



Glycyrrhizin, Curcumin and Cinnamon Prevent From Concanavalin-A and Acetaminophen-Induced Liver Injury and Oxidative Stress in Mouse Model

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Abstract

Daily exposure to a number of xenobiotics is the major contributing factor to liver injury. The present study investigated the mechanism of hepatoprotection by glycyrrhizin, curcumin and cinnamon in two distinct models of hepatotoxicity i.e., by using Concanavalin-A (ConA) and Acetaminophen (APAP). For this evaluation, *balb/c* mice were pretreated with glycyrrhizin (200 mg/kg i.g.), curcumin (100 mg/kg i.g.) and cinnamon (200 mg/kg i.g.) extracts for 14 days followed by administration of ConA (15 mg/kg i.v.) and APAP (200 mg/kg i.p.) for 8 hours. At the end of the experiment, mice were dissected and blood and liver samples were collected for biochemical and histopathological analysis. Statistical analysis by using one-way ANOVA followed by DMR test was performed for the significance of results. The results showed that pre-treatment of glycyrrhizin, curcumin and cinnamon ameliorated the damaging effects of ConA and APAP on the liver as indicated by the serum transaminase enzymes and total protein levels. In addition, ConA and APAP exerted severe damage on liver tissues as confirmed from the histopathological analysis. However, glycyrrhizin, curcumin and cinnamon prevented liver injury, possibly through antioxidant mechanism. In conclusion, glycyrrhizin, curcumin and cinnamon possessed antioxidant properties with therapeutic potential in liver injury related to oxidative stress.

Keywords: Cinnamon, Curcumin, Glycyrrhizin, Histopathology, Liver injury, Oxidative stress.

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Contribution of this paper to the literature

The main focus of the study is the use of phytochemicals in oxidative stress-induced ailments. In this study, liver injury was induced by concanavalin-A and acetaminophen in experimental mice and we found that the mechanism of liver injury involves oxidative stress. Then we used phytochemicals i.e., glycyrrhizin, curcumin and cinnamon as pre-treatment for liver injury. We found that glycyrrhizin, curcumin and cinnamon prevent from concanavalin-A- as well as acetaminophen-induced acute liver injury through antioxidant mechanism.

1. Introduction

Chemical and drug-induced toxicity is a major contributing factor in many diseases as it became a major challenge to health [1]. The exact prevalence is difficult to define due to inadequate observation, underreporting and incomplete diagnosis or detection [2]. Due to inevitable side-effects of prescribed drugs, the threat of evolving hepato-renal injury is common. Drug toxicity is mostly involved in liver injuries characterized by fibrosis and scar-formation (cirrhosis), which may progress to liver failure [3]. Prescribed medications e.g., acetaminophen, aspirin, ibuprofen, naproxen and diclofenac are responsible about 50% cases of liver failure [4] and according to data analyses in Iceland and France, liver injury rises about 14-19 per 100,000 populations annually [5]. About >900 drugs, chemicals and herbs are described to cause liver injury [6]. So, these chemicals are purposely used for evaluation in a defined experimental animal models to explore the possible mechanisms of liver injury and some novel therapies for human illnesses [7, 8]. Hepatotoxicity is assessed by certain biomarkers e.g., by measuring serum enzyme activity, including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). In drug or chemical-induced toxicity, serum levels of these markers may increase 10-100 times the upper limit of normal [9].

Concanavalin-A (ConA)-induced acute liver injury is a well-established experimental model of immune cells resembling the autoimmune and viral hepatitis in humans [10]. ConA is a plant lectin obtained from *Canavalia brasiliensis* that induces hepatitis in experimental models by activating T-cells and the release of many inflammatory cytokines (IFN- γ , TNF- α and IL-6) by stimulating Kupffer cells [11, 12]. Overproduction of these inflammatory mediators causes asymptomatic increase in liver transaminase enzyme levels as well as serum proteins, the principal indicators of liver injury [13]. Acetaminophen (APAP), the extensively used antipyretic and analgesic drug, is safe at the prescribed dosage, but an overdose of APAP causes injury of liver tissues resulting in acute and chronic liver failure [14]. APAP-induced acute liver injury in mice is also a widely studied model to detect stress-induced inflammation and acute hepatitis [14, 15]. Conversely, the use of conventional herbal remedies for therapeutic purpose is on the rise. Glycyrrhizin, a glycoside, is obtained from *Glycyrrhiza glabra* (*G. glabra*), generally known as “Liquorice” or “Mulethi”, an important herbaceous plant of the family *Fabaceae* [16, 17]. Curcumin, an alkaloid, is mainly obtained from herbaceous rhizomatous plant *Curcuma longa* (*C. longa*), generally known as “Turmeric” or “Haldi”, an important plant of the family *Zingiberaceae* [18]. Both glycyrrhizin and curcumin have strong anti-oxidant properties of free radicals scavenging activity that may improve the overall oxidative enzyme status and inhibit plasma membrane lipid peroxidation in living systems [19-21]. Cinnamon is commonly used herb and spice in many countries, aboriginal to southeast Asia. This herb is accessible nowadays in two leading forms, i.e., Ceylon and Cassia (*Cinnamomum zeylanicum* and *Cinnamomum cassia* respectively), belong to the family *Lauraceae* [22]. In conventional ayurvedic system of medicine, cinnamon is regarded as an important therapy for digestive, respiratory and gynecological complications [23]. Presently, there is inadequate research for evaluating the ameliorative efficacy of the above mentioned plants constituents, thereby the present study was planned to explore whether a single pretreatment with glycyrrhizin, curcumin and cinnamon protect mice from ConA- and APAP-induced acute liver injury.

