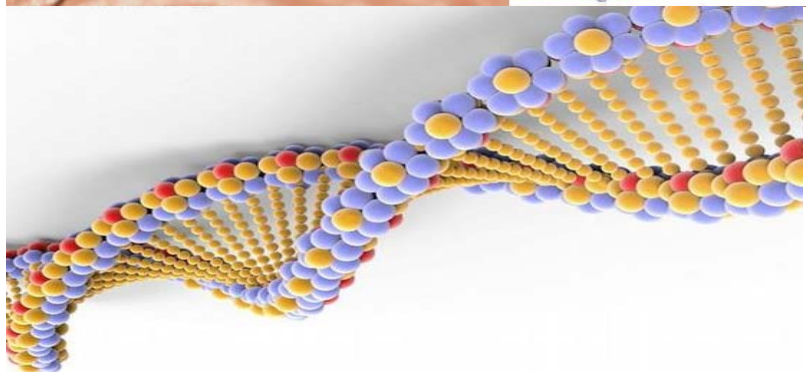
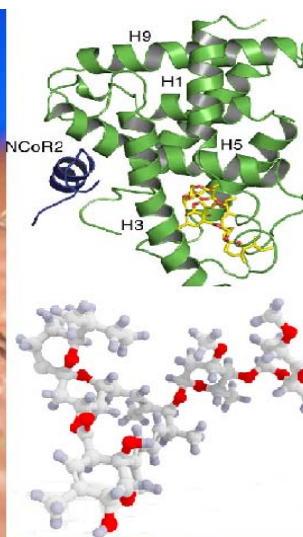


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Chemical composition and antimicrobial potency of essential oils from roots of *Pinus* growing in Algeria

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Abstract. The objective of this study is to determine the chemical composition and antimicrobial potency of essential oils of three roots of genus *Pinus* (*P. halepensis*, *P. pinea* and *P. pinaster*) growing in Algeria for the first time. The essential oils used in the present study were isolated by hydrodistillation using a Cleavenger-type apparatus according the European Pharmacopoeia, and identified by GC and GC-MS. 14, 12, 11 constituents were identified, representing an average of 98.8 %, 91.3 % and 83.6 % of the total oil of roots of *P. halepensis*, *P. pinea* and *P. pinaster* respectively. The chemical profile reveals the dominance of monoterpenes compounds, although some quantitative variance was noticed. The main constituents of the oil root of *P. halepensis* were α -Pinene (87.4 %) and trans-p-Caryophyllene (3.5 %). The oil of *P. pinea* was dominated by Limonene (66.3 %), Trans-p-Caryophyllene (9.6 %), Myrcene (3.2 %) and Osmorhizol (3.2 %). The oil of *P. pinaster* was rich by α -Pinene (22 %), β -Pinene (27.1 %) and Trans-p-Caryophyllene (21.4 %). The antimicrobial activities of the essential oils from roots of *P. halepensis*, *P. pinea* and *P. pinaster* were tested against a panel of bacteria and one yeast strain using the agar well diffusion technique and dilution methods. The diameters of zones of inhibition exhibited by the essential oils were between 7 and 25 mm. The minimal inhibitory concentration was 28.4 μ g/mL of *P. halepensis* and *P. pinaster* against *S. aureus* and *C. albicans* respectively.

Key Words: Pinaceae; *Pinus halepensis*; *Pinus pinea*; *Pinus pinaster*; Roots; Essential oils; GC; GC-MS; Antimicrobial Activity

1. Introduction

Plants are considered to have a rich source of naturally occurring bioactive compounds with high medicinal activities against various diseases. Also the use of medicinal plants for local remedies is a traditional custom. As we know medicinal plants have the potency to combat all these diseases [1, 2], so the current study is carried out on three medicinal plants *P. halepensis*, *P. pinea* and *P. pinaster*. The local people of the area use these plants to treat respiratory diseases cough, bronchitis, colds and rheumatic pains [3]. It is believed that part of these traditional and pharmaceutical uses of pines are due to their essential oils, which have been shown to possess good antimicrobial properties [4, 5].

The *Pinus* is an important part of the economies of Mediterranean countries, and also serve as an effective tool in the conservation of forests and other ecosystems. *Pinus* is currently highly prized in

international markets meaning that their cones have become the most important product of stone pine forests providing higher incomes to their owners than any other forest resource (timber, grazing, hunting rights, etc.). The chemical composition of various pine species has been the subject of numerous studies, the majority of this studies focused on the needles and cones species [3-15]. Reviewing the current literature, there are no previous studies of chemical composition and biological activity of root of *Pinus*, these cause we are interested to study the chemical composition of essential oils from roots of *Pinus*.

