Volume 9, Issue 6, 2019, 4560 - 4566 ISSN 2069-5837

Biointerface Research in Applied Chemistry

www.BiointerfaceResearch.com

https://doi.org/10.33263/BRIAC96.560566

Original Research Article Open Access Journal

Received: 07.09.2019 / Revised: 22.10.2019 / Accepted: 23.10.2019 / Published on-line: 25.10.2019

Comparative study of antibacterial inhibitory effect of silver nanoparticles and garlic oil nanoemulsion with their combination

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ABSTRACT

Silver can inhibit bacterial activity. Previous studies showed that high concentration of silver nanoparticles (AgNPs) in compared to its lower concentrations is toxic for human health. However, by decreasing concentration of AgNPs, antibacterial activity also decreases. In this study, we investigated synergistic inhibitory activity of the combination of AgNPs and garlic oil nanoemulsion (GONE) for increase antibacterial activity of AgNPs at lower concentrations. AgNPs and GONE with sizes of 30.7 and 19.3 nm were synthesized and prepared by chemical reduction and low energy method, respectively. Physicochemical properties of AgNPs and GONE were investigated. The minimum inhibitory concentration (MIC) of samples was assessed using the standard microdilution method against *pseudomonas aeruginosa* and *staphylococcus aureus*. *P. aeruginosa* was suspected to AgNPs and GONE at all concentration, but in the case of *S. aureus*, antibacterial activity was revealed at ≥29.1% (v/v) and ≥ 36.4 ppb concentration of GONE and AgNPs, respectively. In addition, at low concentration, *S. aureus* was unsuspected to AgNPs and GONE. Combination of AgNPs and GONE (CAG) demonstrated synergistic inhibitory effects at low concentration (≥29.1% (v/v) and ≥ 36.4 ppb concentration). Also, CAG revealed antibacterial activity against *S. aureus* at low concentration. These results indicate that combination of GONE and AgNPs has potential as a green antiseptic agent.

Keywords: *Silver nanoparticle; garlic oil nanoemulsion; antibacterial inhibitory effect; green antiseptic agent, green preservative agent.*

1. INTRODUCTION

Today, many antibacterial agents exist against specific/unspecific bacterial species. Some of them are natural agents that called green antibacterial agents, including silver [1], zinc oxide and copper oxide [2], herbal oil [1] and etc. The antibacterial activity of silver is well known. Except for a few rare strains, the silver ion is effective against a wide range of microorganisms [3-5]. Silver can inhibit bacterial activity in various medical applications, including dental work [6, 7], catheters [8], and the burn wound healing [9].

Reduce size of material may change their properties such as antibacterial activities [1]. Antibacterial activity of silver nanoparticles (AgNPs) is more than silver bulk [10]. Silver nanoparticles (AgNPs) have an enormous specific surface area that assists more quickly dissolution of ions than the equivalent bulk silver [11]. Bacterial cells that treated with AgNPs showed several structural abnormalities including cell size, outer cell layers, cytoplasmic membrane and contents [12]. Further, AgNPs can interact with active site of bacterial enzymes [13] and nucleic acids [14] that ultimately caused inhibited bacterial cell growth and division [15].

Previous study demonstrated that the toxicity of AgNPs against human cells is considerably lower than bacteria [16]. In addition, low concentrations of silver showed that it is non-toxic to human cells [17-19]. Thus, the decreasing concentration of AgNPs can cause to decrease risk to health. In contrast, it was reported that the antimicrobial activity of AgNPs was dependent on its concentration; decreasing AgNPs concentration can lead to decrease antibacterial activity [20]. Therefore, a solution is needed to resolve this paradox. One approach is combination AgNPs with other antibacterial agents, which can reveal antibacterial synergistic effects. The combination AgNPs with antibiotic showed antibacterial synergistic effect against bacterial cells [21-23]. One of the antibiotic alternatives is herbal oil such as garlic oil.

