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Hybrid Handwash with Silver Nanoparticles from *Calotropis gigantea* Leaves and Patchouli Oil: Development and Properties

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Abstract

When washing hands, handwashing is one way to prevent diseases caused by bacteria such as *Staphylococcus aureus* and *Escherichia coli*, the most common bacteria that can cause infections. The production of handwash utilizing silver nanoparticles as an active antibacterial agent remains a relatively infrequent practice. The synthesis of silver nanoparticles from the leaves of *Calotropis gigantea*, which grows in the geothermal area of Ie Seu-um Aceh Besar, has been carried out using the green synthesis method and hybrid green synthesis with patchouli oil. Handwash with active ingredients such as silver nanoparticles was successfully formulated, evaluated, and tested against *S. aureus* and *E. coli*. The organoleptic characteristics, pH, viscosity, foam height measurements, density, irritation, and antibacterial activity against *S. aureus* and *E. coli* were evaluated. The results showed that the organoleptic properties of the handwash with silver nanoparticles were not changed during a 30-day storage period, with pH values in the range of 9.7-10.3, and did not cause irritation upon using silver nanoparticle handwash. The best formula for handwashing with silver nanoparticles in inhibiting the growth of *S. aureus* and *E. coli* bacteria was F2, with inhibition zones of 12.9 ± 2.85 mm and 10.95 ± 0.8 mm, respectively. The formulated handwash with silver nanoparticles met the requirements of good liquid soap according to the Indonesian National Standard (SNI) with potent antibacterial activity.

1. Introduction

Staphylococcus aureus and *Escherichia coli* are the most common pathogenic bacteria that infect humans, and can enter the body through the palms, nose, mouth, digestive system, urinary tract, or genital organs [1, 2]. They are even behind the emergence of global endemic and epidemic diarrhea [3]. According to the WHO (World Health Organization) and UNICEF (United Nations Children's Fund), there are approximately 2 billion cases of diarrhea, and 1.9 million children under the age of five die from diarrhea worldwide each year [4].

Figure 1. *Calotropis gigantea* plant (personal documentation).

S. aureus and *E. coli* can be found on the human skin. Although *S. aureus* is harmless to human skin, this Gram-positive coccus bacterium can generally cause various infections with severity ranging from mild to fatal, such as skin infections, toxic shock syndrome [5], psoriasis [6], atopic dermatitis (AD) [7–10], food poisoning [11], allergic asthma [12], pneumonia [13], cystic fibrosis (CF) [14], chronic granulomatous disease (CGD) [15], osteomyelitis [16], diabetic foot infections (DFI), and many other diseases. *E. coli* has been associated with intestinal disorders such as ulcerative colitis, colorectal cancer, and celiac disease [17]. In addition to causing diseases involving the gastrointestinal tract, *E. coli* is also involved in bloodstream infections [18].

One of the easiest, simplest, most effective, and most practiced methods to prevent diseases caused by bacteria is to maintain personal hygiene by washing hands with running water and soap [19]. Liquid soap is more practical and has an appealing form compared to other soaps, leading to a rapid increase in its usage. Soap production can be carried out through various chemical processes or natural methods. In the context of using natural ingredients, making soap with plant extracts is known to have several advantages, including less irritant effect, cleaning effectiveness, environmental friendliness, and the potential to enhance antimicrobial effects [20]. Several studies on soap production were reported using green betel leaves (*Piper betle)* [21], red betel leaves (*Piper ornatum*) [22], clove leaves (*Syzygium aromaticum*) [23], cayenne pepper leaves (*Capsicum frutescens*) [24], kaffir lime leaves (*Citrus hystrix*) and robusta coffee (*Coffea canephora*) [25]. Research on soap production using metal nanoparticles has not been widely reported. Silver is the most used metal among all metals due to its antibacterial properties.

Silver nanoparticles (AgNPs) are currently one of the main alternative antimicrobials for controlling microorganisms [26, 27]. AgNPs were reported to cause structural and physiological changes in microbial cell membranes, such as accumulation and changes in membrane permeability and potential, as well as inhibition of respiratory proteins attached to the membrane, disrupting cell homeostasis and ultimately leading to microbial cell death [28]. AgNPs were previously synthesized using an endemic plant (*Calotropis gigantea*) from the geothermal manifestation area of Ie Seu-um, Aceh province, Indonesia [29]. AgNPs synthesized using *C. gigantea* leaves (AgNPs-LCg) showed antibacterial activity against *S. aureus*, *E. coli*, and the fungus *C. albicans* of 10.60 ± 0.22 mm, 8.40 ± 0.33 mm, and 8.90 ± 0.25 mm, respectively [30]. Further research indicated that patchouli oil used to coat AgNPs-LCg enhanced the antibacterial activity against *E. coli* and *S. aureus* [31]. Patchouli oil is known to form a capping layer on the corona of AgNP-LCg obtained from the green synthesis process, protecting AgNPs from subsequent degenerative changes, thus resulting in increased antibacterial activity. AgNPs-LCg coated with fractionated patchouli oil into high fraction patchouli oil (HP) is subsequently called HP-AgNPs-LCg.

