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HEPATOPROTECTIVE ACTION OF ETHANOLIC EXTRACT OF *CYPERUS PERTENUIS* IN CHRONIC INTOXICATED ALBINO RATS

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ABSTRACT

Objective: The objective of this research work was to evaluate the ability of *Cyperus pertenuis* dried roots in providing protection against paracetamol induced hepatotoxic chronic models of albino rats.

Methodology: Ethanolic extract of dried roots of *Cyperus pertenuis* was prepared by maceration which was then evaluated for phytoconstituents. Male albino rates were divided in five groups (n=6) i.e. Group-I or Vehicle control group was only administered with 5% CMC (10ml/kg). Group-II was disease induced group which was given paracetamol (500mg/kg) for seven days. Group-III was standard group given silymarin 100mg/kg prior to administration of paracetamol 500mg/kg daily for seven days. Group-IV and V were given plant extract (200 and 400mg/kg respectively) prior to administration of paracetamol 500mg/kg daily for seven days. On 8th day animals were sacrificed to get their blood and liver. Serum was used to evaluate hepatic enzymes i.e. AST, ALT, ALP and TB and liver biopsies were used for histopathological analysis. **Results:** Paracetamol significantly increased hepatic enzymes while a significant reduction in level of hepatic enzymes were observed in plant extract treated rats. Two way ANOVA test was applied which declared that extracts in dose of 400mg/kg produced significant (P<0.001) results. Moreover,

histopathological slides of disease group were appeared to be damaged by necrosis while no physical damage was observed in the liver of extract treated animals. **Conclusion:** Results concluded that *Cyperus pertenuis* plant can be used to protect livers against paracetamol poisoning.

Keywords: ALP, Histopathology, Hepatocytes, Paracetamol, AST

INTRODUCTION

Every day exposure of toxic substances to body may have deleterious effect on the health of human body [1]. Liver is mainly targeted by toxins and chemical substances while first passing by hepatic portal circulation [2] Some drugs prescribed on daily basis may also contribute to the damage of liver like paracetamol, nimesulide [3], diclofenac and indomethacin etc [4], So a powerful hepatoprotective agent is always needed by the body to cope with the harm of these hepatotoxic drugs. Currently, silymarin is available in market to protection of liver from toxic effects of chemicals [5]. Trend is shifting towards the use of conventional remedies to protect the hepatocytes from damage. In india a huge there is a huge list of plants which are used in conventional medicines for the protection of liver and for detoxification purposes [6]. *Capparis spinosa* [7], *daccus carota* [8], *euphorbia antisyphilitica* [9], *hygrophyla auriculata* [10], *lycium chinensis* [11], *rubia cordifolia* [12], *silybum marianum and zingiber officinale* [8, 13], have been extensively studied for their hepatoprotective activities. *Cyperus pertenuis* is a herb that belongs to family Cyperaceae and it has been traditionally used with combination of others plants in gastrointestinal and liver disorder. This plant is first time evaluated for its hepatoprotectivity. The aim of this study was to evaluate the hepatoprotective effect of ethanolic extract of *Cyperus pertenuis* dried roots in chronic intoxicated rats.

MATERIALS AND METHODS

Chemicals and Equipments: Diagnostic kit of ALT, AST, ALP and TB were obtained from Merck, ethanol, Formalin, n-hexane, methanol, Sodium bicarbonate, silymarin, xylene, paraffin, hematoxylin, eosin dye. All the chemicals of analytical grade were used. Paracetamol was donated by Consolidated Chemical Laboratories Pvt. Ltd. Silymarin was purchased from Abbott Laboratories, Pakistan. Digital electronic balance (FA2004B,Yoke Galvano), Centrifuge machine (Pro-economic, Vortex Mixer (VM-300), Grinder (Waves, Pakistan), Selectra-Pro-s (Merck, Germany), Microwave oven (DHG-9053A), Microscope, Spectrophotometer (UV1900, Yoke Galvano).

