ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 15, Issue 3, 2022

Online - 2455-3891
Print - 0974-2441

Research Article

PREVENTION OF DNA SUGAR, HUMAN PERIPHERAL LYMPHOCYTES AND ERYTHROCYTES DAMAGES FROM FREE RADICAL INDUCED OXIDATION BY NATURAL ANTIOXIDANTS

OMKAR NG[1], PREM KUMAR G[2]*, BHAGYALAKSHMI V[3], DINESHA RAMADAS[4]

[1]Department of Physiology, Shridevi Institute of Medical Sciences and Research Hospital, Tumkur, Karnataka, India. [2]Department of Biochemistry, Andra Medical College, Visakhapatnam, Andhra Pradesh, India. [3]Department of Biochemistry, Rangaraya Medical College, Kakinada, Andhra Pradesh, India. [4]Department of Biochemistry, Adichunchanagiri Institute for Molecular Medicine, Central Research Laboratory-Adichunchanagiri Institute of Medical Sciences, Adichunchanagiri University, Mandya, Karnataka, India.
Email: dr.premkumargera@yahoo.co.in

Received: 30 November 2021, Revised and Accepted: 31 January 2022

ABSTRACT

Objective: The present study focused on the identification of particular extract which shows signification protection of DNA sugar against excessive oxidation.

Methods: The different extracts (water, alcohol, alcohol: water, and hexane) of Agathi seeds (*Sesbania grandiflora Linn)* were evaluated using various antioxidant and other relevant assays like DNA sugar protection and antioxidant activities.

Results: The alcohol: water (1:1) extract of *S. grandiflora Linn* seeds showed the highest antioxidant and free radical scavenging activity. It inhibited membrane lipid peroxidation by 55% at 50 µg/ml, scavenged approximately 69% of hydroxyl and 1,1-diphenyl-2-picrylhydrazayl radicals at 2–3 fold lower concentrations compared to the other extracts. In addition, the alcohol: water extract inhibited ferrous sulfate: ascorbate-induced sugar oxidation of DNA and also showed non-toxic nature against lymphocytes.

Conclusion: These results establish the antioxidant potential of the extract, which could be used as natural antioxidant source.

Keywords: DNA, Lymphocytes, Fenton, Erythrocytes, Agathi, Antioxidants.

 DOI: http://dx.doi.org/10.22159/ajpcr.2022v15i3.43726. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Natural diet is rich sources of active compounds polyphenols, carotenoids, flavonoids, vitamins, proteins, polysaccharides, and certain important trace metals such as zinc and selenium [1-3]. These play a major role as an antioxidant source in reduce cellular damages [4]. Antioxidants are man-made or natural substances, diet high in green leafy vegetables and fruits, which are good sources of antioxidants, have been found to be healthy [5]. Antioxidant substances could be natural or synthetic. Natural antioxidants are obtained from natural sources are safe, non-toxic, inexpensive, and have been used in food, cosmetics, and other related industries. On the other hand, synthetic antioxidants are substances created from chemical process; in excessive usage, they are reported as toxic [6]. Hence, day by day, researchers are in the search of new source of natural antioxidants.

The plant Agathi (*Sesbania grandiflora*) belongs to the family *Fabaceae* of genus *Sesbania* and species Grandiflora. It is widely grown in India, Indonesia, Myanmar, the Philippines, Thailand, and South-East Asian countries. The leaves are bitter are rich in Vitamin C, calcium, sterols, saponin, quercetin, myricetin, and other chemical antioxidants [7]. It is reported that, the seeds of *S. grandiflora* rich with natural antioxidant Vitamin E and Phytocerol contents. Hence, herein we made an attempt to find the DNA sugar protectant and non-toxic nature of antioxidant content of ethanol-water extract of *S. grandiflora* [8].

METHODS

Agathi seeds were obtained from authentic source, Bangalore Karnataka state, India. 100 g of well dried Agathi seeds were grounded into a fine powder using a domestic electric grinder.

Preparation of extracts of agathi seeds

One gram of the Agathi seeds powder was added to 100 ml of double-distilled water, ethyl alcohol: water (1:1), ethyl alcohol, and hexane. The ethyl alcohol and hexane were analytical grade. The solutions were homogenized and the resultant suspension was centrifuged using refrigerated centrifuge for 10 min at 4°C. The supernatants were filtered using Whatman No. I filter paper. The water extract of Agathi seeds powder (Ethanol-water Agathi seed extract) was lyophilized, ethyl alcohol-water (1:1) extract and hexane extracts were rotary evaporated and concentrated. Similarly, other solvent extracts the ethyl alcohol extract of Agathi seeds and the hexane extract of Agathi seeds were concentrated separately using rotary evaporator. Each dried extract (was dissolved mg) in 0.1 ml of the respective extracting solvent or solvent mixture and made up to 10 ml with water. The solution was filtered using glass wool followed with 0.45 µm microbial filter and stored at 4°C for further studies.

