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Research Article

IN VITRO ANTIOXIDANT ACTIVITY OF ETHYL ACETATE FRACTION OF WATER EXTRACT OF FLOWERS OF *COUROUPITA GUAIANENSIS*

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ABSTRACT

Couroupita guaianensis Aublet (Family: *Lecythiadaceae*) commonly known as Naglingam is a tree found throughout the plains of India. The flowers are used to cure cold, intestinal gas formation and stomachache. Ethyl acetate fraction of water extract of *Couroupita guaianensis* flowers (EAFWE) was screened for *in- vitro* antioxidant activity using DPPH assay, superoxide scavenging effect, reducing power and *in-vitro* lipid peroxidation.

EAFWE was found to be extremely effective in scavenging DPPH (EC_{50} =24.41 µg/ml) and superoxide radical (EC_{50}=10.65 µg/ml) whereas inhibition of lipid peroxidation was moderate (EC_{50}=199.70). This fraction also exhibited anti oxidant activity in terms of significant reducing power.

Keywords: Antioxidant activity, DPPH, Superoxide, Reducing power.

INTRODUCTION

Many medical and scientific researchers are convinced that uncontrolled free radical activity in the body is directly associated with a number of health problems. Free radical reactions have been implicated in the pathology of many human diseases including atherosclerosis, ischemic heart disease, the aging process, inflammation, diabetes, immunodepression, the neurodegenerative condition and other disease conditions[1].

Free radicals are continuously produced in our body. However, these are rigorously controlled by antioxidants. When this precarious balance is broken, in favour of free radicals, it causes an oxidative stress. This oxidative stress can attack lipids, which constitute the cellular membranes, bases of the DNA, and amino acids of proteins. Antioxidants fight free radicals, and therefore may be able to help prevent the diseases that free radicals promote. Free radical scavengers (anti-oxidants) are key elements in the defense system, which the body uses in order to neutralize the activity of these dangerous and, over the long-term, deadly free radical enemies. Drugs with multiple mechanisms of protective action, including antioxidant properties, may be one way forward in minimizing tissue injury in human disease [2].

As plants produce a lot of antioxidants to control the oxidative stress, they can represent a source of new compounds with antioxidant activity. A number of plants and plant isolates have been reported to protect free-radical induced damage in various experimental models.

Couroupita guianensis Aublet (Family: *Lecythiadaceae*) commonly known as Naglingam is a tree found throughout the plains of India. The flowers are used to cure cold, intestinal gas formation and stomachache[3]. From the flowers of *C.guianensis,* an aliphatic hydrocarbon and stigmasterol have been isolated[4]. Different extracts of flowers of the plant have been screened for immunomodulatory activity[5]. Methanol extract derived fractions of the plant inhibited the growth of microorganisms[6]. Petroleum ether and chloroform extracts of this plant exhibited larvicidal activity against vectors[7]. Aim of the present study was set to evaluate ethyl acetate fraction of water extract (EAFWE) of flowers of the plant *C.guianensis* for its *in-vitro* antioxidant activity, as there is scarcity of data available on the same.

METHODS

Plant material

Flowers of *C.guaianensis* were collected from the outfield of Baroda city, India and authenticated in Botany Department of M.S.University, Baroda, India.

Preparation of test fraction (EAFWE)

Air dried powder of flowers was subjected to extraction with distilled water. Extract was filtered and then centrifuged. Water extract was reduced to half and then polyssaccaride content was removed by precipitation with alcohol. Precipitate was removed by centrifugation and the filtrate was repeatedly shaken with ethyl acetate in a separating funnel. All ethyl acetate fractions were combined and concentrated in vacuum and further used for antioxidant screening. Preliminary phytochemical screening showed presence of phenolics, tannins and flavonoids in EAFWE.

Animals

The study protocol was approved by Institutional Animal Ethical Committee. Rats of either sex, weighing 160-200g were used for the experiment. The animal was sacrificed and a 10% w/v liver homogenate was prepared and used for the *in vitro* estimations.

In-vitro antioxidant activity

Assay for antiradical activity with DPPH

Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of colored 1, 1, diphenyl picryl hydrazine brought about by sample[8]. A stock solution of DPPH was prepared by dissolving 4.4 mg in 3.3 ml methanol. Test medium included 150µl of DPPH solution along with different concentration of samples in 3 ml methanol. Blank was prepared in the same way, with no sample added. The decrease in absorbance caused by the presence of sample was noted after 15 minutes. EC_{50} was calculated as the 50% reduction in absorbance brought about by sample compared with blank.

