ANTI-INFLAMMATORY AND HEPATOPROTECTIVE ACTIVITIES OF ETHANOLIC EXTRACT OF *EUPHORBIA THYMIFOLIA* LINN.

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Summary

Euphorbia thymifolia Linn. (Euphorbiaceae) is a small prostate, hispidly pubescent, annual weed, which is commonly found in India and tropical countries.The plant is reported to be used in traditional medicine for the treatment of diarrhea, jaundice, pain etc. The aim of the present study was to evaluate the anti-inflammatory and hepatoprotective activities of ethanolic extract of whole plant. The anti-inflammatory effect was evaluated by carrageenan and cotton pellet induced rat models and hepatoprotective activity was accessed on CCl_4-induced liver damage in rats. A dose dependent anti-edematogenic activity of ethanolic extract of *E. thymifolia* (dose of 200 mg/kg, p.o.) was observed and was comparable to Ibuprofen (dose of 50 mg/kg, p.o.; $p < 0.01$) used as standard. The extract of the plant, screened for its hepatoprotective activity in CCl_4 (0.5 ml/kg, p.o.) induced liver damage in rats at a dose of 100 mg/kg, p.o. The extract significantly ($P < 0.01$) normalized the serum enzymes alanine amino transferase (ALT), asparate amino transferase (AST), and lipid peroxidation (LPO) elevated by CCl_4-toxicity, which was also supported by the histological examination of liver tissues. The hepatoprotective effect of the extract was comparable to that of silymarin (50 mg/kg, p.o.) used as reference standard. The preliminary phytochemical screening of the ethanolic extract showed the presence of phenolics, terpenes and flavonoids. Thus, the results validate the use of *E. thymifolia* in traditional medicines for the treatment of jaundice and inflammation related disorders.

Keywords: Anti-inflammatory, Euphorbia thymifolia, Euphorbiaceae, Hepatoprotective

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Introduction

Euphorbia thymifolia Linn. (Euphorbiaceae), also known as *Chamaesyce thymifolia* L., is a small prostate, hispidly pubescent, annual weed with horizontally spreading branches occurring in red and green forms. It is widely distributed in India and throughout tropics. This plant is reported to have antibacterial [1-2], anti-fungal [3-4], antioxidant [5-6], antiviral [5,7-8]. It is also used for astringent actions, as stimulant, in worm infection [9], pain and jaundice [10-11]. The presence of polyphenolics [12], flavonoids [7] and alkaloids [1] has been reported from *Euphorbia thymifolia* Linn. The plants of genus *Euphorbia* contain diversified classes of compounds in both free and conjugated form viz. tannins, phenolics [13-14], flavonoid glycosides [15], terpenes [16-19] and steroids [20]. Plants of genus *Euphorbia* are reported to be used as purgative, anthelmintic, antianaphylactic [21] anti-arthritic [22], antidiarrhoeal [23], anti-inflammatory [24-25], in warts treatment, antiasthmatic [26], astringent, narcotic, diuretic [27], and immunosuppressive properties [28]. The genus *Euphorbia* is also reported for cytotoxic activity [16, 18, 29], antinociceptive activity [30], antidepressant [17] and antioxidant activities [5-6, 31]. Oxidation processes are important for living organisms, the reduced oxygen species mediate important physiological processes and may induce cellular damage including hepatotoxicity. A great deal of interest is being generated towards the bioactive polyphenolics from natural source to be used as dietary source of anti-oxidants. In the present study, the phenolics rich ethanolic extract of *E. thymifolia* was used to evaluate its anti-inflammatory and hepatoprotective activities, which was not reported earlier.

Materials and methods

Plant material, extraction and isolation

The whole plants of *Euphorbia thymifolia* were collected from Tamil Nadu and were authenticated by Prof. K.N. Dubey (Department of Botany, Banaras Hindu University, Varanasi, India). A voucher specimen (specimen No. PCRL 36) has been deposited in the Pharmacutical Chemistry Research Laboratory, Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi, India, for future reference. The shade-dried powder of whole plant was passed through sieve No. 40 & 600 g was extracted (soxhlation) with petroleum ether (60-80ºC) for 24 h and subsequently with ethanol (30 h). The extractives were concentrated *in vacuo* (total yield 11 % w/w). Qualitative determination of ethanolic extract was done for the presence of tannins, flavonoids, sterols, terpenes and alkaloids using standard methods [32].

