

## Hepatoprotective activity of ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves against CCl<sub>4</sub>-induced liver injury in rats

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### ABSTRACT

**Background:** The botanical Latin name of the plant, *Turnera aphrodisiaca*, describes its ancient use as an aphrodisiac.

**Methods:** The aim of the present study is to evaluate the protective effect of ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves against carbon tetrachloride (CCl<sub>4</sub>)-induced liver damage in male Wistar rats.

**Results:** Administration with ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves (200 and 400 mg/kg) for 7 days significantly reduced the impact of CCl<sub>4</sub> toxicity on the serum markers of liver damage, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase in a dose dependent manner. In addition, treatment of both the extracts 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 both extracts markedly reduced the toxicity of CCl<sub>4</sub> and preserved the histoarchitecture of the liver tissue to near normal.

**Conclusion:** Thus, the results suggest that ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves acts as a potent hepatoprotective agent against CCl<sub>4</sub> induced hepatotoxicity in rats.

**Keywords:** Aqueous extract of *Turnera aphrodisiaca* leaves, Carbon tetrachloride, Ethanolic extract of *Turnera aphrodisiaca* leaves, Hepatoprotective *Turnera aphrodisiaca*

### INTRODUCTION

In ancient Indian literature, it is mentioned that every plant on this earth is useful for human beings, animals and other plants. The liver is the key organ regulating homeostasis in the body. It is involved with almost all the biochemical pathways related to growth, fight against diseases, nutrient supply, energy provision and reproduction.<sup>1</sup> The liver is expected not only to perform physiological functions but also to protect the hazards of harmful drugs and chemicals. In spite of tremendous scientific advancement in the field of hematology in recent years, liver problems are on the rise. Jaundice and hepatitis are two major hepatic disorders

that account for a high death rate.<sup>2</sup> Number of deleterious metabolic changes in the liver is induced by alcohol. Steatosis, alcoholic hepatitis and cirrhosis resulting in weight and volume changes are caused by excessive use of alcohol for a long-time. Minimum 80% of heavy drinkers had been reported to develop steatosis, 10-35% alcoholic hepatitis, and approximately 10% liver cirrhosis. In recent studies, the animal models suggest that liver injury in chronic alcoholics is due to oxidative stress that leads to fibrosis and impaired liver functions and increased apoptosis.<sup>3</sup> *Turnera aphrodisiaca* ward (synonym *T. diffusa* Willd., family *Turneraceae*) is commonly known as damiana. The leaves of *T. aphrodisiaca* have been used traditionally as a stimulant, aphrodisiac, tonic, diuretic,

nerve tonic, laxative and in kidney, menstrual and pregnancy disorders.<sup>4,5</sup> The British Herbal Pharmacopoeia lists specific indications for damiana as anxiety neurosis associated with impotency, and includes other indications such as depression, nervous dyspepsia, atonic constipation and coital inadequacy.<sup>6</sup> Damiana has achieved some repute in the treatment of sexual impotence where it is used in conjunction with strychnine, phosphorus or some other stimulants in homoeopathic formulations. The leaf infusion of damiana has been used as a traditional remedy in the diseases related to the gastrointestinal and respiratory system, reproductive organs, and for the treatment of gonorrhoea in Latin American societies. Mother tincture (85% ethanol extract) of damiana is an important homoeopathic medicine for the treatment of sexual debility, and nervous prostration.<sup>7-10</sup> Phytochemical reports on *T. aphrodisiaca* indicate that the plant contains tetraphyllin B (cyanoglycoside), gonzalitosin I (flavonoid), arbutin (phenolic glycoside), damianin, tricosan-2-one, hexacosanol (hydrocarbons); a volatile oil containing  $\alpha$ -pinene,  $\beta$ -pinene, p-cymene and 1,8-cineole; and  $\beta$ -sitosterol (phytosterol).<sup>11-15</sup>

