In Vitro Antioxidant Activity of *Enicostemma axillare*

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Four successive extracts of the whole plant of *Enicostemma axillare* (*E. axillare*), were examined for *in vitro* antioxidant activity using nine different methods. In the 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) method, all the four extracts of *E. axillare* showed potent antioxidant activity with IC_{50} values ranging from 13.26 to 24.36 μg/ml. The chloroform extract has shown potent antioxidant activity in H_2O_2, nitric oxide, and hydroxyl radical using the deoxyribose and lipid peroxidation methods, with IC_{50} values of 16.99 ± 0.38, 60.66 ± 0.30, 25.06 ± 0.12, and 94.66 ± 2.40 μg/ml, respectively. Potent activity was also observed for the petroleum ether extract with the deoxyribose, *p*-nitroso dimethyl aniline (*p*-NDA), and H_2O_2 methods and for the ethyl acetate extract with the H_2O_2 and nitric oxide methods. All extracts showed moderate total antioxidant capacity using the phosphomolybdenum method. The activity was not correlated with the total phenol content of the extracts.

Key words —— *Enicostemma axillare*, antioxidant, total phenol, Gentianaceae

INTRODUCTION

Enicostemma axillare (*E. axillare*) Lam Raynal (synonym—*Enicostemma littorale* Blume, Gentianaceae) is a perennial herb found throughout India and common in coastal areas. The plant is used in folk medicine to treat diabetes mellitus, rheumatism, abdominal ulcers, hernia, swelling, itching and insect poisoning.[1] Its antiinflammatory,[2] hypoglycemic,[3–5] and anticancer[6] activities have been reported. These reported activities and many of the ethnomedical uses of the plant are related to its antioxidant activity. Swertiamarin, alkaloids, steroids, triterpenoids, saponins, flavonoids, xanthones, phenolic acids, *etc*. were isolated from the plant.[4] Many such compounds have protective effects due to their antioxidant properties.[7] Several antioxidants of plant origin were experimentally confirmed and used as effective protective agents against free radical-mediated toxicity.[8] Studies have implicated the role of free radicals in the causation of inflammation, diabetes and cancer.[9] Hence, the present study focused on the evaluation of the antioxidant activity of *E. axillare* using nine different methods.

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MATERIALS AND METHODS

Plant Collection —— The whole plants of *E. axillare* were collected during full bloom in September 2005 from Bidar district, Karnataka, India, and authenticated by Dr. S. Rajan, Medicinal Plants Survey and Collection Unit, Government Arts College Ootacamund, India.

Extraction and Phytochemical Analysis —— The whole plants were shade-dried, powdered, and extracted (300 g) successively and separately with 1.5 l each of petroleum ether (60–80°C), chloroform, ethyl acetate, and methanol in a Soxhlet extractor for 18–20 hr. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40–50°C) in a rotavapor. All the extracts were subjected to qualitative chemical tests for the identification of various phytoconstituents.[10] The total phenol content was determined using Folin-Ciocalteu reagent,[11] and the total flavonol content was estimated using the aluminum chloride method.[12]

Preparation of Test and Standard Solutions —— Four successive extracts of *E. axillare* and the standard antioxidants [ascorbic acid, rutin, butylated hydroxyanisole (BHA)] were dissolved in distilled

dimethyl sulfoxide (DMSO) separately and used for *in vitro* antioxidant assays with eight different methods, excluding the hydrogen peroxide method. For the hydrogen peroxide method, in which DMSO interferes, the extracts and standards were dissolved in distilled methanol before use. The stock solutions were serially diluted with the respective solvents to obtain lower dilutions.

***In Vitro* Antioxidant Activity** —— In all these methods, a specific concentration of the extract or standard solution was used to give a final concentration of 1000 μg/ml to 0.45 μg/ml. Absorbance was measured against a blank solution that contained the extract or standard, but without the reagent. A control experiment was performed without adding the extract or standard. The IC_{50} value, which is the concentration of the sample required to scavenge 50% of free radicals, was calculated.

Scavenging of 2,2'-Azino-bis(3-Ethylbenzo-Thiazoline-6-Sulfonic Acid) Diammonium Salt (ABTS) Radical Cation —— To 0.2 ml of various concentrations of the extract or standard solution, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution (2 mM) were added and incubated for 20 min. Absorbance of these solutions was measured spectrophotometrically at 734 nm.[13]

2,2-Diphenyl-1-Picryl Hydrazyl (DPPH) Radical Scavenging Method —— The extract or standard solution (10 μl) was added to DPPH in methanol solution (100 μM, 200 μl) in a 96-well microtiter plate (Tarsons, Kolkata, India). After incubation at 37°C for 30 min, the absorbance of each solution was determined at 490 nm.[13]

