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Research Article
Curcumin Ameliorates Carbon Tetrachloride-induced Liver Injury in Rats Through Modulating Various Biological Activities

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Abstract

Background and Objective: Curcumin, an active compound derived from turmeric (*Curcuma longa*) was noticed to have health promoting effects through modulating various biological activities. The present study was carried out to assess the hepatoprotective effect of curcumin in CCl_4-induced hepatotoxicity rats. **Materials and Methods:** Liver function enzymes activity was evaluated using the spectrophotometric method. The levels of inflammatory markers were measured using the enzyme-linked immunosorbent assay (ELISA) method. Moreover, antioxidant enzymes (Catalase, glutathione-S-transferase) were assayed in the serum calorimetrically. Haematoxylin-eosin staining was performed to examine the histopathological changes. In addition, expression of vascular endothelial growth factor (VEGF) protein was evaluated through immunohistochemical staining. Statistical comparison between groups was made via using SPSS software by matching analysis of variance. A $p<0.05$ was measured to be statistically significant. **Results:** Oral administration of CCl_4 into rats for 8 weeks had resulted in significant decrease of the serum level of the antioxidant enzymes including superoxide dismutase (SOD) and catalase. It had also significantly increased the serum level of the liver function enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and proinflammatory markers. Oral administration of 50 mg kg^{-1} b.wt., of curcumin evidences a significant protection against CCl_4 induced liver damage as measured in terms of liver function enzymes, antioxidant enzymes, inflammatory markers and histopathological parameters. Moreover, curcumin treatments protected against CCl_4-induced liver damage by maintaining histological damage. **Conclusion:** Based on these findings, it is concluded that antioxidant effect of curcumin could protect liver injury and normalize the architecture of hepatocytes against CCl_4 induced toxicity

Key words: Carbon tetrachloride, hepatotoxicity, antioxidant enzymes, histopathological changes, liver function enzymes

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Carbon tetrachloride, an organic solvent, commonly used industrial solvent and it is the well-recognised animal model of xenobiotic-induced and oxidative stress-mediated hepatotoxicity[1,2]. The CCl_4 induction causes various types of changes in the liver tissues and alters the liver enzymes level as well as antioxidant enzymes levels.

Drug-induced liver injury (DILI) is a common adverse drug reaction and it can lead to liver failure and even death[3-5]. Single oral doses of CCl_4 showed increased liver weight, elevated levels of fat, serum urea, liver enzyme activities and clear histopathological evidence of liver damage with cell necrosis[6]. Previous study demonstrated that CCl_4 undertakes metabolic activation via hepatic microsomal cytochrome P450 enzymes to form trichloromethyl radical with the power of oxidative stress that causes hepatocytes damage and finally causes of disturbance of hepatic functions[7]. Moreover, metabolites initiate a lipid peroxidation chain reaction and ultimately lead to several chronic diseases including liver injury[8,9]. Various types of drugs are used to treat liver diseases but such drugs cause adverse effects and alter the various biochemical and physiological process. However, an alternative safer treatment is needed to overcome such problem. In this context, it was evidenced that natural products or active compounds of medicinal plants play a significant role in disease management including liver diseases. Earlier studies demonstrated that a number of polyphenolic compounds present in foods or fruits displayed antioxidant activities against tissue damage caused through free radicals[10,11].

Turmeric is an old Indian spice with a potent medicinal compound called curcumin. Curcumin, a compound of turmeric and has been proven to show anti-inflammatory, anti-oxidant and anticancer effects, it is pharmacologically safe and has minimal toxicity to tissue[12,13]. Curcumin play a significant role in the inhibition of pathogenesis of diseases through its rich source of antioxidant and reactive oxygen species scavenging properties. Moreover, it has been revealed to kill cancer cells and modulating various genetic pathways[14,15]. The present study was carried out to measure the hepatoprotective effect of curcumin in CCl_4-induced hepatotoxicity through liver function markers, antioxidant enzymes level, histopathology, ultrastructural changes and immunohistochemistry staining.

MATERIALS AND METHODS

Study area: The current study was performed in the Lab of Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University from December, 2018 to May, 2019.

