ANTIFUNGAL ACTIVITIES OF *CUNNINGHAMIA LANCEOLATA* HEARTWOOD EXTRACTIVES

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Three extractives from China-fir were obtained by a sequential extraction processes with hexane, ethyl acetate, and methanol. The components of the three extractives were analyzed: (1) The gas chromatography-mass spectrometry (GC-MS) analysis showed that in addition to the presence of cedrol, naphthalenes comprised a relatively large percentage of both the hexane extract (10.39%) and the ethyl acetate extract (9.43%). (2) Total phenolic contents analysis showed that phenols took up 6.66 % of the ethyl acetate extract and 22.8% of the methanol extract. All extracts, even with low concentrations, presented fair antifungal activities against two white-rot fungi, *Trametes versicolor* and *Irpex lacteus* and two brown-rot fungi, *Postia placenta* and *Gloeophyllum trabeum*. Cedrol and naphthalenes were partly responsible for the bioactivities. The synergistic effect of phenols and antifungal compounds also contributed to the wood decay resistance.

Keywords: Antifungal activity; China-fir heartwood; Extractives; Components; Wood preservatives

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INTRODUCTION

Timber products are extensively used. However, wood is susceptible to degradation caused by wood decay fungi and insects. Wood durability constitutes a major factor in applications. Some widely used wood preservatives, such as chromated copper arsenate (CCA) have negative impact on the environment. From the environmental perspective, finding naturally existing constituents in highly durable tree species and understanding their mechanisms can help to achieve wood protection while preserving the environment (Chang et al. 2000). Many scientists have studied wood extractives and their bioactivities.

China-fir, *Cunninghamia lanceolata* (Lambert) Hooker, is a fast-growing native Chinese species and has been extensively planted in Southern China. The wood of China-fir is famous for its durability. In China, lumber produced from the tree is commonly used for wooden boats, coffins, and other products where its natural resistance to termites and rot is a necessity (Lu et al. 1987). Zhou (1981) tested the natural resistance of 161 types of heartwood of Chinese species against *Polyporus versicolor* Fr. and *Lensites trabea* Pers ex Fr., and China-fir was naturally resistant against these decay fungi. It was also reported that China-fir wood was rated as highly resistant against *Trametes versicolor* (L. ex Fr.) Pilat and *Gloeophyllum trabeum* (Pers ex. Fr.) Murr (Freitag et al.

2006). There have been some studies that focused on the essential oil of China-fir (Lu and Wang 1999; Shieh and Wu 2007). Although the essential oil showed antifungal (against *Lenzites trabea*) and antitermite (against *Reticulitermes chinensis*) activities, with the mass loss of *Pinus massoniana* sapwood treated with 2.8%, the essential oil from Chinese fir was much higher than that of Chinese fir heartwood. This result showed that some weak-volatile or involatile components may play a vital role in the natural resistance of Chinese fir (Lu et al. 1987). However, there has been no report on the analyses of properties of these medium and strong polarity extractives against wood decay fungi.

The objective of this research was to extract the heartwood of China-fir sequentially with hexane, ethyl acetate, and methanol, which are low, medium, and strong polar solvents, respectively, evaluate the antifungal activities of three extractives and the combination of these three extracts against *Irpex lacteus*, *Trametes versicolor*, *Gloeophyllum trabeum*, and *Postia placenta*, and understand the relationship between the constituents of the extractives and their antifungal activities.

EXPERIMENTAL

Materials

Plant material, China-fir heartwood, was obtained from Fu Jian Province. China-fir heartwood chips and cubes were freshly prepared. The test fungi included two brown-rot fungi, *Gloeophyllum trabeum* (Pers ex Fr.) Murr. and *Postia placenta* (Fr.) M.J. Larsen & Lombard, and two white-rot fungi, *Irpex lacteus* (Fr.) Fr. and *Trametes versicolor* (L.) Pilát. These fungi were sub-cultured and grown in potato dextrose agar (PDA). Folin–Ciocalteu reagent was made by Sigma. Solvents used for extraction were of standard analytical grade after distillation. Other chemicals were of analytical grade.

