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Research Article

The Immunomodulator Effects of Biosynthetic Nanosilver Based on Immunoglobulin G Level of Mice Induced by Measles Vaccine Compared with Immunostimulant Agent

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ABSTRACT

Inulin is a carbohydrate class contained in gembili tuber (*Dioscorea esculenta* (Lour.) Burkill) as a source of prebiotics to maintain immunity. Inulin can be used as a nanosilver bioreductor and increase the solubility of gembili's inulin. This study aims to determine the effectiveness of gembili's inulin nanosilver as an immunomodulator based on IgG levels of vaccine-induced Mice. Nanosilver at doses of 2 mg/KgBW, 4 mg/KgBW, 8 mg/KgBW, and 12 mg/KgBW was compared with synthetic immunostimulants to observe the type of immunomodulatory activity. This research was conducted in several stages, including the biosynthesis process, characterization, determination of nanosilver dose, animal conditioning, animal treatments, taking blood from retro-orbital sinuses, and ELISA reader. Blood serum obtained from the animals was analyzed for IgG levels using Mice IgG ELISA Kit and Spectrophotometer UV-VIS. The pH variation test was also conducted in this study which aims to determine the stability of the nanosilver solution at pH 8, 10, and 12. The results of the ELISA reader showed that the dose of 4 mg/KgBW. had the highest Optical Density value compared to other doses. Then, the comparison with synthetic immunostimulants produces p values of 0.13 >0.05, which is not significantly different. Nanosilver inulin at 4 mg/KgBW. is the chosen dose as an immunomodulator with immunostimulant activity. The stability results showed that increasing pH changes do not affect the pH change of gembili's inulin nanosilver solution. It is because nanosilver's pH control and adjustment are still in the alkaline range.

INTRODUCTION

The immune system works to recognize and kill foreign objects that enter the human body (Larenas-Linnemann *et al.,* 2020). Vaccination is one way to maintain the immune system against infectious agents. The measles vaccine is one type of live vaccine with a strain-weakened virus, so protective antibodies are formed when exposed to the measles virus (Yahmal, 2021). The measles vaccine has an efficacy of approximately 85%, so a weak immune system can be infected with measles again (Kemenkes RI, 2017). Accordingly, supplements that have an immunostimulant effect or can increase the activity of the immune system is necessary (Larenas-Linnemann *et al.,* 2020). Nanoparticles are solid colloidal particles with diameters ranging from 1-1000 nm (Tiyaboonchai, 2003). Silver is a metal developed in the medical world with the most effective antibacterial activity in animal cells. Silver ions can cause toxicity in organ tissue because it produces thiol affinity, which is high in the liver and other organs (Tiwari *et al*., 2011). When silver is made into nanoparticles, there is an increase in contact with bacteria that cause the

effectiveness of antibacterial activity (Ahmed *et al.,* 2016) and reduces toxicity (Mohanraj and Chen, 2006). Physical, chemical, and biological methods can synthesize Nanosilver. Synthesis of Nanosilver with biological methods is preferred because it is more environmentally friendly and minimizes waste and the dangerous use of inorganic materials. This method is better known as a bioreactor because it is done using reducing agents from plant extracts (Fabiani *et al*., 2018). Biosynthesis of Nanosilver using plant extracts has been widely done (Dubey *et al.,* 2010).

Gembili tubers are local Indonesian tubers used as a silver-reducing agent to produce Nanosilver and increase the body's immunity (Fabiani *et al.,* 2018). Gembili tuber (*Dioscorea esculenta* (Lour.) Burkill) contains 14.77% of Inulin, one of the oligosaccharides groups, which can enhance the mechanism as an immunostimulant (Khasanah *et al.,* 2019). *Inulin* is a carbohydrate class that contains plant fiber and is widely used in health as a source of prebiotics to increase body immunity (Azhar, 2009). Gembili's Inulin includes long carbohydrates chain of 2-60 units. Long-chain Inulin (22-60 units) is less soluble and more viscous (Dewanti and Rahayuni, 2013). Nanoparticle preparations have the advantage of increasing the solubility of gembili's Inulin. Small particle size is used to design, build, and manipulate materials to produce materials with new properties and functions (Albrecht *et al.,* 2006).

