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Chemical composition and antifungal activity of essential oil isolated from *Chamaecyparis formosensis* Matsum. wood

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Abstract

The chemical composition of the essential oil of *Chamaecyparis formosensis* wood has been examined. GC-MS data and retention indices for reference samples were used to identify 32 constituents. α-Eudesmol (18.06%), β-guaiene (8.0%), (–)-β-cadinene (7.89%), γ-costal (7.03%), α-muurolol (6.49%), 4α-hydroxy-4β-methyldihydrocostol (5.52%), σ-selinene (4.78%), santolina triene (4.60%), eremophilene (4.32%), humulene (4.11%), myrtenol (4.11%), and τ-cadinene (3.25%) were the most abundant components. Tests with the typical wood decay fungi, *Laetiporus sulphureus* and *Trametes versicolor*, proved the antifungal activity of the oil, as the growth of *L. sulphureus* and *T. versicolor* was inhibited at concentrations of 50 and 100 μg ml^{-1}, respectively. The following characteristic volatile compounds were isolated and purified from ethyl acetate fractions: *epi*-cubenol, chamaecynone, myrtenol, *cis*-myrtanol, 12-hydroxyisointermedenol and 4α-hydroxy-4β-methyldihydrocostol. Chamaecynone possessed the strongest antifungal activity, with an antifungal index of 88.2% and 67.3% for *L. sulphureus* and *T. versicolor* at a dose of 50 μg ml^{-1}, respectively.

Keywords: *Chamaecyparis formosensis*; essential oil; α-eudesmol; chamaecynone; antifungal activity; *Laetiporus sulphureus*; Trametes versicolor.

Introduction

Wood preservatives are widely used all over the world. Identification of the bioactive constituents of highly durable wood species and elucidation of the mechanisms of biodeterioration can contribute to the development of environmentally friendly wood protection systems. In our previous studies (Chang et al. 1998, 1999, 2000, 2001) we demonstrated that Taiwania wood (*Taiwania cryptomerioides*), one of the most important plantation softwoods in Taiwan, is a species with excellent antifungal and antitermitic properties.

Chamaecyparis formosensis Matsum is also a precious wood in Taiwan because of its good wood quality and fragrance and its outstanding durability. It is an endemic tree that grows at elevations of 1500–2150 m in Taiwan's central mountains (Liu et al. 1988) and it is known as the Taiwan red cypress. Many traditional Japanese-style houses are constructed of *C. formosensis*. Its fragrance is an indication of the presence of essential oils, which are primarily composed of terpenes and their oxygenated derivatives. Besides its use in perfumes, there is a long history of its medicinal use. The oil shows antibacterial and antifungal effects.

Kafuka and Ichikawa (1931) studied the volatile constituents of the leaves of *C. formosensis* and found α-pinene to be the major constituent (85%). In addition, several terpenoids, including camphene, dipentene, cineol, α-terpinene, β-terpinene, borneol, bornyl acetate, bornyl formate, humulene, and cadinene, were identified. Fang et al. (1986a) re-analyzed the leaf essential oils and identified 41 terpenes. The predominant compound was also found to be α-pinene and other major constituents included β-pinene, 3-carene, α-terpinene, γ-muurolene, and kaurene. The root, bark, wood, cones, and leaves of *C. formosensis* have also been studied (Nozoe et al. 1966; Fang et al. 1986b; Hsu et al. 1995). However, the relationship between the essential oils of *C. formosensis* wood and its antifungal properties has not been investigated in detail. The aim of this study was to examine this relationship using *Trametes versicolor* and *Laetiporus sulphureus* for antifungal testing.

Materials and methods

General instruments

High-performance liquid chromatography (HPLC) was carried out using a Jasco model PU-980 pump equipped with an RI-930 detector and a Hibar Lichrosorb Si 60 (25×1 cm i.d.) column. IR spectra were recorded on a Bio-Rad FTS-40 instrument. Mass spectra (MS) were obtained on a Finnigan MAT-95S mass spectrometer. NMR spectra were recorded on Bruker Avance 400- and 500-MHz Fourier transform (FT) NMR instruments.

