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Research Article

Hepatoprotective Effects of *Chionanthus Virginicus* Bark Tincture and Saffron Stigma Extract Against CCl_4 Induced Hepatotoxicity in Female Albino Rats

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Abstract

The liver is a vital organ, and allopathic medicines used to treat liver disorders are often inefficient and associated with adverse effects. Therefore, it is crucial to develop safe and effective therapies for hepatic disorders. The present study evaluated the hepatoprotective effects of saffron stigma extract and the herbal medicine *Chionanthus virginicus* (bark tincture), both individually and in combination, against carbon tetrachloride (CCl_4)-induced hepatic injury in female white albino rats. Experimental rats were divided into five groups (n = 5 per group): Group 1, normal control; Group 2, toxicant control, administered a 1:1 (v/v) mixture of CCl_4 in olive oil (0.187 mL/kg body weight) via intraperitoneal injection for 2 weeks; Group 3, treated orally with saffron stigma extract (0.25 mL/kg/day) + CCl_4; Group 4, treated orally with *C. virginicus* (0.2 mL/kg/day) + CCl_4; and Group 5, co-treated orally with saffron and *C. virginicus* + CCl_4. Hepatoprotective effects were assessed by measuring serum hepatic enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin. Lactate dehydrogenase (LDH) was evaluated as a marker of oxidative stress to monitor hypoxic conditions. Rats receiving the treatments showed significant recovery in body weight and a decline in hepatic enzyme levels compared to the CCl_4 -only group. These results indicate that saffron and *C. virginicus* crude bark tincture exhibit significant hepatoprotective effects, mitigating CCl_4-induced liver injury.

Keywords: Hepatoprotective, carbon tetrachloride, *C. virginicus*, Saffron.

1. Introduction

Since past three decades chronic liver diseases have become one of the key health concerns. Far and wide, cirrhosis and hepatocellular carcinoma are considered as prompt factors to comprehend the incidence and prevalence of liver diseases through the whole world. In addition to non-alcoholic fatty liver diseases, increased intake of alcohol, poor drug control and novel viral outbreaks are primarily involved life threatening liver damage [1]. Drug induced liver (DILI) is a significant clinical challenge due to its complex mechanisms and lack of specific biomarkers [2]. DILI is generally a prospective study that offers clinical data and biological samples of specimens to diagnose hepatopathology on the basis of abnormal hepatic functions [3]. DILI is most commonly known to induce through carbon tetrachloride (CCl_4). In numerous experimental research studies CCl_4 is utilized as a model hepatotoxicant in mice and rat species to induce oxidative hepatocyte injury that confers transmutation of CCl_4 to highly reactive trichloromethyl free radicals ($^oCCl_3$) via cytochrome P450 enzymatic system which consequently affect various cellular mechanisms [4]. CCl_4 intoxication lead to an elevation in hepatic-serum enzymes i.e. ALT, AST and ALP

and may produce remarkable hepatocytes lesions coupled to decrease in body weight [5].

Although certain allopathic medications are available to cure hepatic infections but their lethal side effects foreground the necessity of alternative therapeutic approaches. A number of herbal formulations have been recognized to be potentially advantageous in control and treatment of hepatic ailments due to their safe mode of action and relatively fewer side effects than synthetic drugs. Botanical extracts have been a part of traditional medicine for decades. These herbal extracts have different organic materials that play a key role in fighting and prohibiting liver diseases via their specific active ingredients[6]. Considerable attention has been focused on naturally occurring plants containing anti-oxidant and anti-carcinogenic agents[7]. Since ancient times, bark tincture of *Chionanthus virginicus* (fringe tree) had been utilized as hepatic stimulant to cure liver hypertrophy. The bark contains some phytochemically significant agents i.e. lignin's and secoiridoids responsible for its anti-oxidant properties [8]. *C. virginicus* can inhibit the development of tumor blood vessel (anti-angiogenic) owing to its phytochemical constituents i.e. saponin that possesses anti-inflammatory and anti-oxidant properties [9]. In traditional fork medicine it was utilized to treat jaundice, liver injury and congestion of central portal vein[10]. Most effectively, it is one of the prominent herbal medicines that play a significant role to stimulated bile flow in combination with other herbs such as barberry and blood synthesis for spleen and other secretory vessels due to its significant flavonoids[11].

Saffron, *Crocus sativus*, member of family *Iridaceae*; A bibliographical survey on saffron biochemical properties in 13 major Islamic Traditional Medicine books demonstrated that it exhibit wide variety of pharmalogical properties i.e. oxytcic, anti-cancerous, anti-asthmatic, anti-depressant etc. It further proclaimed that saffron along with its major bioactive ingredients it contain more than 150 volatile and several other non volatile components to enhance bioavailability and absorption of various other drugs [12]. On the basis of research data available, saffron is composed of mainly three major metabolically active ingredients: Water-soluble carotenoids (*Crocins*) that are known to be anti-inflammatory and retards oxidative stress[13]. One of the major constituent of *Crocins* is (Crocetin) i.e. esters of a polyene dicarboxylic acid that reduce the level of marker of cardiac oxidative stress Lactate dehydrogenase [14], anti- inflammatory in action; (Picrocrocin) Volatile oil that is involve in apoptosis of tumor cells; (Safranal) it is antiangiogenic in its action reacting against cancerous cells [15]. Additionally, proteins, amino acids, sugars, vitamins, flavonoids, minerals and other chemical constituents are present in saffron as by-products. Saffron reduces oxidative stress via scavenging free radicals through its anti-tissue and hypolipidemic properties [16].

The current research work was designed to evaluate the hepatoprotective action of alcoholic extract of *C. virginicus* and saffron stigma extract alone and when co-administrated in an experimental model of carbon tetrachloride induced hepatic intoxication in albino rats.

