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The Activity of Unripe Wood Apple (*Limonia acidissima* Groff) Peel and Flesh Extracts in Inhibiting Diarrhe-Causing Bacteria

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Abstract: Diarrhea is characterized by having liquid stool more often than usual, typically occurring three or more times within a 24-hour period. While rarely fatal, it can disrupt daily life and is often treated with antibiotics due to pathogens like *Bacillus cereus*, *Shigella flexneri*, and *Salmonella typhimurium*. An overgrowth of *Escherichia coli* can also be a contributing factor. Recent studies suggest that unripe wood apples may have antibacterial properties. This study evaluates the antibacterial activity of extracts from the peel and flesh of the fruit against the bacteria, as mentioned earlier. Using disk diffusion, microdilution, and bioautography methods, it was found that the ethanol extract inhibited *Bacillus cereus* and *Shigella sonnei*, while the flesh extract affected *Bacillus cereus* and *Escherichia coli.* The bioautography test, however, showed no antibacterial activity from either extract, likely due to their low concentration. In conclusion, the peel and flesh extracts of unripe wood apples exhibit potential antibacterial activity against diarrhea-causing bacteria, particularly *Bacillus cereus*.

Keywords: antibacterial activity, wood apple, *Bacillus cereus*, *Shigella flexneri*, *Salmonella typhimurium*

Abstrak: *Diare ditandai dengan buang air besar yang konsistensinya lebih cair dari biasanya, terjadi tiga kali atau lebih dalam waktu 24 jam. Meskipun diare jarang menyebabkan kematian, diare dapat mengganggu aktivitas sehari-hari secara signifikan. Pengobatan sering kali melibatkan pemberian antibiotik, karena diare dapat disebabkan oleh berbagai bakteri patogen, seperti Bacillus cereus, Shigella flexneri, dan Salmonella typhimurium. Penyakit ini juga bisa disebabkan oleh pertumbuhan berlebih flora normal, seperti Escherichia coli. Beberapa penelitian telah menunjukkan bahwa buah kawista muda memiliki sifat antibakteri, sehingga menunjukkan potensinya sebagai agen antibakteri alami, terutama melawan bakteri penyebab diare. Penelitian ini bertujuan untuk mengevaluasi aktivitas antibakteri ekstrak kulit dan daging buah kawista muda terhadap bakteri penyebab diare, khususnya Bacillus cereus, Escherichia coli, Shigella flexneri, dan Salmonella typhimurium. Aktivitas antibakteri ekstrak etanol buah kawista muda diuji dengan metode difusi cakram, mikrodilusi, dan bioautografi. Hasil penelitian menunjukkan bahwa ekstrak kulit buah kawista muda mempunyai efek penghambatan terhadap Bacillus cereus dan Shigella sonnei. Kemudian, ekstrak daging buah kawista muda menunjukkan efek penghambatan terhadap Bacillus cereus dan Escherichia coli. Uji bioautografi menunjukkan tidak adanya aktivitas antibakteri pada ekstrak kulit dan daging buah kawista muda, kemungkinan karena konsentrasi ekstrak yang rendah. Kesimpulannya, ekstrak kulit dan daging buah kawista muda mempunyai potensi aktivitas antibakteri terhadap bakteri penyebab diare, terutama terhadap diare yang disebabkan Bacillus cereus.*

Kata kunci: *aktivitas antibakteri, kawista, Bacillus cereus, Shigella flexneri, Salmonella typhimurium*

INTRODUCTION

The World Health Organization defines diarrhea as having three or more loose or watery stools daily. Under normal circumstances, the gut maintains a balanced process of absorbing and secreting water and electrolytes. Diarrhea occurs when this balance is disrupted (Kelly *et al*. 2018). Diarrhea is a symptom caused by infections from various bacterial, viral, and parasitic organisms, most spreading through water contaminated with feces. These infections are more prevalent in areas with inadequate sanitation, hygiene, and access to safe drinking, cooking, and cleaning water. In low-income countries, rotavirus and *Escherichia coli* are the two most common causes of moderate-to-severe diarrhea. *Shigella sonnei* causes bloody diarrhea, fever, and abdominal pain. It can be life-threatening for children under five and may result in stunted growth (Torraca *et al*. 2020). Other important pathogens include *Cryptosporidium, Shigella species, Bacillus cereus*,

and *Salmonella typhimurium* (WHO 2017). To address antibiotic resistance in treating diarrhea, natural products can be utilized as alternatives. One such option is wood apple.