Animals

Male albino rats (Charles foster strain) weighing 100-140 g were obtained from M/S Asian Fauna Store, Varanasi. Animals were randomly housed in groups of five in polypropylene cages at an ambient temperature with a 12 h light: 12 h dark cycle. The animals were allowed free access to laboratory diet (M/s Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. The animals were fasted overnight before the experiment. Experiments were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals [33].

Reagents and Chemicals

Lambda-carrageenan (Sigma, Type IV, Steinheim, Germany) and carbon tetrachloride (CCl_4) were purchased from Merck India Ltd, Mumbai. Silymarin was obtained from Ranbaxy Laboratories Limited, India. Span diagnostic kits were used for the spectrometric enzyme assays. All other chemicals used were of analytical grade.

Acute toxicity studies

The acute toxicity for all test compounds was carried out in albino rats weighing 100-140 g which were fasted overnight. The dosage was varied from 50-2000 mg/kg body weight. The animals

were observed for 24 h for any signs of acute toxicity such as increased/decreased motor activity, tremors, convulsion, sedation, lacrimation etc. No mortality of the animals was observed even after 24 h. Hence, the LD_{50} cut off value of the test compounds was fixed as 2000 mg/kg and $1/10^{th}$ of cut off value (i.e. 200 mg/kg) was taken as maximum screening dose for the evaluation of anti-inflammatory and hepatoprotective activity.

Anti-inflammatory activity

Carageenan- induced hind paw edema in rats

The inhibitory activity of the ethanolic extract of *E. thymifolia* on carrageenan-induced rat paw oedema was determined according to the method of Winter et al.[34]. The experimental animals were divided into five groups, each containing five animals. Group-I was marked as control (received an equal volume of distilled water), group-II as standard (received Ibuprofen, 50 mg/kg, p.o.) and groups III-V were given ethanolic extract of 50, 100 & 200 mg/kg p.o. respectively. After 30 min., all the groups were injected with carrageenan (0.1 ml, 1% w/v in normal saline) subcutaneously in the subplanter region of the left hind paw. The paw volume was measured by the difference of volume with mercury plethysmometer before and 1, 2 and 3 h after carrageenin injection. Percentage of inhibition of edema was determined for each animal by comparison with control and calculated by the following formula [35]:

$$\% \text{ Inhibition} = 1 - \left(\frac{dt}{dc}\right) \times 100$$

where d*t* is the difference in paw volume in the drug-treated group and d*c* the difference in paw volume in the control group.

Cotton pellet induced Granuloma

The method of Mossa et al. [36] was used for this study. This involves surgical insertion of sterilized cotton pellet (30 mg in weight) subcutaneously into the groin of rats using ether as an anaesthetic agent. Five groups of five rats in each group were included in the study. After shaving off fur, the animals were anaesthetized and administered the same doses of extracts, vehicle or Ibuprofen as in the carrageenan-induced rat paw oedema test.

After the surgical insertion, sterilized cotton pellet (30 mg) was implanted in the groin region of each rat. The extracts (50, 100 or 200 mg/kg), vehicle and Ibuprofen (50 mg/kg) were administered to respective groups of the animals for seven consecutive days. All the animals were sacrificed on the eighth day with an over dose of ether. The pellet and the surrounding granuloma were dissected out carefully, made free from extraneous tissues and dried overnight in an oven at 60ºC to a constant weight. The weight of the granuloma tissue was obtained

by determining the difference between the initial (30 mg) and the final weight of the cotton pellet with its attached granulomatous tissue. The mean weight of the granuloma tissue formed in each group and the percentage inhibition were determined.

Carbon tetrachloride-induced liver damage

The rats were randomly divided into four groups of five animals each. Group I was marked as control and given daily single dose of 1% of polyethyleneglycol (PEG) at a dose of 2 mL/kg, p.o. Group II -intoxicated with a single dose of CCl_4 (CCl_4: PEG in 1:1 ratio; 0.5 mL/kg, p.o.). Group III & IV received a daily single dose of silymarin (50 mg/kg in 1% of PEG, p.o.) and ethanolic extract in 1% PEG (100 mg/kg, p.o.) for 7 consecutive days respectively. Group III served as standard. All animals except control received CCl_4 on fifth day. Rats were sacrificed after 24 h of CCl_4 administration and blood was collected by cardiac puncture and was centrifuged at 2500 rpm for 15 min. to separate serum.The hepatoprotective activity was measured by determining serum enzyme concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lipid peroxidation (LPO) and liver was removed for the histopathology.

