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IN VITRO ANTIOXIDANT POTENTIAL EVALUATION OF *Euphorbia hirta* L.

Nilesh Kumar Sharma, Sreela Dey, Ramasare Prasad*

Molecular Biology and Proteomics Laboratory
Department of Biotechnology
Indian Institute of Technology Roorkee, Roorkee, Uttaranchal, India 247667
*E mail-rapdyfbs@iitr.ernet.in

Summary

Aqueous extract of *Euphorbia hirta* L. was prepared in hot water and crude extract yield (7%w/w) after lyophilization was used for antioxidant potential determination. The quantitative estimation of major constituents of crude extract was carried out using standard methods and contained total sugar (29.53 mg glucose equivalent), total phenol (52.92 mg gallic acid equivalent) and total flavonoid (18.57 mg rutin equivalent) per gram crude extract. The total antioxidant potential of crude extract was determined using phosphomolybdenum complex and ferric reducing power (FRAP) assays, which showed 185 µmol of ascorbic acid and 398 µmol Fe (II) equivalent per gram crude extract, respectively. The crude extract exhibited significant free radical scavenging activity of 247 µmol Trolox equivalent per gram crude extract and IC 50 value 0.175 mg crude extract / ml, as revealed by ($ABTS^{\bullet *}$) [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging assays, respectively. Hydroxyl radical scavenging activity was performed using deoxyribose assay as well as calf thymus DNA oxidative damage assays and results showed significant hydroxyl radical scavenging activity (IC 50 value 0.162 mg crude extract/ml). Lipid peroxidation inhibition potential of crude extract evaluated using mouse brain tissue homogenate, showed significant inhibition potential IC50 value 0.143mg crude extract per ml. In Indian traditional medicine, importance of *E. hirta* L. is well evidenced from earlier literature, but this is first *in vitro* report showing *E. hirta* L. as potent source of natural antioxidants. Therefore, on the basis of present research findings, we suggest *E hirta* L. as promising plant source in health, food and cosmetic industry.

Key Words: Antioxidant, Scavenger, lipid peroxidation, hydroxyl radical, oxidative damage

The formation of reactive oxygen (ROS) may cause oxidative stress and destruction of unsaturated lipids, DNA, proteins and other essential molecules and considered to have major role in mutagenesis, carcinogenesis, ageing, and neurodegenerative diseases, such as Parkinson's and Alzheimer's (5, 6). Besides implications in health aspects, free radical is also responsible for deterioration of food and cosmetic product quality. It has been well documented that, in addition to endogenous antioxidant defense system, external supply of both synthetic as well plant derived natural antioxidants appears to play significant role in oxidative stress imbalances (7, 8).

There is high demand for natural antioxidants in the food, cosmetic and therapeutic industry, due to their low cost, high stability, high compatibility with dietary intake and no harmful effects inside the human body, like some synthetic antioxidants BHA (Butylated Hydroxyl Anisole) and BHT (Butylated Hydroxy Toluene) are carcinogenic in nature. On the basis of above facts, natural antioxidants would be promising alternative for synthetic antioxidants (9, 10). The development of alternative natural antioxidants such as those found in plants origin is of worthy consideration for our health industry and hold promising commercial potential. *Euphorbia hirta* L. (family Euphorbiaceae), a wild herbaceous plant is very common in all tropical countries, including India and has been widely acknowledged for use in traditional medicine like cough, coryza, hay asthma, bronchial infections, bowel complaints, worm infestations, kidney stones (1). A number of reports have been well documented in earlier research work including sedative and anxiolytic activity (2), analgesic, antipyretic, anti-inflammatory, antidepressant for blood pressure (3), antihypertensive (4). Although, use of *Euphorbia hirta* L is reported for different biomedical use, the present work is being made to explore the *in vitro* antioxidant potential.

Methods

Chemicals

All chemicals were of analytical and highest purity and were purchased from Sigma Chemical Co. (St., Louis, USA), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA). Hi-Media, India Ltd. and, Merck Co. (Germany).

