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Research Article

FREE RADICAL SCAVENGING ACTIVITY OF EUPHORBIA HIRTA LINN. LEAVES AND ISOLATION OF ACTIVE FLAVONOID MYRICITRIN

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ABSTRACT

Extract from the leaves of *Euphorbia hirta* Linn. was investigated for antioxidant activity. Methanolic extract and ethyl acetate fraction revealed the presence of flavonoids, hence considered for the further study. Methanolic extract also showed the presence of alkaloids, steroids and carbohydrates. The methanolic extract of *Euphorbia hirta* Linn. has DPPH scavenging activity of 89.75±0.032% and hydroxyl radical scavenging activity of 83.5±0.046% at 100µg/ml, while ethyl acetate fraction have DPPH scavenging activity of 91.88±0.060% and hydroxyl radical scavenging activity of 85.53±0.023%. These values were compared with standard ascorbic acid, which showed DPPH radical scavenging activity of 94.26±0.049% and hydroxyl radical scavenging activity of 89.69±0.064% at 10µg/ml. As ethyl acetate fraction was showing significant activity, it was further used for in-vivo antioxidant activity. Ethyl acetate fraction showed significant in-vivo antioxidant activity, 1.207±0.10, 45.85 ±5.2, 0706±0.03 and 0.0106±0.005 for Glutathione, Superoxide dismutase, Catalase activity and Lipid peroxidation respectively. Compound A1 was isolated which was further confirmaed as a myricitrin by Uv, Ir, and ^{H}NMR spectral analysis. *Euphorbia hirta* Linn. Showed In-vitro and In-vivo powerful antioxidant activity may be responsible for it's wide and popular traditional use.

Key words: *Euphorbia hirta*, Phytochemical, Antioxidant, DPPH, *In-vivo*, *In-vitro*.

INTRODUCTION

Euphorbia hirta Linn. belonging to family euphorbiaceae which is large families of trees, shrubs, and herbs of rainforest Guinean, Soudanian & xerophylatic habitat. Although the plants belonging this family have important economic uses as food stuff, medicinal & industrial purposes particularly as a source of rubber & timber. [1] Free radical has been implicated in human diseases such as lung disease, heart failure, hepatotoxicity, nephrotoxicity, inflammation and diabetes. All biomolecules may be attacked by the free radicals but lipids are probably the most susceptible one; this oxidative destruction is known as lipid peroxidation and it may include many pharmacological events.[2] Current hypothesis favors the concept that lowering oxidative stress has various health benefits. [3] Natural products including plants have been the basis of treatment of human diseases for thousands of years. Almost 60% of all new chemical entities introduced world wide as a drug in last two decades may be traced to or inspired by natural product. [4] Many plants contains substantial amount of antioxidants like Vit C, Vit. E, carotenoids, flavonoids and tannins. These phytoconstituents can be utilized to scavenge the excess free radicals from the human body. [5] The present work has been designed to evaluate the antioxidant potential of the methanolic extract & ethyl acetate fraction of the leaves of *Euphorbia hirta.* Linn.

MATERIALS AND METHODS

Plant Material

Plant Material: leaves of Euphorbia hirta were collected from the surroundings of Hubli & Dharwad, Karnataka (India) and identified at Department of Botany Kothambari College Hubli. Leaves were air-dried packed and stored.

Preparation of extract

The dried leaves were pulverized and 100 gm of leaves sample was first defatted with 700 ml of Petroleum ether(40/60) followed by successive extraction with 500 ml of methanol by hot maceration for 72 hrs. The methanolic extract was concentrated & dried under reduced pressure, further it is dissolved in water and fractionated in to ether, chloroform, ethyl acetate and n-butanol. Every extract and fraction was concentrated & dried over anhydrous sodium sulphate & were further used for phytochemcial and pharmacological investigations. [6]

Chemicals

DPPH(2,2 diphenyl -1- picryl hydrazyl) ascorbic acid, methanol, BHT (butylated hydroxyl toluene), potassium dihydrogen phosphate, potassium hydroxide, deoxyribose, ferric chloride, ascorbic acid, hydrochloric acid, EDTA (ethylene diamine tetra acetic acid), TCA (trichloracetic acid), TBA (thiobarbituric acid) & hydrogen peroxide, acetic acid, n-butanol, pyridine, 5,5 dithiobis(2-nitrobenzoic acid), phosphate buffer, ethanol, chloroform and epinephrine. All the chemicals used were AR grade and were obtained from SD fine chemicals Delhi.

