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GC-MS ANALYSIS AND BIOLOGICAL ACTIVITY OF ESSENTIAL OIL OF FRUITS, NEEDLES AND BARK OF *PINUS PINEA* GROWN WILDLY IN JORDAN

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Abstract: Essential oils from needles, fruits and bark were extracted from *Pinus pinea* L. (stone pine) grown wildly in Jordan. The chemical composition, antibacterial activity, antioxidant activity of essential oils were evaluated. The chemical compositions were identified using Gas-Chromatography-Mass spectrometry (GC-MS) and retention indices (Van den Dool & Kratz). The results show that the essential oil obtained from needles composed mainly of guaiol (12.7%), limonene (11.42%), and β-caryophyllene (7.61%), while fruit contains limonene (32.56%), and α-pinene (6.78%). The essential oils from barks were rich in β-caryophyllene (16.51%), limonene (14.83%), caryophyllene oxide (11.83%), and longifolene (7.51%). *In vitro*, the antibacterial activity of the essential oils was evaluated using the agar-well diffusion method against three different strains of bacteria (Gram-positive bacteria: *Staphylococcus aureus* and Gram-negative bacteria: *Klebsiella pneumoniae* and *Escherichia coli*). The results indicated that essential oil exhibited appreciable antibacterial activity against *S. aureus*. The essential oil from fruit exhibited weak antibacterial activity against *E. coli* and *K. pneumoniae*. Essential oils of *P. pinea* showed appreciable antioxidant activity *in-vitro*.

Keywords: Essential oils, *Pinus pinea*, antioxidant activity, antibacterial activity

Pinus pinea L. (Italian stone pine) belongs to the family Pinaceae which consists of 250 species of the plants. In recent classification *P. halepensis, P. brutia, P. heldreichii, P. pinaster, P. pinea, P. canariensis, and P. roxburghii* are grouped within the subsection of pinaster, also known as the Mediterranean pine group (1, 2). *Pinus pinea* is widely spread in the northern hemisphere, especially in the Mediterranean region, Europe, Asia, South Africa, and North and Central America (3). *P. halepensis* and *P. brutia* can be adopted in different environmental conditions.

In Arab countries, it is usually located in the mountain regions of Lebanon, Syria, Algeria, Libya, and the Moroccan countryside. It is abundantly grown in Jordan reaching to a height of 30 m. Due to its medicinal properties, the essential oils of *Pinus pinea* have been used in folk medicine for the treatment of skin diseases such as eczema and psoriasis and as an analgesic and anti-inflammatory agent. The essential oil from the cones and needles of *Pinus pinea* showed significant wound healing activity and had a remarkable effect on rheumatic pain (4). Essential oils derived from *Pinus pinea* grown in Lebanon (5) showed cytotoxic activities against drug-sensitive CCRF/ADR5000 leukemia cells with IC_{50} values ranging from 29.5 μg/mL to 61.4 μg/mL.

The volatile composition of *Pinus pinea* L. needles (6) showed significant variation in the chemical constituents. Several studies have shown a significant difference in the volatile composition of the essential oil which may be due to the geographic location, climatic conditions, and time of collection. The variation in the chemical constituents may also affect its antioxidant activity. Scavengers like flavonoids, polyphenols, and phenolic acids are excellent antioxidant compounds that scavenge free radicals like peroxide and hydroperoxide, and thus inhibit the oxidative stress that causes diseases (7).

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Synthetic drugs and synthetic antioxidants produce adverse effects apart from their free radical scavenging activity (8). Consumption of natural food supplements, phytochemicals, and natural antioxidants can significantly lower the adverse effects. Food supplements of natural origin are the best way to combat these adverse effects (9). The purpose of this study was to investigate the chemical constituents present in the essential oils from the needles, fruits, and bark of *Pinus pinea* L. that grow wild in Jordan and to evaluate the antioxidant and antibacterial activity.

