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Preliminary studies on the antioxidant activity of *Tribulus terrestris* and *Eclipta alba*

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ABSTRACT
Tribulus terrestris L. and *Eclipta alba* L. are commonly used plants in Indian folklore medicine. The present study investigates the antioxidant potential of ethanolic extract of the fruits of *Tribulus terrestris* and the methanolic extract of the aerial parts of *Eclipta alba*. They were investigated for hydroxyl radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free radical scavenging activity and for inhibition of lipid peroxidation. It was found that both the extracts exhibited significant free radical scavenging capacity for DPPH and for hydroxyl radical. Further, the extracts showed considerable inhibition of lipid peroxidation.
KEYWORDS*: Tribulus terrestris*, *Eclipta alba*, antioxidant activity, free radicals, lipid peroxidation

INTRODUCTION

Reactive oxygen species (ROS) such as O_2^-, H_2O_2 and $HO^\cdot$ are harmful to the living cells. Oxidative stress that results from the increased generation of ROS has been implicated in major human ailments like cardiovascular disease [1], cancer [2], neural disorders [3, 4] and in the process of ageing [5]. Normally the cellular antioxidant enzymes and free radical scavengers protect a cell from toxic effects of ROS. However the oxidative damage of cellular macromolecules occurs when generation of ROS overtakes the antioxidant defense [6]. Exposure of cell membrane to free radicals stimulates the process of lipid peroxidation. Lipid peroxidation leads to cell necrosis, which is implicated in a number of pathophysiological conditions. The most reactive free radical is the hydroxyl radical which is known to initiate lipid peroxidation and cause fragmentation of DNA leading to mutations [7]. Along with this new understanding of the injurious role of free radicals in disease pathology, there is an increasing appreciation for the need for both fundamental and clinical research on antioxidants. The role of potential preventive and therapeutic benefits of antioxidants is now being foreseen for a variety of antioxidant nutritional and pharmacological interventions. Hence the therapeutic use of antioxidant compounds in diseases would perhaps be one of the approaches to control the ROS mediated pathogenesis.

Large numbers of medicinal plants containing flavonoids, saponins, and polyphenolic constituents etc. have potential for antioxidant activity [8]. Several plants like *Withania somnifera* [9], *Tinospora cordifolia* [10], *Emblica officinalis* [11], *Glycyrrhiza glabra* [12] etc, have been studied extensively for antioxidant activity. However an array of plants remains unexplored for potential antioxidant activity.

Tribulus terrestris L. (Zygophyllaceae) is an annual plant native of mediterranean region. In India, it is called "Gokhru". *T. terrestris* is an important herb commonly used in the folk medicine of many countries for different purposes. The fruits of the plant *T. terrestris* has been shown to exhibit diuretic [13], anti-urolithiatic [14], CNS stimulant [15], antimicrobial [16] and antifungal activities in rats [17]. Recently Ojha et al [18] and Phillips et al [19] have confirmed the antioxidant and antihypertensive activity in rat heart.

Eclipta alba L. (Asteraceae) grows in tropical and subtropical countries at an altitude of up to 2000 meters. In India, it is called "Bhringraj". The plant *E. alba* has been reported to show hepatoprotective effect on experimental liver damage in rats and mice [20]. In ayurveda, it is used for the treatment of liver cirrhosis and infective hepatitis [21]. *E. alba* has also been found to exhibit nootropic [22], analgesic [23] and antimicrobial activity [24].

Although both the plants are rich in polyphenolic constituents [25, 26] there has been a dearth of studies reported to explore their antioxidant potential. This prompted us to evaluate the *in vitro* antioxidant activity of *T. terrestris* and *E. alba*. On the basis of earlier reports the methanolic extract of *E. alba* [27, 28] and ethanolic extract of *T. terrestris* [29, 30] were selected for the investigations. The extracts were investigated for inhibition of lipid peroxidation and hydroxyl radical as well as 1,1-diphenyl-2-picrylhydrazyl stable free radical (DPPH) scavenging activity.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), and adenosine-5-diphosphate (ADP) were obtained from Hi-media (Mumbai) while ascorbic acid was obtained from Thomas Baker Chemicals Ltd.(Mumbai). All other chemicals and reagents used were of analytical grade available commercially.

