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## Original Article

# Antioxidant and hepatoprotective effects of *Capparis spinosa* L. fractions and Quercetin on tert-butyl hydroperoxide- induced acute liver damage in mice



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## ABSTRACT

The present study investigates the antioxidant and hepatoprotective effects of *Capparis spinosa* L. and Quercetin in tert-butyl hydroperoxide (t-BHP) induced acute liver damage. Different fractions of *C. spinosa* were examined for total phenolic content and antioxidant property. Among these fractions, hydroalcoholic extract was used to assess the hepatoprotective effect in tert-butyl hydroperoxide (t-BHP) induced hepatotoxicity model by determining serum biochemical markers, sleeping time and antioxidant assay such as reduced glutathione (GSH) as well as histopathological examination of liver tissues. The total phenolic and Quercetin contents of hydroalcoholic fraction were significantly higher than other fractions. It also showed high antioxidant activity. Pretreatment with hydroalcoholic fraction at the dose of 400 mg/kg and Quercetin at the dose of 20 mg/kg showed liver protection against t-BHP induced hepatic injury, as it was evident by a significant decrease in serum enzymes marker, sleeping time and MDA and an increase in the GSH, SOD and CAT activities confirmed by pathology tests. The final results ascertained the hepatoprotective and antioxidant effects of *C. spinosa* and Quercetin in a dose-dependent manner. Moreover, this study suggests that possible mechanism of this protection may be associated with its property of scavenging free radicals which may be due to the presence of phenolic compounds. © 2018 Center for Food and Biomolecules, National Taiwan University. Production and hosting by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Liver is the largest internal organ in the human body that plays a major role in metabolism and detoxification of various chemicals, drugs and other toxic compounds.<sup>1</sup> Oxidative stress is a biochemical condition that occurs in the body producing several types of reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS).<sup>2</sup> Oxidative stress is regarded as one of the pathological mechanisms that causes initiation and progression of liver damage through inducing irreversible alteration of lipid

membranes, proteins and DNA and, more importantly, through modulating pathways that control biological function.<sup>3</sup> A number of pro-oxidants are implicated in the oxidative stress and cell injury that result from their intracellular metabolism to free radical intermediates.<sup>4</sup> tert-butyl hydroperoxide (t-BHP) is an organic lipid hydroperoxide analogue which is often used as a model compound to induce oxidative stress during *in vitro* and *in vivo* studies. t-BHP can be metabolized into free radical intermediates by cytochrome P-450 which subsequently initiates lipid peroxidation and depletes cellular reduced glutathione (GSH) content. t-BHP caused leakage of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and formation of malondialdehyde (MDA) in hepatocyte. It also mediated DNA base damage in mammalian cells.<sup>5–7</sup>

The genus *Capparis* belonging to the Capparaceae family includes nearly 250 species; many of which are distributed in the

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Mediterranean regions.<sup>8</sup> *Capparis spinosa* L. has been used for pharmaceutical, cosmetic, and nutritional purposes. In many countries, the flower buds, fruits, roots, and seeds of the *C. spinosa* have been used in folk medicine as an anti-rheumatic, tonic, expectorant, anti-spasmodic, diuretic and analgesic agents.<sup>9</sup> They are very good sources of glucosinolates (glucocapparin, glucoiberin, sinigrin and glucobrassicin), flavonoids, phenolic acids and alkaloids; all of which are known to provide health-improving benefits due to their various biological activities such as antioxidant, anti-carcinogenic, antimicrobial and antimutagenic.<sup>10</sup> Furthermore, leaves of *C. spinosa* is used to prepare Liv-52 which is employed to treat alcoholic liver disease, viral hepatitis, and liver cirrhosis.<sup>11</sup>

Quercetin is a flavonoid found in many fruits and vegetables including green apple, onion, green tea, lemon as well as in medicinal botanic plants. In addition, *C. spinosa* is one of the rich sources of Quercetin. It is one of the most active antioxidants owing to its high ability to scavenge free radicals.<sup>12</sup>

The present study was conducted to investigate the antioxidant activity and phenolic content of *C. spinosa* fractions. We have also examined the mechanism of *C. spinosa* and Quercetin as hepatoprotective agents by studying their effects on serum liver function, oxidative stress biomarkers and hepatic histopathology of mice subjected to t-BHP-induced hepatotoxicity.

## 2. Materials and methods

### 2.1. Chemicals

Quercetin, chloroform, ethyl acetate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), trichloro acetic acid (TCA), thio-barbituric acid (TBA), bovine serum albumin (BSA) and Bradford reagent were purchased from Sigma–Aldrich chemical company (St. Louis, MO), USA. All chemicals and reagents used were analytical grade. Tert-butyl hydroperoxide (t-BHP) was also purchased from Roche chemical company (Germany).

### 2.2. Plant extraction

Plant was collected from the Iranian province of Khuzestan during spring 2015. Plant materials were taxonomically identified by central herbarium of Ahvaz Jundishapur University of Medical Sciences, Iran. The leaves of *C. spinosa* were shade-dried, ground and soaked in 80% aqueous-ethanol, ethyl acetate and chloroform in separate containers for three days with occasional shaking.

