Original article

Inhibitory test of andaliman (*Zanthoxylum achantopodium* DC) extract mouthwash against dental plaque bacteria

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ABSTRACT

Background: *Andaliman (Zanthoxylum achantopodium DC) is an endemic plant that is found in the province of Sumatera Utara, Indonesia. It contains secondary metabolites, such as alkaloids, flavonoids, glycosides, saponins, tannins, and triterpenoids/steroids, which can potentially be used as a mouthwash. Streptococcus sanguinis and Staphylococcus aureus are the primary colonizing bacteria in plaque formation. Bacterial plaque is known to be the main cause of periodontal disease but can be controlled mechanically and chemically using mouthwash.* **Purpose:** *This study aimed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of andaliman extract mouthwash (2%, 4%, 8%) against Streptococcus sanguinis ATCC®10556™ and Staphylococcus aureus ATCC® 25923™.* **Methods:** *This is a laboratory study with a post-test control-only design. The sample consists of andaliman extract mouthwash (2%, 4%, 8%), a positive control (chlorhexidine gluconate 0.2%), and a negative control (mouthwash formulation without andaliman extract) with three repetitions for each group. Data were analyzed with the one-way ANOVA test and post hoc LSD test.* **Results:** *The andaliman extract mouthwash with concentrations of 2%, 4%, and 8% significantly reduced the number of Streptococcus sanguinis and Staphylococcus aureus colonies (p<0.05), and there was a significant difference in the andaliman extract mouthwash with concentrations of 2%, 4%, and 8% compared to the negative control.* **Conclusion:** *Andaliman extract mouthwash with a concentration of 8% was more effective in inhibiting Streptococcus sanguinis growth than Staphylococcus aureus. The MIC values for both bacteria were 2%, but the study could not determine the MBC value.*

Keywords: andaliman; plaque; Streptococcus sanguinis; Staphylococcus aureus

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INTRODUCTION

Periodontal disease is a chronic inflammatory process accompanied by the destruction of the surrounding connective tissue and alveolar bone as well as tooth loss in some cases.[1] In general, periodontal disease is caused by bacterial plaque on a tooth's surface, where plaque is a thin layer of biofilm containing a collection of pathogenic microorganisms.[2] These pathogenic microorganisms can cause direct damage to periodontal tissue by activating the immune–inflammatory response but could also be beneficial bacteria in the periodontal pocket by providing sources of nutrition.[3]

Supragingival plaque plays an important role in the growth, accumulation, and pathogenesis of subgingival plaque, especially in the early stages of the periodontal diseases gingivitis and periodontitis.[4] Supragingival plaque formation is mediated by bacteria that can form extracellular polysaccharides that allow these bacteria to attach to teeth and interact with other bacteria,[5] i.e., *Streptococcus sanguinis* and *Staphylococcus aureus.*

Streptococcus sanguinis is facultative anaerobic gram-positive bacteria and normal flora found in the human oral cavity, and it plays a role in the initial colonization of plaque formation. *Streptococcus sanguinis* is known as a pioneer in forming dental plaque. The attachment of

Streptococcus sanguinis is mediated by fimbriae, pilus proteins, lipoproteins, and the enzyme glucosyltransferase, which gives it a greater adhesion ability to participate in the process of plaque maturation that contributes to the development of periodontal disease.[6]

Staphylococcus aureus is a normal bacterial flora, but in certain circumstances, it can turn into a disease-causing pathogen due to predisposing factors such as poor oral hygiene. *Staphylococcus aureus* can exacerbate periodontitis by getting into the periodontal pocket formed due to abnormal gingival sulcus depth.[7] *Staphylococcus aureus* plays an important role in causing periodontal diseases by forming a biofilm on dental plaque and exacerbating periodontal diseases by secreting various pathogenic factors.[8]

Plaque control is vital to prevent plaque formation and reduce the progression of periodontal disease caused by bacteria. It can be performed mechanically with a toothbrush, dental floss, or interdental brush, and chemically by using mouthwash.[9] Currently, the active ingredient in mouthwash that is widely used in Indonesia is chlorhexidine, and 0.2% chlorhexidine gluconate is an antimicrobial and gold standard in reducing plaque formation. The use of chlorhexidine mouthwash has various reversible side effects such as discoloration of teeth and restorations, changes in taste sensation, and it can even trigger desquamative lesions.[10]

