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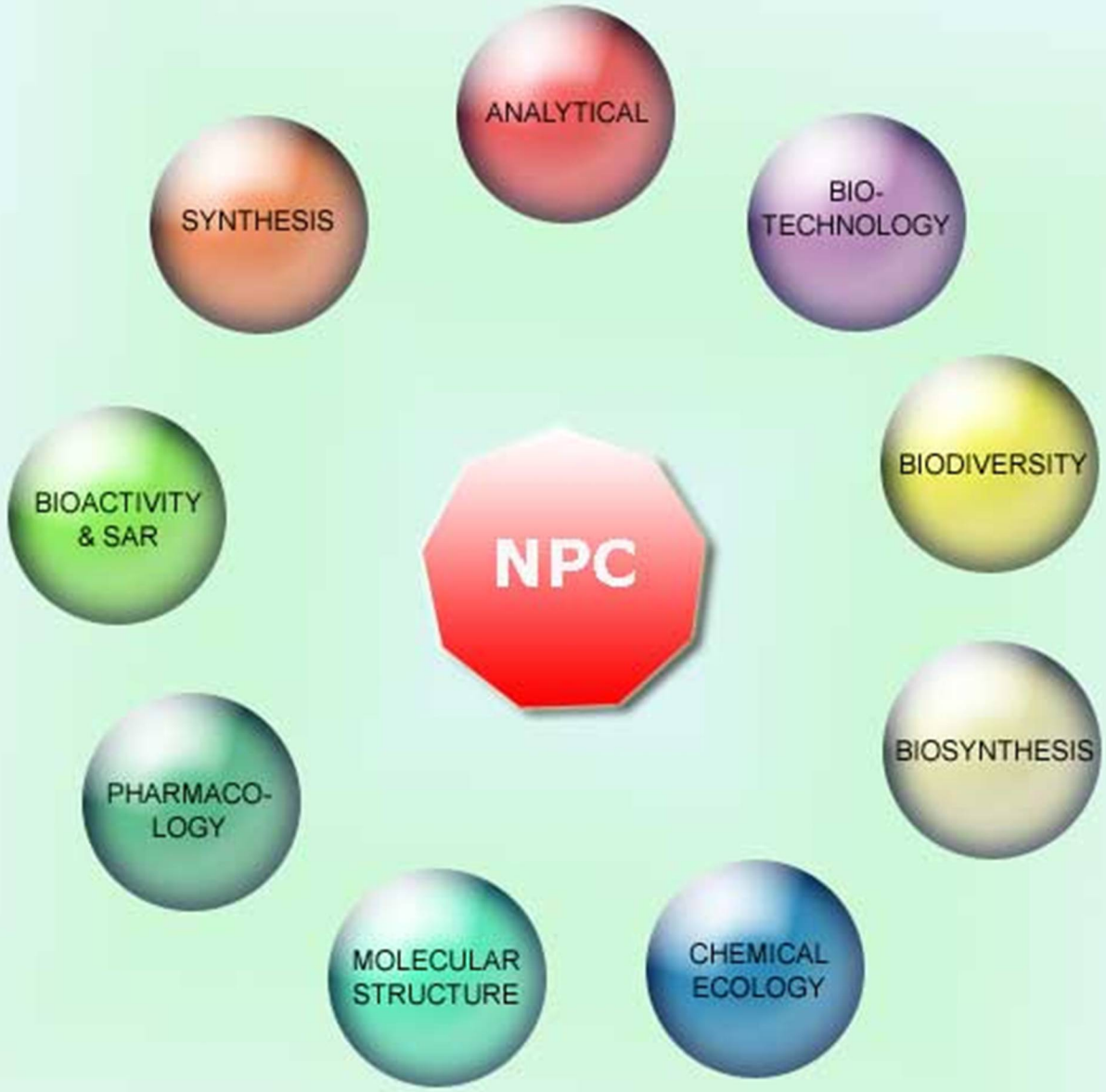

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Composition and Antimicrobial Activities of the Leaf Essential Oil of *Machilus kusanoi* from Taiwan

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The hydrodistillated leaf essential oil of *Machilus kusanoi* was analyzed to determine its composition and yield. Twenty-three compounds were identified, the main components being β-caryophyllene (23.3%), β-eudesmol (17.1%), α-terpineol (16.0%), *n*-dodecanal (14.2%), and *n*-decanal (10.4%). Sesquiterpene hydrocarbons (28.1%) and non-terpenoids (25.0%) were the predominant groups of compounds. The leaf oil exhibited excellent antimicrobial and anti-wood-decay fungal activities.

Keywords: *Machilus kusanoi*, Lauraceae, essential oil, antimicrobial activity, anti-wood-decay fungal activity, β-caryophyllene, β-eudesmol.