This study aims to evaluate the formulation of handwash preparations with AgNP-LCg and AgNP-LCg hybridized with patchouli oil. The handwash was evaluated for its antibacterial activity against Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*). Additionally, the handwash was subjected to several tests, including organoleptic, pH, viscosity, foam height, density, and irritation tests, to ensure that the handwash preparation meets the standards.

2. Materials and Methods

2.1. Sample Preparation

Leaves of the *Calotropis gigantea* plant were used in this study. This plant grows in the geothermal area of Ie Seu-um, Aceh Besar District, Aceh Province, Indonesia [32]. The sampling location was 5°32’48.9” N, 95°32’49.07” E at 97 m above sea level (Figure 1).

Samples of *C. gigantea* leaves were taken and washed under running water to remove dirt and impurities. This plant has white latex, which can easily be cleaned with water when fresh. After washing, the samples were cut into pieces to increase the surface area, speed up the drying process, and avoid the rotting of water trapped between the leaves. The chopped leaves were air-dried for a few days before the extraction process.

2.2. Sample Extraction

The extraction process of *C. gigantea* leaves was carried out by boiling 10 g of the leaf samples in 100 mL of distilled water for 20 minutes, followed by filtration using

Table 1. Formulation of the handwash [33].

Components	Concentration (%)				
	F0	F1	F2	F3	F4
AgNPs-LCg	-	20	30	-	-
HP-AgNPs-LCg	-	-	-	20	30
Glycerine	10	10	10	10	10
HPMC	0.3	0.3	0.3	0.3	0.3
CAPB	5	5	5	5	5
SLS	1	1	1	1	1
Sodium benzoate	0.2	0.2	0.2	0.2	0.2
Potassium hydroxide	1	1	1	1	1
Stearic acid	1	1	1	1	1
Lemon fragrance	0.2	0.2	0.2	0.2	0.2
Aquadest	100	100	100	100	100

Descriptions: F0: Basis Formula; F1: Formula 1; F2: Formula 2; F3: Formula 3; F4: Formula 4; AgNPs-LCg: Silver nanoparticles *C. gigantea* Leaves; HP-AgNPs-LCg: Silver nanoparticles *C. gigantea* leaves patchouli oil coating.

filter paper. The leaf extract of *C. gigantea* was labelled as LCg [30].

2.3. Green Synthesis of AgNPs-LCg

AgNPs-LCg was synthesized by mixing 90 mL of $AgNO_3$ (4 mM) with 10 mL of LCg. The reaction was incubated at room temperature (25 ± 1 °C) with constant stirring using a shaker for 48 hours. The *C. gigantea* leaf extract synthesized silver nanoparticles were labeled as AgNPs-LCg [30].

2.4. Green Synthesis of Hybrid HP-AgNPs-LCg

The green synthesis of silver nanoparticles using the hybrid method was performed by mixing 90 mL of $AgNO_3$ (4 mM) with 10 mL of LCg. The reaction was incubated at room temperature (25 ± 1 °C) with constant stirring using a shaker for 24 hours. After 24 hours, 2 mL of high-fraction patchouli oil was added, and continuous stirring was maintained for another 24 hours. The formed compound was labeled as HP-AgNPs-LCg [30].

2.5. Handwash Formulation

Handwash was formulated with the active substances AgNPs-LCg and HP-AgNPs-LCg along with additional substances of glycerine, HPMC (hydroxypropyl methylcellulose), CAPB (Cocamidopropyl betaine), SLS (sodium lauryl sulfate), sodium benzoate, potassium hydroxide, stearic acid, lemon fragrance, and distilled water. Handwash was prepared in 5 formulas where F0 was the base formula, which did not contain the active substances AgNPs-LCg or HP-AgNPs-LCg. The other four formulas contained the active substances at different concentrations, as listed in Table 1.