Herbal ingredients are currently used as alternative materials that have the antibacterial ability to prevent the formation of dental plaque. One of the herbal plants with antibacterial activity and the potential to be used as a mouthwash is andaliman (*Zanthoxylum achantopodium* DC), which originated in North Sumatera, Indonesia. It is widely found in Dairi, North Tapanuli, Tobasa, Humbang, Silindung, and Toba Holbung. Antibacterial compounds identified from andaliman are alkaloids, terpenoids, flavonoids, saponins, and glycosides. Andaliman has been reported for its strong antimicrobial activity and acts as a natural preservative to prevent the growth of pathogen bacteria.[11]

Shasti's research found that testing andaliman extract against *Staphylococcus aureus* showed the largest inhibition zone, 18.98 mm, at 8%.[12] The 25 mg/mL and 12.5 mg/mL of andaliman extract also had an inflammatory effect that resulted in a reduction in the TNF-α and IL-6 levels of fibroblast infected by *Streptococcus sanguinis*.[13]

The novelty of this study is to examine andaliman extract formulated into mouthwash, thereby inhibiting *Streptococcus sanguinis* and *Staphylococcus aureus*. This study aims to observe the andaliman extract mouthwash as an antibacterial against *Streptococcus sanguinis* and *Staphylococcus aureus* in terms of its MIC and MBC values.

MATERIALS AND METHODS

This is experimental laboratory research (true experimental design) with a post-test-only control group design. This research has received approval from the ethics committee of the University Sumatera Utara Hospital (No. 309/KEPK/USU/2022).

Andaliman was confirmed by a Herbarium Medanese (MEDA) Laboratory, Medan, Indonesia. The sample used in this study was taken from one of the andaliman fruit producers in Sipira Village, Onan Punggu district, Toba Samosir Regency. Extracts were made using the maceration method: 2.5 kg of andaliman was dried and refined to produce simplicia powder, which was immersed in 96% ethanol solvent (1:10) and left for 18 hours. It was then filtered to obtain a macerate, and the maceration process was repeated twice. The maceration results were combined and concentrated in a rotary vacuum evaporator to obtain a thick extract. Phytochemical tests were carried out to identify alkaloids, flavonoids, glycosides, saponins, tannins, steroids, and triterpenoids.[14] Next, the formulation of andaliman extract mouthwash was performed according to Table 1.

Cultures of *Streptococcus sanguinis* ATCC®10556™ and *Staphylococcus aureus* ATCC® 25923™ were taken from the Microbiology Laboratory in the Faculty of Pharmacy at the University Sumatera Utara. The bacteria were rejuvenated before conducting the antibacterial test. The bacterial suspension was made with a concentration of 10^{-3} Mcfarland. The antibacterial effectiveness of the andaliman extract mouthwash was carried out using the dilution method to obtain the MIC and MBC values. Eight ml of nutrient broth media was put into a test tube, then 1 ml of andaliman extract mouthwash with three concentrations (2%, 4%, and 8%) was added along with a positive control (chlorhexidine gluconate 0.2%) and a negative control (mouthwash formulation without andaliman extract).

Table 1. Andaliman extract mouthwash formulation

Materials	Negative control	Concentration (w/w)			Positive control
		2%	4%	8%	
Andaliman extract	-	2 gr	4 gr	8 gr	
Glycerin	4 gr	4 gr	4 gr	4 gr	
Sorbitol	9 gr	9 gr	9 gr	9 gr	0.2% Chlorhexidine gluconate
CMC-Na	0.3 gr	0.3 gr	0.3 gr	0.3 gr	
Aquadest	86.7 gr	84.7 gr	82.7 gr	78.7 gr	
Total	100 gr	100 gr	100 gr	100 gr	

After that, 1 ml of bacterial suspension was added to each tube and vortexed. All tubes were incubated at 37 °C for 24 hours.[15] Observations were made on each tube by looking at turbidity levels. The MIC value was the lowest concentration that showed a clear area in the tube.[16] All test tubes were transferred to solid media. *Streptococcus sanguinis* ATCC®10556™ was streaked on the blood agar medium using the streak plate method. At the same time, the tube containing the *Staphylococcus aureus* ATCC® 25923™ bacterial suspension was subcultured on the plate count agar (PCA) medium using the pour method. After incubation at 37 °C for 24 hours, the number of bacterial colonies was calculated using a colony counter.[15] The lowest concentration that did not indicate the presence of bacterial colonies was defined as the MBC value.[16] The data were analyzed using the Shapiro–Wilk test because the results were homogenous and normal, then continued with one-way ANOVA and post hoc least significant difference (LSD) tests to compare the differences between all groups.

