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The Characteristic and Antibacterial Activity of Nanosilver Biosynthetic using Sweet Orange

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Abstract: Sweet orange contains citric acid and ascorbic acid that acts as a silver ion bioreducer to form nanosilver (AgNPs). Silver ion green-synthesis using sweet orange peel resulted in smaller particle sizes than *C. lemon* and *C. limeta*. Therefore, this study aims to determine the characteristic of nanosilver biosynthetic using a combination of sweet orange juice-peel infusion as bioreductor and its antibacterial activity. Silver nitrate solution of 1.0 mM was mixed with sweet orange juice-peel infusion in a ratio of 3.0: 0.0, 1.5: 1.5, and 0.0: 3.0 mL. The process was conducted using a water bath at 60 ˚C for 45 minutes. The formation of AgNPs is indicated by a color change from colorless to yellowish-brown using UV/Vis spectrophotometer and PSA. The stability test of AgNPs is determined for 30 days of storage at room temperature and their antibacterial activity against *S. aureus* and *S. epidermidis*. The results showed that sweet orange juice accelerated the color change at the SPR range of AgNPs at 438-459 nm, but it is less stable. The mixed solution of sweet orange juice and peel infusion of 1.5: 1.5 mL is chosen ratio because stable and promising as an antibacterial agent compared with another formula. The AgNPs produced had a particle size of 41±10 nm and a spherical shape.

Keywords: sweet orange, nanosilver, citric acid, antibacterial activity

1. INTRODUCTION

Nanosilver is one of the most widely used nanomaterials in health care products, cosmetics, the food industry, medical equipment, and electronic equipment [1]. It has been found that silver is a metal that has broad antibacterial activity. It is effective and less toxic to animal cells when compared to other metals, which also have antibacterial activity. In addition, it is used as nitrate to induce an antibacterial effect, and when made into nanoparticles, there is an increase the area of contact with bacteria which causes the antibacterial activity to be more effective [2]. Physical technique is one of some methods to produce metal nanoparticles, such as laser ablation, lithography, and high energy irradiation. Chemical techniques developed some methods such as chemical reducing agents, electrochemistry, and photochemical reduction. However, the techniques used to synthezise metal nanoparticles are expensive and not environmentally friendly due to the use of hazardous, toxic, flammable chemicals and high energy requirements [3]. Another method as an alternative for synthesis is biological techniques that used plant extracts. Plants are selected because they are environmentally friendly and cost-effective materials. Leaves, roots, stems, seeds, and fruits

are used as biosynthetic agents of metal nanoparticles. Plant extracts can be bioreducer in the green synthesis of metal nanoparticles because of the active components. The naturally contained compound in plant extracts are reducing sugars, ketones/aldehydes, amine groups, water-soluble heterocyclic compounds, and proteins. Protein either act as reducing agents. Reducing agents that reduce metal ions as well as capping agents or stabilizing agents that maintain the stability of metal nanoparticles [4].

The results of research by Kaviya *et al.* (2011) stated that sweet orange peel (*C. sinensis*) was proven as a bioreducer in silver ion green synthesis that produced at a size of 10±1 nm at 60 ˚C for 45 minutes. The research developed by Kahrilas *et al*. (2014) carried out sweet orange peel as a bioreducer. It is heated using a microwave during 15 minutes of biosynthesis process that obtained nanosilver with a size of 7.36 ± 8.06 nm. Besides sweet orange peel, sweet orange juice has also been used as a bioreducer in nanosilver green synthesis. Sweet orange juice is used as a bioreducer because it contains citric acid and ascorbic acid that can act as reducing metal and as an effective stabilizer in the synthesizing metal nanoparticles [7].

However, the stability of nanosilver resulting from the research of Kaviya *et al.* (2011) and Kahrilas *et al*. (2014) has not been reviewed about the stability of the nanosilver formed. All parts of the sweet orange fruit, such as fruit juice and sweet orange peel, combined as a bioreducer in nanosilver biosynthesis have not been conducted. The combination of these two parts of sweet orange as a bioreducer can be a choice and has great potential in nanosilver biosynthesis. The phytochemical composition in sweet oranges varies between parts of the fruit, essential oils, sugars, organic acids, phenolic acids, and flavonoids are compounds in sweet orange juice. Meanwhile, the peel of the sweet orange fruit consists of compounds such as phenolic acids, organic acids, flavonoids, peptides, and essential oils [5]. In this study, fruit juice and sweet orange peel infusion combined to maximize the compounds contained in sweet oranges as reducing agents and stabilizing agents in nanosilver biosynthesis. The aim is to obtain a stable nanosilver and a relatively short reaction time of color change, producing a nanoparticle size and antibacterial activity against *S. aureus* and *S. epidermidis* bacteria.