Methods

Extraction

China-fir heartwood chips were oven-dried (50 ^{o}C) and weighed (to the nearest 0.01 g, recorded as W_1). The chips were sequentially immersed in hexane, ethyl acetate, and methanol for a week at room temperature (RT) to avoid changing some heat sensitive substances during extraction, and then filtered. Solvents were changed every 24 h. However, this extraction method could only remove some of the extractives, not all of them. Extractives were collected after vacuum recycling of solvents at about 35 ^{o}C, and weighed as W_2. Yields of all extractives were calculated according to the following formula:

$$\text{Yield}_{\text{RT}} = W_2 / W_1 \times 100\% \quad (1)$$

The 19 mm China-fir heartwood cubes went through a sequential extraction processes with hexane, ethyl acetate, and methanol for a week at room temperature (RT). Solvents were changed every 24 h. The extract-free cubes were used for decay resistance tests.

GC-MS analysis

Both hexane and ethyl acetate extracts were subjected to gas chromatographic-mass spectrometry (GC-MS) analysis using an Agilent 6890N-5973 insert GC-MS to identify the volatile components of the hexane extract and the ethyl acetate extract. The components of the methanol extract were not analyzed by GC-MS analysis because these components were of strong polarity and involatile. Separation was achieved using a DB–17MS capillary column (30 m long, inner diameter of 0.25 mm, film thickness of 0.25 μm) using helium as the carrier gas at a flow rate of 1 ml min^{-1} and splitless injection. Operating conditions were as follows: injector temperature of 270 ^oC, initial oven temperature was 40 ^oC which was held for 4 min, raised at a rate of 10 ^oC min^{-1} to 165 ^oC, held for 15 min, raised at 5 ^oC min^{-1} to 200 ^oC, held for 2 min, then raised at 10 ^oC min^{-1} to 250 ^oC and held for 10 min. Mass spectrometer conditions were: ionization mode of EI, electron energy of 70 eV, interface temperature of 280 ^oC, ion source temperature of 230 ^oC and mass scan range of 15-500 AMU.

Determination of total phenolic contents

Total phenolic contents (TPCs) of the ethyl acetate extract and the methanol extract were determined by the Folin–Ciocalteau colorimetric method according to Zovko et al. (2010). Briefly, 0.5 mL of the extract solution was mixed with the Folin–Ciocalteau reagent (0.5 mL) and 100 mg mL^{-1} Na_2CO_3 (0.5mL). After 1 h of incubation at room temperature the absorbance was measured against water at 760 nm. TPCs was calculated from calibration curve of gallic acid and expressed as gallic acid equivalents.

Evaluation of antifungal activities

Antifungal assays were performed based on the methods of Gao et al. (2008) and Cheng et al. (2006). The solvents for the hexane extract, the ethyl acetate extract and didecyl dimethyl ammonium chloride (DDAC) were hexane, ethyl acetate, and water, respectively. Methanol served as solvent for the methanol extract and the combination of three extracts, ethanol served for naphthalene and cedrol. Solutions of five concentrations of each sample were added into sterilized potato dextrose agar (PDA) to yield a final concentration range of 0.125, 0.25, 0.5, 1.25, and 2.5 mg mL^{-1} in triplicate, when the agar was still in liquid form. Then fungal plugs from the edge of actively growing cultures were transferred onto the center of the Petri dishes and incubated in the dark at 28 ^oC and 70% relative humidity until the fungal mycelium reached the edges of their solvent control dishes. The antifungal index expressed as % inhibition was calculated by the following formula,

$$\text{Antifungal index } \% = (D_2 - D_1)/ D_2 \times 100\% \quad (2)$$

where D_1 is the diameter of growth zone in the experimental dish and D_2 is the diameter of the growth zone in the solvent control dish. The IC_{50} values (the concentration in mg mL^{-1} that inhibited 50% of the mycelium of fungus growth) were calculated by probit analysis. Didecyl dimethyl ammonium chloride (DDAC), a commercial fungicide, was used as a positive control.

