

Total Antioxidant Capacity of Labdane and Pimarane Diterpenoids of *Juniperus phoenicea* L

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Abstract: Although *Juniperus phoenicea* L is a widely distributed wild tree in the south of Saudi Arabia, but its phytochemical and physiological evaluation is still poor. The chromatographic separation of the CH₂Cl₂/MeOH, 1:1 extract of *J. phoenicea* L. fruits gave β -sitosterol, stigmasterol, four labdane and two pimarane diterpenoids, including new labdane diterpenoid. Additionally, the volatile compounds of the main petroleum ether extract, as well as the steam-volatile constituents were identified by GC/MS. The separated compounds were identified by spectral tools. The new diterpenoid was identified as 5,9,10-triopicupressic acid.

A previous false identification of sandracopimaric acid, from the fruits of *Juniperus phoenicea* L. grown in Egypt, was detected and declared to be revised.

Total antioxidant capacity of extracts was estimated using total antioxidant capacity kit of Biodiagnostic, based on Koracevic et al., for biological fluids, with slight modification to suit extracts. Ascorbic acid was used as a reference antioxidant compound. Reasonable results were obtained by applying the modified method on petroleum ether extract, methylene chloride extract and ethyl acetate extract of *Juniperus phoenicea* L.

Keywords: *Juniperus phoenicea* L.; labdane diterpenoids; pimarane diterpenoids; 5,9,10-triopicupressic acid; total antioxidant capacity.

1. INTRODUCTION

Different extracts of *Juniperus phoenicea* L. were found to be highly physiologically active. Oils of berries and leaves showed very high cytotoxic activities against many cell lines. Additionally, the antimicrobial activity and minimum inhibitory concentration of leaf and berry oils showed high activity against most of the tested strains [1, 2]. The essential oils obtained by supercritical fluid extraction at different pressures were active as antiviral, antiproliferative and antimicrobial [3]. The antioxidant activity by using DPPH and ABTS assays of *J. phoenicea* was determined [5,4].

J. phoenicea leaf extracts show a remarkable effect in enhancing liver and kidney functions and may thus be of therapeutic potential in treatment hepatotoxicity and nephrotoxicity [6].

The aqueous extract of *J. phoenicea* caused a dose dependent protection of rats against castor oil induced diarrhoea and reduced castor oil induced enteropooling. The extract also caused a dose

dependent decrease in intestinal transient and showed a significant dose dependent relaxant effect on rat ileal smooth muscle [7].

Many phytochemical investigations of *J. phoenicea* indicated the separation of essential oils [1, 3, 4, 8], labdane and pimarane diterpenoids, with remarkable unsaturation [2, 9], furanone glucosides [10, 11], and phenyl propanoids [9, 12, 13]. Although many chemical research publications were found on *J. phoenicea*, from diverse countries; Tunisia [4, 5, 8], Morocco [9], Egypt [1], Italy [3], and France [10-13], but only one research publication was found on *J. phoenicea* from Saudi Arabia [14], which has reported the isolation of some sesquiterpenes and diterpenes. In this research article, we aimed to phytochemically and physiologically reinvestigate *J. phoenicea*. We could report the isolation of four labdane and two pimarane diterpenoids, including a new labdane diterpenoid, in addition to the identification of the volatile compounds of petroleum ether extract by GC/MS. Additionally,

the total antioxidant capacity of extracts was estimated using total antioxidant capacity kit of Bio-diagnostic, based on Koracevic et al. [15], for biological fluids, with slight modification to suit extracts.

