CHAPTER-7

General summary
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Reactive Oxygen Species (ROS) are constitutively produced in small quantities aimed at killing invading pathogens through activation of natural killer cells (Morrel, C.N., 2008). They are very transient species due to their high chemical reactivity that leads to lipid peroxidation and oxidation of DNA and proteins. (Mates, M., 2000, Droge, 2002). The endogenous antioxidants can combat the oxidative stress only to a limited extent when overwhelming quantities are produced. The above leads to oxidative cellular injury by changing the balance between pro-oxidant and antioxidant factors (Sayre et al., 2007, Agarwal, et al., 2012). In order to protect the tissues and cells from oxidative stress antioxidants can be provided externally. Evidence exists for those synthetic antioxidants like BHA, BHT are limited in their scope due to carcinogenicity at more than 0.1% dosage (EFSA, 2012).

The World Cancer Research Fund/American Institute for Cancer Research in 2007 reports that, the complex role of diet in prevention or curing of chronic diseases. A typical diet provides more than 25,000 bioactive food constituents many of which may modify a multitude of processes that are related to these diseases. Earlier studies in our laboratory have reported that, dietary components such as turmeric, Sundakai, Curry leaves and spice/vegetable extracts could effectively quench the ROS, and exert their protective effect at cellular levels (Shalini and Srinivas, 1987; Srinivas and Shalini, 1991; Sujatha and Srinivas, 1995, Sivapriya and Leela Srinivas, 2007, Ningappa et al., 2008). Earlier from our laboratory, a 6 kDa protein, 8 kDa protein (US process / product patent, US 8,389,677B2) and 14 kDa protein were isolated, characterized, sequenced (P85278- UNIPROT) from Turmeric (Chethankumar M and Leela Srinivas, 2008, Shalini et al., 1992). The above information initiated us to search for a new antioxidant protein from Turmeric (Curcuma longa L).

Hydroxyl radicals are highly reactive; they can damage virtually cellular macromolecules such as DNA, lipid and proteins. Lipid peroxidation is oxidative degradation products of lipids, in which free radicals take electrons from the lipids incell membranes, resulting in cell damage and leads to a free radical chain reaction mechanism. 1,1-Diphenyl-2-picrylhydrazyl, (DPPH) is a cell-permeable, stable free radical that acts as a hydrogen radical scavenger. It is useful as a screening tool for detecting free radical scavenging capacity of other
antioxidants. The superoxide radical is a free radical, in which two oxygen molecules are connected together and one oxygen has an extra electron and this radical damage cell membranes.

The second chapter reveals the screening of widely used, eight popular spices for their antioxidant activity. In an effort to identify the efficient antioxidant fraction from these spices, we evaluated the antioxidant properties of spice extracts of ambient temperature water, boiling water, ethanol, methanol, hexane and petroleum ether using various in vitro model systems where BHA, Curcumin and alpha tocopherol used as standard antioxidants as shown in Fig. 2.1 to 2.8. The following extracts of spices show more antioxidant activity in four antioxidant model systems. The ethanol extract of black pepper and boiling water extract of Curcuma longa (64%) showed more hydroxyl radical scavenging activity. The boiling water extract (68%) of Curcuma longa showed more lipid peroxidation inhibition activity. The boiling water extract of Curcuma longa (58%) showed more DPPH radical scavenging activity and the ethanol extract of Curcuma aromatica (56%) superoxide radical scavenging activity. Finally when all these extracts of spices were subjected to thermal stability test by keeping in boiling water bath for 60 minutes and then their antioxidant activity towards lipid peroxidation inhibition was analyzed. The results indicates that, only boiling water extract of Turmeric was retained its most of antioxidant activity. Hence, boiling water extract of Turmeric was selected for further studies to identify the active principle of the extract.

