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Antibacterial activity on hand sanitizer spray of ethanolic leaf *Sambiloto* extract (*Andrographis paniculata*) against *Staphylococcus aureus*

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Abstract. *Aminah D, Susilowati A, Setyaningsih R. 2023. Antibacterial activity on hand sanitizer spray of ethanolic leaf Sambiloto extract (*Andrographis paniculata*) against* Staphylococcus aureus*. Asian J Trop Biotechnol 20: 50-55. Sambiloto* leaves, known as the king of bitters, contain andrographolide and flavonoid compounds that can be antibacterial. *Sambiloto* leaf extract is formulated into spray preparations as antibacterial active ingredients derived from herbal plants and is an alternative to alcohol-based antiseptics. This study aimed to determine the characteristics of a hand sanitizer spray preparation of *Sambiloto* leaf extract and its activity in inhibiting the growth of *Staphylococcus aureus* bacteria. Bitter leaf extract was added to the formulation of hand sanitizer spray with concentrations of 10, 15, and 20% with carbopol 940 base, triethanolamine, propylparaben, propylene glycol, and methylparaben. Physical evaluation of hand sanitizer spray was examined based on color, texture, aroma, pH, homogeneity, and adhesion. The inhibitory power of spray hand sanitizer was determined based on the diffusion method's diameter of the inhibition zone. The physical evaluation showed that the hand sanitizer results followed the good spray criteria. The three spray formulations can inhibit S. aureus with a weak inhibitory category (<0.05); the highest reduction in microbes was recorded in formulation 3 (59.25%). Based on ANCOVA analysis, there were significant differences between spray treatments, which were influenced by differences in covariates. The result of paired t-test analysis showed that there was a significant difference between the number of bacteria before and after spray treatment.

Keywords: Antibacterial, hand sanitizer, *Sambiloto* leaves, *Staphylococcus aureus*

INTRODUCTION

Infectious diseases are a problem in the health sector that keeps on increasing from time to time and are most common in everyday life (Hossain et al. 2021). Skin is a part of the body that is very susceptible to infection because it is the outermost layer covering the body, always in contact with the external environment, be it sunlight, climate, or chemical factors. Skin is also a medium for transmitting microbes through the hands that occur from humans to the environment or vice versa. *Staphylococcus aureus* is a bacteria that often causes skin infections (Bloom and Cadarette 2019).

The *S. aureus* is a gram-positive bacterium that is round and usually arranged in irregular clusters like grapes (Hossain et al. 2021). The *S. aureus* is a major pathogen for humans. Nearly everyone experiences some S. aureus infection, ranging from food poisoning or mild skin infections to severe, life-threatening infections (Mussard et al. 2020). The *S. aureus* grows rapidly on several types of bacteriological media under aerobic or microaerobic conditions. It actively metabolizes, ferment carbohydrates, and produce a variety of pigments from white to dark yellow. The *S. aureus*, in general, often causes skin infections characterized by redness, swelling, pain, and pus in skin wounds (Sri et al. 2018).

Preventing infectious diseases due to *S. aureus* requires the improvement of the environment and skin that makes direct contact so that it is always sterile at all times. Hand sanitizer is more effective than washing hands with soap because it is more practical and efficient, absorbs, and dries quickly (Lestari et al. 2020). Spray preparation is one of the new preparations that has the advantage that it can be applied directly to the skin to reduce the possibility of contamination or other infections. Spray preparations can last longer when applied to the skin due to the gelling agent (Estikomah et al. 2021). Hand sanitizer spray consists of two or more substances, mainly a homogeneous solute and a solvent (Lestari et al. 2020). Hand sanitizer spray products generally contain the active ingredient alcohol, which triggers microbial resistance. Furthermore, using alcohol in hand sanitizer is also less safe because alcohol is flammable and causes skin irritation with repeated use (Sahabuddin et al. 2017). Hence, an innovation in the form of a hand sanitizer spray, whose active ingredients come from herbal plants, namely *Sambiloto*, known as bitters. Natural active ingredients derived from the bitter plant replace alcohol in a spray formulation as an antibacterial agent.

