Experimental Article

Crocin mitigates carbon tetrachloride-induced liver toxicity in rats

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Abstract

Objectives: Carbon tetrachloride (CCl4) is one of the most dangerous hepatotoxic environmental pollutants thus this study aimed at investigating the potential preventive effect and mechanism of crocin against CCl4-induced hepatotoxicity.

Methods: Forty Male rats were allocated for two weeks treatment with; corn oil, CCl4 in corn oil, crocin (100 mg/kg), or crocin plus CCl4. At time of euthanasia liver was removed, weighted and processed for histopathological evaluation and estimation of liver contents of active caspase3, lipid peroxidation (MDA) and reduced glutathione (GSH). We also evaluated antioxidant enzymes activities [superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT)], phase I metabolizing enzyme [cytochrome P450 sub family 2E1 (CYP2E1)] an Phase II metabolizing enzyme, [glutathione-S-transferase (GST)] in liver tissue. Blood samples were used for evaluation of liver function tests and inflammatory cytokines [interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α)].

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Introduction

Carbon tetrachloride (CCl4) is a common industrial solvent which is well known for its hepatotoxicity.1–3 CCl4 is used in the synthesis of chlorinated organic compounds including chlorofluorocarbon refrigerants, agricultural fumigant, in the production of semiconductors, in the processing of fats, oils and rubber and in laboratory applications.4,5 Occupational exposure to carbon tetrachloride may occur in the chemical industry, in laboratories, and during degreasing operations.6,7 Numerous poisonings and fatalities have occurred due to high exposure to carbon tetrachloride. The major pathological changes have been seen in the liver even at levels that were generally below 5 ppm (32 mg/m3).8,9 Liver damage can occur after 24 h and in serious cases resulted in hepatic coma and death.10,11 Through the investigation of acute CCl4-induced liver damage in animal models, it is now generally accepted that CCl4 toxicity resulted from free radical generation by cytochrome P450 system in liver microsomes and consequently causes lipid peroxidation of membranes that leads to liver injury.12–15

One of the possible popular successful approaches for the protection and even treatment of liver damage due to oxidative stress could be achieved through use of phytochemicals with antioxidant properties.13,16,17 One such phytochemical is crocin, a main constituent of the Crocus sativus (Saffron), exhibits a variety of pharmacological effects including inhibition of skin tumor growth in mice,18 improvement of learning behavior previously impaired by ethanol,19 anti-hyperlipidemic effects,20 anti-atherosclerotic effects,21 and anti-cancer effect.22,23 Interestingly, recent studies indicated, potential free radical scavenging, antioxidant and lipid peroxidation inhibition properties of crocin.24,25

Note worthy, study by Lin and Wang26 pointed out a first clue for partial hepatoprotective effect of crocin against liver carcinogenesis induced by diethylnitrosamine and aflatoxin B1. Our previous study documented a possible protective utility of crocin against brelyum chloride-induced brain and liver injury.27 As a continuation for efforts to establish an effective hepatoprotective modality utilizing nature substance against CCl4 as an environmental toxin, it is pertinent to study crocin in well-designed animal model for liver toxicity, which will answer questions regarding its protective utility and potential mechanism. Therefore, the global aim of this proposal is to evaluate the potential protective utility of crocin against hepatic toxicity induced by carbon tetrachloride. Moreover, exploring the possible mechanisms whereby this agent mediated its beneficial effects.

Materials and Methods

Animals and study protocol

Forty male Sprague–Dawley rats (200–250 g) were kept at 20–25°C in a 12 h light/12 h dark cycle with free access to food and water. The animals were feed standard chow and water ad libitum. The experimental protocols were approved and carried out according to guidelines for the use and care of experimental animals.

Design of the work

After a period of adaption, rates were classified into four groups (n = 10) as follow:

➢ Group I: Negative control; treated intraperitoneal (i.p.) with corn oil in a dose of 0.2 ml/100 g animal.
➢ Group II: crocin group; injected i.p. with crocin in a dose of 100 mg/kg/day.
➢ Group III: CCl4 group; Injected i.p. with CCl4, dissolved 1:1 in sterile corn oil. In a dose of 0.2 mL/100 g animal for two consecutive days/week.
➢ Group IV: Combination group; injected I.P with both crocin and CCl4 using the same doses schedule mention before.

