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Antibacterial Activity of Nanocomposite Chitosan-Silver Nanoparticle with *Cymbopogon citratus* Extract as a Bioreductor against *Staphylococcus aureus*

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Abstract

Nanocomposites are materials formed by combining two components, one or both of which are on the nanometer scale. The nanocomposite in this study is a combination of chitosan and silver nanoparticles produced through the synthesis of silver nitrate using *Cymbopogon citratus* extract. Silver nanoparticles have antibacterial abilities that can be utilized to overcome various diseases. However, their antibacterial properties may be reduced due to the tendency of silver nanoparticles to agglomerate. This can be overcome by the addition of chitosan as a stabilizing agent to prevent agglomeration and maintain the antibacterial effectiveness of silver nanoparticles. This study aims to evaluate the antibacterial activity of a nanocomposite formed by combining chitosan and silver nanoparticles synthesized using *Cymbopogon citratus* extract against *Staphylococcus aureus* through the diffusion method. The samples used included nanocomposites at concentrations of 6.25 mg/mL, 12.5 mg/mL, 15 mg/mL, 25 mg/mL, and 50 mg/mL, amoxicillin as a positive control, Acetic acid, and distilled water as negative controls. The results of antibacterial activity testing showed that all nanocomposite test concentrations had the ability to inhibit the growth of *Staphylococcus aureus* as evidenced by the formation of an inhibition zone around the disc paper. However, the highest antibacterial activity shown by the nanocomposites was still lower compared to the antibacterial activity of amoxicillin.

Keywords: Antibacterial activity, chitosan, Cymbopogon citratus, silver nanoparticles, Staphylococcus aureus

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Nanokomposit adalah bahan yang terbentuk dari penggabungan dua komponen, di mana salah satu atau keduanya memiliki skala nanometer. Nanokomposit pada penelitian ini merupakan penggabungan dari kitosan dan nanopartikel perak yang dihasilkan melalui sintesis perak nitrat dengan ekstrak *Cymbopogon citratus*. Nanopartikel perak memiliki kemampuan antibakteri yang dapat dimanfaatkan untuk mengatasi berbagai penyakit. Namun, sifat antibakteri yang dimilikinya dapat berkurang akibat kecenderungan nanopartikel perak untuk teraglomerasi. Hal ini dapat diatasi dengan penambahan kitosan sebagai agen penstabil untuk mencegah aglomerasi dan mempertahankan efektivitas antibakteri nanopartikel perak. Penelitian ini dilakukan dengan tujuan untuk mengetahui kemampuan antibakteri dari nanokomposit hasil penggabungan kitosan dan nanopartikel perak yang disintesis menggunakan ekstrak *Cymbopogon citratus* terhadap bakteri *Staphylococcus aureus* menggunakan metode difusi. Sampel yang digunakan adalah nanokomposit dengan konsentrasi 6,25 mg/mL; 12,5 mg/mL; 15 mg/mL; 25 mg/mL dan 50 mg/mL, amoksisilin sebagai kontrol positif, serta asam

asetat dan aquades sebagai kontrol negatif. Hasil pengujian aktivitas antibakteri menunjukkan bahwa seluruh konsentrasi uji nanokomposit memiliki kemampuan dalam menghambat pertumbuhan bakteri *Staphylococcus aureus* yang dibuktikan dengan terbentuknya zona hambat disekitar kertas cakram. Namun, aktivitas antibakteri terbesar yang ditunjukkan oleh nanokomposit masih lebih kecil jika dibandingkan dengan aktivitas antibakteri yang dimiliki amoksisilin.

Kata Kunci: Aktivitas antibakteri, Kitosan, Cymbopogon citratus, Nanopartikel perak, Staphylococcus aureus

INTRODUCTION

Nanocomposites are materials formed by combining two components, at least one of which has a nanometer scale dimension [1]. One component serves as the matrix or binder material, while the other functions as the filler. Nanofillers are added to the matrix during the nanocomposite fabrication process to make nanoparticles with better properties than the original materials that made up the matrix. The better performance is because the smaller particle size makes the surface area bigger, which allows for more extensive interactions and leads to better material properties [2].