## METHODS

### Chemicals

CCl<sub>4</sub> 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 Span Diagnostics, India. 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 and authenticated by Botanical Survey of India, Deccan Regional Centre, Hyderabad wide file no. BSI/DRC/2017-18/Identification/133. Shade dried *Turnera aphrodisiaca* leaves 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 *Turnera aphrodisiaca* leaves 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.<sup>16</sup> 20 mg extract was dissolved in 2 ml distilled water and filtered. 2 ml FeCl<sub>3</sub> was added to

the filtrate, blue black precipitate indicated the presence of tannins.<sup>16</sup> To 0.5 ml of the filtrate obtained in alkaloids test 5 ml distilled water was added. Frothing persistence indicated the presence of saponins.<sup>16</sup> 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.<sup>17</sup> Salkovski test was performed using a small amount of extract solution. To this solution 5 drops of conc. H<sub>2</sub>SO<sub>4</sub> and 1 ml chloroform were added. Change of yellow color into red indicated the presence of terpenoids.<sup>17</sup> A small amount of material was extracted in ethanol and evaporated to dryness. Residue was dissolved in distilled water and 0.5 ml Folin-ciocalteu reagent was added followed by 2 ml 20 % Na<sub>2</sub>CO<sub>3</sub> solution. Development of bluish color indicated the presence of phenols.<sup>18</sup>

### Animals

Adult male Wistar rats weighing 220-230 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 photo-cycle 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 six rats. In order to study the liver protective effect of ethanolic and aqueous extracts of *Turnera aphrodisiaca* leaves in rats, dose levels of 400 and 200 mg/kg BW p.o were used respectively.

- Vehicle control (n=6, Treated with normal saline only for 7 days).
- Hepatotoxic control (n=6, Treated with CCl<sub>4</sub>/Olive oil (1:1 v/v, 0.7 ml/kg, i.p.) for 7 Days).
- Standard group (n=6, Treated with silymarin (25 mg/kg, p.o.) and CCl<sub>4</sub>/Olive oil (1:1 v/v, 0.7 ml/kg, i.p.) alternate days for 7 days).
- Treatment group I (n=6, Treated with EETA (400 mg/kg bw p.o) and CCl<sub>4</sub>/Olive oil (1:1 v/v, 0.7 ml/kg, i.p.) alternate days for 7 days).
- Treatment group II (n=6, Treated with EETA (200 mg/kg bw p.o) and CCl<sub>4</sub>/Olive oil (1:1 v/v, 0.7 ml/kg, i.p.) alternate days for 7 days).

- Treatment group III (n=6, Treated with AETA (400 mg/kg bw p.o) and CCl<sub>4</sub>/Olive oil (1:1 v/v, 0.7 ml/kg, i.p.) alternate days for 7 days.
- Treatment group IV (n=6, Treated with AETA (200 mg/kg bw p.o) and CCl<sub>4</sub>/Olive oil (1:1 v/v, 0.7 ml/kg, i.p.) alternate days for 7 days.<sup>19</sup>

At the end of the treatment, rats were sacrificed by cervical dislocation, blood samples were collected by direct cardiac puncture. The serum was used for the evaluation of marker enzymes. Liver was dissected out and washed with ice-cold saline and a homogenate was prepared in 0.1N Tris HCL buffer (pH 7.4). The homogenate was used for the assay of antioxidant marker enzymes.

### **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 and CAT were assayed in the liver.<sup>20,21</sup>

SOD was assayed by the inhibition of the formation of NADH-phenazine methosulphate nitroblue 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<sub>2</sub>O<sub>2</sub>) consumed in the reaction. The remaining H<sub>2</sub>O<sub>2</sub> was reacted with dichromate- acetic acid reagent and monitored spectrophotometrically at 590 nm. Activity was expressed in μmol H<sub>2</sub>O<sub>2</sub> 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 and E). The liver sections were evaluated histologically with a camera attached to a light microscope. The extent of CCl<sub>4</sub>-induced liver damage was evaluated based on pathologic lesions in liver sections stained with H and 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.<sup>22</sup> The liver pathology was scored as described by French et al. 2000 as follows:<sup>23</sup>

- 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**

### **Body weight and weight of liver**

No animals died during CCl<sub>4</sub> administration period. The administration of CCl<sub>4</sub> caused a significant decrease in the body weight of rats as compared with the control rats (Table 1).

