

Ethyl Acetate Extract of Red Melinjo (*Gnetum Gnemon* L.) Peel as Antibacterial Compound

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ABSTRACT

Melinjo (*Gnetum gnemon* L.) is a native plant in Indonesia. Melinjo peel is generally disposed of, although it contains tannin, flavonoid and saponin, which can be used as antimicrobial compounds. The purpose of this research was to analyze the antimicrobial activity of melinjo peel extract. Maceration method was conducted to extract the melinjo peel, using ethyl acetate as the solvent. The extract at concentration of 4, 8, 12, and 16% (w/v) could inhibit *Escherichia coli* ATCC 8739, *Bacillus cereus* ATCC 10876, and *Pseudomonas aeruginosa* ATCC 9027, but could not inhibit *Rhizopus oligosporus* ATCC 22959. The extract MIC and MBC value was ranged from 0.50-0.69% and 2.00-2.76%, respectively. Alkaloids, saponin, phenolic, flavonoids and glycosides were found in the extract. The inhibition of selected extract (12%) had a similar level to 1000 ppm Colistin against *E. coli* and *P. aeruginosa*. Low pH (pH 4) increased the inhibition of the extract, while neutral pH decreased the inhibition. Heating extract at 65 °C for 30 minutes increased the inhibition, whereas heating at 75, 85, and 95 °C decreased the inhibition. The present of salt and sugar at concentration of 1-5% and 10-50%, respectively, increased the inhibition. The extract could damage cell morphology and was confirmed by the presence of ions (Ca²⁺, K⁺, dan Mg²⁺) outside of the cells.

Keywords : melinjo, extract, antimicrobial, cell damage

I. INTRODUCTION

Melinjo (*Gnetum gnemon* L.) is one of the native plants in Southeast Asia and Western Pacific Ocean from Assam, through Indonesia, Malaysia, Philippines and Fiji [1]. The production of melinjo has increased from 197.648 tons in 2014 to 213.025 tons in 2015 [2].

Melinjo peel is sometimes consumed as vegetables but is often discarded [3]. Melinjo peel contains bioactive compound such as tannin, flavonoid and saponin [4]. These compounds serve as medicine, antibody, an antimicrobial compound, pigment, and anti-inflammation [3]. Therefore, further utilization of melinjo peel is needed as an antimicrobial compound.

Antimicrobial substances are biological or chemical compounds that can inhibit microbial growth and activity [5]. Reference [3] study showed that melinjo peel with concentration of 15% using ethyl acetate solvents could inhibit *P. aeruginosa* bacteria as much as 6.73 mm, greater than Penicillin G antibiotics that were not able to inhibit *P. aeruginosa*.

In this study red melinjo peel was used. This is based on the results of another research, which showed that red melinjo peel contains a higher total phenolic content

(0.386 mg GAE/ g sample) than yellow (0.103 mg GAE/ g sample) and green Melino peel (0.095 mg GAE/ g sample) [6]. Phenolic is an antibacterial compound due to the presence of reactive hydroxyl group [7]. Melinjo peel extraction was carried out by the maceration method using ethyl acetate solvents. Maceration is an extraction method without heating and carried out at room temperature, so it can prevent damage to the phytochemical compound [8]. The results showed that ingredients extracted by ethyl acetate contains higher flavonoid content than the ingredients extracted with ethanol [9]. Reference [3] study showed that ethyl acetate extract of melinjo peel at a concentration of 15% was able to inhibit *P. aeruginosa* bacteria with a diameter of 6.64 mm, greater than ethanol extract of melinjo peel which could not inhibit *P. aeruginosa* at all at the same concentration.

The antimicrobial assay was carried out using four types of microbes, namely *E. coli*, *B. cereus*, *P. aeruginosa* and *R. oligosporus*. These microbes are pathogenic microbes that are commonly found in the environment. Each microbe represents Gram-negative bacteria, spore forming Gram-positive bacteria, and molds, so the antimicrobial assay can be determined by its effectivity based on the type of microbes.

Ethyl acetate extract of red melinjo peel would be analyzed for the antimicrobial activity against four types of

microbes at different concentrations, then the selected concentration of extracts would be determined. Selected extract would then be analyzed further for the phytochemical component qualitatively, compared with antibiotic compounds, and the stability of selected extracts will be tested against pH, heating temperature, salt and sugar concentration. In addition, the selected ethyl acetate extract of red melinjo peel would be tested for its effect on ion leakage and microbial morphology by Atomic Absorption Spectroscopy (AAS) and Scanning Electron Microscope (SEM) methods.

