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Hepatoprotective Potential of *Blepharis persica* (Burm. F.) O. Kuntze

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ABSTRACT

This study aimed to assess the hepatoprotective activity of ethanol (90%) extract of Blepharis persica seeds (BPEE) in rats. The plant extract showed a remarkable hepatoprotective activity against acetaminophen-induced hepatotoxicity as judged from the serum marker enzymes and histopathological studies on liver tissues. The acetaminophen-induced group shows a significant rise in the serum enzymes. Treatment of rats with different doses of plant extracts significantly altered serum marker enzymes levels to near normal against acetaminophen treated rats. The activity of the extracts was comparable to the standard drug, silymarin. Histopathological changes of liver sample were compared with respective control. Results indicate the hepatoprotective properties of Blepharis persica against acetaminophen induced hepatotoxicity in rats.

Keywords: *Blepharis persica*, Acetaminophen, Hepatoprotective, Oxidative stress, Transaminases.

INTRODUCTION

The liver is the vital organ of paramount importance involved in the maintenance of metabolic function and detoxification from the exogenous and endogenous challenges, like xenobiotic, drugs, viral infection and chronic alcoholism. If during all such exposures to the above mentioned challenges the natural protective mechanisms of the liver are overpowered, the result is hepatic

injury. Liver damage is always associated with cellular necrosis, increase in tissue liquid peroxidation and depletion in the tissue GSH levels. In addition serum levels of many biochemical markers like SGOT, SGPT, ALP and billirubin are elevated [1,2]. In spite of phenomenal growth of modern medicine, there are no synthetic drugs available for hepatic disorder. However there are several herbs/herbal formulation claimed have possess beneficial activity in treating hepatic disorder.

Blepharis persica (Burm. f.) O. Kuntze. (Acanthaceae) commonly known as Uttingana, Sunishannaka, Chaupatia and Borahu is a soft grey-pubscent perenial herb [3]. It is indigenous to India (Punjab, Western Rajasthan, Malwa region of Madhya Pradesh), Pakistan, Iran, Africa (Thar) [4]. It is used as purgative, tonic, aphrodisiac [5], diuretic, expectorant and used in treatment of urinary discharges, leukoderma, ascites, disorders of liver and spleen. It contains saponin, tannins, flavonoids and glycoside (blepharin) [6,7]. It has delayed seed dispersal and rapid germination property [8]. The traditional formulations are available in the name of Kumaryasava in Ayurvedic system and Majoon Bandkushad in Unani medicine system.

In spite of tremendous advances in modern medicine, there are no effective drug available the stimulate liver function, offer protection to the liver from damage or help to regenerate hepatic cells [9,10]. In absence of reliable liver protective drugs in modern medicine, there exists a challenge for pharmaceutical scientists to explore the potential of hepatoprotective activity in plants on the basis of traditional use [11]. Therefore, to justify the traditional claims; the seeds of *B. persica* has been selected for hepatoprotective effect against acetaminophen intoxicated rats.

MATERIALS AND METHODS

Plant material

The seeds of the plant were collected in the month of April from Patiala, Punjab and authenticated by Dr. Sunita Garg, Chief Scientist, NISCAIR, New Delhi, India (Ref. No.-NISCAIR/RHMD/Consult/2013/2311/91 dated 13/09/2013). The seeds were shade dried, coarsely powdered and stored in an air tight container till use.

Extraction and fractionation

The plant material was extracted by cold maceration with ethanol (90%) – BPEE (*Blepharis persica* ethanol extract) and distilled water – BPAE (*Blepharis persica* aqueous extract) till exhausted completely. The extracts so obtained were freed off solvent under vacuum and used for further studies.

Phytochemical screening

The preliminary phytochemical screening of ethanolic (90%) extract of *B. persica* seeds showed the existence of alkaloids, flavonoids, tannins and phenolic compounds which were confirmed by the positive reaction with the respective testing reagents. Similarly, the screening of seeds aqueous extract of *B. persica* exhibited the presence of carbohydrates, proteins, amino acids, phenolic compounds, glycosides and saponins [12].