RESULTS

The phytochemical tests showed that the metabolite compounds found in andaliman extract are alkaloid, flavonoid, glycoside, saponin, tannin, and triterpenoid/steroid (Table 2). The antibacterial activity of the andaliman extract mouthwash against *Streptococcus sanguinis* and *Staphylococcus aureus* with concentrations of 2%, 4%, and 8%, a positive control, and a negative control was observed. It could be seen that all of the tubes were turbid due to the color of the concentrated andaliman extract mouthwash (Figure 1). Furthermore, to discover if turbidity was caused by bacterial growth or not and to determine the values of MIC and MBC, all the diluted tubes were transferred to solid media and incubated for 24 hours. Then, a calculation was carried out on a petri dish with the number of bacterial colonies observed using a colony counter (Figures 2 and 3).

As the concentration increased, the results showed a decrease in the number of colonies for *Streptococcus sanguinis* and *Staphylococcus aureus.* The highest average number of *Streptococcus sanguinis* and *Staphylococcus aureus* colonies were in the negative control group (mouthwash without andaliman extract) at 1,896.33 CFU/ml and 4,849 CFU/ml, respectively. The concentration of 2% was the lowest concentration that began to inhibit the growth of *Streptococcus sanguinis* and *Staphylococcus aureus,* with the average number of bacterial colonies being 703.33 CFU/ml and 3,878 CFU/ml, respectively. The comparison of antibacterial activity of andaliman extract mouthwash for each concentration showed more effectiveness in inhibiting the growth of *Streptococcus sanguinis* with a fewer average number of bacterial colonies than *Staphylococcus aureus* with a more significant average number of bacterial colonies (Table 3).

Post hoc LSD tests on antibacterial activity against *Streptococcus sanguinis* revealed that the concentrations of 2%, 4%, 8%, and a positive control had no significant differences (p>0.05) in each concentration, while the negative control group showed a significant difference (p<0.05). Meanwhile, LSD tests on antibacterial activity against *Staphylococcus aureus* showed that the concentrations of 2% and 4% and 4% and 8% had no significant difference (p>0.05). At the same time, in the other group, there were

Table 2.   Phytochemical screening test results for andaliman extract

Secondary metabolites	Result
Alkaloid	+
Flavonoid	+
Glycoside	+
Saponin	+
Tannin	+
Triterpenoid / Steroid	+

Figure 1.   Observation results of the andaliman extract mouthwash dilution test with three repetitions: A. *Streptococcus sanguinis*; B. *Staphylococcus aureus.*
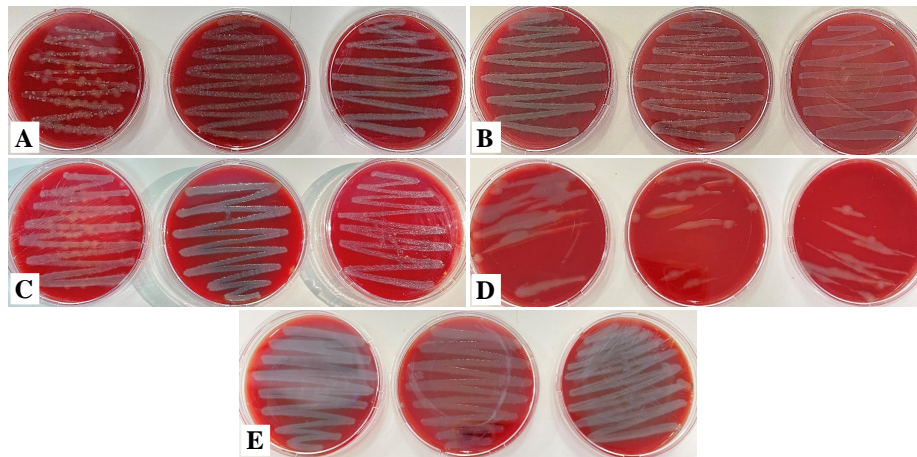
Figure 2. Results of andaliman extract mouthwash and *Streptococcus sanguinis* cultured on blood agar media: A. 2% concentration; B. 4% concentration; C. 8% concentration; D. positive control E. negative control.
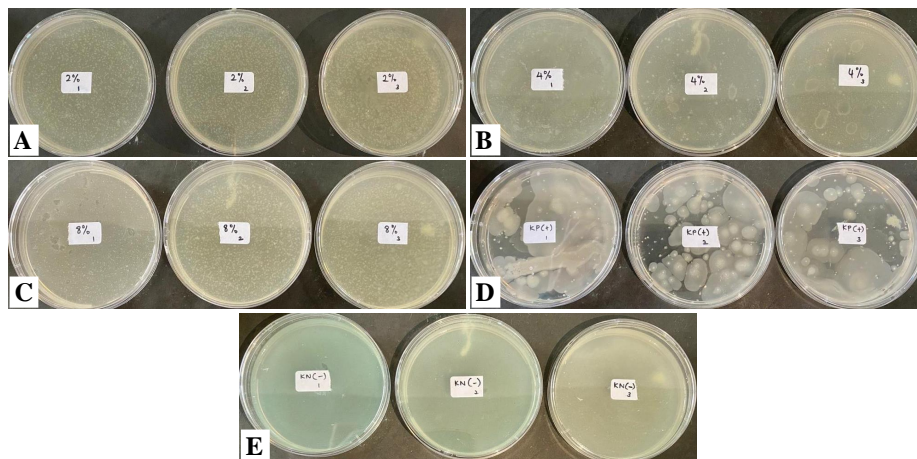

