Evaluate the Biological Activities of Biosynthesized ZnO- Nanoparticles Using *Escherichia Coli*

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Abstract

About 50 isolates of *E. coli* were identified (83(٪ from 60 stool sample, and 30 test bacteria which formed biofilm. ZnO- NPs synthesis by *Escherichia coli* and a white cluster pellet appeared, and absorption peak of UV-Vis. spectroscopy observed at 268 nm. XRD pattern showed the lattice planes of (100), (002), (101), (102), (110), (103) and (112) agreed 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 are 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 zone around ZnO NPs as follows 5, 4, 2, 2, and 2mm for *P.aeruginosa*, *S.aureus*, *A.baumannii*, *K.pneumoniae*, and *E. coli* respectively. Also, ZnO NPs was able to decrease biofilm, and results showed that inhibition percentage 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. A549 cells viability was decreased with increasing the concentration of the ZnO NPs, and the IC_{50} values of the A549 and WRL cells were 105.8 and 167.3 µg/mL respectively. In this study, the synthesized ZnO NPs using nonpathogenic *E.coli* showed antibacterial, antibiofilm and anticancer activities against studied 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.*

Introduction

Nanoparticles (NPs) is one of the roads to nanotechnology that is related with 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 [(1)]. Zinc is an important nutrient in living organisms [(2)]. In addition, ZnO has been registered as "Generally Recognized as Safe" (GRAS) by the US Food and Drug Administration [(3)] owing to its non-toxic properties [(4)]. Researches showed that ZnO NPs possess a great possibility in biological applications, such as the antimicrobial agents [(5)]. Moreover, many studies have been described on the efficacy of ZnO- NPs in preventing the growth of broad-spectrum of pathogens [(6)], which possibly could substitute the conventional antibiotic [(7)]. The effect of zinc oxide on highly resistant biofilms, distinguishing degrees of operation for treatment depending on the bacteria used [(8)]. 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 [(9)], these findings indicated that zinc oxide nanoparticles may selectively induce cancer cell apoptosis, which could be promising candidates for cancer care [(8)]. This study aims to

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biosynthesis ZnO- NPs from nonpathogenic *E.coli*, and used these nanoparticles as antibacterial, antibiofilm against multidrug resistant isolates, in-addition to that used 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) years old.

Test bacterial isolates

About 30 isolates of test bacteria (*P.aeroginosa, A.baumannii, K.pneumoniae, E.coli,* and *S.aureus)* were obtained from AL-Mustansiriyah university laboratories.

Detection of Biofilm production

Congo red test

Pathogenic bacteria were screened to detection their ability to biofilm production as in [10].

Microtiter plate method

To explore the susceptibility of bacteria to biofilm formation using the Microtiter plate method according to [11].

Biological synthesis of ZnO- Nps

Method of biosynthesis was according to [12], and the creation of a white precipitate at the bottom of the flask is an indication of the formation of nanoparticles [13].

Characterization of biosynthesized ZnO nanoparticles

Morphological study of ZnO NPs was identified using SEM (TESCAN-VEGA/USA). While, Optical properties were studied using UV-Vis. spectroscopy (Metertech sp. 8001) at (200-900) nm. Also, 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, 25C°, and 37 C° for 7 months, then UV− Vis. spectrophotometer and FTIR were performed.

Minimum inhibitory concentration of ZnO NPs

MIC of the synthesized ZnO nanoparticles was evaluated by the microtiter plate dilution method according to [14] with modification.

Effect of ZnO-Nps on Biofilm Formation

Congo red agar method

ZnO NPs used in different concentration (100, 50, and 25 mg/ml) and the method as in [15] with modification.

Microtiter plate method

The procedure was used as described by [16] with modification, and the inhibition of biofilm formation was calculated as equation described by [17].

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

Anticancer effect of ZnO NPs

Cancer cells (A549) and normal cells (WRL68) were used and the concentration ZnO NPs (25, 50, 100, 200, 400 µg/mL). Statistical analysis was performed to calculate the IC50 according to the following equation:

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

Statistical analysis

All statistical analysis were carried out using one way analysis of variance ANOVA(Duncan), and the statistical significance was fined as ≤ 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 of *E. coli* were identified (83(٪ from 60 stool sample, and documentation as described by (18). *Escherichia coli* under acidic environment eosin Y is precipitated and formed an amide bond among eosin Y and methylene blue in the medium (19).

