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Harnessing Chitosan-Stabilized Silver Nanoparticles in *Cymbopogon citratus* Nanocomposites: A Promising Antibacterial Solution Against *Streptococcus mutans*

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ABSTRACT

Nanotechnology explores materials at the nanometer scale and has broad applications, including dentistry. One of the emerging innovations is nanocomposites. Nanocomposites consist of two or more materials, including filler materials and stabilizing agents, and possess antimicrobial activity. Meanwhile, silver nanoparticles (AgNPs) synthesized using lemongrass leaf extract (Cymbopogon citratus) through a green method have potential as antibacterial agents, particularly against oral cavity bacteria. However, AgNPs tend to aggregate, necessitating the use of stabilizing agents such as chitosan. This study evaluated the antibacterial efficacy of AgCh nanocomposites with C. citratus against S. mutans. The study employed the disc diffusion method to test the antibacterial effectiveness of nanocomposites at concentrations of 6.25 mg/mL, 12.5 mg/mL, 15 mg/mL, 25 mg/mL, and 50 mg/mL, along with 0.2% chlorhexidine as the positive control and acetic acid and distilled water as negative controls. Observations at 24, 48, and 72 hours revealed that no inhibition zones formed at 24 hours. However, inhibition zones began to form at 48 and 72 hours, with higher nanocomposite concentrations resulting in larger inhibition zones. The 50 mg/mL concentration produced the largest inhibition zone. This study demonstrates that AgChCc nanocomposites demonstrated significant antibacterial activity, with the optimal concentration for highest effectiveness against S. mutans being 50 mg/mL.

Keywords:
Silver nanoparticles (AgNPs); Cymbopogon citratus; Chitosan; Antibacterial activity; Streptococcus mutans

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1. Introduction

Nanotechnology explores matter at the nanometer scale and has revolutionized fields like medicine and dentistry. With the ability to control and manipulate matter at the nanometer-scale level, nanotechnology has paved the way for the development of various applications that have great potential in various fields, such as electronics, medicine, food industry, and others. One of the emerging nanoscale technologies is nanocomposites. Nanocomposites are composite materials that have nanometer-sized components, promising significant improvements in function and performance

compared to materials with larger particle sizes [1]. In dentistry, nanotechnology addresses limitations of conventional materials, such as chlorhexidine's side effects. Nanocomposite materials in dentistry can be used as mouthwash. The commonly used mouthwash is chlorhexidine 0.2%, but chlorhexidine has side effects of tooth discoloration, impaired taste perception, and increased calculus formation in supragingival [2], therefore an alternative material derived from natural materials is needed such as the use of nanocomposites in mouthwash. Nanocomposites consist of two or more materials, namely *filler* material and stabilizing agent. Commonly used stabilizing agents are *titanium dioxide, zinc dioxide, silicon dioxide, polyvinyl alcohol*, cellulose acetate, and chitosan [3].

Chitosan is obtained from the deacetylation of chitin, which is a major component in the shells of crustaceans, fungi, and insects such as the horned beetle *Xylotrupes gideon (X. gideon*). Chitosan has become a very instrumental material in the manufacture of nanocomposites. Chitosan has biocompatible, *biodegradable*, and non-toxic properties, which have antimicrobial, antioxidant, anti-inflammatory, and anticancer activities. The mechanism of antimicrobial action of chitosan involves interaction with the microbial cell membrane , leading to cell leakage and cell death. These properties make chitosan an ideal candidate for applications in dentistry [4].

Filler material in a nanocomposite is a component that is added to the main material to improve its properties. The *filler* material is the result of synthesizing silver particles with *Cymbopogon citratus* which produces AgNPs that are more effective and more environmentally friendly [5].The synthesis of AgNPs using ethanol extract of *C. citratus*, which has been carried out in previous studies, is used as a bioreductor to convert Ag^{+} ions into Ag^{0}. This green synthesis process is considered safer, environmentally friendly, and economical compared to chemical-physical synthesis which is expensive and causes environmental pollution [6]. AgNP synthesis using *C. citratus* can be a safer and more environmentally friendly alternative to produce AgNPs that are effective in inhibiting bacterial growth [7].

