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Red Laser-Activated Silver Nanoparticles from Green Synthesis Extract of Butterfly Pea for Antimicrobial Photodynamic Therapy Against *Staphylococcus aureus*

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Abstract

This study investigated the potential of photodynamic therapy (PDT) using green-synthesized silver nanoparticles (AgNPs) derived from butterfly pea extract (*Clitoria ternatea L.*) to combat *Staphylococcus aureus* (*S. aureus*). The use of a red diode laser as a method for enhancing the antimicrobial activity of AgNPs presents a novel approach to treating bacterial infections. The red diode laser is crucial, as it activates the AgNPs, enhancing their antimicrobial properties. This combination of light, natural extract, and nanoparticles underscores the innovative approach of using PDT in treating bacterial infections. By integrating these elements, the study aims to provide insights into effective, biocompatible treatments for antibiotic-resistant bacteria. The primary objective of this study is to synthesize and characterize AgNPs using butterfly pea extract and evaluate their effectiveness against *S. aureus* when combined with red laser irradiation. Silver nanoparticles were synthesized using an environmentally friendly method that processes butterfly pea extract as the reducing agent for the synthesis of the nanoparticles. Using UV-Vis spectrophotometry to track the creation of silver nanoparticles (AgNPs), it was determined that the butterfly pea extract was an effective source of nanoparticles. The particle size distribution and peak absorbance wavelength were determined by characterization utilizing a Particle Size Analyzer (PSA). Tryptic soy agar (TSA) plates were used to investigate the antibacterial activity of AgNPs against *Staphylococcus aureus (S. aureus)*. The effectiveness of photoinactivation against S. aureus was evaluated by exposing AgNPs at a concentration of 1 mM to a red diode laser for 90 seconds. The results showed that the produced AgNPs had potential antibacterial capabilities when combined with red light therapy. The results demonstrated that the synthesized silver nanoparticles can effectively kill or inhibit the growth of *Staphylococcus aureus (S. aureus)* when exposed to a red diode laser for 90 seconds. The findings suggest that photodynamic therapy using green-synthesized AgNPs and red laser irradiation could be a promising approach to controlling bacterial infections like *S. aureus*. Further research is recommended to explore the underlying mechanisms of photoinactivation and to optimize treatment parameters for in vivo applications on experimental animals.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium responsible for many clinical diseases. Infections caused by this pathogen are prevalent in both community and hospital settings. Treatment remains challenging due to the emergence of multidrug-resistant strains, such as Methicillin-Resistant *Staphylococcus aureus* (M.R.S.A.). According to WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) 2020 data, 40% of *S. aureus* strains are resistant to MRSA.[1]

One promising approach is the development of nanotechnology-based antimicrobial therapies, particularly the green synthesis of nanoparticles. Nanotechnology involves the production of materials at the nanoscale, typically ranging from 1 to 100 nm.[2] Nanoparticles can be produced from gold, silver, platinum, and palladium materials. Silver nanoparticles (AgNPs) are especially favoured due to their ease of production, unique properties, and strong affinity for binding to various biomolecules, making them effective in eradicating bacteria while being non-toxic to humans, animals, and plants.[3]

The benefits of nanoparticles include increased reactivity and focused medication administration with fewer adverse effects because of their tiny size and large surface area. Technology and medicine are progressing because of their special qualities, which are useful in imaging, diagnostics, and environmental applications. Silver nanoparticles are synthesized by introducing reducing agents through physical, chemical, biological, and green synthesis methods. Green synthesis is a method using organisms such as algae, plants, bacteria, fungi, and their metabolities. The method of synthesis with plant extracts is effective in preventing oxidation and aggregation of synthesized AgNPs while reducing cost and chemical usage.[4] The success of silver nanoparticle formation depends on the presence of many phytochemicals of plant extract which is fully responsible for AgNPs synthesis.[5] A green synthesis approach is employed to mitigate the dangers associated with these reductants,[6] utilizing natural materials such as plant extracts derived from roots, stems, leaves, seeds, fruits, and flowers.[7]

One of the plant extracts with functional benefits for the human body is the butterfly pea (*Clitoria ternatea L.*). The butterfly pea (*Clitoria ternatea L.*), which has antibacterial, antidiabetic, anti-obesity, anticancer, and anti-inflammatory qualities, is one of the plant extracts with practical uses. When these advantages are delivered as nanoparticles, their bioavailability and therapeutic efficacy increase. All parts of the butterfly pea plant are claimed to offer various health benefits. For instance, the petals are known for their antioxidant, antidiabetic, anti-obesity, anticancer, anti-inflammatory, and antibiotic properties.[8] Butterfly pea also exhibits pharmacological potential as an antimicrobial, antidepressant, anthelmintic, anticancer, and antidiabetic agent, with anthocyanin pigments that are water-soluble and produce colors ranging from red to blue.[9]

