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Effect of methanolic extract of *Piper nigrum* fruits in Ethanol-CCl_4 induced hepatotoxicity in Wistar rats

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ABSTRACT

Piper nigrum popularly known as 'Black Pepper' contains abundant amount of piperine alkaloids. Piperine alkaloids have been implicated in Hepatoprotective activity. To evaluate effect of methanolic extract of Piper nigrum fruits in Ethanol-CCl_4 induced hepatotoxicity Wistar rats. The methanolic extract of P. nigrum (MEPN) (100 and 200 mg/kg, p.o., 15 days) and Piperine (PPR) (50 mg/kg, p.o., 15 days) was gavaged daily to the rats along with Ethanol [40%,2ml/100gm,p.o. for 15 days, twice a day] & on 14th Day CCL_4 [1:1 in groundnut oil, 0.1 ml/kg,s.c.]. The levels of Triglycerides (TG), aminotranferases (AST, ALT), alkaline phosphatase (ALP), Bilirubin and Superoxide dismutase (SOD), Catalase (CAT), Glutathione reductase (GSH) and Lipid peroxidation (TBARS) levels in liver were measured. Morphological and histopathological indices in the liver of healthy and Ethanol-CCl_4 treated rats were also measured. In the underlying study, Ethanol- CCl_4 exhibited increase in the hepatic biomarkers (TG, AST, ALT, ALP, and Bilirubin), LPO which were significantly decreased after pretreatment with MEPN (100, 200 mg/kg) and PPR (50 mg/kg). Ethanol- CCl_4 significantly decreased levels of SOD, CAT, and GSH which were restored with MEPN and PPR. The results were similar to that of Liv52 [1ml/kg, p.o. for 15 days], which served as a reference standard. Histopathological studies were also in agreement of above. The study indicates that P. nigrum possess potential hepatoprotective activity which may be attributed to its piperine alkaloids, having therapeutic potential in treatment of liver disorders.

Keywords: piperine alkaloids**,** triglycerides, black pepper.

INTRODUCTION

The liver is the key organ regulating homeostasis in the body. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction .[1] Humans are continuously exposed to different kinds of chemicals such as food additives, industrial chemicals, pesticides and other undesirable contaminants in the air, food and soil. [2, 3] Most of these chemicals induce a free radical-mediated lipid peroxidation leading to disruption of biomembranes and dysfunction of cells and tissues. [4] Injury may result from direct toxicity, occur via hepatic conversion of a xenobiotic to an active toxins, or be produced by immune mechanisms, usually by the drug or a metabolite acting as a hapten to convert a cellular protein into an immunogen. [5] Ethanol consumption is considered to be a risk factor in the development of liver damage. Ethanol when administered chronically is known to potentiate hepatotoxicity of carbon-tetrachloride. Alcoholic liver disease (ALD) remains one of serious health problems. ALD is the common consequence of prolong and heavy alcohol intake. The fatal changes in the liver include fatty liver, hepatitis and hepatic cirrhosis. [6] Multiple mechanisms are likely to be involved in these pathogenesis especially by toxic substances generated during alcohol metabolism.

Reactive oxygen species (ROS) and other free radicals believe to be the key mechanism of ALD. [7] These ROS are generated from many sources such as the activation of Kupffer cells [8] which explains progression of ALD. CCl_4 is an extensively used xenobiotic to induce lipid peroxidation and toxicity. [9] It is well established that CCl_4 is metabolized in the liver to highly reactive trichloromethyl radical which initiate free radical-mediated lipid peroxidation of the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane leading to accumulation of lipid-derived oxidants causing liver injury. [10] It also induces hydropic degeneration, centrilobular necrosis, fatty changes, cirrhosis and hepatoma. [11] In the absence of reliable liver protective drugs, herbs may play role in relieving liver disorders. Many plants demonstrate hepatoprotective activity. Piperine is a nitrogenous pungent substance present in black pepper, obtained from *Piper nigrum* L. (Piperaceae).It has been shown that piperine reduces inflammation and pain processes [12] anticonvulsant and antiulcer activity [13] and protects the liver. [14]

The present study aims to investigate the protective effect of methanolic extract and methanolic fraction of *Piper nigrum* on rat liver damage induced by ethanol-CCl_4.

