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HYDROGEN PEROXIDE INDUCED DNA DAMAGE: PROTECTION BY ANTIOXIDANT COLEUS AROMATICUS

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ABSTRACT

The antioxidant properties of boiling water extract of *Coleus aromaticus* were evaluated using lipid peroxidation inhibition activity, hydroxyl radicals scavenging activity, 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging effect and Superoxide radical scavenging activity. The extracts of *Coleus aromaticus* effectively protects H_2O_2 (100µM) induced cell death in human peripheral lymphocytes. Further *Coleus aromaticus* extract prevents H_2O_2 (1mM) mediated calf thymus DNA damage as evidenced by agarose gel electrophoresis. The present study reveals that, the boiling water extract of *Coleus aromaticus* showed significant antioxidant and DNA damage protectant activity when compared to antioxidants like BHA, Curcumin and α-tocopherol.

KEY WORDS: Antioxidants, *Coleus aromaticus*, Cytotoxicity, DNA damage, H_2O_2.

INTRODUCTION

Reactive oxygen species (ROS) cause wide oxidative damage to cellular biomolecules like DNA, proteins and lipids and they contribute to the oxidative stress-related diseases[1,2], though several promising synthesized antioxidants available, but their toxicity and side effects avoids their recommendation [3]. Hence, there is great interest in the use of naturally occurring antioxidants towards inhibition or prevention of oxidative stress-related diseases[4]. The administration of an antioxidant source comprising of multiple components could offer protection against cancer[5,6] and combat oxidative stress-induced physiological malfunctions[7]. According to President of Foundation for innovation in Medicine, if a food or

its part is having medical /health benefits and resulting in the prevention /cure of diseases then it is called as Nutraceutical. *Coleus aromaticus* is a large succulent herb, aromatic, branched with distinctive smelling leaves. The taste of this leaf is pleasant with refreshing odour. The leaves are strongly flavoured and make an excellent addition to meat dishes. It is also used as a vegetable, also as herb in the food trade. It has traditional medicinal properties and hence used for the treatment of coughs, sore throats and nasal congestion, infections, rheumatism and flatulence. In traditional medicine, it is used to treat malarial fever, hepatopathy, renal and vesical calculi, cough, chronic asthma, hiccough, ronchitis, helminthiasis, colic, convulsions, and epilepsy henoy and others [8,9,10,11].

MATERIALS AND METHODS

Coleus aromatic leaves obtained from Sasyakashi, maintained by Adichunchanagiri Maha Samsthana Mutt, Bellur cross, Nagamangala Taluk, Mandya District, Karnataka state, India. DPPH (2,2-Diphenyl-2picrylhydrazyl, Superoxide dismutase (SOD), NBT (Nitro blue tetrazolium), DMSO (Dimethyl sulphoxide), Bovine serum albumin (BSA), Calf thymus DNA, Linolenic acid and Gallic acid from Himedia Private Ltd India. Hydrogen peroxide was purchased from SRL, India. All other chemicals unless otherwise mentioned were of analytic grade and procured from Merck (Dermastadt, Germany). Solvents were distilled before use.

Boiling water extract of *Coleus aromaticus* leaves

Ten gram of the cleaned, fresh selected *Coleus aromaticus* leaves crushed with 100 ml of hot water and boiled for five minutes. It was vortexed for 2 hrs at room temperature. The solution was centrifuged at 6000 rpm for 20mins at room temperature. The supernatant was filtered using Whatman No. 1 filter paper, followed by 0.22m Sortorius microbial filter. Then the supernatant was freeze dried and lyophilized and stored at 4°C for further studies. The efficacy of the extract reported here was quantified based on the dry weight of the whole extract per volume of assay solution.

Proximate analysis

The proximate analysis such as protein[12], total sugars [13], polyphenols[14], flavonoids[15] was done for the *Coleus aromaticus* extract.

Antioxidant activity

Antioxidant activity of aqueous and boiling water extract of Coleus aromaticus was studied in different model system to study their antioxidant activities.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was assessed according to the method of Shimada et al. 1992[16]. The α-tocopherol at various concentrations ranging from 0 to 100 M 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 mixtures were incubated at 37C for 30 min and measured at 517 nm. α-tocopherol (400 µM) was used as positive control under the same assay conditions. Negative control was without any inhibitor or extract of Coleus aromaticus extract. Lower absorbance at 517 nm represents higher DPPH scavenging activity. The % DPPH radical scavenging activity of Coleus aromaticus extract was calculated.