In these work, essential oils, extracted from the roots of Algerian *Pinus* (*halepensis*, *pinea* and *pinaster*) were identified by gas chromatography and were tested for their antimicrobial action in order to attempt to correlate between the chemical components of the oils and their antimicrobial activity.

2. Materials and methods

2.1. Plant Material

The roots of *P. halepensis*, *P. pinea* and *P. pinaster* were collected from Nedroma region near Tlemcen (West Northern of Algeria). Botanical identification of plant was conducted by Professor Noury BENABADJI and a voucher specimen of the plant was deposited in the Herbarium of the Laboratory of Botany, Department of Biology, and University of Abou Bekr Belkaïd -Tlemcen, Algeria.

2.2. Essential oil isolation

Each fresh root (400-500g) was submitted to hydrodistillation for 5 h using a Clevenger-type apparatus according to the *European Pharmacopoeia* [16]. The essential oils were stored in sealed vials in the dark at + 4°C before analysis and bioassays tests. The essential oils obtained were yellow with an aromatic odour.

2.3. Gas Chromatography (GC)

GC analysis was performed using a Perkin-Elmer Clarus 600 GC apparatus (Walton, MA, USA) equipped with a single injector and two flame ionization detectors (FID). The analysis was carried out using two fused silica capillary columns (60 m 0.22 mm i.d.; film thickness 0.25 µm) with different stationary phases: Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). The operating conditions were as follows: Injector and detector temperatures were maintained at 280 °C. Helium was used as carrier gas (1 mL/min), the injection volume was 0.1 µL, split ratio was adjusted at 1:80, the oven temperature was programmed from 60 °C to 230 °C at the rate of 2 °C/min and then held isothermally at 230 °C for 30 min.

2.4. Gas Chromatography - Mass Spectrometry (GC-MS)

The oils were investigated using a Perkin Elmer TurboMass quadrupole analyzer, directly coupled to a Perkin Elmer Autosystem XL equipped with two fused-silica capillary columns (60 m x 0.22 mm, film thickness 0.25 µm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). Other GC conditions were the same as described above. Ion source temperature: 150 °C; energy ionization: 70 eV; electron ionization mass spectra were acquired with a mass range of 35 – 350 Da; scan mass: 1s. Oil injected volume: 0.1 µL.

2.5. Component Identification

Identification of the components was based (i) on the comparison of their GC retention indices (RI_a and RI_p) on non polar and polar columns, respectively determined the retention time of a series of *n*-alkanes with linear interpolation, with those of authentic compounds or literatures data [17, 18]; (ii)

on computer matching with commercial mass spectral libraries [19, 20], and comparison of spectra with those of Corsican laboratory-made library.

2.6. Antimicrobial assays

2.6. 1. Micro-organisms and culture conditions

The essential oils from roots of *Pinus* were tested against seven microorganisms, including Gram positive: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 49452, *Listeria monocytogenes* ATCC 15313, *Bacillus cereus* ATCC 10876, Gram negative: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 70063 and a yeast: *Candida albicans* ATCC 10231. The microorganisms were obtained from the Pasteur Institute in Paris. Two different techniques were used to test the antimicrobial activity: the paper disc diffusion and the dilution agar method. The minimum inhibitory concentration (MIC) was determined by the later method. The bacteria were cultivated in Brain Heart Infusion (BHI) Broth at 37 °C. *C. albicans* was cultivated in Sabouraud dextrose agar (DAS) at 27 °C. The inoculi were prepared by the direct inoculation of colonies in 1 mL of sterile saline solution and adjusted to the 0.5 standard of the McFarland scale, corresponding to 1.5×10^8 CFU/mL for the bacteria and 2 to 5×10^6 CFU/mL for fungal strains [21].

2.6. 2. Agar disc diffusion method

The standard agar disc diffusion method [22] was used to evaluate the inhibitory spectrum of the essential oil against the micro-organisms analyzed in the present study. The bacterial inoculi were seeded on Mueller-Hinton agar solidified in Petri dishes, in such a way as to produce uniform growth throughout the dish. Once the dishes were prepared, 6 mm-diameter discs of filter paper containing 10 SL of the undiluted essential oil were pressed lightly against the surface of the agar. After 30 min at room temperature, the dishes were incubated in a bacteriological oven at 37 °C for 24 h. For the cultures of *C. albicans*, incubation time was 48 h, at 27 °C and the substrate was Sabouraud dextrose agar. At the end of the test period, the diameter of the inhibition zone formed over the agar culture was measured in millimeters. All tests were conducted in triplicate and the inhibition zones formed in the experimental dishes were compared with those of the controls.

