

In vitro Antioxidant and RBC membrane Stabilization Activity of *Euphorbia wallichii*

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ABSTRACT

Background: *Euphorbia wallichii* belongs to family Euphorbiaceae is used to treat various diseases on folklore levels in Kashmir valley. Objective of the study is to explore antioxidant potential and anti-inflammatory activities of *Euphorbia wallichii*. **Materials and Methods:** Antioxidant potential of extracts was evaluated by means of total phenolics, DPPH, reducing power, microsomal LPO, and hydroxyl radical scavenging activity by using standard procedures. Anti-inflammatory activity was assessed using hypotonic solution induced human erythrocyte haemolysis. **Results:** The highest phenolic content of 465 mg GAE/g was observed in methanol extract followed by ethyl acetate (359 mg GAE/g) and aqueous extract (291 mg GAE/g). At concentration of 700 µg/mL, DPPH radical scavenging activity of methanol extract was (94.85%) IC₅₀ (160 µg/ml), ethyl acetate (92.68%) IC₅₀ (200 µg/ml) and aqueous (90%) IC₅₀ (250 µg/ml). The reducing power of the extracts increased in a concentration dependent manner. At concentration of 70 µg/mL, 92.72, 80.74 and 75.75% inhibition was observed with methanol, ethyl acetate and aqueous extract on microsomal LPO with IC₅₀ values 31.5, 34.5 and 42 µg/ml. Superoxide radical scavenging activity of *Euphorbia wallichii* extracts increased in a dose dependent manner with IC₅₀ values 36.05 µg/ml (methanol), 45 µg/ml (ethyl acetate)

and 34.5 µg/ml (aqueous extract). *Euphorbia wallichii* extracts exhibited antioxidant effects on Calf thymus DNA damage. At the higher concentration of plant extracts (12 µg/ml), 90, 86 and 78% increase in RBC membrane stabilization was observed with methanol, ethyl acetate and aqueous extracts of *Euphorbia wallichii*. **Conclusion:** These results clearly indicate that *Euphorbia wallichii* extracts possesses the free radical scavenging activity as such can be employed as potential antioxidant and anti-inflammatory agent against various oxidative stress related pathological conditions.

Key words: *In vitro* antioxidant, Correlation, Lipid peroxidation, DNA damage, HRBC membrane stabilization.

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INTRODUCTION

The aim of the present study was to determine the phytochemical screening, total phenolic content and to characterize the antioxidant and RBC membrane stabilizations assays of the different extracts of *Euphorbia wallichii* which is currently used in folk medicine for treatment of edema, skin disease, cutaneous anthrax and exanthema.

Biological combustion involved in the respiration process produces harmful intermediates called reactive oxygen species (ROS). ROS including superoxide anions, hydroxyl radicals, hydrogen peroxide, alkoxyl radicals cause damage to the biological macromolecules (DNA, proteins, lipids, carbohydrates etc.) resulting in various degenerative diseases like cancer, atherosclerosis, cardiovascular diseases.¹ The balance between antioxidant and oxidation is believed to be a critical concept for maintaining a healthy biological system.² Dietary antioxidants can stimulate cellular defenses and help to prevent cellular components against oxidative damage.^{3,4} Because of potential health risk and toxicity associated with synthetic antioxidants a widespread agreement needed to replace the synthetic antioxidants with natural antioxidants.⁵ Therefore, the search for antioxidants from natural sources has received much attention, and efforts have been made to identify new natural resources for active antioxidant compounds. Several assays have been frequently used to estimate antioxidant capacities in plant extracts including DPPH, reducing power, superoxide radical, hydrogen peroxide radical, hydroxyl radical and lipid peroxidation assays.⁶ These techniques have shown different results among plants tested and across laboratories.⁷

Inflammation is the reaction of living tissues to injury, infection or irritation. Lysosomal enzymes released during inflammation produce a variety of disorders which leads to the tissue injury by damaging

the macromolecules and lipid peroxidation of membranes which are assumed to be responsible for certain pathological conditions as heart attacks, septic shocks and rheumatoid arthritis etc. Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane.⁸ HRBC or erythrocyte membrane is analogous to the lysosomal membrane⁹ and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an *in vitro* measure of anti inflammatory activity of the drugs or plant extracts.

MATERIALS AND METHODS

Plant material collection

The whole plant of *Euphorbia wallichii* was collected from Kangdoori Gulmarg area of Jammu and Kashmir during the month of June 2014, identified by the Centre of Plant Taxonomy, Department of Botany, University of Kashmir, and authenticated by Akhter Hussain Malik. A reference specimen has been retained in the herbarium under reference number 2076-KASH.

Extraction

The plant material was dried in the shade at 30 ± 2°C. The dried material was ground into a powder using mortar and pestle and passed through a sieve of 0.3 mm mesh size. The powder obtained was extracted in different solvents using a Soxhlet extractor (60-80°C). The extracts were then

concentrated with the help of rotary evaporator under reduced pressure and the solid extract was stored in refrigerator for future use.

Phytochemical screening of the crude extracts

Phytochemical screening was performed by using various standard procedures.

Total phenolic content (TPC)

The total phenolic content of different extracts of *E. wallichii* was determined by Folin-Ciocalteu reagent according to the method of Singleton *et al.*¹⁰ following quantification on the basis of standard curve of gallic acid. Results are presented in milligrams (mg) gallic acid (GAE) equivalent, per gram plant on dry weight basis.

DPPH radical scavenging activity

The assay was conducted on the basis of scavenging activity of the stable DPPH free radical following the method described by Braca *et al.*¹¹ with some minor modifications. To 1 mL of the sample (10, 20, 30, 40, and 50 µg/mL) was added 3 mL of a 0.1 mmol/L methanol solution of DPPH. The absorbance of all the samples was determined at 517 nm (UVD 2960, Labomed, Inc.) after an incubation period of 30 min. Percentage inhibitory activity was determined according to the following formula:

$$\% \text{ inhibition} = [1 - (A_e/A_0)] \times 100$$

Where A_0 is the absorbance without sample and A_e is the absorbance with sample.

