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Hepatoprotective Effects of *Clitoria ternatea* and *Vigna mungo* against Acetaminophen and Carbon tetrachloride-induced Hepatotoxicity in Rats

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ABSTRACT

The present study was undertaken to investigate hepatoprotective activity of *Clitoria ternatea* seed and root and *Vigna mungo* seed against acetaminophen- and carbon tetrachloride-intoxicated rats. The liver functioning was evaluated by measuring serum marker enzymes and hepatic fibrosis was accessed by measuring collagen content in terms of p-hydroxyproline levels. Mast cell infiltration and potentiation of phenobarbitone-induced sleeping time were also measured. In addition, serum bilirubin, creatinine, lipid peroxidation and antioxidant parameters were also estimated. *C. ternatea* and *V. mungo* seed extracts significantly ($p<0.05$) decreased SGOT, SGPT, ALP and total bilirubin in both acetaminophen and CCl_4 - intoxicated rats. The *C. ternatea* root extract, showed similar results only in CCl_4 - intoxicated rats. These findings were further supplemented by histopathological studies of liver tissues. Hepatic collagen content as evident from decreased ($p<0.05$) hydroxyproline levels and hepatic mast cell infiltration were significantly decreased in extracts pre-treated animals. In addition, *C. ternatea* and *V. mungo* seed extracts significantly ($p<0.05$) reduced hepatic lipid peroxidation as evident from the decreased MDA, increased antioxidant enzymes activities and GSH levels in the liver tissues. The *V. mungo* seed extract significantly potentiated barbiturate-induced sleeping time. The findings of study suggested that *C. ternatea* and *V. mungo* possess potent hepatoprotective activity. The hepatoprotective activity of *C. ternatea* could be attributed to antioxidant properties and prevention of pre-inflammatory changes. The hepatoprotective activity of *V. mungo* could be attributed partly to the hepatic microsomal enzyme inhibition and partly to the antioxidant properties.

Key words: Acetaminophen, carbon tetrachloride, hepatotoxicity, hydroxyproline, mast cells

INTRODUCTION

Liver is a key organ of the body playing major role in maintaining homeostasis. It is involved in almost all the metabolic pathways of the body related to growth, immunity, energy supply and reproduction. It also plays a significant role in detoxification of variety of exogenous materials. Therefore, the maintenance of a healthy liver is vital to overall health and well being. Unfortunately, liver is exposed to variety of toxins in our day-to-day life leading to severe liver injuries. The hepatic carcinoma, jaundice and hepatitis are the major liver disorders that account

for a high death rate. In spite of tremendous scientific achievements in the field of hepatology in recent years, there is not a single drug that stimulates liver functions, offer protection to the liver from damage or help regeneration of hepatic cells (Chatterje, 2000). Modern medicinal agents used in liver disorders are also associated with some severe side effects. Hence, herbal plants have been investigated in search of better hepatoprotective agents. Plant extracts have been used by traditional medical practioners for the treatment of liver disorders for centuries (Schuppan *et al*., 1999). Extracts of about 25 different plants have been reported to cure liver disorders (Mohamed Saleem *et al*., 2010). Plant extracts as such (Mounnissamy *et al*., 2008; Ali *et al*., 2009) or as polyherbal formulations (Satyapal *et al*., 2008) have shown promising hepatoprotection. Legumes have been reported to have hepatoprotective activities (Wu *et al*., 2001). *Clitoria ternatea* seed and roots were reported to have nootropic, anti-depressant, anti-diabetic, anti-inflammatory, anti-hyperlipidemic, etc (Mukherjee *et al*., 2008). *Vigna mungo* was found to have immunostimulatory activity (Solanki and Jain, 2010). *Clitoria ternatea* L. (butterfly pea) and *Vigna mungo* L. (Fabaceae) were reported to be used in variety of disease conditions of liver in Indian traditional system of medicine (Anonymous, 2003). However, no scientific and methodical investigations have so far been reported in literature regarding their actions on liver. Hence, the present investigation was undertaken to evaluate the hepatoprotective activity of *C. ternatea* and *V. mungo* against acetaminophen and carbon tetrachloride-induced hepatotoxicity in rats.

MATERIALS AND METHODS

Plant collection and identification: The plant *C. ternatea* is available in two varieties - blue and white. The blue variety has been used for present investigation. The plant was collected during summer (April-May, 2007) from the fields and road side of the Charotar region of the Gujarat state, India. The seeds of *V. mungo* were purchased from the local market of the same region. Both the plants were botanically identified by Dr. G. C. Jadeja, Professor and Head, Department of Agricultural Botany, B. A. College of Agriculture, Anand, India. The quality of the plants was ascertained as per Ayurvedic Pharmacopoeia of India by determining foreign matters, total ash, acid insoluble ash, alcohol soluble extractive and water soluble extractive values (Anonymous, 2003).

Extraction: The dried powdered (mesh No. 40) seeds of both the plants were defatted with petroleum ether and then extracted with 50% v/v alcohol. The solvents were evaporated at 60°C to have pasty mass referred to as seed extracts, respectively. The roots of *C. ternatea* were directly extracted with 50% v/v alcohol by maceration and evaporated to have hydroalcoholic extract of roots.

Chemicals and reagents: All the chemicals used were of analytical grade. Silymarin and acetaminophen were received as gift samples from Zydus Research Centre, Ahmedabad, Gujarat. The solvents and reagents were purchased from S. D. Finechemicals Limited, Mumbai.

Pharmacological evaluation

Animals: Albino rats (Wistar strain) weighing 150-200 g were obtained from the central facility and acclimatized for 6 days. Throughout the studies, all rats were housed individually under

specific pathogen-free conditions in polypropylene cages at ambient temperature (25±1°C), relative humidity (55±5%) and under a 12/12 h light-dark cycle. Animals had free access to standard commercial pellet diet (Pranav Agro Industries Ltd., Sangli, India) and water *ad libitum* throughout the study period. This study was approved by the institutional animal ethics committee in accordance with the guidelines of Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA) (CPCSEA, 2003).

Acute toxicity studies: Albino rats were kept fasting for overnight providing only water, after which the extracts were, administered orally 400 mg kg^{-1} and observed for 72 h. If mortality was observed in 2 out of 3 animals, then the dose administered was assigned as toxic dose. If the mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher dose up to 1000 mg kg^{-1} for each extracts.

Hepatoprotective studies

Acetaminophen-induced hepatotoxicity: The acetaminophen (ACP) hepatotoxicity was induced in rats by method of Muriel *et al*., (1992). Wistar albino rats of either sex weighing 150-200 g were selected for the study. Animals were divided in different groups each of six animals.

- **Group-I:** Normal control: the animals received only aqueous suspension of 1 mL of 1% CMC as vehicle orally at 0, 6, 12 and 18 h without any treatment
- **Group-II:** ACP control: the animals were - intoxicated with ACP (500 mg kg^{-1} body weight, p.o.) at 0, 6, 12 and 18 h
- **Group-III:** Animals received silymarin (25 mg kg^{-1} body weight, suspended in 1% CMC solution, p.o.) 2 h before ACP administration
- **Group-IV:** Animals received 50% hydroalcoholic extract of seeds of *C. ternatea* (500 mg kg^{-1} body weight, p.o.) 2 h before ACP administration
- **Group-V:** Animals received 50% hydroalcoholic extracts of roots of *C. ternatea* (500 mg kg^{-1} body weight, p.o.) 2 h before ACP administration
- **Group-VI:** Animals received 50% hydroalcoholic extract of seeds of *V. mungo* (500 mg kg^{-1} b.wt.p.o.) 2 h before ACP administration

The blood samples were collected by the retro-orbital puncture method under light ether anesthesia at 24 h. At the end of the study, animals were anaesthetized and liver was dissected quickly, collected, washed thoroughly in normal saline, bloated and preserved at -40°C for further analysis. The liver tissues were subjected to biochemical and histopathological investigations.

