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Formulation of Silver Nanoparticle Mouthwash and Testing of Antibacterial Activity Against *Staphylococcus aureus*

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Abstract

Silver Nanoparticles (AgNP) are silver particles of no more than 100 nm in size. Silver nanoparticles have antimicrobial characteristics and have been applied to various fields as antibacterial agents. This study aims to formulate and examine the antibacterial activity in preparing silver nanoparticle mouthwash on *Staphylococcus aureus*. The silver nanoparticles are synthesized using a chemical reduction method, of which the wavelength is then characterized using UV-Vis spectrophotometry, and the PSA instrument is used for particle size. Silver nanoparticles are formulated for a mouthwash with various concentrations such as 0%, 60%, 70%, and 80% consecutively as formula 1, 2, 3, and 4. The observation is then performed on the organoleptic, pH, stability, and bacterial activity of the *Staphylococcus aureus* using the disk diffusion method. The study results indicate that the preparation of silver nanoparticle mouthwash has a good organoleptic; the average pH of formula 1, 2, 3, and 4 consecutively is 3.40, 3.40, 3.46, and 3.54; however, it is not stable during the storage stage. The result of the antibacterial activity test on *Staphylococcus aureus* bacteria shows that formula 2 has the most oversized average inhibition zone diameter that is 13.14±0.31 mm compared to formulas 1, 3, and 4, namely 5.20±0.44; 12.40±0.74; and 8.40±0.89 mm. The active formula of mouthwash preparation to inhibit the growth of *Staphylococcus aureus* bacteria is formula 2.

Keywords: nanoparticles, nanosilver, mouthwash, antibacterial, *Staphylococcus aureus*

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1 Introduction

Oral disease is the sixth-highest disease suffered by the Indonesian people based on the survey of Household Health in 2011, the number of oral diseases in Indonesia has reached 79.6%. The illness that Indonesian people predominantly suffer from is Recurrent aphthous stomatitis (RAS), a recurrent stomatitis named sprue [1]. RAS is not a deadly disease, but it can cause the death of mouth tissue and is even very risky to develop oral cancer or mouth cancer if it is not treated correctly [2].

One of the causes that can prolong or worsen RAS is a normal flora of the mouth. Some bacteria living in the mouth are *Staphylococcus aureus, Streptococcus mutans, Streptococcus viridians, Staphylococcus aureus epidermis,* and *Staphylococcus pneumonia*. Those bacteria are normal flora of the mouth, and it has a relationship with the human being called commensalism. Still, it can be a pathogen or infect humans with declining immunity. For example, *Staphylococcus aureus* bacteria are often natural flora germs on human skin and mucous membrane. However, these bacteria may cause bacteremia and systemic infection in particular cases with symptoms such as necrosis, inflammation, and abscess formation [3-4]. Besides, *Staphylococcus aureus* causes infectious diseases in the mouth, such as abscess, *gingivitis*, *parotitis*, *Staphylococcal mucositis*, *denture stomatitis*, *angular cheilitis*, and endodontic infection [5-6]. Meanwhile, *Streptococcus mutants* bacteria are the primary bacteria causing dental caries/tooth decay [7-8].

RAS can be prevented and treated by keeping the mouth clean and healthy. One of the ways is to gargle using antibacterial substances to control the number of bacteria inside the mouth. One of the substances that can be used as an antibacterial substance is silver nanoparticles (AgNP). It has a good ability as an antimicrobial on bacteria, viruses, and eukaryotic microorganisms. A study on silver nanoparticles antibacterial activity test on *Staphylococcus aureus*, isolated from patients who suffered from stomatitis, has been conducted. The inhibition zones on the concentration of 20%, 40%, 60%, 80%, and 100% consecutively are 6, 6.25, 7.5, 10.25, and 17 mm. The results indicate that the bigger the concentration of silver nanoparticles, the more influential the antibacterial activity becomes. Thus, various additional concentrations of silver nanoparticles in mouthwash preparation are 60%, 70%, and 80%.

2 Experimental section

2.1 Tools and Materials

$AgNO_3$ (Merck), sodium citrate ($Na_3C_6H_5O_7$) (Sigma-Aldric), aquadest, Glycerin, PEG 40 hydrogenated castor oil (PT. Cognis, Indonesia), menthol, sodium saccharin (PT. Bratachem), sodium benzoate (PT. Bratachem), sodium nitrate (PT. Bratachem), nutrient agar media (NA), potato beef, peptone and blank disk.