Screening for hepatoprotective potential

Drugs and Chemicals/Instruments

Reduced glutathione (GSH), malondialdehyde (MDA), sodium dodecyl sulphate (SDS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), disodium ethylene diamine tetra acetic acid (EDTA), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), tris-HCl, sodium citrate, acetic acid, butanol, pyridine, acetaminophen, silymarin were procured from Sigma (MO, USA), CDH (New Delhi, India) and Microlabs (Mumbai, India), Cooling Centrifuge (REMI, Vasai), Rotary evaporator (Equitron Roteva, Mumbai), Ultrasonic Cleaner (Steryl Med Equip systems) and UV/Visible Spectrophotometer (UV 1700, Pharmaspec, SHIMADZU, Japan) were used.

Preparation of test material

The ethanolic (90%) and aqueous extracts were dissolved separately in distilled water with a concentration of 50 mg/ml of respective suspension/solution.

Silymarin was prepared in distilled water to the concentration of 50 mg/ml solution.

Acetaminophen administered orally [13].

Experimental animals

The albino wistar rats of either sex (100-150 g) were procured from Panacea Biotech, Lalru after the permission from CPCSEA. The animals were housed at Rayat & Bahra Institute of Pharmacy, Sahauran (Reg. No. 1380/a/10/CPCSEA) with the approved protocol no. RBIP/IAEC/CPCSEA/2014/25 during all the stages of the experimentation. The animals were placed in the room with air conditioned (24 ± 4ºC) for light & dark cycles (12 hr each). All animals were access to the standard chow diet bought from Ashirwad Industries, Ropar and the water *ad libitum*. The experiments were planned and performed as per the ethical norms accepted by Ministry of Social Justices and Empowerment, Government of India and implemented by the Institutional Animal Ethics Committee (IAEC) of RBIP, Sahauran.

Experimental groups

Each experimental group consisted of five animals per group. Group I receive distilled water throughout the experiment. Group II acetaminophen orally and then received distilled water for 7 days. Groups III will receive acetaminophen and then the standard drug for 7 days. All the groups from IV to XI will receive acetaminophen and then the test drug was administered for all the 7 days. The animals will be sacrificed on Day 8^{th} by the method of cervical dislocation. The liver will be removed quickly and then immediately perfused with ice-cold saline i.e., 0.9% NaCl. The liver was homogenised for estimation of tissue parameters. Just before the sacrifice, blood samples for the separation of serum will be collected by the puncture of retro-orbital sinus under light diethyl ether anesthesia for the estimation of various biochemical parameters [13].

Estimation of serum parameters

AST [14], ALT [14], ALP [15], Total bilirubin [16], Total proteins [17] and Albumin [18] levels in the serum were estimated using commercially available kits of *Carol* Company, Goa.

Estimation of tissue parameters (Antioxidative effects)

Lipid peroxidation: It was determined by measuring the amounts of malondialdehyde (MDA) produced primarily, according to the method of Ohkawa's 1979. The levels of lipid peroxides were expressed as nM of thiobarbituric acid reactive substances (TBARS)/mg protein [19].

Estimation of GSH: It was determined by measuring the amounts of reduced glutathione (GSH) level, according to the method of Ellman's 1959. The levels of reduced glutathione were expressed as µM of reduced glutathione/mg protein [19].

Histological analysis of liver

Liver tissue of the treated and non treated rats were isolated and fixed in 10% phosphate buffered formalin for at least 24 h. Then the paraffin sections were prepared and cut into 5 µm thick sections in a rotary microtome and mounted on the slide. The sections were then stained with Haematoxylin–Eosin dye and were studied for histopathological changes, i.e., necrosis, fatty changes, ballooning degeneration, lymphocytes and Kupffer cells infiltration [20].

Safety evaluation study

As per OECD guidelines (423) the safety evaluation study was carried out. In this study three rats of same weight and age were engaged in a single dose as highest one up to 2000 mg/kg orally. The animals were monitored for 1 hr continuously. The observed the animals after every 4 hr and finally after every 24 hr up. The observation of animals after every 24 hr was continued up to 15 days for any mortality or behavioural changes [21].

Statistical analysis

Results were reported as means ± SD with one way ANOVA method to evaluate differences between the groups. The differences among the means were investigated by Tukey-Kramer test for multiple comparisons using computerized program at 95% ($P < 0.05$) confidence level [22].

RESULTS

The yield of the ethanol (90%) extract of *B. persica* (seeds) was 12.80% w/w and its aqueous extract was found to be 19.75% w/w by Cold maceration in triplicate form. Phytochemical investigations revealed the presence of carbohydrates, proteins, alkaloids, Tannins, phenolic compounds and flavonoids in the ethanol (90%) extract. Aqueous extract also showed the presence of glycosides and saponins.

