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RESEARCH ARTICLE

IN VITRO ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF *EUPHORBIA HIRTA* LINN

Goldie Uppal*, Vijay Nigam

SRET College of Pharmacy, Barsar, Hamirpur (H. P.), India

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ABSTRACT

The antioxidant properties of ethanolic extract of *Euphorbia hirta* Linn was tested using standard *in vivo* and *in vitro* models. *Euphorbia hirta* Linn (Euphorbiaceae) is commonly used in traditional medicines for the treatment of various ailments. In anti-lipid peroxidation assay, extract showed a decrease in the absorbance which was comparable with the standard ascorbic acid. In DPPH free radical scavenging assay, there was a decrease in the absorbance with increase in concentration, which indicate the antioxidant activity of *Euphorbia hirta* Linn. In reducing power assay, there was an increase in the absorbance with an increase in the concentration of *Euphorbia hirta* Linn, which indicate the reducing capacity of *Euphorbia hirta* Linn. In nitric oxide free radical scavenging assay, there was a decreased in the absorbance with increase in concentration, which was comparable with the standard ascorbic acid. In anti-lipid peroxidation assay, the *Euphorbia* extract showed a decrease in the absorbance which was comparable with the standard ascorbic acid.

INTRODUCTION

Antioxidants counter the action of free radicals by several mechanisms. These mechanisms include: (1) enzymes that degrade free radicals, (2) proteins such as transferrin that can bind metals which stimulate the production of free radicals, and (3) antioxidants such as vitamins C and E that act as free radical scavengers (Picot *et al*, 1994). Numerous studies have demonstrated that antioxidant vitamins and supplements can help lower the markers indicative of oxidant stress and lipid peroxidation in diabetic subjects and animals (Sreeenivasa *et al*, 2009). Oxidative stress is a well known mechanism that is responsible for the development of vascular damage. Reactive oxygen species (ROS) are normally produced throughout oxygen metabolism and play a major role in physiological and pathological cell redox signaling. Oxidative stress results from an imbalance due to overproduction of ROS by the different cellular sources. Antioxidants inhibit the generation of ROS and the subsequent formation of lipid peroxidation products, thereby preventing both oxidative and carbonyl stress. Most antioxidants prevent LDL oxidation in cell-dependent and cell-free systems (Nadji Belkheiri *et al*, 2010; Sies H., 1991).

Recently, there has been a considerable interest in finding natural antioxidants from plant materials to replace synthetic ones. Data from both scientific reports and laboratory studies show that plants contain a large variety of substances that possess antioxidant activity. Phytochemicals with antioxidant effects include some cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins and triterpenes (Stocker R., 1999; Lingert H. *et al*, 1979). It is conceivable that antioxidant/free radical scavenging activity of *Euphorbia hirta* is unlikely to be the only mechanism associated with its antidiabetic effects. The occurrence of oxidative mechanisms in plants may explain why an abundance of antioxidant compounds have been identified in plant tissue (Chanda S., *et al* 2009). Therefore it seems that plants particularly those with high levels and strong antioxidant compounds have an important role in improvement of disorders involving oxidative stress such as diabetes mellitus. There are many investigations which have studied the effects of these plants and their antioxidant ingredients on diabetes and its complications and achieved good results (Mohammad Abdollahi *et al*, 2005).

MATERIALS AND METHODS

Anti-lipid peroxidation effect (Kumar, *et al*, 2005; Mondal and Muzumder, 2006; Wade, *et al*, 1985)

Rat liver homogenate was used as the source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. Liver was collected immediately after sacrificing the animal by cervical dislocation under ether anaesthesia. The liver was isolated, and 10% (w/v) homogenate was prepared in the 40 mM of Tris hydrochloride buffer using a tissue homogenizer under ice cold (0-4°C) conditions. The liver homogenate was used for the study of *in vitro* lipid per oxidation. Various solutions of potassium chloride (0.15 M), ferric chloride (1 mM), thiobarbituric acid (0.38% TBA, 15%

Corresponding author: uppalgoldie2@gmail.com

TCA in 0.25 N HCl) and ascorbic acid (1000 µg/ml) were prepared by as per standard procedures. From the stock solution of ascorbic acid 20, 40, 60, 80 and 100 µg/ml ascorbic acid solutions were prepared. Sample solutions were prepared in similar manner and the filtrates were used for the experiment.