C. citratus, which has long been used in traditional medicine, is known to have various therapeutic properties, including the ability to fight bacteria. *C. citratus* leaf extract has been known to have good antibacterial activity against several types of bacteria, one of which is *Streptococcus mutans. S. mutans* is a Gram-positive bacterium as one of the main causes of dental caries. *S. mutans* has the ability to form *biofilms* and produce an acidic environment through carbohydrate fermentation, which is one of the main causes of dental caries [8]. Nanocomposite is a synthesis of $AgNO_3$ with *C. citratus* extract stabilized with chitosan. Chitosan can be used as an effective reducing agent in the synthesis of AgCh nanoparticles. *C. citratus* leaf extract can help increase the surface area of AgCh particles. Therefore, the use of *C. citratus* leaf extract in the synthesis of AgCh chitosan nanocomposites can enhance the antibacterial activity of the resulting nanocomposites [9].

Research on AgNP synthesis with *C. citratus* has been shown to inhibit the growth of *Salmonella paratyphi, Shigella flexneri, Vibrio cholerae, Bacillus cereus, and Escherichia coli* bacteria [10]. Synthesis of AgNPs with natural materials has low stability and is able to easily undergo aggregation into a larger size, so a stabilizing material is needed, namely chitosan [11]. However, no research has been conducted using chitosan as a stabilizing agent against bacteria in the oral cavity. Therefore, this study aims to synthesize AgNPs with *C. citratus* and the use of horn beetle chitosan as a stabilizing agent (AgChCc) in increasing antibacterial activity against *S. mutans* bacteria using the diffusion method.

2. Methods

Materials and tools used in this study are: $AgNO_3$ 0.1 M (Sigma Aldrich), distilled water, acetic acid, *S. mutans* bacteria (microbiology laboratory, Faculty of Medicine, YARSI University), *C. citratus* extract (BPSI TROA), chitosan *X. gideon* (chemistry laboratory, Faculty of Marine and Fisheries, IPB), chlorhexidine 0.2% (Minosep, Indonesia), 70% ethanol solution (SAE Alcohol 70%, Indonesia), BA (*Blood Agar)* media obtained from the Faculty of Yarsi University, BHI media, stirring rod, Petri dish, glassware, measuring cup, incubator, vernier, paper discs, Erlenmeyer flask, *magnetic stirrer*, micro pipette, tweezers, test tube rack, marker pen, and test tube.

Ethical approval for this study was obtained from the Health Research Ethics Committee of Faculty of Dentistry Universitas Trisakti, with approval number 800/S1/KEPK/FKG/6/2024.

Preparation of *C. citratus* ethanol extract

The process of making ethanol extract from *C. citratus* starts with drying the leaves for two weeks. Next, extraction was carried out using the maceration method. Dried *C. citratus* was soaked with 70% ethanol in a ratio of 1:10 for 72 hours at room temperature. In the maceration process, the solution was shaken manually for 15 minutes at every multiple of eight hours for three days. The solution obtained was filtered using Whatman number one filter paper. The liquid was put into a *rotary evaporator* at 50-60°C to evaporate, thus obtaining *the crude extract* [12].

Preparation of AgCh nanocomposite

The preparation of AgCh nanocomposites began by dissolving 1.7 g of $AgNO_3$ (Sigma Aldrich) into 100 mL of distilled water to produce a concentration of 0.1 M $AgNO_3$ solution. A total of 100 mL of the solution was mixed with two grams of *C. citratus* extract, then the mixture was heated at 40°C for 30 minutes. AgNP solution was formed, then add 1.5% (b/v) chitosan solution, by weighing 1.5 g chitosan powder dissolved in acetic acid and adding distilled water up to 100 mL. The mixture was heated at 40°C for 30 minutes. Next, let stand for 24 hours to produce nanocomposites. The resulting nanocomposite solution was centrifuged at room temperature at 9000 rpm for three minutes. Then, washed three times using distilled water and centrifuged again at the same speed. After the precipitate was obtained, dilutions were made using distilled water at concentrations of 6.25; 12.5; 15; 25; and 50 mg/mL [13], [14].