The handwash preparation was carried out by adding glycerin in a beaker, then gradually adding a potassium hydroxide solution into the beaker while heating at 50 °C under continuous stirring until a paste was formed. Stearic acid, HPMC, SLS, CAPB, sodium benzoate, and lemon fragrance were added to the paste under continuous stirring until a homogenous mixture was formed. Active ingredients, such as AgNPs-LCg and HP-AgNPs-LCg, were added according to the formula under continuous stirring until a homogenous mixture formed. Finally, distilled water was added to reach a volume of 50 mL. The handwash was then poured into a clean container.

2.6. Evaluation of the Handwash Formulation

2.6.1. Organoleptic Test

Organoleptic tests were carried out by observing the form, color, and odor of the handwash formulas containing AgNPs-LCg and HP-AgNP-LCg [34].

2.6.2. pH Measurement

pH Measurement was carried out using a pH meter at 25 ± 2 °C. The instrument was calibrated in a neutral solution with a pH of 7.0 and a *buffer* of pH 4.0, followed by rinsing the electrode with distilled water and drying. The calibrated electrode was put in the sample until a constant pH was shown [34]. pH measurements were carried out on days 0, 7, 14, 21, and 30.

2.6.3. Viscosity Measurement

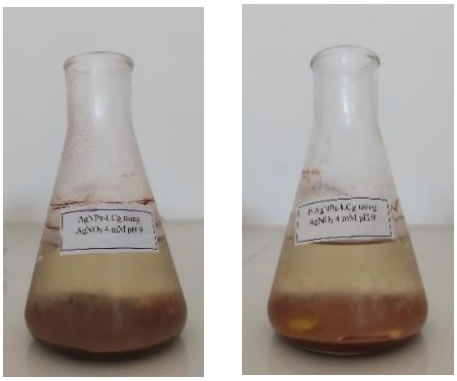
A viscosity test was performed using a Lamy Rheology viscometer type CP-4000 with spindles no. 20 and 40 at 300 rpm for 30 seconds. The results were recorded [35]. The measurements were carried out on days 0, 7, 14, 21, and 30.

2.6.4. Foam Height Measurement

A sample of 1 gram was dissolved in 10 mL of distilled water and poured into a measuring glass. The measuring glass was shaken regularly for 20 seconds, and the height of the foam formed was measured. After 5 minutes, the foam height was measured again [36]. The measurements were carried out on days 0, 7, 14, 21, and 30.

2.6.5. Density Measurement

A clean and dry pycnometer was calibrated by determining the weight of the empty pycnometer (a). Next, distilled water and handwash were added to the



(a) (b)

Figure 2. (a) AgNPs-LCg (b) HP-AgNPs-LCg.

pycnometer using a dropper pipette, and the pycnometer was closed. The weight of the pycnometer containing water (b) and the pycnometer containing the sample (c) were determined. The treatment was conducted at 25 ± 2 °C [34]. The results were calculated using Equation 1:

$$Density\ (g/mL) = \frac{c-a}{b-a} \quad (1)$$

where a represents the weight of the empty pycnometer, b is the weight of the pycnometer filled with distilled water, and c represents the weight of the pycnometer containing the sample.

2.6.6. Irritation Test

The inclusion criteria for participants for the irritation test include being physically and mentally healthy, over 18 years old, with no previous history of skin allergies, and not sick (fever) when the test took place. The formulation was applied to the inner forearm at the attachment site (2.5 × 2.5 cm), then left open and observed. The present test was performed twice daily (morning and evening) for three days. The participants completed a questionnaire based on the presence or absence of an irritation reaction. A positive irritation reaction was indicated by the presence of redness, itching, or swelling on the skin of the forearm being treated [37].

2.6.7. Antimicrobial Activity Test

The microbial isolates used as bioindicators in this research were *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. These isolates were collected from the Aceh Fundamental Sains Laboratorium, which has undergone rejuvenation and reidentification according to standard protocols [38].

The antimicrobial activity test was performed using the disk diffusion method (Kirby-Bauer). The turbidity of the microbial isolate suspensions was adjusted to the 0.5 McFarland standard, resulting in a final inoculum of 1.5 x 10^8 CFU/mL for the bacteria [38]. The bacterial suspension of *S. aureus* and *E. coli* were each inoculated onto Mueller Hinton Agar's (MHA) surface in a petri dish using a sterile swab. The agar was then allowed to absorb the microbial isolate suspension for 15 minutes at room temperature. The sterilized paper discs (6 mm in diameter) were first soaked for 15 minutes in each sample (positive control, F0, F1, F2, F3, and F4). The positive control used in the antibacterial test was the hand and body wash U-Hansa by ARC (Atsiri Research Center USK). In contrast, this study's negative control (F0) was the handwash formulation without adding AgNPs-LCg and HP-AgNPs-LCg. The paper discs were placed on Petri dishes inoculated with isolates and incubated at 37 °C for 24 hours. Vernier caliper measured the inhibition zone (in mm) around the discs to determine the diameter of the clear zone around the disc. The antimicrobial activity test was conducted in duplicate to confirm the diameter of the inhibition zone.

