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(Research Article)

Inhibitory effect of spiny amaranth (*Amaranthus spinosus*) on the growth of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*

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Abstract

Periodontal disease is a chronic inflammation of periodontal tissue as a result of complex interactions between subgingival periodontal pathogenic bacteria and the host immune system. *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are the periodontopathogenic bacteria that can increase the host inflammatory response and cause tissue damage. Antibacterial agent is needed to inhibit the growth of pathogenic bacteria so that the periodontal tissues health in periodontitis patients is improved. Objective: To determine the inhibition of spiny amaranth extract (*Amaranthus spinosus*) on the growth of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. Methods: This research is an in vitro laboratory experimental research. Spiny amaranth extraction was carried out by maceration. The metabolites content of spiny amaranth extract was analyzed by qualitative and quantitative phytochemical analysis. The inhibition test was carried out by diffusion method. *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* were cultured in BHIB media and then grown in MHA media. Spiny amaranth leaf extract with concentration from 100% to 1.56% was used as independent variable. The samples were incubated at 37^{0}C for 24 hours and then the inhibition zone was measured. Results: Spiny amaranth leaf extract showed an inhibitory effect against *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* bacteria at concentrations of 25%, 50%, and 100%. While the extract concentrations of 25%, 12,5%, 6,25%, 3,125%, 1,56%, and negative control did not form an inhibition zone. The findings of the statistical analysis showed a significance level of 0.00000 ($p<0.05$). Conclusion: Spiny amaranth leaf extract can inhibit the growth of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*.

Keywords: Spiny amarant; Antibacterial activity; *Porphyromonas gingivalis; Aggregatibacter actinomycetemcomitans*; Minimal Inhibitory Concentration (MIC)

1. Introduction

The subgingival biofilm and the human immune system interact complexly to cause periodontal disease which causes an inflammatory state in the periodontal tissue due to the presence of periodontal pathogenic bacteria (10). Based on data from WHO (World Health Organization) the world's population suffers from periodontal disease is around 10-15%. Based on Riskesdas data in 2018, 74.1% of the population suffered from periodontitis. The key bacteria that cause periodontal disease are *Aggregatibacter actinomycetemcomytans* and *Por-phyromonas gingivalis* (5, 14).

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* are gram-negative anaerobic bacteria. The bacterium *A. actinomycetemcomitans* is thought to be the primary pathogen causing aggressive periodontitis. In patients with aggressive periodontitis, an increase in the number of *A. actinomycetemcomitans* bacteria was found and an increase in proinflammatory cytokines, especially prostaglandin E2, in response to toxins from bacteria. *A. actinomycetemcomitans* bacteria produce endotoxin in the form of lipopolysaccharide (LPS) and exotoxin in the form of

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leukotoxin and genotoxin cytolethal distending toxin that can disrupt the balance of the host immune response (14). *P. gingivalis* bacteria are able to express virulence factors such as fimbriae, capsules, lipopolysaccharides, and gingipain which cause periodontal tissue damage. *P. gingivalis* bacteria are able to interact on external media and colonize and form a complex microorganism environment in the form of biofilms that protect the body's defense system. These bacteria act as secondary colonizers in subgingival plaque that can bind to *Streptococcus mutans* and *Prevotella intermedia* (5, 12).

The inflammatory process in the periodontal tissues is initiated by the interaction between bacteria and host immune cells such as lymphocytes, neutrophils, and macrophages. Neutrophils are the body's first line of defense in the periodontal tissues, which migrate to the gingival sulcus when stimulated. Neutrophils can stimulate the secretion of proinflammatory cytokines (IL-1α, IL-1β, IL-6). Macrophages can also secrete proinflammatory cytokines such as IL-1β, IL-6, IL-23, TNF-α and matrix metalloproteinase (MMP) which play a role in the process of osteoclastogenesis and collagen degradation. This imbalance in host immune system activity can initiate periodontal tissue damage (7).

The main treatment for periodontitis is scaling and root planing (SRP) which is part of phase I (non-surgical) therapy. Using mechanical tools, scaling and root planing are processes that remove calculus and supragingival and subgingival biofilm from the tooth surface. After the SRP procedure, there was a decrease in the number of pathogenic bacteria including *A. actinomycetemcomitans* and *P. gingivalis* (10). However, SRP has limitations in clearing bacterial plaque in deep pockets, infrabony defects, or furcation areas and thus requires additional therapy. Topical antiseptics such as mouthwashes and antibiotics can be used as adjunct therapy (6, 13).

