Hepatoprotective Constituents from *Cleome droserifolia*

Maged Saad Abdel-Kader,* Saleh Ibrahim Alqasoumi, and Areej Mohammad Al-Taweel

Department of Pharmacognosy, College of Pharmacy, King Saud University; P.O. Box 2457, Riyadh 11451, Kingdom of Saudi Arabia. Received January 28, 2009; accepted March 17, 2009; published online March 19, 2009

The effect of ethanol extract from aerial parts of *Cleome droserifolia* was investigated against carbon tetrachloride induced liver injury. The hepatoprotective activity was evaluated through the quantification of biochemical parameters and confirmed using histopathology analysis. Efficient hepatoprotective effect was achieved by crude extract, fractions and some pure compounds. The phytochemical studies showed that the petroleum ether fraction afforded two known guaiane sesquiterpenes buchariol (1) and teucladiol (2) in addition to daucosterol (β-sitosterol glucoside) (3). The $CHCl_3$ fraction afforded three known flavonoid derivatives; 5,3′-dihydroxy-3,6,7,4′,5′-pentamethoxyflavone (4), 5′-hydroxy-3,6,7,3′,4′,5′-hexamethoxyflavone (5) and luteolin (6) and a known dolabellane diterpene (1*R*,2*R*,3*E*,7*E*,11*R*,12*S*)-2-*O*-acetyl-16-*O*-(3-hydroxy-3-methylglutaryl)-dolabella-3,7-dien-2,16,18-triol (7). The active parts of the MeOH fraction afforded the previously unreported 3′-methoxy-3,5,4′-trihydroxy flavone-7-neohesperidoside (8) and a known megastigmane norterpene; (6*S*,9*R*)-roseoside (9).

Key words *Cleome droserifolia*; hepatoprotection; flavonoid; terpene

Cleome droserifolia (Forssk) Del. (Cleomaceae), known locally in Saudi Arabia as Al-Samwa, is distributed on rocky slopes and ledges in northwestern Saudi Arabia.[1] *C. droserifolia* leaves extract have anti-schistosomiasis activity.[2] The plant extract also has a hypoglycemic effect through potentiation of peripheral, hepatic insulin sensitivity and diminishing intestinal glucose absorption.[3,4] Treatment of hepatocytes culture with the plant extract resulted in reduction in cell viability; however, the level of albumin was not affected.[5] Previous phytochemical study of *C. droserifolia* indicated the presence of sesquiterpenes, steroids and flavonoids.[6—8]

Screening of plants used in Saudi folk medicine as remedy for liver problems is one of our research interest.[9—11] In the present study biologically directed phytochemical study of the ethanol extract of *C. droserifolia* was conducted.

Results and Discussion

In our search for hepatoprotactive compounds from plants used in Saudi folk medicine as remedy for liver problems, the ethanol extract of the aerial parts of *C. droserifolia* showed 35.87, 26.8, 21.9 and 34.8% reduction at a dose of 500 mg/kg body weight in the level of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and bilirubin, respectively. The ethanol extract was subjected to liquid–liquid partitioning; each fraction was tested for hepatoprotective activity. Biologically directed chromatographic purification of the active fractions was conducted and resulted in the isolation of **1**—**9**. Compounds **2**—**8** were tested biologically, while the amount of **1** and **9** were not enough for biological study.

Compounds **1** and **2** were identified as the guaiane sesquiterpenes buchariol previously isolated from an Egyptian collection of *C. droserifolia*[7] and 6-hydroxynardol (teucladiol) isolated from *Teucrium leucocladum*.[12] Compounds **4**—**6** were identified as 5,3′-dihydroxy-3,6,7,4′,5′-pentamethoxyflavone, 5′-hydroxy-3,6,7,3′,4′,5′-hexamethoxyflavone; both previously reported from *C. droserifolia*[13]; and luteolin,[14] respectively. Sitosterol-3-*O*-[β-D-glucopyranoside] (daucosterol) (**3**) was identified by comparison with literature data.[15] Compound **7** was identified as the dolabellane diterpene (1*R*,2*R*,3*E*,7*E*,11*R*,12*S*)-2-*O*-acetyl-16-*O*-(3-hydroxy-3-methylglutaryl)-dolabella-3,7-dien-2,16,18-triol previously isolated from *Chrozophora oblique*,[16] while **9** proved to be (6*S*,9*R*)-roseoside,[17] a norterpenoid with the megastigmane skeleton isolated for the first time from the Cleomaceae.

