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LC-QTOF-MSMS Analysis: Metabolites Compounds from The Crude Extract of Kalamansi Oranges Fruit (*Citrofortunella microcarpa*)

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ABSTRACT— Kalamansi orange is the main and superior commodity from Bengkulu Province, Indonesia. This orange has been used by the community as a source of beverages managed by local industries. However, there is no data reporting the chemical compounds in kalamansi oranges from Bengkulu Province. The purpose of this study was to analyze the chemical compounds detected in the extract of kalamansi orange from Bengkulu Province using LC-QTOF-MS/MS. Measurements with LC-QTOF-MS/MS carried out in the Laboratory of PT. Saraswanti Indo Genetech (SIG), Bogor, Indonesia. The results of this study show that the extract of kalamansi orange contains alkaloid compounds in the form of nortopane and cadambine (2α, 3β, 6exo-trihydroxy nortropane, 2α, 3β, dihydroxy nortropane and 3α-dihydrocadambine). In addition, the kalamansi orange extract also has 19 bioactive compounds from the flavonoid group. The results of the analysis of vitamin C levels in the orange extract using High Performance Liquid Chromatography (HPLC) showed that the vitamin C content in the extract was 0.04 ± 0.0002%. Information about the bioactive compounds in orange extract is expected to be the first step for greater utilization for this typical citrus commodity of Bengkulu Province.

KEYWORDS: Bengkulu, Kalamansi, Metabolites, Oranges, LC-QTOF-MS/MS

1. INTRODUCTION

Kalamansi orange is a leading citrus commodity from Bengkulu Province, Indonesia. This type of orange has been widely cultivated by the people of Bengkulu Province. This type of orange has been trusted by local people to have many benefits. Some of them are antihypertensive [2], antidiabetic [16] and various other properties that are often associated with the antioxidant mechanism of the orange and its chemical compounds. Chemical compounds obtained from plants are known as phytochemicals. Some of these phytochemical compounds are secondary metabolites that do not play an important role in plant growth but are related to plant defense mechanisms against microbes. Phytochemical compounds that are usually examined are alkaloids, flavonoids, tannins, terpenoids and saponins [15]. Calamondin oranges from Bengkulu Province, Indonesia are one variety with calamondin oranges from the Philippines and Mandarin. Previous research has shown data analysis of flavonoid levels contained in this orange [9]. In addition, other data from the same variety oranges show the analysis of folate components, physicochemical, sugar components and organic acids [5]. However, no data has been reported regarding the chemical compound content of kalamansi oranges from Bengkulu Province, Indonesia. Previous studies have identified citrus compounds from several citrus commodities from Mandarin. The study also showed that the same citrus species but planted on different lands could have different phytochemical content. This can be influenced by nutrients, humidity and other factors

that affect plant growth [17]. Therefore, this research is important to be carried out in order to identify the phytochemical compounds contained in kalamansi oranges from Bengkulu Province. This bioactive compound data will be the basis for further biological analysis on the citrus, both in silico, in vitro and in vivo analysis.

2. Material and Methods

2.1 Sample Preparation and Extraction

Citrus samples were taken from the Putri Bengkulu farmer group in Central Bengkulu Regency. The oranges selected as research samples are oranges with a medium maturity level. The kalamansi oranges obtained were then taken to the laboratory to be processed into simplicia. The drying process was carried out with a protected drying technique so that the sample was not exposed to direct sunlight. The dried samples will then be mashed to form citrus simplicia. A total of 45 grams of citrus simplicia then macerated with 96% ethanol at room temperature. The maceration process was carried out for 5x24 hours. After that, the maceration results will be processed using a rotary evaporator to obtain a paste of kalamansi orange extract.

2.2 Identification of Bioactive Compound

Bioactive compounds in kalamansi orange extract were identified in the laboratory of PT. Saraswanti Indo Genetech, Bogor, Indonesia. The identification is carried out using a standardized screening test method for active natural ingredients [12] with contract number SIG.Mark.R.X.2019.018559. Identification of bioactive compounds using qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS/MS). Examination and identification begins with the preparation of standard biotin and chloramphenicol assays. The 1 mg/L biotin standard was prepared by dissolving 25 L of the 1000 mg/L biotin standard in a 25 mL volumetric flask. The mixture was then homogenized and calibrated until the volume was exactly 25 mL. The 1 ppm chloramphenicol standard was prepared by dissolving 25 L of 1000 mg/L chloramphenicol standard into a 25 mL volumetric flask. The mixture was homogenized and the volume was made up to 25 mL. The 1 ppm chloramphenicol standard was prepared by dissolving 25 L of 1000 mg/L chloramphenicol standard into a 25 mL volumetric flask. The mixture was homogenized and the volume was made up to 25 mL. The sample was weighed as much as 0.1 g and added methanol solvent until the volume was 10 mL. After that, the mixture was homogenized and filtered using filter paper with a 0.22 m GHP/PTFE membrane filter. Thus, the sample is ready to be injected into the LC-QTOF-MS/MS system. Each sample will be done in duplo. The LC settings on the instrument include the selected column C18, column temperature 40^0C, autosampler temperature 15^0C, and injection volume of 10 L. The mobile phase used was 0.1% formic acid in acetonitrile and 0.1% formic acid in aquabides. The flow rate used is 0.6 ml/min, gradient. The MS settings on the instrument are $TofMS^E$ mode of operation, Ionization ESI (-)/ESI (+) and acquisition range 50-1200 Da.