Animals

Albino rats (Wistar strain) of either sex weighing (150-200 gm) were obtained from the Animal house of KLES's college of Pharmacy Vidyanagar, Hubli. Animals were divided in to Three groups, Contol , standard and test treated including six animals each groups.

Animals were housed at temperature 25 ±1 ^{0}C and relative humidity 41.55% and provided food and water ad libitum.

Isolation of Myricitrin

Ethyl acetate plant fraction was applied on precoated silica gel G plate and run in solvent system chloroform and methanol (80:20) shown three different distinct spots.

Then ethyl acetate fraction was subjected for column chromatography using silica gel G and Chloroform and methanol (80:20) as a mobile phase. Where compound A1 was isolated.

Free radical scavenging activity: *In-vitro*

1. DPPH radical scavenging activity

Samples for the experiment were prepared in different concentrations in the range of 10-100µgm/ml in AR grade methanol. These samples of above concentrations were mixed with 3ml of 100µM of DPPH prepared in AR grade methanol and finally the volume was made up to 4ml with AR grade methanol if necessary.The absorbance of the resulting solutions and the blank (prepared similarly by omitting the sample) were recorded after 20mins at room temperature against BHT (10-100µgm/ml in AR grade methanol) and ascorbic acid (1-10µgm/ml in AR grade methanol). The disappearance of DPPH was read spectrophotometrically at 517nm using a UV-Visible Spectrophotometer. Radical Scavenging Capacity (RSC) in percentage was calculated by the following equation.

RSC (%) =100 x A_{blank} - A_{sample} / A_{blank}

⇒ RSC = Radical Scavenging Capacity
⇒ A_{blank} = Absorbance of blank.
⇒ A_{sample}= Absorbance of sample.

From the RSC values obtained the IC_{50} were calculated, which represents the concentration of the scavenging compound that caused 50% neutralization.[7] (Savadi RV et al. 2008).

2. Hydroxyl radical scavenging activity

All the reagents were dissolved in freshly prepared phosphate buffer. The reaction mixture [deoxyribose (2.8mM), KH_2PO_4-KOH buffer of pH 7.4 (20mM), FeCl3 (0.1mM), EDTA (0.1mM), H_2O_2 (1mM) & ascorbate (0.1mM)] taken was 3ml and drug 1ml for each variable concentration. The reaction mixture along with drug was incubated for 1hr at 37^{0}C. After incubating the reaction was stopped by adding 2ml of ice cold 0.25N HCl containing 15% trichloroacetic acid, 0.38% thiobarbituric acid and 0.05% BHT. Following heating at 80^0 for 15mins, samples were cooled and centrifuged at 1000rpm for 10min; the absorbance of the supernatant was measured at 532nm. . Test compounds were dissolved in 0.05N NaOH and the pH was adjusted to 7.4 with 0.1N HCl. The absorbance was read against blank (buffer solution only) at 532nm.The absorbance was used for the calculation of the percentage dissolution of 2-deoxy-D-ribose degradation by the sample by using the following formula.

RSC (%) =100 x A_{blank} - A_{sample} / A_{blank}

⇒ RSC = Radical Scavenging Capacity
⇒ $A_{blank =}$ Absorbance of blank.
⇒ A_{sample}= Absorbance of sample...
⇒ BHT was used as a positive control.

From the RSC values obtained, the IC_{50} values were calculated, which represent, the concentration of the scavenging compound that caused 50% neutralization.[7]

Free radical scavenging activity: *In-vivo*[8]

The experimental animals were divided into four groups of six animals each.

