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Chemotaxonomy of Indonesian *Citrus maxima* based on Leaf Essential Oils

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Abstract: Pummelo (*Citrus maxima*) is one of true citrus species originated from South East Asian regions. Pummelo is known as having high morphological diversity, but lacking of comprehensive publications documenting chemical diversity. The objectives of this study were to reveal essential oil diversity and its implication on the chemotaxonomy of the species. Leaves from forty individual plants were collected from seven districts representing pummelo distribution areas in Indonesia. Isolation of essential oils was done using maceration and solvent extraction procedures. Qualitative and quantitative analysis of the essential oils was conducted using GC-MS. Thirty one compounds were identified, consisted of monoterpenes, sesquiterpenes, diterpenes, ketones, alkenes and fatty acids. Assessment on chemotaxonomical importance of essential oils was carried out using cluster analysis and principal component analysis on volatile compounds. Three chemotypes were recognized, defined as "nerol/loliolide/allo-aromadendrenechemotype", "alpha-selinenechemotype" and "alpha-pinene/delta-carenechemotype". Three chemotypes defined in this study was the first reported chemotaxonomic analysis for Indonesian *C. maxima.*

Keywords: Chemotaxonomy, Essential Oils, Phytochemistry, *Citrus maxima*

Introduction

Citrus maxima (Burm.)Merr.,or pummelo, is a fruit crop species originated from Southeast Asian region. From taxonomical perspective, *C.maxima* is one of "true Citrus species" within the genus *Citrus*. This taxonomic status has been proven from various studies based molecular data (Pang *et al*., 2007; Kyndt *et al*., 2010). *C. maxima* shows morphological variability and recent study indicated that its variability lead to the recognition of infraspecific grouping (Susandarini *et al*., 2013). However, there is no chemotaxonomical study focusing on the essential oil variability on this species.

Essential oils are one of the phytochemical compounds used in chemotaxonomic studies (Gonzales *et al*., 2001; Merle *et al*., 2004). Reserach on *Citrus* essential oils are generally conducted in relation to their aromatic properties applied in food industry, cosmetics and perfume (Sawamura *et al*., 2001; Gogorcena and Ortiz, 2006; Tao *et al*., 2008). Several studies revealed the use of essential oils from various plant species as food preservatives, antimicrobial agent and natural pesticides. The potential of essential oils as food preservatives has been reported by Romeo *et al*. (2010) who studied the antibacterial properties of essential oils from *Lippiacitriodora, Cupressussempervirens and Melissa officinalis* against *Listeria* strain. Meanwhile, the application of essential oils in pest management has been noted by Campolo *et al*. (2014) who reported that a mixture of kaolin and *Citrus sinensis* essential oil might be an alternative chemical pesticide for durum wheat. Regarding to the potential of essential oils as natural pesticides, Ziaee *et al*. (2014) investigated the effect of microencapsulation of *Cuminum cyminum* essential oil on its persistence and insecticidal efficiency againts bettle pests.

Essential oils are generally produced in special glands or secretory tissues and may differ considerably among various plant organs (Nagegowda and Dudareva, 2007). The occurrence of essential oils could be species-specific in terms of their accumulating cells, tissues and organs within particular plant species. Previous studies showed that chemical composition of essential oils on some *Citrus* species were species-specific, or even

cultivar-specific (Gonzales *et al*., 2001; Merle *et al*., 2004; Tao *et al*., 2008).

Essential oil compositions from peel and leaves of various *Citrus* species have been studied for their role as chemical markers for distinguishing taxa at species. Tomi *et al*. (2008) reported the use of essential oils in determining hybrid status by comparing oil profiles between hybrid plants and their putative parents. Previous studies on the use of essential oils for clarifying taxonomic status and hybrid identification on *Citrus* have been reported (Sawamura *et al*., 2001; Gogorcena and Ortiz, 2006; Tao *et al*., 2008). In this study, the variability of essential oil composition among 40 samples of Indonesian *C. maxima* cultivars and landraces was examined along with their taxonomic implication in defining infraspecific grouping within the species.

Materials and Methods

Plant materials for this study were collected from 7 districts in 3 different islands representing pummelo's distribution areas in Indonesia. A total of 40 samples were obtained, consisted of both registered cultivars and landraces. Fully developed leaves from healthy individual trees were washed using water and wiped with 70% ethanol prior to extraction procedure to ensure that the samples were free of contaminations. Plant samples used in this study was listed in Table 1.