3. Results and Discussion

3.1. Sample Extraction

Sample extraction was carried out using water as the solvent. Water is a commonly used solvent in the green synthesis of AgNPs-LCg [30, 39, 40]. It is inexpensive and non-toxic, making it safer for applications related to health and the environment.

3.2. Green Synthesis AgNPs-LCg and HP-AgNPs-LCg

The silver ions (Ag^+) in the $AgNO_3$ solution were reduced to silver (Ag^0) by the plant extract, indicated by a color change to brown [30, 39, 40]. Evaluating this color change is a basic indicator of the success of the green synthesis of AgNPs-LCg. The synthesis of AgNP-LCg in this research phase showed the expected color change (Figure 2a). The hybrid reaction of AgNPs-LCg using patchouli oil heavy fraction also resulted in a brown color but with some oily texture (Figure 2b).

3.3. Evaluation of Handwash

Stability testing involves long-term (real-time) and accelerated testing conducted on batches according to the established stability testing protocol or to confirm re-testing periods of a substance or the shelf life of a finished product. For long-term (real-time) storage conditions, stability testing is generally conducted every three months during the first year, every six months during the second year, and once a year after that until the established shelf life [41]. In this study, the real-time stability testing was performed for a shortened 1 month, with tests conducted on days 0, 7, 14, 21, and 30.

Table 2. Results of the organoleptic test.

		Color	Odor
Day 0	F0	Clear	Lemon
	F1	Orange-brown	Lemon with a distinctive aroma
	F2	Orange-brown	Lemon with a distinctive aroma
	F3	Greenish brown	Lemon with a distinctive aroma
	F4	Dark greenish brown	Lemon with a distinctive aroma
Day 30	F0	Clear	Lemon
	F1	Orange-brown	Lemon with a distinctive aroma
	F2	Reddish brown	Lemon with a distinctive aroma
	F3	Greenish brown	Lemon with a distinctive aroma
	F4	Dark brown	Lemon with a distinctive aroma

Descriptions: F0: Basic Formula; F1: Formula containing 20% AgNPs-LCg; F2: Formula containing 30% AgNPs-LCg; F3: Formula containing 20% HP-AgNPs-LCg; F4: Formula containing 30% HP-AgNPs-LCg.

Table 3. Results of the pH measurement.

Formula	Day 0	Day 7	Day 14	Day 21	Day 30
F0	10	10	9.9	9.9	10.1
F1	10.1	10	10.1	10.2	10
F2	9.9	10	10.2	10.3	10.1
F3	10	9.9	9.9	9.9	9.7
F4	9.9	9.9	9.9	9.9	9.9

Descriptions: F0: Basic Formula; F1: Formula containing 20% AgNPs-LCg; F2: Formula containing 30% AgNPs-LCg; F3: Formula containing 20% HP-AgNPs-LCg; F4: Formula containing 30% HP-AgNPs-LCg.

Table 4. Results of the viscosity measurement.

Formula	Viscosity (cPs)				
	Day 0	Day 7	Day 14	Day 21	Day 30
F0	660.4	520.2	485.9	299.2	615.4
F1	907.3	918	979.3	901.6	548.3
F2	756	574.6	626.3	859.1	493.8
F3	855.5	704	577.5	568.2	405
F4	873.3	801.2	728.4	606.9	498.1

Descriptions: F0 : Basis Formula; F1: Formula containing 20% AgNPs-LCg; F2: Formula containing 30% AgNPs-LCg; F3: Formula containing 20% HP-AgNPs-LCg; F4: Formula containing 30% HP-AgNPs-LCg.

3.2.1. Organoleptic Test

Based on Table 2, the color of the handwash formulation did not significantly change from day 0 to day 30 (Figure 3). Slight color variations were noted between each formulation, likely due to the different concentrations of AgNPs-LCg and HP-AgNPs-LCg in each formula. Overall, the formulations were dark due to the active ingredients, AgNPs-LCg and HP-AgNPs-LCg, which are brownish.