Turmeric is one of most popular spice all over the world with a long and distinguished human use particularly in the Eastern civilization (Ravindran, 2007). Apart from its culinary uses, turmeric has been used widely in the traditional medicine because of its several health beneficial properties (Chattopadhyay et al., 2004, Damalas, C.A., 2011). The third chapter represents the isolation and characterization of new antioxidant protein from Turmeric (Curcuma longa L) and this protein named by us as “BGS-Haridrin”. BGS-Haridrin was found to be acidic as evidenced by native gel and appeared as homogenous band on SDS PAGE of molecular weight approximately around 28 kDa and the purity was further confirmed by rp-HPLC, which showed single peak and further molecular weight was confirmed by MS MALDI. The biochemical and biophysical nature of BGS-Haridrin was studied and the proteinaceous nature was confirmed by various tests. The antioxidant activity of
BGS-Haridrin seems to be reasonable when stored at 4°C and -20°C. PAS staining confirmed that, BGS-Haridrin was a glycoprotein. The antioxidant activity of BGS-Haridrin was studied in comparison with known antioxidants such as α-tocopherol, Curcumin, Ascorbate and synthetic antioxidant, BHA. BGS-Haridrin (0.1µM) showed antioxidant activity of 80% at low dose towards scavenging hydroxyl radicals. It inhibits lipid peroxidation against significantly to the tune of 76.4%. This result confirms that, BGS-Haridrin is an effective inhibitor of lipid peroxidation at a low dose. The antioxidant activity of BGS-Haridrin was comparable to α-tocopherol (400µM), Curcumin (400µM) and BHA (400µM) with very low of 4000 folds less dosage. It exhibited powerful stable DPPH radical scavenging activity to the tune of 73% at a dosage of 0.43nM (12µg) and it was compared with the Ascorbic acid at dosage of 56.77nM (10µg). BGS-Haridrin inhibited the superoxide radicals which are generated during enzymatic activities up to 92% at a dose of 0.285nM (8µg) whereas, Curcumin inhibits the same 90% at a dose of 19nM (7µg). This shows the efficiency of BGS-Haridrin at a very low dose towards inhibiting superoxide radicals. The ferric ion reducing activity and ferrous ion chelating activity of BGS-Haridrin are comparable to standard molecules. The products of protein oxidation in biological samples are the protein carbonyl derivatives of Pro, Arg, Lys, and Thr (Wong et al., 2006). These derivatives are chemically stable and serve as markers of oxidative stress for most types of ROS. The amount of protein carbonyls formed is estimated by Protein carbonyl assay, where BSA was used as standard. The amount of protein carbonyl formed in BGS-Haridrin was 90.9 nmol/mg, this results showed that, BGS-Haridrin was chemically stable.

To establish whether a synergy of complimentary antioxidant activity exists between the three antioxidant components of Turmeric namely BGS-Turmerin, BGS-Haridrin and Curcumin, an interaction study was done by keeping BGS-Haridrin as constant, to this an increasing amount of BGS-Turmerin was added and the reverse was also studied. In the same way, the interaction study was done using BGS-Haridrin and Curcumin. BGS-Turmerin is a 14 kDa protein isolated, purified and sequenced in our laboratory. The antioxidant synergy between BGS-Haridrin and BGS-Turmerin was studied, where; BGS-Haridrin was kept as constant at its IC-50 dose of 0.05µM. It provided inhibition of 40% by scavenging hydroxyl radicals. To this, BGS-Turmerin was added in increasing dose of 0 to 0.175 µM. Addition of
BGS-Turmerin (0.175 µM) to BGS-Haridrin increases the antioxidant activity by 49% at a mole ratio 3:1. Similarly, the reverse was studied by keeping BGS-Turmerin as constant at its IC-50 value 0.090µM and BGS-Haridrin was added in increasing dose of 0 to 0.1µM. It gave an inhibition of 38% at a dosage of BGS-Turmerin with an IC-50 value of 0.09µM alone. The addition of BGS-Haridrin (0.1µM) to BGS-Turmerin (0.09µM) increases the antioxidant activity by 58% at a mole ratio 1:1. The synergistic antioxidant seems to be better when BGS-Hariderin is added to BGS-Turmerin possibly indicating a better molecular interaction between 28kDa BGS-Haridrin and 14 kDa protein BGS-Turmerin. In the second part of the experiment, the antioxidant synergy between BGS-Haridrin and Curcumin was studied. BGS-Haridrin was kept as constant with an IC-50 value 0.05µM. BGS-Haridrin alone gave an inhibition of 40% by scavenging hydroxyl radicals and Curcumin was added in increasing dose of 0 to 400 µM. The cocktail of BGS-Haridrin (40%) and Curcumin 400µM increases the antioxidant activity by 38% at a molar ratio of 1:4000. Similarly, the reverse was studied by keeping Curcumin as constant with an IC-50 value 200µM. It gave an inhibition of 34% as shown in the figure 3.17. BGS-Haridrin was added in increasing dosage of 0 to 0.1µM. The cocktail of Curcumin 200µM and BGS-Haridrin 0.1µM increases the antioxidant activity by 55%. The synergistic antioxidant seems to be better when Haridrin is added to Curcumin in increasing order indicating a better interaction between Curcumin and BGS-Haridrin. These combination forms the multi potent antioxidant activity and ability of scavenging free radicals in a lower dosage. It also shows that, the fat soluble Curcumin and the water soluble Haridrin may possibly protect the lipid membrane and the internal components in the cytosol like vitamin E and vitamin C.

Reactive oxygen species, such as hydroxyl radical, hydrogen peroxide, superoxide, peroxynitrite and others, are major sources of oxidative stress in cells, damaging proteins, lipids, and DNA (Orrenius et al., 2007, Perron et al., 2008). In fourth chapter, the study was made to evaluate inhibitory effects of BGS-Haridrin on oxidative DNA damage caused by H₂O₂, t-BOOH, Fenton reactants, UV rays, Cigarette smoke, bidi smoke and organic fuel smoke condensates. These causes a series of DNA lesions, including single-strand, double-strand breaks, cross linking of DNA and damage to bases. It is noteworthy that, BGS-Haridrin at a lower dose of 0.1µM extends very good protection to DNA, though there is protection offered by
other antioxidants like BHA or α-tocopherol and Curcumin at a dose of 400µM, which is 4000 times more and added to the above BGS-Haridrin is non toxic.

