ORIGINAL ARTICLE

# Cytotoxicity and antibacterial activity of actinomycetes-mediated biogenic silver nanoparticles against methicillin-resistant *Staphylococcus aureus* (MRSA)

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## ABSTRACT

***Background:*** *MRSA is one of the most life-threatening pathogens, thus its prevention has become a crucial public health concern.* ***Objectives:*** *This study aims to determine the prevalence of MRSA among coagulase-positive staphylococci and evaluate the cytotoxicity and anti-MRSA efficacy of biogenic silver nanoparticles (AgNPs).* ***Methodology:*** *Phenotypic detection of MRSA among 220 coagulase-positive Staphylococcus spp. isolates was investigated by CHROMagar™ MRSA, and their identification was confirmed by MALDI-TOF and detection of nuc and mecA genes. Furthermore, the actinomycetes-mediated biosynthesized AgNPs were characterized, and their cytotoxicity and anti-MRSA potential were assessed.* ***Results:*** *Of 220 isolates, 92 (41.8%) were presumptively identified as MRSA by CHROMagar MRSA, however, MALDI-TOF discriminated them into S. aureus, S. pseudintermedius, S. cornubiensis, and S. delphini. Results of molecular characterization revealed the presence of nuc and mecA genes in all confirmed MRSA isolates. In addition, a promising AgNPs-producing Streptomyces virginiae SNPPA6 was isolated, identified and utilized for extracellular biosynthesis of spherical AgNPs with an average size of 11.18 nm. The characterization of the AgNPs by XRD and FTIR revealed their crystalline nature and the presence of proteinaceous capping agents. The biogenic AgNPs exhibited promising anti-MRSA activity with MIC values ranging from 4 to 64 µg/mL. Cytotoxicity assessments using MTT assay indicated the cytotoxic impact of AgNPs on MDA-MB-231, A549 and HSF cell lines with $IC_{50}$ values of 24.5, 29.2 and 36.3 µg/mL, respectively.* ***Conclusion:*** *Our findings highlighted the potential application of the biogenic AgNPs produced by S. virginiae SNPPA6 as a promising candidate for fighting MRSA at non-cytotoxic concentrations.*

## INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a pathogenic bacterium that is resistant to multiple drugs and is responsible for causing various types of infections.[1] Currently, MRSA is regarded as one of the most life-threatening in humans, causing infections that have gained significant importance in both hospital and community environments, and their prevention has become a crucial public health concern.[2] Given the significant impact of antibiotic resistance in MRSA on public health, it is imperative to develop new and inventive approaches for fighting their infections. Recently, there has been a considerable emphasis on noble metal nanoparticles owing to their unique physical and chemical characteristics. Among the metallic nanoparticles, silver nanoparticles (AgNPs) have grabbed the scientific interest in several sectors, especially in the biomedical fields as effective anticancer, anti-inflammatory, and antimicrobial agents; as well as drug delivery systems.[3] The superior antibacterial activity against various bacterial pathogens including MRSA is well documented and their antibacterial mechanisms thought to be attributed to disruption of membrane permeability and integrity, induced oxidative stress and DNA fragmentation.[4–6] It has been assumed that AgNPs produced using microbial- and plant-mediated extracts provide advantages since they are cost-effective, easy to obtain, energy efficient, and less toxic, compared with those produced by chemical and physical approaches.[7] Although the biosynthesis of AgNPs by various microorganisms and plant extracts has been described in numerous literature, the biosynthesis via actinomycetes could provide additional beneficial merits to the

produced AgNPs. Actinomycetes are bacterial members recognized for producing bioactive substances, particularly antibacterial and anticancer agents that can be incorporated into the produced AgNPs as capping agents, providing unique antimicrobial and anticancer properties of the bioinspired AgNPs.[8–10]

This study aims to investigate the cytotoxicity and anti-MRSA activity of actinomycetes-mediated AgNPs. Despite the potential benefits of using these nanoparticles in combating drug-resistant bacteria, it is crucial to understand their potential adverse effects on human health. By evaluating both the cytotoxicity and antimicrobial properties of these nanoparticles, we hope to provide valuable insights for future research and development in the field of nanotechnology. Here in, we address extracellular biosynthesis and characterization of AgNPs produced by *Streptomyces virginiae* SNPPA6 with the assessment of their efficacy as an antibacterial agent against MRSA with an insight into their potential cytotoxicity.

