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Optimization of Ultrasonic-Assisted Extraction Parameters and Antibacterial Activity of *Zanthoxylum acanthopodium* D.C against *Cutibacterium acnes*

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ABSTRACT

Acne is a disease that has affected many populations globally. One of the causes of acne is the presence of acne-causing bacteria, namely *Cutibacterium acnes*, which is reportedly resistant to several antibiotics. Andaliman fruit (*Zanthoxylum acanthopodium* DC.) is traditionally used by Indonesian people as a spice which has antibacterial potential. This study aims to evaluate the antibacterial activity of the ethanolic extract of Andaliman fruit against *C. acnes* using the microdilution method. The extraction method for andaliman fruit was the Ultrasonic-Assisted Extraction with variations in frequency (30, 40, and 50 kHz) and duration (10, 15, and 20 minutes). Phytochemical screening showed the presence of flavonoids, alkaloids, tannins, steroids, and saponins in the extract. The results showed the antibacterial activity of Andaliman fruit extract against *C. acnes*, with the Minimum Inhibitory Concentration (MIC) observed in the extract with a frequency of 40 kHz and an extraction duration of 10 and 15 minutes at a concentration of 5000 ppm. However, the Minimum Bactericidal Concentration (MBC) test showed negative results, indicating that Andaliman extract did not have a bactericidal effect on these bacteria. Statistical analysis using the Kruskal–Wallis and Dunn tests revealed significant differences ($p < 0.05$) between extraction treatments and the positive control in MIC and MBC values. These findings indicate that the ethanolic extract of andaliman fruit possesses inhibitory but not bactericidal activity against *C. acnes*, suggesting its potential as a natural anti-acne agent for further formulation and mechanistic studies.

INTRODUCTION

Acne is one of the most common diseases, affecting by nearly 9.4% of the global population (Tan & Bhate, 2015). Both infectious and non-infectious factors can cause acne that appears on the body. Bacteria that play a role in acne are *Staphylococcus epidermidis, Cutibacterium acnes* (previously known as *Propionibacterium acnes), Staphylococcus aureus* (Meilina & Hasanah, 2018).

Cutibacterium acnes is a gram-positive bacterium that can form spores on the skin that contain many sebaceous glands. It contains chemotactic factors and produces lipolytic enzymes that contribute to the formation of sebum, a lipid-rich substance in the skin. *C. acnes* will produce lipase, protease, and hyaluronidase that hydrolyze sebum triglycerides and turn them into free fatty acids. The free fatty acids will then cause hyperkeratosis, retention, and

formation of microcomedones that lead to acne (Samara et al., 2023).

One of the therapies commonly used in the treatment of acne is antibiotics. Antibiotics can work by inhibiting the synthesis of bacterial cell walls, protein synthesis, and nucleic acids synthesis (Byrne et al., 2019). Antibiotics commonly used to treat acne include clindamycin, tetracycline, and erythromycin (Daud et al., 2018). However, according to research by Fitrianingsih et al. (2019), *Cutibacterium acnes* has been reported to experience resistance to these antibiotics.

Zanthoxylum acanthopodium DC. fruit, known as "Lada Batak", is one of Indonesia's endemic natural resources that offers numerous benefits. This fruit is an endemic plant that grows in the highlands of North Sumatra. Andaliman fruit is often used as a spice and preservative by the Batak people. According to research by Husni (2023), andaliman fruit contains secondary metabolite compounds that are useful as anti-inflammatory, anti-cancer, antioxidant, anti-diabetic, and antibacterial agents. The andaliman plant offers various pharmacological benefits due to the presence of flavonoids, alkaloids, steroids, tannins, and saponins (Sepriani et al., 2019). Chemical compounds with antibacterial potential in andaliman fruit are typically extracted using an appropriate solvent (Lestari et al., 2020). Ultrasonic extraction is a conventional extraction method that utilizes ultrasonic waves to enhance the extraction process. The sonic waves break the cell walls, thereby increasing the release of secondary metabolite compounds into the solvent (Ivasenko et al., 2021). The ultrasonic method has several advantages, namely the short extraction duration and the use of relatively less solvent. The solvent used is 96% ethanol because this solvent can attract chemical compounds that are polar, semi-polar, and non-polar (Stevani et al., 2021).

