Profiling of untargeted compounds from the ethanolic extract of green melinjo (*Gnetum gnemon* L.) peel

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Abstract. Melinjo (*Gnetum gnemon*) is one of the plants that is quite well known in Indonesia. The seeds of melinjo fruits are commonly processed as chips named as emping melinjo, while the fruit peel and leaves are consumed as vegetables. Extracts from melinjo peels have been reported to exhibit antioxidant activity and are utilized as ingredients in functional beverages. In addition, melinjo peel extracts have also been reported to have potential antihyperuricemic effects. The compounds suspected to contribute to antioxidant and antihyperuricemic activities belong to the phenolic compound group. Other compounds with potential antioxidant and antihyperuricemic properties from melinjo peel have not yet been identified. Therefore, this study aimed to explore the untargeted metabolite content in green melinjo peel. Ethanol extracts of green melinjo peel were analyzed using LC-HRMS. This analysis provides qualitative information for structural or molecular formula identification as well as quantitative data for each compound. The untargeted metabolites identified in retention time the ethanol extract of green melinjo peel were classified into 10 groups: amino acids-peptides, choline, vitamin, piperidines, phorbin, purines, phenolics, polyols, lipids, and alkaloid. The four dominant untargeted metabolites were choline (428.00 ppm), 2-hydroxy-2-butenedioicacid (213.20 ppm), phenylalanine (189.60 ppm), and Tempol_H (184.00 ppm).

1 Introduction

Melinjo (*Gnetum gnemon* L.) is a well-known plant in Indonesia. Several regions such as East Java, Central Java, West Java, and the Special Region of Yogyakarta are producers of melinjo throughout Indonesia. Melinjo is a seasonal plant that is usually harvested twice a year, in May-July and October-December.

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The seeds of the melinjo fruit are commonly processed into chips named as emping melinjo, while the fruit peel and leaves are used as vegetables [1]. During the harvest season, melinjo peel is widely sold in the market at a low price. If not sold, the melinjo peel will be wasted and easily rot, which can pollute the environment. Therefore, this shall be addressed immediately.

Currently, melinjo peel is not only used as a vegetable. Melinjo peel extract is reported to have antioxidant activity and is used as an ingredient in functional drinks. In addition, melinjo peel extract has also been stated to have potential as an antihyperuricemic, for it is able to reduce uric acid levels in hyperuricemic rats. Compounds suspected to act as antioxidants and antihyperuricemic are a group of phenolic compounds ([1] [2], [3], [4]). However, other compounds which have potential antioxidant and antihyperuricemic properties from melinjo peel are still unknown. The melinjo peel available in te market varies in color, namely green, yellow, and red, which indicates the level of fruit maturity. Green peel indicates the immature stage, yellow represents the semi-mature stage, and red at the mature stage [5]. All three types of melinjo peel are used as vegetables, however, people prefer the green peel because it is softer compared to the yellow and red peels. In addition, according to Sucipto et al. [6], green melinjo peel contains higher levels of flavonoids compared to the yellow and red peels. Therefore, melinjo with green peel is a priority in this research and will be the starting point for exploration activities for the development of functional compounds during the development of the peel of the melinjo fruit.

Previous studies have reported that the best extraction process for melinjo peel uses ethanol as a solvent to extract phenolic and flavonoid compounds due to its amphiphilic properties. Ethanol is relatively non-toxic, environmentally friendly, and easily evaporates ([7], [8]). The use of ultrasonic-assisted extraction methods in this extraction process aims to shorten the extraction time. Ultrasonic waves generate cavitation bubbles that collapse and cause particle collisions, leading to fragmentation of the cellular structure. This facilitates the dissolution of bioactive components into the solvent. The energy applied is relatively less damaging to the bioactive components compared to microwave-assisted extraction methods ([8-10]). Exploratory research to help discover new compounds from ethanolic extract of green melinjo peel that have not been previously identified but may possess potential biological activities, such as antioxidant, antihyperuricemic, antihypertensive and antidiabetic agent. Compounds exploration was conducted using High Resolution Mass Spectrometry (HRMS). This instrument is capable of providing qualitative information on the identification of the structure or molecular formula, as well as quantitative information on a compound. Therefore, this study aims to explore the untargeted metabolite compounds present in the ethanolic extract of green melinjo peel to confirm that the melinjo peel, which is still in the young fruit development stage, has potential for utilization and future research development.

