Ahmad Dahlan Medical Journal
VOL 7, No. 1, 09-19
http:// http://journal2.uad.ac.id/index.php/admj

Article

In *Vitro* Evaluation of Antibacterial Potential BioMint D-Spray Derived from *Anredera cordifolia* and *Mentha spicata* Extracts on Diabetic Wound Pathogens

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ARTICLE INFO

Article history
Received 05 Mar 26
Revised 02 Apr 26
Accepted 15 Apr 26

Keywords
Antibacterial
Anredera cordifolia
Mentha spicata
Diabetic Foot Ulcer
Well Diffusion Method

ABSTRACT

Diabetes mellitus is a metabolic disorder associated with a high risk of complications, particularly Diabetic Foot Ulcers (DFU). Indonesia recorded approximately 20.4 million diabetes cases in 2024, increasing the demand for effective and affordable wound care. *Anredera cordifolia* and *Mentha spicata* contain bioactive compounds including flavonoids, saponins, carvone, and phenolic acids with proven antibacterial and anti-inflammatory properties, making them promising natural candidates for diabetic wound management. This study employed a true experimental design using a combined extract of *Anredera cordifolia* and *Mentha spicata* leaves at a 1:1 ratio prepared via maceration. Phytochemical screening and pH testing were performed to characterize the extract. Antibacterial activity was assessed using the well diffusion method at concentrations of 60%, 70%, 80%, 90%, and 100%, followed by incubation for 12 and 24 hours and observed. Inhibition zone diameters were analyzed using the Shapiro-Wilk normality test followed by One-Way ANOVA at $\alpha = 0.05$. Phytochemical analysis confirmed the presence of flavonoids, alkaloids, and tannins, with all concentrations exhibiting an acidic pH of 4.0. Antibacterial activity against *Staphylococcus aureus* was observed across all concentrations, with 60% established as the minimum inhibitory concentration (MIC). Inhibition zone diameters ranged from 14.20 mm to 15.40 mm across observation periods. The combined extract demonstrated intermediate antibacterial activity against *Staphylococcus aureus* based on CLSI standards, with inhibition zones of 14.20 to 15.40 mm compared to novobiocin (22 mm). No statistically significant difference was observed between concentrations. Further in vivo studies are recommended to evaluate pharmacological effects and optimal concentration in biological systems.

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INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by hyperglycemia resulting from underutilization of glucose as an energy source and its overproduction through inappropriate gluconeogenesis and glycogenolysis[1]. Based on data from the International Diabetes Federation (IDF) in 2024, the prevalence of diabetes in adults in Indonesia reached 11.3% or around 20.4 million cases[2]. This high number implies an increased risk of complications, namely Diabetic Foot Ulcers (DFU). Diabetic foot ulcers are open wounds that occur due to partial or full thickness tissue damage, which can extend to tendons, muscles, bones, or joints in diabetes patients. One of the main causes of DFU is peripheral neuropathy, namely the loss of protective sensation (LOPS) in the lower extremities. As a result, sufferers are unable to feel minor trauma such as friction or pressure, either from foot deformities or foreign objects[3].

The high incidence of diabetic foot ulcer complications in Indonesia requires effective, affordable, and acceptable treatment. *Anredera cordifolia* (Binahong) is a local herbal plant that is readily available, inexpensive, and high in antioxidants. *Anredera cordifolia* (Binahong) leaves contain flavonoids, saponins, alkaloids, and polyphenols, which act as anti-inflammatory and antimicrobial agents. Flavonoid compounds in binahong function as antioxidants to reduce oxidative stress and inflammation in wounds, while saponins function to increase collagen production and tissue regeneration[4].

