The antifibrotic effect of *Zilla spinosa* extracts targeting apoptosis in CCl_4-induced liver damage in rats

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Background/aim
Liver fibrosis and its end-stage cirrhosis are the main reasons of morbidity and mortality all over the world. The current study aimed to evaluate the efficacy of *Zilla spinosa* (*Z. spinosa*) on CCl_4-induced liver fibrosis, apoptosis, and oxidative stresses in rats.
Materials and methods
Extract of aerial part of *Z. spinosa* was used in this study. Thirty male Sprague? Dawley rats were enrolled in this study and divided into five groups (six each): group 1 served as control and groups 2–5 were treated with CCl_4 (1 ml/kg intraperitoneal twice a week for 8 weeks), where group 2 served as a control positive, group 3 received silymarin (50 mg/kg) daily, and groups 4 and 5 were administrated with *Z. spinosa* (100 and 200 mg/kg, respectively) daily for 8 weeks. At the end of each experiment, liver function tests were analyzed in serum, whereas malondialdehyde (MDA), Nitric oxide (NO), Glutathione (GSH), and hydroxyproline (HA) were analyzed in liver tissues. Liver fibrosis was confirmed histopathologically, and collagen content, caspase-3, and α-smooth muscle actin (α-SMA) were assayed immunhistochemically.
Results
Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, MDA, NO, and HA levels were increased ($P<0.05$), whereas total protein and GSH were decreased ($P<0.05$) in CCl_4-administrated rats. Histopathological results showed loss of lobular structure, fibrosis with expansion of portal tract by fibrous tissue together with inflammatory changes confined to portal tract and central vein, and intense centrilobular necrosis and remarkable fatty hydropic degeneration. In addition, extensive accumulation of connective tissue, marked depletion of glycogen, strong expression of α-SMA, and increased of caspase-3 were found in CCl_4-administrated rats. Oral administration of *Z. spinosa* at 100 or 200 mg/kg restored the normal levels of liver function parameters, MDA, NO and GSH; decreased HA; and reduced collagen, glycogen content, caspase-3, and α-SMA in liver tissue of rats. The high dose of 200 mg/kg showed more potent effect than low dose of 100 mg/kg when compared with silymarin treatment group.
Conclusion
The present study clarified that *Z. spinosa* extract has antioxidant and antiapoptotic properties in CCl_4-induced liver fibrosis in rats, and may be able to exert a therapeutic effect on developing hepatic fibrosis; moreover, high dose of 200 mg/kg appeared to be more potent than low dose (100 mg/kg).

Keywords:
α-smooth muscle actin, antioxidant, histopathology, liver fibrosis, *Z*illa *spinosa*

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Introduction

Liver fibrosis and its end-stage cirrhosis are the main reasons of morbidity and mortality all over the world [1]. Liver fibrosis is known as the wound healing procedure that occurs as a result of the extent of chronic liver injury. Liver fibrosis may occur owing to hepatitis viral infections, alcoholism [2], and CCl_4 exposure [3]. Moreover, many environmental toxins cause chronic liver diseases, nutritional troubles, autoimmune circulatory disturbances, cholestasis, and long-term drug administrations [4]. Carbon tetrachloride-induced liver injury is a common model in rats. CCl_4 is metabolized by cytochrome P450 to toxic free radicals. These radicals covalently bind to cellular macromolecules and lead to membrane lipid peroxidation, with progression of liver damage hypomethylation of nucleic acids, disorder of calcium homeostasis, extreme production of inflammatory

cytokines, fibrosis, cirrhosis, cell death, and cancer, depending on the dose and exposure time [5].

Liver fibrosis is distinguished by the overaccumulation of extracellular matrix (ECM) proteins, possessing type I and type III collagen proteins; this leads to disorder of hepatic architecture and function, and therefore prominent characteristic of cirrhotic liver [6]. Liver fibrosis and fibrogenic cells that produce the scarring response are recognized as hepatic stellate cells (HSCs). The activated HSCs convert into myofibroblast-like cells, which then proliferate and generate an ECM as a result of continual chronic inflammation [7]. The HSCs are activated by reactive oxygen species (ROS), inflammatory cytokines, and growth factors. Therefore, the treatment strategy should focus on diminishing the exposure of HSCs to these oxidative and inflammatory stimuli, consequently, to slow down the progression of fibrosis process [1].

Liver fibrosis is a reversible reaction that appears in almost all pathological conditions concerned with chronic hepatic injury. However, in chronic liver diseases, there is a fundamental relationship between injury, hepatocyte death through apoptosis or necrosis inflammation, and fibrosis, and it results in the formation of apoptotic bodies, and engulfment of these bodies by stellate cells enhances stellate cell activation [8,9]. Thus, there is promotion of hepatocyte apoptosis in different liver diseases in humans, and therapeutic strategy becomes effective when inhibiting hepatocyte apoptosis, which stops progression of liver diseases [10]. In addition, it has been reported that fibrosis has a dynamic bidirectional nature [11].

In recent years, herbal medicines have acquired wide attention and popularity for the treatment of liver disease because of their safety and efficacy [12]. Among the herbal products, *Zilla spinosa* (*Z. spinosa*) possesses a rich source of bioactive ingredient. *Z. spinosa* is one of the most common plant species of family *Cruciferea*, owing to its important uses in the folk medicine; it is used as a drink against kidney stones [13]. Phytochemical studies have reported that *Z. spinosa* contains flavonoids, carbohydrates, glucosinolates, free sinapine, sterols, and triterpenes. Many research studies have shown that *Z. spinosa* has numerous biological efficacies such as antidiabetic, antibacterial [14], antifungal, anticancer, antirheumatic, and powerful hepatoprotective and antiviral activities [15].

Phenols and flavonoids as natural antioxidants have protective effect in different models of toxin-induced oxidative stress. Silymarin is a natural antioxidant and contains flavonoid components; it has antioxidative, anti-inflammatory, and anticarcinogenic activities. It is a powerful antioxidant used to improve liver damage induced by various chemicals or toxins, including phenyl hydrazine and carbon tetrachloride [16]. The bioactive components from almost all plants decrease oxidative stress via preventing mitochondrial pathway of apoptosis [17].

The present study aims to investigate the efficacy of *Z. spinosa* on CCl_4-induced liver fibrosis in rats. The biochemical investigation includes liver function tests and the status of antioxidants. Liver fibrosis was confirmed histopathologically, and collagen content, caspase-3, and α-smooth muscle actin (α-SMA) were assayed.

Materials and methods

CCl_4 and silymarin used

CCl_4 was obtained from El-Gomhorya Company (Cairo, Egypt). Silymarin was obtained from the pharmacy as sachets produced by SEDICO Pharmaceutical Co. (6 October City, Egypt). Each sachet contains 140 mg silymarin (calculated as silybin). It was freshly prepared and administered by dissolving the content of each sachet in water (50 ml). All other chemicals used throughout the experiments were of the highest analytical grade available.

Extraction and isolation

Air-dried ground aerial part of *Z. spinosa* (500 g) were defatted with petroleum ether (40–60°C), and extracted three times at room temperature with C_2H_5OH : H_2O (7 : 3). The combined extracts were filtered and evaporated under reduced pressure and lyophilized (25 g). Twenty grams of the dry residue was used for pharmacological studies [15].

Animals and ethical approval

Male Sprague? Dawley rats were obtained from the Experimental Animal Center (National Research Center) and had a weight range of 150? 200 g. All animals were housed in plastic cages at a room temperature of 22±1°C, relative humidity of 50 ±20%, and under a 12? h light/dark cycle. Animals were fed on basal diet in accordance with Reeves et al and National Research council. Nutrient [18,19] and water were supplied *ad libitum*. Rats were acclimatized to laboratory conditions one week before beginning of the experiment. The studies were performed in accordance with the guidelines for the humane treatment of animals as set forth by the Association of Laboratory Animal Sciences and the

Center for Laboratory Animal Sciences at National Research Center. This study was approved by the Ethics Committee of National Research Center, with approval no 18005.

