Original article:

HEPATOPROTECTIVE ACTIVITY OF CINNAMON ETHANOLIC EXTRACT AGAINST CCL4-INDUCED LIVER INJURY IN RATS

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ABSTRACT

The inner bark of cinnamon (*Cinnamomum zeylanicum* L.) is commonly used as a spice and has also been widely employed in the treatment and prevention of disease. The aim of the present study is to evaluate the protective effect of cinnamon bark extract against carbon tetrachloride (CCl_4)-induced liver damage in male Wistar rats. Administration with cinnamon extracts (0.01, 0.05 and 0.1 g/kg) for 28 days significantly reduced the impact of CCl_4 toxicity on the serum markers of liver damage, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase. In addition, treatment of cinnamon extract resulted in markedly increased the levels of superoxide dismutase and catalase enzymes in rats. The histopathological studies in the liver of rats also supported that cinnamon extract markedly reduced the toxicity of CCl_4 and preserved the histoarchitecture of the liver tissue to near normal. Thus, the results suggest that cinnamon extract acts as a potent hepatoprotective agent against CCl_4 induced hepatotoxicity in rats.

Keywords: Carbon tetrachloride, *Cinnamomum zeylanicum* L., cinnamon, hepatoprotective, rat

INTRODUCTION

The liver as a vital organ in the body is primarily responsible for the metabolism of endogenous and exogenous agents. It plays an important role in drug elimination and detoxification and liver damage may be caused by xenobiotics, alcohol consumption, malnutrition, infection, anaemia and medications (Mroueh et al., 2004). Hepatotoxicity is defined as injury to the liver that is associated with impaired liver function caused by exposure to a drug or another non-infectious agent (Navarro and Senior, 2006). Hepatotoxic agents can react with the basic cellular components and consequently induce almost all types of liver lesions. Toxins and drugs are among the basic etiopathogenetic agents of acute liver failure in Western countries (Grattagliano et al., 2009). Nevertheless, chemical toxins (including acetaminophen, carbon tetrachloride, galactosamine and thioacetamide) are often used as the model substances causing experimental hepatocyte injury in both in vivo and in vitro conditions (Domenicali et al., 2009; Kucera et al., 2006; Ledda-Columbano et al., 1991; Rousar et al., 2009). Despite the fact that hepatic problems are responsible for a significant number of liver transplantations and deaths recorded worldwide, available pharmacotherapeutic options for liver diseases are very

limited and there is a great demand for the development of new effective drugs. A number of studies have shown that the plant extracts having antioxidant activity protect against CCl_4 hepatotoxicity by inhibiting lipid peroxidation and enhancing antioxidant enzyme activity (Shahjahan et al., 2004; Sheweita et al., 2001).

Cinnamon (*Cinnamomum zeylanicum* L., Lauraceae) is a tropical evergreen tree and grows wild in Sri Lanka, Madagascar, India and Indochina. The inner bark of the tree has been used in ethno-medicine and flavoring for foods (Bakkali et al., 2008; Baytop, 1999). In addition to its culinary uses, cinnamon has been employed in traditional herbal medicine to treat a variety of health conditions (Gruenwald et al., 2010). Some studies showed that extracts and its constituents from cinnamon also posses antimicrobial (Carmo et al., 2008; Chao et al., 2000; Dusan et al., 2006; Ranasinghe et al., 2002; Shahverdi et al., 2007), insecticidal (Yang et al. 2005), acaricidal (Fichi et al., 2007), antityrosinase (Marongiu et al., 2007), antioxidant and antimutagenic (Jayaprakasha et al., 2007) activities. In addition, other evidence suggests that cinnamon may be effective in the treatment of cancer (Hyeon et al., 2003; Nishida et al., 2003) and infectious diseases (Hayashi et al., 2007; Premanathan et al., 2000), and that it also shows anti-inflammatory (Hong et al., 2002; Tung et al., 2008), antioxidant (Su et al., 2007; Murcia et al., 2004; Okawa et al., 2001), hypotensive (Preuss et al., 2006), and cholesterol-lowering effects (Khan et al., 2003; Subash Babu et al., 2007). The present study investigates the hepatoprotective potential of cinnamon ethanolic extract treatment against CCl_4-induced liver toxicity in rats.

