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The Potentials of Limau (*Citrus amblycarpa* Hassk. Ochse) as A Functional Food and Ornamental Mini Tree based on Metabolomic and Morphological Approaches

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Abstract

Limau (*Citrus amblycarpa* (Hassk.) Ochse) is one of the native *Citrus* species of West Java that is grown in the local backyard, thus is easily found in local cuisines. Limau has great potentials for use as a potted plant in home gardens for its aesthetic value and fragrance. The objective of this research was to describe the metabolites fingerprint and morphology of Limau as a potential functional and ornamental mini tree. Methanol leaf extracts from one-year-old Limau originated from Bogor were analyzed using untargeted Gas Chromatography Mass Spectrometry (GCMS). Methanol leaf extract was dominated by vitamin E and composed of other 18 metabolites including phytosterols, fatty acids and terpenes. The major portion of fruit extract consisted of 6-octadecenoic acid, palmitate and various fragrance compounds such as alpha sinensal, alpha limonene, beta citronellal, citronellol, and sabinene. Limau fruits are also a functional food due to its rich antioxidant and aromatic content. The morphology of limau tree is suitable as a potted ornamentals or mini-fruits tree for its small crown, sparse branching with unifoliate aromatic leaves and small spines, and plentiful aromatics mini fruits with the diameter of around 2-4 cm.

Keywords: GCMS, Indonesian native citrus, metabolites fingerprinting, mini-fruits tree, vitamin E

Introduction

Citrus is a highly demanded and is one of the economically important horticulture produces in the world (FAO, 2016), including in Indonesia (Pusdatin, 2015). The rise of public income and awareness of a healthy lifestyle are thought to be a trigger for an increasing demand for citrus (Darmawan et al., 2014). Citrus has been selected by the Indonesian government as one of three strategic horticultural commodities to improve economic growth (Dirjenhort, 2016). Various species of citrus are grown in Indonesia. Recently, the Indonesian Citrus and Subtropical Fruit Research Institute (ICSFRI) reported that about 241 citrus genotypes from all around the country have been collected and maintained (Budiyati, 2014). Most of the citrus species is popularly used by consumers as table fruits that can be directly consumed, such as mandarin, tangerine and pummelo. However, there are other citrus species that are more favorable as flavor or ornament rather than table fruit (Lv et al., 2015).

Limau (*Citrus amblycarpa* (Hassk.) Ochse) is one of Indonesia's native citrus, specifically native to West Java, and is well known for flavoring cuisines rather than fresh fruit consumption (Lim, 2012) due to its sour taste and pleasantly fragranced fruit juice. Most Indonesians use the juice to enhance the flavor of local cuisines, particularly for making "sambal" (chilli sauce) which is apparently an origin of Indonesia. In addition to the use of Limau, the dreg of the fruits can be mixed into perfumed hand-washing water after meals, since it is the habit of many locals to eat without using cutlery (Irsyam, 2015).

The fruits are the main attraction of the Limau plant. Healthy trees produce a large number of small (20-40mm) fruits. In addition, Limau has aromatic leaves which have not been widely used; the aromatic leaves are highly potential to be used as a mixture of cooking spices (Setiadi and Pariamin, 2004), flavoring and aromatic oil.

The potentials of Limau as functional food can be evaluated through metabolomic approach. Metabolomic refers to a new field of 'omics' research that uses sophisticated analytical technologies to identify the type, location and amounts of metabolites, mostly in the form of low molecular weight molecules, at specific time or condition inside living organism or its product (Ramsden, 2009; Putri et al., 2013a; Kosmides et al., 2013; Clish, 2015). Metabolomic approach involves comprehensive and simultaneous systematic determination of metabolites at certain time or condition (Hall, 2006). Various metabolomic instruments have been recently developed, such as Nuclear Magnetic Resonance (NMR), Supercritical Fluid Chromatography Mass Spectrometry (SFCMS), Capillary Electrophoresis Mass Spectrometry (CEMS), Liquid Chromatography Mass Spectrometry (LCMS), High Performances Liquid (HPLC) and Gas Chromatography Mass Spectrometry (GCMS) (Putri et al., 2013b). GCMS is highly suitable and is widely used to study low molecular weight and volatile compounds in various types of plants and their derivatives such as coffee (Choi et al., 2010; Jumhawan et al., 2013), potatoes (Uri et al., 2014), clary sage, lavender, lemon and orange (Wu et al., 2014). Untargeted GCMS analysis is feasible to describe metabolites fingerprinting of Limau and thus can be used as early information for further study regarding its potential as a functional food.

