Characterization and Stability of Red Melinjo (*Gnetum gnemon* L.) Extract as Antibacterial Compound

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ABSTRACT

Melinjo (*Gnetum gnemon* L.), a species widely found in Southeast Asia including Indonesia, has peels rich in bioactive molecules like flavonoids, tannins, and saponins, all of which exhibit antimicrobial activity. However, the peel is usually discarded. The present research focuses on evaluating the antimicrobial properties of extracts derived from melinjo peel. The red melinjo peel was extracted using the maceration method in ethyl acetate at room temperature for 24 hours. Extract concentrations of 4–16% (w/v) were able to inhibit the growth of *Pseudomonas aeruginosa* ATCC 9027, *E. coli* ATCC 8739, and *B. cereus* ATCC 10876, but showed no inhibitory effect against *R. oligosporus* ATCC 22959. 0.50 to 0.69% was the Minimum Inhibitory Concentration (MIC) while the Minimum Bactericidal Concentration (MBC) value ranged from 2.00 to 2.76% of the extract. In 12% concentration, the extract has a similar inhibitory capacity of 1000 ppm Colistin against *P. aeruginosa* and *E. coli*. In pH 4, the inhibition of the extract increased and decreased in neutral pH. 30 minutes of 65^{o}C heat treatment increased the antibacterial activity but decreased at 75 to 95^{o}C. Salt and sugar with concentrations of 1 to 5% and 10 to 50%, also increased the antibacterial activity of the extract.

Keywords: Antimicrobial, Cell damage, *Gnetum gnemon* L., Melinjo

INTRODUCTION

Melinjo (*Gnetum gnemon* L.) is one of Southeast Asia's native plant and the western Pacific region, cultivated widely from Assam to Fiji. In Indonesia, melinjo is well known as the main ingredient in *emping* (chips made from its seeds) and used as a vegetable in various traditional dishes (Barua et al., 2015). According to data from the Badan Pusat Statistik (2015), melinjo output climbed from 197,648 tons in 2014 to 213,025 tons in 2015. As production expanded, the amount of discarded melinjo peel also grew, despite the peel's considerable potential as a source of bioactive materials that remains largely untapped.

Melinjo peel, although occasionally consumed as a vegetable, is generally discarded as waste. However, it contains diverse bioactive compounds such as tannins, flavonoids, saponins, phenolic compounds, β-carotene, lycopene, carotenoids, and vitamin C (Barua et al., 2015; Angel & Parhusip, 2024). These compounds exhibit strong biological activities including antibacterial, antioxidant, anti-inflammatory, and pigment-related properties. Phenolic compounds serve as antibacterial agents due to the reactive hydroxyl groups in their structure that can disrupt bacterial membranes (Wulandari et al., 2018). Among the various types, red melinjo peel has been reported to contain the highest total phenolic content compared to the yellow and green varieties. Parhusip et al. (2019) demonstrated that an ethyl acetate extract of red melinjo peel at a 15% concentration effectively inhibited *Pseudomonas aeruginosa* growth, producing a clear zone of 6.73 mm—greater than that of Penicillin G, which showed no inhibition.

In previous studies, Angel & Parhusip (2024) tested four indicator microorganisms: *E. coli* (Gram-negative), *B. cereus* (spore-forming Gram-positive), *P. aeruginosa* (Gram-negative), and *R. oligosporus* (mold), while Parhusip et al. (2019) focused on *Staphylococcus aureus*, *Salmonella Typhi, Listeria monocytogenes*, and *Candida albicans,* which represent both Gram-positive and Gram-negative bacteria, molds and yeasts, as such providing a broader understanding of antimicrobial activity. However, there has been no research specifically investigating the stability of ethyl acetate extracts of red melinjo peel under various food processing conditions, using representative foodborne pathogenic and spoilage microorganisms, combined with morphological damage analysis via Scanning Electron Microscopy (SEM).