Limonia acidissima, also known as the wood apple, is a tropical fruit that is commonly found in Southern Asia and is often underutilized (Pant *et al*. 2020). This fruit has shown effectiveness against several severe conditions, including cancer, diabetes, hyperlipidemia, and microbial infections (Sharma & Tenguria 2021; Parvez & Sarker 2021; Shah *et al*. 2020). Several studies have shown that ripe and unripe wood apples demonstrate significant pharmacological activity. Ethanol and acetone extracts from the peel of wood apple fruit exhibited moderate inhibitory effects on several bacteria, including *Klebsiella oxytoca, Vibrio metschnikovii, Escherichia coli, Bacillus subtilis*, and *Staphylococcus aureus*. Additionally, these extracts demonstrate significant analgesic properties, antidiarrheal effects, and moderate antimicrobial activity (Islam *et al*. 2020). Methanol extract of wood apple fruit flesh and seeds at a concentration of 100% was classified as the concentration with the highest effectiveness in inhibiting the growth of *Escherichia coli* (Ansari *et al*. 2023). Another research indicates that wood apple peel extract, when used at a 100% concentration, was highly effective in inhibiting the growth of *Salmonella* sp. bacteria. Furthermore, its efficacy was comparable to that of tetracycline, demonstrating similar results in combating this type of bacteria (Prastyaningtias & Dian 2023).

This study aimed to evaluate the antibacterial activity of the skin and flesh extracts of unripe wood apples against diarrhea-causing bacteria, specifically *Bacillus cereus, Escherichia coli, Shigella flexneri*, and *Salmonella typhimurium*.

MATERIALS AND METHOD

Materials

The samples used for research were the skin and flesh of an unripe wood apple (*Limonia acidissima*) sourced from Subang Regency, West Java. Test bacteria used in this work were *Bacillus cereus*, *Escherichia coli, Shigella flexneri, and Salmonella typhimurium, Shigella sonnei*. *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Shigella sonnei* ATCC 25931. *Shigella flexneri* and *Salmonella typhimurium* obtained from Biomedical Laboratory, Faculty of Medicine, Padjadjaran University.

All chemicals used in this study, including bacterial growth media, were sourced from Merck.

Methods

Sample Preparation & Identification

Sample determination

Determination of the skin and flesh of wood apple (*Limonia acidissima Groff*) was carried out at the Jatinangor Herbarium, Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences.

Simplicia preparation

The process began with wet sorting, which separates the fruit from foreign objects, such as damaged fruit. After this, the fruit was washed thoroughly with running water until clean. Next, the washed fruit was dried and then chopped. The skin and flesh of the unripe fruit were separated using a hammer and pulp scraper. Finally, the fruit was dried in an oven to prevent the formation of microorganisms, such as mold. The skin and flesh of the unripe wood apple were dried at 40–42ºC for ±7 days until dry. After that, a dry sorting process was carried out to separate any foreign components still present. Each simplicia was then grounded using a grinder, then sieved with a 60 mesh sieve to obtain uniform powder particle size.

Extract Preparation

600 grams of dry powder from the skin and flesh of unripe wood apples were weighed and extracted separately. For the flesh, extraction was conducted using the Soxhlet method over seven cycles with a solvent ratio of 1:10 and 96% ethanol. In contrast, the wood apple skin was extracted using the reflux method, with a solvent ratio of 1:5 using the same ethanol concentration.

The extraction results were then filtered using a combination of batis cloth and filter paper until a clear extract was obtained. The resulting extract was concentrated using a rotary evaporator at a temperature of 60°C. Finally, the percent yield was calculated, ensuring that the concentrated extract was no less than 10%.

