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FREE RADICAL INDUCED CELL DAMAGE: PROTECTED BY PURIFIED *COLEUS AROMATICUS* LEAVES PROTEIN

Santhosh Kumar Nune [1], Aliya Nusrath [2] and Dinesha Ramadas [*3]

Department of Biochemistry [1], Shridevi Institute of Medical Sciences & Research Hospital, Tumkur - 572106, Karnataka, India.
Department of Biochemistry [1, 2], Adichunchanagiri Institute of Medical Sciences, B.G. Nagara, Mandya - 571448, Karnataka, India.
Adichunchanagiri Institute for Molecular Medicine [3], CRL-AIMS, Adichunchanagiri University, B.G. Nagara, Mandya - 571448, Karnataka, India.

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Correspondence to Author:
Dinesha Ramadas

Scientific Officer/Research Scientist, Adichunchanagiri Institute for Molecular Medicine, Adichunchanagiri University, B.G. Nagara, Mandya-571448, Karnataka, India.

E-mail: r.dinesha@gmail.com

ABSTRACT: Objective: Many studies have demonstrated the antioxidant properties of extracts of *Coleus aromaticus*. The purified *Coleus aromaticus* leaves protein was studied to investigate its *in-vitro* antioxidant activity, preventing oxidative DNA damage induced by hydrogen peroxide (H_2O_2) and its protective effect on lymphocyte cell viability. **Materials and Methods:** *In-vitro* antioxidant capacity by DPPH assay, hydrogen peroxide (H_2O_2) used to damage the calf thymus DNA, the purified *Coleus aromaticus* leaves protein (CALP) at 25 µg concentration was used to prevent the H_2O_2 induced DNA damage, which was demonstrated on submarine agarose gel electrophoresis. The protective effect of purified CALP on cell viability of human peripheral lymphocytes against damage induced by H_2O_2 was studied by the Trypan dye blue exclusion method. Standard antioxidants like gallic acid, ascorbic acid, and butylated hydroxyanisole were used. **Results:** The purified CALP protein showed significant antioxidant activity when compared to Gallic acid. At 25 µg concentration of purified CALP provided almost equal protection as given by standard antioxidant Ascorbic acid at 400 µM concentrations against H_2O_2 induced DNA fragmentation in submarine agarose gel electrophoresis. The cell viability study showed that human peripheral lymphocytes treated with the purified CALP (25 µg) had 71 ± 2% viability, BHA (400 µM) had 68 ± 3% viability, and Ascorbic acid (400 µM) had 78 ± 2% viability when compared to untreated lymphocytes (83 ± 4%). **Conclusion:** The study demonstrated that the purified CALP possesses significant antioxidant activity, protective effect on H_2O_2 induced DNA damage, and lymphocyte cell damage, which showed itself is not toxic.

INTRODUCTION: Many studies have reported, antioxidants play a vital role in the prevention of oxidation induced by factors like UV rays, oxidative stress factors like Hydrogen peroxide, Tertiary Butyl Hydroperoxides (t-BOOH), Ferrous Sulphate: Ascorbate mixture, *etc.* [1]

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The synthetic antioxidants like BHA, BHT, or natural antioxidants from dietary resources, herbs, and spices may be the choice for preventing oxidative damage [2]. The added advantage of natural antioxidants over synthetic products is that they are nontoxic even on consumption in milligram quantity.

In India, ages back practice of using dietary sources such as herbs, and spices as medicines and cosmetics is prevailing, due to their potential to treat different diseases [3]. Reactive oxygen species (ROS) like H_2O_2 and O_2- cause a series of DNA lesions, including single-strand, double-strand

breaks, cross-linking of DNA and damage to bases. The conversion of these ROS to highly reactive OH- radical is responsible for DNA damage [4]. Natural antioxidants such as vitamin C, vitamin E, flavonoids, and phenolic acids fight against free radical species that cause DNA damages. Medicinal Herbs have high potential due to their antioxidant activity [5].

Studies have shown that the extract of medicinal plant *Coleus aromaticus* has been used to treat ailments from the common cold to several diseases [6-8]. Hence, the different solvent extracts of *Coleus aromaticus* have been studied earlier to investigate its properties towards protection against UV rays, H_2O_2, t-BOOH [9, 10]. Crude protein obtained from *Coleus aromaticus* extract showed to be rich in protein content, low in concentration of phenols, flavonoids, sugars, which was reported earlier by [11]. In this study, the purified *Coleus aromaticus* leaves protein is selected to investigate its antioxidant, protective activity against H_2O_2 induced DNA damage and its effect on lymphocyte cell viability.