Each solvent was filtered through a filter paper (Whatman No. 2) and then was removed under vacuum in a rotary evaporator until dryness. The percentage yields of extracts in different solvents were 15.75% (w/w) for dried hydroalcoholic, 4.51% for ethyl acetate and 3.9% for chloroformic fraction, respectively.

### 2.3. HPLC analysis

A high performance liquid chromatography system (HPLC) (Shimadzu-02-0600, Japan) was used for quantitative and qualitative analysis of Quercetin. Analyses were conducted using a C18 column (4.6 mm × 150 mm, 5 μm diameter particle sizes, Waters, Symmetry, Milford, USA). The mobile phase used was methanol: acetonitrile: water (10:10:80) with a flow rate of 1 ml/min and injection volume was 20 μl. An amount of 0.5 g of each extracts were dissolved in 10 ml of solution (methanol–acetic acid–water (1:2:1)) for 1 h on a shaker at laboratory temperature. 2 ml of the solutions were centrifuged for 10 min at 2000 × g. Then, the solutions were filtered through a micro filter with regenerated cellulose membranes of the pore size 0.22 μm. The filtrate was applied

for HPLC. Standard solutions of Quercetin were also prepared by dissolving different amount in methanol. Suitable concentrations were injected to find the RT (retention time) values and check the linearity between concentration and peak areas. The spectrum was detected at 370 nm using a UV detector.

### 2.4. Total phenolic content (TPC)

Total phenolic contents of crude extracts were determined by a spectrophotometer using the Folin-Ciocalteu's reagent.<sup>13</sup> Different dilutions of each dry extract (3.75, 6.25, 12.5 and 25 mg/ml) were prepared in 10 ml of their own solvent. Moreover, different concentrations of tannic acid (0.02, 0.04, 0.06 and 0.08 mg/ml) were prepared as standards. Then, 0.5 ml of extracts or standards was transferred into a 5 ml volumetric flask and was swirled with 2.5 ml of Folin-Ciocalteu's reagent (diluted 1:10, v/v). After 5 min, 2 ml of Na<sub>2</sub>CO<sub>3</sub> (7.5%, v/v) solution was added and mixed. The solution was thoroughly mixed and placed at ambient temperature for 2 h until the characteristic blue color was developed. The absorbance was measured at 765 nm after 30 min. Quantification of TPC was based on a standard curve generated with tannic acid (TAC) at 765 nm.

All tests were conducted in triplicate and averaged. Results were expressed as tannic acid equivalent (mg tannic acid/g dried extract). Additional dilution was done in case the absorbance value measured was over the linear range of the standard curve.

### 2.5. In-vitro free radical scavenging activity

#### 2.5.1. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Radical scavenging activity was determined by a spectrophotometric method based on the reduction of the methanol solution of DPPH.<sup>14,15</sup> Tests were carried out in triplicate. A methanol solution (0.1 ml) of the test sample at various concentrations was added to 3.9 ml of a DPPH solution (25 mg/lit). The decrease in absorbance at 515 nm was ceaselessly determined every 1 min with a UV–Vis spectrophotometer for 30 min. The percentage inhibition (I %) of DPPH radical was calculated in the following way:

$$\text{Inhibition of DPPH\%} = \left[ 1 - \frac{A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

A blank is the absorbance of the control reaction (containing all reagents except the test compound) while A sample is the absorbance of the test compound. The percentage of inhibited DPPH radical was calculated by the above equation; then, it was plotted against the sample/standard concentration to obtain the amount of antioxidant necessary to decrease the initial concentration of DPPH by 50% (IC<sub>50</sub>). Based on the parameter IC<sub>50</sub>, the result was expressed in terms of mg dry matter of sample/standard equivalent g<sup>-1</sup> DPPH in the reaction medium.

#### 2.5.2. Ferric reducing antioxidant potential (FRAP) assay

The FRAP assay was performed according to Benzie and Strain method.<sup>16</sup> The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. Briefly, the FRAP reagent contained 2.5 ml of a 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine, sigma) solution in 40 mmol/L HCl plus 2.5 ml of 20 mmol/L FeCl<sub>3</sub>·6H<sub>2</sub>O and 25 ml of 0.3 mol/L acetate buffer, pH 3.6 and was prepared freshly and warmed at 37 °C. First, absorption of fresh FRAP reagent were measured at the wavelength of 593 nm. Then, 100 μl sample or standards were mixed with 300 μl distilled water and 3 ml FRAP reagent and the absorbance of reaction mixture at 593 nm was measured spectrophotometrically. Finally, the absorbance was

measured again after 30 min incubation at 37 °C. The FRAP values were achieved by standard calibration curve obtained by using different concentrations of FeSO<sub>4</sub>·7H<sub>2</sub>O. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol/L FeSO<sub>4</sub> (equivalent concentration 1 = EC<sub>1</sub>).

We then selected the most potent antioxidant extract for *in vivo* examination.

## 2.6. Animals

56 male swiss albino mice (6–8 weeks old, 25–30 g) were obtained from animal house of Ahvaz Jundishapur University of Medical Science, Iran. Mice were kept in polycarbonate cages under standard condition (temperature 25 ± 2 °C) with 12 h light/dark cycle. They were provided with standard pellet diet and free access to drinking water *ad libitum*. Animals were acclimated to the environment for a minimum of seven days prior to the experiment. The investigation was performed according to the Animal Ethics Committee Guidelines of Ahvaz Jundishapur University of Medical Sciences for the use of experimental animals.

