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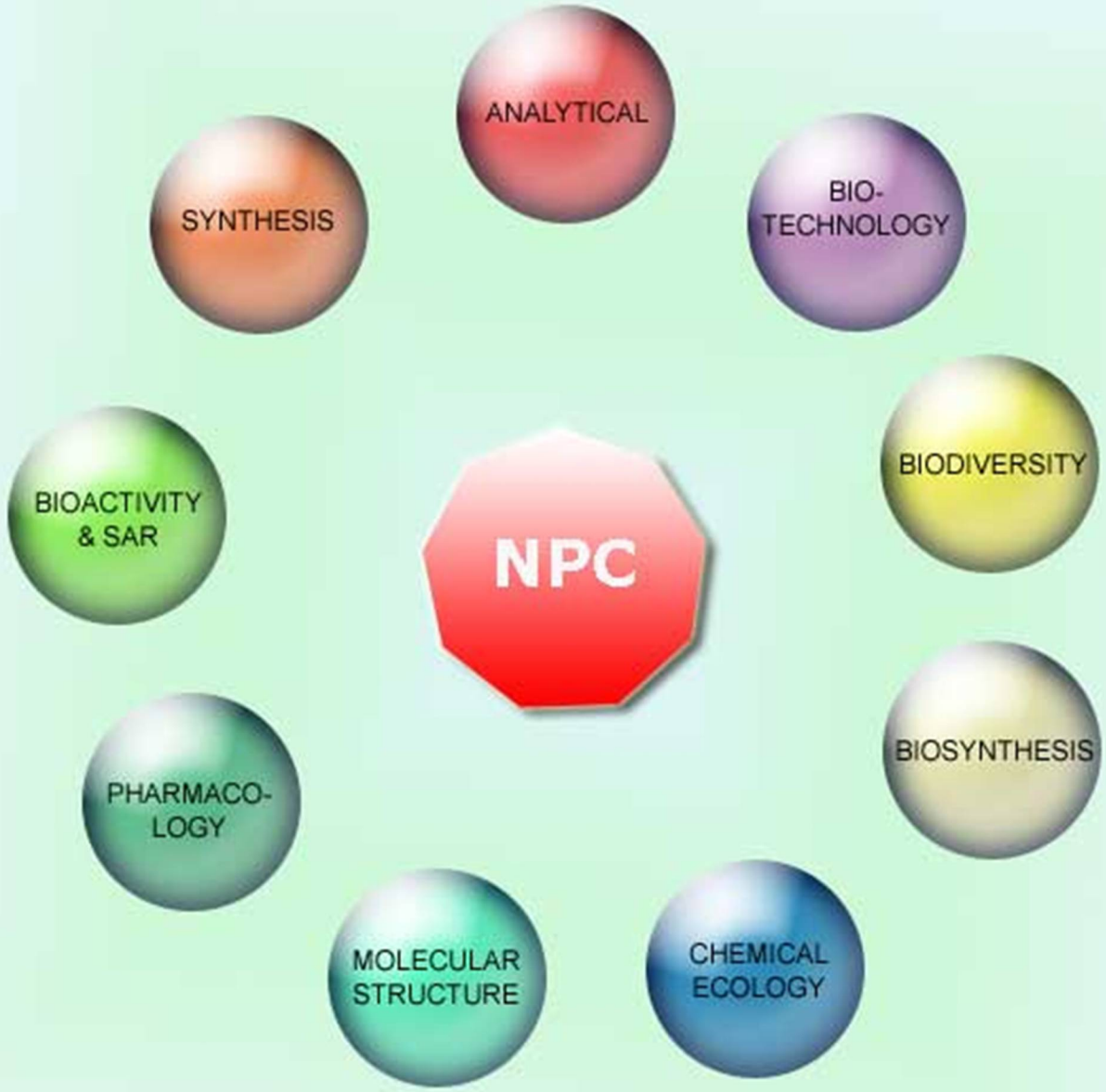

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Composition and Antimicrobial and Anti-wood-decay Fungal Activities of the Leaf Essential Oils of *Machilus pseudolongifolia* from Taiwan

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The hydrodistillated leaf essential oil of *Machilus pseudolongifolia* was analyzed to determine its composition and yield. Seventy compounds were identified, the main components being β-eudesmol (26.8%), α-cadinol (20.8%), viridiflorene (8.9%), α-caryophyllene (5.3%), globulol (4.6%) and β-caryophyllene (4.2%). Oxygenated sesquiterpenes (60.1%) and sesquiterpene hydrocarbons (31.4%) were the predominant groups of compounds. The leaf oil exhibited excellent antimicrobial and anti-wood-decay fungal activities.

