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**ABSTRACT**

The objective of this work is to develop cost-effective, reliable, and large-scale production of metallic nanoparticles (NPs) by adopting green chemistry principles for industrial applications. In that view, we have studied the phytochemical reducibility of silver nitrate by making use of *Calotropis Procera* (Ait.) R. Br root extract, and based on its medicinal properties, an attempt was made to evaluate the therapeutic potentials of silver (Ag) NPs containing this plant extract towards the clinical strains of bacteria. The optimization studies on reducing the potentials were done considering the concentration, pH, temperature, and reaction period of both the extract and the metal precursor. The nanoarchitecture elements were interpreted using visual, spectroscopic, and microscopic analyses cohorting the antibacterial potentials towards the clinically significant strains. The antimicrobial activity exercised by these Ag NPs towards 10 different strains of medically important bacteria at a given concentration was proved to be significant. The antimicrobial potential was further validated quantitatively, and the MIC/MBC concentration values were determined. Finally, the cytotoxicity of Ag NPs when tested against the HEPK cell line indicated that the metal-
phytochemical moiety exhibited the maximum therapeutic efficacy and thereby paving the way for the development of disruptive technologies in the field of nanomedicine.

1. Introduction

In recent years, the increased incidence and emergence of antibiotic resistance has not only threatened the hospital environment but also remaining a global economic menace [1]. The major contributor to this public health concern is attributed by bacteria (~54%), envisioned to exceed the global mortality rate triggered by cancer. As a defense mechanism, various antimicrobials have been formulated against bacteria, fungi, viruses, protozoa, etc. to impede essential cellular processes either to inhibit or eliminate the microbial attacks. But their efficiency is compromised due to the microbial strategies striving for their survival and also inheriting the same for their progeny. A collection of pathogens termed ESKAPE (Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species) were found to be the most susceptible strains in the course of acquiring antibiotic resistance [2,3]. Alongside, the resistance pattern towards multiple classes of antibiotics are due to their indiscriminate use and was best studied in microbes such as Escherichia coli. It was reported that the spontaneous mutation and horizontal gene transfer remains to be the principal mechanism conferring antimicrobial resistance property [4]. As a result, the quantity of antibiotics intake gets reduced via alteration in the target sequence and efflux pumps. In the process of striking the resistance mechanism, novel antibiotics have been developed and actively been pursued but those formulations were decrypted by the microcosmonauts.

On the other hand, the metallic nanoparticles (NPs) that exhibit improved properties based on their size, distribution, architecture, chemical assembly, colloidal stability and biocompatibility have been devised to counter balance this resistance pattern via nano-composite assembly and surface modification [5]. In addition, the NP’s high surface area to volume ratio allows easy interaction with other particles or living cells and thereby bringing about the desired effect. So, the optical properties of nanoforms of noble metals namely gold, silver, platinum, etc. were employed in biomedical and therapeutic applications [6]. Meanwhile, the metallic NPs have low colloidal stability which tends them to aggregate in the biological environment and to solve this problem, the usage of chemicals in the synthesis protocol has been replaced with phytochemical moieties of indigenous plant such as Calotropis procera and the surface modification of which increases the biocompatibility of these materials [7].

C. procera (Ait.) R. Br. belongs to the Asclepiadaceae family and is an extensively grown plant in India and other warm, dry places due to the very interesting medicinal properties that are used in many ayurvedic formulations and as a traditional system of medicines. The leaves are perceived as a valuable antidote for snakebite, sinus, rheumatism, mumps, etc. and the root barks are used to treat a variety of illnesses such as leprosy, menorrhagia and high fever [8]. They are traditionally used to treat diarrhoea, jaundice and the best remedy for skin diseases and for that reason, various reports on the pharmacological activities of the plant namely antihelmintic, antidiabetic, antimalarial, antimicrobial, anticancer and hepato-protective are being developed [9–11].

The surface-modified NPs with pharmacologically active phytochemicals of Calotropis sp. would challenge conventional drugs encountering clinically significant strains [12].
Herein, we report a tailored phyto-based silver NPs (AgNPs) using *Calotropis* root extract, where the characterisation and bactericidal activity experiments were carried out using sophisticated analytical techniques and clinically significant bacterial isolates to highlight the biological activity of synthesised system. The schematic representation of AgNPs from *Calotropis* root extract is shown in Figure 1.