Proximate analysis

Estimation of protein content

The total protein content of the crude extract was determined as per the standard protocol [9]. Various concentrations of bovine albumin (0–100 μg/mL) or agathi leaves 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 samples was determined from the calibration curve.

Estimation of total sugar

The total sugar of the agathi ethanol-water extract was estimated by the phenol-sulphuric acid method [10]. Different aliquots of the extract

(0–25 μL) were made up to 1mL with distilled water. To this 1 mL of 5% phenol and 5 mL of concentrated sulphuric acid were added keeping the mixture ice-cold water bath. Orangishred color developed was read at 520 nm immediately. The sugar concentration of the extract was calculated according to the standard glucose calibration curve.

Determination of total phenol content

The total phenolic content of the agathi ethanol-water extract was determined according to the method of Folin-Ciocalteu reaction [11] with appropriate modifications, using gallic acid as standard. An aliquot of the samples (10–40 μL) was mixed with 50% Folin-Ciocalteu reagent; the volume was made up to 1mL with methanol: water mixture (50:50 v/v). Further, the mixture was then allowed to stand for 10min at room temperature followed by the addition of 20% Na_2CO_3. Further, the absorbance was measured at 725 nm. Results were expressed as milligrams of gallic acid equivalents per gram.

Antioxidant activity

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

DPPH radical scavenging activity was assessed as per the standard method [12]. DPPH, α-tocopherol, and Ascorbic acid were procured from Sigma (St. Louis, USA). Different extracts of Agathi seeds at various concentrations ranging from 10 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 colorimetrically at 517 nm in a Shimadzu ultraviolet (UV)-1601 spectrophotometer (Tokyo, Japan). Standard antioxidants such as α-tocopherol and Ascorbic acid, all at 400 µM and 100 µM respectively, 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.

$$\% \text{ Viability} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined using Deoxyribose assay [13] with some modification. The reaction mixture contained $FeCl_3$ and ascorbate (100 μM), H_2O_2 (1 mM), EDTA (100 μM), 2- deoxy-D-ribose (2.8mM), and 1mL of 0.1mM potassium phosphate buffer (pH 7.4) mixed in various concentrations of Agathi seeds extract (50–400 μg/mL). The reaction mixture was incubated for 1 h at 37°C. The reaction was terminated by adding 1 mL each of Trichloroacetic acid (2.8%) and Thiobarbituric acid (0.5%); this mixture was placed in boiling water bath for 15 min. After cooling, the reaction mixture was centrifuged for 5 min at 5000 rpm. The control was without any test compound and the readings were taken at 535 nm. The percentage hydroxyl radical scavenging activity was determined by comparing with control. Decreased absorbance of the reaction mixture indicated decreased oxidation. Consider the following:

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

Determination of antioxidant activity using erythrocyte ghost and linolenic acid micelles

The erythrocyte membranes (ghosts) preparation was carried out as per the standard procedure [14]. In brief, fresh venous human blood samples were drawn with ACD anticoagulant (acid citrate dextrose) mixed well and refrigerated centrifuged, the obtained pellet was washed 3 to 5 times using isotonic phosphate buffer saline (PBS 5 mM, pH 7.4, and 150 mM NaCl). Further, the cell pellet was suspended in hypotonic (PBS 5 mM, pH 7.4 at 4°C) for hemolysis to take place. Further, contents were refrigerated centrifuged at 12,000 rpm for 20 min. The obtained erythrocytes were separated from plasma and buffy coat again washed with fresh hypotonic phosphate buffer and centrifuged at 1500 rpm to remove unlysed RBC cells. The membranes were dispersed pale yellowish pink "ghost" suspended in isotonic 5 mM phosphate buffer, pH 7.4. By Bradford's method, the protein content of ghost was estimated [6]. Ghost suspension (200 μg) and linolenic acid (1.8 μmole) were subjected to peroxidation by Fenton reactants (ferrous sulfate and ascorbic acid) (10:100 μmole) in a final volume of 0.5 mL Tris-buffered saline (TBS 100 mM, pH 7.4, and 0.15 M NaCl) with increasing concentration of agathi seeds extract (10 to 50 μg); the contents were incubated for an hour and 1% TBA was added. Finally, the contents were kept in a boiling water bath for 15 min and then cooled, centrifuged to remove precipitate if any. The color developed was read at 535 nm using UV visible spectrophotometer.