Experimental design

Thirty rats were used in this study and divided into five groups as follows (six each):

(1) Group 1 (normal control group): rats were subcutaneously injected with olive oil 0.5 ml/kg twice a week for 8 weeks.
(2) Group 2 (CCl_4-treated group): group of rats received 50% CCl_4 solution (CCl_4 : oil=1 : 1) intraperitoneal at a dose of 1 ml/kg twice a week for 8 weeks.
(3) Group 3 (CCl_4+slyimarin): group of rats that were treated with CCl_4 for 8 weeks as in group 2 and treated with silymarin daily at dose of 50 ml/kg for 8 weeks.
(4) Group 4 (CCl_4+100 mg *Z. spinosa*/kg): group of rats that were treated with CCl_4 for 8 weeks as in group 2 and received *Z. spinosa* daily at dose of 100 ml/kg for 8 weeks.
(5) Group 5 (CCl_4+200 mg *Z. spinosa*/kg): group of rats that were treated with CCl_4 for 8 weeks as in group 2 and received *Z. spinosa* daily at dose of 200 ml/kg for 8 weeks.

Collection of blood samples

At the end of the experiment, blood samples were collected after 16 h of fasting using the orbital sinus technique of Sanford [20]. Blood samples were left to clot in clean dry test tubes, and then centrifuged at 3000 rpm for ten minutes. The clear supernatant serum was then separated and frozen at −20°C for the biochemical analysis.

Preparation of liver homogenate

Immediately after blood sampling, animals were killed by cervical dislocation under light ether anesthesia, and livers were collected for biochemical and histopathological examinations. Liver tissues were rapidly removed, washed in ice-cooled saline, plotted dry, and weighed. A weighed part of each liver was homogenized, using a homogenizer (MPW-120 laboratory homogenizer, MPW Medical Instruments, Warsaw, Poland), with ice-cooled saline (0.9% NaCl) to prepare 20% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 5 min at 4°C (2k15; Laborzentrifugen, Sigma, Germany). The clear supernatant was then separated and frozen at −20°C for the biochemical analysis.

Biochemical analyses methods

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were estimated using the kits from Vitro Scient, Hannover, Germany [21–23]. Serum total bilirubin was determined using the kit from Biodiagnostic Co. (Cairo, Egypt) [24]. Serum total protein, was estimated using the kits purchased from Spectrum (Hannover, Germany) [25].

Liver homogenate was used to determine malondialdehyde (MDA) according to Ohkawa *et al.* [26], Nitric oxide (NO) according to Montgomery and Dymock [27], Glutathione (GSH) according to Beutler *et al.* [28] using the kits from Biodiagnostic Co., and hydroxyproline (HA) determination was done by enzyme-linked immunosorbent assay according to the method of Bancroft and Gamble [29], using Glory science kit (Biodiagnostic, Cairo, Egypt).

Histopathological examination of liver sections

Then, the rest of liver tissue was fixed in 10% phosphate buffered formalin (dehydrated, cleared in xylene), and then the liver specimens were processed into paraffin blocks and sections of 5-μm thickness. Histopathological examination of liver sections stained with hematoxylin and eosin staining was performed to assess histopathological changes [30], whereas Masson staining was used to detect collagen deposition for assessment of fibrosis.

Histochemical and immunohistochemical examination

Sections of 5-μm thickness produced were stained with periodic acid Schiff (PAS) to histochemically demonstrate glycogen in the liver sections [30].

Liver sections were deparaffinized in xylene and rehydrated in graded alcohol. The tissues were pretreated with 10 mmol/l citrate buffer, pH 6.0, and kept in microwave oven at 500 W for 10 min for antigenic retrieval. The slides were washed with PBS, for 5 min. Sections were incubated overnight at 4°C in a humidified chamber with one of the following primary antibodies: mouse monoclonal antibody to α-SMA diluted 1 : 100 and caspase-3 antibody diluted 1 : 50. The sections were rinsed again with PBS and then incubated with a biotinylated goat anti-rabbit and mouse antibody for 10 min. The sections were rinsed again with PBS. Finally, sections were incubated with streptavidin peroxidase. To visualize the reaction, slides were incubated for 10 min with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA). The slides were counterstained with hematoxylin and then dehydrated and mounted. Primary antibodies were omitted and replaced by PBS for negative controls.

Image analysis of the area occupied by collagen fibers

Quantitative assessment of liver fibrosis was performed on sections stained with Masson trichrome stain using computerized image analysis (Leica Qwin 500) in Image Analyzer Unit, Pathology Department, National Research Centre. The data were obtained using Image software (LEICA Imaging Systems Ltd, Cambridge, England) computer program. In each chosen picture, the Masson trichrome-stained [26] area was enclosed inside the standard measuring frame and then the red colored area was masked by a blue binary color to be measured. The percentage of the area of fibrosis over the whole observed field was assessed to represent the degree of hepatic fibrosis. The degree of fibrosis was expressed as the mean of ten fields sampled from each slide.

Statistical analysis

Data were analyzed using the statistical package for the social science (SPSS/Windows version 16; SPSS Inc., Chicago, Illinois, USA). The degree in variability of results will be expressed as means±SEM. Data were evaluated by one-way analysis of variance followed by Tukey's multiple comparisons test. The level of significance will accept at P less than 0.05.

Results

Biochemical results

Markers of liver damage (ALT, AST, ALP, and total bilirubin) were increased, and total protein was decreased significantly ($P<0.05$) by CCl_4 administration when compared with control group. *Z. spinosa* treatment at dose of 100 or 200 mg/kg lowered the increased liver markers and restored them almost to the normal levels ($P<0.05$). Total protein level was also increased by *Z. spinosa* administration at a dose of 200 mg/kg compared with CCl_4-treated group. ALT, AST, ALP, and total bilirubin also reduced significantly ($P<0.05$) in silymarin-treated group when compared with CCl_4-treated group (Table 1).

CCl_4-treated group showed significant elevation in MDA, NO, and HA levels, whereas showed severe depletion in GSH level in liver homogenates compared with control group. On the contrary, CCl_4-treated group receiving *Z. spinosa* at dose of 100 or 200 mg/kg had significant reduction in MDA, NO, and HA levels compared with CCl_4 group. Furthermore, *Z. spinosa* improved GSH depletion caused by CCl_4 treatment. MDA, NO, and HA levels decreased significantly ($P<0.05$), whereas GSH level increased significant ($P<0.05$), in silymarin-treated group when compared with CCl_4-treated group (Table 2).

Histopathological results

The present histopathological results confirm with the biochemical analysis. The control rat's liver showed normal structure demonstrate by central vein with hepatocytes, rounded and vesicular nuclei, and blood sinusoids (Fig. 1a).