Indonesia is a tropical country that is rich in natural resources. One of the nutritious plants that can grow in Indonesia is spiny amaranth (*Amaranthus spinosus*). Spiny amaranth is weeds or wild plants from America that generally live in tropical areas such as Indonesia (15, 11). Traditionally, spiny amaranth is used to treat jaundice, diarrhea, peptic ulcers, mouth ulcers, bronchitis, and so on (8). Many active substances found in spiny amaranth, including alkaloids, flavonoids, terpenoids, saponins, betalains, tannins, and carotenoids, offer various systemic health advantages (4).

Previous research has shown that spiny amaranth extract has the potential to inhibit the growth of gram-positive and gram-negative bacteria, namely *Staphylococcus aureus*, *Bacillus subtilis, Bacillus megaterium, Streptococcus mutans, Escherichia coli, Shigella dysenteriae, Pseudomonas aeruginosa,* and *Salmonella typhi* (9, 1). However, research data regarding the inhibition of spiny amaranth extract on *P. gingivalis* and *A. actinomycetemcomitans* bacteria are still not available. The purpose of this study was to determine the inhibition of spiny amaranth extract (*Amaranthus spinosus*) on the growth of *P. gingivalis* and *A. actinomycetemcomitans* bacteria.

2. Material and methods

2.1. Material and methods

The Ethics Committee of Universitas Airlangga's Faculty of Dental Medicine gave its approval to the study. This study was carried out at Universitas Airlangga's Research Center Faculty of Dental Medicine, Pharmacy Laboratory of Universitas Widya Mandala, and Faculty of Science and Technology Laboratory of Universitas Airlangga. This research is an in vitro laboratory experimental research with post test-only control group design. The sample used was *Porphyromonas gingivalis* ATCC 33277 and *Aggregatibacter actinomycetemcomitans* ATCC 43718 bacteria cultured in BHIB media at Universitas Airlangga's Research Center Faculty of Dental Medicine. The sample population was calculated using the Federer formula. Thus, this study required 4 replications per sample group.

2.2. Spiny amarant extraction

Spiny amaranth plants were washed using water. After being separated, the spiny amaranth leaf and stem were dried in an oven set to 45 °C for two 24-hour periods. The dried leaf and stem were crushed by using a blender to form a fine powder. A total of 305.5 grams of spiny amaranth stem and 322 grams of spiny amaranth leaf powder were macerated by immersing them in 2 L of methanol (CH_3OH) as a solvent. The maceration process was completed in 3x24 hours. The methanol filtrate was filtered from the residue using Buchner funnel with filter paper. The extract was evaporated at 65 °C for 5 days to separate the methanol solution with the active constituents in the leaf and stem extract. significance· (UNESCO 2002). Although there are national and regional registers, the international one is the most visible for its attachment to UNESCO in general.

2.3. Bacteria culture praparation

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* bacteria were put into two different test tubes containing BHIB media and incubated for 24 hours at 37 °C in an anaerobic environment in an anaerobic jar. The turbidity of the bacteria culture was then observed with standard 0.5 McFarland.

2.4. Inhibitory test of spiny amaranth extract on the bacteria growth

The serial dilution method was used to obtain differences in the concentration of spiny amaranth leaf extract. Eight test tubes each containing extracts with different concentrations, i.e. 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56% were prepared. The extract were tested to detect antibacterial properties against *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* bacteria by using disc diffusion method. Bacterial cultures of *A. actinomycetemcomitans* and *P. gingivalis* were grown on Muller Hinton Agar (MHA) media using the spreading technique. Paper discs of 6 mm diameter were prepared and dripped with 10 µl of various extracts concentrations using micropipette. The prepared paper disc was then placed on the MHA media in petri dish containing bacteria using sterile tweezers according to the label. The negative control group without administration of spiny amaranth extract were also prepared. The bacteria in the media were incubated in an anaerobic atmosphere at 37^{0}C for 24 hours. Using a vernier caliper, the diameter of the inhibition zone surrounding the paper disc on the petri dish was measured in order to visualize the inhibition zone.