## 2.7. Study design

Plant extracts were dissolved in normal saline prior to be applied to the mice. t-BHP was diluted in normal saline, and then; it was administered intraperitoneally in a single dose of 1.5 mM/kg. Animals were distributed into seven groups, each containing 8 mice. Group 1, as the negative control group, received normal saline for 5 days while group 2 received oral hydroalcoholic fraction of *C. spinosa* (400 mg/kg) for five consecutive days. Group 3 as the positive control received t-BHP (1.5 mM/kg/IP) only on the 5th day and groups 4 to 7 received *C. spinosa* orally in doses of 100, 200 and 400 mg/kg and Quercetin (20 mg/kg/po) respectively for 5 days and t-BHP (1.5 mM/kg/IP) on the 5th day, 1 h after the last dose of extract and Quercetin administration.

## 2.8. Hexobarbital-induced sleeping time

On the 6th day, hexobarbital was administered at the dose of 40 mg/kg body weight I.P, and the sleeping time was recorded in minutes from the onset of sleep to its natural arousal, i.e. loss of righting reflex to its recovery.<sup>17</sup>

## 2.9. Sample collection

On the 6th day, 24 h after t-BHP administration, animals were anaesthetized with diethyl ether and their blood samples were collected by cardiac puncture. They were then sacrificed by decapitation and their livers were isolated and washed with saline quickly. Afterward, the liver was weighed and divided into two parts. One part was used for pathological studies and the other part was homogenized for tissue antioxidant assays.

## 2.10. Biochemical assays

The blood samples were left to clot for 40 min at room temperature. Serum was separated by centrifugation at 1050 × g at 24 °C for 5 min and was stored at –20 °C until further analyses. Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were determined employing the Reitman-Frankel method.<sup>18</sup> Alkaline phosphatase (ALP) was estimated according to king's method.<sup>19</sup>

In order to estimate the tissue biochemical parameters, the liver tissues were chopped into small pieces with scissors. Then, it was

homogenized in ice-cold phosphate buffer (pH 8) at a concentration of 10% (weight by volume) using a wisetis HG-150 homogenizer. The unbroken cells and cell debris were removed by centrifugation at 200 × g for 10 min using a (HettichZentrifugen, Germany) refrigerated centrifuge. Protein content in homogenates was measured by the method of Bradford<sup>20</sup> using crystalline BSA as standard. The supernatant was used for the evaluation of GSH, malondialdehyde (MDA), superoxide dismutase (SOD) and catalase employing standard methods.

The GSH content in the tissue was determined using a modified version of the method described by Ellman, based on the formation of a yellow colored complex with Ellman's reagent (DTNB).<sup>21</sup> Free endogenous GSH was assayed in a 3 ml volume glass by the addition of 40 µl of 0.5 mM DTNB prepared in 0.2 M phosphate buffer (pH = 8) to 2 ml phosphate buffer and 40 µl of the supernatant. The absorbance was read at 412 nm using a UV–visible spectrophotometer.

The lipid peroxidation was expressed by measuring the amounts of malondialdehyde (MDA) based on the thiobarbituric acid (TBA) color reaction and using the method described by Buege and Aust.<sup>22</sup> Briefly, 0.5 ml of liver supernatant was mixed with 1.5 ml of TCA (10%, w/v). The samples were centrifuged at 3000 × g for 10 min; and 1.5 ml of each sample supernatant was transferred to a test tube containing 2 ml of TBA solution (0.67%, w/v). The mixture was kept in boiling water for 30 min, forming a pink color solution. The mixture was then immediately cooled and the absorbance was measured at 532 nm by spectrophotometer. The concentration of MDA was calculated based on the absorbance coefficient of the TBA–MDA complex ( $\epsilon = 1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ ) and it was expressed as nmol/mg protein.

Catalase (CAT) activity was measured by the method of Aebi and Bergmeyer.<sup>23</sup> 100 µl of liver supernatant was added to a quartz cuvette and the reaction was initiated by the addition of 1.9 ml of freshly prepared H<sub>2</sub>O<sub>2</sub> (30 mM) in phosphate buffer (50 mM, pH 7.0). The decomposition rate of H<sub>2</sub>O<sub>2</sub> was measured spectrophotometrically at 240 nm during 60s. The activity of CAT was expressed as µmol H<sub>2</sub>O<sub>2</sub>/mg tissue/min.

Superoxide dismutase SOD activity was measured using a calorimetrically enzymatic assay kit (ZellBio GmbH, Ulm, Germany). In this assay, the SOD activities were expressed as units per microgram of liver protein.

The levels of MDA, SOD and GSH were normalized with protein.

## 2.11. Histopathological assessments

For the histological examination, the livers were fixed in 10% formalin solution for at least 24 h. Then, the liver tissues were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 µm sections and stained with hematoxylin and Eosin dye (H&E stain). These sections were then examined under a photomicroscope for the presence of cell necrosis, fatty change, ballooning degeneration, edema and mononuclear/poly-nuclear cell infiltration.

## 2.12. Statistical analysis

Data analysis was carried out using GraphPad Prism software version 5. Results were expressed as Mean ± SEM and all statistical comparisons were made by means of one-way ANOVA test followed by Tukey's post hoc analysis. p-value less than 0.05 was considered to be significant.