Research by Tiwari *et al.* (2011) concluded that Nanosilver with a dose of 10 mg/Kg BW is a safe and recommended health treatment. The immune system can be detected based on the levels of antibodies formed in the body. Antibodies are immunoglobulin proteins secreted by antigen-fixed B cells. One of the antibodies, Immunoglobulin Gamma (IgG), plays an important role. IgG is an isotope of the primary antibody in blood and extracellular fluid that controls its occurrence of infection in body tissues. The formation of IgG antibodies provides a possibility that the immune system has formed antibodies against infection (Abdul-Hamid *et al.,* 2021). This research aims to know the immunomodulatory effect of nanosilver based on IgG levels as a reference for analyzing the increase in body immunity. The method used for diagnosing IgG levels is Enzyme-Linked Immunosorbent Assay Analysis (ELISA) using a Mice IgG ELISA Kit.

The ELISA method is one of the immunological techniques that aims to measure levels based on activity or expression response protein and the immune reaction of an individual. The advantage of the ELISA method is that it uses antibodies with high specificity so that the accuracy of materials or analytes can be relied on (Santosa, 2020). The working principle of colorimetry-based ELISA is the light intensity absorbed in a colored solution with waves-specific values with readable absorbance (Santoso *et al.,* 2013). The test animal's serum was analyzed using the ELISA reader method to determine IgG levels in the test mice's blood serum after being induced by the measles vaccine. After that, the results were compared with the levamisole immunostimulant to determine the type of nanosilver immunomodulatory activity. The Nanosilver solution is expected to remain stable in the research process for four weeks. The length of storage time will impact changes in the pH nanosilver solution. Therefore, to determine the stability of nanosilver preparations with gembili's inulin bioreductor, the stability test of the preparation was also conducted at different pH conditions.

METHODS

Instrumentation

Hematocrit (Vitrex Medical), box ice, Plate (well), vortex (Barnstead), stopwatch, sealer, hotplate (Maspion), digital scale (Precisa), refrigerator (Aqua, Japan), Centrifuge (Hettich Zentrifugen Mikro 22), Elisa Reader (Spark, Tecan), incubator (Sakura, EM200T), ultraviolet-visible (UV-VIS) spectrophotometer (Genesys-150), pH meter (Lutron PH-207), Particle Size Analyzer (Malvern Panalytical). Materials: gembili's inulin powder (UMKM Plalangan, Gunungpati, Semarang, Central Java, Indonesia), silver nitrate ($AgNO_3$) (Merck, Germany), standard inulin (Aldrich SIGMA, USA), dry measles vaccine (Biofarma, Rajawali Nusindo), Mice IgG ELISA Kit (Bioassay Technology Laboratory), Levamisole (Konimex), Prednisone (Phapros), water, male mice Balb-C strain (CV Dunia Kaca, Surakarta, Indonesia), and NaCl (Merck KGaA).

Ethical clearance

Ethical clearance is an instrument for measuring the ethical acceptability of research processes. Ethical clearance is a reference for researchers to ensure integrity, honesty, and fairness in conducting research. Ethical clearance aims to protect researchers from ethical demands in research (LIPI, 2022). This study received ethical clearance from the "Laboratorium Penelitian dan Pengujian

Terpadu" Universitas Gadjah Mada, Yogyakarta, Indonesia.

Biosynthetic proces

Gembili's inulin powder of 10 g is dissolved in 250 mL aquadest at room temperature 25 °C (solution A). Silver nitrate powder of 85 mg dissolved in 500 mL of distilled water at 40 °C (solution B). Mix 7.0 mL of solution A and 36 mL of solution B at 60 °C for 15 minutes. The mixed solution was stored at room temperature for 24 hours (Ermawati *et al.,* 2020). A Nanosilver solution of 1.0 mL was inserted into the cuvette to measure the maximum wavelength in the nanosilver SPR range at 400-500 nm using a UV-VIS spectrophotometer. Results measurements were compared to inulin's standard nanosilver.