Using natural extracts as reducing and stabilizing agents in the synthesis of AgNPs is environmentally friendly and enhances the biocompatibility of the nanoparticles produced. Photodynamic therapy (PDT) is a promising alternative that combines light of specific wavelengths with photosensitizers and oxygen to generate reactive oxygen species (ROS) capable of killing microorganisms. In this context, silver nanoparticles (AgNPs) have shown significant potential as photosensitizers due

to their antimicrobial properties and ability to produce ROS when light activates them. The red laser-activated synthesis of butterfly pea (*Clitoria ternatea L.*) extract could be a novel approach to enhancing the effectiveness of photodynamic antimicrobial therapy.[10] *S. aureus* is efficiently eliminated by red laser-activated PDT because of its deep tissue penetration and enhanced generation of ROS. Strong photosensitizer properties of the butterfly pea extract improve antibacterial activity, especially against resistant bacteria like MRSA.[11]

This study aims to determine the antimicrobial effectiveness of AgNPs derived from butterfly pea extract (*Clitoria ternatea L.*) through the photodynamic therapy of bacteria by combining red laser light. This combination is expected to produce reactive oxygen species that cause biological damage to the target *S. aureus*. This study's long-term benefit is providing valuable knowledge to stakeholders about using silver nanoparticles from butterfly pea extract as photosensitizers in photodynamic therapy to reduce *S. aureus* bacteria.

MATERIALS AND METHODS

This research was conducted at the Biophysics Laboratory of Universitas Airlangga from March to May 2024. The materials used in this experiment included butterfly pea (*Clitoria ternatea L.*) extract, distilled water, liquid $AgNO_3$ (at concentrations of 1 mM, 1.5 mM, 2 mM, and 3 mM), tryptic soy agar (TSA), tryptic soy broth (TSB), *S. aureus* bacteria, and physiological saline (distilled water and pure NaCl solution).

The important materials and tools utilized in this experiment are clearly listed, and each item is associated with a trademark which included CNC Red-Ozone Diode Laser, Total Plate Counter, Biobase dynamic light scattering nanometer particle size analyzer, and UV-Vis spectrophotometer.

CNC Red-Ozone Diode Laser, which is essential for causing silver nanoparticles to activate. This laser's emission produces reactive oxygen species (ROS) at certain wavelengths, which increases the antibacterial efficiency against *Staphylococcus aureus*. Provide comprehensive diode laser specs to guarantee maximum treatment efficacy and repeatability.

The mechanism of this research method is described as follows in Figure 1.

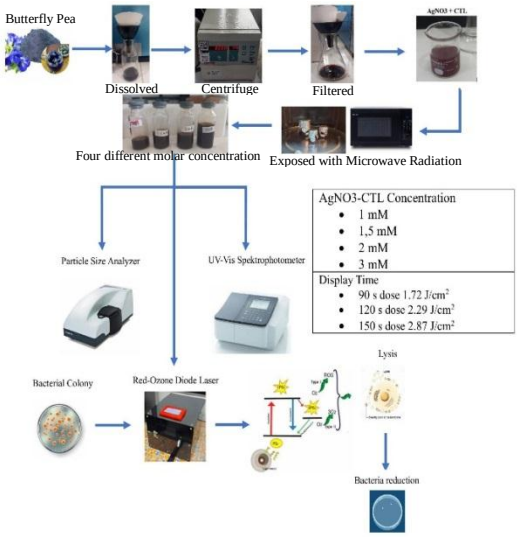

Figure 1. Mechanism of Research Methods

Silver Nanoparticles Butterfly Pea

The process of making silver nanoparticles concentrated using the extract from butterfly peas (*Clitoria ternatea L.*) as a reducing agent. A clear extract solution was produced after dissolving 25 grams of butterfly pea powder in 50 milliliters of distilled water and centrifuging the mixture to get rid of any debris. Rather than going into depth about how the concentrated extract was made, it was decreased to 12 milliliters and used to create silver nanoparticles. The emphasis of the final AgNPs is their

ability to attack *Staphylococcus aureus* effectively.

To determine the effect of $AgNO_3$ concentration, the filtered solution was mixed with $AgNO_3$ at four different molar concentrations: 1 mM, 1.5 mM, 2 mM, and 3 mM. Butterfly pea extract as the filtered solution was produced in a 1:9 ratio to $AgNO_3$ for each molar concentration. After that, the mixture was exposed to 450 watts of microwave radiation for five minutes, which produced silver nanoparticles.