## 3. Results

The physical properties and percentage (%) yield of different extracts are demonstrated in Table 1.

**Table 1**  
Physical properties, total phenolics and Quercetin content of different extract of *C. spinosa*.

Fraction	Color	Consistency	% Yield (w/w)	Quercetin content (mg/g)	Total phenolics (mg/g)
Hydro-alcoholic	Greenish brown	Semi-solid	15.75	7.415 ± 0.405	26.58978 ± 1.191
Ethyl acetate	Dark green	Semi-solid	4.51	2.897 ± 0.312 <sup>a</sup>	6.53532 ± 0.425 <sup>a</sup>
Chloroform	Dark green	Semi-solid	3.9	4.986 ± 0.309 <sup>a</sup>	13.65529 ± 1.361 <sup>a</sup>

Values are presented as mean ± S.E.M for 3 replications.

<sup>a</sup> Significantly different compared to hydro-alcoholic fraction ( $p < 0.05$ ).

### 3.1. HPLC analysis

Evaluation of Quercetin in different fractions of *C. spinosa* by HPLC revealed that hydroalcoholic fraction had the highest Quercetin content (7.415 ± 0.405 mg/g) followed by chloroformic (4.986 ± 0.309 mg/g) and ethyl acetate fraction (2.897 ± 0.312 mg/g). The results are shown in Table 1. Moreover, the chromatograms of Quercetin standard solution and hydroalcoholic fraction of *C. spinosa* are shown in Fig. 3.

### 3.2. Total phenolic content (TPC)

The amount of total phenolics is varied in different extracts (Table 1). The highest total phenolic levels were detected in hydroalcoholic extract (26.58978 ± 1.191 mg/g extract) followed by chloroformic (13.65529 ± 1.361 mg/g) and ethyl acetate extract (6.53532 ± 0.425 mg/g).

### 3.3. In-vitro free radical scavenging activity

#### 3.3.1. DPPH radical scavenging activity

The evaluation of antioxidant activities of extracts and Quercetin by DPPH assay showed that Quercetin has better scavenging activity and has the lower IC50 followed by hydroalcoholic extract, chloroformic and ethyl acetate extract, respectively. The antioxidant activities of Quercetin, hydroalcoholic and chloroformic extracts were similar ( $p > 0.05$ ) while ethyl acetate extract exhibited a weak antioxidant activity (Table 2).

#### 3.3.2. FRAP assay

The FRAP assay measures the antioxidant activity of any substance in the reaction medium by its reducing ability. Antioxidant potential of different fractions of *C. spinosa* and Quercetin was estimated according to their ability to reduce the TPTZ-Fe (III) complex to a TPTZ-Fe (II) complex and was expressed as EC1. The hydroalcoholic fraction of *C. spinosa* and Quercetin showed highest FRAP antioxidant activity as recorded in DPPH methods. The order of FRAP activity of respective samples is as follows: Quercetin > hydroalcoholic > chloroformic, followed by ethyl

acetate extract (Table 2). As in the case of DPPH, the ferric reducing antioxidant capacity of ethyl acetate extract was found to be significantly lower than other samples ( $p < .005$ ).

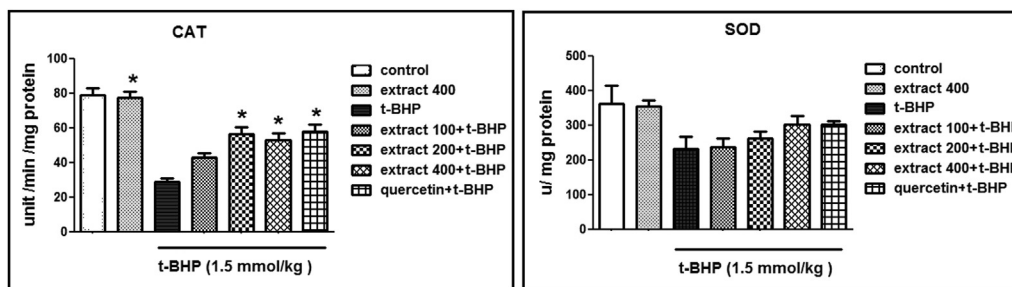
In addition, a significant correlation was observed between TPC and the FRAP assays ( $R^2 = 0.699$ ; data not shown). Moreover, a significant correlation of  $R^2 = 0.586$  was calculated between TPC and the IC 50 of DPPH radical-scavenging activity.

### 3.4. Biochemical changes

The hepatic enzymes of serum ALT, AST and ALP are well known as biomarkers for early acute hepatic damage. The effects of pretreatment with *C. spinosa* extracts and Quercetin on the t-BHP-induced elevation of ALT, AST and ALP are shown in Table 3. Data analyses demonstrated that t-BHP (2 mmol/kg/ip) intoxication develop severe hepatic damage as it was evident from a significant increase in the serum activities of ALT, AST and ALP ( $p < 0.05$ ) as compared to the normal control group (Table 3). Pretreatment with *C. spinosa* (100, 200 and 400 mg/kg) and Quercetin (20 mg/kg/po) significantly decreased the elevation of AST and ALT ( $p < 0.05$ ). In addition, *C. spinosa* decreased ALP in all doses by a dose-dependent manner; however, high dose of *C. spinosa* extract (400 mg/kg) and Quercetin (20 mg/kg/po) significantly reduced the elevation of ALP. Furthermore, no significant difference was observed between the control and second group which received high doses of *C. spinosa* (400 mg/kg).

