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RESEARCH ARTICLE

HEPATOPROTECTIVE POTENTIAL OF TUBERS OF *HABENARIA INTERMEDIA* D.DON. AGAINST CARBONTETRACHLORIDE INDUCED HEPATIC DAMAGE IN RATS

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ABSTRACT

Habenaria intermedia D.Don. (Orchidaceae) is commonly known as Vrddhi in Indian system of medicine. The purpose of the study was to assess the effects ethyl acetate and ethynolic extracts of tubers of *Habenaria intermedia* against Carbon tetrachloride (CCl_4, 0.7 ml/kg.p.o. in liquid paraffin 1:1, i.p.) induced hepatotoxicity in rats. The hepatoprotecitve effect of extracts was evaluated by the assay of liver function and biochemical parameters such as SGOT, SGPT, SALP, serum bilirubin and cholesterol. Ethyl acetate and ethanol extracts (100 and 200 mg/kg p.o) exhibited significant ($p<0.001$), hepatoprotecitve activity by restoration of increased levels of serum bilrubin, cholesterol and enzymes in CCl_4 induced hepatotoxic animals compared to the normal and the standard drug Silymarin treated groups. Histopathlogical studies of liver sections of animals treated with extract showed regeneration of hepatocyts, absence of necrosis and fatty infiltration, which further give evidence for the hepatoprotecitve activity.

INTRODUCTION

The liver plays a vital role in drugs, xenobiotic metabolism and biological equilibrium of the organisms. Liver, functioning in the removal of substances from the portal circulation, is susceptible to the toxicity from drugs xenobiotic and oxidative stress, culminating in liver dysfunction. Inspite of tremendous stride in modern medicine there is no specific treatment to counter the menacing impact of dreaded diseases (Chaterjiee, 2000). There are few drugs that stimulate liver function, offering protection to the liver from damage and regeneration of hepatic cells. Due to this fact, attempts are being made to find suitable curative agents, less toxic and free from side effects than synthetic drugs, originating from natural product of plants and minerals for the treatment of liver diseases (Bhandarkar and Khan, 2004).

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Liver damage induced by carbon tetrachloride is the best model of xenobiotic induced hepatotoxicity in human beings. Carbon tetrachloride causes liver and kidney damage through free radical mediated process. Scientists are searching for the traditional system of medicine for possible remedies to cure various hepatic disorders, as there are no potential drugs to cure completely all liver damage (Lee *et al*., 2004). *Habenaria intermedia* D. Don (Family: Orchidaceae) is commonly known as Vrddhi in Indian system of medicine. It is endangered medicinal orchid, perennial growing in Assam, Eastern Himalayan Nepal forests at an elevation of 1500-2800 meters. The edible tubers are sweet, emollient and used as intellect promoting, aphrodisiac, depurative, anthelmintic, rejuvenating and tonic. Tubers are also useful in asthma, leprosy and skin diseases. This plant is an important ingredient of Chyavanprasha and well known polyherbal rejuvenator (Warrier *et al.,* 1994; Kirtikar and Basu, 1994). The tubers of the plant contain steroids, flavanoids Coumarin glycosides and tannins. Tannins being polyphenolic in nature are also known

to exhibit antioxidant property (Candan and Sokmen, 2004). Antioxidant activity of polyherbal formulations containing tubers of *Habenaria intermedia* was investigated in nitric oxide scavenging activity (Jagetia *et al.,* 2004). The present investigation was designed to evaluate the hepatoprotecitve activity of *Habenaria intermedia* D.Don in CCl_4 induced toxicity in rat liver.

MATERIALS AND METHODS

Chemicals

All the solvents and chemicals used were of analytical grade. Standard kits for SGOT, SGPT and Bilirubin (Teco Diagnostic, USA) and Cholesterol (Span Diagnostics, India), Standard drug Silymarin (Micro laboratory, India), were used in the present study.

Plant material and extraction

Tubers of *Habenaria intermedia* D. Don were obtained from Forest research institute, Dehra Dun, India and authenticated by qualified taxonomist, Department of Botany, Kuvempu University, Shankaraghatta. The collected tubers were washed with running water and chopped into small pieces and shade dried. Dried tubers were coarsely powdered and exhaustively extracted with ethyl acetate and ethanol (95%) using Soxhlet extractor. The extracts were concentrated by rotary flash evaporator under reduced pressure and controlled temperature, followed by freeze drying and stored in a desicator. The average yield of ethyl acetate extract, EAHI (Yellowish black-semisolid), was 0.9% w/w and ethanol extract, EtHI (Brown-sticky solid) was 2.5% w/w. Suspensions of each extract were prepared using Tween-80 and distilled water (2:8). The suspensions were used to assess hepatoprotecitve activity in CCl4 induced liver toxicity.

