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Tamarix dioica (Ghaz) Protective Potential in the Carbon Tetrachloride-Induced Hepatotoxicity Animal Model

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ABSTRACT

Introduction: Hepatic diseases remain the leading cause of death worldwide. Despite overall advancements in health care, mortality due to hepatic diseases is constantly growing. More than 2 million people globally are estimated to die each year from liver diseases, and current treatment offers little for its management. Thus, it is essential to find more effective and less toxic pharmaceutical alternatives for the treatment of liver diseases.

Aims & Objectives: *Tamarix dioica*, a shrub broadly used in herbal medicine for the treatment and prevention of various diseases. The current study was designed to analyze the hepatoprotective effect of *T. dioica* in BALB⁄cmice against CCl4-induced acute liver damage.

Place and duration of study: The study was conducted in NIH, Islamabad, Pakistan, for six months in 2016-2017.

Material & Methods: For *in vivo* evaluation, the animals (n= 42) were randomly divided into seven groups (n=6), three control (i.e. Group, I or normal control, group II or induction control received 0.9% normal saline orally, and Group III or positive control received silymarin 100 mg/kg per oral), and four treatment groups (i.e. IV, V,VI and VII were treated with oral *T.dioica* 200 mg/kg/day, 300mg/kg/day methanol extract, 200mg/kg/day and 300mg/kg/day of aqueous extracts respectively for six days, followed by intraperitoneal administration of CCl4 on the seventh day. The blood samples were collected for analysis of LFTs, and hepatic tissue was taken for histological analysis. Data was analyzed using SPSS version 16, one-way ANOVA with Duncan's Multiple Range Test (DMRT).

Results: CCl4 induction in Group 2 resulted in severe hepatic derangement manifested as highly elevated mean LFTs (ALT 7245.56, AST 3292.11, ALP 340.09 U/L, bilirubin 4.64 mg/dl) as compared to healthy controls (ALT 38.97, AST 50.20, ALP 57.17 U/L, bilirubin 1.25 mg/dl: (Group 1) levels $p<0.001$. Pretreatment with different extracts of *T.dioica* for 6 days before CCl4 administration produced varying degrees of hepatoprotection. 300mg/kg aqueous extract *T.dioica* (Group7) prevented damage with maximal hepatoprotection, reduced LFTs (ALT: 339.95 , AST: 242.90 , ALP: 116.86 U/L, bilirubin: 1.38 mg/dl) and normalized liver histology as compared to Group 2 and standard drug silymarin 100mg/kg, (ALT: 6483.23, AST: 2567.69, ALP: 272.19 U/L, bilirubin: 2.84 mg/dl: Group 3) $p<0.001$. Lesser hepatoprotection was provided by *T.dioica* aqueous extract 200mg/kg (ALT: 439.93, AST: 367.87, ALP: 180.62 U/L bilirubin: 1.53 mg/dl: Group VI) and least by 300mg/kg & 200mg/kg methanolic extracts Groups V & IV (ALT: 6338.06, 6443.91, AST: 2800.81, 3012.34, ALP: 242, 248 U/L & bilirubin: 2.82 & 3.62 mg/dl) respectively. Further, no drug-induced toxicity symptoms were observed 24 hours after administration of the high dose oral *T. dioica* 2000 mg/kg/body weight aqueous and methanolic extracts were administered.

Conclusion: Pretreatment with *T. dioica* extracts especially 300mg/kg aqueous extract reduced acute CCl4-mediated liver damage, ameliorated histopathological as well as biochemical parameters and was free of toxicity in 2000mg/kg /body weight dose in the mice experimental model. *T. dioica* has potential in hepatoprotective drug research.