2 Materials and Methods

2.1 Material and Tools

This study used fresh green melinjo peel obtained from Gunung Kidul Regency, Yogyakarta. For extraction, 90% technical ethanol was used. LC-HRMS analysis required methanol (*HPLC-grade*), a 0.2 µm nylon filter, water with 0.1% formic acid (*Fisher Chemical, Optima LC-MS Grade*), and acetonitrile with 0.1% formic acid (*Fisher Chemical, Optima LC-MS Grade*). The equipment used included grinder (*Orion MG-100B*, Indonesia), centrifuge (*Sorvall Biofuge Primo, Thermo Fisher Scientific,* Germany), ultrasonic bath (*Model K2-D10,* China) was applied for extraction, while compound profiling was performed using a

UHPLC system (*Thermo Scientific™ Vanquish™ Horizon with Binary Pump,* Germering, Germany) coupled to a high-resolution mass spectrometer (*Thermo Scientific™ Orbitrap™ Exploris 240 HRMS,* Bremen, Germany).

2.2 Method

2.2.1 Green melinjo peel extraction

The extraction process was carried out by modifying the procedure from previous studies ([1], [3], [5], [7]). Green melinjo peel was washed, blanched at 95 °C for 3 minutes, drained, cut, and dried in drying oven at 50 °C for 24 hours. After melinjo peel was dried, it was ground and sieved using a 60-mesh sieve. The green melinjo peel powder was added with 90% ethanol (1 : 20 ratio, w/v) and extracted using an ultrasonic bath at 40 kHz frequency, 240 W power, and 30 °C for 20 minutes. The extract and peel powder residue were separated by centrifuge. The obtained green melinjo peel extract was subsequently analyzed for untargeted metabolite compounds.

2.2.2 Metabolite compound analysis through LC-HRMS

Analysis of untargeted compounds from the ethanolic extract of green melinjo peel was conducted at Corpora Science, Yogyakarta. The filtrate of green melinjo peel extract was filtered with a 0.22 µm nylon filter and then analyzed using a Thermo Scientific™ Vanquish™ Horizon UHPLC system with Binary Pump coupled with a Thermo Scientific™ Orbitrap™ Exploris 240 HRMS mass spectrometer. The compound separation was carried out on a Thermo Scientific™ Hypersil GOLD™ analytical column (150 mm length x 2.1 mm ID x 1.9 µm particle size, Lithuania) with a mobile phase consisting of phase A: MS grade Water with 0.1% Formic Acid and B: MS grade Acetonitrile with 0.1% Formic Acid. Flow rate 0.2 mL/min, total runtime : 23 minutes, gradient elution set at 0% B and increased gradually to 15% in 4 min, 32% in 14 min, 50% in 19 min, 100% in 21 min, hold at 100% for 21 min and back to 0% B in 21 min, hold at 0% in 23 min. Column temperature 35 °C. Injection volume is 5 µL. The HRMS data was collected in the range of 70-1000 m/z with a mass resolution of 60,000 FWHM. The compound was identified using Thermo Scientific™ Compound Discoverer 3.3 software (San Jose, USA).

2.2.3 Data Analysis

The study on the profiling of untargeted compounds from the ethanolic extract of green melinjo peel was exploratory research. Therefore, the data analysis focused on the investigation and interpretation of compounds detected using the LC-HRMS instrument. The analytical results were obtained in the form of a Total Ion Chromatogram (TIC), which represents the overall chemical profile of the sample. Each major peak in the chromatogram was further analyzed to determine its m/z value, retention time, and resulting fragmentation pattern. Data analysis was performed using Microsoft Office Excel 2021.

3 Results and Discussion

3.1 LC–HRMS untargeted metabolomic profiling analysis of green melinjo peel ethanolic extract

The total ion chromatogram (TIC) from LC-HRMS analysis represents the intensity of all ions detected in a sample. Identification of specific ions that form peaks in the TIC can be performed through fragmentation spectra, which are generally obtained from tandem mass spectrometry (MS/MS). Each signal corresponds to a different ion or metabolite, so fragmentation analysis enables understanding of the structural characteristics of those compounds [11].