Keywords: *Machilus pseudolongifolia*, Lauraceae, essential oil, antimicrobial activity, anti-wood-decay fungal activity, β-eudesmol, α-cadinol.

Machilus pseudolongifolia Hayata, (Lauraceae) is an endemic species of Taiwan and grows at mountainous regions at 600 to 1800 m elevations in the northern part of the island and at 1400 to 2300 m elevations in the southern part of the island [1]. There are no literature reports on the chemical composition and biological activities of the essential oils or other extractives from this species. Therefore, we used hydrodistillation to collect the leaf oil, which was analyzed by GC/FID and GC/MS. In order to prevent the widespread in-hospital infection, we selected 10 microbial strains for testing. In addition, the warm and humid climate of Taiwan can easily cause decay of wood product. Therefore in order to prevent wood decay, we also applied the essential oils to 4 strains of commonly found white rot fungi and brown rot fungi in Taiwan to examine their interdiction efficacies. As a consequence, the second part of the study examined the antimicrobial and anti-wood-decay fungal activities of the oil. The purpose of this study was to establish a chemical basis for the effective multipurpose utilization of the species.

Hydrodistillation of *M. pseudolongifolia* leaves gave a yellow-colored oil with a yield of 2.13 ± 0.04 mL/100 g, based on the dry weight of leaves. The identified constituents are presented in Table 1, where all compounds are listed in order of their elution from the DB-5 column. Seventy components were identified, representing 100% of the oil. Among the groups, oxygenated sesquiterpenes predominated (60.1%), followed by, sesquiterpene hydrocarbons (31.4%), monoterpene hydrocarbons (4.3%), non-terpenoids (1.8%), oxygenated monoterpenes (1.2%), and diterpenes (0.1%). Among the oxygenated sesquiterpenes, β-eudesmol (26.8%), α-cadinol (20.8%), and globulol (4.6%) were the major compounds. Of the sesquiterpene hydrocarbons, viridiflorene (8.9%), α-caryophyllene (5.3%) and β-caryophyllene (4.2%) were the main components.

The essential oil of *M. pseudolongifolia* was tested against three Gram-positive and five Gram-negative bacteria, as well as two fungi. The results, presented in Table 2, indicated that except for *Aspergillus niger,* a medium to strong growth suppression activities were observed for the other 8 bacteria and one yeast. The most sensitive microorganisms were *Bacillus cereus*, *Staphylococcus aureus*, *S. epidermidis* and *C. albicans* with inhibition zones of 31 to 52 mm and MIC values of 125 to 250 μg/mL, respectively. The essential oil showed better suppressive activity toward the Gram-positive bacteria than the Gram-negative bacteria and the fungi. These observations were similar to those

Table 1: Chemical composition of the leaf oil *M. pseudolongifolia*.