Animals

Adult mice (25-30g) and wister rats (180-200g) were used in the present study. The animals were procured from disease free animal house, National Institute of Pharmacy, Shivamogga, Karnataka, India. All the animals were kept in quarantine for 10 days under standard husbandry conditions. (27.3°C, RH–65°C±10%) for 12hr in dark and light cycle respectively and were given standard food (Hindustan lever) and water *adlibitum*. The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) and care of laboratory animals was taken as per CPCSEA guidelines.

Acute toxicity study

Acute toxicity study was conducted on Swiss albino mice weighing between 20-25g using stair case/up and down method. The extracts were orally administered to different groups of mice at dose of 50-2000 mg/kg. p.o. Convulsion, sedation, body temperature, mortality and behavioral changes if any were observed (Reid and Munch, 1938**).** Extracts of *Habenaria interme*dia did not produce any toxicity up to dose level of 2000 mg/kg. p.o.

Experimental Protocol

Rats were divided into seven groups comprising of six animals in each group. Group I served as normal control and received normal saline (5 ml/kg p.o) for seven days. Group II was administered with CCl_4 in liquid paraffin (0.7 ml/kg, 1:1, v/v, i.p, on alternate days (Singh *et al*., 1999; Muzumdhar *et al*., 1998). Group III was administered with Silymarin (100 mg/kg p.o) simultaneously with toxicant (Rege *et al*., 1984). Group IV and Group V were treated with ethyl acetate extract (100 and 200 mg/kg p.o). Group VI and VII were administered with ethanol extract (100 and 200 mg/kg p.o).

Assessment of Hepatoprotecitve activity

On the seventh day after administration of last dose of extracts, the rats were anesthetized by light ether anesthesia and blood was collected from the retro-orbital plexus. It was allowed to coagulate for 30 min and serum was separated by cold centrifugation at 2500 rpm. The centrifugate was used to estimate the serum glutamate pyruvate transminase (SGPT), Serum glutamate oxaloacetate (SGOT) (Rietman, 1957) and serum alkaline phosphatase (SALP**)** (King, 1959).Total and direct bilirubin and cholesterol (Jendrassik and Grof, 1938) were also determined.

Histopathlogical studies

For histopathological observation, sections were taken from each lobe of liver immediately. The tissue was fixed in 10% neutral formalin, dehydrated in graded ethanol and embedded in paraffin, cut into 4-5µ thick sections, stained with Haematoxylin and Eosin for photomicroscopic assessment (Galigher and Kozloff, 1971).

Statistical analysis

The data were expressed as mean ± SE (n=6). The data were analyzed using one way ANOVA followed by Dunnet's multiple comparison tests. $p<0.01$ were considered statistically significant (Dunnet, 1964).

RESULTS

Phytochemical analysis

Preliminary phytochemical tests and thin layer chromatographic studies ethyl acetate and ethanol extracts of tubers of *Habenaria intermedia* revealed the presence of flavanoids, coumarin glycosides, carbohydrates, steroids, alkaloids and tannins (Khandelwal, 2008; Kokate, 1994)

Acute toxicity studies

Habenaria intermedia extracts (EAHI and EtHI) did not show any toxicity and behavioral changes in mice up to 2000 mg/kg.p.o. Hence, the doses selected were 100 and 200 mg/kg.p.o.

Hepatoprotecitve activity

Rats were treated with CCl_4 (0.7 ml/kg in liquid paraffin, 1:1, i.p.) suffered from hepatotoxicity. The serum levels of SGOT,

SGPT, SALP (Table 1 and Figures 1-3), bilirubin (Total and direct) and cholesterol levels (Table 2 and Figures 4-6) were significantly elevated. Ethyl acetate and ethanolic extracts (100 and 200 mg/kg, p.o.) of tubers of *Habenaria intermedia* exhibited significant hepatoprotecitve activity ($p<0.001$) by decreasing the elevated enzymes and bilirubin level against CCl_4 induced hepatotoxicity. Results were further supported by histopathological studies. Results were also comparable with standard drug Silymarin (100 mg/kg.p.o.)