AgNPs-CTL Characterization Method

Reactive oxygen species are produced when a diode laser is used because the light it generates has certain wavelengths that silver nanoparticles can absorb.[10] These very reactive chemicals, known as ROS, can harm proteins, nucleic acids, and bacterial cell membranes, eventually leading to bacterial inactivation.[11] This technique demonstrates how well photodynamic treatment targets and eradicates bacteria while improving antibacterial results when combined with diode lasers and silver nanoparticles.[12]

UV-Vis spectrum analysis was conducted using a U.V-Vis spectrophotometer (Shimadzu, 1800) covering a wavelength range of 300 to 1100 nm. The reduction of pure Ag+ ions was monitored by observing the spectrum at room temperature. The maximum characteristic peak of AgNPs is observed at around 400–500 nm.[9]

The test involved placing silver nanoparticles in a glass bottle, sealing it, and storing it in a dark room at room temperature. The extract preparation process involves using butterfly pea powder. The powder is dissolved in distilled water and stirred until thoroughly mixed. Then, it is centrifuged and filtered to obtain a clear extract solution. This standardized extract is then used to synthesize silver nanoparticles, ensuring consistency and reproducibility in subsequent experiments. The stability of the silver nanoparticles was assessed by checking the color change and monitoring the wavelength of the absorbance peak with UV-Vis daily for one week.

The absorbance test was continuously conducted from the first to the seventh day to determine the stability of the silver nanoparticles over time. The stability of the colloidal silver nanoparticles can be inferred from changes in the absorbance peak.[13] A shift in the absorption peak to a longer wavelength indicates low stability due to agglomeration, characterized by a color change and a corresponding shift in the peak wavelength.[12]

The next step in nanoparticle characterization was measuring the particle size distribution using the dynamic light scattering (DLS) method with a particle size analyzer (PSA). This method is considered more accurate than SEM or TEM as it provides the particle size distribution within the sample. PSA can measure particles ranging from 0.6 nanometers to 7 micrometres. The principle of DLS is based on the Brownian motion of suspended particles, which are generated from thermal collisions with the solvent, causing fluctuations in the intensity of the light scattered by a laser.

The analysis of these fluctuations determines the speed of Brownian motion and particle size using the Stokes-Einstein equation. PSA works by emitting light scattered by the particles in the sample, where the scattering intensity is inversely proportional to the particle size. The measurement results are processed into digital data for mathematical analysis.

Particle size and distribution measurements were conducted at the Institute of Biological Sciences and

Engineering (LIHTR), Airlangga University, using a Particle Size Analyzer (PSA). The testing involved inserting a sample of silver nanoparticles (AgNPs) into the PSA and setting the wavelength and absorbance value according to the highest result from the previous absorbance test with a UV-Vis Spectrophotometer. The measurement results included the single particle size distribution, average size, and Polydispersity Index (PdI) values.

Measurements using PSA provide average particle size distribution results, assuming a spherical shape, with the size expressed in radii.[14] PSA can measure particle sizes ranging from 0.6 nanometers to 7 micrometres. This revision improves the clarity and readability of the original text while preserving the citation placement.[15]

Antibacterial Activity Test

This test assessed the antibacterial properties of AgNPs-CTL using the disk diffusion method, a standard approach for evaluating antibacterial activity. This approach was carried out aerobically since *S. aureus* bacteria normally prefer situations with plenty of oxygen, which makes it perfect for evaluating the efficacy of antibiotics in settings that closely mimic human illnesses. The process began by culturing the bacteria in sterile Tryptic Soy Broth (TSB) and incubating them for 24 hours. Following the culturing process, bacterial growth was monitored to ensure it reached the target density. Then, 50 µL of the bacterial culture was equally distributed onto 9 cm diameter Petri plates filled with Tryptic Soy Agar (TSA) medium to guarantee constant bacterial coverage for the following tests.

After the media surface had dried, ten microliters of AgNPs were added to four 0.6-cm-diameter paper discs. These discs were then placed over the TSA medium in the Petri dishes that contained TSB and *S. aureus* bacteria. The discs were placed equidistantly within the dish, aligned in four quadrants, and incubated for 24 hours. The antibacterial activity of the butterfly pea flower extract was indicated by the presence of an inhibition zone around the paper discs, and the diameter of these zones was measured after incubation.

To prepare the bacterial culture of *S. aureus*, a sterile solution, was made by mixing 0.3 g of TSB with 10 mL of distilled water and adding *S. aureus* bacteria. Subsequently, 5 mL of the sterile TSB solution was transferred into a test tube, and one dose of bacterial isolate was mixed into it, followed by a vortex homogenization. Once homogeneous, the TSB in the reaction tube was poured into a 25 mL Erlenmeyer flask and incubated for 24 hours.

Irradiation of Bacteria with Laser

After the bacteria are grown with the photosensitizer (silver nanoparticles) for 30 minutes, the bacteria on the microplate are ready for light exposure using a red diode laser. The irradiation is performed perpendicular to the sample at a fixed ideal distance of 10 mm from the light source. A red laser with a wavelength of 600 nm was used, characterized by a Jasco CT-10 monochromator, to measure the peak wavelength. The OMM-6810B-220V power meter was used to measure the power output, which came out to be 2.49 mW. Diode laser radiation was applied for 90, 120, and 150 seconds at different intervals The irradiation stage is crucial because it must maximize the antibacterial impact while limiting harm to adjacent tissues and achieving optimal bacterial elimination via energy density optimization. The objective is to identify the optimal irradiation settings in photodynamic antimicrobial therapy to

improve treatment effectiveness.[30] Different time intervals resulted in different energy densities for each treatment.

