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Research Article

ANTIOXIDANT EFFECTS OF 28KDA ANTIOXIDANT PROTEIN FROM TURMERIC (*CURCUMA LONGA L*)

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ABSTRACT

A 28 kDa glyco protein isolated, purified and characterized from boiling water extract of Turmeric (Curcuma longa L) and named as BGS-Haridrin. The protein showed sharp single peak in RP-HPLC with a retention time of 32.6 min. The apparent molecular weight of BGS-Haridrin was w28 kDa showed in SDS PAGE and MALDI/MS analysis. BGS-Haridrin scavenged hydroxyl, DPPH radicals, superoxide radicals 76-82 % about and inhibited lipid peroxidation about 78% at a maximum dosage of 0.9 nM concentration when compared to BHA, Curcumin (400 µM) and α-tocopherol (400µM). BGS Haridrin effectively protects H_2O_2 (100µM) induced cell death in human peripheral lymphocytes. Further BGS-Haridrin prevents H_2O_2 (1mM) caused calf thymus DNA damage as evidenced by agarose gel electrophoresis. In summary, the results represent *in vitro* antioxidant effects of BGS-Haridrin antioxidant protein from Turmeric (*Curcuma longa L*).

Key words: Antioxidants, Turmeric protein, BGS-Haridrin, Lipid peroxidation, DNA damage, H_2O_2.

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 [1, 2, 3, 4].

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 [5, 6]. Dietary antioxidants, such as proteins, ascorbate, α-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 [7,8]. 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 Koeneigii*), Sundakai (*Solanum torvum*), Methi leaves (*Trigonella Foenum Graecum*) effectively inhibit ROS-induced lipid peroxidation and DNA damage [11, 12, 13, 14, 03, 15, 02, 16].

Turmeric (*Curcuma long 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 [17]. The Curcumin present in Turmeric is reported as antioxidant, anti-carcinogen, anti-HIV, anti-bacterial, anti-fungal, and anti-inflammatory activities [18]. Many active protein antioxidants are reported from Turmeric are excellent antioxidants, anti-tumour promoter, DNA damage protectant, anti-inflammant [11, 12, 15].

In this study, the glycoprotein isolated, purified and characterized that lead to an efficient natural antioxidant and hence prevents H_2O_2 induced DNA damage and also protects lymphocytes.

MATERIALS AND METHODS:

Butylated hydroxyl anisole (BHA), α-tocopherol, acrylamide, bisacrylamide, N,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 (Dermastadt, Germany). Shimadzu UV-1601 spectrophotometer (Tokyo, Japan) was used for calorimetric analysis and Kubota 6800 (Kubota Co., Osaka, Japan) was used for centrifugation, transilluminator (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. 5□l of HPLC quality water was injected and a blank gradient was run to identify any background noise and to balance baseline. BGS-Haridrin (5□g in 50□l 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 [19]. BGS-Haridrin (15□g) and medium range molecular weight marker mix (15□g) 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 distained 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 spectrometry. 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 [20]; 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 [25]. The reaction mixture containing $FeCl_3$ (100 µM), EDTA (104 µM), H_2O_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 without any antioxidant was considered 100% oxidation. The percentage 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 [26] with minor modifications. The evaluation of oxidation was done by measuring the TBA reactive substances[27]. The human erythrocyte ghost was isolated according to the method of Dodge et al. (1963) [28]. 100 µl of ghost suspension (300 µg membrane protein equivalent) was subjected to peroxidation by ferrous sulphate and ascorbic acid (10:100 µmol) (Fenton, 1984)[29] in final volume of 1 ml of Tris buffered saline (20 mM, pH 7.4,150 mM NaCl).

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, 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)[30] 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 acetate 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.

