Assessment of Molecular Mechanism of Gallate-Polyvinylpyrrolidone-Capped Hybrid Silver Nanoparticles against Carbapenem-Resistant Acinetobacter baumannii

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ABSTRACT: Acinetobacter baumannii is an opportunistic nosocomial pathogen and causes bacteremia, urinary tract infections, meningitis, and pneumonia. The emergence of drug-resistant strain makes most of the current antibiotics ineffective. It is high time to screen some therapeutics against drug-resistant strains. Plant-based medicines have recently emerged as one of the important therapeutic choices. Therefore, in the present study, we have screened the metabolites of Phyllanthus emblica, Ocimum tenuiflorum, and Murraya koenigii for their antibacterial effect against carbapenem-resistant strain (RS-307) of A. baumannii. The result showed that the methanolic extract of P. emblica inhibits the growth of RS-307. The composition of this extract was determined using phytochemical screening and nuclear magnetic resonance (1D and 2D-NMR). The mechanism of action of the plant extract was validated by estimating reactive oxygen species (ROS), lipid peroxidation, protein carbonylation, and membrane damage. The result showed that treatment with this extract showed a significant elevation in the production of ROS generations, lipid peroxidation, and protein carbonylation. This confirms that plant extract treatment confirmed ROS-dependent membrane damage mechanism. The NMR result showed the presence of ethyl gallate, ellagic acid, chebulagic acid, quercetin, flavonoid, and alkaloid. To validate the antimicrobial activity of the secondary metabolite (i.e., gallic acid), we synthesized gallate-polyvinylpyrrolidone-capped hybrid silver nanoparticles (G-PVP−AgNPs) and characterized using Fourier transform infrared spectroscopy (FTIR). G-PVP−AgNPs showed good antimicrobial activity against RS-307, and its mechanism of action was investigated using fluorescence and transmission electron microscopy and FTIR that confirmed ROS-dependent killing mechanism. Therefore, the present study highlighted and recommended the use of G-PVP−AgNPs as suitable therapeutics against carbapenem-resistant A. baumannii.

1. INTRODUCTION

Acinetobacter baumannii is an opportunistic pathogen that mainly causes hospital-acquired infections and emerges in the military patients in Iraq and Kuwait. It causes infections such as pneumonia, bloodstream, and urinary tract infections in the ICUs of hospitals.2−5 It has developed antimicrobial resistance because of mutations that lead to change in target, development of enzymes that inactivate antibiotics, and reduction of access to bacterial targets.2,4 A. baumannii has been termed as an ESKAPE pathogen by the Infectious Diseases Society of America and WHO because of its ability to escape antibiotics.2 Till present, it has become resistant to most of the antibiotics; however, clinical data showed that carbapenem, such as doripenem, meropenem, and imipenem, are effective against A. baumannii, but resistance has emerged against these drugs.6−7 Hospital-acquired infections caused by resistant pathogens are responsible for high morbidity and mortality;6−8 hence, their cure and prevention are required at an urgent basis. Various approaches11−18 have been tried to control the drug-resistant A. baumannii, but the eradication of the carbapenem-resistant A. baumannii is difficult.19 Different plant extracts are traditionally used to control infections and are readily applicable, economic, and reasonable; hence, to eradicate carbapenem-resistant A. baumannii, various plant extracts can play a crucial role. Therefore, in the present study, we have screened different extracts of medicinal plants (P. emblica, M. koenigii, and O. tenuiflorum) for their antimicrobial activity against the carbapenem-resistant A. baumannii. We have identified the secondary metabolites in the selected plants using nuclear magnetic resonance (NMR) and explored their molecular mechanism. The identified secondary metabolite was tagged on PVP-capped silver nanoparticles (PVP−AgNPs), and the molecular mechanism of its antibacterial activity was investigated using biochemical tests, Fourier transform infrared spectroscopy (FTIR), fluorescence microscopy, and transmission electron microscopy (TEM).

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2. RESULTS

In the present study, we have used a pre-isolated clinical strain of *A. baumannii*, that is, RS-307. This strain is a multidrug-resistant clinical strain isolated from the AIIMS, New Delhi. 