The aroma in each formulation containing AgNPs-LCg and HP-AgNPs-LCg remained unchanged during storage, with a lemon scent due to the addition of lemon fragrance along with the distinctive aroma of the *C. gigantea* leaf extract, which originates from the AgNPs-LCg and HP-AgNPs-LCg synthesized from *C. gigantea* leaf aqueous extract. The handwash formulation was a homogeneous liquid soap with a characteristic color and distinctive scent, which were by the standards for a homogenous liquid [34].

3.2.2. pH Measurement

The pH value significantly influences the formulation's absorption into the skin [34]. A pH value that is too low can irritate the skin, while a pH value that is too high can affect the skin's moisture levels. Therefore, maintaining a balanced pH is essential and must meet the established standards for a good formulation.

Based on the pH test results in Table 3, the handwash tends to be alkaline, ranging from 9.7 to 10.3. Some soaps with a similar pH are those synthesized with cayenne pepper leaves [24] and red betel leaves [25]. This pH range meets the requirements of the Indonesian National Standard (SNI) for a good formulation, which is between pH 8 and 11 [34].

3.2.3 Viscosity Measurement

Based on Table 4, the viscosity of the handwash formulation ranged from 405 to 907.3 cPs. During storage, the viscosity of each formulation fluctuated. A decrease in viscosity may occur due to glycerin, which is hygroscopic [42]. A higher concentration of glycerin resulted in lower viscosity, and a lower concentration resulted in higher viscosity [43]. Therefore, a decrease in viscosity may occur due to glycerin's ability to attract and retain water in the formulation, making the handwash thinner and lower viscosity.

Another factor that may contribute to fluctuations in viscosity is the temperature during storage. Higher temperatures decrease viscosity, making the sample more liquid, which indicates an inverse relationship between temperature and viscosity [44]. The handwash formulation is stored in different rooms each week, and the temperature may vary, affecting the viscosity. Although the viscosity values vary, they still meet the requirements of liquid soap viscosity according to SNI, which is 400–4000 cPs [34]. However, in formulation F0 on day 21, the lowest viscosity reading was 299.2 cPs, which does not fall within the acceptable viscosity range according to SNI.

3.2.4. Foam Height Measurement

Each formula's foam height is measured by shaking, and the foam height is measured at 0 and 5 minutes. Based

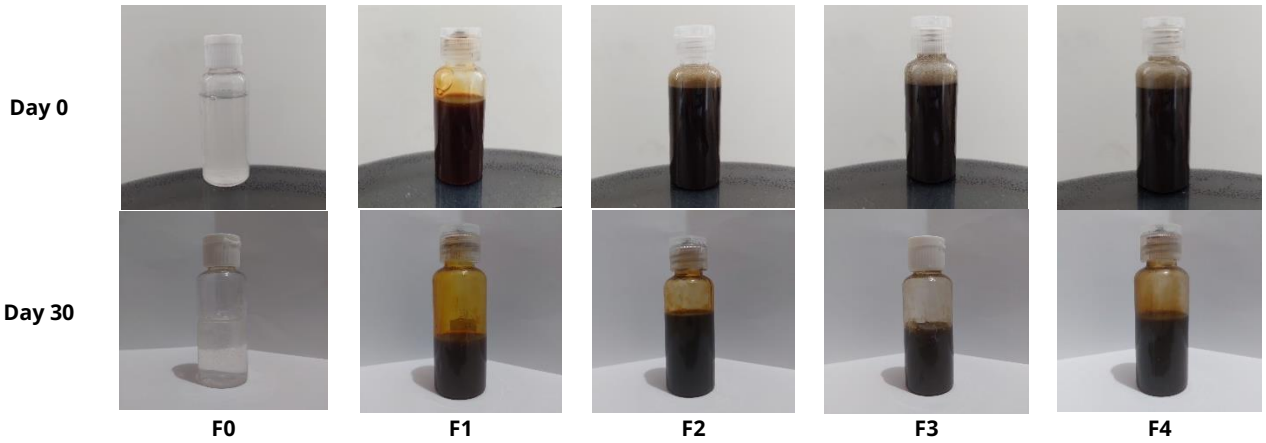


Figure 3. Organoleptic test of handwash F0: Basic Formula; F1: Formula containing 20% AgNPs-LCg; F2: Formula containing 30% AgNPs-LCg; F3: Formula containing 20% HP-AgNPs-LCg; F4: Formula containing 30% HP-AgNPs-LCg.

