Hepatoprotective Potential of Flavonoid Rich Fraction of *Enhydra Fluctuans* Against CCl_4-Induced Oxidative Damage in Rats

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Summary

The present study evaluated the hepatoprotective potential of *Enhydra fluctuans* Lour. (Compositae) against carbon tetrachloride-induced oxidative damage in rats. The hepatoprotective effect of ethyl acetate fraction of *E. fluctuans* (EAEF) was evaluated by assaying the serum biochemical parameters glutamate pyruvate transaminase (ALT), glutamate oxaloacetate transaminase (AST), alkaline phosphatase (ALP), bilirubin, and total protein. Malondialdehyde (MDA) level, as well as the activities of superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT) were determined to explain the possible mechanism of the activity. In addition, histopathology of liver tissue was investigated to observe the morphological changes. The elevated levels of serum ALT, AST, ALP, and total bilirubin were restored significantly towards normal by EAEF. MDA concentration was decreased, while the liver antioxidative enzyme activity was elevated in all EAEF treated rats. All the results were compared with standard drug silymarin. Histological study showed the reduction of fatty degeneration and liver necrosis. The experimental results of this study revealed that the flavonoid rich ethyl acetate fraction has significant hepatoprotective activity. This effect may be due to the ability of EAEF to inhibit the lipid peroxidation and increase in the anti-oxidant enzymatic activity.

Key words: Antioxidant, *Enhydra fluctuans* Lour, hepatoprotective activity, lipid peroxidation, tetrachloride.

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Introduction

The liver is an organ of paramount importance and appears to be a sensitive target site for substances modulating biotransformation. The impairment of the liver function is generally caused by xenobiotics, excessive exposure to various pharmacological and chemical agents, and protozoal or viral infections. Carbon tetrachloride (CCl_4) has been widely used in animal models to investigate chemical toxin-induced oxidative liver damage [1]. The magnitude of liver damage by disease or hepatotoxins is generally measured by the level of glutamate pyruvate transaminase (ALT), glutamate oxaloacetate transaminase (AST), alkaline phosphatase (ALP), bilirubin, and some pathological characteristics like fatty liver, cirrhosis, and necrosis. These effects are observed due to formation of reactive intermediates such as trichloromethyl free radicals ($CCl_3^{\cdot}$) metabolized by the mixed function cytochrome p450 in the endoplasmic reticulum [2]. These free radicals induce lipid peroxidation and generate lipid peroxides which bind covalently to microsomal lipids and proteins [3]. This phenomenon results in the excess generation of reactive oxygen species (ROS) like the superoxide anion $O_2^{\cdot-}$, H_2O_2 and the hydroxyl radical, $OH^{\cdot}$. Under this induced stress condition the defense capacities against ROS become insufficient [4] and which is considered as an important factor in acute and chronic liver injury [5]. The liver injury induced by CCl_4 is a result of free radicals [6] and lipid peroxidation that causes hepatic cell damage.

Numerous medicinal plants and their formulation are used for liver disorders in ethnomedical practice as well as traditional system of medicine in India [7]. Herbs play a vital role in the management of various liver disorders [8]. In the absence of a reliable liver protective drug in the modern medicine, a number of medicinal preparations in Ayurveda are recommended for the treatment of liver disorders [9].

Enhydra fluctuans Lour. (Compositae), an edible semi aquatic herbaceous vegetable plant with serrate leaves, grows all over India. The leaves which are slightly bitter, are used to treat inflammation, skin diseases, and small pox [10]. The leaves are also antibilious and are used in nervous diseases [11] and in torpidity of liver [12]. The plant possesses nutritional value and its methanol extract has been reported to have analgesic activity [13] and antidiarrheal activity [14]. Recently the free radical scavenging potential of crude extract and different fractions was reported [15]. The leaves of *E. fluctuans* have been reported to have hypotensive activity [16]. Chemical constituents such as different sesquiterpene lactones were isolated from the petroleum ether extract of *E. fluctuans* [17-20]. Gibberelin [21] and cholesterol derivatives [22] have also been reported from this plant.

The present investigation evaluated the hepatoprotective and antioxidant potential of flavonoid rich portion of *Enhydra fluctuans* against CCl_4-induced oxidative damage in rats.

