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Silver Nanoparticles Synthesised with Red Betel Leaf (*Piper crocatum*) Extract as a Photosensitiser for Inactivation of *Escherichia coli* and *Staphylococcus aureus*

(Nanopartikel Perak Disintesis dengan Ekstrak Daun Sirih Merah (Piper crocatum) sebagai Fotopemeka untuk Menyahaktifkan Escherichia coli dan Staphylococcus aureus)

SURYANI DYAH ASTUTI[1,*], DAMITA KARREN[1], YUNUS SUSILO[2], AHMAD KHALIL YAQUBI[3], ANDI HAMIM ZAIDAN[1] & NASRUL ANUAR ABD RAZAK[4]

[1]Department of Physics, Faculty of Science and Technology, Airlangga University, Surabaya, 60115, East Java, Indonesia
[2]Faculty of Engineering Dr Soetomo University, Surabaya 60118, Indonesia
[3]Doctorate Degree, Faculty of Science and Technology, Airlangga University, Surabaya, 60115, Indonesia
[4]Department of Biomedical Engineering, Faculty of Engineering, Universiti Malaya, 50603 Kuala Lumpur, Malaysia

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ABSTRACT

Infectious diseases caused by *Escherichia coli* and *Staphylococcus aureus* are major health concerns in Indonesia, exacerbated by antibiotic resistance and biofilm formation. This study explores the use of betel leaf-synthesised silver nanoparticles (AgNPs) combined with red laser irradiation to enhance antimicrobial effectiveness against these resistant bacteria. The study included four groups: a control group (T0) without laser irradiation, *E. coli* groups (A1 and A2), and *S. aureus* groups (A3 and A4) treated with varying concentrations of AgNPs and irradiated with a 665 nm diode laser. The photosensitiser group (A2 and A4) received AgNPs synthesised from red betel leaf extract (AgNPs-Pc), followed by a 10-min incubation. The samples were then irradiated at four different times: 90, 120, 150, and 180 min, using a laser with a wavelength of 665 nm to evaluate the antimicrobial effects. The findings showed that AgNPs-Pc combined with a red laser significantly reduced *E. coli* growth compared to the control group without photosensitiser. For *S. aureus*, growth occurred after irradiation without photosensitiser (PS). Irradiation of *S. aureus* with AgNPs-Pc at concentrations of 1 mM, 1.5 mM, and 2 mM for 150 s resulted in bacterial death reductions of 89.74%, 91.24%, and 89.05%, respectively. The effective inactivation of *E. coli* was 87.29 ± 2.68% at an energy density of 22.68 J/cm². *S. aureus* required a higher energy density of 37.80 J/cm² for 91.24 ± 2.76% inactivation. This study shows that red betel leaf extract-synthesised silver nanoparticles combined with red laser irradiation effectively combat antibiotic-resistant *E. coli* and *S. aureus*.

Keywords: *Escherichia coli;* photoinactivation; photosensitiser; red diode laser; *Staphylococcus aureus*

ABSTRAK

Penyakit berjangkit yang disebabkan oleh *Escherichia coli* dan *Staphylococcus aureus* merupakan masalah kesihatan utama di Indonesia, diburukkan lagi dengan kerintangan antibiotik dan pembentukan biofilem. Penyelidikan ini mengkaji penggunaan nanozarah perak hasil sintesis daun sirih (AgNPs) yang digabungkan dengan penyinaran laser merah untuk meningkatkan keberkesanan antimikrob terhadap bakteria rintang antibiotik. Kajian ini melibatkan empat kumpulan: kumpulan kawalan (T0) tanpa penyinaran laser, kumpulan *E. coli* (A1 dan A2) dan kumpulan *S. aureus* (A3 dan A4) yang dirawat dengan pelbagai kepekatan (AgNPs) dan disinari dengan laser diod 665 nm. Kumpulan fotopemekaan (A2 dan A4) menerima AgNPs yang disintesis daripada ekstrak daun sirih merah (AgNPs-Pc), diikuti dengan eraman selama 10 min. Sampel tersebut kemudiannya disinari pada tempoh masa yang berbeza: 90, 120, 150 dan 180 min, menggunakan laser dengan panjang gelombang 665 nm untuk menilai kesan antimikrob. Hasil menunjukkan bahawa AgNPs-Pc yang digabungkan dengan laser merah secara signifikan mengurangkan pertumbuhan *E. coli* dibandingkan dengan kawalan tanpa fotopemekaan. Untuk *S. aureus*, pertumbuhan terjadi setelah sinaran tanpa fotopemekaan (PS). Sinaran ke atas *S. aureus* dengan AgNPs-Pc pada kepekatan 1 mM, 1.5 mM dan 2 mM selama 150 saat mengurangkan kematian bakteria masing-masing sebanyak 89.74%, 91.24% dan 89.05%. Penyahaktifan berkesan bagi *E. coli* adalah 87.29 ± 2.68% pada ketumpatan tenaga 22.68 J/cm². *S. aureus* memerlukan ketumpatan tenaga yang lebih tinggi sebanyak 37.80 J/cm² untuk penyahaktifan sebanyak 91.24 ± 2.76%. Hasil ini menunjukkan bahawa nanozarah perak hasil sintesis ekstrak daun sirih merah yang digabungkan dengan sinaran laser merah adalah berkesan untuk melawan *E. coli* dan *S. aureus* yang rintang terhadap antibiotik.