3. Results

Qualitative phytochemical tests were carried out on stem and leaf extract of spiny amaranth. The experiments were conducted based on the substances to be identified including alkaloids, tannins, saponins, terpenoids, and flavonoids. The results of the analysis are shown in table 1.

Table 1 Qualitative phytochemical analysis results

Compound group	Spiny amaranth	
	Stem extract	**Leaf extract**
Alkoloid	Positive	Positive
Tannin	Negative	Negative
Saponin	Positive	Positive
Terpenoid	Positive	Positive
Flavonoid	Positive	Positive

The percentage of secondary metabolite compounds in each spiny amaranth leaf and stem extract was determined by quantitative phytochemical analysis following the conclusion of the qualitative analysis of secondary metabolites found in the leaf and stem extract. The following is the result of quantitative phytochemical analysis of leaves and stem extract of spiny amaranth.

Table 2 Quantitative phytochemical analysis result

Parameter	Spiny amaranth	
	Stem extract	**Leaf extract**
Alkaloid (%)	8.68	10.01
Saponin (%)	10.4	15.44
Terpenoid (%)	1.7	3.2
Flavonoid (%)	1.06	1.81

Analyzing the outcomes of quantitative phytochemical tests, it can be concluded that the spiny amaranth leaf extract contains higher concentrations of alkaloids, saponins, terpenoids, and flavonoids than the spiny amaranth stem extract. Spiny amaranth leaf extract then continued in the inhibition test on the growth of *P. gingivalis* and *A. actinomycetemcomitans* bacteria. Research on the inhibition of spiny amaranth leaf extract on the growth of *A. actinomycetemcomitans* and *P. gingivalis* bacteria was carried out using the disc diffusion method.

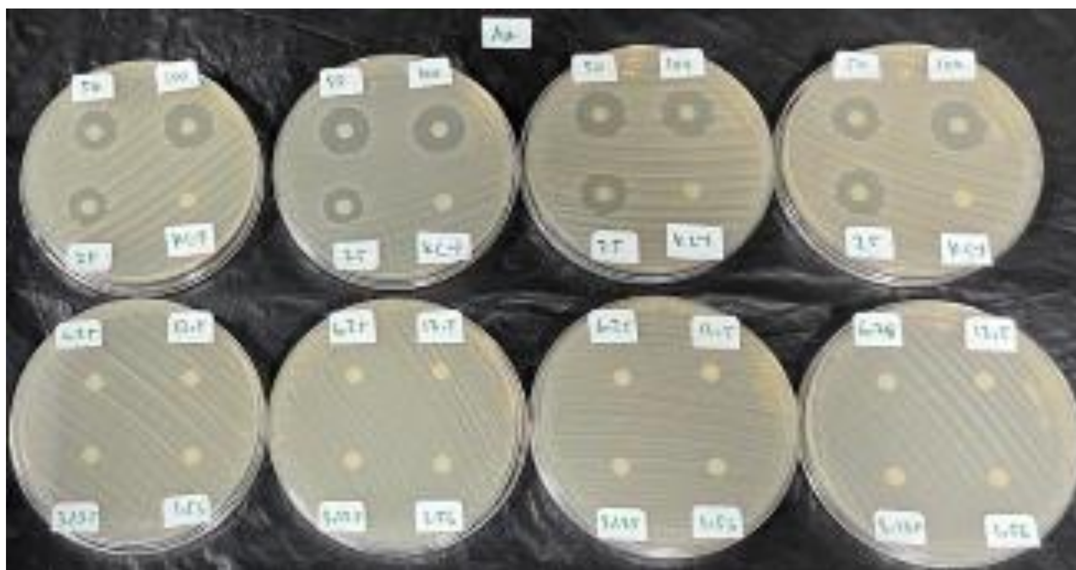

Figure 1 Inhibition zone of spiny amaranth leaf extract against *A. actinomycetemcomitans* bacteria