2.3 Measurement of Concentration of Vitamin C Extract with HPLC

Measurement of the concentration of Vitamin C in kalamansi orange extract was carried out at the Laboratory of the Department of Food Science and Technology, Faculty of Agricultural Technology, Bogor Agricultural University. A total of 1 gram of kalamansi orange extract was analyzed with Luna 5u C18(2) 100 A (4.6 x 250 mm) HPLC. This examination uses a UV-Vis PDA detector with a wavelength of 242 nm. This examination used a pro-HPLC methanol mobile phase with a flow rate of 1 mL/minute. Measurement of vitamin C concentration will be carried out twice on each sample.

2.4 Data Analysis

The screening process for active substances from natural ingredients using LC-QTOF -MS/MS is carried out

using UNIFI software, which includes a mass spectrum library of active natural ingredients from the waters database. UNIFI software can identify the mass spectrum of the compound in the sample which is then matched with the mass spectrum in the library. Bioactive compounds will be identified if they meet the following requirements: i) mass error of analyte reading 5 ppm error; ii) isotope match MZ RMS PPM 6 ppm and isotope match MZ RMS % 10 %; iii) Analyte intensity 300; and iv) There is one fraction with brake value < 4 in the fragment match elution system.

3. Results

Analysis of the results of identification of bioactive compounds showed that the examined citrus extract contained alkaloids and flavonoids. A total of three bioactive compounds were detected in the alkaloid group, namely 2α,3β,6exo-Trihydroxy nortropane, 2α,3β-Dihydroxy nortropane and 3α-Dihydrocadambine (Figure 1). Alkaloids are a group of nitrogenous base compounds, most of which are heterosilkyl compounds and are found in plants. However, other nitrogen compounds such as amino acids, peptides, proteins, amino sugars, antibiotics, nucleic acids and nucleotides are not included in this group. In this study, two compounds detected were the nortropane group and one group of compounds was the cadambine group.

