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Full Length Research Paper

Chemical composition and antioxidant activity of essential oil of pine cones of *Pinus armandii* from the Southwest region of China

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The essential oil of an endemic species, *Pinus armandii*, gathered from China, was extracted by hydrodistillation and solid phase micro-extraction (SPME). The oils have been studied by GC and GC-MS. A total of 57 compounds have been identified in the pine cone oil extracted by hydrodistillation, the principal components being *α*-pinene (20.92%) and D-limonene (15.78%), *β*-pinene (4.91%) and *trans*-pinocarveol (4.76%), while the oil extracted by SPME showed *α*-pinene (41.59%), D-limonene (17.8%), *β*-caryophllene (11.02%) and *β*-pinene (7.54%) as the principal components. SPME extracts indicated that *α*-pinene and *β*-caryophllene were in greater concentration in the head space vapours than in the oil. The antioxidant activity of the oils from *P. armandii* was evaluated using the DPPH. This is the first report describing the essential oil composition and antioxidant activity of this species.

Key words: *Pinus armandii*, essential oils, SPME, GC-MS, antioxidant activity, DPPH.

INTRODUCTION

The genus Pinus belongs to the Pinaceae family and comprises about 250 species. *Pinus armandii* are economically important conifers that are indigenous to the southwest region of China.

Pine is used in ethnomedical practice throughout the world. Indians use a boiled extract of the inner bark from *P. strobes* (White pine) as an astringent for diarrhea or in cough remedies. In 19th century North America, *P. sylvestris* (Scots pine) was employed as a diuretic and to induce perspiration and thus help break a fever. *P. brutia* is used in folk medicine in Turkey, and recently the antimicrobial activity of tar obtained from the roots and stems of *P. brutia* against *Staphylococcus aureus*, *Streptococcus pyrogenes*, *Escherichia coli*, and *Candida albicans* was reported (Sakagami, 1991; Unten, 1989). Previous studies on *Pinus* species reported the diterpenoids (Cheung, 1993; Fang, 1991), triterpenoids (Fang, 1991), flavonoids (Fang, 1988), and lignans (Feng, 2002) content.

Pine oils are widely used as fragrances in cosmetics, flavoring additives for food and beverages, scenting agents in a variety of household products, and intermediates in the synthesis of perfume chemicals. They are also used for medicinal purposes in aromatherapy as carminative, rubefacient, emmenagogue, and abortifacient agents. *Pines* are among the most important forest trees in the Mediterranean region. There are numerous studies dealing with the essential oils of conifer species and especially those of *Pinus*. These studies deal with the reasons for the chemical variation of pine oils from the geographical (Rezzi, 2001), seasonal (Isidorov, 2003), genotypic (Lazutka, 2001), and environmental (Kupcinskiene, 2008) points of view.

We have focused our attention on the biologically active compounds of the cones of coniferous trees which have been treated as wastes in the forestry industry. And we have recently reported diterpenes (Yang, 2005; 2008) from the pine cone of *P. koraiensis* and *P. armandii*. The aim of the present study is to investigate the chemical composition and antioxidant properties of terpenes and aroma volatiles from pine cones of *P. armandii* as a

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potential source for large scale production of essential oils and flavour essences. To the best of our knowledge, this is the first report on the chemical composition and antioxidant activity of the essential oil of *P. armandii* from Southwest China.

MATERIALS AND METHODS

Plant material

P. armandii (Pinaceae) cones collected from Yunnan province of the People's Republic of China in October 2008 were identified by Professor Ding Yi, Tsinghus University. The voucher specimen (No. Pa-081012) has been deposited in the Herbarium of School of Food Science and Engineering, Harbin Institute of Technology.

Oil isolation

Hydrodistillation

The aerial parts of *P. armandii* were left to dry at room temperature and 250 g of the plant material were coarsely minced and placed in a flask containing 2000 ml of water and steam distilled in a Clevenger-type apparatus for 5 h. The essential oil was dried over anhydrous magnesium sulfate and stored at 4°C in the dark. Essential oil yield was 1.12% based on dried weight of sample.