## METHODOLOGY

The media utilized in this investigation were purchased from Condalab (Madrid, Spain). All fine chemicals were obtained from Sigma-Aldrich.

**Identification of MRSA**

Two hundred and twenty non-repetitive coagulase-positive staphylococcal isolates obtained from the NCI were screened for MRSA by the chromogenic MRSA agar method.[11] In brief, a loopful of each isolate was inoculated onto CHROMagar™ MRSA and incubated at 37 °C. After 24 h, mauve-colored colonies were preliminarily identified as MRSA, picked up and identified by MALDI-TOF/MS following the previously described protocol.[12] The molecular confirmation of MRSA was conducted by detecting *nuc* and *mecA* genes; the genomic DNA was extracted following rapid lysis method, and the polymerase chain reaction (PCR) was conducted using previously described specific primers and amplification protocols.[13,14]

**Isolation and identification of AgNPs-producing Actinomycetes**

To isolate actinomycetes, sediment samples were collected from the River Nile in Giza, Egypt, and then mixed with a sterile saline solution (0.9% NaCl). Afterward, 100 µL portions of the diluted soil suspensions were uniformly spread over the surfaces of humic-acid–vitamin agar that contained nalidixic acid (25 µg/mL) and cycloheximide (25 µg/mL).[15] The plates were incubated at 28 °C for 7 to 14 days. Afterward, actinomycetes colonies with typical morphology were chosen and purified using the sub-culturing method.

To screen the ability of actinomycetes isolates to produce AgNPs, each isolate was inoculated in ISP2 broth and incubated at 28 °C, 200 rpm for 96 h. Subsequently, the culture was centrifuged at 10,000 x*g* for 15 min and the supernatant was collected. The supernatant was filtered by passing through a filter with a pore size of 0.22 µm and the cell-free extract was added to an equal amount of 2 mM $AgNO_3$ solution. The biosynthesis of AgNps was assessed by observing the color change to brown after incubating at 28°C for 96 h with shaking at 200 rpm in the absence of light. The earliest indication of AgNPs biosynthesis was the visual observation of the reaction mixture's color change to brown, which was further validated by UV-Vis absorbance. Consequently, a proficient isolate designated SNPPA6 was selected and identified by phylogenetic analysis based on the 16S rRNA gene according to the previously mentioned method.[16,17]

**Biosynthesis of AgNps**

The biosynthesis of AgNPs was achieved by cultivating the isolate SNPPA6 in ISP2 broth at 28 °C for 96 h with shaking (200 rpm). After centrifugation at 10,000 xg for 15 min, the rsultant supernatant was filtered using 0.22 µm filters and mixed with the same volume of 2 mM $AgNO_3$. Following an incubation period of 96 h at 28 °C in the dark, the reaction mixture was centrifuged at 40,000 xg for 15 min. Afterward, the AgNPs pellets were suspended again in ultrapure water and subjected to centrifugation at 40,000 xg for 15 min to eliminate unwanted impurities. Finally, the biogenic AgNPs pellet was dried at 50 °C for 24 h.

**Characterization of AgNPs**

The absorption spectrum of the biogenic AgNPs was scanned at 300–700 nm using an UV-Vis spectrophotometer. The size and shape of the biogenic AgNPs were investigated by transmission electron microscopy (TEM). Moreover, the X-ray diffraction (XRD) was investigated by a D8 Discover diffractometer. The biogenic AgNPs were mixed potassium bromide, and the pressed pellet was scanned by Fourier transform infrared (FT-IR) spectrometry using a Nicolet 6700 FT-IR spectrometer in the range of 400–4000 $cm^{-1}$ according to previously described method.[18]

**Antibacterial activity**

The anti-MRSA activity of the biogenic AgNPs was investigated by determining the minimum inhibitory concentration (MIC) in 96-well microtitre plate using resazurin-based assay. To prepare the bacterial suspension stock, the bacterial isolates (MRSA) were cultured in Mueller Hinton broth (MHB) at 37 °C for 24 h. Subsequently, the culture was centifuged at 8,000 x*g* for 5 min and the bacterial cells were resuspended in sterile saline and diluted to adjust $OD_{600}$ to 0.2–0.25. In a 96-well microtitre plate, the AgNPs were mixed with MHB and added to column 1. Then, the nanoparticles were subjected to serial dilution (columns 2 through 11 with 3 replicates per concentration in rows A to C). Subsequently, 10 µL of bacterial suspension stock was added to each well. Column 12 contained bacterial suspension only (Control). Wells containing the

nanoparticles at each dilution (without bacteria) were the color/turbidity controls (row D). Afterward, the plates were incubated at 37 °C for 24 h. Then, 10 µL resazurin (0.5 mg/mL) were added to each well, and the cultures were further incubated for 3 h at 37 °C. Subsequently, the plates were visually inspected for color change; where the change in the color from blue to pink denotes the reduction of resazurin by viable bacteria, and the MIC was determined as the lowest concentration of AgNPs that prevented the change in the color of resazurin.