MATERIAL AND METHODS

Chemicals

Modified Griess reagent, DPPH·, ferrozine, sodium nitroprusside, β-carotene, and other chemical were purchased from Sigma-Aldrich (USA) and used without purification.

Plant material

The needles, fruits and barks of *P. pinea* species were collected during July and August in 2015 from different trees in Jordan, planted in Al-Salt governorate (Al-Ahliyya Amman University), and in Amman governorate (Iraq Al-Ameer, Amman). The plants were identified by Taxonomist (Dr. Dawud Mohammad Al-Eisawi, University of Jordan, Amman).

Isolation of the essential oils

The air-dried needles, fruits and bark of the collected plant were ground to about 0.8 mm particle size (18-20 mesh). Ground plant material (200 g) was accurately weighed transferred to a double-necked round-bottomed flask (2 L). Distilled water was added to it (1.25 L) and it was subjected to hydro-distillation using Clevenger-type apparatus for 3 hours (n = 3). The hydro-distilled essential oils were collected and dried over anhydrous sodium sulfate. The essential oils were transferred to amber colored vials, flushed with nitrogen, sealed and stored in a refrigerator until required.

Analysis of essential oils (EOs)

Analysis of EOs and fraction were carried out using Varian Chrompack CP-3800 GC/MS/MS-2200 equipped with a split-spitless injector, DB-5MS fused silica column (5% phenyl, 95% polydimethylsiloxane 30 m × 0.25 mm, film thickness 0.25 µm). A linear temperature program was used to separate the different oil components. Temperature programming was applied at 3°C/min heating rate starting from 60°C (initial temperature) to 250°C (final temperature) and held at 250°C for 5 min with a total run time of 70 min. Injector temperature was 250°C with a split ratio of 1 : 10; injection volume (1 µL), carrier gas: helium; MS source temperature/detector temperature: 250°C, ionization energy (EI): 70eV; amu gain -492, amu offs. -67, ionization current 60 µm; scan range 40-400 amu.

Mass spectrum of every chemical constituent was compared with corresponding reported spectrum (in NIST, Wiley Mass Spectral Database -1995 and ADAMS-2007 libraries) for GC-MS and published references. Identification of compounds was also confirmed by comparing their retention indices (RI) relative to n-alkanes (C_8-C_{20}) with reported values in the literature including Adam's library. Samples were analyzed in triplicate.

Antimicrobial activity

The antibacterial activity of the essential oils, fraction and isolated compound was evaluated by agar diffusion method against three bacterial species, two gram-negative strains (*Klebsiella pneumoniae* and *Escherichia coli* ATCC 8739) and one-gram positive strain (*Staphylococcus aureus* ATCC 6538a). Samples were vortexed, circular discs of filter paper (6 mm) were sterilized and placed in the samples (33% EO in 30% aqueous DMSO (dimethylsulfoxide) for soaking (24 h). The plates were inoculated with bacteria and the samples were placed over the media. The plates were incubated at 37.0 ± 0.5°C for 24-48 h. After the desired incubation time, the diameter of the zone of inhibition was measured. DMSO (30%) was used as negative control and different antibiotics discs were used as standard. The antibiotics were tobramycin (30 µg/disc), penicillin G (10 U/disc), lincomycin (2 µg/disc), chloramphenicol (30 µg/disc) and cefaclor (30 µg/disc).

DPPH free radical scavenging activity[10]

The free-radical scavenging activity of the EOs was measured as a decrease in the absorbance of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). A stock solution of DPPH (0.002% w/v) was prepared in methanol and different concentrations of the EOs were added (2–250 µg/mL). After incubation at room temperature for 30 min, the pale pink color developed was measured at 517 nm using a spectrophotometer and compared with the standard (1–100 µg/mL ascorbic acid). Free radical scavenging activity (9) was expressed as the percentage inhibition calculated using the following formula:

$$\%\ Free\ radical\ scavenging\ activity = [1 - \frac{Abs_{sample}}{Abs_{control}}] \times 100$$

Ferrous ion-chelating activity

The samples were estimated for the ferrous ion-chelating activity as described by Chua et al. (11). Briefly, 750 µL of the sample in methanol mixture (2-250 µg/mL) were incubated with 50 µL ferrous chloride solution ($FeCl_2$) (2 mM). The reaction was initiated by the addition of 50 µL ferrozine solution (5 mM) into the mixture and allowed to stand for 10 minutes in dark. The absorbance of the reaction mixture was measured at 562 nm. Rutin was used as a positive control in this assay.