Sample preparation and compound isolation

Logs from 80-year-old *C. formosensis* Matsum were collected from the experimental forest of National Taiwan University. Heartwood chips were prepared from a green cut tree. A sample

of 2 kg of the air-dried wood chips was subjected to hydrodistillation for 8 h using a Clevenger-type apparatus. The oil (1.6%, v/w) was dried above anhydrous Na_2SO_4. In a parallel experiment, 10 kg of wood chips was exhaustively extracted with methanol (MeOH). The extracts were condensed to 420 g and then extracted with ethyl acetate (EtOAc) and after evaporation the EtOAc-soluble fraction was obtained. The EtOAc fraction (80 g) mixed with silica gel (160 g) was chromatographed on a silica gel column (1.0 kg) by elution with gradients of hexane (*n*-C_6H_{14}), EtOAc and MeOH. An aliquot of 500 ml of eluate was collected for each fraction. TLC analyses were used to monitor the chemical composition of each fraction. Fractions with similar compositions were pooled to 45 fractions (EA-1 to EA-45). Compounds **1–6** were obtained by HPLC separation and purification. *epi*-Cubenol (**1**) (Kuo et al. 2002) [retention time (RT) 13.2 min] and chamaecynone (**2**) (Nozoe et al. 1966) (RT 14.8 min) [Si-60 column, mobile phase EtOAc/*n*-C_6H_{14} (23:77), flow rate 1.5 ml min^{-1}] were isolated from the EA-7 fraction. Myrtenol (**3**) (Deagostino et al. 2001) (RT 23.0 min) and *cis*-myrtanol (**4**) (Kim and Lee 1997) (RT 25.0 min) were obtained from EA-11; however, the mobile phase was changed to EtOAc/*n*-C_6H_{14} = 25:75. 12-Hydroxyisointermedenol (**5**) (Ahmed and Mahmoud 1998) was purified from the EA-18 fraction with the same HPLC system at 20.0 min; the mobile phase was EtOAc/*n*-C_6H_{14} = 50:50 at a flow rate of 2.0 ml min^{-1}. 4α-Hydroxy-4β-methyldihydrocostol (**6**) (Gonzalez et al. 1992) in the form of colorless needle crystals was separated from the EA-22 fraction during condensation. Structures of six compounds isolated from *C. formosensis* were identified using NMR, FTIR, and MS spectrometry. The spectral data are consistent with those reported in the literature. The structures of compounds isolated from *C. formosensis* are presented in Figure 1.

GC-MS analysis of essential oil

A Finnigan Trace GC–Polaris Q mass instrument (Finnigan-Spectronex, USA) was used for GC-MS analysis with a fused silica column (30 mm × 0.25 mm i.d.) coated with SPB™-50 (film thickness 0.25 μm). The temperature program was as follows: 40°C for 1 min, then increased at 5°C min^{-1} to 250°C and held for 10 min. The other parameters used were: injector temperature, 250°C; ion source temperature, 200°C; EI, 70 eV; carrier gas, He at 1 ml min^{-1}; injection volume, 1 μl; split ratio, 1:50; and mass range, 35–650 m/z. Quantification was by percentage peak area. Identification of individual components was carried out using the Wiley/NBS Registry of Mass Spectral Database and a NIST MS Search. Chromatographic results expressed as area percentages were calculated with a response factor of 1.0.

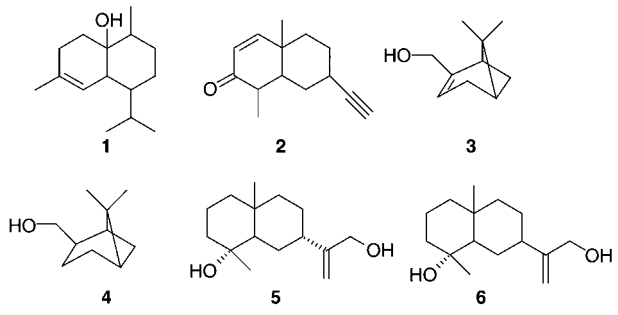

Figure 1 Compounds isolated from *C. formosensis*. *epi*-Cubenol (**1**), chamaecynone (**2**), myrtenol (**3**), *cis*-myrtanol (**4**), 12-hydroxyisointermedenol (**5**), 4α-hydroxy-4β-methyldihydrocostol (**6**).

Antifungal assays

The fungi used were *Trametes versicolor* (L. ex Fr.) Quel. (BCRC 35253) and *Laetiporus sulphureus* (B. ex Fr.) Bond. (BCRC 35305). Antifungal assays were carried out in triplicate and the data were averaged. Different concentrations of the essential oil (50, 100, and 200 μg ml^{-1}) were added to sterilized potato dextrose agar (PDA). Compounds isolated from the EtOAc fraction were tested for their antifungal activity at a dose of 50 μg ml^{-1}. The test plates were incubated at 27°C. When the mycelium of fungi reached the edge of the control plate, the antifungal index was calculated as follows:

$$\text{Antifungal index (\%)} = (1 - Da/Db) \times 100$$

where Da is the diameter of the growth zone in the experimental dish (cm) and Db is the diameter of the growth zone in the control dish (cm).