II. METHODS

The research was conducted in the University of Pelita Harapan, between August-December 2019. This research used IBM Statistical Package for the Social Sciences (SPSS) ver. 22, application to analyze the data. The design of the experiment was using a completely randomized design with one and two factors. Completely randomized design with two factors was used to analyze the comparison of extract with Colistin antibiotics, the extract stability against pH, heating temperature, salt and sugar concentration. Meanwhile, completely randomized design with two factors was used to compare extract with Penicillin G antibiotics.

a. Materials and Equipment

Materials used for this research were red melinjo peel obtained from Bogor, ethyl acetate, demineralized water, crystal violet, lugol, 96% alcohol, safranin, immersion oil, Nutrient Agar (NA) medium, Nutrient Broth (NB) medium, *B. cereus* ATCC 10876 from Institut Pertanian Bogor (IPB), *E. coli* ATCC 8739 from IPB, *P. aeruginosa* ATCC 9027 from IPB, *R. oligosporus* ATCC 22959 from IPB, magnesium ribbon, amyl alcohol, 5% and 1% iron (III) chloride (FeCl_3), chloroform, ammonia, concentrated sulfuric acid (H_2SO_4), 4 N H_2SO_4 , Dragendorff reagent reaction, Meyer reagent reaction, Wagner reagent reaction, acetic acid anhydrous, ethanol, eter, Whatman filter paper no. 1, concentrated chloric acid (HCl), 1 M and 2 N HCl, 0,5 M potassium hydroxide (KOH), 5% hydrogen peroxide, benzene, KH_2PO_4 , sodium chloride solution, sugar solution, and aluminium foil.

Equipment used for this research were cabinet dryer, autoclave, ose needle, glass slide, bunsen burner, pipette, microscope, hairdryer, petri dish, incubator, micropipette, test tube, threaded test tube, vortex, water bath, heater, magnetic stirrer, rotary evaporator, evaporating dish, bulb pump, Mohr pipette, pH meter, oven, analytical balance, 10 mL volumetric flask, stirring rod, separating funnel, Erlenmeyer flask, centrifuge, SEM, and AAS.

b. Powder Making and Extraction of Red Melinjo Peel

Red melinjo was washed and peeled, seed was disposed, and the peel was dried using a cabinet dryer at 50°C for 24 hours. Dry red melinjo peel was crushed using a dry blender, then sifted using an abrasive screener (ϕ : 35 Mesh) to obtain powdered red melinjo peel. Red melinjo

peel powder was extracted using ethyl acetate with a ratio between melinjo peel to solvent of 1:4. The extraction was carried out using a shaker at 110 rpm. The mixture was then filtered with Whatman filter paper no. 1 so that the filtrate and dregs is obtained. Dregs were disposed, while the filtrate was evaporated using a rotary evaporator at 55°C, then blasted with nitrogen gas to obtain ethyl acetate extract of red melinjo peel [10] (with modification).

c. Antimicrobial Assay

This test was carried out using well diffusion method with modification. Ethyl acetate extract of red melinjo peel of 0, 4, 8, 12, and 16% (w/v) concentrations were each put into a well with a diameter of 6 mm as much as 60 μL on NA media containing the test microorganisms. The petri dish was incubated at 37°C for 24 hours, and the inhibition zone diameter was measured [11] [12].

d. Qualitative Determination of Phytochemical Compound

Qualitative determination of phytochemical compound included flavonoids [13], phenolic [14], alkaloids [15], steroids and triterpenoids [15], terpenoids [16], tannins [15], saponin [15] [17], and glycosides [18].

e. Antibiotics Testing Comparison

This test was carried out using well diffusion method [12]. The antibiotics used were Penicillin G and Colistin. The antibiotics with concentrations of 10, 100, and 1000 ppm were put into a well with a diameter of 6 mm as much as 60 μL on NA. The petri dish was incubated at 37°C for 24 hours, then the inhibition zone diameter was measured.

f. Stability Test of Selected Extract against pH, Heating Temperature, Salt and Sugar Concentration

The stability test of selected extracts at various pH was carried out by mixing selected extracts with solutions at pH 4, 5, 6, and 7, then antimicrobial activity was tested by a well diffusion method [19].

The stability test of the selected extract at various heating temperatures was carried out by heating the extract at 65, 75, 85, and 95°C for 30 minutes using a water bath. Then the extract was tested for its antimicrobial activity by the well diffusion method [20] (with modification).

Stability testing of selected extracts at various salt concentrations was carried out by mixing selected extracts into sterile salt solutions with concentrations of 1, 2, 3, 4, and 5%, Testing of antimicrobial activity carried out by well diffusion method [19].

The stability test of the selected extract at various sugar concentrations was carried out by dissolving the selected extract into sugar solutions with concentrations of 10, 20, 30, 40, and 50%. Antimicrobial activity was tested by the well diffusion method [20].

g. Atomic Absorption Spectroscopy (AAS)

The AAS test aimed to determine bacterial cell leakage, which focuses on the amount of calcium ions (Ca^{2+}), potassium (K^+), and magnesium (Mg^{2+}). The bacterial culture was rejuvenated with Nutrient Broth (NB) media for 24 hours. Then, ethyl acetate extract of red melinjo peel was added to the bacterial culture as much as 12%, then incubated at 37°C for 24 hours. Samples were sent to Pusat Penelitian Ilmu Pengetahuan dan Teknologi (Puspiptek) to analyze Ca^{2+} , K^+ , and Mg^{2+} ions. The number of Ca^{2+} ions was analyzed by AAS at a wavelength of 422.7 nm, K^+ ions at a wavelength of 766.5 nm, and Mg^{2+} ions at a wavelength of 285.2 nm [3] (with modification).

h. Scanning Electron Microscope (SEM)

The SEM method was used to determine the morphological changes of bacteria that had been treated with the selected ethyl acetate extract of red melinjo peel. A bacterial culture that had been refreshed in NB media at 37°C for 24 hours, were added with 12% concentration of ethyl acetate extract of red melinjo peel. The mixture was homogenized with a vortex, then incubated at 37°C for 24 hours. Then the sample was put into a centrifuge at a speed of 15,000 rpm for 7 minutes so that the precipitate and filtrate were obtained. The filtrate was discarded, while the precipitate was sent to Institut Teknologi Bandung for analysis. The precipitate was applied to a carbon-coated plate. Then, the plate was coated with aurum under vacuum condition, and the sample was ready to be inserted into the SEM instrument [3] (with modification).