**Assessment of cytotoxicity**

The cytotoxicity of the *Streptomyces*-synthesized AgNPs was assessed in vitro against various cell lines, including breast cancer (MDA-MB-231), non-small cell lung cancer (A549) and human skin fibroblast (HSF) cell lines. The cell lines were cultured in RPMI-1640 medium supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL) and cytotoxicity activity was assessed using the colorimetric cell viability MTT assay.[19] Briefly, MDA-MB-231, A549 and HSF cell lines were treated with different concentration of AgNPs. Briefly, AgNPs-treated and control (Untreated cells) cell lines were incubated for 24 h. Afterwards, 10 µL of the MTT reagent (0.5 mg/mL) were added to each well and the microplates were incubated for 4 h. Then, 100 µL of the solubilization solution was added into each well and the absorbance was determined 570 nm using a microplate reader.

## RESULTS

In this study, of 220 non-repetitive coagulase-positive staphylococcal isolates obtained from the NCI, 92 (41.8%) were presumptively positive for MRSA according to their growth on CHROMagar™ MRSA. Based on MALDI-TOF results, the presumptive MRSA isolates were identified as *S. aureus* (88/92), *S. pseudintermedius* (2/92), *S. cornubiensis* (1/92), and *S. delphini* (1/92). The molecular characterization revealed that all MRSA isolates (88/88) harbored both *nuc* and *mecA* genes, confirming the identification at the species level and the methicillin resistance, respectively.

**Table 1: Prevalence of methicillin resistant coagulase-positive *Staphylococcus* spp.**

| ***Staphylococcus* spp.** | **n** | **%** |
|---|---|---|
| *Staphylococcus aureus* | **88** | **95.65** |
| *Staphylococcus pseudintermedius* | **2** | **2.17** |
| *Staphylococcus cornubiensis* | **1** | **1.08** |
| *Staphylococcus delphini* | **1** | **1.08** |
| **Total** | **92** | **100** |

In an attempt to isolate efficient AgNPs-producing actinomycetes, sediment samples collected from the Nile River were used as a potential source for actinomycetes. Twenty-five actinomycetes isolates recovered from the sediments were screened for their capability to produce AgNPs using the cell-free extracellular extracts. Of 25 isolates, only 3 exhibited changing of the $AgNO_3$ solution color to brown. One strain designated SNPPA6 demonstrating the characteristic resonance at 410-430 nm was chosen for further investigations. The selected strain was identified by amplification and sequencing of its 16S rRNA as *Streptomyces virginiae*. The 16S rRNA sequence of the isolate was submitted to the GenBank (accession number OL587806), and the phylogenetic tree was constructed (Fig. 1). The culture supernatant of *S. virginiae* SNPPA6 was incubated with $AgNO_3$ solution in dark conditions and biofabrication of AgNPs was indicated by the development of brown color as the primary indication of the biosynthesis. The control without *S. virginiae* SNPPA6 culture supernatant did not produce any color after the same incubation period (Fig. 2).

The UV-Vis spectrum of the biogenic AgNPs revealed a characteristic absorption peak at 412 nm (Fig. 3). TEM analysis confirmed the fabrication of monodispersed spherical AgNPs of sizes ranging from 9.27 to 15.9 nm with an average size of 11.18 nm (Fig. 4). The XRD pattern showed five intense peaks at 38.26, 44.47, 64.71, 77.73 and 81.91° endorsing the crystallinity of AgNPs (Fig 5). FTIR spectrum showed characteristic bands at 1468, 1678, 1468, and 1678 $cm^{-1}$ (Fig. 6).

