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C. aromaticus leaf extract mediated synthesis of Zinc oxide nanoparticles and their antimicrobial activity towards clinically multidrug-resistant bacteria isolated from pneumonia patients in nursing care

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Abstract

Current study examined the potential of Zinc Oxide Nanoparticles (ZnO-NPs) fabricated using the Caryophyllus aromaticus (C. aromaticus) leaf extract to inhibit multidrug-resistant (MDR) Acinetobacter baumannii infection. Analytical results confirmed the stable ZnO-NPs fabrication with mean particle size of about 18 nm. Zeta potential and Fourier transform infrared (FTIR) findings proved the ZnO-NPs capping with polyphenols of phyto-extract. Selected area electron diffraction (SAED) and x-ray diffraction (XRD) studies displayed the synthesized ZnO-NPs crystalline nature. The findings revealed that ZnO-NPs fabricated by leaf extract of C. aromaticus induced-bacterial cell-death, triggered DNA condensation of MDR A. baumannii and showed an obvious effect on the production of biofilm, cells grown in biofilm, microbial cell attachment as well as invading human lung cells depending on the concentration. The ZnO-NPs fabricated using C. aromaticus plant leaf extract did not show any obvious effect on the human lung cell viability. Furthermore, approximately 90% MDR A. baumannii infection was inhibited by the fabricated ZnO-NPs without cytotoxic effect at 0.028 µg ml⁻¹ EC₅₀. Thus, fabricated ZnO-NPs were considered as potent anti-MDR A. baumannii agent for efficient therapy and further lead to advancement of various efficient biomedical applications in nursing care.

1. Introduction

Nanoscience has proved that mostly distinct and dissimilar properties namely electrical conductivity, optical, chemical reactivity, hardness and active surface area will be obtained by decreasing the particle size to nanometers [1, 2]. Among the most significant and various characteristics of NPs, a few of them are due to their anti-microbial metal nanoparticles namely zinc, copper, silver and titanium [3, 4]. Anti-bacterial nanoparticles are the innovative biomaterial groups with distinctive effects to enhance the common human health standards. For bacterial deactivation, the nanoparticles react with the bacterial cells active components which results in the death of bacterial cells. On the other side, cost effective and most accurate chemicals are conventionally introduced to generate nanoparticles. However, for the health of public and environment, the high-energy that is utilized for these methods and the pollution of chemicals could lead to severe issues [5].

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Nanoparticles that are produced utilizing biological methods by means of biological pathways have attracted scientific attention in the recent times [6]. The NPs synthesis involving the use of natural resources has been described as bio-synthesis. In addition, a common interest between nanotechnology and biotechnology was developed by nanoparticles biofabrication that caused in improvement of new material class which was utilized in various fields of science. Copper oxide [7], ZnO [8, 9], iron oxide [10–12], titanium and silver [13, 14] are the few significant nanomaterials that are investigated, and can be fabricated with the help of physical, biological and chemical processes. Few microbes, yeasts, plants and fungi have showed their utmost capability in nanoparticle synthesis [15, 16] and the biofabrication can be intra-cellular [17] or extra-cellular [18, 19]. The significant benefit of extra-cellular method is their eco-friendliness, simplicity and the fabrication capability in commercial scale. Because of the above reasons, investigators prefer the nanoparticles synthesis using biological systems, coupling with environment-friendly, biocompatible (simple) and low environmental risks fabricated methods. The significant characteristics of nanoparticles namely particle size, optical properties, electrical and so on, could be monitored by controlling the biofabrication by changing parameters such as substrate exposure time, pH, temperature and substrate concentration [20–22].

The biomolecules adsorption on the surface of nanoparticles results in their stabilization by avoiding agglomeration and therefore enhances their aqueous dispersibility that makes the eco-friendly methods more advantageous towards the preparation of composites. On the other hand, the nanoparticles fabricated using chemical reagents can result in new toxicity related issues because of these harmful chemicals adsorption on the graphene sheets surface causing it less attractive for biological and environmental applications. The above-mentioned properties demonstrate the major advantages of biofabricated nanoparticles in biological applications like cancer therapy and drug delivery [23–25].

