Antioxidant Activity of Ethanolic Extract of Peel and Seed Melinjo (*Gnetum gnemon*) Based on Color Variations

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Abstract. Melinjo (*Gnetum gnemon* Linn) is a plant of the genus Gnetacea that has been identified as having various biological activities, including antioxidants. This study aimed to analyze the biological activity of Gnetum gnemon for the determination of total phenolics, anti-free radicals (antioxidants), and the identification of chemical compounds contained in ethanol extract from the peel and inner seeds (endosperm). The total phenol and antioxidant activity of the peel and endosperm of G. gnemon seeds based on color variations have been investigated. The total phenolic content was analyzed by the Folin-Ciocalteu method. Antioxidant activity was analyzed by NO, FRAP, and Trolox as comparison standards which showed antioxidant activity. Identification of compounds in the extract using Thin Layer Chromatography (TLC) and Gas Chromatography-Mass Spectroscopy (GCSM). The results showed that the endosperm extract of the yellow seed G. gnemon had the highest total phenolic content, at 99.96 mg GAE/g sample and the lowest was in the red peel extract at 27.53 mg GAE/g sample. Likewise, the green seed extract had the highest NO scavenging activity of 43.80% and the lowest was the red peel extract which detected no inhibition of antioxidant activity. On the other hand, in the FRAP test, the activity of G. gnemon peel extract had a stronger reducing power than the endosperm seed extract in all color variations. The compound profile in the test extract showed a fairly clear spot of the compound under UV light at 366 nm and the stain appearance. About 10 compounds were detected in the GC-MS spectrum, which had a similarity index equal to or greater than 90%. However, there was no relationship between the maturity level of the melinjo color and the antioxidant activity tested.

Keywords: Gnetum gnemon · endosperm seeds · peel · color variations · antioxidants

1 Introduction

The melinjo plant (*Gnetum gnemon* Linn.) is a type of open-seeded annual tree often found in tropical regions including Indonesia. Melinjo has been cultivated for food purposes and has good activities in maintaining health. Melinjo is widely available in

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Indonesia and consumed in large quantities [1]. All parts of melinjo have high nutritional value, besides carbohydrates, there are also fats, proteins, minerals, and vitamins [2]. The part of melinjo that is often used is leaves, fruits, or seeds, both of which are known to have a fairly high number of antioxidants. Melinjo seeds are often consumed as a basic ingredient for making emping chips and herbaceous drinks such as tea or herbal powder as nutrients that are rich in benefits [3]. The seeds of the melinjo plant are in the range of 2.0–2.5 cm in length, are ellipse-shaped, have short tapered ends, and consist of three layers of coat. The outer coat layer (Sarcostesta) as young as the green color gradually becomes yellow, then turns red after old age and ripens. The peel melinjo seed has been used in wet or fresh form, to be processed into vegetables or fried into chips [4]. Peel melinjo seed contains ascorbic acid, tocopherol, and polyphenols which have antioxidant activity and have high potential as anti-inflammatory and anti-aging [5]. Stilbenoid compounds are the main secondary metabolite types found in melinjo. According to [6], the type of stilbenoid component isolated from 50% ethanol and methanol extracts in melinjo seeds is rich in polyphenol components and is named resveratrol. Resveratrol compounds are polyphenols that have a dual role as antioxidants as well as antibacterials and are commonly used as natural preservatives. Previous research revealed that one of the bioactive contents in melinjo seeds, namely resveratrol, was proven to have antioxidant activity [7].

The natural antioxidant compounds are increased of them for food, medicine, and health supplements along with increasing knowledge about the free radical activity [8]. Free radicals are molecules where the electrons in their outer orbit are unpaired and are reactive causing damage to the body. The existence of this damage causes various degenerative problems [9]. The causes of degenerative problems arise due to hydroxyl radicals that work in the body's biochemical mechanisms [10]. One way to prevent the destructive effects of free radicals is by choosing suitable nutritional sources, such as antioxidants, vitamins, minerals, carotene, and others. Anti-free radicals play a role in protecting cells from damage caused by radical hydroxyl molecules [11]. The ripening rate of melinjo seeds and the concentration of solvents affect the phenolic profile, flavonoids, resveratrol, and antioxidant activity [12]. Differences in harvest time associated with the ripening of fruits *Bunchosia glandulifera*, reportedly affect phenolic and physicochemical content [13]. The content of metabolite constituents and antioxidant activity based on the degree of ripening of green, yellow, and red melinjo seeds is still unclear. Therefore, our study evaluated the metabolite content of melinjo seed ethanol extract and antioxidant activity, including the identification of color difference-based compounds.