2. Experimental detail

2.1. Materials

Fresh roots of *C. procera* plant were obtained from a local farm in Chennai (Lat. 10°7’12” N; Long. 77°33’0” E), Tamilnadu, India. Silver nitrate (AgNO₃ ≥ 99.8%; AR grade) was procured from Sigma-Aldrich (Bengaluru, India) and used without further purification. All the glassware used was cleansed using chromic acid (HiMedia, Mumbai, India), sterilised using an autoclave and dried in a hot air oven. All the experiments were performed in triplicates and the results are expressed as mean ± SD (standard deviation) of all concentrations.

2.2. Preparation of *Calotropis* root extract

For the preparation of *Calotropis* root extract, the fresh and healthy *Calotropis* roots obtained were thoroughly washed and dried in shade for 2–3 days, finely chopped into 50 g size followed by converting into fine powder using a blender. A specified quantity of the powdered sample was boiled along with 150 mL of deionised water (Millipore, Model 8200, Germany) for 20 min, filtered through muslin cloth followed by Whatman No. 1 filter paper (Merck, Mumbai, India). The phytochemical analysis of filtered root extract was done using the standard procedures [13]. The filtered *Calotropis* root extract was used within 4 h for synthesising the AgNPs until which it was stored in a refrigerator.

2.3. Green synthesis of AgNPs

In a typical synthesis protocol, an aqueous solution of 1 mM AgNO₃ prepared was used for the synthesis of AgNPs and for that, the root extract was added dropwise to 1 mM
AgNO₃ at 1:10 ratio and left for about 5–10 min under the stirring condition at various temperatures (100, 150 and 200 °C) and observed for the changes in colour. This experiment was performed in dark to prevent the photolytic effect of the metal precursor. Further, the separation of AgNPs from the dispersion medium was carried out by centrifugation (10,000 rpm/15–20 min). The supernatant was discarded, and the pellet was washed several times with deionised water, dried, powdered and stored at 4 °C for further experimentation (Figure 2).

### 2.4. Characterisation studies

UV–Vis spectrophotometer (Varian Cary 300, Agilent, CA, USA) was used to monitor the concentration of AgNPs, where the spectra measured in the wavelength range of 300–800 nm using silver nitrate as control and the absorbance maxima typical for AgNPs was recorded. Further detailing on the crystal structure, phase and texture of the synthesised AgNPs was monitored using a Rigaku MultiFlex X-ray diffractometer (Rigaku, Tokyo, Japan) through Cu Kα rays at a diffraction angle (2θ) from 20° to 100°. The mean size of AgNPs was calculated taking the full width at half maximum (FWHM) of (111) plane using the Debye–Scherrer equation,

\[ D = \frac{K\lambda}{\beta \cos \theta} \]

where \( K \) is the Scherrer constant (0.9); \( \lambda \) is the wavelength of the X-rays; \( \beta \) is the FWHM and \( \theta \) is the Bragg’s angle in radians.

The functional groups responsible for the reduction of silver to stable nanosilver was detected using Fourier-transform infrared (FTIR) spectrophotometer (FT/IR 4600, JASCO, Japan). The analysis was performed using KBr pellets (1:100) and the spectrum recorded in the range from 400 to 4000 cm⁻¹ at 4 cm⁻¹ resolution. To determine the morphology and arrangement of AgNPs, a drop of AgNPs was dispersed onto a carbon-coated copper grid, dried and examined using Zeiss Supra 55 (Carl Zeiss, Germany) at an accelerating voltage of 25 kV. The size of the particles and its distribution were determined using ImageJ software (National Institutes of Health) measuring atleast 200 particles. The elemental composition pertaining to the purity of nanosilver was evidenced using the Energy-dispersive X-ray spectroscopy (EDX) (Oxford instruments X-MAX). The hydrodynamic diameter of the synthesised nanostructures was validated using Malvern zeta sizer Nano ZS (Malvern Instruments, Worcestershire, UK) 90 using deionised water as a dispersing agent at neutral pH.
2.5. Antibacterial study—In vitro approach

