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Synthesis of Silver Nanoparticles by Sodium Borohydride Reduction Method: Optimization of Conditions for High Anti-staphylococcal Activity

Ifeanyi T. Nzekwe[1*], Chukwuma O. Agubata[2], Chukwuebuka E. Umeyor[1], Ifeanyi E. Okoye[1] and Chidalu B. Ogwueleka[1]

[1]Department of Pharmaceutics and Pharmaceutical Technology, Nnamdi Azikiwe University, Awka, Nigeria.
[2]Department of Pharmaceutical Technology and Industrial Pharmacy, University of Nigeria, Nsukka, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author ITN designed the study and wrote the final draft. Author CEU reviewed the study design, methods and results while author COA reviewed design and protocol, edited manuscript and approved final version submitted. Author CBO carried out protocols and prepared the first draft. Author IEO was involved in directing method and evaluating results to meet set objectives and in funding research.

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ABSTRACT

Aim: The aim of this research was to optimize the reaction conditions for the production of silver nanoparticles sufficiently bioactive for incorporation into pharmaceutical gels.
Methods: Silver nanoparticles were prepared by silver nitrate-sodium borohydride reduction method under different stoichiometric conditions and differences in their spectral properties were investigated while bioactivities were studied against different bacterial species. The most bioactive sample was characterized using transmission electron microscopy, atomic force microscopy and photon correlation spectroscopy, and the influences of varying reaction conditions (pH, solvent

**Corresponding author: E-mail: it.nzekwe@unizik.edu.ng;*

type and solvent temperature) were investigated using the stoichiometric ratio with best bioactivity.
Results: The 25: 25 batch (sodium borohydride and silver nitrate in equal volume ratio) demonstrated the highest bioactivity which was significantly different (P = 0.05) from others. Physicochemical characterization revealed a hydrodynamic size of 17.46 d.nm (pdi 0.62) and a peak resonance mode at 450 nm. Bioactivity was highest at neutral pH conditions of reaction and improved with precursor solvent temperature, up to 45°C. Whereas the control sample of ciprofloxacin demonstrated no activity against *Staphylococcus aureus*, silver nanoparticles in methanol exhibited very good activity against *Staphylococcus aureus*.
Conclusion: It is hereby concluded that reaction conditions can affect the antimicrobial activity of silver nanoparticles, possibly by influencing the size and yield of silver nanoparticles using the silver nitrate-sodium borohydride reduction method.

Keywords: Bioactivity; reaction conditions; microscopy; particle size.

1. INTRODUCTION

The increasing cost of healthcare due to emergence of microorganisms with multi-drug resistance have encouraged many researchers to engage in developing new effective antimicrobials with improved activity and new mechanisms of action. Of all the chemical elements with oligodynamic effect against bacteria, fungi and algae, silver is the least toxic, and silver-based materials including silver nitrate, silver proteinate and silver nanoparticles are useful as antiseptics based on the ability of the biologically active silver ion (Ag+) to irreversibly damage key enzyme systems in the cell membranes of pathogens [1]. Reviews [2,3] have shown that silver-impregnated dressings can improve the short-term healing of wounds and ulcers, and that silver dressings improved wound healing and quality of life in chronic non-healing wounds, cause greater reduction in wound size, with leakage and odour control [4].

Silver nanoparticles and nanostructures have a high potential for increased application in wound healing and antisepsis. Silver compounds when used as antibacterial agents are toxic to cells and can sometimes be harmful to normal human cells, but silver nanoparticles are far less reactive. The silver nanoparticles not only interact with the surface of a membrane but can also penetrate inside the bacteria [5].

Different reducing agents have been used in aqueous or non-aqueous solutions, leading to formation of metallic silver (Ag), which is followed by assembly into nanoparticles. The sodium borohydride method is a facile synthetic method which can be adapted in resource-poor settings for preparing silver nanoparticles for different antimicrobial applications. In this work, given the increasing menace posed by Staphylococcus in wound management [6,7], we seek insight into how reaction conditions can influence antimicrobial (especially anti-staphylococcal) activity of silver nanoparticles in order to make them more useful in wound management.

