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Original article

Variations in volatile oil quantity and chemical composition of *Litsea cubeba* (Lour.) Pers. from northern Thailand

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ABSTRACT

Litsea cubeba (Lour.) Pers. is known as an aromatherapy plant. Volatile oil from fruit has been used commercially as a citral source. Based on the capacity of the volatile oil fraction and chemical isolate conversion into products for the flavor and fragrance industries, the United States, Western European countries and Japan are the major global importers.

This study evaluated the chemical composition and variation of the volatile oil of *Litsea cubeba*. Lower montane forest in northern Thailand is where most natural *L. cubeba* is distributed and 74 fruit samples were harvested from seven locations six in Chiang Mai province and one in Phitsanulok province. Hydro-distillation was used for fruit oil extraction. Gas chromatography/ mass spectroscopy (GC/MS) were used to determine and characterize the components of the volatile oil. Analysis of the composition of samples from the different locations revealed 12 main compounds citral, 70.95%; D-limonene, 3.59%; linalool, 1.76%; polegone, 1.39%; citronella, 1.32%; 6-methyl 5 hepten -2- one, 0.81%; β-myrcene, 0.63%; sabinene, 0.61%; eucalyptol, 0.46%; geraniol, 0.39%; a- pinene, 0.31%; and caryophyllene, 0.29%. Comparisons of the volatile oil contents involving location factors showed significant differences. *L. cubeba* oil from different locations had high yields with different constituents. The results from the present study can be used for future conservation planning and breeding programs of *L. cubeba* especially for the specific volatile oil constituent.

Variations in the chemical composition of *L. cubeba* oil between locations were found. Citral-rich sources were from Den Ya Khad in Chiang Mai (85%) and Phu Hin Rongkla, Phitsanulok (80%). Moreover, *L. cubeba* also provided high D-limonene with the Doi Inthanon sample producing a high content of this constituent (9%) as well as being rich in linalool (4.7%). Thus, locations should be distinguished to select plus trees for a future breeding program of this species. An advantage for conservation and utilization of *L. cubeba* would be to introduce this species to hill tribes for planting as an economic tree crop instead of annual crops.

Keywords: *Litsea cubeca*, chemical composition, variation, citral, GC/MS

INTRODUCTION

Litsea cubeba (Lour.) Pers., an aromatic evergreen tree in the family Lauraceae, is widely distributed naturally in montane forest from East Asia to Southeast Asia (Nor Azah and Susiarti, 1999). It is native to China, Indonesia and some other parts of Southeast Asia, where it occurs mainly in mountainous regions. Smitinand (1980) listed botanical and vernacular names of 19 species of *Litsea* in Thailand and the Forest Herbarium, Department of National Parks Wildlife and Plant Conservation reported 37 species of *Litsea* in the revised edition 2014 of the same publication. Ngernsaengsaruay *et al*. (2011) reported that *L. cubeba* is a small-to-medium-sized, dioecious tree, 4-15 m tall. Huang *et al.*(2008) reported on the variety of the species in the flora of China, namely, *cubeba* and *formosana*. Phupan *et al.* (2002) also reported that the two varieties are found in northern Thailand. The dependency on topographical habitat of 17 Lauraceae species in the lower montane forest in northern Thailand has been examined. *L. cubeba* can be grouped as a member of species associated with a ridge habitat (Sri-Ngernyuang *et al.*2003). In order to implement genetic resources conservation, Eiadthong (2011) reported on the demographic status of the Laccifera Lacquer Tree (*Gluta laccifera* (Pierre) Ding Hou) in Thailand and noted planting density was low (1-7 stem/ha); thus, this species is currently considered to be “threatened” and it is recommended that basic data on its ecology and geographical distributions should be thoroughly detailed for its sustainable management and utilization in the future. Thus the status of *L. cubeba* is similar to that of the Lacquer Tree.

