Antibacterial Activity of Bidara Arab Leaves (*Ziziphus spina-christi L)* Extract Against Gram-negative Anaerobic Subgingival Bacteria

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ABSTRACT

Background: *Gram-negative anaerobic subgingival bacteria play an important role in the initiation and progression of periodontitis. The secondary metabolites contained in Bidara Arab leaves (Ziziphus spina-christi L) have potential to be antibacterial and inhibited the growth of pathogenic bacterial. The purposed of this study to evaluated the antibacterial activity of Bidara Leaves extract against gram-negative anaerobic subgingival bacteria.*

Method: *Experimental laboratories using Mueller hinton agar to determine the antibacterial activity of Bidara leaves extract 40% and 60% against Porphyromonas gingivalis, Aggregatibacter ctinomycetemcomitans, and Fusobacterium nucleatum, with a comparison of the negative control group ethanol 96% and tetracycline as positive control. The production of extracts through maseration techniques and the subsequent phytochemical testing through tube reaction test*.

Result: *The results of phytochemical analysis identified the presence of flavonoids, tannins, saponins, steroids. The inhibition test showed that the Bidara leaves extract exhibited strong inhibition and a concentration of 60% was more effective than 40% against Gram-negative anaerobic subgingival bacteria including Porphyromonas gingivalis, Aggregatibacter ctinomycetemcomitans, and Fusobacterium nucleatum.*

Conclusion: *The Bidara Arab leaves (Ziziphus spina-christi L) extract at concentration 60% was more effective than 40% against gram-negative anaerobic bacteria.*

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INTRODUCTION

Periodontitis is a chronic inflammation of the periodontal tissue that is caused by bacteria. Plaque accumulation of bacteria triggers the immune response, which subsequently initiates the development of periodontitis. Plaques containing pathogenic bacteria and toxins initiate the process of periodontal tissue damage in periodontitis. Gram-negative bacteria commonly found in periodontal pockets include *Aggregatibacter actinomycetemcomitans, Eikenella corrodens, Fusobacterium nucleatum, Prevotella intermedia, Porphyromonas gingivalis, and Tannerella forsythia*. In chronic periodontitis, bacteria and their products, such as lipopolysaccharides (LPS), peptidoglycans, lipoteichoic acid, and proteases, are capable of increasing local cytokines, which can further modulate the host response, thus accelerating alveolar bone resorption. [1,2]

In the United States, the prevalence of periodontitis is 47% among adults over the age of 30, with 38% being moderate or severe. The intensity of periodontal disease in Asia and Africa is higher than in Europe, America, and Australia. [3] In 2018, basic health research results revealed that the prevalence of dental and oral diseases in the Indonesian population was 57.6%, and periodontitis prevalence was 74.1%. [4] Periodontitis is the leading cause of tooth extraction and permanent tooth loss.[5]

Indonesia is endowed with an extensive plant kingdom with traditional medicinal potential and a tropical climate. As well as being naturally occurring, traditional medicine consists of a substance that is employed to treat a wide range of ailments. As a result of safety concerns, the utilization of plants and their formulations as botanical medicines is on the rise. Pure natural ingredients in herbal medicines, which are derived from plants, have significantly fewer adverse effects, hazards, and risks than synthetic substances. Microbes that are exposed to chemical antimicrobials insufficiently will develop resistance to them. Bacterial resistance mechanisms encompass a range of activities such as target modification, permeability alteration, antibiotic inactivation, and metabolic pathway bypass. [6]

Bidara Arab Leaves (*Ziziphus spina-christi L*.) is one specific plant utilized in traditional medicine and exhibit promise as antibacterial agents. [7,8] Ethanol leaf extract with an ethanol solvent contains alkaloids, flavonoids, saponins, tannins, ketones, steroids, and triterpenoids. Previous studies have also shown that Bidara arab leaves have the highest antioxidant activity compared to fruit and seed extracts. Traditional Chinese medicine commonly uses Bidara Arab leaves to treat skin infections.[9]

