Original Research Article

Hepatoprotective and antioxidant activity of *Enicostemma littorale* against CCl_4 induced liver damage in rats

Abstract:

Objective

To investigate the hepatoprotective and antioxidant effects of ethanol extract of *Enicostemma littorale* Blume (Ens) against CCl_4 induced hepatic injury in albino rats.

Methods

Male albino rats of six numbers in each group were undertaken for study. Hepatoprotective and antioxidant effect of *E. littorale* Blume (Ens) ethanol extract at a dosage of 100 & 200 mg/kg body weight was evaluated.

Results

The degree of hepatoprotection was assessed by measuring the activity levels of the marker enzymes such as serum aspartate transaminase (AST), alanine transaminase (ALT) alkaline phosphatase (ALP) , acid phosphatase (ACP) and total bilurubin. Free radicals generated lipid per oxidation was assessed by measuring the activity levels of the tissue antioxidant enzymes such as glutathione peroxidase,(GPX) catalase (CAT), superoxide dismutase (SOD). The CCl_4 administered rats recorded elevated activity levels of serum AST,ALT,ALP and ACP revealing CCl_4 induced hepatotoxicity. In the groups treated with 100mg/kg and 200mg/kg of the extract, the above bio-chemical markers of hepatotoxicity were found to be decreased when compared to CCl_4 treated control group. Both the doses of EEEL used in the study showed significant protective property than control. (*$p<0.01$, **$p<0.001$ vs. control)..In the groups treated with 100mg/kg and 200mg/kg of the extract, GPX, SOD and catalase were found to be increased when compared to CCl_4 treated control group.($p<0.01$ vs control).

Conclusions

It can be concluded that the ethanol extract of *E. littorale* Blume is not only hepatoprotective but also possess significant antioxidant property.

Keywords: Hepatoprotective, Antioxidant enzymes, *Enicostemma littorale* Blume,

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. Carbon tetrachloride (CCl_4) is one such hazardous chemical which induces hepatopathy through membrane lipid per oxidation by its free radical derivative, (CCl_3 ·, CCl_3O_2 ·). Excessive production of the reactive species manifests in tissue-thiol depletion, lipid per oxidation, plasma membrane damage etc., culminating into severe hepatic injury [1]. Much of the cell damage that occurs during liver degeneration is believed to be caused by free radicals, highly reactive oxygen species liberated during alcohol metabolism. These radicals react with cell membrane and induce lipid peroxidation, which has been implicated as important pathological mediation[2] in many clinical disorders such as heart disease, diabetes, cancer and liver disease. The management of liver diseases is still a challenge to the modern medicine. 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. Plant drugs are frequently considered to be less toxic and free from side effects.[3] Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices as well as in traditional systems of medicine in India. There are a number of evidences indicating that natural substances from edible and medicinal plants exhibit strong antioxidative activity, and could work against hepatic toxicity caused by various toxicants [4-5] *Enicostemma littorale*(*E. littorale*) Blume (Family: Gentianaceae) is a perennial, tropical traditional medicinal herb with sessile lanceolate leaves, flowers arranged in clusters, fruit in a capsule. The plant is locally used for its medicinal properties in Tamilnadu, Kerala India, such as antiinflammatory, antiulcer activity[6], hypoglycaemic[7], and antimalarial activities[8]. The present study aims to investigate the hepatoprotective and antioxidant effects of *E. littorale* on CCl_4 induced hepatotoxicity in male albino rats.

Materials and methods:

Plant material:

The whole plant of *E. littorale* were collected during January (2018) in and around Palakkad District, Kerala, India. The plant were cleaned, shade dried, authenticated by Dr Jayaraman, Plant anatomy research centre, Chennai. A voucher specimen was deposited in the Department of Pharmacognosy, Sanjo college of Pharmaceutical studies, Palakkad, Kerala. (SCOPS/P.COG/07/2018)

Preparation of plant extract:

For preliminary phytochemical analysis, extract was prepared by weighing 500 grams of the dried powdered leaves were subjected to hot successive continuous extraction using Soxhlet apparatus with different solvents as per the polarity, petroleum ether, benzene, chloroform, ethanol and finally with aqueous. The extracts were filtered in each step using Whatman filters paper. The filtrate was concentrated using a rotary evaporator at low temperature (40-45 ^{o}C) and pressure. The presence or absence of the primary and secondary phytoconstituents was detected by usual prescribed method [9]

Chemicals and Drugs:

Carbon tetra chloride and Ecoline kits for serum aspartate transaminase (AST), alanine transaminase (ALT) alkaline phosphatase (ALP) and acid phosphatase (ACP), total bilirubin, were purchased from Sigma Co. (Sigma St. Louis, MO). Standard Silymarin was obtained fron Ranbaxy Ltd, New Delhi. Absolute ethanol was of analytical grade and was purchased from Merck (German).The other reagents were of analytical grade.

Animals

Western albino rats 180-230 gm maintained in the Animal house facility of the Department of Pharmacology, Sanjo College of pharmaceutical studies, were used in these experiments. The animals were maintained on standard small animal feeds (Excel feed, Ilorin) and water *ad libitum*. This research was carried out in accordance with the rules governing the use of laboratory animals as accepted internationally. The experiment was conducted between the hours of 900 h and 1600 h. The experimental groups consisted of six animals. They were maintained at constant room temperature (22° ± 1 °C) and submitted to12 h light/dark cycle with free access to food and water.

Experimental procedure

Acute oral toxicity study

Acute oral toxicity was conducted as per OECD guidelines (Organization of Economic Cooperation and Development) 423 (Acute toxic class method). The acute toxic class method is a step wise procedure of three animal of a single sex per step. Depending on the mortality and / or moribund status of animals, on the average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance. This procedure results in the use of a minimal number of animals while allowing for acceptable data based scientific conclusion. The method uses defined doses, (5, 50, 300, 2000 mg/kg body weight) and the results allow a substance to be ranked and classified according to the globally harmonized system (GHS) for the classification of chemicals which causes acute toxicity. The method previously described by Lorke [10] was adopte

Hepatoprotective activity:

The method of Ko et al [11] was used for screening the hepatoprotectivity of the test extract. Adult Wistar rats of either sex were randomly assigned into 5 groups of 6 animals. The animals of Group I served as normal control and received only the vehicle normal saline (10 ml/kg i.p). Group II served as toxic control and administered CCl_4 (1ml/kg) by subcutaneous injection. The animals of Group III and IV received *Enicostemma littorale* extract (100 mg/kg BW and 200mg/kg BW p.o. respectively) for 15 days. Group V served as Standard and was treated with Silymarin (25 mg/kg BW i.p., for 15 days). Animals (except Group 1) were treated CCl_4 at a dose of 1 ml/kg BW by subcutaneous injection . Blood samples were collected after last dose CCl_4 administration by direct cardiac puncture under light ether anesthesia and animals were sacrificed by cervical decapitation and hepatic tissue was collected. Heparinized blood sample were taken and assessed for serum enzyme markers and hepatic tissue was taken and subjected to histopathological study and further tissue was analyzed for Glutathione and lipid per oxidation.

Group 1-Normal animals (10mg/kg i.p)

Group 2-CCl_4 (1ml/kg s.c) treated animals

Group 3-CCl_4 + EEEL (100mg/kg ,p.o.) treated animals.

Group 4-CCl_4 + EEEL (200mg/kg, p.o.) treated animals.

Group 5-CCl_4 + Silymarin (25mg/kg i.p.) treated animals

Estimation of biochemical parameters:

Separated serum was analyzed. serum aspartate transaminase (AST), alanine transaminase (ALT) alkaline phosphatase (ALP) and acid phosphatase (ACP), and total bilirubin are

estimated[12] the tissue levels of enzymatic antioxidants *viz*. superoxidase dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), were estimated.[13-16]

Statistical analysis

The results are analysed by one way ANOVA followed by Dunnet's test and p value <0.001 was considered significant

Results:

Acute toxicity:

The results showed no clinical signs and mortality of the animal therefore an LD_{50} > 2000 mg/kg body weight may be assume.