One of the researchers examined nanoparticles genotoxicity and cytotoxicity effects with varied particle sizes (like 113, 80, 20 nm) and showed that the larger nanoparticles are less toxic over the smaller nanoparticles (20 nm) [26]. The nanoparticles cytotoxicity was directly proportional to nanoparticles concentration, which means as the NPs concentration increases, the NPs cytotoxicity also increases. The most challenging part during the NP biosynthesis is the identification of resources from the nature as a reference from the biological source. Different shaped and sized natural biomolecules mediated nanostructures which possess a rapid process of synthesis and were even more stable. Additionally, ZnO-NPs are utilized in various fields namely gas sensors, chemical adsorbents, catalysts for light demolution, semiconductor manufacturing, varnishes and rubber industry, paints, light emitting diodes, glass sunscreen, lasers, solar cells, piezoelectric devices, UV light absorbing materials, photodiode, UV filter feeders, deodorizing, diabetes treatment, cosmetics, optical sensors, visible light, ultraviolet, catalysis and generally in medical and pharmaceutical fields [27–29]. Various types of physico-chemical methods are available for ZnO-NPs synthesis [30] which have a few drawbacks that include advanced apparatus need for production, low efficiency, lack of solution reliability and non-uniform particle size [31].

*Coleus aromaticus* Lour. is a well known species that belong to the family of Lamiaceae. It is a widely available medical plant used in the current research to biofabricate zinc oxide nanoparticles (Zno-NPs). It is a highly aromatic, tender and fleshy pubescent herb with distinct smell containing several phytochemicals like caryophyllene (bicyclic sesquiterpene), patchoulene and carvacrol (monoterpenoid) including flavonoids such as apigenin, quercetin, genkwanin, salvigenin, and luteolin, [32]. It is also utilized for the treatments of hepatopathy, malarial fever, vesical and renal calculi, chronic asthma, cough, bronchitis, hiccough, colic, anthelmintic, and convulsions [33].

Current study examined the potential of ZnO-NPs fabricated using the *C. aromaticus* leaf extract to inhibit multidrug-resistant (MDR) Acinetobacter baumannii infection. ZnO-NPs fabricated by the leaf extract of *C. aromaticus* induced-bacterial cell-death, triggered DNA condensation of MDR *A. baumannii* and showed an obvious effect on the production of biofilm, cells grown in biofilm, microbial cell attachment as well as invading human lung cells depending on the concentration. The ZnO-NPs fabricated using *C. aromaticus* plant leaf extract did not show any obvious effect on the human lung cell viability.

### 2. Materials and methods

#### 2.1. Materials

The required resources for the fabrication in ZnO-NPs are: Zinc Nitrate (Zn(NO$_3$)$_2$·6H$_2$O), and the precursor of zinc purchased from Sigma Aldrich, Shanghai, China. Deionized water was obtained commercially.

#### 2.2. Plant extract preparation

*C. aromaticus* leaves are washed using distilled water followed by drying in an oven at a temperature of 40 °C. Electrical blender was used to ground the obtained dried leaves in to powder. Milli Q water was used to obtain...
the extract from the leaf powder. The extract of *C. aromaticus* was prepared by mixing 200 ml of Milli Q water with 40 g *C. aromaticus* dried leaves powder. Later, the mixture was allowed for stirring for about 4 h and then kept on a water-bath at a temperature of 60 °C for a period of 40 min which was then transferred into a refrigerator for further use.

### 2.3. Preparation of zinc oxide NPs

0.2 M Zinc acetate dehydrate was made into a mixture with 50 ml leaf extract of *C. aromaticus* with the aid of magnetic stirring for around 60 min at a temperature of 60 °C. A solution, which is light yellow in color was obtained upon the completion of reaction, which was later allowed to settle down its temperature to the room level and then centrifuged at a speed of 8000 rpm for 20 min followed by washing with methanol and distilled water repeatedly for eliminating the impure particles. Thus, the product, which was washed earlier, had been dried at a temperature of 80 °C with the help of an oven and after that treated in the Muffle furnace at a temperature of 350 °C for around 3 h for calcinations.

### 2.4. Characterization

Measurements of Optical absorption for the synthesized NPs were recorded with the help of Thermo Scientific, Evolution—220 UV–vis spectrophotometer. The shape and size characterization of the products of microstructure was carried out by using Tecnai-30 Transmission electron microscope (TEM). The analysis of X-ray powder diffraction is recorded with the help of diffractometer (Shimadzu-7000), which was further helpful in determining the purity of phase in the NPs. Every experiment was carried out in triplicate and each of the obtained data was evaluated by using the software, Orogon Pro 7.5 SRO (Origin Lab Corporation, USA).

Spectrum of Perkin Elmer 1000 (in the mode of attenuated total reflectance (ATR)) was used to record the measurements of FTIR.