III. RESULTS AND DISCUSSION

a. Ethyl Acetate Extract of Red Melinjo Peel

Red melinjo that was used in this research was *Gnetum gnemon* L., Gnetaceae genus, from Bogor. The percentage of peel on red melinjo fruit was 36.10%. Fresh red melinjo peel with moisture content 73.30% decreased to 5.58% after drying process. The yield of red melinjo peel powder was 89.24%, with moisture content of 9.87%. The yield of extract produced was 1.41%.

b. Antimicrobial Assay

Antimicrobial assay of ethyl acetate extract of red melinjo peel was conducted using well diffusion method with 0, 4, 8, 12, and 16% concentrations. According to Figure 1, it can be seen that ethyl acetate extract of red melinjo peel could inhibit *E. coli*, *B. cereus*, and *P. aeruginosa* bacteria, while it could not inhibit *R. oligosporus* mould. The extract could not inhibit *R. oligosporus* due to the presence of ergosterol, a sterol that is only found in the fungal membrane. The interaction between essential oil and ergosterol will cause damage to the cell membrane, causing leakage of fungal cell [21]. There is a possibility that red melinjo peel does not contain essential oil.

Gram-negative bacteria (*E. coli* and *P. aeruginosa*) had a lower inhibition zone diameter than Gram-positive bacteria

(*B. cereus*). Gram-negative bacteria consists of two membranes (inner and outer membrane) that sandwich a layer of peptidoglycan. Meanwhile, Gram-positive bacteria has a thicker layer of peptidoglycan that surrounds a single cytoplasmic membrane. The inner membrane of Gram-negative bacteria is as same as cytoplasmic membrane of Gram-positive bacteria that contains phospholipids and proteins. The outer membrane of Gram-negative bacteria contains lipoproteins and glycolipids (in the outer leaflet consists lipopolysaccharides) which are not present in the cytoplasmic membrane [22]. The presence of outer membrane could inhibit the antimicrobial compounds to diffuse inside bacterial peptidoglycan membrane and cell [3].

Figure 2 shows that the increase of extract concentration would increase the inhibition zone diameter. This was due to the higher content of antimicrobial compounds in the extract, therefore increasing the inhibition zone. The inhibition zone diameter of more than 20 mm is classified as very strong, the inhibition zone diameter of 10-20 mm is classified as strong, the inhibition zone diameter of 5-10 mm is classified as moderate and the inhibition zone diameter of 5 mm or less is classified as weak [23]. The ethyl acetate extract of red melinjo peel could be classified as strong. Due to efficiency, the selected extract of ethyl acetate of red melinjo peel was at 12% concentration.

c. MIC, MBC, and MFC Value of The Extract

Table 1 shows the MIC, MBC, and MFC value of ethyl acetate extract of red melinjo peel against the test microbes (mikroba uji). The inhibition of the extract was more effective against Gram-positive bacteria (*B. cereus*) than Gram-negative bacteria (*E. coli* and *P. aeruginosa*). This was due to the presence of the outer membrane of Gram-negative bacteria [22] that inhibits the antimicrobial compounds to diffuse inside bacterial peptidoglycan membrane and cell [3]. According to the results of reference [4], the MIC and MBC value of ethanol extract of melinjo peel was 1.40 and 5.58% respectively, greater than ethyl acetate extract of red melinjo peel. Moreover, the ethyl acetate extract of red melinjo peel against *P. aeruginosa* bacteria has the MIC and MBC values of 1.36 and 5.43% respectively which is greater than the value in this study.

Table 1. MIC, MBC, and MFC Values of Ethyl Acetate Extract of Red Melinjo Peel Against 4 Types of Microbes

Microbes	MIC (%)	MBC (%)	MFC (%)
<i>E. coli</i>	0.69	2.76	-
<i>B. cereus</i>	0.50	2.00	-
<i>P. aeruginosa</i>	0.67	2.68	-
<i>R. oligosporus</i>	-	-	0

The differences between the MIC and MBC value can be caused by environmental factors. Phenolic compounds will be produced by plants in response to environmental stress [24]. The mineral content in the soil (such as aluminum, potassium, sulfur, sodium, and manganese),

and climate also affected the plant phenolic content [25]. The higher content of antimicrobial compounds in plants will decrease the value of MIC and MBC. The lower value of MIC and MBC shows that the component has a strong inhibition activity.