MATERIALS AND METHODS

Animals

Albino rats (Wistar strain) of either sex weighing between 200-250 g, were obtained from Bharat Serum and Vaccines Ltd., Thane. Animals were housed into groups of five under standard laboratory conditions of temperature 25 ± 1ºC with free access to food and water. The experiments were performed during the light portion. The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethical Committee of MGV's Pharmacy College, Nashik.

Plant material and extraction

Fruits of *P. nigrum* (1Kg) were brought from local market and authenticated by Mr. P. G. Diwakar, Botanical Survey of India, Pune. Voucher specimen has been retained there (ARBAPI1). The fruits were powdered and then the powdered *Piper nigrum* L. was defatted with the Petroleum Ether. The Petroleum ether was removed and powder was air dried. Thereafter extraction was carried out with the help of methanol. The extract was concentrated and evaporated to dryness for getting the crude methanolic extract of *Piper nigrum* L. The yield was 15%w/w. The phytochemical testing of extract was also carried out.

Drugs and Treatment schedule

Ethanol and CCl_4 was purchased from Qualigens Fine chemicals Ltd, Mumbai. Liv- 52 was purchased from Himalaya Drug Company, Mumbai. AST kit (Pathozyme Diagnostics, India), ALP kit (Accurex Biomedical Pvt. Ltd., India), Bilirubin kit (Biolab Diagnostics (I) Pvt. Ltd., India), ALT kit (Span Diagnostics Ltd., India) were used for the study. All drug solutions were freshly prepared in saline before each experiment. MEPN and PPR was dissolved in distilled water and administered orally. In Ethanol-CCl_4 treated groups, Ethanol [40%,2ml/100gm,p.o. for 15 days, twice a day] & on 14th Day CCL_4 [1:1 in groundnut oil, 0.1 ml/kg,s.c.] and sacrificed on the 15th day. The animals were divided in following experimental groups, 5 animals in each.

Group I: Vehicle (Normal saline1 ml/kg, p.o., 15 days), Group II: Ethanol [40%,2ml/100gm,p.o.] for 15 days, twice a day & on 14th Day CCL_4 [1:1 in groundnut oil, 0.1 ml/kg,s.c.] , Group III: Liv-52 [1ml/kg,p.o.] for 15 days, Ethanol [40%,2ml/100gm,p.o.] for 15 days, twice a day & on 14th day CCL_4[1:1in groundnut oil,0.1ml/kg,s.c], Group IV: MEPN [100mg/kg,p.o.] for 15 days, Ethanol [40%,2ml/100gm,p.o.] for 15 days, twice a day &on 14th day CCL_4[1:1 in groundnut oil,0.1ml/kg,s.c], Group V: MEPN [200mg/kg,p.o.] for 15 days, Ethanol [40%,2ml/100gm,p.o.] for 15 days, twice a day &on 14th day CCL_4[1:1 in groundnut oil,0.1ml/kg,s.c.] , Group VI: Piperine [50mg/kg,p.o.] for 15 days, Ethanol [40%,2ml/100gm,p.o.] for 15 days, twice a day &on 14th day CCL_4[1:1 in groundnut oil,0.1ml/kg,s.c.].

After Ethanol-CCl_4 treatment, animal was sacrificed by cervical dislocation and blood samples were collected by heart puncture method for determination of various biochemical parameters. The upper liver lobe tissues were immediately dissected out, washed with ice-cold 0.9% saline and weighed. They were then subjected to antioxidant and histopathology studies.

Estimation of body and liver weight

In each group, body weight of rats was taken before and Ethanol-CCl_4 treatment. Isolated liver was weighed after keeping them in ice-cold saline and squeezing out the blood.