Superoxide scavenging activity by alkaline DMSO method

The Superoxide radical ($O2^{\bullet-}$) scavenging activity of *Coleus aromaticus* was measured according to the method of Lee *et al.* 2002[17]. The reaction mixture containing 100µl of 30mM EDTA (pH 7.4), 10µl of 30mM hypoxanthine in 50mM NaOH, and 200µl of 1.42mM nitro blue tetrazolium (NBT) with or without *Coleus aromaticus* extracts. SOD serving as positive control at various concentrations ranging from 50-300µg. After the solution was pre-incubated at ambient temperature for 3min, 100µl of xanthine oxidase solution (0.5U/ml) was added to the mixture and incubated for one hour at 37°C, and the volume was made up to 3ml with 20mM phosphate buffer (pH 7.4). The solution was incubated at room temperature for 20 minutes, and absorbance was measured at 560 nm. Appropriate controls were included to rule out the artifacts induced reaction. The control was without any inhibitor. Inhibitory effect of *Coleus aromaticus* on superoxide radicals was calculated as follows.

$$\% \text{ Superoxide radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

Cyto-toxicity study of the *Coleus aromaticus* extract

Human peripheral lymphocytes were isolated from 10ml of venous blood drawn from young, healthy donors. Blood was collected in ACD (85mM citric acid-71mM trisodium citrate-165mM D-glucose) in the ratio of 5:1.4 volumes of solution A (hemolysing buffer-150mM NH_4Cl in 10mM Tris buffer, pH 7.4) was added, mixed well, incubated at 4°C for 30 min.

Centrifuged at 1200 rpm for 12 min, the supernatant (hemolysate) was discarded, pellet was washed again with 5ml of hemolyzing buffer and the pellet containing cells were washed thrice with 10 ml of solution B (250mM m-inositol in 10mM phosphate buffer pH 7.4) and suspended in same solution. The cell viability was determined by Trypan dye blue exclusion method[18]. 10µl of lymphocyte sample added 10µl of Tryphan blue (0.02%) and the cells were charged to Neuberg's chamber and the cell number was counted. The dead cells being permeable to Tryphan blue appear blue against white color of the viable cells. The survival rate of lymphocytes was determined at time intervals 20th, 40th and 60th minutes of incubation. Viability was tested by Tryphan blue exclusion and exceeded 96% in each isolation. Percentage viability was calculated by the formula.

$$\% \text{ viability} = \frac{\text{Total no. of viable cells}}{\text{Total no. of viable cells + dead cells}} \text{ X } 100$$

Protective effect of *Coleus aromaticus* extract on H_2O_2 induced DNA damage

The DNA damage induced by H_2O_2 was analyzed on 0.6% submarine agarose gel according to the method of Sultan et al, 1995[19]. Calf thymus DNA (10 mg) was mixed well in one ml of 20 mM Potassium phosphate buffer, at pH 7.4, 150 mM NaCl and store at 4C. The sheared 15 µg of calf thymus DNA was treated with 1mM of H_2O_2 with or without *Coleus aromaticus* and Ascorbic acid (400µM) in 100µl potassium phosphate buffer (20mM, pH 7.4). The reaction mixture was mixed with 10 µl of sample loading buffer (0.5% bromophenol blue, 50% glycerol in water) and then the reaction mixture was incubated at 37C for 30 min and then placed on ice for 10 min to stop the reaction. 20µl of the reaction mixture (15 µg of DNA) was run on 0.6% agarose with ethidium bromide (1 µg/ml). The electrophoresis was carried out using 20mM TBE 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.

Statistical analysis

Statistical analysis was done using student's t-test and a $p < 0.05$ was considered as statistically significant when compared with controls and results refer to mean ± SD.

