Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 3
Open Access

Phyllosphere Bacteria of Andaliman (Zanctoxylum acanthopodium DC.) as Potential Antimicrobial Compounds Source against Pathogenic Bacteria

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Cite this paper as: Debie Rizqoh, Sipriyadi, Noual Mas'ud, Indah Dwi Rahmawati, Shella Sharon, Utari Hartati Suryani, Novriantika Lestari, Oktoviani, Risky Hadi Wibowo (2024) Phyllosphere Bacteria of Andaliman (Zanctoxylum acanthopodium DC.) as Potential Antimicrobial Compounds Source against Pathogenic Bacteria. *Frontiers in Health Informatics, 13* (3),6399-6408

ABSTRACT

Infectious diseases are diseases that can be life-threatening if left untreated and can be caused by pathogenic microbes such as Escherichia coli, Bacillus subtilis and Staphylococcus aureus. The irrational use of antimicrobials has caused many pathogenic microbes to adapt and become resistant to antimicrobials. Andaliman (Zanthoxylum acanthopodium DC.) and phylosphere bacteria are known to have antimicrobial activity due to the compounds they contain. This study aims to determine the antimicrobial potential of Andaliman phyllosphere bacteria (Z. acanthopodium DC.) against E. coli, B. subtilis and S. aureus. This research was an experimental laboratory research with qualitative data collection methods. The antagonist test was carried out by using spot technique. The five best isolates were identified molecularly based on the 16s rRNA gene. Then, crude extracts of potential phyllosphere bacteria of Andaliman were carried out through secondary metabolite screening and Minimum Inhibitory Concentration (MIC) test with disc diffusion method. From the antagonist test results, six isolates inhibited the growth of S. aureus and 8 isolates inhibited B. subtillis. In the MIC test results, the best inhibitory zone was obtained with a concentration of 70% in the AF43 isolate measuring 15.79 mm, which was included in the strong category against S. aureus bacteria and the MIC test results were also obtained at a concentration of 70% in the AF43 isolate measuring 13.57 mm. mm which falls into the strong category against B. subtilis bacteria. AF43 isolate extract is an isolate that has inhibitory power against S. aureus and B. subtilis bacteria, where a concentration of 100% produces the most significant inhibitory power, measuring 17.2 mm against B. subtilis bacteria and 23.4 mm against S. aureus bacteria.

KEYWORDS : *Andaliman (Zanthoxylum acanthopodium DC.), Antimicrobial, pathogen bacteria,*

phyllosphere bacteria

INTRODUCTION

Infection is a possible disease that threatens lives, and the incidence of infection in humans has increased in two decades (Barreto et al., 2006). Microorganisms like bacteria, fungi, parasites and viruses can cause infection in humans. Infections caused by bacteria can occur and attack various organ systems in the human body (Novard et al., 2019). Indonesian Basic Health Researches 2018 data shows infectious disease prevalence in Indonesia, such as Acute Respiratory Tract Infection (ARTI) (9.3%), pneumonia (4.0%), diarrhea (8.0%), and diarrhea in toddlers (12.3%) (Kemenkes RI, 2018). Bengkulu became the third province with the highest prevalence incidence of diarrhea, with a prevalence reaching more than 9% (Kemenkes RI, 2018).

Staphylococcus aureus is a Gram-positive bacteria that is cocci-shaped and arranged like wine. *S. aureus,* known as the microbiome in humans, can be found in the human respiratory tract, digestion tract and skin and can cause diseases in humans (Syahrurachman et al., 2010). Infection of *S. aureus* in humans can cause various diseases, including bacteremia, endocarditis, skin and soft tissue infection, osteomyelitis, infection tool prosthetics, gastroenteritis, urinary tract infections and others (Taylor & Unakal, 2024).

Escherichia coli is a rod-shaped, Gram-negative bacteria that has become a microbiome in the human intestine. It can cause primary infection of the intestine, for example, diarrhea in children and other organ tissue infections outside the intestines. *E. coli* infection can also cause poisoning of food, diarrhea, urinary tract infections, sepsis, and meningitis in children (Syahrurachman et al., 2010).

B. subtilis is a Gram-positive bacteria that can form endospores. *B. subtilis* is normal microbiota in the gut (Djaenuddin & Muis, 2015). *B. subtilis* produces an extracellular toxin known as subtilisin. *B. subtilis* can cause diseases like bacteremia, endocarditis, meningitis, respiratory tract infections, urinary tract infections, and gastrointestinal tract (Utami et al., 2017).