The first step involved irradiation preparation by mixing 500 microliters of bacterial culture with the photosensitizer and diluting it in 4.5 mL of sterile physiological water, then vortexing to achieve homogeneity. Fifty microliters of the dilution and 50 microliters of AgNPs-CTL were added (1 mM, 1.5 mM, 2 mM, and 3 mM). This process was repeated five times for each AgNPs-CTL concentration, resulting in 20 wells in the microplate. The same procedure was performed for the control group (without AgNPs-CTL).

Once the microplate was prepared, it was incubated for 30 minutes at 37°C, followed by red laser irradiation for 90, 120, and 150 seconds. The irradiated samples were poured into 3.5 cm diameter Petri dishes containing sterile TSA media. The process was homogenized by forming a figure-eight pattern in each treatment group, followed by a 24-hour incubation at 37°C with the Petri dishes placed upside down. The final step involved reading the *S. aureus* antibacterial test results using a colony counter.

Data analysis

The efficacy of red laser irradiation and silver nanoparticles in suppressing Staphylococcus aureus growth was assessed by analyzing data from bacterial tests and silver nanoparticle characterization. The observation data were analyzed statistically using the Two-Way ANOVA Factorial test in IBM SPSS. The Two-Way ANOVA Factorial test is a parametric statistical test used to compare the means of multiple samples, mainly when two or more factors categorize the samples.

RESULTS AND DISCUSSION

This research uses butterfly pea flower extract in the green method of synthesizing silver nanoparticles with varying solution concentrations of 1 mM, 1.5 mM, 2 mM, and 3 mM. It uses photodynamic red laser light therapy to test the effectiveness of S. aureus bacteria at varying durations of exposure. different, namely 90 seconds, 120 seconds, and 150 seconds.

Synthesis of Silver Nanoparticles from Butterfly Flower Extract

Figure 2 shows the Butterfly Flower Extract Solution.

Figure 2. Butterfly Flower Extract Solution

Silver nanoparticles (AgNPs) were synthesised using a natural bioreductant from butterfly pea flower extract.[16] Butterfly pea flower extract contains a phenolic concentration of 16.20 µg GAE/100% concentration and a total flavonoid content of 4.88 µg QE/100%. Butterfly pea flower extract contains active compounds such as flavonoids, tannins, saponins, anthraquinones, terpenoids, and alkaloids.[8]

The anthocyanin pigment in butterfly pea flowers is water-soluble, producing colors that range from red to blue. This is evident when a solution of butterfly pea flower extract mixed with distilled water appears purplish-blue.

The color changed to brownish-yellow when $AgNO_3$ solutions with varying molarity concentrations were added and irradiated in a microwave for 5 minutes. The formation of silver nanoparticle colloids can be visually observed, with the colloid transitioning from yellow to brownish after adding butterfly pea flower extract, as shown in the following picture.

Visually, the colloidal silver nanoparticles formed are brown. The concentration variations were 1 mM, 1.5 mM, 2 mM, and 3 mM. Higher concentrations of silver nitrate precursor resulted in more pronounced color changes.[17] This occurs because higher concentrations of silver nitrate led to the formation of more silver nanoparticles in the presence of bioreactors from butterfly pea flower extract. The mechanism for reducing silver ions into colloidal silver nanoparticles can be seen in equation (1).

$$Ag^{+} + e^{-} \rightarrow Ag^{0} \qquad (1)$$

The electron source used for Ag^{+} ion reduction is predicted to come from phenol compounds which have conjugated double bonds. These phenolic compounds are functional groups attached to the structure of the flavonoid compounds contained in butterfly pea flower extract. Butterfly pea flowers contain a total of phenolic compounds ranging from 53-460 mg or the equivalent of acid gallate/g dry extract as well as compounds such as tannins, saponins, triterpenoids, phenols, flavonoids, flavonol glycosides, alkaloids, anthraquinones and steroids.[18]

Flavonoid compounds can be used as natural reducing agents in the synthesis of silver nanoparticles (AgNps). Phenolic compounds in flavonoids function as natural bioreductors for the formation of Ag^{+} ions into Ag^{0}. Apart from functioning as a natural bioreductors, butterfly pea flower extract also functions as a capping agent for silver nanoparticles so that the particle size remains stable on the nanometer scale.

