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The Antibacterial and Antibiofilm Efficacy of *Citrullus lanatus* Rind Ethanol Extract Against *Aggregatibacter actinomycetemcomitans*

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Abstract

Watermelon (*Citrullus lanatus*) rind contains secondary metabolite compounds which exhibit antimicrobial effects against Gram-positive and Gram-negative bacteria. *Aggregatibacter actinomycetemcomitans* can cause tissue damage and bone resorption, leading to aggressive periodontitis. Chlorhexidine as golden standard antimicrobial mouthwash can cause tooth staining. There is a need for herbal remedies with fewer side effects as an alternative treatment for periodontitis. This study aims to determine the antibacterial and antibiofilm efficacy of *C. lanatus* rind ethanol extract against *A. actinomycetemcomitans*. This is an experimental laboratory in vitro with a post-test-only control group design. The antibacterial test was conducted using the plate count method and the antibiofilm test using the microtiter plate biofilm assay method. The samples used were ethanol extract of *C. lanatus* rind 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%, chlorhexidine 0.2% as positive control, distilled water, and BHI-B as negative control. As a result, extract concentrations from 12.5% to 100% exhibited antibacterial effects on *A. actinomycetemcomitans* equivalent to chlorhexidine. During the 3-hour incubation period, extract at 25% and 100% concentrations on all incubation times showed a better antibiofilm effect than chlorhexidine. It can be concluded that ethanol extract of *C. lanatus* rind had the potential as an alternative antibiofilm agent against *A. actinomycetemcomitans*.

Keywords: *Aggregatibacter actinomycetemcomitans*; antibacterial; antibiofilm; ethanol extract of *Citrullus lanatus* rind; herbal medicine.

Abbreviations: *A. actinomycetemcomitans*: *Aggregatibacter actinomycetemcomitans*, ATCC: American Type Culture Collection, ANOVA: Analysis of Variance, BHI-A: Brain Heart Infusion-Agar, BHI-B: Brain Heart Infusion-Broth, BPSI-TROA: Instrument Standardization Testing Center for Medicinal and Aromatic Spice Plants, C: Celcius, CFU: colony-forming unit, *C. lanatus*: *Citrullus lanatus*, MiCore: Microbiology Center of Research and Education, OD: Optical Density, PBS: Phosphat Buffered Saline, w/v: weight by volume.

INTRODUCTION

Watermelon (*Citrullus lanatus*) is a popular fruit due to its refreshing flavour, nutritional value, and low calories (Romdhane et al., 2017). It is the second most produced fruit globally (11%) following bananas (FAOSAT, 2021). *C. lanatus* is grown in tropical and subtropical regions with abundant soil organic material (Sorokina et al., 2021). The pulp and seeds of *C. lanatus* are often consumed because they contain many beneficial properties for the body. *C. lanatus* pulp has antihypertensive, anti diabetic, antacid, laxative, anticancer, and antimicrobial properties. Moreover, *C. lanatus* seed shows antihypertensive, neuroprotective, anticancer, and antimicrobial activity (Ismael et al., 2022). Unfortunately, *C. lanatus* rind is considered food waste. Nevertheless, it contains secondary metabolite compounds such as alkaloids, flavonoids, glycosides, phenolics, saponins, steroids, tannins, and triterpenoids, which exhibit antihypertensive, antiulcer, anticancer, and antimicrobial effects (Ismael et al., 2022; Kumar et al., 2020).

Previous studies showed that ethanol extract of *C. lanatus* rind demonstrated antibacterial and antibiofilm properties against Gram-positive and Gram-negative bacteria such as *Staphylococccus aureus*, *Bacillus subtilis*, *Streptococcus. pneumoniae*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Against periodontitis, ethanol extract of *C. lanatus* rind could inhibit *Porphyromonas gingivalis* and *Treponema denticola* (Egbuonu, 2015; Gotama, 2022; Mohammed et al., 2020). Another bacteria can lead to periodontitis is *Aggregatibacter actinomycetemcomitans* (Gasner & Schure, 2022). It is a facultative anaerobic Gram-negative bacteria with virulence factors such as cytolethal distending toxin (CDT), leukotoxins, and lipopolysaccharides (LPS). The virulence factor of *A. actinomycetemcomitans* can modulate the host's immune response and produce cytokines that are the main cause