2. Materials and Methods

ConA (99% purity) and APAP (99% purity) were procured from Sigma Aldrich. Glycyrrhizin, curcumin and cinnamon plant materials were purchased from the local botanic choice company (Pak) and plant extracts were prepared according to the set protocols. *G. glabra* plant material was identified and deposited in the Herbarium (voucher specimen number 194-1-18), Botany Department, University of Agriculture Faisalabad, Pakistan. Glycyrrhizin was extracted from roots of *G. glabra* by following previously described procedure [24]. Curcumin was obtained from commercially available capsules of *C. longa* (Sigma). The plant material of *C. zeylanicum* was prepared, identified and deposited in the Herbarium (voucher specimen number 244-1-18), Botany Department, University of Agriculture Faisalabad, Pakistan and the extract was prepared by following previously described procedures [25, 26]. The crystalline powdered ConA was reconstituted (3 mg) in 1 ml of phosphate buffered saline solution. In accordance with total dose calculation, 16 mg of ConA was dissolved in 5.33 ml of phosphate buffered saline. The final solution was mixed for 10 minutes by vortex mixer (Mini tube vortexer, AS-MTV-1-1009) and aliquots were prepared in eppendorf tubes (1.5 ml, natural polypropylene conical). Acetaminophen was reconstituted in water. The solution was vortexed using vortex mixer (Mini tube vortexer, AS-MTV-1-1009) and aliquots were prepared in subsequent eppendorf tubes. All the components were prepared in the post-graduate physiology lab of Institute of Physiology and Pharmacology, University of Agriculture Faisalabad, Pakistan.

2.1. Animals

Healthy adult Balb/c mice of both sex (20 ± 3 g weight) were arranged and caged group-wise (n=6). Mice were maintained in the animal housing facility at a temperature $25 \pm 4^\circ\text{C}$ and relative humidity of $55 \pm 2\%$. The animals were observed daily and body weight was determined at regular intervals throughout the experimental period. The experimental mice were provided with local feed (mouse chow diet # 14) *ad libitum* till the completion of trial.

2.2. Experimental Design and Drug Administration Protocol

After 1 week of adaptation, mice were divided randomly into various groups and subjected to treatments. To explore the protective effects of glycyrrhizin, curcumin and cinnamon, mice were subjected to pretreatment of plant

constituents for 14 days. Then at 15th day, the groups were further exposed to administration of vehicle, ConA (15 mg/kg) and APAP (200 mg/kg) during 8 hours. At the end of trial, blood and liver tissue samples were obtained for investigation.

2.3. Blood and Liver Samples Collection

Mice were dissected subsequently at the end of trial. Blood samples were collected in clot activator tubes (Gel & Clot Activator, Xinle, China) and centrifuged (80–2 Centrifugal Machine, China) at 1010 x g for 15 min. The collected serum was placed in eppendorf tubes and stored in the biomedical freezer (Sanyo Biomedical Freezer, MDF-U333, Japan) at -30°C until analysis. The collected liver samples were weighed and placed in freshly prepared buffered solution of formalin (10%) for 1 week. The organ sections were evaluated histopathologically with compound microscope (IM-910 IRMECO GmbH & Co; Germany) equipped with a camera (TOUPCAM, ToupTek Photonics Co., Ltd; China). The extent of organ injury was assessed on the basis of individual pathologic lesions in each section stained with H&E staining.

2.4. Biochemical Parameters

The serum sample was utilized for evaluation of liver function markers by using automated biochemistry analyzer (BioLab-310) and microplate spectrophotometer (Thermo Scientific Multiskan GO™ equipped with SkanIt software version 4.1). The ALT and AST levels were measured in accordance with previously described methods [27-29] by using commercial biochemical kits; ALT (DiaSys GmbH, Germany) and AST (DiaSys GmbH, Germany) respectively. Serum total protein was determined by using commercial biochemical kit; Total Protein (BioRays Lab, Pakistan).

2.5. Histopathological Procedure

Histopathological analysis of collected tissue samples was performed in accordance with previously described method [30, 31], started from fixation, dehydration, clearing, infiltration, embedding, sectioning, mounting and then staining.

2.6. Statistical Analysis

The effect of drug treatments was assessed statistically by applying one-way ANOVA and DMR post hoc test. For two groups' comparison, Mann-Whitney t-test was applied. Data were expressed as Mean±SE with the level of significance set at 5%, using SPSS software (21.0).

3. Results

3.1. Glycyrrhizin and Curcumin Ameliorated ConA-Induced Liver Injury

To evaluate the protective potential of glycyrrhizin and curcumin, mice were pre-treated with glycyrrhizin and curcumin intragastrically for 14 days before single dose exposure of ConA (15 mg/kg). The significant increase ($p < 0.05$) in transaminase levels (both ALT and AST) was noted after ConA administration in the vehicle-treated group, whereas these levels were decreased significantly ($p < 0.001$) in the glycyrrhizin- and curcumin-pretreated groups as compared to that of vehicle control group Figure 1. Moreover, glycyrrhizin and curcumin pretreatment ameliorated the ConA-induced high levels of serum protein and albumin Figure 2. Gross examination of liver samples from glycyrrhizin and curcumin pretreatment mice groups appeared normal with no apparent damage or coloration Figure 3a. Histopathological examination showed that the degenerative changes at 8 hrs ConA-post exposure were attenuated by glycyrrhizin and curcumin pretreatment Figure 3b.

