ANGIOTENSIN CONVERTING ENZYME INHIBITOR ACTIVITY OF MELINJO (GNETUM GNEMON L.) SEED EXTRACTS AND MOLECULAR DOCKING OF ITS STILBENE CONSTITUENTS

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ABSTRACT

Objectives: To evaluate the angiotensin converting enzyme (ACE) inhibitory activity of melinjo (Gnetum gnemon) seed extract and to study molecular docking of stilbene contained in melinjo seeds.

Methods: Melinjo seed powders were extracted with n-hexane, dichloromethane, ethyl acetate, methanol, and water successively. The extracts were evaluated ACE inhibitory activities using ACE kit-Wist and the phenolic content using Folin–Ciocalteu method. The extract demonstrated the highest ACE inhibitory activity was subjected to liquid chromatography-mass spectrometry (LC-MS) to know its stilbene constituent. The stilbene constituents in melinjo seed were performed molecular docking using AutoDock Vina, and ligand-receptor Interactions were processed using Ligand Scout.

Results: The ethyl acetate extract demonstrated the highest ACE inhibition activity with inhibitory concentration 50% value of 9.77 × 10⁻⁶ μg/mL and the highest total phenolic content (575.9 mg gallic acid equivalent/g). Ultra-performance LC-MS analysis of ethyl acetate extract has detected the existency of resveratrol, gnetin C, ε-viniferin, and gnemonoside A/B. These compounds displayed similar physiochemical properties to lisinopril (ACE inhibitor), as in silico molecular docking studies demonstrated that they fit into the lisinopril receptors.

Conclusion: In vitro analysis ethyl acetate extract from melinjo seeds demonstrated the highest ACE inhibitory activity. Molecular docking analysis indicated that resveratrol dimers, gnetin C and gnemonoside A can be considered ACE inhibitor.

Keywords: Angiotensin converting enzyme inhibitor, Gnetum gnemon, Melinjo, Total phenolic, Antihypertension, Molecular docking.

INTRODUCTION

Indonesia abounds with natural resources including 30,000 plant species of 40,000 species in worldwide, while the 940 of them are medicinal plants [1]. However, only a few of them that had been studied and proved benefits also the mechanism of action scientifically. Medicinal plants and vegetables provide stilbene with the interesting biological activity including apoptosis induction of colon cancer [2] and blood glucose reduction [3]. One of stilbene derivates, resveratrol which was known to provide anti-aging activity [4], antiobesity, cardioprotective, antioxidant, anticancer [5], antidiabetic [6], anti-inflammatory, and antithrombotic [7] so that many studies had been conducted to find the other possible potential effect which could benefit human life.

Genus Gnetum (Gnetaceae family) has been known to contains stilbene, such as resveratrol, in high concentration [3,8,9]. One of those plants is Gnetum gnemon L. or locally known as Melinjo, which widely cultivated in Southeast Asia. The fruit, seed, leave, and flowers of melinjo can be consumed. This plant is the staple food in some places. Melinjo is rich in resveratrol, so very helpful in health aspect because the effect was associated with protection of cardiovascular disease, and some of cancers such as breast cancer, prostate cancer, and colorectal cancer [10-13].

The part of melinjo plant which has health benefits were the seed, leave, and root parts. The main component of melinjo seed is stilbene compound consisting gnemonoside A, gnemonoside C, gnemonoside D, gnetin C, gnetin L, and resveratrol [14]. Stilbene and flavonoid are polyphenolic compounds. The availability of polyphenolic compound in this melinjo thus indicated that melinjo has many biological activities which benefit on human body. According to that reason, we evaluated the angiotensin converting enzyme (ACE) inhibitory activity test and determined total phenolic content of melinjo seed extracts.

METHODS

Materials

Melinjo seeds were obtained from Melinjo Production Center, Banten Province and authenticated by Plant Conservation Center of Indonesia Science Agencies, Indonesia. The voucher specimen was deposited in Herbarium Laboratory of Pharmacognosy-Phytochemistry, Faculty of Pharmacy, Universitas Indonesia. ACE kit (Dojindo, and Japan), gallic acid, methanol, dichloromethane, n-hexane, ethyl acetate, sodium chloride, and dimethyl sulfoxide (DMSO) were purchased from Merck, Germany.

