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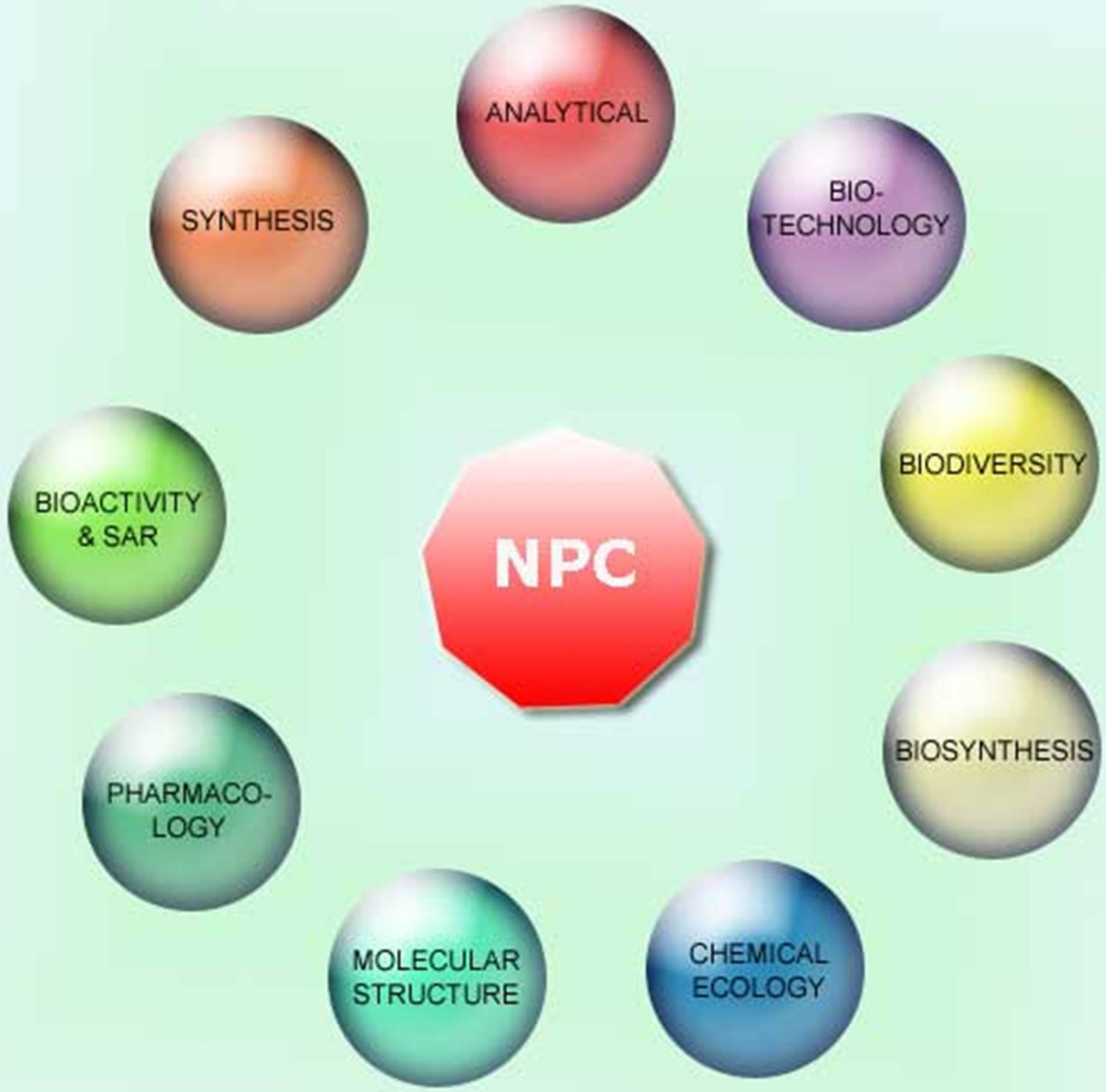