2. EXPERIMENTAL

2.1 General

¹H NMR spectra were taken on Bruker 600 MHz at King Fahd Center for Medicinal Research, KAU, Jeddah, Saudi Arabia; GC/MS analysis was performed on A Perkin Elmer Clarus 500 GC-MS (Perkin Elmer, Shelton, CT, USA). The software controller/integrator was TurboMass version 5.4.2.1617. An Elite-1 GC capillary column, Crossbond® 100% dimethyl polysiloxane (30-meter × 0.25 mm ID × 0.25 µm df, Perkin Elmer) was used. The carrier gas was helium (purity 99.9999%) and flow rate was 0.9 mL/min. Source (EI+): source temperature, 270°C. GC line temperature was 210°C. Electron energy was 70 eV, and trap-emission was 100 v. The oven was programmed as follows: initial temperature was 100°C (hold 2 min) to 250°C (rate °15C/min, hold 4.0 min). The injector temperature was 260°C. The MS scan was from 40 to 500 m/z (500 scan/sec). The injection volume was 1.0 µL, and the Split ratio was .40:1. Samples were acquired by applying the total ion chromatogram (TIC) scan modes. An average TIC scan of each peak, at definite retention times, was saved using the TurboMass software to characterize the closed peaks obtained from the MS chromatogram of the analyzed samples. The screw-capped (PTFE/silicon) 1-ml autosampler vials (12 mm × 32 mm) used for recovery were purchased from Waters (Milford, MA, USA).

2.2. The plant material

Juniperus phoenicea L. fruits were collected from Nemas City, near Abha, KSA, in August 2012 by the third Author, and identified by Faraj Elghamdi, Assistant Professor of Plant Taxonomy, Botany Department, Faculty of Science, KAU, Saudi Arabia. A voucher specimen was deposited under the number HKAU 1791 in the Herbarium of the Biology Department, Faculty of Science, King Abdulaziz University.

2.3 Processing of the plant material

The dried and grinded fruits, 377 g, were extracted by soaking at room temperature in CH₂Cl₂/MeOH, 1:1, 1800 mL. The CH₂Cl₂/MeOH extract was obtained by filtration and washing the plant material with CH₂Cl₂/MeOH mixture, 240 ml. The collected filtrate was left in fume cupboard with suction for overnight to be concentrated to its ¼ volume. Dilution with water, 200 ml, and exhaustive solvent extraction, using a separatory funnel, by petroleum ether (40-60°C), followed by CH₂Cl₂, then AcOEt and finally n-BuOH gave the crude extracts Jp1, 13.6 g, Jp2, 1.6 g, Jp3, 17.4 g and Jp4, 7.03 g, respectively.

2.4 Separation of compounds

The pet. ether extract, Jp1, 5.6 g of which, was steam distilled. The distillate was extracted with diethyl ether and the ether extract was dried over anhydrous Na₂SO₄ to give the steam volatile fraction, Jp1a, 220 mg, which was analysed by GC/MS. A sample from the pet. ether extract, Jp1 was analysed by GC/MS. The steam nonvolatile material left in the distillation flask was extracted with diethyl ether and the ether extract was dried over anhydrous Na₂SO₄ to give the steam non-volatile fraction, Jp1b, 2.34 g.

The non-volatile fraction, Jp1b, was separated on silica gel CC, eluted with pet. ether (40-60°C) then pet. ether containing increased amounts of diethyl ether. The fractions 4 and 5 (out of 5), eluted by 2% ether in pet. ether and 1, 2 and 3 (out of 6), eluted by 5% ether in pet. ether, 267 mg, contained a mixture of 1 and 4 (4:1). The fraction 1 (out of 5), eluted by 10% ether in pet. ether, 14 mg, contained a mixture of 1, 5 and 6 (6:3:1). The fraction 4 (out of 11), eluted by 20% ether in pet. ether, 70 mg, contained 3. The fraction 10 (out of 11), eluted by 20% ether in pet. ether, 10 mg, contained β-sitosterol, and stigmasterol, (1:1).

The CH₂Cl₂ extract, Jp2, contained main component 2, which was purified by TLC (silica gel, diethyl ether/petroleum ether 1:50, Rf 0.72). To get rid of Na₂SO₄ crystals from AcOEt extract, as well as to fractionate it, it was loaded on 100 g silica gel (0.063-0.200 mm)/alumina, 1:2, in an addition funnel, and eluted by CHCl₃/pet. ether, 3:1, then CHCl₃ and finally 5% MeOH in CHCl₃ to give two fractions Jp31 and Jp32, 110 and 59 mg, respectively. The fraction Jp31 gave by TLC (silica gel, diethyl ether/petroleum ether 1:50) compound 2 (110 mg, Rf 0.5). The fraction Jp32 contained fats (saturated fatty acids). The n-BuOH extract, Jp4, contained sugars and glycolipids.

