



HEPATOPROTECTIVE ACTIVITY OF LEAVES OF *PARKINSONIA ACULEATA* LINN AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

Free radicals are generated during the metabolism of synthetic chemical substances/drugs by liver can cause hepatotoxicity. Supplementation with exogenous antioxidants, including alkaloid compounds from plant sources, may be useful for protecting liver against free radical induced hepatotoxicity. *P. aculeata* has been reported to have potent anti oxidant activity. With this background the present study has been undertaken to explore the *in vivo* hepatoprotective action of *P. aculeata* leaves. In the present work leaf extracts of *P. aculeata* (Fabaceae) was selected to determine its *in vitro* and *in vivo* hepatoprotective activity, where hepatotoxicity was induced by CCl₄ (20 mM) for *in vitro* study and by oral administration of Paracetamol (2 gm/kg) for *in vivo* study. Extract was administered orally at a daily dose of 200 mg/kg and 300 mg/kg, for 7 days (*in vivo*). *In vitro* hepatoprotective activity was assessed by checking the viability of the cells by using Trypan blue dye and by measuring release of cytosolic enzymes in the medium and *In vivo* hepatoprotective activity was assessed by measuring serum biochemical parameters and endogenous anti oxidant enzymes. The levels of cytosolic enzymes, serum enzymes and endogenous antioxidant enzymes, used as a marker of oxidative damage to hepatocytes, was reversed to the same level as in Normal group in dose dependent manner. No obvious signs of toxicity were observed 300 mg/kg treatment dose.

Key words: Fabaceae, Hepatoprotective, *Parkinsonia Aculeata*, liver and *Parkinsonin-A*,

INTRODUCTION

Liver is the vital organ of metabolism and excretion. Many synthetic chemical substances/drugs are metabolized mainly by liver. During this process free radicals are generated and can cause hepatotoxicity by damaging the cell membrane and cell constituents. Human beings possess inbuilt natural endogenous antioxidant defence system against this oxidative stress by scavenging the generated free radicals. The inbuilt scavenging systems are the Glutathione (GSH), Superoxide dismutase (SOD) and Catalase (CAT). But prolonged stressful condition produces large amount of free radicals that cannot be handled by our inbuilt mechanism. Thus many of the drugs

have greater tendency to cause hepatotoxicity as well other organ toxicities.

Paracetamol is a common antipyretic agent, which is safe in therapeutic doses but can produce fatal hepatic necrosis in man and rodents with toxic doses [Mitchell et al., 1973] Paracetamol hepatotoxicity is caused by its highly reactive metabolite, N-acetyl-p-benzo quinoneimine (NAPQI) [Neils et al., 1996]. NAPQI is again metabolised by microsomal cytochrome P450 reductase in to two metabolites, Semiquinone and hydroquinone. Semiquinone, a one-electron reduction metabolite, is highly reactive whereas hydroquinone, a two-electron reduction metabolite is non reactive. The semiquinone radical can bind directly with cellular macromolecules

leading to hepatotoxicity by causing oxidative stress and GSH depletion [Diadelis et al., 1995].

As synthetic drugs are reported for various adverse drug reactions and organ toxicities, research is directed towards the exploration of herbal medicines as new remedial measures to treat various drug induced ailments. Traditional medicine is used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses [WHO, 2000]. In many developed countries, 70 to 80% of the population is using some form of alternative or complementary medicine. Especially in some Asian and African countries like India, more than 80% of people depend on plant based traditional medicine for primary health care [WHO, 2002]. Herbs are useful for protecting various organs owing to their potential antioxidants constituents such as quercetin, β -carotene, tocopherol and vit-C. *P. aculeata* is a small, spiny tree, belongs to the family Leguminosae and is native to tropical America, extending from Mexico to South America. In India it is found in all dry regions, particularly western parts. Plantations of this species are also being raised in the arid and semi-arid tracts of western Uttar Pradesh, Rajasthan and Gujarat.