### 2.5. The conditions of bacterial strains and growth

10 clinical strains of MDR *A. baumannii* are collected from the infected patient’s sputum samples. The MDR patterns in clinically isolated strains of *A. baumannii* are classified with the help of disk-diffusion agar process. In addition, *A. baumannii* (ATCC 19606) has been used as a reference strain. Moreover, all the isolated microbial strains were grown on tryptic soy agar (TSA) for a day at a temperature of 37 °C. Five colonies grown on TSA were moved to 3 ml sterile tryptic soy broth (TSB) followed by incubation at 150 rpm with continuous shaking at a temperature of 37 °C for 6 h till exponential phase of growth has reached.

### 2.6. Cell culture

RPMI-1640 medium added with 100 units/ml streptomycin or penicillin and 10% of heat-inactivated fetal bovine serum (FBS) was used to culture the human lung carcinoma cell line, A549 (ATCC Cat No. CCL-185) at a temperature of 37 °C with 5 percent CO₂ in humidified atmosphere.

### 2.7. Determination of Anti-microbial effect of fabricated ZnO-NPs using leaf extract of *C. aromaticus* over clinically isolated strains of MDR *A. baumannii*

For the determination of minimal bactericidal concentration (MBC) and minimal inhibitory concentration (MIC), the fabricated ZnO-NPs using *C. aromaticus* leaf extract were tested over 10 isolated strains of *A. baumannii* ATCC19606 and MDR *A. baumannii*. According to the guidelines of Clinical and Laboratory Standard Institute (CLSI), the assay was carried out in 96-well plates by modified broth micro-dilution method [10]. In brief, 5 bacterial colonies were added to 3 ml Mueller Hinton Broth (MHB) and then cultured for 6 h at 37 °C until it reaches the exponential phase. Bacterial suspension was adjusted to a final reference wavelength OD600 measurement of one equivalent to 1 × 10⁸ CFU ml⁻¹ approximately. 100 μl bacterial suspension was added to microtiter plates containing 100 μl two-fold serial dilutions in MHB medium of ZnO-NPs fabricated using leaf extract of *C. aromaticus*. Later, incubation of the plates was carried out for 18 h at 37 °C. Addition of 10 μl resazurin obtained from Sigma-Aldrich Chemicals followed by the incubation for 2 h in dark, was done to observe the change in colour. Lowest concentration before the change of color was taken as MIC value. For the determination of MBC value, 10 μl broth with greater than or similar concentrations to MIC value was poured on the Mueller Hinton Agar (MHA) plates and later subjected to incubation overnight at 37 °C. MBC value was observed where growth of colonies was not present. Moreover, colistin was utilized as a quality control. This study was performed thrice independently with duplicate assay.

### 2.8. Study of bacterial DNA damage using immunofluorescence

*A. baumannii* (ATCC19606) and MRD *A. baumannii* (NPBRCOE 160575) were treated with 0.09 μg ml⁻¹ MIC of synthesized ZnO-NPs and incubated at 37 °C. After 2 h of treatment, the bacterial suspension was added to the
2.10. Effect of ZnO-NPs fabricated using aqueous leaf extract of C. aromaticus on the formation of biofilm

With the help of microtiter plate assays, the efficacy of fabricated ZnO-NPs on the formation of biofilms through MDR A. baumannii was evaluated. In brief, a portion of 100 μl bacterial cells were added to a 96-well flat bottomed plate. An aliquot of double serial dilutions (100 μl) of ZnO-NPs fabricated using leaf extract of C. aromaticus and colistin was in dilution with TSB medium supplemented with glucose (0.25%) which was then added to the well plates to final concentrations ranging from 1/8 MIC to 1/2 MIC (0.012 μg ml⁻¹ to 0.045 μg ml⁻¹). Following incubation at a temperature of 37 °C for a day, the bacterial dispersion was removed and the well plates were cleaned using PBS twice. The well plates were dried and later stained using 200 μl crystal violet (0.1%) at a temperature of 37 °C for a period of 30 min. Later the well plates were cleaned using PBS and then the stained biofilms were solubilized using 200 μl DMSO. Absorbance indicates the formation of biofilms measured by micro plate absorbance reader with OD595 as a reference wavelength.