Protective effect of BGS-Haridrin on H_2O_2 induced DNA damage:

The extent of DNA oxidation by H_2O_2 was analyzed on 0.6% submarine agarose gel according to the method of Sultan et al., (1995)[31] with minor modifications. Calf thymus DNA (10 mg was mixed well in one ml of 20 mM phosphate buffer, at pH 7.4, 150 mM NaCl and store at 4□C. It was sheared using a 21-gauge needle to obtain low molecular weight species. Then 15 µg of calf thymus DNA was treated with 1mM of H_2O_2 with or without BGS-Haridrin (0.9nM), BHA (400µM) and Curcumin (400µM) in 100µl final volume of 20 mM potassium phosphate buffer, at pH 7.4. The reaction mixture was mixed with 10 µl of loading buffer (0.5% bromophenol blue, 50% glycerol in water) and then the reaction mixture was incubated at 37□C for 30 min and then placed on ice for 10 min to stop the reaction. 20µl (15 µg) of DNA was run on 0.6% agarose with ethidium bromide (1 µg/ml). The electrophoresis was conducted in TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, 18 mM NaCl, pH 8) at 60 V for 3 h and DNA was visualized under a UV transilluminator.

Prevention of H_2O_2 induced cell death by BGS-Haridrin:

Human peripheral lymphocytes were isolated from freshly drawn human blood and cell viability was monitored by tryphan blue dye exclusion method according to H.J. Phillips, (1973) method [32]. The initially isolated lymphocytes isolated were tested for its viability by distributing equally, such that each tube contains $1X10^6$ cells and considered as control. The rest was subjected to H_2O_2 (1 mM) in 250 ml of HBSS pH-7.4 with and without antioxidant, BGS-Haridrin (0.9nM), Curcumin (400µM) and BHA (400 µM). It was incubated at 37□C. At different time intervals (10, 20, and 30 min, 1 h, 3 h and 5 h) an aliquot of reaction mixture was taken mixed with tryphan blue and loaded into hemocytometer. The toxicity study was also done for BGS-Haridrin at different concentration (0.2 to 1.0 nM).

Statistical analysis:

Statistical analysis was done using SPSS (Windows version 10.01; SPSS Inc. Chicago,1L) using a one way student's t-test and a $p < 0.05$ was considered as statistically significant when compared with controls and results refer to mean ± SD.

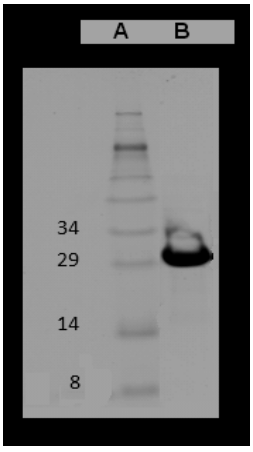

Fig. 1: SDS PAGE of BGS-Haridrin

Electrophoretic profile of BGS-Haridrin on SDS-PAGE, BGS-Haridrin (15µg) and medium range molecular weight markers (15µg) loaded on gel, electrophoresis carried at 80V for 3% stacking gel, 40V for 10% resolving gel. After electrophoresis the gel stained with Coomassie brilliant blue R250 stain for 8h. Lane A= medium range molecular weight markers and lane B = BGS-Haridrin (15µg) respectively.

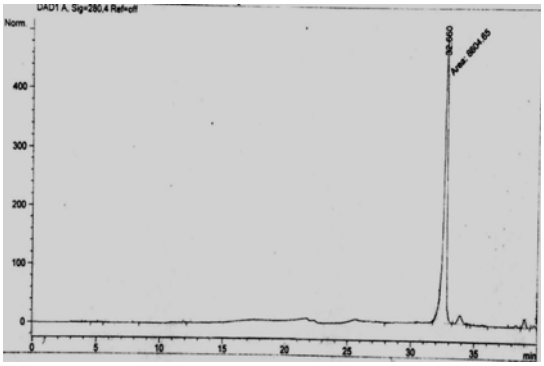

Fig. 2: Reversed phase-HPLC of BGS-Haridrin

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).