2.5.1. Agar diffusion method

The antibacterial efficacy of AgNPs was determined using the agar diffusion method [14] against clinically significant bacteria namely *Salmonella typhi*, *Shigella flexneri*, *Bacillus subtilis*, *E. coli*, *S. aureus*, *Enterococcus sp.*, *K. pneumoniae*, *P. aeruginosa*, *Staphylococcus epidermidis*, and *E. faecalis*. The strains used are typical representatives of two large bacterial taxonomical lineages and were obtained from a private clinical laboratory in India. Prior to the assay, each bacterium was refreshed on nutrient agar stocks and fresh overnight grown suspensions in nutrient broth were preferred. The inoculum size of each strain from overnight suspensions was prepared using 0.8% NaCl and the turbidity adjusted to OD$_{600} = 0.1$. Sterile disks were loaded with 5, 10, 50, 100 μL of AgNPs (1 mg/mL) and control disk with the 1 mM solution of AgNO$_3$. The inoculated plates were incubated at 37°C for 24 h for any inhibitory effect and at the same visualised and measured the extent of inhibition using the calipers.

2.5.2. Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of phyto-fabricated AgNPs were analysed following CLSI 2015 guidelines [15]. The MIC test was performed in a 96-well microtiter plate using a standard broth microdilution method and MBC test on the Mueller–Hinton Agar (MHA) plates. The bacterial inoculums were adjusted to the concentration of $10^6$ CFU/mL. For the MIC test, 100 μL of the synthesised AgNPs stock solution (1000 μg/mL) was added and diluted twofold in 100 μL of MHA started from column 1 to column 10. Column 1 of the microtiter plate contained the highest concentration of AgNPs, while column 10 contained the lowest concentration. Columns 11 and 12 served as negative and positive controls respectively containing only medium and medium with bacterial inoculums. Each well of the microtiter plate was added with 30 μL of the resazurin solution (0.02% w/v) and incubated at 37°C for 24 h [16]. Any change in colour was observed namely blue or purple colour indicated no growth, while pink or colourless indicated bacterial growth. The lowest concentration of antibacterial agent inhibiting the bacterial growth was considered as its MIC value. MBC test was performed by plating the suspension from each dilution of microtiter plates into the MHA plate. The plates were incubated at 37°C for 24 h, where the lowest concentration with no visible growth on the MHA plate was considered as its MBC value.

2.6. Cytotoxicity studies—MTT assay

The cytotoxic effect of phyto-fabricated AgNPs was tested against HEPK (Keratinocytes) cells procured from NCCS, Pune, India. The HEPK cells were seeded in a 96-well plate at a density of 1 × 104 cells per well in 200 μL of Dulbecco’s modified eagle’s medium containing 10% foetal bovine serum and 1% penicillin–streptomycin antibiotics and were cultured at 5% CO$_2$ and 37°C for 24 h. The growth medium in the wells was replaced after 24 h with medium containing AgNPs (0.78–100 μg/mL) and incubated for another 24 h. The medium was removed thereafter and replaced with 100 μL of medium containing MTT reagent or 3(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide and incubated for 4 h. The unreduced MTT was aspirated and 200 μL of dimethyl sulfoxide was added to each well to dissolve the MTT formazan crystals. The content was mixed properly, and absorbance measured at 595 nm in an ELISA microplate reader (Invitrogen JOURNAL OF EXPERIMENTAL NANOSCIENCE 221
Bioservices, Bengaluru, India) [17]. The test was for the comparison, TGF-β was used as positive control and the cells without any treatment served as the negative control.

3. Results and discussion

The green chemistry principles remain to be one of the promising strategies to override the hurdles associated with conventional methods in the event of NPs synthesis. The biological extract particularly plants origin has been the most sought after reducing agent for its non-toxic and rich metabolic content. Accordingly, the root extract prepared from the C. procera plant was phytochemically probed prior to the NPs synthesis.

