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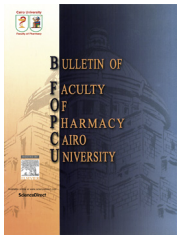

ORIGINAL ARTICLE

Hepatoprotective and antioxidant polyphenols from a standardized methanolic extract of the leaves of *Liquidambar styraciflua* L.

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Abstract The methanolic extract of the leaves of *Liquidambar styraciflua* L. (Altingiaceae) (LSE) was evaluated for hepatoprotective and antioxidant activities in carbon tetrachloride liver-damaged rats. Hepatotoxicity was induced via intraperitoneal injection of CCl_4 1:9 in olive oil, at a dose of 0.5 ml/kg b.wt. The animals received the extract, orally, at two dose levels (250 and 500 mg/kg b.wt.) The administration regimen was twice a week, for six consecutive weeks. LSE exhibited a significant dose-dependent protective effect by lowering the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), malondialdehyde (MDA) and ameliorating the level of serum protein. In addition, LSE showed antioxidant activity through improving the levels of blood glutathione (GSH), vitamin C, vitamin E and hepatic total protein contents. The LSE revealed activity approached that of silymarin, a known hepatoprotective agent. These biochemical observations were supported by examination of the histopathological features of the liver. Chromatographic fractionation of LSE afforded seven phenolic compounds. These were identified on the basis of chromatographic, chemical and spectroscopic analyses as: gallic acid (**1**), isorugosin B (**2**), casuarictin (**3**), quercetin-3-O-β-D-$^4C_1$-glucopyranoside (**4**), myricetin-3-O-α-L-$^1C_4$-rhamnopyranoside (myricetrin) (**5**), quercetin (**6**) and myricetin (**7**). The isolated phenolics probably account for the antioxidant and hepatoprotective effects exhibited by the parent extract. Furthermore, a validated RP-HPLC method was devised for standardization of LSE, in view to fulfill the requirements of efficient research methodology for evaluation of bioactive herbal drugs.

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The above findings suggested that LSE could be considered as a standardized herbal product with antifibrotic, hepatoprotective and antioxidant potential.

1. Introduction

Liver disease is considered a global problem especially in developing countries including Egypt. Hepatitis, viral infections, food additives, alcohol, toxic industrial chemicals, air and water pollutants are the major risk factors of liver toxicity.[1] Treatment of liver diseases *via* synthetic medication is usually disappointing, due to severe undesirable effects produced on prolonged administration. Therefore, herbal products had gained more interest in this issue and recent studies aimed at characterizing the health promoting properties of many plants, especially those rich in phenolics known to possess remarkable antioxidant and hepatoprotective potencies.

Liquidambar (Sweetgum) is a genus of four species of flowering plants belonging to family Altingiaceae.[2] Numerous studies have been conducted on both chemical composition and bioactivities of its members. Several types of phytoconstituents have been isolated namely; tannins,[3–5] iridoids,[6] flavonoids,[7,8] di- and triterpenoids.[9–13] Reports on the biological activities were concerned with wound healing,[14] anti-ulcerogenic,[15] antifungal,[16] antimicrobial and antioxidant[17,18] effects.

Regarding *Liquidambar styraciflua* L., earlier studies on its secondary metabolites reported the isolation of gallic and ellagic acids,[19] shikimic acid[20,21] as well as triterpenoids.[22] Moreover, its cytotoxic,[23] cancer chemopreventive[24,25] and antihypertensive[26] effects have been evaluated. In a previous publication, the authors investigated the influence of seasonal variation on the chemical composition, antioxidant and anti-inflammatory activities of the leaf and stem essential oils.[27] In Taiwan, the related species *Liquidambar formosana* is traditionally claimed to be useful for management of hepatitis; besides, its antihepatotoxic activity has been evidenced.[11] Yet, data concerning either the *in vivo* antioxidant potential or hepatoprotective effect of *L. styraciflua* are lacking. In addition, plants of family Altingiaceae are known to be enriched in polyphenols, which owing to their antioxidant effect, exert a wide variety of biological potentialities.

The current study was thus planned in view to evaluate the antioxidant and hepatoprotective activities of the methanolic extract of the leaves of *L. styraciflua*, naturalized in Egypt, against CCl_4-induced hepatic damage in rats, as well as, to isolate and identify its components. Furthermore and in compliance with research protocols for assessment of herbal drugs, a standardization procedure based on quantitative reversed phase-high performance chromatography (RP-HPLC) was developed and validated to estimate the amount of bioactive principles.

2. Materials and methods

2.1. Plant material

Leaves of *L. styraciflua* L. were collected, in December 2010, from Alzohreya Botanical Garden, Cairo, Egypt. The plant was kindly authenticated by Agricultural Engineer Therese Labib (Herbarium Section, Orman Garden, Giza, Egypt) and Dr. Mohamed El Gebali (Plant Taxonomy and Egyptian Flora Department, National Research Center, Dokki, Giza, Egypt). Voucher specimen of the plant material was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University (PHE.1217-1224).

2.2. Plant extract

Air-dried powdered leaves of *L. styraciflua* (4 kg) were exhaustively extracted by percolation, at 50 °C, with 80% methanol (3×6 L). The resulting extract was evaporated under vacuum, at a temperature not exceeding 40 °C, until almost free from methanol. The final concentrate was then subjected to lyophilization to yield a dry greenish brown powder (93.4 g). The dried *L. styraciflua* methanolic extract (LSE) was then carefully saved in a dark brown tightly closed container for further biological and phytochemical investigation.

2.3. Chemicals and biochemical kits

Folin–Ciocalteu reagent, gallic acid, quercetin, sodium carbonate, vitamin E and vitamin C were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All chemicals and solvents were of analytical grade. Polyamide 6 for column chromatography was obtained from Reidal-De Haen AG (Seezle Hannover, Germany). The following biochemical kits were purchased from their respective sources: Glutathione kit (Wak-Chemie Medical, Germany) and Transaminase kits (BioMerieux Co.) were used for the assessment of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzymes.

2.4. Experimental animals

Male Wistar albino rats (100–120 g) were obtained from the Animal House, National Research Center (NRC), Egypt. All animals were kept in a controlled environment of air and temperature with access to water and diet *ad libitum*. Anesthetic procedures and animal handling were in compliance with the ethical guidelines of Medical Ethics Committee of the National Research Centre in Egypt and performed to ensure that animals did not suffer at any stage of the experiment (Approval No. #10031).