Using antimicrobials irrationally has caused many pathogen microbes to adapt to the environment and become resistant to drugs (Candrasari, 2014). In 2013, over 700,000 deaths occurred all over the world as a result of. The estimated number of deaths in 2050 will be 10 million as a consequence of antibiotic resistance, and 4.7 million of them are Asian population. The significant impact of antibiotic resistance is an increase in morbidity and mortality because resistant bacteria infection risk spread and costs more expensive treatment (CDC, 2022).

Based on a study by Asbur (2018) shows that fruit Andaliman (*Zanthoxylum acanthopodium* DC.) extract has bactericidal ability to *Bacillus stearothermophilus*, *Pseudomonas aeruginosa*, *Vibrio cholera*, and *Salmonella typhimurium.* (Asbur, 2018) Based on research conducted by Maysarah (2009) shows that the endophyte fungus of Andaliman is capable of inhibiting the growth of Aspergillus *sp*. (Maysarah, 2009). Besides the plant, antimicrobial compounds can also originate from secondary metabolism results of interaction between two phyllosphere bacteria (Rizqoh et al., 2016). A number of phyllosphere bacteria can produce bioactive compounds to compete with other microorganisms to obtain space and nutrition for growing (Lindow & Brandl, 2003). Based on research results by Rizqoh *et al.* (2016) stated that bacteria phyllosphere from Reundeu (*Stauroyne longata)* can produce antimicrobial compounds and inhibit the growth of 4 pathogen microbes that is *E. coli, S. aureus, Candida tropicalis* and *C. albicans.*

Research conducted by Aritonang (2016) shows the ability of phyllosphere bacteria to inhibit the growth of fungi from the genera *Aspergillus, Alternaria* and *Fusarium*. Rizqoh et al. (2021) have carried out studies in isolation and characterization of Andaliman phyllosphere bacteria. The results of this research are advanced from this research, which aims to determine the antimicrobial activity of Andaliman phyllosphere bacteria, identify potential isolates of Andaliman phyllosphere bacteria, determine the minimum inhibitory concentration (MIC) of Andaliman phyllosphere bacteria crude extract and identify chemicals compound contained in Andaliman phyllosphere bacteria.

MATERIALS AND METHODS

Reculture of Target Microbes

Target bacteria (*E. coli*, *S. aureus* and *B. subtilis*) are reculture in Tryptic Soy Broth (TSB), then incubated at temperature 37 ^{0}C for 24 hours. Its turbidity was measured with spectrophotometry (OD = 0.3 concentration 10 6 – 10 7 cells /mL).

Antagonist Test

Antagonist tests of phyllosphere bacteria isolates against target microbes are carried out with a two-layer agar media technique consisting of semi-solid NA media and solid NA media. Target microbes that have been measured its turbidity with spectrophotometry, mixed with semi-solid NA media, and then poured in on solid media that has been frozen previously on the plate. After that, phyllosphere bacteria isolates are dotted on it. The culture of antagonist tests was incubated for 24 hours at room temperature. Observation is done after the test culture has been incubated for 24 hours. A positive bacteria isolate has the potential to produce antimicrobial compounds if the isolate forms an inhibition zone. The diameter of the inhibition zone was measured with vernier calipers, and the power of inhibition activity was assessed based on Surjowardojo et al. (2015) categories (Table 1).

Isolation of Phyllosphere Bacteria Genome

Isolate bacteria cultured in Kings'B media for 24 hours. Amount 1.5 ml of culture was centrifuged at 10,000 rpm for 10 minutes. Genomic DNA bacteria are extracted by following a bacterial DNA Presto Mini gDNA Bacterial Kit (Geneid) according to the instructions from the manufacturer.