Machilus kusanoi Hayata, (Lauraceae) is an endemic species of Taiwan and is distributed from the lowlands to 1400 m [1]. There are no literature reports on the chemical composition and biological activities of the essential oils or other extractives from this species. However, the essential oil composition and antimicrobial activity of other species of this genus have been previously reported [2-7]. Therefore, we used hydrodistillation to collect the leaf oil, analyzed for its chemical composition, and evaluated for its antimicrobial and anti-wood-decay fungal activities. The purpose of this study was to establish a chemical basis for the effective multipurpose utilization of the species.

Hydrodistillation of *M. kusanoi* leaves gave a yellow-colored oil with a yield of 2.83 ± 0.03 mL/100 g, based on the dry weight of leaves. Twenty-three compounds were identified (Table 1), of which sesquiterpene hydrocarbons were predominant (28.1%), followed by non-terpenoids (25.0%), oxygenated sesquiterpenes (19.5%), oxygenated monoterpenes (18.9%), and monoterpene hydrocarbons (8.4%). Among the sesquiterpene hydrocarbons, β-caryophyllene (23.3%) was the major compound, and of the non-terpenoids, *n*-dodecanal (14.2%) and *n*-decanal (10.4%) were the chief components. Among the oxygenated sesquiterpenes, β-eudesmol (17.1%) was the main components, and of the oxygenated monoterpenes, α-terpineol (16.0%) was the chief components.

Although the leaf oil constituents of *M. kusanoi* was primarily sesquiterpenoids, like those of *M. pseudolongifolia* [2], *M. philippinensis* [3], *M. obovatifolia* [4], *M. velutina* [5] and *M. thunbergii* [6], their main components differed. Further comparison with the leaf oil of *M. japonica* [7] was predominantly monoterpenoids and, therefore, differed from the leaf oil of *M. kusanoi*.

The essential oil of *M. kusanoi* was tested against three Gram-positive and five Gram-negative bacteria, as well as two fungi. The results, presented in Table 2, indicated that a medium to strong growth suppression against all ten microbes studied. The most sensitive microorganisms were *Bacillus cereus*, *Staphylococcus aureus*, *S. epidermidis* and *Candida albicans* with inhibition zones of 32 to 42 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 of the literature [2,4,8-11]. Comparing the antimicrobial activities of the essential oils from *M. obovatifolia* [4], *Litsea. nakaii* [8], *L. kostermansii* [9], *L. laevigata* [10], and *Cinnamomum subavenium* [11] the leaf essential oil of *M. kusanoi* was superior. The results verify that *M. kusanoi* leaf oil has excellent antimicrobial activity. However, in order to ascertain the source compounds of antimicrobial activity from *M. kusanoi*, the main components were individually tested for their antimicrobial activities. The results indicated that the active source compounds were *n*-dodecanal and β-eudesmol. These results were similar to those of Ho *et al*. [8,9]. There are also studies supporting the contention that these compounds have high activity in suppressing microbial growth [13-16].

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

Compound	RI	Conc. (%)	Identification
α-Pinene	939	0.5	KI, MS, ST
α-Phellandrene	1003	2.4	KI, MS, ST
p-Cymene	1025	0.5	KI, MS, ST
β-Phellandrene	1030	0.4	KI, MS
cis-Ocimene	1037	4.1	KI, MS, ST
trans-Ocimene	1050	0.5	KI, MS, ST
p-Methyl-acetophenone	1183	0.7	KI, MS, ST
α-Terpineol	1189	16.0	KI, MS, ST
n-Decanal	1202	10.4	KI, MS, ST
Verbenone	1205	0.4	KI, MS, ST
Carvenone	1258	1.2	KI, MS
(*E*)-Patchenol	1331	0.7	KI, MS
α-Copaene	1377	0.3	KI, MS, ST
(*Z*)-Trimenal	1398	0.5	KI, MS
n-Dodecanal	1409	14.2	KI, MS, ST
β-Caryophyllene	1419	23.3	KI, MS, ST
α-Humulene	1455	3.5	KI, MS, ST
Viridiflorene	1497	0.7	KI, MS
δ-Cadinene	1523	0.3	KI, MS, ST
Caryophyllene alcohol	1572	0.5	KI, MS, ST
Caryophyllene oxide	1583	1.5	KI, MS, ST
β-Eudesmol	1651	17.1	KI, MS, ST
α-Eudesmol	1654	0.4	KI, MS, ST
Compound identified			
Monoterpene hydrocarbons (%)		8.4	
Oxygenated monoterpenes (%)		18.9	
Sesquiterpene hydrocarbons (%)		28.1	
Oxygenated sesquiterpenes (%)		19.5	
Others		25.0	
Oil Yield (mL/100 g)		2.83 ± 0.03	

[a] Retention index on a DB-5 column with reference to n-alkanes [12].
[b] MS, NIST and Wiley library spectra and the literature; RI, Retention index; ST, authentic standard compounds. [c].trace < 0.1%

The essential oil of *M. kusanoi* 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* [17] and *M. philippinensis* [3], the leaf oil of *M. kusanoi* was superior. The results verified that *M. kusanoi* leaf oil has excellent anti-wood-decay fungal activities.

