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# Hepatoprotective activity of *Piper longum* traditional milk extract on carbon tetrachloride induced liver toxicity in Wistar rats

[Actividad hepatoprotectora del extracto tradicional lácteo de *Piper longum* en ratas Wistar]

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## Abstract

*Piper longum* Linn. (Piperaceae) (fruits and roots powder) is given with boiled milk in the Indian traditional system of medicine for the treatment of liver ailments and jaundice. However, the biochemical basis and mechanism of hepatoprotective action of *Piper longum* milk extract is not scientifically studied. Thus, the present study was designed to investigate the hepatoprotective activity of *Piper longum* milk extract. Carbon tetrachloride ($CCl_4$) was used as a hepatotoxin at a dose of 0.5 ml/kg p.o. with olive oil (1:1) thrice a week for 21 days to produce the chronic reversible type of liver necrosis. Following treatment with *Piper longum* milk extract (200 mg/day p.o. for 21 days), a significant hepatoprotective effect was observed in $CCl_4$ induced hepatic damage as evident from decreased level of serum enzymes, total bilirubin and direct bilirubin. The hepatoprotective effect of *Piper longum* is comparable to the standard drug silymarin (25 mg/kg/day p.o. for 21 days).

**Keywords:** *Antioxidant enzymes; carbon tetrachloride; hepatoprotective activity;* Piper longum.

## Resumen

En el sistema de medicina tradicional india, *Piper longum* Linn. (Piperaceae) (frutos y polvo de la raíz) es dado con leche hervida para el tratamiento de ictericia y dolencias del hígado. Sin embargo, las bases bioquímicas y el mecanismo de acción hepatoprotectora del extracto lácteo de *Piper longum* no se ha estudiado científicamente. El presente estudio fue designado para investigar la actividad hepatoprotectora del extracto lácteo de *Piper longum*. El tetracloruro de carcono ($CCl_4$) se usó como hepatoxina a una dosis de 0.5 ml/kg p.o. en aceite de oliva (1:1) tres veces a la semana por 21 días para producir la necrosis hepática crónica reversible. El tratamiento con el extracto lácteo de *Piper longum* (200 mg/día p.o. por 21 días) tuvo un efecto hepatoprotector significativo evidenciado por el decrecimiento en suero de enzimas, bilirrubina directa y bilirrubina total. El efecto hepatoprotector de *Piper longum* es comparable al fármaco de referencia silimarina (25 mg/kg/día p.o. por 21 días).

**Palabras Clave:** Cassia angustifolia; Senna alexandrina; *sen; laxantes naturales; antranoides; Fitoterapia*.

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## INTRODUCTION

Liver is a versatile organ of the body that regulates internal chemical environment. Liver injury induced by various hepatotoxins has been recognized as a major toxicological problem for years. Because of its unique metabolic functions and relationship to the gastrointestinal tract, liver is an important target of toxicity to xenobiotics, oxidative stress, ethanol and toxic chemicals (antibiotics, chemotherapeutics, aflatoxins, carbon tetrachloride, chlorinated hydrocarbons, etc.). There are numerous plants and polyherbal formulations claimed to have hepato-protective activity. Nearly 150 phytoconstituents from 101 plants have been claimed to possess liver protecting activity. (Subramonium and Pushpangadan, 1999) *Piper longum* Linn. (Piperaceae), is a well known traditional medicine, promotes physical and mental health and also improves defense mechanisms of body. Nearly two-thirds of all traditional ayurvedic formulas contain a special blend of ingredients, which includes *Long pepper (Piper longum)* for this purpose. (Kirtikar and Basu, 1980). It is advised in Ayurveda that the fruits of this plant should be extracted as a milk decoction. The milk extract is reported to be nearly 27 times more active than the aqueous extract (Shankar et al., 2007, Sudha et al., 2004). Ethanolic extract of the fruits of *Long pepper* (*Piper longum*) was used for its hepatoprotective activity (Jalalpure et al., 2003). Also the main active constituent of *Piper longum*, Piperine is reported to have hepatoprotective property (Koul and Kapil, 1993, Khare, 2004). However, no systematic attempts have been made to establish the scientific basis of these beneficial effects of *Piper longum* milk extract. Hence, the aim of the present study was to investigate the hepatoprotective activity of *Piper longum* milk extract on carbon tetrachloride induced liver toxicity in rats.

## MATERIALS AND METHODS

### Animals

Healthy untreated wistar rats of either sex weighing 200-300 g were selected. The animals were housed 4 per cage, under standard environmental conditions like controlled light and dark cycle every 12 hours, temperature 22 ± 2 ˚C, 60-70% relative humidity. They were fed with standard rodent diet and water ad libitum. The animals were acclimatized for 5 days before starting experiment. All the experiments were conducted in accordance with the CPCSEA guidelines (Rule 5 (a) of the "Breeding of and Experiments on animals, Rules 1998", Government of India).