2.11. Eradication of established biofilm through ZnO-NPs fabricated using leaf extract of C. aromaticus plant

The efficacy of fabricated ZnO-NPs on preformed biofilm of A. baumannii using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was evaluated. The established biofilm was cultured as described previously with slight modifications [34]. Bacterial culture (1 × 10⁶ CFU ml⁻¹) of 200 μl quantity was added to a flat bottom microplate (96-well) and later incubated for a day and then 5 days at a temperature of 37 °C. For 5th day biofilm, aspiration of the used stationary medium and planktonic biofilm cells was performed and then replaced using 200 μl of fresh TSB medium supplemented with glucose (0.25%) daily. After incubating at day 1 and day 5, the culturing medium was removed gently and then well plates were washed using PBS twice. ZnO-NPs fabricated using aqueous leaf extract of C. aromaticus ranging from 1 to 8 MIC (0.09 μg ml⁻¹–0.072 μg ml⁻¹) was diluted using TSB medium supplemented with glucose (0.25%) then seeded into well plates and incubated for a day at a temperature of 37 °C. Subsequently, the culturing medium was discarded and later replaced by 10 μl of 5 mg ml⁻¹ MTT solution (Invitrogen). Mitochondrial succinic dehydrogenase enzyme activity was assessed by incubating at a temperature of 37 °C for 60 min and later the crystals of insoluble formazan were solubilized using 200 μl DMSO. Absorbance indicates the cell viability number assessed with the help of micro plate absorbance reader with OD595 as a reference wavelength.

2.12. Microbial attachment and invasive assay

To determine the effect of ZnO-NPs fabricated using leaf extract of C. aromaticus plant on attachment of MDR A. baumannii to human lung carcinoma cells, we performed an earlier reported process [35, 36]. Treatment of five clinical strains of ATCC 19606 and MDR A. baumannii with ZnO-NPs fabricated using aqueous leaf extract of C. aromaticus at 1/8 to 1/2 MIC (0.045 μg ml⁻¹–0.012 μg ml⁻¹) was performed for a period of 18 h. Centrifugation of the bacterial dispersions was carried out at a speed of 6000 rpm for about 10 min and later washed thrice using PBS solution. The cells were again suspended in PBS solution and later adjusted with PBS to a final reference wavelength OD600 of 1.0 prior continuing with microbial cell adhesion and bacterial invasion assays. Furthermore, A549 cell lines were grown in a 6-well plates till the cells achieved 90% confluence (1 × 10⁶ CFU ml⁻¹) at standard conditions. Then, the culture medium was discarded and the bacterial cells
were cleaned using PBS solution, then incubated with 5 strains of ATCC 19606 and MDR A. baumannii (1 × 10⁷ CFU ml⁻¹) for 30 min at a temperature of 37 °C. Thereafter, non-adherent bacterial cells were discarded and washed thrice using PBS solution. Then the bacterial cells were removed using (0.25%) trypsin/EDTA for 5 min and later lysed using Triton X-100 (0.025%) for about 10 min. Later, the viable bacterial cells were diluted serially 10 times and the bacterial number was evaluated by calculating colony forming units (CFU) on TSA well plates following an overnight incubation.

To determine bacterial invasion, 5 clinical isolates of MDR A. baumannii and ATCC 19606 were subjected to fabricated ZnO-NPs at 1/8 to 1/2 MIC (0.045 μg ml⁻¹ to 0.012 μg ml⁻¹) for a period of 18 h. Thereafter, using these bacteria, A549 cell lines have been infected for 1 h at a temperature of 37 °C. The extracellular bacteria have been separated by cleaning thrice using PBS solution following incubation of cells for 1 h with gentamicin in order to kill all the remaining extracellular A. baumannii bacterial cells. Cell death was occurred by using Triton X-100 (0.025%) and (0.25%) trypsin /EDTA. The sustainable bacteria have been diluted serially 10 times and the bacterial number was evaluated with the help of CFU on TSA well plates following overnight incubation. The experimentations were carried out independently in three-fold using duplicate assay.

2.13. Cytotoxicity MTT assay of ZnO-NPs fabricated using aqueous leaf extract of C. aromaticus
With the help of MTT cell proliferation assay, cell viability was evaluated. In a 96-well culture plates, A549 cell lines were cultured until the cells attained 90% confluence approximately at standard conditions. The culture medium was discarded and later incubated with fabricated ZnO-NPs diluted by complete medium at 0.01 μg ml⁻¹ to 0.72 μg ml⁻¹ concentrations. The cells then were grown at standard conditions for one day. Thereafter, the culture medium was separated and was replaced by 100 μl of fresh complete culture medium. Mitochondrial succinic dehydrogenase enzyme activity was measured followed by incubation for 4 h at 37 °C in the MTT solution and water-insoluble formazan crystals were solubilized with sodium dodecyl sulphate (SDS) diluted by 0.01 M HCl. Absorbance was calculated with the help of microplate absorbance reader at 570 nm. Positive controls (5% DMSO) and negative control (only cell growing medium) were included. The experimentations were carried out independently in three-fold by duplicate assay.
2.14. To determine the antimicrobial effect of ZnO-NPs fabricated using \textit{C. aromaticus} leaf extract over intracellular \textit{A. baumanii}