Headspace solid-phase micro-extraction (HS-SPME)

A PDMS coated fiber (100 mm) and a manual SPME holder (SUPELCO Inc.) were used for sample extraction. In a blank run, the fibre was exposed to the GC inlet for 5 min for thermal desorption at 250°C before headspace sampling. One gram of each sample was sealed in a 10 ml screw top vial with phenolic cap and PTFE/ silicone septa (SUPELCO Inc.) and stored in a drying cabinet at 75°C for 30 min. The SPME fibre was exposed to each sample for 5 min by manually penetrating the septum (0.25 cm depth). After the exposure, the fused silica fiber was introduced for 30 s into a sealed 25 ml vessel containing 0.5 ml of C_5-C_{25} *n*-alkanes in *n*- hexane. This additional operation makes it possible to calculate chromatographic retention indices (RI) of organic compounds recorded on chromatograms. When the sampling was finished, the fiber was withdrawn into the needle and transferred to the injection port of the GC and GC-MS system, operating in the same conditions used as above both for the identification and the quantification of the constituents, apart from the splitless injection mode and the injector temperature (250°C).

Gas chromatography

An Agilent 6890N gas Chromatograph system was used for analysis, fitted with a DB-5 capillary column (60 m × 0.25 mm ID, 0.25 µm film thickness). The oven temperature was held at 80°C for 5 min and then programmed from 80 to 250°C at a rate 5°C/min with helium as carrier gas (1 ml/min). The injector temperature was 250°C and detection was performed by FID, at 250°C. Quantitative data were obtained by electronic integration of peak areas without the use of correction factors.

Gas chromatography-mass spectrometry

Analyses of both samples (oil and SPME extracts) were performed using a Chromatograph Agilent 6890 interfaced to an Agilent 5973 mass spectrometer system operating in the EI mode at 70 ev, equipped with a split/splitless injector. The temperature of injector and ion source was set at 250 and 230°C, respectively. The interface was kept at 270°C. Helium was used as the carrier gas (1 mL/min) and the capillary column used was DB-5 MS column (60 m × 0.25 mm ID, 0.25 µm film coating). The temperature programme was the same as that used for the GC-FID analysis; injection mode was splitless; scan time, 1 s; mass range, 50 - 500 amu. The injected volume was 1 µl. In both cases, injections were made of a mixture of oil and *n*-alkanes (C_5–C_{25}).

Qualitative and quantitative analyses

Most constituents were identified by gas chromatography by comparison of their GC retention indices (RI) with those reported in literature (Adams, 1995) or with those of standards purchased, synthesized or identified in oils of known composition. Further identification was confirmed when possible by comparison of their mass spectra with those stored in MS databases (NIST and Wiley libraries). Relative component concentrations were obtained directly from GC peak areas and appear in Table 1 as percentage composition.

Antioxidant activity

The antioxidant activity of the volatile compounds from *P. armandii* was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH (Brand-Williams, 1995). A methanolic stock solution (50 ml) of the antioxidant was placed in a cuvette, and 2 ml of 6×10^{-5} M methanolic solution of DPPH was added. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined using a Pgeneral-T6 spectrophotometer after 1 h for all samples. Methanol was used to zero the spectrophotometer. The absorbance of the DPPH radical without antioxidant, that is, the control, was measured daily. Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution (Blois, 1958). All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated (Yen, 1994).

RESULTS AND DISCUSSION

The components identified from *P. armandii* oil, their retention indices and their percentage composition are summarized in Table 1 where all the compounds are arranged in order of their elution on the DB-5 column. The principal compounds (>10%) appear in bold face. The DB-5 column has been used in the GC-MS analysis to identify the majority of the components. Most of the constituents have been identified from their mass spectra and retention indices on the non-polar column, and their retention indices on this column appear in Table 1.

The oil extracted by hydrodistillation was characterized by a large amount of mototerpenes (84.09%) made up of mototerpenes hydrocarbons (50.99%) and oxygenated mototerpenes (33.1%). Sesquiterpenes represented only 4.31% of the oil. The diterpene, abietadiene (1.6%), was also found in the oil. The principal compounds were found to be *α*-pinene (20.92%) and D-limonene (15.78%). Other representative compounds were identified as *β*-pinene (4.91%) and *trans*-pinocarveol (4.76%), while *p*-menth-1-en-3-ol (2.74%), verbenone (2.57%), cis-carveol (2.38%), myrtenol (2.35%), camphor (2.1%), verbenene (2.0%), *p*-cymene (2.0%), 4-isopropenyltoluene (1.94%), carvone (1.85%), myrtenal (1.73%), caryophyllene oxide (1.59%), *β*-caryophyllene (1.58%), α-campholenal (1.5%),

Table 1. Percentage component of the pine cone oils of *Pinus armandii*.