In addition to functional purposes, potted Limau tree has the potentials to be commercialized as an ornamental potted plant for its attractive physical properties, its wide adaptability to diverse environment and does not need intensive maintenance (Setiadi and Parimin, 2004). The potentials of Limau for ornamental purposes can be identified based on the morphological approach (Dos Santos et al., 2015). Morphological identification is relatively easy, simple, time- and cost-saving so the conventional botanist were able to apply without using sophisticated laboratory instruments (Dorji and Yapwattanaphun, 2011). Morphological observation is important to describe the plant's morphology at vegetative and generative stages.

Ornamental plant refers to various types of plant that are intentionally planted for decorating rooms, home gardens and landscapes. Most citrus researchers only focus on the yield improvement and continuity of fruit production, with less focus on its potentials as ornamental pot or mini fruit trees. The use of citrus as ornamentals has been developing worldwide, in particular Europe (Del Bosco, 2003). However, apart from kumquat (*Citrus japonica*) there have been limited reports about commercialization of citrus as an ornamental pot plant. This research aimed to evaluate the potentials of Limau as both functional and ornamental plant based on its morphological identification and metabolites fingerprinting approaches.

Materials and Methods

Experimental Site

The experiment started from January until April 2017 at Pasir Kuda experimental farm of Bogor Agricultural University, Bogor, Indonesia. The altitude, latitude and longitude of the location were 263 meters above sea levels, 6°36'32.6" South latitude and 106°47'0.9" East longitude. The daily temperature, relative humidity and monthly rainfall during the experimental period varied from 23-31°C (average 25.8 °C), 82–88% (average 85.9%) and 370-525 mm (average 423 mm) (BMKG, 2017).

Plant Materials

A year-old limau grafted onto Japanese citroen rootstock was collected from the commercial nursery of Bogor Agricultural University, Agropromo, and then transferred to the Pasir Kuda farm. Limau was grown in 30 cm polybags filled with a well-mixed media of soil, compost and rice husk with an equal ratio (by volume). Nitrogen-phosphorus-potassium compound fertilizer at a rate of 20 grams (Yara, Norway), and micronutrients (Growmore, USA) at a rate of 2 grams per pot was applied monthly through soil drench and foliar feeding, respectively. Irrigation was not applied during the experiment due the rainy periods during the experiments.

Scoring

Metabolites fingerprinting was conducted using GCMS instrument at the Laboratory of Ministry of Health Jakarta, Indonesia. The instrument used was an Agilent 7890 gas chromatograph interfaced to a 5975 mass selective detector and equipped with chemstation database system. Samples were leaves or fruits that were harvested from five potted Limau plants. The picked samples from each individual plant were pooled to provide composite samples. Leaf samples were collected from the third to the fifth position on a dormant branch. Fruits were collected at a mature stage prior to ripening because most consumers preferred mature rather than ripe fruits. Samples were collected starting at 9.00 am and were quenched by directly transferred onto cool box containing ice gel and transported to the laboratory for extraction. Ten grams of leaves and fruit samples were weighed, and dried for three days at 60°C. The dried samples were blended prior to maceration with 99.9% ethanol analysis for about five days. Ten mL of macerated extracts were transferred into a tube for subsequent evaporation at 60°C for about an hour. The evaporated samples were redissolved in 200 µl extract prior to injection into GCMS (Labkesda, 2017).

Growth habit, morphology of trunk, leaf, flower, fruit and seeds were scored from five potted Limau plants based on descriptors for citrus (IPGRI, 1999) and plant morphology (Tjitrosoepomo, 2009). Evaluation of limau ornamental potentials was based on morphological features described in Dos Santos et al (2015).

