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Essential oils composition and antimicrobial activity of six conifers harvested in Lebanon

by Layal Fahed[a)b)], Madona Khoury[a)b)], Didier Stien[a)c)], Naïm Ouaini[b)], Véronique Eparvier[a)], and Marc El Beyrouthy*[b)]

[a)] CNRS - Institut de Chimie des Substances Naturelles, 1 Avenue de la Terrasse, Gif-sur-Yvette, FR-91198 France

[b)] Department of Agricultural Sciences, Holy Spirit University of Kaslik, Kaslik, Jounieh, B.P. 446 Lebanon

[c)] Laboratoire de Biodiversité et Biotechnologies Microbiennes (LBBM), Observatoire Oceanologique, UPMC Univ Paris 06, CNRS, Sorbonne Universités, Banyuls/Mer, FR-66650 France

Abstract:

The chemical composition and antimicrobial activity of the essential oils (EOs) of six conifers harvested in Lebanon, *Abies cilicica*, *Cupressus sempervirens*, *Juniperus excelsa*, *Juniperus oxycedrus*, *Cedrus libani* and *Cupressus macrocarpa* gold crest, were investigated. The EOs were obtained by hydrodistillation using a Clevenger-type apparatus and characterized by GC and GC-MS analyses. A principal components analysis based on Pearson correlation between essential oils chemical analyses was also conducted. The minimum inhibitory concentrations (MICs) of these essentials oils were determined against a range of bacteria and fungi responsible for cutaneous infections in human, using the broth microdilution technique. The EOs showed the most interesting bioactivity on the dermatophytes species (MIC values 32-64 µg/ml). Each of the major compounds of *C. macrocarpa* as well as an artificial reconstructed EO were tested on *Trichophyton rubrum* showing a contribution of the minor components to the overall activity**.**

Keywords:

Chemical composition, Antimicrobial activity, Essential oils, Conifers, Lebanon

Introduction

Conifers are woody plants with most of them being evergreen trees. About 630 species of conifers can be found as wild populations in all continents except Antarctica [1]. In Lebanon they are represented by ten species belonging to five genera [2]. *Cedrus libani* A.Rich. , the national emblem of Lebanon, was mentioned in the Old Testament of the Holy Bible over 75 times and in the Epic of Gilgamesh, the oldest recorded legend [1]. Its wood was intensively exploited by ancient civilizations in the building of temples, palaces, and boats [3], and its essential oil was used by the Egyptians in the mummification process [4]. Lebanese conifer species are an important part of the folk medicine systems. *Abies cilicica* (Antoine & Kotschy) Carrière a plant endemic to Lebanon, Syria and asiatic Turkey is traditionally used in Lebanon for the treatment of rheumatism and lumbago, and is used in Turkey against gastrointestinal diseases, asthma and bronchitis [5] [6]. *Cupressus sempervirens* L. f. *horizontalis* (Mill.) Voss is known as an antiasthmatic, antitussive, antiodontalgic, antiulcer and antirheumatic herbal remedy, while *Juniperus* species *J. excelsa* M. Bieb. and *J. oxycedrus* L. are priced for their antirheumatic and antineuralgic properties [7]. Beside indigenous species, many conifers are introduced in Lebanon generally for ornamental purposes. This is the case of *Cupressus macrocarpa* Hartw. goldcrest, a plant used popularly in interior landscaping due to its bright color and agreeable lemon fragrance.

Antimicrobial resistance is a global concern requiring urgent actions across all concerned sectors. This phenomenon is accelerated by the excessive and inappropriate use of antimicrobial agents [8].

In an attempt to search for alternatives to conventional antibiotics and antifungals for the treatment of topical, non severe infections, the essential oils of the six above-mentioned conifer species were subjected to chemical analysis and evaluation of their antimicrobial activity.

Results and Discussion

Chemical Analysis

The chemical compositions of the six conifers essential oils are presented in table 1. The yields of the EOs obtained by hydrodistillation were as follows: 0.08% (*J. oxycedrus*), 0.29% (*C. sempervirens*), 0.34% (*C. libani*), 0.68% (*C. macrocarpa*), 0.93% (*A. cilicica*) and 1.1% (*J. excelsa*). GC and GC-MS analyses led to the identification of 91 components accounting for 81.6% (*A. cilicica*) to 94.3% (*C. macrocarpa*) of the oils.