Extract Phytochemical Screening

The phytochemical compounds of the extract were qualitatively determined with standard procedures (Nortjie *et al*. 2022). The analyzed compounds were alkaloids, flavonoids, saponins, tannins, terpenoids, and steroids.

Antibacterial Activity Assay

To prepare for the test, the bacteria were rejuvenated on Mueller Hinton Agar (MHA) media by incubating them for 24 hours at 37°C. The bacterial turbidity was then adjusted to a McFarland standard of 0.5 by adding physiological saline (NaCl). The extract was mixed with 5% DMSO to achieve a final concentration of 50% (w/v) and was called extract stock.

Disk diffusion assay

Agar plate preparation was done through 10 mL of MHA media at a temperature of approximately 50°C was poured into a petri dish and allowed to solidify. Subsequently, 10 µL of a bacterial suspension (0.5 McFarland standard) was evenly

smeared across the surface of the agar medium using a sterile cotton swab. To optimize the concentration of ciprofloxacin, four paper discs, each 6 mm in diameter, were placed on the surface of the agar. Then, 20 µL ciprofloxacin was dropped on the disc at concentrations 1000, 100, 10, and 1 ppm, respectively.

Then, agar plates were prepared using the same previous procedure to analyze antibacterial activity. Six paper discs were then placed on the agar. On each disc, 20 µL of Ciprofloxacin (as the positive control) at its optimized concentration, 20 µL of media (as the negative control), and 20 µL of unripe wood apple skin extract at concentrations of 500,000 ppm, 400,000 ppm, 200,000 ppm, 100,000 ppm, 50,000 ppm, and 25,000 ppm were successively added. The petri dish was then incubated at 37°C for 18-24 hours. After incubation, the diameter of the inhibition zones formed around each paper disc was observed and measured. The same procedure was repeated for the unripe wood apple pulp extract.

Microdilution assay

The microdilution assay is a method used to determine a substance's minimum inhibitory concentration (MIC). This assay is divided into several groups: blanks, inhibitory negative control, inhibitory positive control, and the sample tests (extract). The inhibitory negative control indicates the absence of inhibitory activity, while the inhibitory positive control demonstrates 100% inhibitory activity. In this study, several types of blanks were set in column 1, A5-A12 wells, D5-D12 wells, and G5-G12 wells. Then, inhibitory positive control was set in H5-H12 wells, and negative control was set in column 3. The microplate well-filling map for this assay is described in Figure 1.

In wells 12a-12c, an extract stock made from unripe wood apple peel was added in varying concentrations through serial dilution. 100 µL of this extract stock was placed in wells 12a-12c. After homogenizing, 100 µL was transferred to fill wells 11a-11c. This procedure continued sequentially until wells 5a-5c were reached. To ensure equal volume, 100 µL of the solution from each column was then discarded. As a result, wells 5a-5c contained the lowest final concentration of the extract, 128 ppm, while wells 12a-12c had the highest final concentration, 16,400 ppm. This serial dilution procedure was also applied to an extract stock of unripe wood apple flesh in wells 5d-12f. and the antibiotic ciprofloxacin in wells 5g-12h. However, for antibiotics, the lowest final concentration of 0.39 ppm and the highest final concentration of 50 ppm were used.

In general, percent inhibition can be calculated using the following formula:

$$\%\ inhibition = \left(1 - \frac{Abs\ sample}{Abs\ inhibitory\ negative\ control}\right) x\ 100\%$$

Where:

Abs sample: the absorbance of the sample that is subtracted by its blank.
Abs inhibitory negative control: the absorbance of test bacteria growth that is subtracted by its blank.
Blank: every component other than the target.

