

Hepatoprotective properties of *Caesalpinia sappan* Linn. heartwood on carbon tetrachloride induced toxicity

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Aim of the study was to investigate the methanol and aqueous extracts of heartwood of *C. sappan* for its hepatoprotective activity against CCl₄ induced toxicity in freshly isolated rat hepatocytes and animals. Freshly isolated rat hepatocytes were exposed to CCl₄ (1%) along with/without various concentrations of methanolic and aqueous extract of *C. sappan* (1000-800 µg/ml) and the levels of selected liver enzymes were estimated. Antihepatotoxic effect of methanolic extract was observed in freshly isolated rat hepatocytes at concentrations 1000-800 µg/ml and was found to be similar to that of standard drug silymarin. Wistar strain albino rat model was used for the investigation of *in vivo* hepatoprotective properties of aqueous and methanolic extract of *C. sappan* (100 and 200 mg/kg body weight). Liver damage was induced by ip administration of CCl₄ (30%) suspended in olive oil (1 ml/kg body weight). Both the tested extracts showed potent hepatoprotective activity at 200 mg/kg body weight test dose which was comparable with that of the standard silymarin used in similar test dose. The methanolic and aqueous extract was able to restore the biochemical levels to normal which were altered due to CCl₄ intoxication in freshly isolated rat hepatocytes and also in animals.

Keywords: *Caesalpinia sappan*, Heartwood, Hepatocytes, Hepatoprotective

Caesalpinia sappan Linn., a small thorny tree, 6-9 m high¹⁻² is found in India, Peru, Malaya etc. It is being used traditionally for large number of ailments and reported to have a wide variety of medicinal properties. Its anticonvulsant³, anti-inflammatory⁴, anti-proliferative⁵, antimicrobial⁶, antiviral⁷, anticoagulant⁸, immunostimulant⁹, vasorelaxing¹⁰ and antioxidant¹¹ activities have been reported³⁻¹¹. The heartwood of the plant is reported to have greatest medicinal value. The wood is orange-red, hard and very heavy. According to Ayurveda, the heartwood is useful in vitiated conditions of *Pitta*, burning sensation, wounds, ulcers, leprosy, skin diseases, diarrhea, dysentery, epilepsy, menorrhagia, leucorrhoea, diabetes etc. A decoction of the heartwood is commonly used in Kerala, India for its antithirst, blood purifying, antidiabetic properties and the plant is one of the ingredients in many traditional Ayurvedic formulations¹¹.

Brazilin, an isolated compound from this plant is reported to reduce bromo carbon trichloride (BrCCl₃)

induced toxicity on cultured rat hepatocytes *in vitro*¹². The methanolic and aqueous extract of heartwood of *Caesalpinia sappan* is reported to possess potent antioxidant activity¹¹. Since many hepatic injuries are free radical mediated, it is possible that the heartwood of the plant may show potent hepatoprotective activity. Hepatoprotective activity of this plant has not been reported so far. In this study *in vitro* and *in vivo* hepatoprotective property of aqueous and methanolic extracts of heartwood of *Caesalpinia sappan* in CCl₄ induced toxicity is being reported.

Materials and Methods

Chemicals and reagents—All routine chemicals were obtained from SD-fine chemicals, Mumbai, India. 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), collagenase, insulin, dexamethasone, F-12 Coons media, Ham's F12 medium, New born calf serum, Silymarin and antibiotics were purchased from Sigma Chemicals Co., St. Louis, MO, USA. Ecoline diagnostic kits were purchased from E-Merck, India.

Plant material—The heartwood of *Caesalpinia sappan* Linn. was collected during October 2001 from the campus of M.G. University, Kottayam, India and

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authenticated by S. Rajan, Medicinal Plants Survey and Collection Unit, Govt. Arts College, Ootacamund, India. A voucher specimen has been preserved for further reference.

Preparation of plant extract—The heartwood of *C. sappan* (38 g) was mechanically powdered and subjected to single Soxhlet extraction using methanol (500 ml). The extract was concentrated to dryness under reduced pressure and controlled temperature to yield a light brown colored mass (3.5 g, 9.2%). The aqueous extract was prepared by boiling the powder of heart wood (38 g) with water (1000 ml) repeatedly for 48 hr. Then the extract was concentrated by lyophilization to yield dark brown colored substance (4 g, 10.52%). Preliminary phytochemical tests indicated the presence of flavonoids, glycosides, triterpenoids and tannins in both methanol and aqueous extracts.