Biofilm Formation

Result exhibited that biofilm formed by (3) isolates of *S. aureus*, while (2) isolates of *P. aeruginosa*, (3) isolates of *E. coli*, (4) isolates of *K. pneumoniae*, (4) isolates of *A. baumannii* 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 biofilm and 2 isolates weak biofilm production, and the 3 isolates of *S. aureus* produce strong biofilm.

Duarte *et al*, (20) observed that 74.7٪ of *A. baumannii* isolates were capable to produce biofilm. Karigoudar *et al*., (21) showed 69٪ of *E. coli* were able to produce biofilm, and Murugan *et al*., (22) reported that *P. aeruginosa* and *S. aureus* has a very high capacity for biofilm production. Nirwati *et al* (23) 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 of NPs regulated by general conditions such as metal ions are restriction in the microbial cells or on the microbial surface in the presence of enzymes, thereby reduced to form NPs (24).

ZnO-Nps Characterization

An absorption peak observed at 268 nm refer to the effective biosynthesis of ZnO NPs. Ifeanyichukwu *et al*., (25) 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) agreed to 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 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 (26) (Figure 1).

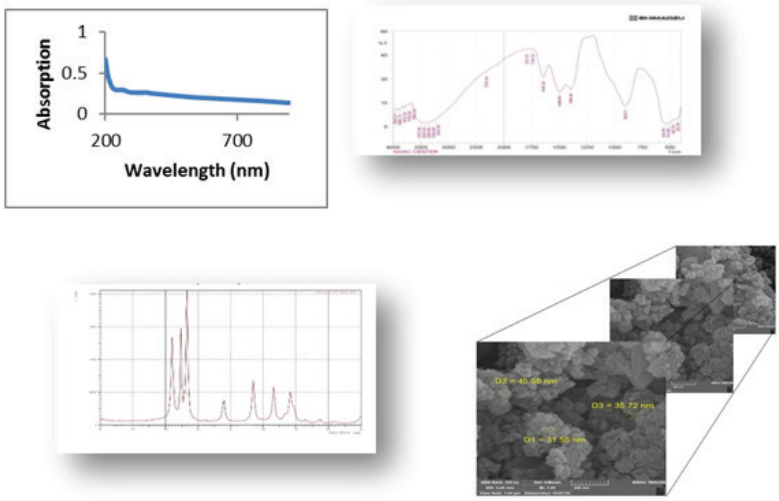

Figure (1) ☐ A- UV−visible absorption spectrum, B-FTIR result, C- XRD pattern, D-SEM picture of ZnO NPs Determination of shelf life of biosynthesized ZnO NPs

The Zinc Oxide nanoparticles stored for 7 months at different temperatures and remained stable without change in color. The results in Figure (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.

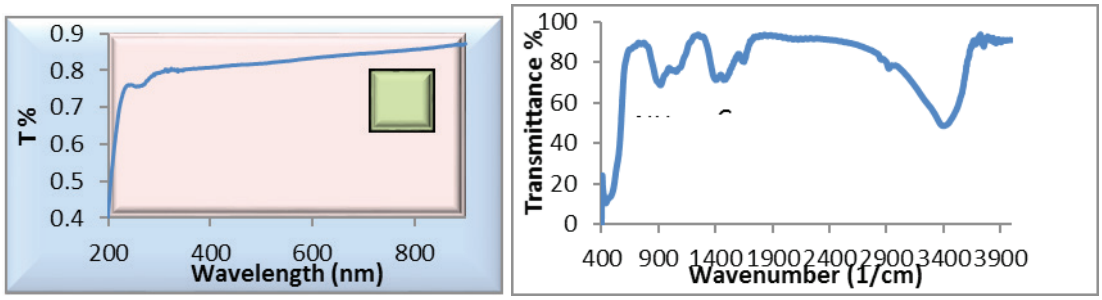

Figure (2) □ A- UV−visible transmittance spectrum, B- FTIR result of ZnO NPs

Similar results were also reported by [27]. The good stability of ZnO NPs because of the free amino and carboxylic groups and it is interacted with the surface of ZnO NPs, and the amide group obtained from the protein acted as capping agent of the ligands of the ZnO NPs [28].

Minimal Inhibitory Concentration of ZnO nanoparticles

Results showed that the MIC of biosynthesized ZnO NPs was found to be 12.5 mg / ml against each *E.coli*, *A.baumannii*, and *K.pneumoniae*. While the MIC against *P.aeroginosa* was 50 mg/ml, and 25mg/ml for *S. aureus.* The diameter of inhibition zones around the filter paper saturated with sub MIC ZnO NPs as flows (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 manifested by MIC of nanoparticles probably results from the differences in the tested genus and species [29]. The mechanism of antimicrobial activity of ZnO NPs may include release of Zn^{2+} and generation of ROS and damage to cell membrane [30].

