STABILITY AND ANTIBACTERIAL ACTIVITY TEST OF NANOSILVER BIOSYNTHETIC HYDROGEL

DIAN EKA ERMAWATI1, ADI YUGATAMA2, BERLIANA RIZKA RAMADHANI3, INTAN PERTIWI1, ANISWATUN ROSIKHOH1, SAFIRA RAHMA NOVACHIRIA2

1Department of Pharmacy, Pharmacy Diploma Study Program, Vocational School, Sebelas Maret University, Surakarta, Indonesia,
2Department of Pharmacy, Bachelor of Pharmacy Study Program, Faculty of Mathematics and Natural Sciences, Sebelas Maret University, Surakarta, Indonesia

Email: dianeekaerma@gmail.com

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ABSTRACT

Objective: This study aims to formulate nanosilver (AgNPs) biosynthetic hydrogel for topical antibacterial treatment and its stability and antibacterial activity.

Methods: The mixture of silver nitrate solution and Turmeric juice (Zingiber officinale) was stirred at room temperature for 24 h; afterward, it was analyzed using UV-VIS spectrophotometry, particle size analysis, and TEM. The carbopol 940 was selected as a gelling agent with an AgNPs concentration of 5%, 10%, 20%, and 30%. Furthermore, the gel preparation was tested for stability using the cycling test method and antibacterial activity. The antibacterial activity against Staphylococcus aureus, Staphylococcus aureus, and Escherichia coli, and Pseudomonas aeruginosa.

Results: The nanosilver biosynthetic has a yellow-brown color with the maximum wavelength peak at 433 nm, and a particle size of 157.4-166.7 nm. TEM analysis showed that AgNPs have a round shape, while the antibacterial activity of hydrogel preparations was moderately inhibited. Furthermore, the hydrogel was evaluated for pH, viscosity, dispersibility, and antibacterial activity before and after the cycling test. Formula with 30% AgNPs is chosen formula with pH value of 5.87±0.65; viscosity of 4833.3±2.82 c.PS; and dispersibility of 5.50±0.15 after cycling test.

Conclusion: The high concentration of AgNPs will increase the viscosity, pH, and dispersibility. Formula with 30% AgNPs have the highest antibacterial activity. Furthermore, all hydrogel preparations meet the requirements of Indonesian Standard Product (SNI) No. 06-2588-1992 for good gel stability before and after the cycling test.

Keywords: Antibacterial, Cycling test, Turmeric juice, Nanosilver, Hydrogel

INTRODUCTION

Nanosilver (AgNPs) is a potential antibacterial and also an alternative of topical antibiotics that have caused bacterial resistance to antibiotics. Furthermore, a previous study was conducted to determine the characterization of biosynthetic silver using turmeric juice, and it was discovered that the selected formula meets the requirements. It was a formula composed of turmeric juice and silver nitrate with a ratio of 5:38 ml. Silver is a metal that has recently attracted attention due to its broad antibacterial activity. In addition, the study on silver was developed. Silver ions are formulated to become silver nanoparticles (AgNPs) to improve antibacterial activity and decrease toxicity [1]. In this study, silver nitrate (AgNO3) which is conducted by green synthesis method using plant extracts, induce antibacterial effects [2]. Also, this method is advantageous because it is safe, simple, reduce waste, and is environmentally friendly.

Active ingredients of plant extracts such as terpenoids, phenolics, flavonoids, and other components with carboxylic acid, amide, and aldehyde functional groups are used as bioreductor agents which are selected based on the presence of metabolite compounds that converts silver ion (Ag+) to Ag0 (silver) [3]. Turmeric (Curcuma longa L.) which is one of the plants used as AgNPs biosynthetic agent. Turmeric was selected because it contains aldehyde (CHO) groups in flavonoid compounds for reducing silver ions [4]. Based on previous studies on AgNPs biosynthetic using powder infusion of turmeric rhizome as bioreductor. The profile of the inhibition zone produced by the nanosilver of turmeric infection against Staphylococcus aureus bacteria is 14 mm (for 1.0 mmol AgNO3 concentration). Also, the particle size of the AgNPs produced through the biosynthesis of turmeric infusion is between 20-50 nm and has a round shape [5], while the particle size of the AgNPs produced through the biosynthesis of dry turmeric rhizome is between 5-35 nm with a particle size of 10±0.56 nm [6]. In this study, turmeric juice is used as bioreductor to form AgNPs.

