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Characterization and Antimicrobial Activity of Piper Betel L. (Betel vine) Extract-Biosynthesized Silver Nanoparticles

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Abstract

Biosynthesis is a promising and environmentally safe technique for producing effective antibacterial silver nanoparticles (AgNPs). These particles have been used for a long time to combat various bacterial strains and are regarded as an efficient method for addressing the emergence of antibiotic-resistant bacteria. In this study, *Piper betel* plant extract was tested as an agent for the biosynthesis of silver nanoparticles. Spectrophotometry was employed to determine the optimal extract concentration for biosynthesis while scanning electron microscopy was used to assess the size and shape of the nanoparticles. Broth microdilution was used to measure their antibacterial efficacy against *Methicillin-Resistant Staphylococcus aureus (MRSA)*. The highest yield of biosynthesized AgNPs was obtained using a 10% extract preparation. Characterization revealed that the nanoparticles ranged in size from 300-1300 nm and had a branched shape, which is known to enhance antimicrobial effectiveness due to the sharp edges. The mean minimum inhibitory concentration (MIC) against MRSA was determined to be 19.53 µg/mL, while the mean minimum bactericidal concentration (MBC) was 21.0 µg/mL. *Piper betel* extract is an effective agent for the biosynthesis of AgNPs.

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Contents

1 Introduction

Due to the emergence and increasing prevalence of antibiotic-resistant bacteria, the search for alternative strategies has led scientists to either revisit traditional medicinal modalities or develop novel technologies. One of the fields where these two strategies meet is the biosynthesis of antimicrobial silver nanoparticles (Barlow, 2018; Ahmed et al., 2023).

Nanotechnology is a field of science that consists of designing and producing useful materials that range in size from 1 to 100 nanometers or molecules with dimensions as small as 1 millionth of a millimeter. A wide variety of applications has been developed for nanoparticles in the field of bioengineering and pharmaceuticals (Saores et al., 2018). One of these, silver nanoparticles (AgNPs), are effective antimicrobials. Due to its large surface area relative to its extremely small size, AgNPs can penetrate bacterial cell walls and easily bind or react with cellular components (Beyene et al., 2017; Sagee et al., 2012). The damage caused to the cell includes disruption of cell membrane structure, inhibition of enzyme systems, damage to DNA, and formation of reactive oxygen species (ROS). These benefits, however, are not without risks. The same mechanisms that enable AgNPs to kill bacteria can potentially cause damage to a person's cells. Care must be taken to determine how much nanoparticles can be used effectively and safely (Liao et al., 2019; Siddiqi et al., 2018).

Different methods for the synthesis of AgNPs have been reported. These methods are grouped under three major categories: physical, chemical, and biological. Physical methods such as evaporation-condensation and laser ablation have advantages over other methods in areas such as product purity and yield. Unfortunately, these technologies are expensive and consume a great amount of energy. Alternative physical methods such as irradiation pose additional hazards (Abbasi et al., 2016; Iravanit et al., 2014).

The most common of the three major technologies is chemical reduction, using either organic or inorganic reducing agents. In chemical reduction, metal ions are treated with a reducing agent in the presence of a stabilizer or capping agent. Although more practical than physical methods, studies have indicated that chemical reduction is not eco-friendly and can still be expensive. Additionally, many of these processes involve toxic chemicals and producing hazardous byproducts (Abbasi et al., 2016; Liao et al., 2019; Siddiqi et al., 2018).

Considering these challenges, researchers were prompted to develop more economical and safer biologically based methods, also known as biosynthesis. Biosynthesis can be accomplished using several techniques ranging from reducing bacteria and fungi to algae or plant extracts (Hulkoti & Taranath, 2014; Asmathunisha & Kathiresan, 2013). Extracts from different species of plants have been shown to effectively biosynthesize metal nanoparticles by providing phytochemicals that, in a similar way to chemical reduction, serve as reducing agents and stabilizers. These however are eco-friendlier and more economical (Iravanit et al., 2014; Gour & Jain, 2019).

