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Evaluating the biological activities of biosynthesized ZnO nanoparticles using *Escherichia coli*

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ABSTRACT

About 50 isolates (83%) of *Escherichia coli* were identified from 60 stool sample, and 30 examined bacteria formed biofilm. ZnO NPs was synthesized by *E. coli* and a white cluster pellet appeared, followed by observing absorption peak of UV-Vis. spectroscopy at 268 nm. XRD pattern showed the lattice planes of 100, 002, 101, 102, 110, 103 and 112 compatible to the 2θ values of 32.45˚, 34.73˚, 36.56˚, 47.70˚, 55.86˚, 62.12˚and 63.10˚ respectively, and the diffraction peaks were assigned with the hexagonal phase, while SEM images exhibited that size of the particles ranged between 31.55-45.9 nm. ZnO NPs displayed antibacterial potentiality against pathogenic bacteria, and inhibition zones around ZnO NPs were as follows: 5, 4, 2, 2, and 2 mm for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *E. coli* respectively. In addition, ZnO NPs was able to decrease biofilm, revealing that after 48 h of incubation, inhibition percentage were 18.6, 27.7, 39.4, and 19.6 % against *S. aureus*, *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, respectively. A549 cells viability was decreased by elevating the concentration of ZnO NPs, and the IC_{50} values of the A549 and WRL cells were 105.8 and 167.3 µg mL^{-1} respectively. In this study, the synthesized ZnO NPs using nonpathogenic *E.coli* showed antibacterial, antibiofilm and anticancer activities against the examined pathogenic bacteria. So, these nanoparticles can be further used in biomedical, pharmaceutical and other applications as an effective antimicrobial and anti-cancerous agent.

Key words: Biosynthesized ZnO NPs, *E.coli*, Antibacterial, antibiofilm, Anticancer.
Article type: Research Article.

INTRODUCTION

Nanoparticles (NPs) is one of the roads to nanotechnology that is related to the nanoscale materials through very small particles size ranging between 1 to 100 nm. NPs display distinctive properties due to their very small size also high surface area to volume ratio, which have attributed to the significant differences in the properties over their bulk counterparts (Singh *et al.* 2011). There are several studies reported about nanoparticles around the world (Bagherzadeh Lakani 2016, Johari 2016 a, b; Bozorgpanah Kharat 2018) Zinc is an important nutrient in living organisms (Sturikova *et al.* 2018). In addition, ZnO has been registered as "Generally Recognized as Safe" (GRAS) by the US Food and Drug Administration (FDA 2016) owing to its non-toxic properties (Pulit-Prociak *et al.* 2016). Researches showed that ZnO NPs possess a great possibility in biological applications, such as the antimicrobial agents (Sirelkhatim *et al.* 2015). Moreover, many studies have been described on the efficacy of ZnO NPs in preventing the growth of broad-spectrum of pathogens (Jayaseelan, *et al.* 2012), which possibly could substitute the conventional antibiotic (Yusof *et al.* 2019). The effect of zinc oxide was examined on highly resistant biofilms, distinguishing degrees of operation for treatment depending on the bacteria used (Martínez-carmona *et al.* 2018). ZnO NPs also have been widely used in cancer therapy and have been reported to stimulate selective cytotoxic effects on cancer cell proliferation. Zinc oxide nanoparticles may be the most cytotoxic to cancer cells than adipocyte cells (Chandrasekaran & Pandurangan 2016). These findings indicated that zinc oxide nanoparticles may selectively induce cancer cell apoptosis, which could be promising candidates for cancer care (Martínez-carmona *et al.* 2018). This study aims to biosynthesis ZnO NPs from nonpathogenic *E.coli*,

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and used these nanoparticles as antibacterial, antibiofilm against multidrug-resistant isolates, as well as applying them as anticancer against human epithelial alveolar cells (A549).

MATERIALS AND METHODS

Collection of *E.coli*

Sixty stool samples were collected from non-infectious persons to obtain of *E.coli* bacteria, and ages ranged from 6- 60 year olds.

Test bacterial isolates

About 30 isolates of examined bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Escherichia coli*) were obtained from AL-Mustansiriyah University Laboratories.

Detection of Biofilm production

Congo red test

Pathogenic bacteria were screened to detect their ability to biofilm production as by Niveditha *et al.* (2012).