Similarly, RS-307 has high MIC for different antibiotics such as amikacin (128 μg/mL), ciprofloxacin (128 μg/mL), erythromycin (128 μg/mL), meropenem (64 μg/mL), colistin (64 μg/mL), polymyxin B (64 μg/mL), minocycline (16 μg/mL), and gentamicin (32 μg/mL), which confirms its multidrug-resistant (MDR) nature.

2.1. Methanol Extract of *P. emblica* Showed Antimicrobial Activity against RS-307. Different plant extracts such as methanol, chloroform, and petroleum ether have been prepared using a Soxhlet extractor for *P. emblica*, *M. koenigii*, and *O. tenuiflorum*. The antimicrobial activity of different plant extracts was tested against carbapenem-resistant strain of *A. baumannii* (RS-307) via the disc diffusion assay method. Disc diffusion assay confirms the best antimicrobial activity in the methanol extract of *P. emblica* (10 μL) with a zone of inhibition of 1.2 cm. In contrast, there was no zone of inhibition for *O. tenuiflorum* and *M. koenigii*. The methanol extract of *P. emblica* was further analyzed for the growth curve of RS-307 in triplicates (result is prepared as mean). The growth curve also confirms the antimicrobial activity of the methanol extract of *P. emblica* (Figure 1).

![Figure 1.](image)

Figure 1. Growth kinetic of *A. baumannii* strain RS-307 was determined in the presence and absence of the methanol extract of *P. emblica* by using a UV–vis spectrophotometer at 605 nm. Data are expressed as mean ± SEM of 3 values (n = 3) (C_{avg}; average of the control, T_{avg}; average of the methanol extract of the *P. emblica* test).

2.2. Methanol Extract of *P. emblica* Enhances Reactive Oxygen Species Production. As the methanol extract of *P. emblica* showed antimicrobial activity, its molecular mechanism of action was determined. NBT assay for untreated (control) and treated *A. baumannii* strain with the methanol extract of *P. emblica* showed a significant increase in the reactive oxygen species (ROS) production in the treated samples as compared to the untreated bacterial samples (Figure 2a). This suggests the contribution of the ROS in the antimicrobial activity of the *P. emblica* extract.

2.3. High Lipid Peroxidation after Treatment with the Methanol Extract of *P. emblica*. The enhanced ROS leads to lipid peroxidation; hence, it was also estimated after treatment with the methanol extract of *P. emblica*. Lipid peroxidation has been estimated for untreated (control) and treated samples in triplicates, and the result are shown in Figure 2b. The result confirms the high lipid peroxidation in RS-307 after treatment with the methanol extract of *P. emblica*.

2.4. Methanolic Extract of *P. emblica* Increases Protein Carbonylation. Protein carbonylation is protein oxidation of amino acid side chains that can be promoted by ROS and can be detected by reaction to produce DNPH-detectable protein products. The protein concentration was estimated via Bradford assay, and 150 μg/mL protein was used in the present experiment. Protein carbonylation was detected by the DNPH method. The result showed that the methanol extract of *P. emblica* enhances the carbonyl content of RS-307 as compared to the untreated sample (Figure 2c). The result further supports the ROS-dependent mechanism.

2.5. Enhanced Membrane Leakage of Reducing Sugars and DNA. The formation of ROS and lipid peroxidation will cause membrane damage; hence, we have estimated the membrane damage of RS-307 in the presence of the methanol extract of *P. emblica*. The membrane damage will cause the leakage of the reducing sugar and DNA of bacterium, and the result showed that membrane leakage of reducing sugars and DNA was found to be increased after treatment with the methanol extract of *P. emblica*.

2.6. Phytochemical Components of *P. emblica*. The methanol extract of *P. emblica* showed good antimicrobial activity against RS-307; hence, we performed phytochemical screening. The phytochemical screening showed that it has tannins, phenol, glycosides, alkaloids, anatheraquinone, phytosterol, cuomarin, and chalcone; hence, it is speculated that the antimicrobial activity of *P. emblica* may be associated with these compounds (Table 1).