### Plant material

Authenticated crude dried powder of *Piper longum linn.* was collected from Ms. Lallu Vrajlal Gandhi Ayurvedic store, Ahmedabad. The drug was authenticated by Prof. Vimalkumar, Head of Pharmacognosy Division. Voucher Specimen (PL-02/05-06) has been retained in the Institute of Pharmacy, Nirma University, India. It was mainly the dried powder of fruits and roots part of the herb. The powder was stored in polyethylene bags at room temperature until needed.

### Extraction of plant material

Powdered drug was mixed with milk which was diluted with equal volume of water and then boiled to reduce the volume to half of the original. *Piper longum* (200 mg/kg p.o. for 21 days) was administered as milk extract along with the undissolved particles present in the vehicle. The extract was prepared fresh everyday for the entire treatment period of 21 days.

### Experimental procedure

In the preliminary toxicity study, none of the animals showed any signs of toxicity up to 2 g/kg, p.o. dose and hence $1/10^{th}$ of the maximum dose administered (i.e. 200 mg/kg, p.o.) was selected for the present study.

The rats were divided into five groups of 7 animals each. Group I served as control 1 group and received only boiled milk (0.25 ml/kg/day, p.o. for 21 days; milk was diluted with equal volume of water and then boiled to reduce the volume to half of the original). Group II rats served as control 2 group and only olive oil (Vehicle for $CCL_4$) was given (0.05 ml/kg/day, p.o.) for 21 days. Group III rats received $CCL_4$ (0.05 ml/kg p.o. thrice a week) in olive oil (5:5) for 21 days. Group IV animals received $CCl_4$ (as in Group III) and standard reference drug silymarin (25 mg/kg/day, p.o.) for 21 days. Group V animals received $CCl_4$ (as in Group III) and P*iper longum* milk extract (200 mg/kg/day, p.o.) for 21 days.

After the completion of treatment period, blood samples were collected from the retro-orbital plexus under light ether anesthesia in anticoagulant free vials. 15 min later, samples were centrifuged at 3500-4000 rpm for 20 minutes to separate serum which was used for various liver function tests. All the animals were sacrificed under light ether anesthesia and livers were

quickly excised, rinsed in cold saline, blotted, weighed and immediately stored at -2 to -8 ˚C and used for various biochemical estimations (like Serum SGOT, SGPT, ALP, total and direct bilirubin levels, antioxidant parameters, etc.). A portion of liver was stored separately in formalin buffer after washing with ice-cold saline for histopathology studies.

### Statistical analysis

Results are presented as mean ± standard error of mean. Statistical difference between the means of various groups were analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A value of $P<0.05$ was used as the criterion of significance. The calculations were made with computer-assisted analysis using the SPSS software.

## RESULTS

Administration of $CCL_4$ produced significant hepatotoxicity in experimental animals, as is evident by an elevation of the serum marker enzymes namely SGOT, SGPT and ALP in $CCL_4$ treated rats. Treatment of the animals with either boiled milk or olive oil, alone, did not influence either of the serum marker enzymes and hence acted as respective control groups (refer Figs. 1-3).

### Effect on serum Enzymes

### Effect on serum glutamate pyruvate transaminase (SGPT) levels

As shown in Fig. 1A, $CCl_4$ administered rats (0.5 ml/kg p.o. thrice a week for 21 days) showed significant increase in SGPT levels (159.30 U/L ± 6.86) when compared to both - control 1 (71.73 U/L ± 4.69) and control 2 animals (69.67 U/L ± 4.25), respectively. Administration of *Piper longum* milk extract (200 mg/kg/day p.o. for 21 days) significantly decreased SGPT levels (78.86 U/L ± 2.43), which were comparable with the standard drug silymarin (25 mg/kg/day p.o. for 21 days) (70.19 U/L ± 2.09).

### Effect on serum glutamate oxaloacetate transaminase (SGOT) levels

As shown in Fig. 1B, $CCl_4$ administered rats (0.5 ml/kg p.o. thrice a week for 21 days) showed significant increase in SGOT levels (104.09 U/L ± 10.42) when compared to both, control 1 (52.68 U/L ± 3.21) and control 2 animals (50.58 U/L ± 1.72), respectively. Administration of *Piper longum* milk extract (200 mg/kg/day p.o. for 21 days) significantly decrease SGOT levels (67.89 U/L ± 3.69), which were comparable to silymarin (25 mg/kg/day p.o. for 21 days) (61.29 U/L ± 6.89).