Collection of Plant and Extracts preparation: Dried roots of *Cyperus pertenuis* was purchased from local market of Lahore and were identified by botanist of Department of Botany, University of the Punjab Lahore. Plant specimen was preserved in herbarium of Riphah Institute of Pharmaceutical Sciences Lahore which was given voucher no RIU-02-07-33. Dried roots were crushed into coarse particle sizes powder with the help of grinder and its known weight (1kg) was soaked in ethanol for five days. After five days it was filtered and residue was again soaked in ethanol for another five days and then filtered. Filtrates were subjected to rotary evaporator for evaporation of solvent. Finally semi solid paste was obtained which was kept in air tight glass container and preserved into refrigeration after proper labeling. The %age yield of plant extract was determined by using formula;

% yield=weight of extract / total weight of powdered material × 100

Solubility test: Different solvents such as water, ethanol, hexane, normal saline, chloroform, di-methyl sulfoxide and ethyl acetate were taken and plant extract w/v was dissolved to find its solubility. The result was expressed in terms of soluble, insoluble and slightly soluble.

Phytochemical screening: Chemical tests were carried out on the ethanolic extract of plant and on the powdered specimens using standard procedures to identify the constituents.

Test for tannins: 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration**.**

Test for Alkaloids: 500-600 mg of crude extract was treated with 8 ml of 1% HCl, warmed on water bath and then filtered and divided in to four test tubes.

    a. ***Hager's test:*** 2 ml of filtrate was mixed with few drops of Hager's reagent. Saturated solution confirmed the presence of alkaloids.

    b. ***Wagner's test:*** 2 ml of filtrate was mixed with few drops of Wagner's reagent. Appearance of reddish brown precipitates showed the presence of alkaloids.

    c. ***Dragendorff test****: 2 ml of filtrate was mixed with Dragendorff's reagent. Turbidity or precipitates indicated the presence of alkaloids.

    d. ***Mayer's test:*** 2 ml of filtrate was mixed with Mayer's reagent. Appearance of turbidity or precipitates indicated the presence of alkaloids [14].

Test for saponin: About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5 ml of

distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Test for steroids: Two ml of acetic anhydride was added to 0.5 g of each sample with 2 ml H2S04. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for cardiac glycosides (Keller-Killani test): 5ml of extract plus 2ml of glacial acetic acid plus one drop FeCl3 solution was mixed and then it was under layed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer **[15]**.

Tests for terpenes and Sterols Libermann-Burchard Test: 30 ml of crude extract was added in petroleum ether. Petroleum ether was evaporated to get dry residue. Residue was extracted with 20 ml of chloroform and the chloroform layer was then treated with anhydrous Sodium Sulphate. 0.5 ml of acetic anhydride was mixed with 5 ml of chloroform layer. Then two drops of concentrated H_2SO_4 was added which gave green, blue and pink to purple colors. Green to pink color indicated the presence of sterols while pink to purple colors is proof of presence of triterpenes **[16]**.

Test for carbohydrates:

a. ***Molisch's Test:*** A test tube was taken and 2 ml of plant extract was added then 2 drops of α-naphthol solution. Inclined the tube with great care and pour drop wise conc. H_2SO_4, using a dropper, along the sides of the tube. Violet color at the junction of the two liquids indicate positive test.

b. ***Fehling's Test:*** In a test tube, 2 ml plant extract was added and then poured equal volumes of Fehling

A & Fehling B and place it in a boiling water bath for few minutes.. When the content of the test tube came to boiling, mixed them together and color changed or precipitate formation was observed. The appearance of yellow 'or brownish-red precipitate of cuprous oxide showed the presence of reducing sugars in the given sample.

Test for proteins:

a. ***Biuret test:*** A liquid plant extract is treated with an equal volume of 1% strong base (sodium or potassium hydroxide most often) followed by a few drops of aqueous copper sulfate. If the solution turns purple,

protein is present. (5–160 mg/mL can be determined).

b. *Ninhydrin test:* Took5 drop 0.1% ninhydrin's solution in test tube and added with 2 ml plant extract and warmed in a boiler approximately for 10 minutes.

Test for fixed oil and lipid:

a. *Grease spot test:* Several drops of the test solution were placed on a sheet of paper and the solvent was allowed to evaporate, the formation of translucent spot on the paper indicated the presence of triglycerides.

Experimental animals: Male albino rats of weight 150-180g were selected for this study, which were purchased from the University of Veterinary and Animal Sciences (UVAS) Lahore. The animals were kept in animal house of Riphah Institute of pharmaceutical Sciences (RIPS) where they were provided with standard condition of temperature (25°C), humidity (50%) and 12/12 h of light and dark cycles and fed with standard pellet diet and water. They were housed individually in cages containing sterile paddy husk throughout the experiment and had free access to sterile food and water. The study was started after getting approval for the usage of animals from departmental research and

ethical committee with protocol no RIU-ACE-118.