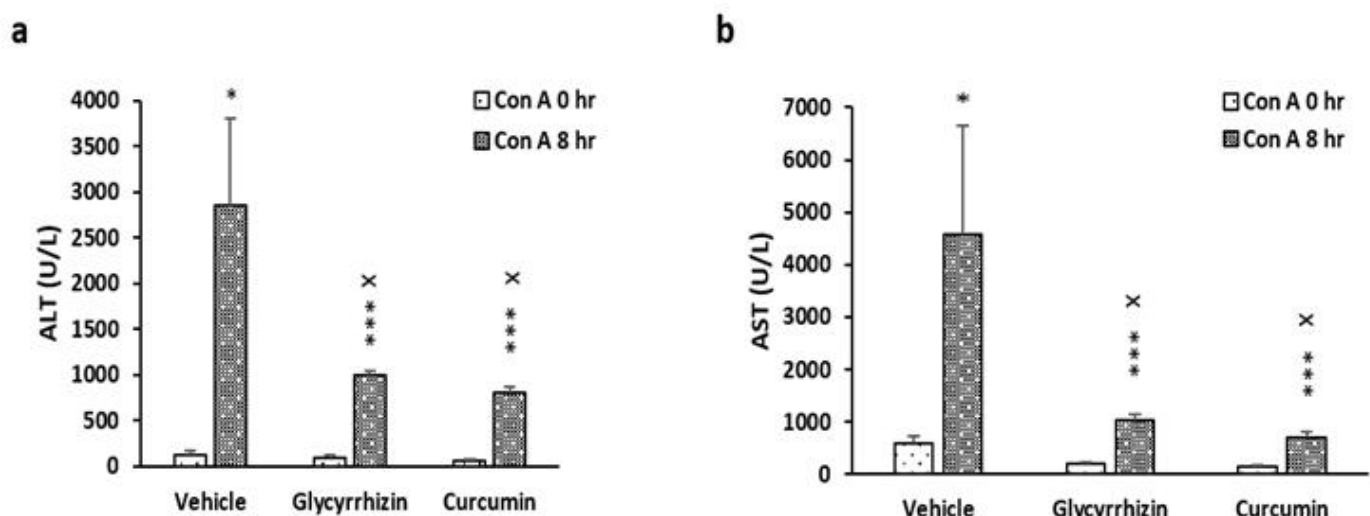


Figure 1. Effect of glycyrrhizin and curcumin pretreatment on serum liver function markers.

Note: (a) ALT and (b) AST. * $P < 0.05$ and **** $P < 0.001$ when compared at 0 and 8 hour points, while at x $P < 0.05$ when compared at similar time points.

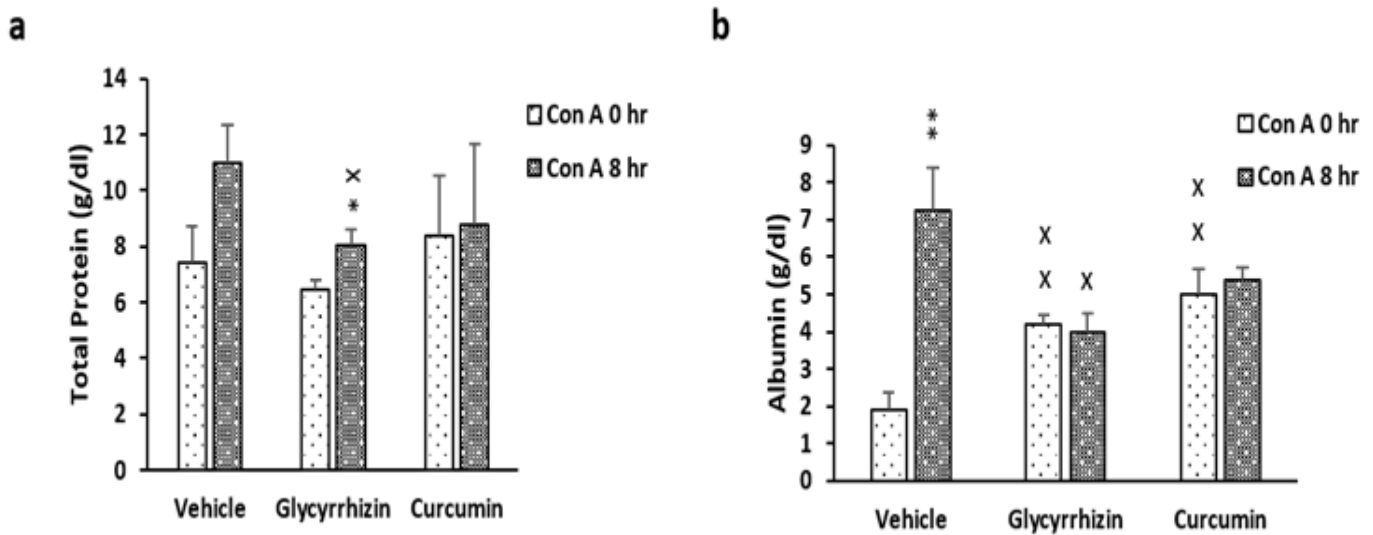


Figure 2. Effect of glycyrrhizin and curcumin pretreatment on serum total proteins.

Note: (a) Total protein and (b) albumin. *P < 0.05 and **P < 0.01 when compared at 0 and 8 hour points, while at x P < 0.05 and xx P < 0.01 when compared at similar time points.