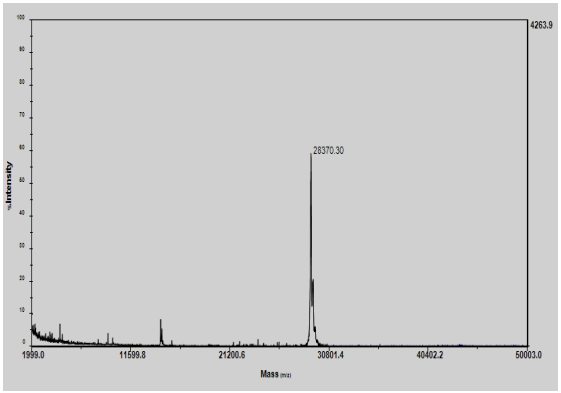

Fig. 3 : MALDI/MS analysis of BGS-Haridrin

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

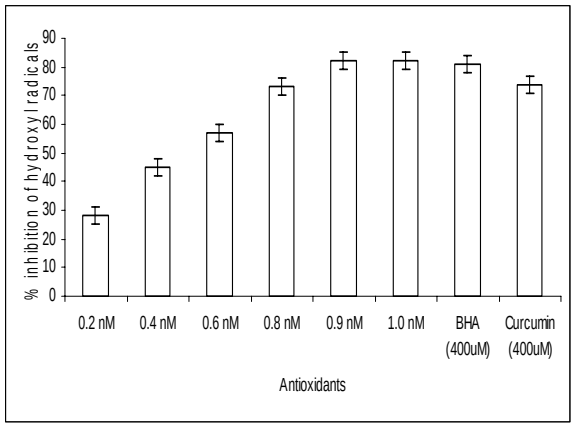

Fig. 4: Hydroxyl radical scavenging activity of BGS-Haridrin

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 (100:100µM) + H_2O_2 (1 mM) + EDTA ± various concentrations of BGS-Haridrin (0.2 to 1.0nM) in 1.0 ml PB (0.02M phosphate buffer, pH 7.4), incubating at 37°C for 60 min. Values are means ± SD of triplicates.

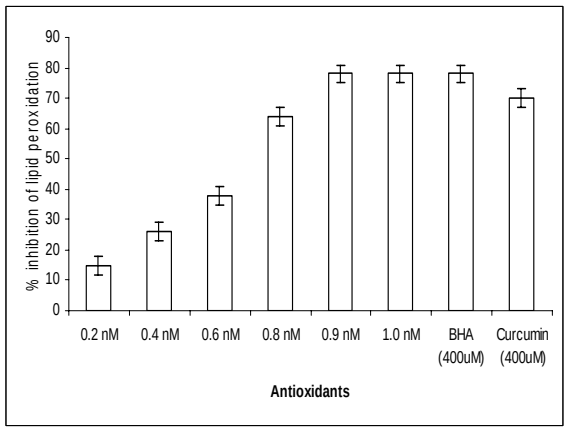

Fig. 5: Dose-dependent Lipid peroxidation inhibition activity of BGS-Haridrin

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.

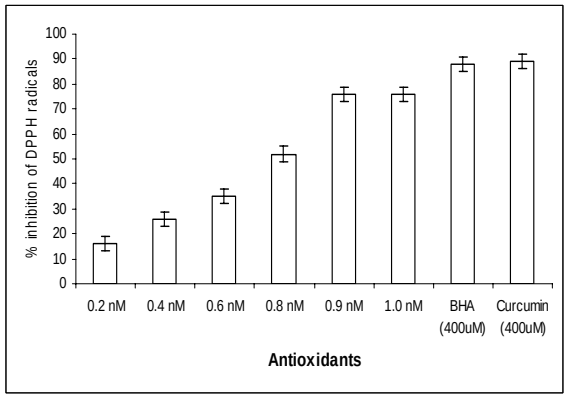

Fig. 6: Dose dependent DPPH radical scavenging activity of BGS-Haridrin

DPPH (0.5mM) + different concentrations of BGS-Haridrin (0.2 to 1.0 nM). Mixture incubated at 37°C for 30 min and the absorbance read at 517 nm using spectrophotometer. Values are means ± SD of triplicates.

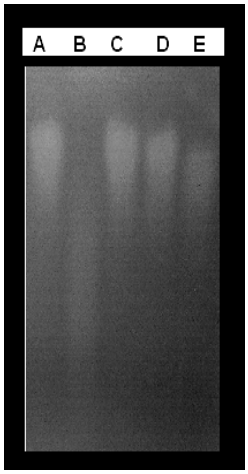

Fig. 7: Inhibition of peroxides induced DNA degradation by BGS-Haridrin.

