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ORIGINAL ARTICLE

Geothermal Flora and AgNPs Synergy: A Study on the Efficacy of *Lantana camara* and *Acrostichum aureum*-Infused Hand Sanitizers

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Abstract

Hand hygiene is an important factor that needs to be observed in controlling the spread of diseases transmitted through hand-to-hand contact. Synthesis of silver nanoparticles from tembelekan (*Lantana camara*) and paku laut (*Acrostichum aureum*) using the green synthesis method has good antibacterial activity against Staphylococcus aureus and Escherichia coli bacteria. Therefore, a preparation formulation was made, namely hand sanitizer, which is still rarely used. Formulations that have successfully entered the evaluation stage include organoleptic tests, homogeneity tests, spreadability tests, adhesion tests, viscosity tests, pH tests, accelerated stability tests, and irritation tests. Antibacterial activity was evaluated against bacteria *Staphylococcus aureus* and *Escherichia coli.* The hand sanitizer is formulated to contain 5% tembelekan AgNPs (F1); paku laut AgNPs 5% (F2); and a combination of 2.5% paku laut AgNPs and 2.5% tembelekan AgNPs. The resulting hand sanitizer has good organoleptic characteristics, except for the color of the preparation, which changed during the accelerated stability test. Test results for pH, adhesion, spreadability, viscosity, and homogeneity of *hand sanitizer* meet the requirements of a good test. Irritation tests on ten volunteers showed no irritation reaction. Antibacterial tests show that *hand sanitizer* has bacterial antibacterial activity with an average ± standard deviation of the inhibition zone *Staphylococcus aureus* is 6.605±0.459(F1); 6.665±0.615(F2); 6.380±0.282(F3) dan *Escherichia coli* namely 6.575 ± 0.219 (F1); 6.860 ± 0.155 (F2); 6.810 ± 0.056 (F3). Making *hand sanitizer* AgNPs-based ingredients from plants can be used as hand *sanitizer*, but stabilizers are required to prevent color changes during storage.

Introduction

Hand hygiene is an important concern in controlling disease and preventing infection because hands can be a medium for spreading microorganisms directly and indirectly [1,2]. Microorganisms such as bacteria can cause infections and diseases that majorly impact health [3,4]. *Staphylococcus aureus* and *Escherichia coli* are bacteria present on the skin [5]. Diseases caused by *Staphylococcus aureus* bacteria include lung infections, abscesses, bacteremia, endocarditis, and osteomyelitis [5]. *Escherichia coli* found outside the gastrointestinal tract can cause urinary tract infections (UTI), bacteremia, pneumonia, peritonitis, and diarrhea [6]. One way to prevent the spread and contraction of disease is by maintaining hand hygiene using hand sanitizer products [7].

Hand-cleaning products such as soap can reduce the amount of dirt and microbes on the skin. However, water availability is an obstacle to washing hands so that alternative hand sanitizers can be used [1]. Hand sanitizer is a product used to remove pathogens generally found on hands without using water [8]. The advantages of hand sanitizers are that they dry quickly, have better antimicrobial activity, and, after application, the hand sanitizer gel can provide longer protection [7]. Currently, most hand sanitizer products on the market contain alcohol, which can potentially negatively impact the lipid structure of epidermal keratinocytes. Thus, the skin becomes irritated, and the skin of the hands becomes dry [7]. There are several cases due to the use of hand sanitizers that contain alcohol, such as cases of burns [9,10]. Other cases occurred in children under 12 years in 2020, which caused nausea and lethargy, confusion, respiratory arrest, and even death in the child. Therefore, it is important to find alternative preparations of non-alcoholic hand sanitizers that are safe and environmentally friendly, one of which is by using natural resources and plants [11–15]. Several studies regarding the production of hand sanitizers using active plant ingredients have been reported, including *Moringa oleifera* [11], *Aromatic Eugenia* [16], *Aloe vera* [11], *Myristica fragrans* [17] and *Melaleuca alternifolia* [18]. Research on hand sanitizers using metal nanoparticles as active ingredients is still little reported, while there is increasing interest from the research community and consumers towards nanoformulations [7,19,20].

Nanoparticles range from 1 to 100 nm [21–23]. Silver nanoparticles (AgNPs) have garnered significant attention due to their potent antibacterial, antifungal, and antiviral properties [21,22,24–26]. AgNPs disrupt normal cellular membrane function by causing structural damage, altering membrane permeability, leading to loss of cell function, and ultimately causing cell death [27]. Silver nanoparticles can be synthesized through various methods, including chemical, physical, and biological processes [21,28]. The biological synthesis of nanoparticles, particularly through green synthesis methods, has seen rapid development. This approach often involves using plant-based reductants, which offer advantages such as cost-effectiveness, environmental friendliness, and enhanced antibacterial properties [25,29].

