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Amelioration of *Trichosanthes lobata* in Paracetamol-Induced Hepatic Damage in Rats: A Biochemical and Histopathological Evaluation

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Abstract

The aim of the present work is to investigate the possible therapeutic potential of *Trichosanthes lobata* in paracetamol induced hepatic damage in rats. The hepatotoxicity was induced in rats by administration of paracetamol at higher dose (2 gm/kg) reflected in term of increase lipid peroxidation, impairment in antioxidants [Superoxide Dismutase (SOD), Catalase (CAT) and Reduced Glutathione (GSH)] along with elevation of serum marker enzymes. The possible ameliorative effect of *Trichosanthes lobata* Leaf extracts mainly Aqueous (TLAQ) and Alcoholic (TLAL) were administered at various doses for 7days and found to restored the depleted antioxidants (SOD, GSH, CAT) attenuated lipid peroxidation (MDA formation) and prevented elevated marker enzymes significantly. Further, histopathological alterations were also improved with TLAQ and TLAL in dose dependent manner. Thus, the hepatoprotective activity of TLAQ and TLAL may be correlated with amelioration of oxidative stress by improving endogenous antioxidant defence ability of hepatocytes and histopathological alterations in rats. The ameliorative effect of *Trichosanthes lobata* in paracetamol-induced hepatic damage in rats was studied.

Keywords: Flavonoids, Hepatic Damage, Paracetamol, *Trichosanthes lobata*

1. Introduction

Hepatic damage occurs mainly due to either liver infection or with administration/ingestion of antibiotics in therapeutic ranges. Various chemicals mainly chlorinated hydrocarbons, benzene, alcohol, mercury, arsenic etc are known to induce hepatic damages, leading to overall decline in metabolic functions of liver[1]. Some of these toxicants formed their active metabolites in liver and thus responsible for lipid peroxidation, protein dysfunction and finally oxidative stress. These cellular consequences disturb the vital physiological process which results into cell death and organ dysfunction[2]. The major concerns to noticed the liver diseases includes; firstly almost impossible as liver is extremely regenerative organ in which repairing action of damaging events mostly take place simultaneously; secondly, currently employed allopathic agents does not promise/guarantee for cure and/or prevention of liver diseases. Hence there is urgent need of hepatoprotective agent with least side effect[3,4].

Trichosanthes lobata Roxb (Family-Cucurbitaceae) known as Patola (Sanskrit) and wild snake gourd (English), is largely found in Maharashtra, India. Traditionally, the plant used as bitter tonic, laxative,

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depurative, digestive, cardiotonic, anthelmentic, and in treatment of jaundice[5]. Documented reports suggest the presence of various phytoconstituents viz; cucurbita-5, 24-dienol, α, β carotene, lycopene, lutein, vitamin C and β-sitosterol in *Trichosanthes lobata*[6]. However, there are no scientific reports unavailable indicating hepatoprotective effects of *Trichosanthes lobata.* Thus based on the presence of chemical constituents in *Trichosanthes lobata,* the present studies was undertaken to investigate the hepatoprotective effect of *Trichosanthes lobata* in PCM induced hepatic damage in rats and to understand the underlying mechanism of action.

2. Materials and Methods

2.1 Plant Material

The plant *Trichosanthes lobata* (Family- Cucurbitaceae) was collected in the month of June from local region of Lonavala region of Maharashtra, state, India. The plant herbarium was taxonomically identified at Botanical Survey of India (BSI), Pune. A voucher specimen (BSI/WC/Tech/2008/354-RRW/TL-2.) has been deposited for future reference.

2.2 Drugs and Chemicals

Paracetamol and silymarin were obtained as gift sample from Torent Research Centre, Ahmedabad and Cadila Pharma Ltd. India, respectively. Thiobarbituric Acid (TBA), reduced glutathione, oxidised glutathione and nicotinamide adenine dinuleotide (NADPH) were obtained from Himedia Laboratories, Mumbai, India, 5, 5-dithiobis (2-nitrobenzoic acid)-(DTNB) and epinephrine were purchased from Sigma chemical Co, St. Louis, MO, USA. Standard reagents and kits for determination of AST, ALT, ALP, LDH, Total Proteins and Total bilirubin were purchased from Span Diagnostics, Surat, India and Ranbaxy laboratories, Delhi, India. All other chemicals and reagents were of analytical grade obtained from local suppliers.

