Original Research Article

Ferrous sulphate - Ascorbate induced DNA damage and protection by natural compounds

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Abstract

Objectives: The *in vitro* antioxidant and inhibition of Ferrous sulphate: Ascorbate induced fragmentation of DNA by crude proteins of Agathi seeds (*Sesbania grandiflora Linn*) evaluated using various relevant assays. **Materials and methods:** Assays like DPPH, Hydroxyl radicals, Ferric ion reducing, Ferrous ion chelation, Cytochrome C and Ferrous sulphate-Ascorbate complex induced DNA damage prevention studies were done to evaluate the antioxidant capacity of Agathi seed protein. **Results:** Agathi seeds (*Sesbania grandiflora Linn*) protein showed more antioxidant and free radical scavenging activity. It scavenged approximately 71% of hydroxyl and 1,1-diphenyl-2-picrylhydrazayl radicals 61%. In addition, it reduced cytochrome c and ferric ion levels, chelated ferrous ions and inhibited Ferrous sulfate:Ascorbate-induced fragmentation and sugar oxidation of DNA. **Conclusion:** These results establish the antioxidant potential of crude proteins of (*Sesbania grandiflora Linn*).

Keywords: Agathi seeds (*Sesbania grandiflora Linn*); Antioxidant activity; Free radical scavenging; DNA damage, Cytochrome C, Ferric ion; Ferrous ion.

Introduction

Oxidative stress is play a role in several pathogenesis of cardiovascular diseases, neurodegeneration, cancers, diabetes and so on[1]. The sources of antioxidants may be natural or artificial. Plant-based antioxidants are a kind of phytonutrient[2]. Dietary fruits and green leafy vegetables are rich in antioxidants[3]. Antioxidants protects the physiological system through direct reduction of oxidative stress[4]. Antioxidant works in concert through several different mechanisms like ROS scavenging, inhibition of lipid peroxidation[5]. Plant-derived antioxidants are natural products having radical-scavenging or reducing capacity[6]. Due to their therapeutic and preventive actions, these compounds receive a attention of researchers[7].

Sesbania Grandiflora or Agathi or the 'vegetable hummingbird,' is belonging to the family Fabaceae and genus Sesbania[8]. The parts like flowers, seeds, leaves, fruits and so on are known to have medicinal properties and are edible. Lot of research work has been done on plant leaves of Agathi where as very few research activities have been done on its seeds[9]. Herein the researchers made an attempt to evaluate the antioxidant and DNA protective activity of Agathi seeds.

Materials and methods

Agathi seeds (*Sesbania grandiflora Linn*) were procured from authentic source. The required chemicals like Ammonium sulphate, butylated hydroxyl anisole (BHA), α-tocopherol, calf thymus DNA, ferrous sulphate, ascorbic acid, 2-deoxy ribose, ferric chloride, linoleic acid, were purchased from Sigma (St. Louis, USA).

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All other chemicals unless otherwise mentioned were procured from Merck (Dermastadt, Germany). Shimadzu UV-1601 spectrophotometer (Tokyo, Japan) was used for photometric analysis and Kubota 6800 (Kubota Co., Osaka, Japan) was used for centrifugation.

Extraction of Agathi seeds protein (ASP)

Five gram of dried Agathi seeds were powdered and was homogenized in 20 ml of 10mM Tris buffer of pH 7.0 and the volume was made up to 100 ml with the same buffer. The suspension was incubated overnight at 4°C with constant stirring. The homogenate was filtered through Whatman No. I filter paper and centrifuged at 13,000 rpm at 4°C for 20 min. The resultant residue was discarded and the supernatant was brought to 65% saturation of ammonium sulphate. The precipitated protein content subjected to dialysis using 2kDa molecular cutoff membrane against double distilled water for duration of 72 hours with an interval of 6 hours. Further the dializedsalt free protein was subjected to proximate analysis.

Total Protein content

The total protein content of the crude extract was determined as per the standard protocol[10]. Various concentrations of bovine albumin (0–100 μg/mL) or Agathi seeds extract at the concentration ranging from 0 to 20 μL were added to series of tubes and the volume was made up to 100μL with 0.15M NaCl. 1 mL Bradford's reagent was added to all the tubes and mixed well. The absorbance was measured at 595 nm. The concentration of the protein in the extract was determined from the calibration curve.