### Effect on serum alkaline phosphatase (ALP) levels

Fig. 1C shows that $CCl_4$ administered rats (0.5 ml/kg p.o. thrice a week for 21 days), exhibited significant increase in alkaline phosphatase levels (393.30 U/L ± 3.08) when compared to control 1 (163.0 U/L ± 4.32) and control 2 rats (157.25 U/L ± 2.11), respectively. Administration of *Piper longum* milk extract (200 mg/kg/day p.o. for 21 days) significantly decreased alkaline phosphatase levels (192.77 U/L ± 1.36) which were comparable with silymarin (25 mg/kg/day p.o. for 21 days) (170.58 U/L ± 1.16).

### Effect on serum total bilirubin and direct bilirubin levels

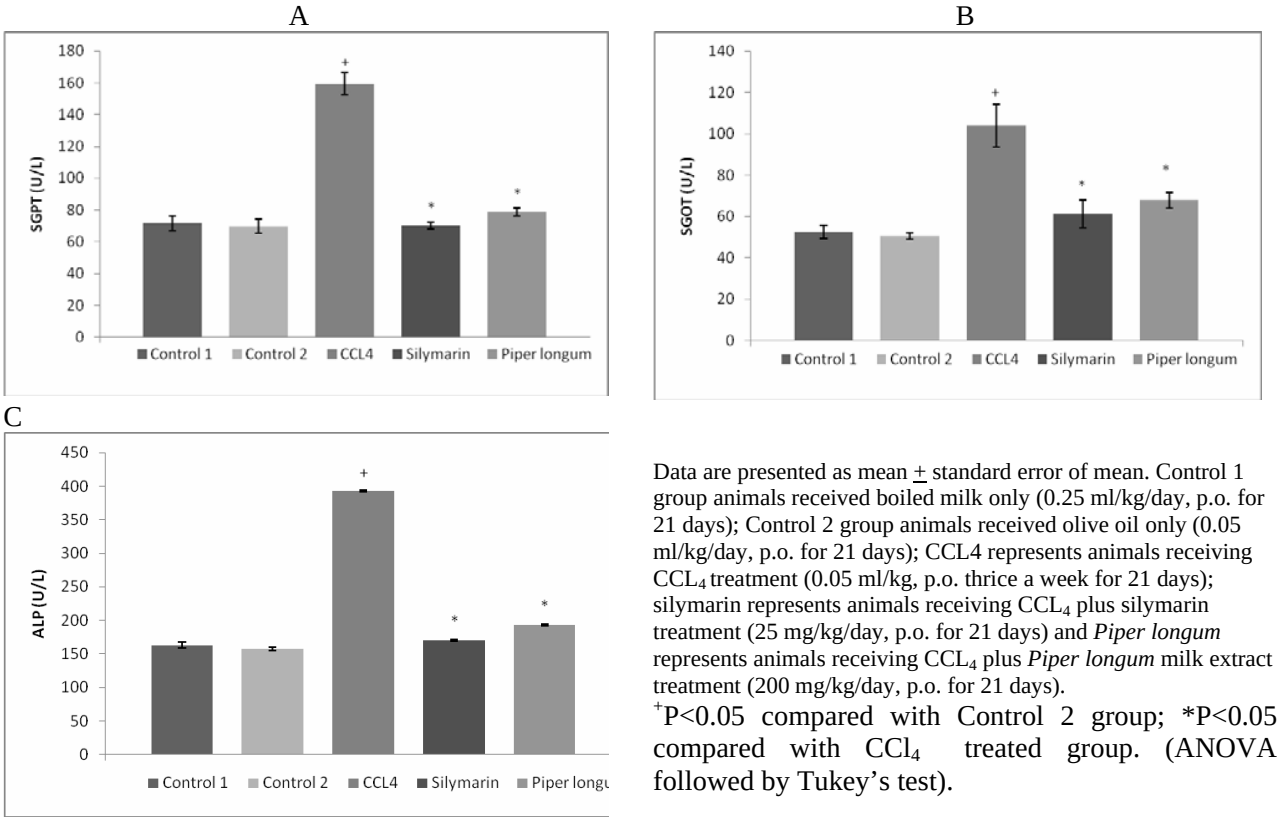
### Effect on serum total bilirubin levels

Fig. 2A exhibits that $CCl_4$ administered rats (0.5 ml/kg p.o. thrice a week for 21 days) significantly elevated serum total bilirubin levels (3.27 mg/dl ± 0.15) when compared to control 1 rats (0.61 mg/dl ± 0.02) and control 2 rats (0.47 mg/dl ± 0.02) respectively. *Piper longum* milk extract (200 mg/kg/day p.o. for 21 days) significantly reduced total bilirubin levels (0.93 mg/dl ± 0.02) as compared to $CCl_4$ treated animals. Silymarin (25 mg/kg/day p.o. for 21 days) also significantly reduced total bilirubin levels (0.65 mg/dl ± 0.02).

