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ANTIFUNGAL ACTIVITY TEST OF NANOSILVER IN SERUM COSMETIC PREPARATIONS

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Abstract: Serum is one of the cosmetic products that people often use today. Along with the times and technological developments, innovations in serum products are also increasing with the addition of active ingredients. Nanosilver is one of the nanotechnology findings and is known as a good antifungal. In this study, serum products were made with the addition of nanosilver as an antifungal, with variations in the acquisition of 20 ppm nanosilver by 5, 10, and 15%. This study aims to determine the characteristics of nanosilver synthesized using UV-Vis Spectrophotometer and TEM, determine the physical and chemical characteristics of nanosilver serum preparations, and determine the antifungal activity of nanosilver in serum cosmetic preparations. In this study, the synthesis of nanosilver using a chemical reduction method with sodium citrate as a reducing agent and stabilizer. Characterization of nanosilver with UV-Vis Spectrophotometer showed that the synthesized nanosilver was relatively stable, and the results of description with TEM showed that the nanosilver produced was on the nanoscale with an average diameter of 35.075 nm and spherical shape. The physical and chemical characteristics of the serum preparation carried out by organoleptic testing showed that the five samples were homogeneous, non-irritating, and had a pH of 6. Testing of nanosilver antifungal activity was carried out by the DMC (Direct Microscopic Count) method with direct counting techniques under a microscope for eight weeks. Based on the calculation results, from week 0 to week 8, it shows that the serum preparation with the addition of nanosilver can inhibit fungi with the growth of the number of hyphae in F1, F2, F3, F4, F5 respectively 24.25; 22.25; 3.25; 3; and 2.75%.

Keywords: *Serum, Nanosilver, Antifungal Activity, DMC, UV-Vis Spectrophotometer, TEM*

INTRODUCTION

Cosmetics are commodities used by almost all levels of society in Indonesia. One of the causes of cosmetic damage is caused by microbial contamination. Contamination of microbes that often occurs in cosmetics is contamination caused by fungi [1], [2]. The addition of preservatives in cosmetics is significant to prevent the occurrence of fungal contamination. Common and frequently used preservatives are paraben esters such as methylparaben, propylparaben, ethylparaben, and butylparaben. The use of paraben esters in cosmetics recommended by the FDA (Food and Drug Administration) and Indonesian BPOM regulations is 0.4% for a single preservative. The use of paraben preservatives is limited because parabens can cause side effects such as skin redness and allergic reactions [1]. Some studies [3-8] report that parabens have estrogenic properties, and estrogen is known to play a central role in the growth and development of breast cancer [9]. Therefore, in this study, an alternative preservative was made that can replace the part of paraben preservatives that are harmful to the body. This preservative is one of the applications of nanoparticle technology with its activity as a new antimicrobial in cosmetic preparations [10].

Nanotechnology is an innovation that controls shape and size on the nanometer (nm) scale, ranging from 1 nm - to 100 nm. The cosmetic industry widely uses the nanoscale to improve the quality of preparations [11]. One example of nanotechnology findings is nanosilver. Nanosilver is used chiefly as an antimicrobial agent [12]. Nanosilver can kill microbes such as viruses and bacteria by continuously releasing silver ions, which can be considered a microbe-killing mechanism [13]. In addition to killing viruses and bacteria, nanosilver can also inhibit fungal growth and cause fungal death. According to [14] and [15], nanosilver will release silver ions and bind to specific protein groups that affect the function of membrane proteins and disrupt cell permeability. Silver ions will also inhibit mycelial growth in fungi by inhibiting conidia germination and suppressing their development. Nanosilver ions have a genotoxic effect that suppresses the activity of respiratory chain enzymes and destroys DNA, eventually leading to cell death. Previous studies have shown that nanosilver can inhibit the growth of Candida albicans [10], *Aspergillus niger* [16], and *Fusarium oxysporum f. sp. radicis-lycopersici* (FORL) strain [17].

In this study, the synthesis of nanosilver was carried out using the chemical reduction method. Chemical reduction is the most frequently applied method for synthesizing silver nanoparticles as a stable colloidal dispersion in water or organic solvents [18]. In this nanosilver synthesis, sodium citrate is used as a reducing agent and stabilizer [19]. Research [20] said that synthesizing nanosilver using sodium citrate as a reducing agent and stabilizer will produce nanosilver particles with a spherical shape with particle sizes ranging from 30-60 nm.