Amplification of the 16S rRNA Gene by Polymerase Chain Reaction (PCR)

Amplification of the 16S rRNA gene was conducted on some of the best isolates that have antimicrobial activity. Gene amplification is carried out with mix 12.5 PCR buffer GC II, 4 µl dNTPs (2.5 mM/dNTP), 1 µl primer 63 F (CAGGCCTAACACATGC-AAGTC), 1 µl primer 1387 R (GGGCGGWGTGTACAA-GGC), 4 µl DNA template, and 0.25 µl Taq DNA polymerase (GreenTaq) and 2.25 µl ddH2O. Amplification was done in the PCR machine for 30 cycles. The Amplification Stages regulated are predenaturation for 5 minutes and denaturation for 1 minute at 94 ºC, annealing for 1 minute at 55 ºC, and 1-minute polymerization and 2 minutes post PCR at 72 ºC. Visualization of 16S rRNA amplicons was performed through electrophoresis. This PCR gene amplification was then sequenced at 1st BASE PTE Ltd, Singapore.

Analysis 16S rRNA sequence

Bacterial DNA sequences are analyzed to identify phyllosphere bacteria. Species identification is done with homology sequence analysis using the Blast-N program from the NCBI website (http://www.ncbi.nlm.nih.gov/). Then, the sequences were analyzed for their relationship to each other with phylogenetics analysis. 16S rRNA gene sequences were aligned using the Mega 11 application program. The phylogenetic tree was also constructed with the Mega 11 application program.

Extraction Isolate Bacteria Philosphere Andaliman Using Ethyl Acetate

Culture is performed to potential Andaliman phyllosphere bacteria isolates. The isolates were cultured in 500 ml NB media and incubated on a shaker *at* 170 RPM (Rotary Per Minute) at 30ºC for 72 hours. Next, 500 ml of the ethyl acetate solvent was added to the culture, incubated at 30°C for 24 hours, and stirred for 20 minutes. After waiting 10 minutes on the funnel, the liquid is separated between the medium. The top layer part was

taken and evaporated using a vacuum rotary evaporator HS-2005V at a temperature of 40 ºC with a speed of 90 rpm. The crude extract was stored at 20ºC for the next step usage (Müller & Ruppel, 2014).

Minimum Inhibitory Concentration (MIC) Test Andaliman Phyllosphere Bacteria Crude Extract to Pathogen Microbes

Minimum Inhibitory Concentration (MIC) is the lowest concentration of antimicrobial agents that can inhibit other organism growth. MIC test in this research was conducted using the disc diffusion method. At the beginning of the MIC test, 12 treatments were used, with each treatment done three times repetition. Andaliman phyllosphere bacteria crude extracts are made concentration 10% (0.1 g/ml), 20% (0.2 g/ml), 30% (0.3 g/ml), 40% (0.4 g/ml), 50% (0, 5 g/ml), 60% (0.6 g/ml), 70% (0.7 g/ml), 80% (0.8 g/ml), 90% (0.9 g/ml), and 100% (1 g/ml)—each concentration of crude extracts diluted with using 7% DMSO. The positive control of the fungi test used ketoconazole, the positive control of the bacteria test used amoxicillin, and the negative control used DMSO 7%.

First, 1 ml of suspension bacteria or fungi was added to a liquid NA medium (for bacteria), then homogenized with a magnetic stirrer and poured into a petri dish to solidify. Next, a 6-mm-diameter paper disc was inserted into a petri dish test using tweezers. Then, the paper disc was dripped with 5 µl crude extract of Andaliman phyllosphere bacteria isolates with different concentrations and incubated for 3x24 hours at 37°C. After that, the clear zone that appears around the paper disc was observed. A clear zone around the paper disc indicates that the crude extract can inhibit targeted microbes. The inhibition zone calculation measured the clear zone around the paper disc. The inhibition zone was measured on three sides, then averaged from the results measurement and reduced with a paper diameter disc used. Furthermore, the inhibition zone category is classified based on Table 1.

Tabel 1. Category of Inhibition Zone

Diameter of Inhibition Zone	Category
≥ 21 mm	Powerful
11-19 mm	Strong
5-10 mm	Moderate
≤ 5 mm	Weak

RESULTS

Inhibited Ability of Phyllosphere Bacteria to Pathogenic Microbes

Phyllosphere bacterial antagonist tests have been conducted against *E. coli, S. aureus, B. subtilis,* and *C. albicans*. Of the 64 isolates, only 51 isolates of phyllosphere bacteria were tested for antagonism against these microbes. That is because as many as 13 isolates of phyllosphere bacteria did not grow well, so they were not tested. Phyllosphere bacteria with antimicrobial activity form a clear zone in the antagonistic test (Figure 1). Several phyllosphere bacteria showed antimicrobial activity: 8 isolates were antagonistic against *B. subtilis* and 6 were antagonistic against *S. aureus* (Table 2). There is no antimicrobial activity against *E. coli* in Andaliman phyllosphere bacteria.