Figure 3. Results of andaliman extract mouthwash and *Staphylococcus aureus* cultured on PCA media: A. 2% concentration; B. 4% concentration; C. 8% concentration; D. positive control E. negative control.

Table 3. The average number of *Staphylococcus aureus* and *Streptococcus sanguinis* bacterial colonies

| Concentrations | The average number of bacteria (CFU/ml) | | | |
| | *Streptococcus sanguinis* | | *Staphylococcus aureus* | |
	Mean ± SD	ANOVA	Mean ± SD	ANOVA
2%	703.33±105.19		3,878 ± 634.863	
4%	524.67±163.37		3,638.66± 332.726	
8%	204.33±108.45	0.001*	2,982 ± 259.963	0.000*
PC (+)	460.67±137.59		811.33 ± 440.312	
NC (-)	1,896.33±744.23		4,849 ± 203.315	

Notes**:** * a significant difference (P<0.05)

Table 4. The difference in the average number of bacterial colonies in the andaliman extract mouthwash against the growth of *Streptococcus sanguinis* and *Staphylococcus aureus* for each concentration

| Concentrations | | P-value | | | | |
		2%	4%	8%	PC (+)	NC (-)
Streptococcus sanguinis	2%		0.549	0.114	0.419	0.002*
	4%			0.292	0.829	0.001*
	8%				0.394	0.000*
	PC (+)					0.001*
Staphylococcus aureus	2%		0.485	0.022*	0.000*	0.015*
	4%			0.075	0.000*	0.004*
	8%				0.000*	0.000*
	PC (+)					0.000*

Notes: Post hoc LSD *a significant difference (P<0.05)

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significant differences in each concentration (p<0.05). The antibacterial tests for *Streptococcus sanguinis* and *Staphylococcus aureus* showed significant differences in each concentration against the negative control group. The andaliman extract mouthwash effectively inhibited the growth of both *Streptococcus sanguinis* and *Staphylococcus aureus* (Table 4).

DISCUSSION

In this study, andaliman extract (*Zanthoxylum acanthopodium* DC) was dissolved and macerated in 96% ethanol solvent. Sepriani et al.[17] used 96% ethanol solvent because it is a universal solvent that can be polar or non-polar so that the metabolite compounds found in the andaliman plant can be extracted. The formulation of andaliman extract mouthwash consists of four main ingredients: glycerin, CMC-Na, sorbitol, and Aqua Dest. Glycerin is a humectant component to prevent the active substances in the andaliman extract mouthwash from evaporating into the air. The addition of CMC-Na binds the mouthwash ingredients so that all components will be homogeneous. Sorbitol is used to provide a sweet taste to compensate for the bitterness of andaliman. Aqua Dest is used as a solvent.[18]