Key words: *Tamarix dioica*, Hepatoprotective, Silymarin, Carbon tetrachloride

INTRODUCTION

Liver regulates the majority of body functions, including detoxification, metabolism, antimicrobial defenses, and immune response whereas, any disturbance in its normal functioning may lead toward serious consequences.[1] The extensive metabolic capacity of the liver makes it more susceptible to chemicals includes liver injuries.[2] Carbon tetrachloride (CCl4) is identified as a possible human carcinogen. In animals, it is rapidly absorbed into the systemic circulation via the gastrointestinal tract.[3,4]CCl4 administration by inhalation, gastric infusion, and oral bolus gave its peak concentration in fats, liver, kidney, brain, and lung, therefore extensively used to induce experimental hepatopathy.[5,6] In experimental murine

models, the cytochrome P450 2E1 is predominantly involved in the bio activation of CCl4.[7] Accidental exposure to CCl4 can cause acute hepatic and renal failure followed by dialysis when exposed to 200 ppm CCl4 for less than 3 hours.[8] Furthermore, hepatic tumors were observed at higher CCl4 doses followed by loss of cellular Ca^{+2} sequestration and disruption of Ca^{+2} homeostasis with subsequent cell damage.[9]CCl4 also stimulates fibrosis by activating nitric oxide (NO), tumor necrosis factor α (TNF-α), and transforming growth factor (TGF)-α/β in the cell and pushes the cell toward apoptosis.[10] Hepatitis cirrhosis, alcoholic liver disease, hemochromatosis, and hepatomas are some examples of liver diseases and current medical treatments for such hepatic diseases are either too expensive or associated with adverse effects.[11] Medicinal plants have been used worldwide for their therapeutic purposes since ancient times. Extracts of several medicinal plants show permissible results against various diseases without any critical side effects.[12] Multiple studies validated the usage of traditional medicine in the management of various diseases.[13] *Tamarix dioica* (*T. dioica*) is a small shrub that belongs to the family *Tamaricaceae*.[14] The plant is well-known in ancient and present herbal remedies for its anti-diabetic, anti-fungal, anti-infective and anti-dermatosis, carminative, anti-inflammatory, and diuretic properties.[15,16] Nonetheless, there are still few clinical studies for the therapeutic effectiveness of *T. dioica*. Keeping in view these facts, the present study is designed to evaluate the hepatoprotective potential of *T. dioica* plant aqueous and methanolic extracts against CCl4-induced toxicity into BALB/c mice.

MATERIAL AND METHODS

Plant material

Leaves of *T. dioica* were obtained and identified by the Department of Botany, PMAS-Arid Agricultural University Rawalpindi, Pakistan.

Chemicals and reagents

Carbon tetrachloride, silymarin, olive oil, methanol, distilled water, serum bilirubin and diagnostic kits (Huma Star 600) of Pharmacopoeia grade were obtained from National Institute of Health (NIH), Islamabad, Pakistan.

Animal selection and maintenance

BALB⁄c mice body weights 35-45g on standard laboratory diet were obtained from NIH Islamabad, Pakistan, and were kept at 25±1ºC temperature, the relative humidity of 10% with normal light and dark schedule. The research proposal was evaluated and approved by Research Ethical Committee and a lot reference number 10-M.PH/LCWU-17981.

Extract preparation

Tamarix dioica aqueous extract

Dried *T. dioica* aqueous extraction were prepared by grinding followed by soaking in distilled water (20g/200ml) for overnight and then boiled at 100ºC. The extract was cooled at room temperature and supernatant was then filtered through Whatman® Grade 42 filter paper.[17]

Tamarix dioica methanolic extract

Dried plant material was ground and extracted with 80% methanol (20g/200ml) to obtain the crude methanolic extract. The extract was concentrated by rotary evaporation and stored in the refrigerator at 4ºC for future study and dose preparation.[17]

Biological Study

Drug administration protocol and animal grouping

Animals were divided into seven groups (n=6). All groups received standard food orally throughout the study period. Group, I (Normal control or NC) and group II (Induction control) were administered 0.9% normal saline orally for six days. Group III (Positive control) received silymarin 100 mg/kg per oral. Two treatment groups (IV and V) were treated with methanol extract (ME), and the other two groups (VI and VII) received aqueous extracts (AE) orally at the dose of 200 and 300 mg/kg respectively, for six days. All groups, except normal control, received an intraperitoneal injection (i.p.) of CCl4 in olive oil (1:1) at the dose of 0.5 mg/kg for hepatotoxicity induction on the seventh day of study.

Acute toxicity studies

Male mice were selected for the acute toxicity testing according to OECD guidelines-423.[18] *T.dioica* aqueous and methanolic extracts at the dose of 2000 mg/kg body weight were administered p.o while distilled water was given to the control group.