MATERIALS AND METHODS

Chemicals

CCl_4 was obtained from Merck, Germany. Assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, superoxide dismutase (SOD) and catalase (CAT) were purchased from Parsazmoon Company of Iran. All other reagents used in the experiment were of analytical grade.

Preparation of the plant powder and extraction

The plant materials were obtained from the local market. Shade dried cinnamon bark was milled and extracted using ethanol 80 % in Soxhlet apparatus for 8 h. Then, the extract was evaporated to dryness and the final dry extract was stored in dark at -20 °C until used for the experiments. The percentage yield of extract was 17.7 % (w/w) of the initial raw material.

Phytochemical analysis

The qualitative phytochemical analysis of the crude extract of *Cinnamomum zeylanicum* L. bark was carried out to determine the active phytochemical constituents which were responsible for the hepatoprotective activity. Some of these methods were as follows:

20 mg extract was dissolved in 10 ml ethanol and filtered. 0.5 ml conc. HCl and magnesium ribbon were added to 2 ml filtrate. Development of pink-tomato red color indicated the presence of flavonoids (Parekh et al., 2006). 20 mg extract was dissolved in 2 ml distilled water and filtered. 2 ml $FeCl_3$ was added to the filtrate, blue-black precipitate indicated the presence of tannins (Parekh et al., 2006). To 0.5 ml of the filtrate obtained in alkaloids test 5 ml distilled water was added. Frothing persistence indicated the presence of saponins (Parekh et al., 2006). 20 mg extract was dissolved in 2 ml distilled water and filtered. To the filtrate, 2-4 drops of 1 % HCl was added and steam was passed through it. To the 1 ml of this solution 6 drops of Wagner's reagent was added. Brownish-red precipitate indicated the presence of alkaloids (Finar, 2003). Salkovski test was performed using a small amount of extract solution. To this solution 5 drops of conc. H_2SO_4 and 1 ml chloroform were added. Change of yellow color into red indicated the presence of terpenoids (Finar, 2003). A

small amount of material was extracted in ethanol and evaporated to dryness. Residue was dissolved in distilled water and 0.5 ml Folin-ciocalteau reagent was added followed by 2 ml 20 % Na_2CO_3 solution. Development of bluish color indicated the presence of phenols (Sadasivam and Manickam, 1996).

Animals

Adult male Wistar rats weighing 200-230 g were used in the study. The animals were maintained in an air conditioned animal house at a temperature of 22 ± 2 °C, relative humidity of 57 ± 2 % and photocycle of 12:12 h light and dark. The animals were provided with standardized pelleted feed and drinking water ad libitum. All the experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Ethics Committee. The animals were observed daily for any signs of toxicity. Body weight was recorded at regular intervals throughout the experimental period.

Experimental groupings

After acclimatization a period of one week, the animals were randomly divided into seven groups each consisting of nine rats. Group I served as the normal control and was given olive oil (i.p. 0.5 ml/kg b.w.) as vehicle twice a week intraperitoneally for a period of four weeks. Group II served as the cinnamon extract control and was given cinnamon extract dissolved in distilled water orally at a dose level of 0.05 g/kg b.w. daily for a period of four weeks. For inducing hepatotoxicity, animals of Groups III, IV, V, VI, and VII were intraperitoneally administered 0.5 ml/kg body weight of carbon tetrachloride (50 % CCl_4 in olive oil) twice a week for a period of four weeks. After CCl_4 intoxication, Group III served as the CCl_4 control. Groups IV-VII were orally administered cinnamon extract dissolved in distilled water at doses of 0.005, 0.01, 0.05 and 0.1 g/kg, respectively, daily for a period of four weeks.