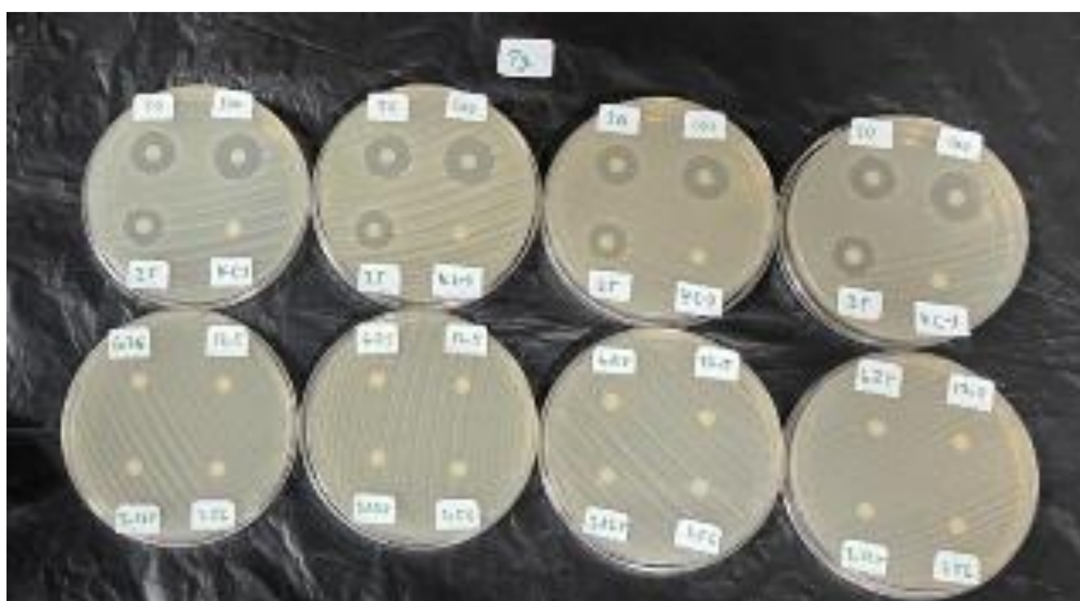

Figure 2 Inhibition zone of spiny amaranth leaf extract against *P. gingivalis* bacteria

The potential of spiny amaranth leaf extract to inhibit the growth of *A. actinomycetemcomitans* and *P. gingivalis* bacteria was demonstrated by the diameter of the clear zone surrounding the paper disc, which was calculated with a vernie caliper. Spiny amaranth leaf extract with concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 1.56% and the negative control group as the independent variable with 4 repetitions in inhibiting the growth of bacteria *P. gingivalis* and *A. actinomycetemcomitans*.

Spiny amaranth leaf extract showed the ability to inhibit the growth of *P. gingivalis* and *A. actinomycetemcomitans* bacteria at concentrations of 25%, 50%, and 100%. A vernie caliper was used to measure the diameter of the inhibitory

zone surrounding the paper disc after it had been visually examined. The average and standard deviation were measured according to the following table and diagram:

Table 3 Inhibition zone diameter (mm) of *A. actinomycetemcomitans* bacteria in each concentration group

No.	K (-)	1,56%	3,125%	6,25%	12,5%	25%	50%	100%
1.	-	-	-	-	-	15.6	18.4	21.4
2.	-	-	-	-	-	15.55	18.95	21.2
3.	-	-	-	-	-	16.8	19.8	21.6
4.	-	-	-	-	-	17.2	19.35	22.8
Mean	-	-	-	-	-	16.29	19.13	21.75
SD	-	-	-	-	-	0.83902	0.59512	0.71880

Table 4 Inhibition zone diameter (mm) of *P. gingivalis* bacteria in each concentration group

No.	K (-)	1,56%	3,125%	6,25%	12,5%	25%	50%	100%
1.	-	-	-	-	-	15.4	18.2	21.2
2.	-	-	-	-	-	15.75	19.05	21.6
3.	-	-	-	-	-	15.6	17.8	21.15
4.	-	-	-	-	-	16.2	18.8	21.8
Mean	-	-	-	-	-	15.74	18.46	21.44
SD	-	-	-	-	-	0.340037	0.567707	0.314576