2.5. Biological study

2.5.1. Acute toxicity study

Three groups of rats (8 rats, each) were examined for the acute toxicity of the plant extract. One oral dose of LSE at concentrations of 250, 500 and 1000 mg/kg b.wt. was administered to rats. The mortality rate was calculated 24 h after administration of LSE. Only one dead animal was observed after 24 h

at a dose of 1000 mg/kg b.wt. Therefore, two doses had been selected to conduct this study; 250 and 500 mg/kg b.wt.

2.5.2. Experimental design[28,29]

LSE (15 g) was suspended in 0.5% carboxy methyl cellulose (CMC) in distilled water at the appropriate concentration prior to oral treatment. The administration regimen was twice a week for six consecutive weeks. Fifty-six male Wistar albino rats were divided into seven groups (8 animals, each).

Group 1 (normal control): was orally treated with the vehicle (0.5 ml CMC) and received 0.5 ml olive oil by i.p. injection.

Groups 2 and 3 (normal healthy): received LSE, orally, at two different doses 250 and 500 mg/kg b.wt., and were i.p. injected with 0.5 ml olive oil.

Group 4 (CCl_4 group): was orally ingested with the vehicle (0.5 ml CMC) and i.p. injected with 0.5 ml/kg b.wt. CCl_4 (in olive oil 1:9 v/v).

Groups 5 and 6: were given LSE, orally, at 250 and 500 mg/kg b.wt.; and forced with 0.5 ml/kg b.wt. CCl_4 at the same time, for the same duration.

Group 7 (Silymarin): was treated, orally, with silymarin (100 mg/kg b.wt.) and forced with CCl_4 (0.5 ml/kg b.wt. i.p.) at the same time, for the same duration.

2.5.3. Sample preparation

Blood samples were collected from the animal, by puncturing the sublingual vein, in clean and dry test tubes, left 10 min to clot and centrifuged at 3000 rpm (4 °C) for serum separation. The separated serum was stored at −80 °C for further determination of liver function enzymes. Liver tissue was divided into two portions. The first portion was used for histopathological examination and the second was homogenized in normal physiological saline solution (0.9% NaCl) (1:9 w/v). The homogenate was centrifuged at 4 °C for 5 min at 3000 rpm and the supernatant used for estimation of hepatic oxidative stress markers.

2.5.4. Biochemical assays

2.5.4.1. Oxidative stress markers. The serum level of malondialdehyde, as a product of polyunsaturated fatty acid oxidation was determined spectrophotometrically, at 535 nm.[30] Glutathione was assayed using dithiobis-2-nitrobenzoic acid (DTNB) in PBS, and color absorbance measured at 412 nm.[31] Vitamin C was estimated using Folin reagent; measurements were done at 760 nm.[32] Vitamin E was determined colorimetrically based on measuring the intensity of the pink colored complex produced by reacting ferrous ions with bathophenanthroline, at 536 nm.[33]

2.5.4.2. Serum biomarkers for liver function tests. Aspartate and alanine aminotransferase (AST and ALT) activities were measured according to Gella et al.[34]; whereas, alkaline phosphatase (ALP) was determined by adopting the procedure of Rosalki et al.[35]

2.5.4.3. Serum and hepatic total protein content. Total protein was assayed based on which Coomassie Brilliant Blue dye reacts with Bradford reagent giving a blue complex at 595 nm.[36]

2.5.4.4. Histopathological examination. Liver slices were fixed in 10% paraformaldehyde and embedded in paraffin wax blocks. Sections of 5 μm thick were stained with hematoxylin & eosin (H&E) and Masson's trichrome, then examined under a light microscope for the determination of pathological changes.[37]

2.5.4.5. Statistical analysis. All data were expressed as mean ± SD of eight rats in each group. Statistical analysis that was carried out by one way analysis of variance (ANOVA) among various treatments was followed by the least significance difference (LSD) test at $p < 0.05$. SPSS ver. 14.0 (Chicago, IL, USA) was used for the statistical calculation:

$$\%\text{Change} = \text{control mean} - \text{treated mean}/\text{control mean} \times 100.$$

$$\%\text{Improvement} = \text{treated mean} - \text{injured mean}/\text{control mean} \times 100.$$

2.6. Phytochemical investigation

2.6.1. Determination of total phenolic content

Total phenolic content was determined using Folin–Ciocalteu reagent.[38] Absorption was measured, at 760 nm, by means of HP 8451 spectrophotometer (Hewlett-Packard, Cambridge, UK) and compared to gallic acid calibration curve. Results were expressed as mg gallic acid equivalents (GAE)/g extract. Each assay was carried out in triplicate.

2.6.2. Extraction, isolation and identification of phenolics

Fractionation of LSE (65 g) was done on a polyamide 6 column (200 g, 100 cm × 5.5 cm) by eluting with distilled H_2O, followed by gradients of H_2O and MeOH. Fractions (200–500 ml) were collected into 12 main fractions (I–XII). The compounds within subfractions II (1.02 g), V (0.3 g) and XII (1.5 g) were separated through repeated preparative paper chromatography over Whatmann No. 3 MM using the appropriate developing system (*n*-butanol:acetic acid:water (4:1:5, v/v, BAW, upper layer). Sub-fraction II (eluted with 40% MeOH) yielded three major compounds: **1** (42 mg), **2** (35 mg) and **3** (26 mg); while subfraction V (eluted with 60% MeOH) yielded two compounds (**4**, 26 mg) and (**5**, 20 mg); and finally subfraction XII (eluted with MeOH) afforded two major compounds **6** (37 mg) and **7** (28 mg). All compounds were identified by conventional analytical methods (qualitative chromatography and UV spectroscopy) and confirmed through ^1H and ^{13}C NMR spectral data.

2.6.3. RP-HPLC standardization of LSE

2.6.3.1. Sample preparation. An accurately weighed amount of LSE (0.1078 g) was introduced into a 20 ml volumetric flask. The sample was sonicated and the volume adjusted with MeOH (w/v). All sample solutions were filtered through 0.45 μm membrane filter into sample vials and degassed, before HPLC analysis.

2.6.3.2. Construction of the standard calibration curves. Standard stock solutions of gallic acid and quercetin were prepared by weighing accurately and dissolving in MeOH to final concentrations of 1.28 and 0.12 mg/ml, respectively. The stock solutions were serially diluted for preparation of final concentration ranges of 0.128–0.512 mg/ml for gallic acid and 0.012–0.048 mg/ml for quercetin. Standard calibration curves were established based on four concentrations. Each sample was repeated in triplicates.