Kata kunci: *Escherichia coli;* fotopemekaan; laser diod merah; penyahaktifan foto; *Staphylococcus aureus*

INTRODUCTION

Infectious diseases are one of the biggest health problems in Indonesia. Bacteria that often cause human infections, such as *Escherichia coli* and *Staphylococcus aureus*, lead to diarrhoea and skin infections. These bacteria are usually the cause of nosocomial infections in hospitals. Research conducted at a hospital in Indonesia showed that *E. coli* were among the many found in the intensive care unit (ICU) and hospital accommodation rooms (Rohde 2019). As the immune system develops antibiotic resistance, diseases caused by bacteria become more serious and deadly. Bacterial resistance poses a significant problem because it can increase morbidity, mortality, and healthcare costs. Research into new methods for developing antimicrobials continues for more effective results at lower costs. Therefore, nanotechnology has been developed to be used for antibacterial purposes (Deepak et al. 2011).

Nanotechnology is a technology based on engineering the properties of nanometre-sized materials. It involves using nano-sized materials, typically 1-100 nm (Deepak et al. 2011). The very small size of nanoparticles results in a large surface area relative to their volume, making it easier for them to interact with other particles and increasing their antibacterial properties (Li et al. 2008). Metal nanoparticles, such as gold, silver, iron, metal oxides, and zinc, have great potential for application in the biomedical field due to their high surface-to-volume ratio and excellent conduction ability (Güzel & Erdal 2018).

The antibacterial activity of AgNPs is influenced by several factors, including the concentration, shape, and size of the nanoparticles, the type of bacteria, the number of bacterial colonies, and the contact time of the nanoparticles with the bacteria. Factors affecting particle size in synthesis include solution temperature, reducing agent, and reaction time. Silver nitrate ($AgNO_3$) is commonly used as the silver salt in the synthesis of silver nanoparticles. AgNPs have a strong absorption spectrum at wavelengths between 400 nm and 500 nm (Amourizi, Dashtian & Ghaedi 2020).

Silver nanoparticles (AgNPs) are widely used in biomedical devices due to their antibacterial, antifungal, and antiviral properties. The synthesis of AgNPs can be carried out through two main approaches: the top-down and bottom-up methods. These methods involve various techniques, including chemical, physical, and biological processes. An example of a bottom-up approach is metal reduction, where metal ions are reduced to form nanoparticles (Setyawati et al. 2023).

Green synthesis produces nanoparticles by utilising materials such as bacteria, fungi, plant extracts, and small biomolecules like vitamins and amino acids. This method does not cause significant environmental impacts because it is environmentally friendly and non-toxic during synthesis. This method has three main factors: solvent, reducing agent, and non-toxic material. The benefit of using plants for nanoparticle synthesis is that they are readily available and contain a variety of active functional groups that can facilitate the reduction of silver ions. The main compounds that ensure the reduction of nanoparticles are biomolecules such as polysaccharides, tannins, saponins, phenolics, terpenoids, flavonoids, alkaloids, proteins, enzymes, vitamins, amino acids, and alcohol components (Thomas et al. 2004). The red betel plant (*Piper crocatum*) is a medicinal plant that contains tannins, alkaloids, and flavonoids (Rohde 2019). Red betel leaf extract has an absorption spectrum peak of around 300 to 800 nm (Mahmudah, Muntaha & Muhlisin 2019). The use of medicinal plants in the synthesis of AgNPs is not only for controlling size and shape but also to impart plant antimicrobial properties to AgNPs (Garini et al. 2021).

E. coli is a Gram-negative bacterium that can become pathogenic, causing symptoms like diarrhoea, fever, and nausea. Its cell wall has less peptidoglycan than Gram-positive bacteria and contains an outer membrane with lipopolysaccharides, contributing to antibiotic resistance, although its mechanical resistance is weak (Niculescu & Grumezescu 2021). *S. aureus* is a Gram-positive bacterium commonly found in the mouth and respiratory tract, but it can cause skin infections like acne, boils, and abscesses (Papageorgiou et al. 2000). Its cell wall, composed of peptidoglycan and teichoic acid, provides structural rigidity and makes it more susceptible to penicillin, though its mechanical strength is high due to the large amount of peptidoglycan (Yoon, Li & Shim 2013).

Photodynamic Inactivation (PDI) is a method used for microbial inactivation through light irradiation (Keiser 2022). PDI consists of three main components: A light source, photosensitiser as material, and Radical Oxygen Species (ROS) (Méndez-Pfeiffer et al. 2019). Photosensitiser (PS) with an absorption spectrum that matches the wavelength spectrum of light can absorb light energy to trigger photochemical reactions and produce ROS (Al-Sharqi et al. 2019). ROS is responsible for causing cell damage and death (Puspita, Safithri & Sugiharti 2019). The light emitted by the laser has a coherent, monochromatic beam and contains light aligned with a specific wavelength (Kusuma, Hendriani & Genta 2017). The wavelength of the red laser ranges from 620-750 nm. The advantage of the red laser lies in its ability to penetrate structures with a slightly thick layer, thereby causing greater damage to cells and tissues compared to lasers with lower wavelengths (Mittal, Chisti & Banerjee 2013).