The oral administration of the ethanol (90%) extract and aqueous extract the seeds caused neither any behavioural changes nor mortality up to 2000 mg/kg. Table 1 shows the levels of serum enzymes (AST, ALT and ALP), total bilirubin, total proteins and albumin in normal group of rats, *viz*., AST (60.45 ± 4.654 U/L), ALT (50.11 ± 3.546 U/L), ALP (163.30 ± 5.399 U/L), TB (0.278 ± 0.096 mg/dl), TP (6.292 ± 0.518 g/dl) and ALB (3.427 ± 0.422 g/dl).

The Acetaminophen (2 g/kg; p.o.) challenged group of rats showed increase in the serum levels of enzymes AST (165.85 ± 5.274 U/L), ALT (155.22 ± 3.64 U/L), ALP (255.40 ± 4.867 U/L), TB (0.596 ± 0.169 mg/dl) and reduction in the serum levels of TP (3.470 ± 0.788 g/dl) and ALB (2.030 ± 0.159 g/dl) indicating the development of hepatotoxicity caused by Acetaminophen (Table 1).

Pre-treatment of rats with the ethanol (90%) extract at 200 and 400 mg/kg showed reduction in the rise of AST (109.85 ± 5.089↓ and 81.04 ± 6.281↓), ALT (93.45 ± 3.791↓ and 79.81 ± 3.388↓), ALP (219.90 ± 4.325↓ and 205.05 ± 4.489↓), TB (0.545 ± 0.025↓ and 0.493 ± 0.018↓) and showed the increase in the level of TP (4.184 ± 0.084 and 5.367 ± 0.080↑) and ALB (2.554 ± 0.457 and 2.797 ± 0.081↑) respectively in comparison to Acetaminophen control group. The groups of rats pretreated with the aqueous extract at 200 and 400 mg/kg showed reduction in the rise of AST (125.08 ± 6.324 and 108.05 ± 5.351↓), ALT (100.45 ± 3.18 and 88.81 ± 2.285↓), ALP (225.55 ± 3.005 and 215.05 ± 3.185↓), TB (0.565 ± 0.015 and 0.515 ± 0.023↓) and showed the increase in the level of TP (3.885 ± 0.155 and 4.835 ± 0.063↑) and ALB (2.440 ± 0.043 and 2.625 ± 0.065↑) respectively in comparison to Acetaminophen control group. Pre-treatment of group of rats with silymarin at 50 mg/kg showed reduction in the rise of AST (69.02 ± 3.546↓), ALT (56.01 ± 2.205↓), ALP (191.9 ± 4.962↓) and TB (0.413 ± 0.118↓) and increase in the level of TP (6.200 ± 0.610↑) and ALB (3.065 ± 0.416↑) under the same investigational conditions in comparison to Acetaminophen control group (Table 1 and Figures 1-6).

Table 1 and Figure 7 shows the increase in MDA level in acetaminophen control group (50.47 ± 4.088 nM/mg protein) compared to normal group (20.00 ± 2.988 nM/mg protein). Pre-treatment of groups of rats with the ethanol (90%) and aqueous extracts at 200 and 400 mg/kg showed reduction in the rise of MDA level by 45.68 ± 1.990, 37.15 ± 1.033↓ and 46.85 ± 2.145, 40.55 ± 3.045↓ respectively compared to acetaminophen control group. The group pretreated with silymarin at 50 mg/kg showed 31.6 ± 1.600↓ reduction of MDA level with reference to acetaminophen control group.

Table 1 and Figure 8 shows the reduction in glutathione (GSH) content in the liver homogenate of acetaminophen control group (48.5 ± 4.037 µM/g of liver) as compared to the normal group (106.20 ± 7.360 µM/g of liver). Pre-treatment of groups of rats with the ethanol (90%) and aqueous extracts at 200 and 400 mg/kg showed increase in GSH level by 62.83 ± 2.483, 75.83 ± 3.430↑ and 56.22 ± 2.857, 68.88 ± 3.252↑ respectively as compared to acetaminophen control group. Pre-treatment of rats with silymarin at 50 mg/kg showed increase in the level of GSH by 87.17 ± 4.446↑ under the same investigational conditions in comparison to acetaminophen control group.