2.6. 3. Determination of the minimum inhibitory concentration (MIC)

The MIC assays were performed by standard dilution methods [23]. The MIC was defined as the lowest product concentration that prevented visible growth of bacteria. All tests were performed on Muller-Hinton agar. Briefly, 30 SL of 2-fold serial dilutions in DMSO (dimethylsulfoxide, Sigma-Aldrich) was added to 15 mL of agar to obtain concentrations ranging from 0.1 to 5 mg/mL tested product. The resulting agar solutions were mixed at high speed for 15 s, immediately poured into sterile Petri dishes, and then allowed to set for 30 min.

3. Result and Discussion

3.1. Chemical Characterization

The yield of essential oils was: 0.2 %, 0.19 % and 0.3 % for the essential oils of *P. halepensis*, *P. pinea* and *P. pinaster* respectively.

The chemical compositions of essential oils from roots of *P. halepensis*, *P. pinea* and *P. pinaster*, were investigated by GC-RI and GC-MS analysis. 14, 12, 11 components accounted for 98.8 %, 91.3 %, 83.6 %, were identified in the essential oils from roots of *P. halepensis*, *P. pinea* and *P. pinaster* respectively (Table 1). Their retention indices and normalized % abundances are shown in Table 1. All components were identified by comparison of their EI-MS and GC retention indices with those of Corsican laboratory-produced “Aromes” library, with the exception of one component that was identified by comparison with spectral data and retention indices from the literature (Table 1). Among them, 7

hydrocarbons sesquiterpene, 10 hydrocarbons monoterpene and 3 no-terpinic oxygenated compounds were identified. The chromatographic profile of essential oils of three roots from *Pinus* showed that the essential oils from roots of *P. halepensis*, *P. pinea* and *P. pinaster* were characterized by large amount of monoterpenes (93.2 %, 73.4 % and 52.9 %) respectively. Comparing the present data (Table 1) with those previously reported in literature, the studied of essential oils displayed different chemical profiles, although monoterpene hydrocarbons have been reported as the main constituents of the essential oils of aerial parts of the genus *Pinus* [3-15].

However, the essential oil from the root of *P. halepensis* was characterized by a large amount of hydrocarbon compounds (98.8 %) made up of monoterpenes (93.2 %) and sesquiterpenes compounds (5.5 %). The principal compounds were α -Pinene (87.4 %) and Trans-*p*-Caryophyllene (3.5 %). 11 constituents (91.3 %) were identified in root of *P. pinea* oil: Limonene (66.3 %), Myrcene (3.2 %), Trans-*p*-Caryophyllene (9.6 %) and Osmorhizol (3.2 %) were the most abundant constituent in this oil. The oil of *P. pinaster* was characterized by the presence of α -Pinene (22), β -Pinene (27.1 %) and Trans-*p*-Caryophyllene (21.4 %).

Table 1: Chemical composition of essential oils from roots of *Pinus*.

No. ^a	Components	IR _{Lit} ^b	IR _a ^c	IR _p ^d	Essential oils (%) ^e			Identification ^f
					<i>P. halepensis</i>	<i>P. pinea</i>	<i>P. pinaster</i>	
1	α -Pinene	931	932	1023	87.4	2.9	22	RI, MS
2	Camphene	943	944	1066	0.8	tr	1.1	RI, MS
3	β -Pinene	970	971	1108	1.3	0.6	27.1	RI, MS
4	Myrcene	970	983	1159	1	3.2	1.1	RI, MS
5	3-Carene	1005	1006	1147	1.5	nd	nd	RI, MS
6	Limonene	1020	1021	1195	0.7	66.3	1.6	RI, MS
7	β -Phelandrene	1023	1025	1208	0.1	0.2	0.3	RI, MS
8	Terpinolene	1078	1082	1247	0.4	nd	nd	RI, MS
9	α -Terpinolene	1161	1164	1583	0.1	nd	nd	RI, MS
10	Estragol	1176	1176	-	nd	0.2	nd	RI, MS
11	Bornyle acetate	1266	1269	1515	nd	nd	1.7	RI, MS
12	α -Longipinene	1359	1359	1475	nd	0.9	nd	RI, MS
13	NI	-	-	-	nd	nd	3.2	-
14	α -Copaene	1379	1373	1475	0.3	nd	0.9	RI, MS
15	Osmorhizol	1383	1380		nd	3.2	nd	RI, MS, Ref
16	Longifolene	1411	1404	1571	nd	2.7	nd	RI, MS
17	Trans- <i>p</i> -Caryophyllene	1424	1418	1583	3.5	9.6	21.4	RI, MS
18	α -Humulene	1456	1449	1651	0.6	1.5	3.2	RI, MS
19	Germacrene D	1480	1474	1692	0.3	nd	nd	RI, MS
20	α -Murolene	1496	1492	1709	0.8	nd	nd	RI, MS
	Total identification %				98.8	91.3	83.6	
	Yields% (w /w)				0.2	0.19	0.3	
	% Monoterpene hydrocarbons				93.2	73.4	52.9	
	% Sesquiterpene hydrocarbons				5.5	14.7	25.5	
	% No terpinic oxygenated				0.1	3.2	1.7	