The phytochemical screening of C. procera root for the presence of secondary metabolites was investigated using ethanolic and aqueous extract, respectively, and the constituents identified based on the polarity, colour intensity and precipitates formed (Table 1). The presence of phytochemical constituents was indicated in terms of scores of +, ++ and +++ using standardised values based on the prepared root extract. A score of ‘+’ corresponds to 0.01 to 0.1%, ‘++’ corresponds to 0.1 to 0.3% and ‘++++’ corresponds to more than 0.3% based on the dry weight of plant material. It is quite obvious from Table 1, the root extract of C. procera showed the presence of alkaloids scoring ‘+’, saponins, flavonoids, sterols and triterpenoids scoring ‘++’ and cardiac glycosides with ‘++++’ score which is in agreement with the previous reports [18]. Subsequently, the extraction efficiency was higher when ethanol was used compared to the aqueous extract that showed slight to the mere presence of above-mentioned secondary metabolites. Thus, the ethanolic extract was retained for fabricating the AgNPs.

The synthesis and stability of the reaction mixture were monitored from the colour change and UV–Vis spectra. There was a gradual change in colour from yellowish-green to golden yellow and finally yellowish-brown colloid within 10 min of the addition of extract. This clearly indicated the initiation of the reduction of silver salt by the phytochemical moieties to the nano form. Secondly, the spectral analysis focussed on the wavelength range of 400–460 nm (Figure 3) with its absorbance maxima (\( \lambda_{\text{max}} \)) at 455 nm attributed to the typical SPR property of silver (Ag\(^0\)) at 100 °C [19]. A few hours later, no further colour change was observed indicating a complete reduction of silver salts. Moreover, the AgNPs thus formed remained stable showing a very little aggregation. Meanwhile, the NPs synthesised at increased temperatures namely 150 and 200 °C showed little broadening inferring agglomeration of particles. Besides the broadening of plasmon bands, the absorption tail was found prominent at longer wavelengths and at higher temperatures indicative of size distributions of NPs [20]. The time taken for AgNPs synthesis was much less and this fact can be explained taking into account the role of bio-molecules

| Phytochemical moiety | Ethanol | Water |
|----------------------|---------|-------|
| Alkaloids            | +       | +     |
| Tannins              | −       | −     |
| Saponins             | ++      | ++    |
| Flavonoids           | ++      | ++    |
| Anthraquinones       | −       | −     |
| Sterol               | ++      | +     |
| Triterpenoids        | ++      | +     |
| Cardiac glycosides   | +++     | +     |

−, Absent; +, 0.01–0.1%; ++, 0.1–0.3%; ++++, > 0.3%.

Table 1. Phytochemical screening of C. procera root extract.
such as flavonoids and phenolic compounds present in the extract that might have provided the high reductive ability [21].

The powdered XRD remains one of the best analytical techniques to examine the crystal nature and purity of a compound after 12 h for different temperatures. The prepared AgNPs showed diffraction patterns (Figure 4) with prominent peaks at $2\theta = 38.21^\circ$, $44.32^\circ$, $77.65^\circ$ and $81.72^\circ$, $98.2^\circ$ that matched well with (111), (200), (220), (311) and (222), (400) planes of Ag typical for FCC symmetry (JCPDS 04-0783) at all temperatures. The highest peak intensity of (111) plane with narrow FWHM at all temperatures illustrated the crystalline nature of synthesised AgNPs [22]. The average crystallite size of AgNPs was calculated using the Debye–Scherrer equation and found to be $\sim 22$ nm at $100^\circ$C. It is at this tiniest scale, the particles tend to nucleate, grow and multiply with their lowest energy (111) which corroborates well with the previous reports.