of tissue damage and bone resorption, leading to localized aggressive periodontitis (Bao et al., 2018; Missoum, 2020; Rajendra, 2022). Those virulence factors also play a role in bacterial attachment to the oral cavity surface and support biofilm formation. Biofilm is a group of microorganisms, mainly bacteria, that adhere to the tooth surface and are covered in the extracellular polymer (Soesanto et al., 2023). Poor oral hygiene supports microbial dysbiosis, allowing the pellicle layer of biofilm to develop into pathogenic biofilm (Larsen & Fiehn, 2017). These bacteria interactions (quorum sensing) involve signalling molecules (autoinducers) that can regulate gene expression and support the pathogenic process of biofilm formation (Oluwole, 2022).

The management of periodontitis includes chlorhexidine as an adjunct with scaling and root planing. Chlorhexidine is used as a golden standard antimicrobial mouthwash, but its long-term use might demonstrate adverse reactions such as tooth staining, xerostomia, and changes in taste (Deus & Ouanounou, 2022). Hence, there is a crucial need for herbal remedies that have fewer side effects than chlorhexidine and are easily accessible, thus providing an alternative treatment option for periodontitis. To address this gap, our study aims to investigate the antibacterial and antibiofilm efficacy of *C. lanatus* ethanol extract rind against *A. actinomycetemcomitans*. This study shows potential in providing valuable insights into how herbal extracts could be used as alternative therapeutic agents in managing periodontitis.

MATERIALS AND METHODS

This is an in vitro study with an experimental post-test-only control group design. The ethical clearance was exempted from the Ethics Committee for Health Research at the Faculty of Dentistry, Universitas Trisakti, Jakarta (647/S1/KEPK/FKG/7/2023).

Materials

The materials used in this research were ethanol extract of *C. lanatus* rind at concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%. Chlorhexidine 0.2% was used as a positive control. Distilled water was used as a negative control for the antibacterial test and Brain Heart Infusion-Broth (BHI-B) (Sigma Aldrich) was used as a negative control for the antibiofilm test.

Methods

Sample Preparation

The ethanol extract of *C. lanatus* rind was obtained from the Instrument Standardization Testing Center for Medicinal and Aromatic Spice Plants (BPSI-TROA) in Bogor, West Java, Indonesia. The ethanolic extract of *C. lanatus* rind was prepared using the maceration method. One thousand five hundred grams of *C. lanatus* rind was dried at 37°C for three days, ground into powder, soaked with 96% ethanol solution for 24 hours, filtered, and evaporated with a rotary evaporator to obtain extract at 100% concentration. Serial dilution was performed using distilled water to extract at concentrations of 50%, 25%, 12.5%, 6.25%, and 3.125%.

Phytochemical Tests

Qualitative phytochemical tests were performed at BPSI-TROA to identify secondary metabolites in the ethanolic extract of *C. lanatus* rind, such as alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids, and glycosides.

Bacterial Culture

A. actinomycetemcomitans ATCC 29522 was acquired from the Microbiology Center of Research and Education (MiCore) laboratory, Faculty of Dentistry, Universitas Trisakti, Jakarta. *A. actinomycetemcomitans* ATCC 29522 were cultured on BHI-B. The culture was homogenized using a vortex (Biosan) and then incubated in an anaerobic jar (Oxoid) at 37°C for 24 hours. The bacterial absorbance value was measured using a microplate reader (Safas) at 490 nm and equal to the McFarland standard of 0.5 (1.5×10^8 CFU/mL).

Antibacterial Test

Antibacterial test were conducted using microdilution and plate count methods. A total of 100 µL of bacterial suspension and each test solution were distributed into 96-well plates (Nest Biotech) and then diluted 10,000 times. Each test solution was taken five µL and spread on Brain Heart Infusion-Agar (Oxoid) media. BHI-A was incubated for 24 hours and then the total number of bacterial colonies was counted. This test was repeated four times.