Garlic has potent antibacterial activity [24-28]. Garlic (fresh weight) is composed mainly of water (60–70 g/100 g) and sulfur-containing compounds (11–35 mg/100 g) [29]. Garlic oil demonstrated wide-spectrum antibacterial activity [30]. The most significant components of garlic are the organosulfur-containing compounds [29], such as Alliin, Allicin and diallyl disulphid that have the main role at antimicrobial activity of garlic [24, 25, 31]. Garlic oil showed high levels of antibacterial activity against *Helicobacter pylori* [25]. The emulsification of garlic oil (i.e., at nanoemulsion system) maybe cover and protect its active volatile components. In addition, solubility of Garlic oil can increase at nanoemulsion form.

The aim of this study was the evaluation of synergistic antibacterial effect of AgNPs along with garlic oil nanoemulsion against *staphylococcus aureus* (gram-positive model) and *pseudomonas aeruginosa* (gram-negative model).

2. MATERIALS AND METHODS

2.1. Chemicals and Materials.

Garlic oil (*Allium Sativum L*) was purchased from Noshad (Tehran, Iran). Bacterial species of *Staphylococcus aureus* (*S. aureus*) (ATCC 25923) and *Pesudomonas aeruginosa* (*P.aeruginosa*) (ATCC 27853) were provided from Pasteur Institute of Iran (Tehran, Iran). Phosphate buffer saline (PBS), nutrient broth and agar, sodium borohydride, trisodium citrate and other chemicals were purchased from Merck Chemicals Co. (Darmstadt, Germany). Silver nitrate ($AgNO_3.2H_2O$) was obtained from Sigma-Aldrich.

2.2. Preparation of Garlic Nanoemulsion.

GONE were prepared using low-energy method. Garlic oil (as oil phase), a mixture of Tween 85 and Tween 80 (as surfactant agents), ethanol (as co-surfactant agent), and distilled water (as water phase) were used to prepare GONE. Briefly, 5% (v/v) oil, 20% (v/v) Tween 85 and 5% (v/v) Tween 80 were mixed and stirred at 200 RPM for 5 minutes. Followed by adding 10% (v/v) ethanol and 60% (v/v) distilled water, and mixtre was stirred at 800 RPM for 60 minutes.

2.3. Stability test.

2.3.1. Long time stability.

For long time stability, the optimized GONE was stored in dark place at room temperature for one month. Then, GONE was checked for any sign of phase separation/turbidity.

2.3.2. Accelerated Stability Study.

Centrifugation, heating–cooling cycles and freeze–thaw stress tests were applied for the evaluation stability of optimized GONE:

2.3.2.1. Centrifugation. GONE was centrifuged at 12000 RPM for 30 min to analyze any phase separation.

2.3.2.2. Heating–cooling cycles. Heating–cooling cycles were carried out by keeping the GONE at 4 and 40°C, alternating each temperature for 24 h and it was repeated thrice. Then, the sample was checked visually for phase separation/turbidity.

2.3.2.3. Freeze–thaw stress. For freeze–thaw study, the optimized GONE was kept alternatively at -21°C and +25°C for 24 h and it was repeated thrice. Followed by visual evaluation for phase separation/turbidity.

2.4. Silver Nanoparticle Synthesis.

AgNPs were synthesized by using sodium borohydride as a reductant and trisodium citrate as stabilizing agent. To synthesis of AgNPs, 0.5 ml of trisodium citrate 0.01 M were mixed with 12 ml of silver nitrate solution (0.5 mM) and stirred at 200 rpm. Then, 0.5 ml of ice cooled solution of $NaBH_4$ (0.01 M) was rapidly added into flask.

2.5. Particle Size Analysis.

The average particle size of GONE and AgNPs was measured by DLS (Dynamic Light Scattering, Scatteroscope I, K-ONE LTD, Korea) at 25°C.