Characteristic of nanosilver

Characteristic of nanosilver includes scanning at SPR range of nanosilver and particle size. The nanosilver solution was scanned at 400-500 nm wavelength using UV-VIS spectrophotometry. Particle size was observed with Particle Size Analysis (PSA) instrument. A Nanosilver solution of 1.0 mL was analyzed using PSA. Results measurements were compared to inulin's standard nanosilver.

The pH stability test

The pH stability test of gembili inulin's nanosilver solution was conducted based on a shift in the maximum wavelength. The nanosilver solution's absorbance results after pH adjustment using a UV-VIS spectrophotometer. The procedure was done in an adjusted nanosilver solution. The pH value of the medium was added with 0.1 mol/L NaOH solution (Putro *et al.,* 2022). In this study, the control pH was 8, and the pH variation of the medium solution was 10 and 12.

Animal treatments

The test animals were grouped into two large groups: the treatment group and the control group. The treatment group was divided into four groups, each group consisted of three mice (nanosilver dose of 2 mg/Kg body weight (BW); 4 mg/KgBW; 8 mg/KgBW; and 12 mg/KgBW). The control group was divided into five, with each control consisting of three mice (aquadest control, inulin control, $AgNO_3$ control, prednisone control, and levamisole control). In the neutral control group, mice were given aquadest at a dose of 0.5 mL/20g BW. The prednisone-positive control group contained the compound immunosuppressants at a dose of 0.06 mg/20g BW. In the positive control group, levamisole contains an immunostimulatory compound at 0.455 mg/20g BW (Ermawati *et al.,* 2016). The treatment was done for seven days before the test animals were vaccinated, until the experiment was complete.

Vaccination was conducted using the measles vaccine intraperitoneal at a dose of 6.5 µL/20g BW. The vaccine is dissolved in 0.9% NaCl solution, so it is isotonic with blood in the test animal's body. Giving the measles vaccine is recommended twice (Yahmal, 2021). Therefore, in this study, all test animals were given the vaccination treatment twice on the 7th and 14th days. The test animal's blood sample of 0.25 mL was taken from the retro-orbital sinus in the eye as a baseline. Blood sampling was done again on the 12th and 19th days. The blood sample was isolated and centrifuged to obtain the serum expected to contain IgG. The blood sample of each test animal is included in the Eppendorf 2.0 mL and conditioned at room temperature for about 1 hour. Then, it was centrifuged at 14000 rpm for 15 minutes (Manurung and Saptini, 2020). The resulting serum was stored at -20 °C until the serum was ready for reading antibody levels.

A standard solution of 50 µL was put in well standard. A serum test of 50 µL was put into the serum well. Add 50 µL of biotinylated antigen to each well and mix slowly. The plate was closed using a sealer and incubated for 60 minutes at 37°C. After incubation, the sealer was opened, and the liquid in the well was thrown away. The wells were washed five times with 300 µL Wash Buffer manually. The plate was stamped upside down 4-5 times on the material absorbent to ensure no liquid sticks to the well. Avidin-HRP solution of 50 µL was added to the standard and sample wells. The plate was closed using a sealer and incubated for 60 minutes at 37°C. After incubation, the cover was removed, and the plate was washed. A substrate-A solution of 50 µL was added to each well, and a substrate-B solution of 50 µL was added to each well. The plates were incubated for 10 minutes at 37 °C in a dark room. After incubation, each well was added 50 µL stop solution. The blue color of the solution will change to yellow after the addition of the stop solution. Results are well-read on ELISA to determine IgG levels in each serum sample (Elabscience, 2021).

Data analysis

Analysis using the SPSS 21 (SPSS Corp., College Station, TX) program with the one-way

ANOVA test to find out significant differences between group's treatment (Cleophas *et al.,* 2012). Meanwhile, the stability test results from data of nanosilver's pH changes were analyzed using the program SPSS 21 with the Wilcoxon test. If the *p*-value is <0.05, this result indicates a significant difference, whereas if the *p*-value is >0.05, this indicates the result is not significantly different.