2. Materials and methods

2.1. Preparation of *C. virginicus* bark tincture

The bark of *Chionanthus virginicus* foregathered during late autumn, it was then washed, dried, powdered and extracted with 20mL ethanol (96% v/v) and kept at 4℃ overnight. The mixture was centrifuged for 15 minutes at 4000 rpm, the supernatant was filtered using Whatman filter paper no.1 and filtered ethanolic extract was prepared with distilled water in 1:1 (v/v) [17].

2.2. Preparation of aqueous extract of saffron stigma

Dried stigma of saffron were extracted and aqueous extract was prepared by maceration method, 8g of stigma powder was macerated in 300 mL distilled water for period of 72h, refrigerated and centrifuged to transfer in a freeze-drier. Then the aqueous extract was evaporated at 40^{o}C, dried and stored in freezer prior to use[18].

2.3. Experimental animals

Twenty five female white albino rats weighing between 120-160g were purchased from Animal breeding Centre UVAS, Lahore, Pakistan. The animals were harbored in polypropylene cages with absolute access on rat pellets and water. The

animals were procured under controlled environmental conditions i.e. temperature (25ºC), humidity (60±10 ºC), following the standard hygienic procedures of rat house. This experimental work is performed in accordance with animal safety protocols established by animal care committee University of Lahore, Pakistan.

2.4. Experimental design

The animals were divided into five groups comprising of five rats each (n=5) for different experimental trials. Group 1 served as normal control, this group was fed with rat pellets and water only for 2 weeks. Group 2 (intoxicated group) were given intraperitoneal dose of CCl_4 (0.187 ml/kg body weight of 1:1 volume dissolved in olive oil). Group 3 was treated with saffron stigma extract at oral dosage of 0.25 ml/kg body weight along with intoxication of CCl_4.Group 4 rats used to receive 0.2 ml/kg body weight of *C. virginicus* bark extract via oral gavage orally for 2 weeks followed by CCl_4, respectively. Group 5 was co-administrated with both natural extracts at their respective dosages.

2.5. Animal dissection

Animals were weighed and sacrificed by cervical dislocation using ether anesthesia at fifteenth day of experiment after being fasted overnight. Blood samples were collected from heart into non-heparinized bottles. The blood was allowed to coagulate and centrifuged at 3000 rpm for 5 minutes. The hemolysis free serum samples were obtained for serum enzyme analysis (i.e. ALT, AST, ALP and bilirubin) and LDH activities using commercially available assay kits.

2.6. Biochemical assay

Following kits were used to perform histochemical assays: **ALT:** SGPT reagent test kit (Method: IFCC-international federation for clinical chemistry and laborartory medicine)/Germany. AST: SGOT reagent test kit (Method: IFCC-international federation for clinical chemistry and laborartory medicine)/Germany. ALP: SR reagent test kit (Method: IFCC-international federation for clinical chemistry and laborartory medicine)/Germany. Total Bilirubin: Diasys: (Diagnostic system GmbH) /Germany. LDH: Roche Diagnostic GmbH (Method: IFCC-international federation for clinical chemistry and laborartory medicine)/Germany.

2.7. Histopathology

After gathering liver samples, they were excised and washed with normal saline solution. The record of each liver was retained carefully, with respect to liver architecture i.e. size, shape, color and existence or absence of any nodule. The specimens placed in fixing agent i.e. Formalin were processed to produce paraffin sections, these samples were fixed for 24 hours to permit chemical and physical alterations to harden and preserve tissue specimens. Following fixation five selected specimens one from each group were dissected and dehydrated by immersing samples within series of alcohol-chain reactions. After that "clearing agent" Xylene displaced ethanol in specimens and lastly it was replaced by paraffin wax. The block fixed with cassette were removed from mound and subjected to microtome. These prepared liver sections were analyzed using microscopic for histopathological alterations.

2.8. Statistical analysis

All data were expressed as Mean±SEM. Significant difference between the studies groups was evaluated using one way analysis of variance (ANOVA) followed by post hoc multiple comparisons test (95% confidence interval), least significant difference (LSD) with SPSS. The sequential difference among means at level of $p<0.05$ was acknowledged to be statistically significant.

3. Results

3.1. Experimental groups and study design

This research work is executed using five experimental groups. A total of 25 albino rats were divided into five groups of five rats each (n=5).These groups were labelled as G_1= Control group; animals were kept under normal conditions, they had free assess to rat feed and distil water, G_2= Carbon tetrachloride (CCl_4); liver injury was induced in rats using intraperitoneal injection of CCl_4, G_3= Saffron stigma extract + CCl_4; as CCl_4 is utilized as an hepatoxicant, toxicity induced via its administration is cured through oral administration (gavage) of saffron, G_4= *C. virginicus* bark tincture + CCl_4; this curative extract was also given orally with help of gavage and G_5= Saffron + *C. virginicus* bark tincture + CCl_4; co-administrated

Table 1. Increase or decrease in body weight and Relative liver weight (RLW) following administration of Saffron and *C. virginicus* alone and in combination to CCl_4 exposed rats following 2 weeks treatment (RLW: CCl_4 > Control).

Groups	No. of rats (n)	(WTI*)g	(WT2*)g	(RLW***)g
G_1(Control)	5	120	132	5.3
G_2(CCl_4)	5	138	113	5.9
G_3(saffron)	5	147	153	7.1
G_4(*C. virginicus*)	5	128	132	6.7
G_5(Combination)	5	150	156	7.0

Note: *Mean weight at Day 1 of experiment. **Mean weight at Day 15 (dissection). ***Relative Liver Weight.