2.6. Concentration Analysis of AgNPs.

AgNPs concentration was evaluated by inductively coupled plasma - optical emission spectrometry (ICP-OES).

2.7. pH Measurement.

pH of GONE and AgNPs was determined by pH indicator at 25°C.

2.8. Concentration of Samples for Antibacterial Assay.

Figures 1 to 3 show concentration of AgNPs, GONE and combination of them (CAG). Concentration of GONE and AgNPs was range from 3.6 to 32.7 % (v/v) and 4.55 to 40.91 ppb, respectively. Concentration of CAG was similar to GONE and AgNPs (3.6 % (v/v)/4.55 ppb to 32.7% (v/v)/40.91 ppb).

2.9. Microorganisms and growth condition.

P. aeruginosa and *S. aureus* had been stored in glycerol 10% at -20°C. They were cultured in nutrient broth at 37°C for 16 h. Then, a single bacterial colony was separated from the stock cultures by loop and cultured onto nutrient agar medium for 24 h. Subsequently, a few bacterial colonies inoculated into 10 ml of physiological serum for preparation of ~0.5 McFarland standard of bacterial suspension (0.85% NaCl).

2.10. Determination of Inhibitory Activity.

Inhibitory activity of GONE, AgNPs and CAG was measured against *S. aureus* and *P. aeruginosa* by 96-well plate microdilution method [1]. Serial dilutions of each sample were prepared by PBS. Concentrations of GONE were ranging from 3.6-32.7 % (v/v). Concentrations of AgNPs were ranging from 4.5-40.9 (ppb). Bacteria-free wells applied as blank control. In addition, sample products-free wells applied as negative controls (growth control). Briefly, all wells were filled with 50 µl of sterile nutrient broth (2X) (n=3). Following, 50µl of sample solutions (GONE, AgNPs or CAG) were added to all wells. Finally, 10 µl of 0.5 McFarland of bacterial suspension was added to each well, and plate incubated for overnight at 37°C. By microplate reader (BioTek Instruments, Inc., USA), absorption was recorded at 630 nm and data were normalized with equation 1:

Equation 1:

$$Growth\ (normalized) = \frac{absorbsion\ of\ treatment\ wells}{absorbtion\ of\ negative\ control\ wells} \times 100$$

2.11. Statistical analysis.

All results were reported as mean average ± standard deviation. One-way ANOVA analysis and LSD (least significant difference) comparison tests between the samples was applied to evaluate inhibitory activity of GONE, AgNPs and CAG. Also, the antibacterial synergistic effect of CAG was evaluated at a significant level of $P<0.05$.

3. RESULTS

3.1. Characterization of GONE and AgNPs.

3.1.1. Particle size and pH.

The d_{50} (median hydrodynamic diameter) of optimized GONE and AgNPs were 19.3 nm and 30.7, respectively. pH of both samples was about 5 at 25°C.

3.1.2. Long time Stability.

GONE and AgNPs showed more than 30 days stable at room temperature in a dark place.

3.1.3. Thermodynamic stability studies.

Heating-cooling cycle, freeze-thaw cycles and centrifugation showed that GONE had good physical stability without any flocculation, phase creaming and separation.

3.2. Inhibitory activity Analysis.

The broth microdilution method was applied for evaluation of inhibitory activity of GONE, AgNPs, and CAG on two strains of *S. aureus* and *P. aeruginosa* [1].

3.2.1. Inhibitory activity of GONE.

Figure 1 showed inhibitory activity of GONE against *P. aeruginosa* and *S. aureus*. the results showed that significant differences may be observed between negative control (growth control) and GONE on *P. aeruginosa* (*P<0.001*) and *S. aureus* (*P<0.001*). According to Figure 1, a positive significant differences exist between negative control and GONE against *P. aeruginosa* that suggested GONE had inhibitory activity (*P<0.001*). However, result showed statistically negative sign in the case of *S.aureus*. It was suggested that GONE had proliferation effect against S.aureus (*P<0.001*). The results demonstrated that by increasing concentration of GONE, the inhibitory activity of GONE against *P. aeruginosa* was increased. In the case of S. aureus, by increasing concentration of GONE from 3.6 to 14.5 % (v/v), did not show any inhibitory activity. But, by increasing concentration above 14.5 % (v/v), inhibitory effect was revealed.