Materials and Methods

Plant material

Fresh aerial part of the plant was collected locally in the rural belt of Bankura, West Bengal, India in the month of December, 2006 and identified by Dr. H. J. Chowdhury, Joint Director, Botanical Survey of India, Howrah, West Bengal, India. The voucher specimen (SS/2007/01) has been deposited in our laboratory for further reference. The plant material was shade dried and milled in mechanical grinder for further studies.

Drugs and chemicals

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatatse (ALP), total bilirubin, and total protein determination kits were purchased from Span Diagnostics Ltd., Surat, India. All the reagents and solvents used in the study were of analytical grade and commercially available include 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB) and carbon tetrachloride (Sisco Research Laboratory, Mumbai), nicotinamide adenine dinucleotide reduced (NADH), nitro blue tetrazolium, phenazine methosulphate, trichloro acetic acid, thiobarbituric acid (Loba Chemie, Mumbai).

Extraction and fractionation

Air-dried and powdered aerial part (1.8 kg) was extracted successively with petroleum ether (60-80°C) and methanol using Soxhlet apparatus. The solvents were then removed under reduced pressure and sticky residues were obtained. The crude methanol extract (105 g), after removal of the solvent, was dissolved in 10% sulfuric acid solution and subsequently partitioned with chloroform, ethyl acetate, and *n*-buatnol successively to give chloroform, EtOAc, *n*-BuOH soluble fractions. The ethyl acetate fraction (EAEF) was evaporated under reduced pressure and concentrated *in vacuo* to obtain a flavonoid rich fraction (12.9 g). Weighed amount of EAEF was suspended in Tween 80 prior to administration.

Determination of total flavonoid in ethyl acetate fraction

Total amount of total flavonoid in the ethyl acetate fraction was determined according to the method described by Moreno et al [23]. The sample solution (1 ml) contains 1 mg EAEF, in methanol was added to test tube containing 0.1 ml of 10% aluminium nitrate, 0.1 ml of 1 M potassium acetate and 3.8 ml of methanol. After 40 min at room temperature, the absorbance was determined at 415 nm. The total amount of flavonoids was calculated according to the equation obtained from the standard quercetin graph:

Absorbance = 0.0342 μg quercetin +0.01338 (R^2 = 0.9965)

Animals

Adult male Wistar albino rats weighing 150-200 g were used for the present investigation. They were housed in clean polypropylene cages and were fed with standard pellet diet and water *ad libitum* with a 12 h light-dark cycle. All the studies were approved by Jadavpur University Animal Ethical Committee, Kolkata, India.

Acute toxicity studies

The acute toxicity study EAEF was performed using albino rats. The animals were fasted for 3 h prior to the experiment and maintained under standard conditions. The EAEF suspension was administered orally in increasing doses up to 2000 mg/kg and observed for mortality and toxicity for up to 14 days.

Study protocol

Animals were divided into five groups (n = 6) and treated as follows. Group I treated as normal group which received liquid paraffin. Group II to group V were treated with CCl_4 in liquid paraffin (1:2) at the dose of 1 ml/kg body weight (b.w.) intraperitoneally once in every 72 h for 14 days. Ethyl acetate fraction of *E. fluctuans* (EAEF) at the doses of 200 and 400 mg/kg, b.w. were administered orally to the animals in group three and four daily for 14 days. Group V received sillymarin as a standard drug at the dose of 25 mg/kg, b.w. daily for 14 days.

Preparation of serum

Animals were anaesthetized under light ether anesthesia 24 h after the last treatment. The thoracic region was opened to expose the heart. Blood was obtained via cardiac puncture by means of a 5 ml hypodermic syringe and needle and placed in ice-cold 2 ml microcentrifuge tubes. It was allowed to clot and then centrifuged at 5000 r.p.m for 5 min. The serum samples were collected and left standing on ice until required estimation. Liver tissues were collected for biochemical and histopathological examination.

Biochemical estimations

Serum biochemical parameters like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatatse (ALP), total bilirubin and total protein were determined by using commercially available kits (Span Diagnostic Limited, Surat, India).

Lipid peroxidation

Liver tissues were homogenized in 1.15% KCl and the homogenate was centrifuged at 10000 *g* at 4°C for 20 min. From this microsomal fraction, lipid peroxidation in liver was ascertained by the formation of malondialdehyde (MDA) and measured by the thiobarbituric acid reactive substance (TBARS) method according to Ohkawa et al [24]. The levels of lipid peroxides were expressed as 'n' moles of thiobarbituric acid substances (MDA)/g of tissue using extinction co-efficient of 1.56 $x10^5$ $M^{-1}cm^{-1}$.