Previous research demonstrated that the addition of red betel leaf extract (*P. scrotum*) effectively inhibited bacterial growth (Kher et al. 2024). The treated bacteria, including *Bacillus subtilis, Pseudomonas aeruginosa* (Yaqubi et al. 2024), *E. coli, S. aureus*, and *Candida albicans*, exhibited varying degrees of inhibition (Yaqubi et al. 2022). The stronger inhibition of Gram-positive bacteria by silver nanoparticles synthesised using green betel leaves (*Piper betle* L.) can be attributed to several factors. One key factor is the differences in the cell wall structure between Gram-positive and Gram-negative bacteria.

Gram-positive bacteria have a thicker peptidoglycan layer in their cell walls, which can interact more readily with nanoparticles, enhancing their antimicrobial effect. In contrast, Gram-negative bacteria have an additional outer membrane that acts as a barrier, making it more difficult for the nanoparticles to penetrate and exert their antimicrobial effects (Astuti et al. 2019b). Using a 395 nm purple LED and a radiation dose of 524 J/cm^2 against *E. coli* reduced 94.3% (Parasuraman et al. 2020). Radiation with a wavelength of 658 ± 0.05 nm and a dose of 54.8 J/cm^2 combined with the exogenous photosensitiser methylene blue reduced *S. aureus* biofilm by 92.01% (Enwemeka, Baker & Bumah 2021). A 629 nm red LED reduced *S. aureus* by 22% with the help of an endogenous photosensitiser.

Betel leaf-synthesised AgNPs offer several innovations by providing an eco-friendly and sustainable method for nanoparticle synthesis using the plant's natural bioactive compounds. These AgNPs act as antimicrobial agents and effective photosensitisers, enhancing their antibacterial activity when combined with light exposure. This synergy, especially under red laser irradiation, promotes the production of ROS, making them more effective against drug-resistant bacteria and capable of disrupting biofilms resistant to traditional antibiotics. This approach holds promise for advancing photodynamic therapy. Because it enhances the antimicrobial effects via photodynamic treatment, the combination of red laser irradiation and AgNPs made from betel leaves is significant because it addresses antibiotic resistance and biofilm formation. This method closes a gap by treating resistant infections, particularly those involving biofilms, which are difficult to target with conventional antibiotics, more successfully while providing a natural, environmentally acceptable substitute for chemical production.

This study explores the combined effects of red laser irradiation and AgNPs synthesised from *P. scrotum* extract, a plant not extensively studied for this purpose. Unlike previous research that focuses on individual treatments, this work investigates the synergistic potential of red laser and AgNPs-Pc on *E. coli* (Gram-negative) and *S. aureus* (Gram-positive), offering a comparative analysis of their antibacterial efficacy. Additionally, the study emphasizes biofilm disruption, an area less explored in the context of laser-activated nanoparticles. It aims to provide insights into real-world applications, particularly in nosocomial infection control.

MATERIALS AND METHODS

PREPARATION AND SYNTHESIS OF RED BETEL (*Piper crocatum*) SILVER NANOPARTICLES

The materials used in this research were natural ingredients, namely red betel leaves (*P. crocatum*). Red betel leaf extract was prepared by dissolving 0.5 g of red betel leaf powder in 10 mL of ethanol-distilled water (70:30) solvent (Kusuma, Hendriani & Genta 2017). The solution was heated using a microwave at 90 watts for 9 min and then centrifuged at 5000 rpm for 30 min. Afterwards, the supernatant was separated from the sediment and mixed with 10 mL of ethanol-distilled water (70:30).

The synthesis of silver nanoparticles - *P. crocatum* (AgNPs-Pc) was conducted using a green synthesis method with silver nitrate ($AgNO_3$) and red betel leaf extract (Pc). Three variations of AgNPs concentration were used: 2 mM, 1.5 mM, and 1 mM. To synthesise AgNPs, 45 mL of $AgNO_3$ solution and 5 mL of red betel leaf extract were mixed in an Erlenmeyer flask, and the concentration was standardised by preparing a stock solution, measuring its concentration, and diluting it to the desired level for the experiment. The flask was then covered with aluminium foil and heated in a microwave at 360 watts for 5 min. The synthesis of AgNPs-Pc was considered complete when the solution changed to a yellowish-brown colour and became homogeneous. This step was repeated for each concentration of AgNPs (Keiser 2022).

The absorption spectrum of red betel leaf extract was measured using a Shimadzu UV-VIS 1800 spectrometer. Subsequently, the red betel leaf extract underwent testing with a Particle Size Analyser (PSA) using the Dynamic Light Scattering (DLS) method to determine the particle size distribution.

BACTERIAL CULTURE

E. coli (ATCC 25922) and *S. aureus* (ATCC 25923) bacteria were cultured in Tryptone Soy Broth (TSB) (Garini et al. 2021). Then, they were incubated for 24 h at 37 °C until the colony reached the 1.0 McFarland standard.