Antibacterials are substances that can inhibit or kill bacteria that cause infection. Bacteria, or pathogenic microorganisms, cause infection by entering body tissues and reproducing within them. It is known that the Bidara Arab leaves can kill a number of bacteria, including *Escherichia coli, Staphylococcus epidermidis, Pseudomonas aeruginosa, Salmonella typhimurium, Proteus mirabilis, Klebsiella pneumonia, Enterobacter spp., Acinetobacterium, and Serratia spp*. In addition, the plant also has antifungal activity against *Candida albicans, Trichophyton rubrum, Tricophytone mentagrophytes, Microsporum canis, and Aspergillus fumigatus.* [10,11]

There is currently no research on the inhibitory activity of Bidara Arab leaf extract against anaerobic gram-negative bacteria, which are known to contribute to periodontal disease such as *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum*. Therefore, further research is needed to investigate the antibacterial efficacy of Bidara Arab leaf extract against subgingival bacteria. The purposed of this study to evaluated the antibacterial activity of Bidara

Leaves extract against gram-negative anaerobic subgingival bacteria including *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum*.

RESEARCH METHOD

Materials

Bidara Arab leaves (*Ziziphus spina-christi L*) utilized in this investigation were procured from the Probolinggo District, East Java, Indonesia. Extraction and phytochemical analysis were conducted at the Faculty of Pharmacy, University of Mahasaraswati Denpasar. *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum* bacterials were obtained and cultivated from Faculty of Dentistry, University of Airlangga.

Methods

The study was conducted using an experimental method to evaluate the antibacterial activity of Bidara Arab leaves extract in vitro. The type of research used was experimental post only control group design with 4 groups; group 1: control with ethanol 96%, group 2: positive control with tetracycline, group3: Bidara Arab leaves extract (*Ziziphus spina-christi L*) 40%, group 4: Bidara Arab leaves extract (*Ziziphus spina-christi L*) 60%. The number of samples is determined using the Federer formula, where there are 4 groups and each group is repeated 6 times, resulting in a total of 24 samples per bacterium.

Production of Bidara Arab leaves extract (*Ziziphus spina-christi L*)

The leaves of Bidara Arab (*Ziziphus spina-chrisit L*) are collected and washed clean with running water. Subsequently, the delicate Bidara Arab leaves (*Ziziphus spina-chrisit L*) are then maserated with 96% ethanol and inhaled for 3x24 hours, filtered every 24 hours, and occasionally mixed repeatedly. The results of the maseration are filtered with a Buchner corong coated with paper. After filtering the result, store it in a location away from sunlight.

Preparation of Bidara Arab leaves (*Ziziphus spina-christi L*) extract concentration of 40% and 60% using a 96% ethanol solvent. Concentration of 40% means that the solution consists of 0,40 ml of Bidara Arab leaves extract (*Ziziphus spina-christi L*) and 0,60 ml of ethanol solution. Concentration of 60% means the solution is composed of 0,60 ml of Bidara Arab leaves extract (*Ziziphus spina-christi L*) and 0,40 ml ethanol.

Phytochemical Testing

Phytochemical screening tests on Arab spina leaf extract (*Ziziphus spina-christi* L) using tube reaction tests, including tests of alkaloid compounds, flavonoids, tannins, quinones, saponins, and steroids,and quinons. These tube tests are useful for preliminary screening of plant extracts for the presence of bioactive compounds. Positive reactions indicate the presence of the respective classes of phytochemicals, which are often linked to various biological activities, including antimicrobial effects.

Identification of Flavonoid: the insertion of an alkaline solution (sodium hydroxide) into the extract typically results in a yellow hue that intensifies after standing. Significance: The formation of an alkaline complex frequently identifies flavonoids through this color change. Identification of Tannins:

The presence of tannins results in the formation of a blue, green, or blackish precipitate when ferric chloride ($FeCl_3$) is added to an extract. Significance: The presence of tannins can be detected by the formation of complex compounds that are formed when iron binds with them. Identification of Saponins: The presence of saponins is indicated by the formation of a persistent froth that persists for several minutes after the extract is shook with water. Significance: The foaming property is a result of the surface-active agents in saponins, which decrease the surface tension of water. Identification of Steroids: The addition of sulfuric acid and acetic anhydride results in a color change, which is typically a transition from green to blue or violet. Significance: The formation of a steroidal complex with sulfuric acid is the cause of this color change, which is a recognizable reaction for the detection of steroids. Identification of Alkaloids: In the presence of alkaloids, the addition of Mayer's reagent (potassium mercuric iodide) or Dragendorff's reagent (bismuth nitrate) results in the formation of a precipitate that is typically milky white (Mayer's) or orange (Dragendorff's). Significance: These reactions are predicated on the formation of insoluble complexes between the alkaloids and the reagents. Identification of Quinones: Once the extract is exposed to an alkaline solution (e.g., NaOH), it develops a red or yellow hue. Significance: These unique colors are the result of redox reactions that quinones endure. [12,13]