Table 1 Serum levels of liver function parameters of group of rats treated with and/or without CCl_4 and *Z. spinosa* extract or silymarin for 8 weeks

Groups	Parameters				
	ALT (IU/l)	AST (IU/l)	ALP (IU/l)	Total bilirubin (mg/dl)	TP (g/dl)
Control	28.4±1.12[a]	46.2±0.56[a]	72.4±1.40[a]	0.33±0.01[a]	7.1±0.09[a]
CCl_4	96.2±1.30[b]	137.1±1.30[b]	212.6±2.10[b]	1.9±0.004[b]	4.12±0.05[b]
CCl_4+silymarin	25.3±0.97[a]	44±0.80[a]	80.3±1.10[c]	0.41±0.007[c]	7.4±0.02[a]
CCl_4+*Z. spinosa* 100 mg/kg	44.8±0.54[c]	99.3±1.10[c]	120.7±0.87[d]	0.92±0.006[d]	6.2±0.045[c]
CCl_4+*Z. spinosa* 200 mg/kg	30.5±1.00[d]	50.4±0.98[d]	96.2±0.79[e]	0.51±0.010[e]	7.0±0.023[d]

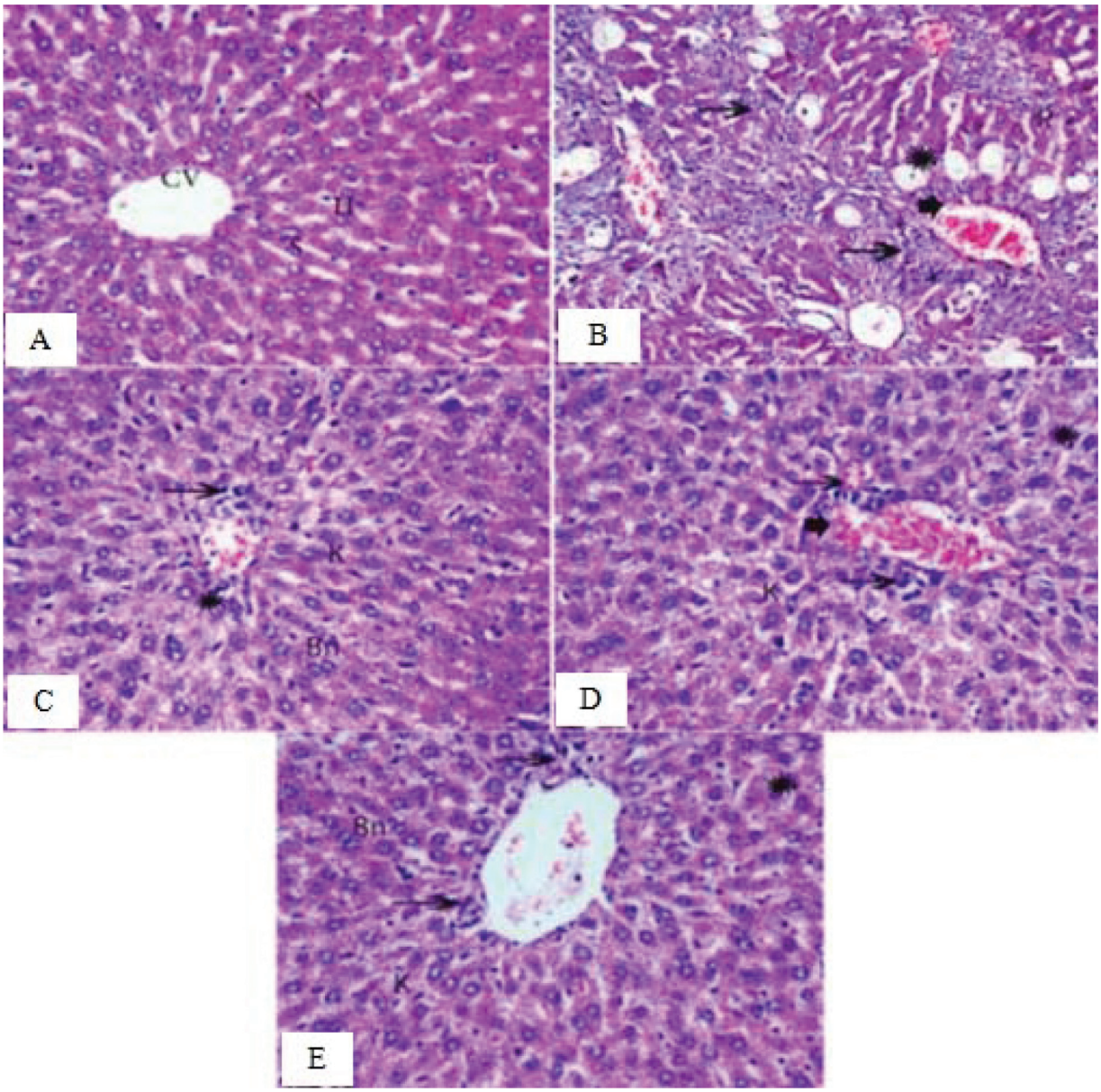
ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; TP, total protein. All data are expressed as mean±SE. The different letters (a, b, c, d, e) are significantly different using ANOVA test at $P<0.05$.

Table 2 Oxidant/antioxidant parameters and hydroxyproline content in liver tissues of group of rats treated with or without CCl_4 and *Z. spinosa* extract or silymarin for 8 weeks

Groups	Parameters			
	MDA (nmol/g)	NO (nmol/g)	GSH (mg/g)	Hydroxyproline (μmol/g)
Control	48.2±0.88[a]	374±1.3[a]	2.80±0.05[a]	140±1.7[a]
CCl_4	189.4±0.68[b]	750±1.7[b]	1.20±0.07[b]	901±2.1[b]
CCl_4+silymarin	55.8±0.53[c]	383±1.2[c]	3.10±0.04[c]	256±1.4[c]
CCl_4+*Z. spinosa* 100 mg/kg	132±0.79[d]	512±1.2[d]	2.10±0.08[d]	618±1.8[d]
CCl_4+*Z. spinosa* 200 mg/kg	54±0.83[c]	400±1.3[e]	2.80±0.06[a]	305±1.5[e]

ANOVA, analysis of variance; GSH, glutathione; MDA, malondialdehyde; NO, nitric oxide. All data are expressed as mean±SE. The different letters (a, b, c, d, e) are significantly different using ANOVA test at $P<0.05$.