RESULTS AND DISCUSSION

Nanosilver biosynthesis process is done by dissolving 7.0 mL gembili's inulin solution in 36 mL silver nitrate solution at 60 °C for 15 minutes. Research by Kaviya *et al.* (2011) stated that biosynthesis at 60 °C has a fast reaction process and produces a smaller particle size than at room temperature. Biosynthesis produces a homogeneous gembili's inulin nanosilver solution. It happens because nanoparticles can increase the solubility of gembili's inulin (Albrecht *et al.,* 2006). In addition, there was a color change to yellowish-brown in the solution after 15 minutes of the biosynthesis process. Then, the mixed solution was conditioned at room temperature for 24 hours to maximize the biosynthetic process (Ermawati *et al.,* 2020). A color change in yellowish-brown indicates the success of the nanosilver biosynthesis process using plant extracts. The color change occurs because of the R-CHO group, which can react with Ag+ form Ag0 silver nanoparticles (Figure 1). The results of the PSA analysis showed that nanosilver has a Z-average value of 481.4 nm. As for nanosilver preparations, inulin's standard has a Z-average of 109.6 nm. Z-average was the main result of PSA, which shows the average value of particle distribution (Scientific, 2012). Although there were differences in the results of the Z-average values between nanosilver gembili's inulin with inulin's standard, the results of the Z-average values were still in the range of nanoparticles (1-1000 nm). Based on the particle size characterization results, the biosynthetic process using gembili's inulin formed a nanosilver. The maximum wavelength obtained in the SPR range of nanosilver was 418 nm, which follows the SPR range for the nanosilver between 400–500 nm (Kaviya *et al.,* 2011).

Stability Test

The effect of pH on the stability of nanosilver was evaluated to determine the difference in the pH value of the solution when the pH of the media was adjusted. The test consisted of three pH variation conditions: pH 8 as the control pH and pH treatments 10 and 12. The nanosilver solution color was dark yellow, so its readings were in the visible wavelength range of 380-780 nm using an UV-Vis spectrophotometer. Based on the measurement results, the higher the pH value, the more significant the maximum wavelength shift. Analysis of pH stability test results was also conducted statistically using the Wilcoxon Test to determine whether there is a statistically significant difference between the control pH (pH 8) with pH adjusted solutions (pH 10 and pH 12). The Wilcoxon Test is used because the Shapiro-Wilk data indicated Sig <0.05, which means the data were not normally distributed.

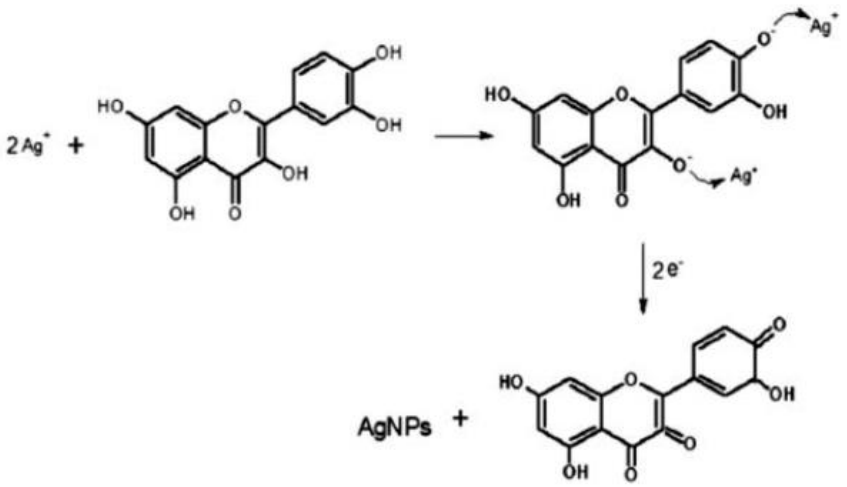


Figure 1. The possible mechanism of silver ion reduction by plant extract (Jain *et al*., 2017)

