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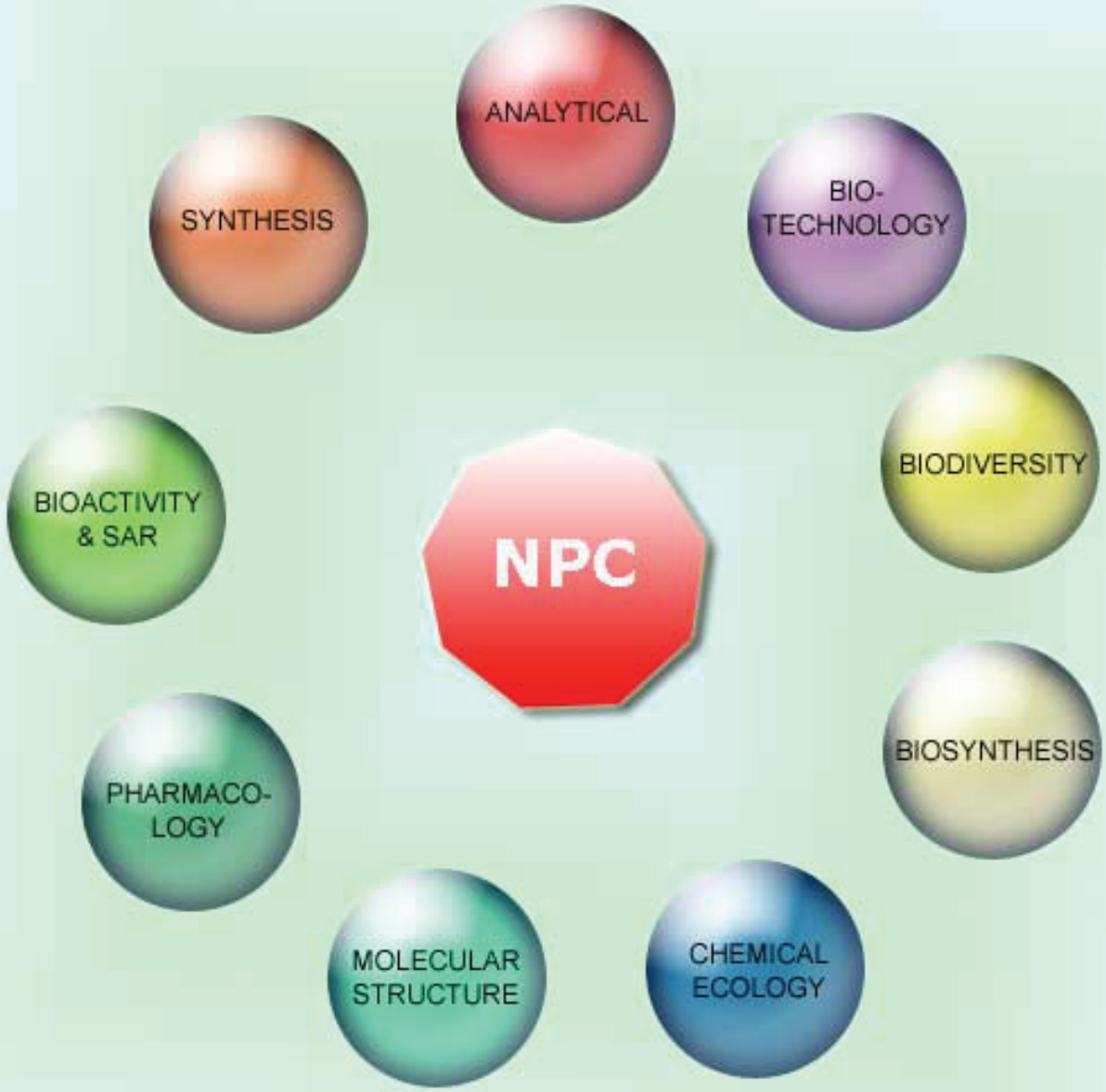

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Composition and Antipathogenic Activities of the Twig Essential Oil of *Chamaecyparis formosensis* from Taiwan