Hepatoprotective study in chronic models: Male albino rats were divided into five groups with six animals in each group. Group-I was kept as normal or vehicle control group which was administered only with 0.5%CMC (10ml/kg) once daily for 7 days. Group-II was assigned as disease control group and was administered only with paracetamol suspension (500mg/kg/p.o) once daily for seven days. Group-III was standard control group administered with silymarin in dose of 100mg/kg/p.o. an hour before administration of paracetamol suspension (500mg/kg/p.o) once daily for seven days. Group-IV was experimental control-I which was administered with ethanolic extract suspension of *C. pertenuis* in dose of 200 mg/kg/p.o. once daily for 7 days one hour before administration of paracetamol suspension (500mg/kg/p.o). Groups-V was marked as experimental control-II and administered with same extract of *C. pertenuis* (400 mg/kg/p.o.) once daily for 7 days one hour before administration of paracetamol suspension (500mg/kg/p.o). Animals were sacrificed 24 h after the administration of last dose and their blood was collected. Blood was allowed to clot for 15 minutes and serum was separated. Then the liver of rats were removed and washed with normal saline then preserved

in formalin solution for later on histopathological studies. The pieces of liver were processed and embedded in paraffin wax. Sections of about 3-4μm were cut down and were fixed on glass slides. They were stained with hematoxylin and eosin and were used for histopathological evaluations [17].

Effect on Body Weight: Initially the weights of animals were noted before the start of study at day one and then on daily basis weights were noted before administration of dose. Then finally weights of animals were found just before the induction of anesthesia. Then difference in their mean body weights was calculated and observations were recorded in table 5.

Histopathological slides preparation and Grading: Liver slides were prepared by assessing of specimens and then tissues were fixed on glass slides. They were dehydrated and cleared from fixing solutions and water. Tissue was then impregnated and embedded after sectioning. Slides were stained out and specimens were generally graded into four categories [18, 19] which were periportal necrosis, intralobular necrosis, portal inflammation and fibrosis. Scoring is described in table 1 as follow;

Table 1:   HAI (Histology Activity Index) for Grading of slides [19]

Indication	Stage	Score	Omitted number
Normal	0	0	0
Periportal ±Bridging	Mild	1	2,7,8,9
necrosis	Moderate	3	
	Marked	4,5,6,10	
Portal inflammation	Mild	1	2
	Moderate	3	
	Marked	4	
Fibrosis	Mild	1	2
	Moderate	3	
	Marked	4	
Focal necrosis and	Mild	1	2
Intralobular degeneration	Moderate	3	
	Marked	4	

Statistical Analysis: All results were expressed by Mean±S.D and were statistically analyzed by using one way/two way analysis of variance (ANOVA) test. A value with *P*<0.05 was considered significant.

RESULTS

Percentage yield, solubility and Phytoconstituents: Ethanolic extract of *Cyperus pertenuis* dried roots yielded 2.4% semisolid paste. Results of solubility studies and phytochemical studies are shown in table 2 and 3 respectively.

Evaluation of hepatoprotectivity: 200mg/kg and 400mg/kg of ethanolic extract doses were administered to evaluate hepatoprotective action in chronic diseased model. Paracetamol induced toxicity and enzyme levels i.e. ALT, AST, ALP and TB in sera which were raised to 215.7±5.46u/l, 173.7±2.31u/l, 688±9.45u/l and 0.9±0.03mg/dl when compared to

vehicle group 110±3.03u/l, 109.8± 2.85u/l, 226±4.60 u/l and 0.5±0.04mg/dl, respectively. The results are shown in Table 4 in which there is significant ($p <$ .05) reduction in serum enzymes level. Treatment of rats with ethanolic extract at dose of 200mg/kg and 400mg/kg/ p.o. 1hr prior to administration of paracetamol showed significant fall in serum level of ALT (160.3±3.71 and 144±3.471u/l), AST (155.3±4.60 and 137.7±1.3u/l), ALP (540±5.43 and 520±11.04u/l) and TB (0.6±0.04 and 0.7± 0.04mg/dl) respectively, having significant difference ($p <$ .05) as compared to vehicle and standard groups. While there the more significant difference ($p <$ .001) was seen when compared to disease group. In chronic model extract, effect at dose of 400mg/kg was found to be more potent than ethanolic extract at dose of 200mg/kg

in lowering serum level of ALT, AST and total bilirubin. 400mg/kg lowered serum enzymes comparable to Standard group that reduced ALT (132±3.15u/l), AST (136.3±1.96 u/l), ALP (346±12.46 u/l) and TB (0.06±0.03mg/dl).

