Comparative Study on Volatile Compounds of *Alpinia japonica* and *Elettaria cardamomum*

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Abstract: The volatile compounds obtained from the ether extracts, headspace gases and steam distillates of *Alpinia japonica* and *Elettaria cardamomum* were analyzed by GC/MS. Both species were rich sources of naturally rare fenchane-type monoterpenoids, fenchene, fenchone, fenchyl alcohol and its acetate, together with 1,8-cineole. The distributions of volatile sesquiterpenoids were very poor in both species. Chiralities of fenchone in *A. japonica* and *E. cardamomum* were 99% of (1*S*,4*R*)-(+)-form. Camphor in *A. japonica* is composed of a mixture of (1*R*,4*R*)-(+)-form (94.3%) and (1*S*,4*S*)-(-)-form (5.7%). On the other hand, *E. cardamomum* produced only (1*R*,4*R*)-(+)-camphor (99%).

Key words: *Alpinia japonica*, *Elettaria cardamomum*, Zingiberaceae, monoterpenoids, fenchanes

1 INTRODUCTION

Alpinia genus including *Alpinia japonica*, *A. katsumadai*, *A. zerumbet*, *A. conchigera*, *A. galanga*, *A. hainanensis*, *A. ligulata*, and *A. nieiwenhuizii*, among others, is the largest, most widespread, and taxonomically most complex genus within the Zingiberaceae family, with more than 230 species occurring throughout tropical and subtropical Asia. Many of them are used as foods, spices, flavors as well as traditional medicines, to cure rheumatism, arthritis and fungal infection¹⁻⁶. Almost all of *Alpinia* species contain volatile terpenoids in their rhizomes and leaves¹⁻⁶. The whole parts and the rhizomes of *A. japonica* which elaborates a strong sweet aroma have been used as folk medicines and to cure stomachache, muscle pain, menoxenia, nematemesia and diarrhea. Itokawa *et al.*^{7,8} and Morita *et al.*⁹ reported the isolation of several sesquiterpenoids which showed spasmolytic activity from this plant. Li *et al.*⁶ described that the labdane diterpenoids isolated from the Chinese *A. japonica* showed inhibitory activity of nitric oxide production. However, the volatile components of the different organs of this plant have not yet been fully investigated.

Elettaria cardamomum belonging to the Zingiberaceae whose seed oil has been used as spice and for helping cure muscle, and skin and hair care¹⁰. In addition to its wide use for culinary purpose, cardamom has been used in tradi-

tional medicine for asthma, constipation, colic, diarrhea, dyspepsia, hypertension, epilepsy and is considered useful as antibacterial, antifungal, antiviral, carminative, diuretic, and stomachic¹¹.

The present paper concerns with the analysis of the volatile components present in different organs of *Alpinia japonica* and *Elettaria cardamomum* by GC/MS. Little attention has been paid to the headspace (HS) gas sampling of the volatile components with Solid Phase Micro Extraction (SPME) for these plant species. The aim of the present paper was comparative analysis of the volatile compounds obtained by HS-SPME, steam distillation and extraction with diethyl ether. The chiralities of fenchone and camphor found in the dried rhizomes of *A. japonica* and *E. cardamomum* were also reported.

2 MATERIALS AND METHODS

2.1 Plant material

A. japonica (12 kg) and *E. cardamomum* (7 kg) (identified by Yoshinori Asakawa (YA)) were collected by YA in Nakatsumine, and Tokushima Bunri University (TBU) botanical garden, Tokushima, Japan in May and June 2013, respectively and the voucher specimens were deposited in the Institute of Pharmacognosy, TBU. The fresh plant of *A.*

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japonica was divided into flowers, leaves, stems, rhizomes, fruits, and roots, while *E. cardamomum* into rhizomes, leaves, and roots. The plant material was dried in room temperature.