Biochemical estimation

Serum was separated from collected blood using centrifuge at 3000 *g* for 15 min and used for estimation of TG, [15] AST, [16] ALT, [17] ALP,[18] Bilirubin[19]. The excised liver was then weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10%w/v. The homogenates were centrifuged at 10,000 *g* for 20 min. The clear supernatants were used for the assays of Superoxide dismutase (SOD), [20] Catalase (CAT), [21] reduced glutathione (GSH), [22] and Extent of lipid peroxidation (LPO) [23] was evaluated in terms of thiobarbituric acid reactive substances (TBARS). [24]

Histopathological studies

The liver was fixed in 10% formalin. The specimens were then processed for standard procedure and were embedded in paraffin wax. The blocks were then sectioned according to hematoxylin and eosin method. Five-micrometer thick histological sections were obtained from the paraffin blocks. The sections were examined under the light microscope and photographs were taken under 10X using Moti camera.

Statistics

The mean ± SEM values were calculated for each group. One-way ANOVA followed by Dunnett's multiple comparison tests were used for statistical analysis. Values of $p<0.05$ were considered statistically significant.

RESULTS

The changes in weights of rats among the experimental group after 15 days were found to be significant. Significant reduction ($p<0.05$) were observed in the body weight and isolated liver weight of Ethanol-CCl_4 treated group compared to vehicle treated group. This failure to thrive in animals pretreated with PPR (50 mg/kg), MEPN (100 mg/kg, 200 mg/kg), and Liv-52 (1 ml/kg p.o.) was probably due to Ethanol-CCl_4. Pretreatment with PPR, MEPN and Liv-52 exhibited significant ($p<0.05$) elevation in the body weight and isolated liver weight in these groups compared to Ethanol-CCl_4 [Table1].

Ethanol-CCl_4 treated rats showed raised serum activities of TG, AST, ALT, ALP, and Bilirubin when compared to vehicle treated group. A significant reduction ($p<0.05$) was observed in the serum markers in the animals treated with PPR (50 mg/kg), MEPN (100 mg/kg, 200 mg/kg), and Liv-52(1 ml/kg p.o) compared to Ethanol-CCl_4 treated group [Table 2].

A significant (<0.05) reduction in SOD, CAT, GSH and elevation of LPO were observed in Ethanol-CCl_4 treated group compared to vehicle treated group. A significant (<0.05) elevation in SOD, CAT, GSH and reduction in TBARS were observed in animals treated with PPR (50 mg/kg), MEPN (100 mg/kg, 200 mg/kg), and Liv-52 (1 ml/kg p.o) compared to Ethanol-CCl_4 treated group [Fig.1, 2, 3, 4].

Table 1: Effect of *Piper nigrum* on body weight and isolated liver weight in Ethanol-CCl_4 treated rats

Treatment (mg/kg)	Final body weight (g)	Isolated liver weight (g)
Normal saline	242.8 ± 3.587	2.304 ± 0.071
Ethanol-CCl_4	197.2 ± 4.084*	1.380 ± 0.024*
Liv-52 + Ethanol-CCl_4	242.3 ± 1.890#	2.200 ± 0.021#
MEPN(100)+Ethanol-CCl_4	214.6 ± 2.515#	1.572 ± 0.052#
MEPN(200)+Ethanol-CCl_4	217.2 ± 1.639#	1.741 ± 0.037#
PPR (50) + Ethanol-CCl_4	234.3 ± 2.979#	1.993 ± 0.037#

