Original Article:

The Antimicrobial Effects of Ciprofloxacin Combined with Green Synthesized Glutathione-coated Silver Nanoparticles on Biofilm Formation of *Pseudomonas Aeruginosa*

Mohammad Ghorbani[1], Raheleh Safaeijavan[2,*], Shohreh Zare Karizi[3]

[1] Department of Biology, Islamic Azad University, Varamin-Pishva Branch, Iran
[2] Department of Biochemistry and Biophysics, Islamic Azad University, Varamin-Pishva Branch, Iran
[3] Department of Genetics, Islamic Azad University, Varamin-Pishva Branch, Iran

*Corresponding Author email address: safaeijavan@gmail.com(R. Safaeijavan)

ABSTRACT

Introdaction: Nowadays, antibiotic resistance is rising at an alarming rate. Essentially, one of the important ways for bacteria such as *P. aeruginosa* to survive in the presence of antibiotics is biofilm formation. In the current report, we have focused on inhibiting the microbial biofilm formation of *P. aeruginosa* through combining glutathione (GSH) coated silver nanoparticles (AgNPs) and Ciprofloxacin (Cip).**Materials and Methods:** AgNPs were biosynthesized using *Eucalyptus Camaldulensis* leaf extracts and surface modification of AgNPs was done, using glutathione. Synthesized nanoparticles were characterized by FTIR, XRD, DLS, SEM, and CHN tests. Then, 50 isolates of *P. aeruginosa* were originated from different samples of hospitalized patients from Sina hospital and 24 isolates were selected as a strong biofilm producer using microtiter plate method for further studies. Finally, the synergistic effect of GSH-coated AgNPs and Cip was investigated on biofilm formation of *P. aeruginosa*. **Result:**The images of SEM represent the spherical structure of silver nanoparticles with a smooth surface. Also, the results of FTIR, XRD, DLS, SEM, and CHN of AgNPs before and after surface coating confirmed the formation of GSH-coated AgNPs. GSH-coated AgNPs and Cip at a concentration of 1/2 and 1/4 MIC had an inhibitory activity on biofilm formation of 87.5% and 83.4% of *P. aeruginosa* isolates respectively. **Conclusion:**This study illustrated that the combination of GSH-coated AgNPs and Cip has a synergistic inhibitory activity on *P. aeruginosa* biofilm formation.

Keywords: Biofilm; *Eucalyptus camaldulensis*; Silver nanoparticles; Synergistic; Ciprofloxacin

INTRODUCTION

Among Gram-negative bacteria, *Pseudomonas aeruginosa* (*P. aeruginosa*) is a greatly susceptible one to genetic modifications, causing antibiotic resistance [1]. *P. aeruginosa* is an opportunistic pathogen which is involved in the high rate of morbidity and mortality [2]. It is known as one of the important agents in hospital infections such as urinary tract infections and Gastroenteritis and in most cases, it has been observed that the pathogenesis resulting from them is dependent on the development of biofilms [3].Gastroenteritis means the inflammation of stomach, and small and large intestines. Severe gastroenteritis results in approximately 800,000 deaths annually by some bacteria (i.e. *Staphylococcus aureus, Pseudomonas aeruginosa Escherichia coli, Clostridium difficile, Yersinia enterocolitica, Salmonella, Shigella, and Campylobacter,* parasites such as *Giardia, Cryptosporidium* and viruses [4, 5 and 6]. Biofilms are sessile microbial communities which grow on surfaces, mostly embedded in a matrix of extracellular polymeric substances [7]. Formation of biofilms protects organisms from antibiotics, disinfectants, and allows them to survive in the hostile environment [8]. Thus,