2.2 Solvent extraction of volatile compounds

The fresh flowers, leaves, stems, fruits, and roots of *A. japonica* (each 10 g sample) were crushed by pestle in mortar, followed by extraction with redistilled diethyl ether immediately. The dried plant materials of *A. japonica* and *E. cardamomum* were ground to give the powders and then extracted with diethyl ether for a week. 50 mL of each crude extract (from fresh and dried plant samples) was filtered through the Pasteur pipette packed with celite, followed by evaporation of the solvent at room temperature to give the strong fragrant extracts.

2.3 HS-GC/MS analysis of Steam-Distillates

Each fresh leave (1.5 kg) of *A. japonica* and *E. cardamomum* was dried for three months, followed by steam distillation using the home made steam-distilled apparatus for one hour to obtain the steam-distilled water (each 500 mL), which contained essential oils. Each 10 g of distilled water was kept in a 20 mL vial with stopper and then equilibrated for 30 min at 80°C by a HS-20 GC (Shimadzu). Each 1 mL of HS gas from distilled water containing essential oils was injected into a GC/MS-QP2010 Ultra (Shimadzu) equipped with a BC-WAX fast column (20 m × 0.18 mm i.d., 0.18 µm film thickness). The oven temperature program was set at 40°C with 2 min initial hold and then raised to 230°C at a rate of 20°C/min. The carrier gas was helium with a constant flow of 1 mL/min by split ratio of 100:1. Electron impact ionization was employed for the mass spectrometry at an ionization energy of 70 eV in scan mode. The injector and the ion source temperatures were set at 250°C and 200°C, respectively.

2.4 HS-SPME-GC/MS analysis

The dried rhizomes and flowers of *A. japonica* were cut into small pieces (0.1 g), and they were kept in a vial (20 mL) which was completely closed. The HS gas was absorbed on a PDMS/DVB type fiber (Supelco) at 60°C for 20 min. Then, the fiber was introduced into an injector of 7890A GC System (Agilent Technologies) equipped with the GC Science order made BC-WAX column (20 m × 0.18 mm i.d., 0.18 µm film thickness). The oven temperature program was set at 45°C with 1 min initial hold and then raised to 230°C at a rate of 20°C/min. The carrier gas was helium with a constant flow of 1 mL/min by split ratio of 10:1. A 5975C inert XL EI/CI MSD (Agilent Technologies) was operated under electron impact condition at an ionization energy of 70 eV in scan mode. The injector and the ion source temperatures were set at 250°C and 200°C, respectively.

2.5 GC/MS analysis

GC/MS analyses of all ether extracts were carried out by using a 6890N gas chromatograph coupled with a 5973 mass selective detector (Agilent Technologies) on an HP-5MS column (30 m × 0.25 mm i.d., 0.26 µm film thickness). The oven temperature program was set at 50°C with 3 min initial hold and then raised to 250°C at a rate of 5°C/min. The injector and the ion source temperatures were set at 250°C and 230°C, respectively. The carrier gas was helium with a constant flow of 1 mL/min. The mass selective detector was operated under electron impact condition at an ionization energy of 70 eV in scan mode. The retention indices were calculated relative to C₈-C₂₇ *n*-alkanes. Compounds were identified using a computer supported library¹²⁾, mass spectra of references compounds^{13, 14)} and mass spectra from the literature and our own library databases.

2.6 Enantioselective GC/MS analysis

The dried and fresh rhizomes of *A. japonica* and *E. cardamomum* were cut into small pieces (0.1 g), and they were kept in a vial (20 mL) and sealed with a rubber cap. The headspace gas was absorbed on a PDMS/DVB type fiber at 40°C for 30 min. Then, the fiber was introduced into an injector of a MDGC/GCMS-2010 (Shimadzu) to conduct the chiral analyses of fenchone and camphor. With this system, a heart-cut of the relevant fractions can be made and transferred from the first polar column to the second chiral one via a switching element. The first GC was equipped with a BC-WAX column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The injector and the flame ionization detector temperatures were both set at 250°C. The carrier gas was helium with a constant pressure held at 180 kPa. The first oven temperature program was set from 70°C to 230°C at a rate of 5°C/min. The second GC was equipped with a γ-DEX 225 column (30 m × 0.25 mm i.d., 0.25 µm film thickness) and the second oven was initially set at 70°C for 10 min and then raised to 120°C at a rate of 1°C/min. Electron impact ionization was employed for the mass spectrometry at an ionization energy of 70 eV in scan mode. The GC/MS data was checked by retention index, sensitivity, and MS fragmentation every week using the standard samples.

