

***Bacillus licheniformis* (MN900686) Mediated Synthesis, Characterization and Antimicrobial Potential of Silver Nanoparticles**

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Abstract: The use of bacteria in the synthesis of silver nanoparticles (AgNPs) emerges as an ecofriendly and exciting approach. In the present study, we reported the biosynthesis of AgNPs by using culture supernatant of the bacteria *Bacillus licheniformis* (MN900686). The biogenically synthesized AgNPs were confirmed by the change in the color of the culture filtrate from yellow to brown after the addition of AgNO₃. Further characterization performed by means of UV vis-spectroscopy showed absorption peak at 414 nm which confirmed the formation of AgNPs. Fourier Transfer infrared (FTIR) confirmed the involvement of biological molecules in the formation of nanoparticles (NPs). The SEM revealed that the NPs have approximately 38 nm size. The agar well diffusion assay was used to determine antibacterial activity while tube dilution method was used to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The human pathogenic bacterial strains i.e., *P. aeruginosa* (MN900691) and *B. subtilis* (MN900684), were used as test strains. The anti-bacterial assay against test strains revealed that these NPs showed concentration dependent increased zone of inhibition (ZOI). The maximum ZOI at 25 µL of AgNPs was 20 mm against *B. subtilis* after 24 hours of incubation. One-way ANOVA test showed significant ZOI ($p \leq 0.05$) against *B. subtilis*. The MIC was ranged from 4.3-6.6 µg/mL while MBC ranged from 8.3 to 6.6 µg/mL. Overall, this study suggested that the biogenically synthesized NPs are an effective alternative source of antimicrobials against pathogenic bacteria.

Key words: *Bacillus licheniformis*, silver nanoparticles, characterization, antibacterial activity, MIC and MBC determination

1 Introduction

Infectious diseases caused by different pathogenic microorganisms and occurrence of resistance against commercially available antimicrobial agents has become serious threat to the life of human being. Therefore, there is a need to find the new antimicrobial agents from inorganic and natural substances. Nanomaterials have emerged as unique antimicrobial agents due to their novel physical and chemical properties¹. Nanotechnology is the emerging as rapidly growing with its very much applications in science and technology for the synthesis of new materials². By using advanced nanotechnology techniques, humans have

become able to synthesize nanoparticles (NPs) in the labs³. There are many types of NPs synthesized using metals such as Au, Ag, Ce, Pt, Pd and Zn⁴, but among them, most important are silver nanoparticles (AgNPs). AgNPs have potential applications in medical nano-engineering and pharmaceutical fields for the development of therapeutic agents, chronic diseases diagnostics and biosensors⁵. The AgNPs have extensively been studied for their activities particularly antimicrobial activities^{6, 7} and emerging as promising nano antibiotics now a days⁸. AgNPs have broad spectrum based antibacterial abilities so can be utilized in biomedical and medical devices and textile fields⁹. By

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using an ecofriendly approach, AgNPs applications have been increased in preparation of large number of products such as pests, electronic devices and in controlling microbe's growth and infections^{10,11}.

The use of silver as suspension and in nano-particulate form has a dramatic revival in nanotechnology. It has great antibacterial potency against human pathogens. The main task in NPs synthesis is the control of their physical properties like uniform particle size, similar shape, chemical composition, morphology and crystal structure. AgNPs have great effectiveness against most of the microbial pathogens particularly against the multi-drug resistance (MDR) bacteria. Microorganisms have been probed as potential bio-factories for metallic NPs synthesis such as silver, copper, zinc and gold¹².

The antibacterial activity of biogenic NPs in combination with antibiotics enhances their importance in controlling the MDR pathogenic bacteria in planktonic and biofilm mode¹³. Biogenically synthesized NPs are easy to produce biocompatible, economic, environmental friendly and offer different catalytic abilities compared to chemically synthesized ones. They have anticancer and antioxidant properties. Furthermore, they have more stability, as the natural organic material (citrate, sodium dodecyl sulfate) of bacteria work as natural capping layers surrounding the biogenic NPs, make these AgNPs active, stable and reusable¹³⁻¹⁵. AgNPs act as promising antimicrobial agent due to their long term stability and biocompatibility¹⁶.

