

# Hepatoprotective activity of *Ehretia microphylla* on paracetamol induced liver toxic rats

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**ABSTRACT:** World wide liver toxicity is the most prevalent healthcare problem. We need to address this with herbal based medicine because herbal products have high efficacy and less side effects. Many of the *Ehretia* genus (Boraginaceae) have the hepatoprotective property. But *Ehretia microphylla* (EM) was not reported yet for its hepatoprotective potential. Therefore the current research was aimed to evaluate the hepatoprotective action of EM. The hepatoprotective activity of EM was assessed against the paracetamol induced liver toxic rats. The chloroform and ethyl acetate extracts of EM were selected for the investigation. The dose was selected by using OECD423 guidelines. The paracetamol intoxicated rats were elevated the liver functional parameters like serum SGOT, SGPT and ALP levels and also triglyceride and total cholesterol. These elevated parameters were significantly lowered in chloroform and ethyl acetate extracts of EM pretreated rats when compared with silymarin. However the antioxidant markers including CAT, SOD, GSH and GPx were significantly increased in EM extract treated rats when compared with paracetamol induced toxic rats. It revealed the plant extracts of EM has significant hepatoprotective activity. Further, this research will be useful for the isolation of active principles from EM and determining its molecular mechanisms.

**KEYWORDS:** Liver; silymarin; antioxidant; Boraginaceae; *Ehretia*.

## 1. INTRODUCTION

Liver is the vital organ in the human body which consists of many of the metabolic enzymes which metabolize the carbohydrate, proteins and fats etc. Liver is also involved in the immune function to maintain body integrity [1]. The world was affected by chronic liver disease irrespective of sex, age, region or race. Reports of WHO revealed the 46% of global diseases and 59% of the mortality is reason for chronic diseases (cardiovascular diseases; mainly heart disease and stroke; cancer; respiratory diseases; liver diseases and diabetes) and almost 35 million people in the world die because of chronic diseases [2]. The UK National statistics reports that the liver diseases are the fifth common death causing ailment [3,4]. Medicinal plants and herbal extracts play an important role in hepatoprotective activity [5].

*Ehretia microphylla* (EM) (Family: Boraginaceae) is one of the most important medicinal plants in the Philippines and other Asian Countries, and it has a variety of medicinal values. The decoction of the leaves is used as a cough suppressant, and it is prescribed for the treatment of diarrhea with bloody discharge and dysentery [6, 7]. The antioxidant [8], antimicrobial [9], anti-hypercholesterolemic [10], anti-allergic [11] activities have been reported in scientific platforms.

Many of the herbals from *Ehretia* genus have been reported for their hepatoprotective activity [12]. Moreover the anticancer activity of quercetin isolated from EM against HepG2 human liver cancer cell line has been reported [13]. And also, many of the anticancer herbals against hepatocellular carcinoma (liver cancer) have been reported for their hepatoprotective activity [14, 15, 16]. However, there are no scientific reports for hepatoprotective activity of EM. Preliminary studies conducted by our lab have reported the antioxidant

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and free radical scavenging activity of EM [17]. Furthermore free radical scavenging and antioxidant mechanisms are associated with hepatoprotective activity [18]. Therefore the current research work was designed to evaluate the hepatoprotective activity of EM.

## 2. RESULTS AND DISCUSSION

The results of acute oral toxicity study are shown in Table 1.

