Study of the hepatoprotective effect of *Juniperus phoenicea* constituents

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Abstract: Different fraction obtained from the aerial parts of *Juniperus phoenicea* showed significant activity as hepatoprotective when investigated against carbon tetrachloride induced liver injury. The hepatoprotective activity was evaluated through the quantification of biochemical parameters and confirmed using histopathology study. Phytochemical investigation of the petroleum ether, chloroform and methanol fractions utilizing different chromatographic techniques resulted in the isolation of five known diterpenoids namely: 13-epicupressic acid (**1**), imbricatolic acid (**2**), 7α-hydroxysandaracopimaric acid (**3**), 3β-hydroxysandaracopimaric acid (**4**), isopimaric acid (5), four flavonoid derivatives: cupressuflavone (**6**), hinokiflavone (**7**), hypolaetin-7-O-β-xylopyranoside (**9**), (-) catechin (**10**), inaddition to sucrose (**8**). Both physical and spectral data were used for structure determination and all isolates were evaluated for their hepatoprotective activity. Compounds **2** and **6** were effective, however; **7** was the most active. Hepatoprotective activity of **7** is comparable with the standard drug silymarin in reducing the elevated liver enzymes and restoring normal appearance of hepatocytes. Hepatoprotective effect of combination of **6**, **7** and silymarin with the diterpene sugiol was also explored.

Keywords: *Juniperus phoenicea*; diterpenoids; flavonoids; Hepatoprotective; combined effect.

INTRODUCTION

Juniperus phoenicea is a tree or shrub up to 8 meters tall known locally as "Arayar". In Saudi Arabia, this species extends from the Mediterranean region southwards to as far south as Taif (Chaudhary, 1999). Previous phyto-chemical studies of *J. phoenicea* revealed that diterepenes are the main secondary metabolites of the plant (Muhammad *et al*., 1992; Mossa *et al*., 2004; Muhammad *et al*., 1995; Muhammad *et al*., 1996). The diterpene sandaracopimaric acid showed a lipoxygenase inhibition effect (Muhammad *et al*., 1996). Several biflavanoids including amentoflavone, cupressuflavone, robusta-flavone, hinokiflavone and mono-O-methylhinokiflavone were isolated from the leaf extract of *J. phoenicea* (Fatma *et al*., 1979). Phenylpropanoids (Hussein *et al*., 2003; Comte *et al*., 1996c; Comte *et al*., 1997), flavonoids (Maatooq *et al*., 1998) and furanones (Comte *et al*., 1996a; Comte *et al*., 1996b) were also isolated from the plant. Recently, amentoflavone, cupressuflavone, myricitrin, quercetin, cosmosin, quercitrin, *p*-coumaric acid and caffeic acid were isolated from the leaves of *J. phoenicea* growing in Egypt (Ali *et al*., 2010). Deoxypo-dophyllotoxin and *β*-peltatin-A methyl ether were identified as the major cytotoxic components of *J. phoenicea* (Cairnes *et al*., 1980). The aqueous decoction of the berries showed antihepatotoxic effect (Aboul-Ela *et al*., 2005). The ethanol extract and fractions obtained by liquid-liquid fractionation of the aerial parts showed promising hepatoprotective effect (Alqasoumi *et al*., 2009).

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MATERIALS AND METHODS

General

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). Centrifugal preparative TLC (CPTLC) was preformed using Chromatotron (Harrison Research Inc. model 7924): 1 mm silica gel P254 disc. Non UV active components were detected on TLC plates using

vanillin/H_2SO_4 spray reagent followed by heat at 105°C for 5 minutes.

Plant materials

The aerial parts (twigs, leaves, fruits) of *Juniperus phoenicea* (L.) sub. sp. *phoenicea* (Cupressaceae) were collected at 15km south of Al Hadda, near Taif, Saudi Arabia, in January 2004, 2009. 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. A voucher specimen (# 14166) was deposited at the herbarium of this center.

Extraction, fractionation and purification

The dried ground aerial parts (2600g) were extracted to exhaustion by percolation at room temperature with 90% ethanol (16L), and the extract was evaporated *in vacuo* to leave 577g of residue. A portion of this residue (502g) was dissolved in 1200ml of 20% aqueous methanol and defatted with petroleum ether (500ml×3) to yield 174.82g petroleum ether soluble fraction. The aqueous methanol fraction was diluted with water until a 40% aqueous methanol mixture was produced and this was partitioned with chloroform (500ml×3) to yield 72.83g of chloroform soluble fraction and 254.35g of aqueous methanol soluble fraction. Preliminary hepatoprotective screening of the crude extract and all fractions (table 34 and 35) showed that the hepatoprotective activity of the ethanol extract was distributed between all phases.

Petroleum ether fraction

Ten grams of the petroleum ether extract were fractionated using vacuum liquid chromatography (VLC) technique on silica gel (15×10 cm i.d., 250g) eluting with hexane containing 10% increments of ethyl acetate to yield seven fractions. Fraction 1 (1.2/g) was further purified using CPTLC (4/mm silica gel GF_{254} disk, solvent: 15% EtOAc in hexane) to give 230/mg of **1** as amorphous powder (R_f=0.39, solvent system: 30% EtOAc in hexane) and **2** as an oily residue (R_f=0.48, solvent system: 50% EtOAc in hexane). Preparative TLC of fraction 2 (130 mg) on C_{18} TLC plates using 20% H_2O in CH_3CN as developing system resulted in the isolation of 17/mg of **3** as amorphous powder. Fraction 3 (0.5g) was rechromatographed on silica gel CC (30×1.5 cm i.d., 35g) eluting with 10% MeOH in $CHCl_3$ to give seven fractions A-G after TLC screening. Further purification of fractions E and F by Chromatotron (1mm silica gel GF_{254} disk, solvent: 5% MeOH in $CHCl_3$) led to the isolation of compound **4** (67/mg) as amorphous powder (R_f=0.36; solvent system: 10% MeOH in $CHCl_3$).