***Carbon tetrachloride*-induced hepatotoxicity:** The method of Agarwal *et al*. (2006) was used to produce carbon tetrachloride-induced hepatotoxicity. Albino rats (Wistar strain) of either sex weighing 150-200 g were selected for the study. Animals were divided in different groups each of six animals.

- **Group-I:** Normal control: the animals received only aqueous suspension of 1% CMC as vehicle orally without any treatment for 7 days
- **Group-II:** CCl_4 control: the animals were - intoxicated with 1:1 (v/v) mixture of CCl_4 in Arachis oil (1.5 mL kg^{-1} b.wt., twice a week on day 3 and day 6). The animals were sacrificed 24 h after the last CCl_4 treatment
- **Group-III:** CCl_4 treated animals received silymarin (25 mg kg^{-1} b.wt., suspended in 1% CMC solution, p.o.) once daily for 7 days
- **Group-IV:** CCl_4 treated animals received 50% hydroalcoholic extract of seeds of *C. ternatea* (500 mg kg^{-1} b.wt., p.o.) once daily for 7 days
- **Group-V:** CCl_4 treated animals received 50% hydroalcoholic extracts of roots of *C. ternatea* (500 mg kg^{-1} b.wt., p.o.) once daily for 7 days
- **Group-VI:** CCl_4 treated animals received 50% hydroalcoholic extract of seeds of *V. mungo* (500 mg kg^{-1} b.wt., p.o.) once daily for 7 days

At the end of the study, the blood was collected by retro-orbital puncture method under light ether anesthesia and serum was separated by centrifuging at 2000 rpm for 15-20 min. At the end of the study, animals were anaesthetized and liver was dissected quickly, collected, washed thoroughly in normal saline, bloated and preserved at - 40°C for further analysis.

Assessment of liver function: At the end of the study, the blood was collected by retro-orbital puncture under light ether anesthesia and serum was separated by centrifuging at 2000 rpm for 15-20 min. The serum levels of aspartate aminotransferase (SGOT) (Tietz, 1970), alanine aminotransferase (SGPT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (King and King, 1954), creatinine (Bonsnes and Taussky, 1945) and total bilirubin (Malloy and Evelyn, 1937) were estimated colorimetrically using UV Visible spectrophotometer (Shimadzu-UV-1601, Japan). All the tests were carried out with serum diagnostic kits supplied by Span Diagnostic Ltd., India.

Lipid peroxidation and anti-oxidant parameters in liver: The liver homogenates were prepared in tris-hydrochloride buffer (0.1 M, pH 7). They were subjected to estimation of protein (Lowry *et al.*, 1951), malondialdehyde (MDA) (Ohkawa *et al.*, 1979), superoxide dismutase (SOD) (Misra and Fridovich, 1972), catalase (Aebi, 1974) and reduced glutathione (GSH) (Beutler *et al.*, 1963).

***Phenobarbitone-induced* sleeping time:** To investigate the hepatic microsomal enzymes inhibition, potentiation of the Phenobarbitone-induced sleeping time was measured in rats (Walker and Parry, 1949). Animals were divided into different groups, each consisting of six. The control group received single dose of phenobarbitone (80 mg kg^{-1}, i.p.). The treatment groups received 500 mg kg^{-1} b.wt, p.o. of each extracts one hour before phenobarbitone injection. The animals were observed for righting reflex. If the animals failed to maintain normal posture when placed on one side within 30 sec; it was considered as loss of righting reflex.

Determination of hepatic hydroxyproline content: Hydroxyproline (HYP) was determined colorimetrically in duplicates from 0.2 g of liver tissues using a modified method of Jamall *et al.* (1981). Briefly, the frozen tissue was homogenized in 4 mL of 6 N HCL and hydrolyzed at 110°C for 16 h. the hydrolysate was filtered and then 30 μL aliquot of these samples was evaporated under vacuum, the sediment was dissolved in 1.2 mL of isopropanol and incubated with 0.2 mL of 0.84% chloramine-T in acetate-citrate buffer (pH 6.0) for 10 min at room temperature. Then, 1.0 mL of Ehrlich's reagent was added and the mixture was incubated at 60°C for 25 min. The absorbance of the sample solution was measured at 560 nm wavelength (Simadzu-UV-1601, Japan). Next, the hydroxyproline content in 100 mg of liver was calculated from the standard curve of 4-hydroxy-L-proline (Sigma, USA) and expressed as μg/100 mg liver weight.

Number of mast cells in liver tissues: It was carried out at Dr. Shah's pathology laboratory, Ahmadabad, India. Toluidine blue staining for mast cells was performed by immersion of liver sections in 0.01% toluidine blue (Sigma, USA) for 1 min at room temperature. The number of mast cells was quantified in 25 randomly selected, non-overlapping fields and expressed as the number of mast cells/mm^2.

Histopathological studies: The rats were sacrificed under deep anesthesia and the livers were excised quickly and fixed in 10% buffered neutral formalin. Paraffin sections (5-10 μ) were prepared, stained with haematoxylin-eosin and finally mounted in neutral medium. Histopathological studies were carried out in the pathology laboratory.

Statistical analysis: All the data are expressed in Mean±SEM. Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Tukey's test to compare the significance between all the groups. $p<0.05$ was considered to be significant. All the statistics were estimated by using SigmState™ 2.03 statistical soft ware.

RESULTS

Acute toxicity study: The acute toxicity study in rats showed that all the extracts were safe orally up to the dose of 1000 mg kg^{-1} b.wt.

Effects of various treatments on acetaminophen-induced hepatotoxicity: Acetaminophen significantly increased serum SGOT, SGPT and ALP levels in the control group suggesting the severe liver injuries. All the treatment groups, except *C. ternatea* seeds extract, significantly reduced serum SGOT, SGPT and ALP levels (Table 1). These effects were comparable to that of silymarin, a reference hepatoprotective drug. The serum total and bound bilirubin levels were also significantly reduced by all the extracts, except *C. ternatea* root extract. None of the treatment affected serum creatinine levels (Table 2).

Acetaminophen also induced significant increase in the liver lipid peroxidation, SOD and catalase activities and decrease in serum GSH levels (Table 3). All the treatments significantly reduced lipid peroxidation, SOD, catalase and increased GSH levels. However, *C. ternatea* seeds

Table 1: Effects of *C. ternatea* and *V. mungo* extracts on serum enzymes levels in acetaminophen - intoxicated rats

Groups (n = 6)	SGOT (IU L^{-1})	SGPT (IU L^{-1})	ALP (KA units L^{-1})
Normal	61.50±3.47	57.33±3.31	58.32±2.24
ACP Control	184.33±4.69*	146.33±3.71*	134.66±4.39*
ACP + Silymarin[§]	48.33±2.55**	45.17±2.27**	53.36±2.11**
ACP + CT seed extract[†]	95.17±3.92**	82.33±2.40**	82.61±4.12**
ACP + CT root extract[†]	180.00±5.91	142.00±4.52	145.06±5.52
ACP + VM seed extract[†]	121.17±0.83**	99.33±1.61**	59.067±1.85**

All values represent Mean±SEM, statistical analysis was carried out using one way ANOVA followed by Tukey's multiple range. $p<0.05$ was considered statistically significant. *Significant when compared with the normal group, **Significant when compared with the control group. ACP: Acetaminophen, CT: *Clitoria ternatea* and VM: *Vigna mungo*, IU: International Units, KA: Kilo Activity. [†]Dose: 500 mg kg^{-1}, p.o., [§]Dose 25 mg kg^{-1}, p.o.

