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Chemical composition and biological activity of the essential oil from the wood of *Pinus heldreichii* Christ. var. *leucodermis*

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Abstract The chemical composition of the essential oil obtained by steam distillation from the wood of *P. heldreichii*, collected from north Greece area was determined by GC and GC/MS for the first time. Forty constituents (corresponding to 96.3% of the total weight) were identified. The main components were: limonene, cembrene, longifolene, α-pinene, methyl chavicol, kaurene and cembrene A. The antimicrobial activity of the oil was evaluated against six Gram positive and Gram negative bacteria and three human pathogenic fungi, using the agar dilution technique. Strong activities against most of the tested microorganisms were exhibited. Moreover, the oil showed a very promising antioxidant activity through Rancimat method.

Chemische Zusammensetzung und biologische Aktivitäten des ätherischen Öls aus dem Holz von *Pinus leucodermis*

Zusammenfassung Das ätherische Öl aus Holz von *P. heldreichii*, (Nordgriechenland) wurde mittels Wasserdampfdestillation gewonnen und die chemische Zusammensetzung mit GC und GC/MS analysiert. Vierzig verschiedene Inhaltsstoffe (entsprechend bis 96.3 % des Gesamtgewichts) konnten identifiziert werden. Die Hauptinhaltsstoffe waren: Limonen, Cembren, Longifolen, α-Pinen, Methylchavicol, Kauren und Cembren A. Die antimikrobielle Aktivität des ätherischen Öls wurde an sechs Gram positiven und negativen Bakterien sowie drei pathogenen Pilzen mit der Agardiffusionsmethode bestimmt. Das ätherische Öl zeigte eine deutliche antimikrobielle Aktivität gegen die meisten geprüften Mikroorganismen. Die antioxidative Wirkung des ätherischen Öls, die mit der Rancimat Methode getestet wurde, war vielversprechend.

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1 Introduction

The genus *Pinus* belongs to the family Pinaceae and the monotypic subfamily Pinoideae. There are about 115 species of *Pinus* and their natural distribution ranges from arctic and subarctic regions of Eurasia and North America south to subtropical and tropical regions of Central America and Asia (Farjon 1984; Silba 1986; Rushforth 1987).

The species was first described as *Pinus heldreichii* by the Swiss botanist K. Hermann Christ in 1863 from specimens collected on Mount Olympus, and then described a second time as *P. leucodermis* in 1864; the author of the second description was the Austrian botanist F. Antoin. Some minor morphological differences have been claimed between the two descriptions (leading to the maintenance of both as separate taxa by a few botanists), but this is not supported by modern studies of the species, which show that both names refer to the same taxon and are synonyms.

Pinus heldreichii Christ. var. *leucodermis* is a forest species endemic to the Balkan peninsula and one of the least

studied species, often called white bark pine. It is encountered in the mountains of southeastern Europe, in southwestern Bulgaria, Bosnia, Albania, the former Yugoslav Republic of Macedonia, Serbia, northern Greece and locally in southern Italy, growing at 900–2,500 m altitude (Tsoumis 1983). It is an evergreen tree, usually up to 25–35 m high, that has up to 2 m trunk diameter.

The tree is called Bosnian pine [English], Panzerkiefer [German], pin d'ecorce blanche [French], il pino loricato [Italian], robolo [Greek], cherna mura [Bulgarian, Serbian], whitebark pine, or Heldreich pine.

P. heldreichii wood is commonly used in Greece as a valuable material for making wine barrels. It is a rare, but excellent species for the timber industry, which is known to have a remarkable natural durability (Petrovic and Miric 1981).

Lange et al. (1994) investigated the xylem oleoresin which was tapped from several *P. heldreichii* trees in Kosovo and thunbergol and cembrol (84.8%) were identified as its main components. In addition, this work revealed that limonene (79.4%), α-pinene (11.2%) and longifolene (5.6%) were the main components of gum turpentine oil. Obst (1998) also reported that 83% of the Bosnian pine turpentine is composed of limonene.