Chloroform fraction

Part of the chloroform soluble fraction (67.83g) was fractioned using Sephadex LH-20 column (100 × 5 cm i.d., 700g). The column was eluted with the following sequence of solvents mixture 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) to afford, in order of elution, three fractions [1 (20.41g), 2 (36.05g) and 3 (11.37g)]. The hepatoprotective assay showed that the activity was apportioned between fraction 2 (6.5g) and 3 (15.4g).

Fraction 2 (3.4 g) was subjected to a silica gel column (80 × 3 cm i.d., 105 g), eluted with $CHCl_3$ and Me_2CO with increasing concentration of Me_2CO [$CHCl_3$; $CHCl_3$–Me_2CO /9:1, 8:2, 7:3, 6:4, and Me_2CO]; fractions 250 ml each were collected, screened by TLC (solvent system: 50% EtOAc in hexane) and similar fractions were pooled to give 3 fractions. Fraction A (1.3g) was subsequently purified by CPTLC (4mm silica gel GF_{254} disk, solvent: 20% EtOAc in hexane) to give 90mg of **1** (R_f = 0.69, solvent system: 40% EtOAc in hexane) as amorphous powder. Fraction B (1.5g) was rechromatographed on LiChroprep® RP-18 (25-40µm, MERCK) column (60 × 2.5 cm i.d., 60g) using 100% CH_3CN as eluant to give 55mg of **5** as amorphous powder (R_f= 0.37, solvent system: 100% CH_3CN, RP-18 plates).

Portion of fraction 3 (6.5 g) was rechromatographed on a Sephadex LH-20 column (80×4 cm i.d., 200 g) and eluted with MeOH–H_2O (3:1, 2L) to yield **6** and **7** as amorphous powders (R_f= 0.36 and R_f= 0.50 respectively, solvent system: 10% MeOH in $CHCl_3$ containing 0.1% HCO_2H).

Methanol fraction

The methanol fraction (249.35g) was fractionated by chromatography on Sephadex LH-20 (100×5 cm i.d., 700g) 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 (12.9g), 2 (125.95g), 3 (60.4g) and 4 (50.1g)]. The hepatoprotective assay indicated that the activity was mainly distributed between fraction 2 (125.95g) and 3 (60.4g); see table 41.

A sample of fraction 2 (5g) was subjected to silica gel CC (90×3 cm i.d., 130g) eluted with $CHCl_3$ followed by mixtures of $CHCl_3$ and MeOH. Polarity was increased by increasing the MeOH contents to obtain 7 fractions. The fractions 2-4 (1.6g) were subjected to a Sephadex LH-20 CC (60×3 cm i.d., 90g) eluting with CH_2Cl_2-MeOH (4:1, 500 ml) and CH_2Cl_2-MeOH (3:2, 500ml). Fractions eluted with CH_2Cl_2-MeOH (3:2, 500/ml) were further purified over LiChroprep® RP-18 CC (60×2.5/cm i.d., 30g) eluting with 18% CH_3CN-H_2O to provide compound **8** (356/mg) as white crystals.

Fraction 3 (25 g) was dissolved with H_2O and partitioned with EtOAc affording an EtOAc soluble fraction (2.7 g) and a water soluble fraction (22.3 g). The EtOAc-soluble fraction was subjected to silica gel CC (60×4 cm i.d., 90g) using a $CHCl_3$-MeOH gradient (from 19:1 to 0:1 v/v); fractions were collected, screened by TLC (solvent

system: 25% MeOH in $CHCl_3$) and pooled into two fractions. Fraction A (800mg) was further purified on a Sephadex LH 20 column (60×3 cm i.d., 60g) using MeOH as eluant followed by crystallization from MeOH to give **9** (49/mg). Fraction B (1.2g) was first subjected to silica gel CC (60×1.5 cm i.d., 40g) eluted with $CHCl_3$-MeOH (from 18:2 to 14:6 v/v). the semi pure fractions were further purified over Sephadex LH 20 column (60×3 cm i.d., 60g) using MeOH as eluant to yield pure **10** (175 mg).

13–Epicupressic acid (**1**). M.P. 104-105 °C. $[\alpha]_D$ = +22.6 (*c* = 0.031, $CHCl_3$). IR ν_{max} (KBr): 3510, 3090, 1720, 1645, 1250, 1225, 1150, 990, 925 and 875 cm^{-1}. ^{1}H- and ^{13}C-NMR ($CDCl_3$): tables 1 and 2. ESIMS: 343 (100, $[M + Na]^+$), calc. for $C_{20}H_{32}O_3$.

Imbricatolic acid **(2)**. $[\alpha]_D$ = +45.1 (*c* = 0.032, $CHCl_3$). IR ν_{max} (film): 3500, 3000, 2934, 2873, 1740, 1694, 1645, 1240 and 890 cm^{-1}. ^{1}H- and ^{13}C-NMR ($CDCl_3$): Tables 1 and 2. ESIMS: 345 (100, $[M + Na]^+$), calc. for $C_{20}H_{34}O_3$.

7α–Hydroxysandaracopimaric acid (**3**). M.P. 132-134 °C. $[\alpha]_D$ =-80.0 (*c* = 0.023, MeOH); IR ν_{max} (KBr): 3400, 3070, 1720, 1640, 1245, 995 and 905 cm^{-1}. ^{1}H- and ^{13}C-NMR (CD_3OD): Tables 1 and 2. ESIMS: 341 (100, $[M + Na]^+$), 357 (45, $[M + K]^+$), calc. for $C_{20}H_{30}O_3$.