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

Isolation of lymphocyte and their protection

Human peripheral lymphocytes were isolated according to the standard protocol [15]. Fresh venous blood (10 mL) mixed with four volumes of hemolyzing buffer (150 mM NH_4Cl in 10 mM tris buffer, pH 7.4) and mixed well. The contents were incubated for 30 min at 4°C and centrifuged at 1200 rpm for 20 min and the supernatant was discarded. The pellet was washed twice to thrice with 10 mL of 250 mM m-inositiol in 10 mM phosphate buffer, pH 7.4, and re-suspended in the same solution. The cell viability was determined by tryphan dye blue exclusion method. Percentage viability was calculated as follows;

$$\% \text{ Viability} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

Determination of DNA sugar damage by spectrophotometric method

Fenton's reactants induced oxidative DNA sugar damage was determined according to the standard protocol [16]. In brief, the reaction mixture in a total volume of 1 mL containing 1 mg calf thymus DNA was treated with Fe^{3+} (10 mM), EDTA (10 mM) and H_2O_2 (2 mM) without or with various concentrations of the extract (10–50 µg) in potassium phosphate buffer (20 mM, pH 7.4). Ascorbic acid (10 mM) was added to the reaction mixture and was incubated at 37°C for 1 h in water bath with shaker. To 1 mL of the above mixture 1 mL of Trichloro acetic acid and 1 mL of 1% TBA were added and boiled for 20 min. The contents were cooled and the pink color absorbance was read spectrophotometrically at 523 nm.

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

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.

Table 1: Cytotoxicity of ethanol-water extract of agathi (*Sesbania grandiflora Linn)* seeds toward human blood lymphocytes

Concentration (mg/mL)	Viability (%)
No treatment	97.0±3.21
Fenton reactant along	35.0±0.82
Fenton reactant+0.2 (extract)	94.0±1.23
Fenton reactant+0.4 (extract)	95.0±1.24
Fenton reactant+0.6 (extract)	96.0±1.77[a]
Fenton reactant+0.8 (extract)	96.0±1.78[b]

Data are expressed as the mean standard deviation (n=3). Means with different letters (a–b) are significantly different ($p<0.05$)

Table 2: Diphenyl-2-picrylhydrazyl radical scavenging activity of ethanol-water extract of Agathi (*Sesbania grandiflora Linn*) seeds

Extraction	Concentration (µg/mL)	Percentage DPPH[a] radical scavenging activity
Negative control	No	0
Water	100	40±1.2
Ethanol	100	51±2.0
Hexane	100	46±2.2
Ethanol – water	25	88±1.7
Alpha-tocopherol	85.5 (400 µM)	82±1.5
Ascorbic acid	100	78±2.2

Each value is expressed as mean±standard deviation (*n*=3). [a]PPH, 1,1-dephenyl-r-picrylhydrazyl (DPPH) radical. DPPH Diphenyl-2-picrylhydrazyl

RESULTS AND DISCUSSION

The different extracts of Agathi seeds were subjected to proximate analysis and DPPH radical scavenging activity. The proximate analysis showed that the ethanol-water extract contains Polyphenols, proteins, and negligible amount of sugars (data not shown). The antioxidant and DNA protective nature and non-toxic nature to cells study results are as follows.

DPPH radical scavenging effect

The DPPH radical scavenging activity of ethanol-water extract of Agathi seeds on Fe^{3+} dependent hydroxyl radical generation was reported and confirmed using a direct approach [17,18]. The results obtained are shown in Table 2, the ethanol-water extract of Agathi seeds exhibited powerful DPPH radical scavenging activity of 88% at 25 µg, which was much more than water, ethanol, hexane extract (at 100 µg dosage each), which showed DPPH radical scavenging activity of 40%, 41%, 51%, and 46%, respectively. The well-known antioxidants such as alpha-tocopherol (85.5 µg) and ascorbic acid (100 µg) showed 82 and 78% DPPH radical scavenging activity respectively. The results indicate that ethanol-water extract is a powerful free radical scavenger compared to other extracts and known antioxidants. Hence, for further studies only ethanol-water extraction of Agathi seeds considered.

Hydroxyl radical scavenging activity

The short-lived hydroxyl radicals are highly reactive of all the reduced form of dioxygen and in excess, initiate cell damage in *in vivo* model [19,20]. The antioxidant effect of Agathi seeds extracts on hydroxyl radicals generated by Fe^{3+} ions was measured by the extent of deoxyribose degradation, which is an indicator of TBA–MDA adducts formation. Among the various extracts tested (Fig. 1). The ethanol-water extract showed maximum hydroxyl radical scavenging activity by 69% at 50 µg which is comparatively good when compared to standard antioxidant Ascorbic acid (400 µM) and alpha-tocopherol (400 µM) which were 75% and 71% respectively. This implies that ethanol-water extract could be an effective hydroxyl radical scavenger.