Histopathlogical observations

Histopathology of normal rat liver shows prominent central vein, normal arrangement of hepatic cells (Figure 7). Microscopic examination of CCl_4 treated liver section shows centrilobular necrosis, kupffer cells around the central vein and fatty degeneration (Figure 8). Liver section treated from Silymarin protected the structural integrity of hepatocyte cell membrane and showed recovery of hepatic cells (Figure 9).

Table 1. Effect of *Habenaria intermedia* D. Don on Biochemical parameters

Group	Dose mg/kg	SGOT (µ/1)	SGPT(µ/1)	ALP (µ/1)
Normal	5ml (Saline)	164.3 ± 3.66	71.85 ± 4.40	205.1 ± 7.99
CCl_4	0.7	692.0 ± 11.87[a]	325.5 ±10.18[a]	386.9 ± 7.18[a]
Silymarin CCl_4	100	294.9 ± 2.12[b]	78.64 ± 0.92[b]	236.4 ± 3.03[b]
Ethyl acetate Extract+ CCl_4	100	310.6 ± 2.82[b]	137.8 ± 3.58[b]	246.9 ± 2.80[b]
Ethyl acetate Extract+ CCl_4	200	304.3 ± 2.16[b]	132.3 ± 3.21[b]	240.2 ± 2.34[b]
Ethanolic Extract+ CCl_4	100	315.5 ± 4.33[b]	152.0 ± 3.09[b]	251.0 ± 3.10[b]
Ethanolic Extract+ CCl_4	200	309.2 ± 4.12[b]	146.2 ± 3.01[b]	247.4 ± 3.02[b]

Values are mean ±SE from 6 animals in each group;
[a]p*<0.01 as compared with Normal Control group;
[b]p* <0.00 1 as compared with CCl_4 and control group.

Table 2. Effect of *Habenaria intermedia* D. Don on cholesterol, total and direct bilirubin levels in rats

Group	Dose mg/kg	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Serum Cholesterol (mg/dl)
Normal	5ml (saline)	1.205 ± 0.63	0.4773 ± 0.02	64.96 ± 0.69
CCl_4	0.7	2.239 ± 0.12[a]	0.9402 ±0.07[a]	115.2 ± 2.51[a]
Silymarin CCl_4	100	0.8865 ± 0.04[b]	0.60 ± 0.011[b]	76.14 ± 1.37[b]
Ethyl acetate Extract+ CCl_4	100	1.53 ± 0.03[b]	0.62 ± 0.02[b]	68.79 ± 0.85[b]
Ethyl acetate Extract+ CCl_4	200	1.46 ± 0.01[b]	0.58 ± 0.01[b]	66.35 ± 0.64[b]
Ethanolic Extract+ CCl_4	100	1.62 ± 0.02[b]	0.60 ± 0.02[b]	77.76± 0.85[b]
Ethanolic Extract+ CCl_4	200	1.59 ± 0.02[b]	0.56 ± 0.02[b]	67.35 ± 0.56[b]

Values are mean ±SE from 6 animals in each group;
[a]p* <0.01 as compared with Normal Control group;
[b]p* <0.001 as compared with CCl_4 and control group.