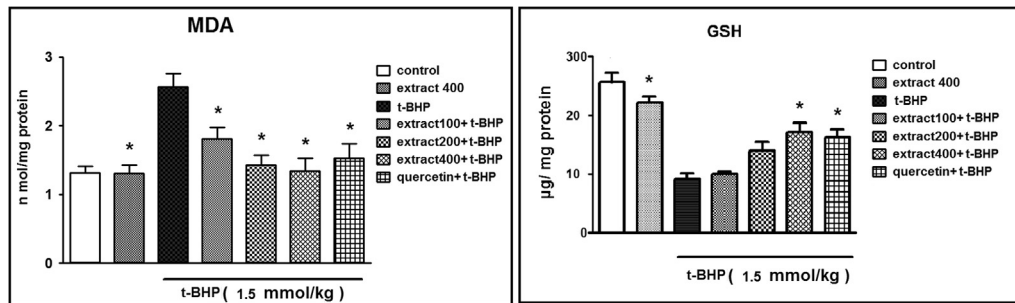
### 3.5. Effect on oxidative stress

In order to further evaluate the possible mechanism involved in the protective effect of *C. spinosa* and Quercetin on hepatotoxicity, MDA formation, GSH content and antioxidant enzymes (SOD and CAT) were measured. As seen in Figs. 1 and 2, t-BHP treatment increased MDA formation and depleted liver GSH level. Compared to the control group, t-BHP also significantly decreased SOD and CAT activities. Regarding the inhibition of lipid peroxidation, we observed that the groups which were pretreated with *C. spinosa* and Quercetin significantly reduced the amount of MDA in liver homogenate, as compared with the t-BHP-intoxicated group

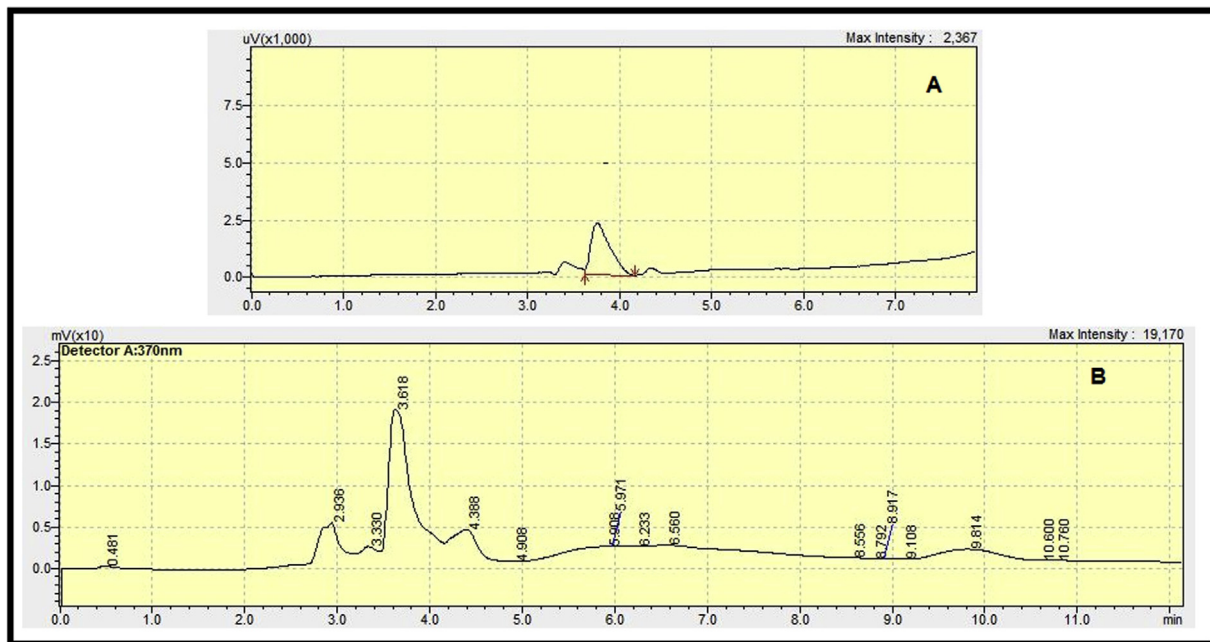


**Fig. 1.** Pretreatment effects of *C. spinosa* and Quercetin on the hepatic SOD and CAT activity. Each value represents means ± S.E.M. of 8 mice per group. \*: Significantly different ( $p < 0.05$ ) from the positive control group.





**Fig. 2.** Pretreatment effects of *C. spinosa* and Quercetin on the hepatic GSH and MDA content. Each value represents means  $\pm$  S.E.M. of 8 mice per group. \*: Significantly different ( $p < 0.05$ ) from the positive control group.



**Fig. 3.** (A): HPLC chromatogram of 100 ng/ml Quercetin standard solution. (B): HPLC Chromatogram of hydroalcoholic extract of *C. spinosa* containing Quercetin.