Therefore, this study aims to characterize the bioactive components extracted with ethyl acetate and evaluate their antimicrobial activity against representative foodborne microbes, namely *B. cereus* (Gram-positive), *E. coli* (Gram-negative), *P. aeruginosa* (pathogenic and spoilage bacterium), and *R. oligosporus* (mold). Furthermore, the study examines the stability of the extract under different food processing factors, including pH (4, 5, 6, and 7), heating temperature (65, 75, 85, and 95°C for 30 minutes), salt concentration (1–5%), and sugar concentration (10–50%). These parameters simulate common conditions in food processing, where salt levels above 5% and sugar levels exceeding 40% can act as preservatives (Utomo et al., 2015; Yusmita, 2018). The morphological alterations of microbial cells after extract exposure were analyzed using SEM to provide deeper insight into the mode of action of the extract

MATERIALS AND METHODS

Materials and Equipment

The materials used in this research consisted of red melinjo peel collected from Bogor, along with several solvents such as ethyl acetate, ethanol, amyl alcohol, chloroform, benzene, ether, and demineralized water. Chemical reagents included concentrated and 4 N sulfuric acid (H_2SO_4), concentrated hydrochloric acid (HCl) along with its 1 M and 2 N solutions, ammonia, acetic acid anhydrous, sodium chloride solution, 0.5 M potassium hydroxide (KOH),

sugar solution, 5% hydrogen peroxide, KH_2PO_4, and iron(III) chloride at concentrations of 1% and 5%. Phytochemical identification was supported by Dragendorff, Meyer, and Wagner reagents. Microbiological analysis employed Nutrient Agar (NA) and Nutrient Broth (NB) media, together with reference cultures acquired from the Institut Pertanian Bogor (IPB), including *E. coli* ATCC 8739, *B. cereus* ATCC 10876, *R. oligosporus* ATCC 22959, *P. aeruginosa* ATCC 9027. Staining processes made use of 96% alcohol, crystal violet, lugol, immersion oil, and safranin, Additional laboratory supplies and materials, such as magnesium ribbon, No. 1 Whatman filter paper, and aluminum foil were also used.

The main equipment included a cabinet dryer, autoclave, microscope, incubator, rotary evaporator, centrifuge, pH meter, analytical balance, water bath, oven, magnetic stirrer, and analytical instruments such as AAS and SEM.

Preparation and Extraction of Red Melinjo Peel Powder (Ahmad et al., 2018 with modification)

Red melinjo peel was washed, peeled, and the seeds were discarded. The peel was dried at 50°C for 24 hours, ground, and sieved through a 35-mesh screen. Extraction was done by mixing the dried powder with ethyl acetate in a 1:4 (w/v) proportion and shaking it at 110 rpm. The mixture was subsequently filtered through Whatman No. 1 filter paper. The filtrate was evaporated at 55°C using a rotary evaporator, and the concentrated residue was dried with nitrogen gas to yield the red melinjo peel ethyl acetate extract.

1. Characterization Test of Ethyl acetate in Red Melinjo Peel Extract
   A. Antimicrobial Assay

      Different concentrations of the red melinjo peel ethyl acetate extract (0, 4, 8, 12, and 16%) were dispensed into 6-mm wells, with each well receiving 60 µL on NA plates inoculated with the test microbes. The plates were then incubated at 37°C for 24 hours, after which the diameter of the resulting inhibition zones was recorded.

   B. Phytochemical Compound Qualitative Determination

      Phytochemical compound that was determined includes phenolic (Parhusip, et al., 2022), flavonoids (Nugrahani et al., 2016), alkaloids, steroids and triterpenoids, terpenoids, tannins (Parhusip, et al., 2020), saponin (Asmara, 2017), and glycosides (Parhusip, et al., 2020).

   C. Antibiotics Testing Comparison

      The comparison of antibiotic activity was examined using the well-diffusion method, following the approach of Parhusip et al. (2020). Penicillin G and Colistin were selected as reference antibiotics. Each antibiotic solution was prepared at concentrations of 10, 100, and 1000 ppm, and 60 µL of each was introduced into 6-mm wells on Nutrient Agar plates. The plates were incubated at 37°C for 24 hours, and afterward the inhibition zones surrounding each well were measured.