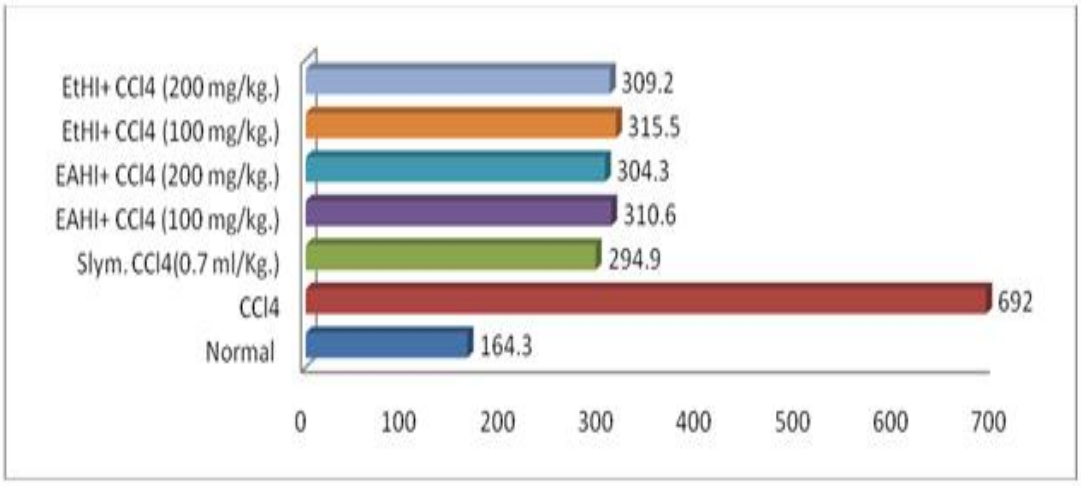

Figure 1. Effect of *H. intermedia* extract on SGOT (µ/l.)

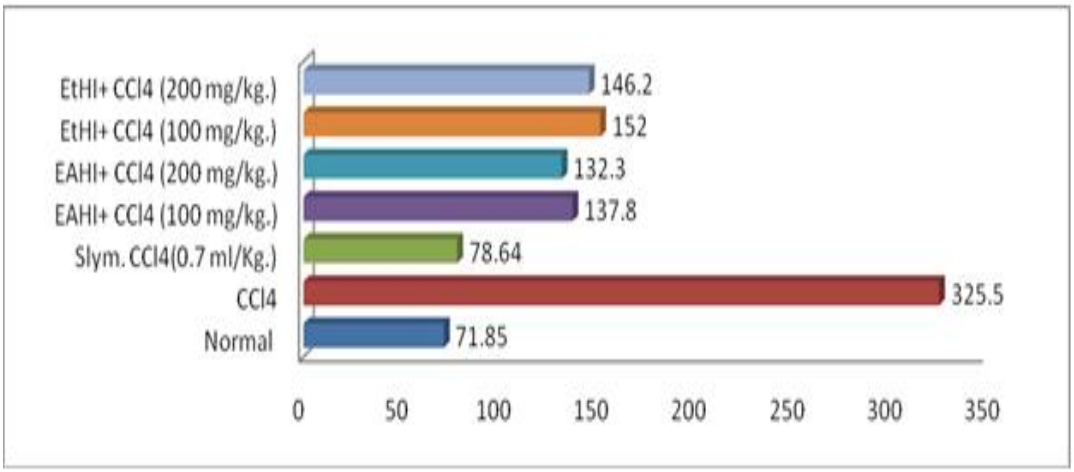

Figure 2. Effect of *H. intermedia* extract on SGPT (µ/l.)

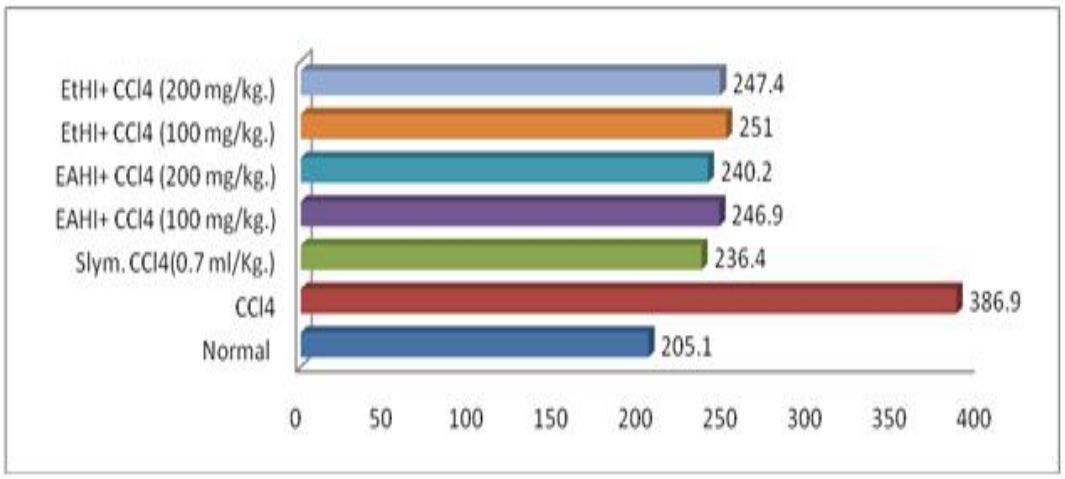

Figure 3. Effect of *H. intermedia* extract on SALP (µ/l.)