2.6.3.3. HPLC analysis[39]. HPLC analysis was performed on Agilent instrument (Agilent technologies, USA) equipped with quaternary pump G1311A, vacuum degasser G1322A, standard preparative autosampler G1329A with an injection volume of 20 µl and Diode Array and multiple wavelength detector (Agilent technologies) as well as a reverse phase ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm) and a ZORBAX Eclipse XDB-C18 guard column (4.6 × 12.5 mm, 5 µm).

The mobile phase consisted of MeOH (Solvent A) and AcOH in H_2O, 1:25 (Solvent B). The gradient elution program began with 100% B for 4 min, 50% A for 6 min, 80% A for 10 min and then A was reduced to 50% again for 2 min. Total run time was 22 min. The flow rate was 0.6 ml/min. The detection was made at 280 nm and 330 nm. Data were processed using Chemstation 4.02 software.

2.6.3.4. Validation of the quantification method[40].

2.6.3.4.1. Linearity. Linearity was determined for the calibration curves obtained by HPLC analysis on four concentrations (0.128–0.512 mg/ml for gallic acid and 0.012–0.048 mg/ml for quercetin). The correlation coefficients were calculated by the linear regression method.

2.6.3.4.2. Precision. A sample was prepared as previously described and analyzed six times on the same day to evaluate intra-daily variation and on three consecutive days to assess inter-daily variation (nine measurements). The precision was expressed as relative standard deviation (RSD%).

2.6.3.4.3. Accuracy. The accuracy was tested by separate spiking with a known amount of the standard gallic acid (1.1 mg) and quercetin (2.6 mg) to the extract samples and analyzed in triplicates. The average recovery was calculated according to the formula: Recovery (%) = [(net measured amount − original amount)/spiked amount] × 100.

2.6.3.4.4. Limit of detection (LOD) and limit of quantitation (LOQ)[41]. Limit of detection (LOD) and limit of quantitation (LOQ) were determined according to the International Conference on Harmonization Guidelines and calculation was based on the standard deviation of the response (σ) and the slope of the calibration curve (S). LOD = 3.3 * σ/S and LOQ = 10 * σ/S.

2.6.3.4.5. Stability. Stability was evaluated by analyzing the same sample at 0, 2, 4, 7, 24 and 48 h after its preparation.

Table 1 Intra-day and inter-day precision of the developed RP-HPLC method for determination of polyphenolics in the methanolic extract of *L. styraciflua* L.

Parameter	Gallic acid		Quercetin	
	Content*	RSD (%)	Content*	RSD (%)
Intra-day [a]	68.419 ± 0.0107	2.9012	0.9297 ± 0.00040	0.7905
Inter-day [b]	68.777 ± 0.0134	3.6150	0.9264 ± 0.00020	3.9924
Stability [c]	68.386 ± 0.0146	3.9667	0.9373 ± 0.00019	3.7336

* Mean ± SD (mg/g extract).
[a] Sample analyzed five times during one day, (n = 6).
[b] Sample analyzed each day over three consecutive days, (n = 9).
[c] Sample analyzed at 0, 2, 4, 7, 24, 48 h (n = 6).

3. Results and discussion

3.1. Biological study

With respect to the oxidative stress markers, healthy control rats showed insignificant changes in GSH, MDA, vitamin C and E (Table 2). A significant decrease in GSH, vitamin C and E was observed in CCl_4-injured rats by 32.37%, 32.79% and 27.20%, respectively; while MDA was significantly increased by 25.62%. Rats treated with 250 mg/kg b.wt. of LSE exhibited amelioration in GSH, MDA, vitamins C and E levels by 13.70%, 9.62%, 18.57% and 14.77%, respectively. Administration of higher doses of LSE (500 mg) resulted in better improvement of all parameters (by 19.42%, 20.59%, 20.64% and 16.45%, respectively). Meanwhile, administration of silymarin (reference herbal hepatoprotective)

Table 2 Effect of the methanolic extract of *Liquidambar stryaciflua* L. on oxidative stress markers in normal and CCl_4 treated rats.

Parameters	Control	Control treated (250 mg/kg b.wt.)	Control treated (500 mg/kg b.wt.)	CCl_4	CCl_4 treated (250 mg/kg b.wt.)	CCl_4 treated (500 mg/kg b.wt.)	CCl_4 treated with silymarin	$p <$
GSH	6.64 ± 3.50^{a} –	7.06 ± 0.65^{a} (+6.32)	7.09 ± 0.58^{a} (+6.77)	4.49 ± 0.56^{b} (−32.37)	5.40 ± 0.44^{c} (−18.67)	5.78 ± 0.48^{c} (−12.95)	5.97 ± 0.03^{c} (−10.09)	0.0001
MDA	6.75 ± 0.50^{d} –	$6.94 \pm 0.43^{c,d}$ (+2.81)	6.73 ± 0.53^{d} (+0.29)	8.48 ± 0.58^{a} (+25.62)	$7.83 \pm 0.34^{c,d}$ (+16.00)	7.09 ± 0.46^{b} (+5.03)	$7.42 \pm 0.34^{b,c}$ (+9.92)	0.0001
Vitamin C	$4.36 \pm 0.31^{a,b}$ –	4.58 ± 0.46^{a} (+5.04)	$4.69 \pm 0.16^{b,c}$ (+7.56)	2.93 ± 0.30^{c} (-32.79)	3.74 ± 0.19^{c} (−14.22)	3.83 ± 0.38^{c} (−12.15)	3.74 ± 0.18^{c} (−14.22)	0.003
Vitamin E	7.72 ± 0.48^{a} –	7.17 ± 0.23^{b} (−7.12)	7.73 ± 0.46^{b} (−7.12)	5.62 ± 0.55^{b} (−27.20)	6.76 ± 0.57^{b} (−12.43)	6.89 ± 0.49^{b} (−10.75)	6.79 ± 0.22^{b} (−12.04)	0.0001

All values are means ± SD of eight rats in each group. Data are expressed as µg/mg protein for GSH, Vit. C, Vit. E and µmol/mg protein for MDA.
Unshared letters between groups are the significance values at $p < 0.05$; values between brackets are percentage change over control group.
Statistical analysis is carried out using one way analysis of variance (ANOVA), CoStat Computer Program accompanied with LSD test at $p < 0.05$.

Table 3 Effect of the methanolic extract of *Liquidambar stryaciflua* L. on liver function enzymes, serum and tissue protein in normal and CCl_4 treated rats.