### 3.1. FTIR spectral analysis

The bio-molecular interaction of the root extract in the event of nanosilver reduction was identified using FTIR analysis (Figure 5), where the spectrum demonstrated prominent peaks around 3482, 2911, 2352, 1794, 1459, 1151 and 830 cm$^{-1}$. There are stretching vibrations at 3482 cm$^{-1}$ (strong, broad O–H), 2911 cm$^{-1}$ (strong N–H stretch, amine), 2841 cm$^{-1}$ (medium C–H stretch, alkane), 1794 cm$^{-1}$ (around 1780 cm$^{-1}$ C=O stretch, carboxylic acid), 1459 cm$^{-1}$ (around 1450 cm$^{-1}$ C–H bend), 1151 cm$^{-1}$ (weak O–H stretch, alcohol) and 830 cm$^{-1}$ (medium C=C bend, alkene). This indicates the presence of amine, phenolic, alcoholic aromatics from the root extract of *C. procera* involved as reducing and/or capping agent in the AgNPs synthesis [23–24]. However, the FTIR spectrum of AgNPs prepared by boiling the extract to $200^\circ$C highlighted peaks only at 3482, 2911 and 1794 cm$^{-1}$ attributing to stretching vibrations of O–H, N–H and C–H, respectively. As the metal NPs in the colloidal form are easily influenced by van der Waals forces of attraction, the possibility of coagulation is much faster at higher temperatures (150 and $200^\circ$C). It was presumed that these moieties behave as steric or electrostatic barriers around the particle surface conferring a highly dispersive nature of particles [25].
3.2. Scanning electron microscopy and Energy-dispersive X-ray spectroscopy (EDX) analysis

Electron microscopy is a useful tool that bestows the topographical insights namely size, shape and distribution of particles. In this accord, the fabricated NPs revealed elliptical to nearly spherical and square shaped particles (Figure 6) in the size range of 38–44 nm. The variable shapes of particles may be correlated with different sizes. When resolved, the NPs were not found in direct contact even within the aggregates, indicating the stabilisation of particles by the capping agent. The EDAX spectra recorded in Figure 7 showed that the chemical composition of colloid constituted pure silver with 75.2% as its weight percentage.

Figure 4. Comparison of the powdered XRD patterns of AgNPs synthesised using the root extract of *C. procera* at different temperatures.

Figure 5. FTIR spectral comparison of AgNPs synthesised using *C. procera* root extract at different temperatures.
3.3. Dynamic light scattering (DLS) and zeta potential analysis

The DLS technique was used to measure the size distribution of NPs (aggregation) in deionised water and based on this, the DLS measurements shown in Figure 8(a) indicates the dispersive nature of NPs at physiological temperatures. The AgNPs apparent size distribution towards smaller value (20–30 nm) was found consistent with the XRD studies. This may be explained in terms of the enhancement of strong interparticle electrostatic interactions and chaperone-like activity of phytochemicals. Alongside, the measurements of zeta potential (Figure 8(b)) gave information about the stability of dispersed NPs and the observation of AgNPs higher zeta potential value in between −50 and −100 mV correlating to higher stability of their dispersions [26] and the possibility of strong electrostatic interaction between the NPs and phytochemical moieties.

3.4. Antibacterial activity

The obtained results of antibacterial activity testing for AgNPs are shown in Table 2. From the analysis of results, the AgNPs exhibited significant antibacterial action in a...
dose-dependent fashion for all bacteria under optimised laboratory conditions (pH 7; 37°C; 1 mM). For the same bacterial concentrations, the increase of NPs concentration resulted in an increased bactericidal effect. This trend could be observed as there were fewer NPs that encountered each bacterial cell at their lowest concentration. Among the tested strains, *E. coli* and *P. aeruginosa* exhibited maximum zone of inhibition (ZoI) of 17 mm as evident from the zone size followed by *S. flexneri* (16 mm), *S. typhi* (15 mm) and *K. pneumoniae* (15 mm). The comparison between the ZoI of bacteria exposed to different concentrations of AgNPs showed significant differences at 10, 25, 50 and 100 μg concentrations in an increasing trend when statistically analysed using one-way analysis of variance (ANOVA; *p* < 0.05). This was substantiated by running a control test using only AgNO₃ which exhibited lower growth inhibition than the AgNPs.

