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RESEARCH ARTICLE

EVALUATION OF ANTIHEPATOTOXIC AND ANTIOXIDANT POTENTIAL OF *Hiptage benghalensis* IN CCl_4 INDUCED ALBINO RATS

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Abstract

The hepatoprotective and anti-oxidant potential of ethanolic leaf extracts of *Hiptage benghalensis* was investigated against CCl_4 induced hepatotoxicity in albino wistar rats. CCl_4 induction resulted in an increase in serum aspartate transferase, alanine amino transferase, alkaline phosphatase and bilirubin levels indicating liver damage and a collective depletion in the levels of antioxidants superoxide dismutase, glutathione reductase, glutathione peroxidase, glutathione-S-transferase and catalase which in turn led to increased LPO levels suggestive of loss of structural integrity in the hepatocytes when compared with the normal control groups. Treatment with ethanolic leaf extract of *Hiptage benghalensis* (ELEHb) in doses of 100, 200 and 300 mg/kg bw. brought about a significant decrease ($p \leq 0.05$) in the levels of serum enzymes, a significant increase ($p \leq 0.05$) in the enzymatic antioxidant values and subsequent decrease of LPO levels

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INTRODUCTION

Liver disease is a major health problem in India. Hepatic viral infections especially are of serious concern among cases of liver disease. It is estimated that 4.7% of the population of India is a carrier of hepatitis B virus (HBV)**.** Liver disease is considered to be the fourth important cause of mortality in the most productive period of life (15-45 years)**.** It is estimated that 15 - 40% of those affected eventually form serious complications, such as cirrhosis or liver cancer. Hepatitis C also remains significant as a cause of liver disease.

There is a prevalence rate of 26% in South India and 10-15% in North India with 16-20% accounting in Mumbai, which is considered as the financial capital of India. Global mortality prevalence due to liver cancer associate to about 5,98,000 deaths while 8-10 million deaths are estimated due to other liver diseases such as cirrhosis, cholestatis, fatty liver, hepatic encephalopathy, fulminant hepatic failure and chronic hepatitis (Handa, 1990). Consumption of contaminated food or exposure to chemicals in the occupational environment, environmental toxins, poor eating habits, alcohol consumption, prescribed and over-the-counter drug usage can damage and weaken the liver and eventually lead to hepatitis, cirrhosis, alcoholic liver diseases which will affect the structure and function of the liver as well as its physiological role such as the biotransformation of lipophilic compounds into water soluble derivatives.

Drug induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures. More than 900 drugs have been implicated in causing liver injury and it is the most common reason for a drug to be withdrawn from the market (Arundel and Lewis, 2006). There are ample experimental and epidemiological studies supporting the involvement of oxidative stress in the pathogenesis and progression of several chronic diseases (Maxwell, 1995). The reactive metabolites such as trichloromethyl (CCl_3) and trichloromethylperoxy radicals emanated from CCl_4, initiate peroxidation of membrane unsaturated fatty acids which cause lipid peroxidation of membrane that seriously impair the function of liver.

The use of natural remedies for the treatment of liver diseases has a long history and medicinal plants and their derivatives are still used all over the world in one form or another for this purpose. *Hiptage benghalensis*, a stout, high-climbing liana of the Malpighiaceae family, holds a reputed position in Indian medicine. Bark, leaves and flowers of this plant possess astringent, expectorant, cardio tonic, anti-inflammatory, wound healing potential and are used in treating burning sensation of the body, foul ulcers, hepatoprotective, cough, asthma, cardiac debility, rheumatism, hyperdipsia, obesity, instrinsic hemorrhage (Verma et al., 1993). Eventhough folklore claims on hepatoprotective action of this plant are available, thorough scientific validations about the same are far and few. Hence the present study is aimed at investigating the antioxidant potential and its contribution to the hepatoprotective ability of the ethanolic leaf extract of *H.benghalensis* against CCl_4 induced hepatotoxicity.

MATERIALS AND METHODS

Preparation of plant extract

The plant material under study was shade dried and coarsely powdered. About 500 gms of plant material was soaked in ethanol for 48hrs. After 48 hrs of soaking the solvent was distilled off under reduced pressure at 50°C and dried in vacuum.