Superoxide dismutase (SOD) activity

Hepatic SOD activity was measured according to Kakkar et al [25]. The liver tissues were homogenized in phosphate buffer (67 mM, pH 7.8). The homogenate was centrifuged at 1600 rpm for 15 min. An adequate amount of the liver supernatant was mixed with the reaction mixtures, contained 0.2 ml of EDTA (6 mM) with 0.1 ml of sodium cyanide (NaCN, 3 mM), 0.05 ml of riboflavin (2 mM), 0.1 ml of nitro blue tetrazolium (NBT, 50 mM) and phosphate buffer in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp for 15 min, and the absorbance was measured at 530 nm. One unit of SOD is defined as the amount of enzyme required to reduce NBT by 50%. The specific activity of SOD was expressed as a unit mg^{-1} protein in supernatant.

Glutathione content (GSH)

For measure GSH, tissue fragments were thawed and homogenized on ice in 1 ml of homogenizing buffer (250 mM sucrose, 20 mM Tris-HCl, 1mM dithiothreitol, pH 7.4), using glass-teflon homogenizers. The homogenates were centrifuged at 15,000 rpm at 4°C for 2 h. The reduced glutathione was determined by the method of Ellman [26].

Catalase activity (CAT)

Liver tissue was homogenized in M/150 phosphate buffers in ice and centrifuged at 2000 rpm for 10 min at 4°C. From the supernatant, catalase activity was assayed by the method of Aebi [27].

Histological studies

Liver tissue was collected, washed in normal saline and fixed by using fixative (picric acid, formaldehyde 40% and glacial acetic acid) for 24 h. The fixed tissues were dehydrated with alcohol and then embedded in paraffin, cut into 3-5 μm sections, stained with the haematoxylin-eosin dye and finally, observed under a photomicroscope and morphological changes such as cell necrosis, ballooning degeneration, fatty changes or inflammation of lymphocytes were observed.

Statistical analysis

The results were expressed as mean ± S.D. (n = 6). The statistical significance of differences between groups was determined by one way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons among groups by using GraphPad Prism4, La Jolla, CA 92037, USA. Differences of $p<0.05$ were considered statistically significant.

Results

Amount of total flavonoids

Total amount of flavonoids in the faction was expressed as µg of quercetin equivalent per mg of the sample. The flavonoid content in the ethyl acetate fraction was found as 92.32 ± 12.25 µg-mg^{-1}.

Acute toxicity study

EAEF did not show any sign and symptoms of toxicity and mortality up to 2000 mg/kg dose.

Biochemical estimations

The effect of ethyl acetate fraction of *E. fluctuans* on AST, ALT, ALP, total bilirubin, and total protein in CCl_4 intoxicated rats are summarized in Table 1. AST, ALT, ALP, and bilirubin levels were significantly higher in rats receiving CCl_4 alone than the normal rats. There was a significant decrease ($p<0.01$) in all the hepatic enzymes in rats receiving EAEF (200 and 400 mg/kg) and silymarin. The total protein level comes to normal in drug treated rats when compared to CCl_4 treated group.

Lipid peroxidation

The effect of ethyl acetate fraction on the oxidative damage by CCl_4 is shown on Figure 1A. Malondialdehyde (MDA) is the product of lipid peroxidation and is a common marker of lipid peroxidation. The content of MDA was significantly ($p<0.01$) increased in the liver of CCl_4-treated rats (65.3 ± 1.94 nM/g tissue) as compared with the normal group (25.6 ± 1.77 nM/g tissue). Treatment with EAEF at the doses 200 and 400 mg/kg significantly ($p<0.05$) suppressed the formation of TBARS in the liver, suggestive of less oxidative damage of liver.

SOD activity

The effect of EAEF on hepatic SOD activity is shown in Figure 1B. SOD activity of the CCl_4 treated control group (15.26 ± 3.36 U/mg tissue) was found to be significantly lower than the normal group (36.32 ± 2.36 U/mg tissue). However, a significant dose dependent reversal of the SOD level was observed EAEF treated groups.