The results of the phytochemical tests on andaliman extract (*Zanthoxylum acanthopodium* DC) in this study showed the presence of alkaloid compounds, flavonoids, glycosides, saponins, tannins, and triterpenoids/steroids. This agrees with Muzafri's research, which states that the results of phytochemical screening of andaliman extract show that it contains alkaloid compounds, flavonoids, glycosides, saponins, tannins, triterpenoids/steroids, and anthraquinone glycosides.[19] Alkaloids as antibacterial compounds can inhibit bacterial nucleic acid and protein synthesis, metabolism, and efflux pumps.[20] The mechanism of flavonoids in inhibiting bacteria is almost similar to glycosides, which can form complex compounds with extracellular proteins, so *Streptococcus sanguinis* and *Staphylococcus aureus* cannot maintain the shape of their cell membranes and pathogenicity, which are essential for bacterial growth.[21] Saponin compounds can increase membrane permeability, which can inactivate bacterial enzymes in the metabolic process and cause the death of *Streptococcus sanguinis* and *Staphylococcus aureus*. Tannins are phenolic compounds that can damage the function of bacterial genetic material and inhibit enzymes so that bacteria cells are not formed.[22] Terpenoids as antibacterial compounds can form bonds with porins. Damaged porins inhibit the nutrient transport process so that bacteria will lack nutrients, which results in bacterial inhibition or death in *Streptococcus sanguinis* and *Staphylococcus aureus*.[23]

The antibacterial effectiveness test used in this study was the dilution method, which was carried out by calculating the values of MIC and MBC. Each test tube that had andaliman extract mouthwash with concentrations of 2%, 4%, 8%, a positive control, and a negative control was given a bacteria suspension of *Streptococcus sanguinis* and *Staphylococcus aureus*. The observations showed that each test tube had the same level of turbidity that was difficult to determine via the dilution method (Figure 2). This was influenced by the color of the andaliman extract mouthwash, which was concentrated. Therefore, this study had to calculate the number of bacterial colonies by continuing the culture of diluted results in solid media, namely, blood agar and PCA.

The MIC value of the andaliman extract mouthwash was determined by a petri dish with a smaller number of bacterial colonies than the petri dish of the negative control group. The MBC value was determined by a petri dish with no bacterial growth. The results showed a decrease in bacterial colonies as the concentration increased. Khalishah's research states that the higher the concentration of antibacterial substances, the higher the ability to inhibit the growth of bacteria.[24] The results of this study showed that the MIC value was at the concentration of 2% because it was the lowest concentration that began to inhibit the growth of *Streptococcus sanguinis* and *Staphylococcus aureus* compared to the negative control. Meanwhile, this study's MBC value could not be determined because bacterial colonies were still found at the concentration of 8%.

The andaliman extract mouthwash inhibited the growth of *Streptococcus sanguinis* and *Staphylococcus aureus* in this study. Lubis found that 4% andaliman extract mouthwash was effective because it reduced the number of *Streptococcus mutants*.[13] Furthermore, research by Shasti et al.[12] proved the ability of 8% andaliman extract to inhibit the growth of *Staphylococcus aureus*. Sitanggang et al.[23] researched the inhibitory activity of andaliman extract on the growth of *Escherichia coli*. Based on the analysis of inhibitor diameter, the MIC value was found at a concentration of 60%. This proves that andaliman extract mouthwash (*Zanthoxylum acanthopodium* DC) has a bacteriostatic effect on gram-positive anaerobic bacteria that cause periodontal disease. The concentration of antibacterial compounds affects the ability to inhibit bacterial growth. Darajat stated that the higher the concentration of antibacterial substances, the more antibacterial compounds contained, which means bacteria will be killed quickly at higher concentrations.[25]

In this study, bacterial growth was found at each concentration of 2%, 4%, and 8%, and several technical and biological factors could cause this in the control group. The technical factors can mainly be controlled by researchers; these include the large inoculum, pH, incubation length, temperature, and medium. Biological factors consist of bacterial cell wall structure and resistance.[26] Gram-positive bacteria, such as *Streptococcus sanguinis* and *Staphylococcus aureus*, have thicker peptidoglycan layers on the cell wall than gram-negative bacteria, forming a rigid structure. The presence of wider peptidoglycan structures in gram-positive bacteria makes the antimicrobial

compounds more challenging to penetrate gram-positive cell walls than gram-negative cell walls.[27] Another biological factor is resistance. Bacteria are likely to become resistant during antibacterial tests because resistance is an adaptation bacteria naturally make to survive. Resistance is a fundamental factor that cannot be controlled.[26]

In conclusion, there was a decrease in the number of bacterial colonies for both *Streptococcus sanguinis* and *Staphylococcus aureus* from the highest to the lowest concentration. The concentration of 8% was shown to be more effective in inhibiting *Streptococcus sanguinis* growth than *Staphylococcus aureus*. The MIC value that began to inhibit bacterial growth for both bacteria was 2%, while the MBC could not be determined in this study.

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