Parameters	Control	Control treated (250 mg/kg b.wt.)	Control treated (500 mg/kg b.wt.)	CCl_4	CCl_4 treated (250 mg/kg b.wt.)	CCl_4 treated (500 mg/kg b.wt.)	CCl_4 treated with silymarin	$p <$
AST	2.90 ± 0.15^{b} –	2.85 ± 0.16^{b} (−1.75)	2.86 ± 0.15^{b} (−1.37)	4.20 ± 0.17^{a} (+44.82)	2.96 ± 0.23^{b} (+2.07)	2.93 ± 0.21^{b} (+1.03)	2.98 ± 0.19^{b} (+2.75)	0.0078
ALT	2.02 ± 0.13^{bc} –	2.00 ± 0.07^{b} (−0.99)	1.88 ± 0.03^{c} (−6.93)	2.53 ± 0.13^{a} (+25.25)	2.19 ± 0.24^{b} (+8.41)	2.14 ± 0.03^{b} (+5.94)	$2.04 \pm 0.33^{b,c}$ (+0.99)	0.0001
ALP	116.72 ± 14.11^{c} –	108.60 ± 9.88^{d} (6.95)	101.80 ± 3.34^{d} (−12.78)	158.00 ± 5.24^{a} (+35.36)	138.80 ± 6.97^{b} (+18.91)	135.80 ± 10.00^{b} (+16.34)	134.20 ± 3.76^{b} (+14.97)	0.0001
Serum protein	$1.63 \pm 0.03^{b,c}$ –	1.73 ± 0.06^{b} (+6.13)	1.68 ± 0.05^{c} (+3.06)	1.93 ± 0.17^{a} (+18.40)	1.69 ± 0.11^{b} (+3.68)	1.67 ± 0.06^{b} (+2.45)	$1.65 \pm 0.14^{b,c}$ (+1.22)	0.0005
Tissue protein	60.80 ± 3.19^{e} –	61.20 ± 1.92^{a} (+0.65)	64.00 ± 2.34^{a} (+5.26)	52.40 ± 4.60^{c} (−13.81)	56.60 ± 2.70^{d} (−6.91)	58.20 ± 4.21^{b} (−4.27)	$57.60 \pm 2.30^{c,d}$ (−5.26)	0.0001

All values are means ± SD of eight rats in each group; data are expressed as Unit/L (AST, ALT, ALP); mg/ml (serum protein); mg/g liver (tissue protein).
Unshared letters between groups are the significance values at $p < 0.05$; values between brackets are percentage change over control group.
Statistical analysis is carried out using one way analysis of variance (ANOVA), CoStat Computer Program accompanied with LSD test at $p < 0.05$.

led to amelioration by 22.28%, 15.70%, 18.57% and 15.16% of GSH, MDA, and vitamins C and E levels, respectively.

The liver function enzymes (Table 3) demonstrated insignificant changes following the treatment of control healthy rats with either 250 or 500 mg/kg b.wt. of LSE. Meanwhile, CCl_4-injured rats showed a significant increase in AST, ALT and ALP enzyme activity by 44.82%, 25.24% and 35.36%, respectively. Treatment of CCl_4-injured rats with LSE at 250 mg/kg b.wt. reduced the elevated AST, ALT and ALP activities by 42.76%, 16.83% and 16.45%, respectively. Moreover, treatment with LSE at 500 mg/kg b.wt. showed better amelioration (by 43.79%, 19.30% and 19.02%, respectively). In comparison the standard drug, silymarin, lowered enzymatic activities by 42.07%, 24.26% and 20.39% for AST, ALT and ALP, respectively.

Serum and hepatic total protein contents were insignificantly changed in normal rats treated with both LSE doses (Table 3). Obviously, CCl_4-injured rats demonstrated a significant increase in serum protein content by 18.40%, while that of hepatic protein was significantly reduced by 13.81%. Treatment with 250 mg of LSE ameliorated serum and hepatic protein levels by 14.72% and 6.90%, respectively; this improvement was raised (15.95% and 9.54%, respectively) by increasing the dose to 500 mg LSE. On the other hand, silymarin administration improved serum protein by 17.18%, and hepatic total protein by 8.55%.

Histological analysis of liver sections of normal control rats showed normal hepatic lobular architecture. The hepatocytes were within normal limits and arranged in thin plates; they were separated by narrow blood sinusoids lined by endothelial cells. Portal tracts extended with no fibrous tissue or lymphocyte deposition (Fig. 2a and b). Healthy rats treated with LSE (250 and 500 mg/kg b.wt.) showed normal hepatic lobular architecture. The hepatocytes were within normal limits and neither hydropic nor steatosis changes were observed. Portal tracts were normal and with no sign of fibrosis (Fig. 2c–f).

Liver injured with CCl_4 showed portal loss of hepatic lobular architecture. Ballooning of hepatocytes, deformed cord arrangement and disturbed sinusoids were seen. The hepatocytes showed a marked degree of hydropic and steatotic changes, and massive necrosis. Portal tracts were extended with marked numbers of chronic inflammatory cells and fibrous tissue. There were portoportal and portocentral fibrosis (Fig. 3a and b). Treatment of injured liver with LSE (250 mg/kg b.wt.) partly preserved the hepatic normal architecture; mild degrees of steatosis and hydropic changes were recorded; the hepatocytes were still swollen with narrow sinusoids; and portal tracts were extended with the appearance of moderate fibrous tissue (Fig. 3c and d). Treatment with LSE at the higher dose (500 mg/kg b.wt.) showed well-formed nucleated hepatocytes arranged in cord with obvious sinusoidal arrays; minimal fat vacuoles and inflammatory lymphocyte infiltrations were observed (Fig. 3e and f). Treatment with silymarin showed swelling and foamy appearance of hepatocytes (Fig. 3g and h); besides, hydropic and steatosis changes and fibrotic tissues were obviously seen.

Carbon tetrachloride (CCl_4) is a potent environmental hepatotoxin; it enhances the formation of free radicals through bioactivation in phase I of the cytochrome P450 system to form the reactive metabolic trichloromethyl radical ($^{\cdot}CCl_3$) and trichloromethyl peroxy radical ($^{\cdot}OOCCl_3$) which can bind with polyunsaturated fatty acids to generate lipid peroxides