All parts of *L. cubeba* contain volatile oil. Fruit-derived oil is used commercially. The fruit is rich in valuable volatile oils, namely May Chang essential oil. China is the main distributor of this industrial crop. Volatile oil from leaves and fruit are used for several purposes, for example, anticancer activities (Ho *et al.,*2010), medicinal products such as cardiac arrhythmia, inflammatory mediators and in clinical aromatherapy as an aromatic and fragrance cosmetic as an aromatherapy volatile oil, soap, shampoo and skin lotion. The considerable traits are due to the major components of the volatile oil obtained from *L. cubeba*. Approximately 70% of the composition is *E*-citral (geranial), *Z*-citral (neral) and D-limonene, which are sources of vitamin A for food and cosmetic products. The citral content is the most important indicator of oil quality. The specified minimum value (international standard, ISO) of the citral content is 74%. In Thailand, the properties of volatile oil have been studied for more than a decade. Roongrattanakul *et al.* (2002) developed formulas for a mosquito repellent fluid with 2-6 hours protection and Thawatsin *et al*. (2006) also undertook similar studies. Moreover, its use as a termite repellent (Lin and Yin, 1995), insecticide (Ko *et al*., 2009) and antioxidant activities of *L. cubeba* oil have been reported (Hwang *et al.,* 2005, Trisonthi, 2007 and Puk-uthai, 2008).

The quantity of citral and other compounds in the oil affect its quality and price. Consequently, study on the genetics, quantity and chemical variation of *L. cubeba* oil from different areas is useful to determine the influence of location on the oil quality and quantity.

The purpose of this study was to assess the chemical composition variation of *L. cubeba* in northern Thailand using Gas Chromatography and Mass Spectroscopy (GC/MS) and to evaluate the influence of location on the chemical composition variation of *L. cubeba* oil.

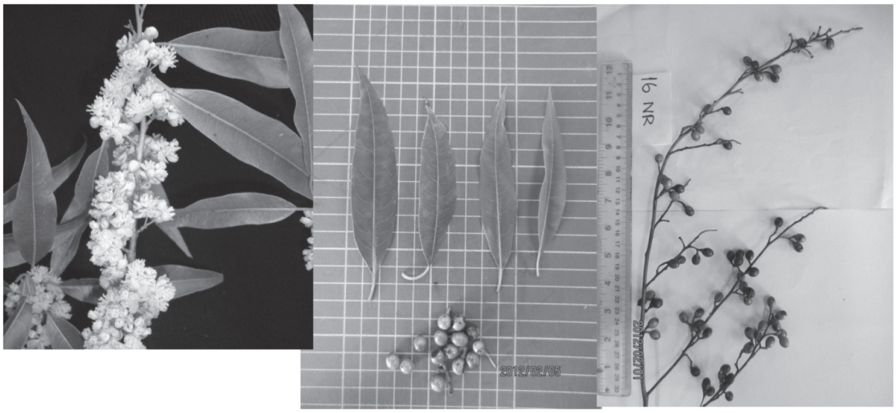

Figure 1 Flower, leaf and fruit samples of *Litsea cubeba* in Thailand.

MATERIALS AND METHODS

The variations in the chemical composition of the volatile oil obtained from the fruit of *Litsea cubeba*, collected from 7 locations were determined using GC/MS analysis. In order to determine the variations in the chemical composition, the comparative yield or peak area percentages of each chemical compound were calculated and compared.