Six well culture plates were used to culture the A549 cells and later grown at typical conditions until the cells attained about 90% confluence (\(1 \times 10^6\) cells/ml). By MDR \textit{A. baumanii}, the cells were infected for 2 h at 37 °C and later cleaned thrice using PBS. Consequently, addition of complete medium, comprising of different concentrations which ranges from 1/8 MIC-1/2 MIC (0.012 μg ml\(^{-1}\)–0.045 μg ml\(^{-1}\)) of fabricated ZnO-NPs was carried out and later the cells were incubated at standard conditions. After incubating for a day, the cells are cleaned using PBS thrice. In 0.25% EDTA/trypsin, the cells are incubated for a duration of 5 min followed by incubating for 10 min in Triton X-100 (0.025%) for cell lysis. The viable bacterial cells were diluted 10 times serially and the bacterial number was estimated by calculating the CFU on TSA plates after incubating them overnight. All the stages of the experiments were done three times independently using duplicate assay.

\textbf{Figure 3.} XRD pattern of the biosynthesized ZnO-NPs.

\textbf{Figure 4.} FT-IR spectrums (A) and Zeta potential (B) of ZnO-NPs.
2.15. Determining by immunofluorescence method of cell viability of the cells infected with *A. baumannii* on treatment with ZnO-NPs fabricated using *C. aromaticus* leaf extract

On the cover slips, A549 cells were cultured to reach 90% confluence for a day at standard conditions. The culture medium of the cells was discarded and later cleaned using PBS. By MDR *A. baumannii*, the cells are infected and treated with fabricated ZnO-NPs as explained above. The cells were washed two times using PBS, fixed for 10 min in pre-cooled methanol followed by the protocol of immunofluorescence as explained above. All the stages of the experiments were done three times independently.

3. Result and discussion

Ultraviolet-Visible Spectrophotometer (UV–vis) was utilized for determining the nanoparticles (NPs) structural characterization by assessing the optical absorbance and showed the plant extract ability in forming the ZnO-NPs. As displayed in figure 1, the bands of absorption at a wavelength 320 nm showed the ZnO-NPs formation. Therefore, the synthesized ZnO-NPs optical absorption spectra will have outstanding blue-shift confirming the particles size should not be larger than that of exciton-Bohr radius (r Bohr). This absorption peak is a significant indication of the formation of Zinc-oxide, whereas the blue-shift proved confinement of particles in nanoscale.

Also, the band gap for prepared ZnO-NPs was calculated from the absorption spectrum using the following equation, which was found to be 3.2 eV.

\[ \alpha h\nu = D(h\nu - E_g)n \]

h = energy of the photon, \( E_g = \) band gap of ZnO NPs and D = constant. The transition data delivers the best linear fit in the banded region for \( n = 1/2 \).

The photoluminescence (PL) spectrum of ZnO-NPs exhibits emission peaks at 480, 470, 418 and 368 nm, when measured at room temperature (figure 2). The presence of emission peak at about 418 nm could be because of the electron-hole recombination. In addition, the PL spectrum displays a characteristic blue band edge emission with broad and small intense peaks at about 368 nm [37]. Furthermore, the presence of this emission could be possibly because of the oxygen deficiency in the ZnO-NPs. The major edge noticed at about 420 nm is because of the quantum confinement effect.

XRD pattern of ZnO-NPs showed the diffraction pattern of points at scattering angles of 76.4° (201), 74.2° (102), 35.2° (100), 72.1° (004), 31.4° (101), 62.4° (103), and 57.8° (110) correspondingly (figure 3). Hence, the XRD pattern clearly confirms the ZnO-NPs formation by using bio-materials. Similarly, these peaks of diffraction of ZnO-NPs showed the hexagonal structure of wurtzite and the development of NPS with respect to
JCPDS NO: 008, 05-0664 and 79-2205 have also been confirmed. Furthermore, crystalline ZnO-NPs formation was also proved by the existence of the narrow peak of diffraction [8]. Also, the crystalline size of prepared NPs was calculated by following Debye–Scherrer’s formula given as follows, which is found to be 15 nm.

\[
D = K\lambda /\beta \cos \theta 
\]

D = mean particle size, \( \theta \) = Bragg angle, K = constant, \( \lambda \) = wavelength of x-ray and \( \beta \) = full width at half maximum intensity.