Preparation of suspension—10 mg of both the aqueous and methanolic extracts of *C. sappan* were dissolved separately in 1 ml of Dimethyl sulphoxide (DMSO) and volume was made up to 10 ml with F12 Coon's/MEM to obtain a stock solution of 1 mg/ml concentration and stored at -20°C prior to use. Further dilutions were made to obtain different concentrations ranging from 1000-800 µg/ml with the respective media and used for *in vitro* investigations. Suspensions of standard silymarin powder was also prepared (1000 µg/ml) in a similar manner. Both the extract and standard silymarin were suspended in sodium CMC (0.3%) in distilled water and used for *in vivo* investigations.

Isolation of culture of hepatocytes—Liver cells were isolated by a modified procedure of Seglen 1994¹³. The calcium free HEPES buffer and collagenase solutions were warmed in a water bath (37°C). The abdomen of the rat was opened under phenobarbital sodium (35 mg/kg body weight) anesthesia. A midline incision was made and a loosely tied ligature was placed around the portal vein approximately 5 mm from the liver and the cannula was inserted up to the liver and then the ligature was tightened and heparin was injected into the femoral vein (1000 IU). The inferior venacava was cut below the renal vein. Perfusion was performed for 20 min (37°C) with calcium free HEPES buffer, containing 1% bovine serum albumin fraction V at a flow rate of 30 ml/min. The liver swells during this time, slowly changing its color from dark red to grayish white. The swollen liver was then perfused with TPVG solution

(50 ml) followed by perfusion with calcium free HEPES buffer, which contained additional collagenase (0.075%) and calcium chloride (4 mM) at a flow rate of 15 ml/min for 20 min.

After the perfusion, the lobes were removed and transferred into a sterile petri dish containing calcium free HEPES buffer and dispersed gently. It was transferred into a sterile conical flask and the crude cell suspension was stirred with the help of a magnetic stirrer for 5 min to release hepatocytes into the solution. The cell suspension was filtered through a nylon mesh (250 µ) and the cell suspension was centrifuged at 1000 rpm for 15 min. The supernatant was aspirated off and the loosely packed cell pellet was resuspended in calcium free HEPES buffer. This washing procedure was repeated three times. Cell viability was determined by the trypan blue dye exclusion method¹⁴. These isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% newborn calf serum, antibiotics, 10⁻⁶M dexamethasone and 10⁻⁸ bovine insulin. The cell suspension was incubated at 37°C for 30 min in a humidified incubator with 5% CO₂.

Carbon tetrachloride (CCl₄) induced in vitro hepatocytes injury—CCl₄ induced hepatic injury was carried out by the method determined by Yoshinobu *et al.*¹⁵. After an incubation of 24 hr, the hepatocytes were exposed to the fresh medium containing CCl₄ (1%) along with/without various concentrations of the extracts or the medium alone (as normal). The little percentage of DMSO present in the wells (maximum 0.2%) was found not to affect the experiment. After 60 min of CCl₄ challenge, concentrations of aspartate amino transferase (ASAT), alanine amino transferase (ALAT), alkaline phosphatase (ALP), triglycerides (TGL), total proteins, albumin, total bilirubin, direct bilirubin and cholesterol in the medium were measured as an indication of hepatocytes necrosis using Ecoline diagnostic kits.

In vivo hepatoprotective effect of C. sappan—Colony bred Wister stain adult albino rats (150-200 g) of either sex were used for the investigations. All the animals were maintained under standard husbandry conditions with food and water *ad libitum*. The experimental procedures were approved by the Committee for the Purpose of Control and Supervision of Experiments of Animals (CPCSEA), Chennai (Proposal No. JSSCP/IAEC/M.Pharm/Ph.Biotech/02/2002-2003). The animals were divided into 7 groups of 6 animals in each group and the

treatment schedule is as in Table 2. Liver damage was induced by ip administration of 30% CCl₄ suspended in olive oil (1 ml/kg body weight, ip)¹⁶. Animals received the treatments by the oral route for a period of 7 days. On the seventh day except group I (control), all other groups received 30 percent CCl₄ suspended in olive oil (1 ml/kg body weight) ip. After 24 hr of intoxication, on the 8th day, blood was withdrawn and collected in sterile centrifuge tubes and allowed to clot. Serum was separated and used for the estimation of ASAT, ALAT, ALP, TGL, total proteins, albumin, total bilirubin, direct bilirubin and cholesterol using Ecoline diagnostic kits.