RESULTS

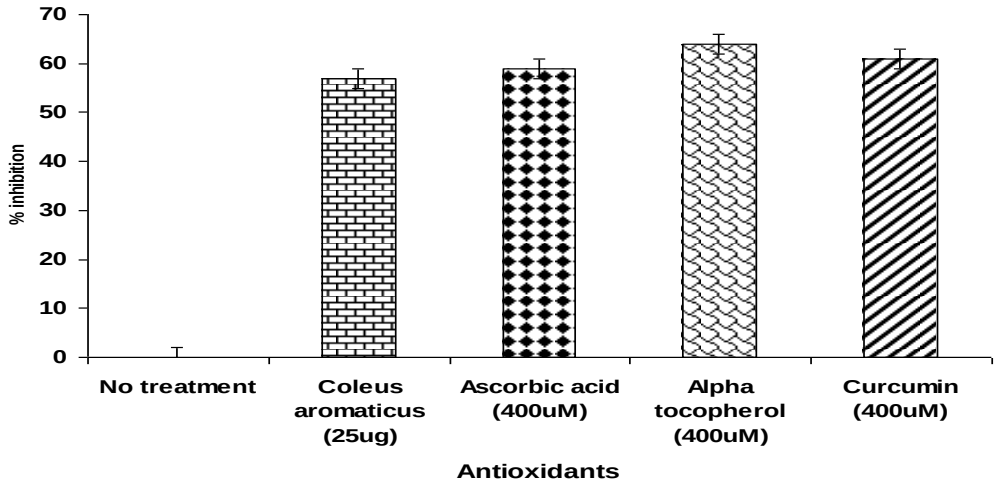

Figure-1: DPPH radical scavenging activity of *Coleus aromaticus* extract

DPPH (0.5mM) + With or without *Coleus aromaticus* extract (25µg) / α-tocopherol /Ascorbic acid/Curcumin (400µM). Mixture incubated at 37°C for 30 min and the absorbance read at 517 nm using spectrophotometer.

Values are means ± SD of triplicates.

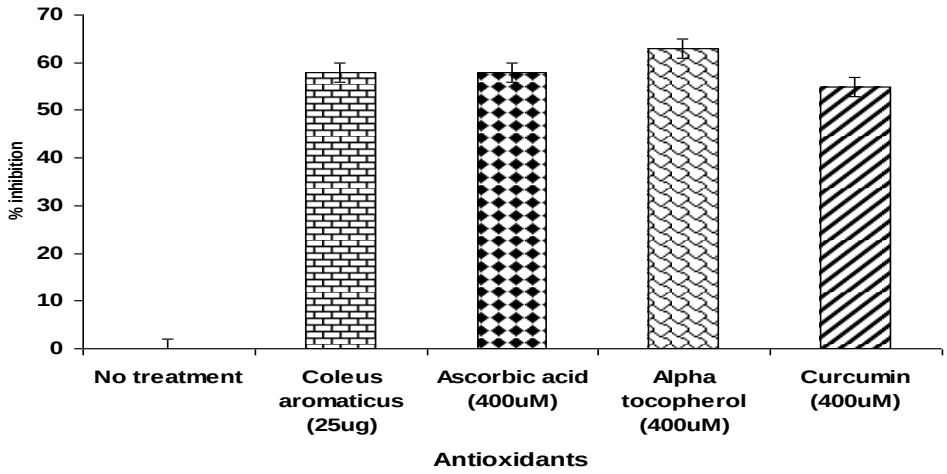

Figure-2: Superoxide scavenging activity of *Coleus aromaticus* extract

Hypoxanthine (30mM) + Nitro blue tetrazolium (NBT) (1.42mM) + With or without *Coleus aromaticus* extract (25µg) / α-tocopherol /Ascorbic acid/Curcumin (400µM). Mixture incubated at 37C for 20 min and the absorbance read at 560 nm using spectrophotometer.

Values are means ± SD of triplicates.