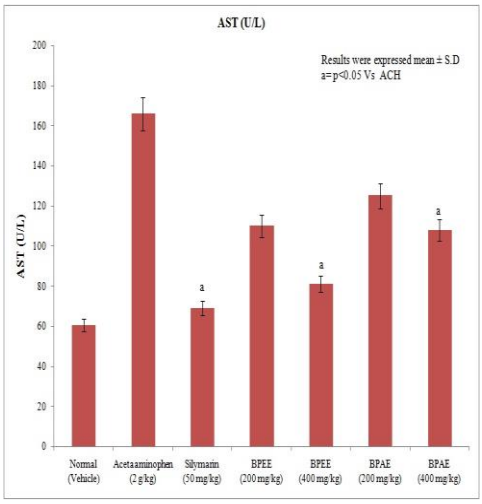
Figure 9 showed the histopathology of liver showed clear nucleus contained normal arrangements of hepatocytes. Sinusoids in the periphery region are merged in a reticulum. The arrangements of hepatic cells are present in the series and laminae were anastomosed in a labyrinth form with sinusoidal spaces. The cytoplasm of normal group hepatocytes contained clear eosinophilic with specific nuclei. The microscopical examination of Acetaminophen induced liver section confirmed the pathological changes like cloudy swelling and hydropic changes which leaded to the hepatic cells necrosis and centrilobular fatty changes with clear

spacing of lipid. Liver segments of the rats pretreated with the ethanolic (90%) and aqueous extracts of the *B. persica* showed the absence of necrosis and space of lipids. The liver segments of the group pretreated with silymarin did not showed parenchymal injury.

Table 1: Hepatoprotective activity of *Blepharis persica* against acetaminophen induced hepatotoxicity in rats.

Variables	**Serum parameters**						**Tissue parameters**	
Groups	**AST (U/L)**	**ALT (U/L)**	**ALP(U/L)**	**TB(mg/dl)**	**TP(g/dl)**	**ALB(g/dl)**	**TBARS (nM/mg Protein)**	**GSH (µM/mg Protein)**
Normal (Vehicle)	60.45 ± 4.654	50.11 ± 3.546	163.35 ± 5.399	0.278 ± 0.096	6.292 ± 0.518	3.427 ± 0.422	20 ± 2.988	106.2 ± 7.360
Acetaminophen(2 g/kg b.wt., p.o.)	165.85 ± 5.274[a]↑	155.22 ± 3.64[a]↑	255.40 ± 4.867[a]↑	0.596 ± 0.169[a]↑	3.470 ± 0.788[a]↓	2.030 ± 0.159[a]↓	50.47 ± 4.088[a]↑	48.5 ± 4.037[a] ↓
Silymarin (50 mg/kg b.wt., p.o.)	69.02 ± 3.546[b] ↓	56.01 ± 2.205[b]↓	191.9 ± 4.962[b] ↓	0.413 ± 0.118[b]↓	6.200 ± 0.610[b]↑	3.065 ± 0.416[b]↑	31.6 ± 1.600[b] ↓	87.17 ± 4.446[b]↑
BPEE extract (200 mg/kg b wt., p.o.)	109.85 ± 5.089[c]↓	93.45 ± 3.791[c] ↓	219.90 ± 4.325[c]↓	0.545 ± 0.025[c]↓	4.184 ± 0.084	2.554 ± 0.457	45.68 ± 1.990	62.83 ± 2.483
BPEE extract (400 mg/kg b wt., p.o.)	81.04 ± 6.281[c] ↓	79.81 ± 3.388[c] ↓	205.05 ± 4.489[c]↓	0.493 ± 0.018[c]↓	5.367 ± 0.080[c]↑	2.797 ± 0.081[c]↑	37.15 ± 1.033[c] ↓	75.83 ± 3.430[c]↑
BPAE extract (200 mg/kg b wt., p.o.)	125.08 ± 6.324	100.45 ± 3.18	225.55 ± 3.005	0.565 ± 0.015	3.885 ± 0.155	2.440 ± 0.043	46.85 ± 2.145	56.22 ± 2.857
BPAE extract (400 mg/kg b wt., p.o.)	108.05 ± 5.351[c]↓	88.81 ± 2.285[c] ↓	215.05 ± 3.185[c]↓	0.515 ± 0.023[c]↓	4.835 ± 0.063[c]↑	2.625 ± 0.065[c]↑	40.55 ± 3.045[c] ↓	68.88 ± 3.252[c]↑

Note: The results are stated as the Mean ± SD of five rats/group and One way ANOVA by Tukey's multiple test was followed; Significantly different results are symbolically represented by a = in comparison of the normal group ($P < 0.01$); b = in comparison of the Acetaminophen group ($P < 0.01$) and c = in comparison of the Acetaminophen group ($P < 0.05$).