The use of antibiotics topically leads to increased bacterial resistance and limits the potential effectiveness of antimicrobial agents [7]. Resistance is not inhibition of bacterial growth by administration of therapeutical doses of antibiotics or with minimal inhibitory levels. Generally, resistance often occurs in Streptococcus pneumonia, Staphylococcus aureus, and Escherichia coli bacteria [8]. According to a study by Gao [9], nanosilver has lower toxicity than silver ions because of its nano size. Furthermore, the toxicity of nanosilver to bacteria increases due to a large specific surface area [10]. Nanosilver is more toxic to bacteria and less toxic to human tissues. Also, its advantages as an antimicrobial treatment include safety, long life of the preparation, reduce waste, practical, and provides a sense of comfort for long-term use [11].

The nanosilver biosynthetic uses turmeric juice as bioreductor agent, then dispersed into a hydrogel preparation to facilitate its application on skin. Its formulates with types of gelling agents, such as methylcellulose, carboxymethylcellulose (CMC), hydroxypropyl methylcellulose (HPMC), and carbopol 940. In this study carbopol 940 was used as a gelling agent because it is easy dispersed in water at low concentration range of 0.05-2.00%, and has sufficient viscosity as a hydrogel base [13]. Furthermore, it does not cause primary irritation, sensitivity, or allergic reaction. The characterization of silver nanoparticles was carried out using Particle Size Analyzer (PSA) and UV-Vis spectrophotometer, whereby the hydrogel preparations were tested for physicochemical properties and antibacterial activity before and after the stability test. The physicochemical test includes the organoleptic, viscosity, pH test, and dispersibility, while the antibacterial activity test was performed on Staphylococcus epidermidis ATCC 25923, Staphylococcus aureus ATCC 12228, Escherichia coli ATCC 27853, and Pseudomonas aeruginosa ATCC 25922.

MATERIALS AND METHODS

The materials that were used include Turmeric rhizome from Pasar Gede, Surakarta, Central Java, Indonesia that was identified with
number of 014835/S.Tb./II/2020 in Taxonomy of Plant Laboratory, Biology Faculty, Universitas Gadjah Mada, Yogyakarta, Indonesia, AgNO₃ (Merck), Carbopol 940, TEA, Glycerin (Repacking by PT Bratachel, Indonesia), (bacteria from Microbiology Laboratory of Medical Faculty, UNS, Surakarta: American Type Culture Collection (ATCC) 25923, ATCC 12228, ATCC 27853; ATCC 25922; aqueast; aluminum foil; Whatman paper No. 1. Instruments: Spectrophotometer UV-VIS (Genesys™), Fourier Transform-IR (Shimadzu), Transmission Electron Microscope (JEOL/EO 1400), Sentrifugator (mini Spin Plus), Scanning Electron Microscope (FEI Quanta 200), oven (Memmert), incubator (Thermo Scientific Series 8000 WJ), digital calipers (Krisbow), thermometer, hot plate (IKC MAG), pH meter, magnetic stirrer (Labtech ST 6), analytic neraca (Precisa), and Viscometer (Viskotester VT-04, Kokubunji: Japan).

Sample preparation
Silver nitrate (AgNO₃) solution of 1.0 mmol was prepared by weighing 85 mg of AgNO₃ powder, which is then dissolved with aqua dest at a temperature of 40 °C in a 500 ml volumetric flask. Turmeric rhizome was washed with water, after which it was aerated, blended, and filtered using Whatman paper No.1. Subsequently, the turmeric juice is centrifuged at 10,000 rpm for 10 min at room temperature, and the clear yellow liquid (top) is separated and collected in a falcon disk [14].

The turmeric juice was mixed with 1.0 mmol AgNO₃ solution, and the comparison of volume ratio between the turmeric juice and AgNO₃ solution is 5:38 ml. The biosynthesis process was performed at room temperature and protected from light, while the mixture was stirred for 24 h using a magnetic stirrer at room temperature; afterward, the color change was observed [6].

