American Journal of Essential Oils and Natural Products

Available online at www.essencejournal.com

AJEONP American Journal of Essential Oils and Natural Products

AkiNik

ISSN: 2321-9114
AJEONP 2020; 8(2): 01-05

Received: 01-01-2020
Accepted: 02-03-2020

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Composition of the essential oil of *Cedrus brevifolia* needles Evaluation of its antimicrobial and antioxidant activities

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Abstract
The chemical composition of the previously unknown essential oil of *Cedrus brevifolia* A. Henry ex Elves & A. Henry (Pinaceae) has been studied. The essential oil of the needles was obtained by hydrodistillation and was analyzed by GC and GC-MS. Identification of the substances was made by comparison of mass spectra and retention indices with literature data. A total of 49 different compounds were identified. The main components were α-pinene (56.8%) and limonene (7.8%). The essential oil and its main constituents were evaluated against Gram-positive/-negative bacteria and fungi and revealed strong antimicrobial activity. In addition, their inhibitory potencies on lipid peroxidation, lipoxygenase activity and their interaction with 1,1-diphenyl-picrylhydrazyl (DPPH) activity are discussed. The essential oil presented moderate antioxidant activity, while α-pinene interacted significantly with DPPH free radicals and limonene inhibited the lipid peroxidation, but both in high concentrations (1 mM).

Keywords: *Cedrus brevifolia*; essential oil, α-pinene; GC-MS antimicrobial activity, antioxidant activity

1. Introduction
The genus of true cedars, *Cedrus* L., consists of four closely related species with geographically separated distributions, *i.e.*, *C deodara* (Roxb.) Loud in the Hindu Kush, Karakoram and Indian Himalayas, *C. libani* A. Rich in Turkey, Lebanon and Syria, *C. brevifolia* (Hook. f.) Henry in Cyprus and *C. atlantica* (Endl.) Manetti ex Carrière in North Africa (Algeria, Morocco) [1]. *C. brevifolia* is an important narrow endemic tree of Cyprus flora and it is well-differentiated from other species of the genus based on morphological and Eco physiological traits, such as short needles and slow growth, resistance to aphids, and the highest tolerance to drought in all cedar species [2, 3]. The essential oils of *Cedrus* genus are used for a variety of purposes from scenting soap to medicinal practices due to their significance pharmacological activities [4]. So far, no information exists about the volatile constituents of the needles of *C. brevifolia*, therefore we have undertaken the present study in order to investigate the chemical composition of the essential oil (EO) extracted from the needles and its potential antimicrobial and antioxidant activities.

2. Materials and methods
2.1 Plant material
Needles of *C. brevifolia* were collected in April 2013 by Dr. K Nicolaou from a native population in the Valley of Cedars (Paphos, Cyprus). A voucher specimen is kept at the Herbarium of Department of Forests, Cyprus, under the number: CYP 1467.
2.2 Isolation and identification of the essential oil
Air-dried plant material (20 g) was cut in small pieces, and the essential oil was obtained by hydro distillation in 500 mL H_2O for 1.5 h in a modified Clevenger apparatus with a water-cooled oil receiver to reduce artifacts produced during distillation by over-heating [5]. The oil, taken in 2 mL of capillary GC grade *n*-pentane and dried over anhydrous sodium sulfate, was subsequently analyzed by GC and GC-MS and stored at -20 ºC.
Gas chromatography (GC) analysis was carried out using an Agilent 7890B GC-FID system, equipped with DB-5 capillary. The integration of the peaks was calculated according to the area % as reported from the Peak Simple software. Two replicates of the oil sample were processed in the same way.
The composition of the volatile constituents was established by GC-MS analyses, performed