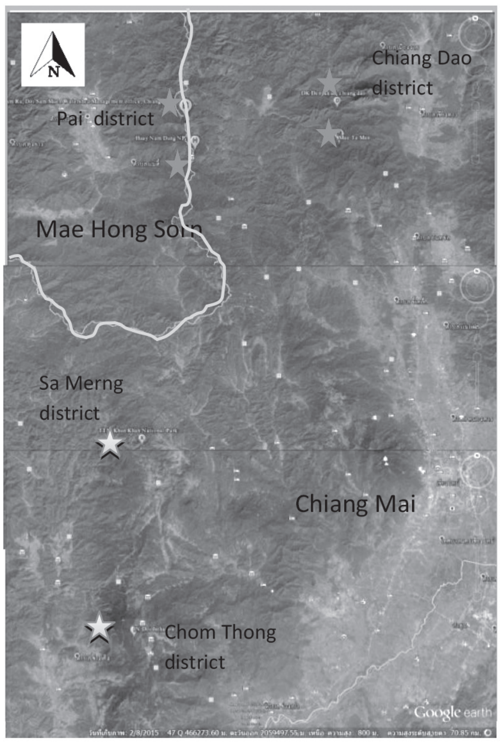

Figure 2 Map of study locations in upper (red) and lower (blue) Chiang Mai provinces.

Fruit parts were collected within a year from 7 locations in northern Thailand-6 locations in Chiang Mai province and 1 location in Phitsanulok province (Figure 1). The details of the locations are described in Table 1 and province locations are shown in Figures 2 and 3. Sample trees were at least 1 km apart and the complete distribution range was covered.

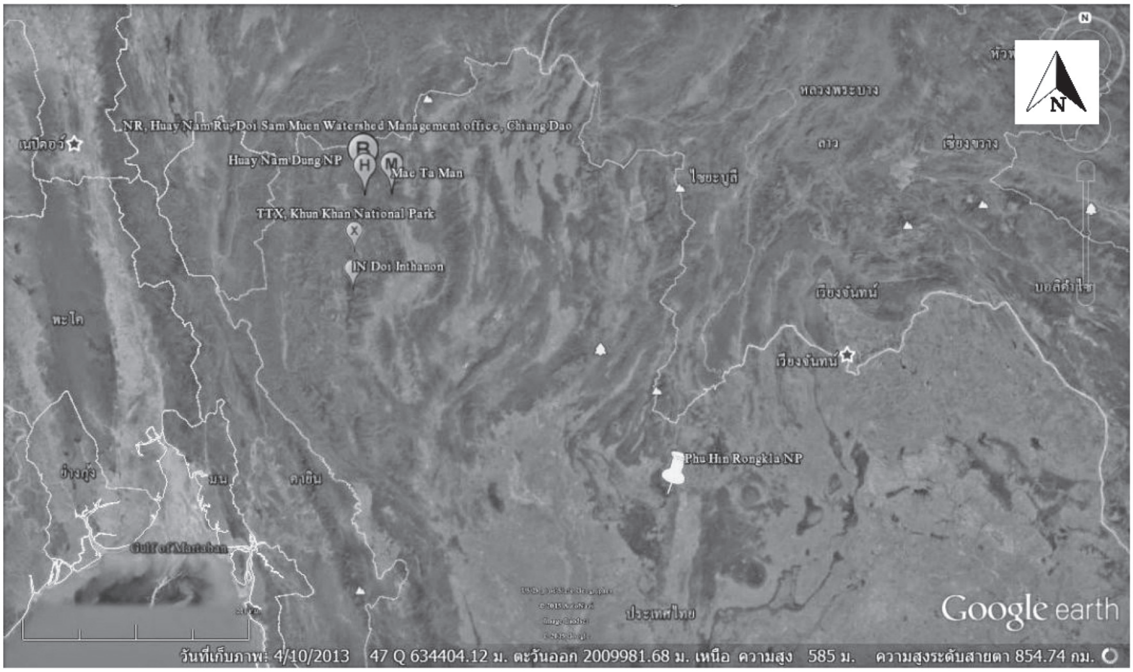

Figure 3 Map of study locations and positions of Chiang Mai (red) and Phitsanulok (yellow) provinces.

Seventy four fruit samples from different trees were collected during the fruiting period (June-September 2014). Fruit of each sample tree was collected separately during the fruiting period (June-September) due to the close relationship of the genetic and chemical components. Fruit samples were transferred to the laboratory under dry conditions and were stored at -60 °C in a freezer for further analysis. The analytical grade standard chemicals and reagents were supplied by Sigma-Aldrich (Thailand).