Secondly, upon analysing the antibacterial effect observed between groups using independent *t*-test (*t* = 0.0381; df = 8; *p* > 0.05), not much significant difference in the inhibitory effect could be appreciated when the same AgNPs concentration was treated against Gram-positive and Gram-negative bacterial strains (*N* = 5). This clearly indicates that the highest toxicity is observed for the highest AgNPs concentration (100 μg) irrespective of bacterial strains. This bactericidal effect may be attributed to the binding affinity of silver ions with various bacterial cell components namely DNA and protein causing injury to

Figure 8. DLS spectrum (a) and zeta potential (b) of AgNPs.
the cell resulting in the cytoplasmic outpour [27]. It is also found that the damage incurred by AgNPs in the DNA might influence bacterial metabolic processes such as respiration by combining with oxygen and sulfhydryl (S–H) groups on the cell wall, which consumes the energy currency ATP, ultimately leading to cell death [28].

Prior to the preliminary antibacterial screening of AgNPs using agar diffusion test, MIC and MBC were performed to validate the antibacterial potential. The recorded MIC values at which no visible growth of test bacterial strains is found are presented in Table 3. This range of concentration was selected prior to an initial antimicrobial screening where the inhibitory phenomenon was most prominent. The antimicrobial effect for AgNPs towards a panel of clinical isolates was appreciated by the amount of fluorescent Resoruﬁn produced in proportion to the viable bacterial cell concentrations. Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye that gets irreversibly reduced to Resoruﬁn producing pink ﬂuorescence by the action of oxidoreductases indicative of viable cells. However, in the non-viable cells, this Resoruﬁn is further reduced to a colourless and a non-ﬂuorescent molecule, hydroresoruﬁn [29]. Among the tested pathogens, S. flexneri, E. coli, P. aeruginosa and E. faecalis were found more sensitive to AgNPs with a MIC value of 3.12 μg/mL followed by S. typhi and B. subtilis with an MIC value of 6.25 μg/mL. Whilst S. aureus, Enterococcus sp., K. pneumoniae and S. epidermidis were found resistant to AgNPs among the tested strains showing a MIC value of 12.5 μg/mL. This difference in MIC values could have resulted due to differences in their metabolic activity. In addition, the tendency of bacteria to form ﬁlms might limit the accessibility of ﬂuorophores to bacteria within the bioﬁlm preventing the emission of ﬂuorescence signal [30].

| Test organisms  | 5 μg  | 10 μg | 25 μg | 50 μg | 100 μg | Control |
|-----------------|-------|-------|-------|-------|--------|---------|
| S. typhi        | –     | 6 ± 1.5| 9 ± 0.95| 12 ± 2| 15 ± 1.3| 10 ± 1  |
| S. flexneri     | –     | 7 ± 1.2| 10 ± 1.2| 13 ± 1.3| 16 ± 1.1| 9 ± 0.84|
| B. subtilis     | –     | 6 ± 0.96| 9 ± 0.96| 12 ± 0.8| 14 ± 2  | 9 ± 1   |
| E. coli         | –     | 8 ± 0.9| 10 ± 1.3| 13 ± 1.5| 17 ± 0.9| 11 ± 1.5|
| S. aureus       | –     | 7 ± 1.2| 9 ± 0.75| 12 ± 0.82| 14 ± 1.4| 10 ± 0.96|
| Enterococcus sp. | –     | 7 ± 1.1| 8 ± 0.76| 11 ± 0.69| 14 ± 0.9| 10 ± 1  |
| K. pneumoniae   | –     | 8 ± 0.85| 9 ± 0.28| 12 ± 1  | 15 ± 1.5| 9 ± 0.44|
| P. aeruginosa   | –     | 7 ± 1.3| 9 ± 1   | 13 ± 1.5| 17 ± 1.5| 11 ± 1.3|
| S. epidermidis  | –     | 7 ± 0.8| 9 ± 0.25| 12 ± 0.7| 14 ± 0.9| 8 ± 1.4 |
| E. faecalis     | –     | 9 ± 1.2| 11 ± 0.9| 14 ± 2  | 17 ± 1.5| 12 ± 0.9|

−, No zone.
The experiment was performed in triplicates and the ZOI expressed as mean ± SD.

