European Journal of Medicinal Plants
14(1): 1-14, 2016, Article no.EJMP.24952
ISSN: 2231-0894, NLM ID: 101583475

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Antioxidant Activity of *Euphorbia hirta* Linn Leaves Extracts

S. Asha[1], P. Thirunavukkarasu[2], V. Magendira Mani[3] and A. Mohamed Sadiq[4*]

[1]Department of Biochemistry, D.K.M. College for Women (Autonomous), Sainathapuram, Vellore, Vellore DT, Tamil Nadu-632001, India.
[2]Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu-608502, India.
[3]Department of Biochemistry, Islamiah College (Autonomous), Vaniyambadi, Vellore DT, Tamil Nadu-635751, India.
[4]Department of Biochemistry, Adhiparasakthi College of Arts and Science (Autonomous), G.B. Nagar, Kalavai, Vellore DT, Tamil Nadu-632506, India.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2016/24952
Editor(s):
(1) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Mindaugas Liaudanskas, Lithuanian University of Health Sciences, Lithuania.
(2) Justin Kabera, National Industrial Research and Development Agency, Rwanda.
Complete Peer review History: http://sciencedomain.org/review-history/14065

Original Research Article

Received 10th February 2016
Accepted 26th February 2016
Published 7th April 2016

ABSTRACT

In the present study, the objectives were to determine the total phenolic content, total flavonoid content and antioxidant activities of aqueous, ethanol and methanol extracts of the leaves of *Euphorbia hirta*. The crude extracts were investigated for its total phenolic and flavonoid content by using Folin-Coicalteu assays and Aluminium chloride colorimetric method. It was found that *Euphorbia hirta* ethanol extract had highest total phenolic and flavonoid content compared to other extracts. In addition, the antioxidant activity of three extracts was tested by DPPH (1,1'-diphenyl-1-picrylhydrazyl) free radical scavenging assay, superoxide radical scavenging assay and hydroxyl radical scavenging assay. From the result, it is concluded that ethanolic extract has a strong percentage of inhibition with lower IC_{50} value than aqueous and methanol extract. The results indicate that there was a direct correlation between total phenol and antioxidant activity. This study

**Corresponding author: E-mail: mohamed68@rediffmail.com;*

confirmed that the leaves of *Euphorbia hirta* possess antioxidant property to scavenge the free radical.

Keywords: 1,1-Diphenyl -2- Picryl hydrazyl free radical scavenging activity; superoxide anion scavenging assay; hydroxyl radical scavenging activity; total phenol content; total flavonoid content; antioxidant activity.

1. INTRODUCTION

Transfer of electrons from a substance to an oxidizing agent is known as oxidation. Approximately 1-3% of the consumed oxygen in living cells is converted to several harmful reactive oxygen species [ROS] and free radicals under physiological conditions. The major site of ROS production in the cell is a mitochondrial respiratory chain. Therefore, mitochondria are suggested as prime targets for oxidative damage [1]. Reactive oxygen species [ROS] are greatly reactive molecules, which includes superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radicals (Roo^-), reactive hydroxyl radicals (OH^-) as most common reactive oxygen species and nitric oxide, peroxynitrite as the nitrogen derived free radicals. An antioxidant can be defined as "Any substance that is capable of delaying or inhibiting the oxidation of the oxidizable substrate when present in a low concentration compared to that of an oxidizable substrate. [2,3]. To inhibit the oxidative chain reaction, adequate antioxidants are supplied as natural or synthetic food additives to humans and animals as natural or as septic preventive pharmaceuticals. However, synthetic antioxidants such as butylated hydroxyl anisole [BHA], butylated hydroxyl O-toluene [BHT], propyl gallate [PG], metal chelating agents [EDTA], tertiary butyl hydroquinone [TBHQ], nordihydroguaretic acid [NDGA] have many side effects. Hence, plant based natural antioxidants are supplied in the form of leafy vegetables, fruits, seeds, cereals and algae, acts as a good source to produce a wide range of natural antioxidants. Plants contain a wide variety of natural antioxidants termed 'phytochemicals'. These include flavonoids, classes of phenolic compounds, carotenoids, terpenoids, vitamin C and E and polyphenols. Among the phytochemicals of plants, phenolics are the most abundant natural antioxidant acts as reducing agents, hydrogen donators, free radical scavengers and singlet oxygen quenchers and as cell saviors. The relationship between level of a phenolic compound and antioxidant potential of plants has been reported previously. The phenolic compound with antioxidant activity is predominantly due to their redox properties [4]. The aim of the present study is to determine the total phenol, total flavonoid content and antioxidant activity of *Euphorbia hirta*, seems to have many traditional and pharmacological activities as mentioned in our previous report [5].