Blood and liver sample collection

The experimental animals were sacrificed for blood and liver collection. Blood was collected directly from the aorta followed by centrifugation for five minutes at 12000 rpm/min. The supernatant was collected and preserved at -20ºC for further analysis. Livers samples were preserved in 10% formalin solution and stored for subsequent analysis.[19]

Liver biochemical assessment

Liver function tests (LFTs) including aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine transaminase (ALT), and total bilirubin were determined via commercially available diagnostic kits (HumaStar 600).

Histopathological observation

Animal liver samples were obtained for histopathological examination and preserved in

formalin (10% v/v) solution followed by paraffin embedding and cutting into 5µm thick sections with a microtome and were stained subsequently with hematoxylin and eosin (H&E) dye and were observed under the light microscope (IM-910 IRMECO GmbH & Co. Germany).

Statistical analysis:

Statistical analysis was done by using SPSS software version 16.0, one-way analysis of variance (ANOVA) with Duncan's Multiple Range Test (DMRT). All results were expressed as mean ± SE and 5% level of significance ($P \leq 0.05$).

RESULTS

Effect of *T. dioica* extracts on bilirubin levels against CCl4-induced acute liver injury in BALB/c mice:

The results depicted that the bilirubin levels in the induction group (II) significantly increased, $p< 0.05$, (4.64 ± 0.029) as compared to the control group (1.25 ± 0.018). It shows that CCl4 induces hepatotoxicity. The groups that received the pre-treatment with ME and AE have statistically significant lower levels of bilirubin ($p<0.05$), as shown in Table-1 and Fig-1.

Effect of *T. dioica* on liver parameters

A significant increase level of AST and ALT ($p\leq 0.05$) was observed in the carbon-tetrachloride treated (II) group as compared to the normal control group (Table-1). The ME and AE pretreatment and standard drug treatment have significantly ($p<0.05$) reduced the upregulated serum liver markers (AST and ALT), which showed the hepatoprotective effects of *T. dioica*. As shown in Table-2 and Fig-2. Furthermore, among various treatment groups, maximum protection was observed in AE treated group against CCl4-induced acute liver injury. As shown in Table-3 and Fig-3.

***Tamarix dioica* protects mice from CCl4-induced acute liver injury**

Liver histopathological examination showed significant changes on liver section exposed with CCl4 (B) when compared to the control (NC) group (A) shown in Fig-4. The changes in CCl4 exposed liver section revealed abnormal morphological characteristics, vacuolated hepatocytes, fat accumulation, mitotic figures and the severity of hepatic damage. The histopathological examination of liver sections of standard group (C) showed to have significantly reduced mitotic figures, vacuolated hepatocytes and no fat accumulation. The pretreated mice groups with AE (D: 200mg/kg & E: 300mg/kg) and ME (F: 200mg/kg & G: 300mg/kg) found to have significantly organized liver tissues, highly significant lower fat accumulation and vacuolated hepatic cells as compared to CCl4 exposed (B) group. The animal group treated with AE 300mg/kg showed to have maximum liver protection. Furthermore, no toxicity symptoms were observed after 24 hours when a high dose of 2000 mg/kg body weight of *T. dioica* aqueous and methanolic extracts was administered p.o. compare to the control group.

Serum Parameters (U/L)	ALP	ALT	AST	Total Bilirubin (mg/dl)
NC (Group I)	57.17 ± 0.731	38.97 ± 0.54	50.20 ± 0.69	1.25 ± 0.018
Induction Control (Group II)	340.09 ± 5.709	7245.56 ± 84.40	3292.11 ± 38.51	4.64 ± 0.029
Positive Control (Group III)	272.19 ± 3.386	6483.23 ± 28.24	2567.69 ± 28.29	2.84 ± 0.13
Treatment-ME 200 (Group IV)	248 ± 3.396	6443.91 ± 36.74	3012.34 ± 36.40	3.62 ± 0.094
Treatment - ME 300 (Group V)	242 ± 3.715	6338.06 ± 44.64	2800.81 ± 29.58	2.82 ± 0.133
Treatment-AE 200 (Group VI)	180.62 ± 5.349	439.93 ± 0.86	367.87 ± 7.54	1.53 ± 0.039
Treatment-AE 300 (Group VII)	116.86 ± 2.681	339.95 ± 2.91	242.90 ± 3.49	1.38 ± 0.109