Characteristic of nanosilver
To determine the success of the biosynthesis process, the absorbance result of mixture ratios was measured using spectrophotometer UV-VIS with a wavelength range of 300-700 nm. Furthermore, to determine the size and distribution of nanosilver particles, analysis was performed using PSA; the nanosilver colloid of 1.0 ml was analysed using a particle size analyzer. The solution resulting from biosynthesis was analyzed for the size and shape of the particle produced using a TEM.

Hydrogel formulation
Carbopol 940, which acts as a gelling agent, is dissolved with water at a temperature of 70 °C, after which it was stirred until homogeneous, the AgNO₃ was added as a new gelating agent to make a gel. Also, glycerin was added as humectants and nanosilver biosynthetic. Lastly, phenoxethanol was added as a preservative, and the mixtures were stirred until homogeneous. The hydrogel formula with a variation of nanosilver biosynthetic is seen in table 1.

Table 1: The hydrogel formula with variation of nanosilver biosynthetic concentration

| Material          | Composition (grams) | Base formula | F1   | F2   | F3   | F4   |
|-------------------|---------------------|--------------|------|------|------|------|
| Nanosilver        | 0.0                 | 5.0          | 10.0 | 20.0 | 30.0 |
| Carbopol 940      | 0.5                 | 0.5          | 0.5  | 0.5  | 0.5  |
| Triethanolamine   | 1.0                 | 1.0          | 1.0  | 1.0  | 1.0  |
| Glycerin          | 1.00                | 1.00         | 1.00 | 1.00 | 1.00 |
| Phenoxethanol     | 0.5                 | 0.5          | 0.5  | 0.5  | 0.5  |
| Aquadest          | 8.80                | 8.30         | 7.80 | 6.80 | 5.80 |

(F1, F2, F3 and F4 represent the variation of nanosilver biosynthetic concentration)

Antibacterial activity test
Bacteria were cultured in media and then placed in a well with a diameter of 10 mm. Furthermore, the total nanosilver solution and hydrogel preparation of 50 ml was poured into the well. The agar medium was then incubated for 24 h at 37 °C, and the clear zone was exhibited, which indicated the ability of nanosilver biosynthetic to inhibit bacteria. Also, tests were performed on nanosilver solutions, hydrogel preparations, AgNO₃ solutions and water, while the bacteria used were Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa. The bacteria from Microbiology Laboratorium of Medical Faculty, UNS, Surakarta, Indonesia there are: ATCC 25923, ATCC 12228, ATCC 27853; ATCC 25922, respectively.

Stability test by cycling test method
The stability test was performed by storing the biosynthetic agent of the nanosilver using a hydrogel preparation of turmeric juice at a temperature of 4 °C and 40 °C for six cycles, respectively with 24 h for each cycle. Furthermore, the stability tests using the cycling method were conducted to determine the rapid changes that generally occur under normal conditions. The results of observations of gel preparations are conducted before and after the cycling test for six cycles. Gel preparations were performed in a one-cycle process, in which they are stored at a cold temperature of 4 °C for 24 h and then placed at a temperature of 40 °C. All these series of processes are counted as one cycle.

Viscosity was measured using a Rikon viscometer with spindle number-1, whereby the cup was filled with the preparations of the hydrogel. The rotor is placed in the center of the cup. After turning ON the tool, wait for 1 min and record the viscosity value. Meanwhile, the viscosity value that meets the requirement of gel preparations is 500-20,000 cPs [15]. Also, the pH meter was calibrated using buffers standard with pH values of 4.01 and pH 6.86, and the electrode was inserted into the hydrogel preparation and then stirred until a constant pH value is reached. After which, the pH value was observed and recorded. The pH value that meets the requirement of gel formulations was no significant difference in the color change after stirring for 24 h at room temperature. The success of the nanosilver formation is
visually proved by a color change from clear yellow to brown. This is due to the oxidation and reduction process of an organic compound, whereby the ion reduces to a nanosilver (Ag°). The dark brownish color produced showed that more organic compounds are oxidized and more silver ions are reduced to nanosilver (Ag°), thereby increasing the concentration of nanosilver formed. In addition, stirring during the reaction process accelerates the reaction between the AgNO₃ solution and the compound that reduces silver ion, thereby increasing the concentration of nanosilver formed [19]. In this study, the color change from the biosynthesis process after 24 h was not significantly different because the initial color of the biosynthesis was brown. Nanosilver using turmeric juice as a bioreducer in this study had a clear yellowish initial color and brownish at final color (fig. 1).