2. MATERIALS AND METHODS

2.1 Quantitative Analysis

2.1.1 Determination of total phenol content

The amount of total phenol content present in the different solvent extracts of *Euphorbia hirta* leaves was determined by following the colorimetric method, Folin-Ciocalteau reagent method [6,7]. Each extract of 0.5 mL [1.0 mg/mL] in methanol and 0.1 mL of Folin - Ciocalteu reagent [10%v/v] was mixed, added into the test tubes. The mixture was allowed to stand for 15min. To the mixture, 2.5 mL of saturated carbonate solution was added and mixed well. The mixture was further incubated for 30 min at room temperature. The total phenolic content was determined at 760 nm using UV-VIS spectrophotometer. A calibration curve was made by preparing 1 mL aliquots of 100, 200, 300, 400 and 500 µg mL^{-1} solutions of Gallic acid and the results were expressed as gallic acid equivalents in milligram per gram [mg GAE/g] of the sample. Absorbance = mx + a. Gallic acid acts as a common reference compound. The estimation was carried out in triplicate.

The amount of phenol in plant extracts was calculated by the following formula:

$$T = C_1 \times V/M$$

Where,

T = Total Phenolic content mg g^{-1} of extracts in GAE [Gallic acid equivalent];

C_1 = The Concentration of Gallic acid established from the calibration curve mg mL^{-1};

V = The Volume of extract solution [mL]

M = The Weight of the plant extract [g].

2.1.2 Determination of total flavonoid content

The aluminium chloride colorimetric technique was used for total flavonoids determination [8]. *Euphorbia hirta* extracts [0.5 mL of 1:10 g/mL] in methanol; 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water constitutes the reaction mixture. The mixture was allowed to stand for 30 min at room temperature without any disturbance. The absorbance of all the mixtures was measured using UV-VIS spectrophotometer at 415 nm against blank containing water instead of the sample. Quercetin acts as a standard compound for the quantification of total flavonoid. A standard curve was prepared with quercetin of known concentrations. The total flavonoid content was determined as mg/g of quercetin equivalents [mg QE/g] of plant material in triplicate.

The total content of flavonoid in plant extracts was calculated by the following formula:

$$T = C_1 \times V/M$$

Where,

T = Total flavonoid content mg g^{-1} of extracts in quercetin equivalent;

C_1 = The Concentration of quercetin solution established from the calibration curve mg mL^{-1};

V = The Volume of extract solution [mL]

M = The Weight of the pure plant extract [g].

2.1.3 Antioxidant activity

Antioxidant activity was assessed by 1,1-diphenyl-2-picrylhydrazyl [DPPH] assay, superoxide anion scavenging assay and hydroxyl radical scavenging assay. All the data collected for each assay were the average of three determinations of three independent experiments.

2.1.3.1 1,1-Diphenyl -2- Picrylhydrazyl free radical scavenging activity:

The hydrogen atom or electron contribution ability of the *Euphorbia hirta* extracts and standard on scavenging DPPH free radicals was determined by following the method described by [9]. DPPH, the purple colour stable free radical readily shows a maximum absorption at 517 nm and undergo reduction by an antioxidant. DPPH [1,1-diphenyl-2-picrylhydrazyl] purple colour is converted to 1-1-diphenyl-2-picrylhydrazine, a yellow colour by reacting with antioxidants. This test provides information on the free radical scavenging potential of the *Euphorbia hirta* extract. Different volumes [2, 4, 6, 8, 10 µL] of alcoholic and water extracts were mixed with 1.0 mL of 0.1 mM DPPH radical in methanolic solution. The reaction mixture was shaken vigorously incubated in room temperature in the dark for 30 min. After 30 mins, of reaction period the decrease in absorbance at 517nm was monitored by placing in a UV-VIS spectrophotometer. Ascorbic acid [Sigma-Aldrich], a stable antioxidant was used as a standard reference positive control. A decrease in DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. The inhibition percentage [1%] of DPPH radical scavenging activity was calculated according to the equation.

DPPH radical scavenging activity [I%] = [Abs control –Abs sample] / [Abs control] × 100

[10] where Abs control and Abs sample are the absorbance values of the blank sample and of the tested samples (Aqueous, Ethanol and Methanol extracts of *Euphorbia hirta*). The antiradical activity was expressed in terms of the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50% [IC_{50}]. The IC_{50} value for each extract or reference compounds was determined graphically by plotting the percentage of DPPH scavenging as a function of extract concentration.

2.1.4 Superoxide anion scavenging assay

Superoxide anion scavenging activity was measured by the method determined by [11]. Phenazine Metho Sulfate – Nicotinamide Adenine Dinucleotide (PMS-NADH), a non – enzymatic system generates superoxide anion radicals through the reaction of PMS, NADH and oxygen. The generated superoxide anion O_2 reduces NBT to form a blue formazan. The blue formazan formed was determined spectrophotometrically at 560 nm.

0.3 ml of *Euphorbia hirta* extract at different concentration [20, 40, 60, 80, 100 µg/mL] was mixed with the reaction mixture. The reaction mixture [3.0 ml of Tris HCl buffer 100 mm, pH 7.4] constitute 0.75 mL of nitroblue tetrazolium [NBT] 300 µm, 0.75 ml of nicotinamide adenine

dinucleotide [NADH] 936 µM solution and 0.75 mL phenazine methosulfate 120 µM, respectively. The absorbance of the reaction mixture was recorded at 560nm after 5minute against control samples. BHT acts as a standard positive control. The degree of scavenging activity was calculated as a scavenging percentage.