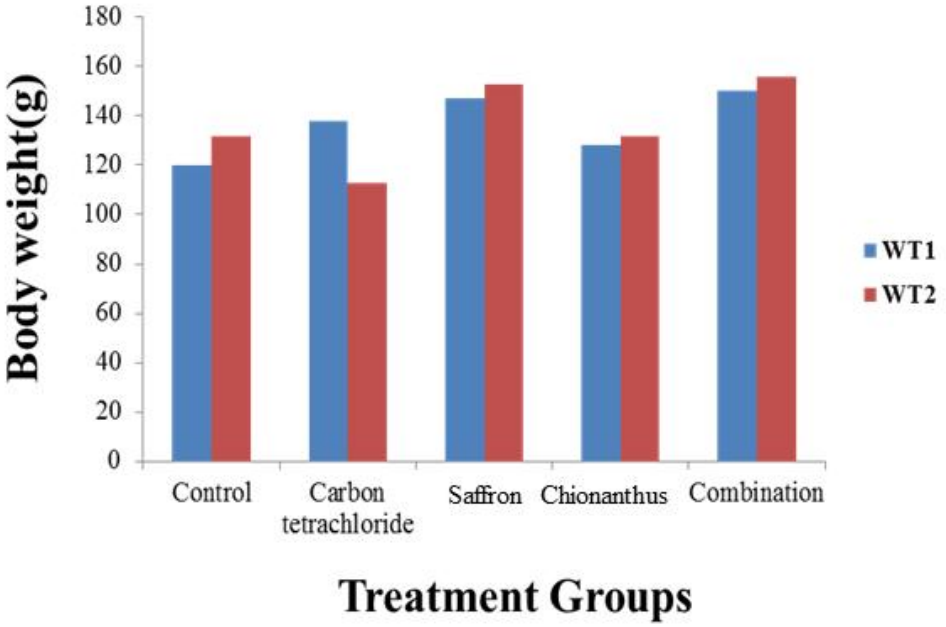

Figure 1. Increase or decrease in body weight following administration of Saffron and *C. virginicus* alone and in combination to CCl_4 exposed rats at day 1 and day 15 of experiment. Order of Body weight recovery is as follow: Combination > *Saffron* > *C. virginicus*.

Table 2. Values of different parameters studied, shown as Mean±SEM for a total of five rats in each group (n=5).

Parameters	ALT (U/L)	AST (U/L)	ALP (U/L)	BIL (mg/dl)	LDH (U/L)
G_1 (Control)	51.6±1.0*	245±7.1*	475±9.6*	0.10±0.0	479±27.7*
G_2 (CCl_4)	57.8±2.8*	241±7.2*	523±20.0*	0.12±0.2*	747±87.6*
G_3 (CCl_4 + Saffron)	56.8±4.0*	239±13.8*	486±31.8*	0.10±0.0	594±44.6*
G_4 (CCl_4 + *C. virginicus*)	46.2±1.8*	219.6±5.7*	486±31.8*	0.10±0.0	508±103*
G_5 (CCl_4 + Combination)	45.0±1.4*	213±3.5*	421±11.1*	0.10±0.0	375.8±86*
*p value	0.004*	0.04*	0.01*	0.43	0.02*

at respective doses. This study prolonged for a period of 14 days, on 15th day animals were weighed and sacrificed, livers were removed and blood was collected for further analysis.

3.2. Body weight and relative liver weight (RLW)

There has been significant increase or decrease in body weight at day 1 and day 15 as well as RLW calculated after sacrificing animals shown in table 1. Significant recovery of body weight had been observed in all treatment groups except CCl_4 inoculating rats. These changes in weights of rats had also been illustrated through Figure 1 using bar graph. It was evaluated that RLW in case of rats with CCl_4 induced toxicity was increased when compared to normal control, which indicated ailment of liver.

3.3. Liver function enzymes and biochemical parameters

The proposed treatment showed significant values for all liver function enzymes except bilirubin whose values remained constant for all groups. In current study values shown via Alanine amino transferase for groups following treatment is like normal control, however rise in values is observed in case of toxicant group.

