Composition, antifungal and antifumonisin activity of *Pinus wallichiana, Pinus monticola* and *Pinus strobus* essential oils from Patagonia Argentina

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ABSTRACT

The essential oils composition of *P. wallichiana P. monticola* and *P. strobus* was determined by GC-FID and GC-MS analysis. *Pinus wallichiana* was the species with the highest essential oils production with an average yield of 0.97%, followed by *P. monticola* and *P. strobus* with 0.58 and 0.50% (v/dry wt).

The main compounds with concentration higher than 5.0% were the monoterpene hydrocarbons:α-pinene (14,8 - 21%), β-pinene (22,8 - 34%), limonene (4,6 - 17,8 %) and myrcene (1,6 -12,3%).The essential oils from *P. wallichiana* and *P. monticola* in comparison to *P. strobus* have been characterized by a higher content of limonene (17,8, 14,0 and 4,6%, respectively). On the other hand, the essential oil of *P. strobus* showed high levels of myrcene and oxygenated and hydrocarbons sesquiterpenes. All of the evaluated essential oils showed some inhibitory effects on the growth of *F. verticillioides.* However, none of the evaluated EOs significantly altered the FB_1 production under the experimental conditions.

KEYWORDS

Pinus wallichiana, Pinus monticola, Pinus strobus, essential oils composition, antifungal, fumonisin.

INTRODUCTION

The genus *Pinus* is widely distributed in the northern hemisphere, specially the Mediterranean region, Caribbean areas, Asia, Europe, North and Central America. In Argentina, the sub-antarctic forests phytogeographic area has a microclimate where ecological conditions are suitable for exotic conifers cultivation, usually *Pinus* species, from other regions and continents (Dimitri, 1982). In this phytogeographic area, several thousands of hectares of *Pinus ponderosa*, *P. contorta*, *P. radiata* and *Pseudotsuga menziesii* has been established (Godoy, *et. al.*, 2007). Pines are important forest species primarily for timber. So, it would be very important from the commercial point of view, to extent the cultivation of white pines, on account of their ability to flourish and survive under harsh climatic conditions as well as in poor soils. Besides, they have good adaptation, fast growth and wood quality for various applications in the wood industry. Previous studies have shown that *Pinus wallichiana* A.B. Jackson *(= P. excelsa* Wall. ex D. Don, *P. griffithii* Mc Clell.*)*, *P. monticola* Dougl. Ex D. Donand and *P. strobus* L. grown in Argentina have a high potential in afforestation projects (Godoy, *et. al.*, 2007; Guerra, *et. al.*, 2009).

There are a large number of references in the literature on the chemical composition of turpentine (oleoresins) or volatiles terpenes (headspace) from *Pinus* species. However, the composition of essential oils (EOs) from the needles of *P. wallichiana*, *P. monticola* and *P. strobus* grown in Argentina is little known. Pine needle oils are widely used for medicinal purposes in aromatherapy and as fragrances in cosmetics, flavouring additives for food and beverages (Yang, et. al., 2010). Previous studies describing the essential oils composition of *Pinus* species and their antifungal properties have been reported (Krauze-Baranowska, *et. al.*, 2002; Ustun, *et. al.*, 2006; Dob, *et. al.*, 2005). Nevertheless, the lack of records of previous

chemical studies on the essential oil composition and antifungal activity of these three species of *Pinus* grown in Argentina, prompted us to study their essential oil chemical composition and biological activity.

MATERIALS AND METHODS

Plant material

Young needles of *Pinus wallichiana* A.B. Jackson *(= P. excelsa* Wall. ex D. Don, *P. griffithii* Mc Clell.*), Pinus monticola* Douglas and *Pinus strobus* Linne were randomly collected during autumn of 2007. *Pinus wallichiana* was collected from Esquel (42° 58' S; 71° 22' W; 568 m), and *Pinus monticola* and *Pinus strobus* were collected from Valle Chico (42° 44' S; 71° 45' W; 550 m). Voucher specimens are kept in the Herbarium of the UNPAT sede Esquel under code numbers: BF-N°343 (*P. wallichiana*), BF-N°344 (*P. monticola*) and BF-N°345 *(P. strobus*).

