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Hepatoprotective and antioxidant activity of *Euphorbia antiquorum*

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ABSTRACT

Aqueous extract of the aerial parts of *Euphorbia antiquorum* Linn. was evaluated for its hepatoprotective and antioxidant activity to validate its use in traditional therapeutic indications. This extract exerted significant antioxidant activity (at 20μg, 40μg, 60μg, 80μg and 100μg/ml in-vitro) as evidenced by its reducing power, hydroxyl and superoxide anion radical scavenging activities. This *in vitro* efficacy was reinforced by a significant dose dependent hepatoprotection (at 125 mg/kg and 250mg/kg dose) by decreasing the activity of serum enzymes, bilirubin, cholesterol, triglycerides and lipid peroxidation while it significantly increased the reduced Glutathione levels of tissue in a dose dependant manner. The Hepatoprotective and Antioxidant activities of the extract are being comparable to standards Silymarin and Sodium metabisulphite respectively. The results obtained in the present study indicate that *Euphorbia antiquorum* is a potential source of natural hepatoprotectives and antioxidants. The hepatoprotective property may be attributed to the antioxidant potential and the antioxidant principles of the plant. The present study justifies the claim of the native practitioner that the decoction of the plant is useful in treating jaundice.

KEY WORDS : Antioxidant activity, CCl_4 *Euphorbia antiquorum*, Hepatoprotectivity,.

INTRODUCTION

Hepatic system is very vital organ system involved in the body's metabolic activities. As a result the chemical reactions in the liver may generate several reactive species like free radicals. These reactive species form covalent bond with the lipids of the tissue. However inbuilt protective mechanisms combat the hazardous reactions associated with the free radicals. Due to excessive exposure to hazardous chemicals, the free radicals generated will be so high such that they overpower the natural defensive system leading to hepatic damage and cause jaundice, cirrhosis and fatty liver, which remain one of the serious health problems. Carbontetrachloride (CCl_4) is one such hazardous chemical which induces hepatopathy through membrane lipid peroxidation by its free radical derivative, ($CCl_3^{\cdot}$, $CCl_3O_2^{\cdot}$). Excessive production of the reactive species manifests in tissue-thiol depletion, lipid peroxidation, plasma membrane damage etc., culminating into severe hepatic injury [1]. In the background of the above, it is realized that antioxidant activity or inhibition of generation of free radicals plays a crucial role in providing protection against such hepatic damage.

Several herbs and herbal products are known to possess antioxidant principles and may be useful as organ protective agents. Herbs belonging to Euphorbiaceae are reported have antioxidant principles like flavonoids and shown organ protective properties [2, 3]. *Euphorbia antiquorum* is a large shrub or small tree growing throughout the hotter parts of India and Ceylon. The juice of the plant is acrid, anti-inflammatory, purgative and is useful in rheumatism, dropsy, gout, neuropathy, deafness, cough [4]. The juice, which flows from the branches is a popular application to warts and other cutaneous affections [5]. Isolated compounds from the plant include euphol, antiquol, euphorbol, isohelinol, camelliol [2,6]. Preliminary phytochemical investigation showed the presence of triterpenes & flavonoids, both of which are reported to possess hepatoprotective and antioxidant activity [7,8]. Similarly there were claims from a local native practitioner that the decoction of the test plant is highly useful in treating jaundice. Since the

pharmacological profile of the plant is not completely established. Therefore this plant is taken for the present study. With this scientific information, the present study was designed with an aim to assess the antioxidant and hepatoprotective activity of the aqueous extract of aerial parts of *Euphorbia antiquorum* Linn. (EA), against CCl_4 induced liver damage.

MATERIALS AND METHODS

Plant material

The aerial parts of EA was collected from the out fields of Harapanahalli, India in the month of September/October 2005 and authenticated by Prof. K. Prabhu, Department of Pharmacognosy, SCSCP. A voucher specimen is currently deposited in the Department of Pharmacognosy (SCSCP – PH – 01/2007).