Reducing power

The assay was conducted according to the method of Oyaizu.¹² According to this method, the reduction of Fe^{3+} to Fe^{2+} was determined by measuring absorbance of Pearl's Prussian blue complex. This method is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess relative to the antioxidants. To different concentrations of the extracts (10, 20, 30, 40, and 50 µg/mL) were added 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$]. The mixture was incubated at 50°C for 20 min and 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was added to (2.5 mL) distilled water and FeCl_3 (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. BHT was used as the standard and the percentage reduction was calculated using the following formula:

$$\% \text{ reduction} = [1 - (1 - A_c/A_s)] \times 100$$

Hydroxyl radical scavenging

The assay was conducted according to the method of Halliwell *et al.*¹³ It is based on the measurement of thiobarbituric acid reactive species (predominantly malondialdehyde producing pink color on reaction with thiobarbituric acid) generated from the degradation of deoxyribose on exposure to hydroxyl radical. Hydroxyl radical is generated from Fe^{3+} -ascorbate- H_2O_2 system (Fenton reaction). The reaction mixture contains 25 mM deoxyribose, 10 mM ferric chloride, 100 mM ascorbic acid, 2.8 mM H_2O_2 in 10 mM KH_2PO_4 (pH 7.4), and various concentrations of plant extracts (10–50 µg/mL). The reaction mixture was incubated at 37°C for 1 h. 1% thiobarbituric and 3% trichloroacetic acid (1 ml each) were added, and the mixture was heated at 100°C for 20 min. The gradation in color intensity was measured spectrophotometrically 532 nm. The results were expressed as percentage inhibition of deoxyribose oxidation using the formula below:

$$\% \text{ inhibition} = (A_c - A_e/A_c) \times 100$$

Where A_c is the absorbance in the presence of control and A_e is the absorbance in the presence of the extract.

Superoxide radical scavenging activity

The assay was based on the capacity of the extracts to inhibit formazan formation by scavenging the superoxide radicals generated in Riboflavin-light-NBT system.¹⁴ The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 µg riboflavin, 12 mM EDTA, and NBT 0.1 mg/3 mL, added in sequence. Reaction was started by illuminating the reaction mixture with different concentrations of sample extract/standard for 90 sec. Immediately after illumination, the absorbance was measured at 590 nm. BHT was used as positive control.

The percentage of superoxide anion scavenged was calculated by using the following equation:

$$\% \text{ inhibition} = (1 - A_s/A_c) \times 100$$

Hydrogen peroxide radical scavenging activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch *et al.*¹⁵ The principle of this method is that there is a decrease in absorbance of H_2O_2 upon oxidation of H_2O_2 . A solution of 43 mM H_2O_2 was prepared in 0.1 M phosphate buffer (pH 7.4). 1 ml extract (each at different concentrations) in 3.4 mL phosphate buffer was added to 0.6 mL of H_2O_2 solution (43 mM), and absorbance of the reaction mixture was recorded at 230 nm. A blank solution contained the sodium phosphate buffer without H_2O_2 .

The percentage of H_2O_2 scavenging by the extracts and standard was calculated using the following equation:

$$\% \text{ inhibition} = (1 - A_s/A_c) \times 100$$

Where A_c is the absorbance of the control and A_s is the absorbance in the presence of extracts.

Preparation of liver microsomes

Liver from the freshly killed rats were perfused and kept in an ice cold normal saline 0.9% NaCl and extraneous material was removed. All operations were performed at 4°C. Tissue was blotted between the folds of a filter paper and weighed. 20% (w/v) homogenate was prepared in 0.25 M sucrose. The homogenate was filtered through a muslin cloth and centrifuged at 12000 g for 20 min at 4°C to separate nuclear debris. The supernatant so obtained was diluted 1:5 with 0.125 M sucrose containing 8 mM CaCl_2 and kept on ice for 50 min with constant stirring. The pellet obtained after centrifugation at 12000 g for 10 min was washed with the washing solution containing 0.15 M KCl, 1 mM EDTA and 0.01 M NaH_2PO_4 and was again centrifuged at 12000 g for 10 min to get the microsomal pellet.

The microsomal pellet was then resuspended in a minimum volume of 0.25 M sucrose and stored at -80°C for experimental use.

Lipid peroxidation assay (Liver Microsomes)

The assay of lipid peroxidation was done using the method of Chang *et al.*¹⁶ Liver microsomes were incubated for 5 min in presence and absence of plant extract (50 µ-1000 µg) prior to addition of 100 µM FeSO_4 and 50 µM H_2O_2 and then incubated for 20 min (37°C) in 0.15 M NaCl (pH 7). Control incubation received vehicle only and the induced incubation contained vehicle plus liver microsomes but had no addition of plant extract. The reaction was terminated by the addition of TCA-TBA reagent (5% w/v) and the lipid peroxidation content of the samples was determined as malondialdehyde (MDA) formed per mg of protein at 532 nm absorbance. Percentage inhibition was calculated using the formula;

$$\% \text{ inhibition} = 1 - (\text{Induced-Treated}/\text{Induced-Control}) \times 100$$

Antioxidant activity against oxidative damage to DNA

Hydroxyl radicals generated by Fenton reaction were used to induce oxidative damage to DNA. The reaction mixture (15 mL) contained 25 mg

of calf thymus DNA in 20.0 mM phosphate buffer saline (pH 7.4) and different concentrations of plant extract (10 mg, 30 mg, 50 mg and 80 mg) were added and incubated with DNA for 15 min at room temperature.¹⁷ The oxidation was induced by treating DNA with 20 mM ferric nitrate and 100 mM ascorbic acid and incubating the mixture for 1 h at 37°C. The action was terminated by the addition of loading buffer bromophenol blue (0.25%) and glycerol (30%) and the mixture was subjected to gel electrophoresis in 0.7% agarose/TAE buffer run at 100 V. DNA was visualized and photographed by Gel Doc.

Human red blood cell (HRBC) membrane stabilization method

Fresh whole human blood was collected and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min and packed cells were washed three times with isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline.

Membrane stabilizing activity of the extracts was assessed using hypotonic solution induced human erythrocyte haemolysis.¹⁸ The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extracts with concentrations (50-2000 µg/ml) and standard drug Diclofenac sodium. The control sample consisted of 0.5 ml of RBC mixed with hypotonic buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 560 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated according to formula.

$$\text{Hemolysis} = (\text{Optical density of Test sample} / \text{Optical density of Control}) \times 100$$

Statistical analysis

Determination of total phenolic content, DPPH radical, superoxide radical, hydrogen peroxide, hydroxyl radical and lipid peroxidation assays were conducted in triplicates. The value for each sample was calculated as mean \pm standard deviation (SD). The results were evaluated by using the SPSS (version 12.0) and Origin 8.1 version softwares and evaluated by one-way ANOVA. Correlation coefficient were calculated by using Origin 8.1 version also.