To increase effectiveness, we combined binahong with *Mentha spicata* (spearmint). *Mentha spicata* (spearmint) contains a diverse array of bioactive phytochemicals, including phenolic acids (rosmarinic acid, chlorogenic acid, caffeic acid), flavonoids (luteolin derivatives, apigenin derivatives, kaempferol derivatives, narinrutin), as well as terpenoid compounds predominantly consisting of carvone as the major component of its essential oil, along with trans-carveol, β-caryophyllene, and germacrene D[5]. *Mentha spicata* essential oil has been shown to possess significant antibacterial activity against multiple strains of *Staphylococcus aureus*, including biofilm-producing strains and methicillin-resistant *S. aureus* (MRSA), with MIC values ranging from 0.125% to 0.25% and inhibition zones reaching up to 35.67 ± 6.81 mm in the disc diffusion assay, suggesting its potential application as a complementary natural antimicrobial agent[6].

In addition to its antibacterial properties, spearmint also has a distinctive aroma that provides a fresh, calming effect, which is expected to reduce the unpleasant odor of diabetic wounds, thus increasing user comfort. Through a combination of these two natural ingredients, we developed BioMint D-spray, based on Binahong leaf extract and spearmint, intended as an potential antibacterial spray for diabetic wounds. It is hoped that this spray can provide an effect in preventing wound infections while helping to accelerate the healing of diabetic wounds.

METHODS

This study employed a true experimental in vitro design to evaluate the antibacterial effectiveness of BioMint D-Spray, a formulation derived from *Anredera cordifolia* (binahong) and *Mentha spicata* (spearmint) extracts, on diabetic wound bacteria. The research was conducted from May to August 2025 at the Microbiology Laboratory, Department of Medical Laboratory Technology, Poltekkes Kemenkes Surabaya. The test microorganism, *Staphylococcus aureus* (ATCC 25923), was obtained from the Center for Health Laboratory (BBLK). The primary materials consisted of simplisia (141.5 g each) of *Anredera cordifolia* (binahong) and *Mentha spicata* (spearmint) in a 1:1 ratio. Extraction was performed using the maceration method with 850 mL absolute ethanol (C_2H_6O, CAS No. 64-17-5) at a 1:3 (b/v) ratio for five days at room temperature with periodic stirring[7,8]. The resulting filtrate was evaporated under reduced pressure using a rotary evaporator to obtain a concentrated extract, which was then diluted with dimethyl sulfoxide (DMSO, CAS No. 67-68-5) to produce concentrations of 60%, 70%, 80%, 90%, and 100%.

Phytochemical screening was conducted qualitatively to identify the presence of flavonoids, alkaloids, and tannins. Flavonoid content was determined using the Shinoda method. A sufficient aliquot of the combined *A. cordifolia* and *M. spicata* extract was transferred into a test tube and mixed with 5 mL of distilled water, followed by heating for 1 minute. The mixture was then filtered through Whatman filter paper, and the resulting filtrate was collected for analysis. To the filtrate, 0.1 g of magnesium powder (Mg) was added, followed by 2 mL of hydrochloric acid (HCl) solution (1:1), 2 mL of alcohol, and 2 mL of 96% ethanol. The mixture was homogenized and allowed to separate into two distinct layers. The appearance of a pink or magenta-red color, particularly in the upper amyl alcohol layer, indicates the presence of flavonoid compounds in the sample. A positive result was recorded upon observation of red, yellow, or orange coloration in the amyl alcohol layer. The presence of alkaloids was assessed using Dragendorff's reagent. A sufficient aliquot of the combined extract was placed into a test tube, and Dragendorff's reagent was added dropwise. The formation of an orange to black precipitate upon addition of the reagent was taken as a confirmatory positive result indicating the presence of alkaloid compounds. Tannin content was evaluated through a ferric chloride ($FeCl_3$) colorimetric reaction. A sufficient aliquot of the combined extract was placed into a test tube and treated with 0.1% $FeCl_3$ solution. The mixture was homogenized for 10 seconds. The formation of brownish green or blue black coloration was taken as an indication of the presence of tannin compounds in the extract[9]. The pH of each extract concentration was measured using pH paper to assess the formulation stability.