If the general formula was derived, the percentage inhibition for each test group was as follows:

The absorbance of Inhibitory negative control:

$$Abs\ N - Abs\ M$$

%inhibition of unripe wood apple peel extract:

$$\left(1 - \frac{Abs\ P - Abs\ O}{Abs\ N - Abs\ M}\right) x\ 100\%$$

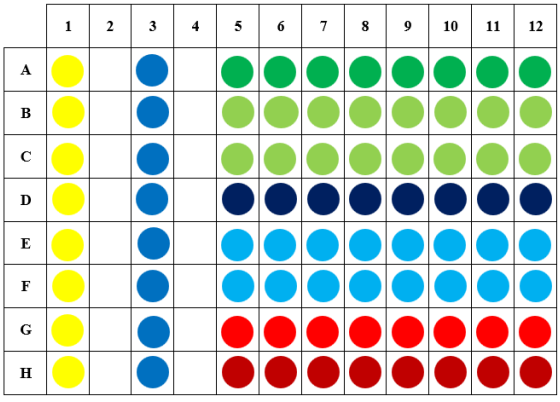

Figure 1. Microplate well-filling map for minimum inhibitory concentration testing, where: 100 µL MHB media (M); 50 µL MHB media + 50 µL test bacteria (N); 100 µL MHB media + 100 µL unripe wood apple peel extract (O); 100 µL MHB media + 95 µL unripe wood apple peel extract + 5 µL test bacteria (P); 100 µL MHB media + 100 µL unripe wood apple flesh extract (Q); 100 µL MHB media + 95 µL unripe wood apple flesh extract + 5 µL test bacteria (R); 100 µL MHB media + 100 µL ciprofloxacin (S); 100 µL MHB media + 95 µL ciprofloxacin + 5 µL test bacteria (T)

Table 1. TLC eluent composition

Eluent formula	Eluent mixture (mL)		
	Formic acid	Chloroform	Ethyl acetate
F1	10	0	0
F2	0	0	10
F3	0	10	0
F4	5	0	5
F5	0	5	5
F6	5	5	0
F7	3.3	3.3	3.3
F8	1.6	6.6	1.6
F9	1.6	1.6	6.6
F10	6.6	1.6	1.6

%inhibition of unripe wood apple flesh extract:

$$\left(1-\frac{Abs\ R-Abs\ Q}{Abs\ N-Abs\ M}\right) x\ 100\%$$

%inhibition of Inhibitory positive control (ciprofloxacin):

$$\left(1-\frac{Abs\ T-Abs\ S}{Abs\ N-Abs\ M}\right) x\ 100\%$$

Thin-Layer Chromatography (TLC)–Bioautography

The TLC-bioautography method's initial step involved optimizing extracting components' separation using Thin Layer Chromatography (TLC). The stationary phase used was a silica plate, while the mobile phase comprised a mixture of three solvents, formic acid, chloroform, and ethyl acetate, in varying concentrations (Table 1). 10 mL of this solvent mixture was added to the chromatography chamber and allowed to saturate for 30 minutes. The silica plate on which the sample had been spotted was then inserted into the chamber and eluted with the mobile phase until the solvent reached 0.5 cm from the top edge of the plate. After removing the plate, it was dried, and the separation was visualized using UV light at 254 nm and 366 nm, followed by staining with cytoborate.

The second step of bioautography was measuring the ability of the compound component that separated on the TLC plate against the test bacteria. A total of 15 mL of MHA medium was poured into a Petri dish and allowed to solidify. The chromatogram from the optimization process was placed on the solid MHA medium and left undisturbed for 30 minutes. After this time, the chromatogram was removed from the medium. The medium was then incubated for 24 hours at 37°C.

RESULT AND DISSCUSION

Sample Preparation & Identification

The initial step in sample preparation involved identifying the plant, which verified the identity of the sample. This analysis confirmed that the tested samples were the skin and flesh of the wood apple fruit. The second step was simplicial preparation and its extraction. This step resulting a sample recovery of 17.38% for unripe wood apple peel and 48.55% for unripe wood apple flesh. The low yield from extracting the skin of the kawista fruit is likely due to its tough texture. This hardness makes it more challenging to recover the skin during extraction than the fruit's flesh. The third step involved identifying the compound components of the ethanol extract from the sample through phytochemical screening. The results indicate that the ethanol extract of unripe wood apple peel and flesh contains alkaloids, flavonoids, saponins, tannins, and terpenoids but does not contain steroids.