2 Materials and Methods

2.1 *Gnetum gnemon* Sample Preparation and Extraction Process

The melinjo sample was taken from an agricultural area in Bojong Gede, Bogor-West Java, Indonesia (Fig. 1). The samples were washed and cleaned with running water and then dried in the sun for 2–4 days. The melinjo seed sample was peeled first before being crushed. Peeling is intended to separate the peel and the seeds of melinjo. The inside of the melinjo seed (endosperm) is removed from the shell, then the endosperm will be


Fig. 1. Melinjo plant (*Gnetum gnemon* Linn) (Bhat and Yahya, 2014)

analyzed on the melinjo seed. Furthermore, the sample is chopped into small pieces and the small particle size of the sample is carried out with a blender which aims to make the sample particles more homogeneous.

The extraction process for each part of the sample uses the maceration method, which is soaking the sample in the organic solvent while stirring at a speed of 150 rpm. The sample extraction process used a material and solvent ratio of 1:30 (w/v) and each part of the sample was separated based on color variations, namely green, yellow and red. The solvent used in the sample extraction was 70% ethanol. The extraction method refers to Phukan et al [14]. with slight modifications. The process of filtering the sample extract solution using filter paper. The filtered melinjo extract was concentrated in a vacuum with an evaporator at 45 °C to obtain a thick extract. The thick extract was weighed and placed in a vial for the next series of tests. Quantitative tests were carried out by determining total phenol and free radical (antioxidant) activity. While the qualitative test was carried out by analyzing the compounds using TLC and GCMS.

2.2 Total Phenolic Compound Test

The determination of total dissolved phenolic content in the sample was determined by the modified Folin Ciocalteu method according to Kunarto et al., [12]. A total of 20 ul samples were added to each 100 ul folin ciocalteu (FC) reagent with a dilution of 1:10 v/v then mixed homogeneously and 80 ul sodium carbonate (7.5% w/v) was added. The sample mixture was vortexed and incubated for 90 min at 30 °C. All samples were prepared in the dark room in triplicate (triple). The absorbance measurement of the sample in the micro reader plate at the absorbance of 760 nm. The calibration curve was carried out by measuring the standard solution of gallic acid at a serial concentration of 20, 40, 60, 80, and 100 mg/L. The measurement results are expressed as mg gallic acid equivalent (GAE) per gram of dry sample.

2.3 Nitric Oxide (NO) Scavenger Activity Test

The principle of the NO test is based that sodium nitroprusside in an aqueous solution at physiological pH spontaneously forms nitric oxide and interacts with oxygen to produce nitrite ions in Gries reagent (1% sulfanilamide, 2% H3PO4, and 0.1% N-(1-naphthyl)

ethylenediamine dihydrochloride. The scavenging of nitric oxide competes with oxygen resulting in reduced nitrite ion production. Sodium nitroprusside reagent (10 mM), diphosphate-buffered saline was homogenized in a series of different sample concentrations, dissolved in water, and incubated at room temperature for 150 min. The mixture on a microplate reader in the same way without sample but replaced with an equivalent amount of water- served as a negative control. After incubation, 0.5 ml of Griess reagent was added. An absorbance chromophore was formed and read at a wavelength of 546 nm. The calibration curve was carried out by measuring the standard solution of Curcumin at a serial concentration of 12.5, 25.0; 50.0;100.0; and 200 mg/L. The measurement results are expressed as mM Trolox equivalent per mg dry weight extract [14].