2.3 Preparation of Extracts

The powdered plant material (500 gm) was subjected to successive solvent extraction with the solvents in the order of increasing polarity in ratio of (1:2) viz; petroleum ether (40-60 °C), followed by chloroform and alcohol. Aqueous extract was prepared by cold maceration process using chloroform water 1996 as extracting solvent wherein, chloroform servers as preservative[7]. The extracts were dried under reduced pressure using rotary evaporator afforded semi solid extracts. It was then stored in ambered glass bottle until used.

2.4 Preliminary Phytochemical Analysis

The preliminary phytochemical analyses were carried out for the presence of various chemical constituents in alcoholic and aqueous extract of *Trichosanthes lobata* respectively[8].

2.5 High Performance Thin Layer Chromatography (HPTLC) Study

The aqueous and alcoholic extracts of *Trichosanthes lobata* were dissolved in respective HPTLC grade ethanol and water which were used for sample application on precoated silica gel GF 254 aluminium sheet (Made-Merck, Germany). The samples (5 µL) were spotted in the form of bands of width 6 mm with a 100 µL sample using a Hamilton syringe on silica gel which was precoated on aluminium plate GF-254 plates (20 cm X 10 cm) with the help of Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software. The linear ascending development of chromatogram was carried out in a (20 cm X 10 cm) twin trough glass chamber saturated with the mobile phase (Ethyl acetate:Formic acid:glacial acetic acid:Water (99:12:11:27 v/v/v). The developed plate was dried by hot air to evaporate solvents from the plate. The plate was sprayed with anisaldehyde sulphuric acid and 5 % Ferric chloride as spray reagent and dried at 100 °C in hot air oven for 3 min. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and images were taken at 214 nm. captured the images under UV light at 214 nm, respectively. The Retention factor (R_f) values and finger print data were recorded (WIN CATS software). Quercitin was used as reference standard for comparison.

2.6 Experimental Animals

Wistar rats of either sex (150-200 gm; 10–12weeks old) were procured from National Toxicology Centre, Pune. Animals were housed (6/group) in polycarbonate cages with standard conditions: humidity (50 ± 5 %),

temperature (25 ± 2 ºC) and light (12 hr light: 12 hr dark cycle) in our departmental animal house and were fed with a standard diet (Amrut lab Animal feed Pune, India) and water *ad libitum*. Experimental protocols was reviewed and approved by the Institutional Animal Ethics Committee KLE University's College of Pharmacy, Belagavi, Karnataka. (IAEC Reg. No.: 627/02/a/CPCSEA).

2.7 Acute Oral Toxicity Studies

Acute toxicity studies of aqueous and alcoholic extract were carried as per OECD-423 guidelines)[9]. The extracts at various doses were administered up to 2000 mg/kg, p.o. Animals were observed for toxic effect, behavioural changes and mortality, if any for a period of 72 hr. The doses of extracts were selected on the basis of findings of pilot experiments.

2.8 Dose Selection and Drug Administration

The aqueous (TLAQ) and alcoholic (TLAL) extracts were dissolved/suspended in 1% CMC and administered by oral route. The various doses of TLAQ and TLAL were administered to PCM induced rats and serum marker enzymes were measured for all the groups. The 200, 400 mg doses were found to be effective and hence used for further hepatoprotective activity evaluation.

3. Paracetamol-Induced Hepato-toxicity in Rats

Paracetamol (Acetaminophen; Torent Research Centre, Ahmedabad) was suspended in 1 % CMC and administered per oral (p.o); at a dose of 2 gm /kg. Animals, after acclimatization (6-7 days) in the animal quarters, were fasted overnight and randomly divided into seven groups (n = 6) and treated in the following way. Group I served as Normal (vehicle) control and fed orally with CMC (10 ml/kg b.w; p.o). Group II vehicle control administered with PCM in CMC. Animals of Group III were treated with, Silymarin (100 mg.kg b.w; p.o). Group IV and V rats were treated with (200 mg and 400 mg/kg b.w) of TLAQ and Group VI and VII rats were treated with (200 mg and 400 mg /kg b.w) of TLAL respectively. The drug treatment and vehicle were administered once a day for 7 day to the respective group. On the 7th day, paracetamol suspension was given by oral route, in a dose of (2 gm/kg) in 1% CMC to all rats except the rats in group I[10].