on a Hewlett-Packard 5973-6890 system operating in EI mode (70 eV) equipped with a split/splitless injector (220 ºC), a split ratio 1/10, using a fused silica HP-5 MS capillary column (30 m × 0.25 mm I.D, film thickness: 0.25 µm). The temperature program was from 60 ºC (5 min) to 280 ºC at a rate of 4 ºC/min. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. Injection volumes of each sample were 2 µL. Retention indices for all compounds were determined according to the Van den Dool approach [6], using *n*-alkanes as standards. The identification of the components was based on comparison of their mass spectra with those of Wiley and NBS Libraries [7] and those described by Adams (2007), as well as by comparison of their retention indices with literature data [8]. In many cases, the essential oil was subject to co-chromatography with authentic compounds (Fluka, Sigma). Optical rotation value was determined in a Perkin-Elmer 341 Polarimeter at 589 nm (20 ºC) in GC grade pentane.

2.3 Evaluation of Antimicrobial Activity

2.3.1 Antibacterial activity

The essential oil and its main constituents were screened using the micro-dilution method [9] against the Gram-negative bacteria: *Enterobacter cloacae* (human isolate), *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC 13311); as well as against the Gram-positive bacteria: *Bacillus cereus* (clinical isolate), *Listeria monocytogenes* (NCTC 7973), *Micrococcus flavus* (ATCC 10240) and *Staphylococcus aureus* (ATCC 6538). Bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. The inocula were prepared daily and stored at 4 °C until use. Dilutions of the inocula were cultured on Tryptic Soy Agar (TSA) to verify the absence of contamination and to check the validity of the inoculum. The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtiter plates. The samples were dissolved in the appropriate solvents and added (at a concentration ranging from 1-10 mg/mL) in Tryptic Soy Broth (TSB) (100 µL) containing bacterial inoculum (1.0×10^4 CFU per well), in order to achieve the appropriate concentrations. Microplates were incubated at a rotary shaker (160 rpm) for 24 h at 37 °C. The following day, 30 µL of 0.2 mg/mL solution of *p*-iodonitrotetrazolium violet (INT) was added to the plates, followed by 30 min incubation to ensure adequate color reaction. Inhibition of bacterial growth was indicated by a clear solution or a definite decrease in color reaction [10]. The lowest concentrations without visible growth (at the binocular microscope) were defined as the concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by serial sub-cultivation of 2 µL into microtitre plates containing 100 µL of TSB per well, followed by an additional 24 h incubation period. The lowest concentration without visible growth at the binocular microscope was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by microplate manager 4.0 (Bio-Rad Laboratories) and compared with that of the blank and positive control. The antibiotics streptomycin and ampicillin were used as positive controls (1 mg/mL in sterile physiological saline) for all tested bacteria. All determinations were carried out at least three times, and in triplicate.

2.3.2 Antifungal Activity

The antifungal assay was carried out by a modified micro-dilution technique [11] against the following fungi: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium*. The fungi were maintained on MA (malt medium) and the cultures were stored at 4 °C and sub-cultured once per month. The fungal spores were washed from the surface of the agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted, with sterile saline, to a concentration of approximately 1.0×10^5, in a final volume of 100 µL per well. The inocula were stored at 4 °C for further use. Dilutions of the inoculum were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum. MIC determinations were performed by a serial dilution technique using 96-well microtiter plates. The examined samples were dissolved in 30% of ethanol (essential oil: 1 mg/mL, components: 3 mg/mL) and added in broth MA containing inoculum. Microplates were incubated at a rotary shaker (160 rpm) for 72 h at 28 °C. The lowest concentrations without visible fungal growth at the binocular microscope were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of 2 µL of the tested samples dissolved in broth MA, and inoculated for 72 h, into microtiter plates containing 100 µL of broth per well, followed by an additional 72 h incubation at 28 °C. The lowest concentration without visible growth was defined as MFC indicating 99.5% killing of the original inoculum. The commercial antifungal agents, bifonazole and ketoconazole, were used as positive controls (at a concentration ranging from 1-3500 µg/mL). All determinations were carried out at least in triplicate.