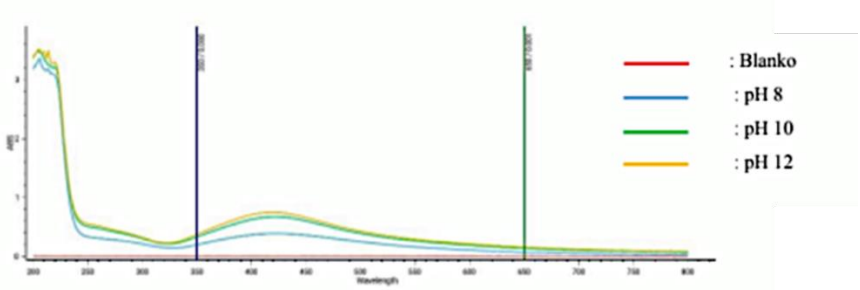


Figure 2. The results of scanning wavelength shift of nanosilver solution in various pH media

Based on the results of the Wilcoxon Test, the average maximum λ position of the nanosilver at pH 10 and pH 12 compared to pH 8 shows the Asymp value. Sig >0.05 means there was no significant difference when pH adjustments were done to the maximum λ position of nanosilver. However, the UV-Vis spectrophotometer readings on all three replications showed that the maximum λ shift is quite significant at pH 10 and 12 compared to a pH of 8. pH changes increasingly in alkaline conditions affect the maximum lambda shift of the nanosilver solution, but the result was not significant. This is because nanosilver's control pH and pH adjustment were still in the alkaline range.

Immunomodulator Test

This research was declared to have met the ethical requirements for research on experimental animals with document number 00029/04/LPPT/VIII/2021 by the Ethical Clearance Commission, Laboratory Integrated Research and Test, Universitas Gadjah Mada, Yogyakarta, Indonesia. The effectiveness study of nanosilver gembili's inulin doses was done on the Balb-C strain male mice aged 4-8 weeks weighing 20-25 g. Test animals were conditioned for one week to adapt to the test environment. The test animals were given oral treatment according to the dose group. Gembili's inulin nanosilver solution was made in four dosage ratings to observe their most effective dose. The dose of nanosilver consisted of 2 mg/KgBW; 4 mg/KgBW; 8 mg/KgBW; and 12 mg/KgBW. Group control consisted of control Prednisone, Levamisole, inulin, silver nitrate ($AgNO_3$), and aquadest. Prednisone was an immunosuppressant control to suppress/normalize abnormal immune reactions (Baratawidjaja and Rengganis, 2012). Levamisole was an immunostimulant control activity to increase the body's immune reaction (Baratawidjaja and Rengganis, 2012). Inulin control was used to compare the effectiveness of inulin as an immunomodulator of water-soluble fiber and nanosilver. $AgNO_3$ control was used for comparison of the activity of $AgNO_3$ as an immunomodulator before and after the biosynthesis process becomes nanosilver. Aquadest control was used as a neutral control. The solution was given orally daily in test animals according to the treatment group.

Vaccination against measles was recommended twice in administration (Yahmal, 2021). Vaccination was conducted intraperitoneally on the 7th and 14th days. Intraperitoneal vaccination was chosen because it produces the highest immune response (Sugiani *et al.,* 2015). In contrast, blood was taken on the retro-orbital sinus section on the 0th, 12th, and 19th days. Taking blood in the retro-orbital sinus was a blood sampling method with experimental animals that could survive. The sinus retro-orbital was located behind the eyes (Handajani, 2021). Blood drawn on day 0 was used as a baseline to compare changes in the immune response to the vaccine during treatment. Blood sampling on the 12th day was used to see the immune response to the vaccine given for the first time and blood drawn on the 19th day was used to see the increase in antibodies after administration of the second vaccine. Blood samples were centrifuged to obtain serum containing IgG of test animals. The main principle of centrifugation is to separate substances based on molecular weight by providing centrifugal force so that the heavier substances will settle while the lighter substances are at the upper layer of the solution. The centrifugation technique is done in a centrifuge machine at various speeds (Masruroh *et al.,* 2018). The centrifugation process was conducted at 14,000 rpm for 15 minutes. The centrifugation will separate the clear-colored serum from yellowish and red blood cells, where the serum will be at the upper layer. The serum was stored at -20 °C and then used for the antibody level reading.