Biochemical evaluation

At the end of the treatment period all animals were fasted for 12 h, then blood samples were collected by cardiac puncture using sterile disposable syringes under diethyl ether anesthesia. Sera were separated out by centrifugation at 3000 rpm for 15 min and stored at -20 °C until analysis. After the collections of blood samples, animals were killed and samples of the liver of each animal were dissected, weighed and homogenized for the determination of SOD and CAT activities. Liver damage was assessed by the estimation of serum activities of ALT, AST, and ALP using commercially available test kits. The results were expressed as units/liter (IU/L). In addition, the serum levels of total protein were estimated in the experimental animals by using commercial kit.

Measurement of SOD and CAT in liver homogenate

Liver homogenates were prepared in cold Tris-HCl (5 mmol/L, containing 2 mmol/L EDTA, pH 7.4) using a homogenizer. The unbroken cells and cell debris were removed by centrifugation at 10,000 g for 10 min at 4 °C. The activities of SOD (Kakkar et al., 1984) and CAT (Sinha, 1972) were assayed in the liver. SOD was assayed by the inhibition of the formation of NADH-phenazine methosulphate nitro-blue tetrazolium formazan which was measured at 520 nm. One unit of SOD activity is defined as the enzyme concentration required inhibiting the chromogen production by 50 % in 1 min under the assay conditions. CAT activity was determined by measuring the amount of hydrogen peroxide (H_2O_2) consumed in the reaction. The remaining H_2O_2 was reacted with dichromate-acetic acid reagent and monitored spectrophotometrically at 590 nm. Activity was expressed in µmol H_2O_2 consumed/ min/mg protein.

Histopathological techniques

A fresh piece of the liver (right distal lobe) from each rat, previously trimmed to

approximately 2 mm thickness, was rapidly fixed in 10 % neutral formalin. The fixed tissues were then embedded in paraffin, sectioned (5 µm) with a rotary microtome and stained with haematoxylin and eosine (H&E). The liver sections were evaluated histologically with a camera attached to a light microscope (Nikon E400). The extent of CCl_4-induced liver damage was evaluated based on pathologic lesions in liver sections stained with H&E method. Score system was used for histopathological examinations. Hepatocyte necrosis, fatty change, hyaline degeneration, ballooning degeneration, and infiltration of inflammatory cells were prominent in the histological findings (Valeer, 2003). The liver pathology was scored as described by French et al. 2000 as follows:

Score 0 = no visible cell damage
Score 1 = focal hepatocyte damage on less than 25 % of the tissue
Score 2 = focal hepatocyte damage on 25-50 % of the tissue
Score 3 = extensive, but focal, hepatocyte lesions
Score 4 = global hepatocyte necrosis.

The morphology of any lesions observed was classified and registered (Gray, 1958).

Statistical analysis

Statistical analysis was performed using the SPSS for Windows statistical package, version 10.0 (SPSS Inc. Chicago, IL, USA). Data were expressed as means ± S.E.M. The effects of drug treatments were evaluated statistically using the one-way analysis of variance (one-way ANOVA) followed by the Dunnett post-hoc test to correct for multiple comparison treatments. Statistical significance was set at the $p < 0.05$ level.

RESULTS

Phytochemical analysis

Phytochemical analysis of *Cinnamomum zeylanicum* L. bark extract demonstrates the presence of flavonoids, tannins, saponins, alkaloids, terpenoids and phenols.