The UV data of **8** in MeOH (λ_{max}: 354, 268, 255 nm) was diagnostic for a flavonol skeleton.[18] In the ^{1}H-NMR spectrum (experimental), the two *meta*-coupled doublets at δ_H 6.46 and 6.85 (J=2.0 Hz) correlated to two methines at δ_C 99.8 and 95.1 and were assigned to C-6 and C-8, respectively. The ABX system at δ_H 6.92 (d, J=8.5 Hz), 7.57 (dd, J=2.0, 8.5 Hz), and 7.96 (d, J=2.0 Hz) was assigned to a disubstituted ring B. In the ^{13}C-NMR spectrum the chemical shifts of the two oxygenated carbons at δ_C 147.4 and 150.1 indicated 3′,4′-dioxygenation.[19] In addition, the ^{1}H-NMR spectrum showed a methoxyl signal at δ_H 3.85 and two sugars as indicated from the two anomeric protons at δ_H 5.56 and 5.58 ppm. The UV bathochromic shifts with NaOMe (λ_{max}: 398 nm), $AlCl_3$/HCl (λ_{max}: 401, 359, 299 and 268 nm) indicated 3,5,4′ free hydroxyl groups. The failure of **8** to produce any shift in band II with NaOAc indicated a substituted C-7.[18] The position of the methoxyl group at C-3′ was indicated from the heteronuclear multiple bond correlations (HMBC) of its protons at δ_H 3.85 with C-3′ at δ_C 147.4. The two sugars consequently must be attached to C-7. This fact was supported by the HMBC correlation between the anomeric proton at δ_H 5.58 and C-7 at δ_C 162.1. The presence of a methyl signal at δ_H 1.12 (d, J=5.5 Hz) and δ_C 18.4 in both ^{1}H- and ^{13}C-NMR indicated the presence of a rhamnosyl moiety. The chemical shifts of the sugar carbons was in complete agreement with those reported for 2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose (neohesperidose).[19,20] ESI-MS showed an M^++Na at 647 *m/z* and M^++H at 625 consistent with the molecular formula $C_{28}H_{32}O_{16}$, further supporting the structure of **8** as the previously unreported 3′-methoxy-3,5,4′-trihydroxyflavone-7-neohesperidoside (Fig. 1).

Hepatoprotective Activity Total ethanol extract, all fractions resulted from liquid–liquid fractionation and chromatographic purification were subjected to hepatoprotective activity evaluation using silymarin as standard. The use of