2. MATERIALS AND METHODS

2.1. Instruments

UV/Vis spectrophotometer (Genesys, Thermo Fisher Scientific: Walthman, MA), analytical balance (Mettler Toledo AL204, d=0.0001 g, Columbus, Ohio), digital balance (Precisa XB620C, d=0.01 g, Moosmattstrasse, Switzerland), centrifuge (Heraeus Fresco 17, Thermo Fisher Scientific, Germany), SEM (JEM-1400 Flash Electron Microscope, Jeol, Japan), hotplate (Maspion, Indonesia), PSA (Horiba, Japan), and glasswares. Materials: Sweet orange fruit Pacitan, Indonesia (Citrus sinensis (L.) Osbeck, aqua bidestilata, silver nitrate 99.8% (Merck repackaged by CV Nitra Kimia, Yogyakarta), NaOH, Staphylococcus aureus bacteria ATCC 25923 and Staphylococcus epidermidis ATCC 12228 (Cultured in Microbiology Laboratorium, Medical Faculty, UNS Surakarta), nutrient agar, Clindamycin disc standard (Oxoid, DA-2).

2.2. Sample preparation

Sweet orange juice was obtained by squeezing the fruit and then filtered with Whatman filter paper no 1. The juice was centrifuged at 10,000 rpm for 10 minutes, and the supernatant from the centrifuge was stored for the next process [7]. Meanwhile, sweet orange peel infusion is obtained by boiling sweet orange peel in hot aquabidest. An orange peel of 4.0 g was put into 40 mL of hot

aquabidest for two minutes. The infusion solution obtained was filtered using Whatman filter paper no 1 and saved for the next process [8].

2.3. The biosynthesis process

Nanosilver green synthesis is conducted out by mixing 3 mL of a sweet orange juice - peel infusion into 40 mL of $AgNO_3$ solution 1.0 mM. The biosynthesis process was carried out in a water bath at 60 ℃ for 45 minutes (Konwarh et al, 2014). Comparison of the volume of fruit juice-sweet orange peel infusion is a combination of bioreducers that are called independent variables in ratio of 3.0: 0.0; 1.5: 1.5; and 0.0: 3.0 mL.

2.4. Characterization of nanosilver biosynthetic

The nanosilver biosynthetic characterization is carried out using UV/Vis spectrophotometer and TEM instruments. UV/Vis spectrophotometer is used to confirm the formation of nanosilver biosynthetic by analyzing at the position of the maximum wavelength (λ max) at the absorbance spectra of surface plasmon resonance (SPR) range. The combined solution of fruit juice and infusion of sweet orange peel was used as a blank. The sample was measured with a wavelength between 200-800 nm. Meanwhile, TEM is used to determine the nanosilver's morphology of the nanosilver, shape, particle distribution, and particle size [9].

The stability test of the nanosilver is carried out by observing the presence of a maximum wavelength shift (λ max) in the absorbance spectra, and the UV/Vis spectrophotometer is after the nanosilver is stored for several days. The nanosilver biosynthetic is stored in a refrigerator at 4 ℃, and the 1st, 7th, 14th, 21st, and 28th days of storage, the absorbance spectra were scanned using a UV/Vis spectrophotometer [4]. Meanwhile, the effect of the media's pH on the snanosilver's tability was adjusted by adding 0.1 mol/L NaOH until the pH values of the media were 6, 8, and 10 respectively.

2.5. Antibacterial activity of nanosilver biosynthetic

The nanosilver's antibacterial activity is tested by using the well diffusion method against Staphylococcus aureus and Staphylococcus epidermidis bacteria. Each bacterial strain is cultured by flattening using a sterile ose rod into the prepared nutrient agar medium. Furthermore, the well with a diameter of 10 mm in the media is poured with 100 μL of nanosilver and aquadest as a negative control. Standard clindamycin discs are selected for positive controls. The media is incubated at 37 ℃ for 24 hours, and then the diameter of the inhibition zone formed is measured using a caliper [8].