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In this study, antipathogenic activities of the twig essential oil and its constituents from *Chamaecyparis formosensis* Matsum were evaluated *in vitro* against six plant pathogenic fungi. The essential oil from the fresh twigs was isolated using hydrodistillation in a Clevenger-type apparatus, and characterized by GC–FID and GC–MS. Twenty-five compounds were identified, representing 98.9% of the oil. The main components were β-eudesmol (25.1%), τ-muurolol (21.6%), elemol (15.0%), totarol (14.9%), and α-cadinol (12.4%). The twig oil (500 μg/mL) showed growth inhibitory activity against the phytopathogenic fungi, *Fusarium oxysporum*, *Pestalotiopsis funereal*, and *Ganoderma austral*, with antifungal indices of 92.7%, 71.1%, and 87.7%, respectively. In addition, the oil suppressed totally the growth of *Rhizoctonia solani*, *Colletotrichum gloeosporioides,* and *Fusarium solani.* In order to ascertain the source compounds of these antipathogenic activities*,* the main components were individually evaluated. τ-Muurolol and α-cadinol exhibited excellent activity against *F. oxysporum, R. solani*, *C. gloeosporioides,* and *F. solani*, with IC_{50}< 50 μg/mL. These compounds also efficiently inhibited the mycelial growths of *P. funereal* and *G. austral*. Thus, α-cadinol and τ-muurolol could be considered as potential natural fungicides for controlling fungal pathogens and worth.

Keywords: *Chamaecyparis formosensis*, Cupressaceae, Essential oil, Antipathogenic activity, α-Cadinol, τ-Muurolol.

There are a multitude of plant pathogens, such as fungi, bacteria, nematodes and viruses that can cause disease or damage of plants [1,2]. Pathogenic fungi cause yield losses in numerous economically important crops [3]. According to Oreke *et al*. [4] and Agrios [5], the pre- and post-harvest losses due to fungal disease in world crops production may amount to more than 12% in developing countries. The damping off pathogens (*Fusarium oxysporum*, *Rhizoctonia solani*), leaf spot pathogens (*Pestalotiopsis funereal*, *Colletotrichum gloeosporioides*) and root rot pathogens (*Ganoderma austral*, *Fusarium solani*) are well-known important plant pests [6-9]. In recent years, fungicides used in chemical mediation of plant diseases are mostly synthetic. Although highly effective in controlling postharvest diseases in various vegetables, fruits and trees, overuse of the chemicals can lead to drug-resistance, danger of chemical residues on food, and are generally harmful to both ecology and human health [10-12]. Increasing recognition of the importance of fungal infections, and the difficulties encountered in their treatment has stimulated the search for alternatives to synthetic chemical fungicides. As a consequence, fungicides derived from plants, or agro-chemicals, that offer protection to a "green" food production system have received the attention of many scientists. Certain of these natural chemicals, for example, essential oils, are present in varying amounts in different plant tissues. The components of essential oils have low molecular weights, are volatile at room temperature, and possess strong fragrances or odors. Recent studies indicated that essential oils are safe [13], and with antibacterial and antifungal activities [8]. Our research team has proven on numerous occasions the antifungal efficacies of various essential oils [14-16].

Chamaecyparis formosensis Matsum (Cupressaceae) is one of the five most valuable conifers in Taiwan [17]. Previous studies have reported the composition of the root, bark, wood, heartwood, cone, and leaf essential oils of *C. formosensis* [18-20]. A few reports noted that *C. formosensis* wood essential oil had antifungal and insecticidal activities [21-22]. No prior study has investigated the chemical composition and biological activity of the twig essential oil of *C. formosensis*. Therefore, the aim of the present study was (a) to examine the chemical composition of the essential oil isolated from the twigs of *C. formosensis* by GC–FID and GC–MS; (b) to evaluate the antipathogenic activity of this essential oil against certain important phytopathogens.

Hydrodistillation of *C. formosensis* twigs gave a dark-yellow oil with a yield of 1.21 ± 0.02 mL/100 g, based on the dry weight of twigs. The identified constituents are presented in Table 1, where all compounds are listed in order of their elution from the DB-5 column. Twenty-five compounds were identified (Table 1), representing 98.9% of the oil. Among the groups, oxygenated sesquiterpenes were predominant (80.0%), followed by diterpenes (16.8%), sesquiterpene hydrocarbons (1.6%), and oxygenated monoterpenes (0.5%). Among the oxygenated sesquiterpenes, β-eudesmol (25.1%), τ-muurolol (21.6%), elemol (15.0%) and α-cadinol (12.4%) were the major compounds, and of the diterpenes, totarol (14.9%) was the chief component.

