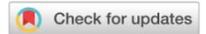




Determination of the antagonistic efficacy of silver nanoparticles against two major strains of *Mycobacterium tuberculosis*



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Abstract: Tuberculosis (TB) is considered one of the most prominent diseases across the globe. This present study aims to inspect the impact of silver nanoparticles (AgNP) against *Mycobacterium tuberculosis*, which is the causative vector of TB. The efficacy of the AgNP was conducted based on the minimum inhibitory concentration (MIC) of the AgNPs through microplate Alamar blue assay. The preparation of the AgNPs involved chemical synthesis. The state and the size of the AgNPs were determined and confirmed by using ultraviolet-visible (UV-Vis) absorption spectroscopy, X-ray diffraction (XRD) spectroscopy, and Transmission electron microscopy (TEM). This study evaluated two strains: *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis*. In addition, another multiple drug-resistant *Mycobacterium tuberculosis* strain was also considered in this study, along with the clinically obtained isolates from *Mycobacterium tuberculosis* H37Rv and *Mycobacterium tuberculosis bovis*. The synthesized nanoparticles were found to be tetrahedral in shape with an average particle size of 45±3 nanometre (nm). The obtained results indicated that the proliferation of all the strains (two reference strains and one MDR strain) was resisted by the action of the synthesized AgNPs. The MIC of the MDR strain was noted within the range of 2-12 µg/ml, whereas the MIC for the *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* was noted in the range of 2-14 and 3-30 µg/ml, respectively. Accordingly, this study proposed a novel approach to combat tuberculosis, which is considered a global threat to humankind, indicating the present study's novelty.

Introduction

The world is suffering from various critical problems (Roy and Ray, 2019; Roy and Ray, 2020). Among such problems, environmental issues, water scarcity, and human health-related issues are of notable cognizance (Roy et al., 2022; Roy et al., 2022). The issues related to human health are of notable significance in this regard (Roy et al., 2022; Song et al., 2022; Su et al., 2022).

Microorganisms and their associated products (like enzymes) are regarded as the causative agents for such disorders (Ghoshal et al., 2022; Vipparla et al., 2022). Tuberculosis is considered one of the major causes of global deaths. Among various causative agents of TB, *Mycobacterium bovis* and *Mycobacterium tuberculosis* are notable (Reddington et al., 2011). These two vectors have enough potential to impart TB into any humans and



animals with high efficacy. The reports by the World Health Organization (WHO) indicated that TB causes more than a few million deaths annually throughout the globe. In addition to that, around 10 million people get affected by TB, which in turn causes serious health concerns. The *Mycobacterium bovis* has been reported as a global threat since it causes TB in cattle (WHO, 2016). Such infections are prevalent in more than 130 countries. It has been reported that *Mycobacterium bovis* is mostly zoonotic, and its presence was not discovered within humans. However, such infection was found to spread to humans through the consumption of milk obtained from the infected cattle. The cardinal bypass route to prevent the spreading of such infections was to pasteurize the milk (Esch et al., 2013).

The elongated time period of the treatment of tuberculosis, along with the increased frequency of infection and side effects of the drugs used to suppress tuberculosis in turn, indicated that significant research attention needs to be paid to finding alternative treatment strategies to combat tuberculosis. The over usage of conventional drugs for suppressing tuberculosis for a prolonged time has also resulted in the development of multi-drug resistance Tuberculosis strains which has also attracted the attention of scientists worldwide to combat such a critical scenario (Ducati et al., 2006).

The enhanced susceptibility of the *Mycobacterium bovis* and *Mycobacterium tuberculosis* within the global population has become a threat, especially for developing countries. In this regard, the antagonistic efficacy of the various metal-based nanoparticles has gained notable research cognizance. From time immemorial, silver has been used as an antimicrobial agent. However, with the emergence of the medical field, the application of silver in the medical domain has reduced drastically (Basak et al., 2020).