β–Hydroxysandaracopimaric acid (**4**): M.P. 249-251°C. $[\alpha]_D$=+29.5 (*c*=0.025, MeOH). IR ν_{max} (KBr): 3495, 3280, 2550, 1696, 1085, 988, 910 and 858 cm^{-1}; ESIMS (*m/z* 341 $[M + Na]^+$ and 357 9100, $[M + K]^+$), calc. for $C_{20}H_{30}O_3$.

Isopimaric acid (**5**): M.P. 132-134 °C. $[\alpha]_D$ = –14.8 (*c* = 0.025, $CHCl_3$). IR ν_{max} (KBr): 3300–2500, 1690 (COOH), 3090, 1640, 1000, 910 and 900 cm^{-1}; ^{1}H- and ^{13}C-NMR (CD_3OD): Tables 1 and 2. ESIMS: 325 (100, $[M + Na]^+$), 341(36, $[M + K]^+$), calc. for $C_{20}H_{30}O_2$.

Cupressuflavone (**6**). M.P. 392 °C Decomp. UV λ_{max} (MeOH) 329, 276 and 226 nm. IR ν_{max} (KBr): 3440, 1640, 1610, 1580 and 1165 cm^{-1}. ^{1}H- and ^{13}C-NMR (DMSO-d_6): Tables 3 and 4. ESIMS: 539 (100, $[M + 1]^+$), 561 (74, $[M+Na]^+$), 577 (32, $[M+K]^+$), calc. for $C_{30}H_{18}O_{10}$.

Hinokiflavone (**7**): M.P. 362-363°C. UV λ_{max} (MeOH) 419, 338 and 270 nm. IR ν_{max} (KBr): 3500, 1635, 1610, 1570 and 1170 cm^{-1}. ^{1}H- and ^{13}C-NMR (DMSO-d_6): Tables 3, 4. ESIMS: 539 (100, $[M + 1]^+$, 561 (64, $[M + Na]^+$), 577 (28, $[M + K]^+$,calc. for $C_{30}H_{18}O_{10}$.

Sucrose (**8**). M.P. 183-184 °C. $[\alpha]_D$ = +68.0 (*c* = 0.25, H_2O). ESIMS: 365 (100, $[M + Na]^+$), 381 (53, $[M + K]^+$), calc. for $C_{12}H_{22}O_{11}$).

Hypolaetin-7-O-β-xylopyranoside (**9**). M.P. 199-201°C. UV λ_{max} (MeOH) 340, 301 and 275 nm; (NaOMe) 392, 335 and 265 nm; ($AlCl_3$) 363, 316 and 278 nm; ($AlCl_3$/HCl) 356, 314 and 281 nm; (NaOAc) 400, 330 and 273 nm. ESIMS: 435 (100, $[M + 1]^+$), 457 (67, $[M + Na]^+$), calc. for $C_{20}H_{18}O_{11}$.

(–)Catechin (**10**). M.P. 155-156 °C; $[\alpha]_D$ = –6.0 (*c* = 0.027, MeOH). UV λ_{max} (MeOH) 280 nm. ESIMS: 291 (100, $[M + 1]^+$), calc. for $C_{15}H_{14}O_6$.

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* (Alqasoumi *et al*., 2009). The experiments and procedures used in this study were approved by the Ethical Committee of the College of Pharmacy, King Saud University and the Ethical Committee of the College of Pharmacy, Salman Bin Abdulaziz University.

Chemicals

Silymarin (Sigma-Aldrich, St. Louis, MO, USA).

Hepatoprotective activity

Male Wistar rats were divided into seven groups six animals each. *Group I* received normal saline and was kept as a control group. *Groups II- IX* 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 mmole/ kg). Groups *IV* and *V* were treated with 250 and 500 mg/kg of fractions or 20.7 mmole/ kg of pure compounds. Group *VI* received 5.6 mg of **6** and 3 mg of sugiol (10.35 mmole/ kg). Group *VII* received 5.6 mg of **7** and 3 mg of sugiol (10.35 mmole/ kg). Group *VIII* received 10 mg of silymarin and 6 mg of sugiol (20.7 mmole/ kg). Group *IX* received 11.2 mg of **7** and 6 mg of sugiol (20.7 mmole/kg). Drug treatment was started 5 days prior to CCl_4 administration and continued till the end of the experiment. After 48 h, following $CC1_4$ administration the animals were sacrificed using ether anesthesia. Blood samples were collected by heart puncture and the serum was separated for evaluating the biochemical parameters. The liver was immediately removed and a small piece was fixed in 10% formalin for histopathological assessment.

Determination of enzyme levels

The biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and total bilirubin were estimated by reported methods (Edwards and Bouchier, 1991). The enzyme activities were measured using Reflotron® diagnostic strips (Roche, Basel, Switzerland) and were read on a Reflotron® Plus instrument (Roche).

Estimation of NP-SH

Non-protein sulfhydryl groups (NP-SH) were quantified according to the Sedlak and Lindsay method (Sedlak and Lindsay, 1968). The liver was cooled in a beaker immersed in an ice bath. 200/mg of liver were homogenized in 8/ml of 0.02 *M* ethylenediaminetetraacetic acid (EDTA). Aliquots of 5ml of the homogenate were mixed in 15 ml test tubes with 4 ml of distilled water and 1/ml of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at approximately 3000 rpm to precipitate the protein. 2/ml of the supernatant were mixed with 4/ml of 0.4 *M* Tris buffer, pH 8.9 and 0.1 ml of 0.01 *M* DTNB [5, 5`-dithio-bis-(2-nitrobenzoic acid)] added and the sample was shaken. The absorbance was measured spectrophotometrically within 5 min of addition of DTNB at 412 nm against a reagent blank with no homogenate.