2.6. Statistical analysis

Research data include the maximum wavelength (λ max) of the UV/Vis spectrophotometer characterization and are tested statistically with the Kruskal-Wallis Test. Meanwhile, the λ max data of stability test are obtained from five nanosilver formulas during 28 days of storage and the effect of pH adjustment. The λ max of nanosilver is tested statistically with the Wilcoxon Test. All statistical tests are conducting using SPSS.v.25 software. The mean and standard deviation are obtained from three replicated samples [6].

3. RESULTS AND DISCUSSION

Nanosilver biosynthetic in this experimental study is carried out by mixing a combination of bioreducer, namely fruit juice – sweet orange peel infusion in a mixture's ratio of F1 (3:0 mL), F2 (1.5:1.5 mL), and F3 (0:3 mL) in $AgNO_3$ solution 1.0 mM, then reacted on water bath at 60 ℃ during a reaction time of 45 minutes. Based on observations, each formula showed variation in the color change of the solution. The results showed that the solution with a high ratio of sweet orange juice (F1) had a faster color change after five minutes the reaction. On the other hand, the high ratio of

sweet orange peel infusion (F3) resulted in colorless solution for nine minutes. The color change of the solution is closer to the study conducted by Kaviya *et al* (2011) resulting in a colorless solution with a duration of nine minutes. This may be due to phytochemical differences between fruit juices and sweet orange peels. In comparison, citric acid and ascorbic acid are high in fruit juices than in sweet orange peels [5]. In contrast, flavonoid compounds, such as hesperidin are abundant in sweet orange peels [10]. In sweet oranges, another groups of compounds can also be found in one part. For example, carbohydrates/sugars that are mostly found only in fruit juices or peptides which are mostly found only in fruit peels.

Figure 1. The result of color change after the biosynthesis process showed that variation ratio (fruit juice and sweet orange peel infusion-silver nitrate solution) produced a different color, and SEM analysis.

Color change from colorless to a yellow-brown solution after 10 minutes indicates the presence of nanosilver. The formation of nanosilver from the reduction of Ag+ metal ions cannot be separated from the role of metabolite compounds in sweet citrus fruits such as terpenoids, flavonoids, sugars, phenolic acids, polyphenols, and proteins. Based on previous studies, the proton dissociation of the –OH group in terpenoid group compounds triggers a chemical structure resonance which can result in an oxidation process of the compound. On the other hand, the tautomeric transformation of flavonoid compounds from the enol form to the keto form which can be another mechanism in the formation of nanosilver from Ag+ ions. Flavonoids can also chelate metal ions such as Ag+ ions and are involved in the nucleation and aggregation processes or the growth phase. The aldehyde group found in monosaccharides such as glucose is also known to act as a reducing agent. The oxidation process of the aldehyde group contained in the sugar structure through the addition of nucleophile –OH, triggers the reduction of metal ions. Meanwhile, compounds such as proteins, polysaccharides, organic ligands, and other biological components on the surface of nanoparticles have been known to increase particle stability. Metabolites contained in sweet oranges are both reducing agents and stabilizing agents.

The presence of electrostatic interactions such as electron donor from electronegative oxygen in the aquadest solvent can contribute to fluctuations in the position. According to the results of physical observations of the three solutions of the nanosilver formula, it is found that on the 7th day after biosynthesis, agglomerates are formed based on nanosilver solution of F1. The formation of agglomerates at the bottom where the nanosilver is stored is an early indication of the deteriorating stability [11]. Therfore, the high volume of sweet orange juice (F1) compared to a bioreducer will form more agglomerates during a short storage time, affecting the nanosilver's stability. From the results of measuring the maximum position of the nanosilver and physical observations of the three formulas for 28 days of storage, it can be concluded that the nanosilver biosynthetic of F2 is stable for 28 days

of storage. This is seen from the shift in the maximum position observed in the range of no more than 1-4 nm, and during storage on the bottom of the flacon, no agglomerates were formed.