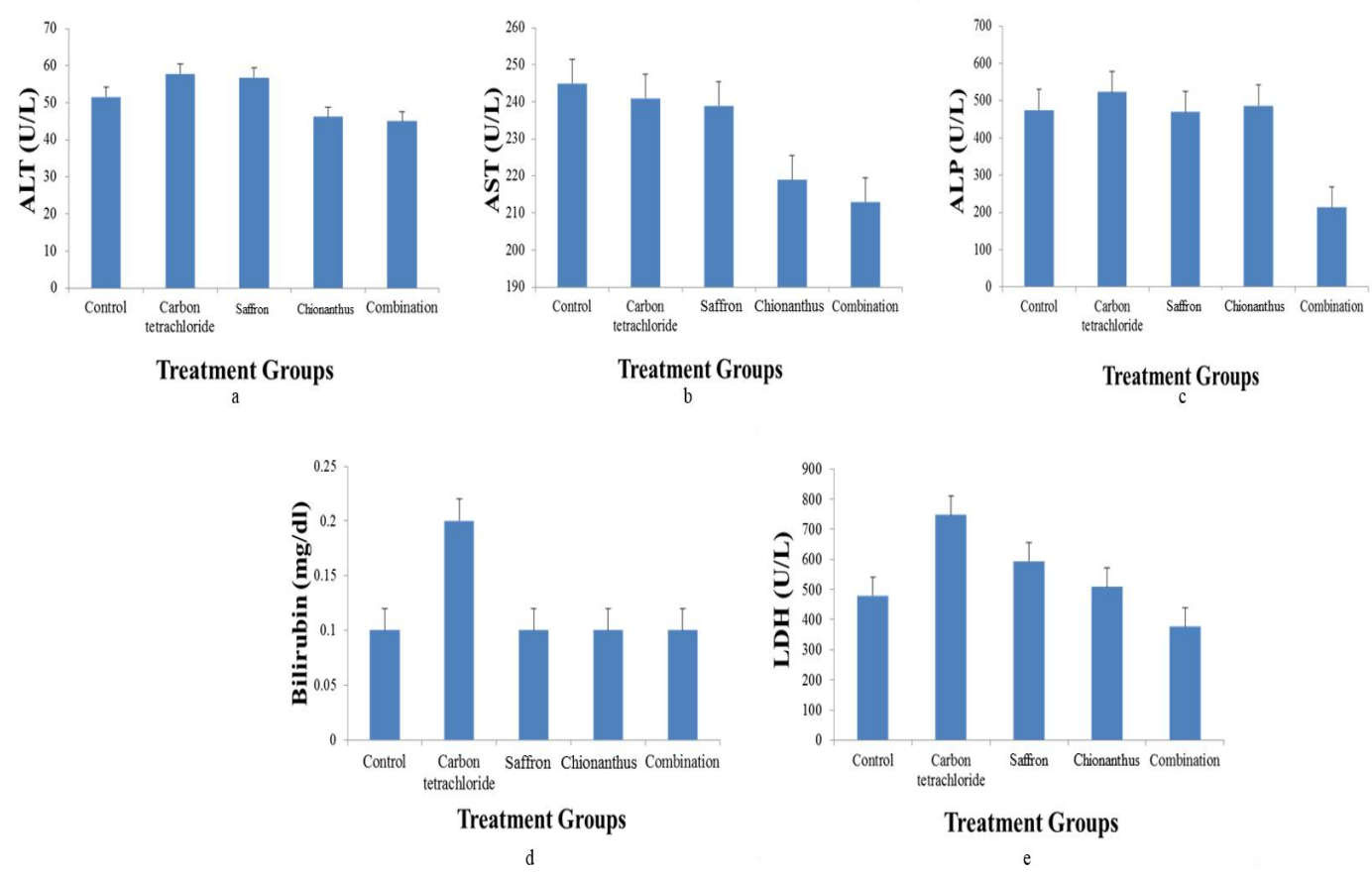

Figure 2. (a) Alanine aminotransferase results following administration of Saffron and *C. virginicus* alone and in combination to CCl_4 exposed rats following 2 weeks treatment. Order of protectivity depicted in graph is as follow: (Combination > *C. virginicus* > Saffron). (b) Aspartate Transaminase results following administration of Saffron and *C. virginicus* alone and in combination to CCl_4 exposed rats following 2 weeks treatment. Order of protectivity is as follow: (Combination > *C. virginicus* > Saffron). (c) Alkaline Phosphate results following administration of Saffron and *C. virginicus* alone and in combination to CCl_4 exposed rats following 2 weeks treatment. Order of protectivity is as follow: (Combination >Saffron > *C. viriginicus*). (d) Bilirubin concentrations remained same in all other groups except for toxicant control group with minute alterations elicited in bars following administration of Saffron and *C. virginicus* tincture alone and in combination to CCl_4 exposed rats. (e) Lactate Dehydrogenase results following administration of Saffron and *C. virginicus* alone and in combination to CCl_4 exposed rats. Order of protectivity is as follow: (*C. virginicus* > Saffron > Combination)

The liberation of these enzymes into the circulation is provoked by hepatocellular injury and not necessarily cell mortality which is a sign of liver damage. Following conclusions demonstrate hepatic success of these compounds against CCl_4 aroused toxicity.

Figure 2 (a), Liver Functional enzyme, ALT activity has long been considered as an indicator of liver injury, in the 14 days experiment for control group is (51.6±1.02), that has been elevated in case of CCl_4 (toxicant control group) to (57.8±2.85) which indicates marked increase when compared with control animals. Hepatic damage changes metabolic function and membrane permeability, consequently leading to release of serum enzymes. This would result in decrease in level of ALT in hepatocytes but elevation in serum ALT. Further, decline of ALT activity is also observed in Saffron (56.8±4.02), *C. virginicus* (46.2±1.88) and co-administration of saffron stigma extract and herbal extract (45.0±1.41) in contrast to toxicant control one.

The values of combination group were more statistically significant than other treatment groups when compared to normal control ($p<0.01$*). Comparing treatment groups with control animals revealed slow healing process of proposed compounds however if the treatment period may prolong they can absolutely recover. Here, the order of protectivity in treatment groups is as follow: (Combination > *C. virginicus* > Saffron). Figure 2(b) demonstrated that AST activity was also increased in CCl_4 mediated group resulting in fatty degeneration; however values were lowered in treatment groups with statistically significant outcomes when compared to control one. AST values in normal control (245±7.1) while in case of CCl_4 it was found (241±7.2) with elevated levels than normal, this observation is consistent with hypothesis of present study. Therefore, maintenance of serum AST to normal values using hepatoprotective agents is of great need. Saffron administrated in 14 days showed the recovery (239.6±13.8) and *C. virginicus* manifested a value of (219.6±5.75) while combination of two exhibit (213.6±3.50). The order of hepatoprotectivity for this liver functional parameter of study is observed under the following trend: (Combination > *C. virginicus* > Saffron).

The values are statistically significant ($p<0.05$*) because treatment groups exhibit mean values lower than intoxicated one and more closely related to control group. The significant value for said parameter is 0.04*.

Figure 2(c) depicted that ALP level was significantly increased in CCl_4 inoculated animals but decrease and recovery of ailment is observed when we moved towards treatment groups of study. ALP has shown value of (474.8±9.6) for normal control, while CCl_4 showed an increased value of (523±20.0) that demonstrated liver injury. Saffron, *C. virginicus* and combination of both showed slight hepatoprotective effect with values (469.6±5.6), (480±31.8) and (421±11.1) respectively. Order of relative protectivity is as follow: (Combination > Saffron > *C. viriginicus*). It showed slow healing mechanism of disease in treatment groups, yet animals are proceeding towards recovery of liver disease. The values observed for following three treatment groups appeared to be statistically significant with ($p<0.05$*) that is 0.01**. Hence, it is depicted that treatment which is utilized to cure liver injury is effective against CCl_4 inoculation.

