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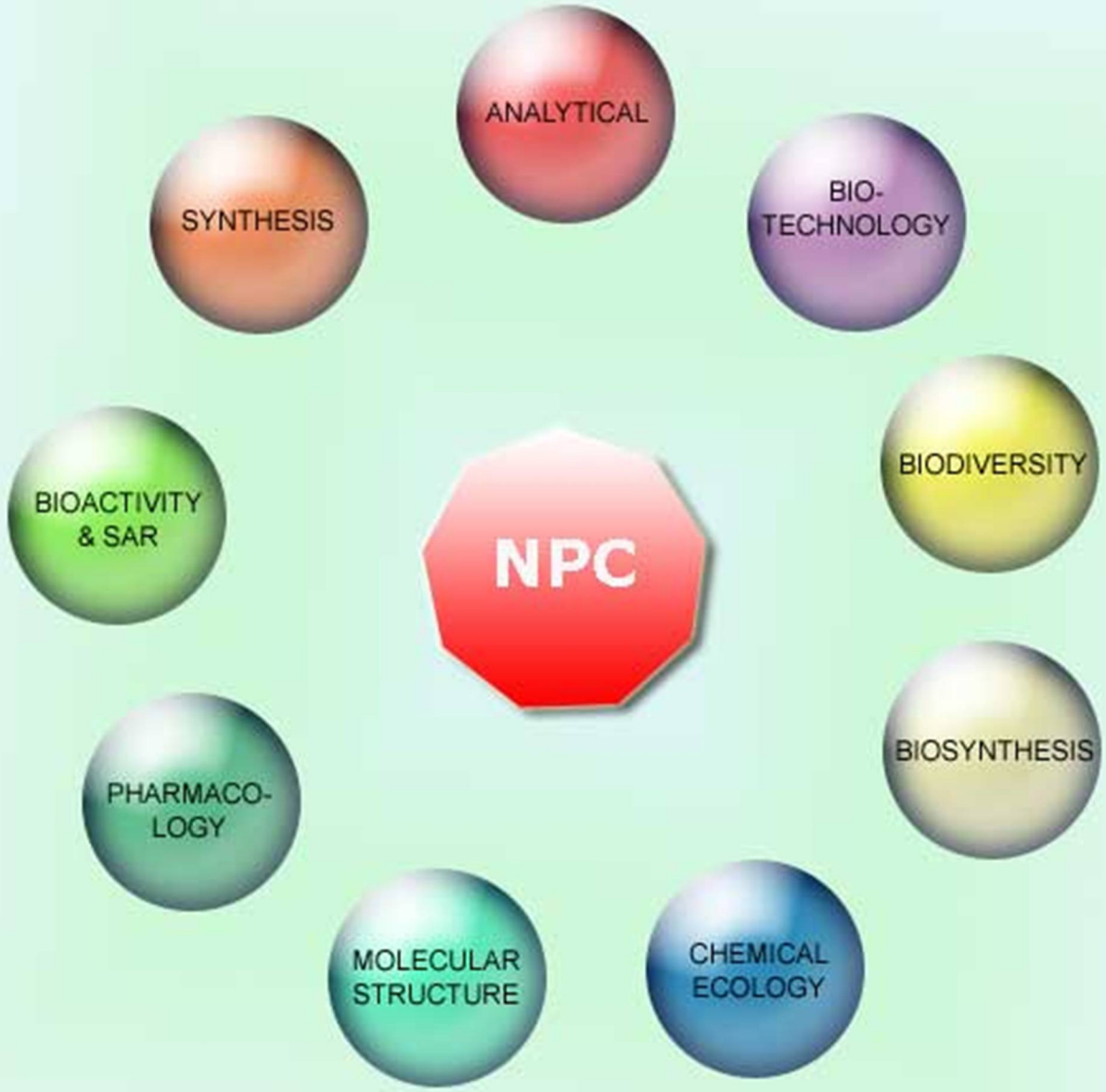