* To whom correspondence should be addressed. e-mail: mpharm101@hotmail.com

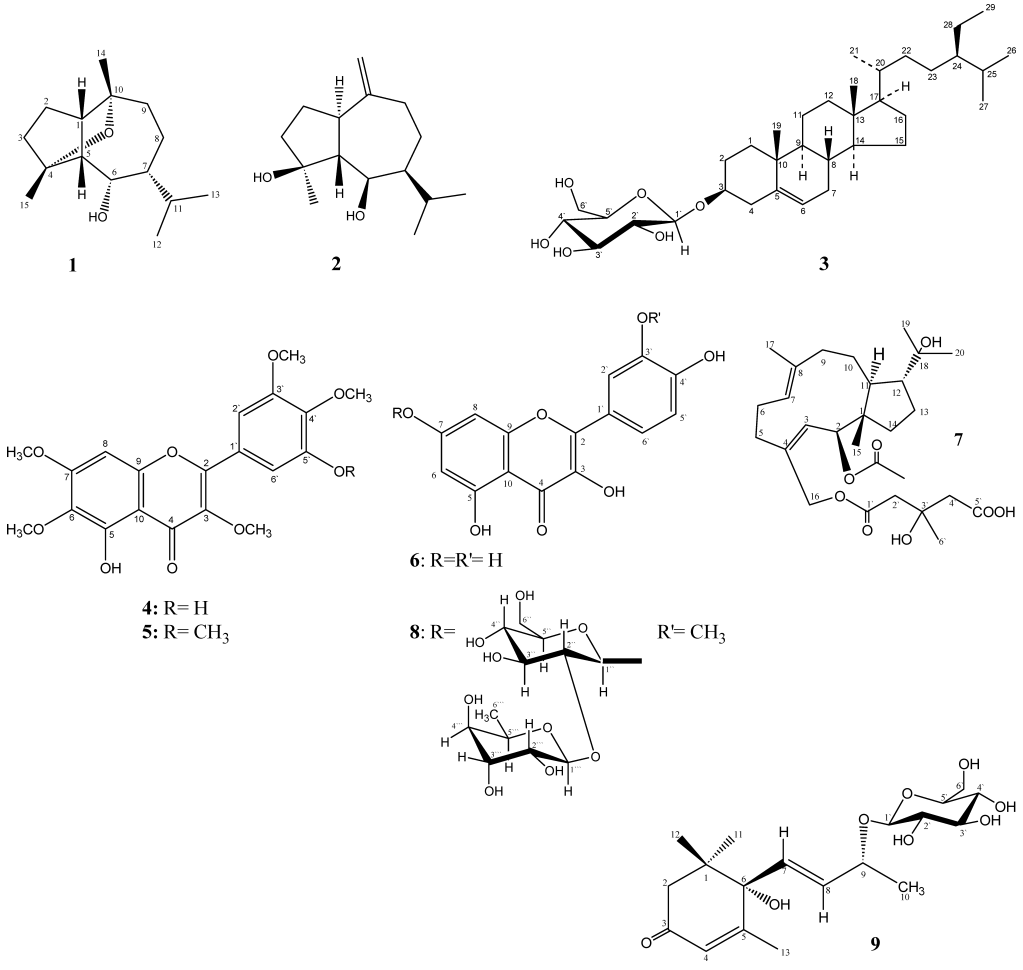

Fig. 1. The Structures of the Isolated Compounds **1**—**9** from *C. droserifolia*

silymarin, at a dose of 10 mg/kg (20.7 μmol/kg) prior to the administration of CCl_4 resulted in a significant decrease in the elevated SGOT, SGPT, ALP and bilirubin level in rats. All tested compounds were used at a 20.7 μmol/kg dose. Pretreatment of rats with **2**, **3** and **7** failed to decrease the elevated levels of the enzymes and bilirubin. Treatment of rats with **4**, **5**, and **8** resulted in a significant decrease in the levels of the four tested parameters (Table 1). They exhibited a good protection against CCl_4. Compound **6** caused a significant ($p<0.05$) reduction in SGOT and bilirubin levels, whereas SGPT and ALP levels were reduced but not by a statistically significant values.

The histological appearance of the hepatocyte reflects their damage conditions.[21] Exposure of hepatocytes to toxic agents such as CCl_4 leads to histopathological changes from the normal histological appearance (Fig. 2A). The hepatocytes of rat treated with a single dose of 1.25 ml CCl_4/kg, showed centrilobular necrosis and extensive fatty change was observed on the midzonal or entire lobe at 24 h after treatment (Fig. 2B). Effective hepatoprotective agents will protect the hepatocytes from the histopathological changes caused by the toxic agents. Liver tissues of rats treated with CCl_4 and silymarin showed no necrosis or fatty deposition but had only minimal portal inflammation (Fig. 2C) reflecting good protection of the known hepatoprotective drug silymarin. Histological changes in the liver of rats treated with 20.7 μmol/kg of **8** and CCl_4 showed a significant recovery except cytoplasmic vascular degenerations around portal tracts, mild inflammation and foci of lobular inflammation (Fig. 2D).

The results of the biological study revealed that flavonoids **4**, **5** and **8** showed moderate hepatoprotective activity as compared with silymarin. They are all flavonoidal in nature. The flavonoid derivative **6** is less effective, while the other compounds belonging to other chemical classes were not effective. These results might be connected to the antioxidant activities of flavonoid derivatives.

Experimental

General Experimental Procedures Melting points were determined in open capillary tubes using Thermosystem FP800 Mettler FP80 central processor supplied with FP81 MBC cell apparatus, and were uncorrected. Ultraviolet absorption spectra were obtained in methanol and with different shift reagents on a Unicum Heyios α UV–Visible spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DRX-500 (Central Lab at the College of Pharmacy, King Saud University) spectrometer operating at 500 MHz for proton and 125 MHz for carbon, respectively. The chemical shift values are reported in δ (ppm) relative to the internal standard TMS or residual solvent peak, the coupling constants (J) are reported in Hertz (Hz). 2D-NMR experiments (COSY, HSQC, HMBC and NOESY) were obtained using standard Bruker program. MS were obtained using Liquid Chromatography/Mass Spectrometer (Quattro micro API) equipped with a Z-spray electrospray ion source (Micromass®, Quattro micro™, WATERS) and Gas Chromatography/Mass Spectrometer (6890N GC/5973 Inert MS; Agilent Technologies).