RESULTS

Phytochemical screening of *Euphorbia wallichii* extracts

The results of the phytochemical analysis of the various extracts of *Euphorbia wallichii* in various solvents has shown a remarkable variation in phytochemical compounds. The detailed investigations of phytochemicals in various solvents are shown in Table 1. The study revealed that the extracts of *Euphorbia wallichii* are showing maximum presence of tannins in hexane, chloroform, ethyl acetate and methanol extracts, but inadequately present in aqueous extract. Flavanoids and phenols were maximally present in chloroform, ethyl acetate and methanol extracts, whereas in hexane, ethyl acetate and methanol extracts, terpenoids and cardiac glycoside are maximum, but inadequately present in aqueous extract. Cardenolides are maximum in hexane and ethyl acetate extracts, but are completely absent in chloroform extract. Steroids on other hand were maximally present in hexane, chloroform, methanol and aqueous extracts, but absent in ethyl acetate extract. Volatile extracts are completely absent in hexane, ethyl acetate, methanol and aqueous extracts and inadequately present in chloroform extract.

DPPH radical scavenging activity of *Euphorbia wallichii* extracts

DPPH radical scavenging activity is one of the most widely used method for screening the antioxidant activity of plant extracts. (Figure 1) shows the antioxidant activities of methanol, ethyl acetate and aqueous extracts of *Euphorbia wallichii* and assessed by using the DPPH radical scavenging method. 100-700 µg/ml of extracts was used and the percentage inhibition was recorded in a dose dependent manner. The highest DPPH scavenging activity was observed in methanol extract (94.85%) followed by ethyl acetate (92.68%) and aqueous extract (90.02%) at the highest concentration of plant extract (700 µg/ml). The IC₅₀ values of the three extracts was recorded as 160, 200 and 250 µg/ml respectively which were inversely related to the antioxidant capacity (Table 2). Ascorbic acid a known antioxidant showed 98.03% DPPH radical scavenging activity at the concentration of 700 µg/ml.

Reducing power of *Euphorbia wallichii* extracts

Reducing power activity of *Euphorbia wallichii* methanolic, ethyl acetate and aqueous extracts as a function of their concentrations is shown in (Figure 2). The reducing power capability of the extracts was excellent and increased with dose dependent manner. As shown in (Figure 2), a higher absorbance value indicates a stronger reducing power of the samples. The reducing power activity of methanolic extract at 50-300 µg/ml was increased from 0.240-1.768 respectively. The reducing power activity of the ethyl acetate and aqueous extracts also increased in a concentration dependent manner. At the higher concentration of the extracts (300 µg/ml), we observed absorbance of 1.287 and 1.050 respectively (Figure 2).

Superoxide radical scavenging activity of *Euphorbia wallichii* extracts

Superoxide radicals generated from riboflavin-light-NBT system and can be measured by their ability to reduce NBT. The decrease in absorbance at 590 nm with the plant extracts and the reference compound BHT indicates their abilities to quench superoxide radicals in the reaction mixture. As shown in (Figure 3), superoxide radical scavenging increased in concentration dependent manner. At the higher concentration of the plant extract (60 µg/ml), we observed 79.82, 61.20 and 51.11% inhibition with methanol, ethyl acetate and aqueous extract respectively. The IC₅₀ values (Table 2) of the plant extracts on superoxide scavenging activity were 34.05, 36.05 and 45.5 µg/ml, respectively. BHT a known antioxidant showed 89.47% inhibition at the concentration of 60 µg/ml.

Hydrogen peroxide scavenging capacity of *Euphorbia wallichii* extracts

Scavenging ability of methanol, ethyl acetate and aqueous extracts of *Euphorbia wallichii* on hydrogen peroxide is shown (Figure 4) and compared with BHT as standard. The *Euphorbia wallichii* extracts were capable of scavenging hydrogen peroxide in an amount dependent manner. 250 µg/ml of each extracts of *Euphorbia wallichii* exhibited 84.76% (methanol), 70.29% (ethyl acetate) and 53.67% (water) scavenging activity on hydrogen peroxide. On the other hand, using the same amounts, BHT exhibited 94.51% hydrogen peroxide scavenging activity. Results show that the scavenging activity values on hydrogen peroxide of 50-250 µg/ml of the extracts of *Euphorbia wallichii* decreases than that of BHT in the order of BHT (32-94%) > methanol extract (29.8-84.76%) > ethyl acetate extract (11.93-70.29%) > aqueous extract (7.38-53.67%). The IC₅₀ values (Table 2) of the plant extracts on hydrogen peroxide scavenging activity were 99, 152 and 236 µg/ml, respectively.

Microsomal lipid peroxidation of *Euphorbia wallichii* extracts

Antioxidant capacity of *Euphorbia wallichii* extracts in the biological lipid peroxidation system was examined using rat liver microsomes. Addition of FeSO₄ and H₂O₂ to the microsomal suspension increased TBARS time-dependently up to 20 min. When the microsomes had been pre-treated with each tested extracts, the production of TBARS was inhibited markedly in a dose dependent manner. At the higher

Table 1: Phytochemical analysis of various extracts of *Euphorbia wallichii*

Parameters	Hexane Extract	Chloroform extract	Ethyl acetate extract	Methanolic extract	Aqueous extract
Tannins	++	++	++	++	+
Alkaloids	--	--	++	+	+
Flavanoids	+	++	++	++	+
Phenols	++	++	++	++	+
Steroids	++	++	--	++	++
Terpenoids	++	--	++	++	+
Saponins	--	--	--	++	++
Phlobtannins	--	--	--	--	--
Cardiac glycosides	++	--	++	++	--
Cardenolides	++	--	++	+	+
Volatile oils	--	+	--	--	--
Anthraquinone	--	++	--	+	+

Table 2: IC₅₀ values (µg/ml) of *Euphorbia wallichii* extracts

Extracts	DPPH	Superoxide Radical	Hydrogen peroxide	Lipid Peroxidation	Hydroxyl radical
Methanolic extract	160 ± 0.12	34.05 ± 0.65	99 ± 0.08	31.5 ± 0.90	33.33 ± 0.67
Ethyl acetate extract	200 ± 1.45	36 ± 0.89	152 ± 1.34	34.5 ± 1.05	43.10 ± 0.89
Aqueous extract	250 ± 1.23	45.5 ± 0.87	236 ± 1.89	42 ± 1.45	60 ± 1.90

The data were presented as means ± S.D of three parallel measures and evaluated by one way ANOVA. Differences were considered to be statistically significant if p<0.05.