Sambiloto is a medicinal plant used in traditional medicine that can thrive and is cultivated in various parts of the world, including Indonesia. *Sambiloto* is an annual plant with a 50-90 cm height and many rectangular branches (Wilyawan 2018). The main compounds in the leaves of the bitter plant are andrographolide and flavonoids, which act as antibacterial. In addition, bitter

leaves also contain saponins, alkaloids, and tannins (Islam et al. 2018).

The ethanolic extract of *Sambiloto* leaves is formulated into a spray preparation using a hand sanitizer spray formulation, which refers to research by Puspita et al. (2020). This study aimed to determine the characteristics of a hand sanitizer spray preparation of *Sambiloto* leaf extract and its activity in inhibiting the growth of *S. aureus* bacteria.

MATERIALS AND METHODS

Study area

This research was conducted from March to June 2022 at the Laboratory 3 and 4 of the Biology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia.

Instruments and materials

The equipment used in this research were: petri dishes, stir bar, test tube, volume pipette, micropipette, incubator, autoclave, dark bottle, oven, blender, rotary evaporator, bunsen lamp (spirtus lamp), analytical balance, hot plate stirrer, Erlemeyer, beakers, measuring cups, spray bottles and needles, pH strips, Biology Safety Cabinet (BSC), and colony counter.

The materials used were bitter leaf (*Andrographis paniculata* (Burm.fil.) Nees), 70% ethanol, aquadest, Mueller-Hinton Agar (MHA) media powder, carbopol 940, pure culture of *S. aureus*, filter paper, aluminum foil, and commercial hand sanitizer spray (Antis brand) as the positive control, triethanolamine, propylene glycol, propyl paraben, methyl paraben, citri oleum, cotton swab, disc paper, erythromycin, plate-count agar media powder, and Nutrient Broth media powder, and Plate Count Agar (PCA) media.

Preparation of bitter leaf (*Andrographis paniculata*) simplicia

Good quality *Sambiloto* leaves were taken as much as 3 kg. *Sambiloto* leaves were washed with running water and drained. After that, bitter leaves were chopped and dried using an oven at 50ºC for 2x24 hours and blended until smooth. The simplicia was then stored in a dark, closed container to avoid light (Brigitta et al. 2021).

Preparation of ethanolic extract from bitter leaf

The extraction method used in this study was maceration with 70% ethanol as solvent. The simplicia of bitter leaves was weighed as much as 500 grams and transferred into a beaker with a capacity of 1 L. The simplicia was then dissolved in 70% ethanol and tightly covered with aluminum foil. Soaking was carried out for 48 hours without exposure to sunlight while being stirred every 8 hours, and then the mixture was filtered and separated thrice until the color faded. The filtrate obtained was then put in a rotary evaporator at 70 rpm at a temperature of 60°C for one hour and then increased to a temperature of 70°C to evaporate the solvent and obtain a concentrated extract (Mardiana and Handayani 2016).

Antibacterial test of ethanolic extract of bitter leaf

This was a preliminary test to determine the variation in the concentration of bitter leaf's ethanol extract, formulated into a hand sanitizer spray preparation. The *S. aureus* bacteria were subcultured into MHA media using the streak method and then incubated for 24 hours in an incubator at 37°C. Two up to three colonies of *S. aureus* bacteria were taken using an ose needle and transferred into a 0.9% physiological saline solution of ±2 mL in a test tube. The suspension was vortexed so that bacterial cells were evenly distributed. The suspension turbidity was compared with a standard McFarland solution of 0.5. Furthermore, a sterile cotton swab was dipped into the suspension and then pressed against the tube wall above the surface of the suspension to remove excess fluid. The cotton swab was applied evenly to the entire surface of the MHA media, and then a well was made (5 mm) with the help of a cork borer. Each hole was filled with 50 microliters of ethanol extract of bitter leaf (extract concentrations of 5%, 10%, 15%, and 20%), DMSO as a negative control, and 15 g erythromycin as a positive control. Variations in the extract concentration were tested to determine whether it could be formulated into a hand sanitizer spray and inhibit the activity of *S. aureus* bacteria. Then, Petri plates were incubated for 24 hours at 37°C. Determination of the antibacterial activity of ethanolic extract of bitter leaf was measured by calculating the clear zone area around the well with a caliper. This test was repeated 3 times. The concentration of ethanolic extract that can inhibit the growth of *S. aureus* bacteria was used to determine the effective concentration variations in the hand sanitizer spray formulation of *Sambiloto* leaf extract.