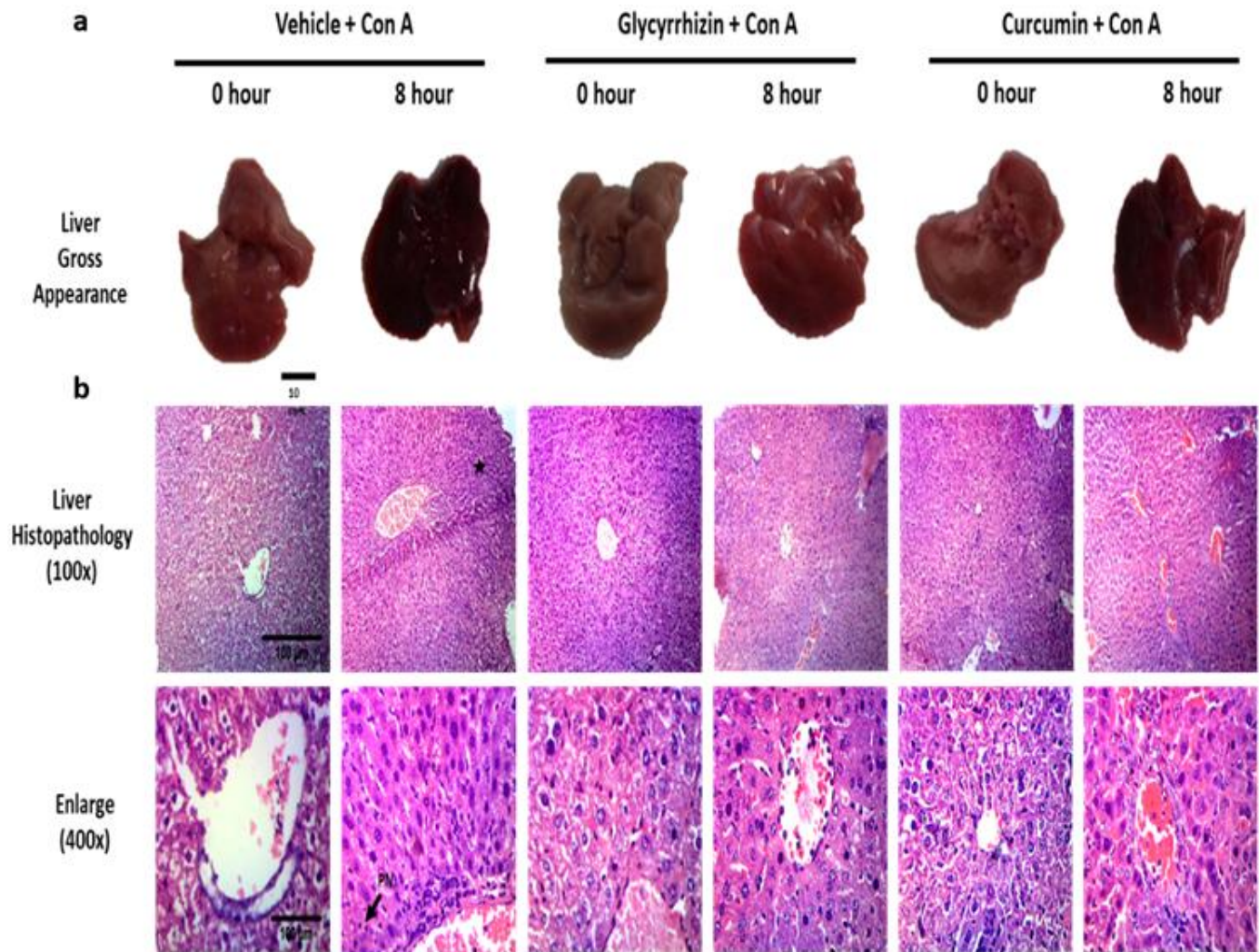


Figure 3. Effect of glycyrrhizin and curcumin pretreatment on liver tissue structure.

Note: (a) Macroscopic view of representative liver from ConA-administered mice group after 8 hours showed rough surface of the liver and the development of nodular firm liver with dark reddish brown color. The ameliorative effect of glycyrrhizin and curcumin pretreatment in ConA-induced liver injury was assessed by (b) histopathological examination of representative liver sections. Glycyrrhizin and curcumin pretreated groups exhibited minimal hepatocytes degeneration (H and E stain, 100x and 400x) with restoration of tissue parenchyma. PN, pyknotic nuclei; Star, cellular infiltration.

3.2. Glycyrrhizin and Curcumin Possess Antioxidant Properties

To investigate whether the hepatoprotective effects of glycyrrhizin and curcumin involve antioxidant mechanisms, we evaluated serum levels of TOS and TAC. While ConA exposure caused oxidative stress, glycyrrhizin and curcumin pretreatment for 14 days significantly decreased ($p < 0.001$) the oxidative stress and increased the overall antioxidant capacity ($p < 0.01$) in mice **Figure 4**. These results indicated that glycyrrhizin and curcumin exert hepatoprotection through antioxidant mechanism.

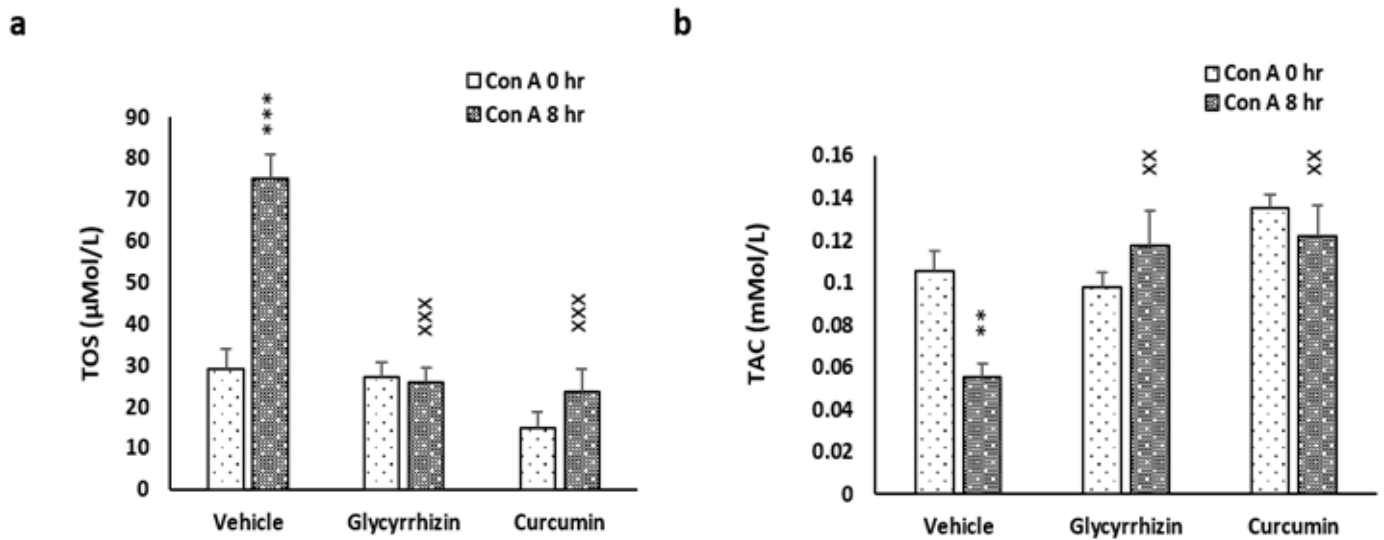


Figure 4. Effect of glycyrrhizin and curcumin pretreatment on serum oxidant/antioxidant status.

Note: (a) TOS and (b) TAC. **P<0.01 and ***P<0.001 when compared at 0 and 8 hour points, while at xx P<0.01 and xxx P<0.001 when compared at similar time points.