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Composition and *in vitro* Anticancer Activities of the Leaf Essential Oil of *Neolitsea variabillima* from Taiwan

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This study investigated the chemical composition and *in vitro* anticancer activities of the essential oil isolated from the leaf of *Neolitsea variabillima*. The essential oil was isolated using hydrodistillation in a Clevenger-type apparatus, and characterized by GC–FID and GC–MS. Sixty-seven compounds were identified, representing 100% of the oil. The main components identified were *trans*-β-ocimene (13.4%), α-cadinol (10.5%), terpinen-4-ol (9.3%), τ-cadinol (9.2%), β-caryophyllene (8.8%), and sabinene (6.7%). The anticancer activities of oil were evaluated. The results showed that the oil exhibited cytotoxic activity against human oral, liver, lung, colon, melanoma, and leukemic cancer cells. The presence of β-caryophyllene, τ-cadinol, and α-cadinol significantly contributed to the anticancer activities of *N. variabillima* leaf oil.

Keywords: *Neolitsea variabillima*, Essential oil, Anticancer activity, β-Caryophyllene, τ-Cadinol, α-Cadinol.

Neolitsea variabillima (Hayata) Kaneh. & Sasaki (Lauraceae) (=*N. aciculata* var. *variabillima* J.C. Liao) is an endemic species of Taiwan and is distributed from the lowlands to 1500 m [1]. Few studies have investigated the chemical composition and biological activities of the essential oils or other extracts from this species. Thus, we used hydrodistillation to collect the leaf oil, and it was analyzed by GC–FID and GC–MS. In the second part of the study, we examined the *in vitro* anticancer activities of the leaf oil. The purpose of this study was to establish a chemical basis for effective multipurpose utilization of the tree species.

Hydrodistillation of *N. variabillima* leaves gave a yellow oil with a yield of 1.08 ± 0.02 mL/100 g, based on the dry weight of leaves. The identified constituents are presented in Table 1, where all compounds are listed in order of their elution from the DB-5 column. Sixty-seven compounds were identified (Table 1), representing 100.0% of the oil. Among the groups, monoterpene hydrocarbons were predominant (36.1%), followed by oxygenated sesquiterpenes (25.9%), sesquiterpene hydrocarbons (23.2%), and oxygenated monoterpenes (14.9%). Among the monoterpene hydrocarbons, *trans*-β-ocimene (13.4%) and sabinene (6.7%) were the major compounds. Of the oxygenated sesquiterpenes, α-cadinol (10.5%) and τ-cadinol (9.2%) were the chief compounds, whereas of the sesquiterpene hydrocarbons, β-caryophyllene (8.8%) was the major component. Terpinen-4-ol (9.3%) was the chief components among the oxygenated monoterpenes. Other representative compounds were α-pinene (3.1%), β-pinene (2.6%), 1,8-cineole (2.5%), γ-terpinene (3.7%), and bicyclogermacrene (2.0%).

Although the leaf oil constituents of *N. variabillima* was primarily monoterpenoids, like those of *N. oblongifolia* and *N. umbrosa* [2], their main components differed. Further comparison with the leaf oil of *N. aciculate* [3a], *N. parvigemma* [3b], *N. pallens* [3c], *N. australiensis*, *N. brassii*, *N. dealbata* [3d], *N. sericea* [3e], *N. foliosa* var. *caesia* [3f] and *N. fischeri* [3g], were predominantly sesquiterpenoids and differed from the leaf oil of *N. variabillima*.

To evaluate the anticancer activities of leaf essential oil of *N. variabillima* from Taiwan, we tested the effect of essential oil 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 38.9, 42.6, 36.9, 16.8, 8.8, and 8.6 μg/mL, respectively (Table 2). This is the first report on the anticancer activities of *N. variabillima* leaf essential oil.