Inhibition (%) = [Absorbance Control – Absorbance Sample / Absorbance Control] × 100 where Ac and As were the absorbance values for control and test sample, respectively. IC_{50} value was calculated.

2.1.5 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of *Euphorbia hirta* extract was assayed by following the 2-deoxyribose oxidation method. The 2-deoxyribose gets oxidized and degraded to melandialdehyde in the presence of hydroxyl radical generated by the Fenton reaction [12]. Therefore, the hydroxyl scavenging effect of *Euphorbia hirta* extracts on hydroxyl radicals was estimated by malondialdehyde chromogen formation due to 2-deoxy-2-ribose degradation.

0.2 mL KH_2PO_4-KOH [100 mµ], 0.2 mL deoxyribose [1.5 mM], 0.2 mL $FeCl_3$ [500 mM], 0.1 mL EDTA [1 mM], 0.1 mL ascorbic acid [1 mM] and 0.1 mL H_20_2 [10 mM] constitutes the reaction mixture. $FeCl_3$ and EDTA are mixed before adding to the reaction mixture. 0.1mL of aqueous, ethanol and methanol extract were mixed with the reaction mixture. The mixture was incubated for 1 h at temperature 37ºC. To the reaction mixture, 1.0 mL of TBA [1% W/V], and 1.0 mL of TCA [2.8% W/V] was added after incubation. Then the mixture was heated on a water bath at 80ºC for 20 minutes to develop the colour. A pink colour was developed. After cooling, the absorbance of the solution was measured at 532 nm spectrophotometrically against blank. Quercetin acts as a standard. The hydroxyl radical scavenging activity of extracts, standard [I%] was determined and IC_{50} value was also calculated.

Scavenging effect (%) = [(Control absorbance – Sample absorbance) / (Control absorbance)] x 100

2.2 Statistical Analysis

All the data were presented as mean ± S.D from three separate observations using SPSS 13.0 program. Data on the total phenolic and flavonoid contents of standard and extracts were analysed using the correlation and regression by Microsoft Excel program. The IC_{50} values were determined using the Graph pad Prism 5 software. For antioxidant assays, one-way ANOVA test followed by Tukey's test and Dunnet's Multiple Range test was used. A probability of $P < 0.05$ was considered as significant among IC_{50} of various extracts and standard for different antioxidant assays.

3. RESULTS

3.1 Total Phenol Content and Total Flavonoid Content

The total Phenol content of *Euphorbia hirta* was measured by Folin - Ciocalteau reagent and are presented in Fig. 1. Table 1– summarizes the total phenolic content of different extracts of *Euphorbia hirta*, expressed as milligrams of gallic acid equivalents [GAE]. The absorbance of various dilutions of gallic acid was found as standard curve equation with y= 0.003x + 0.020, R^2 = 0.993. The total phenol contents in aqueous extract, ethanol extract and methanol extract were calculated as 275.64±2.45, 291.74±2.46, and 285.41±3.00 mg/g, respectively. Analysis of the phenolic contents in all extracts of *Euphorbia hirta* revealed that the ethanolic extract contained the maximum phenolic content in terms of gallic acid equivalents, followed by methanol and the aqueous extract.

Table 1. Total phenolic content and total flavonoid content of different crude extracts of *E. hirta*

Extract	Total phenols (mg of GAE/g of crude extract)	Flavonoid content (mg of QE/g of crude extract)
Aqueous	275.64±2.45	26.37±0.37
Ethanol	**291.74±2.46**	**40.32±1.67**
Methanol	285.41±3.00	32.29±6.30

Total phenols are expressed as Gallic acid equivalent
Total flavonoid are expressed as mg of total flavonoid content/g of samples based on quercetin as standard
values represent mean ± SD. (n=3)
GAE = Gallic acid equivalent
QE = Quercetin equivalent

The total flavonoid content of the plant sample *Euphorbia hirta* measured by Folin-Ciocalteu reagent was calculated as quercetin equivalent/g extract. The total flavonoid content of extracts with reference to standard curve was reported to

be [Y = 0.012x + 0.008 and R^2 = 0.993] (Fig. 2) 26.37±0.37 QE mg/g for aqueous, 40.32±1.67QE mg g^{-1} for ethanol and 32.29±6.30 QE mg g^{-1} for methanol extracts respectively (Table 1). According to the results of this study, total flavonoid contents of ethanol extract had the highest amount of flavonoid contents followed by methanol extract and aqueous extract.