DMC, or direct microscopic counting, is a method of counting the number of cells in a population under a microscope. The purpose of DMC is to determine the number of fungal cells and determine the development of fungi in various circumstances, determine the presence of fungal and bacterial growth on the surface of the sample, and collect a mixture of microbes that can grow on the surface of the model that is usually found in the environment so that the observation results become real [21].

Serum is a cosmetic with a high concentration of active substances and low viscosity, which delivers a thin film of active ingredients on the skin surface. Serums are formulated with low density and are less clear (semi-transparent), with a higher dynamic ingredient content than typical topical preparations [22]. One of the advantages of serum preparations is that there are more active substances in serum than in other cosmetic preparations. Hence, the serum is faster and more effective in treating skin problems [23]. Serum cosmetics began to be developed due to lifestyle changes where consumers want to simplify cosmetics to save time, concentrated forms that provide better effects than other topical preparations, and the development of production techniques in the cosmetics industry [24].

Previous research Jalestri & Taufikurohmah (2016) conducted antifungal testing against Candida albicans with the addition of 10, 15, 20% nanosilver in morning cream preparations with the results of 20% nanosilver concentration morning cream preparations showing the most optimal antifungal activity against *Candida albicans* [10]. In addition, researchers Tantyani & Taufikurohmah (2020) conducted antifungal testing against Candida albicans with nanosilver concentrations of 30, 40, 50, and 60 ppm with the result that a nanosilver concentration of 60 ppm showed the most optimal antifungal activity against *Candida albicans* [25]. Based on the description above, it is known that the antifungal activity of nanosilver in serum cosmetic preparations has not been reported; therefore, researchers are interested in researching the antifungal activity of nanosilver in serum cosmetic practices with nanosilver concentration variations of 5%, 10%, and 15%.

RESEARCH METHODS

Synthesis and Characterization of Nanosilver

Nanosilver was synthesized from colorless 1000 ppm $AgNO_3$ base material. The 1000 ppm AgNO3 solution was obtained by dissolving 1.57 g of AgNO3 crystals (white) into a 1000 mL volumetric flask, then diluted with *aquabidest* to the limit. A 1000 ppm AgNO3 solution was formed, ready to be used as an ingredient in the synthesis of nanosilver [25]. Nanosilver synthesis was carried out by bottom-up method by heating 1000 mL of distilled water in a beaker, heated to 60ºC. Before boiling, the aquadest was reduced by 20 mL, and 2 grams of sodium citrate and 20 mL of 1000 ppm AgNO3 were added. Heating was continued until the solution was grayish-yellow (stable). Furthermore, the nanosilver colloids that have been synthesized are characterized by UV-Vis Spectrophotometer and Transmission Electron Microscopy (TEM) [10].

Preparation of Serum

Serums are made using the following formulation [24].

Table 1. Serum Formulation

Materials	F1 (-)	F2 (+)	F3	F4	F5
Nanosilver (%)	-	-	5	10	15
Carbomer (%)	0.45	0.45	0.45	0.45	0.45
TEA (%)	0.2	0.2	0.2	0.2	0.2
Propilen Glykol (%)	10	10	10	10	10
Methylparaben (%)	-	0,2	-	-	-
Ad *aquadest* (%)	100	100	100	100	100

The serum was prepared by weighing the carbomer and dissolving it in distilled water. After that, it was left to swell for 24 hours. Then, propylene glycol was added and stirred constantly (mixture A), dissolved methylparaben, and put into propylene glycol (mixture B). Mixture A and mixture B were mixed until homogeneous. Then TEA was added drop by drop until a slightly thick mass was formed and stirred until homogeneous. After the serum base was finished, nanosilver was added to the serum base and stirred until homogeneous.

Physical and Chemical Characteristics of Serum Cosmetic Preparations

Serum that has been made is tested with several tests, as follows

1. Organoleptic Test

   Organoleptic tests were performed visually on the serum preparations, including color, odor, and texture. This test used 15 panelists who were asked to assess the scent, color, and texture of the serum to measure the panelist's level of liking for the serum.

2. Homogeneity Test

   Serum homogeneity testing is carried out to show that the serum that has been made is homogeneous and there are no visible coarse grains in the serum. The serum samples are applied to glass objects to see whether there are coarse grains [26].

3. pH Test

   Determination of serum pH was carried out using universal pH by dripping the serum onto the pH paper and then observing the color

change that occurred. The color change that occurs indicates the pH of the serum [27].