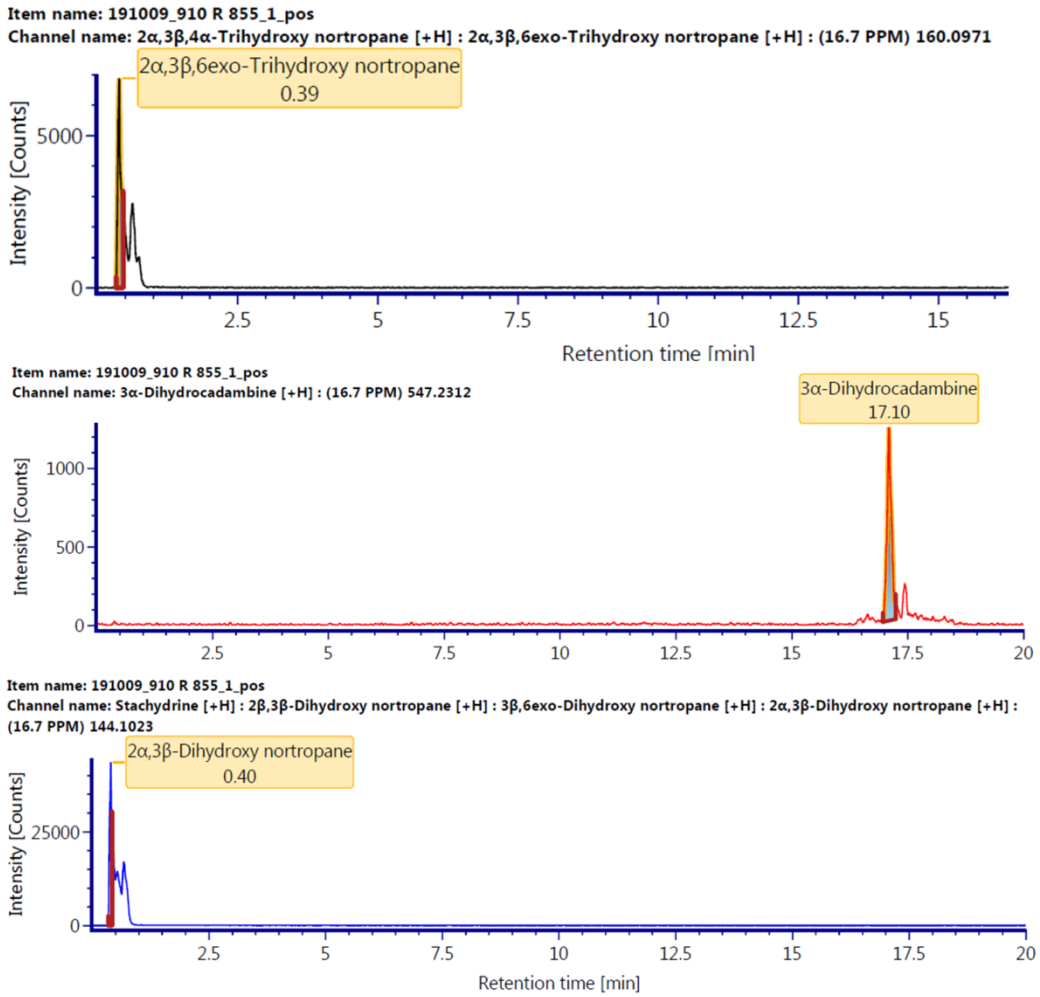

Figure 1 Alkaloid Compounds from Kalamansi Orange Extract

The most detected compounds from the samples examined were flavonoid compounds. Nineteen compounds were detected in this study (Table 1). Flavonoids are one of the most abundant groups of secondary metabolites found in plants in addition to the phenolic, terpenoid, and alkaloid groups. This group of compounds is rich

in hydroxyl groups (-OH) and is often characterized by a structure (C6-C3-C6) which consists of three carbons and is flanked by two aromatic structures that cause flavonoid compounds to experience resonance. In this study, the flavonoid group (including compounds from the flavone group) has a very large variety of compounds. A total of 7 compounds were identified under positive ESI conditions, and 12 compounds were identified in negative ESI conditions. Measurement of vitamin C concentration in this study showed that the concentration of vitamin C in the extract was 0.04 ± 0.0002%. These flavonoid and vitamin C group compounds are usually components that play a role in the antioxidant activity of extracts

Table 1. Flavonoid compounds in the ethanolic extract of Kalamansi Orange Extract

RT (min)	Molecular formula	Adducts	Number of high energy fragments identified	Isotope MTCH intensity RMS percent	Compound name
16.50	$C_{22}H_{24}O_9$	+H	1	4.03	3,5,6,7,8,3',4'-Heptemethoxyflavone
16.66	$C_{21}H_{22}O_8$	+H, +Na	1	2.93	3',4',5',5,7,8-Hexame-thoxy flavone
16.68	$C_{20}H_{20}O_7$	+H, +Na	1	4.22	5,7,8,3',4'-Pentametho-xyflavone
16.46	$C_{19}H_{18}O_6$	+H	1	13.10	4',5,6,7-Tetramethoxy-flavone
16.67	$C_{21}H_{22}O_9$	+H	5	12.81	Isoliquiritigenin-4′-O-β-D-glucopyranoside
16.86	$C_{36}H_{58}O_{11}$	+H	1	32.57	Chebuloside II
17.17	$C_{27}H_{34}O_7$	+H	2	14.55	Neokurarinol
6.63	$C_{27}H_{30}O_{15}$	-H	1	2.36	Apigenin-6,8-di-C-glucoside
8.80	$C_{23}H_{26}O_{11}$	-H	3	6.09	(-)-Epiafzelechin-3-O-(6"-O-acetyl)-β-D-allosepyranoside
10.34	$C_{28}H_{34}O_{15}$	-H	1	1.74	Hesperidin
8.20	$C_{34}H_{42}O_{20}$	-H	1	18.33	Typhaneoside
8.80	$C_{21}H_{22}O_9$	-H	2	13.72	Liquiritin
8.81	$C_{27}H_{32}O_{14}$	-H	3	29.71	Kushenol J
16.79	$C_{21}H_{22}O_6$	-H	1	176.36	Noranhyoicaritin
8.26	$C_{28}H_{32}O_{16}$	-H	1	54.74	Isorhamnetin-3-O-β-rutinoside_1
8.43	$C_{27}H_{30}O_{14}$	-H	1	10.44	Patuletin-7-O-(6″-isobutyryl)-glucoside
8.87	$C_{29}H_{34}O_{17}$	-H	2	21.14	Malvidin 3,5-diglucoside
9.04	$C_{28}H_{32}O_{15}$	-H	1	22.53	Spinosin
10.34	$C_{28}H_{34}O_{15}$	-H	1	1.74	Hesperidin