Bacteria and fungi were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute of Biological Research "Siniša Stanković", University of Belgrade, Serbia.

The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program and the results are expressed as mean values and standard deviation (SD).

2.3.3 Evaluation of antioxidant activity

2.3.3.1 Interaction with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) stable free radical (Reducing ability RA)

From the stock solution of the essential oil (5 mg/0.1 mL), 20 µL were diluted in absolute ethanol to a final volume of 1 mL and then added to 1 mL DPPH (0.1 mM, in absolute ethanol). Stock solutions (10 mM) for α-pinene and limonene were also performed. The same experimental procedure was followed (final concentration 1 mM). The reaction mixture was allowed at room temperature and the optical density (OD) of the solution was measured after 20 and 60 min at 517 nm. No changes were observed in the RA% values. The optical densities of the samples without the presence of DPPH were recorded and subtracted from the corresponding OD with DPPH. Nor-Dihydroguaiaretic acid (NDGA) and butylated hydroxyl toluene (BHT) were used as reference compounds under the same experimental conditions [12].

2.3.3.2 Inhibition of linoleic acid lipid peroxidation (anti-lipid peroxidation)

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a free radical initiator. Linoleic acid sodium salt solution (10 mL of the 16 mM) was added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4 pre-thermostated at 37 ºC. The oxidation reaction was initiated at

37 ºC under air by the addition of 50 mL of 40 mM AAPH solution. Oxidation was carried out in the presence of 10 µL of the examined samples. In the assay without antioxidant, lipid oxidation was measured in the presence of the same level of solvent. The rate of oxidation at 37 ºC was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. Trolox was used as a standard [13].

2.3.3.3 Soybean lipoxygenase inhibition activity (LOX inhibition)

The tested samples 10 µL from the stock solutions (essential oil, α-pinene, limonene) were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline) at a final volume of 1 mL. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor (NDGA 0.1 mM 84 % inhibition) [13].

All results were expressed as the mean ± standard deviation (SD). SPSS (version 11.0) statistical program was used for data analysis (Pearson's correlation coefficient).

3. Results & Discussion

The constituents of *C. brevifolia* needles EO are presented in Table 1a. The main components were α-pinene (56.8%) and limonene (7.8%), followed by manoyl oxide (7.6%) and β-pinene (4.2%). The EO presented high amounts of monoterpene hydrocarbons (76.6%), while its content in oxygenated monoterpenes and diterpenes was lower, 8.6% and 8.3%, respectively (Table 1b). In contrast, the sesquiterpene hydrocarbons (1.6%) and oxygenated sesquiterpenes (0.6%) were present in distinctly small amounts (Table 1b). The antibacterial and antifungal activities of the obtained EO and its main constituents, *i.e.*, α-pinene and limonene, are presented in Tables 2 and 3, respectively. It can be seen that all samples possess strong antibacterial and antifungal activities and showed similar or better effects than positive controls. In addition, the EO alone showed the highest antifungal activity compared to pure compounds, revealing that also other constituents contribute to its activity. The results from the antioxidant assays are summarized in Table 4. The samples were studied with regard to their antioxidant ability, such as a) interaction with the stable free radical DPPH, b) interaction with the water-soluble azo compound AAPH and c) to their ability to inhibit soybean LOX. The EO presented moderate antioxidant activity through all the assays. It is noteworthy that α-pinene interacted significantly with DPPH free radical at 1mM, which is a high concentration, and exhibited moderate anti-lipid peroxidation activity. Limonene inhibited the lipid peroxidation at 1 mM, whereas no response was given with DPPH. Both compounds did not present any reducing ability at 0.1 mM. Their inhibitory activities against soybean lipoxygenase were found to be similar. So far, the lipoxygenase inhibition, superoxide and hydroxyl radicals scavenging activities of *Cedrus brevifolia* bark extracts have been examined with significant results [14]. Furthermore, the chloroform extract of *C. deodara* exhibited significant antioxidant potential mainly due to the presence of sesquiterpenes [15]. Compared to the EOs of the other two Mediterranean cedars, *i.e.*, of *C. libani* [16, 17] and of *C. atlantica* [18], there are some similarities concerning the presence of α-/β-pinenes, while it is totally distinct compared to the EO of *C. deodara* [19-21] or the percentage of α-pinene is significantly lower [22].