4. Irritation Test

Testing was carried out by testing serum samples on human skin. This irritation test was conducted on 15 panelists; each tested each formula preparation. The serum will be dropped as much as one drop on the upper part of the arm because the upper part is a sensitive part like the face, so it can be used for irritation testing. Then, observe the symptoms that arise, such as redness, itching, and skin swelling [28].

5. Scanning Electron Microscopy (SEM) Test

Scanning Electron Microscope (SEM) was used to observe the size and morphology of the silver nanoparticles in the serum. The liquid serum was prepared semi-solid before being tested with the SEM instrument. The test results will be obtained as images of the sample surface.

Antifungal Activity Test

Antifungal activity testing uses the DMC (Direct Microscopic Count) method by counting the number of fungi in the population under a microscope. Testing is done by adding 1 mL of serum sample to the glass object, covering the model with a protected glass, and then marking to separate each spot.

Sample testing was carried out by observing samples under a microscope for eight weeks, starting from week 0 to week 8, in an open room. The microscope magnification used in the observation is 40x and 100x Magnification [29]. A microscope takes pictures of fungi that grow during observation with an Optilab viewer and counts the number of hyphae fungi produced.

RESULTS AND DISCUSSION

This study aims to determine the characteristics of nanosilver synthesized using UV-Vis Spectrophotometer and TEM, determine the physical and chemical characteristics of nanosilver serum preparations, and determine the antifungal activity of nanosilver in serum cosmetic preparations. This research was conducted in 3 stages: synthesis and characterization of nanosilver, physical and chemical characterization of nanosilver serum cosmetic preparations, and antifungal activity test.

Synthesis and Characterization Nanosilver 20 ppm

In this study, 20 ppm nanosilver was synthesized using a chemical reduction method by adding reducing agents and stabilizers in sodium citrate. Sodium citrate, as a reducing agent, converts the silver Ag^+ ion solution into a colloidal solution of silver Ag^0 particles [30]. As a stabilizer, sodium citrate controls the homogeneous formulation of nanoparticles with the desired size and geometric shape and prevents agglomeration and possible precipitation of nanoparticles [31]. The synthesis process was stopped after the color change of the solution to a stable yellow as follows.

Figure 1. Synthesis Nanosilver.

The reaction that occurs in the synthesis of nanosilver is as follows:

$$2\ Ag^+ + C_6H_5O_7^{3-} \rightarrow 2Ag^0 + C_5H_4O_5^{2-} + H^+ + CO_2$$

[32]

The reaction during the synthesis process is that sodium citrate as a reducing agent will undergo hydrolysis when put into distilled water to form citric acid, reducing Ag^+ ions to Ag^0. Silver ions (Ag^+) are concentrated in an aqueous solution, receiving electrons from the reducing agent to switch from a positive valence state to a zero valence state (Ag^0), followed by nucleation and growth. This leads to coarse agglomeration into oligomeric clusters to produce colloidal AgNPs [33].

A UV-Vis Spectrophotometer characterized the synthesized nanosilver to confirm its stability. The characterization of silver nanoparticles using a UV-Vis spectrophotometer is based on the absorption of UV-Vis light energy by the outermost electron layer of silver metal so that it is excited to produce waves that move transversely and are known as surface plasmon resonance (SPR) [34]. Tests were conducted twice, at week 0 and week 8. The nanosilver's absorbance range (λ max) is from 380 to 450 nm [35].

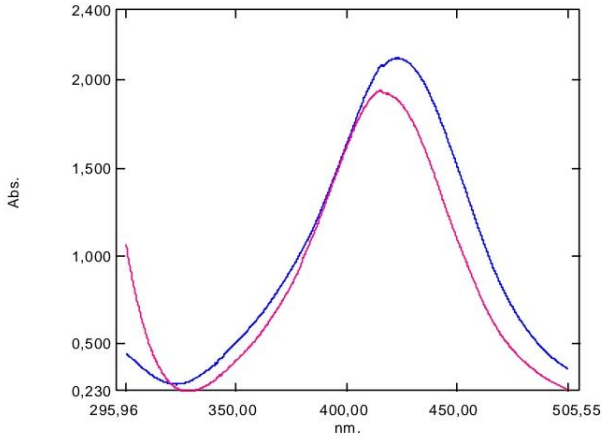

Figure 2. Surface Plasmon Resonance Spectra of Nanosilver Over 2 Months of Storage

Based on Figure 2, the wavelength peak and absorbance are listed in the following table.

