SUPPLEMENTARY MATERIAL

Antioxidant activity and chemical composition of *Juniperus excelsa* ssp. *polycarpus* wood extracts

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Extracts from the wood of *Juniperus excelsa* ssp. *polycarpus* were analyzed for their antioxidant activity using the DPPH method and compared with ascorbic acid (AA) and butylated hydroxytoluene (BHT). The most active extracts were analyzed for their chemical composition using gas chromatography-mass spectrometry. Acetone extract was found to be the moderate active as an antioxidant agent at 58.38%, which was lower than the value of vitamin C (98.56%) at the concentration of 14.20 mg/mL. The major components identified in the acetone extract as trimethylsilyl (TMS) derivatives were pimaric acid TMS (24.56%), followed by α -D-glucopyranoside,1,3,4,6-tetrakis-O-(TMS)- β -D-fructofuranosyl-2,3,4,6-tetrakis-O-(TMS) (21.39%), trifluoromethyl-bis-(TMS)methyl ketone (9.32%), and cedrol (0.72%). The dissolved water:methanol (1:1 v/v) partitioned from acetone extract afforded 12 fractions; among them, the F9 fraction was found to have good antioxidant activity (88.49%) at the concentration of 14.20 mg/mL. The major compounds identified in F9 fraction were α -D-glucopyranoside,1,3,4,6-tetrakis-O-(TMS) (20.22%), and trifluoromethyl-bis-(TMS)methyl ketone (5.10%).

Keywords: *Juniperus excelsa* ssp. *polycarpus* wood; Acetone extract; Fraction; GC/MS; Antioxidant activity

3. Experimental

3.1. Plant materials

Fresh stem of *J. excelsa* ssp. *polycarpus* was collected from Sira, Chalous road, and Alborz, Iran in February of 2015. The plant material was identified by Khosrow Ashrafi, Assistant Professor, Department of Wood Science and Paper Technology, Karaj Branch, Islamic Azad University, Karaj, Iran, and a voucher specimen number 1893 was deposited in the Herbarium College of Agricultural and Natural Resources, Karaj Branch, Islamic Azad University, Karaj, Iran. The inner bark was separated from the stems and air-dried to achieve 8.0% moisture content. Because, these species are not widely available, and their utilization is limited, therefore we prepared the wood samples with smaller diameters and the age is approximately 10 years.

3.2. Extraction and fractionation

The wood of stem was cut into small pieces and chopped using a laboratory electrical rotary mill to obtain wood flour. The flour size was between 40 and 60 mesh. Approximately 100 g of this flour was placed into

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the 10 extraction thimbles, and then 10 independent extracted using pure acetone (400 mL in a 500-mL balloon) and a Soxhlet-type apparatus for 8 h. The combined extract was concentrated using a Heidolph Laborota 4001 rotary-evaporator apparatus (at 40 °C to reach total solvent evaporation) for approximately 15 min. Then, the extracts were collected, dried over anhydrous sodium sulphate, and stored at 4 °C until further analysis. The solid extractive weight was 3.50 g. Subsequently, 2.87 g of the solid extractives were dissolved in water:methanol (1:1 v/v) (in triplicate, separately) where the marc or the residue was discarded then the supernatant was poured into a separatory funnel, followed by the addition 50 mL of *n*-hexane.

The mixture was shaken by hand for 10 min. Half a gram of the water:methanol aqueous supernatant extractive was used for column chromatography with silica gel, Merck KGaA 64271 Darmstadt, Germany. The 12 fractions were labeled F1 to F12 (Figure S2) and the solid weight of fractions after solvent evaporation are presented in Table S5.

The 3 × 10 mL eluent volume was used in the chromatographic separation for each solvent. The solid weights of all the fractions after solvent evaporation are presented in Table S5. We would like to notice that the amount of extracted materials of F7, F8, F11, and F12 is very little. Thus, we didn't prepare the concentrations of these fractions.

3.3. Free radical scavenging activity by DPPH assay

The free radical scavenging activities of the acetone and water:methanol extracts, as well as the fractions from F1 to F12 of the stem inner bark powders, were determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Salem et al. 2015). For this method, a stock solution was prepared by dissolving 2.4 mg of DPPH free radical in 100 mL of methanol. The stock solution was stored at 20 °C. The working solution was prepared by diluting the DPPH stock solution with methanol. Then, 1250 µL of the working solution was combined with 250 µL of the methanol extract from the medicinal plant (1 mg/mL). Serial dilutions were carried out with the stock solutions (1 mg/mL) of tested extract to obtain concentrations of 0.44, 0.89, 1.78, 3.55, 7.10, and 14.20 mg/mL. The experiment was performed in triplicates, and the average absorbance was recorded for each concentration. The reaction mixture was mixed for 10s and left to stand at room temperature in a dark place for 30 min. The absorbance was measured at 517 nm, using a UV scanning spectrophotometer. Ascorbic acid (AA) and butylated hydroxytoluene (BHT) were used as the reference standards and were dissolved in methanol to make the stock solutions with the same concentration (1 mg/mL). The control samples were prepared with the same volume of solution, without test compounds and the referenced standards. Pure methanol (Sigma-Aldrich, Germany) was used as a blank. The DPPH free radical scavenging activity (%) was calculated using the following equation:

$$\text{Inhibition} = 100(A_c - A_s)/A_c$$

where the percentage inhibition value was calculated from the absorbance of the control, (A_c), and of the sample (A_s). The controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses.

3.4. Analysis of extracts

Gas chromatography-mass spectrometry (GC/MS) analysis of the acetone and F11 extracts were performed using split mode (50:1) injection. One microlitre of the silylated extract, 300 μL N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), 30 μL 1% trimethylchlorosilane (TMCS) reagent, and approximately 60 μL of pyridine were run on a HP 7890 (Hewlett Packard, USA) gas chromatograph fitted with a cross-linked Polysiloxane HP-5 capillary column (dimensions: 30 m x 0.25 mm, 0.25 μm coating thickness) and coupled with a model 5975C mass detector. The GC/MS operation conditions were as follows: injector temperature 250 $^{\circ}\text{C}$; transfer line 280 $^{\circ}\text{C}$; oven temperature program 50 to 250 $^{\circ}\text{C}$ (5 $^{\circ}\text{C}/\text{min}$); carrier gas: He at 1.4 mL/min; mass spectra: electron impact (EI+) mode 70 eV with a mass range of 40 to 450 m/z; and ion source temperature at 250 $^{\circ}\text{C}$. Individual components were identified using Wiley 275 L and NIST05 mass database matching and by comparing the retention times and mass spectra of constituents with published data (Julian and Konig 1988; Adams 1995, 2001).

3.5. Statistical analysis

Data of antioxidant activity were statistically analyzed using the SPSS program using factorial experimental design with two factors (treatments and concentrations).

Table S1. Statistical analysis of the effect of treatments and concentrations on the antioxidant activity of wood extractives of *J. excelsa* ssp. *Polycarpus*.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	149538.428	77	1942.058	45.422	0.000
Intercept	437127.080	1	437127.080	10223.820	0.000
Treatment	86517.956	12	7209.830	168.628	0.000
Concentration	48639.825	5	9727.965	227.524	0.000
Treatment * Concentration	14380.647	60	239.677	5.606	0.000
Error	6669.897	156	42.756		
Total	593335.405	234			
Corrected Total	156208.325	233			

Table S2. Mean $\pm$ SD of the antioxidant activity (%) as affected by different treatments of wood extractives of *J. excelsa* ssp. *polycarpus* and comparing them with BHT and Vitamin C.

Treatment	Concentration (mg/mL)					
	0.44	0.89	1.78	3.55	7.10	14.20
F1	0.34 $\pm$ 4.57	0.42 $\pm$ 10.24	1.35 $\pm$ 9.99	2.37 $\pm$ 9.14	3.55 $\pm$ 8.46	4.77 $\pm$ 13.20
F2	0.34 $\pm$ 4.74	0.51 $\pm$ 4.74	3.47 $\pm$ 0.09	4.06 $\pm$ 4.74	18.02 $\pm$ 9.73	36.89 $\pm$ 1.36
F3	14.47 $\pm$ 6.01	29.02 $\pm$ 1.95	49.49 $\pm$ 7.87	54.15 $\pm$ 1.70	65.57 $\pm$ 11.76	75.30 $\pm$ 17.77
F4	32.99 $\pm$ 0.17	43.40 $\pm$ 1.44	58.04 $\pm$ 2.71	64.81 $\pm$ 7.11	67.09 $\pm$ 6.68	68.19 $\pm$ 14.04
F5	9.90 $\pm$ 0.42	17.51 $\pm$ 4.66	37.14 $\pm$ 2.45	49.58 $\pm$ 5.93	57.78 $\pm$ 4.99	60.15 $\pm$ 8.04
F6	12.86 $\pm$ 4.23	34.77 $\pm$ 7.02	48.82 $\pm$ 0.09	49.24 $\pm$ 2.20	50.68 $\pm$ 0.09	59.73 $\pm$ 5.08
F9	22.59 $\pm$ 13.79	27.75 $\pm$ 9.48	59.81 $\pm$ 1.95	76.99 $\pm$ 0.34	85.45 $\pm$ 1.52	88.49 $\pm$ 0.17
F10	9.14 $\pm$ 4.40	12.61 $\pm$ 14.98	50.93 $\pm$ 11.84	60.49 $\pm$ 4.15	62.61 $\pm$ 11.00	65.74 $\pm$ 8.72
Acetone	22.25 $\pm$ 4.99	24.20 $\pm$ 17.26	41.79 $\pm$ 0.51	44.59 $\pm$ 1.44	53.38 $\pm$ 1.61	53.98 $\pm$ 1.53
water:methanol	11.42 $\pm$ 2.45	19.04 $\pm$ 3.98	34.26 $\pm$ 0.09	54.15 $\pm$ 8.80	58.29 $\pm$ 3.98	58.38 $\pm$ 4.23
n-Hexane	28.09 $\pm$ 1.52	32.06 $\pm$ 12.10	36.72 $\pm$ 0.68	43.74 $\pm$ 2.28	50.00 $\pm$ 2.12	52.28 $\pm$ 0.17