Preparation of extract

Melinjo powder (1 kg) was refluxed with n-hexane and then filtered. The same procedure was repeated 2 times. The organic solvent was combined, and evaporated using rotary vacuum evaporator at 40°C, and dried using oven vacuum to get hexane extract. The same procedure was applied using dichloromethane, ethyl acetate, methanol and water, successively to get dichloromethane, ethyl acetate and methanol, excluded water extract. The water solution was dried using freeze dryer to get water extract.

%Extract yield = \frac{Extract weight}{Herbweight} \times 100

In vitro ACE inhibition activity test

Dry extracts were dissolved in DMSO and distilled water and made in a final concentration of 100 μg/mL. Each extract was subjected...
Results and Discussion

The result of authentication of the sample stated that the sample was G. gneton L. Gnetaceae family. The sample was sorted to eliminate contaminant. First, we did wet sortation to separate the unwanted plants part. After that, we washed to clean the plant part from the dirt. The cleaned part then cut into small parts to ease and fasten the drying process. The next process was drying process. The chosen drying process was preventing direct sun exposure in the drying process so that hopefully the secondary metabolite contained in the study plant will not be destroyed [20]. Then, we did dry sortation to separate the herb from other unwanted things. The red fruit (ripe) was separated from the seed skin in brown which then separated again to obtain the yellow seed.

The last step of study plant was the minimizing herb size using instruments that appropriate for the herb characteristics. The minimizing process of herb was conducted using grinder, to obtain the higher extraction area so that it provided maximum process. The filtering process was conducted using sieve paper. The too small herb powder (soft powder) was not wanted because the soft powder could be released in the filtering process. While the too big size also unwanted because this will disturb the maximum extraction process. The herb then saved in a dry and protected container from the sunlight to maintain the quality [21]. We obtained 0.89 kg herb from 4 kg wet melinjo.

Melinjo dry powder (1 kg) was extracted using reflux method using gradual dissolution. The chosen method was extracted using reflux method because this method was fast and had been widely used [22]. The gradual extraction technique used to maximize the extraction process, whereas the compounds will be extracted based on the polarity properties.

In the extraction process, we obtained methanol extract provided the highest yield number with 6.37% followed by water extract with 3.90%, n-hexane, ethyl acetate, and dichloromethane with the respective yields of 1.28%, 1.12%, and 0.002% (Table 1). Methanol provided the highest yield number because methanol was a universal solvent with the polar property. After that, the extract with the second highest yield was found in water extract with 3.90%. Water yield was not as much as methanol caused by the polarity properties of water was very high. There were not that many compounds with very high polarity properties and it was estimated that the polysaccharides were found in the water extract. These results were similar to the literature which found that the polysaccharides content such as carbohydrate in melinjo was high enough with 64.1% [23]. While the yield values of the n-hexane, ethyl acetate, dichloromethane could be estimated that melinjo seeds did not contain lots of non-polar compounds. The non-polar compound was extracted in n-hexane solvent, while the semipolar compounds were not extracted with dichloromethane and ethyl acetate solvents.

ACE inhibition test and IC\textsubscript{50} measurement test

We conducted the in vitro ACE inhibition test of the melinjo seed extract. The study procedure was based on the ACE inhibitory test using ACE kit-WST which had been done in the previous study [24]. In this study, the ACE inhibitory activity was stated as inhibitory percentage on pharmacophoric feature points instead of chemical structure.

\textbf{Table 1: The result of extraction process}

| Solvent         | Crude extract (g) | Yield (%) |
|-----------------|-------------------|-----------|
| n-Hexane        | 11.33             | 1.28      |
| Dichlorom ethane| 1.62              | 0.002     |
| Ethylacetate    | 9.98              | 1.12      |
| Methan3         | 56.35             | 6.37      |
| Water           | 34.56             | 3.90      |
values. Ethyl acetate extract demonstrated the highest ACE inhibitory activity, but still too lower that captopril as a standard (Table 2). In this study, resveratrol showed low ACE inhibitory activity, with inhibition of 41.12%. The result is consistent with the previous study [25]. Su et al. 2015 studied ACE inhibitory activity of stilbene from Ampelopsis brevipedunculata var. hancei [25].