Extraction of essential oils was performed using modified maceration and solvent extraction procedure adopted from Merle *et al.* (2004). Two grams o fresh leaves were manually ground into powder. A volume of 3 mL petroleum benzene was added into leaf powder and after setled down for 10 min the mixture was centrifuged at 12,000 rpm for 10 min. The resulted solution was then poured into glass tube. The left over debris was treated with the same procedure for 2 more times. The bulk of solutions collected from three extraction procedures was air-dried for 24 h. Sample dilution using 500 µL of petroleum benzene was done right before injecting oil samples to the GC.

GC-MS analysis of essential oil was carried out using GC HP5890 Series II and Mass Selective Detector HP5972 Series, fitted with HP-5MS 5%-phenyl methyl silicone capillary column (30 m ×25 mm ×0,25 µm). The analysis was performed using splitless method and run with the following program: injection volume 1 µL, injector temperature at 250°C, MS interface temperature at 280°C, initial temperature at 80°C, initial time 1 min, increment rate of 10°C/min, final temperature at 220°C and final time 16 min. Identification of essential oil compounds was carried out by matching mass spectral fragmentation patterns with those from GC-MS data systems and spectral WILEY library.

Data Analysis

The data used for subsequent numerical taxonomy analysis were composition of essential oil compounds and their relative amounts among samples. Calculation of relative amounts for each compound was done on the basis of peak-area ratios. Taxonomic similarities among samples were formulated using Gower's general similarity coefficient. The resulting similarity matrix was then subjected to cluster analysis using Unweighted Pair-Group Method using Arithmetic averages (UPGMA). Principal component analysis was then employed to discern taxonomic value of each compound in determining the grouping of samples. Cluster analysis and principal component analysis were performed using Multivariate Statistical Program version 3.1 (Kovach, 2007).

Table 1. Plant samples used in essential oil analysis

No.	Sample code	Collection site (District)	No.	Sample code	Collection site (District)
1.	ACH-1	Bireuen	21.	JGY-10	Gunungkidul
2.	ACH-2	Bireuen	22.	JGY-12	Gunungkidul
3.	ACH-4	Bireuen	23.	JGY-13	Gunungkidul
4.	ACH-5	Bireuen	24.	JGY-14	Bantul
5.	ACH-7	Bireuen	25.	JGY-15	Bantul
6.	ACH-8	Bireuen	26.	JTM-1	Magetan
7.	JTG-1	Karanganyar	27.	JTM-2	Magetan
8.	JTG-3	Karanganyar	28.	JTM-6	Magetan
9.	JTG-5	Karanganyar	29.	JTM-8	Magetan
10.	JTG-6	Karanganyar	30.	JTM-10	Magetan
11.	JTG-7	Kudus	31.	JTM-12	Magetan
12.	JTG-8	Kudus	32.	SLW-4	Pangkep
13.	JGY-1	Gunungkidul	33.	SLW-5	Pangkep
14.	JGY-2	Gunungkidul	34.	SLW-6	Pangkep
15.	JGY-3	Gunungkidul	35.	SLW-7	Pangkep
16.	JGY-4	Gunungkidul	36.	SLW-9	Pangkep
17.	JGY-5	Gunungkidul	37.	SLW-10	Pangkep
18.	JGY-6	Gunungkidul	38.	SLW-11	Pangkep
19.	JGY-7	Gunungkidul	39.	SLW-13	Pangkep
20.	JGY-8	Gunungkidul	40.	SLW-14	Pangkep

Results

A total of 40 samples were collected for leaf essential oils analysis. The resulting chromatogram profiles from GC-MS analysis showed considerable variations. Results of compound identification showed that leaf essential oils of *C. maxima* consisted of 31 compounds. Determination of compound was based on the matching of mass spectral patterns with the minimum similarity of 85%. The 31 identified compounds were comprised of monoterpenes, sesquiterpenes, diterpenes, ketons, alkanes and fatty acids (Table 2).

Qualitative comparison among three groups of terpenes showed that sesquiterpenes had the highest proportion. Meanwhile, in terms of relative amount, phytol was the found to be a compound with the highest percentage. From the proportion of six compound groups found in leaf essential oil of *C. maxima*, it was obvious that monoterpenes and sesquiterpenes were dominant.