3.2 Antioxidant Activity

3.2.1 DPPH free radical scavenging activity

This method is based on the free radical scavenging activity of leaf extracts of *Euphorbia hirta* on DPPH radical. The reaction capability was determined by a decrease in absorbance at 517 nm. At 2 to 10 µg concentrations, the percentage inhibition of samples and standard on DPPH radical increases with increase in concentration. Hence, the extract exhibited a concentration- dependent radical scavenging activity that is, higher the concentration, higher the radical scavenging activity. The DPPH radical scavenging activity of *Euphorbia hirta* leaf extracts is shown in Table 2 and Fig. 3. Results revealed that ethanol extract 86.70±0.60% showed potent inhibition of DPPH radical compared to methanol extract 79.31±0.24% and aqueous extract 64.18±0.14% at 10 µg/ml concentration. The percentage inhibition of Ascorbic acid was found to be 90.76±0.21%. The scavenging effects of samples and standard on the DPPH radical were in the following order Ascorbic acid > Ethanol > Methanol > Aqueous. The concentration of extract required to scavenge 50% of the DPPH· radicals, the Ic_{50} values are presented in (Table 5 and Fig. 6). In general, the lower the IC_{50} value, the stronger the scavenging activity. In the present investigation, the IC_{50} value of DPPH radical scavenging activity for the aqueous extract was 7.07±0.06 µg/mL, ethanol extract was 2.81±0.04 µg/mL and methanol extract was 4.34±0.04 µg/mL whereas that of the standard, ascorbic acid exhibited 2.13±0.02 µg/mL. These observations revealed that methanol and aqueous extract have a weaker antioxidant activity than ethanol extract. The present result could be attributed to the presence of phenolic compounds.

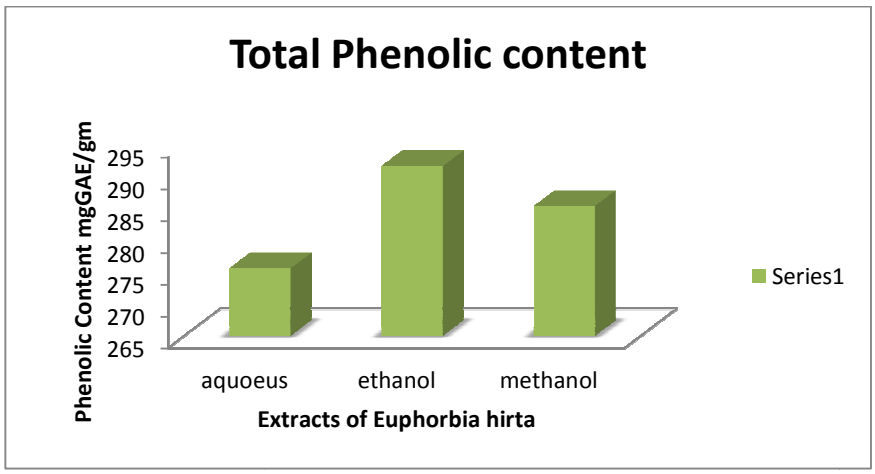

Fig. 1. Quantitaive analysis of total phenolic content of *E. hirta* leaf extracts

Table 2. DPPH radical scavenging activity of different extracts of *E. hirta* Linn

Concentration µg/ml	% of inhibition			
	Ascorbic acid	Aqueous	Ethanol	Methanol
2	48.17±0.03	24.47±0.29	44.17±0.11	36.60±0.14
4	60.85±0.07	33.53±0.18	56.78±0.06	48.58±0.27
6	70.58±0.07	44.59±0.40	66.52±0.05	58.51±0.38
8	81.84±0.09	54.69±0.17	77.77±0.06	70.46±0.33
10	90.76±0.21	64.18±0.14	86.70±0.06	79.31±0.24

Values are expressed as mean ± S.D

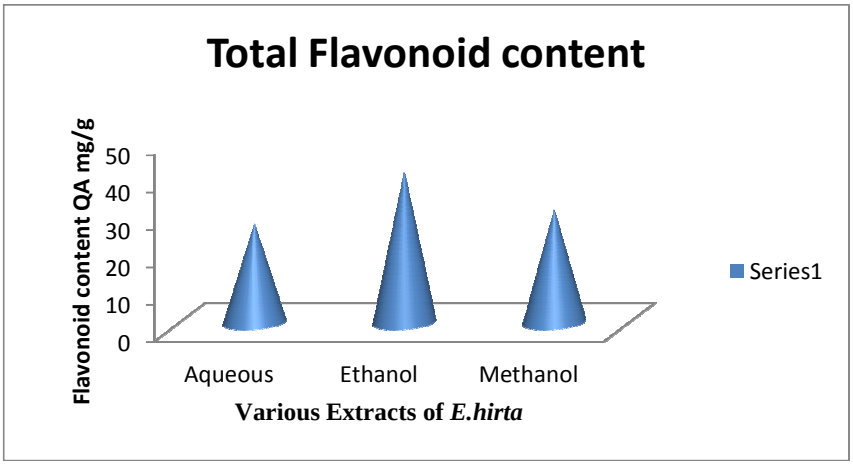

Fig. 2. Quantitaive analysis of total flavonoid content of three different extracts of *E. hirta*