Extract preparation

Aerial parts of EA was shade dried, ground to coarse powder and subjected to successive extraction by using different solvents in the increasing order of their polarity (pet ether, $CHCl_3$ and methanol) in soxhlet apparatus, until the eluent became colourless and then macerated with chloroform water. The aqueous extract was dried under reduced pressure at a yield of 15% (*w*/*w*). From this extract, on evaporation of water *in vacuum*, a brown coloured substance was obtained which was kept at 4° C until use.

Preliminary phytochemical investigation

All the extracts were subjected to preliminary phytochemical tests [9]. All the tests reveal that the plant possesses steroids, glycosides, triterpenoids, tannins and flavonoids. Since aqueous extract has shown the better results for the presence of polyphenolic compounds and triterpenoids, this extract was selected for further study.

Animals

Adult wistar rats (180-220 g) and swiss albino mice (18-22g) were used in this study. They were housed in well-ventilated rooms under standard conditions (23 ± 2° C, humidity 65-70 %, 12 h light / dark cycle), fed with standard rodent pellet diet (Lipton India Ltd. Mumbai) and with tap water *ad. Libitum*. Albino mice are used for acute toxicity studies and rats for Hepatoprotective activity. Approval from the institutional animals ethical committee was obtained prior to the beginning of the study.

Reducing power

The reducing power was determined according to the method of Oyaizu [10]. Different doses of EA extract (20-100µg) were mixed in 1ml of distilled water. This was mixed with phosphate buffer (2.5ml, 0.2M, pH6.6) and potassium ferricyanide (2.5ml, 1%). This mixture was incubated at 50°C for 20 minutes. A portion (2.5ml) of trichloroacetic acid (TCA) (10%) was added to the mixture, which was then centrifuged at 3000rpm for 10 minutes. The upper layer of the solution (2.5ml) was mixed with distilled H_2O (2.5ml) and $FeCl_3$ (0.5ml, 0.1%) and the absorbance was measured at 700nm (Elico SL 164 double beam UV spectometer).

Superoxide-anion scavenging activity

Measurement of superoxide anion scavenging activity was done based on the method described by Nishimiki [11] and slightly modified.

About 1ml of Nitroblue tetrazonium solution (156µM NBT in 100mM phosphate buffer, pH 7.4), 1ml NADH solution (468µm in 100mM phosphate buffer, pH 7.4) and 0.1ml of sample solution of EA in water were mixed. The reaction is started by adding 100ml of Phenazinemethosulphate (PMS) solution (60M) in 100mM phosphate buffer, (pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5minutes and the absorbance at 560nm (Elico SL 164 double beam UV spectometer) was measured against blank samples.

Hydroxyl radical scavenging activity

Hydroxyl radical generation by Phenylhydrazine has been measured by the 2-deoxyribose degradation [12]. In 50mM phosphate buffer (pH 7.4) 1mM Deoxyribose, 0.2mM Phenylhydrazine hydrochloride and the extract at different doses (20-100µg) were added in a total volume of 1.6ml. Incubation was terminated after lhour or 4hour and 1ml each of 2.8% TCA and 1% (W/V) thiobarbutric acid (TBA) were added to the reaction mixture & heated for 10minutes, in a boiling water bath. The tubes were then cooled briefly and absorbance taken at 532nm (Elico SL 164 double beam UV spectometer) *Acute Toxicity Study*

The test extract (Aqueous extract) was screened for acute toxicity as per CPCSEA guideline No 420, i.e. fixed dose method in albino mice. Since, none of the animals died at 2500 mg/kg dose, $1/20^{th}$ (125mg/kg) and $1/10^{th}$ (250mg/kg) of this cut off dose were selected for further studies.