Table 2: Effects of *C. ternatea* and *V. mungo* extracts on total bilirubin, direct bilirubin and creatinine levels of acetaminophen - intoxicated rats

Groups (n = 6)	Total bilirubin (mg dL^{-1})	Direct bilirubin (mg dL^{-1})	Ceeatinine (mg dL^{-1})
Normal	0.92±0.04	0.23±0.02	2.29±0.05
ACP + Control	4.70±0.08*	1.87±0.09*	1.69±0.04
ACP + Silymarin[§]	1.35±0.04**	0.38±0.03**	2.37±0.12
ACP + CT seed extract[†]	2.28±0.04**	0.67±0.05**	2.42±0.11
ACP + CT root extract[†]	4.39±0.13	1.71±0.07	2.42±0.05
ACP + VM seed extract[†]	1.84±0.06**	0.46±0.03**	2.20±0.07

All values represent Mean ± SEM, statistical analysis was carried out using one way ANOVA followed by Tukey's multiple range test. $p<0.05$ was considered statistically significant. *Significant when compared with the normal group, **Significant when compared with the control group. ACP: Acetaminophen, CT: *Clitoria ternatea* and VM: *Vigna mungo*. [†]Dose: 500 mg kg^{-1}, p.o., [§]Dose 25 mg kg^{-1}, p.o

Table 3: Effects of *C. ternatea* and *V. mungo* extracts on liver anti-oxidant parameters of acetaminophen - intoxicated rats

Groups (n = 6)	Protein (mg g^{-1} of tissue)	MDA (nmol mg^{-1} protein)	SOD (Units/min/mg protein)	Catalase (Units/min/mg protein) $x10^{-3}$	GSH (µg mg^{-1} protein) $x10^{-3}$
Normal	19.67±0.57	1.52±0.28	1.04±0.11	13.80±0.85	80.70±4.50
ACP + Control	14.96±0.73*	4.22±0.38*	3.02±0.19*	22.40±1.49*	4.34±0.17*
ACP + Silymarin[§]	21.57±0.49**	2.44±0.09**	0.38±0.09**	7.00±0.48**	80.90±2.12**
ACP + CT seed extract[†]	18.61±0.12**	2.49±0.20**	0.87±0.04**	6.87±0.77**	48.40±1.33**
ACP + CT root extract[†]	16.78±1.06	3.83±0.13	2.77±0.21	5.61±0.77**	42.20±2.56**
ACP + VM seed extract[†]	19.85±0.25**	2.73±0.18**	1.35±0.04**	13.40±0.61**	59.12±1.66**

All values were expressed as Mean±SEM, statistical analysis was carried out by one way ANOVA followed by Tukey's multiple range test. $p<0.05$ was considered statistically significant. *Significant when compared with the normal group, **Significant when compared with the control group. ACP: Acetaminophen, CT: *Clitoria ternatea* and VM: *Vigna mungo*. [†]Dose: 500 mg kg^{-1}, p.o., [§]Dose 25 mg kg^{-1}, p.o.

extract did not affect lipid peroxidation, SOD activities and GSH levels in the liver tissues of acetaminophen treated rats.

Effects of various treatments on CCl_4-induced hepatotoxicity: Rats-intoxicated with CCl_4 showed a significant increase in the activities of SGOT, SGPT and ALP (Table 4). It also produced significant decrease in the bilirubin levels. The creatinine levels were not significantly affected,

indicating that there is minimum renal toxicity. All the treatment groups significantly reduced the activities of SGOT, SGPT and ALP. The serum total and bound bilirubin were also significantly reduced by all the treatments. The creatinine levels were not affected by any of the treatments (Table 5).

The carbon tetrachloride intoxication resulted in significant increase in liver lipid peroxidation, SOD activities, catalase activities and decrease in GSH levels in control group (Table 6). All the treatments significantly lowered lipid peroxidation, SOD, catalase and increased GSH levels.

Table 4: Effects of *C. ternatea* and *V. mungo* extracts on serum enzymes levels of CCl_4 - intoxicated rats

Groups (n = 6)	SGPT (Units L^{-1})	SGOT (Units L^{-1})	ALP (KA units L^{-1})
Normal	11.33±1.20	13.00±1.34	19.85±0.49
CCl_4 Control	212.67±5.99*	222.00± 6.07*	41.07±0.80*
CCl_4 + Silymarin[§]	46.33±4.99**	48.33±5.64**	22.24±0.82**
CCl_4 + CT seed extract[†]	98.67±5.62**	107.00±6.08**	29.36±0.68**
CCl_4 + CT root extract[†]	183.00±5.65**	190.67±5.86**	33.25±0.93**
CCl_4 + VM seed extract[†]	95.17±8.78**	102.33± 9.07**	24.26±0.83**

All values represent Mean±SEM, statistical analysis was carried out using one way ANOVA followed by Tukey's multiple range test. $p<0.05$ was considered statistically significant. *Significant when compared with the normal group, **Significant when compared with the control group. CT: *Clitoria ternatea* and VM: *Vigna mungo*. [†]Dose: 500 mg kg^{-1}, p.o., [§]Dose 25 mg kg^{-1}, p.o.

Table 5: Effects of *C. ternatea* and *V. mungo* extracts on total bilirubin, direct bilirubin and creatinine levels in CCl_4 - intoxicated rats

Groups (n = 6)	Total bilirubin (mg dL^{-1})	Direct bilirubin (mg dL^{-1})	Creatinine (mg dL^{-1})
Normal	0.96±0.06	0.32±0.02	2.00±0.15
CCl_4 Control	5.48±0.39*	1.70±0.05*	2.02±0.09
CCl_4 + Silymarin[§]	1.07±0.09**	0.50±0.03**	2.37±0.12
CCl_4 + CT seed extract[†]	1.86±0.11**	0.84±0.03**	2.34±0.09
CCl_4 + CT root extract[†]	3.03±0.14**	1.94±0.07**	2.42±0.05
CCl_4 + VM seed extract[†]	2.17±0.17**	0.63±0.03**	2.13±0.06

All values represent Mean±SEM, statistical analysis was carried out using one way ANOVA followed by Tukey's multiple range test. $p<0.05$ was considered statistically significant. *Significant when compared with the normal group, **Significant when compared with the control group. [†]Dose: 500 mg kg^{-1}, p.o., [§]Dose 25 mg kg^{-1}, p.o.