Air-dried samples of 200 g were extracted using hydro-distillation until no more oil was produced. Then, 1 µl of the oil was mixed with 1.0 ml of propan-2-ol (solvent) in a 2 ml vial before injection for analysis using GC/MS.

Table 1 Details of sample populations of *Litsea* cubeba from seven locations used for GC/MS analysis.

No.	Source of population	Administrative area	Altitude (m)	Symbol	Sample size
1	Khun Khan National Park	Samerng District, Chiang Mai	1,437	TTX	15
2	Huay Nam Dung National Park	Mae Taeng District, Chiang Mai	1,418	T	11
3	Phu Hin Rongkla National Park	Nakhon Thai District, Phitsanulok	1,623	RK	14
4	Huay Nam Ru or Doi Sam Muen Watershed Management Office	Border of Pai and Mae Tang District, Chiang Mai	1,649	NR	7
5	Mae Ta Man Watershed Management Office	Chiang Dao District, Chiang Mai	1,550	MM	10
6	Inthanon National Park	Chom Thong District, Chiang Mai	1, 565	IN	3
7	Den Ya Khud Chiang Dao Wildlife Sanctuary,	Chiang Dao District, Chiang Mai	1,379	DK	14
				Total	74

GC analysis was carried out using a gas chromatograph (Agilent 7890A) and MS analysis was carried out using a mass spectrometer (Agilent 5975 C), equipped with a DB-5MS column (30 m. × 0.25 mm i.d. × 250 µm film thickness) (J&W Scientific, Folsom, CA). The injection volume was 1 µl, the injector temperature was 50 °C and the detector temperature was 270 °C, with a split ratio of 1:50. The oven program consisted of: 50 °C for 5 min, then 5 °C/min to 80 °C for 2 min, then 10 °C to 140 °C for 0 min, then 40 °C/min to 270 °C for 2 min, and the run time was 19.75 min. Helium was used as the carrier gas at a pressure of 9.2821 psi at a flow rate of 1.16 ml/min and an average velocity of 36.595 cm /sec. Effluent from the GC column was introduced directly into the source of the MS via a transfer line (290 °C). Ionization was obtained by electron impact (70eV, source temperature 230 °C). The scan range was 50-550 amu. The compounds were tentatively identified by comparison of the mass spectra of each peak with those of authentic samples in the NIST08.L MS library. From the GC/MS analysis, the yields of chemical components or peak area percentages were reported automatically

Statistical analysis using descriptive statistics (mean value, minimum, maximum value, standard deviation and number of samples) was performed for each chemical character for different locations. The significance of different amounts of each component between locations was tested using analysis of variance (ANOVA) in the SPSS (2005) statistical software package, Version 13.0. and was represented by critical values from F-values and statistical significance (p). ANOVA was performed on the yield (%) of each chemical composition by sample location. The means of those yields (%) were plotted using the Excel package in Windows 97.

RESULTS AND DISCUSSION

For quantitative testing, the average peak area percentages based on retention time were calculated. The overall mean proportions of chemical components are illustrated in Figure 4. The differentiation of the main components between the 7 locations are shown in Figure 5(a-g).

Statistical analyses of the chemical contents of the 74 volatile oil sample of the complete dataset indicated mean values (range in parentheses) of the percentage peak area of 12 main compounds as citral 70.95% being for geranial + neral, with geranial or *E*-citral 39.15% (18.25-49.82%), neral or *Z*-citral 31.80% (14.87-40.52%), D-limonene 3.59% (0.42-15.32%), linalool 1.71% (0.64-11.77%), pulegone 1.39% (0.60-1.89%), citronella 1.32% (0.45-4.76%) 6-methyl 5 hepten -2- one 0.81% (0.19-6.73%), β-myrcene 0.63% (0.08-1.63%), sabinene 0.61% (0.05-4.92%), eucalyptol 0.46% (0.09-2.99%), geraniol 0.39% (0.13-1.24%), α- pinene 0.31% (0.08-0.91%) and caryophyllene 0.29% (0.05-0.63%) (Table 2 and Figure 4).