Sambiloto leaf extract hand sanitizer spray formulation

In this study, ethanolic extract of *Sambiloto* leaves with different concentrations of 10%, 15%, and 20% were arranged according to the method of Pushpita et al. (2020). The hand sanitizer spray formulation was made with a combination of carbopol 940 as a gelling agent, triethanolamine as a base, propylene glycol as a humectant, methylparaben, and propylparaben as a preservative, citri oleum as a flavoring, and aquadest as a solvent (Table 1).

Table 1. Formulation of hand spray sanitizer of *Sambiloto* leaf extract

Ingredients	Concentrations (%)		
	Formula I (FI)	Formula II (FII)	Formula III (FIII)
Bitter leaf ethanolic extract	10%	15%	20%
Carbopol 940	0.5	0.5	0.5
Triethanolamine	0.5	0.5	0.5
Propyleneglycol	10	10	10
Methyl paraben	0.18	0.18	0.18
Propyl paraben	0.02	0.02	0.02
Citri oleum	0.5	0.5	0.5
Aquadest (total volume)	100	100	100

Preparation of hand sanitizer spray preparation of *Sambiloto* leaf extract

This carbopol was dispersed in cold water, and hot water was added until the carbopol was completely dispersed; then, triethanolamine was added to form a transparent gel mass. Propylene glycol, methylparaben, propylparaben, citri oleum, and ethanol extract of bitter leaf were added to the mixture and stirred until all ingredients were mixed. The preparation was placed into a glass beaker to which 100ml of distilled water was added, stirred until homogeneous, and poured into a spray bottle.

Physical test of hand sanitizer spray

Physical properties testing of hand sanitizer spray of *Sambiloto* leaf extract included organoleptic tests, homogeneity, pH, and adhesion (Anindhita and Oktaviani 2020). An organoleptic test was conducted to observe hand sanitizer's physical appearance (color, aroma, and texture). The homogeneity of hand sanitizer spray was tested by spraying the sanitizer onto a piece of transparent glass and then observing the presence or absence of particles or compounds that were not mixed evenly. The preparation mixture was categorized as homogeneous, with no solid particles and agglomerates. The pH test of the spray preparation was carried out using a universal pH indicator; ideally, the pH value of the mixture was 4.5-7. The adhesion test was carried out on the skin by spraying it on the arm from a distance of 3 cm, and the result was observed after 10 seconds (Anindhita and Oktaviani 2020).

Antibacterial activity test of hand sanitizer spray of ethanolic extract of *Sambiloto* leaves

The *S. aureus* bacteria were subcultured on MHA media using the streak method. 2-3 colonies of *S. aureus* were taken using an ose needle and transferred into a 0.9% physiological saline solution of ±2 mL in a test tube. The suspension was vortexed so that bacterial cells were evenly distributed. The turbidity of the suspension was compared with a standard McFarland solution of 0.5. Furthermore, a sterile cotton swab was dipped into the suspension and pressed against the tube wall above to drain excess fluid. The cotton swab was applied evenly to the entire surface of the MHA media, and then a well was made with a cork borer with a size of 5 mm. Each hole was filled with 50 microliter hand sanitizer-spray preparation of *Sambiloto* leaf extract (formulation 1, formulation 2, formulation 3). The spray formulation without adding ethanol extract of *Sambiloto* leaf was the negative control, and the commercial hand sanitizer spray with Antis brand was the positive control. Then, the plates were incubated for 24 hours at 37°C. Determination of the antibacterial activity of ethanolic extract of bitter leaf was measured by calculating the clear zone area around the well with a caliper. This test was repeated 3 times. Antibacterial activity was determined by calculating the clear zone area around the well with a caliper.

Hand microbial reduction test

The test for reducing the number of microbes on the hands was carried out to determine the number of microbial colonies before and after being treated with spray hand sanitizer. Therefore, swabbing was done with 3 respondents for each hand sanitizer spray formulation. The criteria for respondents being swabbed was that they did not have open wounds or indications of irritation and other skin diseases in the area of the palms and fingers of both hands. First, respondents were asked to wash their hands with running water for 20 seconds and then rub their palms together to make the microbes on their hands uniform. Then, a sterile cotton swab was dipped into the NB medium and rubbed firmly on one finger from the distal to the proximal direction 3 times. The cotton swab was then streaked evenly on Plate Count Agar media on a petri dish (Radji et al. 2007; Nurwaini and Saputri 2018; Situmeang and Sembiring 2019).