Table 3: Effect of plant extract on RBC membrane stabilization

Concentration (mg/ml)	Diclofenac	Aqueous extract	Ethyl acetate	Methanol extract
1 mg/ml	75.32 ± 0.44	52.92 ± 0.86	53.6 ± 0.97	70.53 ± 0.70
3 mg/ml	95.32 ± 0.64	61.07 ± 0.59	73.66 ± 0.98	82.3 ± 0.77
6 mg/ml	96.14 ± 0.93	73.38 ± 1.02	74.92 ± 0.83	87.66 ± 0.83
9 mg/ml	97.15 ± 0.78	77.27 ± 1.08	81.23 ± 0.49	89.77 ± 0.71
12 mg/kg	98.23 ± 0.81	78.8 ± 0.90	86.6 ± 0.53	90.58 ± 0.93

The data were presented as means ± S.D of three parallel measures and evaluated by one way ANOVA. Differences were considered to be statistically significant if p<0.05.

Table 4: Correlation coefficient (R²) between different assays

	DPPH	Superoxide radical	H ₂ O ₂ radical	OH radical
Superoxide radical	0.7449			
H ₂ O ₂ radical	0.9915	0.7274		
OH radical	0.8995	0.8975	0.7983	
Lipid peroxidation	0.8966	0.9630	0.8490	0.9667

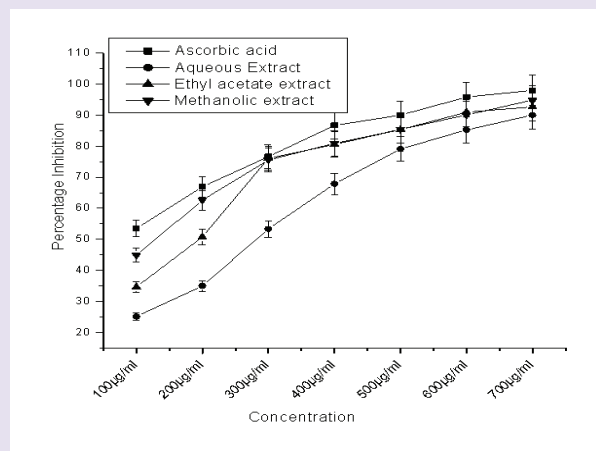


Figure 1: The effect of different extracts of *Euphorbia wallichii* and known antioxidants on DPPH radical scavenging activity. The results represent mean \pm SD of 3 separate experiments.

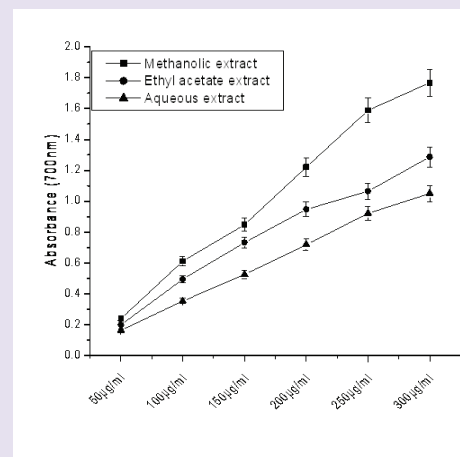


Figure 2: The effect of methanol, ethyl acetate and aqueous extracts of *Euphorbia wallichii* on reducing power method. The results represent mean \pm SD of 3 separate experiments.

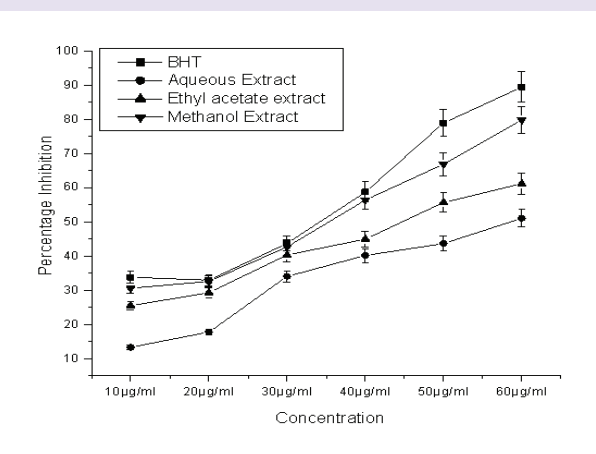


Figure 3: The effect of different extracts of *Euphorbia wallichii* and known antioxidant BHT on super oxide anion radical scavenging activity. The results represent mean \pm SD of 3 separate experiments.

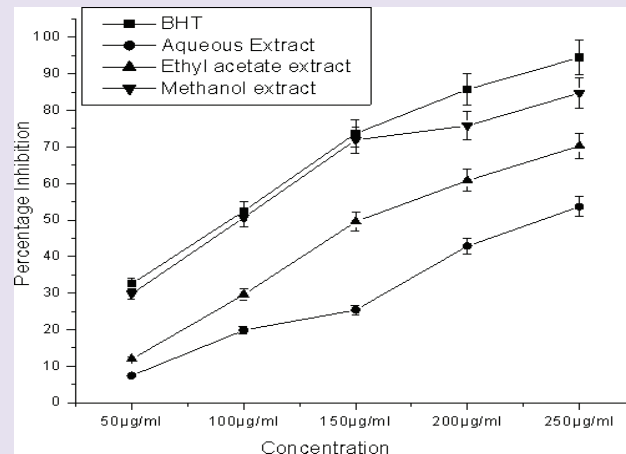


Figure 4: The effect of methanol, ethyl acetate and aqueous extracts of *Euphorbia wallichii* and known antioxidant BHT on hydrogen peroxide radical scavenging activity. The results represent mean \pm SD of 3 separate experiments.

concentration of the plant extracts (70 $\mu\text{g/ml}$), we observed about 92.72% inhibition in TBARS formation with methanolic extract, followed by ethyl acetate extract (80.74%) and 75.7% inhibition with aqueous extract (Figure 5). The half inhibitory concentration (IC_{50}) of the extracts in this system was 31.5 $\mu\text{g/ml}$ (methanol), 34.5 $\mu\text{g/ml}$ (ethyl acetate) and 42 $\mu\text{g/ml}$ with aqueous extract (Table 2). BHT a known antioxidant used in the study inhibits the TBARS formation up to 96.85% at the concentration of 70 $\mu\text{g/ml}$.