Figure 2(d) graphical bars showed that Bilirubin values were within the normal range in all treatment groups except for CCl_4 treated with negligible differences which indicates abnormality. Bilirubin concentration is used to assess chemically induced hepatic injury. Liver excretes bilirubin into bile besides its other normal functions, bilirubin content of the blood remained almost same for Control, Saffron, *C. virginicus* and combination of oil and herbal extract with a value (0.10±0.0) except CCl_4 with negligible difference of (0.12±0.02). Similar sized bars shown in figure 2(d) proposed that positive treatment group showed equity in effectiveness against injury in just like control animals but values for this parameter of study are not statistically significant.

Figure 2(e) marked for LDH concentrations that had been tested as a potential biomarker to measure oxidative stress. LDH is marker of oxidative stress inside liver and heart tissue. Increased levels of LDH is a projection of lipid peroxidation, a deteriotive phenomenon of lipids, in hepatic cells of CCl_4

mediated animals values were elevated by (747±87.6) when compared to control group (479.4±27.7) as shown in table 2, LPO activity reserved back by treatment with Chionanthus virginicus herbal extract on CCl_4 induced toxicity. However, no marked difference was observed in case of combination of saffron and bark tincture being applied (375.8±86.14). Treatment with saffron is also affective for LDH (593.6±44.6) yet, *C. virginicus* extract proved to be the best among all other in protecting liver from peroxidative injury. The p value 0.02 ($p<0.05$*) was observed which is statistically significant.

LDH level is probably exceptional prognostic marker ensuring hypoxic conditions within tumor cells in case of CCl_4 treated animals which have been significantly reversed back to normal in C. virginicus as compared to all other treatment groups.

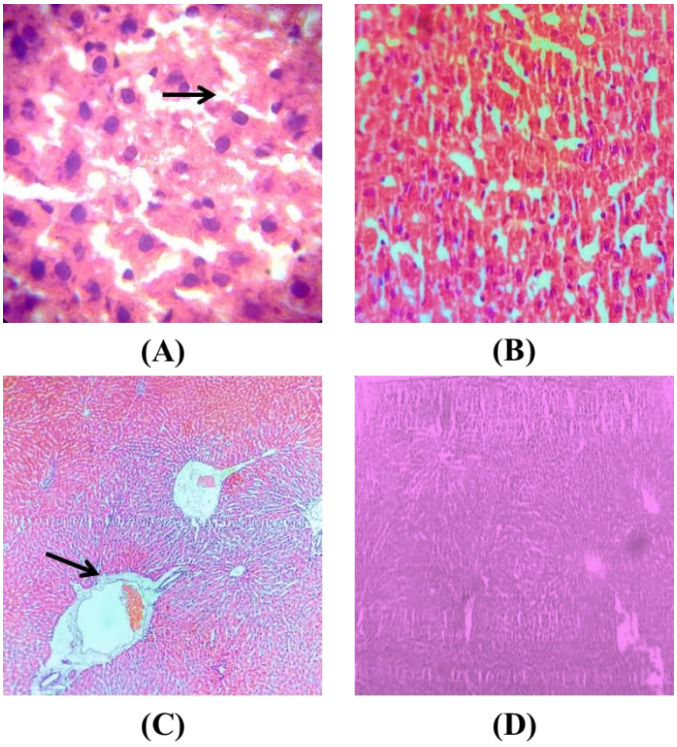

Figure 3. Histopathology of Liver section of rat (Normal Control), normal cytoarchitecture of liver showing central vein, single cell thick, cuboidal shaped hepatocytes separated via sinusoidal spaces along with nucleus located in center which contains one or more nucleoli (observed at A: 100X, B:60X, C: 40X ,D: 10X).

Certain natural compounds including herbs, vegetables, fruits, seeds, roots and other plant parts contain chemical ingredients that exhibit several biological and metabolic activities to be utilized in drug discovery and development. Presently, numerous natural products are clinically offered as powerful hepatoprotective medicines. These formulations showed potential as modern therapeutic agents to cure hepatic ailments after biological evaluation.

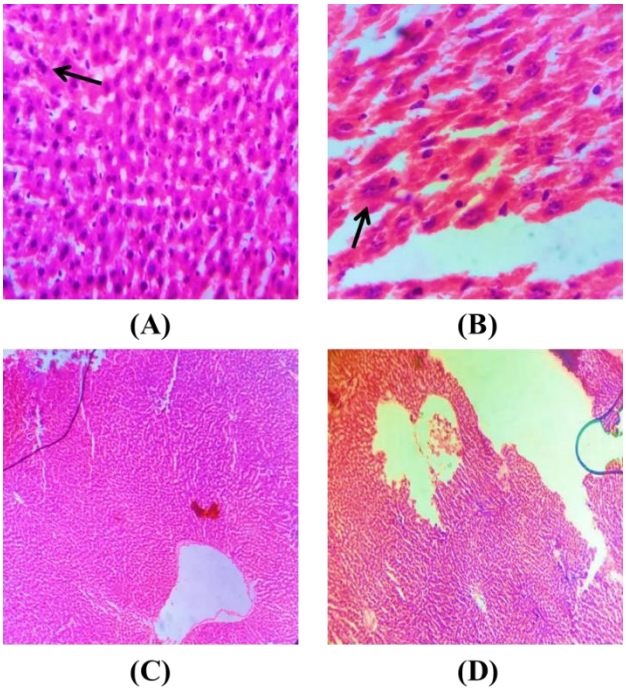

Figure 4. Histopathology of liver section of rat (Toxicant control), showing multinucleated hepatic cells, fat accumulation, active necrosis, inflammation and disarray of hepatocytes (observed at A: 100X B: 60X, C: 40X, D: 10X).

3.4. Histopathological examination

Histopathological examination of liver (Hematoxylin and Eosin H and E stain) from the control group showed that there was no evident noxious liver damage. Animals administrated with CCl_4 at dosage 0.187ml/kg per body weight endowed elevated levels of the liver necrosis in comparison to control group. Microscopic study of Saffron stigma extract has revealed marked improvement in hepatic cells after severe hepatic fibrosis induced by CCl_4. Its cellular array is more or less similar to the normal group. There was no atypical or malignancy in the hepatocytes of liver treated by saffron. *C. virginicus* slide showed no structural abnormality in cells, i.e. no irregularity in cells is described.

