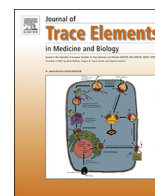




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## Toxicology

## Bactericidal assessment of nano-silver on emerging and re-emerging human pathogens

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## ABSTRACT

With the threat of the growing number of bacteria resistant to antibiotics, the re-emergence of previously deadly infections and the emergence of new infections, there is an urgent need for novel therapeutic agent. Silver in the nano form, which is being used increasingly as antibacterial agents, may extend its antibacterial application to emerging and re-emerging multidrug-resistant pathogens, the main cause of nosocomial diseases worldwide. In the present study, a completely bottom up method to prepare green nano-silver was used. To explore the action of nano-silver on emerging *Bacillus megaterium* MTCC 7192 and re-emerging *Pseudomonas aeruginosa* MTCC 741 pathogenic bacteria, the study includes an analysis of the bacterial membrane damage through Scanning Electron Microscope (SEM) as well as alternation of zeta potential and intracellular leakages. In this work, we observed genuine bactericidal property of nano-silver as compare to broad spectrum antibiotics against emerging and re-emerging mode. After being exposed to nano-silver, the membrane becomes scattered from their original ordered arrangement based on SEM observation. Moreover, our results also suggested that alternation of zeta potential enhanced membrane permeability, and beyond a critical point, it leads to cell death. The leakages of intracellular constituents were confirmed by Gas Chromatography-Mass Spectrometry (GC-MS). In conclusion, the combine results suggested that at a specific dose, nano-silver may destroy the structure of bacterial membrane and depress its activity, which causes bacteria to die eventually.

## 1. Introduction

Antibiotics deserve much of the credit for the dramatic increase in life expectancy around the world in the 20th century. A famous physician expert once noted that the discovery of penicillin in the early 1940s showed more curative power to a lone provider than the collective talent of all the physicians in New York at that time [1]. Unfortunately, it is inevitable that, after sometime, bacteria develop resistance to existing antibiotics, making infectious disease more difficult to treat [2]. The resistance profile has probably been smouldering issue for years, but nowadays it's almost like a switch got triggered. Infectious disease experts are alarmed by the prospect that effective antibiotics may not be available to control the re-emergence of previously deadly infections and the emergence of new infections caused by drug-resistant bacteria in near future. Recent outbreaks of pneumonia (*Pseudomonas aeruginosa*) and tuberculosis (*Mycobacterium tuberculosis*), for example, both of which have re-emerged within the region itself, have also focused regional, national and international attention on the threat developed by re-emerging infectious diseases and in particular

on southeast Asia as the epicentre of these types of diseases. Indeed, tuberculosis and pneumonia are re-emerging in a drug-resistant form [3,4].

Southeast Asia is also a hotspot for emerging infectious disease in particular, zoonotic diseases as a result of many factors including population growth, mobility and environmental changes. Severe Acute Respiratory Syndrome (SARS), Nipah virus and the most recent pandemic influenza A H1N1 are only a few of many examples of emerging infectious diseases in the southeast Asia; each of these diseases has caused global societal impact related to unexpected illnesses [5]. Other emerging infectious diseases caused by bacteria are less terrible than these examples; however, they nonetheless may take a significant increase in morbidity as well as cost related to prolong treatments. In recent years, the infection caused by bacteria in man to have probably emerged in china was brain abscess during infection with *Bacillus megaterium* [6]. To the best of our knowledge, infections caused by these bacteria are rare and have not been reported as the cause of any important clinical diseases [7,8]. While it is not known which new infectious diseases will emerge tomorrow, populations have to be

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protected by staying one step ahead of the microbes by searching a new antimicrobial drugs. Moreover, researchers also discovered some bacterial species that are resistant to different classes of antibiotics, including new synthetic drugs, even though these bacteria have never exposed to antibiotics [9,10]. These studies support a hypothesis that antibiotic resistance is genetically rich natural phenomenon, deeply embedded in the bacterial pan-genome in which bacteria evolve; it can be slowed but not stopped. If these types of non-pathogenic bacteria create any health problems, then it's very difficult to control them by conventional antibiotics. Hence, we need new antibiotics to control resistant bacteria, which must act at several cellular and molecular levels. The mode of action of nano-silver is not known for specific at single sight but to influence bacterial membrane permeability and many metabolic pathways at same time.

The present study aimed to understand the antibacterial activity and acting mechanism of nano-silver, the mechanism of inhibition against emerging *B. megaterium* MTCC 7192 and re-emerging *P. aeruginosa* MTCC 741 through alternation of overall surface charges and destroying membranous structure with consequent leakages of important intracellular constituents such as amino acids, fatty acids, organic acids and sugar acids. The study represented a potential template for the design of novel antibacterial agents to overcome the issue of emerging and re-emerging infectious diseases.