Antibacterial Activity Assay

Disk diffusion assay

Characterizing the compound content in the skin and flesh of unripe wood apples has not been widely conducted; therefore, the disc diffusion method was very suitable for testing its antibacterial activity.

Based on the activity test results, it was found that the unripe wood apple peel extract at a concentration of 25,000-500,000 ppm could inhibit all test bacteria, exhibiting moderate to strong activity. The most significant effect of unripe wood apple peel extract was inhibiting *Bacillus cereus*. Similarly, the unripe wood apple flesh extract, at the same concentration range, demonstrated moderate to strong inhibitory activity, although its effectiveness was lower than that of the peel extract. So far, no specific tests on the compound content have been conducted on the peel of the kawista. However, the antibacterial properties of the kawista's flesh and pulp are likely due to a combination of several compounds, including citric acid. Notably, the citric acid content is higher in unripe kawista compared to the ripe fruit. According

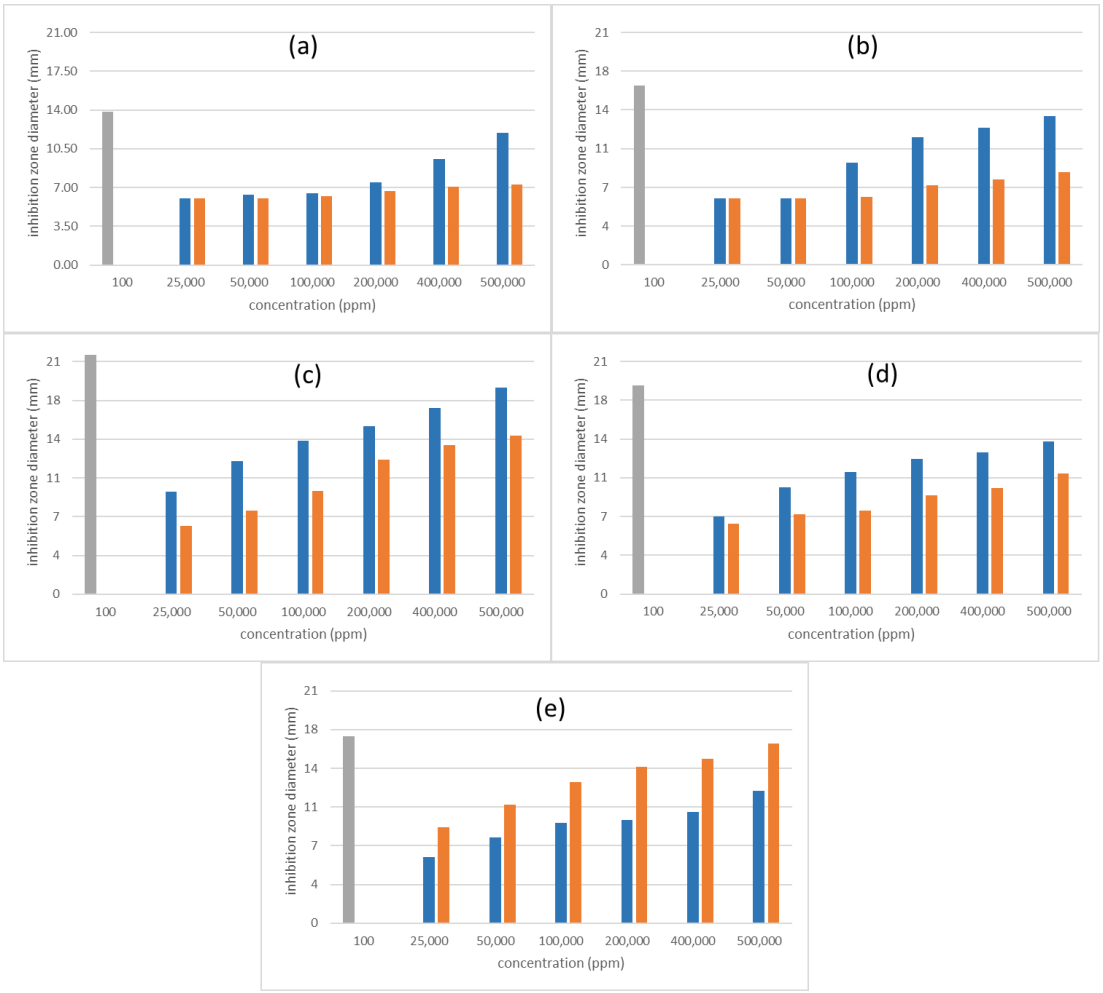

Figure 2. Inhibitory power of extract of unripe wood apple peel (■) and flesh (■) compared with the inhibitory positive control, ciprofloxacin (■), resulting from disk diffusion assay against tes bacteria: *Salmonella typhimurium* (a), *Shigella flexneri* (b), *Bacillus cereus* (c), *Shigella sonnei* (d) and *Escherichia coli* (e)

to various studies (Burel *et al*. 2021; Shah *et al*. 