It has been suggested that biogenic AgNPs produce reactive oxygen species and free radicals which cause cell death through apoptosis and prevent bacterial replication. Since AgNPs are smaller than the microorganisms, they diffuse into cell and rupture the cell wall. It has also been observed that smaller NPs are more effective than the bigger ones because of their quick penetration in the bacterial cell¹⁷.

In this present work, microbial production of AgNPs was investigated by using culture supernatant of the bacterial strain *Bacillus licheniformis* as a reducing agent. The biogenically synthesized AgNPs were characterized by UV-vis spectroscopy, fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and scanning electron microscopy (SEM). The synthesized AgNPs were further evaluated for the antibacterial efficacy using agar well diffusion method against two pathogenic bacteria (both Gram negative and Gram positive) such as *P. aeruginosa* (MN900691) and *B. subtilis* (MN900684). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was also determined.

2 Materials and Methods

2.1 Microorganisms source

Bacillus licheniformis (MN900686) was obtained from microbiology lab, Government College University, Lahore to be used for the synthesis of AgNPs, cultured on nutrient agar slant and incubated at 37°C for 24 hrs. The antimicrobial activity was checked against two human pathogenic bacteria (both Gram negative and Gram positive) such as *P. aeruginosa* (MN900691) and *B. subtilis* (MN900684), obtained also from microbiology lab, Government College University, Lahore.

2.2 Supernatant collection

Sterilized nutrient broth was inoculated with fresh culture of the bacterial strain *B. licheniformis*. The culture flasks were incubated for 24 hours in rotatory shaker incubator at 37°C. After the incubation period, the bacterial culture was centrifuged at 12000 rpm for 15 minutes. The supernatant was saved and the pellet was discarded.

2.3 Extracellular synthesis of AgNPs

10 mM silver nitrate (AgNO₃) solution was added in the supernatant for AgNPs synthesis in 2:1 (2 ratio was supernatant and 1 ratio was AgNO₃ solution) while another reaction mixture without AgNO₃ was used as control. The solutions were incubated for 72 hours on rotatory shaker at 150 rpm at 37°C and kept in dark to prevent any photochemical reaction during the experiment. After 72 hrs, the solutions color was turned from yellow to brown having mixture of AgNO₃ and bacterial suspension while no color change was observed in control supernatant without AgNO₃. The AgNPs were purified by centrifugation at 12000 rpm for 15 minutes twice and collected for further characterization.

2.4 Characterization of AgNPs

The appearance of brown color indicated that the AgNPs formation occurs in the reaction mixture due to reduction of Ag ions^{1,18}. The mixture was further characterized, by UV-Visible spectroscopy, fourier transform-infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), and X-ray diffraction (XRD) in order to authenticate the formation and check the specificity, size *etc.* of AgNPs.

2.4.1 UV-visible spectroscopy

The supernatant was tested qualitatively by UV-visible spectroscopy using AE-S70-1U UV-visible spectrophotometer and silver nitrate solution was used as control. UV-vis spectrophotometer from 370 to 970 nm operated at a resolution of 1 nm was used as a function of wavelength for spectral analysis of AgNPs. The peak of AgNPs varies from 400 to 470 nm. Occurrence of peak between 400-470 nm showed formation of more AgNPs and reduction of silver nitrate.

2.4.2 Fourier transform-infrared (FTIR) spectroscopy

The FTIR spectral analysis establishes the bio-molecules which are responsible for stabilization and capping of AgNPs as well as to check the functional groups of the AgNPs. The completely dried samples of AgNPs were used in order to perform FTIR. The spectrum was recorded on FTIR: IR Prestige-21 (P/N 206-72010. SHIMADZU Fourier transform infrared in the transmission range of 500 - 4000 cm^{-1}).

2.4.3 Scanning electron microscopy (SEM)

The size and morphology of the AgNPs were examined by scanning electron microscope (EM6200).

2.4.4 X-ray diffraction (XRD)

The crystalline nature of the AgNPs was characterized by XRD technique using an X-ray diffractometer (Phillips PW 1729/40) operated at 40 kv, 40 mA, step size of 0.2 over the 2θ range of 20-80°. Glass slides coated with AgNPs were tested.

