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Composition, Antioxidant and Antimicrobial Activities of the Leaf Essential Oil of *Machilus japonica* from Taiwan

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The chemical composition, and antioxidant and antimicrobial activities of the essential oil isolated from the leaf of *Machilus japonica* from Taiwan have been investigated. The essential oil from the fresh leaves was isolated using hydrodistillation in a Clevenger-type apparatus, and characterized by GC–FID and GC–MS. A total of 97 compounds were identified, representing 100% of the oil. The main components identified were α-phellandrene (14.5%), α-pinene (12.8%), thymol (12.6%), β-pinene (8.3%), α-terpineol (6.5%) and carvacrol (6.0%). The antioxidant activity of the oil was tested by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capability test. The results showed that the IC_{50} was 51.8 μg/mL. The antimicrobial activity of the oil was tested by the disc diffusion and micro-broth dilution methods against ten microbial species. The oil exhibited strong growth suppression against Gram-positive bacteria and yeast, with inhibition zones of 48~54 mm and MIC values of 16.12~32.25 μg/mL, respectively. For the antioxidant and antimicrobial activities of the oil, the active source compounds were determined to be thymol and carvacrol.

Keywords: *Machilus japonica*, Lauraceae, Essential oil, Antioxidant activity, Antimicrobial activity, Thymol, Carvacrol.

Machilus japonica Sieb. & Zucc. (Lauraceae) is a large evergreen tree widely distributed in the broad leafed forests from the lowlands to an altitude of 1300~2000 m throughout southern Japan, the Ryukyus, south Korea and Taiwan [1]. Scanty references were found regarding the chemical compositions of this species [2-6]. However, in Taiwan, there is no report of the essential oil composition and bioactivities for *M. japonica*. Worldwide, only two reports, are known, one by Komae and Hayashi [5] in Japan, and the other by van Khien *et al*. [6] in Vietnam. Furthermore, both papers only dealt with the compositions, and there was no comment on their bioactivities. Therefore, in this study, the essential oil from the leaves of *M. japonica* was first isolated using hydrodistillation, and then analyzed. The second part of the study examined the antioxidant and antimicrobial activities of the leaf oil. The purpose of this work was to establish a chemical basis for the effective multipurpose utilization of the species.

Hydrodistillation of *M. japonica* leaves produced a yellow-colored oil with a yield of 2.86 ± 0.05 mL/100 g, based on the dry weight of leaves. Ninety-seven compounds were identified (Table 1), of which monoterpene hydrocarbons were predominant (50.5%), followed by oxygenated monoterpenes (31.7%), sesquiterpene hydrocarbons (13.0%), oxygenated sesquiterpenes (3.6%), and non-terpenoids (1.3%). Among the monoterpene hydrocarbons, α-phellandrene (14.5%), α-pinene (12.8%), and β-pinene (8.3%) were the major compounds, and of the oxygenated monoterpenes, thymol (12.6%), α-terpineol (6.5%) and carvacrol (6.0%). Among the sesquiterpene hydrocarbons, β-caryophyllene (5.1%) was the main component. Relevant literature on the leaf essential oils of *M. japonica* is rare. Only Komae and Hayashi [5] and van Khien *et al*. [6] have made previous studies. The former [5] studied the leaf oil from Japan. They identified a total of 9 compounds with the main components being caryophyllene (18.6%) and β-phellandrene (14.7%). van Khien *et al*. [6] reported on *M. japonica* leaf oil from Vietnam. They identified a total of 18 compounds with the main ones being α-phellandrene (60.2%), β-phellandrene (10.5%), and *p*-cymene (20.3%). Comparing the main components reported in the two reports, the *M. japonica* leaf oils showed markedly divergent compositions.

From the results presented above, the leaf oil constituents of *M. japonica* were primarily monoterpenoids, which differed from *M. kusanoi* [7], *M. pseudolongifolia* [8], *M. philippinensis* [9], *M. obovatifolia* [10], *M. velutina* [11], and *M. thunbergii* [12], all of which contained mostly sesquiterpenoids.

