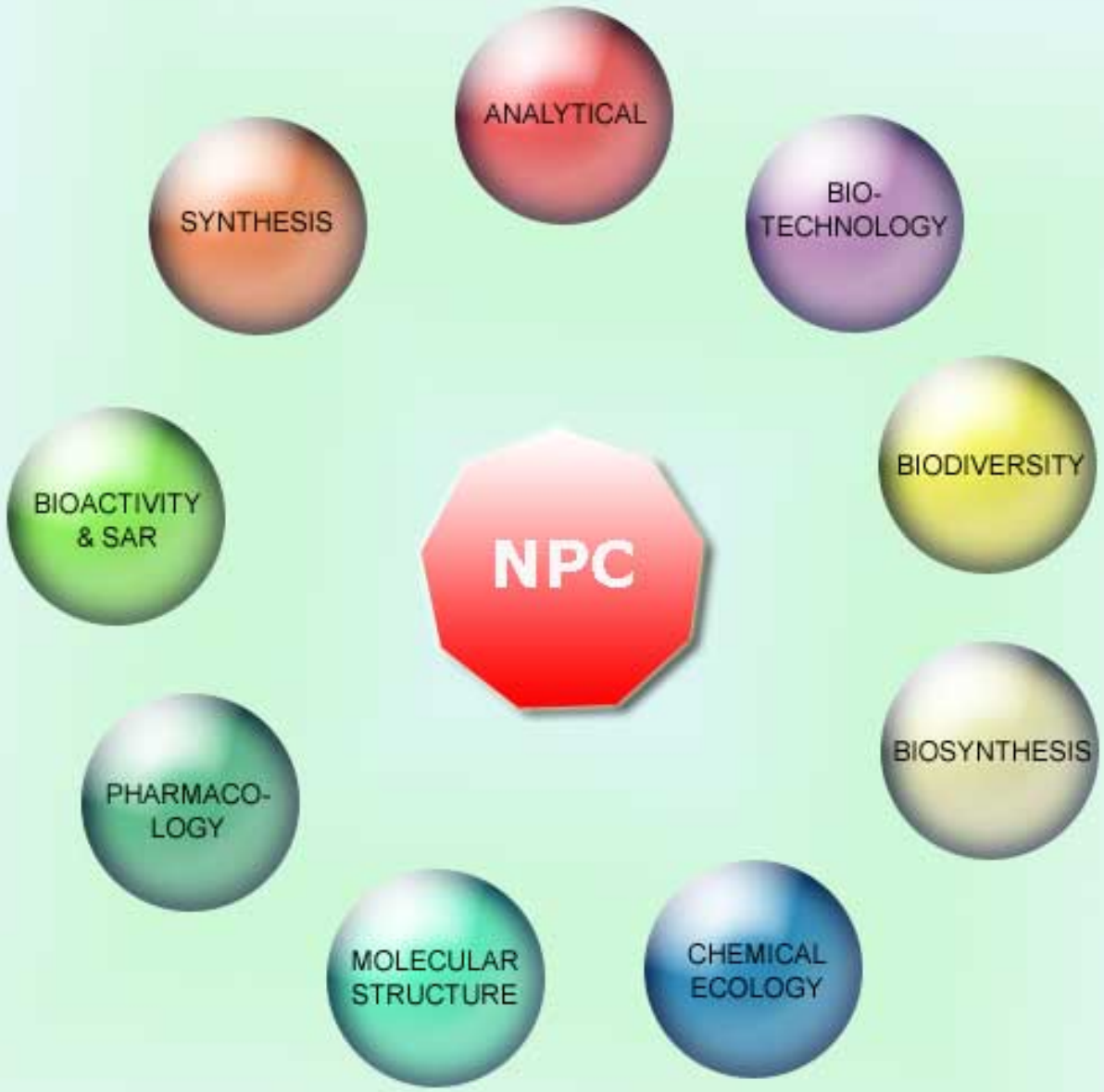
NATURAL PRODUCT COMMUNICATIONS

An International Journal for Communications and Reviews Covering all Aspects of Natural Products Research



Volume 5. Issue 4. Pages 507-666. 2010
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

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NPC Natural Product Communications

2010
Vol. 5
No. 4
617 - 620

Compositions and *in vitro* Anticancer activities of the Leaf and Fruit Oils of *Litsea cubeba* from Taiwan

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Received: January 14th, 2010; Accepted: March 3rd, 2010

The leaf and fruit essential oils of *Litsea cubeba*, extracted by hydrodistillation, were assessed for anticancer activities. A total of 53 and 50 compounds were identified, respectively from the leaf and fruit oils, and their yields were 13.9 ± 0.09% and 4.0 ± 0.03%, v/w, of the oven-dried materials, respectively. The main compound in the leaf oil was 1,8-cineol, and in the fruit oil, citral. The fruit oil, but not that of the leaf, exhibited cytotoxic activity against human lung, liver and oral cancer cells.