Hepatoprotective and antioxidant activity:

The estimated values of serum AST, ALT, ALP, ACP and Total bilurubin values in control (saline) group of rats are tabulated in Table 1. A remarkable elevation was observed in Serum AST, ALT, ALP, ACP, total bilirubin and values in CCl_4 intoxicated rats (Toxic Control group). In the groups treated with 100mg/kg and 200mg/kg of the extract, the above bio-chemical markers of hepatotoxicity were found to be decreased when compared to CCl_4 treated control group. Evidently, the hepatoprotective effects of higher dose of Ethanolic extracts of *Enicostemma littorale* (200mg/kg) were near to that of standard i.e. Silymarin (25mg/kg). Both the doses of EEEL used in the study showed significant protective property than control. (*p<0.01, **p<0.001 vs. control) However the test extract was found to be less potent than that of standard,

TABLE: 1 SERUM ENZYME PROFILE

Treatment	AST(U/I)	ALT(U/I)	ALP (IU/L)	ACP(U/L)	Total Bilurubin(mg/100ml of blood)
Control (saline)	97.3±1.18	35.08±0.2	15.92±0.72	10.5±0.064	0.39±0.04
CCl_4 (1ml/kg)	186.7±1.82	136.9±1.94	98.3±7.9	38.6±2.9	0.89±0.76
EEEL(100mg/kg)	138.64±5.92	72.4±6.8	63.9±5.8	25.4±0.95	0.74±0.06
EEEL(200mg/kg)	106.8±8.28	47.5±4.1	43.6±3.4	16.5±0.18	0.57±0.04
Silymarin25mg/kg	105.3±4.3	49.4±3.6	34.8±2.9	16.2±1.2	0.24±0.03

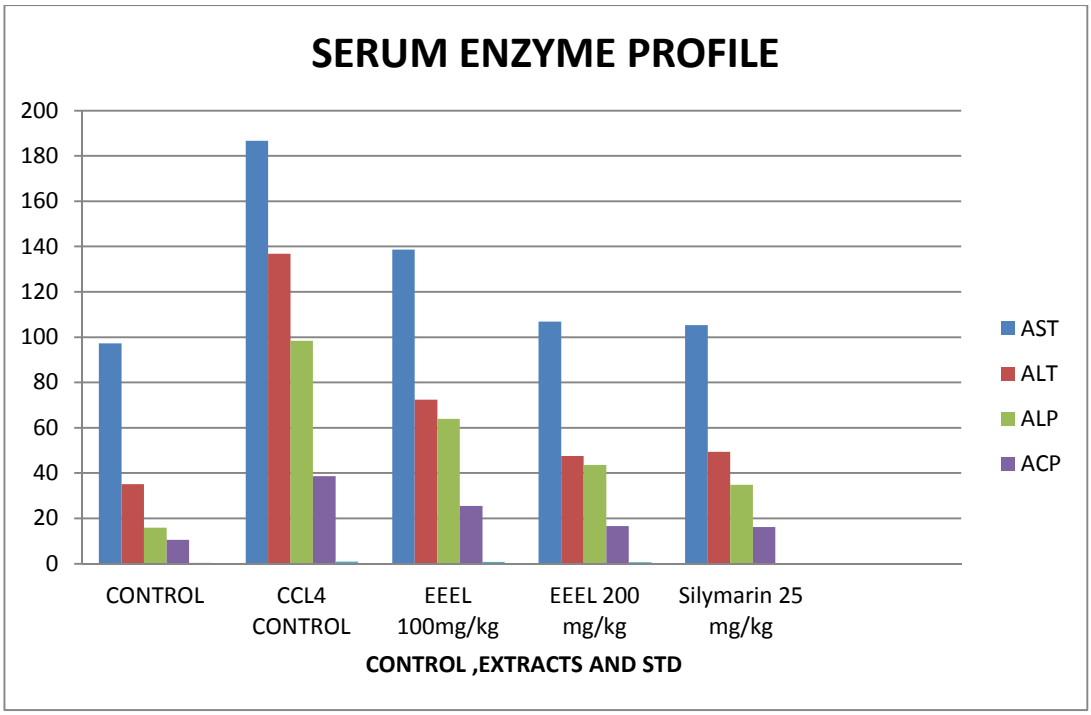
Data are expressed as mean ±S.E (n=6) *p<0.01 vs.control, **p<0.001 vs. control