Animals grouping and treatment: Rats (Male wistar, 6-8 weeks old, 175-225 g b.wt.) were obtained from King Saud University, Saudi Arabia. All animal experimental protocols involving animals were approved by the institutional ethical committee of CAMS, Qassim University. Thirty-two rats (n = 8 for each group) were categorised into four groups: (1) Control normal control group received sterile distilled water (2) CCl_4 (50 mg kg^{-1} b.wt., in vehicle solution, oral gavage) (3) CCl_4+curcumin (40 mg kg^{-1} b.wt., in vehicle solution, oral gavage) (4) curcumin (40 mg kg^{-1} b.wt.) only. The treatment was given orally three times in a week for 8 weeks. Upon completion of treatment, rats were culled and blood and liver tissue sample were collected for analysing liver function markers, antioxidant enzymes level, inflammatory markers, histopathology, ultra-structural changes and immunohistochemistry parameters.

Biochemical assay: For the estimation of ALT, ALP and AST activities in serum samples, commercially available enzymatic kits were used. ALT, ALP and AST activity was evaluated using the spectrophotometric method. The results are expressed as Units/litre (U L^{-1}) and results are interpreted accordingly.

Measurement of inflammatory markers: Serum TNF-α and IL-6 levels were measured using the enzyme-linked immunosorbent assay (ELISA) method with the appropriate commercial kits (Abcam, USA) and results were interpreted accordingly.

Measurement of antioxidant enzymes: Antioxidant enzymes (Catalase, superoxide dismutase (SOD)) were assayed in the serum calorimetrically using Abcam kit (UK), according to the instruction of the manufacturer. The calculations of catalase and glutathione-S-transferase activity concentration were evaluated through the suitable equation of the kit.

Histopathological analysis: Liver tissues were collected and fixed in 10% formalin solution (Neutral buffered saline) and processed for histopathological analysis as per the standardized procedure[16]. Five micrometer (5 µm) thick sections obtained from the paraffin blocks were stained by haematoxylin-eosin for assessing features of inflammation, haemorrhages, edema, blood vessel dilation and necrosis processes. Moreover, Masson trichrome stain was used to analyse the fibrosis in the liver tissues.

Immunohistochemical analysis: Expression of VEGF was evaluated immunohistochemical analysis as previously described methods[17-19]. A thin liver tissue section was made through microtome and deparaffinised was performed with xylene. Endogenous peroxidase activity was done via 0.3% hydrogen peroxide in methanol for 30 min to block the endogenous peroxidase activity. Antigen retrieval was performed in the citrate buffer (pH 6.0) for 20-30 min. Then, blocking agent was added on tissue to lock the unwanted site. Monoclonal mouse anti-VEGF (Abcam, UK) was applied at 1: 250 dilutions for overnight at 4°C temperature in humid chamber. Following incubation with secondary antibody for one hour, followed by incubation with streptavidin-biotin enzyme complex was applied for 45 min. Finally, diaminobenzidine (DAB) as chromogen was used to detect VEGF protein using the mouse monoclonal antibody. The sections were counter stained with haematoxylin, photographed and the results were interpreted accordingly.

Statistical analysis: Data from each treated group were explained as Mean±SEM. Statistical comparison between groups was made via using SPSS software by matching analysis of variance. A $p<0.05$ was measured to be statistically significant.

RESULTS

Effect of curcumin on liver enzymes measurement: The liver function enzymes including ALT, AST and ALP was measured and result was compared with control group to evaluate the hepatoprotective effect of curcumin. Evaluation of the liver enzymes on rats treated with CCl_4 alone or in combination with curcumin. ALT, AST and ALP levels in the CCl_4 treated group were significantly higher when compared with control group ($p<0.05$).