Experimental animals

Healthy adult Swiss albino male rats, weighing 120-150g were obtained from Tamilnadu Veterinary and Animal Sciences University, Chennai,Tamilnadu. The animals were allowed to acclimatize under laboratory conditions for a period of 5 days prior to the experiment. Animals were housed in standard polypropylene cages. Six animals were housed per cage, so as to provide them with sufficient space, and to avoid unnecessary morbidity and mortality. Animals were maintained under standard condition of 12: 12 hrs. light/dark cycle and at an ambient temperature at 23±2°C, with 65±5 % humidity. Animals were fed with standard rat chow pellet obtained from Sai Durga foods and feeds, Bangalore, India and water ad libitum. All the studies were conducted according to the ethical guidelines of CPCSEA after obtaining necessary clearance from the committee (Approval No: 790/03/ac/CPCSEA).

Acute Toxicity Studies

Oral acute toxicity study was conducted as per the guidelines of Organization for Economic Co-operation and Development (OECD) (OECD, 2001). The ethanolic leaf extract of *H.benghalensis* (ELEHb) was prepared and subjected to toxicity studies in 5 different groups with each group consisting of 6 animals. A single dose of 1, 2, 3, 4, 5 g/ Kg (bw.) were given to five different groups of animals and observed continuously for 21 days.

Experimental Design

The animals were divided into six groups with each group containing six rats each. Group I rats served as normal control rats. Group II animals comprised disease control rats and were administered CCl_4 (at a dosage of 0.5 ml/150g bw. in olive oil in the ratio1:1) orally for three days. In groups III, IV and V, administration of CCl_4 was followed by treatment with ethanolic leaf extract of *H. benghalensis* (ELEHb) at dose levels of 100mg, 200mg and 300mg/kg bw. orally for 21 days. In group VI, the experimental animals were treated with Silymarin at a dose of 25mg/kg bw. orally for 21 days.

At the end of experimental period, the animals were sacrificed by cervical decapitation and blood was collected in EDTA containing vials. Then it was centrifuged 3000rpm for 10 minutes. The serum was collected and used for the analysis of various biochemical estimations such as AST, ALT, ALP (King, 1965), total protein, A/G ratio & total bilirubin (Malloy and, Evelyn, 1937). The liver tissue was excised, homogenized in ice-cold phosphate buffer/carbonate buffer and utilized for antioxidant studies such as superoxide dismutase (SOD) (Misra and Fridovich, 1972), glutathione reductase (GSH) (Beutler et al., 1963), glutathione peroxidase (GPx) (Rotruck et al., 1973), glutathione-S-transferase (GST) (Habig et al., 1974) and catalase (CAT) (Sinha, 1970).

Histopathological Study

Liver samples were dissected and excised from the experimental animals of each group, washed with the normal saline, fixed in 10% formalin and processed for paraffin embedding following the microtome technique. The sections of 5µ thickness were taken, processed in alcohol-xylene series and were stained with alum-haematoxylin and eosin. The sections were then examined microscopically to observe histopathological changes (Sujai Suneetha, 1993).

Statistical Analysis

Results are expressed as Mean ± SEM. Multiple comparison of the significant ANOVA was performed by Duncan's multiple comparison test, $p \leq 0.05$ were considered as statistically significant. All data were analyzed with the aid of statistical package programme aid SPSS 17.0 for window.

RESULTS

Acute toxicity studies

Acute toxicity studies showed that the plant extract did not show any toxicity and mortality up to a dose of 5g/Kg bw. in experimental animals. Three submaximal doses (100, 200 & 300mg/kg.bw. which were found to be safe in rats were employed for further pharmacological and biochemical investigations.

Effect of CCl_4 and ELEHb on levels of Hepatic enzymes and Bilirubin

The results of the effect of CCl_4 and ELEHB on the levels of hepatic enzymes AST, ALT, ALP and bilirubin are tabulated in Table 1. The hepatic injury in experimental animals induced by CCl_4 resulted in an increase in serum AST (36.7%), ALT (43.6%), ALP (33%) and LDH (41%) levels when compared with the normal control groups. However, a significant decrease ($p \leq 0.05$) in elevation of serum enzymes was noted following treatment with ELEHb in a dose dependent fashion and was comparable with the standard drug treated groups. There was a 28% increase in bilirubin levels in CCl_4 induced experimental animals which was significantly brought down ($p \leq 0.05$) to normalcy on treatment with ELEHb in increasing doses of 100,200,300 mg/kg bw (Table 2).

