *et al.,* (1994) with slight modifications using a chromogenic assay based on the oxidation of N,N,N,N-tetra methyl-p-phenylene diamine (TMPD) during the reduction of PGG_2 to PGH_2. The assay mixture contains 100 mM Tris-HCl buffer (pH 8.0), 15 µM hematin, 3 µM EDTA, 100 µg COX-1 or COX-2 enzyme and various concentrations of NBM extract. The mixture was pre-incubated at 25 °C for 15 min and then the reaction was initiated by addition of arachidonic acid, TMPD, in a total volume of 1 ml. The enzyme activity was determined by estimating the velocity of TMPD oxidation for the first 25 seconds of the reaction by following the increase in absorbance at 603 nm. A low rate of non-enzymatic oxidation observed in the absence of COX-1 and COX-2 was substracted from the experimental value while calculating the percent inhibition.

2.3. Results

2.3.1. Antimicrobial activity of rice bran

Rice bran methanolic extracts from four rice varieties were screened for their antimicrobial properties against gram negative bacteria (*Escherichia coli, Salmonella typhi and Pseudomonas aeruginosa*), gram positive bacteria (*Staphylococcus aureus, Bacillus subtilis*) and fungi (*Candida albicans*). Results obtained in the present study revealed that the tested samples from all the four rice varieties showed antimicrobial activity against all the tested microbes. All the rice samples showed MIC values, less than 25 µg/ml against all the tested microbes (Figure 16).

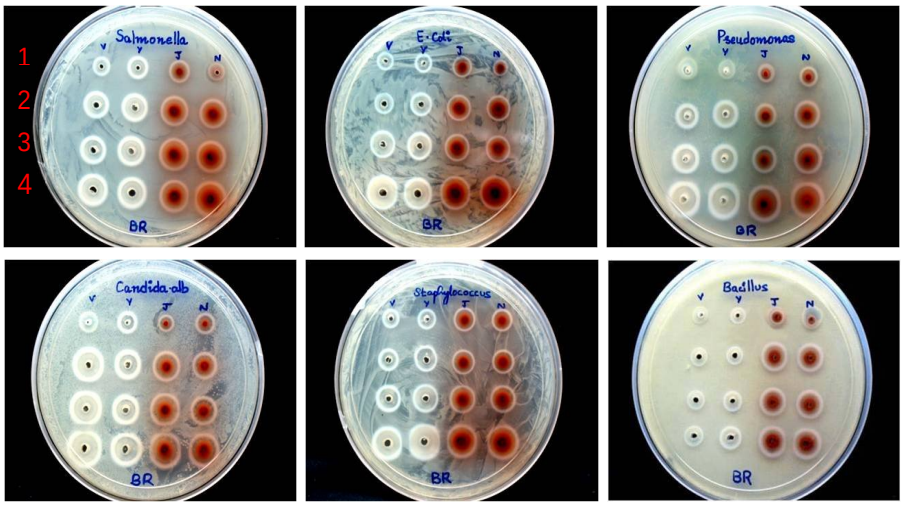


Figure 16: Antimicrobial activity of bran samples from the four rice varieties. Numbers 1, 2, 3 and 4 indicates the concentrations of 25, 50, 75 and 100 µg/ml respectively. V = Vasumathi, Y= Yamini, J = Jyothi, N= Njavara.

2.3.2. Antimicrobial property of polished rice

Among the four rice varieties used for the antimicrobial property of methanolic extracts from polished rice flour, only Njavara variety showed stronger antimicrobial activities against all the tested microbes with maximum zone of inhibitions compared to

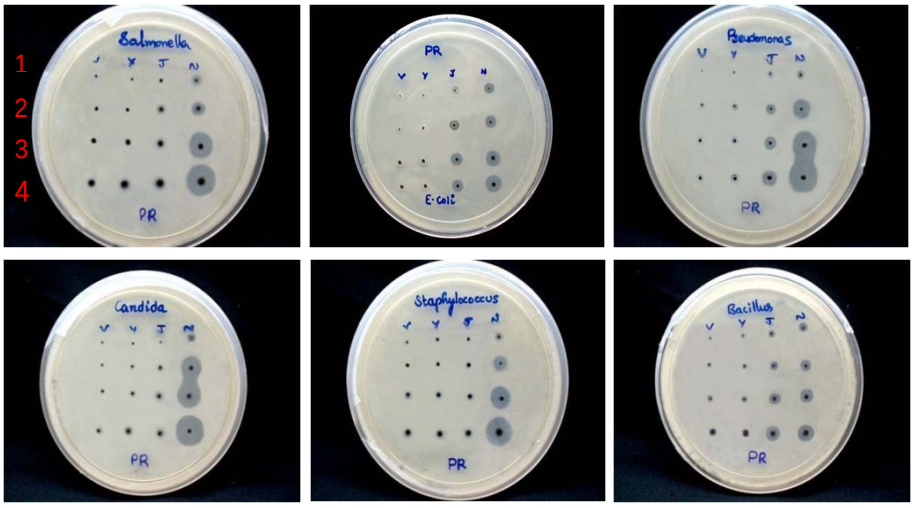


Figure 17: **Antimicrobial activity of polished rice samples** from the four rice varieties. Numbers 1, 2, 3 and 4 indicates the concentrations of 25, 50, 75 and 100 µg/ml respectively. V = Vasumathi, Y= Yamini, J = Jyothi, N= Njavara.

the other three rice samples (Table 6). Jyothi variety also showed significant antimicrobial activity against all the tested microbes. Vasumathi and Yamini didn't showed any significant antimicrobial activity (Figure 17).

Table 6: **Zone of inhibition of polished rice (PR) and bran (BR)** samples from the four rice varieties. The diameter of zone of inhibition as expressed in millimeters (mm)

Microbial Strain	Vasumathi		Yamini		Jyothi		Njavara	
	PR	BR	PR	BR	PR	BR	PR	BR
Candida albicans	----	12.2	----	12.1	6.0	13.1	14.0	12.4
Staphylococcus areus	----	11.1	----	13.2	6.5	12.2	10.1	12.8
Bacillus subtillis	----	09.5	5.5	09.0	7.3	11.9	07.4	12.2
Salmonella typhi	----	13.2	----	12.9	7.0	13.2	11.2	14.4
Escherechia coli	----	13.1	6.7	12.5	5.3	14.0	08.2	14.5
Pseudomonas areugenosa	----	13.5	----	13.2	6.6	13.7	10.8	14.1

Data were mean ± SD of three independent experiment (n=3)

2.3.3. *In vitro* α-glucosidase and amylase inhibitory study

The *in vitro* α-glucosidase and amylase inhibitory studies demonstrated that phenolic-rich NBM extract had α-glucosidase and amylase inhibitory activity. The percentage inhibition of both the enzymes at 25, 50, 75 and 100 µg /ml concentrations

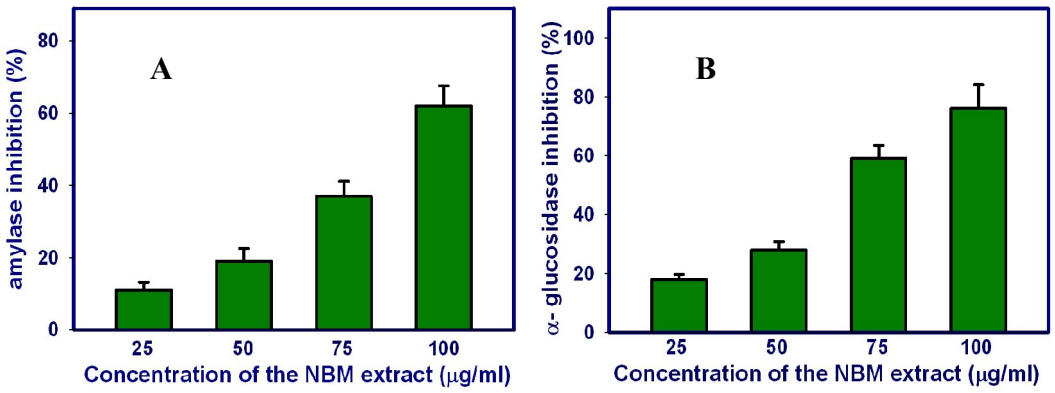


Figure 18: Effect of NBM on amylase and α-glucosidase. Inhibitory effect of different levels of NBM extract phenolics on (A) pancreatic amylase (0.33 units, 30 minutes incubation), (B) α-glucosidase (0.02 units, 30 minutes). Data were mean ± SD of three independent experiment (n=3)

of NBM extract showed a concentration-dependent reduction in percentage inhibition (Figure 18). IC_{50} values of NBM extract for α-glucosidase inhibition and amylase inhibition were 48 and 85 µg/ml respectively. Acarbose, a well known standard drug for postprandial hyperglycemic patients showed inhibitory activity on α-glucosidase and amylase with an IC_{50} value of 10 and 13 µg/ml respectively.

2.3.4. Effect of NBM on oral glucose tolerance test in normoglycemic rats

Overnight fasting normoglycemic rats were orally administered with glucose (2 g/kg). A significant hyperglycaemia was observed during 120 min. Pretreatment with NBM extract significantly prevented glucose-induced hyperglycaemia in a dose dependent manner (Figure 19). NBM, at a dosage of 500 mg/kg body weight of rat, showed the blood glucose levels similar to the control normoglycemic rats.

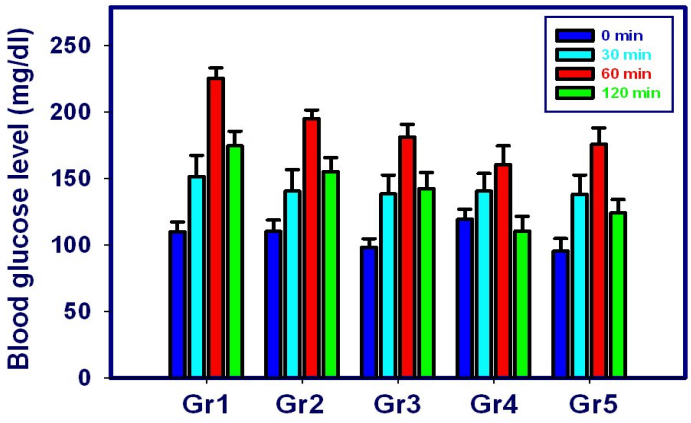


Figure 19: Oral glucose tolerance test in normal rats. Effect of NBM extract on blood glucose levels upon glucose load. Group (Gr) 1 (control), Gr 2 Gr 3 and Gr 4 are NBM treated with 100, 300 and 500 mg/kg/bw and Gr 5 acarbose (2 mg/kg/bw) treated rats. Data were mean ± SD of three independent experiment (n=3).

2.3.5. Antihyperglycemic effect of NBM on alloxan induced diabetic rats

The daily administration of vehicle (1 ml/kg bw) did not significantly change the blood glucose level in alloxanic rats. Oral administration of NBM extract (100, 300,

500 mg/kg/day) during 14 days induced an antihyperglycaemic effect in diabetic rats. The maximum antihyperglycemic effect was seen in 500 mg/kg/day NBM treated rats which was comparable to the standard drug acarbose (Figure 20).

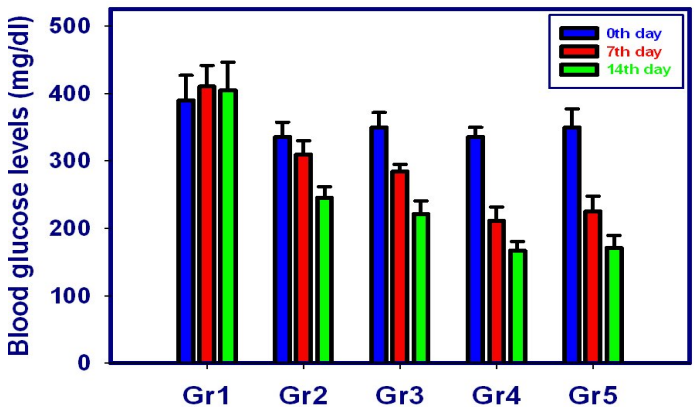


Figure 20: Antihyperglycemic effect of NBM. Diabetic rats were treated for 14 days with different concentrations of NBM extract and the blood glucose levels were determined on 0^{th}, 7^{th} and 14^{th} day. Group (Gr) 1 (control), Gr 2, Gr 3 and Gr 4 are NBM treated with 100, 300 and 500 mg/kg/bw and Gr 5 acarbose (2 mg/kg/bw) treated rats. Data were mean ± SD of three independent experiment (n=3).

2.3.6. Postprandial antihyperglycemic effect of NBM on diabetic rats

Glucose (2 g/kg) was orally administered to the diabetic rats which were pretreated for 14 days with different concentrations of NBM extract and the blood glucose levels were measured at 0, 30, 60, 120 min. Blood glucose levels increased from 380-560 mg/dl in group 1 rats. In group 2 and 3 blood glucose levels increased upto 60 min and then decreased by 120 min (Figure 21). In group 4 and 5 blood glucose levels increased upto 30 min and then decreased. Group 4 rats showed blood glucose levels 140 mg/dl at 120 min, which is almost similar to the blood glucose levels before glucose load (Figure 21).