2020), citric acid inhibits bacteria by disrupting bacterial cell membranes and lowering the pH of the cell's interior. This can lead to cell death. The most significant effect of unripe wood apple flesh extract was inhibiting *Bacillus cereus* and *Escherichia coli*. All these findings were compared to the antibiotic ciprofloxacin at a concentration of 100 ppm, which was identified as the optimal concentration for inhibiting the test bacteria (Figure 2). The classification of inhibitory activity based on the disc diffusion method is weak (a clear zone smaller than 5 mm), medium (a clear zone ranging from 5 mm to 10 mm), strong (a clear zone ranging from 11 mm to 20 mm), and powerful (a clear zone larger than 20 mm) (CLSI 2024).

Microdilution assay

Unripe wood apples' skin and flesh extracts have a minimum inhibitory concentration of 4100 ppm. However, the graph showing the percentage of inhibitory power indicates that the unripe wood apple skin extract has greater inhibitory power than the flesh extract. This is evident from the graph, which shows that the inhibitory power of the unripe wood apple peel extract tends to increase as the concentration of the extract increases. In contrast, the unripe wood apple flesh extract exhibits increased inhibitory power up to a particular concentration, after which its inhibitory power declines with further increases in concentration (Figure 3).

Compared to the standard antibiotic ciprofloxacin, the unripe wood apple peel and flesh extracts have a much higher minimum inhibitory concentration. Nonetheless, since wood apple was a fruit consumed regularly, it was considered relatively safer than synthetic chemical antibiotics. This suspicion warrants further research.

Bioautography

The thin-layer chromatography (TLC) bioautography method combines the separation and analysis techniques of TLC with the detection of biological activity (Rütten *et al*. 2022). The separated extract compound components are shown by stains on the TLC plate identified by cytoborrate staining

