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Preparation and Vibrio sp Antibacterial Activity of Silver Nanoparticles Mediated by *Chromolaena odorata* Leaf Extract using Different Temperatures

Emsal Yanuar [a], Khotibul Umam [b], Wirya Sarwana [a], Ihsanul Huda [a], Dani Wijaya [b], Roto Roto [c*] and Mudasir Mudasir [c*]

[a] Department of Metallurgy, Sumbawa University of Technology, Sumbawa, Indonesia.
[b] Department of Biotechnology, Sumbawa University of Technology, Sumbawa, Indonesia.
[c] Department of Chemistry, Gadjah Mada University, Sekip Utara, Yogyakarta, Indonesia.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This research aims to synthesize silver nanoparticles (AgNPs) using Kirinyuh (*Chromolaena odorata*) leaf extract with different temperature and evaluate its activity in Vibrio sp.
The synthesis was carried out by combining between silver nitrate solution and Kirinyuh leaf extract at room temperature (AgNPs 200) and hydrothermal process at 60^{o}C (AgNPs 200*). Based on the results of LC-MS analysis, most of the chemical content of an extract was a flavonoid group compound. The color of the solution changed from colorless to red-brown, and a new peak around 435 nm showed that silver nanoparticles have been successfully obtained. The hydrothermal process exhibits sharp peaks rather than without hydrothermal treatment. The silver nanoparticles have a definite crystalline structure. The FTIR spectroscopy analysis indicated the organic compound was responsible for capping and stabilizing agents. In addition,TEM analysis showed that AgNPs 200 and AgNPs 200* exhibited average particle size of 32.89 nm and 27.82 nm, respectively. The presence of AgNPs can enhance the zone of growth inhibition of Vibrio sp approximately 300% compared to Kirinyuh leaf extract. Silver nanoparticles is estimated to possess the potential to be further evolved and utilized to inhibit the growth of Vibrio sp bacteria in shrimp agriculture.

**Corresponding author: E-mail: mudasir@ugm.ac.id, roto05@ugm.ac.id;*

Keywords: Green synthesis; Chromolaena odorata; silver nanoparticles; vibrio sp; temperatures.

1. INTRODUCTION

The decreased production of pond activists, especially shrimp, has an impact on the availability of national shrimp demand. 20% of shrimp ponds that experience annual losses due to disease illnesses by bacteria. Shrimp infected with vibrio sp cause a red spot on the body (white spot) [1]. This disease is known as vibriosis, which is the primary opponent of farmers because the infection will prompt shrimp to die in a short time [2]. The harvest efforts were executed to minimize the incident of shrimp deaths, such as the addition of antibiotics into the water pond. Nevertheless, the bacteria that are given antibiotics will be able to adapt to boost immunity and eventually become resistant continuously [3]. In addition to the actuality of regulations on excessive use of antibiotics can inhibit the shrimp marketing process. The usage of antibiotics is only prevented in the downstream domain, but it is not the source of the disease in the upstream area. Therefore, a comprehensive strategy is necessary needed in overcoming the problem of vibriosis through preventing the growth of Vibrio sp.

One of the materials is interest and recommences to develop both the synthesis process and its application is silver nanoparticles. Silver nanoparticles have a robust antibacterial activity. The electrostatic interaction between the positive charge of silver nanoparticles and the negative charge on the bacterial cell effects damage to the cell membrane so that it will cause bacteria to die [4]. Silver nanoparticles have superior antibacterial activity against Staphylococcus Aureus [5], Escherichia Coli [6], L. Monocytogenes [7], Pseudomonas spp, Bacillus sp, Staphylococcus sp, and Aspergillus Niger sub sp, Aspergillus Flavus sub sp, Penicillium sp [8]. Besides, silver nanoparticles have also studied for their activity in Vibrio sp bacteria in Shrimp agriculture [9]. However, the use of silver nanoparticles to control bacterial growth in aquatics is still limited studied. In contrast, the occupation of Vibrio sp bacteria in significant frequencies is a prominent problem for the extension of Shrimp pond because it will cause infection, which expires in harvest failure or death.

Green synthesis of silver nanoparticles is continuously studied and developed. This method utilizes plant extracts as a reducing agent and stabilizer of silver nanoparticles. According to Kumar Sur et al., [10], green synthesis can be an alternative method in gaining silver nanoparticles. Because it is environmentally friendly, low cost, simple process, and plenty of materials used sufficiently so that silver nanoparticles can be produced on a large scale [10]. Currently, silver nanoparticles have been successfully synthesized using a variety of plant extracts such as *Enicostemma Axillare* Leaves [11], Shikakai leaves and Reetha [10], Melissa Officinalis leaves [5] and Sesbania grandiflora leaves [8]. These plant extracts contain flavonoid and phenolic chemical compounds that have a responsible actor in the process of reducing Ag^{+} to Ag^{0}. Besides, these compounds can be utilized to prevent the agglomeration of silver nanoparticles. In the other hand, the temperature also will influence the reaction time and size of the nanoparticles. With the temperature increases, the size of silver particles obtained is smaller [12]. Meanwhile, the rate of reduction of silver ion to silver metal increases [13].