## 2. Materials and methods

### 2.1. Bacterial strains and reagents

The medicinal plant, *Tinospora cordifolia* was selected from Gir forest region of Gujarat, India on the basis of its medicinal property [11]. Silver nitrate, AR grade was procured from Thermo Fisher Scientific, Vadodara, India. The two bacterial strains one Gram positive (*Bacillus megaterium* MTCC 7192) and one Gram negative (*Pseudomonas aeruginosa* MTCC 741) are clinical isolates that were obtained from the microbial type culture collection (MTCC), Institute of microbial technology, Chandigarh, India. Bacterial strains were cultivated on Nutrient broth medium (pH 7.3 ± 0.1; HiMedia, Vadodara, India) at 37 °C with shaking at 150 rpm. Bacterial cell suspensions were diluted with a sterile Milli-Q water (pH 7) to obtain a final concentration of 10<sup>8</sup> CFU/ml. Four broad spectrum antibiotics, namely, amoxicillin (Sun pharmaceutical, Ankleshwar, India), chloramphenicol (Alps pharmaceuticals, Uttarakhand, India), trimethoprim-sulfamethoxazole (Abbott Healthcare, Mumbai, Maharashtra), and linezolid (Glenmark pharmaceutical, Ankleshwar, India) were used for bactericidal assay. All other chemicals used in this study were analytical grade (Sigma Aldrich, Ahmedabad, India).

### 2.2. Synthesis and characterization of green nano-silver

For the preparation of aqueous extract, 10 g of dry stem powder was boiled in a conical flask with 150 ml of deionised water for 10 min at 70 °C on magnetic stirrer at 500 rpm, then the mixture was filtered through WhatmanNo.1 filter paper, and the stem extract was collected and stored at 4 °C for further use in synthesis of nano-silver.

Aqueous solution (10<sup>-3</sup> M) of silver nitrate (AgNO<sub>3</sub>) was prepared and stem extract of *T. cordifolia* was added (1:9 ratio) in dark condition for the reduction of silver nitrate at room temperature [12]. The bio-reduction of silver nitrate was confirmed by UV-vis spectrophotometric (Agilent Technologies, Cary 60) analysis. Dynamic Light Scattering (DLS) technique was used to determine the size and the potential stability of the nano-silver in the suspension using Nanotracc Wave (S3500). The surface morphology of nano-silver along with elemental analysis was determined by scanning electron microscope (Zeiss, EVO-18) coupled with an energy dispersive X-ray analysis (EDX) facility by applying an acceleration voltage of 15 kV.

### 2.3. Comparative in vitro antibacterial activity of nano-silver with broad spectrum antibiotics

The comparative antibacterial activities of nano-silver and broad spectrum antibiotics was effectively accessed against emerging pathogens *Bacillus megaterium* MTCC 7192 and re-emerging pathogens *Pseudomonas aeruginosa* MTCC 741 using agar well diffusion assay method [13]. The drug of choice for each tested strain was used to prove the unique bactericidal action of nano-silver compared to conventional broad spectrum antibiotics, namely, amoxicillin (Inhibits bacterial cell wall synthesis), trimethoprim-sulfamethoxazole (Inhibits synthesis of proteins and nucleic acids), chloramphenicol (Inhibits bacterial protein synthesis by preventing elongation process) and linezolid (Inhibits the initiation process in bacterial protein synthesis at a very earlier stage) [14]. The concentration of nano-silver and antibiotics was adjusted to 100 µg/ml, and the concentration of bacterial cells was 10<sup>8</sup> CFU/ml. After incubation at 37 °C for 24 h the zones of inhibition was measured [15]. The assays were performed with three replications and standard deviation between replication was calculated.

### 2.4. Bacterial morphology influenced by nano-silver

Samples from bacterial cultures (*Bacillus megaterium* MTCC 7192 and *Pseudomonas aeruginosa* MTCC 741), mixed with 100 µg/ml of nano-silver, were collected at 3 h and pre-fixed with 2.5% glutaraldehyde for 30 min; these were then washed two times in the same buffer and post-fixed for 2 h in 1% osmium tetroxide. After washing with buffer, dehydration process was conducted with 30, 50, 70, 80, 90 and 100% of ethanol. Prior to analysis by SEM (Zeiss, Model EVO-18), the samples were dried at 40 °C and subjected to analysis with 15 kV accelerating voltage. Control experiment was conducted in absence of nano-silver [16].

### 2.5. Nano-silver bacteria interfacial potential measurement

Bacterial surfaces are characterised by their surface electric charge, which allows the measurement of zeta potential through Nanotracc Wave (Model S3500). The bacterial cultures were harvested by centrifugation at 10,000 rpm for 10 min, and the cell pellets washed three times with 0.1 mM potassium phosphate buffer solution (pH 7.2) and finally re-suspended in the same buffer to the final concentration of 10<sup>8</sup> CFU/ml. The washed bacterial cell suspensions were incubated with 100 µg/ml of nano-silver for 3 h [17]. Zeta potential was also carried out for normal and autoclaved (at 121 °C, 15 psi for 20 min) bacterial cells [18]. The average bacterial size of the sample was also measured in Nanotracc Wave (Model S3500) for autoclaved, non-treated and treated bacterial cells at ambient temperature (28 °C).