Leaves of *P. aculeata* are found to contain various flavonoids such as C-glycosides (*epi-orientin*, *Parkinsonin-A*, *Parkinsonin-B*, *Parkintin*) and flavone C-glycoside (*Luteolin*), *orientin*, *iso-orientin*, *vitexin*, *iso-vitexin*, *lucenin-II*, *vicenin-II*, *diosmetin 6-C- β -glucoside*, *apigenin*, *luteolin*, *kaempferol* and *chrysoeriol* which are reported as potent antioxidants [Bhatia et al., 1966; El-sayed et al., 1991]. Further leaf extract of *P. aculeata* has been reported for its potent in vitro antioxidant activity [Mruthunjaya & Hukkeri, 2008]. Hence *P. aculeata* was selected to evaluate the hepatoprotective potential against CCl_4 induced (*in vitro*) and Paracetamol induced (*in vivo*) hepatotoxicity. In the present paper we demonstrated the in vitro protective activity of extract on isolated toxin challenged hepatocytes by assessing the viability of hepatocytes as well as by estimating the cytosolic enzymes such as Glutamate Oxaloacetate Transaminases (GOT), Glutamate Pyruvate Transaminase (GPT) and Lactate Dehydrogenase (LDH). The *in vivo* activity of the extract in drug induced toxicity has been assessed by estimating serum biochemical parameter such as Serum Glutamate Oxaloacetate Transaminases (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALP), Total Protein, Total Bilirubin and liver endogenous antioxidant enzymes such as SOD, CAT and GSH

MATERIALS AND METHODS

Chemicals: Silymarin was obtained from Sigma Aldrich, USA. Paracetamol was obtained from Micro labs, Bangalore (INDIA) as a gift sample. CCl_4 was obtained from Rankem, Bangalore (INDIA). The kits for all biochemical estimations were purchased from Merck Ltd, Kalyan-Badlapur Road, Ambarnath (INDIA). The solvents and other chemicals used were of analytical grade and obtained from local dealers.

Collection of plant material: The leaves of *P. aculeata* were collected from Devara Kotta village of Hiriyura District, Karnataka and authenticated by Dr. K. Mruthunjaya, Dept. of Pharmacognosy, JSS College of Pharmacy, Mysore INDIA.

Animals: The experiments were carried out on Wistar rats of either sex weighing 170 ± 20 g. Animals were maintained in standard laboratory conditions of temperature ($27 \pm 2^\circ\text{C}$), humidity. The rats were fed on standard food pellets and water *ad libitum*. The studies conducted were approved by the Institutional Ethical Committee, JSS College of Pharmacy, Mysore, Karnataka, INDIA.

Preparation of extract: The leaves were shade-dried at room temperature and the alcoholic extract of *P. aculeata* (PAL) was obtained with 95% v/v alcohol for 30 cycles, using soxhlet apparatus. The aqueous extract of *P. aculeata* (PAQ) was prepared with the remaining dried coarse powdered crude drug (100 g) by a maceration process for 24 h with intermittent shaking. The extracts were decanted, filtered and concentrated and dried on water bath and stored in dessicator till use.

In-Vitro hepatoprotective activity: Overnight fasted male Wistar rat weighing 200 g was used. Animal was anaesthetized by using ketamine and 1% of sod.citrate was injected (i.p.) to prevent blood clotting. Rat was sacrificed by cervical dislocation and Liver lobes were isolated after cardiac and liver perfusion with Ca^{2+} - Mg^{2+} free Hanks buffer salt solution (pH 7.4) for 15 min. Single cell suspension of hepatocytes was prepared by using (0.75% in HBSS) collagenase digestion method. Fixed number of hepatocytes (1×10^6) was incubated in the medium with toxin (CCl_4) and with or without PAL/ PAQ (at conc.1, 10 and 100 $\mu\text{g/ml}$) at 37°C for 3 h. Hepatoprotective activity was assessed by checking the viability of the cells after 3 h of incubation using Trypan blue dye and by measuring release of cytosolic enzymes like GPT, GOT and LDH in the medium using semiautoanalyser.