**Table 1.** Acute toxicity study of ethyl acetate and chloroform extracts of *Ehretia microphylla*.

S. no	Response	Head		Body		Tail	
		Before	After	Before	After	Before	After
1	Alertness	Normal	Normal	Normal	Normal	Normal	Normal
2	Grooming	Absent	Absent	Absent	Absent	Absent	Absent
3	Torch response	Normal	Normal	Normal	Normal	Normal	Normal
4	Pain response	Normal	Normal	Normal	Normal	Normal	Normal
5	Tremor	Absent	Absent	Absent	Absent	Absent	Absent
6	Convulsion	Absent	Absent	Absent	Absent	Absent	Absent
7	Righting reflex	Normal	Normal	Normal	Normal	Normal	Normal
8	Gripping strength	Normal	Normal	Normal	Normal	Normal	Normal
9	Pinna reflex	Present	Present	Present	Present	Present	Present
10	Corneal reflex	Present	Present	Present	Present	Present	Present
11	Writhing	Absent	Absent	Absent	Absent	Absent	Absent
12	Pupils	Normal	Normal	Normal	Normal	Normal	Normal
13	Urination	Normal	Normal	Normal	Normal	Normal	Normal
14	Salivation	Normal	Normal	Normal	Normal	Normal	Normal
15	Skin colour	Normal	Normal	Normal	Normal	Normal	Normal
16	Lacrimation	Normal	Normal	Normal	Normal	Normal	Normal

Acute toxicity studies of ethyl acetate and chloroform extracts of EM revealed the safety dose of dose until 2000 mg/kg. Hence the dose fixed for study as (1/20<sup>th</sup>) 100 mg/kg as low dose and (1/10<sup>th</sup>) 200 mg/kg as high dose for EM.

Liver necrosis in paracetamol (PCM) induced rats increases the activities of Serum Glutamic Pyruvate Transaminase (SGPT), Serum Glutamic Pyruvate Oxaloacetate (SGOT) and Serum Alkaline Phosphatase (ALP) in plasma by leakage of these enzymes from liver cytosol into the blood stream [19]. These biochemical parameters of various treatment groups were shown in Table 2.

The Serum SGOT, SGPT and ALP levels were increased significantly ( $p < 0.001$ ) in PCM induced rats compared to normal controlled rats. The standard and extracts treated groups significantly ( $p < 0.05$ ) reduced these level compared to PCM induced group. The PCM intoxicated rats showed significant increase of triglyceride (TG) and total cholesterol (TC). The possible mechanism for hyperlipidemic condition might be worsening the liver cells which are essential for lipid metabolism. Another probable mechanism for increased serum lipid level may be recognized by increased synthesis of hepatic cells and decreased liver degradation [20]. Values of treatment groups were decreased towards normal when compared with PCM control animals. Serum bilirubin level is the key parameter to find the normal liver function [21]. Values of treatment groups were decreased towards normal when compared with PCM control animals.

The reactive oxidant species and/or lipid peroxidase are normally deactivated by the antioxidant enzymes like Catalase (CAT), Superoxide dismutase (SOD), Reduced glutathione (GSH) and Glutathione peroxidase (GPx). These antioxidant enzyme activities are drastically decreased in PCM intoxicated rats, the plant extracts protect significantly decreased CAT, SOD, GSH and GPx levels when compared to the rats treated with control groups [22]. PCM intoxicated rats showed acute liver necrosis thereby increased level of lipid peroxidation (LPO). This is counteracted by plant extracts and standard. The CAT, SOD, GSH and GPx values of extracts and standard treated groups are shown in Table 3.

**Table 2.** Effect of plant extracts and standard against PCM induced liver toxic rats (serum biochemical markers).

Treatment Group	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	HDL (mg/dl)	TG (mg/dl)	TC (mg/dl)	TB (mg/dl)	DB (mmol/l)
Group I	66.16 ±7.98	99 ±2.09	170.51 ±11.6	53 ±3.90	103 ±2.08	80 ±2.03	0.8 ±0.01	0.2 ±0.02
Group II	398 ±3.07## #	153 ±5.08###	318.61 ±3.95#	28 ±1.28##	379 ±3.81##	347 ±1.22##	2.8 ±0.01##	0.8 ±0.01###
Group III	261 ±3.47***	125 ±3.96***	250 ±1.68***	47 ±2.97***	203 ±3.95***	113 ±1.98***	1.3 ±0.02***	0.196 ±0.03***
Group IV	227 ±2.68**	102 ±1.37***	195 ±4.08**	49 ±7.04**	158 ±2.66**	107 ±3.08**	1.2 ±0.01**	0.261 ±0.03***
Group V	250 ±2.95**	115 ±3.67***	263 ±2.95**	37 ±5.08*	243.7 ±2.81**	128 ±2.96**	1.5 ±0.03**	0.255 ±0.01***
Group VI	216 ±5.65**	105 ±3.56**	225.8 ±3.07*	43 ±4.93**	191.7 ±5.03*	126.6 ±3.28*	1.22 ±0.03**	0.189 ±0.02***
Group VII	230 ±3.62*	118 ±2.78**	255 ±7.08**	40 ±3.28**	187 ±3.33**	115.4 ±3.77**	1.47 ±0.02**	0.38 ±0.01**
Group VIII	215 ±5.91**	103 ±3.35**	210 ±5.83**	45 ±2.68**	160.9 ±2.55**	109.98 ±2.52*	1.2 ±0.03**	0.3 ±0.01**