3.1. Stability test of nanosilver

The stability of the nanosilver from the biosynthesis results with bioreducer combined of fruit juice – sweet orange peel infusion is observed for 28 days of storage at room temperature. Observation of the stability is carried out several times, on the 1st, 7th, 14th, 21st, and 28th days of storage after the green synthesis process. The maximum wavelength position is used as one of the parameters to determine the stability of the three nanosilver formulas. In addition, physical observations of the solution are also carried out to determine the presence of agglomerates formed during storage. Data on the position of maximum wavelength of nanosilver for the five formulas during 28 days of storage can be seen in Table 1. Based on the results obtained, during 28 days of storage, the position experienced quite a variety of shifts. The fluctuation of the position of more than 1-4 nm is seen in nanosilver F1 and the largest fluctuation occurred in nanosilver of F3. Statistical analysis conducted on the position stability data of the three formulas showed that from the normality test, the F3 data were not normally distributed as seen from the Shapiro-Wilk Sig. value of 0.04 < 0.05. Meanwhile, non-parametric statistical test using the Wilcoxon test is selected to compare the average position of maximum wavelength of nanosilver biosynthetic. This is achieved using a combined of fruit juice and sweet orange peel infusion (F3) as bioreducer with the average position of maximum wavelength. The results of the biosynthesis using sweet orange juice (F1) or sweet orange peel infusion (F3) for 28 days of storage using the Wilcoxon Test between nosilver F2 against nanosilver F3 showed the Asymp Sig. value of 0.01 < 0.05. This means there is a significant difference between the average position of the maximum wavelength of nanosilver F2 with the average position of the maximum wavelength of nanosilver F3. Similar Wilcoxon Test results were also shown between nanosilver F2 against nanosilver F1 for the Asymp Sig. value of 0.01 < 0.05, while between F2 and F1 showed value of 0.355 > 0.05. Thereofer, adding 1.5 mL of sweet orange juice combined with 1.5 mL of sweet orange peel infusion as a bioreducer did not provide a statistically significant difference compared to using sweet orange juice (F1). From the results of physical observations of the three solutions of nanosilver formulas, agglomerates are formed on the F1 nanosilver solution on the 7th day after biosynthesis.

Table 1. The results data on the position of maximum wavelength of nanosilver for the three formulas during one month of storage

Formulas	Maximum Wavelength (nm)				
	Day-1	Day-7	Day-14	Day- 21	Day- 28
F1	446 ± 3	441 ± 2	447 ± 1	447 ± 2	445 ± 1
F2	438 ± 1	440 ± 2	438 ± 1	440 ± 1	439 ± 1
F3	458 ± 0	462 ± 2	452 ± 0	452 ± 1	451 ± 1

Table 2. The maximum wavelength data for nanosilver F3 and F4 for one month of storage with pH adjustment

Formula	pH	Maximum Wavelength (nm)				
		Day 1	Day 7	Day 14	Day 21	Day 28
F2	6	441 ± 1	443 ± 0	441 ± 1	441 ± 0	443 ± 1
	8	440 ± 1	439 ± 2	438 ± 1	435 ± 1	436 ± 1
	10	440 ± 0	428 ± 16	433 ± 10	440 ± 11	449 ± 8

The effect of pH test on the stability of nanosilver for 28 days of storage is shown at table 1. The test is applied to two-volume variations formulas for a combination of fruit juice and orange peel infusion, F2 proven to have good stability. After going through the nanosilver biosynthesis process, the two formulas selected then adjusted the pH of the solution to pH 6, 8, and 10. The maximum wavelength data for nanosilver F2 for 28 days of storage with pH adjustment can be seen in Table 2. Based on the test results, the two formulas have pH value more than the maximum wavelength shifts to a blue shift. The increase pH value in both formulas cannot maintain the stability of nanosilver during one month of storage, as seen from irregular fluctuations in the position at pH 10. On the other hand, at pH 6, fluctuations of value change of maximum wavelength for F2 were not more than 1-4 nm. Thus, confirmed that the nanosilver biosynthetic with combined bioreducer of 1.5: 1.5 mL at pH 6 is stable for one month of storage. The Wilcoxon Test is also selected to analyze the test data for the effect of pH to determine the significant difference between the control formula (pH 6) and the formula adjusted for pH (pH 8 and pH 10). Based on the results, the average position of maximum wavelength of nanosilver F2 at pH 8 and pH 6 and the average position of F4 at pH 8, pH 10 and pH 6 showed the Asymp Sig. value of 0.01 < 0.05. This indicated a significant difference when adjusting the pH to the average position of the maximum nanosilver during 28 days of storage.

