Indian Journal of Biochemistry & Biophysics
Vol. 46, June 2009, pp. 247-252

Evaluation of antioxidant potential of *Clitoria ternata* L. and *Eclipta prostrata* L.

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Received 22 October 2008; revised 25 March 2009

Free radical-mediated oxidative stress is believed to be the primary cause of many disorders, such as cardiovascular diseases, brain dysfunction, cataract, diabetes mellitus, arthritis, cancer, ageing etc. In treatment of these diseases, antioxidant therapy has gained an utmost importance in the recent years. Current research is now directed towards finding naturally occurring antioxidants of plant origin. In Indian system of medicine, *Clitoria ternata* L. and *Eclipta prostrata* L. are the important medicinal plants, which have a wide range of applications. In the present study, the antioxidant potential of aqueous extracts of *C. ternata* and *E. prostrata* was evaluated by determining the levels of enzymatic and non-enzymatic antioxidants. I*n vitro* antioxidant capacity was also determined using different assays and the results were compared with standard antioxidants such as butylated hydroxy toluene (BHT), ascorbic acid and rutin. Our results showed that both plant extracts possessed significant levels of enzymatic and non-enzymatic antioxidants and also exhibited antioxidant capacity. However, *C. ternata* showed higher levels of enzymatic and non-enzymatic antioxidants, as compared to *E. prostrata*. In addition, the antioxidant capacity of *C. ternata* was observed to be significant as compared to *E. prostrata.*

Keywords: Free radicals, Antioxidants, *Clitoria ternata*, *Eclipta prostrata*, Radical scavenging activity.

Free radicals are fundamental to many biochemical processes and represent an essential part of aerobic life and metabolism[1]. Reactive oxygen species (ROS)-mediated oxidative damage to macromolecules such as lipids, proteins and DNA has been implicated in the pathogenecity of major diseases like cancer, rheumatoid arthritis, degeneration process of aging and cardiovascular disease etc. Antioxidants have been reported to prevent oxidative damage caused by free radicals by interfering with the oxidation process through radical scavenging and chelating metal ions[2]. Novel natural antioxidants from plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties[3].

Clitoria ternata (family *Caesalpinaceae*), a herb and common garden flower plant found all over India is used as laxative, diuretic, anti-catharatic agent, in hectic fever, used in the treatment of eye infections, skin diseases, urinary troubles, ulcers and has antidotal properties[4]. It is also used to treat neurological disorders and is considered to be wholesome for the intellect[5]. Polyacyl glycosides of delphinidin-type[6] and eight anthocyanin ternatin pigments[7] have been isolated from the plant.

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Eclipta prostrata (family *Asteraceae*) is an herb, widely distributed throughout India. Its leaves are used in the treatment of hepatic and spleen enlargement, scorpion and snake bite and jaundice and root extract is used as antiseptic to ulcers and wounds. The plant is used in the treatment of hyperlipidemia and hepatic diseases in traditional medicine[8]. Antibacterial, antifungal, antimyotoxic and antihemorrhagic activities have been reported in the leaf extract[9]. The plant is also used in the treatment of liver ailments[10], wounds, ulcers, skin diseases and has shown anti-inflammatory property[11]. A flavonoid diosmetin, two isoflavonoids 3'-hydroxybiochanin A and 3'-*O*-methylorobol[12] and five oleanane-type triterpenoids[13] have been isolated from the methanol extract of plant.

In the present study, we have evaluated the antioxidant property and potential of the aqueous extracts of *C. ternata* and *E. prostrata* by determining various enzymatic and non-enzymatic antioxidants and antioxidant capacity assays.

Materials and Methods

Thiobarbituric acid and 2, 2'-diphenyl-3-picryl hydrazyl (DPPH) were obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. 2, 4, 6-Tri pyridyl-s-triazine, 5, 5'-dithio-bis 2-nitrobenzoic acid

(DTNB), nitroblue tetrazolium (NBT), reduced glutathione, butylated hydroxy toluene (BHT) were procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used were of analytical grade obtained from commercial sources.