3.1 Biochemical Studies

On 8th day, under light ether anaesthesia blood was withdrawn directly from the heart and thereafter rats were sacrificed by euthanasia. The liver tissues were removed, washed with cold normal saline and preserved at -20 ºC. The serum was separated by centrifugation at 1200 rpm (Remi, USA) below 30 ˚C for 15 min and used for the assay of liver marker viz; Serum alanine aminotransferase (ALT), Serum aspartate aminotransferase (AST)[11], alkaline phosphate (ALP)[12], Lactic Dehydrogenase (LDH)[13], Total Bilirubin (TB)[14] and Total Proteins (TP)[15] were determined by standard methods using enzyme assay kits.

3.2 Antioxidant Assays

The liver homogenate (10% w/v) was prepared in Triss buffer at (pH-7.4) and then centrifuge (Remi, Pvt. Ltd.) at 6000 rpm for 15 min and the supernatants used for the measurement of antioxidants. The Lipid peroxidation was assayed in homogenate by determining the formation of MDA[16], Catalase (CAT)[17], Superoxide dismutase (SOD)[18], and Reduced Glutathione (GSH)[19].

3.3 Histopathological Studies

The remaining liver tissue preserved in 10 % w/v formalin was embedded in paraffin wax and cut into 3-4 µm thick section on microtome and sections were stained using haematoxylin and eosin, and observed under microscope for histoarchitecture alterations.

3.4 Statistical Analysis

The Experimental data was analysed by One way ANOVA followed by student 't' test and results were expressed as Mean ± Standard Error Mean (SEM). Differences were considered statistically significant when $p<0.05$.

4. Results

4.1 Preliminary Phytochemical Analysis

Based on preliminary phytochemical analysis of TLAQ showed the presence of flavonoids, tannins and

polyphenolic compounds whereas, TLAL showed the presence of flavonoids, tannins, saponins and alkaloids.

4.2 HPTLC Analysis

Optimized HPTLC chromatogram of TLAQ and TLAL extracts showed presence of quercitin as major phytoconstituents at 214 nm which is comparable with standard quercitin and exhibited as blackish (visible) band in the R_f range of 0.47 to 0.52. Figure 1 showed a good linear relationship (r^2 = 0.98 and 0.98 with respective to height and peak area) at the concentration of 5µl/spot. Thus it is possible that standard quercitin and extract showed R_f value at same wavelength i.e., 214 nm. Hence our extracts might contain quercitin as important flavonoid.