( $p < 0.05$ ). Moreover, the group pretreated with 400 mg/kg of *C. spinosa* had even lower MDA levels than the other treatment group. GSH forms the main intracellular antioxidant to scavenge the free radicals. Administration of t-BHP significantly reduced the levels of GSH. Pretreatment with *C. spinosa* and Quercetin significantly recovered the levels of GSH depletion produced by t-BHP. Likewise, 400 mg/kg of *C. spinosa* and Quercetin showed the best result.

Antioxidant enzymes form the first line of defense against free radical-induced toxicity. In the present study, SOD and CAT

activities were significantly decreased ( $p < 0.05$ ) in the t-BHP group when compared to the control group, indicating that t-BHP damaged Antioxidant defence. Quercetin and *C. spinosa* in all doses raised SOD and CAT activities as compared to the mice treated with t-BHP. The increase was also significant in CAT activity.

### 3.6. Histopathology

The histopathological pictures of liver are shown in Fig. 4. The pattern of liver in the negative control group and the second group with only crude extract showed normal hepatic architecture. The hepatocytes are within normal limits and are separated by narrow blood sinusoids. Structure of liver lobules was preserved and no cell necrosis, fatty change and inflammation were seen (Fig. 4.1 and 4.2). t-BHP intoxicated group exhibited severe histopathological changes such as centrilobular hepatic necrosis, fatty changes, vacuolization, and infiltrating lymphocytes (Fig. 4.3).

Pretreatment with 100 mg/kg of *C. spinosa* extract showed mild improvement and congestion, diffuse necrosis, accumulation of lymphocytes in the portal. Vacuolization and severe edema were also observed in hepatocytes (Fig. 4.4). Administration of 200 mg/kg of *C. spinosa* extract showed mild to moderate congestion and necrosis, moderate lymphocytic aggregation in portal, sporadic and

**Table 2**

*In vitro* antioxidant activity of *C. spinosa* extracts and Quercetin as determined by the DPPH and FRAP assays.

Extract	DPPH (IC 50 value; mg/ml)	FRAP (EC1 value; mg/ml)
Hydro-alcoholic	0.034891 $\pm$ 0.0017	1.655187 $\pm$ 0.1219
Ethyl acetate	0.277039 $\pm$ 0.0122 <sup>a, b</sup>	15.90301 $\pm$ 1.082 <sup>a, b</sup>
Chloroformic	0.0392 $\pm$ 0.0011	3.908373 $\pm$ 0.3171 <sup>a</sup>
Quercetin	0.01158 $\pm$ 0.00107	0.4903 $\pm$ 0.05911

Values are presented as mean  $\pm$  S.E.M for 3 replications.

<sup>a</sup> Significantly different compared to Quercetin ( $p < 0.05$ ).

<sup>b</sup> Significantly different compared to hydro-alcoholic fraction ( $p < 0.05$ ).

**Table 3**Effects of the pretreatment with hydroalcoholic fraction of *C. spinosa* and Quercetin on the serum activities of AST, ALT, ALP and sleeping time in t-BHP-induced hepatotoxicity.