Hydroxyl radical scavenging activity

To determine the hydroxyl radical scavenging activity in crude protein[11-12] the deoxyribose assay was used. The reaction mixture containing $FeCl_3$ (100 µM), EDTA (104 µM), H_2O_2 (1 mM), 2-

deoxy-D-ribose (2.8 mM) were mixed with or without various concentrations of ASP (10 to100 µg) in 1 ml final reaction volume, made with 20 mM potassium phosphate buffer, at pH 7.4 and incubated for 1 h at 37°C. The mixture was heated to 95°C in a 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 5319g in a Kubota 6800 (Kubota Co., Osaka, Japan) for 15 min. The absorbance of the supernatant was measured at 532 nm. The negative control was without any antioxidant or ASP. The percent hydroxyl radical scavenging activity of extracts was determined accordingly in comparison with the 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[13]. Different dosages of ASP and ascorbic acid, at various concentrations (20 to 100 µg) were mixed in 1 ml of freshly prepared 0.5 mM DPPH ethanolic solution and 2 ml of 0.1 M acetate buffer at pH 5.5. The resulting solutions were then incubated at 37°C for 30 min and measured at 517 nm in spectrophotometer. Standard antioxidants such as α-tocopherol, and L-ascorbic acid, all at 80 µg, were used as positive controls under the same assay conditions. Negative control was without any inhibitor or extract. Lower absorbance at 517 nm represents higher DPPH scavenging activity. Percent DPPH radical scavenging activity of the extracts was calculated accordingly from the decrease in absorbance at 517 nm in comparison with the negative control.

Cytochrome-c reduction capacity

The cytochrome c reducing capacity of ASP was determined according to the method of Suter and Richter, 2000[14]. Unaltered cytochrome c has a characteristic spectra with λ_{max} of 550 nm, due to its active heme group, which contains a ferrous ion. When subjected to oxidation by oxygen saturated phosphate buffer (0.1 mM, pH 7), the peak at 550 nm will reduce quantitatively. In the assay, cytochrome c (15 µM) was subjected to oxidation by oxygen saturated phosphate buffer (0.1 mM, pH 7), then ASP (5 to 30 µg)/BHA (800 µM)/ascorbic acid (1 mM) was added and incubated at ambient temperature for 30 min. Absorbance at 550 nm was measured using spectrophotometer. Absorbance increases with increase in reduction of oxidized cytochrome c suitable controls were maintained.

Test for ferric ion reducing capacity (Fe^{3+} to Fe^{2+})

The ferric ion reducing capacity was determined by using Potassium ferricyanide solution (100µl; 4 mM) was mixed with phosphate buffer (200µl; 20mM; pH 6.5), with or without ASP/BHA at various concentrations (5 to 100µg). The contents were incubated at 50°C for 20 min. Trichloroacetic acid (200µl; 10%) was added to the reaction mixture and centrifuged at 10000 rpm. The resulting supernatant was taken and mixed with Ferric chloride solution (100µl; 2 mM) and final volume was made up to 1 ml with water and then incubated at ambient temperature for 10 min. The absorbance was recorded at 700 nm using spectrophotometer. Absorbance increases with an increase in ferric ion reducing capacity[15].

Test for ferrous ion chelating activity (binds Fe^{2+})

Ferrous ion chelating activity was measured by using Potassium ferricyanide, ferric chloride and EDTA were procured from Sigma (St. Louis, USA) and double distilled water was used. The reaction solution containing ferrous chloride (200 µM) and potassium ferricyanide (400 µM), with or without Agathi seeds protein (ASP) / EDTA at various concentrations ranging from 20 to 100 µg, was made to 1 ml with double distilled water and mixed. The reaction mixture was incubated at 20°C for 10 min. Formation of the potassium hexacyanoferrate complex was measured at 700 nm using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). The assay was carried out at 20°C to prevent Fe^{2+} oxidation. Lower absorbance indicated a higher iron chelating capacity. The negative control was without any chelating compound or test sample. The percent ferrous ion chelating activity was calculated accordingly by comparing the absorbance of the test samples with that of the negative control[16].