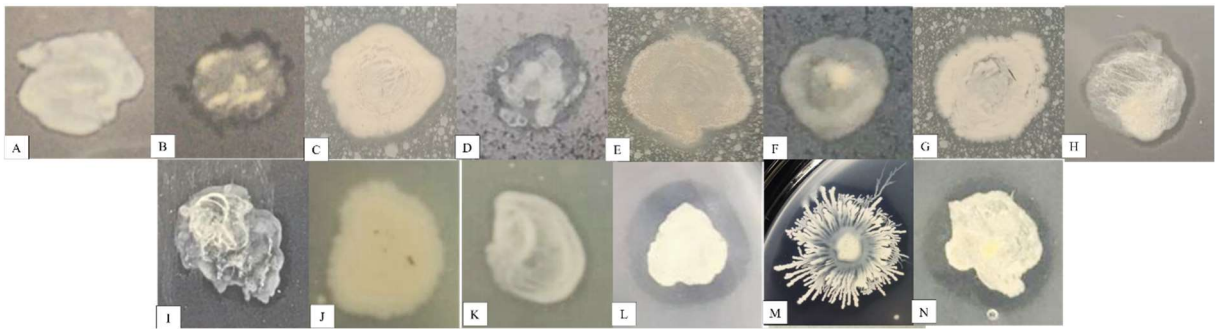

Figure 1. Inhibition Zone of the Andaliman Philosphere bacterial isolates antagonism test against pathogenic microbes; A-H: inhibition to *B. subtilis* ((A) AF 3, (B) AF 6, (C) AF 26, (D) AF 43, (E) AF 49, (F) AF 50, (G) AF 53, (H) AF 58); I-N: inhibition to *S. aureus* ((I) AF 23, (J) AF 26, (K) AF 49, (L) AF 58, (M) AF 63, (N) AF 64).

Table 2. Potential antimicrobial activity of Andaliman Phyllosphere Bacteria

Isolate Code	Antimicrobial activity of Andaliman Phyllosphere Bacteria					
	B. subtilis			*S. aureus*		
	Inhibition zone (+/-)	ZOI Diameters (mm)	Category	Inhibition zone (+/-)	ZOI Diameters (mm)	Category
AF 3	+	1,2	Weak	-	-	-
AF 6	+	4,0	Weak	-	-	-
AF 23	-	-	-	+	1,2	Weak
AF 26	+	2,6	Weak	+	0,9	Weak
AF 43	+	5,1	Moderate	-	-	-
AF 49	+	1,2	Weak	+	1,1	Weak
AF 50	+	2,5	Weak	-	-	-
AF 53	+	1,0	Weak	-	-	-
AF 56	-	-	-	-	-	-
AF 58	+	1,0	Weak	+	5,1	Moderate
AF 63	-	-	-	+	3,2	Weak
AF 64	-	-	-	+	3,5	Weak
Total	8			6		

Some Andaliman phyllosphere isolates showed inhibitory activity against more than one microbe. The widest antimicrobial spectrum was AF 49, which could inhibit *B. subtilis* and *S. aureus*. Furthermore, AF 26 and AF 58 could inhibit *B. subtilis* and *S. aureus*. Then, AF 50 and AF 53 could inhibit *B. subtilis*.

Molecular Identification of Potential Isolate of Andaliman Phyllosphere Bacteria

The known base sequences of the 16S rRNA coding gene are used to determine bacterial species based on data in the gene bank and create a phylogenetic tree based on previously identified nearby organisms. From matching with data in the gene bank, it is known that AF26, AF43, AF49, AF50, and AF58 have the closest similarity to *Brevundimonas* sp. (99.24%), *Pseudomonas* sp. (71.45%), *Brevundimonas* sp (99.10%), *Pseudomonas* sp. (80.58%) and *Bacillus altitudinis* (98.52%). Based on phylogenetic analysis, isolates AF26 and AF49 are closely related, as are AF43 and AF50 (Figure 2).