Principle

Decomposition of lipid membrane in the body leads to the formation of malondialdehyde (MDA) along with other aldehydes and enals as the end product. This reacts with thiobarbituric acid to form colored complexes. Hence these are called as the Thiobarbituric Acid Reactive Substances (TBARS). The complex of TBA-MDA is selectively detected at 532 nm using UV spectrophotometer.

Procedure (Ilavarasan and Mallika, 2005)

0.5 ml of homogenate was taken and to it 1 ml of 0.15 M KCl and 0.5 ml of test drugs at different concentrations (20, 40, 60, 80, 100 µg/ml) were added. Lipid peroxidation was initiated by adding 100 µl of 1 mM ferric chloride. The reaction was stopped by adding 2 ml of ice cold 0.25 N HCl containing 15% trichloroacetic acid, 0.38% thiobarbituric acid, and 0.2 ml of 0.05% butylated hydroxyl toluene. These reaction mixtures were heated for 60 min at 80ºC then cooled and centrifuged at 6900 rpm for 15 min. The absorbance of supernatant was measured at 532 nm against blank, which contained all reagents except liver homogenate and drug. Same experiments were performed to determine the normal (without drug and $FeCl_3$) and induced (without drug) lipid peroxidation level in the tissue. The percentage of anti-lipid peroxidation effect (% ALP) was calculated by the following formula (Feldman, *et. al.*, 1999).

$$\% \text{ Anti lipid peroxidation effect} = \frac{FeCl_3 \text{ O.D.} - \text{Sample O.D.}}{FeCl_3 \text{ O.D.} - \text{Normal O.D.}} \times 100$$

Free radical scavenging activity by DPPH method (Mondal and Muzumder, 2006; Kumar, *et. al.*, 2005)

DPPH solution was prepared by dissolving 33 mg of DPPH in 1 Liter methanol and was kept in dark amber colored bottle so as to protect it from sunlight. Sample Stock solution of 1000 µg/ml was prepared by dissolving 10 mg of test drug in 100 ml of solvent and 10, 20, 40, 60 and 80 µg/ml solutions were prepared, the filtrates were used for the experiment. Ascorbic acid solution was prepared by dissolving 10 mg of ascorbic acid in 10 ml of solvent to prepare 1000 µg/ml solution. From the stock solution 10, 20, 40, 60 and 80 µg/ml ascorbic acid solutions were prepared.

Principle

Scavenging free radical potentialities were evaluated against a methanolic solution of 1, 1- diphenyl-2-picryl hydrazyl (DPPH), and a stable free radical. Anti oxidant react with DPPH and convert it to 1, 1- diphenyl- 2-picryl-hydrazine and the degree of discoloration indicates the scavenging activity of drug. The change in the absorbance produced at 517 nm, has been used as a measure of antioxidant activity (Blois, 1958).

Procedure (Ilavarasan and Mallika, 2005)

Different concentrations of test drug solution and standards were prepared. To this 5 ml of methanolic solution of DPPH was added, shaken well and mixture was incubated at 37°C for 30 min. A blank was prepared in the similar way and the absorbance was measured at 517 nm. Scavenging activity was expressed as the percentage inhibition calculated using the following formula.

$$\% \text{ Anti radical activity} = \frac{\text{Absorbance of control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Reducing power assay (Oyaizu, 1986; Mondal and Muzumder, 2006)

Phosphate Buffer pH 6.6, potassium ferricyanide $K_3Fe(CN)_6$ (1%), trichloroacetic acid (10%) and ferric chloride (0.1%) solutions were prepared by standard procedure. Sample solution was prepared from stock of 1000 µg/ml and 10, 20, 40, 60, 80 µg/ml solutions were prepared, and the filtrates were used for the experiment. Ascorbic acid solution was prepared by dissolving 10 mg of ascorbic acid in 10 ml of solvent to prepare 1000 µg/ml solution. From the stock solution 10, 20, 40, 60 and 80µg/ml ascorbic acid solutions were prepared.