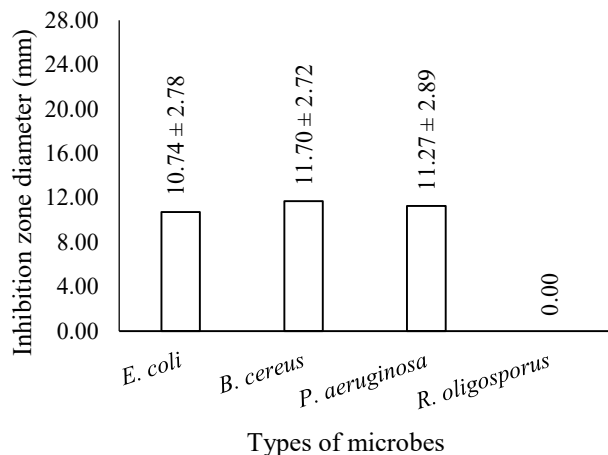


Figure 1. Inhibition Zone Diameter of Ethyl Acetate Extract of Red Melinjo Peel against 4 Types of Microbes

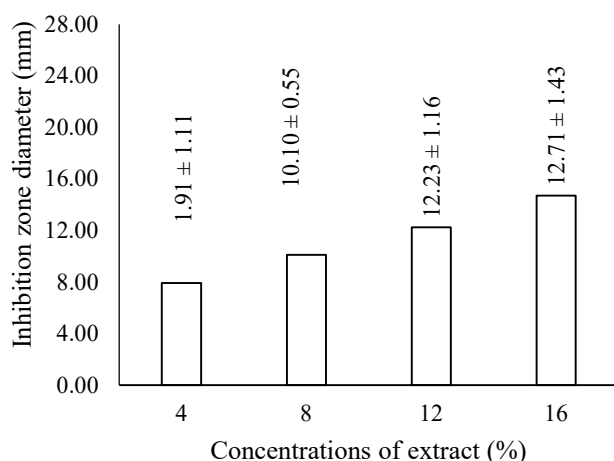


Figure 2. Average Inhibition Zone Diameter of Ethyl Acetate Extract of Red Melinjo Peel at Various Concentrations

d. Qualitative Analysis of Phytochemical Compound in Extract

The results of the qualitative analysis of phytochemical compound in the extract can be seen in Table 2. The analysis showed positive results for alkaloids, saponin, phenolics, flavonoids, and glycosides. The presence of these compounds showed that ethyl acetate could extract the compounds based on the polarity. Ethyl acetate is a semi-polar solvent that can extract alkaloids, aglycone, glycosides, sterols, terpenoids, and flavonoids [26].

e. Comparison of Selected Extract and Antibiotics

The results of ANOVA statistical analysis showed that there was significant differences ($p < 0.05$) between 1000 ppm Colistin antibiotics inhibition zones diameter with selected ethyl acetate extract of red melinjo peel (12%). Figure 3 shows the inhibition zone diameter of selected ethyl acetate extract of red melinjo peel compared to Colistin antibiotics against *E. coli* and *P. aeruginosa* bacteria. Ethyl acetate extract of red melinjo peel has an inhibition zone diameter 1.44 times greater than 1000 ppm Colistin against *E. coli*, and 1.50 times greater against *P. aeruginosa*. Overall, the inhibition zone diameter of ethyl acetate extract of red melinjo peel was 1.47 times greater than 1000 ppm Colistin.

Colistin or Polymyxin E is amphiphatic (has polar and nonpolar parts), and interacts like detergent against cell membranes, which can disrupt the structure of the cell membranes. The bond between a cationic polypeptide (Colistin) and anionic lipopolysaccharide (the outer membrane of Gram-negative bacterial cell) causes disruption of the bacterial cell membrane. Colistin replaces magnesium and calcium ions (ions that serve as lipopolysaccharide molecules stabilizer) in anionic lipopolysaccharides, resulting in an unstable cell membrane structure, increased cell permeability, leakage of cell contents, and cell death [27].

The results of ANOVA statistical analysis showed that there was a significant difference ($p < 0.05$) between 1000 ppm Penicillin G antibiotics inhibition zone diameter and selected ethyl acetate extract of red melinjo peel (12%). Based on Figure 4, ethyl acetate extract of red melinjo peel has inhibition diameter 0.74 times smaller than 100 ppm Penicillin G antibiotics. Penicillin G works as transpeptidase enzyme inhibitor, one of the Penicillin binding proteins (PBPs), that builds bacterial cell wall [28]. Penicillin is a structural analog in the form of D-alanyl-D-alanyl, so it may bind with transpeptidase (acyl-D-alanyl-D-alanyl structure) that catalyzes the crosslinking reaction.

f. Stability of Selected Extract against pH

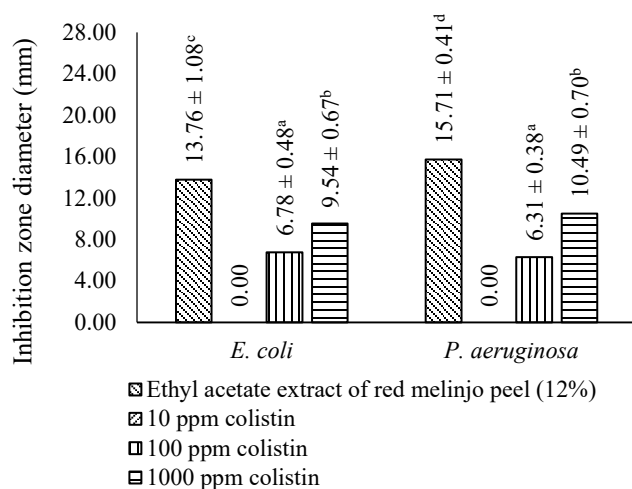
The results of ANOVA statistical analysis showed that pH has a significant effect ($p < 0.05$) on the inhibition zone diameter. Based on Figure 5, it can be seen that at pH 4 the selected ethyl acetate extract of red melinjo peel has a greater inhibition zone diameter than pH 7. This showed that the lower the pH, the greater the inhibition zone diameter of ethyl acetate extract of red melinjo peel.