Effect on Protein and A/G ratio

There was a 3 fold decrease in the levels of protein in CCl_4 induced experimental animals which had a direct impact on the level of A/G ratio which was also decreased when compared with the normal control groups. But treatment with ELEHb resulted in elevated levels of protein and A/G ratio (Table 3).

Effect on Antioxidant and LPO levels

The present study showed a collective depletion in the levels of antioxidants SOD, GR, GSH, GPx, GST, CAT which had a direct impact on the levels of LPO which was elevated (41%). Levels of SOD, GR, GSH, GPx, GST, CAT showed a depletion of (42%, 40%, 42%, 37%, 50% and 53% respectively) when compared with control animals. Treatment with ELEHb showed a significant increase ($p \leq 0.05$) in the enzymatic antioxidant values and a significant decrease ($p \leq 0.05$) in the LPO levels comparable to standard treated groups (Table 4).

Histopathological Examination

Histopathological examination of liver tissues of normal experimental animals (Group I) stained with Hematoxylin and Eosin showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein. In the liver sections of the rat intoxicated with CCl_4 there was disarrangement and degeneration of normal hepatic cells with intense centrilobular necrosis, sinusoidal hemorrhages and dilatation. Treatment with ELEHb showed very mild fatty changes. The hepatic lesions produced by the toxin were reduced. The extent of tissue necrosis was reduced in a dose dependent manner. The liver sections of the experimental animals treated with ELEHb at a concentration of 300mg/kg bw. and standard drug silymarin showed less vacuole formation, reduced sinusoidal dialation, less disarrangement and degeneration of hepatocytes.

TABLE 1: EFFECT OF ETHANOLIC LEAF EXTRACT OF *H. benghalensis* ON HEPATIC MARKER ENZYMES LEVELS

Groups	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	LDH (IU/L)
Group I	$59.61 \pm 1.14^{*}$	$42.23 \pm 1.09^{*}$	$88.6 \pm 0.89^{*}$	$191.10 \pm 1.58^{*}$
Group II	$162.21 \pm 2.28^{*,**,a}$	$96.69 \pm 0.89^{*,**,a}$	$266.14 \pm 3.16^{*,**,a}$	$465.61 \pm .14^{*,**,a}$
Group III	148.23 ± 1.41	77.89 ± 1.92	214.56 ± 3.08	392.32 ± 0.70
Group IV	108.25 ± 1.78	70.54 ± 4.33	160.83 ± 2.28	283.85 ± 0.04
Group V	$74.23 \pm 2.28^{**,a,b}$	$48.65 \pm 0.89^{**,a,b}$	$99.87 \pm 3.19^{**,a,b}$	$201.67 \pm .60^{**,a,b}$
Group VI	$64.23 \pm 1.78^{a,b}$	$45.20 \pm 0.70^{a,b}$	$95.64 \pm 3.57^{a,b}$	$198.81 \pm 2.28^{a,b}$

* - Significant when compared between Group 1 and Group 2 (p≤ 0.05, n=6)
** - Significant when compared between Group 2 and Group 5 (p≤ 0.05, n=6)
a - Significant when compared between Group2, Group 5 and Group 6 (p≤ 0.05, n=6)
b - Non significant when compared between Group 5 and Group 6 (p≤0.01, n=6)

TABLE 2: EFFECT OF ETHANOLIC LEAF EXTRACT OF *H. benghalensis* ON BILIRUBIN LEVELS

Groups	Bilirubin mg/dl
Group I	0.80 ± 0.01*
Group II	2.90 ± 0.01^{*,**,a}
Group III	2.51 ± 0.02
Group IV	1.92 ± 0.03
Group V	1.11 ± 0.04$^{**, a, b}$
Group VI	0.96 ± 0.05 $^{a, b}$

* - Significant when compared between Group 1 and Group 2 (p≤ 0.05, n=6)
** - Significant when compared between Group 2 and Group 5 (p≤ 0.05, n=6)
a - Significant when compared between Group2, Group 5 and Group 6 (p≤ 0.05, n=6)
b - Non significant when compared between Group 5 and Group 6 (p≤0.01, n=6)