BHT	57.95±0.26	58.38±0.00	61.00±0.09	62.86±0.09	73.52±0.09	73.86±0.09
Vitamin C	60.66±0.08	61.76±0.00	67.26±0.09	74.28±0.00	93.23±0.00	98.56±0.09

Table S3. The suggestable chemical composition of acetone wood extract of *J. excelsa* ssp. *polycarpus*.

Components	Retention Time (min)	Area (%)
Trisiloxane,octamethyl	6.608	2.17
Trifluoromethyl-bis-(TMS)methyl ketone	8.819	9.32
Tetrasiloxane,deca methyl	12.445	2.03
TMS ether of glycerol	19.653	0.45
Hexasiloxane,tetradecamethyl	22.140	0.30
Cedrol	27.513	0.72
Androstan-3-one,1,17-dimethyl-17-(TMS)oxy)-O-methyloxime,(1.α,5.α,17.β)	30.686	0.63
D-Fructose,1,3,4,5,6-pentakis-O-(TMS)	32.599	9.97
β-D-Galactofuranose,1,2,3,5,6-pentakis-O-(TMS)	33.439	1.57
Galactopyranose,1,2,3,4,6-pentakis-O-(TMS)-β-d-	34.323	12.10
D-Glucose,2,3,4,5,6-Pentakis-O-(TMS)	36.136	11.30
Hexadecanoic acid, TMS ester	36.601	0.78
9,12-Octadecadienoic acid(Z,Z),trimethylsilyl ester	39.541	0.46
Oleicacid, TMS ester	39.641	0.34
Octadecanoic acid, TMS ester	40.094	0.27
Pimaric acid TMS	41.085	24.56
α-D-Glucopyranoside,1,3,4,6-tetrakis-O-(TMS)-β-D-fructofuranosyl2,3,4,6-tetrakis-O-(TMS)	45.555	21.39
Total	-	98.36

Table S4. The suggestable chemical composition of F9 wood extract of *J. excelsa* ssp. *polycarpus*.

Component	Retention Time (min)	Area (%)
Trifluoromethyl-bis-(TMS)methyl ketone	8.818	5.10
Silanamine,N,N-methaneteraylbis[1,1,1-trimethyl]	9.503	0.36
Propanoic acid,2-[TMS]oxy] TMS ester	12.996	0.18
TMS ether of glycerol	19.663	1.83
Hexasiloxane, tetradecamethyl	22.139	0.24
Malic acid,O-(TMS)-bis(TMS) ester	25.246	0.24
β -D-Galactofuranose,1,2,3,5,6-pentakis-O-(TMS)	33.438	2.81
Glucopyranose,1,2,3,4,6-pentakis-O-(TMS)	34.521	4.93
Benzoic acid,3,4,5-tris(TMS)- TMS ester	35.361	0.32
D-Glucose,2,3,4,5,6-pentakis-O-(TMS)	36.146	9.05
Hexadecanoic acid, TMS ester	36.611	0.60
α - D-Glucopyranoside,1,3,4,6-tetrakis-O-(TMS)	45.565	20.22
2H-1-Benzopyran,3,4-dihydro-2-[3,4 bis[(TMS)oxy]phenyl]	52.718	0.49
Total	-	46.37

Table S5. The solid weight of fractions after solvent evaporation.

Fractions	Solid weight (g)	Fractions	Solid weight (g)
F1	0.179	F7	0.026
F2	0.262	F8	0.005
F3	0.186	F9	0.113
F4	0.114	F10	1.096
F5	0.119	F11	0.043
F6	0.085	F12	0.002

Figure S1. Isolation scheme of active constituents of *J. excelsa* ssp. *polycarpus* from the water:methanol extract of wood.

Figure S2. The antioxidant activity of extracts from the wood of *J. excelsa* ssp. *Polycarpus*.

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