Plant material

Aerial parts of *E. alba* and fruits of *T. terrestris* were obtained from and authenticated by Dr. Ashish Phadke, Head, Department of Draavyaguna (Herbal Pharmacology), Y.M.T. Ayurvedic Medical College, Khargar, Navi Mumbai, Mumbai, India. A voucher specimen of the drugs has been retained at the Department of Pharmacology of Bombay College of Pharmacy, Mumbai.

Extraction

The parts of the plants were dried at room temperature and powdered. The powder of aerial parts of *E. alba* was extracted with methanol (yield: 15.7%, w/w) and of fruits of *T. terrestris* was extracted with 95% ethanol (yield: 20.32%, w/w) in a soxhlet extractor until extraction was complete and the solvent was evaporated under reduced pressure at 40°C in a rota-evaporator. Qualitative phytochemical analysis indicated the presence of saponins, tannins and alkaloids in the ethanolic extract of *T. terrestris*. While the methanolic extract of *E. alba* indicated the presence of tannins and saponins.

Animals

Male Wistar rats weighing 180-220 g were used. Ethical clearance for the study was obtained from the Institutional Animal Ethics Committee of the Bombay College of Pharmacy. They were housed in standard environmental conditions and allowed free access to standard laboratory chow and water *ad libitum*.

Inhibition of lipid peroxidation in rat liver homogenate

For preparing liver homogenate (10%), Wistar rats were killed by decapitation. The liver was exhaustively perfused with ice-cold saline through the portal vein. The liver was separated and then homogenized in 10 volumes of ice-cold 0.15 M KCl [31].

The level of lipid peroxidation in the rat liver was measured *in-vitro* as thiobarbituric acid reactive substances (TBARS) [32]. Fresh liver homogenates (0.2 mL) was incubated with 0.1 mL of 0.15 M KCl, Tris HCl buffer (pH 7.5) and 0.05 mL of 40 µM adenosine diphosphate with different concentrations of the test extracts dissolved in dimethyl sulphoxide (DMSO) separately (10 to 400 µg/mL) in 0.05 mL. After 5 min, the reaction was started by the addition of 0.1 mL each of 10 µM $FeSO_4$ and 100 µM ascorbic acid and the reaction mixture was incubated at 37°C for 1 h. The reaction was terminated by addition of 2 mL thiobarbituric acid reagent, boiled for 15 min at 95°C, cooled under tap water and then, centrifuged. The absorbance of the supernatant was measured at 535 nm against the respective blanks, which contained all reagents and corresponding extract except the liver homogenate. Control samples contained the liver homogenate with all the reagents but the extract was replaced by DMSO. α-Tocopherol (47.27 µg/mL) was used as a reference standard for comparison, final concentration in reaction mixture was 100µM. Malondialdehyde thus formed was quantified using the molar extinction coefficient of 1.56×10^5 $cm^{-1}M^{-1}$. The inhibition of lipid peroxidation was determined by calculating the percent decrease in the amount of the Malonaldehyde (MDA) formed. Percent inhibition of lipid peroxidation was calculated using the formula:

% inhibition of lipid peroxidation = $((A_0 - A_1) / A_0) \times 100$

Where A_0 was the absorbance of the control and A_1 was the absorbance of the test or reference standard.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging potential of the test extracts was measured by using ascorbic acid, iron-EDTA model of hydroxyl radical generating system [33]. The formaldehyde formed during the oxidation of DMSO by the Fe^{+3}- ascorbic acid system was used to detect hydroxyl radicals. The reaction mixture contained 50 mM phosphate buffer, pH 7.4, 167 µM iron- EDTA (as a 1:2 mixture), 0.1 mM EDTA solution, 2 mM ascorbic acid and 33 mM DMSO in a final volume of 3 mL. The test extracts were incubated for 10 min with the reaction mixture at various concentrations (10 to 100 µg/mL). Mannitol (9.10 mg/mL) was used as reference standard, final concentration in reaction mixture was 50mM. After 30 min the reaction was

terminated by the addition of 1 mL of ice-cold 17.5% (w/v) trichloroacetic acid. Nash reagent (4 mL) was added and the mixture was incubated for 40 min at 37ºC [34]. The absorbance was measured at 412 nm against the respective blanks, which contained all reagents and corresponding extracts except DMSO. Control samples contained all the reagents but the extract was replaced by phosphate buffer. The formaldehyde formed was assayed spectrophotometrically by the method of Nash. Extinction co-efficient of 8.0 cm^{-1} mM^{-1} was used to calculate the amount of formaldehyde produced. The hydroxyl radical scavenging activity was determined by calculating the percent decrease in the amount of the formaldehyde formed.