3 RESULTS AND DISCUSSION

Table 1 shows the distribution of volatile compounds of the ether extracts in each organ of *Alpinia japonica*. The major volatile in all of the different organs of *A. japonica* is fenchone: In the fresh and dried leaves and dried rhizomes contained 41-45% of fenchone and even the fresh roots also contain fenchone as a large amount. It is noteworthy that the fresh flowers elaborate much more

Table 1 GC/MS analysis of volatile components of different organs of *A. japonica* and *E. cardamomum*.

Compounds	RI (HP-5MS)	Relative percentage [%]											
		<i>A.j</i> Fl ^{*b}	<i>A.j</i> Fr ^{*b}	<i>A.j</i> Lf ^{*a}	<i>A.j</i> Lf ^{*b}	<i>A.j</i> St ^{*a}	<i>A.j</i> St ^{*b}	<i>A.j</i> Rh ^{*a}	<i>A.j</i> Rt ^{*a}	<i>A.j</i> Rt ^{*b}	<i>E.c</i> Rh ^{*a}	<i>E.c</i> Rt ^{*a}	<i>E.c</i> Lf ^{*a}
Tricyclene	927	0.4											
α -Pinene	936	5.0	3.2	1.6		3.2		2.3	2.3		6.4	3.6	4.6
α -Fenchene	941	27.0	1.5	1.1		5.2		1.6			10.7	t	10.7
Camphene	950	0.5		1.6		t		1.8			7.9	10.3	1.1
β -Pinene	978		4.6										
α -Phellandrene	1002		2.8										
Limonene	1024	2.2											
1,8-Cineole	1025	10.5	69.4	30.6		8.6	8.7	12.7		10.0	22.2	10.0	6.5
(<i>E</i>)- β -Ocimene	1041		0.7										
Fenchone	1069	13.0	0.7	42.2	45.0	23.2	37.7	41.2	1.1	31.2	1.0	t	1.6
α -Fenchol	1099	7.0		2.0		6.0	8.8	3.0			0.5	2.0	
Camphor	1123	0.7		3.2		1.8	3.9	2.1		t	5.7	0.6	3.4
Pinocarvone	1137					t				t			
Bornenol	1150									t	1.0	3.1	
Myrtenal	1172									t			
α -Terpineol	1176		1.5							t		t	
Verbenone	1183									t			
β -Fenchyl acetate	1224	0.9		3.3	11.0	9.2	11.4	8.6	7.4	6.0	13.6	40.0	
Bornyl acetate	1270	0.6				t						t	
α -Cubebene	1355					t							
β -Elemene	1389	0.8		0.8		1.2		2.0			2.0	t	
β -Caryophyllene	1421	4.0	6.3	0.4							1.0	1.0	0.9
γ -Maaliene	1428	1.0						3.1				t	
α -Humulene	1455	0.6	6.3	1.4		1.7						1.0	
α -Panasinsene	1455	2.3											
α -Amorphene	1477				3.5				3.5				
Eremophilene	1486	0.8											
δ -Selinene	1490	0.4		0.5								t	
α -Selinene	1494	1.0										t	
γ -Guaiene	1499				0.9			3.4	0.9				
γ -Cadinene	1507	1.3		1.0		6.5				t			
<i>cis</i> -Calamenene	1517							1.5		t			
Guai-6,9-diene	1565	2.0											
β -Caryophyllene oxide	1578	2.2	0.9				3.8			t			
Guaiol	1593	0.8				3.2						t	
γ -Eudesmol	1618	1.0										t	
α -Santalol	1669				1.1				1.1				
α -Cyperone	1741									t			
Unknown diterpene	1862	3.4								67.3	15.0	16.9	58.3
Total (%)		89.4	97.9	89.7	61.5	69.8	74.3	83.2	83.6	47.2	87.0	88.5	87.1
Monoterpene hydrocarbons		35.1	12.8	4.3	—	8.4	—	5.7	2.3	—	25.0	13.9	16.4
Oxygenated monoterpenoids		32.7	71.6	81.3	56.0	48.8	70.5	67.6	8.5	47.2	44.0	55.7	11.5
Sesquiterpene hydrocarbons		12.2	12.6	4.1	4.4	9.4	—	10.0	4.4	t	3.0	2.0	0.9
Oxygenated sesquiterpenoids		6.0	0.9	—	1.1	3.2	3.8	—	1.1	t	—	t	—
Other		3.4	—	—	—	—	—	—	67.3	—	15.0	16.9	58.3