CHARACTERIZATION OF RED LASER DIODE (665 NM) FOR VARYING IRRADIATION TIMES

The laser diode light source is a red laser with a wavelength of 665 nm. Characterisation was conducted using a Jasco CT-10 monochromator to determine the peak wavelength. The power output was measured at 32.76 mW using an OMM-6810B-220V power meter. The size of the spot beam area is 0.13 cm^2. Diode laser irradiation was performed with varying exposure times of 90, 120, 150, and 180 s. To calculate the energy density value, the irradiation time was determined as follows (Caires et al. 2020):

$$\textit{Energy density (J.cm}^{-2}\textit{)} = \textit{Intensity (W. cm}^{-2}\textit{)} \times \textit{Irradiation time} \text{ (s)}$$

ANTI-BACTERIAL ACTIVITY TEST

The antibacterial activity test was conducted using the disc diffusion method. A fresh bacterial culture in Tryptic Soy Broth (TSB) was incubated overnight at 37 °C, diluted to OD600 = 0.1 (~10^6 CFU/mL), and 50 µL was spread on Tryptic Soy Agar (TSA) plates. The paper disc was treated with 10 µL of red betel leaf extract at a concentration of 1 mM, 1.5 mM, and 2 mM, while control discs were treated

with distilled water or left untreated. Once the paper disc absorbed the solution, it was placed onto the surface of the agar medium in a petri dish. The petri dish was then incubated for 24 h (Yoon, Li & Shim 2013). The presence of an inhibitory zone around the paper disc indicates the antibacterial ability of red betel leaf extract (Yoon, Li & Shim 2013).

PHOTODYNAMIC INACTIVATION OF BACTERIA USING AgNPs AND 665 NM LASER

The study included four sample groups: (T0) a control group without laser irradiation; (A1 and A2) *E. coli* groups irradiated with a 665 nm diode laser at varying times and AgNPs-Pc concentrations; and (A3 and A4) *S. aureus* groups irradiated under similar conditions. Groups A2 and A4 received AgNPs-Pc as a photosensitiser, followed by 30 min of incubation and laser irradiation for 90, 120, 150 or 180 s. The treated samples were cultured on the TSA media, incubated at 37 °C for 24 h, and bacterial colonies were counted using a Quebec colony counter.

STATISTICAL ANALYSIS

Statistical analysis in this study used the Two-Way ANOVA Factorial test with IBM SPSS to determine the influence of each factor and the interactions between factors. Data are deemed to have differences (H0) and are rejected if the significance value $p < \alpha = 0.05$. Then, the Tukey post hoc test was conducted to determine the differences in each sample factor with the condition $p < 0.005$.

RESULTS

The results of the absorption spectrum characterisation of AgNPs-Pc are shown in Figure 1. UV-Vis absorption spectrum measurements were carried out in the 325 nm to 800 nm wavelength range. AgNPs-Pc exhibit an absorption spectrum around 350 nm to 500 nm, but in this study, a red laser was used to determine its effectiveness. Then, the particle size results (Figure 2) are based on the Particle Size Analyser (PSA) test on AgNPs-Pc with concentrations of 1 mM, 1.5 mM, and 2 mM, resulting in diameters of 118.36 nm, 100.79 nm, and 128.71 nm, respectively. These

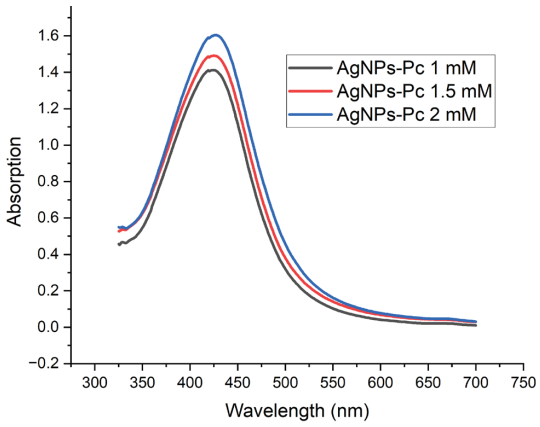

FIGURE 1. UV-Vis absorbance spectrum of AgNPs-Pc at concentrations of 1 mM, 1.5 mM, and 2 mM

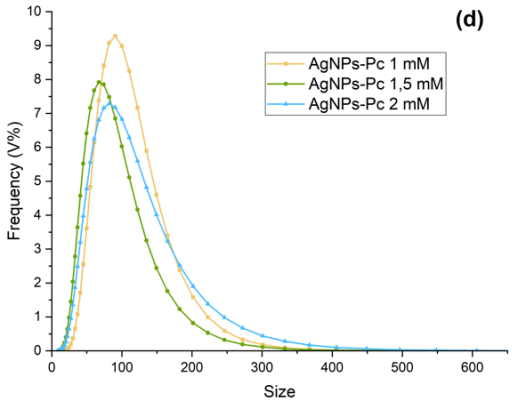

FIGURE 2. PSA test results of AgNPs-Pc at a concentration of 1 mM, 1.5 mM, and 2 mM

results showed that AgNPs-Pc at 1 mM, 1.5 mM, and 2 mM concentrations are classified as having slightly larger diameters than the general nanoparticle range, which is typically 1-100 nm in size.

The antibacterial test against *E. coli* and *S. aureus* is used to determine the diameter of the inhibition zone for the growth of bacterial colonies. Each sample was tested using the diffusion well method. Based on Figure 3, the zone of inhibition with antibacterial AgNPs-Pc at concentrations of 1 mM, 1.5 mM, and 2 mM is 0.56 mm, 0.84 mm, and 0.92 mm, respectively, for *E. coli*. Meanwhile, the zone of inhibition with antibacterial AgNPs-Pc at concentrations of 1 mM, 1.5 mM, and 2 mM is 1.34 mm, 1.44 mm, and 1.59 mm, respectively, for *S. aureus*.