Body weight and weight of liver

No animals died during CCl_4 administration period. The administration of CCl_4 caused a significant decrease in the body weight of rats as compared with the control rats (Table 1). The animals co-treated with cinnamon extract (0.01, 0.05 and 0.1 g/kg b.w.) for four weeks also gained weight during the experimental period. Liver weights and liver index (the ratio of liver weight to body weight) were higher in CCl_4-treated animals than in control animals. Co-treatment with cinnamon extract (0.01, 0.05 and 0.1 g/kg b.w.) resulted in both liver weights and liver index that were significantly reduced compared to those of CCl_4-treated rats. There was no significant alteration in control rats treated solely with cinnamon extract (0.05 g/kg b.w.).

Biochemical analysis

CCl_4 is activated by phase-II detoxifying enzymes in liver cell endoplasmic reticulum to form trichloromethyl and peroxytrichloromethyl free radicals. These can react covalently with several biomolecules such as protein, nucleic acid and lipid, resulting in cellular membrane degeneration, increased permeability, and leakage of cytoplasmic ALT, AST and ALP. Serum levels of ALT, AST and ALP should serve as hepatotoxicity indexes. Indeed, CCl_4 administration produced significant elevations of serum ALT and AST compared to the normal control group. However, pretreatment of rats with 0.01, 0.05 and 0.1 g/kg b.w. cinnamon extract significantly decreased these serum biochemical indices as compared with the CCl_4 treatment group. Hepatic SOD and CAT activities in the CCl_4 treatment group were reduced compared to the normal control group. These antioxidant enzyme activities were all statistically significantly greater in the group treated with cinnamon extract (0.01, 0.05 and 0.1 g/kg b.w.) compared with the CCl_4 treatment group. There was no significant alteration in control rats treated solely with cinnamon extract (Table 2).

Table 1: Body weight, liver weight and liver index of acute CCl_4 (50% CCl_4/olive oil)-treated rats with or without cinnamon ethanolic extract

Parameters	Control	Cinnamon extract (0.05 g/kg b.w.)	CCl_4	CCl_4 + Cinnamon extract (g/kg b.w.)			
				0.005	0.01	0.05	0.1
Initial body weight (g)	224 ± 10.4	216 ± 9.8	223 ± 13.2	219 ± 12.5	221 ± 11.2	218 ± 8.9	220 ± 11.4
Final body weight (g)	256 ± 15.3	249 ± 17.2	226 ± 10.5 +++	232 ± 8.5	239 ± 13.6	240 ± 17.4 *	245 ± 15.2 **
Weight gain (g)	32 ± 4.9	33 ± 3.7	3 ± 0.7 +++	13 ± 3.4	18 ± 6.3 *	22 ± 5.9 *	25 ± 4.1 **
Liver weight (g)	5.3 ± 0.34	5.2 ± 0.41	8.5 ± 1.2 +++	6.9 ± 0.64	6.5 ± 0.71 **	6.3 ± 0.57 **	5.9 ± 0.83 ***
Liver index	2.07 ± 0.04	2.09 ± 0.03	3.76 ± 0.07 +++	2.97 ± 0.05	2.72 ± 0.04 **	2.63 ± 0.03 **	2.41 ±0.03 ***

Values are mean ± S.E.M. (n = 9 rats per each group). The liver index was calculated as liver weight / body weight × 100, * $p <0.05$, ** $p<0.01$, ***, $p<0.001$ significantly different from the group treated with CCl_4. +++. $p<0.001$ significantly different from the control

Table 2: Effects of cinnamon ethanolic extract on serum and liver biochemical indices in CCl_4-induced hepatotoxicity in rats