However, hepatic degradation in this experimental group could be as a result of animal's enormous activity to get void of

intoxication. Further, mild necrotic alterations were observed in liver cells of rats following co-administration after the induction of hepatotoxicity in rats by CCl_4.

Figure 3 showed, from hepatic histological analysis of control animals' distinct portal cords, rows of hepatocytes and typical array of hepatocyte arranged back to back, nuclei can be seen. Control group exhibit normal hepatic design with discrete liver cells and space of disse separating cuboidal hepatocytes from each other. It was observed that, the hepatocytes were polyhedral in shapes having large rounded vesicular nuclei; hepatocytes appeared to be in proper arrangement and separated from one another by blood sinusoidal spaces. No malignancy, irregularity in cells or pathological change was observed.

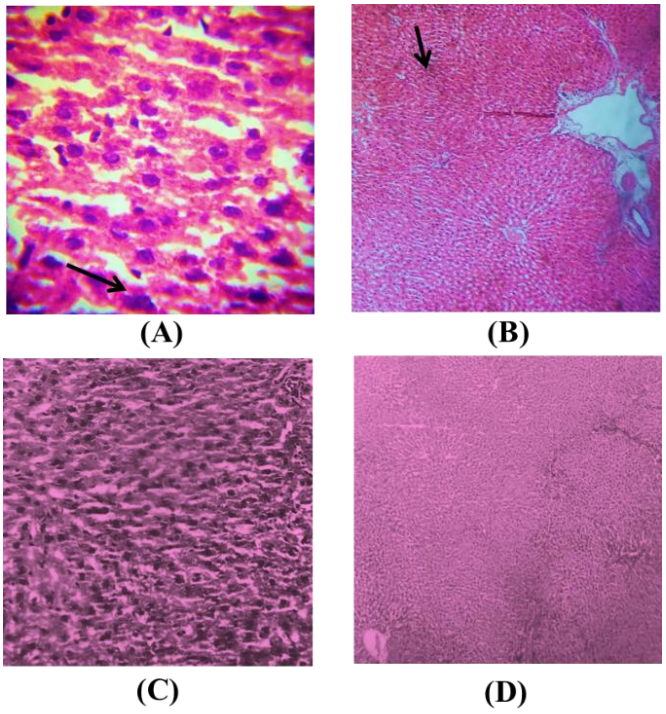

Figure 5. Histopathology of liver section of saffron treated rat, although normal architecture of hepatocytes some areas showed congestion, observed at (A: 100X, B: 60X, C: 40X, D: 10X).

Figure 4, liver histology of CCl_4 treated rats revealed intense centrilobular necropsy, sinusoidal congestion with fatty lesions and hydropic changes and destruction of liver cells in response to injury. Other histopathological changes such as extensive accumulation of connective tissue, few necrotic debris and inflammatory cells that results in the formation of continuous fibrosis septa were detected. Liver steatosis was also observed that leads to liver fibrosis following liver cirrhosis.

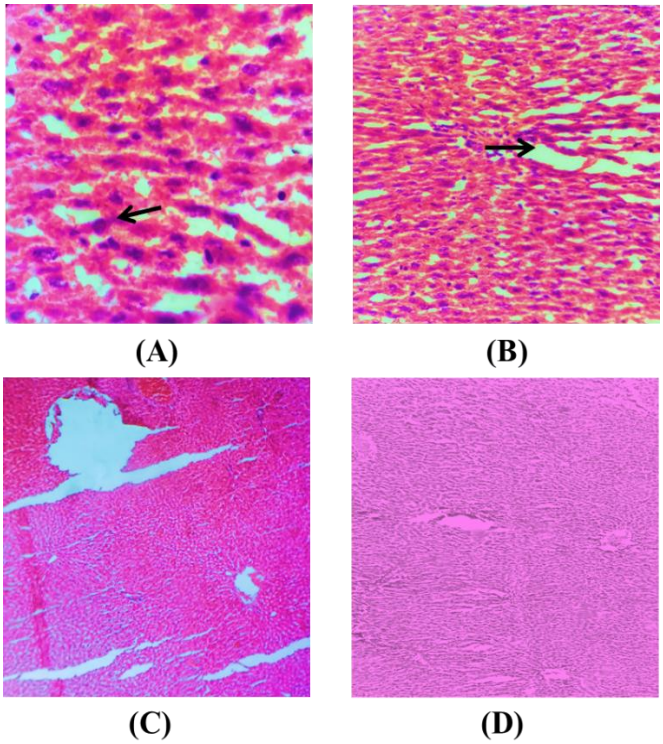

Figure 6. Histopathology of liver section of *C. virginicus* treated rats; liver cell morphology exhibits glandular appearance, with some dilated areas and aggregation of intermediate filaments in cytoplasm (at A: 100X, B: 60X, C: 40X, D: 10X).

Figure 5 depicted that, histological profile of section from Saffron stigma extract (oral) + CCl_4 injected (IP) rats exhibited prominent decline of inflammation, least deterioration of liver tissue lacking necrotic alteration and rare fatty degradation. Liver histopathology of rats treated with saffron observed at different magnification of microscope to interpret any marked changes by this hepatoprotective strategy. Sinusoidal spaces at some areas of liver with small congestion and dilation are observed.

Figure 6 revealed, using contrasting magnification power of microscope it was evaluated that there are some histopathological changes caused after administration of *C. virginicus*. Histological summary of hepatic regions from CCl_4 + *C. virginicus* cured animals showed minute ventricular degradation of hepatocytes, few congested areas with minimal fatty changes. Histopathological overview of this group

demonstrates that *C. virginicus* resorted animals retrieved towards recovery.