However, to ascertain the compounds responsible for the anticancer activities of *N. variabillima* leaf oil, the main components were individually tested for their anticancer activities. Sabinene, *trans*-β-ocimene, terpinen-4-ol, β-caryophyllene were purchased from the Fluka Co. (Milwaukee, USA), τ-cadinol, and α-cadinol were isolated from the essential oil of *M. philippinenensis* according to the method proposed by Ho *et al*. [4a]. The results showed that the active compounds were β-caryophyllene, τ-cadinol, and α-cadinol. The IC_{50} values of the three compounds against the six cancer cells were 24.0, 18.9, and 9.9 μg/mL against OEC-M1 cells; 111.2, 38.6, and 12.1 μg/mL against J5 cells; 31.3, 18.6, and 10.8 μg/mL against A549 cells; 9.8, 30.6, and 0.8 μg/mL against HT-29 cells; 3.2, 2.5, and 1.3 μg/mL against UACC-62 cells; and 4.6, 3.6, and 2.8 μg/mL against K562 cells, respectively (Table 2). β-Caryophyllene is reported to be cytotoxic against a number of human cancer cell lines including MCF-7, MDA-MB-468, UACC-257, A549, Hela, and HT-29 [4b, 4c]. τ-Cadinol has been reported to be cytotoxic to A549, MCF-7, and HT-29 [4d]. α-Cadinol is also reported to be cytotoxic against three human cancer cell lines, including A-549, MCF-7, and HT-29 [4e]. The presence of β-caryophyllene, τ-cadinol, and α- cadinol significantly contributed to the anticancer activities of *N. variabillima* leaf oil.

Experimental

Plant materials: Fresh leaves of *N. variabillima* were collected in June 2012 from Lienhuachih Research Center of the Taiwan Forestry Research Institute in central Taiwan (Nantou County, elevation 600 m, N 23º 55′ 08″, 120º 52′ 85″). The samples were compared with specimen no. ou 10896 from the Herbarium of National Chung-Hsing University and positively identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH-028) was deposited in the NCHU herbarium. Leaves of the species were collected for subsequent extraction and analysis.

Isolation and Analysis essential oil: Leaves of *N. variabillima* (1 Kg) was hydrodistilled for 8 h with 3 L of distilled water. The

Table 1: Chemical composition of the leaf essential oil of *N. variabillima*.