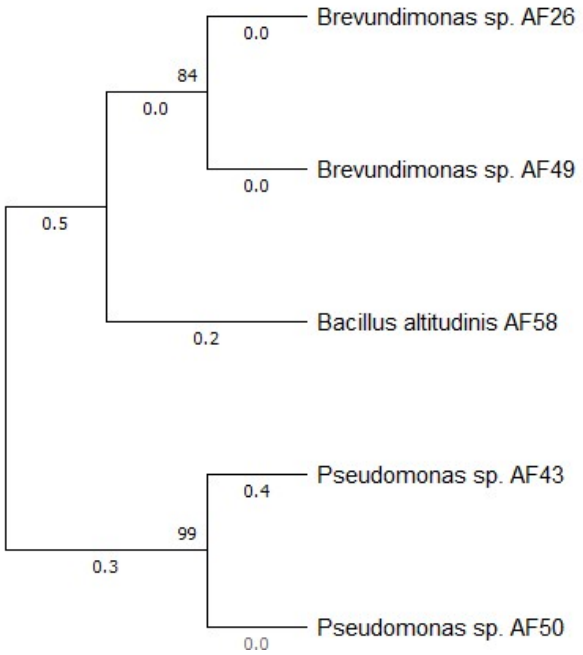

Figure 2. The phylogenetic tree shows the relationships between the five best Andaliman phyllosphere bacterial isolates that produce antimicrobial bioactive compounds.

Extraction of Andaliman Phyllosphere Bacterial Isolates Using Ethyl Acetate

In the previous step, the best isolates of Andaliman phyllosphere bacteria were isolated: AF26, AF43, AF49, AF50, and AF58. Table 3 shows the extraction process results of 5 Andaliman phyllosphere isolates.

Table 3. Extraction results of Andaliman phyllosphere isolates using ethyl acetate solvent.

Isolate Code	Volume of extract (ml)
AF26	6,7
AF43	6,6
AF49	6,6
AF58	6,2
AF50	5,9

Minimum Inhibitory Concentration (MIC) Test of Five Isolates Phyllosphere Bacteria Extract Andaliman To *B. subtilis and S. aureus*

MIC test results from five Andaliman phyllosphere bacteria extracts to *B. subtilis* and *S. aureus* can seen in Tables 4 and 5. Based on Table 3, it can be seen that not all concentrations of Andaliman phyllosphere bacterial isolate extracts have antibacterial activity against *B. subtilis.* Isolates AF26, AF49, and AF58 can not inhibit *B. subtilis* growth. Isolate AF43 has the power to inhibit *B. subtilis* at concentrations of 70%, 80%, 90%, and 100%. MIC test results on isolate AF50 showed antibacterial activity in bacteria *B. subtilis* at 100% concentration.

Based on Table 5, it can be seen that not all concentrations of phyllosphere bacterial isolate extracts activity against *S. aureus.* Isolate AF26, AF49, AF50, and AF58 do not have antibacterial activity against *S. aureus* at all concentrations. Isolate AF43 showed inhibitory activity at 70%, 80%, 90%, and 100% concentration. So, the MIC value of AF43 is 70%.

DISCUSSION

The formation of an inhibition zone around the phyllosphere bacterial isolates inoculated onto the test media showed that the Andaliman Phyllosphere bacteria have antimicrobial activity. These results align with research conducted by Asbur (2018) which shows that Andaliman fruit extract is bactericidal against *Bacillus* sp. Andaliman plants have several biological activities such as larvicides, anti-inflammatory, analgesic, antimicrobial, antioxidant, and antifungal. The bioactive compounds in Andaliman, such as essential oils, alkaloids, flavonoids, saponins, and tannins, have an antimicrobial effect that can reduce the number of bacterial colonies (Rizqoh et al., 2024). According to Pelczar & Chan (2005) the mechanism of action of antimicrobial compounds is generally carried out by damaging cell walls, changing membrane permeability, disrupting protein synthesis, and inhibiting enzyme action.

The results of the antagonist test in this study found that the Andaliman philosphere bacteria had higher inhibiting activity against *B. subtilis* than *S. aureus* . The defence of the Gram-positive bacterial cell wall is relatively more vulnerable because most of it is only composed of a peptidoglycan layer (Gow et al., 2017).