The viability of human erythrocyte cells is a good model system to evaluate the toxicity of test compound and also the protection affected by the protectants. Freshly isolated human erythrocyte cells are classified into five groups. First group of lymphocytes are called control and their viability was analyzed using Trypan blue dye exclusion method. The viability of lymphocytes in control group ranges between 81 to 86%. The 2nd group of lymphocytes treated with cell death inducers, ranges from 23 to 61%. The third group of lymphocytes treated with cell death inducers along with antioxidants BHA or alpha tocopherol, the % of viable cells ranges from 66 to 70%. The fourth group of lymphocytes treated with Curcumin along with cell death inducers. The viability of cells ranges from 45 to 78%. The fifth group of lymphocytes treated with cell death inducers along with the protectant BGS-Haridrin. The % viable cells ranges from 70 to 78% protected from cell death inducers. The above results confirm the potency of antioxidant BGS-Haridrin towards recuding / preventing % of cell death and indicate its non toxicity by itself.

The studies in the chapter V reveal that, the antibacterial and antiprotease properties of BGS-Haridrin. The antibacterial properties of BGS-Haridrin was analyzed against human pathogenic bacteria, Escherichia coli, Vibrio cholerae, Streptococcus sps, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhimurium, Proteus vulgaris and Pseudomonas. The dose dependent study showed that BGS-Haridrin inhibits the growth of Escherichia coli, Vibrio cholerae, Klebsiella pneumoniae, Staphylococcus aureus, Proteus vulgaris, and Streptococcus sps. It results found to be moderately effective when compared to standard antibiotic Streptomycin. The results of zone of inhibition indicated that BGS-Haridrin at 15 µg/ disc exhibited broad spectrum of antibacterial activity by producing clear zones of inhibition ranges between 13 to 16 mm when compared to standard antibiotic Streptomycin which give a ranges of 14 to 18mm. The minimum inhibitory concentration (MIC) provides BGS-Haridrin is having antibacterial property at least dose and the special feature of thermal stability of BGS-Haridrin. The antiprotease activity of BGS-Haridrin was checked against serine protease Trypsin in different model systems. First it was compared with other standard protease inhibitors like EDTA, EGTA and PMSF to confirm that, BGS-Haridrin was a
serine protease inhibitor. The caseinolytic activity was checked, PMSF used as standard inhibitor. At 120µg dose of BGS-Haridrin and PMSF, a maximum inhibition of trypsin protease activity of 85% and 90% respectively was noted. In the recalcification ability study was done to find the anticoagulant property of BGS-Haridrin and a standard anticoagulant PMSF (10 to 50µg) using PPP isolated from fresh human blood and by adding CaCl\textsubscript{2}. BGS-Haridrin showed very negligible amount of recalcification activity and the clotting time was reduced from 84 to 68 seconds and whereas, PMSF reduced the clotting time from 80 to 60 seconds. The dose dependent hydrolysis of human fibrinogenolytic activity by BGS-Haridrin was done. At a maximum dosage of 50µg of BGS-Haridrin, the hydrolysis of human fibrinogenolytic activity induced by serine protease was completely prevented.

Some exogenous sources of free radicals are UV- radiation, smoking, alcohol, iron-overload, pesticides, etc. The imbalance between these production and elimination of free radicals may cause oxidative stress. Free radicals can be scavenged by several enzymes like glutathione peroxidase, catalase, superoxide dismutase as well as by the non-enzymatic antioxidant defence system like tocopherol, β-carotene, vitamin C, glutathione, lipoic acid, uric acid, bilirubin, which quench their activity. Therefore, much attention is now focused on the role of the enhancement of the defences against ROS (Palmieri and Sblendorio, 2007, Machlin and Bendich, 1987, Sies,H., 1993). Chapter VI describes another line of study done to investigate the protective role of BGS-Haridrin against iron overload induced oxidative stress in male Swiss Wistar mice. The treatment of BGS-Haridrin, Curcumin and Ascorbic to the iron overload group significantly protects the level of enzymic antioxidants such as SOD, Catalase, GST, GPx and non-enzymic antioxidants such as vitamin C, vitamin E and reduced glutathione in the serum and the various organs of the mice namely liver, lung, heart and kidney. The level of MDA was estimated by lipid peroxidation which shows that the iron overloaded group animals had an increased level of MDA whereas the animals treated with BGS-Haridrin showed significantly decreased levels of MDA.

Thus all our lines of investigation reveal that, BGS-Haridrin to be a possible potent antioxidant capable of ameliorating oxidative stress at various cellular levels from cellular membrane to DNA. Attempts were made to sequence the protein, but could not succeed as the ionization was not enough to detect amino acid residues with certainty. However, this is being taken up as a separate project.
Reference

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