The antibacterial test was performed using the well diffusion assay against *Staphylococcus aureus* (ATCC 25923). The bacterial suspension was adjusted to match the turbidity of 0.5 McFarland standard (≈1.5×10^8 CFU/mL) in 0.9% NaCl solution. Then, 20 µL of each test solution along with positive control (novobiocin) and negative control (distilled water) was pipetted into 6 mm diameter wells using a sterile cork borer on Mueller Hinton Agar (MHA, Oxoid, UK) plates[10]. The plates were incubated at 37°C for 12 and 24 hours. Minimum Inhibitory Concentration zones were measured using a digital caliper and recorded in millimeters (mm). The presence of an inhibition zone around the well indicating that the extract was able to inhibit bacterial growth (bacteriostatic effect). The inhibition zone diameter was calculated using the formula: $\frac{(Dv-Dc)+(Dh-Dc)}{2}$, where Dv is the vertical diameter, Dh is the horizontal diameter, and Dw is the well diameter. The antibacterial activity was interpreted based on CLSI criteria.

Table 1. Inhibition Zone Diameter(mm) base on CLSI 2020

Category	***Inhibition Zone Diameter (mm)***	***Interpretation***
Resistent	≤ 12 mm	No inhibition of bacterial growth
Intermediate	12 – 16 mm	Moderate antibacterial activity
Sensitive	≥ 16 mm	Strong inhibition of bacterial growth

The data were collected from inhibition zone measurements for each BioMint D-Spray concentration. Normality and homogeneity tests were conducted to assess data distribution. If the data were normally distributed (p-value > 0.05), further analysis was performed using a One-Way ANOVA to determine significant differences among treatment groups.

RESULTS

The results of this study are presented in accordance with the objective, which is to determine the phytochemical content, pH, and antibacterial activity of *Anredera cordifolia* (binahong) and *Mentha spicata* (spearmint) extracts. Phytochemical test done for identifying compound metabolite secondary includes flavonoids, alkaloids, and tannins in the extract Binahong (*Anredera cordifolia*) and Spearmint (*Mentha spicata*). The test results are presented in table 2 as follows:

Table 2. Phytochemical Test Results Binahong Leaf Extract (Anredera cordifolia) and Spearmint Leaf Extract (Mentha spicata)

Compound	***Results***
Flavonoid	Positive (+)
Alkaloid	Positive (+)
Tannin	Positive (+)

pH measurements were performed to determine level acidity in each extract at various concentrations (60%, 70%, 80%, 90%, and 100%). The results showed that in all variation concentration has the same pH, namely 4.0 as seen in table 3.

Table 3. Results of pH measurements on Binahong Leaf Extract (Anredera cordifolia) and Spearmint Leaf Extract (Mentha spicata)

Concentration	***pH***
60%	4.0
70%	4.0
80%	4.0
90%	4.0
100%	4.0

The antibacterial activity test was conducted using the well diffusion method to determine the inhibitory effect of various extract concentrations on bacterial growth. Observations were carried out at the 12th and 24th hours. The diameter of the inhibition zone was calculated using the following formula, as recommended by Clinical and Laboratory Standards Institute (CLSI): Inhibition Zone Diameter = $\frac{(Dv-Dc)+(Dh-Dw)}{2}$.