### 2.6. Gas Chromatography- Mass Spectrometry (GC-MS) analysis

GC-MS profile was carried out to detect the leakages of intracellular constituents from non-treated and nano-silver treated bacterial cells (same as zeta potential). The known volume of bacterial supernatant (water 100 v/v) were dried at 40 °C in nitrogen turbo evaporator (Biotage, TurboVap®LV); followed by derivatized with 80 µl of a 20 mg/ml methoxyamine hydrochloride solution in 1 ml of pyridine for 90 min at 37 °C on shaker. Subsequently, the samples were silylated for 60 min at 37 °C with 160 µl *N,O*-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) on shaker. The derivatized bacterial supernatants were analyzed with Gas Chromatograph-Mass Spectrometer (GCMS-QP2010 Plus, Shimadzu, Japan) coupled with Shimadzu GCMS-QP2010 SE mass selective detector. About 1 µl aliquots of the supernatants were injected into a DB-17 MS capillary column (60 m × 0.25 mm I.D., 0.25 µm film thickness) using split less injection (230 °C, 1.5 min). Helium gas was used as a carrier gas at a flow rate of 1 ml/min. Leakages of intracellular compounds were identified by mass spectra analysis (50–700 m/z

range) through the National Institute Standard and Technology (NIST) library 2012 and GCMS solution software to calculate the similarity index and above 90% match was considered acceptable [19]. The mass spectrum of identified intracellular compounds of treated bacteria was compared with the spectrum of the non-treated bacteria (control) and identified leakage constituents due to nano-silver treatment on bacterial surface. The GC–MS profile of aqueous stem extract of *T. cordifolia* was also carried out. The common compounds between *T. cordifolia* aqueous extract and nano-silver treatment of bacterial culture as well as between bacterial suspension without silver and nano-silver treatment were identified and subtracted to identify unique compounds (linkages) release due to nano-silver treatment on bacterial surface.

### 3. Results and Discussions

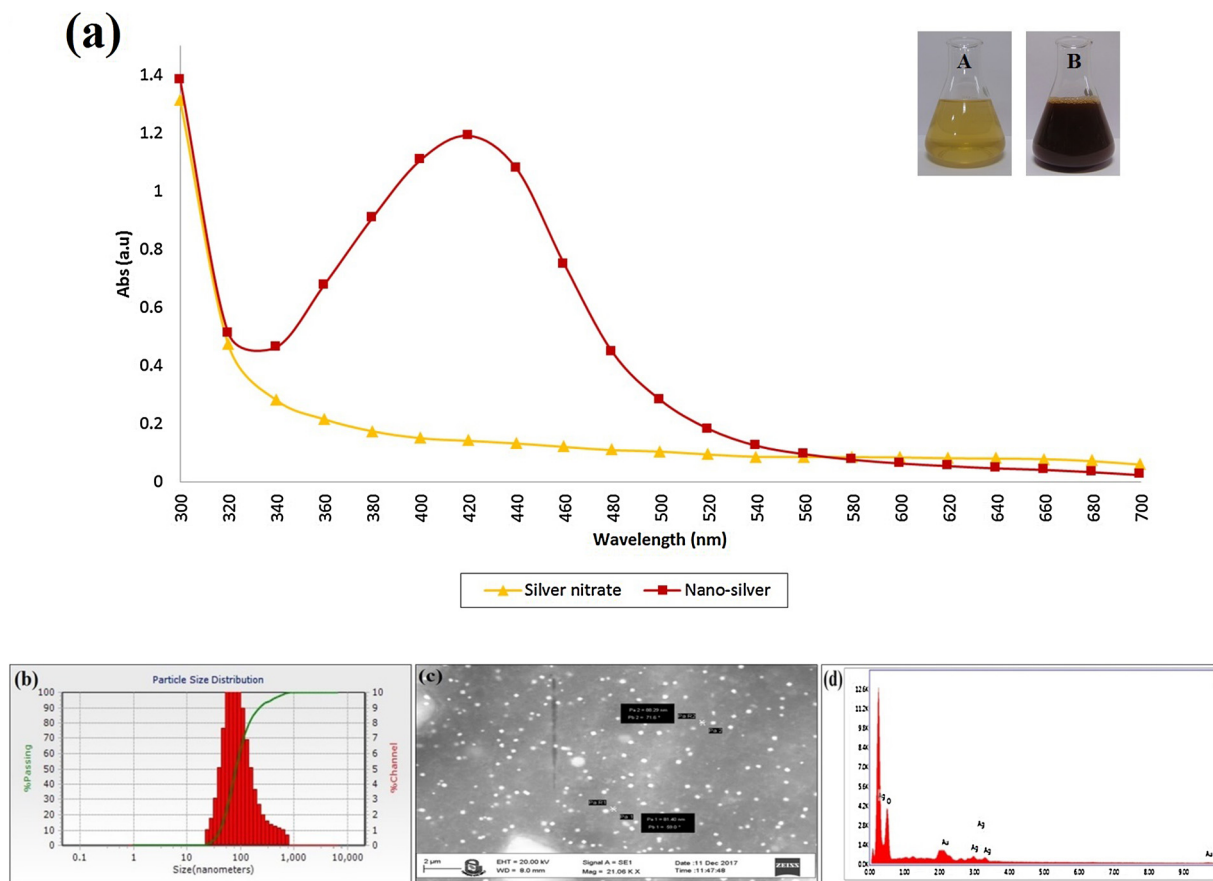
#### 3.1. Synthesis and characterization of nano-silver