Afterward, each formulation of the hand sanitizer spray and positive control was sprayed evenly on the fingers. Hand sanitizer spray Formula I was sprayed on the middle finger, Formula II on the ring finger, and Formula III on the little finger. Swabbing with cotton buds was done again on each finger after hand sanitizer spray dried. Next, the cotton swab was scratched evenly on the PCA media, and all petri dishes were incubated at 37°C for 24 hours. Plate Count Agar (PCA) is a bacteriological medium used to determine the total number of viable aerobic bacteria in a sample. These general-purpose media are routine culture media that microbiology laboratories use to cultivate a broad spectrum of microorganisms. The number of bacteria is expressed per ml (CFU/mL) in liquid samples. In addition, microbial cultures from swabbing hands before and after each treatment that grew on petri dishes were counted using a colony counter, and each calculated microbial number was recorded.

RESULTS AND DISCUSSION

Antibacterial activity of ethanolic leaf extract of *Sambiloto* against *staphylococcus aureus*

The ethanolic extract of *Sambiloto* leaves obtained from the maceration process was 36.4 grams from 165 grams of simplicia *Sambiloto* leaves. The resulting extract was dark green, with an extract yield of 22%. The inhibitory power of ethanolic extract of *Sambiloto* leaves against *S. aureus* can be observed in Table 2. The antibacterial activity of ethanolic extract of *Sambiloto* leaves against *S. aureus* showed the extract's average diameter of inhibition zone was weak at 5%, 10%, 15%, and 20% concentrations.

All variations of the concentration of bitter leaf extract can inhibit the growth of *S. aureus* bacteria in the category of weak inhibition. The 5% concentration extract had the lowest inhibitory power of 12.51 mm, and the 20% concentration extract had the highest inhibition of 15.90 mm. Furthermore, it can be observed that the higher the concentration of the extract, the greater the antibacterial inhibition produced. The ethanolic extract of *Sambiloto* leaves with higher concentrations had a better inhibitory ability against the growth of *S. aureus* bacteria due to the high content of extract active compounds.

Physical characteristics of spray hand sanitizer ethanolic extract of *Sambiloto* leaves

The physical characteristics of spray hand sanitizer ethanolic extract of *Sambiloto* leaves at the beginning and end of storage for 4 weeks are shown in Table 3.

The preparation of spray handsanitizer ethanolic extract of *Sambiloto* leaves was observed to have good physical characteristics at the beginning and end of storage for 4 weeks. The organoleptic characteristics of the three hand-sanitizer spray formulations were as follows: ethanol extract of *Sambiloto* leaves were dark green, a liquid texture like water due to the aquadest composition in the spray, and a citrus aroma that comes from the addition of citri oleum essence. Furthermore, the homogeneity of the three formulations was homogeneous, indicated by the absence of agglomerated particles on the slide. The homogeneity of preparation indicates that the ingredients used in the dosage formula were mixed well. The homogeneity of the mixture produces a good preparation because the active substance in the preparation is dispersed with other ingredients in the formula so that the preparation contains the same amount of active ingredient (Suena et al. 2017).

The pH of all three spray formulations was 6. The pH of spray gel preparation must comply with the pH requirement range for topical application, i.e., 4.5-7; if the pH is too alkaline, it causes scaly skin, while skin infection may occur if the pH is too acidic (Suyudi 2004). The stickiness of hand sanitizer spray preparation of ethanol extract of *Sambiloto* leaves can be seen in Table 3. The three formulations were attached to the skin for more than 10 seconds after being sprayed on the skin of the upper arm. The ability to adhere to the skin shows that spray preparation can maintain the active substance on the skin to increase its effectiveness. Based on the results of the physical quality test of the preparation, the hand sanitizer spray formula of *Sambiloto* leaf extract can be used as an alternative hand sanitizer.

Antibacterial activity of spray hand sanitizer ethanolic extract of *Sambiloto* leaf

The antibacterial activity of hand sanitizer spray preparation of ethanolic extract of *Sambiloto* leaves against *S. aureus* is shown in Table 4 and Figure 1.