METHODS

Research Material

The primary equipment used in this research included an ultrasonic water bath for extraction, a vacuum rotary evaporator for solvent removal, an incubator for microbial cultivation, and a microplate reader with 96-well plates for determining MIC and MBC. Additional essential laboratory equipment, including an autoclave, biosafety cabinet, and analytical balance, was also utilized during the experimental procedures. The materials used in the research were andaliman fruit (*Zanthoxylum acanthopodium* DC.) from Hariarapintu village, Samosir Regency, North Sumatra, clindamycin, ethanol 96%, concentrated HCl, magnesium, $FeCl_3$ 1%, concentrated H_2SO_4, Dragendorff reagent, Mayer reagent, Liebermann-Burchard's reagent, Bouchardat reagent, NaCl 0.9%, *Cutibacterium acnes*, DMSO (Dimethyl sulfoxide) 0.5%, Mueller Hinton Broth, Mueller Hinton Agar, aquadest, and 0.5 McFarland standard.

Sample Preparation

Andaliman fruits were harvested by wet sorting to separate impurities present in the fruit. Samples collected were washed using running water, then wet weighing is performed. After that the samples were dried and weighed. The dried fruit was then blended and sieved using a 60 mesh sieve (Sitanggang et al., 2019).

Extraction

The dried andaliman fruit was extracted using a modified ultrasonic method based on the (Malau et al., 2021), namely by weighing 10 grams of dried fruit and 100 ml of 96% ethanol solvent (1:10). This research was conducted using the Randomized Group Design method with 2 factors, namely frequency variation (30 kHz, 40 kHz, 50 kHz) and variation in extraction duration (10 minutes, 15 minutes, 20 minutes) (Loghmanifar et al., 2022). The filtered extract was then evaporated using a rotary evaporator with a temperature of 50°C to obtain a viscous extract (Rienoviar & Setyaningsih, 2018).

Flavonoid Test

The viscous extract of andaliman fruit was added with 0.1 gram of magnesium powder and 1 ml of concentrated HCl. The flavonoid test is positive if the extract solution turns yellow or orange to a reddish color (Bhandary et al., 2012).

Alkaloid Test

Alkaloid testing used three methods: Dragendorff, Mayer, and Bouchardat tests. To prepare the sample, viscous andaliman fruit extract was mixed with 1 mL of 2N HCl and 9 mL of aquadest. The mixture was heated in a water bath for 2 minutes, cooled, and filtered. The filtrate was divided into three test tubes. Each tube received two drops of either Dragendorff, Mayer, or Bouchardat reagent. A positive alkaloid test was indicated by an orange or beige precipitate with Dragendorff reagent, a white precipitate with Mayer reagent, and a brown-black precipitate with Bouchardat reagent (Sulistyarini et al., 2020).

Tannin Test

The viscous extract of Andaliman fruit was added with two drops of $FeCl_3$ 1%. The tannin test is positive if the extract solution produces a green to black color (Pringgenies et al., 2018).

Steroid and Triterpenoids Test

The viscous extract of andaliman fruit was added to 9 ml of diethyl ether then shaken. The diethyl ether layer was separated and then 2-3 drops of Liebermann-Bouchard reagent were added. Positive triterpenoid testing if the solution is blue, and positive steroid testing if the solution is green (Rienoviar et al., 2019).

Saponin Test

The viscous extract of andaliman fruit was added to 10 ml of hot water in a test tube. Then shaken for ± 1 minute. Andaliman fruit is positive for saponins if a stable foam is formed for no less than 10 minutes and does not disappear when 1 drop of HCl 2N is added (Anggraeni, 2020).