In this study only volatile components of essential oils were defined as target compound, primarily due to their biological significance as bioactive compound with various medicinal properties. It was therefore, terpenes became groups of target compound and thus were used in the numerical taxonomic analysis for determining the existence of chemotypes within *C. maxima*. The determination of the chemotypes was based on the taxonomic affinity of cultivars and landraces. A dendrogram resulted from cluster analysis of 21 volatile compounds figuring taxonomic affinity among 40 *C. maxima* samples showed two major clusters (Fig. 1). The first cluster, designated as cluster A, consisted of 34 samples whereas the second cluster assigned as cluster B comprised of 6 samples. The first cluster could be divided into two subclusters, A1 and A2.

Table 2. Composition of essential oil compounds from *C. maxima* leaves

No.	Compound	RI*	Percentage**
1.	*alpha-pinene*	982	0.53-1.54
2.	*trans-isolimonene*	983	0.23-0.77
3.	*delta-carene*	1004	0.45-1.32
4.	*trans-ocimene*	1097	0.20-0.53
5.	*nerol*	1228	0.78-3.12
6.	*citronellol*	1229	1.03-3.71
7.	*beta-caryophyllene*	1420	1.25-5.24
8.	*calarene*	1431	0.38-1.23
9.	*alpha-humulene*	1453	0.39-1.53
10.	*patchoulene*	1457	0.32-1.57
11.	*allo-aromadendrene*	1459	0.44-1.45
12.	*germacrene D*	1475	0.12-0.44
13.	*beta-ionone*	1485	0.68-2.28
14.	*alpha-selinene*	1493	1.55-3.70
15.	*alpha-farnesene*	1496	0.21-1.12
16.	*delta-cadinene*	1523	0.48-1.85
17.	*nerolidol*	1560	1.57-4.89
18.	*lauric acid*	1571	0.13-0.63
19.	*caryophyllene oxide*	1580	0.89-4.46
20.	*spathulenol*	1640	8.88-20.49
21.	*heptadecane*	1700	0.25-0.40
22.	*myristic acid*	1767	0.51-0.97
23.	*loliolide*	1772	0.54-1.24
24.	*neophytadiene*	1842	0.90-3.20
25.	*hexadecanoic acid, methyl ester*	1926	0.42-1.30
26.	*palmitic acid*	1968	2.56-4.37
27.	*margaric acid*	2022	0.32-0.92
28.	*phytol*	2116	15.44-35.71
29.	*ethyl linoleolate*	2159	1.11-1.77
30.	*stearic acid*	2178	1.29-1.93
31.	*9,12,15-octadecatrienoic acid, methyl ester*	2186	0.48-0.83

RI*: Retention indices reported in the literature
Percentage**: Range of percent composition calculated from peak-area ratios

Table 3. Eigen values of 21 volatile compounds in *C. maxima* leaf essential oils

		Eigen value		
Compound code	Volatile compound	PC 1	PC 2	PC 3
C1	*alpha-pinene*	-0.072	0.245	0.023
C2	*delta-carene*	-0.129	0.085	-0.106
C3	*trans-isolimonene*	0.046	0.033	0.226
C4	*beta-ocimene*	0.002	0.034	0.039
C5	*nerol*	0.372	0.028	-0.046
C6	*citronellol*	0.024	-0.094	-0.033
C7	*loliolide*	0.238	-0.581	0.153
C8	*beta-caryophyllene*	0.345	0.404	0.054
C9	*alpha-humulene*	0.146	-0.093	0.101
C10	*germacrene*	0.032	0.021	-0.106
C11	*alpha-selinene*	0.258	0.351	-0.087
C12	*alpha-farnesene*	0.261	0.065	0.098
C13	*delta-cadinene*	0.327	0.205	-0.083
C14	*calarene*	0.025	0.088	-0.059
C15	*patchoulane*	0.155	0.181	0.181
C16	*allo-aromadendrene*	0.311	-0.348	-0.079
C17	*spathulenol*	0.073	0.028	-0.051
C18	*caryophyllene oxide*	0.157	0.197	0.036
C19	*nerolidol*	0.351	-0.168	-0.700
C20	*neophytadiene*	0.349	-0.115	0.557
C21	*phytol*	0.006	-0.015	0.123