Statistical analysis—The statistical analysis was carried out by one way analysis of variance (ANOVA). The values are represented as mean ± S.E.M. Comparison of mean values of different groups treated with different dose levels of the two extracts and positive control with normal were estimated by Turkey's Multiple Comparison Test. $P < 0.05$ was considered significant.

Histopathology—Liver was removed fixed overnight in 10% buffered formalin and paraffin-embedded. The sections were stained with hematoxylin and eosin (H&E) for histological evaluation and examined under light microscope. In brief, 4 µm thick sections of paraffin-embedded rat liver were dewaxed in xylene, rehydrated in graded alcohol series, and washed with distilled water for 2 min. Subsequently, the sections were stained with hematoxylin for 5 min at room temperature. After 15 min, the sections were counterstained with eosin for 2 min, dehydrated in graded alcohol series, washed with

xylene, and blocked by rosin. H & E stained slides were observed under microscope at ×40 magnifications.

Results and Discussion

Hepatoprotective effects in freshly isolated rat hepatocytes—The effects of the aqueous and methanolic extracts of *C. sappan* on freshly isolated rat hepatocytes intoxicated with CCl₄ are recorded in Table 1. A significant increase in the levels of ASAT, ALAT, ALP, total bilirubin, direct bilirubin and a significant reduction in levels of TGL, total proteins, albumin and cholesterol were observed in hepatocytes exposed to CCl₄ when compared to normal rats. These cells when treated with the aqueous and methanolic extract of *C. sappan*, significant dose dependent restoration of the altered biochemical parameters towards the normal was observed. A similar result was obtained when CCl₄ intoxicated hepatocytes were treated with the standard silymarin. Hepatoprotective effect of methanolic extract was slightly better than aqueous extract and similar to that of silymarin used.

In vivo hepatoprotective activity—Effects of aqueous and methanolic extracts of *Caesalpinia sappan* on CCl₄ intoxicated rats are recorded in Table 2. Intoxication of rats treated with CCl₄ significantly altered the biochemical parameters when compared with the normal control rats ($P < 0.001$). In CCl₄ intoxicated rats there was elevation in the levels of ASAT, ALAT, ALP, TGL, total bilirubin, direct bilirubin and decrease in total proteins, albumin and cholesterol levels on comparison with control. Liver injured rats when treated with 200 mg/kg body weight

Table 1— Effect of treatments of aqueous and methanol extract of *Caesalpinia sappan* on the biochemical parameters of CCl₄ intoxicated freshly isolated rat hepatocytes
[Values are mean ± SE of 6 replicates]