Wood decay resistance

The extracted cubes were redried and weighed to a constant weight. After being conditioned at 20 ^{o}C and 65% relative humidity (RH), these cubes were sterilized by exposure to 2.5 mrad of ionizing radiation from a cobalt 60 source. The cubes were then exposed to each of the fungi in a soil-block test according to procedures described in American Wood-Preservers, Association Standard E10 (AWPA 2006). The cubes were incubated for 12 weeks. The difference between initial and final weight was used to determine weight loss, which served as a measure of extract efficacy.

RESULTS AND DISCUSSION

Extraction

After sequential extraction of chips, three extractives were obtained. Their color and yields are shown in Table 1. With the sequential extraction, the color of extractives was deeper and the yields were higher. It is possible that phenols and pigments existed in the ethyl acetate extract and the methanol extract.

Table 1. Color and Yields of All Extractives

Solvent	Color	$Yield_{RT}$ (%)
Hexane	Yellow	0.8
Ethyl acetate	Orange red	1.0
Methanol	Dark red	2.8

Analysis of Extracts

GC-MS analysis

The GC–MS analysis of the hexane extract and the ethyl acetate extract of *Cunninghamia lanceolata* heartwood led to the identification and quantification of a total of 16 components (Table 2) accounting for 48.29% of the hexane extract and 18 components (Table 3) accounting for 66.06% of the ethyl acetate extract.

Cedrol (19.10%), 1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]- (6.429%) and 1-Naphthalene-propanol, .alpha.-ethenyldecahydro-.alpha.,5,5,8a-tetramethyl-2-methylene-, [1S-[1.alpha.(S*),4a.beta.,-8a.alpha.]] (5.821%) were the three most abundant components of the hexane extract. Cedrol (19.87%), 1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]- (15.796%) and 3-Cyclohexene-1-methan-ol, .alpha,.alpha.4-trimethyl- (8.281%) were the three most abundant components of the ethyl acetate extract. Furthermore, it is found that naphthalenes accounted for 10.39% of the hexane extract and 9.43% of the ethyl acetate extract.

Table 2. Components of the Hexane Extract from *Cunninghamia lanceolata* Heartwood by GC/MS Analysis

No.	RT[a]	Compound	Molecular formula	Peak area In GC-MS (rel. %)
1	15.139	3-Cyclohexene-1-methanol, .alpha., .alpha.,4-trimethyl-, (S)-	$C_{10}H_{18}O$	0.775
2	18.816	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S(1.alpha.,2.beta.,4.beta.)]-	$C_{15}H_{24}$	1.519
3	19.158	3-Cyclohexene-1-methanol, .alpha., .alpha.,4-trimethyl-, propanoate	$C_{13}H_{22}O_2$	0.601
4	19.619	1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]-	$C_{15}H_{24}$	6.429
5	19.842	Caryophyllene	$C_{15}H_{24}$	2.374
6	20.064	1H-3a,7-Methanoazulene, octahydro-3,8,8-trimethyl-6-methylene-, [3R-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]-	$C_{15}H_{24}$	2.937
7	21.808	1,6-Cyclodecadiene,1-methyl-5-methylene-8- (1-methylethyl)-, [s-(E,E)]-	$C_{15}H_{24}$	1.204
8	21.945	Naphthalene, decahydro-4a-methyl-1-methylene-7- (1-methylethenyl)-, [4aR-(4a.alpha.,7.alpha.,8a.beta.)]-	$C_{15}H_{24}$	0.751
9	22.090	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	$C_{15}H_{24}$	0.776
10	22.612	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	$C_{10}H_{16}$	0.782
11	22.945	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1- (1-methylethyl)-, (1S-cis)-	$C_{15}H_{24}$	0.601
12	26.365	Cedrol	$C_{15}H_{26}O$	19.103
13	27.596	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	$C_{15}H_{24}$	0.831
14	29.477	1H-Benzocycloheptene,2,4a,5,6,7,8-hexahydro-3,5,5,9-tetramethyl-, (R)-	$C_{15}H_{24}$	2.170
15	33.573	Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methyl-2,4-pentadienyl)-, [4aS-(4a.alpha.,5.alpha.,8a.beta.)]-	$C_{20}H_{32}$	1.612
16	35.359	1-Naphthalenepropanol, .alpha.-ethenyldecahydro-.alpha.,5,5,8a-tetramethyl-2-methylene-, [1S-[1.alpha.(S*),4a.beta.,8a.alpha.]]	$C_{20}H_{34}O$	5.821