Histopathology

Histopathological analysis was carried out by fixing liver tissue in 10% buffered formalin saline, processed, embedded in paraffin wax to cut into 3 μm using microtome, and then stained with both haematoxylin and eosin. Observations were made under 400x magnification in light field microscope.

Statistical analysis

The mean ± standard error of the mean (S.E.M.) was determined for each parameter. The data was subjected to one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. The results were considered significant if $p < 0.05$.

Results

Preliminary phytochemical analysis showed the presence of tannins, flavonoids, and terpenes in the ethanolic extract of *E. thymifolia*.

Anti-inflammatory activity:

Carrageenan induced inflammation

In carrageenan induced animal models, the ethanolic extract of *E. thymifolia* at the doses of 50, 100, and 200 mg/kg (p.o.), inhibited the edema formation in first hour by 29.14% ($p < 0.05$), 29.43%, and 45.71% ($p < 0.01$) respectively in a dose dependent manner. The effect also extended and significantly increased in second and third hour ($p < 0.05$). The reference drug (Ibuprofen) group significantly inhibited the edema formation by 59.71%, 67.57% and 68.92% at first, second and third hour respectively ($p < 0.05$). The inhibition of paw edema produced by ethanolic extract (200 mg/kg, p.o.) in third hour was found to be comparable to that of Ibuprofen (50 mg/kg, p.o.; $p < 0.01$). Results are shown in Table 1.

Table1. Effects of ethanolic extract of *E. thymifolia* on carrageenan- induced edema in rats.

Groups	Dose (mg/kg, p.o.)	Mean increase in paw volume (mL) at time T (h)		
		T(1)	T (2)	T (3)
Control	-	0.700 ± 0.026 (-)	0.740 ± 0.023(-)	0.740 ± 0.023(-)
Ibuprofen	50	0.282 ± 0.127*** (59.71)	0.240 ± 0.094** (67.57)	0.230 ± 0.027** (68.92)
Ethanolic extract	50	0.496 ± 0.065*(29.14)	0.490 ± 0.098*(33.78)	0.460 ± 0.083* (37.84)
	100	0.494 ± 0.063** (29.43)	0.462 ± 0.061** (37.57)	0.390 ± 0.062** (47.30)
	200	0.380 ± 0.163** (45.71)	0.340 ± 0.126** (54.05)	0.310 ± 0.049** (58.11)

Values were expressed as mean ± S.E.M. (n= 5). *$p < 0.05$, **$p < 0.01$ are compared with the control group (ANOVA followed Dunnett's *t*-test.). Values given in parentheses represent Percentage of Inhibition.

Cotton pellet induced granuloma

In cotton pellet granuloma, the ethanolic extract of *E. thymifolia* inhibited the granuloma formation by 32.1%, 40.8%, and 45.4% ($p < 0.05$) at the doses 50, 100 and 200 mg/kg, respectively (Table 2).

Table 2. Effects of ethanolic extract of *E. thymifolia* on cotton pellet induced granuloma in rats

Groups	Dose (mg/kg, p.o.)	Mean increase in weight of pellets (mg)	% Inhibition
Control	-	64.5 ± 3.4	-
Ibuprofen	50	25.6 ± 2.1	60.3*
Ethanolic extract	50	43.8 ± 1.1	32.1*
	100	38.2 ± 1.2	40.8*
	200	35.2 ± 2.3	45.4*

Values were expressed as mean ± S.E.M. (n= 5).*$p < 0.05$ is compared with the control group (ANOVA followed Dunnett's *t*-test).

Hepatoprotective Activity:

The results of hepatoprotective activity of ethanolic extract (100 mg/kg, p.o.) in the CCl_4-induced liver damage are shown in Table 3. The CCl_4-intoxication significantly elevated the ALT, AST and LPO levels in the serum against the control group. The ethanolic extract & silamyrin (standard) treated groups showed significant fall in the elevated ALT, AST and LPO levels.