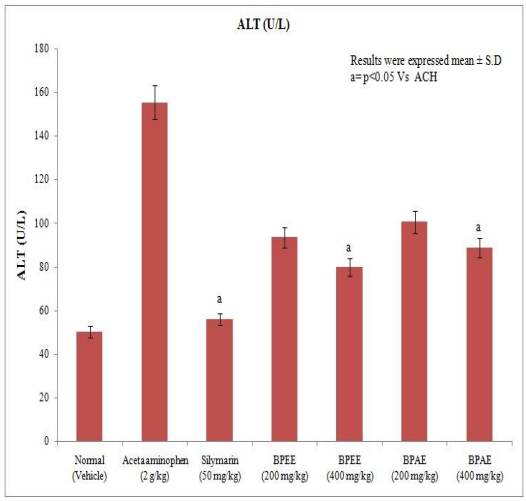
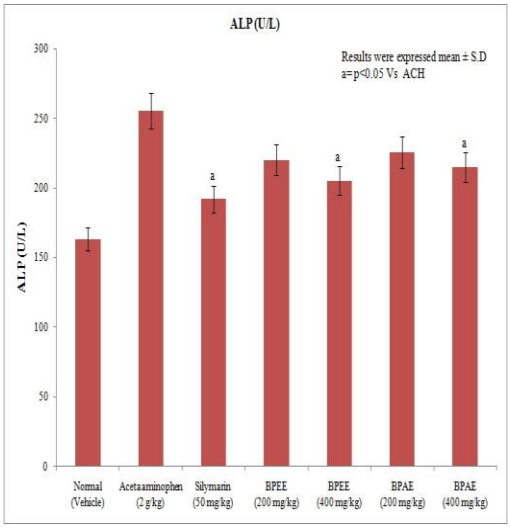