Exploration of the use of plants as materials for synthesizing silver nanoparticles taken from the Ie Seu-Um geothermal manifestation area, Mount Seulawah Agam, Aceh. The Ie Seu-Um geothermal manifestation area is a geothermal area due to volcanic activity. These extreme environmental conditions can influence the formation of plant secondary metabolite biosynthesis pathways. Extreme environments can increase plants' secondary metabolite compounds [19,30]. Water conditions rich in minerals and spread throughout the environment also influence soil mineral composition and plants' biosynthesis in geothermal areas [25]. The research reported in the Ie Seu-Um geothermal area is the synthesis of *Calotropis gigantea* [25,30]. Further research on other plants that have high antibacterial activity, such as tembelekan (*Lantana camara*) and paku laut (*Acrostichum aureum*), is necessary [19].

This study aims to formulate hand sanitizer preparations using silver nanoparticles synthesized from *Lantana camara* and *Acrostichum aureum*, as well as a combination of both, derived from the Ie Seu-Um geothermal manifestation area of Mount Seulawah Agam, Aceh. The development of these AgNP-based hand sanitizers aims to provide an innovative, alcohol-free alternative that is both effective against bacteria such as *Staphylococcus aureus* and *Escherichia coli* and gentle on the skin. The hand sanitizers will be evaluated according to established standards, including organoleptic properties, homogeneity, spreadability, adhesion, viscosity, pH, accelerated stability, and irritation potential. Additionally, the antibacterial efficacy of the formulations will be assessed.

The study contributes to the growing body of knowledge on alcohol-free hand sanitizers by exploring the potential of plant-based silver nanoparticles as an effective alternative, particularly in light of their enhanced antibacterial properties, cost-effectiveness, and environmental friendliness. This research also expands our understanding of the biosynthesis of nanoparticles in extreme environments and their application in healthcare products.

Materials and Methods

Sample Preparation

The selected tembelekan and paku laut leaves are pest-free, intact, and in good condition. Tembelekan leaves, and paku laut were obtained from Ie Seu-Um, Aceh. The leaves of both plants were also obtained from Ie Seu-Um, Aceh. First, the leaves were washed with water to remove dirt. Paku laut leaves were cut into small pieces to expedite the drying process, while tembelekan leaves were left whole due to their small and thin size. The leaves were subsequently dried for three days.

Production of Tembelekan and Paku Laut Leaf Extracts

Before extraction, the tembelekan and paku laut leaves must be collected, washed with water, cut into small pieces, and dried for three days. Extraction is performed at room temperature (25 °C ± 2 °C). To prepare the extracts, 10 g of each leaf type is placed in a beaker and mixed with 100 mL of distilled water [30]. The mixture is then boiled for 1 hour at 70 °C while maintaining the ambient room temperature at 25 °C ± 2 °C. After boiling, the solution is filtered using Whatman No. 1 filter paper. The resulting filtrates are referred to as *Lantana camara* leaf water extract (LLc) and *Acrostichum aureum* water extract (LAa).

Green Synthesis AgNPs-LLc and AgNPs-LAa

10 mL of LLc was alkalized by adding 0.1 N NaOH until the pH reached 8. A 100 mL volumetric flask was prepared, and 4.35 mM of $AgNO_3$ was dissolved in 100 mL of distilled water. 10 mL of LLc was added to an Erlenmeyer flask, followed by 90 mL of $AgNO_3$ powdered solution. The mixture was then stirred in the dark at room temperature (25 °C ± 2 °C) at a speed of 220 rpm for 24 hours. The same procedure was applied to the LAa, with the pH adjusted to 9 and using a 5 mM $AgNO_3$ solution. The resulting silver nanoparticles were designated as AgNPs-LLc (Silver nanoparticles *Lantana camara*) dan AgNPs-LAa (Silver nanoparticles *Acrostichum aureum*).

Hand Sanitizer Formulation

Table 1. Hand sanitizer formulation.

Sample	Function	Formulation (%)			
		F0	F1	F2	F3
AgNPs-LLc[a]	Active ingredients	0	5	0	2,5
AgNPs-LAa[b]	Active ingredients	0	0	5	2,5
Carbopol	Base gel	0,5	0,5	0,5	0,5
TEA[c]	Alkalizer	0,2	0,2	0,2	0,2
Glycerin	Humectant	2	2	2	2
Methylparaben	Preservative	0,12	0,12	0,12	0,12
Propylparaben	Preservative	0,02	0,02	0,02	0,02
Aquades	Solvent	ad 100 mL			

Note: [a]: Silver nanoparticles *Lantana camara*; [b]: Silver nanoparticles *Acrostichum aureum*; [c]: Triethanolamine; F0: basic; F1: *Hand sanitizer* 5% AgNPs-LLc; F2: *Hand sanitizer* 5% AgNPs-LAa; F3: *Hand sanitizer* 2,5% AgNPs-LLc dan 2,5% AgNPs-LAa.

The hand sanitizer formulation utilizes AgNPs-LLc and AgNPs-LAa as active ingredients, and the excipients used in the formulations include carbopol, TEA, glycerin, methylparaben, and propylparaben. Four different formulations were prepared. Formulation F0 (formulation 0) was made without active ingredients, while the other three formulations incorporated varying concentrations of AgNPs-LLc and AgNPs-LAa, as detailed in Table 1.