This research found that phyllosphere bacteria species in Andaliman consist of *Brevundimonas* sp., *Pseudomonas* sp., and *Bacillus altitudinis*. Steven et al. (2018) characterized *Pseudomonas* and *Enterobacteriaceae* as the dominant taxa of apple. Several studies reveal *Pseudomonas* as the most abundant genus in the phyllosphere region.(Aleklett et al., 2014; Kecskeméti et al., 2016; Steven et al., 2018) Seed coat-associated bacteria that have been reported in the phyllosphere are mainly *Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria*.(Johnston-Monje & Raizada, 2011; Rodríguez et al., 2018; Sivakumar et al., 2020)

Ethyl acetate is a good solvent used for extraction because it is capable of easy evaporation, is not hygroscopic, and has low toxicity. Ethyl acetate is also a solvent with characteristic semi-polar ones that can interest polar or non-polar compounds and have low toxicity. Ethyl acetate is a solvent that can attract alkaloids, flavonoids, saponins, tannins, polyphenols, and triterpenoids (Putri et al., 2013).

In this research, the antagonist test of isolates AF26, AF43, AF49, AF50, and AF58 have inhibition ability against the bacteria *B. subtilis* and *S. aureus*. However, only AF43 and AF50 had inhibition ability against the *B. subtilis* and *S. aureus* in the MIC test. The inhibition ability of antibacterial substances was influenced by several factors, including (1) the concentration substance antibacterial; (2) storage time; (3) environment temperature; (4) physical properties and chemical food, including water content, pH, and number of compounds in it.(Ati, 2018) The strongest possibility that influences the results of this research is storage time because these Andaliman phyllosphere bacterial isolates have been stored for two years.

In this research, the MIC value of Andaliman phyllosphere bacteria crude extract is around 70-100% with a diameter of up to 23,40 mm (powerful) (Table 4-6). In another research by Sihombing (2019), MIC of Andaliman fruit extract testing against *Salmonella typhi* obtained a concentration of 75 % with the biggest inhibition zone diameter of 19 mm (strong). MIC test of Andaliman fruit extract carried out by Muzafri (2018) against *Salmonella thypii* obtained a concentration of 100 %, with the most optimal resistance of 17.76 mm. Meanwhile, Rahmawati *et al. (2022)* found the antifungal activity of Andaliman extract against *C. albicans* with an MIC value of 10%. Based on studies conducted by Rizqoh *et al*. (2016), the isolation of phyllosphere bacteria from medicinal plants such as Reundeu (*Staurogyne longata*) has antibacterial activity against *S. aureus, E. coli, C. albicans* and *Candida tropicalis*.

Phyllosphere and endophytic bacteria are known to produce various secondary metabolite compounds with various biological activities including antioxidant, antifungal, antibacterial, antiviral and antihelminthic (Gouda et al., 2016; Kasaei et al., 2017). These antifungal compounds are in the form of flavonoids, tannins, saponins and alkaloids which are obtained by the extraction process (Megawati et al., 2023). Phyllosphere bacterial populations are known to be influenced by plant genotype, immune system and species, soil type,

climatic conditions, and geographic location (Copeland et al., 2015). So it can be said that Factors from the metabolite components of phyllosphere bacteria that resemble the andaliman plant and also the use of ethyl acetate solvent influence the ability to properly inhibit some microbes (Devi et al., 2015; Janatiningrum et al., 2021; Sitanggang et al., 2019).

CONCLUSIONS

Some Andaliman phyllosphere bacteria isolates could potentially produce antimicrobial bioactive compounds that could inhibit the growth of *B. subtilis* and *S. aureus*. From molecular identification based on 16s rRNA gene, it is known that AF26, AF43, AF49, AF50 and AF58 have the closest similarity to *Brevundimonas* sp. (99.24%), *Pseudomonas* sp. (71.45%), *Brevundimonas* sp (99.10%), *Pseudomonas* sp. (80.58%) and *Bacillus altitudinis* (98.52%). The Minimum Inhibitory Concentration (MIC) of Andaliman phyllosphere bacterial isolate extract, which has the potential to inhibit *B. subtilis* has the smallest activity at a concentration of 70% in isolate AF43 and 100% in isolate AF50 with a strong category. The MIC of AF43 isolate extract could also inhibit *S. aureus* at a concentration of 70%.

Acknowledgement

Thanks for the support from the Faculty of Medicine, University of Bengkulu. This research is supported by a grant from the Non-tax Revenue of the Faculty of Medicine, University of Bengkulu.

Funding Source

This research is supported financially by a grant from the Non-tax Revenue of the Faculty of Medicine, University of Bengkulu.

Conflict of Interest

Author declared that there is no conflict of interest.

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