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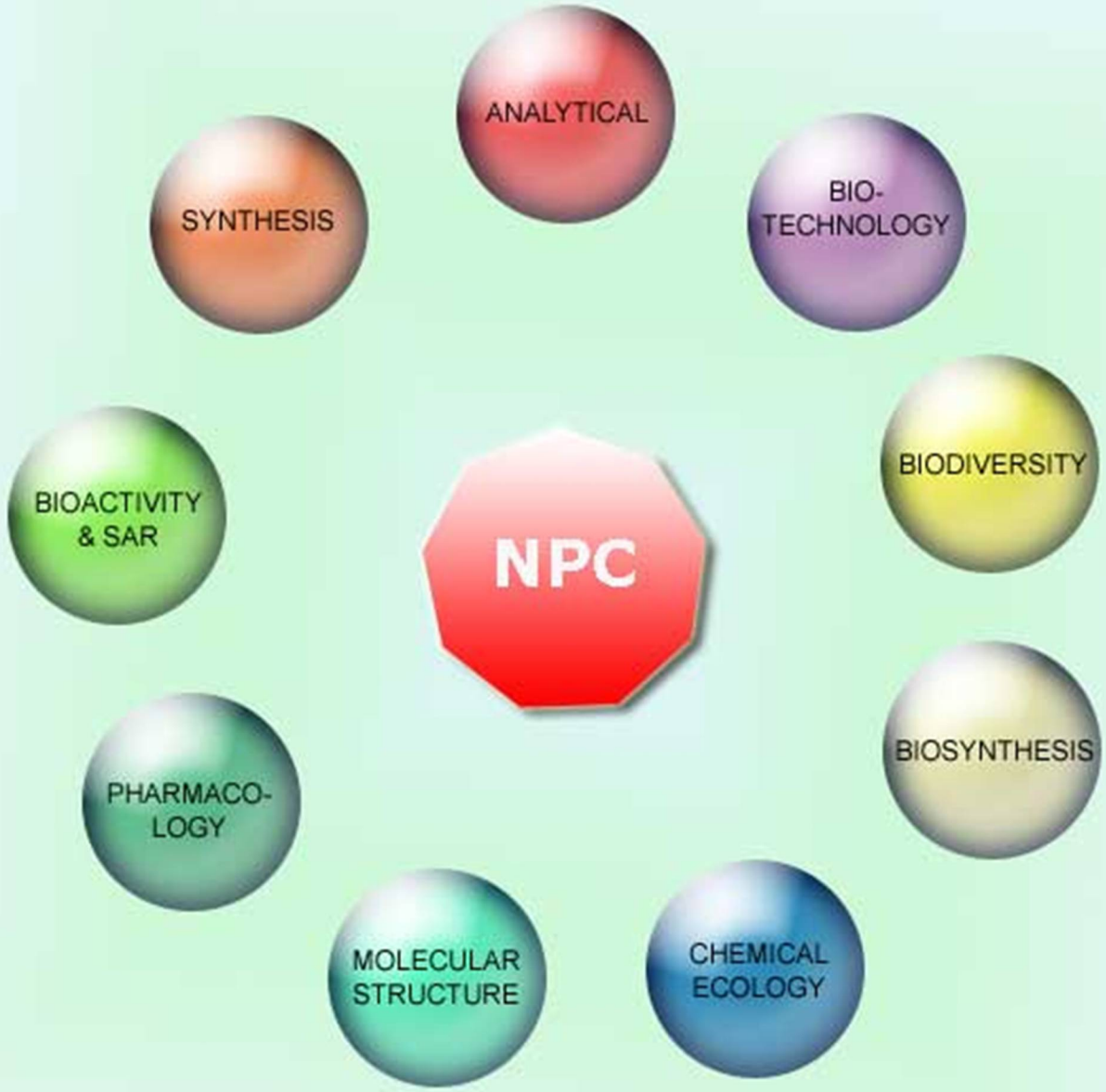

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Composition, Antioxidant and Antimicrobial Activities of Leaf and Twig Essential Oils of *Litsea akoensis* from Taiwan

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This study analyzed the hydrodistilled essential oils in the leaves and twigs of *Litsea akoensis* to determine composition and yield. Seventy-one and 40 compounds were identified in the leaf and twig oils, respectively. The main components of leaf oil were limonene (18.5%), thymol (10.1%), *p*-cymene (9.6%), β-caryophyllene (8.9%), and carvacrol (8.2%). The main components of twig oil were β-phellandrene (43.7%) and *trans*-β-ocimene (10.4%). The results demonstrated that leaf oil had excellent antioxidant and antimicrobial activities, superior to those of twig oil.

