Indonesian Journal of Chemical Research

http://ojs3.unpatti.ac.id/index.php/ijcr Indo. J. Chem. Res., 13 (1), 94-101, 2025

Antibacterial Activity Test of Silver Nanoparticle Composites With Gandaria Seed (Bouea macrophylla G.) Bioreductor and Ouw Natural Clay Matrix (ONC)

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Received: March 2025
Received in revised: April 2025
Accepted: May 2025
Available online: May 2025

Abstract

An antibacterial activity test of silver nanoparticle composite with bioreductor of gandaria seeds (*Bouea macrophylla* G.) and Ouw natural clay (ONC) matrix was conducted. Silver nanoparticles (AgNPs) were synthesized by forming the colloidal silver solution by chemical reduction method using gandaria seed bioreductor. The formation of AgNPs can be known through UV-Vis absorption when there is a color change. Based on the results, the maximum wavelength of colloidal NPP is 435-444 nm. Silver nanoparticles synthesized through the preparation process were tested to determine their effectiveness as antibacterial agents against Escherichia coli and Staphylococcus aureus. The test results showed that the NPP formulation, containing an aqueous extract of gandaria seeds with the addition of polyvinyl alcohol (PVA), produced an inhibition zone of 10.6 mm against E. coli, indicating strong antibacterial activity. In contrast, the activity against S. aureus was classified as moderate, with an inhibition zone measuring 6.8 mm. For the antibacterial activity test with Ag/ONC nanocomposite samples on both test bacteria, there was no inhibition zone or antibacterial activity.

Keywords: Gandaria seeds, nanoparticles, antibacterial, natural clay, zone of inhibition.

INTRODUCTION

The green synthesis method is a synthesis method that uses natural materials from plants and microorganisms from the earth and sea to form metal nanoparticles. This method relies on bio-based materials, particularly plant-derived extracts, which act as natural reducing agents. These extracts typically contain antioxidant compounds that facilitate the reduction of silver ions into silver nanoparticles in a sustainable and non-toxic manner (Arifin, Harjono, & Wijayati, 2016; Catherina M. Bijang et al., 2023; R, Zakir, & Budi, 2020; Rusnaenah, Zakir, & Budi, 2017). The process uses plants containing secondary metabolite compounds such as terpenoids, ketones, aldehydes, amides, and carboxylates (Mulfinger et al., 2007).

Plant extracts that are available as bioreductors in the synthesis of silver nanoparticles are plants that contain secondary metabolite compounds that can act as reducing agents to convert Ag^+ ions into Ag^0 (Isaac et al., 2013). One of the plants that can be used as a bioreductor is Gandaria (*Bouea macrophylla G.*) (Kiat et al., 2024, Bijang et al., 2024). Gandaria is an endemic plant in Maluku with uneven distribution across all islands and found only in particular regions, such as Ambon island as the largest production center and Central Maluku Regency, namely Saparua and West Seram islands (Tanasale, 2011).

Nanoparticle synthesis can be done using gandaria plant extract because it contains potent antioxidants. However, silver nanoparticles have a tendency to aggregate the during the synthesizing process. The aggregation causes silver nanoparticles to stick together, forming larger silver particles and reaching bulk size. Hence, the addition of polyvinyl alcohol (PVA) is essential as a stabilizing agent in the synthesizung silver nanoparticles (Prasetiowati, Prasetya, & Wardani, 2018). Gandaria seed water extract's role in forming silver nanoparticles is in the plant's antioxidant ability to reduce Ag^+ ions into Ag^0. The antioxidant content in gandaria seeds is flavonoids, which are distributed in the form of anthocyanins, responsible for the purple color of gandaria seeds.