The leaf oil of *M. japonica* was tested for its DPPH free radical scavenging capability. Ascorbic acid was used as a positive control. The IC_{50} for the DPPH free radical scavenging capability of the leaf oil was 51.8 μg/mL. The individual main components of the leaf essential oil, α-pinene, β-pinene, α-phellandrene, *p*-cymene, α-terpineol, thymol, carvacrol, and β-caryophyllene, were also compared for their DPPH free radical scavenging capability. The results showed that the DPPH free radical scavenging capabilities in a decreasing order were thymol (IC_{50} = 31.4 μg/mL), carvacrol (IC_{50} = 38.7 μg/mL), α-pinene, β-pinene, α-phellandrene, *p*-cymene, α-terpineol, and β-caryophyllene (IC_{50}> 2000 μg/mL). Hence, we deduced that the phenolic compounds were mainly responsible for the radical scavenging. The results are in congruency with the conclusions of several other reports [14-19]. When the DPPH free radical scavenging capabilities of the leaf essential oil were compared with those of leaf oils of different provenances from Taiwan, such as *Cinnamomum osmophloeum*, with IC_{50} values ranging from 33.4 to 708.5 μg/mL [20], the leaf essential oil was within the same range. The threshold concentration also compared favorably with the IC_{50} values of 460 μg/mL for the leaf oil of *Nigella sativa* [21] and 500 μg/mL for the leaf oil of *Curcuma zedoaria* [22].

The essential oil of *M. japonica* was tested against three Gram-positive and five Gram-negative bacteria, as well as two fungi. The results, presented in Table 2, indicated a moderate to strong growth