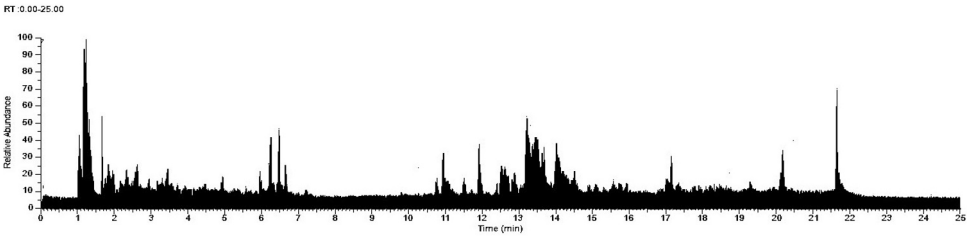

Fig. 1. Total Ion Chromatogram of ethanol extract of green melinjo peel

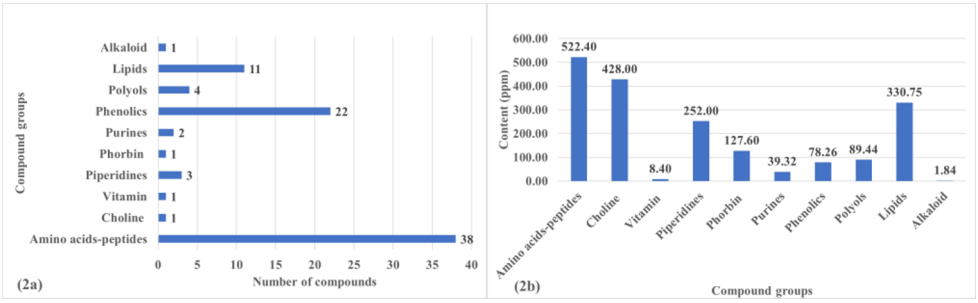

Fig. 2. Compound groups identified in ethanol extract of green melinjo peel

Figure 1 shows the total ion chromatogram (TIC) of the green melinjo peel extract, presenting the metabolic profile detected within a retention time range of 0–25 minutes. The compound grouping is presented in Figure 2a, it displays 10 chemical groups for the 84 identified compounds : amino acids-peptides, choline, vitamin, piperidines, phorbin, purines, phenolics, polyols, lipids, and alkaloid. The groups with the highest number of compound variations were amino acids-peptides, phenolics, and lipid groups. Figure 2b. illustrates the content percentage of each compound group. Amino acids-peptides, choline, lipids, and piperidines are the dominant compound groups. Each major group also features a dominant individual compound.

3.2 Amino acids and Peptides

The ethanolic extract of green melinjo peel was identified as containing 7 types of amino acids, 22 peptides, and 9 amino acid derivatives. The dominant amino acids were

phenylalanine (189.60 ppm), arginine (61.60 ppm), and isoleucine (116.40 ppm), while the dominant peptides were leucine-proline (10.70 ppm), L-α-Aspartyl-L-lysyl-L-isoleucyl-L-histidyl-L-proline (8.67 ppm), and γ-L-glutamyl-L-leucine (4.04 ppm). The three peptides have the potential to possess functional properties. Based on the BIOPEP-UWM Database of Bioactive Peptides, the sequence consisting of leucine-proline (LP) has the potential to be an Angiotensin-Converting Enzyme (ACE) inhibitor, inhibitor of lactocepin, and dipeptidyl peptidase (DPP) IV inhibitor. L-α-Aspartyl-L-lysyl-L-isoleucyl-L-histidyl-L-proline (DKIHP) sequence has the potential to be an ACE inhibitor and γ-L-glutamyl-L-leucine (EL) as antioxidative peptide. Figure 3 and Figure 4 show Extracted Ion Chromatogram from Phenylalanine and Leucyl-Proline which indicates the retention time and molecular weight of those compounds.

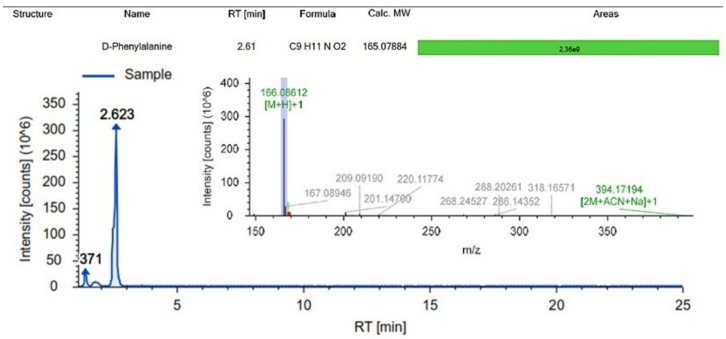

Fig. 3. Extracted ion chromatogram of Phenylalanine observed from total ion chromatogram of ethanol extract of green melinjo peel