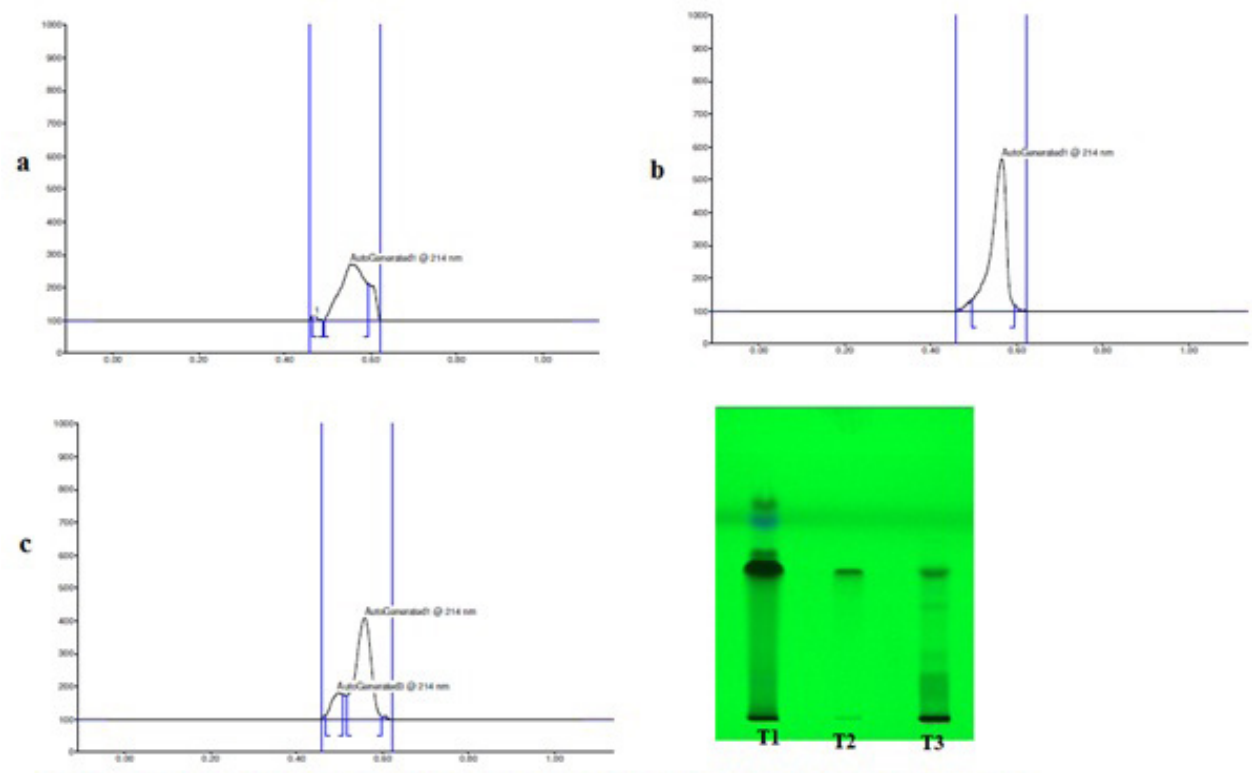

Fig. 1. **(a)** HPTLC peak showing presence of Quercitin at 214 nm in alcoholic extract of *Trichosanthes lobata*. **(b)** Peak of standard Quercitin at 214 nm. **(c)** Peak showing presence of Quercitin at 214 nm in aqueous extract of *Trichosanthes lobata*. T2 Quercitin, T1 and T3 replicate spots for TLAL and TLAQ on HPTLC plate 5 µl ml of sample was applied and compared with standard quercitin, at 214 nm.

4.3 Acute Oral Toxicity in Rats

The acute toxicity studies on aqueous and alcoholic extract of *Trichosanthes lobata* were not carried out by oral route at doses 2000 mg/kg, mortality nor were toxic clinical symptoms observed. Furthermore, the pilot experiment was performed for the effective dose for hepatoprotective activity evaluation, based on findings of pilot experiment and oral acute toxicity studies various doses viz. 200 and 400 mg/kg were selected for detailed hepatoprotective activity evaluation.

4.4 Hepatoprotective Activity Screening

Rats administered with paracetamol (2 gm/kg) induced significant ($p<0.05$) hepatic damage as evidence from significant elevation of AST, ALT, ALP, LDH and total bilirubin with marked decreased in total protein concentration compared to paracetamol treated group. Pre-treatment with TLAQ and TLAL (200 and 400 mg/kg) and Silymarin (100 mg/kg) prevented the elevation of serum marker enzymes AST, ALT, ALP, LDH and total bilirubin with marked restoration of total proteins in paracetamol treated rats. (Table 1 and 2).

Table 1: Effect of an aqueous (TLAQ) and alcoholic (TLAL) extracts of *Trichosanthes lobata* on serum marker enzymes in paracetamol-induced liver toxicity in rats

Treatment and dose (mg/kg, p.o)	Serum marker enzymes (Mean ± SEM; n=6)			
	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)
Vehicle treated 10 ml/kg	21.83 ± 2.48	16 ± 3.4	3.1 ± 0.3	221.5 ± 19.58
PCM 2 gm/kg	127.33 ± 6.59##	72.8 ± 5.1##	54.6± 5.5##	861.5 ± 89.5##
Silymarin 100 mg/kg	43.33 ± 4.13**	37.1 ± 6**	15.6 ± 2.3**	338 ± 26**
TLAQ 200 mg/kg	113.83 ± 6.5*	83.8 ± 4.9*	47.4 ± 3.2*	766 ± 54.82*
TLAQ 400 mg/kg	70.6 ± 6.1**	53.8 ± 6**	42.4 ± 3**	575 ± 62.4**
TLAL 200 mg/kg	113.3 ± 9.8*	64.3 ± 4.5*	47 ± 3.7*	757 ± 47.86*
TLAL 400 mg/kg	73.5 ± 6.8**	51.5 ± 4**	41 ± 3.5**	639 ± 54.7**

*p < 0.05 and **p<0.01 compared with PCM treated group. ## p<0.05 compared with vehicle treated group. PCM: Paracetamol.