Constituents	KI [a]	Conc.(%)	Identification [b]
α-Thujene	930	t [c]	KI, MS, ST
α-Pinene	939	0.8	KI, MS, ST
Camphene	954	0.2	KI, MS, ST
β-Pinene	979	0.3	KI, MS, ST
Myrcene	991	0.1	KI, MS, ST
α-Phellandnene	1003	0.2	KI, MS, ST
α-Terpinene	1017	0.1	KI, MS, ST
p-Cymene	1025	0.3	KI, MS, ST
β-Phellandrene	1030	1.1	KI, MS, ST
1.8-Cineole	1031	0.2	KI, MS, ST
cis-Ocimene	1037	0.3	KI, MS, ST
trans-Ocimene	1050	0.8	KI, MS, ST
γ-Terpinene	1060	t	KI, MS, ST
Terpinolene	1089	t	KI, MS, ST
Linalool	1097	0.7	KI, MS, ST
n-Nonanal	1101	t	KI, MS, ST
α-Terpineol	1189	0.1	KI, MS, ST
Estragole	1195	0.2	KI, MS
n-Decanal	1202	0.9	KI, MS, ST
Citronellol	1226	0.1	KI, MS, ST
δ-Elemene	1338	0.1	KI, MS
Citronellyl acetate	1353	t	KI, MS
iso-Ledene	1376	0.3	KI, MS
α-Copaene	1377	0.7	KI, MS
β-Elemene	1391	0.2	KI, MS
Z-Trimenal	1398	0.3	KI, MS
Methyl eugenol	1404	0.4	KI, MS
α-Gurjunene	1410	0.9	KI, MS
β-Caryophyllene	1419	4.2	KI, MS, ST
β-Cedrene	1421	0.2	KI, MS
E-Trimenal	1421	0.2	KI, MS
β-Gurjunene	1434	0.1	KI, MS
γ-Elemene	1437	0.2	KI, MS
Aromadendrene	1441	1.8	KI, MS, ST
cis-Muurola-3.5-diene	1450	0.3	KI, MS
trans-Muurola-3.5-diene	1454	0.3	KI, MS
α-Caryophyllene	1455	5.3	KI, MS, ST
allo-Aromadendne	1460	0.6	KI, MS, ST
γ-Gurjunene	1477	3.1	KI, MS
α-Amorphene	1485	0.2	KI, MS
β-Selinene	1490	1.1	KI, MS
δ-Selinene	1493	0.7	KI, MS
Viridiflorene	1497	8.9	KI, MS, ST
(E,E)-α-Farnesene	1506	0.2	KI, MS
δ-Amorphene	1512	0.2	KI, MS
γ-Cadinene	1514	0.2	KI, MS
δ-Cadinene	1523	1.4	KI, MS
Zonarene	1530	0.1	KI, MS
α-Cadinene	1539	0.1	KI, MS
α-Calaconene	1546	0.1	KI, MS
Elemol	1550	0.5	KI, MS
Ledol	1569	0.3	KI, MS, ST
epi-Globulol	1570	1.8	KI, MS
Caryophyllene alcohol	1572	1.5	KI, MS, ST
Spathulenol	1578	0.3	KI, MS
Globulol	1585	4.6	KI, MS, ST
Guaiol	1601	1.4	KI, MS
β-Eudesmol	1651	26.8	KI, MS, ST
α-Cadinol	1654	20.8	KI, MS, ST
Bulnesol	1676	1.0	KI, MS
epi-α-Bisabolol	1685	t	KI, MS
α-Bisabolol	1686	t	KI, MS
Eudesma-7(11)-en-4-ol	1700	0.1	KI, MS
(2*Z*, 6*E*)-Farnesol	1701	0.8	KI, MS
(2*E*, 6*Z*)-Farnesol	1746	0.2	KI, MS
Phytol	1943	0.1	KI, MS
Monoterpene hydrocarbons (%)		4.3	
Oxygenated monoterpenes (%)		1.2	
Sesquiterpene hydrocarbons (%)		31.4	
Oxygenated sesquiterpenes (%)		60.1	
Diterpenes (%)		0.1	
Others (%)		1.8	
Oil Yield (mL/100 g)		2.13 ± 0.04	

[a] Retention index on a DB-5 column with reference to n-alkanes [2].
[b] MS, NIST and Wiley library spectra and the literature; RI, Retention index; ST, authentic standard compounds. [c].trace < 0.1%

of the literature [3-6]. Comparing the antimicrobial activities of the essential oils from *Cinnamomum subavenium* [3], *Litsea. nakaii* [4], *L. kostermansii* [5], and *L. laevigata* [6], the leaf essential oil of *M. pseudolongifolia* was superior. The results verify that *M. pseudolongifolia* leaf oil has excellent antimicrobial activity. However, in order to ascertain the source compounds of antimicrobial activity from *M. pseudolongifolia*, the main components were individually tested for their antimicrobial activities. The results indicated that the active source compounds were β-eudesmol and α-cadinol. These results were similar to those of Ho *et al*. [4,5]. There are also studies supporting the contention that these compounds have high activity in suppressing microbial growth [7-9].

The essential oil of *M. pseudolongifolia* was tested against 2 white rot fungi (*Trametes versicolor, Phanerochaete chrysosporium*) and 2 brown rot fungi (*Phaeolus schweinitzii, Lenzites sulphureu*). The anti-wood-decay fungal indices presented in Table 3 are a clear demonstration of the excellent anti-wood-decay fungal property of the oil. The growth of *T. versicolor, Phane. chrysosporium, Phaeo. schweintizii* and *L. sulphureu* was completely inhibited at concentrations of 75, 75, 75, 25 μg/mL, respectively. Comparing the anti-wood-decay fungal activities of the essential oils from *Chamaecyparis formosensis* [10] and *M. philippinensis* [11], the leaf oil of *M. pseudolongifolia* was superior. The results verified that *M. pseudolongifolia* leaf oil has excellent anti-wood-decay fungal activities.