A nanocomposite is a mixture combines a discontinuous phase and a matrix. Silver nanoparticles act as the discontinuous phase of the nanocomposite, while clay is the matrix (Motshekga, Ray, Onyango, & Momba, 2013; Roy et al., 2022). Clay is among the natural resources that have not been utilizing optimally. One of the clay producers in Maluku is located on Saparua island, precisely in Ouw Village. Generally, people in Ouw village only use natural clay for pottery (sempe) and bricks. Ouw Natural Clay (ONC) has physical and chemical properties that can be modified. Additionally, ONC has the potential to be an adsorbent for laboratory waste (Bijang and Telussa, 2008), a rhodamine B degrader (Teddy, 2018), and a cation exchanger (C.M. Bijang, Telussa, & Koritelu, 2014). Clay acts as a binder for nanofillers (fillers) in nanocomposites (Pezzin et al., 2011). Nanocomposites have several applications, such as, antimicrobial agents in food packaging (Qu & Luo, 2021), and due to their ability to inhibit the growth of foodborne pathogens, extend shelf life, and enhance the mechanical and barrier properties of the packaging materials. Incorporation of nanoparticles such as silver (Ag), zinc oxide (ZnO), titanium dioxide (TiO_2), and clay-based materials into biopolymer matrices has shown significant antimicrobial efficacy against a wide range of microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*. For instance, silver nanoparticles (AgNPs) are well-known for their broad-spectrum antimicrobial activity, They have been widely studied in polymer-based nanocomposites for food packaging applications (Rai, Yadav, & Gade, 2009).

The application of silver nanoparticles using a bioreductor is not limited to the formation of the nanocomposite but also applied in antibacterial activity tests. Pathogenic bacteria are more dangerous and cause infections both sporadically and endemically, among them are *Escherichia coli* and *Staphylococcus aureus* (Pratiwi, 2017). *E. coli* and *S. aureus* were chosen as the target for the test because they are pathogenic bacteria from gram-positive and gram-negative bacteria often found in humans and able to cause infections.

Tehuayo (2020) successfully synthesized silver nanoparticles with gandaria seed water extarct (*Bouea macriphylla G.*) as a bioreductor. The optimal concentration of $AgNO_3$ as the precursor of the green synthesis was found to be 1 mM, and with the addition of PVA increased the stability of silver nanoparticle formation.

Nalawati et al., (2022) synthesized silver nanoparticles using $AgNO_3$ and *Jatropha curcas L.* seed extract as the reducing agent, which achieved particle sizes ranging from 33 to 116 nm with demonstrated antimicrobial properties. This study focuses on testing the antibacterial activity of silver nanoparticle composites synthesized using gandaria seed bioreductor and Ouw natural clay matrix.

METHODOLOGY

Materials and Instrumentals

The tools used in this research are as follows: A set of glassware (Pyrex), Blender (Philips), Analytical balance (Ohaus Adventurer TM Pro), Petri dishes (Pyrex), Magnetic stirrer (Ikamag), Micro pipet (DragonLAB), 100 mesh sieve, Mortar and pestle, Spatula, Desiccator, Buret and stand, UV-Vis spectrophotometer (Shimadzu UV-1800), Inoculation loop, Sterile punch, Autoclave (Tomy ES-315), Incubator shaker, Vortex, Oven (Mammert), Hot plate (Cimarec 2), Ruler.

The materials used in this research are as follows: Gandaria seeds, Ouw natural clay, Aqua destilata (distilled water), Aqua bidestilata (double-distilled water), Escherichia coli bacteria (ATCC 7839), *Staphylococcus aureus* bacteria (ATCC 6538), Nutrient Agar (NA), Nutrient Broth (NB), H_2SO_4 (p.a Merck), $AgNO_3$ (p.a Merck), Polyvinyl Alcohol (PVA), Amoxicillin, Filter paper, Cotton, Aluminum foil.

Methods

Gandaria Seed Extraction

50 g of gandaria seeds are washed, dried under sunlight, crushed, and pulverized using a blender. 4.004 g of gandaria seed powder is placed into an Erlenmeyer flask, and then 400 mL of double-distilled water is added. Boiled at 100^{o}C while stirred with a magnetic stirrer for 3 hours until completely extracted, then filtered. Obtained gandaria seed water extract with a concentration of 1%(w/v) (Tehuayo A, 2020).