2.5 The GC/MS analysis

The pet. ether extract, Jp1, gave by GC/MS α-thujene (Rt 3.05 min, 0.5%), α-pinene (Rt 3.14 min, 4.8%), sabinene (Rt 3.49 min, 1.4%), β-pinene (Rt 3.55 min, 1.2%), 3-carene (Rt 3.90 min, 1.9%), bornyl acetate (Rt 6.64 min, 1.0%), 4-terpenyl acetate (Rt 7.13 min, 1.2%), α-terpenyl acetate (Rt 7.22 min, 3.6%), α-cubebene (Rt 7.38 min, 1.0%), β-cedrene (Rt 7.92 min, 1.7%), cedrol (Rt 9.45 min, 3.3%), geranyl geraniol (Rt 12.55min, 4.3%), a mixture of 1, 2, 3 (Rt 13.72 min, 10.4%), a mixture of 4, 5 (Rt 14.48min, 28.4%), and 6 (Rt 14.85 min, 35.5%). The steam-volatile fraction of pet. ether extract, Jp1a, gave by GC/MS trans citral (Rt 5.55 min, 0.9%), terpinen-4-ol (Rt 5.59 min, 0.9%), α-terpineol (Rt 5.65 min, 1.6%), bornyl acetate (Rt 6.64 min, 1.8%), α-terpenyl acetate (Rt 7.13 min, 0.5%), β-cedrene (Rt 7.92 min, 1.4%), cedrol (Rt 9.45 min, 4.5%), α-cadinol (Rt 9.79 min, 1.8%), geranyl linalool (Rt 12.19 min, 4.1%), Z,E-E-geranyl geraniol (Rt 12.30 min, 3.2%), E,E-E-geranyl geraniol (Rt 12.55 min, 18.2%), linoleic acid (Rt 13.21 min, 2.3%), linolenic acid (Rt 13.29

min, 2.3%), stearic acid (Rt 13.70 min, 11.4%) unknown fatty acid (Rt 13.99 min, 6.8%), unknown fatty acid (Rt 14.71 min, 7.7%), unknown fatty acid (Rt 15.61 min, 12.7%), unknown fatty acid (Rt 16.70 min, 14.5%).

2.6 5,9,10-triepi-cupressic acid, 3:

Amorphous material, ¹H NMR: δ 5.90, 1H, dd (17.4, 10.8), H-14, 5.21, 1H, dd (17.4, 1.2); 5.06, 1H, dd (10.8, 1.2), H-15, H-15', 1.27, 3H, br s, H-16, 4.83, 1H, br s; 4.49, 1H, br s, H-17, H-17', 1.23, 3H, s, H-18, 0.59, 3H, s, H-20; ¹³C NMR: δ 39.35, C-1, 20.10, C-2, 38.20, C-3, 44.39, C-4, 56.57, C-5, 26.29, C-6, 38.94, C-7, 148.30, C-8, 56.72, C-9, 40.89, C-10, 18.13, C-11, 41.58, C-12, 73.96, C-13, 145.23, C-14, 111.95, C-15, 29.23, C-16, 106.78, C-17, 28.30, C-18, 183.60, C-19, 12.95, C-20.

2.7 Total Antioxidant Capacity

Total antioxidant capacity of extracts was estimated using total antioxidant capacity kit of Bio-diagnostic [16], based on Koracevic et al. [15], for biological fluids, with slight modification to suit extracts.

2.8 Reagents

Substrate (H₂O₂, R1 from Bio-diagnostic)

Chromogen (3,5-dichloro-2-hydroxybenzene sulphionate, R2 from Bio-diagnostic)

Enzyme-Buffer (superoxide dismutase - Sodium phosphate buffer: 100 mmol/litre, pH 7.4, R3 from Bio-diagnostic)

2.9 Procedure

R1 was diluted with distilled water (1:1000) immediately before use (10 µl R1 was mixed with 10 ml distilled water). Equal volumes of R2 and R3 were mixed immediately before use (working reagent). The sample, 50 mg, was dissolved in methanol, 10 ml (pet. ether extract, CH₂Cl₂ extract, AcOEt extract). Ascorbic acid, as a reference antioxidant compound, 50 mg, was dissolved in methanol, 10 ml.