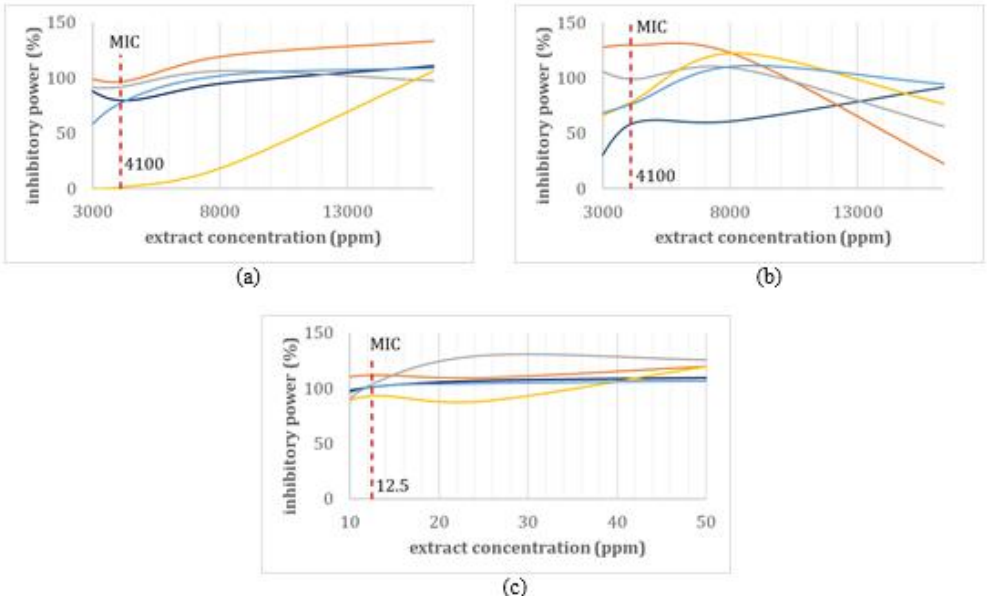

Figure 3. Inhibitory power of wood apple peel extract (a), wood apple flesh extract (b), and ciprofloxacin (c) against test bacteria: *Salmonella typhimurium* (—); *Shigella flexneri* (—); *Bacillus cereus* (—); *Shigella sonnei* (—); *Escherichia coli* (—)

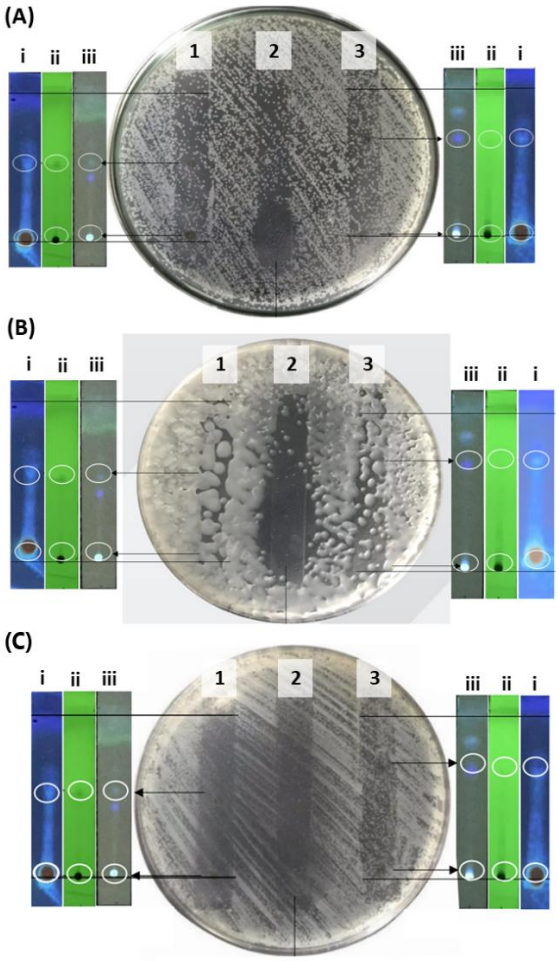

Figure 4. The activity of the extract components in unripe wood apple skin (1) and its flesh (3) extracts were compared with ciprofloxacin (2) against the test bacteria: *Escherichia coli* (A), *Bacillus cereus* (B), *Shigella sonnei* (C). The extract component was separated by TLC and visualized by cytoborrate staining (i), UV 254 nm (ii), and UV nm 366 (iii) in wavelength

(i), UV 254 (ii), and UV 366 (iii).

To assess the potential presence of compounds with antibacterial activity, a process of separating the compounds from the extract of the skin and flesh of unripe wood apple was conducted using thin-layer chromatography (TLC). The results revealed several compounds that were successfully separated and visualized on the TLC plate (Figures 4A(i), (ii), (iii); 4B(i), (ii), (iii); and 4C(i), (ii), (iii)). The compounds identified on the TLC plate were tested against *Escherichia coli, Bacillus cereus*, and *Shigella sonnei*. These three bacterial strains were selected based on previous diffusion tests, which indicated that the extract from the skin and flesh of unripe wood apple exhibited inhibitory effects against them. Using the TLC bioautography method, the extract compounds showed no inhibitory activity against the three test bacteria compared to ciprofloxacin.