Activation of Ouw Natural Clay with Sulfuric Acid

The activation procedure began by combining 100 grams of Ouw Natural Clay (ONC) powder with 500 mL of 2 M sulfuric acid (H_2SO_4), followed by continuous stirring for a duration of 24 hours. The mixture is then washed with hot distilled water until no residual acid remains (verified using a 5% $BaCl_2$ test), then filtered with filter paper and dried in an oven at 80^{o}C. The dried residue is placed in a desiccator, then finely ground and passed through a 100-mesh sieve to obtain a uniform particle size (Bijang et al., 2015).

Synthesis of Silver Nanoparticles Using Gandaria Seed Water Extract and PVA

5 mL of 2% PVA solution is placed into an Erlenmeyer flask, then 3 mL of gandaria seed water extract is added. The mixture is titrated with 10 mL of 1 mM $AgNO_3$ solution with a burette until the color changes. The solution is stirred with a magnetic stirrer and then left for 2 days. The volume ratio of $AgNO_3$: gandaria seed water extract: PVA is 10:3:5 mL (Tehuayo A, 2020).

Synthesis of Ag/ONC Nanocomposite Using Gandaria Seed Water Extract

100 mL of $1x10^{-3}$ M $AgNO_3$ solution is added to 2 g of activated Ouw Natural Clay (ONC). The mixture is stirred using a shaker for a continuous period of 24 hours at ambient temperature. Subsequently, 25 mL of the aqueous extract obtained from gandaria seeds was introduced into the mixture and stirred using a magnetic stirrer for 1 hour to ensure thorough blending. The resulting nanocomposite suspension is taken by separating the precipitate an then dried at 40°C (Shameli et al., 2011).

Antibacterial Activity Test

Preparation of Test Bacterial Suspension

The preparation of bacterial suspension of the test colonies *E. coli* and *S. aureus* was done by taking one inoculation loop of colonies from solid Nutrient Agar (NA) media into an Erlenmeyer flask that containing 30 mL liquid Nutrient Broth (NB). The falsk was then incubated for 24 hours at 37°C. Bacterial concentration was then adjusted to a cell density of 10^6 CFU/mL.

Antibacterial Testing Using the Well Diffusion Method (Balouiri, Sadiki, & Ibnsouda, 2016)

Purified and rejuvenated *Escherichia coli* and *Staphylococcus aureus* along with a double-layer nutrient agar medium were used in this antibacterial activity test. The first layer served as a nutrient base and the second layer was a mixture of the medium and the pathogenic bacterial suspension with a cell density of 10^6 CFU/mL. Wells with a 6-7 mm diameter were made in the upper (second) layer of the medium using a sterile punch. The double-layer nutrient agar was placed in two different petri dishes for each test bacterium. Then, 40 µL silver nanoparticle suspension synthesized with gandaria seed water extract and PVA was dropped into 3 wells on each of the *E. coli* and *S. aureus* plates. The same procedure was applied for the Ag/ONC nanocomposite with gandaria seed water extract. In separate petri dishes for each test bacterium, one well was filled with 40 µL of distilled water as negative control while the other is filled with 40 µL of liquid amoxicillin as positive control. Then, the agar media were incubated for 24 hours at 37 °C. The diameter of the inhibition zones formed around the wells was measured after incubation.

RESULTS AND DISCUSSION

Activation of Ouw Natural Clay with sulfuric acid

The activation of ONC with sulfuric acid is done to increase the surface area and to remove impurities between the clay layers that can block the active side of the clay, hence enhancing the nanoparticle synthesis process. Additionally, the activation process causes ion exchange and impurity removal on clay crystal surfaces, improving the clay's adsorption capacity and increasing surface area. Activation also homogenizes the cation distribution within the clay with the cations present in the activating compounds (Sari, Nurbaeti, & Pratiwi, 2016). The active side of the clay includes the external surface, interlayer spaces, and the edges of the aluminium crystals (Fatimah., 2014).