Rats in Group I (control) receive ethanol only (1ml/200gm), Group II standard receive ethanol and standard drug(Ranitidine 30mg/kg), Group III received ethanol and test drug(ethyl acetate fraction 200mg/kg).

In-vivo free radical scavenging activity was carried out in ethanol induced gastric ulcer model. Prior induction of gastric ulcer Group III animals were feed with the ethyl acetate fraction in a dose of 200mg/kg body weight one hour prior administration of 90 %ethanol. Ulcer was induced by oral administration of 90 %ethanol v/v 1ml/200gm. Rat were killed by overdose of chloroform and the stomach was removed. The gastric tissue was homogenized centrifuged and supernatant was used for further study.

1. Lipid peroxidation (LPO) was assayed by the method of Ohkawaet al. (1979) in which the malondialdehyde (MDA) releasedserved as the index of LPO. 1, 1, 3, 3-Tetra ethoxypropane malondialde-hyde bis (diethyl acetal) was used as standard. To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA were added. The mixture was made up to 4.0 ml with water and then heated in a water bath at95.8oC for 60 min using glass ball as a condenser. After cooling, 1.0ml of water and 5 ml of n-butanol/pyridine mixture were added andshaken vigorously. After centrifugation at 4000 rpm for 10 min, theorganic layer was taken and its absorbance was measured at 532 nm. The level of lipid peroxides was expressed as nmoles of MDAformed/mg of protein

2. Reduced glutathione (GSH) was estimated by the method of Ellman, (1959). 0.1 ml of tissue homogenate was precipitated with 5% TCA (trichloroacetic acid). The contents were mixed well for complete precipitation of proteins and centrifuged. To 0.1 ml of supernatant, 2.0 ml of 0.6 mM DTNB [5, 5 dithiobis (2-nitrobenzoic acid)] reagent and 0.2 M phosphate buffer (pH 8.0) were added to make up to a final volume of 4.0 ml. The absorbance was read at 412 nm against a blank containing TCA (trichloroacetic acid) instead of sample. A series of standards treated in a similar way also run to determine the glutathione content. The amount of glutathione was expressed as nmoles/g heart tissue. 5-sulphosalicylic acid was used to prevent the oxidation of glutathione.

3. Catalase was assayed by the method of Takahara et al. (1960). To 1.2 ml of 50 mM phosphate buffer pH 7.0, 0.2 ml of the tissue homogenate was added and reaction was started by the addition of 1.0 ml of 30 mM H2O2 solution. The decrease in absorbance was measured at 240 nm at 30 s intervals for 3 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as ▯ moles of H2O2 decomposed/min/mg protein.

4. Superoxide dismutase was assayed by the method of Misra and Fridovich, (1972). (50mg/kg body weight/day) and injected with adriamycin, as described 0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate-bicarbonate (pH 10.2) buffer. The reaction was initiated by the addition of 0.5 ml of 1.8 mM epinephrine (Freshly prepared) and the increase in absorbance at 480 nm was measured in a Shimadzu UV-1601 spectrophotometer. One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine autoxidation. [8](Rajprabhu D. et al., 2007)

Statistical Analysis

Data were expressed as ± SEM of triplicate determinations.

RESULTS AND DISCUSSION

The A1 compound was yellow crystalline and identified as Myricitrin by various spectral analysis as follows:

A) UV spectra have shown maximum absorption at 340.2 nm

B) IR spectra have shown wave number for A1

- 3293.74 (OH stretching)
- 2949.91 (CH stretching of aromatic CH)
- 2839.58 (CH stretching of CH3)
- 1715.90 (C=O stretching)
- 1448.65 (C=C stretching)

C) NMR spectra has shown delta values for A1

- δ (7.7) aromatic protons.
- δ (8.68) CH3
- δ (5.31) OH
- δ (2.15) CH2

D) MS base peak for A1 found at 353.3 and molecular ion peak at 463.4.