Characterization of Nanoparticles using UV-Vis

The stability of AgNPs-CTL from a physical perspective can be observed visually through changes in the color of the solution. The color change in the green synthesis AgNPs-CTL is a crucial indicator of the reduction of silver to nanoparticles, as shown in Figure 2. According to Figure 2, the color changes from brownish-yellow to a darker brownish hue. There was no visible agglomeration in the sample, indicating that the particle size in the solution was small and stable and did not tend to form large clumps. This information is crucial for ensuring the effectiveness of nanoparticles in various applications. Over time, as the solution is left in the dark at room temperature, the color of AgNPs-CTL changes from yellow-brown to dark brown.

The formation of silver nanoparticles is marked not only by a change in solution color but also by the appearance of $\lambda maks$.[13] Silver nanoparticles exhibit the Surface Plasmon Resonance (SPR) phenomenon,[19] which can be observed in the spectrum of wavelength and absorbance peak measurements using a UV-Vis spectrophotometer. The absorbance and wavelength were measured using a UV-Vis spectrophotometer to confirm the ongoing reduction reaction. The formation of silver nanoparticles is characterized by an absorbance peak and a wavelength in the range of 385-515 nm. AgNPs-CTL UV-Vis analysis with variations (1 mM, 1.5 mM, 2 mM, 3 mM) is illustrated in Figure 3a (first day) and Figure 3b (seventh day).

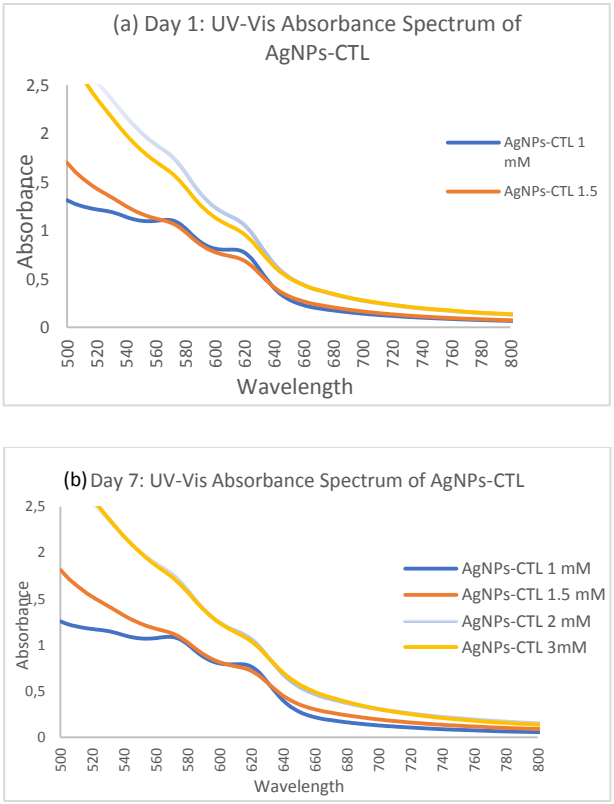

Figure 3. UV-Vis Spectrum of Solution Synthesis of Silver Nanoparticles $AgNO_3$ concentration 1 mM, 1.5 mM, 2 mM, 3 mM first day (a) and seventh day

The stability of the absorbance and wavelength of AgNPs-CTL was analyzed using a UV-Vis spectrophotometer on day 1 and day 7 after synthesis. The absorbance peak and wavelength of AgNPs-CTL with different concentrations (1 mM, 1.5 mM, 2 mM, 3 mM) show two peak wavelengths with the following descriptions: (1) 1 mM has peak wavelengths of 574 and 618 nm with absorbances of 1.052 and 0.708, (2) 1.5 mM has peak wavelengths of 571 and 621 nm with absorbances of 1.075 and 0.678, (3) 2 mM has peak wavelengths of 575 and 618 nm with absorbances of 1.699 and 0.982, and (4) 3 mM has peak wavelengths of 575 and 619 nm with absorbances of 1.531 and 0.972.

According to Figure 3, on the seventh day of measurement, there are two peaks in wavelength and absorbance as follows: (1) 1 mM has peak wavelengths of 573 and 612 nm with absorbances of 1.074 and 0.71; (2) 1.5 mM has peak wavelengths of 571 and 619 nm with absorbances of 1.176 and 0.741; (3) 2 mM has peak wavelengths of 575 and 618 nm with absorbances of 1.699 and 0.982; and (4) 3 mM has peak wavelengths of 576 and 619 nm with absorbances of 1.732 and 1.051.

The UV-Vis test results indicate that the wavelength stability of AgNPs-CTL is within the range of 570–620 nm. Non-agglomerated AgNPs-CTL improves the antibacterial effectiveness due to their superior stability and reactivity. Large clumps decrease the efficacy of therapy because they have less surface area and responsiveness. The first wavelength range of 570 nm shows the characteristic value of silver nanoparticles. The second wavelength range of 620 nm represents absorption used as a reference for measuring red diode laser irradiation, which has a wavelength spectrum of 620–759 nm.[12] This observation, as depicted in Figure 3, shows no significant shift in wavelength, indicating relative stability. Meanwhile, the decrease in absorbance at a concentration of 1 mM suggests a change in the composition of the solution, potentially due to the oxidation of phytochemicals in the extract that may have contributed to the absorption at 570 nm.[20] Table 1 shows the PSA Test Results.