MATERIALS AND METHODS: Calf thymus DNA, Agarose, Gallic acid, DPPH, Ethidium bromide, SDS, H_2O_2, BHA, Thiobarbutiric acid, was from Sigma Chemical Company USA. Ascorbic acid was from HIMEDIA, India. EDTA, Ferrous sulphate, Hydrogen peroxide was from S.D. fine Chem. Ltd. India. NaCl, Potassium acetate, Sodium acetate were purchased from SRL, India. All the other chemicals were of analytical R-grade. The concentrations of the solvents used in this study were not more than 0.1%, and the effective concentrations of antioxidants were used based on the literature of [9]. The present study was undertaken to show the *in-vitro* antioxidant activity, established assay 2, 2-diphenyl–1-picryl hydroxyl (DPPH) radical assay, the protective effect of purified CALP against DNA damage and also protective effect on cell viability of human peripheral lymphocytes against damage induced by H_2O_2.

***In-vitro* Antioxidant Activity:**

DPPH Radical Scavenging Assay: DPPH radical scavenging activity was assessed according to the method of Shimada *et al.,* (1992) with minor modifications [12]. In this assay, the radical scavenger activity of purified CALP decolorizes ethanolic DPPH solution from purple to yellow colour. This is due to the reduction of the stable DPPH radicals to diphenyl- picrylhydrazine in the presence of hydrogen-donating antioxidant. The color changes allow the detection of the scavenging activity at 517 nm.

The purified CALP at a concentration ranging from 20 to 160 µg mixed in 1 ml of freshly prepared 0.5 mM DPPH ethanolic solution and 2 ml of 0.1 M sodium acetate buffer pH 5.5. The resulting solutions were then incubated at 37 °C for 30 min. Gallic acid was used as standard antioxidant under the same assay conditions. Control was without any standard or test protein. The % DPPH radical scavenging activity of the protein was calculated from the decrease in absorbance at 517 nm and compared with control. The % DPPH activity was calculated using the following formula.

% Inhibition of DPPH Scavenging Activity = Abs of control – Abs of samples/ Abs of control × 100

DNA Protectant Activity of *Coleus Aromaticus* Protein:

Submarine Agarose Gel Electrophoresis for DNA Separation: The DNA damage induced by H_2O_2 was analyzed on a submarine agarose gel according to the method of Sultan *et al.,* [13]. DNA submarine gel electrophoresis was carried out by using 0.6% agarose prepared in TAE buffer and bands were visualized under U.V transilluminator [14].

H_2O_2 Induced DNA Damage: Protection by Purified *Coleus Aromaticus* Protein: Calf thymus DNA (10 mg) was mixed in 1 ml of 20 mM Potassium phosphate buffer, at pH 7.4, as stock and stored at 4 ºC. The sheared 10 µg concentrated calf thymus DNA was treated with 1 mM of H_2O_2 with and without Purified CALP and Ascorbic acid (400 µM) in 100 µl potassium phosphate buffer (20 mM, 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 37 ºC for 30 min and then placed on ice for 10 min to stop the reaction.

20 µl of the reaction mixture was run on 0.6% agarose with ethidium bromide (1 µg/ml). The electrophoresis was carried out using 20 mM 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 [14].

Assessment of Lymphocyte Viability and Protective Effect of Purified *Coleus Aromaticus* Protein against H_2O_2 Induced Damage:

Isolation of Human Peripheral Lymphocyte: Isolation of human peripheral lymphocyte procedure was done according to the method described by [9]. Human peripheral lymphocytes were isolated from 10 ml of venous blood drawn from young, healthy donors. Blood was collected in ACD (85 mM citric acid-71 mM trisodium citrate-165 mM D-glucose) tube in the ratio of 5:1. 4 ml of haemolysing buffer solution (150 mM NH_4Cl in 10 mM Tris buffer, pH 7.4) was added, mixed well, incubated at 40 °C for 30 min and Centrifuged at 1200 rpm for 12 min.

The supernatant (hemolysate) was discarded, the pellet was washed again with 5 ml of hemolyzing buffer and the pellet containing cells were washed thrice with 10 ml of Hank's balanced salt solution (HBSS: 250 mM -inositol in 10 mM sodium phosphate buffer pH 7.4) and suspended in same solution [15].

Assessment of Human Peripheral Lymphocyte Viability: The cell viability was determined by Tryphan dye blue exclusion method [16, 17]. The survival rate/ viability of lymphocytes was determined at 60 min of incubation. The % viability of Lymphocytes in presence or absence of BHA /Ascorbic acid /Purified CALP by Trypan blue exclusion by viable cells was determined the dead cells being permeable to Trypan blue appear blue against white color of the viable cells.

10 µl of lymphocyte sample were treated with H_2O_2 (10 µg) in the presence or absence of BHA (400 µM) / Ascorbic acid (400 µM) / purified CALP (25 µg) in 1 ml of HBSS. 10µl of Trypan blue (0.02%) was added to all the tubes. After the desired incubation time of 60 min, the cells were charged to Neuberg's chamber and the cell number was counted [18]. The % viability was calculated by the formula.