Glutathione content

Figure 1C shows the effect of EAEF on the content of GSH in CCl_4 induced hepatotoxicity in rats. The administration of CCl_4 alone significantly decreases the total glutathione content of the liver homogenate. CCl_4 administration with the tested compounds significantly ($p<0.05$) inhibited the depletion of GSH compared to the control group.

Catalase activity

Catalase activity in the liver homogenate is shown in figure 1D. CAT activity of the CCl_4 treated control group (152.45 ± 15.89 U/mg tissue) was significantly ($p<0.01$) lower than the normal group (274.36 ± 21.56 U/mg tissue). The CAT activities in EAEF and silymarin treated rats were significantly ($p<0.05$) higher compared to CCl_4 treated group.

Histological assessment

The hepatoprotective effect of EAEF was supported by histopathological assessment of the liver tissue. Liver tissue of normal rats showed no abnormal state in the liver architecture (Fig. 2A), while CCl_4 treated control group showed centri-lobular necrosis with large globule fatty degeneration (Fig. 2B). Treatment with EAEF along with CCl_4 decreased the inflammation and liver necrosis (Fig 2C, D).

Discussion

The elevated levels of serum enzymes are indicators of cellular leakages and loss of functional integrity of cell membrane in liver. Serum ALP and bilirubin levels on the other hand are related to the function of hepatic cells [28]. EAEF significantly decreased the AST, ALT towards normal level, indicating that EAEF preserved the structural integrity of the hepatocellular membrane and liver cell damage caused by CCl_4, which is confirmed by histopathological studies. The decrease in elevated bilirubin level and increase in the total protein level indicate the reduced injury to hepatic parenchyma.

MDA is one of the end products in the lipid peroxidation process. Increase in MDA content by CCl_4 oxidative damage, suggests enhanced lipid peroxidation leading to liver damage and failure of antioxidant defense mechanisms. Lipid peroxides are not only noxious to living organism, but also some of their stable breakdown products such as MDA have been recognized to cause some cell alterations by modifying protein structures. It has been shown that MDA is mutagenic to human cells [29] and play a significant role in DNA damage, sister-chromatid exchanges (SCEs) and carcinogenesis [30]. The significant reduction in MDA content in animals treated with EAEF suggests the protection of liver through its inhibitory action on lipid peroxidation.

SOD is an important antioxidant enzyme for stabilizing oxidative reactions. SOD plays a role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. The observed increase of SOD activity suggests that the EAEF have an efficient protective mechanism in response to ROS and also, these findings indicate that EAEF may be associated with decreased oxidative stress and free radical-mediated tissue damage.

Glutathione is an intracellular reductant and plays major roles in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds. Deficiency of GSH within living organisms can lead to tissue disorder and injury. For example, liver injury induced by alcohol [31] or by acetaminophen [32], lung injury by smoking [33] and muscle injury by intense physical activities [34] are all known to be correlated with low tissue levels of GSH. The GSH reduction in control group may be explained by increased utilization of GSH for removal of ROS and lipid damaged products. In our study, treatment with EAEF restored the depleted glutathione levels in a dose dependent manner. EAEF may act by inducing the detoxifying enzymes, which might detoxify the free radicals produced following carbon tetrachloride intoxication.

Catalase decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals. Therefore reduction in the activity of the enzyme may result in a number of deleterious effects due to the accumulation of hydrogen peroxide. Administration of EAEF restored the activities of catalase in CCl_4 intoxicated rats. Our results revealed that EAEF prevented excessive free radicals accumulation and protected the liver from CCl_4 intoxication.

The histopathological findings of liver tissue suggest that treatment with EAEF reduced the liver injury. Cytoplasmic vacuolation of liver cells was due to excess accumulation of lipid [35] and glycogen [36]. Hepatoprotective activity of EAEF can also be explained on the basis of histological examination.

Wide varieties of naturally occurring compounds can protect the liver and other tissues from damage [37]. It is conceivable that these active principles like flavonoids, alkaloids and saponins were alone or in combination with others responsible for protecting the liver against CCl_4 induced damage, as demonstrated in this study. Fractionation of the methanol extract with ethyl acetate pulled up the flavonoids present in the extract to give a flavonoid rich fraction. Ethyl acetate fraction also found as potent source of polyphenolics and exhibits promising free radical scavenging activity [15]. Recently the protective effect of total flavonoids on animal liver injury and liver fibrosis has been proved by some authors [38-40].