Table 1. PSA Test Results

Concentration AgNPs-CTL	Average (nm)	Standar Deviation (nm)
1 mM	5.35	4.90
3 mM	4.04	3.22

Particle Size Analyzer Characterization

The Particle Size Analyzer (PSA) test aims to determine the particle size distribution using the Dynamic Light Scattering (DLS) method, which utilizes infrared scattering. DLS is also known as photon correlation spectroscopy. The particle size measured by DLS is the diameter of a circle of particles that diffuse at the same speed during the measurement. In this study, we tested AgNPs-CTL at 1 mM and 3 mM concentrations.

From the PSA test results, as shown in Table 1, the average particle diameter size for AgNPs-CTL 1 mM is 5.35 nm, while for AgNPs-CTL 3 mM, it is 4.04 nm. These results indicate that AgNPs-CTL 1 mM and 3 mM fall within the nanoparticle category, as their sizes are between 1-100 nm. These findings correspond with the nanoparticle category for the other two molarities tested.

Figure 4 shows the PSA Test Results AgNPs-CTL 1 mM and 3 mM. Figure 4(a) illustrates the particle size distribution of AgNPs-CTL 1 mM, which ranges from 1.22 nm to 18.23 nm. Figure 4(b) shows the particle size distribution of AgNPs-CTL 3 mM, which ranges from 1.22 nm to 12.22 nm. The distribution indicates that most of the particles are concentrated in the smaller size range, as evidenced by the peaks at low diameter values.

Antibacterial Activity Test

Antibacterial test against *Staphylococcus aureus* was carried out to determine the diameter of the inhibition zone for the growth of bacterial colonies. Each sample was tested using the disk diffusion method. The results showed an inhibition zone with antibacterial AgNPs-CTL of 1 mM, 1.5 mM, 2 mM, and 3 mM, respectively, for *Staphylococcus aureus*, a Gram-positive bacterium. Measurement of the inhibition zone of AgNPs-CTL with *Staphylococcus aureus* showed good antibacterial activity with an average inhibition zone of 1.40 mm.

Figure 5 shows photographic images of bacterial inhibition zones against *S. aureus*, produced by silver nanoparticles prepared with different molar ratios of $AgNO_3$. These results show good antibacterial activity with an average inhibition zone diameter of 1.51 mm.

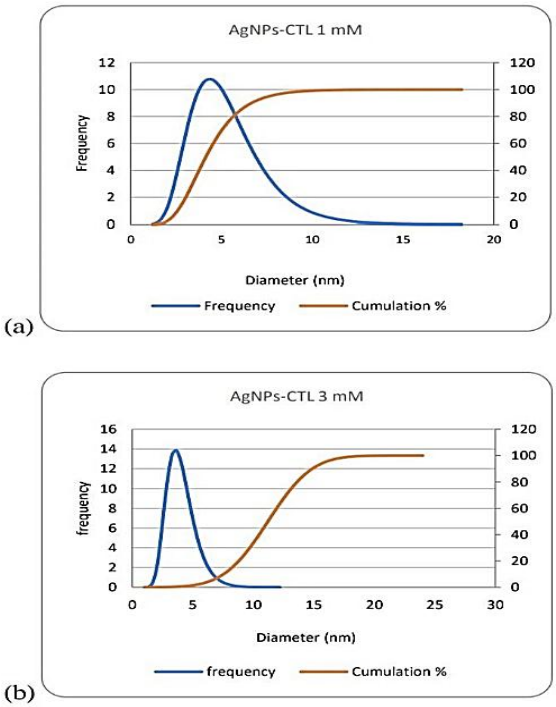

Figure 4. PSA Test Results AgNPs-CTL 1 mM and 3 mM

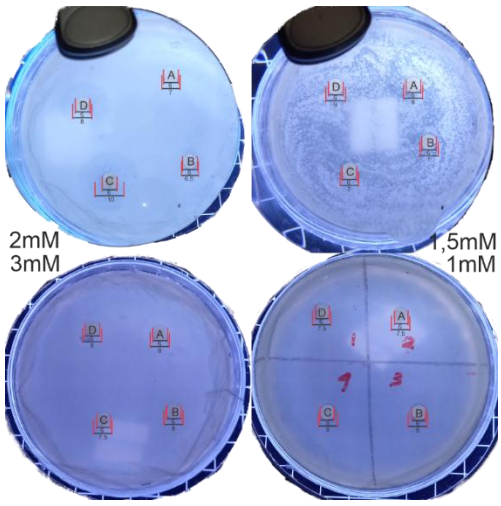

Figure 5. Disc Diffusion Method Results

Based on the inhibition zone test results presented in Table 1, it can be seen that the average diameter of the inhibition zone is the highest there is inhibition of AgNPs-CTL of *S. aureus* bacteria with a concentration variation of 3 mM, which is 1.8 mm. This shows that AgNPs-CTL has good antibacterial effectiveness.[21] Figure 5 shows the transparent zone (inhibition zone) formed using the disc diffusion method, indicating the antibacterial effectiveness of different treatments against *Staphylococcus aureus.*