Many plants have produced organic compounds such as flavonoids, ketones, aldehydes, terpenoids, amides and carboxylic acids which are directly needed to synthesize silver nanoparticles as reducing and stabilizing agents [14]. The Kirinyuh is one of the plants have flavonoid, phenol and aldehyde compounds in the leaves. Kirinyuh plants are found wild in the Sumbawa area. This plant has also been used by society as a traditional medicine to treat wounds in Sumbawa. Some of the flavonoid compounds found in the Kirinyuh leaf extract are the quercetin and kaempferol groups. According to Jain and Maheta [15], one of the compounds that can reduce Ag^{+} ions to Ag^{0} is the quercetin group [15]. Therefore, in the present research, the green synthesis of silver nanoparticles has been carried out using Kirinyuh extract as a reducing and stabilizing agent. The silver nanoparticles obtained were investigated by the performance for activity against Vibrio sp. The Vibrio sp was collected and isolated by shrimp in the industry of Sumbawa Caridea AV 7, Sumbawa, West Nusa Tenggara, Indonesia.

2. MATERIALS AND METHODS

2.1 Materials

The silver nitrate (Merck), Thiosulfate Citrate Bile Salts (TCBS) (Himedia), sodium hydroxide

(Merck), paper disc, filtered paper, distilled water, shrimp (Sumbawa Caridea AV7), and Kirinyuh leaves.

2.2 Preparation of Kirinyuh Leaf Extract

Kirinyuh leaves were collected from the local area of Sumbawa, West Nusa Tenggara, Indonesia. The leaves were rinsed with distilled water to remove dust, then the leaves were dried at room temperature for one week to remove the water content. The dried Kirinyuh leaf was crushed by blender to get a thin Kirinyuh powder. 6.25 grams of powder sample was added into 250 ml of boiled distilled water at the temperature of 60^{o}C and stirred for 15 minutes. After that, the solution was filtered to separate the extract with leaf mash. The filtrate obtained was stored in a closed dark bottle at room temperature. Kirinyuh leaf extract was characterized using a UV-VIS spectrophotometer to determine the absorption area of the chemical compounds and analyzed by LC-MS to determine the organic compounds in the extract.

2.3 Green Synthesis of Silver Nanoparticles

The synthesis of silver nanoparticles was carried out at room temperature (AgNPs 200) and hydrothermal at 60^{o}C (AgNPs 200 *). 10 ml of 1000 ppm silver nitrate solution was added with 1 mL of Kirinyuh leaf extract and added with 39 mL of distilled water. The color change of the solution from colorless to red-brown indicates that silver nanoparticles formed. The preparation of AgNPs 200* follows the procedure of AgNPs 200. However, The solution was then subjected the hydrothermal method at 60^{o}C. The produced AgNPs were characterized by Ultra Violet-Visible Spectroscopy (UV-VIS), Transmission Electron Microscope (TEM), and Particle Size Analysis (PSA). The powder of silver nanoparticles was obtained and identified by Fourier-Transform Infrared Spectroscopy (FTIR) to distinguish responsible for the organic compound in the extract as a capping agent and X-Ray Diffraction (XRD) to determine the structure crystal.

2.4 Evaluation Activity of Silver Nanoparticles

The shrimp was crushed by a mortar to achieve a smooth sample. 0.1 gram of sample was added with distilled water and then homogenized. After that, the sample was diluted 1000 times with NaOH 0.05 M. 0.5 µL the sample was put into 88 gram/L Thiosulfate Citrate Bile Salts (TCBS) media. TCBS media is a selective media for the growth of Vibrio sp (Fig. 1).