The nano-silver was successfully synthesized from aqueous stem extract of *T. cordifolia* by mixing with 1 mM silver nitrate solution. Additions of plant extract into the silver nitrate solution resulted in the gradual change of the colour of silver nitrate solution from light yellow to dark brown, indicating the formation of nano-silver (Fig. 1a). Similar colour changes were reported in previous studies and hence confirmed the completion of reaction between silver nitrate and stem extract [20]. The synthesis of nano-silver was further confirmed by UV–vis spectrophotometer. A strong specific peak for the synthesized nano-silver was obtained at 420 nm (Fig. 1a). Dynamic light scattering data showed that the synthesized nano-silver were in the range of 82.60 nm  $\pm$  0.8 (Fig. 1b), which is well in agreement with SEM result (Fig. 1c). The surface zeta potential value of nano-silver was measured to be slightly

positive and was found to be 16.2 mV (data not shown in Fig. 1). The positive charge of nano-silver prevents them from aggregation and increases their stability, as well as help to enhance their antibacterial activity by interacting with negatively charged biomolecules of bacterial membrane [21]. The SEM image of the sample showed the formation of spherical nano-silver in aggregated form with average 84.84 nm size, which was confirmed to be silver by EDX analysis (Fig. 1c and 1d). The EDX analysis showed a strong peak at 3 keV, which is typical for the absorption of metallic nano-crystallites silver due to unique surface plasmon resonance (SPR), thereby confirming the formation of nano-silver [22]. The presence of additional peak for oxygen indicates that some nano-silver got oxidized by forming silver oxide [23].

#### 3.2. Comparative in vitro antibacterial activity of nano-silver and broad spectrum antibiotics

The comparative antibacterial activities of the nano-silver and broad spectrum antibiotics were assessed against emerging *B. megaterium* (MTCC 7192) and re-emerging *P. aeruginosa* (MTCC 741) bacteria. A direct comparison of the nano-silver and the broad spectrum antibiotics under the same dose range (10  $\mu$ g/100  $\mu$ l for 10<sup>8</sup> CFU/ml inoculum) showed that the tested microorganisms developed resistance to antibiotics belonging to different classes (Fig. 2). The mean of three replicates of the diameter of inhibition zone around each well of nano-silver and broad spectrum antibiotics is represented in Table 1. The inhibition zones of nano-silver obtained in this study indicated that a nano-silver has potential to control emerging and re-emerging multi-drug-resistant pathogens compared to tested antibiotics. These findings further agree with previous results by other researchers, where it was



**Fig. 1.** Characterization of green synthesized nano-silver (a). UV–vis spectra (Inset: Colour changed before-A and after-B synthesis of nano-silver), (b). Particle size distribution by dynamic light scattering, (c). SEM image and (d). EDX spectrum (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