TABLE 3: EFFECT OF ETHANOLIC LEAF EXTRACT OF *H. benghalensis* ON PROTEIN LEVELS AND A/G RATIO

Groups	Protein g/dl	A/G ratio mg of AG
Group I	6.26 ± 0.01*	2.11 ± 1.02*
Group II	2.13 ± 0.02$^{*,**, a}$	1.21 ± 0.08$^{*,**, a}$
Group III	2.92 ± 0.01	1.35 ± 0.06
Group IV	4.71 ± 0.01	1.78 ± 1.08
Group V	6.01 ± 0.02$^{**, a, b}$	1.92 ± 0.05$^{**, a, b}$
Group VI	6.12 ± 0.01 $^{a, b}$	1.96 ± 0.07 $^{a, b}$

* -

Significant when compared between Group 1 and Group 2 (p≤ 0.05, n=6)
** - Significant when compared between Group 2 and Group 5 (p≤ 0.05, n=6)
a - Significant when compared between Group2, Group 5 and Group 6 (p≤ 0.05, n=6)
b - Non significant when compared between Group 5 and Group 6 (p≤0.01, n=6)

TABLE 4: EFFECT OF ETHANOLIC LEAF EXTRACT OF *H. benghalensis* ON THE LEVELS OF SOD & CATALASE

Groups	SOD mM of epinephrine oxidised/min/g tissue	Catalase mmoles of hydrogen peroxide utilized/min/mg of protein
Group I	90.32 ± 1.58 [*]	67.32 ± 0.58 [*]
Group II	37.45 ± 1.14 [*,**, a]	35.39 ± 1.15 [*,**, a]
Group III	49.61 ± 0.89	41.07 ± 0.59
Group IV	67.56 ± 1.11	55.89 ± 1.40
Group V	81.25 ± 1.92 [**, a, b]	62.09 ± 1.65 [**, a, b]
Group VI	84.34 ± 2.54 [a, b]	68.34 ± 1.75 [a, b]

* - Significant when compared between Group 1 and Group 2 (p≤ 0.05, n=6)
** - Significant when compared between Group 2 and Group 5 (p≤ 0.05, n=6)
a - Significant when compared between Group2, Group 5 and Group 6 (p≤ 0.05, n=6)
b - Non significant when compared between Group 5 and Group 6 (p≤0.01, n=6)

TABLE 5: EFFECT OF ETHANOLIC LEAF EXTRACT OF *H. benghalensis* ON GSH, GPX, GST AND LPO LEVELS

Groups	Glutathione reductase µm of glutathione oxidised /min/g tissue	Glutathione peroxidase µmoles of GSH oxidised / min/g tissue	Glutathione-S-transferase U/min/g tissue	LPO nmoles of MDA/g tissue
Group I	10.98 ± 0.19 [*]	717.62 ± 1.81 [*]	1824.22 ± 10.83 [*]	413.45 ± 1.58 [*]
Group II	4.63 ± 0.04 [*,**, a]	265.65 ± 1.67 [*,**, a]	916.45 ± 6.51 [*,**,a]	986.46 ± 1.14 [*,**, a]
Group III	5.85 ± 0.03	395.23 ± 2.58	1129.67 ± 7.41	892.68 ± 0.89
Group IV	7.27 ± 0.04	525.67 ± 3.78	1385.85 ± 6.18	722.92 ± 2.07
Group V	9.48 ± 0.09 [**, a, b]	696.73 ± 3.80 [**, a, b]	1773.76 ± 5.70 [**, a,b]	496.89 ± 3.16 [**, a, b]
Group VI	10.12 ± 0.13 [a, b]	707.43 ± 2.30 [a, b]	1800.98 ± 3.53 [a, b]	421.25 ± 1.78 [a, b]

* - Significant when compared between Group 1 and Group 2 ($p \leq 0.05$, n=6)
** - Significant when compared between Group 2 and Group 5 ($p \leq 0.05$, n=6)
a - Significant when compared between Group2, Group 5 and Group 6 ($p \leq 0.05$, n=6)
b - Non significant when compared between Group 5 and Group 6 ($p \leq 0.01$, n=6)