2. MATERIALS AND METHODS

2.1 Materials

Silver nitrate (Merck, England), sodium borohydride powder (Sigma Aldrich, USA), sodium hydroxide crystals (BDH, England), concentrated hydrochloric acid (Sigma Aldrich, USA), absolute ethanol (BDH, England), methanol (BDH, England), nutrient agar (Oxoid), nutrient broth (Oxoid), Mueller Hinton Agar (Oxoid). Strains of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Bacillus subtillis* were obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka, Nigeria.

2.2 Preliminary Synthesis of Silver Nanoparticles

Chemical reduction method [8] was used, with slight modification. Silver nitrate (18.16 mg) was dissolved in 200 ml of ice cold distilled water, to give a 1.2 mM solution. At the same time, a 2.4 mM aqueous solution of sodium borohydride ($NaBH_4$) was prepared by adding 40.77 mg of $NaBH_4$ (molar weight=37.83 mg/mol) to 200 ml of ice cold distilled water. Use of a 1:2 molar ratio is expected to promote stability [9]. Several batches of silver nanoparticles were formed by reacting silver nitrate solution with sodium borohydride in the following ratios: 5:25, 10:25, 15:25 and 25:25 respectively.

2.3 Determination of Bioactivity of Synthesized Silver Nanoparticles

The bioactivities of the different batches were determined using the agar well diffusion method. Briefly, twenty millilitres (20 ml) of the molten Mueller Hinton Agar was poured into sterile petri dishes (90 mm) and allowed to set. A Standardized concentration (Mcfarland 0.5) of overnight cultures of test isolates (*Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumonia* and *Bacillus subtilis)* were swabbed aseptically on the agar and holes (6 mm) were made in the agar plates using a sterile metal cork borer. Twenty microlitres (20 µl) of the various samples and controls were introduced in well-labelled holes in replicates under aseptic conditions. The samples were initially allowed to diffuse into the agar for 1 h at room temperature, before incubation at 37℃ for 24 h. Ciprofloxacin (5 µg/ml) was used as positive control while DMSO or sterile water was used as the negative control. The inhibition zone diameters were subsequently measured.

2.4 Characterization of the Nanoparticles

2.4.1 UV-VIS spectrophotometry and pH evaluation

The UV-VIS spectra of the nanoparticulate solutions were obtained using UV-VIS spectrophotometer (Jenway 6505, China) and the pHs of the different batches were measured with a pH meter (HANNA, Padova, Italy).

2.4.2 Atomic force microscopy

A topographical image of the most bioactive batch (25:25 mixture) was obtained using atomic force microscopy. This was also used to determine the particle size of the silver nanoparticles. Topographical images of the silver nanoparticles were obtained with an Agilent 5500 Atomic Force Microscope employing the contact mode technique with a cantilever having resonant frequency of 17 KHz and spring constant of 0.08 N/m. Sheet of mica was used as a substrate for placing the nanoparticles.

2.4.3 Photon correlation spectroscopy

Photon correlation spectroscopy was used to determine the hydrodynamic diameters of the formulated silver nanoparticles. The hydrodynamic diameters were determined using a ZetaSizer Nano spectrometer (Malvern Instruments, Worcestershire, UK) at 25℃. The instrument uses dynamic light scattering (DLS) technology.

2.4.4 Transmission electron microscopy (TEM)

The shape and morphology of the particles were also determined using the transmission electron microscope. The transmission electron microscope was operated at an accelerating voltage of 80 Kv with direct magnification of 80,000x. TEM images depend on differences in electron density between the sample and surrounding substrate.

2.4.5 Effect of solvent

The reactants (silver nitrate and sodium borohydride) were dissolved in water, ethanol or methanol, respectively, and reacted as previously described. The antimicrobial activities of the resulting nanoparticles were compared using the agar well diffusion method.

2.4.6 Effects of precursor solution pH on antimicrobial activity of silver nanoparticles

Silver nitrate solution was prepared as previously described and divided into several parts. The pH conditions of the solutions were adjusted to 4.5, 7.9 or 9 with 0.1N sodium hydroxide or 0.1N hydrochloric acid. These were then reacted with sodium borohydride as described.

2.4.7 Effect of temperature

The Silver nitrate solutions were equilibrated at different temperatures: 5℃, 18℃, 25℃ and 45℃. These solutions were then reacted with sodium borohydride as stated.