Table 2. Wavelength Peak and Absorbance of Nanosilver

No	Week	Maximum Wavelength (nm)	Absorbance
1.	0	415.20	1.9400
2.	60	423.30	2.1254

Based on the cluster diameter values in the table, it shows that the 20 ppm nanosilver is still in the nanoscale range, which is between 1-100 nm, so it can be said that the 20 ppm nanosilver particles are pretty stable even though they are stored until the 8th week. The stability of nanosilver needs to be observed because nanosilver tends to aggregate. Aggregation occurs through addition reactions on single nanoparticles with growing clusters. In addition to the addition reaction, collection can be formed due to the assimilation process of a group into a more extensive set. The existence of interparticle solid forces on nanosilver makes the particles come closer and gather to form a larger cluster over time [36].

Furthermore, the nanosilver was characterized using a TEM (Transmission Electron Microscopy) instrument. Testing using this TEM instrument aims to determine the synthesized nanosilver's morphology, structure, and particle size.

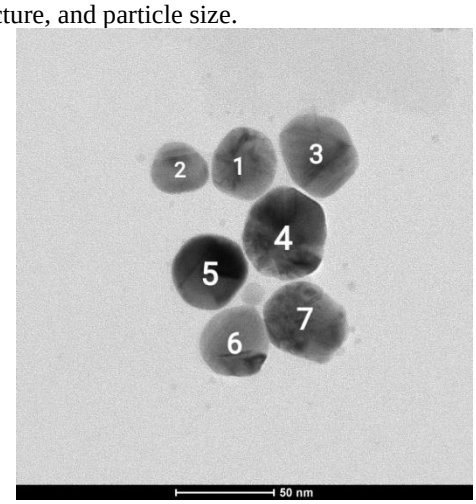

Figure 3. Characterization of 20 ppm Nanosilver Colloids with 80,000x Magnification Using TEM

Based on the results of TEM testing, the nanosilver's morphology obtained from this test is spherical. This is by research conducted by [20]; the morphology of nanosilver obtained shows a spherical shape when nanosilver is synthesized with sodium citrate as a reducing and stabilizing agent.

In addition to particle morphology, the particle size of nanosilver can also be determined from characterization with TEM. The particle diameter size of nanosilver in Figure 3 is shown in Table 3.

Table 3. Diameter Size of Nanosilver Particles

No	Diameter (nm)
1	31.548
2	25.566
3	36.572
4	42.615
5	38.860
6	35.000
7	35.364
Average	35.075

Based on the results showed that 20 ppm nanosilver has a cluster diameter size that varies from 25-42 nm. The size range of 20 ppm nanosilver particle diameters falls into the field of human skin pore diameters of around 20-50 nm, so if nanosilver is added to cosmetic formulations and is suitable for application to human skin because nanosilver can penetrate the pores of human skin [12].

Physical and Chemical Characteristics of Serum Cosmetic Preparations

1. Organoleptic Tests

The results of organoleptic testing of serum preparations by panelists with variations in the addition of nanosilver as much as 5%, 10%, and 15% with negative control and positive control in each preparation labeled F1, F2, F3, F4, and F5 in week one including texture, color, and aroma can be seen in this figure.

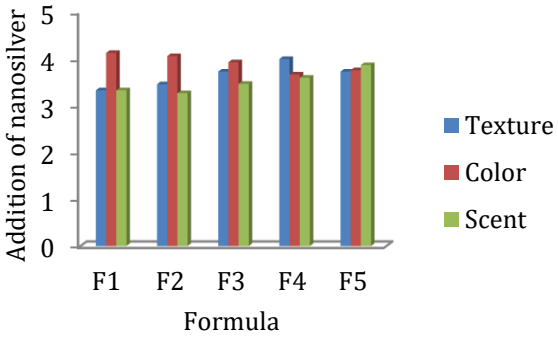

Figure 4. Organoleptic Test Results – Week 1

The diagram of organoleptical observations in week 1 shows that the sample most favored by panelists in terms of texture is the serum sample labeled F4 with the addition of nanosilver as much as 10% with an average value of 4. Then, in terms of color, the sample most preferred by the panelists was the F1 label serum sample, which was the negative control, without the addition of methylparaben and nanosilver preservatives, resulting in a clear white serum sample with an average value of 4.13. Furthermore, regarding aroma, the most preferred sample by the panelists was the serum sample labeled F5, namely with the addition of 15% nanosilver with an average value of 3.87.