2.4 Ferric-Reducing Antioxidant Power (FRAP) Activity Test

The reducing characteristic of iron in the test sample was determined by the method developed by Habu and Ibeh [15]. A total of 1 ul of sample extract was mixed with 2.5 ml of potassium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1 g/100 ml). After that, the mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (10%) was added to the mixture to stop the reaction. Equal volumes of distilled water were added followed by 0.5 ml of iron chlorate (0.1 g/100 ml) (FeCl3). In the same way without sample but replaced with an equivalent amount of water- served as a negative control. The procedure was carried out in triplicate and allowed to stand for 30 min before measuring the absorbance at a wavelength of 700 nm. The above procedure was repeated in the same manner using Trolox as a positive control. The percentage of antioxidant activity in the FRAP test is calculated according to the formula:

$$\text{Antioxidant activity } (\%) = \frac{(\text{A1} - \text{A0})}{\text{A1}} \times 100\%$$

A1: positive control
A0: negative control

2.5 Profile of the Compound by Thin Layer Chromatography (TLC)

Application of the test sample on the TLC plate was carried out by diluting the sample to 100 ppm and as much as 3 ul of the sample was developed in the TLC plate with a chloroform-methanol (4:1) mobile phase system and silica gel G60 F254 stationary phase using a chromatographic vessel until saturated. After the spot sample propagates up to the upper limit then the plate is dried and sprayed with 10% H2SO4 reagent while being heated at 90 °C for 5 min. The sample's brownish-red spot color appears to indicate that the profiles of some compounds in the test sample are representative [16].

2.6 Spectrum Interpretation by Gas Chromatography Mass Spectrum (GC-MS)

Analysis of the test samples identified by gas chromatography-mass spectroscopy (GCMS). The 5 ul test sample was run in a 5 ms RTX glass column (30 m x 0.20 mm ID x 0.11 m). The operation of the instrument profile is as follows: column temperature

150 °C, injection temperature 220 °C, ion source temperature 230 °C quadrupole pressure 140 °C 13.4 Psi, split ratio (8:1), source gas: helium, total flow 7.0 mL/min, time end 40.00 min, star m/z: 25 and end m/z: 300 [9].

3 Results

3.1 Total Phenol

Phenolic compounds dissolved in melinjo ethanol extract were expressed with mg (GAE)/g sample gallic acid equivalent (GAE)/g. The standard solution of gallic acid is a phenol group compound with a simple, pure and stable structure [7]. The results of the total phenol content of melinjo extract, on the peel and endosperm seeds according to color variations showed a range of 27.53−34.50 mg GAE/g samples and 85.67−99, respectively (Fig. 2). The highest total phenolic content comes from the endosperm extract of yellow seeds, while the lowest comes from the red peel extract. The total phenolic content of the endosperm seeds of melinjo from large to small is yellow > red > green colors, respectively. While the total phenolic extract of the peel is successively yellow > green > red colors. In this study in general, the value of total phenolic content of the peel ethanol extract and endosperm seed is not related to the degree of maturity/color of melinjo.

3.2 Antioxidant Activity (NO Assay)

The antioxidant activity of ethanol extract is carried out with different levels of color variation in the NO free radical inhibition test in Fig. 3. The results showed that all extracts, including the melinjo peel, and endosperm of the seeds had different NO inhibition patterns. According to Fig. 3, it is known that the activity of seed endosperm ethanol extract has a moderate and higher NO inhibition than melinjo peel extract, which is range from

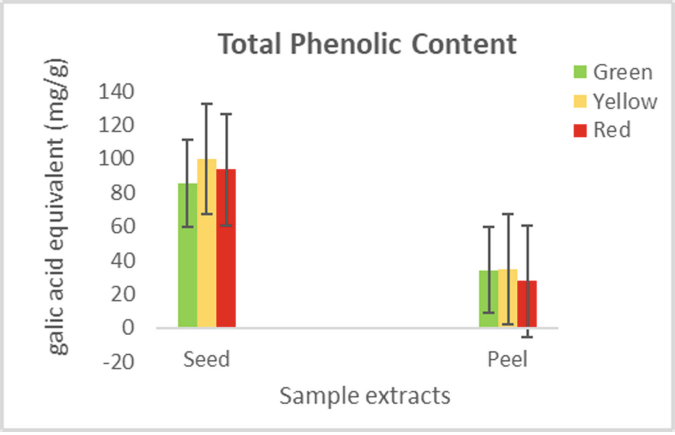

Fig. 2. The total phenol content of endosperm seeds and melinjo peel ethanol extract. Bars indicate the standard deviation of triplicate determination (n = 3).