Table 6: Effects of *C. ternatea* and *V. mungo* extracts on liver anti-oxidant parameters in CCl_4 - intoxicated rats

Groups (n = 6)	Protein (mg g^{-1} tissue)	MDA (nmol mg^{-1} protein)	SOD (Unit/min/ mg protein)	Catalase (Unit/min/mg protein) $x10^{-3}$	GSH (µg mg^{-1} protein) x 10^{-3}
Normal	4.43±0.11	5.58±0.65	1.17±0.03	1.15±0.07	26.12±0.01
CCl_4 Control	3.17±0.16*	17.80±1.03*	3.21±0.19*	3.71±0.14*	0.05±0.01*
CCl_4 + Silymarin[§]	4.78±0.24**	8.46±0.42**	1.30±0.06**	1.29±0.08**	27.33±0.07**
CCl_4 + CT seed extract[†]	4.41±0.18**	8.86±0.36**	1.99±0.20**	1.47±0.14**	34.20±0.02**
CCl_4 + CT root extract[†]	4.22±0.05**	9.86±0.40**	2.32±0.13**	2.52±0.14**	30.12±0.01**
CCl_4 + VM seed extract[†]	4.31±0.10**	9.58±0.23**	1.60±0.14**	1.21±0.10**	33.36±0.07**

All values were expressed as Mean±SEM, statistical analysis was carried out by one way ANOVA followed by Tukey's multiple range test. $p<0.05$ was considered statistically significant. *Significant when compared with normal group, **Significant when compared with CCl_4 control group, CT: *Clitoria ternatea* and VM: *Vigna mungo*. [†]Dose: 500 mg kg^{-1}, p.o., [§]Dose 25 mg kg^{-1}, p.o.

Table 7: Effects of *C. ternatea* and *V. mungo* on hepatic hydroxyproline content in acetaminophen - intoxicated rats

Groups (n = 6)	Hydroxyproline (µg/100 mg of liver)	Mast cell count (Cells mm^{-2})
Normal	16.72±0.45	7.12±0.33
ACP Control	32.17±0.62*	34.30±0.86*
ACP + Silymarin[§]	17.99±0.29**	10.37±0.45**
ACP + CT seed extract[†]	23.55±1.01**	21.10±0.78**
ACP + CT root extract[†]	31.99±0.57	32.03±0.56
ACP + VM seed extract[†]	19.99±0.38**	14.78±0.75**

All values represent Mean±SEM, statistical analysis was carried out using one way ANOVA followed by Tukey's multiple range. $p<0.05$ was considered statistically significant. *Significant when compared with the normal group, **Significant when compared with the control group. ACP: Acetaminophen, CT: *Clitoria ternatea* and VM: *Vigna mungo*. [†]Dose: 500 mg kg^{-1}, p.o., [§]Dose 25 mg kg^{-1}, p.o.

Table 8: Effects of *C. ternatea* and *V. mungo* on hepatic hydroxyproline content in CCl_4 - intoxicated rats

Groups (n = 6)	Hydroxyproline (Mg/100 mg of liver)	Mast cell count (Cells mm^{-2})
Normal	19.28±0.68	15.00±0.41
CCl_4 Control	40.72±0.46*	39.93±1.34*
CCl_4 + Silymarin[§]	19.01±0.51**	13.80±0.78**
CCl_4 + CT seed extract[†]	29.28±0.61**	23.95±0.73**
CCl_4 + CT root extract[†]	31.99±0.57**	30.45±0.75**
CCl_4 + VM seed extract[†]	30.27±0.33**	21.32±1.10**

All values represent Mean±SEM, statistical analysis was carried out using one way ANOVA followed by Tukey's multiple range. $p<0.05$ was considered statistically significant. *Significant when compared with the normal group, **Significant when compared with the control group. CT: *Clitoria ternatea* and VM: *Vigna mungo*. [†]Dose: 500 mg kg^{-1}, p.o., [§]Dose 25 mg kg^{-1}, p.o.

Effects on hepatic collagen content: Collagen contents of liver tissue were quantified by determination of the hydroxyproline content (Table 7). The hydroxyproline contents were significantly increased in both CCl_4 treated and ACP treated groups when compared with the respective normal groups. All the treatment groups significantly reduced liver hydroxyproline contents. However, *C. ternatea* root extract did not affect hepatic hydroxyproline levels in ACP- intoxicated rats.

Effects on hepatic mast cells: Mast cell counts were significantly increased in both CCl_4 treated group and ACP treated group as compared to respective normal groups. All the treatments, except *C. ternatea* root extract in ACP - intoxicated rats, significantly decreased liver mast cell counts (Table 8).

Effects on barbiturate-induced sleeping time: Barbiturates are metabolized extensively in liver by microsomal enzymes. Hepatic microsomal enzyme inhibitors are known to potentiate barbiturate-induced sleeping time. Hence, to evaluate hepatic microsomal enzyme inhibition as a protective mechanism, we studied effects of various treatments on phenobarbitone-induced sleeping time. The phenobarbitone-induced sleeping time was significantly increased by *V. mungo* seeds extract as compared to control group. The *C. ternatea* seeds extract significantly reduced the phenobarbitone-induced sleeping time (Table 9).

Table 9: Effect on phenobarbitone-induced sleeping time

Groups (n = 10)	Treatments	Induction time (min)	Sleeping time (min)
Control	Vehicle + Phenobarbitone (80 mg kg^{-1}, i.p.)	50.6±1.69	78.6±2.71
CT seed extract	*C. ternatea* seeds extract (500 mg kg^{-1}, p.o.) + Phenobarbital (80 mg kg^{-1}, i.p.)	51.2±2.21	45.3±1.78†
CT root extract	*C. ternatea* Roots extract (500 mg kg^{-1}, p.o.) + Phenobarbitone (80 mg kg^{-1}, i.p.)	54.5±1.99	79.7±4.08
VM seed extract	*V.* seeds extract (500 mg kg^{-1}, p.o.) + Phenobarbitone (80 mg kg^{-1}, i.p.)	25.3±3.91*	307.0±4.18*

All values represent Mean±SEM of 10 rats. Statistical analysis was carried out using one way ANOVA followed by Tukey's multiple range. $p<0.05$ was considered statistically significant, *: significant when compared with control group, †: significant decrease in sleeping time when compared with control group. CT: *Clitoria ternatea* and VM: *Vigna mungo*

Histopathological study: The liver section of animals treated with ACP showed a high degree of hepatic damage characterized by cell vacuolation, pyknotic and degenerated nuclei and wall of bile capillaries (Fig. 1B). In the liver sections of animals treated with hydroalcoholic extracts of *C. ternatea* seeds (Fig. 1D) and hydroalcoholic extract of *V. mungo* seeds (Fig. 1F), the nuclei were not very clear as in normal hepatocytes (Fig. 1A); however when compared with ACP treated group, the number of hepatocytes with normal nucleus was more in numbers and there was less damage observed to the cell membrane and the wall of the bile capillaries. The recovery was comparable to that with silymarin, a standard hepatoprotective agent (Fig. 1C). In the liver section of the animals treated with hydroalcoholic extract of *C. ternatea* roots (Fig. 1E); no improvement was observed in the architecture of the hepatocytes when compared with the ACP treated group.

A comparison of the liver sections of animals treated with CCl_4 showed a high degree of damage characterized by cell vacuolation, pyknotic and degenerated nuclei and nearly complete loss of hepatocytes architecture. The normal architecture of the liver was lost. Also the intra-lobular vein was badly damaged with wide spaces at some sinusoids. In the liver section of the animals treated with hydroalcoholic extracts of *C. ternatea* seeds (Fig. 2D), roots (Fig. 2E) and *V. mungo* seeds (Fig. 2F), the nuclei were not very clear as in normal hepatocytes; however, when compared with the CCl_4 - intoxicated group, the numbers of hepatocytes with normal nucleus were much more in numbers. The endothelium was also found disrupted in places. Pyknotic nucleus and vacuolation in cytoplasm were observed to be low, as compared to the CCl_4 group (Fig. 2B). The recovery was comparable to that with silymarin, a standard hepatoprotective agent (Fig. 2C).