Plant collection and preparation of extract

Clitoria ternata and *Eclipta prostrata* were collected from the Regional Agricultural Research Station, Anakapalli, Andhra Pradesh, India and authenticated by Dr M Venkaiah, Associate Prof, Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India. Preparation of plant extracts was done according to the previously described procedure[14]. Fresh leaves of both plants were separately collected during the month of September, cleaned with distilled water, cut into small pieces, mashed separately in pre-cooled mortar and pestle. The 10 ml of ice cold 0.1 *M* phosphate buffer, *p*H 7.6 containing 0.1 m*M* EDTA was added to obtain different concentrations (25, 50 and 100 mg/ml) of extracts. The extracts were filtered through a muscline cloth and centrifuged at 15,000 rpm for 15 min. The supernatants obtained were used for the determination of enzymatic, non-enzymatic antioxidants and antioxidant capacity of individual plant extract.

Assay of enzymatic antioxidants

The assay of superoxide dismutase (SOD) was carried out by the previously described method[15]. In brief, 0.5 ml of plant extract, 1.0 ml of 0.125 *M* sodium carbonate, 0.4 ml of 25 µM NBT and 0.2 ml of 0.1 m*M* EDTA were added. The reaction was initiated by adding 0.4 ml of 1.0 m*M* hydroxylamine hydrochloride and absorbance was measured at 560 nm using spectrophotometer (Hitachi, Germany). Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed as units per mg protein.

The catalase (CAT) activity was assayed by the titrimetric method[16]. To 2.5 ml of 0.1 *M* phosphate buffer, *p*H 7.5, 2.5 m1 of 0.9% H_2O_2 (v/v) in the same buffer and 0.5 ml of the plant extract was added and incubated at room temperature for 3 min. The reaction was arrested by adding 0.5 ml of 2 *N* H_2SO_4 and the residual H_2O_2 was titrated with 0.1 *N* potassium permanganate solution. Units of enzyme activity were expressed as ml of 0.1 *N* potassium permanganate equivalents of H_2O_2 decomposed per mg protein.

Peroxidase activity assay was carried out according to the previously described procedure[17]. To 3.5 ml of 0.1 *M* phosphate buffer, *p*H 6.5, 0.2 ml of plant extract and 0.1 ml of freshly prepared 0.1% *o*-dianisidine solution was added. The reaction was initiated by adding 0.2 ml of 0.2 *M* H_2O_2 and the absorbance was read at 430 nm for 3 min with an interval of 30 s. A graph was plotted with the increase in absorbance against time. From the linear phase, the change in absorbance per min was calculated and activity was expressed as units per mg of protein.

Glutathione peroxidase (GPx) was assayed as described elsewhere[18]. Briefly, 0.2 ml each of 0.8 m*M* EDTA, 10 m*M* sodium azide, 1.0 m*M* GSH, 2.5 m*M* H_2O_2, 0.32 *M* phosphate buffer, *p*H 7.0 and plant extracts were mixed in the final volume of 1.2 ml and incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 ml of 10% TCA and the tubes were centrifuged at 5000 rpm for 5 min. To 0.5 ml of supernatant, 3.0 ml of 0.33 *M* phosphate solution and 1.0 ml of 0.6 m*M* DTNB reagent were added and the absorbance was read at 420 nm. Graded amount of standards were also treated similarly. GPx activity was expressed as µg of glutathione utilized per mg protein.

Assay of ascorbate oxidase activity was carried out according to the previously described procedure[19]. Briefly, to 3.0 ml of ascorbate solution (0.003%), 0.1 ml of plant extracts was added and change in absorbance at 265 nm was measured at an interval of 30 s for a period of 5 min. One unit of enzyme activity was expressed as 0.01 OD (optical density) change per mg of protein.