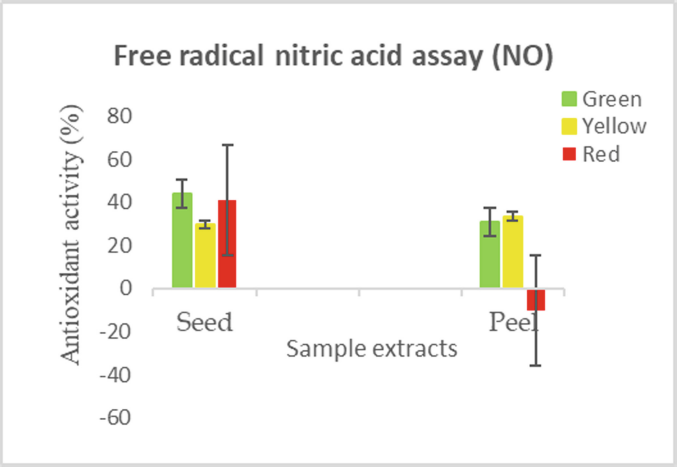

Fig. 3. Anti-free radical activity NO extracts of melinjo ethanol (endosperm of seeds and peel). Bars indicate the standard deviation of triplicate determination (n = 3)

high to low 43.8% (green color), 40.92% (red color), and 29.54% (yellow color). In contrast, melinjo peel extract has successive inhibitions of 33.38% (yellow color), and 30.95% (green color), and there is no NO inhibitory effect on red peel extract. Based on these results, melinjo seed endosperm ethanol extract is more efficient at inhibiting NO radicals than melinjo peel extract.

3.3 Antioxidant Activity (FRAP Assay)

The determination of the results of the antiradical activity of the FRAP method showed a different pattern, namely that the melinjo seed coat ethanol extract is quite good at reducing free radicals compared to seed endosperm extract. However, the results of both are just a little bit different. The FRAP test results of melinjo peel extract are in the inhibitory range between 197.65%−239.88%, while the endosperm extract of melinjo seeds inhibits between 171.85%−179.14%. This study explained that the antioxidant strength in the FRAP test can reduce ethanol extract higher than in the NO test (Fig. 4).

3.4 Profile of Compound by TLC

The determination of the chemical compound profile of the melinjo peel ethanol extract and seed endosperm were traced with TLC using the silica gel stationary phase 60 F254 and the chloroform-methanol mobile phase (4:1). The eluent systems are used because they separate more samples. The sample spots that appear on the TLC silica plates represent a large number of well-extracted compound components. The overall identification results of the melinjo peel and endosperm extract of seeds are presented in Fig. 5. The identification results clearly showed that several compounds were detected under UV light of 366 nm (left) and the appearance of stains with 10% sulfuric acid (right).

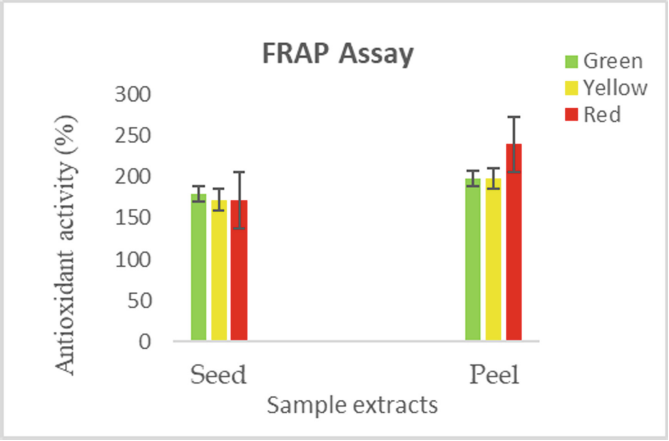

Fig. 4. The anti-free radical activity of FRAP ethanol extract melinjo (endosperm of seeds and peel). Bars indicate the standard deviation of triplicate determination (n = 3)

Fig. 5. Chromatogram results in TLC spot the melinjo endosperm extract, green, yellow, red (1–3), and endosperm green, yellow, red (4–6) peel.