FTIR studies were utilized to detect possible functional groups which are involved in ZnO-NPs fabrication. Figure 4(A) displayed the ZnO-NPs FT-IR spectra. The broad absorption peak observed at about 3442 cm\(^{-1}\) showed the presence of \(-\text{OH}\) functional groups. The oscillational band observed at about 2943 cm\(^{-1}\) is characteristic to the presence of \(-\text{CH}\) (Alkane) functional groups. Furthermore, the band observed at about 2848 cm\(^{-1}\) is due to the stretching functionalities of C–H bond. The presence of peak noticed at about 1741 cm\(^{-1}\) is due to the C=O functional groups, indicating the utility of \(-\text{OH}\) (hydroxyl) functionalities of phyto extract biological molecules for ZnO-NPs reduction [38–41]. Additionally, ZnO-NPs FTIR spectrum displayed the bands at 1125 cm\(^{-1}\) and 1455 cm\(^{-1}\) corresponding to C–O bond stretching and C–H bond bending, respectively. The above findings have proved the ZnO-NPs capping with the polyphenols of plant extract.

Moreover, measurements of electro kinetic (zeta) potential for the synthesized ZnO-NPs indicated the -ve surface energy of around \(-25\) mV (depicted in figure 4(B)). The -ve surface electro kinetic potential of synthesized ZnO-NPs is because of the phyto polyphenols surface capping which are adsorbed on the surface of the ZnO-NPs following reduction. We all know that plant polyphenols are -vely charged which causes -ve charge on the ZnO-NPs surface following their adsorption upon the NPs surface. The above findings confirmed the phyto polyphenols capping upon the surface of synthesized NPs.

Transmission electron microscopy (TEM) graphs showed that the fabricated nanoparticles are mostly oval and spherical in shape with particle sizes ranging from 15–20 nm and the mean diameter of nanoparticles obtained is found to be around 18 nm (figures 5(A), (B)). Additionally, images of TEM displayed that the

Table 1. Minimum bactericidal concentration (MBC) and MIC of ZnO-NPs against MDR A. baumannii and A. baumannii ATCC 19606 (\( n = 10 \)).

|                    | ZnO-NPs | Colistin |
|--------------------|---------|---------|
|                    | MBC     | MIC     | MIC\(_{90}\) | MIC\(_{50}\) | MBC | MIC | MIC\(_{90}\) | MIC\(_{50}\) |
| A. baumannii ATCC 19606 | 0.35    | 0.08    | —           | —           | 1   | 1   | —           | —           |
| MDR clinical isolates | 0.35–>0.73 | 0.04–0.19 | 0.19       | 0.08       | 1–2 | 1–2 | 2           | 2           |

Figure 6. Possible mechanism of formation of ZnO-NPs using plant extract.
fabricated nanoparticles are anisotropically shaped which exhibit hexagonal and spherical nanoparticles. The ZnO-NPs selected area electron diffraction (SAED) pattern is indicated in figure 5(C). The selected area electron diffraction pattern proved that the fabricated ZnO-NPs nature is polycrystalline. Some of the previous reports have also displayed similar kind of results for the fabrication of ZnO-NPs [3, 8]. Further, DLS size histogram studies were shown in figure 5(D), which indicated that the average particle size of ZnO-NPs was found to be 15 nm, which is in agreement with TEM and XRD results [42].

The possible mechanism which is involved in the fabrication of ZnO-NPs utilizing extract of C. aromaticus is that these biomolecules may react with zinc ions (Zn^{2+}) resulting in zinc-polyphenolic complex formation. The subsequent as-formed complex was placed upon the substrate, accompanied by drying and annealing. During the process of annealing, the complex is changed into ZnO-NPs [43]. One of researcher reported the high reduction ability of the metallic salts, with corresponding metal possessing a high tendency of donating electrons [44]. Furthermore, the resultant metallic ion (M^{2+}) is reduced to zerovalent (M^0) with the help of the broad...
spectrum of biomolecules present in the plant extract \cite{44, 45}. The possible mechanism of formation of ZnO-NPs by using plant extract is shown in figure 6.

3.1. Biological studies

3.1.1. Determination of MBC and MIC

The fabricated ZnO-NPs were assessed for their antibacterial effect over MDR A. baumannii that were separated clinically. The values of MBC and MIC were in the range of 0.36 μgm l⁻¹ to >0.72 μgm l⁻¹ and 0.05 μgm l⁻¹ to 0.18 μgm l⁻¹, correspondingly (table 1). The MICs needed to inhibit 90% and 50% of clinically separated MDR A. baumannii (MIC90 and MIC50) were 0.18 μgm l⁻¹ and 0.09 μgm l⁻¹, correspondingly. All the clinical MDR A. Baumannii isolates were sensitive towards colistin. The both MBC and MIC values of colistin with clinically isolated MDR A. baumannii were 1 μgm l⁻¹–2 μgm l⁻¹, which had less antibacterial effect than fabricated ZnO-NPs.