2.4.8 Gel preparation and evaluation

Silver nanoparticles in carbomer and carbomer-mucin gels were prepared by adding 2.5% of carbomer gels, to silver nanoparticle solutions to form 0.5% carbomer dispersions. The pHs of the gels were subsequently varied with either 0.1N NaOH or 0.1N HCl, to produce four different batches labelled A to D. The viscosities of the gels were also determined using a viscometer.

3. RESULTS AND DISCUSSION

Silver nanoparticles were prepared from the reduction of silver nitrate to atomic silver, with subsequent agglomeration into nanoparticles. Differences in colour were observed for the nanoparticle solutions prepared from the different reaction stoichiometries. These differences in colour and resonant frequencies can be linked to differences in particle shapes and sizes. The absorption wavelength arises from the s-p electron transitions, which in turn depend on the size and shape of the nanoparticle, both of which determine the mean freedom of motion. For example, the 5:25 ratio solution appears as a brightly yellow solution (with an SPR of 500 nm), while the 25:25 ratio solution is a greenish yellow solution (SPR of 450 nm). The latter is characteristic of silver nanoparticles. Different UV-Vis spectra were also observed with differences in reaction ratios (Fig. 1). The position of the resonant wavelength correlates with the size average of the particles and from Fig. 1, it may be predicted that the 5:25 batch particles are slightly bigger than the 25:25 batch particles. Also, the absorbance of the former was slightly lower than that of the latter at the peak wavelengths. The particle size of the optimized 25:25 batch with bioactivity was confirmed by transmission electron microscopy and photon correlation microscopy (Figs. 2 and 3). Also, Fig. 2 revealed that the nanoparticles prepared with a 25:25 reactants ratio were compact with higher number of particles per unit area relative to the 5:25 batch.

The 25:25 batch (sodium borohydride and silver nitrate in equal volume ratio) demonstrated the highest bioactivity and was significantly different (P = 0.05) from others (Table 1). Due to the lack of activity observed for the 5:25 batch (Table 1), it is concluded that bioactivity is influenced by particle size and surface free energy. The design and manufacture of devices with sizes between 10-1000 nm has provided a variety of applications [10].

The activity of the 25:25 batch of silver nanoparticles against both *Staphylococcus aureus* and *Escherichia coli* agrees with previous researches [11,12]. The differences in bioactivity between the 10:25 and 25:25 batches, in spite of similar spectral patterns, suggest that the effect may be due to different yields of the nanoparticles in solution. It may therefore be suggested that increase in the ratio of silver nitrate improves the yield of the silver nanoparticles. Reports have shown that the size, morphology/shape, chemical composition and surface chemistry of nanoparticles [13] may affect the stability and behaviour in biological microenvironment and cellular distribution [14]. Nanoparticles can be taken up by cells via endocytosis to enable cellular internalisation of the drug [15], but this involves size and shape specificity.

Atomic force microscopy indicated an average particle size of 13 nm, while photon correlation spectroscopy indicates 17.46 nm (PDI 0.616). The fair level of agreement observed between electron microscopy and dynamic light scattering can be attributed to the spherical nature of the particles, such that the hydrodynamic radius approaches real particle radius.

The pH measurements in Tables 1 and 2 indicate that highest bioactivity is exhibited at around neutral pH, and least in acidic media. The reduced activity in acid medium is in agreement with the findings of other researchers [16], who synthesized silver nanoparticles using *Bacillus brevis* under varying extracellular pH conditions. In varying the pH of preformed silver nanoparticles, we report that the activity at neutral pH is better than that obtained at pH 9 (Table 2). The effect of pH has been explained in terms of speed of nucleation, which is slower at low pH, leading to formation of silver nanocrystals of low amount and large size, whereas at higher pH conditions, fast nucleation process occurred because of the accessibility of –OH ions, leading to formation of a high amount of small size particles [16,17].