Antibacterial Test

Mueller Hinton Agar (MHA)

15.2 grams of MHA media were dissolved in 400 mL of deionized water (Aquadest). Then heated in a water bath until boiling while stirring. The dissolved media was sterilized using an autoclave at 121°C for 20 minutes. Sterilized MHA media was poured into petri dishes aseptically and allowed to stand at room temperature until it solidified (Utomo et al., 2018).

Mueller Hinton Broth (MHB)

2.1 grams of MHB media were dissolved in 100 mL of deionized water (Aquadest). Then the media was sterilized using an autoclave at 121°C for 20 minutes (Rosmania & Yanti, 2020).

Bacterial Culture and Inoculum Standardization

Using a sterile inoculation loop, collect a small fragment of Cutibacterium acnes and streak it on MHA. The bacteria were then incubated for 24 hours at 37°C (Fitrianingsih et al., 2019).

Preparation of Bacterial Suspension

A small fragment of bacteria that had been subcultured was taken and placed into a test tube containing 10 mL of sterile 0.9% NaCl, and then homogenized using a vortex. The turbidity level of the suspension was compared with McFarland 0.5 solution (Novelni et al., 2023).

Preparation of Extract Solution

The solution of andaliman fruit extract was prepared with a concentration of 10,000 ppm by weighing 100 mg of thick extract and dissolving it in 0.5% DMSO in a 10 ml volumetric flask, followed by homogenization.

Preparation of Clindamycin Solution

Clindamycin powder was weighed out to 10 mg and dissolved in 10 mL of sterile distilled water in a 10 mL test tube.

Minimum Inhibitory Concentration (MIC)

The MIC assay was carried out using the microdilution method on a 96-well plate. 100µL of Mueller Hinton Broth (MHB) was put into the wells in columns B1-B12, C1-H3, C5-H7, and C9-H11. A total of 100µL of andaliman fruit extract that has been diluted using DMSO is added to column C1. Then, the dilution was performed by transferring 100 µL from the well of column C1 to the well of column D1 using a micropipette. After that, 100µL from the well of column D1 was transferred to the well of column E1 with a micropipette. The step is repeated until column H1 is reached. Then, in the column containing

Mueller-Hinton Broth (MHB), 100 µL of a *Cutibacterium acnes* bacterial suspension was added. In wells A1-A12, 100µL of clindamycin was added (positive control). The microdilution plate was incubated at 37°C for 24 hours. Then, the turbidity was observed by comparing the treated suspension with the control. Three replications were performed on each sample group (Situmorang et al., 2024).

Minimum Bactericidal Concentration (MBC)

The MBC assay is performed using the streak plate method. A sterile inoculating loop took a small amount of bacteria from the sample group that shows MIC, then scratched a zigzag on MHA. Positive MBC is characterized by 99.9% dead bacteria (Balouiri et al., 2016).

Data Analysis

Statistical testing employs a one-way ANOVA with an α level of 0.05 and a 95% confidence interval. However, before this test was carried out, a normality test must be carried out using the Shapiro-Wilk test and a variance test using Levene's test (Hasanah et al., 2023; Sandy et al., 2021). The data was not normally distributed, then a non-parametric test, namely the Kruskal-Wallis test, was carried out and followed by the Post-Hoc Dunn Test (Rozi et al., 2022).

RESULTS AND DISCUSSION

The extraction process of Andaliman Fruit (*Zanthoxylum acanthopodium* DC.) begins with wet sorting, drying, dry sorting, pulverizing, and sieving.