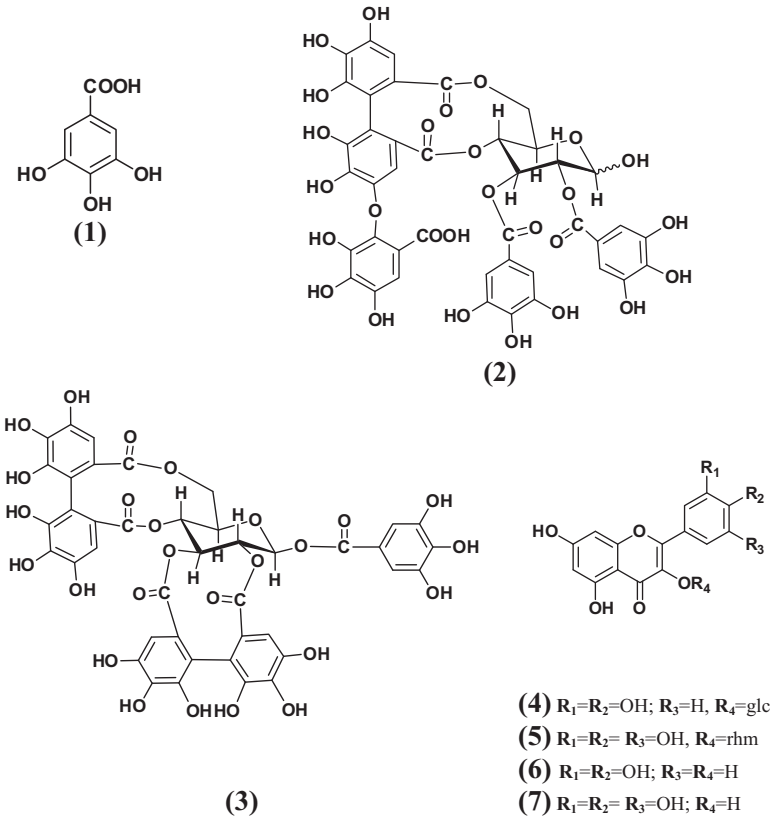

Figure 1 Structures of the phenolic compounds isolated from the methanolic extract of *Liquidambar styraciflua* L.

and ROS that induce injury or necrosis with corresponding health problems.[42,43] Moreover, CCl_4 is known to decrease glutathione (GSH) of phase II enzymes and to reduce antioxidant enzymes and substrates resulting in induction of oxidative stress, which is an important factor in acute and chronic injuries in various tissues.[44] Consequently, the accumulated free radicals could further stimulate lipid peroxidation process and therefore increase malondialdehyde content. The observed decrease in GSH activity might be due to a decrease in availability of GSH produced during enhanced lipid peroxidation processes. Treatment of CCl_4 injured rats with LSE significantly ameliorated the disturbed GSH and MDA contents thus supporting the role of the plant extract as antioxidant and free radical scavenger.

As regards vitamin C and coinciding with the present findings, Frei et al.[45] reported that peroxyl radicals are trapped by ascorbate resulting in a decrease in the level of both enzyme and vitamin during the free radical scavenging process. Similarly, the reduction of vitamin E as a result of oxidative stress is due to that the vitamin acts as a soluble antioxidant to protect biological membranes against oxidative stress which leads to maintenance of cell function.[46]

The significant rise in ALT, AST and ALP subsequent to CCl_4 intoxication reflected the rise in enzyme activities[28,43,47] due to an increase in hepatic cell membrane fragility that led to enzyme release into circulation. These enzymes, being cytoplasmic in location, are released into the circulation after autolytic breakdown or cellular necrosis due to the damaged structural integrity of the liver.

According to Romero et al.[48], CCl_4 intoxication induced changes in the process of protein synthesis. Hence, increase in serum total protein content can be considered as a useful index of the severity of cellular dysfunction in liver diseases. This was obvious in the present study, where hepatic protein level was decreased and its serum concentration increased. Stimulation of protein synthesis has been advanced as a contributory self-healing mechanism, which accelerates liver regeneration process[49].

In conclusion, treatment with the methanolic extract of *L. styraciflua* L. noticeably attenuated the increased level of serum enzymes and caused a subsequent recovery toward normalization. This gives an additional support that this extract is able to: condition the hepatocytes, accelerate regeneration of parenchyma cells, protect against membrane fragility and decrease leakage of enzymes into circulation.

Histopathological studies revealed that CCl_4 induced extensive fatty change, blood vessel congestion, cellular hypertrophy and infiltration, necrotic foci, destruction of the lobular architecture, fibrosis, and nuclear degeneration in some areas.[43] This was in accordance with the present findings reflected as massive deformation of hepatic cells architecture after CCl_4 induction. Treatment with LSE ameliorated the hepatocyte degeneration forms where necrosis and infiltration of inflammatory cells were all in fact ameliorated. Minimal deposition of fat vacuoles and mild fibrous tissue were apparently observed. This gave an additional support that the methanolic extract of *L. styraciflua* may exert an antifibrotic effect beside its role as hepatoprotective and antioxidant through improving liver function enzymes and oxidative stress markers. Conversely, silymarin failed to improve the histopathological picture of the liver where the liver still showed a marked degree of fibrosis, inflammation and steatosis.