Groups	Concentration (µg/ml)	ASAT U/L	ALAT U/L	ALP U/L	TGL mg/dl	Total Protein g/dl	Albumin F/L	Total Bilirubin mg/dl	Direct Bilirubin g/dl	Cholesterol mg/dl
Control	—	12±0.01	15±0.46	29±0.39	160±9.08	3.092±0.04	0.691±0.06	0.921±0.002	0.089±0.005	31.92±0.52
CCl ₄	1%	78±0.53 ^a	95±2.86 ^a	96±0.48 ^a	71±3.06 ^a	1.621±0.06 ^a	0.300±0.01 ^a	1.961±0.03 ^a	0.324±0.01 ^a	12.96±0.13 ^a
Silymarin	1000	14±1.24 ^b	22±0.97 ^b	33±0.83 ^b	181±12.47 ^b	2.991±0.02 ^b	0.570±0.03 ^b	0.598±0.002 ^b	0.082±0.02 ^b	33.42±0.26 ^b
	1000	19±1.22 ^{b,c}	36±0.98 ^b	40±0.92 ^{b,d}	204±10.08 ^b	2.692±0.02 ^b	0.491±0.04 ^b	0.922±0.002 ^{b,d}	0.092±0.04 ^{b,c}	42.06±0.44 ^b
Aqueous extract	900	21±0.66 ^b	40±1.39 ^b	43±0.73 ^{b,c}	196±8.90 ^b	2.609±0.03 ^b	0.470±0.07 ^b	1.114±0.002 ^b	0.116±0.01 ^b	39.35±0.16 ^b
	800	23±1.02 ^b	47±1.34 ^b	49±0.51 ^b	174±9.81 ^b	2.009±0.03 ^b	0.430±0.05 ^b	1.201±0.001 ^b	0.136±0.02 ^b	38.66±0.25 ^b
	1000	17±1.25 ^b	29±1.76 ^b	47±0.51 ^b	152±5.52 ^b	2.921±0.02 ^b	0.460±0.05 ^b	0.901±0.003 ^b	0.117±0.02 ^b	30.01±0.29 ^b
Methanol extract	900	22±0.44 ^b	32±1.80 ^b	52±0.58 ^b	141±6.73 ^b	2.679±0.03 ^b	0.432±0.04 ^b	1.006±0.003 ^b	0.134±0.02 ^b	26.93±0.28 ^b
	800	24±1.02 ^b	33±1.78 ^b	59±0.66 ^b	136±5.12 ^b	2.342±0.03 ^b	0.420±0.04 ^b	1.112±0.001 ^b	0.162±0.02 ^b	25.21±0.22 ^b

P values: a = < 0.001, when compared to normal group, b = < 0.001, when compared to CCl₄ group, c = < 0.05, d = < 0.01, e = < 0.001, when compared to silymarin.

Table 2—Effect of treatment of aqueous and methanol extract of *Caesalpinia sappan* on the biochemical parameters of CCl₄ intoxicated rats [Values are mean ± SE of 6 rats]

Groups	Concentration (mg/kg)	ASAT U/L	ALAT U/L	ALP U/L	TGL mg/dl	Total Protein g/dl	Albumin F/L	Total Bilirubin mg/dl	Direct Bilirubin g/dl	Cholesterol mg/dl
Control	-	58.83±1.3	7.33±0.9	267.06± 7.13	256.33±16.76	7.83±0.65	4.96±0.44	0.45±4.28	0.15±2.23	121±12.88
CCl ₄	1ml/kg	96.83±1.92 ^d	59.98±2.37 ^d	408.3±15.96 ^d	128.83±10.76 ^d	5.0±0.68 ^b	3.46±0.2 ^b	1.2±7.74 ^d	0.4±3.33 ^d	47.83±5.12 ^d
Silymarin	200	64.8 ±1.42 ^{bz}	28.24±1.33 ^{az}	263.4 ±11.53 ^{az}	256.6 ±5.72 ^{az}	6.6 ±0.39 ^{aw}	4.84±0.3 ^{ay}	0.42±3.74 ^{az}	0.16±2.44 ^{az}	92.8±5.35 ^{az}
Aqueous extract	200	65.8±1.42 ^{cz}	31.84±0.61 ^{bz}	309.52 ±17.35 ^{ay}	256.8±1.99 ^{az}	7.0 ±0.7 ^{aw}	5.66±0.27 ^{az}	0.64±2.44 ^{cz}	0.26±2.44 ^{cz}	66.2±1.11 ^{cx}
	100	77.33±1.62 ^{dz}	37.06±3.7 ^{bz}	355.18±3.65 ^{dx}	179.33±3.69 ^{cx}	6.33±0.33 ^{aw}	4.11±0.13 ^{ax}	0.85±4.28 ^{dy}	0.33±2.1 ^{cx}	49.83±3.18 ^{dw}
Methanol extract	200	68.8±1.24 ^{dz}	33.98±1.27 ^{bz}	321.48±16.99 ^{by}	230.2±7.7 ^{az}	6.6±0.67 ^{aw}	5.24±0.21 ^{az}	0.78±3.16 ^{cz}	0.26±2.44 ^{cy}	52±5.02 ^{cw}
	100	81±1.65 ^{dz}	37.15±1.03 ^{dz}	362.78±1.03 ^{dz}	146±14.1 ^{dw}	5.66±0.33 ^{bw}	3.78±0.18 ^{bw}	0.86±4.21 ^{dy}	0.35±2.35 ^{dw}	44.16±1.92 ^{dw}