The addition of AgNPs-Pc to the bacterial samples demonstrated its antibacterial properties. Bacterial growth results were obtained by calculating the number of bacterial colonies grown with AgNPs-Pc on a plate. Figure 4 shows the percentage reduction in the number of *E. coli* and *S. aureus* bacterial colonies. The laser irradiation process was carried out at varying times, namely 90, 120, 150, and 180 s. This variation in time provides different energy densities for each treatment. The success of this laser irradiation can be seen from the presentation of a decrease in the number of bacterial colonies resulting from laser irradiation in the control group. Figure 5 shows the percentage of *E. coli* bacterial death with the addition of AgNPs-Pc, which indicated the relationship between exposure time and bacterial viability.

Based on the statistical test results in Table 1, the significance value is $p < 0.05$, which shows a significant difference in each variation of time given to each treatment. The results of the Two-Way ANOVA factorial test indicate that treatment using a laser and the addition of 1 mM AgNPs-Pc with a duration of 90 s gave the highest percentage of *E. coli* bacterial death at 87.29%.

For *S. aureus*, 47% AgNPs-Pc was added at each variation of concentration. Figure 6 shows that the treatment of the three groups resulted in a relationship between exposure time and bacterial viability regarding colony numbers. The death percentage of S. *aureus* bacteria with the addition of AgNPs-Pc is shown in Figure 6.

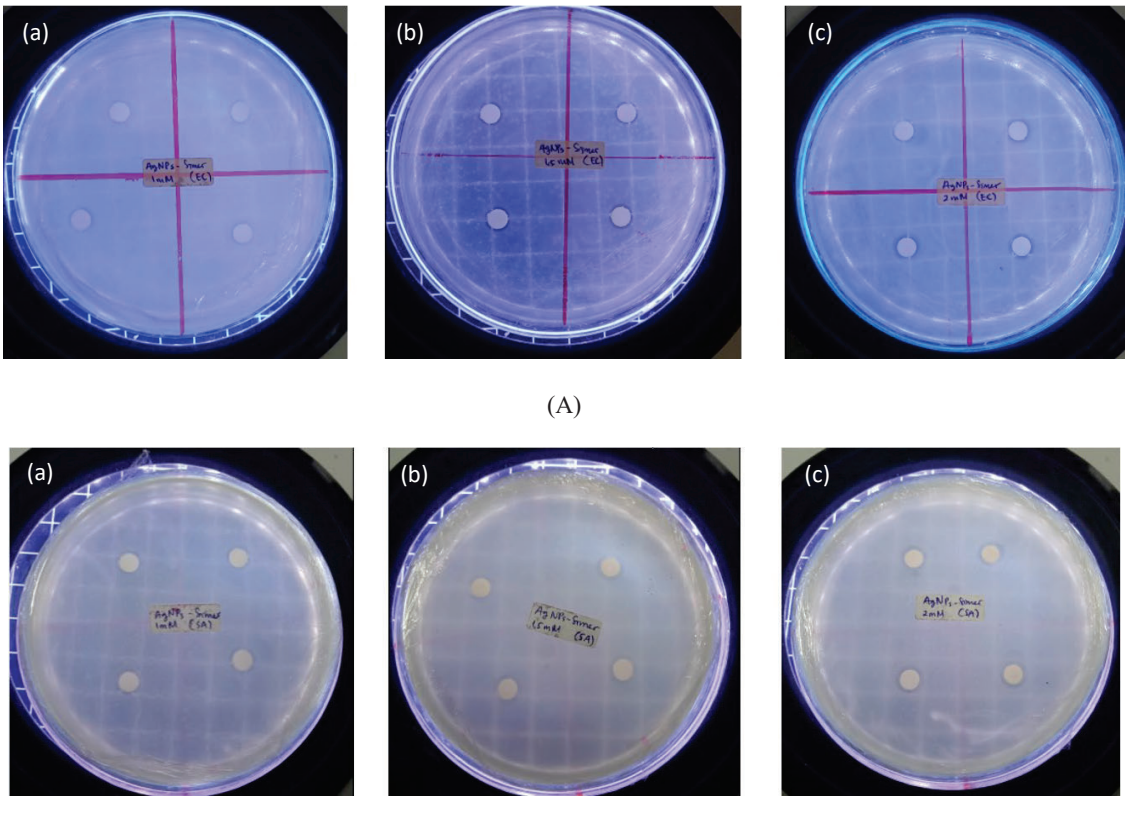

FIGURE 3. Anti-bacterial test results of (A) *E. coli* (B) *S. aureus* bacterial disk diffusion method with AgNPs-Pc reducing agent (a) 1 mM, (b) 1.5 mM, and (c) 2 mM