Lane A: Calf thymus DNA untreated;
Lane B: Calf thymus DNA + H_2O_2
Lane C: Calf thymus DNA + H_2O_2 + BGS-Haridrin (15µg)
Lane D: Calf thymus DNA + H_2O_2 + BHA (100µg)
Lane E: Calf thymus DNA + H_2O_2 + Curcumin (100µg)

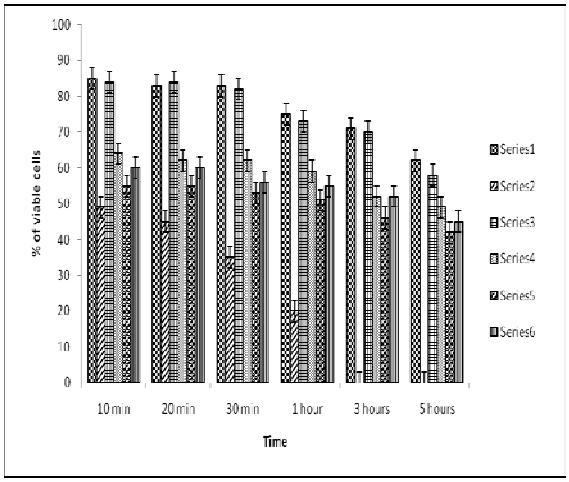

Fig. 8: Prevention of H_2O_2 induced cell death in Lymphocytes by BGS-Haridrin and other standard antioxidants

Lymphocytes (1 X 10^6) with or without H_2O_2 (1 mM)/ BGS-Haridrin (0.9nM)/ BHA (400 µM) / Curcumin (400µM) is taken in 500 µl of HBSS and incubated at 37 °C. Aliquot of lymphocytes taken at different time interval (0–5 hours) and charged into hemocytometer to determine the percentage of cell death using Tryphan blue exclusion method.

A. Lymphocytes alone;
B. Lymphocytes + H_2O_2 (1 mM);
C. Lymphocytes + BGS-Haridrin (0.9 nM);
D. Lymphocytes + H_2O_2 (1 mM)+ BGS-Haridrin (0.9nM).
E. Lymphocytes + H_2O_2 (1 mM)+ BHA (400 µM);
F. Lymphocytes + H_2O_2 (1 mM)+ Curcumin (400 µM).

Table 1: Comparison of antioxidant activity of BGS-Haridrin along with other standard antioxidants

Antioxidants	Concentration	% of inhibition			
		Deoxyribose assay	TBARS assay	DPPH radical scavenging assay	Superoxide radical scavenging assay
BGS-Haridrin	0.9 nM	82 ± 2.5	78 ± 2.7	76 ± 2.1	75 ± 2.9
BHA	400µM	81 ± 3.1	78 ± 3.2	88 ± 3.1	76 ± 3.7
Curcumin	400µM	74 ± 3.5	70 ± 3.6	89 ± 3.3	69 ± 3.5
SOD	300µg	-	-	-	86 ± 1.2

Comparison of antioxidant activities of BGS-Haridrin against other standard antioxidants such as BHA, Curcumin and SOD in different model system used. BGS-Haridrin (0.9nM) in comparison with other standard antioxidants like BHA (400µM), Curcumin (400µM) and SOD (300µg) in different antioxidant model systems.

RESULTS AND DISCUSSION

This study describes the antioxidant and DNA damage protectant 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)[34]. 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 **(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)**.

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[33]. Further, we studied the effect of the BGS-Haridrin on hydroxyl radicals generated by Fe^{3+} 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. The inhibition was statistically significant when compared to that without any inhibitor. The scavenging potential was compared with known antioxidants, such as BHA, and Curcumin. Each of these antioxidants inhibited lipid peroxidation by 81% and 74% at 400 µM concentration, respectively (Figure -4).

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 -5).**

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 H_2O_2 induced DNA damage:

Oxidative damage of DNA damage resulted in various degenerative diseases (Halliwell & Gutteridge, 1981). When calf thymus DNA was treated with H_2O_2 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 protectant activity at 15µg (lane C) (Figure-7). The effectiveness of BGS-Haridrin to prevent H_2O_2 induced DNA damage was associated to its hydroxyl radical scavenging activity and also lipid peroxidation inhibition activity as

observed by Deoxyribose assay and TBARS assay. This suggests that BGS-Haridrin may prevent H_2O_2 mediated oxidative DNA damage.

Prevention of H_2O_2 induced cell death by BGS-Haridrin:

The inhibition ability of BGS-Haridrin against H_2O_2 induced cell death in lymphocytes was tested. The maximum cell death induced by H_2O_2 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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