Note: numbers in bold type indicate prominent taxonomic value

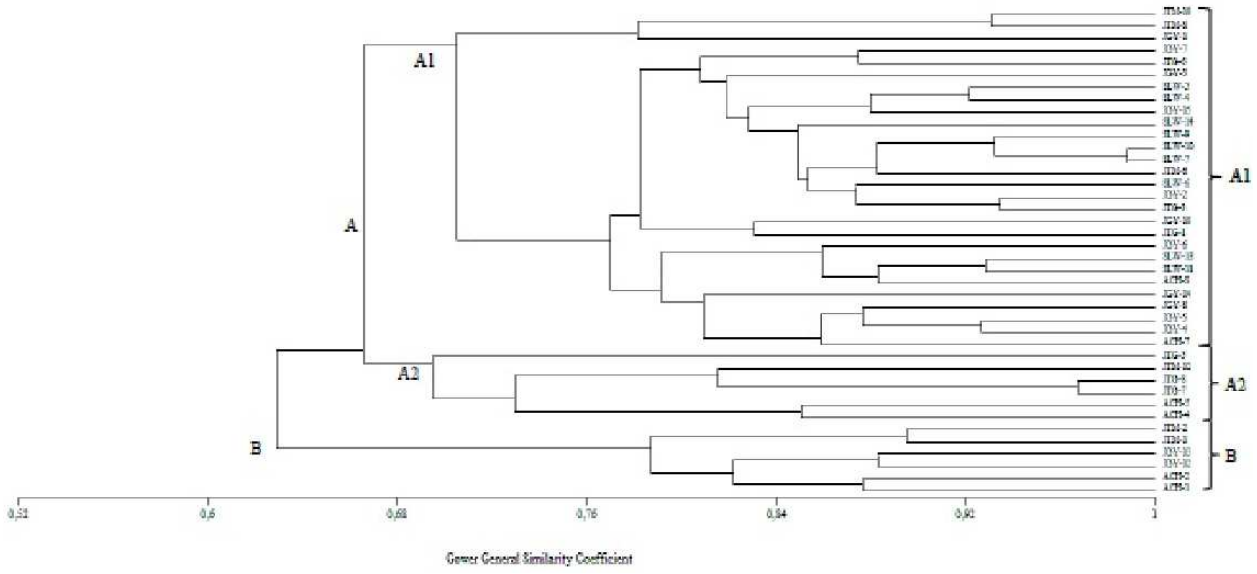

Fig. 1. Dendrogram showing taxonomic affinity of 40 *C. maxima* samples based on leaf volatile compounds

Dendrogram topology with bifurcating pattern formed at similarity value of 0.624 indicated that cluster A and B had distinctive characteristics that clearly differentiate between them. In this regards, the volatile compounds differentiated those two clusters were categorized as taxonomic distinguishing characters. These characters could be identified by observing the eigen value from three Principal Components (PCs) resulted from principal component analysis (Table 3). The percentage of explained variance on the first principal component were 29.94, while the second principal component explained

49.95% of total varienace. A biplot of PCA results (Fig. 2) showed distribution patterns of samples in two dimentional graph as well as represented relative contribution of each essential oil compunds in differentiating samples into groups of chemotypes.

Criteria for particular compound to be categorized as distinguishing character adopted in this study, or herewith as chemical marker, was at least having eigen value of 0.2 (absolute value). Based on the eigen values, five volatile compounds were found to be differentiating characters as indicated by their eigen values. These compounds were nerol, loliolide, allo- aromadendrene, nerolidol and neophytadiene. They represented five major compounds separating cluster A from B and thus considered as distinguishing chemical characters. Referring to the combining results of cluster analysis and principal analysis, it was noticeably recognized the existence of three groups representing three chemotypes within *C. maxima*, defined here as subcluster A1, subcluster A2 and the smaller cluster B. Since the recognition of chemotypes should be based on chemical markers, then the eigen value from principal component analysis was used for this purpose.

Observation on the results of cluster analysis and principal component analysis indicated that cluster B could be denoted as "nerol/loliolide/allo-aromadendrene" chemotype (chemotype I). Subcluster A2 was defined as "alpha-selinene" chemotype (chemotype II), whereas subcluster A1 which showed the most diverse members was identified as "alpha pinene/delta carene" chemotype (chemotype III). The grouping of samples into three chemotypes was summarized in Table 4.