STATISTICAL ANALYSIS

For each set of experiments where two or more than two groups were compared, an analysis of variance (ANOVA) test were used to determine the significance of the differences. Differences between the control and CCl_4-treated group were compared for significance using Dunnette test for non paired samples (Woolson and Clarke, 2002). 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 infiltration by immersion in different concentrations of ethanol (70- 100%), xylene (3 times, 1hr each) and finally paraffin wax (4 times, 1hr 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 (3μm). 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 (Prophet *et al*., 1994). The slides were then deparaffinized, hydrated and to stained in Mayer's hematoxylin solution for 15 minutes. The slides were then washed in lukewarm running tap water for 15 minutes 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 minutes. The slides were then dehydrated and cleared through two changes each of 95% ethyl alcohol, absolute ethyl alcohol, and xylene (2 minutes each) and finally mounting with resinous medium.

RESULTS

^{1}H- and ^{13}C-NMR data used for the identification of compounds **1- 5** are presented in tables 1 and 2. ^{1}H- and ^{13}C-NMR data of compounds **6**, **7** and **9** are presented in tables 3 and 4. Effect of the isolated compounds on serum biochemical parameters using silymarin as standard is presented in table 5. Effects of combination of **6**, **7** and silymarin with sugiol on serum biochemical parameters and nonprotein sulfhydryl groups (NP-SH) are presented in tables 6- 9. Histopathological changes of liver cells treated with CCl_4, CCl_4 and silymarin, CCl_4 and compounds **2**, **6**, **7**, combinations of **7** and silymarin with sugiol comparing with normal liver cells are presented in fig. 1.

Table 1: ^{1}H-NMR chemical shift values of diterpenes **1-5**

H	* 1	* 2	† 3	† 4	* 5
1	1.10 (brd, 14.0), 1.79 (m)	1.08 (m); 1.86 (m)	1.22 (m), 1.78 (m)	1.06 (m), 1.24 (m)	1.05 (m), 1.78 (m)
2	1.42 (m), 1.76 (m)	1.54 (m); 1.86 (m)	1.44 (m), 1.60 (m)	1.37 (m), 1.40 (m)	1.48 (m), 1.70 (m)
3	1.04 (dd; 4.0; 13.0) 2.16 (brd; 13.0)	1.06 (dd; 4.0; 13.0) 2.17 (brd; 13.0)	1.60 (m), 1.90 (m)	4.02 (dd; 4.5, 11.0)	1.60 (m), 1.70 (m)
5	1.23 (m)		2.44 (dd; 2.5, 13.0)	1.53 (m)	1.88 (m)
6	1.81 (m), 1.88 (m)	1.34 (m)	1.47 (m), 1.70 (m)	1.00 (m), 1.30 (m)	1.64 (m), 1.93 (m)
7	1.85 (m), 2.41 (brd; 9.0)	1.90 (m); 1.98 (m)	4.11 (brs)	1.84 (m), 2.20 (m)	5.24 (s)
9	1.47 (m)	1.91 (m) 2.42 (dd; 2.5, 8.5)	2.23 (dd; 6.0, 12.5)	1.60 (m)	1.68 (m)
11	1.23 (m), 1.49 (m)		1.44 (m), 1.60 (m)	1.39 (m), 1.43 (m)	1.28 (m), 1.48 (m)
12	1.23 (m), 1.63 (m)	1.54 (m)	1.48 (m), 1.49 (m)	1.15 (m), 1.24 (m)	1.28 (m), 1.41 (m)
14	5.92 (dd; 11.0, 17.5)		5.52 (s)	5.25 (s)	1.84 (m), 1.88 (m)
15	5.08 (dd; 1.0, 11.0) 5.22 (dd; 1.0, 17.5)	1.27 (m), 1.52 (m)	5.82 (dd; 10.5, 17.5)	5.76 (dd; 11.0, 18.0)	5.73 (dd; 10.5, 17.5)
16	1.20 (s)	0.96 (m) 1.45 (m)	4.93 (d, 10.5) 5.01 (d, 17.5)	4.63 (d, 11.0) 4.68 (d, 18.0)	4.79 (d, 10.5) 4.85 (d, 17.5)
17	4.50 (s), 4.85 (s)	1.54 (m)	1.07 (s)	1.05 (s)	0.79 (s)
18	1.16 (s)	1.34 (m); 1.65 (m)	—	—	—
19	—	3.64–3.72 (m)	1.19 (s)	1.14 (s)	1.19 (s)
20	0.51 (s)	0.92 (d; 6.5)	0.86 (s)	0.87 (s)	0.83 (s)

*$CDCl_3$ and †CD_3OD, δ in ppm, J in Hz.

Table 2: ^{13}C-NMR chemical shift values of diterpenes **1- 5**

Carbon	* 1	* **2**	† **3**	† **4**	* **5**
1	39.1	39.2	39.5	38.3	38.8
2	19.9	19.9	19.3	19.8	17.9
3	37.9	38.0	38.1	76.4	37.0
4	44.2	44.2	48.0	54.7	46.3
5	56.4	56.4	43.3	51.8	45.0
6	26.0	26.1	33.2	25.5	25.2
7	38.7	38.8	73.8	36.6	121.0
8	148.0	148.3	139.8	137.6	135.6
9	56.5	56.7	47.4	51.5	52.0
10	40.6	40.6	39.1	38.6	35.0
11	17.9	21.2	19.3	27.9	20.0
12	41.3	36.4	35.3	35.7	36.1
13	73.7	30.3	38.5	38.5	36.8
14	145.0	39.6	135.1	130.4	46.1
15	111.7	61.3	149.3	149.9	150.3
16	28.0	19.8	111.3	110.8	109.2
17	106.5	106.4	26.3	26.6	21.5
18	29.0	29.0	182.5	181.3	184.9
19	183.5	183.4	17.5	11.7	17.1
20	12.7	12.8	15.1	15.7	15.2

*$CDCl_3$ and †CD_3OD, δ in ppm, J in Hz.