Table 2: Alterations in the values of Total Proteins (TL) and Total Bilirubin (TB) with treatment of *Trichosanthes lobata* (TL) leaves extracts in paracetamol (PCM) induced liver toxicity in rats

Treatment and dose (mg/kg, p.o)	Parameters (Mean ± SEM; n=6)	
	Total Proteins (mg/dL)	Total Bilirubin (mg/dL)
Vehicle treated 10 ml/kg	13.3 ± 1	0.88 ± 0.13
PCM 2 gm/kg	6.4 ± 0.9##	2.21 ± 0.24##
Silymarin 100 mg/kg	11.98 ± 1.1**	1.16 ± 0.24**
TLAQ 200 mg/kg	8.2 ± 0.77*	1.83 ± 0.19*
TLAQ 400 mg/kg	10.53 ± 0.87**	1.23 ± 0.28**
TLAL 200 mg/kg	7.96 ± 0.73*	1.83 ± 0.15*
TLAL 400 mg/kg	9.71 ± 0.8**	1.28 ± 0.24**

*p < 0.05 and **p<0.01 compared with PCM treated group. ## p<0.05 compared with vehicle treated group. PCM: Paracetamol

The MDA formation (an index of lipid peroxidation) was increased significantly in paracetamol treated rats and subsequently prevented (p<0.01) the increased MDA formation significantly compared to vehicle control group (Table 3). Depletion of GSH, and the SOD and CAT were restored by pre-treatment by TLAQ and TLAL dose dependently. The effect of TLAQ (200 mg/kg) and TLAL (200 mg/kg) were comparable to that of silymarin 100 mg/kg (Table 3).

Furthermore, in histopathological studies paracetamol treated liver sections showed necrosis, lymphocytes infiltration, congestion and haemorrhage of hepatocytes. However, treatment with TLAQ and TLAL at the dose of (200 and 400 mg/kg) almost prevented the infiltration of lymphocytes and congestion as compared to PCM treated rats (Figure 2(a)-(g)).

Table 3: Effect of an aqueous and alcoholic extracts of *Trichosanthes lobata* on MDA formation, SOD, GSH, CAT in paracetamol induced hepatotoxicity in rats

Treatment and dose (mg/kg, p.o)	Parameters (Mean ± SEM; n=6)			
	SOD U/mg of Protein	GSH µg/g of Protein	CAT µmole of H_2O_2/mg of Protein	MDA nmole/gm of Protein
Vehicle treated 10 ml/kg	33.66 ± 3	2.76 ± 0.27	29 ± 2.3	4.2 ± 0.34
PCM 2 gm/kg	13.5 ± 1.64##	1.33 ± 0.9##	10.15 ± 1.48##	29 ± 3.6##
Silymarin 100 mg/kg	26.33 ± 2.58**	2.5 ± 0.2**	24.5 ± 2**	9.26 ± 1.1**
TLAQ 200 mg/kg	17.6 ±1.3*	1.7 ± 0.24*	13.8 ± 1.9*	25.7 ± 2.7*
TLAQ 400 mg/kg	21.1 ± 3**	2.2 ± 0.2**	20 ± 2**	19.83 ± 1.7**
TLAL 200 mg/kg	17.3 ± 1.2*	1.7 ± 0.1*	13.6 ± 1*	25.6 ± 2.1*
TLAL 400 mg/kg	22.5 ± 2.1**	2.25 ± 0.22**	22.5 ± 2.1**	21.8 ± 1.7**

*p < 0.05 and **p<0.01 compared with PCM treated group. ## p<0.05 compared with vehicle treated group. PCM: Paracetamol

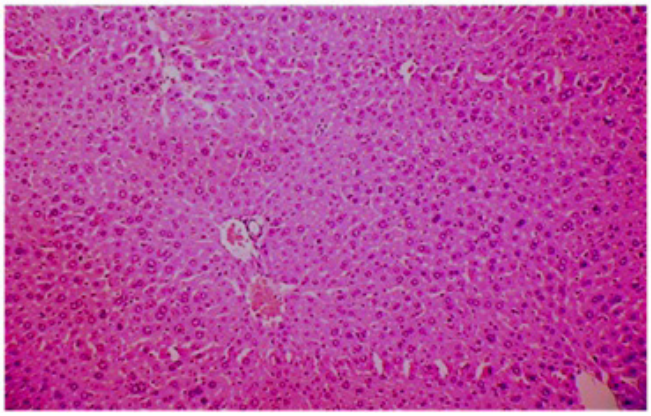

Fig. 2A. Section of liver of normal control rats showed normal hepatic cells with nuclei and cytoplasm.