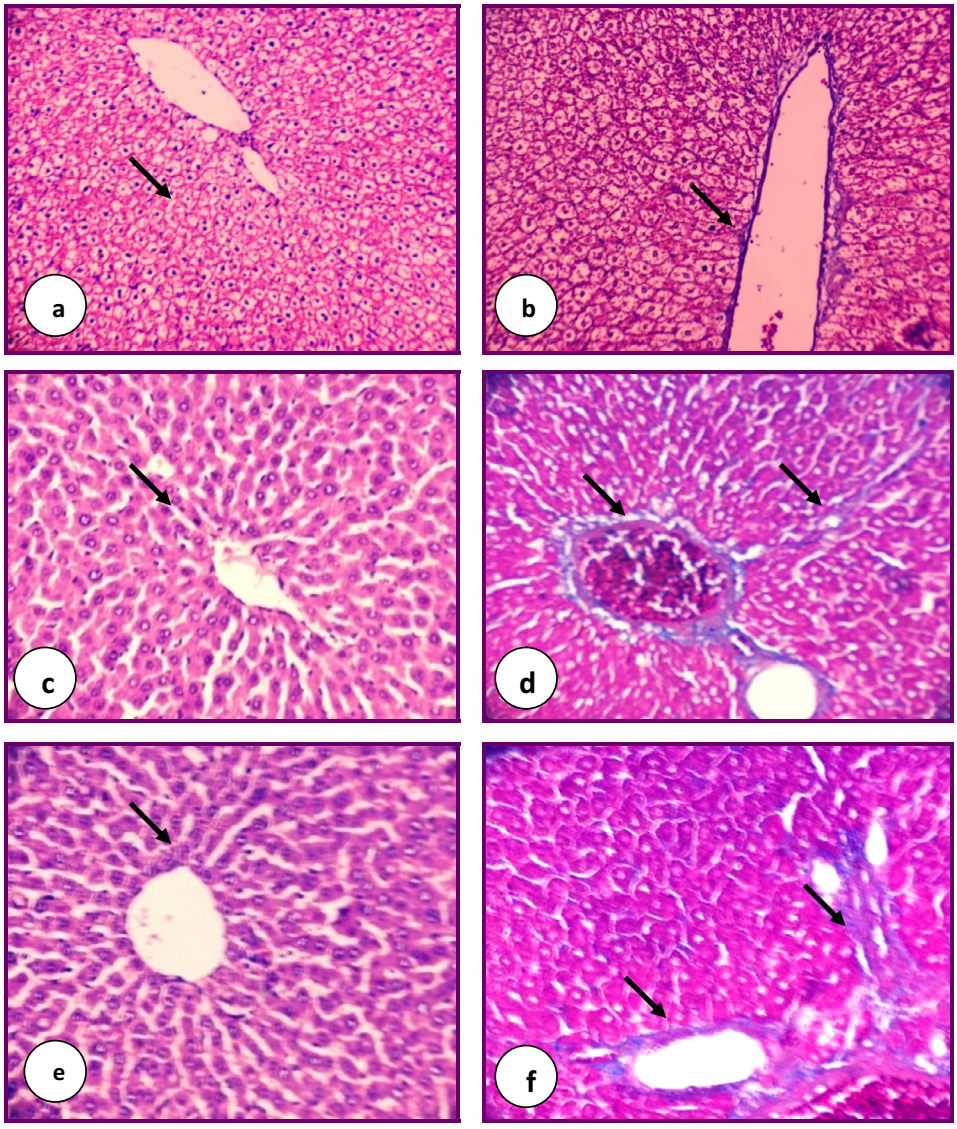

Figure 2 Photomicrographs of H&E and Masson's trichrome stained liver sections (100×) of control (a and b), control treated with 250 mg/kg b.wt. of *L. styraciflua* methanolic extract (c and d), control treated with 500 mg/kg b.wt. of *L. styraciflua* methanolic extract (e and f).

It is evident that, the antioxidant and hepatoprotective activities exhibited by LSE closely approached those of silymarin necessitating a deeper phytochemical exploration of its bioactive constituents.

3.2. Phytochemical investigation

3.2.1. Total phenolic content

The content of total phenolics determined colorimetrically in the methanolic extract of the leaves of *L. stryaciflua* amounted to 111.5 mg GAE/g extract.

3.2.2. Isolated phenolic compounds

Fractionation of LSE resulted in isolation of seven phenolic compounds (**1**–**7**) (Fig. 1). All of these were identified based on chromatographic, chemical and spectroscopic analyses as well as through comparison with published data.

Compound (**1**) was obtained as an off white amorphous powder (69 mg); on co-chromatography (PC) alongside an authentic sample of gallic acid, it appeared as a violet spot under UV and blue color with ferric chloride T.S. R_f (×100) 78 (BAW) and 54 (6% acetic acid). Compound (**1**) was identified as gallic acid.

Compound (**2**) was obtained as white amorphous powder (56 mg) that appeared as a violet spot under UV and turns blue upon spraying with ferric chloride T.S.; R_f (×100, PC) 12 (BAW) and 0 (6% acetic acid). UV Spectral data λ_{max} (nm) MeOH: 272. ^{1}H-NMR spectral data δ (ppm) (MeOD): indicates that this tannin forms an anomer mixture (α- and β-anomers) and that it possesses two galloyl groups with signals at 7.03, 6.97 (*s*, 1H each, α-anomer) and 7.04, 6.76 (*s*, 1H each, β-anomer) and a valoneoyl group (7.3, 6.54, 6.36 [*s*, 1H each (α-anomer); 7.5, 6.55, 6.3 (*s*, 1H each, (β-anomer)]) were observed together with signals of the glucose moiety at 5.38 (1H, *d*, J = 2.5 Hz, H-1), 5.0 (1H, *dd*, J = 7.5 &