Table 4. The grouping of *C. maxima* samples into three chemotypes

Chemotype grouping	Typical compound	Chemotype members (sample code)
Chemotype I	• *nerol* • *loliolode* • *allo-aromadendrene*	ACH-1, ACH-2, JGY-12, JGY-13, JTM-1, JTM-2
Chemotype II	• *alpha-selinene*	ACH-4, ACH-5, JTG-7, JTG-8, JTG-5
Chemotype III	• *alpha-pinene* • *delta-carene*	ACH-7, JGY-4, JGY-5, JGY-8, JGY-14, ACH-8, SLW-11, SLW-13, JGY-6, JTG-1, JGY-10, JTG-3, JGY-2, SLW-6, JTM-6, SLW-7, SLW-10, SLW-9, SLW-14, JGY-15, SLW-4, SLW-5, JGY-3, JTG-6, JGY-7, JGY-1, JTM-8, JTM-10

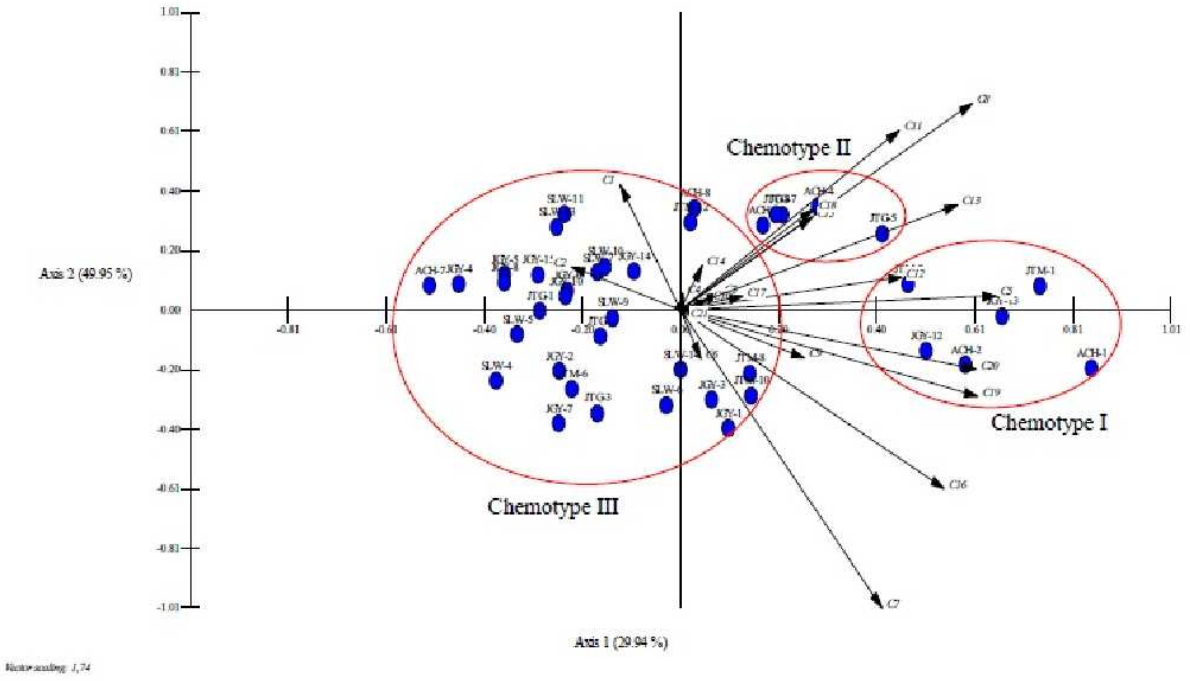

Fig. 2. Biplot graph of PCA results showing the gruping of samples into three chemotypes

Discussion

Variations on the GC-MS profiles of essential oils extracted from *C. maxima* leaves indicated infraspecific variability of the species. The observed variability was due to differences in the composition of essential oil compounds as well as their relative amount among individual samples. Differences in the composition of essential oil within plant species are common. López *et al.* (2009) pointed out that composition of essential oil may vary due to differences on geographical origin, altitude, infraspecific variation, soil type, nutrient status of the plants and phenology.