The literature indicated that the antagonistic impact of metallic silver can be improved to several folds by reducing its size to nano levels through the reduction of their surface areas. Such size reduction may involve the alteration of their physical and chemical properties to a notable extent owing to their volume-to-surface ratio. Previously conducted studies indicated that the silver nanoparticles within the ranges of 10-100 nm size displayed notable cognizance towards the inhibition of the growth of the TB-causing agents even those that have developed resistance against multi-drug resistance (Raza et al., 2021). The cardinal notion behind such action may be corroborated due to the high empathy of silver towards nitrogen and sulfur, which are present in plenty within the cell membrane proteins of the microorganism. The

nanoparticles composed of silver can react with the amino acids containing sulfur located either inside or outside the cell membrane, thereby affecting the viability of the bacterial cell. Furthermore, silver in the cationic form has been reported to be released from their respective nanoparticle conjugates and interact with the phosphorous moieties located within the DNA of the microorganisms, which in turn results in the hindrance of the DNA replication, or along with the proteins-rich in the sulfur moiety. All such actions result in the inactivation of the enzymatic function, and as a result, the infectious activity of the respective microbe ceases (Joshi et al., 2020).

Accordingly, the objective of the present study was to evaluate the minimum inhibitory (MIC) of the synthesized silver nanoparticles against two different TB-causing vectors (*Mycobacterium bovis* and *Mycobacterium tuberculosis*) through the determination of microplate Alamar blue assay. The authors truly believe that adopting such a strategy will benefit the various stakeholders and result in a better diagnosis of Tb, which is considered one of the major threats to the existence of humankind.

Methodology

Bacterial Strain

The two different bacterial strains used in this study were obtained from ATCC. One reference strain of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Furthermore, ten clinical isolates were also obtained for *Mycobacterium bovis* (accession number MB1–MB10). Two of the isolates belonged to SB0268. Ten more clinical isolates for *Mycobacterium tuberculosis* (accession number MT1 – MT10), among which two isolates belonged to the type SB0223.

One of the clinical isolates of the multi-drug resistant strain of *Mycobacterium tuberculosis*, which was diagnosed through the simplified version indirect proportion method against 40 µg/ml of rifampicin, 0.2 µg/ml of isoniazid, 4 µg/ml streptomycin, and 2 µg/ml ethambutol by strictly adhering to the standard literature reported protocols.

Furthermore, the minimum inhibitory concentrations values of rifampicin, isoniazid, streptomycin, and ethambutol were determined against the ten different clinical isolates of the *Mycobacterium bovis* and *Mycobacterium tuberculosis* through a simplified version using indirect proportion methodology. The minimum inhibitory concentration of streptomycin was noted within the range of 6-21 µg/ml, 0.6-21 µg/ml for ethambutol, and 5-16 µg/ml for rifampicin. All the strains

were kept maintained on the Lowenstein-Jensen medium, which was subcultured freshly before every evaluation. The chemicals and media were procured from Sigma Aldrich.

Silver Nanoparticles Synthesis

The synthesis of the silver nanoparticles was carried out through the method of chemical reduction. The synthesis was carried out by using polyvinylpyrrolidone (of PVP having a molecular weight of 40000) and sodium borohydride (NaBH_4). The PVP was used as the stabilizing agent, whereas the NaBH_4 was used as the reducing agent. 0.5 ml 30 millimolar trisodium citrate in the form of an aqueous solution was taken in a flask along with 50 ml of deionized water. Additionally, 0.5 ml, 5 mM silver nitrate was added to their aqueous solution. Subsequently, freshly prepared 50 mM, 0.5ml NaBH_4 in the form of the aqueous solution was added immediately. We waited until the resulting mixture displayed a light pale-yellow colour. After another 45 seconds, the aqueous solution of PVP was added. We further waited until the colour of the resulting suspended solution turned into a darker yellowish colour (El Hotaby et al., 2017).

Characterization Silver nanoparticles

The synthesized nanoparticles' morphology and size were determined using a transmission electron microscope (TEM). The make and model of the instrument (TEM) is JOEL 1210, Joel Ltd., Tokyo, Japan). The optical absorption feature of the colloidal silver was determined by using Ocean Optics USB2000+VIS-NIR fiber-optic spectrophotometer. Finally, the X-ray diffraction analysis of the synthesized nanoparticles was determined by considering a minute quantity and drying them on a quartz plate.