Table-1: Effect of silymarin and *T. dioica* pretreatment at seventh day on liver function tests of CCl4-intoxicated mice

Serum Parameters (U/L)	Positive Control	Treatment-ME 200	Treatment-ME 300	Treatment-AE 200	Treatment-AE 300
ALP	20.40%	40.13%	43.47%	18.98%	93.13%
ALT	98.33%	85.62%	82.21%	99.07%	100%
AST	96.29%	93.34%	91.98%	94.21%	98.89%
Total Bilirubin (mg/dl)	100%	89.65%	68.96%	93.1%	75.86%

Table-2: Percentage protection after *T. dioica* extracts pretreatment compares to the standard treatment group

Treatment groups	Percentage protection
Silymarin (100mg/kg) + CCl_4	78.75 %
Methanolic extract (200mg/kg) + CCl_4	77.2 %
Methanolic extract (300mg/kg) + CCl_4	71.65 %
Aqueous extract (200mg/kg) + CCl_4	76.34 %
Aqueous extract (300mg/kg) + CCl_4	91.97 %

Table-3: Percentage protection of silymarin and *T. dioica* leaves extracts against CCl4-induced liver injury

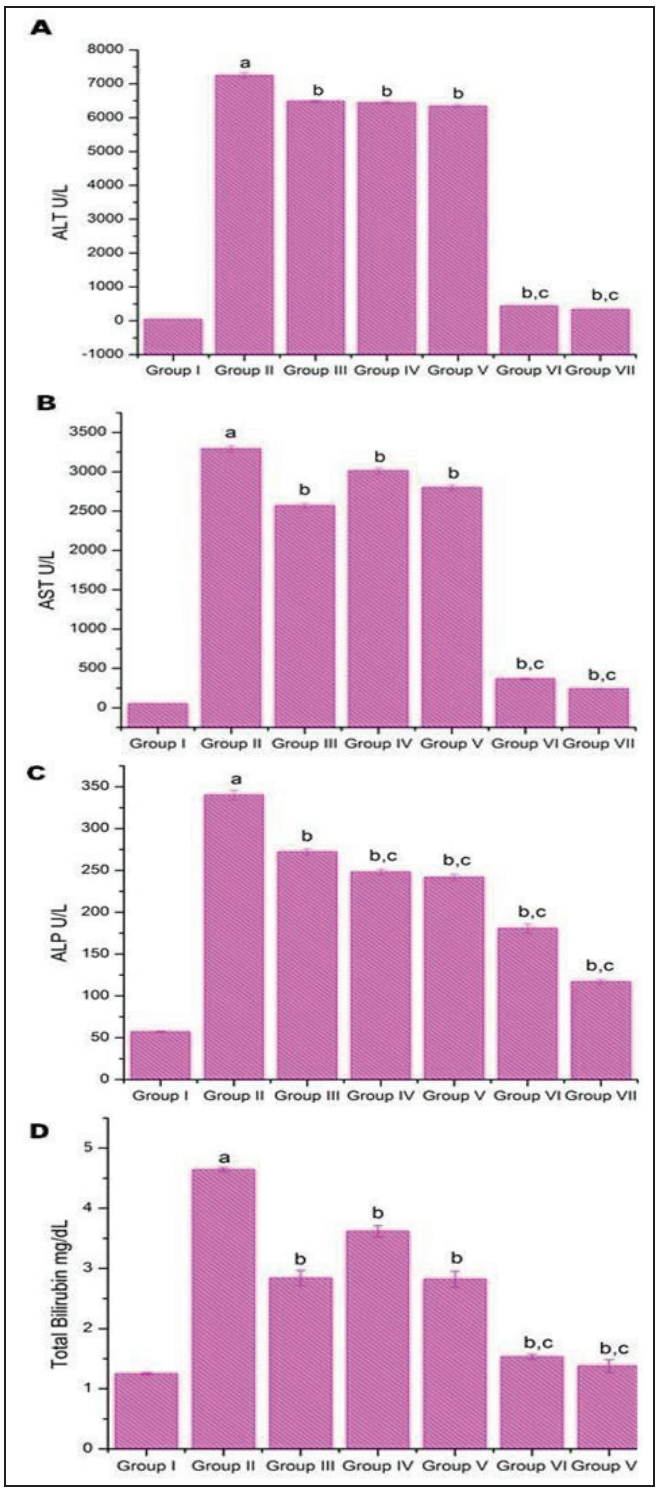

Fig-1: *T. dioica* protects experimental animals from CCl4-induced liver damage and significantly alters the level of serum biomarkers in treatment groups.