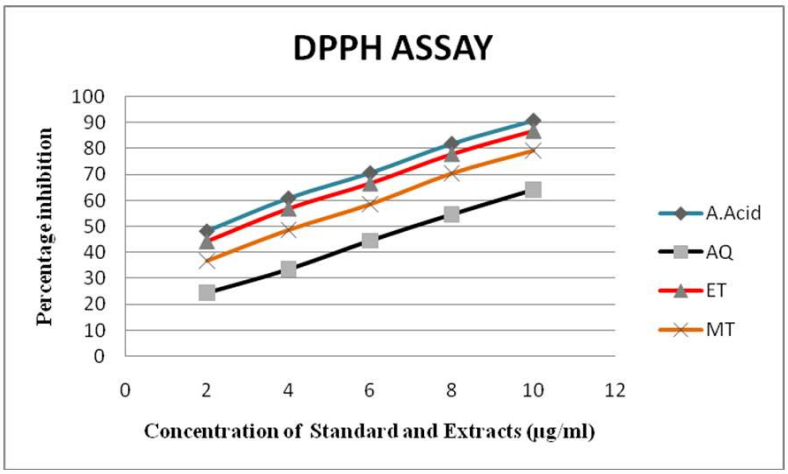

Fig. 3. DPPH radical scavenging activity of extracts of *E. hirta* Linn leaf

Results are expressed as mean ± S.D. (n=3)
A. Acid- Ascorbic acid acts as a reference compound.
AQ- Aqueous extract of E. hirta Linn.
ET- Ethanol extract of E. hirta Linn.
MT- Methanol extract of E. hirta Linn.

3.2.2 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity measures the degree of inhibition of the 2-deoxyribose degradation by the Fonton reaction. The hydroxyl radical scavenging activity of *Euphorbia hirta* extract was shown in Table 3 and Fig. 4 and the results were ranked as Ethanol extract [80.05 ± 0.11%] > Ascorbic acid [73.77±0.22%] > Aqueous extract [70.05±0.112%] > Methanol extract [68.54±0.12%]. The ethanolic extract of *Euphorbia hirta* to quench the hydroxyl radical mediated deoxyribose damage seems to be directly related to the propagation prevention in the process of lipid per oxidants. The activity was assessed at a concentration of 5, 10, 15, 20, 25 µg/mL and the sample exhibits minimum activity at 5 µg/mL and maximum activity at 25 µg/mL. The ethanol extract seems to be a good scavenger of reactive oxygen species. In the present investigation, the IC_{50} value of hydroxyl radical scavenging activity for ethanol, methanol was 13.92±0.03µg/ml, 18.03±0.07 µg/ml while for aqueous was 16.94±0.04 µg/mL (Table 5 and Fig. 6). These antioxidant activities are comparable to that of IC_{50} value of L- ascorbic acid 15.72±0.04 µg/mL, which serves as a positive control. The ethanolic plant extract

showed antioxidant activities with an increased absorbance, thus proving their capacity to scavenge hydroxyl radicals.

3.2.3 Superoxide radical scavenging activity

In this assay, superoxide ions [O_2^-] are produced by phenazine methosulphate / beta – nicotinamide adenine di nucleotide which converts NBT to a reduced NBT-diformazon, a blue coloured formation, measured at 560nm. The antioxidant reduces the rate of NBT-diformazion formation, which leads to a declined absorbance. Ethanolic extract of *Euphorbia hirta* displayed a superior superoxide scavenging activity with an IC_{50} value 33.61±0.036 µg/mL than other extracts of *Euphorbia hirta*. Ethanol extract at 20, 40, 60, 80 and 100 µg/mL concentration produced 42.58±0.493, 53.78±0.12, 64.76±0.168, 76.83±0.068, 90.78±0.14 percentage of inhibition. The aqueous and methanol extract showed a inhibition of 80.25±0.17%, 85.34±0.14% at the concentration of 100 µg/mL with a IC_{50} of 53.25±0.04 µg/mL and 44.90±0.045 µg/mL. The superoxide scavenging activity of an extract of *Euphorbia hirta* and standard BHT is shown in Table 4 and Fig. 5. BHT was used as standard and produced 95.28±0.57% inhibition at the concentration of 100 µg/ml. IC_{50} value of BHT was found to be 25.75±0.05 µg/ml (Table 5 and Fig. 6). The superoxide scavenging activities of samples and standard exist in the order of Butylated Hydroxytoluene > Ethanol > Methanol > Aqueous.

4. DISCUSSION

4.1 DPPH

DPPH, a stable synthetic free radical has been widely used to determine antioxidant activity of natural compound [13,14,15,16,17,18,19,20]. The DPPH method is an ideal method because it is fast, easy and consistent and also does not disintegrate with water, methanol or ethanol. In the present study, *Euphorbia hirta* was screened for its antioxidant and radical scavenging activity by DPPH [21]. DPPH scavenging assay is based on the reduction of methanolic DPPH· solution in the presence of hydrogen –donating antioxidant, leads to the formation of a non-radical form DPPH-H [Diphenyl picrylhydrazine]. There occur a change in colour from purple to yellow and this is proportional to the concentration and scavenging potential of the extract [22] in the term of hydrogen donating ability [23]. The DPPH free radical has its maximum absorbance at 517 nm and was measured by spectrophotometrically. The decrease in absorbance of DPPH at 517 nm [24], indicates a significant antiradical activity of plant extracts. Ascorbic acid used as a standard. Lower IC_{50} indicates a higher antioxidant acivity.