Carbopol as a gel base in gel preparations provides clear results [31]. Glycerin is commonly used as a humectant to retain moisture in the skin [32]. Methyl and propyl paraben act as preservatives to prevent microbial contamination, which is crucial due to the high water content in hand sanitizers [31]. TEA is employed to balance the pH and stabilize the formulation because the carbopol base, with a pH range of 2.5-3.0, can be irritating when applied topically. TEA helps to adjust the pH and increase the viscosity of the carbopol [31,32].

To prepare the gel, 0.5 grams of carbopol are weighed and placed in a mortar containing hot water at 70 °C. The mixture is stirred with a pestle until homogeneous at room temperature (25 °C ± 2 °C). Next, 0.2 mL of TEA is added gradually while stirring until homogeneous. Plant-based AgNPs are added gradually, followed by 0.12 grams of methylparaben, 0.02 grams of propylparaben, and 2 mL of glycerin. The mixture is stirred continuously until homogeneous. Finally, distilled water is added to bring the total volume to 100 mL, with constant stirring until the formulation is fully integrated [32].

Organoleptic Test

The organoleptic test was conducted on hand sanitizers containing AgNPs-LLc and AgNPs-LAa [33]. Observations of the shape, color, and smell were recorded over six cycles.

Homogeneity Test

The homogeneity test was performed with 0.5 g of hand sanitizer preparation. The sample was placed between two glass plates and observed for any inconsistencies. According to the standard for the homogeneity test, no grains or lumps should be visible after the preparation is pressed or wiped between the glass plates [34,35]. This test was conducted over six cycles.

pH test

pH measurement was conducted using a pH meter at 25 °C ± 2 °C. The pH meter was calibrated with a buffer solution of pH 7, followed by calibration with a buffer of pH 4. After calibration, the meter was rinsed and dried [36]. To test the pH, 0.5 g of the hand sanitizer preparation was dissolved in 10 mL of distilled water, and the pH value was measured using the pH meter. The desired pH range for the preparation is 4.5 – 6.5 [20,34,35]. This test was conducted over six cycles.

Spreadability Test

0.5 g of the preparation was weighed, placed on a glass plate, and pressed with another glass plate for 1 minute. The diameter of the resulting preparation was measured in three directions (vertical, horizontal, and diagonal). The preparation was then subjected to additional pressing with loads of 50 g, 100 g, 150 g, and 200 g for 1 minute. The diameter was measured within 5-7 cm [37]. This test was conducted over six cycles.

Adhesion Test

A total of 0.5 g of hand sanitizer gel preparation is placed on a glass plate, and another preparation glass is placed on top. A load of 80 grams is then added on top of the upper glass plate for 1 minute, after which the load is removed. The time is then measured from when the two glass plates are simultaneously released until the hand sanitizer preparation returns to its original state [38]. Good adhesion for hand sanitizer gel is more than 1 second [39]. Hand sanitizer testing was carried out for six cycles.

Viscosity test

The viscosity test is conducted to determine the viscosity of the gel preparation and its resistance to flow. A gel is suitable if its viscosity falls within the 1,000-10,000 mPa.s (medium viscosity gel) [40]. Viscosity test using a Rheometer (Lamy Rheology Instruments CP-4000 Plus Cone Plate System) at 60 rpm for 30 seconds. The desired viscosity range is 2000 – 4000 cP [37,41]. Tests are carried out both before and after the accelerated stability test.

Accelerated Stability Test

Accelerated stability tests are performed over six cycles. The hand sanitizer gel preparation is stored at a low temperature of ± 4°C for 24 hours, then removed and placed at a high temperature of ± 40°C; this constitutes one cycle. The parameters evaluated in this accelerated stability test include the gel preparation's organoleptic properties, homogeneity, pH, adhesion, and spreadability [17].

Irritation Test

The irritation test was conducted on ten panelists aged 20-30 years, using the inner forearm as the test site. The hand sanitizer preparation was applied to an area of 2.5 x 2.5 cm on the inner forearm, which was left uncovered. Observations were made to monitor any reactions. Each panelist underwent three repetitions of the test over one day. Positive irritation reactions were characterized by redness, itching, or swelling on the treated skin [42–44].

Panelists provided informed consent and agreed to the irritation test protocols. They completed a questionnaire regarding any reactions they experienced. Panelists selected for the test had no prior history of allergies, infections, trauma, or visible clinical signs on their skin. The formulation was evaluated based on the skin's reaction after applying the hand sanitizer. Reactions were categorized as follows: redness was marked (+), itching (++), burning sensation (+++), swelling (++++), and no reaction was recorded (-).

Antibacterial Activity Test

The bioindicators used in this research are *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. The isolate was obtained from the Aceh Basic Science Laboratory, which followed the procedures and protocols for rejuvenation and identification [45].