The change in color of the media from green to yellow indicates the growth of Vibrio sp. The Vibrio isolate was used to evaluate the antibacterial activity of silver nanoparticles. To understand silver nanoparticles activity has been

Table 1. Shows the chemical compounds in Kirinyuh leaf extract

No	Compound name	Formula purposed of molecular	Retention Time (minutes)	M/Z
1	Skullcapflavone Ⅱ	$C_{19}H_{18}O_8$	0.40	375.1073
2	Undulatoside A	$C_{16}H_{18}O_9$	3.77	353.0872
3	Apigenin-6,8-di-C-glucoside	$C_{27}H_{30}O_{15}$	6.81	593.1517
4	Cyclolaudenol	$C_{33}H_{40}O_{21}$	7.08	771.1987
6	Quercetin-3-gentiobioside	$C_{27}H_{30}O_{17}$	7.41	625.1400
7	3-O-[β-D-Glucopyra-nosyl-(1→2)]-β-D-glucopyranosyl-7-O-α-L-glucopyranosyl-kaempferol	$C_{33}H_{40}O_{20}$	7.62	755.2030
8	Quercetin-3-O-(2G-α-L-rhamnosyl)-rutinoside	$C_{32}H_{40}O_{20}$	7.78	757.2194
9	3-O-[β-D-Glucopyra-nosyl-(1→2)]-β-D-glucopyranosyl-kaempferol	$C_{27}H_{30}O_{16}$	8.60	610.2308
10	Quercetin-3-O-β-D-glucopyranoside	$C_{21}H_{20}O_{12}$	8.82	463.0876
11	Kaempferol-3-O-rutinoside_1	$C_{27}H_{30}O_{15}$	9.53	593.1513
12	3-Methoxy-distylin	$C_{16}H_{14}O_7$	10.71	317.0666
13	3′-O-Methylviolanone	$C_{18}H_{18}O_6$	14.44	331.1172
14	5,7,2'-Trihydroxy-6-methyl-8-methoxy-3(R)-(4'-methoxybenzyl) chroman-4-one	$C_{19}H_{20}O_7$	15.20	160.0975
15	Genkwanin_1	$C_{16}H_{14}O_5$	16.67	285.0765

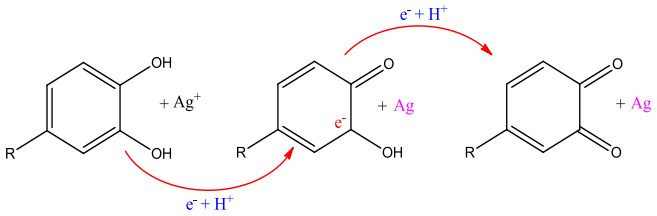


Fig. 1. Proposed mechanism of the reduction Ag^+ to be Ag^0 (Albukhari et al., 2019)

utilized some samples such as water as a negative control, Kirinyuh leaf extract, AgNPs 200 and AgNPs 200* and amoxicillin as a positive control was used. A paper disc immersed all samples for 3 minutes. Furthermore, the softened paper disc was placed and make a sample code point, then incubated during overnight (24 hours). The calculation of the inhibition zone was identified by measuring the clear zones formed on TCBS media.

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening of Kirinyuh Leaf Extract

Analysis of the organic chemical compounds in the extract was carried out by the LC-MS instrument [16]. Table 1. shows the content of the chemical compound along with formulas and mass molecules in Kirinyuh leaf extract. The compounds in the extract will be partially separated in the column depending on the properties of the compound. The retention time and molecular mass of compounds can be used as a reference to find out the species and classes of compounds. Most of the chemical compounds contained in the extract are of the flavonoid groups. The flavonoid compound consists of 15 carbon atoms which have the structure of 2 phenyl rings and one heterocyclic ring. The mechanism of formation of silver nanoparticles consists of 3 stages, namely the reduction process from Ag^+ to be Ag^0 followed by the process of collecting (coalescence) and subsequently the growth of particles. Fig. 1 illustrates the mechanism of the reaction of Ag ions with the hydroxy group to produce silver nanoparticles. According to Albukhari, S et al. [17], Ag^+ ions react with OH groups attached to aromatic rings in flavonoid compounds for reducing silver ions to silver metal [16], besides flavonoid compounds also performs as capping or stabilizing agents to prevent agglomeration of silver nanoparticles. As additional information, Liu and Guo [18] have conducted studies on the mechanism of the reaction of the reduction of Ag^+ to Ag by the quercetin molecule, where one quercetin molecule can reduce two molecule silver ions [18]. Therefore, Kirinyuh leaf extract can be used to synthesize silver nanoparticles as a reducing and stabilizing agent.

3.2 Analysis of Silver Nanoparticles Using UV-VIS

UV-Vis spectroscopic analysis aims to determine the interaction between Ag^+ ions and organic compounds in the Kirinyuh leaf extract. Based on measurements, with increasing reaction time to synthesize silver nanoparticles, the color of solution changes from initially colorless to red-brown. This phenomenon has been confirmed a reduction reaction between Ag^+ and organic compounds in the extract [11]. Fig. 2 shows the effect of time on the absorbance peak of silver nanoparticles using UV-Vis spectroscopy and the solution color of silver nanoparticles.