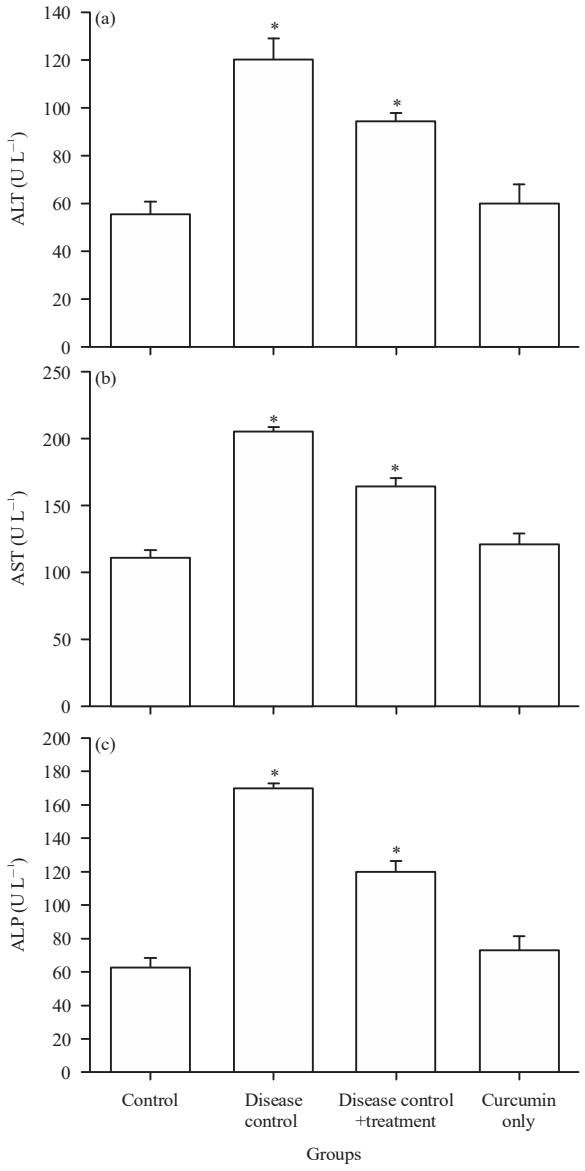

Fig. 1(a-c): Evaluation of the liver enzymes on rats treated with CCl_4 alone or in combination with curcumin, (a) ALT, (b) AST and (c) ALP

ALT, AST and ALP levels in the CCl_4 treated group were significantly higher when compared with control group (*$p<0.05$ vs. control) ($p<0.05$), CCl_4+curcumin group showed significantly decreased ALT, AST and ALP level than the CCl_4 treated group only (*$p<0.05$ vs. diseases control) ($p<0.05$)

The CCl_4+curcumin group showed significantly decreased ALT, AST and ALP level than the CCl_4 treated group only ($p<0.05$) (Fig. 1). This finding suggested that curcumin act as hepato-protectant through decreasing the liver function enzymes level.

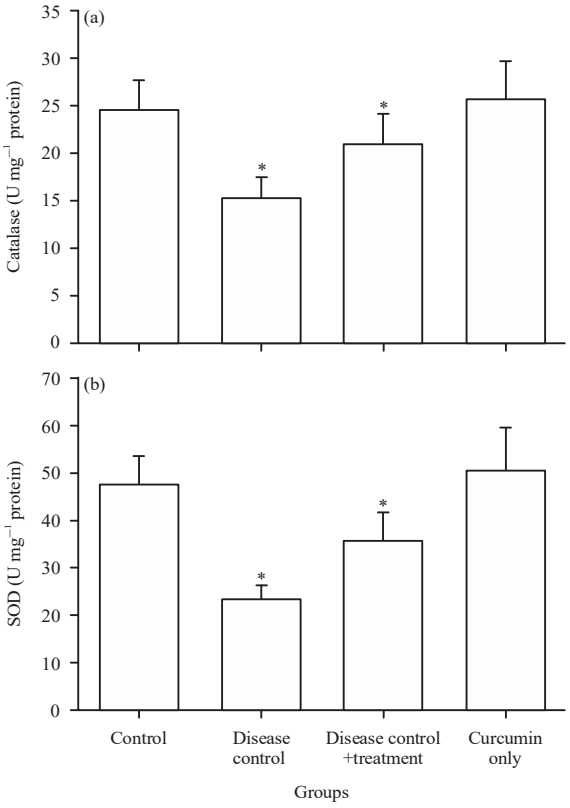

Fig. 2(a-b): Evaluation of the antioxidant enzymes on experimental groups, (a) Catalase and (b) SOD

