Terpenoids from *Juniperus procera* with hepatoprotective activity

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Abstract: The petroleum ether fraction of *Juniperus procera* 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 analysis. Phytochemical investigation of the petroleum ether fraction utilizing different chromatographic techniques resulted in the isolation of six known diterpenoids namely: 4-*epi*-abietol **(1)**, ferruginol **(2)**, hinokiol **(3)**, sugiol **(4)**, Z-communic acid **(5)** and hinokiol-1-one 3β,12-dihydroxyabieta-8,11,13-triene-1-one) **(6)**, in addition to the sesquiterpene 8α-acetoxyelemol (**7**). Both physical and spectral data were used for structure determination and all isolates were evaluated for their hepatoprotective activity. Compounds **1** and **4** were the most effective in reducing the elevated liver enzymes as indication for liver protection.

Keywords: *Juniperus procera*; diterpenoids; sesquiterpene; hepatoprotective.

INTRODUCTION

Juniperus procera Hochst. ex Endl. known locally as "Arar" is a tree upto 8 m tall extends southwards from south Saudi Arabia into Yemen and then across the Red Sea into Africa (Collenette, 1999). Previous phytochemical study of different parts of *Juniperus procera* resulted in the isolation of different classes of diterpenes and sesquiterpenes. The two lignans β-peltatin A Me ether and deoxypodophyllotoxin were isolated from the bark of *J. procera* (Muhammad *et al*., 1995). Several antimicrobial diterpenes were isolated from the bark and leaves of *J. procera* including isocupressic acid, (+)-Z-communic acid, (+)-totarol and sugiol (Muhammad *et al*., 1995; Muhammad *et al*., 1996). A bioassay-guided fractionation of *J. procera* berries yielded antiparasitic, nematicidal and antifouling constituents, including a wide range of known abietane, pimarane and labdane diterpenes (Samoylenko *et al*., 2008). The phenolic diterpene totarol, isolated from *J. procera* showed synergistic effect with INH against four species of *Mycobacterium* (Mossa *et al*., 2004). The essential oil of *J. procera* was effective as antioxidant and OH-radical-scavenging agents when assessed in the deoxyribose degradation assay (Burits, 2001).

Screening of plants used in Saudi folk medicine as remedy for liver problems is one of our research interest (Abdel-Kader *et al*., 2009a; Abdel-Kader *et al*., 2009b; Abdel-Kade *et al*., 2010). In the present study biologically directed phytochemical study of the petroleum ether fraction of *J. procera* was conducted.

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*): 4 mm silica gel P254 disc.

Plant materials

The aerial parts of *Juniperus procera* (Hochst. ex Endl.) were collected 35 km south of Baljurashi, Saudi Arabia, in February 2004. 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 (# 13497) was deposited at the herbarium of this center.

Extraction, fractionation and purification

The dried ground aerial parts of *J. procera* (800 g) were extracted to exhaustion by percolation at room temperature with 90% ethanol (12 L), and the extract was evaporated under reduced pressure to leave 191.2 g) of

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1

2 $R_1=R_2=R_3=R_4=R_5=$ H
3 $R_1=R_2=R_4=R_5=$ H $R_3=$ OH
4 $R_1=R_2=R_3=$ H $R_4, R_5=$ O
6 $R_1, R_2=$ O $R_3=$ OH $R_4=R_5=$H

5

7

Scheme

the total extract. A portion of this residue (186.2 g) was dissolved in 1200 mL of 20% aqueous methanol and defatted with petroleum ether (500 mL × 3) to yield 64.2 g of 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 (500 mL × 3) to yield 20.2 g of chloroform soluble fraction. The 40% aqueous methanol yielded 101.8 g residue after evaporation to dryness. Hepatoprotective screening showed that the petroleum ether fraction had a significant hepatoprotective effect against CCl_4 induced toxicity in rats.