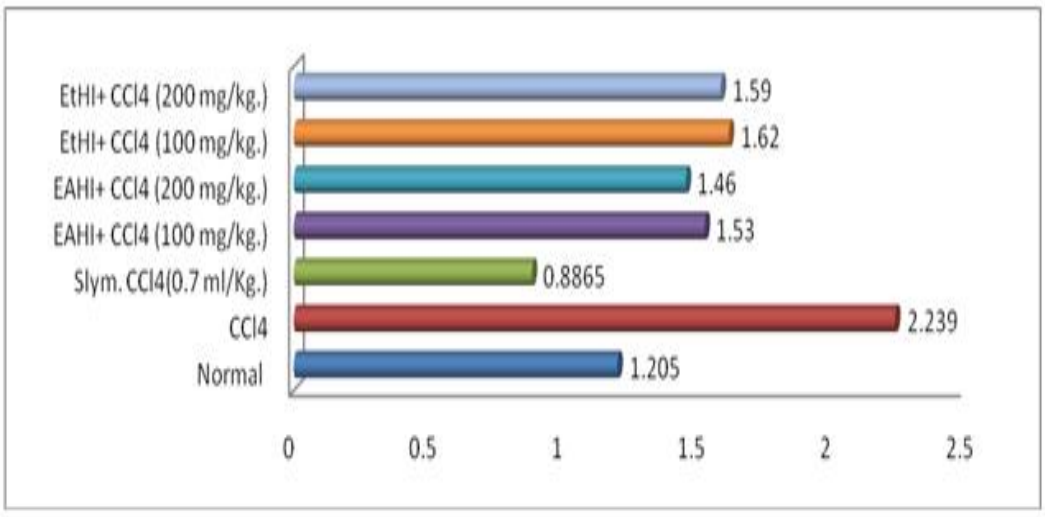

Figure 4. Effect of *H. intermedia* extract on Total Bilirubin (mg/dl.)

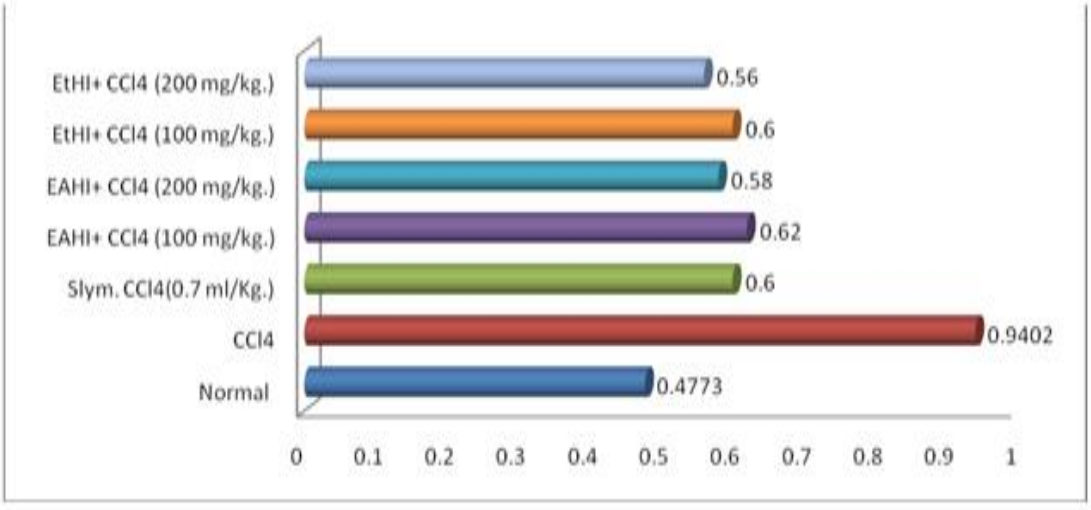

Figure 5. Effect of *H. intermedia* extract on direct Bilirubin (mg/dl.)