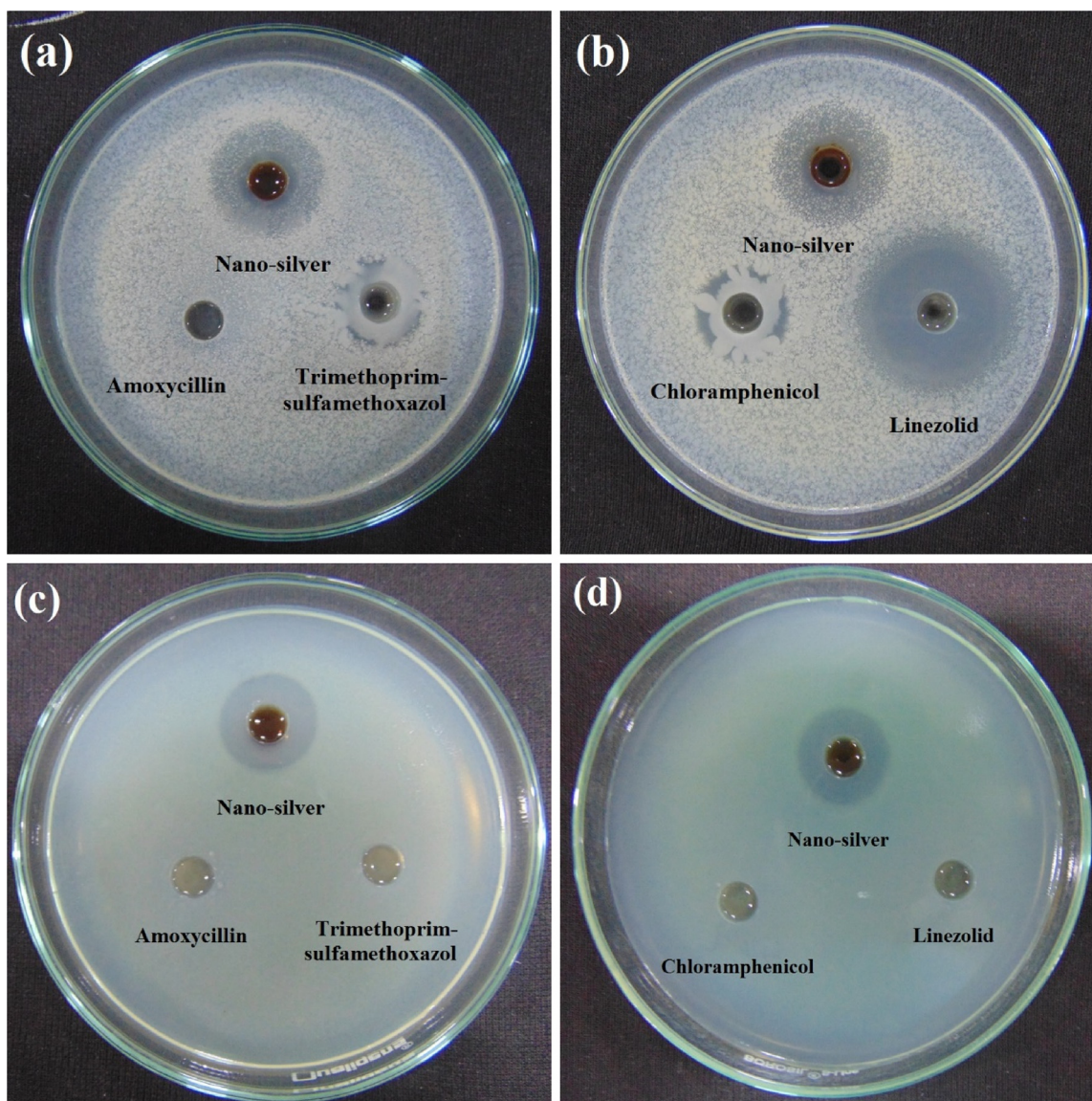


Fig. 2. Showing comparison of antibacterial efficiency of nano-silver with different broad spectrum antibiotics against *B. megaterium* MTCC 7192 (a, b) and *P.aeruginosa* MTCC 741 (c, d).

**Table 1**  
Zone of inhibition of different bioactive agents against emerging *B. megaterium* (MTCC 7192) and re-emerging *P. aeruginosa* (MTCC 741) pathogens.

Bioactive agents	Zone of inhibition (mm)	
	Emerging <i>B. megaterium</i> (MTCC 7192)	Re-emerging <i>P.aeruginosa</i> (MTCC 741)
Amoxicillin	ND	ND
Trimethoprim-sulfamethoxazole	ND	ND
Chloramphenicol	ND	ND
Linezolid	20 ± 1.5	ND
Nano-silver	16 ± 1.2	● ± 1.1