Compound	RI	Oil (%)	SPME (%)	Identification
α-Thujene	931	0.27	0.37	RI_2, MS
α-Pinene	944	20.92	41.59	RI_2, MS
Camphene	959	1.19	1.4	RI_2, MS
Verbenene	964	2	0.92	RI_1, MS
β-Cymene	979	0.21	0.16	RI_2, MS
β-Pinene	988	4.91	7.54	RI_2, MS
β-Myrcene	991	tr	2.34	RI_2, MS
α-Phellandrene	1011	0.95	0.2	RI_1, MS
3-Carene	1018	0.4	0.15	RI_2, MS
p-Cymene	1030	2	0.91	RI_1, MS
D-Limonene	1037	15.78	17.8	RI_2, MS
1,8-Cineole	1040	0.62	0.19	RI_1, MS
o-Cymene	1045	0.3	0.09	RI_1, MS
γ-Terpinene	1064	0.12	0.06	RI_1, MS
4-Isopropenyltoluene	1095	1.94	0.51	RI_1, MS
Fenchol	1123	0.22	tr	RI_1, MS
α-Campholenal	1134	1.5	0.57	RI_1, MS
4-Acetyl-1-methylcyclohexene	1137	0.11	tr	RI_1, MS
trans-Pinocarveol	1152	4.76	1.09	RI_1, MS
Camphor	1158	2.1	0.23	RI_2, MS
Pinocarvone	1162	0.47	tr	RI_1, MS
Oxygenated monoterpene $C_{10}H_{16}O$	1173	tr	0.19	-
Oxygenated monoterpene $C_{10}H_{16}O$	1175	4.9	0.56	-
Borneol	1178	0.65	0.12	RI_1, MS
Pinocamphone	1187	0.57	0.14	RI_1, MS
1-(3-Methylphenyl)-ethanone	1191	1.02	tr	RI_1, MS
p-Menth-1-en-3-ol	1198	2.74	tr	RI_1, MS
p-Menth-8-en-2-ol	1202	0.26	tr	RI_1, MS
Myrtenol	1207	2.35	0.2	RI_2, MS
Myrtenal	1208	1.73	0.73	RI_1, MS
cis-Carvotanacetol	1216	0.27	0.06	RI_1, MS
Verbenone	1221	2.57	0.96	RI_1, MS
cis-Carveol	1226	2.38	0.22	RI_1, MS
3-Isopropybenzadehyde	1230	0.24	0.08	RI_1, MS
tans-Carveol	1237	1.05	0.2	RI_1, MS
Cuminaldehyde	1250	0.12	tr	RI_1, MS
Carvone	1252	1.85	0.19	RI_1, MS
p-Menth-1(7)-en-2-one	1257	0.21	tr	RI_1, MS
Borneol acetate	1295	0.28	0.28	RI_1, MS
Perillalaldehyde	1306	0.13	tr	RI_1, MS
α-Cubebene	1363	tr	0.09	RI_1, MS
α-Ylangene	1389	tr	0.05	RI_1, MS
α-Copaene	1394	tr	0.32	RI_1, MS
β-Bourbonene	1405	tr	0.21	RI_1, MS
β-Caryophyllene	1443	1.58	11.02	RI_1, MS
β-Cubebene	1449	tr	0.09	RI_1, MS
Cyclosativene	1459	tr	0.08	RI_1, MS
α-Caryophyllene	1477	0.31	0.09	RI_1, MS
γ-Muurolene	1494	0.12	0.45	RI_1, MS
Germacrene D	1504	tr	0.18	RI_1, MS
Eremophilene	1511	tr	0.07	RI_1, MS
α-Muurolene	1514	tr	0.06	RI_1, MS
γ-Cadinene	1518	tr	0.19	RI_1, MS
δ-Cadinene	1535	tr	0.15	RI_1, MS

Table 1. Contd.