### Effect on serum direct bilirubin levels

In Fig. 2B can be observed that $CCl_4$ administered rats (0.5 ml/kg p.o. thrice a week for 21 days) significantly elevated serum direct bilirubin levels (0.854 mg/dl ± 0.02) as compared to control 1 group (0.41 mg/dl ± 0.01) and control 2 rats (0.32 mg/dl ± 0.02), respectively. *Piper longum* milk extract (200 mg/kg/day p.o. for 21 days) significantly reduced direct bilirubin levels (0.60 mg/dl ± 0.01) as compared to $CCl_4$ treated group. Silymarin (25 mg/kg/day p.o. for 21 days) also significantly reduced direct bilirubin levels (0.50 mg/dl ± 0.02).

**Figure1**. Effects of *Piper longum* milk extract on serum SGPT levels (A), serum SGOT levels (B); and serum alkaline phosphatase levels (C).




Data are presented as mean ± standard error of mean. Control 1 group animals received boiled milk only (0.25 ml/kg/day, p.o. for 21 days); Control 2 group animals received olive oil only (0.05 ml/kg/day, p.o. for 21 days); CCL4 represents animals receiving $CCL_4$ treatment (0.05 ml/kg, p.o. thrice a week for 21 days); silymarin represents animals receiving $CCL_4$ plus silymarin treatment (25 mg/kg/day, p.o. for 21 days) and *Piper longum* represents animals receiving $CCL_4$ plus *Piper longum* milk extract treatment (200 mg/kg/day, p.o. for 21 days).

$^{+}P<0.05$ compared with Control 2 group; $*P<0.05$ compared with $CCl_4$ treated group. (ANOVA followed by Tukey's test).

**Effect on antioxidant enzyme activity in liver**

**Effect on malondialdehyde contents in liver**

Fig. 3A shows taht $CCl_4$ significantly increased malondialdehyde (MDA) levels in liver (42.47 µM/mg of protein ± 0.57) compared to control 1 group (14.78 M/mg of protein ± 0.51) and control 2 group (13.60 µM/mg of protein ± 0.2), respectively. *Piper longum* milk extract significantly reduced the levels of MDA (22.47 µM/mg of protein ± 0.98). Silymarin also reduced MDA levels (16.76 µM/mg of protein ± 0.46).

**Effect on superoxide dismustase levels in liver**

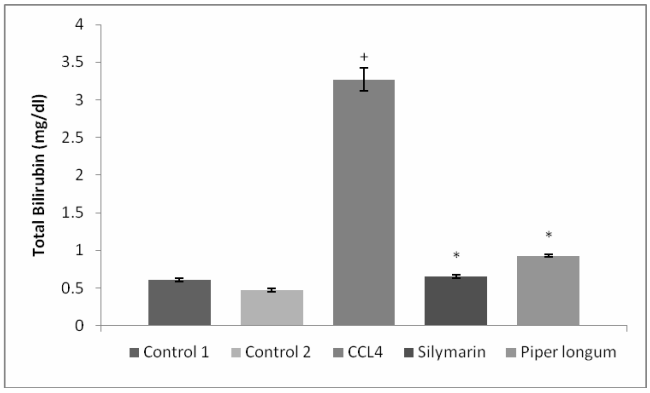
In Fig. 3C is observed that $CCl_4$ significantly reduced superoxide dismustase (SOD) levels (0.08 Units/mg of protein ± 0.02) in liver compared to control 1 group (0.24 units/mg of protein ± 0.01) and control 2 group (0.25 Units/mg of protein ± 0.01) respectively. *Piper longum* milk extract increased SOD levels (0.16 Units/mg of protein ± 0.01) as compared to $CCl_4$ treated group. Silymarin also increased SOD levels significantly (0.20 Units/mg of protein ± 0.01).

**Effect on catalase levels in liver**

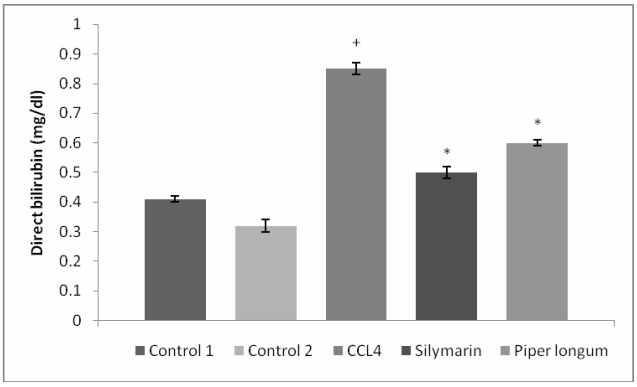
According with Fig. 3C, $CCl_4$ also reduced catalase levels (2.31 Units/min/mg of protein ± 0.11) as compared to control 1 group (7.88 Units/min/mg of protein ± 0.36) and control 2 group (8.55 Units/min/mg of protein ± 0.15) respectively. *Piper longum* milk extract and silymarin increased catalase levels significantly (3.86 Units/min/mg of protein ± 0.47) and (4.54 Units/min/mg of protein ± 0.20), respectively.

