In vitro Assessment of Two Species of the Genus *Pinus* Growing in Algeria for their Antimicrobial and Antioxidant Activity

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Summary

A comparative study of the hydrodistilled oils of *Pinus pinea* L. and *Pinus pinaster* Aiton by GC and GC-MS was conducted. The combined essential oil of *P. pinea* was rich in limonene (56.5%), followed by α-pinene (6.5%) and β-phellandrene (6.2%). The major compounds from the combined essential oil of *P. pinaster* were: α-pinene (36.4%), β-pinene (12.3%) and (E)-β-caryophyllene (7.1%). The results showed that the oils have a great potential as antibiotics against some microorganisms: *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Candida albicans*. The maximum zone of inhibition of *P. pinea* was obtained against *Candida albicans* (23 mm). Antioxidant capacity was assessed by *in vitro* testing using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and significant activity was found for the various oils. These results suggested that essential oils of *P. pinea* and *P. pinaster* possess antimicrobial and antioxidant properties, and could, therefore, present potential source of active ingredients for food and pharmaceutical industry.

Key words

Pinus pinea, *Pinus pinaster*, essential oils, GC, GC-MS, biological activities

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Introduction

Essential oils are complex mixtures made up of volatile compounds with strong odors, that are synthesized in several plant organs, including buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Bakkali et al., 2008; Franz and Novak, 2010). These volatile compounds have diverse ecological functions, acting as defensive substances against microorganisms and herbivores, but can also be important to attract insects for the dispersion of pollens and seeds (Bakkali et al., 2008). Essential oils have found therapeutic applications in human medicine due to their anticancer, antinociceptive, antiphlogistic, antiviral, antimicrobial and antioxidant properties (Edris, 2007; Buchbauer, 2010; Teixeira et al., 2013).

In folk medicine, various parts of *Pinus species* (bark, needle, cone and resin) have been used for treatment of diseases such as rheumatism, bronchitis and low blood pressure. Furthermore, *Pinus* is known for its anti-inflammatory, antioxidant, antiseptic and antimicrobial activities (Dob et al., 2005). Essential oils from *Pinus* species have been reported to have various therapeutic properties (Fuentes et al., 2006; Kozan et al., 2006). The chemical compositions of essential oil from many *Pinus* species have been reported by many research groups (Dagne et al., 1999; Stevanoic et al., 2004; Dob et al., 2005; Fekih et al., 2014; Nam et al., 2014), and have shown variations in compositions according to their regional sources and locations. The presence and the concentration of certain chemical constituents also fluctuate according to the season, climate conditions and site of plant growth (Bakkali et al., 2008).

In this study, we aimed at evaluating the antimicrobial and antioxidant activities of *Pinus pinea* L. and *Pinus pinaster* Aiton essential oils. Additionally, the intra-species variations of essential oils and the volatile fractions obtained from twenty locations of two *Pinus* species are discussed using statistical analysis.

Material and methods

Plant Materials

The aerial parts of *P. pinea* and *P. pinaster* (needles, twigs and buds) were collected from twenty regions near Tlemcen (northwestern part of Algeria; Table 1). The voucher specimens of the plants were deposited in the Herbarium of the Laboratory of Botany, Department of Biology, University of Tlemcen (Algeria).

Essential oil isolation

Each fresh aerial part (400-500 g) was submitted to hydrodistillation for 5 h using a *Clevenger*-type apparatus according to the *European Pharmacopeia* (Council of Europe, 1997). The essential oils were stored in sealed vials in the dark at +4°C before analysis and bioassays tests. The obtained essential oils were yellow, with an aromatic odor.

Gas Chromatography (GC) Analysis

GC analysis was performed using a Perkin-Elmer Clarus 600 GC apparatus (Walhton, 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 follow: injector and detector temperatures were maintained at 280°C. Helium was used as carrier gas (1 $mL.min^{-1}$), 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 with an increase rate of $2°C.min^{-1}$ and then held isothermally at 230°C for 30 min.

Gas Chromatography - Mass Spectrometry (GC-MS) Analysis

The oils were analyzed using a Perkin Elmer Turbo Mass 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.

Component Identification and Quantification

Identification of the components was based (i) on the comparison of their GC retention indexes (RI_{ap} and RI_{p}) on non-polar and polar columns, respectively determined by the retention time of a series of *n*-alkanes with linear interpolation, with those of authentic compounds or literature data (Mc Lafferty and Stauffer, 1994; König et al., 2001); (ii) on computer matching with commercial mass spectral libraries (NIST, 1999; Brereton, 2003), and comparison of spectra with those of our laboratory made library.

Statistical analysis

Data analyses were performed using principal component analysis (PCA) and cluster analysis (Massart, 1998). Both methods aim at reducing the multivariate space in which objects (essential oil samples) are distributed but are complementary in their ability to present results (Yilmaz et al., 2013). Indeed, PCA provides the data for diagrams in which both objects (essential oil samples) and variables (essential oil components) are plotted while cluster analysis informs a classification tree in which objects (sample locations) are gathered. PCA was carried out using functional 'PCA' from the statistical R software. The variables (volatile components) were selected using a function from the statistical software. The cluster analysis produced a dendrogram (tree) using Ward's method of hierarchical clustering, based on the Euclidean distance between pairs of essential oil samples.