The composition of essential oils which comprised of monoterpenes, sesquiterpenes, diterpenes, ketons, alkanes and fatty acids was in accordance to Mondello *et al*. (2005) who reported that *Citrus* essential oils had two components, the volatile and non volatile compounds. The volatile compounds were mainly consisted of monoterpenes and sesquiterpenes with their oxygenated derivates, whereas the non volatile compounds were aliphatic hydrocarbons, aldehydes, ketons, acids and esters. Regarding on the number of compounds, Bakkali *et al*. (2008) noted that plant essential oils generally consisted of 20 to 60 components with various concentrations. Quantitaive analysis of various compound groups indicated that monoterpenes and sesquiterpenes were dominant. This result was similar to the composition of essential oils from *Citrus nobilis*, in which monoterpenes and sesquiterpenes were dominant (Liu *et al*., 2013). Similar report was found on the essential oils of *Citrus limetta*, with sesquiterpenes being the most abundant compared to other compounds (Colecio-Juárez *et al*., 2012).

The grouping of samples resulted from cluster analysis, combined with results of principal component analysis demonstrated the existence of three chemotypes within *C. maxima* based on leaf essential oils. Determination of chemotypes based on the observed variations of essential oil composition has been the subject of discussion by several authors. Grayer *et al*. (1996) suggested a combination of major essential oil components rather than a single and dominant compound as a basis of defining chemotypes. Similar argument was pointed out by Labra *et al*. (2004) by mentioning that chemotype determination should not be based on a single essential oil component since plants generally have two or more major compounds of relatively equal amounts. The use of principal component analysis in determining chemotypes has been suggested by Boushama *et al*. (2006) for *Lavanduladentata*. In the same way, Wheeler *et al*. (2007) used principal component analysis as a basis for examining infraspecific variation and defining chemotypes based on leaf essential oils for *Melaleucaquinquenervia*. From taxonomical perspective, there were criteria for the recognition of chemotype when dealing with variations observable within a species. A chemotype is defined as individuals within a species which have distinctive chemical phenotype differed from the other members of the species (Keefover-Ring *et al*., 2009).

A number of studies on the use of essential oils for describing chemotypes in various *Citrus* species have been reported. In *C. reticulata* three chemotypes were identified based on analysis of leaf essential oils from 58 cultivars. They were recognized as “sabinene/linalool”, “c-terpinene/linalool” and “methyl N-methylanthranilate” chemotypes, respectively (Lota *et al*., 2001). Similarly, analysis on leaf essential oils of *C. limon* resulted in characterization of two chemotypes, whereas four chemotypes were identified on *C. aurantifolia* (Lota *et al*., 2002). Another chemotaxonomic study on *C. reticulata* based on 13 major compounds of essential oils resulted in the classification of cultivar groups known as “sabinene/delta-carene”, “gamma-terpinene” and “sabinene” chemotypes (Kasali *et al*., 2010). These studies indicated the existence of variations on essential oils at species level and their chemotaxonomic applications on genus *Citrus.*

Observation on the grouping of samples into three chemotypes did not correspond to their geographical origin. This result implied that infraspecific variation on leaf essential oils defined as chemotypes in this study indicated the existence of a genetic background. This inference was formulated referring to an argument that genetically-determined chemotypes was indicated when individuals representing populations from different geographical origin with different environmental conditions clustered within one chemotype (De Martino *et al*., 2009; Chauhan *et al*., 2011). Result of this study corresponded to previous chemotaxonomic study on *Citrus* cultivars from various countries which confirmed that instead of affected by geographical origin, variations on essential oils suggested differences in chemotypes (Bhuiyan *et al*., 2009). An important aspect on taxonomic value of essential oil variations within a species was revealed in this study, suggesting the role of essential oils as potential chemical marker in plant classification.

Conclusion

Qualitative and quantitative analysis of leaf essential oils in this study demonstrated the identification of three chemotypes within *C. maxima*. Results of this study, therefore, supported the recognition of essential oils as chemotaxonomic marker. Phytochemical analysis in this study added scientific value of *C. maxima* leaf essential oils, especiallytheir potential use as aromatic components in food, aromatherapy and perfume.

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Author's Contributions

Ratna Susandarini: The first author is the person responsible for conducting research, which covers sample collection, laboratory experiments, data analysis, and manuscript preparation.

Rugayah: The second author provides taxonomic analysis of the results as presented in part of discussion section.

Lauretius Hartanto Nugroho: The third author holds the responsibility in interpreting essential oil data for the evaluation of phy t ochemical aspect in this study.