Principle

The reducing capability was measured by the transformation of Fe^{3+} to Fe^{2+} in the presence of different test drugs at 700 nm as per the reported method. Increased absorbance of the reaction mixture indicates increased reducing power.

Procedure

Reducing power of *Euphorbia hirta* was determined on ability of antioxidants to form colored complex with potassium ferricyanide. Different concentration of the *Euphorbia hirta* (10, 20, 40, 60, 80 µg/ml) were mixed with 2.5 ml phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1%). The mixture was incubated at 50ºC for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5ml of water and 0.5 ml of $FeCl_3$ (0.1%) were added to it and absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Nitric oxide radicals scavenging activity (Ilavarasan and Mallika, 2005; Kumar, *et. al.*, 2005; Sreejayan and Rao, 1997; Rajlakshmi and Banerjee, 2003)

Sodium nitroprusside, phosphate buffered saline and Griess reagent were prepared as per standard procedures. Sample solution was prepared from stock of 1000 µg/ml was prepared by dissolving 10 mg of test drug in 10ml of solvent and 10, 20, 40, 60, 80 µg/ml solutions were prepared, the filtrates were used for the experiment. Standard ascorbic acid solution was

prepared and from the stock solution 10, 20, 40, 60 and 80 µg/ml ascorbic acid solutions were prepared.

Principle

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH, reacts with molecular oxygen to form nitrate ions. Sulfanilamide is quantitatively converted to a diazonium salt by reacting with nitrate in acidic condition (5% phosphoric acid). This diazonium salt coupled with N-(1-napthyl)-ethylenediamine (NED), forming an azo dye that can be measured quantitatively at 542 nm.

Procedure

Different concentrations of sample solutions were prepared in 100 ml volumetric flasks. To this 0.1489 g of sodium nitroprusside (5 mM) was added and kept for incubation. At different time intervals 5.6 ml was taken, 0.2 ml of Griess reagent A was added, and kept for incubation at 30° C for 10 min. After incubation 0.2 ml of Griess reagent B was added and kept for incubation at 30° C for 20 min. After incubation, absorbance was measured at 542 nm against blank. Concentration of $NO^{\bullet}$ was calculated from standard calibration curve.

RESULTS AND DISCUSSION

It was observed that, *Euphorbia hirta* Linn and standard drug significantly inhibit the production of Malondialdehyde (MDA) and showed the antioxidant activity (Fig. I). *Euphorbia hirta* Linn showed percentage inhibition of 32.47%, 40.19%, 46.33%, 64.95% and 81.78% respectively with IC_{50} value of 44.84 where as IC_{50} values for ascorbic acid was 51.36 respectively (Table I).

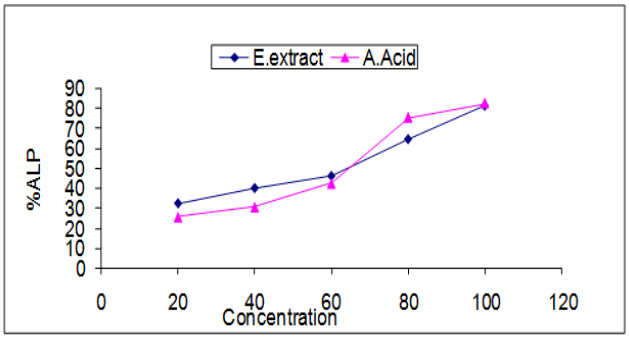

Fig I: Percentage ALP vs. Concentration plot for Ethanolic Extract of *Euphorbia hirta* Linn and Ascorbic acid by anti lipid peroxidation method

The results showed that *Euphorbia hirta* Linn has concentration dependant scavenging activity against DPPH free radicals. *Euphorbia hirta* Linn at the concentration of 10, 20, 40, 60 and 80 µg/ml showed concentration dependant reduction in absorbance and percentage inhibition of 15.18%, 24.82%, 55.18%, 66.75% and 73.98% respectively (Table II) which is comparable to standard drug ascorbic acid at the same concentration range (Fig II).

