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RESEARCH ARTICLE

Antibacterial and antibiofilm efficacy of green synthesized ZnO nanoparticles using *Saraca asoca* leaves

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Abstract

Biofilms are made up of bacterial colonies and their extracellular polymeric substances (EPS) matrix, which protects the bacteria from adverse environmental conditions. The increasing drug resistivity of pathogenic bacteria is becoming an emergency for developing new antibacterial agents. In this study, we have synthesized the zinc oxide nanoparticles (ZnO NPs) using the leaf extract of *Saraca asoca* plant, and the antibacterial and antibiofilm activity of green synthesized ZnO NPs was measured against the biofilm-producing bacteria *Bacillus subtilis*. The disk diffusion data reveals that the zone of inhibition (ZOI) starts at a concentration of 0.5 mg/mL and minimum inhibition concentration (100 µg/mL) and minimum bactericidal concentration (150 µg/mL) values were also evaluated for green synthesized ZnO nanomaterials. Crystal violet test and microscopic examination were used to assess the impact of produced nanoparticles on biofilm development. The findings indicated a nearly 45%, 64%, and 83% suppression of biofilm development at 0.5 × MIC, 0.75 × MIC, and 1 × MIC value, respectively. The biofilm biomass of the preformed or matured biofilms by the ZnO NPs was evaluated to be 68%, 50%, and 33% at concentrations of 0.5 × MIC, 0.75 × MIC, and 1 × MIC which was concentration-dependent. Moreover, flow cytometry results suggest damage to the bacterial cell membrane. The data indicated that the proportion of dead cells increased with NP concentration in comparison to the control. Therefore, it can be concluded that the green synthetic ZnO nanoparticles showed excellent antibacterial and antibiofilm activity against the *Bacillus subtilis* bacteria that produce biofilms and that they could be a promising substitute agent for the treatment of biofilms and drug-resistant bacteria.

Keywords Biofilm · ZnO nanoparticles · Antibacterial · Green synthesis

Introduction

Every year, millions of people suffer from illness due to bacterial infections globally. Over the last few years, there is an excess and misuse of traditional antibiotics and antibacterial agents causing drug resistance to pathogenic bacteria, which is a serious public health concern (Samreen et al. 2021). So, there is an urgent need for an alternative antibacterial agent to prevent this crisis. It is well known that most bacteria live in either a planktonic state or grow in biofilm form. According to a report by the national institute of health (NIH), approx. 80% of bacterial infections are caused and spread by bacterial biofilms (Donlan 2001; Schachter 2003). The biofilm is a very complex structure, which starts to form due to the adhesion of planktonic bacteria on biological or non-biological surfaces in search of nutrients (Garrett et al. 2008). After the adhesion, the bacteria start colonization and secrets a matrix of extracellular polymeric substances (EPS), which surround the bacterial colonies and provide a protective environment from external stress (López et al. 2010). So basically, biofilm is a complex, multi-layered 3D structure, which consists of bacterial colonies and EPS inside them. Apart from bacterial infections, there are many more

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disadvantages of biofilms in different areas such as medical care units, membrane wastewater treatments, marine industries, water transport systems, and many more (Flemming 2020). The conventional treatment methods for the control and disruption of bacterial biofilms have become less effective, costly, and waste-producing. The chemical and mechanical route of treatment like water jet-spraying, high-pressure water, ultrasonication, etc., are not always feasible and produce more waste and water scarcity (Boudarel et al. 2018; Yu et al. 2020). Also, the drug resistance of the bacteria and the cost of the compounds minimize the use of antibiotics and other biocides. So, there is a lack of novel antimicrobial agents against drug-resistant bacteria and a serious need for an alternative agent for the treatment of biofilms.