Effect of *Cyperus pertenuis* extracts on body weight: In chronic study initial weights of vehicle control, disease control and experimental groups were 155.2±2.007g, 156.0±2.380g, 155.8±2.414g, 159.3±0.954g and 158.7±1.892g and on day seven after completion of experiment mean weight of all groups were 165.5±2.36g, 148.0±2.11g, 165.3±2.73g, 170.0±1.13g,and 169.2±1.89g respectively. This indicated that weight of disease group animal was reduced while in all other groups the gain in weight was recorded.

Table 2: Solubility testing of *Cyperus pertenuis* extracts

Solvents	Water	Normal saline	Ethanol	n-hexane	Ethyl acetate	DMSO	Chloroform
Solubility	Insoluble	Insoluble	Soluble	Insoluble	Slightly Soluble	Soluble	Soluble

Table 3: Phytochemical results of *Cyperus pertenuis* extracts

Phytochemical	Test performed	Ethanolic extract
Tannin	Ferric chloride test	+
Alkaloids	Hagar's test	+
	Wagner test	+
	Mayer test	+
	Dragondorff test	+
Saponin	Foam test	-
Steroid	Ring test	+
Cardiac glycosides	Ring test	-
Terpenes	Salkowski test (modified)	+
Carbohydrates	Molish test, Fehling Test	+
Proteins and amino acid	Biuret test, Ninhydrin test	-
Lipids and fixed oil	Spot test	+
Phenols	FC method	+
Flavonoids	Aluminum chloride method	+
Phytosterol	Libermann Burchard Test	+

+ : Detected; - : Not detected

Table 4: Evaluation of hepatoprotectivity

Groups	ALT (u/l)	AST(u/l)	ALP(u/l)	TB (mg/dl)
Vehicle group	110±3.03	109.8±2.85	226±4.60	0.5±0.04
Induced group	215.7±5.46[a]	173.7±2.31[a]	688±9.45[a]	0.9±0.03[a]
Standard group	132±3.15[***]	136.3±1.96[***]	346±12.49[***]	0.6±0.03[***]
Experimental group-I	160.3±3.71[***]	155.3±4.60[***]	540±5.43[***]	0.6±0.04[***]
Experimental group-II	144±3.47[***]	137.7±1.30[***]	520±11.04[***]	0.7±0.04[***]