Distillation of the essential oils and GC-MS analysis

The essential oils were obtained by hydrodistillation according to Zygadlo *et. al.*, (1996). Analyses were performed in a Shimadzu GC-R1A (FID) gas-chromatograph, fitted with a 30 m x 0.25 mm (0.25 µm film thickness) fused silica capillary column coated with a phase 5% phenyl 95% dimethylpolysiloxane, non polar DB-5 column and then a polar Supelcowax 10 coated with a phase polyethylene glicol. The GC operating conditions were as follows: oven temperature programmed from 40 -230^{0}C at 2^{0}C /min, injector and detector temperatures 240^{0}C. The carrier gas was nitrogen at a constant flow of 0.9 ml/min. The constituents of the essential oils were identified on the basis of their GC retention index (RI) with reference to an homologous series of

n-alkanes (C_9 - C_{25}), by comparison of their retention times with those of pure authentic samples from Sigma and Fluka Companies, peak enrichment on co-injection with authentic standards wherever possible, by GC-MS library search (Nist) and using visual inspection of the mass spectra from literature (Adams, 1995), for confirmation. GC/MS analyses were performed with a Perkin Elmer Q-700 equipped with a SE-30 capillary column (60m x 0.25 mm; coating thickness 0.25 µm film). The analytical conditions were: oven temperature from 40^0C to 230^0C at 2^0C/min, the carrier gas was helium at a constant flow of 0.9 mL/min, the source was at 70 eV.

Fungal strain

An isolate of *Fusarium verticillioides* MRC 826 from the Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, Republic of South Africa (PROMEC), grown on carnation leaf agar by monosporic isolation, was used in all the experiments.

Testing for antifungal activity. Minimum inhibitory concentration (MIC)

For the evaluation of antifungal activities, experiments were performed according to the semisolid agar antifungal susceptibility method (SAAS) (Provine and Hadley, 2000) modified. Briefly, five-ml/ aliquots of semisolid brain-heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 0.5% agar, w/v (Bacto Agar, Difco Laboratories), pH 7.4 (without dextrose, buffer or indicator) were prepared in sterility, in 16 by 125 mm glass tubes with and without the addition of essential oils. These essential oils were dissolved with dimethyl sulfoxide (DMSO), and then added to the different tubes in order to obtain concentrations 5, 10, 20, 40,100, 300, 500, 1000, 2000, 4000 and 5000 µl/L of culture medium. The final concentration of DMSO was adjusted to 4.5 µl/ml in all the tubes. As control, a free essential oil-medium with a 4,5 µl/ml

final concentration of DMSO was used. Volatile compounds were mixed with the medium at 45 ºC, and stored at 4 ºC until solidification. In addition, one tube with uninoculated essential oils - free medium, was included as a sterility control. A conidia suspension (1×10^6 /ml) prepared with a *F. verticillioides* culture grown in Czapek-dox for one week were used as inoculums. A standard loopful (0.001 ml) of this conidia suspension was inserted deeply into each tube of medium containing a known concentration of essential oils, as well as essential oil-free medium, by a centered down-up motion to form a two dimensional inoculum. Sterile mineral oil (0.5 ml) was layered on the inoculated medium to inhibit sporulation, and then the tubes were tightly capped.. All cultures were incubated for 48 h at 28 ºC or until good growth was apparent in the essential oils-free control. Within 48 h, when by visual inspection a good growth of the *F. verticillioides* in the essential oils -free medium was detected, the growth in all tubes was visually compared with that of the essential oil-free control in order to determine inhibition. The growth was scored in the following manner: 4+, growth comparable to that of the essential oils - free control; 3+, growth approximately 75% that of the control; 2+, growth approximately 50% that of the control; 1+, growth 25% or less that of the control; and 0, no visible growth. Each treatment had 5 replications whose average gave degree of mycelial development.