Fig. 2a shows the Ag^+ solution has no absorption in the visible area because the Ag^+ is an ionic bond and the d10 configuration attribute of the Ag^+ [19]. Kirinyuh leaf extract solution has a strong absorption at the wavelength region of 250-350 nm. Flavonoid organic compounds have chromophore groups like conjugated bonds and benzene bound with hydroxyl and ketones cause electrons to transition from n to π*. Increasing time shows increasing absorbance at 435 nm that indicates the amount of silver nanoparticles increases because the possibility of collision between Ag^+ ions and reduction agent is expanding [17]. This state will enhance the intensity of electron excitation from ground level orbitals to a higher orbital so that the absorption value of silver nanoparticles increases. This phenomenon is following Lambeer Beer's law that with increasing concentration, the absorbance value also increases. The area at wavelength 270-350 nm decreases when the increase in absorbance at 400-450 nm, due to reducing the number of hydroxyl groups (OH) because it has reacted with Ag^+ in solution.

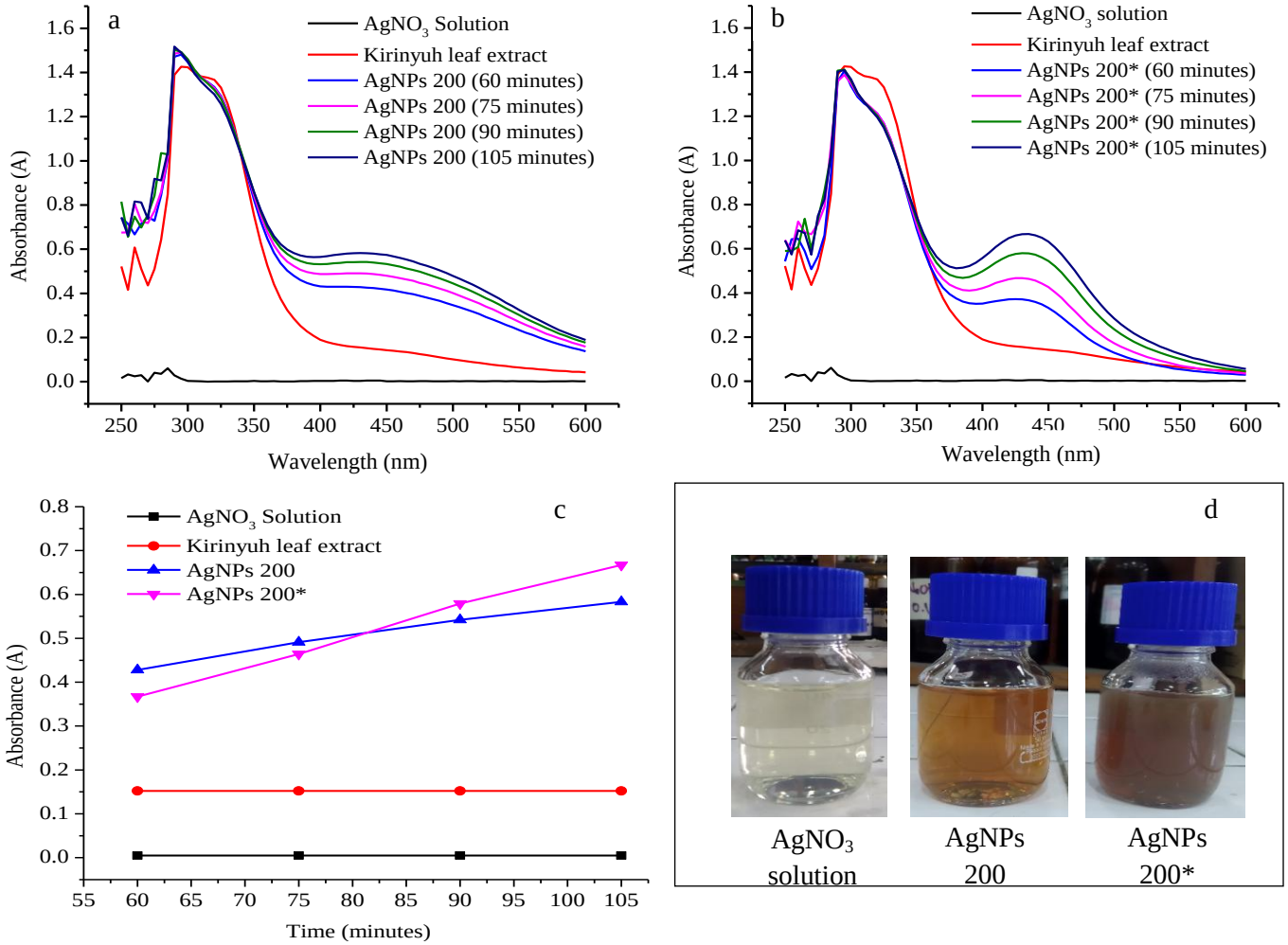


Fig. 2. Shows about results of UV-Vis spectroscopy analysis (a) AgNPs 200 (b) AgNPs 200* (c) comparison of absorbance of AgNPs 200 and AgNPs 200* and (d) The color of silver nanoparticles solution