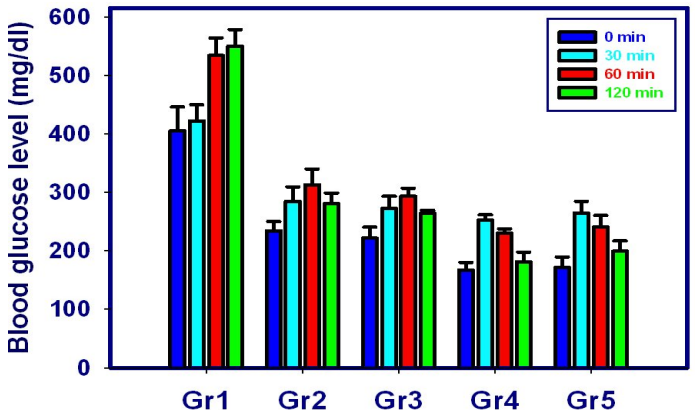


Figure 21: Oral glucose tolerance in diabetic rats. 14 days NBM treated diabetic rats were challenged with glucose load and the blood glucose levels were determined upto 120 min. Group (Gr) 1 (control), Gr 2, Gr 3 and Gr 4 are NBM treated with 100, 300 and 500 mg/kg/bw and group 5 acarbose (2 mg/kg/bw) treated rats. Data were mean ± SD of three independent experiment (n=3)

2.3.7. NBM induced cytotoxicity, reduced proliferation and clonogenic efficiency of U373 and C6 glioma cell lines

To assess the effect of NBM on proliferation of glioma cell lines, a series of experiments were conducted. First, U373 and C6 cells were treated with the indicated concentrations of NBM for 24 h and subjected to MTT assay to determine the cytotoxic effect of NBM. As shown in Figure 22 (A), NBM treatment significantly

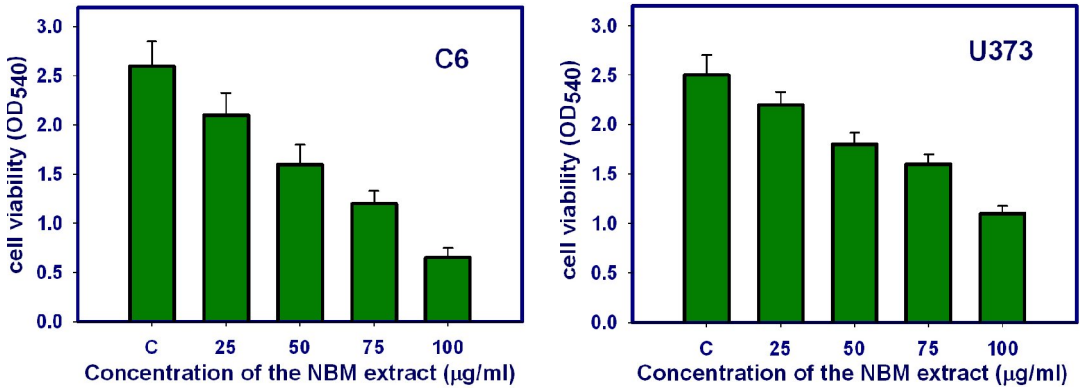


Figure 22 (A): Effect of NBM on cytotoxicity. C6 and U373 glioma cells were treated with vehicle (0.1 % DMSO) or indicated concentrations of NBM for 24 h and subjected to MTT assay. Dose dependent growth inhibition was observed in the tested cell lines. Data were mean ± SD of three independent experiment (n=3)

reduced the cell viability in a dose dependent manner. Further, to determine the effect of NBM on proliferation, U373 and C6 cells were treated with indicated concentrations

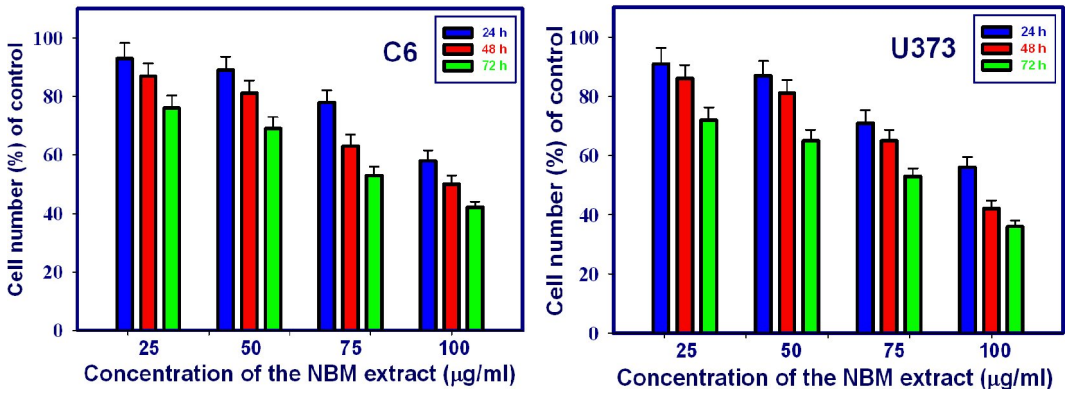


Figure 22 (B): Effect of NBM on cell viability. U373 and C6 glioma cells were seeded in 6 well plates and treated with vehicle (0.1 % DMSO) or indicated concentrations of NBM for 24 h, 48 h and 72 h and total viable cell numbers were counted. Data were mean ± SD of three independent experiment (n=3)

of NBM for 24 h, 48 h, and 72 h and number of trypan-blue excluding viable cells were counted at each time period. As shown in Figure 22 (B), the proliferation rates of U373

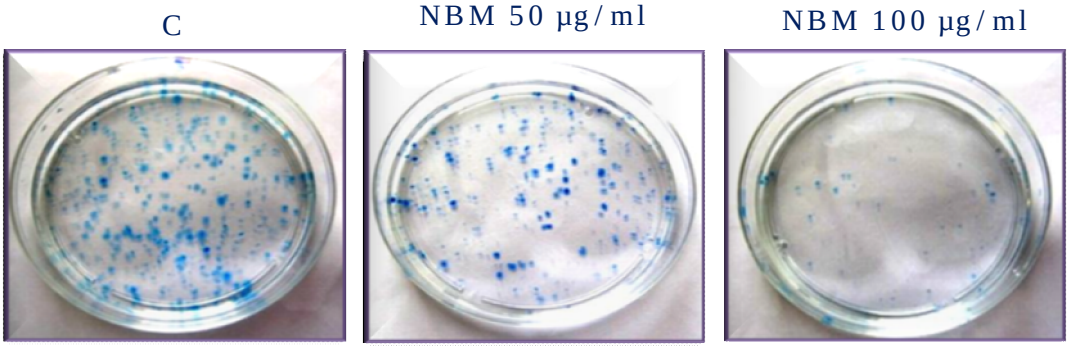


Figure 22 (C): Effect of NBM on cloning efficiency. 500 C6 cells were plated in 100-mm culture dishes and after 24 h cells were treated with vehicle (0.1 % DMSO) or indicated concentrations of NBM for 24 h. After 7 days, colonies were stained with methylene blue and colonies that contain ≥ 50 cells were counted. 'C' denotes the vehicle treated control cells.

and C6 cells were significantly reduced in a dose and time dependent manner following NBM treatment. To further characterize the effect of NBM on colony forming ability, U373 and C6 cells were subjected to clonogenic assays. As shown in Figure 22 (C), NBM treatments greatly reduced the clonogenic efficiency of C6 cells in a dose dependent manner compared to vehicle treated which attained about 80 % cloning efficiency. Overall, these experiments demonstrated that NBM significantly reduced the growth of U373 and C6 cell lines and causes cytotoxicity.

2.3.8. Inhibition of COX activity by NBM

The effect of NBM on COX-1 and COX-2 activity; *in vitro,* was measured by TMPD assay. The IC_{50} values obtained for COX-1 and COX-2 enzymes were 92 and 39 µg/ml respectively. From this data, it was clearly evident that COX-2 is more inhibited than COX-1 by NBM (Figure 23).

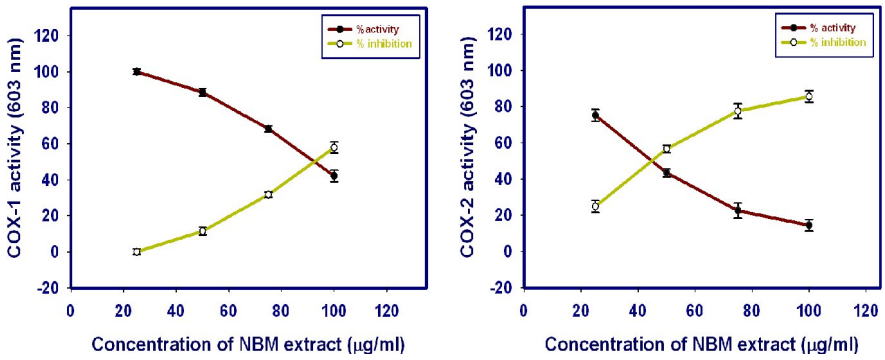


Figure 23: Effect of NBM on COX-1 and COX-2 activities. NBM preferentially inhibited COX-2. The IC_{50} values for COX-1 and COX-2 are 92 and 39 µg/ml respectively. Data are mean ±SEM of three independent experiments (N=3)

2.4. Discussion

Despite dietary recommendations to increase whole grain intake, little research has been conducted on the physiological effects of diets high in whole grains. Compared to refined grains, whole grains are much superior in bioactive principles including simple phenols, flavonoids, anthocyanins, alkaloids, tannins apart from high minerals, vitamins, good protein, lipid and insoluble fiber (Slavin, 2000; Russo *et al.*, 2010). Scientific data suggests that whole grain consumption decreases the incidences of life style-related disorders such as type II diabetes and cancer. Our previous study showed that bran from rice contains more phytochemicals than brown rice or polished rice. These bioactive principles are highly correlated to the antiradical scavenging activity. Available literature suggests that plant extracts with high radical scavenging activity are beneficial for the treatment of oxidative stress related disorders. Hence we checked the antiproliferatory, antidiabetic and antimicrobial properties of phytochemical rich Njavara rice bran methanolic extract (NBM).

The antimicrobial property of polished rice and bran from four rice varieties were examined. The results of antimicrobial activity were shown in Table 6, Figures 16 and 17. Bran samples from all the four rice varieties exhibited prominent antimicrobial activity against all the microbes used for the study whereas only polished rice samples from pigmented varieties (Njavara and Jyothi) exhibited significant antimicrobial activity against all the microbes tested. This difference in the antimicrobial activity of different extracts of tested samples could be due to the phytochemical variation in components among them. The superior antimicrobial activity, in comparision with other three rice varieties, shown by Njavara samples could be due to its high phytochemical content and radical scavenging property. Medicinal plants with high radical scavenging

and antimicrobial properties are being used as food preservatives (Al-Bakri and Afifi, 2007). Therefore, Njavara rice bran phyotochemicals can also be exploited as food preservatives for increased safety and quality of shelf life.

Since NBM extract is superior in terms of phytochemical content and free radical scavenging activity, further antidiabetic and antiproliferatory studies were carried out only with NBM. Postprandial hyperglycemia could induce the non-enzymatic glycosyation of various proteins, resulting in the development of chronic complications (Pari and Saravaran, 2004). Therefore control of postprandial plasma glucose levels is critical in the early treatment of diabetes mellitus and in reducing chronic vascular complications. Inhibition of enzymes involved in the metabolism of carbohydrates including α-glucosidase and amylase is one of the therapeutic approaches for reducing postprandial hyperglycemia. Our *in vitro* studies demonstrated an appreciable α-glucosidase and amylase inhibitory activity of NBM with IC_{50} values 48 and 85 µg/ml respectively. The *in vitro* inhibitory activity may not always correlate with the *in vivo* studies (Ye *et al.*, 2002). So, it is necessary to confirm the *in vivo* action after oral administration to live animals, which is an important step in screening plant extracts for physiological and pharmacological effects.

In the *in vivo* experiments, aqueous dispersions of NBM at the doses 100, 300, and 500 mg/kg showed marked decrease in plasma blood glucose (PBG) levels in a dose dependent manner after the glucose load to the normoglycemic rats as well as diabetic rats (Figures 19 and 21). The tendency of the NBM extract to suppress the PBG demonstrates inhibitory activity against carbohydrate digestive enzymes and preventing the rise in blood glucose levels.

Plant phenolic compounds modulate the enzymatic breakdown of carbohydrates by inhibiting amylases and glucosidases (McDougall *et al.*, 2005). Whole grain cereal diets have been recommended for diabetics and epidemiological reports indicate a lower incidence of diabetes amongst whole grain consumers (Venn and Mann, 2004). However, the mechanisms of action were not clearly reported. The results of this investigation suggest that, the phenolic compounds present in the NBM extract may regulate the glucose uptake from the intestinal lumen by inhibiting carbohydrate digestion and absorption, leading to normal glucose homeostasis. It is also possible that, phenolic compounds may regulate the glucose absorption by mechanisms other than enzyme inhibition.

Different mechanisms of action to reduce blood glucose levels with the help of plant extracts already exist. Some plants exhibit properties similar to the well-known sulfonylurea drugs like glibenclamide; they reduce blood glucose in normoglycaemic animals. Some other plants act like biguanides such as metformin which is an antihyperglycaemic compound; they do not affect blood glucose in normal state. We hypothesized that NBM extract could have a biguanides-like mechanism since it decreased blood glucose only in hyperglycaemic rats. It is also known that alloxan selectively destroys insulin-secreting β-cells in the islets of Langerhans and their effects are irreversible. In the present study, the dose of alloxan (120mg/kg, bw) was selected in order to partially destroy the pancreatic β-cells. Under these conditions, insulin was secreted but not sufficiently to regulate the blood glucose. NBM extract was able to decrease the glycaemia in alloxan permanent hyperglycaemic rats (Figure 20). This suggests that the increase of blood glucose obtained with alloxan results from a diminution but not due to the total abolition of insulin secretion. Overall NBM extract

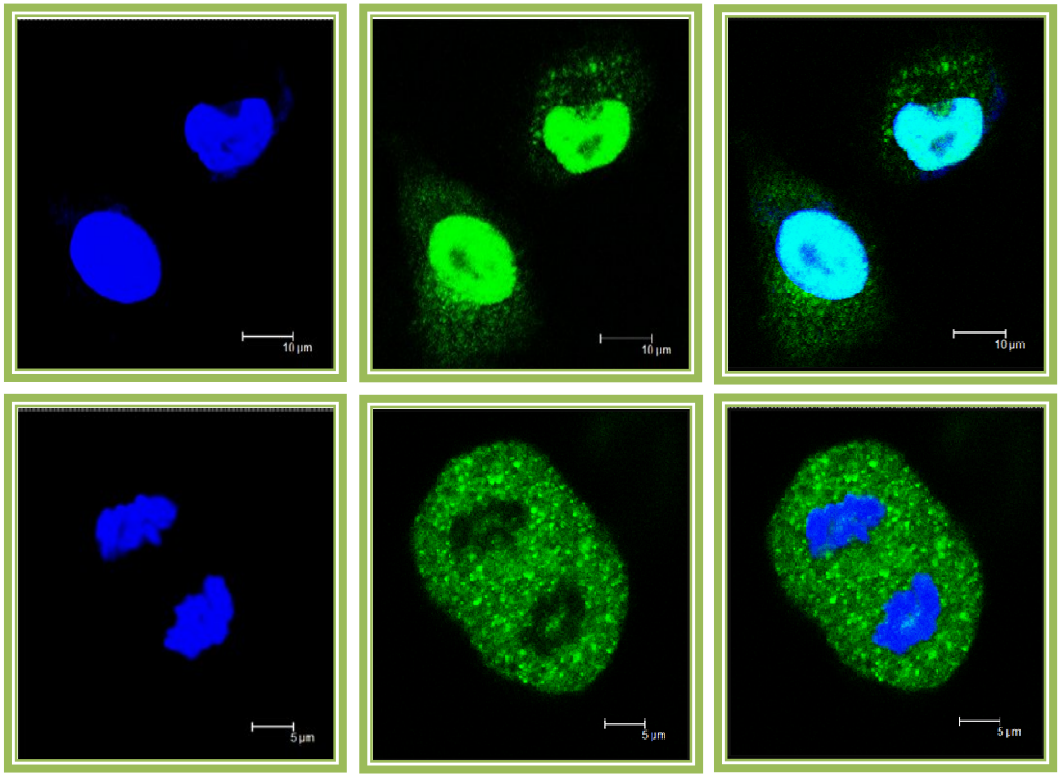
showed inhibitory activity against carbohydrate digestive enzymes and decreased the postprandial hyperglycemia both in normal and diabetic rats. NBM extract also showed antihyperglycemic effect on alloxan induced diabetic rats.