2.5 Antibacterial activity of AgNPs

The antibacterial activity of *B. licheniformis* synthesized AgNPs was analyzed through agar well diffusion method against the test pathogens i.e., *P. aeruginosa* and *B. subtilis*^{13,19-21}. Muller Hinton agar plates were prepared and well of 6 mm diameter was made by using sterile cork borer. The test pathogens culture was adjusted to 0.5 McFarland turbidity standard and was spread on the media plate uniformly. The AgNPs at different concentrations (10 $\mu\text{g/mL}$, 15 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$, and 25 $\mu\text{g/mL}$) were used. A well loaded with DMSO without AgNPs was maintained as negative control (Fig. 1). The plates were incubated in an inverted position for 24 hrs at 37°C. The susceptibility of the test organisms was determined by measuring the diameter of the zone of inhibition around each well and measured in mm to test the antibacterial activity.

2.6 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of synthesized AgNPs was measured using tube dilution method and following²² with little changes. 3 mL nutrient broth was added into the test tubes. Almost 10

μL of bacterial fresh culture which was already adjusted to 0.5 McFarland turbidity standard was added to the tubes having broth. After that, different concentrations of AgNPs were added in these test tubes. The test tubes were incubated at 37°C for 24 hours. Following incubation, MIC was recorded as the lowest most concentration without any visible growth. For MBC determination, 10 μL of lower most MIC was spread on nutrient agar plates. The concentration at which the 99% of the growth was inhibited was recorded as MBC.

2.7 Statistical analysis

Experiments were run in triplicates. Microsoft Excel 2019 was used to draw graphs while SPSS version 10 was used to calculate Means, Standard error and ANOVA test. The figure of FTIR data was made on origin 2019A. The pictures of SEM and XRD were from one replicate.

3 Results

3.1 Biogenic synthesis of AgNPs

Synthesis of AgNPs by using *B. licheniformis* was confirmed by the change of color from yellow to brown after 72 hours incubation of the reaction mixture. The formation of AgNPs indicated that certain reducing agent were present in the supernatant released by the tested bacteria are actually involved in the reduction of Ag^+ ions to AgNPs. In control group, the reduction of Ag^+ ions did not occur due to the absence of reducing agent produced by bacteria. It is assumed that the Ag^+ ions required NADPH-dependent nitrate reductase enzymes for their reduction, which were released by bacteria in their extracellular environment²³.

3.2 UV-visible spectroscopy

The AgNPs were analyzed using UV visible spectrophotometer. The absorption spectrum for AgNPs was measured from 370 nm - 970 nm. The absorption peak was observed around 414 nm (Fig. 1). The AgNPs showed broad peaks due to different sizes of the NPs.

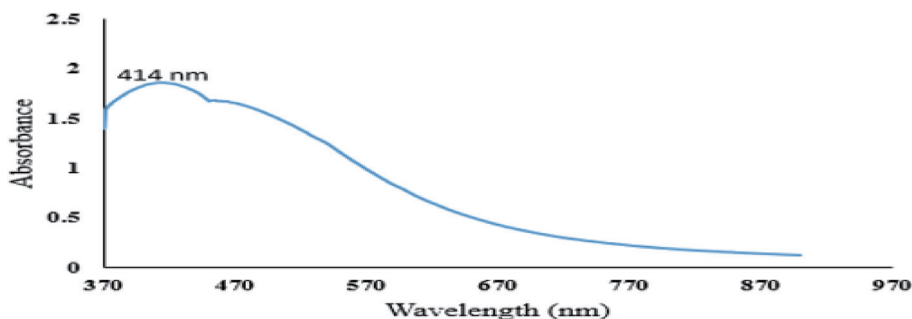


Fig. 1 UV-vis spectra of *B. licheniformis* synthesized AgNPs. The formation of absorbance peak at 414 nm indicated the reduction of silver nitrate and formation of AgNPs.