Carbon tetra - chloride induced toxicity

The method of Ko et al [13] was used for screening the hepatoprotectivity of the test extract. The animals were randomly assigned into 5 groups of 6 animals. Group I and II served as normal & intoxicated control and received only the vehicle (normal saline). Group III served as standard, was treated with Silymarin (100 mg / kg / day BW for 3 days) The animals of Group IV and V received EA extract (125 mg/kg BW and 250mg / kg

BW respectively) for 3 days. Twenty-four hours after the last dosing, animals (except Group 1) were treated orally with CCl_4 (11 % *v/v* in olive oil) at a dose of 1 ml / kg BW. Animals were sacrificed 24 hrs, after CCl_4 treatment, hepatic tissue and heparinized blood sample were taken and assessed for serum enzyme and Glutathione estimation.

Serum enzymes, which were assessed, include Serum gluataic oxaloacetate transaminase (SGOT) and Serum glutamic pyruvic tranaminase (SGPT) [14], Total Bilirubin and Direct Billirubin [15], Cholesterol, Triglycerides and Alkaline phosphate (ALP) content.

Tissue Glutathione measurements were performed using a modification of the Ellman procedure [16, 17]. Tissue samples were homogenized in ice cold TCA (lgm tissue plus 10 ml 10 % TCA) in a homogeniser. Briefly after centrifugation at 3000 rpm for 10 minutes, 0.5 ml supernatant was added to 2 ml of 0.3 M Disodium hydrogen phosphate solution. A 0.2ml solution of DTNB (5,5 Dithio-bis 2- nitrobenzoic acid) (0.4 mg in 1 ml of 1 % Sodium nitrate) was added and the absorbance at 412 nm was measured immediately after mixing.

Extent of lipid peroxidation was done by combining 1.0ml of biological sample (0.1 – 2.0 mg of membrane protein or 0.1 – 0.2 μmol of lipid phosphate) with 2.0 ml of TCA-TBA-HCl and thoroughly mixed. The solution was heated for 15min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000rpm for 10min. The absorbance of the sample is determined at 535nm against blank that contains all the reagents without the lipid [18].

Statistical analysis

Data were analyzed by ANOVA followed by Bonferroni's multiple variance test. Results with $P<0.05$ were considered statistically significant.

RESULTS

In vitro Antioxidant Activity

Reducing power

Table I shows the dose dependant increase in the % of absorbance, indicating that the EA possesses dose dependant reducing power. All the doses of EA demonstrated greater absorbance than control and the difference were found to be significant ($P<0.01$).

Superoxide anion scavenging activity

In the PMS / NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS / NADH coupling reaction, reduces NBT. The decrease in absorbance at 560nm with EA thus indicates the consumption of superoxide anion radical in the reaction mixture. 100μg of the sample possess 73% of inhibition as compared to the standard Sodium Metabisulphite (25μg) which showed 86% inhibition / scavenging activity.

Hydroxyl radical scavenging activity

Results presented indicate that phenylhydrazine in solution generates OH* radicals as measured by 2-deoxy ribose degradation. It's found that 100μg (This is a in vitro study and varying doses have been studied. The activity at 100 μg is given here whereas activity at lower doses are recorded in the table.explain the rationale behind selecting this dose in the methods section) of aqueous extract of EA and 25μg of Sodium metabisulphate scavenge the OH* radicals and inhibit the production of TBA reactive material significantly over period 1hour (Table II).

Hepatoprotectivity

The estimated values of serum GOT, GPT, ALP, Cholesterol, Triglycerides, Total Bilirubin and Direct bilirubin values in control (saline + vehicle) group of rats were found to be 102 ± 5.48, 51 ± 1.87, 198 ± 3.59, 111 ± 2.21, 221 ± 7.88, 0.923 ± 0.012, 0.235 ± 0.010 respectively (Table III).

A remarkable elevation was observed in serum GOT, GPT, ALP, cholesterol, Triglycerides, Total bilirubin and Direct bilirubin values in CCl_4 intoxicated rats (Toxic Control group). In the groups treated with 125mg/kg and 250mg/kg of the (EA) extract, the above biochemical markers of hepatotoxicity were found to be decreased when compared to CCl_4 treated control group. Evidently, the hepatoprotective effects of higher dose of EA (250mg/kg) were near to that of standard i.e. Silymarin (100mg/kg). Both the doses of EA used in the study showed significant protective property than control. However the test extract was found to be less potent than that of standard drug.