Additionally, several studies have been carried out on the chemical analyses of the essential oil from the needles of *P. heldreichii*. Maric et al. (2007) found that Bosnian pine needles material from Herzegovina is rich in limonene (52.8%), germacrene D (15.8), α-pinene (10.2%) and caryoplyllene (7.7%). Quite similar results were reported by Nikolic et al. (2007) in a research study carried out on Bosnian pine needle volatiles from different populations in Serbia and Montenegro, as well as by Petrakis et al. (2001) in a study on the needles' oils from trees growing in Greece, where they concluded the following chemical profile: limonene $\gg$ α-pinene $>$ germacrene-D $>$ β-caryophyllene $>$ β-pinene.

Since the 16th century, a high interest has arisen in *P. heldreichii* wood, apart from wine barrel production, also as the main resource for housing, dairying and storage of goods (Todaro et al. 2007).

However, information describing its chemical properties is very limited in the literature. In this study here, the chemical composition as well as the antioxidant and the antimicrobial profile of the essential oil from the wood of *P. heldreichii* is reported for the first time.

2 Material and methods

2.1 Plant material and isolation of the essential oil

Straight-grained mature heartwood from ten trees of *P. heldreichii*, with an average number of 6–8 annual rings cm^{-2}, was collected from a high-altitude forest area of Pindos in north-west Greece. After a slow air-drying process of six months, small, clear wood specimens were prepared. Voucher specimen is kept in the Laboratory of Pharmacognosy & Chemistry of Natural Products, University of Athens. The dried wood was subjected to hydro-distillation for 4 h, in 0.4 l of water, in a Clevenger-type apparatus, with a water-cooled oil receiver to reduce formation of artifacts due to overheating during hydro-distillation (British Pharmacopoeia 1993). The wood essential oil was collected over water and dried over anhydrous sodium sulfate (Panreac Quimica S.A. Barcelona, Spain) and it was stored at 4–6 °C until analyzed.

2.2 Essential oil analysis

The oil was analyzed by GC on a Perkin-Elmer 8500 gas chromatograph with FID, fitted with a Supelcowax-10 fused silica capillary column (30 m x 0.32; film thickness, 0.25 μm). The column temperature was programmed from 75 to 200 °C at a rate of 2.5 °C/min. The injector and detector temperatures were programmed at 230 °C and 300 °C, respectively. Helium was used as carrier gas at a flow rate of 0.6 ml/min. The GC-MS analysis was carried out using a Hewlett Packard 5973-6890 GC-MS operating on EI mode (equipped with a HP 5MS 30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness capillary column). Helium (1 ml/min) was used as carrier gas. Temperature program: the initial temperature of the column was 60 °C (for 5 min), then raised to 280 °C within 3 °C/min, and held there for 30 min (total time: 93.33 min).The compounds were identified by comparison of their retention indexes (RI) (Massada 1976), retention times (RT) and mass spectra with those of authentic samples and/or the NIST/NBS, NIST02, Wiley 575 libraries spectra and the literature (Adams 2007). The percentage composition of the essential oil is based on peak areas obtained without FID factor correction.

2.3 Antimicrobial activity

Antimicrobial activity of the essential oils against bacteria and fungi was determined by using the agar dilution technique. The microorganisms included two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228); four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 227853); and the pathogenic fungi *Candida albicans* (10231), *C. tropicalis* (13801) and *C. glabrata* (28838). Standard antibiotics (netilmicin, amoxicillin and clavulanic acid) were used in order to control the sensitivity of the tested bacteria and 5-flucytocine, amphotericin B and itraconazole were used in

order to control the tested fungi. Pure limonene, α-pinene and cembrene were also tested to compare their antimicrobial activities with the ones of the assayed oil. All technical data have been described previously (Runyoro et al. 2010). Minimum inhibitory concentrations (MICs) were determined for the oil sample and the standard pure compounds under identical conditions, for comparison purposes. The experiments in all cases were repeated three times.

2.4 Antioxidant activity

The method used was a modification of the Rancimat method reported by Lalas and Tsaknis (2002). About three grams of oil consisting of purified sunflower oil and pinus oil (0%-Control, 1%, and 2%) were accurately weighed into the reaction vessel of the Rancimat 743 (Metrhom LTD, Herisau, CH 9101, Switzerland). The reaction vessels were placed in the apparatus. The conditions were set at 70 °C and 7 L/h. The protection factor (P.F.) was calculated as P.F. = (induction period with antioxidant)/(induction period without antioxidant). A protection factor greater than 1 indicates inhibition of the lipid oxidation. The higher the value, the better is the antioxidant activity (Lalas and Dourtoglou 2003).