Data is expressed as mean ± SEM, n=6, One way ANOVA followed by Dunnett's test Compared with normal control; # p<0.05, ## p<0.01, ### p<0.001, Compared with disease control; \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

**Table 3.** Effect of plant extracts and standard against PCM induced liver toxic rats (Antioxidant markers).

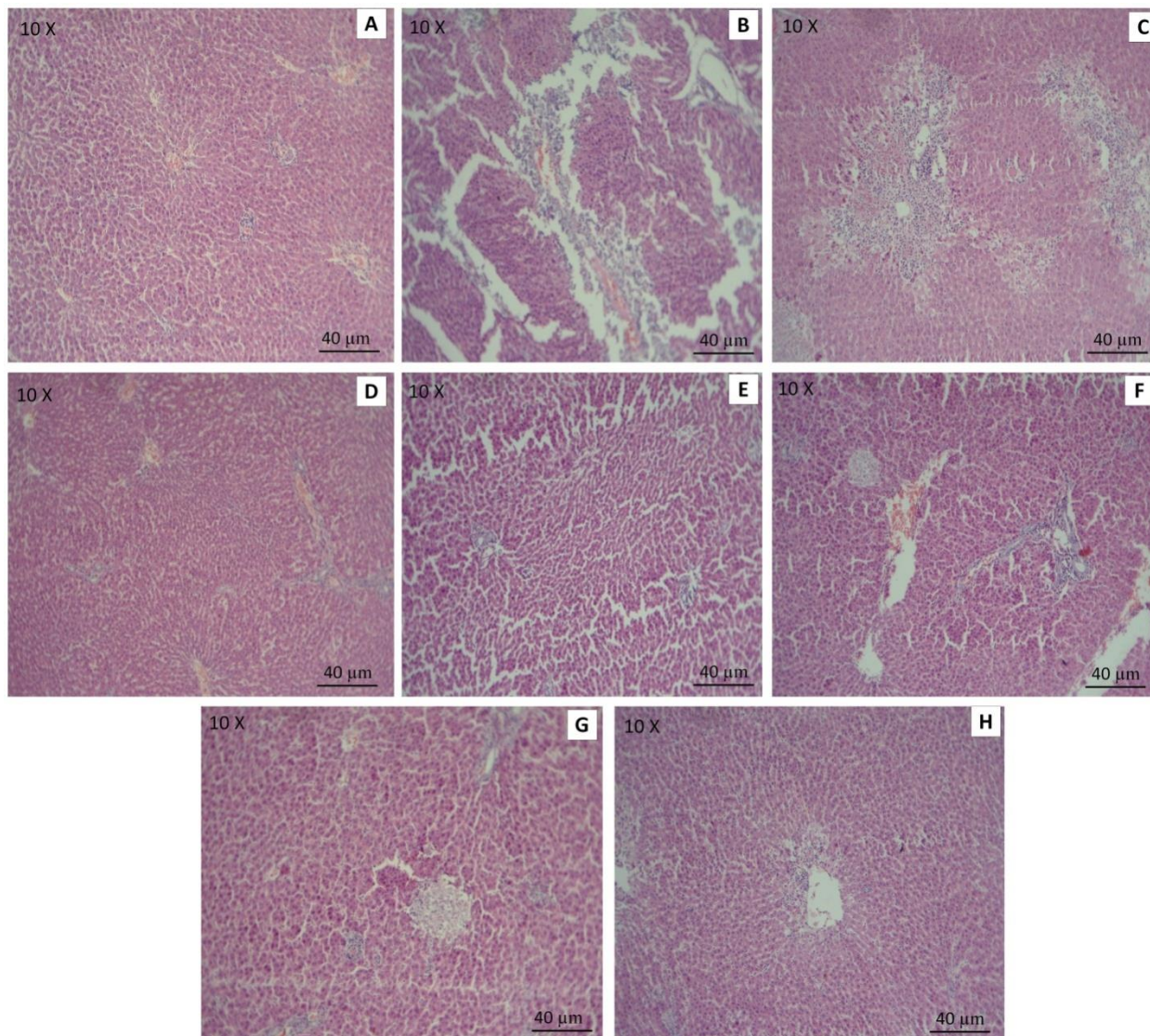
Treatment Group	CAT (H2O2 consumed/min/mg of protein)	SOD (unit/min/mg protein)	GSH (unit/min/mg of protein)	GPx (GSH consumed/min/mg protein)	LPO (MDA per mg of protein)
Group I	276.12±7.16	90.41±3.65	41.67±1.29	115.07±1.16	4.28±0.81
Group II	91.71±15.15###	37.32±2.11###	19.86±2.13###	41.67±2.19###	85.19±0.91###
Group III	219.61±6.76***	69.13±3.15***	36.18±1.76**	91.73±3.12***	10.16±1.21***
Group IV	265.12±5.67***	85.91±2.91***	40.36±3.61***	110.09±2.16***	5.93±0.16***
Group V	146.12±1.25***	42.02±1.94***	24.25±0.26**	59.45±0.41***	20.12±0.36***
Group VI	242.18±0.12***	62.10±1.95***	29.16±0.25***	95.21±0.28***	10.02±0.34**
Group VII	168.11±3.12**	51.92±4.51*	34.73±2.91***	79.67±3.16***	14.91±0.12***
Group VIII	262.19±2.15**	81.12±2.96**	39.18±1.28***	107.31±1.29***	6.01±0.24***