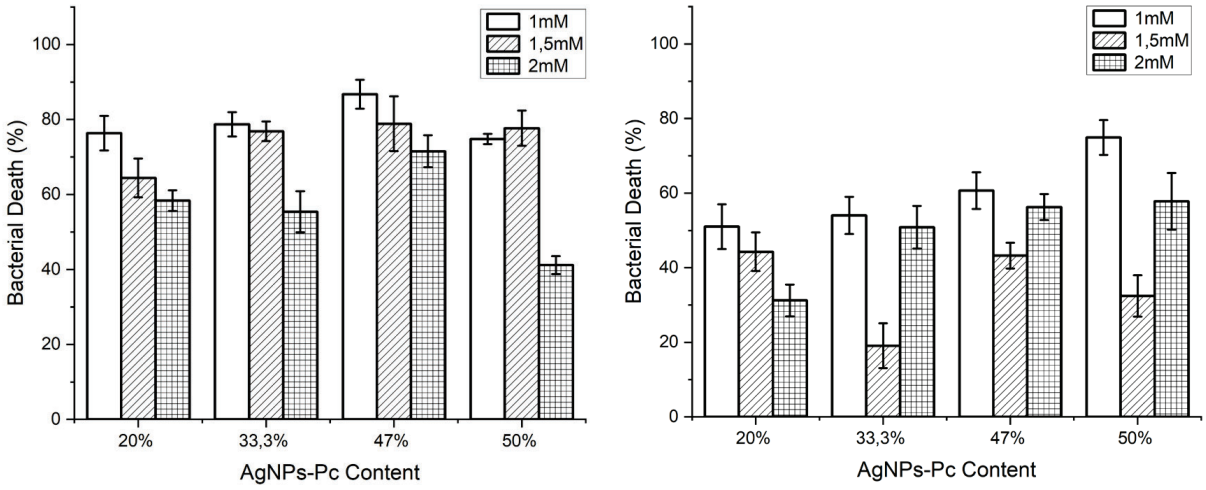

FIGURE 4. Percentage reduction of (a) *E. coli* and (b) *S. aureus* at various concentrations of AgNPs-Pc

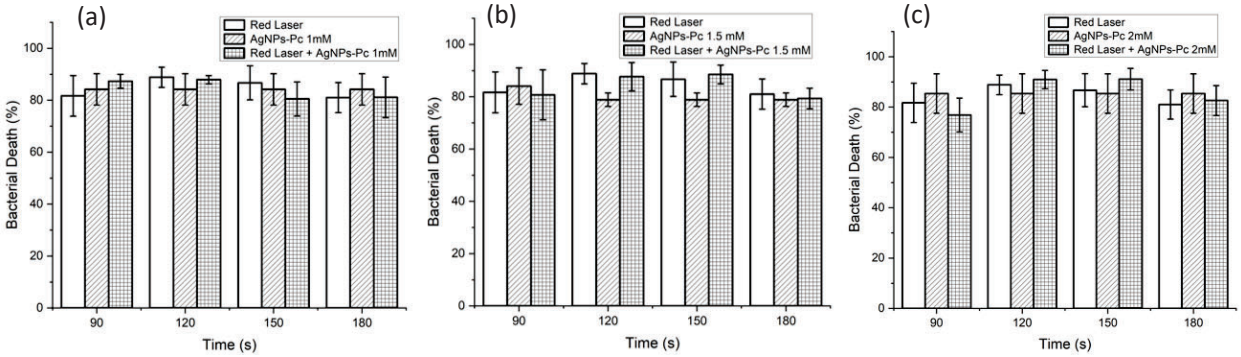

FIGURE 5. Percentage reduction of *Escherichia coli* observed with AgNPs-Pc at the following concentrations: (a) 1 mM, (b) 1.5 mM, and (c) 2 mM

Based on the statistical test results in Table 2, the significance value is $p < 0.05$, indicating a significant difference in each variation of time given to each treatment. The results of the Two-Way ANOVA factorial test show that treatment using a laser and the addition of 1.5 mM AgNPs-Pc with a duration of 150 s gave the highest percentage of death of *S. aureus* at 92.24%.

DISCUSSION

The use of silver in the form of small particles or colloids has been known for centuries. In the last few decades, research and development in nanotechnology, involving collaboration and contributions from chemistry, physics, biology, and materials engineering experts, have resulted in many new techniques for producing AgNPs with controllable sizes and properties (Yaqubi et al. 2023). One environmentally friendly technique is the green synthesis or biosynthesis method using natural ingredients such as plant extracts (Ding et al. 2016). In the synthesis process, instead of conventional heating, a microwave irradiation method is applied, which can speed up the reaction time, does not cause significant changes in the chemical reaction, and produces more homogeneous heating. Microwave heating also reduces energy consumption and produces better results by preventing aggregation in the formation of particles (Sorinolu et al. 2022).

The synthesis of red betel leaf extract silver nanoparticles (AgNPs-Pc) in this study demonstrates antibacterial properties. At low concentrations, AgNPs-Pc did not have a significant effect compared to higher concentrations (Kashef, Huang & Hamblin 2017). At a concentration of 2 mM, AgNPs-Pc achieved a bacterial death percentage of 85.37% for *E. coli* and 72.73% for *S. aureus* (Hakimov et al. 2022). These findings are consistent with earlier studies indicating that

smaller nanoparticle sizes, resulting from higher $AgNO_3$ concentrations, provide a larger surface area for interaction with bacterial cell walls, thereby enhancing antibacterial effects (ElZorkany et al. 2019). This aligns with Monteiro et al. (2020), who observed that the increased surface-to-volume ratio of smaller nanoparticles amplified their bactericidal efficiency.