α-Cadinene	1540	tr	0.27	RI_1, MS
Caryophyllene oxide	1611	1.59	1.28	RI_1, MS
Oxygenated Sesquiterpene($C_{15}H_{24}O$)	1663	0.36	0.08	-
Hexahydrofarnesylactone	1847	tr	0.05	RI_1, MS
trans-*α*-Bisabiolene epoxide	1880	0.35	tr	RI_1, MS
Abietadiene	2098	1.6	0.29	RI_1, MS
Monoterpene hydrocarbons		50.99	74.04	
Oxygenated monoterpenes		33.1	6.01	
Sesquiterpene hydrocarbons		2.01	13.32	
Oxygenated sesquiterpenes		2.3	1.41	
Diterpene hydrocarbons		1.6	0.29	
Total identified		90	95.07	

tr: Traces (<0.05%); RI: retention index according to *n*-alkanes (C_5–C_{25}) on the DB-5 column; MS: mass spectra data; RI_1: retention data according to literature values; RI_2: retention data according to authentic standards.

camphene (1.19%), *trans*-carveol (1.05%) and 1-(3-methylphenyl)-ethanone (1.02%) were minor constituents of the oil.

The oil vapour adsorbed by headspace SPME showed higher amounts of monoterpenes (80.05%) than sesquiterpenes (14.73%). As in the hydrodistilled oil, mototerpenes hydrocarbons (74.04%) were found in higher amounts than the oxygenated monoterpenes (6.01%) and also sesquiterpene hydrocarbons (13.32%) appeared as dominant in comparison with oxygenated sesquiterpenes (1.41%). In this analysis, the principal compounds were found to be *α*-pinene (41.59%) and D-limonene (17.8%) and *β*-caryophllene (11.02%). Other characteristic compounds of the oil were identified as *β*-pinene (7.54%), *β*-myrcene (2.34%), camphene (1.4%), caryophyllene oxide (1.28%), *trans*-pinocarveol (1.09%).

Quantitative but not qualitative differences have been found in the chemical composition of both analysed samples depending on the extraction method. *α*-Pinene (20.92–41.59%) was found as the principal component of this species but it was found in greater concentrations in the SPME extracts than in the hydrodistillation ones. Other compounds that appeared in higher concentration in SPME extract were sesquiterpenes, such as *β*-caryophyllene (1.58-11.02%), *α*-copaene (t-0.32%), *β*-bourbonene (t-0.21), *γ*-muurolene (0.12 - 0.45%) and *α*-cadinene (t-0.27%). Comparing both techniques, it seems that SPME is more sensitive for compounds of low molecular mass that are more volatile while steam distillation yields the majority of compounds from the plant. This is normal because the SPME extraction was at 75°C while steam distillation needed boiling water with the resultant higher temperature. It is worth mentioning that we have only used one kind of fibre in the SPME extraction, so more comparative studies should be done with different fibres to be sure about the sensibility of both methods.

In addition, relatively stable organic radical DPPH· has been widely used in the determination of the antioxidant activity of single compounds as well as the different plant extracts. The method is based on the reduction of alcoholic DPPH· solutions in the presence of a hydrogen donating antioxidant. DPPH· solutions show a strong absorption band at 517 nm appearing as a deep violet color. The absorption vanishes and the resulting decolourization is stoichiometric with respect to degree of reduction. The remaining DPPH·, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant. The method was used to evaluate the antioxidant properties of *P. armandii* essential oil in comparison with those of known natural and synthetic antioxidants, ascorbic acid and BHT (Table 2).

Table 2. Radical scavenging activity of the pine cone oils of *Pinus armandii*, BHT and ascorbic acid with DPPH.

Material	IC_{50}[a]
The oil of *Pinus armandii*	378.51±7.13
BHT	23.71±1.25
Ascorbic acid	63.04±4.17

[a]Concentration (µg/ml) for a 50% inhibition. Values represents average of triplicates ± standard deviation.

The ascorbic acid and the BHT showed the highest radical scavenging activity, while the activity of the *P. armandii* was much lower. For slow reacting compounds the influence was attributed to the complex reacting mechanism. In our study, probably, the constituents from *P. armandii* was involved one or more secondary reactions, which resulted in the slower reduction of DPPH· solutions.

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