The activation result the exchange of interlayer cations such as Ca^+, Na^+, and K^+ with H^+ ions from H_2SO_4. H_2SO_4 also serves to dissolve organic and inorganic impurities, thereby increasing the surface area and pore volume of ONC (Bijang et al., 2015).

Synthesis of Silver Nanoparticles using Gandaria Seed Water Extract (GSWE) and PVA

Synthesis of AgNP using gandaria seed water extract that is reacted with 1 mM $AgNO_3$ solution as the silver nanoparticle precursor and 2% PVA as a stabilizer. The volume ratio of GSWE:$AgNO_3$:PVA is 3:10:5. From this reaction, characteristics related to several parameters are obtained, including the color change of the solution, UV-Vis spectrum, the effect of contact time, and the influence of adding 2% PVA on the nanoparticle formation reaction. The principle of the AgNP formation reaction using GSWE is based on the ability of functional groups present in GSWE to act as bioreductors, reducing Ag^+ to Ag^0 (Fabiani, Silvia, Liyana, & Akbar, 2019). The color change observed in the solution from yellowish-brown to reddish-brown is one of the indications that the AgNP formation reaction has occurred.

Qualitative analysis of AgNP growth can be determined based on the specific SPR characteristics of the nanoparticles. Surface Plasmon Resonance (SPR) refers to the vibration induced by light in nanometer-sized structures and the excitation of electrons in the conduction band around the nanoparticle surface, known as localized surface plasmon resonance (LSPR). SPR greatly depends on the size, shape, and dielectric properties of the particle and its

surroundings, so each type of particle has a different SPR range (Wendri, Rupiasih, & Sumadiyasa, 2017). The SPR absorption band of AgNPs occurs at a wavelength of 400–450 nm (Vijayan et al., 2014).

Silver nanoparticles tend to aggregate, as qualitatively indicated by a broad absorbance spectrum. This aggregation tendency arises the solutions Brownian motion and Van der Waals interactions. Aggregation leads to non-uniform nanoparticle sizes or diameters (Acharya, Mohanta, Deb, & Sen, 2018). A stabilizing agent is required to maintain aggregation and ensure uniform size distribution (stability). In this study, polymer-based stabilization is achieved by using polyvinyl alcohol (PVA). PVA, a polymer, stabilizes AgNPs through steric stabilization. This occurs when polymer molecules adsorb onto the nanoparticle surfaces, forming a coating layer that generates repulsive forces between particles, preventing them from clumping together (Shafaei, 2007).

Steric stabilization works by having polymer chains create a protective barrier around nanoparticles, generating repulsive forces that oppose the Van der Waals attractions within the solution. When colloidal particles come close to each other, Van der Waals forces tend to pull them together. In the absence of these repulsive forces, particles will clump, causing instability in the colloidal system. Stability is maintained because these repulsive interactions counterbalance the attractive forces, much like a stable mechanical system that returns to equilibrium after being disturbed (Kopeliovich, 2013).

The following is the absorbance spectrum of the silver nanoparticle colloid at a 1 mM $AgNO_3$ modified with 2% PVA concentration.

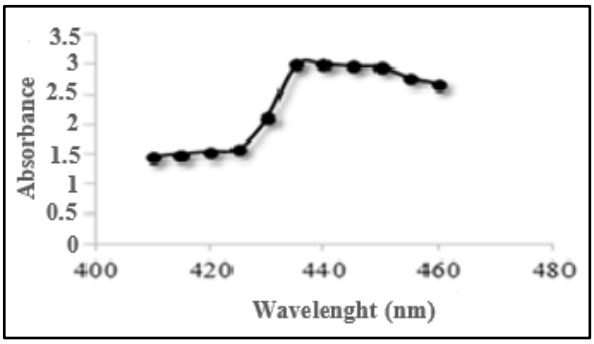

Figure 1. Absorbance vs. wavelength graph of $AgNO_3$ 1 mM silver nanoparticleswith the addition of 2% PVA.