A blank experiment was conducted by mixing 20 µl distilled water and 0.5 ml diluted R1, standing 10 minutes, adding 0.5 ml of working reagent, standing 5 minutes, dilution with 1 ml distilled water and reading the absorption at 512 nm.

Ascorbic acid solution experiments were conducted by mixing 20, 10, 5 µl, separately, with 0.5 ml diluted R1, standing 10 minutes, adding 0.5 ml of working reagent, standing 5 minutes, dilution with 1 ml distilled water and reading the absorption at 512 nm.

Sample experiments were conducted by mixing 40, 20, 10, µl solution, separately, with 0.5ml diluted R1, standing 10 minutes, adding 0.5 ml of working reagent, standing 5 minutes, dilution with 1 ml distilled water and reading the absorption at 512 nm.

2.10 Calculation

$$\% \text{Inhibition} = [(AB - AS) / AB] \times 100$$

where: AB is the absorbance of the blank sample, and AS is the absorbance of tested extract.

3. RESULTS AND DISCUSSIONS

The chromatographic separation of the extract of *J. phoenicea* L. fruits gave β-sitosterol, stigmasterol, four labdane 1-4 and two pimarane diterpenoids, 5 and 6, including a new labdane diterpenoid, 3. Additionally, the volatile compounds of the main petroleum ether extract, as well as the steam-volatile constituents were identified by GC/MS.

Compound 1 as E-communic acid was isolated previously from *Juniperus taxifolia* by Muto et al. [17]. Compound 2 was identified as 13-epi-torulosal, isolated previously from *Juniperus rigida* by Woo et al. [18]. Compound 4 was identified as 15-norlabda_{12,(17)}8-E-diene 14-carboxaldehyde 19-carboxylic acid, isolated previously from *Platycladus orientalis* by Kuo and Chen [19]. Compound 6 was identified as isopimara-7-en-18-oic acid, isolated previously from *Pinus armandii* heartwood [20]. Compound 5 as sandracopimaric acid was identified by spectral data and confirmed by comparing ¹H NMR data with the corresponding compound isolated previously from *Juniperus taxifolia* [17] and with the 4-epimer, isolated previously from *Illicium jiadifengpi* [21]. In the 4-epimer, the methyl group (C-18) absorbs at 1.26 ppm instead of 1.21 for the methyl group (C-19). Sandracopimaric acid 5 and 4-episandracopimaric acid differ also in the chemical shift value of the methyl group (C-20), where in the former it absorbs at 0.84 ppm, versus 0.73 in case of the 4-epimer [21]. El-Sawi and Motawe [2] reported a diterpenoid as sandracopimaric acid from the fruits of *Juniperus phoenicea* L. grown in Egypt. They reported H-14, H-15, H-16 and H-20 at unjustified δ values of 6.85, 3.59, 4.20 and 1.59 ppm, respectively. The identification of this compound is doubtful and should be revised. Compound 3 was separated from fraction 4 of the pet. ether nonvolatile fraction, Jp1b, eluted by 20% ether in pet. ether. It gave M⁺ at m/z 320, in agreement with C₂₀H₃₂O₃. The structure of 3 was elucidated by ¹H NMR. The ¹H NMR spectrum of 3 (Table 1) was almost similar to that of 2, with the aldehydic proton signal no longer present and the methyl signal of C-18 shifted down-field from 0.97 to 1.23 ppm in 3, in agreement with 19-carboxylic acid. The good co-incidence between the signals of the side chain (H-14, H-15, H-15', H-16) in both compounds (2 and 3) indicated the same constitution of the side-chain at C-9 (Table 1). Comparing the ¹H NMR data of 3 with those of 13-epi-cupressic acid [18], 4,13-diepi-cupressic acid [22] and 4-epi-cupressic acid [23] indicated that H-16 in 13-epi-cupressic acid [18] shifted up-field by 0.09 ppm. Thus, the opposite configuration at C-13 is more probable.