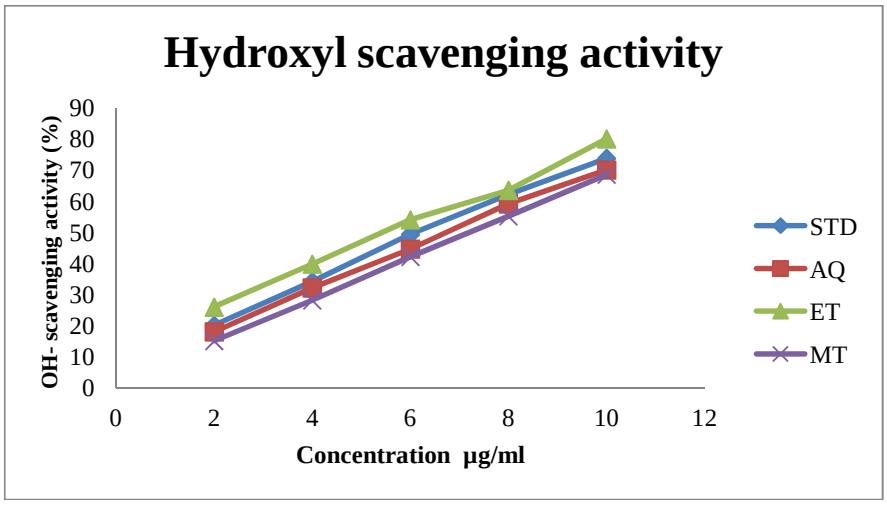

Fig. 4. Hydroxyl scavenging activity of *E. hirta* Linn leaf extracts with reference to a standard compound

STD – Standard Ascorbic acid.
AQ - Aqueous extract of E. hirta Linn.
ET- Ethanol extract of E. hirta Linn.
MT- Methanol extract of E.hirta Linn

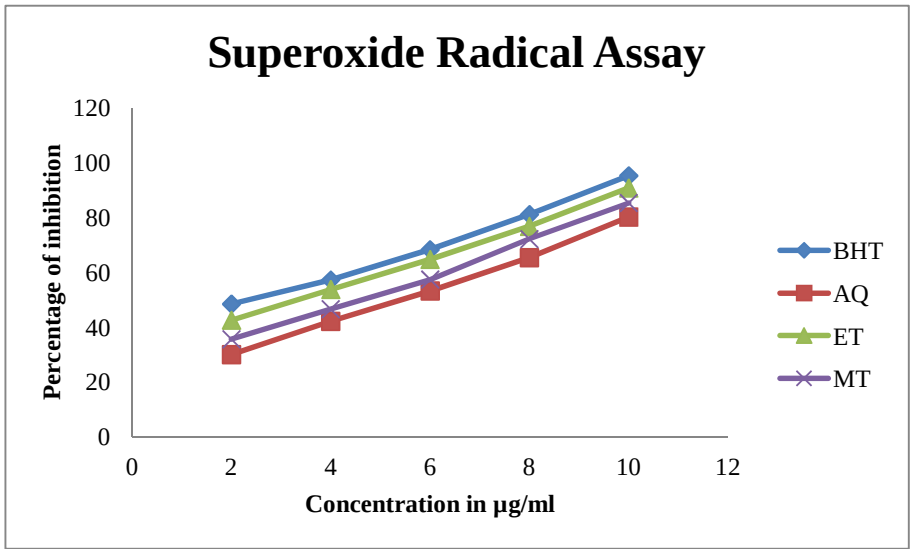

Fig. 5. Superoxide radical scavenging activity of standard and different extracts of *E. hirta* Linn

Results are in triplicate measurement (n=3), expressed as mean ± Standard deviation.
BHT- Butylated hydroxytoluene
AQ- Aqueous extract of E. hirta Linn.
ET- Ethanol extract of E. hirta Linn.
MT- Methanol extract of E. hirta Linn.

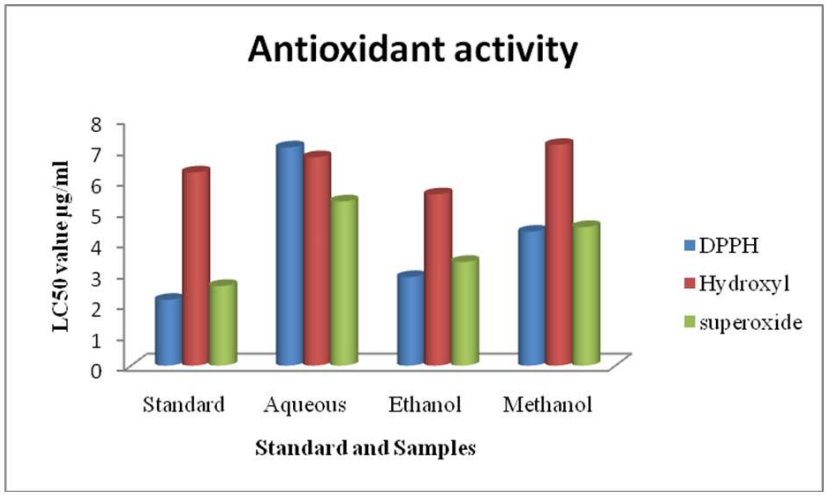

Fig. 6. Antioxidant activities of the *E. hirta* extract on DPPH, OH^- and So^{2-}