*Fl: flower, Fr: fruit, Lf: leaf, St: stem, Rh: rhizome, Rt: root. ^a: dried sample, ^b: fresh sample; t: trace (< 1.0%)

α -fenchene than fenchone. α -Fenchene was also found in the rhizomes and leaves of *E. cardamomum*. 1,8-Cineole is predominant component both in *A. japonica* and *E.*

cardamomum. β -Fenchyl acetate is also rich volatile component both in two plants. Especially, the roots of *E. cardamomum* elaborate this ester in 40%.

Table 2 GC/MS analysis of volatile components obtained from headspace gases of the dried rhizomes and the flowers by SPME method.

Compounds	RI (BC-WAX)	<i>A. japonica</i> (dried rhizome) ^b	<i>A. japonica</i> (dried flowers) ^b
α -Pinene	1043	t	t
Camphene	1095	t	t
β -Pinene	1119	2.1	
Limonene	1207	1.7	2.3
1,8-Cineole	1220	4.5	17.1
4-Cymene	1264	t	t
Fenchone	1389	16.2	28.5
α -Fenchyl acetate	1464	t	t
β -Fenchyl acetate	1490	3.9	2.6
Camphor	1499	1.9	3.0
Linalool	1531	t	1.4
Pinocarvone	1552	t	
β -Fenchol	1561	1.7	1.5
α -Fenchol	1568	3.4	3.5
Terpinen-4-ol	1586		t
β -Caryophyllene	1589		1.9
Guaia-6,9-diene	1603	t	
Myrtenal	1608	t	
9-Epi-(<i>E</i>)-caryophyllene	1669	1.4	
γ -Selinene	1677	t	
γ -Murolene	1677	t	
α -Terpineol	1677	t	1.5
Valencene	1705	6.4	
β -Selinene	1707		6.1
α -Murolene	1710	t	
<i>trans</i> -Dihydroagarofuran	1712	4.5	1.9
Carvone	1716	t	1.1
7-Epi- α -selinene	1748	1.5	
γ -Cadinene	1752	2.4	
<i>cis</i> -Calamenene	1813	2.5	
α -Agarofuran	1876	1.6	
<i>trans</i> - β -Caryophyllene oxide	1971	1.0	1.6
10-Epi- γ -eudesmol	2088	3.7	
Hinesol	2193	1.1	
Valerianol	2198	2.4	
β -Eudesmol	2208	1.4	
Jinkoheremol	2224	1.5	
α -Hydroxydihydroagarofuran	2259	1.1	
Total (%)		67.9	74.0

t: trace (< 1.0%)

Table 3 Volatile components of hydrodistilled dried plant material of *Alpinia japonica* and *Elettaria cardamomum*.

Compounds	RI (BC-WAX)	<i>A. japonica</i> (dried leaves)	<i>E. cardamomum</i> (dried leaves)
α -Pinene	1043		4.1
Camphene	1095		3.1
β -Pinene	1119		15.8
Limonene	1207		2.1
1,8-Cineole	1220	41.5	40.8
Terpinolene	1285		1.6
Heptan-2-ol	1307		1.4
Fenchone	1389	32.1	
β -Fenchyl acetate	1490	1.9	
Camphor	1499	1.6	8.8
Linalool	1531		3.1
β -Fenchol	1561	1.4	
α -Fenchol	1568	4.7	
β -Caryophyllene	1589	1.2	
Methyl (<i>E</i>)-cinnamate	2054		2.2
Total (%)		84.4	83.0