Figure 1

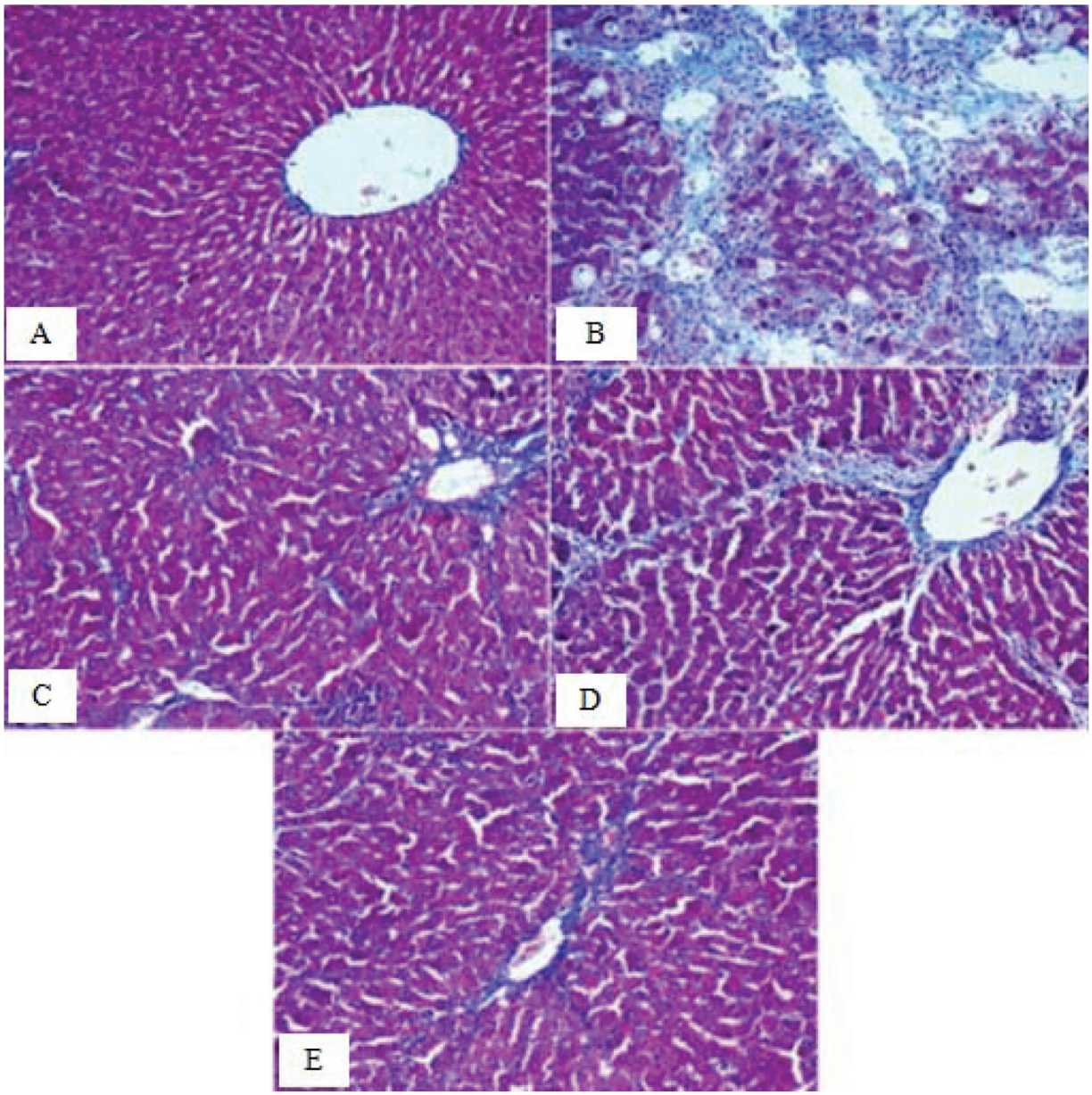


Photomicrograph of the liver sections of a rat stained with haemotoxylin and eosin (H&E). (a) Control group showing normal histological structure of hepatic lobules central vein (CV), hepatocytes (H), blood sinusoids (S), and nuclei (N). (b) CCl_4 showing disruption of the liver tissue with loss of lobular arrangement, bridging fibrosis with collagenous septa formation expanded portal tract to central vein (arrow) with mononuclear cells, vacuolar degeneration and necrosis of hepatocytes (star). Dilated and congested central vein was observed (arrowhead) and pyknotic nuclei (P). (c) CCl_4+sylimarin (50 mg/kg b.w.) showing mild inflammatory cells infiltrations around central vein (arrow), vacuolar degeneration, and necrosis of hepatocytes (star). Binucleated (Bn) and activation Kupffer cells were noticed (K). (d) CCl_4+*Z. spinosa* (100 mg/kg b.w.) showing moderate inflammatory cells infiltrations around central vein (arrow), and centrilobular hepatic necrosis with mild vacuolar degeneration of hepatocytes (star). Dilated and congested central vein and activation Kupffer cells (K) were observed (arrowhead). (e) CCl_4+*Z. spinosa* (200 mg/kg b.w.) showing maintained hepatic architecture, with only few inflammatory cells infiltrations around central vein (arrow), and centrilobular hepatic necrosis with mild vacuolar degeneration of hepatocytes (star). Dilated and congested central vein (arrowhead). Binucleiated (Bn) and activated Kupffer cells were noticed (K) (H&E, ×400).

Rats that received CCl_4 for 8 weeks showed large disruption of the liver tissue with loss of lobular structure, fibrosis with expansion of portal tract by fibrous tissue together with inflammatory changes confined to portal tract and central vein, excess fibrosis extended into hepatic parenchyma in the form of bridging fibrosis, and pseudolobular formation (Fig. 1b). Moreover, CCl_4-treatment group appears as having intense centrilobular necrosis, remarkable fatty hydropic degeneration of the hepatocytes and hemorrhage, which were detected throughout the hepatic parenchyma. Numerous megalohepatocytes, with enlarged nuclei and apoptotic cells were detected (Fig. 1b).

Histopathological investigation of the group treated with CCl_4+slyimarin (50 mg/kg) showed repair in

Figure 2



Photomicrograph of the liver sections of rat stained with Masson's trichrome (MT) is identified by their blue color. (a) Control group showing no signs of collagen deposition. (b) CCl_4 showing extended collagen deposition and appearance of bridging fibrosis with formation of pseudo-lobular. (c) CCl_4+sylimarin (50 mg/kg b.w.) showing mild collagen deposition. (d) CCl_4+*Z. spinosa* (100 mg/kg b.w.) showing moderate positivity to the stain all over the hepatic lobule. (e) CCl_4+*Z. spinosa* (200 mg/kg b.w.) showing small collagen deposition as thin collagenous septa formation. (Masson's trichrome, ×400).

the most of liver tissues and the collagen fibers appeared thinner than those observed in CCl_4 group (Fig. 1C).

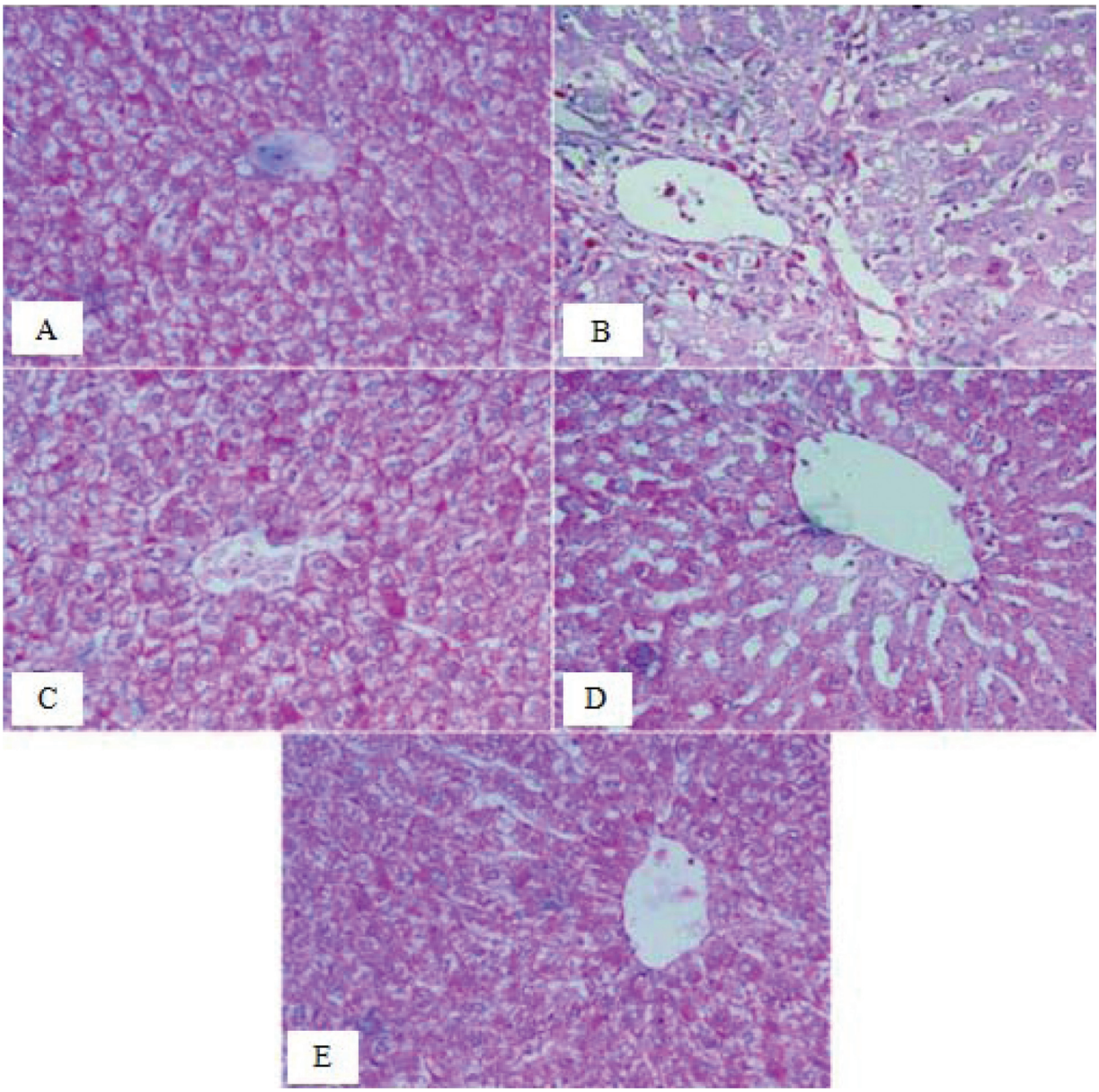
Histopathological examination of the group treated with CCl_4+*Z. spinosa* (100 mg/kg) showed variable degrees of protection. Some hepatocytes showed mild centrilobular necrosis, vacuolated cytoplasm, and darkly stained nuclei. Slightly dilated blood sinusoids and thin collagen fibers were still existent (Fig. 1d). However, CCl_4+*Z. spinosa* (200 mg/kg) treatment group showed reduction in histopathological changes observed in CCl_4 group and the architecture of the liver extends from normal to little periportal fibrosis, with minimal collagen fibers seen around central vein and in the portal tracts (Fig. 1e). Moreover, intact central vein and reduced inflammatory cells were seen. Some cells were vacuolated and slightly dilated; blood sinusoids were noticed, suggesting that *Z. spinosa* at a dose of 200 mg/kg was more effective when compared with the dose of 100 mg/kg or silymarin and that *Z. spinosa* could ameliorate the liver from chronic CCl_4-induced hepatic fibrosis.