Antimicrobial activity

The antimicrobial activity of two essential oils was tested against twelve strains of bacteria: four gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10876), *Enterococcus faecalis* (ATCC 49452), *Lysteria monocytogenes* (ATCC 15313); eight gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 13311), *Acinetobacter baumanii*

Table 1. Origins, latitudes and longitudes of Algerian *Pinus pinea* and *Pinus pinaster*

Areas	Samples Regions		Latitudes	Longitudes	Altitudes
Area 1	Yaghomracen	S14-S17	34°56'11.16"	1°17'40,15"	814 m
	Ain defla	S1	34°53'13.43"	1°18'34.86"	771 m
	Chatouane	S3	34°53'15.27"	1°18'48.06"	784 m
	Bouhannak	L13-L17, S4	35°52'55.8"	1°22'05.31"	766 m
	Djebala	S6-S7, S9-S11	34°55'59.32"	1°49'35.40"	777 m
Area 2	Zenata	S2	35°02'19.21"	1°27'32.62"	280 m
	Oueled Hasna	S12, S21	35°03'01.79"	1°18'37.93"	241 m
	Bedar	S12-S13	35°55'14"	1°17'29"	131 m
	Behira	L1-L11	35°03'36.78"	1°58'11.07"	198 m

*S - *P. pinea* collecting sites; L - *P. pinaster* collecting sites

(ATCC 19606), *Citrobacter freundii* (ATCC 8090), *Proteus mirabilis* (ATCC 35659), *Klebsiella pneumonia* (ATCC 700603) and *Enterobacter cloacae* (ATCC 13047), and one yeast: *Candida albicans* (ATCC 444), *Candida albicans* (ATCC 10231), *Candida albicans* (ATCC 26790). The microorganisms were obtained from "Institut Pasteur de Paris (IPP)".

The antimicrobial activity was tested by the agar-well diffusion method (Mighri et al., 2010). All bacterial cultures were first grown on MHI agar (Mueller-Hinton infusion) plates at 37°C for 18–24 h prior to inoculation onto the nutrient agar. One or several colonies of similar morphology of the respective bacteria were transferred into API suspension medium and adjusted to 0.5 McFarland turbidity standard with a Densimat.

The inoculums of the respective bacteria were streaked onto MHI agar plates using a sterile swab. A sterile filter disc (diameter 6 mm, Whatman paper No. 3) was placed. The disc was impregnated by the tested essential oils (10µL/disc). The treated Petri dishes were placed at 4°C for 1–2 h and then incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the zone of growth inhibition around the discs after 24 h of incubation at 37°C.

The diameter of the inhibition zones of each of the discs was taken as measure of the antimicrobial activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded. The scale of measurement was as follows (disc diameter included) (Sheng-Hsien et al., 2007): ≥ 8 mm: good activity; 7.5-7.9 mm: average activity; 7-7.4 mm: moderate activity; 6.5-6.9: low activity; ≤ 6.4 mm: no activity.

Antioxidant Activity

The hydrogen atoms or electron-donating ability of the corresponding essential oil was determined from the bleaching of purple colored methanol solution of DPPH (Badarinath et al., 2010). This spectrophotometric assay uses the stable radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) as a reagent (Alam et al., 2013). A solution of 0.135 mM DPPH in methanol was prepared and 1.0 mL of this solution was mixed with 1.0 mL of extract in methanol at different concentrations (0.2 to 3 mg mL^{-1}). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 3 h. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid (AA) and gallic acid (GA) were used as positive controls. The ability to scavenge DPPH radical was calculated by the following equation:

$$\% \text{ DPPH radical scavenging} = [(A_0 - A_t)/A_0] \times 100$$

A_0: Absorbance of blank;

A_t: Absorbance of tested sample solution.

Results and discussion

In Table 2 are presented the components identified in the essential oils of *P. pinea* and *P. pinaster* aerial parts. To identify the relationship between the chemical compositions of oils, taxonomic classification and environmental parameters, principal component analysis (PCA) and cluster analysis (CA) were applied to a matrix linking essential oil composition to subspecies identity. The data mentioned in Table 2 and presented in Figures 1 - 4 were obtained from the correlation matrix and the standardized matrix. Preliminary analysis of twenty oil samples of each *P. pinea* and *P. pinaster* showed that their GC chromatograms were qualitatively similar but differed by the abundances of their main components. GC and GC-MS analysis of both combined essential oils of *P. pinea* and *P. pinaster* allowed for the identification of 52 and 64 compounds, respectively, accounting for 96.0% and 92.3% of the samples of essential oils (Table 2). The majority of the identified metabolites in *P. pinea* combined oil were found to be monoterpene hydrocarbons and sesquiterpene hydrocarbons. Monoterpene hydrocarbons account for about 75.8%. The principal component is limonene (56.5%), followed by α-pinene (6.5%), β-phellandrene (6.2%) and myrcene (2.3%). All essential oils contained other monoterpene hydrocarbons in trace amounts, which did not reach 2% in the oils. Sesquiterpene hydrocarbons (8.9%) found in the studied oils are mainly constituted of (E)-β-caryophyllene (4.6%). For the combined oil of *P. pinaster* among these compounds, monoterpene hydrocarbons (64.5 %), sesquiterpene hydrocarbons (14.4%), oxygenated monoterpenes (4.9%) and oxygenated sesquiterpenes (1.4%) were identified. The main components of this oil were: α-pinene (36.4%), β-pinene (12.3%), (E)-β-caryophyllene (7.1%), 3-carene (5.2%) and myrcene (4.9%). Previous studies found similar chemical compositions for *P. pinea* and *P. pinaster* essential oils, but in different proportions (Roussis et al., 1995; Hmamouchi, et al., 2001; Tumen et al., 2010; Nasri et al., 2011; Amri et al., 2012).