Animal treatment was continued for 2 weeks then the experiment was concluded and animals were killed under anesthesia, blood samples were collected and livers were rapidly removed then weighted to calculate relative liver weight to body weight. Liver and blood samples were processed for measuring the following parameters:

Biochemical analysis

Each blood sample was placed in dry clean centrifuge tube, and then centrifuged for 10 min at 3000 revolutions per minute (rpm) to separate the serum. Serum was carefully separated into clean dry Wassermann tubes by using a Pasteur pipette and used for determination of serum liver function tests [(aspartate aminotransferase (AST), alanine aminotransferase (ALT) alkaline phosphatase (ALP) and total bilirubin.] using standard techniques.
Histological evaluation:
Liver samples were fixed in 4% buffered formaldehyde, embedded in paraffin, and stained with hematoxylin-eosin according to Bancroft and Stevens.31 Histologic evaluation was performed twice by two pathologists blinded to the protocol.

Estimation of liver metabolizing enzymes activities
Cytosol and microsomes fractions were prepared at 0—4 °C from liver tissue homogenate according to method of Benson et al.,32 and used for evaluation of metabolizing enzymes. Liver microsomal phase I metabolizing enzyme; CYP2E1 activity was assayed using p-nitrophenol as a substrate33. Cytosolic liver phase II metabolizing enzyme; Glutathione –S-transferase (GST) activity was determined using 1-chloro 2,4 dinitrobenzene (CDNB) as a substrate according to method of Habig et al.34 GST and CYP2E1 activities were normalized against protein content and presented as percentage of corresponding control values.

Measurement of liver oxidative stress status
Liver tissue malondialdehyde (MDA), an indicator of lipid peroxidations; superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GSH-Px); and reduced glutathione (GSH) measurements were performed as described previously35–38 to determine the oxidative status in the liver specimens.

Evaluation of inflammatory cytokines
Plasma Interleukin-6 (IL-6) was determined colorimetrically using rat Elisa Kit (IBL Co., Ltd. Hamburg, Germany) in accordance with the manufacturer’s instructions. Plasma tumor necrosis factor alpha (TNF-α) level was determined via a commercial ELISA kit (IBL Co., Ltd. Hamburg, Germany) using standard curve according to supplier protocol.

Evaluation of active Caspase3
The caspase-3 enzyme activity was measured in liver tissues collected from rats in the four treatment groups using the CaspACE assay system (Promega Corp., Madison, WI) based on the ability of the caspase 3 enzyme to release the yellow chromophore p-nitroaniline (pNA) from the colorimetric substrate (Ac-DEVD-pNA) provided in the CaspACE assay system. Relative caspase-3 activities for each sample and sample plus inhibitor were calculated from the standard curve as described previously.39 Caspase-3 activity values were normalized against sample protein content and presented as percentage of control value.

Statistical analysis
Results were expressed as mean ± SE, and the significance of differences were assessed by one-way ANOVA and Tukey’s test as post hoc. The differences were accepted as statistically significant when P value was less than 0.05.

Results
Effect of treatment on body weight and relative liver weight to body weight
As illustrated in Table 1; the ratio of liver weight to 100 g body weight was significantly increased by sole administration of CCl4 (5.8 ± 0.25, p < 0.01) compared to control animals showing 4.5 ± 0.10. Interestingly treatment with both crocin and CCl4 exhibited liver weight/100 g body weight ratio of 4.7 ± 0.17 which is significantly lower than CCl4 group (p < 0.01) and was close to normal value (Table 1). Comparing the animal total body weight at the end of experiment to its corresponding initial value, only, CCl4 group exhibited a significant decrease compared to its corresponding initial weight (Table 1). Note northerly, the body weights exhibited by combination group had higher values compared to both it initial body weight and body weights exhibited by CCl4-treated group however it is still less than the control values (Table 1).