Antibiofilm Test

This antibiofilm test was conducted using the microtiter plate biofilm assay method. 200 µL of bacterial suspension was incubated at 37°C under anaerobic conditions for 48 hours in 96-well plates. The supernatant was removed, leaving a thin layer of biofilm then rinsed with Phosphate Buffered Saline (PBS) (Biomatik) twice. In each treatment group, five wells with a biofilm layer and two without a biofilm layer were inserted with 200 µL of each test solution and re-incubated for 1, 3, and 24 hours. The wells were then rinsed with PBS, dried, fixated, and stained with crystal violet (0.05% w/v). The wells were rinsed and dried for 15 minutes, then 200 mL of 96% ethanol was added to each well. The intensity of crystal violet staining was measured in optical density (OD) units using a microplate reader with a wavelength of 490 nm.

Data analysis

The normality test was conducted using Shapiro-Wilk. For the antibacterial test, one-way Analysis of Variance

(ANOVA) and post hoc Tukey HSD were used to identify significant differences among the test groups ($P<0.05$). Kruskal-Wallis test was performed for antibiofilm test, followed by post hoc Mann-Whitney tests ($P<0.05$).

RESULTS AND DISCUSSION

Antibacterial test

The antibacterial test result indicates that the ethanol extract of C. *lanatus* rind has antibacterial properties against *A. actinomycetemcomitans*. There was a significant reduction in the total colony count of *A. actinomycetemcomitans* in all concentrations. The highest antibacterial effect was found at 100% concentration with a total number of bacteria of $(2\pm0.5)x10^6$ CFU/mL. Extract concentrations from 12.5% to 100% exhibit antibacterial effects on *A. actinomycetemcomitans* equivalent to positive control. (Figure 1 & 2; Table 1).

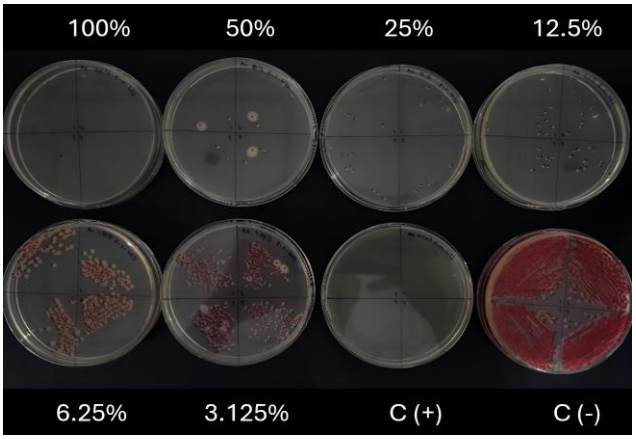

Figure 1. Antibacterial test results based on plate count method.

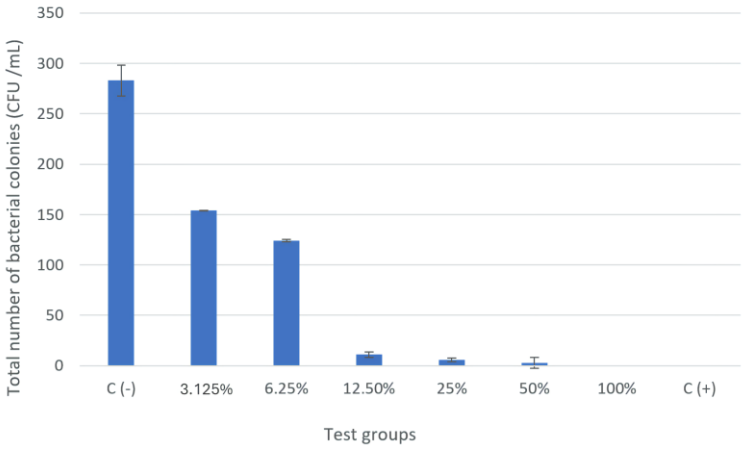

Figure 2. Antibacterial test results of *C. lanatus* rind ethanol extract against *A. actinomycetemcomitans*. Chlorhexidine as positive control and distilled water as negative control.

Table 1. Post hoc test results (Tukey HSD) of antibacterial test toward negative and positive control. *P<0.05; **P<0.01.