Please insert table 1 here

The data matrix was used to generate a biplot superimposing principal component analysis (vectors) with a map of the variables (i.e. molecules) (*Fig. 1*). Loading factors 1 and 2 in PCA described 77.5% of the variability in the dataset. It can be seen that 4 out of 6 EOs clustered in one chemical group, with a high relative proportion of α-pinene and a lower proportion of β-pinene. Two outliers are present in the dataset, *A. cilicica* and *C. macrocarpa* EOs. *A. cilicica* EO contains a significant amount of α-pinene (8.5%) and is therefore not orthogonal to the group of α-pinene rich oils. What makes it peculiar in this dataset is the high proportion of δ-3-carene, as indicated by the

biplot. As regards *C. macrocarpa* EO, it is orthogonal to the others and its specific descriptors are sabinene, terpinen-4-ol and citronellol. These compounds are either absent or minorly present in the other EOs.

Please insert figure 1 here

Antimicrobial activity

The results of the antimicrobial activity of the conifers EOs are reported in table 2. EOs with minimal inhibitory concentrations (MICs) of 128 µg/ml and below are generally considered active [9] [10].

S. aureus was significantly sensitive to *J. excelsa*, *J. oxycedrus* and *C. libani* EOs (MIC of 64 µg/ml) and resistant to three others (MIC > 512 µg/ml). According to the MICs values reported in the literature, α-pinene, the main component of the active oils, couldn't be responsible for their activities [11] [12]. This goes along with the fact that α-pinene is also mainly present in two of the inactive oils. On the other hand, none of the EOs was active against *P. aeruginosa*, *E. coli* or *C. albicans* (MIC ≥ 512 µg/ml). Conversely, *Trichophyton* species were sensitive to most of the tested EOs (MICs from 32 to 512 µg/ml).

Please insert table 2 here

In order to elucidate the origin of the interesting activity of *C. macrocarpa* on the dermatophytes (MICs from 32 to 64 µg/ml), the activity of its major components were evaluated individually as well as a reconstructed EO against *Trichophyton rubrum* (*Table 3*). The artificial EO was prepared using pure terpenes of the major compounds

in the same proportions, whereas the minor ones were replaced by DMSO (25.8%). Terpinene-4-ol was the most active compound (MIC of 64 µg/ml), suggesting a strong contribution to the antifungal activity of the oil. The other components showed less significant activities (MICs from 256 to 512 µg/ml).

The reconstructed EO was less active than the natural one (256 µg/ml *vs* 64 µg/ml), therefore the activity of *C. macrocarpa* EO couldn't be explained by a synergy between its major compounds. These results suggest that the minor constituents have played a significant role in the antimicrobial potential of the oil.

Please insert table 3 here

Conclusions

Lebanese conifers essential oils showed interesting antimicrobial activity particularly against *S. aureus* and *Trichophyton species*. These EOs should therefore be considered for a possible integration in antimicrobial preparations intended to be used in the treatment of superficial infections.

Acknowledgments

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Experimental Part

Plant Material

Specimens of the six conifers were collected as follows:

- Leaves from *Abies cilicica* were collected from Fneidek, North Lebanon (34°27'54.48" N 36°13'10.74" E), at approximately 1560 m altitude, in January 2013.

- Cones from *Cedrus libani* were collected from Bcharre, North Lebanon (34°10'40.91" N 36°02'55.89" E), at approximately 1916 m altitude in September 2011.

- Leaves from *Cupressus macrocarpa* were collected from Achkout, Mount Lebanon (33°59'33.16" N 35°41'59.48" E), at approximately 1048 m altitude, in January 2013.

- Leaves from *Cupressus sempervirens* were collected from Kartaba, Mount Lebanon (34° 5'42.71" N 35°50'51.18" E), at approximately 1052 m altitude in December 2012.

- Twigs from *Juniperus excelsa* were collected from Kartaba, Mount Lebanon, (34°05'55.35" N 35°50'27.77" E), at approximately 1400 m altitude in October 2011.

- Twigs from *Juniperus oxycedrus* were collected from Kartaba, Mount Lebanon, (34°05'55.35" N 35°50'27.77" E), at approximately 1400 m altitude in September 2011.

The plants identification was based on 'Nouvelle flore du Liban et de la Syrie' by Paul Mouterde [13]. Air drying of the plant materiel was performed in a shady place for two weeks at room temperature.

Essential Oil Extraction

The extraction of the essential oils was carried out by hydrodistillation for three hours using a Clevenger-type apparatus according to the European Pharmacopoeia, 1997 [14].