Comparing the ¹³C NMR data of 3 with those of 13-epi-cupressic acid [18] (Table 2) indicated obvious shifts in δ_c values of C-4, C-5, C-6 (- 0.29, - 0.17, +0.11, respectively), as well as those of C-10, C-20 (- 0.49, - 0.75, respectively) and those of C-13, C-16

(- 0.96, + 0.47, respectively). This indicated more probably the opposite configuration at C-5, C-10 and C-13. Comparing the ^{13}C NMR data with those of 4-epi-cupressic acid [23] (Table 2) indicated obvious shifts in δC values of C-5, C-6 (- 6.17, + 1.21, respectively), as well as those of C-10, C-20 (- 1.09, + 2.25, respectively) and those of C-3, C-4, C18, C19 (+ 0.60, + 3.51, - 10.70, - 2.30, respectively). This indicated more probably the opposite configuration at C-5, C-10 and C-4.

The NOESY experiment showed clear noe effects between H-20, H-17, H-17', H-16, H-18, H-11, H 2α and H7 α , as well as between H16, H18, H-14, H-15, H-17, H-7 α and H-11. Thus 3 was identified as 5,9,10-triepicupressic acid.

The total antioxidant capacity considers the cumulative effect of all antioxidants present. Total antioxidant capacity of extracts was estimated using total antioxidant capacity kit of Bio-diagnostic [16], based on Koracevic et al. [15], for biological fluids, with slight modification to suit extracts. Ascorbic acid was used as a reference antioxidant compound. Reasonable results were obtained by applying the modified method on petroleum ether extract, methylene chloride extract and ethyl acetate extract of *J. phoenicea* L.

Table 3 indicated the volumes taken from petroleum ether extract, methylene chloride extract and ethyl acetate extract of *J. phoenicea* L. and the corresponding absorptions at 512 nm. It is clear that

as the quantity of extract increased, the (OH) inhibition% increased and the absorption at 512 nm decreased. Table 4 and figure 2 indicated the (OH) free radical inhibition % of ascorbic acid at concentrations 100, 50, 25 $\mu\text{g/ml}$. Table 5 and figure 3 indicated the (OH) free radical inhibition % of petroleum ether, methylene chloride and ethyl acetate extracts of *J. phoenicea* L. at concentrations 200, 100, 50 $\mu\text{g/ml}$. A remarkable increase of (OH) inhibition % happened when the concentration of the extract has been risen from 50 to 100 $\mu\text{g/ml}$. After that, the duplication of the extract concentration from 100 to 200 $\mu\text{g/ml}$ has a little effect on the (OH) inhibition %.

If we take into account that the molar mass of ascorbic acid is 176 g/mol, while average molar mass of *J. phoenicea* diterpenoids is about 310 g/mol; and also that extracts are not pure diterpenoids, we will realize that the *J. phoenicea* diterpenoids have remarkable total antioxidant capacity. The total antioxidant capacity of *J. phoenicea* diterpenoids could be attributed to the large unsaturation that accommodates the free radicals.

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Table 1: ^1H NMR data (600 MHz, CDCl_3 , δ value, multiplicity (J value in Hz)) of 5,9,10-triepi-cupressic acid (3), compared with those of 2, 13-epi-cupressic acid [5], 4,13-diepi-cupressic acid [10] and 4-epi-cupressic acid [11].

H-atom number	^1H NMR data of compound 3	Compound 2	13-Epi-cupressic acid	4,13-Diepi-cupressic acid	4-Epi-cupressic acid
14	5.90, 1H, dd (17.4, 10.8)	5.90, 1H, dd (17.2, 10.8)	5.88, 1H, dd (17.0, 10.5)	5.81, 1H, dd (17.2, 10.8)	5.92, 1H, dd (17, 10)
15, 15'	5.21, 1H, dd (17.4, 1.2); 5.06, 1H, dd(10.8, 1.2)	5.21, 1H, br d(17.2); 5.06, 1H, br d(10.8)	5.18, 1H, dd (18.0, 2); 5.02, 1H, dd(10.5, 2)	5.13, 1H, d(17.2); 4.98, 1H, d(10.8)	5.20, 1H, br d(17); 5.07, 1H, br d(10)
16	1.27, 3H, br s	1.27, 3H, s	1.18, 3H, s	1.22, 3H, br s	1.26, 3H, s
17, 17'	4.83, 1H, br s ; 4.49, 1H, br s	4.87, 1H, br s; 4.53, 1H, br s	4.82, 1H, s ; 4.51, 1H, s	4.79, 1H, s ; 4.48, 1H, s	4.84, 1H, br s; 4.52, 1H, br s
18	1.23, 3H, s	0.97, 3H, s	1.22, 3H, s	-	-
19	-	9.74, 1H, s	-	1.18, 3H, s	1.13, 3H, s
20	0.59, 3H, s	0.56, 3H, s	0.61, 3H, s	0.54, 3H, s	0.70, 3H, s