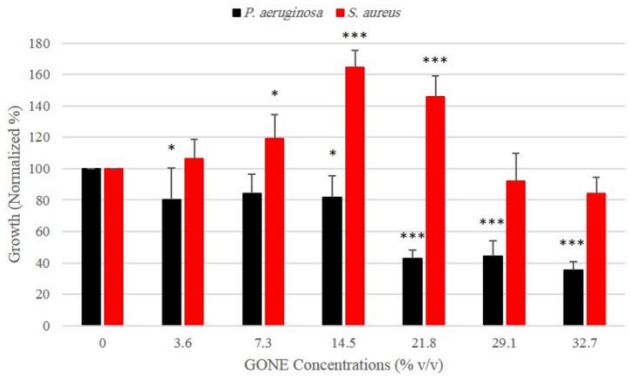

Figure 1. Inhibitory activity of GONE at different concentration was shown. GONE showed better inhibitory activity against *P. aeruginosa* in compared to *S.aureus*. * and *** represent *P<0.05* and *P<0.001*, respectively, in treatment group versus control group.

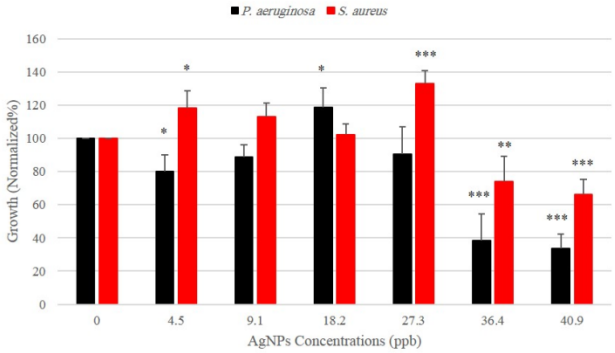

Figure 2. Inhibitory activity of AgNPs was shown. AgNPs showed better inhibitory activity against *P. aeruginosa* in compared to *S.aureus*. *, ** and *** represent *P<0.05*, *P<0.01*.

3.2.2. Inhibitory activity of AgNPs.

Figure 2 showed inhibitory activity of AgNPs against *P. aeruginosa* and *S. aureus*. From the details, significant differences between negative control group and AgNPs treatment group on *P. aeruginosa* (*P<0.001*) and *S. aureus* (*P<0.001*) was observed.

The results demonstrated that by increasing concentration of AgNPs, similar to GONE results, the inhibitory activity of AgNPs against both *P. aeruginosa* and *S. aureus* was increased.

3.2.3. Inhibitory activity of CAG.

Figure 3 illustrates inhibitory activity of CAG against *P. aeruginosa* and *S. aureus*. From the data, a significant differences exist between negative control and CAG against *P. aeruginosa* (*P<0.01*) and *S.aureus* (*P<0.01*). The results showed that CAG has inhibitory activity against *P. aeruginosa* and *S.aureus* at all concentrations, even at lower concentrations.