Nowadays, nanotechnology has become a prominent and emerging tool in each discipline of science due to its nanoscale size which gives them unique physicochemical properties compared to its bulk size. The nanoscale size (ranging from 1 to 100 nm) increases their surface area to volume ratio and catalytic activity, providing a promising material as an antimicrobial agent (Hajipour et al. 2012). Inorganic nanomaterials such as metal and metal oxides are widely used due to their antimicrobial activity. Metal-based NPs such as gold (Au), silver (Ag), copper (Cu), iron (Fe), and magnesium (Mg) along with their oxides like zinc oxide (ZnO), copper oxide (CuO), iron oxides (Fe_3O_4 or Fe_2O_3), magnesium oxide (MgO), and titanium dioxide (TiO_2) have shown the ability to inhibit the growth of bacteria and biofilms (Chatzimitakos et al. 2016). ZnO nanostructures are one of them and have a profile for being less toxic, affordable, and possessing high thermal stability, selectivity, and durability (Kumar Jangir et al. 2017). ZnO nanomaterials are widely used in various applications including biosensing (Shetti et al. 2019; Sharma et al. 2021), gas sensing (Agarwal et al. 2019b), solar cells, pH sensing (Sharma et al. 2020), wastewater treatment (Sheikh et al. 2020; Agrawal et al. 2021), dye degradation (Gnanamozhi et al. 2020), seed germination (Sharma et al. 2022), and nanomedicine where they have shown great potential in interacting well with bio-membranes and displaying antibacterial and anticancer properties (Manna 2012). This is because of their special optical, chemical, electrical, and large band gap properties. Cells are resistant to low concentrations of ZnO nanoparticles, although they are supposed to be environmentally friendly and biodegradable (Natarajan et al. 2016). ZnO nanoparticles in the size range of 10–250 nm may be used as a powerful bactericidal agent against gram-positive and gram-negative pathogens such as *E. coli*, *Bacillus*, *Staphylococcus*, *Pseudomonas*, and other species (Jiang et al. 2009). The impact of ZnO nanoparticles on *Bacillus subtilis* bacteria-produced biofilm was investigated (Awasthi et al. 2020a). The results showed a dose-dependent decrease in biofilm mass and a change in the bacterial membrane. Gram-positive bacteria were shown to be more susceptible to ZnO than gram-negative bacteria in research by Tayel et al. (2011) which examined the impact of ZnO nanoparticles against foodborne pathogenic bacteria. Studies suggest that the antibacterial activity of ZnO nanoparticles may be caused by the production of reactive oxygen species, the release of Zn^{2+} ions, or electrostatic contact of the particles on the microbial surface. According to Tam et al. (2008), the breakdown of ZnO may liberate Zn^{2+} ions, which would then be responsible for the antibacterial action. SEM analysis of the morphological changes in *E. coli* treated with ZnO NPs was done by Zhang et al. (2007). They proposed that the interaction between ZnO NPs and cell membranes was in charge of the antibacterial effect since exposure to ZnO NPs seemed to harm and eventually disintegrate *E. coli* cell membranes (Jiang et al. 2016).

There are many alternative ways to synthesize ZnO nanoparticles like hydrothermal, solvothermal, microwave irradiation, chemical precipitation, sol–gel, and green synthesis, (Agarwal et al. 2019a; Kumar et al. 2021). When synthesizing nanoparticles by the chemical approach, harmful chemical substances are used as capping and reducing agents, which have several negative impacts, including toxicity. As a result, the role of plant extracts in the formation of metal oxide nanoparticles has increased. This method provides a quicker reaction time and is more environmentally friendly than conventional chemical methods (Chennimalai et al. 2021). Plant extracts provide a wide range of bioactive compounds that help to reduce and stabilize nanoparticles (Demissie et al. 2020). Plants and phytochemicals function as stabilizers for nanoparticles in addition to acting as natural reductants for the production of nanoparticles (Awasthi et al. 2020b; Basri et al. 2020). Metal nanoparticles made from plant extracts have been used successfully in biology, electronics, and medicine. These nanoparticles do not significantly endanger the environment and are less hazardous to humans. Many plant extracts, including *Trifolium pratense* (Dobrucka and Długaszewska 2016), *Vitex negundo L.* (Ambika and Sundrarajan 2015), *Aloe vera* (Rasli et al. 2020), *Azadirachta indica* (Iqbal et al. 2021), *Passiflora caerulea* (Santhoshkumar et al. 2017), *Camellia sinensis* (MalligArjuna Rao et al. 2021), *Crocus Sativus* (Owais Mushtaq et al. 2022), and many more, have been used to prepare ZnO nanoparticles and their bactericidal capabilities have also been investigated. The Indian medical system has long utilized *Saraca asoca* to treat a variety of conditions, including fever, pain, urogenital tract illnesses, and uterine, genital, and other reproductive diseases in women (Gupta et al. 2014).

In this study, we have synthesized the green zinc oxide nanoparticles (ZnO NPs) from the leaves extract of *Saraca asoca* plant using our previously used method (Sharma et al. 2022). The synthesized material was characterized using

UV–Vis spectroscopy, XRD, and SEM to confirm the morphology of green ZnO nanoparticles. The main aim of this study was to evaluate the antibacterial and antibiofilm activity against the biofilm-producing bacteria. For this purpose, *Bacillus subtilis* was chosen as the model bacteria and the antibacterial and antibiofilm experiments were done against it. Disk diffusion, MIC, and MBC were examined for antibacterial activity. The effect of nanoparticles on biofilm formation was studied using a crystal violet assay. The effect of ZnO NPs on the bacterial cell was also determined by using the flow cytometry.

Materials and methods

Green synthesis and characterization of ZnO nanoparticles

The zinc oxide nanoparticles were synthesized using similar protocols as reported earlier (Sharma et al. 2022). For this purpose, the leaf extract of *Saraca asoca* plant (RUBL 21255) was prepared. The 10 mL of leaf extract was mixed with 20 mL of 0.002 M zinc nitrate hexahydrate solution under constant stirring. After proper mixing, 20 mL of sodium hydroxide solution (0.001 M) was added dropwise into the above solution and left on a magnetic stirrer for 2 h. A change in the color of the above solution indicates the formation of green ZnO nanoparticles. Then, the solution was centrifuged and washed several times with deionized (DI) water and ethanol for removing any impurities. The pellets were dried to make powder for further use. The UV–Vis spectrophotometer (Multiskan GO, Thermo Scientific) was used to record the absorbance spectra and confirm the synthesis of green ZnO nanoparticles. The morphology of the synthesized nanoparticle was investigated using a field emission scanning electron microscope (JSM-7610FPlus, JEOL).