Table 1. Inhibition zone diameters (mm) produced by silver nanoparticles prepared from varying ratios of silver nitrate and sodium borohydride

Batch	pH	*S. aureus*	*E. coli*	*Klebsiella spp*	*B. subtilis*
5:25	8.41	-	-	-	-
10:25	8.24	-	-	-	-
25:25	7.04	1±0.00	1±0.00	2±0.00	1±0.00

Key: '-' means no activity; 15: 25 was discontinued

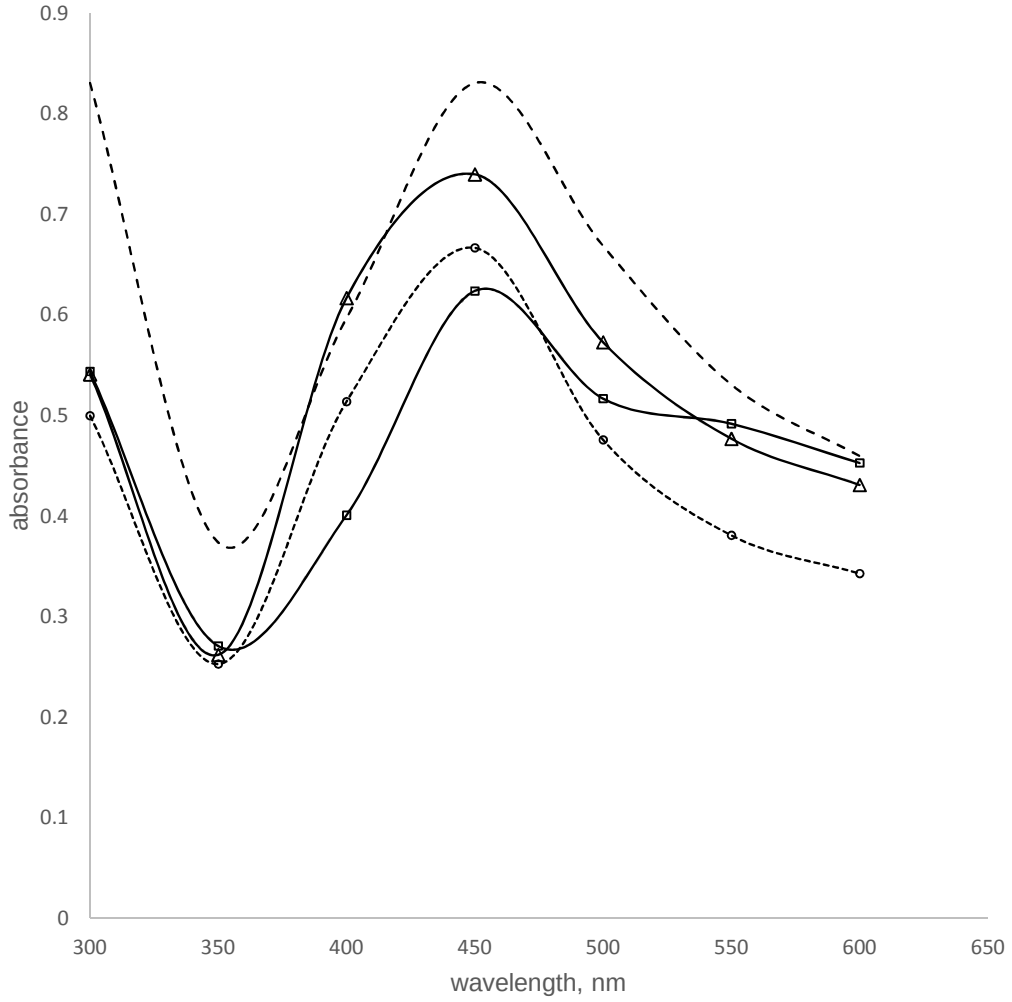


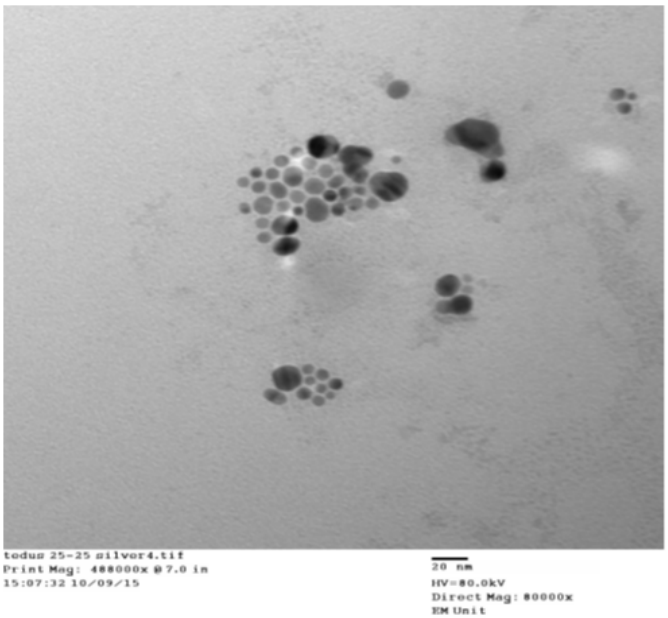
Fig. 1. UV-VIS spectra of silver nanoparticles prepared by reacting varying volume ratios of sodium borohydride and silver nitrate
-□-5:25; --10: 25; -Δ- 25: 25; -○- 15:25