Estimation of non-enzymatic antioxidants

Reduced glutathione (GSH)

GSH was determined as described elsewhere[21]. In brief, 1.0 ml of the each plant extract was treated with 4.0 ml of precipitating solution containing 1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of NaCl in 100 ml water. After centrifugation for 5 min at 5000 rpm, 2.0 ml of protein-free supernatant was mixed with 0.2 ml of 0.4 *M* disodium hydrogen phosphate and 1.0 ml of DTNB reagent. Absorbance was read at 412 nm within 2 min. GSH concentration was expressed as n mol per mg protein.

Estimation of vitamin C

Ascorbic acid content was determined by the procedure described previously[20]. Briefly, to 5.0 ml of ascorbate solution (10 µg per ml), l0 ml of 4% oxalic

acid was added and titrated against 0.026% dichlorophenol indophenol. The amount of the dye consumed was equivalent to the amount of ascorbic acid present in the plant extracts. Similar titration was carried out with 5.0 ml of plant extracts.

Analysis of total phenolics

The total phenolics were determined using the Folin-Ciocalteau reagent[22]. Briefly, 50 µl of the plant extract, 2.5 ml diluted Folin-Ciocalteau reagent and 2.0 ml 7.5% (w/v) sodium carbonate were added and incubated at 45°C for 15 min. The absorbance of all samples was measured in a spectrophotometer (Hitachi, Germany) at 765 nm and the results were expressed as mg of gallic acid equivalents per g weight.

Antioxidant capacity assays

Ferric reducing or antioxidant power assay (FRAP)

The total antioxidant power of the sample was assayed by the method as described previously[23]. In brief, 3.0 ml of FRAP working reagent (2.5 ml of 0.1 m*M* acetate buffer, *p*H 3.6, 0.25 ml of 0.3 m*M* 2, 4, 6-tri pyridyl-s-triazine (TPTZ) solution and 0.25 ml of 10 m*M* $FeCl_3.6H_2O$) was taken in a test tube then 100 µl of plant extracts were added, vortex mixed and the absorbance was read at 593 nm against a reagent blank after 1 min. The results expressed as ascorbic acid equivalents (µ moles/ml) or FRAP units.

Reducing activity or reducing power assay

The ability of the extracts to reduce iron(III) to (II) was assessed by the method described elsewhere[24]. Briefly, 1.0 ml plant extracts was mixed with 2.5 ml 0.2 *M* phosphate buffer, *p*H 6.6, and 2.5 ml 1% aqueous $K_3Fe(CN_6)$ solution. After 30 min of incubation at 50°C, 2.5 ml 10% trichloroacetic acid (TCA) was added and the mixture was centrifuged at 650 rpm for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml water and 0.5 ml 0.1% aqueous $FeCl_3$ and the absorbance was recorded at 700 nm. The results were expressed as ascorbic acid equivalents (AscAE) in mg of ascorbic acid per g of extract. Butylated hydroxy toluene (BHT) and ascorbic acid were used as positive controls.

DPPH Assay

The DPPH (diphenyl picryl hydrazyl) radical scavenging assay was carried out as described earlier[25]. Briefly, 5.0 ml of DPPH solution (0.004%) in methanol was added to 50 µl of plant extracts. After 30 min of incubation at 37°C, the absorbance was read against control at 517 nm. BHT and rutin were used as positive controls. Percentage of inhibition (I) was calculated as follows:

$$\text{Percentage of inhibition (I)} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging was carried out by measuring the competition between deoxyribose and the extract for hydroxyl radicals generated Fenton reaction[26]. Briefly, 0.1 ml of plant extracts was added to the reaction mixture containing 0.1 ml 3.0 m*M* deoxyribose, 0.5 ml 0.1 m*M* FeCl3, 0.5 ml of 0.1 m*M* EDTA, 0.5 ml 0.1 m*M* ascorbic acid, 0.5 ml 1.0 m*M* H_2O_2 and 0.8 ml 20 m*M* phosphate buffer, *p*H 7.4, in a final volume of 3.0 ml. The reaction mixture was incubated at 37°C for 1 h. The thiobarbituric acid reactive substances (TBARS) formed were measured by treating with 1.0 ml of thiobarbituric acid (TBA) (1.0%) and 1.0 ml of TCA (2.8%) at 100°C for 20 min. After cooling the mixtures, absorbance was measured at 532 nm against a control. Percentage of inhibition (I) was calculated as follows:

$$\text{Percentage of inhibition (I)} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Inhibition of lipid peroxide formation