CCl_4 treated group showed low level of SOD and catalase as compared to control group (*p<0.05 vs. control), CCl_4+curcumin group increased the level of SOD and catalase when compared with CCl_4 treated only group (*p<0.05 vs. disease control)

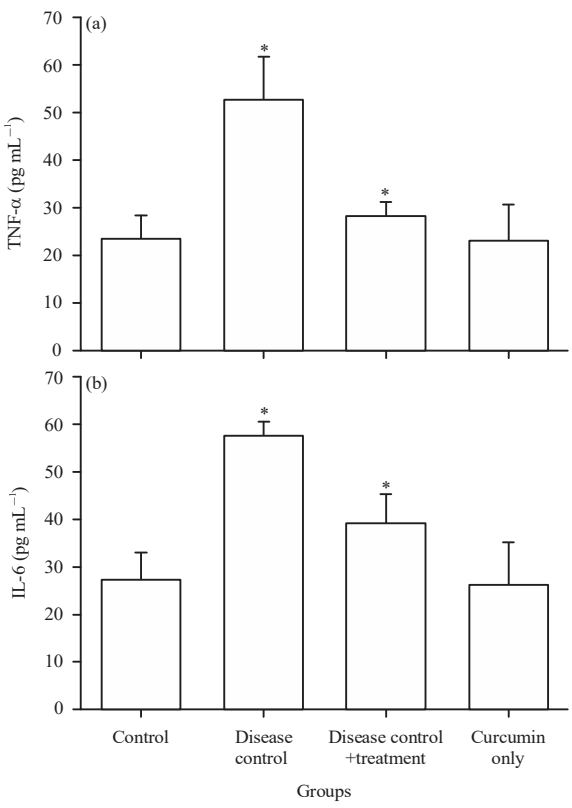

Fig. 3(a-b): Evaluation of the pro-inflammatory cytokines on experimental groups, (a) TNF-α and (b) IL-6

CCl_4 treated group showed enhance level of TNF-α and IL-6 as compared to control group (*p<0.05 vs. control), CCl_4+curcumin group significantly decreased the level these two inflammatory markers when compared with CCl_4 treated only group (*p<0.05 vs. disease control)

Effect of curcumin on antioxidant enzymes: The antioxidant enzymes including SOD and catalase were measured in all treated group and results were compared with control groups. As shown in Fig. 2, CCl_4 treated group caused significant decreased of antioxidant enzymes such as SOD and catalase as compared to control group ($p<0.05$). Moreover, CCl_4+curcumin group significantly increased the antioxidant enzymes level of SOD and catalase when compared with CCl_4 only treated group ($p<0.05$).

Effect of curcumin on pro-inflammatory cytokines: As shown in Fig. 3, IL-6 and TNF-α levels in CCl_4-induced hepatotoxicity were significantly higher as compared to control group ($p<0.05$). Treatment with curcumin significantly decreased the level of IL-6 and TNF-α when compared with CCl_4 treated only group ($p<0.05$).

Histopathological studies: As shown in the Fig. 4, histology of the liver section from the control group showed normal hepatic cells with normal cytoplasm, prominent nucleus and central vein. Compared with the control group, liver tissue in CCl_4-treated group revealed severe injury characterized by infiltration of inflammatory cells, haemorrhages, blood vessel dilation, edema and necrosis. Moreover, the group CCl_4+curcumin significantly maintained the architecture of hepatocytes and mild alterations were observed.

Collagen fiber of liver tissue was examined through Masson's Trichrome staining. More collagen fibers in CCl_4-treated group was seen as compared to CCl_4+curcumin group (Fig. 5).