Table 2. Inhibition zone diameters (mm) produced by silver nanoparticles adjusted to different final pH conditions

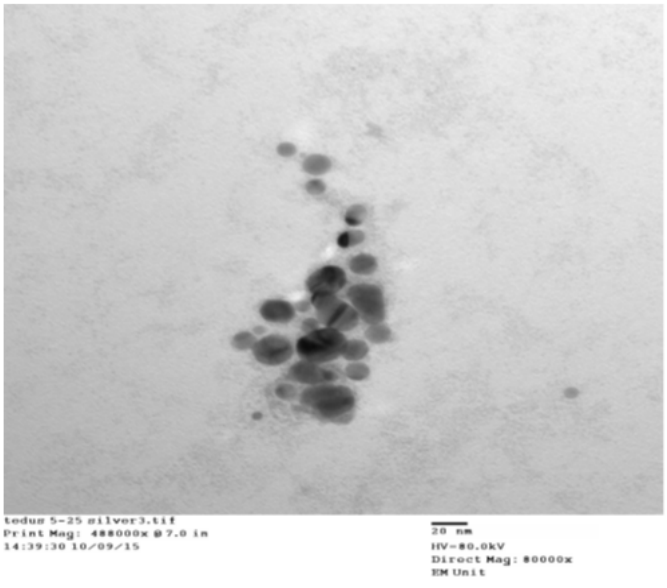
Variable	Inhibition zone diameter (mm) organism			
pH	***S. aureus***	***E. coli***	***Klebsiella spp***	***B. subtilis***
4.50	4.33±0.58	2.00±0.00	2.00±0.00	1.67±0.58
7.99	6.33±0.58	3.33±0.58	2.33±0.58	3.67±0.58
9.00	5.67±0.58	3.33±0.58	2.33±0.58	2.33±0.58
Control (Ciprofloxacin)	0.00±0.00	10.00±0.00	9.67±0.58	9.00±0.00

In Table 3, however, it is seen that increasing the pH of carbomer-stabilized systems may induce viscosity changes making diffusion of the nanoparticles in agar less favourable. This results in poor antimicrobial activity, *in vitro*. In formulating nanosilver gels, therefore this must be factored in, as well as the potential for skin toxicity in low pH systems.

The effect of solvent on bioactivity (Table 4) is seen to be highest with methanol and least with ethanol. Whereas the highest inhibition is recorded by silver nanoparticles in methanol against *Staphylococcus aureus*, no activity is recorded by ciprofloxacin against the organism. *Staphylococcus aureus* is a major cause of nosocomial infections [18,19] and in a recent report, it was the predominant organism isolated from surgical wounds of hospitalized patients in Nigeria [6]. A high rate of resistance to frontline antibiotics such as fluoroquinolones may translate into an increasing rate of failure of wound management with conventional antibiotics, which implies that silver nanoparticles remain an old and reliable tool in wound treatment.



(a)



(b)

Fig. 2. Electron micrographs showing differences in nanoparticles prepared from 25:25 (a) and 5:25 (b) stoichiometric (by volume) ratios of sodium borohydride and silver nitrate