The major volatile of the fresh fruits of *A. japonica* was 1,8-cineole (69.4%) and only small amount (1.5%) of α -fenchene was detected in the crude ether extract of this plant. The major components of the ether crude extract of the fresh flower of *A. japonica* was α -fenchene, however, the dried flower did not contain α -fenchene, instead, fenchone and 1,8-cineole were the predominant volatiles as shown in **Table 2**. The distribution and content of the volatile components in the dried flower of *A. japonica* is not similar to those of the fresh flower but much similar to the dried rhizome.

Thus *A. japonica* is rich source of fenchone which plays an important role in the strong sweet odor of this species. The less potent fragrance of the latter species might be the absence of fenchone, in spite of a large quantity of β -fenchyl acetate.

Gochev *et al.*¹⁵⁾ reported that the major compounds of cardamom seed oil were 1,8-cineole and α -terpinyl acetate which might be significant for the typical aroma of this oil. We could not detect α -terpinyl acetate in all of the different organs of the cultivated *E. cardamomum* in the green house of TBU. The distribution of volatile sesquiterpenoids in two plants was very poor in all of their different organs. It is noteworthy that both plants biosynthesize a large amount of an unknown diterpenoid having the molecular weight of 302 (in mass spectrum, the base peak 137). The volatile components of the roots of *E. cardamomum* is closely related chemically to the Indian *A. galanga* since

this plant predominantly produces β -fenchyl acetate (40.9%)³⁾. The Malaysian *A. ligulata* and *A. nieuwenhuzii* are rich sources of methyl(*E*)-cinnamate¹⁾, however, such an ester has not been detected in the Japanese *A. japonica* and *E. cardamomum* although the steam-distilled water of *E. cardamomum* contains a small amount of this aromatic ester, as shown in **Table 3**.

Thirty-five and nineteen volatile compounds were found in HS gas of the dried rhizome and the dried flowers of *A. japonica*, respectively as shown in **Table 2**. The predominant volatile compounds were fenchone and 1,8-cineole, and the second major group was limonene, β -fenchyl acetate, camphor, α -Fenchol, and *trans*-dihydroagarofuran.

The steam distilled water of *A. japonica* and *E. cardamomum* contained 1,8-cineole predominantly. This chemical profile resembled that of volatiles obtained from the leave extract of *A. japonica* and the rhizome extracts of *E. cardamomum*, respectively.

In order to determine the chirality of fenchone and camphor found in *A. japonica* and *E. cardamomum*, γ -DEX 225 was used for this purpose. Fenchone in both the dried and fresh rhizomes of *A. japonica* showed 99% of (1*S*,4*R*)-(+) -form. The same monoterpene ketone found in both the dried and fresh rhizomes of *E. cardamomum* also showed the same chirality, 99%. Furthermore the chirality of camphor found in both aromatic fresh and dried *A. japonica* and *E. cardamomum* were measured

by the same GC column, indicating that the fresh and dried *A. japonica* produced the same ratio of (1*R*,4*R*)-(+) -camphor as 94.3%. The rest was (1*S*,4*S*)-(-)-camphor in 5.7% in GC/MS using the same column as used in fenchone.

4 CONCLUSION

The volatile components of two Zingiberaceae plants, *Alpinia japonica* and *Elettaria cardamomum* were analyzed by GC/MS. The characteristic scent of both plant was due to the presence of fenchane-type monoterpenoids which were predominant in all parts of organs of two species, except of the presence of 1,8-cineole in the fruits of the former plant. The presence of (1*S*,4*S*)-(-)-camphor in *A. japonica* was the first example in the plant kingdom although the naturally occurring camphor is absolutely its enantiomer, like that isolated from camphor tree (*Cinnamomum camphora*).

A. japonica and *E. cardamom* produce a very unstable oxygenated diterpenoid ($[M]^+ m/z$ 302) whose structure remained to be clarified, however, this compound is also one of the most characteristic chemical markers of both species with the presence of a large amount of fenchane monoterpenoids.