Table 2. Antibacterial activity of ethanolic extract of bitter leaf against *Staphylococcus aureus*

Treatments	Zone inhibitory diameter (mm)	Inhibitory power category
Extract 5%	12.51	Weak
Extract 10%	13.60	Weak
Extract 15%	15.65	Weak
Extract 20%	15.90	Weak
Control – (DMSO)	Not detected	Weak
Control + (Erythromycin)	32.44	Strong

Table 3. Physical characteristics of spray hand sanitizer ethanolic extract of *Sambiloto* leaves

Test parameters	Formulations		
	I (10%)	II (15%)	III (20%)
Color	Dark green	Dark green	Dark green
Smell	Orange	Orange	Orange
Texture	Liquid	Liquid	Liquid
Homogeneous	Homogenous	Homogenous	Homogenous
pH	6	6	6
Adhesion (seconds)	19.36	18.54	19.14

Table 4. Inhibitory power of spray hand sanitizer ethanolic extract of *Sambiloto* leaves against *Staphylococcus aureus*

Treatments	Zone inhibitory diameter (mm) ± SD	Inhibitory power category
Spray **Formula I**	9.01 ± 0.50^{a}	Weak
Spray **Formula II**	9.23 ± 0.86^{a}	Weak
Spray **Formula III**	11.42 ± 0.73^{b}	Weak
Negative control	0.00 ± 0.00^{c}	Weak
Commercial spray (Positif control)	7.07 ± 0.84^{d}	Weak

Note: a, b, c, d: The same letter rank in the same column shows that the mean is not significantly different between treatment groups based on Tukey HSD post hoc ($P<0.05$)

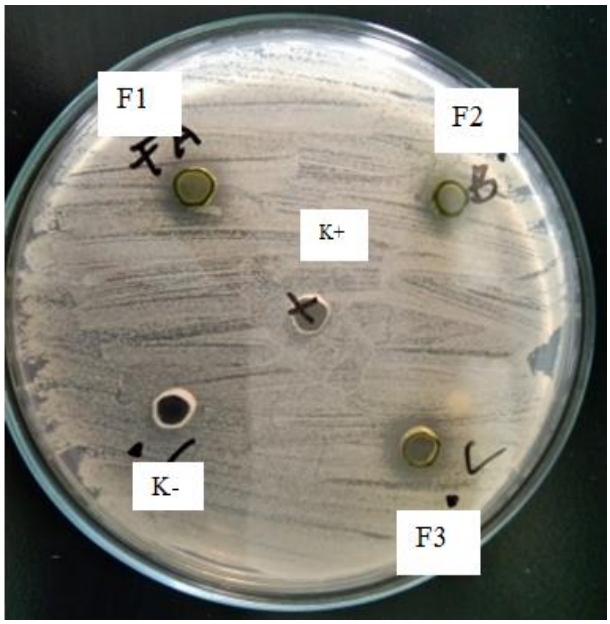

Figure 1. Inhibition zone of hand sanitizer ethanolic extract of *Sambiloto* leaves against *Staphylococcus aureus*. (K+): Positive control; (K-): Negative control; F1: Formula I; F2: Formula II; F3: Formula III

All formulations of hand sanitizer spray can inhibit the growth of *S. aureus* bacteria in vitro and have a higher inhibitory power than commercial hand sanitizer spray. Formula I with 10% ethanolic extract concentration had the lowest inhibitory diameter of 9.01 mm, and Formula III with 20% extract concentration had the highest inhibitory diameter of 11.42 mm. The mean inhibition zone diameter on *S. aureus* was increased with higher concentrations of ethanolic extracts of the three formulations. This indicates that spray hand sanitizer with a higher concentration of ethanolic extract of *Sambiloto* leaves had a better inhibitory ability against the growth of *S. aureus* bacteria. The inhibition of spray hand sanitizer treatment of *Sambiloto* leaf extract was higher than the inhibition of the commercial spray hand sanitizer treatment because the active compound of the ethanolic extract of *Sambiloto* leaves contained in the formulation was able to inhibit the growth of *S. aureus* bacteria better than commercial sprays. The commercial spray product used as a positive control contains alcohol as the active ingredient and DP 300 irgasan as an antibacterial agent. This composition could evaporate during the incubation process, reducing its antibacterial activity and resulting in weaker inhibition. Based on its inhibitory activity against *S. aureus*, this formulation of the spray hand sanitizer extract of *Sambiloto* leaves has the potential to be developed as an alternative to hand sanitizer products with natural non-alcoholic active ingredients.