Plant Material

Euphorbia hirta L. (family Euphorbiaceae), was collected from Shantikunj pharmacy nursery, Haridwar, Uttarranchal was authenticated and voucher specimen kept in department of biotechnology herbarium. Aqueous extract of fresh leaves of *Euphorbia hirta* L. was prepared by boiling for two hour in phosphate buffer (pH 7.4, 50 mM) and extract was filtered with double layer cheese cloth. The filtrate was centrifuged at 10000xg for 45 minute and clear supernatant was collected and freeze dried. Crude extract yield was (w/w 7%) and kept at -20^{0}C for further use *in vitro* antioxidant potential investigation.

Total phenol, total flavonoid and total sugar estimation

Preliminary analysis of crude extract was done for total phenol (11), total flavonoids (12) and total sugar (13) according to standard assay methods.

Total antioxidant and total ferric reducing power determination

Total antioxidant activity was determined in terms of mg equivalent of ascorbic acid per g crude extract using phosphomolybdate complex assay (14). The assay is spectrophotometer method, which is based on the reduction of Mo (VI) to Mo (V) by the sample antioxidant constituents and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH which has absorbance at 695 nm. The ferric reducing antioxidant power (FRAP) of plant samples is precise interpretation of antioxidant potential in both aqueous and organic solvents medium. The FRAP assay was performed as per procedure of (15) with slight modification. The FRAP reagent was prepared by mixing acetate buffer (pH 3.6), 10 m mol TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 m mol HCl and 20 mmol ferric chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP regent was warmed to 37^{0}C for one hour in water bath before use in assay. Crude extract with varied concentration (150 µL) was added to 3.0 ml of the FRAP reagent.

The absorbance of the reaction mixture was taken at 593 nm after 6 min. Iron (II) sulfate solution (100 µM-2000 µM) concentration was used as standard compound, and results were expressed as µmol Fe (II)/g crude extract.

Assay for free radical scavenging activity using ABTS and DPPH assay

The free radical 2,2'-azinobis 3-ethyl benzothiazoline-6-sulfonic acid ($ABTS^{*+}$) was produced reacting ABTS solution (7 mM) with (2.45 mM) potassium persulfate and mixture was allowed to stand in dark for 12-16 hours before use. For aqueous extract ABTS was diluted with PBS (7.4 pH) to an absorbance of 0.700±0.002 at 734 nm (16) and Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid) was used as standard for calibration curve and activity was expressed in terms of µmol TEAC (Trolox Equivalent Antioxidant Capacity) value. Free radical scavenging activity was also performed by DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay. In this assay, 2 ml of $6x10^{-5}$ M methanol solution of DPPH was mixed with 50 µL of crude extract with varied concentration. The decrease in absorbance was recorded at 517 nm (17) and percentage inhibition was calculated (18). BHT (Butylated Hydroxy Toluene) was used as positive standard.

Hydroxyl radical scavenging assay

The hydroxyl radicals were generated on the basis of Fenton reaction, reaction mixture included following reagents, 10 mM KH_2PO_4-KOH, pH 7.4, 100 µM $Fe(SO_4)_2$ $(NH_4)_2$-EDTA(Ethylene Diamine Tetra Acetic acid), 1.42 mM H_2O_2, 0.0-1.0 mg/ml antioxidant and 2.8 mM deoxyribose in a final volume of 1.0 ml. The extent of deoxyribose degradation due to generation hydroxyl radical was estimated directly from the aqueous phase by the TBA method (19). Catechin(+) was used as positive standard. Crude extract was also tested for protection against hydroxyl radical mediated calf thymus DNA damage (20). In brief, reaction mixture contained following components including, 100 µM $FeSO_4$, 1 mM H_2O_2 and calf thymus DNA (1 mg/ml).

Assay for lipid peroxidation using mouse brain homogenate

The brain of normal rats were dissected and homogenized with a polytron in ice cold Tris-HCl buffer(25 mM, pH 7.4) to produce a 1/10 homogenate. The homogenate was centrifuged at 20000xg for 20 min. 0.5 ml of supernatant was incubated with different concentration of crude extract powder in the presence of 25 µL $FeSO_4$ and 100 µL H_2O_2 at 37 ^{0}C for one hour. The reaction was stopped by 1 ml trichloroacetic acid (w/v 10 %) and 1.5 ml 2-thiobarbituric acid (2-TBA, 1% w/v) was added to follow by heating at 100^{0}C for 15 min. The decrease in absorbance was recorded at 532 nm for lipid peroxidation inhibition potential (21).