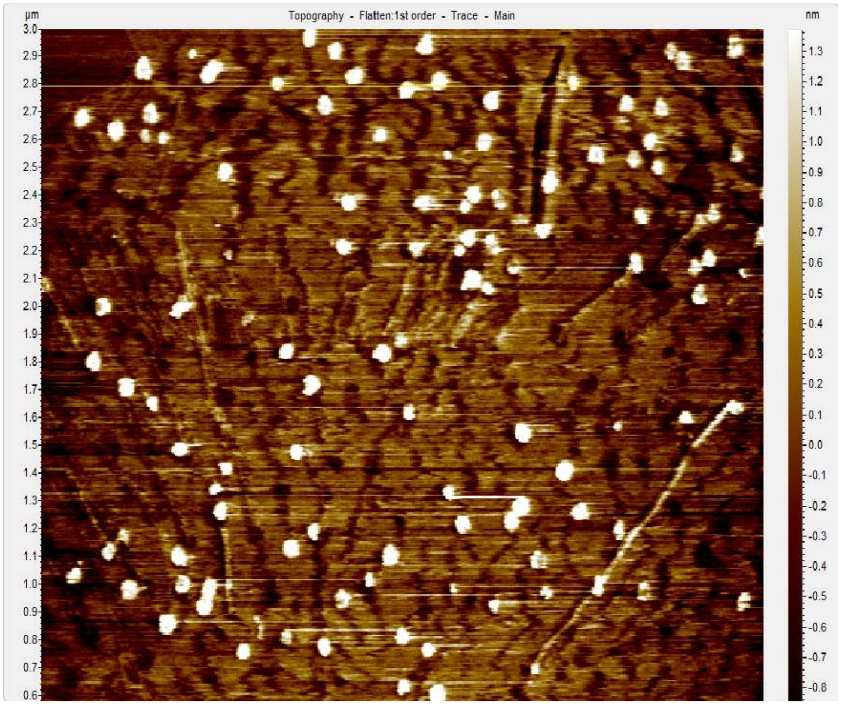


Fig. 3. Topographic image of silver nanoparticles prepared by sodium borohydride silver nitrate reduction method

Table 3. Inhibition zone diameters (mm) of silver nanoparticles in carbomer gels of varying pH

Sample	pH	Viscosity (mPa.s)	Inhibition zone diameters (mm)			
			S. aureus	*E. coli*	*Klebsiella spp*	*B. subtilis*
A	2.19	97	4±0.00	-	-	-
B	4.51	67	4.5±2.12	-	-	-
C	7.01	114	-	-	-	-
D	9.14	174	-	-	-	-

The destabilization of silver nanoparticles by ethanol had been reported previously [8]. Addition of ethanolic silver nitrate to sodium borohydride causes development of a greyish colouration, different from the characteristic greenish yellow colouration of silver nanoparticles in water.

The effect of precursor solution temperature on the bioactivity of the synthesized silver nanoparticles can be observed as increase in bioactivity with temperature (Table 5), and this increase averaged 20 % in going from 5℃ to 45℃, except for *B. subtilis*. Higher temperatures had been associated with a faster rate of formation of nanoparticles [20], and even though this is expected to cause an increase in the diameter of the nanoparticles, the overriding effect of the high yield of nanoparticles at the higher temperature lead to higher bioactivity against *Staphylococcus aureus*.

Table 4. Inhibition zone diameters (mm) produced by silver nanoparticles synthesized in different solvents

Solvent	Inhibition zone diameter (mm)			
	S. aureus	*E. coli*	*Klebsiella spp*	*B. subtilis*
AgN-ethanol	5.67±0.58	2.00±0.00	2.00±0.00	2.00±0.00
AgN-methanol	9.67±0.58	4.00±0.00	3.00±0.00	3.67±0.58
AgN-water	6.67±1.15	3.00±0.00	2.00±0.00	2.67±0.58

Table 5. Inhibition zone diameters (mm) produced by silver nanoparticles prepared with water at different solvent temperatures

Temperature	Inhibition zone diameter (mm)			
	S. aureus	***E. coli***	***Klebsiella spp***	***B. subtilis***
5℃	6.67±0.58	3.33±0.58	2.67±0.58	3.00±0.00
18℃	5.67±0.58	3.33±0.58	2.67±0.58	2.00±0.00
25℃	7.67±0.58	4.00±1.00	3.33±0.58	3.33±0.58
45℃	8.00±1.00	4.00±1.00	3.33±0.58	3.33±0.58

4. CONCLUSIONS

In conclusion, it may be inferred that in the face of rising resistance posed by *Staphylococcus aureus* which is a common cause of nosocomial infections, silver nanoparticles applied as solutions on dressings are an important intervention. In order to potentiate anti-staphylococcal activity, the preparation should be presented in a low viscosity vehicle buffered to neutral pH. If intended for application on surfaces for disinfection of Staphylococcus, the recommended vehicle is methanol, in which the particles have demonstrated a high level of anti-Staphyloccocal activity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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