Preparation of AgCh nanocomposite

To test the antibacterial activity in this study, we started by taking bacterial culture from BA media culture. Next, the bacterial suspension was adjusted to the McFarland 0.5 standard, which is equivalent to the number of bacterial suspensions of 1.5×10^8 CFU/mL. A bacterial suspension of 200 µl was taken using a micropipette, and leveled on BA media with the *spread plate* method using a sterile *cotton swab* [15]. Take a paper disk with a diameter of 6 mm using sterile tweezers and place it on the surface of BA media. Then, 20 µl of test material concentrations of 6.25; 12.5; 15; 25; and 50 mg/mL were dripped on the disc paper [16]. Then, put the agar medium into a jar to eliminate oxygen, then incubate for 24, 48, and 72 hours at 37°C. Observe the formation of the inhibition zone by measuring the clear area on the BA medium using a digital caliper. The formula used to determine the inhibition zone is [17]:

$$L = \frac{(D1-D3)+(D2-D3)}{2} \quad (1)$$

Description:
L : Width of zone of inhibition
D1 : Diameter of horizontal inhibition area (mm)
D2 : Diameter of vertical inhibition area (mm)
D3 : Disc paper diameter (mm)

Data analysis

The results of the observations that have been made are compiled in Excel format. Statistical software SPSS version 2.3 was used to analyze the data. Then the Shapiro-Wilk test was used to perform the normality test. If the p value was >0.05, a parametric test was performed, namely *one-way analysis of variance* (ANOVA). If significant differences were found ($p < 0.05$), further testing was carried out using *Tukey's post hoc* test.

3. Results and Discussion

Inhibition zone observation at 24 hours

Research on the antibacterial activity of AgChCc nanocomposite towards the formation of inhibition zone of *S. mutans* at concentrations of 6.25; 12.5; 15; 25; and 50 mg/mL observed at 24, 48 and 72 hours. The results of observations made at 24 hours showed no inhibition zone formation (**Figure 1**).

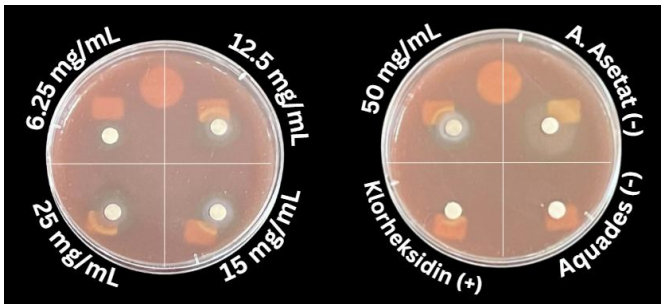

Figure 1 . Zone of inhibition of *S. mutans* at 24 hours

Inhibition zone observation at 48 hours

The observation results were analyzed using the Shapiro-Wilk normality test. Tests were carried out on 48 and 72 hour observations, the results obtained a value of P>0.05 which indicates normally distributed data. The results of the Shapiro-Wilk test can be seen in **Table 1**.

Table1 . Normality test of zone of inhibition at 48 and 72 hours

Group	Shapiro Wilk (48 hours)			Shapiro Wilk (72 hours)		
	Statistic	df	Sig.	Statistic	df	Sig.
Positive control	1.000	3	1.000	1.000	3	1.000
Composite 6.25 mg/mL	1.000	3	0.978	1.000	3	0.987
12.5 mg/mL Composite	1.000	3	0.964	0.993	3	0.843
15 mg/mL Composite	1.000	3	1.000	1.000	3	1.000
25 mg/mL Composite	1.000	3	1.000	1.000	3	1.000
50 mg/mL Composite	1.000	3	1.000	1.000	3	0.962

The statistical test results from the 48-hour observation were normally distributed, then continued with parametric tests using *One Way Anova*. The test results showed a value of P < 0.05, indicating a significant difference in the test group, so further tests were carried out with *post hoc Tukey*. The results of the inhibition zone observation after 48 hours can be seen in **Figure 2**.