3.1.2. Induction of cell death in bacteria by fabricated ZnO-NPs using C. aromaticus leaf extract

Cell death caused by apoptosis in bacteria and yeast can be triggered by ZnO-NPs \cite{46, 47}. Further, to investigate the induced cell death in bacteria, MDR clinical isolates of A. baumannii were subjected to 0.09 μgm l⁻¹ MIC of fabricated ZnO-NPs and Hoechst 33342 dye was utilized for staining. As depicted in figure 7, bacterial cells with no treatment exhibited nucleus with a single distinct round spot whereas, in the bacterial cells after induction with the fabricated ZnO-NPs, a dense chromatin condensation was observed. The positive control used was MDR A. Baumannii treated by Milli Q water. Condensation of chromatin is considered as a characteristic hallmark for inducing cell death \cite{48}. The staining reagent, Annexin V/7-AAD was used to assay the effect of fabricated ZnO-NPs on cell death of A. Baumannii. Prior incubation of MDR A. baumannii clinical isolates and A. baumannii ATCC 19606 with 0.09 μgm l⁻¹ MIC of fabricated ZnO-NPs displayed respectively 16.3% and 19.8% elevation in the cell death of bacteria (figure 8). The outcomes showed that the cell death in bacteria increased significantly on incubating with fabricated ZnO-NPs in both the MDR A. baumannii clinical isolates and A. baumannii ATCC 19606.

3.1.3. Inhibition of biofilm formation by fabricated ZnO-NPs using C. aromaticus leaf extract

MDR A. baumannii was recognized as a strong producer of biofilms \cite{49}. The anti-biofilm efficiency of fabricated ZnO-NPs was assessed over five clinical strains of A. baumannii ATCC 19606 and MDR A. baumannii, parallel to colistin. 96 well plates were used to culture the bacteria for a day in presence of fabricated ZnO-NPs at 1/8 MIC to 1/2MIC (0.012 μgm l⁻¹ –0.045 μgm ml⁻¹) of ZnO-NPs succeeded by standard crystal violet assay. The outcomes indicated that the treatment of fabricated ZnO-NPs at 1/8 MIC (0.012 μgm ml⁻¹) was sufficient to reduce the biofilm production (figure 9). The fabricated ZnO-NPs at 1/2 and 1/4 MIC (0.045 and 0.023 μgm ml⁻¹) considerably reduced the biofilm formation in all the clinically isolated strains. The outcomes...
revealed that the treatment with fabricated ZnO-NPs inhibited the formation of biofilm effectively by MDR A. baumannii, which was similar to the colistin activity.

3.1.4. Effect of fabricated ZnO-NPs using C. aromaticus leaf extract on established biofilm
Effect of fabricated ZnO-NPs on MDR A. baumannii cell viability in the established biofilm was evaluated. Biofilm was produced by five MDR A. baumannii for a day and five days and then treated with fabricated ZnO-NPs at 1 MIC to 8 MIC (0.09 μg ml⁻¹–0.72 μg ml⁻¹). As shown in figure 10(A), at 2 to 8 MIC (0.18 μg ml⁻¹–0.72 μg ml⁻¹), a considerable decrease in the cell viability of bacteria in treated biofilms grown for one day was observed. Similarly, in figure 10(B), treatment of biofilms grown for five days with fabricated ZnO-NPs at 8 MIC (0.72 μg ml⁻¹) might considerably decrease the viability of clinically isolated MDR A. baumannii present in the established biofilm. The outcomes confirmed that fabricated ZnO-NPs reduced the cell viability of bacteria in the established biofilm depending upon the dose.
3.1.5. ZnO-NPs fabricated using leaf extract of C. aromaticus plant inhibit the attachment of MDR A. baumannii to A549 cells

The study was conducted for investigating the inhibition of clinically isolated MDR A. baumannii attachment with host cells by fabricated ZnO-NPs. For investigation, lung epithelial cell line (A549) was preferred due to its high susceptibility to A. baumannii. The fabricated ZnO-NPs were used for direct incubation of bacteria at 1/8 MIC—1/2 MIC (0.012 μg ml⁻¹—0.045 μg ml⁻¹) for a period of 18 h at a temperature of 37 °C and then the determination of bacterial attachment to cells was done by calculating the CFU. Because of the obtained low MIC values, the concentration of fabricated ZnO-NPs utilized in this study were lower than MIC to confirm that the cells associated with bacteria were not killed by inhibition activity of NPs. From figure 11, the outcomes...
displayed that fabricated ZnO-NPs at 1/8 MIC (0.012 μg ml⁻¹) decreased the cells associated with bacteria in all the isolates. Treatment of bacterial cells with fabricated ZnO-NPs at 1/4 MIC and 1/2 MIC (0.023 μg ml⁻¹–0.045 μg ml⁻¹) were capable to reduce the adherent bacterial cell significantly in MDR A. baumannii in comparison to untreated control. In case of colistin, similar observations were identified.