The effect of NBM on cell proliferation was also studied in this investigation. In this study first, we examined whether NBM has significant anti-proliferative effect on glioma cell lines. MTT assay, cell viability and clonogenic assays revealed that NBM extract had significant anti-proliferative effects on U373 and C6 glioma cell lines. NBM also showed the *in vitro* inhibitory property against COX-1 and COX-2 with IC_{50} values 92 and 39 µg/ml respectively (Figure 23) which suggests that NBM showed preference towards COX-2 inhibition rather than COX-1.

In summary, our studies have clearly demonstrated the pharmacological importance of Njavara rice bran. NBM is a potent radical scavenger with significant antimicrobial activity, protective mechanism for type II diabetes and also showed antiproliferative property against glioma cell lines.

3

Chapter



Antiproliferative and proapoptotic effects of Njavara rice bran methanolic extract on glioma cells

3.1. Introduction

Cancer is a major cause of human suffering, with more than 10 million new diagnosis of cancer and more than 6 million cancer related deaths occur annually. Researchers worldwide have been working on developing drugs for cancer treatments, particularly plant-derived anticancer compounds (Adeolu *et al.,* 2009). Despite remarkable advances in the therapeutic strategies, treatment towards malignant gliomas is still a debate. Gliomas are the common form of primary brain tumors and are likely to arise from the transformation of astrocytes or their precursors (Singh *et al.,* 2004). According to WHO classification, gliomas are divided into four clinical grades based on their prognosis and histology. Among these, GBM is highly invasive and recurrent in nature and patients bearing these tumors exhibit bleak prognosis and having life expectancy of about one year. GBMs are likely to recur even after optimal treatment with surgery, radiation, and chemotherapy (Simmons *et al.,* 2001; Song and Moon, 2006; Mason *et al.,* 2007; Stojic *et al.,* 2008; Kevin *et al.,* 2009). The persistent resistant nature of GBM to standard therapies signifies a major clinical impediment that ruthlessly limits life expectancy and is yet to be poorly understood phenomenon. Many factors support the highly resistant and persistent nature of GBM to therapies such as genetic abnormalities can defend the tumor from conventional therapies, the blood-brain barrier limits the entry of chemotherapeutic agents to tumors, GBM is highly angiogenic and has highly invasive potentials (Giese *et al.,* 1996; Iwamoto *et al.,* 2009; Ahmed *et al.,* 2010; Reithmeier *et al.,* 2010). However the environmental and obscure genetic factors underlying gliomagenesis remain poorly understood.

3.1.1. NF-κB and cancer

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that plays a crucial role in regulating the expression of genes involved in immune responses, apoptosis, and cell cycle. Inaccurate regulation of NF-κB may cause inflammation, autoimmune diseases, viral infection and cancer. Five mammalian NF-κB family members have been identified:

- NF-κB1 (also called p50)
- NF-κB2 (also named p52)
- RelA (also known as p65)
- RelB
- c-Rel

They all share a highly conserved Rel homology domain, responsible for their dimerization and binding to DNA and IκB (inhibitor of NF-κB). The transcription factor NF-κB operates only when two members form a dimer. The most abundant activated form consists of a p50 or p52 subunit and a p65 subunit.

Under normal physiological conditions, NF-κB/Rel dimers are localized in the cytoplasm along with IκB. In response to stimulus, such as TNF-α and LPS, the IκB proteins are phosphorylated by an activated IκB kinase (IKK) complex followed by polyubiquitination and degradation by 26S proteasome releasing free NF-κB dimers. The NF-κB complex is then translocated to nucleus and binds to their target gene promoters and drives their transcription (Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002; Karin *et al.,* 2002; Nishikori, 2005). Previously it was reported that NF-κB is constitutively expressed in GBM and is associated with enhanced growth, cell cycle progression, and inducible chemo resistance (Wang *et al.,* 1999; Ansari *et al.,* 2001;

Nagai *et al.,* 2002; Park *et al.,* 2009). Besides, as NF-κB is preferentially over-expressed in GBM, but not in the normal brain, these factors may represent NF-κB as an attractive therapeutic targets for GBM tumor cells, but not normal brain tissue.

Mitogen activated protein (MAP) kinases are strongly activated in response to stressful stumili and plays a crucial role in regulating cell growth, differentiation and apoptosis (Dijsselbloem *et al.,* 2004; Lee *et al.,* 2007). Recently Rasheed *et al.,* (2009) showed the inhibition of NF-κB and MAP kinases activation in KU812 cells treated with polyphenol rich pomegranate fruit extract. Aqueous and methanolic extracts of Bangladeshi medicinal plant extracts showed cytotoxic property against gastric and breast cancer (Uddin *et al.,* 2010). Annabie *et al.,* (2009) showed COX-2 overexpression in glioma cells and its expression was inhibited by inhibiting NF-κB activity. Gambogic acid, a major ingredient of traditional medicinal plant, Gamboga showed anticancer property against oral cancer (He *et al.,* 2009). Recently we have shown the antiproliferatory property of Njavara rice bran methanolic extract against glioma cells (Rao et *al.,* 2010).

3.1.2. Objective

The aim of the present study was to investigate the antiproliferative and proapoptotic effects of Njavara rice bran methanolic extract (NBM) on glioma cells and the underlying signaling pathway involved in regulating proliferation and apoptosis.

3.2. Materials and methods

3.2.1. Plant material and chemicals

Seeds of Njavara variety were obtained from Kerala Agricultural University, Kerala. U373 and C6 cell lines were obtained from National Center for Cell Science (Pune, India). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (CA, USA). TNF-α, Trypsin-EDTA, protease inhibitor cocktail, Caspase-3 and -8 substrates (Ac-DEVD-AFC and Ac-IETD-AMC respectively) and BCIP/NBT were purchased from Sigma Chemicals (St Louis, MO, USA). p65, p50, PARP, pJNK, pERK1/2, iNOS, COX-2, HDAC1 and β-tubulin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). TUNEL assay kit was obtained from Takara Bio Inc (Japan). Lipofectamine^{TM}2000 was obtained from Invitrogen (CA, USA). VECTA SHIELD mounting medium was from Vector Laboratories (Burlingame, CA, USA). All secondary antibodies were purchased from Genei Pvt Ltd (Bangalore, India). Ready-To-Glow^{TM} NF-κB secreted Luciferase Reporter System was purchased from Clontech (USA) and pRL-TK vector was purchased from Promega (Madison, WI, USA).

3.2.2. Rice bran preparation, stabilization and NBM sample preparation.

Njavara rice bran preparation, its stabilization and NBM sample preparation were done by the procedure as mentioned earlier (Chapter-1, section 1.2.3 & 1.2.4)

3.2.3. Cell line and cell culture

U373 and C6 glioma cell lines were cultured in RPMI media, supplemented with 10 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5 % CO_2 at 37 °C.

3.2.4. Flow cytometry analysis

C6 cells were seeded in 6 well plates ($1x10^5$ cells/well) and after overnight incubation, cells were treated with NBM or vehicle (0.1 % DMSO) for 24 h. Then, the cells were harvested with trypsin, fixed in 70 % ethanol, stained with 50 µg/ml propidium iodide (PI) and incubated for 20 min in the dark at room temperature. Then, PI stained-cells were subjected to flow cytometry using a FACS Calibur (BD Biosciences). Cell cycle parameters were analyzed using Modfit software.

3.2.5. Caspase assay

Caspase-3 and Caspase-8 activities were performed as described by Divya *et al* (2009). After treating with 100 µg/ml NBM for 24 h, the cells were lysed in the lysis buffer and 50 µg of the cellular protein and 8 µM of fluorogenic substrate (Ac-DEVD-AFC for Caspase-3, Ac-IETD-AMC for Caspase-8) were added to 1 ml of the assay buffer and incubated for 1 h at 37 °C. Measurements were made on a spectrofluorimeter with a λ_{ex} of 380 nm and a λ_{em} of 460 nm for Caspase-8 and with a λ_{ex} of 400 nm and λ_{em} of 480–520 nm for Caspase-3.

3.2.6. *In situ* terminal-deoxytransferase mediated dUTP nick end labeling (TUNEL) assay

In situ apoptosis detection kit was used to detect DNA fragmentation according to the manufacturer's protocol. C6 cells were seeded on to glass coverslips in 24 well-plates and after overnight incubation, cells were treated with NBM for 24 h. Then, cells were fixed with 4 % paraformaldehyde followed by incubation with a reaction mix containing biotin-dUTP and terminal deoxynucleotidyl transferase for 60 min. Fluorescein-conjugated avidin was applied to the samples, and were then incubated in

the dark for 30 min. Positively stained fluorescein-labeled cells were visualized using Leica confocal microscope and DAPI was used to localize the nuclei.

3.2.7. Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear fractions were prepared as reported previously (Divya *et al.,* 2009). Briefly, C6 cells were pretreated with varying concentrations of NBM and stimulated with TNF-α (10 ng/ml) for 30 min. Cells were then harvested and washed in ice-cold PBS, lysed in 400 µl of cold buffer A [HEPES 10 mmol/L (pH 7.9), KCl 10 mmol/L, 1 mM EDTA, phenylmethanesulphonylfluoride (PMSF) 1 mmol/L, 1 mM EGTA, dithiothreitol (DTT) 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L and pepstatin A 1 mg/L]. After 15 min of incubation on ice, 0.1 % NP-40 was added to the lysates and the tubes were vigorously rocked for 1 min and centrifuged (20,800 g, 5 min) at 4 °C. The supernatant was collected as cytosolic fraction. Then, nuclear pellets were washed with cold buffer A, resuspended in 50 µl of cold buffer B (HEPES 20 mmol/L (pH 7.9), NaCl 420 mmol/L, edetic acid 0.1 mmol/L, egtazic acid 0.1 mmol/L, PMSF 1 mmol/L, DTT 1 mmol/ L, aprotinin 1 mg/L, leupeptin 1 mg/L and pepstatin A 1 mg/L) and vigorously rocked for 30 min at 4 °C followed by centrifugation at 20,800 g for 5 min and the supernatant was collected as nuclear fraction.

3.2.8. Western blotting

Protein samples were mixed with SDS sample buffer, boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis and resolved proteins were transferred on to nitrocellulose membranes. The membranes were blocked with 5 % non-fat dry milk in Tris Buffered Saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl)

for 1 h at room temperature. After blocking, membranes were incubated for overnight in primary antibodies at 4 °C. Membranes were then incubated with respective secondary antibodies for 1-2 h at room temperature and immunoreactivity was detected using BCIP/NBT solution. Before and after incubation of membranes with secondary antibodies, membranes were washed with TBS and TBST (TBS containing 0.1 % Tween-20).

3.2.9. Immunofluorescence

C6 cells were seeded on sterile cover slips in 24-well plates and treated with NBM (100 µg) for 24 h. Then, cells were stimulated with TNF-α (10 ng/ml) for 30 min, and fixed with 4 % paraformaldehyde for 10 min at room temperature followed by permeabilization with 0.2 % Triton X-100. After blocking with 5 % goat serum for 1 h, cells were incubated with p65 primary antibody for 1 h followed by incubation with anti-FITC conjugated rabbit secondary antibody for 1 h at room temperature. Cells were washed thrice in PBS and cover slips were mounted with VECTA SHIELD mounting medium and fluorescence was captured under a Leica confocal microscope.

3.2.10. Transient transfection and Luciferase assays

To examine the effect of NBM on TNF-α induced NF-κB reporter activity, C6 cells were seeded in 24 well plates and transiently transfected with 1 µg of pNFκB-MetLuc2-Reporter, pMetLuc2-Control and 0.5 µg of pRL-TK (for normalization of transfection efficiency) vectors by using lipofectamine 2000. After 6 h, cells were treated with NBM (100 µg) for 24 h followed by TNF-α (10 ng/ml) treatment. The cell culture medium was harvested after 24 h and subjected to metridia luciferase activity

according to the manufacturer's protocol. For renilla luciferase activities, cells were lysed in passive lysis buffer and measured the luciferase activities with luminometer. NF-κB metridia luciferase activates were normalized with that of renilla luciferase values.

3.2.11. Statistics

All data were expressed as mean ± standard deviation (SD) obtained from at least three independent experiments. Statistical comparisons among two groups were carried out by student's t-test. Differences were considered to be statistically significant at a *P* value of <0.05.