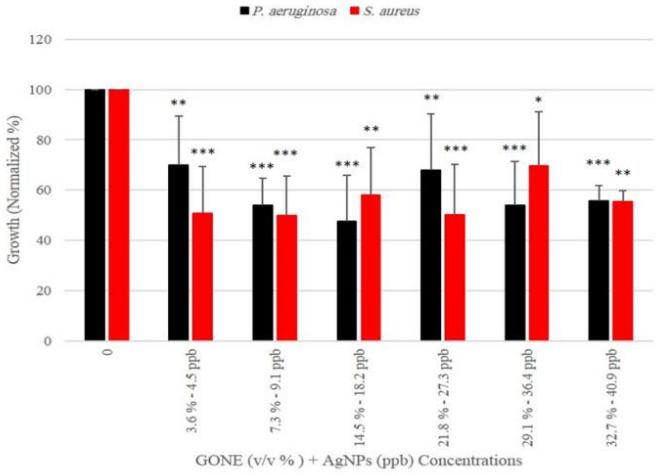

Figure 3. Inhibitory activity of CAG was shown. Concentration of GONE and AgNPs showed as % (v/v) and ppb, respectively, in each sample. CAG showed different inhibitory activity against *P. aeruginosa* and *S.aureus* at various concentration. *, ** and *** represent *P<0.05*, *P<0.01* and *P<0.001*, respectively, in treatment group versus control group.

3.3. Multiple inhibitory activity comparisons.

One-way ANOVA analysis was carried out for better understanding about the significant differences of inhibitory activities between GONE, AgNPs and CAG at a significance level of *P<0.05* (Tables 1 and 2).

3.3.1. GONE vs. AgNPs.

Tables 1 and 2 show multiple comparisons of GONE with AgNPs against *P. aeruginosa* and *S. aureus*. According to analysis, antibacterial activity of GONE in compared to AgNPs is not significant against *P. aeruginosa* and *S. aureus* (*P>0.05*).

3.3.2. GONE vs. CAG.

CAG showed improved antibacterial activity in comparison to GONE but only in the case of *S. aureus* was significant (*P<0.01*).

3.3.3. AgNPs vs. CAG.

Statistical analysis demonstrated that CAG has significant inhibitory activity against only *S. aureus* (*P<0.01*) in comparison with AgNPs. In the case of *P. aeruginosa*, *p* level was bigger than 0.05 (*P=0.341*).

Table 1. Multiple comparisons between inhibitory activities of GONE, AgNPs and CAG against *P. aeruginosa*.

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
GONE	AgNPs	-11.85714	13.66244	.397	-40.5609	16.8466
	CAG	2.57143	13.66244	.853	-26.1323	31.2751

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AgNPs	GONE	11.85714	13.66244	.397	-16.8466	40.5609
	CAG	14.42857	13.66244	.305	-14.2751	43.1323
CAG	GONE	-2.57143	13.66244	.853	-31.2751	26.1323
	AgNPs	-14.42857	13.66244	.305	-43.1323	14.2751

*. The mean difference is significant at the 0.05 level.

Table 2. Multiple comparisons between inhibitory activities of GONE, AgNPs and CAG against *S. aureus*.

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
GONE	AgNPs	15.14286	13.00846	.260	-12.1869	42.4726
	CAG	54.00000*	13.00846	.001	26.6702	81.3298
AgNPs	GONE	-15.14286	13.00846	.260	-42.4726	12.1869
	CAG	38.85714*	13.00846	.008	11.5274	66.1869
CAG	GONE	-54.00000*	13.00846	.001	-81.3298	-26.6702
	AgNPs	-38.85714*	13.00846	.008	-66.1869	-11.5274

*. The mean difference is significant at the 0.05 level.

The present study was designed to evaluate any synergistic effect of antibacterial inhibitory effect of a combination of GONE with AgNPs in compare with individually GONE and AgNPs. AgNPs has great antibacterial activity against a wide spectrum of bacterial species [32, 33]. Our results showed that AgNPs with 30.7 nm size had significant antibacterial inhibitory effect at concentrations upper than 36.4 ppb against *P. aeruginosa* and *S. aureus* (*P<0.001*). The antimicrobial mechanism of silver ions is related to their interaction with thiol (sulfhydryl) groups of cell membrane, cytoplasmic wall, intracellular structures (i.e., ribosomes and mitochondria [34]), biomolecules (i.e., DNA and RNA [35, 36], enzymes and proteins [12, 37, 38]). Further, AgNPs accumulated in the bacterial membrane and led to membrane permeability (i.e., the release of K^+ ions) which resulting in cell death [12, 39, 40]. Also, AgNPs can interact with the bases in DNA strand [41]. More, it can interact with thiol groups of enzymes (i.e., active site or functional groups) and proteins by form disulfide bonds [12, 42] that may decrease or disrupt their function. AgNPs generate reactive oxygen species (ROS) that can disturb the function of biomolecules such as DNA, proteins and enzymes [11, 40].