Keywords: *Litsea akoensis*, Lauraceae, essential oil, antioxidant activity, antimicrobial activity, limonene, thymol.

Litsea akoensis Hayata (Lauraceae) is a medium-sized evergreen tree, endemic to Taiwan, and distributed throughout broad leaved forests at low to medium altitudes [1]. There are reports suggesting that the root extractive has an antimycobacterial activity [2], and stem bark extractive has a cytotoxic activity against P-388, KB16, A 549, HT-29, MCF-7, NCI-H460, and SF-268 cancer cell lines [3-4]. However, there are no literature reports on the chemical composition and biological activities of the essential oil from this species. This study deals with the extraction and analysis of essential oils obtained followed by examination of the antioxidative and antimicrobial activities. The purpose of this study was to establish a chemical basis for the effective multipurpose utilization of the species.

Based on the dry weight of leaves and twigs, hydrodistillation of *L. akoensis* produced yellow-colored oils with yields of 2.36 ± 0.03 and 1.88 ± 0.04 mL/100 g, respectively. All compounds are listed in order of their elution from the DB-5 column (Table 1). A total of 71 compounds were identified from the hydrodistilled leaf oil of *L. akoensis*. Among the leaf oil compounds, monoterpene hydrocarbons were predominant (33.6%), followed by oxygenated monoterpenes (29.8%), oxygenated sesquiterpenes (20.2%), sesquiterpene hydrocarbons (15.9%), and non-terpenoids (0.5%), Among the monoterpene hydrocarbons, limonene (18.5%) and *p*-cymene (9.6%) were the major compounds. Of the oxygenated monoterpenes, thymol (10.1%) and carvacrol (8.2%) were the main components. Caryophyllene oxide (3.3%) was the chief component among the oxygenated sesquiterpenes.

Forty components were identified from the twig oil. Among the component groups, monoterpene hydrocarbons were the most dominant (68.9%), followed by sesquiterpene hydrocarbons (14.5%), oxygenated sesquiterpenes (13.9%), and oxygenated monoterpenes (2.6%). β-Phellandrene (43.7%) and *trans*-β-ocimene (10.4%) were the major compounds of the monoterpene hydrocarbons.

The compounds of *L. akoensis* leaf oil, like those of the leaf oil of *L. laevigata* [5] were primarily monoterpenoids, which differed from *L. mushaensis* [6], *L. linii* [6], *L. coreana* [7], *L. kostermansii* [8], *L nakaii* [9], *L. resinosa*, *L. rasilipes*, and *L. paludosa* of the same genus [10] containing mostly sesquiterpenoids.

The leaf and twig oils of *L. akoensis* were tested for their DPPH free radical scavenging capability. Ascorbic acid was used as a positive control. The IC_{50} of the DPPH free radical scavenging capability of the leaf and twig essential oils were 68.5, > 2000 μg/mL, respectively. The results demonstrated clearly that the leaf oil had antioxidant activities superior to those of twig oil. The individual main components of the leaf essential oil, carvacrol, thymol, limonene, *p*-cymene 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), limonene, *p*-cymene and β-caryophyllene (IC_{50}> 2000 μg/mL). Hence, we deduced that the phenolic compounds were mainly responsible for the radical scavenging. The

Table 1: Chemical composition of the leaf and twig oils of *L. akoensis*.