Antibacterial studies of green synthesized ZnO nanoparticles

Disk diffusion method

The antibacterial activity of green ZnO nanoparticles for *Bacillus subtilis* (MTCC 121) was evaluated using the disk diffusion method. For this purpose, a single colony of model bacteria was picked from the mother plate and inoculated into 10 mL of fresh nutrient broth culture. Then the culture was incubated overnight in an incubator shaker at 37 °C. The 1:100 dilution of overnight grown culture was used as inoculum to spread on fresh agar plates. On the other hand, nanoparticles loaded disks were prepared with green synthesized ZnO nanoparticles. For this, different concentrations of green synthesized ZnO nanoparticles (ranging from 0.5 to 20 mg/mL) were prepared and 20 µL from each was loaded onto UV-sterilized Whatman filter paper disks (diameter 6 mm). After the spreading of inoculum, nanoparticles loaded disks were placed on agar plates with negative (DI water dipped disk) and positive control (Gentamicin loaded disk). Then, the agar plates were incubated overnight at 37 °C and the zone of inhibition was measured to evaluate the antibacterial efficacy of green synthesized ZnO nanoparticles.

Determination of MIC and MBC

The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of green synthesized ZnO nanoparticles were evaluated using both resazurin dye-based assay and broth dilution method. Resazurin dye-based assay is a simple and easy process for the determination of the MIC value of any material against bacteria as resazurin is dye blue color dye, which is reduced to pink color (resorufin) in the presence of active bacteria (Elshikh et al. 2016; Chakansin et al. 2022). In this study, different concentration of green ZnO NPs (varying from 25 to 200 µg/mL) was prepared into the wells of A, B, and C rows of 96-well plates with control (without NPs) and blank (only nutrient media) with a final volume of well 100 µL as described in our previous paper (Owais Mushtaq et al. 2022). Then, the prepared wells were inoculated with 1:100 dilution of the overnight-grown culture of *Bacillus* bacteria and incubated in an incubator shaker at 37 °C for 18 h. One row (D) from 96-well plates was only with nanoparticles to see any effect of it on resazurin dye. After the incubation period, 20 µL of the resazurin dye (concentration 0.5 mg/mL) was dispensed into each well of the plate and again incubated at 37 °C for 3 h in the dark. After the incubation, the color change in the plate was observed to evaluate MIC.

For confirmation analysis, the MIC of synthesized nanoparticles was also measured by the broth dilution method. For this, 15 mL culture tubes with a final volume of 10 mL of nutrient broth, where nutrient broth without ZnO NPs serves as a control. The prepared tubes were inoculated with 1:100 dilution of the overnight-grown culture of *Bacillus* bacteria and incubated in an incubator shaker at 37 °C for 24 h. Before incubation, we measured the optical density of each sample tube and compared it with the optical density of samples after the incubation period. After the incubation period, 100 µL culture was taken out from MIC and higher than MIC values and spread on fresh agar plates to determine MBC value.

Antibiofilm studies of green synthesized ZnO nanoparticles

Pre-treatment of nanoparticles on biofilm

The effect of synthesized nanoparticles on biofilm formation was determined using the method described previously

(Awasthi et al. 2020a). In this experiment, washed and UV-sterilized coverslips (22 × 44 mm size) were placed into boiling tubes and autoclaved with 5 mL of nutrient media. Then, the tubes were inoculated with the overnight grown culture (1:100 diluted) of *Bacillus* and incubated at 37 °C for 24 h. In the tubes, there was one blank (only nutrient broth), one control (without treatment), and three treated with different values of ≤ MICs, which were 0.5 × MIC (50 µg/mL), 0.75 × MIC (75 µg/mL), and 1 × MIC (100 µg/mL).

After the incubation period, the coverslips were removed from the tubes and washed with DI water. The coverslips were stained with 0.1% crystal violet dye for 15 min and again washed with DI water to remove excess staining of dye. Then, the coverslips were dried and quantified by measuring the absorbance of solubilized the dye of coverslips in 30% acetic acid at 550 nm. The percentage of biofilm biomass was calculated through the following equation (Banerjee et al. 2020).

$$\%\ \text{Biofilm biomass} = \left[\{\text{Testsample OD 550nm/control OD 550nm}\} \times 100\right].$$

Post-treatment of nanoparticles on biofilm

In this experiment, the effect of synthesized nanoparticles was evaluated against the pre-formed or matured biofilms. For this, first, the biofilm was grown on coverslips (without treatment) using the above-described method and then the coverslips were transferred into the different tubes, which contain a different concentration of nanoparticles, i.e., 0.5 × MIC (50 µg/mL), 0.75 × MIC (75 µg/mL), and 1 × MIC (100 µg/mL) and incubated again for the next 24 h. After the incubation period, the coverslips were washed, stained, and quantified in a similar procedure as described above.

Biofilm imaging

After staining with crystal violet, washed and dried coverslips of both pre-treated and post-treated were observed under the microscope (Leica DFC-450C DM-1000) in the bright field at the different magnifications and images captured at the 40 × using a digital camera.