2.7. NMR Spectroscopy Result Confirms Different Metabolites in the Methanolic Extract of *P. emblica*. The NMR spectroscopy result confirmed the presence of different primary and secondary metabolites in the methanol extract of *P. emblica*. It contains primary metabolites such as sucrose, alpha-glucose, beta-glucose, citrate, proline, GABA, alanine, threonine, valine, and beta-sitosterol and secondary metabolites such as ethyl gallate (a phenolic acid), ellagic acid (a phenol), chebulagic acid (a tannin), quercetin (a flavonoid), and alkaloid (Figure 3). The gallic acid derivative (ethyl gallate) is the major secondary metabolite identified in the methanolic extract of *P. emblica*.

2.8. Gallic Acid is Capped to the PVP–AgNPs. PVP–AgNPs have shown antimicrobial activity against carbapenem-resistant *A. baumannii*. Hence, gallate-capped PVP–AgNPs were synthesized and validated for their antimicrobial activity against this strain. The gallic acid-capped PVP–AgNPs (G-PVP–AgNPs) are characterized by FTIR. The various modes of vibrations were recognized and given to define the different functional groups present in the given samples. FTIR spectra of the G-PVP–AgNPs is characterized by the presence of the major band at 3438, 2928, 1600, 1383, 1289, 1223, and 1079 cm⁻¹ and minor peaks at 2875, 1432, and 578 cm⁻¹ (Figure 4). The 3438 cm⁻¹ band corresponds to the stretching vibration of O–H and N–H groups, while the 2928 cm⁻¹ band is due to the O–H stretching of carboxylic acid and C–H stretching of alkane. The band at 1600 cm⁻¹ corresponds to C=C stretching of alkene and C–H bending of an aromatic compound. The band 1383 cm⁻¹ is due to the O–H bending of alcohol, C–H bending of aldehyde, O–H bending of
phenol, and C–H bending of alkane. A band at 1289 cm$^{-1}$ corresponds to the C–N stretching of aromatic amines and C–O stretching of the aromatic ester. The band at 1223 cm$^{-1}$ corresponds to the C–O stretching of alkyl aryl ether or vinyl ether and C–N stretching of amine. The band at 1079 cm$^{-1}$ corresponds to the C–O stretching of primary alcohol and C–N stretching of amines. The FTIR result confirms the presence of $\text{–OH}$, $\text{–NH}$, C=$\equiv$C, and $\text{–COOH}$ groups and the aromatic ring that indicates the presence of gallic acid and PVP. The FTIR pattern is similar to the PVP-capped AgNPs but the more intense peak at 3438 and 1383 cm$^{-1}$.

**2.9. Gallate-Capped PVP–AgNPs Have Better Anti-microbial Activity than PVP–AgNPs Alone.** We have tested the antimicrobial activity of G-PVP–AgNPs, and the result showed that G-PVP–AgNPs have better antimicrobial activity as compared to the PVP–AgNPs or methanol extract of *P. emblica* alone. Therefore, it is suggested that G-PVP–AgNPs might be better than PVP–AgNPs to control the carbapenem-resistant *A. baumannii*.

**2.10. Fluorescence Microscopy Confirms More Dead Cells after Treatment with G-PVP–AgNPs.** Viability assays using the fluorescent dyes propidium iodide (PI) and acridine orange (AO) have been developed for the concurrent determination of both nonviable and viable cells. PI is impervious to unbroken membranes but easily permeates the membranes of nonviable cells and binds to RNA or DNA, generating orange-red fluorescence. AO is a cationic dye that can pervade through the membrane and shackle to nucleic acids of viable cells and those at low concentrations, causing green fluorescence. When PI and AO are used concurrently,
was a significant shift in the FTIR spectra of amide phospholipids carbohydrates, proteins, and nucleic acids. The untreated samples related to FTIR spectra of molecules such as scale bar is shown in pixels (px) where 1 px is 264.58 µm. Also results in the damage of lipids, which is evident from the shift in spectra from 2928 to 2922 cm⁻¹ in the treated samples. Treatment of G-PVP–AgNPs also resulted in a shift of alpha-helix in a range of 1659–1637 cm⁻¹. There was also a significant change in phospholipids that can be seen from the shift in the peak from 1236 cm⁻¹ in the control to 1239 cm⁻¹ in the G-PVP–AgNP-treated sample. Similarly, change in the polysaccharides is also observed, which is evident from a shift in the peak from 1064 cm⁻¹ in the control to 1053 and 1055 cm⁻¹ in the G-PVP–AgNPs and PVP–AgNPs, respectively. All the results showed that the treatment of G-PVP–AgNPs (Figure 6D) showed the alteration in the membrane components as compared to the control (Figure 6A).