| Test organisms  | MIC and MBC of AgNPs on clinical isolates (μg/mL) |
|-----------------|---------------------------------------------------|
| S. typhi        | –        | –        | MBC | MIC | − | + | + | + | + | + | + | + | + | + | − |
| S. flexneri     | –        | –        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |
| B. subtilis     | –        | −        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |
| E. coli         | –        | −        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |
| S. aureus       | –        | −        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |
| Enterococcus sp. | –      | −        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |
| K. pneumoniae   | –        | −        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |
| P. aeruginosa   | –        | −        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |
| S. epidermidis  | –        | −        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |
| E. faecalis     | –        | −        | −   | MBC | MIC | + | + | + | + | + | + | + | + | + | − |

−, No growth; +, bacterial growth; NC, negative control; PC, positive control.
Subsequently, the MBC values for the respective strains exposed to different concentrations of AgNPs are shown in Table 3. This parameter determines the lowest concentration of antimicrobial, in this case, AgNPs that brings about bacterial cell death. The growth pattern of bacterial colonies upon sub-culturing AgNPs treated strains was observed. Among the tested strains *S. aureus*, *Enterococcus* sp., *K. pneumoniae* and *S. epidermidis* showed an MBC value of 25 µg whereas *S. typhi* and *B. subtilis* showed MBC value at 12.5 µg followed by *S. flexneri*, *E. coli*, *P. aeruginosa* and *E. faecalis* with an MBC value 6.25 µg. This ensured the complete protection of bacteria at higher concentrations namely 50 and 100 µg. The higher potency of AgNPs as mentioned above (MBC @ 6.25 µg) might attribute to a local dosage effect, where these NPs could simultaneously encounter a bacterium in a tiny area [31]. This superior performance of AgNPs was well reflected in MIC and MBC showing a fourfold strengthened bactericidal effect.

### 3.5. Cytotoxicity assay

The success of any antibacterial agents for clinical applications depends on the level of toxicity induced to the cells. In this study, cytotoxicity of phyto-fabricated AgNPs was explored against HEPK keratinocytes using MTT assay and from the results, the phyto-fabricated AgNPs as compared against the positive control of TNF-β are found to be non-toxic (Figure 9) to mammalian cells with significant antibacterial activity against clinically important bacteria. A possible explanation could be due to the difference in the composition of the cell membrane of bacteria and human cells. The bacterial cell membrane predominantly consists of peptidoglycan layer comprising as many saccharide units and whose hydrolysis of bonds brings about cell death. As for the mechanism of action is concerned, the antibiotic permeates the bacterial cell wall and inhibits enzymes (transpeptidases and carboxypeptidases) responsible for the synthesis of peptidoglycan layer snuffing out bacterial cell [32].

It was inferred from earlier reports that β-lactam antibiotics mimics D-alanylalane peptide fragment, an enzyme-substrate that facilitates the binding of penicillin-binding proteins.
(PBPs). These PBPs are found anchored in the cell membrane and are involved in the cross-linking of the bacterial cell wall. The antibiotic irreversibly binds to the active site of PBP disrupting cell wall synthesis. As there is a complete absence of such membranous architecture and enzyme machinery in human cells, antibiotics could not harm them [33].

4. Conclusion

In conclusion, we indicate a simple, cost-effective and eco-friendly approach towards the synthesis of AgNPs from Calotropis root extract and explored its therapeutic potential. Typical nano characteristics of silver were achieved at optimised laboratory conditions. The therapeutic efficiency of AgNPs against clinical pathogens was found significant as evident from the ZoI (agar diffusion method), MIC and MBC. The mechanism of action of AgNPs towards Gram-positive and Gram-negative strains remained unbiased at a given concentration which varied significantly with that of the antibiotics. Further, the phyto-fabricated AgNPs were found to be not cytotoxic to the HE PK cells even at higher concentrations (100 µg) rather toxic to bacterial strains treated at the same concentration. This could be one such strategy to combat antimicrobial resistance wherein AgNPs could diffuse easily within biofilms and encounter them. Hence, the AgNPs could be a promising candidate to design non-toxic functionalised NPs which can be used in antimicrobial, anticancer, and drug delivery applications.

Disclosure statement

No potential conflict of interest was reported by the authors.

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