Part of the petroleum ether fraction (18 g) was subjected to column chromatography over silica gel (540 g, cm i.d.) using a gradient of petroleum ether (40-60°C/EtOAc. Fractions 200 mL each were collected and screened by TLC and similar fractions were collected. Fractions eluted with 0.5% EtOAc in petroleum ether provided 1100 mg of pure gummy compound **1**. Fractions eluted with 6% EtOAc in petroleum ether (630 mg) were further purified by CPTLC (2 mm silica gel GF_{254} disk, solvent: petroleum ether-Me_2O-NH_3; 49:1:0.1) to give 343 mg of **2** as colorless prisms on crystallization from $CHCl_3$. More polar fractions eluted with 8% EtOAc in petroleum ether (200 mg) were further purified by CPTLC (2 mm silica gel GF_{254} disk, solvent: EtOAc-CH_2Cl_2-HOAc; 0.5:99.5:0.1) to yield 80 mg of white plates of **3** on crystallization from $CHCl_3$**.** Fraction eluted with 10% EtOAc in petroleum ether (700 mg) showed two major spots on their TLC. These fractions were subjected to CPTLC, using 2 mm silica gel GF_{254} disks (solvents: 1% Me_2O-$CHCl_3$). This treatment after TLC screening and pooling gave two separate crude fractions each containing one of the two spots. Each crude fraction was separately further purified by CPTLC (1 mm silica gel GF_{254} disk, solvent: 2% EtOAc-CH_2Cl_2) to give **4** (250 mg) as colorless crystals and **5** (60 mg) as needle shape crystals on crystallization from $CHCl_3$. Fractions eluted with 15% EtOAc in petroleum ether (420 mg) yielded 300 mg of **6** as colorless needles upon crystallization from hot EtOAc. Fractions eluted with 20% EtOAc in petroleum ether (123 mg) were subjected to CPTLC (1 mm silica gel GF_{254} disk, solvent: 4% EtOAc in CCl_4) to give 55 mg of oily compound **7**.

4-epi-Abietol (4-epi-abietinol) (**1**). $[\alpha]_D = -137$ (c = 0.15, $CHCl_3$). ^{1}H- and ^{13}C-NMR: Table 1. CIMS: 289 (100, $[M + 1]^+$).
Ferruginol (**2**). M.P. 45-47^0. $[\alpha]_D = + 45$ (c = 0.05, $CHCl_3$). ^{1}H- and ^{13}C-NMR: Table 1. EIMS: 286 (100, $[M]^+$), 271 (89), 229 (28), 215 (18), 201 (55), 189 (81), 175 (87), 159 (25), 149 (36), 141 (20), 128 (20), 115 (20), 91 (14), 55 (14)
Hinokiol (**3**)**.** M.P. 240-242^0. $[\alpha]_D$= +66 (c = 0.11, EtOH); UV (MeOH): 220 and 283. ^{1}H- and ^{13}C-NMR: table 1. EIMS: 302 (90, $[M]^+$), 281 (50), 269 (100), 227 (25), 216 (10), 187 (15), 175 (22), 147 (22), 133 (18), 115 (18), 55 (10).
Sugiol (**4**). M.P. 297-299^0. $[\alpha]_D$ = + 24 (c = 0.015, MeOH). ^{1}H- and ^{13}C-NMR: Table 1. ESIMS: 323 (100, $[M + Na]^+$), 301 (34, $[M + 1]^+$).

Z–Communic acid (**5**). M.P, 120-122^0. $[\alpha]_D$ = + 33 (*c* = 0.07, EtOH). UV (MeOH): 220 and 235. ^{1}H- and ^{13}C-NMR: Table 1. ESIMS: 303 (100, $[M + 1]^+$)

Hinokiol-1-one (**6**). M.P. 222-224^0. $[\alpha]_D$ = + 203 (*c* = 0.11, MeOH). UV (MeOH): 230 and 300. ^{1}H- and ^{13}C-NMR: Table 1. ESIMS: 339 (100, $[M + Na]^+$).