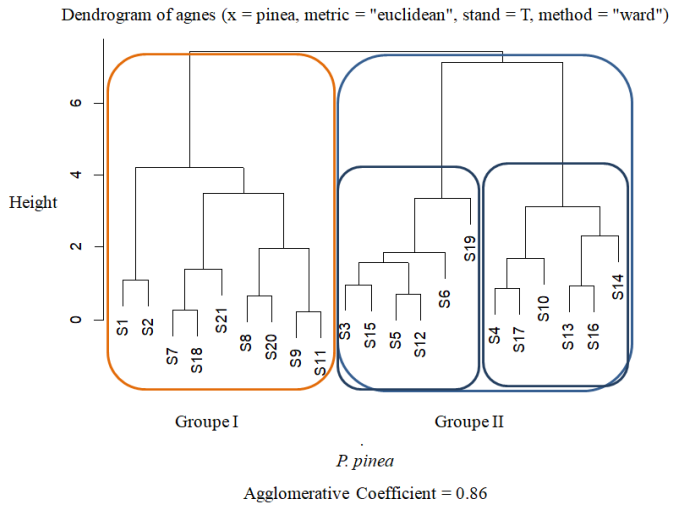

Figure 1. Cluster Analysis of chemical composition of essential oil of *P. pinea* from Algeria

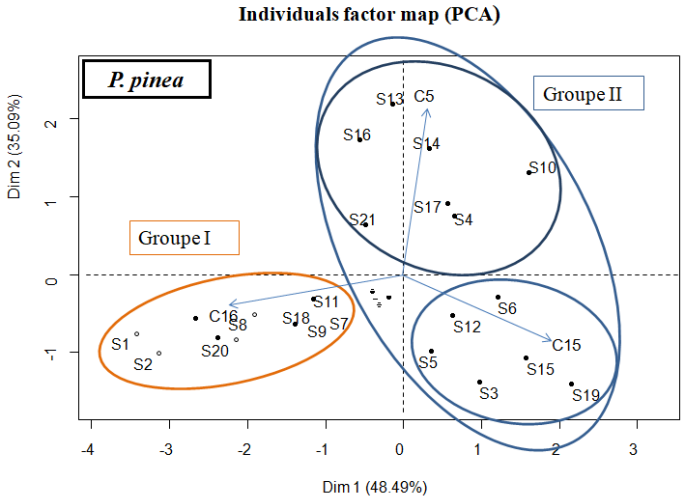

Figure 2. PCA of chemical composition of essential oils of *P. pinea* from Algeria

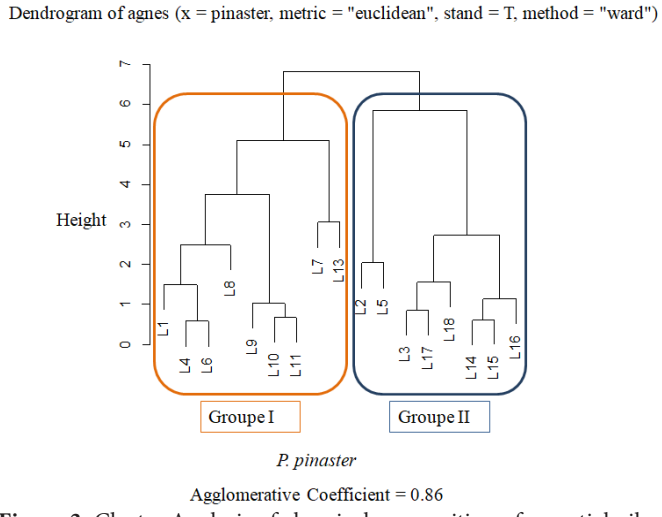

Figure 3. Cluster Analysis of chemical composition of essential oils of *P. pinaster* from Algeria

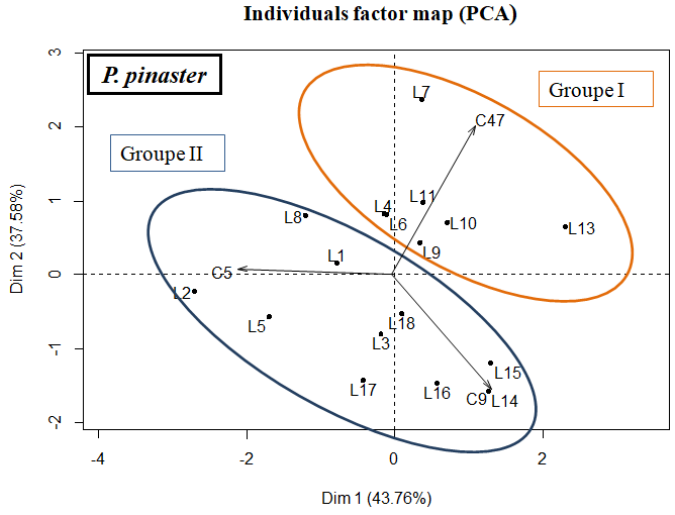

Figure 4. PCA of chemical composition of essential oils of *P. pinaster* from Algeria