4. Conclusions

This study has demonstrated an overview analysis of the volatile composition of *C. brevifolia* needles, performed by GC and GC-MS. To our knowledge, this is the first time that this essential oil is analyzed and compared with the composition of EOs of related species, regarding its qualitative and quantitative characteristics, as well as its antimicrobial and antioxidant activities have been evaluated. It is noteworthy to point out that the EO of *C. brevifolia* needles possesses high antimicrobial properties, which justifies its traditional uses.

Table 1a: Chemical composition of *Cedrus brevifolia* essential oil

Compound	RI[a]	Composition (%)
tricyclene	922	0.2
α-pinene	941	56.8
camphene	950	1.4
verbenene	952	0.5
sabinene	969	0.2
β-pinene	977	4.2
myrcene	985	1.8
α-phellandrene	1003	0.2
δ-3-carene	1005	0.3
α-terpinene	1012	0.1
p-cymene	1019	0.5
limonene	1027	7.8
β-terpinene	1028	1.1
γ- terpinene	1050	0.2
α-terpinolene	1078	0.9
p-cymenene	1083	0.4
fenchol	1112	0.1
chrysanthenone	1114	0.1
α-campholenal	1119	0.7
trans-pinocarveole	1133	0.7
trans-verbenole	1137	0.2
trans-pinocamphone	1151	0.2
pinocarvone	1154	0.4
p-mentha-1,5-dien-8-ol	1163	0.4
cis-pinocamphone	1170	0.1
terpinen-4-ol	1171	0.3
trans-dihydrocarvone	1197	0.2
trans-carveol	1209	0.3
thymol, methylether	1228	0.1
carvone	1233	0.2
bornyl acetate	1274	3.0
trans-pinocarvyl acetate	1284	0.6
myrtenyl acetate	1311	0.5
α-terpinyl acetate	1320	0.3
neryl acetate	1365	0.2
β-bourbonene	1369	0.1
β-caryophyllene	1404	0.3
germacrene D	1462	0.5
(*E*,*E*)-α-farnesene	1485	0.1
cis-α-bisabolene	1520	0.6
(*E*)-nerolidol	1541	0.6
1-ethyldibenzothiophene isomer	1711	0.2
1-ethyldibenzothiophene isomer	1716	0.2
benzyl benzoate	1760	0.2
sandaracopimara-8(14),15-diene	1956	tr[b]
manool oxide	1991	7.8
abieta-8,11,13-triene	2046	0.2
abietadiene	2078	0.2
dehydro-abietal	2259	0.2
methyl abietate	2383	tr
abieta-8,13(15)-dien-18-al	2396	0.1
Total		96.5

[a] Retention Index calculated against C9-C24 n-alkanes on the HP 5MS column capillary column, tr = traces (≤ 0.01%)

Table 1b: Grouped components (% v/v)

Monoterpene hydrocarbons	76.6
Oxygenated monoterpenes	8.6
Sesquiterpene hydrocarbons	1.6
Oxygenated sesquiterpenes	0.6
Aromatic compounds	0.2
Diterpenes	8.5
Miscellaneous compounds with 13 C	0.4

Table 2: Antibacterial activity of *Cedrus brevifolia* needles essential oil and its main components (MIC and MBC in mg/mL)