8α–Acetoxyelemol (**7**). $[\alpha]_D$ = + 39.9 (*c* = 0.08, $CHCl_3$). ^{1}H- and ^{13}C-NMR: Table 1. ESIMS: 303 (100, $[M + Na]^+$)

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* (Abdel-Kader *et al*., 2009a; Abdel-Kader *et al*., 2009b; Abdel-Kader *et al*., 2010). The experiments and procedures used in this study were approved by the Ethical Committee of the College of Pharmacy, King Saud University.

Chemicals

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

Table 1: ^{1}H- and ^{13}C-NMR data in ppm of **1- 7** (multiplicity, J in parentheses in Hz)

Pos	1 [a])		2 [a])		3 [b])		4 [c])		5 [a])		6 [c])		7 [a,d])	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	—	39.2	1.61 (m), 1.94 (m)	38.9	1.61 (m), 1.64 (m)	37.3	1.38 (m) 2.06 (brd, 12.5)	37.7	1.07 (dd; 3.5, 13.5), 2.22 (m)	39.2		211.4	5.78 (dd, 11.0, 17.2)	148.3
2	—	18.4	1.47 (m), 1.87 (m)	19.2	1.58 (m), 1.77 (m)	28.3	1.40 (m), 1.56 (m)	18.8	1.55 (m)	19.9	3.21(dd, 10, 12.5)	46.2	4.94 (d; 11.0); 4.92 (d; 17.2)	112.8
3	—	35.5	1.30 (m), 1.47 (m)	41.7	4.41 (d, 4.5)	77.1	1.32 (m), 1.09 (m)	41.3	1.40 (dd, 4, 13.5), 1.88 (m)	37.9	3.89 (dd, 4.5, 12.5)	77.0	4.87 (brs); 4.62 (brs)	110.8
4	—	27.5	—	33.7	—	39.1	—	33.0	—	44.2		39.0	—	146.2
5	—	51.1	1.41 (m)	50.3	1.38 (m)	50.1	1.75 (dd, 3.5, 10)	49.6	1.37 (dd, 2.5, 11.5)	56.2	1.73 (brs)	49.4	1.50 (t; 12.5)	52.2
6	—	24.0	1.21 (m), 2.06 (m)	19.3	1.14 (m), 2.07 (m)	19.2	2.67 (brd, 4) 2.78 (brd, 4)	36.1	1.96 (m)	25.8	1.78–1.87 (m)	19.3	1.77 (m)	29.1
7	7.16 (t; 8.5)	121.1	2.70 (m), 2.78 (m)	29.7	2.65 (m), 2.76 (m)	30.1	—	196.9	1.91 (m), 2.47 (m)	38.5	2.73–2.83 (m)	30.1	1.80 (m)	52.1
8	—	135.5	—	127.2	—	125.0	—	123.5	—	147.9	—	126.2	5.09 (ddd; 4.0, 10.7, 10.7)	72.8
9	—	51.3	—	148.6	—	147.6	—	156.3	1.77 (s)	56.7	—	139.9	2.04 (d; 4.0)	44.8
10	—	37.9		37.5	—	39.5	—	37.8		40.4	—	52.6	—	41.1
11	—	22.8	6.66 (s)	111.0	6.61 (s)	110.9	7.11 (s)	109.8	2.17 (m), 2.41 (m)	22.3	7.76 (s)	116.3	—	72.7
12	—	28.1	—	150.7	—	152.6	—	161.4	5.33 (t;, 6)	131.5	—	152.9	1.21 (s)	24.6
13	—	145.3		131.4	—	131.9	—	133.7	—	131.7	—	133.6	1.22 (s)	26.3
14	5.78 (brs)	122.4	6.85 (s)	126.6	6.69 (s)	126.2	8.37 (s)	126.3	6.81 (dd, 10.5, 17.2)	133.8	7.09 (s)	126.6	1.09 (s)	28.5
15	3.30 (m)	34.8	3.14 (sept)	26.8	3.08 (m)	26.6	3.60 (sept)	27.0	5.20 (d, 17.2) 5.10 (d, 10.5)	113.3	3.68 (sept)	27.2	1.73 (brs)	17.5
16	1.01 (d, 6.5)	20.8	1.26 (d, 7.5)	22.7	1.12 (d, 7.5)	22.9	1.35 (d, 7.5)	22.4	1.79 (brs)	19.7	1.40 (d, 7.0)	22.7	—	—
17	1.03 (d, 6.5)	21.4	1.24 (d, 7.5)	22.5	1.10 (d, 7.5)	23.1	1.34 (d, 7.5)	22.5	4.87 (brs) 4.51 (brs)	107.8	1.38 (d, 7.0)	22.9	—	—
18	0.98 (s)	26.7	0.96 (s)	21.6	0.97 (s)	16.2	0.84 (s)	32.3	0.66 (s)	29.0	1.16 (s)	17.3	—	—
19	3.53 (d, 10.5) 3.91 (d, 10.5)	64.9	0.94 (s)	33.4	0.77 (s)	28.7	0.80 (s)	21.1	—	184.1	1.20 (s)	29.1	—	—
20	0.78 (s)	14.6	1.19 (s)	24.7	1.08 (s)	25.2	1.21 (s)	23.1	1.26 (s)	12.8	1.64 (s)	25.5	—	—