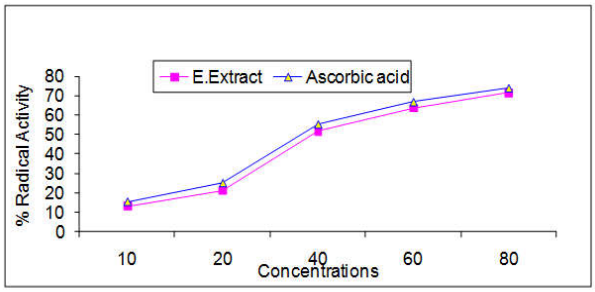

Fig. II: Percentage Radical Activity vs Concentration plot for Ethanolic Extract of Euphorbia hirta Linn and Ascorbic acid by DPPH radical scavenging activity

It was observed that absorbance of the test sample and standard sample was increased with increase in concentration of test and standard. It shows significant reducing capabilities of test sample (Fig. III). So, *Euphorbia hirta* Linn showed concentration dependant reducing capacity (Table III).

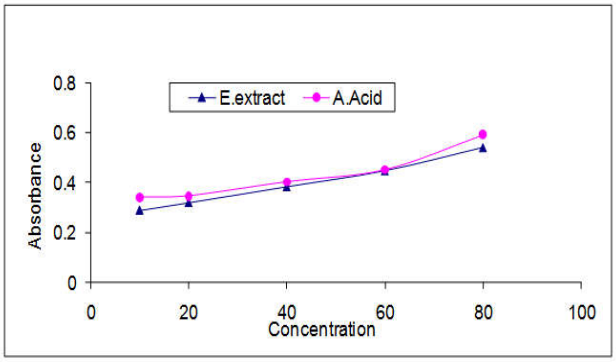

Fig. III: Absorbance vs Concentration plot for Ethanolic Extract of *Euphorbia hirta* Linn and Ascorbic acid by reducing power assay

The results showed concentration dependant decrease in absorbance of test and standard sample. *Euphorbia hirta* Linn at the concentration of 10, 20, 40, 60 and 80 µg/ml showed percentage inhibition of 22.61%, 30.38%, 46.56%, 58.76% and 75.61% respectively (Table IV) which is comparable to standard drug Ascorbic acid at the same concentration range (Fig. IV). So, *Euphorbia hirta* Linn has concentration dependant inhibition of nitric oxide free radicals.

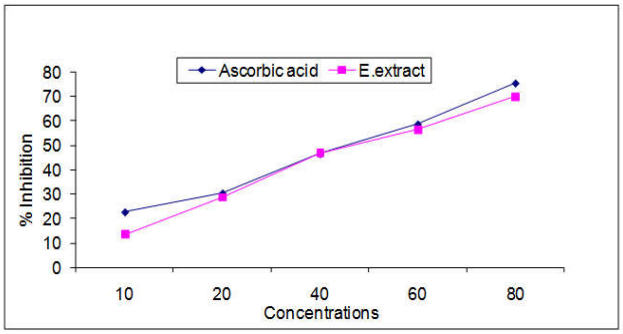

Fig. IV: Percentage inhibition vs Concentration plot for Ethanolic Extract of *Euphorbia hirta* Linn and Ascorbic acid by Nitric oxide free radical scavenging method

Table I:-Anti Lipid peroxidation effect of Ethanolic extract of *Euphorbia hirta* Linn.