there is a lot of interest in developing methods that can prevent the formation of biofilms [9]. Ciprofloxacin, an antibacterial agent of the fluoroquinolone family, is the fifth biggest generic antibiotic produced in the world that holds 24% of the market for medicines. It has the most antibacterial activity against Gram-negative bacteria and better safety profile and good effectiveness against resistant pathogenic bacteria as compared to other antibiotics [10, 11]. Ciprofloxacin is used in the urinary and respiratory tract, skin, bone, and joint infections [12].Silver and its products have been used in water and air purification, cosmetics, clothing and biomedical applications, including diagnosis, treatment, and drug delivery because of its broad‑spectrum antimicrobial features. With the rapid advancement of nanotechnology, the applications of nanoscale materials has also been expanded. Nowadays, silver is the most used material in engineered nanomaterial [13]. It has also been proved that silver nanoparticles (Ag NPs) have a fatal effect against various bacteria such as *E. coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *P. aeruginosa* [14].Recently, AgNPs have emerged as a strong tool to treat microbial infections including multi-resistant pathogens [15] and have been used as a molecular method for targeted drug delivery. Currently, nano- antibiotics have attracted attention as an alternative treatment method [16]. Biosynthesis of nanoparticles, using plant extract is a useful technique due to its cost-effective and eco-friendly approach with no use of any toxic chemicals in the synthesis process [17]. The action of plant extracts may be both reducing and stabilizing in AgNPs synthesis [18].*Eucalyptus camaldulensis* belongs to a large genus of the Myrtaceae family. In traditional medicine, it has been used as anti-inflammatory and analgesic drug and for treatment of bacterial infections of the lung and urinary tracts worldwide. Essential oils of eucalyptus have various functions. It has been used as an insect repellant and microbial activity inhibitor of some fungi and bacteria [19, 20].Glutathione (a tripeptide biomolecule (γ-Glu-Cys-Gly)) as a reducing or coating agent can produce uniformly sized water-soluble nanoparticles which can easily attach to antibiotics. This is interesting and important in medical applications. GSH contains a –SH group that can be easily adsorbed onto the surface of silver nanoparticles. It is also bound to toxins, such as heavy metals and pesticides, and converts them into forms that can be excreted in urine or bile [21]. Considering the importance of this biological molecule, many investigations have recently been conducted to figure out the glutathione's reaction in the presence of nanoparticles [22].Hence, the present study aims at synthesizing silver nanoparticles by the green method using *Eucalyptus camaldulensis* leaf extracts; the antibacterial effect of Cip in the presence of GSH-coated AgNPs was also investigated on biofilm formation of *P. aeruginosa*.

MATERIALS AND METHODS

Materials

Silver nitrate, Crystal violet, Muller Hinton (MH) agar, Tryptic soy broth (TSB), tryptic soy agar (TSA), Methyl Red-Voges Proskauer (MR-VP) broth and SIM medium were obtained from Merck, Germany. Glutathione from Sigma-Aldrich, USA and Ciprofloxacin was purchased from Padtan Teb Co., Iran. Moreover, fresh leaves of *Eucalyptus camaldulensis* were purchased from Iranian Biological Resource Center.

Bacterial Isolates and Identification of P. aeruginosa

In this experimental study, 50 clinical samples including wound, urine, phlegm, body fluid and blood were taken from the medical laboratory of Sina hospital, Tehran, Iran during Jan-May, 2017 and were identified and confirmed as *P. aeruginosa* by microbiological and biochemical tests such as oxidase, catalase, triple sugar iron (TSI) agar, sulfide indole motility (SIM), oxidation fermentation (OF) test and MR-VP.

Green Synthesis of Silver Nanoparticles

The alcoholic extract solutions of plant leaves were prepared by 5 gr of *Eucalyptus camaldulensis* powder in a beaker, containing 96% alcohol. After an overnight stirring, *Eucalyptus camaldulensis* alcoholic extract was filtered. The amount of 100 ml aqueous solution of 5 mM $AgNO_3$ was added dropwise to 140 mL of the resulted alcoholic extract

solution [23]. The darkening of the solution color marks the formation of silver nanoparticles. Furthermore, the formation of AgNPs was confirmed by spectrophotometric analysis by a (SHIMADZU Prestige-21, Japan) instrument [24]. The solutions were diluted to a ratio of 1:4 with 96% alcohol and centrifuged at 4000 rpm for 10 min. The wavelengths were read before adding silver nitrate, 2 hours after adding silver nitrate and 24 hours after adding silver nitrate. Then, the obtained solution was centrifuged at 4000 rpm for 20 min; then, it was once washed with 96% alcohol and twice with deionized water. Finally, the biosynthesized AgNPs were dried in a dark place and were properly stored.

Preparation of GSH-coated AgNPs

GSH aqueous solution (0.5%, 20 cc) was mixed with (0.01 g) AgNPs. The mixture was sonicated for one hour and placed on the shaker for five hours [22]. The precipitate was separated through centrifugation at 4000 rpm for 5 min and placed in a dark place to dry for a week.

Characterizations of AgNPs and GSH-coated AgNPs

The morphology of AgNPs and GSH-coated AgNPs were investigated by Scanning Electron Microscopy (Philips XL30 SEM, Netherlands). For XRD studies, dried nanoparticles were coated on XRD grid and the patterns were recorded by using (Panalytical X'Pert PRO, Netherlands) instrument. The functional groups on the surface of AgNPs and GSH-coated AgNPs were determined by FTIR analysis using (FT_IR 8400S Shimadzu, Japan) equipment. Dynamic light scattering (DLS) measurements were performed, using a Malvern Zeta size analyzer instrument (ZEN 3600, UK) to investigate the size distribution pattern and average particles size of the nanoparticles. Besides, synthesized nanoparticles were tested to measure Carbon (C), Hydrogen (H) and Nitrogen (N), using CHN analyzer (Biotek Cytation 3, USA).