DISCUSSIONS

In the present study, the hepatoprotective effects of *C. ternatea* seed and root extracts and *V. mungo* were evaluated against paracetamol and CCl_4-induced liver injuries in rats (Fig. 3, 4). The liver functioning was tested measuring serum SGOT, SGPT and ALP levels. The effects on lipid peroxidation and anti-oxidant enzymes and GSH were studied. The mast cell infiltration, hepatic collagen synthesis and microsomal enzymes inhibition were determined.

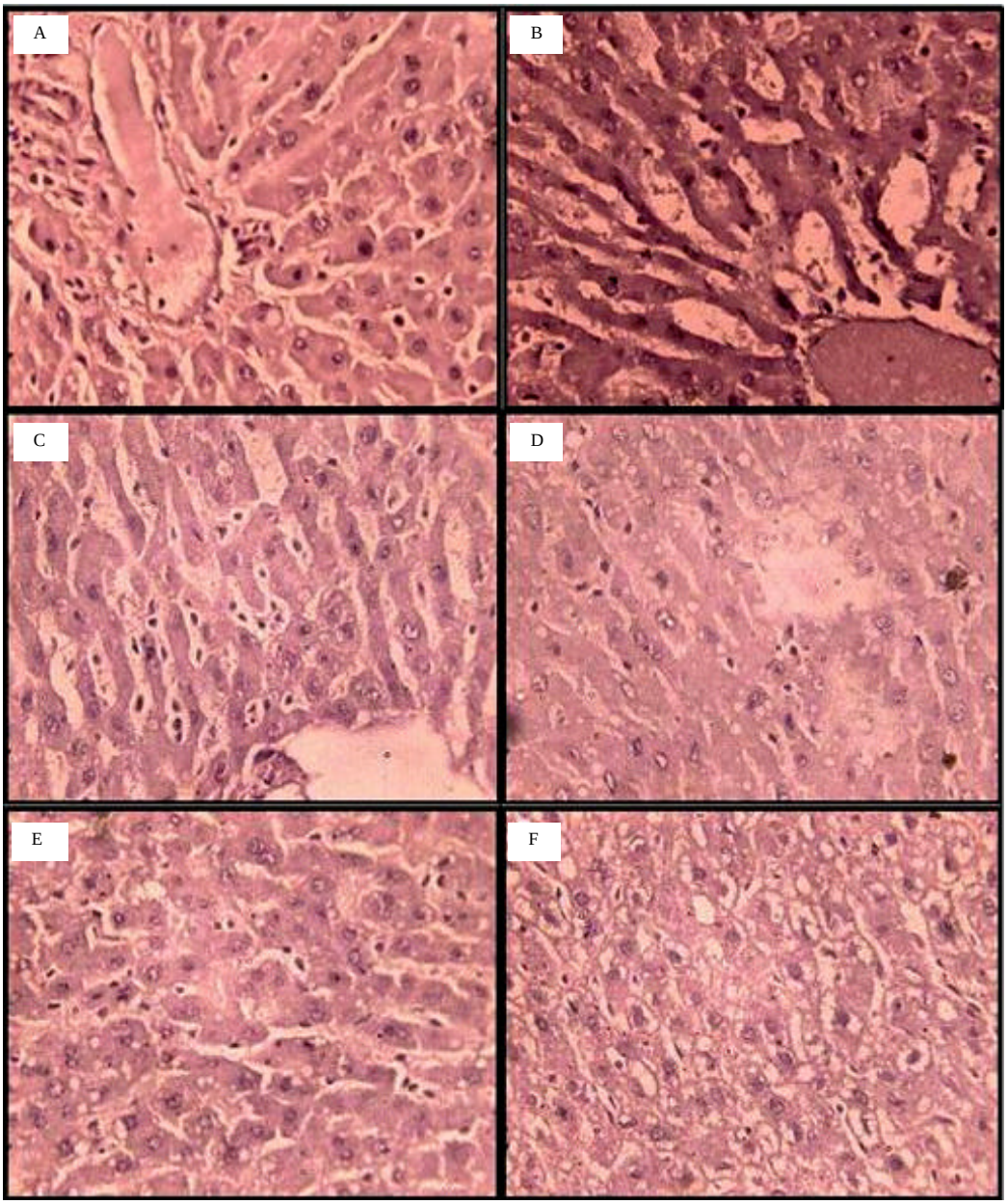

Fig. 1: Histopathological studies of liver tissues of acetaminophen - intoxicated rats. Photographs showing histopathology of liver sections (5-10 μ), 40X, Haematoxylin-eosin stain. Liver tissue of, A) Control rat showing normal histology, B) Acetaminophen treated rat showing cellular degeneration, hydropic changes, fatty changes with wide spread hepatocellular necrosis, C) Silymarin (25 mg kg^{-1}) treated rat showing very little necrosis or degeneration, D) *C. ternatea* seeds extract (500 mg kg^{-1}) treated showing no hepatocellular damage except areas of focal degeneration and sinusoidal dilation, E) *C. ternatea* roots extract (500 mg kg^{-1}) treated showing hepatocellular damage, fatty changes and loss of liver architecture and F) *V. mungo* seeds extract (500 mg kg^{-1}) treated showing minimal hepatic necrosis, cellular degeneration, hydropic changes and fatty changes