Protective effect of Agathi seeds protein (ASP) on Fenton reactant-induced DNA sugar damage

Oxidative DNA sugar damage was determined according to the method of Sultan et al., 1995[17]. The reaction mixture contains potassium phosphate buffer (1000 µl; 20 mM; pH 7.4), which contained well sheared calf thymus DNA (1mg) was treated with Ferrous sulfate: Ascorbate (5:50 µmole) as the standard Fenton reagent (Fenton, 1984; Shalini *et al.*, 1994) with or without Agathi seeds protein (ASP) at concentrations ranging from 20 to 100 µg/ml. The reaction mixture was incubated at 37°C for 1 h in a water bath shaker. The colour was developed by adding TCA (1 ml of 2.8%) and TBA (1 ml of 1%) and boiled for 20 min, cooled on ice and centrifuged at 5319g for 10 min. The absorbance was read at 535 nm using spectrophotometer. The negative control was without any test sample. The percent inhibition of DNA oxidation was calculated accordingly by comparing the absorbance of the test sample with a negative control.

$$\% \text{ inhibition} = \frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Control}}} \times 100$$

Statistical analysis

Statistical analysis was done in SPSS (Windows Version 10.0.1 Software Inc., New York) using a one-sided student's t-test. All results refer to means ± SD. $P < 0.05$ was considered as statistically significant when compared to relevant controls.