This study has similar results to another research, that ethyl acetate extract of akway bark has a greater inhibition zone diameter at pH 4 than pH 7 [29]. In acid condition, the pH of cell cytoplasm will decrease, causing disruption of enzyme activity inside the cell [30]. Acid can cause damage of the outer cell membrane and lead to penetration of hydrophobic antibacterial compound inside cell [31].

Table 2. The Results for Qualitative Analysis of Phytochemical Compound in Ethyl Acetate Extract of Red Melinjo Peel

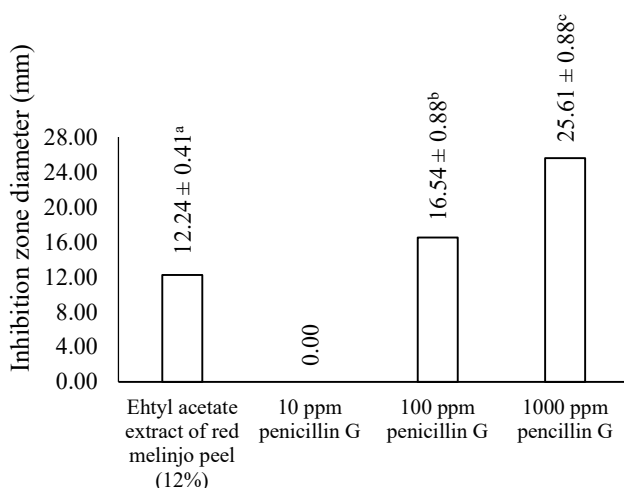
Phytochemicals analysis	Results
Alkaloids	+
Saponin	+
Tannin	-
Phenolics	+
Flavonoids	+
Triterpenoids	-
Steroids	-
Glycosides	+

Note: (+) : positive, (-) : negative



Note: ^{a,b,c,d} same superscripts indicate there is no significant difference at $\alpha = 5\%$

Figure 3. Inhibition Zone Diameter of Ethyl Acetate Extract of Red Melinjo Peel Compared with Colistin Antibiotics



Note: ^{a,b,c} same superscripts indicate there is no significant difference at $\alpha = 5\%$

Figure 4. Inhibition Zone Diameter of Ethyl Acetate Extract of Red Melinjo Peel Compared with Penicillin G Antibiotics

g. Stability of Selected Extract against Heating Temperature

The results of ANOVA statistical analysis showed an interaction between type of microbes and heating temperature significantly affect ($p < 0.05$) to the inhibition zone diameter. Figure 6 shows the inhibition zone diameter of the ethyl acetate extract of red melinjo peel at various heating temperatures. The extract that was heated at 65°C has the greatest inhibition zone diameter than the other treatments. This was caused by the activation of flavonoids that was heated up to 70°C [32]. This study has a similar result, which heating at 75, 85, and 95°C would lead to damage of the active compounds, causing the inhibition zone diameter to decrease [32].

Extract that was heated at 95°C has the lowest inhibition zone diameter, which shows that the antimicrobial compounds are damaged. This study has similar results to another research, that heated garlic extract at 100°C showed no inhibition zone diameter compared to the extract that was heated at 65°C [33]. The result of another research also showed that increasing heating temperature will damage the flavonoids compounds [34].

h. Stability of Selected Extract against Salt Concentrations

The results of ANOVA statistical analysis showed interaction between type of microbes and salt concentrations significantly affect ($p < 0.05$) the inhibition zone diameter. Figure 7 shows the inhibition zone diameter of ethyl acetate extract of red melinjo peel at various salt concentrations against *E. coli*, *B. cereus*, and *P. aeruginosa*. Increasing salt concentrations will increase the inhibition zone diameter. Therefore, it can be stated that there is synergism between ethyl acetate extract of red melinjo peel and salt concentrations.

Salt has the bactericidal (killing) and bacteriostatic (inhibits) properties. Salt will inhibit microbes bacteriological and enzymatic activities. Sodium chloride salt can increase substrate's osmotic pressure, causing the water in the cell to come out, the cell shrinks, and inhibits microorganism activity. The ionization of sodium chloride will produce chlorine ions which are toxic to microorganisms, which also block the respiration system [35].