Compounds		*B.c*	*M.f*	*S.a*	*L.m*	*E.c*	*En.cl*	*P.a*	*S.t*
Essential oil	MIC	0.012±0.0007^{a}	0.012±0.0007^{a}	0.025±0.002^{a}	0.04±0.000^{a}	0.018±0.0007^{a}	0.04±0.002^{a}	0.012±0.0003^{a}	0.012±0.0008^{a}
	MBC	0.025±0.000^{a}	0.025±0.002^{a}	0.05±0.003^{a}	0.05±0.02^{a}	0.025±0.000^{a}	0.05±0.002^{a}	0.05±0.003^{a}	0.05±0.01^{a}
α-pinene	MIC	0.04±0.002^{b}	0.06±0.002^{b}	0.04±0.0007^{b}	0.08±0.002^{b}	0.06±0.002^{b}	0.12±0.01^{b}	0.04±0.000^{b}	0.04±0.003^{b}
	MBC	0.08±0.002^{b}	0.08±0.000^{b}	0.08±0.003^{b}	0.16±0.003^{b}	0.08±0.002^{b}	0.16±0.003^{b}	0.08±0.003^{b}	0.08±0.000^{a}
limonene	MIC	0.04±0.001^{b}	0.06±0.001^{b}	0.04±0.0007^{b}	0.12±0.007^{c}	0.06±0.002^{b}	0.16±0.003^{c}	0.04±0.003^{b}	0.04±0.002^{b}
	MBC	0.08±0.000^{b}	0.08±0.002^{b}	0.08±0.000^{b}	0.16±0.000^{b}	0.08±0.003^{b}	0.24±0.003^{c}	0.08±0.002^{ab}	0.08±0.003^{ab}
	MBC	0.12±0.01^{cd}	0.25±0.007^{c}	0.12±0.01^{d}	0.50±0.02^{e}	0.25±0.007^{c}	0.25±0.01^{c}	0.12±0.000^{c}	0.12±0.003^{c}
Streptomycin	MIC	0.09±0.003^{d}	0.15±0.000^{d}	0.04±0.003^{b}	0.15±0.007^{d}	0.15±0.000^{c}	0.25±0.01^{e}	0.15±0.01^{d}	0.15±0.003^{d}
	MBC	0.15±0.01^{d}	0.35±0.007^{d}	0.09±0.003^{b}	0.35±0.01^{d}	0.35±0.007^{d}	0.50±0.02^{d}	0.35±0.02^{d}	0.35±0.02^{d}
Ampicillin	MIC	0.25±0.02^{e}	0.25±0.01^{e}	0.25±0.01^{d}	0.40±0.02^{f}	0.25±0.01^{e}	0.40±0.000^{f}	0.75±0.02^{e}	0.40±0.02^{e}
	MBC	0.40±0.02^{e}	0.40±0.02^{e}	0.40±0.000^{e}	0.50±0.02^{e}	0.50±0.03^{e}	0.75±0.007^{e}	1.25±0.02^{e}	0.50±0.02^{e}

B.C: *Bacillus cereus*, *M.f.*: *Micrococcus flavus*, *S.a.*: *Staphylococcus aureus*, *L.m.*: *Listeria monocytogenes*, *E.c.*: *Escherichia coli*. *En.cl*: *Enterobacter cloacae*, *P.a.*: *Pseudomonas aeruginosa*, *S.T.*: *Salmonella typhimurium*.
Different letters in each column and for each sample and standard indicate significant differences among mean values of each MIC or each MBC (p<0.05).