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Composition, Anticancer, and Antimicrobial Activities *in vitro* of the Heartwood Essential Oil of *Cunninghamia lanceolata* var. *konishii* from Taiwan

Yu-Chang Su[a], Kuan-Ping Hsu[b], Eugene I-Chen Wang[b] and Chen-Lung Ho[b*]

[a]*Department of Forestry*, *National Chung Hsing University*, *250 Kuo Kuang Rd.*, *Taichung*, *Taiwan 402*
[b]*Division of Wood Cellulose*, *Taiwan Forestry Research Institute. 53*, *Nanhai Rd., Taipei*, *Taiwan 100*

chenlung@tfri.gov.tw

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This study investigated the chemical composition, anticancer, and antimicrobial activities *in vitro* of the essential oil isolated from the heartwood of *Cunninghamia lanceolata* var. *konishii* from Taiwan. The essential oil was isolated using hydrodistillation in a Clevenger-type apparatus, and characterized by GC–FID and GC–MS. Thirty-seven compounds were identified, representing 100% of the oil. The main components identified were cedrol (58.3%), α-cedrene (11.8%), α-terpineol (4.2%) and β-cedrene (3.5%). The oil exhibited cytotoxic activity against human lung, liver and oral cancer cells. The active source compound was cedrol. 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 42~50 mm to MIC values of 31.25~62.5 μg/mL, respectively. For the antimicrobial activities of the oil, the active compound was determined to be cedrol.

Keywords: *Cunninghamia lanceolata* var. *konishii*, Essential oil, Cedrol, Anticancer activity, Antimicrobial activity.

Cunninghamia lanceolata var. *konishii* (Hay.) Fujita (Taxodiaceae) is an indigenous species of Taiwan. It is one of the five most valuable conifers in Taiwan [1]. The species distributes in northern and central Taiwan at 1300~2000 m. Previous studies have reported the composition of wood, bark, and leaves of *C. lanceolata* var. *konishii* [2-11]. However, only one report noted that the wood essential oil had anti-wood-decay-fungal and antipathogenic activities [11]. No prior study has investigated the chemical composition and biological activity of the heartwood essential oil. Thus, we first obtained this by hydrodistillation and analyzed its chemical composition. In the second part of the study, we examined the *in vitro* anticancer and antimicrobial activities of the essential oil. The purpose of this study was to establish a chemical basis for effective multipurpose utilization of the tree species.

Hydrodistillation of *C. lanceolata* var. *konishii* heartwood gave a dark-yellow oil with a yield of 1.08 ± 0.02 mL/100 g, based on the dry weight of heartwood. The identified constituents are presented in Table 1, where all compounds are listed in order of their elution from the DB-5 column. Thirty-seven compounds were identified (Table 1), representing 100.0% of the oil. Among the groups, oxygenated sesquiterpenes were predominant (67.6%), followed by sesquiterpene hydrocarbons (19.5%), oxygenated monoterpenes (10.5%), diterpenes (1.6%), and monoterpene hydrocarbons (0.9%). Among the oxygenated sesquiterpenes, cedrol (58.3%) was the major compound, and of the sesquiterpene hydrocarbons, α-cedrene (11.8%) was the chief component.

To evaluate the anticancer activities of the essential oil, we tested it on the viability of three human cancer cell lines: human oral squamous cancer OEC-M1, human hepatocellular carcinoma J5, and human lung adenocarcinoma A549 cells. Cells were incubated with various concentrations of essential oils for 48 h, and then the cell viabilities were measured by the alamarBlue® proliferation assay. The results showed that treatment for 48 h with the oil reduced the viability of OEC-M1, J5, and A549 cells with IC_{50} values of around 37.5, 52, and 80 ppm, respectively. This is the first report on the anticancer activities of *C. lanceolata* var. *konishii* heartwood essential oil against human lung, liver and oral cancer cells.