Table 5. Results of the foam height measurement.

	Foam Height (cm)									
	F0		F1		F2		F3		F4	
Time	**0**	**5**	**0**	**5**	**0**	**5**	**0**	**5**	**0**	**5**
Day 0	6	5.3	7.4	5.9	8.4	7.5	9.2	7.5	8.8	7
Day 7	9.5	8.5	9.6	8.5	7.3	7	8.2	7.5	8.9	7.5
Day 14	8.5	7	8.5	7	9	7.5	9.2	7.5	9	7.5
Day 21	6	5.5	6	5.3	8.5	8	7.6	7	7.2	6.5
Day 30	6	5.6	6	5.6	6.3	5.5	7.4	6.6	6.5	5.8

Descriptions: F0: Basic Formula; F1: Formula containing 20% AgNPs-LCg; F2: Formula containing 30% AgNPs-LCg; F3: Formula containing 20% HP-AgNPs-LCg; F4: Formula containing 30% HP-AgNPs-LCg; Time: in minute.

Table 6. Results of the density measurement.

Formula	Density (g/mL)
F0	1.03
F1	1.04
F2	1.06
F3	1.04
F4	1.05

Descriptions: F0: Basic Formula; F1: Formula containing 20% AgNPs-LCg; F2: Formula containing 30% AgNPs-LCg; F3: Formula containing 20% HP-AgNPs-LCg; F4: Formula containing 30% HP-AgNPs-LCg.

on Table 5, the foam height of the handwash formulation ranged from 5.3-9.5 cm, which meets the SNI requirements with a range of 1.3-22 cm [34].

The foam formation in the handwash formulation results from the combination of surfactants, such as SLS and CAPB. SLS (sodium lauryl sulfate) is an anionic surfactant commonly used in nonparental pharmaceutical formulations and cosmetics [42]. CAPB (Cocamidopropyl betaine) is used in the cosmetic industry to produce products that are good for skin safety [45]. Using CAPB as a co-surfactant enhances foam production, making it more stable. This effect occurs because CAPB is compatible with other surfactants, such as SLS. CAPB is an amphoteric surfactant with both an anionic (negative charge) and a cationic (positive charge) end. When combined with SLS (which also has an anionic group), these surfactants work synergistically to produce abundant and stable foam [35]. Hence, the combination of SLS and CAPB ensures the foam height of the handwash formulation meets the required standards.

3.2.5 Density Measurement

Based on the results in Table 6, formulations F2 and F4 demonstrated the highest specific gravity at 1.06 g/mL and 1.05 g/mL, respectively, followed by F1 and F3 at 1.04 g/mL and, lastly, formulation F0 at 1.03 g/mL [34].

Formulations F2 and F4, which each contain 30% of AgNPs-LCg and HP-AgNPs-LCg from the total formulation, have the highest specific gravity value. Following these, formulations F1 and F3 contain 20% of AgNPs-LCg and HP-AgNPs-LCg, and finally, formulation F0 contains neither AgNPs-LCg nor HP-AgNPs-LCg. All handwash formulations meet the specific gravity requirements of a good liquid soap according to SNI, which falls within the range of 1.01 – 1.10 g/mL [34].

3.2.6. Irritation Test

Irritation is a local response on the skin caused by a reaction after exposure to a chemical substance, leading to inflammation or injury. This study evaluated the types of irritation erythema and edema. Erythema is a skin inflammation that manifests as redness caused by the dilation of capillaries due to chemical toxins or sunburn. Conversely, edema is the excessive accumulation of

Table 7. Results of the irritation test.

Participants	Erythema	Edema
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	-	-
Total	0	0

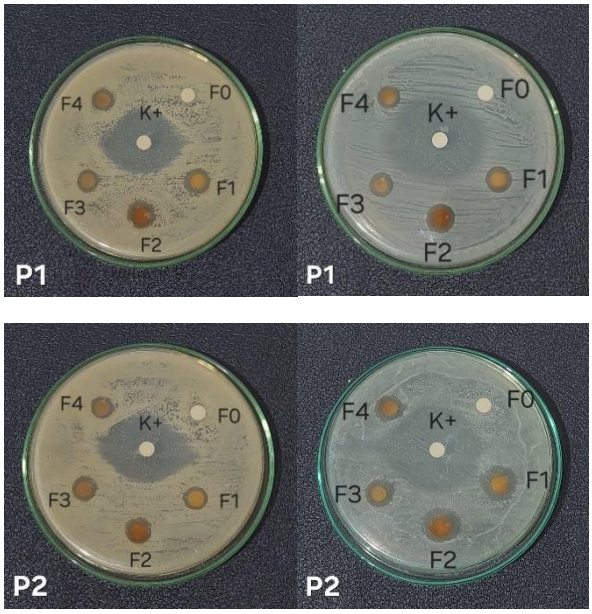
Table 8. Inhibition zone category [46].