Nitric oxide radical scavenging activity[12]

The aqueous sodium nitroprusside (SNP) solution reacts with oxygen and generates nitrite ions, these ions can be quantitated by Modified Griess reagent. In brief, the reaction mixture contained 10 mM SNP (0.25 mL), phosphate-buffered saline (pH 7.4, 0.40 mL) and various concentrations of the test solution (0.10 mL) in test tubes, after incubation at 25°C (150 min.), the 0.25 mL of Griess reagent (1x, Sigma-Aldrich, USA) was added. The color generated during the diazotization of nitrite ions was measured spectrophotometrically at 546 nm. Quercetin (10-175 µg/mL) was used as a standard compound for comparison.

β-carotene bleaching (BCB) Assay[13]

A solution of β-carotene was prepared by dissolving 5 mg of β-carotene in 50 mL of chloroform. An aliquot of the 3 mL was added to 40 mg linoleic acid and 400 mg of tween 40. It was mixed and set aside for 2 minutes. The chloroform was evaporated off using nitrogen gas. The residue was reconstituted in 100 mL of distilled water using vortex. Immediately after preparation, the absorbance of this solution was recorded at 470 and 700 nm. Different solutions of oil (50 µg/mL to 1000 µg/mL) were prepared in methanol (with the aid of 0.05% Tween-40). β-Carotene-linoleic acid emulsion (1 mL) was mixed with different solutions of oil (0.25 mL). All the solutions (control and test) were capped and incubated (50°C) for 1 hour. The control sample contains anequivalent amount of methanol (0.05% Tween-40). The absorbance of the solutions (λ_{470} and λ_{700} nm) was determined after 60 min. All determination was carried out in triplicate; the degradation rate (DR) and antioxidant activity were calculated.

$$\text{Degradation rate (DR) of β-carotene} = \text{Ln}\,(A_{initial}/A_{sample})/60$$

$$\text{Antioxidant activity (\%)} = [1 - \frac{\textit{Degradation rate of sample}}{\textit{Degradation rate of control}}] \times 100$$

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Graph-Pad Prism 5 (San Diego, CA, USA) for Windows was used for statistical analyses of experimental data.

RESULT AND DISCUSSION

Chemical composition of essential oils

The simultaneous use of mass spectroscopy and arithmetic retention index (14) correlation allowed for unequivocal identification of more than 72.9, 78.2 and 75.6% of the components of the essential oils obtained from needles, fruits and bark of *Pinus pinea* under study which are determined by the GC-FID and GC-MS. The essential oils yield using hydro-distillation from needles, fruits and bark were 0.19%, 0.50% and 0.21% v/w, respectively. The total number of identified compounds were 28, 29 and 27 in the essential oil obtained from needle, fruits and bark respectively (Table 1). The main constituents of the essential oil from needles were guaiol (12.7%), limonene (11.4%), and β-caryophyllene (7.6%). Hmamouchi et al. (15) reported that the main component of the EOs obtained from *P. pinea* grown in Morocco was α-pinene (37.0%). *P. pinea* grown in Jordan showed that guaiol (12.7%) as the major component, while the percentage of α-pinene was 0.51% in the same sample. Amri et al. (16) reported that the limonene (54.1%) was the main constituent in the *Pinus pinea* grown in Tunisia. The essential oil of needles of *Pinus pinea* grown in Greece was rich in limonene (39.05%) and β-phellandrene (13.8%). The other components were α-pinene (5.13%), guaiol (2.79%), and β-pinene (2.65%) (17). *Pinus pinea* grown in Italy and Turkey were rich in limonene and β-phellandrene content (18, 19). The percentage of limonene in Italy and Turkey were 58.9% and 55.0% respectively. While the percentage of β-phellandrene ranged from 6.7 to 7.4% The percentage of α-pinene was 6.2% in *Pinus pinea* grown in Italy (18).