Microtiter plate method

To explore the susceptibility of bacteria to biofilm formation using the Microtiter plate method according to Babapour (2016).

Biological synthesis of ZnO- Nps

Method of biosynthesis was according to Daneshvar (2008), and the creation of a white precipitate at the bottom of the flask is an indication of the formation of nanoparticles (Hu & Chen 2008).

Characterization of biosynthesized ZnO nanoparticles

Morphological study of ZnO NPs was identified using SEM (TESCAN-VEGA/USA). Optical properties were studied using UV-Vis. spectroscopy (Metertech sp. 8001) at 200-900 nm. In addition, FTIR 4000-400 cm^{-1} (Shimadzu/Japan) was used to determine the functional groups, and XRD (7000- Shimadzu Maxima) to determine the crystalline structure of ZnO NPs.

Determination of shelf life of biosynthesized ZnO NPs

Biosynthesized ZnO NPs were stored at 4 °C, 25 °C and 37 °C for seven months, then UV-Vis. spectrophotometer and FTIR were performed.

Minimum inhibitory concentration (MIC) of ZnO NPs

MIC of the synthesized ZnO nanoparticles was evaluated by the microtiter plate dilution method with modification.

Effect of ZnO NPs on Biofilm Formation

Congo red agar method

ZnO NPs used in different concentration including 100, 50, and 25 mg mL^{-1} and the method as in Hasan (2016) with modification.

Microtiter plate method

The procedure was used as described by Abd Ulkareem (2012) with modification, and the inhibition of biofilm formation was calculated as equation described by Namasivayam & Karthick (2013).

$$\text{Inhibition of biofilm formation (\%)} = \frac{OD\ control - DO\ tratment}{OD\ control} \times 100$$

Anticancer effect of ZnO NPs

Cancer cells (A549) and normal cells (WRL68) were used by the concentrations of ZnO NPs including 25, 50, 100, 200, 400 µg mL^{-1}. Statistical analysis was performed to calculate the IC_{50} according to the following equation:

$$\text{Viability (\%)} = \frac{optical\ density\ of\ sample}{optical\ density\ of\ control} 100\%$$

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Statistical analysis

All statistical analysis were carried out using One-Way ANOVA and Duncan, and the statistical significance was found as $p \leq 0.05$. The data was determined using Graph Pad Prism version 6 (Graph Pad Software Inc., La Jolla, CA).

RESULTS AND DISCUSSION

Isolation and Identification of *Escherichia coli*

About 50 isolates (83%) of *Escherichia coli* were identified from 60 stool sample, and documentation as described by Saadi Al-Baer & Hussein (2017). *E. coli* is precipitated under acidic environment eosin Y and formed an amide bond among eosin Y and methylene blue in the medium (Antony *et al.* 2016).

Biofilm Formation

Result exhibited that biofilm formed by isolates of *S. aureus*, while isolates of *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii* were formed by Congo red agar method. Results of Microtiter plate method showed that 4 isolates of *P. aeruginosa*, *A. baumannii*, *E. coli* and *K. pneumoniae* produced strong biofilm, while 2 isolates produced moderate and 2 isolates weak biofilms. On the other hand, the 3 isolates of *S. aureus* produced strong biofilms. Duarte *et al.* (2016) observed that 74.7% of *A. baumannii* isolates were capable to produce biofilm. According to Karigoudar *et al*. (2019), 69% of *E. coli* were able to produce biofilm, while Murugan *et al*. (2016) reported that *P. aeruginosa* and *S. aureus* has a very high capacity for biofilm production. It was also true for Nirwati *et al.* (2019) who observed that 85.63% of *K. pneumoniae* isolates were able to produce biofilm.

Biosynthesis of ZnO Nanoparticles

ZnO-NPs synthesis by *Escherichia coli* as white cluster pellet, and the dry weight was evaluated. The biosynthesized NPs were regulated by general conditions such as metal ions which restrict the microbial cells or on the microbial surface in the presence of enzymes, thereby reducing to form NPs (Khan *et al.* 2020).