Production of bacterial suspension

The bacteria that have been vaccinated are taken with a sterile oxygen needle and then suspended in a tube containing a 0,9% solution of NaCl in 2 ml until the same durability as the 0,5 McFarland equivalent standard is obtained. The treatment was done on every type of test bacteria.

Preparation of Agar Medium

Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and *Fusobacterium nucleatum* bacterials corrosion suspension equivalent to a 0,5 McFarland equivalent solution, taken using a cotton swab. Then the disc that has contained the Bidara Arab leaves extract (*Ziziphus spina-christi L*) with a concentration of 40%, 60%, positive control, and negative control is placed on the Mueller Hinton Agar medium that contains the *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum* bacterials and incubated in an incubator at 37°C for 24 hours.

Observation and Measurement of Inhibition Zones

After 24 hours of incubation, measurements and observations are conducted. The extent of the inhibition zone is indicative of the bacterial sensitivity to antibiotics or other antibacterial substances used as test materials, as indicated by the clear area. With the aid of a calliper, the inhibition zone's diameter is determined in millimeters. The diameter of the inhibition zone was then categorized according to the Davis and Stout (1971) classification of its antibacterial strength as follows. [14]

1. A barrier zone diameter of 20 mm or more means that the inhibition force is very strong.
2. The diameter of the 10–20 mm inhibition zone means a strong inhibition force.
3. The diameter of the inhibition zone is 5–10 mm, which means a moderate inhibition force.
4. A inhibition zone diameter of 2–5 mm means weak inhibition power.

Data Analysis

The Shapiro-Wilk test for normality indicated that the inhibitory zone data of *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum* bacterials were distributed normally (p>0,05). The *Levene* test for homogeneity identified the existence data of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were non-homogen (p<0,05), and *Aggregatibacter actinomycetemcomitans* data was homogen (p>0,05).

The mean inhibitory zone data of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were analyzed using One-Way Anova test, and to determine between which groups differences existed, the Gomes-Howell test was conducted. The data on the mean inhibitory zones of *Aggregatibacter actinomycetemcomitans* were analyzed using the One-Way Anova test. In order to identify any significant differences between the groups, the Tukey Post Hoc test was performed.

RESULTS

Phytochemical Analysis

The phytochemical analysis of an extract derived from the Bidara Arab leaf (*Ziziphus spina-christi L*) identified the presence of flavonoids, tannins, saponins, steroids, however, no alkaloids, quinons were detected (Figure 1).

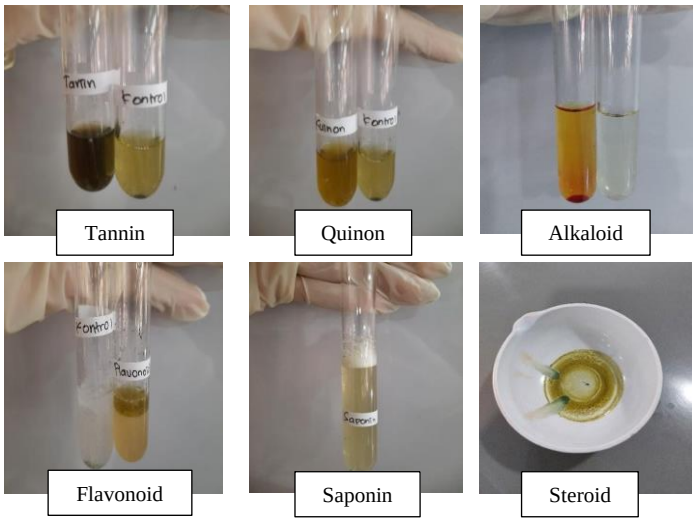

Figure 1. Phytochemical screening tests on Arab spina leaf extract (*Ziziphus spina-christi L*) using tube reaction tests