GC Analyses

Analytical gas chromatography was performed on a Thermo Electron Corporation gas chromatograph fitted with a flame ionization detector (FID), a DB-5 MS capillary column (30 m × 0.25 mm) with 0.1 µm film thickness or a fused silica HP Innowax polyethylene glycol capillary column (50 m × 0.20 mm, film thickness 0.20 µm). Helium was the carrier gas (0.7 ml/min). The column temperature was initially set to 35 ˚C before being gradually increased to 85 ˚C at 5 ˚C/min, held for 20 min at 85 ˚C, raised to 300 ˚C at 10 ˚C /min and finally held for 5 min at 300 ˚C. Diluted 1 µl samples (1/100, v/v) were injected at 250 ˚C manually and in the splitless mode. Flame ionisation detection (FID) was performed at 310 ˚C.

GC–MS Analyses

The GC/MS analyses were performed using an Agilent 6890 gas chromatograph coupled with 5975 Mass Detector. The 7683 B auto sampler injected 1 µL of each diluted oil sample (1/100, v/v). A fused silica capillary column DB-5 MS (30 m × 0.25 mm internal diameter, film thickener 0.1 µm) or a fused silica HP Innowax polyethylene glycol capillary column (50 m × 0.20 mm, film thickness 0.20 µm) was used. Helium was the carrier gas (0.7 ml/min). The oven temperature program was identical to that described above (cf. GC Analysis). The mass spectra were recorded at 70 eV with an ion source temperature of 310 ˚C and a transfer line heated to 320 ˚C. The acquisition was recorded in full scan mode (50 – 400 amu).

Qualitative and Quantitative Analyses

Most constituents were identified by gas chromatography by comparing their retention indices (RI) with those from the literature [15] [16], or with those of authentic compounds obtained from Sigma-Aldrich (Lebanon and France). The retention indices were determined relatively to a homologous series of *n*-alkanes (C8 to C24) analysed under the same operating conditions. Concomitantly, their mass spectra on both columns were compared with those provided in the NIST and Wiley 275 libraries, our home-made library constructed with pure compounds and EOs of known composition or with mass spectra from the literature [15] [17].The relative concentration of each compound was calculated based on the GC–FID peak areas without using correction factors.

Statistical analysis

A principal components analysis based on Pearson correlation between essential oils chemical analyses was conducted with XLstat version 2014. In the biplot, samples (EOs) are displayed as vectors and EO components are displayed as points.

Antimicrobial activity

Gram (–) bacterial strain *Pseudomonas aeruginosa* CIP 82118, Gram (+) bacterial strain *Staphylococcus aureus* ATCC 29213, yeast *Candida albicans* ATCC 10231 and the clinical isolates of dermatophyte Trichophyton rubrum SNB-TR1, *Trichophyton mentagrophytes* SNB-TM1, *Trichophyton soudanense* SNB-TS1, *Trichophyton violaceum* SNB-TV1 and *Trichophyton tonsurans* SNB-TT1 [18], were used in this study.

The antimicrobial activity of the EOs was measured using a broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines with minor modifications [19 – 24]. The essential oils and its major components were diluted in DMSO and were tested at concentrations ranging from 512 to 16 μg/ml. The maximum DMSO concentration didn't exceed 5% (v/v). Sterility and positive controls in Müller-Hinton broth (MHB) or Roswell Park Memorial institute (RPMI) medium alone and with 5% of DMSO (v/v) were included. Positive control wells contained microorganisms without antibiotics or antifungals. The microplates were incubated at 37°C for 24 h for bacteria, and at 35°C for 48 h for yeasts and 5 days for dermatophytes. The minimal inhibitory concentrations (MIC) refer to the lowest concentrations preventing visible microbial growth (*Table 2*, *Table 3*). Oxacillin and gentamicin (16 – 0.03 μg/ml) were used as reference antibiotics, while itraconazole (16 – 0.03 μg/ml) and fluconazole (64 – 0.125 μg/ml) were used as positive controls for the antifungal assays. The antimicrobial standards were purchased from Molekula – Dorset, UK and the pure terpenes and the culture media from Sigma-Aldrich, France.

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Table 1. Chemical composition of six conifers essential oils