2.12. Scanning Electron Microscopy Confirms Membrane Disruption by G-PVP–AgNPs. Field emission scanning electron microscopy (FESEM) analysis of A. baumannii bacterial cells showed an observable change in the cellular morphology of treated bacterial samples (Figure 7) as compared to the control. The result showed that the treatment resulted in the loss of cellular integrity and membrane rupture, which further supports the results from other techniques such as FTIR. The result showed that in all the treated samples, G-PVP–AgNPs showed the best antimicrobial activity. There is complete lysis of the bacterium after G-PVP–AgNP treatment that can be seen in the collection of the death cell/damage membrane (Figure 7D,H).

3. DISCUSSION

A. baumannii causes hospital-acquired infections such as pneumonia and bloodstream infections. Carbapenem is a last-resort antibiotics agent, and emergence of multidrug resistance, mainly carbapenem resistance, is becoming a very serious public health risk. Antibiotics are being used against it, but it has acquired resistance against most of the antibiotics. 

Traditionally used medicinal plants can be an alternative to the present therapeutics as they do not have any side effects. These plant extracts have shown antibacterial activity against various bacteria. In the present study, we have explored P. emblica, M. koenigii, and O. tenuiflorum for their bioactive compounds against the carbapenem-resistant A. baumannii. Disc diffusion assay of A. baumannii has shown varied effects in the presence and absence of different plant extracts. The large zone of inhibition was observed in the methanol extract of P. emblica, while the smaller zone of inhibition was seen during treatment with O. tenuiflorum and M. koenigii. The methanol extract of P. emblica was further investigated for growth analysis and found that the treatment decreases the growth of the RS-307. To validate the molecular mechanism of action, we have investigated the production of ROS, peroxidation of lipid,
CARBONYLATION OF PROTEINS, AND LEAKAGE OF THE MEMBRANE. ROS ESTIMATION CONFIRMS THE SIGNIFICANT INCREASE IN ROS PRODUCTION AFTER TREATMENT WITH THE METHANOL EXTRACT OF P. EMBLICA. ROS CAN CAUSE MODIFICATION OF MACROMOLECULES SUCH AS LIPIDS AND PROTEINS, WHICH LEADS TO LOSS OF FUNCTION, MEMBRANE DAMAGE, AND ELEVATED MUTAGENESIS THAT LEADS TO BACTERIAL DEATH WHEN ROS PRODUCTION CrossES BACTERIUM’S DETOXIFICATION AND REPAIR CAPABILITY. UPREGULATED ROS PRODUCTION FURTHER ENHANCES LIPID PEROXIDATION, PROTEIN CARBONYLATION, AND MEMBRANE RUPTURE THAT CAN BE SEEN FROM INCREASE IN THE LEAKAGE OF REDUCED SUGAR AND DNA. MEMBRANE DAMAGE DISTURBS ENERGY PRODUCTION BY OXIDATIVE PHOSPHORYLATION AND OTHER MEMBRANE-ASSOCIATED ACTIVITY. TO IDENTIFY THE ACTIVE SECONDARY METABOLITE IN THE METHANOL EXTRACT RESPONSIBLE FOR ROS PRODUCTION, WE HAVE PERFORMED THE QUALITATIVE PHYTOCHEMICAL SCREENING USING DIFFERENT CHEMICAL METHODS; IT IDENTIFIES