Antibacterial Evaluation

Based on Davis and Stout's (1971) classification of antibacterial strength, Figure 2 shows that the Bidara Arab leaf extract (*Ziziphus spina-christi L*) at a concentration of 40% and 60% are strong against *Porphyromonas gingivalis (Pg), Aggregatibacter actinomycetemcomitans (Aa), and Fusobacterium nucleatum (Fn)* bacteria, whereas group 2 positive control with tetracyclines has very strong inhibition power, and 96% ethanol as negative control group 1 has no inhibition power.

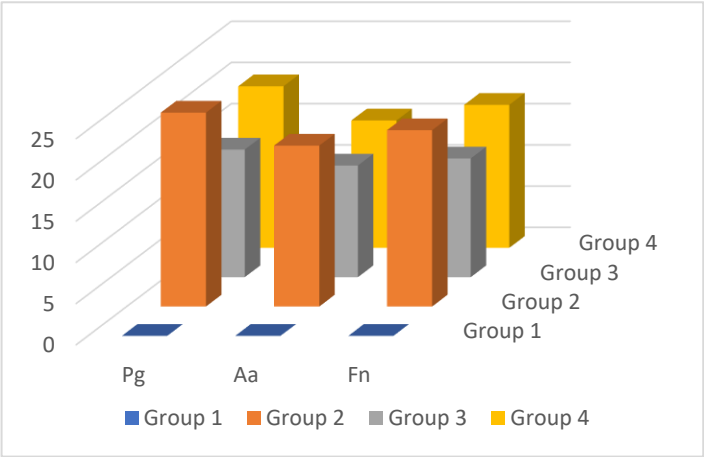

Figure 2. Graphic of the inhibition zone's mean of Bidara Arab leaf extract (*Ziziphus spina-christi L*) 40% and 60% against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Fusobacterium nucleatum bacteria*.

The results of a differences test of *Porphyromonas gingivalis* bacteria using One-way Anova (Table 1) and a post hoc test Games-Howell (Table 2) indicated that there were significant differences among the four groups ($p<0.05$). A significant difference was observed in Table 2 between group 2 and 3 ($p < 0.05$), as well as between group 2 and 4 ($p \leq 0.05$). This indicated that tetracyclines inhibited *Porphyromonas gingivalis* more effectively than the Bidara Arab leaf extract (*Ziziphus spina-christi L*) 40% and 60%. A significant difference ($p<0.05$) exists between group 3 dan 4, indicating that Bidara Arab leaf extract (*Ziziphus spina-christi L*) 60% exhibit greater efficacy in inhibiting *Porphyromonas gingivalis* bacteria compared to 40%.

Table 1. Differences Test's Results of Inhibitory Zone of *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and *Fusobacterium nucleatum* Bacterials

Group	n	*Pg* Mean (mm)	*Aa* Mean (mm)	*Fn* Mean (mm)
Group 1	6	0,00±0,00	0,00±0,00	0,00±0,00
Group 2	6	23,51±0,19	21,38±0,22	19,51±0,23
Group 3	6	15,48±0,26	14,40±0,23	13,54±0,14
Group 4	6	19,60±0,23	17,35±0,14	15,45±0,24
P		0,000*	0,000*	0,000*

Table 2. The Significant Differences Test of *Porphyromonas gingivalis* Bacteria Between Two Groups

Group	Mean Differences	p
Group 1 and Group 2	-23,51	0,000*
Group 1 and Group 3	-15,48	0,000*
Group 1 and Group 4	-19,60	0,000*
Group 2 dan Group 3	8,03	0,000*
Group 2 dan Group 4	3,91	0,000*
Group 3 dan Group 4	-4,12	0,000*

The findings from a comparison test of *Aggregatibacter actinomycetemcomitans* bacteria, using One-way Anova (Table 1) and Tuket (Table 3) as post hoc tests, revealed statistically significant differences among the four groups ($p<0.05$). A notable differentiation was identified in Table 3 between groups 2 and 3 ($p < 0.05$), as well as 2 and 4 ($p < 0.05$). This demonstrated that tetracyclines inhibited *Aggregatibacter actinomycetemcomitans* bacteria more effective than the Bidara Arab leaf extract (*Ziziphus spina-christ L*) 40% and 60%. A statistically significant difference ($p<0.05$) is observed between group 3 and group 4, suggesting that the 60% concentration of Bidara Arab leaves extract (*Ziziphus spina-christi L*) is more effective than the 40% concentration in inhibiting *Aggregatibacter actinomycetemcomitans* bacteria.