Superscripts a, b, c and d denote statistical significance in comparison to normal group $P < 0.5$, < 0.05 , < 0.01 and < 0.001 respectively. Superscripts w, x, y and z denote statistical significance in comparison to CCl₄ group at $P < 0.5$, < 0.05 , < 0.01 and < 0.001 , respectively.

of aqueous extract of *C. sappan* showed significant increase in ASAT, ALAT, ALP, TGL, albumin, total bilirubin, direct bilirubin and a decrease in total proteins and cholesterol when compared with the normal group. Treatment with aqueous and methanol extract of heartwood of *C. sappan* at both 100 and 200 mg/kg body weight restored the biochemical parameters towards normal. The significant changes in the levels of biochemical parameters refer to the effect of plant extracts in protecting the liver by restoring the altered levels in rats. The liver injured rats that received 200 mg/kg body weight of the standard silymarin restored the altered levels considerably when compared with the CCl₄ group.

Liver injuries induced by CCl₄ are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs¹⁷⁻²⁰. Since the changes associated with CCl₄ induced liver damage are similar to that of acute viral hepatitis²¹, CCl₄ mediated hepatotoxicity was chosen as the experimental model. It has been established that CCl₄ is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P450 dependent monooxygenases to form a trichloromethyl radical (CCl₃). The CCl₃ radical alkylates cellular proteins and other macromolecules with simultaneous attack on polyunsaturated fatty acids, in presence of oxygen, to produce lipid peroxides, leading to liver damage²². Thus, antioxidant or free radical generation inhibition is important in protection against CCl₄ induced liver lesions²³. Hepatotoxic compounds such as CCl₄ are known to cause marked elevation in serum enzymes and bilirubin levels. It causes marked decrease in TP

levels. Silymarin is used as standard hepatoprotective compound since it is reported to have a protective effect on the plasma membrane of hepatocytes²⁴. To our knowledge, this is the first study which reveals the hepatoprotective effect of methanolic and aqueous extract of heartwood of *C. sappan* against CCl₄ induced toxicity in isolated rat hepatocytes and in animals.

CCl₄ has been found to induce extensive liver damage within a period of 24 hr following ip administration. As a result of this, accumulation of fat in the liver and necrosis in the centrilobular region of the liver occurs. As a consequence, the microsomal enzyme activities are found to decrease and due to lipid peroxidation, the water-soluble enzymes leak into plasma from the liver. It is shown by the significant decrease in triglycerides and proteins in CCl₄ intoxicated rat hepatocytes or animals in the present studies. Treatment with the both methanolic and aqueous extract of heartwood of *C. sappan* exhibited significant restoration of the altered biochemical parameters towards normal in CCl₄ intoxicated rat hepatocytes and in rats. The effect of the methanolic extract at 1000 µg/ml was comparable to that of standard silymarin at the same concentration on rat hepatocytes. Its hepatoprotective effect with *in vivo* studies at 200 mg/kg body weight was comparable to that of silymarin at 200 mg/kg body weight, positively supported by the histopathology results (Fig. 1).

Several phenolic compounds and flavonoids have been isolated from heartwood of this plant²⁵⁻²⁶. Brazilin, a major constituent of heartwood is reported to have hepatoprotective activity^{12,25}. Hence, the hepatoprotective effect showed by heartwood of *C. sappan* Linn. in this study may be due to the