% viability = Total no. of viable cells / Total no. of cells × 100

Statistical Analysis: All data are expressed as mean ± standard deviation of three replicate (n=3). The significance of the experimental observation was checked by the student's t-test, and $P < 0.05$ was considered as statistically significant when compared to relevant controls.

RESULTS:

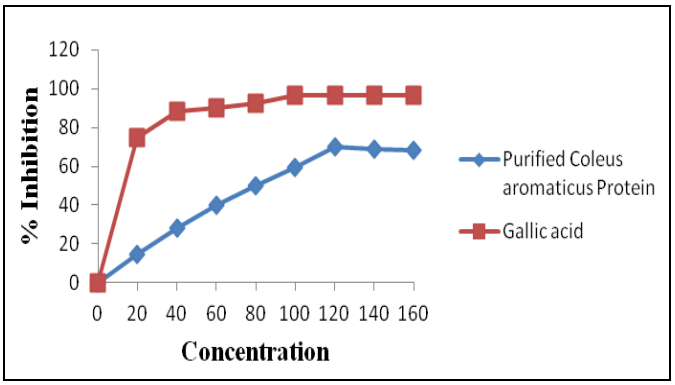

FIG. 1: % INHIBITION OF DPPH SCAVENGING ACTIVITY AT DIFFERENT CONCENTRATIONS OF PURIFIED CALP AND GALLIC ACID Data are expressed as mean ± standard deviation of three replicate (n=3)

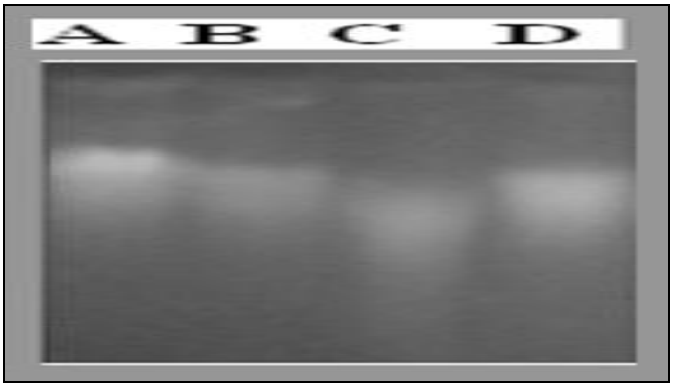

FIG. 2: DNA PROTECTANT ACTIVITY OF PURIFIED CALP AGAINST H_2O_2

Lane A: Calf thymus DNA sheared (10 µg)
Lane B: DNA + H_2O_2+ purified CALP (25 µg)
Lane C: DNA + H_2O_2 (144 µM)
Lane D: DNA + H_2O_2+ Ascorbic acid (400 µM)

TABLE 1: THE CELL TOXICITY INDUCED BY H_2O_2 AND PROTECTION BY ANTIOXIDANTS

Lymphocytes	% Viability
Lymphocytes alone (10 µl)	83 ± 4
Lymphocytes (10 µl) + H_2O_2 (144 µM)	58 ± 2
Lymphocytes + H_2O_2+ purified *CALP* (25 µg)	71 ± 2
Lymphocytes + H_2O_2+ BHA (400 µM)	68 ± 3
Lymphocytes + H_2O_2+ Ascorbic acid (400 µM)	78 ± 2

Data are expressed as mean ± standard deviation of three replicate (n=3)

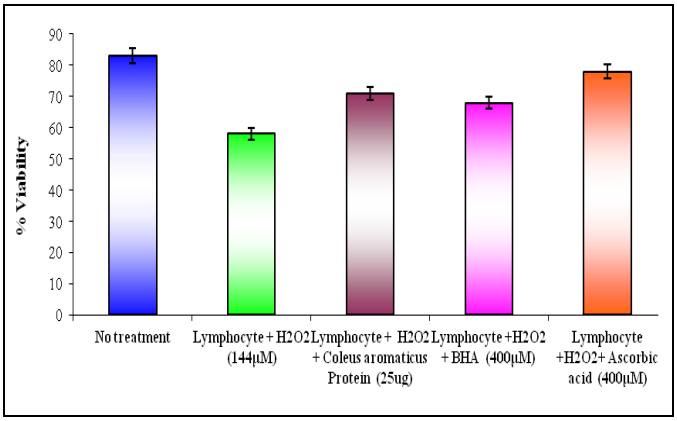

FIG. 3: THE CELL TOXICITY INDUCED BY H_2O_2 AND PROTECTION BY ANTIOXIDANTS These results indicate that the efficiency of each individual antioxidant tested exhibits protection against H_2O_2 induced cell death

DISCUSSION: Direct approach with DPPH radicals, a stable radical, is being used to evaluate free radical scavenging activities of natural antioxidants. As shown in **Fig. 1**, at 20 to 160 µg dose-dependent DPPH radical scavenging activity of purified CALP with gallic acid as antioxidants, this CALP significantly inhibited the DPPH radicals. The % inhibitory activity of CALP ranges a minimum of 14.43% at 20 µg/ml to a maximum of 69.13% at 120 µg/ml, whereas the standard antioxidant gallic acid showed % inhibitory activity ranges from a minimum at 74.81% at 20 µg/ml to a maximum of 96.54 at 100 µg/ml.