Hydroxyl radical scavenging activity of *Euphorbia wallichii* extracts

A significant decrease in concentration of hydroxyl radical was observed due to *Euphorbia wallichii* extracts (Figure 6). All the extracts exhibited significant activity, above 60% in a concentration dependent manner with maximal inhibition of $75.97 \pm 2.57\%$ with methanol extract, 70.46% with ethyl acetate and 67.54% with aqueous extract at 70 $\mu\text{g/ml}$ with IC_{50} value of 33.33 $\mu\text{g/ml}$ (methanol), 43.10 $\mu\text{g/ml}$ (ethyl acetate) and 60 $\mu\text{g/ml}$ with aqueous extract (Table 2). BHT used as standard inhibits the hydroxyl radical up to 83.37% with the same concentration.

Calf thymus DNA damage protection activity of *Euphorbia wallichii* extracts

Protective effect of *Euphorbia wallichii* extracts on DNA damage was studied using Calf thymus DNA. (Figure 7) shows the electrophoretic pattern of Calf thymus DNA after the Fenton's reagent induced damage both in the presence and absence of *Euphorbia wallichii* extracts. The Fenton's reaction involves the reaction between hydrogen peroxide and Fe^{2+} to form hydroxyl radical. The results showed complete degradation of DNA treated with Fenton's reagent which is indicated in lane 2. The action of plant extracts on the DNA damage caused by Hydroxyl radical was indicated from lane 3 to lane 8. The intensity of the DNA damage was reduced on a concentration dependent manner of methanolic, ethyl acetate and aqueous extracts towards DNA which shows the protective effect of the extracts towards hydrogen peroxide induced damage. At the concentration of extracts (10-80 $\mu\text{g/ml}$) the DNA damage was protected in a dose dependent manner which is indicated on (Figure 7). As compared with our above results, it is again indicated that methanolic

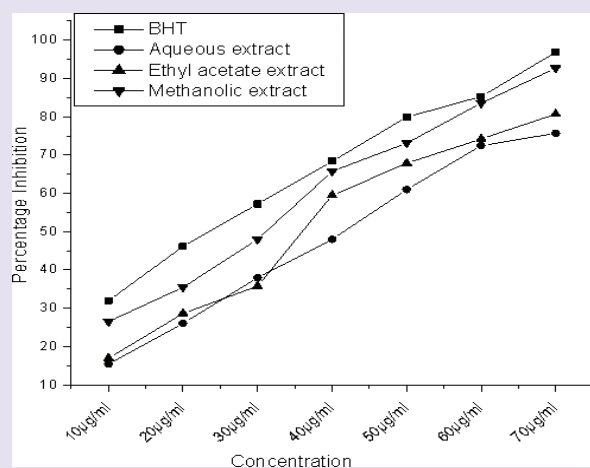


Figure 5: The effect of methanol, ethyl acetate and aqueous extracts of *Euphorbia wallichii* and known antioxidant BHT on lipid peroxidation radical scavenging activity. The results represent mean \pm SD of 3 separate experiments.

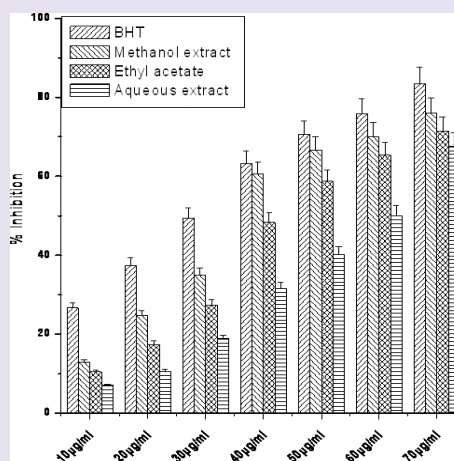


Figure 6: The effect of different extracts of *Euphorbia wallichii* and known antioxidant BHT on hydroxyl radical scavenging activity. The results represent mean \pm SD of 3 separate experiments.

extract of *Euphorbia wallichii* showed strong DNA damage protection once compared with that of ethyl acetate and aqueous extract, may be due to the possible ability of polyphenolic compounds of *Euphorbia Wallichii* plant that could be responsible for the protection against oxidative damage to DNA.

Effect on erythrocyte membrane stability of *Euphorbia wallichii* extracts

All the three extracts of *Euphorbia wallichii* significantly protect the human blood erythrocyte membrane against lysis induced by hypotonic solution. At the concentration of 1-12 mg/ml, we observed a dose dependent increase in the RBC membrane stabilization. Highest concentration of the plant extract (12 mg/ml) afforded $90.58 \pm 0.93\%$ inhibition with methanol extract, 86.6 ± 0.53 with ethyl acetate and 78.8 ± 0.90 with aqueous extract (Table 3). Diclofenac showed a significant protection of the human RBC against the damaging effect of hypotonic solution. At a concentration of 12.0 mg/ml, $98.23 \pm 0.81\%$ inhibition of RBC haemolysis was observed as compared with plant extracts (Table 3).

Correlation between assays

To correlate the results obtained with the different methods, regression analysis was performed (i.e. correlation coefficient r^2) (Table 4) (Figure 8). Significant correlation were found between various methods to determine the antioxidant potential, especially DPPH was correlated with superoxide radical, hydrogen peroxide radical and lipid peroxidation assays. The lowest correlations were observed between DPPH and superoxide radical assay ($r^2 = 0.744$) (Figure 8A) and between superoxide radical and H_2O_2 assays ($r^2 = 0.727$) (Figure 8E). A strong correlation was found between the DPPH and H_2O_2 scavenging assays ($r^2 = 0.9915$) (Figure 8C) and between superoxide radical and lipid peroxidation ($r^2 = 0.966$) (Figure 8G).

DISCUSSION

Euphorbia wallichii is used ethnopharmacologically for the treatment of various complaints. The therapeutic benefit of medicinal plants is usually contributed to their antioxidant properties. Phytochemical investigation reported that *Euphorbia wallichii* constitute an incredible antioxidant compounds like tannins, phenols, steroids etc and exhibited remarkable IC_{50} values in different extracts (Table 1), (Table 2). Antioxidants fight against free radicals and protect us from various diseases. They exert

their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms.¹⁹ In this study, *in vitro* antioxidant and anti inflammatory activity of different extracts of *Euphorbia Wallichii* were tested for their antioxidant activity using DPPH, H_2O_2 , OH and lipid peroxidation assays. *In vitro* anti-inflammation activities of various extracts were determined on human RBCs. The DPPH test is a widely used method to evaluate the free radical scavenging effect of plant extracts. This method is based on the reduction of DPPH solution in the presence of antioxidant resulting in the formation of non radical DPPH-H by the reaction. The stable DPPH were reduced by all the extracts and, thus changing the color from purple to yellow to varying degree depending on the presence of antioxidant compounds. The degree of discoloration indicates the scavenging potential of the extract. In the present study, among all the extracts tested, the highest capacity to neutralize DPPH radicals was found for the methanolic extracts followed by ethyl acetate and aqueous extract. Our results revealed that there is a strong and significant correlation between TPC and DPPH• free radical scavenging activity of different extracts of *Euphorbia wallichii*.