Results

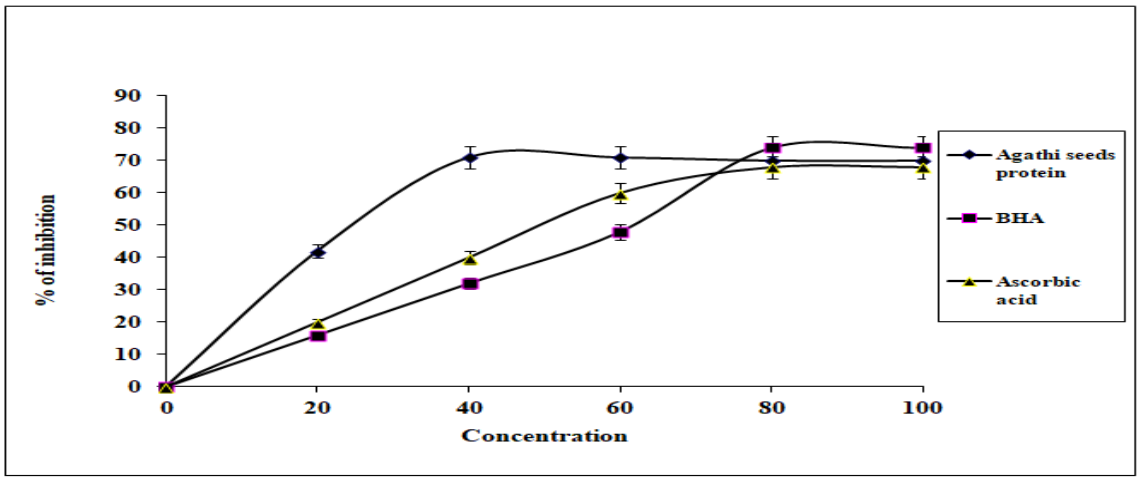

Fig. No.1: Hydroxyl radical scavenging activity of Agathi Seeds protein

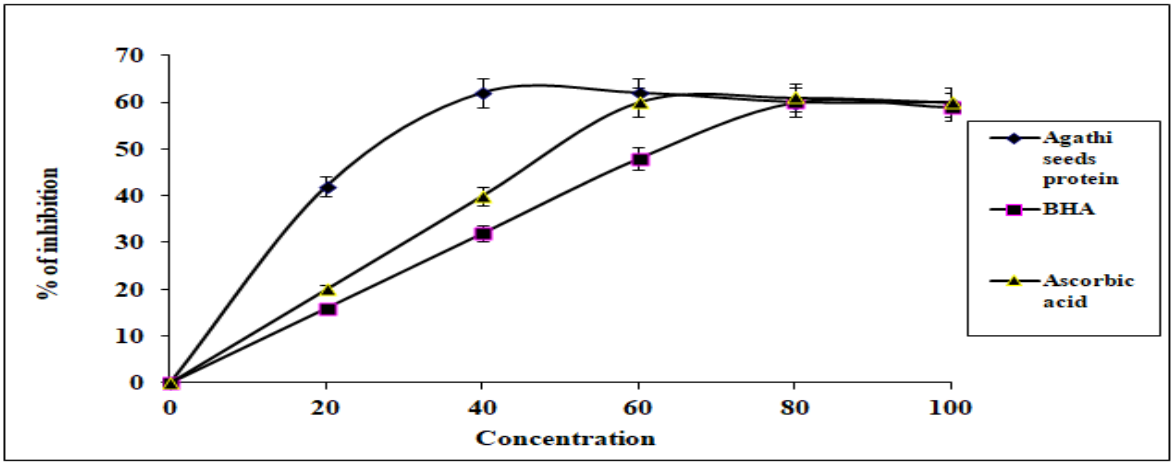

Fig. No.2: DPPH radical scavenging activity of Agathi Seeds protein

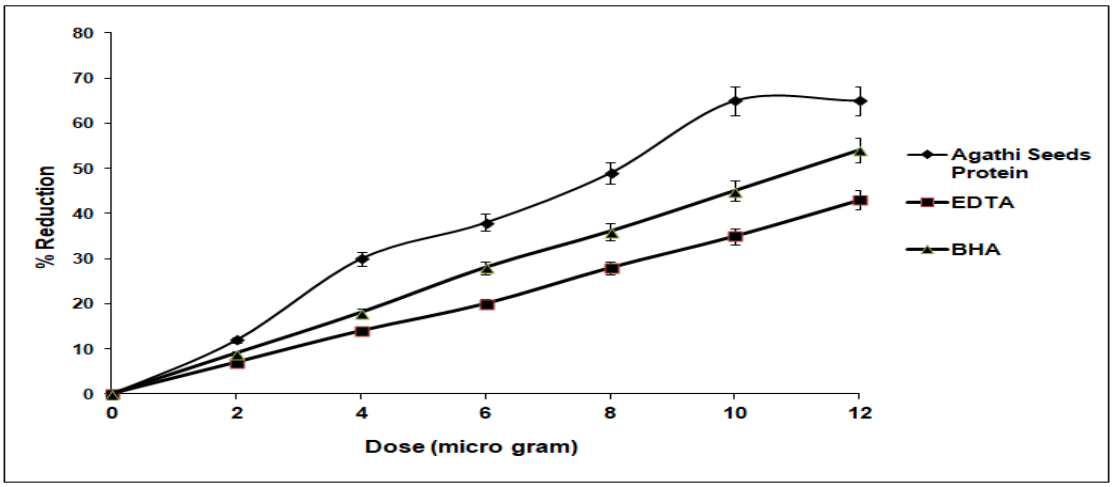

Fig.-3: Ferric ion reducing power of Agathi Seeds protein

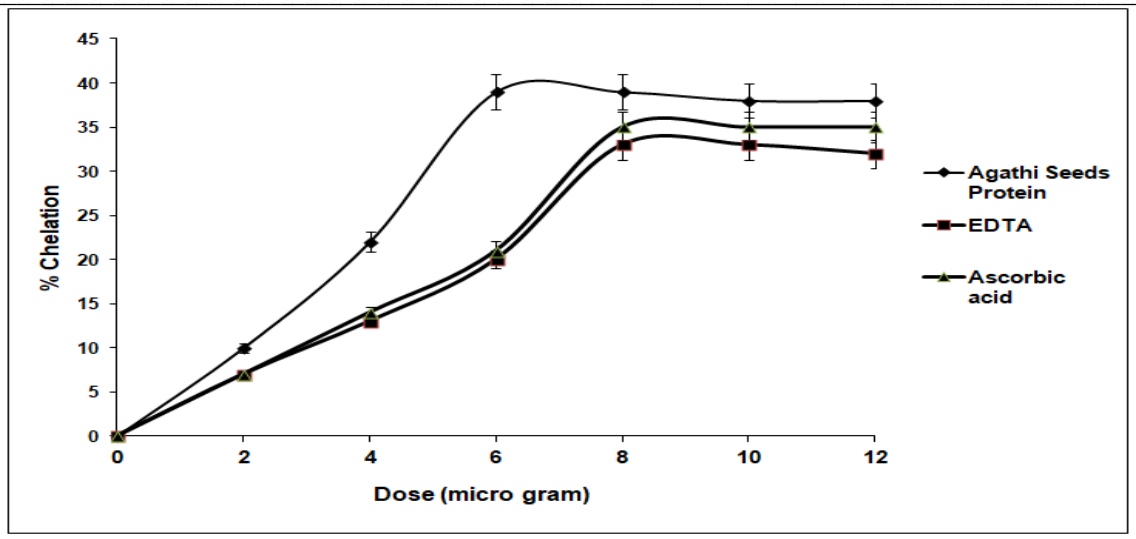

Fig.-4: Ferrous Ion chelation activity of Agathi Seeds protein

Discussion

Hydroxyl radical scavenging activity

Hydroxyl radicals are most reactive of all the reduced forms of dioxygen (Rollet-Labelle *et al*., 1998). Further, ASP on hydroxyl radicals generated by Fe^{3+} ions was measured by the extent of deoxyribose degradation; an indicator of TBA–MDA adducts formation. ASP showed **(Fig.-1)** maximum hydroxyl radical scavenging activity by 71% at 40µg which was lower in concentration whereas, BHA (80µg) showed 78% and Ascorbic acid (80µg) showed 68% hydroxyl radical scavenging activity. This gives an impression that ASP could be an effective hydroxyl radical scavenger.

DPPH radical scavenging activity

The free radical scavenging activity of ASP using a direct approach with DPPH radicals Chang et al. (2001). ASP exhibited powerful DPPH radical scavenging activity of 62% at 40µg, which was comparatively good when compared to known antioxidants such as Ascorbic acid (80 µg), BHA (80µg), showed 60% and 61% DPPH radical scavenging activity, respectively **(Fig.-2)**. The results indicate that ASP is a powerful free radical scavenger.

Ferric ion reducing power

It has been shown that the antioxidant effect exponentially increased as a function of the development of the reducing power (Tanaka, Kuie, Nagashima, & Taguchi, 1988). As shown in (**Fig. 3)**, the maximum absorbance for ASP was up to 1.2, at 5-fold lesser concentration (20µg), compared to BHA which showed absorbance of 1.2. The reducing capacity might be due to their hydrogen donating ability (Shimada et al., 1992).

Cytochrome c reduction