However, in order to ascertain the source compounds of the anticancer activities from *C. lanceolata* var. *konishii*, the main components, α-terpineol, α-cedrene, β-cedrene, and cedrol were individually tested for their cytotoxic activities. The results showed that cedrol treatment for 48 h reduced the viability of OEC-M1, J5, and A549 cells with IC_{50} values of around <25 ppm. The results were in agreements with Jiang *et al.* [12], who found that cedrol had the best efficacies against A549 cells.

The heartwood oil of *C. lanceolata* var. *konishii* was also 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 suppression against all ten microbes. The most sensitive microorganisms were *Bacillus cereus*, *Staphylococcus aureus*, *S. epidermidis*, and *Candida albicans*, with inhibition zones of 42 to 50 mm to MIC values of 31.25~62.5 μg/mL, respectively. The essential oil showed superior suppressive activity toward the Gram-positive bacteria than that of 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 [14]. 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 [15]. In comparison with the antimicrobial activity of the essential oils from *Metasequioa glyptostroboides* [16], *Litsea kostermansii* [17], *L. akoensis* [18], *Machilus pseudolongifolia* [19], and *M. kusanoi* [20] the antimicrobial activity of the heartwood essential oil of

Table 1: Chemical composition of the heartwood essential oil of *Cunninghamia lanceolata* var. *konishii*.

Compound	RI[a)]	Concentration(%)	Identification[b)]
α-Pinene	939	0.7	MS,KI,ST
Limonene	1029	0.1	MS,KI,ST
Terpinolene	1086	0.1	MS,KI,ST
Fenchone	1087	0.1	MS,KI,ST
exo-Fenchol	1122	0.9	MS,KI,ST
Camphor	1146	1.4	MS,KI,ST
Isoborneol	1162	0.1	MS,KI
Borneol	1169	2.2	MS,KI,ST
4-Terpineol	1177	0.1	MS,KI,ST
p-Cymen-8-ol	1183	0.1	MS,KI,ST
α-Terpineol	1189	4.2	MS,KI,ST
endo-Fenchyl acetate	1220	0.1	MS,KI
exo-Fenchyl acetate	1233	0.1	MS,KI
Bornyl acetate	1289	0.2	MS,KI,ST
α-Terpinyl acetate	1349	1.0	MS,KI,ST
α-Duprezianene	1389	0.4	MS,KI
β-Elemene	1391	1.1	MS,KI,ST
α-Cedrene	1412	11.8	MS,KI,ST
α-*cis*-Bergamotene	1413	0.1	MS,KI
β-Cedrene	1421	3.5	MS,KI,ST
β-Duprezianene	1423	0.1	MS,KI
dehydro-Sesquicineole	1471	0.5	MS,KI
β-Chamigrene	1478	0.2	MS,KI
ar-Curcumene	1481	0.4	MS,KI
β-Selinene	1490	0.8	MS,KI,ST
Cuparene	1503	1.1	MS,KI,ST
Caryophyllene oxide	1583	0.2	MS,KI,ST
Cedrol	1601	58.3	MS,KI,ST
epi-Cedrol	1619	1.7	MS,KI
γ-Eudesmol	1632	0.9	MS,KI,ST
α-Acorenol	1633	0.4	MS,KI,ST
τ-Cadinol	1640	0.7	MS,KI,ST
β-Eudesmol	1651	0.8	MS,KI,ST
α-Eudesmol	1654	1.9	MS,KI,ST
α-Bisabolol	1686	0.6	MS,KI,ST
Cedryl acetate	1769	1.7	MS,KI
Totarol	2314	1.6	MS,KI,ST
Monoterpene hydrocarbons (%)		0.9	
Oxygenated monoterpenes (%)		10.5	
Sesquiterpene hydrocarbons (%)		19.5	
Oxygenated sesquiterpenes (%)		67.6	
Diterpenes (%)		1.6	
Oil yield (mL/100 g)		1.08 ± 0.02	

[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. lanceolata var. *konishii* was superior. The results validated the excellent antimicrobial activity of *C. lanceolata* var. *konishii* heartwood essential oil. However, to ascertain the source compounds of antimicrobial activity from *C. lanceolata* var. *konishii* heartwood oil, the main components were individually tested for antimicrobial activities. The results indicated that the active source compound was again cedrol. Various studies support the argument that these compounds are highly active in suppressing microbial growth [21-23].