Determination of membrane damage by flow cytometry

The bacterial membrane damage with the treatment of ZnO NPs was investigated by flow cytometry analysis using propidium iodide (PI) dye. PI can enter bacterial cell membranes only when it is damaged (dead cells). PI cannot enter intact cells (live cells). So PI enters into dead cells, binds with DNA, and emits red fluorescence when stimulated with a blue laser.

Preparation of sample for the experiment

In this experiment, the mid-log phase culture of *Bacillus* (0.1 OD) was treated with various concentrations of nanoparticles and untreated serves as a control. After 6 h of treatment, cells were centrifuged at 8000 rpm for 10 min. Then, the supernatant was discarded and the pellets were washed with PBS. The pellets were resuspended again into PBS to make a homogenous suspension. One milliliter suspension of the treated and untreated groups was taken out into 1.5 mL of the centrifuge tube, and subsequently, the cells were incubated with 10 µL of PI dye (100 µg/mL) for 15–20 min at room temperature. After incubation, the cells were washed twice with PBS to remove excess unbound PI, and finally, the cells were resuspended again into 1 mL of PBS.

Flow cytometry analysis

After PI staining, samples were acquired in a flow cytometer (Cytoflex, Beckman Coulter) and at least 10,000 events were recorded for each sample. The red fluorescence (excitation 485 nm and emission 630 nm) of PI was collected through PE (585/42 BP) filter. Cells were plotted against FSC-A and SSC-A to set the scaling factor, threshold, and gates. The SSC-A and PE-A parameters were selected to differentiate PI-positive and PI-negative cells. All the data were analyzed and plotted using CytExpert 2.0® software.

Statistical analysis

The data was analyzed in Microsoft Excel. The data was provided as the standard deviation of three replications. To evaluate if there were significant changes between the treatments, a probability level of 0.05 ($P < 0.05$) was used.

Result and discussion

Characterization of green synthesized zinc oxide nanoparticles

The synthesized dried powder of ZnO material was characterized by the UV–Vis spectrophotometer and scanning electron microscope (SEM) to confirm the synthesis of green ZnO nanoparticles. The UV–Vis spectra of

the synthesized zinc oxide material were taken in the 300–800 nm range (Fig. 1). The synthesized material showed an absorption peak of around 360 nm, which confirms the synthesis of green ZnO nanomaterial.

The surface appearance and geometrical properties of the green synthesized zinc oxide nanostructures were studied using a scanning electron microscope (SEM). The scanning of the samples was carried out in the presence of an electron beam with a high level of energy. The morphology of synthesized ZnO NPs was analyzed with the help of a JSM-7610FPlus, JEOL instrument. The NPs had a range of size distribution from 20 to 80 nm (Fig. 2A) and had an average size of 40–50 nm with spherical geometry and were agglomerated into larger structures (Fig. 2B). The characterization results of green synthesized ZnO nanoparticles were almost similar to previously synthesized nanoparticles (Sharma et al. 2022).

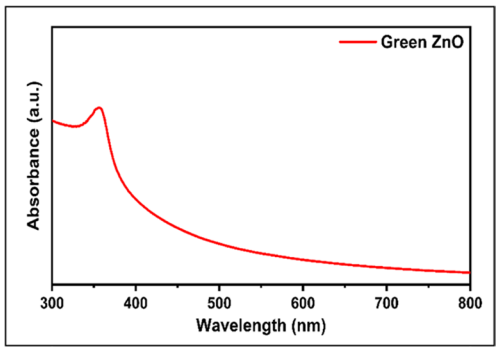

Fig. 1 UV–Vis results of green synthesized ZnO NPs

Antibacterial studies of green synthesized ZnO nanoparticles

The disk diffusion method is a simple process to check the antibacterial activity of any material. In this experiment, we evaluated the antibacterial activity of green synthesized ZnO NPs against *Bacillus subtilis.* The zone of inhibition was measured for different concentrations of ZnO NPs. Figure 3 shows the minimal zone of inhibition (ZOI) of ZnO NP-treated bacterial cells which starts with a concentration of 0.5 mg/mL and increases with the concentration up to 5 mg/mL. After this, the ZOI remains similar for higher concentrations of ZnO NPs, i.e., 10 mg/mL, 15 mg/mL, and 20 mg/mL.

This demonstrated that initially, the ZOI increases in a dose-dependent manner, but after an optimal concentration (5 mg/mL), there was no significant effect on ZOI on increasing the dose. This may be due to the maximum adsorption of nanoparticles by a disk. The ZOI for different concentrations of ZnO NPs was measured and presented in Fig. 4.

In this study, the ZOI starts at a very low concentration (0.5 mg/mL) of the green synthesized ZnO NPs compared to other studies. Owais Mushtaq et al. (2022) synthesized ZnO nanoparticles through the plant extract of *Crocus sativum* and evaluate their antibacterial activity against *Bacillus subtilis*. The results showed that their ZOI starts at the 2 mg/mL concentration, which is 4 × higher than our study. In another study by Raghavendra et al. (2022), the green synthesized ZnO NPs using *Areca catechu* plant for wastewater treatment. They evaluated the antibacterial activity against many bacteria including *Bacillus subtilis.* The results demonstrated good antibacterial activity against all of them.