Preparation of oil for Rancimat method: Sunflower oil (Sol, Elais S.A., Athens, Greece) was purified from trace metals and other prooxidants via adsorption chromatography to yield purified sunflower oil triacylglycerol fractions according to the method described by Fuster et al. (1998). A glass column (40 × 2.5 cm i.d.) (wrapped with aluminum foil to prevent light-induced oxidations during the purification process), plugged with glass wool, was packed with 250 g of alumina (activated at 100 °C for 8 h and then at 200 °C for 12 h) suspended in *n*-hexane, capped with sea sand, and conditioned by prewashing with 200 ml of *n*-hexane. The oil (100 ml) was dissolved in an equal volume of hexane and passed through the column, which was then washed with 200 ml of *n*-hexane. The hexane (total volume 300 ml) was evaporated using a rotary evaporator and the triacylglycerols were collected in an aluminium foil-wrapped flask.

2.5 Statistical analysis

The results were expressed as average values. Data are expressed as means ± S.D.

3 Results and discussion

The results obtained from the chemical composition are summarized in Table 1. The identified components represent 96.30% of the total oil. The percentages of the constituents

Table 1 Main components of the essential oil from *P. heldreichii* wood
Tab. 1 Hauptinhaltsstoffe des ätherischen Öls aus dem Holz von *P. heldreichii*

	Compound*	% in ess.oil	HP-5§	Method of identification
1	**α-pinene**	**6.43**	931	a, b, c
2	camphene	0.37	942	a, b, c
3	β-pinene	0.25	966	a, b, c
4	myrcene	1.09	975	a, b, c
5	**limonene**	**28.70**	1040	a, b, c
6	α-terpinolene	0.34	1085	a, b, c
7	exo-fenchol	0.63	1121	a, b, c
8	β-terpineol	0.11	1150	a, b, c
9	camphene hydrate	0.20	1154	a, b, c
10	borneol	0.83	1170	a, b, c
11	terpinen-4-ol	0.26	1177	a, b, c
12	methyl chavicol	5.94	1195	a, b, c
13	γ-terpineol	2.48	1199	a, b, c
14	trans-carveol	0.15	1217	a, b, c
15	cis-carveol	0.06	1231	a, b, c
16	carvacrol methyl ether	0.05	1236	a, b, c
17	carvone	0.16	1242	a, b, c
18	iso-bornyl acetate	0.05	1282	a, b, c
19	α-longipinene	0.86	1350	a, b, c
20	α-ylangene	0.13	1367	a, b, c
21	longicyclene	0.27	1372	a, b, c
22	sativene	0.35	1389	a, b, c
23	**longifolene**	**6.89**	1412	a, b, c
24	trans-β-farnesene	0.25	1453	a, b, c
25	β-chamigrene	0.12	1472	a, b, c
26	β-selinene	1.05	1489	a, b, c
27	α-selinene	0.42	1495	a, b, c
28	β-himachalene	0.07	1498	a, b, c
29	β-bisabolene	0.06	1506	a, b, c
30	juniperol	0.51	1601	a,c
31	**cembrene**	**23.82**	1936	a, b, c
32	cembrene A	5.58	1946	a, b, c
33	kaurene type	0.24	1957	c
34	phenanthrene type	0.22	1974	c
35	kaur-15-ene	0.16	1997	a, b, c
36	kaurene	5.88	2043	a, b, c
37	abietadiene	0.39	2083	a, b, c
38	sandaracopimarinal	0.37	2182	a, b, c
39	dehydro abietal	0.06	2270	a, b, c
40	4-epi-abietal	0.47	2307	a, b, c
Total		**96.3%**		

*Compounds listed in order of elution from a HP-5 MS column

§ Retention indices (KI) on HP-5 MS capillary column

a = Retention time; b = Retention Index; c = mass spectra

are based on normalization of peak areas without application of the response correction factor. The major components are: limonene 28.70%, cembrene 23.82%, longifolene 6.89%, α-pinene 6.43%, methyl chavicol 5.94%, kaurene 5.88% and cembrene A 5.58%.