Results and discussion

C. formosensis wood yielded 1.6% essential oil by steam distillation. The following volatile compounds were isolated, purified, and identified in the EtOAc fraction of MeOH extracts: *epi*-cubenol (**1**); chamaecynone (**2**); myrtenol (**3**); *cis*-myrtanol (**4**); 12-hydroxyisointermedenol (**5**); and 4α-hydroxy-4β-methyldihydrocostol (**6**). The results of GC and GC/MS analyses of the *C. formosensis* essential oil are compiled in Table 1: α-eudesmol (18.06%), β-guaiene (8.0%), (–)-β-cadinene (7.89%), γ-costal (7.03%), α-muurolol (6.49%), 4-α-hydroxy-4β-methyldihydrocostol (5.52%), σ-selinene (4.78%), santolina triene (4.60%), eremophilene (4.32%), humulene (4.11%), myrtenol (4.11%), and τ-cadinene (3.25%) were the main constituents. α-Eudesmol with a yield of 18.06% is the predominant compound. According to the results of Fang et al. (1986a), the major compounds in leaf oil of *C. formosensis* were: α-pinene (57.32%), β-pinene (3.25%), 3-carene (5.61%), α-terpinene (0.06%), γ-muurolene (1.54%), and kaurene (1.80%). Accordingly, there is a significant difference between wood oil and leaf oil. Monoterpenes, such as α-pinene, β-pinene, camphene, carene, limonene, etc., are predominant in the leaf oil (Fang et al. 1986a). In the wood oil, however, we identified only the monoterpenes myrtenol, myrtenal, and santolina triene. We found chamaecynone (a norsesquiterpene, C_{14}) that does not seem to be present in the leaf oil. Chamaecynone is a termiticide (Harayama and Inubushi 1977). It is the first example of a natural acetylenic compound of terpenoid origin (Nozoe et al. 1966).

In this study, 26 sesquiterpenes were identified. These can be classified into 12 skeletal types, namely: copaene, elemane, aromadendrane, guaiane, germacrane, humulane, eremophilane, eudesmane, cadinane, longifolane, acorane, and aristolane type (Figure 2).

Two representative fungal strains of *Trametes versicolor* (white rot fungus) and *Laetiporus sulphureus* (brown rot fungus) were selected to test the antifungal activity of the essential oils. The antifungal indices presented in Table 2 are a clear demonstration of the excellent antifungal property of the oil. The growth of *L. sulphureus* and *T. versicolor* was completely inhibited at concentra-

Table 1 Composition of wood essential oil from *C. formosensis.*

Peak No.	Compound	RT (min)	Concentration (%)
1	*cis*-Myrtenol	17.02	4.11
2	Myrtenal	17.90	1.90
3	Santolina triene	19.03	4.60
4	(–)-β-Elemene	20.35	2.32
5	Isoledene	20.74	0.86
6	4,5-Dehydro-isolongifolene	21.94	0.13
7	Germacrene D	22.46	0.29
8	α-Gurjunene	22.95	1.53
9	τ-Muurolene	23.07	0.78
10	(–)-Alloaromadendrene	23.37	1.35
11	Valencene	23.52	2.45
12	α-Muurolene	23.73	1.42
13	τ-Cadinene	24.23	3.25
14	(–)-β-Cadinene	24.51	7.89
15	(–)-α-Cubebene	24.70	1.01
16	(–)-Calamenene	25.20	0.40
17	Humulene	25.61	4.11
18	Cadala-1,3,8-triene	26.09	0.25
19	τ-Elemene	26.30	0.28
20	(+)-Calarene	26.91	0.76
21	7-(5-Hexynyl)-tricyclo[4.2.2.0(2,5)]dec-7-ene	27.43	1.39
22	*epi*-Cubenol	27.59	2.63
23	σ-Selinene	27.89	4.78
24	α-Muurolol	28.08	6.49
25	β-Guaiene	28.46	8.00
26	α-Eudesmol	28.58	18.06
27	Eremophilene	28.86	4.32
28	Chamaecynone	29.32	1.79
29	4α-Hydroxy-4β-methyldihydrocostol	29.99	5.52
30	2α-Hydroxycostol	30.40	2.68
31	Unidentified	30.70	0.28
32	12-Hydroxy-isointermedenol	31.21	2.22
33	γ-Costal	31.83	7.03
34	Unidentified	32.67	0.11
35	Unidentified	34.93	0.36