EEEL- Ethanolic extract of *Enicostemma littorale*

AST-Aspartate transminase ACP-Acid phosphatase

ALT- Alanine transaminase ALP- Alkaline phosphatase

FIG : 1 SERUM ENZYME PROFILE



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 2. In the groups treated with 100mg/kg and 200mg/kg of the extract, GPX,SOD and catalase were found to be increased when compared to CCl_4 treated control group. Evidently, the hepatoprotective effects of higher dose of Ethanolic extracts of *Enicostemma littorale* (200mg/kg) were near to that of standard i.e. Silymarin (25mg/kg). Both the doses of EEEL used in the study showed significant protective property than control. (*$p<0.01$, vs. control) However the test extract was found to be less potent than that of standard drug.

TABLE-2: ANTIOXIDANT ACTIVITY

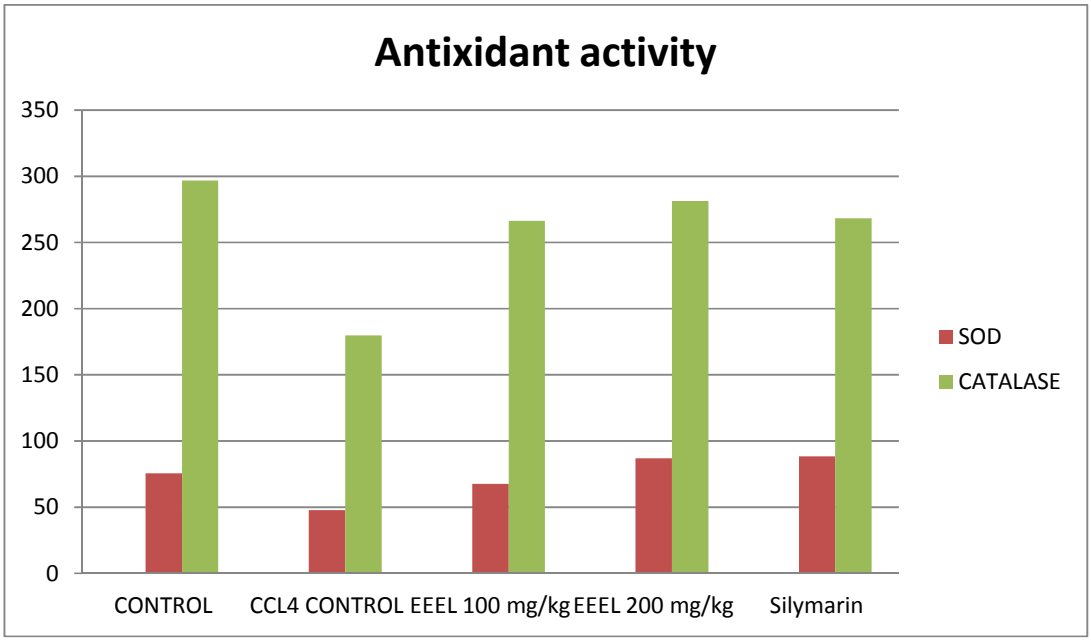
Treatment	Glutathione peroxidase (mg liver protein)$^{-1}$	SOD (mg liver protein)$^{-1}$	Catalase (mg liver protein)$^{-1}$
Control (saline)	0.992±0.05	75.81±1.94	296.83±10.05
CCl_4 (1ml/kg)	0.61±0.03	47.84±0.50	179.73±5.78
EEEL(100mg/kg)	0.85*±0.07	67.73*±0.54	266.27*±8.74
EEEL(200mg/kg)	0.92*±0.06	86.97*±0.75	281.38*±9.92
Silymarin25mg/kg	0.95*±0.03	88.34*±2.54	268.27*±6.46