Effect of curcumin on serum VEGF protein expression: The VEGF, an angiogenic factor protein expression was measured through immunohistochemical staining in all experimental

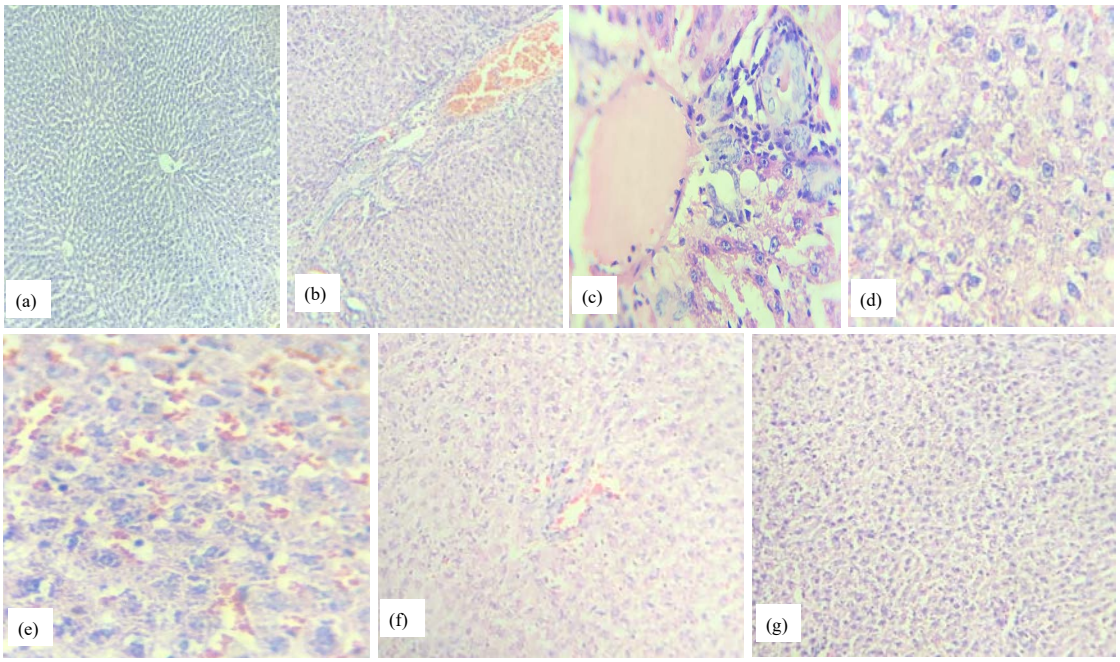

Fig. 4(a-g): Microscopic examination of liver tissues showing (a) Control group: the normal architecture of hepatocytes as hepatic cells with normal cytoplasm, prominent nucleus and central vein, (b-e) CCl_4-treated group tissues showing haemorrhage and increased inflammatory cells, haemorrhages, edema and necrosis, (f) Curcumin treated tissue-where the liver tissue alteration was significantly lowered as compared to CCl_4-treated group and (g) Curcumin only treated group showed normal hepatocyte