% inhibition = $((A_0 - A_1) / A_0) X100$

Where A_0 was the absorbance of the control and A_1 was the absorbance of the test or reference standard.

Reduction of 2,2-diphenyl-1-picrylhydrazyl radical

DPPH radicals are widely used in the model system to investigate the scavenging activities of several natural compounds. The radical scavenging potential of the test extracts were assessed by adding to 1.5 mL ethanolic solution of DPPH (100 µM) an equal volume of test extracts (10 to 400 µg/mL) dissolved in ethanol. Appropriate controls were maintained. Ascorbic acid (50.0 mg/mL) was used as reference standard, final concentration in reaction mixture was 252 µM. The absorbance was measured at 517 nm after 20 min at room temperature [35] against the respective blanks, which contained the respective concentration of extract in ethanol. Control samples contained only DPPH in ethanol. The free radical scavenging activity was determined by calculating the percent decrease in the absorbance of DPPH. The degree of DPPH scavenging activity of both extracts was evaluated by using the following formula:

% inhibition = $((A_0 - A_1) / A_0) X100$

Where A_0 was the absorbance of the control and A_1 was the absorbance of the test or reference standard.

Protein estimation

The protein content of the rat liver homogenate was determined by the method of biuret reaction using bovine serum albumin as a standard [36].

Statistical analysis

The result of test and corresponding reference standard were statistically analysed and compared with that of control using student's *t*-test. $p<0.001$ was considered as statistically significant.

RESULT AND DISCUSSION

In the present study the ethanolic extract of fruits of *T. terrestris* and methanolic extract of aerial parts of *E. alba* showed significant dose dependent inhibition of lipid peroxidation and DPPH scavenging activity with increasing concentration. Further, the extracts of *T. terrestris* and *E. alba* exhibited a non-dose dependent and dose dependent inhibition of the oxidation of DMSO, respectively in the Fe^{3+}- ascorbic acid system for $^{\cdot}OH$ generation.

Inhibition of lipid peroxidation

Lipid peroxidation is a degenerative process, which affects the polyunsaturated fatty acids of membrane phospholipids. Ferrous sulphate was used for the initiation of reaction which generates free radicals. Free radicals react with polyunsaturated fatty acid moieties of cell membrane phospholipids resulting in lipid hydro-peroxides being produced [37]. Lipid hydro-peroxide can be decomposed to produce alkoxy and peroxy radical which eventually yield numerous carbonyl products such as malondialdehyde (MDA). MDA forms adduct with thiobarbituric acid which is detected spectrophotometrically. Ethanolic extract of fruits of *T. terrestris* and methanolic extract of aerial parts of *E. alba* showed significant dose dependent inhibition of lipid peroxidation with increasing concentration from 10-400 µg/mL (Table 1). Both the extracts were found to inhibit lipid peroxidation up to 86.57 % and 79.16%, respectively at the concentration of 400 µg/mL. In comparison α-tocopherol, the reference standard used in this assay was found to inhibit malondialdehyde formation by around 91 % with respect to control. The extracts of *T. terrestris* and *E. alba* were found to inhibit $FeSO_4$ induced lipid peroxidation significantly with IC_{50} values of 60 µg/mL and 130 µg/mL, respectively.

Hydroxyl radical scavenging activity

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [38]. The model used in this study was ascorbic acid-iron-EDTA model of $^{\cdot}OH$ generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate the hydroxyl radical. The extract of *T. terrestris* exhibited a non dose dependent inhibition of oxidation of DMSO indicating hydroxyl radical scavenging activity up to 30.51 % at concentration of 100 µg/mL. While the extract of *E. alba* exhibited dose dependent inhibition of oxidation of DMSO indicating hydroxyl radical scavenging activity up to 22.11 % at the concentration

Table 1: Effect of ethanolic extract of T. terrestris and methanolic extract of E. alba on lipid peroxidation in vitro.