![Fig. 1: The comparison samples color, the color change of nanosilver biosynthetic (b), silver nitrate solution (c) and sweet orange infusa (a); and the maximum wavelength of nanosilver biosynthetic at SPR range of 400-500 nm](image)

Nanosilver biosynthesis results were analyzed using spectrophotometry UV-Vis to determine the spectra and absorption peak that shows the success of nanosilver form with turmeric juice as a bioreducer. Furthermore, Prathna [14] stated that the success of nanosilver formation is known from the maximum absorbance in the range of 400-500 nm. The maximum absorbance range is caused by the Surface Plasmon Resonance (SPR) between light waves and electrons on a metal surface such as silver that is detected at a specific wavelength. Also, Shameli [4] stated that the formation of nanosilver has a spherical shape which is characterized by absorption spectra in the range of 400-420 nm. The spectra produced from the nanosilver with turmeric juice as a bioreducer, and volume of turmeric juice-AgNO₃ solution with a ratio of 5:38 ml had absorption peaks at 433 nm. In addition, the nanosilver spectra showed the successful formation of nanosilver, which was characterized by peaks in the wavelength range of 400-500 nm. The peak absorption around 350-385 nm is the absorption range of flavonoid in turmeric [20], and an increased absorption value shows a high proportion of the nanosilver formed [19].

TEM analysis was performed to determine the surface morphology and particle size of the sample. The sample results in fig. 2, showed that AgNPs using turmeric juice as bioreducer with a ratio of 5:38 ml has an average particle size of 17.97 nm with a spherical shape without any aggregation at a magnification of 250,000 times. In this study, all particles are evenly distributed in the mixed matrix of turmeric juice and AgNO₃, and an average particle size of AgNPs using turmeric juice produced a smaller size when compared with AgNPs using turmeric infusion by Kurian [5] and AgNPs using water extract of dry rhizome by Alsammarraie [6]. The particle size in this study is a little bit bigger than AgNPs using water extract of dry rhizomes by Shameli [4]. Therefore, it was concluded that biosynthesis using turmeric juice produces smaller particle sizes when compared to turmeric infusion or water extract of dry turmeric rhizome.

![Fig. 2: Reaction mechanism of silver ion reduction by flavonoid compounds](image)
Nanoparticles are defined as particles with a size between 1-100 nm. Furthermore, the diameter of a particle of nanosilver biosynthetic with turmeric juice in this study was characterized using PSA and replicated three times. The result showed a diameter ranging from 157.4-166.7 nm. According to research by Campbell [21, 36], nanosilver with a particle size of 20-200 nm does not penetrate the lower layer of the epidermis; therefore, it is safe for topical pharmaceutical and cosmetics preparations. Based on this, the range of biosynthetic yields of nanosilver in this study was confirmed with a particle size of 157.4-166.7 nm in the nanoparticle range, which is safe as a topical preparation. According to Nidhin [22], particles that have a Polydispersants Index value (PDI) in the range of 0.1 to 0.5-0.7 are categorized as monodisperse (particle size homogeneously dispersed), when the value of the Polydispersants Index (PDI) is more than that of 0.7 including polydisperse (particle size is not homogeneously dispersed). The Polydispersants Index (PDI) value obtained in this study was based on the data of 3 times replication of the PSA test of 0.369-0.436. Based on these data, the value of the Polydispersants Index (PDI) is less than 0.5. Therefore, the particles are into the monodisperse category (homogeneous dispersed).