3.3. Cinnamon ameliorated APAP-Induced Liver Injury

Results indicated that cinnamon pre-treatment protected the mice from APAP-induced liver injury due to an increased levels of serum ALT and AST Figure 5. Analysis of serum for proteins indicated a significant increase in total protein levels as well as albumin levels in APAP-treated mice groups (p<0.01 and 0.001). In the cinnamon pretreated mice group, no significant increase in total serum proteins and albumin levels was observed as was in APAP-treated mice group (p>0.05) as shown in Figure 6. Investigation of the gross and histopathological alterations in the liver tissues resulted that cinnamon pretreatment reduced the extent of liver damage by normalizing the liver structure. Histopathological analysis showed that cinnamon pretreatment restored the parenchymal structure of the liver tissue Figure 7.

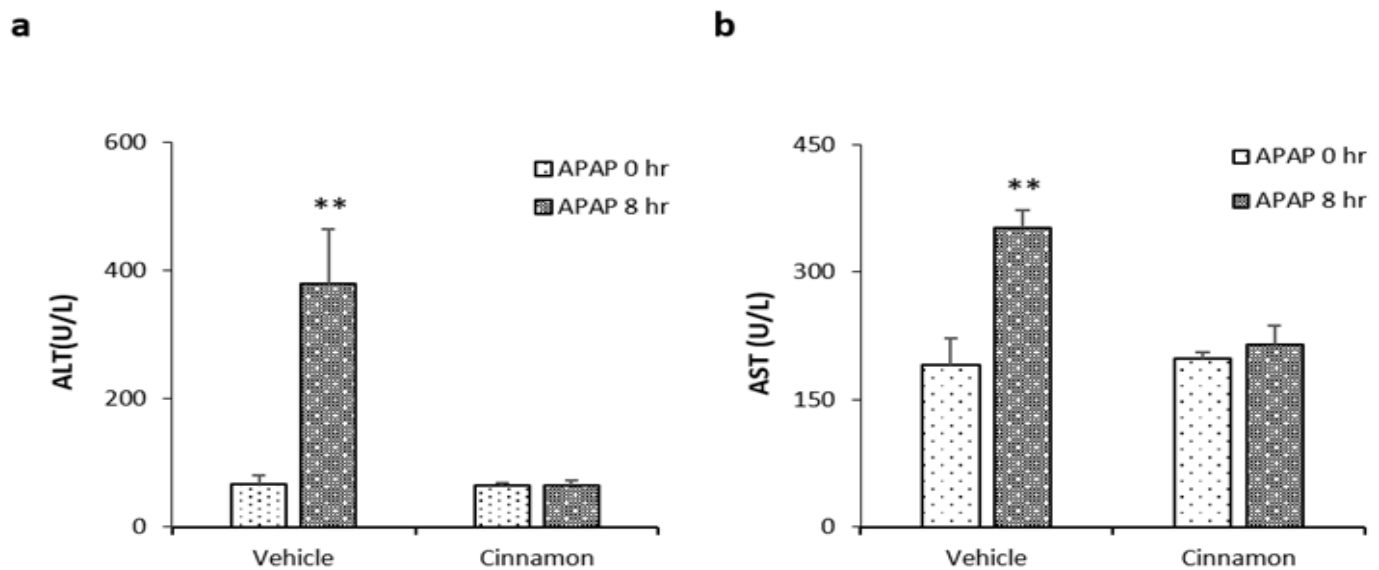


Figure 5. Effect of cinnamon pretreatment on serum liver function markers.

Note: (a) ALT and (b) AST. **P<0.01 when compared 0 and 8 hr points.

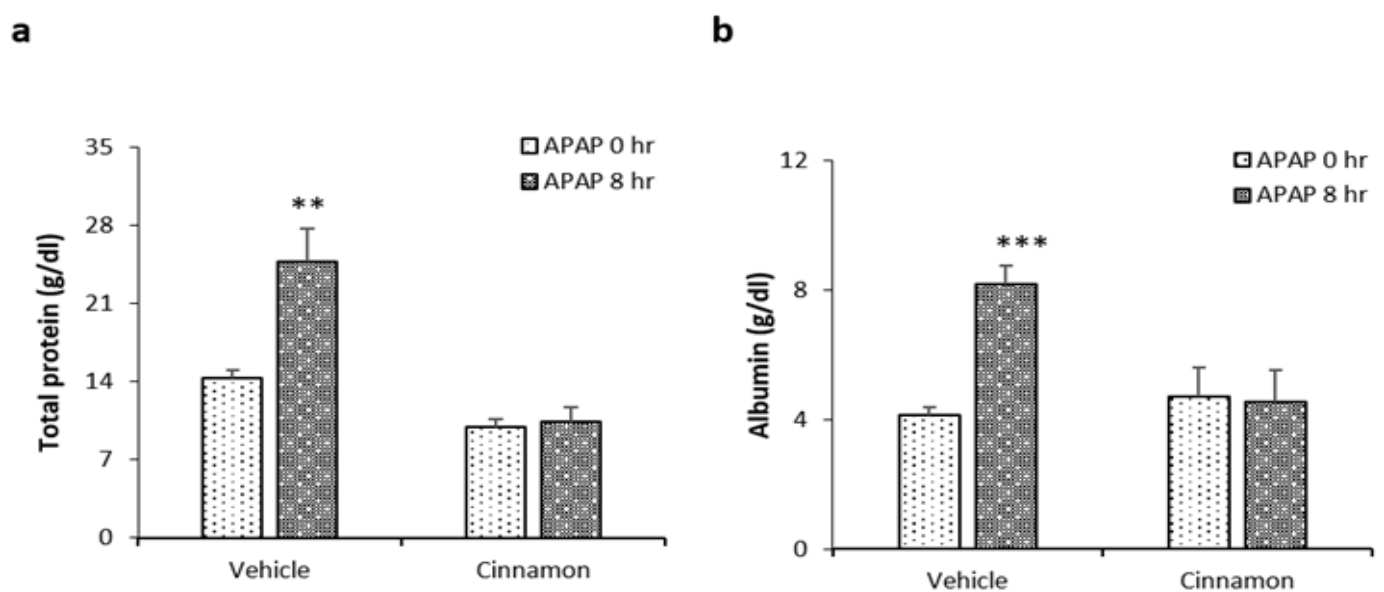


Figure 6. Effect of cinnamon pretreatment on APAP-induced serum total protein alteration.