**Figure 2**. Effects of *Piper longum* milk extract on serum total bilirubin levels (A) and serum direct bilirubin levels (B).

A



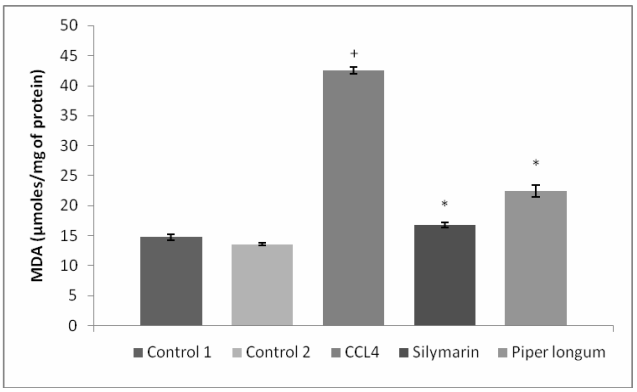

B



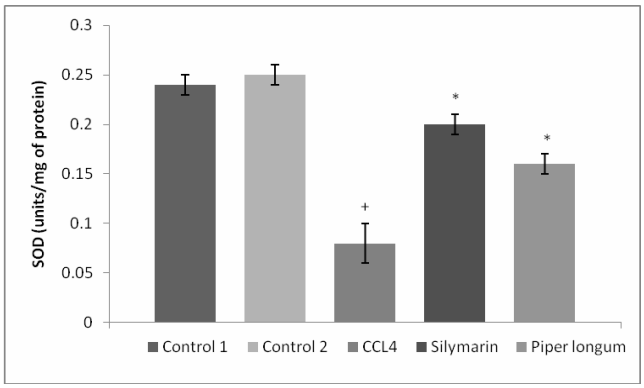

Data are presented as mean ± standard error of mean. Control 1 group animals received boiled milk only (0.25 ml/kg/day, p.o. for 21 days); Control 2 group animals received olive oil only (0.05 ml/kg/day, p.o. for 21 days); CCL4 represents animals receiving $CCL_4$ treatment (0.05 ml/kg, p.o. thrice a week for 21 days); silymarin represents animals receiving $CCL_4$ plus silymarin treatment (25 mg/kg/day, p.o. for 21 days) and *Piper longum* represents animals receiving $CCL_4$ plus *Piper longum* milk extract treatment (200 mg/kg/day, p.o. for 21 days).
$^+$ P<0.05 compared with Control 2 group; *P<0.05 compared with $CCl_4$ treated group (ANOVA followed by Tukey's test).

**Figure 3**. Effects of *Piper longum* milk extract on malondialdehyde contents in liver (A), superoxide dismustase levels in liver (B), and catalase levels in liver (C)

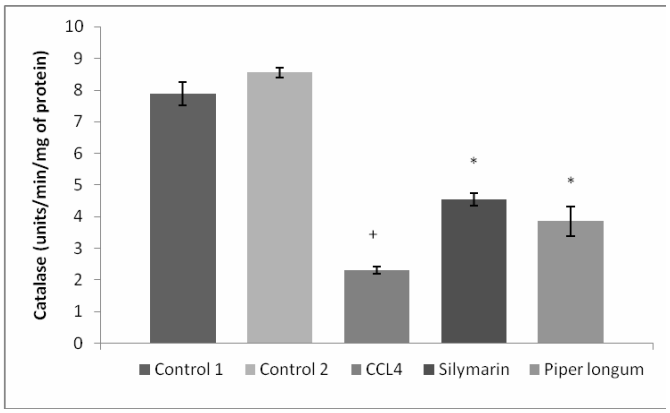
A




B




C




Data are presented as mean ± standard error of mean. Control 1 group animals received boiled milk only (0.25 ml/kg/day, p.o. for 21 days); Control 2 group animals received olive oil only (0.05 ml/kg/day, p.o. for 21 days); CCL4 represents animals receiving $CCL_4$ treatment (0.05 ml/kg, p.o. thrice a week for 21days); silymarin represents animals receiving $CCL_4$ plus silymarin treatment (25 mg/kg/day, p.o. for 21 days) and *Piper longum* represents animals receiving $CCL_4$ plus *Piper longum* milk extract treatment (200 mg/kg/day, p.o. for 21 days).
$^+$ P<0.05 compared with Control 2 group; *P<0.05 compared with $CCl_4$ treated group. (ANOVA followed by Tukey's test).