The animals co-treated with ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves (200 and 400 mg/kg BW) for three 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<sub>4</sub>-treated animals than in control animals. Co-treatment with ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves (200 and 400 mg/kg BW) resulted in both liver weights and liver index that were significantly reduced compared to those of CCl<sub>4</sub>-treated rats. However, comparing the two extracts of *Turnera aphrodisiaca* leaves (200 and 400 mg/kg BW), ethanolic extract shows more significant results when compared with aqueous extract.

**Table 1: Body weight, liver weight and liver index of acute CCl<sub>4</sub> (50% CCl<sub>4</sub>/olive oil)-treated rats with or without cinnamon ethanolic extract.**

Parameters	Control	CCl <sub>4</sub>	CCl <sub>4</sub> + EETA (mg/kg BW)		CCl <sub>4</sub> + AETA (mg/kg BW)	
			400	200	400	200
Initial body weight (g)	224±10.4	223±13.2	221±11.2	219±12.5	217±10.2	221±11.2
Final body weight (g)	256±15.3	226±10.5 <sup>+++</sup>	239±13.6	232±8.5	241±11.4	242±6.5
Weight gain (g)	32±4.9	3±0.7 <sup>+++</sup>	18±6.3*	13±3.4	16±5.0*	11±4.3*
Liver weight (g)	5.3±0.34	8.5±1.2 <sup>+++</sup>	6.5±0.71**	6.9±0.64	5.5±0.71**	4.9±0.64*
Liver index	2.07±0.04	3.76±0.07 <sup>+++</sup>	2.72±0.04**	2.97±0.05	2.82±1.04**	2.82±1.0*

Values are Mean±S.E.M. (n=6 rats per each group). The liver index was calculated as liver weight/body weight×100, \* p <0.05, \*\* p<0.01, significantly different from the group treated with CCl<sub>4</sub>. +++p<0.001 significantly different from the control, EETA-Ethanolic extract of *Turnera aphrodisiaca* leaves, AETA- Aqueous extract of *Turnera aphrodisiaca* leaves

**Table 2: Effects of EETA on serum and liver biochemical indices in CCl<sub>4</sub>-induced hepatotoxicity in rats.**

Groups	Billirubin	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	SOD (U/mg protein)	CAT (U/mg protein)	Total protein (g/dL)
Control	0.70±0.03	44.81±3.28	125.81±10.28	280.45±13.44	9.4±1.7	4.05±0.05	62.66±2.6
Toxic Control	2.42±0.17	595.33±18.4 <sup>+++</sup>	671.80±19.63 <sup>+++</sup>	559.00±17.17 <sup>+++</sup>	5.8±1.3 <sup>+++</sup>	0.61±0.01 <sup>+++</sup>	26.27±0.81 <sup>+++</sup>
Standard	1.10±0.03**	78.41±3.28	188.12±11.28	323.11±10.20	8.2±1.5**	3.33±0.03**	54.34±2.4**
EETA 400	1.68±4.62**	266.00±14.91**	331.66±20.60**	384.16±25.36**	7.6±1.3**	2.45±0.05**	43.07±3.38**
EETA 200	2.06±2.20**	358.66±28.83**	335.83±38.10**	420.00±26.35**	6.9±1.5*	2.55±0.05*	41.52±1.83**
AETA 400	1.88±3.62**	298.20±11.11**	412.11±10.11**	424.06±15.16**	7.1±1.2**	2.75±0.07**	48.07±3.18**
AETA 200	3.06±4.20**	358.10±18.83**	535.13±18.01**	520.10±10.50**	5.9±1.2*	2.95±0.06*	51.12±1.13**

Values are Mean±S.E.M. (n= 6 rats per each group). \*p <0.05, \*\*p<0.01 significantly different from the group treated with CCl<sub>4</sub>, +++p<0.001 significantly different from the control, EETA-Ethanolic extract of *Turnera aphrodisiaca* leaves, AETA- Aqueous extract of *Turnera aphrodisiaca* leaves

### Biochemical analysis

CCl<sub>4</sub> is activated by phase-II detoxifying enzymes in liver cell endoplasmic reticulum to form trichloromethyl and peroxy trichloromethyl 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<sub>4</sub> administration produced significant elevations of serum ALT and AST compared to the normal control group. However, pretreatment of rats with 400 and 200 mg/kg BW ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves significantly decreased these serum biochemical indices as compared with the CCl<sub>4</sub> treatment group.