Furthermore, in order to ascertain the source compounds of the *M. pseudolongifolia* essential oil, we also tested the anti-wood-decay fungal activities of its major component compounds. The results indicated that the sources of activities were also α-cadinol and β-eudesmol. The IC_{50} values of the 2 compounds (α-cadinol and β-eudesmol) against the 4 decay fungi were 16 and 48 ppm against *T. versicolor*; 18 and 38 ppm against *Phane. chrysosporium*; 20 and 23 ppm against *Phaeo. Shweinitzii*; and 18 and 20 ppm against *L. sulphureu*, respectively. At a 50 μg/mL concentration, α-cadinol showed total growth inhibition against all white-rot and brown-rot fungi tested; while β-eudesmol at 50 μg/mL concentration could completely inhibit brown-rot fungi but partially inhibit white-rot fungi. The results agree with those of Kondo and Imamura [7] and Mori *et al*. [12]. Thus, the excellent wood-decay-fungi inhibitive activities exhibited by the *M. pseudolongifolia* leaf oil could well be contributed by the presence of compounds such as α-cadinol and β-eudesmol etc.

Experimental

Plant materials: Fresh leaves of *M. pseudolongifolia* were collected in July 2008 from the Dahanshan at an

elevation of 1200 m in southern Taiwan (N 23º 56′ 38″, E 120º 53′ 19″, Pingtung County). The samples were compared with specimen no. ou4393 from the Herbarium of National Chung-Hsing University and positively identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH-004) has been deposited in the NCHU herbarium. Leaves of the species were collected for subsequent extraction and analysis.

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

Essential oil analysis: The method of Su *et al*. [13] was adopted. 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 5ºC/min. Injector temperature: 270ºC. Carrier gas: He with a flow rate of 1 mL/min. Detector temperature: 250ºC split ratio: 1:10. One μL sample was injected. 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. All data were the average of triplicate analyses.

Component identification: Identification of the leaf essential oil constituents was based on comparisons of retention index (RI) [14], retention times (RT), and mass spectra with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [2,15].

Antimicrobial activity: The *in vitro* antibacterial and antifungal activities of the oil were evaluated by the disc diffusion method using Mueller-Hinton agar for bacteria and Sabouraud dextrose agar for fungi [16]. Discs containing 15 μL and 30 μL of the oil, which was dissolved in dimethylsulphoxide (DMSO), were placed on the inoculated plates with test microorganisms. Growth inhibition zones (including disc diameter of 6 mm) were measured after 24 h and 48 h of incubation at 37°C and 24°C for bacteria and fungi, respectively. Gentamicine and tetracycline for bacteria, and nystatine for fungi were used as positive controls [4,5].

Microbial strains were obtained from the Culture Collection and Research Center of the Food Industry Research and Development Institute, Hsinchu City, Taiwan. The microbial strains included 5 Gram-negative bacteria: *Escherichia coli* (IFO 3301), *Enterobacter aerogenes* (ATCC 13048), *Klebsiella pneumoniae* (ATCC 4352), *Pseudomonas aeruginosa* (IFO 3080), and *Vibrio parahaemolyticus* (ATCC 17803); 3 Gram-positive bacteria: *B. cereus* (ATCC 11778), *S. aureus* (ATCC 6538P), and *S. epidermidis* (ATCC 12228); 1 fungus: *A. niger* (ATCC 16404) and 1 yeast: *C. albicans* (ATCC 10231). Minimum inhibitory concentration (MIC) values were measured by the microdilution broth susceptibility assay recommended by NCCLS [17]. Stock solutions of the oil were prepared in DMSO. Dilution series were prepared from 1000 μg/mL to 50 μg/mL in sterile distilled water in micro-test tubes, from where they were transferred to 96-well microtitre plates. Bacteria grown in double-strength Mueller-Hinton broth and fungi grown in double-strength Sabouraud dextrose broth were standardized to 10^8 CFU/mL. The last row, containing only the serial dilutions of sample without microorganisms, was used as a negative control. Sterile

Table 2: Antimicrobial activity of the essential oil of *M. pseudolongifolia*.