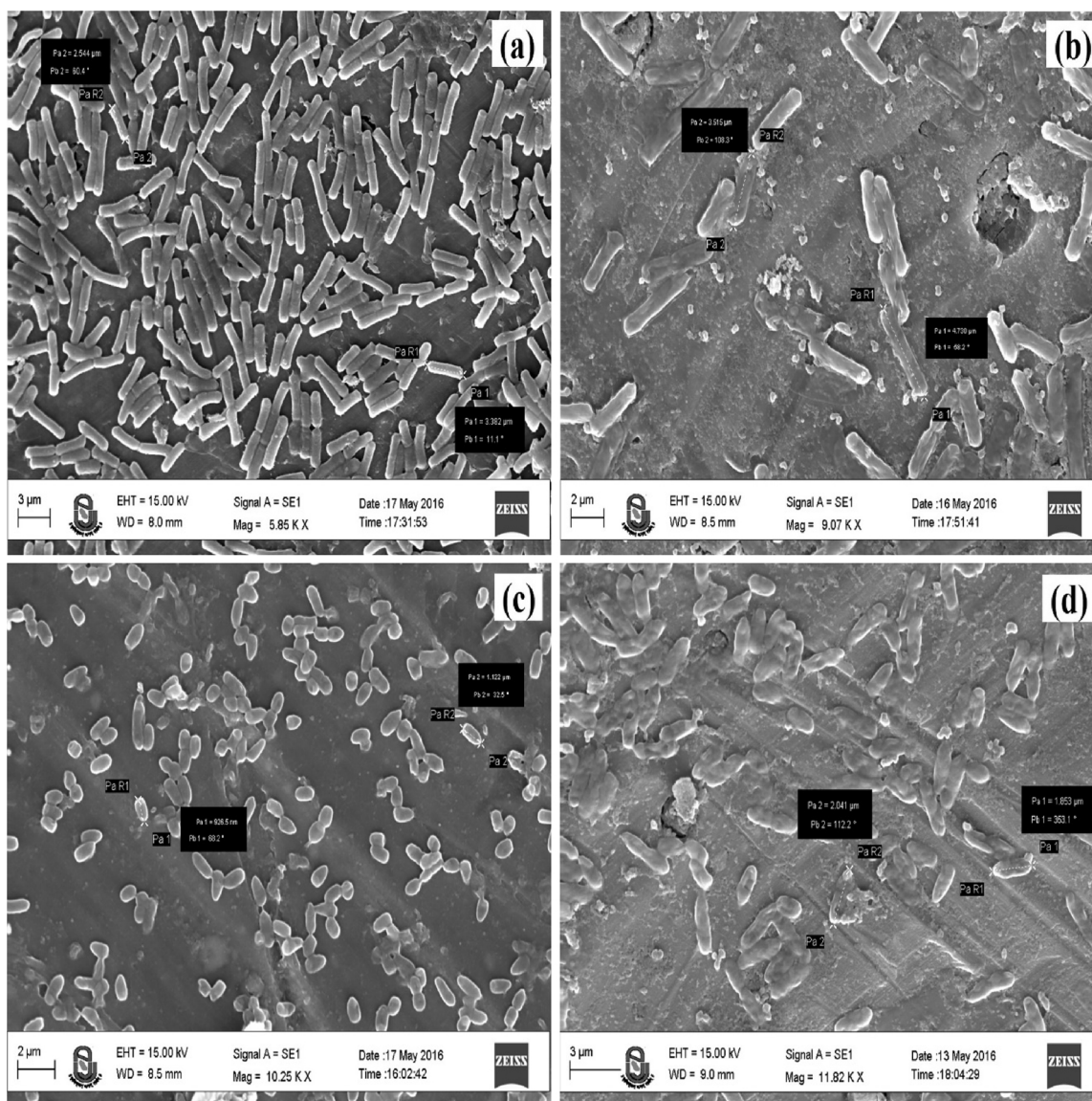
ND = Not Detected.

Number after ± indicates standard deviation.

proven that nano-silver exert the same result on multidrug-resistant bacteria [24].

Our findings showed that *B. megaterium* MTCC 7192 developed resistance to all tested antibiotics, except linezolid (Fig. 2a and 2b).

Linezolid is a new class of antibiotics called the oxazolidinones that are useful to control the emergence of drug resistance Gram-positive organisms [25]. Unfortunately, *B. megaterium* MTCC 7192 developed resistance to trimethoprim-sulfamethoxazol, which has been valuable combinatorial therapy for treating a variety of infections. This emerging resistance to trimethoprim-sulfamethoxazol is disturbing because it is a combination of two broad spectrum antibiotics that act synergistically against a wide variety of urinary and respiratory tract infections [26]. There are limited data available to the infections caused by *B. megaterium*, there have been no previous data on resistant to antibiotics, except cloxacillin and streptomycin and thus, careful investigation is required [27,28]. The increasing bacterial resistance among Gram-positive species is concerning because they are responsible for 1/3 of nosocomial infectious diseases [29]. On the other hand, *P. aeruginosa* MTCC 741 developed resistance to all tested broad spectrum antibiotics (Fig. 2c and 2d). Our findings of bactericidal activity of nano-silver against multidrug-resistant *P. aeruginosa* are exactly in accordance with findings shown by Lara et al. [30]. In recent years, *P. aeruginosa* bacteria that are resistant to different classes of antibacterial agents is one of the most feared bacteria that causes pneumonia [31,32]. The high antibiotic prescribing rate is one of the leading causes for the



**Fig. 3.** Visualization of nano-silver treated *B. megaterium* MTCC 7192 and re-emerging *P. aeruginosa* MTCC 741 surface by SEM: (a, c) normal, (b, d) membrane damage, and bacterial aggregation in nano-silver treated cells.

development of antibiotic resistant microbes in case of re-emergence of infectious diseases. However, the speed of the resistance development varies depending on the antibiotics and microbes. Overall, our findings suggest that there was significant difference between the bactericidal efficacy of nano-silver and antibiotics on emerging *B. megaterium* MTCC 7192 and re-emerging *P. aeruginosa* MTCC 741, demonstrating that the bactericidal mechanism of nano-silver was not affected by those known resistance mechanisms that differentiate these strains from susceptible strains. The data presented in the study are novel as nano-silver exhibit excellent bactericidal effect towards emerging and re-emerging bacterial infections regardless of their drug resistant mechanisms.