Further experiment was conducted on ethyl acetate extract and captopril standard to get their IC₅₀. In this study, IC₅₀ of the ethyl acetate extract is 9.77 × 10⁻⁸ μg/ml, while the IC₅₀ for captopril was 1.13 × 10⁻¹² μg/ml (Table 3). This showed that this plant has potential in ACE inhibition activity. According to literatures, IC₅₀ value for captopril was 6.87 nM [16]; 1.0 × 10⁻¹¹ μg/ml [25]. The difference result of IC₅₀ value from the literature and another study could be caused by many factors such as the method of experiment, study conditions, enzyme source, and the association between inhibitor and enzyme sources [16].

### Total phenolic content

The total phenolic content determination of the melinjo seed extract was performed using Folin–Ciocalteu method. Folin–Ciocalteau reagent was chosen because the phenolic compounds could react with Folin creating blue solution which the absorbance value could be obtained. The calibration curve used as the comparison was obtained from gallic acid which was measured in 765 wavelength. Gallic acid used as standard because it was a general phenolic compound contained in plant [26,27]. According to gallic acid analysis, we obtained calibration curve with the linear regression equation expressed as y = 0.1035x + 0.0316 with the correlation coefficient (R²) = 0.9958.

GAE was a general reference to measure phenolic compound contained in materials [28]. In this study, ethyl acetate extract provided the highest total phenolic with 575.884 mg GAE/g (Table 4). Polyphenol has been known to demonstrate ACE inhibitory activity in vitro and in vivo [29]. Phenolic compounds solubility depended on the solvent used. Polyphenol component had a broad spectrum with various solubility properties. The high total phenolic content in ethyl acetate solvent was expected because of the polyphenol groups such as flavonol. Ethyl acetate solvent was highly suitable for phenolic extraction. Therefore, the ethyl acetate solvent was used to extracting the phenolic compound from noni fruit (Morinda citrifolia L.) [30]. Besides that, ethyl acetate extract of corn silk (Zea mays L.) also reported having the highest total phenolic content obtained from methanol extract, water fraction, and n-hexane fraction [31].

### Mass analysis

LC-MS showed that extract contains eight prominent peaks with molecular weight 149.06, 184.11, 213.15, 413.314, 414.316, 550.61, 646.44, and 756.55 au (Fig. 1). Apart from these dominant peaks, there are small peaks which may also contribute to enhance the extract.

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**Table 2: ACE inhibitory activity of Melinjo seed extracts**

| Sample       | Concentration (ppm) | Inhibitory activity (%) |
|--------------|---------------------|-------------------------|
| n-Hexane     | 100                 | 79.28                   |
| Dichloromethane | 100               | 89.92                   |
| Ethylacetate | 100                 | 92.10                   |
| Methanol     | 100                 | 90.64                   |
| Water        | 100                 | 89.90                   |
| Resveratrol  | 100                 | 41.12                   |
| Captopril    | 1×10⁻⁸              | 93.35                   |

*IC₅₀: Inhibitory concentration 50%

**Table 3: IC₅₀ of captopril and the ethyl acetate extract**

| Sample               | IC₅₀ (µg/ml) |
|----------------------|-------------|
| Ethyl acetate extract| 9.97×10⁻⁸   |
| Captopril            | 1.13×10⁻¹²  |

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**Fig. 1: (a and b) Liquid chromatography–mass spectrometry (MS)/MS chromatogram of ethyl acetate extract**
activity. Kato et al. (2002) also reported some chemical constituents from melinjo seed extract such as gnnemonoside B (m/z-779.24); gnetin C (m/z-455.14); and resveratrol (m/z-229.0625) which are responsible for their pharmacological activities. In this study, molecular weights m/z-227 au was very similar to resveratrol, m/z 779.248 was similar to gnnemonoside B, and m/z-455.19 was similar to gnetin C [32].

**Molecular docking**

Docking results were calculated, and the best-docked conformation was chosen based on the number of conformations in a cluster and estimated free energy of binding. The lowest binding energy indicated better affinity of ACE inhibitor. As shown in Table 5, resveratrol gave a free energy of binding as high as affinity in kcal/mol, −7.90. In contrast, the other stilbene, gnetin C, gnnemonoside A, and gnnemonoside D with lower free energy of binding, docked better than the original ligand in the lisinopril receptor and ACE inhibitor. Molecular docking study of tea polyphenol on ACE reported that catechin showed good affinity the active site of ACE [33].