Microbial species	*M. pseudolongifolia*		Compounds [c]						Antibiotics		
			1	2	3	4	5	6	Tetracycline	Gentamicine	Nystatine
	IZ [a]	MIC [b]	MIC	MIC	MIC	MIC	MIC	MIC	IZ	IZ	IZ
Bacillus cereus	31 ± 0.8	250	500	>1000	750	>1000	125	125	22 ± 0.8	-	nt
Staphylococcus aureus	39 ± 0.4	125	250	1000	500	1000	62.5	62.5	21 ± 0.4	-	nt
Staphylococcus epidermidis	52 ± 0.4	125	250	1000	500	1000	62.5	62.5	34 ± 0.4	-	nt
Escherichia coli	20 ± 0.8	750	1000	>1000	>1000	>1000	750	500	-	22 ± 0.8	nt
Enterobacter aerogenes	28 ± 0.4	375	750	>1000	>1000	>1000	250	125	10 ± 0.4	-	nt
Klebsiella pneumoniae	26 ± 0.8	375	750	>1000	>1000	>1000	250	125	-	21 ± 0.8	nt
Pseudomonas aeruginosa	18 ± 0.4	750	>1000	>1000	>1000	>1000	750	500	-	12 ± 0.8	nt
Vibrio parahaemolyticus	16 ± 0.8	1000	>1000	>1000	>1000	>1000	1000	1000	-	13 ± 0.8	nt
Aspergillus niger	10 ± 0.4	>1000	>1000	>1000	>1000	>1000	>1000	1000	nt	nt	17 ± 0.8
Candida albicans	32 ± 0.8	250	250	>1000	>1000	>1000	125	125	nt	nt	19 ± 0.8

[a] Inhibition zone diameter (mm), including diameter of sterile disk 6 mm; values are given as mean ± SD.[b] Minimum inhibitory concentration values as μg/mL.
[c] 1. β-caryophyllene (≥ 98.5%), 2. globulol (≥ 98.5%), 3. α-caryophyllene (≥ 98%), 4. viridiflorene (≥ 98%), 5. α-cadinol (100%), 6. β-eudesmol (≥ 98%). Compound 1 to 4 were purchased from the Fluka Co. (Milwaukee, USA), and Compound 6 was purchased from the Wako Co. (Tokyo, Japan), where as the Compound 5 was from an isolate of the Ho *et al*. [19] study on *Machilus philippinenesis* essential oil.
Essential oil tested at 15 μL/disc for bacteria and 30 μL/disc for fungi.(-), Inactive; (7-14), moderately active; (>14), highly active; nt, not tested.

Table 3: Anti-wood-decay fungal indices of leaf essential oil from *M. pseudolongifolia*

Dosage (μg/mL)	Anti-wood-decay fungal index (%)			
	Trametes versicolor	*Phaneochaete chrysosporium*	*Phaeolus schweintizii*	*Lenzites sulphureu*
12.5	46 ± 3.3	26 ± 3.3	29 ± 3.3	68 ± 3.3
25	69 ± 6.6	48 ± 3.3	49 ± 3.3	100 ± 0
50	91 ± 6.6	86 ± 3.3	89 ± 6.6	100 ± 0
75	100 ± 0	100 ± 0	100 ± 0	100 ± 0
100	100 ± 0	100 ± 0	100 ± 0	100 ± 0

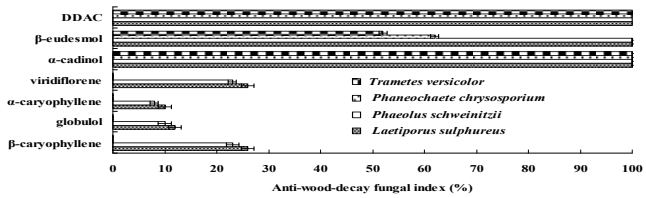

Figure 1: Anti-wood-decay fungal indices of the six main compounds (50 μg/mL) of the leaf essential oil of *M. pseudolongifolia*

distilled water and medium served as a positive control After incubation at 37°C for24 h and 24°C for 48 h, the MIC values were determined. All experiments were performed in triplicate.

Anti-wood-decay fungal assays: The method of Su *et al.* [11] was adopted. The fungi used were *T. versicolor* (L. ex Fr.) Quel. (BCRC 35253), *Phane. chrysosporium* Burdsall (BCRC 36200), *Phaeo. schweinitzii* (Fries) Paterson (BCRC 35365) and *L. sulphureus* (B. ex Fr.) Bond. (BCRC 35305). Microbial strains were obtained from the Culture Collection and Research Center of the Food Industry Research and Development Institute, Hsinchu City, Taiwan. Anti-wood-decay fungal assays were carried out in triplicate and the data were averaged. Different concentrations of the essential oil (12.5~100 μg/mL) were added to sterilized potato dextrose agar (PDA). The test plates were incubated at 27ºC. When the mycelium of fungi reached the edge of the control plate, the anti-wood-decay fungal index was calculated as follows: Anti-wood-decay fungal 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).

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Natural Product Communications
2010
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Contents

Continued inside backcover