			Compounds (%)					
Compounds	**RI[a]**	**RI[b]**	***Abies cilicica***	***Cedrus libani***	***Cupressus macrocarpa* Gold crest**	***Cupressus sempervirens***	***Juniperus excelsa***	***Juniperus oxycedrus***
Santene	889	1001	1.5					
Tricyclene	925	1030	0.7			0.2		
α-Thujene	929	1035			1.0			
α-Pinene	938	1076	**8.5**	**39.7**	1.6	**57.5**	**38.9**	**44.7**
Camphene	953	1076	**7.4**	0.5	0.2		0.7	0.1
Sabinene	973	1132		0.2	**24.9**	0.5	0.1	
β-Pinene	980	1118	2.1	**14.7**		**3.0**	0.5	2.4
Myrcene	993	1174	0.9	1.4	2.4	2.4	0.3	0.5
α-Phellandrene	1005	1188	0.7	0.1	0.4			
δ-3-Carene	1013	1159	**14.5**	0.1			**4.2**	0.2
α-Terpinene	1013	1188			**3.8**	0.3		
p-Cymene	1025	1280	0.6	0.3	1.3		0.4	0.1
Sylvestrene	1027	1176			2.4			
Limonene	1030	1203		1.3		**3.7**	0.9	2.6
β-Phellandrene	1033	1218	**3.4**					
cis-β-ocimene	1045	1269				0.2		
γ-Terpinene	1057	1255	0.8	0.1	**6.5**	0.4	0.1	
cis-Sabinene hydrate	1058	1556			0.7			
α-Fenchone	1079	1401		0.1				
Terpinolene	1086	1265	1.4	0.2	2.4	1.7	0.6	0.3
trans-Sabinene hydrate	1096	1474			0.4			
Linalool	1098	1553	0.7		1.9	0.8		0.1
cis-p-Mentha-2-en-1-ol	1117	-		0.1	0.9			
α-Campholenal	1128	1487					0.7	0.9
trans-Pinocarveol	1138	1664		0.4			0.6	1.9
trans-p-Mentha-2-en-1-ol	1139	-			0.4			
Camphor	1145	1532			**3.1**		0.5	
trans-Verbenol	1152	1683					1.1	
Citronellal	1155	1486			**4.9**			
Pinocarvone	1163	1587					0.1	
Borneol	1167	1719	0.5	0.2			0.1	1.4
Terpinen-4-ol	1176	1611	0.8	0.3	**20.7**	1.4		0.8
p-Cymen-8-ol	1182	1864		0.1			0.2	0.4
α-Terpineole	1189	1706	0.6	1.4	1.6	0.6		3.1
cis-Piperitol	1192	1674			0.2			
Myrtenal	1193	1648		0.2				0.2
Myrtenol	1196	1804		0.2				0.1
trans-Verbenone	1217	1725		0.3			0.3	0.6
trans-Piperitol	1217	1741			0.3			
trans-Carveol	1218	1845		0.2			0.3	0.5

Citronellol	1220	1772			**10.3**			
Myrtenyl acetate	1227	1698		0.1				
Carvone	1241	1752						0.3
Linalyl acetate	1259	1665	0.7					
Bornyl acetate	1285	1597	**4.4**	0.1		0.3	0.1	1.6
2-Undecanone	1298	1585			0.2			
Carvacrol	1299	2239						0.3
Citronellic acid	1312	-			0.5			
α-Terpinyl acetate	1333	1709	0.8			**3.0**		
α-Cubebene	1352	1466		0.1				**4.5**
α-Longipinene	1355	-	**3.0**					
Longicyclene	1368	-	0.8					
Ylangene	1372	1493	0.6					
α-Copaene	1377	1497		0.5				1.3
Neryl acetate	1379	1760		0.3				
Longifolene	1407	1574	2.4					
Cedrene	1411	1568		0.3			1.4	
β-Caryophyllene	1415	1612	**5.6**	0.2		0.6		1.1
Himachala-2,4-diene	1434	-	**6.3**					
trans-α-Bergamotene	1436	1573					0.1	
α-Himachalene	1447	1648	0.9					
α-Humulene	1452	1668	2.0	0.2		0.4		0.4
β-Farnesene	1455	1689	0.8	0.5				1.4
Germacrene D	1477	1726				0.7	0.2	**3.5**
γ-Muurolene	1478	1704		0.2				
α-Curcumene	1483	1784						1.4
10s,11s-Himachala-3(12), 4-diene	1486	-	2.5					
β-Himachalene	1499	-	1.6					
α-Muurolene	1500	1740		0.1				
β-Bisabolene	1508	1741	0.7					
α-Bisabolene	1510	1778		1.4				
γ-Cadinene	1515	1776		0.1				1
δ-Cadinene	1525	1772		0.3		0.3	0.1	1.7
α-Calacorene	1541	1918	0.6					
Nerolidol	1566	2050	0.7					1.6
Caryophyllene oxide	1580	2152	0.6	0.1				1.9
Viridiflorol	1591	2104					1.9	
Longiborneol	1597	2100	0.6					
α-Cedrol	1599	2120				**4.5**	**35.7**	
epi-Globulol	1629	2025				0.3		
T-cadinol	1640	2188				0.3		**5.5**
Himachalol	1648	2247	0.9					
α-Cadinol	1650	2256				1.3		
8-Cedren-13-ol	1702	2323		0.2				