The lipid peroxidation was induced by $FeS0_4$-ascorbate system in sheep liver homogenate[27] and the formed TBARS were estimated[28]. The reaction mixture consisting of 0.1 ml each of 25% (w/v) sheep liver homogenate in 40 m*M* Tris-HCl buffer, *p*H 7.0, 30 m*M* KCl, 0.16 m*M* ferrous iron ($FeSO_4$), plant extracts or positive control and 0.06 m*M* ascorbic acid. The reaction mixture was then incubated at 37°C for 1 h and 0.4 ml of the reaction mixture was treated with 0.2 ml 8.1% (w/v) sodium dodecyl sulfate, 1.5 ml of 1.0% TBA, and 1.5 ml 20% acetic acid, and adjusted to the *p*H 3.5. The total volume was then made up to 4.0 ml by adding distilled water and the reaction mixture was kept in a water bath at 95°C for 1 h. To the precooled reaction mixture, 1 ml of distilled water and 5 ml n-butanol and pyridine mixture (15:1 v/v) were added and shaken vigorously.

After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. Percentage of inhibition (I) was calculated as follows:

$$\text{Percentage of inhibition (I)} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Estimation of total protein

Total protein was estimated by the method described earlier[29] Briefly, 0.1 ml of plant extract and different concentrations of standards were taken and the volume was made up to 1.0 ml with distilled water. To all the tubes, 5.0 ml of alkaline copper reagent was added and left at room temperature for 10 min. Then 0.5 ml of Folin's phenol reagent was added and the blue colour developed was read after 20 min at 620 nm against a reagent blank. Protein concentration was expressed as mg/g of sample, mg/ml (or) µg/ml.

Results and Discussion

Enzymatic antioxidant levels

The results obtained on the enzymatic antioxidants of *C. ternata* and *E. prostrata* are presented in Table 1. All the enzymatic antioxidant activities increased with increasing concentrations ranging from 25 to 100 mg/ml. Higher SOD and CAT activities were detected in *C. ternata* at a concentration of 100 mg/ml, as compared to *E. prostrata* (2.92 ± 0.040 and 1.18 ± 0.025 units/mg protein respectively). Similar activity was reported earlier in *Allium* spp[30]. Similarly, at 100 mg/ml plant extracts, the higher CAT and peroxidase activities in *C. ternata*, as compared to *E. prostrata*. GPx activity was low in *E. prostrata* as compared to *C.ternata*. GPx, a selenium containing enzyme plays a key role in regulating the concentration of H_20_2 and wide range of organic peroxides. Ascorbate oxidase activity was higher in *E. prostrata*, as compared with *C. ternata*; the significant ascorbate oxidase activity in *E. prostrata* might be associated with low levels of SOD, CAT, peroxidase and GPx activities, emphasizing the importance of ascorbate system in *E. prostrata*.

Antioxidant defense enzymes SOD, CAT, peroxidase, GPx and ascorbate oxidase protect the aerobic cells against oxygen toxicity and lipid peroxidation. SOD plays an important role in protecting cells against ROS. CAT is particularly efficient at high concentration of H_20_2 and detoxifies H_20_2 to H_20 and 0_2. Peroxidase and GPxs catalyze the degradation of various peroxides by oxidizing glutathione with the formation of its conjugates. These peroxidases scavenge the high reactive lipid peroxide in the aqueous phase of cell membrane.