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

Compound	RI[a]	Concentration(%)	Identification[b]
(*3Z*)-Hexenol	859	t[c]	KI, MS, ST
Santolina triene	909	t	KI, MS, ST
Tricyclene	927	t	KI, MS, ST
α-Thujene	930	t	KI, MS, ST
α-Pinene	939	12.8	KI, MS, ST
Camphene	954	1.4	KI, MS, ST
β-Pinene	979	8.3	KI, MS, ST
Myrcene	991	1.6	KI, MS, ST
α-Phellandrene	1003	14.5	KI, MS, ST
p-Mentha-1(7),8-diene	1004	t	KI, MS, ST
α-Terpinene	1017	0.8	KI, MS, ST
p-Cymene	1025	5.4	KI, MS, ST
δ-3-Carene	1031	2.7	KI, MS, ST
1,8-Cineole	1031	0.5	KI, MS, ST
cis-Ocimene	1037	0.4	KI, MS, ST
trans-Ocimene	1050	0.4	KI, MS, ST
γ-Terpinene	1060	0.2	KI, MS, ST
n-Octanol	1068	t	KI, MS, ST
p-Mentha-3,8-diene	1073	t	KI, MS, ST
Terpinolene	1089	1.9	KI, MS, ST
Linalool	1097	0.5	KI, MS, ST
n-Nonanal	1101	0.1	KI, MS, ST
endo-Fenchol	1117	0.7	KI, MS, ST
cis-*p*-Menth-2-en-1-ol	1122	0.1	KI, MS
allo-Ocimene	1132	t	KI, MS
trans-*p*-Menth-2-en-1-ol	1141	0.1	KI, MS
Camphor	1146	t	KI, MS, ST
Camphene hydrate	1150	0.1	KI, MS, ST
Isoborneol	1162	0.1	KI, MS
Borneol	1169	0.6	KI, MS
cis-Pinocamphone	1175	0.4	KI, MS
Terpinen-4-o1	1177	0.5	KI, MS, ST
α-Terpineol	1189	6.5	KI, MS, ST
Methyl chavicol	1194	0.5	KI, MS, ST
n-Decanal	1202	0.4	KI, MS, ST
trans-Piperitol	1208	0.1	KI, MS, ST
Citronellol	1226	0.4	KI, MS, ST
Pulegone	1237	0.1	KI, MS, ST
Isobornyl formate	1239	0.1	KI, MS
trans-Piperitone epoxide	1256	0.4	KI, MS
(*2E*)-Decenal	1264	t	KI, MS
(*E*)-Cinnamaldehyde	1270	t	KI, MS
Isobornyl acetate	1286	0.5	KI, MS
Thymol	1290	12.6	KI, MS, ST
Menthyl acetate	1295	t	KI, MS
Carvacrol	1299	6.0	KI, MS, ST
Undecanal	1307	0.3	KI, MS, ST
Veloutone	1311	0.2	KI, MS
Dihydro citronellol acetate	1321	t	KI, MS
neo-Verbanol acetate	1321	0.2	KI, MS
Citronellyl acetate	1353	0.3	KI, MS
Decanoic acid	1373	0.5	KI, MS, ST
α-Copaene	1377	0.4	KI, MS, ST
Geranyl acetate	1381	0.2	KI, MS, ST
Modheph-2-ene	1384	t	KI, MS
β-Elemene	1391	0.1	KI, MS, ST
(*Z*)-Trimenal	1398	0.1	KI, MS, ST
Dodecanal	1409	t	KI, MS, ST
α-Gurjunene	1410	0.1	KI, MS, ST
β-Caryophyllene	1419	5.1	KI, MS, ST
β-Gurjunene	1434	0.1	KI, MS, ST
α-Guaiene	1440	t	KI, MS, ST
Aromadendrene	1441	0.2	KI, MS, ST
α-Himachalene	1451	t	KI, MS, ST
trans-Muurola-3.5-diene	1454	0.1	KI, MS
α-Caryophyllene	1455	0.9	KI, MS, ST
allo-Aromadendrene	1460	0.1	KI, MS, ST
Drima-7,9(11)-diene	1473	t	KI, MS
γ-Muurolene	1480	1.1	KI, MS
Germacrene D	1485	0.1	KI, MS, ST
β-Selinene	1490	1.1	KI, MS, ST
α-Selinene	1498	1.6	KI, MS, ST
α-Muurolene	1500	0.2	KI, MS, ST
Germacrene A	1509	0.1	KI, MS
γ-Cadinene	1514	0.2	KI, MS
7-*epi*-α-Selinene	1522	t	KI, MS
δ-Cadinene	1523	1.1	KI, MS
trans-Cadina-1(2),4-diene	1535	0.1	KI, MS
α-Cadinene	1539	0.1	KI, MS
α-Calacorene	1546	0.1	KI, MS
α-Agarofuran	1550	0.1	KI, MS
ledol	1569	0.2	KI, MS, ST
Caryophyllene alcohol	1572	0.2	KI, MS, ST
Spathulenol	1578	t	KI, MS, ST
Globulol	1585	0.7	KI, MS, ST
Viridiflorol	1593	0.2	KI, MS, ST
5-*epi*-7-α-Eudesmol	1608	0.2	KI, MS
10-*epi*-γ-Eudesmol	1624	0.1	KI, MS
1-*epi*-Cubenol	1629	0.2	KI, MS
γ-Eudesmol	1632	0.2	KI, MS
τ-Cadinol	1640	0.6	KI, MS, ST
δ-Cadinol	1646	0.1	KI, MS
α-Cadinol	1654	0.7	KI, MS
Bulnesol	1676	t	KI, MS
epi-α-Bisabolol	1685	t	KI, MS
(*2Z*, *6E*)-Farnesol	1701	t	KI, MS
(*2E*, *6E*)-Farnesol	1725	0.1	KI, MS
Compound classes			
Monoterpene hydrocarbons		50.5	
Oxygenated monoterpenes		31.7	
Sesquiterpene hydrocarbons		13.0	
Oxygenated sesquiterpenes		3.6	
Others		1.3	
Yield (mL/100 g)		2.86 ± 0.05	

[a] Retention index on a DB-5 column with reference to *n*-alkanes [13].

[b] MS, NIST and Wiley library spectra and the literature; RI, Retention index; ST, authentic standard compounds. [c].trace < 0.1%.

suppression against all ten microbes. The most sensitive microorganisms were *Bacillus cereus*, *Staphylococcus aureus*, *S. epidermidis*, and *Candida albicans*, with inhibition zones of 48 to 54 mm and MIC values of 16.12~32.25 μg/mL, respectively. The essential oil showed superior suppressive activity against the Gram-positive bacteria than either the Gram-negative bacteria or 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 [23]. 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 [24,25]. Comparing the antimicrobial activities of the leaf essential oil of *M. japonica* with those of *M. kusanoi* [7], *M. pseudolongifolia* [8], and *Litsea linii* [26], and the twig oil extracted from *L. mushaensis* [26], the leaf essential oil of *M. japonica* was superior. The results verify that *M. japonica* leaf oil has excellent antimicrobial activity. However, to ascertain the source compounds of the antimicrobial activity from *M. japonica*, the main components were individually tested for antimicrobial activities. The results indicated that the active source compounds were thymol and carvacrol. Various studies support the argument that these compounds are highly active in suppressing microbial growth [27-29].