It has to be noted, the presence in the oil of a total 37.18% of monoterpene hydrocarbons and 36.07% of diterpenes followed by oxygenated monoterpenes 10.92% and sesquiterpenes 10.47% (Table 2). High level of monoterpenes ($\sim$ 60%) was also detected in the essential oil from the needles of *P. heldreichii* growing in Greece (Petrakis et al. 2001).

Comparing the results of this study with previously reported ones on essential oils from different parts of the tree, both wood and needles oil have limonene and α-pinene as the most abundant components, while the rest major constituents are not comparable. So, in the wood oil, cembrene and longifolene (23.82% and 6.89% respectively) are among its main constituents, while they are almost absent in the needle oil (Table 3).

On the other hand, the composition of the wood oil in comparison with the xylem oleoresin (Lange et al. 1994) shows a completely different chemical profile. Wood oil has only some similarities with gum turpentine oil (Lange et al. 1994) regarding the presence of its main constituents (α-pinene, β-pinene, limonene, methyl chavicol longifolene) but with totally different percentages (limonene $\sim$ 80% in gum oil but only 29% in wood oil) while cembrene, cembrene A and kaurene appeared only in wood oil (Table 3).

The wood oil was tested against Gram ($\pm$) bacteria and fungi and exhibited a wide profile of antimicrobial activity (Table 4) against all tested microorganisms (MIC values 2.00–3.74 mg/ml) while pure limonene was almost inactive and α-pinene showed a moderate antimicrobial profile (MIC values 2–15 mg/ml) in accordance with previously reported results (Runyoro et al. 2010). The expressed quite strong activity of the wood oil of *P. heldreichii* could be mainly attributed to the content in cembrene (23.82%) which shows a strong activity against Gram-positive bacteria and pathogenic fungi (Table 4) as it has been also previously reported (Chen et al. 2009). Cembrene which is the main constituent only of the wood oil of the tree seems to be recognized as the most valuable compound for its antimicrobial activity.

The protection of *P. heldreichii* wood oil on purified sunflower oil was also studied (Table 5). In all ratios used, wood oil improved significantly the resistance of sunflower oil to oxidation. The results obtained, using the Rancimat method to evaluate the antioxidant activity, showed that *P. heldreichii* wood oil can be considered as a good natural source with significant antioxidant activity. This activity can be attributed to the main constituents and/or to synergy among

Table 2 Composition of different classes of terpenes in the essential oil of *P. heldreichii* wood
Tab. 2 Zusammensetzung der verschiedenen Terpenklassen im ätherischen Öl von *P. heldreichii* Holz

Compounds	% in essential oil	
Monoterpene	37.18	
Sesquiterpene	10.47	
Diterpenes	36.07	
Hydrocarbons		83.72
Oxygenated monoterpenes	10.92	
Oxygenated Sesquiterpene	0.51	
Oxygenated Diterpenes	0.90	
Oxygenated compounds		12.33
Others		0.22
Total		96.27

Table 3 Main components (% in essential oil) from different parts of *P. heldreichii*
Tab. 3 Hauptinhaltsstoffe (Anteil im ätherischen Öl in %) verschiedener Pflanzenteile von *P. heldreichii*

Compounds	Wood	Xylem oleoresin (Lange et al. 1994)	Gum turpentine oil (Lange et al. 1994)	Needles (Petrakis et al. 2001)	Needles (Maric et al. 2007)	Needles (Nikolic et al. 2007)
α-pinene	6.43	–	11.16	13.8	10.2	17.51
β-pinene	0.25	–	0.47	4.2	3.0	5.66
limonene	28.70	–	79.44	34.3	52.8	26.30
methyl chavicol	5.94	–	0.18	–	–	–
longifolene	6.89	1.1	5.62	–	–	–
β-caryophyllene	–	–	0.09	8.4	7.7	10.41
germacrene D	–	–	–	12.8	15.8	13.53
cembrene	23.82	5.6 or 4.1	–	0.2	0.1	–
cembrene A	5.58	–	–	–	–	–
kaurene	5.88	–	–	0.3	–	–
thunbergol/ cembrol (1:2)	–	84.8	–	–	–	–

Table 4 Antimicrobial activities (MIC mg/ml) of the studied *P. heldreichii* essential oil and its main components
Tab. 4 Antimikrobielle Aktivität (MIC mg/ml) des ätherischen Öls von *P. heldreichii* und seiner Hauptinhaltsstoffe