Binding mode of compound ACE reductase (PDB 1O86) by docking studies using AutodockVina, visualization by ligandScout, was shown in Fig. 2. There are nine important amino acids which are given similarity with lisinopril, listed in Table 6. In this study, dimer of resveratrol showed better ACE inhibitory binding affinity than that monomer. This phenomena also showed stilbene from A. brevipedunculata var. hancei [25]. According to the study, tetramer of showed the highest ACE inhibitory binding affinity, followed by that trimer, dimer, and monomer [25].

Further research is needed to clarify the ACE inhibitory activity of the compounds contained in the melinjo seeds and also to evaluate ACE inhibitory activity in vivo of the ethyl acetate extract to confirm its traditional use.

**CONCLUSION**

Ethyl acetate extract of melinjo seeds demonstrated the strongest ACE inhibitory activity. From molecular docking, the ACE inhibitory activity from melinjo suggested that dimer of resveratrol is responsible for ACE inhibitory activity. Further, preclinical and clinical studies are needed to explore the role of this extract and their bioactive compounds from melinjo seeds in the prevention and management of hypertension.

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| Sample                  | Total phenolic content (mg GAE/g) |
|-------------------------|-----------------------------------|
| n-Hexane                | 30.611                            |
| Dichloromethane         | 104.102                           |
| Ethylacetate            | 575.884                           |
| Methanol                | 398.551                           |
| Water                   | 41.224                            |

GAE: Gallic acid equivalents

| Sample                  | Affinity (kcal/mol) |
|-------------------------|---------------------|
| Lisinopril (co-crystallized) | -8.10               |
| trans-Resveratrol       | -7.90               |
| Gnetol                  | -8.10               |
| Isorhapontigenin        | -8.20               |
| trans-Piceid            | -10.10              |
| Gnetin C                | -10.90              |
| ε-viniferin             | -11.00              |
| Gnamonol M              | -11.00              |
| Gnamonol K              | -12.00              |
| Gnamonoside D           | -12.20              |
| Gnamonoside C           | -12.20              |
| Gnamonoside B           | -12.50              |
| Latifolol               | -12.70              |
| Gnamonoside A           | -13.00              |

**Table 4: Determination of total phenolic content**

**Table 5: Affinity/free energy of binding (kcal/mol) of lisinopril (original ligand); and stilbenein melinjo seed ethyl acetate extract**

**Table 6: List of important amino acid in binding conformation**

**Ligands**

| Ligands | Amino acids |
|---------|-------------|
| Lisinopril | glu162, thr166, trp279, gln281, thr282, glu376, asp377, lys511, tyr520 |
| trans-Resveratrol | asn70, glu143, val351, his383, phe512, his387, glu411, val518, arg522 |
| Gnetol | glu143, ser355, trp357 |
| Isorhapontigenin | ser355, zr701, glu411, ala356, val351, tyr523 |
| trans-Piceid | tyr520, glu162, ala354, val380, val379, thr282, glu281 |
| Gnetin C | asn70, glu143, val351, ala356, glu384, his387, phe512, arg522 |
| ε-viniferin | glu143, ala256, his387, glu384, arg522, asn70, trp357 |
| Gnamonol M | ala354, ser517, val518, tyr523 |
| Gnamonol K | arg522, ala356, glu162, asp277, ala354, val380, tyr523 |
| Gnamonoside D | asn70, phe512, glu384, val518 |
| Gnamonoside C | ala356, his387, glu384, his353, val518 |
| Gnamonoside B | ser517, tyr523, ala354, val380 |
| Latifolol | thr282, glu162, glu411, ala356, asn66, tyr523, val380, ala354, gly2000, glu376 |
Fig. 2: Binding mode of compound angiotensin-converting enzyme reductase (PDB 1086) by docking studies using AutoDock Vina, visualition by LigandScout, (a) Lisinopril_1086, (b) trans-resveratrol_1086, (c) gnetol_1086, (d) isorhapontigenin_1086, (e) trans-piceid, (f) gnetin C_1086, (g) ε-Viniferin_1086, (h) gnemonol M_1086, (i) gnemonolK_1086, (j) gnemonoside D_1086, (k) gnemonosideC_1086, (l) gnemonosideB_1086, (m) latifolol_1086, (n) gnemonosideA_1086
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