2.2 Silver Nanoparticles Synthesis

500 mL of $AgNO_3$ solution is heated (100°C) until boiled. Each drop of 50 mL of sodium citrate ($Na_3C_6H_5O_7$) is then added while stirred using a magnetic stirrer. The heating process is stopped when the solution turns yellow, but it is mixed until the temperature is similar to the room temperature. The colloidal silver nanoparticles (AgNP) are characterized using UV-Vis spectrophotometry to determine the silver nanoparticles formulation if the absorbance peak occurs in the wavelength in the range of 400 nm. The characterization using PSA aims to identify the particle size of the synthesis result.

Table 1 The Formula of Mouthwash

Ingredients	Function	Concentration % (b/v)			
		F1	F2	F3	F4
Silver nanoparticles (v/v)	Active agent	-	60	70	80
PEG 400	Stabilizer and solvent	2	2	2	2
Menthol	Flavor	0.5	0.5	0.5	0.5
Sodium saccharin	Flavor	0.1	0.1	0.1	0.1
Sodium benzoate	Preservative	0.3	0.3	0.3	0.3
Citric acid	Buffer	0.5	0.5	0.5	0.5
Glycerin	Co-solvent	15	15	15	15
Aquadest	Solvent	ad 100	ad 100	ad 100	ad 100

2.3 Mouthwash Formula

Mouthwash is made through four formulations (Table 1), and each formulation is replicated three times.

2.4 The Physicochemical Characteristic Evaluation of Silver Nanoparticle Mouthwash

The physicochemical characteristics of silver nanoparticle mouthwash preparation are evaluated. The characteristics include organoleptic, pH, and stability. Organoleptic evaluation is done by observing the shape, color, smell, and taste or sensation of the Pharmacy program students studying UIN Maulana Malik Ibrahim Malang. pH is measured using the pH meter. The stability test used the Cycling Test. The test is done by keeping the silver nanoparticle mouthwash at a temperature of 4^{o}C for 24 hours and then putting it at 40^{o}C for 24 hours. The method involving two different temperatures is considered as one cycle. It is done for six periods or 12 days and is observed for its organoleptic and pH on days 0 and 12.

2.5 Activity Test of Inhibition on Staphylococcus aureus

The study involved six treatment groups in the antibacterial activity test: aquadest as the negative control, silver nanoparticle colloid (AgNP) as the positive control, and four mouthwash formulas as the test group. Antibacterial activity testing is done by splitting the media in the lower part of the petri dish into four sections. Each of them is identified using the label. Each disk paper is soaked in aquadest, silver nanoparticle colloid, and mouthwash preparation of formula 1, 2, 3, and 4 for ±25 minutes. The six disks are put on the media of agar inoculated with *Staphylococcus aureus* according to the label under the dish petri. Each treatment is repeated four times. Then, it is heated in the incubator at 37^{o}C for 24 hours. After that, the clear inhibition zone around the disk is observed. Its diameter is measured using the caliper.

3 Results and Discussion

The synthesis of silver nanoparticles employed the chemical reduction method by reacting 500 mL of $AgNO_3$ 0,001M solution with 50 mL of $Na_3C_6H_5O_7$ 1% solution. The $AgNO_3$ solution is heated (100°C) until it reaches its boiling point, then sodium citrate ($Na_3C_6H_5O_7$) is added drop by drop and mixed using a magnetic stirrer. The heating stopped when the solution turned yellow. However, the stirring is continued until it reaches room temperature [7-8]. The yellow color indicated silver nanoparticles. The following chemical reaction (equation 1) has occurred during the reduction process of $AgNO_3$ [9].

$$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag^{0} + C_6H_5O_7H_3 + 3Na^{+} + H^{+} + O_2$$ (Equation 1)

Table 2 The measuring result of silver nanoparticles using spectrophotometer UV-Vis and PSA

Name	Wavelength	Abs	particle size (nm)	Polydispersity index
AgNP replication 1	429.00	0.316	42.86	0.42
AgNP replication 2	425.50	0.246	73.26	0.46
AgNP replication 3	426.00	0.230	46.35	0.48
	426.83 ± 0,03	0.264 + 0,23	54.16 ± 14.23	0.45 ± 0.06

Table 3.The Evaluation Result of the Physicochemical Characteristics of Mouthwash Preparation