The major constituents of essential oil from fruits were limonene (32.56%) and α-pinene (6.78%). Macchioni et al. (18) have reported that EO from cones contains mainly 61.6% of limonene in variety grown in Italy, while in the Jordanian variety the percentage of limonene was 32.56%. The percent of α-pinene (19.4%) was also higher than the Jordanian variety (6.78%). The other constituents present were β-caryophyllene (2.8%), β-pinene (2.1%), α-terpineol (1.4%), longifolene (1.3%). In Turkey, Tumen et al. reported that EO of cones contained mainly limonene and β-phellandrene which

Table 1. Chemical composition of essential oils from needles, fruits and barks of *Pinus pinea* grown in Jordan.

Rt	RRI	RI	Compound	Relative percent content		
				Needles	Fruit	Bark
6.2	933	932	α-pinene [a,c]	0.51	6.78	0.48
7.5	977	977	Sabinene [a,c]	0.40	1.13	—
7.8	988	988	β-pinene [a,c]	0.36	4.66	0.37
9.1	1024	1024	*p*-cymene [a,c]	—	0.88	—
9.3	1031	1030	Limonene [a,c]	11.42	32.56	14.83
9.9	1044	1044	E-ocimene [a,c]	0.77	—	—
11.6	1089	1089	*p*-cymenene [f]	—	1.04	—
12.9	1120	1121	E-*p*-Mentha-2,8-diene-1-ol [a,d]	—	—	—
13.1	1125	1125	E-verbenol [a,d]	—	0.53	—
13.3	—	1129	α-campholenal [a,d]	—	0.83	—
13.5	1137	1135	Z-*p*-Mentha-2,8-diene-1-ol [a,d]	—	0.65	—
13.7	1140	1140	E-pinocarveol [a,d]	—	1.19	—
13.9	1145	1145	Z-verbenol [a,d]	—	1.48	—
14.6	1160	1160	Pinocarvone [a,d]	—	0.71	—
16.0	1195	1194	α-terpineol [a,d]	1.04	1.62	0.49
16.6	1206	1206	Verbenone [a,d]	—	1.26	—
17.0	1217	1218	E-carveol [a,d]	—	2.87	0.63
17.6	1235	1228	Thymol methyl ether [a,d]	1.61	1.31	0.71
18.2	1242	1242	Carvone [a,d]	—	1.72	0.61
19.9	1286	1282	Bornyl acetate [a,d]	—	0.99	—
22.0	1333	1332	δ-elemene [b,c]	0.62	—	—
22.6	1353	1346	α-longipinene [b,c]	0.80	0.99	2.12
25.0	1402	1402	Methyl eugenol [d,f]	0.21	—	—
25.2	1411	1405	Longifolene [b,c]	1.05	2.35	7.51
25.5	1415	1415	β-caryophyllene [b,c]	7.61	2.31	16.51
25.6	1419	1417	Aromadendrene [b,c]	—	—	—
26.7	—	1443	6,9-guaiadiene [b,c]	1.66	—	—
26.7	1445	1445	γ-muurolene [b,c]	—	—	0.29
27.2	—	1457	α-humulene (α-caryophyllene) [b,c]	1.97	0.61	2.97
28.0	1478	1476	Germacrene-D [b,c]	5.52	0.88	1.28
28.3	1479	1482	δ-selinene [b,c]	4.14	—	0.88
28.5	1490	1488	Z-β-guaiene [b,c]	2.70	—	—
28.6	1494	1494	α-muurolene [b,c]	0.27	—	0.32
29.3	—	1509	Phenol,2,4-bis (1,1-dimethylethyl) [d,f]	—	—	0.72
29.6	1497	1513	δ-cadinene [b,c]	1.01	—	0.36
30.7	—	1543	E-α-bisabolene [b,c]	0.64	—	0.49
32.0	1576	1576	Caryophyllene oxide [b,d]	1.56	3.73	11.83
32.6	1592	1591	Guaiol [b,d]	12.70	1.22	3.13
32.8	1592	1598	Longiborneol [b,d]	—	—	1.18
33.1	1606	1603	Humulene epoxide II [b,d]	—	—	1.64
33.4	—	1614	Rosifoliol [b,d]	0.73	—	—
33.5	1617	1617	10-epi-γ-eudesmol [b,d]	2.36	—	—
33.8	—	1625	Humulane-1,6-dien-3-ol [b,d]	—	—	0.52