This study used the Indirect ELISA technique to search antibodies using specific antigens. The diluted standard was added to the sample, which sets the curve to determine the formula for determining the sample content. The incubation process aims to produce antigen-antibody complexes optimally. Washing aims to remove unbound antibodies. A secondary antibody is added and labeled with the enzyme resulting in an antigen-antibody binding complex with an enzyme-labeled antibody. The addition of substrate aims to produce color precipitates. A stop solution was added at the final stage, which works for a stop reaction. The absorbent was read on an ELISA reader and qualified using the expert curve on standard absorbance values (Santosa, 2020). The result of the reading using the ELISA reader is an absorbance value or Optical Density (OD) (Figure 3 and Table 1). Measuring the absorbance with the calibration curve is to determine the relationship between concentration and absorbance. The formation of a linear line on the graph shows the concentration and absorbance, which are related to each other linearly. The graph of ELISA reader results in this study shows a linear line which means the OD value is directly proportional to the level of IgG in serum.

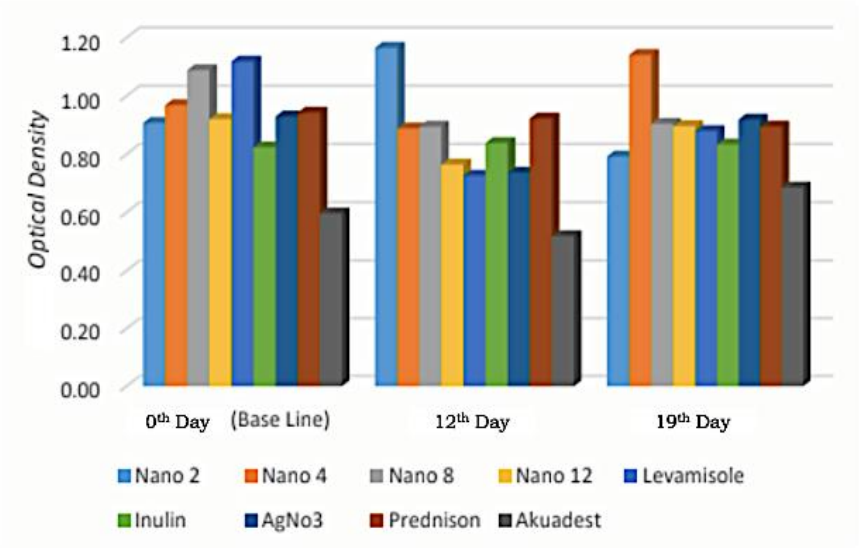


Figure 3. The results of average value of OD from ELISA Reader Analysis.

Table 1. The results of OD value based on ELISA Reader analysis

Treatment's Group	The average value of OD		
	Base line	12th Day	19th Day
Nanosilver 2 mg/KgBW	0.91±0.06	1.17±0.29	0.79±0.09
Nanosilver 4 mg/KgBW	0.97±0.05	0.89±0.08	1.14±0.15
Nanosilver 8 mg/KgBW	1.09±0.12	0.89±0.05	0.90±0.09
Nanosilver 12 mg/KgBW	0.92±0.13	0.76±0.04	0.90±0.05
Levamisole®	1.12±0.16	0.73±0.09	0.88±0.14
Prednisone®	0.94±0.19	0.92±0.10	0.89±0.06
Gembili's Inulin	0.82±0.11	0.84±0.33	0.83±0.11
Silver Nitrate	0.93±0.24	0.74±0.20	0.92±0.05
Aquadest	0.60±0.52	0.52±0.48	0.69±0.14

*Numeric data presented as mean±SD, standard deviation.
OD, Optical Density.