Figure 1: AST **Figure 2:** ALT



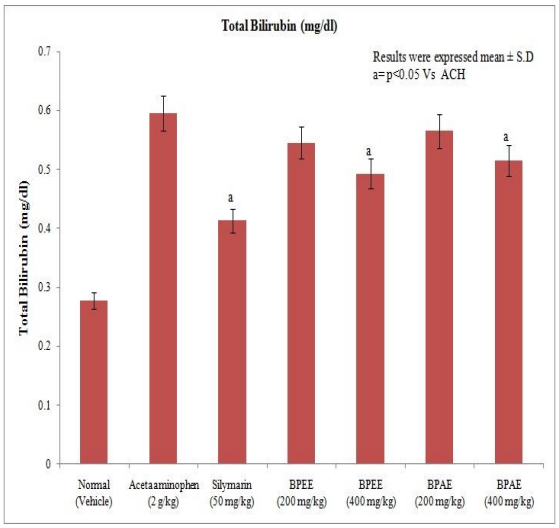


Figure 3: ALP **Figure 4:** Total Bilirubin

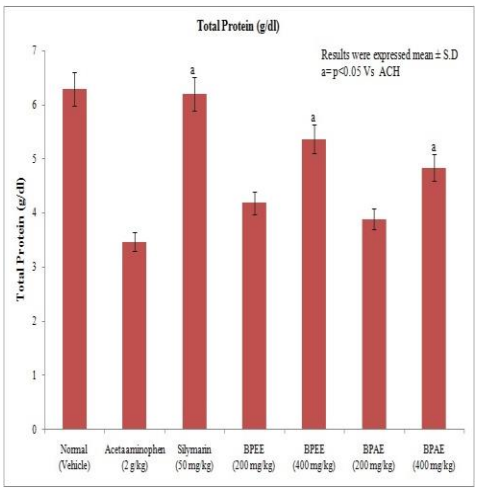


Figure 5 : Total Protein

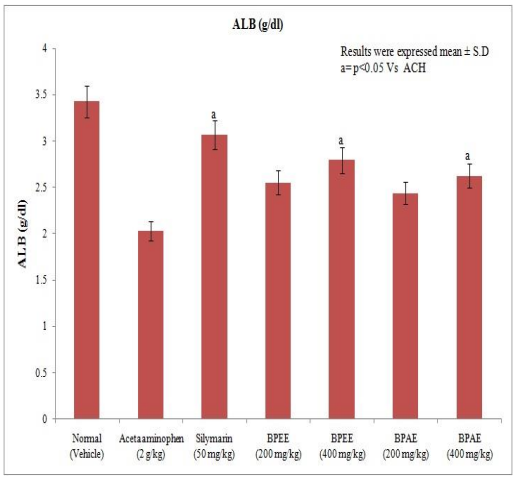


Figure 6 : ALB

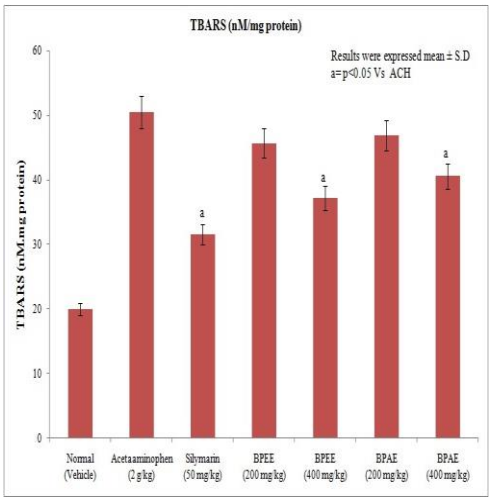


Figure 7 : TBARS

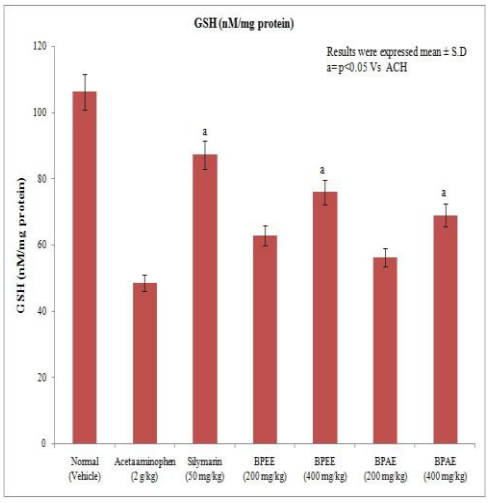


Figure 8 : GSH

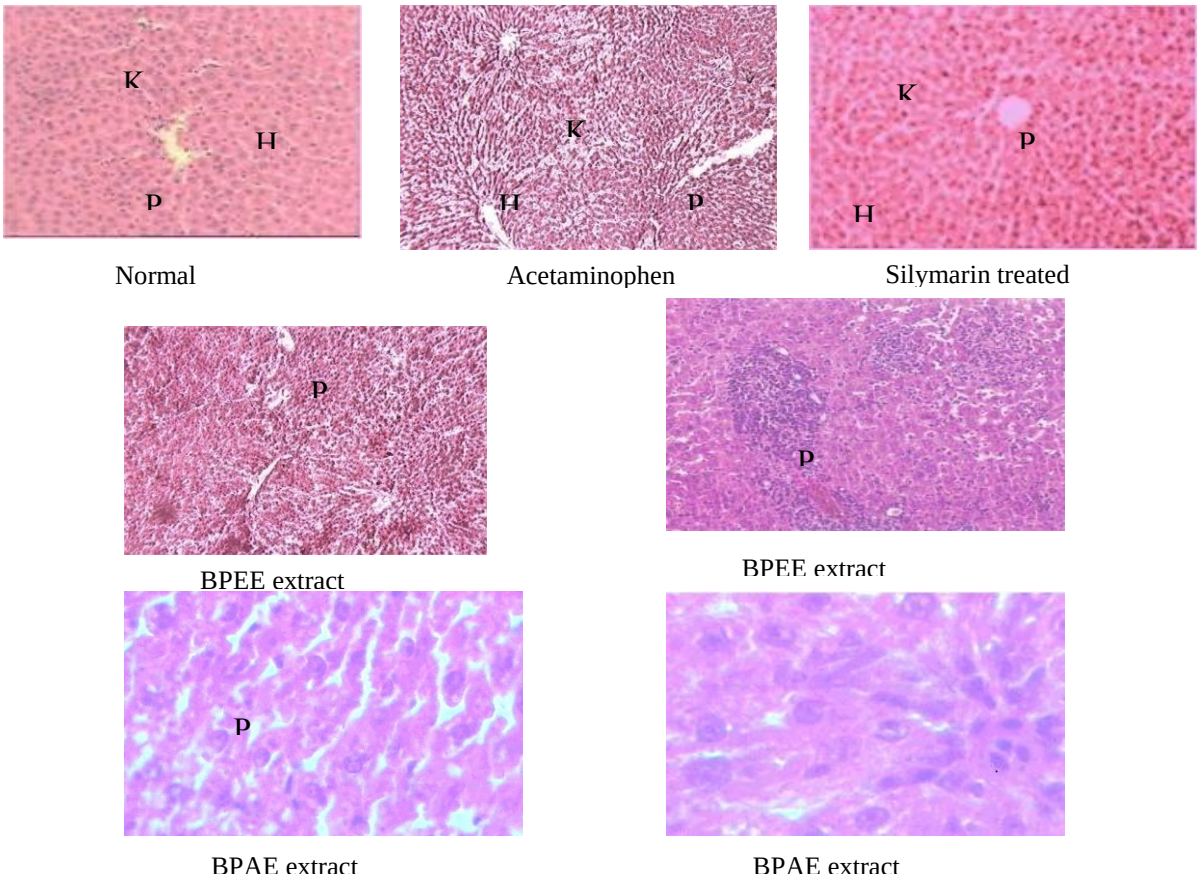


Figure 9: Histopathological studies provided supportive evidence for the biochemical analysis KC- Kupffer cells, HC- Hepatic cells, PV-Portal veins

DISCUSSION

The yield of the ethanol (90%) extract of *B. persica* (seeds) was 12.80% w/w and its aqueous extract was found to be 19.75% w/w by Cold maceration in triplicate form. Phytochemical investigations revealed the presence of carbohydrates, proteins, alkaloids, Tannins, phenolic compounds and flavonoids in the ethanol (90%) extract. Aqueous extract also showed the presence of glycosides and saponins. The LD50 of the seeds was found to be above than 2000 mg/kg. Thus, it would be safe to use this drug as a hepatoprotective. The dose level can be increased to 1000 mg/kg to get more effects *at par* with the silymarin. Overdose of acetaminophen produces a potentially fatal and hepatic centrilobular necrosis. The hepatotoxicity of acetaminophen occurred by cytochrome P4502E1 which produces a toxic metabolite, NAPQI (N-acetyl-p-benzoquinoneimine) [23].

In the present investigation, Acetaminophen administration orally resulted in elevated activities of AST (60.45 ± 4.654 to 165.85 ± 5.274), ALT (50.11 ± 3.546 to 155.22 ± 3.645) and ALP (163.35 ± 5.399 to 255.40 ± 4.867) in Group II serum level against