2. Stability Test of Ethyl Acetate Extract from Red Melinjo Peel Against Several Food Processing Factors
   A. Stability Assessment of the Extract under pH, Thermal Treatment, and Salt–Sugar Conditions

      The stability of the selected ethyl acetate extract was tested under different conditions using the well diffusion method (Parhusip et al., 2020). The extract was evaluated at pH 4–7, heating temperatures of 65, 75, 85, and 95°C (30 min in a water bath), salt concentrations of 1–5%, and sugar concentrations of 10, 20, 30, 40, and 50% to assess changes in antimicrobial activity.

   B. Atomic Absorption Spectroscopy (AAS) (Parhusip and Sitanggang, 2024, with modification)

The AAS analysis was conducted to evaluate the leakage of intracellular components by measuring the release of calcium (Ca^{2+}), potassium (K^{+}), and magnesium (Mg^{2+}) ions. Bacterial cultures were first reactivated in Nutrient Broth (NB) for 24 hours. Afterward, 12% of the red melinjo peel ethyl acetate extract was introduced into the culture and incubated again at 37°C for another 24 hours. The treated samples were then delivered to the Pusat Penelitian Ilmu Pengetahuan dan Teknologi (Puspiptek) for ion quantification. Calcium ions were detected using AAS at 422.7 nm, potassium at 766.5 nm, and magnesium at 285.2 nm.

3. Morphological Evaluation of Cellular Damage
   A. Scanning Electron Microscope (SEM) (Parhusip and Sitanggang, 2024 with modification)

For the SEM analysis, the technique was employed to observe structural alterations in bacterial cells exposed to the selected ethyl acetate extract from red melinjo peel. Cultures previously grown in NB at 37°C for 24 hours were mixed with the extract at a concentration of 12% and homogenized using a vortex mixer. After being incubated at 37°C for 24 hours, the culture was centrifuged at 15,000 rpm for 7 minutes to isolate the pellet. The supernatant was removed, and the pellet was forwarded to Institut Teknologi Bandung (ITB) for analysis. The pellet was mounted on a carbon-coated stub, coated with a thin layer of gold under vacuum conditions, and then placed into the SEM instrument for imaging.

Experimental Design

There were two factors in the first stage of the study: the concentration of the extract (0, 4, 8, 12, and 16%) and the type of test microbial culture (*Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Rhizopus oligosporus*). Each treatment was replicated twice. A Completely Randomized Design (CRD) was applied as the experimental design in this research.

RESULT AND DISCUSSION

Antimicrobial Assay

To test the antimicrobial activity of the ethyl acetate extract of red melinjo peel, well diffusion method was used using 0, 4, 8, 12, and 16% concentrations. As shown in Figure 1a, the extract inhibited *Escherichia coli*, *Pseudomonas aeruginosa,* and *Bacillus cereus*, but not *Rhizopus oligosporus*. The lack of inhibition against *R. oligosporus* may be due to ergosterol in fungal membranes and the low essential oil content of red melinjo peel (Parhusip et al., 2020).

Gram-negative bacteria (*E. coli* and *P. aeruginosa*) showed smaller inhibition zones than the Gram-positive *B. cereus* because their outer membrane, rich in lipopolysaccharides, limits antimicrobial penetration (Baurain et al., 2016).

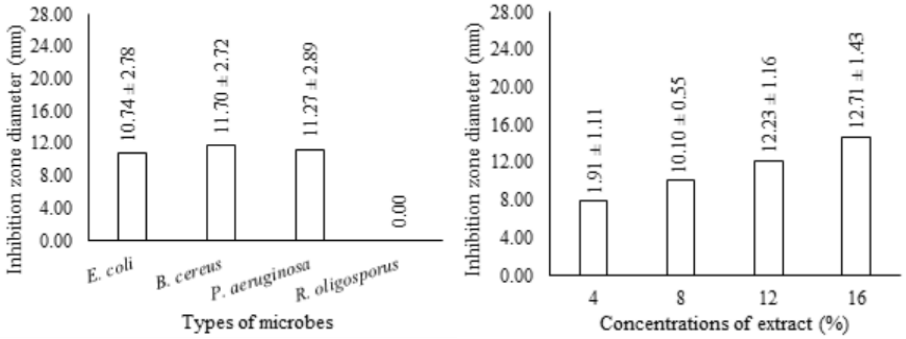


Figure 1a. Inhibition Zone Diameter the Peel Extract against the 4 Microbes, 1b. Average Inhibition Zone Diameter the Peel Extract at Various Concentrations

As shown in Figure 1b, the inhibition zone increased with higher extract concentrations, indicating a greater presence of antimicrobial compounds. Based on Widyasanti et al. (2016), inhibition zones above 20 mm are considered very strong, 10–20 mm strong, 5–10 mm moderate, and below 5 mm weak. The red melinjo peel extract demonstrated a strong inhibitory effect. The 12% concentration was selected for further analysis due to its effectiveness and efficiency.