Effect of phenolic compounds on FB_1 production

The FB_1 production was determined using healthy maize as substratum. Corn grain free from FB_1 (300 g), was placed in 1.000-ml dark Erlenmeyer flasks at 35% humidity and sterilized for two consecutive days in autoclave for 15 minutes at 121 ºC. Autocleaved maize was inoculated with 200 µl of conidia suspension of *F. verticillioides* prepared as described in Testing for

antifungal activity. Incubation lasted 28 days in the dark at 25 ºC, with manual stirring the first 5 days. The AE were applied on a sterilized paper disk Whatman No. 3 (14 mm diameter), which was placed over corn grain on the 5^{th} day post-inoculation. The EOs concentrations used were 100 µl/kg of maize (100 ppm). Control flasks were prepared following the same procedure, however, no EOs were added on paper disk. Five replications of each treatment were done. The experiment was performed twice.

Fumonisin B_1 quantification

Separation and purification of the toxin were performed in the fermented maize following the methodology of Voss *et. al.,* (1990) modified. Briefly, after incubation, fermented maize was sterilized in an autoclave for 15 min at 121 ºC and dried in a vacuum oven at 60 ºC until constant weight was achieved. Later, 10 g of dried maize was finely ground. The FB_1 was extracted with ultrapure water by shaking the powder and water for 2 h in an orbital shaker. The aqueous extracts were centrifuged at 9000g, and filtered through filter paper (Whatman no. 4, Whatman International, Maidstone, UK). Samples (100 mL) from the aqueous extracts were diluted with acetonitrile (100 µL). The quantification of the extracts was performed following the methodology proposed by Shephard *et. al.,* (1990). Briefly, an aliquot (50 µL) of the diluted extracts was derivatized with 200 µL of *o*-phthaldialdehyde solution. This solution was obtained by adding 5 ml of 0.1 M sodium tetraborate and 50 µL of 2-mercaptoethanol to 1 ml of methanol containing 40 mg of *o*-phthaldialdehyde. The derivatized samples were then analyzed by means of a Hewlett Packard HPLC equipped with a fluorescence detector. The wavelengths used for excitation and emission were 335 nm and 440 nm, respectively. An analytical reverse phase column C18 (150 mm × 4.6 mm internal diameter and 5 µm particle size) connected to a

precolumn C18 (20 mm × 4.6 mm and 5 µm particle size) was also used. The mobile phase was methanol and NaH2PO4 0.1 M (75:25), with the pH being set at 3.35 ± 0.2 with orthophosphoric acid, and a flow rate of 1.5 ml/min used. The quantification of FB_1 was carried out by comparing the peak areas obtained from samples with those corresponding to the analytical standards of FB_1 (PROMEC, Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg; Republic of South Africa).

Statistical evaluation

Data from these studies were analyzed by one-way analysis of variance (ANOVA) and DGC Multiple Comparison test (Di Rienzo, et. al., 2003). Results giving P values < 0.05 were considered significantly different.

RESULTS AND DISCUSSION

The essential oils composition of *P. wallichiana*, *P. monticola* and *P. strobus* was determined by GC-FID and GC-MS analysis. *Pinus wallichiana* was the pine specie with the highest EO production with an average yield of 0.97% (v/dry wt), followed by *P. monticola* and *P. strobus* with 0.58 and 0.50% (v/dry wt), respectively. Seventy one compounds, representing more than 95% of the oils, were identified (Table I). Oils were predominantly composed of monoterpene hydrocarbons (70.3-80.2%), with β-pinene as major constituent (22.8-34%), followed by α-pinene (14.8 - 21%), limonene (4.6-17.8 %) and myrcene (1.6-12.3%) in the three pinus species studied. *P. wallichiana* and *P. monticola* EOs were characterized by a high content of limonene (17.8, 14.0 and 4.6%, respectively) while *P. strobus* oil showed high content of myrcene (12.3%)

and was dominated by oxygenated and hydrocarbons sesquiterpenes (19.0 and 6.6%, respectively) (Table I).