Pretreatment with AE of *T. dioica* (300 mg/kg/day p.o) for six days prior to CCl4 exposure (0.5 mg/kg) on seventh day significantly protects hepatocytes in Balb/c mice (n = 6) from liver injury due to the CCl4 as assessed by measuring (A) serum ALT, (B) AST, (C) ALP, and (D) total bilirubin activity. Each value represents mean ± S.E.M. (n=6) at $p<0.001$. [a]$p<0.001$ as compared to group I (normal control). [b]$p<0.001$ as compared to group II (induction control). [c]$p<0.001$ as compared to group III (positive control).

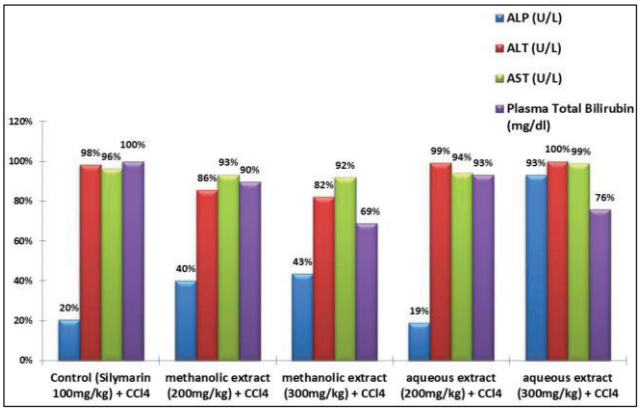

Fig-2: Percentage protection of various biochemical parameters against CCl4-induced hepatic injury.

Percentage protection of treatment groups, when compared to that of induction control group, revealed that aqueous extract (300mg/kg) produced maximum hepatocellular protection as depicted by values of all serum biomarkers.

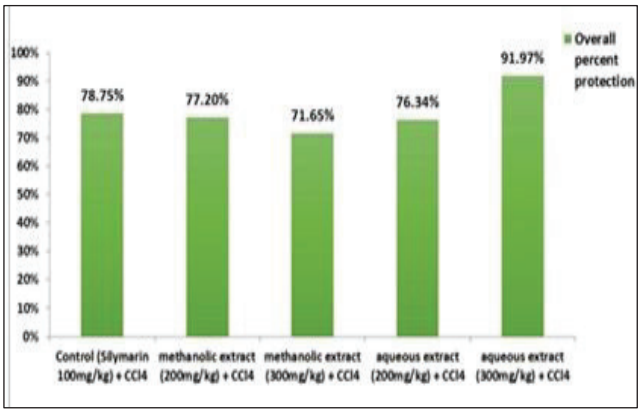

Fig-3: Effects of *T. dioica* extract on hepatic cells protection

T. dioica aqueous extract (300mg/kg) exhibited the maximum overall protection to hepatic cells (91.97 %) when compared to the positive control group (silymarin 100mg/kg) and methanolic extracts (ME-200 and ME-300) respectively.

Fig-4 shows; (A) Normal cells, (B) CCl4-induced cells, (C) Standard or silymarin-induced cells (200 mg/kg), (D) ME-200 + CCl4 treated cell, (E) ME-300 + CCl4 treated cell, (F) AE-200+ CCl4 treated cell, (G) AE-300+ CCl4 treated cell. These results show that pretreatment with *T. dioica* methanolic (D& E) and aqueous (F & G) extracts significantly protects the hepatic cells from injury compared to silymarin (C) and CCl4-induce (B) groups.