The results of the DPPH scavenging activity of *Euphorbia hirta* Linn. extract compared to standard ascorbic acid & BHT is shown in Figure 1-2. Methanolic extract and ethyl acetate fraction both showed potent antioxidant activity compared to BHT. The IC50 values were found to be higher than BHT, the ethyl acetate fraction exhibited statistically highly significant free radical scavenging capacity on DPPH radical (IC_{50} value-13.93µg/ml) than the standard BHT (IC_{50} value-20.64µg/ml). as shown in Table 1 and Figure 3.

The results of the hydroxyl radical scavenging activity of *Euphorbia hirta* Linn. extract compared to standard ascorbic acid & BHT is shown in Figure 4-5. Methanolic extract and ethyl acetate fraction both showed potent antioxidant activity compared to BHT. The hydroxyl radical scavenging activity of ethyl acetate fraction (IC_{50} value-32.86 µg/ml) & that of standard BHT (IC_{50} value-43.87µg/ml). as shown in Table 1 and Figure 6. Ethyl acetate fraction also shown significant In-vivo antioxidant activity (Table 2 and Figure 7).

The present work also revealed that the extract from the leaves of *Euphorbia hirta* Linn. possesses potent antioxidant activity

presumably presumably because of its phytochemical constituents. These facts justify the medicinal use of the plant for the treatment of various diseases. However further work is necessary to ascertain the clinical safety of methanolic extract and ethyl acetate fraction from the plant and to determine the appropriate concentration for therapy so as to safeguard the health of the teeming mass of traditional users who more often do not take these factors in to consideration.

Table 1: IC_{50} values of Methanolic extract and Ethyl acetate fraction

Extract/Fraction	*In-vitro* Models	
	DPPH Radical IC50	OH Radical IC50
Methanolic extract	21.42±0.005	33.25±0.005
Ethyl acetate Fraction	32.86±0.004	33.25±±0.005

Compound A1
Name: Myricitrin
Synonyms: Myricetin-3-rhamnoside; 5,7-Dihydroxy-3-((2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyl-tetrahydro-pyran-2-yloxy)-2-(3,4,5-trihydroxy-phenyl)-1-benzopyran-4-one

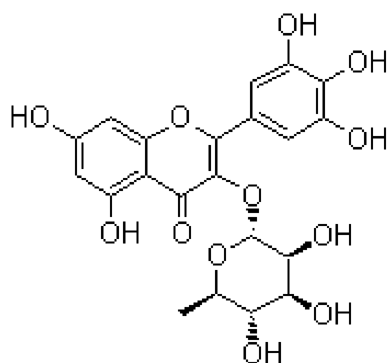


Table 2: Estimation of GSH, SOD, CAT and Lipid peroxidation (Mean ± SEM)

Group	Glutathione (n mol/mg wet gland)	Superoxide dismutase	Catalase activity	Lipid peroxidation
Control	0.751±.0.0773	71.21±2.474	0.1605±0 .02422	0.05583±0.015 71
Standard	1.848± 0.2639	29.71± 4.340	0.9230± 0.02115	0.00585±0.001 132
Extract	1.152±0.09361	52.02±1.337	0.4937±0 .04493	0.007258±0.00 3873