Parameters	Control	Cinnamon extract (0.05 g/kg b.w.)	CCl_4	CCl_4 + Cinnamon extract (g/kg b.w.)			
				0.005	0.01	0.05	0.1
ALT (IU/L)	44.81 ± 3.28	54.00 ± 4.71	595.33± 18.04 +++	449.28 ± 67.45	266.00 ± 14.91 ***	358.66 ± 28.83 ***	417.40 ± 44.28 *
AST (IU/L)	125.81 ± 10.28	197.25 ± 5.20	671.80 ± 19.63 +++	572.71 ± 30.34	331.66 ± 20.60 ***	335.83 ± 38.10 ***	512.00 ± 23.15 **
ALP (IU/L)	280.45 ± 13.44	270.75 ± 15.28	559.00 ± 17.17 +++	593.00 ± 22.89	384.16 ± 25.36 ***	420.00 ± 26.35 ***	401.71 ± 10.37 ***
SOD (U/mg protein)	9.4 ± 1.7	9.1 ± 2.1	5.8 ± 1.3 +++	6.4 ± 0.9	6.9 ± 1.5 *	7.6 ± 1.3 **	8.3 ± 1.1 ***
CAT (U/mg protein)	4.05 ± 0.05	3.75 ± 0.05	0.61 ± 0.01 +++	3.15 ± 0.05	2.45 ± 0.05 ***	2.55 ± 0.05 ***	0.95 ± 0.15 ***
Total protein (g/dL)	62.66 ± 2.6	57.66 ± 3.57	26.27 ± 0.81 +++	35.50 ± 1.84	41.52 ± 1.83 ***	43.07 ± 3.38 ***	45.01 ± 4.52 ***

Values are mean ± S.E.M. (n= 9 rats per each group). * $p <0.05$, ** $p<0.01$, *** $p<0.001$ significantly different from the group treated with CCl_4. +++. $p<0.001$ significantly different from the control

Histopathological findings

The central vein, hepatocyte and portal space were observed to be normal in the control group (Figure 1 A-G). CCl_4 is a hepatotoxicant known to produce a characteristic centrilobular pattern of degeneration and necrosis. In the present study, CCl_4 application constituted histopathological changes in the liver. Severe hyperemia was observed in the area surrounding the central veins. Wide vacuolar degeneration of hepatocytes and lymphocyte infiltration were observed. Derangement of the hepatocyte cord and necrosis at the periphery of central vein were also determined in CCl_4 group (Table 3). The histological appearance of the cinnamon extract-treated groups was quite similar to that of the control group, and tissue damage and necrosis were of less extent in this group than the CCl_4 group. Minimal tissue degeneration was observed at the periphery of the central vein. No derangement was observed at hepatocyte cords. Lymphocyte infiltration was not detected in the cinnamon extract-treated group. Hydropic and vacuolar degeneration were found only at the periphery of the central vein. Moderate degenerative changes, vacuolar degeneration of hepatocytes, and lymphocyte infiltration at the periphery of the central vein were determined in this group.

DISCUSSION

The present study demonstrates the hepatoprotective, curative and antioxidant effects of cinnamon ethanolic extract against CCl_4-induced liver injury in rats. The liver is one of the vital organs in our body responsible for detoxification of toxic chemicals and drugs. Thus it is the target organ for all toxic chemicals. Numerous studies noted that CCl_4 is widely used to induce liver damage because it is metabolized in hepatocytes by cytochrome P_{450}, generating a highly reactive carbon-centered trichloromethy radical, leading to initiating a chain of lipid peroxidation and thereby causing liver fibrosis (Fang et al., 2008; Weber et al., 2003; Ashok et al., 2001; Bahceioglu et al., 1990; Aleynik et al., 1997; Halliwell and Gutteridge, 1998). CCl_4 not only initiates lipid peroxidation but also reduces tissue CAT and SOD activities, and this depletion may result from oxidative modification of these proteins (Augustyniak et al., 2005). Our results showed that administration of cinnamon extract effectively protected against the loss of these antioxidant activities after CCl_4 administration, and it is well known to serve diverse biological functions, including protection of cells from oxidative damage by ROS and free radicals (Nakamura et al., 2001; Gabele et al., 2009). Phytochemicals have also been shown to stimulate synthesis of anti-

Table 3: Histological injury score of liver under different doses of cinnamon extract in rats treated with CCl_4