Consituents	KI[a]	Content(%)	Identification[b]
α-Thujene	930	0.9	MS, KI, ST
α-Pinene	939	3.1	MS, KI, ST
Camphene	954	0.1	MS, KI, ST
Sabinene	975	6.7	MS, KI, ST
β-Pinene	979	2.6	MS, KI, ST
β-Myrcene	990	0.9	MS, KI, ST
α-Phellandrene	1002	0.1	MS, KI, ST
α-Terpinene	1017	1.6	MS, KI, ST
p-Cymene	1024	0.2	MS, KI, ST
Limonene	1029	1.4	MS, KI, ST
1,8-Cineole	1031	2.5	MS, KI, ST
cis-β-Ocimene	1037	0.6	MS, KI, ST
2-Heptyl acetate	1043	0.1	MS, KI
trans-β-Ocimene	1044	13.4	MS, KI, ST
γ-Terpinene	1060	3.7	MS, KI, ST
Terpinolene	1088	0.8	MS, KI, ST
Linalool	1096	0.7	MS, KI, ST
cis-Thujone	1102	0.3	MS, KI
2-Isopropyl-5-methyl-(*2E*)-hexenal	1104	0.3	MS, KI
2-Isopropyl-5-methyl-(*2Z*)-hexenal	1114	0.2	MS, KI
Terpinen-4-ol	1177	9.3	MS, KI, ST
α-Terpineol	1189	0.8	MS, KI, ST
trans-Piperitol	1208	0.1	MS, KI, ST
Bornyl acetate	1288	0.3	MS, KI, ST
Linalool propanoate	1337	0.0	MS, KI
δ-Elemene	1338	0.3	MS, KI
α-Cubebene	1345	0.2	MS, KI, ST
Neryl acetate	1361	0.2	MS, KI, ST
β-Elemene	1390	1.3	MS, KI, ST
α-Gurjunene	1409	0.3	MS, KI, ST
α-Cedrene	1411	0.2	MS, KI
β-Caryophyllene	1419	8.8	MS, KI, ST
β-Cedrene	1420	0.1	MS, KI
p-Cymen-7-ol acetate	1422	0.2	MS, KI
β-Copaene	1432	0.1	MS, KI, ST
Aromadendrene	1441	0.7	MS, KI, ST
cis-Muurola-3,5-diene	1449	0.1	MS, KI
trans-Muurola-3,5-diene	1453	0.3	MS, KI
α-Humulene	1454	0.9	MS, KI, ST
allo-Aromadendrene	1460	0.4	MS, KI
trans-Cadina-1(6),4-diene	1476	0.5	MS, KI
γ-Muurolene	1479	0.5	MS, KI
Germacrene D	1485	0.4	MS, KI, ST
β-Selinene	1490	0.9	MS, KI
Valencene	1496	0.8	MS, KI
Bicyclogermacrene	1500	2.0	MS, KI
α-Muurolene	1500	0.1	MS, KI
γ-Cadinene	1513	1.0	MS, KI
cis-γ-Bisabolene	1515	0.4	MS, KI
δ-Cadinene	1523	1.8	MS, KI
Zonarene	1529	0.3	MS, KI
γ-Cuprenene	1533	0.2	MS, KI
trans-Cadina-1,4-diene	1534	0.2	MS, KI
α-Cadinene	1538	0.2	MS, KI
α-Copaen-11-ol	1541	0.4	MS, KI
Elemol	1550	0.2	MS, KI, ST
Germacrene B	1561	0.5	MS, KI
Caryophyllenyl alcohol	1572	0.2	MS, KI, ST
Spathulenol	1578	1.3	MS, KI, ST
Caryophyllene oxide	1583	0.4	MS, KI, ST
Globulol	1590	0.7	MS, KI, ST
Ledol	1602	0.6	MS, KI
Humulene epoxide II	1608	0.2	MS, KI
1-*epi*-Cubenol	1628	0.7	MS, KI
τ-Cadinol	1640	9.2	MS, KI, ST
τ-Muurolol	1642	1.6	MS, KI, ST
α-Cadinol	1654	10.5	MS, KI, ST
Monoterpene hydrocarbons (%)		**36.1**	
Oxygenated monoterpenes (%)		**14.9**	
Sesquiterpene hydrocarbons (%)		**23.2**	
Oxygenated sesquiterpenes (%)		**25.9**	
Yield (mL/100g)		**1.1 ± 0.03**	

[a] Kovats index on a DB-5 column with reference to *n*-alkanes [5]. [b] MS, NIST and Wiley library spectra and the literature; KI, Kovats index; ST, authentic standard compounds.

essential oil obtained was dried with anhydrous sodium sulfate. The oil yield and all test data are the average of triplicate analyses. 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: 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) [15], retention times (RT), and mass spectra with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [5, 6].

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 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 data were the average of triplicate analyses [7].

Table 2: IC_{50} values of *N. variabillima* leaf oil and it's main constituents against cancer cell lines.

Cell lines[a]	IC_{50}(µg/mL)						
	Essential oil	Compounds[b]					
		1	2	3	4	5	6
OEC-M1	38.9	>200	>200	>200	24	18.9	9.9
J5	42.6	>200	>200	>200	111.2	38.6	12.1
A549	36.9	>200	>200	>200	31.3	18.6	10.8
HT-29	16.8	>200	>200	>200	9.8	30.6	0.8
UACC-62	8.8	>200	>200	>200	3.2	2.5	1.3
K562	8.6	>200	>200	>200	4.6	3.6	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] 1. Sabinene ; 2. *trans*-β-Ocimene; 3.Terpinen-4-ol; 4. β-Caryophyllene; 5. τ-Cadinol; 6. α-Cadinol.

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