The results showed a decrease in the average OD value on 12th day compared to day 0 (Base Line). The average OD value experienced an increase on the 19th day. On the first vaccination, the primary immune response forms IgM predominately earlier than IgG. After that, in the secondary immune response, IgG will reach the highest titer value. After giving the second vaccination, antibodies in the blood rise sharply and reach maximum levels compared to the primary response. This is due to cell expansion memory formed by the primary response (Santoso, 2020). Based on the OD value, the highest IgG level was formed in blood serum taken after the second vaccination, on the 19th day. Nanosilver at a dose of 4 mg/Kg BW had the highest OD value on the 19th day compared to other nanosilver doses and the control group. The OD score of nanosilver at a dose of 2 mg/Kg BW showed a significant decrease on the 19th day. Nanosilver did not increase significantly at 8 mg/KgBW and 12 mg/KgBW. Research by Tiwari *et al.* (2011) concluded that the safe dose of nanosilver is in the range of 1-10 mg/Kg BW. ELISA reader results in the $AgNO_3$ and Levamisole control group showed immunomodulatory activity, marked by a decrease in value OD on the 12th day and an increase on the 19th day. This was because Levamisole has immunostimulant activity (Baratawidjaja and Rengganis, 2012), and $AgNO_3$ has immunomodulatory activity (Kumar *et al.,* 2018). Gembili's inulin control showed no significant change in OD values. This was presumably because gembili's inulin is a long-chain carbohydrate that was less soluble and more viscous (Dewanti and Rahayuni, 2013), so the immunomodulatory activity was less effective. The OD value of the Prednisone control decreased which was suspected to be an immunosuppressant that functions to suppress the reaction of abnormal immunity (Baratawidjaja and Rengganis, 2012).

The results of the OD values on the 19th day were tested statistically using One-Way ANOVA to determine the differences in the OD values between treatment groups. In the Shapiro-Wilk test and homogeneity, the data indicated a result >0.05, which means that it is normally distributed and homogeneous. The ANOVA test results showed sig 0.017 (<0.05), meaning the data were significantly different between groups. The Post Hoc test found that nanosilver with a dose of 4 mg/KgBW has a significantly different OD value compared to other treatment groups. Based on statistical tests of gembili's inulin nanosilver at a dose of 4 mg/KgBW proved to be the most effective dose as an immunomodulator. This finding was in line with the research of Tiwari *et al.* (2011), that tested AgNPs toxicity and resulted in data indicating a dose of 4 mg/Kg BW was the best dose for therapeutic application. To determine the type of immunomodulatory activity of nanosilver gembili's inulin, a statistical test was conducted to compare the OD value between nanosilver at a dose of 4 mg/Kg BW and levamisole which was a synthetic immunostimulant. Statistical testing was conducted in the form of two-way ANOVA. In the normality test, a Sig value of >0.05 was produced, which means data were normally distributed. The results of the homogeneity test also showed a sig value >0.05. The variation of the data was homogeneous. The two-way ANOVA test results show a comparison of nanosilver dose of 4 mg/Kg BW with levamisole with a Sig value of 0.13 (> 0.05), which means it was not significantly different. Based on test results of nanosilver inulin gembili at a dose of 4 mg/KgBW is an immunomodulator with activity as an immunostimulant.

CONCLUSIONS

Testing of the effectiveness of gembili's inulin nanosilver doses on mice's IgG levels resulted in showing that the nanosilver gembili's inulin at a dose of 4 mg/KgBW was an effective dose as an immunomodulator with immunostimulant activity. The IgG level of mice was increased after vaccine-induced compared to synthetic immunostimulants. Comparison between nanosilver gembili's inulin at a dose of 4 mg/KgBW with synthetic immunostimulant shows that nanosilver was an immunomodulator with immunostimulant activity. The results showed that changes in pH under conditions increasingly do not affect the pH change of nanosilver solution. This finding is because nanosilver's pH control and pH adjustment are still in the alkaline range.

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CONFLICT OF INTEREST

No conflict of interest between all authors

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