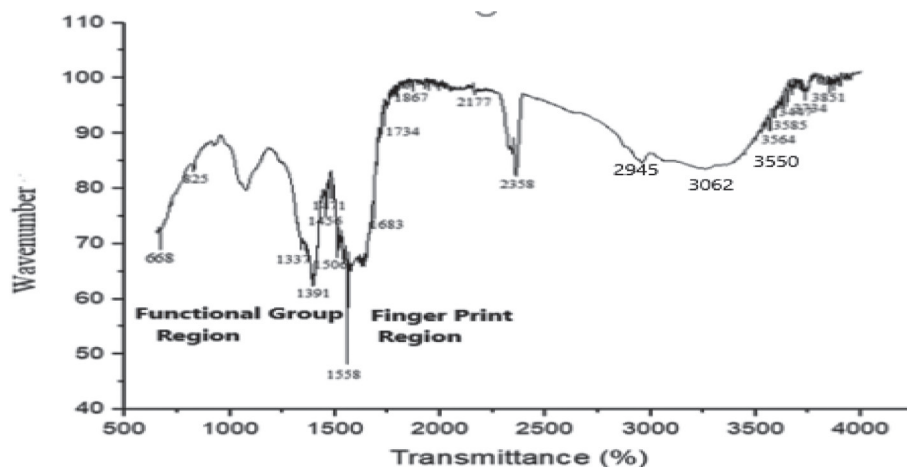


Fig. 2 FTIR spectra of *B. licheniformis* synthesized AgNPs. The different bands indicate various functional groups.

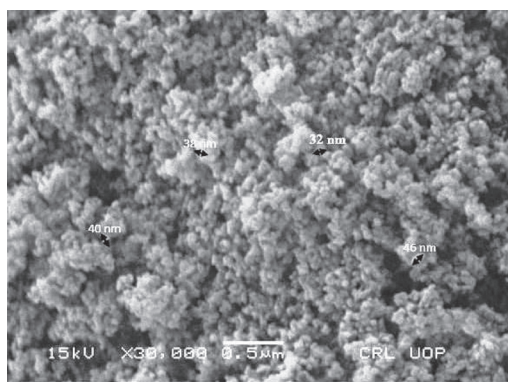


Fig. 3 SEM micrograph of *B. licheniformis* synthesized AgNPs taken at scale of 0.5 μm. AgNPs of spherical shape can be seen.

3.3 Fourier transform-infrared (FTIR) spectroscopy

The FTIR showed number of bands in the region 500-4000 cm^{-1} . The analysis of AgNPs showed absorption bands at various peaks (Fig. 2). The bands present at 3550, 3062, 2945, 2358, 1337 and 1391 cm^{-1} corresponded to the stretching vibrations of alcohol (O-H), primary amines (N-H), alkane (C-H), amine (C-N) and alcohol (C-O) groups

respectively. Amides containing carbonyl groups ($\text{C}=\text{O}$) were observed at 1683 and 1508 cm^{-1} . The other peaks at 1558, 1456 and 1411 cm^{-1} can be assumed to the C-O stretching vibrations aromatic and aliphatic amines, respectively (Fig. 2). The above information confirms the presence of stabilizing agents, which provide stabilization to the AgNP.

3.4 Scanning Electron Microscopy (SEM)

The size and morphology of AgNPs were examined by SEM analysis. The synthesized NPs were mostly spherical in shape with size ranging from 30-46 nm in a scale bar of 0.5 μm (Fig. 3). There were certain NPs aggregation suggesting that the protein molecules play important role as capping agents for biosynthetic NPs by preventing agglomeration and providing stability to the synthesized NPs.

3.5 X-ray diffraction (XRD)

The XRD pattern of the AgNPs showed unique diffraction peaks at 2θ of about 37, 44.8, 64.9, and 77 which indicated the presence of 111, 200, 220 and 311 orientations, respectively (Fig. 4). The sharp peaks of AgNPs appeared as a result of capping agents, which stabilize the NPs. The