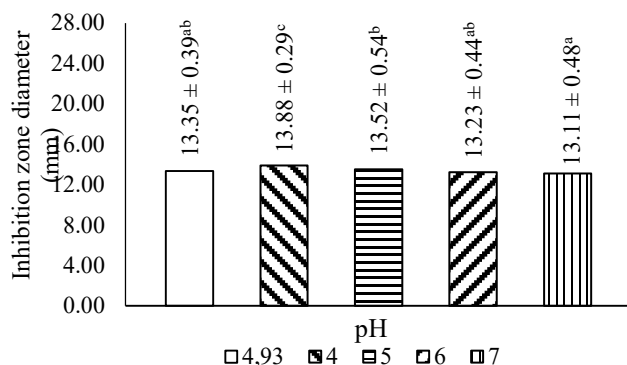
i. Stability of Selected Extract against Sugar Concentrations

The results of ANOVA statistical analysis showed that type of microbes has a significant effect ($p < 0.05$) on the inhibition zone diameter. The type of microbes (Gram-positive and Gram-negative bacteria) will affect the inhibition zone diameter. Figure 8 shows the inhibition zone diameter of ethyl acetate extract of the red melinjo peel against *E. coli*, *B. cereus*, and *P. aeruginosa*. Gram-negative bacteria (*E. coli* and *P. aeruginosa*) has the greater inhibition zone diameter than Gram-positive bacteria (*B. cereus*). This was due to the presence of the outer membrane of Gram-negative bacteria [22] that

inhibits the antimicrobial compounds to diffuse inside bacterial peptidoglycan membrane and cell [3]. The presence of sugar bonds causing the flavonoids to be more soluble in water, so that the ethyl acetate fraction easily diffused and penetrate to *B. cereus* cell wall that contains protein (polar), phospholipids and lipoproteins (nonpolar) [36].

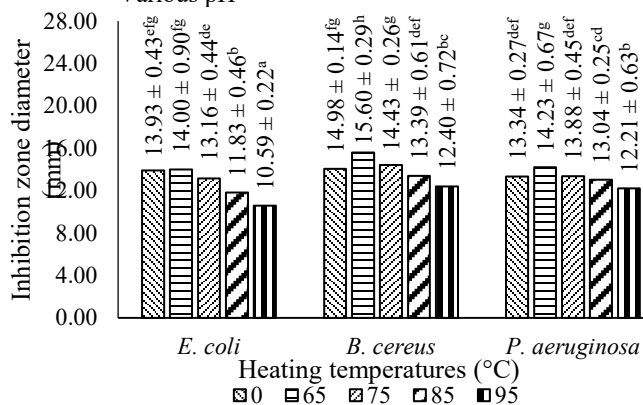
Figure 9 shows that the increase of sugar concentrations will increase the inhibition zone diameter of ethyl acetate extract of red melinjo peel. Reference [37] stated that 10% sugar solution can increase the osmotic pressure, causing the water inside of the cell to come out. The cell will lack of water and leading cell death by lysis.

Sucrose is a disaccharide that is used as a preservative [38]. Increasing sugar concentrations will increase the osmotic pressure outside of the cell, causing the water inside of the cell to come out, and leading to cell death by lysis [39]. If the osmotic pressure outside of the cell is higher than inside as the sugar addition, then the water inside of the cell will be pulled out, causing the cytoplasm membrane to detach form the cell wall (plasmolysis). As the water release inside of the cell causing the cell to shrink [38].



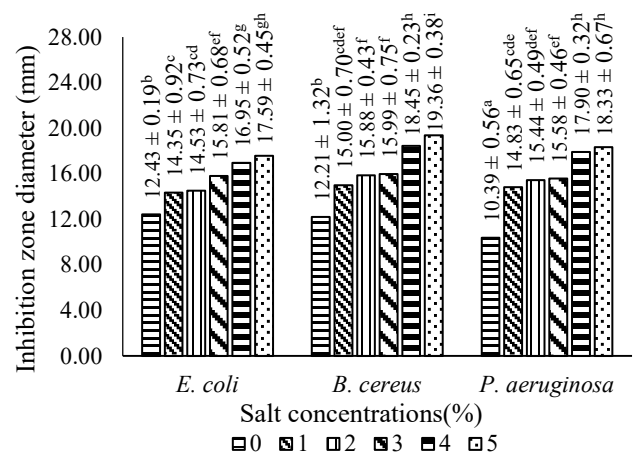
Note: ^{a,b,c} same superscripts indicate there is no significant difference at $\alpha = 5\%$

Figure 5. Average Inhibition Zone Diameter of Selected Ethyl Acetate Extract of Red Melinjo Peel at Various pH



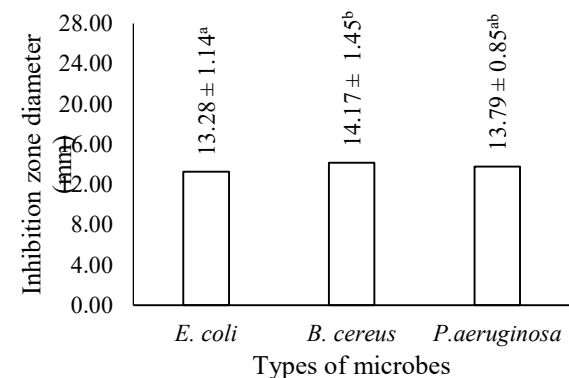
Note: ^{a,b,c,d,e,f,g,h} same superscripts indicate there is no significant difference at $\alpha = 5\%$

Figure 6. Inhibition Zone Diameter of Selected Ethyl Acetate Extract of Red Melinjo Peel at Various Heating Temperatures