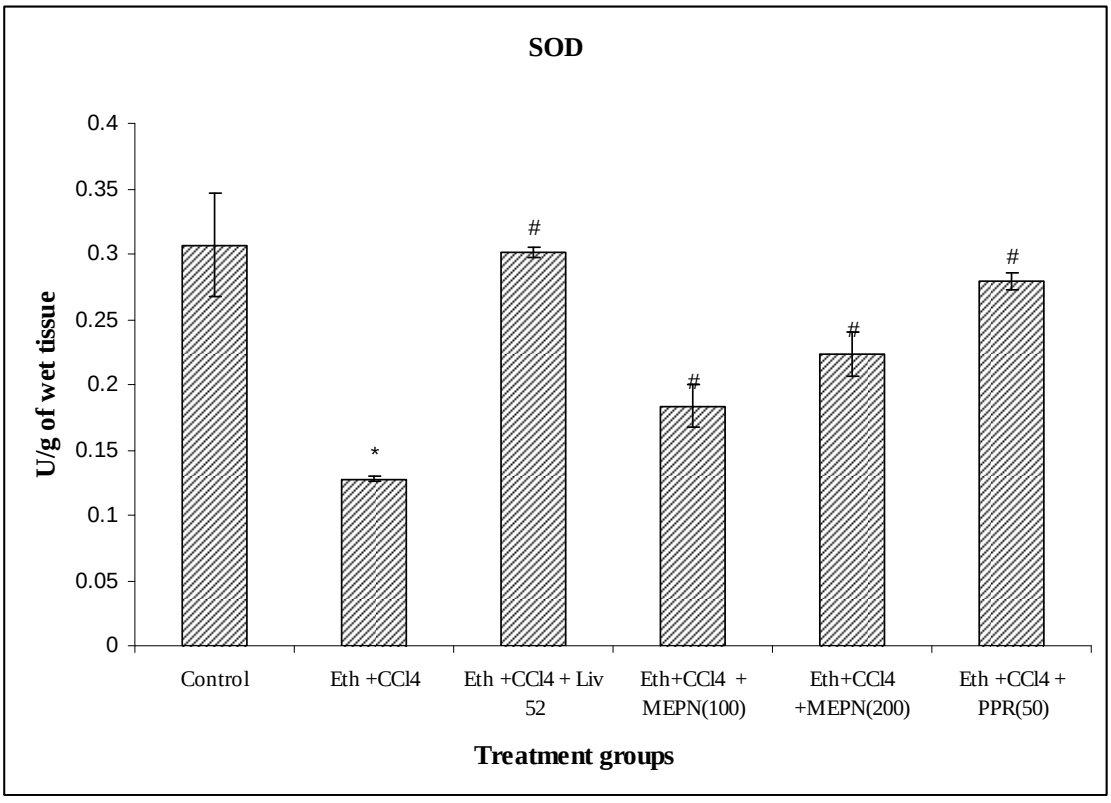
*N=5. The observations are mean ±SEM. * p<0.05 as compared to control and # p<0.05 as compared to Ethanol-CCl_4 (ANOVA followed by Dunnett's test). MEPN: Methanolic extract of Piper nigrum, PPR: Piperine.*

Table 2: - Effect of *Piper nigrum* on activity of liver biomarkers in Ethanol-CCl_4 treated rat

Treatment Groups	Triglyceride (mg/dl)	Alkaline Phosphatase	SGOT (U/L)	SGPT (U/L)	Bilirubin (mg/dl)	
					Direct	Total
Normal saline	190.9± 1.10	59.85± 2.07	46.90± 2.77	22.18± 0.36	0.303± 0.02	0.220± 0.02
Ethanol-CCl_4	321.2± 1.0*	171.5±8.30*	96.19± 2.98*	88.87± 3.841*	10.28± 0.32*	9.921± 0.31*
Liv-52 + Ethanol-CCl_4	211.7± 0.68#	90.3±3.41#	56.92± 2.92#	36.66± 1.38#	2.234± 0.07#	1.912± 0.03#
MEPN(100)+Ethanol-CCl_4	257.8± 1.23#	118.3±2.98#	74.26± 2.84#	67.39± 0.97#	3.618± 0.39#	3.507± 0.28#
MEPN(200)+Ethanol-CCl_4	243.1± 1.70#	109.2±7.05#	66.39± 1.68#	55.52± 0.81#	3.216± 0.03#	2.883± 0.12#
PPR (50) + Ethanol-CCl_4	254.9± 1.80#	93.42±3.73#	63.95± 1.42#	45.06± 1.76#	3.377± 0.07#	2.798± 0.14#