Biofilm Formation in the 96-Well Microtiter Plate

Microtiter plate method is a qualitative method for biofilm detection described by Christensen et al [25]. *P. Aeruginosa* isolates were grown on trypticase soy agar with 2% glucose at 37°C for 24 hours. The culture was diluted in trypticase soy broth in test tubes and adjusted to 0.5 McFarland standards. Then, 200 µL of bacterial suspension was transmitted to every three wells of a sterile 96-well plate and were incubated overnight at 37 °C. A well containing only TSB was regarded as negative control. After incubation, wells were washed with phosphate buffer saline and dried. Afterward, the plates were stained with crystal violet (1%) for 5 minutes, rinsed with distilled water and air dried. Finally, 100 µL of 33% glacial acetic acid was added to each well and the OD was measured at 570 nm, using an ELISA reader (Biotek Cytation3, USA). According to the criteria of Stepanovic et al, the biofilm formation was interpreted as shown in Table 1.

Table 1. Explanation of biofilm formation. Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control

Average OD value	Biofilm production
≤ ODc / ODc < ~ ≤ 2x ODc	Non / Weak
2x ODc < ~ ≤ 4x Odc	Moderate
> 4x Odc	Strong

MIC Determination of GSH-coated AgNPs and Cip

Susceptibility tests with GSH-coated AgNPs and Cip were done, using a standard broth microdilution method following Clinical and Laboratory Standards Institute guidelines (CLSI, 2017). Two 96-well microtitre plates were used for nanoparticles and antibiotic. Aliquots of 100 ml of the Mueller–Hinton (MH) broth was added into the wells of each two plates. Subsequently, in one of the plates, 100 µL of the serially diluted antibiotic was added in the first well and in another plate 100 µL of the serially diluted nanoparticles was added. The bacterial suspension was then added to them (100 µL) and the plates were incubated overnight at 37°C.

The MIC of Cip was described according to CLSI, 2017 (table 2). All tests were done in duplicate and also the positive control wells (medium + *P. aeruginosa* ATCC827852) and negative control (medium + antibiotic and nanoparticle) were used.

Table 2. The MIC of Cip (CLSI, 2017)

Antibiotic	MIC of Cip (µg/mL)
Susceptible	1≤
Moderate	2
Resistant	≥4

Synergistic MIC of GSH-coated AgNPs Combined with Cip

To investigate the synergistic effect of GSH-coated AgNPs and Cip, bacterial cells were grown to form biofilms and then treated with 1/2 and 1/4 MIC of GSH-coated AgNPs and Cip alone and then, together with combinations [26].

Statistical Analysis

The data were statistically analyzed by SPSS software (version 17). Also chi-square test was used.

RESULTS

Characterization of Green Synthesized AgNPs and GSH-coated AgNPs

The color change of the plant extract (fig. 1) during the reaction indicated the formation of AgNPs, which was confirmed by UV–Vis spectrophotometry.

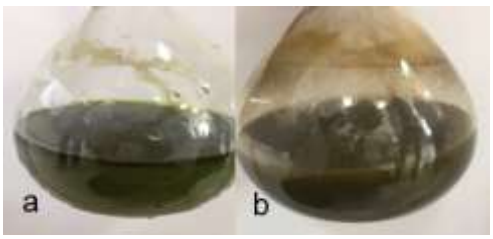


Figure1. a. aqueous solution of leaf extract b. Aqueous solution of AgNPs in leaf extract

UV–Vis spectral analysis was done in a wavelength range between 350 and 800 nm. The reduction of silver ions (Ag+) to silver Nanoparticles was monitored through measuring the UV–Vis spectrum of the extracts before adding silver nitrate, 2 hours after adding silver nitrate and 24 hours after adding silver nitrate. The results showed maximum absorbance at 495 nm (fig. 2). Bashir et al., in their study of biosynthesis of AgNPs using *Eucalyptus camaldulensis* leaf extracts and their properties, reported a maximum absorption of 425 nm [27].