The formation of silver nanoparticles can be identified not only by the color change of the solution but also by the appearance of a maximum wavelength (λmax) in the range of 400–450 nm, characteristic of silver nanoparticles (Mulfinger et al., 2007). Figure 1 shows that the λmax of silver nanoparticles synthesized from 1 mM $AgNO_3$ appears at 435–444 nm with an absorbance value of 3.000. Measurements using a UV-Vis spectrophotometer indicate that nanoparticles formed from a 1 mM $AgNO_3$ concentration are stable. According to Saware et al., (2014), if a larger amount of Ag^+ is reduced, an excess of extract results, which leads to more stable nanoparticles. Using of PVA solution in the nanoparticle synthesis process can also contribute to achieving stable results. Furthermore, the absorbance value can indicate the tendency of nanoparticle size. Qualitatively, the higher the absorbance value, the greater the number of nanoparticles formed, or the higher the nanoparticle concentration in the solution (Mulfinger et al., 2007).

The Analysis of The UV-Vis Spectrophotometer of The Ag/ONC Nanocomposite with Gandaria Seed Water Extract

The formation of silver nanoparticles is accompanied by an increase in Surface Plasmon Resonance (SPR). SPR is highly dependent on the size, shape, and dielectric properties of the particles and their surroundings, so each type of particle has a different SPR range. The synthesis of silver nanoparticles in the ONC sample was analyzed using a UV-Vis spectrophotometer at 420–460 nm wavelenghts. The UV-Vis spectrum of the Ag/LAO nanocomposite measurement is shown in Figure 2.

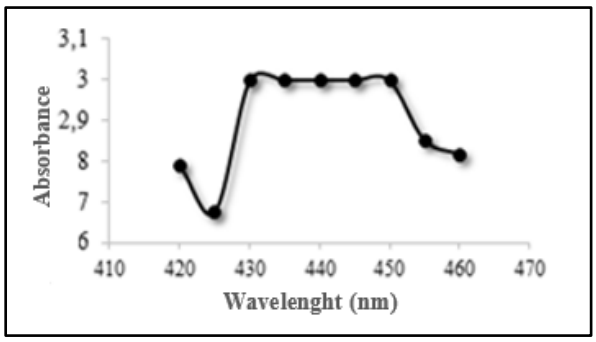

Figure 2. UV-Vis Spectrum of the Ag/LAO Nanocomposite

Antibacterial Activity Testing Using the Well Diffusion Method

Antibacterial activity testing using the well diffusion method. The principle of this method involves creating wells in agar that has been inoculated with bacteria and then dropping the test solution into the wells. Inhibition of microbial growth is observed as a clear zone (inhibition zone) around the wells. This method has the advantage of making it easier to

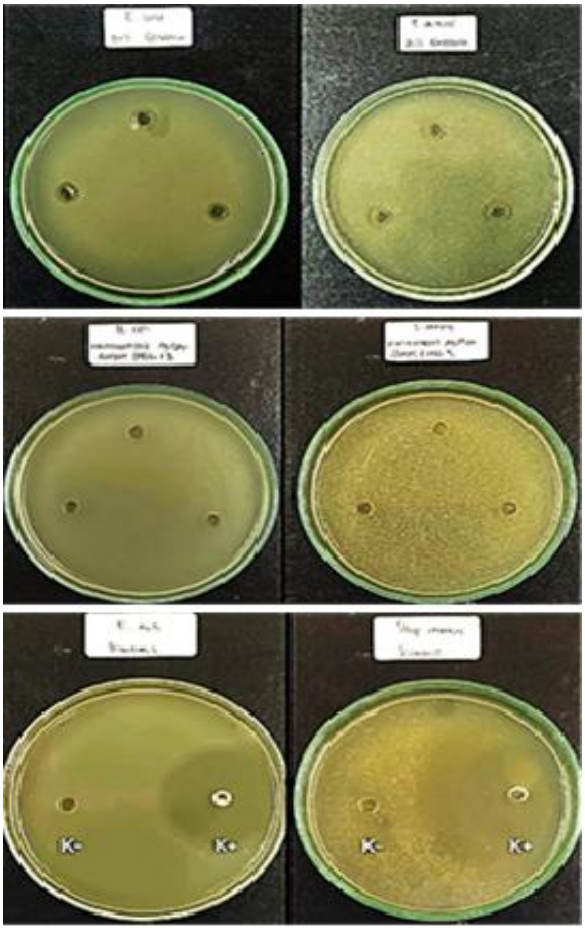

Figure 3. Antibacterial activity against *E. coli* and *S. aureus* (a) GSWE with the addition of PVA, (b) Ag/LAO nanocomposite and GSWE, (c) positive control (+), negative control (−)