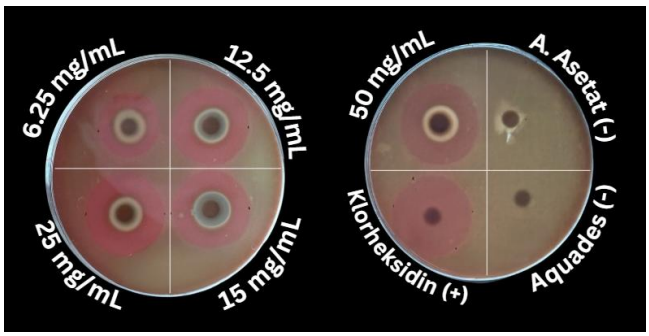

Figure 2 . Zone of inhibition of *S. mutans* at 48 hours

The results of further testing of the inhibition zone of S. mutans at 48 hours showed a significant difference with a value of P < 0.05. In the AgChCc nanocomposite group, the concentrations of 6.25; 12.5; 15; and 25 mg/mL showed significant differences (P<0.05) with the positive control group. The difference in the inhibition zone formed in the AgChCc 6.25; 12.5; 15; and 25 mg/mL groups showed a smaller inhibition zone compared to the control group. While the 50 mg/mL AgChCc concentration group did not show a significant difference in P value = 0, 986 with the control group, although the 50 mg/mL AgChCc concentration did not show a significant difference, the inhibition zone formed in the 50 mg/mL AgChCc concentration was higher than the control. The zone of inhibition at 48 hours observation from the smallest to the largest in order were concentrations of 6.25; 15; 12.5; 25 mg/mL, and 50 mg/mL. The results of the average zone of inhibition in each group can be seen in **Table 2**.

Table2 . Zone of inhibition of *S. mutans*

Group	Zone of Inhibition (mm) ± SD		*P value Anova*
	48 hours	72 hours	
Positive control	23.74 ± 0,76[a]	24.4 ± 0.27[a]	P<0.05
Nanocomposite 6.25 mg/mL	13.96 ± 0,25[c]	14.18 ± 0.42[c]	
Nanocomposite 12.5 mg/mL	19.74 ± 0.15[b]	19.62 ± 0.03[b]	
Nanocomposite 15 mg/mL	18.84 ± 1.9[b]	19.05 ± 1.93[b]	
Nanocomposite 25 mg/mL	20.8 ± 0.02[b]	20.98 ± 0.34[b]	
50 mg/mL Nanocomposite	24.18 ± 0.34[a]	24.45 ± 0.14[a]	

[a-c] *in the same column indicates significant differences*

Inhibition zone observation at 72 hours

The observation of the inhibition zone at 72 hours showed a significant difference ($p<0.05$) between the 6.25; 12.5; 15; and 25 mg/mL groups and the control group, with the formation of a lower inhibition zone compared to the control. While the 50 mg/mL AgChCc group was not significantly different ($p>0.05$) compared to the control group. The AgChCc group at 72 hours observation showed the formation of inhibition zones from the smallest to the largest successively from the AgChCc 6.25; 15; 12.5; 25; and 50 mg/mL groups. The results of the inhibition zone observation at 72 hours can be seen in **Figure 4**.

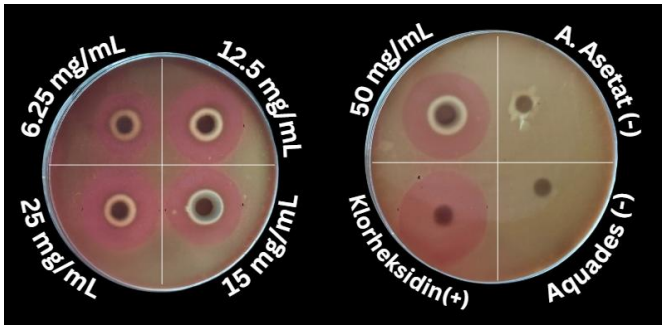

Figure 3. Zone of inhibition of *S. mutans* at 72 hours

Nanocomposite is a material formed from a combination of two or more materials [4]. In this study, the nanocomposite uses a combination of three materials, namely $AgNO_3$, *C. citratus*, and chitosan, each of which has antibacterial activity. The results of the antibacterial activity test showed that at the 24-hour observation AgChCc was unable to inhibit the growth of *S. mutans* by not forming an inhibition zone. The results of this study are supported by previous research which states that *S. mutans* requires an incubation time of more than 24 hours to adapt to new environments such as changes in pH [18], nutrient availability, and oxidative stress [19]. The 48 and 72 hour observations showed the formation of inhibition zones directly proportional to the concentration used, the inhibition zone formed was getting bigger as the concentration increased.