Silver nanoparticles are nanomaterials that have excellent catalytic activity, stability, and conductivity [3]. Silver nanoparticles can be produced through the reduction of precursor agents such as silver nitrate [4]. Silver nitrate ($AgNO_3$) is an inorganic compound with a broad spectrum of activity and is effective against various types of microorganisms [5]. Antibacterial activity is enhanced when silver is in nanoparticle form, as the particles become more reactive and easier to ionize at the nanoscale [6]. Silver nanoparticles can be synthesized through various approaches, including chemical (bottom-up), physical (top-down), and biological (biosynthesis) methods [7]. Synthesis by chemical and physical methods has disadvantages such as requiring high energy, producing toxic products, and having a negative impact on the environment. Therefore, many biological syntheses are developed using reducing agents from natural materials such as plant extracts [8]. One of the plant extracts that has ability to as a reducing agent is *C. citratus* extract [9].

In previous studies, *C. citratus* extract has successfully been used as a bioreductor to produce silver nanoparticles. However, silver nanoparticles have a tendency to agglomerate, which can lead to uneven particle size distribution, the formation of precipitates, and a reduction in their antibacterial efficacy [4, 9]. To address this issue, the addition of stabilizing agents is necessary to achieve size uniformity and prevent particle agglomeration [10]. One such stabilizing agent is chitosan. Chitosan is a deacetylated derivative of chitin, which can be derived from beetles like *Xylotrupes gideon* [11]. Chitosan functions as a stabilizer by coating silver nanoparticles with a positive charge, thereby facilitating electrostatic interactions that result in homogeneous nanoparticles. Therefore, the inclusion of chitosan is anticipated to inhibit agglomeration and preserve the antibacterial effectiveness of silver nanoparticles [10, 12].

MATERIALS AND METHODS

Materials

The materials used in this study include distilled water, acetic acid (SMARTLAB), 96% alcohol (SAE Medical), $AgNO_3$ powder (Sigma Aldrich), C. *citratus* extract powder, chitosan powder from X. *gideon*, amoxicillin discs (10 µg/disc), *S. aureus* bacteria, 0.9% NaCl, Nutrient Agar and Nutrient Broth media, magnetic stirrer, beaker glass, inoculating needle, alcohol lamp, sterile swabs, disc paper, petri dishes, micropipette, test tubes, incubator, and caliper.

Methods

Preparation of nanocomposites

A total of 1.7 g of $AgNO_3$ was dissolved in 100 mL of distilled water to prepare an $AgNO_3$ solution. Then, 90 mL of this solution was mixed with 2 g of *C. citratus* extract and heated at 40 °C for 30 minutes to form a silver nanoparticle solution. Next, 1.5 g of chitosan in a mixture of 30 mL acetic acid and 100 mL distilled water was added to the silver nanoparticle solution and heated for 30 minutes at 40 °C. The resulting nanocomposite solution was obtained after allowing the mixture to stand for 24 hours [13].

Preparation of test solutions

Dilution was performed to obtain nanocomposite solutions at five different concentrations: 6.25 mg/mL, 12.5 mg/mL, 15 mg/mL, 25 mg/mL, and 50 mg/mL. This process was carried out by mixing the nanocomposite solution with acetic acid as the solvent in specific volumes until the desired concentrations were achieved.

Preparation of bacterial suspension

Staphylococcus aureus bacterial colonies were transferred from the culture into a test tube containing 5 mL of Nutrient Broth medium and incubated for 24 hours at 37°C. After that, the bacterial suspension was taken from the test tube and transferred into a new

sterile tube containing 0.9% NaCl solution. Next, the turbidity of the suspension was adjusted to the McFarland 0.5 standard. If the suspension was too turbid, sterile 0.9% NaCl was added to dilute it. Conversely, if the turbidity was insufficient, additional bacterial culture from the Nutrient Broth medium was added until the turbidity is in accordance with the McFarland 0.5 standard [14].

Antibacterial activity test

The antibacterial activity was tested using the disc diffusion method against *S. aureus* ATCC 12600. The process begins by spreading bacteria on the surface of the nutrient agar plate using a sterile swab. Next, paper disks were placed on the surface of the nutrient agar plate using tweezers, and 20 µL of the test solution was applied onto the disc using a calibrated micropipette. As a positive control, an amoxicillin disc with a concentration of 10 µg/disc was also placed on the nutrient agar surface. The Petri dish was then incubated at 37°C. After incubation, the antibacterial activity was observed at 24, 48, and 72 hours to evaluate the effect of the test material on *S. aureus*. This was assessed by measuring the inhibition zone horizontally and vertically using a caliper, with the results interpreted based on the classification system proposed by Davis and Stout [15].