Table 2: ^{13}C NMR data (150.9 MHz, CDCl_3 , δ value, multiplicity) of 5,9,10-triepi-cupressic acid (3), compared with those of 13-epi-cupressic acid [5] and 4-epi-cupressic acid [11].

C-atom number	^{13}C NMR data of compound 3	13-Epi-cupressic acid	4-Epi-cupressic acid
1	39.35	39.3	38.0
2	20.10	20.0	19.3

3	38.20	38.3	38.8
4	44.39	44.1	47.9
5	56.57	56.4	50.4
6	26.29	26.4	27.5
7	38.94	38.7	38.6
8	148.30	148.6	148.9
9	56.72	56.8	58.0
10	40.89	40.5	39.8
11	18.13	18.0	18.5

12	41.58	41.5	42.5
13	73.96	73.0	73.1
14	145.23	145.1	147.3
15	111.95	110.8	111.5
16	29.23	26.7	29.1
17	106.78	105.7	107.6
18	28.30	28.4	181.3
19	183.60	183.5	17.6
20	12.95	12.2	15.2

Table 3: The volumes taken from petroleum ether, methylene chloride and ethyl acetate extracts of *J. phoenicea* L. and the corresponding absorptions at 512 nm.

Sample	Volume taken in μl	Absorption at 512 nm	Inhibition %
Blank		0.292	0
PE extract	40	0.089	69.5
	20	0.095	67.5
	10	0.218	25.3
CH ₂ Cl ₂ extract	40	0.077	73.6
	20	0.099	66.1
	10	0.273	6.5
AcOEt extract	40	0.134	54.3
	20	0.156	46.6
	10	0.217	25.5

Table 4: The ($\cdot\text{OH}$) free radical inhibition % of ascorbic acid at concentrations 100, 50, 25 $\mu\text{g/ml}$.

conc. $\mu\text{g/ml}$	ascorbic acid
100	97.86
50	96.41
25	95.72

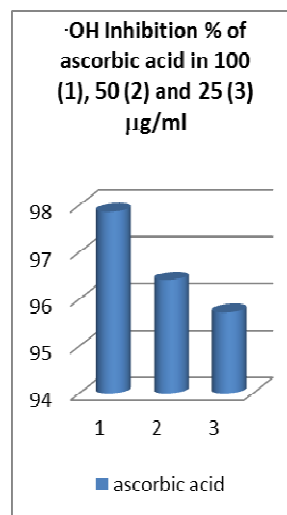
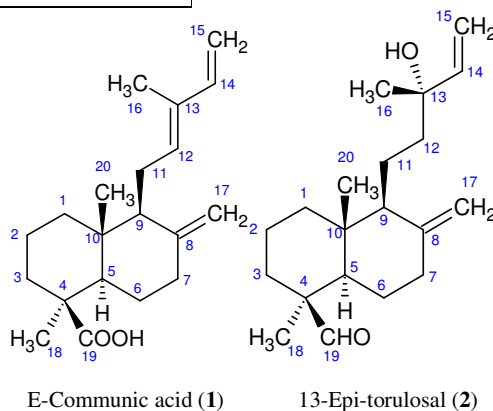


Fig 2: The ($\cdot\text{OH}$) free radical inhibition % of ascorbic acid at concentrations 100, 50, 25 $\mu\text{g/ml}$.

Table 5: The ($\cdot\text{OH}$) free radical inhibition % of petroleum ether, methylene chloride and ethyl acetate extracts of *J. phoenicea* L. at concentrations 200, 100, 50 $\mu\text{g/ml}$.