The essential oil of *P. pinea* from Greece was studied and the major compounds were found to be limonene (39.05%), α-pinene (5.13%), β-phellandrene (13.8%) (Roussis et al., 1995). Analysis of essential oil of this plant collected from Turkey was also reported and the characteristic components were limonene (54.1%), α-pinene (7.7%), and β-pinene (3.4%) (Tumen et al., 2010; Amri et al., 2012). Nasri et al. (2011) analyzed the needle's essential oil of *P. pinea* from Tunisia. The most abundant components were limonene (35.9%) and α-pinene (6.4%). Other studies revealed different main compounds, e.g. the most abundant compound in the needles of *P. pinea* oil was β-pinene (37.0%), and β-caryophyllene (22.2%), in *P. pinaster* (Hmamouchi et al., 2001). This variability in chemical composition can be attributed to the species, part of the plant, geographical region, climate conditions, period of collection of the plant, and the method of essential oil extraction (Viljoen et al., 2005; Jordan et al., 2006; Mejri et al., 2010).

To identify the relationship between the chemical composition of essential oils from *P. pinea* and *P. pinaster*, volatile fractions and environmental PCA (Figs. 2 and 4) and cluster analysis (Figs. 1 and 3, Tables 3 and 4) were applied to a matrix linking volatile compositions to sample location from discriminate family compounds. The principal factorial plane constructed using axes 1 and 2 accounts for 58.59% and 51.60% of the variance of *P. pinea* and *P. pinaster*, respectively.

The antimicrobial activities of *P. pinea* and *P. pinaster* essential oils originating from the northwestern part of Algeria were evaluated by paper disc diffusion method against fifteen microorganisms. Table 5 showed that these oils have variable antimicrobial activities against all tested strains. From our results, the variation in quantities of different components might be responsible for the different antimicrobial activities. The inhibition zones of essential oils of *P. pinea* and *P. pinaster* were in the range of 6 to 23 mm and 6 to 12 mm, respectively. Most of the bacterial strains showed low susceptibility to the essential oil of *P. pinaster*. The essential oil of *P. pinea* however showed good bacteria inhibitory effects on some tested microorganisms. The maximum zone of inhibition was recorded against *C. albicans*

Table 2. Chemical composition of *Pinus pinea* and *Pinus pinaster*

N°	Compounds	IR_{Lit} [a]	IR_{ap} [b]	IR_{p} [c]	Combined Oil[d]	Simple Oils[e]		Combined Oil[d]	Simple Oils[e]		Identifications[f]
					P. pinaster	Min	Max	*P. pinea*	Min	Max	
1	Hexanal	770	774	1055	0.1	0.1	0.1	0.6	0.1	2.4	*RI, MS*
2	Heptanal	877	876	1187	0.1	nd	0.1	nd	nd	nd	*RI, MS*
3	Tricyclene	914	920	1020	0.1	nd	0.1	nd	nd	nd	*RI, MS*
4	α-Thujene	917	922	1023	0.1	nd	0.1	nd	nd	nd	*RI, MS*
5	α-Pinene	931	928	1022	36.4	18.0	72.7	6.5	3.4	11.2	*RI, MS*
6	Camphene	940	944	1066	0.9	nd	1.7	0.1	0.1	0.1	*RI, MS*
7	Thuja-2,4(10)-diene	942	946	1123	0.1	nd	0.3	nd	nd	nd	*RI, MS*
8	Sabinene	960	964	1120	nd	nd	0.3	0.7	0.1	2.1	*RI, MS*
9	β-Pinene	966	970	1110	12.3	nd	33.6	1.8	0.8	3.4	*RI, MS*
10	Myrcene	976	976	1159	4.9	nd	13.6	2.3	0.1	4.0	*RI, MS*
11	α-Phellandrene	989	997	1164	0.1	nd	0.2	0.3	0.1	0.5	*RI, MS*
12	3-Carene	1002	1005	1147	5.2	nd	15.0	nd	nd	nd	*RI, MS*
13	*p*-Cymene	1006	1011	1268	0.1	nd	0.3	0.1	0.1	0.3	*RI, MS*
14	α-Terpinene	1013	1010	1178	nd	nd	nd	0.2	0.1	0.4	*RI, MS*
15	Limonene	1015	1020	1199	2.4	0.1	7.8	56.5	44.4	73.8	*RI, MS*
16	β-Phellandrene	1023	1025	1208	1.1	nd	2.1	6.2	2.6	11.0	*RI, MS*
17	(E)-β-Ocimene	1041	1037	1247	0.1	nd	0.1	0.5	0.1	1.6	*RI, MS*
18	γ-Terpinene	1047	1049	1243	0.1	nd	0.2	0.1	0.1	0.3	*RI, MS*
19	trans-Linalol oxide	1058	1058	1435	nd	nd	nd	0.2	0.1	0.4	*RI, MS*
20	*p*-Cymenene	1075	1072	1432	0.5	nd	1.3	0.3	0.1	0.6	*RI, MS*
21	Terpinolene	1082	1080	1275	0.1	nd	1.7	0.2	0.1	0.5	*RI, MS*
22	Linalool	1086	1084	1544	0.3	nd	1.8	0.3	0.1	1.4	*RI, MS*
23	Camphor	1117	1123	1517	0.3	nd	0.6	nd	nd	nd	*RI, MS*