Table 1. Effects of Compounds **2**—**8** on Serum Biochemical Parameters

Treatment ($n=6$)	Biochemical parameters							
	SGOT (units/l)		SGPT (units/l)		ALP (units/l)		Bilirubin (mg/dl)	
	Mean ±S.E.	% Decrease	Mean ±S.E.	% Decrease	Mean ±S.E.	% Decrease	Mean ±S.E.	% Decrease
Normal (control)	100.45±15.84		38.45±7.55		427.66±51.14		0.70±0.11	
CCl_4 only	423.33$^{a)}$±24.51***		367.00$^{a)}$±27.12***		1109.50$^{a)}$±33.51***		4.00$^{a)}$±0.23***	
Silymarin+CCl_4	154.00$^{b)}$±27.61***	63.6	123.85$^{b)}$±29.34***	66.2	482.16$^{b)}$±41.00***	56.5	1.15$^{b)}$±0.19***	71.2
2+CCl_4	399.66$^{b)}$±34.56	5.6	330.00$^{b)}$±25.46	10.1	1005.00$^{b)}$±34.71	9.4	3.66$^{b)}$±0.37	8.5
Normal (control)	102.81±18.90		40.96±10.57		596.16±38.70		0.71±0.10	
CCl_4 only	594.16$^{a)}$±28.30***		474.66$^{a)}$±36.33***		1206.66$^{a)}$±36.32***		4.05$^{a)}$±0.37***	
Silymarin+CCl_4	203.50$^{b)}$±26.17***	65.7	121.26$^{b)}$±18.84***	74.4	757.66$^{b)}$±33.42***	37.2	1.36$^{b)}$±0.19***	66.41
3+CCl_4	590.00$^{b)}$±20.05	—	405.00$^{b)}$±26.73	14.7	1098.66$^{b)}$±19.33*	8.9	3.81$^{b)}$±0.32	5.9
Normal (control)	110.98±14.47		40.30±14.54		471.16±33.17		0.61±0.07	
CCl_4 only	584.00$^{a)}$±26.23***		428.66$^{a)}$±30.73***		1102.66$^{a)}$±39.96***		3.85$^{a)}$±0.23***	
Silymarin+CCl_4	207.00$^{b)}$±18.94***	64.5	135.16$^{b)}$±24.49***	68.4	615.00$^{b)}$±31.86***	44.2	1.32^{b}±0.17***	65.7
4+CCl_4	349.66$^{b)}$±32.40***	40.1	278.33$^{b)}$±28.36**	35.1	904.16$^{b)}$±30.50**	18.0	3.13$^{b)}$±0.20*	18.7
Normal (control)	100.45±15.84		38.45±7.55		427.66±51.14		0.70±0.11	
CCl_4 only	423.33$^{a)}$±24.51***		367.00$^{a)}$±27.12***		1109.50$^{a)}$±33.51***		4.00$^{a)}$±0.23***	
Silymarin+CCl_4	154.00$^{b)}$±27.61***	63.6	123.85$^{b)}$±29.34***	66.2	482.16$^{b)}$±41.00***	56.5	1.15$^{b)}$±0.19***	71.2
5+CCl_4	282.00$^{b)}$±29.07**	33.4	200.83$^{b)}$±35.95***	45.3	837.16$^{b)}$±45.62***	24.5	2.24$^{b)}$±0.42**	44.0
Normal (control)	104.96±24.27		33.10±10.31		497.00±31.91		0.67±0.12	
CCl_4 only	415.50$^{a)}$±23.01***		362.50$^{a)}$±41.89***		1105.33$^{a)}$±41.96***		4.22$^{a)}$±0.25***	
Silymarin+CCl_4	176.33$^{b)}$±31.23***	57.5	106.68$^{b)}$±22.73***	70.6	569.33$^{b)}$±29.30***	48.5	1.08$^{b)}$±0.17***	74.4
6+CCl_4	303.50$^{b)}$±30.03*	26.9	283.16$^{b)}$±28.76	21.8	1018.66$^{b)}$±41.38	7.8	3.20$^{b)}$±0.26*	24.2
Normal (control)	110.98±14.47		40.30±14.54		471.16±33.17		0.61±0.07	
CCl_4 only	584.00$^{a)}$±26.23***		428.66$^{a)}$±30.73***		1102.66$^{a)}$±39.96***		3.85$^{a)}$±0.23***	
Silymarin+CCl_4	207.00$^{b)}$±18.94***	64.5	135.16$^{b)}$±24.49***	68.4	615.00$^{b)}$±31.86***	44.2	1.32$^{b)}$±0.17***	65.7
7+CCl_4	490.83$^{b)}$±25.91*	15.9	364.33$^{b)}$±46.31	15.0	1143.83$^{b)}$±40.75	—	3.83$^{b)}$±0.24	—
Normal (control)	102.81±18.90		40.96±10.57		596.16±38.70		0.71±0.10	
CCl_4 only	594.16$^{a)}$±28.30***		474.66$^{a)}$±36.33***		1206.66$^{a)}$±36.32***		4.05$^{a)}$±0.37***	
Silymarin+CCl_4	203.50$^{b)}$±26.17***	65.7	121.26$^{b)}$±18.84***	74.4	757.66$^{b)}$±33.42***	37.2	1.36$^{b)}$±0.19***	66.41
8+CCl_4	445.83$^{b)}$±37.67*	24.9	302.66$^{b)}$±21.50**	36.2	1019.83$^{b)}$±43.53**	15.5	2.71$^{b)}$±0.36*	33.1