[a]) Collected in $CDCl_3$, [b]) Collected in DMSO-d_6, [c]) Collected in pyridine- d_5, [d]) CH_3CO: δ_H 2.07 (s), δ_C 21.6 (CH_3), 169.7 (CO).

Table 2: Effects of *Juniperus procera* fractions and pure isolates 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)	81.83 ± 11.82		37.18 ± 11.82		362.66 ± 28.61		0.75 ± 0.07	
CCl_4 only	349.33[a] ± 27.06***		307.00[a] ± 39.91***		807.50[a] ± 19.33***		1.99[a] ± 0.13***	
Silymarin + CCl_4	233.83[b] ± 25.59***	33.0	105.88[b] ± 15.63***	65.5	574.83[b] ± 54.89**	28.8	1.05[b] ± 0.08***	47.2
Pet. Ether 250mg + CCl_4	326.50[b] ± 19.34***	6.5	298.66[b] ± 19.35***	2.7	794.00[b] ± 10.58***	1.67	1.52[b] ± 0.14**	23.6
Pet. Ether 500mg + CCl_4	270.50[b] ± 17.03***	22.5	200.50[b] ± 12.32**	34.7	685.50[b] ± 39.95***	15.1	1.25[b] ± 0.09*	37.2
Normal (control)	108.43 ± 18.62		36.78 ± 11.47		394.16 ± 40.40		0.70 ± 0.09	
CCl_4 only	400.66[a] ± 24.05**		337.16[a] ± 25.09**		904.33[a] ± 27.63***		3.12[a] ± 0.22***	
Silymarin + CCl_4	191.66[b] ± 26.33***	52.1	125.28[b] ± 22.22**	62.8	465.33[b] ± 27.84***	48.5	1.15[b] ± 0.16***	63.1
1 + CCl_4	259.66[b] ± 31.48**	35.2	140.50[b] ± 25.56***	58.3	506.33[b] ± 32.69***	44.0	1.25[b] ± 0.13**	59.9
2 + CCl_4	329.66[b] ± 23.71***	17.7	275.50[b] ± 36.34**	18.3	842.50[b] ± 40.98***	6.8	3.19[b] ± 0.31**	29.8
5 + CCl_4	317.33[b] ± 32.98***	20.8	326.16[b] ± 24.87**	–	881.16[b] ± 24.05**	–	3.57[b] ± 0.28**	–
7 + CCl_4	365.00[b] ± 29.33***	8.9	334.83[b] ± 32.12***	–	939.16[b] ± 26.53**	–	3.28[b] ± 0.25**	–
Normal (control)	106.56 ± 14.52		45.17 ± 11.20		429.66 ± 18.73		0.64 ± 0.10	
CCl_4 only	405.83[a] ± 20.18***		343.66[a] ± 30.40**		1026.66[a] ± 51.47***		3.99[a] ± 0.19***	
Silymarin + CCl_4	145.83[b] ± 24.64***	64.1	106.10[b] ± 17.24***	72.0	515.00[b] ± 30.12***	49.8	0.96[b] ± 0.18**	75.9
3 + CCl_4	305.16[b] ± 19.43**	24.8	303.50[b] ± 16.92*	11.7	1011.83[b] ± 37.64**	–	2.90[b] ± 0.37***	27.3
4 + CCl_4	273.33[b] ± 19.34***	32.6	136.48[b] ± 21.57***	60.3	642.50[b] ± 43.96***	37.4	2.52[b] ± 0.28***	36.8
6 + CCl_4	335.66[b] ± 35.15*	17.3	292.00[b] ± 22.37**	15.0	1005.66[b] ± 37.78*	–	3.16[b] ± 0.21*	20.8