Experimental

Plant materials: Fresh heartwood of *C. lanceolata* var. *konishii* was collected in June 2011 from Chilan Mt in northeast Taiwan (Yilan County, elevation 1500 m, N 24º 40´ 89˝, 121º 40´ 85˝). The samples were compared with specimen no. ou 8588 from the Herbarium of National Chung-Hsing University and positively identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH-021) was deposited in the NCHU herbarium. Leaves of the species were collected for subsequent extraction and analysis.

Isolation of the heartwood essential oil: Heartwood of *C. lanceolata* var. *konishii* (1 Kg) was hydrodistilled for 8 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 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 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. Data are expressed as the means ± SD of 3 independent experiments.

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 [13,24].

Cell culture: Human lung adenocarcinoma A549, human hepatocellular carcinoma J5, and human oral squamous cancer OEC-M1 cells were obtained from ATCC (Rockville, MD, USA) and propagated in RPMI-1640 medium supplemented with 10% heated-inactivated FCS and 2 mM L-glutamine (Life Technologies, Inc., MD), and cultured in a 37°C, 5% CO_2 incubator.

Table 2: Antimicrobial activity of the heartwood essential oil of *C. lanceolata* var. *konishii*.

Microbial species	*Cunninghamia lanceolata* var. *konishii* Heartwood		Compounds[c]				Antibiotics		
			1	2	3	4	Tetracycline	Gentamicin	Nystatin
	IZ[a]	MIC[b]	MIC	MIC	MIC	MIC	IZ	IZ	IZ
Bacillus cereus	50 ± 0.8	31.25	>1000	>1000	>1000	15.625	22 ± 0.8	-	nt
Staphylococcus aureus	42 ± 0.4	62.5	>1000	>1000	>1000	62.5	21 ± 0.4	-	nt
Staphylococcus epidermidis	48 ± 0.8	31.25	>1000	>1000	>1000	15.625	34 ± 0.4	-	nt
Escherichia coli	36 ± 0.8	250	>1000	>1000	>1000	250	-	22 ± 0.8	nt
Enterobacter aerogenes	32 ± 0.8	250	>1000	>1000	>1000	250	10 ± 0.4	-	nt
Klebsiella pneumoniae	29 ± 0.4	375	>1000	>1000	>1000	250	-	21 ± 0.8	nt
Pseudomonas aeruginosa	26 ± 0.8	375	>1000	>1000	>1000	375	-	12 ± 0.8	nt
Vibrio parahaemolyticus	26 ± 0.4	375	>1000	>1000	>1000	375	-	13 ± 0.8	nt
Aspergillus niger	28 ± 0.4	375	>1000	>1000	>1000	250	nt	nt	17 ± 0.8
Candida albicans	42± 0.4	62.5	>1000	>1000	>1000	62.5	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 (≥ 99.5%), 2. α-cedrene (≥ 99.0%), 3. β-cedrene (≥ 99.0%), 4. cedrol (≥ 99.0%). Compounds 1 to 4 were purchased from the Sigma Co. (USA). Essential oil tested at 15 μL/disc for bacteria and 30 μL/disc for fungi.(-), Inactive; nt, not tested.

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 replacing 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 values are given as means ± SD. Data analysis involved one-way ANOVA with subsequent Scheffe´s test.

Antimicrobial activity [25]: 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. 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 [26] and as reported earlier [17]. Data are expressed as the means ± SD of 3 independent experiments.

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