In order to test the antipathogenic activities of the twig essential oil, it was mixed in PDA medium for conducting bioassays. The plant pathogenic fungi tested were 2 seedling pathogens, *Fusarium oxysporum* (*F.o.*), and *Rhizoctonia solani* (*R.s.*); 2 leaf pathogens *Pestalotiopsis funereal* (*P.f.*), and *Colletotrichum gloeosporioides* (*C.g.*); and 2 root and stem pathogens *Ganoderma austral* (*G.a.*), and *Fusarium solani* (*F.s.*). Figure 1 shows the antifungal indices of *C. formosensis* twig oil (at a concentration of 500 μg/mL) against plant pathogenic fungi. At this concentration, the antifungal indices of the twig oil against damping-off pathogens, *F.o.* and *R.s.* were 92.7% and 100%, respectively. As for the leaf pathogens, *P.f.* and *C.g.*, the antifungal indices of the twig oil were71.0% and

Table 1: Chemical composition of the twig essential oil of *Chamaecyparis formosensis*.

Compound	R.I. [a)]	Conc. (%)	Identification [b)]
cis-Myrtanol	1254	0.3	MS, RI, ST
Myrtenyl acetate	1327	0.2	MS, RI, ST
δ-Elemene	1338	0.2	MS, RI
α-Cedrene	1412	0.4	MS, RI, ST
β-Chamigrene	1478	0.3	MS, RI, ST
β-Selinene	1490	0.3	MS, RI
Valencene	1496	0.3	MS, RI, ST
δ-Cadinene	1523	0.1	MS, RI
Elemol	1550	15.0	MS, RI, ST
Spathulenol	1578	0.3	MS, RI
Juniperol	1599	0.2	MS, RI
Cedrol	1601	1.5	MS, RI, ST
Sesquithuriferol	1605	0.7	MS, RI
10-*epi*-γ-Eudesmol	1624	0.3	MS, RI
Hinesol	1642	1.7	MS, RI
τ-Muurolol	1642	21.6	MS, RI, ST
Agarospirol	1648	0.2	MS, RI
β-Eudesmol	1651	25.1	MS, RI, ST
α-Cadinol	1654	12.4	MS, RI, ST
Valerianol	1658	0.3	MS, RI
Occidenol	1678	0.7	MS, RI
Abietatriene	2057	1.3	MS, RI
Kaurene	2043	0.7	MS, RI
Totarol	2314	14.9	MS, RI, ST
Compounds identified		98.9	
Monoterpene hydrocarbons (%)		0	
Oxygenated monoterpenes (%)		0.5	
Sesquiterpene hydrocarbons (%)		1.6	
Oxygenated sesquiterpenes (%)		80.0	
Diterpenes (%)		16.8	
Oil yield (mL/100 g)		1.21 ± 0.02	

[a] Retention index on a DB-5 column with reference to *n*-alkanes [23].

[b] MS, NIST and Wiley library spectra and the literature; RI, Retention index; ST, authentic standard compounds.

100%, respectively. The antifungal indices of the oil against root rot pathogens, *G.a.* and *F.s.* were 87.7% and 100%, respectively.

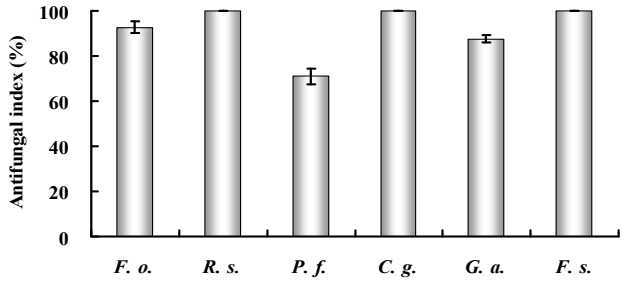

Figure 1: Antipathogenic activities of the twig oil (500 μg/mL) from *Chamaecyparis formosensis* against the plant pathogenic fungi.

The results obtained showed that the twig oil had excellent antifungal activities with values > 71.0%, and suppressed totally the growth of *R.s*., *C.g.* and *F.s.*.