All values were expressed as Mean±S.D and two way ANOVA test was applied. Sign [a] indicated significantly different from vehicle group with *P* < .05. Sign * indicated significantly different from disease group with *P* < .001.**
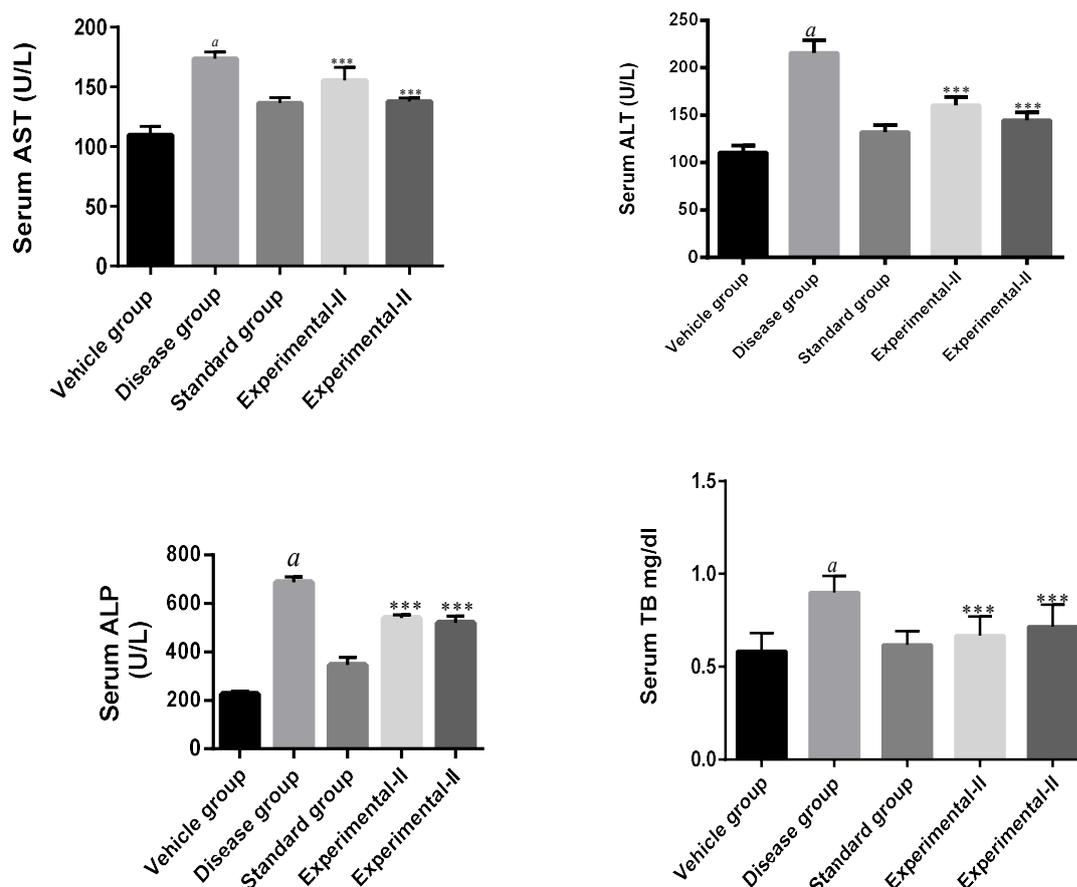

Figure 1: Graphical representation of serum markers
All values were expressed as Mean±S.D and two way ANOVA test was applied. Sign [a] indicated significantly different from vehicle group with *P* < .05. Sign * indicated significantly different from disease group with *P* < .001.**

Table 5: Effect of *Cyperus pertenuis* extracts on body weight of experimental animals of chronic toxicity model

Groups	1st day weight (g)	7th day weight (g)
Vehicle group	155.2±2.007	165.5±2.36
Disease group	156.0±2.380	155.3±1.87[**]
Standard group	155.8±2.414	165.3±2.73[a]
Experimental group A	159.3±0.954	170.0±1.13[a]
Experimental group B	158.7±1.892	169.2±1.89[a]

All values were expressed as Mean±S.D and sign [a] represents insignificant change in mean body weight compared to first day with *P* < .05. Sign[**] shows significant change in mean body weight compared to first day values with *P* < .01.
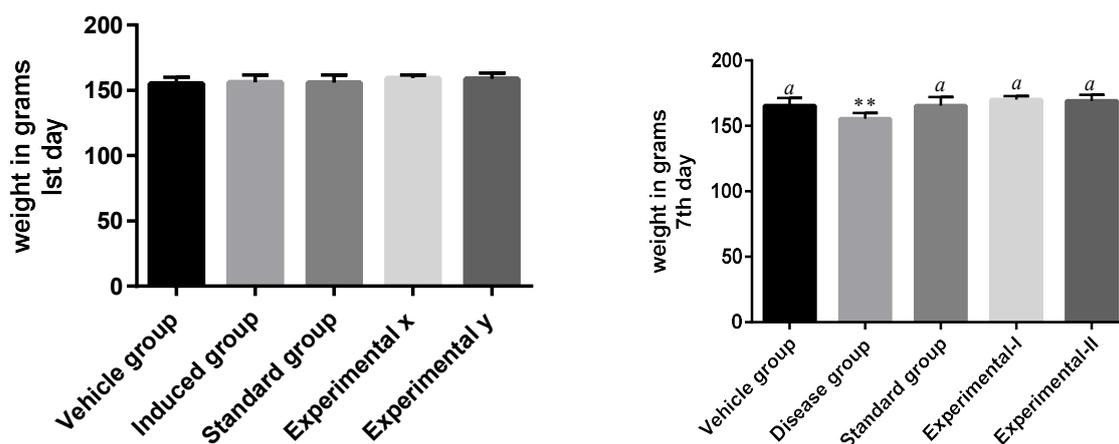
Figure 2: Effect of *Cyperus Pertenius* extract on body weight of experimental animals