Results and Discussion

Metabolites Fingerprinting of Limau

Early documentation of metabolites is needed to evaluate the potential of Limau plants to provide functional metabolites. Gas chromatography (GC) was served as metabolites separator while mass spectrometer was the metabolites detector. The principle of GC as metabolites separator depends upon the interaction power of metabolites with stationary phase. The weaker the interaction, the earlier the elution of the metabolites and vice versa. The time from injection up to elution was called the retention time (RT). The metabolites were ionized, separated according to their mass-to-charge ratio ($m.z^{-1}$) and detected as ion intensity then identified based on retention time obtained from GC and mass spectrum obtained from MS. Our GCMS was integrated with an online chemstation database that provided metabolites reference for the identification of detected metabolites. Generally, there are two types of GCMS analysis, namely targeted and untargeted techniques.

The present study used untargeted GCMS analysis to elucidate the abundance of functional metabolites within aromatic leaves and fruits of Limau plants. The amount of certain metabolite in the sample, also called as relative concentration (RC), is proportional to its peak area that showed in chromatogram. The peak area (PA) is treated as a triangle, thus is calculated by multiplying the height times its width at half height. The PA of certain metabolite divided by the total of PA indicated the percent of its metabolite in the sample (Chempages, 2017).

Based on our experiments, 56 metabolites were detected and identified within methanol fruit and leaf extract of Limau plants. The leaf extract composed of 19 metabolites (Table 1), while fruit extract composed of 39 metabolites (Table 2). There were two similar metabolites detected in both plant organs, namely

Table 1. Metabolites fingerprinting of Limau leaf based on untargeted GCMS approach

No	RT	Quality	Metabolites	RC (%)
1	36.140	89	Vitamin E	23.38
2	40.704	66	Taraxasterol	15.16
3	40.863	55	Benzo[b]naphtho[2,3-d]furan	14.85
4	29.437	94	Phytol isomer	10.99
5	29.851	99	Linolenic acid	5.13
6	32.906	99	Squalene	4.91
7	39.125	91	Beta-sitosterol	3.70
8	32.520	90	Nonanoic acid, 9-(3-hexenylidenecyclopropy lidene)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester	3.28
9	35.064	93	Gamma-Tocopherol	3.15
10	27.114	99	Neophytadiene	3.15
11	39.201	95	Dihydrolanosterol	2.75
12	33.754	94	2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane	2.30
13	33.409	51	Methyl linolenate	2.09
14	28.789	99	Palmitate	1.62
15	28.665	81	1-[2-methyl-2-(4-methyl-3-pentenyl) cyclopropyl] ethanol	0.95
16	33.326	53	(+)-1-menthene	0.89
17	32.216	72	Butyric acid, ester with citronellol	0.84
18	31.471	49	Mehp	0.66
19	39.677	95	Beta amyrin	0.19
Total of peak areas				99.99

palmitate and neophytadiene. Palmitate ($C_{16}H_{32}O_2$), also known as palmitic acid, is a long-chain saturated fatty acid that could act as an antioxidant, a hypocholesterolemic and a nematicide (Sermakkani and Thangapandian, 2012). Neophytadiene ($C_{20}H_{38}$) is a volatile metabolite that is mostly found as a flavor compound in tobacco and may potentially possess some antimicrobial activity (Stojanovic et al., 2000). Neophytadiene was also found in medicinal plants alike Kirinyuh (*Eupatorium odoratum*) and reported to have pharmacological properties such as antipyretic, analgesics, anti inflammatory, anti microbial, and anti oxidant (Raman et al., 2012).