Data is expressed as mean ± SEM, n=6, One way ANOVA followed by Dunnett's test Compared with normal control; # p<0.05, ## p<0.01, ### p<0.001, Compared with disease control; \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

Values of treatment groups were increased towards normal when compared with PCM Control animals. Across all the extract treatment groups "PCM + EAEM 200mg/kg Group" showed maximum activity, when compared with "PCM + SLY 100 mg/kg, Group".

The results of the histopathological study are shown in Figure 1. Group I Normal control, Liver shows normal lobular architecture, hepatocytes show no significant pathology, Group II PCM induced control hepatocytes show cytoplasmic vacuolation, periportal inflammation and central vein shows congestion destruction, Group III SLY 50 mg/kg treated PCM control- portal traid shows mild periportal inflammation, Group IV SLY 100 mg/kg treated PCM control liver shows normal portal tracts, the hepatocytes in the hepatic parenchyma are normal, Group V CEM 100mg/kg treated PCM control shows mild hydropic changes in the hepatocytes of centrilobular region, the sinusoids are mildly dilated, Group VI CEM 200 mg/kg treated PCM control liver shows extensive necrosis in the centrilobular region, Group VII EAEM 100 mg/kg treated PCM control- liver section shows extensive necrosis in the centrilobular region and Group VIII EAEM 200 mg/kg treated PCM control shows sinusoids are dilated, there is no significant necrosis seen.

The liver damage associated with PCM overdose is due to the formation of the hepatotoxic metabolite. The liver toxicity in rats, damages the normal morphology of liver cells and formation of cytoplasmic vacuolation, periportal inflammation, central vein destruction, hydropic changes and dilated sinusoids. These morphological damages were regained by CEM and EAEM. It might be associated with the phytoconstituents (Flavonoids, phenolic compounds, glycosides, and phytosterols) present in the extracts.



**Figure 1.** Histopathology of Liver. A: Group I, B: Group II, C: Group III, D: Group IV, E: Group V, F: Group VI, G: Group VII, H: Group VIII. Magnifications at 10X.