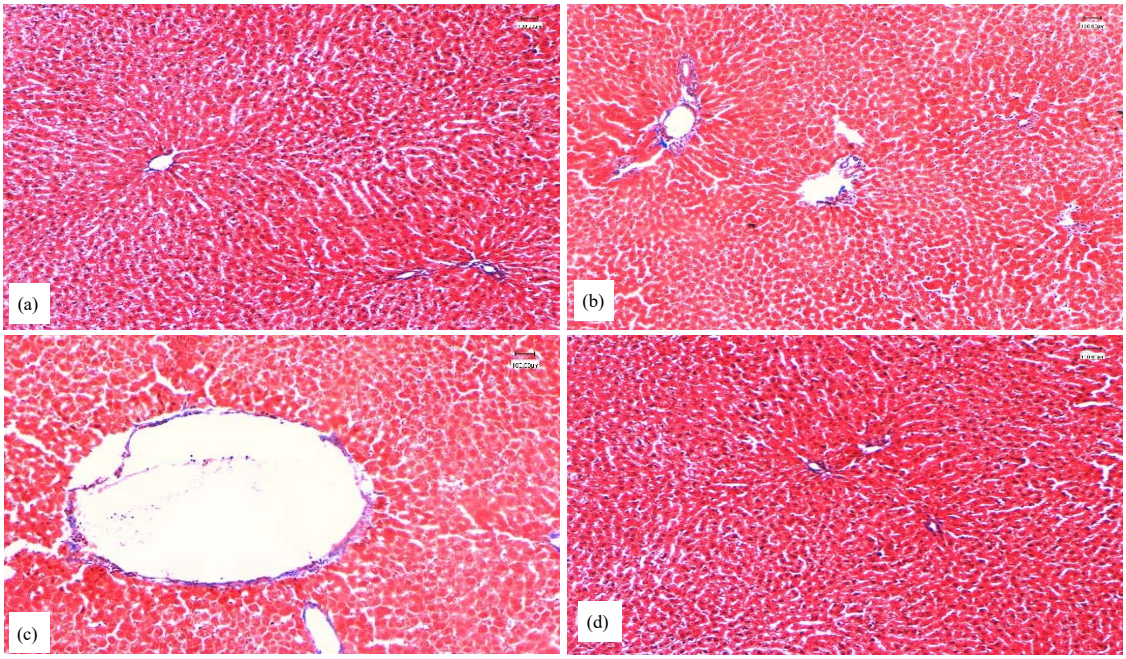

Fig. 5(a-d): Masson's Trichrome staining of collagen within the liver tissue, (a) Normal distribution of collagen fibres, stained blue in control group, (b) More collagen fibres in CCl_4-treated group, (c) Normal distribution of collagen fibres, stained blue, in CCl_4 plus curcumin group and (d) Normal collagen fibres distribution in curcumin only treated group (Masson's trichrome, ×40)

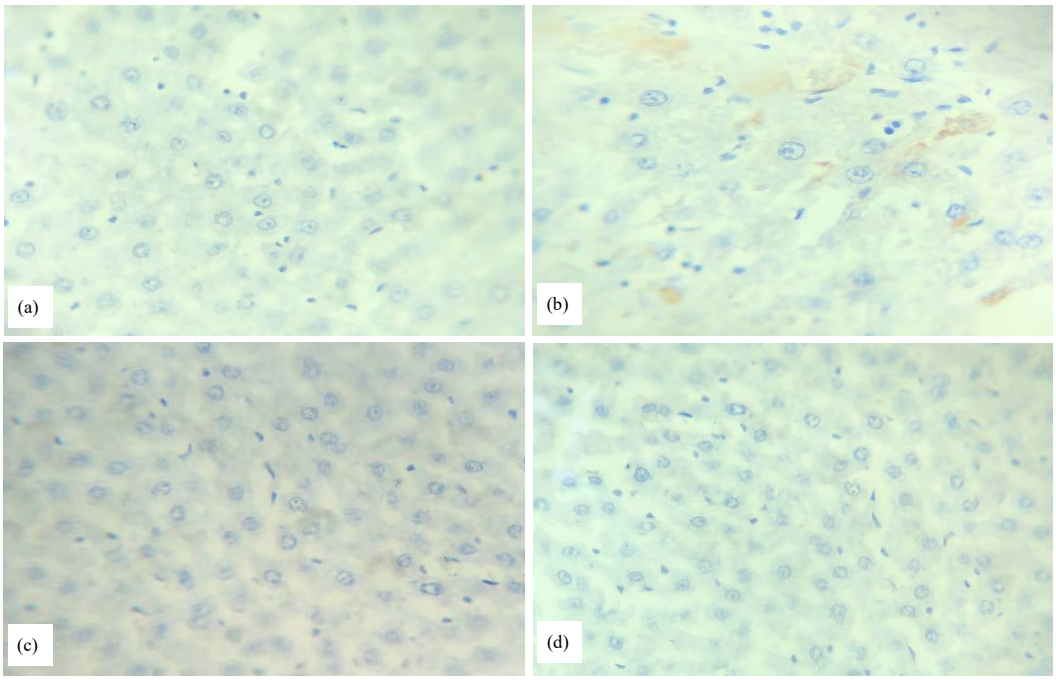

Fig. 6(a-d): Expressional evaluation of VEGF protein in experimental group. VEGF protein expression was measured in experimental groups. (a) Control group did not show VEGF expression, (b) CCl4-treated group displayed cytoplasmic expression of VEGF protein, (c) VEGF protein expression was mild or undetectable in CCl4-treated group+curcumin treated group only and (d) VEGF protein did not show expression in curcumin-only group

groups. As shown in Fig. 6, it was noticed that VEGF protein expressed in the CCl_4-treated group whereas control and curcumin only group did not show any expression of VEGF protein. However, curcumin+CCl_4-treated group treated showed very low or undetectable expression of VEGF.