THE PRESENCE OF TANNINS, PHENOL, GLYCOSIDES, ALKALOIDS, ANTHRAQUINONE, PHYTOSTEROL, COUMARIN, AND CHALCONE. TO QUANTITATE THE SECONDARY METABOLITE, 1D AND 2D-NMR WERE RUN FOR THIS METHANOL EXTRACT OF P. EMBLICA AND THE RESULT CONFIRMS THE PRESENCE OF THE GALLATE DERIVATIVE (ETHYL GALLATE) IN THE HIGHEST QUANTITY. WE HAVE PREVIOUSLY SHOWN THAT THE PVP−AGNPS ARE ACTIVE AGAINST THE CARBAPENEM-RESISTANT A. BAUMANNII;15 HENCE, WE HAVE SYNTHESIZED GALLATE-COATED PVP−AGNPS (G-PVP−AGNPS) AND TESTED THEIR ANTIMICROBIAL ACTIVITY. THE RESULT SHOWED THAT G-PVP−AGNPS HAVE BETTER ANTIMICROBIAL ACTIVITY THAN PVP−AGNPS. THE MOLECULAR MECHANISM OF THESE AGNPS WAS ALSO IDENTIFIED USING BIOCHEMICAL ASSAY, FLUORESCENCE MICROSCOPY, FTIR, AND TEM. THE HYBRID-CAPPED G-PVP−AGNPS RESULT IN MORE ROS PRODUCTION, LIPID PEROXIDATION, AND MORE PROTEIN CARBONYLATION AS COMPARED TO OTHER ANTIMICROBIAL TESTED ONES AND THE CONTROL. THE RESULT SHOWED THAT THERE ARE MORE DEAD CELLS AFTER G-PVP−AGNPS (FLUORESCENCE MICROSCOPY), MEMBRANE DISRUPTION (TEM RESULT), AND CHANGE IN MEMBRANE COMPOSITIONS (FTIR RESULT) AS COMPARED TO OTHER TREATMENTS AND THE CONTROL. THIS STUDY ENLIGHTENS THE ROLE OF THE HERBAL COMPONENT OF P. EMBLICA AND G-PVP−AGNPS TO CONTROL THE INFECTION OF THE CARBAPENEM-RESISTANT STRAIN OF A. BAUMANNII AND CAN BE FURTHER EXPLORED TO DEVELOP SOME ALTERNATIVE THERAPEUTICS.

4. CONCLUSIONS

The present study concludes that the methanol extract of the P. emblica is active against carbapenem-resistant A. baumannii. It was also observed that the methanol extract of P. emblica is more effective as compared to various other solvents used for...
the preparation of the plant extracts. The present study also emphasized the significance of G-PVP–AgNPs against multi-drug-resistant A. baumannii. It significantly enhances the ROS production that causes leakage of the membrane, elevation in the lipid peroxidation, and an increase in protein carbonylation in carbapenem-resistant A. baumannii and hence follows an ROS-dependent killing mechanism. Hence, the herbal-active compound (gallate) of P. emblica when tagged with AgNPs may play a significant role to eliminate A. baumannii and control the nosocomial infection caused by carbapenem-resistant strain of A. baumannii.

5. MATERIALS AND METHODS

5.1. Reagents. Luria Bertani (LB) broth, sodium dodecyl sulfate, sodium chloride, 2-thiobarbituric acid (TBA), guanidine hydrochloride, trichloroacetic acid (TCA), ethanol, nitroblue tetrazolium (NBT), phosphate buffer, 2,4-dinitrophenyl hydrazine (DNPH), and methanol were purchased from HiMedia, India. Hydrochloric acid, glycine, Tris-HCl, and NaCl were purchased from Merck, India. Ethyl acetate, methanol, petroleum ether, and chloroform were purchased from Fisher Scientific. Leaves of plants such as P. emblica (identification no. RUBL211692), M. koenigii (Idt. no.: RUBL211706), and O. tenuiflorum (Idt. no. RUBL211707) were obtained locally and identified.