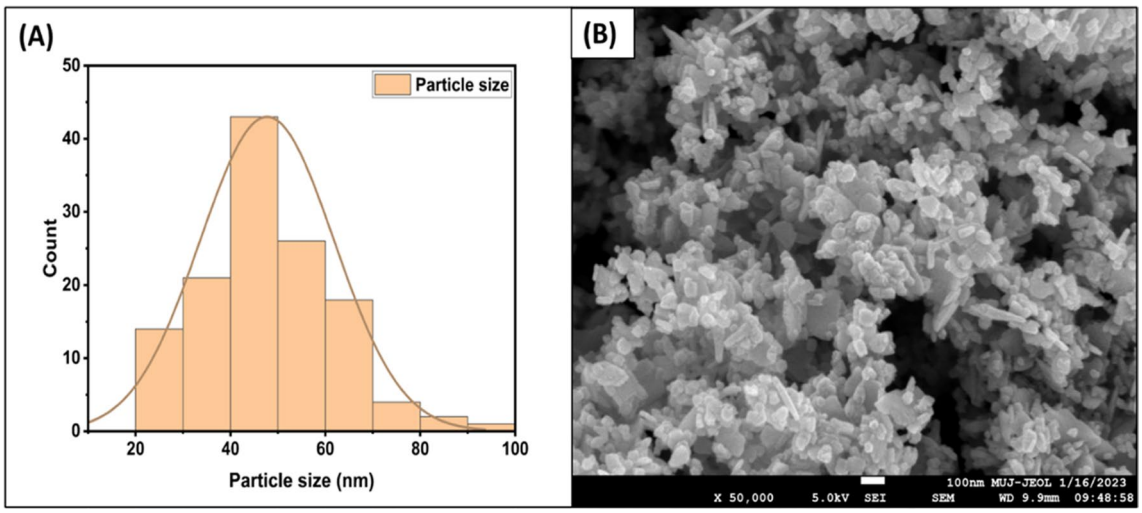

Fig. 2 **A** Size distribution graph and **B** SEM images of green synthesized nanoparticles

Determination of MIC and MBC

The MIC is a minimum concentration of any material, which inhibits the growth of bacteria and the MBC is a minimum concentration of any material, which kills the 99.99% of bacteria in the media. In the resazurin dye-based assay, the minimum concentration where the blue color remains the same is known as the MIC value. In Fig. 5, it can be seen that at 100 µg/mL concentration of ZnO NPs, the blue color does not change which is considered MIC. In the broth dilution method, the optical density of samples was compared before the incubation and after the 24-h incubation. The results demonstrated that at 100 µg/mL concentration, there was no specific change in the optical density, which was similar to the above results. The MBC value can be the same as the MIC value or may be higher than it. For this, 100 µL from the MIC and its higher concentration were taken out and spread onto agar plates with further incubation for 24 h.

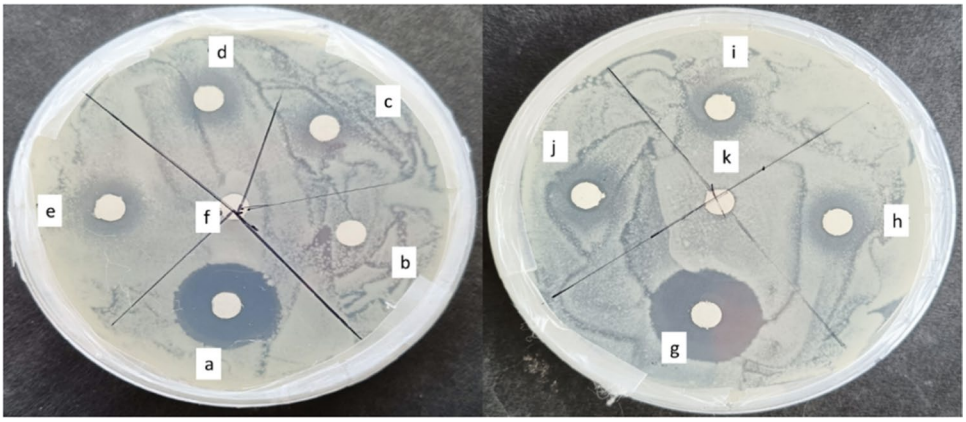

Fig. 3 Disk diffusion data of green synthesized ZnO nanoparticles: (**a**) gentamicin loaded disk serving as a positive control (**b**) 0.5 mg/mL, (**c**) 1 mg/mL, (**d**) 2 mg/mL, (**e**) 5 mg/mL, (**f**) DI water disk serving as a negative control, (**g**) gentamicin loaded disk, (**h**) 10 mg/mL, (**i**) 15 mg/mL, and (**j**) 20 mg/mL, (**k**) DI water disk