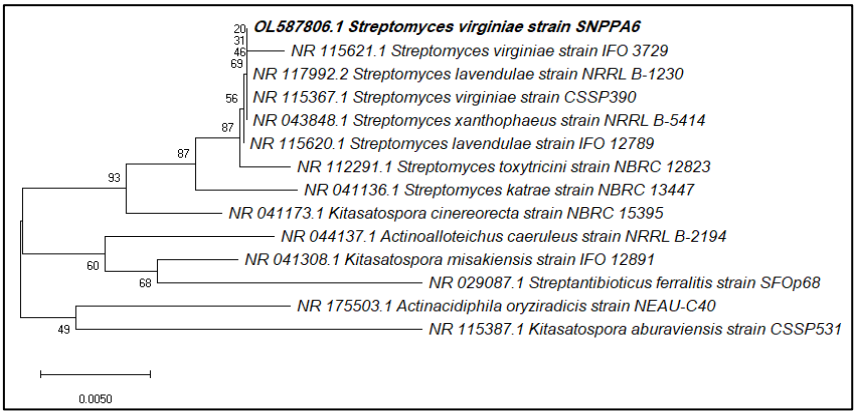


**Fig. 1:** Phylogenetic tree showing the relationships between *S. virginiae* SNPPA6 and the most closely related species.

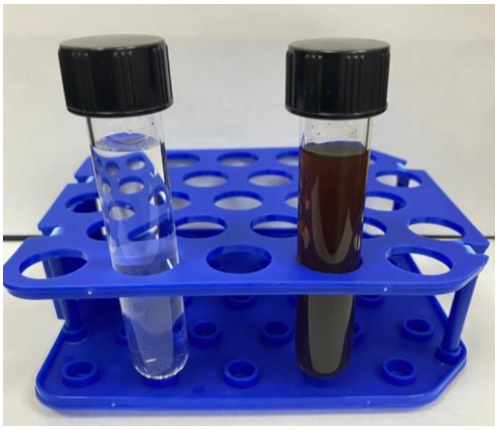

**Fig. 2:** Bio-fabrication of AgNPs by cell-free supernatant of *S. virginiae* SNPPA6 (Right). The control without *S. virginiae* SNPPA6 supernatant (Left).

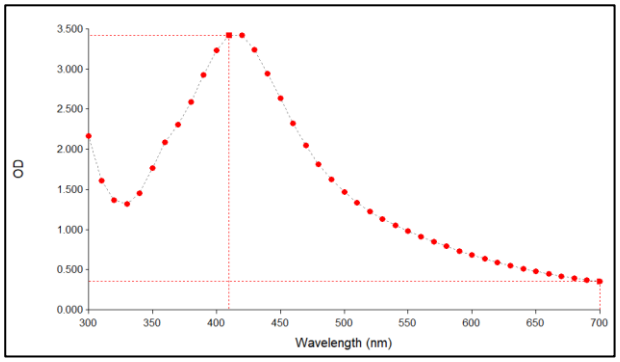


**Fig. 3:** UV-visible spectral analysis of AgNPs produced by *S. virginiae* SNPPA6.

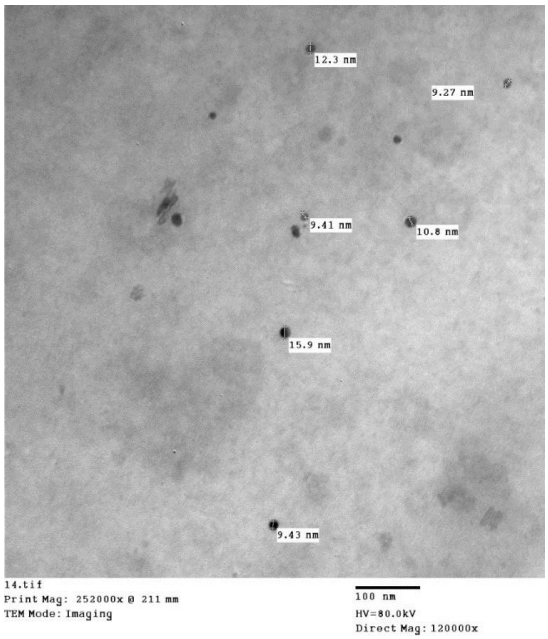


**Fig. 4:** Transmission electron micrograph showing the spherical shape and size of AgNPs produced by *S. virginiae* SNPPA6.