Result of Masson trichrome staining

Masson's trichrome staining was carrying out to evaluate collagen fiber distribution in liver tissue. The liver sections of the control group showed little amount of collagen fibers around central vein and portal tract (Fig. 2a). The CCl_4 group revealed extensive accumulation or deposition of connective tissue resulting in the formation of pericentral and periportal collagen deposition with abundant septa seen radiating from portal tracts and central veins, making bridging fibrosis, and pseudolobule formation (Fig. 2b).

In CCl_4+silymarin-treated group, few collagen fibers were apparent around the central veins and portal area (Fig. 2c). The CCl_4+*Z. spinosa* (100 mg/kg) group showed decreased pericentral and periportal collagen deposition, mild septa radiating from portal tracts and central veins, and mild bridging fibrosis as compared with CCl_4-treated group (Fig. 2d). However, microscopic examination revealed that *Z. spinosa* (200 mg/kg) remarkably decreased the degree of liver fibrosis and ameliorated CCl_4-induced hepatic fibrosis except few fibrous tissues around central vein (Fig. 2e).

Figure 3



Photomicrograph of the liver sections of rat stained with Periodic acid Schiff stain (PAS), identified by their magenta color. (a) Control group showing strong PAS reaction in the form of red granules in hepatic tissues. (b) CCl_4 showing wide areas that give negative results with PAS stain. (c) CCl_4+sylimarin (50 mg/kg b.w.) showing increase in positivity of cells to the stain. (d) CCl_4+*Z. spinosa* (100 mg/kg b.w.) showing moderate positivity to the stain all over the hepatic lobule. (e) CCl_4+*Z. spinosa* (200 mg/kg b.w.) showing increase in positivity of cells to the stain and more or less normal content of glycogen (PAS, ×400).

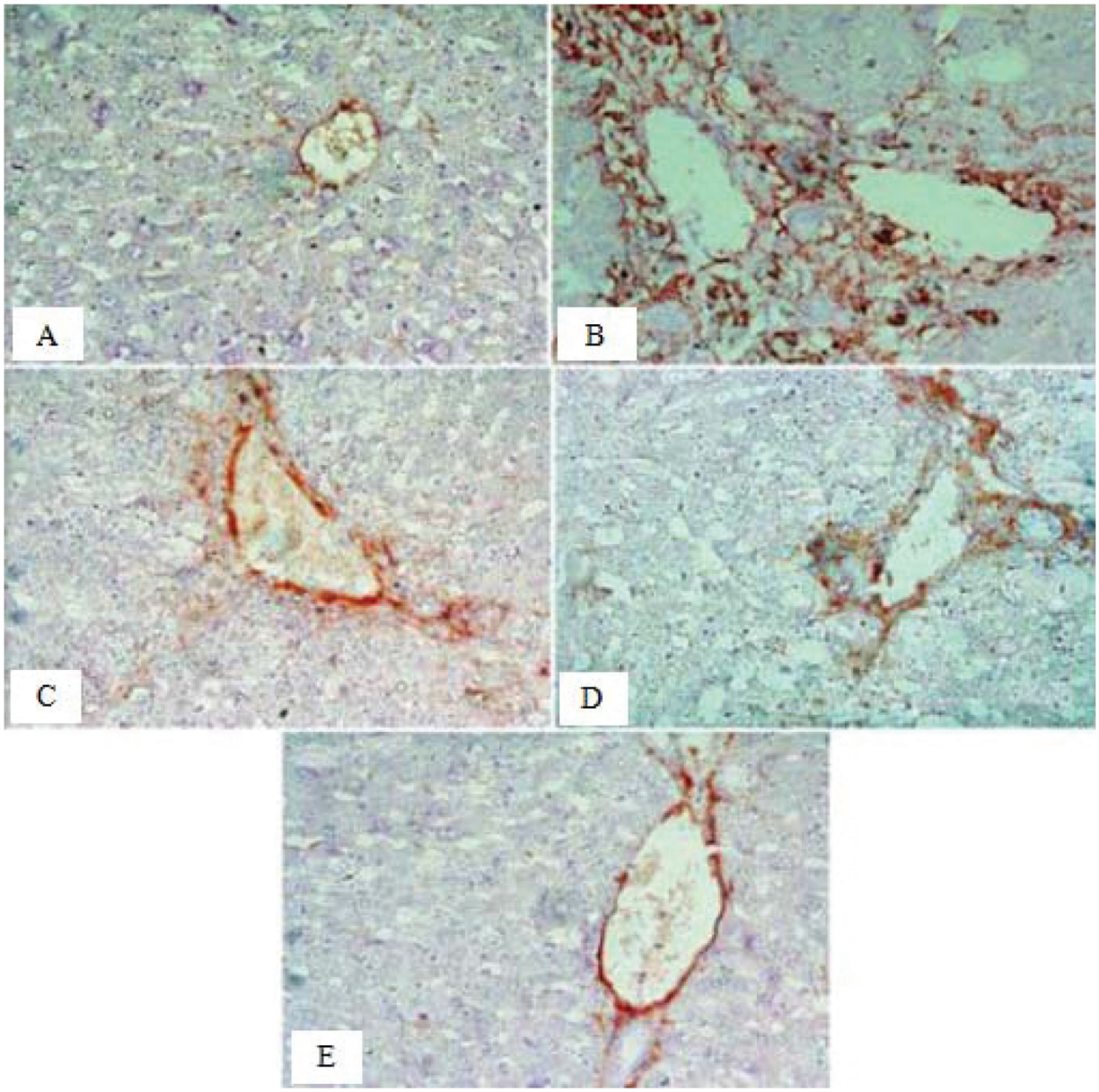
Histochemical result

The liver sections of the control group showed positive reaction of glycogen in hepatic tissues especially in the hepatocytes around the central vein (Fig. 3a). Carbon tetrachloride also resulted in marked depletion of glycogen within the liver cells together with increased amount of fibrous tissue (Fig. 3b). These effects were slightly improved in the group treated with CCl_4+*Z. spinosa* (100 mg/kg) (Fig. 3d), whereas marked amelioration was observed in CCl_4+*Z. spinosa* (200 mg/kg)-treated rats (Fig. 3e). However, the silymarin group resulted in improvement in glycogen content of hepatocytes (Fig. 3c).