	3.125%	6.25%	12.5%	25%	50%	100%
C (-)	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**
C (+)	<0.001**	<0.001**	0.210	0.803	0.999	1.000

Antibiofilm test

The antibiofilm result showed that the ethanol extract of *C. lanatus* rind had antibiofilm properties against *A. actinomycetemcomitans* (Figure 3 and Table 2). Compared to the negative control, There was a decrease of OD in 1, 3, and 24 hours in incubation time. At 3 hours of incubation, the OD decreased significantly in all concentrations ($P<0.01$). Compared to the positive control, extract of 100% concentration at 1 hour and 24 hours incubation showed a lower OD value significantly ($P<0.01$).

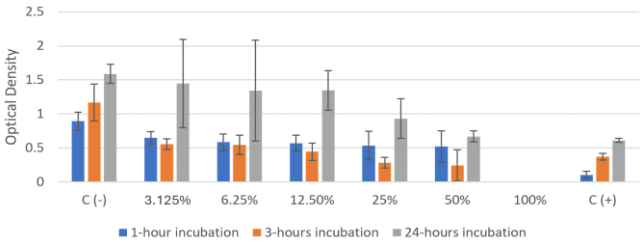

Figure 3. Antibiofilm test results of *C. lanatus* rind ethanol extract against *A. actinomycetemcomitans*. Chlorhexidine as positive control and BHI-B as negative control.

Table 2. Post hoc test results (Mann-Whitney) of antibiofilm test toward negative and positive control at 1, 3, and 24 hours of incubation. *P<0.05; **P<0.01

	Concentration	3.125%	6.25%	12.5%	25%	50%	100%
C (-)	1-hour	0.009**	0.009**	0.009**	0.009**	0.076	0.009**
	3-hours	0.009**	0.009**	0.009**	0.009**	0.009**	0.009**
	24-hours	0.602	0.602	0.175	0.009**	0.009**	0.009**
C (+)	1-hour	0.754	0.917	0.917	0.917	0.917	0.009**
	3-hours	0.009**	0.028*	0.251	0.047*	0.347	0.009**
	24-hours	0.009**	0.009**	0.009**	0.009**	0.047*	0.009**

Phytochemical test

Phytochemical test results showed that ethanol extract of *C. lanatus* rind contained secondary metabolite compounds such as alkaloids, flavonoids, glycosides, phenolics, saponins, steroids, tannins, and triterpenoids (Table 3).

Table 3. Phytochemical test result of *C. lanatus* rind ethanol extract.

No	Secondary metabolite compound	Results
1	Alkaloids	+
2	Flavonoids	+
3	Glycosides	+
4	Phenolics	+
5	Saponins	+
6	Steroids	+
7	Tannins	+
8	Triterpenoids	+

Discussion

This antibacterial test uses the plate count method due to its cost-effectiveness, simple procedures, and the ability to count bacteria without requiring special tools or materials (Bankier et al., 2018). Distilled water was used as a negative control since it does not affect the bacteria growth and thereby does not affect the course of the experiment (Widowati et al., 2022). As a positive control, chlorhexidine 0.2% is used in this study because it is the gold standard of antimicrobial used as adjuvant therapy for periodontitis. Its bacteriostatic and bactericidal properties reduce plaque and prevent periodontal disease effectively (Deus & Ouanounou, 2022). Since this is a preliminary study, the concentration in this study ranges from 3.125% to 100%. Therefore the effective concentrations for antibacterial and antibiofilm effect are currently unknown.

This study showed that the antibacterial effect of *C. lanatus* rind ethanol extract increased along with the concentration. This result is similar to a previous study regarding the antibacterial effect of *C. lanatus* rind ethanol extract against *Streptococcus mutans* using the agar well diffusion method (Govindaraj et al., 2022). Using the disc diffusion method, another study also showed an increase in the diameter of the inhibition zone against *Streptococcus mutans* in line with a higher concentration of *C. lanatus* rind ethanol extract (Ghozaly & Balqis, 2022). Another result of this antibacterial test was that extract concentrations from 12.5% to 100% exhibited antibacterial effects on *A. actinomycetemcomitans* equivalent to chlorhexidine. It means that in 100% concentration, *C. lanatus* was as effective as chlorhexidine against *A. actinomycetemcomitans.*