ZnO-Nps Characterization

An absorption peak observed at 268 nm refer to the effective biosynthesis of ZnO NPs. Ifeanyichukwu *et al*. (2020) reported that absorption peak of ZnO NPs synthesized from pomegranate leaf at 284 nm, while FTIR gave information about functional group related with the synthesized nanoparticles. XRD pattern observed lattice planes of 100, 002, 101, 102, 110, 103 and 112 compatible with the 2θ values of 32.45°, 34.73°, 36.56°, 47.70°, 55.86°, 62.12°and 63.10° respectively, with the hexagonal phase of ZnO. SEM images showed that the size of ZnO-NPs particles ranged between 31.55-45.9 nm, and aggregated as uneven round structure, which is similar to that reported by Muhammad (2019; Fig. 1).

Determination of shelf life of biosynthesized ZnO NPs

The Zinc Oxide nanoparticles stored for 7 months at different temperatures and remained stable without change in color. The results in Fig. 2 illustrates the transmittance spectrum of the ZnO NPs as a wavelength (263 nm) and the band at 434 cm^{-1} is confirmed the stretching vibration of ZnO NPs.

Similar results were also reported by Saputrais (2017). The good stability of ZnO NPs is due to the free amino and carboxylic groups which is interacted with the surface of ZnO NPs, while the amide group obtained from the protein functions as capping agent of the ligands of the ZnO NPs (Peletiri *et al.* 2012).

Minimal Inhibitory Concentration of ZnO nanoparticles

Biosynthesized ZnO NPs was found to be 12.5 mg mL^{-1} against each *E.coli*, *A.baumannii*, and *K.pneumoniae*, while the MIC against *P.aeroginosa* was 50 mg mL^{-1}, and 25 mg mL^{-1} for *S. aureus.* The diameter of inhibition zones around the filter paper saturated with sub MIC ZnO NPs as follows: 5, 4, 2, 2, and 2 mm for *P. aeruginosa*, *S.* aureus *A.baumannii*, *K.pneumoniae*, and *E.coli* respectively. The variation in the antimicrobial activates of ZnO NPs as exhibited by MIC of nanoparticles probably results from the differences in the examined genus and species (Alaskaree 2018). The mechanism of antimicrobial activity of ZnO NPs may include release of Zn^{2+} and generation of ROS, hence damage to cell membrane (Thakur *et al.* 2021).

Antibiofilm on Congo red method

Pink colonies in the presence of Zinc oxide nanoparticles implied a loss of biofilm formation capability in all pathogenic bacterial isolates of the study (*A. baumannii*, *P. aeroginosa*, *K. pneumoniae*, and *S. aureus*; Fig. 3).

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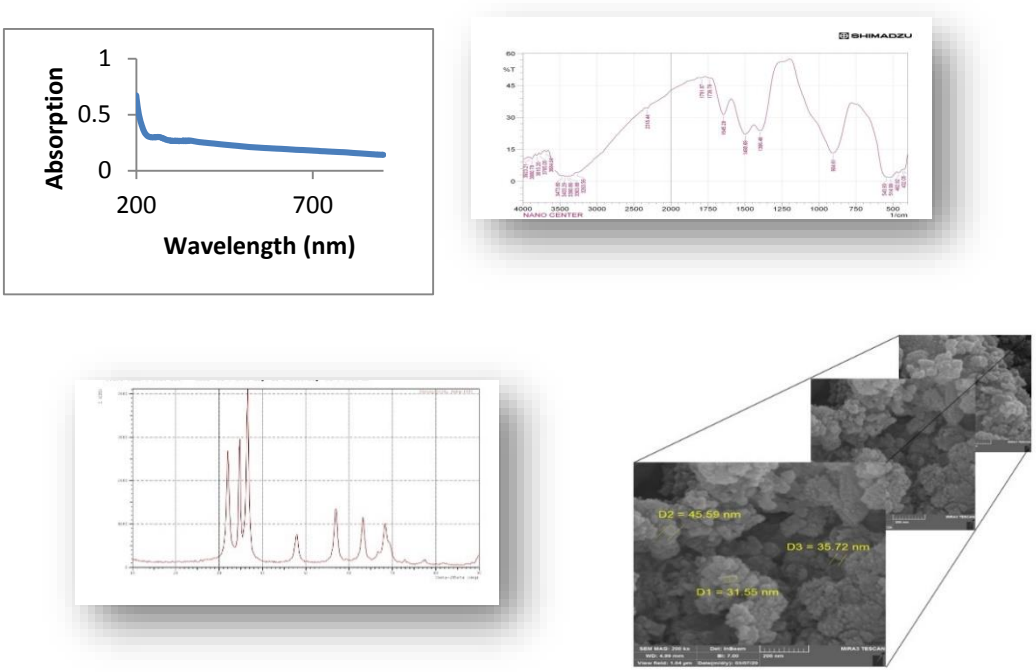