Siti Subandiyah: The fourth author provides academic consultancy in writing up results of the research and preparing the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

References

Bakkali, F., S. Averbeck, D. Averbeck and M. Idaomar, 2008. Biological effects of essential oils-a review. Food Chem. Toxicol., 46: 446-475. DOI: 10.1016/j.fct.2007.09.106

Bhuiyan, M.N.I., J. Begum, P.K. Sardar and M.S. Rahman, 2009. Constituents of peel and leaf essential oils of *Citrus medica* L. J. Scientific Res., 1: 387-392. DOI: 10.3329/jsr.v1i2.1760

Boushama, L., J.B. Boti, F.A. Bekkara, V. Castola and J. Casanova, 2006. Infraspecific chemical variability of the essential oil of *Lavandula dentata* L. from Algeria. Flavour Fragr. J., 21: 368-372. DOI: 10.1002/ffj.1659

Campolo, O., R.V. Romeo, A. Malacrinò, F. Laudani and G. Carpinteri *et al*., 2014. Effects of inert dusts applied alone and in combination with sweet orange essential oil against *Rhyzopertha dominica* (Coleoptera: Bostrichidae) and wheat microbial population. Industr. Crops Products, 61: 361-369. DOI: 10.1016/j.indcrop.2014.07.028

Chauhan, A., R.S. Verma and R.C. Padalia, 2011. Aroma in plants: Can it serve as a criterion in delimitation of taxa at infraspecific level? Curr. Sci., 101: 489-489.

Colecio-Juárez, M.C., R.E. Rubio-Núñez, J.E. Botello-Álvarez, G.M. Martínez-González and J.L. Navarrete-Bolaños *et al*., 2012. Characterization of volatile compounds in the essential oil of sweet lime (*Citrus limetta*Risso). Chil. J. Agric. Res., 72: 275-280. DOI: 10.4067/S0718-58392012000200017

De Martino, L., V. De Feo, C. Formisano, E. Mignola and F. Senatore, 2009. Chemical Composition and Antimicrobial Activity of the Essential Oils from Three Chemotypes of *Origanum vulgare* L. ssp. *Hirtum* (Link) Ietswaart Growing Wild in Campania (Southern Italy). Molecules, 14: 2735-2746. DOI: 10.3390/molecules14082735

Gogorcena, Y. and J.M. Ortiz, 2006. Characterisation of sour orange (Citrus aurantium) cultivars. J. Sci. Food Agric., 48: 275-284. DOI: 10.1002/jsfa.2740480304

Gonzales, C.N., F. Sanchez, A. Quintero and A. Usubillaga, 2001. Chemotaxonomic value of essential oil compounds in *Citrus* species. ISHS ActaHortic, 576: 49-51.

Grayer, R.J., G.C. Kite, F.J. Goldstone, S.E. Bryan and A. Patont *et al*., 1996. Infraspecific taxonomy and essential oil chemotypes in sweet basil, *Ocimumbasilicum*. Phvtochemistry, 43: 1033-1039. DOI: 10.1016/S0031-9422(96)00429-3

Kasali, A.A., O.A. Lawal, O.T.F. Abanikannda, A.A. Olaniyan and W.N. Setzer, 2010. *Citrus* essential oils of Nigeria part IV: Volatile constituents of leaf oils of mandarins (*Citrus reticulate* Blanco) from Nigeria. Rec. Nat. Prod., 4: 156-162.

Keefover-Ring, K., J.D. Thompson and Y.B. Linhart, 2009. Beyond six scents: Defining a seventh *Thymus vulgaris* chemotype new to southern France by ethanol extraction. Flavour Fragr. J., 24: 117-122. DOI: 10.1002/ffj.1921

Kovach, W.L., 2007. MVSP-a multivariate statistical package, 3.1. Kovach Computing Services, Pentraeth, Wales.

Kyndt, T., T.N. Dung, P. Goetghebeur, H.T. Toan and G. Gheysen, 2010. Analysis of ITS of the rDNA to infer phylogenetic relationships among Vietnamese *Citrus* accessions. Genet. Resour. Crop Evol., 57: 183-192. DOI: 10.1007/s10722-009-9460-0

Labra, M., M. Miele, B. Ledda, F. Grassi and M. Mazzei *et al*., 2004. Morphological characterization, essential oil composition and DNA genotyping of *Ocimum basilicum* L. cultivars. Plant Sci., 167: 725-731. DOI: 10.1016/j.plantsci.2004.04.026

Liu, C., D. Jiang, Y. Cheng, X. Deng and F. Chen *et al*., 2013. Chemotaxonomic study of citrus, poncirus and fortunella genotypes based on peel oil volatile compounds-Deciphering the Genetic Origin of Mangshanyegan (Citrus nobilis Lauriro). PLoS One, 8: e58411-e58411. DOI: 10.1371/journal.pone.0058411

López, M.L., J.S. Dambolena, M.P. Zunino and J.A. Zygadlo, 2009. GC and GC-MS of Terpenoids. In: Isolation, Identification and Characterization of Allelochemicals/Natural Products, Sampietro, D.A., C.A.N. Catalan, M.A. Vattuone and S.S. Narwal (Eds.), Science Publishers, pp: 203-234.