Based on experimental data, it can be concluded that flavonoid rich ethyl acetate fraction of *E. fluctuans* had significant hepatoprotective effects. The possible mechanisms of protection include the inhibition of lipid peroxidation and increase in the content of enzymatic defense system, which cause the recuperation of biological parameters and the integrity of the tissue. The present results can attest for the traditional uses of the plant in the treatment of liver diseases.

Table 1.

Effect of ethyl acetate fraction of *E. fluctuans* and silymarin on serum biochemical parameters[#].

Biochemical parameters	Normal	CCl_4 1 ml/kg	EAEF 200 mg/kg	EAEF 400 mg/kg	Silymarin 25 mg/kg
ALT (U/l)	33.84 ± 4.1[a]	91.76 ± 18.36	40.22 ± 12.39[a]	35.09 ± 5.86[a]	41.34 ± 12.15[a]
AST (U/l)	109.94 ± 18.05[a]	372.72 ± 72.95	164.42 ± 28.85[a]	146.33 ± 19.33[a]	186.96 ± 53.38[a]
ALP (U/l)	120.59 ± 25.52[a]	400.74 ± 34.07	211.53 ± 16.52[a]	199.72 ± 10.68[a]	161.70 ± 21.79[a]
Total protein (mg/dl)	8.39 ± 1.19[a]	4.72 ± 0.54	6.12 ± 0.24[*ns*]	7.26 ± 0.21[a]	7.63 ± 0.47[a]
Total bilirubin (mg/dl)	1.29 ± 0.19[a]	4.66 ± 0.24	2.86 ± 0.24[a]	2.39 ± 0.33[a]	2.12 ± 0.13[a]

[#] Values are mean ± s.d. (n = 6) [a] $p<0.01$, *ns* = not significant

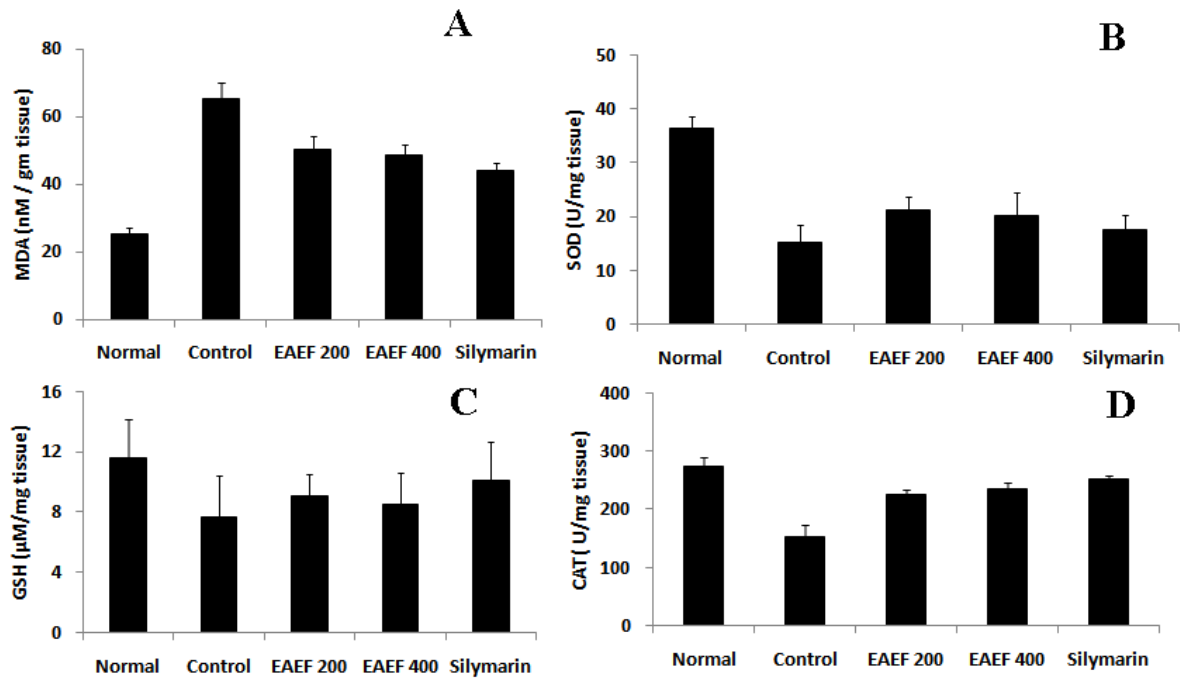

Figure 1. Effect of EAEF on lipid peroxidation (A), SOD activity (B), GSH level (C) and CAT activity (D).