Inhibition Zone	Category
< 5 mm	Weak
5-10 mm	Moderate
> 10-20 mm	Strong
> 20-30 mm	Very strong

Table 9. Results of the antimicrobial activity test.

Microbes	Formula	Inhibition zone (mm)		
		P1	P2	Mean ± SD
S. aureus ATCC 25923	K+	35.55	37.67	36.61 ± 1.49
	F0	6.78	7.02	6.9 ± 0.16
	F1	9.98	13.89	11.93 ± 2.76
	F2	10.88	14.92	12,9 ± 2.85
	F3	8.81	10.89	9.85 ± 1.47
	F4	9.09	11.64	10.36 ± 1.80
E. coli ATCC 25922	K+	30.52	32.70	31.61 ± 1.54
	F0	6.44	6.20	6.32 ± 0.16
	F1	10.56	9.37	9.96 ± 0.84
	F2	11.72	10.19	10.95 ± 08
	F3	8.90	8.89	8.89 ± 0
	F4	9.31	9.18	9.24 ± 0.09

Descriptions: K+: Positive control; F0: Basic Formula; F1: Formula containing 20% AgNPs-LCg; F2: Formula containing 30% AgNPs-LCg; F3: Formula containing 20% HP-AgNPs-LCg; F4: Formula containing 30% HP-AgNPs-LCg; P1: Repetition 1; P2: Repetition 2.



(a) (b)

Figure 4. Antimicrobial test against (a) *E. coli* (b) *S. aureus.*

serous fluid or water within cells, tissues, or serous cavities.

The irritation test results showed no irritation symptoms or reactions (Table 7). This was evaluated based on erythema (redness) and edema (swelling). Therefore, the handwash formulation produced in this study is relatively safe to use and does not irritate.

3.2.7. Antimicrobial Activity Test

The antimicrobial activity testing of the handwash formulation was conducted using the Kirby-Bauer disk diffusion method. The purpose of the antibacterial activity test was to assess the ability of the handwash formulation to inhibit *S. aureus* and *E. coli* by measuring the diameter of the clear zones. The strength level category of the sample inhibition zone falls into one of the following categories. If the inhibition zone was < 5 mm, it was considered in the weak category, 5-10 mm was in the medium category, > 10-20 mm was in the strong category, and >20-30 mm was in the very strong category (Table 8). The results of the antibacterial activity evaluation are presented in Table 9 and Figure 4.

Based on Table 9, it was found that F1 exhibited a strong inhibitory zone against *S. aureus* with a value of 11.93 ± 2.76 mm and a moderate inhibitory zone against *E. coli* with a value of 9.96 ± 0.84 mm. F2 showed a strong inhibitory zone against both bacteria, with *S. aureus* (12.9 ± 2.85 mm) and *E. coli* (10.95 ± 0.8 mm). F3 demonstrated a moderate inhibitory zone against *S. aureus* (9.85 ± 1.47 mm) and *E. coli* (8.89 ± 0 mm). Lastly, F4 exhibited a strong inhibitory zone against *S. aureus* (10.36 ± 1.80 mm) and a moderate inhibitory zone against *E. coli* (9.24 ± 0.09 mm). These inhibitory zone values are below the positive control, which showed a very strong inhibitory zone against both *S. aureus* and *E. coli*, with values of 36.61 ± 1.49 mm and 31.61 ± 1.54 mm, respectively.

Based on Table 9, it was found that F1 exhibited a strong inhibitory zone against *S. aureus* with a value of 11.93 ± 2.76 mm and a moderate inhibitory zone against *E. coli* with a value of 9.96 ± 0.84 mm. F2 showed a strong inhibitory zone against both bacteria, with *S. aureus* (12.9 ± 2.85 mm) and *E. coli* (10.95 ± 0.8 mm). F3 demonstrated a moderate inhibitory zone against *S. aureus* (9.85 ± 1.47 mm) and *E. coli* (8.89 ± 0 mm). Lastly, F4 exhibited a strong inhibitory zone against *S. aureus* (10.36 ± 1.80 mm) and a moderate inhibitory zone against *E. coli* (9.24 ± 0.09 mm). These inhibitory zone values were below the positive control, which showed a very strong inhibitory zone against both *S. aureus* and *E. coli*, with values of 36.61 ± 1.49 mm and 31.61 ± 1.54 mm, respectively.