Laser Irradiation Results

The laser irradiation process was carried out with varying times, namely 90, 120 and 150 seconds. These different time intervals result in different energy densities for each treatment. The effectiveness of this laser irradiation can be observed by reducing the number of bacterial colonies compared to the control group.

The addition of AgNPs-CTL to bacterial samples shows that the synthesis used is antibacterial. Bacterial growth results were obtained by calculating the number of bacterial colonies grown with AgNPs-CTL in a plate.

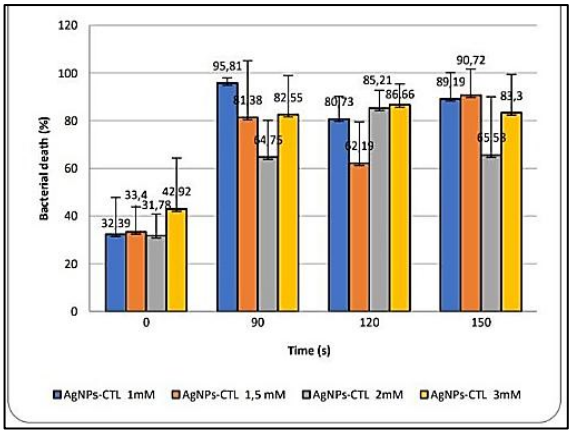

Figure 6. Percentage of Death of *Staphylococcus aureus* Bacteria

Figure 6 shows the percentage increase in death of *Staphylococcus aureus* bacteria. It can be seen that compared to the addition of AgNPs-CTL without irradiation (0s), the addition of AgNPs-CTL becomes effective with laser irradiation. There was an increase in bacterial death at each variation in AgNPs-CTL concentration.

Analysis of the Effectiveness of Red Laser against Staphylococcus aureus Bacteria

The observation data obtained were then analyzed statistically using the Two-Way ANOVA Factorial test on IBM SPSS. Based on the results of the statistical analysis in Table 2, a significance value of ($p = 0.094$) (where $\alpha = 0.05$) was obtained from the data normality test, indicating that the data is usually distributed. The subsequent analysis using the Two-Way ANOVA Factorial test yielded a significance value of ($p = 0.043$) for the variations in AgNPs-CTL concentration and ($p = 0.039$) for the interaction between AgNPs-CTL concentration and exposure time, both of which are less than ($\alpha = 0.05$). This indicates significant differences in both cases. A post hoc test determined which treatment groups showed significant differences. The results revealed that AgNPs-CTL concentrations of 1 mM and two mM produced significantly different outcomes, with the highest percentage of bacterial death being 88.58% for the one mM concentration.

Additionally, the interaction between AgNPs-CTL concentration and exposure time showed that the highest percentage of bacterial death was achieved with a combination of 1 mM for 90 seconds (1A), yielding a value of 95.81%.

Other notable results included combinations of 1.5 mM for 150 seconds (2C) with 90.72% bacterial death and 1 mM for 150 seconds (1C) with 89.19% bacterial death.

These results suggest that lower concentrations with shorter exposure times can effectively kill bacteria. Overall, based on this analysis, the most optimal treatment combination for bacterial inactivation is a concentration of 1 mM for 90 seconds.

Conversely, for variations in exposure time, the Two-Way ANOVA Factorial test showed a significance value of ($p = 0.790$) (where ($\alpha = 0.05$), indicating that variations in exposure time do not produce a significant difference.

Photodynamic inactivation involves three main aspects: visible light, reactive oxygen species (R.O.S.), and photosensitizers that act as light sensitizers. This process requires an alignment between the absorption spectrum of visible light and the photosensitizer. Lasers are commonly used as light sources in photodynamic inactivation due to their advantages, such as uniform (monochromatic) and parallel light emission.[23] This research used a red laser diode with a wavelength range of 620 – 759 nm.