The antibiofilm test in this study used the microtiter plate biofilm assay method since it is able to test many samples in one test, using simple equipment, and affordable prices (Azeredo et al., 2017). For the negative control, BHI-B was used since it is a good medium for the bacteria to form biofilm (Adame-Gómez et al., 2020). The antibiofilm test in this study used an incubation period of 1, 3, and 24 hours as it represents the biofilm formation phase, starting with the pellicle formation phase in the first 2 hours, then the initial adhesion phase in 2 to 4 hours, and the maturation phase for 24 hours (Martínez-Hernández et al., 2023; Widyarman & Lazaroni, 2019).

Ethanol extract of *C. lanatus* rind began to inhibit biofilm formation at 1 hour of incubation (pellicle formation phase) with the highest OD shown at extract, with a concentration of 100%. The most effective antibiofilm effect was shown at 3 hours of incubation. This means that the ethanol extract of *C. lanatus* rind works best against *A. actinomycetemcomitans* in the initial adhesion phase of the biofilm. Furthermore, extracts with a concentration of 100% in all incubation periods and 25% in the 3-hour incubation period had a more effective antibiofilm effect compared to chlorhexidine.

This study also indicates an increase in the antibiofilm effect in line with the concentration during each incubation period. This result is similar to the research on the antibiofilm effect of *C. lanatus* rind ethanol extract against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* using biofilm formation inhibition method. This result showed a smaller OD number as the concentration rose (Ijewereme et al., 2018). Compared to incubation time of 1 and 3 hours, the decrease in OD during the 24-hour incubation period indicated that the

biofilm had formed completely and the resistance had increased 1,000-1,500 times stronger than the planktonic bacteria state (Goswami et al., 2023). The increased activity of *A. actinomycetemcomitans* in the 24-hour incubation time showed the bacteriostatic effect of *C. lanatus* rind ethanol extract.

In the maceration process, 96% ethanol was used as solvent since it is relatively less toxic than methanol. It also could extract high bioactive compounds comparable to methanol due to its high polarity for extracts with high water content (Rezagholizade-Shirvan et al., 2023; Toupal & Coşansu, 2023; Utami & Putri, 2020). The antibacterial and antibiofilm effect of *C. lanatus* rind ethanol extract due to its secondary metabolite compounds such as alkaloids, flavonoids, glycosides, phenolics, saponins, steroids, tannins, and triterpenoids. Alkaloids suppress cell wall synthesis, metabolism, protein synthesis, and nucleic acids, and alter bacterial cell membrane permeability (Yan et al., 2021). Flavonoids can change the permeability of bacterial cell membranes, interfere with nucleic acid synthesis and bacterial metabolism (Shamsudin et al., 2022). Glycosides can degrade the phospholipid membrane of bacterial cells. Phenolics are able to inhibit nucleic acid synthesis and damage bacterial cell proteins (Widowati et al., 2022). Saponins may trigger bacterial cell lysis, increase bacterial cell membrane permeability, and inhibit bacterial adhesion (Khan et al., 2018). Tannins also act by inhibiting the metabolism and attachment of bacteria on the surface of the host (Kaczmarek, 2020). Steroids and triterpenoids can suppress cell wall synthesis and disintegrate bacterial cell membranes (Widowati et al., 2021).

An ethanol extract of *C. lanatus* rind is proven to have an antibacterial and antibiofilm effect against *A. actinomycetemcomitans*. However, further study is needed to determine the toxicity of this extract.

CONCLUSIONS

Ethanol extract of *C. lanatus rind* contains alkaloids, flavonoids, glycosides, phenolics, saponins, steroids, tannins, and triterpenoids that potentially inhibit the growth and formation of *A. actinomycetemcomitans* biofilm *in vitro*. The growth of *A. actinomycetemcomitans* is inhibited by the lowest concentration of 3.125% and the inhibition of biofilm formation was most effective during biofilm initial adhesion phase.

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***Competing Interests*:** The authors declare that there are no competing interests.

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