			Formula 1	Formula 2	Formula 3	Formula 4
Organoleptic		Homogeneous solution	100%	100%	100%	100%
	Form	Non-homogeneous solution	-	-	-	-
		Others	-	-	-	-
		E	100%	-	10%	24%
	Color	F	-	95%	85%	76%
		G	-	5%	5%	-
		Menthol	100%	100%	86%	86%
	Aroma	Unflavored	-	-	14%	14%
		Other Scents	-	-	-	-
		Refreshing and sweet	100%	100%	100%	100%
	Taste and Sensation	Not refreshing and sweet	-	-	-	-
		Refreshing and not sweet	-	-	-	-
		Not refreshing and not sweet	-	-	-	-
pH ± SD			3.40 ±0.00	3.40 ±0.10	3.46 ±0.05	3.54 ±0.11
Stability Test	Form		Stable	Stable	Stable	Stable
	Color		Stable	Unstable	Unstable	Unstable
	Aroma		Stable	Stable	Unstable	Unstable
	Taste and Sensation		Stable	Stable	Stable	Unstable
	pH		Unstable	Unstable	Unstable	Unstable

The measurement using spectrophotometer UV-Vis (table 2) shows a synthesis of silver nanoparticles. It is proven with the emergence of absorbance peaks on the 400-450 nm wavelength. The synthesis result sample showing its peak on the 400 - 450 nm wavelength is silver nanoparticles and silver ions on the 370 - 400 [10].

The measurement of silver nanoparticles aims to determine the size and homogeneity of silver nanoparticles being formed. The silver nanoparticles ranged from 1-100 nm and have antibacterial characteristics. The silver nanoparticles in the research (Table 2) have a suitable size and good homogeneity. The value of PDI or polydispersity index shows a homogeneity level. When the PDI closes to 0, it shows homogeneous particle disperse. On the other hand, PDI is more significant than 0.50 and offers a high heterogeneity [11].

The synthesized silver nanoparticle colloid is formulated into a mouthwash. It dissolves 13.2 mL of glycerin and 2.2 grams of PEG 40 hydrogenated castor oil into a small aquadest. 0.55 grams of menthol is dissolved into a small amount of ethanol 70%, 0.11 grams of sodium saccharin, 0.33 grams of sodium benzoate, and 0.55 grams of citric acid dissolved in a small aquadest. Then, the glycerin and PEG 40 hydrogenated castor oil, menthol, sodium saccharin solution, sodium benzoate, and citric acid are mixed in a bowl using a magnetic stirrer until homogenous. Then, the silver nanoparticle colloid is added gradually and stirred until all of them are homogenous and is added until the volume reaches 100 mL.

The evaluation result of the physicochemical characteristics of silver nanoparticle mouthwash is described in Table 3. It includes the mouthwash's shape, color, aroma, taste, and sensation. The characteristics are observed by seven students of 8 semesters of Pharmacy of UIN Malang attending organoleptic testing.

The observation result of silver nanoparticle mouthwash (Table 3) shows that four formulas are homogeneous solutions. All respondents answered that the keys in formulas 1, 2, 3, and 4 are comparable. Observing the preparation color shows the four formulas have almost the same colors. The most chosen color code is code F in formula 2 (amount 95%), formula 3 (amount 85%), and formula 4 (amount 76%), but it is a little bit different in the color of formula 1, G color code is brighter than others codes. Others say that the G color code is 5% in formula 2, the E color code is 10% in formula three, the G color code is 5% in formula 3, and the E color code is 24% in formula 4.

The observation result of the aroma of silver nanoparticle mouthwash (Table 3) shows that almost all respondents say they have a menthol aroma. In contrast, the others say they are not flavorful. All respondents say that formula 1 and formula 2 have a menthol aroma. However, only 86% of respondents say that Formula 3 and 4 have menthol aroma, while 14% say they are not flavorful. The observation result of the taste and sensation of mouthwash

preparation (Table 3) shows that all respondents have a sweet and refreshing taste.

The measuring result of the pH value of silver nanoparticle mouthwash (table 3) falls into the standard pH range of oral development. It is equal to 2-9 [12]. Meanwhile, organoleptic and pH observation on the silver nanoparticle mouthwash stability test shows that it is unstable due to the change of color, aroma, taste, sensation, and pH during the storage phase measured on days 0 and 12.

