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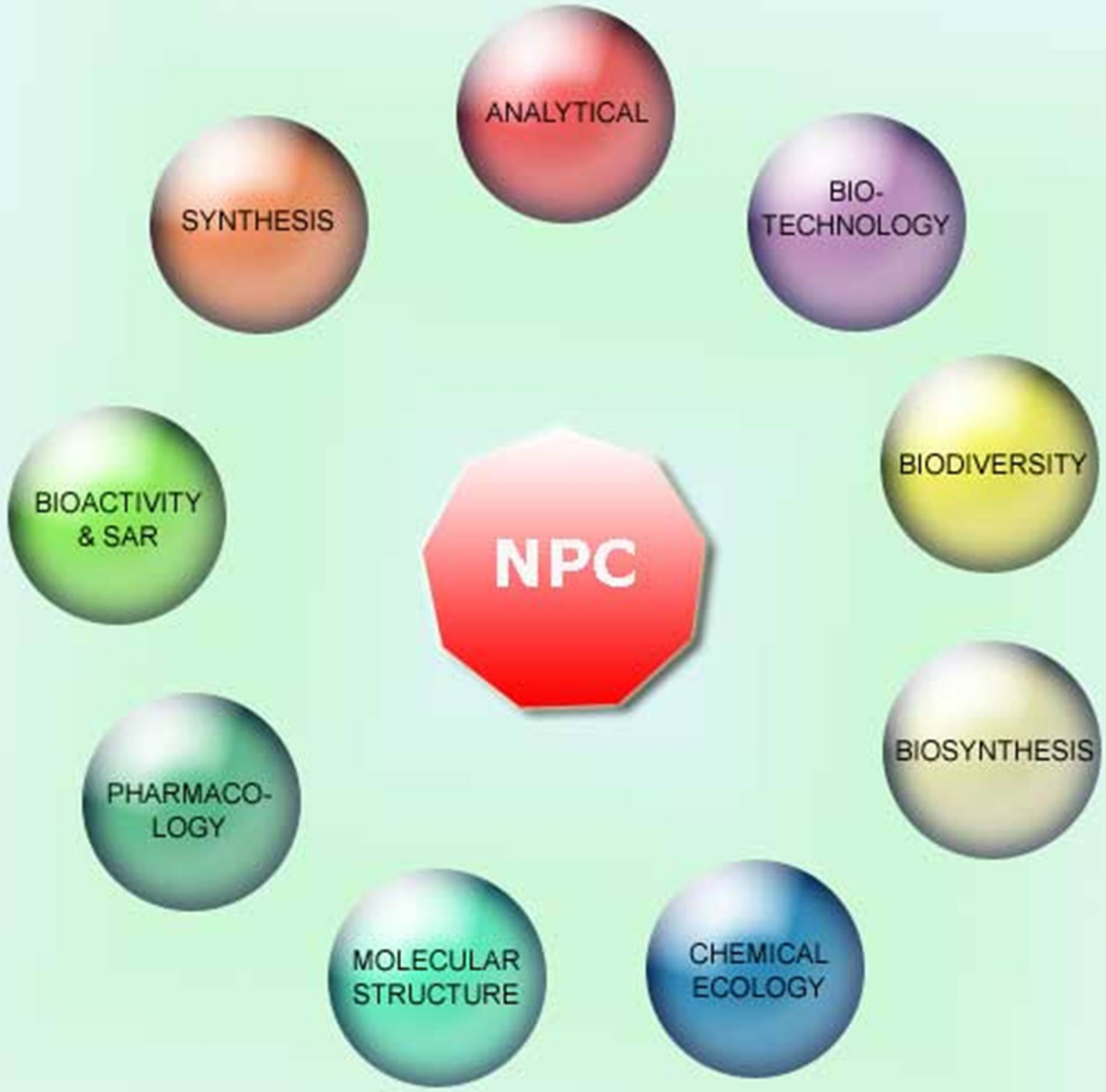

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Composition, *in-vitro* Anticancer, and Antimicrobial Activities of the Leaf Essential Oil of *Machilus mushaensis* from Taiwan

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This study investigated the chemical composition, *in-vitro* anticancer, and antimicrobial activities of the essential oil isolated from the leaf of *Machilus mushaensis* from Taiwan. The essential oil was isolated using hydrodistillation in a Clevenger-type apparatus, and characterized by GC–FID and GC–MS. Forty compounds were identified, representing 100% of the oil. The main components identified were *n*-decanal (61.0%), and α-cadinol (20.8%). The oil exhibited cytotoxic activity against human oral, liver, lung, colon, melanoma, and leukemic cancer cells. The antimicrobial activity of the oil was tested by the disc diffusion and micro-broth dilution methods against ten microbial species. The oil exhibited moderate growth suppression against Gram-positive bacteria and yeast with inhibition zones of 25-29 mm to MIC values of 375-500 μg/mL, respectively. α-Cadinol was found to show promising anticancer and antimicrobial activities.