N°	Compounds	IR_{Lit} [a]	IR_{ap} [b]	IR_{p} [c]	Combined Oil [d]	Simple Oils [e]		Combined Oil [d]	Simple Oils [e]		Identifications [f]
					P. pinaster	Min	Max	*P. pinea*	Min	Max	
24	cis-Verbenol	1124	1127	1655	0.1	nd	0.2	0.1	0.1	0.1	*RI, MS*
25	(E)-Limonene-1,2-epoxyde	—	1124	1456	nd	nd	nd	0.1	0.1	0.2	*RI, MS Ref*
26	Pinocarvone	1133	1136	1558	0.2	nd	0.3	nd	nd	nd	*RI, MS*
27	Lyratol	1140	1150	1780	0.2	nd	0.1	nd	nd	nd	*RI, MS*
28	Terpinen-4-ol	1154	1161	1600	0.2	nd	0.5	0.4	0.1	1.0	*RI, MS*
29	*p*-Cymen-8-ol	1154	1161	1833	0.2	nd	0.3	nd	nd	nd	*RI, MS*
30	Cryptone	1160	1156	1667	0.2	nd	0.3	0.2	0.1	0.5	*RI, MS*
31	Myrtenal	1164	1172	1628	2.0	nd	7.5	0.3	0.1	0.5	*RI, MS*
32	α-Terpineol	1176	1700	1700	0.8	nd	2.1	0.8	0.1	3.0	*RI, MS*
33	Verbenone	1176	1184	1707	0.2	nd	0.6	nd	nd	nd	*RI, MS*
34	Decanal	1180	1185	1498	0.1	nd	0.1	nd	nd	nd	*RI, MS*
35	Thymyl methyl oxyde	1210	1214	1589	0.1	nd	0.1	1.2	0.8	2.1	*RI, MS*
36	cis-3-Hexenyl 2-methylbutyrate	1213	1220	1457	0.1	nd	0.1	nd	nd	nd	*RI, MS*
37	Hexyl isovalerate	1217	1220	1442	0.1	nd	0.1	nd	nd	nd	*RI, MS*
38	Perillaldehyde	1248	1246	1784	nd	nd	nd	0.5	0.1	1.4	*RI, MS*
39	Bornyl acetate	1264	1269	1575	nd	nd	0.5	0.3	0.2	0.5	*RI, MS*
40	Tridecane	1300	1292	1300	0.1	nd	0.3	nd	nd	nd	*RI, MS*
41	α-Cubebene	1344	1350	1452	0.2	nd	0.4	nd	nd	nd	*RI, MS*
42	Geranyl acetate	1357	1361	1752	0.3	nd	0.8	nd	nd	nd	*RI, MS*
43	α-Copaene	1371	1379	1488	0.6	nd	3.8	nd	nd	nd	*RI, MS*
44	α-Ylangene	1376	1371	1476	nd	nd	nd	0.2	0.1	0.3	*RI, MS*
45	Dodecanal	1389	1382	1708	0.1	nd	0.4	nd	nd	nd	*RI, MS*
46	Longifolene	1400	1413	1571	0.9	nd	3.4	1.2	nd	1.1	*RI, MS*
47	(E)-β-Caryophyllene	1414	1424	1591	7.1	0.3	25.1	4.6	1.7	8.1	*RI, MS*

N°	Compounds	IR_{Lit} [a]	IR_{ap} [b]	IR_{p} [c]	Combined Oil [d]	Simple Oils [e]		Combined Oil [d]	Simple Oils [e]		Identifications [f]
					P. pinaster	Min	Max	*P. pinea*	Min	Max	
48	α-Guaiene	1440	1443	1583	nd	nd	nd	0.4	0.1	1.5	*RI, MS*
49	α-Humulene	1446	1456	1665	2.6	tr	30.7	0.6	0.1	1.2	*RI, MS*
50	2-Phenylethyl isovalerate	1463	1465	1980	3.3	0.4	10.4	0.6	0.1	2.5	*RI, MS*
51	Germacrene	1473	1480	1704	1.3	nd	4.7	0.7	0.2	1.3	*RI, MS*
52	α-Muurolene	1496	1490	1719	0.2	nd	0.4	nd	nd	nd	*RI, MS*
53	γ-Cadinene	1507	1513	1752	0.6	0.1	5.0	0.2	0.1	0.6	*RI, MS*
54	(E)-α-Bisabolene	1530	1513	1753	0.1	nd	0.4	0.5	0.1	1.5	*RI, MS*
55	β-Elemol	1541	1533	2044	nd	nd	nd	0.3	0.1	1.1	*RI, MS*
56	trans-Calamenene	1566	1576	1980	0.5	nd	1.9	nd	nd	nd	*RI, MS*
57	(E)-Nerolidol	1586	1591	—	0.2	nd	0.7	nd	nd	nd	*RI, MS*
58	Globulol	1589	1579	2074	nd	nd	nd	0.6	0.1	1.6	*RI, MS*
59	Viridiflor	1592	1592	2089	nd	nd	nd	0.3	0.1	0.8	*RI, MS*
60	Guaiol	1593	1584	2090	0.2	nd	0.5	0.3	0.1	0.8	*RI, MS*
61	Humulene epoxide II	1602	1602	2044	0.1	nd	0.4	0.2	0.1	0.4	*RI, MS*
62	Phenylethyl tiglate	1613	1608	2031	0.2	nd	0.3	nd	nd	nd	*RI, MS*
63	Dodecanoic acid	1624	1627	2096	0.5	nd	2.1	nd	nd	nd	*RI, MS*
64	β-Eudesmol	1641	1651	2232	nd	nd	nd	0.1	0.1	0.2	*RI, MS*
65	Benzyl benzoate	1730	1723	—	nd	nd	nd	0.6	0.1	0.6	*RI, MS Ref*
66	(E, E)-Farnesyl acetate	1816	1793	2123	0.2	nd	0.5	nd	nd	nd	*RI, MS*
67	Caryophyllene oxide	1816	1793	2123	0.5	nd	1.3	0.8	0.1	2.7	*RI, MS*
68	Geranyl 2-methylbutyrate	1828	1817	—	0.1	nd	0.4	nd	nd	nd	*RI, MS*
69	Hexadecanoic acid	1951	1953	—	nd	nd	nd	0.7	nd	3.3	*RI, MS Ref*
70	Manoyl oxide	2007	1997	2346	nd	nd	nd	0.8	0.1	2.9	*RI, MS*
71	Abieta-8,12-diene	2010	2046	—	1.4	nd	3.4	nd	nd	nd	*RI, MS*