$*p<0.05$; $**p<0.01$; $***p<0.001$, *a*) as compared with the normal saline (control) group; *b*) as compared with the CCl_4 only group.

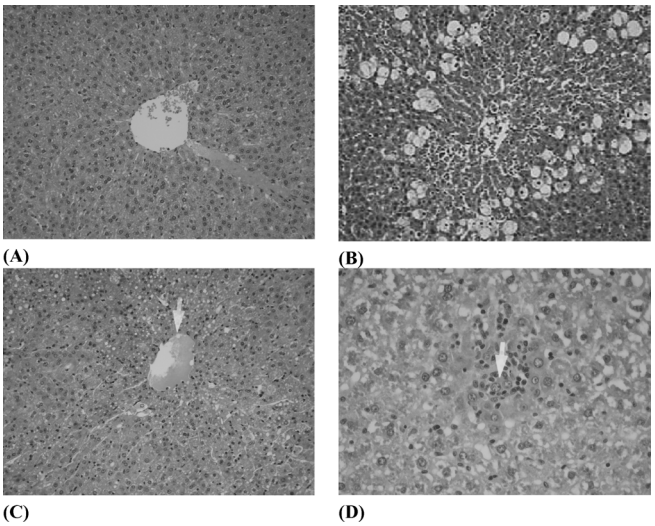

Fig. 2. Histopathological Appearance of Liver Cells

(A) Normal cells; (B) liver cells of rats treated with CCl_4 showed centrilobular necrosis and extensive fatty change was observed on the midzonal or entire lobe at 24 h after treatment; (C) liver cells of rats treated with CCl_4 and silymarin showed no necrosis or fatty deposition but had only minimal portal inflammation; (D) liver cells of rats treated with CCl_4 and **8** showed a significant recovery except cytoplasmic vascular degenerations around portal tracts, mild inflammation and foci of lobular inflammation.

Plant Material The aerial parts of *Cleome droserifolia* (Cleomaceae) were collected at 75 km south of Tabuk, Saudi Arabia, in April 2003. The plants were identified by Dr. Mohammad Atiqur Rahman, taxonomist of the Medicinal, Aromatic and Poisonous Plants Research Center (MAPPRC), College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Voucher specimen (#13490) was deposited at the herbarium of this center.