Preparation of Silver nanoparticles

The two-fold serial dilution of the synthesized nanoparticle was prepared within the range of 0.25-250 $\mu\text{g}/\text{ml}$.

Determination of the Minimum Inhibitory Concentration of the synthesized Silver Nanoparticles by Adopting Microplate Alamar Blue Assay

The two different bacterial strains evaluated in this study were assessed for MIC through microplate Alamar blue assay. The two respective bacterial inoculums were

taken out from the Lowenstein-Jensen media tube. This was added to Middle brook 7H9 broth media (5ml) which

comprised 0.5% glycerol and 0.1% casitone and was supplemented with albumin, dextrose, oleic acid, and catalase (7H9-S) (Becton Dickinson Microbiology Systems, USA). A few glass beads were also into the resulting mixture.

After incubating for seven days at $37\pm 2^\circ\text{C}$, the mixture tube was vortexed for a time span of 180 seconds and was allowed to rest for 20 minutes. The resultant supernatant was transferred to another fresh test tube, and the resultant mixture's turbidity was adjusted to the scale of 1 on a McFarland metric of a nephelometer. The resulting suspension was thereafter diluted 1:6 in 7H9-S and was used as the test inoculum (Guzel et al., 2019).

The microplate Alamar blue assay was carried out by following the standard literature-reported protocol. For this purpose, 200 μl of autoclaved de-ionized water was poured into a sterile 96-well microtitre plate which was procured from Becton Dickinson Labware, USA. For each respective strain evaluated, 100 μl 7H9-S containing nanoparticle dilution was distributed to the container wells and inoculated with 100 μl mycobacterial suspension in the diluted form. Thereafter, the resulting plates were sealed and covered with parafilm and were kept in incubation for five days at $37\pm 2^\circ\text{C}$. After that, 25 μl freshly prepared 1:1 mixture of 10% Tween 80 and AB reagent was added into the resulting solution and further incubated for 24 hours. The presence of blue colour in the resulting mixture indicated no bacterial growth, whereas the appearance of the pink colour indicated growth of the microorganisms. The MIC was evaluated as the minimum lowest concentration of the drug, preventing the colour change from blue to pink (Collins et al., 1997). As the control set, standalone media, AB + media, drug dilution + AB + media, and bacterial cell + media + AB were taken. All these experiments were carried out within a biological safety cabinet.

Results

Silver nanoparticles: Characterization

The silver nanoparticles evaluated in this work were synthesized by following the method of chemical reduction. The transmission electron microscopic images have been provided in Figure 1, indicating that the mean radius of the synthesized silver nanoparticles was within the range of 40-60 nm. Moreover, it was also discovered that the synthesized nanoparticles were mostly spherical and tetrahedral in shape.

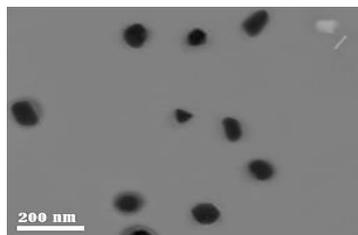


Figure 1. Images obtained from Transmission Emission Microscopy of the synthesized Silver Nanoparticles.

Impact of the silver nanoparticles on the mycobacterial species

In order to determine the efficacy of the synthesized silver nanoparticles against the two mycobacterial strains (through in-vitro analysis) at various concentrations, within the range of 0.25–250 μ l adopting minimum inhibitory concentration AB assay. The obtained results indicated the effective antagonistic activity of the silver nanoparticles against the two mycobacterial strains evaluated in this study. The minimum inhibitory concentration of the synthesized silver nanoparticles against the reference strain *Mycobacterium bovis* was 4 μ l/ml and 1 μ l/ml against *Mycobacterium tuberculosis H37Rv*. Accordingly, it was depicted that the minimum inhibitory concentration range against the clinical isolate of *Mycobacterium bovis* was found within the range of 3–30 μ l/ml. The minimum inhibitory concentration for the clinical isolates of *Mycobacterium tuberculosis* against the synthesized silver nanoparticles was noted within the range of 2–15 μ l/ml.