Table 3. Effect of ethanolic extract of *E. thymifolia* on the biochemical parameters of CCl_4-intoxicated rats (n = 5, mean ± S.E.M.)

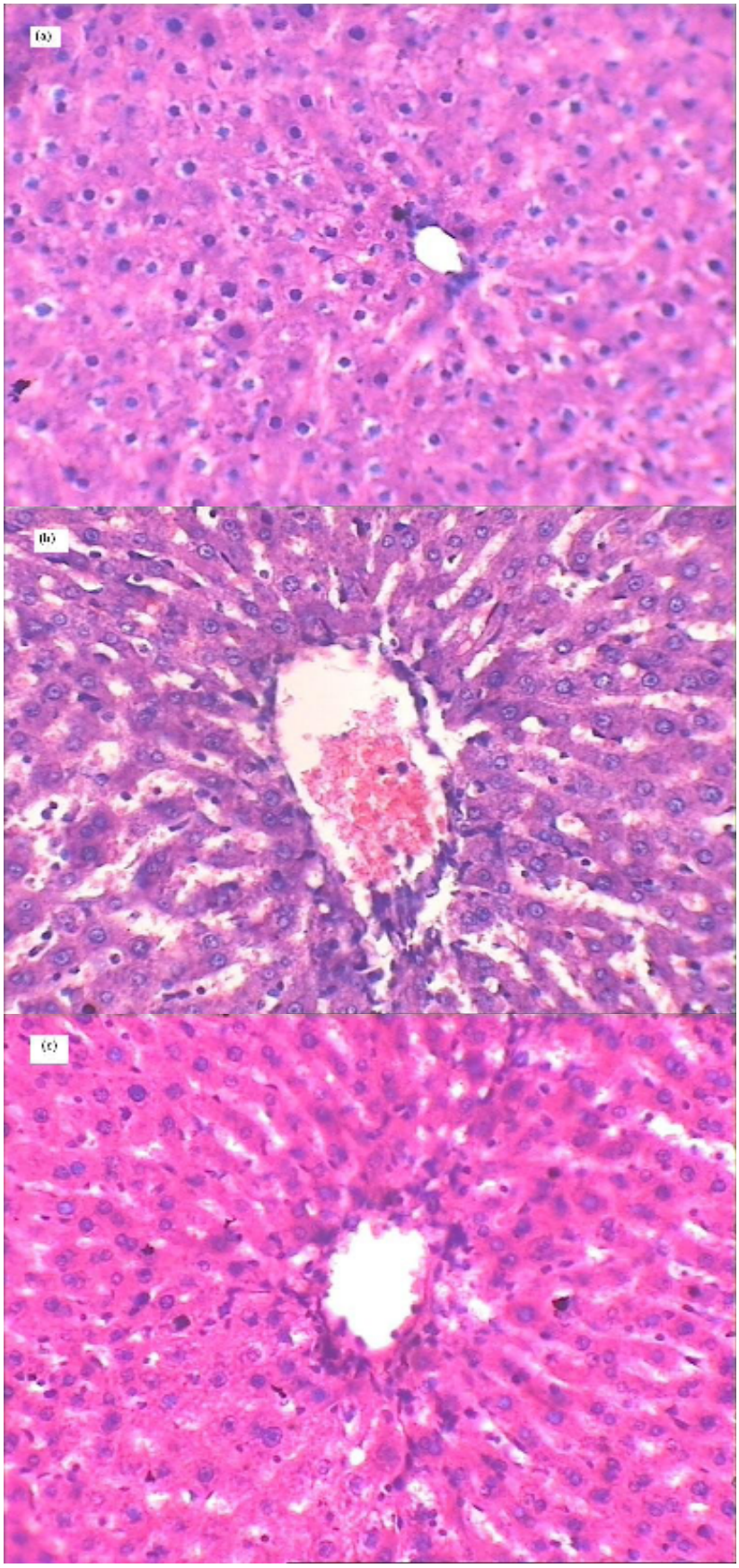
Groups	Dose	ALT (U/L)	AST (U/L)	LPO (% Inhibition)
Control	2.0 mL/kg, p.o	31.69 ± 1.13 [a, b]	23.09 ± 1.01 [a, b]	18.8 ± 1.96 [a, b]
CCl_4	0.5 mL/kg, p.o	160.04 ± 2.0 [b, c]	129.60 ± 6.42 [b, c]	85.65 ± 1.64 [b, c]
Std. + CCl_4	50 mg/kg, p.o	11.37 ± 1.92 [a, c]	75.35 ± 3.03 [a, c]	41.17 ± 4.86 [a, c]
Ethanolic extract + CCl_4	100 mg/kg, p.o.	131.21 ± 2.14[a,b,c]	97.14 ± 1.80 [a, b, c]	31.31 ± 1.90 [a, d]