Extraction and Fractionation The dried ground aerial parts (1000 g) were extracted to exhaustion by percolation at room temperature with 90% ethanol (12 l), and the extract was evaporated *in vacuo* to leave 139 g of residue. Hepatoprotective assay showed 35.9, 26.8, 21.9 and 34.8% reduction in the SGOT, SGPT, ALP and bilirubin levels, respectively at a dose of 500 mg/kg. The total extract was fractionated using liquid–liquid partitioning and the resulted fractions were tested biologically. A portion of the total ethanol extract (135 g) was dissolved in 1200 ml of 20% aqueous methanol and defatted with petroleum ether (500 ml×3) to yield 12.6 g of petroleum ether soluble fraction (45.3, 39.8, 18.9 and 36.7% reduction in SGOT, SGPT, ALP and bilirubin, respectively at a dose of 500 mg/kg). The aqueous methanol fraction was diluted with water until a 40% aqueous methanol mixture was produced and this was partitioned with chloroform (500 ml×3) to yield 66.2 g of chloroform soluble fraction (36.0, 40.1, 23.9 and 35.9% reduction in SGOT, SGPT, ALP and bilirubin, respectively at a dose of 500 mg/kg) and 56.1 g of aqueous methanol soluble fraction (43.0, 30.0, 25.6 and 36.4% reduction in SGOT, SGPT, ALP and bilirubin, respectively at a dose of 500 mg/kg).

Chromatographic Purification and Isolation All fractions resulted from chromatographic purification of the $CHCl_3$ and MeOH fractions were tested for hepatoprotective activity at 250 mg/kg and active fractions only were selected for further purification. Due to lack of materials the petroleum ether sub-fractions were not tested.

Petroleum Ether Fraction Part of the petroleum ether extract (5.6 g) was fractionated on a VLC column (30×4 cm i.d.) of silica gel (125 g), eluted with petroleum ether, $CHCl_3$, EtOAc and MeOH, in increasing proportions until 5% MeOH–EtOAc. Fractions, each of 200 ml were collected. Fraction A: 1.2 g, eluted with $CHCl_3$, was further purified by CPTLC (4 mm silica gel GF_{254} disk, solvent: 8% Me_2CO in $CHCl_3$). Fractions 16 ml each were collected, screened by TLC and similar fractions were pooled. Fraction

A-1 (combined Frs. 7—12, 660 mg) was further purified on a Chromatotron (2 mm silica gel GF_{254} disk), eluted with 4% Me_2CO in $CHCl_3$ to give compound **1** (23 mg, oily residue, *Rf*=0.44; solvent system: 25% Me_2CO in $CHCl_3$). Fraction A-2 (Frs. 40—66, 350 mg) was further purified on a silica gel CC, eluted with, $CHCl_3$, $CHCl_3$/Me_2CO, followed by purification on CPTLC (1 mm silica gel GF_{254} disk, solvent: 20% EtOAc in hexane) to give compound **2** (30 mg, oily residue, *Rf*=0.28; solvent system: 40% EtOAc in hexane). Fraction B (400 mg, eluted with EtOAc) was further purified on a Chromatotron (2 mm silica gel GF_{254} disk), eluted with 5% MeOH in $CHCl_3$ to give compound **3** (120 mg, fine colorless needles, *Rf*=0.38; solvent system: 15% MeOH in $CHCl_3$).

$CHCl_3$ Fraction Further fractionation of the $CHCl_3$ fraction (61.2 g) by CC (Sephadex LH-20, 100×5 cm i.d., 700 g) and using CH_2Cl_2–Me_2CO (4 : 1, 2 l), CH_2Cl_2–Me_2CO (3 : 2, 2 l), CH_2Cl_2–MeOH (1 : 4, 1 l) and MeOH (2 l) as eluants afforded four fractions [1 (6.5 g), 2 (32.6 g), 3 (15.4 g) and 4 (6.7 g)]. Bioassay revealed that the hepatoprotective activity was retained in fraction 1 (6.5 g) (28.9, 26.9, 28.5 and 48.2% reduction in SGOT, SGPT, ALP and bilirubin, respectively) and 3 (15.4 g) (35.0, 22.6, 24.6 and 26.8% reduction in SGOT, SGPT, ALP and bilirubin, respectively).