Farnesol	1724	1946			0.9			
Tetradecanoic acid	1768	2672						0.3
Phytol	1950	2603						0.2
Isophyllocladene	1967	-			0.4			
Sclarene	1974	2408		**5.7**				
Abieta-8,11,13-triene	2055	-		**7.5**				
Abieta-7,13-diene	2083	-		**9.4**				
Total identified			81.6	89.4	94.3	84.4	90.1	88.9

Bold numbers indicate the percentages higher than 3% to show main components

[a]) Retention index on a DB-5MS column.

[b]) Retention index on an HP Innowax column

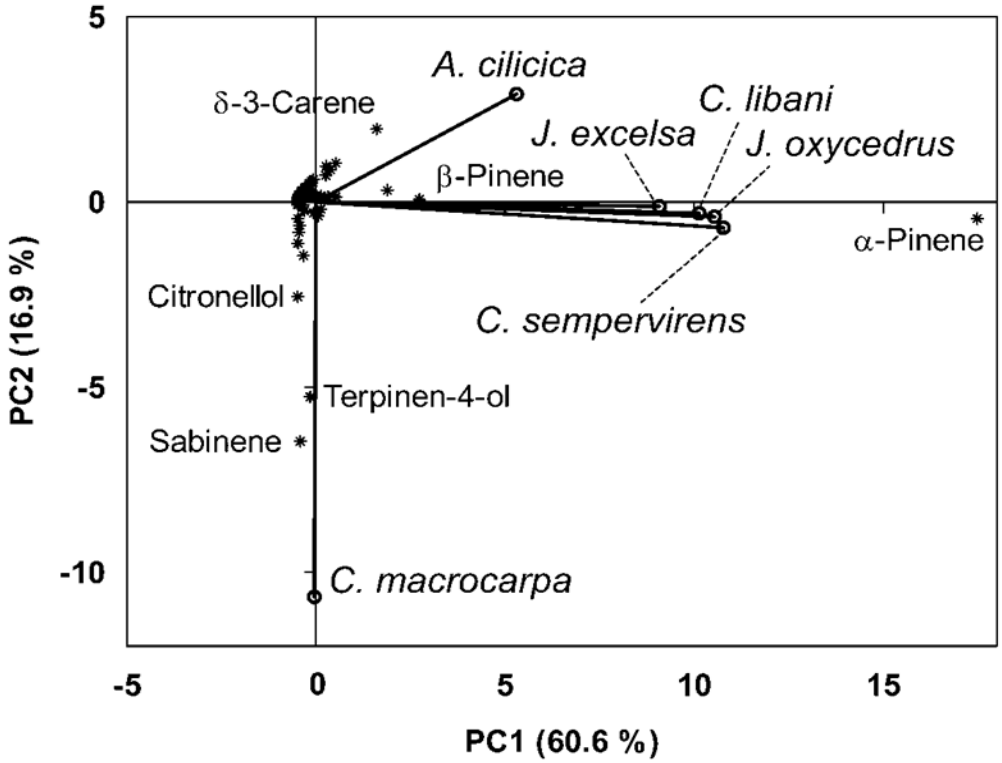

Figure 1. Biplot from the principal component analysis of the six conifers EOs

Table 2. Antimicrobial activity (MIC in µg/ml) of the six conifers essential oils

	S. aureus	*P. aeruginosa*	*C. albicans*	*T. rubrum*	*T. mentagrophytes*	*T. soudanense*	*T. violaceum*	*T. tonsurans*
A. cilicica	> 512	> 512	> 512	64	64	32	32	64
C. libani	64		512	64				
C. macrocarpa	> 512	> 512	512	64	64	32	32	64
C. sempervirens	> 512	> 512	> 512	512				
J. excelsa	64		512	128				
J. oxycedrus	64		512	256				
Positive control	1[a]	0.25[b]	1[c]	2[c]	1[d]	2[c]	2[c]	4[c]

[a]) Oxacillin; [b]) Gentamicin [c]) Fluconazole; [d]) Itraconazole

Table 3. Antimicrobial activity (MIC in µg/ml) of *C. macrocarpa* major compounds and a reconstructed essential oil prepared with the combination of these compounds.

	Sabinene	Terpinen-4-ol	Citronellol	□-Terpinene	Citronellal	□-Terpinene	Camphor	*C. macrocarpa* reconstructed EO (74.2%)
T. rubrum	512	64	256	512	512	512	512	256