Scavenging of Hydroxyl Radical in the Deoxyribose Method —— To the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride (0.1 mM, 0.2 ml), ethylenediaminetetraacetic acid disodium salt (EDTA) (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml), and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer (pH, 7.4, 20 mM), was added 0.2 ml of various concentrations of the extract or standard in DMSO to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37°C. After incubation, ice-cold trichloroacetic acid (0.2 ml, 15% w/v), and thiobarbituric acid (0.2 ml, 1% w/v) in 0.25 N hydrochloric acid were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled, and the absorbance was measured at 532 nm.[13]

Scavenging of Hydroxyl Radical in the *p*-Nitroso Dimethyl Aniline (*p*-NDA) Method —— To a solution containing ferric chloride (0.1 mM, 0.5 ml), EDTA (0.1 mM, 0.5 ml), ascorbic acid (0.1 mM, 0.5 ml), H_2O_2 (2 mM, 0.5 ml), and *p*-NDA (0.01 mM, 0.5 ml) in phosphate buffer (pH 7.4, 20 mM) were added various concentrations of the extract or standard in distilled DMSO (0.5 ml) to produce a final volume of 3 ml. Absorbance was measured at 440 nm.[14]

Scavenging of Hydrogen Peroxide —— A solution of hydrogen peroxide (20 mM) was prepared in Phosphate buffer saline (PBS) (pH 7.4). Various concentrations of the extract or standard in methanol (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min, the absorbance was measured at 230 nm.[15]

Nitric Oxide Radical Inhibition Assay —— The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), PBS (1 ml), and the extract or standard solution (1 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed, 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completion of the diazotization reaction, 1 ml of naphthyl ethylene diamine dihydrochloride was added, and the mixture was allowed to stand for 30 min in diffused light. The absorbance was measured at 540 nm.[13]

Scavenging of Superoxide Radical with the Alkaline DMSO Method —— To the reaction mixture containing 0.1 ml of nitro blue tetrazolium (1 mg/ml in DMSO) and 0.3 ml of the extract or standard in DMSO was added 1 ml of alkaline DMSO (1 ml of DMSO containing sodium hydroxide 5 mM in 0.1 ml of water) to give a final volume of 1.4 ml, and the absorbance was measured at 560 nm.[14]

Evaluation of Total Antioxidant Capacity —— An aliquot of 0.1 ml of extract or standard solution in DMSO was combined with 1 ml of reagent solution (sulfuric acid 0.6 M, sodium phosphate 28 mM, and ammonium molybdate 4 mM) in an Eppendorff tube. The tubes were capped, incubated in a water bath at 95°C for 90 min, cooled to room temperature, and the absorbance of each solution was measured at 695 nm.[16] The results are expressed as millimolar equivalents of ascorbic acid.

Lipid Peroxidation Inhibitory Activity —— Egg yolk was separated and washed with acetone until the yellow color was removed. The creamy white powder obtained was egg lectin. Lipid peroxidation was induced by adding ferric chloride 10 μl (400 mM) and L-ascorbic acid 10 μl (400 mM) to a mixture containing egg lectin (3 mg/ml) in phosphate buffer solution and different concentra-

Table 1. Extraction, Phytochemical Analysis, and *In Vitro* Antioxidant Activity of *E. axillare* in Different Methods

Extract/Standard	Extraction			
	Characterstics (% yield)	Phytochemical analysis	Total phenol mg/g[a,b]	Total flavonol mg/g[a,c]
Petroleum ether extract	Blackish green sticky residue (2.4%)	Steroids, triterpenoids	—	—
Chloroform extract	Blackish green sticky residue (2.8%)	Alkaloids, flavonoids, phenols, triterpenoids	80.00 ± 1.15	17.73 ± 0.81
Ethyl acetate extract	Brown semisolid (3%)	Flavonoids, phenols, saponins, iridoid glycosides, tannins	73.33 ± 5.81	28.80 ± 0.83
Methanol extract	Brown semisolid (4%)	Alkaloids, flavonoids, phenols, iridoid glycosides, tannins	97.33 ± 4.80	31.33 ± 2.19
Ascorbic acid			—	—
Rutin			—	—
BHA			—	—

Extract/Standard	IC_{50} values ± SEM (μg/ml)[a,e] by method			
	ABTS	DPPH	Deoxyribose	*p*-NDA
Petroleum ether extract	13.26 ± 0.49	460.73 ± 9.65	54.30 ± 1.42	119.00 ± 2.08
Chloroform extract	24.36 ± 0.29	396.60 ± 3.01	25.06 ± 0.12	209.66 ± 0.88
Ethyl acetate extract	13.86 ± 1.33	113.16 ± 7.00	149.33 ± 2.04	> 1000
Methanol extract	18.48 ± 0.56	325.30 ± 5.68	> 1000	> 1000
Ascorbic acid	11.25 ± 0.49	2.69 ± 0.05	—	> 1000
Rutin	0.52 ± 0.05	3.91 ± 0.10	—	> 1000
BHA	—	—	86.16 ± 4.04	> 1000