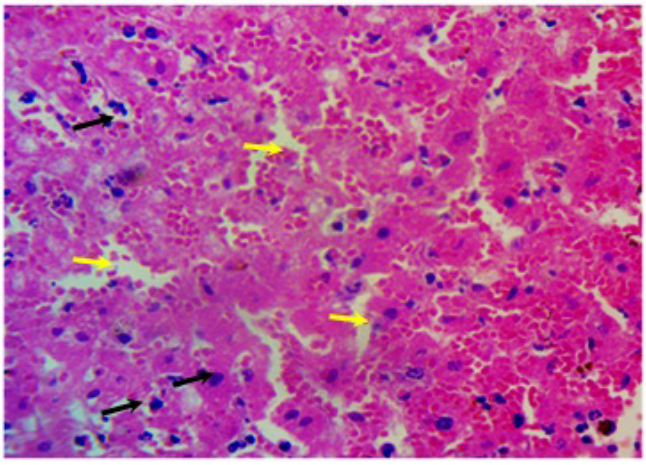

Fig. 2B. Section of paracetamol (PCM) overdose-treated rat liver showed marked necrosis, (yellow arrows), lymphocytes infiltration (black arrows), congestion and hemorrhages.

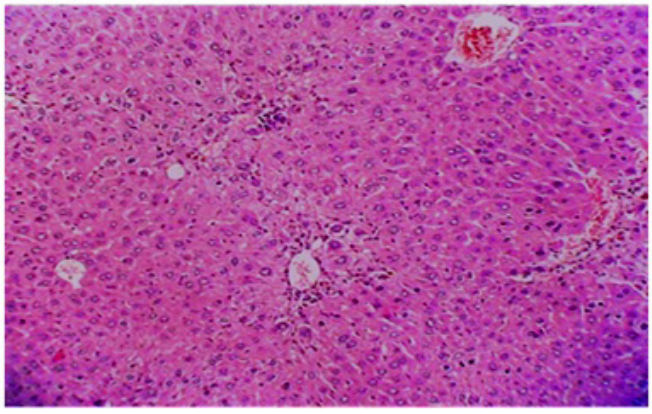

Fig. 2C. Silymarin (100 mg/kg) + paracetamol (2 g/kg mg/kg) treated group showed mild congestion, lymphocytic infiltration and regenerating architecture of hepatocytes with mild necrosis.

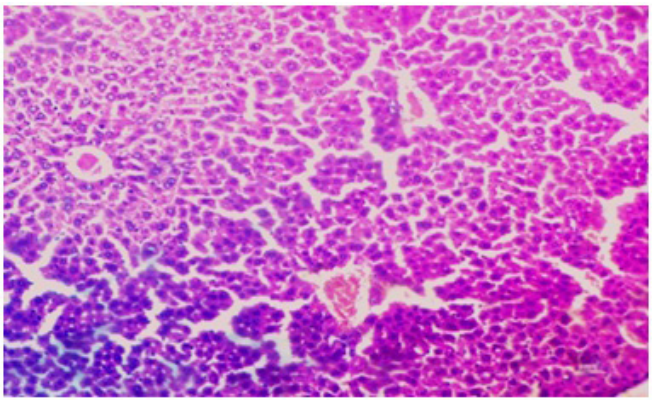

Fig. 2D. TLAQ (200 mg/kg) + PCM treated group showed vascular dilatation, mild infiltration of lymphocytes.

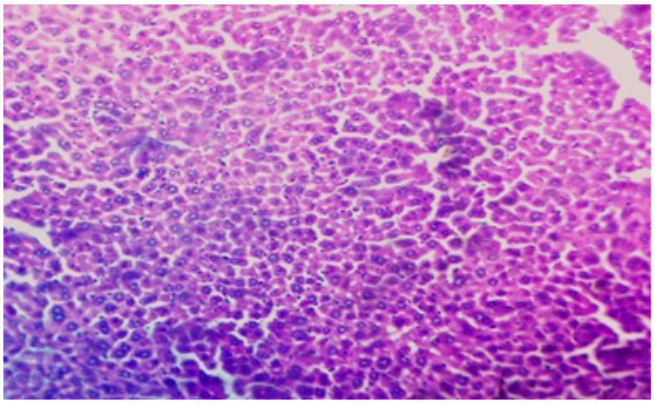

Fig. 2E. TLAQ (400 mg/kg) + PCM treated group, showed regeneration of hepatocytes with prominent nucleus and no signs of necrosis or inflammatory infiltrate and are close to normal.