Table 1. Yield Value of Ethanol Extract of Andaliman (*Zanthoxylum acanthopodium*) Fruit

Frequency	Time	Yield (%)
	10 min	5.00
30 kHz	15 min	5.00
	20 min	5.33
	10 min	6.70
40 kHz	15 min	7.30
	20 min	8.06
	10 min	6.10
50 kHz	15 min	6.30
	20 min	7.9

Note: The weight of dry sample = 10 g

The extraction of andaliman fruit dried powder in this study used the ultrasonic method due to the relatively short extraction time and the use of less solvent (Kristina et al., 2022). The solvent used in this research is 96% ethanol, as it offers several advantages, including being more neutral, which makes it non-toxic, and can extract more chemical compounds than methanol and water (Wendersteyt et al., 2021). The mean extract yields were presented in **Table 1**. The yield of extract from Andaliman fruit was affected by both frequency and duration of extraction. As shown in **Table 1**, extraction at 30 kHz yielded the lowest yields, ranging from 5% to 5.33%, indicating limited cavitation intensity at this frequency. Increasing the frequency to 40 kHz enhanced the extract yield, reaching a maximum of 8.06% at 20 minutes. This increase reflects that higher cavitation energy at 40 kHz enables more efficient disruption of plant cell walls, improving solvent penetration into the cellular matrix and thereby facilitating the release of soluble phytoconstituents (Muzykiewicz-Szymańska et al., 2024). However, an increase in frequency to 50 kHz did not produce a proportional increase in yield; somewhat, the yield decreased slightly, 6.1-6.3%. This result is likely due to excessive cavitation at higher frequencies, which causes microbubbles to collapse before optimal energy transfer occurs, thereby reducing extraction efficiency. Additionally, prolonged or intense ultrasonication may partially degrade thermolabile compounds, further contributing to the reduced yield (Kumar et al., 2021; Shen et al., 2023).

The result demonstrates that 40 kHz for 20 minutes represents the optimal extraction condition under the tested parameters, as it balances effective cell wall disruption with the preservation of extractable constituents. These findings align with previous studies, which report that moderate ultrasonic frequencies often achieve higher extraction efficiency in phytochemical-rich plant matrices due to enhanced mass transfer and reduced solvent resistance.

Phytochemical screening is a method used to identify secondary metabolite compounds present in the extract (Baharuddin, 2019). The results of phytochemical screening on andaliman

fruit extract are positive for flavonoids, alkaloids, steroids, tannins, and saponins (**Table 2**), where the results are in accordance with the research of Sepriani et al. (2019).

Table 2. Phytochemical Screening Results of Andaliman Fruit

Phytochemical	Result
Flavonoids	+
Alkaloids	+
Tannins	+
Steroids	+
Saponin	+

Testing for flavonoid metabolites in andaliman fruit extract involves first adding magnesium and HCl, which reduces flavonoid compounds and causes a change in color from red to orange (Kopon et al., 2020). For alkaloid tests, Dragendorff, Mayer, and Bouchardat reagents are used in sequence. Initially, HCl is added to the extract to isolate alkaline alkaloids (Ergina et al., 2014). The Dragendorff reagent is then added, resulting in a beige precipitate as alkaloid compounds react with tetraiodobismuthate(III) ions. Next, the Bouchardat reagent is used, forming a brown precipitate due to covalent bonds between alkaloids and K+ ions, which result in potassium-alkaloid bonds (Sulistyarini et al., 2020)

The next phytochemical screening is testing for tannin compounds in andaliman fruit extract. Testing is performed with the addition of 1% $FeCl_3$. The test results of tannin compounds in andaliman fruit extract are positive, as evidenced by the formation of a blackish green color due to the reaction between tannin compounds and Fe^{3+} ions (Oktavia & Sutoyo, 2021). Fe^{3+} ions bind six pairs of free electrons of the O atom in the tannin compound to form a tannin-iron complex compound (Koopmann et al., 2020). These results are in accordance with the research of Putri et al. (2024), the addition of 1% $FeCl_3$ reagent to the extract will cause a blackish or bluish-green color.