These results indicate that the CALP is an antioxidant protein towards scavenging DPPH radicals and comparable with gallic acid. The advantage is that it is a chain-breaking antioxidant, which does not turn into a radical itself at the end of the chain reaction. A similar study by Annapurni. S *et al.*, (2001) reported the antioxidant potential of the protein fraction of the *Coleus aromaticus* leaves shows the protein content was found to be 1.375 mg/g and recommended the herb as a natural and economical source of antioxidants against free radical-induced toxicity [19, 20].

Reported that the hydro-alcoholic *C. aromaticus* extract of the leaves scavenged the DPPH radicals in a concentration-dependent manner with maximum scavenging activity of 80% and further Govindaraju Subramaniyan *et al.*, (2014) [21] showed that, IC_{50} values for scavenging of DPPH by the essential oil of *C. aromaticus* was found to be 54.23 µl/ml, while for Ascorbic acid it was 50.44 µl/ ml [21].

In this study, the protective effect of purified CALP was comparable to the protective effect of ascorbic acid against H_2O_2 induced DNA damage. In **Fig. 2**, Lane A shows the sheared calf thymus DNA without any treatment, Lane B shows sheared Calf thymus DNA treated with H_2O_2 and protection offered by CALP at a dosage of 25 µg. Lane C shows damage caused by H_2O_2 (144 µM) to sheared calf thymus DNA resulting in low molecular weight species of DNA. Oxidative damaged DNA being low molecular weight moves faster in electrophoresis. The protection provided by purified CALP (25 µg) is almost equal to the protection given by Ascorbic acid (400 µM) as shown in Lane D. Excess oxidative stress target the DNA, which attacks bases and sugar moieties, creating strand breaks, altering gene expression, and ultimately lead to mutagenesis. Oxidative damaged DNA has been thought to be a critical contributor to the development of aging and some degenerative diseases. Moreover, continuous oxidative damage to DNA is believed to be a significant contributor to the development of many cancers [22].

A study by Ramadas D *et al.*, (2015) [10] showed a protective effect of *Coleus aromaticus* (25 µg) extract on tertiary butyl hydroperoxide induced DNA damaged [10]. A similar Study by [9] also observed a protective effect of boiling water extract of *Coleus aromaticus* on H_2O_2 induced DNA damage [9]. Further, a study by Rao *et al.*, (2006) [23] reported a hydroalcoholic extract of *Coleus aromaticus* offered protection against radiation-induced DNA damage in Chinese hamster fibroblast (V79) cells [23]. The present specifically demonstrates the protective effect of *Coleus aromaticus* protein on DNA damage by hydrogen peroxide. The viability of human cell assay is to evaluate the toxicity of test compounds and will also show whether the extract is toxic to human cells or not [24]. As shown in **Table 1** and **Fig. 3**, untreated human peripheral lymphocytes had 83 ± 4% viability, whereas there was a decrease in viability of lymphocytes induced by H_2O_2 after one-hour incubation (58 ± 2). However in the presence of purified CALP at 25 µg, BHA at 400 µM and ascorbic acid at 400 µM the lymphocyte viability increased to 71 ± 2%, 68 ± 3% and 78 ± 2% respectively. The results of the present study indicate that the efficiency of purified CALP

protection against H_2O_2 induced cell death, similar to other standard antioxidants such as Ascorbic acid and BHA. A study by [25] also reported an aqueous extract of *Coleus aromaticus* leaves had protective effects on the viability of the cells in a fibroblast culture system [25]. Thus, the protective mechanism against cell death by antioxidants such as Ascorbic acid, BHA, and purified CALP can neutralize the primary effect of the free radicals and the subsequent cell death.

CONCLUSION: This study demonstrated that the purified CALP possesses significant antioxidant activity and protective effect on H_2O_2 induced DNA damage and also lymphocyte cell damage, similar to the standard antioxidant BHA and ascorbic acid. Additionally, this study indicates that the purified protein of *Coleus aromaticus* leaves by itself is also not toxic to the cells.

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CONFLICTS OF INTEREST: There are no conflicts of interest.

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