Garlic oil demonstrated a wide-spectrum antibacterial activity [30]. From our findings, GONE showed a significant inhibitory activity (*P<0.05*) compared to negative control against *P. aeruginosa*. But in the case of *S. aureus*, inhibitory effect did not show significant differences (*P<0.05*). Our results showed that inhibitory activity of GONE was increased by increasing oil concentration. Antibacterial activity of garlic oil is related to the abundance of sulfur-containing compounds which may react with SH groups of cellular proteins by formation of disulfide bonds that decrease or disrupted their function [24, 43-45]. Another antibacterial mechanism of garlic oil is increase cell membrane permeability by attack oil to the phospholipid of membrane, that lead eventually to bacterial death [46]. More, NEs can induce generation of ROS and make oxidative stress in the cell cytoplasm [47].

According to the results, all concentrations of GONE and AgNPs (expect concentration of 18.2 ppb) showed antibacterial activity against *P. aeruginosa* that is gram-negative bacteria. However, low concentrations of GONE (3.6 to 21.8 % (v/v)) and AgNPs (4.5 to 27.3 ppb) did not present any antibacterial activity against *S. aureus*. Another study also showed that AgNPs has better antibacterial inhibitory at the low concentration against *E. coli* (gram-negative model) compared to *S. aureus* (gram-positive model) [40]. It may be related to different at compositional and structural of between gram-negative and -positive bacteria.

Our finding suggested that CAG has synergistic antibacterial effect against *P. aeruginosa* and *S. aureus*. Also, CAG showed significantly antibacterial effect against *S. aureus* (*P<0.01*) even at lowest concentration (3.6 % (v/v) of GONE and 4.5 ppb of AgNPs). In CAG formulation, due to presence of surfactants in NE structure as an outer layer, AgNPs can aggregate on surfactant and provides a positive surface charge on the NE vesicles [48]. It leads to increase attraction of NE to the negatively charged of bacterial membrane surface [48]. Also, surfactants can considerably decrease surface hydrophobicity of bacterial cell that can damage the bacterial cell wall [49]. Another possible mechanism is increased fluidity on cells of organism by non-ionic surfactants [50]. Pervious study showed that interaction NE with bacterial cell leads to release of RNA and DNA to extracellular space [51-53]. So, NEs could increase permeability on bacterial cell wall [51-53]. Maybe it can facilitate passing AgNPs from extracellular to intracellular space and increase damage cell by increasing interaction AgNPs with intracellular structures and biomolecules. Also, small size of NEs helps them better penetrate into the bacterial cells [49]. Therefore, GONE as carrier, can enhance delivery of AgNPs into cytoplasmic space of bacterial cell.

4. CONCLUSIONS

In the current study, GONE and AgNPs were prepared and synthesized with sizes of 19.3 and 30.6 nm, respectively. The results showed that combination of AgNPs with GONE (CAG) can increase antibacterial activity in compared to AgNPs and GONE individually in a lower concentration. In addition, CAG showed synergistic inhibitory effects against *S. aureus*, wherein AgNPs and GONE individually did not show any inhibitory antibacterial activity. Thus, antibacterial inhibitory effect of

AgNPs can increase at lower concentrations by combination with GONE. These results indicate that combination of GONE and AgNPs has potential as a green antiseptic and preservative agent.

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6. ACKNOWLEDGEMENTS

This research has been supported by Tehran University of Medical Sciences and health Services grant by number 98-01-61-40840.