Table 3. Hydroxyl radical scavenging activity of *E. hirta* Linn leaf extracts

Concentration µg/ml	% of inhibition			
	Ascorbic acid	Aqueous	Ethanol	Methanol
2	20.16±0.12	18.06±0.05	26.02±0.08	15.11±0.04
4	34.20±0.12	32.16±0.05	39.82±0.08	28.16±0.05
6	49.33±0.17	44.65±0.11	54.06±0.11	42.13±0.05
8	56.17±0.11	59.15±0.12	63.54±0.07	55.15±0.11
10	73.77±0.22	70.05±0.11	80.05±0.11	68.54±0.12

Values are expressed as mean ± S.D

Table 4. Superoxide radical scavenging activity of leaf extracts of *E. hirta* Linn

Concentration µg/ml	% of Inhibition			
	BHT	Aqueous	Ethanol	Methanol
2	48.47±0.35	30.00±0.13	42.58±0.49	35.64±0.10
4	57.28±0.18	42.18±0.29	53.78±0.12	46.63±0.17
6	68.32±0.22	53.22±0.35	64.76±0.16	57.41±0.15
8	81.44±0.61	65.36±0.29	76.83±0.68	72.20±0.17
10	95.28±0.57	80.25±0.17	90.78±0.14	85.34±0.14

Values are expressed as mean ± S.D

Table 5. *E. hirta* Linn leaves extracts at different concentration

Leaves extract	IC50 value (µg/ml)		
	DPPH free radical assay	Hydroxyl radical assay	Superoxide radical assay
Standard	2.13±0.02	6.26±0.04	2.58±0.05
Aqueous extract	7.07±0.06	6.75±0.04	5.32±0.04
Ethanol extract	2.87±0.04	5.55±0.03	3.36±0.03
Methanol extract	4.34±0.040	7.15±0.07	4.49±0.04

Each value represents a mean ± S.D. (n = 3)

Antioxidants with DPPH radical scavenging activity donate hydrogen [25] to free radicals, particularly to the lipid peroxides or hydroperoxides radicals, major propagators of the chain autoxidation of lipids, to form a non-radical species, results in the lipid peroxidation propagation phase inhibition.

Here, IC_{50} means the concentration of sample required to scavenge 50% DPPH radicals in the specified time. The IC_{50} value of *Euphorbia hirta* leaves extracts varied from 2.91±0.04 µg/mL to 7.07±0.06 µg/mL and that of the standard 2.15 µg/mL. Different parts of *Cichorium intybus* L. [26]; hydroalcoholic extract of chicory [*Cichorium intybus* L.] leaves [27]; ethanol extract of *Diospyros kaki* L. leaves [28]; ethanol extract of *Desmodium gangeticum* leaves [29] also showed the similar results in leaves with higher DPPH radical inhibition and lower IC_{50} value representing a best free radical scavenging activity in accordance with our findings.

However, the hydrogen donating capabilities were not the same among the *Euphorbia hirta* extracts. The difference in inhibition percentage was due to the difference in secondary metabolites. Similar results and the relationship between the secondary metabolites and antioxidant activity were also previously been reported by [30,31,32,33]. In the present study, among the tested extracts, ethanol showed higher inhibition percentage than the other extracts. [34] also reported that the ethanol extract had maximum radical scavenging capacity of 13.86% at the lowest concentration. The ethanol extract of *Ulmus davidiana* stem bark exhibited the highest free radical scavenging activity compared to other extracts [35]. The free radical scavenging activities of various extracts of *Euphorbia hirta* depends on the ability of antioxidant compounds to lose hydrogen and the structural confirmation of these compounds [36]. Since all the crude extracts from this plant showed antioxidant activity it could be used as a medicine for the treatment of various diseases.

4.2 Hydroxyl Scavenging Activity

Hydrogen peroxide itself is not very reactive, but it gives rise to hydroxyl radical. Therefore, hydrogen peroxide removal is a very important process in a cell system [37]. Hydroxyl radical is short lived extremely reactive free radicals, formed in the biological systems, capable of damaging a wide range of biomolecule found in living cells, such as sugars, aminoacids, lipids and nucleotides [38,39] by diffusion limited reaction. Since this radical causes damage to DNA strand, it leads to carcinogenesis, mutagenesis and cytotoxicity. Moreover, hydroxyl radicals are the strong reactive species, capable of initiating lipid peroxidation process by obtaining hydrogen atoms from unsaturated fatty acids. Therefore, it is very important to identify the compounds that have the excellent scavenging capacity to protect living systems from OH·. The hydroxyl radical scavenging capacity of an extract seems to be directly

interrelated to the prevention of propagation of lipid peroxidation [40]. Under certain pathological and stress conditions hydroxyl produces radicals are in turn involved in enormous biological damage.