Table 1. Classification of inhibition zone diameter by Davis and Stout

Inhibition Zone Diameter	Inhibitory Activity
> 20 mm	Very Strong
10 – 20 mm	Strong
5 – 10 mm	Medium
< 5 mm	Weak

Data analysis

The data obtained from the study are presented as the mean ± standard deviation (SD). Data were analyzed statistically using ANOVA (α 0.05), followed by Tukey HSD Post Hoc Test

RESULTS AND DISCUSSION

Antibacterial activity

The antibacterial activity of chitosan-silver nanoparticle nanocomposites with *C. citratus* extract as a bioreductor was evaluated using the disc diffusion method against *S. aureus*. *Staphylococcus aureus* was selected as the test microorganism for this study due to its role as a major pathogen responsible for human infections, its high virulence, and its tendency to develop resistance to various antibiotics [16]. This makes it essential to assess the potential of nanocomposites as an alternative antibacterial agent [17].

Based on the results of this study, it can be concluded that the nanocomposite has antibacterial activity against *S. aureus*. This activity is evident from the formation of a clear area called the inhibition zone around the disc paper (**Figure 1**).

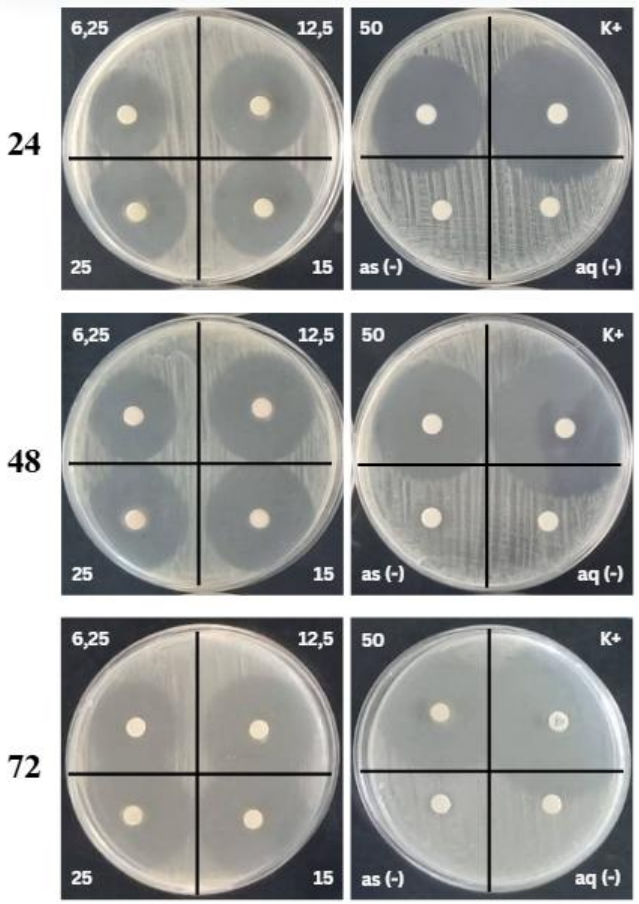

Figure 1. Inhibition zone of *S. aureus* growth at 24, 48, and 72 hours

The antibacterial activity was observed at three different time points to evaluate the effect of the test material on *S. aureus*. A test material is considered bactericidal if the inhibition zone increases in size and clarity over time. Conversely, if the inhibition zone decreases or becomes less distinct, the material is classified as bacteriostatic that only inhibits bacterial growth without killing the bacteria [18]. Based on the results of this study, the formation of inhibition zones at all tested nanocomposite concentrations showed an increase in size over time, indicating that all tested nanocomposite concentrations exhibit bactericidal properties against *S. aureus*.