their Group I control values. Similarly, the serum bilirubin level was raised significantly due to acetaminophen toxicity (0.278 ± 0.096 to 0.596 ± 0.169) showed in Group II. However, the total serum protein level was decreased in response to acetaminophen administration when compared with the control values (6.292 ± 0.518 to 3.470 ± 0.788) (Table 1). Abnormally higher activities of serum enzymes after acetaminophen administration are considered to be the signs for the development of hepatic toxicity, which leads to discharge of cellular enzymes in the blood. When the liver plasma membrane gets injured, the enzymes situated in the cytosol are discharged into the circulation [24].

Oral administration of different doses of *B. persica* ethanolic (90%) and aqueous extract to acetaminophen administered rats resulted in gradual regularization of the activities of AST (BPEE 200 mg/kg - 109.85 ± 5.089, BPEE 400 mg/kg - 81.04 ± 6.281, BPAE 200 mg/kg- 125.08 ± 6.324 and BPAE 400 mg/kg - 108.05 ± 5.351), ALT (BPEE 200 mg/kg - 93.45 ± 3.791, BPEE 400 mg/kg - 79.81 ± 3.388, BPAE 200 mg/kg- 100.45 ± 3.185 and BPAE 400 mg/kg - 88.81 ± 2.285) and ALP (BPEE 200 mg/kg - 219.90 ± 4.325, BPEE 400 mg/kg - 205.05 ± 4.489, BPAE 200 mg/kg- 225.55 ± 3.005 and BPAE 400 mg/kg - 215.05 ± 3.185).

Groups of rats pretreated with the ethanolic (90%) extract at dose of 400 mg/kg revealed more significant ($P < 0.05$) improvement in levels of the AST, ALT and ALP. The Group III pretreated with the silymarin (50 mg/kg) showed well significant ($P < 0.01$) reduction in increase in the serum enzymes level, *viz*. AST (69.02 ± 3.546), ALT (56.01 ± 2.205) and ALP (191.9 ± 4.962) in comparison to acetaminophen control group.