Inhibitory effect of AWEC on fenton reactant-induced DNA sugar damage

To find the chelating and the inhibitory effect of ethanol-water extract of Agathi seeds against iron-dependent oxidation of calf thymus DNA sugar was tested by a TBARs assay. As shown in Fig. 2, the extract offered effective inhibition by 55% at 50 µg against ferrous sulfate: ascorbate-induced [16]. DNA sugar damage using erythrocyte ghost as a source of lipids, when compared to Ascorbic acid (400 µM) and Alpha-tocopherol (400 µM), showed 62% and 71% respectively. When Linolenic acid micelle is used as a source of lipids, the maximum protection provided by the ethanol- water extract is 55% when compared to Ascorbic acid (400 µM) and Alpha-tocopherol (400 µM), showed 66% and 71%, respectively. Similar studies have reported that extracts of Curry leaves, Turmeric, *Coleus aromaticus* leaves extract exhibit chelating effect of ferrous ions and a reducing capacity [16,19,21,22].

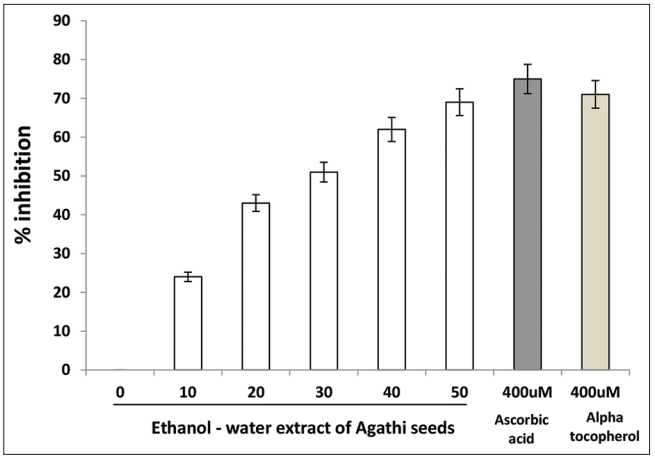

Fig. 1: Hydroxyl radical scavenging activity of ethanol-water extract of Agathi seeds, ascorbic acid and alpha tocopherol. Data are expressed as the mean±standard deviation (n=3). Mean values p<0.005

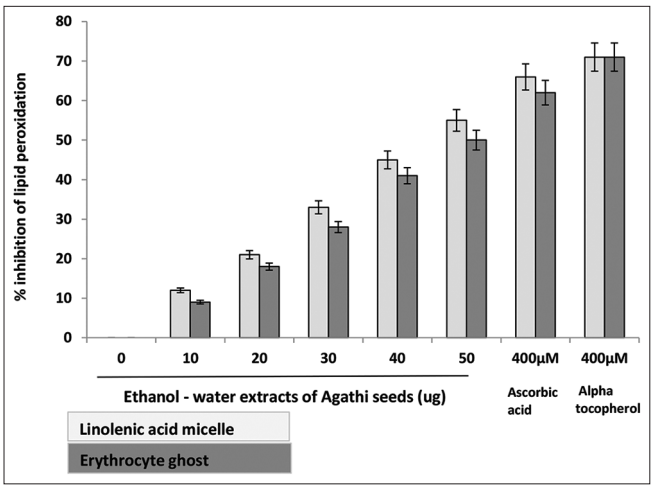

Fig. 2: Inhibition of lipid peroxidation in linolenic acid micelle and erythrocyte ghost. Data are expressed as mean standard deviation (n=3); Means values p<0.05

Lymphocytes protection studies

We also investigated the lipid peroxidation induced cell death by Fenton reactants (Table 2). Treatment with Fenton reactants on lymphocyte cells significantly showed cell toxicity. The maximum cell death was induced by Fenton reactant noticed at 30 min. The cell death induced by the Fenton reactant is alone about 35%. The protection offered by ethanol-water extract was 96% at both 0.6 and 0.8 mg/ml. This will show the protective nature and non-toxic nature of the extract. The high percentage of viable cell clearly indicates that ethanol-water extract of Agathi seeds is non-toxic protein with no cytotoxicity toward human lymphocytes (Table 1).

CONCLUSION

This work highlights the importance of curry leaves which have been traditionally used in the Indian culinary system from time immemorial, as a rich source of antioxidants. More specifically, the extraction of curry leaves in a 1:1 ethyl alcohol: water mixture, compared to other solvent systems, showed maximum antioxidant and free radical scavenging activities under in vitro conditions. Thus, the ethyl alcohol: water (1:1) extract of curry leaves could be a very good lead for the extraction of an effective natural nutraceutical or antioxidant drug. Further investigation into ethyl alcohol: water (1:1) extract of curry leaves for its lead active compounds and *in vivo*, antioxidant mechanisms are warranted.

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.

FUNDING

This study was done without any external financial support.

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