Species-essential oils	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*E. cloacae*	*K. pneumoniae*	*E. coli*	*C. albicans*	*C. tropicalis*	*C. glabrata*
P. heldreichii	2.20	2.45	3.00	3.25	3.74	3.12	2.75	2.20	2.00
Limonene	>20	>20	>25	>25	>25	>20	>25	>25	>25
α-Pinene	7.50	9.50	6.00	15.00	8.00	2.00	4.00	4.00	2.00
Cembrene	1.40	1.54	4.72	>25	5.26	6.70	4.84	3.71	3.27
Itraconazole	NT	NT	NT	NT	NT	NT	1×10^{-3}	0.1×10^{-3}	1×10^{-3}
5-Flucytocine	NT	NT	NT	NT	NT	NT	0.1×10^{-3}	1×10^{-3}	10×10^{-3}
Amphotericin B	NT	NT	NT	NT	NT	NT	1×10^{-3}	0.5×10^{-3}	0.4×10^{-3}
Netilmicin	4×10^{-3}	4×10^{-3}	8.8×10^{-3}	8×10^{-3}	8×10^{-3}	10×10^{-3}	NT	NT	NT
Amoxicillin	2×10^{-3}	2×10^{-3}	2.4×10^{-3}	2.8×10^{-3}	2.2×10^{-3}	2×10^{-3}	NT	NT	NT
Clavulanic acid	0.5×10^{-3}	0.5×10^{-3}	1×10^{-3}	1.6×10^{-3}	1×10^{-3}	1.2×10^{-3}	NT	NT	NT

NT = not tested

Table 5 Protection factor of various concentrations of *P. heldreichii* wood oil on purified sunflower oil
Tab. 5 Antioxidativer Effekt verschiedener Konzentrationen des ätherischen Öls aus dem Holz von *P. heldreichii* auf Sonnenblumenöl

Sample	Oil in sunflower oil (%)	Protection factor
P. heldreichii oil	1	2.17 (±0.06)
P. heldreichii oil	2	2.08 (±0.02)

Values are means of triplicate determinations and standard deviation (SD) is given in parenthesis

the different oil components. The measured antioxidant power depends on the chosen method, the concentration and the nature and physicochemical properties of the studied antioxidant (Martos-Viuda et al. 2010). However, the increase in the quantity of wood oil added to the mixture did not increase the induction period. Studies indicate that some dietary compounds may have concentration-dependent antioxidant or prooxidant activities. It has been observed that the activity of some antioxidants does not increase linearly with increasing concentration. At sufficiently high levels of addition, it may even become a pro-oxidant (Schuler 1990). It is well known that phenolic compounds are strong antioxidant agents, but recently it has been published that essential oils rich in nonphenolic compounds may also exhibit antioxidant properties (Kordali et al. 2005). It has been published in recent literature, that compounds like limonene, α-pinene, β-pinene and kaurene type diterpenoids, which are among the most abundant in the studied wood oil, have shown strong to moderate antioxidant activity (Ruberto and Baratta 2000; Thirugnanasampandan et al. 2008), so the exhibited antioxidant activity could suggest to be attributed mainly to them.

4 Conclusion

The GC and GC/MS analyses of the essential oil from *P. heldreichii* wood led to the identification of forty constituents (corresponding to 96.30% of the total weight) among which limonene, cembrene, longifolene and α-pinene have been found as the most abundant ones.

Besides, the oil exhibited a broad spectrum of strong antimicrobial activities especially against Gram positive bacteria (MIC values 2.20–2.45 mg/ml) and human pathogenic fungi (MIC values 2.0–2.5 mg/ml). This activity could be mainly attributed to the high content of cembrene identified in significant amounts ($\sim$ 24%) only in the wood oil of *P. heldreichii*, among the previous studied volatiles, from different parts of the tree. However, further investigation should be carried out on new series of pathogenic microorganisms, in order to validate a potent antiseptic use in the field of cosmetics.

Moreover, the antioxidant activity of *P. heldreichii* oil could find further potential application in the area of food protection mostly in the process of food storage.

Finally, except for the well known extensive use of *P. heldreichii* wood in the timber industry, this study contributes to the further exploitation of the wood oil in the above mentioned different commercial areas of high interest.

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