[a] Retention time (min)

Table 3. Components of the Ethyl Acetate Extract from *Cunninghamia lanceolata* Heartwood by GC/MS Analysis

No.	RT[a]	Compound	Molecular formula	Peak area In GC-MS (rel. %)
1	14.353	Borneol	$C_{10}H_{18}O$	0.760
2	15.225	3-Cyclohexene-1-methanol,.alpha.,.alpha.4-trimethyl-	$C_{10}H_{18}O$	8.281
3	19.166	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate	$C_{12}H_{20}O_2$	1.023
4	19.628	1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]-	$C_{15}H_{24}$	15.796
5	19.825	Caryophyllene	$C_{15}H_{24}$	1.931
6	20.064	1H-3a,7-Methanoazulene, octahydro-3,8,8-trimethyl-6-methylene-, [3R-(3.alpha.,3a.beta.,7.beta., 8a.alpha.)]-	$C_{15}H_{24}$	6.778
7	21.398	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	$C_{15}H_{24}$	0.698
8	21.936	Naphthalene, decahydro-4a-methyl-1-methylene-7- (1-methylethenyl)-, [4aR-(4a.alpha.,7.alpha.,8a.beta.)]-	$C_{15}H_{24}$	1.472
9	22.090	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	$C_{15}H_{24}$	1.556
10	22.603	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	$C_{10}H_{16}$	0.693
11	22.937	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1- (1-methylethyl)-, (1S-cis)-	$C_{15}H_{24}$	0.649
12	26.254	Cedrol	$C_{15}H_{26}O$	19.867
13	27.314	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethylidene)-, (4aR-trans)-	$C_{15}H_{24}$	0.756
14	27.579	Naphthalene, decahydro-4a-methyl-1-methylene-7- (1-methylethylidene)-, (4aR-trans)-	$C_{15}H_{24}$	0.642
15	29.443	1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3.alpha.,3a.beta.,7.beta., 8a.alpha.)]-	$C_{15}H_{24}$	0.808
16	33.564	Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methyl-2,4-pentadienyl)-, [4aS-(4a.alpha.,5.alpha.,8a.beta.)]-	$C_{20}H_{32}$	2.143
17	33.966	Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methyl-2,4-pentadienyl)-, [4aS-(4a.alpha.,5.alpha.,8a.beta.)]-	$C_{20}H_{32}$	0.850
18	35.282	Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methyl-2,4-pentadienyl)-, [4aS-(4a.alpha.,5.alpha.,8a.beta.)]-	$C_{20}H_{32}$	1.360

[a] Retention time (min)

TPCs analysis

Total phenolic contents analysis showed that phenols comprised 6.66% of the ethyl acetate extract and 22.8% of the methanol extract.