Extract/Standard	IC_{50} values ± SEM (μg/ml)[a,e] by method			Total anti
	H_2O_2	Nitric oxide	Lipid peroxi-dation	oxidant capacity[d]
Petroleum ether extract	37.76 ± 5.14	194.50 ± 0.28	100.00 ± 1.52	0.63 ± 0.03
Chloroform extract	16.99 ± 0.38	60.66 ± 0.30	94.66 ± 2.40	0.72 ± 0.01
Ethyl acetate extract	24.03 ± 1.41	69.47 ± 0.63	> 1000	0.85 ± 0.02
Methanol extract	60.86 ± 0.20	219.90 ± 2.68	> 1000	0.59 ± 0.04
Ascorbic acid	187.33 ± 3.93	—	—	—
Rutin	36.66 ± 0.22	65.44 ± 2.56	—	—
BHA	24.88 ± 0.16	—	112.66 ± 1.32	—

a) Average of three determinations, mean ± SEM, *b*) gallic acid, and *c*) rutin, equivalent in mg/g of the extract. *d*) The total antioxidant capacity expressed as millimolar equivalents of ascorbic acid. In the alkaline DMSO method, no extract showed activity.

tions of the extracts (100 μl). After incubation for 1 hr at 37°C, the reaction was stopped by adding 2 ml of 0.25 N hydrochloric acid containing 15% w/v trichloroacetic acid and 0.375% w/v thiobarbituric acid, boiled for 15 min, cooled, centrifuged, and absorbance of the supernatant was measured at 532 nm.[17)]

RESULTS AND DISCUSSION

In the ABTS method, all four extracts of *E. axillare* showed potent antioxidant activity, with IC_{50} values ranging from 13.26 to 24.36 μg/ml (Table 1). However, the standards rutin and ascorbic acid exhibited better results with lower IC_{50} values. In the H_2O_2 method, the petroleum ether, chloroform, and ethyl acetate extracts showed potent activity greater than or comparable to the standard rutin, and all the extracts showed more potent activity when compared with standard ascorbic acid. In the nitric oxide method, the chloroform and ethyl acetate extract showed comparable activity to that of the standard rutin. In the deoxyribose, *p*-NDA, and lipid peroxidation methods, the petroleum ether and chloroform extracts showed more potent or comparable antioxidant activity to those of the standards used. In the alkaline DMSO method, since the IC_{50} values were found to be greater than 1000 μg/ml, all the extracts were found to be inactive. All the extracts exhibited moderate total antioxidant capacity in the phosphomolybdenum method and low activity in the DPPH method.

Free radical and reactive oxygen species are well known inducers of cellular and tissue pathogenesis leading to several human diseases, such as cancer, inflammatory disorders, and diabetes mellitus, as well as in the aging process.[18)] Many plant species with antioxidant activities act as protective agents against these diseases.[7)] In the present study, potent antioxidant activity was observed using many methods for all extracts of *E. axillare*. Among the extracts, the petroleum ether, chloroform, and ethyl acetate extracts exhibited potent activities. However, the efficacy of each extract differed against various free radicals depending on the specific assay methodology, reflecting the complexity of the mechanisms and diversity of the chemical nature of the phytoconstituents present.

The total phenol and flavonol contents in the methanol extract were found to be higher than in the other extracts. However, the methanol extract was found to be the least active among the four extracts. Hence, no correlation exists between the observed antioxidant activity and total phenol and flavonol contents. The preliminary phytochemical analysis indicated the presence of steroids and triterpenoids in the petroleum ether extract; alkaloids, flavonoids, phenols and triterpenoids in the chloroform extract; flavonoids, phenols, saponins, iridoid glycosides, and tannins in the ethyl acetate extract; and alkaloids, phenols, saponins, iridoid glycosides, and tannins in the methanol extract. The observed antioxidant activity may be due to the presence of any of these constituents in the extracts. *E. axillare* is known to have potent antiinflammatory, antidiabetic, and anticancer activities.[3–6)] These reported activities may due to the antioxidant properties of the plant. However, further studies are required to confirm this.

In conclusion, the successive petroleum ether, chloroform, and ethyl acetate extracts of *E. axillare* exhibited potent *in vitro* antioxidant activity. The plant merits further investigation in animal models to confirm its antioxidant activity and to isolate the active constituents, especially due to their non-polar nature.

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