Table 3. The Significant Differences Test of *Aggregatibacter actinomycetemcomitans* Bacteria Between Two Groups

Group	Mean Differences	p
Group 1 and Group 2	-21,38	0,000*
Group 1 and Group 3	-14,40	0,000*
Group 1 and Group 4	-17,35	0,000*
Group 2 dan Group 3	6,97	0,000*
Group 2 dan Group 4	4,03	0,000*
Group 3 dan Group 4	-2,94	0,000*

The statistical analysis revealed significant differences among the four groups ($p<0.05$) based on the outcomes of a One-way Anova test for *Fusobacterium nucleatum* bacteria (Table 1) and a Games-Howell post hoc test (Table 4). Table 4 revealed a statistically significant differences between groups 2 and 3 ($p < 0.05$), as well as group 2 and 4 ($p \leq 0.05$). According to these results, tetracyclines exhibited a greater inhibitory effect against *Fusobacterium nucleatum* compared to the Bidara Arab leaf extract (*Ziziphus spina-christi*), at 40% and 60%, respectively. A notable differences ($p<0.05$) can be observed between groups 3 and 4, suggesting that the efficacy of Bidara Arab leaf extract (*Ziziphus spina-christi L*) at 60% is more effective to that of 40% in inhibiting *Fusobacterium nucleatum* bacteria.

Table 4. The Significant Differences Test of *Fusobacterium nucleatum* Bacteria Between Two Groups

Group	Mean Differences	p
Group 1 and Group 2	-19,51	0,000*
Group1 and Group 3	-13,54	0,000*
Group 1 and Group 4	-15,45	0,000*
Group 2 dan Group 3	5,97	0,000*
Group 2 dan Group 4	4,06	0,000*
Group 3 dan Group 4	-1,91	0,000*

DISCUSSION

The antibacterial content of the Bidara Arab leaves (*Ziziphus spina christi-L*) in this research was extracted by maseration with 96% ethanol organic solvent. This is consistent with the results of a study by Ali et al. (2016), which found that ethanol extracts of Bidara Arab leaves (*Ziziphus spina-christi L*) are more

significantly effective against various species of Gram-positive and negative bacteria compare to the methanolic extracts. The potential of ethanolic extracts as efficient antimicrobial agents against harmful bacterial strains has been highlighted by their higher antibacterial activity when compared to methanolic extracts from all sections of *Ziziphus spina-christi.* Numerous investigations have confirmed this, showing that ethanolic extracts have a greater ability to stop bacterial development, making them a viable substitute for treating bacterial infection [16]

The results of this study showed that the Bidara Arab leaves (*Ziziphus spina-christi L*) extract at concentration 60% was more effective than 40% against gram-negative anaerobic subgingival bacteria, this result supported by [15] which found that the antibacterial effect of *Ziziphus spina-christi L* extract, increased by increasing the concentration. An observed correlation was found between the rise in concentration and the corresponding increase in inhibitory impact on the growth of bacteria. [17]

Periodontal disease is caused by anaerobic subgingival bacteria, including *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Fusobacterium nucleatum*. The Bidara Arab leaves were intended to inhibit this bacteria in this research. This is comparable to a study conducted by Hamad, (2018) that investigated the mechanism by which Bidara Arab leaves eliminated the bacteria responsible for gingivitis. Effective inhibition of Bidara Arab on gram-negative bacteria that cause gingivitis, such as *Pseudomonas aeruginosa, Escherichia coli, Klebsiella spp., and Pseudomonas monilli*, was reported [17]. Another investigation, Bidara Arab leaves extract was selectively effective against *Proteus vulgaris* and *Vibrio parahaemolyticus* among the gram-negative bacteria [18]. The Bidara Arab leaves (*Ziziphus spina-christi L*) extract is also expected to be effective in inhibiting *Streptococcus oralis*, a bacterium that causes periodontal disease and is present in dental plaque. [19]