N°	Compounds	IR_{Lit} [a]	IR_{ap} [b]	IR_{p} [c]	Combined Oil[d]	Simple Oils[e]		Combined Oil[d]	Simple Oils[e]		Identifications[f]
					P. pinaster	Min	Max	*P. pinea*	Min	Max	
72	*n*-Heneicosane	2100	2099	2100	nd	nd	nd	0.5	0.1	0.5	*RI, MS*
73	Abieta-8(14),13(15)-diene	2133	2152	—	0.5	nd	2.3	nd	nd	nd	*RI, MS*
74	*n*-Docosane	2200	2199	—	nd	nd	nd	0.3	0.1	0.3	*RI, MS*
75	*n*-Tricosane	2301	2299	—	nd	nd	nd	0.3	0.1	0.3	*RI, MS*
76	Pentacosane	2498	2499	—	nd	nd	nd	0.4	0.1	0.4	*RI, MS*
% Total Identification					*92.3*			*96.0*			
% Yields (w/w)					0.08			0.24			
% Hydrocarbon compounds					*78.9*			*84.7*			
% Monoterpene hydrocarbons					64.5			75.8			
% Sesquiterpene hydrocarbons					14.4			8.9			
% Oxygenated compounds					*6.3*			*4.8*			
% Oxygenated monoterpenes					4.9			2.4			
% Oxygenated sesquiterpenes					1.4			2.4			
% Oxygenated no-terpenic compounds					*5.3*			*5.5*			
% Other derivatives					*1.8*			*1.0*			

[a]: retention indices of literature on the apolar column (RI_{Lit}) reported from König et al. (2001); [b]: retention indices on the apolar Rtx-1 column; [c]: retention indices on the polar Rtx-wax column; [d]: normalized percentages abundance are given on the apolar column except for component with identical *RI* (percentages are given on the polar column); [e]: minimum and maximum normalized percentages abundances from simple oils; tr: trace (<0.05%); [f]: RI: retention Indices; MS: Mass Spectra in electronic impact mode; *Ref*: compounds identified from literature data: König et al. (2001).

(ATCC 444): 23 mm followed by *C. albicans* (ATCC 26790: 12 mm. In contrast, the oils did not show bacterial inhibitory effects on *E. cloacae* (ATCC 13047) and *P. aeruginosa* (ATCC 27853).

The antimicrobial activity of *P. pinea* essential oil is seemingly related to the oxygenated monoterpenes. However, it is difficult to attribute the activity of a complex mixture to a single or particular constituent. Major or trace compounds might give rise to the antimicrobial activity. In oils, a possible synergy of two or more compounds is very likely to play an important role in bacterial inhibition (Cox et al., 2000).

Table 3. Clustering of *Pinus pinea* oils samples by statistical analysis

Components	Group I (S12,S13,S19,S21)		Group II (S3-S11,S15-S18)	
	Range[a]	Average[a]	Range[a]	Average[a]
C5 α-Pinene	3.4-11.2	6.8	4.73-8.07	6.3
C8 β-Pinene	1-3.4	2.2	0.8-2.1	1.5
C9 Myrcene	0-2.5	0.5	0-4	2.4
C13 Limonene	51.4-73.8	58.5	44.4-65.3	57.1
C14 β-Phellandrene	5-8.6	6.5	3.3-8	5.3
C26 α-Terpineol	0-0.1	0.04	0-3	0.3
C34 (E)-β-Caryophyllene	1.9-4.4	3.4	1.7-8.2	5.2
C53 Hexadecanoic acid	0-3.3	1.01	0-3.2	1.8