Keywords: *Machilus mushaensis*, Essential oil, α-Cadinol, Anticancer activity, Antimicrobial activity.

The Lauraceae family contains approximately 45 genera and 2250 species [1].The *Machilus* genus is comprised of deciduous trees and shrubs. There are about 100 species in the genus, which are widely distributed, mainly in Eastern Asia. [2]. In Taiwan, there are eight endemic *Machilus* trees, *M. konishii* Hayata, *M. kusanoi* Hayata, *M. mushaensis* Lu, *M. obovatifolia* (Hayata) Kaneh. et. Sasaki, *M. obovatifolia* var. *taiwensis* Lu & Chen, *M. philippinensis* Merr., *M. pseudolongifolia* Hayata., *M. thunbergii* Siebold & Zucc. and *M. zuhoensis* Hayata [3]. All these species have a fragrant odor, and certain species possess bioactivity. In our previous report, the leaf essential oils of *M. zuihoensis* [4], *M. kusanoi* [5], *M. pseudolongifolia* [6], *M. philippinensis* [7], and *M. obovatifolia* [8], were extracted and found to have antimicrobial activities. *M. mushaensis* is an endemic species of Taiwan and is distributed from the lowlands to 1500 m [3]. No studies have investigated either the chemical composition or biological activities of the essential oils from this species. Therefore, hydrodistillation was used to collect the leaf oil, which was analyzed by GC–FID and GC–MS. Malignant tumors, in particular oral, liver, lung, colon, melanoma, and leukemia, are a principal cause of death in Taiwan, as also in many industrialized countries. Ten microbial strains and six cancer cell lines were chosen for antimicrobial and anticancer activities. The purpose of this study was to establish a chemical basis for effective multipurpose utilization of the tree species.

Hydrodistillation of *M. mushaensis* leaves produced a yellow-colored oil with a yield of 3.2 ± 0.03 mL/100 g oven dried leaves. All compounds are listed in order of their elution from the DB-5 column (Table 1). A total of 40 compounds were identified among which non-terpenoids were predominant (65.0%), followed by oxygenated sesquiterpenes (24.9%), sesquiterpene hydrocarbons (8.6%), diterpenes (1.0%), monoterpene hydrocarbons (0.6%), and oxygenated monoterpenes (0.1%). Among the non-terpenoids, *n*-decanal (61.0%) was the major compound, and of the oxygenated sesquiterpenes, α-cadinol (20.8%) was the main component. Other representative compounds were *z*-trimenal (3.7%), viridiflorene (2.9%), β-caryophyllene (1.7%) and globulol (1.3%). This is the first study on the chemical characterization of the leaf oil.

From the results presented, the leaf oil constituents were primarily non-terpenoids. Intra-genus leaf oil comparisons indicated that many *Machilus* trees, such as *M. zuihoensis* [4], *M. kusanoi* [5], *M. pseudolongifolia* [6], *M. philippinensis* [7], *M. obovatifolia* [8], *M. velutina* [9], and *M. thunbergii* [10], all have predominately sesquiterpenoids as their main constituents and, therefore, differed from the leaf oil of *M. mushaensis*.

To evaluate the anticancer activities of the leaf essential oil of *M. mushaensis*, we tested its effect on the viability of six human cancer cell lines: OEC-M1 (human oral squamous) cells, J5 (human hepatocellular carcinoma) cells, A549 (human lung adenocarcinoma) cells, HT-29 (human colon) cells, UACC-62 (*human* melanoma) cells, and K562 (human leukemic) cells. Cells were incubated with various concentrations of essential oil for 48 h, and then cell viabilities were measured by the alamarBlue® proliferation assay. The results showed that oil treatment for 48 h reduced the viability of OEC-M1 cells, J5 cells, A549 cells, HT-29 cells, UACC-62 cells, and K562 cells, with IC_{50} values around 48.6, 58.8, 50.8, 3.8, 6.8, and 14.8 μg/mL, respectively (Table 2). This is the first report on the anticancer activities of *M. mushaensis* leaf essential oil.

However, in order to ascertain the source compounds of the anticancer activities, the main components of *M. mushaensis* oil, *n*-decanal, *z*-trimenal, viridiflorene, and α-cadinol were individually tested for their cytotoxic activities. The results showed that the active compound was α-cadinol. α-Cadinol treatment for 48 h reduced the viability of OEC-M1 cells, J5 cells, A549 cells, HT-29 cells, UACC-62 cells, and K562 cells with IC_{50} values < 15 ppm (Table 2). α-Cadinol is reported to be cytotoxic against three human cancer cell lines, including A-549, MCF-7, and HT-29 [11]. The presence of α-cadinol significantly contributed to the anticancer activities of *M. mushaensis* leaf oil.