Note: (a) Total protein and (b) albumin levels. **P<0.01 and ***P<0.001 when compared at 0 and 8 hr points.

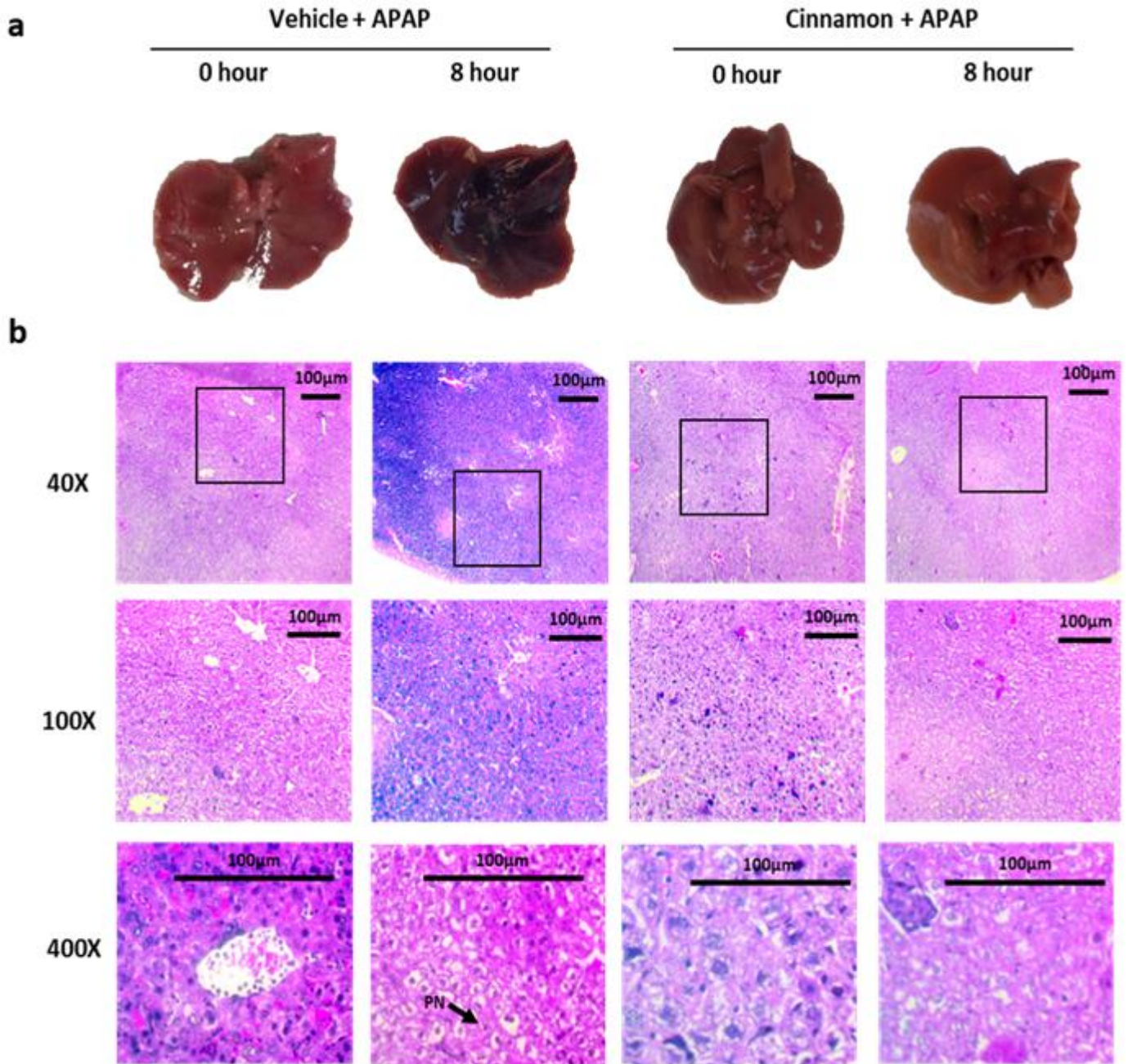


Figure 7. Effect of cinnamon pretreatment on liver tissue structure.

Note: (a) Macroscopic and (b) histopathological examination of the liver from APAP-administered mice group after 8 hours showed the development of rough surface with dark reddish brown color. The ameliorative effect of cinnamon pretreatment against APAP was assessed histopathologically of liver sections (H and E stain, 40x, 100x and 400x). APAP, acetaminophen; PN, pyknotic nuclei.