Result of α-SMA expression

Hepatic α-SMA expression was used as an indicator of HSC activation. Liver sections of control group showed no immunoreactive expression of α-SMA (Fig. 4a). The immunohistochemical-stained sections of liver treated with CCl_4 showed strong immunoreactive expression of α-SMA (fibrogenic marker) where clearly in cytoplasmic stained dark brown color and apparent as expands along collagen

Figure 4



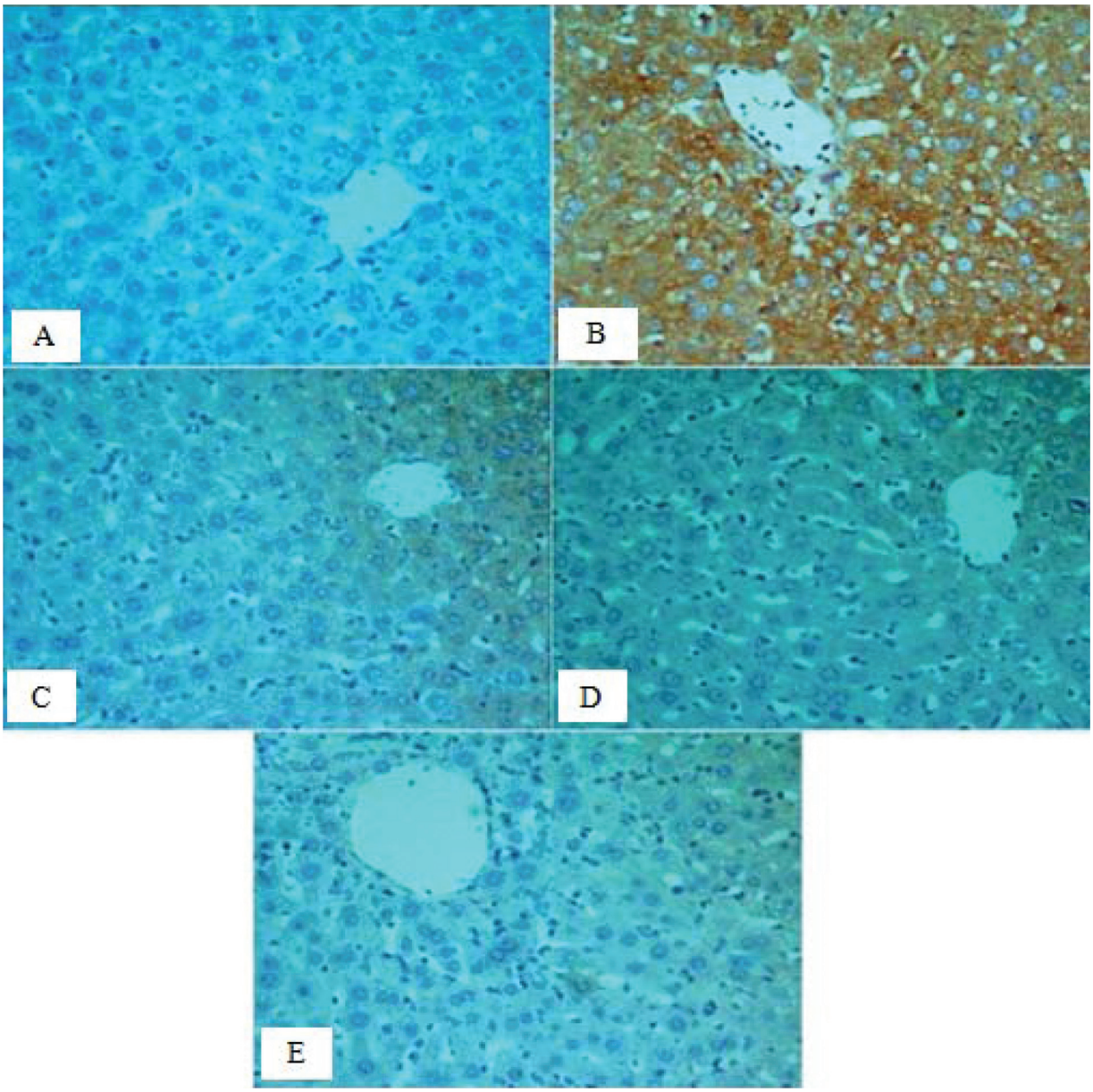
Photomicrograph of the liver sections of rat stained with α-SMA, identified by their brown color. (a) Control group showing no expression of α-SMA positive staining, (b) CCl_4 showing wide strong immunoreactive expression of α-SMA is mainly observed in the fibrous septa. (c) CCl_4+sylimarin (50 mg/kg b.w.) showing small number of α-SMA positive staining cells around portal triad and central vein indicated less fibrosis. (d) CCl_4+*Z. spinosa* (100 mg/kg b.w.) showing mild α-SMA positive staining cells around portal triad and central vein are present. (e) CCl_4+*Z. spinosa* (200 mg/kg b.w.) showing weak α-SMA positive staining cells around portal triad and central vein are present and more or less normal (immunohistochemistry α-SMA, ×400).

septa bridging portal areas and central areas (Fig. 4b). Mild positive immune reaction for α-SMA was seen around central vein and in-between hepatocytes in *Z. spinosa* (100 mg/kg)-treated group (Fig. 4d). In addition, the *Z. spinosa* (200 mg/kg)-treated group showed little brown coloration scattered around central vein and less positive reaction in the fibrous tissue bands as compared with the CCl_4-treated group (Fig. 4e). The silymarin-treated group showed decreased positive reaction in the fibrous tissue bands as compared with the CCl_4 group (Fig. 4c).