5.2. Preparation of Plant Extracts. The plant samples were collected and air-dried. Air-dried plant samples (1 g) were used in a Soxhlet extractor using methanol, chloroform, and petroleum-ether as solvents. The plant extracts were air-dried, weighed, and resuspended in distilled water (for the methanol and chloroform extracts) and distilled water with 1% DMSO (for the petroleum ether extract).

5.3. Growth Kinetics and Disc Diffusion Test. In the present study, pre-isolated clinical carbapenem-resistant strain (RS-307) of A. baumannii was used, which is accessible in our laboratory at the Central University of Rajasthan. MIC showed that RS-307 strain has high resistance to carbapenems (meropenem and imipenem) and other antibiotics; hence, they are multidrug resistant strains. Growth kinetics was analyzed by taking absorbance at 605 nm at a time interval of 30 min to check the effect of different plant extracts against A. baumannii, using a UV–vis spectrophotometer. Treatment of 100 μL of the plant extract per 100 mL of bacterial culture was given at 0.4 OD. Disc diffusion tests of different extracts of P. emblica, M. koenigii, and O. tenuiflorum were performed on RS-307 as per published methods.

5.4. Phytochemical Screening of Plant Extracts Using Chemical Methods. The methanol extracts of plants showed antimicrobial activity; hence, they were analyzed for the presence of tannins, saponins, terpenoids, chalcone, coumarins, glycosides, cardiac glycosides, steroids, alkaloids, quinones, anthraquinone, phenols, and anthocyanin as per our published protocol.

5.5. Identification of Metabolites in P. emblica Using 1D-NMR and TOCSY 2D NMR. The methanol extract of P. emblica showed the best antimicrobial activity; hence, its metabolite was characterized by NMR. The 1H NMR spectra of the methanol extract were recorded using a 700 MHz NMR (Agilent, USA) spectrometer equipped with a room-temperature triple-resonance probe. 1D and 2D NMR spectra of dry extracts were carried out by dissolving the samples in 600 μL of D2O in 5 mm NMR tubes with deuterated trimethylsilyl propionate (TSP) in a coaxial insert as an external reference. 1D 1H NMR spectrum with water suppression was acquired with a single 90° pulse using a spectral width of 9124.1 Hz, a relaxation delay of 14 s, 32 K data points, an acquisition time of 3.591 s, and 64 numbers of scans with 8 dummy scans. The free induction decays (FIDs) were multiplied by an exponentially decaying function prior to Fourier transformation with a line broadening of 0.3 Hz. The 1H NMR spectra of plant extracts were manually phased, and an automated baseline was corrected using Vnmr 3.2A (Agilent, USA). The 1H NMR spectra were referenced to the TSP resonance at 0.0 ppm. To confirm the assignments, two-dimensional 1H–1H total-correlated spectroscopy (zTOCSY) was performed. Assignments were further confirmed by comparing them with the existing literature values, spectral library of phytochemicals, and spectral data libraries.

5.6. Estimation of ROS via Nitro Blue Tetrazolium Assay. The reactive oxygen production in the bacterial cell was estimated on a multowell ELISA reader using the in vitro conversion of nitroblue tetrazolium (NBT) to blue/purple-colored formazan crystals via the superoxide anion as per the published method.

5.7. Estimation of Lipid Peroxidation Using TBRAS (TBA Reactive Substance) Assay. ROS causes peroxidation of the polyunsaturated fatty acid yielding α,β-unsaturated aldehydes such as malondialdehyde (MDA). MDA interacts with thiobarbituric acid (TBA) and produces a pink-colored dimeric compound, quantified by the ELISA reader.

5.8. Estimation of Protein Carbonylation. Protein carboxyls are created by oxidation of side chains of amino acid. The protein carbonyl group (C=O) on oxidized amino acids is quantified by dinitrophenylhydrazine (DNPH) that forms a stable dinitrophenyl (DNP) hydrazine product. The published method is used for the estimation of protein carbonylation.

5.9. Effect of Plant Extracts on Membrane Leakage of Sugars and DNA. The effect of the plant extract on membrane leakage of sugars (reducing) and DNA from the cells was quantified using the published protocol.