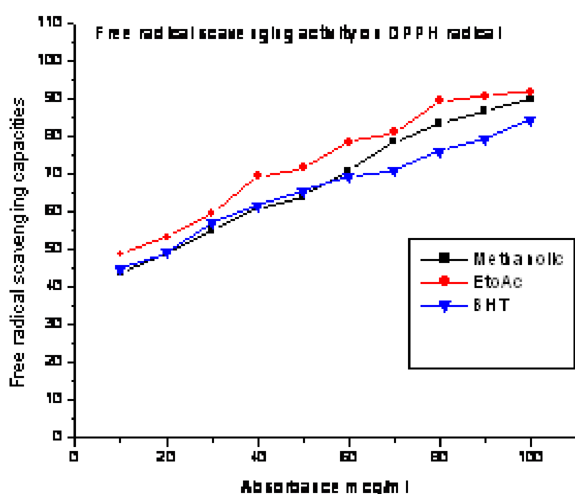


Figure 1: Free radical scavenging activity of various extract on DPPH radical

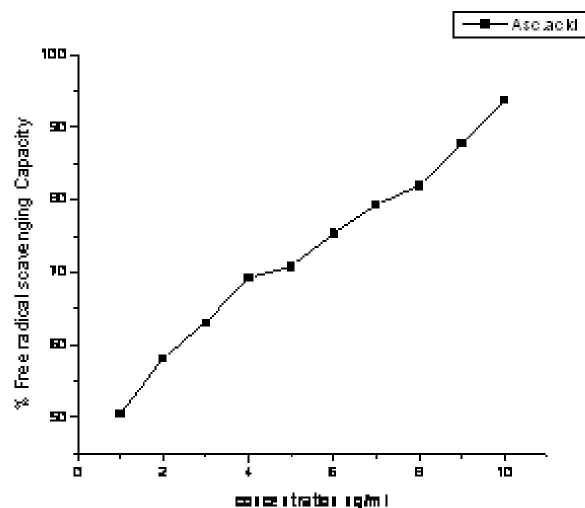


Figure 2: Free radical scavenging activity of Ascorbic acid on DPPH radical

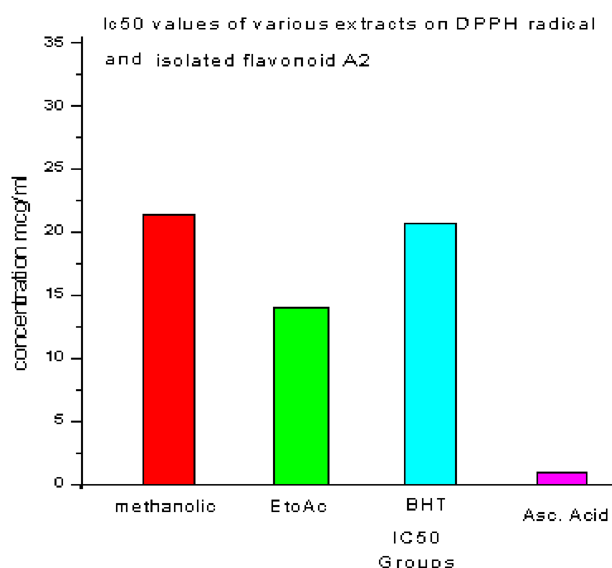


Figure 3: IC_{50} values of various groups on DPPH radical

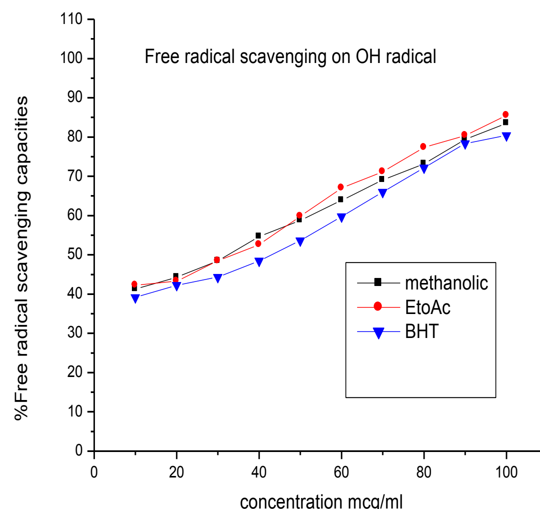


Figure 4: Scavenging of Hydroxyl radicals by various groups

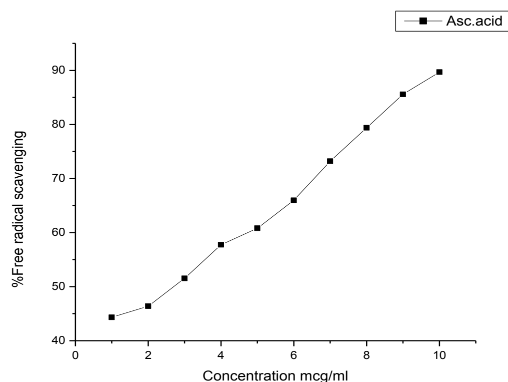