Cytochrome c, a major electron transport protein of the respiratory chain, was used as a model protein to investigate the direct reductive capacity of ASP as one of the reaction mechanisms of antioxidant activity. ASP significantly reduced the oxidised cytochrome c, up to 100%, in a time dependent manner at 20 min (data not shown) and in a dose dependent manner. The direct cytochrome c reductive capacity of ASP (10 µg) was comparable to BHA (400µM) and Ascorbic acid (1000 µM). These results indicate that ASP is a potent reducing agent for the active heme group of cytochrome c.

Ferrous ion chelation

The chelation of Fe^{2+} ions by the ASP was studied with potassium ferricyanide where iron–hexacyanoferrate complex formed. The activity was monitored at 700 nm spectrophotometrically. As shown in Fig. 4, ASP exhibited maximum chelating effect of 60% at 6µg and its iron chelating effect is comparable with EDTA (10µg) and Ascorbic acid (10µg).

Protection of DNA sugar damage by Agathi Seeds Protein:

The inhibitory effect of Agathi seeds protein (ASP) against iron dependent oxidation of calf thymus DNA sugar was tested by a TBARs assay. As explained in the methods, the protein provides effective inhibition by 68% at 40µg against Ferrous sulfate:Ascorbate-induced [16-17]. DNA sugar damage using linolenic acid as a source of lipids, when compared to Ascorbic acid (400µM) and Alpha-tocopherol (400µM), showed 59% and 65% respectively.

Similar studies have reported that proteins from *Murraya koenigii*, *Curcuma longa L*, *Curcuma aromatica*, *Coleus aromaticus* leaves, *Muntingia calabura*, *Withania somifera*, *Moringa oleifera*, *Mint L*, *Illicium verum* exhibits excellent antioxidant and other inhibitory activities[18-28].

Conclusion

The above results indicate the antioxidant capacity and nature of Agathi Seeds protein. Further studies required develop as a rich source of antioxidant in the field of nutraceutical and pharmaceutics.

Authors' contributions

We hereby declare that, all the authors contributed equally in preparing and finalizing this review manuscript.

Conflict of interest

The authors declare no conflict of interest.

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