One plant that shows promise is the evergreen vine *Piper betle L*., locally known in the Philippines as "ikmo" or Betel vine. Ikmo has several uses in local tradition. Its leaves have been used as herbal medicine for its antimicrobial properties and as an ingredient for a traditional chewing mixture known as "nga-nga" when used with lime and areca nut (De Castro-Ontengco & Capal, 2019). Betel leaves have been shown to have high amounts of reducing substances, which are important components for the biosynthesis of AgNPs (Biswas et al., 2022; Periakaruppan & Danaraj, 2022).

This study investigated whether the leaf extract of *Piper betle L.* can be used to successfully synthesize AgNPs from silver nitrate ($AgNO_3$). Characterization of the nanoparticles was determined using scanning electron microscopy (SEM). Further, the antimicrobial activity of the resulting product was tested against methicillin-resistant *Staphylococcus aureus* through the determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) by broth microdilution technique.

2 Materials and Methods

Materials

Fresh piper betel leaves were sourced from Subic, Zambales, and authenticated by the Jose Vera Santos Memorial Herbarium of the University of the Philippines – Diliman. Analytical grade $AgNO_3$ (Loba Chemie India) was provided by the Chemistry Department of the Adventist University of the Philippines while the Mueller-Hinton Agar (Himedia) and Tryptic Soy Agar (Himedia) were provided by the Medical Laboratory Science Department of the same university. Mueller-Hinton broth media was purchased in Sta. Cruz, Manila.

Four clinical isolates of Methicillin-Resistant *Staphylococcus aureus* (MRSA) were provided by the Mary Mediatrix Medical Center in Lipa city (Cuny et al., 2010; Nimmo & Coombs, 2008). These isolates come from different clinical samples namely, blood, eyelid abscess, wound abscess, and pus from an infected foot. The isolates were sub-cultured into thioglycolate broth at 35^{o}C then maintained at 4 °C until further use.

Preparation of Aqueous Extract

Piper betel leaves were washed with sterile distilled water and then oven-dried at 100^{o}C for 10 minutes. The dried leaves were ground using a blender until they reached a powdery consistency. A total of 5.3 grams of powdered dry leaves were obtained (Gupta et al., 2023; Singh et al., 2023).

The powdered leaves were mixed with 100 mL of sterile distilled water in a beaker and heated at 60^{o}C for 20 minutes with constant agitation using a magnetic stirrer. It was left to stand at room temperature for an additional 10 minutes before it was filtered using Whatman paper No. 1. The resulting filtrate was stored at 4^{o}C and utilized for the biosynthesis of AgNP.

Biosynthesis of AgNP

Five milliliters of the aqueous piper betel extract was mixed with 45 mL of 0.001 M solution of $AgNO_3$ in an Erlenmeyer flask. The mixture was stirred continuously at 35^{o}C for 24 hours using a magnetic stirrer following the procedure by Jalab et al. (2021). The progressive reactivity and formation of the AgNPs was determined based on the color alteration of the mixture from light yellow to reddish brown. To confirm the reaction, the light absorption peak was measured using a UV-Vis spectrophotometer (Hitachi U-5100) between the 300 – 600 nm range.

Characterization of AgNP Morphology and Size

To determine the surface morphology and size of the synthesized nanoparticles, the products were examined under a scanning electron microscope (Thermo-Scientific^{TM} Phenom XL) in De La Salle University, Taft Ave., Manila.

Determination of MIC and *MBC*

To measure the antimicrobial activity of the biosynthesized AgNPs, broth microdilution method was employed using CLSI standard procedure. Briefly, serial two-fold dilutions of biosynthesized AgNP ranging from 600 µg/mL to 1.17 µg/mL were incubated with standardized inoculums of the four MRSA isolates. The tests were performed in triplicates. MICs were determined as the lowest concentration of AgNP that resulted in growth inhibition indicated by the lack of turbidity in the wells after 24 hrs of incubation at 35°C. MBCs were determined as the lowest concentration of the AgNP that showed no growth after 24 hrs of incubation at 35°C when an aliquot from the wells was sub-cultured onto Tryptic Soy Agar plates (Mandal & Mandal, 2011; Magana et al., 2008).

3 Results and Discussions

Morphology and Size

Scanning electron microscopy revealed the resulting AgNPs have varying shapes and sizes. Some particles were more spherical while others appeared like long plates demonstrating sharp edges. While most of the particles were <0.1 µm (<100 nm) in size, others were larger than 2 µm (2000 nm). The heterogenous nature of the resulting particle shape and size may influence its antibacterial activity. Figure 1 presents electron micrograph images of the resulting particles.