**Figure 4.** Microarchitecture of liver sections of control 1 rats (only boiled milk treated; 0.25 ml/kg/day, p.o. for 21 days). It shows central vein and normal parenchymal cells. Photographs to 10X.

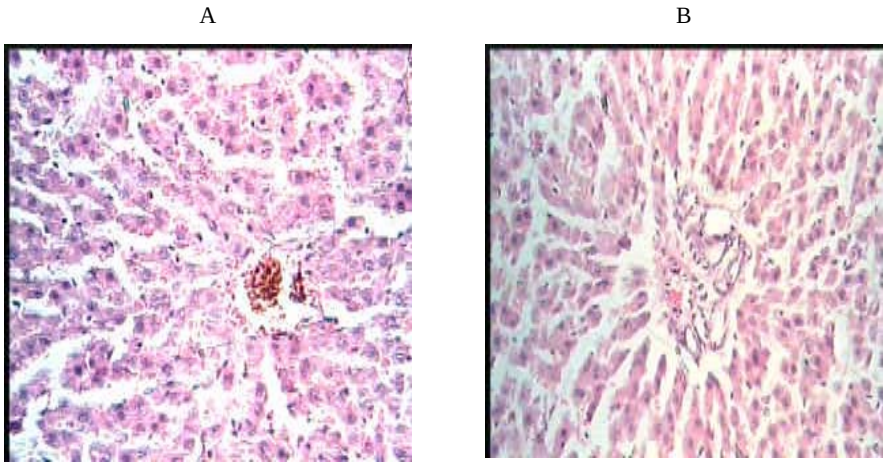



**Figure 5.** Microarchitecture of liver sections of control 2 (only olive oil treated; 0.05 ml/kg/day, p.o. for 21 days) rats. It shows central vein and normal parenchymal cells. Photographs to 40X (A) and 10X (B)

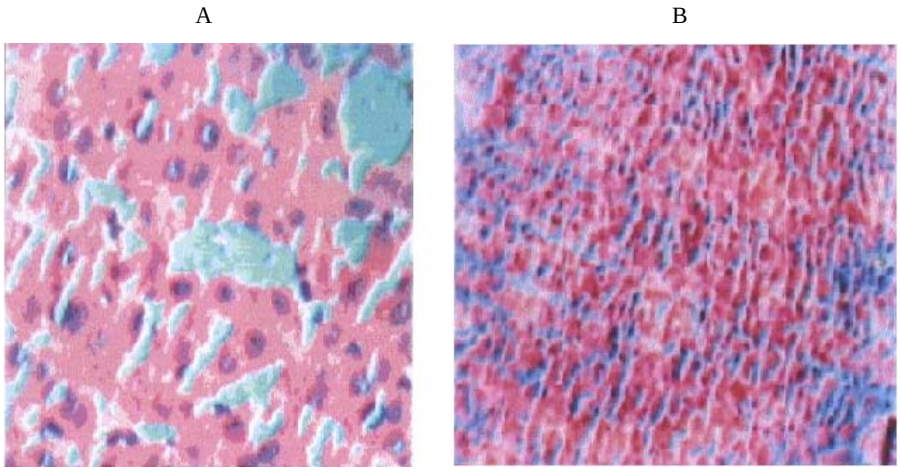



**Figure 6**. Microarchitecture of liver sections of CCl4 treated (0.05 ml/kg, p.o. thrice a week for 21 days) rats. It shows development of large septa of connective tissue flowing together and penetrating into parenchyma, resulting into extensive necrosis. Photographs to 10X.

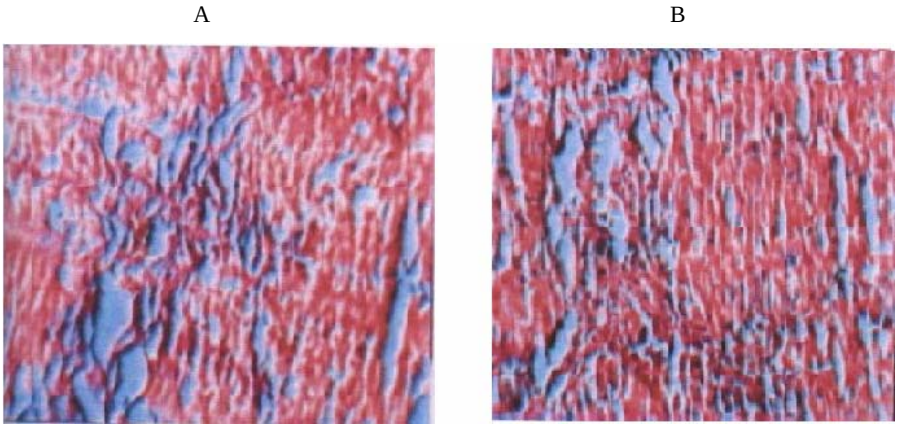


**Figure 7**. Microarchitecture of liver sections of silymarin treated (25 mg/kg/day, p.o. for 21 days) rats. It shows more number of regenerating liver cells around the necrotic area. Photographs to 40X.