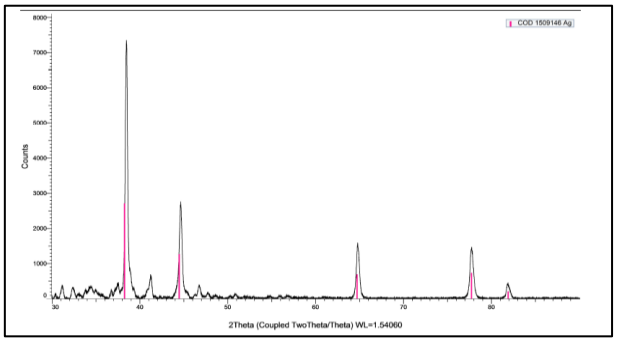


**Fig. 5:** XRD spectrum of the biogenic AgNPs.

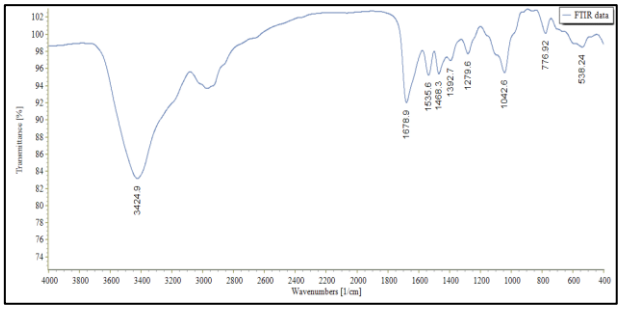


**Fig. 6:** FTIR spectrum of the biogenic AgNPs.

The anti-MRSA potential of the *Streptomyces*-mediated AgNPs was inspected by resazurin-based microtitre dilution assay to determine the lowest concentration of AgNPs that inhibited MRSA. Results revealed that the *Streptomyces*-mediated biogenic AgNPs inhibited the growth of all investigated MRSA isolates with MIC values ranging from 4 to 64 µg/mL. The MIC value of was less than 16 µg/mL against most MRSA isolates (74/88). On the other hand, the MIC values were 32 and 64 µg/mL against 11 and 3 MRSA isolates, respectively.

The influence of AgNPs on the proliferation of MDA-MB-231, A549, and HSF cell lines following the exposure to AgNPs determined using MTT assay was concentration-dependent. The half maximal inhibitory concentration ($IC_{50}$) of AgNPs on HSF was found to be 36.3 µg/mL, while its values were 24.5 and 29.2 µg/mL on MDA-MB-231 and A549 cells, respectively.

## DISCUSSION

This article addresses the implementation of MALDI-TOF/MS for identification of the recovered methicillin-resistant coagulase-positive staphylococcal isolates and highlights the anti-MRSA activity of the bioinspired AgNPs produced by S. virginiae SNPPA6 with an insight into their potential cytotoxicity. In this study, the phenotypic detection of MRSA was investigated by CHROMagar™ MRSA. Likewise, several recent investigations reported the use of the chromogenic media CHROMagar™ MRSA for rapid

and accurate detection of MRSA in various samples.[20,21] In this study, the prevalence of MRSA among the coagulase-positive staphylococci was 41.8%. This results in consent with previous reports regarding the prevalence of MRSA.[22] However, the global prevalence of MRSA varied from 10 to 78% depending on the geographic regions, isolation sources, and detection method.[23–25] In our study we used the MALDI-TOF/MS to identify the phenotypically detected MRSA and to distinguish between various coagulase-positive methicillin-resistant *Staphylococcus* spp. Based on the results, four different species belonging to the genus *Staphylococcus* were identified as *S. aureus*, *S. pseudintermedius*, *S. cornubiensis* and *S. delphini*. It has been reported that the species of the coagulase-positive *Staphylococcus intermedius* group (*S. intermedius*, *S. delphini*, *S. cornubiensis*, and *S. pseudintermedius*) appear like *S. aureus* complex (*S. aureus*, *S. argenteus*, and *S. schweitzeri*) biochemically but can be discriminated by MALDI-TOF/MS.[26] In this context, the emergence of the coagulase-positive methicillin-resistant *S. pseudintermedius* (MRSP) has been reported as a zoonotic pathogen that could be transmitted from dogs to humans.[27,28] In this study, *nuc* and *mecA* genes were detected in all MRSA isolates confirming the phenotypic findings. These results are in good harmony with previous investigations that reported the detection on both genes in MRSA isolated from various sources.[29,30]