The results of the observation of the diameter of the inhibition zone at 48 and 72 hours fell into the classification of strong and very strong inhibition zones. Classification of antibacterial activity based on inhibition zone according to Davis and Stout criteria, divided into: 1) no inhibition zone; 2) weak, i.e. inhibition zone less than 5 mm; 3) moderate, i.e. inhibition zone 5-10 mm; 4) strong, i.e. inhibition zone 11-20 mm; and 5) very strong, i.e. inhibition zone 21-30 mm [20].

The AgChCc composite has stronger antibacterial activity than the individual materials. The AgChCc composite formed by ionic gelation method produces AgChCc in the form of nanoparticles with cross-links between the three materials in the stirring process using a *magnetic stirrer*. Materials in the form of nanocomposites can facilitate the controlled release of antibacterial agents and increase contact with bacterial cells, so that the bacterial cell wall is damaged [21], [22], [23]

The incorporation of three materials into a composite, consisting of $AgNO_3$, *C. citratus*, and chitosan has synergy to form a bond. $AgNO_3$ is reduced by active compounds in *C. citratus* such as saponins, alkaloids, flavonoids, and tannins which are

known to have antibacterial activity [24], [25]. $^{+}$The active compounds contained in *C. citratus* act as bio reductants by donating electrons contained in the active compounds to convert Ag ions into Ag ^{0} by phenolic compounds [6].

The active flavonoid compounds contained in *C. citratus* can inhibit nucleic acid synthesis and damage the permeability of bacterial cell walls, microsomes, and lysosomes through interactions with bacterial DNA [26]. Saponins have the potential as bacterial inhibitors with a mechanism that disrupts the permeability of bacterial cell membranes, causing cell death. This disruption of the cell membrane can trigger damage that results in the release of cell components, such as nucleic acids, proteins, and nucleotides, which ultimately causes lysis of bacterial cells [27]. In addition, polyphenol and phenolic compounds are known to disrupt cytoplasmic function and membrane permeability, damage DNA, and inhibit nucleic acid synthesis in bacterial cells [28].

The synthesized AgNPs are not stable, so a stabilizer such as chitosan is needed. Chitosan contains amine (-NH_2) and hydroxyl (-OH) groups that are not only able to act as reducers by donating ions, but also able to stabilize the synthesized AgNPs so that aggregation does not occur. The formation of AgChCc nanocomposites consisting of three materials, each of which has antibacterial activity with different mechanisms. The mechanism that inhibits bacterial growth occurs through cell wall damage by antibacterial active substances. This damage results in disruption of the integrity of cellular components, so that the process of bacterial respiration cannot take place [24].

AgNPs as an antibacterial starts by AgNPs accumulating on the surface of the bacterial membrane until they enter the bacterial cell. This changes the permeability of the bacteria, damages the DNA, and ultimately kills the bacteria. The size, shape and surface parameters of silver nanoparticles affect the damage to the bacterial membrane. Due to their small size, AgNPs can interact with the peptidoglycan that protects bacteria more easily [29],[30].

Chitosan has antibacterial activity against *S. mutans* because it is a Gram-positive bacterium that has a higher peptidoglycan and lipid content than Gram-negative bacteria. The amine group possessed by the polycationic nature of chitosan allows interaction with lipids in the cell wall, thus damaging the bacterial defense. In addition, the presence of negative charge and acidic nature of teichoic acid in the cell wall of Gram-positive bacteria interact with chitosan, which ultimately damages the structure of the bacterial cell wall [31]. Each of the ingredients that make up the nanocomposite has good antibacterial activity and the synergy of the three ingredients can increase its activity as an antibacterial with the formation of strong and very strong inhibition zones against *S. mutans.*

Inhibition zone observations at 48 and 72 hours showed that several concentration groups showed an increase in the inhibition zone at 72 hours observation compared to 48 hours but at a concentration of 12.5 mg/mL showed a decrease in the inhibition zone. The increase in the inhibition zone in many nanocomposite groups indicates the potential as a bacteriocide at that concentration, while the decrease in the inhibition zone at 72 observations indicates the potential of the concentration is only bacteriostatic. Observation of the inhibition zone from the results of the study cannot conclude the nature of the AgChCc nanocomposite against the growth of *S. mutans* bacteria whether it is bacteriostatic or bactericidal because the zones formed there are increasing and decreasing, so future studies should assess time-kill kinetics to confirm bactericidal vs. bacteriostatic effects.