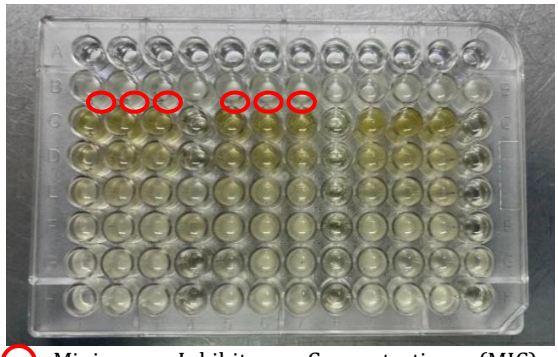
The next test involved steroids testing, which was performed by adding diethyl ether to isolate non-polar compounds from the extract (Ghasemzadeh et al., 2014). After that, Liebermann-Burchard reagent is added, and a green ring will form, indicating the presence of steroid compounds in the extract. The saponin test on andaliman fruit extract was validated by shaking the extract and producing a stable foam for 10 minutes by adding HCl. The formation of foam is due to a hydrolysis reaction characterized by the formation of foam in the extract (Bhernama, 2021).

Antibacterial activity of the ethanol extract of andaliman fruit against *Cutibacterium acnes* as tested using the liquid microdilution method, which requires a small sample, offers good sensitivity, and provides qualitative results(Sari et al., 2021). This method determines the Minimum Inhibitory Concentration (MIC) in a well plate. Clindamycin, an antibiotic used to treat moderate to severe acne by inhibiting protein synthesis, served as the positive control (Hikmah & Hasanah, 2023).

Table 3. Minimum Inhibitory Concentration (MIC) Test Results

Sample Frequency	Time	MIC (ppm)
30 kHz	10 minute	0
	15 minute	0
	20 minute	0
40 kHz	10 minute	5000
	15 minute	5000
	20 minute	0
50 kHz	10 minute	0
	15 minute	0
	20 minute	0
Clindamycin	-	1000

The MIC test results (**Table 3**) showed that the ethanol extract of andaliman fruit exhibited inhibitory activity at a concentration of 5000 ppm when extracted for 10 and 15 minutes at an ultrasonic frequency of 40 kHz (**Figure 1**).



○=Minimum Inhibitory Concentration (MIC) of Andaliman Fruit Ethanol Extract 40kHz 10 minutes; 40kHz 15 minutes

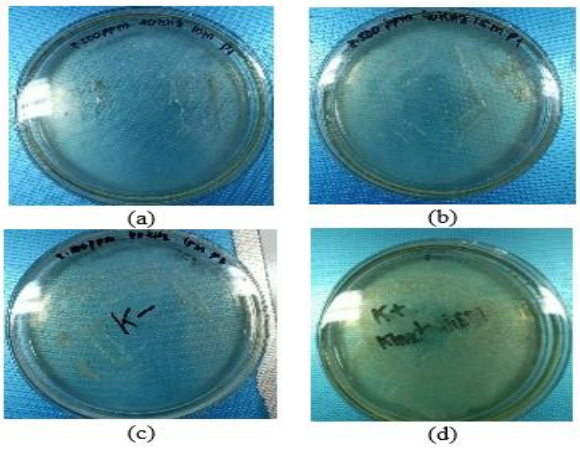
Figure 1. Minimum Inhibitory Concentration (MIC) Test Results

This outcome is likely due to the ability of the 40 kHz frequency to generate sufficient cavitation

energy to effectively disrupt the cell walls of the andaliman fruit, thereby facilitating the release of bioactive constituents without causing structural degradation.

In contrast, extraction at 30 kHz may produce suboptimal cavitation intensity, resulting in less efficient release of active compounds, whereas at 50 kHz, cavitation bubbles collapse too rapidly to sustain optimal extraction conditions (Dadi et al., 2019). Compared with extracts of *Durio zibethinus* rind, which demonstrated an MIC of 4000 ppm against P. acnes (Fitrianingsih et al., 2019), the andaliman extract exhibits moderate antimicrobial potency.

The next test is the Minimum Bactericidal Concentration test which aims to see the ability of andaliman fruit extract to actually kill bacteria (**Figure 2**). This test was conducted using the streak plate method. The results (**Table 4**) obtained from the treatment are andaliman extract is KBM >5000 ppm because the extract that shows MIC is not able to kill *Cutibacterium acnes* bacteria. This happens because it is possible that the concentration of 5000 ppm andaliman fruit extract has not been able to achieve a bactericidal effect and the ability of bacteria to form biofilms which are the basis for bacteria to survive (Gyawali & Ibrahim, 2014).