Fig. 1. A: UV-visible absorption spectrum, B: FTIR result, C: XRD pattern, D: SEM picture of ZnO NPs.

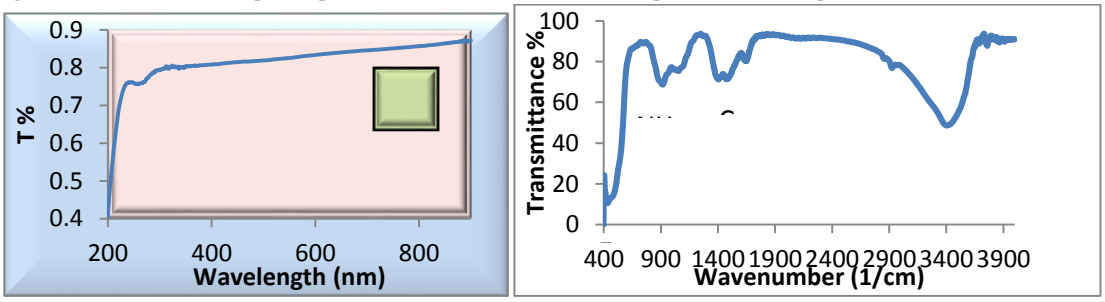

Fig. 2. A: UV-visible transmittance spectrum, B: FTIR result of ZnO NPs.

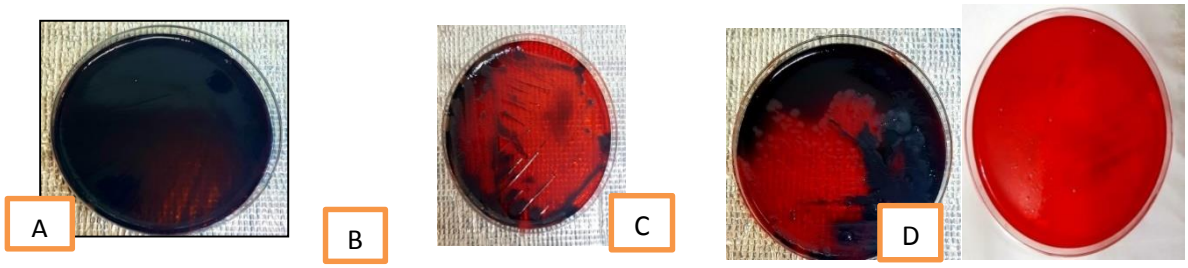

Fig. 3. Antibiofilm activity of ZnO NPs. A: Control; B: Antibiofilm of 25 mg mL^{-1} ZnO NPs; C: Antibiofilm of 50 mg mL^{-1} ZnO NPs; D: Antibiofilm of 100 mg mL^{-1} ZnO NPs.

Antibiofilm on Microtiter plate method

ZnO NPs was able to decrease biofilm formation from MDR bacterial isolates. Results showed that inhibition percentage of biofilm were (18.6, 27.7, 39.4, and19.6) % against *S.aureus*, *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, respectively after 48 hours of incubation Table 1. The resistant properties of biofilm, lead to eradication of biofilm related disease is challenging ZnO nanoparticles have bioactivity properties like regulator of biofilms formation according to the concentration of this nanoparticles, as it has been as promising antibacterial agents than conventional antibiotics.