Fig 1: HISTOPATHOLOGICAL STUDIES

Fig 1(a): Normal rat liver section

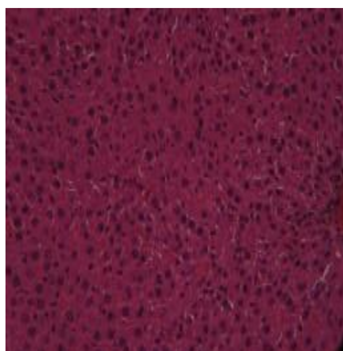


Fig 1(b): Liver section of rats intoxicated with CCl_4

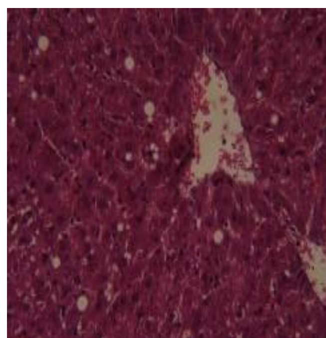


Fig 1(c): Liver section of rat treated with ELEHb (100 mg/kg bw.)

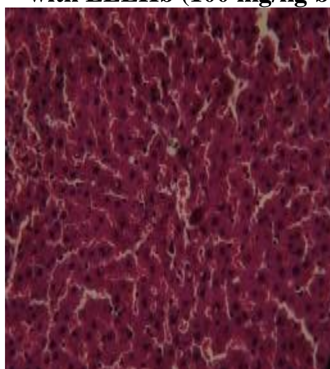


Fig 1(d): Liver section of rat treated with ELEHb (200 mg/kg bw.)

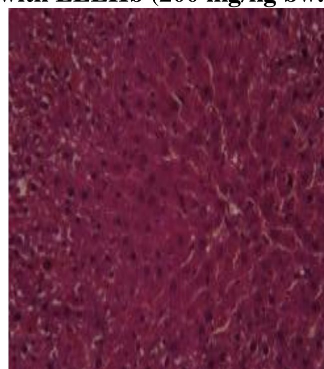


Fig 1(e): Liver section of rat treated with ELEHb (300 mg/kg bw.)

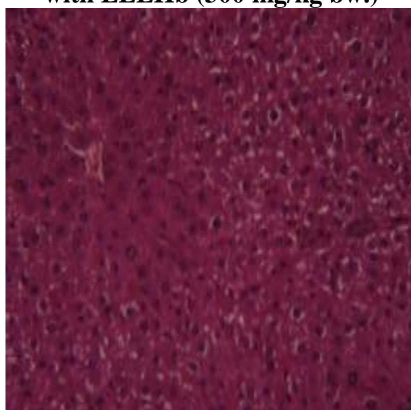
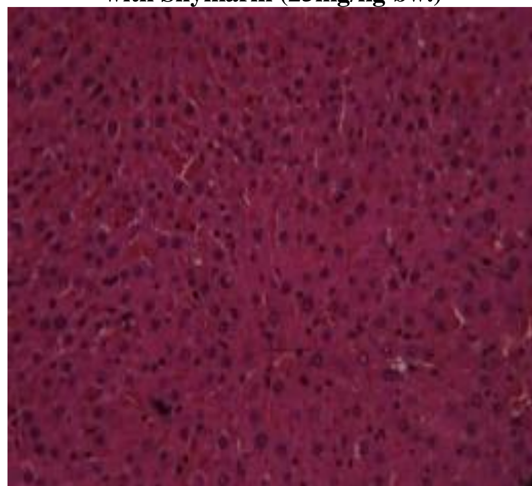


Fig 1(f): Liver section of rat treated with Silymarin (25mg/kg bw.)



DISCUSSION

Hepatotoxicity induced by CCl_4 is the most commonly used model system for the screening of hepatoprotective activity of plant extracts/drugs (Srivastava and Shivanandappa, 2010). The changes associated with CCl_4 induced liver damage are similar to that of acute viral hepatitis (Rubinstein, 1962). The hepatotoxicity of CCl_4 is attributed to the formation of trichloromethyl and trichloromethyl peroxyl radicals, initiating lipid peroxidation and resulting in fibrosis and cell necrosis (Kadiiska et al., 2000). Long-term administration of CCl_4 causes chronic liver injury (Hernandez-Munoz et al., 1990).