Table 4. The Result of Measurement of Inhibition Zone Diameter of Mouthwash Preparation

	Formula 1	Formula 2	Formula 3	Formula 4	AgNP	Aquadest
Diameter of inhibition zone (mm) ±SD	5.20 ±0.44	13.14 ±0.31	12.40 ±0.74	8.40 ±0.89	8.80 ±0.83	0

The minor inhibition zone value in formula 1 was 5.20 mm because, in formula 1, no silver nanoparticles were added. There is an inhibition zone value in Formula 1 due to adding a preservative or antimicrobial ingredient, namely sodium benzoate. Adding preservatives allows the diameter of the inhibition zone to occur in Formula 1. However, if we compare it with the positive control, AgNP provides more minor inhibition. The most significant inhibitory zone value should be found in Formula 2. The addition of colloidal silver nanoparticles in formula 2 is the greatest compared to formulas 1, 3, and 4. Increasing the silver nanoparticle concentration should increase the antibacterial inhibitory power [4]. Based on the differences in the results of this research, that is not appropriate. It is caused by an interaction between silver nanoparticles and the activity of preservatives, namely sodium benzoate [13]. The possible communication happens because silver nanoparticles have antibacterial activity, and so does sodium benzoate. Thus, the antibacterial activity of silver nanoparticles in formulas or action from sodium benzoate decreases.

This is similar to the research result stating that there is no synergistic effect when sodium benzoate is combined with potassium sorbate, which also functions as a preservative [10]. Sodium benzoate is a preservative with bacteriostatic and fungistatic properties in acidic conditions. Its preservative mechanism begins with the absorption of benzoic acid into cells, then the intracellular pH of the cell becomes acidic. If the intracellular pH of cells decreases to 5 even lower, anaerobic fermentation of glucose decreases dramatically. It causes growth, and the development of the microorganism cells is inhibited [14]. While the antibacterial activity of potassium sorbate inhibits enzymes containing sulphydryl, it is done using sorbic acid reacting slowly with cysteine by using an addition reaction with thiol groups[21]. It is known that the antibacterial activity of potassium sorbate is the same as the mechanism of the antibacterial action of silver nanoparticles, that is, by damaging bacterial cell walls, interfering with cell metabolism, and inhibiting bacterial cell synthesis. The point of this mechanism is that silver ions will replace the hydrogen cation (H +) of the thiol sulphydryl group to produce a more stable S-Ag group on the cell surface, causing bacterial death [15].

The largest inhibitory zone value should occur in formula 4 because adding colloidal silver nanoparticles is the most important compared to formulas 2 and 3. As the concentration of silver nanoparticles increases, the antibacterial inhibitory power will be higher [16]. However, this is different from the results of tests on the antibacterial activity of silver nanoparticle mouthwash, which experienced a decrease in the diameter of the inhibition zone and the addition of silver nanoparticles' colloidal concentration. This incompatibility is caused by the interaction between silver nanoparticles and the preservative activity, sodium benzoate [17]. An exchange may occur because silver nanoparticles have activity, so the antibacterial activity of silver nanoparticles in the formula or the action of sodium benzoate decreases. The results of this study are similar to the results of research where there was no synergistic effect when sodium benzoate as a preservative was combined with potassium sorbate, which also functions as a preservative [18].

4 Conclusions

The results of physicochemical characteristics of the silver nanoparticles-based mouthwash preparation in the form of organoleptic test and pH test with an average value of 3 times repetitions of measurements in formulas 1, 2, 3, and 4, respectively at 3.40; 3.40; 3.46; and 3.54 is good enough. It is suitable for the standard preparation requirements intended for a mouth. However, it has poor stability because there are changes during the storage period on the 0^{th} and 12^{th} days. While the antibacterial activity of *Staphylococcus aureus* in the silver nanoparticles-based mouthwash preparation decreases. It occurs along with an increasing concentration of silver nanoparticles in mouthwash preparations. The level of 60% silver nanoparticles in mouthwash can effectively inhibit the growth of *Staphylococcus aureus* bacteria.

5 Declarations

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5.2 Author Contributions

Concept – Rahmi Annisa; Firdausi Zahrah; Design – Rahmi Annisa, Begum Fauziyah, Dewi Sinta Megawati, Firdausi Zahrah; Collection and Processing – Rahmi Annisa; Firdausi Zahrah; Analysis– Rahmi Annisa, Begum Fauziyah, Dewi Sinta Megawati, Firdausi Zahrah; Writing – Rahmi Annisa.; Critical Reviews – Dewi Sinta Megawati.

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5.4 Conflicts of Interest

The authors declare no conflict of interest.

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