Histological findings
Liver sections of control rats showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Figure 1). Liver sections obtained from CCl4 treated rat showed disarrangement of normal hepatic cells, pale stained hepatocytes around the central vein (C) with extensive vacuolization around the central vein massive fatty degeneration, multifocal centrilobular hepatic cell death, and cellular infiltration, and massive numbers of inflammatory cells infiltration (Figure 1). Conversely, mild vascular congestion (V) was detected in the crocin sections with no histopathological alteration appearing in hepatocytes. Compared to CCl4 group, interestingly, livers of rats treated with crocin and CCl4 revealed better preservation of the normal liver architecture and rare generalized vacuolization of the cytoplasm of hepatocytes, with apparently normal nuclei, very few inflammatory cells infiltration (Figure 1).

Effect of treatment on liver function tests
The serum levels of liver functions (ALP, ALT, AST and total bilirubin) are presented in Table 2. In the CCl4 treated

<table>
<thead>
<tr>
<th>Body weight (% of initial)</th>
<th>Liver weight/100 g body weight</th>
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<tbody>
<tr>
<td>Control 116.64 ± 3.7*</td>
<td>4.5 ± 0.10</td>
</tr>
<tr>
<td>Crocin 113.5 ± 4*</td>
<td>4.4 ± 0.20</td>
</tr>
<tr>
<td>CCl4 94.17 ± 2.6b,c</td>
<td>5.8 ± 0.25b</td>
</tr>
<tr>
<td>Combination 100.47 ± 3.2ab</td>
<td>4.7 ± 0.17b</td>
</tr>
</tbody>
</table>

- Data were calculated as relative weight of liver to 100 g animal body weight at the end of experiment.
- Data are presented as mean ± standard error of 10 animals/group.
- a, b or c indicates significant difference from control, crocin or corresponding initial body weight respectively at p ≤ 0.01 using Tukey’s test as post ANOVA test.

Table 1: Effects of crocin on body and liver weights of carbon tetrachloride (CCl4) treated rats at the end of study (2 weeks).
group, the serum levels of ALP, ALT, AST and total bilirubin significantly, \( p < 0.01 \), increased to 369 ± 8.5, 390 ± 6.9, 461 ± 11.3 and 2.5 ± 0.007 respectively, compared to negative control group values of 66 ± 2.7, 42 ± 3.9, 36 ± 3.2 and 0.28 ± 0.005 respectively. Pretreatment of CCl4-treated rats with crocin significantly, \( p < 0.01 \), decreased the CCl4 induced elevation of these markers levels to 160 ± 7.4, 183 ± 9.6, 138 ± 8.5 and 0.5 ± 0.009 respectively (Table 2). Interestingly, sole crocin administration does not exhibit any significant change from control values of liver functions.

**Effect of treatment on metabolizing enzymes in liver tissue**

The effects of crocin and/or CCl4 on activities of phase I metabolizing enzyme; (CYP2E1) and Phase II metabolizing
enzymes (GST) are compiled in Figure 2. Administration of CCl4 resulted in a significant, \( p < 0.01 \), increase in CYP2E1 activity to 260% \( \pm 6.8 \) of control value with concomitant significant decrease in GST activity to 45% \( \pm 2.2 \) of control value respectively, \( p < 0.01 \). Conversely sole treatment with crocin exhibited an opposite effect. Crocin treated rate exhibited 60% \( \pm 1.7 \) and 210% \( \pm 8.1 \) of control values for CYP2E1, and GST respectively. Interestingly, addition of crocin to CCl4 treatment abrogated CCl4-induced disturbance of metabolizing enzymes and normalized all values.

**Effect on lipid peroxidation and GSH level in liver tissue**

CCl4 caused a substantial increase in liver MDA content to 19.3 \( \pm 0.5 \) with concomitant significant fall in liver GSH content 76 \( \pm 2.2 \) compared to control group (Table 3). Administration of crocin alone showed a non-significant decrease in liver MDA content, 4.5 \( \pm 0.5 \) (Table 3), while exhibited a significant increase in GSH content 168 \( \pm 3.3 \) compared to control group (Table 3). Combined administration of crocin and CCl4 resulted in a significant reduction in the liver MDA content (8.4 \( \pm 0.75 \)) with significant increase in liver GSH content (120 \( \pm 4.4 \)) compared to CCl4 treated group (Table 3).