Acute toxicity study [OECD Guidelines 425, 2006]: Acute toxicity studies were conducted as per the OECD guidelines 425, 2006 to determine the safe dose. According to the guideline female Wistar rats were used for the study. The suspension of PAL was prepared by suspending in 0.25% CMC and a single dose of PAL (2000 mg/kg) was administered orally to single animal and observed for the 24 h for mortality. Based on the presence or absence of mortality further experiment was planned as per the guide lines.

***In vivo* hepatoprotective activity** [Mitra et al, 1998; Gupta et al, 2006]: Experimental animals were randomly divided into five groups of six each Group – I, Group – II, Group – III, Group – IV and Group – V. The animals of Group – I and Group – II were administered vehicle for seven consecutive days p.o. The animals of group III and IV were administered PAL (200 mg/kg and 300 mg/kg, p.o.) respectively, for seven consecutive days. The animals of group V were administered Silymarin (100 mg/kg, p.o.) for seven consecutive days.

On 7th day after 2 h of treatment, acute liver damage was induced in animals of group I, III, IV and V by oral administration of 2 gm/kg of Paracetamol. After 48 h of Paracetamol administration blood was collected from retro orbital sinus and allowed to coagulate for 30 min followed by centrifugation at 2500 rpm for 5 minutes. Separated serum was used for estimating serum biochemical parameters. After blood collection, animals were sacrificed by cervical dislocation. The whole liver was then perfused *in situ* with ice-cold saline, dissected out, blotted dry and immediately weighed. A 10% liver homogenate was prepared with 150 mM KCL using Teflon– glass homogenizer (Yamato L.S. G. L.H-21, Japan). The tissue homogenate was centrifuged at 14,000 rpm for 1 h at 4°C. The supernatant was used for evaluation of liver endogenous antioxidant enzymes. The remaining part of the liver was washed in normal saline and fixed in 10% formalin for 2 days. Then 5 μ thickness microtome sections were made. The sections were processed in alcohol-xylene series and stained with haematoxylin and eosin. The slides were studied under a light microscope for any histological damage/protection.

STATISTICAL ANALYSIS: All the values were expressed as mean \pm SEM. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test using Graph Pad Prism Ver 5.0 software. A value of $P < 0.05$ was considered significant.

RESULTS

From *in vitro* study, CCL₄ treatment significantly reduced the percentage viability of hepatocytes when compared to Normal (Table. 1). Incubation of toxin challenged hepatocytes with PAL/ PAQ for 3 h significantly reversed the CCl₄ induced hepatotoxicity. The IC₅₀ value of PAL and PAQ was 7.49 and 53.57 μ g/ml respectively. Further CCl₄ caused significant increase in the GOT, GPT and LDH levels in the media when compared to Normal. This increase in GOT, GPT and LDH levels by CCl₄ was significantly reversed by both the extracts of *P. aculeata* in a dose dependent manner. Among two extracts alcoholic extract as found more potent than aqueous extract in reversing the toxin induced elevated enzyme levels (Table. 2).

From the acute toxicity study it was observed that oral administration of PAL at 2000 mg/kg did not cause mortality in the first animal. The extract was found safe in four more animals dosed at 2000 mg/kg indicating the LD50 above 2000 mg/kg.

From *in vivo* study Paracetamol treatment significantly elevated the levels of SGOT, SGPT, LDH, ALP and Total Bilirubin, while Total Protein level was significantly decreased when compared to Normal (Table. 3). Treatment with PAL at both the tested doses (200 and 300 mg/kg) and Silymarin (100 mg/kg) significantly reversed SGOT, SGPT, LDH, ALP, Total Bilirubin and Total Protein levels when compared to positive Control. The treatment with PAL at 300 mg/kg was found similar to standard Silymarin in reducing the elevated LDH levels. However PAL at 300 mg/kg was found less effective than standard Silymarin in reversing the other biochemical parameters.