The tissue glutathione was found to be depleted upon CCl_4 intoxication, indicate that the tissue damage is due to over powering the inbuilt free radical scavenger mechanisms. This tissue GSH depletion was inhibited by the pretreatment with test extract in a dose dependant manner. Similarly lipid peroxidation induced by CCl_4 treatment was reversed by test extract in a dose dependant manner. The results are compiled in table IV.

DISCUSSION

Since the extract has demonstrated dose dependant anti-oxidant activity in all the models of the study, the aqueous extract was taken for assessing the *in vivo* hepatoprotective properties. Pretreatment with the test extract has reduced the elevated levels of biochemical markers of hepatoxicity. Further it was also observed that the tissue GSH depletion due to CCl_4

Table-I: Reducing power of aqueous extract of aerial parts of EA doses and SMS (n=3) and superoxide anion scavenging activity by PMS / NaOH-NBT method (n= 03).

Treatment	Dose (µg/ml)	Reducing Property (Abs)	Increase (%)	PMS-NaOH System (Abs)	Inhibition (%)
Control	-	0.229 ± 0.0012	-	0.863 ± 0.0076	-
SMS	25	0.417± 0.0105*	82	0.121± 0.0012*	86
EA	20	0.310± 0.0032*	26	0.614± 0.0116*	29
EA	40	0.329± 0.0070*	44	0.519± 0.0012*	40
EA	60	0.357 ± 0.0037*	56	0.423± 0.0016*	51
EA	80	0.375±0.0010*	64	0.348±0.0081*	60
EA	100	0.396±0.112*	73	0.234±0.01123*	73

* *P – Value <0.001 Vs control group, Bonferronis test. ; SMS : Sodium metabisulphate.*

Table II : Hydroxyl radical scavenging activity of aqueous extract of aerial parts of EA by 2-deoxyribose degradation assay (n= 03).

Incubation system	Hydroxyl Determination By 2-deoxyribosedegradation Assay (Abs 532 nm)	
	Abs	Inhibition (%)
2-deoxyribose/ phenylhydrazine	0.448 ± 0.0035	-
2-deoxyribose/ phenylhydrazine / SMS 25µg/ml	0.088 ± 0.0076*	80
2-deoxyribose/ phenylhydrazine / EA 20µg/ml	0.335 ± 0.0133*	25
2-deoxyribose/ phenylhydrazine / EA 40µg/ml	0.277 ± 0.0026*	38
2-deoxyribose/ phenylhydrazine / EA 60µg/ml	0.215 ± 0.0050*	52
2-deoxyribose/ phenylhydrazine / EA 80µg/ml	0.150 ±0.0087*	67
2-deoxyribose/ phenylhydrazine / EA 100µg/ml	0.121 ±0.0038*	73

* *P-Value <0.001 Vs 2-deoxyribose/ phenylhydrazine treated, Bonferroni's test. ; SMS: Sodium metabisulphate.*

Table No III : Effect of aqueous extract of the aerial parts of EA and CCl_4-induced hepatoxicity (n=6)

Groups	SGOT levels (U/L) Mean ± SE	SGPT levels (U/L) Mean ± SE	ALP (mg/dl) Mean ± SE	Cholesterol (mg/dl) Mean ± SE	Triglycerides (mg/dl) Mean ± SE	Total Bilirubin (mg/dl) Mean ± SE	Direct Bilirubin (mg/dl) Mean ± SE
Group 1	102 ± 5.48	51 ± 1.87	198±3.59	111±2.21	221±7.88	0.923±0.012	0.235±0.010
Group 2	355 ± 4.95	167 ±3.69	1036±6.32	179±2.27	766±4.57	5.036±0.035	1.84±0.056
Group 3	183 ± 4.93*	88 ±2.46*	483±2.52*	121±1.69*	463±2.03*	1.838±0.026*	0.248±0.006*
Group 4	304 ± 2.85*	145 ±2.27*	962±4.56*	153±2.17*	693±2.97*	3.490±0.032*	0.632±0.0025*
Group 5	257 ± 2.32*	103 ±2.81*	710±4.01*	135±1.77*	574±4.03*	2.289±0.024*	0.393±0.010*