Assay for superoxide radical scavenging activity

The assay was based on capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-nitro blue tetrazolium (NBT) system[9]. The reaction medium contains 2.5ml of phosphate buffer (pH 7.6), 100µl riboflavin (20µg), 200µl EDTA (12mM), 100µl NBT (0.1 mg) and different concentrations of sample contained in 100µl of methanol. The reaction was started by illuminating the reaction mixture for 5 minutes. The absorbance was measured at 590 nm. Blank was performed in the same way, with 100µl of methanol instead of test substance. EC_{50} was calculated as 50% reduction in absorbance brought about by sample compared with blank.

Determination of reducing power

The reducing power of EAFWE was determined according to the method of Oyaizu[10]. Samples were mixed with 5 ml phosphate buffer

(2M, pH 6.6) and 5 ml potassium ferricyanide (1%). The mixture was then incubated at 50° C for 20 minutes. 5 ml trichloroacetic acid (10%) was added and the mixture was centrifuged at 4000 rev./ min. The upper 5 ml solution was then mixed with 5 ml distilled water and 1 ml ferric chloride (0.1%). The absorbance was then measured at 700 nm. An increase in absorbance of the reaction mixture indicated an increase in the reducing power. Ascorbic acid (0.3 mg) was used as standard.

Measurement of effect on lipid peroxidation on rat liver homogenate

Rat liver homogenate was prepared by homogenizing the tissue in chilled Tris buffer (10mM, pH 7.4) at a concentration of 10% w/v. Peroxidation was induced in liver tissue by Iron-ADP complex in the presence of ascorbic acid. The incubation medium constituted of 0.5 ml of the liver homogenate (10% w/v), 100 µM $FeCl_3$, 1.7 µM ADP, 500 µM of ascorbate and different concentrations of samples in 2 ml of total incubation medium. The medium was incubated for 20 min. at 37°C. Extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) content[11]. Results were expressed in terms of decrease in MDA formation by the sample extract. Ascorbic acid was used as positive control.

RESULTS

***In-vitro* antioxidant activity**

EAFWE showed a concentration dependent antiradical activity by inhibiting DPPH radical with an EC_{50} value of 24.41µg/ml (Table 1). EAFWE showed almost two times more inhibitory activity on DPPH radical than the standard curcumin which showed an EC_{50} value of 52.71 µg/ml.

EAFWE was also found to scavenge superoxide radicals generated in riboflavin-NBT-light system *in-vitro.* The EC_{50} value of EAFWE was found to be 10.65 µg/ml. EAFWE was found to be two times more potent than ascorbic acid, which had an EC_{50} of 23.52 µg/ml (Table 2).

As shown in Table 3, EAFWE (15mg/ml) showed reducing power that was comparable to the standard.

In the present study EAFWE showed potent and concentration dependent inhibition of lipid peroxidation induced by Iron/ADP/Ascorbate complex in the rat liver homogenate. The EC_{50} value for EAFWE was found to be 199.70 µg/ml; whereas the standard, ascorbic acid showed an EC_{50} value of 30.05 µg/ml (Table 4).

Table 1: Antiradical activity of EAFWE of flowers of *C.guaianensis* observed with DPPH

Samples	Concentration (µg/ml)	% inhibition	EC 50 (µg/ml)
	5	10.93 ± 1.71	
	10	16.07 ± 1.26	
EAFWE	20	36.93 ± 1.31	24.41
	30	74.78 ± 1.45	
	40	85.93 ± 3.50	
Curcumin			52.71

Values are mean ± S.E.M. of three replicate analyses.

Table 2: Superoxide anion scavenging activity of EAFWE of flowers of *C.guaianensis* observed with a riboflavin-light-NBT system

Samples	Concentration (µg/ml)	% inhibition	EC 50 (µg/ml)
	5	38.94 ± 1.90	
	10	50.66 ± 0.17	
EAFWE	20	66.00 ± 0.91	10.65
	30	69.96 ± 0.33	
	40	72.77 ± 1.03	
Ascorbic acid			23.52

Values are mean ± S.E.M. of three replicate analyses.