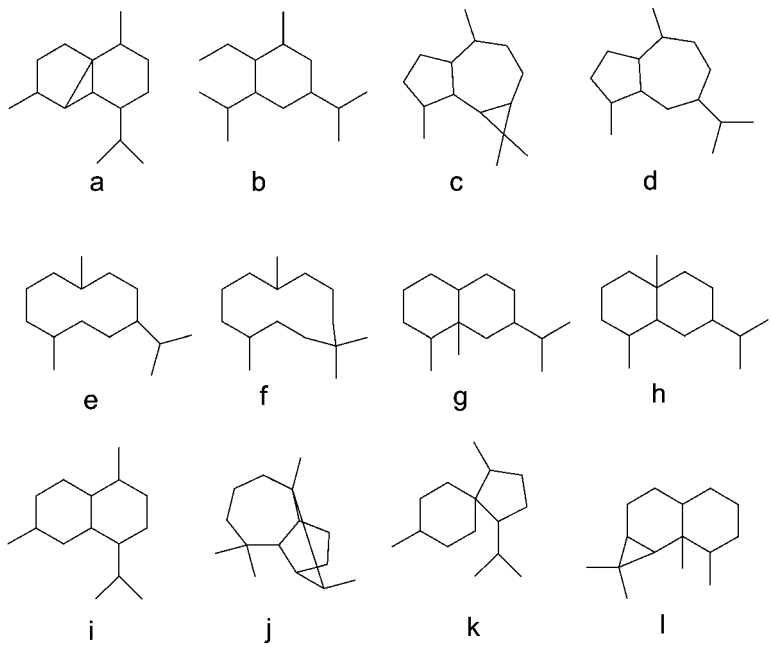

Figure 2 Main sesquiterpenes skeletons of essential oils from *C. formosensis*. (a) copaane type, (b) elemane type, (c) aromadendrane type, (d) guaiane type, (e) germacrane type, (f) humulane type, (g) eremophilane type, (h) eudesmane type, (i) cadinane type, (j) longifolane type, (k) acorane type, and (l) aristolane type.

Table 2 Antifungal indices of wood essential oil from *C. formosensis.*

Dosage (μg ml^{-1})	Antifungal index	
	T. versicolor	*L. sulphureus*
50	62.4±6.6	100±0
100	100±0	100±0
200	100±0	100±0

tions of 50 and 100 μg ml^{-1}, respectively. The literature also contains reports on similar effects of essential oils. Tellez et al. (2000) studied the composition and biological activities of the essential oil obtained by steam distillation from *Callicarpa americana* and demonstrated that α-eudesmol (9.4%) and humulene (10.0%) are the active substances. Gurjunene is one of the major antifungal constituents of the essential oil of *Calea clematidea* (Flach et al. 2002). Substances such as copaene, myrtenol, germacrene D and α-muurolol were also reported to be antifungal (Saito et al. 1996; Chang et al. 2000; Gallori et al. 2001; Krauze-Baranowska et al. 2002). The terpenoids from the EtOAc fraction were subjected to in-depth analysis. Figure 3 shows the results for *C. formosensis* at a dose of 50 μg ml^{-1}. All compounds were active, except *epi*-cubenol. Chamaecynone revealed a significant inhibitory effect against *T. versicolor* and *L. sulphureus*, as its antifungal index was 67.3% and 88.2% at a dose of 50 μg ml^{-1}, respectively. The pure compounds isolated were less active than the crude essential oil. This observation may be due to a synergistic effect of all the constituents, or there may be other antifungal compounds in the oil not yet detected.

Conclusions

The oil of *C. formosensis* is a fungicide. It was demonstrated that volatile constituents of the wood oil contribute to the high decay resistance of this wood. Several typical terpenoids with an antifungal effect were isolated from *C. formosensis*; however, their antifungal performance was not as effective as that of the crude wood oil.

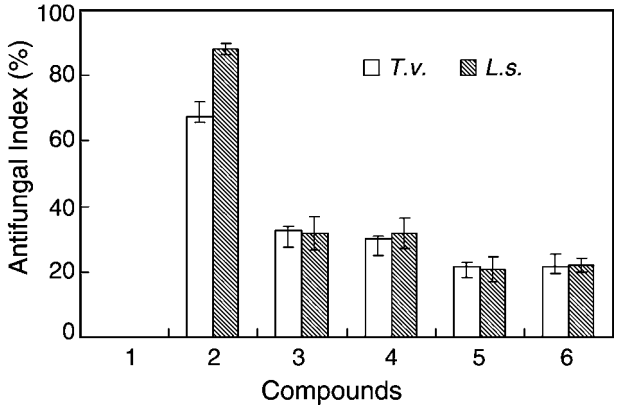

Figure 3 Antifungal activity of the constituents (50 μg ml^{-1}) of *C. formosensis* against the white rot fungus *Trametes versicolor* (white bar) and the brown rot fungus *Laetiporus sulphureus* (black bar). Compound **1**: *epi*-cubenol; Compound **2**: chamaecynone; Compound **3**: myrtenol; Compound **4**: *cis*-myrtanol; Compound **5**: 12-hydroxyisointermedenol; Compound **6**: 4α-hydroxy-β- methyldihydrocostol.

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