The mean and standard deviation values of the percentage peak area of the main compounds in the oils derived from each sample at the selected locations are shown in Table 2.

The oil from Den Ya Khud (DK), Chiang Mai location (Figure 5 g) represented the highest average percentage peak area of both geranial and neral as equal to 47.27 and 38.46%, respectively, followed by oil from Phu Hin Rongkla (RK), Phitsanulok equal to 43.98 and 36.22%, respectively (Table 2).

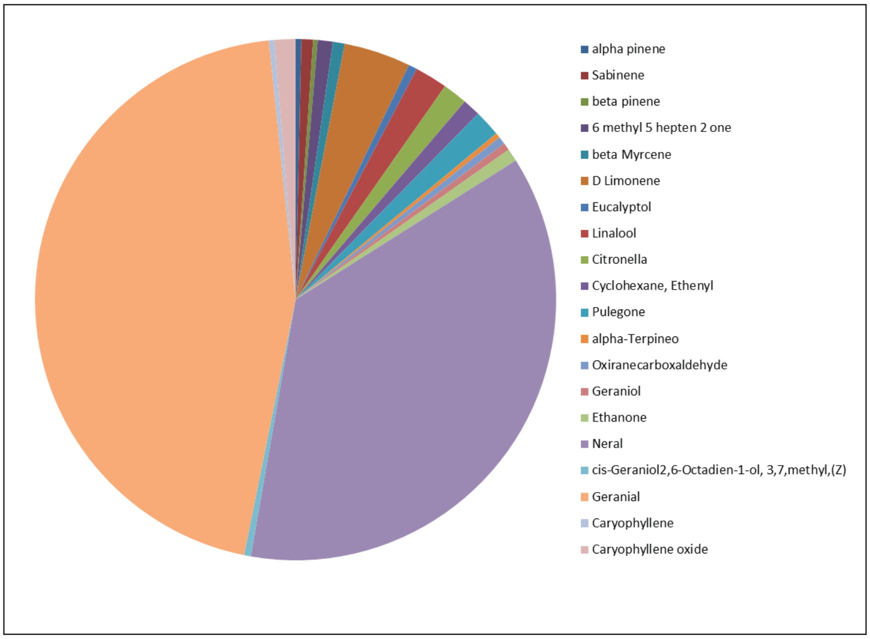

Figure 4 Overall mean main constituents composition of volatile oil of *Litsea cubeba* from seven locations in upper and lower northern Thailand.

The Doi Inthanon sample produced *L.* cubeba fruits containing an outstanding amount of D-limonene (Figure 5), while the other habitats showed not significant differences in the amount. In addition, Doi Inthanon (IN) contained not only high D-limonene but also linalool, 6 methyl 5 hepten 2 one, β- mercene, β-pinene and a-pinene.

The comparative peak area percentages of each chemical component for the 7 locations of *L .cubeba* are shown in Figure 5(a-g).

Figure 5 Chemical composition of volatile oil of *L.cubeba* fruit from 7 locations.

Table 2 Mean percentage peak area of chemical compounds obtained from volatile oil of *L. cubeba* fruit from seven different locations.