Figure 5: Free radical scavenging activity of Ascorbic acid on Hydroxyl radical

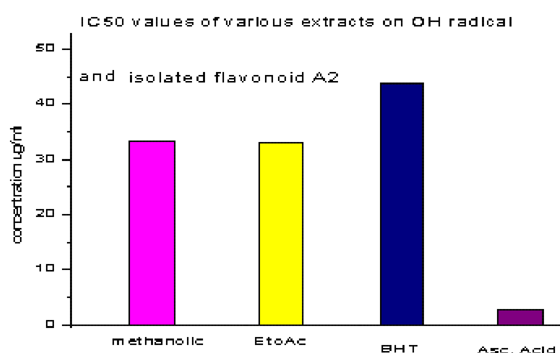
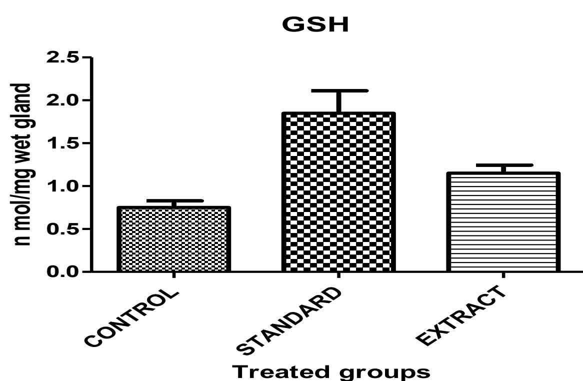
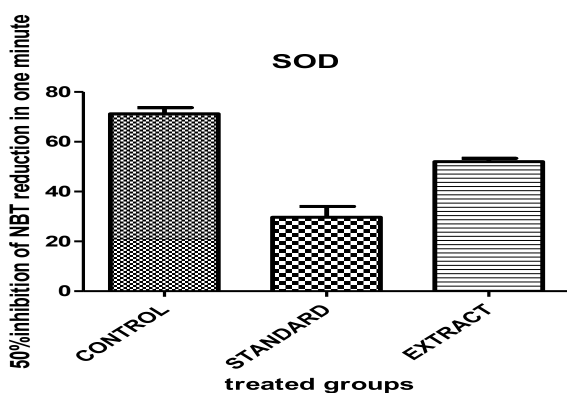


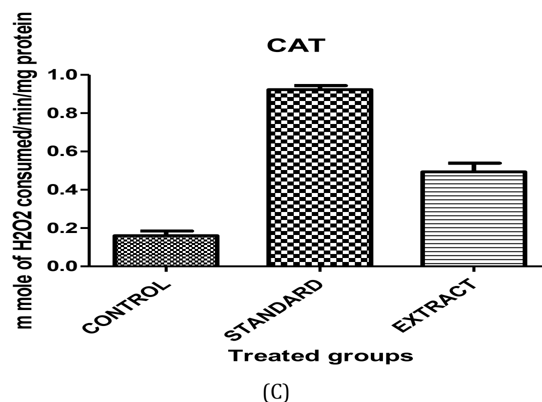
Figure 6: Histogram showing IC_{50} values of various groups on hydroxyl radical



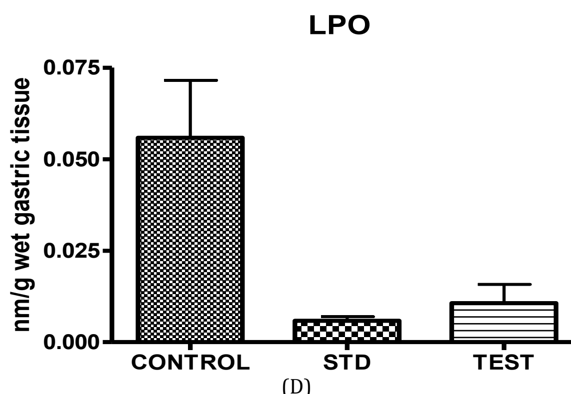
(A)



(B)



(C)



(D)

Figure 7: Histogram showing (A) GSH, (B) SOD, (C) CAT and (D) Lipid peroxidation

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