Keywords: *Litsea cubeba*, anticancer activities, 1,8-cineol, citral.

Litsea cubeba Pers., family Lauraceae, is a deciduous bush or small tree, mostly distributed in East Asia. In Taiwan, the plant is distributed in the central and eastern mountainous regions ranging in elevation from 300 to 2,300 m. The whole plant possesses a pungent gingery odor. The 1977 compilation of the Chinese herbal medicine dictionary recorded antibacterial and anti-inflammatory activities of *L. cubeba* [1]; subsequently, a few reports also noted that *L. cubeba* essential oils had extensive antibacterial and antifungal activities [2~4]; however, the anticancer activity of *L. cubeba* essential oils has not been reported. Thus, we first endeavored to examine the essential oils from the leaves and fruits of Taiwan grown plants using the hydrodistillation method and then analyzing their compositions. The foremost cause of death in Taiwan is malignant tumors, of which liver, lung, and oral cancers are the most prevalent types. In the second part of the study we tested the essential oils from these two plant parts for their *in vitro* anti-cancer activities. The information thus obtained may serve as the basis for a multipurpose approach of developing *L. cubeba* for practical applications.

The oil yields from *L. cubeba* leaves and fruits were 13.9 ± 0.09 and 4.0 ± 0.03%, v/w, respectively. Comparing our results with the literature [5~10], there were substantial differences; we obtained the highest leaf oil yield. The yields of fruit oil were comparable to those of the literature [6,8,9,11-12].

The identified constituents are presented in Table 1, where all compounds are listed in order of their elution from the DB-5 column. A total of 53 compounds were identified from the hydrodistilled leaf oil. The main constituents were 1,8-cineol (57.6%), sabinene (12.3%), α-terpinyl acetate (9.8%), α-pinene (4.1%), and β-pinene (3.2%). Oxygenated monoterpenes predominated (72.7%), followed by monoterpene hydrocarbons (25.1%), sesquieterpene hydrocarbons (1.9%), and oxygenated sesquiterpenes (0.3%). Literature pertaining to *L. cubeba* leaf oil refers it to the cineole type [6-8,10], the linalool type [5,9,10], and the linalool/citronellal type [5]. Our sample belongs to the cineole type and had a composition similar to those reported by Lin [6] and Cheng and Cheng [7].