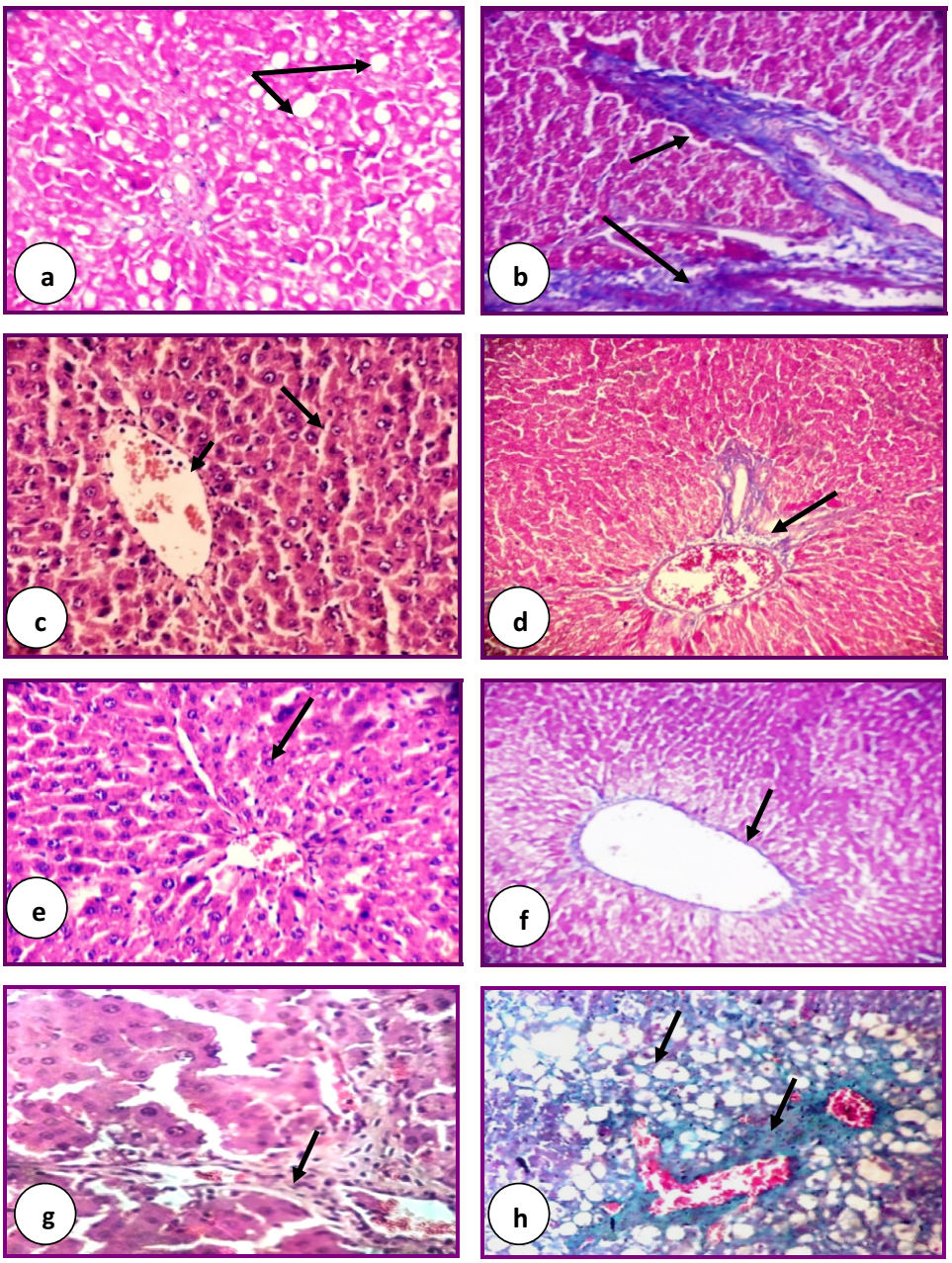

Figure 3 Photomicrographs of H&E and Masson's trichrome stained liver sections (100×) of CCl_4 group (a and b), CCl_4 treated with 250 mg/kg b.wt. of *L. styraciflua* methanolic extract (c and d), CCl_4 treated with 500 mg/kg b.wt. of *L. styraciflua* methanolic extract (e and f), CCl_4 treated with silymarin (g and h).

3.5 Hz, H-2), 5.7 (1H, *t*, *J* = 7.5 Hz, H-3), 4.88 (*m*, H-4), 4.57 (*m*, H-5), 5.12 (*m*, H-6) (α-anomer) and at 6.02 (1H, *d*, *J* = 7.5 Hz, H-1), 5.81 (1H, *t*, *J* = 7.5 Hz, H-2), 5.91 (1H, *t*, *J* = 7.5 Hz, H-3), 5.72 (1H, H-4), 4.44 (*d*, H-5), 3.84 (m, H-6) (β-anomer). On the basis of the above evidence, the structure of compound (**2**) was elucidated as isorugosin B.[5]

Compound (**3**) was isolated as an amorphous off-white powder (44 mg), which appeared violet under UV light. It gave blue color after spraying with ferric chloride T.S. Its R_f (PC) was 72 (6% acetic acid) and 34 (BAW). UV Spectral data λ_{max} (nm) MeOH, 278 revealed an ellagitannin molecule. ^{1}H-NMR spectral data δ (ppm) (MeOD); signals of two hexahydroxydiphenoyl moieties (HHDP) appeared at 6.35 6.46, 6.75, 6.77 (1H, each *s*) and one galloyl moiety at 6.93 (2H, *s*). Those of α, β-anomeric protons of glucose appeared at:5.23 (1H, *d*, *J* = 2.5 Hz, H-1α), 4.85 (1H, *d*, *J* = 8 Hz, H-1β), 5.11 (1H, *t*, *J* = 8 Hz, H-3α), 5.05 (1H, *t*, *J* = 8 Hz, H-3β), 4.72 (1H, *dd*, *J* = 8 & 2.5 Hz, H-2α), 4.45 (1H, *t*, *J* = 8 Hz, H-2 β), 3.18–3.83 (*m*, overlapping other sugar and hydroxyl proton signals). Compound (**3**) was isolated in a mixture form of alpha and beta linkage and identified as casuarictin.[50]

Compound (**4**) was obtained as a pale yellow amorphous powder (51 mg) that appeared as a dark purple spot on PC under UV light changing to yellow upon exposure to ammonia vapors and stained dirty green on spraying with ferric chloride T.S. with R_f (×100): 70.9 (BAW) and 19.3 (6% acetic acid). UV

λ max: MeOH (257, 358), MeOH/NaOAc (273, 399), MeOH/NaOAc/H_3BO_3 (262, 379), MeOH/$AlCl_3$ (273, 309, 364), MeOH/$AlCl_3$/HCl (259, 355). ^{1}H NMR data (MeOD): δ ppm 3.1–3.7 (*m*, sugar protons), 5.4 (1H, *d*, J = 7.5 Hz glucose H-1″), 6.1 (1H, *d*, J = 2.5 Hz, H-6), 6.3 (1H, *d*, J = 2.5 Hz, H-8), 6.8 (1H, *d*, J = 6.6 Hz, H-5′), 7.54 (1H, *dd*, J = 2.1, 6.6 Hz, H-6′) and 7.45 (1H, *d*, J = 2.1, H-2′). ^{13}C NMR data: δ ppm 156.7 (C-2), 133.7 (C-3), 177.9 (C-4), 161.7 (C-5), 99.1 (C-6), 164.6 (C-7), 94 (C-8), 156.8 (C-9), 104.4 (C-10), 121.6 (C-1′), 116.6 (C-2′), 145.3 (C-3′), 148.9 (C-4′), 115.7 (C-5′), 122.1 (C-6′), 101.3 (C-1″), 74.5 (C-2″), 78 (C-3″), 70.3 (C-4″), 76.9 (C-5″) and 61.4 (C-6″). The structural formula of compound (**4**) was established as quercetin-3-O-β-$^4C_1$-glucopyranoside.[51]

Compound (**5**) was obtained as yellow powder (32 mg) that appeared as a dark purple spot on PC under UV light, turning yellow when fumed with NH_3. R_f (×100) 54 (BAW) and 42 (6% acetic acid). On spraying with ferric chloride T.S., it was stained green. The UV absorption spectrum in MeOH exhibited two major bands at λ_{max} (MeOH) 259, 363 nm indicating a flavonol nucleus. ^{1}H-NMR (MeOD) spectral data showed: signals of aglycone moiety δ (ppm) at 6.18 (1H, *d*, J = 2.1 Hz, H-6), 6.35 (1H, *d*, J = 2.1 Hz, H-8), 6.93 (2H, *s*, H-2′, H-6′); and sugar moiety δ (ppm) at 5.2 (*d*, J = 2.1 Hz, H-1″), 3.1–3.9 (*m*, sugar protons) and 0.95 (3H, *d*, J = 6.2 Hz, H-6″). ^{13}C NMR data: δ ppm, aglycone: 157.1 (C-2), 134.9 (C-3), 178.3 (C-4), 161.7 (C-5), 98.7 (C-6), 164.5 (C-7), 93.4 (C-8), 158.1 (C-9), 104.5 (C-10), 120.5 (C-1′), 108.3 (C-2′), 145.4 (C-3′), 136.5 (C-4′), 145.4 (C-5′), 108.3 (C-6′); rhamnose moiety: 102.2 (C-1″), 70.5 (C-2″), 70.7 (C-3″), 72.0 (C-4″), 70.1 (C-5″) and 16.3 (C-6″). Compound (**5**) was identified as myricetin-3-O-α-L-$^1C_4$-rhamnopyranoside (myricetrin).[52]

Compounds (**6**) (54 mg) and (**7**) (36 mg) were identified through matching their spectroscopic and physical data with those published in the literature and confirmed by comparison with those obtained for authentic samples of quercetin and myricetin, respectively.