However, in order to ascertain the source compounds of the antipathogenic activities of *C. formosensis*, the main components, elemol, τ-muurolol, β-eudesmol, α-cadinol, and totarol were individually tested for their antifungal activities. Figures 2a-c show the antifungal indices of the main compounds (100 μg/mL) against damping-off, leaf spot, and root rot pathogens, respectively. The results indicated that τ-muurolol and α-cadinol exhibited excellent activity against *F.o., R.s., P.f., C.g., G.a.,* and *F.s.* with the highest antifungal indices ranging from 67 ~ 100%. IC_{50} values of τ-muurolol against *F.o., R.s., P.f., C.g., G.a.,* and *F.s.* were 30.8, 18.3, 80.6, 18.8, 88.9, and 18.8 μg/mL. For α-cadinol, the following IC_{50} values were obtained against the 6 pathogenic fungi: 38.6, 28.9, 45.9, 10.8, 40.8, and 30.8 μg/mL (Table 2). The results obtained for controlling the fungal pathogens are worthy of further investigation.

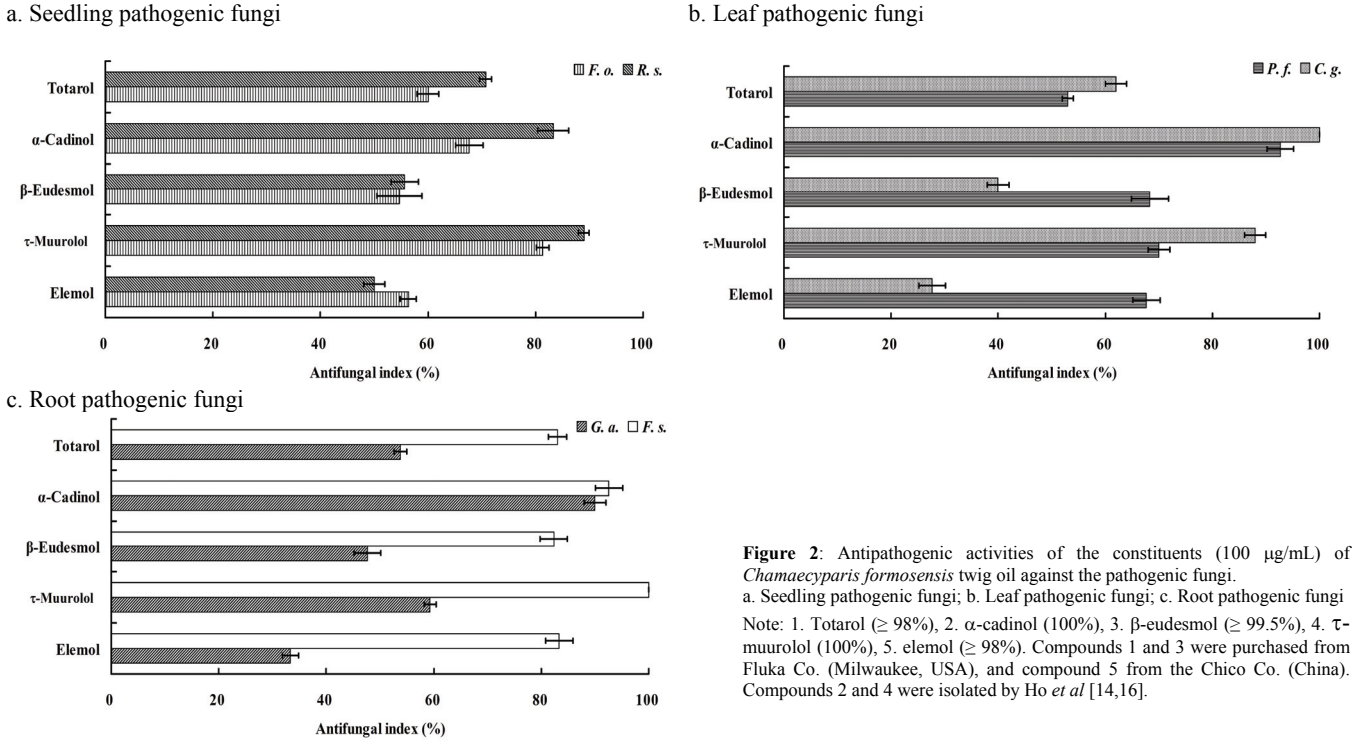

Figure 2: Antipathogenic activities of the constituents (100 μg/mL) of *Chamaecyparis formosensis* twig oil against the pathogenic fungi.
a. Seedling pathogenic fungi; b. Leaf pathogenic fungi; c. Root pathogenic fungi
Note: 1. Totarol (≥ 98%), 2. α-cadinol (100%), 3. β-eudesmol (≥ 99.5%), 4. τ-muurolol (100%), 5. elemol (≥ 98%). Compounds 1 and 3 were purchased from Fluka Co. (Milwaukee, USA), and compound 5 from the Chico Co. (China). Compounds 2 and 4 were isolated by Ho *et al* [14,16].