D_v= diameter vertikal zona hambat (mm)

D_h= diameter horizontal zona hambat (mm)

D_w= diameter sumur (well) (mm)

Table 4. Antibacterial Testing by Diffusion Method well Concentration 60%

Sample	***12 Hour Observation***		***24 Hour Observation***		***Average***	
	Information	***Inhibition Zone (mm)***	***Information***	***Inhibition Zone (mm)***	***12 Hours***	***24 hours***
MH1	DV : 20mm DH : 24mm DC : 7mm	15.00	DV : 21mm DH : 22mm DC : 7mm	14.50	**14.60 mm**	**14.20 mm**
MH2	DV : 20mm DH : 23mm DC : 7mm	14.50	DV : 21mm DH : 22mm DC : 7mm	14.50		
MH3	DV : 22mm DH : 22mm DC : 7mm	15.00	DV : 21mm DH :21mm DC :7mm	14.00		

MH4	DV DH DC	: 22mm : 23mm : 7mm	15.50	DV DH DC	: 22mm : 21mm : 7mm	14.50	
MH5	DV DH DC	: 20mm : 20mm : 7mm	13.00	DV DH DC	: 20mm : 21mm : 7mm	13.50	

Dv = Vertical Diameter; Dh = Horizontal Diameter; Dc = Disc Diameter

Table 5. Antibacterial Testing by Diffusion Method well Concentration 70%

Sample	***12 Hour Observation***		***24 Hour Observation***		***Average***	
	Information	***Inhibition Zone (mm)***	***Information***	***Inhibition Zone (mm)***	***12 Hours***	***24 hours***
MH1	DV: 23mm DH: 24mm DC: 7mm	15.50	DV: 24mm DH: 25mm DC: 7mm	16.50	**14.90 mm**	**15.00 mm**
MH2	DV: 21mm DH: 21mm DC: 7mm	14.00	DV: 22mm DH: 22mm DC: 7mm	15.00		
MH3	DV: 23mm DH: 23mm DC: 7mm	16.00	DV: 21mm DH: 22mm DC: 7mm	14.50		
MH4	DV: 22mm DH: 22mm DC: 7mm	15.00	DV: 22mm DH: 22mm DC: 7mm	15.00		
MH5	DV: 22mm DH: 20mm DC: 7mm	14.00	DV: 21mm DH: 22mm DC: 7mm	14.50		

Dv = Vertical Diameter; Dh = Horizontal Diameter; Dc = Disc Diameter

Table 6. Antibacterial Testing by Diffusion Method well Concentration 80%

Sample	***12 Hour Observation***		***24 Hour Observation***		***Average***	
	Information	***Inhibition Zone (mm)***	***Information***	***Inhibition Zone (mm)***	***12 Hours***	***24 hours***
MH1	DV: 21mm DH: 22mm DC: 7mm	14.50	DV: 23mm DH: 22mm DC: 7mm	15.50	**14.40 mm**	**15.30 mm**
MH2	DV: 20mm DH: 22mm DC: 7mm	14.00	DV: 22mm DH: 23mm DC: 7mm	15.50		

MH3	DV: 21mm DH: 22mm DC: 7mm	14.50	DV: 22mm DH: 21mm DC: 7mm	14.50	
MH4	DV: 22mm DH: 22mm DC: 7mm	15.00	DV: 23mm DH: 23mm DC: 7mm	16.00	
MH5	DV: 21mm DH: 21mm DC: 7mm	14.00	DV: 22mm DH: 22mm DC: 7mm	15.00	

Dv = Vertical Diameter; Dh = Horizontal Diameter; Dc = Disc Diameter

Table 7. Antibacterial Testing by Diffusion Method well 90% concentration

Sample	***12 Hour Observation***		***24 Hour Observation***		***Average***	
	Information	***Inhibition Zone (mm)***	***Information***	***Inhibition Zone (mm)***	***12 Hours***	***24 hours***
MH1	DV: 19mm DH: 19mm DC: 7mm	12.00	DV: 20mm DH: 22mm DC: 7mm	14.00		
MH2	DV: 22mm DH: 23mm DC: 7mm	15.50	DV: 24mm DH: 25mm DC: 7mm	17.50		
MH3	DV: 20mm DH: 22mm DC: 7mm	14.00	DV: 20mm DH: 22mm DC: 7mm	14.00	**14.20 mm**	**15.00 mm**
MH4	DV: 22mm DH: 21mm DC: 7mm	14.50	DV: 22mm DH: 22mm DC: 7mm	15.00		
MH5	DV: 22mm DH: 22mm DC: 7mm	15.00	DV: 22mm DH: 21mm DC: 7mm	14.50		

