In vivo Hepatoprotective and Antioxidant Activity of *Enicostemma littorale* against Ccl_4 Induced Liver Damage in Rats

Dhanapal Venkatachalam[1*], B. Samuel Thavamani[1], K. Muddukrishniah[1] and Sukanya Vijayan[1]

[1]Department of Pharmacognosy, Sanjo College of Pharmaceutical Studies, Velappara, Palakkad, Kerala – 678 702, India.

Original Research Article

ABSTRACT

To investigate the hepatoprotective and antioxidant effects of ethanol extract of *Enicostemma littorale* Blume (Ens) against CCl_4 induced hepatic injury in albino rats. 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. 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 bilirubin. Lipid peroxidation caused by free radicals 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

**Corresponding author: E-mail: vddpaul@gmail.com;*

administered rats recorded elevated activity levels of serum AST, ALT, ALP and ACP revealing CCl_4 induced hepatotoxicity. In the groups treated with 100 mg/kg and 200 mg/kg of the extract, the above biochemical markers of hepatotoxicity were found to be decreased when compared to CCl_4 treated control group. Both the doses of ethanol extract of *Enicostemma littorale* (EEEL) used in the study showed significant protective property than the 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. 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.

1. INTRODUCTION

The hepatic system is a very vital organ system involved in the body's metabolic activities. Hence, 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 remains one of the serious health problems. Carbon tetrachloride (CCl_4) is one such hazardous chemical which induces hepatopathy through membrane lipid peroxidation by its free radical derivative like $CCl_3\cdot$, $CCl_3O_2\cdot$. Excessive production of the reactive species manifests in tissue-thiol depletion, lipid peroxidation, plasma membrane damage etc., culminating into severe hepatic injury [1]. Much of the cell damage that occurs during liver degeneration is believed to be caused by free radicals which are highly reactive oxygen species liberated during alcohol metabolism. These radicals react with cell membrane and induce lipid peroxidation, which has been implicated as an important pathological mediation [2] in many diseases such as heart disease, diabetes, cancer and liver disease. Management of liver diseases is still a challenge to modern medicine. In such a background, 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 constituents and may be used 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 and fruit in a capsule. The plant is locally used for its medicinal properties such as anti-inflammatory, antiulcer activity [6], hypoglycaemic [7] and antimalarial activities [8] in Tamilnadu and Kerala, India. The present study aims to investigate the hepatoprotective and antioxidant effects of *E. littorale* on CCl_4 induced hepatotoxicity in male albino rats.

2. MATERIALS AND METHODS

2.1 Plant Material

The whole plant of *E. littorale* was collected during January (2018) in and around Palakkad District, Kerala, India. The plant was cleaned, shade dried and 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).

2.2 Preparation of Plant Extract

For preliminary phytochemical analysis, extract was prepared by weighing 500 grams of the dried powdered leaves and subjected to hot successive continuous extraction using Soxhlet apparatus with different solvents like petroleum ether, benzene, chloroform, ethanol and water in the increasing order of polarity. The extracts were filtered in each step using Whatman filter paper. The filtrate was concentrated using a rotary evaporator at low temperature (40-45°C)

and pressure. The presence or absence of the primary and secondary phytoconstituents was detected by usual prescribed method [9].

2.3 Chemicals and Drugs

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

2.4 Animals

Wistar albino rats of 180-230 gm, maintained in the Animal House facility of the Department of Pharmacology, Sanjo College of Pharmaceutical Studies, Palakkad were used in these experiments. The animals were fed on standard small animal feeds (Excel feed, Ilorin) and water *ad libitum*. This research was carried out by following 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.

2.5 Experimental Procedure

2.5.1 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 animals of single sex per step. Depending on the mortality and / or moribund status of animals, on an 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 adopted. The LD50 was found to be 2000 mg/kg body weight.

2.6 Hepatoprotective Activity

The method of Ko et al. [11] was used for screening the hepatoprotectivity of the ethanol extract of *Enicostemma littorale* prepared as mentioned above. Adult Wistar rats of either sex were randomly assigned into 5 groups of 6 animals each. 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). The 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 of 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 was 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 peroxidation.

Group 1-Normal animals (10 mg/kg i.p)
Group 2-CCl_4 (1ml/kg s.c) treated animals
Group 3-CCl_4 + EEEL (100 mg/kg ,p.o.) treated animals.
Group 4-CCl_4 + EEEL (200 mg/kg, p.o.) treated animals.
Group 5-CCl_4 + Silymarin (25 mg/kg i.p.) treated animals

2.7 Estimation of Biochemical Parameters

Serum aspartate transaminase (AST), alanine transaminase (ALT) alkaline phosphatase (ALP) and acid phosphatase (ACP), and total bilirubin were estimated in the separated serum [12]. The tissue levels of enzymatic antioxidants *viz.* superoxidase dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), were also estimated [13-16].