Table 1. Chemical compositions of the leaf and fruit oils of *L. cubeba*

Consituents	R. I [a]	Concentration (%)		Identification [b]
		Leaf	Fruit	
α-Thujene	930	0.4	- [c]	MS, KI, ST
α-Pinene	939	4.1	0.6	MS, KI, ST
3-Methyl-cyclohexanone	952	-	t [d]	MS, KI
Camphene	954	0.1	t	MS, KI, ST
6-Methyl-heptan-2-ol	965	t	-	MS, KI, ST
Sabinene	976	12.3	0.1	MS, KI, ST
β-Pinene	979	3.2	0.5	MS, KI, ST
6-Methyl-5-hepten-2-one	986	0.1	2.6	MS, KI, ST
β-Myrcene	990	1.1	1.3	MS, KI, ST
6-Methyl-5-hepten-2-ol	992	-	t	MS, KI, ST
m-Metha-1(7),8-diene	1001	t	-	MS, KI
α-Phellandrene	1003	t	-	MS, KI, ST
α-Terpinene	1017	0.4	-	MS, KI, ST
p-Cymene	1025	0.1	t	MS, KI, ST
Limonene	1029	2.4	11.3	MS, KI, ST
1,8-Cineol	1031	57.6	1.1	MS, KI, ST
cis-β-Ocimene	1037	-	t	MS, KI, ST
Benzene acetaldehyde	1042	-	t	MS, KI, ST
trans-β-Ocimene	1049	t	-	MS, KI, ST
Bergamal	1057	0.1	-	MS, KI, ST
Γ-Terpinene	1060	0.6	t	MS, KI, ST
cis-Sabinene hydrate	1070	0.1	-	MS, KI, ST
trans-Linalool oxide (furanoid)	1073	-	t	MS, KI, ST
Terpinolene	1089	0.2	t	MS, KI, ST
Linalool	1097	0.3	1.7	MS, KI, ST
Perillene	1101	-	t	MS, KI
trans-*p*-Mentha-2.8-dien-1-ol	1123	-	t	MS, KI, ST
cis-Limonene oxide	1137	-	t	MS, KI, ST
cis-*p*-Mentha-2,8-dien-1-ol	1138	0.1	0.1	MS, KI
trans-*p*-Mentha-2-en-1-ol	1141	0.1	-	MS, KI
Camphor	1146	t	0.4	MS, KI, ST
iso-Pulegol	1150	-	0.1	MS, KI, ST
Citronellal	1153	0.2	1.7	MS, KI, ST
cis-Chrysanthenol	1164	t	1.0	MS, KI
δ-Terpineol	1166	0.1	-	MS, KI
α-Phellandrene-8-ol	1170	-	0.1	MS, KI
Terpinen-4-ol	1177	1.7	1.9	MS, KI, ST
p-Cymen-8-ol	1182	t	t	MS, KI, ST
α-Terpineol	1189	1.0	0.2	MS, KI, ST
cis-Piperitol	1196	-	t	MS, KI, ST
n-Decanal	1202	0.1	-	MS, KI, ST
trans-Piperitol	1208	-	0.1	MS, KI, ST
trans-Carveol	1217	-	t	MS, KI, ST
z-Cinnamaldehyde	1219	t	-	MS, KI, ST
Citronellol	1226	-	t	MS, KI, ST
Methyl nonanate	1227	t	-	MS, KI
Nerol	1230	t	0.7	MS, KI, ST
exo-Fenchyl acetate	1233	0.1	-	MS, KI
Neral	1238	t	31.3	MS, KI, ST
Geraniol	1253	0.1	1.2	MS, KI, ST
Geranial	1267	0.3	37.6	MS, KI, ST
iso-Pulegyl acetate	1278	0.1	-	MS, KI, ST
Methyl nerolate	1283	t	0.2	MS, KI, ST
Bornyl acetate	1289	0.1	0.1	MS, KI, ST
iso-Verbanol acetate	1310	0.8	-	MS, KI, ST
neoiso-Isopulegyl acetate	1313	-	t	MS, KI
Methyl geranate	1325	-	0.1	MS, KI, ST
E-Hasmigone	1336	-	0.2	MS, KI, ST
α-Terpinyl acetate	1349	9.8	0.8	MS, KI, ST
Neryl acetate	1362	0.1	1.0	MS, KI, ST
α-Copaene	1377	-	0.7	MS, KI, ST
Geranyl acetate	1381	t	0.4	MS, KI, ST
β-Elemene	1391	0.2	-	MS, KI, ST
β-Caryophyllene	1419	1.2	0.4	MS, KI, ST
β-Copaene	1432	-	0.1	MS, KI, ST
α-Caryophyllene	1455	0.2	t	MS, KI, ST
α-Acoradiene	1466	0.1	-	MS, KI
trans-Cadina-1(6),4-diene	1477	t	-	MS, KI
γ-Muurolene	1480	0.2	-	MS, KI
cis-β-Guaiene	1493	0.2	-	MS, KI
Caryophyllene oxide	1582	0.3	t	MS, KI, ST
Yield %, v/w		13.9±0.09	4.0 ±0.03	

[a] Retention index on a DB-5 column with reference to *n*-alkanes [13].
[b] MS, NIST and Wiley library spectra and literature; RI, Retention index; ST, authentic standard compounds.
[c] not detected.
[d] trace < 0.1%.

L. cubeba fruit oil yielded 50 identified compounds, with citral (68.9%) predominating [neral (31.3%) and geranial (37.6%)]. Other main components included limonene (11.3%) and 6-methyl-5-hepten-2-one (2.6%). Oxygenated monoterpenes accounted for 84.9%, followed by monoterpene hydrocarbons (13.9%), sesquiterpene hydrocarbons (1.2%), and oxygenated sesquiterpenes (trace). Literature on *L. cubeba* fruit oil designated three chemotypes, viz. the citral type [6,7,11,14], the limonene type [8], and the citronellal type [12,14]. Our results indicated a citral type.

To evaluate the anticancer activities of the essential oils, we tested their effect on the viability of three human cancer cell lines: human oral squamous OEC-M1 cells, human hepatocellular carcinoma J5 cells, and human lung adenocarcinoma A549 cells. Cells were incubated with various concentrations of essential oils for 24 h and 48 h, and the cell viability was measured by the alamarBlue® proliferation assay. The results showed that fruit oil treatment for 24 h reduced the viability of OEC-M1 cells, J5 cells, and A549 cells, with IC_{50} values of around 50, 50, and 100 ppm, respectively (Fig. 1). This is the first report on the anticancer activities of *L . cubeba* fruit essential oil against human lung, liver and oral cancer cells. Citral is the main ingredient of the oil, and we have found one prior report on the anticancer activity of citral against human breast cancer cell line MCF-7 [15]. Here we report that citral probably possesses anticancer activities against human lung, liver and oral cancer cells. Essential oils from *Hedyosmum arborescens* and *Allium sativum* also showed cytotoxicity to A549 lung cancer cells and J5 liver cancer cells, respectively [16,17].