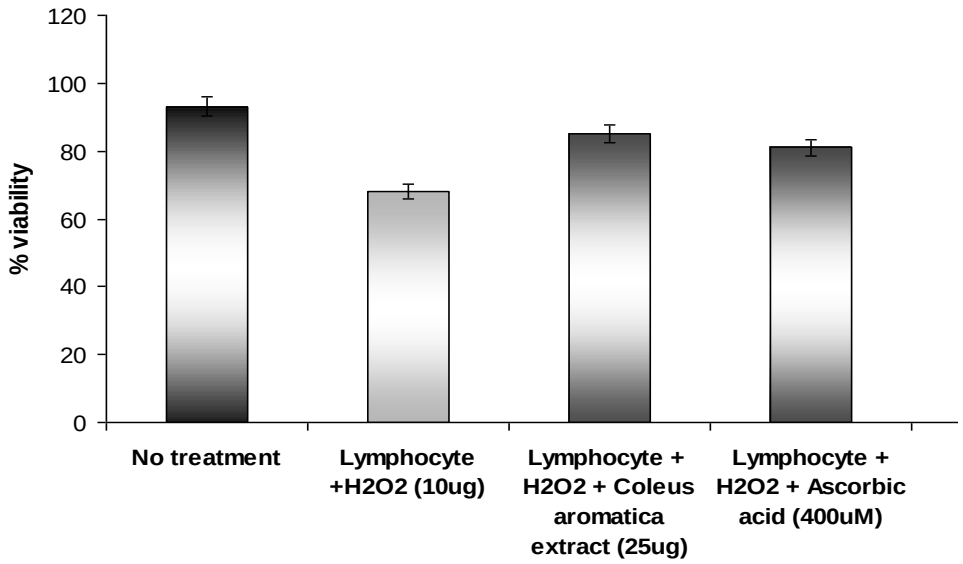

Figure-3: Cytotoxicity study of *Coleus aromaticus* extract

Lymphocytes (10^6cells) pretreated with or without antioxidants at indicated concentrations in 0.5ml HBSS pH 7.4, incubated at 37°C for 20min., then H_2O_2 (144µM) was added, incubated at 37°C for 60 minutes in final volume of 1ml HBSS, pH 7.4. After the desired incubation time (60 minutes), viability of the cells was determined by Tryphan blue exclusion and the percentage of viable cells was calculated as mentioned in methods. Values are means ± SD of triplicates.

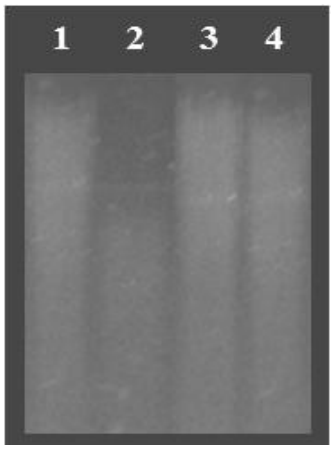

Figure 4: Inhibition of Hydrogen peroxide induced DNA degradation by extracts of Star Anise.

Lane 1: Calf thymus DNA untreated;

Lane 2: As lane (1) + H_2O_2

Lane 3: As lane (2)+ *Coleus aromaticus* extract (15µg)

Lane 4: As lane (2)+ of Ascorbic acid (15µg)

DISCUSSION

Proximate Analysis

The extraction and proximate analysis of the *Coleus aromaticus* extract was done as reported earlier[20]

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

As shown in Figure-1, a direct approach with DPPH radicals - a stable radical used to evaluate the antioxidant activity of extract of *Coleus aromaticus*. It is evidenced that, 57% scavenging activity at 25ug concentration of extract. The *Coleus aromaticus* extract exhibited a statistically significant DPPH radical-scavenging activity when compared to the group without any inhibitor. At the concentration of 400µM standard antioxidants like Ascorbic acid, α-tocopherol and Curcumin showed 59%, 64% and 61% DPPH radicals respectively.

Superoxide scavenging activity

Superoxide anions are the most common free radicals generated in biological systems and the concentration of superoxide anions increases under oxidative stress. The assay was carried to investigate the superoxide radical scavenging activity of extract of *Coleus aromaticus*. As shown in Figure-2, the superoxide scavenging activity of *Coleus aromaticus* extract (25ug) was found to be 58%. Ascorbic acid, α-tocopherol and Curcumin were used as the positive control which offered 58%, 63% and 55% scavenging activity at a concentration of 400µM.

Cytotoxicity study of *Coleus aromaticus* extract

As shown in Figure 3, The study was done to check and confirm the cytotoxic nature of the extract and other standard antioxidant Ascorbic acid. The results confirmed that, the extract of *Coleus aromaticus* and antioxidant Ascorbic acid are not toxic to cells and provide protection to lyphocytes against Hydrogen peroxide.

Protective effect of extracts of *Coleus aromaticus* on H_2O_2 induced DNA damage

As shown in figure 4, the calf thymus DNA was treated with H_2O_2 for 30 min, extensive DNA fragmentation due to oxidation by hydroxyl radicals was seen on submarine agarose gel by the enhanced mobility (Lane 2) as compared to the untreated DNA (Lane 1). The lane 3 showed that, DNA treated with H_2O_2 along with Ascorbic acid (15µg) protected DNA damage in comparison to the untreated DNA (Lane 1). The effectiveness of the extract of Coleus aromaticus to prevent H_2O_2 induced DNA damage was associated to its hydroxyl

radical scavenging activity. This suggests that the *Coleus aromaticus* extract could prevent H_2O_2 mediated oxidative DNA damage.

CONCLUSION

The above results indicates that, the boiling water extract of *Coleus aromaticus* acts as an effective antioxidant at a dosage of 25µg and it provide protection to DNA against peroxides. Its antioxidants activities could be mainly due to combined effect of phytochemicals present in it.

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