Table 1. Continued.

Rt	RRI	RI	Compound	Relative percent content		
				Needles	Fruit	Bark
33.9	1626	1626	γ-eudesmol [b,d]	0.83	—	—
34.8	1650	1650	α-eudesmol [b,d]	5.19	1.49	2.24
35.3	1669	1664	14-hydroxy-9-epi-E-caryophyllene [b,d]	—	1.33	1.61
40.0	1794	1798	Ethyl myristate [d,f]	1.56	—	—
44.9	—	1943	Phthalic acid butyl hexyl ester [d,f]	—	—	0.65
46.3	1995	1982	Manoyl oxide [d,e]	3.61	1.03	1.21
TOTAL				72.89	78.16	75.61
Monoterpene hydrocarbon				13.47	46.00	15.68
Oxygenated monoterpene				2.64	15.18	2.44
Sesquiterpene hydrocarbon				28.01	7.14	32.73
Oxygentedsesquiterpene				23.38	7.77	22.17
Diterpene				3.61	1.03	1.21
Nonterpene				1.77	1.04	1.37

Rt: Retention time (in minutes), RRI: Relative retention indices, RI: retention indices, Concentration of constituents reported as Mean percent of triplicate analysis of 3 samples, —Not present, [a]monoterpene, [b]sesquiterpene, [c]hydrocarbon, [d]oxygenated, [e]diterpene, [f]nonterpene

Table 2. Antibacterial evaluation of essential oils obtained from needles, fruits and bark of Pinus pinea grown in Jordan.

Sample	Zone of inhibition (mm)		
	S. aureus	*E. coli*	*K. pneumoniae*
EO from needles (33% essential oil in 30% DMSO)	12	-	-
EO from fruits (33% essential oil in 30% DMSO)	19	7	7
EO from bark (33% essential oil in 30% DMSO)	15	-	-
Control (30% DMSO)	-	-	-
Tobramycin (30 µg)		28	36
Penicillin G (10 U)	20	-	-
Lincomycin (2 µg)	31	-	-
Chloramphenicol (30 µg)	-	-	38
Cefaclor (30 µg)	-	20	-

Diameter of disks were 6 mm, (n = 2)

Table 3. DPPH radical scavenging, ferrous ion chelating, ß-carotene bleaching and SNP scavenging activity of essential oils from needles, fruits and barks of *Pinus pinea*.

Sample	IC_{50} (DPPH radical) µg/mL	IC_{50} (Fe^{2+} assay) µg/mL	IC_{50} (ß-carotene bleaching assay) µg/mL	IC_{50} (SNP radical scavenging activity) µg/mL
EO from Needles	45.1 ± 1.5	51.1 ± 0.9	110.5 ± 1.3	175.0 ± 2.5
EO from Fruits	40.5 ± 0.7	48.0 ± 1.1	115.4 ± 2.1	185.1 ± 1.9
EO from Bark	48.4 ± 1.2	55.0 ± 0.5	138.2 ± 2.3	201.2 ± 1.7
Ascorbic acid	5.0 ± 0.4	-	-	-
Rutin	-	7.5 ± 0.5	42.4 ± 1.2	-
Quercetin	-	-	-	55.4 ± 0.8

Values are expressed as mean ± SD (n = 3)

constitutes around 69.5% of essential oil (20). The yield of oil from *P. pinea* bark was 0.2% (v/w). The results showed that β-caryophyllene (16.5%) as the major compound. The other constituents identified were limonene (14.83%), caryophyllene oxide (11.83%) and longifolene (7.51%). This difference or variations in the concentration may be due to their geographical location, climatic condition, time of collection and other factors.