The chromatographic investigation of the LSE which exhibited antioxidant and hepatoprotective activities resulted in isolation of seven phenolic compounds *viz*., gallic acid (**1**), isorugosin B (**2**), casuarictin (**3**), quercetin-3-O-β-D-$^4C_1$-glucopyranoside (**4**), myricetin-3-O-α-L-$^1C_4$-rhamnopyranoside (myricetrin) (**5**), quercetin (**6**) and myricetin (**7**).

Our results are in accordance with those of Spencer et al.[14] who isolated gallic acid from the bark and sapwood of *L. styraciflua* L. Likewise isoquercetrin,[7] casuarictin,[3] isorugosin B[5] have been previously isolated from *L. formosana* leaves. On the contrary, Hanato et al.[5] isolated myricetin 3-O-glucoside from *L. formosana* leaves, whereas myricetin-3-O-α-L-$^1C_4$-rhamnopyranoside (myricetrin) was herein isolated from the extract of those of *L. styraciflua* L.

The antioxidant and hepatoprotective activities of the tested extract could be attributed to the compounds isolated therefrom. Domitrović et al.[53] and Maalik et al.[54] reported that flavonoids namely, quercetin can ameliorate acute liver damage by acting as scavengers of free radicals, inhibiting the inflammatory response, manifested by a decreased deposition of fats in liver cells; thereby it protects liver cells from fibrosis and reduces plasma concentration of alanine aminotransferase thus exerting high antioxidant and anti-inflammatory activities and antifibrotic action and may be partially responsible for beneficial effects observed in injured liver tissue. In addition, Pandy et al.[55] mentioned that myricetin significantly protected the membrane lipid peroxidation and protein oxidation subjected to oxidative stress, as evidenced by a decrease in the MDA level and protein carbonyl content. Also, the antioxidant activity may also be attributed to isoquercitrin which decreased the oxidative stress markers, such as levels of ROS, protein carbonylation and lipid peroxidation, and inducing the superoxide dismutase activity in order to increase survival of cells and leading to protection of the cells.[56] On the other hand, the antihepatotoxic activity of tannins had been evidenced by Hikino et al.[57], who reported that the hydrolysable and condensed tannins generally inhibited the action of glutamic–pyruvic transaminase (ALT) and the increase of the number of the galloyl grouping potentiated the antihepatotoxic activity. In hydrolyzable tannins, increase of the galloyl grouping led to potentiation of the enzyme inhibitory activity such as casuarictin, while in the low-molecular weight polyphenols such as gallic acid, the presence of 1,2dihydroxy or 1,2,3-trihydroxy grouping on the benzene ring appears to be required.[57] Furthermore, gallic acid was reported to protect the integrity of plasma membrane and to increase the regenerative and reparative capacity of the liver[58]; in addition, it showed protective effects against serum GPT and GOT which are released into the blood when plasma membrane of hepatic cells is damaged.[59]

Therefore, it can be deduced that the antioxidant effects of the phenolic components of the methanolic extract of *L. styraciflua* leaves (LSE) effectively decreased oxidative stress and consequently produced a significant hepatoprotective activity in liver damaged animals. Therefore, it was deemed necessary to quantify these compounds within the extract to facilitate its standardization, as a requirement to guarantee its therapeutic value.

3.2.3. Standardization of LSE by RP-HPLC

3.2.3.1. Validation of the quantification method. The calibration curves indicated good linearity (R^2 = 0.9896 and 0.9962), within the tested range for gallic acid and quercetin respectively.

The limit of detection (LOD) (0.0534, and 0.01268 mg/ml) and limit of quantitation (LOQ) (0.1619, 0.0384 mg/ml) were determined for gallic acid and quercetin, respectively.

The analytical precision from the results of intra-day (six times a day) and inter-day (over three consecutive days) was calculated as shown in Table 1. The relative standard deviations (RSDs), which were both less than 5% for gallic acid and quercetin, respectively lie within the same range which were both less than 5%. The stability tests revealed that the RSDs (3.967%, 3.7333%) for the determined compounds were also less than 5% and the mean recovery (mean ± SD) of gallic acid and quercetin was 98.99 ± 0.3211 and 102.4646 ± 0.0268 (RSD are 0.8041% and 0.7552%), respectively. The results indicated that the method was precise for simultaneous determination of the two components.

3.2.3.2. Determination of polyphenolic compounds in LSE by HPLC analysis. Seven phenolic compounds *viz*., (**1**) to (**7**) were identified and quantified in LSE for standardization purpose, based on comparison with isolated compounds. Quercetin glycosides were quantified as quercetin (**6**), whereas pyrogallol tannins were quantified as gallic acid (**1**). Compounds **1**–**3** were

calculated as gallic acid, amounting to 33.84 mg /gram extract while compounds **4**–**7** were calculated as quercetin and reached up to 0.88 mg/g extract.

The development of a simple, precise validated RP-HPLC-UV procedure will facilitate the standardization of the methanolic extract of *L. styraciflua* leaves. The proposed method represents a contribution to quantification of active phenolics in this extract, using quercetin and gallic acid as markers. On the other hand, the previous findings revealed the value of the analyzed extract as a promising source of health promoting polyphenols and encourage their inclusion in food supplements as alternatives to the traditional leaf extracts.

4. Conclusion

Results acquired from the present study suggest that oral administration of the methanolic extract of the leaves of *L. styraciflua* L. obviously prevents oxidative damage to major biomolecules, decreases severity of fibrosis, normalizes hepatic cell architecture and affords significant protection against CCL_4-induced oxidative stress and liver damage in rats.

The phenolic compounds isolated from the extract, and quantified by a validated RP-HPLC method, might be responsible for its activity either individually or in mixture through synergism. Hence, *L. styraciflua* leaves enriched with such effective antioxidants could be considered as a promising source of natural hepatoprotective agents. However, further application in medical practice should be confirmed by conveying pharmacological and preliminary placebo-controlled clinical studies.

5. Conflict of interest

The author has no conflicts of interest to disclose.

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