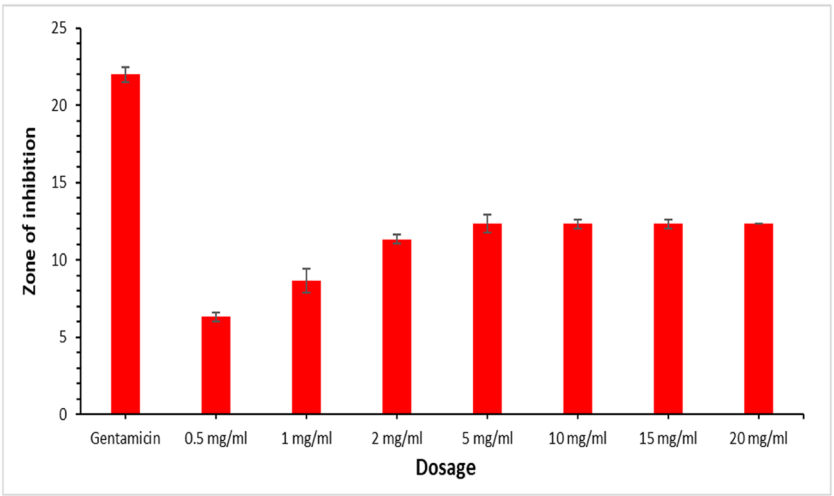

Fig. 4 ZOI produced by different concentrations of ZnO NPs. The error bars represent standard deviation

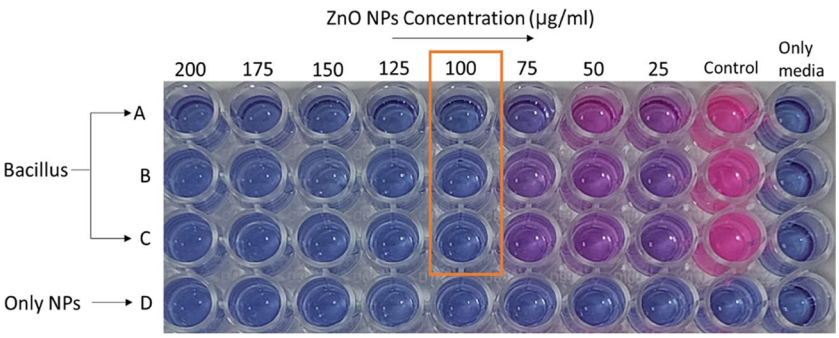

Fig. 5 MIC determination of green synthesized ZnO NPs using resazurin dye-based assay

The results showed that there were no colonies grown at 150 µg/mL concentration, so it was assumed to be the MBC value for green ZnO NPs.

The MIC and MBC values were quite low for these ZnO NPs against *Bacillus subtilis* bacteria. Ifeanyichukwu et al. (2020) synthesized ZnO nanoparticles using the *Punica granatum* plant extract to measure the antimicrobial efficiency. The antimicrobial activity results showed the MIC value of 1200 µg/mL for *Bacillus* bacteria. In another work, Nazir et al. (2021) green synthesized ZnO nanoparticles by plant extract of *Eriobotrya japonica*. The synthesized nanoparticles were assessed for antimicrobial activity against *E. coli* and *B. subtilis*, and the MIC was 364 µg/mL and 311 µg/mL, respectively. In a recent study by Owais Mushtaq et al. (2022), the antibacterial activity of green ZnO nanoparticles synthesized through saffron extract was assessed and MIC obtained from the results was 312 µg/mL against *Bacillus subtilis*. So, it is demonstrated that our green synthesized ZnO nanoparticles have good antibacterial properties.

Antibiofilm studies of green synthesized ZnO nanoparticles

Pre-treatment and post-treatment of nanoparticles

The effect of green synthesized ZnO NPs on biofilm formation and preformed/matured biofilms was examined by using a crystal violet assay. In the pre-treatment method, the biofilm formation on the coverslip was investigated along with the treatment of various concentrations of ZnO NPs. In the post-treatment method, the biofilm was first formed on coverslips without exposing them to NPs and then the effect of nanoparticles was investigated on preformed biofilms. In this experiment, the crystal violet dye was quantified, and the percentage of biofilm biomass was calculated using the equation described earlier. The data showed that in the pre-treatment method, the % biofilm biomass for 0.5×MIC, 0.75×MIC and MIC values was 54.65 ± 2.80, 36.26 ± 1.70, and 12.47 ± 0.80, respectively (Fig. 6A). In the post-treatment method, the % biofilm biomass for 0.5×MIC, 0.75×MIC and MIC values was 67.23 ± 1.80, 49.18 ± 2.30, and 32.5 ± 1.15, respectively (Fig. 6B). The results demonstrated that as the concentration of nanoparticles increases, the percentage (%) of biofilm biomass reduces in both pre-treatment and post-treatment methods. This reveals that the synthesized ZnO NPs showed a very good antibiofilm effect on both types of treatment and the antibiofilm activity increases in a dose-dependent manner.

Microscopic images of biofilms

The images of biofilms grown on pre-treated and post-treated coverslips were captured under the microscope in the bright field at 40×, to visualize and compare the effect of the green synthesized ZnO NPs at their MIC and Sub-MICs values. In both types of treatment, the biofilm formation was inhibited in a concentration-dependent manner. As the biofilm formation starts with the monolayer adhesion of planktonic bacterium on the surface of the coverslips and association with other bacteria initiates the formation of a microcolony, further which leads to macro colonies and then a fully connected 3-dimensional network of bacteria covered with EPS matrix (Roy et al. 2018).