Table 3: ^{1}H-NMR chemical shift values of Compounds **6**, **7** and **9** (DMSO-d_6)

Position	**6**-I	**6**-II	**7**-I	**7**-II	**9**
3	6.80 (s)	6.80 (s)	6.85 (s)	6.84 (s)	6.63 (s)
5	13.1 (s) (OH)	13.1 (s) (OH)	12.89 (s) (OH)	13.22 (s) (OH)	—
6	6.48 (s)	6.48 (s)	6.20 (d; 2.0)		6.48 (s)
7	10.8 (s) (OH)	10.8 (s) (OH)	11.30 (s) (OH)	10.86 (s) (OH)	—
8			6.48 (d; 2.0)	6.74 (s)	—
2`	7.51 (dd; 2.0, 8.5)	7.51 (dd; 2.0, 8.5)	8.01 (d; 8.5)	7.96 (d; 8.5)	7.40 (s)
3`	6.76 (dd; 2.0, 8.5)	6.76 (dd; 2.0, 8.5)	7.05 (d; 8.5)	6.95 (d; 8.5)	—
4`	10.3 (s) (OH)	10.3 (s) (OH)		10.39 (s) (OH)	—
5`	6.76 (dd; 2.0, 8.5)	6.76 (dd; 2.0, 8.5)	7.05 (d; 8.5)	6.95 (d; 8.5)	6.81 (brd; 8.0)
6`	7.51 (dd; 2.0, 8.5)	7.51 (dd; 2.0, 8.5)	8.01 (d; 8.5)	7.96 (d; 8.5)	7.39 (brd; 8.0)

Xyl: 4.86 (d; *J*=7.5, (H-1'), 3.29 (m, H-2'), 3.20 (m, H-3'), 3.31 (m, H-4'), 3.29 (m); 3.73 (m, H-5').

Table 4: ^{13}C-NMR chemical shift values of Compounds **6, 7** and **9** (DMSO-*d)*

Position	**6**-I	**6**-II	**7**-I	**7**-II	**9**
2	163.0	163.0	163.1	164.2	164.2
3	103.1	103.1	103.9	102.6	102.7
4	182.5	182.5	181.7	182.1	182.2
5	161.5	161.5	161.3	153.1	152.2
6	99.0	99.0	98.9	124.7	98.5
7	164.1	164.1	164.2	157.3	150.8
8	98.8	98.8	94.0	94.6	127.1
9	155.3	155.3	157.1	153.7	144.5
10	104.2	104.2	103.8	104.1	105.2
1`	121.7	121.7	124.2	121.1	121.6
2`	128.4	128.4	128.3	128.5	113.6
3`	116.3	116.3	115.3	115.9	145.7
4`	161.4	161.4	161.4	160.6	149.8
5`	116.3	116.3	115.3	115.9	116.0
6`	128.4	128.4	128.3	128.5	119.1

Xyl: 101.7 (C-1'), 73.0 (C-2'), 75.6 (C-3'), 69.3 (C-4'), 65.8 (C-5').

Table 5: Effects of compounds **2, 6** and **7** 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	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
2 + CCl_4	210.83[b] ± 20.44*	49.2	138.78[b] ±23.51*	61.7	614.00[b] ±23.07*	44.4	1.61[b] ±0.31*	61.8
Normal	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
6 + CCl_4	171.66[b]±32.58*	59.4	124.74[b] ±24.58*	66.0	508.66[b] ±23.23*	54.1	1.15[b]±0.23*	71.2
Normal	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*	69.2	121.26[b] ±18.84*	74.4	757.66[b] ±33.42*	37.2	1.36[b] ±0.19*	66.41
7 + CCl_4	183.16[b]±29.19*	65.7	132.66[b] ±21.74*	72.0	765.70[b]±34.00*	36.5	1.56[b] ±0.30*	61.5

*p<0.001, [a]as compared with the normal saline (control) group; [b] as compared with the CCl_4 only group

Table 6: Effects of combination of **6, 7** and sugiol at 10.35 mmole/kg 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	97.26±11.31		28.85±5.86		385.83±23.04		0.58±0.05	
CCl_4 only	428.83[a]±34.38 **		354.33[a] ±30.44**		914.33[a] ±41.79**		4.05 [a] ±0.17**	
Silymarin + CCl_4	140.83[a]±13.64*	67.15	106.18[a] ±13.85**	70.03	467.83[a] ±29.26**	48.83	1.07 [a] ±0.13 **	73.58
6+ Sugiol +CCl_4	264.00[b]±23.83*	38.43	207.5[b]±18.10*	41.43	681.50[b] ±27.63**	25.46	2.37 [b] ±0.25**	41.48
7+ Sugiol +CCl_4	196.83[b]±22.71**	54.10	165.5[b] ±21.36**	53.29	595.5[b] ±21.87**	34.87	1.98 [b] ±0.20**	51.11

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

Table 7: Effects of combination of Silymarin, **7** and sugiol at 20.7 mmole/kg 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	85.18±11.22		32.93±6.23		308.6±13.72		0.59±0.05	
CCl_4 only	398.00[a] ±21.50		340.16 [a]±30.96		990.5 [a] ±35.36		3.89 [a]±0.27	
Silymarin + CCl_4	122.43[a]±14.96*	69.23	122.83 [a]±10.36*	63.89	426.5 [a]±25.04*	56.94	1.2 [a]±0.26*	69.15
Silymarin+ Sugiol+CCl_4	108.46[b]±14.63*	72.74	101.61 [b]±15.63*	70.12	378.83 [b]±23.61*	61.75	1.09 [b]±0.17*	71.97
7 + Sugiol +CCl_4	123.00[b] ±13.40*	69.09	115.66 [b]±15.17*	65.99	403.83 [b]±23.26*	59.22	1.26 [b]±0.13*	67.60