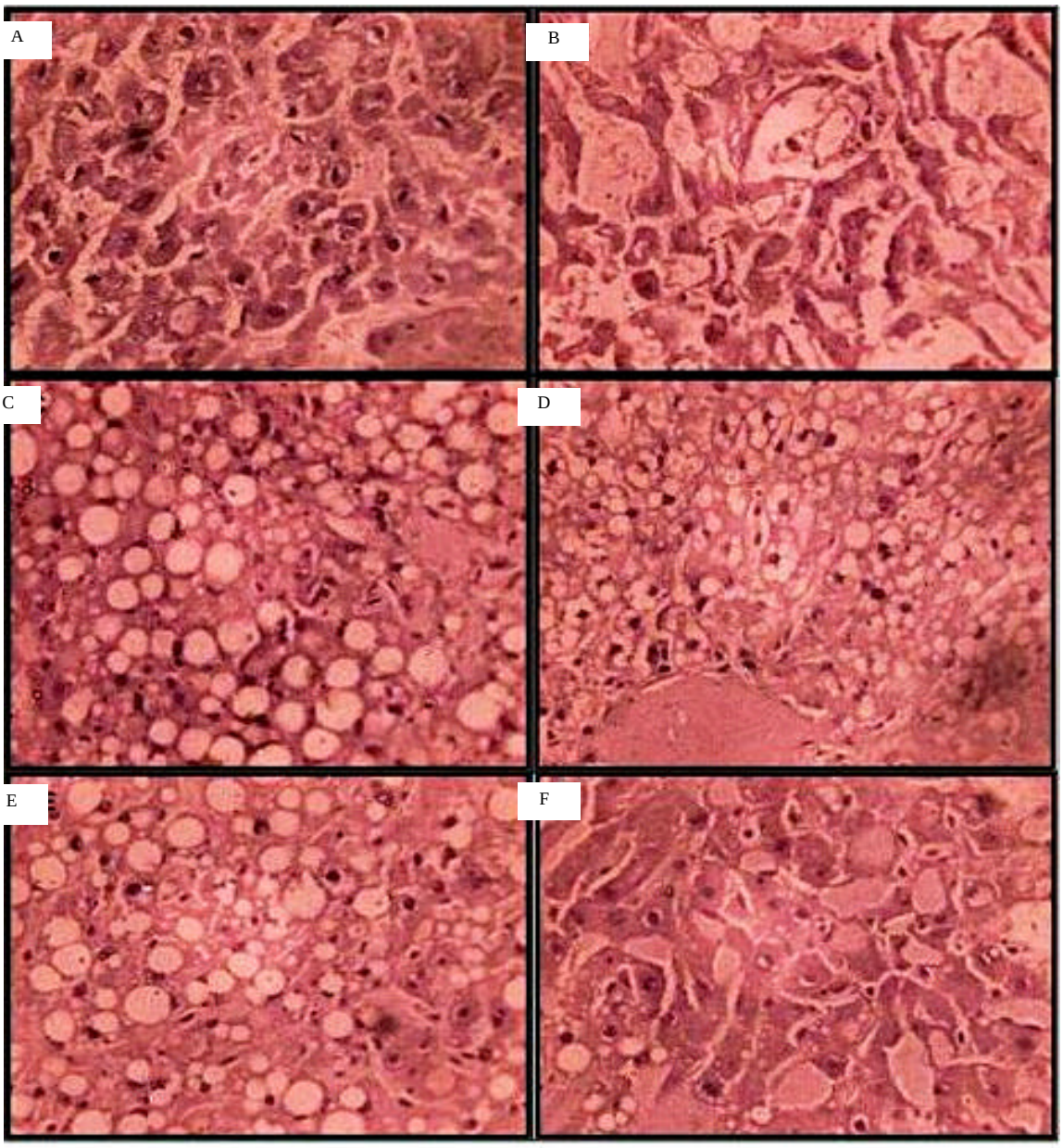

Fig. 2: Histopathological studies of liver tissues of CCl_4 - intoxicated rats. Photographs showing histopathology of liver sections (5-10 μ), 40X, Haematoxylin-eosin stain. Liver tissue of, A) Control rat showing normal histology, B) Carbon tetrachloride treated rat showing cellular degeneration, hydropic changes, fatty changes with wide spread hepatocellular necrosis, C) Silymarin (25 mg kg^{-1}) treated rat showing very little necrosis or degeneration, D) *C. ternatea* seeds extract (500 mg kg^{-1}) treated showing no hepatocellular damage except areas of focal degeneration and sinusoidal dilation, E) *C. ternatea* roots extract (500 mg kg^{-1}) treated showing less hepatocellular damage and F) *V. mungo* seeds extract (500 mg kg^{-1}) treated showing minimal hepatic necrosis, cellular degeneration, hydropic changes and fatty changes

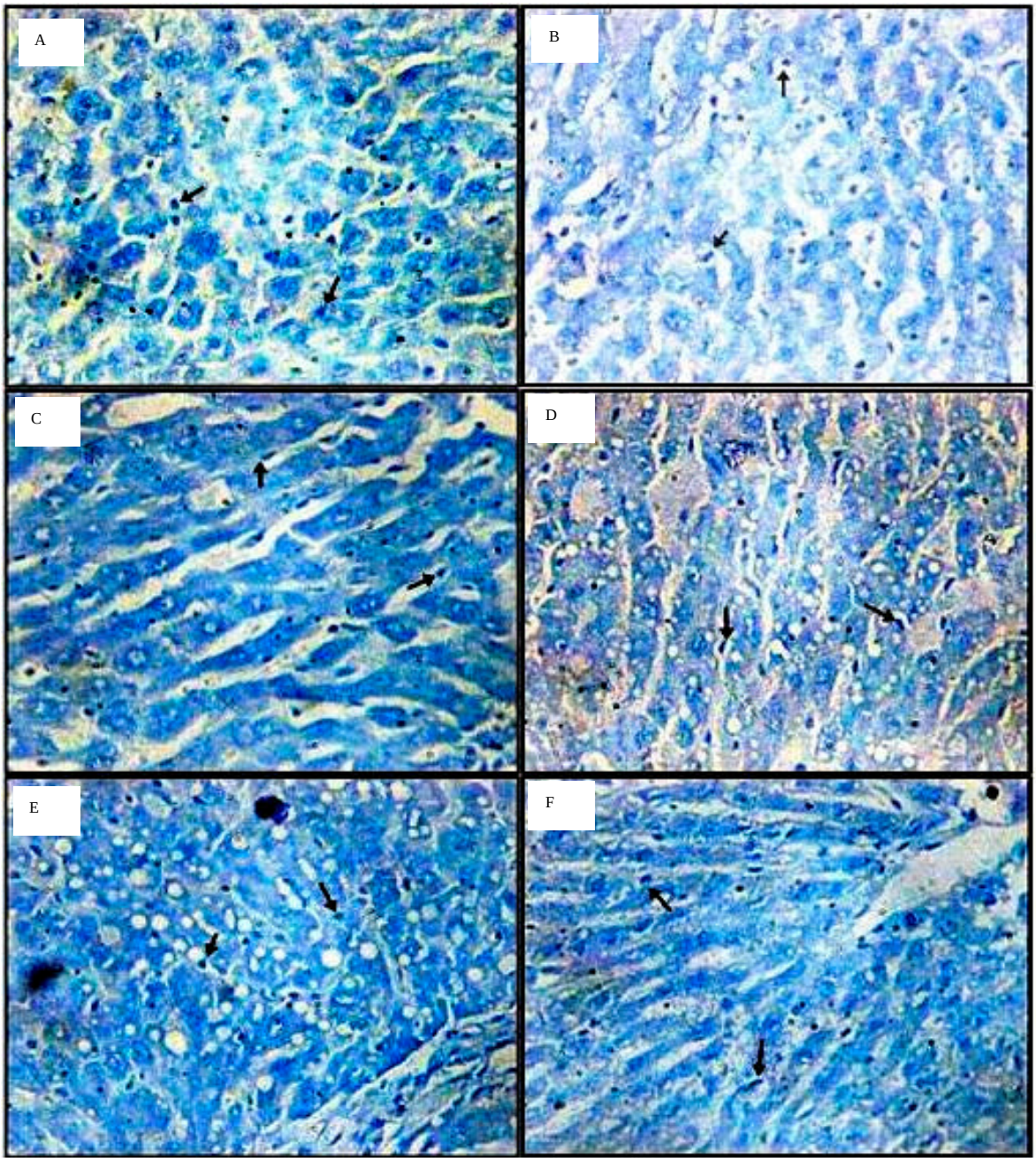

Fig. 3: Effects on mast cell infiltration in liver tissues of acetaminophen - intoxicated rats. Photographs showing histopathology of liver sections (5-10 μ), 40X, Toluidine blue stain. Liver tissues of A) control rats showing normal hepatocytes with mast cells stained blue, indicated with arrow, B) acetaminophen treated rat showing numerous mast cells, C) silymarin (25 mg kg^{-1}) treated rats showing mast cells similar to that of normal, D) *C. ternatea* seed extract (500 mg kg^{-1}) treated showing relatively less mast cells, E) *C. ternatea* root extract (500 mg kg^{-1}) treated showing mast cells similar to acetaminophen treated and F) *V. mungo* seeds extract (500 mg kg^{-1}) treated showing less mast cells similar to silymarin