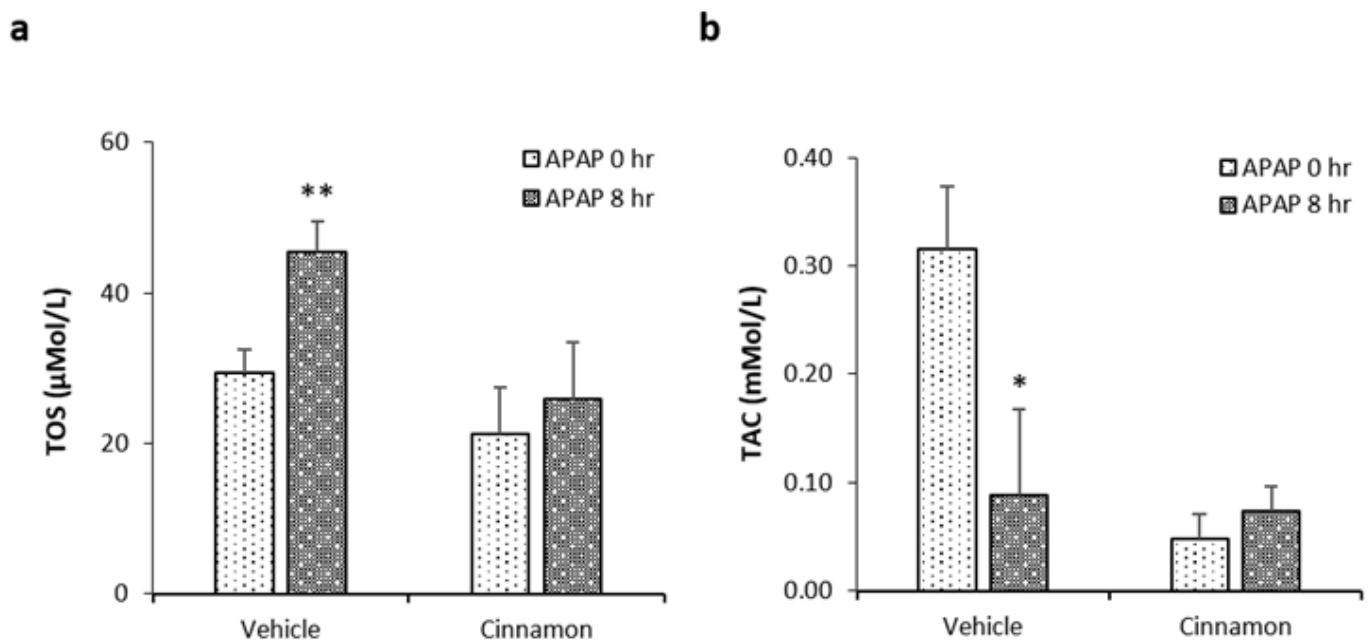


Figure 8. Effect of cinnamon pretreatment on APAP-induced oxidative stress.

Note: (a) TOS and (b) TAC. *P<0.05 and **P<0.01 when compared at 0 and 8 h points.

3.4. Cinnamon Possesses Antioxidant Properties

Exploring APAP-induced organ injury involves oxidative stress, we speculated if the ameliorative potential of cinnamon is owing to its antioxidant properties. So, we evaluated serum oxidant/antioxidant markers and observed that TAC was decreased in APAP group, whereas cinnamon pretreated group presented significant increase in

antioxidant capacity. Likewise, APAP exposure caused significant increase in TOS that was decreased by pretreatment of cinnamon [Figure 8](#).

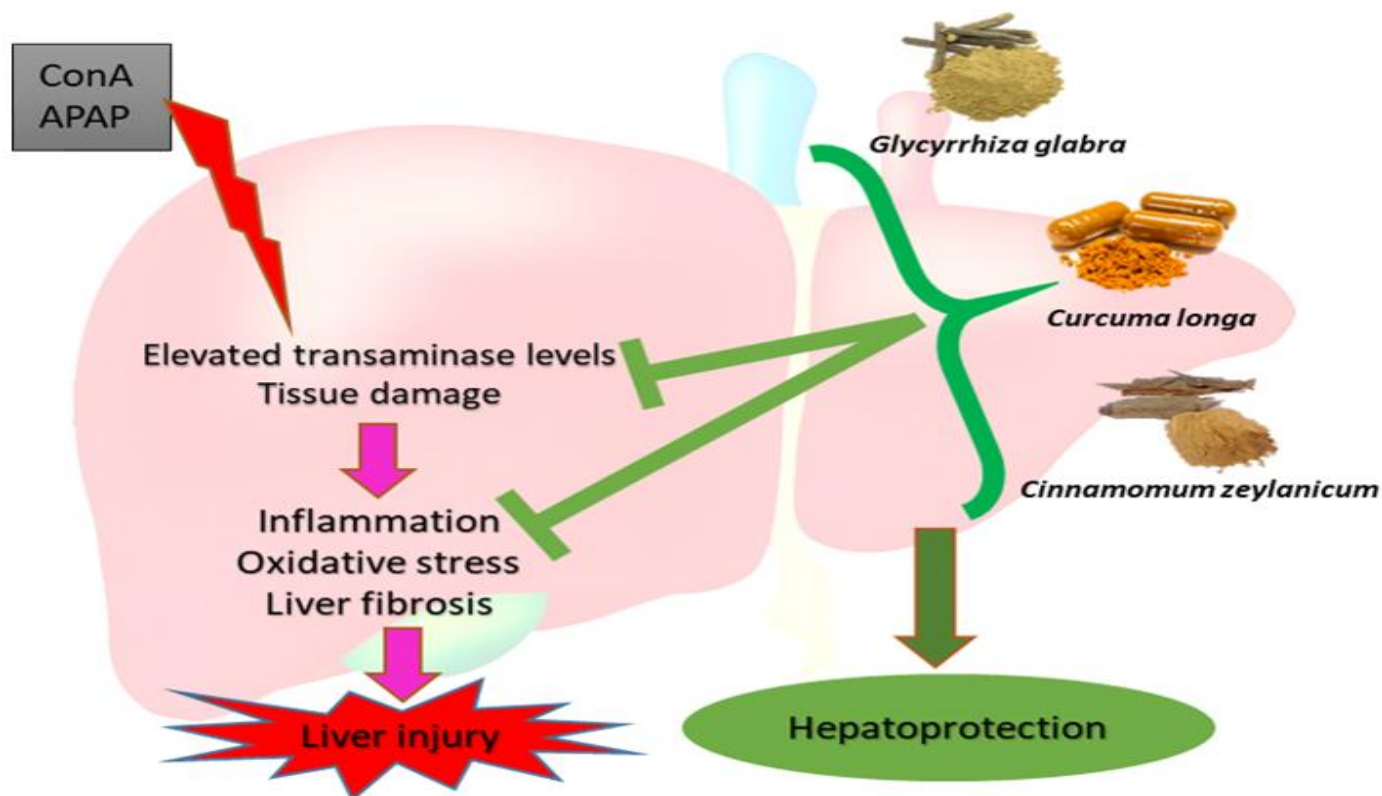


Figure 9. ConA- and APAP- induced acute liver injury is ameliorated by glycyrrhizin, curcumin and cinnamon. Liver injury is indicated by elevated transaminase levels as well as oxidant/antioxidant parameters of tissue damage. The use of glycyrrhizin, curcumin and cinnamon display hepatoprotective effects by decreasing the elevated transaminase levels as well as oxidative stress. ConA: concanavalinA, APAP: acetaminophen.