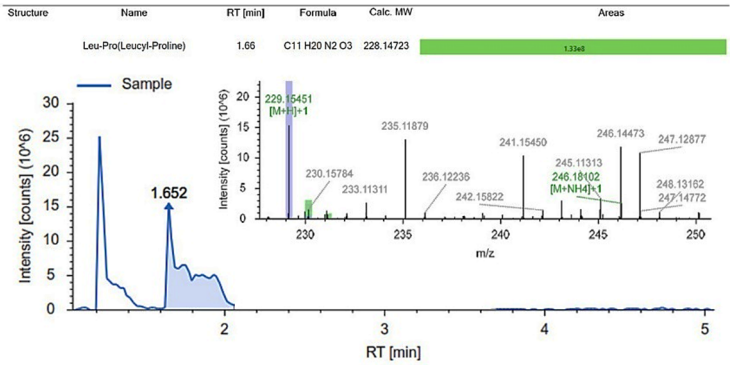

Fig. 4. Extracted ion chromatogram of Leucyl-Proline observed from total ion chromatogram of ethanol extract of green melinjo peel

In another study, peptides from faba beans and wheat gluten were reported to have antioxidant and ACE inhibitory activities ([12], [13]). A peptide from *Larimichthys polyactis* with the sequence Trp–Asp–Asp–Met–Glu–Lys–Ile–Trp has been identified as xanthine oxidase inhibitor (IC_{50} value of 3.16 ± 0.03 mM) [14]. The green melinjo peel extract contains the Ile–Trp sequence, indicating the need for further investigation to evaluate its potential as xanthine oxidase inhibitor. There has been no research explaining the bioactive peptides from melinjo peel, making it very possible to explore their potential.

3.3 Choline and Vitamin

The ethanolic extract of green melinjo peel also contains choline and vitamins, as indicated by a peak retention time of 1.176 minutes for choline (428.00 ppm) and pantothenic acid (8.42 ppm) at 2.705 minutes. Figure 5 shows Extracted Ion Chromatogram from Choline which indicates the retention time and molecular weight of the compound. Choline is an essential nutrient, but the human body can only synthesize it in limited amounts. Therefore, humans require choline intake from dietary sources to supplement endogenously produced choline and prevent deficiency. Choline deficiency can have serious consequences, as it risks disrupting the migration, proliferation, apoptosis, and differentiation of fetal progenitor cells, which can ultimately negatively affect brain development [15]. Vitamin B5 (pantothenic acid) and choline have a specific role in regulating the formation of adipocytes or brown fat cells through cellular energy metabolism. These brown fat cells are potential targets in the treatment of various metabolic diseases associated with obesity. Plant-food sources high in choline include dried soybeans (1160 ppm) and wheat germ (1520 ppm) while vitamin B5 can be found including whole grains, nuts, mushrooms, avocados, potatoes, and broccoli ([15], [16]). The green melinjo peel, which is a by-product of melinjo seeds, can also provide choline and vitamin B5 as an essential nutrient.

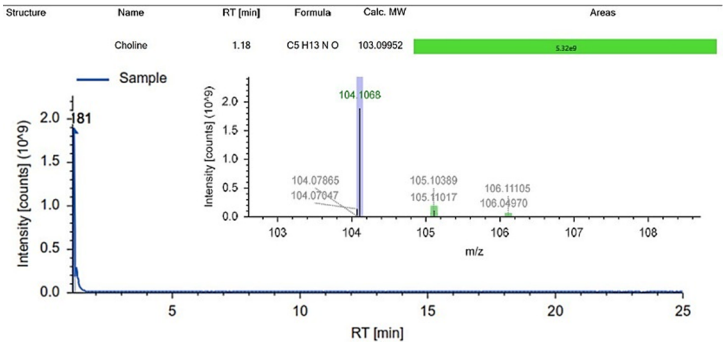

Fig. 5. Extracted ion chromatogram of Choline observed from total ion chromatogram of ethanol extract of green melinjo peel