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

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 μmole/kg) Groups IV and V were treated with 250 and 500 mg/kg of fractions or 20.7 μmole/kg of pure compounds. Drug treatment was started 5 days prior to CCl_4 administration and continued till the end of the experiment. After 48h, 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).

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.

Table 3: Effect of *Juniperus procera* fractions on the level of nonprotein sulfhydryl (NP-SH) groups in the liver of rat treated with CCl_4.

Treatment (n=6)	Nonprotein sulfhydryls (NP-SH)		
	Mean ± S.E. (μmol/gm wet weight tissue)	% increase with reference to the content of CCl_4-treated liver	% effectiveness for reversing the NP-SH content to normal
Normal (control)	2.34 ± 0.15		
CCl_4 only (toxicity control)	1.02 ± 0.05***		
Silymarin + CCl_4	2.25 ± 0.19***	120.6	91
Pet. Ether 250 mg + CCl_4	1.28 ± 0.12	25.5	6
Pet. Ether 500 mg + CCl_4	1.85 ± 0.16***	81.37	51
$CHCl_3$ 250 mg + CCl_4	1.55 ± 0.16**	51.9	21
$CHCl_3$500 mg + CCl_4	1.84 ± 0.22**	80.4	50
MeOH 250 mg + CCl_4	1.36 ± 0.18	33.3	2
MeOH 500 mg + CCl_4	1.59 ± 0.25**	55.8	25

*) p<0.05; **) p<0.01; ***) p<0.001, as compared with the CCl_4 only group.