5.10. Synthesis of Gallate-Coated PVP–AgNPs and Their Antimicrobial Activity. We have previously shown that the PVP–AgNPs are active against carbapenem-resistant A. baumannii. The NMR result confirms that the gallate derivative is the most important component of the methanol extract of P. emblica. Hence, we have capped PVP–AgNPs with gallate as per the published protocol. In brief, 5 mL of AgNO3 (0.01 M) was mixed with 10 mL of 2-methoxyethanol and stirred for two h at 37 °C. Polyvinylpyrrolidone (PVP, 0.2 g) was added and stirred for 10 min. This is followed by the addition of 280 μL of 5 mM gallic acid (prepared in 25 mM NaOH) and stirring for 10 min. The capped G-PVP–AgNPs were characterized by UV–vis and FTIR spectroscopy.
The characterized G-PVP–AgNP was also tested for its antimicrobial activity against RS-307 using disc diffusion assay as per our published method.12

5.11. Fluorescence Microscopy. We determined the live/dead cells in the same treated and untreated bacterial sample by performing fluorescence microscopy as per the published method.29 First, 1 mL of bacterial culture was harvested by centrifugation at 13,000 rpm for 10 min. The pellet was suspended in 500 μL of PBS containing 1% Triton X-100. AO (27 μL, 1 mg/mL) and 50 μL PI (1 mg/mL) were added to this solution and incubated in the dark for 30 min at room temperature. The solution was centrifuged at 7,000 rpm for 10 min. The pellet was dissolved in 100 μL of PBS. Subsequently, a thin smear (10 μL) of this suspension was prepared on the glass slides and observed under a fluorescence microscope using a blue filter.

5.12. Field Emission Scanning Electron Microscopy. FESEM is a useful technique to study the morphological features of bacteria.30 SEM analysis was performed as per the published method.29 The bacterial suspension were grown in LB broth until the O.D. reached 0.4, and divided into four sets. One set was kept untreated. Other sets were treated with 100 μL of the methanol extract of P. emblica, PVP–AgNPs, and G-PVP–AgNPs. All the bacterial culture were incubated at 37 °C until the OD of the control reached 1.0. Bacterial cells (4 mL, treated and untreated) were centrifuged at 15,000 rpm for 15 min at 4 °C. The obtained pellets were washed with 100 μL of PBS (2 times) to remove the remaining medium before the pellets were fixed with glutaraldehyde. A solution (100 μL) of 2.5% glutaraldehyde in PBS (pH 7) was added to the pellet and incubated for 2.5 h at 4 °C. After fixation, cells were centrifuged at 15,000 rpm for 15 min. The pellet was washed four times with PBS (100 μL, 15,000 rpm, 15 min) at 4 °C. Finally, 20 μL of PBS was added to the pellet and mixed, and one drop of this suspension was placed on the glass coverslip. The smear was allowed to air dry and then dehydrated with ethanol gradients (35, 50, 75, 95, and 100%) and air-dried overnight. The fixed bacterial smears were then coated with a layer of gold (Quorum), and the conductive samples were examined with SEM (Nova NanoSEM 450).

5.13. FTIR Spectroscopy. Membrane changes were also monitored using FTIR spectra as per the published method.29 Bacterial samples in different conditions were fixed and dehydrated as per methods used above for SEM. The FTIR spectra were recorded in the transmission mode by the potassium bromide (KBr) disc method with the PerkinElmer spectrophotometer version 10.4 with deuterated triglycerine sulphate (DTGS) detector elements. The samples pellet was mixed with potassium bromide (KBr) and grinded in a mortar and pestle. FTIR data were recorded in the wavelength range 4,000–400 cm⁻¹. The obtained background from the KBr disc was automatically subtracted from the prepared KBr and sample disc spectra.

5.14. Statistical Analysis. Data were analyzed by a Student’s t-test, and a value of p < 0.05 was considered significant. The analysis was performed using GraphPad Prism (version 6) and Microsoft Excel.

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