Discussion

Antibacterial agents play a vital role in various industries, such as textile, medicine, water purification, and food & beverage processing. (Makvandi et al., 2021). Nanomaterials have been found to play a vital role as antimicrobial agents since they have been reported to bind and disrupt the integrity of the bacterial cell membrane. The mechanism of such disruption involves binding the silver ions within the nitrogen, oxygen, and sulfur moieties present within the amino acids of the membrane protein. The membrane disruption results in the release of the contents present within the cells leading to their death (Gupta et al., 2019).

The literature indicated that nanoparticle-based conjugates had been reported to diagnose tuberculosis. These conjugates can mitigate various problems, such as the bioavailability of the drugs, the requirement of frequent dosage, the appearance of multi-drug resistance, and imparting of better bactericidal activity. Accordingly, it was evident that prevention and cutting tuberculosis with silver nanoparticles have notable cognizance (Dahiya et al., 2020). A lot of work has been carried out to determine the antagonistic efficacy of silver

nanoparticles against different mycobacterium species. However, no work has been done to determine the impact of silver nanoparticles on *Mycobacterium bovis*, which indicates the novelty of the present work.

The present study indicated that the different strains of mycobacterium species have different degrees of susceptibilities against the synthesized silver nanoparticles. The minimum inhibitory concentration of the synthesized nanoparticles had efficacy against the *Mycobacterium bovis* and *Mycobacterium tuberculosis*. The MIC values obtained for *Mycobacterium tuberculosis* and *Mycobacterium bovis* were recorded as 4 and 16 μ l/ml, respectively. This observation, in turn, indicated the higher level of antibiotic resistance for *Mycobacterium tuberculosis* as compared to *Mycobacterium bovis*. The extended observation noted from the obtained results was that an elevated concentration of silver nanoparticles was required to neutralize *Mycobacterium bovis* as compared to *Mycobacterium tuberculosis*. Accordingly, the obtained results indicated that the synthesized silver nanoparticles have enough potential to inhibit the growth of the various strains of Mycobacterium species.

The literature reported that the efficacy of the silver nanoparticles banks on several parameters to suppress the growth of microorganisms. And the optimization of the different process parameters to define the optimum efficiency. A few of the parameters that notably impact the antagonistic property of different microorganisms are particle, size, surface area, and the number of silver ions released in the aqueous state (Islam et al., 2018). The smaller particle size, lower surface area, and potential to release more silver ions in the form of Ag^+ collaboratively exhibit better anti-microbial efficacy. A recently conducted study indicated that in an in-vivo experiment conducted with 65 white mice were infected for two weeks with the virulent strain of *Mycobacterium tuberculosis*. After that, the animals were treated with silver nanoparticles and isoniazid. The experiments' findings indicated that the infected mice' survival rates were around 95% (Krishna et al., 2017). The previously conducted studies indicated that the therapy adopted for tuberculosis is daunting. Generally, tuberculosis in both animals and humans is treated with a similar drug. However, this solution is not always considered a practical solution. Accordingly, the results obtained in this study proposed a novel approach to cure tuberculosis in both animals and humans. But more detailed research regarding defining the mechanism of action of the silver nanoparticles along with the optimization of the various process parameters toward neutralizing the causative

agent of tuberculosis may be regarded as the future scope of this study (Dey et al., 2022; Roy et al., 2022; Roy and Ray, 2022).

Conclusion

The present study indicated that the silver nanoparticles synthesized in the laboratory have ample potential to suppress the growth and propagation of the two different strains of tuberculosis, namely *Mycobacterium bovis* and *Mycobacterium tuberculosis*. However, more elaborative research needs to be paid to commercializing those products. In addition, this study should be conducted with a greater sample size to get insights regarding their potential as an anti-microbial agent. The authors truly believe that this study will benefit various stakeholders and open new avenues of treatment of tuberculosis.

Conflicts of interest

The reporting and publication of this study do not include any conflicts of interest for the authors.

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