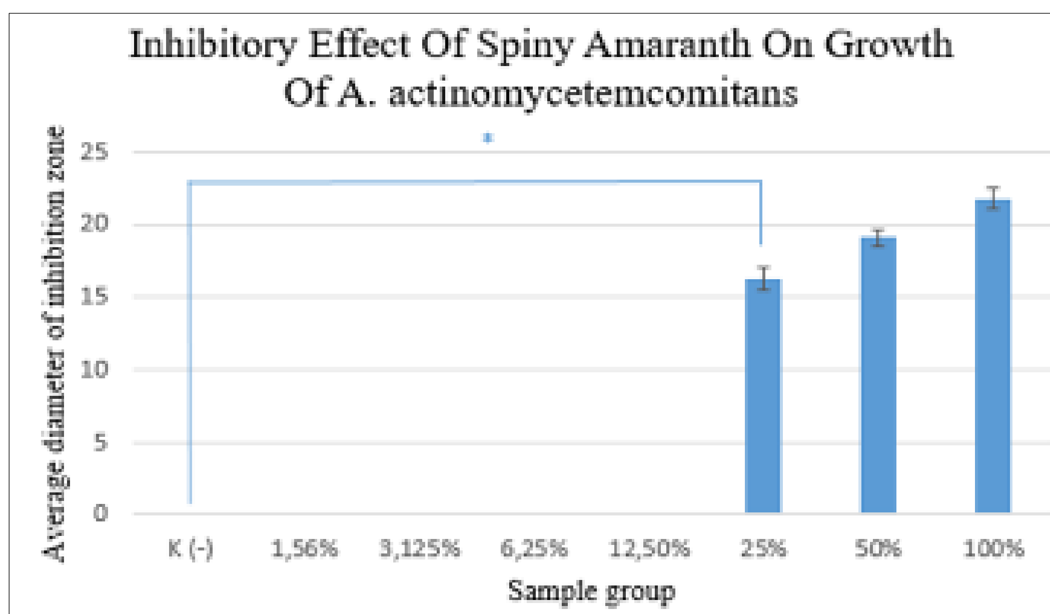

Figure 3 Diagram of the inhibition zone of *A. actinomycetemcomitans* bacteria

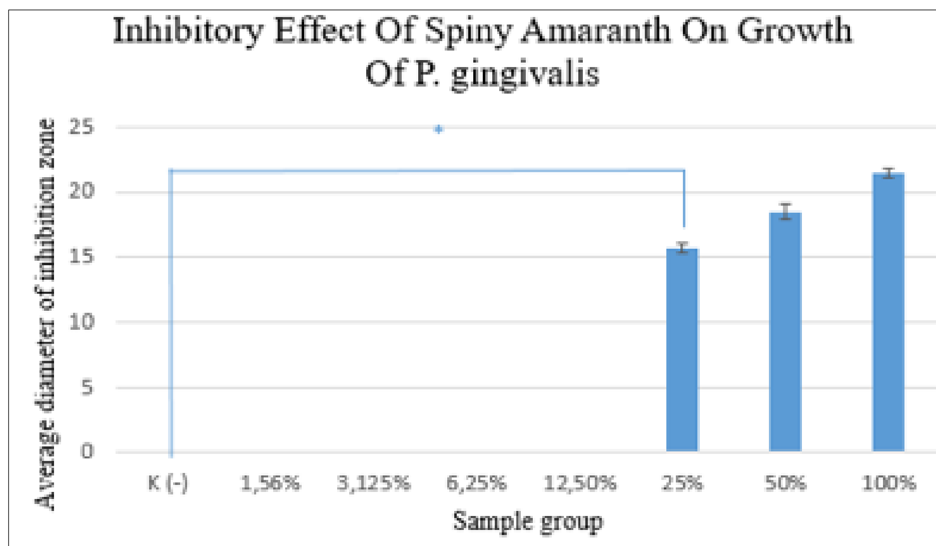

Figure 4 Diagram of the inhibition zone diagram of *P. gingivalis* bacteria

Based on Figures 4 and 5 above, the inhibition test of spiny amaranth leaf extract on the growth of *A. actinomycetemcomitans* and *P. gingivalis* bacteria had a significant difference in the sample group with a concentration of 25% and K (-) as a negative control. Bacteria inhibition test was first seen in the sample group of leaf spiny amaranth extract with a concentration of 25% as the lowest concentration indicating the ability to inhibit bacterial growth or minimal inhibitory concentration (MIC).