MIC, MBC, and MFC Value of The Extract

Table 1 presents the MIC (Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentration), and MFC (Minimum Fungicidal Concentration) of the extract against the tested microorganisms. The extract displayed stronger inhibitory activity against the Gram-positive *B. cereus* compared to the Gram-negative *E. coli* and *P. aeruginosa*. This difference is likely related to the additional outer membrane present in Gram-negative bacteria, which can restrict the entry of antimicrobial agents (Baurain et al., 2016).

Table 1. MIC, MBC, and MFC of Red Melinjo Peel Ethyl Acetate Extract for Four Microbes

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

In comparison, Octavia (2010) documented MIC and MBC values of 1.40% and 5.58% for the ethanol-based extract of melinjo peel. The ethyl acetate extract evaluated in the present work exhibited superior antimicrobial activity, as reflected by its lower values when tested against *P. aeruginosa*. These differences may result from environmental factors, as Rezende et al. (2015) noted that phenolic production is influenced by stress, soil mineral content, and climate-factors that affect antimicrobial compound levels and, consequently, MIC and MBC values.

Qualitative Analysis of Phytochemical Compounds in the Extract

The qualitative analysis of phytochemicals in the ethyl acetate extract is summarized in Table 2. The extract tested positive for flavonoids, alkaloids, phenolics saponins, and glycosides. These results suggest that ethyl acetate, a semi-polar solvent, effectively extracts a wide range of bioactive compounds. According to Parhusip, et al., (2020), ethyl acetate can dissolve alkaloids, aglycones, glycosides, sterols, terpenoids, and flavonoids.

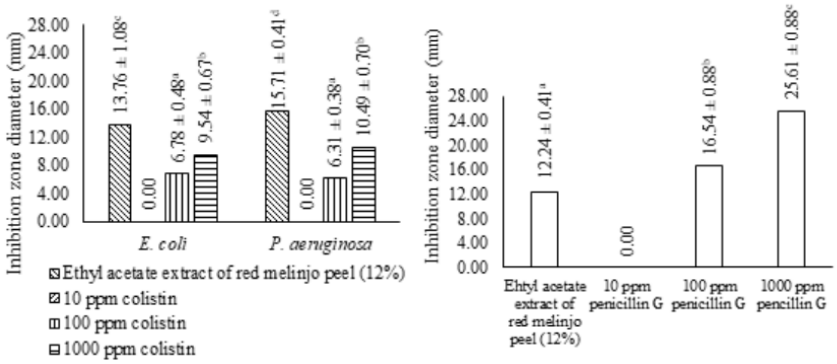
Table 2. Results of Phytochemical Compounds Present in the Red Melinjo Peel Ethyl Acetate Extract

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

Comparison of Selected Extract and Antibiotics

The ANOVA analysis revealed that the inhibition zones formed by the 1000 ppm Colistin treatment differed significantly ($p < 0.05$) from those produced by the 12% ethyl acetate

extract of red melinjo peel. As illustrated in Figure 2a, the inhibitory activity of the extract was compared with Colistin against *Pseudomonas aeruginosa* and *Escherichia coli*. The extract produced an inhibition zone that was approximately 1.44 times larger than that of 1000 ppm Colistin when tested against *E. coli*, and about 1.50 times larger against *P. aeruginosa*. When averaged across both bacterial species, the extract demonstrated an inhibition diameter that was roughly 1.47 times greater than the value recorded for 1000 ppm Colistin.



Note: Identical superscript letters (a–d) denote groups that are not statistically different at α = 0.05.