In reducing power assay, antioxidants cause the reduction of Fe^{3+} into Fe^{2+} , thereby changing the solution in to various shades from green to blue, depending on the reducing power of compounds.²⁰ The ferric reducing power activity of different extracts of *Euphorbia wallichii* seems to be due to presence of polyphenols which is correlated to its total phenolic content and phytochemical screening. Methanolic extract with high TPC (465 ± 5.55 mg/GAE) increases the reducing power capacity of *Euphorbia wallichii* in a dose dependent manner. Hence, methanolic extract of *Euphorbia wallichii* may act as electron donors and could react with free radicals to convert them into more stable products and then terminate the free radical chain reactions. Our results are in tune with the results observed by Irshad *et al.*, were studying the antioxidant activity of *Cassia fistula* extracts.²¹

Superoxide anion ($O_2^{\cdot-}$) is one of the most important representatives of free radicals. It acts as a precursor of more reactive oxidative species such as single oxygen and hydroxyl radicals that have the potential of reacting with biological macromolecules and thereby inducing tissue damage, and plays a vital role in peroxidation of lipids.²² Methanol extract with IC_{50} value of 34.05 ± 0.65 μ g/ml showed strong super oxide radical scavenging activity than ethyl acetate and aqueous extract, however

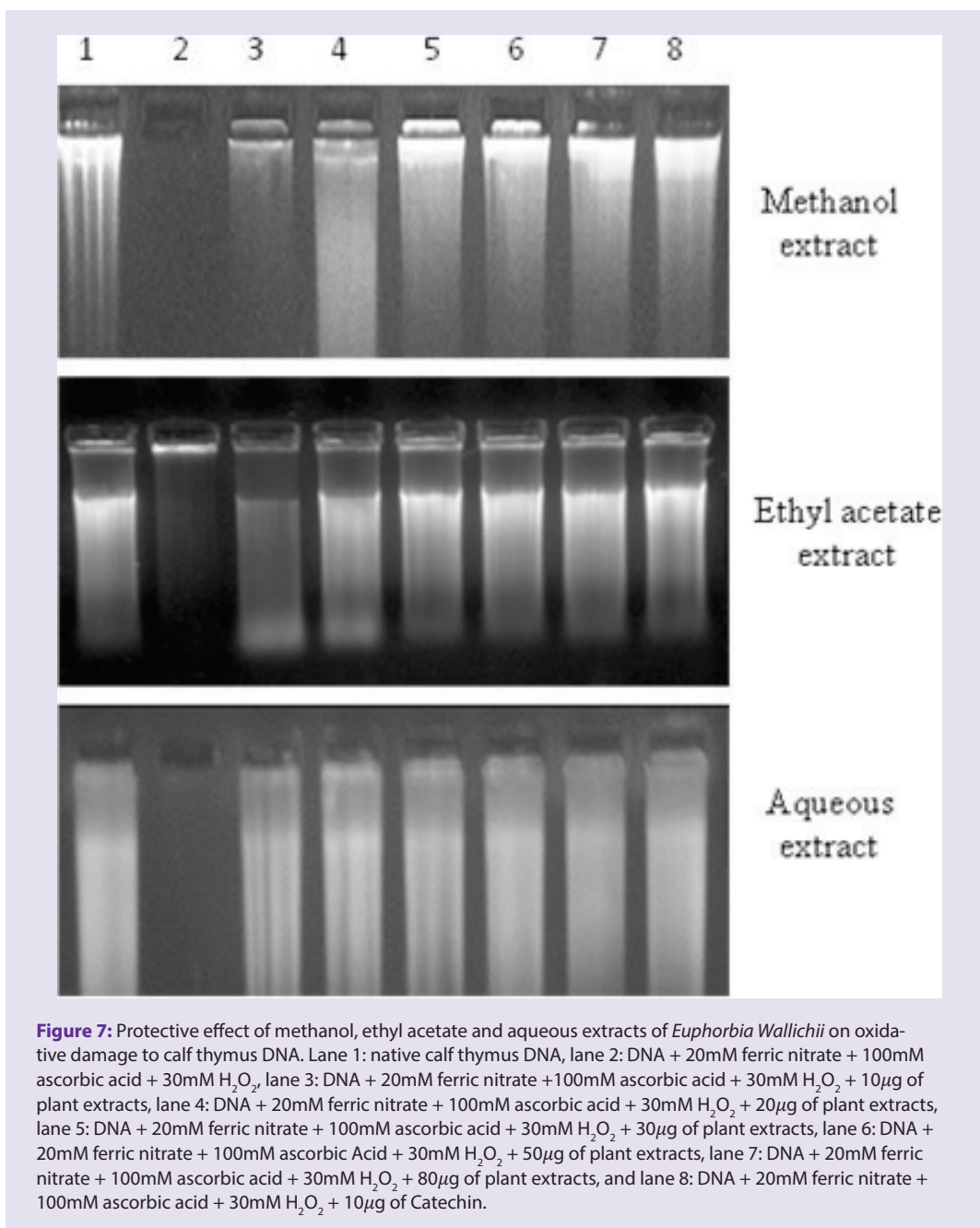


Figure 7: Protective effect of methanol, ethyl acetate and aqueous extracts of *Euphorbia Wallichii* on oxidative damage to calf thymus DNA. Lane 1: native calf thymus DNA, lane 2: DNA + 20mM ferric nitrate + 100mM ascorbic acid + 30mM H₂O₂, lane 3: DNA + 20mM ferric nitrate + 100mM ascorbic acid + 30mM H₂O₂ + 10µg of plant extracts, lane 4: DNA + 20mM ferric nitrate + 100mM ascorbic acid + 30mM H₂O₂ + 20µg of plant extracts, lane 5: DNA + 20mM ferric nitrate + 100mM ascorbic acid + 30mM H₂O₂ + 30µg of plant extracts, lane 6: DNA + 20mM ferric nitrate + 100mM ascorbic acid + 30mM H₂O₂ + 50µg of plant extracts, lane 7: DNA + 20mM ferric nitrate + 100mM ascorbic acid + 30mM H₂O₂ + 80µg of plant extracts, and lane 8: DNA + 20mM ferric nitrate + 100mM ascorbic acid + 30mM H₂O₂ + 10µg of Catechin.