Non-enzymatic antioxidant levels

All the non-enzymatic antioxidant levels increased in concentration-dependent manner from 25 to 100 mg/ml. Significantly high levels of GSH were found in *C. ternata*, as compared with *E. prostrata* (98.28 ± 0.77 and 11.0 ± 0.52 nanomoles/mg protein respectively) as shown in Fig. 1A. In plants, the role of glutathione as radical scavenger and membrane stabilizer is reported[31]. In the present study, the high levels of GSH and GPx activity observed in *C. ternata* was a notable result, keeping in view that they play an important role in the prevention of lipid peroxidation[31].

Table 1—Enzymatic antioxidant levels of *Clitoria ternata* and *Eclipta prostrata*

[Values represent average of three determinations and expressed as mean ± S.D]

Plant	Conc. of extract (mg/ml)	SOD (U/mg)	Catalase (U/mg)	Peroxidase (U/mg)	GPx (U/mg)	Ascorbate oxidase (U/mg)
C. ternata	25	0.98 ± 0.02	0.19 ± 0.002	0.105 ± 0.03	59.5 ± 1.40	0.19 ± 0.04
	50	1.86 ± 0.05	0.26 ± 0.004	0.403 ± 0.03	109.6 ± 1.31	0.20 ± 0.02
	100	2.92 ± 0.04	0.38 ± 0.008	0.605 ± 0.01	139.8 ± 1.47	0.26 ± 0.05
E. prostrata	25	0.68 ± 0.020	0.021 ± 0.01	0.172 ± 0.01	39.4 ± 0.81	0.47 ± 0.2
	50	0.99 ± 0.022	0.035 ± 0.02	0.205 ± 0.02	59.9 ± 0.77	0.59 ± 0.3
	100	1.18 ± 0.025	0.044 ± 0.01	0.275 ± 0.01	79.4 ± 0.87	0.87 ± 0.2

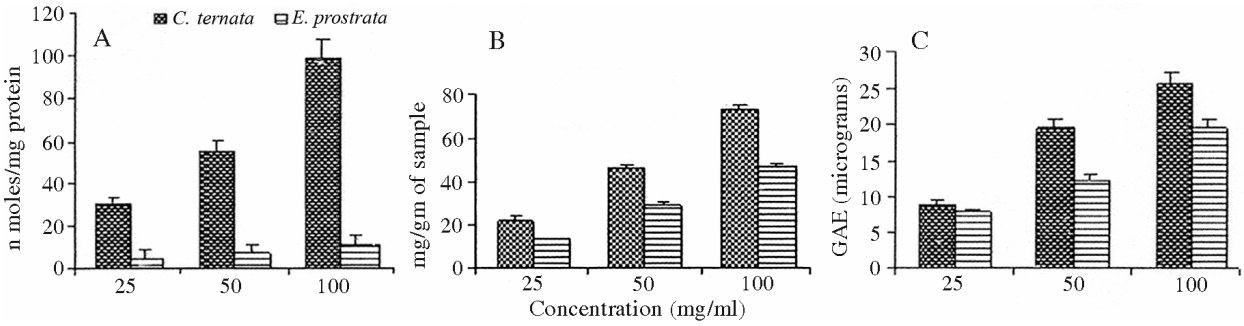

Fig. 1—Levels of Non-enzymatic antioxidants, includes GSH, expressed as nano mole/mg protein (A), Vitamin C expressed as mg/g of sample (B) and Total phenolics expressed as µg Gallic acid acid equivalents (C). All th values are an average of triplicates and expressed as mean ± S.D.