3.3. Results

3.3.1. NBM altered the cell cycle distribution and induced apoptosis

Altered and deregulated cell cycle is one of the prominent features of glioma cell lines. In order to ascertain the effect of NBM on cell cycle distribution, C6 cells

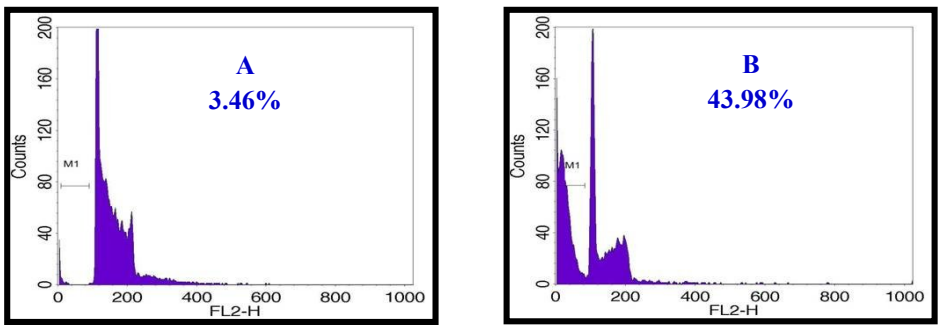


Figure 24: Effect of NBM on cell cycle distribution. C6 cells were seeded in 6 well plates and treated with (A) vehicle (0.1 % DMSO) or (B) NBM (100 µg/ml) for 24 h, fixed in 70 % ethanol, stained with propidium iodide and subjected to FACS analysis. The number of sub-G0/G1 phase cells is expressed as percentage of the total number of cells.

were treated with indicated concentrations of NBM for 24 h, subjected to FACS analysis and quantified the cell population at different phases of cell cycle. As shown in Figure 24, NBM treatment significantly increased the sub G1 cell population (apoptotic cells) at the expense of G1 population in a concentration dependent manner, indicating

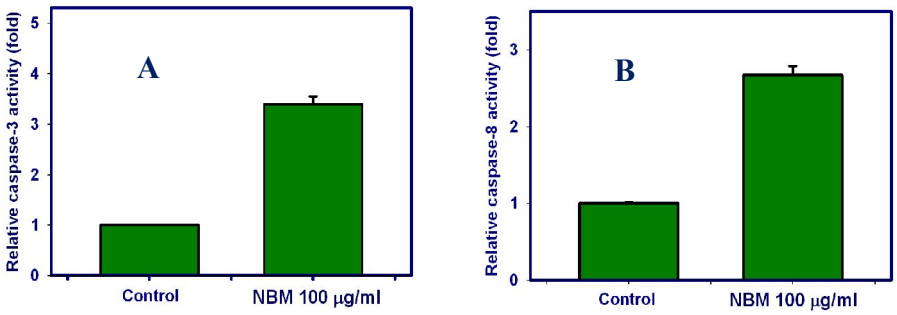


Figure 25: Effect of NBM on caspases-3 and caspases-8 activities. C6 cells were seeded in 100 mm culture plates and treated with NBM for 24 h and estimated the caspases-3 and -8 enzyme activities as described in materials and methods. All data presented are the mean ± SD and are representative of three independent experiments.

that NBM promoted apoptosis in glioma cells. To further confirm the effect of NBM on apoptosis, we analyzed the Caspase-3, Caspase-8 activities and PARP cleavage. Several lines of evidences suggest that caspases are important modulators of apoptosis, induce apoptotic features by acting on several cellular substrates, including PARP. As shown

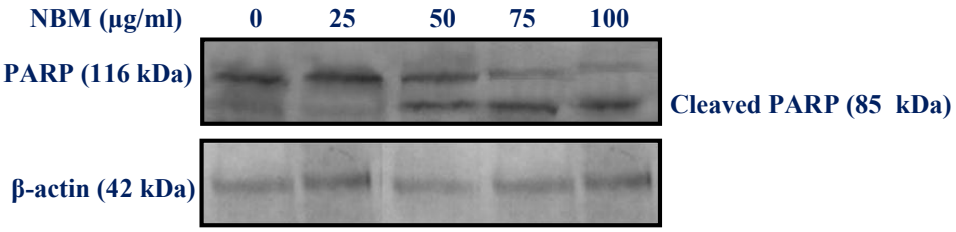


Figure 26: Effect of NBM on PARP cleavage. C6 cells were seeded in 100mm culture plates and treated with indicated concentrations of NBM for 24 h. Then, cells were harvested and whole cell lysates were subjected to western blotting for cleaved PARP. β-actin used as loading control and the blots were representatives of three independent experiments.

in Figure 25, caspase-3 and caspase-8 activities were significantly increased in a dose dependent manner following NBM treatment. In addition, NBM treatment significantly increased the PARP cleavage in a concentration dependent manner (Figure 26). To further determine whether NBM induces apoptosis in GBM cells, the extent of DNA fragmentation as determined by TdT-mediated dUTP biotin nick end labeling (TUNEL) assay was performed. As shown in the Figure 27, NBM- treated C6 cells were TUNEL positive (~64 %) compared to vehicle (0.1 % DMSO) treated cells (~4 %). These results suggest that NBM treatment induced caspase-dependent apoptotic cell death in glioblastoma cells. Our data strongly suggeste that cytotoxic and growth inhibitory effects observed in response to NBM treatment were also associated with the induction of apoptosis.

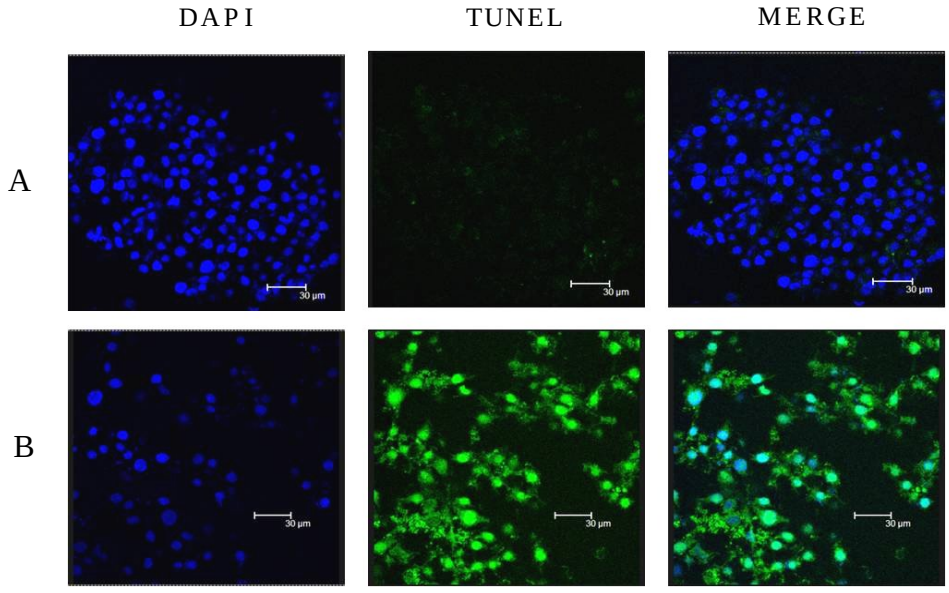


Figure 27: Effect of NBM on DNA fragmentatation. C6 cells were seeded on glass coverslips and treated with vehicle (0.1 % DMSO) **(A)** or with NBM **(B)** for 24 h. Then cells were subjected to TUNEL assay as described in materials and methods. DAPI used as to identify nuclei and data are representatative of three independent experiments.

3.3.2. NBM inhibited the TNF-α-induced nuclear translocation of p50 and p65

NF-kB is a dimeric transcription factor which plays a crucial roles on

inflammation, carcinogenesis and apoptosis. Oncogenic role for NF-kB was evident in several human malignancies including gliomas. In order to know the effect of NBM on TNF-α-induced nuclear translocation of NF-κB, C6 cells were treated with varying concentrations of NBM and determined the nuclear levels of p50 and p65. As shown in Figure 28, nuclear p50 and p65 levels were increased following TNF-α stimulation compared to unstimulated cells and further treatment with NBM significantly reduced the p50 and p65 levels in a dose dependent manner. To better characterize the inhibition of nuclear translocation of NF-kB, C6 cells were treated with NBM followed by TNF-α

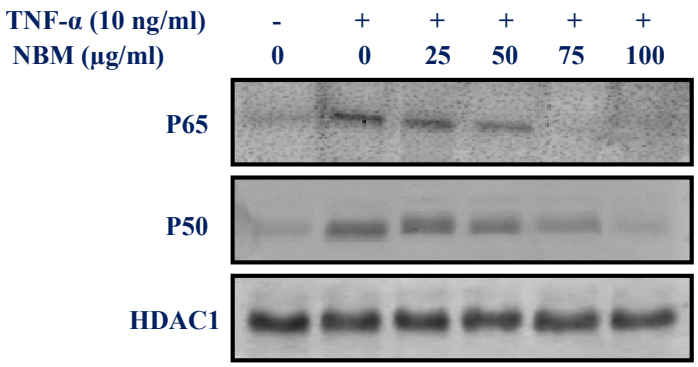


Figure 28: Effect of NBM on TNF-α induced nuclear translocation of p50 and p65. C6 cells were seeded in 100-mm dishes and treated with indicated concentrations of NBM for 24 h followed by TNF-α stimulation for 30 min. Nuclear lysates were prepared and subjected to western blotting for p50 and p65. HDAC-1 used as loading control and the blots were representatives of three independent experiments.

stimulation and subjected to immunofluorescence analysis. As shown in Figure 29, p65 was translocated to nucleus following TNF-α stimulation in C6 cells. However, treatment with NBM resulted in the inhibition of nuclear translocation of p65 which might be retained in the cytosol following TNF-α stimulation. These findings clearly suggest that NBM significantly inhibits the TNF-α-induced nuclear translocation of p65.

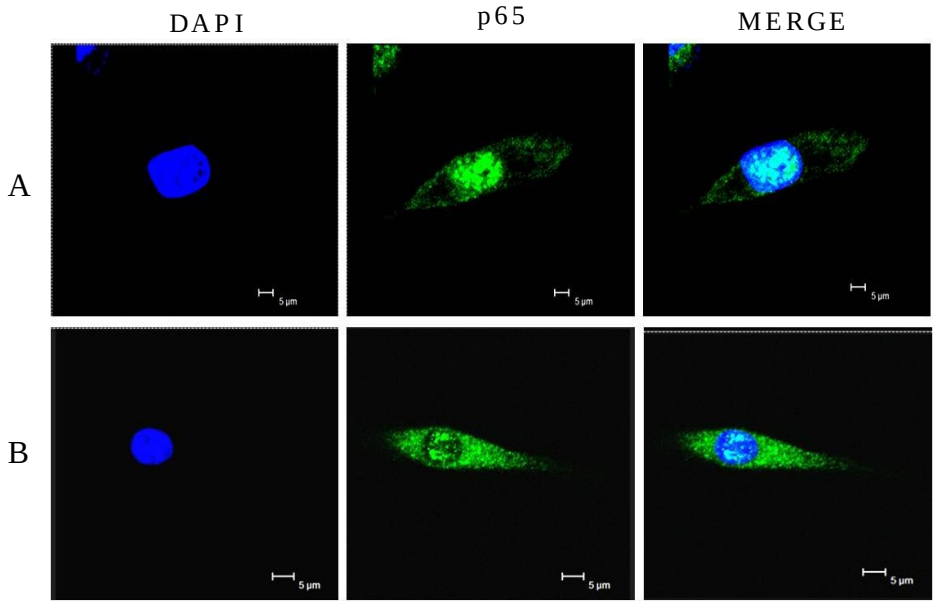


Figure 29: Immunofluorescence analysis of p65. C6 cells were seeded on to coverslips in 24 well plates and pretreated with (A) vehicle (0.1%) DMSO and (B) NBM (100 µg) for 24 h, followed by stimulation with TNF-α (10 ng/ml) for 30 min. Cells were then fixed in 4% paraformaldehyde and incubated with p65 primary antibody and anti-FITC secondary antibodies for 1 h at room temperature. Fluorescence was captured under Leica confocal microscope. DAPI was used to visualize the nuclei. Data are the representative of three independent experiments.

3.3.3. NBM down-regulated the NF-κB reporter gene expression

Once IκB inhibitory effects are relieved, p50/65 complex is translocated to nucleus and binds to and activate expression of their target genes. To know the effect of NBM on NF-κB activation, C6 cells were transiently transfected with pNF-κB-MetLuc2-Reporter, and pRL-TK (for normalization of transfection efficiency) plasmids, and treated with NBM followed by TNF-α stimulation. As shown in Figure 30, on stimulation with TNF-α, NF-κB reporter activity was greatly enhanced compared to unstimulated cells and this enhanced reporter activity is potentially inhibited by NBM treatment in a dose dependent manner. These results demonstrate that NBM attenuated the NF-κB dependent reporter gene expression.

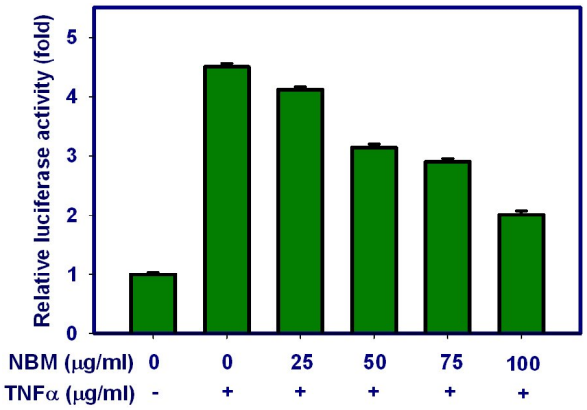


Figure 30: Effect of NBM on NF-κB dependent reporter gene expression. C6 cells were seeded in 24 well plates and transiently transfected with pNF-κB-MetLuc2-Reporter and pRL-TK (for normalization of transfection efficiency) plasmids using lipofectamine 2000 as described in materials and methods. After 6 h cells were treated with indicated concentrations of NBM for 24 h followed by TNF-α stimulation. Culture medium was then harvested after 24 hrs and analyzed for metridia luciferase activity and cells were lysed in passive lysis buffer and analyzed for renilla luciferase activity. The metridia luciferase activities were normalized against renilla luciferase activities. Data expressed as mean ± SD and are representative of three independent experiments ($P < 0.05$).

3.3.4. NBM inhibited MAP kinases activation and downregulated iNOS, COX-2

MAP kinases play a crucial role in regulating cell growth, differentiation and apoptosis in response to diverse extracellular stimuli in eukaryotic cells. As shown in Figure 31, treatment with NBM inhibited the activation of pERK 1/2 and pJNK in a dose dependent manner and also inhibited the expression of downstream proteins, iNOS and COX-2.

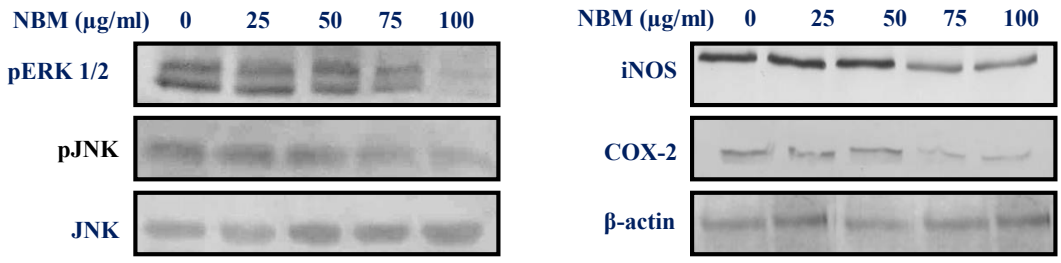


Figure 31: Effect of NBM on MAP kinases, iNOS and COX-2. C6 cells were treated with NBM with indicated concentrations for 24 hours. NBM inhibited phosphorylation of pERK ½, pJNK and also inhibited the downstream proteins iNOS and COX-2 expression.