The difference in the concentration of the ethanolic extract of *Sambiloto* leaves formulated into hand sanitizer spray, i.e., Formula I, Formula II, and Formula III, significantly affected the diameter of the inhibition zone of *S. aureus*. This was indicated by a significance value of 0.000 <0.05 in the one-way ANOVA statistical analysis test. Furthermore, based on Tukey HSD statistical analysis, the Formula I treatment was not significantly different from Formula II; in contrast, the Formula III treatment was significantly different from the treatment of Formula I and 2.

Reduction of microbes on hands before and after using hand sanitizer spray

The test of decreasing the number of microbial colonies after using the hand sanitizer spray formulation of ethanolic extract of *Sambiloto* leaves on 3 respondents. The data on the percentage decrease in the number of microbial colonies on the hands is shown in Table 5.

Table 5. The number of microbial colonies on hands decreases before and after being treated with spray hand sanitizer

Treatments	Percentage of decline (%)
Basic formulation of spray hand sanitizer (Negative control)	4.58
Formula I (10%)	30.23
Formula II (15%)	53.81
Formula III (20%)	59.25
Commercial hand sanitizer spray (Positive control)	77.51

The concentration of ethanolic extract of *Sambiloto* leaves in the formulation of spray hand sanitizer was directly proportional to the percentage of microbial decline. Hand sanitizer spray with Formula III (20% concentration) resulted in the highest (59.25%) reduction in the microbial colony. The less decline in microbial colonies was observed in formulas 1 and 2, i.e., 30.23% and 53.81%, respectively. The decrease in microbes showed that the active ingredient of ethanolic extract of *Sambiloto* leaves contained in the spray hand sanitizer preparation could kill bacteria on the hands. It was observed that only a few bacteria live on the hands while wiping or swabbing after using the spray hand sanitizer of ethanol extract of *Sambiloto* leaves. The percentage decrease in microbial numbers in commercial sprays was higher than the decrease in microbial numbers from the three spray formulations (not following the inhibition test results) because the spray was applied directly to the hand media in this test. The types of bacteria on the hands were very diverse, causing the spray of *Sambiloto* leaf extract to decrease their effectiveness.

Statistical analysis showed a significant difference between the spray treatments given (as evidenced by the sig value or probability of 0.011), with the covariate also having a significant difference (as evidenced by the sig value or probability of 0.008). This means that the difference in treatment between spray formulations of ethanol extract of *Sambiloto* leaves resulted in differences in the number of microbial colonies. Yet, the number of microbial colonies influenced this difference before spray treatment (covariate differences influenced differences between spray treatments). Based on the statistical analysis of the T-test, it can be observed that there was a significant difference between the number of microbial colonies before and after spray treatment (as evidenced by a significance value of 0.000 <0.05).

Spraying hand sanitizer with ethanolic extract of *Sambiloto* leaves can reduce the number of microbes on the hands because it contains active compounds. *Sambiloto* leaves contain large amounts of andrographolide and flavonoid compounds that can be antibacterial to inhibit bacterial growth (Pujiasmanto 2008). The flavonoid compounds work as antibacterial with the mechanism inhibiting nucleic acid synthesis, inhibiting cytoplasmic membrane function, and inhibiting energy metabolism of bacteria (Ansel 2008). Due to the inhibition of microbial growth on the hands, when swabbing was done, spraying hand sanitizer with ethanol extract of Sambilotto leaves on the skin surface of the hands reduced the number of germs as they died (Dewi 2013).

In conclusion, hand sanitizer of ethanolic extract of *Sambiloto* leaves showed good physical characteristics and stability. The spray hand sanitizer of ethanolic extract of *Sambiloto* leaves can inhibit the growth of *S. aureus in vitro* with weak inhibitory power. Formula III, with a 20% concentration, showed a higher inhibitory power of 11.42 mm. Formula III also resulted in a percentage reduction in the number of microbial colonies, 59.25%, but the reduction effectiveness was less than commercial spray (77.51%).

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