Compound	Conc. (µg/ml)	Absorbance			Mean± SEM	% Inhibition	IC_{50} µg/ml
		I	II	III			
Ethanolic Extract	20	0.363	0.367	0.358	0.363±0.002	32.47	44.84
	40	0.321	0.327	0.325	0.324±0.002	40.19	
	60	0.292	0.295	0.293	0.293±0.001	46.33	
	80	0.198	0.201	0.197	0.199±0.001	64.95	
	100	0.111	0.113	0.117	0.114±0.002	81.78	
Ascorbic Acid	20	0.400	0.397	0.398	0.398±0.001	25.54	51.36
	40	0.374	0.371	0.375	0.373±0.001	30.49	
	60	0.313	0.308	0.317	0.313±0.003	42.37	
	80	0.144	0.148	0.142	0.145±0.002	75.64	
	100	0.133	0.107	0.108	0.109±0.002	82.77	

Table II: Absorbance, % inhibition and IC_{50} values of standard solution and ethanolic extract in DPPH Radical Scavenging Activity

Compound	Conc. (µg/ml)	Absorbance			Mean± SEM	% Inhibition	IC_{50} µg/ml
		I	II	III			
Ethanolic Extract	10	0.361	0.358	0.367	0.362±0.003	12.77	
	20	0.328	0.331	0.329	0.329±0.001	20.72	
	40	0.201	0.198	0.206	0.202±0.002	51.33	51.90
	60	0.157	0.148	0.151	0.152±0.003	63.37	
	80	0.118	0.123	0.115	0.119±0.002	71.32	
Ascorbic Acid	10	0.352	0.348	0.356	0.352±0.002	15.18	
	20	0.316	0.311	0.309	0.312±0.002	24.82	55.63
	40	0.184	0.187	0.188	0.186±0.001	55.18	
	60	0.134	0.138	0.142	0.138±0.002	66.75	
	80	0.106	0.111	0.108	0.108±0.001	73.98	

Table III: Absorbance, % inhibition and IC_{50} values of standard solution and ethanolic extract in reducing power assay Activity

Compound	Conc. (µg/ml)	Absorbance			Mean± SEM
		I	II	III	
Ethanolic Extract	10	0.283	0.296	0.294	0.291±0.0040
	20	0.321	0.320	0.324	0.322±0.0012
	40	0.388	0.382	0.388	0.386±0.0020
	60	0.458	0.443	0.450	0.450±0.0043
	80	0.538	0.548	0.543	0.543±0. 0028
Ascorbic Acid	10	0.343	0.339	0.346	0.343±0.002
	20	0.346	0.340	0.355	0.347±0.004
	40	0.405	0.404	0.403	0.404±0.001
	60	0.458	0.451	0.451	0453. ±0.002
	80	0.612	0.577	0.594	0.594±0.010

Table IV: Absorbance, % inhibition and IC_{50} values of standard solution and ethanolic extract in nitric oxide scavenging activity

Compound	Conc. (µg/ml)	Absorbance			Mean± SEM	% Inhibition	IC_{50} µg/ml
		I	II	III			
Ethanolic Extract	10	0.398	0.386	0.388	0.391±0.004	13.30	
	20	0.324	0.319	0.322	0.322±0.002	28.60	
	40	0.237	0.242	0.242	0.240±0.002	46.78	50.74
	60	0.195	0.194	0.198	0.196±0.001	56.54	
	80	0.131	0.137	0.138	0.135±0. 002	70.06	
Ascorbic Acid	10	0.342	0.354	0.351	0.349±0.004	22.61	
	20	0.312	0.316	0.315	0.314±0.001	30.38	54.36
	40	0.242	0.238	0.243	0.241±0.002	46.56	
	60	0.188	0.187	0.183	0.186±0.002	58.76	
	80	0.109	0.112	0.108	0.110±0. 001	75.61	

Conclusion

Reactive oxygen species (ROS) play a relevant role in the etiology and pathogenesis of diabetes mellitus and its complications. In the present study, the percentage inhibition of peroxide formation was increased in a dose dependant manner. The ethanolic extract showed promising free radical scavenging effect of DPPH in a concentration dependant manner up to a concentration of 80 µg/ml. The reference standard ascorbic acid also demonstrated a significant radical scavenging potential. The reductive ability was also found to be increasing in a dose dependant manner, with ethanolic extract showing the maximum absorbance. The *Euphorbia hirta* extract showed significant free radical scavenging action

against nitric oxide (NO) induced release of free radicals at the concentration from 10 µg/ ml to 80 µg / ml.

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