Fig. 1b shows the effect of hydrothermal treatment on the time of the formation of silver nanoparticles. The hydrothermal treatment produced a sharper peak of silver nanoparticles, and a high absorbance wavelength value at 435 nm opposed the height of AgNPS 200 samples (Fig. 1a). The rate reaction was affected by temperature treatment in the synthesis of silver nanoparticles. When the temperature raised, the movement of Ag ions and organic compounds in the solution is increasingly active, the intensity of the collision between the two is high and finally the reaction rate of the formation of silver nanoparticles increases [20].

Fig. 1c illustrates the effect of time with the absorbance of silver nanoparticles. In this situation, silver nanoparticles are found to have a maximum wavelength of 435 nm. This maximum wavelength is following the results of recent studies that range of absorption silver nanoparticles which are around 420-450 nm [5,8,10,11]. This maximum wavelength will be used to calculate the effect of time on the absorbance value. In the AgNO3 solution and Kirinyuh leaf extract, it decided that the reaction did not show the absorbance change at the wavelength of 435 nm. The concentrations of organic compounds and Ag^{+} in solution are sufficient so that the reaction rate of the formation of silver nanoparticles rises [20].

3.3 Analysis of Silver Nanoparticles Using XRD

The crystal structure of silver nanoparticles was analyzed using X-ray diffraction. Fig. 3 shows the XRD pattern of silver nanoparticles exhibited a high-intensity peaks at 2 Theta 38.20 and 44.28o, correspond to the characteristics of planes (111) and (200) [21,22,23]. The silver nanoparticles pattern was well indexed to the JCPDS database file no. 04-0783. The distance between the lattice on silver nanoparticles obtained approximately 2,348 and 2,030. Thus the crystal structure of silver nanoparticles has a face center cubic (fcc) structural system [23]. Besides, the pattern results also showed that the presence of peaks at 2 Theta 28.06, 32.16, 46.28, 54.76 and 57.47 confirmed the existence of the crystalline structure of organic compound powder in Kirinyuh leaf extract [21,24]. From the pattern data, silver nanoparticles in plane 111 obtained a particle size of approximately 23 nm. These results were calculated using the Debye-Scherrer equation below:

$$D = \frac{0.9\,\lambda}{\beta \cos \theta}$$

Where D is the average particle size obtained (nm), β is the value of the width at half intensity (radians), Θ is the Bragg angle (degree), and λ is the x-ray wavelength.

