INTRODUCTION

Reactive oxygen species (ROS) are highly reactive derived from the metabolism of oxygen. ROS may required for normal cell function in a small quantity, but excessive production of ROS can be detrimental to cells and may cause oxidative damage to lipids, proteins, and DNA. This oxidative damage caused by a disturbance of the balance between the antioxidant defense mechanisms of the human organism and the level of reactive oxygen species (ROS) has been associated with many pathological disorders such as atherosclerosis, diabetes and cancer.

Supplementation with exogenous antioxidants may help to restore this balance. Synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butylated Hydroxyl Toluene (BHT) are widely used as antioxidants but there is a need in identification of effective natural antioxidants which is are inexpensive and easily available. Dietary antioxidants, such as proteins, ascorbate, tocopherol. Phenolics compounds, flavonoids from spices, herbs, fruits and vegetables could help body to protect the cells from damage caused by oxidative stress and to secure the defense system against degenerative diseases.

Many Indian medicinal plant, herbs, spices are considered potential sources of antioxidant compounds and in some cases, their active constituents are known (9,10). Earlier studies in our laboratory shown that, antioxidant proteins isolated, purified and characterized from dietary components, such as turmeric (Curcuma longa), Curry leaves (Murraya koenigii), Sundakai (Solnum torum), Methi leaves (Trigonella Foenum Gracum) effectively inhibit ROS-induced lipid peroxidation and DNA damage.

Turmeric (Curcuma longa L) belongs to Zingiberaceae family, is widely used as a spice, colouring agent and for its flavour in Indian and South East Asian diet. The rhizome is extensively used in Ayurveda and traditional medicine. The Curcumin present in Turmeric is reported as antioxidant, anti-carcinogen, anti-HIV, anti-bacterial, anti-fungal, and anti-inflammatory activities. Many active protein antioxidants are reported from Turmeric are excellent antioxidants, anti-tumour promoter, DNA damage protective, anti-inflammatory.

In this study, the glycoprotein isolated, purified and characterized that lead to efficient natural antioxidant and hence prevents H2O2 induced DNA damage and also protects lymphocytes.

MATERIALS AND METHODS:

Butylated hydroxyl anisole (BHA), α-tocopherol, acrylamide, bisacrylamide, N,N',N'-tetramethylethylenediamine (TEMED), trichloro acetic acid (TCA) thiobarbituric acid (TBA), calf thymus DNA, ferrous sulphate, ascorbic acid, 2-deoxy ribose, ferric chloride, disodium ethylenediamine tetra acetic acid (EDTA) and nitro blue tetrazolium (NBT) were purchased from Sigma (St. Louis, USA). All other chemicals unless otherwise mentioned were procured from Merck (Darmstadt, Germany), Shimadzu UV-1601 spectrophotometer (Tokyo, Japan) was used for calorimetric analysis and Kubota 6800 (Kubota Co., Osaka, Japan) was used for centrifugation, transiluminator (Sydney, Australia) was used to visualize the DNA damage.

Purification of protein:

In the present investigation, Turmeric powder was obtained from local market and BGS-Haridrin was isolated as described previously by Dinesha R and Leela Srinivas, 2010.

Reversed Phase–HPLC of BGS-Haridrin

Reversed Phase HPLC (RP-HPLC) of BGS-Haridrin was done using Shimadzu double pump HPLC system. SBI of HPLC quality water was injected and a blank gradient was run to identify any background noise and to balance baseline. BGS-Haridrin (50g in 500l HPLC quality water) was injected onto C18 µ-Bondpak column. The fractions were collected at the flow rate of 1ml/min and eluted by gradient elution using (0-90%) acetonitrile:water as the mobile phase (with 0.1%TFA). The peak was monitored by fluorescence emission at 345nm (Excitation at 285nm).

SDS-PAGE of BGS-Haridrin

Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) of BGS-Haridrin was carried out in slab gels according to the method of Laemmli. BGS-Haridrin (15g) and medium range molecular weight marker mix (15lg) were loaded onto the gel and the electrophoresis was carried out at 50V for 3% stacking gel and 90V for 10% resolving gel. After electrophoresis the gel was stained with 0.05% (w/v) Coomassie blue R250 dye in acetic acid:methanol:water (60:30:10, v/v) for 8 hrs. The gel was stained in the same solution without the dye.

Determination of molecular mass of BGS-Haridrin by Q-Tof Mass Spectrometer:

Electro spray ionization spectra of BGS-Haridrin were recorded on a Hewlett-Packard (Model HP-1100) electro spray mass spectrometer. Electro spray ionization was carried out using a capillary with an ID of 0.1mm. The tip was held at 5000V in a positive ion detection mode. Nebulization was assisted by Nitrogen gas (99.8%) at a flow rate of 10 L/min. the spray chamber was held at 300°C. Data was acquired over a suitable mass range using a conventional quadrupole detector with cycle time of 3s.
Estimations:
The total protein content of the crude extract was determined by Bradford’s method \(^{(2)}\); the total sugar was estimated by the phenol-sulphuric acid method \(^{(21)}\). Total phenolic content was determined by the Folin-Ciocalteau reagent \(^{(22)}\). The curcuminoids content was determined according to Ravindranath et al \((1981)\) method \(^{(23)}\). The flavonoid content was determined according to Cheon et al \((2000)\) method \(^{(24)}\).