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References

- 1) Yusoff, M.M.; Ibrahim, H.; Hamid, N.A. Chemical characterization and antimicrobial activity of rhizome essential oils of very closely allied Zingiberaceae species endemic to Borneo: *Alpinia ligulata* K. Shum. and *Alpinia nieuwenhuizii* Val. *Chem. Biodiv.* **8**, 916-923 (2011).
- 2) Nam, P.; Hu, Y.; Zhao, J.; Fneg, Y.; Zhong, Y.Z. Chemical composition of two *Alpinia* species from Hainan island, China. *Naturforsch.* **59c**, 157-160 (2004).
- 3) Jirovetz, L.; Buchbauer, G.; Shafi, M.P.; Leela, N.K. Analysis of the essential oils of the leaves, Stems, Rhizomes and roots of the medicinal plant *Alpinia galanga* from southern India. *Acta Pharm.* **53**, 73-81 (2003).
- 4) Faridah, Q.Z.; Abdelmageed, A.H.A.; Nor Hazirah, A.N.; Muhaman, Y. Comparative study of essential oil composition of leaves and rhizomes of *Alpinia conchigera* Griff. at different post-harvest drying periods. *J. Med. Plants Res.* **4**, 2700-2705 (2010).
- 5) Ali, S.; Sotheeswaran, S.; Tuiwawa, M.; Smith, R.M. Comparison of the composition of the essential oils of *Alpinia* and *Hedychium* species-essential oils of Fijian plants, Part 1. *J. Essent. Oil Res.* **14**, 409-411 (2002).
- 6) Li, Q.M.; Luo, J.G.; Yang, M.H.; Kong, L.Y. Terpenoids from rhizomes of *Alpinia japonica* inhibiting nitric oxide production. *Chem. Biodiv.* **12**, 388-396 (2015).
- 7) Itokawa, H.; Morita, H.; Watanabe, K.; Mihashi, S.; Iitaka, Y. Agarofuran-, eudesmane- and eremophilane-type sesquiterpenoids from *Alpinia japonica* (Thunb.) Miq. *Chem. Pharm. Bull.* **33**, 1148-1153 (1985).
- 8) Itokawa, H.; Watanabe, K.; Morita, H.; Mihashi, S.; Iitaka, Y. A novel sesquiterpene peroxide from *Alpinia japonica* (Thunb.) Miq. *Chem. Pharm. Bull.* **33**, 2023-2027 (1985).
- 9) Morita, M.; Nakanishi, H.; Morita, H.; Mihashi, S.; Itokawa, H. Structures and spasmolytic activities of derivatives from sesquiterpenes of *Alpinia speciosa* and *Alpinia japonica*. *Chem. Pharm. Bull.* **44**, 1603-1606 (1996).
- 10) Articles.mercola.com/herbal-oils/cardamom-seed.as.px
- 11) Duke, J.A.; Bogenschutz-Godwin, M.J.; DuCelliar, J.; Duke, P.K. Hand book of medicinal herbs. 2nd ed. Boca Raton, CRC Press, pp. 153-154 (2002).
- 12) Joulain, D.; König, W.A. The atlas of spectral data of sesquiterpene hydrocarbons. E.B.-Verlag, Hamburg (1998).
- 13) MassFinder: In Hochmuth, D.H. (Ed.) Version 4.0, Scientific Consulting, Germany (2008).
- 14) Stein, S.E. *NIST Chemistry WebBook*, NIST Standard Reference Database 69. Linstrom, P.J.; Mallard, W.G. (Eds.). National Institute of Standards and Technology, Gaithersburg MD, 20899, (<http://webbook.nist.gov>) (2011).
- 15) Gochev, V.; Grova, T.; Stoilova, I.; Atanasova, T.; Nenov, N.; Stancev, V.; Soyanova, A. Low temperature extraction of essential oil bearing plant by liquefied gases. 7. Seeds from cardamon (*Elettaria cardamomum* (L.) Maton). *J. Biosci. Bioch.* **1**, 135-139 (2012).