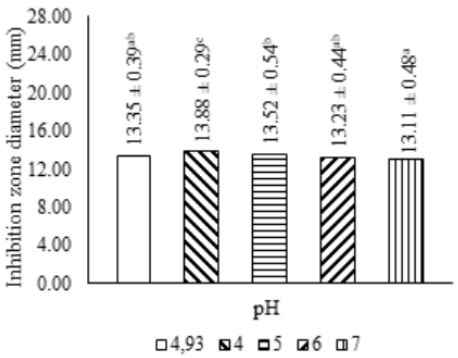
Figure 2a. Comparison of inhibition zones between the peel extract and Colistin; Figure 2b. Comparison of inhibition zones between the peel extract and Penicillin G.

Colistin (polymyxin E) is amphipathic and disrupts bacterial membranes by binding to anionic lipopolysaccharides in Gram-negative bacteria. It replaces stabilizing magnesium and calcium ions, increasing membrane permeability, causing leakage, and leading to cell death (El-Sayed et al., 2020).

ANOVA analysis indicated that the inhibition zone diameter of 1000 ppm Penicillin G differed significantly ($p < 0.05$) from that of the 12% red melinjo peel ethyl acetate extract. As shown in Figure 2b, the extract inhibition zone was 0.74 times smaller. Penicillin G inhibits transpeptidase enzymes (PBPs) that cross-link peptidoglycan, blocking bacterial cell wall synthesis (Dowling et al., 2017).

Effect of pH Variation on the Stability of the Selected Extract

ANOVA revealed a significant impact of pH on the size of the inhibition zone ($p < 0.05$). According to Figure 3, the extract's inhibitory effect was greater at pH 4 than at neutral pH, indicating that the antimicrobial potency of the ethyl acetate extract improves in more acidic environments.



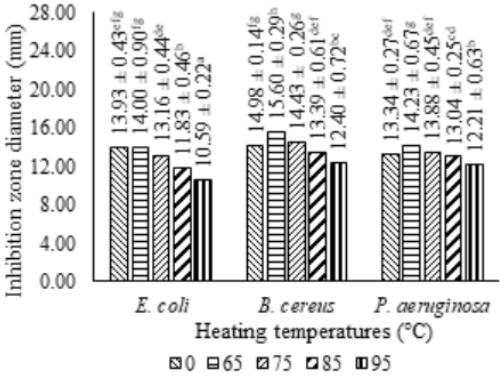
Note: Identical superscript letters (a–c) denote groups that are not statistically different at α = 0.05.

Figure 3. Average Antimicrobial Inhibition Zones Produced by the Selected Peel Extract Under Different pH Conditions for *E. coli*, *B. cereus*, and *P. aeruginosa*

This aligns with the work of Cepeda (2015), which showed that akway bark extract in ethyl acetate displayed higher antimicrobial activity at pH 4 than at pH 7. According to Ma et al. (2024), acidic conditions disrupt microbial enzyme activity and damage outer membranes, enabling hydrophobic antimicrobial compounds to penetrate cells.

Effect of Temperature Variation on the Stability of the Selected Extract

ANOVA results showed that microbial type and heating temperature significantly affected the inhibition zone diameter ($p < 0.05$). As shown in Figure 4, the extract heated at 65°C produced the largest inhibition zone, likely due to flavonoid activation up to 70°C (Jahan et al., 2015). Higher temperatures (75–95°C) reduced activity because of compound degradation, consistent with Jahan *et al.* (2015), who reported loss of antimicrobial effect at 100°C, and Aulia & Widjanarko (2018), who found that increased temperatures degrade flavonoids.



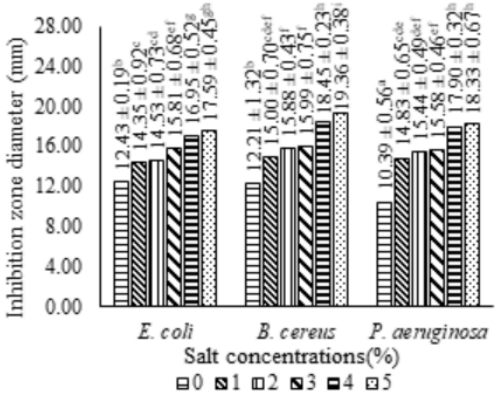
Note: Identical superscript letters (a–h) denote groups that are not statistically different at $\alpha = 0.05$.