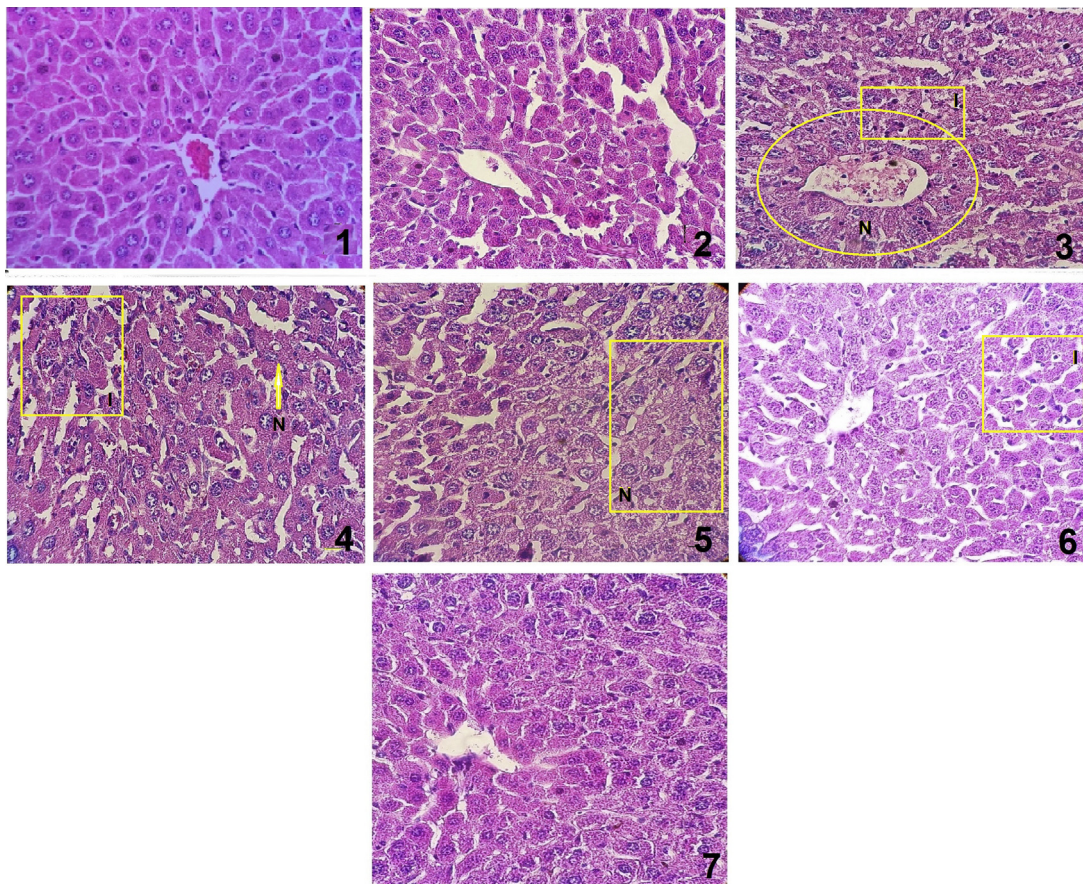
Groups	ALT (U/l)	AST (U/l)	ALP (U/l)	Sleeping time (min)
1 – Normal saline (control)	77.35 ± 5.073	119.1 ± 7.898	303.9 ± 7.426	15.44 ± 1.613
2 – Extract (400 mg/kg)	90.94 ± 3.54 <sup>b</sup>	113.3 ± 6.617 <sup>b</sup>	301.4 ± 11.17 <sup>b</sup>	18.29 ± 1.604 <sup>b</sup>
3 – t-BHP (50 mg/kg)	357.33 ± 25.94 <sup>a</sup>	491.7 ± 26.53 <sup>a</sup>	430.9 ± 15.25 <sup>a</sup>	43.53 ± 2.977 <sup>a</sup>
4 – Extract (100 mg/kg) + t-BHP	241.41 ± 17.19 <sup>a, b</sup>	308.5 ± 22.23 <sup>a, b</sup>	405.2 ± 6.501 <sup>a</sup>	38.85 ± 2.667 <sup>a, b</sup>
5 – Extract (200 mg/kg) + t-BHP	160.21 ± 17.72 <sup>a, b</sup>	311.4 ± 16.43 <sup>a, b</sup>	378.8 ± 13.67 <sup>a</sup>	30.97 ± 1.771 <sup>a, b</sup>
6 – Extract (200 mg/kg) + t-BHP	129.80 ± 15.93 <sup>b</sup>	236.8 ± 16.49 <sup>a, b</sup>	340.7 ± 21.67 <sup>b</sup>	24.60 ± 2.011 <sup>b</sup>
7 – Quercetin (200 mg/kg) + t-BHP	192.42 ± 14.90 <sup>a, b</sup>	219.4 ± 12.70 <sup>a, b</sup>	326.7 ± 21.44 <sup>b</sup>	26.80 ± 2.092 <sup>a, b</sup>

Animals in group 1 received normal saline solution, while group 2 received *C. spinosa* extract (400 mg/kg, po) for 5 days, group 3 received t-BHP (0.18 mM/kg, ip) on the fifth day. The mice in groups 4, 5 and 6 were pretreated with *C. spinosa* extract (100, 200 and 400 mg/kg, p.o, respectively) once daily for five consecutive days. Group 7 were pretreated with Quercetin (1.5 mM/kg, p.o), once daily for five consecutive days. One hour after the final treatment, the mice were treated with t-BHP (0.18 mM/kg, ip). Hepatotoxicity was determined 24 h later by quantifying the sleeping time and serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) as well as alkaline phosphatase (ALP).

Each value represents the mean ± SEM for eight mice.

<sup>a</sup> Significantly different from the control ( $p < 0.05$ ).

<sup>b</sup> Significantly different from t-BHP group ( $p < 0.05$ ).



**Fig. 4.** Histopathological observations of liver sections stained with hematoxylin and eosin ( $\times 100$ ). (1 and 2) Liver sections of mice treated with saline and hydroalcoholic extract of the *C. spinosa* (400 mg/kg, p.o.), respectively, showing normal architecture and central vein and normal hepatic cells; (3) Liver section of mice treated with t-BHP (1.5 mmol/kg, i.p.) showing centrilobular necrosis, congestion and severe inflammation; (4) Liver section of *C. spinosa* (100 mg/kg, p.o.) + t BHP (1.5 mmol/kg, i.p.)-treated mice, showing small areas of necrosis and partially severe inflammation.; and (5) Liver section of mice treated with *C. spinosa* (200 mg/kg, p.o.) + t BHP (1.5 mmol/kg, i.p.) showing partially preserved hepatocytes as well as the architecture with small areas of necrosis and inflammatory cell infiltration. (6 and 7) Liver section of mice treated with *C. spinosa* (400 mg/kg, p.o.) and Quercetin (20 mg/kg, p.o.), respectively + tBHP (1.5 mmol/kg, i.p.) showing well preserved hepatocytes and architecture with a few to milder degree of inflammation. N: necrosis I: inflammatory cell infiltration.

brief fatty changes in hepatocytes and partial edema (Fig. 4.5). In the group treated with 400 mg/kg of *C. spinosa* extract, slight congestion, mild lymphocytic accumulation and edema were seen (Fig. 4.6). The pattern of hepatic tissue in the Quercetin group was similar to the group received 400 mg/kg of *C. spinosa* (Fig. 4.7).