Organoleptical observations were continued until week 4.

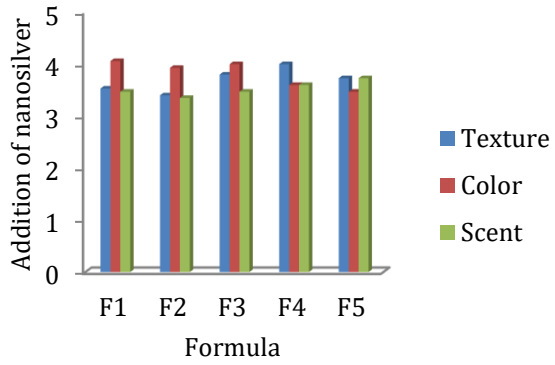

Figure 5. Organoleptic Test Results – Week 4

Based on the diagram of organoleptic observation in week 4, it shows that the sample most favored by panelists in terms of texture is the serum label F4 sample with the addition of 10% nanosilver with an average value of 4. Then, in terms of color, the most preferred sample by panelists was the serum label F1, which was the negative control, without adding methylparaben and nanosilver preservatives with an average value of 4.06. Furthermore, regarding aroma, the most preferred sample by panelists is the serum sample labeled F5, with the addition of 15% nanosilver with an average value of 3.73.

Based on the organoleptic testing conducted, it shows that the addition of nanosilver does not affect the texture, aroma, and color of the serum preparation during four weeks of storage.

2. Homogeneity Tests

Serum homogeneity testing determines whether the serum preparation is homogeneous and has no visible coarse grains. Serum homogeneity testing is done with a certain amount of sample applied to a piece of glass or other suitable transparent material and observing whether coarse particles are on the glass object [26]. The results of the serum preparation homogeneity test are as follows:

Table 4. The results of the serum preparation homogeneity test

Sample	Homogeneity
F1 (Control Negative)	Homogenous
F2 (Control Positive)	Homogenous
F3 (NS 5%)	Homogenous
F4 (NS 10%)	Homogenous
F5 (NS 15%)	Homogenous

Based on the homogeneity test that has been carried out, the five serum samples show the absence of coarse particles on the glass surface. So, it can be concluded that the five serum preparation samples are homogeneous.

3. pH Test

In this study, it is necessary to test the pH of the serum because this serum is applied topically to the skin, so the pH value of the serum preparation must be followed with the pH of the skin so as not to irritate the skin. The standard pH of the skin is in the range of 4.5 - 6.5 [23]; [37]. One of the requirements for cosmetic preparations when applied to the skin is that the pH of the preparation should not be too acidic or too basic. If the pH of the preparation is too acidic, it can cause the skin to become inflamed and cause acne; if the preparation's pH is too alkaline, it can cause the skin to become dry and sensitive [23].

Table 5. Serum pH Testing Results

Serum Samples	pH values
F1 (Control Negative)	6
F2 (Control Positive)	6
F3 (NS 5%)	6
F4 (NS 10%)	6
F5 (NS 15%)	6

The results obtained can be concluded that the results of the serum pH test with active substance variations of 5%, 10%, and 15%, and negative control and positive control are good because they enter the skin pH range so that the serum is safe when applied to the skin. This is because all serum formulations fall within the skin pH range in the field of 4.5 - 6.5 [23]; [37].

4. Skin Irritation Tests

Irritation testing on the skin determines how much irritation is caused by the serum produced. Testing is done by testing serum samples on human skin. Serum preparations will drop as much as one drop on the arm's upper part because it is sensitive to the face, so it can be used for irritation testing. Then, observe symptoms such as redness, itching, and burning on the skin [28]. In this study, the skin irritation test was conducted on 15 panelists; each panelist tested each formula preparation.

The tests carried out by the 15 panelists on each variant of the serum preparation formula show that none of them experienced irritation in the form of redness, itching, or burning when applied to the skin. This shows that the serum that has been made is safe to use and does not cause irritation to the skin because all five serum samples have a pH value of 6, which is by the pH of the skin is in the range of 4.5 - 6.5 [23]; [37].

5. Scanning Electron Microscope (SEM) Test

Testing using a Scanning Electron Microscope (SEM) instrument was used to observe the size and morphology of the silver nanoparticles present in the serum. The sample analyzed using SEM is a serum sample with the addition of 15%

nanosilver. The following are the results of testing nanosilver serum samples using SEM.