3.4 Piperidines

Piperidine group compounds were identified at retention times of 1.839; 2.335; and 12.327 minutes from the ethanolic extract of green melinjo peel, they were piperidine (66.80 ppm), Tempol-H (184.00 ppm), and piperidinyl-peptide (1.20 ppm), respectively. Tempol-H is the dominant piperidine compound in this extract. Figure 6 shows Extracted Ion Chromatogram from Tempol-H, which indicates the retention time and molecular weight of the compound. Piperidine is a heterocyclic compound with a six-membered ring, consisting of one nitrogen atom and five carbon atoms, all of which are sp3-hybridized. Piperidine derivatives are known as one of the most important fundamentals in drug design. Several piperidine derivatives have the potential to act as antioxidant and anti-inflammatory agents. Piperidine derivatives are also found in chillies and black pepper. Black pepper contains about 5–9% piperine, which is a derivative compound of piperidine [17]. Tempol-H, a piperidine derivative, can play as an antioxidant to prevent experimental liver damage [18]. The presence of piperidine and its derivatives in green melinjo peel is expected to serve as an alternative antioxidant and anti-inflammatory agent. Therefore, further elaboration is needed.

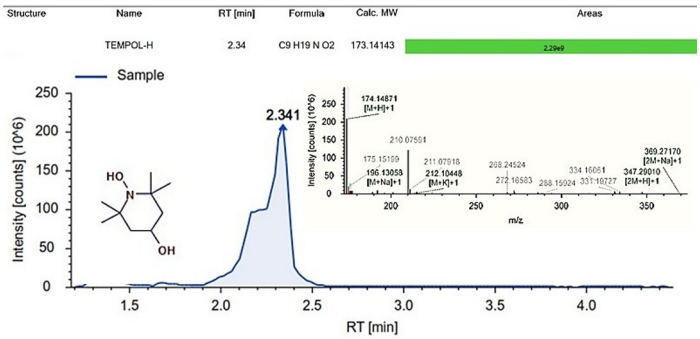

Fig. 6. Extracted ion chromatogram of Tempol-H observed from total ion chromatogram of ethanol extract of green melinjo peel

3.5 Phorbin

At a retention time of 17.140 minutes, it showed the presence of phorbin compounds with an area percentage of 127.60 ppm and a molecular formula of $C_{35}H_{36}N_4O_5$. According to Brown et al. [19], phorbin is a pigment derived from algae and photosynthetic bacteria that can be preserved in organic lake sediments in the form of degradation products derived from original chlorophyll. Phorbin is the core framework of chlorophyll. This proved that the green color of the melinjo peel is a chlorophyll pigment.

3.6 Purines

Adenine and guanine are purine nitrogen bases, the main components of nucleic acids and present in all living cells. Purines can come from endogenous (biosynthetic) and exogenous (dietary) sources. Purine catabolism produces uric acid (UA). Abnormal UA metabolism can lead to hyperuricemia, an increase in uric acid levels in the blood. The chromatogram results showed adenosine appearing at a retention time of 1.655 minutes and guanosine at 1.6544 minutes, with area percentages of 34.40 ppm and 4.80 ppm, respectively. The purine content in melinjo peel is lower than melinjo seeds. According to [20], melinjo seeds contain 2220 ppm of purine. In the study, although melinjo peel contains purines, it also contains phenolics and peptides which may contribute to the inhibition of xanthine oxidase activity and uric acid formation.

3.7 Phenolics

The total ion chromatogram (TIC) results showed that the ethanolic extract of green melinjo peel also contains phenolic compounds of 78.26 ppm, both in the form of flavonoid and non-flavonoid compounds. Flavonoid compounds contained in this extract include quercetin, kaempferol, rutin, luteolin, and chalcone, while non-flavonoid phenolic compounds include resveratrol. The phenolic compounds are contained in broccoli (880 ppm), green tea (830 ppm), and plum red (730 ppm). Phenolic and flavonoids have the potential as antioxidants, anti-inflammatory, antibacterial, antiviral, antidiabetic, and antihyperuricemic [21], while resveratrol is a natural stilbene and has various biological activities, including as an antioxidant, anti-inflammatory, antimicrobial, anticancer, and cardioprotective [22]. The diversity of phenolic compounds in green melinjo peel has great potential to support the need for bioactive compounds in daily consumption.

3.8 Polyols

Ethanol extract of green melinjo peel was identified to contain D-chiro-inositol (80.80 ppm) and D-myo-inositol (2.40 ppm). Inositol is a polyol compound, an organic molecule containing six hydroxyl (-OH) groups within a single molecule. D-chiro-inositol is more involved in the regulation of insulin and androgen metabolism, whereas myo-inositol is associated with reproductive functions, including ovulation, fertility, and estrogen synthesis. D-Chiro-inositol can be found in various food sources such as legumes, pumpkin, grapes, citrus fruits, and dandelion. The intake of myo-inositol in humans is approximately 1 gram per day, mainly obtained from foods such as cereals, legumes, seed oils, and similar products. However, most myo-inositol is actually produced endogenously in the body, ranging from about 1 to 4 grams per day, depending on individual needs and dietary patterns ([23], [24]). Therefore, green melinjo peel is important in fulfilling inositol requirements so that it is suitable for consumption as a vegetable in the daily diet.