3.5 Interpretation of Mass Spectrum by GC-MS

The components of the chemical compounds constituting melinjo extract that are predominantly active as antioxidants / anti-free radicals will be further identified. Melinjo seed endosperm ethanol extract was chosen for GC-MS analysis as a potential source of antioxidants. Furthermore, the seed endosperm's dry extract was dissolved with a methanol concentration of 0.1 mg/ml, filtered, and injected into the GCMS instrument.

Table 1. Analysis of GC-MS chemical components of melinjo seed endosperm ethanol extract

No	Retention Time	Area (%)	Compound Component	Molecular Formula	Similarity Index (%)
1	3.19	7.12	Ascorbic acid 2,6-dihexadecanoate	C38H68O8	92
2	4.76	2.70	beta-D-Glucopyranose	C6H10O5	93
3	5.47	18.90	Alpha-Methyl-D-mannopyranoside	C7H14O6	90
4	7.49	0.16	Pentadecanoic acid	C15H30O2	91
5	8.79	0.24	Hexadecanoic acid, methyl ester	C17H34O2	90
6	10.28	0.34	n-Heptadecanol-1	C17H36O	95
7	10.81	0.27	Heptadecatetraene	C17H36O	95
8	10.95	0.15	Octadecanoic acid	C18H36O2	91
9	11.36	0.12	Eicosyl acetate	C22H44O2	92
10	14.24	1.03	1-Hexadecanol	C16H34O	96

The results of the sample spectrum analysis will be compared with mass spectrum fragmentation based on the NIST library database. According to the analysis in Table 1, the main component (major) with the largest percentage is found in the melinjo seed endosperm, namely alpha-methyl-D-mannopyranoside (18.90%) which belongs to the glycoside group, followed by ascorbic acid 2, 6-dihexadecanoate (7.12%) belongs to the ascorbic acid group, and other fatty acids.

4 Discussion

In this study, we determined the potential antioxidant activity and identification of metabolites that play a role in the ethanol extract of melinjo seeds (endosperm seed and peel) based on color diversification. The endosperm seed extract is a more effective component than melinjo peel extract in vitro, considering that the activity of the extract is predominantly active in suppressing free radicals. Phytochemicals of plants are complex and medicinal plants are very rich in antioxidant compounds. Therefore, antioxidant activity in plant extracts can not only be evaluated by one single method [9]. The study [17] revealed that the antioxidant activity in the ethanol fraction of melinjo peel and seeds is in line with the high total values of phenols and total flavonoids. The maturity level of the seeds and the concentration of solvents significantly affect the antioxidants in the ethanol extract of the glutinous rice seeds. Another study states that the ripening of melinjo seeds successfully stimulates the activity of enzymes in phenolic biosynthesis. The increasing amount of phenol content in ripe fruit melinjo is due to the high production of phenylalanine ammonia [18]. Evaluation of the primary metabolites of ethanol extract of *Newbouldia laevis* leaves in in-vitro antioxidant testing was successfully carried out. The activity of NO free radicals indicates the potential of the extract as an antidote to free radicals with important metabolites associated with its ethnomedicinal use [15]. The main component of the melinjo seed endosperm is known

to have the potential for antioxidant bioactivity (there is an ascorbic acid group). Phenol compounds in melinjo seed ethanol extract affect antioxidant activity [13]. Detection of endosperm compounds of melinjo seeds with GCMS is expected that all volatile and non-volatile compounds will become out so that important compounds that play a role in antioxidant activity will be detected. GCMS analysis on melinjo seed endosperm extract revealed the presence of two compounds alpha-methyl-D-mannopyranoside and ascorbic acid 2,6-dihexadecanoate, which may be antioxidant phytochemicals. Antioxidant activity is closely related to secondary metabolite levels compared to the ripening effect of melinjo fruit.

5 Conclusion

The peel- and the endosperm of *G. gnemon* seed-derived ethanol extract contain various phenolic contents. Furthermore, they elicit antioxidant properties—except that of the red peel counterpart-- as shown through NO and in vitro FRAP assays. In particular to the endosperm of *G. gnemon* seed, our GCMS analysis detected two substances referred to as alpha-methyl-D-mannopyranoside and ascorbic acid 2, 6-dihexadecanoate.

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