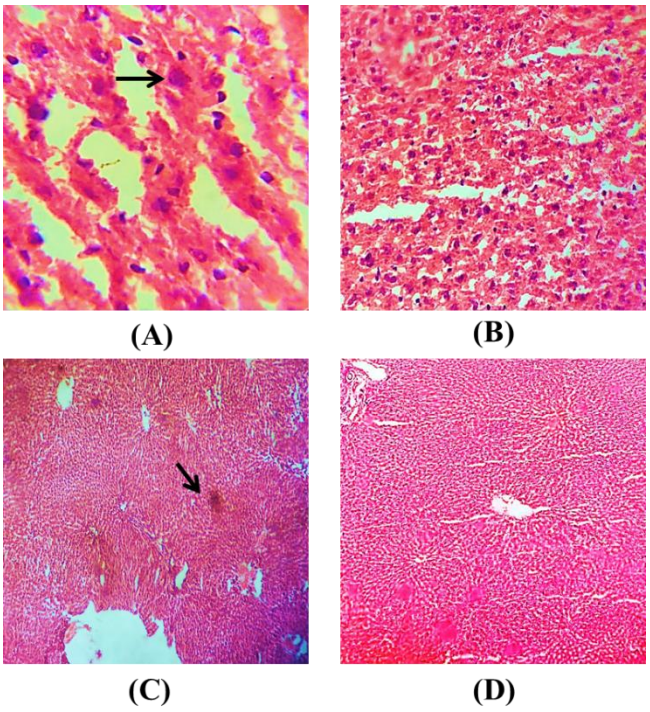

Figure 7. Histopathology of liver section of rats co-administrated with Saffron and *C. virginicus*, exhibiting normal structure however minute dilations along with moderate fatty changes and disarray of neoplastic hepatic cords are observed at (A: 100X, B: 60X C: 40X D: 10X).

Figure 7, Liver sections biopsy of CCl_4 + Combination i.e. co-treated with saffron as well as *C. virginicus* reported very rare hepatocytes destruction, few congested portions beside minimal fatty changes. Hepatocytes with normal array, arranged back to back with their normal shapes are observed just like normal control. Excellent recovery from ailment is observed in this case. Below diagrams are showing histochemistry of rats co-treated with saffron and *C. virginicus*.

4. Discussion

Various research studies validate that DILI is most common cause of hepatic disorders. However, it requires a number of assays to demonstrate whether the possible cause of disease is any signature clinical drug. The susceptibility to DILI is demonstrated by genetic and environmental factors as well. During clinical practices, liver function test (LFT) is used as principle measure to identify liver toxicity. The pathology of DILI is characterized by elevation in serum enzymological markers and histological examination of hepatocytes from demise of minor injury to acute necrosis[19].

An earlier study proposed that administration of CCl_4 at dosage of 0.7 ml/kg body weight for one week resulted in hepatic damage that was evident by rise in serum hepatic functional biomarkers. CCl_4 was also observed to be immunosuppressive in its action as it caused chemotactic migration and cell adhesion in affected areas[20].

Here, experimental model taken for study liver injury is CCl_4 provoked acute hepatotoxicity. The potency of any hepatoprotective drug depends upon how well it can minimize the intoxication and retains standard hepatic physiological conditions that are imbalanced through injecting hepatotoxin. It was investigated from animal models analysis that inoculation of liver injury via administration of CCl_4 depicts an increase in hepatic serum marker enzymes resulting in an acute hepatic destruction. Exceptional elevation in values of serum biochemical parameters and markers of oxidative stress through CCl_4 administration suggest alterations of the liver morphology which is a confirmation of previous studies on the hepatotoxicity of CCl_4. Histochemistry results of present study illustrate rats administrated with CCl_4 show advancement in serum contents of hepato specific enzymes. [21].

The hepatoprotective effects of saffron stigma extract have been investigated against CCl_4 toxicity in female albino rats and it has resulted in significant reduction in levels of hepatospecific serum enzymes, this study highlighted the activity of saffron as a potential anti-cancer agent. Saffron displayed phenolic and flavonoid contents which are responsible for its anti-oxidant activity. Even studies have confirmed that saffron along with its major biological active carotenoid is potent enough to cure various cancers[22].

In search of pharmaceutical substitutes to preclude hepatic damage nutraceuticals or plant based medications have been the subject of many published reviews. These compounds are evolving as a source to offset the harmful effects of allopathic suppositories [23]. The present study aims to provide a scientific basis to effectively cure hepatic injuries.

Table 1 showed increase or decrease in body weight at day 1 and day 15 as well as RLW calculated after sacrificing animals. Significant recovery of body weight had been observed in all treatment groups except CCl_4 inoculating rats. These changes in weights of rats had also been illustrated through Figure 1 using bar graph. It was evaluated that RLW in case of rats with CCl_4 induced toxicity was increased when compared to normal control, which indicated liver injury. A study carried out by Masuda manifested that CCl_4 provoked liver toxicity is a challenging subject for researchers, this compound has played significant role in tissue damage and necrosis involving metabolic activation of reactive free radical oxygen species, eventually leading to decrease in body weight, abnormal metabolism of body and death [24].

Table 2, statistical preview of this study proposed that 2 weeks treatment following oral administration of saffron and *C. virginicus* extract alone and in combination at respective dosages next to CCl_4 administration reversed changes induced via CCl_4. Reduced metabolic activation of carbon tetrachloride by cytochrome activity would going to lower commencement of trichloromethyl free radical formation thus, declined lipid peroxidation that's really necessary in protection against hepatic injury in relation to the current research study. [25]. Earlier works have been publicized on existence of saponin, alkaloids, flavonoids, and glycosides in *C. virginicus* bark tincture. The results of following study plan demonstrate outstanding hepatoprotective action has expressed via ethanol extract of *C. virginicus*. Perhaps it is concerned to bulk of flavonoids available. Initial investigations revealed marked liver defensive action is exhibited by flavonoid compounds that additionally affirmed to own free radical scavenging characteristic. The present work provides evidence of above report [26].