4. Discussion

Liver injury is comprised with the elevated transaminase levels arise from liver into the systemic circulation because of an increased fluidity and permeability of membranes around organelles and cells [32]. Metabolic alterations in the liver function outcome in elevated serum transaminase levels [33]. The presence of high globulin and albumin in blood circulation is also a distinctive feature of acute and chronic liver diseases. Even a single dose exposure of ConA and the overdose of APAP to experimental animals induces liver injury [34], pathogenesis of which includes cell damage and oxidative stress provocation. In our experiment, we found antioxidant and preventive potential of glycyrrhizin, curcumin and cinnamon against ConA- and APAP-induced liver injury.

In the present study, following ConA-administration to mice, we measured serum ALT and AST levels along with serum total proteins after 8 hours of exposure. The increased levels of transaminase, total protein and albumin after ConA administration indicated ConA-induced liver injury. Glycyrrhizin and curcumin pretreatment inhibited the ConA-dependent increased levels of transaminase, total protein and albumin. Both glycyrrhizin and curcumin are good hepatoprotective agents and have been used effectively in clinical trials to treat viral hepatitis [35, 36]. Likewise, curcumin treatment inhibited the increased levels of AST and ALT in chemical-induced hepatotoxic model [37]. On the other hand, an overdose of APAP causes glutathione depletion and the accumulation of toxic metabolite (NAPQI) that is responsible for the formation of cytotoxic protein adducts and causes hepatocytic necrosis [38] as a result of elevated transaminase levels. We found that cinnamon significantly restores the elevated transaminase levels.

As known, liver synthesizes majority of the circulating proteins [39], albumin being the most abundant one (60% of total serum protein) involved in the maintenance of body fluids, oncotic pressure and transportation of hormones and drugs [40]. Its level increases in viral hepatitis [41] and liver toxicity [42]. Glycyrrhizin and curcumin prevents liver fibrosis in many induced injuries of the liver [43-46]. In our experiment, serum levels of protein and albumin were increased at 8 hours after ConA and APAP exposure in mice. These elevated levels were returned to normal range by glycyrrhizin, curcumin and cinnamon pretreatment.

Gross appearance of liver showed a degenerated pattern with appearance of rough nodular surface and scar formation, the characteristic hallmarks of ConA-toxicity. Glycyrrhizin and curcumin pretreatment prevented from liver scarring and maintained the liver architecture. The histopathological analysis of liver sections identified marked inflammatory pattern and areas of degenerative parenchyma of ConA-toxicity that were ameliorated with glycyrrhizin and curcumin pretreatment. Similarly, cinnamon pretreatment also restored the normal architecture of the liver that was degenerated due to APAP overdose. In this way, pretreatment with glycyrrhizin, curcumin and cinnamon ameliorated the acute liver injury. After biochemical and histopathological analysis, we speculated if the protective effects of glycyrrhizin, curcumin and cinnamon might be owed to antioxidant potential. So, we measured the serum levels of TOS and TAC. We found that pre-treatment with glycyrrhizin, curcumin and cinnamon decreased oxidative stress presumably by phase II enzymes activation that are responsible for keeping cellular oxidant/antioxidant balance [17, 47]. Thus, the antioxidant properties of glycyrrhizin, curcumin and cinnamon were verified [Figure 9](#). The overall representation of the research project is shown in [Figure 10](#).

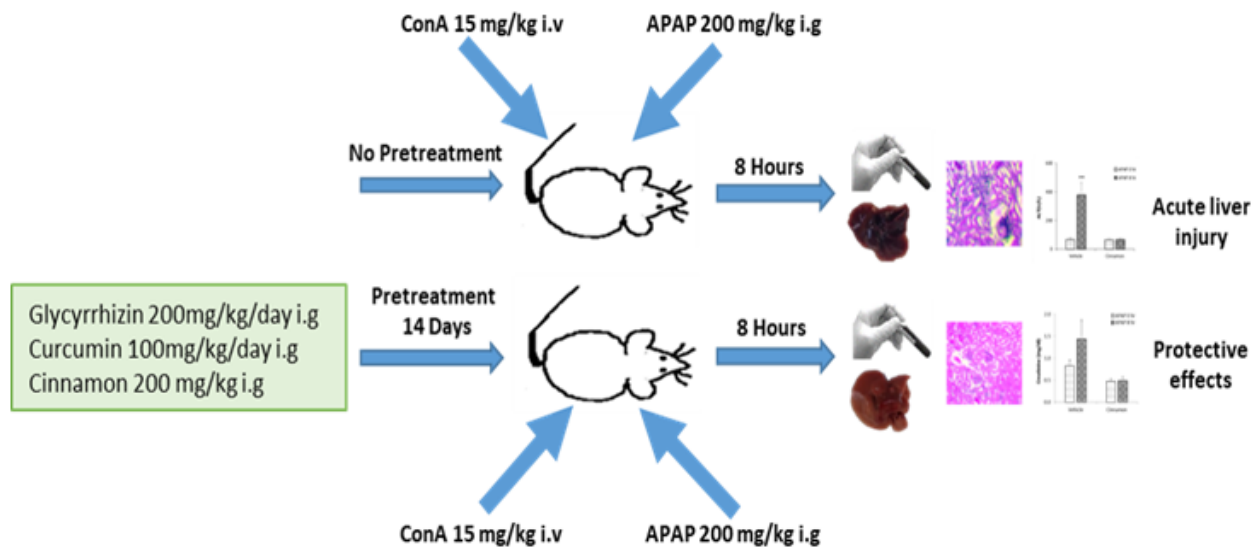


Figure 10. Mice were pretreated with glycyrrhizin, curcumin and cinnamon for 14 days followed by administration of a single dose of concanavalin-A and acetaminophen for 8 hours and results showed protective effects of glycyrrhizin, curcumin and cinnamon against concanavalin-A- and acetaminophen-induced liver injury.

5. Conclusions

We found that ConA- and APAP-induced liver injury are accompanied by oxidative stress. Pretreatment with glycyrrhizin and curcumin prevented from ConA-induced acute liver injury by antioxidant mechanism in the same way as cinnamon pretreatment ameliorated APAP-induced acute liver injury and oxidative stress.

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