The phytochemical screening tests (Figure 1) showed that the Bidara Arab leaves (*Ziziphus spina-christi L*) extract contained secondary metabolite compounds, such as flavonoids, tannins, saponins, steroids, however, no alkaloids, quinons were detected. In contrast to these reports, alkaloids were reportedly found in the leaves collected from Sukoharjo District, Central Java, Indonesia [20] and from Palembang, Indonesia. [21] According to the findings of this study, the alkaloids were not identified in leaves from Nigeri States, Nigeria. [22] By contrast, Suliman and Mohammed (2018) found that the phytochemical components of leaves from Sudan do not contain steroids. [23] The variety in phytochemical content is mostly attributed to disparities in climate and environmental conditions specific to the plant's growth location. Environmental and climatic factors, particularly temperature, soil composition, and plant maturity, exert influence over the chemical composition and functional characteristics of herbs. [24] The leaves were discovered to possess substantial biological activity, which is attributable to their high concentrations of polyphenol compounds. [22]

The phytochemical components of Bidara Arab leaves extract (*Ziziphus spina-christi L*) may have antibacterial properties. For instance, flavonoids possess antibacterial, antioxidant, and anti-inflammatory properties. This investigation did not identify any alkaloids, despite their known antibacterial and antifungal properties. Saponins possess antibacterial, antioxidant, anticancer, and antidiabetic properties, in addition to serving as barriers against pathogenic microbes and enhancing immunity. Tannin is a compound that attaches to proteins and generates water-insoluble compounds. Tannins, in their role as antibacterials, bind to proteins within the bacterial cell wall and cause them to coagulate, thereby preventing bacterial proliferation. Steroids, including cholesterol and ergosterol, compose cell membranes. Fusidic acid is a steroid compound that has the ability to prevent either gram-positive or gram-negative bacterial infections. [20] Researchers demonstrated that

a saponin derivative from the leaf of Bidara Arab leaves extract (*Ziziphus spina-christi L*) inhibits the three target macromolecules in *Escherichia coli, Staphylococcus epidermidis, and Candida albicans*. This molecule is a *Christinin* compound. [10]

Christinin, a saponin glycoside derived from *Ziziphus spina-christi* (also known as Christ's thorn or jujube), has been highlighted in several studies for its potential antimicrobial properties. Research shows that various extracts of *Ziziphus spina-christi*, including those containing christinin, display antibacterial activity against both Gram-positive and Gram-negative bacteria. For example, the butanol extract containing christinin demonstrated significant inhibition zones against bacterial strains such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, as well as fungal strains like *Candida albicans*. These effects are attributed to the bioactive compounds, especially the saponins and polyphenols, found in the plant extracts, which disrupt bacterial cell membranes and prevent bacterial growth. [25]

The antimicrobial properties of *Ziziphus spina-christi* against Gram-negative anaerobic bacteria have been widely explored in scientific literature. Studies have shown that extracts from various parts of the plant, particularly the stem bark and seed oil, contain bioactive compounds, such as saponins, alkaloids, tannins, and glycosides. These compounds exhibit multiple mechanisms of action: (1) Membrane Disruption: Saponins, in particular, are known to damage bacterial cell membranes, resulting in leakage of intracellular materials and subsequent inhibition of bacterial growth. (2) Enzyme Inhibition**:** Alkaloids and flavonoids present in *Ziziphus spina-christi* are capable of inhibiting key bacterial enzymes involved in metabolism, thereby impeding bacterial proliferation (3) Biofilm Disruption**:** Tannins have been shown to disrupt biofilm formation, a crucial mechanism for bacterial survival, especially in anaerobic conditions. These antimicrobial actions have been demonstrated against various pathogenic bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa*, which are commonly associated with Gram-negative infections. [13]

CONCLUSION

The Bidara Arab leaves (*Ziziphus spina-christi L*) extract at concentration 60% was more effective than 40% against gram-negative anaerobic bacteria.

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