Lota, M., D.R. Serra, F. Tomi and J. Casanova, 2001. Chemical variability of peel and leaf essential oils of 15 species of mandarins. Biochem. Syst. Ecol., 29: 77-104. DOI: 10.1016/S0305-1978(00)00029-6

Lota, M., D.R. Serra, F. Tomi, C. Jacquemond and J. Casanova, 2002. Volatile components of peel and leaf oils of lemon and lime species. J. Agric. Food Chem., 50: 796-805. DOI: 10.1021/jf010924l

Merle, H., M. Morón, M.A. Blázquez and H. Boira, 2004. Taxonomical contribution of essential oils in mandarins cultivars. Biochem. Syst. Ecol., 32: 491-497. DOI: 10.1016/j.bse.2003.09.010

Mondello, L., A. Casilli, P.Q. Tranchida, P. Dugo and G. Dugo, 2005. Comprehensive two-dimensional GC for the analysis of citrus essential oils. Flavour Fragr. J., 20: 136-140. DOI: 10.1002/ffj.1506

Nagegowda, D.A. and N. Dudareva, 2007. Plant Biochemistry and Biotechnology of Flavor Compounds and Essential Oils. In: Medicinal Plant Biotechnology: From Basic Research to Industrial Applications, Kayser, O. and W.J. Quax (Eds.). WILEY-VCH Verlag GmbH and Co. KGaA, Weinheim.

Pang, X.M., C.G. Hu and X.X. Deng, 2007. Phylogenetic relationships within *Citrus* and its related genera as inferred from AFLP markers. Genet. Resour. Crop Evolut., 54: 429-436. DOI: 10.1007/s10722-006-0005-5

Romeo, F.V., S. De Luca, A. Piscopo, E. De Salvo and M. Poiana, 2010. Effect of some essential oils as natural food preservatives on commercial grated carrots. J. Essential Oil Res., 22: 283-287. DOI: 10.1080/10412905.2010.9700325

Sawamura, M., H.S. Song, H.S. Choi, K. Sagawa and H. Ukeda, 2001. Characteristic aroma components of Tosa-buntan (*Citrus grandis* Osbeck forma *Tosa*) fruit. Food Sci. Technol. Res., 7: 45-49. DOI: 10.3136/fstr.7.45

Susandarini, R., S. Subandiyah, Rugayah, B.S. Daryono and L.H. Nugroho, 2013. Assessment of taxonomic affinity of indonesian pummelo (*Citrus maxima* (Burm.) Merr.) based on morphological characters. Am. J. Agric. Biol. Sci., 8: 182-190. DOI: 10.3844/ajabssp.2013.182.190

Tomi, F., M. Barzalona, J. Casanova and F. Luro, 2008. Chemical variability of the leaf oil of 113 hybrids from *Citrus clementina* (Commun) x *Citrus deliciosa* (Willow Leaf). Flavour Fragr. J., 23: 152-163.

Tao, N., Y. Liu, J. Zhang, H. Zeng and Y. Tang *et al*., 2008. Chemical composition of essential oil from the peel of Satsuma Mandarin. Afr. J. Biotechnol., 7: 1261-1264. DOI: 10.5897/AJB08.033

Wheeler, G.S., P.D. Pratt, R.M. Giblin-Davis and K.M. Ordung, 2007. Intraspecific variation of *Melaleuca quinquenervia* leaf oils in its naturalized range in Florida, the Caribbean and Hawaii. Biochem. Syst. Ecol., 35: 489-500. DOI: 10.1016/j.bse.2007.03.007

Ziaee, M., S. Moharramipour and A. Mohsenifar, 2014. MA-chitosan nanogel loaded with *Cuminum cyminum* essential oil for efficient management of two stored product beetle pests. J. Pest Sci., 87: 691-699. DOI: 10.1007/s10340-014-0590-6