EM is one of the important medicinal plants widely used in tribes of Philippines and other Asian Countries for the management of many diseases. EM has reported many biological activity in scientific platform and has not been reported its hepatoprotective activity. The results of the present research revealed that the EMEM and CEM have significant hepatoprotective activity. This research work is the first report for hepatoprotective activity of EM. PCM induced liver toxicity is the widely used model for screening of hepatoprotective drugs. Due to the overdose of PCM lead to cause liver injury in rat through hepatic necrosis caused by the reactive N-acetylimidoquinone metabolite of PCM[23]. The EM treated animals shown significant action on lower the toxicity.

Our previous research reported for antioxidant activity of EM. The overproduction of Reactive oxygen species due to liver necrosis can worsen the quantum of oxidative stress leading to combining mechanism of injury associated with liver damage. Antioxidant enzymes such as SOD, CAT, and GPx play a significant role in the prevention of tissue damage. In current research PCM toxic rats declined the antioxidant enzymes

in liver homogenate and EM shows significant improvement of antioxidant enzymes. This result suggest that the antioxidant mechanism of EM might be the reason for its hepatoprotective activity. Furthermore we have already quantified the total phenolic and flavonoid contents. These flavonoids and phenolic compounds are having potent antioxidant capacity in literature[24]. So that the hepatoprotective activity responsible compounds from EM is maybe the phenolics or flavonoids derivative.

### 3. CONCLUSION

In conclusion, the chloroform and ethyl acetate extract of EM produced significant hepatoprotective activity against PCM toxic rats. The hepatoprotective activity of EM might be the presence of phenolic and flavonoid compounds. Further study needed to isolation of active principles and development of formulation for commercialization.

### 4. MATERIALS AND METHODS

#### 4.1. Materials

The aerial parts of EM was collected locally Sattankulam at Thuthukudi District in Tamil Nadu state in India. PCMan and Silymarin (SLY) were purchased from Sigma Aldrich, USA. Petroleum ether, chloroform, ethyl acetate and methanol were obtained from SD Fine Chemicals Pvt. Ltd, Mumbai, India. Ketamine hydrochloride was obtained from KMCH Pharmacy, Coimbatore. The experimental animals were procured from Sri Venkateshwara Enterprises, Bangalore, India. Sodium carboxyl methyl cellulose, formalin, glacial acetic acid, n-butanol, sodium azide, sodium dodecyl sulfate, hydrochloric acid and Tris-HCl were procured from Merck (India) Ltd., Mumbai. SGOT, SGPT, ALP, total bilirubin (TB), direct bilirubin (DB), TC, TG, high-density lipoproteins (HDL)- cholesterol level and low-density lipoproteins (LDL) cholesterol level kits were purchased from Span diagnostics, Surat, India. Ellman's reagent, Tri chloro acetic acid (TCA), glutathione reduced solution, sodium pyrophosphate buffer, phenazine methosulphate, nitroblue tetrazolium, Nicotinamide adenine dinucleotide (NADH), thiobarbituric acid (TBA), 1,1,3,3,-tetra-ethoxypropane and hydrogen peroxide were purchased from Himedia Laboratories, Mumbai, India.

#### 4.2. Preparation of plant material

The aerial parts of EM was authenticated by Dr. V. Chelladurai, Survey of Medicinal and Aromatic plants Unit Siddha, CCRAS (Central Council for Research in Ayurvedic Sciences), Palayamkottai, Tamil Nadu, India, and voucher specimen (SBTH 11.12) was deposited in the Department of School of Biotechnology, NITC, Calicut, Kerala for future reference. After removing of the earthy matters, the aerial parts were washed and shade dried and powdered using a micro pulverizer and the powder passed through sieve no. 40# [25].

#### 4.3. Extraction of plant material

Powdered plant material was subjected to continuous hot extraction method by using soxhlet apparatus. Increasing orders of polarity solvents (petroleum ether, chloroform, ethyl acetate and methanol) were used[26]. The plant material (1 kg) was of finely powdered and extracted with 5 L of petroleum ether in a soxhlet apparatus for 72 h, after the successive extraction, the obtained marc was further extracted with solvents of increasing order of polarity (Chloroform, Ethyl acetate and Methanol) [27]. After extraction the extracts were separately concentrated under vacuum using rotary vacuum evaporator at 40°C until get viscous solid mass. The obtained crude extracts were weighed and stored at 4°C for the further analysis.