This obviously suggests the defensive result of the extract in recovering the functional reliability of liver cells. The serum bilirubin was observed for the evaluation of hepatic function and a few abnormal rises shows hepatobiliary disease and rigorous trouble of hepatocellular architecture [25]. Acetaminophen administration resulted in raised Group II serum bilirubin level which signifying hepatic injury and proving the hepatotoxic reduced the raised level of the total bilirubin in serum to normalizing its hepatoprotective efficacy (BPEE 200 mg/kg - 0.545 ± 0.025, BPEE 400 mg/kg - 0.493 ± 0.018, BPAE 200 mg/kg- 0.565 ± 0.015 and BPAE 400 mg/kg - 0.515 ± 0.023) with respect to silymarin group (0.413 ± 0.118). Acetaminophen treated group showed decrease in total serum protein level which impaired the protein synthesis. Subsequent treatment of acetaminophen intoxicated rats with ethanolic (90%) and aqueous extracts of *B. persica* increased the total protein (TP) level (BPEE 200 mg/kg - 4.184 ± 0.084, BPEE 400 mg/kg - 5.367 ± 0.080, BPAE 200 mg/kg- 3.885 ± 0.155 and BPAE 400 mg/kg - 4.835 ± 0.063) with respect to silymarin group (6.200 ± 0.610). This further indicates the restorative nature of extract against acetaminophen toxicity. Hepatic lipid peroxidation, stated as Thiobarbituric acid (TBA) reacting substances, increased considerably in acetaminophen toxicity. While, the level of protective enzymes such as MDA and GSH in liver tissue are get lowered after the acetaminophen administration. The reduced level of MDA and GSH is an indication of a hepatic damage due to acetaminophen toxicity. Marked decrease in the activities, MDA (BPEE 200 mg/kg - 45.68 ± 1.990, BPEE 400 mg/kg - 37.15 ± 1.033, BPAE 200 mg/kg- 46.85 ± 2.145 and BPAE 400 mg/kg - 40.55 ± 3.045) and GSH (BPEE 200 mg/kg - 62.83 ± 2.483, BPEE 400 mg/kg - 75.83 ± 3.430, BPAE 200 mg/kg- 56.22 ± 2.857 and BPAE 400 mg/kg - 68.88 ± 3.252), associated with acetaminophen toxicity were significantly inverted to normal on oral administration of ethanolic (90%) and aqueous extracts of *B. persica* in a dose dependent manner confirming the anti-lipid peroxidative capability to the extract.

Acetaminophen was activated as a NAPQI metabolite by cytoP450 [23]. It is detoxified by conjugating with glutathione (GSH). Thus, GSH constituted the protection against acetaminophen persuaded the generation of free radicals [26]. In acetaminophen toxicity, total GSH was lowered by the damage of hepatocytes. As a result, the production of NAPQI glutathione conjugate was

reduced. Administration of ethanolic (90%) and aqueous extracts of *B. persica* effectively reloaded the acetaminophen induced reduction of hepatic GSH probably due to reduced production of NAPQI metabolite by cytoP450.

Acetaminophen induced damage of hepatocytes decreased the glycogen level of the liver tissue. The significant rise in hepatic glycogen level was detected after the administration of extracts indicating development in hepatic status.

CONCLUSION

The histopathological investigation of the Group I revealed the regular and separate hepatic cells (Figure 9). However, the necrosis of hepatocytes was observed after acetaminophen administration in Group II with damage of hepatocytes. Ethanolic (90%) and aqueous extracts of *B. persica* treatment groups to such acetaminophen intoxicated rats showed improvement from necrosis. From this it was revealed that the plant extracts has a potential to revoke the changes persuaded by acetaminophen toxicity return to normal. The healing efficacy of ethanolic (90%) extracts at the dose 400 mg/kg of seeds of *B. persica* was dose dependent as proved by gradual improvement of the distorted values of various biochemical enzymes back to normal. This may be due to promotional activation of anti-oxidative enzymes and rejuvenation of hepatocytes that reinstates the structural and the functional integrity of liver. Thus, the present study confirms the hepatoprotective action of *B. persica* (seeds) against acetaminophen induced hepatotoxicity in rats.

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