Furthermore, in order to ascertain the source compounds of the *M. kusanoi* 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 *n*-dodecanal and β-eudesmol. The IC_{50} values of the 2 compounds (*n*-dodecanal and β-eudesmol) against the 4 decay fungi were 56 and 48 ppm against *T. versicolor*; 42 and 38 ppm against *Phane. chrysosporium*; 25 and 23 ppm against *Phaeo. Shweinitzii*; and 23 and 20 ppm against *L. sulphureu*, respectively. At a 50 μg/mL concentration, *n*-dodecanal and β-eudesmol could completely inhibit brown-rot fungi but partially inhibit white-rot fungi. The results agree with those of Kondo and Imamura [13], Nami [18] and Mori *et al*. [19]. Thus, the excellent wood-decay-fungi inhibitive activities exhibited by the *M. kusanoi* leaf oil could well be contributed by the presence of compounds such as *n*-dodecanal and β-eudesmol etc.

Experimental

Plant materials: Fresh leaves of *M. kusanoi* were collected in July 2009 from the Dahanshan at an elevation of 500 m in southern Taiwan (N 22º 25′ 28″, E 120º 43′ 32″, Pingtung County). The samples were compared with specimen no. ou6851 from the Herbarium of National Chung-Hsing University and positively identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH-011) 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. kusanoi* (1 Kg) 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: 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. Diluted samples (1.0 μL, 1/100, v/v, in ethyl acetate) were injected manually in the split mode.

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

Microbial species	*Machilus kusanoi*		Compounds [c]					Antibiotics		
	Leaf		1	2	3	4	5	Tetracycline	Gentamicine	Nystatine
	IZ [a]	MIC [b]	MIC	MIC	MIC	MIC	MIC	IZ	IZ	IZ
Bacillus cereus	32 ± 0.4	250	>1000	>1000	250	500	125	22 ± 0.8	-	nt
Staphylococcus aureus	42 ± 0.4	125	>1000	1000	125	250	62.5	21 ± 0.4	-	nt
Staphylococcus epidermidis	40 ± 0.8	125	>1000	1000	125	250	62.5	34 ± 0.4	-	nt
Escherichia coli	23 ± 0.8	500	>1000	>1000	500	1000	500	-	22 ± 0.8	nt
Enterobacter aerogenes	28 ± 0.4	375	>1000	>1000	250	750	125	10 ± 0.4	-	nt
Klebsiella pneumoniae	26 ± 0.4	375	>1000	>1000	250	750	125	-	21 ± 0.8	nt
Pseudomonas aeruginosa	23 ± 0.8	500	>1000	>1000	750	>1000	500	-	12 ± 0.8	nt
Vibrio parahaemolyticus	18 ± 0.4	1000	>1000	>1000	1000	>1000	1000	-	13 ± 0.8	nt
Aspergillus niger	18 ± 0.4	1000	>1000	>1000	1000	>1000	1000	nt	nt	17 ± 0.8
Candida albicans	36 ± 0.8	250	>1000	>1000	125	250	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. α-terpineol (≥ 98.5%), 2. *n*-decanal (≥ 98%), 3. n-dodecanal (≥ 98%), 4. β-caryophyllene (≥ 98.5%), 5. β-eudesmol (≥ 98%). Compound 1 to 4 were purchased from the Fluka Co. (Milwaukee, USA), and Compound 5 was purchased from the Wako Co. (Tokyo, Japan). 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. kusanoi*

Dosage (μg/mL)	Anti-wood-decay fungal index (%)			
	Trametes versicolor	*Phaneochaete chrysosporium*	*Phaeolus schweintizii*	*Lenzites sulphureu*
12.5	43 ± 3.3	23 ± 3.3	25 ± 3.3	60 ± 3.3
25	72 ± 3.3	40 ± 3.3	46 ± 3.3	100 ± 0
50	86 ± 6.6	83 ± 6.6	80 ± 6.6	100 ± 0
75	100 ± 0	100 ± 0	100 ± 0	100 ± 0
100	100 ± 0	100 ± 0	100 ± 0	100 ± 0

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) [12], retention times (RT), and mass spectra with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [12,20].

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 [21]. Discs containing 15 μL and 30 μL of the oil, which was totally 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 [2,8,9].

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:

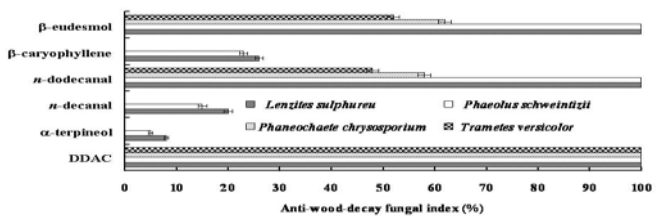

Figure 1: Anti-wood-decay fungal indices of the five main compounds (50 μg/mL) of the leaf essential oil of *M. kusanoi*.
Note: DDAC (didecyl dimethyl ammonium chloride) is a wood preservative for wood decay fungi and is used as a positive control.

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 [22]. 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 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.* [11,23] 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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