3.1.6. ZnO-NPs fabricated using leaf extract of C. aromaticus plant inhibits invasion of MDR A. baumannii into human lung epithelial cells

Invasion of bacteria is an essential phenomenon which contributes to the bacterial infections’ pathogenesis; hence we assessed the fabricated ZnO-NPs effects on MDR A. baumannii into A549 cells. Fabricated ZnO-NPs at 1/8 MIC to 1/2 MIC (0.012 μg ml⁻¹–0.045 μg ml⁻¹) were used to treat bacterial cells and thereafter incubated...
using A459 cells and then immediately treated with gentamycin following infection period. In parallel, determination of colistin treatment as control was done. The outcomes in figure 12 exhibited some activity on the bacterial invasion percentage for 1/8 MIC (0.012 μg ml⁻¹) treated bacteria whereas, the most prominent effects were developed on treatment by fabricated ZnO-NPs with 1/4 MIC (0.025 μg ml⁻¹) and 0.045 μg ml⁻¹). Altogether, these outcomes revealed that fabricated ZnO-NPs efficiently reduced the invasion of A. baumannii into host cells.

3.1.7. MTT cytotoxicity assay of C. aromaticus leaf extract mediated ZnO-NPs
The cytotoxic effects of fabricated ZnO-NPs ranging from 0.01 to 0.72 μg ml⁻¹ was assessed with the help of MTT assay after treatment for a day (figure 13). No considerable cytotoxic effects were identified at 0.72 μg ml⁻¹ maximum concentration. The calculation of 50% cytotoxic concentration from concentration response curve was found to be 5.72 μg ml⁻¹.

3.1.8. Killing effect of fabricated ZnO-NPs using C. aromaticus leaf extract over MDR clinical isolates of A. baumannii in human lung epithelial cells
A. baumannii can invade and live inside the lung epithelial cells, hence killing effect of fabricated ZnO-NPs is a fascinating approach to a novel strategy over bacterial infection. The A. baumannii cells infected A549 cells, which were treated with different concentrations of fabricated ZnO-NPs from 1/8 MIC 1/2 MIC (0.012 μg ml⁻¹–0.045 μg ml⁻¹). As shown in figure 14, assessment of sustainable bacteria in cell lysate exhibited nearly 92%, 80%, 65% MDR A. baumannii reduction, on treating with 1/2, 1/4, 1/8 MIC of fabricated ZnO-NPs respectively. These outcomes confirmed that MDR A. baumannii clinical isolates were sensitive to the fabricated ZnO-NPs activity and the effect of ZnO-NPs action on killing activity of MDR A. baumannii was dependent on its dose. In meantime, for the confirmation of fabricated ZnO-NPs non-toxicity, a parallel study was carried out by nucleus staining of A549 cells that were cultured on cover slips using Hoechst 33342 dye followed by monitoring with fluorescence microscopy. The outcomes displayed no changes in nuclei which indicate that the fabricated ZnO-NPs toxicity on A549 cells was insignificant. This outcome further specified that inhibitory effect on infection of MDR A. baumannii was not because of fabricated ZnO-NPs which induced cell cytotoxicity (figure 15).

4. Conclusions
In conclusion, we displayed the fabrication of ZnO-NPs utilizing C. aromaticus leaf extract. FTIR analysis has proved the ZnO-NPs capping with polyphenols of phyto extract. SAED and XRD findings showed the synthesized ZnO-NPs crystalline nature. ZnO-NPs fabricated using leaf extract of C. aromaticus plant showed a potent antimicrobial effect by activating DNA damage which causes death in bacterial cells, including a decrease in the production of biofilm, MDR A. baumannii cell viability present in established biofilm, cells associated with bacteria, invasion of bacterial cells and microbial infection in human lung epithelial host cells. Although, the fabricated ZnO-NPs provides a major potential, additional studies should be continued for their advancement and fabricated ZnO-NPs may offer potent alternative treatment for reducing infections caused by MDR A. baumannii.
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