DISCUSSION

CCl_4 has been commonly used in animal models to examine chemical toxin-induced liver toxicity and as an admirable model to assess the role of hepatoprotectants[20-22]. Numerous studies based on natural products have been performed to evaluate hepatoprotective potentiality against CCl_4 induced liver toxicity. Several studies have demonstrated that active compounds of medicinal plants are rich in antioxidant, which play a significant role in the prevention of pathogenesis of diseases through scavenging property of free radical. Furthermore, active compound of herbs meaningfully guards the cells against the harmful effect caused by reactive oxygen species (ROS). To achieve the aim of the current hypothesis, experimentation was performed out to evaluate the role of curcumin as a hepatoprotectant against CCl_4-induced hepatotoxicity. In addition, liver damage was analysed using liver function markers, antioxidant enzymes level, pro-inflammatory cytokines, histopathology, ultra-structural changes and immunohistochemistry

In the present study, it was noticed that levels of ALT, AST and ALP enzymes in the CCl_4 treated group only were significantly higher when compared with control group. CCl_4+curcumin group showed significantly decreased ALT, AST and ALP level than the CCl_4 treated group only ($p<0.05$) (Fig. 1). Earlier studies have revealed that CCl_4 increases AST and ALT levels in serum[23] and this finding revealed that the development and progression of liver damage occurs due to CCl_4 exposure. Curcumin act as hepato-protectant through the attenuation of higher enzymes level due to CCl_4 exposure. In accordance to current result, Kyung *et al.*[24] showed that induction of liver damage through dimethylnitrosamine (DMN) resulted on significant increase on the level of AST and ALT enzymes than that observed on the normal control group, indicating the progression of liver injury. Moreover, comparison of treatment groups and the DMN-only group showed that the DMN with curcumin group showed considerably lower AST and ALT values than the DMN-only group[24].

Inhibition of inflammatory cytokines and mediator production or function serve as principal mechanisms in the regulation of inflammation and the materials that decrease the expression of such inflammation-associated genes show therapeutic potential in the treatment of inflammatory diseases[25]. Excitingly, the altered/over expression of pro-inflammatory cytokines accelerates the accumulation of ROS, that causes cell damage[26,27].

The use of natural products plays significant role in liver protection against drug induced damage through action as anti-inflammatory agent. As found in current study, IL-6 and TNF-α levels in CCl_4-induced hepatotoxicity were significantly high as compared to control group ($p<0.05$). Treatment with curcumin significantly decreased the level of IL-6 and TNF-α when compared with CCl_4 treated only group ($p<0.05$). In the support of current finding, previous finding based on natural compound Zerumbone (ZER) was performed to examine its protective role against CCl_4-induced acute liver injury through anti-inflammation. It was reported that serum and liver tissue of TNF-α and IL-6 levels were higher in the CCl_4-group than in the control group and ZER-pre-treatment inhibits the CCl_4-intoxication-induced production of inflammatory cytokines. Moreover, the hepatoprotective effect of ZER was reached by down-regulating the inflammatory response[28]. In this regards, previous finding revealed that curcumin decrease the levels of pro-inflammatory cytokines including IL-1α, IL-6 and TNF-α in monocyte cultures exposed to pre-eclamptic plasma[29].

Oxidative stress plays an important role in liver injury and liver tissues alteration. At the cellular level, oxidative stress causes to a large spectrum of responses, depending on the cell type, the level of ROS achieved, as well as the duration of the exposure[9,30-31]. CCl_4 requires bioactivation through cytochrome P450 system of phase I in liver and produces the reactive metabolic trichloromethyl radical and proxy trichloromethyl radical. These free radicals bind to polyunsaturated fatty acids, form alkoxy and peroxy radicals that can produce lipid peroxide, that cause damage in cell membrane, change enzyme activity and finally induce hepatic injury[32]. In order to resist the oxidative stress, our defence systems hold antioxidant systems, such as nonenzymic system including GSH and a series of antioxidant enzymes like SOD, CAT that work to control the cascades of uncontrolled oxidation and finally protect cells from oxidative damage through scavenging of ROS[33]. Antioxidant activity and the inhibition of free radical generation are principal steps in terms of protecting the liver from the damage and inhibit the pathogenesis of liver associated diseases.