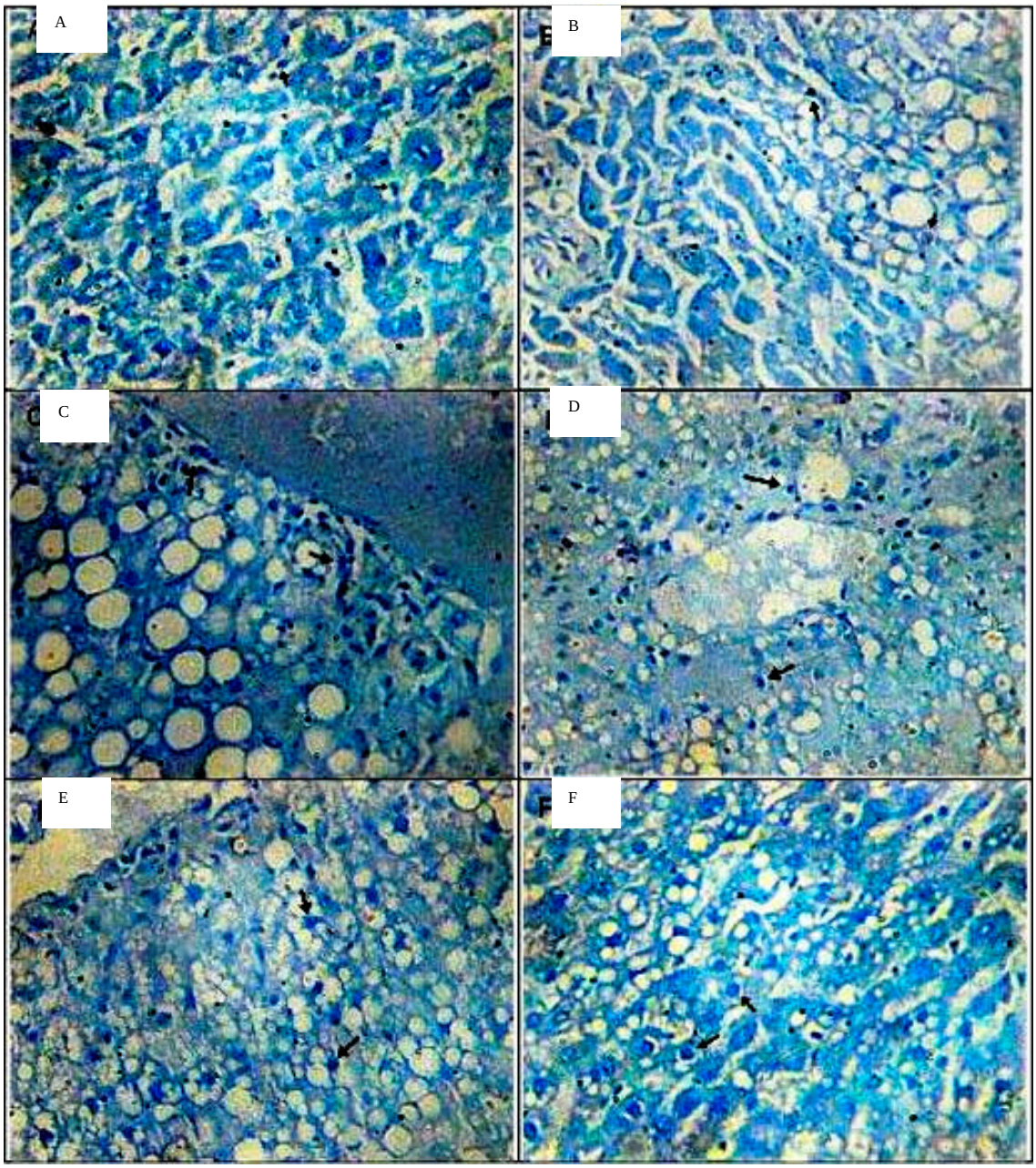

Fig. 4: Effects on mast cell infiltration in liver tissues of CCl_4 - intoxicated rats. Photographs showing histopathology of liver sections (5-10 μ), 40X, Toluidine blue stain. Liver tissues of A) control rats showing normal hepatocytes with mast cells stained blue, indicated with arrow, B) Carbon tetrachloride treated rat showing numerous mast cells in hepatic matrix, C) silymarin (25 mg kg^{-1}) treated rats showing mast cells similar to that of normal, D) *C. ternatea* seed extract (500 mg kg^{-1}) treated showing relatively less mast cells, E) *C. ternatea* root extract (500 mg kg^{-1}) treated showing mast cells similar to acetaminophen treated and F) *V. mungo* seeds extract (500 mg kg^{-1}) treated showing less mast cells similar to silymarin

Paracetamol at therapeutic doses is primarily metabolized and detoxified by glucuronidation and sulphation and subsequently followed by renal excretion. However, when paracetamol is taken in a toxic dose, the compound is converted to a toxic form N-acetyl-p-benzo-quinone imine (NAPQI), which is an electrophilic intermediate, oxidized by Cytochrome P_{450} and converted to a highly reactive and toxic metabolite as in the case of paracetamol over dose (Dahlin *et al*., 1984; Gupta and Misra, 2006). NAPQI can rapidly react with the glutathione (GSH) and lead to a 90% total hepatic GSH depletion in the cells and mitochondria, which can result in hepatocellular death and mitochondrial dysfunction (Mitchell *et al*., 1973). In addition, NAPQI can increase the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxide anion, hydroxyl radical and hydrogen peroxide and nitro oxide and peroxynitrite, respectively. Excess levels of ROS and RNS can attack biological molecules such as DNA, protein and phospholipids, which lead to lipid peroxidation, nitration of tyrosine and depletion of anti oxidant enzymes (SOD, CAT, GPx) that further results in oxidative stress. NAPQI can also induce DNA stand breaks and promote apoptosis and necrosis in paracetamol-induced hepatotoxicity (Bergman *et al*., 1996).

Carbon tetrachloride (CCl_4) is a well known xenobiotic producing hepatotoxicity in various experimental animals (Rudnick *et al*., 2007). Biotransformation of CCl_4 by cytochrome P450 produces hepatotoxic metabolites-trichloromethyl free radicals (CCl3• and/or CCl3OO•) (Brattin *et al*., 1985). Covalent binding of trichloromethyl free radicals to cell proteins is considered to be the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell apoptosis and necrosis (Weber *et al*., 2003). Recently it was reported that immune cells are activated by hepatotoxins including paracetamol and CCl_4, leading to infiltration in damaged liver tissues where they secrete various cytokines (Ramaiah and Rittling, 2007). These cytokines trigger hepatotoxicity via a Fas/ FasL pathway in CCl_4 -induced liver toxicity in rats.

The membrane lipid peroxidation by CCl_4metabolites lead to impairment of membrane functions and integrity resulting in release of cytosolic contents into the serum. The magnitude of hepatic damage is assessed by measuring the levels of released cytosolic contents viz. transaminases like SGOT and SGPT, in circulation (Agarwal *et al*., 2006). SGOT is relatively non-specific and elevated during hepatic, cardiac and muscle injuries. SGPT is more selectively a liver parenchymal enzyme than SGOT and is a better parameter for detecting liver injury (Arnaiz *et al*., 1995). The elevated levels of these serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. In the present study, the significant increase in the serum levels of SGOT, SGPT and ALP in Paracetamol and CCl_4 - intoxicated rats, could be attributed to cytosolic leakage and damaged structural integrity of hepatocytes, because these are cytoplasmic in location and are released in to circulation after cellular damage. The results of the present study demonstrated that pretreatment of rats with *C. ternatea* seed extract effectively protected against paracetamol and CCl_4 - induced hepatotoxicity. However, root extract significantly protected against CCl_4 -induced liver toxicity only. This fact was further confirmed by histological observation. Present results showed that *C. ternatea* seed and root extracts dramatically prevented the CCl_4 -induced morphological changes in the liver sections.