**Effect of treatment on antioxidant enzymes activities in liver tissue**

Table 3 shows the activities of enzymatic antioxidants (CAT, SOD, and GSH-Px) in the rats liver tissue. A significant decrease, \( p < 0.01 \), in the activities of the above mentioned enzymatic antioxidants activities was observed after CCl4 administration (18 \( \pm 1.3 \), 190 \( \pm 12 \) and 8.25 \( \pm 0.7 \) respectively) compared to control values of 32 \( \pm 3.2 \), 380 \( \pm 10 \) and 15.34 \( \pm 0.3 \) respectively. In contrast, crocin alone treated group showed a significant increase in liver CAT, SOD and GSH-Px activities compared to control values. However, concomitant administration of crocin with CCl4 significantly restored these enzyme activities back towards normalcy (27 \( \pm 2.2 \), 360 \( \pm 9 \) and 13.32 \( \pm 0.9 \) for CAT, SOD and GSH-Px respectively) as shown in Table 3.

**Results of plasma interleukin-6 and plasma tumor necrosis factor alpha**

Table 4 showed that there was significant increase in plasma levels of IL-6 (110 \( \pm 6.3 \)) and TNF-\( \alpha \) (188 \( \pm 6.6 \)) in
Croci control value in liver tissue (Figure 3). Sole crocin administration a significant (treated with crocin and CCl4 (combination group) showed 143% /C6 /C6 (98% /C6 had no significant effect on active caspase 3 activity /C6 ).

Part via free radical generation,40 crocin was chosen in our established that, CCl4-induced liver toxicity is mediated in against CCl4-induced liver injury in rats. Since, it is well crocin, a natural compound consumed in human diet, Discussion

Per our results, CCl4 induced liver toxicity in rats which was evidenced by: increased liver weight to body weight elevated liver enzymes and total bilirubin, an indication of structural and functional defects in liver cells,41,42 along with histological disturbance of liver tissue. Our study indicated that, CCl4-induced liver toxicity are possibly mediated through: 1) generation of free radical and oxidative stress, indicated by: a) abrogation of antioxidant enzymes activity and glutathione content, b) elevation of MDA in liver tissues, 2) induction of phase I metabolizing enzymes leading to more production of CCl4-generated free radicals and concomitant inhibition of phase II metabolizing enzymes leading accumulation of CCl4-generated free radicals, 3) induction of inflammation confirmed by: a) elevation of: IL-6 and TNF-α and b) histopathological investigation, 4) activation of caspase cascade of apoptosis indicated by the reported increase

It treated group as compared to normal control group exhibited; 39.7 ± 2.5 and 22 ± 1.4 for IL-6 and TNF-α respectively. On the contrary, crocin treated group had no significant change from control values. Interestingly, animals treated with both crocin and CCl4 exhibited significant mitigation of CCl4-induced changes in IL-6 and TNF-α (62 ± 3.6 and 55 ± 2.7 for IL-6 and TNF-α respectively).

Caspase-3 activity in liver tissue of rats treated with crocin and or CCl4

The CCl4 treated group exhibited a significant (p < 0.01) increase in active caspase-3 content to 416% ± 19.5 of control value in liver tissue (Figure 3). Sole crocin administration had no significant effect on active caspase 3 activity (98% ± 3.7 of corresponding control value). While animals treated with crocin and CCl4 (combination group) showed a significant (p < 0.01) suppression in caspase-3 content, 143% ± 5, compared to CCl4 treated group (Figure 3).

Discussion

This study aimed at evaluation of protective utility of crocin, a natural compound consumed in human diet, against CCl4-induced liver injury in rats. Since, it is well established that, CCl4-induced liver toxicity is mediated in part via free radical generation,40 crocin was chosen in our study as a potential protective agent because of its antioxidant activity.25

Table 3: Antioxidant enzymes activities, reduced glutathione content and lipid peroxidation content in liver tissue of rats treated with crocin and or carbon tetrachloride (CCl4).