Dv = Vertical Diameter; Dh = Horizontal Diameter; Dc = Disc Diameter

Table 8. Antibacterial Testing by Diffusion Method well 100% concentration

Sample	***12 Hour Observation***		***24 Hour Observation***		***Average***	
	Information	***Inhibition Zone (mm)***	***Information***	***Inhibition Zone (mm)***	***12 Hours***	***24 hours***
MH1	DV: 22mm DH: 23mm DC: 7mm	15.50	DV: 21mm DH: 24mm DC: 7mm	15.50	**15.30 mm**	**15.40 mm**

MH2	DV: 24mm DH: 25mm DC: 7mm	17.50	DV: 22mm DH: 24mm DC: 7mm	16.00
MH3	DV: 22mm DH: 20mm DC: 7mm	14.00	DV: 21mm DH: 22mm DC: 7mm	14.50
MH4	DV: 21mm DH: 23mm DC: 7mm	15.00	DV: 22mm DH: 24mm DC: 7mm	16.00
MH5	DV: 21mm DH: 22mm DC: 7mm	14.50	DV: 23mm DH: 21mm DC: 7mm	15.00

Dv = Vertical Diameter; Dh = Horizontal Diameter; Dc = Disc Diameter

The differences in antibacterial effectiveness of BioMint D-Spray against *Staphylococcus aureus* across concentration levels (60%, 70%, 80%, 90%, and 100%) were examined using one-way ANOVA. The analysis yielded $F(4,20) = 1.500$ with a significance value of 0.240 ($p > 0.05$). Based on these results, the null hypothesis was accepted, indicating that no statistically significant differences existed in the mean inhibition zone diameters among the tested concentration groups.

DISCUSSION

Phytochemical test results

Qualitative phytochemical tests showed that Binahong and Spearmint leaf extracts contain secondary metabolites in the form of flavonoids, alkaloids, and tannins. A positive reaction to flavonoids is indicated by a color change from yellow to orange after the addition of HCl and magnesium powder, indicating the formation of flavyllium salts through the Shinoda reduction reaction. Flavonoids inhibit bacterial growth through various mechanisms, including disruption of cell wall synthesis, prevention of biofilm formation, disruption of cell membrane integrity, and inhibition of bacterial efflux pumps[11]. One of their principal molecular actions is to form complexes with proteins through non-specific forces such as hydrogen bonding and hydrophobic effects, as well as through covalent bond formation, thereby inactivating microbial adhesins, enzymes, and cell envelope transport proteins[12]. Flavonoids can also exert antibacterial activity by damaging the cytoplasmic membrane, inhibiting energy metabolism, and inhibiting the synthesis of nucleic acids[13].

A positive alkaloid reaction is indicated by the formation of an orange precipitate upon addition of Dragendorff's reagent. In qualitative identification using Dragendorff's reagent, the reagent reacts with alkaloids to form an orange-to-red precipitation, which confirms the presence of alkaloids in the extract[14]. Alkaloids are nitrogen-containing heterocyclic compounds that represent one of the most important types of natural products owing to their large number,

structural diversity, and complexity[15]. Their antibacterial properties operate through a mechanism that disrupts the peptidoglycan components in the bacterial cell wall, with metabolomic studies confirming that alkaloid compounds inhibit cell wall biosynthesis, specifically targeting peptidoglycan synthesis pathways, which ultimately prevents the formation of an intact cell wall and leads to bacterial cell death[16].