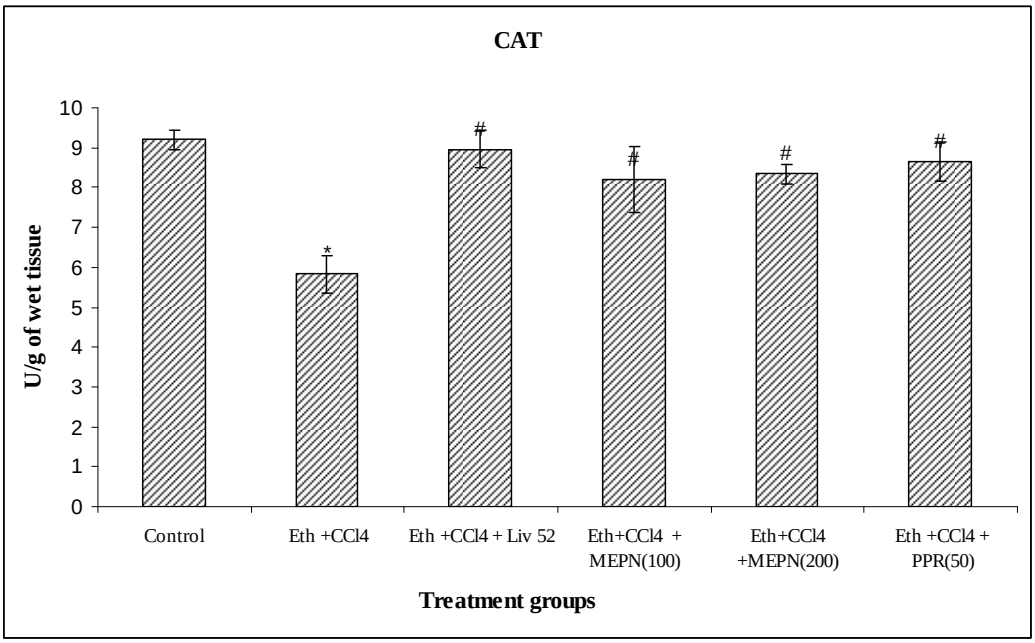
*N = 5, All values are expressed as mean ±SEM. One way ANOVA followed by Dunnett's test. *P< 0.05 against control group, # P< 0.05 against Ethanol+CCl_4 group.*
MEPN=Methanolic Extract of Piper nigrum, PPR: Piperine

Fig.1 Effect of *P. nigrum* on levels of Liver Superoxide dismutase in Ethanol-CCl_4 treated rats



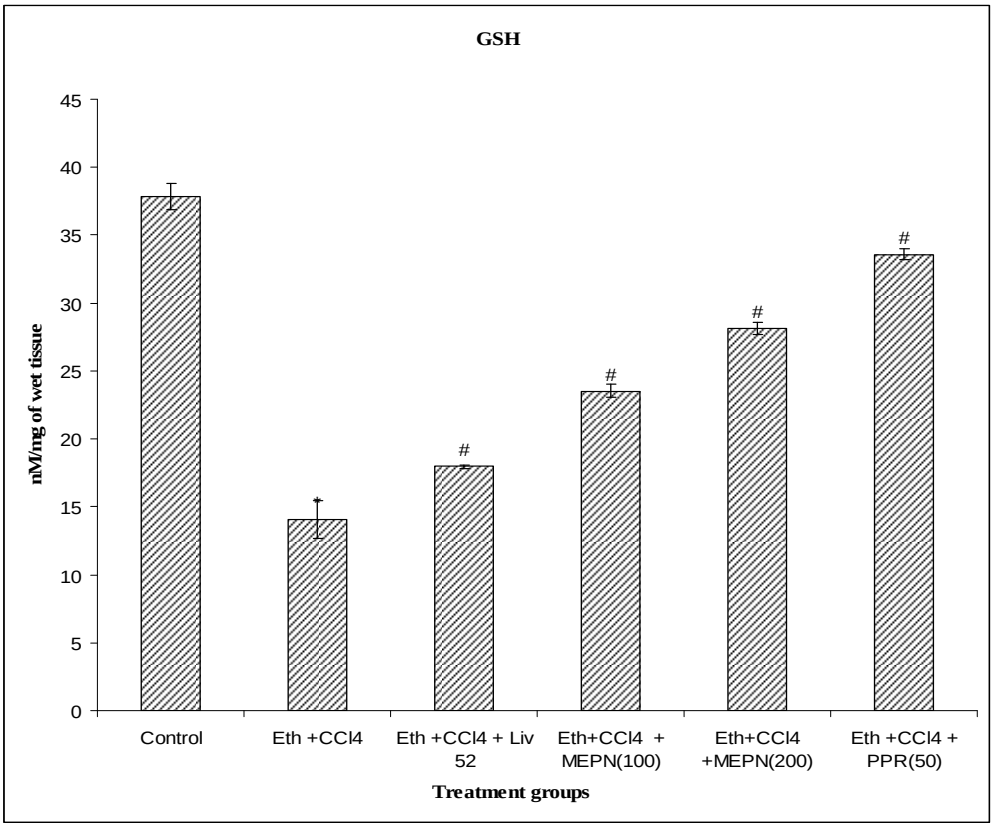
*N=5. The observations are mean ±SEM. * p<0.05 as compared to control and # p<0.05 as compared to Ethanol-CCl_4 (ANOVA followed by Dunnett's test). MEPN: Methanolic extract of Piper nigrum, PPR: Piperine.*