#### 4. Discussion

Increasing evidence has indicated that cellular damage mediated by oxidative stress contribute to the initiation and progression of many liver diseases.<sup>24</sup> Many efforts have been made to develop



the complementary and alternative medicines for reducing oxidative stress and improving liver functions.<sup>25,26</sup> Therapeutic agents from natural sources, such as Quercetin and silymarin, are particularly attractive for treatments as they have the potential to reduce the risk of drug toxicity.<sup>27</sup> Total phenolic content of extract often determines its pharmacological effects such as antioxidant activity. The *in vitro* study demonstrated that hydroalcoholic extract with higher total phenolic content exhibit higher antioxidant activity followed by chloroformic and ethyl acetate extract. Therefore, components like phenolic compounds may play an important role in its antioxidant activity. Several studies have reported a direct relationship between phenolic content and antioxidant activity.<sup>28</sup>

HPLC analysis of *C. spinosa* extracts showed different amounts of Quercetin in these extracts, and the Quercetin content of hydroalcoholic fraction of *C. spinosa* was higher than chloroformic and ethyl acetate extracts. This finding indicated that solvent can affect phytochemical composition of each extract.

T-BHP is a dangerous chemical that is highly reactive, flammable and toxic.<sup>29</sup> The mechanism by which t-BHP causes damage involves the biotransformation of t-BHP by the cytochrome P-450 system into a free radical intermediates, peroxy (tBuOO) and alkoxy (tBuO) radical; all of which cause lipid peroxidation eventuating in cells damage.<sup>30</sup> Aminotransferases (AST and ALT) are the first enzymes to be used in diagnosis of liver damage. Since these are normally located in the cytosol, toxicity affecting the liver with subsequent breakdown in membrane architecture of the cells leads to their spillage into plasma while their concentration rises in the bloodstream.<sup>31–33</sup> Administering t-BHP to mice markedly increases serum transaminase (AST and ALT) and ALP levels. This increase commonly reflects the severity of liver injury.<sup>34</sup> Pretreatment with *C. spinosa* and Quercetin attenuated these increased enzyme activity and was followed by recovery towards normal levels. These findings strongly prove the ability of these substances in protecting hepatocyte against membrane fragility and decreasing the leakage of enzymes into the circulation.

A number of studies have demonstrated that the neutralization of free radical by antioxidant compounds may reduce the amounts of t-BHP induced oxidative damage; and thus, protect the liver.<sup>35,36</sup> Metabolism of t-BHP leads to the formation of highly unstable free radicals to initiate peroxidation and other oxidative damage in the cells.<sup>30</sup> Hence, the antioxidant capacities to scavenge of free radicals can inhibit t-BHP – induced lipid peroxidation.<sup>37</sup>

Both enzymatic and non-enzymatic antioxidant system are essential for cellular response in order to deal with oxidative stress under physiological condition. Therefore, an increase in a variety of oxidative stress biomarkers like MDA and a decrease in other antioxidants like GSH and antioxidant enzyme including CAT and SOD strongly support the presence of oxidative stress in liver damage.<sup>38–40</sup> Antioxidant enzymes (SOD and CAT) form the first line of defense against oxidative tissue-damage.<sup>41</sup> SOD plays an important role in scavenging toxic intermediates and converting O<sub>2</sub> into H<sub>2</sub>O<sub>2</sub>. Moreover, CAT metabolizes H<sub>2</sub>O<sub>2</sub> to non-toxic products.<sup>27</sup> GSH, an important intracellular antioxidant, acts both as a co-factor for glutathione peroxidase and as a direct scavenger to remove reactive species such as hydroxyl radical, peroxynitrite, and singlet oxygen. Enhanced levels of GSH also protect liver against a potential oxidative damage.<sup>42</sup> MDA is one of the lipid peroxidation products which has been used as a biomarker of lipid peroxidation for several decades. It has been used as a facile means for assessing lipid peroxidation in biological materials.<sup>43</sup> Our findings showed that MDA levels were increased in response to t-BHP treatment while GSH, SOD and CAT levels were decreased. These preliminary findings indicate an oxidative damage associated with t-BHP. In this work, Quercetin and *C. spinosa* extract (400 mg/kg) brought the MDA and antioxidant enzymes to the normal levels, implying that

these substances prevent the oxidative damage of t-BHP. In addition, these pretreated groups have shown an increase in hepatic GSH content that indicate their ability to enhance the antioxidant capacity of liver. Moreover, histopathological studies under light microscope confirms the protective effects of *C. spinosa* and Quercetin against the t-BHP-induced liver damage as it was evident by the reversal of centrilobular necrosis, vacuolization and scattered lymphocytes infiltrate in hepatic parenchyma.

## 5. Conclusion

This study demonstrated that *C. spinosa* and Quercetin are effective for the prevention of t-BHP-induced hepatic damage in mice; and therefore, it could be used as a hepatoprotective agent. The protective effects against acute liver injury may be due to the higher antioxidant capacity, free radical scavenging effect, inhibition of lipid peroxidation and increased antioxidant activity. According to the results obtained, abundant flavonoid compounds such as Quercetin are considered as the main hepatoprotective factor in the hydroalcoholic extract of *C. spinosa*.

## Conflict of interest statement

The authors don't have any conflict of interest.

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