Results

Total phenol, total flavonoids and total sugar content

Total phenol, total flavonoids and total sugar content of crude extract presented in Table.1

Table1 Total phenol, total flavonoids and total sugar content of *E. hirta* L. crude extract.

Total phenol mg Gallic acid equivalent/g crude extract of *E. hirta* L.	Total flavonoids mg Rutin equivalent/g crude extract of *E. hirta* L.	Total sugar mg glucose equivalent/g crude extract of *E. hirta* L.
52.92±5.62	18.57±3.87	29.53±2.28

Total antioxidant and ferric reducing power activity

Total antioxidant activity using phosphomolybdate complex assay and ferric reducing antioxidant power(FRAP) of crude extract was also determined and expressed in ascorbic acid and Fe(II) equivalent per gram of crude extract presented in Table. 2.

Table 2. Total antioxidant activity and FRAP estimation of *E. hirta* L. crude extract.

Total antioxidant activity µ mole of Ascorbic acid equivalent per g crude extract of *E. hirta* L	185±4.58
Total antioxidant activity µ mole of Fe(II) equivalent per g crude extract of *E. hirta* L	398±13.88

Free radical scavenging activity

Free radical scavenging potential of crude extract was studied against ABTS and result was obtained from TEAC calibration curve and found to be 247±12.58 µmol (TE) Trolox equivalent per gram crude extract. Further, free radical scavenging potential was assessed against DPPH and result is presented in Table. 3.

Table 3. Scavenging potential against DPPH radical of *E. hirta* L. aqueous crude extract.

Crude extract of *E. hirta* (mg/ml	Absorbance (517 nm)	% scavenging to DPPH free radical
0	0.750	0
0.05	0.612	18.40±1.96
0.10	0.518	30.93±1.58
0.15	0.394	47.46±3.75
0.20	0.321	57.20±4.36
0.25	0.234	68.80±5.21
IC 50 of crude extract = (.175±.098) mg/ml IC 50 of BHT= (.165±.076) mg/ml		

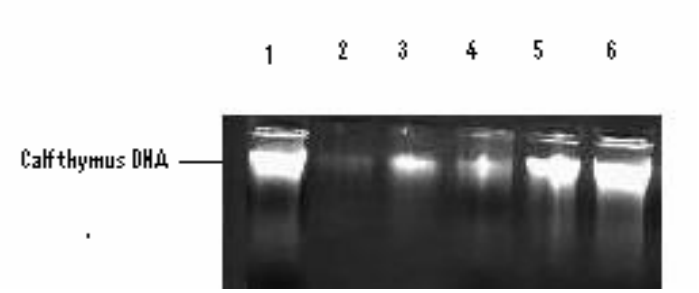
Hydroxyl radical scavenging activity

Crude extract was analyzed for protection against hydroxyl radical mediated oxidative damage to deoxyribose sugar and calf thymus DNA. The results are summarized in Table. 4, Fig. 1.

Table 4. Hydroxyl radical scavenging activity of *E. hirta* L crude extract

Crude extract (mg/ml)	Absorbance (532 nm)	%$OH^{\cdot}$ scavenging
0	0.398	0
0.05	0.314	21.10±3.21
0.1	0.246	38.19±2.58
0.15	0.212	46.73±4.82
0.2	0.135	66.73±3.24
0.25	0.106	73.36±5.21
IC 50 of crude extract=(0.162±.063)mg/ ml) IC 50 of Catechin =(0.078±.063)mg/ ml)		

Fig.1. Lane 1 (only calf thymus DNA), Lane 2 (DNA+ reaction mixture), Lane3-6 (DNA+ reaction mixture+50, 100, 200, 400 µg crude extract/ml). After 1% agarose electrophoresis, stained with ethidium bromide



Inhibition of lipid peroxidation in mouse brain homogenate

Lipid peroxidation inhibition potential of crude extract was studied and IC 50 was found to be 0.143±.084 mg crude extract/ml and inhibition potential was compared with positive standard butylated hydroxy toluene and data is given in Table. 5.