The pre-treatment of nanoparticles reduces the biofilm formation, and suppresses the macro colonies formation compared to the control, as the concentration of green ZnO NPs increases. In Fig. 7A, it is apparent that in the control, a well-developed biofilm is formed and in the 0.5×MIC, the biofilm can be seen in macro colonies form. At 0.75×MIC, fewer macro colonies can be seen in the biofilm and at the MIC value, the biofilm was formed with microcolonies refers to the initial stage of biofilm

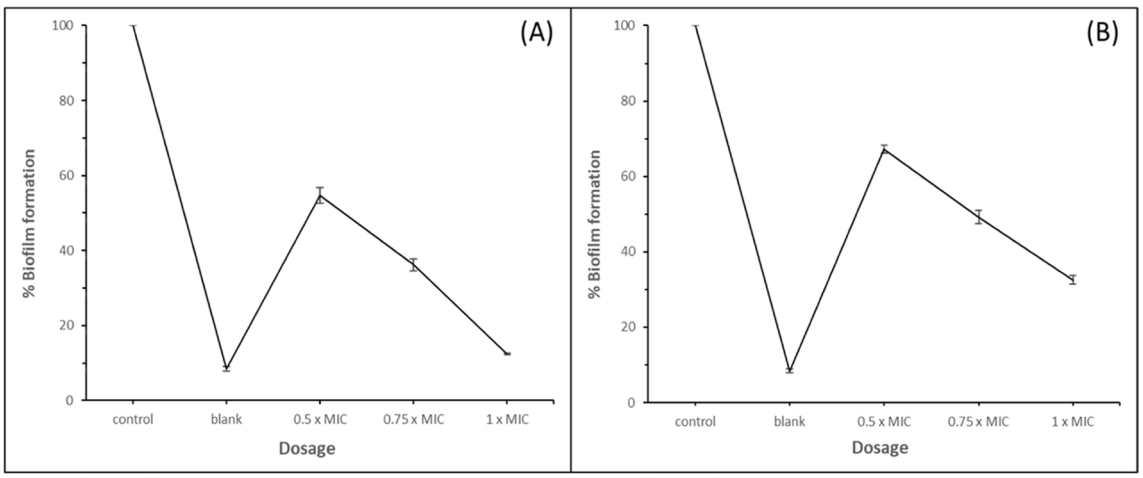

Fig. 6 Line plot of % biofilm biomass treated with ZnO NPs. **A** Pre-treatment. **B** Post-treatment. The error bars represent standard deviation

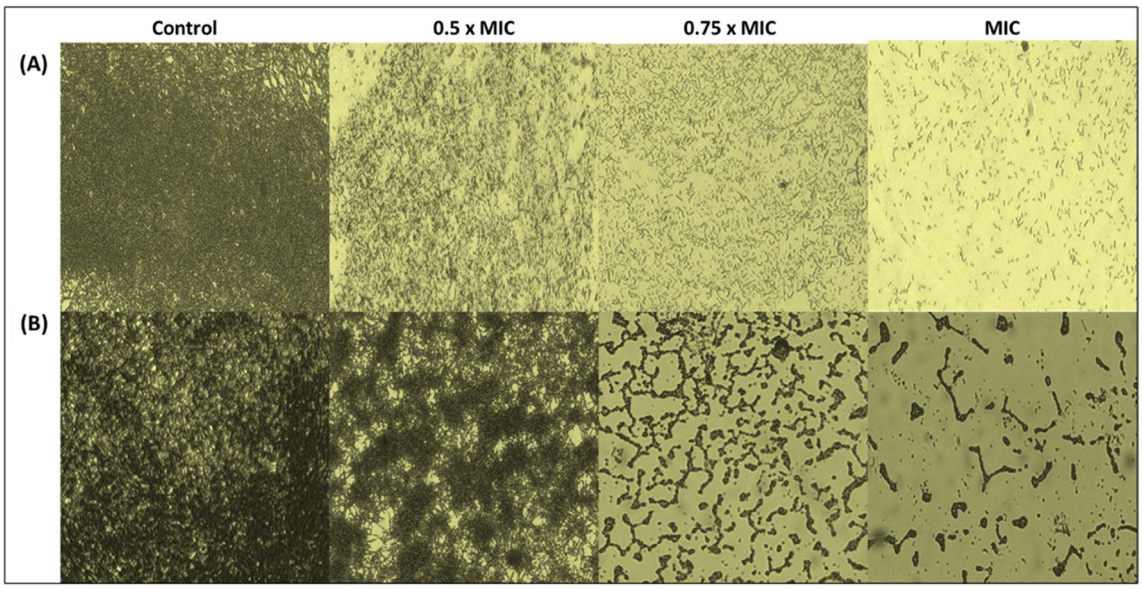

Fig. 7 Microscope images of biofilms on coverslips at 40×. **A** Pre-treated. **B** Post-treated

formation. Similarly, in the post-treatment method, the increasing concentration of nanoparticles affects the preformed biofilms in a dose-dependent manner (Fig. 7B). So, it can be concluded that the green ZnO NPs reduce the adhesion of bacteria on the surface, may delay the formation of biofilm, and disrupt the preformed biofilms in an effective mode in a concentration-dependent manner.