3.9 Lipids

The lipids group identified in the ethanolic extract of green melinjo peel amounted to 330.75 ppm consisting of fatty acids or simple lipids, complex lipids (phospholipids and sphingolipids), and lipid derivatives (sterols). The fatty acids identified include palmitic acid, arachidonic acid, hexadeca-5,9-dienoic acid, octadecatetraenoic acid, eicosenoic acid, 2-hydroxy-2-butenedioic acid, and 9,10,13-trihydroxy-11-octadecenoic acid. Among the lipid compounds, the dominant one is 2-hydroxy-2-butenedioic acid (213.20 ppm). Figure 7 shows Extracted Ion Chromatogram from 2-hydroxy-2-butenedioic acid which indicates the retention time and molecular weight of the compound. Hydroxy fatty acids (HFAs) are fatty acid that contain one or more hydroxyl groups along their main carbon chain. They may occur in either saturated or unsaturated forms. The 2-hydroxy-2-butenedioic acid is suspected to be a derivative of malic acid, which have the potential to act as an antioxidant and anti-inflammatory. According to [25], malic acid from the fermentation of apple and pear juice using symbiotic bacteria and yeast culture can function as an antioxidant and anti-inflammatory agent. Identification of 2-hydroxy-2-butenedioic acid in green melinjo peel requires further elaboration to determine its concentration and potential.

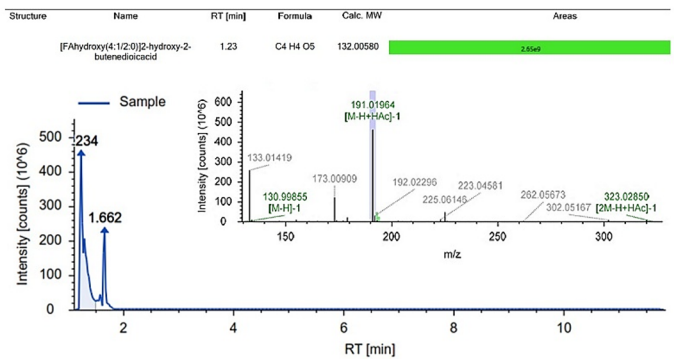

Fig. 7. Extracted ion chromatogram of 2-hydroxy-2-butenedioic acid observed from total ion chromatogram of ethanol extract of green melinjo peel

3.10 Alkaloid

The ethanolic extract of green melinjo peel was identified as containing Tryptoquivalines W (2.00 ppm) at a retention time of 5.669 minutes with the molecular formula $C_{27}H_{30}N_4O_7$. This compound is an alkaloid compound included in the group of tryptoquivaline derivatives. According to [26], tryptoquivalines are usually isolated from molds (fungi), especially the genus Aspergillus, for example Aspergillus flavus and Aspergillus oryzae. Tryptoquivalines have shown potential to mediate cancer chemo-preventive responses with NF-kB inhibitory activity.

4 Conclusion

Green melinjo peel (immature stage) was found to contain several metabolite compounds. The identified compounds were classified into ten groups. Besides phenolics, compounds suspected to have antioxidant potential in the ethanolic extract of green melinjo peel include the peptide γ-L-glutamyl-L-leucine (EL), Tempol-H, and 2-hydroxy-2-butenedioic acid. Meanwhile, the compound potentially acting as an antihyperuricemic agent is the peptide with the sequence Ile–Trp. Newly identified peptide compounds, such as leucine–proline (LP) show potential as angiotensin-converting enzyme (ACE) inhibitors, lactocepin inhibitors, and dipeptidyl peptidase IV (DPP-IV) inhibitors. L-α-Aspartyl-L-lysyl-L-isoleucyl-L-histidyl-L-proline (DKIHP) sequence has the potential to be an ACE inhibitor. It is also known that green melinjo peel contains purines, which can cause hyperuricemia, but at the same time contains anti-hyperuricemic compounds, making it highly interesting for further investigation.

Acknowledgments

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