According to diffusion and microdilution assays, the peel of unripe wood apples exhibits more potent antibacterial activity than the fruit's flesh. However, the bioautography test indicated no antibacterial activity from the peel or the flesh of the unripe wood apple. This lack of activity was likely attributed to the low extract concentration.

CONCLUSION

Tests conducted on the antibacterial activity of the peel and flesh of unripe wood apple against *Bacillus cereus*, *Escherichia coli*, *Shigella flexneri*, and *Salmonella typhimurium* revealed that the peel of the unripe wood apple was particularly effective in alleviating diarrhea, especially that caused by *Bacillus cereus*.

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REFERENCES

Ansari, A., Andriana, A., Rozikin, R. & Adnyana, I.G.A. (2023). Methanol extract inhibition's effectiveness of kawista fruit Var. Dompu on e-coli through in-vitro. *Indonesian Journal of Multidisciplinary Science*. **2(7)**: 2755-2772.

Burel, C., Kala, A. & Purevdorj-Gage, L. (2021). Impact of pH on citric acid antimicrobial activity against Gram-negative bacteria. *Letters in Applied Microbiology*. **72(3)**: 332-340.

CLSI. (2024) Performance Standard for Susceptibility Testing. 34 ed. Clinical and Laboratory Standard Institute.

Islam, F., Azad, A.K., Faysal, M., Azad, M.A.K., Islam, S., Al Amin, M., Sultana, N., Dola, F.Y., Rahman, M.M & Begh, M.Z.A. (2020) Comparative study of analgesic, antidiarrhoeal and antimicrobial activities of methanol and acetone extracts of fruits peels of *Limonia acidissima* L. (*Rutaceae*). *Journal of Drug Delivery and Therapeutics*. **10(1)**: 62-65.

Kelly, L., Jenkins, H., & Whyte, L. (2018) Pathophysiology of diarrhoea. *Paediatrics and Child Health* (United Kingdom). **28(11)**: 520 – 526.

Nortjie, E., Basitere, M., Moyo, D. & Nyamukamba, P. (2022). Extraction methods, quantitative and qualitative phytochemical screening of medicinal plants for antimicrobial textiles: a review. *Plants*. **11(15)**:1-17.

Pant, H., Lobo, V., Santhosh, A. & Verma, S. (2020). Pharmacognosy and medicinal value of some exotic fruits and vegetables consumed in India. *The Asian Journal of Horticulture*. **15(2)**: 39-52.

Parvez, G.M.M. & Sarker, R.K. (2021) Pharmacological potential of wood apple (*Limonia acidissima*): A Review. *International Journal of Minor Fruits, Medicinal and Aromatic Plant*s. **7(2)**: 40–47.

Prastyaningtias, S.D. & Dian, A.S.S. (2023). Antibacterial activity test of kawista fruit peel extract (*Limonia Acidissima* L.) from pasinan village, pasuruan, against *Salmonella* Sp colonies *in vitro*. *International Journal of Ecophysiology*. **5(2)**: 54-61.

Rütten, A., Kirchner, T. & Musiol-Kroll, E.M. (2022). Overview on strategies and assays for antibiotic discovery. *Pharmaceuticals*. **15(10)**: 1-24.

Shah, M.S., Kamble, S.M., Shinde, A.R. & Jagtap, D. R. (2020). Novel utilization and phytopharmacological review of multipotential traditional plant *Limonia acidissima*. *World Journal of Pharmaceutical Research*. **9(7)**: 866-886.

Sharma, P. & Tenguria, R.K. (2021) Phytochemical Properties and Health Benefits of Limonia acidissima: A Review. *International Research Journal of Plant Science*. **12(2)**: 1–6.

Torraca, V., Holt, K. & Mostowy, S. (2020). Shigella sonnei. *Trends in microbiology*. **28(8)**: 696-697.

WHO (2017) Diarrhoeal disease: Key facts. WHO Fact sheet. (May), 4.