2.8 Estimation of Glutathione peroxidase, Superoxide dismutase (SOD), and Catalase (CAT)

Glutathione peroxidase (GPx) was measured by the method described by Rotruck et al. The

activities were expressed as μ g of GSH consumed/ min/mg protein. The SOD activity in cell lysate was determined as per the method followed earlier by Kakkar et al. Enzyme activity was expressed as 1 unit = 50% inhibition/minute/mg of protein. The CAT activity in cell lysate was assayed as per the method reported earlier by Aebi. CAT was expressed in terms of □mol of rogen peroxide decomposed/min/mg of protein [17-19]

2.9 Statistical Analysis

The results are analysed by one way ANOVA followed by Dunnet's test using prism pad software and p value <0.001 was considered to be significant.

3. RESULTS

3.1 Acute Toxicity

The acute toxicity in Wistar albino rat was studied as per the Organization for Economic Cooperation and Development (OECD) guideline 423. The LD_{50} value of EEEL i.e 2000 mg/kg body weight was determined using the method of maximum likelihood.

3.2 Hepatoprotective and Antioxidant Activity

The estimated values of serum AST, ALT, ALP, ACP and Total bilirubin 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 (25 mg/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.

The tissue glutathione was found to be depleted upon CCl_4 intoxication which indicates that the tissue damage is due to over powering the inbuilt free radical scavenger mechanisms. This tissue Glutathione (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 100 mg/kg and 200 mg/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* (200 mg/kg) were near to that of standard i.e. Silymarin (25 mg/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.

4. DISCUSSION

Since the extract has demonstrated for antioxidant property in dose dependant 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 depletion of GSH in the issue was due to CCl_4 challenge and was reversed by

Table 1. Effect of EEEL on serum biochemical parameters

Treatment	AST(U/l)	ALT(U/l)	ALP (IU/L)	ACP(U/L)	Total Bilirubin (mg/100 ml 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 (1 ml/kg)	186.7±1.82	136.9±1.94	98.3±7.9	38.6±2.9	0.89±0.76
EEEL(100 mg/kg)	138.64±5.92	72.4±6.8	63.9±5.8	25.4±0.95	0.74±0.06
EEEL(200 mg/kg)	106.8±8.28	47.5±4.1	43.6±3.4	16.5±0.18	0.57±0.04
Silymarin 25 mg/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 transaminase; ACP-Acid phosphatase; ALT- Alanine transaminase; ALP- Alkaline phosphatase

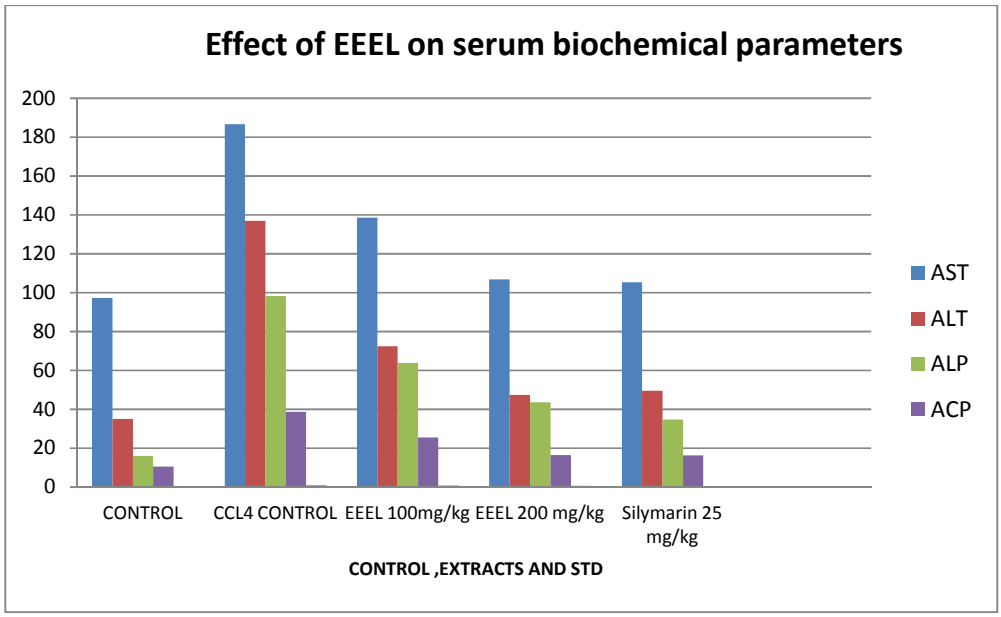

Fig. 1. Effect of EEEL on serum biochemical parameters

Table 2. Antioxidant activity of EEEL

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 (1 ml/kg)	0.61±0.03	47.84±0.50	179.73±5.78
EEEL(100 mg/kg)	0.85*±0.07	67.73*±0.54	266.27*±8.74
EEEL(200 mg/kg)	0.92*±0.06	86.97*±0.75	281.38*±9.92
Silymarin 25 mg/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