Table. 5 Lipid peroxidation inhibition potential of *E. hirta* L. crude extract against mouse brain homogenate

Crude extract of *E. hirta* (mg/ml)	Absorbance (532nm)	% lipid peroxidation Inhibition in mouse brain
0	0.55	0
0.05	0.427	22.36±1.28
0.10	0.292	46.90±2.14
0.15	0.206	62.54±1.94
0.20	0.189	65.63±2.58
0.25	0.124	77.45±4.26
IC 50 of crude extract= (0.143±.084 mg/ml IC 50 of standard BHA = (0.124±.043) mg/ml		

Statistical analysis

All experiments were performed in triplicate and results were expressed as mean± SEM.

Discussion

In the recent frontiers of medicine and spirit forwarded from previous research endeavor, its urge and need for investigation of natural antioxidants from natural sources mainly dietary and medicinal plant sources. As there are several reports on natural antioxidants of plant origin and their importance in health, food and cosmetic industry are well acknowledged. Besides well known and traditionally used natural antioxidants from tea, wine, fruits, vegetables and spices, some natural antioxidant (e.g. rosemary and sage) are already exploited commercially either as antioxidant additives or a nutritional supplements (22). Also many other plant species have been investigated in the search for novel antioxidants (23, 24). The present work was tried to establish in vitro antioxidant potential including ferric reducing antioxidant, free radical scavenging activity, hydroxyl radical scavenging activity and lipid peroxidation inhibition potential using established assay methods. Total antioxidant activity and ferric reducing antioxidant power was also revealed with suitable assay and was found to be significantly high (185±4.58) µmol of ascorbic acid and (398±13.88) µmol Fe (II) equivalent per gram crude extract, respectively.

The result showed comparison to high antioxidant activity (532 µmol Fe (II)/ g dry wt.) reported for aqueous extract of *Rhodiola sacra* during screening work (24). In the line of *in vitro* evaluation, it was necessary to establish free radical scavenging activity of crude extract and was performed using ABTS and DPPH free radical, which revealed appreciable scavenging activity (247±12.58) µmol TE per gram crude extract and IC50 value (175±.098) mg crude extracts / ml, respectively. The result of free radical scavenging activity is in accordance with the free radical scavenging activity against ABTS and DPPH of *Melissae folium* infusion phenolics and comparable to positive standard butylated hydroxy toluene with IC 50 value (0.165±0.076) (17,18,25). Oxidative DNA damage has been implicated to be involved in various degenerative diseases including Alzheimer's disease, Parkinson's disease, Hodgkin's disease and Bloom's syndrome (26). In view to make ascertain hydroxyl radical scavenging activity of crude extract, Fenton reaction generated hydroxyl radical damaging assay to deoxyribose and calf thymus was studied. The present data from our plant *E hirta* L. which is presented in Table.4, Fig. 4 and IC 50 value found to be (0.162±.063) mg crude extract per ml. In comparison to earlier work on dose dependent decrease in the Fenton's reaction-mediated degradation of DNA by the presence of plant extract of *Pothomorphe peltata* with IC 50 value (100-300 µg/ml) and standard catechin(+) with IC 50 values (65µg/ml), our plant *E hirta* L. showed good hydroxyl radical scavenging potential, and involved to scavenge hydroxyl radical scavenging activity (27). Lipid peroxidation, which involves a series of free radical-mediated chain reaction processes, is also associated with several types of biological damage. The role of free radicals and active oxygen mediated through lipid peroxidation is becoming increasingly recognized in the pathogenesis of many human diseases, including cancer, aging and atherosclerosis (28). Inhibition potential against mouse brain lipid peroxidation was studied and result given in Table. 5 showed significant IC 50 value (0.143±.084) mg crude extract per ml, which is comparable to standard synthetic antioxidant BHA IC 50 value (0.124±0.43). Our data is also in significant comparison to other plant like *Pothomorphe peltata* with IC 50 value (100-500 µg/ml) reported earlier (27). Preliminary estimation of total phenol content, total flavonoids and total sugar content was carried out and presented in Table 1. The data showed that amount of phenolic and flavonoids found to be high and have been in tune with previous work that the antioxidant activity of plants might be due to their phenolic compounds (25, 29). In conclusion, in the present era of biomedicine and health medicine, role of natural antioxidants witnessed a credible role in health, food and cosmetic industry.

In keeping such spirit for work on natural antioxidant, we found and strongly recommend *Euphorbia hirta* L. plant as source of natural antioxidant on the basis of in vitro study. In future, we are looking forward to investigate and find out actual constituents responsible for *in vitro* antioxidant potential and their role in hepatoprotective mechanism.

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