Antibacterial activity of nanosilver biosynthetic

The antibacterial test of nanosilver biosynthetic with a ratio of 5:38 ml was performed using the good diffusion method on 1.0 mmol AgNO3 solution. Furthermore, the positive controls include Vancomycin® for gram-positive and Chloramphenicol® for gram-negative bacteria. Meanwhile, water is a negative control. The results showed that the inhibition zone with diameter 20 mm has a very strong category; likewise, the inhibition zone of 10-20 mm has a strong category, the inhibition zone with a diameter of 5-10 mm has moderate, while the inhibition zone with a diameter of 5-10 mm has a weak category. The measurement of the diameter of the inhibition zone was performed after the incubation process for 24 h at 37 °C and using a caliper.

The results of the antibacterial test showed that the nanosilver biosynthetic using turmeric juice had an inhibitory effect on gram-positive and gram-negative bacteria with a strong category. Furthermore, the tests in both groups of bacteria showed that there was an antibacterial activity of nanosilver biosynthetic, whereby nanosilver colloidal inhibits gram-positive and gram-negative bacteria [23]. The purpose of hydrogel formulation is to comfort the use of nanosilver biosynthetic and provide a good therapeutic effect, and this was tested for antibacterial efficacy after the cycling test to determine the antibacterial activity before and after the stability test. Therefore, the results of the inhibition of the nanosilver biosynthetic hydrogel are presented in table 2.

Table 2: The results of the diameter of inhibition zone of nanosilver biosynthetic hydrogel compared with nanosilver biosynthetic solution

| Samples          | S. aureus Before | S. aureus After | S. epidermidis Before | S. epidermidis After | E. coli Before | E. coli After | P. aeruginosa Before | P. aeruginosa After |
|------------------|-----------------|----------------|-----------------------|---------------------|---------------|---------------|----------------------|---------------------|
| AgNO3 solution   | 15.00±0.10      | 17.80±0.15     | 18.00±0.70            | 20.00±0.80          | 18.00±0.30    | 18.00±0.60    | 18.00±0.60           | 18.00±0.60          |
| AgNPs solution   | 15.00±0.10      | 17.80±0.15     | 18.00±0.70            | 20.00±0.80          | 18.00±0.30    | 18.00±0.60    | 18.00±0.60           | 18.00±0.60          |
| AgNPs Gel 0%     | 15.00±0.10      | 17.80±0.15     | 18.00±0.70            | 20.00±0.80          | 18.00±0.30    | 18.00±0.60    | 18.00±0.60           | 18.00±0.60          |
| AgNPs Gel 5%     | 15.00±0.10      | 17.80±0.15     | 18.00±0.70            | 20.00±0.80          | 18.00±0.30    | 18.00±0.60    | 18.00±0.60           | 18.00±0.60          |
| AgNPs Gel 10%    | 15.00±0.10      | 17.80±0.15     | 18.00±0.70            | 20.00±0.80          | 18.00±0.30    | 18.00±0.60    | 18.00±0.60           | 18.00±0.60          |
| AgNPs Gel 20%    | 15.00±0.10      | 17.80±0.15     | 18.00±0.70            | 20.00±0.80          | 18.00±0.30    | 18.00±0.60    | 18.00±0.60           | 18.00±0.60          |
| AgNPs Gel 30%    | 15.00±0.10      | 17.80±0.15     | 18.00±0.70            | 20.00±0.80          | 18.00±0.30    | 18.00±0.60    | 18.00±0.60           | 18.00±0.60          |

(p-value>0.05; all values are mean±SE; n=3)

Generally, all formula has moderate inhibition both before and after the cycling test. Furthermore, these results show that nanosilver biosynthetic produces moderate-strong inhibition before the cycling test and produced moderate inhibition after the cycling test; however, the hydrogel base formula (P0) did not produce any inhibitory bacteria. The higher the concentration of nanosilver used, the larger the inhibition zone. In the antibacterial activity test of the nanosilver biosynthetic hydrogel showed that Gram-negative bacteria are more sensitive than Gram-positive bacteria. The antibacterial activity of nanosilver hydrogel decreased in the inhibition zone compared with the nanosilver biosynthetic solution, and the results show a moderately-strong inhibition. According to a study by Patel [24] which stated that carbopol is the best gelling agent in the release of the active substance compared to CMC-Na, HPMC, and sodium alginate. Also, carbopol as a gelling agent affects the viscosity of the gel preparation, which affects the release of the active substance in the preparation of gel [25]. The nanosilver concentration in the hydrogel preparation also affects the antibacterial activity, and this is supported by the study conducted by Aminia [26], which stated that lower active substances in gel preparations result in a smaller inhibition zone. The decrease in the hydrogel inhibition zone before and after the cycling test showed that the stability of the hydrogel is influenced by environmental conditions.