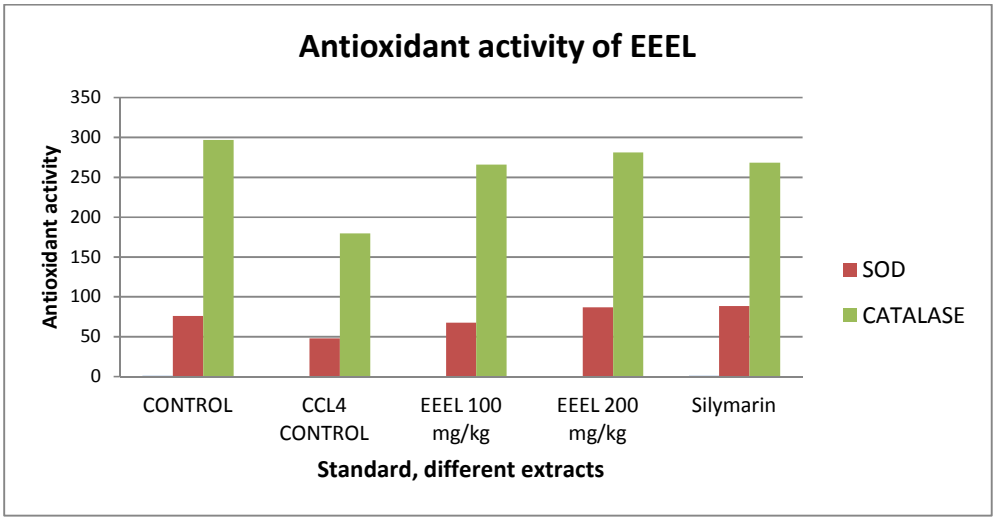

Fig. 2. Antioxidant activity of EEEL

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 endogenous substances such as superoxide dismutase, catalase, and glutathione peroxidase and glutathione reductase 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 [20]. The hydroxyl radicals thus produced may attack the sugar of DNA base causing sugar fragmentation, base loss and DNA stand leakage Ethanolic extract of *E. littorale* 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 does not interfere with the generation of the free radicals but it scavenges off the free radicals. CCl_4 undergoes hepatic metabolism to give rise to tri-chloromethyl 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 malondialdehyde (MDA) formation in hepatic cells after CCl_4 challenge is in accordance with the earlier report which suggests involvement of trichloromethyl and tri-chloromethylperoxy radicals in the propagation of per-oxidation process [21]. Pretreatment with the extract has prevented oxygen free radicals and thereby prevented the formation of peroxy radicals. This aspect of test extract also contributes to the hepatoprotectivity. Unpublished data on the hepatoprotective activity of this plant on other models like paracetamol and thiacetamide induced hepatotoxicity indicated that the hepatoprotectivity of the test extract is not model specific. SOD is metalloproteins catalyzing the dismutation of superoxide anion to hydrogen and oxygen. Studies have shown the importance of SOD in protecting cells against oxidative stress [22]. The SOD activity could be decreased in tissue during alcohol ingestion. This decrease could be due to the feedback inhibition or oxidative inactivation of enzyme protein due to excess ROS generation [23]. CAT, hemeprotein, catalyzes the reduction of hydrogen peroxides [24] acts as preventive antioxidant and plays an important role in protection of tissues against the deleterious effects of lipid peroxidation [25].The activity levels of catalase in tissue decreased in ethanol fed animals. This might be due to the inhibition of CAT activity, which is suggestive of enhanced synthesis of O_2^- during the ingestion of alcohol since O_2^- is a powerful inhibitor of catalase [26]. GPX is an enzyme with selenium in the form of selenocysteine and can catalyze the reduction of hydrogen peroxide and hydroperoxides to nontoxic products. GPX has a well-established role in protecting cells against oxidative injury. GPX is non-specific for H_2O_2 and lipid peroxide generated during alcohol ingestion which are efficiently be 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 CCl4 administered rat. This might lead to decreased antioxidant defense and increased oxidative stress and thereby the tissue injury [27-29].

5. CONCLUSION

The results of the present investigation, 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 principle(s) responsible for such activity are needed to confirm.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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