### 3.3. Surface morphology of bacteria influenced by nano-silver treatment

To observe the membrane deformities upon the nano-silver treatment, we scanned the nano-silver treated and non-treated bacterial cells using SEM. The images indicate clumping of nano-silver on bacterial membrane in treated cells than non-treated cells (Fig. 3). Upon interaction with nano-silver, the surface potential of bacterial membrane is neutralized, resulting into increased in surface tension. After 3 h of treatment, the interactions result in surface potential changes which

lead into the membrane depolarization at the point of clumping. As a result, bacterial membrane become scattered from their original ordered arrangement. The scattered membrane containing cells no longer remain intact, often found in aggregates (Fig. 3b, d).

### 3.4. Surface potential neutralization of bacteria

Surface charge neutralizations of the bacterial cell membrane are important target site of the certain membrane acting compounds particularly with antimicrobial properties, which acts on bacterial cell surface [33]. According to our observations, nano-silver produced alternation of zeta potential in both gram-negative and gram-positive bacteria. As shown in Fig. 4, the initial interaction of nano-silver with a cell surface of *B. megaterium* MTCC 7192 and *P. aeruginosa* MTCC 741 cells display zeta potential of  $-31.2$  and  $-30.5$ , respectively. However, dead (Autoclaved) bacterial cells of *B. megaterium* MTCC 7192 and *P. aeruginosa* MTCC 741 had a lower zeta potential of  $0.5$  and  $-2.8$  mV compared to normal bacterial cells (data not shown in Fig. 4). In our study, it was found that, the zeta potential value moved towards neutral with treatment of nano-silver. The calculated zeta potential values of *B. megaterium* MTCC 7192 and *P. aeruginosa* MTCC 741 at different time

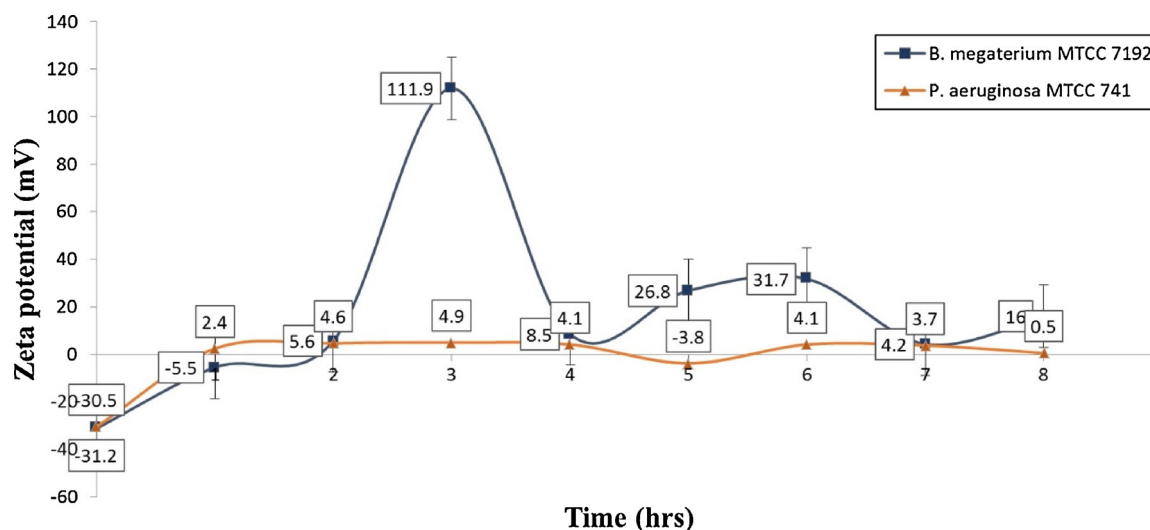


Fig. 4. Change in bacterial Zeta potential in presence of nano-silver. Each data point represents mean  $\pm$  SD for three independent experiments.

intervals are shown in Fig. 4. There were significant differences in the zeta potential of *B. megaterium* MTCC 7192 compared to *P. aeruginosa* MTCC 741, when they were normal or exposed to nano-silver or autoclaved.

Moreover, it was also found that the magnitude of decrease in the negativity of the zeta potential of bacterial cell surface was found to be greater in *B. megaterium* MTCC 7192 and such alternation was found to be time dependent (studied till 8 h) as well. These low values of the zeta potential indicated the interaction between cationic nano-silver and the negative surface potential of bacterial surface. Interestingly, such change in zeta potential through nano-silver can be correlated with the increased bacterial membrane permeability as it was evident from GC–MS analysis (Fig. 5 Supplementary material). The present findings are in conformity with earlier reports, where it has been stated that surface neutralization of the bacterial membrane leads to increase in cell permeability [34].