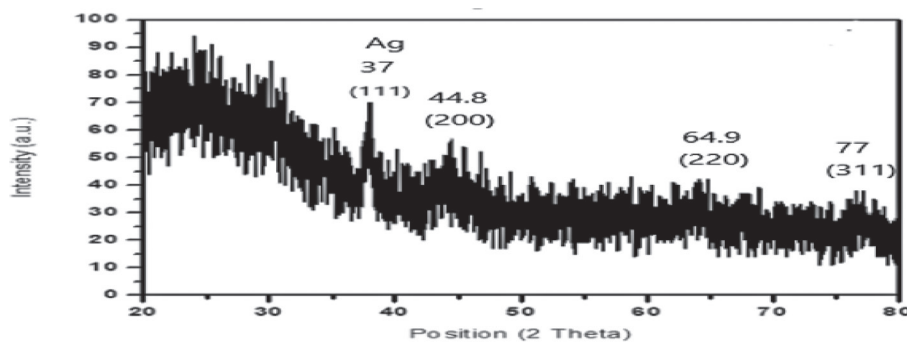


Fig. 4 XRD analysis of *B. licheniformis* synthesized AgNPs. Peaks indicate the different capping agents which give stability to the AgNPs.

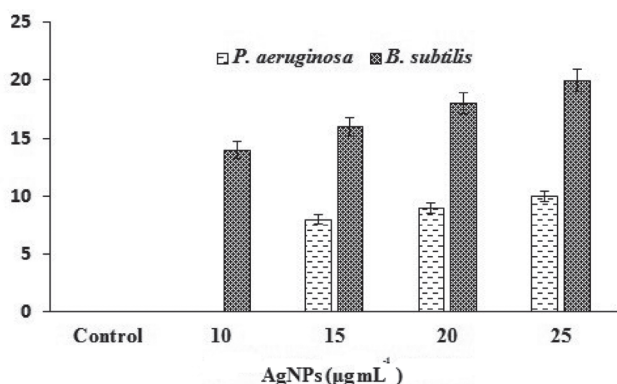


Fig. 5 Antibacterial activity of *B. licheniformis* (MN900686) AgNPs through well diffusion method against (a) *B. subtilis* (b) *P. aeruginosa*.

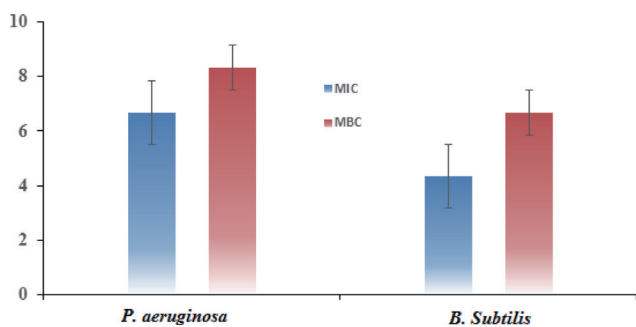


Fig. 6 MIC and MBC value of *B. licheniformis* AgNPs against *P. aeruginosa* and *B. subtilis*.

diffraction peak present at 37 was ascribed to the 111 lattice plane of face centered cubic (FCC). The XRD result shows the crystalline structure of biogenically synthesized AgNPs.

3.6 Antibacterial activity of AgNPs

Bacillus licheniformis synthesized AgNPs showed significant antibacterial activity against human pathogenic strains such as *P. aeruginosa* and *B. subtilis*. DMSO was used as negative control during antibacterial test. AgNPs showed highest zone of inhibition against *B. subtilis*. The maximum ZOI (20 mm) was observed against *B. subtilis*. The negative control (DMSO) was unable to inhibit the growth of any test strain (Fig. 5). One-way ANOVA showed that all the AgNPs activity was significant as compared to control.

3.7 MIC and MBC Determination

The Maximum MIC value (6.6 µg/mL) was noted against *P. aeruginosa* strain while lowest value was 4.3 µg/mL against *B. subtilis* (Fig. 6). The MBC values were calculated following spreading. The maximum MBC value was 8.3 µg/mL against *P. aeruginosa* while minimum value was 6.6 µg/mL against *B. subtilis* (Fig. 6).

4 Discussion

Biogenically synthesized AgNPs have been proved as effective and valuable compounds against pathogenic bacteria. They also have excellent antimicrobial and antiviral activity^{24, 25}. Although, a lot of methods are available which can be used for the synthesis of AgNPs. Much of these techniques are of chemical nature compared to biogenic or green synthesis of AgNPs, a more eco-friendly approach^{25, 26} and non toxic³. The bacterial synthesis of AgNPs seems more budget friendly as compared to chemical synthesis.