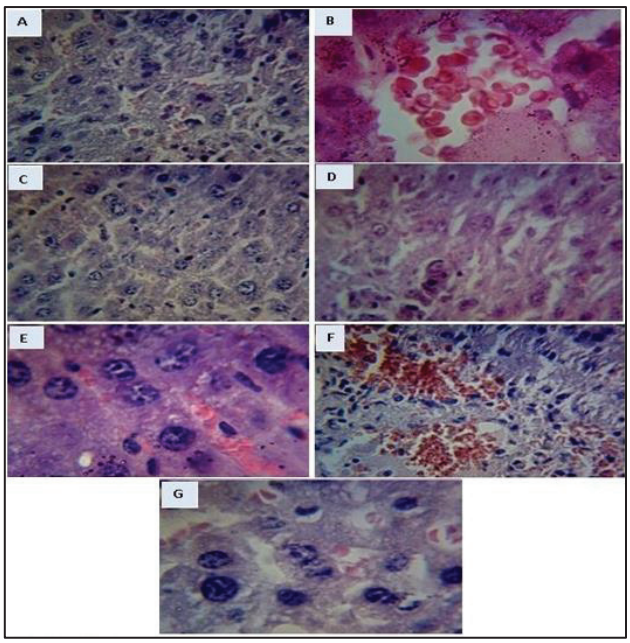

Fig-4: Histopathological examination of liver samples collected from BALB/c mice.

DISCUSSION

Medicinal plants are the most important source of traditional medicine for the majority of the world's population. Natural products have attracted more attention in the last 20 years as potential sources of new antibacterial, antiviral, and phytotoxic chemicals.[20] *T. dioica* requires significant attention due to its biological activities. Previous studies on *T. dioica* phytochemical screening showed that steroids and phlobatannins are present in all parts of the plant, while phenols, flavonoids, and tannins, are present in leaves, flowers, and roots; saponins and terpenoids are present in flowers, leaves, and stems.[21] Studies revealed that plants that contains the highest contents of phenols and flavonoids, are enriched in antioxidants and exhibits their hepatoprotection action against CCl4-induced liver injuries by inhibiting NF-κB, TNF-α, and TGF-α/β signaling pathways.[22,23] Previous studies on phytochemicals screening of *T. dioica* have revealed its strong antiallergic, anti-inflammatory, anticarcinogenic, antiviral, antithrombotic, and hepatoprotective, as well as antioxidant potential.[24]. However, its antioxidant and anti-inflammatory potential supports our findings.[25] The present study on liver-protective action showed that *T. dioica* leaves extracts, both aqueous and methanolic have given promising results against CCl4-induced livers injury in mice and shown relatively great improvement than silymarin. The antihepatotoxic effects of Silybum marianum have been studied several times.[26] *T. dioica* aqueous and methanolic extracts have found non-toxic when administered orally to the animals.[27,28] Animals pretreatment with *Trichosantes dioica* aqueous and methanolic extracts exhibit marked improvement against CCl4-induced hepatotoxicity and also shows significant hepatoprotective effect against CCl4 toxicity in contrast with silymarin.[29] A significantly raised ($p<0.005$) serum level of AST, ALP, ALT, and total bilirubin were observed in CCl4 treated animals (Group II) as compared to the group treated with standard drug or silymarin (Group III).[30] In our study the animal receiving aqueous and methanolic extracts of *T. dioica* (Group IV and VII) show a significant reduction in serum markers levels as a comparison to diseased control. The study further confirms that the administration of *T. dioica* extracts at the dose of 200mg/kg, 300 mg/ kg revert the level of serum enzymes towards normal. *T. dioica* aqueous extracts at the dose of 300 mg/kg show efficient therapeutic effects that are comparable to the standard drug silymarin. No comparative data to support or refute our findings regarding *T. dioica* efficacy against CCl4 were found. Moreover, no drug-induced toxicity symptoms were observed when a high dose of 2000 mg/kg body weight of *T. dioica* aqueous and methanolic extracts were administered p.o. Hence, the study revealed that *T. dioica* extracts are safe and have the potential for future development of advanced hepatoprotective drugs. [31]

CONCLUSION

T. dioica extracts reduced acute CCl4-mediated liver damage in vivo and ameliorated the histopathological as well as biochemical parameters in mice. The *T. dioica* has the potential to alleviate the severity of liver damage caused by carbon tetrachloride. Hence, it is concluded that *T. dioica* can be used as supportive therapy for the treatment of drug-induced and other oxidative stress-mediated hepatoxicity in the future.

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