Deoxyribose assay:
The hydroxyl radical scavenging activity of BGS-Haridrin was done according to the method of Halliwell et al. \((1981)\) with minor modifications. The reaction mixture containing FeCl\(_3\) (100 μM), EDTA (104 μM), H\(_2\)O\(_2\) (1 mM) and 2-deoxy-D-ribose (2.8 mM) were mixed with or without BGS-Haridrin at various concentrations (0.2nM to 1.0 nM) in 1 ml final reaction volume made with potassium phosphate buffer (20 mM pH 7.4) and incubated for one hour at 37°C.

BHA and Curcumin (400μM) were used as positive control. The mixture was heated at 95°C in water bath for 15 min followed by the addition of 1 ml each of TCA (2.8%) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA). Finally the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm using the negative control. The % inhibition of hydroxyl radical scavenging activity of protein was determined.

Lipid peroxidation inhibition assay:
The evaluation of antioxidant activity of BGS-Haridrin based on the inhibition of peroxidation in RBC ghost was done according to Shimazaki et al. \((1984)\) \(^{(25)}\) with minor modifications. The reaction mixture was treated with or without BGS-Haridrin (0.2 nM to 1.0 nM), BHA, α-tocopherol and Curcumin (400μM) were used as positive control. The contents were incubated for 1 h at 37°C. The reaction was terminated by the addition of 10 μl of 5% phenol and 1 ml of 1% TCA. To each system 1 ml of 1% TBA was added and the contents were kept in a boiling water bath for 15 min, cooled and centrifuged at 6000 rpm for 10 min.

The absorbance of supernatants was measured colorimetrically at 535 nm. Appropriate blanks were included for each measurement. The negative control without any test sample was considered as 100% peroxidation. The % inhibition of lipid peroxidation was determined accordingly by comparing the absorbance of the test samples with negative control.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity:
DPPH radical scavenging activity was assessed according to the method of Shimada et al. \((1992)\) \(^{(26)}\) with minor modifications. The BGS-Haridrin at concentrations ranging from 0.2 nM to 1.0 nM was mixed in 1 ml of freshly prepared 0.5 mM DPPH ethanolic solution and 2 ml of 0.1 M acetic buffer pH 5.5. The resulting solutions were then incubated at 37°C for 30 min and measured colorimetrically at 517 nm. BHA and Curcumin (400 μM) was used as positive control under the same assay conditions. Negative control was without any inhibitor or BGS-Haridrin. Lower absorbance at 517 nm represents higher DPPH scavenging activity. The % DPPH radical scavenging activity of BGS-Haridrin was calculated from the decrease in absorbance at 517 nm in comparison with negative control.
Reversed Phase HPLC (RP-HPLC) of BGS-Haridrin (5µg in 50µl HPLC quality water), injected to C18 µ-Bondpak column. Fractions collected at the flow rate of 1ml/min, eluted by gradient elution using (0–90%) acetonitrile:water as mobile phase (with 0.1%TFA).

MALDI/MS analysis of BGS-Haridrin

The chromatogram of purified protein BGS-Haridrin by MALDI/MS analysis (MALDI TOF Bruker Ultra.ex II.) shows single peak at 28.3 kDa.

Hydroxyl radical scavenging activity of BGS-Haridrin

Dose-dependent hydroxyl radical scavenging activity by the antioxidant protein BGS-Haridrin along with other standard antioxidants by deoxyribose method. Deoxy-D-ribose (2.8 mM) + ferric chloride + ascorbic acid (100:100 µM) + H₂O₂; (1 mM) + EDTA ± various concentrations of BGS-Haridrin (0.2 to 1.0 nM) in 1.0 ml PB (0.02M phosphate buffer, pH 7.4), incubating at 37°C for 30 min and the absorbance read at 517 nm using spectrophotometer. Values are means ± SD of triplicates.

Inhibition of peroxides induced DNA degradation by BGS-Haridrin

Erythrocyte ghost (300 µg) + ferrous sulphate + ascorbic acid (10:100 µmol) ± various concentrations of purified protein (0.2–1.0 nM) in 500 µl of TBS (10 mM Tris, pH 7.4, 0.15 M saline), incubating at 37°C for 60 min. Lipid peroxidation was measured by TBARS. Values are means ± SD of triplicates.
Results and Discussion

This study describes the antioxidant and DNA damage protective activities of BGS-Haridrin isolated boiling water extract of Turmeric (Curcuma longa L). BGS-Haridrin was isolated from boiling water extract of Turmeric according Dinesha R and Leela Srinivas (2010) and Srinivas L et al (1992). About 0.22mg/g of BGS-Haridrin protein was recovered after different stages of purification.