Group	Injury of score [a]			
	Fatty Degeneration	Necrosis	Cell swelling	Inflammation
Control	0	0	0	0
Cinnamon extract (0.05 g/kg b.w.)	0	0	0	0
CCl_4	4	3	3	3
Cinnamon extract				
0.005 g/kg b.w. + CCl_4	2	1	2	1
0.01 g/kg b.w. + CCl_4	0	0	0	1
0.05 g/kg b.w. + CCl_4	3	2	2	2
0.1 g/kg b.w. + CCl_4	2	2	2	2

[a] Livers were scored for hepatic injury via light microscopy with score 0 = no visible cell damage; score 1 = focal hepatocyte damage on less than 25 % of the tissue; score 2 = focal hepatocyte damage on 25-50 % of the tissue; score 3 = extensive, but focal, hepatocyte lesions; score 4 = global hepatocyte necrosis.
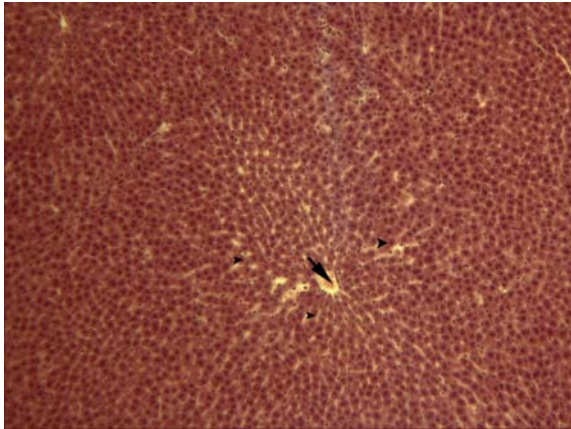
Figure 1A: Liver section of normal control showing normal central vein (arrow) and radiating hepatocytes (arrowhead) (H&E*16)
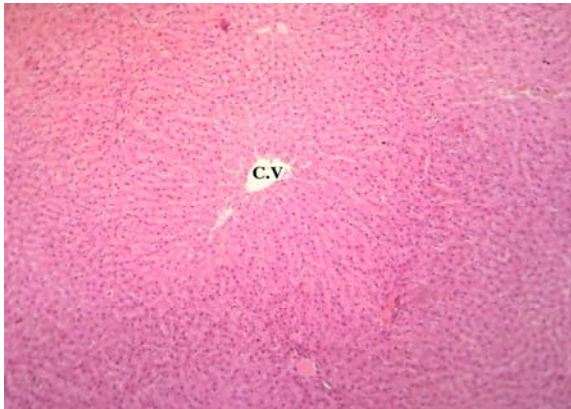

Figure 1B: Liver section from cinnamon extract treated animals shows normal central vein (C.V.) and hepatocyte (H&E*16)
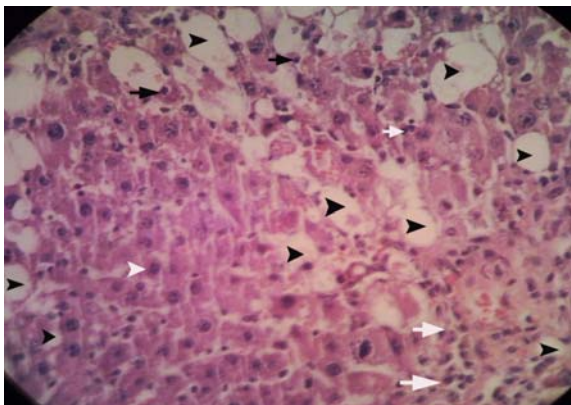