Note: Minimum Bactericidal Concentration (MBC) of Andaliman Fruit Ethanol Extract against Cutibacterium acnes (a) 40 kHz 10 min 5000 ppm (b) 40 kHz 15 min 5000 ppm (c) Negative control (d) Positive control

Figure 2. Minimum Bactericidal Concentration (MBC) Test Result

Table 4. MBC Test Result

Sample		MBC (ppm)
Frequency	**Time**	
40 kHz	10 minute	>5000
	15 minute	
Clindamycin	-	1000

Flavonoids, alkaloids, tannins, and saponins contained in andaliman fruit have a role in inhibiting bacterial growth. The flavonoid mechanism inhibits bacterial growth by inhibiting the process of nucleic acid synthesis, inhibiting the process of energy metabolism, inhibiting cell membrane function, damaging the cell wall in bacteria so that it causes changes in nutrient transport in bacteria which can later cause toxic effects on these bacteria (Azzahra, 2024; Sapoetri et al., 2022).

Alkaloid are also compounds that can function as antibacterials. Based on the research of Lister et al., 2022 the compounds in andaliman fruit extract act as antibacterials are alkaloid compounds which have a mechanism that can inhibit the formation of peptidoglycan in bacteria which results in the process of forming the cell wall layer in bacteria becoming imperfect and will make the bacteria die. The mechanism of action of tannin compounds in inhibiting bacterial growth is by inactivating enzymes and causing lysis of bacterial cells, thus disrupting the formation of bacterial cells and causing cell death (Sapara, 2016). Saponin is also a compound present in andaliman fruit extract. In the research of Sitanggang et al., 2019 saponin compounds contained in andaliman have a role in inhibiting bacterial growth by damaging cell permeability and inhibiting enzyme activity in bacteria so that it can interfere with the process of bacterial growth.

The Kruskal-Wallis non=parametric test was employed due to the non-normal distribution of the dataset. The test produced an Asymp. Sig. value less than 0.05, demonstrating that extraction frequency and duration had a significant effect on MIC and MBC values. Dunn's post-hoc test was subsequently applied to assess pairwise differences among treatment groups (Husain et al., 2022). No significant difference was observed ($p = 1.000$; $p > 0.05$) between the 40 kHz x 10 min and 40 kHz x 15 min groups, indicating a plateau in extraction efficiency within this interval. However, both the 40 kHz x 10 min and 40 kHz x 15 min groups differed

significantly from the positive control ($p = 0.043$; $p < 0.05$), confirming that this extraction conditions resulted in measurable antimicrobial effects compared to the comparator.

CONCLUSIONS

The ethanolic extract of *Zanthoxylum acanthopodium* (andaliman) demonstrated antibacterial activity against Cutibacterium acnes, with the optimum extraction condition obtained at 40 kHz for 10–15 minutes, yielding a Minimum Inhibitory Concentration (MIC) of 5000 ppm and no bactericidal effect (MBC > 5000 ppm). The presence of flavonoids, alkaloids, tannins, and saponins is believed to contribute to the inhibitory activity. Although the potency remains lower than clindamycin, these findings support the potential of andaliman extract as a natural anti-acne candidate. Further work should focus on compound isolation, mechanism elucidation, and in-vivo or formulation studies to confirm therapeutic relevance.

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AUTHORS' CONTRIBUTIONS

RSP: literature search; experimental studies; data analysis; manuscript preparation.

EPR: Concepts or ideas; design; definition of intellectual content; manuscript editing; manuscript review.

EH: Design; manuscript editing; manuscript review.

RR: Manuscript review.

AWS: Manuscript editing; manuscript review.

CONFLICT OF INTERESTS

None to declare

ETHICAL CONSIDERATION

Ethical issues (including plagiarism, data fabrication, double publication, etc) have been completely observed by the author.

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