All values were expressed as Mean±S.D and sign [a] represents insignificant change in mean body weight compared to first day with $p<0.05$. Sign [**] shows significant change in mean body weight compared to first day with $p<0.01$

Histopathological Grading:

Histopathological findings were expressed in the term of numerical scoring by using histology activity index (HAI) of Knodell *et al.* (1981) as shown in table 6. Histopathological scoring of liver transverse sections of Vehicle group showed intact liver architecture with normal hepatocytes and well defined nucleus with mean HAI=0 (Figure 3.A). In contrast, disease group (treated with paracetamol) showed moderate to marked periportal necrosis, moderate periportal and bridging necrosis, mild to moderate hepatocytes degeneration, mild fatty changes and moderate portal inflammation with mean HAI=8.4 (Figure 3.B). Histopathology of standard group, (treated with silymarin 25mg/kg) along with paracetamol, showed intact liver architect with normal hepatocytes and well defined nucleus with mean HAI=0 (Figure 3.C). On histological examination, the transverse sections of liver specimens of rats treated with *Cyperus pertenuis* extract at 200mg/kg along with paracetamol showed moderate to marked periportal necrosis, mild degeneration of hepatocytes and fatty changes with mean HAI=4.10 (Figure 3.D) while no pathological lesions were observed in transverse sections of liver biopsy specimens of rats treated with *Cyperus pertenuis* extract at dose of 400mg/kg. Normal hepatocytes with well-defined nucleus were seen in transverse sections of liver biopsy specimens of experimental group y with mean HAI=0 (Figure 3.E).

Table 6: Histological scoring of transverse sections of liver specimens

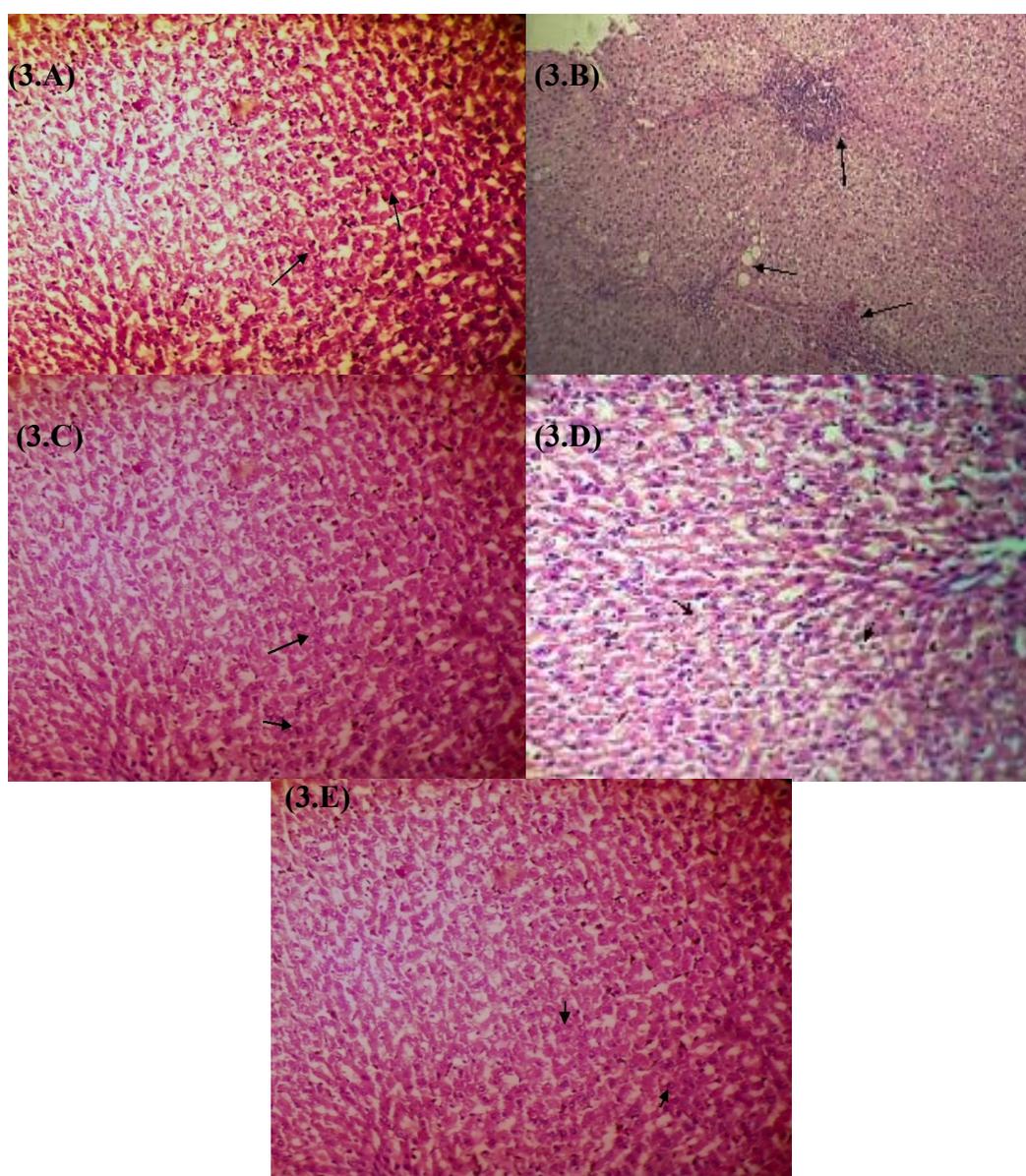| Group (n=6)	Periportal Bridging necrosis	Interlobular degeneration & Focal necrosis	Portal Inflammation	Fibrosis	HAI
		Histopathological Lesions			
Vehicle Group	0	0	0	0	0
Disease Group	4	1.4	3	0	8.4
Standard Group	0	0	0	0	0
Experimental Group-I	3.4	1	0	0	4.4
Experimental Group-II	0	0	0	0	0