In present study, CCl_4 treated group caused significant decreased of antioxidant enzymes as compared to control group. Moreover, CCl_4+curcumin group significantly increased the SOD and catalase level when compared with CCl_4 treated only group. Moreover, curcumin is a rich source of antioxidants and such property of curcumin showed suppression of liver damage through the prevention of oxidative damage. The previous findings were in accordance with this study as CCl_4 treatment induced oxidative stress as noticed by high malondialdehyde and nitric oxide levels and decreased SOD, GPx and glutathione reductase (GR) levels[34]. Moreover, in this milieu, pre-treatment with Mahonia oiwakensis Hayata stems extract showed significant hepatoprotection with respect to CCl_4-induced acute liver injury[34]. Another study reported that significant decrease in the antioxidant enzymes were noticed in liver of rats exposed to lindane and curcumin treatment nearly normalized all changes[35].

To evaluate the hepatoprotective properties of curcumin further, histopathological examination was made on all treated group and results were interpreted accordingly. In the current study, it was seen that histology of the liver section from the control group showed normal hepatic cells with normal cytoplasm, prominent nucleus and central vein. Compared with the control group, liver tissue in CCl_4-treated group caused severe injury characterized by infiltration of inflammatory cells, haemorrhages, blood vessel dilation, edema and necrosis. Moreover, such alterations were significantly attenuated by curcumin treatment and maintained the architecture of hepatocytes and mild alterations were observed. This result agrees with a previous finding that treatment of DMN-only group showed tissue alterations including congestion and destruction of hepatic architecture, massive and severe hepatocyte necrosis. Whereas the severity of histological changes and cirrhotic liver alterations in curcumin treatment groups was significantly lower than that in the DMN-only treated group[24]. The kaempferol glycosides isolated from *C. tinctorius* L. including kaempferol 3-O-rutinoside and kaempferol 3-O-glucoside showed hepatoprotective effect as kaempferol 3-O-rutinoside and kaempferol 3-O-glucoside mitigated the CCl_4-induced liver histological alteration, as evidences through histopathological evaluation[36].

VEGF is another protein that is a potent stimulator of angiogenesis, inducer of endothelial cell migration and vascular permeability[37-39]. The flavonoids and antioxidants of medicinal plants have shown a good role as anti-angiogenesis. In the current study, it was reported that VEGF protein

expressed in the CCl_4-treated group whereas control and curcumin only group did not show any expression of VEGF protein. However, curcumin+CCl_4-treated group treatment showed very low or undetectable expression of VEGF. In this regard, study based on natural products such as resveratrol revealed good effects on suppressing angiogenesis in mice with hepatic cell carcinoma (HCC) xenograft and the mechanism underlying its anti-angiogenesis is through inhibiting VEGF expression[40]. Another study based on Rhizoma paridis saponins reported that CCl_4 intoxication damaged liver function, caused hepatocyte necrosis and upregulated the expression levels of the mRNA and phosphorylated proteins of VEGF, PDGF, ERK1/2 and α-SMA. Whereas, by the oral administration of Rhizoma paridis saponins, the severity of hepatic fibrosis was relieved significantly and the expression levels of the mRNA and phosphorylated protein of VEGF, PDGF, ERK1/2 and α-SMA were decreased[41].

CONCLUSION

In conclusion, it is demonstrated that curcumin could alleviate CCl_4-induced liver injury in rats via normalizing liver function enzymes, enhancing antioxidant enzyme activity and suppression of cytokines activity. Curcumin acts through its antioxidant property to resist the oxidative stress and scavenging of ROS and finally prevents the pathogenesis of liver associated diseases.

SIGNIFICANCE STATEMENT

This study discover that curcumin could alleviate CCl_4-induced liver injury in rats via normalizing liver function enzymes, enhancing antioxidant enzyme activity and suppression of cytokines activity. Moreover, curcumin treatments protected against CCl_4-induced liver damage by maintaining histological damage. This study will help researchers to uncover the critical areas of antioxidant effect of curcumin in amelioration of liver injury and normalize the architecture of hepatocytes toxicity.

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