Fig. 2 Effect of *P. nigrum* on levels of Liver Catalase in Ethanol-CCl_4 treated rats.



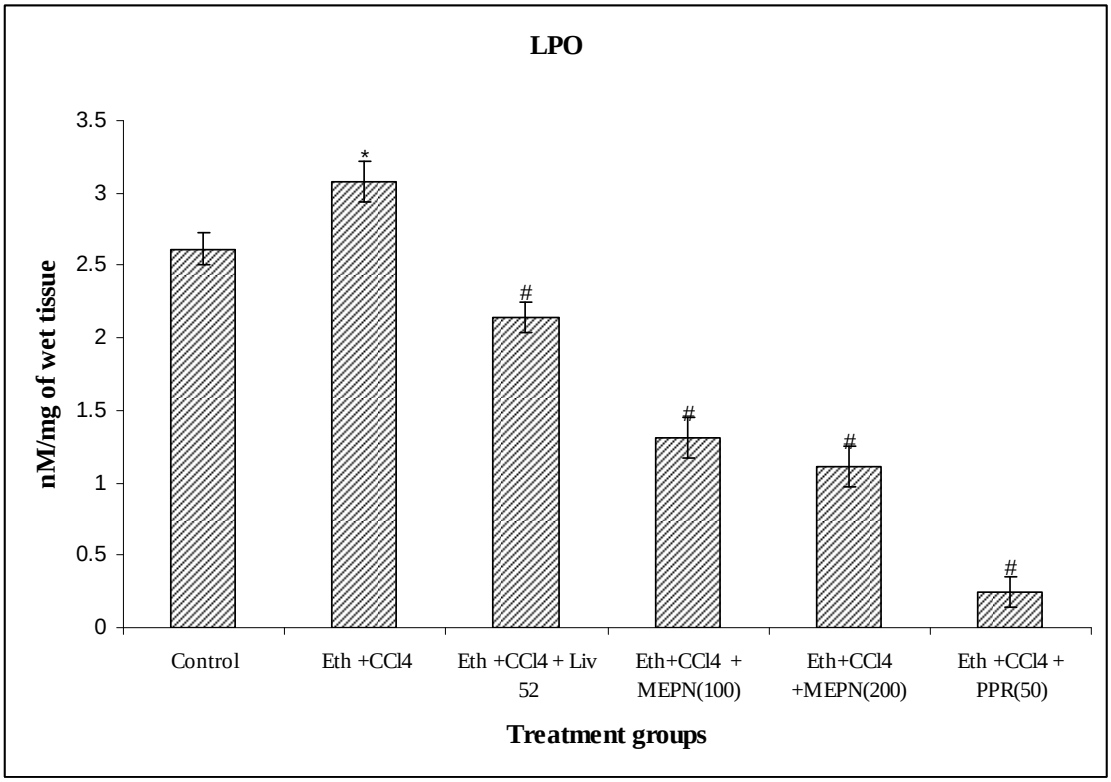
*N=5. The observations are mean ± SEM. * p<0.05 as compared to control and # p<0.05 as compared to Ethanol-CCl_4 (ANOVA followed by Dunnett's test). MEPN: Methanolic extract of Piper nigrum, PPR: Piperine.*

Fig.3 Effect of *P. nigrum* on levels of Liver Glutathione dismutase in Ethanol-CCl_4 treated rats



*N=5. The observations are mean ± SEM. * p<0.05 as compared to control and # p<0.05 as compared to Ethanol-CCl_4 (ANOVA followed by Dunnett's test). MEPN: Methanolic extract of Piper nigrum, PPR: Piperine.*