Figure 1C: Liver section of CCl₄–treated rats revealing fatty degeneration (black arrowhead), hepatocyte necrosis (black arrow), inflammatory cells infiltration (white arrow) and normal hepatocyte (white arrowhead) (H&E*640)
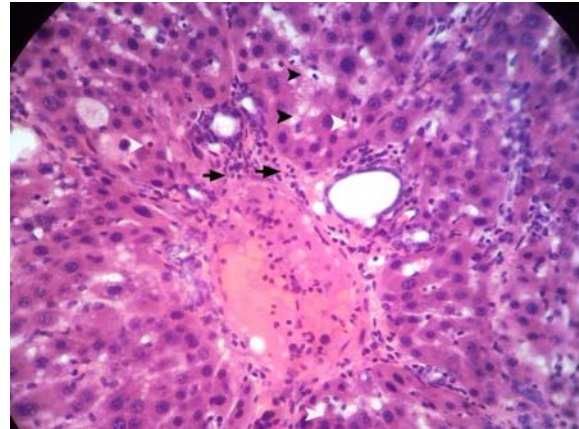

Figure 1D: Liver section from rat treated with 0.005 g/kg b.w. + CCl₄ shows fatty degeneration (arrowhead), necrosis and infiltration of inflammatory cells (arrow) (H&E*640)
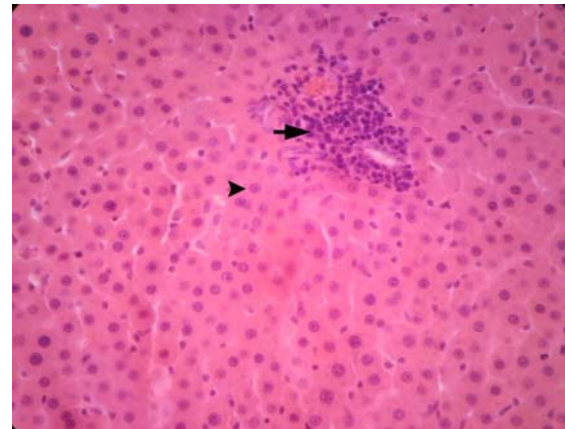

Figure 1E: Liver section from rat treated with 0.01 g/kg b.w. + CCl₄ shows infiltration of inflammatory cells (arrow) with normal hepatocyte (arrow head) (H&E*160)
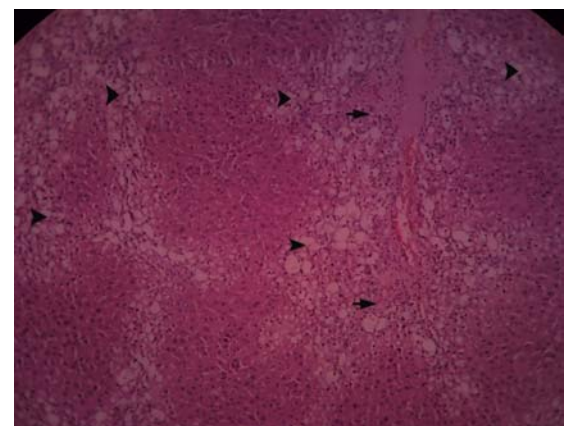

Figure 1F: Liver section from rat treated with 0.05 g/kg b.w. + CCl₄ shows fatty degeneration (arrowhead), infiltration of inflammatory cells (arrow) (H&E*160)
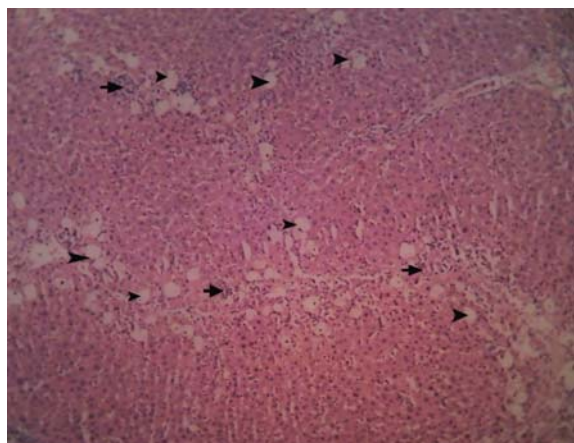
Figure 1G: Liver section from rat treated with 0.1 g/kg b.w. + CCl₄ shows fatty degeneration (arrowhead), infiltration of inflammatory cells (arrow) (H&E*160)

Figure 1: Histopathological changes of liver tissue

oxidant enzymes and detoxification systems at the transcriptional level, through antioxidant response elements (Masella et al., 2005), and to increase γ-glutamylcysteine synthesis (Kim et al., 2007).