Lipid peroxidation by free radical derivatives of CCl_4 is one of the principle mechanisms of CCl_4 -induced liver injury. Increased lipid peroxidation is generally believed to be an important underlying cause of the initiation of oxidative stress related various tissue injuries, cell death and

progression of many acute and chronic diseases. MDA is a major reactive aldehyde that appears during the peroxidation of polyunsaturated fatty acids of biological membrane. Increase in MDA levels in the liver suggests enhanced peroxidation leading to tissue damage and failure of the antioxidant defense mechanisms to prevent the formation of excessive free radicals. In the present study, paracetamol and CCl_4 -induced liver toxicity caused an increase in the MDA levels in liver tissues as compared to normal control group. Treatments with *C. ternatea* seed, root and *V. mungo* seed extracts significantly reversed these changes and decreased MDA levels.

Several studies have demonstrated that anti-oxidant enzymes such as SOD and catalase represent protection against oxidative tissue damage (Wang *et al*., 2004). SOD is an exceedingly effective defense enzyme that converted the dismutation of superoxide anions into hydrogen peroxide (H_2O_2) (Reiter *et al*., 2000). Catalase is a haemoprotein in all aerobic cells that metabolize H_2O_2 to oxygen and water. GSH acts as a non-enzymatic anti-oxidant that reduces H_2O_2, hydroperoxides (ROOH) and xenobiotic toxicity. The GSH is readily oxidized to glutathione disulphide (GSSG) by any of the selenium-containing glutathione peroxidase isozymes, as well as the reaction with ROOH or xenobiotic compounds that may subsequently causes the reduction of GSH levels. The GSSG is either rapidly reduced by glutathione reductase and NADPH or utilized in the protein folding process in the endoplasmic reticulum, where it is recycled by protein disulfide isomerase to form GSH. Because of these recycling mechanisms, GSH is an extremely efficient intracellular buffer for oxidative stress (Cantin *et al*., 2007). In the present study, paracetamol and CCl_4 produced significant increase in SOD and catalase activity indicating the oxidative stress condition during paracetamol and CCl_4 intoxication. Hydroalcoholic extracts of *C. ternatea* seed and root and *V. mungo* seed produced significant decrease in SOD and catalase activities in CCl_4- intoxicated rats. However, the root extracts did not affect MDA and catalase activity in paracetamol - intoxicated rats. The GSH levels were also significantly increased by all the extracts in paracetamol and CCl_4 - intoxicated rats. A significantly higher content of GSH in liver would afford the tissue a better protection against an oxidative stress, thus contributing to the abolishment of hepatotoxicity. There is report that *Azadirecta indiaca* leaf extract showed hepatoprotective effect against paracetamol-induced hepatic damage in rats that is attributed to the presence of quercetin and rutin compounds of plant-showing antioxidant activity (Chattopadhyay and Bandyopadhyay, 2005). Our results are parallel to this report.

Bilirubin is one of the most useful clinical clues to the severity of hepatic necrosis and its accumulation is a measure of binding, conjugation and excretory capacity of hepatocytes. Hepatotoxins like ACP were reported to decrease hepatic conjugation of bilirubin (Davis *et al*., 1976). The present study showed that hydroalcoholic extracts of *C. ternatea* seed and root and *V. mungo* seed significantly decreased both direct and indirect bilirubin levels in serum suggesting their effects on the functional integrity of the hepatocytes.

Creatinine levels are indicative of renal functioning and are increased in severe renal damage. The CCl_4 was found to produce renal toxicity (Ogeturk *et al*., 2005). Hence, in the present study we investigated the effects of CCl_4 on kidney functioning. All the treatments did not alter creatinine levels in both the models of hepatotoxicity. The finding of the study indicated that the selected dose levels of hepatotoxins did not produce renal damage.

Hepatotoxins like paracetamol and CCl_4 have been reported to activate immune cells especially mast cells, leading to their infiltration into the damaged liver where they secretes various cytokines

like TNF-α, leucotrienes and interleukins (Ramaiah and Rittling, 2007). The number of mast cells is reported to increase in chronic liver diseases associated with fibrosis. In the present study we investigated the hepatic mast cell infiltration in paracetamol and CCl_4 - intoxicated rats. The pretreatment with *C. ternatea* seed and root extracts significantly prevented hepatic mast cell infiltration. A well known hepatoprotective drug silymarin has been reported to inhibit mast cell infiltration (Jeong *et al*., 2005). Thus, the inhibition of mast cell infiltration or mast cell stabilization could be one of possible mechanisms attributed to protective effects of *C. ternatea* and *V. mungo* against paracetamol and CCl_4 - intoxicated rats.

Cirrhotic changes are the hallmark of hepatic damage. Fibrosis is found to be associated with many toxin -induced liver injury including paracetamol and CCl_4. In the present study we estimated hepatic fibrosis in terms of collagen content measured as P-hydroxyproline levels. The hydroalcoholic extracts of *C. ternatea* seed and root and *V. mungo* seed significantly decreased hepatic hydroxyproline content in CCl_4 - intoxicated rats. The plants from the same family leguminosae like mung bean, adzuki bean, black bean and rice bean were reported to decrease hepatic collagen content (Wu *et al*., 2001).

Barbiturates are the class of xenobiotics that are extensively metabolized in the liver and their clearance is delayed in case of damaged liver functioning. Hepatic microsomal enzyme inhibitors also delay their clearance and potentiate barbiturate-induced sleeping time. Hence, to investigate hepatic microsomal enzyme inhibition as possible mechanism of hepatoprotective effect, we studied effects of various extracts on phenobarbitone induced sleeping time. The *C. ternatea* seed extract significantly decreased phenobarbitone induced sleeping time instead of potentiation. This can be attributed to brain tonic activity of *C. ternatea* seeds (Rai *et al*., 2005). The findings suggested that protective effects of *C. ternatea* seed and roots could be because of effects other than hepatic microsomal enzyme inhibition. *V. mungo* showed significant potentiation of sleeping time, suggesting that the protective effect of *V. mungo* seed extract is mediated through hepatic microsomal enzymes inhibition.

CONCLUSION

In the light of overall finding, we conclude that *C. ternatea* and *V. mungo* possess significant hepatoprotective effects against both ACP- and CCl_4-induced hepatotoxicity in rats. The *C. ternatea* roots did not produce hepatoprotective effect in ACP - intoxicated rats. The hepatoprotective activity of *C. ternatea* could be attributed to inhibition of lipid peroxidation, antioxidant action, suppression of mast cell infiltration, down regulation of pro-inflammatory pathway and that of *V. mungo* can be attributed further to both hepatic microsomal enzyme inhibition and antioxidant activities. Further studies are required to find out active phytochemicals and their detailed mechanisms of hepatoprotective effect. The present study provided evidence for the traditional use of these plants in traditional Indian system of medicine.

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