[a]: normalized % abundances

Antioxidant properties of essential oils were evaluated with the aim of discovering a new natural source of antioxidant. The potential antioxidant activities of the *P. pinea* and *P. pinaster* essential oils were determined on the basis of the scavenging activity of the free stable radical DPPH (2,2-diphenyl-1-picryl hydrazyl), and the activity was compared with *two* antioxidants, ascorbic acid (AA) and gallic acid (GA), which were used as reference standards. DPPH radical scavenging is considered as a good *in vitro* model widely used to assess antioxidant efficacy within a very short time. In its radical form, DPPH is reduced by an antioxidant compound or a radical species, to become a stable diamagnetic molecule, a process accompanied with a change from purple to yellow; such color change is an indication of the hydrogen-donating ability of the tested sample (Lee et al., 2007; Marxen et al., 2007; Allali et al., 2013). DPPH assay is a stable free radical method. It is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extract (Koleva et al., 2002). The free radical scavenging activity of the tested samples was evaluated based on the ability to scavenge the synthetic DPPH (Rackova et al., 2007; Alizadeh et al., 2013; Sathya et al., 2013). The results obtained at different concentrations are shown in Figure 5. The best percentages of antioxidant activities were observed for the synthetic antioxidants AA and GA, generally used in food industry, more evident for low concentrations. At 12 mg mL^{-1}, the percentages of DPPH reduction reached 94.5% for AA and 92.6% for GA after 15 min of incubation time.

Table 4. Clustering of *Pinus pinaster* oils samples by statistical analysis

Components	Group I (L13-L17)		Group II (L1-L11)	
	Range[a]	Average[a]	Range[a]	Average[a]
C5 α-Pinene	18-40.7	34.6	0-72.7	39.14
C9 β-Pinene	19.6-33.6	*25.4*	0-15.6	4.9
C10 Myrcene	1-8.7	3.5	0-13.6	5.3
C12 3-Carene	0-0.1	0.1	0-15	*7.2*
C29 Myrtenal	0-7.5	4.3	0.1-3.1	0.7
C36 (E)-β-Caryophyllene	0.3-25.1	4.9	2-15	5.7
C37 α-Humulene	0-30.7	1.02	0.1-2.4	4.05
C69 2-Phenylethyl isovalerate	1.1-10.4	5.7	0.4-3.5	1.6

[a]: normalized % abundances

For essential oils, the antioxidant capacity was dependent on the used concentrations. As shown in Figure 5, the antioxidant activity of *P. pinea* increased with the increase of its concentration from 0.46 to 12 mg mL^{-1}. Indeed, for the high concentration of 12 mg mL^{-1} of *P. pinea* essential oil, after 3 h of incubation time at room temperature the percentage of DPPH reduction reached 88.3%, while the percentage was only 13.8% for low concentration (0.46 mg mL^{-1}). The oil of *P. pinaster* showed the highest antioxidant capacity at 12 mg mL^{-1} (47.9% after 3 h incubation time). From these results, we can conclude that the antioxidant activity depends on the chemical composition of *P. pinea* and *P. pinaster*. The results showed that the inhibitory activity of the essential oil of *P. pinea* at concentration 12 mg mL^{-1} was higher than 50%, after 9 min of incubation time at room temperature.

Table 5. Antimicrobial activity of *Pinus pinea* and *Pinus pinaster* essential oils from the north-west of Algeria

Microorganisms	Diameters of inhibition (mm)	
Gram-negative bacteria	*P. pinea*	*P. pinaster*
Pseudomonas aeruginosa ATCC 27853	na	7
Escherichia coli ATCC 25922	6	6
Salmonella typhimurium ATCC 13311	6	6
Acinetobacter baumanii ATCC 19606	6	6
Klebsiella pneumoniae ATCC 700603	12	6
Enterobacter cloacae ATCC 13047	na	na
Citrobacter freundii ATCC 8090	6	6
Proteus mirabilis ATCC 35659	6	6
Gram-positive bacteria		
Staphylococcus aureus ATCC 25923	6	na
Bacillus cereus ATCC 10876	10	6
Enterococcus faecalis ATCC 49452	6	12
Lysteria monocytogenes ATCC 15313	6	6
Yeast		
Candida albicans ATCC 444	23	6
Candida albicans ATCC 10231	6	10
Candida albicans ATCC 26790	12	6