#### 4.4. Experimental animals

Male albino Wistar rats (150–200 g) were used. The animals were procured from Sri Venkateshwara Enterprises, Bangalore, India. All the animals were kept under standard laboratory conditions (temperature 25±2°C and 12 h light/12 h dark cycle) and acclimatized for 1 week before commencement of the experiment. They were allowed to have free access to standard pellet diet (National Institute of Nutrition, Hyderabad, India) and water ad libitum. The protocol used in the present study was approved by Animal Ethics Committee of KMCH College of pharmacy in accordance with the guidelines for the care and use of laboratory animals set by CPCSEA (KMCRET/PhD/01/2013-14).

#### 4.5. Acute oral toxicity study

The OECD-423 guidelines were used for acute oral toxicity study to fixing the dose for *in vivo* study. The rats were fasted overnight with free excess to water and were grouped into four with each group consisting of 3 animals, to which the extract was administered orally at the dose level of 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg body weight. They were observed for mortality; toxic symptoms such as behavioral changes, locomotor activity, convulsions; direct observation parameters such as tremor, convulsion, salivation, diarrhoea, sleep, coma, changes in skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and CNS, somatomotor activity, periodically for 30 min during first 24 h and specific attention given during first 4 h daily for a total period of 14 days.

#### 4.6. *In vivo* hepatoprotective activity

Based on the preliminary study conducted by our lab, CEM (Chloroform extract of EM) and EAEM (Ethyl acetate extract of EM) were selected for *in vivo* evaluation of hepatoprotective activity [16]. Male Wistar albino rats were divided into eight groups of 6 animals each and received orally the following treatment for seven days. The doses were freshly suspended in 1% sodium carboxyl methyl cellulose for each day of treatment.

- Group I: Normal control rats received 1% sodium carboxyl methyl cellulose
- Group II: PCM control rats received 1% sodium carboxyl methyl cellulose
- Group III: Standard low dose rats received SLY 50mg/kg, p.o.
- Group IV: Standard high dose rats received SLY 100 mg/kg, p.o.
- Group V: CEM low dose rats received CEM 100mg/kg, p.o.
- Group VI: CEM high dose rats received CEM 200mg/kg, p.o.
- Group VII: EAEM low dose rats received EAEM 100mg/kg, p.o.
- Group VIII: EAEM high dose rats received EAEM 200mg/kg, p.o.

Above all the groups except Group I was administrated with 1000mg/kg of PCM orally on 7th day of treatment [28]. After 24h of PCM administration, all the animals were anesthetized using ketamine hydrochloride (87 mg/kg, i.p) and blood samples were collected from retro-orbital plexus [29, 30]. The blood samples were allowed to clot for 1 h at room temperature and the serum was separated by centrifugation at 2500 rpm at 4°C for 15 min. Then the rats were sacrificed by cervical dislocation and the liver was excised, washed and perfused with ice-cold normal saline[31]. The perfused liver samples were then homogenized in ice bath using homogenizer in chilled 10 mM Tris-HCl (pH 7.4) to yield 10% (w/v) liver homogenate for the estimation of biochemical parameters. A small portion of the liver tissue was fixed in formalin for histopathological examination [32, 33, 34, 35].

#### 4.7. Estimation of serum biochemical parameters

The SGOT, SGPT, ALP, TB, DB, TG, TC, HDL and LDL levels were estimated by using auto analyzer with the help of standard enzymatic kits and the procedures were performed as per enzymatic kits protocols [36, 37, 38].

#### 4.8. Estimation of *in vivo* antioxidant markers

After treatment with plant extracts and standard, the rats were sacrificed, liver were isolated and washed with normal saline and stored for 12 h for *in vivo* antioxidant studies [39]. The separated liver was homogenized with motor driven Teflon coated homogenizer with 0.1 M Tris-HCl buffer (pH 7.4) to get 10% homogenate.