4. Discussion

Kalamansi, *Citrus microcarpa* is a citrus plant characterized by a smooth and slightly prickly plant, growing to a height of 3 to 5 meters. This plant is widely cultivated in the Philippines as a medicinal plant. Some tribes in the Philippines use a decoction of kalamansi leaves to reduce hypertension. The juice from the fruit is used for coughs and colds [11]. Kalamansi citrus plants (*Citrus microcarpa*) in Indonesia are also widely found in Bengkulu Province and are often used as the main raw material in the fruit syrup processing industry [14]. The flavonoid content of the Kalamansi orange juice from Bengkulu province using UV-VIS spectrophotometry was 10.958 mg/RE [9]. There are about 40 limonoids in calamansi orange with limonin and nomilin being the main compounds. Limonoids have the ability to inhibit tumor formation by stimulating the enzyme glutathione S-transferase (GST) [7]. Based on the phytochemical test results, Kalamansi orange (*Citrus microcarpa*) contains alkaloids, flavonoids, tannins, phenols and saponins [10].

Hydrocarbons are the dominant compounds in the Kalamansi fruit peel extract, regardless of geographical origin. These hydrocarbon compounds consist mainly of monoterpenes (eg, limonene, b-myrcene, b-pinene, a-pinene, b-phellandrene and sabinene) and sesquiterpenes (eg elemental isomers, farnesene and germacrene), which have also been frequently reported in other types of oranges [5]. Until now, there have not been many

studies reporting data regarding the specific compounds contained in the Kalamansi orange extract. The literature review is only limited to activities and phytochemical tests. From the results of the analysis using the LCMS-QTOF instrument, it was found that the Kalamansi orange extract contains 3 compounds of the Alkaloid group (Figure 1) and 19 compounds of the flavonoid group (Table 1).

Alkaloids are a group of secondary metabolites that give plants a bitter taste, composed of heterocyclic nitrogen atoms. The presence of nitrogen atoms makes the structure of alkaloid compounds different from other metabolite compounds. Alkaloids are synthesized from amino acid derivatives via the mevalonic acid pathway or the pyruvic acid pathway. Therefore, alkaloids can be toxic components that protect plants from insects and other animals, neutralizing components of toxins for the plant body, growth regulators, and growth factors [6].

Flavonoid compounds are phenolic groups with structures containing many hydroxyl (OH) substituents and have antioxidant activity. The group of flavonoids that are antioxidants include flavones, flavonols, catexins, and chalcones [3]. Flavanones are very important citrus flavonoids, and several other compounds are responsible for the bitter taste of oranges, such as naringin, neohesperidin, neoeriocitrin and poncharin [8]. Flavonoids, such as poncharin, dydimin, neohesperidin, hesperidin, narirutin, diosmin, and isorhoifolin were obtained from dry calamodin pulp powder extracted with 80% methanol [13].

Three flavonoid compounds from the ethanolic extract of kalamansi orange are 3,5,6,7,8,3',4'-Heptemethoxyflavone, 3',4',5',5,7,8-Hexame-thoxy flavone, 4', 5,6,7-Tetramethoxy-flavone was also detected in the ethanol extract of gerga oranges from Lebong district, Bengkulu province [19]. In addition, the bioactive compounds of the alkaloid group detected in the gerga orange extract amounted to 6 compounds, while in the calamansi orange there were 3 different compounds. Compounds of the flavonoid group found in gerga oranges were 17 compounds, while in calamansi oranges there were 19 compounds. The variation of the flavonoid group of bioactive compounds in the calamansi orange extract was more than that of the gerga orange extract.

The flavone group of compounds has a closed ring flavonoid basic structure and many ether-groups (R-O-R') which makes it very possible for electron delocalization from heteroatom PEB to conjugated double bonds in the aromatic ring. This condition of flavone structure causes this group of compounds to have high antioxidant activity. Flavonoid compounds have a major role as antioxidants mainly because of the structure of the phenolic hydroxyl group attached to the flavonoid ring structure [3]. The antioxidant activity of calamansi orange and gerga orange extracts has previously been reported. Both citrus extracts have antioxidant activity [18]. This is also supported by the concentration of vitamin C possessed by the kalamansi orange extract. Bioactive compounds in the form of flavonoids or other group compounds can act as antioxidants. One of them is andrographolide from bitter [20], curcumin from turmeric and other compounds [4]. Antioxidants prevent oxidative stress by scavenging free radicals, inhibiting lipid peroxidation, or chelating metal ions [1], [3].

5. Conclusion

Kalamansi orange extract has three alkaloid compounds and 19 flavonoid compounds. the concentration of vitamin C from the orange extract of kalamansi obtained in this study was 0.04 ± 0.0002%.

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