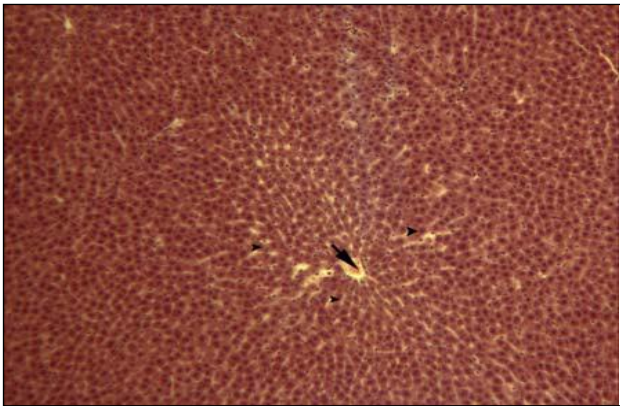
Hepatic SOD and CAT activities in the CCl<sub>4</sub> treatment group were reduced compared to the normal control group.

These antioxidant enzyme activities were all statistically significantly greater in the group treated with ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves (400 and 200 mg/kg BW) compared with the CCl<sub>4</sub> treatment group. There was no significant alteration in control rats treated solely with both extracts of *Turnera aphrodisiaca* (Table 2). However, comparing the two extracts of *Turnera aphrodisiaca* leaves (200 and 400 mg/kg BW), ethanolic extract shows more significant results when compared with aqueous extract.

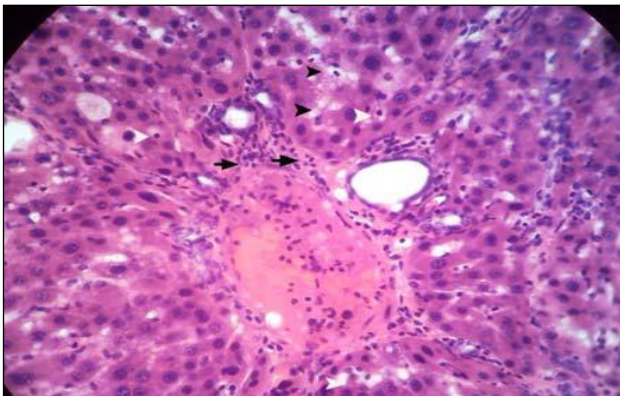
### Histopathological findings

The central vein, hepatocyte and portal space were observed to be normal in the control group (Figure 1 A-G). CCl<sub>4</sub> is a hepatotoxicant known to produce a characteristic centrilobular pattern of degeneration and necrosis. In the present study, CCl<sub>4</sub> application constituted histopathological changes in the liver. Severe hyperemia was observed in the area surrounding the central veins. Wide vacuolar degeneration of hepatocytes and

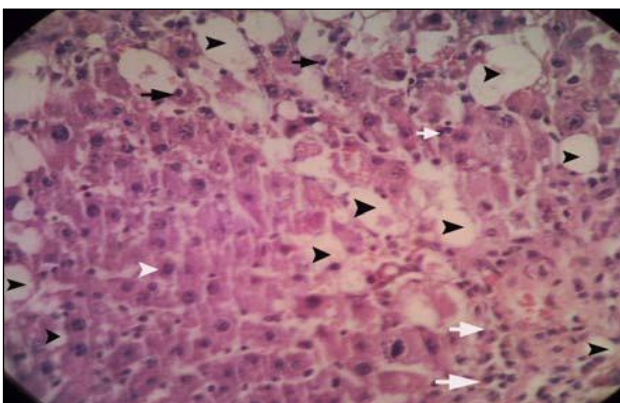
lymphocyte infiltration were observed. Derangement of the hepatocyte cord and necrosis at the periphery of central vein were also determined in CCl<sub>4</sub> group (Figure 1C).