Antibacterial activity was assessed using the disc diffusion method (Kirby-Bauer). The test was carried out twice (Duplo). The turbidity of the bacterial inoculum was visually compared to the 0.5 McFarland standard, which corresponds to approximately 1.5 x 10^8 CFU/mL of bacteria. Mueller-Hinton Agar (MHA) was prepared by dissolving 3.8 grams of the medium in 100 mL of distilled water, then heating and mixing until boiling. The medium was sterilized by autoclaving at 121 °C for 15 minutes at 1 atm pressure. After sterilization, the medium was cooled to 40-45 °C. Subsequently, 25 mL of MHA was poured into sterile petri dishes and allowed to solidify. The bacterial suspensions of *Staphylococcus aureus* and *Escherichia coli* were inoculated onto the entire surface of the MHA using sterile cotton swabs. The inoculated plates were left at room temperature (25 °C ± 2 °C) for 15 minutes. Paper discs (6 mm diameter), soaked with the positive control (commercial hand sanitizer), were placed on the agar. The discs were gently pressed with forceps to ensure contact, and the plates were then sealed. The same procedure was applied for the hand sanitizers AgNP combination *Lantana camara* 2.5% and AgNP *Acrostichum aureum* 2,5%; AgNP *Lantana camara* 5%; AgNP *Acrostichum aureum* 5% and negative control (base). After that, the media containing bacteria was incubated at 37 °C for 24 hours. Before incubation, the plates were labeled. After incubation, the clear inhibition zones around the discs were measured using a caliper to assess antibacterial activity [46].

Results and Discussion

Sample Extraction

The extraction process for producing AgNP-LLc and AgNP-LAa utilizes water as the solvent [20,25,30,47]. Water is a nontoxic solvent, making it relatively safer for human health and the environment and also cost-effective.

Green Synthesis of Silver Nanoparticles

The formation of AgNP-LLc and AgNP-LAa involves the reduction of Ag $ions^+$ to Ag^0. The plant extracts reduce Ag+ in AgNO3 to form Ag^0, characterized by a color change to brown [25,30]. In the green synthesis, AgNP-LLc resulted in a final color change to blackish brown (Figure 1a), while AgNP-LAa changed to reddish brown (Figure 1b).

AgNP-LLc and AgNP-LAa, synthesized using the green synthesis method, exhibit a characteristic round shape. Research on their antibacterial activity, tested against *Staphylococcus aureus* and *Escherichia coli,* showed inhibition of AgNP-LLC (8.3 mm and 7 mm) and AgNP-LAa (7.35 and 7.80).

(a) (b)

Figure 1. (a) AgNP-LLc (b) AgNP-LAa.

Organoleptic Test

Table 2. Organoleptic test results.

Cycling Test	Parameter	F0	F1	F2	F3
Before	Form	Gel	Gel	Gel	Gel
	Color	Clear	Dark yellow	Light yellow	Yellow
	Smell	No smell	No smell	No smell	No smell
Cycle 1	Form	Gel	Gel	Gel	Gel
	Color	Clear	Dark yellow	Light yellow	Yellow
	Smell	No smell	No smell	No smell	No smell
Cycle 2	Form	Gel	Gel	Gel	Gel
	Color	Clear	Dark yellow	Light yellow	Yellow
	Smell	No smell	No smell	No smell	No smell
Cycle 3	Form	Gel	Gel	Gel	Gel
	Color	Clear	Dark yellow	Light yellow	Yellow
	Smell	No smell	No smell	No smell	No smell
Cycle 4	Form	Gel	Gel	Gel	Gel
	Color	Clear	Deep yellow	Reddish orange	Purplish Orange
	Smell	No smell	No smell	No smell	No smell
Cycle 5	Form	Gel	Gel	Gel	Gel
	Color	Clear	Deep yellow	Reddish orange	Purplish Orange
	Smell	No smell	No smell	No smell	No smell
Cycle 6	Form	Gel	Gel	Gel	Gel
	Color	Clear	Deep yellow	Reddish orange	Purplish Orange
	Smell	No smell	No smell	No smell	No smell

Note: F0: Base; F1: Hand sanitizer 5% AgNPs-LLc; F2: Hand sanitizer 5% AgNPs-LAa; F3: Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa.

Organoleptic testing aims to visually assess the quality and stability of the gel. Observations include evaluating the shape, color, and smell [11,48]. Based on Table 2 and Figure 2. Organoleptic F0 is clear in color as it contains no active substances. F1 is dark yellow because of the addition of tembelekan AgNP (*Lantana camara*), which is blackish brown. F2 is light yellow because of the addition of paku laut AgNP (*Acrostichum aureum*), which is reddish brown. F3 is yellow because of the addition of AgNP tembelekan (*Lantana camara*) and paku laut AgNPs (*Acrostichum aureum*). The color of the hand sanitizer preparation differs from that of the active substances because the concentration is only 5%. All formulations are thick gels and do not have a distinctive smell, as the AgNPs used do not impart any significant smell. The active substances and their concentrations influence the color of the preparation.

After cycling tests, the organoleptic properties of the preparations did not change in terms of smell or appearance. Hand sanitizer changes color during storage. F1 is from dark yellow to deep dark yellow, F2 is from light yellow to dark yellow orange-reddish, and F3 is from yellow to orange purplish hue. These color changes, observed under extreme temperatures of 40 °C and -4 °C, suggest that the hand sanitizer preparations are unstable under such conditions. Stabilizers should be added to prevent color changes. Reported AgNP stabilizers include trisodium citrate [49], PVA [47], and EDTA [50].