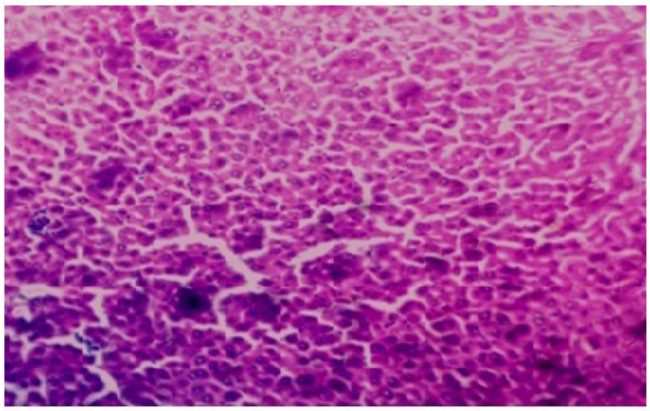

Fig. 2F. TLAL (200 mg/kg) + PCM treated group showed vascular dilatation, mild infiltration of lymphocytes.

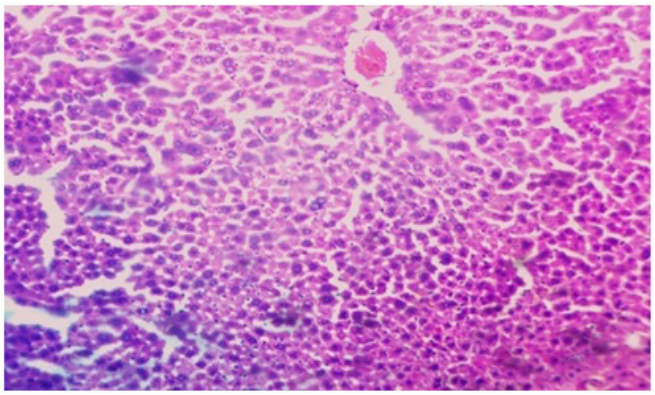

Fig. 2G. TLAL (400 mg/kg) + PCM treated group, showed regeneration of hepatocytes with prominent nucleus and no signs of necrosis or inflammatory infiltrate and are close to normal.

5. Discussion

Paracetamol (Acetaminophen) is widely used clinically as antipyretic and analgesic agent, and found to be safe at therapeutic doses, however, it can induce hepatic damage in human beings, rats and mice at higher doses.[20] Hepatotoxicity of paracetamol has been attributed to the formation of toxic and reactive metabolites N-Aacetyl-P-Benzoquinone Imine (NAPQI). NAPQI is detoxified by conjugation with reduced glutathione (GSH) to form mercapturic acid. If the rate of NAPQI formation exceeds the rate of detoxification by GSH; it oxidizes tissue macromolecules mainly as lipid or thiol group protein and alters the homeostasis of calcium after depleting GSH[21]. The hepatic damage is largely to reflect in terms of leaking of cellular enzymes into the bloodstream due to disturbances caused in the transport functions of hepatocytes. Therefore determination of enzymes in the serum is a useful biological marker of the extent and nature of hepatocelluar damage[22]. In the present experimental findings, the rats treated with paracetamol (2 gm/kg), showed a significant hepatic damage, reflected by elevation of serum marker enzymes (ALT, AST, ALP and LDH) and MDA formation in liver homogenates. Pre-treatment with TLAQ and TLAL extracts at (200 and 400 mg/kg, p.o) significantly prevented the elevation of serum marker enzymes and total proteins. The prevention of elevated serum marker enzymes by TLAQ and TLAL (200 and 400 mg/kg, p.o) might be due to membrane stabilization of hepatocytes which consequently prevents the cystolic released in circulation. These findings are in agreement with the documented fact that, serum transaminase levels return to normal with healing of hepatic parenchyma and regeneration of hepatocytes[23].