The fraction 1 (6.5 g) was fractionated on a silica gel column (80×4 cm i.d., 195 g) using MeOH–$CHCl_3$ solvent system starting with 100% $CHCl_3$. The fractions eluted with 7% MeOH–$CHCl_3$ (3.8 g) were subjected to column chromatography on silica gel (90×3 cm i.d., 120 g). The column was eluted with EtOAc–hexane mixture to afford 30 fractions of 60 ml each. Fractions 12 to 25 (1.3 g) were combined and further purified over LiChroprep® RP-18 column (60×2.5 cm i.d., 80 g). Elution with 20% H_2O–MeOH mixture gave four fractions, 1-a (34 mg), 1-b (226 mg), 1-c (540 mg) and 1-d (500 mg). Fraction 1-c (540 mg) by preparative RP-18 TLC using 20% H_2O–MeOH as a developing solvent afforded **4** (116 mg, *Rf*=0.47, solvent system: 20% H_2O–MeOH) and **5** (11 mg, *Rf*=0.30, solvent system: 20% H_2O–MeOH). Fraction 1-d was subsequently purified by CPTLC (2 mm silica gel GF_{254} disk, solvent: 20% EtOH in $CHCl_3$ containing 0.1% HCO_2H) to give 114 mg of **6**, *Rf*=0.46 (solvent system: 25% EtOH in $CHCl_3$ containing 0.1% HCO_2H) as oily residue.

Part of fraction 3 (3.5 g) was further purified over LiChroprep® RP-18 column (90×2.5 cm i.d., 105 g) using 15% MeOH in H_2O as an eluting system followed by CPTLC (2 mm silica gel GF_{254} disk, solvent: 5—30% MeOH in $CHCl_3$ containing 0.1% HCO_2H) gave 211 mg of light yellow amorphous powder of **7** (*Rf*=0.58, solvent system: 10% MeOH in $CHCl_3$ containing 0.1% HCO_2H).

MeOH Fraction Part of the MeOH fraction (51.1 g) was purified by chromatography on Sephadex LH-20 (100×5 cm i.d., 700 g) using the solvents CH_2Cl_2–MeOH (4 : 1, 2 l), CH_2Cl_2–MeOH (3 : 2, 2 l), CH_2Cl_2–MeOH (1 : 4, 1 l) and MeOH (2 l) to yield four fractions [1 (2.8 g), 2 (4.3 g), 3 (6.4 g) and 4 (37.5 g)]. The hepatoprotective activity appeared in fractions 2 (4.3 g) (36.3, 47.9, 30.7 and 35.5% reduction in SGOT, SGPT, ALP and bilirubin, respectively) and 4 (37.5 g) (40.1, 47.9, 18.8 and 20.6% reduction in SGOT, SGPT, ALP and bilirubin, respectively).

A portion of fraction 2 (2 g) was fractionated over silica gel column (60×2 cm i.d., 60 g) and eluted with EtOAc, and EtOAc/MeOH mixtures in a gradient elution system. Fractions eluted with 15% MeOH in EtOAc gave **8** as a light yellow amorphous powder (390 mg, *Rf*=0.38, solvent system: 35% MeOH in EtOAc) after purification by CPTLC (2mm silica gel GF_{254} disk, solvent: 10—20% MeOH in EtOAc).

Part of fraction 4 (30 g) was subjected to column chromatography over silica gel (100×5 cm i.d., 925 g) and eluted with $CHCl_3$ followed by increasing concentrations of MeOH in $CHCl_3$ (up to 30%) to give three fractions, A, B and C. The active fraction A (2.7 g) was further purified by chromatography on LiChroprep® RP-18 column (60×2.5 cm i.d., 90 g) using 50% H_2O in MeOH as solvent to give **9** as amorphous powder (17.5 mg; *Rf*=0.38, solvent system: 50% H_2O–MeOH).