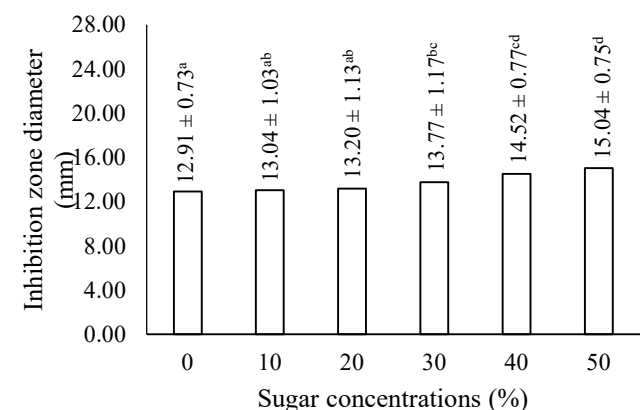
Note: ^{a,b,c,d,e,f,g,h,i} same superscripts indicate there is no significant difference at $\alpha = 5\%$

Figure 7. Inhibition Zone Diameter of Selected Ethyl Acetate Extract of Red Melinjo Peel at Various Salt Concentrations



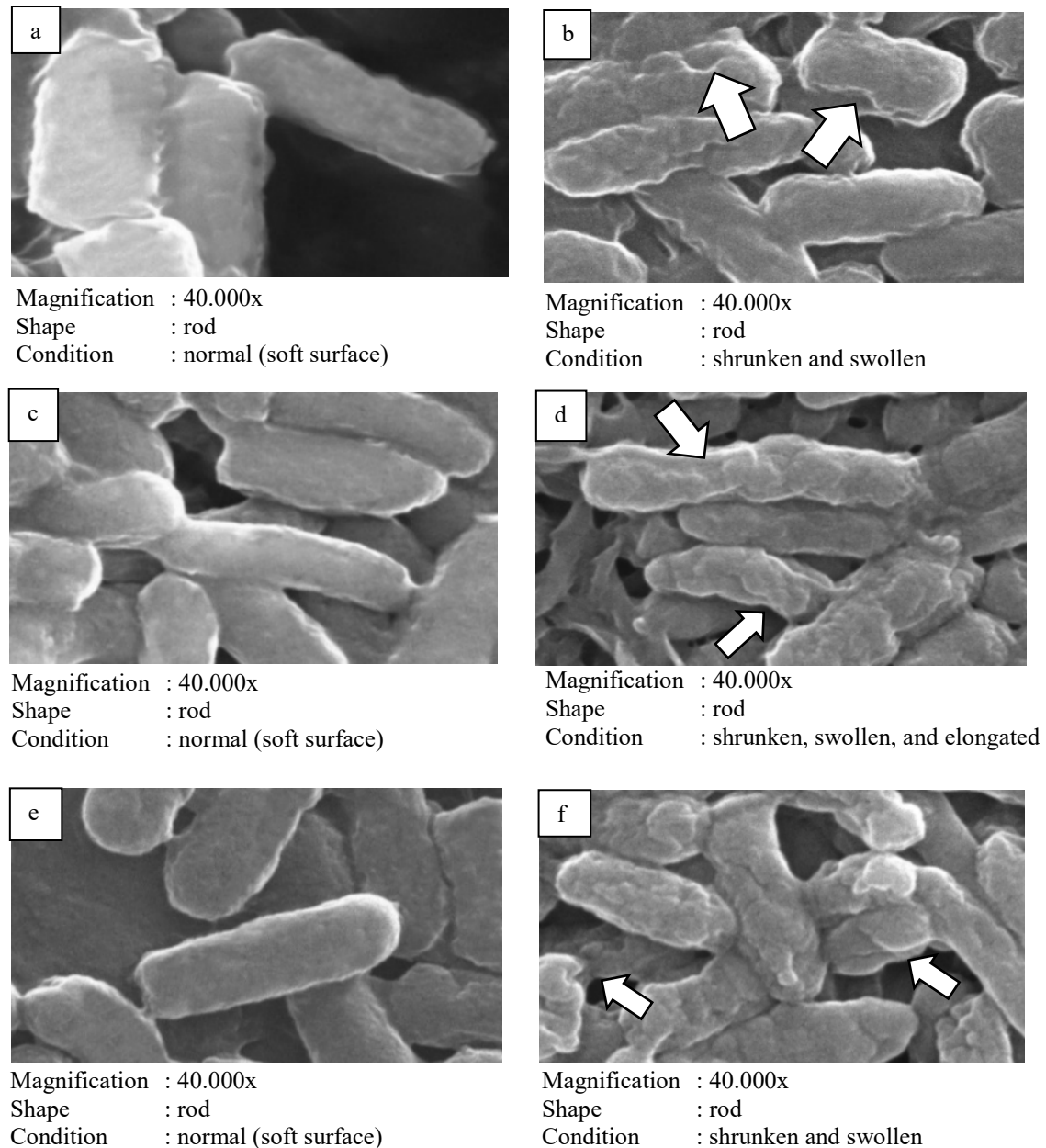
Note: ^{a,b} same superscripts indicate there is no significant difference at $\alpha = 5\%$

Figure 8. Average Inhibition Zone Diameter of Ethyl Acetate Extract of Red Melinjo Peel at Various Sugar Concentrations