Result of caspase-3 content

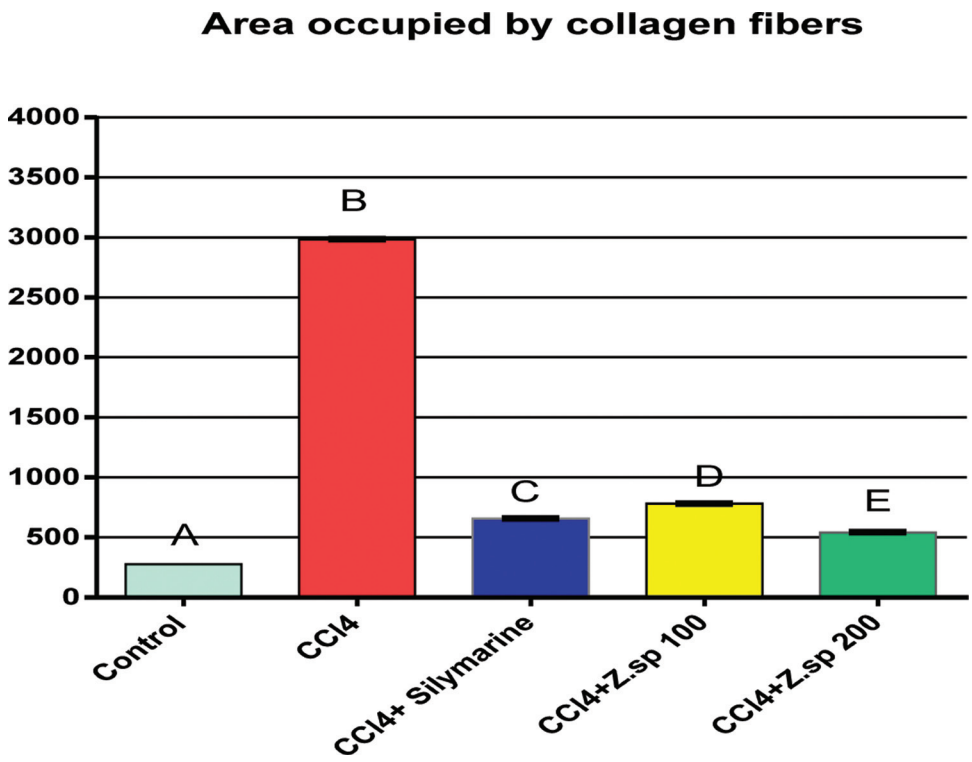
The active caspase-3 content was defined using immunohistochemical technique. In control group, weak active caspase-3 was observed (Fig. 5a). The CCl_4 group showed markedly increased of caspase-3 and appeared as brown stain when compared with the control group (Fig. 5b). The group treated with *Z. spinosa* extract (100 and 200 mg/kg) showed more or less normal appearance of caspase-3 in dose-dependent manner (Fig. 5d and e). The treatment with silymarin showed suppression in caspase-3 content compared with CCl_4-treated group (Fig. 5c).

Figure 5



Photomicrograph of the liver sections of rat stained with caspase-3, identified by their brown color. (a) Control group showing no expression of caspase-3 positive staining. (b) CCl_4 showing strong immunoreactive expression of caspase-3 positive staining cells. (c) CCl_4+sylimarin (50 mg/kg b.w.) small number of caspase-3 positive staining cells (d) CCl_4+*Z. spinosa* (100 mg/kg b.w.) showing mild number of caspase-3 positive cells. (e) CCl_4+*Z. spinosa* (200 mg/kg b.w.) showing small number of caspase-3 positive staining cells more or less normal (immunohistochemistry caspase-3, ×400).

Figure 6



Area occupied by collagen fiber in the liver tissue of control, CCl_4 and treated groups with *Z. spinosa* (n=10 fields/slid/rat); the different capital letters are significantly different using analysis of variance test at $P<0.05$.

Area occupied by collagen fiber (quantitative fibrosis)

In CCl_4 group, there was significant elevation ($P<0.01$) in fibrotic marker when compared with control group. However, *Z. spinosa*-treated group showed significant minimal fibrotic marker compared with fibrosis CCl_4 group ($P<0.001$) in dose-dependent manner. Moreover, silymarin administration significantly reduced collagen fiber formation (Fig. 6).

Discussion

Hepatic fibrosis can lead to the progression of hepatic cirrhosis and hepatocellular carcinoma. The development of approaches to inhibit hepatic fibrosis and find beneficial drugs can prevent or restore liver fibrosis [31]. The newly, research for new antifibrotic drugs has refocused on herbal medicine.

The strategies of therapeutic liver fibrosis have been designed to find antioxidant compounds that can ameliorate the oxidative status, prevent free radicals generation to inhibit ROS-mediated fibrogenesis, and suppress the progression of liver fibrosis [1].

Metabolism of CCl_4 in liver leads to lipid peroxidation and production of free radicals, which causes hepatocytes necrosis, inflammation, and progression of liver fibrogenesis [32]. Oxidative stress and ROS are intermediate factors responsible for fibrosis progression and HSCs activation [33].

In the present study, CCl_4 injection for 8 weeks in rats caused significant elevation of serum AST, ALT, ALP, and total bilirubin. In addition, CCl_4 intoxication produced a significant reduction in total protein level. Moreover, hepatic contents showed significant elevation in MDA, NO, and HP indices, together with reduction in GSH level as a resultant CCl4 oxidative stress. These effects were further confirmed by histopathological examination, which revealed the presence of hepatic degeneration, fatty changes, apoptosis and necrosis.

Yun *et al.* [34] documented that CCl_4 is metabolized by cytochrome p450 (CYP2E1 isoform) into trichloromethyl CCl3• and Cl3COO• (hepatotoxic radicals) that covalently bind to cell constituents leading to lipid peroxidation and elevation of serum parameter (AST and ALT). Moreover, LPO initiates the production of hepatic necrosis, activation of the inflammatory cells including macrophages, activation of HSCs, and the release of fibrogenic mediators. Mainly progression of fibrosis is owing to several

factors such as imbalance between oxidant/antioxidant status and liberation of lipid peroxide metabolites and inflammatory cytokines [35,36].

In the present study, *Z. spinosa* (at 200 mg/kg) successfully improved CCl_4-hepatic damage, and reduced the increased levels of AST, ALT, ALP, total bilirubin, MDA, NO, and HA; besides, it increased GSH level when compared with CCl4-treated group.

El-Toumy *et al.* [15] reported that *Z. spinosa* extract reduced the elevated liver biochemical parameters induced by CCl_4 (AST, ALT, and ALP). Moreover, the present study confirmed the previously findings that the *Amaranthus spinosus* extract has the ability to suppress liver damage and is capable of normalizing the levels of biochemical parameters intoxicated with CCl_4, and displays strong preventing action to minimize peroxidation products and increase antioxidant enzymes in the liver. *Amaranthus spinosus* extract possesses considerable hepatoprotective activity that might be owing to antioxidant defense factors, and phenolics might be the essential constituents responsible for activity [36]. Phytochemical study reported that *Z. spinosa* contained flavonoids, carbohydrates, glucosinolates, free sinapine, sterols and triterpenes, and these compounds have enormous biological effects such as antioxidant, antifungal, hepatoprotective, and antiviral activities [37,38].

Our results indicated that CCl_4 injection for 8 weeks in rats caused severe histopathologic injury in liver such as remarkable fibrosis, architecture deformation, appearance of the fibrotic bridging, and presence of many pseudolobules.

Luo *et al.* [39] reported that the treatment of rats with CCl_4-induced liver fibrosis after 8 weeks. The increase in amount of ECM and bundles of collagen surrounding the lobules produced fibrous septa with distortion of liver tissues and initiated the activation of HSC and genetic overexpression of fibrogenic cytokines also produced by lipid peroxidation [40,41].

In addition, HA is an amino acid predominately found in association with collagen. Quantification of hepatic content of HA is a good method for detection of hepatic fibrosis and evaluation of new potentially antifibrotic agents. In conformity with former studies [42], our results also showed that CCl_4 significantly increased the hepatic content of HA, which indicates high level of collagen production in the liver [43].

Moreover, the degrees of pathological alteration followed chronic intoxication with CCl_4 which were also markedly improved by *Z. spinosa* treatment (at 200 mg/kg). Moreover, after treatment with the extract revealed marked decrease in the hepatic HA and reduced collagen content, indicating *Z. spinosa* has confirmed antifibrotic effects. These results were in conformity with serum parameters level in liver tissues [15]. The potential hepatoprotective mechanisms of aqueous ethanol extract of *Z. spinosa* on CCl_4-induced liver damage in rats may be owing to inhibition of the cytochrome P450-dependent oxygenase activity and stabilization of the hepatocyte membrane [44].

Regarding histochemical results, rats treated with CCl_4 showed decreased PAS content in the cytoplasm of the hepatocytes. These results go in agreement with Poli and colleagues [45,46]. They reported that the oral administration of a single dose of carbon tetrachloride (2.5 mg/kg b.w.) showed remarkable decrease in glycogen content. De *et al.* [47] have confirmed the decrease in reaction of PAS in liver of rats after treatment with CCl4. They assumed that the stress caused by intoxication with CCl_4 leads to increase of glucose level and subsequent production of liver epinephrine and thus increased glycogenolysis, and this could account for the decrease in glycogen content in liver of rats.