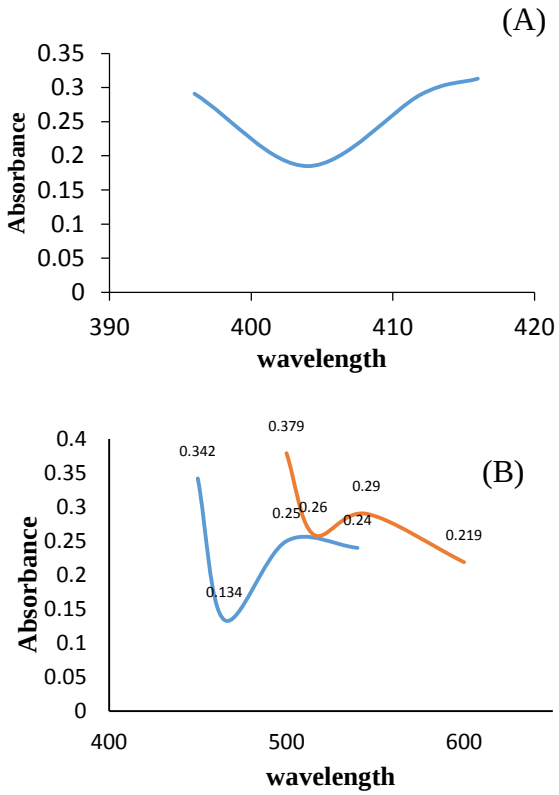


Figure 2. (A) UV–Vis absorption spectrum of the extract. (B) UV–Vis absorption spectrum of the extract 2 hours after adding silver nitrate (blue) and 24 hours after adding silver nitrate (orange).

The images of Scanning Electron Microscopy represent the spherical structure of silver nanoparticles with a smooth surface as it was reported by Velhal et al., in their study in 2015, titled "*Taguchi Design for Parameter Optimization of Size-Controlled Synthesis of Silver Nanoparticles*" [28] Besides after modifying the surface, their spherical structure did not change (figure3).

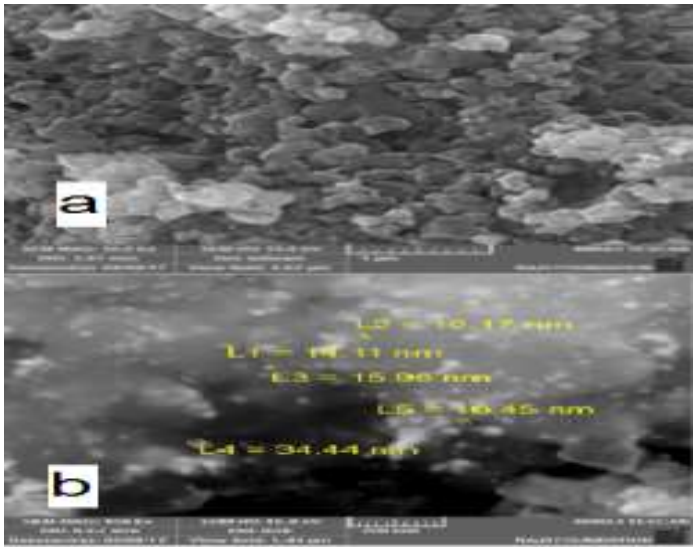
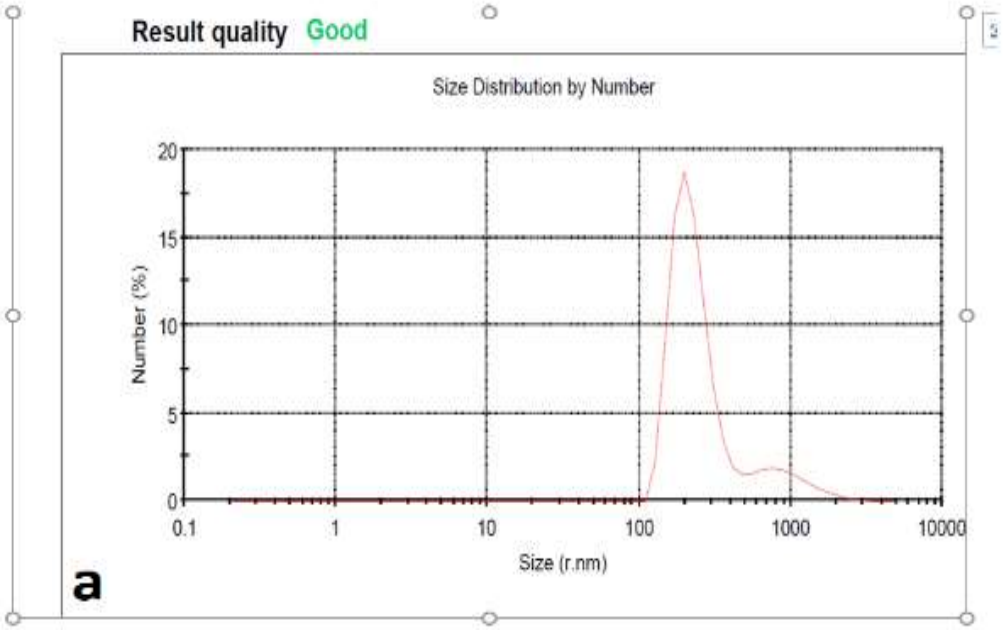