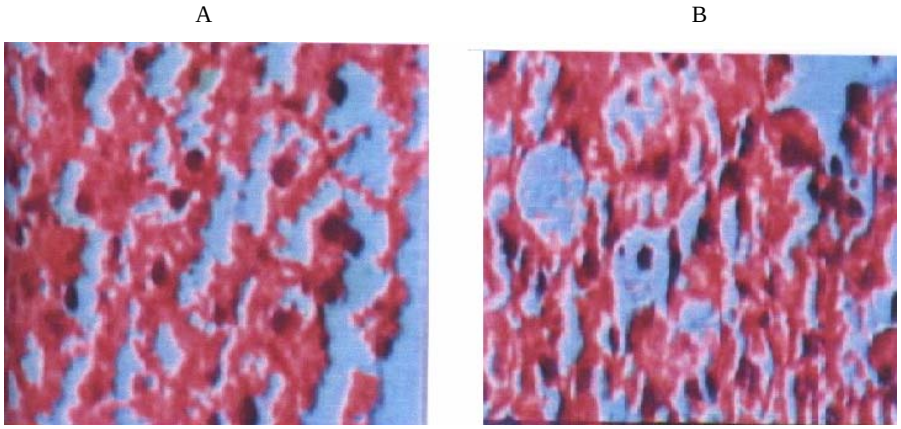
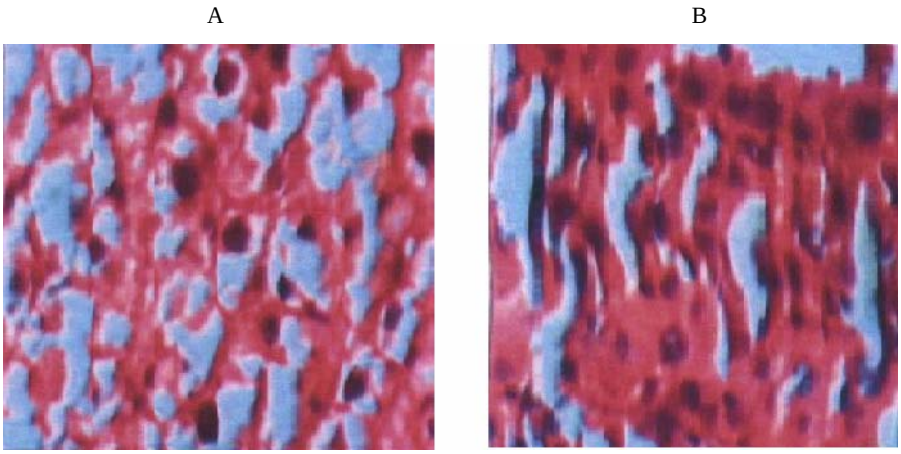



**Figure 8**. Microarchitecture of liver sections of *Piper longum* treated (200 mg/kg/day, p.o. for 21 days) rats. It shows regenerating liver cells around the necrotic area. Photographs to 40X.




### Histopathological studies in liver

Histopathological findings indicated that administration of *piper longum* milk extract offered protection to the hepatocytes from damage induced by $CCL_4$, with mild fatty changes in the hepatic parenchymal cells, which corroborated the changes observed in the hepatic enzymes. It also showed regenerating liver cells around the necrotic area (Figs. 4-8).

## DISCUSSION

Oral administration of $CCl_4$ is one of the easiest, fastest and reliable techniques to develop liver toxicity and can be used to screen hepatoprotective agents. $CCl_4$ gets accumulated in the hepatocytes, where it is activated by oxidases that are involved in the chemolytic breakage of C-Cl bond. This activation occurs in the hepatic endoplasmic reticulum via an enzyme system of electron transport from reduced nicotinamide adenine dinucleotide phosphate to oxygen. $CCl_4$ is activated by cytochrome CYP2E1, CYP2B1, or CYP2B2 and possibly CYP3A to form highly reactive and toxic metabolite trichloromethyl radical. The free radicals locally cause auto-oxidation of the polyenic fatty acids present within the membrane phospholipids. So, organic peroxides are formed after reacting with oxygen (lipid peroxidation). This reaction is autocatalytic in nature so that new radicals are formed from the peroxide radicals themselves. Thus, rapid breakdown of the structure and function of the endoplasmic reticulum is due to the decomposition of lipids. Within less than 30 minutes, there is decline in hepatic protein synthesis and within 2 h, there is swelling of smooth endoplasmic reticulum and dissociation of ribosomes from the rough