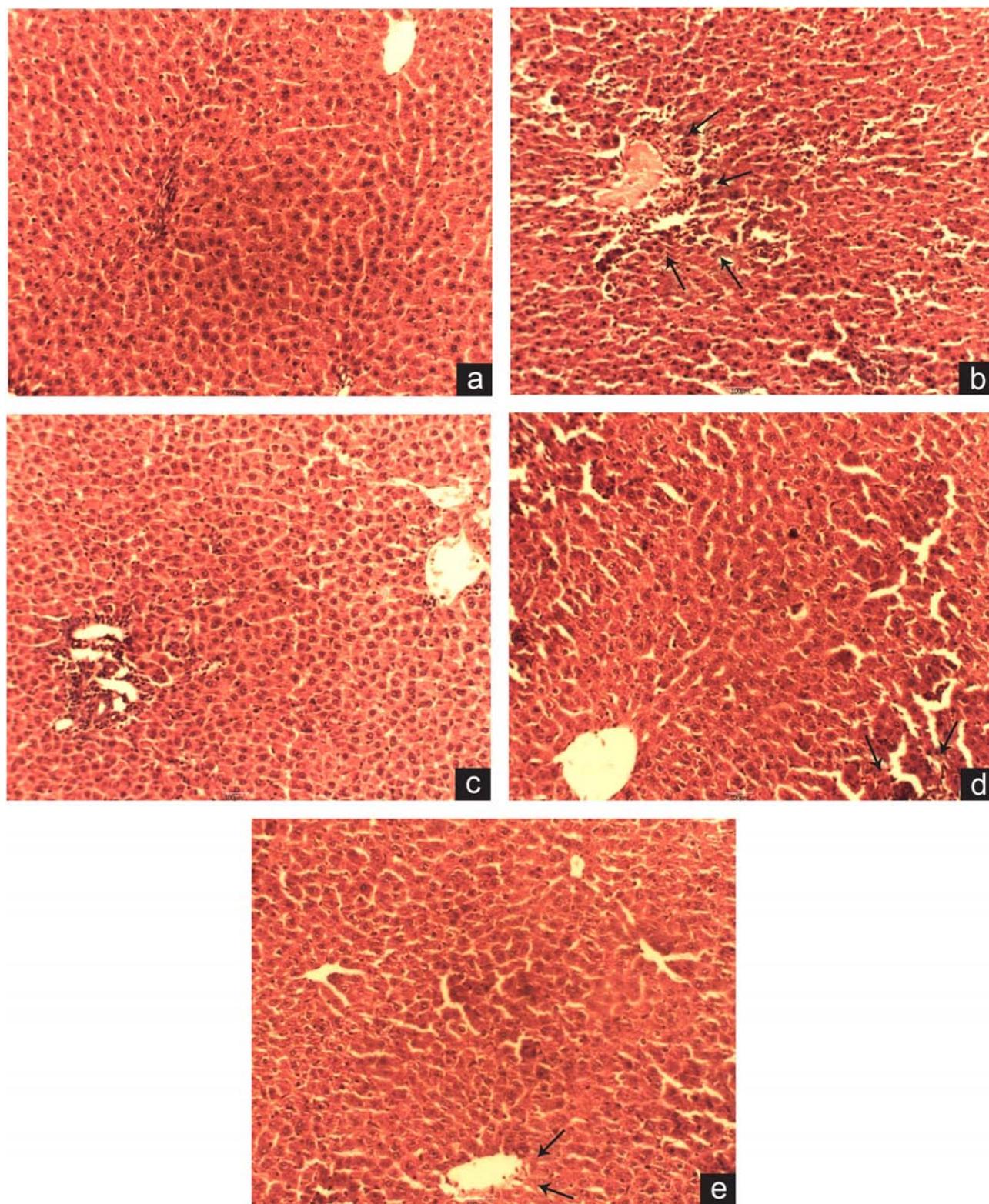


Fig. 1—Histopathology of (a): normal liver having histological structures of normal hepatic lobules; (b): toxicant treated liver (CCl_4 , 1 ml/kg body weight) showing damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis and congestion of hepatic sinusoids; (c): silymarin drug treated liver (200 mg/kg body weight) showing apparently normal hepatocytes; (d): *Caesalpinia sappan* (aqueous extract) treated liver (200 mg/kg body weight) showing mild vacuolization; (e): *Caesalpinia sappan* (methanolic extract) treated liver (200 mg/kg body weight) showing mild vacuolization. H & E $\times 40$.

presence of brazilin, flavonoids and phenolic compounds. The methanolic and aqueous extracts merits further investigation in identifying the active constituents responsible for this activity.

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