<table>
<thead>
<tr>
<th></th>
<th>CAT (U/mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>GSH-PX (U/mg protein)</th>
<th>GSH (umol/mg protein)</th>
<th>MDA (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32 ± 2.20</td>
<td>380 ± 10</td>
<td>15.34 ± 0.3</td>
<td>132 ± 3.5</td>
<td>4.75 ± 0.25</td>
</tr>
<tr>
<td>Crocin</td>
<td>39 ± 1.20a</td>
<td>430 ± 10a</td>
<td>18.39 ± 0.5a</td>
<td>168 ± 3.3a</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>CCl4</td>
<td>18 ± 1.30ab</td>
<td>190 ± 12ab</td>
<td>8.25 ± 0.7ab</td>
<td>76 ± 2.2ab</td>
<td>19.3 ± 0.5ab</td>
</tr>
<tr>
<td>Combination</td>
<td>27 ± 2.25abc</td>
<td>360 ± 9abc</td>
<td>13.32 ± 0.9abc</td>
<td>120 ± 4.4abc</td>
<td>8.4 ± 0.75abc</td>
</tr>
</tbody>
</table>

Animals were treated for 2 weeks.

a, b or c indicate significant change from control, crocin or CCL4 using Tukey’s test as post ANOVA test at p < 0.01.

Data are presented as mean and standard error of 10 animals each group.

Table 4: Effect of treatment with crocin and or CCL4 on plasma inflammatory cytokines levels.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Crocin</th>
<th>CCl4</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml)</td>
<td>39.7 ± 2.5</td>
<td>42 ± 2.5</td>
<td>110 ± 6.3ab</td>
<td>62 ± 3.6abc</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>22 ± 1.4</td>
<td>24 ± 1.5</td>
<td>188 ± 6.6ab</td>
<td>55 ± 2.7abc</td>
</tr>
</tbody>
</table>

Animals were treated for 2 weeks.

a, b or c indicate significant change from control, crocin or CCL4 using Tukey’s test as post ANOVA test at p < 0.01.

Data are presented as mean and standard error of 10 animals each group.

Animals were treated for 2 weeks.

Caspase-3 activity values were normalized against tissue protein content and presented as percentage of control value.

Figure 3: Effect of carbon tetrachloride (CCl4), crocin or both crocin and CCl4 on active caspase-3 activity in rat liver tissue.

Animals were treated for 2 weeks.
in activity of active caspase-3 leading to damage of liver cells. These damaging effects could explain the elevated levels of liver enzymes and disturbance of liver functions reported in our results.\textsuperscript{13,42}

Of particular interest, crocin effectively protected against CCl4-induced hepatotoxicity in rats. These protections are approved via: 1) restoration of relative liver weight to body weight (Table 1), 2) normalization of liver enzymes and serum total bilirubin (Table 2), 3) improvement of liver histological pictures, (Figure 1). According to our study these beneficial effects could be mediated through: 1) inhibition of CCl4 induced disturbance of metabolizing enzymes (Figure 2) leading to reduction of CCl4-induced free radical generation, 2) inhibition of lipid peroxidation manifested by decreased MDA content in liver (Table 3), 3) induction of antioxidant enzymes activity and elevation of reduced glutathione content (Table 3). Furthermore, 4) crocin inhibited CCl4-induced inflammation indicated by abrogation of CCl4-induced elevation of plasma IL-6 and TNF-z levels (Table 4); 5) inhibition of caspase 3 activity, an effect that protects liver cells from death (Figure 3)\textsuperscript{13,47} and 6) some or all of the above. These findings support our earlier report pointed out the hepatoprotective effect of crocin against tyramine chloride-induced liver injury via antioxidant activity.\textsuperscript{25}

CYP2E1, a phase I metabolizing enzyme, catalyzes the bioactivation of CCl4 to its highly reactive trichloromethyl peroxyl radical (CCl3 OO) and superoxide anion free radicals.\textsuperscript{13,44,45} These radicals initiate lipid peroxidation thereby contributing majorly to the pathogenesis of liver toxicity.\textsuperscript{46–48} In contrast to Phase I, Phase II metabolizing enzymes such as GST is involved in detoxification of CCl4 and its reactive metabolites to facilitate their elimination from human organism, \textsuperscript{46,49} Agents that induce Phase II enzymes have been reported to protect against toxic effects of chemicals in rats.\textsuperscript{50–52}