Location	Mean peak area (%)								
	Khun Khan NP	Huay Nam Dung NP	Phu Hin Rongkla NP	Bann Nam Ru	Mae Ta Mann	Inthanon NP	Den Ya Khad		
Chemical compound	**TTX**	**T**	**RK**	**NR**	**MM**	**IN**	**DK**	**Overall mean**	**p-values**
α Pinene	0.226^{b}	0.387^{b}	0.329^{b}	0.170^{b}	0.138^{b}	0.677^{a}	0.315^{b}	0.305	0.001
SD/(Num. of tree)	0.138(8)	0.267(11)	0.189(14)	0.042(2)	0.067(9)	0.178(3)	0.140(6)	0.212(53)	
Sabinene	0.391^{bc}	1.371^{a}	0.948^{ab}	0.174^{c}	0.213^{c}	0.557^{bc}	0.364^{bc}	0.612	0.000
SD/(Num. of tree)	0.345(15)	1.276(11)	0.463(14)	0.078(7)	0.194(10)	0.073(3)	0.344(10)	0.706(70)	
β Pinene	0.159^{d}	0.406^{ab}	0.311^{bc}	0.120^{d}	0.119^{d}	0.460^{a}	0.177^{cd}	0.242	0.000
SD/(Num. of tree)	0.092(14)	0.222(11)	0.101(14)	0.052(3)	0.053(10)	0.111(3)	0.120(10)	8.308(65)	
6 Methyl 5 hepten 2 one	0.423^{c}	1.060^{bc}	1.1764^{b}	0.415^{c}	0.556^{bc}	3.7533^{a}	0.414^{c}	0.811	0.000
SD/(Num. of tree)	0.224(15)	0.478(11)	0.552(14)	0.141(7)	0.193(10)	2.908(3)	0.204(14)	0.904(74)	
β Myrcene	0.313^{d}	0.824^{b}	0.775^{bc}	0.294^{d}	0.508^{cd}	1.363^{a}	0.757^{bc}	0.628	0.000
SD/(Num. of tree)	0.292(15)	0.380(11)	0.281(14)	0.126(7)	0.091(10)	0.231(3)	0.227(14)	0.363(74)	
D Limonene	2.880^{c}	6.135^{b}	3.167^{c}	1.568^{c}	2.318^{c}	9.023^{a}	3.361^{c}	3.585	0.000
SD/(Num. of tree)	1.496 (15)	4.856(11)	1.296(14)	1.099(6)	1.102(10)	2.606(3)	1.494(14)	2.788(73)	
Eucalyptol	0.405^{b}	0.757^{a}	0.919^{a}	0.167^{b}	0.222^{b}	0.280^{b}	0.189^{b}	0.462	0.000
SD/(Num. of tree)	0.174 (15)	0..438 (11)	0.630 (14)	0.057(7)	0.147 (10)	0.026 (3)	0.123(14)	0.440 (74)	
Linalool	1.209^{b}	2.176^{b}	2.229^{b}	1.284^{b}	1.288^{b}	4.709^{a}	1.389^{b}	1.708	0.000
SD/(Num. of tree)	0.300 (15)	0.873 (11)	0.663 (14)	0.319 (7)	0.181 (10)	6.113 (3)	0.124(14)	1.37 (74)	
Citronellal	0.923^{c}	1.830^{ab}	1.648^{abc}	1.200^{bc}	1.038^{bc}	2.403^{a}	1.034^{bc}	1.318	0.002
SD/(Num. of tree)	0.350 (15)	1.017 (11)	1.194 (14)	0.602 (7)	0.381 (10)	0.381 (3)	0.220(14)	0.805 (74)	
Cyclohexene	0.616^{cd}	0.726^{bcd}	1.131^{a}	0.778^{abc}	1.024^{ab}	0.443^{d}	1.220^{a}	0.918	0.000
SD/(Num. of tree)	0.285 (15)	0.201 (11)	0.470 (14)	0.427 (8)	0.328 (10)	0.143 (3)	0.150(14)	0.384 (75)	
Pulegone	1.051^{b}	1.080^{b}	1.738^{a}	1.099^{b}	1.548^{a}	0.603^{c}	1.890^{a}	1.388	0.000
SD/(Num. of tree)	0.399 (15)	0.268 (11)	0.699 (12)	0.383 (7)	0.538 (10)	0.150 (3)	0.225(14)	0.569 (72)	

Table 2 (Continued)