Experimental

Plant materials: Fresh leaves of *M. japonica* were collected in July 2010 from Tiaomikeng (Nantou County, central Taiwan, elevation 1580 m, N 23º 57′ 09″, E 120º 54′ 28″). The samples were compared with specimen no. TAIF 92852 from the Herbarium of the Taiwan Forestry Research Institute and were positively identified by Prof. Yen-Hsueh Tseng of National Chung Hsing University (NCHU). The voucher specimen (CLH-015) was deposited in the NCHU herbarium. Before extraction, the leaves were air dried at room temperature and protected from the light for one week.

Isolation of leaf essential oil: The essential oil of the dry leaves (200 g) was extracted using a Clevenger-type apparatus using a hydrodistillation technique. After extraction, the volume of essential oil obtained was measured, and the essential oil was stored in glass containers hermetically sealed with rubber lids, covered with aluminum foil to protect the contents from light, and kept refrigerated at 8ºC until used. 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. 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) [13], retention times (RT), and mass spectra with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [13,30].

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capability test: The method of Ho *et al*. [31] was used for DPPH assay in this study. Fifty μL of various dilutions of the oils were mixed with 5 mL of a 0.004% methanol solution of DPPH. After an incubation period of 30 min, the absorbance of the samples was determined at 517 nm using a Jasco 7800 spectrophotometer. Tests were carried out in triplicate, and ascorbic acid was used as a positive control.

Antimicrobial activity [32]***:*** Discs containing 15 μL and 30 μL of the oil dissolved in dimethylsulfoxide (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. 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: *Aspergillus 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 [33] and as reported earlier [34].

Table 2: Antimicrobial activities of the leaf oil of *M. japonica*.

Microbial species	*Machilus japonica* Leaf		Compounds								Antibiotics		
			1	2	3	4	5	6	7	8	Tetracycline	Gentamicin	Nystatin
	IZ	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC	IZ	IZ	IZ
Bacillus cereus	54 ± 0.5	16.12	>1000	>1000	>1000	>1000	>1000	2.50	5.00	>1000	22 ± 0.8	-	nt
Staphylococcus aureus	48 ± 0.3	31.25	>1000	>1000	>1000	>1000	>1000	2.50	5.00	>1000	21 ± 0.4	-	nt
Staphylococcus epidermidis	52 ± 0.4	31.25	>1000	>1000	>1000	>1000	>1000	2.50	5.00	>1000	34 ± 0.4	-	nt
Escherichia coli	38 ± 0.4	125	>1000	>1000	>1000	>1000	>1000	31.25	62.5	>1000	-	22 ± 0.8	nt
Enterobacter aerogenes	38 ± 0.8	125	>1000	>1000	>1000	>1000	>1000	31.25	62.5	>1000	10 ± 0.4	-	nt
Klebsiella pneumoniae	36 ± 0.8	125	>1000	>1000	>1000	>1000	>1000	62.5	62.5	>1000	-	21 ± 0.8	nt
Pseudomonas aeruginosa	36 ± 0.8	125	>1000	>1000	>1000	>1000	>1000	62.5	62.5	>1000	-	12 ± 0.8	nt
Vibrio parahaemolyticus	28 ± 0.8	500	>1000	>1000	>1000	>1000	>1000	125	62.5	>1000	-	13 ± 0.8	nt
Aspergillus niger	33 ± 0.4	250	>1000	>1000	>1000	>1000	>1000	125	250	>1000	nt	nt	17 ± 0.8
Candida albicans	50 ± 0.8	31.25	>1000	>1000	>1000	>1000	>1000	0.62	1.25	>1000	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-pinene(≥ 99.5%), 2. β-pinene(≥ 99.5%), 3. α-phellandrene(≥ 99.0%), 4. *p*-cymene (≥99.0%), 5. α-terpineol (≥99.0%), 6. thymol (≥ 99.5%), 7. carvacrol (98.0%), 8. β-caryophyllene (≥ 98.5%), Compounds 1 to 8 were purchased from Fluka Co. (Milwaukee, USA). (-), Inactive.

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