endoplasmic reticulum. (Farber et al., 1971). Lipid export from the hepatocytes is reduced owing to their inability to synthesize apoprotein to complex with triglycerides and thereby facilitate lipoprotein secretion. This results in a fatty liver due to $CCl_4$ poisoning. Mitochondrial injury then occurs and this is followed by progressive swelling of the cells due to increased permeability of the plasma membrane. Plasma membrane damage is thought to be caused by relatively stable fatty aldehydes, which are produced by lipid peroxidation in the smooth endoplasmic reticulum but are able to act at distant sites. This is followed by massive influx of calcium and cell death (Robbins, 2003). The lipid peroxide causes breakdown of the biomembranes at cellular and subcellular levels. As a consequence, the microsomal enzyme activities are found to be decreased and water soluble enzymes leak into the plasma from liver. SGPT is thought to be one of the indices of the degree of cell membrane damage while SGOT is an indicator for mitochondrial damage since mitochondria contains 80% of the enzyme (Dabba and Abdel-Rahman 1998). The increased activity of the liver marker enzymes such as SGPT, SGOT and ALP, as shown in Fig. 1 in the serum of $CCl_4$ induced rats indicate damage to hepatic cells. Both *Piper longum* and silymarin treated rats possess significantly lower SGPT, SGOT and ALP levels as shown in Fig. 1 as compared to $CCl_4$ treated animals. So we can speculate that the protective effect is on both mitochondria and hepatocytes. The normalized levels of the enzymes SGPT, SGOT and ALP after the treatment with *Piper longum* in $CCl_4$ intoxicated rats demonstrated its hepatoprotective action.

Increase in serum bilirubin levels may be found in hepatocellular damage, hemolytic jaundice or hepatitis. $CCl_4$ injury causes significant degeneration of hepatocytes and blockade of the bile ducts which results into significant increase in the serum total bilirubin and direct bilirubin levels (Saraswat et al., 1993). An increase in the levels of serum bilirubin reflected the depth of jaundice caused by $CCl_4$ intoxication as shown in Fig. 2. Pretreatment with *Piper longum* milk extract normalized the elevated total bilirubin and direct bilirubin levels.

The cellular infiltration of activated neutrophilic leukocytes amplifies inflammatory response and cellular injury or depth due to release of superoxide anions and other toxic mediators (Comportti, 1985). The antioxidant enzymes SOD, catalase and peroxidases constitute a mutually supportive team of defense against reactive oxygen species (Bandhopadhay et al., 1999). A decrease in activity of SOD in the liver of $CCl_4$ treated rats in our study might be due to the increased lipid peroxidation or inactivation of the enzyme by cross linking with malondialdehyde (Tabatabaie and Floyd, 1994). This may cause an increased accumulation of superoxide radicals, which could further stimulate lipid per-oxidation. In our study, elevation in the levels of end products of lipid peroxidation in $CCl_4$ treated animals was observed. The increase in MDA levels, decrease in catalase levels and superoxide dismustase levels in liver as seen in Fig. 3, following $CCl_4$ treatment indicates the damage and failure of antioxidant defense mechanism. Treatment with *Piper longum* and silymarin significantly prevented these changes.

Hepatotoxins develop hypoxic conditions which can damage the perivenular zone of the hepatic acinus. The highest expression of cytochrome CYP2E1 in the perivenular region produces oxy-radicals that contribute to the injury. Moreover, hepatocytes in the perivenular area contain less antioxidant factors and enzymes (Kera et al., 1987). Thus, while the lipid peroxidation mediated by oxy-radicals is likely to be the highest in the perivenular area, the detoxifying capacity of the hepatocytes here is reduced, therefore, the production may exceed the detoxification of the perivenular area (Rajesh and Latha 2004). Histopathological studies, as can be seen in Figs. 4 and 5 showed that control 1 and 2 animal groups had normal central vein and liver parenchymal cells. As shown in Fig. 6, the $CCl_4$ intoxicated animals show extensive necrosis, inflammation and infiltration by lymphocytes. In the *Piper longum* treated group (Fig. 8) the areas of regeneration are seen around the necrotic focus. There is more amount of regeneration with mild inflammation and some lymphocytic infiltration in the necrotic area. Standard drug silymarin treated rats showed more number of regenerating liver cells around the necrotic area as shown in Fig. 7. Thus, the histopathological studies substantiated the hepatoprotective effects observed in the biochemical studies and also pinpointed that the beneficial effects are comparable to that of the standard drug silymarin.

## CONCLUSION

The results from the present study demonstrated a significant hepatoprotective and antioxidant activity of *Piper longum* milk extract. Our findings support the reported therapeutic use of this herb in tribal medicine

for liver ailments and jaundice. Further pharmacological investigations are underway to identify the specific constituents of the plant extract responsible for these activities.

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