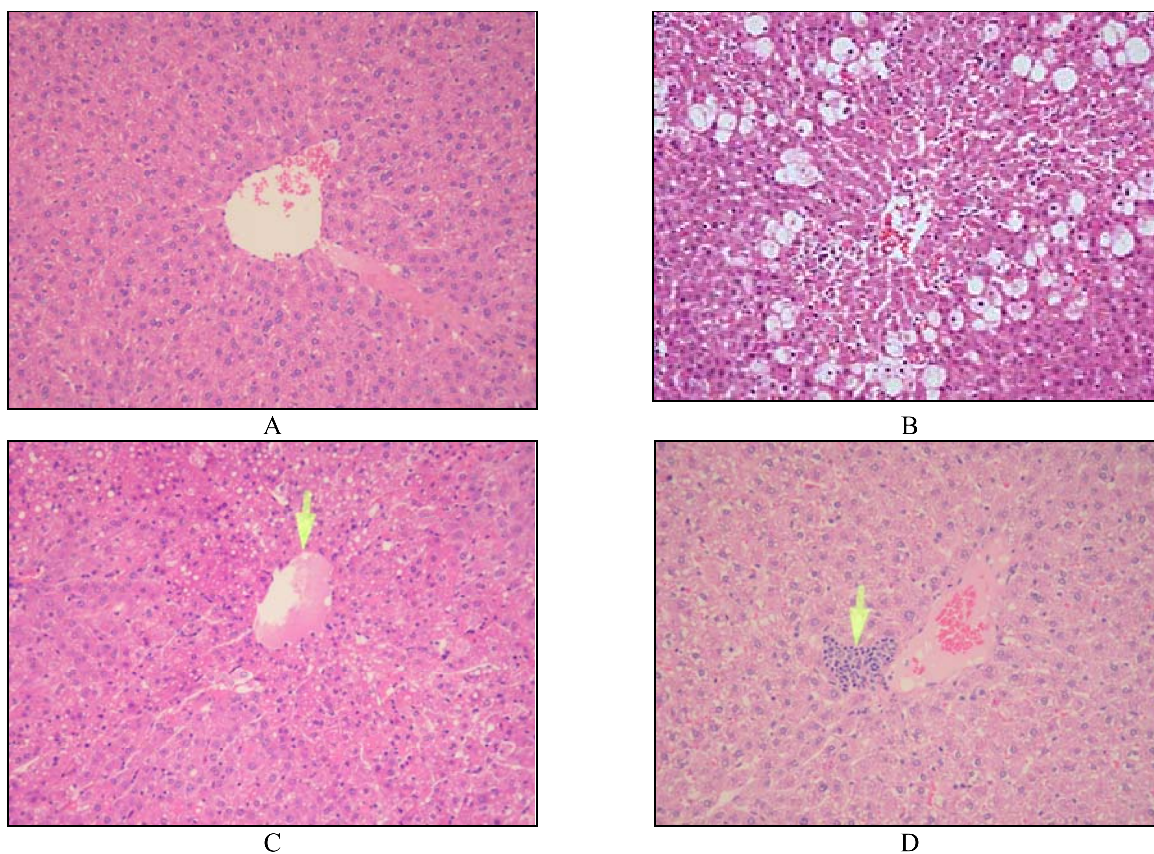

Fig. 1: Histopathological appearance of liver cells; (A) normal cells; (B) liver cells of rats treated with CCl_4 showed centrilobular necrosis and extensive fatty change was observed on the midzonal or entire lobe at 24 h after treatment; (C) liver cells of rats treated with CCl_4 and silymarin showed no necrosis or fatty deposition but had only minimal portal inflammation; (D) liver cells of rats treated with CCl_4 and *Juniperus procera* petroleum ether extract showed mild portal inflammation, focal lobular inflammation, apoptosis and rare foci of inflammation around central veins.

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 **1-7** are presented in table 1. Effect of the isolated compounds on serum biochemical parameters using silymarin as standard is presented in table 2. Effect of *Juniperus procera* fractions on the level of nonprotein sulfhydryl (NP-SH) groups in the liver of rat treated with CCl_4 is presented in table 3. Histopathological changes of liver cells treated with CCl_4, CCl_4 and silymarin, CCl_4 and *Juniperus procera* petroleum ether extract comparing with normal liver cells is presented in fig.1.

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 *J. procera* showed significant activity (Abdel-Kader *et al*., 2009a). The ethanol extract was subjected to liquid–liquid partitioning using petroleum ether, $CHCl_3$ and MeOH. The petroleum ether fraction showed significant hepatoprotactive activity at 500 mg/kg expressed by decreasing the elevated liver enzymes (22.5, 34.7, 15.1 and 37.2 for SGOT, SGOT, ALP and Bilirubin, respectively) (table 2) and restoring of the Nonprotein sulfhydryls (NP-SH) to 51% of the normal level (table 3). Moreover the histopathological study of rat liver treated with 500 mg/kg of the petroleum ether fraction (fig. 1) showed mild portal inflammation, focal lobular inflammation, apoptosis and rare foci of inflammation around central veins indicating a reasonable level of protection (Prophet *et al*., 1994). Consequently, chromatographic purification was conducted for the petroleum ether fraction to identify the active compounds. Repeated chromatography resulted in the isolation of compounds **1-7**. All isolates were tested biologically for their hepatoprotective effect.