3.4. Discussion

Increased consumption of antioxidant-rich whole grains reduces the risk of cancer (Jing *et al.,* 2010). Njavara is one of the important rice variety which is extensively used as whole grain in ayurveda to treat various disorders. Earlier we have shown strong free radical scavenging ability and various pharmacological properties of NBM extract. In the previous chapter, we have shown the antiproliferatory effect of NBM on glioma cell lines (C6 and U373) by performing MTT assay, cell viability and clonogenic assay. In the present chapter, we performed cell cycle analysis to determine the proapoptotic effects of NBM. An analysis of our data indicated that NBM arrest the cell cycle at G2-M phase, and increased the apoptotic sub G1 fraction. The caspases are the critical mediators in regulating apoptosis (Suk, 2005; Yazdi *et al.,* 2010). Caspases signaling is initiated and propagated by proteolytic autocatalysis and by the cleavage of downstream caspases and substrates such as PARP and lamins. Caspase-3 is the executioner of apoptosis and is responsible for the cleavage of PARP (Lee *et al.,* 2006). PARP cleavage results in the cellular disassembly and served as a marker of cells undergoing apoptosis (Lewis-Wambi and Jordan 2009). To further investigate the apoptosis induction following NBM treatment, we determined the effects of NBM on caspase-3 and -8 activities, PARP cleavage and TUNEL assay. The results strongly suggest that NBM induced apoptosis through activating the caspase-3 and -8 and also through the induction of PARP cleavage.

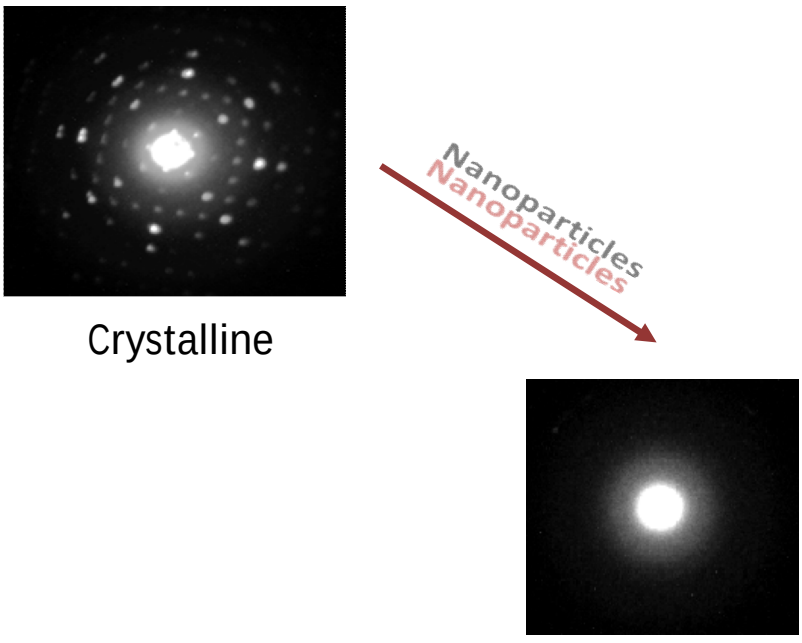
Glial tumors are known to develop due to genetic alterations in low grade lesions, which further acquire additional mutations and progress to more malignant tumors. These genetic alterations disrupt the cell cycle arresting pathways, or activate various signal transduction pathways and contribute to the development of

astrocytomas (Kleihues and Ohgaki, 2007; Louis, 1997; Hayashi *et al.,* 1997). The signaling pathway characterized by MAP kinases (pERK1/2, pJNK and p38) are known to play a crucial role in molecular and cellular events associated with the pathogenesis (Rasheed et al., 2009). The findings herein suggests that compounds present in NBM have the potential to inhibit the activation of MAP kinases (pERK 1/2 and pJNK) and also inhibits the downstream proteins, iNOS and COX-2. Likewise, NF-κB signaling pathway is constitutively activated in glioma which was associated with enhanced growth, cell cycle progression, and inducible chemo resistance. Because of the inhibition of NF-κB activation which has been linked with antitumor activities, we hypothesize that NBM mediates its effect at least partly through inhibition of NF-κB activation. In this study, we elucidated the inhibitory effects of NBM on nuclear translocation of p50 and p65 and their reporter gene expression. Our results showed that TNF-α-induced nuclear translocation of p50 and p65 as well as NF-κB-dependent reporter gene expression which was inhibited following NBM treatment.

In summary, our results demonstrated NBM inhibited cell proliferation and induced apoptosis in glioma cells which was associated with the suppression of NF-κB activation, MAP kinases (pERK 1/2 and pJNK) and the associated downstream proteins, iNOS and COX-2. Therefore, we believe that these findings might help in providing the rationale to initiate *in vivo* studies to examine the efficacy of NBM as chemopreventive agent against gliomas.

4

Chapter



Naringenin induced cytotoxicity in glioma cells and Naringenin nanoparticle preparation

4.1. Introduction

It has been reported that about 40 % of the compounds being developed by the pharmaceutical industry and most of the potent bioactive molecules are poorly water soluble (Radtke, 2001; Arunkumar *et al.,* 2010). Therefore, increasing the dissolution rate of these drugs is a major concern to the pharmaceutical scientists, in order to maximize absorption when it is orally administered (Katteboina *et al.,* 2009; Derle *et al.,* 2010). Over the past 10 years, nanoparticle engineering processes has been developed and reported for pharmaceutical applications. Increasing the solubility and dissolution rate for these poorly soluble drugs by increasing the surface area is a very promising way to improve drug bioavailability. According to the Noyes-Whitney equation, reducing the particle size and thus increasing the surface area will increase the dissolution rate of poorly water soluble drugs (Noyes and Whitney, 1897). Nanoparticles are particularly useful in drug delivery for water insoluble compounds such as cyclosporine A (Dai *et al.,* 2004), ellagic acid (Bala *et al.,* 2006), coenzyme Q10 (Hsu *et al.,* 2003), Griseofulvin (Zili *et al.,* 2005), Quercetin (Wu *et al.,* 2008) because of their size (less than 1000nm) which can increase the absorption and the bioavailability of the drug delivered.

4.1.1. Nanoprecipitation

Nanoprecipitation technique is one of the most popular techniques used for NP preparation due to its easy and simple preparation (Segundo *et al.,* 2006). This process was described and patented by Fessie *et al.,* (1992). In this method, the polymer is dissolved in water–miscible solvent or in a mixture (eg. ethanol or acetone). A lipophilic drug as well as a lipophilic stabilizer (eg. phospholipids) may be added to the

organic solution and poured or injected into an aqueous solution, which may or may not contain a stabilizer (eg. poly vinyl alcohol (PVA) or poloxamer). Organic solvent diffuses immediately into water, allowing the polymer to aggregate in the form of NPs (Segundo *et al.*, 2006).

Previous studies have described the use of pH-sensitive polymers such as hydroxypropylmethylcellulose phthalate (Klipstein *et al.*, 1983), Eudragid®L100, E100 and Eudragid® S100 (Morishita *et al.*, 1993; Jaeghere *et al.*, 2000; Dai *et al.*, 2004) or cellulose acetate phosphate (Lin *et al.*, 1991) to encapsulate antigens or proteins for oral administration. Release of the highly dispersed drug at a specific pH within the gastrointestinal tract, as close as possible to the absorption window of the drug, is expected to increase the probability of the drug absorption and minimize the first-pass metabolism of the drug (Dai *et al.*, 2004). On the basis of the above mentioned considerations, it was thought plausible to combine the advantages of nanoparticles as oral delivery systems with benefits of the pH sensitive property.

4.1.2. Naringenin

Naringenin (NAR), a well known flavonoid distributed ubiquitously in fruits, vegetables, herbs and whole grains (Lee *et al.*, 2004; Renugadevi and Prabu, 2009). NAR has been extensively investigated for its pharmacological effects that induce anti-tumor (Kanno *et al.*, 2005), protects against renal dysfunction in rats during cadmium stress, (Renugadevi and Prabu, 2009) and hepatoprotective activities (Lee *et al.*, 2004). Although NAR is an excellent free radical scavenger with pharmacological activities, clinical studies of this drug have been hampered by its extreme water insolubility. It has been reported that the absolute bioavailability of NAR was achieved only 4 % in rabbits

when the animals were orally administered (Hsiu *et al.*, 2002). To overcome these obstacles, the delivery of NAR using novel dosage forms will likely yield more promising clinical applications of this compound.

4.1.3. Objective

The aim of the present study was to initially evaluate the *in vitro* cytotoxic property of NAR against glioma cell lines as well as preparing Naringenin nanoparticles by using a simple nanoprecipitation technique.

4.2. Materials and methods

4.2.1. Reagents

U373 and C6 cell lines were obtained from National Center for Cell Science (Pune, India). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (CA, USA). Naringenin (NAR), TNF-α, Trypsin-EDTA, protease inhibitor cocktail, Caspase-3 and -8 substrates (Ac-DEVD-AFC, Ac-IETD-AMC respectively) BCIP/NBT, Polyvinyl alcohol (PVA) and Tris-HCl were purchased from Sigma Chemicals (St Louis, MO, USA). p65, p50, PARP, HDAC1 and β-tubulin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). TUNEL assay kit was obtained from Takara Bio Inc (Japan). Lipofectamine^{TM}2000 was obtained from Invitrogen (CA, USA). VECTA SHIELD mounting medium was from Vector Laboratories (Burlingame, CA, USA). All secondary antibodies were purchased from Genei Pvt Ltd (Bangalore, India). Ready-To-Glow^{TM} NF-κB secreted Luciferase Reporter System was purchased from Clontech

(USA) and pRL-TK vector was purchased from Promega (Madison, WI, USA). Aminoalkyl methacrylate copolymers (Eudragid®E100) was kindly given by Roach pharmaceuticals (India). All other chemical reagents were of analytical grade.

4.2.2. Cell culture

U373 and C6 glioma cell lines were cultured in RPMI media, supplemented with 10 % FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5 % CO_2 at 37 °C.

4.2.3. *In vitro* antiproliferative and proapoptotic methods

In vitro antiproliferative and proapoptotic methods including MTT assay, cell viability and clonogenic assay were performed by the procedure as described already (Chapter 2, section 2.2.4). Caspase assay, Flow cytometric analysis, TUNEL assay, western blotting, immunofluorescence of p65, transient transfection and luciferase reporter assay were performed by the procedure as already explained (Chapter 3, section 3.2.4-3.2.9).

4.2.4. Preparation of Naringenin Nanoparticles (NARN)

NARN system was prepared by the nanoprecipitation method (Zili *et al.*, 2005) with slight modifications. 50 mg of NAR and 500 mg of Eudragid®E was dissolved in 25 ml of ethanol. The internal organic phase solutions were quickly injected into the 75 ml of external aqueous solution containing 500 mg of PVA which was kept under sonication. This mixture was sonicated for 3 min and homogenized at 22000 rpm for 25

min. The ethanol was completly removed by rotary vacuum evaporation at 40 °C and the remaining fraction was lyophilized with a freeze dryer.

4.2.5. Particle size and Morphology

Size and external morphology of NARN were determined using transmission electron microscopy (TEM). The samples were prepared by placing one preparation drop on a carbon coated copper grids and the sample was dried completely before analyzing under TEM.

4.2.6. Storage stability

Nanoparticle suspensions were stored under static conditions at room temperature for a period of 3 months. Stability was assessed by comparing the initial size and morphology of the NARN with those obtained after 3 months storage at room temperature. The size and morphology of NARN were checked with TEM.

4.2.7. X-Ray Diffractometry

The patterns of pure NAR and its lyophilized NARN were performed by X-ray diffractometry (Siemens D5000, Germany) using Cu Kα rays with a voltage of 40 kv and a current of 30 mA. The scanned angle was set from $2° \leq 2° \geq 50^{o}$ and the scanned rate was 1^{o} / min.

4.2.8. Reconstitution of lyopilized NARN

Lyophilized NARN was reconstituted by addition of different pH buffer solutions (pH 1.5, 4.5, 7.4) or with milliQ water, followed by sonication in a water bath at room temperature for 3 min.

4.2.9. Fourier transformed infrared (FT-IR) spectroscopy

The FT-IR spectra of the samples were obtained on a Perkin-Elmer 2000 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). Each sample and potassium bromide were mixed by an agate mortar and compressed into thin tablets. The scanning range was 370-4000 cm^{-1} and the resolution was 1 $cm^{-1.}$ Each sample was measured and recorded in triplicate.

4.2.10. Statistics

All data were expressed as mean ± standard deviation (SD) obtained from at least three independent experiments. Statistical comparisons among two groups were carried out by student's t-test. Differences were considered to be statistically significant at a *P* value of <0.05.