Table 2: IC_{50} values (μg/mL) of main constituents of *Chamaecyparis formosensis* twig oil against plant pathogenic fungi.

Constituents	Plant pathognic fungi					
	F. o.	*R. s.*	*P. f.*	*C. g.*	*G. a.*	*F. s.*
Totarol	52.8	46.9	90.8	58.9	92.8	50.3
α-Cadinol	38.6	28.9	45.9	10.8	40.8	30.8
β-Eudesmol	88.3	80.9	86.1	158.9	138.8	58.6
τ-Muurolol	30.8	18.3	80.6	18.8	88.9	18.8
Elemol	92.3	100	86.8	190.8	152.8	42.6

Experimental

Plant materials: Fresh twigs of *C. formosensis* were collected in June 2011 from Chilan Mt in northeast Taiwan (Yilan County, elevation 1050 m, N 24º 40′ 52″, 121º 40′ 15″). The samples were compared with specimen no. ou 6889 from the Herbarium of National Chung-Hsing University and positively identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH- 018) was deposited in the NCHU herbarium. Leaves of the species were collected for subsequent extraction and analysis.

Isolation of the twig essential oil: Twigs of *C. formosensis* (1 Kg) were placed in a round-bottom flask and hydrodistilled for 8 h with 3 L of distilled water. The essential oil obtained was dried with anhydrous sodium sulfate. The oil yield and all test data are the average of triplicate analyses.

Essential oil analysis: A Hewlett-Packard HP 6890 gas chromatograph equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness, J&W Scientific) and a FID detector was used for the quantitative determination of oil components. Oven temperature was programmed as follows: 50ºC for 2 min, rising to 250ºC at 5 ºC/min. Injector temperature: 270ºC. Carrier gas: He with a flow rate of 1 mL/min. Detector temperature: 250ºC, split ratio: 1:10. Diluted samples (1.0 μL, 1/100, v/v, in ethyl acetate) were injected manually in the split mode. Identification of the oil components was based on their retention indices and mass spectra, obtained from GC/MS analysis on a Hewlett-Packard HP 6890/HP5973 equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness, J&W Scientific). The GC analysis parameters listed above and the MS were obtained (full scan mode: scan time: 0.3 s, mass range was m/z 30-500) in the EI mode at 70 eV. Data are expressed as the means ± SD of 3 independent experiments.

Component identification: Identification of the leaf essential oil constituents was based on comparisons of retention index (RI) [28], retention times (RT), and mass spectra with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [23,28].

Antipathogenic assays: The phytopathogenic fungi used were *F. oxysporum* f. sp. *melonis* Snyder & Hansen (BCRC32121), *R. solani* Kuhn (BCRC31626), *P. funerea* (Desmazieres) Steyaert (BCRC35266), *C. gloeosporioides* Penzig (BCRC35003), *Ganoderma australe* (Fries) Paterson (BCRC36246) and *F. solani* (Martius) Saccardo (BCRC32458). Each fungal strain was cultured in potato dextrose agar (PDA, Difco Company). Microbial strains were obtained from the Bioresource Collection and Research Center (BCRC) of Taiwan.

The method of Lee *et al*. [29] was adopted. Essential oil concentration (500 μg/mL) and different concentrations of main compounds (0-250 μg/mL) were added to sterilized potato dextrose agar (PDA) in 9 cm Petri dishes. After transferring the mycelium of one fungus strain, the testing dishes were incubated at 27°C and 70% relative humidity. When the mycelium reached the edge of the control plate, the anti fungal index was calculated as follows:

Antifungal index (%) = (1–Da/Db) x 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).

Each test was repeated 5 times and the data were averaged. The IC_{50} values (the concentration in mg per mL that inhibited 50% of mycelium growth) were calculated by a probit analysis.

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Review/Account

Natural Product Communications
2012
Volume 7, Number 7

Contents

Continued inside backcover