Paracetamol treatment significantly decreased liver endogenous antioxidant enzymes such as SOD, CAT and GSH while lipid peroxidation was significantly enhanced when compared to Normal (Table. 4). PAL treatment (200 and 300 mg/kg) as well as Silymarin treatment (100 mg/kg) significantly reversed SOD, CAT and GSH levels with a subsequent reduction in lipid peroxidation when compared to Control. Treatment with PAL at 300 mg/kg was found similar to standard Silymarin in reversing the SOD levels and lipid peroxidation However it was found less effective than standard Silymarin in reversing the CAT and GSH levels as compared to normal. In case of normal (-ve control), hepatic globular structure, central vein, portal tract and kupffer cells look normal. In case of Paracetamol treated group (+ve control), hepatic cells has shown extensive fatty change and ballooning of hepatocytes, more around

central vein and microvasculisation fatty change. Liver sinusoids were congested. In case of 100 mg/kg silymarin treated group the hepatic globular architecture was normal. There mild inflammatory cells and mild fatty change. There was no congestion. In case of 200 mg/kg PAL there were mild inflammations with portal vein like normal. In case of 300 mg/kg PAL the hepatic architecture was maintained. There was less fatty change.

DISCUSSION

Liver is the vital organ of metabolism and excretion. Many synthetic chemical substances/drugs are metabolized mainly by liver. Free radicals are generated during this phase leading to hepatotoxicity by damaging the cell membrane and cell constituents. The mechanism of CCl₄ induced liver injury is due to the lipid peroxidation caused by the free radical derivatives of CCl₄. CCl₄ is metabolized in endoplasmic reticulum and mitochondria with the formation of CCl₃O by cytochrome P-450. The nascent oxygen O⁻ causes rise in intracellular reactive Fe²⁺ ions, aldehyde and depletion of GSH, and calcium sequestration via lipoperoxidation [Zimmerman & Hayman, 1976; Agarwal et al., 1983].



Paracetamol (N-acetyl-p-aminophenol) is a widely used analgesic and antipyretic drug and is safe when used in therapeutic doses. However, over dosage of paracetamol is known to be hepatotoxic in man and in experimental animals [Dipak et al., 1995]. Paracetamol hepatotoxicity is caused by the reaction metabolite N-acetyl-p-benzo quinoneimine (NAPQI) [Neils et al., 1996]. CCl₄ and Paracetamol are known to cause hepatocellular damage through free radical generation and are commonly employed as experimental hepatotoxic agents. Leaves of *Parkinsonia aculeata* Linn. reported to contain C-glycosides (epi-orientin, Parkinsonin-A, Parkinsonin-B, Parkintin) and flavone C-glycoside (Luteolin), orientin, iso-orientin, vitexin, iso-vitexin, lucenin-II, vicanin-II, diosmetin 6-C-β-glucoside, apigenin, luteolin, kaempferol and chrysoeriol [Bhatia et al., 1966; El-sayed et al., 1991]. It has been reported that *P. aculeata* has potent antioxidant activity [Mruthunjaya & Hukkeri, 2008]. Hence *P. aculeata* was selected to explore its hepatoprotective activity against hepatotoxicity induced by CCl₄ (*in vitro*), Paracetamol and Anti-TB drugs (*in vivo*).

From our present results (*in vitro*), CCl₄ incubation caused an abnormal reduction in the percentage viability of hepatocytes, while abnormal increase in

the GOT, GPT and LDH levels in the media. Treatment with PAL and PAQ significantly reversed all these abnormal changes and thus offered protection against CCl₄ induced toxicity to rat hepatocytes. PAL (at 100 μg/ml) normalized the viability of hepatocytes as well as cytosolic enzymes to near normal indicating that PAL at 100 μg/ml concentration is effective in reversing CCl₄ induced toxicity to rat hepatocytes.

From the *in vitro* study PAL was found most effective when compared to PAQ. An acute toxicity study of PAL was done on Wistar rats owing to its promising *in vitro* activity prior to the hepatoprotective activity. The LD₅₀ of PAL was found to be above 2000 mg/kg. Hence, 1/10th (200 mg/kg) and 1/6.67th (300 mg/kg) of this dose were selected for the *in vivo* study.