The leaf oil of *M. mushaensis* was also tested against three Gram-positive and five Gram-negative bacteria, as well as two fungi. The results, presented in Table 3, indicated moderate growth suppression against all ten microbes. The most sensitive were *Bacillus cereus*, *Staphylococcus aureus*, *S. epidermidis*, and

Table 1: Chemical composition of the leaf essential oil of *Machilus mushaensis*.

Consituents	RI[a]	Concentration(%)	Identification[b]
cis-3-Hexenol	859	0.1	MS, RI, ST
n-Hexanol	871	t	MS, RI, ST
Benzaldehyde	960	0.1	MS, RI, ST
α-Phellandrene	1003	0.1	MS, RI, ST
cis-Ocimene	1037	0.4	MS, RI, ST
trans-Ocimene	1050	0.1	MS, RI, ST
n-Nonanal	1101	0.1	MS, RI, ST
n-Decanal	1202	61.0	MS, RI, ST
2-Ethyl menthone	1283	t	MS, RI
Isobornyl acetate	1286	t	MS, RI
Bornyl acetate	1288	t	MS, RI, ST
Isoledene	1376	0.3	MS, RI
α-Copaene	1377	0.8	MS, RI, ST
β-Elemene	1391	0.1	MS, RI, ST
Z-Trimenal	1398	3.7	MS, RI, ST
α-Gurjunene	1410	0.2	MS, RI, ST
β-Caryophyllene	1419	1.7	MS, RI, ST
Aromadendrene	1441	0.7	MS, RI, ST
α-Himachalene	1451	0.1	MS, RI
α-Humulene	1455	0.6	MS, RI, ST
allo-Aromadendrene	1460	0.2	MS, RI, ST
trans-Cadina-1(6),4-diene	1477	0.3	MS, RI
β-Selinene	1490	0.5	MS, RI
Viridiflorene	1497	2.9	MS, RI, ST
γ-Cadinene	1514	0.3	MS, RI
trans-Cadina-1(2),4-diene	1535	t	MS, RI
α-Cadinene	1539	t	MS, RI
(*E*)-Nerolidol	1563	0.4	MS, RI, ST
Ledol	1569	0.3	MS, RI
Spathulenol	1578	0.1	MS, RI, ST
Caryophyllene oxide	1583	0.1	MS, RI, ST
Globulol	1585	1.3	MS, RI, ST
Viridiflorol	1593	0.9	MS, RI, ST
5-*epi*-7-*epi*-α-Eudesmol	1608	0.5	MS, RI
Isolongifolan-7-α-ol	1619	0.1	MS, RI
10-*epi*-γ-Eudesmol	1624	0.4	MS, RI
α-Cadinol	1654	20.8	MS, RI, ST
Cadalene	1677	t	MS, RI
α-Bisabolol	1686	0.1	MS, RI, ST
Phytol	1943	1.0	MS, RI, ST
Monoterpene hydrocarbons (%)		0.6	
Oxygenated monoterpenes (%)		0.1	
Sesquiterpene hydrocarbons (%)		8.6	
Oxygenated sesquiterpenes (%)		24.9	
Diterpenes (%)		1.0	
Others (%)		65.0	
Oil Yield (mL/100 g)		3.2 ± 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. t, trace<0.1%

Candida albicans, with inhibition zones of 25 to 29 mm to MIC values of 375-500 μg/mL, respectively. The essential oil showed superior suppressive activity toward the Gram-positive bacteria than either the Gram-negative bacteria or the fungi. The probable cause of the susceptibility of Gram-positive bacteria and relative tolerance of Gram-negative bacteria to essential oils has been correlated with the presence of a hydrophilic outer layer [13]. It is presumed that penetration of hydrophobic components in Gram-negative microorganisms is more difficult due to the presence of a second physical barrier formed by the outer membrane [14].

Table 2: IC_{50} values of *Machilus mushaensis* leaf oil and its main constituents against cancer cell lines.

Cell lines [a]	IC_{50} (μg/mL)				
	Essential oil	Compounds			
		n-Decanal	*z*-Trimenal	Viridiflorene	α-Cadinol
OEC-M1	48.6	>200	>200	>200	9.9
J5	58.8	>200	>200	>200	12.1
A549	50.8	>200	>200	>200	10.8
HT-29	3.8	>200	>200	>200	0.8
UACC-62	6.8	>200	>200	>200	1.3
K562	14.8	>200	>200	>200	2.8

[a] Cell lines: OEC-M1 (human oral squamous); J5 (human hepatocellular carcinoma); A549 (human lung adenocarcinoma); HT-29 (human colon), UACC-62 (*human* melanoma); K562 (human leukemic).[b]

However, to ascertain the compounds responsible for the antimicrobial activity of *M. mushaensis* leaf oil, the main components were individually tested for antimicrobial activities. *n*-Decanal, *z*-trimenal, and viridiflorene were purchased from the Fluka Co. (Milwaukee, USA), and α-Cadinol was isolated from the essential oil of *M. philippinenensis* according to the method proposed by Ho *et al*. [6]. The results indicated that the most active compound was α-cadinol. Various studies support the argument that these compounds are highly active in suppressing microbial growth [15-17].