The SEM analysis confirmed the nanosilver formation from the biosynthesis using the F2 bioreductor (1.5: 1.5 mL). The resulting nanosilver is spherical, with the average of particle size is 56 ± 2 nm. As a comparison, the results of the nanosilver characterization from biosynthesis using sweet orange peel extract conducted by Kaviya *et al.* (2011) obtained a particle size of 35 ± 2 nm with synthesis at 25 °C and a particle size of 10 ± 1 nm with synthesis at 60 °C. Using a microwave in the research of Kahrilas *et al.* (2014), the obtained nanosilver shows particles with various diameters where 56 nm is the largest measurement, and the average particle size is 7.36 ± 8.06 nm. Meanwhile, the characterization of nanosilver from biosynthesis using sweet orange peel extract conducted by Annu *et al.* (2018) obtained particle sizes ranging from 9-46 nm. The studies mentioned above showed similarities in the type of particle size distribution and the morphology of the resulting nanosilver. The characterization of SEM from the biosynthesis using sweet orange peel extract (*C. sinensis*) showed that the nanosilver is polydispersed and spherical. The experimental study also confirmed similar results, where the nanosilver is distributed in a polydispersed and spherical manner. The large particles found from the PSA characterization results are due the complex content of compounds in the bioreductant that significantly affect the agglomeration and decomposition properties of the resulting nanosilver. The selected reaction temperature is also important in obtaining the desired nanosilver particle size [12]. However, the resulting nanosilver particle size is still within the average range of the results of the studies mentioned above. The SEM and PSA characterization has shown that the volume variation of the fruit juice and infusion of sweet orange peel as a bioreducer affect the particle size of nanosilver when compared to sweet orange peel extract.

3.2. Antibacterial activity test

The antibacterial activity of nanosilver biosynthetic at F2, which are stable for 28 days of storage against *S. aureus* and *S. epidermidis* bacteria is determined. The well diffusion method is used to test the antibacterial activity of nanosilver with the diameter of the inhibition zone as the measured parameter. The nanosilver showed antibacterial against *S. aureus* and *S. epidermidis* bacteria by

analizing the formation of an inhibition zone for each test bacteria. Based on the tests, F2 produced an inhibition zone diameter of 19.20 mm against *S. aureus* and 18.19 mm against *S. epidermidis* bacteria.

The inhibition zone diameter of gram positif bacteria higher than gram negative bacteria because they have different characteristics. *S. epidermidis* is a coagulase-negative *Staphylococcus* bacteria, and it is different from *S. aureus*, a coagulase-positive *Staphylococcus*. It works by reducing the coagulase enzyme to cause severe illness and death. On the other hand, there is evidence that certain strains of *S. epidermidis* bacteria produce phenol-soluble modules to inhibit the growth of skin pathogens. It can modulate non-host-specific immune responses against pathogens, such as *S. aureus* bacteria. It is also thought to be involved in the pathophysiology of acne through interaction with *P.acnes* bacteria, which is currently renamed *C.acnes* [13]. In healthy skin, *C. acnes* bacteria play a role in inhibiting the growth of *S. aureus*. Acne can form when excessive colonization of these bacteria occurs on the skin [8].

Table 3. Results of the diameter data of the inhibition zone of antibacterial activity of nanosilver biosynthetic at F2

Sample Test	The Inhibition Zone Area (mm)	
	***Staphylococcus aureus* (A)**	***Staphylococcus epidermidis* (B)**
F2	19.20	18.19
Clindamycin	31.38	33.26
Aquadest	0.0	0.0

4. CONCLUSION

The results showed that the addition of sweet orange juice accelerated the color change of the mixed solution. The characterization of nanosilver with UV/Vis spectrophotometer confirmed SPR peak range of 438-459 nm. Furthermore, the stability test showed that nanosilver reduced with sweet orange juice-peel extract (1.5: 1.5 mL) is stable and had the largest inhibition diameter among the other formulas against *S.aureus* of 19.20 mm and *S.epidermidis* of 18.19 mm. PSA analysis confirmed that the particle size of nanosilver biosynthetic is 41 nm with spherical form.

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