Therefore, the toxicity of CCl4 depends, in part, on the balance between the activities of phase I and phase II enzymes. Thus protection could be achieved in part by inhibiting metabolism of CCl4 and enhancing excretion of toxic metabolites secondary to induction of phase II detoxifying enzymes.\textsuperscript{53} This principle has been supported by previous results.\textsuperscript{54–59}

Coping with this principle, our study showed significant induction of Phase I metabolizing enzyme (CYP2E1) and inhibition of Phase II metabolizing enzyme (GST) by CCl4 leading to oxidative stress, confirmed by induction MDA content and abrogation of antioxidant enzymes and glutathione content, and hence liver damage confirmed by H&E staining and manifested by disturbed liver functions.\textsuperscript{46,47,58} Interestingly, treatment with crocin inhibited phase I and stimulated phase II metabolizing enzymes which in turn decreased the level of free radicals generation and abrogated CCl4 induced oxidative stress which would preserve the integrity of liver cells and hence normalized liver histology, relative liver weight to body weight and liver enzymes.

Furthermore, in addition to modulation of metabolizing enzymes by crocin, the inhibition of CCl4-induced oxidative stress by crocin in this study could be ascribed also to its antioxidant characters via induction of enzymatic antioxidant activities (SOD, CAT, GSH-PX) and non-enzymatic antioxidant namely GSH which in turn abrogated the lipid peroxidation as evidenced by diminished MDA level compared to CCl4-treated group.

Noteworthy to mention that, the amount of carbon tetrachloride metabolized in a given tissue is related to the CYP450 content of the tissue.\textsuperscript{59,60} In the liver, the greatest accumulation of carbon tetrachloride metabolites occurs in the centrilobular region, which has high CYP450 levels.\textsuperscript{44,59} These fact could explain the major histological disturbance in this region as illustrated in histological pictures. Furthermore the metabolic rate of CCl4 in humans is more similar to the rate in rats than in other rodent species.\textsuperscript{34}

Tumor necrosis factor-alpha (TNF-z) and IL-6 are central regulators of inflammation\textsuperscript{64} and have been increased in many inflammatory conditions such as chemical toxicity.\textsuperscript{62} The anti-inflammatory effect of crocin has been studied before where crocin showed a suppressive activities on diverse proinflammatory mediators such as NO, IL-1, TNF-z, and reactive oxygen species.\textsuperscript{27,63,64} Our results are in line with these reports where crocin suppressed CCl4-induced elevation of IL-6 and TNF-z, which could contribute for the protective effect of crocin against CCl4-induced liver damage.

The increased level of caspase 3 in liver tissue collected from animal treated with CCl4 might be attributed to oxidative stress and induction of inflammation.\textsuperscript{27,65} Caspase 3 plays the pivotal role in apoptosis where it mediates the virtual apoptotic effect.\textsuperscript{56,67} Administration of CCl4 elevated the caspase 3 in rat liver resulting in liver cell death (Figure 3). Crocin ameliorated CCl4-induced cell death as observed in the decreased centrilobular necrosis and fatty degenerations in combination group (Figure 1). It is plausible that antioxidant, radical scavenging effects and anti-inflammatory actions of crocin, reported in this study, interfered with CCl4 induced elevation of caspase 3 activity and hence the liver cells were preserved, liver weight was normalized and functions were restored, this scenario has been supported by our collective results as well as previous results.\textsuperscript{68–70}

**Conclusion**

In conclusion, our findings revealed that crocin has encouraging protective properties against CCl4-induced hepatotoxicity. These protective effects attributable to more than one mechanisms namely: modulation of metabolizing enzymes favoring low CCl4 generated free radical accumulation, free radical scavenging potential, antioxidant activity, anti-inflammatory effect and inhibition of caspase 3 activity.

**Conflict of interest**

The authors have no conflict of interest to declare.

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Authors’ contributions

BS and HMH conceived idea, established design, interpreted data, performed statistical analysis, revised initial draft, performed the major physical work and addressed comments to reviewers.

AH was responsible for in vivo experiment.

GMM performed histopathological examination of liver tissues and its corresponding data analysis.

EHA contributed in evaluation of inflammatory cytokines and biochemical analysis.

BI purchased chemicals and kits and prepared important solutions.

All authors revised the manuscript critically for important intellectual content and approved for its final form.

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