The results of this study are in line with previous research which revealed that the active fraction of *C. citratus* has antibacterial activity against *S. mutans* bacteria with

the higher the concentration, the greater the diameter of the inhibition zone formed [24]. Previously, research on AgCh nanocomposites synthesized with *Azadirachta indica* was shown to have antimicrobial activity against the growth of *S. mutans* and *Candida albicans* [32]. Furthermore, AgCh nanocomposites synthesized with *Officinale rhizome* showed inhibition of *P. aeruginosa* growth [33]. AgCh nanocomposites synthesized with *Annona squamosa* also proved effective in inhibiting the growth of *E. coli, B. subtilis, X. campestris,* and *S. aureus* [34]. Another study mentioned that the synthesis of AgNPs with *C. citratus* proved effective in inhibiting the growth of *Bacillus cereus, Escherichia coli, Salmonella paratyphi, Shigella flexneri, and Vibrio cholerae* [11].

At 48 and 72 hours of observation, the inhibition zone produced by the positive control was classified as very strong. This is because the positively charged chlorhexidine molecule has the ability to bind strongly with bacterial molecules which are mostly negatively charged. This interaction results in significant adhesion between chlorhexidine and the bacterial cell membrane. Chlorhexidine then disrupts the permeability of the cell membrane, allowing the escape of cytoplasm and low molecular weight cell components through the membrane. As a result, the bacteria experience death [35]. The antibacterial activity test on the negative control using distilled water did not produce an inhibition zone because distilled water is a neutral compound that cannot inhibit bacterial cell growth [36]. Another negative control used is acetic acid, although acetic acid has antibacterial activity, when used as a negative control it does not form an inhibition zone. This can be caused by insufficient concentration to inhibit bacterial growth or the absence of synergistic effects of other active compounds that can affect the antibacterial effect of acetic acid [37].

AgCh nanocomposites synthesized with *C. citratus* potentially have antibacterial activity against *S. mutans* by forming a zone of inhibition that is as large as the positive control at 48 and 72 hours of observation. AgCh nanocomposites can be used as an alternative mouthwash.

This study has several limitations. First, the antibacterial evaluation was conducted *in vitro* against planktonic cultures of *S. mutans*, which may not fully replicate the complex biofilm environment in clinical settings. Second, while the nanocomposite demonstrated concentration-dependent activity, cytotoxicity assessments on human oral cells were not performed, leaving its biocompatibility profile unclear for dental applications. Third, the characterization of nanoparticle size distribution and long-term stability in the composite was limited; advanced techniques like TEM or XRD could provide deeper mechanistic insights. Fourth, the study focused solely on *S. mutans* without evaluating other cariogenic pathogens (e.g., *Lactobacillus* spp.) commonly present in oral biofilms. Lastly, the 72-hour observation period may not reflect prolonged antibacterial efficacy required for clinical use.

4. Conclusion

The results showed that AgChCc nanocomposites have potential as antibacterial against *S. mutans*. The inhibition zone formed is getting bigger as the concentration of AgChCc nanocomposite increases, with a maximum concentration of 50 mg/mL. This nanocomposite shows promise as a natural alternative to chlorhexidine. Further studies with extended observation periods are needed to ensure the bacteriostatic or bactericidal properties of nanocomposites. In addition, further testing is needed to determine the best concentration of nanocomposites as antibacterial agents. Furthermore, antibacterial activity tests need to be carried out against other pathogenic bacteria found in the oral cavity.

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Conflicts of Interest:

The authors declare no conflict of interest regarding the publication of this article.

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