The MS (experimental) and ^{13}C-NMR data (table 1) indicated that compounds **1-6** are diterpenes. The first diterpene **1** was isolated as colourless gum with the molecular formula $C_{20}H_{32}O$ as indicated from the MS (*experimental*) and ^{13}C-NMR data (table 1). The 5 degrees of unsaturation indicated the presence of two double bonds in addition to the 3 ring system. The ^{1}H-NMR spectra (table 1) showed only two olefinic proton singlets at δ_H 6.66 and 6.85 and an isopropyl group at δ_H 1.01 (d, *J*= 6.5, CH_3), 1.03 (d, *J*= 6.5, CH_3) and 3.30 (m, CH). In addition to two methyl singlets at δ_H 0.78 and 0.98 the ^{1}H-NMR spectra of **1** showed CH_2OH as two doublets *J*= 10.5 Hz at δ_H 3.53 and 3.91, δ_c 64.9 ppm. 2D-NMR data and comparison with published data enable the identification of **1** as 4-*epi*-abietol (4-*epi*-abietinol) previously isolated from *J. chinensis*, *J. excelsa* and *J. phoenicea* (Tabacik and Poisson, 1971; Mossa *et al*., 1992; Lee *et al*., 1994).

^{1}H-, ^{13}C-NMR, DEPT, COSY, HSQC and HMBC data of compounds **2**, **3**, **4** and **6** all were diagnostic for a typical abietanes with fully unsaturated C-ring bearing an OH and isopropyl groups at C-12, C-13 respectively. Such arrangement leaves carbons 11 and 14 unsubstituted. In addition to isopropyl methyls, **2**, **3**, **4** and **6** showed another three methyl signals. Compound **2** with the molecular formula $C_{20}H_{30}O$ as indicated from the MS (*experimental*) and ^{13}C-NMR data (table 1) showed no further oxygenation was identified as ferruginol (Harrison and Asakawa, 1987). In comparison with **2**, compound **3** ($C_{20}H_{30}O_2$) has one CH_2 replaced by oxygenated CH (δ_H 4.41 brd, *J*=4.5 Hz, δ_C 77.1 ppm) as indicated from ^{13}C-NMR (table 1), DEPT and HMQC experiments. The data of **3** were identical with the literature data reported for hinokiol previously isolated from *J. thurifera* (San Feliciano *et al*., 1988; Zhao *et al*., 1998). Compound **4** with the molecular formula $C_{20}H_{28}O_2$ as indicated from the MS (*experimental*) and ^{13}C-NMR data (table 1) showed a carbonyl resonance at δ_C 196.9 instead of one CH_2 when compared with **2**. HMBC experiment as well as literature data enable the assignment of the carbonyl group to C-7 and consequently identify **4** as sugiol previously isolated from *J. procera* (Ying and Kubo, 1991; Muhammad *et al*, 1996). Compound **6** ($C_{20}H_{28}O_3$) showed one additional oxygen appeared as carbonyl function at δ_C 211.4 (table 1) when compared with **3**. 2D experiments including COSY and HMBC enable the assignments of the carbonyl group to C-1 while the hydroxyl group is located at C-3. The data of **6** was typical with those

reported for 3*β*, 12-dihydroxyabieta-8, 11, 13-triene-1-one (Hinokiol-1-one) (Mossa *et al.*, 1992).