Before cycling test

Cycle 6

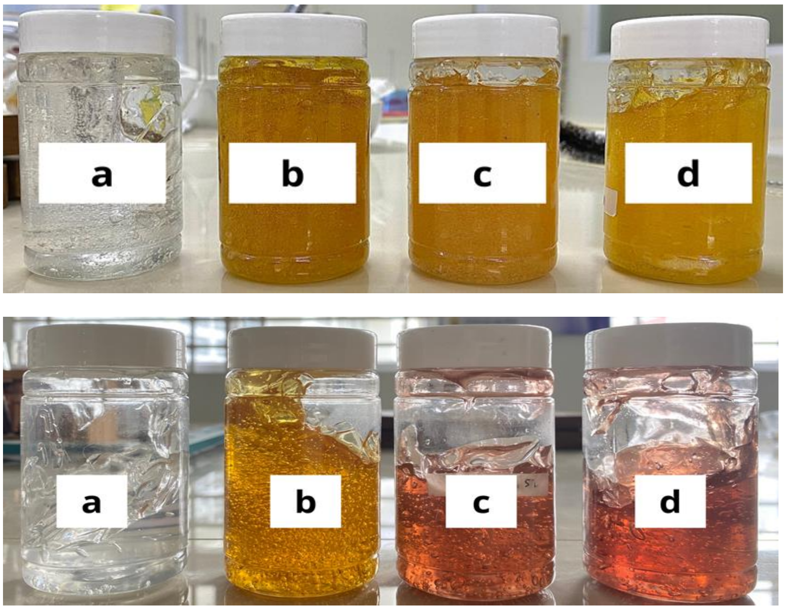


Figure 2. Formulation results *hand sanitizer* (a) Base; (b) Hand sanitizer 5% AgNPs-LLc; (c) Hand sanitizer 5% AgNPs-LAa; (d) Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa.

Homogeneity Test

The homogeneity test for the hand sanitizer is conducted to assess the uniformity of particles within the preparation. According to the results presented in Table 3, no coarse particles were detected in the hand sanitizer. However, air bubbles were observed in the preparation. The hand sanitizer is considered homogeneous if it does not contain coarse particles when spread on transparent glass [11]. A homogeneous gel indicates that the active substance is evenly distributed and stable throughout the preparation [51]. The viscosity level also affects the preparation's homogeneity because the hand sanitizer is not too thick, and the mixture forms more easily [52]. The presence of air bubbles in the preparation is likely due to the addition of triethanolamine immediately after the carbopol is dispersed in water. When neutralized by triethanolamine, the gel can trap air, forming bubbles. However, the air bubbles trapped in the preparation tend to decrease over time with storage [53]. Results from a cycling test, which involved storing the hand sanitizer preparations F1, F2, and F3 for six cycles, showed no settled particles. Additionally, the number of air bubbles decreased after the cycling test. The hand sanitizer formulation that reported air bubble formation contained sandalwood oil [53].

Table 3. Homogeneity test results.

Cycling Test	F0	F1	F2	F3
Before	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Cycle 1	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Cycle 2	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Cycle 3	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Cycle 4	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Cycle 5	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Cycle 6	Homogeneous	Homogeneous	Homogeneous	Homogeneous

Note: F0: Base; F1: Hand sanitizer 5% AgNPs-LLc; F2: Hand sanitizer 5% AgNPs-LAa; F3: Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa.

pH Test

pH testing ensures that hand sanitizer gel preparations are safe for the skin. The ideal pH range for hand sanitizer gel is 4.5 to 6.5, aligning with the skin's acid mantle [20,54]. Carbopol has an acidic pH of 2.5-3.0, which is incompatible with the skin's pH, necessitating the addition of triethanolamine (TEA) to adjust the pH of the hand sanitizer preparation. Higher TEA concentrations result in a higher pH and increased viscosity of the preparation [55]. Therefore, a TEA concentration of 0.2% is sufficient for preparing hand sanitizer. A hand sanitizer with an acidic pH can irritate the skin, while one that is too alkaline can lead to skin scaling.

Table 4. pH test results.

Formulation	Cycling Test (Mean ± SD)						
	Before	S1	S2	S3	S4	S5	S6
F0	5.8 ± 0	5.7 ± 0	5.8 ± 0	5.53 ± 0.06	5.5 ± 0	5.4 ± 0.1	5.4 ± 1.09
F1	5.8 ± 0	5.83 ± 0.06	5.8 ± 0	5.83 ± 0.06	5.9 ± 1.09	5.6 ± 1.09	5.6 ± 1.09
F2	5.8 ± 0	5.8 ± 0	5.83 ± 0.06	5.8 ± 0	5.9 ± 1.09	5.6 ± 1.09	5.6 ± 1.09
F3	5.8 ± 0	5.87 ± 0.06	5.8 ± 0	5.83 ± 0.06	6 ± 0	5.6 ± 1.09	5.6 ± 1.09

Note: F0: Base; F1: Hand sanitizer 5% AgNPs-LLc; F2: Hand sanitizer 5% AgNPs-LAa; F3: Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa; S1: Cycle 1; S2: Cycle 2; S3: Cycle 3; S4: Cycle 4; S5: Cycle 5; S6: Cycle 6; SD: Standard deviation.