Cytotoxicity test of ZnO NPs

The cells viability was decreased by increasing the concentration of the ZnO. The IC_{50} values of the A549 and WRL cells were 105.8, 167.3 µg mL^{-1} respectively (Fig. 4). The results showed that adding ZnO NPs decrease the cell viability of A 549 cells, relating strongly to the concentrations significantly ($p < 0.05$). The percentage of decreasing viability was

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42.3 ± 2.6, 54.1 ± 1.3, 68.2 ± 0.9, 81 ± 1.8 and 93 ± 2.3) in the concentration of 400, 200, 100, 50, 25 respectively, while adding the same concentration to the WRL 68 cells did not show significant effect on viability rate ranging between 62.6 ± 4.4, 71.3 ± 7.1, 88.3 ± 6.2, 87.6 ± 5.2 and 94.2 ± 1.7 (Table 2).

Table 1. Antibiofilm effect of ZnO NPs synthesized by *E.coli* against MDR bacteria.

Bacterial isolates		Biofilm inhibition %
(3)	*S. aureus*	18.6
(1)	*P. aeroginosa*	27.7 39.4 19.6
(6)	*A. baumannii*	
(7)	*K. pneumoniae*	

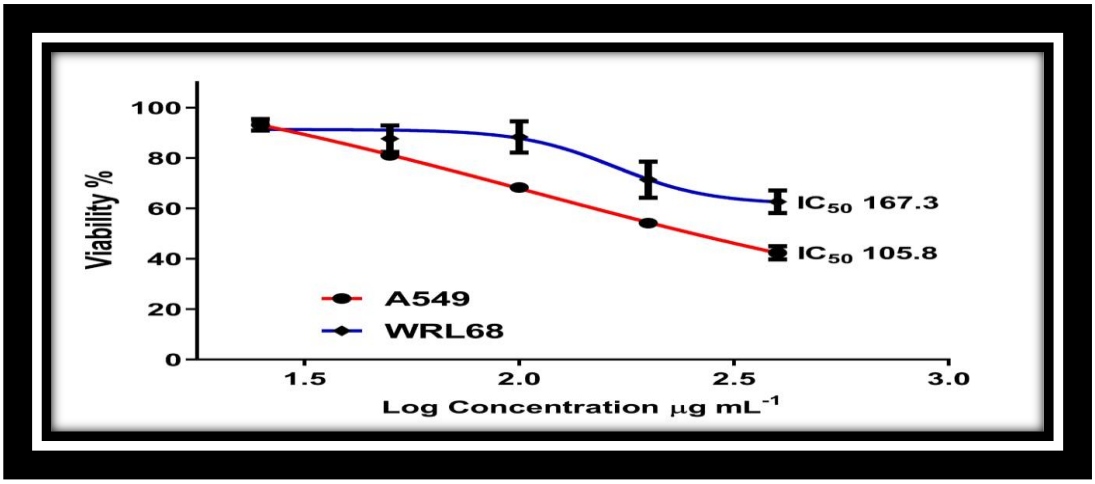

Fig. 4. Cytotoxicity of ZnO NPs on A549 and normal WRL 68. Each point is the mean value of three replicate.

Table 2. Flow cytometric analysis of C549 and WRL68 cells after treated by ZnO-NPs.

Concentration of ZnO NPs (mg mL^{-1})	Viability (%)	
	A 549	WRL
400	42.3 ± 2.6	62.6 ± 4.4
200	54.1 ± 1.3	71.3 ± 7.1
100	68.2 ± 0.9	88.3 ± 6.2
50	81 ± 1.8	87.6 ± 5.2
25	2.3 ±93.1	94.2 ± 1.7

Values are expressed as mean ± SD of three experiments.

Reddy & Srividya (2018) studied the cytotoxicity effect of ZnO NPs against (A549, HEK) human cell lines, and revealed the dose dependent cytotoxicity of zinc oxide nanoparticles using tested cell cultures. The biosynthesized ZnO NPs due to

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its semiconducting nature are reported to induce cytotoxicity in cancer cells by the generation of reactive oxygen species on the surface of the particle, the released Zn^{+2} ions are dissolved in culture media indicating direct interaction of NPs with a membrane of cancer cell resulting in oxidative stress thereby leading to the ultimate death of cancer cells (Miyaue *et al.* 2018).

CONCLUSION

From the results of this study, the biosynthesized ZnO NPs using nonpathogenic *E. coli* showed antibacterial, antibiofilm and anticancer activities against studied MDR bacteria. So, these nanoparticles can be further used in many biotechnological applications as an effective antimicrobial, biofilm disinfectant and anti-cancerous agent.

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