Experimental

Plant materials: Fresh leaves of *M. mushaensis* were collected in June 2012 from Chilan Mt in northeast Taiwan (Yilan County, elevation 1050 m, N 24º 39′ 86″, 121º 40′ 68″). The samples were compared with specimen no. ou 9668 from the Herbarium of the National Chung-Hsing University and identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH-026) was deposited in the NCHU herbarium. Leaves of the species were collected for subsequent extraction and analysis.

Isolation of essential oil: Leaves of *M. mushaensis* (1 Kg) were hydrodistilled for 6 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

Table 3: Antimicrobial activity of the leaf essential oil of *Machilus mushaensis*.

Microbial species	*M. mushaensis*		Compounds				Antibiotics		
			n-Decanal	*Z*-Trimenal	Viridiflorene	α-Cadinol	Tetracycline (30 μg/disk)	Gentamicin (10 μg/disk)	Nystatin (30 μg/disk)
	IZ[a]	MIC[b]	MIC	MIC	MIC	MIC	IZ	IZ	IZ
Bacillus cereus	25 ± 0.8	500	>1000	>1000	>1000	125	22 ± 0.8	-	nt
Staphylococcus aureus	29 ± 0.4	375	>1000	>1000	1000	62.5	21 ± 0.4	-	nt
Staphylococcus epidermidis	28 ± 0.4	375	>1000	>1000	1000	62.5	34 ± 0.4	-	nt
Escherichia coli	16 ± 0.8	1000	>1000	>1000	>1000	750	-	22 ± 0.8	nt
Enterobacter aerogenes	20 ± 0.4	750	>1000	>1000	>1000	250	10 ± 0.4	-	nt
Klebsiella pneumoniae	20 ± 0.8	750	>1000	>1000	>1000	250	-	21 ± 0.8	nt
Pseudomonas aeruginosa	18 ± 0.4	1000	>1000	>1000	>1000	750	-	12 ± 0.8	nt
Vibrio parahaemolyticus	12 ± 0.8	>1000	>1000	>1000	>1000	1000	-	13 ± 0.8	nt
Aspergillus niger	10 ± 0.4	>1000	>1000	>1000	>1000	>1000	nt	nt	17 ± 0.8
Candida albicans	25 ± 0.8	500	>1000	>1000	>1000	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. Essential oil tested at 15 μL/disc for bacteria and 30 μL/disc for fungi.(-), Inactive; nt, not tested.

oil components. Oven temperature was programmed as follows: 50ºC for 2 min, rising to 250ºC at 5ºC/min. Injector temperature: 270ºC. Carrier gas: Helium 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 are 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, 18].

Cell culture: Human oral squamous cancer OEC-M1 cells, human hepatocellular carcinoma J5 cells, human lung adenocarcinoma A549 cells, human colon cancer HT-29 cells, *human* melanoma UACC-62 cells, and human leukemic cell K562 cells were obtained from ATCC (Rockville, MD, USA) and multiplied in RPMI-1640 medium supplemented with 10% heated-inactivated FCS and 2 mM L-glutamine (Life Technologies, Inc., MD), and incubated at 37°C with 5% CO_2 incubator and 95% humidity.

Cell viability assay: The cytotoxicity of the essential oil was assessed using the alamarBlue® proliferation assay according to a protocol from AbD Serotec. Cells (3000 cells/well) were incubated with either essential oils (dissolved in DMSO, final 0.1% DMSO in medium) or vehicle control (0.1% DMSO) for 24 h and 48 h, followed by replacement with fresh medium containing 10% alamarBlue® reagent for an additional 6 h. The absorbances at 570 nm and 600 nm were measured by a microplate reader. All data were the average of triplicate analyses.

Antimicrobial activity: Discs containing 15 μL and 30 μL of the oil dissolved in 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. Gentamicin and tetracycline for bacteria, and nystatin for fungi were used as positive controls, as previously published [19]. 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), *Staphylococcus aureus* (ATCC 6538P), and *S. epidermidis* (ATCC 12228); 1 fungus: *Aspergillus niger* (ATCC 16404) and 1 yeast: *Candida albicans* (ATCC 10231). Minimum inhibitory concentration (MIC) values were measured by the microdilution broth susceptibility assay recommended by NCCLS [20] and as reported earlier [5]. Data are expressed as the means ± SD of 3 independent experiments.

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