Measurement results indicated that the pH decreased after the cycling test (table 4). However, the pH remained within the acceptable range of 4.5 - 6.5. Consistent with previous research, a decrease in pH during storage can occur due to CO_2 reacting with the water content in the gel, leading to a lower pH [32]. Additionally, other reports suggest that pH reduction in hand sanitizer gels can result from the evaporation of silver nanoparticles (AgNP) and from environmental factors such as light, temperature, and humidity [56,57]. Observational data show that hand sanitizer preparations with added active substances (F1, F2, and F3) maintain pH levels more effectively than the base formulation. The pH values indicate that the hand sanitizer gel preparation is safe for skin use. A hand sanitizer with a similar pH value reported is basil [58].

Spreadability Test

Testing the spreadability of hand sanitizer preparations aims to assess how well the hand sanitizer spreads on the skin, affecting active substances' absorption and release rate at the application site. The viscosity of the hand sanitizer preparation plays a crucial role in its spreadability. Lower viscosity results in greater spreading ability, while higher viscosity decreases it. A greater spreading ability allows the substance to cover a larger area, enhancing contact between the active ingredients and pathogens such as viruses or bacteria, thereby increasing the effectiveness of the hand sanitizer. A hand sanitizer preparation is considered good and preferable if it spreads easily on the skin and feels comfortable [32]. Optimal spreadability is indicated by a value of 50-70 mm, which denotes a comfortable and stable condition for the preparation [34,35]. The results obtained showed that the hand sanitizer spread effectively on the skin.

Table 5. Spreadability test results.

Formulation	Cycling Test (Mean ± SD)						
	Before	S1	S2	S3	S4	S5	S6
F0	5.23 ± 0.72	5.43 ± 0.30	5.60 ± 0.61	5.90 ± 0.44	6.51 ± 0.53	5.21 ± 1.02	5.59 ± 0.64
F1	5.79 ± 0.52	5.74 ± 0.50	5.38 ± 0.55	5.99 ± 0.71	6.4 ± 0.47	6.20 ± 0.52	5.8 ± 0.60
F2	5.27 ± 0.24	5.48 ± 0.40	6.10 ± 0.55	5.88 ± 0.75	6.32 ± 0.57	6.5 ± 0.55	6.88 ± 0.70
F3	5.60 ± 0.39	5.80 ± 0.70	5.76 ± 0.57	6.23 ± 0.65	5.72 ± 0.66	6.35 ± 0.66	6.2 ± 0.66

Note: F0: Base; F1: Hand sanitizer 5% AgNPs-LLc; F2: Hand sanitizer 5% AgNPs-LAa; F3: Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa; S1: Cycle 1; S2: Cycle 2; S3: Cycle 3; S4: Cycle 4; S5: Cycle 5; S6: Cycle 6; SD: Standard deviation

The average spreadability power, as shown in Table 5, measured before and after the cycling test, falls within the desirable range of 5-7 cm. After six cycles of the cycling test, there was an increase in the spreadability of the hand sanitizer preparation, although it remained within the good spreadability range. Enhanced spreadability can be attributed to a decrease in viscosity. Lower viscosity results in a larger spreading diameter because the gel flows more easily, leading to a wider distribution diameter. A greater diameter indicates that the gel spreads faster with minimal application, thereby increasing the contact between the preparation and the skin surface [32]. A hand sanitizer with similar increased spreadability reported is basil [58].

Adhesion Test

An adhesion test is conducted to evaluate the ability of a gel preparation to adhere to the skin [55]. Minimizing the adhesive power of hand sanitizer preparations ensures they are not excessively sticky and remain comfortable. Optimal adhesion strength is considered to be ≥1 [32]. Longer-lasting gel preparations are preferable, as they enhance the therapeutic effect of the active substances by allowing them to remain on the skin for a longer period. Adhesion reflects the preparation's ability to stay on the skin, which is related to the persistence of the active substance on the skin [32]. This adhesion test is directly proportional to viscosity; a higher adhesion value generally corresponds to higher viscosity [55]. Based on the results obtained, it was concluded that the hand sanitizer met the criteria for good adhesion.

Table 6. Adhesion test results.

Formulation	Cycling Test (Mean ± SD)						
	Before	S1	S2	S3	S4	S5	S6
F0	2.73 ± 0.60	3.03 ± 0.40	2.4 ± 0.97	2.98 ± 2.23	2.44 ± 0.47	2.00 ± 0.68	2.00 ± 1.07
F1	2.47 ± 0.38	2.49 ± 0.27	2.81 ± 1.08	2.19 ± 0.28	3.12 ± 0.68	2.48 ± 0.79	1.98 ± 0.73
F2	2.59 ± 1.03	2.50 ± 0.24	1.92 ± 0.65	2.12 ± 0.67	2.46 ± 0.51	2.31 ± 0.24	2.09 ± 0.18
F3	3.16 ± 0.57	2.83 ± 0.09	2.32 ± 0.36	1.97 ± 0.15	1.79 ± 0.93	2.53 ± 0.30	2.72 ± 0.39

Note: F0: Base; F1: Hand sanitizer 5% AgNPs-LLc; F2: Hand sanitizer 5% AgNPs-LAa; F3: Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa; S1: Cycle 1; S2: Cycle 2; S3: Cycle 3; S4: Cycle 4; S5: Cycle 5; S6: Cycle 6; SD: Standard deviation

After conducting the cycling test for six cycles, the adhesion test results (Table 6) revealed changes in adhesion power. The observed change in adhesion was due to a decrease in the duration for which the hand sanitizer adhered. This reduction in adhesion may be attributed to a decrease in the viscosity of the preparation. Several factors can influence changes in adhesion to gel preparations, including the concentration of added substances, temperature, mixing method, pH, particle size, and viscosity [32]. A similar decrease in adhesion was reported for hand sanitizers containing cucumber [32] and basil [58].