Fig. 4 Effect of *P. nigrum* on extent of Liver lipid peroxidation in Ethanol-CCl_4 treated rats



*N=5. The observations are mean ± SEM. * p<0.05 as compared to control and # p<0.05 as compared to Ethanol-CCl_4 (ANOVA followed by Dunnett's test). MEPN: Methanolic extract of Piper nigrum, PPR: Piperine.*

DISCUSSION

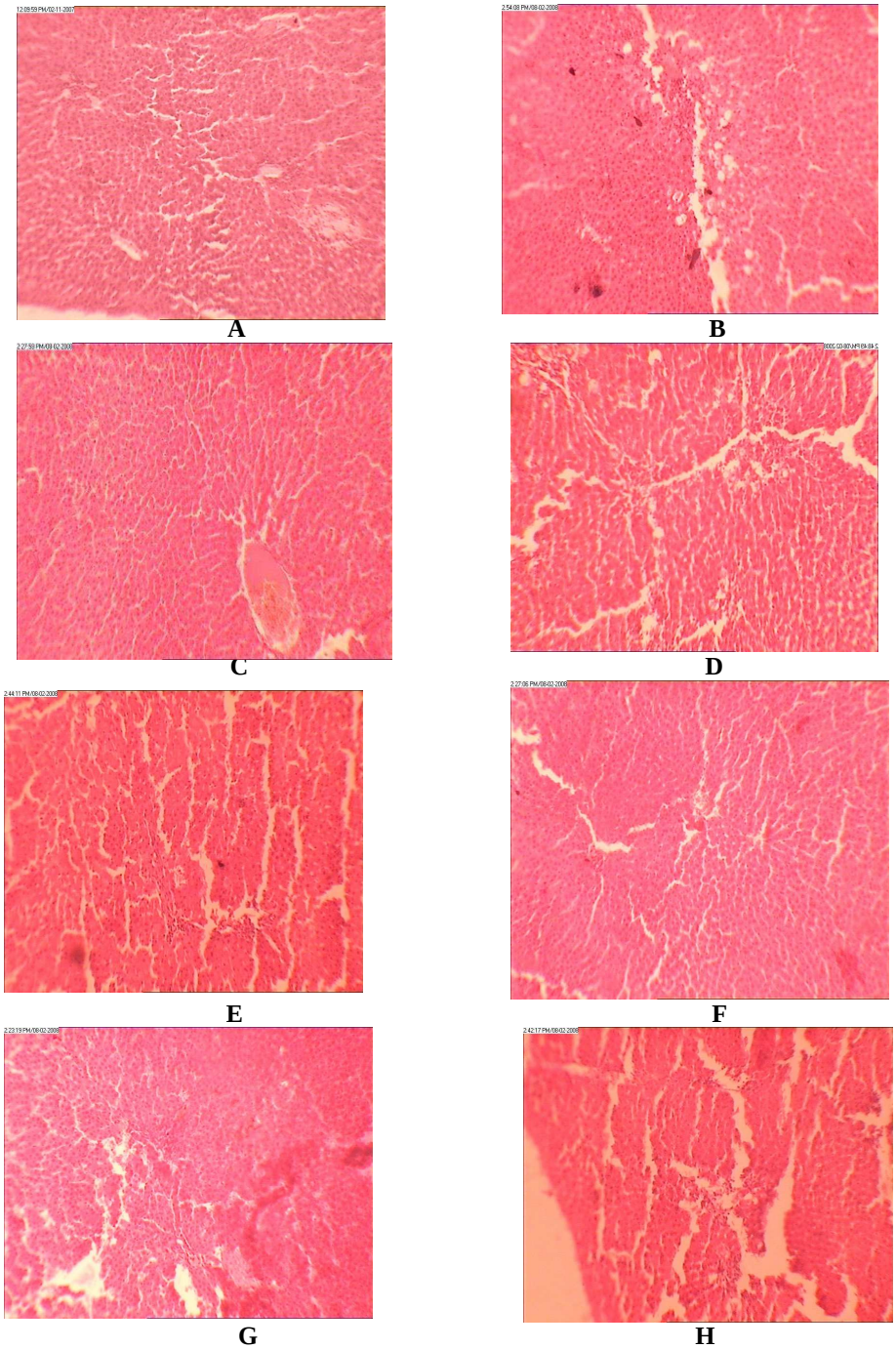
The results obtained in the present study show that the methanolic extract of *Piper nigrum* and its methanolic fraction possess antioxidants, antiulcer, hepatoprotective activity. Oxidative stress is one major factor in etiology of ethanol injury, mainly by Kupffer cell derived ROS. [25] Ethanol activates Kupffer cells primarily through the action of a substance called endotoxin, which is released by certain gram-negative bacteria present in the intestine. [26] Kupffer cell activation generates ROS and proinflammatory cytokines (TNF alpha, IL-1), both of them can lead to liver damage. [27]