Conc. in $\mu\text{g/ml}$	PE extract	CH ₂ Cl ₂ extract	AcOEt extract
200	69.5	73.6	54.3
100	67.5	66.1	46.6
50	25.3	6.5	25.5



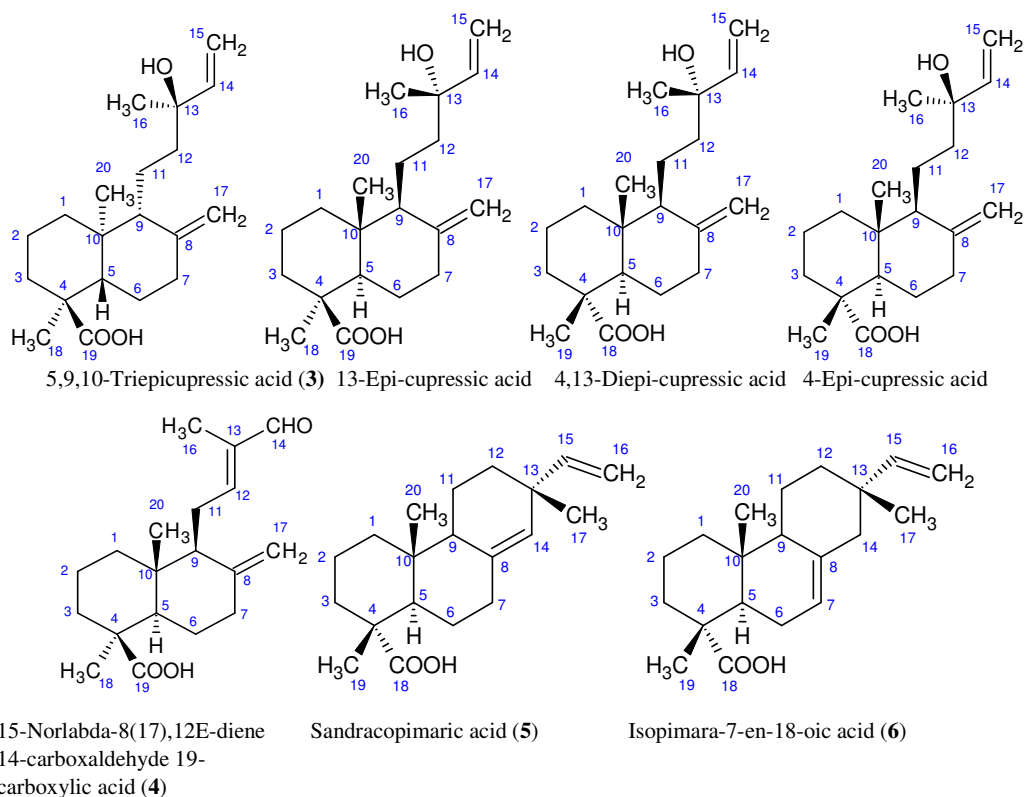


Fig. 1: Isolated diterpenoids from *Juniperus phoenicea* L. fruits

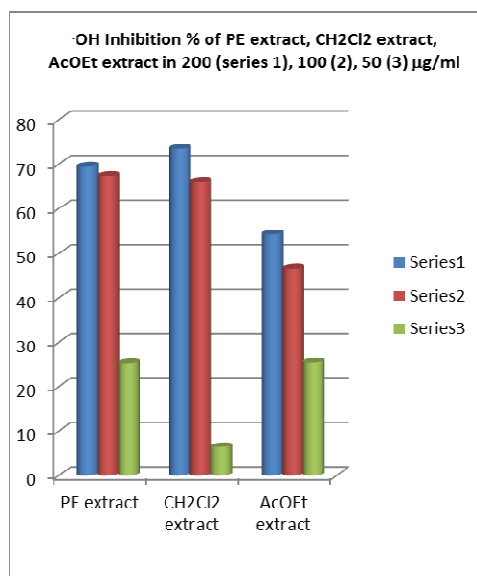


Fig 3: The (.OH) free radical inhibition % of petroleum ether extract, methylene chloride extract and ethyl acetate extract of *J. phoenicea* L. at concentrations 200, 100, 50 µg/ml.

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