It has been well documented over the years that serum levels of marker enzymes are very sensitive indicators employed in the diagnosis of liver diseases and to understand the extent (Ansari et al., 1991) and position of the liver injury. The liver marker enzymes (AST, ALT and ALP) are cytoplasmic in nature but upon liver injury these enzymes enter into the circulatory system due to altered permeability of membrane. SGOT is associated with liver parenchyma cells and is raised in acute liver damage. SGPT is an enzyme found in hepatocytes and its leakage into blood is observed in acute liver damage. ALP is excreted normally via bile by the liver. The liver injury due to toxins can result in defective excretion of bile by hepatocytes which are reflected as their increased levels in serum (Rajesh and Latha, 2004).

In the present study, it was evident from the results obtained that the significant rise ($p \leq 0.05$) in marker enzymes level in CCl_4 induced animals is attributed to damaged structural integrity of the liver. On treatment with ELEHb, the levels of hepatic enzymes significantly reduced ($p \leq 0.05$) which may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. An evident change was noticeable in groups treated with in 300mg/kg bw. of ELEHb and the effect was on par with the effect of the standard drug silymarin.

A significant decrease ($p \leq 0.05$) in the levels of protein is observed after CCl_4 intoxication due to loss of structural integrity of liver cells causing transfer of protein to other parts of the body. Decreased levels of albumin and increased levels of globulin in CCl_4 intoxicated rats are indicative of liver impairment and dysfunction. In the present work, dose dependent treatment treatment with ELEHb resulted in a significant ($p \leq 0.05$) elevation in protein levels indicating regeneration of liver tissues. The restoration of normal level of A/G ratio on treatment with ELEHb showed the restoration of synthetic functions of liver.

Living organisms employ a battery of defense mechanisms such as antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) to prevent or mitigate oxidative tissue damage (Halliwell and Gutteridge, 1989). The antioxidant enzymes SOD, CAT, GSH, GPx and GST constitute a mutually supportive team of defense against ROS (Uday et al., 1999). Superoxide dismutase (SOD) removes the toxic superoxide radical (O^{2-}) formed by the partial reduction of oxygen in tissues. Catalase and GPx deals effectively with a large amount of hydrogen peroxide generated in peroxisomes thus protecting from oxidative stress (Popovici Irina, 2000). GST binds to liphophilic compounds and acts as an enzyme for gluathione (GSH) conjunction reaction. The redox system works effectively to reduce the production of ROS which is responsible for lipid peroxidation and membrane damage.

The reactive metabolites such as trichloromethyl (CCl_3) and trichloromethylperoxy radicals emanated from CCl_4, initiate peroxidation of membrane unsaturated fatty acids. This result in lipid peroxidation of membrane that seriously impair the function of liver. The decrease in activity of SOD in the liver of CCl_4 induced rats may have attributed to the increased lipid peroxidation or inactivation of the enzyme by cross-linking with malondialdehyde. This further caused accumulation of superoxide radicals instigating increased lipid peroxidation. GST, which binds to liphophillic compounds and acts as an enzyme for glutathione (GSH) conjunction reaction was also found to be reduced due to CCl_4 toxicity may be due to decreased availability of GSH. Depletion of GSH also enhanced lipid peroxidation which inturn caused decrease in its levels (Anandan et al., 1999). There was also a notable depletion in the levels of GPx in CCl_4 induced rats indicative of damage to membranes and other cell components. In the present study the activities of SOD, CAT, GSH, GPx and GST were found to be significantly decreased ($p \leq 0.05$) in the CCl_4 induced rats compared with normal groups. Administration of ELEHb restored the activities of these anti-oxidant enzymes to near normalcy when compared to the CCl_4 administered rats. The tendency of these enzymes to return towards a near normal level in groups treated with silymarin and ELEHb is a clear manifestation of their anti-hepatotoxic effect.

Histopathological examination of the liver tissues in experimental animals induced with CCl_4 showed disarrangement and degeneration of normal hepatic cells with intense centrilobular necrosis with sinusoidal hemorrhages and dilatation. The hepatic lesions and necrosis found in CCl_4 administered rats was considerably reduced rats treated with ELEHb and the maximum activity was found in rats treated with 300mg/kg bw indicative of the hepatoprotective action of *H.benghalensis*.

The active principles in the ethanol extract of H. benghalensis may be responsible for its hepatoprotective activity. However, further studies are required in specifying compounds that act against hepatitis in order to establish the hepatoprotective potential of *H. benghalensis*.

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