The MS data of **5** showed an $[M+1]^+$ at m/z 303 for the molecular formula $C_{20}H_{30}O_2$. Both ^{1}H- and ^{13}C-NMR data of **5** (table 1) indicated the presence of 3 double bonds two of which have terminal methylene functions at δ_H 5.20 (d, *J*= 17.2 Hz), 5.10 (d, *J*= 10.5; δ_C 113.3 and δ_H 4.87 (brs), 4.51 (brs); δ_C 107.8. In such case ring C must be opened. It was suggested that **5** have a labdane skeleton and the two methylene were assigned for C-15 and C-17. The two oxygens were involved in the formation of carboxylic group at δ_C 184.1 replacing one of the CH_3 groups. The data of **5** were identical with *Z*-communic acid previously isolated from *J. procera* (Muhammad *et al*., 1995).

The ESI-MS of **7** showed $[M+ Na]^+$ at 303 *m/z* consistent with molecular formulae $C_{17}H_{28}O_3$. Both ^{1}H- and ^{13}C-NMR (table 1) indicated the presence of an acetyl group (CH_3: δ_H 2.07, δ_C 21.6; CO: δ_C 169.7), consequently **7** is acetylated sesquiterpene. The 4 degrees of unsaturation and two terminal methylenes (δ_H 4.92 d, *J*= 17.2 Hz, 4.94 d, *J*= 11.0 Hz; δ_C 112.8 and δ_H 4.62 (brs), 4.87 (brs); δ_C 110.8) indicating the presence of only one ring. The ^{1}H-NMR of **7** exhibited three methyl singlets at δ_H 1.09, 1.21 and 1.22. Two of which are part of a hydroxyl isopropyl moiety. A forth methyl broad singlet at δ_H 1.73 coupled to one of the methylenes at δ_H 4.62 (brs), 4.87 (brs). These data are typical of an elemane sesquiterpene skeleton (Raharivelomanana *et al.*, 2005). The obtained data enable the identification of **7** as 8*α*-acetoxyelemol previously reported from *Juniperus* species (Mata *et al*., 1987; San Feliciano *et al*., 1988).

All compounds were tested at a 20.7 μmol/kg dose for hepatoprotective effect using 20.7 μmol/kg of silymarin as standard hepatoprotective drug. Silymarin act as an antioxidant, increasing the intracellular concentration of GSH and increase cellular membrane stability against xenobiotics injury. It also enhances increase protein synthesis and regeneration of liver cells (Gakova *et al*., 1992; Dehmlow *et al*., 1996a; Dehmlow *et al*., 1996b; Saller *et al*., 2007). The use of silymarin resulted in significant decrease in the elevated levels of SGOT, SGPT, ALP and bilirubin ($p<0.001$) (table 1) when given to rats prior to carbon tetrarchloride.

Pretreatment of rats with **2, 5-7** failed to produce any significant decrease the elevated levels of the liver enzymes and bilirubin. Treatment of rats with **3** resulted in a weak but significant decrease in the levels of SGOT ($p<0.01$) and bilirubin ($p<0.05$) (table 2). Rats treated with **1** and **4** prior to CCl_4 administration showed significant decrease in the elevated level of the four measured parameters ($p<0.01$ and $p<0.001$) (table 2). Compound **1** caused highly significant ($p<0.001$) reduction in SGPT, ALP and bilirubin levels comparable to that produced by silymarin, whereas reduction in SGOT level was weaker than that of silymarin. The reductions of SGOT and bilirubin levels produced by **4** were about half the effect produced by silymarin. The reductions in the levels of SGPT and ALP were closer to that produced by silymarin (table 2). The biological results indicated that **1** is the most active hepatoprotective among the tested compounds. Although most of the compounds belong the same class of diterpenes, however, it is interesting to note that **1** is the only member lacking a fully unsatutrated ring C. The exact mechanism of hepatoprotection of **1** and **4** is not known, however, it might be different from that of silymarin due to their different nature. In such case a combination of these compounds may enhance the activity.

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