4.3. Results

4.3.1. NAR induced cytotoxicity, reduced proliferation and clonogenic efficiency of glioma cell lines

It has been reported that NAR exhibits anti-neoplastic activities against various cancer cell lines. However, its effect on glioma cell lines remains poorly understood. To

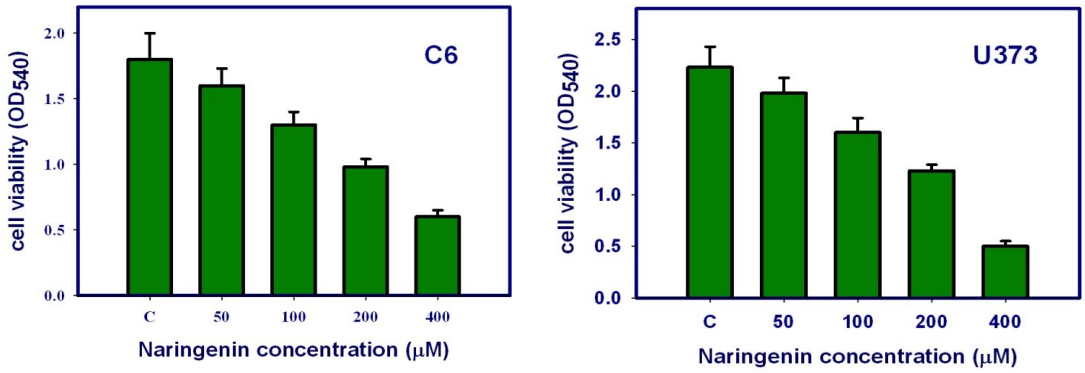


Figure 32: Effect of NAR on cytotoxicity. C6 and U373 glioma cells were treated with vehicle (0.1 % DMSO) or indicated concentrations of NAR for 24 h and subjected to MTT assay. Dose dependent growth inhibition was observed in the tested cell lines. Data were mean ± SD of three independent experiment (n=3).

assess the effect of NAR on proliferation of glioma cell lines, a series of experiments were conducted. First, to determine the cytotoxic effect of NAR, U373 and C6 cells were treated with the indicated concentrations of NAR for 24 h and subjected to MTT assay (Figure 32) NAR treatment significantly reduced the cell viability in a dose dependent manner. Further, to determine the effect of NAR on proliferation, U373 and C6 cells were treated with indicated concentrations of NAR for 24 h, 48 h, and 72 h and number of trypan-blue excluding viable cells were counted period. As shown in Figure 33, the proliferation rates of U373 and C6 cells were significantly reduced in a

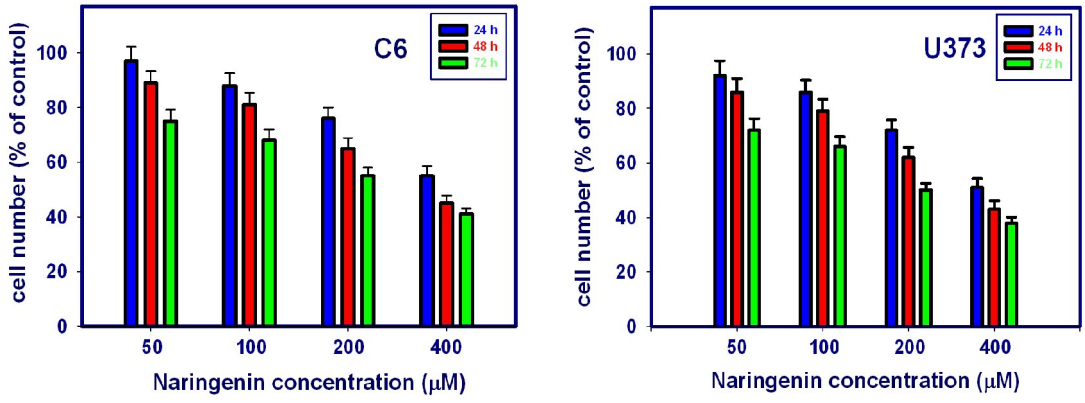


Figure 33: Effect of NAR on cell viability. U373 and C6 glioma cells were seeded in 6 well plates and treated with vehicle (0.1% DMSO) or indicated concentrations of NAR for 24 h, 48 h and 72 h and total viable cell numbers were counted. Data were mean ± SD of three independent experiment (n=3).

dose and time dependent manner following NAR treatment. To further characterize the effect of NAR on colony forming ability, U373 and C6 cells were subjected to clonogenic assays. As depicted in Figure 34, NAR treatments greatly reduced the clonogenic efficiency of U373 and C6 cells in a dose dependent manner compared to vehicle-treated cells which attained about 80 % cloning efficiency. These experiments have clearly demonstrated that NAR significantly reduced the growth of U373 and C6 cell lines and causing cytotoxicity.

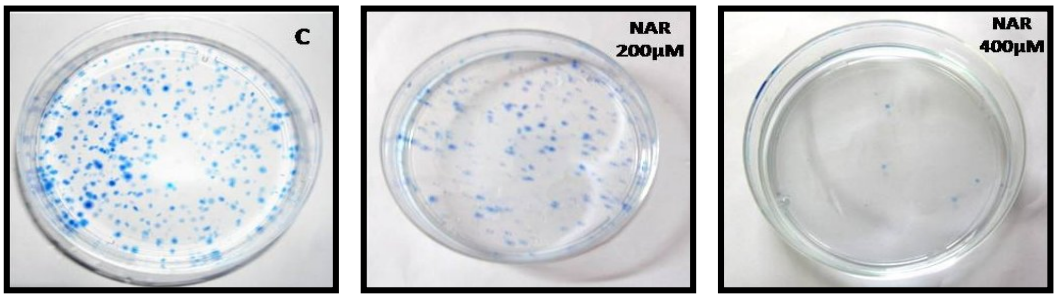


Figure 34: Effect of NAR on cloning efficiency. 500 U373 cells were plated in 100-mm culture dishes and after 24 h cells were treated with vehicle (0.1 % DMSO) or indicated concentrations of NAR for 24 h. After 7 days colonies were stained with methylene blue and colonies that contain ≥ 50 cells were counted. 'C' denotes the vehicle treated control cells.

4.3.2. NAR altered the cell cycle distribution and induced apoptosis

Altered and deregulated cell cycle is one of the prominent features of glioma cell lines. In order to ascertain the effect of NAR on cell cycle distribution, U373 cells were treated with indicated concentration of NAR for 24 h, subjected to FACS analysis and quantified the cell population at different phases of cell cycle. (Figure 35), NAR treatment significantly increased the subG1 cell population (apoptotic cells) at the expense of G1 population in a concentration dependent manner, indicating that NAR promoted apoptosis in glioma cells. To further confirm the effect of NAR on apoptosis

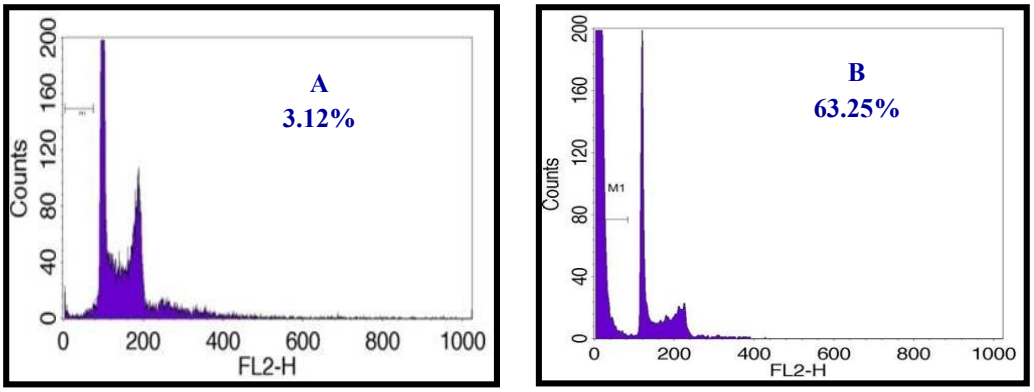


Figure 35: Effect of NAR on cell cycle distribution. U373 cells were seeded in 6 well plates and treated with (A) vehicle (0.1 % DMSO) or (B) NAR (400 µM) for 24 h, fixed in 70 % ethanol, stained with propidium iodide and subjected to FACS analysis. The number of sub-G0/G1 phase cells is expressed as percentage of the total number of cells. Data were mean ± SD of three independent experiment (n=3).

we analyzed the Caspase-3, Caspase-8 activities and PARP cleavage. It is known that caspases are important modulators of apoptosis, induce apoptotic features by acting on several cellular substrates, including PARP. As shown in Figure 36, caspase-3 and caspase-8 activities were significantly increased in a dose dependent manner following NAR treatment. In addition, NAR treatment significantly increased the PARP cleavage

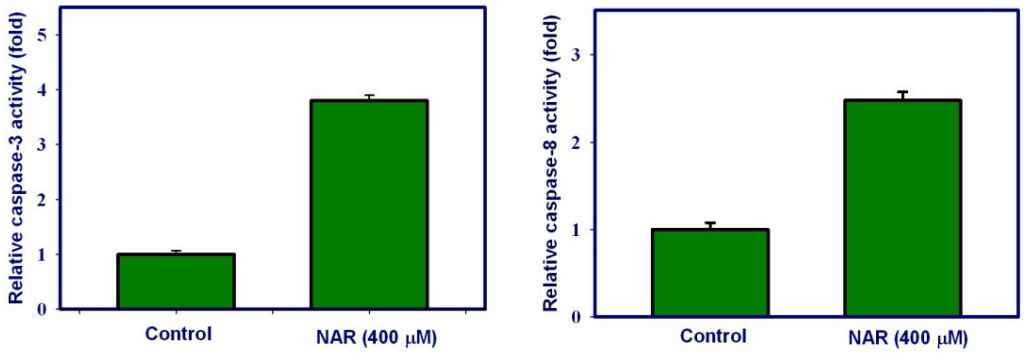


Figure 36: Effect of NAR on caspases-3 and caspases-8 activities. U373 cells were seeded in 100 mm culture plates and treated with NAR (400µM) for 24 h and estimated the caspases-3 and -8 enzyme activities as described in materials and methods. All data presented are the mean ± SD and are representative of three independent experiments.

in a dose dependent manner (Figure 37). To further determine whether NAR induce apoptosis in GBM cells, DNA fragmentation as determined by TdT-mediated dUTP

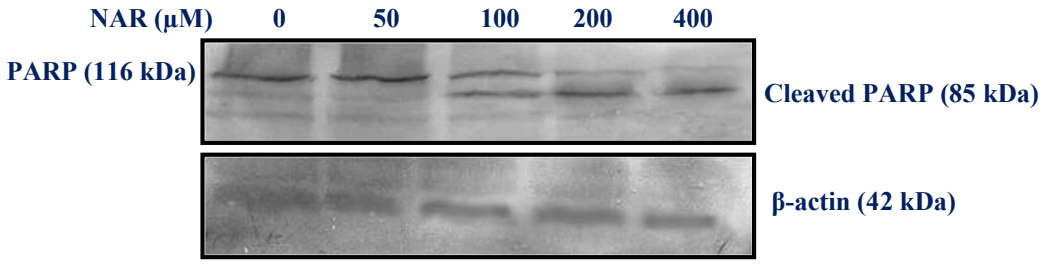


Figure 37: Effect of NAR on PARP cleavage. U373 cells were seeded in 100mm culture plates and treated with indicated concentrations of NAR for 24 h. Then, cells were harvested and whole cell lysates were subjected to western blotting for cleaved PARP. β-actin used as loading control and the blots were representatives of three independent experiments.

biotin nick end labeling (TUNEL) assay was performed. As shown in the Figure 38, NAR-treated U373 cells were TUNEL positive (~78 %) compared to vehicle (0.1 % DMSO)-treated cells (~4 %). These results suggest that NAR treatment induced

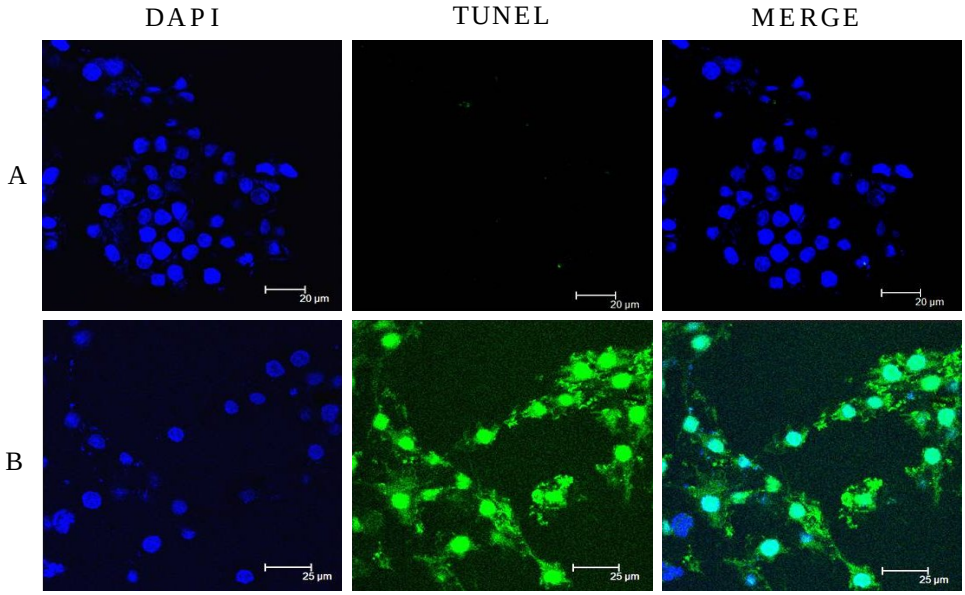


Figure 38: Effect of NAR on DNA fragmentatation. U373 cells were seeded on glass coverslips and treated with vehicle (0.1% DMSO) **(A)** or with 400 μM NAR **(B)** for 24 h. Then cells were subjected to TUNEL assay as described in materials and methods. DAPI used as to identify nuclei and data are representatative of three independent experiments.

caspase-dependent apoptotic cell death in glioblastoma cells. Our study also suggest that cytotoxic and growth inhibitory effects were observed in response to NAR treatment are associated with the induction of apoptosis in glioma cell lines.

4.3.3. NAR inhibited the TNF-α-induced nuclear translocation of p65

NF-kB is a dimeric transcription factor which plays crucial roles in inflammation, carcinogenesis and apoptosis. Oncogenic role for NF-kB was evident in several human malignancies including gliomas. In order to know the effect of NAR on TNF-α-induced nuclear translocation of NF-κB, U373 cells were treated with NAR followed by TNF-α stimulation and subjected to immunofluorescence analysis. As shown in Figure 39, p65 was translocated into nucleus, following TNF-α stimulation in U373 cells. However, treatment with NAR resulted in the inhibition of nuclear translocation of p65 which remained in the cytosol following TNF-α stimulation. These

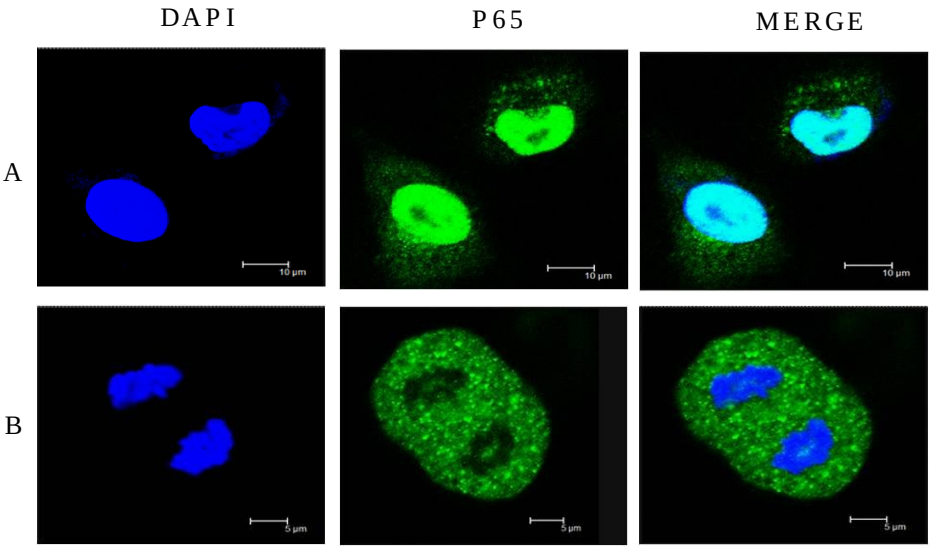


Figure 39: Immunofluorescence analysis of p65. U373 cells were seeded on to coverslips in 24 well plates and pretreated with vehicle (0.1%) DMSO and NAR (400 µM) for 24 h, followed by stimulation with TNF-α (10 ng/ml) for 30 min. Cells were then fixed in 4% paraformaldehyde and incubated with p65 primary antibody and anti-FITC secondary antibodies for 1 h at room temperature. Fluorescence was captured under Leica confocal microscope. DAPI was used to visualize the nuclei.

findings clearly demonstrated that NAR significantly inhibited the TNF-α-induced nuclear translocation of p65.