Methanol leaf extract was dominated by vitamin E, three phytosterols, three terpenes, and five fatty acids. Vitamin E was only found in citrus leaf and seemed to to be absent in the fruits. Vitamin E ($C_{29}H_{50}O_2$), also known as alpha-tocopherol, is one of the essential vitamins for human health because of its strong antioxidant activities against various free radicals (Burton and Traber, 1990). It was also reported to have antioxidant activity in *Alamanda cathartica* L. (Prabhadevi et al., 2012) and *Adiantum capillus-veneris* L. (Kumar et al., 2014). Phytosterols, obtained in form of taraxasterol, beta sitosterol and dihydrolanosterol, were well known as anticancer compounds (Ovesna et al., 2004; Bradford and Awad 2007; Woyengo et al., 2009) and regulated serum cholesterol (Jones and AbuMweis, 2009). Various terpenes such as phytol ($C_{20}H_{40}O$) isomer and beta-amyrin ($C_{30}H_{50}O$) were found to display antimicrobial and anti-inflammatory activities, respectively (Raman et al., 2012; Okoye et al., 2014). Another terpene, such as squalene ($C_{30}H_{50}$) had a similar structure to beta-carotene and was used for various cancer therapy (Kelly, 1999). Five types of fatty acid detected were butyric acid ($C_4H_8O_2$), linolenic acid ($C_{18}H_{30}O_2$), methyl linolenate ($C_{19}H_{34}O_2$), nonanoic acid ($C_9H_{18}O_2$) and palmitate. A number of studies have shown the functional potential activities of these fatty acids, such as antimicrobial, anti-cyanobacterial, anti-inflammatory and may be used as a nematicide (Nakai et al., 2005; Huang et al., 2011; Sermakkani and Thangapandian, 2012).

Methanol fruit extracts composed of 39 metabolites and showed more various metabolites than its leaves. The fruit was dominated by 6-octadecenoid acid and followed by palmitate. 6-octadecenoid acid ($C_{18}H_{34}O_2$), also known as petroselenic acid, an uncommon fatty acid that was firstly found in parsley seeds and showed antimicrobial activities (Placek, 1963). Palmitate ($C_{16}H_{32}O_2$), as previously mentioned, supposed to serve as an antioxidant. Methyl 9-octadecenoate also called methyl oleate ($C_{19}H_{36}O_2$) was used in certain biochemical research as a chromatographic standard, lubricants and solvents (Pubchem, 2017).

Fruit extracts predominantly composed of aromatic compounds that has the potential to be used as flavor and fragrance. In total, there were 13 metabolites identified as flavor compounds, namely alpha sinensal, alpha limonene, alpha-farnesene, benzoic acid, beta bisabolene, beta citronellal, beta-cis-ocimene, beta myrcene, beta pinene, citronellol, 2-methoxy-4-vinylphenol, 2,3-dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one and sabinene. Alpha sinensal ($C_{15}H_{22}O$) was the major flavor compound compared to others so that it supposed to play a role during the formation of aromatic fruit. Alpha-sinensal was reported in minor portion within orange, however, it could contribute to the overall flavor and aroma of its fruit (HMDB, 2017a). Alpha limonene, similar to D-limonene ($C_{10}H_6$), is an aromatic hydrocarbon monoterpenes with antitumor activities and commonly used as aromatherapy, flavors and fragrances (Duetz et al., 2003). Previous report by Othman et al. (2016) confirmed the major portion of limonene in essential oils of pummelo (*Citrus grandis*) and lime (*Citrus aurantiifolia*); and sabinene ($C_{10}H_{16}$) in Kaffir lime (*Citrus hystrix*). Alpha-farnesene ($C_{15}H_{24}$) was used as flavouring agents in the coating solution of apples and pears (HMDB, 2017b). Beta-citronellal ($C_{10}H_{18}O$), citronellol ($C_{10}H_{20}O$), Beta myrcene ($C_{10}H_{16}$), 2-methoxy-4-vinylphenol and 2,3-dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one were reported to have displayed antimicrobial activities (Janssen et al., 1998; Yoshihiro et al., 2004; Kumar et al., 2014).