##### 4.8.1. Catalase (CAT)

The CAT activity was assayed by the method of Sinha[40]. About 0.1 ml of liver homogenate was added with 1 ml of phosphate buffer and hydrogen peroxide. Then the reaction was stopped by the addition of 0.2 ml dichromate acetic acid reagent. The reaction mixture was heated in a boiling water bath for 10 min. The green color developed was read at 570 nm in a Double beam UV-VIS spectrophotometer. Similar procedure was carried out for standard hydrogen peroxide (4 to 20 µl).CAT content present in the liver

homogenates was calculated by interpolation. CAT activity was expressed as H<sub>2</sub>O<sub>2</sub> consumed/min/mg of protein.

#### 4.8.2. Reduced glutathione (GSH)

GSH was estimated by Ellman's procedure[41]. To 250 µl of liver homogenate was mixed with 1 ml of 5% TCA in a 2 ml eppendorf tube, and the mixture was centrifuged at 3000 g for 10 min at room temperature. To 250 µl of the above supernatant was added with 1.5 ml of 0.2 M phosphate buffer and mixed well. Then the reaction was stopped with 250µl of 0.6 mM of Ellman's reagent and the absorbance was measured at 412 nm within 10 min. A standard graph was plotted using glutathione reduced solution (0.1-1 mg/ml) and the amount of GSH present in the liver homogenates was calculated by interpolation. Amount of glutathione expressed as unit/min/mg of protein.

#### 4.8.3. Superoxide dismutase (SOD)

About 250 µl of liver homogenate was mixed with 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazinemethosulphate, and 0.3 mL of nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 ml. Then the mixture was added with 0.2 ml NADH. And the mixture was heated at 30°C for 2 min, the 1 ml glacial acetic acid was added to stop the reaction. The mixture was separated with adding of n-butanol. Then the n-butanol layer color was measured in a colorimeter at 520 nm. The specific activity of the enzyme was expressed as unit/min/mg protein[42].

#### 4.8.4. Estimation of Glutathione peroxidase (GPx)

GPx was measured by the method described by Rotruck et al. About 0.2 ml of liver homogenate was mixed with 0.2 ml 0.4 M phosphate buffer (pH 7.0), 0.1 ml 10 mM sodium azide, 0.2 ml reduced glutathione and 0.1 ml 0.2 mM hydrogen peroxide. The mixture was incubated at 37 °C for 10 min. Then the mixture was added with 0.4 ml 10% TCA to stop the reaction, allowed for 20 min centrifugation at 3200 × g. The supernatant was assayed for glutathione content using Ellman's reagent. The activities were expressed as µg of GSH consumed/ min/mg protein [43].

#### 4.8.5. lipid peroxidation (LPO)

LPO was estimated by the method of Okhawa et al[44]. One ml of liver homogenate was mixed with 0.2 ml 4 % (w/v) sodium dodecyl sulfate, 1.5 ml 20% glacial acetic acid in 0.27 M hydrochloric acid (pH 3.5) and 15 ml of 0.8% thiobarbituric acid (TBA, pH 7.4). The mixture was heated in a hot water bath at 85°C for 1 h. The intensity of the pink colour developed was read against a reagent blank at 532 nm following centrifugation at 1200 g for 10 min. The concentration was expressed as n moles of MDA per mg of protein using 1,1,3,3,-tetra-ethoxypropane as the standard.

### 4.9. Histopathological studies

A small portion of liver was fixed in 10% neutral formalin, dehydrated in alcohol and then implanted in paraffin. Microtome sections of 4-5 µm thickness were made by using a rotary microtome. The sections were stained with haematoxylin-eosin (H&E) dye to observe histopathological changes [45, 46].

### 4.10. Statistical analysis

All the results were expressed as Mean ± SEM. The statistical analysis was carried out by one-way ANOVA followed by Dunnett's multiple comparison tests (Graph pad Prism-5 software).

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**Conflict of interest statement:** The authors declared noconflict of interest.

**Ethics committee approval:** All experiments conducted in this study were approved by Animal Ethics Committee of KMCH College of Pharmacy in accordance with the guidelines for the care and use of laboratory animals set by CPCSEA (KMCRET/PhD/01/2013-14) on 05-04-2014.

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