From the result, it is clear that the extract of *Euphorbia hirta* neutralizes the hydroxyl radical - induced deoxyribose cleavage in a concentration - dependent manner. In the present study, among the leaf extracts, IC_{50} value of ethanol extract [13.92±0.03 µg/mL] was efficient in quenching the hydroxyl radical formation than the other two extracts aqueous [16.94±0.045 µg/mL], methanol [18.03±0.07 µg/mL] and standard, Ascorbic acid [15.72±0.04 µg/mL]. To support our findings, Parameshwari et al. [41] had reported that the antioxidant activity of *Boerhavia erecta* ethanolic extract exhibited a concentration - dependent radical scavenging activity, compared with that of the standard compound against $OH^{\cdot}$ free radicals. Pardeep Sharma et al. [42] also identified a higher activity in an ethanolic extract of the green hull of Juglans regia showed a higher activity.

4.3 Superoxide Anion Radical Scavenging Activity

Superoxide a highly toxic radical, produced in all aerobic cells by several enzymatic and non – enzymatic pathways. These radical have the potential to react with biological macromolecules including DNA and thereby promotes tissue damages [43,44,45]. Although superoxide anion is a weak oxidant it acts as an important precursor for generation of powerful and dangerous other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which initiate free radical chain reaction [46]. Both these contribute to oxidative stress. Superoxide anion, formed from dissolved oxygen by PMS-NADH coupling reaction reduces yellow coloured NBT^{2+} into a blue formazon. The decrease in absorbance at 560nm indicates the consumption of superoxide anion in the reaction mixture and was measured spectrophotometrically. As the data showed, among the different extracts of *Euphorbia hirta* at different concentration [20 – 100 µg/mL], ethanol exhibited good superoxide scavenging activity 33.61±0.036 µg/mL compared to other extracts. The IC_{50} value of standard was found to be 25.75±0.05 µg/mL. The antioxidants are able to inhibit the reduction reaction of blue NBT formation [47,48]. The superoxide scavenging activity of the ethanolic extract of *Euphorbia hirta* has the potential to scavenge superoxide radical ions to form stable radicals, thus terminates the radical chain reaction, as stated earlier in previous reports like Seaweeds [49]; *Boerhavia erecta* [41]; *Rheum ribes* [50]; *Achillea santolina* [51]; *Wagatea soicata* [52].

Phenolics or polyphenols are the most abundant secondary plant metabolites that are ubiquitously present in plants and their products. These phytochemical compounds are derived from phenylalanine and tyrosine [53] and they fall into several categories. Among them, flavonoids are the chief and potent antioxidant compound [54]. The result of the present study showed that leaves of *Euphorbia hirta* are good sources of phenolic compounds. Phenolic compounds react with FCR in Folin Ciocalteau reagent method under the basic conditions and leads to dissociation of the phenolic anion, results in a phenolate anion. The produced phenolate anion reduces molybdenum oxide to a blue coloured molybdenum oxide in FCR with a maximum absorption at 750 nm. The intensity of the blue colour formation is directly proportional to the total quantity of phenolic compounds present in the *Euphorbia hirta* extracts [55,56].

The result of ethanol extract was 291.74±2.46 mg GAE/g, the methanol extract was 285.41±3.00 mg GAE/g and the result of aqueous extract was 275.64±2.45 mg GAE /g. Total phenolic content of ethanol extract was determined higher than another extract. The results correlated well with the previous reports about total phenolic content in *Acacia species* [57,58].

Flavonoids are the naturally occurring secondary metabolites in plants shown to be highly effective scavengers, including singlet, oxygen and various free radicals. The results of *Acacia nilotica* leaves [59] also showed a maximum phenolics and flavonoids content in ethanol extracts as like as our result than other extracts.

Several studies in the literature report a positive correlation between antioxidant activity and the phenolic contents [60,61,62,63,64,65,66,67,68].

In the present result, the antioxidant activity of *Euphorbia hirta* on DPPH scavenging activity, hydroxyl scavenging activity and superoxide radical scavenging activity are mainly due to the presence of phenolic compounds as major components. These phenolic compounds act as primary antioxidant or free radical terminators. These results are in good accordance with

previous studies of [69,70,71,72,73] which showed that high total phenolic content increases the antioxidant activity. *Euphorbia hirta* has been found to have great medicinal importance due to the presence of phenolic compounds.

In this study, different solvents with different polarities have been used to extract different classes of compounds. The results showed that the ethanol extract had highest extraction yield, total phenol and flavonoid content and highest activity in all the antioxidant assays tested. These results revealed that the extracts obtained from ethanol, a polar solvent was found to have maximum activity in comparison with the other extracts obtained. This was in accordance with [74] who reported that some species of Lamiaceae exhibited a high antioxidant activity in ethanol extract.

5. CONCLUSION

In conclusions, the present study indicates that *E. hirta* possess a considerable amount of both phenol and flavonoid content. Comparatively, the data obtained clearly indicate that the ethanol extract possesses higher contents than methanol and aqueous extract. Additionally, the ethanol extract was able to scavenge 1,1-diphenyl-2-picryl-2-picrylhydrazyl, hydroxyl scavenging radicals and superoxide anion radicals effectively when compared with other tested extracts. Our findings provide a basic relationship between total phenol and free radical scavenging activity. Thus, this investigation would be useful to treat and prevent the free radical damages occurring in humans worldwide. Therefore, in future, it is noteworthy to isolate and identify the active components in the ethanol extract of *Euphorbia hirta* Linn.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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