The results of this study align with previous research which reported that the addition of chitosan to AgNPs synthesized using plant extracts effectively inhibits the growth of *S. aureus* [4]. Additionally, other

studies have demonstrated that AgNPs synthesized using *C. citratus* extract exhibit antibacterial activity against *S. aureus* and *P. aeruginosa*. These studies also stated that the addition of a stabilizing agent can prevent AgNP aggregation, thereby preserving its antibacterial effectiveness [19]. In line with these findings, the present study confirms that using chitosan as a stabilizing agent for AgNPs results in antibacterial activity classified as strong to very strong category. This observation is further supported by the quantitative analysis of the inhibition zone diameters, which provides a clearer depiction of chitosan-nanoparticle silver nanocomposites with *C. citratus* extract antibacterial potential (**Figure 2**).

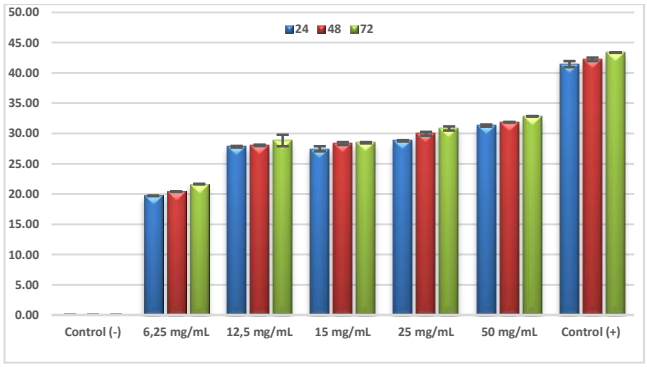

Figure 2. Diagram of the average inhibition zone diameter measurement results against *S. aureus* growth.

The average inhibition zone diameter measurements indicate that among the nanocomposite groups, the highest inhibition zone was observed at a concentration of 50 mg/mL, measuring 31.28 mm at 24 hours, 29.96 mm at 48 hours, and 30.83 mm at 72 hours. Meanwhile, the smallest inhibition zone was recorded for the 6.25 mg/mL nanocomposite, measuring 19.7 mm at 24-hour, 20.42 mm at 48 hours, and 21.65 mm at 72 hours. Overall, the results indicate that inhibition zone size increased with longer incubation periods and higher nanocomposite concentrations.

According to the Davis and Stout classification of inhibition zone diameters, the nanocomposite at a concentration of 6.25 mg/mL exhibited a strong antibacterial effect against *S. aureus* at the 24-hour observation. Over time, its inhibitory effect increased and reached the very strong category with a diameter exceeding 20 mm at the 48 and 72 hour observations. Meanwhile, the positive control group and nanocomposite groups at other concentrations consistently demonstrated inhibition zones classified as very strong throughout all observation periods [15].

Based on the data analysis, the inhibition zone observations at 24, 48, and 72 hours showed a $p<0,05$ indicating a significant difference between all nanocomposite test groups and the control groups. The average inhibition zone diameter produced by all nanocomposite groups was significantly larger than that of the negative control group. This occurred because the nanocomposite contained antibacterial components such as $AgNO_3$, *C. citratus*, and chitosan [4]. On the other hand, the inhibition zone produced by all nanocomposite groups was significantly smaller than that produced by the positive control group. This is because nanocomposites exhibit a less specific and less potent mechanism of action compared to amoxicillin, which directly inhibits bacterial cell wall synthesis [20]. The data analysis also revealed significant differences among all nanocomposite groups, except for the concentrations of 12.5 mg/mL and 15 mg/mL, which showed a $p>0.05$ indicating no significant difference in their antibacterial activity when compared to each other.

CONCLUSION

The test results showed that chitosan-nanoparticle silver nanocomposites with *C. citratus* extract bioreductor at concentrations of 6.25 mg/mL, 12.5 mg/mL, 15 mg/mL, 25 mg/mL, and 50 mg/mL had antibacterial activity against *S. aureus*. Although the effect in inhibiting the growth of *S. aureus* is lower when compared to amoxicillin as a positive control, the inhibitory strength produced by nanocomposites and amoxicillin is classified in the same category, which is very strong according to the zone of inhibition diameter classification by Davis and Stout. The results of this study also showed that all nanocomposite concentrations exhibited growing inhibition zones over time, confirming their bactericidal effect against *S. aureus*. This indicates the potential of nanocomposites as an effective antibacterial agent against *S. aureus*.

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