The role of AgNPs-Pc in this research extends beyond antibacterial activity to functioning as a photosensitiser to enhance bacterial inactivation in the PDI process. Photosensitisers act as light-sensitising substances (Iravani et al. 2014). Similar to Matlou and Nyokong (2020), this study emphasises the importance of matching the visible light spectrum with the photosensitiser's absorption spectrum. However, a key limitation here is that the red laser (665 nm) used did not fully overlap with the AgNPs-Pc absorption spectrum (425 nm). Using the Lambert-Beer equation, the photosensitiser was found to

TABLE 1. Results of statistical analysis on *E. coli*

Treatment	Group	N	Bacterial death (%)		ANOVA
			Average	SD	P-value
AgNPs-PC concentration	1 mM (1)	20	84.20	6.01	0.72
	1.5 mM (2)	20	84.07	6.99	
	2 mM (3)	20	85.37	7.86	
Time	90 s (A)[1]	15	81.68	7.81	0.000
	120 s (B)[1]	15	88.85	3.90	
	150 s (C)[1,2]	15	86.71	6.57	
	180 s (D)[2]	15	81.02	5.81	
Interaction	1A [(1,2)]	5	87.29	2.68	0.008
	1B [(1,2)]	5	87.91	1.58	
	1C [(1,2)]	5	80.50	6.56	
	1D [(1,2)]	5	81.12	7.79	
	2A [(1,2)]	5	80.73	9.56	
	2B [(1,2)]	5	87.68	5.41	
	2C [(1,2)]	5	88.53	3.57	
	2D [(1,2)]	5	79.33	3.92	
	3A [(1)]	5	76.83	6.72	
	3B [(2)]	5	90.95	3.63	
	3C [(2)]	5	91.11	4.31	
	3D [(1,2)]	5	82.61	5.96	

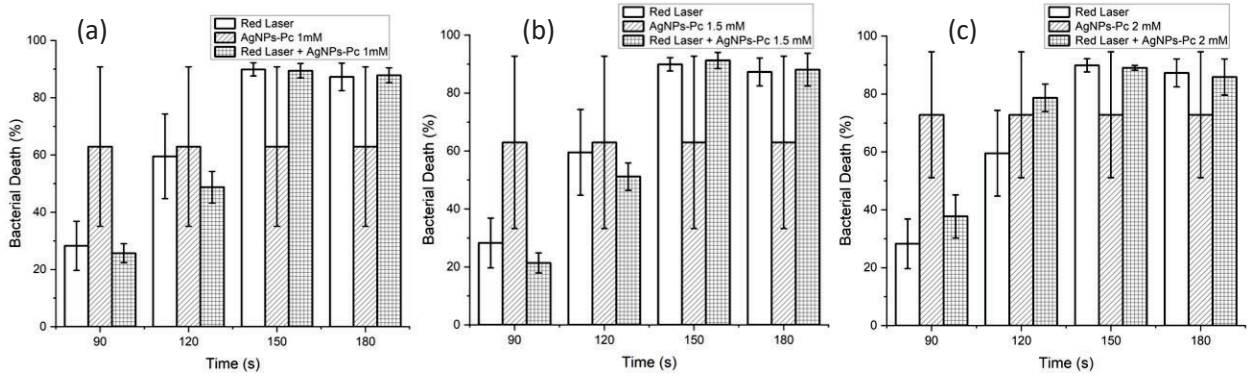

FIGURE 6. Percentage reduction of *S. aureus* bacteria with AgNPs-Pc
(a) 1 mM, (b) 1.5 mM, and (c) 2 mM

TABLE 2. Results of statistical analysis on *S. aureus*

Treatment	Group	N	Bacterial death (%)		ANOVA
			Average	SD	Significance
AgNPs-PC concentration	1 mM (1)[1]	20	62.94	27.88	0.001
	1.5 mM (2)[1]	20	62.98	29.71	
	2 mM (3)[1]	20	72.83	21.73	
Time	90 s (A)[1]	15	28.28	8.56	0.000
	120 s (B)[2]	15	59.53	14.79	
	150 s (C)[3]	15	89.92	2.27	
	180 s (D)[3]	15	87.27	4.81	
Interaction	1A [(1)]	5	25.71	3.31	0.000
	1B [(3)]	5	48.74	5.51	
	1C [(5)]	5	89.46	2.52	
	1D [(4, 5)]	5	87.83	2.60	
	2A [(1)]	5	21.41	3.46	
	2B [(3)]	5	51.18	4.72	
	2C [(5)]	5	91.24	2.76	
	2D [(4, 5)]	5	88.08	5.65	
	3A [(2)]	5	37.71	7.42	
	3B [(4)]	5	78.67	4.75	
	3C [(5)]	5	89.05	0.81	
	3D [(2, 5)]	5	85.89	6.21	

absorb only 0.5% of the emitted laser intensity. This partial mismatch may account for the reduced efficiency compared to studies where absorption spectra were optimally aligned (Matlou & Nyokong 2020).

Photoinactivation occurs due to the photophysical mechanisms involved when the laser light source interacts with the AgNPs-Pc photosensitiser. This mechanism focuses on the electron level in the AgNPs-Pc photosensitiser, which has electron pairs in each molecule (Song & Kim 2009). In the photophysical process, the molecules of the AgNPs-Pc photosensitiser absorb light and cause the molecules to be excited, which is called an excited singlet state. The interaction of the electron spin with the excited singlet electron causes the molecule to switch to an excited triplet state, also known as intersystem crossing (Ormond & Freeman 2013).