Figure 3. SEM images of a) AgNPs and b) GSH-coated Ag

According to the information obtained from the DLS measurements, the Z-average and Pdi of AgNPs were 747.4 and 0.44 respectively (fig. 4, a). Figure. 4, b shows the average particle size of AgNPs after GSH-coating



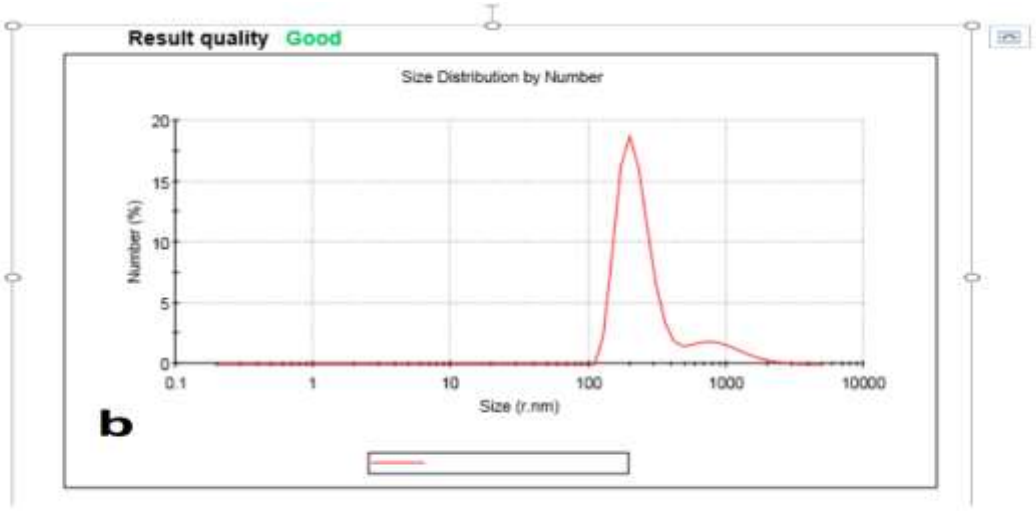


Figure4. DLS spectrum of size distribution observed for a) AgNPs and b) GSH-coated AgNPs.

Figure5 shows XRD patterns of AgNPs. Crystalline silver nanoparticles at 111, 200, 220 and 311, showed peaks at values of 38/16, 46/26, 64/52 and 76/78 respectively, which is perfectly in line with standard AgNPs (JCPDS file no. 04-0783). Also, the XRD pattern of GSH-coated AgNPs confirmed its formation.