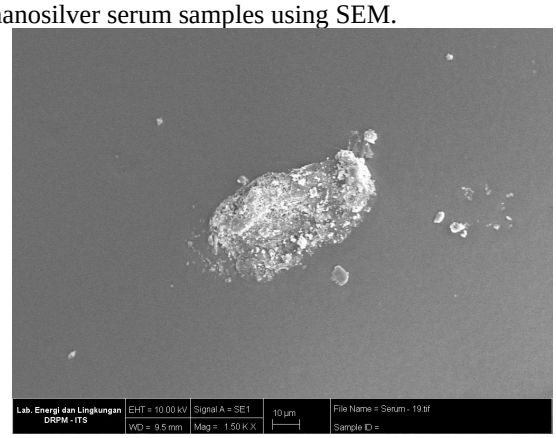

Figure 6. SEM Results of 20 ppm Nanosilver In Serum Sample with 6,000x Magnification

Based on the SEM observation, the nanosilver particles are homogeneously mixed and attached to the serum sample. The morphology of nanosilver particles is not visible in the image, but it can be seen that the particles are formed slightly round. This SEM test was measured with a magnification of 6,000x and a voltage acceleration of 10 kV.

According to [20], characterization using SEM has disadvantages, such as destructive sample preparation and the inability to ensure that the observed image is representative of the sample, leading to biased statistics of heterogeneous size distributions.

Antifungal Activity

In this test, the antifungal activity of nanosilver was tested using the Direct Microscopic Count (DMC) method. This test was conducted to prove that nanosilver has good antifungal ability when applied in serum cosmetics. The DMC method is a method of counting the number of cells in a population under a microscope to determine the development of fungi on the surface of a sample that is usually found in an environment that can experience growth on the surface of the model so that observations are actual [21].

Testing the antifungal activity of nanosilver is done by adding 1 mL of serum sample to the glass object, and then the sample is covered with a glass cover. Sample testing was carried out by observing samples under a microscope for eight weeks, starting from week 0 to week 8, in an open room. The microscope magnification used in the observation is 40x and 100x Magnification because, based on research conducted [29], the fungi can be observed under a microscope with this Magnification.

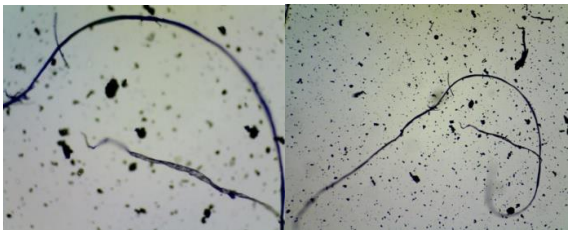

Figure 7. Observation of Antifungal Activity of Nanosilver Serum 40x Magnification (left) 100x Magnification (right)

The results obtained from this test are data on the amount of fungal growth observed for eight weeks. Image Raster can count the growing hyphae of fungi through the image taken using Optilab Viewer software. To determine the percentage rate of inhibition of hyphal growth of fungi can be calculated using the following formula [38]:

$$\% = \left(\frac{\text{Hyphae count}}{\text{Counting Area}}\right) \times \left(\frac{\text{Counting Area}}{\text{Total Area}}\right) \times 100\%$$

From the calculation results using the formula above, the following effects can be obtained:

Based on the values in the table 6, a diagram of the percentage of antifungal activity of nanosilver serum can be obtained.

It can be seen that the inhibition of fungal growth is seen from the number of fungi that decreases during the eight weeks of observation in serum with the addition of 20 ppm nanosilver, both at concentrations of addition as much as 5%, 10%, and 15%. Based on Table 6, serum with the addition of nanosilver could inhibit fungi with the growth of hyphae numbers of 3.25, 3, and 2.75%, respectively. Meanwhile, serum preparations without the addition of preservatives and nanosilver experienced a change in the number of fungal hyphae of 24.25%, and serum with the addition of methylparaben preservative showed an increase in the number of fungal hyphae of 22.25% at week 8.

Testing the antifungal activity of nanosilver was followed by statistical testing using One Way Anova to determine significant differences in the treatment of the samples.

Statistical testing using One Way Anova shows a significant probability result of 0.049 ($p < 0.05$), which means that each sample has a different effect on each model with other treatments. Based on the data analyzed by SPSS using the One-Way ANOVA test, nanosilver inhibits the growth of fungal hyphae in serum preparations.