Figure 4. Average Antimicrobial Inhibition Zones Produced by the Selected Peel Extract Under Different Temperature Conditions for *E. coli*, *B. cereus*, and *P. aeruginosa*

Effect of Salt Concentration Variation on the Stability of the Selected Extract

ANOVA statistical analysis indicated that salt concentration and microbial type significantly affected ($p < 0.05$) the inhibition zone diameter. As seen in Figure 5, the inhibition zone increased with higher salt concentrations, suggesting a synergistic effect between the ethyl acetate extract and salt.

Amalia (2016) stated that salt has bacteriostatic and bactericidal properties. Sodium chloride increases osmotic pressure in the surrounding medium, forcing water out of microbial cells, leading to shrinkage and reduced enzymatic and biological activity. The resulting chlorine ions from salt ionization are also toxic to microbes and interfere with microbial respiration.

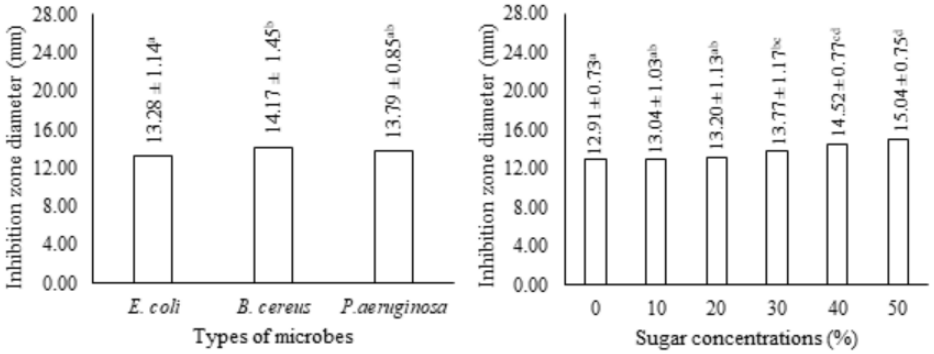


Note: Identical superscript letters (a–i) denote groups that are not statistically different at $\alpha = 0.05$.

Figure 5. Average Antimicrobial Inhibition Zones Produced by the Selected Peel Extract Under Different Salt Concentrations for *E. coli*, *B. cereus*, and *P. aeruginosa*

Effect of Sugar Concentration Variation on the Stability of the Selected Extract

ANOVA results indicated that the microbial species tested had a significant influence on the inhibition zone diameter ($p < 0.05$). Based on Figure 6a, the ethyl acetate extract yielded varying inhibition zones across the three tested microbes: *E. coli*, *B. cereus*, and *P. aeruginosa*. Unexpectedly, *E. coli* and *P. aeruginosa* both Gram-negative displayed larger zones of inhibition than the Gram-positive *B. cereus*. This contrasts with the typical understanding that Gram-negative bacteria's outer membrane limits antimicrobial compound entry (Baurain et al., 2016). The increased water solubility of flavonoids due to sugar bonding may facilitate the diffusion of the ethyl acetate fraction. These compounds can penetrate the *B. cereus* cell wall, which contains both polar protein components and non-polar phospholipid and lipoprotein structures (Naufalin, 2017).



Note: Identical superscript letters (a–d) denote groups that are not statistically different at $\alpha = 0.05$.

Figure 6a. Comparison of average inhibition zones of the peel extract for *E. coli*, *B. cereus*, and *P. aeruginosa* at multiple sugar concentrations; Figure 6b. Effect of sugar concentration on the mean inhibition zone diameter of the peel extract for the same 3 tested microorganisms.

As shown in Figure 6b, increasing sugar concentrations led to increased inhibition zone diameters. This is because sugar solutions (e.g., 10% sucrose) can increase osmotic pressure, drawing water out of microbial cells, and eventually causing cell lysis (Naufalin & Herastuti, 2013).

Sucrose, a disaccharide, is widely used as a natural preservative (Naufalin & Rukmini, 2017). According to Lopez & Hall (2023), the increasing osmotic pressure outside the cell due to added sugar causes intracellular water to be pulled out, resulting in plasmolysis—a condition where the cytoplasmic membrane detaches from the cell wall and the cell shrinks. This ultimately leads to microbial cell death.