Treatment	*T. terrestris* Malondialdehyde µM/mg protein	*E. alba* Malondialdehyde µM/mg protein
Control	0.78 ± 0.047	0.74 ± 0.021
10 µg/mL	0.82 ± 0.049	0.68 ± 0.028
25 µg/mL	0.71 ± 0.026	0.63 ± 0.047
50 µg/mL	0.44 ± 0.11	0.57 ± 0.033*
100 µg/mL	0.17 ± 0.007*	0.47 ± 0.038*
200 µg/mL	0.12 ± 0.001*	0.17 ± 0.027*
400 µg/mL	0.10 ± 0.008*	0.15 ± 0.023*
Vitamin E 47.27 µg/mL	0.06 ± 0.009*	0.06 ± 0.009*

Values are expressed as mean ± S.E., n = 4; *p<0.001 compared with control, Student's t-test

Table 2: Effect of ethanolic extract of T. terrestris and methanolic extract of E. alba on the hydroxyl radical.

Treatment	*T. terrestris* Concentration (mM) of Formaldehyde produced	*E. alba* Concentration (mM) of Formaldehyde produced
Control	0.85 ± 0.031	0.64 ± 0.015
10 µg/mL	0.63 ± 0.017*	0.58 ± 0.020
25 µg/mL	0.68 ± 0.014*	0.57 ± 0.020*
50 µg/mL	0.64 ± 0.047*	0.51 ± 0.026*
100 µg/mL	0.59 ± 0.043*	0.50 ± 0.014*
Mannitol 9.10 mg/mL	0.63 ± 0.008*	0.51 ± 0.011*

Values are expressed as mean ± S.E., n = 4; *p<0.001 compared with control, Student's t-test

Table 3: Effect of ethanolic extract of T. terrestris and methanolic extract of E. alba on the 1,1-diphenyl, 2- picryl hydrazyl (DPPH) free radical

Treatment	*T. terrestris* Absorbance	*E. alba* Absorbance
Control	0.94 ± 0.039	1.07 ± 0.017
10 µg/mL	0.91 ± 0.010	1.01 ± 0.009
25 µg/mL	0.83 ± 0.008	0.94 ± 0.003*
50 µg/mL	0.73 ± 0.038*	0.90 ± 0.002*
100 µg/mL	0.54 ± 0.030*	0.71 ± 0.010*
200 µg/mL	0.27 ±0.089*	0.45 ±0.007*
400 µg/mL	0.06 ± 0.004*	0.23 ± 0.017*
Ascorbic acid 50 µg/mL	0.05 ± 0.005*	0.056 ± 0.003*

Values are expressed as mean ± S.E., n = 4; *p<0.001 compared with control, Student's t-test

of 100 µg/mL. The extract of *T. terrestris* exhibited more inhibition than the 23 % of the reference standard mannitol (Table 2).

Reduction of 2,2-diphenyl-1-picrylhydrazyl radical

1,1-Diphenyl, 2-picrylhydrazyl free radical (DPPH) is a stable free radical having paramagnetic property conferred by its odd electron. Ethanolic solutions of DPPH are densely colored showing absorption band at 517 nm. DPPH can react with antioxidants resulting in decolorization of solution. This decolorization is stoichiometric with respect to the number of electrons taken up [39]. The extracts of both *T. terrestris* and *E. alba* showed significant dose dependent reduction of stable free radical DPPH up to 93.28 % and 77.82 % respectively at the concentration of 400 µg/mL (Table 3). Ascorbic acid, the reference standard used in this assay reduced the OD of DPPH by around 92 % with

respect to that of control. *T. terrestris* and *E. alba* exhibited IC_{50} of 145 µg/mL and 175 µg/mL, respectively for reduction of stable free radical DPPH (Table 3).

Both the extracts have significant ($p<0.001$) DPPH free radical scavenging activity and antiperoxidative activity, while it has shown moderate inhibition of hydroxyl radical scavenging activity. We feel that the antioxidant activity exhibited by *T. terrestris* in the present study may be assigned to the presence of the flavonoids like kaempferol and flavonol glycoside like tribuloside, astragalin and kaempferol-3-rutinoside in the fruits [40]. While the antioxidant activity exhibited by *E. alba* can be ascribed to the coumestans wedelolactone and demethyl wedelolactone, triterpenes and flavonoids like apigenin and luteolin which have been probed for hepatoprotective activity of the plant in the past [41].

The present research work reflects the antioxidant potential of *E. alba* and *T. terrestris*. It also supports the claims in ayurvedic literature for the potential use of these plants in several disorders with underlying pathology of oxidative stress.

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