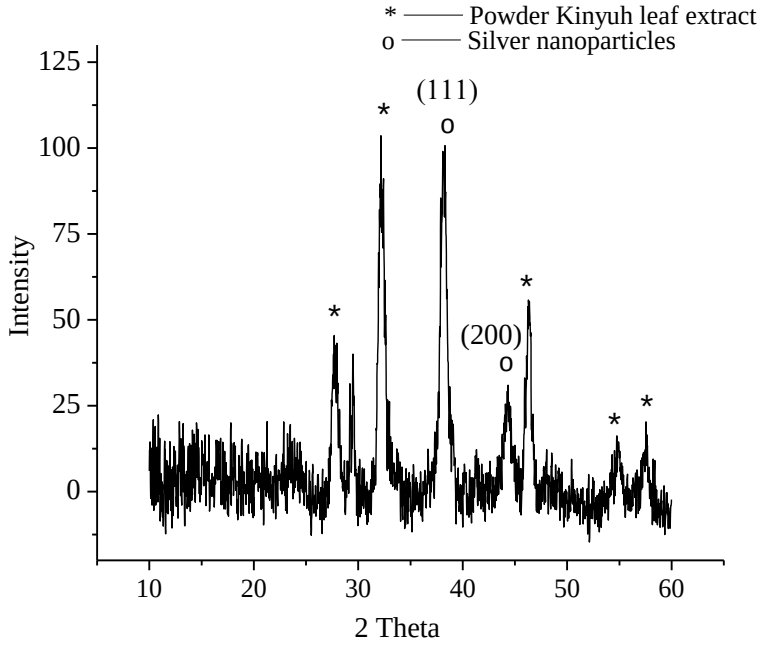


Fig. 3. XRD pattern of silver nanoparticles

3.4 Analysis of Silver Nanoparticles Using FTIR Spectroscopy

Fig. 4 presents the spectra of AgNPs 200 and AgNPs 200* using FTIR spectroscopy. The peaks show the functional group of the organic compound in extract. It is also supported by LC-MS data which shows the extract contains flavonoid, phenol, aldehyde compounds. The occupation of a stiff peak at 3000-3500 cm- confirms the presence of O-H bonds in phenol and alcohol and the width of these peaks is due to the formation of hydrogen bonds [25]. The C=C bound in the aromatic group established by the appearance of a peak at wave number 1607 nm- [21,26]. C = O and C-H groups of aldehydes indicated at the peaks of 1682 nm- and 2918 nm- , respectively. The C-O group identified with a strong appearance at the wavenumber 1030 nm- . There is absorption at wave number 1302 nm- confirming the phenolic group that reacts in response to the Ag^+ ion [27]. Besides that, the existence of the peak in the region of wave number 804 nm- indicates an aromatic group of flavonoid compounds [11]. The spectra of AgNPs 200 peaks and AgNPs 200* do not show significantly different in peaks. The treatment temperature at 60^{o}C has not affected the structure of compounds in the extract. There is a difference in the extent of absorption of OH bonds, which indicates that the number of compounds containing O-H binds to silver nanoparticles at AgNPs 200 * more than AgNPs 200.

3.5 Analysis of Silver Nanoparticles Using Tem and PSA

The particle size is necessary to be analyzed in the nanomaterial field [10].

Fig. 5 shows that the silver nanoparticle shape of the two samples is spherical. However, the AgNPS 200* sample did not display agglomeration indications. According to Liu et al. [20], the size of the silver nanoparticles is influenced by the temperature method. At low temperatures, nanoparticles grow well so that the particle size becomes large, while at high temperatures, it can reduce particle size. Because interactions between organic compounds can prevent agglomeration, this is also encouraged by the UV-VIS analysis that the peak of AgNPs 200* is sharper than AgNPs 200. In other words, the AgNPs 200 sample shows broad peaks, which indicates agglomeration of silver nanoparticles.

Fig. 6a illustrates the size distribution of silver nanoparticles. The average particle size of AgNPs 200 and AgNPs 200* were obtained approximately 32.89 nm and 27.82 nm, respectively. Based on the results of the DLS analysis (Fig 6b-6c), the average size of silver nanoparticles is different from the TEM results of AgNPs 200, and AgNPs 200* approximately 65.1 nm and 64.8 nm, respectively. The DLS measures the hydrodynamic radius, and solvation effect on DLS measurements [28,29].