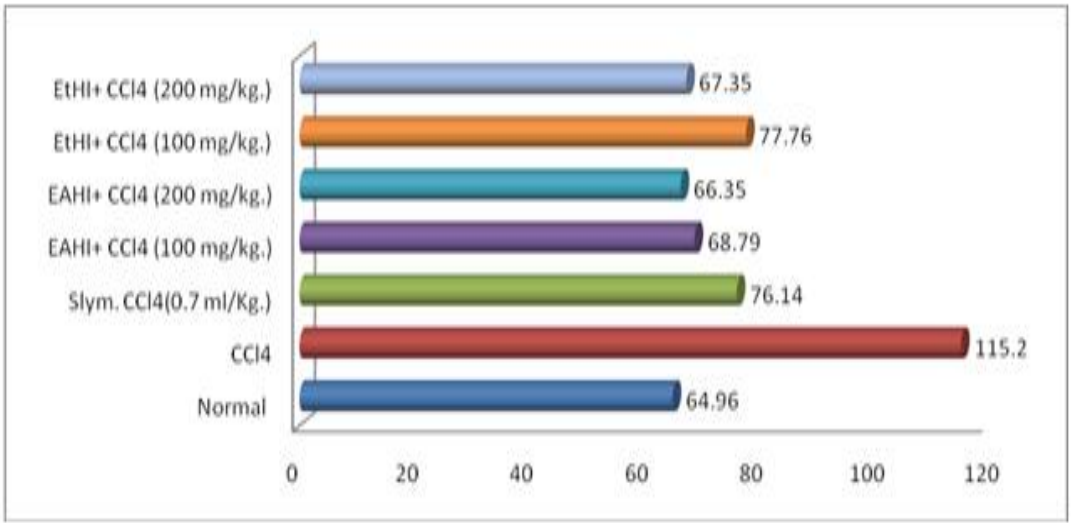

Figure 6. Effect of *H. intermedia* extract on serum cholesterol (mg/dl.)