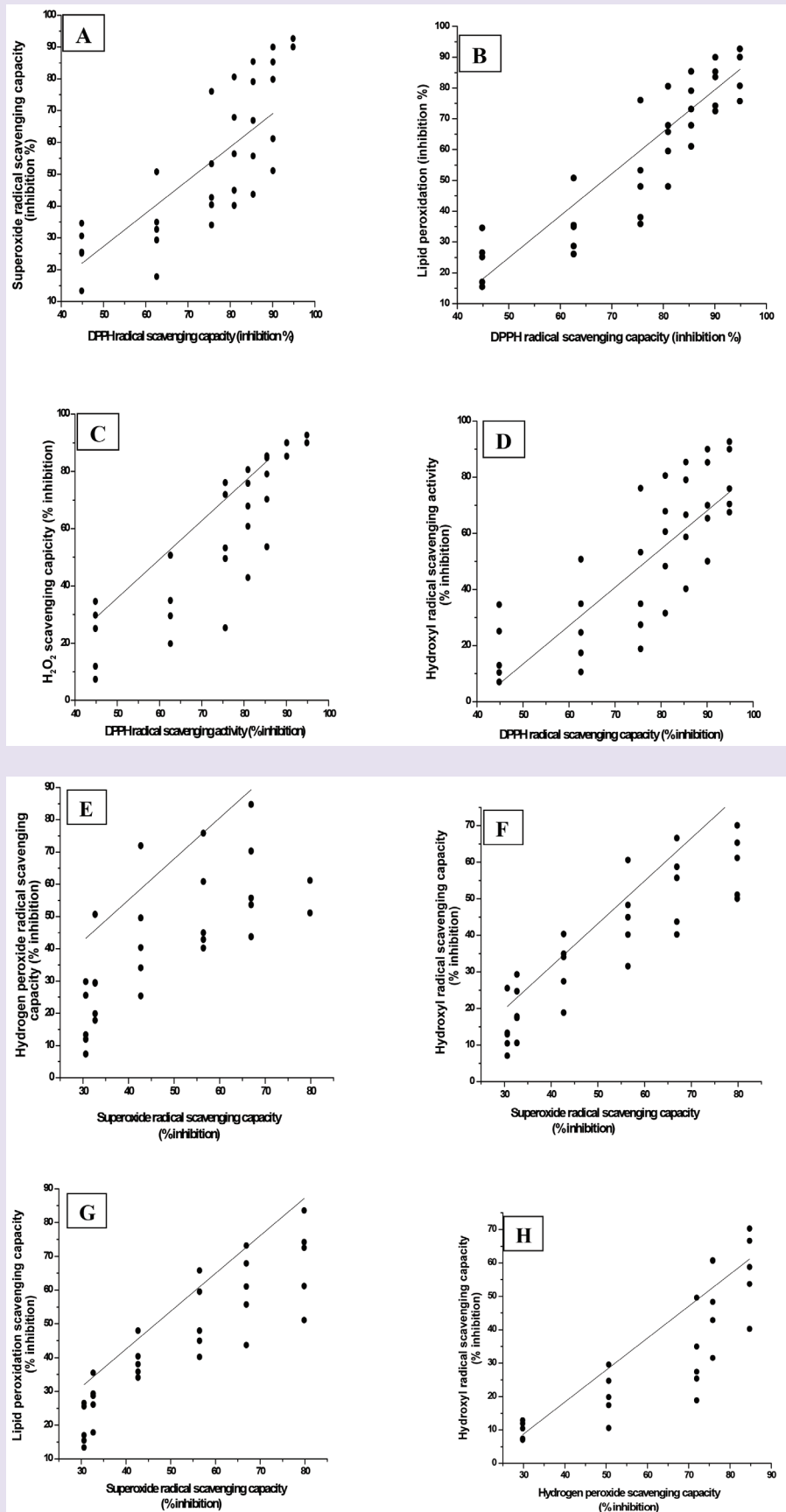
the values remain below the BHT used as known superoxide radical scavenger.

Hydrogen peroxide (H₂O₂) is a biologically relevant, non-radical reactive oxygen species and is inevitably generated as a by-product of normal aerobic metabolism. However, when concentration increases under stress conditions, H₂O₂ could be detrimental for cells²³ and furthermore could be converted into other ROS such as hydroxyl radicals. Thus H₂O₂ scavenging activity becomes a crucial characteristic of total antioxidant activity. In this study, methanol extract (IC₅₀=99 ± 0.08 µg/ml) was found to be efficient scavenger of hydrogen peroxide radical while ethyl acetate extract (IC₅₀=152 ± 1.34 µg/mL) and aqueous extract (IC₅₀=236 ± 1.89 µg/mL) was least efficient. The results strongly suggest that these extracts contain the necessary compounds for radical elimination. As reported already that cytotoxic activity induced by hydrogen

peroxide in mammalian and bacterial cells could be protected by plant phenolics,²⁴ indicating that the observed activity of plant extracts could be used in future to treat cytotoxicity induced by hydrogen peroxide.

In this study, we measured the potential of *Euphorbia wallichii* extracts to inhibit lipid peroxidation in rat liver microsomes induced by the Fe²⁺/ascorbate system. Different extracts protected against lipid peroxidation induced by Fe²⁺ considerably reduced MDA content in a concentration-dependent manner. Methanol extract had the greatest inhibiting activity (92.72%), with the lowest IC₅₀ value 31.5 µg/mL. Similar results were reported by Gul *et al.*, were studying the antioxidant and antiproliferative activities of *Abrus precatorius* leaf extract.²⁵

Hydroxyl radical is generated by a mixture of Fe³⁺-H₂O₂ and ascorbic acid and is assessed by monitoring the degraded fragments of deoxyribose, through malonaldehyde (MDA) formation and DNA strand breaks



continued...