Increased levels of ALT, AST and ALP in serum of the CCl₄-treated animals indicate liver damage as these enzymes leak out from liver into the blood at the instance of tissue damage, which is always associated with hepatonecrosis (Naik and Panda, 2008; Ree and Spector, 1961). With the treatment of cinnamon extract, the levels of these marker enzymes were near normal or only slightly elevated, indicating protection against liver damage. ALP activity is related to the functioning of hepatocytes. Suppression of increased ALP activity suggests the stability of biliary dysfunction in rat liver during chronic hepatic injury with CCl₄. Diminution of total protein and albumin induced by CCl₄ is a further indication of liver damage (Navarro and Senior, 2006). Cinnamon extract has increased the levels of serum total protein towards the respective normal value, which indicates hepatoprotective activity. Stimulation of protein synthesis has been advanced as a contributory hepatoprotective mechanism which

accelerates the regeneration process and the production of liver cells (Rip et al., 1985; Tadeusz et al., 2001).

Additionally, we observed histopathological changes indicating liver damage after CCl₄ administration. It has been reported by previous findings that CCl₄ causes necrosis (Sun et al., 2001; Zalatnai et al., 1991), fibrosis (Mackinnon et al., 1995; Natsume et al., 1999; Zalatnai et al., 1991), mononuclear cell infiltration (Natsume et al., 1999), steatosis and degeneration of hepatocytes, increase in mitotic activity (Teocharis et al., 2001) and cirrhosis (Zalatnai et al., 1991) in liver. It has also been reported that CCl₄ causes apoptosis in liver (Shi et al., 1998; Sun et al., 2001). Therefore, histopathological findings in the liver due to CCl₄ administration are in agreement with previous studies. Pretreatment with cinnamon extract significantly improved the structure of hepatic cells. We therefore suggest that pretreatment with the cinnamon extract markedly reversed hepatotoxicity caused by the CCl₄.

Phytochemical screening of cinnamon bark has revealed the presence of flavonoids, glycosides, coumarins, alkaloids, anthraquinone, steroids, tannins and terpenoids (Shihabudeen et al., 2011). Phytoconstituents like the flavonoids (Baek et al., 1996), triterpenoids (Xiong et al., 2003), saponins (Tran et al., 2001) and alkaloids (Vijayan et al., 2003) are known to possess hepatoprotective activity. The presence of flavanoids in our extract may be responsible for its antioxidant and thus hepatoprotective activity. Numerous studies have suggested that flavonoids commonly function as antioxidants and may protect plants against oxidative stress caused by suboptimal environmental conditions (Bohnert and Jensen, 1996; Rice-Evans et al., 1997; Tattini et al., 2004; Gould and Lister, 2006). The antioxidant capacity of flavones is attributed to the high reactivity of the hydroxyl substituent, with the number of hydroxyl groups on the B-ring being correlated with ROS scavenging capability (Sekher et al., 2001; Burda and Oleszek, 2001; Heim et al., 2002).

In conclusion, the results of this study demonstrate that cinnamon extract was effective for the prevention of CCl₄-induced hepatic damage in rats. Our results show that the hepatoprotective effects of cinnamon extract may be due to both an increase in the activity of the antioxidant-defense system and an inhibition of lipid peroxidation. However, the protective, curative and antioxidant qualities of cinnamon need to be confirmed by characterizing the active ingredient(s) of this plant as well as its mechanism(s) of action.

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