Most of the hepatotoxic chemicals including paracetamol induced damage liver probably by lipid peroxidation directly or indirectly. In higher animals, lipid peroxidation was known to cause destabilization and disintegration of the cell membrane, leading to liver injury, arteriosclerosis and kidney damage. Amongst peroxy radicals are important agents that mediate lipid peroxidation thereby damaging cell membrane[24]. Administration of TLAQ and TLAL extracts at (200 and 400 mg/kg) significantly attenuated the MDA formation; thereby suggesting that free radicals formation/oxidative reactive species are removed/neutralised by the plant constituents present in the extracts and thus prevented hepatic damage.

Glutathione (GSH) is one of the most abundant tripeptide non-enzymatic intracellular biological antioxidant present in liver. It is involved in removal of free radicals such as H_2O_2, superoxide anions and alkoxy radicals, preserving membrane protein thiols and a substrate for glutathione peroxidase and glutathione reductase[25]. In the present experiments, paracetamol administration exhibited lower GSH content in liver, and subsequently pre-treatment with TLAQ and TLAL extracts at (200 and 400 mg/kg,) able to reverse such effects.

The Reactive Oxygen Species (ROS) are the free radicals that initiate the process of hepatic damage due to high oxidative stress so, formed ROS are scavenged/ neutralized by endogenous antioxidant enzymes, thus, there activity get impaired. Since, endogenous defence system activated during such events that include free radical scavengers/Neuratilzers and chain reaction terminators, enzymes such as SOD and CAT[26]. In this study, SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. The restoration of SOD due to pre-treatment with TLAQ and TLAL extracts at (200 and 400 mg/kg) have an efficient protective mechanism in response to ROS during hepatic damage.

CAT is a key component of antioxidant defense system. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular

damage. Excessive production of free radicals may results in alterations in the biological activity of hepatic cellular macromolecules[27]. Pre-treatment with TLAQ and TLAL extracts at (200 and 400 mg/kg) restored CAT activity in paracetamol–induced hepatic damage and thus prevent accumulation of excessive free radicals. The ability of TLAQ and TLAL extracts to protect paracetamol-induced hepatic damage in rats might be attributed to its ability to restored endogenous antioxidants system. Thus, experimental findings suggested that TLAQ and TLAL extracts able to prevent hepatic damage due paracetamol by their antioxidant property. Since, this model of hepatic damage in the rats simulate many of the features of human liver pathology, our finding suggest that natural antioxidants and scavenging agents in *Trichosanthes lobata* leaves extracts might be involved in hepatoprotection.

This was further supported with histopathological changes. Therefore, it seems that TLAQ and TLAL extracts, due to its antioxidant property, might capable of protecting the hepatic tissues from paracetamol-induced injury and inflammatory changes. The TLAQ and TLAL extracts found to rich in flavonoids. Presence of flavonoids in the extracts was confirmed and agreement with our preliminary phytochemical screening and HPTLC studies. Flavonoids are natural products, which have been shown to possess antioxidant property. As *Trichosanthes lobata* leaves extracts contains large amount of flavonoids it may be possible that the hepatoprotective activity may be due to the presence of flavonoids in the extracts.

6. Conclusion

Based on aforementioned finding, we proposed that hepatoprotective effect of *Trichosanthes lobata* leaves extracts might be due to antioxidant effect (elevation of endogenous antioxidant enzymes and total proteins) and membrane stabilization of hepatocytes (reduce AST,ALT, ALP, LDH and total bilirubin) by scavenging/ neutralizing free radicals. The present study thus validate the traditional use of *Trichosanthes lobata* in the treatment of liver diseases and also points out that *Trichosanthes lobata* warrants future detailed investigation as promising hepatoprotective agent.

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CERTIFICATE

This is to Certify that the research project "PHYTOCHEMICAL INVESTIGATION AND HEPATOPROTECTIVE ACTIVITY OF SOME MEDICINAL PLANTS"

Submitted by Mr. Raju R.Wadekarhas been approved in the Institutional Animal Ethics Committee meeting held on 19th December 2008, resolution No. JNMC/IAEC/Res-2/10/2008 and was permitted to use 87 Rats/~~Mice~~/Ra~~bbits~~.

You are hereby informed to strictly adhere to the protocol submitted for approval. In case the project needs to be modified later, the modified version of the protocol should be submitted to the Committee, stating valid reasons for such modifications for fresh approval.

You are required to keep the account of animals used for the project in specified proforma, Form-D.

You have to submit the brief report to the Committee after completion of the project along with **Form-D**

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