From our present results (*in vivo*) Paracetamol treatment caused an abnormal elevation in the levels of serum GOT, GPT, ALP, LDH and bilirubin, while abnormal depletion in liver endogenous antioxidant enzymes in positive control rats along with the marked elevation of lipid peroxidation products due to hepatocellular damage. Treatment with PAL significantly reversed all these abnormal changes and thus offered protection against Paracetamol induced liver damage.

The present study brings about the potential hepatoprotective activity of *P. aculeata* and gives insight into its mechanism of action. Possible mechanism that may be responsible for the protection of Paracetamol induced liver damage by PAL include the following (a) leaves of *P. aculeata* found to contain various flavonoids as listed above [C-glycosides (epi-orientin, Parkinsonin-A, Parkinsonin-B, Parkintin) and flavone C-glycoside (Luteolin), orientin, iso-orientin, vitexin, iso-vitexin, lucenin-II, vicanin-II, diosmetin 6-C-β-glucoside, apigenin, luteolin, kaempferol and chrysoeriol] which are reported as potent antioxidants [Bhatia et al., 1966; El-sayed et al., 1991]. PAL also found to contain flavonoids as tested by preliminary phytochemical tests. So PAL could act as a free radical scavenger intercepting those radicals involved in Paracetamol metabolism by microsomal enzymes. (b) A significantly higher content GSH in liver would afford the tissue a better protection against antioxidative stress, thus contributing to the abolishment of Paracetamol induced hepatotoxicity. (c) Therefore, PAL is a promising hepatoprotective agent. The hepatoprotective action combined with antioxidant activity has a synergistic effect to prevent the process of initiation and progress of hepatocellular damage [Gupta et al., 2006].

CONCLUSION

In conclusion, the present studies indicated that the extracts of leaves of *P. aculeata* possess potent hepatoprotective activity comparable to that of standard Silymarin which is known hepatoprotective agent as evidenced by the serum biochemical

parameter and liver endogenous antioxidant enzymes activity.

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Table 1: Effect of PAL and PAQ on percentage viability of CCl₄ intoxicated hepatocytes by trypan blue exclusion assay

	PAL		PAQ	
	% viability	% protection	% viability	% protection
Control	16.26±4.52 ^b	----	16.26±4.52 ^b	-----
Normal	96.41±0.95	-----	96.41±0.95	-----
1 µg/ml	39.7±2.20 ^{a,b}	37.02	17.33±1.56 ^b	15.35
10 µg/ml	62.30±1.62 ^{a,b}	63.64	41.33±2.53 ^{a,b}	39.66
100 µg/ml	87.75±4.83 ^a	97.00	59.66±4.23 ^{a,b}	71.33

Values are mean±SEM, n=3, ^aP<0.05 as compared with Control. ^bP<0.05 as compared with Normal.

Table 2: Effect of PAL and PAQ on CCl₄ induced toxicity on isolated hepatocytes by assessment of GOT, GPT and LDH release

	PAL			PAQ		
	GPT	GOT	LDH	GPT	GOT	LDH
Control	202.33±6.43 ^b	1779.33±18.85 ^b	183.66±1.45 ^b	202.33±6.43 ^b	1779.33±18.85 ^b	183.66±1.45 ^b
Normal	104.33±5.81	785.00±23.62	103.00±0.57	104.33±5.81	785.00±23.62	103.00±0.57
1µg/ml	170.33±3.4a ^{a,b}	1218.33±16.75 ^{a,b}	136.33±0.88 ^{a,b}	197.33±3.48 ^b	1675.33±16.75 ^b	162.33±0.88 ^b
10µg/ml	153.33±3.75 ^{a,b}	1056.33±21.07 ^{a,b}	123.66±0.88 ^{a,b}	172.33±3.7 ^{a,b}	1329.33±21.07 ^{a,b}	149.66±0.88 ^{a,b}
100µg/ml	130±2.64 ^a	830±20.52 ^a	111±1.15 ^a	158±2.64 ^{a,b}	1185±20.52 ^{a,b}	129±1.15 ^{a,b}