*na: not active

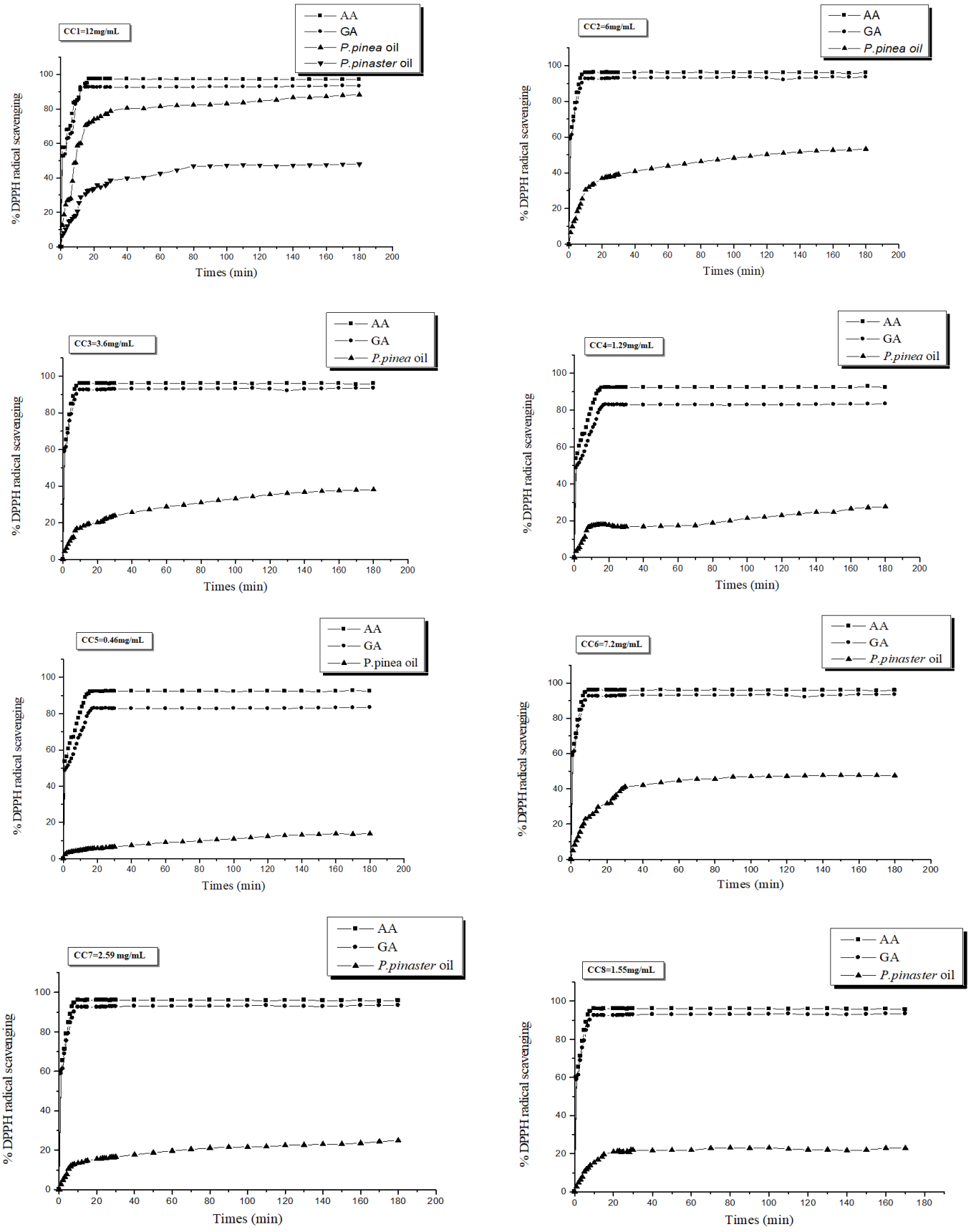

Figure 5. Percentage of DPPH radical scavenging as a function of reaction time for essential oils extracted from the aerial parts of *P. pinea* and *P. pinaster* and tested at different concentrations. AA, ascorbic acid; GA, gallic acid.

It is possible to conclude that the essential oil isolated from *P. pinea* was able to give a proton to the stable radical DPPH. Additionally, the inhibitory activity of the *P. pinea* essential oil (88.3% at 12 mg mL^{-1}) was considerably high for its inhibition ability when compared to the positive controls GA (93.3%) and AA (97.1%).

Antioxidant activities of essential oils from aromatic plants are mainly attributed to the compounds present therein. This may be due to the high percentage of the main compounds, but also to the presence of others constituents in small quantities or to a synergistic effect (Younes et al., 2012).

While the anti-oxidant activity of natural compounds is widely described, little information is reported on their kinetic behavior, which is an important factor in the reduction process. In terms of reaction kinetics, Yen and Duh (1994) postulated that the faster the absorbance decreases, the more potent is the antioxidant activity of the sample. Halliwell (1990) linked the antioxidant power to two parameters: first, the ability to prevent the autoxidation of the free radical at low concentrations and second, the stability of resulting radical after scavenging. Es-Safi et al. (2007) also showed the evolution of the remaining DPPH with time of each compound's family (flavonoids, iridoids, etc.). They showed that compounds have a low kinetic behavior. Figure 5 shows the evolution of the percentage of DPPH reduction with time for each sample at different concentrations. It showed that AA and GA have a rapid kinetic behavior. At 17 and 19 min, they reached the maximum percentage of reduction, respectively. For the AA, this confirms the result obtained by Cal et al. (2003). In contrast, the essential oils at different concentrations have slow kinetic behavior (equilibrium time is greater than 30 min). According to Yen and Duh (1994), the samples AA and GA are more potent than *P. pinea*.

The IC_{50} is conversely related to a compound antioxidant capacity because it expresses the necessary quantity of antioxidant and decreases the concentration of the free radical for 50%. The lower value of IC_{50} has the most important antioxidant activity (Chanda and Dave, 2009). The essential oils of *P. pinea* and *P. pinaste*r could bring back the stable free radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) to the yellow colored diphenylpicrylhydrazine (DPPH) with IC_{50} of 4 mg mL^{-1} and 12 mg mL^{-1} respectively, showing an antioxidant activity lower than that of AA. It seems from these results, that AA is the most effective antioxidant with an IC_{50} of 4 µg mL^{-1} compared to the studied essential oils. The presence of oxygenated compounds and acids in *P. pinea* essential oil may be the reason of their considerable radical-scavenging activity.

Conclusion

The results of current study suggest that the presence of oxygenated compounds and acids in *P. pinea* essential oil may be the main reason of their good bacteria inhibitory effects against *Candida albicans* and their considerable radical-scavenging activity compared to the synthetic antioxidant ascorbic acid and gallic acid that were used as antioxidant references.

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Conflict of interest

None

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