*p<0.001, [a] as compared with the normal saline (control) group; [b] as compared with the CCl_4 only group

DISCUSSION

Our previous study of the hepatoprotective effect of the total extract and fractions obtained by liquid-liquid fractionation of the aerial parts of *J. phoenicea* based on biochemical parameters (serum glutamate oxaloacetate

Table 8: Effects of combination of **6, 7** and sugiol at 10.35 mmole/kg on the level of NP-SH groups

Treatment (n=6)	NP-SH		
	Mean ±S.E. (μmol/gm wet weight tissue)	% Increase with reference to CCl_4-treated liver	% effectiveness for reversing to normal
Normal (control)	4.25 ±0.51		
CCl_4 only (toxicity control)	1.58 ±0.12 *		
Silymarin + CCl_4	3.60 ±0.58 *	128.66	84.71
6 + Sugiol+CCl_4	2.16 ±0.29 *	36.70	50.83
7 + Sugiol+CCl_4	2.30 ±0.23 *	45.56	54.12

*) $p<0.001$, as compared with the CCl_4 only group

Table 9: Effects of combination of Silymarin, **7** and sugiol at 20.7 mmole/kg on the level of NP-SH groups

Treatment (n=6)	NP-SH		
	Mean ±S.E. (μmol/gm wet weight tissue)	% Increase with reference to CCl_4-treated liver	% effectiveness for reversing to normal
Normal (control)	4.86±0.20		
CCl_4 only (toxicity control)	1.57 ±0.18 *		
Silymarin + CCl_4	4.26 ±0.47 *	171.33	87.66
Silymarin + Sugiol+CCl_4	4.29 ±0.45 *	173.24	88.28
7 + Sugiol+CCl_4	3.85 ±0.25 *	145.24	79.22

*) $p<0.001$, as compared with the CCl_4 only group

transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and bilirubin), hepatic nonprotein sulfhydryl group (NP-SH) and histopathological examination showed promising results. Toxicity study were also preformed. According to the obtained results, the methanol sub-fraction is the most safe and active (Alqasoumi *et al*., 2009). Based on these finding we carried out a detailed biologically directed phytochemical study.

Compounds **8** and **10** were identified as sucrose (Popov *et al*., 2006) and (-)catechin (Lu and Foo, 1999) by comparison of their data with the literature as well as direct comparison with authentic samples.

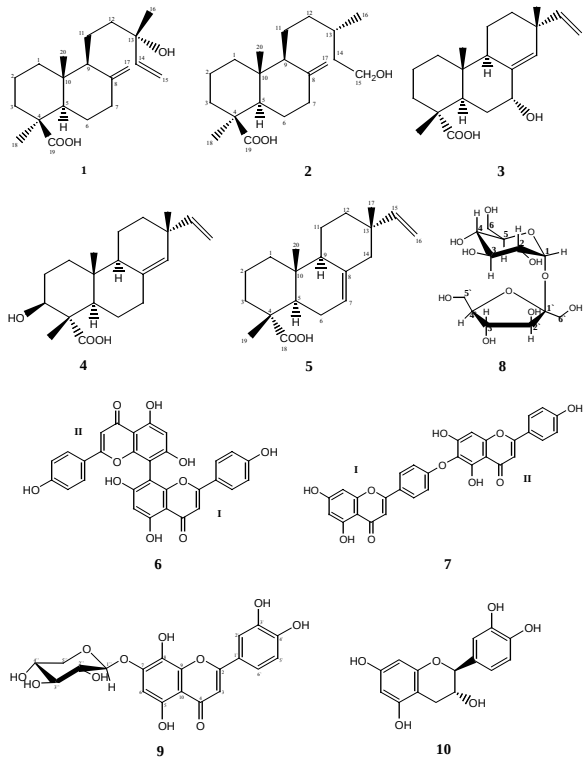
The ESIMS of compound **1** and **2** showed M^++ Na at 343 and 345 *m/z* consistent with molecular formulae $C_{20}H_{32}O_3$ and $C_{20}H_{34}O_3$ respectively. Both ^{1}H- and ^{13}C-NMR (tables 1, 2) of **1** and **2** showed two proton singlets at d_H 4.50, 4.85 and 4.51, 4.85 respectively assigned for H-17. Both compounds showed an 18-CH_3 singlet (d_H 1.16, d_C 29.0)(d_H 1.25 , d_C 29.0); 19-COOH (d_C 183.5)(d_C 183.4); 20-CH_3 singlet (d_H 0.51, d_C 12.7) (d_H 0.61, d_C 12.8) respectively. These data are typical for laban-8(17)-ene skeleton (Su *et al*., 1994). ^{1}H- and ^{13}C-NMR (tables 1, 2) of **1** showed another monosubstituted double bond (CH at d_H 5.92, dd, *J*= 11.0, 17.5 Hz and d_C 145.0 ; CH_2 at d_H 5.08 dd, *J*= 1.0, 11.0 Hz, d_H 5.22 dd, *J*= 1.0, 17.5 Hz and d_C 111.71) assigned for C-14 and C-15. The methyl singlet (d_H 1.20, d_c 28.0) was assigned to C-16 on an oxygenated fully substituted C-13 at d_C 73.7 as indicated from HMBC experiments. The data of **1** was identical with those reported for 13-epicupressic acid (Su *et al*., 1994). MS, ^{1}H- and ^{13}C-NMR data of **2** indicated the lack of the second double bond. The appearance of the C-16 methyl as doublet at d_H 0.92 (*J*=6.5 Hz) and the appearance of CH_2OH (d_H 3.64–3.72 (m), d_C 61.3) indicated a C-15 hydroxyl in **2**. The data of **2** was consistent with those reported for imbricatolic acid (Wenkert and Buckwalter, 1972).