Table 3: Antifungal activity of *Cedrus brevifolia* needles essential oil and its main components (MIC and MFC mg/mL)

Compounds		*A.fum*	*A.v*	*A.o*	*A.n*	*T.v*	*P.f*	*P.o*	*P.v.c.*
Essential oil	MIC	0.025±0.0002^{a}	0.012±0.007^{a}	0.006±0.000^{a}	0.01±0.002^{a}	0.0045±0.0002^{a}	0.01±0.002^{a}	0.01±0.000^{a}	0.012±0.001^{a}
	MFC	0.05±0.003^{a}	0.025±0.002^{a}	0.012±0.0007^{a}	0.012±0.001^{a}	0.006±0.000^{a}	0.012±0.0007^{a}	0.012±0.001^{a}	0.025±0.000^{a}
α-pinene	MIC	0.02±0.002^{a}	0.005±0.0002^{a}	0.01±0.002^{b}	0.04±0.003^{b}	0.01±0.002^{a}	0.02±0.002^{ab}	0.01±0.0007^{a}	0.02±0.0007^{a}
	MFC	0.04±0.003^{a}	0.04±0.003^{ab}	0.04±0.003^{b}	0.08±0.000^{b}	0.04±0.003^{a}	0.04±0.002^{a}	0.04±0.003^{ab}	0.04±0.003^{a}
limonene	MIC	0.04±0.003^{a}	0.04±0.003^{b}	0.02±0.002^{ab}	0.04±0.000^{b}	0.02±0.002^{a}	0.04±0.002^{bc}	0.08±0.007^{a}	0.06±0.002^{ab}
	MFC	0.16±0.01^{b}	0.08±0.003^{bc}	0.04±0.000^{b}	0.16±0.004^{d}	0.04±0.000^{a}	0.08±0.003^{b}	0.16±0.003^{cd}	0.16±0.003^{b}
	MFC	0.50±0.03^{d}	0.25±0.000^{e}	0.25±0.01^{d}	0.50±0.02^{f}	0.06±0.002^{a}	0.25±0.02^{d}	0.25±0.000^{e}	0.50±0.02^{e}
Ketoconazole	MIC	0.20±0.02^{c}	0.20±0.01^{d}	0.15±0.01^{d}	0.20±0.000^{d}	1.00±0.07^{c}	0.20±0.02^{e}	1.00±0.1^{c}	1.50±0.07^{e}
	MFC	0.50±0.000^{d}	0.50±0.03^{f}	0.20±0.01^{c}	0.50±0.02^{g}	1.50±0.07^{c}	0.50±0.02^{e}	1.50±0.07^{f}	2.00±0.000^{f}
Bifonazole	MIC	0.15±0.007^{b}	0.10±0.02^{c}	0.15±0.01^{d}	0.15±0.007^{c}	0.15±0.000^{b}	0.20±0.007^{b}	0.20±0.02^{b}	0.20±0.01^{cd}
	MFC	0.20±0.02^{c}	0.20±0.02^{d}	0.20±0.02^{c}	0.20±0.01^{e}	0.20±0.02^{b}	0.25±0.000^{d}	0.25±0.02^{de}	0.30±0.02^{d}

A.fum: *Aspergillusfumigatus*, *A.v.*: *A. versicolor*, *A.o.*: *A. ochraceus*, *A.n.*: *A. niger*, *T.v.*: *Trichoderma viride*, *P.f.*: *Penicillium funiculosum* *P.o.*: *P. ochrochloron*, *P.v.*: *Penicillium verrucosum* var. *cyclopium*
Different letters in each column and for each sample and standard indicate significant differences among mean values of each MIC or each MFC (p<0.05).

Table 4: Interaction % with 1,1-diphenyl-2-picrylhydrazyl (DPPH), % Inhibitory effect on soybean lipoxygenase; Inhibition of lipid peroxidation

Samples	RA% DPPH (20/60 min)	LOX % inhibition	Anti-lipid peroxidation %
Essential oil	56% @ 5mg/0.1ml	17% @5mg/0.1ml	31% 5mg/0.1ml
α-pinene	65% @ 1mM	15% @ 0.1mM	39% @ 1mM
limonene	No @ 1mM	13% @ 0.1mM	78% @ 1mM
NDGA	97% @ 1mM	84%@ 0.1mM	
Trolox			91%@ 1mM

Results are represented as mean values ± SD from three experiments

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