Values are mean±SEM, n=3, ^aP<0.05 as compared with Control. ^bP<0.05 as compared with Normal

Table 3: Estimation of biochemical parameters in Paracetamol induced hepatotoxicity in rats

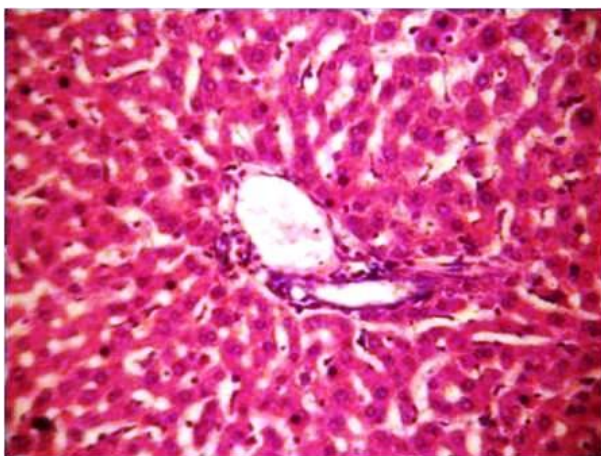
Groups	SGOT	SGPT	ALP	LDH	Total Bilirubin	Total Protein
GROUP I (Control)	285±4.35 ^{b,c}	137±3.21 ^{b,c}	549.33±7.31 ^{b,c}	187.33±3.44 ^{b,c}	1±0.05 ^{b,c}	3.73±0.35 ^{b,c}
GROUP II (Normal)	101±3.78	63.33±2.33	184.33±3.28	112±3.69	0.26±0.03	8.96±0.35
GROUP III (200 mg/kg)	192.5±4.27 ^{a,b,c}	97.5±2.32 ^{a,b,c}	409.75±6.86 ^{a,b,c}	163.33±3.02 ^{a,b,c}	0.72±0.04 ^{a,b,c}	4.92±0.26 ^{b,c}
GROUP IV (300 mg/kg)	151.8±1.82 ^{a,b}	81±3.04 ^{a,b}	279.2±4.47 ^{a,b,c}	124.66±6.02 ^a	0.48±0.03 ^{a,b,c}	6.74±0.29 ^{a,b}
GROUP V (Standard)	118.66±4.05 ^a	68.66±5.36 ^a	211±10.01 ^a	119.33±3.76 ^a	0.43±0.03 ^a	7.26±0.54 ^a

Values are mean±SEM, n=6, ^aP<0.05, when compared with Control, ^bP<0.05 when compared with Normal, ^cP<0.05, when compared with Standard.

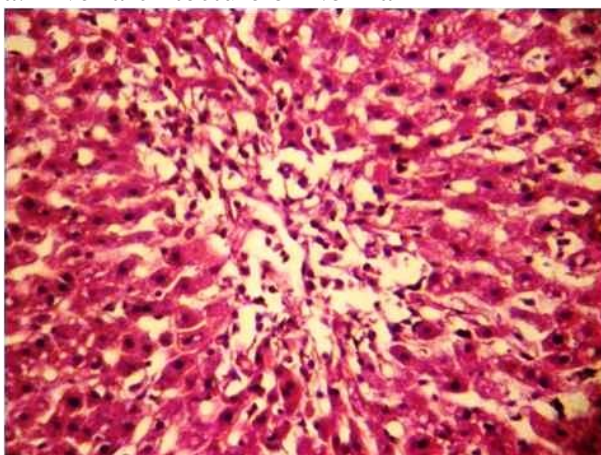
Table 4: Estimation of Tissue Enzyme activity in Paracetamol induced hepatotoxicity