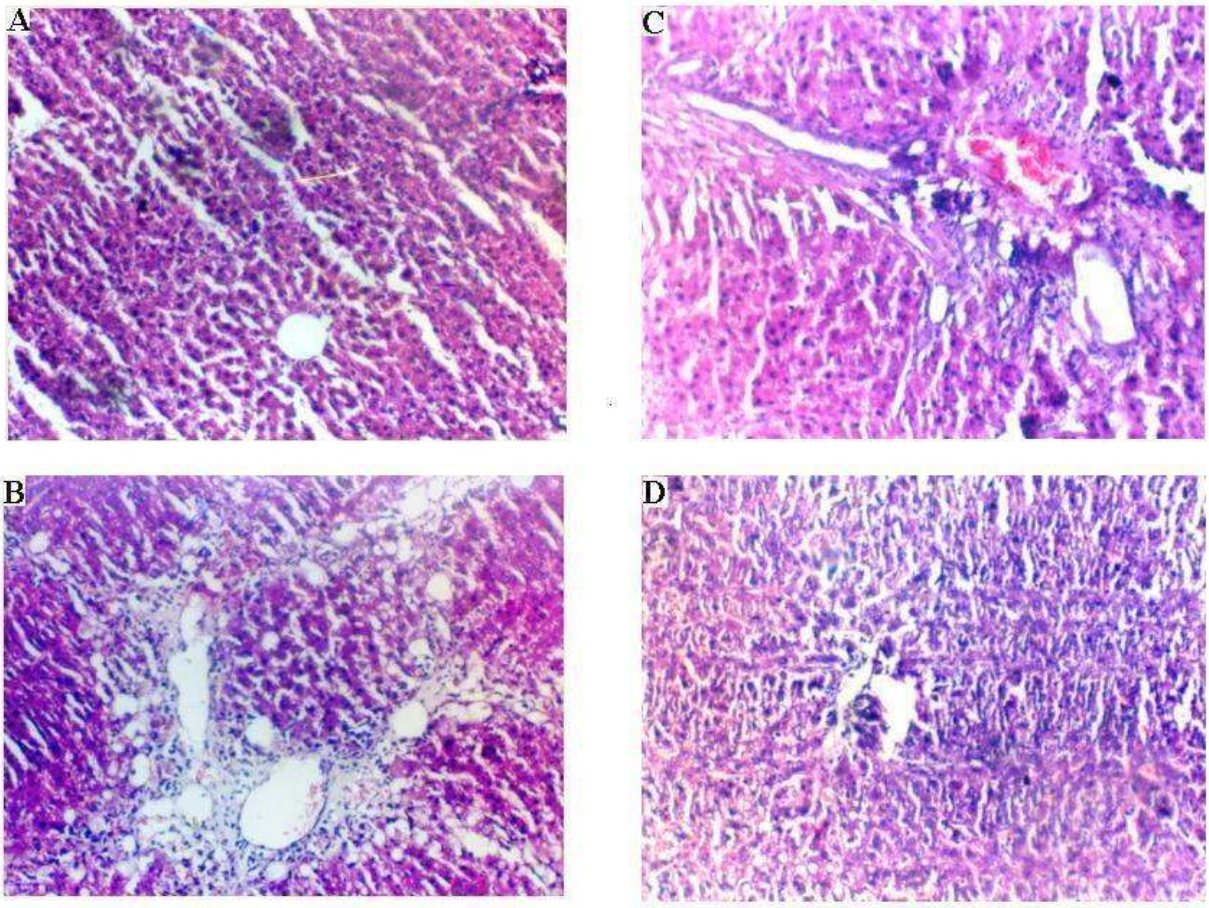

Figure 2. Histopathological examination of liver tissues of different experimental groups. (A) Normal rats (B) CCl_4 treated control rats (C) Rats treated with EAEF200 along with CCl_4 (D) Rats treated with EAEF400 along with CCl_4.

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