Both **3** and **4** (table 1, 2) have a pimarandiene skeleton with a C-8, C-14 double bond (d_C 139.8, 135.1 and d_C 137.6, 130.4 respectively) (Su *et al*., 1994). They also showed same EISMS M^++ Na at 341 *m/z* consistent with the molecular formula $C_{20}H_{30}O_3$. In each compound there were an oxygenated CH (d_H 4.11 brs, d_C 73.8; d_H 4.02 dd, *J*= 4.5, 11.0 Hz, d_C 76.4 respectively). The locations of the hydroxyl groups were assigned to C-7 and C-3 in **3** and **4** respectively based on the HMBC correlations. Comparison of their data with the literature identified **3** as 7α–Hydroxysandaracopimaric acid and **4** as 3β–Hydroxysandaracopimaric acid, respectively (Muhammad *et al*., 1992; Su *et al*., 1994; Wenkert and Buckwalter, 1972).

The molecular formula of **5** ($C_{20}H_{30}O_2$, ESIMS M^++ Na at 325 *m/z*) as well as ^{1}H- and ^{13}C-NMR (tables 1,2) indicated a pimarandiene skeleton with one oxygen less than **3** and **4** (Wenkert and Buckwalter, 1972). In the ^{13}C-NMR spectrum the two carbon signals at d_C 121.0 and d_C 135.6 were assigned for C-7 and C-8 respectively based on HMBC experiment and the compound was identified as isopimaric acid (Wenkert and Buckwalter, 1972; Mihashi *et al*., 1969; Topcu and Ulubelen, 1990).

UV data of **6** indicated a 5, 7, 4'-trihydroxy flavone

(Mabry *et al*., 1970). ^{1}H-NMR (tables 3) showed one singlet for ring A at d_H 6.48, one singlet for H-3 at d_H 6.80 and two doublets each integrated for two protons at d_H 6.76 and 7.51 assigned for 4'-monosubstituted ring B. ^{13}C-NMR data (tables 4) indicated that C-8 appeared as fully substituted at d_C 98.8 bears non-oxygenated substituent (Mabry *et al*., 1970; Agrawal, 1989). However, ESIMS showed peaks at *m*/*z* 539 $[M + 1]^+$, 561 $[M + Na]^+$ and 577 $[M + K]^+$calc. for $C_{30}H_{18}O_{10}$ indicating a dimeric structure. Similarly, the data of **7** indicated 5, 7, 4'-trihydroxy flavone. ESIMS showed *m*/*z* 539 $[M + 1]^+$, 561 $[M + Na]^+$ and 577 $[M + K]^+$calc. for $C_{30}H_{18}O_{10}$. ^{1}H- and ^{13}C-NMR showed two H-3 protons at d_H 6.84 and 6.85. One part of the compound showed two doublets (*J*=2.0 Hz) at d_H 6.20 and 6.48 assigned for H-6 and H-8 respectively. The other part of the compound showed only one singlet for ring A at 6.74 assigned for H-8. Comparison with literature data enable the identification of **6** as cupressuflavone and **7** as hinokiflavone (Markham *et al*., 1987; Kang *et al*., 2005; Williams *et al*.,1987; Chari *et al*., 1977).



The UV data of **9** pointed out to a flavone derivative (λ_{max}^{MeOH} 340 nm) with free 5, 3', 4'-trihydroxy substituents. The lack of any shift with the use of NaOAc indicated a substituted C-7 hydroxyl (Mabry *et al*, 1970). The ^{1}H-NMR data of **9** (table 3) showed two singlets at d_H 6.63 and 6.48 correlated in an HSQC experiment with two carbon signals at d_C 102.7 and 98.5 respectively assigned for H-3 and H-6. Ring B protons appeared as an ABX system at d_H 7.40 (two overlapped protons H-2' and H-6') and d_H 6.81 (d, *J*=8.0 Hz, H-5'). All the assignments were supported by an HSQC and HMBC experiments.

Both ^{1}H- and ^{13}C-NMR data showed signals for pentose moiety. This proposal was supported by the ESIMS M^++ Na at *m*/*z* 457 consistent with the molecular formula $C_{20}H_{18}O_{11}$. The location of the sugar part at C-7 was further supported by the HMBC correlation between the H-1" at d_H 4.86 (d, *J*= 7.5 Hz) and C-7 at d_C 150.8. The chemical shifts of the sugar carbons as well as direct comparison with standard sugars after hydrolysis led to the identification of the C-7 moiety as *b*-D-xylose (Agrawal, 1989). Compound **9** was identified as hypolaetin-7-*O*- *b*-xylopyranoside previously isolated from *Juniperus communis* var. *depressa* (Dai *et al*., 2006; Nakanishi *et al*., 2004).

Treatment of animals with the hepatotoxic agent carbon tetrarchloride resulted in significant increase of transaminases (SGOT and SGPT) and alkaline phosphate levels (ALP) due to hepatocytes damage (Zafar and Ali, 1998). Sever jaundice was reflected by increase level of serum bilirubin (Lin *et al*., 1997). Silymarin act as an antioxidant by scavenging prooxidant free radicals and by increasing the intracellular concentration of GSH. It also exhibits a regulatory action of cellular membrane permeability and increase in its stability against xenobiotics injury; increasing the synthesis of ribosomal RNA by stimulating DNA polymerase-I, exerting a steroid like regulatory action on DNA transcription and stimulation of protein synthesis and regeneration of liver cells (Alqasoumi and Abdel-Kader, 2012b).