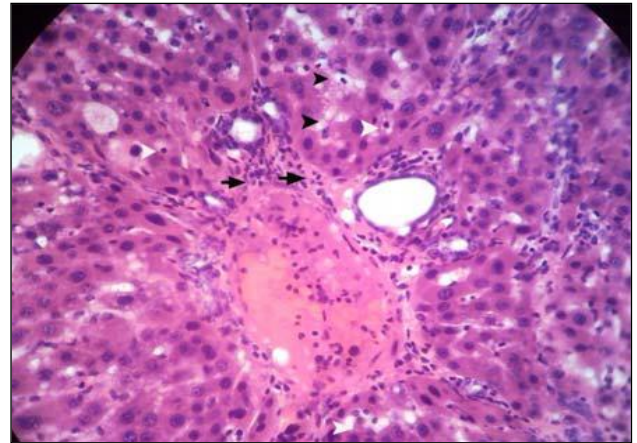
**Figure 1A:** Liver section of normal control showing normal central vein (arrow) and radiating hepatocytes (arrowhead) (H and E\*16).



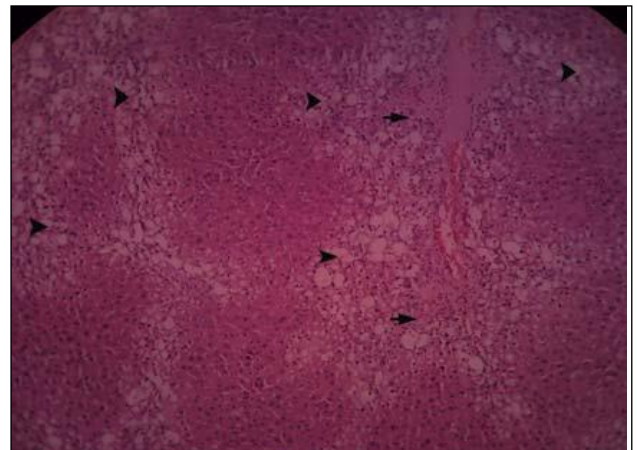
**Figure 1B:** Liver section from silymarin treated animals shows normal central vein (C.V.) and hepatocytes (H and E\*16).



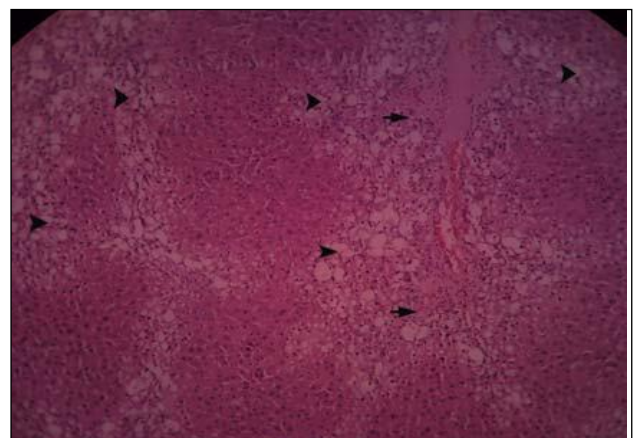
**Figure 1C:** Liver section of CCl<sub>4</sub>-treated rats revealing fatty degeneration (black arrowhead), hepatocyte necrosis (black arrow), inflammatory cells infiltration (white arrow) and normal hepatocyte (white arrowhead) (H and E\*640).