Viscosity test

Viscosity testing is conducted to determine the ease of application of a preparation. The overall viscosity range for hand sanitizer preparations is 2000 – 4000 cP [37,41]. Viscosity describes the thickness of a dosage form, which affects its spreadability and stickiness. Ideally, hand sanitizer should not be too thick; excessive thickness can make it difficult to spread and result in an unpleasant application experience [37]. While high viscosity can decrease spreadability, it can increase the adhesive power of the dosage form and vice versa [55]. Based on the results presented in Table 7, it is concluded that the hand sanitizer meets the requirements appropriately.

Table 7. Viscosity test results.

Formulation	Cycling Test (Mean ± SD)	
	Before	After
F0	3488 ± 387.980	3356.333 ± 27.30079
F1	3520 ± 123.559	3049.667 ± 53.72461
F2	3395.333 ± 247.56	3088 ± 84.04166
F3	3433 ± 58.949	3546 ± 23.38803

Note: F0: Base; F1: Hand sanitizer 5% AgNPs-LLc; F2: Hand sanitizer 5% AgNPs-LAa; F3: Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa; SD: Standard deviation

The viscosity results of the hand sanitizer preparation, both before and after the cycling test, were within the desirable range of 2000 – 4000 cP. As shown in Table 7, the viscosity of the hand sanitizer decreased following the cycling test. However, the F3 hand sanitizer preparation exhibited an increase in viscosity. Despite these changes, all three hand sanitizer formulations remain within the acceptable viscosity range, indicating they should spread well upon application. Temperature fluctuations can influence the viscosity of the preparation. Inappropriate storage conditions or extreme temperature changes can affect the gel's viscosity. High temperatures can cause the hand sanitizer to become more fluid, while low temperatures can make it stiffer [59]. The hand sanitizer that experienced a similar decrease in viscosity was reported to be Palisa [59].

Irritation Test

Table 8. Irritation test results

Formulation	Irritation			
	F0	F1	F2	F3
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-

Note: F0: Base; F1: Hand sanitizer 5% AgNPs-LLc; F2: Hand sanitizer 5% AgNPs-LAa; F3: Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa; - : No reaction occurs; +: Redness; ++: Itching; +++: Burning sensation

Irritation testing of hand sanitizer preparations is conducted to determine their safety for use on the skin. The observed parameters include itching, redness, swelling, and burning sensations [11]. The hand sanitizer was applied to 10 volunteers, each receiving one of three formulations and a base sample. Table 8 shows no reactions such as itching, redness, swelling, or burning sensations were reported. These results are consistent with the pH of the preparation, as a skin pH range of 4.5-6.5 is considered safe for skin application [20,54]. Based on these findings, it can be concluded that the hand sanitizer preparations are non-irritating and safe for use on the skin.

Antibacterial Testing

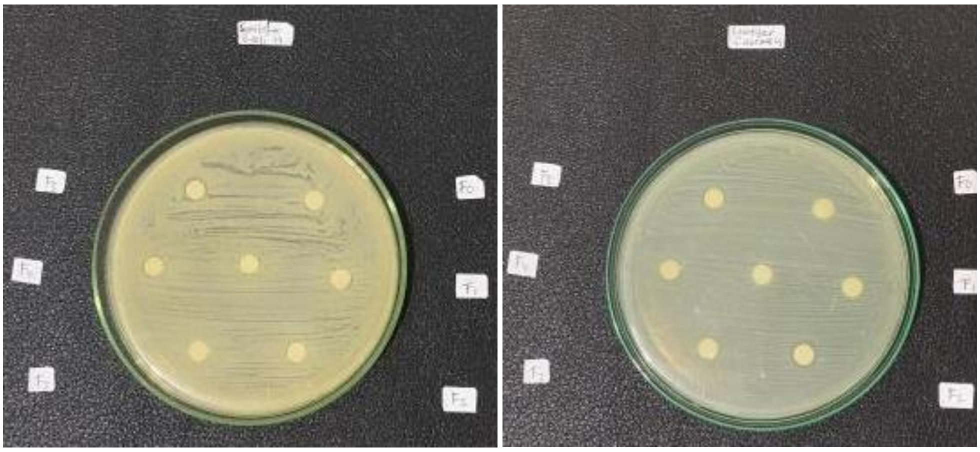
Antibacterial activity testing was conducted to evaluate the inhibitory effects of hand sanitizer preparations against *Staphylococcus aureus* ATCC 25923 (Figure 3b) and *Escherichia coli* ATCC 25922 (Figure 3a). A comparison of antibacterial activity between AgNP preparations without a combination and AgNP preparations with a combination is presented in Table 9. The measured inhibition zones were classified according to the following criteria: ≥21 mm (strong), 11-20 mm (medium), 6-10 mm (weak), and <6 mm (no activity) [60,61].