Evaluation of Antifungal Activities

Table 4. *IC_{50}* Values of Compounds against Brown-rot Fungi *P. placenta* and *G.trabeum* and White-rot Fungi *T. versicolor* and *I. lacteus*

No.	Compounds	Brown-rot fungi		White-rot fungi	
		P. placenta	*G. trabeum*	*T. versicolor*	*I. lacteus*
1	The hexane extract	>2.50	2.06	0.47	>2.50
2	The ethyl acetate extract	0.61	>2.50	0.64	>2.50
3	The methanol extract	1.35	>2.50	0.84	>2.50
4	The combination of three extracts	>2.50	0.98	>2.50	>2.50
5	Cedrol	0.95	1.28	>2.50	0.30
6	Naphthalene	0.19	>2.50	>2.50	0.24
7	DDAC	0.04	0.02	0.01	0.03

Units: mg mL^{-1}

IC_{50} values of compounds against the selected fungi are shown in Table 4. According to the data, all samples exhibited fair bioactivities, although they were not as efficient as DDAC. During the tests, all the samples gave fair antifungal index, and even the concentrations were lower than 2.50 mg mL^{-1}. Each sample showed its characteristic of antifungal activity against two rot fungi. Among the four extract samples, the hexane extract was the most effective against *T. versicolor*, the ethyl acetate extract was against *P. placenta*, and the combination of three extracts was against *G. trabeum.* The methanol extract was rich in phenols, which have antioxidant activity. Schultz and Nicholas (2000 and 2002) demonstrated that in lab decay tests, an antioxidant alone provided no protection against fungal degradation. However, when combined with different organic biocides, greater efficacy was always obtained. This might be the reason that the combination of three extracts was the most effective sample against *G. trabeum*.

Cedrol and naphthalene also presented good bioactivities, especially against *P. placenta* and *I. lacteus*, but low antifungal activity against *T. versicolor*. However, the hexane extract and the ethyl acetate extract, which are composed of about 20% cedrol and 10% naphthalenes, showed great antifungal activity to *T. versicolor*. This implies that there are other constituents responsible for its antifungal activity.

Wood Decay Resistance

Table 5 shows results of the soil tests with the selected fungi. During the test with *P. placenta*, the weight loss was a little high and had high variation. For the most part, China fir heartwood controls had great decay resistance, especially to the two white rot fungi. As mentioned before, all the extract samples gave fair antifungal index, even with the concentrations of lower than 2.50 mg mL^{-1}. In the control cubes, the concentrations of the extracts were many times the *IC_{50}* values. With the sequential extraction processes going, the extracts were removed little by little, and the wood decay resistance was

progressively reduced. At last, the extracted cubes were badly decayed by the two rot decay fungi, and weight losses by the two white rot fungi were also increased. This indicates that the methanol extract does contribute to the wood durability.

Table 5. Weight Loss of Extracted Wood Blocks by Wood Decay Fungi (%)

	P. placenta	*G. trabeum*	*T. versicolor*	*I. lacteus*
Control	19.58 (14.41)	8.88(2.44)	3.29(0.63)	3.59(1.21)
Hexane	35.41 (3.92)	19.33 (5.96)	4.01(0.47)	3.86(0.60)
Ethyl acetate	25.85 (16.67)	21.82 (6.31)	4.89(0.70)	6.57(1.68)
Methanol	40.72 (4.31)	45.99 (11.05)	14.95(4.64)	10.22(4.81)

Note: numbers in ( ) are STDV

CONCLUSIONS

1. Extractives from China-fir heartwood were obtained by a sequential extraction processes with hexane, ethyl acetate, and methanol. GC-MS analysis showed that cedrol was the most abundant component of both the hexane extract (19.10%) and the ethyl acetate extract (19.87%). Naphthalenes accounted for 10.39% of the hexane extract and 9.43% of the ethyl acetate extract. The hexane extract, the ethyl acetate extract, the methanol extract, and the combination of these three extracts exhibited different antifungal activities and had their own superior inhibitory effects. Judging from antifungal properties of extractives and weight loss of extracted wood blocks by wood decay fungi, the three extractives all have some contribution to the durability of China-fir. Cedrol and naphthalenes are partially responsible for the durability of China fir. Other reasons should be investigated further.
2. TPCs analysis showed that phenols comprised 6.66 % of the ethyl acetate extract and 22.8% of the methanol extract. The synergistic effect of phenols and antifungal compounds also contributed to the wood decay resistance.

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