The tannin test using 0.1% $FeCl_3$ solution yielded a positive result marked by the formation of a dark black coloration, indicating the formation of a ferri-tannin complex in which the phenolic hydroxyl groups (–OH) of tannins bind to Fe^{3+} ions. Tannic acid affects enzymatic activities owing to the presence of its phenolic hydroxyl groups; these phenolic compounds play crucial roles in determining antimicrobial effectiveness through mechanisms including reduced bacterial metabolism and disruption of cellular metabolic reactions[17]. Transcriptomic evidence further demonstrates that tannins can inhibit the ribosome pathway in bacteria, disrupting the translation process and inhibiting protein synthesis, with tannin treatment significantly reducing the protein content or gene expression levels of ribosomal subunit components[18].

pH measurement results

The results showed that all sample concentration variants had a pH of 4.0, which is close to the natural skin pH. The typical pH range of human skin is between 4.2 and 5.6, which creates an acidic environment serving as a protective barrier against bacterial overgrowth. In contrast, acute wounds exhibit a neutral to alkaline pH ranging from 6.5 to 8.5, and chronic wounds tend to fall within the range of 7.2 to 8.9. An alkaline environment is detrimental because it increases bacterial proliferation, strengthens protease activity, inhibits fibroblasts, and reduces oxygen supply, thereby significantly slowing wound healing. Conversely, acidic conditions promote fibroblast proliferation, DNA and collagen synthesis, angiogenesis, and macrophage activation, while also reducing MMP activity to maintain tissue structural integrity[19].

Antibacterial test results

The results showed that BioMint D-Spray has antibacterial effectiveness against *Staphylococcus aureus* with inhibition zone values ranging from 14.20–15.40 mm. Inhibition zone analysis showed that a concentration of 100% produced the highest inhibition diameter (15.30 mm at 12 hours and 15.40 mm at 24 hours). This indicates that increasing concentration is directly proportional to antibacterial effectiveness up to a certain point. A concentration of 60% also showed inhibition (14.20 mm), indicating that at this level antibacterial activity has appeared and can be considered as the Minimum Inhibitory Concentration (MIC).

Based on the classification of antibacterial activity, an inhibitory diameter between 10 – 20 mm is considered strong. Thus, all test concentrations exhibited strong antibacterial activity against *Staphylococcus aureus*[20]. The positive control using novobiocin showed an inhibition zone of 22 mm. Based on CLSI standards, an inhibition zone of <12 mm is categorized as resistant, 12–16 mm as intermediate, and >16 mm as sensitive[14]. With an average result of 14–15 mm, *the Anredera cordifolia* (binahong) and *Mentha spicata* (spearmint) extracts are categorized as intermediate, meaning they have antibacterial potential but are still below the effectiveness of standard antibiotics[21].

CONCLUSION

Qualitative phytochemical screening of BioMint D-Spray based on *Binahong* (*Anredera cordifolia*) and spearmint (*Mentha spicata*) leaf extracts confirmed the presence of flavonoids, alkaloids, and tannins. The pH of BioMint D-Spray across all tested concentrations (60%, 70%, 80%, 90%, and 100%) was consistently acidic at 4.0, which is compatible with the natural skin surface pH and may support the inhibition of bacterial growth on diabetic wounds. BioMint D-Spray demonstrated antibacterial activity against *Staphylococcus aureus* ATCC 25923 across all tested concentrations in vitro using the well diffusion method. Inhibition zones ranged from 14.2 to 15.4 mm, classified as strong inhibitory activity according to established criteria. The minimum inhibitory concentration (MIC) was established at 60%, as this was the lowest concentration at which a clear inhibition zone was observed. Statistical analysis using One-Way ANOVA ($p = 0.240 > 0.05$) indicated no significant difference in antibacterial effectiveness among the five concentration groups, suggesting a plateau effect in which the antibacterial activity of BioMint D-Spray reached its maximum capacity at the lowest tested concentration. These findings support the potential of BioMint D-Spray as a natural-based antibacterial spray for the management of diabetic wound infections, though further in vivo studies are recommended to confirm clinical applicability.

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