Table 9. Antibacterial activity of hand sanitizer.

Bacteria	Treatment	Inhibition Zone Diameter (mm)		
		PI	P2	Mean ± SD
Staphylococcus aureus ATCC 25923	K+	6.49	6.10	6.295 ± 0.275
	K- / F0	0	0	0
	F1	6.93	6.28	6.605 ± 0.459
	F2	6.23	7.10	6.665 ± 0.615
	F3	6.18	6.58	6.380 ± 0.282
Escherichia coli ATCC 25922	K+	6.12	6.11	6.115 ± 0.007
	K- / F0	6.26	6.37	6.315 ± 0.077
	F1	6.73	6.42	6.575 ± 0.219
	F2	6.75	6.97	6.860 ± 0.155
	F3	6.77	6.85	6.810 ± 0.056

Note: K+: Positive control; K-/F0: Negative control/ base; F1: Hand sanitizer 5% AgNPs-LLc; F2: Hand sanitizer 5% AgNPs-LAa; F3: Hand sanitizer 2.5% AgNPs-LLc and 2.5% AgNPs-LAa.; SD: Standard deviation; P1; Repetition 1; P2; Repetition 2

The test results presented in Table 9 indicate that the hand sanitizers F1, F2, and F3 exhibit inhibitory activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. However, they fall into the weak inhibition category. For *Staphylococcus aureus* ATCC 25923, F1 and F2 demonstrated better inhibitory effects than F3, with the order of effectiveness being F2 > F1 > F3. For *Escherichia coli* ATCC 25922, F2 was the most effective, followed by F3 and F1, with the order being F2 > F3 > F1. Despite these differences in inhibition, the overall values do not show significant variation. This suggests that the active ingredients, even without combinations, can still provide antibacterial activity.



(a) (b)

Figure 3. (a) *Escherichia coli* (b) *Staphylococcus aureus.*

Inhibition was also observed for F0 against *Escherichia coli* ATCC 25922. This activity in the base formulation (F0) is likely due to several ingredients, such as glycerin, methylparaben, and propylparaben. Glycerin, a humectant, can also have antimicrobial properties. Methylparaben and propylparaben serve as antimicrobials and are included in hand sanitizers to prevent bacterial and fungal growth, given that the gel's primary composition is water, which is more susceptible to microbial contamination. Combining these ingredients enhances their effectiveness as a broad-spectrum preservative, extending the gel's shelf life [62]. No inhibition of *Staphylococcus aureus* ATCC 25923 was observed in F0, which might be attributed to antimicrobial resistance [63]. Previous research has reported that hand sanitizers containing silver nanoparticles derived from *Marchantia sp*. were effective in inhibiting the growth of *Escherichia coli* (19.75 mm ± 1.13) and *Staphylococcus aureus* (16.25 mm ± 0.54) [20].

The antimicrobial activity of AgNPs is generally attributed to four mechanisms: adhesion to cell wall and membrane surfaces, penetration into cells, damage to intracellular structures (such as mitochondria, vacuoles, and ribosomes), and disruption of biomolecules (including proteins, lipids, and DNA). AgNPs can also interact with respiratory enzyme systems, producing reactive oxygen species (ROS) that cause oxidative stress and damage proteins and nucleic acids [63–65]. AgNPs have been reported to attach to bacterial cell membranes, disrupting cell permeability and penetration into bacterial cells. This interaction affects cell membrane integrity and respiration. Bacterial cell membranes contain proteins with sulfur as a key component. AgNPs interact with these sulfur-containing proteins and subsequently with phosphorus-containing compounds such as DNA, leading to damage that can have a lethal effect on microorganisms [56,66].

Research has demonstrated that AgNP-LLc and AgNP-LAa exhibit promising antimicrobial potential against *Escherichia coli* and *Staphylococcus aureus*, making them effective active ingredients for hand sanitizers. Notably, the formulations used in this research did not include alcohol. Consequently, alcohol-free hand sanitizers represent a viable alternative for those seeking to avoid the negative effects associated with alcohol-based hand sanitizers, such as skin sensitivity and risk of accidental fires.

Conclusions

Based on the research results, the hand sanitizer preparations AgNPs-LLc, AgNPs-LAa, and the combination of AgNPs-LLc and AgNPs-LAa that have been evaluated meet the specified parameters, but the color of the preparations changes during accelerated stability. This is possible due to instability in the preparation, so additional stabilizers are required. Silver nanoparticle hand sanitizer has been proven not to irritate users, but further tests need to be carried out in vivo and in vitro. Overall, the formulated hand sanitizer has good antibacterial activity, and the inhibition obtained does not show a significant difference. Even though the difference is insignificant, the best hand sanitizer formulation inhibits the growth of bacteria in F1 against *Staphylococcus aureus* and F2 against *Escherichia coli.*

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