measure the size of the inhibition zone because the active substance diffuses not only on the surface of the agar but also beneath the agar surface. However, this method also has disadvantages, such as the medium being highly susceptible to contamination during sound creation and when opening the petri dish to add the sample.

Nutrient agar media is used in antibacterial testing with the good diffusion method and is previously inoculated with *E. coli* and *S. aureus* bacteria. The samples used are silver nanoparticles (AgNP) synthesized with 1% gandaria seed water extract and 2% PVA, with a volume of 40 µL, and Ag/ONC nanocomposite with 1% GSWE, also 40 µL. The Ag/ONC nanocomposite and GSWE formed earlier were still solid, so they needed to be dissolved in distilled water. Distilled water was the negative control, and amoxicillin was the positive control. Figure 3 shows the antibacterial activity of the samples against *E. coli* and *S. aureus*. The clear zone indicates bacterial inhibition activity. The diameter of the inhibition zones on each petri dish was determined using a ruler and recorded in millimeters. A clear zone signifies antibacterial effects; a larger inhibition zone corresponds more potent antibacterial activity of the extract. On the other hand, the absence of a clear zone indicates that the extract lacks antibacterial properties. Table 1 presents the antibacterial activity results of the Gandaria seed water extract and the Ag/ONC nanocomposite containing 1% GSWE against E. coli and S. aureus.

Table 1. Diameter of the inhibition zone of gandaria seed water extract and Ag/LAO EABG nanocomposite against E. coli and S. aureus bacteria.

Sample	Average Inhibition Zone Diameter (mm)	
	E. coli	*S. aureus*
AgNP with GSWE and PVA	10,6	6,8
Ag/ONC Nanocomposite and GSWE	0	0
Positive Control (+) Amoxicillin	34,5	35
Negative Control (-) Distilled Water	0	0

The results in Table 1 show that the antibacterial activity test of AgNP synthesized from gandaria seed water extract and PVA against *E. coli* and *S. aureus* bacteria exhibits different inhibition zones. This is due to the addition of silver nanoparticles with a 1 mM $AgNO_3$ precursor, which produces nanoparticles with the smallest size and good stability. Silver nanoparticles have a larger specific surface area; therefore, they are capable of penetrating the bacterial cell membrane or attaching to the bacterial surface. When silver nanoparticles come into contact with the bacterial cell wall, they alter its shape and destroy the cell wall and membranes structure (Shang et al., 2020). Differences in the inhibition zones may also be caused by other factors, such as variations in the bacteriostatic properties of the samples against the two bacteria. It is suspected that the sample affects the growth rate of *E. coli* more slowly than *S. aureus*, so during the inhibition zone measurement, the diameter obtained for *E. coli* is larger, while that for *S. aureus* is smaller. This is supported by research from (Septiani, Dewi, &

Wijayanti, 2017), which showed that each bacterium has its own effective inhibition time, and bacterial inhibition effectiveness does not always increase over time due to bacteriostatic properties.

According to Davis and Stout (1971), antibacterial effectiveness is categorized into four levels based on the diameter of the inhibition zone: less than 5 mm is considered weak, 5–10 mm is moderate, 10–20 mm is strong, and more than 20 mm is very strong. Using this classification, the silver nanoparticles (AgNPs) synthesized with gandaria seed water extract and PVA exhibit spotent antibacterial activity against E. coli, with an inhibition zone measuring 10.6 mm. In contrast, the same AgNP formulation shows moderate activity against S. aureus, producing an average inhibition zone of 6.8 mm. Generally, a larger inhibition zone reflects higher antibacterial effectiveness (Kurniawati, 2015). This is suspected to be due to secondary metabolite compounds in the extract that have antibacterial activity. Generally, the action of these secondary metabolites as antibacterial agents can cause changes, leading to damage and inhibition of bacterial cells (Heni, Arreneuz, & Zaharah, 2015).