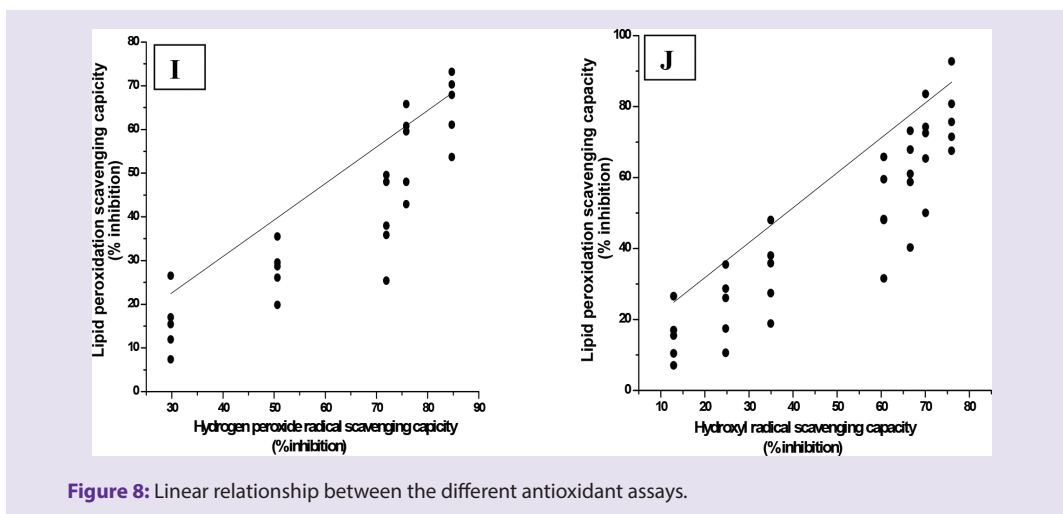


Figure 8: Linear relationship between the different antioxidant assays.

in Calf thymus DNA.²⁶ If any plant extract or drug scavenges the hydroxyl radical, they may either scavenge the radical or may chelate the Fe^{2+} ion, making it unavailable for the Fenton's reaction. Plant extracts containing polyphenols are reported to quench oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical or neutralize free radicals or by their chelating ability due to their high nucleophilic character of the aromatic ring.²⁷ In our study we tested different extracts of *Euphorbia wallichii* and it was observed that extracts exhibit a dose dependent hydroxyl radical scavenging activity and prevents the Calf thymus DNA damage. Similar results were observed by our lab, were studying the antioxidant activity of rhizome of *Podophyllum hexandrum*.²⁸ Our results are in tune with the results reported by Sundararajan and Koduru, while studying the antioxidant activity of *Limnophila heterophylla*.²⁹

It is well known that the vitality of cells depends on the integrity of their membranes. Exposure of red blood cell to injurious substances such as hypotonic medium and phenylhydrazine results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin. The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical -induced lipid peroxidation.³⁰ Compounds with membrane stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reactions, namely the prevention of the release of phospholipases that trigger the formation of inflammatory mediators. Plant saponins and flavonoids are reported to exerted profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro*, while tannins and saponins possess ability to bind cations, thereby stabilizing erythrocyte membranes and other biological macromolecules.³¹

As it is noted from the above results that the extracts of *Euphorbia Wallichii* exhibits good antioxidant activities, the results are highly correlated with the RBC membrane stabilization properties. At the concentrations of 1-12 $\mu\text{g/ml}$, we observed 70-90% inhibition on RBC hemolysis with methanol extract followed by ethyl acetate and aqueous extract respectively. The results of our study suggest that *Euphorbia Wallichii* extracts may offer some beneficial effects in the management of inflammatory conditions. Similar results were reported by Umukoro and Ashorobi, while studying anti-inflammatory and membrane stabilizing property of aqueous leaf extract of *Momordica charantia*.³²

The DPPH, superoxide radical, hydrogen peroxide, hydroxyl radical and lipid peroxidation assays gave comparable results for the antioxidant

activity measured in methanol, ethyl acetate and aqueous extracts of *Euphorbia wallichii*. The highest correlations were found between DPPH, H_2O_2 , superoxide radical, lipid peroxidation and hydroxyl radical assays, especially between DPPH and H_2O_2 , a result previously reported by Stéphanie Dudonné *et al.*³³ These results indicate a relationship between different antioxidant assays, especially once the assays are compared with the DPPH, which is one of the most widely used method for screening the antioxidant activity of plant extract.

CONCLUSION

Overall, it could be concluded that *Euphorbia wallichii* possesses a potent antioxidant activity. During our study we also observed that the extracts stabilize the human RBC membrane in a dose dependent manner, thus reduces the inflammation. The preliminary chemical examination of different extracts of *Euphorbia wallichii* has shown the presence of a number of polyphenols which may be responsible for the antioxidant and RBC membrane stabilization activities. Additional studies are needed to characterize the active compounds and to clarify the *in vivo* potential of this plant.

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CONFLICT OF INTEREST

The authors have no personal or financial conflicts of interest associated with this work.

ABBREVIATION USED

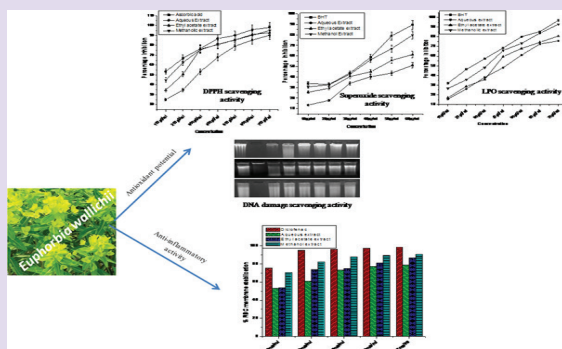
LPO: Lipid peroxidation; **DPPH:** 1, 1-Diphenyl-2-picryl-hydrazyl; **HRBC:** Human red blood cell; **ROS:** Reactive oxygen species; **GAE:** Gallic acid; **BHT:** Butylated hydroxytoluene; **EDTA:** Ethylene diamine tetra acetic acid; **TAE:** Tris acetate; **NBT:** Nitro blue tetra zolium test; **TBARS:** Thiobarbituric acid reactive substances; **TPC:** Total phenolic content; **MDA:** Malondialdehyde; **H_2O_2 :** Hydrogen peroxide.

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PICTORIAL ABSTRACT



SUMMARY

- Methanol extract of *Euphorbia wallichii* showed high content of total phenolic compounds as compared to ethyl acetate and other extract.
- Methanolic extract showed strong *in vitro* antioxidant potential on DPPH radical scavenging activity, reducing power, LPO and other assays.
- Methanol extract exhibit 90% increase in RBC membrane stabilization as compared to other extracts used in the study.

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