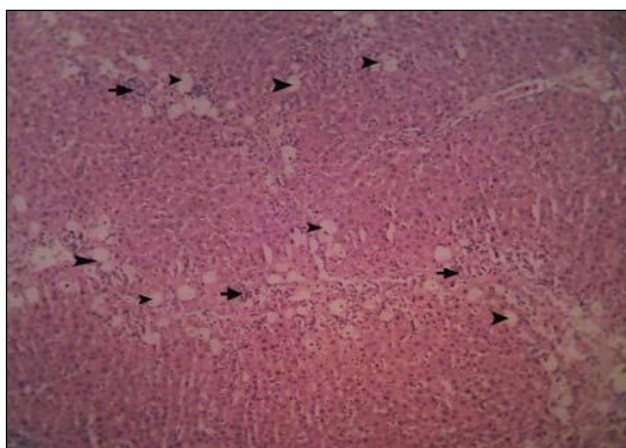
**Figure 1D:** Liver section from rat treated with EETA 400 mg/kg BW+CCl<sub>4</sub> shows fatty degeneration (arrowhead), necrosis and infiltration of inflammatory cells (arrow) (H and E\*640).



**Figure 1E:** Liver section from rat treated with EETA 200 mg/kg BW+CCl<sub>4</sub> shows fatty degeneration (arrowhead), infiltration of inflammatory cells (arrow) (H and E\*160).



**Figure 1F:** Liver section from rat treated with AETA 400 mg/kg BW+CCl<sub>4</sub> shows fatty degeneration (arrowhead), infiltration of inflammatory cells (arrow) (H and E\*160).



**Figure 1G: Liver section from rat treated with AETA 200 mg/kg BW+CCl<sub>4</sub> shows fatty degeneration (arrowhead), infiltration of inflammatory cells (arrow) (HandE\*160).**

The histological appearance of the ethanolic extract of *Turnera aphrodisiaca*-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<sub>4</sub> 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 regeneration 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<sub>4</sub>-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<sub>4</sub> is widely used to induce liver damage because it is metabolized in hepatocytes by cytochrome P450, generating a highly reactive carboncentered trichloromethyl radical, leading to initiating a chain of lipid peroxidation and thereby causing liver fibrosis.<sup>24-29</sup> CCl<sub>4</sub> not only initiates lipid peroxidation but also reduces tissue CAT and SOD activities, and this depletion may result from oxidative modification of these proteins.<sup>30</sup>

Authors results showed that administration of ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves effectively protected against the loss of these antioxidant activities after CCl<sub>4</sub> administration, and it is well known to serve diverse biological functions, including protection of cells from oxidative damage by ROS and free radicals.<sup>31,32</sup> Phytochemicals have also been shown to

stimulate synthesis of anti-oxidant enzymes and detoxification systems at the transcriptional level, through antioxidant response elements and to increase  $\gamma$ -glutamylcysteine synthesis.<sup>33,34</sup>

Increased levels of ALT, AST and ALP in serum of the CCl<sub>4</sub>-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.<sup>35,36</sup> With the treatment of ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves, 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 CCl<sub>4</sub>. Diminution of total protein and albumin induced by CCl<sub>4</sub> is a further indication of liver damage.<sup>37</sup> *Turnera aphrodisiaca* leaves extract (ethanolic and aqueous) 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.<sup>38-39</sup>

Additionally, we observed histopathological changes indicating liver damage after CCl<sub>4</sub> administration. It has been reported by previous findings that CCl<sub>4</sub> causes necrosis fibrosis, mononuclear cell infiltration, steatosis and degeneration of hepatocytes, increase in mitotic activity and cirrhosis in liver. It has also been reported that CCl<sub>4</sub> causes apoptosis in liver. Therefore, histopathological findings in the liver due to CCl<sub>4</sub> administration are in agreement with previous studies.<sup>40-45</sup>

## CONCLUSION

The results of this study demonstrate that ethanolic and aqueous extract of *Turnera aphrodisiaca* leaves was effective for the prevention of CCl<sub>4</sub>-induced hepatic damage in rats.

Authors results show that the hepatoprotective effects of *Turnera aphrodisiaca* leaves extract may be due to both an increase in the activity of the antioxidant-defense system and an inhibition of lipid peroxidation. However, comparing the two extracts of *Turnera aphrodisiaca* leaves (200 and 400 mg/kg BW), ethanolic extract shows more significant results when compared with aqueous extract. The protective, curative and antioxidant qualities of *Turnera aphrodisiaca* 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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