In another study conducted by Iranshahi protective effects of aqueous and ethanol extracts of saffron on hepatic toxicity induced via CCl_4 were observed in mice. It revealed that proposed remedy exhibit anti-oxidant effect and successfully cleaves react oxygen species and fix the hepatic membrane damage [27]. Another study illustrated that saffron stigma contain variety of chemical ingredients such as mineral, vitamins, thiamine, riboflavin, carotene and lycopene. Zeaxanthin along with terpenic essence called safranal is major component of saffron oil. Owing to its medicinal properties, it helped to reduce increased concentration of bilirubin in serum thereby, minimizing the risk of jaundice [28].

Figure 2(a), ALT is a peculiar measure of liver injury, present in its maximum concentrations and it also demonstrated the coherence of hepatocytes. In current study values shown via Alanine amino transferase for groups following treatment is like normal control, however rise in values is observed in case of toxicant group. The liberation of these enzymes into the circulation is provoked by hepatocellular injury and not necessarily cell mortality which is a sign of liver damage.

Figure 2(b), demonstrated that AST activity was also increased in CCl_4 mediated group resulting in fatty degeneration; however values were lowered in treatment groups with statistically significant outcomes when compared to control one. Aspartate amino transferase (AST) is more specifically present in mitochondria of hepatocytes. The increased AST level of CCl_4 treated rats at dose of 0.187ml/kg of body weight is an indication of toxicity. Figure 2(c), depicted that ALP level was significantly increased in CCl_4 inoculated animals but decrease and recovery of disease is observed when we moved towards treatment groups of study. Increase ALP serum level is related to cholestatic liver damage. Decline of serum markers is seen in rats given saffron and *C. virginicus* extract alone and when co-administrated as shown in following research work, it was also in agreement with widely persuaded approach that serum values of these bio-specific enzymes restored back to standard state with the hepatocytes regeneration. Figure 2(d) graphical bars showed that Bilirubin values were within the normal range in all treatment groups except for CCl_4 treated with negligible differences which indicates abnormality.

Figure 2(e) marked for LDH concentrations that had been tested as a potential biomarker to measure oxidative stress. LDH level is probably exceptional prognostic marker ensuring hypoxic conditions within tumor cells in case of CCl_4 treated animals which have been significantly reversed back to normal

in *C. virginicus* as compared to all other treatment groups. LDH activity in HCC has been searched out by different studies, elevated LDH serum levels seem to predict poor results. However, with regard to in vivo hemolysis, it can prove to be a useful precursor, relative abundance of LDH in erythrocytes, results in hemolysis resulting in a marked elevation, which predicts the clinical utility. Increased LDH level is more significantly correlated with tumor size including cell growth and apoptosis[29].

Figure 3 depicted, normal cytoarchitecture of liver showing central vein, single cell thick, cuboidal shaped hepatocytes separated via sinusoidal spaces along with nucleus located in center which contains one or more nucleoli as compared to figure 4, histopathological assay revealed defect extending from heavy tissue necrosis, congested central vein, fatty degradation, atrophy and inflammation of liver cells in CCl_4 injected rats. The hepatoprotective compounds used in following study exhibited sufficient hepatic defense and offers a toxicity barrier as manifested by typical hepatic rows, no necrosis and lesser fatty contents. Figure 5 showed histological overview of saffron treated rats, normal architecture of hepatocytes although some areas were observed with minute congestions. Figure 6, liver cell morphology of *C. virginicus* treated animals' exhibit glandular appearance, with some dilated areas and aggregation of intermediate filaments in cytoplasm. Figure 7, demonstrated cytoarchitecture of animals co-treated with both compounds, normal structure of cells was seen, however minute dilations along with moderate fatty changes and disarray of neoplastic hepatic cords had been noticed. It had been described through series of experimental studies that combination of compounds is always been an effective remedy rather than administrating alone.

5. Conclusion

Evaluation of Saffron and *C. virginicus* alone and when co-administrated for the prevention of CCl_4 induced hepatotoxicity was successfully performed in rats. Both compounds have shown the ability to protect the liver against hepatic injury when CCl_4 was administrated in rats. It was observed from detailed body weight examination, combination of saffron and *C. virginicus* has shown marked recovery in body weight contrasted to administrating these curative compounds individually. Relative body weight (RLW) of CCl_4 treated rats was elevated than control group rats. Moreover, biochemical and histopathological examinations revealed that these compounds have good efficacy in the prevention of hepatic damage caused by CCl_4. Therefore, it is concluded that after treatment with saffron, *C. virginicus* alone and in combination level of hepatospecific serum enzymes ALT, AST, ALP and BIL, marker of oxidative stress LDH and body weight are improved towards normal. This study was exploratory with n = 5 per group; larger, adequately powered studies will be required to confirm these findings and to provide precise estimates of effect sizes.

On the grounds of present research work, it is interpreted that natural protective compounds used in the study possess a promising hepatoprotective effect and both have tendency to be developed as potent hepaprotective drugs in future. This ethno botanical approach would offer promising pharmacological insights by identifying the effect of given natural drug sources on biological pathways, their active ingredients, molecular mechanisms involved and future clinical trials in field of drug development.

Author Contribution Statements

Maharukh Munawar conducted this research work, data collection, formal analysis and preparation of the original draft. Moazama Batool formal analysis and overall manuscript revision with critical guidance. Meerub Sarfraz and Nimra Tahir managed references of the manuscript. Rabia Sundus, Saiqa Rauf and Mamoona Mahmood edited the manuscript.

Ethical approval

The study used only non-invasive techniques, and formal ethical consent was obtained from the Institute of Molecular Biology and Biotechnology, The University of Lahore, Pakistan.

Conflicts of Interest

The authors report no conflicts of interest.

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Data Availability statement

The data presented in this study are available on request from the corresponding author.

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