Groups	SOD	CAT	GSH	L.PEROXI
GROUP I (Control)	0.87±0.25 ^{b,c}	14.78±0.75 ^{b,c}	20.91±0.74 ^{b,c}	50.73±2.23 ^{b,c}
GROUP II (Normal)	10.19±0.69	34.95±0.39	45.07±0.73	20.53±2.28
GROUP III (200 mg/kg)	4.89±0.59 ^{a,b,c}	21.91±0.87 ^{a,b,c}	30.87±0.42 ^{a,b,c}	38.00±2.35 ^{a,b,c}
GROUP IV (300 mg/kg)	8.81±0.80 ^a	31.01±0.38 ^{a,b}	40.98±0.90 ^{a,b}	23.57±2.27 ^a
GROUP V (Standard)	9.99±0.18 ^a	32.01±0.86 ^a	42.99±0.93 ^a	23.04±2.20 ^a

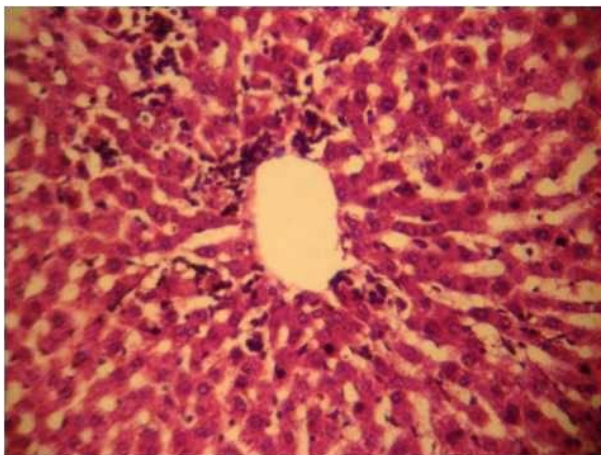
Values are mean±SEM, n=6, ^aP<0.05, when compared with Control, ^bP<0.05 when compared with Normal, ^cP<0.05, when compared with standard.



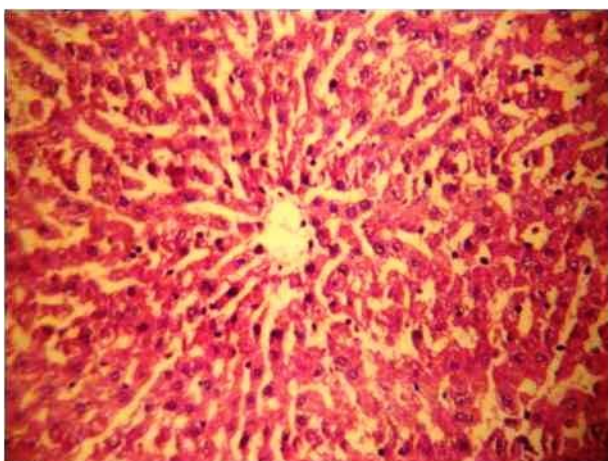
a: Liver architecture of Normal



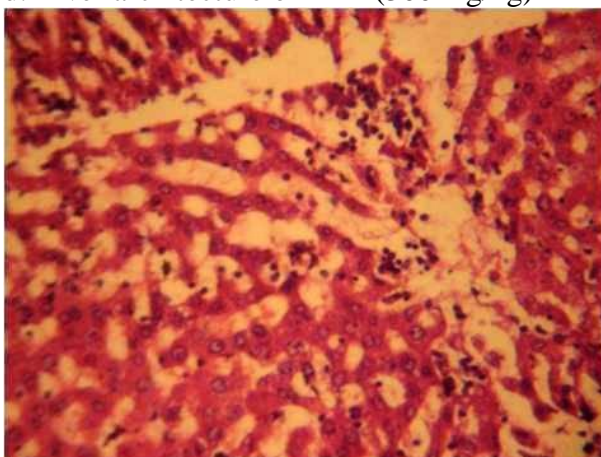
b: Liver architecture of Control (Paracetamol 2 gm/kg)



c: Liver architecture of Standard (100 mg/kg)



d: Liver architecture of PAL (300 mg/kg)



e: Liver architecture of PAL (200 mg/kg)

Figure 1: Histopathological studies in Paracetamol induced hepatotoxicity

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