The SDS-PAGE of BGS-Haridrin on 10% separating gel, showed a molecular mass of ~28kDa when compared to medium range molecular mass markers

Estimations:
The proximate analysis of BGS-Haridrin comprised of much protein content and negligible amount of carbohydrates (data not shown).

Hydroxyl radical-scavenging assay:

Hydroxyl radicals are most reactive and initiate cell damage. Further, we studied the effect of the BGS-Haridrin on hydroxyl radicals generated by Fe³⁺ ions by finding the degree of deoxyribose degradation, an indicator of TBA- Malondialdehyde (MDA) adduct formation. The dose dependent study of the purified protein exhibited an inhibition of the hydroxyl radical scavenging activity. A maximum of 82% inhibition was obtained at 0.9 nM of the protein.

Lipid peroxide inhibition assay:

To find the oxidative stress reducing capability, the lipid peroxidation assay that determines the production of MDA and related lipid peroxides in RBC membrane was carried out. As by-products of lipid peroxidation, ferrous sulfate ascorbate system induced TBA reactive substances are produced. The inhibitory effect of BGS-Haridrin against ferrous sulfate and ascorbic acid-induced ghost lipid peroxidation, BGS-Haridrin showed the highest inhibition of ghost lipid peroxidation, about 78% at 0.9nM concentration. The antioxidant activity of BGS-Haridrin was compared with standard antioxidants, such as BHA and Curcumin. Each of these antioxidants inhibited lipid peroxidation by 78% and 70% at 400 μM concentration, respectively (Figure 4).

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay:

The hydroxyl radical scavenging activity of the BGS-Haridrin was further confirmed using a direct approach with DPPH radicals - a stable radical used to evaluate the antioxidant activity of BGS-Haridrin. As shown in Figure 6, BGS-Haridrin showed a dose-dependent DPPH radical-scavenging activity, and at 0.9 nM dosage the protein showed maximum inhibition of 76%. The protein exhibited a statistically significant DPPH radical-scavenging activity when compared to the group without any inhibitor. At 400μM dosage standard antioxidants like BHA, and Curcumin showed 88% and 89% of scavenging DPPH radicals, respectively.

Protective effect of BGS-Haridrin on H₂O₂-induced DNA damage:

Oxidative damage of DNA damage resulted in various degenerative diseases. When calf thymus DNA was treated with H₂O₂ for 30 min, extensive DNA fragmentation due to oxidation by hydroxyl radicals was seen on agarose gel, by the enhanced mobility (Figure 8, lane B as compared to the untreated DNA (lane A)). Known antioxidants such as BHA and Curcumin (each 100μg) protected DNA damage as assessed by the similar mobility of the DNA (lane D & E) in comparison to the untreated DNA (lane A). BGS-Haridrin exhibits DNA protective activity at 15μg (lane C) (Figure 7). The effectiveness of BGS-Haridrin to prevent H₂O₂-induced DNA damage was associated to its hydroxyl radical scavenging activity and also lipid peroxidation inhibition activity.

### RESULTS AND DISCUSSION

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The SDS-PAGE of BGS-Haridrin on 10% separating gel, showed a molecular mass of ~28kDa when compared to medium range molecular mass markers (Figure 1). RP-HPLC profile of BGS-Haridrin (Figure-2) shows sharp peak and Q-Tof mass spectrum analysis indicated a relative molecular mass of 28.3kDa (Figure 3).

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observed by Deoxyribose assay and TBARS assay. This suggests that BGS-Haridrin may prevent H₂O₂ mediated oxidative DNA damage.

Prevention of H₂O₂ induced cell death by BGS-Haridrin:

The inhibition ability of BGS-Haridrin against H₂O₂ induced cell death in lymphocytes was tested. The maximum cell death induced by H₂O₂ was seen at 30 min. BGS-Haridrin at 0.9 nM concentration offered prevention of cell death (Figure 8). The prevention offered by BGS-Haridrin was 62% at 30 min as against 53-56% offered by BHA and Curcumin (each 400 μM) respectively. It was observed that BGS-Haridrin (0.9 nM) at different time intervals (0–5 h) did not significantly affect the viability of cells. It was found that the viability of cells treated with BGS-Haridrin alone was 82% when compared to that of control cells (Lymphocytes alone).

CONCLUSION

This work highlights the importance of Turmeric which has been traditionally used in India and other countries. The antioxidant protein BGS-Haridrin isolated from boiling water extract of Turmeric (Curcuma longa L) exhibit different radical scavenging activities and thus can be used as an effective antioxidant agent to cellular components. Thus the antioxidant activity of BGS-Haridrin may due to the synergistic action protein. This study also suggests that further understanding the mechanism of action of the antioxidant protein BGS-Haridrin and also its in vivo antioxidant mechanism.

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