The photochemical process in photodynamic inactivation consists of two types: type I and type II. In the type I pathway, electron transfer occurs between excited sensitizer molecules and biological molecules, producing radical ions in the form of reactive oxygen species (ROS). Meanwhile, in the type II pathway, energy is transferred from the excited triplet photosensitizer to the triplet oxygen, producing excited singlet oxygen. ROS can damage the structure of bacterial cell walls, causing bacterial cell lysis. This is the initial stage in bacterial cell death.[24]

ROS and triplet oxygen radicals formed from photochemical processes initiate the peroxidation of unsaturated fatty acids, forming hydroperoxides. Next, singlet and triplet oxygen radicals break hydrogen bonds in saturated fatty acids, producing toxic hydroperoxides, known as lipid peroxidation. This process damages the structure of the bacterial cell wall, resulting in cell lysis.[25]

This research uses *Staphylococcus aureus* as a Gram-positive bacterium with a thick peptidoglycan layer, making it more stress-resistant. A red laser was used to irradiate it for 90, 120, and 150 seconds. The red laser generates reactive oxygen species (ROS) that penetrate the cell wall, damaging proteins, DNA, and lipids, thus effectively inhibiting S. aureus growth. Longer irradiation times enhance the antibacterial effect by increasing ROS production. [26]

This is because *Staphylococcus aureus*, a Gram-positive bacterium, has cell walls consisting of peptidoglycan, teichoic acid and neuraminic acid.[26,27] This polysaccharide-rich cell wall structure is more susceptible to damage during the photoinactivation process.[28,29] So, in this case, there was a significant increase in deaths, namely 42.92%.

Based on Table 2, it is evident that the addition of AgNPs with laser significantly affects the death percentage of *S. aureus*. This is attributed to the fact that the addition of AgNPs can induce bacterial dysfunction and death. *Staphylococcus aureus* bacteria were subjected to the addition of extract, AgNPs, extract+ laser, and AgNPs + laser. According to Figure 6, the highest percentage of death of *Staphylococcus aureus* bacteria in AgNPs-CTL 1mM was observed with AgNPs+laser treatment, reaching 95.81%. Thus, it can be concluded that the addition of AgNPs with a laser significantly affects the death of bacteria on plates containing *Staphylococcus aureus* bacteria.

Based on the results of calculating bacterial death above, statistical analysis can be conducted using SPSS. In this research, two statistical tests were carried

Table 2. Factorial Test Results

Treatment	Group	N	Death Bacteria (%)		Faktorial Test Result	
			Average	SD	Signification	Conclusion
Concentration AgNPs-CTL	1 mM (1)[2]	15	88.58	8.66	P = 0.043	There are different meanings
	1,5 mM (2)[1,2]	15	78.10	24.09		
	2 mM (3)[1]	15	71.85	18.36		
	3 mM (4)[1,2]	15	84.17	12.10		
Total		60				
Time	90 s (A)	20	82.20	11.61	P = 0.790	There is no difference meaning
	120 s (B)	20	78.70	11.83		
	150 s (C)	20	81.12	14.18		
Total		60				
Interaction	1A[(4)]	5	95.81	6.43	P = 0.039	There are different meanings
	1B[(1,2,3,4)]	5	80.73	6.40		
	1C[(3,4)]	5	89.19	21.92		
	2A[(1,2,3,4)]	5	81.38	11.71		
	2B[(1)]	5	62.19	8.10		
	2C[(4)]	5	90.72	26.46		
	3A[(1,2)]	5	64.75	5.65		
	3B[(1,2,3,4)]	5	85.21	7.11		
	3C[(1,2,3)]	5	65.58	1.98		
	4A[(1,2,3,4)]	5	82.55	24.01		
	4B[(2,3,4)]	5	86.66	15.18		
	4C[(1,2,3,4)]	5	83.30	15.56		
Total		60				

out: the normality test and one-way ANOVA.

Based on the results of the normality test using One-Sample Kolmogorov-Smirnov, a significance value of 0.60 was obtained, indicating that the data is usually distributed since p > 0.05. Subsequently, the data was analyzed again with one-way ANOVA, and the results can be seen in Table 2.

Based on Table 2, the addition of AgNPs with laser has a significant effect on the death percentage of *Staphylococcus aureus* bacteria, with a significance value of 0.00. This is attributed to the fact that the addition of AgNPs can induce bacterial dysfunction and death.

CONCLUSIONS

Silver nanoparticles synthesized from butterfly pea flower extract have been successfully employed in bacterial photoinactivation. In vitro tests have shown antibacterial activity. AgNPs-CTL at a concentration of 1 mM significantly increased the death rate of *S. aureus* bacteria by 42.92%. Red laser irradiation resulted in 95.81% bacterial death for *S. aureus* when using AgNPs-CTL at a concentration of 1 mM with an exposure time of 90 seconds.

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CONFLICT OF INTEREST

No conflict of interest.

AUTHOR CONTRIBUTION

SDA: Conceptualization, methodology, validation, writing original draft preparation, supervision, funding acquisition GRAF: Conceptualization, methodology, writing original draft preparation, software UMUS: Conceptualization, Sources, software RA: Conceptualization, writing review and editing. AHZ: Conceptualization, methodology, validation, supervision. AKY: Conceptualization, methodology, validation.

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