Where a = $p < 0.01$ when compared with CCl_4 group; b = $p < 0.01$ when compared with standard, silymarin; c = $p < 0.01$ when compared with control group; d = $p < 0.05$ when compared with control group.

Histopathological examination:

Observations revealed that the CCl_4-intoxication brought critical changes in the normal architecture of rat liver as compared to liver of control group. A massive tissue necrosis, disruption of cytoplasm and cells boundary, and the difference in size of heapatocytes were observed in the CCl_4 treated rats. The ethanolic extract treatment normalized these changes in CCl_4-induced liver damage and the effect was comparable to that of the silymarin treated group used as standard.

Figure 1. Histopathology of (a) normal rat liver (b) CCl_4-intoxicated rat liver (c) ethanolic extract of *E. thymifolia* treated rat liver received equal amount of CCl_4 as in group II.



Discussion and conclusion

The study provides a pharmacological basis for the remedial usage of *E. thymifolia* in inflammation related disorders. Carrageenan induced edema in the hind paw (acute inflammation) and cotton pellet granuloma (chronic inflammation) are widely employed in screening the new antiinflammatory compounds [37]. These studies investigated the antiinflammatory effect of ethanolic extracts of *E. thymifolia* on acute and chronic phases of inflammation. The carrageenan-induced inflammatory process in the rat involves three phases: an initial, second and third phases caused by the release of histamine and serotonin; bradykinin and prostaglandins respectively [38-39]. In the present study the anti-inflammatory activity of ethanolic extract of *E. thymifolia* took place at 1, 2, and 3 h after carrageenan injection, suggesting that its action mechanism may involve multiple anti-inflammatory factors and mediators [40]. This is in consistent with the generally believed thinking that herbal preparations usually have multitargets.

The cotton pellet granuloma method has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation [41]. The dryweight of the implanted cotton pellet correlates well with the amount of granulomatous tissue formed [42]. *E. thymifolia* reduced the dryweights of implanted cotton pellets, indicating that it may inhibit the proliferative phases of inflammation. In this study, the ethanolic extract of *E. thymifolia* inhibited the granuloma formation compared to control group. This may be due to the ability of *E. thymifolia* in reducing the number of fibroblasts and synthesis of collagen and mucopolysaccharide, which are natural proliferative agents of granulation tissue formation.

These studies suggest that the probable anti-inflammatory activity of *E. thymifolia* may be due to its flavonoids (qurecetin and its derivatives) and hydrolysable tannin constituents (viz. corilagin, 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose, gerannin), and their effects on these mediators [43-47]. Further, ethanolic extract of *E. thymifolia* showed ameliorative effect on liver functions, as reflected by histopathological examination and the reduced level of serum enzymes viz. ALT, AST and LPO in CCl_4 damaged liver. Carbon tetrachloride causes hepatic damage due to free radical CCl_3•, formed from CCl_4 by the activation of the NADPH-Cyt. P450 system of liver endoplasmic reticulam [48]. This leads to functional and morphological changes in the cell membrane and results in leakage of hepatic enzymes and thus increases blood serum transaminases and phosphatase activity [49-50]. This cellular necrosis is mediated by lipid peroxidation of membrane lipids due to CCl_3• radicals and resulting in release of peroxides [51]. Therefore, the hepatoprotection may be due to marked antioxidant and inhibitory lipid peroxidation (LPO) activity of *E. thymifolia* [5-6] and also strong inhibitory effect of hydrolysable tannins on ADP plus NADPH-induced lipid peroxidation in microsome [52-53].

Acknowledgements

SKB, TP, B K and HSC are thankful to UGC, New Delhi, India, for the award of Research Fellowship.

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