Nanosilver as an antifungal works by making contact with microorganisms using the surface area of nanosilver attached to the fungal cell wall. Nanosilver will release silver ions and bind to certain protein groups that affect the function of membrane proteins and disrupt cell permeability. Silver ions will also inhibit mycelial growth in fungi by inhibiting conidia germination and suppressing their development. Silver ions also cause structural changes and damage and markedly disrupt vital cell functions such as

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permeability and membrane potential. Silver ions affect mitochondrial membrane potential by increasing gene transcription levels in response to oxidative stress. ROS (Reactive Oxygen Species) generates reactive oxygen species, triggering oxidation reactions catalyzed by silver ions (nanosilver), causing damage to proteins, membranes, and DNA and disrupting nutrient uptake. Nanosilver ions have a genotoxic effect that suppresses the activity of respiratory chain enzymes and destroys DNA, eventually leading to cell death [13-14].

Table 6. Percentage of Antifungal Activity of Serum Nanosilver

Sample	Week							
	1	2	3	4	5	6	7	8
F1 (-)	1.75	1.5	2.5	7.25	10	16. 25	19. 75	24.25
F2 (+)	2.25	3.25	3	5.5	7	14.5	17.5	22.25
NS 5%	1	2.5	3.75	10	7	6.5	5	3.25
NS 10%	0.25	1.75	4.75	8.75	6	5	4.5	3
NS 15%	0.25	0.75	3	6.75	5.25	4.25	3.75	2.75

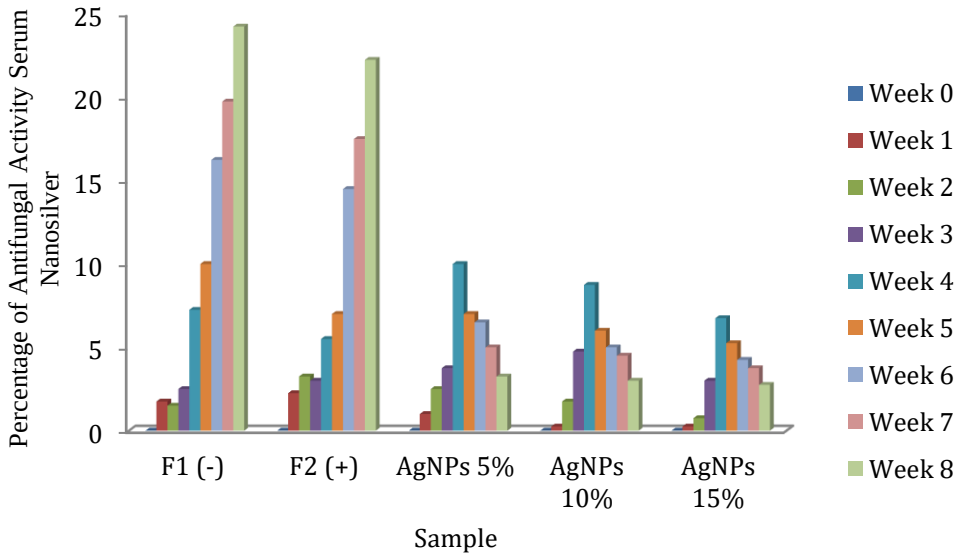

Figure 8. Diagram Percentage of Antifungal Activity Serum Nanosilver

Table 7. SPSS Data Output One-Way Anova Test on Antifungal Activity Testing of Serum Nanosilver

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	330.884	4	82.721	2.664	.049
Within Groups	1086.602	35	31.046		
Total	1417.486	39			

CONCLUSION

Based on the research results, the following conclusions can be obtained: Characterization of synthesized nanosilver with UV-Vis shows that the synthesized nanosilver is relatively stable, with an average diameter of 35.075 nm and spherical shape. The physical and chemical characteristics of the serum preparation carried out by organoleptic testing showed that the five samples were homogeneous, non-irritating, and had a pH of 6. The difference in nanosilver concentration in serum preparations can affect the antifungal activity against fungal growth. Based on observations of fungal growth from week 0 to week 8, it shows that serum preparations with the addition of nanosilver can inhibit fungi with the development of the number of hyphae in F1, F2, F3, F4, F5 respectively 24.25; 22.25; 3.25; 3; and 2.75%.

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