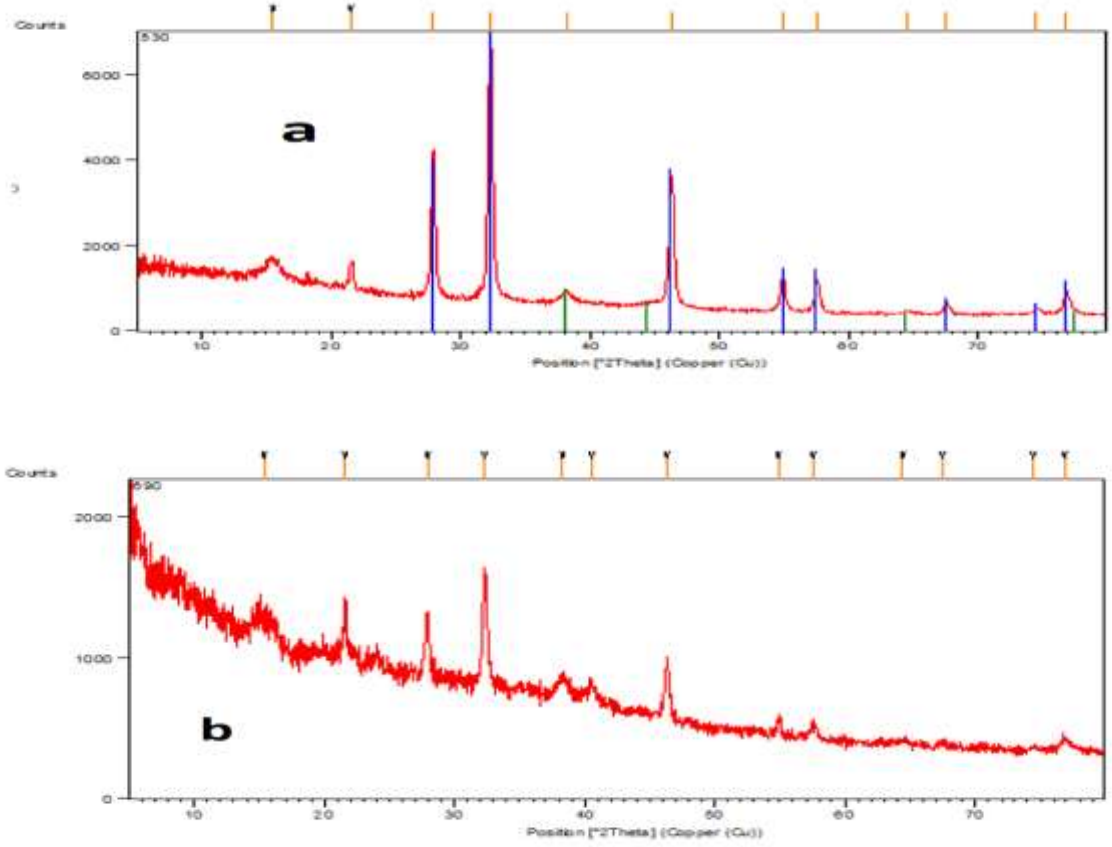
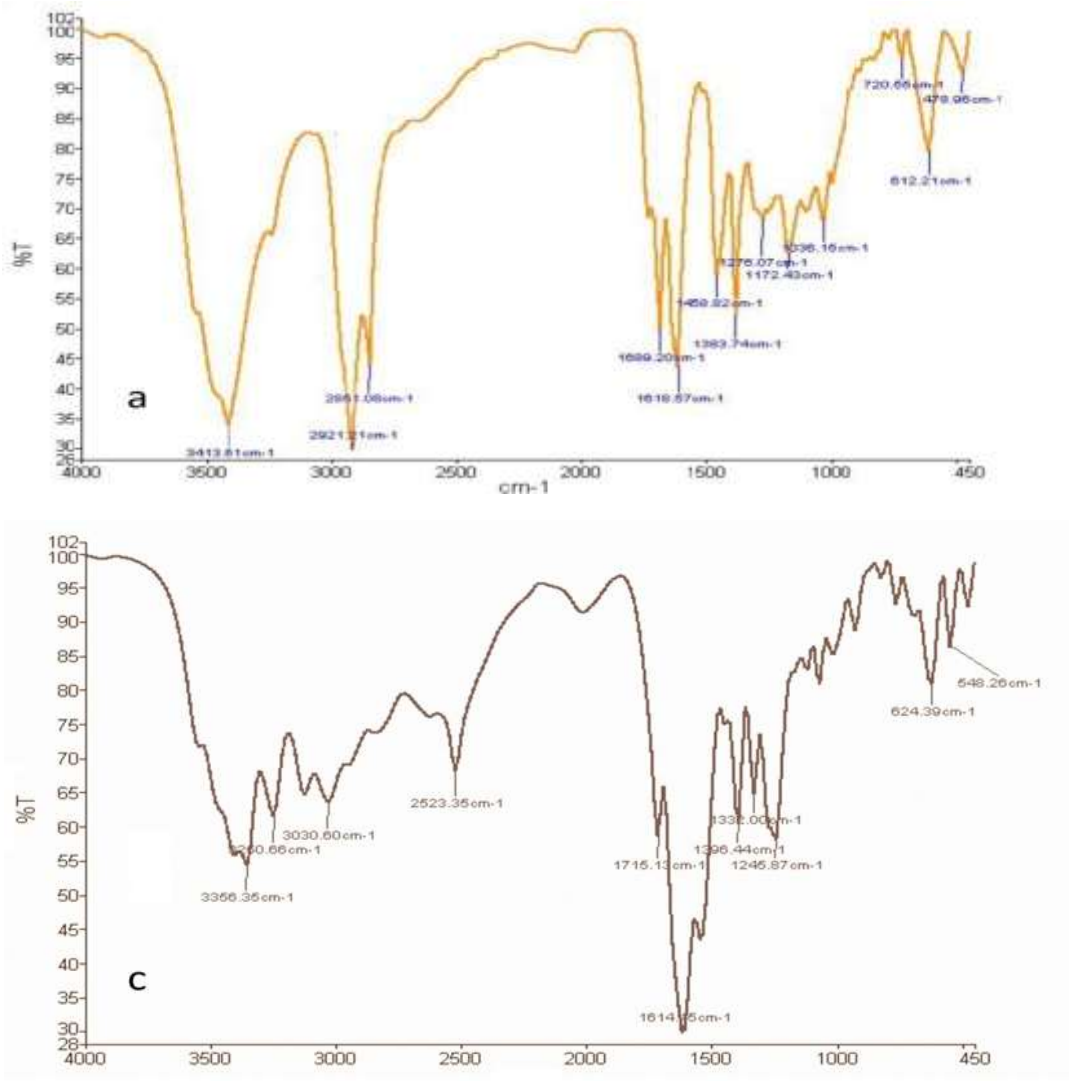