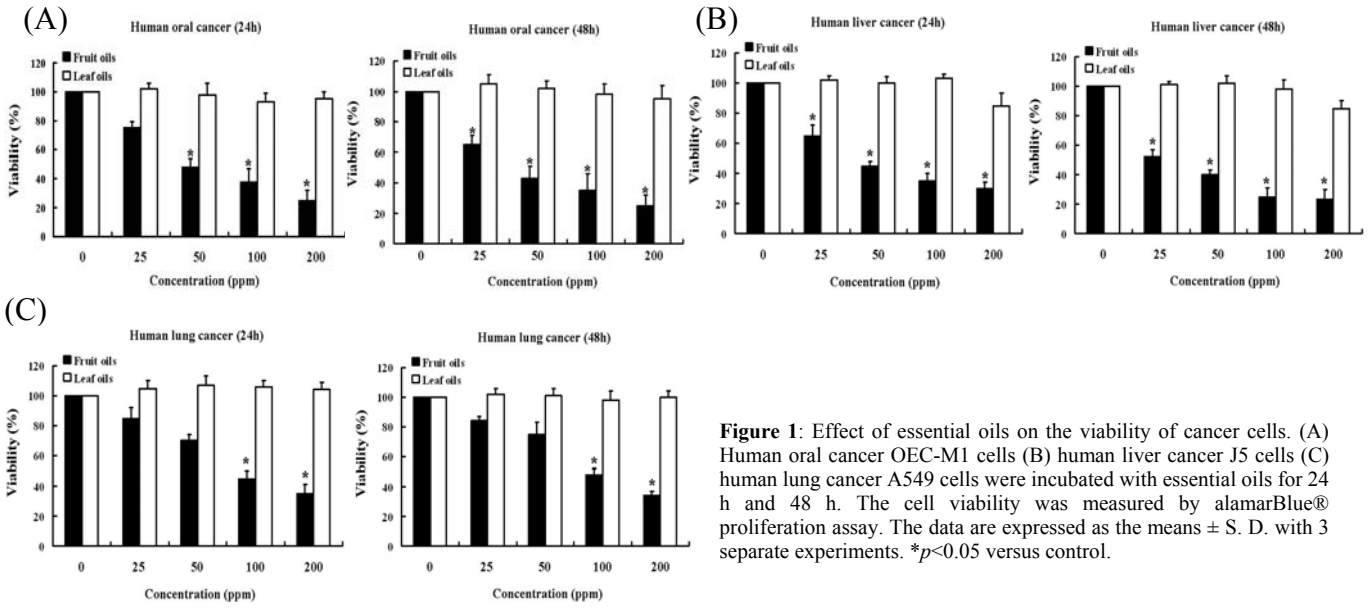

Figure 1: Effect of essential oils on the viability of cancer cells. (A) Human oral cancer OEC-M1 cells (B) human liver cancer J5 cells (C) human lung cancer A549 cells were incubated with essential oils for 24 h and 48 h. The cell viability was measured by alamarBlue® proliferation assay. The data are expressed as the means ± S. D. with 3 separate experiments. *p<0.05 versus control.

Experimental

Plant materials: *Litsea cubeba* was collected from the Lienhuachih Research Center of the Taiwan Forestry Research Institute in central Taiwan between March and June 2009; the leaves were collected in March and the fruit in June. The samples were compared with specimen no. 10188 from the herbarium of the Taiwan Forestry Research Institute and identified by Prof. Yen-Hsueh Tseng of NCHU. The voucher specimen (CLH-005) has been deposited in the Taiwan Forestry Research Institute herbarium. Samples were shipped quickly to our Taipei headquarters and stored at 4ºC until extraction and subsequent analyses.

Isolation of essential oils: The method of Ho *et al.* [18,19] was adopted. Leaves and fruits (1 Kg of each) were placed separately in a round-bottom flask to which 3 L of distilled water was added. The mixture was refluxed for 8 h. The essential oil layer above the water was separated, dried with anhydrous sodium sulfate, placed in specimen bottles, and the yields determined. Each test was repeated 3 times and the data were averaged.

Essential oil analysis: The method of Ho *et al.* [20,21] was adopted. 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. One μL sample was injected. Identification of the oil components was based on their retention indices and MS, 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) [22], retention times (RT), and MS with those obtained from authentic standards and/or the NIST and Wiley libraries spectra, and literature [13,23].

Cell culture: Human lung adenocarcinoma A549 cells, human hepatocellular carcinoma J5 cells, 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 37ºC, 5% CO_2 incubator.

Cell viability assay: The cytotoxicity of essential oils 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 absorbance at 570 nm and 600 nm was measured by a microplate reader

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

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Volume 5, Number 4

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