Antimicrobial activity

EOs of *P. pinea* showed appreciable antibacterial activity compared to the standards on tested microorganisms. EOs obtained from needles, fruits and bark exhibited significant antibacterial activity against *S. aureus*, whereas the EOs obtained from fruit showed weak activity against *E. coli* and *K. pneumoniae*. The EOs obtained from other parts did not exhibit any activity against *E. coli* and *K. pneumoniae* (Table 2). The resistance of Gram-negative bacteria against essential oils (as we see with *E. coli* and *K. pneumoniae*) may be related to the presence of hydrophilic outer membrane surrounding the cell wall that prevents the EO's components from diffusing into the cell wall and causing its inhibitory action (21). There is a significant difference between the present results and the results reported earlier. Hmamouchi et al. (15) reported that EOs of needles of *P. pinea* grown in Morocco have antibacterial activity against *Staphyloccocus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* when compared to our results that showed EO just inhibiting *S. aureus* without any antibacterial activity on *E. coli* and *K. pneumoniae* (15). Similar observations were made by Demirci et al. (19) who reported that EOs obtained from needles of *P. pinea* grown in Turkey had antibacterial activity against *E. coli* and *S. aureus*, whereas our results showed that this EO has antibacterial activity against *S. aureus* only.

Antioxidant activities

The DPPH scavenging activity of the sample was concentration-dependent. In the examined essential oils from various parts of *P. pinea* were capable of scavenging DPPH radical *in-vitro*. The IC_{50} values are mentioned in Table 3. The IC_{50} values were ranged from 40.5 to 48.4 µg/mL. Regarding the Fe^{2+} Chelating assay the IC_{50} values were ranged from 48.0 µg/mL to 55.0 µg/mL. The examined essential oils exhibited weak NO radical scavenging activity with mean IC_{50} value of 175.0 (EO from needles), 185.1 (EO from fruits) and 201.2 µg/mL (EO from bark). The IC_{50} of quercetin was 55.4 ± 0.8 µg/mL. The examined essential oils were also effective in preventing the bleaching of β-carotene using linoleic acid. The IC_{50} of the examined essential oils were ranged from 110.5 to 138.2 µg/mL, while the IC_{50} of rutin was 42.4 ± 1.2 µg/mL. These activities might be due to the presence of phenolic constituents present in the essential oils obtained from needles, fruits and bark. Yener et al. (22) reported that the chelating activity of the needles EO of this species was 17.6 ± 0.1% at a concentration of 1 mg/mL. The reported antioxidant activity was less significant as compared to the antioxidant activity of essential oil of *P. brutia*, *P. halepensis*, and *P. nigra*. The reported IC_{50} values of essential oil against DPPH radical ranged from 0.2 to 1.0 mg/mL (22).

CONCLUSION

The composition of the essential oils from needles, fruits and bark of *P. pinea* L. grown in Jordan were studied and the results indicated that the main component and the concentration of the constituents varied when compared with the same species grown in different or distributed in different geographical and climatic conditions. This difference or variations in the concentration may be due to their geographical location, climatic condition, time of collection and other factors. The studies indicated that the essential oil from fruits of *P. pinea* have significant antibacterial activity against *S. aureus,* while essential oils from fruits, needles and bark exhibited significant antioxidant activity, which may be due to terpene and other compounds. The plant may be further exploited for its active compounds and its therapeutic potentials, as there is a growing interest in new natural antioxidants and antibacterial compounds to replace the synthetic compounds that are being used in food, cosmetic and in pharmaceutical industries.

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Conflict of interest

The authors declare no conflicts of interest.

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