Note: ^{a,b,c,d} same superscripts indicate there is no significant difference at $\alpha = 5\%$

Figure 9. Average Inhibition Zone Diameter of Ethyl Acetate Extract of Red Melinjo Peel at various Sugar Concentrations against *E. coli*, *B. cereus*, and *P. aeruginosa*



Note:

E. coli bacteria before (a) and after (b) contacted with extract

B. cereus bacteria before (c) and after (d) contacted with extract

P. aeruginosa bacteria before (e) and after (f) contacted with extract

Figure 10. The Results of SEM Analysis on *E. coli*, *B. cereus*, and *P. aeruginosa* before and after contacted with Ethyl Acetate Extract of Red Melinjo Peel

j. Leakage Analysis using AAS

The detected ions on spectrophotometer instrument using AAS method indicates the leakage of microbial cell. Based on Table 3, it can be stated that ethyl acetate extract of red melinjo peel could damage the cell membrane and causing the leakage of *E. coli*, *B. cereus*, and *P. aeruginosa*.

Sodium, potassium, calcium, and magnesium are ions contained in bacteria [40]. Potassium ions located in the cytoplasm membrane as membrane transport [41]. Calcium ions serve as a messenger that brings the signal from the surface to the inner part of the cell [42]. Meanwhile, magnesium ions serve as membrane cell constituent [43].

k. SEM Analysis

Before exposure to ethyl acetate extract of red melinjo peel, the bacterial cell has a normal shape and soft surface (Figure 10 (a), (c), and (e)). However, after contacting with extract for 24 hours, the cells were damaged. The damages included shrinkage, cell swelling, cell surface tended to be rough, irregular shape (Figure 10 (b), (d), and (f)), and elongation (10(d)). The cell morphology damage is also supported by the AAS analysis on Table 3, that shows the cell leakage so calcium, magnesium, and potassium ions released from bacteria cell that already contacted with the extract.

E. coli and *P. aeruginosa* shrank and swelled after contacting with extract (Figure 10 (b) and (f)). However, *B. cereus* shrank, swelled, and elongated after contacted with the extract (Figure 10 (d)). The bacterial cell shrinkage is caused by antimicrobial compounds that enter due to the increase of cell membrane permeability. Phytochemical compounds contained in an extract that caused cell damage were alkaloids [44], saponin [45], phenolics [46], and flavonoids [47]. The bacterial cell shrinkage is caused by the differences in osmotic pressure, causing cytoplasm to come out, and leading to crenation (cell shrinkage). Meanwhile, changes of bacterial cell shape are caused by penetration of ethyl acetate extract of red melinjo peel and lead to cell swelling [4].

Table 3. The Results of AAS Analysis against *E. coli*, *B. cereus*, and *P. aeruginosa* after contacted with Selected Ethyl Acetate Extract of Red Melinjo Peel

Microbes	Amount of Ca^{2+} (mg/L)	Amount of Mg^{2+} (mg/L)	Amount of K^{+} (mg/L)
<i>E. coli</i>	79	41.2	314
<i>B. cereus</i>	84.2	33.3	348
<i>P. aeruginosa</i>	82	31.3	327

IV. CONCLUSIONS

Results of the study showed that ethyl acetate extract of red melinjo peel could inhibit *E. coli*, *B. cereus*, and *P. aeruginosa*, but could not inhibit *R. oligosporus*. The average of inhibition zone diameter at 4, 8, 12, and 16% concentrations are 10.74 mm, 11.70 mm, and 11.27 mm respectively. The MIC values against *E. coli*, *B. cereus*, and *P. aeruginosa* are 0.69%, 0.50%, and 0.67% respectively. While the MBC values are 2.76%, 2.00%, and 2.68%. The selected ethyl acetate extract of red melinjo peel was at 12% concentration that was used for the next analysis. Phytochemical compounds contained in the ethyl acetate extract of red melinjo peel was alkaloids, phenolics, flavonoids, saponin, and glycosides, that has antimicrobial properties.

The antibacterial activity of the selected ethyl acetate extract of red melinjo peel 1.47 times greater than 1000 ppm Colistin, while 0.74 times smaller than 100 ppm Penicillin G antibiotics. The ethyl acetate extract of red

melinjo peel has greater antibacterial activity at pH 4 than pH 7. Heating at 65°C would increase the antibacterial activity, while heating at 75, 85, and 95°C would damage the phytochemical compounds, causing the inhibition zone diameter to decrease. Addition of salt up to 5% and sugar up to 50% could increase the antibacterial activity of the ethyl acetate extract of the red melinjo peel.

The results of AAS analysis showed that *E. coli*, *B. cereus*, and *P. aeruginosa* leaked after contacted with ethyl acetate extract of red melinjo peel. Calcium, magnesium, and potassium ions were released from the bacterial cell membrane. As a result of contacting bacterial cells with the selected extract, there was shrinkage, swelling, elongation, and the cell surface became rough.

V. SUGGESTIONS

The suggestions that can be given for further study is to conduct phytochemical analysis quantitatively, to know the alkaloids, phenolics, flavonoids, saponins, and alkaloids content. Temperature and heating time treatment can be added in the extract stability test against heat treatment. In vivo testing of melinjo peel is necessary against experimental animals.

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