The change in the properties of physicochemical of nanosilver biosynthetic during storage is associated with the decrease in pH and high temperatures, which increases the collision rate of nanosilver biosynthetic and further induces faster agglomeration (fig. 3). The change in the size of nanosilver biosynthetic that become bigger is known as agglomeration [27]. Furthermore, the effective surface

Fig. 3: The results of TEM analysis of AgNPs using turmeric juice as bioreductor with a ratio of 5: 38 ml shows an average particle size of 17.97 nm with a spherical shape without any aggregation in magnification 250,000 times.
area in contact with bacterial cells will decrease and result in a decrease in antibacterial activity due to the agglomeration of nanosilver biosynthetic [28]. Nanosilver biosynthetic inhibits bacterial replication by binding and denaturing bacterial DNA [29]. In addition, the nanosilver biosynthetic that accumulates on the surface of bacterial cells will interact with sulphhydril groups, and release a cationic metal (Ag⁺), thereby replacing the hydrogen cation (H⁺) in proteins. This led to the fact that the bacterial proteins became inactive, and the permeability of the cell membrane decreases as a result of the lysis of bacterial cells [30]. Gram-positive consists of several layers of peptidoglycan, so the cell wall structure is thick and rigid, while the Gram-negative bacterial cells consist of one or more layers of peptidoglycan, which are thin, therefore making it more sensitive to antibiotics or other antibacterial agents. Furthermore, it has a porin protein content as an entry and exit channel for an active substance which facilitates cell wall damage by destroying the activity of enzymes in the bacterial cells. However, the lipid content in the cell also increases the permeability cell wall [31].

**Stability test of nanosilver biosynthetic hydrogel**

The color of the hydrogel preparation with the active nanosilver biosynthetic substance—using turmeric rhizome juice has a pale orange color which turns dark due to the greater concentration of the active substance, but the change in the preparation color is not too significant. Hydrogel preparation without an active substance has a transparent color. Meanwhile, all formulas are odorless and viscous after the cycling test.

The viscosity value that met the requirement of gel preparations based on SNI No. 16-4380-1996 is 3,000–50,000 cPs. Furthermore, the result of the viscosity test of nanosilver biosynthetic hydrogel showed that the higher concentration of the active substance affects the lower of viscosity value (table 3), and this occurs when a gel is stored at a high temperature. The polymer chain will release coils with a round ball-like shape (disentangle), and this is a result of gel viscosity (watery). The gel which is stored at cold temperatures, shortens and joins the polymer chains together. Also, it shrivels (entangle), due to changes in viscosity after conditions [32]. TEA as an alkaline agent serves to prevent carbopol, which is easily broken down by heat, thereby causing a decrease in gel viscosity. The preparation during storage becomes more acidic, resulting in a decrease in the number of ionized carboxylate groups, thereby repelling the carboxyl group and causing the structure of the development to decrease in viscosity [33]. Based on the test, all formulas produce viscosity values without any significant difference (p > 0.05) before and after the cycling test. This shows that the hydrogel is stable for six cycles after the cycling test.

**CONCLUSION**

The nanosilver biosynthetic produces yellow-brown color with a maximum wavelength peak at 433 nm, a particle size of 157.4±16.7 nm and has a round shape. The higher concentration of nanosilver biosynthetic as active substances effect inhibition zone has greater. The antibacterial activity of hydrogel preparations was medium-strong inhibition category before the stability test and a moderate category after the stability test. Furthermore, the formula with 30% AgNPs has the highest antibacterial activity. Formula with 30% AgNPs is chosen formula that has pH value of 5.87±0.65; viscosity of 4833.3±2.82 c. Ps; and dispersibility of 5.50±0.15 after cycling test. Therefore, all hydrogel preparations have met the requirements for good gel stability before and after the cycling test.

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