4. Discussion

This research is an experimental laboratory study conducted to determine the effect of spiny amaranth (*Amaranthus spinosus*) extract in inhibiting the growth of *A. actinomycetemcomitans* and *P. gingivalis* bacteria. Phytochemical analysis was carried out on the spiny amaranth leaf and stem, which aims to determine the active compounds in it. The results of qualitative phytochemical analysis showed that the spiny amaranth leaf and stem extract contained alkaloids, saponins, terpenoids, and flavonoids. Meanwhile, tannins were not detected in the leaf and stem extract. The unidentified tannin compounds can be caused by the drying procedure for 1 day in direct sunlight before the extraction procedure at the laboratory. Based on the study by Bernard (2014), drying of plants in direct sunlight that is too long and intensive may result in enzymatic degradation of the plant active compounds (2). Furthermore, quantitative phytochemical analysis of spiny amaranth leaf extract showed a higher content of active compounds compared to spiny amaranth stem extract so that the bacterial inhibitory test was carried out using spiny amaranth leaf extract.

The inhibitory test using disc diffusion method showed that there was an inhibition of the growth of *A. actinomycetemcomitans* and *P. gingivalis* bacteria in the sample group with spiny amaranth leaf extract concentration of 25%, 50%, and 100%. Meanwhile, no inhibition zone was formed in the sample group with extract concentration of 12.5%, 6.25%, 3.125%, 1.56%, and negative control. Thus, the minimal inhibitory concentration (MIC) obtained in spiny amaranth leaf extract with a concentration of 25%.

The ability to inhibit bacterial growth by spiny amaranth leaf extract is supported by the antibacterial activity of the active compounds contained in spiny amaranth, namely alkaloids, flavonoids, saponins, and terpenoids. These active compounds penetrate the bacterial cell membrane involving interactions with the phospholipid bilayer membrane and channels known as porins and then interfere with bacterial cellular activity through various mechanisms (3). Flavonoid compounds have antibacterial activity by inhibiting the bacteria movement thereby preventing bacterial adhesion and colonization, inhibiting nucleic acid and ATP synthesis, against bacteria toxin, and disrupting cell membrane integrity that causes metabolic dysfunction and ruptures bacterial cell membranes (16). The mechanism of alkaloid compounds in inhibiting bacterial growth is by damaging the cell membranes of gram-negative bacteria, inhibiting nucleic acid synthesis, and disrupting bacterial hemostasis (17). Saponins are compounds that can interact with cell membranes causing cell membranes to undergo lipid modification and disrupt the integrity of cell membranes (18, 19). Terpenoids play a role in damaging the outer membrane by damaging bacterial transmembrane proteins that will inhibit bacterial growth (20). This increase in the permeability of the cell membrane causes the cytoplasm to leak and leave the cell, resulting in bacterial cell lysis (21). The cell membrane plays an important role in respiration, osmoregulation, and the

process of cellular transport of bacteria. Disruption of the bacterial cell membrane will cause metabolic dysfunction and result in bacterial death (16).

The larger the diameter of the inhibition zone measured, the higher the activity of inhibiting growth of *A. actinomycetemcomitans* and *P. gingivalis* bacteria by spiny amaranth extract. In this study, it can be seen that the diameter of the inhibition zone of *A. actinomycetemcomitans* and *P. gingivalis* bacteria increases with the increase in the concentration of spiny amaranth leaf extract. An increase in the concentration of the extract indicated there were more secondary metabolites contained in it. Meanwhile, in the negative control group, there was no effect of aquades on bacterial growth, indicating that the activity of inhibiting bacterial growth was derived from secondary metabolites contained in spiny amaranth leaf extract.

With this antibacterial activity, spiny amaranth leaf extract is expected to be used as an antimicrobial agent that can improve periodontal tissue health by inhibiting the growth of periodontal pathogenic bacteria. This in vitro study on the antibacterial activity of spiny amaranth leaf extract is expected to be a reference for further research on the clinical use of spiny amaranth leaf extract in oral tissues as an adjunct therapy for periodontal disease.

5. Conclusion

Based on the results of this research, it is shown that the leaf extract of spiny amaranth (*Amaranthus spinosus*) has an inhibitory effect on the growth of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis bacteria* with a minimum inhibitory concentration (MIC) at a concentration of 25% with an average inhibition zone diameter of 16,287 mm and 15,74 mm.

Compliance with ethical standards

Disclosure of conflict of interest

All authors declare that there are no conflicts of interest regarding the publication of this document.

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