Compound ID	RI[a]	Concentration(%)		Identification[b]
		Leaf	Twig	
α-Thujene	930	0.4	0.3	KI, MS, ST
α-Pinene	939	1.2	2.5	KI, MS, ST
4,4-Dimethyl-2-butenolide	952	0.1	-[d]	KI, MS
Camphene	954	0.1	0.3	KI, MS, ST
β-Pinene	979	0.4	1.1	KI, MS, ST
6-Methyl-5-heptene-2-one	986	t[c]	-	KI, MS, ST
Myrcene	991	2.1	4.9	KI, MS, ST
δ-2-Carene	1002	0.1	-	KI, MS
α-Phellandrene	1003	0.2	1.6	KI, MS, ST
α-Terpinene	1017	0.1	-	KI, MS, ST
p-Cymene	1025	9.6	2.7	KI, MS, ST
Limonene	1029	18.5	-	KI, MS, ST
β-Phellandrene	1030	0.4	43.7	KI, MS, ST
cis-β-Ocimene	1037	0.5	1.2	KI, MS
trans-β-Ocimene	1050	t	10.4	KI, MS
γ-Terpinene	1060	0.1	0.2	KI, MS, ST
cis-Linalool oxide (furanoid)	1072	0.1	-	KI, MS
Linalool	1097	0.5	-	KI, MS, ST
allo-Ocimene	1132	0.1	-	KI, MS
cis-p-Mentha-2,8-dien-1-ol	1137	0.1	0.3	KI, MS
trans-p-Menth-2-en-1-ol	1140	0.2	-	KI, MS
p-Menth-3-en-8-ol	1150	0.7	-	KI, MS
Terpinen-4-ol	1177	1.1	0.5	KI, MS, ST
Cryptone	1185	2.8	0.8	KI, MS, ST
α-Terpieol	1188	0.5	0.5	KI, MS, ST
cis-Piperitol	1196	0.1	-	KI, MS
trans-Piperitol	1208	0.2	-	KI, MS
trans-Carveol	1216	0.3	-	KI, MS
m-Cumenol	1227	0.4	-	KI, MS
cis-Carveol	1229	0.1	-	KI, MS
Cumic aldehyde	1241	1.0	-	KI, MS
Piperitone	1252	0.8	-	KI, MS, ST
Carvenone	1258	0.1	-	KI, MS
Perilla aldehyde	1271	0.2	0.5	KI, MS
p-Menth-1-en-7-al	1275	0.3	-	KI, MS
Bornyl acetate	1288	0.1	-	KI, MS, ST
Thymol	1290	10.1	-	KI, MS, ST
Carvacrol	1298	8.2	-	KI, MS, ST
4-Hydroxy-cryptone	1315	1.6	-	KI, MS
3-Oxo-*p*-menth-1-en-7-al	1333	0.9	-	KI, MS
α-Copaene	1376	0.4	-	KI, MS, ST
β-Elemene	1390	0.6	0.5	KI, MS, ST
β-Caryophyllene	1419	8.9	6.7	KI, MS, ST
Aromadendrene	1441	0.8	-	KI, MS, ST
α-Humulene	1454	0.6	4.1	KI, MS, ST
γ-Muurolene	1478	0.6	-	KI, MS
β-Eudesmene	1490	2.0	0.8	KI, MS
δ-Selinene	1493	t	0.3	KI, MS
α-Selinene	1498	0.5	-	KI, MS
Bicyclogermacrene	1500	t	0.5	KI, MS
α-Muurolene	1500	t	0.3	KI, MS
(*e, e*)-α-Farnesene	1506	t	0.8	KI, MS
γ-Cadinene	1513	0.2	-	KI, MS
δ-Cadinene	1523	0.5	0.2	KI, MS
cis-Calamenene	1529	0.8	0.3	KI, MS
trans-Nerolidol	1563	1.0	0.5	KI, MS, ST
Maaliol	1567	0.7	0.7	KI, MS
Ledol	1569	t	0.1	KI, MS, ST
Spathulenol	1578	3.2	0.8	KI, MS, ST
Caryophyllene oxide	1583	3.3	3.3	KI, MS, ST
Globulol	1590	1.4	2.2	KI, MS, ST
Viridiflorol	1592	3.1	0.9	KI, MS, ST
α-Corocalene	1623	1.7	-	KI, MS
10-*epi*-γ-Eudesmol	1623	0.7	-	KI, MS
1-*epi*-Cubenol	1629	t	1.0	KI, MS
γ-Eudesmol	1632	0.9	0.2	KI, MS
τ-Cadinol	1640	0.8	0.2	KI, MS
τ-Muurolol	1642	0.9	0.3	KI, MS
α-Cadinol	1654	t	0.7	KI, MS
(2*E*,6*E*)-Farnesol	1742	1.3	1.8	KI, MS
all-trans-Farnesyl acetate	1845	1.4	1.3	KI, MS
Compound identified				
Monoterpene hydrocarbons (%)		33.6	68.9	
Oxygenated monoterpenes (%)		29.8	2.6	
Sesquiterpene hydrocarbons (%)		15.9	14.5	
Oxygenated sesquiterpenes (%)		20.2	13.9	
Others		0.5	0.0	
Total identified		100.0	99.9	
Oil Yield (mL/100 g)		2.36 ± 0.03	1.88 ± 0.04	

[a] Retention index on a DB-5 column with reference to n-alkanes [11].

[b] MS, NIST and Wiley library spectra and the literature; RI, Retention index; ST, authentic standard compounds. [c].trace < 0.1%. [d]. not detected

results are also in congruency with the conclusions of several other reports [12-17]. 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 [18], 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* [19], 460 μg/mL for the flower oil of *Origanum vulgare* [20], and 500 μg/mL for the leaf oil of *Curcuma zedoaria* [21].