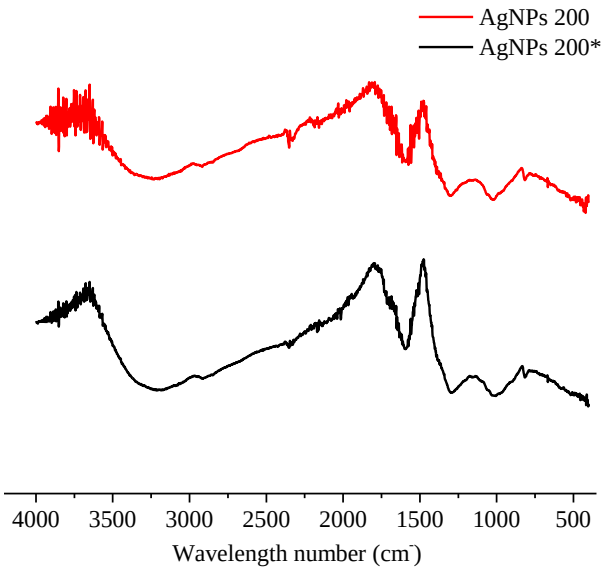


Fig. 4. The FTIR spectra of silver nanoparticles

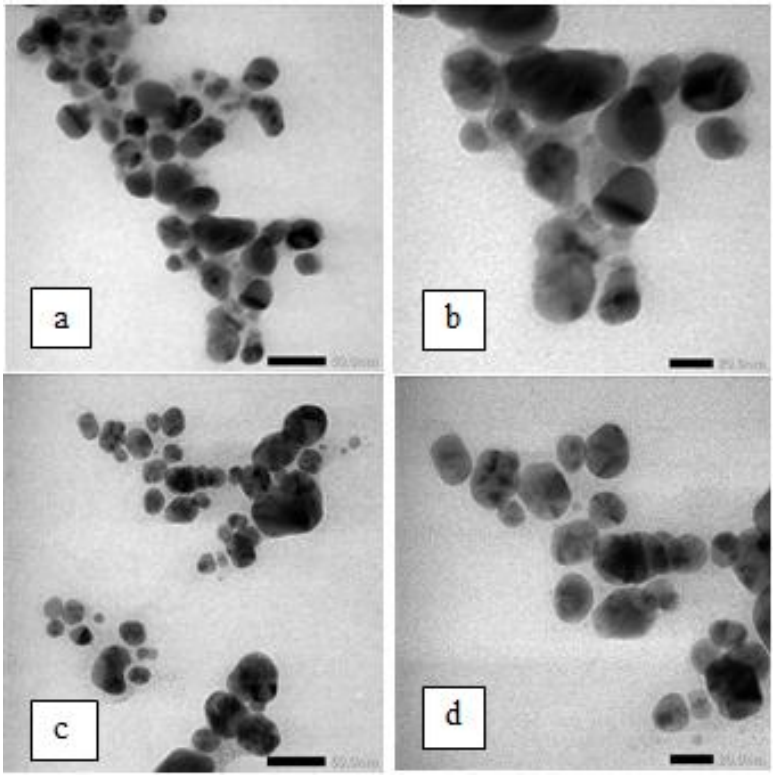
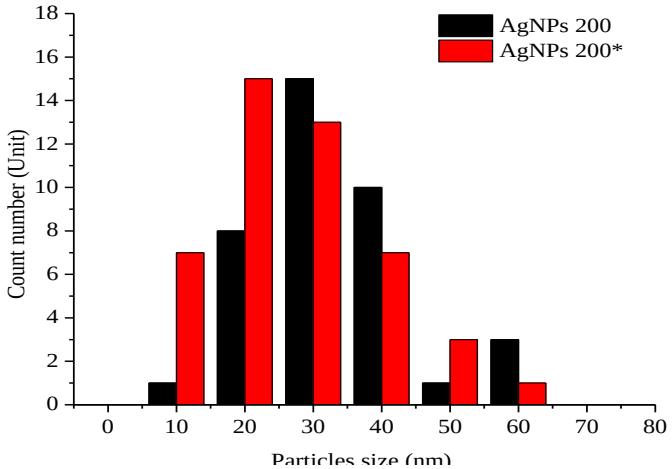


Fig. 5. The particle size and shape of silver nanoparticles by TEM analysis (a-b) AgNPS 200 and (c-d) AgNPs 200*

3.6 Analysis of Silver Nanoparticles Using TEM and PSA

Vibrio sp bacteria are a group of negative bacterial genes that will cause vibriosis in shrimp farming activities. Vibriosis infection distinguished through appearing white on the skin (white spot) of shrimp [1]. In this study, it has isolated vibrio sp bacteria from shrimp using TCBS specific media as growth media. Based on investigations, Vibrio sp bacteria that were successfully isolated had a type of vibrio Harvey sp from shrimp after being incubated for 24 hours. It verified the growth of bacteria that are a colony form, single, round, size 1-3 mm, become yellow spots in the media, and TCBS media change color from green to yellow [30]. The collected bacterial isolates were stored and then used to examine the activity of silver nanoparticles.



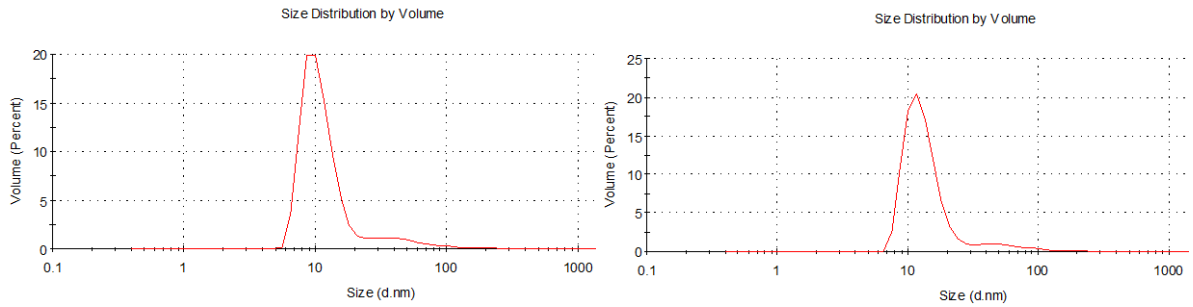


Fig. 6. Particle size distribution (a) size distribution by volume of AgNPs 200* (b) AgNPs 200 (c)

Fig. 7 shows a measurement of the antibacterial activity of Vibrio sp. Water samples established that it cannot inhibit the growth of Vibrio sp, while the Kirinyuh leaf extract has an inhibition zone about 1.12 mm, this appears because the extract contains flavonoid group compounds that can interact with lipids in the cell plasma membrane [31,32]. Plasma membranes in bacterial cells have an essential part in the process of transferring, respiration, transportation, and lipid biosynthesis. When there is interference in contact with the cell membrane or cell destruction, these processes will be disrupted.