Location	Mean peak area (%)								
	Khun Khan NP	Huay Nam Dung NP	Phu Hin Rongkla NP	Bann Nam Ru	Mae Ta Mann	Inthanon NP	Den Ya Khad		
Chemical compound	**TTX**	**T**	**RK**	**NR**	**MM**	**IN**	**DK**	**Overall mean**	**p-values**
α Terpineo	0.211^{bc}	0.312^{b}	0.506^{a}	0.190^{bc}	0.149^{c}	0.143^{c}	0.179^{c}	0.261	0.000
SD/(Num. of tree)	0.064 (15)	0.141(11)	0.210 (14)	0.046 (8)	0.030 (10)	0.015 (3)	0.079(14)	0.170 (75)	
Oxaranecarboxaldehyde	0.478	0.809	0.301	0.180	0.261	n/a	0.273	0.406	0.000
SD (Num. of tree)	0.2100 (12)	0.5735 (11)	0.0770(11)	0.0869 (6)	0.1673 (8)		0.119 (12)	0.341(60)	
Geraniol	0.315^{bc}	0.579^{a}	0.400^{abc}	0.424^{abc}	0.212^{c}	0.510^{ab}	0.466^{ab}	0.398	0.005
SD (Num. of tree)	0.1569 (15)	0.3780 (11)	0.0766(13)	0.162 (8)	0.06996 (9)	0.0360 (3)	0.1958 (5)	0.220(64)	
Ethanone	0.576	1.415	0.400	0.273	0.520	n/a	0.531	0.678	0.001
SD (Num. of tree)	0.2378 (11)	1.042 (10)	0.1283(4)	0.1866 (6)	0.2719 (7)		0.214(11)	0.624(49)	
Neral	26.690^{cd}	24.672^{d}	36.224^{ab}	31.347^{bc}	33.679^{ab}	26.590^{cd}	38.464^{a}	31.802	0.000
SD/(Num. of tree)	8.248 (15)	5.743(11)	0.969(14)	6.162(7)	2.619(10)	4.273(3)	1.178(14)	7.025(74)	
Cis geraniol	0.266	0.090	0.366	0.496	0.359	0.270	0.410	0.357	0.067
SD/(Num. of tree)	0.194 (12)	- (1)	0.103 (11)	0.208 (7)	0.152 (7)	0.086 (3)	0.177 (10)	0.178(51)	
Geranial	33.495^{cd}	31.812^{d}	43.980^{ab}	39.223^{bc}	39.524^{bc}	32.453^{d}	47.269^{a}	39.148	0.000
SD/ (Num. of tree)	10.747(15)	6.469(11)	1.901(14)	7.325(7)	2.165(10)	3.738(3)	2.874(14)	8.308(74)	
Caryophyllene	0.123^{c}	0.283^{b}	0.495^{a}	0.207^{bc}	0.204^{bc}	0.310^{b}	0.274^{b}	0.2898	0.000
SD/(Num. of tree)	0.031 (8)	0.117 (9)	0.102 (13)	0.055 (3)	0.090 (10)	0.191 (3)	0.077 (14)	0.019(60)	
Caryophyllene oxide	0.730	2.867	0.370	0.330	n/a	n/a	0.321	1.114	0.000
SD/(Num. of tree)	0.520 (3)	1.074 (9)	0.107 (9)	0.042 (2)			0.078 (8)	1.281(31)	

Remarks: Mean values in the same row noted by same superscript letter are not significantly different at $p<0.05$ according to Duncan's New Multiple Range Test (DMRT).

The oil derived from fruit in the Chiang Dao district was rich in citral (Figure 5 d, Figure 5 e and Figure 5 g) while the oils derived from Doi Huay Nam Dung (T) (Figure 5 b) and from two locations in lower Chiang Mai, Khun Khan (TTX), Samerng district (Figure 5 a) and Doi Inthanon (IN), Chom Thong district (Figure 5 f) were rich in D limonene and also linalool. The oil derived from Phu Hin Rong Kla fruit (RK) was the only source from Phitsanulok and it produced a moderate amount of almost chemical componeents (Figure 5 c).