The chemical composition of various Pinus oils have been the subject of numerous studies. However, little is known about the EO composition of the *Pinus* growing in southern Argentina. A previous study of the oil of *Pinus* from Argentina, reported a different composition to those found in this study (Guerra, et.al., 2009). Respect to this work, these authors reported a higher content of α-pinene and p-cymene and low content of limonene in *Pinus wallichiana* essential oil, a higher content of β-pinene and δ-3-carene and a low content of limonene in *Pinus monticola* essential, and a higher content of α-pinene, limonene and germacrene D and low content of β-pinene and myrcene in *Pinus strobus.* Besides, *Pinus strobus* essential oil from Poland presented a different composition, with higher content of α-pinene (17.7%) and germacrene D (12.2%) and low content of β-pinene (7.9%) (Krauze-Baranowska, *et. al.,* 2002).

All the essential oils evaluated showed some inhibitory effects on the growth of *F. verticillioides* (Table II), with *P. wallichiana* and *P. monticola* EOs being the most active inhibitors (MIC value of 40 ppm). Under the test conditions, none of the evaluated EOs significantly altered the FB_1 production (Table III). The antifungal and antifumonisin activities of essential oil might be attributable to their main compounds. Therefore, the high antifungal activity of *P. wallichiana* and *P. monticola* in comparison to *P. strobus* essential oils can be attributable to its high limonene content. This is in agreement with Krauze-Baranowska *et. al*., (2002), who reported the low activity of *P. strobus* essential oil on *Fusarium spp.* growth, and concurs with Dambolena *et. al.,* (2008), who reported the high antifungal activity of the limonene monoterpene on the growth of *Fusarium verticilliodes*.

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Table I. Percentage composition of the essential oils of *Pinus wallichiana, Pinus monticola* and *Pinus strobus.*

RI DB-5	RI Supelcowax	Compounds	Pinus wallichiana	Pinus monticola	Pinus strobus	Methods of identification[a]
889		santene			0,1	GCMS
927		tricyclene	0,1			GCMS
930		α-thujene	0,1	0,4	0,4	GCMS, Co
939		α-pinene	**14,8**	**21**	**17,9**	GCMS, Co
953	(1029)	α-fenchene	0.3	T	0.5	GCMS
954	(1053)	Camphene	1,0	3,5	3,2	GCMS
977		β-pinene	**34.0**	**22,8**	**29,1**	GCMS, Co
991		myrcene	1.3	4.2	**12.6**	GCMS
1003		α-phellandrene	0,3	1,8	0,4	GCMS
1017		α-terpinene	0,6	0,3	0,1	GCMS
1025		p-cymene	0,1		0,1	GCMS, Co
1029	(1190)	limonene	**17,8**	**14.0**	4.6	GCMS, Co
1030	(1198)	β-phellandrene		2.0	1.1	GCMS
1031	(1149)	3-carene		4,2		GCMS
1037		cis-ocimene		0,3		GCMS
1060		γ-terpinene		0,6	0,5	GCMS
1089		terpinolene		5,1	T	GCMS, Co
1097		linalool		0,7		GCMS, Co
1099		α-pinene oxide	0,6			GCMS
1122		fenchone	0,1			GCMS
1137		cis-limonene oxide	0,3			GCMS
1139		trans-pinocarveol	2,1			GCMS
1144		cis-pinene hydrate	0,3			GCMS
1145		trans-verbenol	0,5			GCMS
1159		β-pinene oxide	Tr			GCMS
1162		isoborneol			1	GCMS
1165		pinocarvone	1,3			GCMS
1169		borneol			0,1	GCMS
1175		pinocamphone cis	0.4			GCMS
1177		terpinen-4-ol	0,2		0,1	GCMS
1183		p-cymen-8-ol	Tr	Tr	Tr	GCMS
1189		α-terpineol	0,3	1,3	0,3	GCMS
1196	(1667)	myrtenal	2,1			GCMS
1196	(1795)	myrtenol	2,1		0,2	GCMS
1205		verbenone	0,3			GCMS
1217		trans-carveol	0,7			GCMS
1239		isobornyl formate		5,7	0,1	GCMS
1243		carvone	0,5			GCMS, Co
1289		bornyl acetate	Tr			GCMS
1294		undecanone (2)	0,7			GCMS
1298		trans-pinocarvylacetate	0,9			GCMS
1312		cis-pinocarvylacetate	1,9			GCMS
1351		α-cubebene			0,2	GCMS
1368		cis-carvylacetate	0,6			GCMS
1375		α-ylangene	0,1			GCMS