In the fibrotic liver, hepatocytes lose ordinary structure and there is deteriorated functionality and storage of glycogen. Therefore, assessment of hepatocytes' glycogen content can be used as a marker of liver injury [48].

However, the present study revealed that *Z. spinosa* extract (at 200 mg/kg) showed remarkable improvement in glycogen content; therefore, it is able to pass the fenestration in endothelial lining of the sinusoids. Based on aforementioned results, it is preferable to restore glycogen content in fibrosis-treatment strategy using *Z. spinosa* extract [49].

The hepatotoxic effects of CCl_4 stimulate ROS and lower antioxidant defenses, including antioxidant enzymes. There is evidence proving that oxidative stress plays an important role in liver fibrosis; therefore, liver fibrogenesis can be prevented by using antioxidants [50].

Flavonoids are phenolic compounds widely distributed in plants [51]. Polyphenols are free radical scavengers and mediators of peroxidation in the body. Thus,

phenolic constituents contribute to the reduction of free radicals produced by virus or chemical-induced inflammation, which can cause liver damage and fibrosis [52,53]. However, using *Z. spinosa* as an antioxidant source, good additional research needs to be developed regarding it as a viable applicable option in the treatment of liver fibrosis, because the presence of polyhenols are responsible for the observed anti-fibrotic effect [54,55].

Moreover, ROS produced are involved in necrosis and apoptosis of hepatocytes and HSC activation during liver fibrogenesis. In addition, HSCs are considered an important cellular source of ECM during liver fibrosis with the release cytokines and growth factors, and the activation of HSCs produced inflammatory cells and platelets and led to activation of Kupffer cells [56]. In hepatic fibrosis, the activated HSCs are converted into α-SMA-positive myofibroblast, that lead to intensive collagen deposition [57]. Thus, α-SMA is an effective strong marker of fibrosis and could be valuable in the evaluation the efficacy of the antifibrotic therapy [58,59].

In addition, an immunohistochemical study exhibited marked increase of α-SMA expression in CCl_4-fibrotic livers when compared with normal, proving that CCl_4 enhancement stimulated the activation of HSCs in the rat model and agreed with Friedman [60].

The antifibrogenic effects of *Z. spinosa* are likely mediated by upregulation of caspase-3 and α-SMA. Therefore, the effective strategies for the treatment and prevention of hepatic fibrosis focus on activation of HSCs and modify fibrolysis and fibrogenesis.

In the present study, many α-SMA-positive cells were detected in CCl_4-treated group; whereas *Z. spinosa*-treated groups showed few activated cells. Moreover, the present study revealed remarkable improvement in fibrosis marker after treatment with *Z. spinosa* extract. These improvements are owing to high content of polyphenols in *Z. spinosa* extract [15]. This decrease was associated with changes in the redox status and decreased in α-SMA expression; these indicated the inhibited activation of HSCs and suppress production of collagen fibers in the liver tissue. All these results indicate that *Z. spinosa* has therapeutic efficacy against fibrotic rat owing to its antioxidant activity.

Recent research reported that flavonoids from various plants have antioxidant properties and produce their effect on antioxidative enzymes [61,62].

Moreover, polyphenols can act as antioxidants through many potential mechanisms, such as polyphenols can break radical chain reaction with the inhibition of the free radical formation by regulation of enzyme activity or chelating metal ions involved in free radical production. The other potential role of antioxidant effect may be owing to interaction between polyphenolic compounds and other physiological antioxidants [63,64]. Silymarin group has shown decreased fibrosis owing to the known therapeutic effect of this antifibrotic pharmaceutical. As a natural flavonoid, silymarin is known to reduce liver damage through cytoprotection and suppression of Kupffer cell function [65].

The caspase-3 is an essential procedure of programmed cell death involved in cleavage of many apoptosis-related proteins and used in diagnostics for exhibiting of apoptosis in most cell type [66]. Hepatocyte apoptosis displays great ability to phagocytize apoptotic bodies, than being a quiet sequel of liver injury; this may be used to improve liver fibrosis and developing a potential antifibrotic strategy [67,68]. In our result, CCl_4-treated rat showed intensive reaction in caspase-3 which exhibited dense apoptosis when compared with the control group. This may owing to CCl_4 oxidative stress and induction of inflammation process [68].

Moreover, the co-treatment with CCl_4 and Z. *spinosa* or silymarin revealed reduction in caspase-3 expression production. These results indicated that Z. *spinosa* or silymarin has antiapoptotic effect against CCl_4-induced oxidative stress by increasing the antiapoptotic protein production and decreasing the production of apoptotic proteins.

Guangwei *et al.* [69] reported that silymarin has antineoplastic action that suppresses endothelial cells apoptosis through a p53-dependent pathway include Bcl-2/Bax, cytochrome C release, and activation of caspase-3. However, flavonoids can regulate apoptosis and may prevent toxicity and cancer.

Hepatic apoptosis was attenuated by *Z. spinosa* administration from the early stage of chronic liver disease. This is owing to the bioactive composition of *Z. spinosa* extracts, which have efficacy to reduce liver fibrosis targeting apoptosis of hepatocytes and suppression of HSC activation [70,71].

Medicinal plants are able to inhibit the release of hepatocyte-derived apoptotic bodies and damage-associated molecular patterns, some of the initial profibrogenic stimuli that converge to activation and

survival of HSC, while inducing apoptosis of activated HSC; they remove the essential source of ECM. Regulation of mitochondrial pathways of apoptosis by medicinal compounds is the principal induction and protection of apoptosis *in vitro* and *in vivo* [72].

El-Toumy *et al.* [15] reported that *Z. spinosa* contains high amount of Quercetin in addition to polyphenolic and flavonoid compounds almost ubiquitous in plants and plant food sources. Quercetin is considered as a powerful antioxidant owing to its capability to scavenge free radicals and bind transition metal ions. These properties of quercetin permit it to prevent lipid peroxidation [73] and have anti-inflammatory properties [74]. The existence of these compounds could clarify the antioxidant activity found in the crude extract.

The prospective therapeutic ability of silymarin alone or in a combination with vitamin E and/or curcumin against CCl_4-induced liver injury in rats may contribute to their antioxidant, anti-inflammatory and antiapoptotic properties and act on ROS induced by CCl_4 [75]. In addition, silymarin suppresses HSCs through the expression of transforming growth factor-β1 and stabilization of mast cells [76].

All the accomplished results clarify that *Z. spinosa* extract has antioxidant and antiapoptotic properties in CCl_4-induced liver fibrosis; this is owing to the phenolic acids and flavonoids. The flavonoids act as hydrogen donors with metal ion chelators, and the phenolic acids possess good antioxidant activity [77].

From the present study, *Z. spinosa* possesses these two properties: it reduces fibrosis owing to its antioxidant actions, and alleviates fibrosis through reducing the depositions of both α-SMA and collagen fibers. It demonstrates that treatment with *Z. spinosa* was able to exert a therapeutic effect on developing hepatic fibrosis induced by CCl_4, and the inhibitory effect of high dose (200 mg/kg) appeared to be more potent than low dose (100 mg/kg).

Conclusion

The study showed that *Z. spinosa* had inhibitory effects on apoptosis and fibrosis in liver, which were mainly associated with downregulation of HSC activation, thus regulating fibrotic-related factors, such as expression levels of α-SMA, and by inhibiting hepatocyte apoptosis, which may provide potential therapeutic strategies for anti-fibrosis.

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Conflicts of interest
There are no conflicts of interest.

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