^a Order of elution is given on apolar column (Rtx-1).

^b Retention indices of literature on the apolar column (RI_{Lit}) reported from König et al. 2001.

^c Retention indices on the apolar Rtx-1 column (RI_a).

^d Retention indices on the polar Rtx-Wax column (RI_p).

^e Normalized percentages are given on the apolar column except for components with identical RI_a (percentages are given on the polar column). tr= trace (0.05%).

^f RI : Retention indices; MS: Mass Spectra in electronic impact mode; Ref: Compounds identified from literature data[17].

3.2. Antimicrobial activity (assay disc)

The *in vitro* antimicrobial activities of roots of *P. halepensis*, *P. pinea* and *P. pinaster* against the employed microorganisms were qualitatively and quantitatively assessed by the presence or absence of inhibition zones. As shown in Table 2, *C. albicans* was found to be the most inhibited pathogen by the essential oil of *P. pinaster* with a diameter of zone of inhibition from 25 mm. However, the essential oils of *P. halepensis*, *P. pinaster* and *P. pinea* exhibited inhibitory effects against *S. aureus* with diameters of zones of inhibition from 20, 19 and 17 mm respectively, *B. cereus* (10, 12 and 10 mm respectively) and *L. monocytogenes* (10, 7 and 12 mm respectively). The rest of the bacterial strain (*E. coli*, *K. pneumoniae*) showed no inhibition, with diameter of zones of inhibition ranging from 7 to 8 mm (Table 2).

Table 2: Antimicrobial activity of essential oils from roots of *P. halepensis*, *P. pinea* and *P. pinaster*.

Species	Disc diffusion assay (mm)			MIC ($\mu\text{g/mL}$)		
	<i>P. halepensis</i>	<i>P. pinea</i>	<i>P. pinaster</i>	<i>P. halepensis</i>	<i>P. pinea</i>	<i>P. pinaster</i>
Gram-positive bacterium						
<i>S. aureus</i>	20	17	19	28.4	227.7	113.63
<i>L. monocytogenes</i>	10	12	7	-	227.7	-
<i>B. cereus</i>	10	7	12	-	-	-
<i>E. faecalis</i>	12	9	6	-	-	-
Gram-negative bacterium						
<i>K. pneumoniae</i>	7	8	8	-	-	-
<i>E. coli</i>	7	7	8	113.63	227.7	227.7
Yeast						
<i>C. albicans</i>	15	14	25	56.81	113.63	28.4

3.3. Minimum inhibitory concentrations (MIC)

The MIC values of the essential oils against the microbial strains studied are shown in Table 2. These results demonstrated that the essential oils from *Pinus* exhibited potential antimicrobial properties. The MIC values against the microorganisms tested ranged from 28.4 to 227.7 $\mu\text{g/mL}$. The essential oils obtained from roots of *P. pinaster* showed higher activity against *C. albicans* with a MIC of 28.4 $\mu\text{g/mL}$. The most prominent inhibitory action of the essential oil from roots of *P. halepensis* has been observed against *S. aureus* with a MIC of 28.4 $\mu\text{g/mL}$, more interesting than that obtained from the aerial parts [15].

However, *C. albicans* exhibited moderate activity of essential oils of *P. pinea*, with MIC value of 113.6 $\mu\text{g/mL}$. The antimicrobial activity of the essential oils from roots of *Pinus*, could be due to the investigated strain sensitivity to α -Pinene, Sabinene, Limonene and the other lighter components present in essential oils [24-26].

4. Conclusion

It can be concluded by all above reports that the essential oils present in plants are working as active principles to show the antimicrobial activity. Thus the studied plant is potentially a good source of antimicrobial agents and validates their traditional medicinal use and as a source for natural antimicrobial that can serve as an alternative to more expensive conventional medicines and a basis for further pharmacological evaluation.

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Conflict of interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

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