In this research work, the bacterial strain *B. licheniformis* (MN900686) was used for the biogenic synthesis of AgNPs. The color of solution was turned brown from yellow, which confirmed the formation of AgNPs. Zhang *et al.*²⁶ described that the change of color indicates the ability of supernatant to form AgNPs. Previously, Iravani *et al.*²⁷ also reported that the formation of AgNPs changes the color to brown. The more saturated brown color indicates the more AgNPs in the solution²⁸. The change in color was due to the excitation of the surface plasmon vibrations in metal NPs²⁹.

Following visual observation, the solution was further analyzed by UV visible spectrophotometer. UV-visible graph showed a peak at 414 nm which confirmed the formation of spherical-shaped AgNPs. The peak at aforementioned wavelength also confirmed the size ranging less than 100 nm³⁰. The FTIR of the sample was performed to check the involvement of biological molecules. The bands present at 3550, 3062, 2945, 2358, 1337 and 1391 cm⁻¹ were corresponded to the stretching vibrations of alcohol (O-H), primary amines (N-H), alkane (C-H), amine (C-N) and carbonyl (C=O) groups, respectively³¹. The SEM revealed that the AgNPs are of spherical shape and were around 38 nm in size. It is established fact that the things with smaller size have more surface area, hence making them more effective for in-depth potential. The formation of various sharp peaks using XRD, indicated the presence of different molecules which are involved in the stabilization of the AgNPs. Similar results were presented by Kalyanasundaram *et al.*³².

The agar well diffusion method was used to check the antibacterial efficacy of AgNPs against *P. aeruginosa* and *B. subtilis*. AgNPs tested against *P. aeruginosa* at a different concentration of 10, 15, 20, and 25 µg/mL revealed the mean zone of inhibition as 0, 8, 9, and 10 mm in diameters, respectively. Similarly, mean zone of inhibition against *B. subtilis* were recorded as 14, 16, 18, and 20 mm at 10, 15, 20, and 25 µg/mL concentrations, respectively. The antimicrobial activity was found to be maximum for *B. subtilis* and moderate for *P. aeruginosa*. The mean potential as depicted by zones of inhibition of synthesized AgNPs in the screening test determined a less significant effect against growth of Gram-negative bacteria compared to Gram-positive bacteria. This might be due to the structural differenti-

ation of the cell wall compositions of the two groups of bacteria^{33, 34}. Different studies have established that the metal NPs penetrate through the bacterial cell membrane and disturb its functions^{35, 36}. Thus, AgNPs possess significant antibactericidal activity against MDR isolates such as *P. aeruginosa* and *B. subtilis*. Recently³⁷, reported that AgNPs release Ag⁺ ions from the surface and these ions are responsible for the bactericidal efficacy of AgNPs.

The more Ag⁺ ions release will kill more bacteria which results in bigger ZOI. Further, tube dilution method was used for the determination of MIC and MBC. The Maximum MIC value (6.6 µg/mL⁻¹) was noted against *P. aeruginosa* strain while lowest value was 4.3 µg/mL against *B. subtilis*. The maximum MBC value was 8.3 µg/mL against *P. aeruginosa* while minimum value was 6.6 µg/mL⁻¹ against *B. subtilis*. Similar, results were reported by Hamouda *et al.*³⁸. Findings of current study showing AgNPs having lowest minimum inhibitory concentration against pathogenic bacteria suggests the broad spectrum nature of their antimicrobial activity^{39, 40}.

5 Conclusion

Bacillus licheniformis can be utilize to synthesize bioactive AgNPs efficiently using inexpensive substances in an ecofriendly and nontoxic manner. UV-vis spectrophotometer confirmed the reduction of AgNO₃ to AgNPs through reductase enzyme released by *B. licheniformis* in supernatant solution. The FTIR data also confirmed the presence of stabilizing agents, which provide stabilization to the AgNPs. The zone of inhibition in antimicrobial screening test indicated that the AgNPs synthesized via this technique have excellent antimicrobial efficacy against above mentioned pathogenic bacteria. It also suggested that the AgNPs synthesized by this process can be used in various medical fields following toxicity evaluation.

Conflict of Interests

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Author Contributions

IL designed and supervised the study. ST performed all the experimental work and drafted the manuscript. SA, MU, AS, AS helped in data analysis. RI and FA revised the manuscript. All authors approved the final version.

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