Determination of membrane damage by flow cytometry

We performed flow cytometry analysis of green ZnO NP-treated bacterial cells using propidium iodide (PI) stain to determine cell membrane damage. The propidium iodide can enter into and bind the DNA of only those cells, in which the cell membranes are compromised or damaged known as dead cells. The results demonstrated that the increase in the concentration of nanoparticles also increases the number of PI-treated cells. In the control, only 0.78% of cells were PI-positive or dead cells, whereas, in the treated group, the PI-positive or dead cells were 9.43% and 37.70% for 0.5×MIC and 0.75×MIC, respectively (Fig. 8).

Conclusion

In this study, we have synthesized zinc oxide nanoparticles (ZnO NPs) using the leaves extract of *Saraca asoca* plant. The antibacterial and antibiofilm activity of synthesized green ZnO NPs was measured against the biofilm-producing bacteria *Bacillus subtilis*. The disk diffusion and MIC, MBC data revealed that green synthesized ZnO nanoparticles had a great antibacterial efficacy as the zone of inhibition (ZOI)

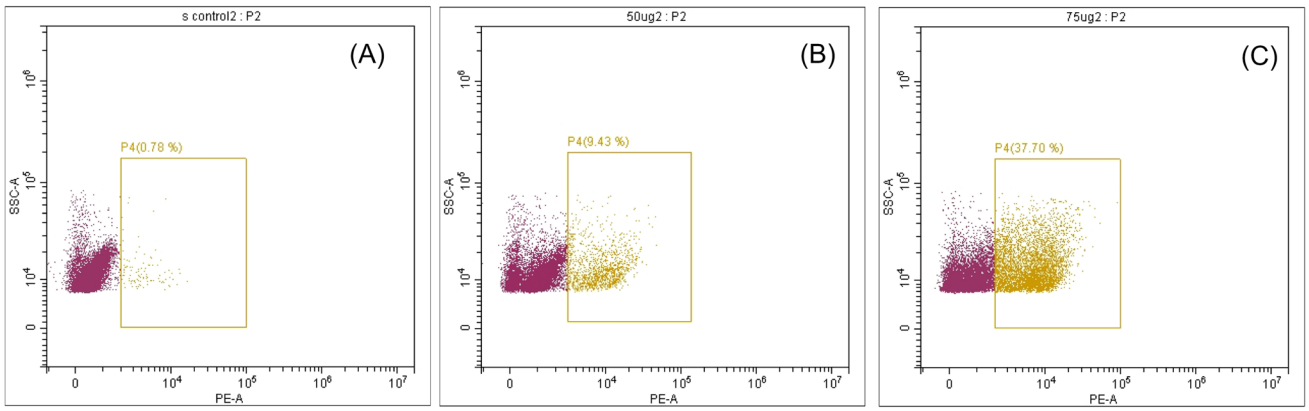

Fig. 8 Flow cytometry data of untreated and treated bacteria: **A** control. **B** 0.5×MIC, and **C** 0.75×MIC

starts at a very low concentration of 0.5 mg/mL and MIC (100 µg/mL) and MBC (150 µg/mL) values were also quite low for green synthesized ZnO nanomaterials. The synthesized nanoparticles exhibited an excellent effect on the biofilm inhibition and stops the biofilm growth at its initial stage as it showed almost 45%, 64%, and 83% inhibition of biofilm growth at 0.5 × MIC, 0.75 × MIC, and 1 × MIC value, respectively. The green ZnO NPs disrupted the preformed or matured biofilms in a concentration-dependent manner and showed a 68%, 50%, and 33% decrease in the % biofilm biomass for the 0.5 × MIC, 0.75 × MIC, and 1 × MIC concentration, respectively. Also, the bacterial cell membrane damage was studied by using flow cytometry. The data reveals that the percentage of dead cells increased with the concentration of NPs in comparison to the control. So, it concluded that the green synthesized ZnO nanoparticles exhibited excellent antibacterial and antibiofilm activity against the biofilm-producing bacteria and it can be a promising alternative agent for the treatment of biofilms and drug-resistant bacteria.

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Author contribution All authors have contributed to the study conception and design Ankush Agrawal: material preparation, data collection and analysis, writing manuscript. Ruhani Sharma: data collection, formal analysis, writing manuscript. Ankita Sharma: data collection, formal analysis. Kailash Chand Gurjar: visualization and editing, Sanjay Kumar: data collection and analysis, writing manuscript. Samit Chatterjee: formal analysis and editing, Harsh Pandey: formal analysis and editing, Kamlendra Awasthi: conceptualization and editing. Anjali Awasthi: conceptualization, experimental designing, supervision and editing. All authors read and approved the final manuscript.

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Data availability All data and materials reported in the manuscripts are available.

Declarations

Ethics approval N/A.

Consent to participate Authors have given their consent to publish the manuscript.

Consent for publication All authors have approved the manuscript for publication.

Competing interests The authors declare no competing interests.

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