3′-Methoxy-3,5,4′-trihydroxyflavone-7-neohesperidoside (8) Yellow crystals; mp 224—226 °C. UV λ_{max} (MeOH): 354, 268 and 255; (NaOMe) 398, 266 and 249 nm; ($AlCl_3$) 398, 362, 299 and 269 nm; ($AlCl_3$/HCl) 401, 359, 299 and 268 nm; (NaOAc) 412 and 262 nm. ESI-MS *m/z*: 647 $[M+Na]^+$, 625 $[M+1]^+$ (Calcd for $C_{28}H_{32}O_{16}$). ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 6.46 (1H, d, *J*=2.0 Hz, H-6), 6.85 (1H, d, *J*=2.0 Hz, H-8), 7.96 (1H, d, *J*=2.0 Hz, H-2′), 6.92 (1H, d, *J*=8.5 Hz, H-5′), 7.57 (1H, dd, *J*=2.0, 8.5 Hz, H-6′), 12.60 (1H, s, 5-OH), 3.85 (3H, s, 3′-OCH_3), 5.58 (1H, d, *J*=7.5 Hz, H-1″), 3.25 (2H, br s, H-2″, 3″), 3.12 (2H, br s, H-4″, 5″), 3.40 (1H, m, H-6″), 3.59 (1H, dd, *J*=7.0, 11.0 Hz, H-6″), 5.56 (1H, br s, H-1‴), 3.43 (1H, m, H-2‴), 3.64 (1H, br s, H-3‴), 3.32 (1H, br s, H-4‴), 3.87 (1H, br s, H-5‴), 1.12 (3H, d, *J*=5.5 Hz, H-6‴). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 157.3 (C-2), 133.8 (C-3), 178.1 (C-4), 161.2 (C-5), 99.8 (C-6), 162.1 (C-7), 95.1 (C-8), 156.5 (C-9), 106.2 (C-10), 121.4 (C-1′), 114.0 (C-2′), 147.4 (C-3′), 150.1 (C-4′), 115.7 (C-5′), 122.8 (C-6′), 101.3 (C-1″), 76.9 (C-2″), 74.8 (C-3″), 70.4 (C-4″), 77.9 (C-5″), 61.2 (C-6″), 98.9 (C-1‴), 70.7 (C-2‴), 70.5 (C-3‴), 72.1 (C-4‴), 70.3 (C-5‴), 18.4 (C-6‴), 56.2 (3′-OCH_3).

Animals Wistar albino rats (150—200 g) roughly the same age (8—10 weeks), obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh were used. The animals were housed under constant temperature (22±2 °C), humidity (55%) and light/dark conditions (12/12 h). They were provided with Purina chow and free access to drinking water *ad libitum*.[9—11]

Chemicals Silymarin (Sigma Chemical Company, U.S.A.).

Hepatoprotective Activity Male Wistar rats were divided into five groups six animals each. Group I received normal saline and was kept as a control group. Groups II, III, IV and V received 0.125 ml of CCl_4 in liquid paraffin (1 : 1) per 100 g body weight intraperitoneally. Group II received only CCl_4 treatment. Group III was administered silymarin at a dose of 10 mg/kg *p.o.* (20.7 μmol/kg) Groups IV and V were treated with 250 and 500 mg/kg of extracts and fractions, respectively or 20.7 μmol/kg of pure compounds. Drug treatment was started 5 d prior to CCl_4 administration and continued till the end of the experiment. After 48 h, following $CC1_4$ administration the biochemical parameters: serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and total bilirubin were measured[22] using diagnostic strips (Reflotron®, ROCHE) and were read on a Reflotron® Plus instrument (ROCHE). The livers of the treated animals were immediately removed and a small piece was fixed in 10% formalin for histopathological assessment.

Statistical Analyses For each set of experiments where two or more than two groups were compared, an analysis of variance (ANOVA) test was used to determine the significance of the differences. Differences between the control and CCl_4-treated group were compared for significance using student's *t*-test for non paired samples.[23] All the values shown are the mean±S.E.

Histopathology The fixed livers samples were placed in cassettes and loaded into tissue baskets. They were subjected to dehydration, clearing and inflatration by immersion in different concentrations of ethanol (70—100%), xylene (3 times, 1 h each) and finally paraffin wax (4 times, 1 h each). The tissues were then transferred into moulds filled with paraffin wax. After orienting the tissues by hot forceps the moulds were chilled on cold plates and excess wax were trimmed off using a knife. The rotary microtome (Leitz 1512) was used for making thin sections (3 μm). The sections were placed onto clean slides that were drained vertically for several minutes before placing them onto a warming table at 37—40 °C.[21] The slides were then deparaffinized, hydrated and stained in Mayer's hematoxylin solution for 15 min. The slides were then washed in lukewarm running tap water for 15 min and placed in distilled water. After they were immersed in 80% ethyl alcohol for one to two minutes then counterstained in eosin-phloxine solution for 2 min. The slides were then dehydrated and cleared through two changes each of 95% ethyl alcohol, absolute ethyl alcohol, and xylene (2 min each) and finally mounting with resinous medium.

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