Figure5. XRD spectra of a) AgNPs and b) GSH-coated AgNPs

The FTIR peaks at 720 cm^{-1} and 624 cm^{-1} indicate the presence of nanoparticles. Peaks observed at 1172, 1383, 1618, 1458, 3413 cm^{-1} are assigned to the functional groups C-O, COO, NH, CH_2 and OH respectively which are related to plant extract residues. In the study of the glutathione spectrum, peaks at 1384, 1616, 3556, 2533 cm^{-1} are related to COO, CH, and OH groups respectively. Also, the observed peak in 2533 cm^{-1} is related to the SH group on the glutathione. In the study of GSH-coated AgNPs spectrum, the disappearance of this peak represents the involvement of this functional group in the bond with nanoparticles (fig. 6).CHN analysis was performed to confirm the formation of GSH-coated AgNPs. According to the results, the amount of carbon was 37.27%, the hydrogen content was 11.88%, and the nitrogen content was 0%. Carbon is a functional group in association with nanoparticles; since nanoparticles alone do not have hydrogen and carbon, their presence indicated the formation of GSH-coated AgNPs.



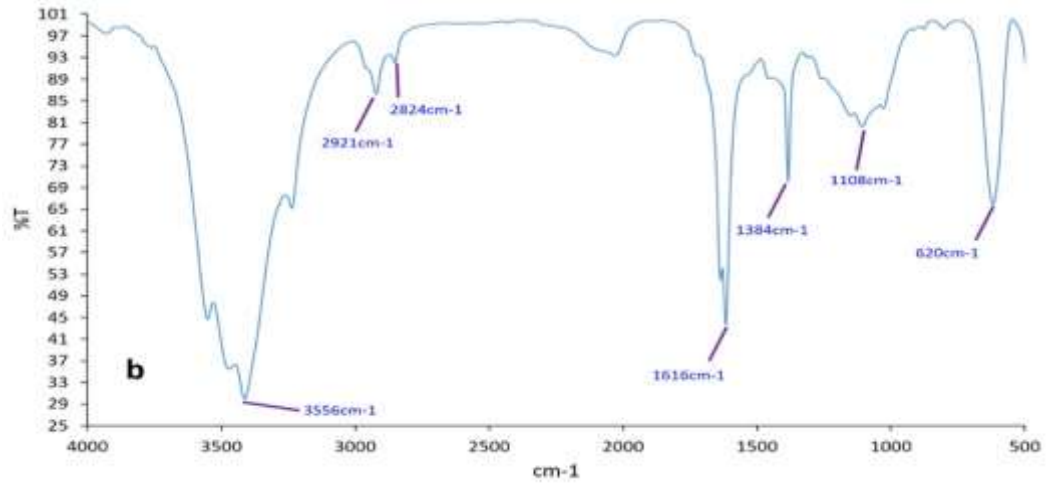


Figure6. Fourier transform infrared spectra of (a) AgNPs (b) GSH and(c) GSH-coated AgNP

Biofilm Formation in the 96-Well Microtiter Plate

All of the 50 studied isolates were classified into four categories: strong, moderate, weak and non-biofilm producers, using microtitre plate method. The 24 isolates were selected as a strong biofilm producer for further studies. Optical density cut-off method was used to examine the results. Fig. 7 shows the abundance of biofilm types.

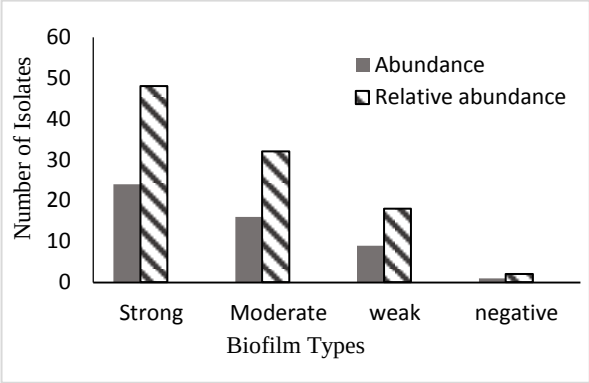


Figure7. abundance of Biofilm types

MIC of Cip and GSH-coated AgNPs

Table. 3 shows the MIC results of Cip based on CLSI 2017 guidelines. The MIC of GSH-coated AgNPs is demonstrated in the table. 4