Limau Morphological Characters

Limau showed ellipsoid crown with erect growth direction and categorized as a shrub rather than tree (Figure 1C). The trunk was a union between a Limau scionwood and a *Japasche citroen* rootstock. The characters of the lower part of scion and rootstock were similar such as brown, ligneous, spherical with no spines. The plant height and the crown width of the observed Limau plants were ranged from 140-160 cm and 80-90 cm, respectively. The crown part consisted of relatively sparse branches and leaves. Branching type of Limau was likely monopodial as indicated by the dominant growth of apical buds forming a central axis rather than the lateral buds. Small (2-5 mm), brownish green, sharp and straight pointed end spines emerged below the leaf petiole in most of the branch nodes. Spines were highly distributed in vegetative shoots rather than generative ones. Although the size was not statistically different, spines emerging in new shoots were smaller and weaker than the old ones. Generally, the development of spines in plants was genetically encoded as a self-defense strategy

Table 2. Metabolites fingerprinting of Limau leaf based on untargeted GCMS approach

No	RT	Quality	Metabolites	RC (%)
1	32.344	99	6-octadecenoic acid	34.40
2	31.089	99	Palmitate	17.33
3	37.419	94	4-(2,3-dihydro-3-methylbutoxy)furo(3,2-g)chromen-7-one	5.11
4	31.689	99	Methyl 9-octadecenoate	5.08
5	28.648	91	Alpha sinensal	4.09
6	5.148	80	Methylmaleinacidanhydride	2.74
7	16.298	91	Cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl	2.67
8	11.940	98	Citronellol	2.07
9	5.576	96	Beta myrcene	1.83
10	33.378	95	5-hydroxyfurocoumarin	1.77
11	8.541	38	8-methoxy-5,5,8-trimethyl-3-nonen-2-one	1.64
12	30.365	99	Palmitic acid, methyl ester	1.60
13	17.215	72	3,8-terpine	1.55
14	15.236	96	2-methoxy-4-vinylphenol	1.50
15	35.653	83	Prangenin	1.48
16	7.707	27	Furanoid linalool oxide	1.43
17	6.224	99	Alpha limonene	1.29
18	22.780	98	Alpha farnesene	1.21
19	6.686	98	Beta cis ocimene	0.91
20	16.733	47	Methoxycitronellal	0.73
21	10.865	47	Sabinene	0.72
22	5.300	96	Beta pinene	0.72
23	9.382	98	Beta citronellal	0.68
24	9.596	91	2,3-dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one	0.67
25	26.386	91	Beta bisabolene	0.63
26	7.321	90	2-(5-methyl-5-vinyltetrahydro-2-furanyl)-2-propanol	0.63
27	10.699	59	Benzoic acid	0.62
28	27.241	38	Alpha patchoulene	0.60
29	14.905	59	Citronellal dimethyl acetal	0.59
30	27.124	95	7-epi-amiteol	0.53
31	10.279	97	1-isopropyl-4-methyl-3-cyclohexen-1-ol	0.52
32	29.537	91	Neophytadiene	0.45
33	12.623	64	4,5-dimethyl-2,6-octadiene	0.43
34	29.041	46	Isospathulenol	0.41
35	25.090	86	2,5-cyclohexadiene-1,4-dione, 2-(methoxymethyl)-3,5-dimethyl	0.36
36	35.205	46	(E,E)-7-methyl-4-(1-methylethylidene)-1,7-cyclodecadienemethanol	0.34
37	29.365	91	Vulgarol a	0.28
38	27.655	70	Bicyclo[5.2.0]nonane 4-methylene-2,8,8-trimethyl-2-vinyl	0.21
39	30.199	83	N-methyl-dibenz(E,G)isoindole	0.18
Total of peak areas				100.00

against herbivores (War et al., 2012; Kariyat et al., 2017).

Limau plants possessed green and pleasantly fragranced compound leaves, specifically known as unifoliate. Unifoliate type was previously documented in *Citrus limon* and *Citrus maxima* as indicated by the presence of wing in petiole (Sevvel, 2016). The shape of petiole wing was either obovate or an inverse of ovate. The shorter petiole of Limau compared to the leaf blade is called brevipetiolate. The leaves possessed pinnate venation, shallow rounded or crenate margin, sharp pointed apex with an angle less than 45°, obtuse base with an angle greater than 90° and ovate shape as proved by the ratio of length to width around 1.84 and also showed the widest part below the middle of leaf blade (NOPD, 2017). The number of leaves arranged alternately; a one-year-old Limau tree has 180 to 200 leaves. The average length and width of leaf blade were 33 and 18 mm, respectively.