4.3.4. NAR down-regulated the NF-κB reporter gene expression

Once IκB inhibitory effects are relieved, p50/65 complex is translocated to nucleus and activate expression of their target genes (Nishikori, 2005). To know the effect of NAR on NF-κB activation, U373 cells were transiently transfected with pNF-κB-MetLuc2-Reporter, and pRL-TK (for normalization of transfection efficiency) plasmids, and treated with NAR followed by TNF-α stimulation (Figure 40), on stimulation with TNF-α, NF-κB reporter activity was greatly enhanced compared

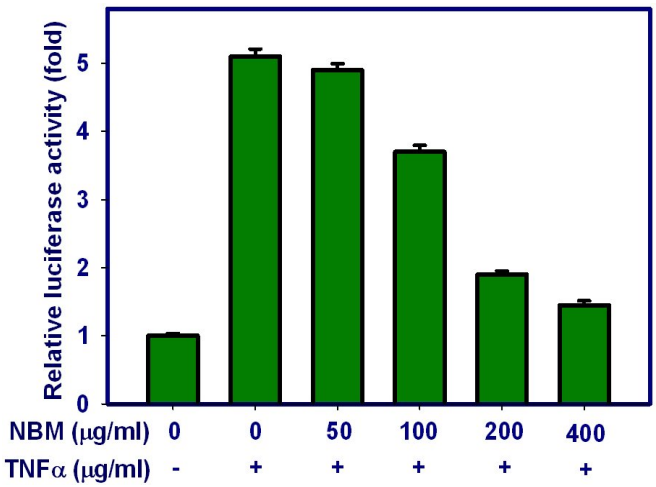


Figure 40: Effect of NAR on NF-κB dependent reporter gene expression. U373 cells were seeded in 24 well plates and transiently transfected with pNF-κB-MetLuc2-Reporter and pRL-TK (for normalization of transfection efficiency) plasmids using lipofectamine 2000 as described in materials and methods. After 6 h cells were treated with indicated concentrations of NAR for 24 h followed by TNF-α stimulation. Culture medium was then harvested after 24 hrs and analyzed for metridia luciferase activity and cells were lysed in passive lysis buffer and analyzed for renilla luciferase activity. The metridia luciferase activities were normalized against renilla luciferase activities. Data expressed as mean ± SD and are representative of three independent experiments.

to unstimulated cells and this enhanced reporter activity is potentially inhibited by NAR treatment in a dose dependent manner. These results clearly demonstrated that NAR attenuated the NF-κB dependent reporter gene expression.

4.3.5. Naringenin nanoparticles (NARN)

4.3.5.1. Physiochemical characterization of NARN

The mean particle diameter of prepared NARN was 45±5 nm and the particles were spherical in shape (Figure 41). The lyophilized NARN were dissolved in different buffers with pH 1.5, 4.5, 7.4 and in milli Q water. NARN dissolved in pH 1.5 and 4.5

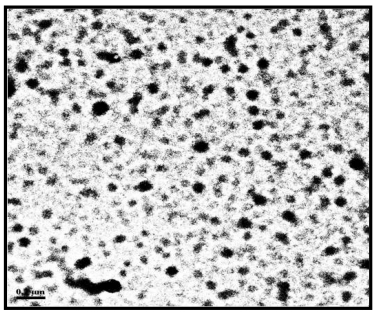


Figure 41: TEM photograph. NARN were prepared by nanoprecipitation method as described in materials and methods using ethanol and water as solvents. Data were representatives of three independent experiments.

showed a clear solution but at 7.4 and in milli Q water NARN formed turbid solution (Figure 42).

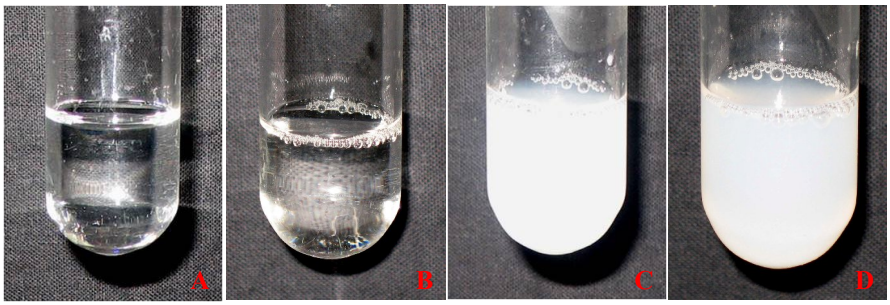


Figure 42: Solubility studies of NARN. appearance of lyophilized NARN reconstituted in (A) pH 1.5, (B) pH 4.5, (C) pH 7.4 and (D) in milli Q water. Data were representatives of three independent experiments.

These observations suggest that the particle size released can depend on the pH of the buffer solution. An explanation for this is that the low pH value could more easily dissolve the gastro-soluble Eudragid E100 polymer from NARN, and thus yield reduced particle size of NAR that is released. The status of incorporated drug in nanoparticle system is an important factor that influences the solubility and bioavailability of the drug. Figure 43, displays the XRD result of the NAR and of its nanoparticles. The characteristics peaks of NAR presented at a diffraction angle of 2θ, 10.95, 12.18, 16.57, 19.68 and 24.55 which could be inferred to the traits of a highly crystalline structure. On the contrary, none of these characteristic peaks were present in the pattern of NARN, suggesting that the crystal structure of NAR was indeed transformed into an amorphous state in NARN.

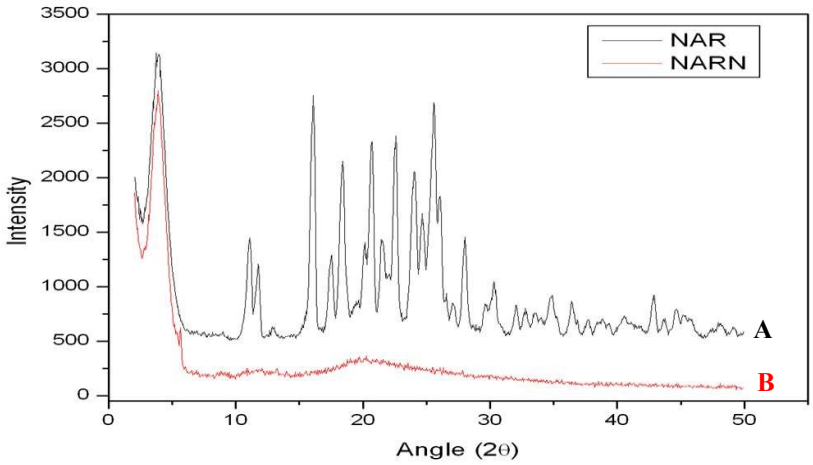


Figure 43: XRD analysis. X-ray diffraction patterns of (A) free naringenin and (B) naringenin nanoparticles. Data were representatives of three independent experiments.

4.3.5.2. Fourier transformed infrared (FT-IR) spectra

The intermolecular interaction of nanoparticles system was establishe by FT-IR. Figure 44, shows the characteristic intensities at OH streach at 3273 cm^{-1} and the OH streach

of NARN which completely disappeared. These results suggested that intermolecular hydrogen bonding occurred in the NARN (Wu *et al.*, 2008).

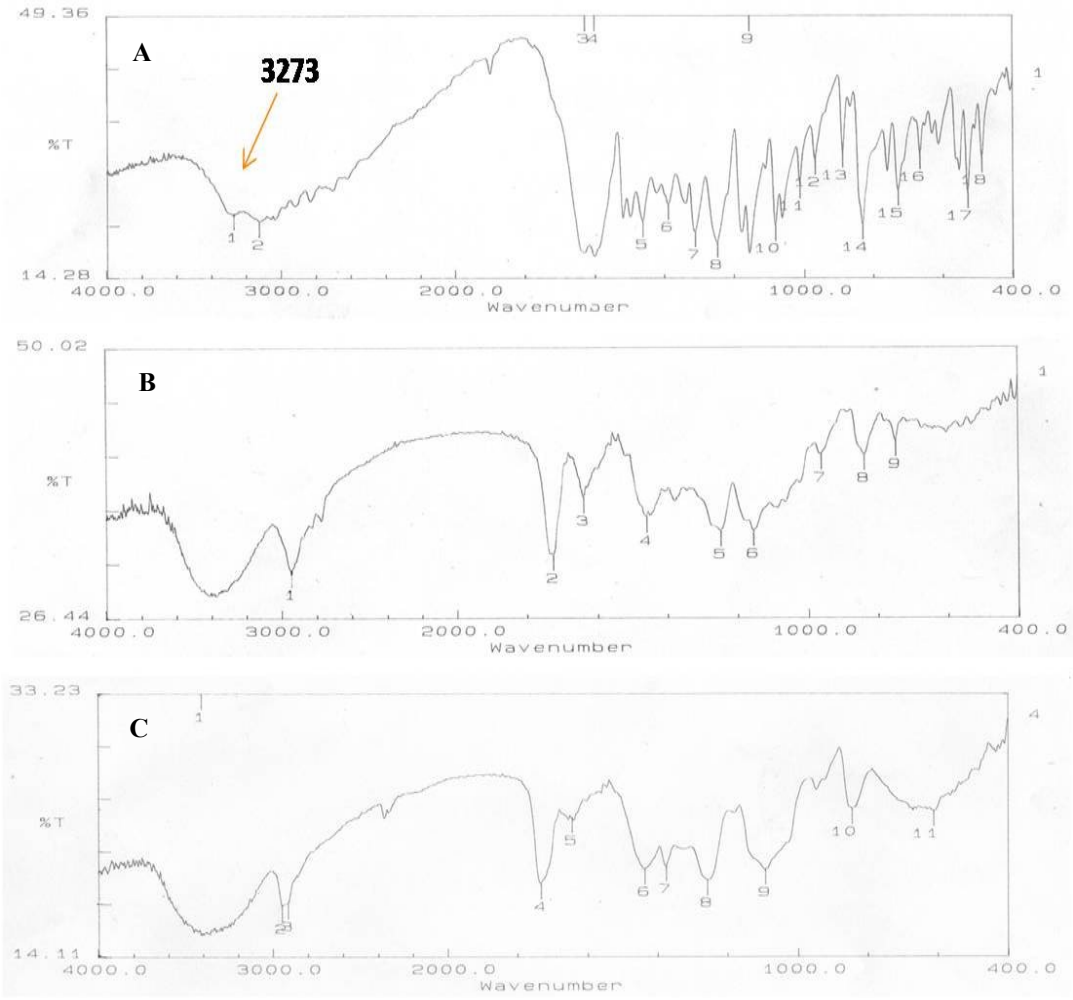


Figure 44: FT-IR analysis. FT-IR spectra of (A) NAR, (B) NARN [NAR (1): Eudragid (10): PVA (10)] and (C) physical mixture [NAR (0): Eudrogid (10): PVA (10)]. Data were representatives of three independent experiments.

4.3.5.3. Storage stability of NARN

Nanoparticle suspensions prepared by us maintained the initial properties with respect to size and external morphology even after 3 months of storage at room temperature (Figure 45).

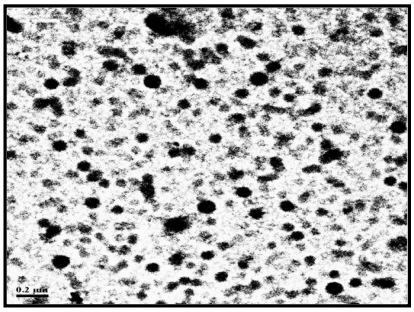


Figure 45: Stability studies of NARN. NARN were prepared by nanoprecipitation method as described in materials and methods using ethanol and water as solvents. TEM photographs were taken after 30 days of NARN preparation. Data were representatives of three independent experiments.

4.4. Discussion

Flavonoids comprise the major dietary group of plant polyphenols (Tapas *et al.*, 2008). Free radical scavenging activity of flavonoids was attributed due to the presence of phenolic hydroxyl groups that are attached to the flavonoid structure (Renugadavi and Prabu, 2009). Literature shows that among the various types of flavonoids the flavanone naringenin (NAR) exhibits wide range of bioactive effects on human health and primarily associated with cancer prevention (Ekambaram *et al.*, 2008; Tapas *et al.*, 2008).

The present study was aimed to investigate the effects NAR on human glioma cells. In this study, we set out to investigate whether NAR have significant anti-proliferative effect on glioma cell lines. MTT assay, cell viability and clonogenic assays revealed that NAR had significant anti-proliferative effects on U373 and C6 glioma cell lines. To determine the proapoptotic effects of NAR, we performed cell cycle analysis. An analysis of our data indicated that NAR arrest the cell cycle at G2-M phase, and increased the apoptotic sub G1 fraction. To further investigate the apoptosis induction

following NAR treatment, we determined the effects of NAR on caspase-3 and -8 activities, PARP cleavage and TUNEL assay. In this study, we have demonstrated that NAR induced apoptosis through activating the caspase-3 and -8, induction of PARP cleavage and DNA fragmentation. We hypothesize that NAR mediates its effect at least partly through inhibition of NF-κB activation. In this study we checked the inhibitory effects of NAR on nuclear translocation of NF-κB and their reporter gene expression. Our results showed that TNF-α-induced nuclear translocation of NF-κB and NF-κB dependent reporter gene expression was inhibited following NAR treatment.

In spite of enormous protective mechanisms of NAR against chronic diseases, its high water insoluble nature makes the drug less affective when orally administered. Therefore novel drug delivery systems (NDDS) can greatly improve the performance of drugs in terms of efficacy, solubility and bioavailability (Derle *et al.*, 2010). We have successfully developed a novel naringenin-loaded nanoparticles (NARN) delivery system using Eudragid ® E 100 and PVA as carriers by a simple nanoprecipitation method. Our findings demonstrate that the prepared stable NARN were amorphous in nature and were highly soluble at acidic environment (pH 1.5).