Table 2—Antioxidant capacity of *C. ternata* and *E. prostrata* compared with standard antioxidants

[Each value is an average of triplicates]

Plant	Conc. (mg/ml)	FRAP Assay (FRAP Units)	Fe(III) to Fe(II) (AscAE)	% Inhibition		
				DPPH radical	Hydroxy radical	Lipid peroxidation
C. ternata	25	546	144	10.31	44.16	48.30
	50	778	188	14.20	49.09	52.60
	100	1240	340	16.87	59.10	57.80
E. prostrata	25	349	17	02.92	12.22	13.96
	50	558	28	03.98	14.09	16.00
	100	940	40	05.00	18.10	18.40
BHT	1 mg/ml	NT	NT	76.20	76.00	82.60
Ascorbic acid	1 mg/ml	NT	NT	NT	76.40	70.00
Rutin	1 mg/ml	NT	NT	40.00	NT	NT

'NT' denotes not tested

Vitamin C content was low in *E. prostrata* as compared to *C. ternata* (47.28 ± 1.13 and 73.28 ± 1.36 mg/g, respectively) as shown in Fig. 1B. Ascorbic acid is found in the chloroplast, cytosol, vacuole and extracellular compartments of the plant cells and acts as a reductant for many free radicals[32]. A number of natural compounds can serve as non-enzymatic antioxidant protectants including ascorbic acid, vitamin E, GSH, carotenoids, uric acid, bilirubin and ceruloplasmin[33].

The total phenolics were higher in *C. ternata* as compared with *E. prostrata* (25.5 ± 0.360 and 19.5 ± 0.458 GAE units, respectively) as shown in Fig. 1C. The higher antioxidant activity of *C. ternata* might be due to higher amount of total phenolics. The strong relationship between the total phenolic content and the antioxidant activity in sweet basil is also reported[34]. The total protein content of *E. prostrata* and *C. ternata* was found to be 14.5 and 8.75 mg/g of wet sample, respectively.

Antioxidant capacity

Results of the antioxidant capacity of aqueous extracts of two plants as determined by FRAP, reducing activity and radical scavenging methods are presented in Table 2. Antioxidant capacity in all the assays was significantly increased with increasing the concentration of the extracts from 25 to 100 mg/ml. The total antioxidant power of the plant extracts determined by the FRAP method was higher in *C. ternata* than *E. prostrata*. Similar studies were reported in Labiatae family[14] and our results very nearly correlated with their findings. Results of antioxidative activity determined by Fe^{+3}/Fe^{+2} reducing activity showed that both plants possessed the iron(III) to (II) reducing activity and the activity was higher in *C. ternata* than *E. prostrata*.

The DPPH free radical scavenging of antioxidants is due to their hydrogen donating ability; the plants with higher hydrogen donating capacity have shown higher DPPH free radical scavenging activity[35]. Both

the extracts showed DPPH radical scavenging activity with percent of inhibition of DPPH radical being 16.8% in *C. ternata* and 5.0% in *E. prostrata* (Table 2), as compared to 40.0% and 76.2% for rutin and BHT respectively (the positive controls). Hydroxyl radicals are one of the quick initiators of the lipid peroxidation process by abstracting hydrogen atom from unsaturated fatty acids or simply autooxidation of polyunsaturated fatty acids, found primarily in membranes[36]. Percentage of inhibition of hydroxyl radical in *C. ternata* and *E. prostrata* was 59.0% and 18.1% respectively, as compared to 76% and 76.4% of inhibition for BHT and ascorbic acid respectively (Table 2). Percentage of inhibition of lipid peroxidation in *C. ternata* and *E. prostrata* was 57.8% and 18.4% respectively, compared to the positive controls BHT and ascorbic acid with 82.6% and 70% of inhibition respectively (Table 2). The radical scavenging and inhibition of lipid peroxidation by the extracts was due to the quenching free radical or reduction of Fe^{+3} to Fe^{+2}, which can be attributed to the presence of a number of polyphenolics such as flavonoids, anthocyanins[37] etc., GSH and vitamin C[33] and enzymatic antioxidants in the extracts.

Based on the results in the present study, it was concluded that *C. ternata* and *E. prostrata* were found to be a good natural antioxidant sources, with *C. ternata* exhibiting higher antioxidant potential, as compared to *E. prostrata.* Further studies are required to identify the active principles responsible for the significant antioxidant effect.

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