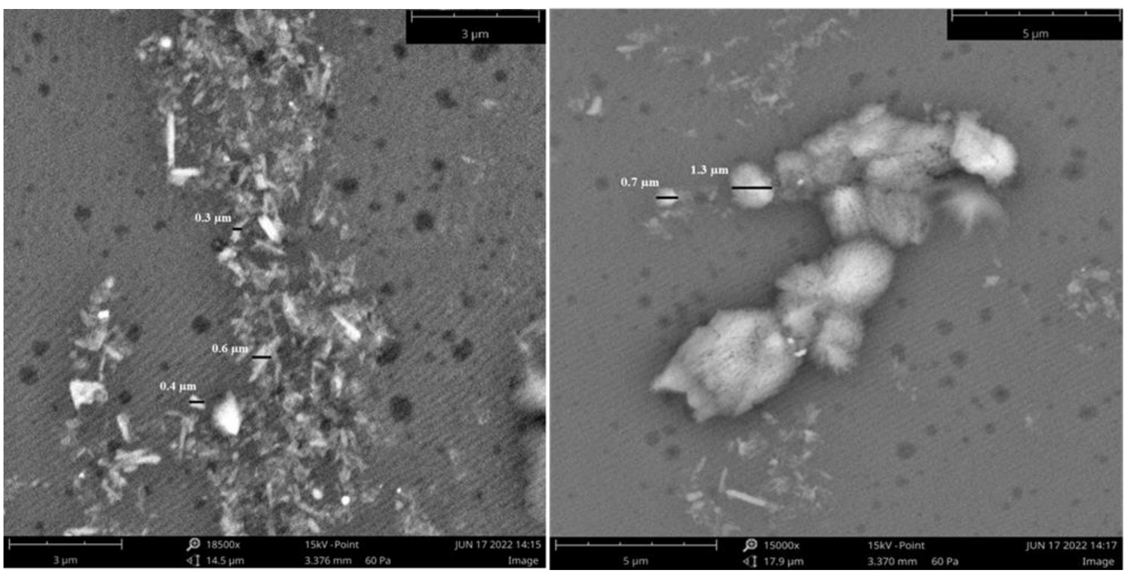

Figure 1. *Scanning Electron Micrograph of Biosynthesized Silver Nanoparticles*

The general understanding is that nanoparticles have more antimicrobial activity at smaller sizes. The small size of the particles is believed to be beneficial to their antimicrobial effect since it allows the particles to penetrate cell walls and membranes (Osonga et al., 2020; Abbasi et al., 2016). However, Truong et al. (2020), in a study comparing the effect of varying sizes determined that larger particles could demonstrate higher antibacterial activity than smaller particles. Shape dependent activity has also been demonstrated previously with spherical nanoparticles showing the highest antimicrobial properties (Cheon et al., 2019).

Antimicrobial Activity Against MRSA

MIC determination through broth microdilution method produced MICs ranging from 9.38 – 28.13 μg/mL with an average of 19.53 μg/mL. Further determination of MBC revealed bactericidal concentrations to range from 12.5 – 28.13 μg/mL with an average of 21.0 μg/mL.

Table 1
MIC and MBC of Biosynthesized AgNPs against MRSA

Isolate Number	MIC (μg/mL)	MBC (μg/mL)
1	28.12	28.12
2	25.0	25.0
3	15.63	18.75
4	9.38	12.5
Mean	19.53	21.0

In contrast, a study by Parvekar et al. (2020), demonstrated an average MBC of 625 μg/mL. This shows that AgNPs biosynthesized using Betel leaf extract was able to inhibit bacterial growth and kill MRSA at much lower concentrations than previously demonstrated.

4 Conclusion

Biosynthesis using aqueous Piper betel leaf extract is effective in conferring antimicrobial activity on silver nanoparticles against methicillin-resistant *Staphylococcus aureus*. Characterization of biosynthesized AgNPs using SEM revealed that the particles have varying shapes and sizes. The AgNPs effectively inhibited growth with a mean MIC of 19.53 μg/mL and mean MBC concentration of 21.0 μg/mL for MRSA isolates.

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