Data are expressed as Mean ±S.E (n=6) *p<0.01 Vs control

EEEL- Ethanolic extract of *Enicostemma littorale*

SOD- Superoxidase dismutase

FIG 2: ANTIOXIDANT ACTIVITY



Discussion:

Since the extract has demonstrated dose dependant anti-oxidant activity in all the models of the study, the ethanol 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

194 challenge was reversed by the test extract and also reduced the extent of lipid peroxidation.
195 Most of the mammals have an effective mechanism to prevent and neutralize the free radical
196 induced damage, which is accomplished by a set of endogenous substances such as
197 superoxide dismutase, catalase, and glutathione peroxidase and glutathione reductase In
198 biochemical system, superoxide radical and H_2O_2 react together to form the hydroxyl radical,
199 this can attack and destroy almost all known biochemicals [17] The hydroxyl radicals thus
200 produced may attack the sugar of DNA base causing sugar fragmentation, base loss and DNA
201 stand leakage Ethanolic extract of *E.littorale* reduced the super oxide anions and also
202 scavenge off the hydroxyl radicals and hence, inhibit the cellular damage. It is apparent from
203 the present study that the test extract does not interfere with the generation of the free radicals
204 but it scavenges off the free radicals. CCl_4 undergo hepatic metabolism to give rise to tri-
205 chloromethyl radicals, which upon reacting with reac-tive oxygen species yields
206 trichloromethyl peroxide radicals, which forms covalent bond with membrane lipids and
207 destroy the membrane integrity. The observation of increased MDA formation in hepatic
208 cells after CCl_4 challenge is in accordance with the earlier report which suggests involvement
209 of trichloromethyl and tri-chloromethylperoxy radicals in the propagation of per-oxidation
210 process [18] The pretreatment with extract has prevented oxygen free radicals and thereby
211 prevented the formation of peroxy radicals. This aspect of test extract also contributes to the
212 hepatoprotectivity. The unpublished data on the hepatoprotective activity of this plant on
213 other models like paracetamol and thiacetamide induced hepatotoxicity indicated that the
214 hepatoprotectivity of the test extract is not model specific. SOD is metalloproteins catalyzing
215 the dismutation of superoxide anion to hydrogen and oxygen. Numerous studies have shown
216 the importance of SOD in protecting cells against oxidative stress[19] The SOD activity could
217 be decreased in tissue during alcohol ingestion. This decrease could be due to the feedback
218 inhibition or oxidative inactivitation of enzyme protein due to excess ROS generation[20] CAT,
219 hemeprotein, catalyzes the reduction of hydrogen peroxides[21] acts as preventive antioxidant
220 and plays an important role in protection against the deleterious effects of lipid peroxidation
221 [22]. The activity levels of catalase in tissue decreased in ethanol fed animals might be due to
222 the inhibition of CAT activity, which is suggestive of enhanced synthesis of O_2^- during the
223 ingestion of alcohol since O_2^- is a powerful inhibitor of catalase [23] GPX is an enzyme with
224 selenium in the form of selenocysteine and can catalyze the reduction of hydrogen peroxide
225 and hydroperoxides to non toxic products. GPX has a well-established role in protecting cells
226 against oxidative injury. GPX is non-specific for H_2O_2 and lipid peroxide generated during
227 alcohol ingestion which are efficiently scavenged by GPX activity. The depression of this

enzyme activity reflects perturbations in normal oxidative mechanism during alcohol ingestion.The cellular antioxidant defense enzymes *viz.* SOD, CAT, and GPX were significantly reduced in the CCl_4 administered rat. This might lead to decreased antioxidant defense and increased oxidative stress and thereby the tissue injury occur. Similar studies also indicate the failure of cellular antioxidant defense system during hepatotoxicity were recorded [24-25]

Conclusion:

The results of the present investigation, it may be concluded that the ethanolic extract of the whole parts of *Enicostemma littorale* possess significant hepatoprotective activity against carbon tetrachloride induced hepatotoxicity and antioxidant activity. The antioxidant potential may be attributed to the presence of polyphenolic compounds. Further studies like isolation and characterization of the active principlel(s) responsible for such activity are needed to confirm.

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