Table I. (continued)

RI DB-5	RI Supelcowax	Compounds	Pinus wallichiana	Pinus monticola	Pinus strobus	Methods of identification[a]
1377		α-copaene	0,1			GCMS
1387		trans-myrtanol cetate	0,3			GCMS
1388	(1539)	β-cubebene	1,3	0,1		GCMS
1388	(1503)	β-bourbonene		0,2	0,2	GCMS
1391		β-elemene		2,8	0,2	GCMS
1408		longifolene	0,1	1,4		GCMS
1409	(1597)	β-caryophyllene	1,8	0,5	3,6	GCMS
1410	(1528)	α-gurjunene	0,4			GCMS
1434		β-gurjunene		0,4		GCMS
1436		nerylacetone	0,9			GCMS
1437		γ-elemene		0,2		GCMS
1441		aromadendrene	1,1			GCMS
1443		β-farnesene Z	0,6			GCMS
1455		alpha humulene	0,5		2,1	GCMS
1460		aromadendrene allo			5	GCMS
1483		γ-curcumene	0,2			GCMS
1493		β-guaiene+	0,1			GCMS
1500		α-muurolene	0,4	3,2	2,7	GCMS
1506	(1760)	farnesene EE	0,6			GCMS
1506	(1734)	β-bisabolene	4,8	0,1		GCMS
1523		δ-cadinene			2,5	GCMS
1561		germacrene B			2,7	GCMS
1576		germacrene D-4-ol			1,5	GCMS
1578		spathulenol	Tr	tr	1,1	GCMS
1585		globulol			1,9	GCMS
1654		α-cadinol	Tr	tr	1,9	GCMS
		Identified compounds	**99,6**	**96,8**	**98**	
		Monoterpene hydrocarbons	**70,3**	**80,2**	**70,6**	
		Oxygenated monoterpenes	**15,2**	**7,7**	**1,6**	
		Sesquiterpene hydrocarbons	**10,8**	**8,8**	**19**	
		Oxygenated sesquiterpenes	**2,5**	**0,1**	**6,6**	

Compounds listed in order of elution from a DB-5 column. RI: Retention Index.

Table II: Minimum inhibitory concentrations of Pinus oils against *F. verticillioides*

Essential oils	Oil centrations (µl/L of culture medium)										
	5000	4000	2000	1000	500	300	100	40	20	10	5
Pinus wallichiana	0	0	0	0	1	1	1	1[a]	3	4	4
Pinus strobus	0	0	1	1	1	1	2	3	4	4	4
Pinus monticola	0	0	0	0	1	1	1	1[a]	3	4	4

0: No visible growth. 1: Growth 25% or less than cntrol. 2: Growth approximately 50% of the control. 3: Growth approximately 75% of the cntrol, 4: Growth about 100% of control. FT: Flowering tops. L: Leaves. n= 5. [a]: Score of minimal inhibitory concentration

Table III: Effects of essential oils FB_1 production.

Essential oils	FB1 ug/g	sd
Pinus strobus	21,1 [a]	2,828303378
Pinus monticola	19,6 [a]	1,643187958
Pinus wallichiana	26,0 [a]	5,676973666
Control	17,5 [a]	4,017022114

. Five replications were done for each treatment. Values having different letters are significantly different from each other according to DGC multiple range test at $P < 0.05$ (n=5).