Leakage Analysis using AAS

Ion leakage from microbial cells was analyzed using Atomic Absorption Spectrophotometry (AAS). As shown in Table 3, the ethyl acetate extract of red melinjo peel caused leakage in *P. aeruginosa, E. coli*, and *B. cereus* as evidenced by the presence of sodium, potassium, calcium, and magnesium ions in the supernatant.

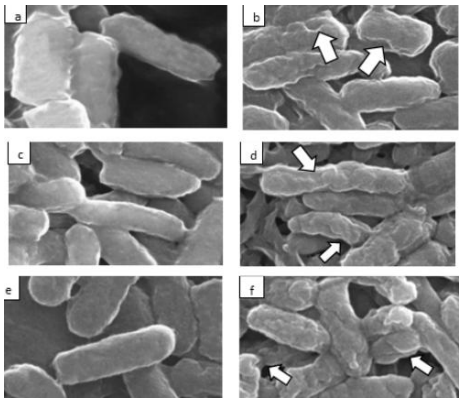
Table 3. Results of AAS Analysis of *P. aeruginosa, B. cereus*, and *E. coli* After Contact with the Selected Ethyl Acetate Extract from Red Melinjo Peel

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

These ions are essential components of bacterial cells. Potassium ions are primarily located in the cytoplasmic membrane and are involved in membrane transport. Calcium ions act as secondary messengers, transmitting signals from the cell surface inward. *Magnesium* ions are a key component of membrane structure, while sodium helps maintain ionic balance (Stautz et al.**,** 2021).

SEM Analysis

Scanning Electron Microscopy (SEM) showed clear morphological changes in *Escherichia coli*, *Bacillus cereus*, and *Pseudomonas aeruginosa* after treatment with the ethyl acetate extract of red melinjo peel. Before exposure, cells appeared normal with smooth surfaces, but after 24 hours, they exhibited shrinkage, swelling, roughened surfaces, and irregular shapes, especially in *B. cereus*. These damages align with ion leakage (Ca^{2+}, Mg^{2+}, K^{+}) recorded in Table 3, indicating increased membrane permeability. Antimicrobial compounds such as saponins, alkaloids, phenolics, and flavonoids in the extract (Parhusip et al., 2020) likely caused this effect. According to Octavia (2010), osmotic imbalance from membrane disruption leads to cytoplasmic loss and cell deformation.



Description: *E. coli* prior to (a) and following (b) exposure, *B. cereus* before (c) and after (d), and *P. aeruginosa* before (e) and after (f) contact with the extract, each observed at a magnification of 100,000×.

Figure 7. SEM images of *B. cereus, E. coli*, and *P. aeruginosa* before and after exposure to the ethyl acetate extract of red melinjo peel.

CONCLUSIONS

The ethyl acetate extract of red melinjo peel showed inhibitory effects against *Escherichia coli*, *Bacillus cereus*, and *Pseudomonas aeruginosa*, but did not suppress the growth of *Rhizopus oligosporus*. At concentrations ranging from 4% to 16%, the extract produced inhibition zones between 10.74 and 11.70 mm. The MIC values obtained were 0.69%, 0.50%, and 0.67%, while the corresponding MBC values were 2.76%, 2.00%, and 2.68%. Based on these results, the 12% extract was chosen for further testing, and phytochemical analysis confirmed the presence of alkaloids, phenolics, flavonoids, saponins, and glycosides. Its antibacterial effect was 1.47 times stronger than 1000 ppm colistin but 0.74 times weaker compared to 100 ppm penicillin G. The extract performed better under acidic conditions (pH 4) than at neutral pH (7), demonstrated increased activity when heated to 65 °C, and declined beyond 75 °C. Additionally, salt levels up to 5% and sugar concentrations up to 50% enhanced its antimicrobial performance. AAS analysis showed the extract caused leakage of Ca^{2+}, Mg^{2+}, and K^{+} ions from bacterial membranes, leading to cell shrinkage, swelling, elongation, and rough surfaces.

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