Analysis of variance (ANOVA) showed the influence of location factors on variation the chemical composition of *L. cubeba* oil, with the quantity of the 12 constituents in the oil being statistically significant when the location of *L. cubeba* natural source was selected as the factor. The ANOVA results show only whether the means are significantly differ from each other or not. To detemine significant differences from one of a susbet of means, the multiple comparison procedure developed by David B. Duncan in 1955 namely, Duncan's new Multiple Range Test (DMRT) is used. The results of the mean differences in the percentage peak area of each chemical component among the 7 different locations are represented by the superscript letters in Table 2. For all samples of *L. cubeba,* the results showed that *L. cubeba* oil sourced from DK and RK had distinctively higher yields of geranial (47% and 44%, respectively), neral (38.5% and 36 %, respectively) and citral (85% and 80%, respectively), while the average amount of citral in oil from all locations was 70.95% (Table 2). Moreover, IN produced a distinctively higher yield of D-limonene of 9% (compared to the mean value of 3.59%), of 4.7% linalool (mean value of 1.71%), of 3.75% - 6 methyl 5 heptane 2 one (mean value of 0.81%) and of 0.68% a-Pinene (mean value of 0.31%) as described in Figure 4. This distinguishing trait of dominant constituents from these three natural sources suggested that genetic improvement could be applied with *Litsea cubeba,* to produce a promising industrial crop with a high quantity of chemical components.

These results could be used in decision making on genetic improvement. The criteria used to determine which individuals should be selected for the specific chemical component combinations and how much net value benefit would be obtained depend on the market prices of the prominent compositions. For example, according to essential oil market prices data from the Sigma-Aldrich company (www.th.aliexpress.com, accessed on March 10, 2015), the linalool price for analytic standard grade (99%) is USD 245/100 ml which is nearly 10 times that of citral (USD 25.8/100 ml). This suggests that if future phenotypic improvement can increase the linalool yield to exceed 7-10% of the total oil, then its net value will be equal to that of citral. However, lavender is the most important plant producing linalool as the main constituent. The amount of linalool obtained from lavender flower essential oil using GC/MS analysis was in the range 23-57% (Shellie *et al.*, 2002). The yield of essential oil from lavender flower ranged from 0.5% to 6.8% (Zheljazkov *et al.*, 2013). In contrast, the yield of oil obtained from *L. cubeba* was very high ranging from 3.66 to 6.0% v/w) obtained from fresh fruit (Tawatsin *et al.,* 2006 and Roonrattanakul *et al.,* 2002) and the linalool produced from *L. cubeba* oil was in the range 1.21% to 4.9%). The comparative results in term of chemical content cost involving these properties could promote *L. cubeba* as a source of linalool as well as citral and D-limonene.

CONCLUSION

Litsea cubeba in northern Thailand showed high commercial production of significant essential oil components, including: citral, D-limonene, linalool, pulegone, citronellal, geraniol and caryophyllene oxide. The studied natural sources of *L. cubeba* that showed dominant amounts of citral were Den Ya Khad and Phu Hin Rongkla. The dominant locations producing high amounts of D-limonene and linalool were Doi Inthanon and Huay Nam Dung. The oils from the Den Ya Khad and Phu Hin Rongkla samples produced high percentages of citral (85% and 80%, respectively) indicating that the phenotype of *L. cubeba* from these two locations could provide a citral content more than six percent greater than the minimum value of the international standard (74%). With different quality characteristics of the oil from *L. cubeba* from different locations, the selection of dominant trees or plus trees of this species is recommended.

Variation in the chemical composition between different locations from this study can be used as an important database in decision making for a gene conservation program and for individual selection. Differentiation of mean values of each component between locations is necessary to inform individual selection.

The fruit samples should be harvested within the same study year as geo-climatic differentiation may influence the amount of each chemical constituent from different types and different locations.

In Thailand, there is still only limited knowledge on how to develop *L. cubeba* into a commercial crop using intensive mass propagation. Study on the culturing technology of *L. cubeba* seedlings is necessary. Furthermore, the prominent resources of breeding populations should be conserved and *in situ* conservation areas are recommended for all the natural distribution areas of *L. cubeba* in northern Thailand as soon as possible.

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