In the excited triplet state, a photochemical process occurs. Photochemistry is divided into two reactions: Type I and Type II. In type I reactions, electron transfer (release of hydrogen) occurs between the excited sensitiser molecule and the surrounding biological molecules to form radical anions and cations in the form of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$). In type II reactions, energy from the T1 energy level is passed directly to $^3O_2$, which excites singlet oxygen ($^1O_2$). In this reaction, the sensitiser returns to the basic energy level because the extra electrons have been transferred to oxygen, forming superoxide radical anions.

ROS and superoxide resulting from Type I and Type II processes will oxidise lipids and proteins in the cell membrane, producing a type of hydrogen peroxide radical which is toxic and will damage the lipids and proteins in the membrane, resulting in cell leakage, which will lead to cell lysis or death. Then, a photobiological reaction occurs, namely the occurrence of free radical peroxides or species that cause damage to the organisation of the cell membrane by forming longer chains and affecting membrane cross-linking, membrane fluidity, and membrane function and structure. Damage caused by photobiology includes damage to DNA, proteins, and membranes (Astuti et al. 2019a).

This research uses *E. coli* bacteria as a representative of Gram-negative bacteria and *S. aureus* as a representative of Gram-positive bacteria. The irradiation process was carried out at varying times, namely 90, 120, 150, and 180 s, or with doses of 22.68 J/cm², 30.24 J/cm², 37.80 J/cm², and 45.36 J/cm². Data analysis shows that irradiating red lasers without a photosensitiser on *E. coli* causes a decrease in the number of colonies. Still, in *S. aureus*, the opposite occurs: An increase in the number of colonies. *S. aureus* were illuminated with a red LED for 192 s, and a dose of 12 J/cm² increased by 327.75% compared

to the control group (without any treatment). Red laser, known as a laser for healing and cell regeneration, could be the reason for the growth of *S. aureus*. Exposure to a red light on human cells can produce positive biochemical effects that strengthen mitochondria, where cellular energy is created so that cells can function more efficiently and rejuvenate and repair themselves.

This phenomenon can also be explained by the difference in cell wall composition between the Gram-negative bacteria *E. coli* and the Gram-positive bacteria *S. aureus*. Gram-negative bacteria, such as *E. coli*, have a double membrane system with a smaller amount of peptidoglycan between the outer and inner membranes of the cell wall. The outer part of the cell wall of Gram-negative bacteria consists of phospholipids and several proteins, making this type more resistant to antibiotics. Still, it has weak mechanical resistance due to its low peptidoglycan content (Astuti et al. 2019a).

Meanwhile, Gram-positive bacteria such as *S. aureus* have cell walls composed of peptidoglycan, resulting in a rigid cell wall. The largest peptidoglycan composition in this type of bacteria is teichoic acid, theichuronic acid, and various polysaccharides. On the outside of the peptidoglycan, there are teichoic acid compounds. Gram-positive bacterial cells are more susceptible to penicillin antibiotics because this antibiotic can damage peptidoglycan. Still, because the amount of peptidoglycan in bacteria is large, it is stronger than mechanical damage (Astuti et al. 2016).

The combination of red diode laser irradiation with AgNPs-Pc can increase ROS, which can damage the biological system of bacteria so that the bacteria experience lysis. The more ROS produced, the greater the bacterial death percentage (Monteiro et al. 2020). Groups A2 and A4 are the groups that are best at reducing bacteria. PDT against *E. coli* was effective when irradiation was carried out at a dose of 22.68 J/cm², and the photosensitiser was AgNPs-Pc 1 mM, resulting in a death percentage of 87.29%. For *S. aureus* bacteria, PDT was effective when irradiation was carried out at a dose of 37.80 J/cm², and the photosensitiser in the form of 1.5 mM AgNPs-Pc produced a percentage of 91.24%. This result is effective because the smallest possible dose can reduce bacterial colonies as much as possible. Using higher doses also does not guarantee effectiveness in inactivating bacteria. This is because, in this study, the samples were not irradiated simultaneously but sequentially, giving the bacterial samples not being irradiated the opportunity to reproduce and repair cells when the red diode laser irradiated the other samples.

CONCLUSIONS

Red betel leaf extract silver nanoparticles (AgNPs-Pc) have been successfully used in the bacterial photoinactivation process because they have been tested as antibacterial and non-toxic. AgNPs-Pc 2 mM increased the death percentage of *E. coli* by 85.37% and *S. aureus* by 72.73%. AgNPs-Pc is more effective for gram-positive bacteria because their cell walls are more easily lysed than Gram-negative bacteria. Red laser irradiation at 665 nm resulted in bacterial death in groups A1, A2, and A4 but not in group A3. In the treatment without AgNPs-Pc, the death of *E. coli* (A1) was 87.29% with irradiation for 90 s. In *S. aureus* (A3), there was an increase in live bacterial colonies, meaning bacterial death was negative. In the treatment group with the addition of 2 mM AgNPs-Pc, the percentage of death of *E. coli* (A2) bacteria was 87.29% with irradiation for 90 s, and 1 mM AgNPs-Pc reduced *S. aureus* (A4) bacteria by 91.24% with irradiation for 150 s. The 665 nm red laser irradiation dose effective for inactivating *E. coli* is 22.68 J/cm². It cannot be determined for *S. aureus* because shining a red laser without an exogenous photosensitiser actually increases the number of these bacteria.

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*Corresponding author; email: suryanidyah@fst.unair.ac.id