Leaf and twig oils of *L. akoensis* were tested against three Gram-positive and five Gram-negative bacteria, as well as two fungi. The results demonstrated clearly that the leaf oil had antibacterial activities superior to those of twig oil (Table 2). Leaf oil showed strong growth suppression activities against all ten microbes studied. The most sensitive microorganisms were *Bacillus cereus*, *Staphylococcus aureus*, *S. epidermidis* and *Candida albicans* with inhibition zones of 46 ~ 52 mm and minimum inhibitory concentration (MIC) values of 31.25 μg/mL. The leaf oil demonstrated stronger growth suppression activities in regards to Gram-positive bacteria as compared to Gram-negative bacteria. These observations are similar to results reported in previous literature [5,6,8,9,22,23]. The antibacterial activities of leaf oil from *L. akoensis* were superior when compared to the antibacterial activities of essential oils from *L. laevigata* [5], *L. mushaensis* [6], *L. linii* [6], *L. kostermansii* [8], *L. nakaii* [9], *Cinnamomum subavenium* [22] and *Machilus pseudolongifolia* [23].

The study results validated the antibacterial activities of *L. akoensis* leaf oil. However, to ascertain the source compounds of the antibacterial activities of *L. akoensis* leaf oil, its main components were individually tested for antibacterial 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 [24-26].

Experimental

Plant materials: Fresh leaves and twigs of *L. akoensis* were collected in July 2009 from Chihshuiying (Pingtung County, southern Taiwan, elevation 1500 m, N 22º 24′ 28″, E 120º 46′ 38″). The samples were compared with specimen no. ou6858 from the Herbarium of National Chung-Hsing University and positively identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH-012) was deposited in the NCHU herbarium. Leaves and twigs of the species were collected for subsequent extraction and analysis.

Isolation of leaf and twig essential oils: Leaves and twigs of *L. akoensis* (1 kg) were placed in a round-bottom flask and hydrodistilled for 8 h with 3L of distilled water. Removed essential oil 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. 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) [11], retention times (RT), and mass spectra with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [11,27].

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capability test: The method of Ho *et al*. [28] 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 [29]***:*** 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: *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 [30] and as reported earlier [31].

Table 2: Antimicrobial activities of the leaf and twig oils of *L. akoensis*.

Microbial species	*Litsea akoensis*				Compounds [c]					Antibiotics		
	Leaf		Twig		1	2	3	4	5	Tetracycline (30 μg/disk)	Gentamicine (10 μg/disk)	Nystatine (30 μg/disk)
	IZ [a]	MIC [b]	IZ	MIC	MIC	MIC	MIC	MIC	MIC	IZ	IZ	IZ
Bacillus cereus	52 ± 0.4	31.25	14 ± 0.8	>1000	>1000	>1000	2.50	5.00	>1000	22 ± 0.8	-	nt
Staphylococcus aureus	46 ± 0.8	31.25	14 ± 0.4	>1000	>1000	>1000	2.50	5.00	>1000	21 ± 0.4	-	nt
Staphylococcus epidermidis	50 ± 0.4	31.25	12 ± 0.4	>1000	>1000	>1000	2.50	5.00	>1000	34 ± 0.4	-	nt
Escherichia coli	36 ± 0.4	125	9 ± 0.8	>1000	>1000	>1000	31.25	62.5	>1000	-	22 ± 0.8	nt
Enterobacter aerogenes	36 ± 0.8	125	9 ± 0.4	>1000	>1000	>1000	31.25	62.5	>1000	10 ± 0.4	-	nt
Klebsiella pneumoniae	32 ± 0.8	250	10 ± 0.8	>1000	>1000	>1000	62.5	62.5	>1000	-	21 ± 0.8	nt
Pseudomonas aeruginosa	32 ± 0.4	250	10 ± 0.4	>1000	>1000	>1000	62.5	62.5	>1000	-	12 ± 0.8	nt
Vibrio parahaemolyticus	26 ± 0.8	500	10 ± 0.8	>1000	>1000	>1000	125	62.5	>1000	-	13 ± 0.8	nt
Aspergillus niger	32 ± 0.4	250	10 ± 0.4	>1000	>1000	>1000	125	250	>1000	nt	nt	17 ± 0.8
Candida albicans	48 ± 0.8	31.25	12 ± 0.8	>1000	>1000	>1000	0.625	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. *p*-cymene (99.0%), 2. limonene (≥ 98.0 %), 3. thymol (≥ 99.5%), 4. carvacrol (98.0%), 5. β-caryophyllene (≥ 98.5%) Compound 1 to 5 were purchased from the Fluka Co. (Milwaukee, USA). (-), Inactive; (7-14), moderately active; (>14), highly active.

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