A great number of plants worldwide showed a strong antioxidant activity [28] and a powerful scavenger activity against free radicals. Keeping this view, we have attempted to study the effect of Piperine (PPR) and its Methanolic extract of *Piper nigrum* (MEPN) on Ethanol-CCl_4 induced Hepatotoxicity. The preliminary phytochemical screening of Methanolic extract of *Piper nigrum* (MEPN) showed the presence of alkaloids, tannins and Proteins.

The results of a present study indicate that the Ethanol [40%, 2ml/100gm, p.o. for 15 days, twice a day] & on 14th Day CCL_4 [1:1 in groundnut oil, 0.1 ml/kg, s.c.] and sacrificed on the 15th day induce pathological changes in serum and biochemical markers, indicative of toxicity and increase in free radical production. In our study, we observed that the isolated liver weight and body weight was significantly decreased in Ethanol-CCl_4 treated group which was prevented by pre treatment with MEPN (100 and 200 mg/kg/day, p.o. for 15 days) at the end of treatment schedule.

Histopathological changes

Fig 5: Sections of liver tissues in various groups



Photomicrographs of histopathological examination (10 X) of liver tissues.

Section A) Control group shows normal architecture.
Section B) and section C) Liver sections of the rats treated with ethanol-CCl_4 showed fatty degeneration, necrosis and infiltration.
Section D) Liver sections of the rats treated with Liv-52 along with ethanol-CCl_4 showed almost normal cellular architecture.
Section E) and Section F) Liver sections of the rats treated with methanolic extract of Piper nigrum (100 & 200mg/kg, resp.) along with ethanol-CCl_4 Showed almost normal histology with mild congestion.
Section G) and Section H) Liver sections of the rats treated with Piperine (50 mg/kg) along with ethanol-CCl_4 showed almost normal histology.

A significant rise in liver marker enzymes is an indicator of abnormal functioning of the liver. Administration of Ethanol-CCl_4 to rats significantly increased serum TG, AST, ALT, ALP, and Bilirubin. In our study, a significant decrease in concentration of SOD and CAT and GSH levels and increased levels of TBARS in Ethanol-CCl_4 treated group was observed. MEPN (100 and 200 mg/kg, p.o. for 15 days) and PPR (50 mg/kg/day, p.o., for 15 days) treatment significantly reversed the changes in antioxidant levels induced by Ethanol-CCl_4 treatment.

The histopathology data has revealed that Ethanol-CCl_4 treatment in liver shows fatty degeneration, necrosis and infiltration. Treatment with MEPN (100 and 200 mg/kg/day, p.o., for 15 days) and PPR (50 mg/kg/day, p.o., for 15 days) has reversed the histopathological features induced by Ethanol-CCl_4 on hepatic tissues.

CONCLUSION

Hepatotoxicity was induced by administering ethanol-CCl_4 as mentioned in method. Our studies showed that the prophylactic treatment with methanolic extract (100 and 200 mg/kg body weight, p.o.) and Piperine (50 mg/kg body weight, p.o.) of *Piper nigrum* for 15 days with Ethanol-CCl_4 treatment offered considerable protection to liver as evidenced from the levels of biochemical parameters (SGOT, SGPT, cholesterol, triglyceride, total proteins, albumin and bilirubin), which was supported by the limited extent of histological damage. Thus it is concluded that the *Piper nigrum* has hepatoprotective activity.

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