### 3.5. GC–MS profile for determination of leakages of intracellular constituents

GC–MS based secretome analysis has important applications in discovering the mode of action of nano-silver and helps unravel the effect of nano-silver on membrane permeability of bacteria. In this study, the leakages of the intracellular constituents from bacterial cells were identified from their mass spectra and retention times, as indicated by the chromatogram of the extra cellular components of the bacterial cells after treatment of nano-silver (Fig. 5; Supplementary material). In case of treated bacterial cells, some constituents were from plant extract, by which we synthesized nano-silver. In general, the total constituents present in extracellular materials in treated cells, non-treated cells as well as plant extract detected in GC–MS were shown in Table 2. An increase in some unique intracellular components in treated cells was observed, compared with those in the plant extract and non-treated cells. The common and unique compounds identified from *Bacillus megaterium* MTCC 7192 and *Pseudomonas aeruginosa* MTCC 741, before and after treatment with nano-silver along with plant extract are depicted in Fig. 5. Total 25 compounds were identified common to Bm\_T, Pa\_T and Pl\_E followed by unique 18 compounds in Bm\_T and 17 in Pa\_T. Moreover, 14 compounds found to be common for Pa\_T and Pl\_E. The nature of compounds identified after treatments were sugars, organic acids, amino acids and fatty acids, which showed significant differences with non-treated cells and plant extracts (Table 3; Supplementary material). This may indicate that nano-silver were found to interact with bacterial cell surfaces and results in membrane damage.

Table 2

Comparison of identified bio-compounds from non-treated and nano-silver treated bacterial cells as well as plant extract based on GC–MS analysis.

List names	Number of total compounds	Number of unique compounds
Bm_C	17	14
Bm_T	88	62
Pa_C	14	10
Pa_T	96	71
Pl_E	99	58
Overall number of unique compounds: 113		

Abbreviation: Bm\_C= *B. megaterium* MTCC 7192\_Control, Bm\_T= *B. megaterium* MTCC 7192\_Treated, Pa\_C= *P. aeruginosa* MTCC 741\_Control, Pa\_T= *P. aeruginosa* MTCC 741\_Treated, Pl\_E= Plant Extract.

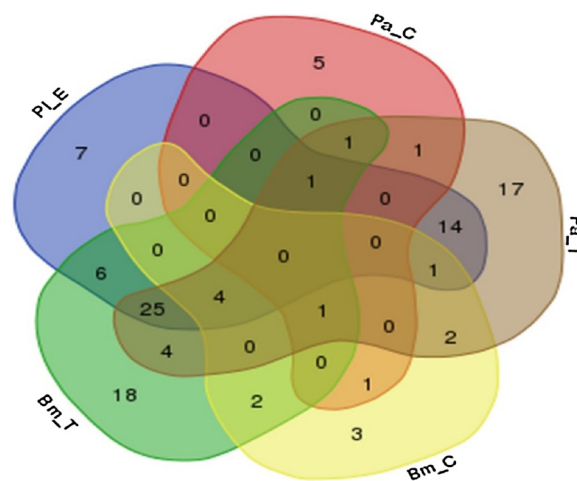


Fig. 5. Venn diagram showing numbers of identified compounds detected from non-treated and nano-silver treated bacterial cells as well as plant extract. The values inside the Venn diagram are the number of metabolites identified by GC–MS.

Abbreviation: Bm\_C= *B. megaterium* MTCC 7192\_Control, Bm\_T= *B. megaterium* MTCC 7192\_Treated, Pa\_C= *P. aeruginosa* MTCC 741\_Control, Pa\_T= *P. aeruginosa* MTCC 741\_Treated, Pl\_E= Plant Extract.

## 4. Conclusions

Nano-silver is considered a novel antibacterial agent, which offers several advantages such as broad spectrum antibacterial activity and lower tendency to induce resistance. The present study demonstrating

the interaction of green synthesized nano-silver exerted a slight stress on the bacterial cell membrane through alternation of zeta potential. The strong antibacterial activity of nano-silver possesses the potential to serve as a platform for developing nano-silver into a novel antibacterial drug to overcome the problem of emerging *B. megaterium* MTCC 7192 and re-emerging *P. aeruginosa* MTCC 741. The antibacterial efficacy of nano-silver against multidrug-resistance indicated that the mode of action of nano-silver is not the same as the mode of action exerted by the antibiotics. Hence, with the threat of multidrug-resistant strains of bacteria, nano-silver could be a good alternative to control emerging and re-emerging infectious diseases.

#### Disclosure statement

The authors declare that they alone are responsible for the writing and content of this paper. The authors report no conflicts of interest in this research work.

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#### Conflict of interest

Authors do not declare any conflict of interest.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jtemb.2018.04.028>.

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