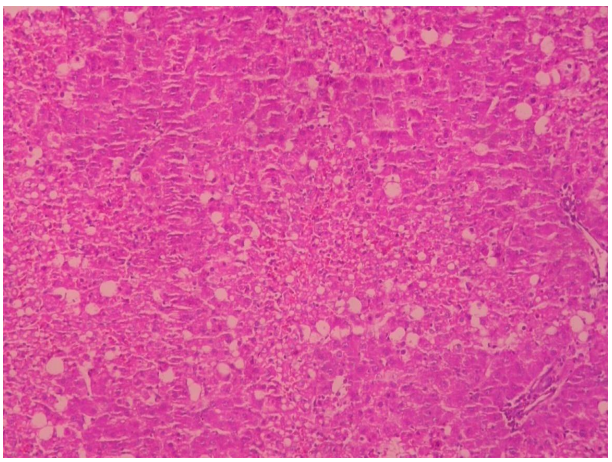

Figure 7. Histopathology of nornal treated group

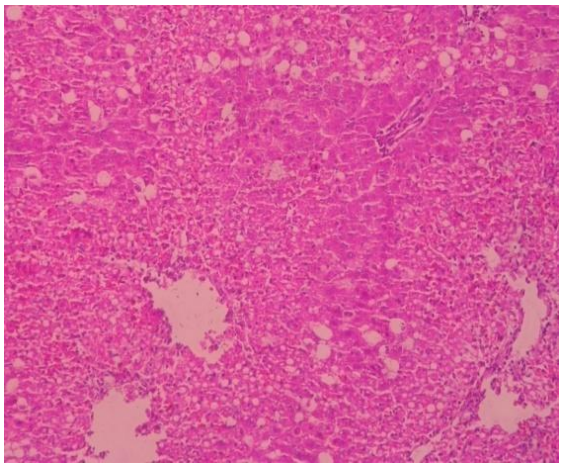

Figure 8. Histopathology of CCl4 treated group

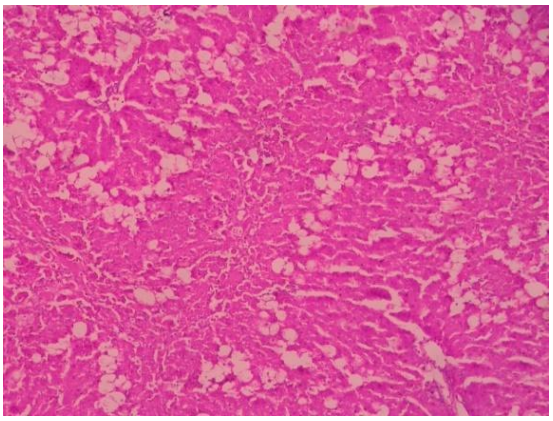

Figure 9. Histopathology of Silymarin treated group

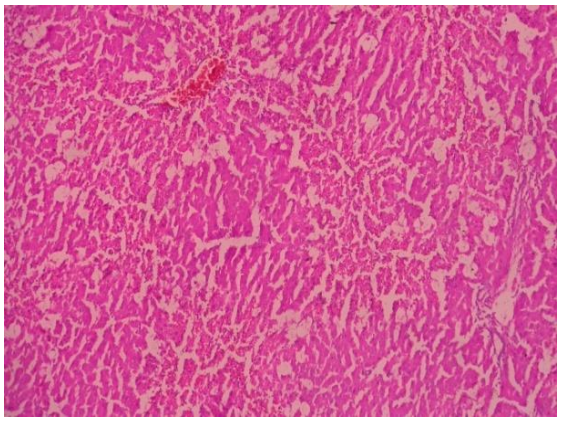

Figure 10. Histopathology of ethyl acetate treated group

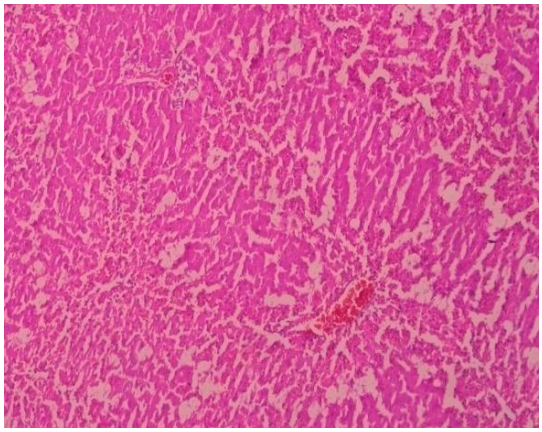

Figure 11. Histopathology of ethonolic treated group

Ethyl acetate and Ethanol extract of tubers of *H. intermedia* treated groups showed mild recovery of hepatocyts, no fatty degeneration, no centrilobular necrosis and exhibited significant protection against CCl_4 induced liver damage in rats (Figure 10 and 11).

DISCUSSION

CCl_4 induced hepatic damage is due to its cytochrome P-450 enzyme system catalyzed hepatic conversion into highly reactive trichloromethyl radical (CCl_3^*) that upon reaction with oxygen radical gives trichloromethyl peroxide radical (CCl_3COO^-) This radical forms covalent bond with sulfohydroxyl group of several membrane molecules like glutathione, which is considered as the initial step in the chain of events leading to lipid peroxidation and hepatic tissue destruction (Brattin and Glende, 1985; Ahmad and Raharah, 2000; Kyung *et al*., 2004; Be-JenWang *et al*., 2004). The degree of hepatotoxicity developed by CCl_4, can be observed by elevated levels of biochemical parameters which are attributed to the generation of trichloromethyl free radical during metabolism by hepatic microsomes which in turn cause peroxidation of lipids of cellular membrane (Recknagel and Glende, 1989).

Hepatocellular necrosis lead to very high level of SGOT, SGPT released from liver in the blood. Among the two, SGPT is a better index of liver injury, as liver SGPT activity represents 90% of total enzyme present in the body (Achilya and Kotgale, 2004). SALP activities on the other hand are related to the functioning of the hepatocyts, increase in its activity is due to increased synthesis in presence of increased biliary pressure (Moss and Butterworth, 1974). Reduction in levels of SGOT and SGPT towards the respective normal value, by EAHI and EtHI, is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damages caused by carbon tetra chloride.

The serum levels of transminase return to normal with healing of hepatic parenchyma and regeneration of hepatocyts. Suppression of increased SALP activity with concurrent depletion of raised bilirubin level suggests the stability of the biliary dysfunction in rat liver during hepatic injury with CCl_4 (Mukerjee, 2002). The suspensions of ethyl acetate(EAHI) and ethanolic(EtHI) extracts (100 and 200 mg/kg p.o) of tubers of *H. intermedia* significantly decreased the CCl_4 induced elevated enzyme levels suggests the protection of structural integrity of hepatocyte cell membrane or regeneration of damaged liver cells by the extracts.

Conclusion

The ethanol and ethyl acetate extracts of tuber of *Habenaria intermedia* are beneficial in the prevention of formation of fatty liver and thereby protect the liver and hepato-enzymes which are commonly involved in combating reactive Oxygen species. Hence tuber of *Habenaria intermedia* can be of enormous use in the management of oxidative stress and treatment of various liver disorders.

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Ethical clearance

The research work was approved by Institutional Animal Ethical Committee (NCP/IAEC/CLEAR/25/02/2009-10, dated 09/03/2010)

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