All isolated compounds were tested for their possible hepatoprotective effect at a 20.7 µmol/kg dose. However, compounds **2**, **6** and **7** were the most effective in reducing elevated levels of SGOT, SGPT, ALP and bilirubin (table 5). All values were highly significant ($p<0.001$). Compound **7** was the most active and closely comparable to silymarin followed by **6**. Histopathological appearance (fig. 1) of liver cells of rats treated with CCl_4 and silymarin showed only minimal portal inflammation. Liver cells of rats treated with CCl_4 and **2** showing a good recovery with absence of necrosis, fatty depositions and only minimal lobular inflammation was observed. Histological appearance of rat liver treated with CCl_4 and **6** showed cytoplasmic vascular degenerations around portal tracts, mild inflammation and foci of lobular inflammation. Liver tissue of rats treated with CCl_4 and **7** showed good recovery with absence of necrosis and fatty depositions. The central vein has minimal portal inflammation. It showed superiority as compared with other groups.

Our previous investigation of *J. procera* showed that the abietane diterpene sugiol was the most effect hepatoprotective compound in the plant (Alqasoumi and Abdel-Kader, 2012a). Compounds **6** and **7** are similar in nature to silymarin and most likely act by the same mechanism; however, sugiol has different nature and may

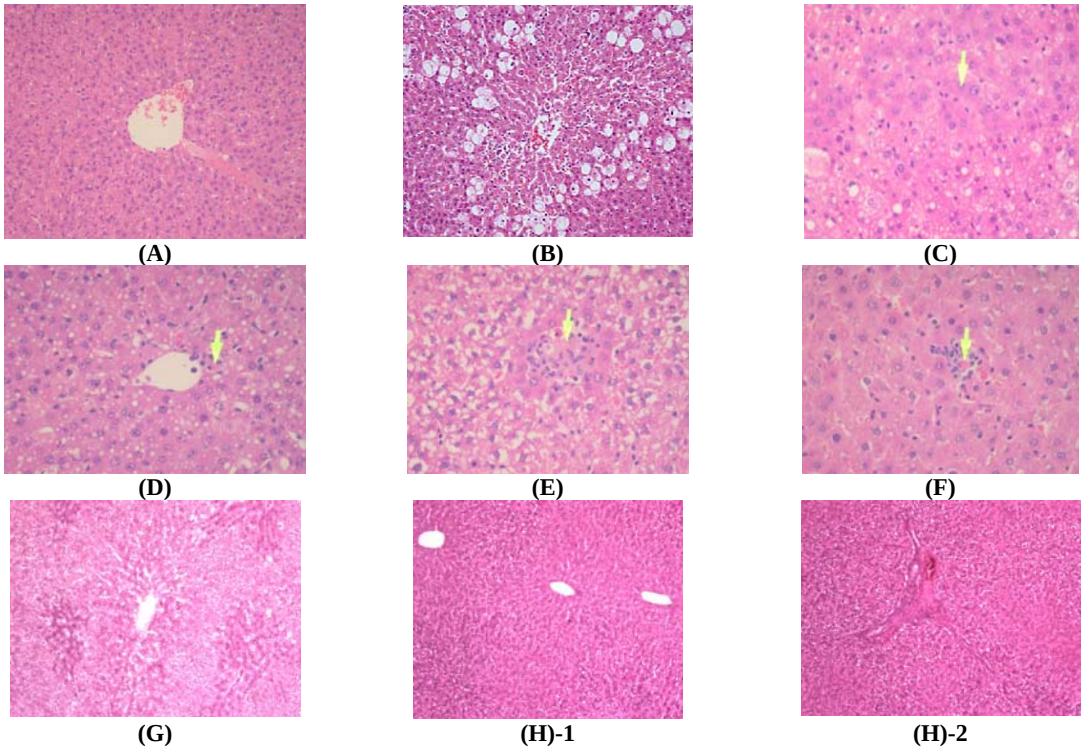


Fig. 1: Histopathological appearance of liver cells; **(A)** normal cells; **(B)** liver cells of rats treated with CCl_4 showed centrilobular necrosis and extensive fatty change was observed on the midzonal or entire lobe at 24 h after treatment; **(C)** liver cells of rats treated with CCl_4 and silymarin showed no necrosis or fatty deposition but had only minimal portal inflammation; **(D)** liver cells of rats treated with CCl_4 and **2** showing a good recovery with absence of necrosis and fatty depositions and indicating a potent hepatoprotective activity. Only minimal lobular inflammation was found; **(E)** Histological appearance of rat liver treated with CCl_4 and **6** showed a significant recovery except cytoplasmic vascular degenerations around portal tracts, mild inflammation and foci of lobular inflammation; **(F)** liver tissue of rats with CCl_4 and **7** showed good recovery with absence of necrosis and fatty depositions. The central vein has minimal portal inflammation; **(G)** liver cells of rats treated with CCl_4 and combination of silymarin and sugiol showed mild central vacuolar degeneration, fatty change and hydropic degeneration **(H)** liver tissue of rats with CCl_4 and combination of **7** and sugiol showed normal lobular pattern with only mild congestion with no significant inflammation.

act by different mechanisms. Consequently, combination of **6**, **7** and silymarin with sugiol were to explore the potential in liver protection. Starting with half doses of **6**, **7** and sugiol (10.35 mmole/ kg) in comparison with silymarin resulted in significant improvement of the enzyme levels and hepatic nonprotein sulfhydryl group (NP-SH) (tables 6 and 8). However, non of these treatments exceeded the protective effect of silymarin. Second combinations tested composed of both **7** and silymarin with sugiol at 20.7 mmole/ kg. The obtained results were highly significant ($p<0.001$) and both combination exceeded the effect of silymarin alone (tables 7 and 9). Histopathological study (fig. 1) showed that treatment with **7** and sugiol resulted in normal lobular pattern with only mild congestion with no significant inflammation. This appearance was the best compared to normal liver cells.

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