According to Tsuchiya [33], there are two interaction mechanisms between flavonoid compounds and the lipid layer (cell membrane), namely the first interaction between the non-polar part of the flavonoid compound and the hydrophobic part of the membrane. The second is the interaction of hydrogen bonds between the polar partition of the flavonoid compound with the group polar lipids [33]. Quercetin-3-O-Rhamnoglucoside compounds are compounds contained in Kirinyuh leaf extracts that can interact with lipids in the plasma membrane to reduce the thickness and disrupt the structure of the monolayer, destroying cell membranes that occur in the process of inhibiting bacterial growth [34].

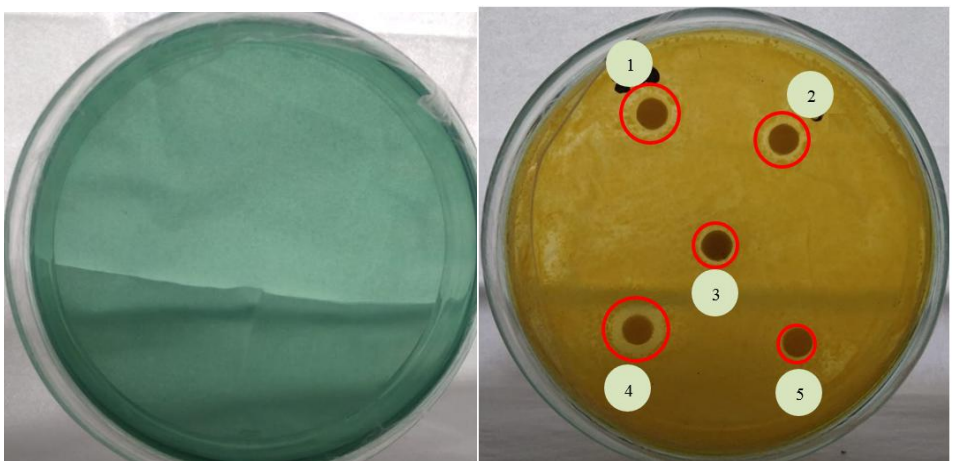


Fig. 7. Analysis results of silver nanoparticle antibacterial activity (a) TCBS media, (b) inhibitory zones of various types of AgNPs 200 * samples (1) AgNPs 200 (2) Kirinyuh leaf extract (3) Amoxicillin and (4) water

Table 2. Shows the results of silver nanoparticles inhibition zones

Sample	Inhibition zone (mm)
Water (Negative control)	0.46
AgNPs 200	4.73
AgNPs 200*	5.13
Kirinyuh leaf extract	1.36
Amoxicillin	7.00

Table 2 shows the inhibition zone of silver nanoparticles against Vibrio sp. The appearance of silver nanoparticles showed a significant influence on the area of growth inhibition of Vibrio sp. In AgNPs 200 and AgNPs 200*, they have inhibition zones of around 4.73 mm and 5.13 mm, respectively. This yield is higher than the inhibition zone of Kirinyuh extract. This inhibition zone was increased by the attendance of silver nanoparticles in media more than 300%. However, the AgNPs inhibitory zone is still small when compared with the amoxicillin, which is about 7 mm. Silver nanoparticles can have antibacterial activity due to interactions between the surface of silver nanoparticles and organic compounds that damage bacterial cell membranes causing reduced bacterial growth. According to Simonelli et al. [35], the reaction of silver nanoparticles with a polar lipid group is electrostatic in the cell plasma membrane [35]. Electrostatic reactions to negative charges on bacterial cells with positive nanoparticle charges cause AgNPs to damage bacterial cells and ultimately cause cell death [36]. Based on the description that silver nanoparticles have a significant influence on the growth of Vibrio sp bacteria and show vigorous activity against Vibrio sp bacteria. Silver nanoparticles may be considered sufficient to be necessary for the industry to inhibit the growth of Vibrio sp.

4. CONCLUSION

Silver nanoparticles with spherical shape have been obtained by green synthesis methods using Kirinyuh leaf extract. This method is straightforward, low cost, environmentally friendly and only used distilled water as a solvent. Hydrothermal treatment can decrease the reaction rate of AgNPs formation. The presence of extract peaks in the XRD and FTIR results indicate that organic compounds respond with silver ions as reducing and stabilizing agents. Silver nanoparticles have a significant impact on the antibacterial activity in Vibrio sp, where the inhibition zone can raise more than 300% as opposed to Kirinyuh extract. The electrostatic reaction with a negative charge on the cell can damage the membrane so that AgNPs can inhibit the growth of Vibrio sp bacteria. Silver nanoparticles have the potential to be further developed and applied to restrain the growth of Vibrio sp bacteria in the agriculture industry.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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