In summary, our results demonstrated that NAR inhibited proliferation and induced apoptosis in glioma cells which has associated with the suppression of NF-κB activation. Therefore, we believe that these findings might help in providing the rationale to initiate *in vivo* studies to examine the efficacy of NAR as chemo-preventive agent against gliomas. The gastric solubility profile of Eudragid® E100 polymer may thus favour the NARN in attaining optimal release of NAR, which may subsequently result in enhanced bioactive efficacy.

Summary and Conclusions

In living systems, free radicals induce oxidative stress in cells and it is considered to be the causative factor for several chronic diseases. These free radicals are produced from both endogenous and exogenous sources. The toxic effects of free radicals are combated regularly by a number of endogenous defense and protective mechanisms which include various enzymes and non-enzymatic antioxidants. These self defense systems may also be supported by antioxidative compounds taken as food and medicines. Though many synthetic compounds like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiarybutylhydroquinone (TBHQ), etc., are efficient antioxidants; their use is being restricted because of their possible toxic and carcinogenic effects. Hence there is a great deal of interest for natural antioxidants obtained from diet, often referred to as "nutraceuticals", in view of their positive health effects. Several studies have shown that the action of the natural antioxidants at the cellular and molecular level involves scavenging of free radicals and protecting against cancer, diabetes and certain microbial infections. In view of this, nutraceuticals are becoming widely accepted as an adjunct to conventional therapies. Bioprospecting or the search for newer bioactive compounds from the nation's biodiversity for better health is a new thrust area in biotechnology.

Clinical trial and epidemiological studies have established an inverse correlation between the intake of whole grain cereals (but not refined ones) and the occurrence of disease such as type II diabetes, cancer and age-related disorders. Compared to refined grains, whole grain cereals are much richer in bioactive molecules, such as simple phenols, flavonoids, anthocyanins and phytoestrogens apart from high insoluble fiber, minerals and vitamins' which are generally recommended as a part of human diet. Among the cereals (including maize, wheat, corn, rice, oats, barley, rye, sorghum), rice

is one of the most important staple food source for over half of the world's population. In Asia alone, more than 2000 million people obtain 60 to 70 % of their calories from rice and its products. Rice bran constitutes about 10 % of rough rice grain. Although rice bran has been recognized as an excellent source of potent phytochemicals, vitamins, minerals, proteins and fiber, it has been under-utilized as a human food and has traditionally been used primarily in animal foods.

It has been recently shown that rice bran contains a unique complex of naturally occurring antioxidant compounds. It was also showed that rice bran has promising health-related benefits in the prevention of different diseases, including cancer, hyperlipidemia, fatty liver, hypercalciuria, kidney stones, heart diseases and more recently whole grain rice is strongly recommended as a staple food for diabetic patients. Thus preparation of products, enriched in the antioxidant components of rice bran, could be of great importance for the treatment of disorders associated with oxidative stress induced by free radicals. Ayurvedic treatise shows the existence of many medicinal rice varieties in India. Thus, proper documentation and research data on these varieties is of great importance. Njavara is one of the most important medicinal rice variety which is exclusively grown in Kerala, South India since ancient times. The dehusked rice is red in colour. It is regarded as a special rice variety with beneficial properties for the circulatory, respiratory and digestive systems, according to the Indian indigenous system of medicine or Ayurveda. It is bestowed with many medicinal properties and is used for the treatment of arthritis, cervical spondylitis, muscle wasting, skin diseases and certain neurological problems. These health benefits of Njavara are preserved only when it is used in the form of brown rice (whole grain). Since the Ayurvedic practice started in India, plant extracts are being used for the

treatment of various disorders associated with oxidative stress. The exact mechanism of these plant-derived preparations is not well understood which requires to be scientifically investigated. Inspite of alleged health benefits of Njavara rice in Ayurveda, scientific studies of this rice are scarce.

In the present study we examined the free radical scavenging properties of Njavara rice bran and compared with the two other commercially available white varieties; Vasumathi and Yamini and with a non-medicinal pigmented rice variety, Jyothi. The free radical scavenging properties were correlated with the total phenolic content (TPC). For taking insight into the effect of solvent property and TPC, Initially Njavara rice bran was used for extracting TPC by using three solvents (MeOH, EtOAc and Hex) with different polarities. Highest TPC was observed in methanolic extract (12.75 µg/g GAE) followed by EtOAc (7.89 µg/g GAE) and Hex (2.92 µg/g GAE). Hence further studies were carried out using MeOH as a solvent for phytochemical extraction.

Inorder to check the importance of whole grain with respect to phytochemical content, studies were carried out with polished rice, brown rice and bran from all the four varieties. The results indicated that the TPC and total flavonoid content (TFC) were more in bran followed by brown rice and polished rice in all the four varieties. An important observation in this study was that TPC content from pigmented rice varieties was 3-4 times higher than the white varieties. However, Njavara variety showed highest TPC and TFC compared to Jyothi, Yamini and Vasumathi. Since bran samples from all the four varieties exhibited greater TPC than brown rice and polished rice, further radical scavenging activities were carried out using bran samples. The radical scavenging activities of 1,1-Diphenyl-2-picrylhydrazyl (DPPH), superoxide, hydroxyl

radical, nitric oxide, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), hydrogen peroxide, reducing power and total antioxidant activity have shown that Njavara rice bran methanolic extract was superior radical scavenger compared to the other three rice varieties. Highly significant correlation between TPC of rice bran and different antiradical scavenging activities was recorded in all the rice varieities.

Our studies proved that Njavara rice bran is a potential source of antioxidants for radical scavenging. The highly positive correlation of antiradical scavenging activity and TPC indicates that polyphenols are the important components in rice bran methanolic extracts which could be used for the free radical scavenging activity. This is the first scientific study on the antiradical efficiency of an Indian medicinal rice, Njavara. The present results formed the basis for selection of Njavara rice bran for further investigation of its pharmacological properties; including antimicrobial, antidiabetic and antiproliferatory activities.

The antimicrobial properties were investigated using polished rice and bran from all the four rice varieties which was determined by well diffusion method. The following microbial strains were used for the study: gram negative bacteria [*Escherichia coli* (ATCC 25922), *Salmonella typhi* (CI), *Pseudomonas aeruginosa* (ATCC 27853)], gram positive bacteria [*Staphylococcus aureus* (CI), *Bacillus subtilis* (QST713)] *and* Fungus [*Candida albicans* (3153A)]. Bran samples from all the four rice varieties exhibited prominent antimicrobial activity against all the microbes used for the study whereas only polished rice samples from pigmented varieties exhibited significant antimicrobial activity against all the microbes tested. This difference in the antimicrobial activity of different extracts of tested samples could be due to the variation in the contents of phytochemical components among the four rice varieties.

The superior antimicrobial activity, in comparision with other three rice varieties, shown by Njavara samples could be due to its high phytochemical content and radical scavenging properties.

Since Njavara rice bran was superior in terms of phytochemical contents and free radical scavenging activity, further antidiabetic and antiproliferatory studies were carried out with Njavara bran. Antidiabetic property of the Njavara rice bran methanolic (NBM) extract was studied. Our *in vitro* studies demonstrated an appreciable α-glucosidase and amylase inhibitory activity of NBM with IC_{50} values 48 and 85 μg/ml respectively. *In vitro* inhibitory activities may not be always relate to the corresponding *in vivo* activity. In the *in vivo* experiments, aqueous dispersions of NBM extracts at the doses of 100, 300, and 500 mg/kg showed marked decrease in plasma blood glucose (PBG) levels in a dose dependent manner after the glucose load to the normoglycemic rats as well as diabetic rats. The results of this investigation suggest that, the phenolic compounds present in the NBM extract may regulate the glucose uptake from the intestinal lumen by inhibiting carbohydrate digestion and absorption, leading to normal glucose homeostasis. It is also possible that, phenolic compounds may regulate the glucose absorption by mechanisms other than enzyme inhibition. In the present study, the dose of alloxan (120mg/kg, bw) was selected in order to partially destroy the pancreatic β-cells. In these conditions, insulin was secreted but not sufficiently to regulate the blood glucose. NBM extract was able to decrease the glycemia in alloxan permanent hyperglycemic rats. This suggests that the increase of blood glucose obtained with alloxan results from a diminution but not due to the total abolition of insulin secretion.

When these alloxan-induced diabetic rats were treated with NBM extract, the PBG levels were decreased. At the concentration of 500 mg/kg NBM, the decrease in PBG levels was almost similar to the standard drug acarbose used for the study. NBM extract showed significant inhibitory activity against carbohydrate digestive enzymes and decreased the postprandial hyperglycemia both in normal and diabetic rats. NBM extract also showed antihyperglycemic effect on alloxan-induced diabetic rats.

Further, the effect of NBM on cell proliferation was also studied. MTT assay, cell viability and clonogenic assays revealed that NBM extract had significant anti-proliferative effects on U373 and C6 glioma cell lines. NBM also showed the *in vitro* inhibitory property against COX-1 and COX-2 with IC_{50} values of 92 and 39 µg/ml respectively, suggesting that NBM showed preference towards COX-2 inhibition rather than COX-1. To determine the proapoptotic effects of NBM, we performed cell cycle analysis. An analysis of our data indicated that NBM arrests the cell cycle at G2-M phase, and increased the apoptotic sub G1 fraction. To further investigate the apoptosis induction following NBM treatment, we determined the effects of NBM on caspase-3 and -8 activities, PARP cleavage and TUNEL assay. The results suggest that NBM induced apoptosis through activating the caspase-3 and -8 and the induction of PARP cleavage.

Glial tumors develop as a result of genetic alterations in low grade lesions, which further acquire additional mutations and progress to more malignant tumors. These genetic alterations disrupt the cell cycle arresting pathways, or activate various signal transduction pathways which contribute to the development of astrocytomas. The signaling pathway characterized by MAP kinases (pERK1/2, pJNK and p38) was known to play a crucial role in molecular and cellular events associated with the

pathogenesis. Our data suggest that compounds present in NBM have the potential to inhibit the activation of MAP kinases (pERK 1/2 and pJNK) and also inhibit the downstream proteins, including iNOS and COX-2. Likewise, NF-κB signaling pathway was constitutively activated in gliomas and associated with enhanced growth, cell cycle progression, and inducible chemo resistance. Because of the inhibition of NF-κB activation, which has been linked with antitumor activities, we hypothesize that NBM mediates its effect, at least partly, through inhibition of NF-κB activation. In this study, we checked the inhibitory effects of NBM on nuclear translocation of p50 and p65 and their reporter gene expression. Our results showed that TNF-α-induced nuclear translocation of p50 and p65 while NF-κB dependent reporter gene expression was inhibited following NBM treatment. Our results demonstrated NBM inhibited proliferation and induced apoptosis in glioma cells which was associated with the suppression of NF-κB activation, MAP kinases (pERK 1/2 and pJNK) and the downstream proteins, including iNOS and COX-2. Therefore, we believe that these findings might help in providing the rationale to initiating *in vivo* studies to examine the efficacy of NBM as chemo preventive agent against gliomas and explain some features of NBM- mediated chemoprevention.

Whole grain cereals are much richer in flavonoids, compared to the refined grains. Our studies also showed that whole grain rice contains 5-10 times higher TFC than the refined grains in all the rice varieties studied. Flavonoids comprise the major dietary group of plant polyphenols. Available literature shows that among the various types of flavonoids, the flavanone naringenin (NAR), exhibits wide range of bioactive effects on human health. In spite of enormous protective mechanisms of NAR against chronic diseases associated with oxidative stress, its high water insoluble nature makes

the drug less effective, when orally administered. Therefore novel drug delivery systems (NDDS) can greatly improve the performance of drugs in terms of efficacy, solubility and bioavailability.

We investigated the effects NAR on human glioma cells. In this study, we set out to investigate whether NAR has significant anti-proliferative effect on glioma cell lines. MTT assay, cell viability and clonogenic assays revealed that NAR had significant anti-proliferative effects on U373 and C6 glioma cell lines. Cell cycle analysis indicated that NAR increased the apoptotic sub G1 fraction, induced apoptosis through activating the caspase-3 and -8, induction of PARP cleavage and DNA fragmentation. In this study we also proved the inhibitory effects of NAR on nuclear translocation of NF-κB and their reporter gene expression. Our data showed that TNF-α-induced nuclear translocation of NF-κB and NF-κB dependent reporter gene expression and was inhibited following NAR treatment.

Inorder to increase the solubility and bioavailability of NAR, we have prepared naringenin nanoparticles (NARN) using Eudragid ® E 100 and PVA as carriers by a simple nanoprecipitation method. The prepared NARN were 45±5 nm in size, spherical in shape and amorphous in nature and they were also stable upto 30 days. These NARN particles were more soluble at pH 1.5 and 4.5 where as at pH 7.4 and in milli Q water, these NARN formed turbid solution. These observations suggest that the size of the particle released can depend on the pH of the buffer solution. The low pH value could more easily dissolve the gastro-soluble Eudragid ® E 100 polymer from NARN and thus yielded a more reduced particle size of NAR that was released.

Our results demonstrated that NAR inhibited proliferation and induced

apoptosis in glioma cells and was associated with the suppression of NF-κB activation. Therefore, we believe that these findings might help in providing the rationale to initiate *in vivo* studies to examine the efficacy of NAR as chemopreventive agent against gliomas. The gastric solubility profile of Eudragid® E100 polymer may thus favour the NARN in attaining optimal release of NAR, which may subsequently result in enhanced bioactive efficacy.

Silent findings in our study

- Njavara rice is a more potent antiradical scavenger, compared to the other three varieties; Vasumathi, Yamini and Jyothi.
- Rice, along with bran, is very effective as antimicrobial agent. Njavara showed potent antimicrobial properties, compared to the other three varieities; Vasumathi, Yamini and Jyothi.
- Inhibition of α-glucosidase and amylase activities, postprandial antihyperglycemic and antihyperglycemic property of NBM extract indicated the usefulness of this variety as orally administered antihyperglycemic agent.
- NBM extract inhibited the proliferation of C6 glioma cells by downregulating MAP kinases, NF-κB pathway and induced apoptosis by caspase dependent mechanism.
- Naringenin inhibited the proliferation of U373 glioma cells by downregulating NF-κB pathway and induced apoptosis in a caspase-dependent mechanism.
- Naringenin nanoparticles were prepared which were spherical in shape with 45±5 nm size and were amorphous.
- The NARN particles were stable for 30 days and they were highly soluble at pH 2.0.

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