Figure 3: Histopathological slides of hepatic tissues of different groups.

DISCUSSION

Acetaminophen or paracetamol is world's most selling OTC drug which is although safe but may produce liver damages when taken in high doses. In liver this is metabolized into N-Acetyl-p-benzoquinone imine (NAPQI) which is normally taken up by glutathiones in normal body and toxicity does not occur. But when paracetamol (PCM) is taken in excess then large amount of NAPQI is formed as a result of metabolism and glutathiones present in body do not suffices to detoxify it [20]. Accumulation of NAPQI results in hepatotoxicity by covalently linking with cysteines [21]. The damage of hepatic cells cause leakage of enzymes i.e. AST (Aspartate aminotransferase), ALT (Alaninie aminotransferase), ALP (Alkaline phosphatase) and TB (total bilirubin) into blood and their plasma level is increased which is actually used as marker to assess hepatotoxicity [22, 23]. Thus increased level of these enzymes in blood is indication of hepatotoxicity and any substance which reduces the level of these enzymes is considered as hepatoprotective. Results indicated that ethanolic extract of plant is enriched with phytoconstituents i.e. tanins, alkaloids, terpenes, flavonoids, phenols and phytosterols. Presence of tannins in plant extract contributes to the hepatoprotection possibly by preventing the damage of membranes [22]. It is also reported that excessive generation of reactive oxygen species (ROS) in mitochondria of liver initiates apoptosis and impairs liver functionality [24, 25]. So prevention of ROS by utilization of an agent may contribute to the protection of liver. Ethanolic extract of *Cyperu pertenuis* is the richest source of phytoconstituents like tannins, alkaloids and terpenes which react with ROS and rendered them unable to harm the cells. Thus hepatoprotective action is achieved by the utilization of ethanolic extract of plant even after the prolong exposure of hepatotoxic substances. Hepatoprotective action of *Cyperu pertenuis* may also be due to that fact that it promotes the level of catalases which [26] which acts as an anti oxidant to protect the liver. Moreover, loss in weight due to administration is also indicative of diseased condition while weight gain in experimental groups is an indication of healthy body tissues. Histopathological slides clearly represented the structural damage which occurred in animals treated with chronic use of PCM. However, standard control group and experimental group-II represented no cellular damage at all. Hepatic cell membranes and nucleus remained intact in experimental group-II which clearly defined the hepatoprotective action of crude plant extract.

CONCLUSION

On basis of result it has been concluded that ethanolic extract of dried roots of *Cyperus pertenuis* strongly inhibit the hepatic cell damage even after chronic administration of PCM. Thus it is strong hepatoprotective agent.

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