*Values are the Mean ± SEM of six rats/ treatment. ; *→ P<0.001 Vs CCl_4 treated group (group 2), Bonferroni's test.*
Group 1-Normal animals (untreated) ; Group 2-CCl_4 (1ml/kg BW) treated animals; Group 3-CCl_4 + Silymarin (100mg/kg BW) treated animals. ; Group 4-CCl_4 + EA (125mg/kg BW) treated animals. ; Group 5-CCl_4 + EA (250mg/kg BW) treated animals.

Table IV : Effect of aqueous extract of aerial parts of EA on hepatic GSH status in rats (n=6) and carbon tetrachloride-induced peroxidation in rats (n=6).

Treatment	Dose (mg/Kg BW)	GHS (Abs412)	Increase (%)	Lipid Peroxidation (Abs 543)	Increase (%)
Normal Saline	-	0.799±0.001	-	0.099±0.001	-
Normal Saline-CCl_4	-	0.239±0.015	-	0.292±0.008	-
Sylamarin- CCl_4	100	0.445±0.006*	86	0.088±0.003*	70
EA-CCl_4	125	0.337±0.004*	41	0.196±0.017*	33
EA-CCl_4	250	0.410±0.003*	72	0.114±0.010*	61

** → P – Value <0.001 Vs normal saline – CCl_4 treated, Bonferroni's test.*

challenge was reversed by the test extract and also reduced the extent of lipid peroxidation.

Most of the mammals have an effective mechanism to prevent and neutralize the free radical induced damage, which is accomplished by a set of endogeneous substances such as superoxide desmutase (SOD) catalase, glutathione peroxidase($GP_{x/}$) and glutathione reductase (GR) .Here, in present study *in vitro* antioxidant acvities shown significant increase in the absorption in reducing power and reduction in absorption in hydroxyl ion and superoxide anion scavenging activities, indicating that the study plant possesses antioxidant activities. In biochemical system, superoxide radical and H_2O_2 react together to form the hydroxyl radical, this can attack and destroy almost all known biochemicals [19]. The hydroxyl radicals thus produced may attack the sugar of DNA base causing sugar fragmentation, base loss and DNA stand leakage [13]. EA extract reduced the super oxide anions and also scavenge off the hydroxyl radicals and hence, inhibit the cellular damage. It is apparent from the present study that the test extract doest not interfere with the generation of the free radicals but it scavenges off the free radicals.

CCl_4 undergo hepatic metabolism to give rise to trichloro methyl radicals, which upon reacting with reactive oxygen species yields trichloromethyl peroxide radicals, which forms covalent bond with membrane lipids and destroy the membrane integrity. The observation of increased malonaldehyde(MDA) formation in hepatic cells after CCl_4 challenge is in accordance with the earlier report, which suggests involvement of trichloromethyl and trichloromethylperoxy radicals in the propagation of peroxidation process [20]. The pretreatment with extract has prevented oxygen free radicals and thereby prevented the formation of peroxy radicals. This aspect of test extract also contributes to the hepatoprotectivity.

Thus, from the results of the present investigation it may be concluded that the aqueous extract of the aerial parts of EA possess significant hepatoprotective and antioxidant activity. It appears that hepatoprotective activity of the test extract is due to its antioxidant potential. The antioxidant potential may be attributed to the presence of polyphenolic compounds. Further studies like isolation and characterization of the active principal(s) responsible for such activity are needed to confirm. However the present study justifies the claim of the native practitioner that the plant is useful in treating jaundice.

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