However, the antibacterial activity of the handwash samples in this study was still above F0, which showed inhibitory zones against both *S. aureus* and *E. coli*. The inhibitory zone measurements confirmed that AgNPs-LCg and HP-AgNPs-LCg added to the handwash formulation showed a synergistic reaction with other components, resulting in strong antibacterial activity. Other research formulations of handwash containing 40% kalimansi orange juice (*Citrofortunella microcarpa*) showed a very strong inhibitory zone against *S. aureus* and *E. coli*, with inhibitory zone diameters of 30.595 and 28.98 mm, respectively [47]. The handwash with 7.5% moringa leaf extract showed a strong inhibitory zone against *S. aureus* and *E. coli*, with inhibitory zone diameters of 15.46 and 14.5 mm, respectively [36].

The use of sodium benzoate in the formulation of handwash prolongs the product's shelf life and prevents microbial growth. Glycerin also possesses antimicrobial properties. When added to the formulation in a concentration of 10%, glycerin acts as a humectant and antimicrobial agent. Glycerin at concentrations below 20% showed antimicrobial properties. Additionally, SLS as a surfactant also showed antimicrobial properties [48]. Thus, sodium benzoate, glycerin, and SLS in the formulation of F0 handwash inhibits bacterial growth, making it effective against *S. aureus* and *E. coli*.

AgNPs (silver nanoparticles) inhibit bacterial growth through various mechanisms [49]. The continuous release of silver ions is a potent microbial killing mechanism [50]. These silver ions can bind to the bacterial cell wall and cytoplasmic membrane due to electrostatic attraction and affinity for sulfur-containing proteins. Once attached, silver ions increase the permeability of the cytoplasmic membrane, leading to disruptions in the bacterial envelope [51]. Respiratory enzymes can also be inactivated when silver ions enter the cell, producing reactive oxygen species (ROS). There is no adenosine triphosphate (ATP) synthesis during this process [52]. ROS can trigger membrane rupture and changes in DNA. Since sulfur and phosphorus are key components of DNA, the interaction of silver ions with these elements can result in issues with DNA replication, cell reproduction, and even the death of the microorganism. Furthermore, silver ions can inhibit protein production by denaturing ribosomes in the cytoplasm [53].

The handwash formulated in this study was more effective against *S. aureus* than *E. coli*. This difference in efficacy is due to the structural differences in the bacterial cell walls. *S. aureus* is a Gram-positive bacterium with a single plasma membrane surrounded by a peptidoglycan wall. This means Gram-positive bacteria have a peptidoglycan layer that is more susceptible to active agents from extracts, resulting in a stronger inhibitory effect than Gram-negative bacteria [54]. On the other hand, *E. coli* is a Gram-negative bacterium with a double membrane system. Its inner plasma membrane is surrounded by an outer membrane that is more permeable. The thick peptidoglycan wall is located between these membranes. The outer membrane of *E. coli* offers protection against antibiotics [55]. Therefore, *S. aureus* is more easily destroyed by antimicrobial agents than *E. coli*, leading to a larger inhibitory zone against *S. aureus* than *E. coli*.

Further research is needed to determine why handwash with HP-AgNPs-LCg has a smaller inhibitory zone than handwash with AgNPs-LCg. While AgNPs-LCg has a lower inhibitory zone than HP-AgNPs-LCg [31], this discrepancy requires further investigation. Additionally, evaluating the synergistic effect of HP-AgNPs-LCg as a base for handwash formulation warrants further examination.

4. Conclusions

The evaluation showed that the handwash formulated using AgNPs-LCg and HP-AgNPs-LCg meets the established parameters. This success has positive implications for daily life because the potential of silver nanoparticles as antibacterial agents can be realized in a practical handwash that can be carried and used anytime and anywhere. Silver nanoparticle handwash proved to be nonirritant to users, but further testing, such as in vivo and in vitro release studies, is needed to determine the combined effects of several physicochemical characteristics, particle or droplet size, viscosity, microstructure arrangement, and aggregation state of the silver nanoparticle liquid soap formulation. The best formula for handwashing with silver nanoparticles in inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria was F2, with inhibition zones of 12.9 ± 2.85 mm and 10.95 ± 0.8 mm, respectively. This study provides a foundation for further research and development of nanoparticle-based antimicrobial products.

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