Here in, we isolated an efficient AgNPs-producing *S. virginiae* SNPPA6 from the sediments of the River Nile that can reduce $AgNO_3$ to AgNPs by the extracellular extract acting as an eco-friendly reducing agent at ambient temperatures. Microbial extracts have been proposed as a potential source of biomolecules that have the ability to reduce the silver salts and transform them into AgNPs.[31] The exposure of microbial communities to heavy metals was thought to exert a selective pressure leading to evolving metal resistance mechanisms to capture, detoxify, and transform these hazardous elements.[18,32] UV-Vis spectrum of AgNPs showed a characteristic narrow absorption peak at 412 nm in good harmony with the surface plasmon resonance characteristic for spherical AgNPs.[33] In addition, TEM revealed the spherical shaped of the biogenic nanoparticles with average size of 11.18 nm. These findings agree with previous investigations reporting the biological synthesis of spherical AgNPs by numerous actinomycetes.[34,35] In our work, XRD analysis suggested the crystalline nature of the bioinspired nanoparticles revealing the characteristic peaks of the standard card of cubic silver (COD card no/file no 1509146).[18,24] FTIR spectrum showed distinctive bands at 1468 and 1678 $cm^{-1}$ that affirm the existence of protein covering the AgNPs. The presence of a peak at 1468 $cm^{-1}$ may be due to the symmetric stretching vibrations of –COO– groups of amino acid residues with free carboxylate groups in the protein. Also, 1678$cm^{-1}$ is assigned to stretching vibrations of –C=O and –C=C suggesting the existence of amide I bonds of the caping proteins. Thus, FTIR analysis indicated the occurrence of proteins among other biomolecules, suggesting their role in the biosynthesis as reducing and stabilizing capping agents.[18,36]

Regarding the antibacterial activity against various clinical isolates of MRSA, the bioinspired AgNPs fabricated by *S. virginiae* SNPPA6 exhibited remarkable antibacterial activity, however, the MIC value varied among the investigated MRSA isolates. The majority of the assessed isolates (84%) were sensitive to low concentrations of AgNPs (≤ 16 µg/mL), while a few isolates (3.4%) were more resistant to AgNPs with MIC value of 64 µg/mL. Likewise, several investigations documented the anti-MRSA activity of biofabricated AgNPs with MIC values ranging from ≤10 to 1500 µg/mL.[37,38] These variations may be attributed to the variation in size, charge, shape, and capping agents. Also, the actinomycetes-mediated AgNPs may be incorporated with antimicrobial metabolites and peptides secreted by the actinomycetes, leading to enhancing their antimicrobial activity.

In this work, the cytotoxicity assessments revealed obvious cytotoxic effects of AgNPs on normal (HSF) and cancer (MDA-MB-231 and A549) cell lines. However, the cancer cells were more sensitive to the biogenic AgNPs than the normal cells. In a similar study, the biofabricated AgNPs produced by *Bacillus funiculus* exhibited cytotoxic impact in MDA-MB-231 cells with an $IC_{50}$ value of 8.7 µg/mL. This antiproliferative effect was suggested to be attributed to the activation of lactate dehydrogenase, caspase-3, and reactive oxygen species (ROS) generation, ultimately leading to induction of apoptosis that was further confirmed through resulting nuclear fragmentation.[39] Our results regarding the cytotoxic impact of the biogenic AgNPs on A549 cells concord with the findings reported by Bano and her colleagues[40] who stated that biogenic AgNPs *Microbacterium proteolyticum* and *Streptomycetes rochei* exerted notable cytotoxic impact on the A549 cells accomplished by significant increase in ROS suggesting that the bio-fabricated AgNPs triggered the apoptosis in the exposed cells. This results explore the anti-MRSA efficacy of biogenic AgNPs produced by *S. virginiae* SNPPA6 against most assessed MRSA isolates at concentrations lower than the cytotoxic concentrations suggesting their prospective application in combating MRSA at non-toxic concentrations.

## CONCLUSION

This investigation demonstrated the prevalence of MRSA among the coagulase-positive staphylococci and shed light on the discriminative power of MALDI-TOF

in distinguishing the coagulase-positive *Staphylococcus* spp. compared with biochemical testing that does not differentiate between *S. aureus*, *S. pseudintermedius*, *S. cornubiensis*, and *S. delphini*. Our results highlighted the potential application of the biogenic AgNPs produced by *S. virginiae* SNPPA6 as a promising candidate for fighting MRSA at non-cytotoxic concentrations.

**Declarations:**
The manuscript has been read and approved by all named authors.
The manuscript is not published elsewhere.
**Ethical Approval:** Not applicable, because this article does not contain any human participants, data or tissues.
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