A single flower of Limau consists of a single whitish yellow pedicel, five whitish yellow calyces, five white petals, an oily yellow stigma, and 20 yellow anthers (Figure 1D). Three to five flowers were arranged alternately surrounding the peduncle and formed a simple cyme inflorescence. The number of flowers within a single potted plant varied and was in the range of 45-60 flowers. Flowering began with the formation of flower buds and then followed by the swelling of flower dome (Figure 1A). Flower retention was four to five days. Limau exhibited constitutive flowering rather than erratic patterns, lead to all year round and multiple harvesting times.

Both fruitlets and mature fruits had rough surface, truncate apex, obloid shape with a convex base (Figure 1B). The peeled fruits are strongly aromatic and consisted of three layers namely bumpy green flavedo as the outermost layer, whitish yellow albedo as the middle layer and soft green segments as the endocarp. Flavedo tends to be thicker than the albedo. There were eight segments filled with juicy soft green pulp. Each segment contained one or two creamy ovoid seeds, therefore a fruit may contain 10-12 seeds in total. The morphology of leaf, inflorescence, flower, fruitlet, mature fruit and seed are in Figure 1D.

A study by Dos Santos et al. (2015) classified the term ornamental plants into four groups: potted plants, mini-fruit plants, hedges and landscaping plants. Limau was likely to fit into two groups; either as potted plants or mini-fruits plant. Limau plant has a relatively short and small crown with the height of <170 cm and width of about 100 cm. In addition, the fruit size of Limau was small (Ø 2-4 cm) compared to most of the marketable citrus fruit such as mandarin, tangerine and even pummelo, thus highly suitable as mini-fruits trees. The presence of small spines in Limau was unfavorable for landscaping. Moreover, such characters make the plant less effective as a hedge which may require large plants with evenly distributed spines within dense branches.

Figure 1. Plant morphology of Limau: A) flowering shoot, B) fruiting shoot, C) entire shoot composed of leaves and branches, D) detached organs consisted of leaf, flower inflorescence, single flower, fruitlet, mature fruit, and seed (from left to right).

Growing Limau as ornamental plant should follow several basic principles such as good plant proportion, pot size suitability and duration of growing before reaching a marketable size. The height and width of the plant canopy was used to evaluate the plant proportion and pot size suitability; plant height should not exceed three times of the pot height in order to keep the balance ornamental proportion (Pennisi, 2017). Because the height and width of Limau varied from 140-160 and 80-90 cm, respectively, the suitable pot sizes were 50 cm in height and 30 cm in diameter. Preparing Limau as potted plant take a shorter time than as mini-fruit trees. As commonly citrus seedling production in Indonesia, Limau is propagated by grafting (Poerwanto and Susila, 2014), and marketable as potted plant after five months after grafting (Setiono, 2016). Mini-fruit tree will take a longer period to reach a marketable size, i.e. for about a year after grafting, and the plants are ready to flower at this age. Exposure to periodic drought may promote flowering of tropical citrus, including Limau (Poerwanto and Susila, 2014).

Conclusion

Limau has good potentials to be commercialized as ornamental pot plant or mini trees; it has brown spherical trunk, aromatic green leaves with winged petiole, white flowers and aromatic green obloid small fruits contained creamy ovoid seeds. Untargeted GCMS analysis had shown the potential of Limau to produce functional compounds as the identified metabolites within leaf and fruit of Limau had antioxidant activities. Vitamin E was found predominantly in the methanol leaf extract, which also contains fatty acids, phytosterols and terpenes. Methanol extract of fruits was dominated by 6-octadecenoid acid and consisted of 13 compounds that had been identified as aromatic flavoring compounds. The presence of small spines on limau branches was unfavorable for landscaping or hedges. Further study is needed to develop more effective techniques for extraction, quantification and examination of limau metabolites for medicinal purposes.

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