According to (Septiani et al., 2017), Secondary metabolites typically exhibit antibacterial effects by targeting the bacterial cell wall, modifying membrane permeability, interfering with protein synthesis, and blocking enzyme functions. In gandaria seeds, flavonoids are identified as the primary secondary metabolites. Flavonoids belong to the most extensive class of phenolic compounds, which are widely recognized for suppressing the growth of various microorganisms, including viruses, bacteria, and fungi (Saputra and Anggraini, 2016)**.** Flavonoids are polar compounds, making it easier for them to penetrate the polar peptidoglycan layer, such as that found in *E. coli* bacteria. Flavonoids exert their antibacterial effects through three primary mechanisms: disruption of nucleic acid synthesis, interference with membrane function, and inhibition of cellular energy production. These compounds compromise the integrity of bacterial cell walls, microsomes, and lysosomes, primarily due to their interaction with bacterial DNA, leading to increased membrane permeability and cellular damage. In addition, flavonoids inhibit cell membrane function by forming complexes with extracellular and soluble proteins, which can damage the bacterial cell membrane and lead to the leakage of intracellular compounds. Flavonoids can also inhibit energy metabolism by blocking the use of oxygen by bacteria. Therefore, bacterial growth can be inhibited in samples of AgNP synthesized from gandaria seed water extract and PVA.

For the Ag/ONC nanocomposite and GSWE samples, both tested against the two bacteria, no inhibition zones were observed, indicating that these samples lack antibacterial activity or active compounds. In the Ag/ONC nanocomposite and GSWE samples, no inhibition zones were found because the silver nanoparticles are trapped within the clay lattice, making it difficult to release Ag ions. As a result, they cannot damage the bacterial cell walls (Kavitake, Devi, Singh, & Shetty, 2016). Additionally, the small amount of extract used also weakens the nanoparticles, affecting their size and making it difficult for them to reach the bacterial cell core, thereby reducing their effectiveness in killing or inhibiting bacteria.

The positive control demonstrated strong antibacterial activity against both tested bacterial strains, with inhibition zones measuring 34.5 mm for E. coli and 35 mm for S. aureus. Amoxicillin, a broad-spectrum antibiotic from the penicillin class, is widely used to treat infections caused by various of Gram-positive and Gram-negative bacteria. The negative control used in this study is sterile distilled water, as it is a neutral compound with no active substances. Distilled water as a negative control demonstrates that the diluent solution has no antimicrobial effect, meaning it is a suitable solvent for dissolving the test samples without influencing the growth of the test bacteria. Therefore, any observed antibacterial activity can be attributed solely to the test samples. As reported by (Lestari, Ardiningsih, & Nurlina, 2016), four key factors determine antibacterial effectiveness: the concentration of the extract, the presence of bioactive constituents, the extract's diffusion capability, and the bacterial strain targeted. These variables collectively influence the average size of the inhibition zones observed during testing.

CONCLUSION

Based on the conducted research, it can be concluded that the antibacterial activity of silver nanoparticles synthesized using gandaria seed extract and PVA against *E. coli* is classified as strong, with an inhibition zone diameter of 10.6 mm. Meanwhile, against *S. aureus*, the antibacterial activity is classified as moderate, with an inhibition zone diameter of 6.8 mm. The antibacterial activity test with the Ag/ONC nanocomposite sample, showed that no inhibition zones were observed against either of the test bacteria, indicating the absence of antibacterial activity.

ACKNOWLEDGMENT

This research was supported by the Directorate General of Higher Education, Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia through the 2023 Higher Education Basic Research Excellence Grant Program.

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