Table 3; Reducing power determination of different concentrations of EAFWE of flowers of *C.guaianensis*

Sample	Reducing powers of different concentrations (mg/ml)			
	0.0	5.0	10.0	15.0
EAFWE	0.028 ± 0.01	0.091 ± 0.03	0.218 ± 0.06	0.303 ± 0.07

Values are mean ± S.E.M. of three replicate analyses.

Ascorbic acid (0.3 mg) was used as standard, giving a reading of 0.430 at 700 nm.

Table 4: Inhibition of lipid peroxidation induced by iron/ADP/ascorbate system in rat liver homogenate by EAFWE of flowers of *C.guaianensis*

Samples	Concentration (µg/ml)	% inhibition	EC 50 (µg/ml)
	50	10.91 ± 2.20	
EAFWE	100	27.07 ± 4.37	199.70
	150	34.10 ± 3.09	
	200	49.71 ± 3.19	
	250	64.09 ± 3.07	
Ascorbic acid			30.05

Values are mean ± S.E.M. of three replicate analyses.

DISCUSSION

The participation of reactive oxygen species in etiology and physiopathology of human disease, such as neurodegenerative disorders, inflammation, viral infection, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and gastric ulcers is already evident. To understand the role of these reactive oxygen species in several disorders and the potential antioxidant protective effect of natural compounds on affected tissues are topics of high current interest. Initially it is necessary to investigate the *in-vitro* antioxidant properties of any natural product or drug to consider it as an antioxidant substance, followed by evaluation of its antioxidant function in biological systems[12].

In the present attempt therefore antioxidant activity of the EAFWE of flowers of *C.guaianensis* was first evaluated *in-vitro*.

DPPH is a stable free radical in aqueous or ethanol solution and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[13]. In order to evaluate antioxidant potency through free radical scavenging with the test samples, the change in the optical density of DPPH radicals is monitored. Hence, $DPPH^{\bullet}$ is usually used as a substrate to evaluate the antioxidant activity[14]. Superoxide radical is known to be very harmful to cellular components as they serve as precursors of more reactive oxygen species[15].

The measurement of reductive ability was done by Fe^{3+}-Fe^{2+} transformation in the presence of EAFWE and standard antioxidant, ascorbic acid. The reducing power is associated with antioxidant activity [14]. Lipid peroxidation is initiated by radicals attacking unsaturated fatty acids, and propagated by a chain reaction cycle[16]. Since unsaturated fatty acids are most important components of biological membranes and impart desirable properties upon the fluidity of cellular membrane structure, the peroxidation of unsaturated fatty acids in biological membranes leads to disruption of membrane structure and function[17]. In particular O^{-2} and $^{\bullet}OH$ induce various injuries to the surrounding organs and play a vital role in some clinical disorders. Therefore removal of O^{-2} and $^{\bullet}OH$ is the most effective defense of the living body against diseases[18]. Any compound, natural or synthetic, with antioxidant properties might totally or partially alleviate this damage.

Recent studies showed that a number of plant products contain polyphenolic substances such as flavonoids and tannins. These natural antioxidative substances usually have a phenolic moiety in their molecular structure. They have been found among flavonoids, tocopherols and catechines. Organic acids, carotenoids, protein hydrolysates and tannins can act as antioxidants or can have synergistic effects when used together with phenolic antioxidants. Phenolic antioxidants are potent free radical terminators[19, 20]. Phenolic compounds, the biologically active components, are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups[21, 22]. In the present study EAFWE revealed the presence of phenolic compounds and flavonoids, and thus one can substantiate its antioxidant activity to its chemical composition.

The exact mechanism of action, however, can only be unfolded after detailed characterization of active moieties from tested fraction. Studies on these lines have already been taken up and shall follow in our future communications.

In conclusion, the results of present study indicate that EAFWE prepared from *C. guaianensis* possesses antioxidant activity. EAFWE was extremely effective in scavenging the stable free radical DPPH and superoxide ion and also possess significant reducing power with inhibitory action on lipid peroxidation. Further *in-vivo* studies on evaluation of drug are warranted and already taken up. It may be concluded that the free radical scavenging activity of flowers of *C.guaianensis* may follow one of the above mechanisms in exhibiting its effectiveness in traditional medicine.

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