Table 3. P. aeruginosa resistance to ciprofloxacin antibiotics

Numbers of isolates	
14	**Susceptible**
1	**Moderate**
9	**Resistant**

Table 4. *P. aeruginosa* resistance to GSH-coated AgNPs

Concentration µg/ml	**Numbers of isolates**
1	-
2	-
4	-
16	-
32	6
64	11
128	7
256	-
512	-

Synergistic Effect of GSH-coated AgNPs and Cip with Concentrations of 1.2 and 1.4

Biofilm formation of *P. aeruginosa* was investigated after growth with 1/2 and 1/4 MIC of GSH-coated AgNPs and Cip.The results indicated that GSH-coated AgNPs and Cip had an inhibitory activity (1/2 MIC) on biofilm formation of 41.6 % and 12.5% of *P. aeruginosa* isolates, respectively. The results showed that 8.4% of isolates did not form biofilms with 1/4 MIC of GSH-coated AgNPs and Cip. While combinations of 1/2 and 1/4 MIC of GSH-coated AgNPs and Cip showed more inhibitory activity than either of them separately (87.5% and 83.4% respectively).

Taglietti et al. examined the antibacterial activity of Glutathione-coated AgNPs on some gram positive and gram negative bacteria and concluded that the coating of AgNPs with glutathione is not suitable and has less antibacterial activity, while in the present study, the combination of Glutathione-coated AgNPs and ciprofloxacin had a good antibacterial activity on the biofilm formation of *Pseudomonas Aeruginosa* [30]. Figure 8 compares the effects of GSH-coated AgNPs and Cip different concentrations and their synergistic effect.

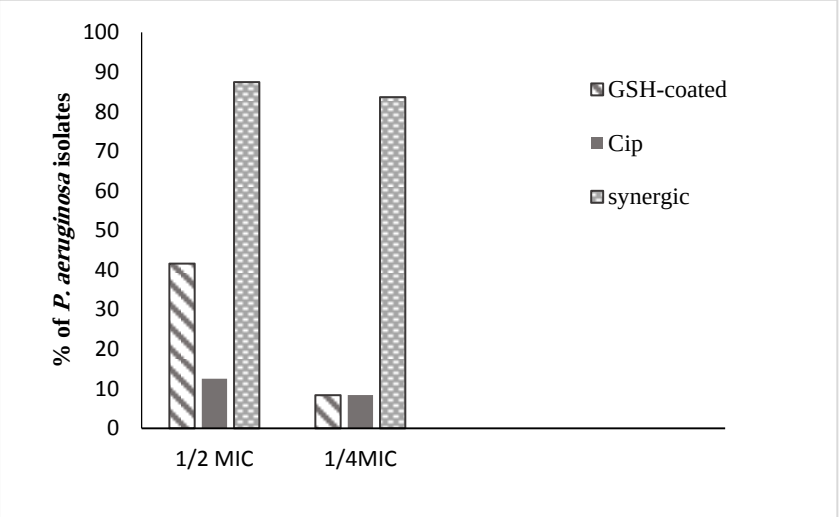


Figure 8. Graph of comparison of the effects of GSH-coated and Cip different concentrations and their synergistic effect

DISCUSSION

In developing countries, the incidence of infectious diseases with a high level of antibiotic resistance is increasing. *Pseudomonas aeruginosa* is an opportunistic pathogen that causes various infections, especially in hospitalized patients. An important indicator of this bacterium is its antibiotic resistance. Factors that affect antibiotic resistance include antibiotic degradation enzymes, multiple pumps, and biofilms.

Finding new agents that could weaken microbial biofilm formation can generate a great change in the treatment of infections with microbes such as *P. aeruginosa*, whose pathogenic potential depends on the formation of biofilms in the host.In 2005, Li et al. showed that antibiotics such as amoxicillin in combination with silver nanoparticles against *E. coli* have synergic activity and effectively inhibit biofilm growth [26]; consequently, AgNPs and antibiotics form a nucleus of nanoparticles surrounded by antibiotic molecules which can be an effective and novel antibacterial system [26].

Ramezani et al. studied the antibacterial effect of some antibiotics and iron oxide nanoparticles on the biofilm formation by *Pseudomonas aeruginosa*. In 20 isolates that were influenced by the combination of iron oxide nanoparticles with antibiotics, 11 isolates did not produce biofilms while in the present study, 3 0f 21 isolates produced biofilm, indicating the synergistic effect of ciprofloxacin and AgNPs [29].Further researches are needed on the efficacy of other metal nanoparticles with various modifications against *Pseudomonas aeruginosa* and on their combined effects and other antibiotics to find the best system.

CONCLUSION

The present study illustrates that the combination of GSH-coated AgNPs and Cip has a synergistic inhibitory activity on *P. aeruginosa* biofilm formation. This study provides helpful insights for improvement of new antimicrobial agents.

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The authors declare no conflict of interest

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