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).

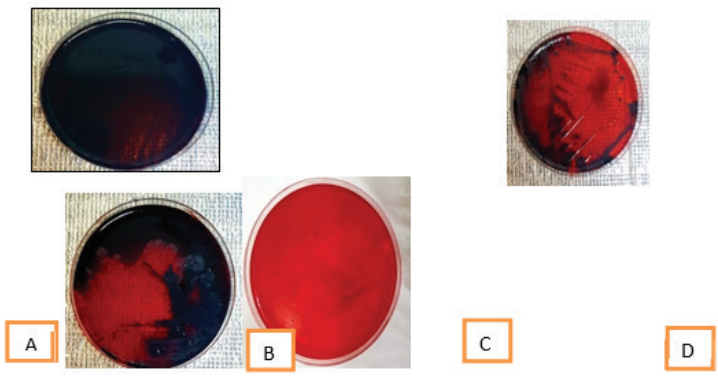

Figure (3) □Antibiofilm activity of ZnO NPs. A−control B−Antibiofilm of 25mg/ml ZnO NPs, C−Antibiofilm of 50mg/ml ZnO NPs D− Antibiofilm of 100mg/ml

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 [31] 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 [31].

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
(6)	A.baumannii	39.4
(7)	K.pneumoniae	19.6

Cytotoxicity test of ZnO NPs:

The cells viability was decreased with increasing the concentration of the ZnO The IC_{50} values of the A549 and WRL cells were 105.8, 167.3 µg/mL respectively as shown in figure (4). The results showed that adding ZnO NPs decrease the cell viability of A 549 cells, and this decreasing related strongly with the concentrations significantly ($P < 0.05$). The percentage of decreasing viability was (42.3 ± 2.6, 54.1 ± 1.3, 68.2 ± 0.9, 81 ± 1.8 and 93 ± 2.3) in concentration (400, 200, 100, 50, 25) respectively, while adding the same concentration to the WRL 68 cells did not show significant effect of viability rate ranged between (62.6 ± 4.4, 71.3 ± 7.1, 88.3 ± 6.2, 87.6 ± 5.2 and 94.2 ± 1.7) as shown in table (2).

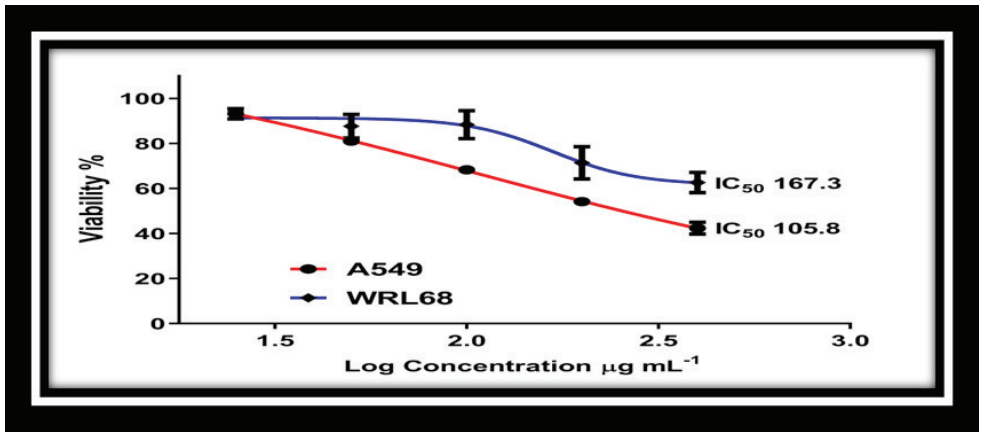

Figure (4) □ Cytotoxicity of ZnO NPs with A549 and normal WRL 68. Each point is the mean value of three replicate

Table (2) □ Flow cytometric analysis of C549 and WRL68 cells after treated with ZnO-NPs.

Concentration of ZnO NPs(mg/ml)	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	93.1±2.3	94.2±1.7
Values are expressed as mean ±SD of three experiments.		

Reddy and Srividya [30] 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 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 [34].

Conclusions

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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Ethical Clearance: Not required

References

1. Singh RP, Shukla VK, Yadav RS, Sharma PK, Singh PK, Pandey AC. Biological approach of zinc oxide nanoparticles formation and its characterization. Adv. Mater. Lett. 2011 Oct 1;2(4):313-7.
2. Sturikova H, Krystofova O, Huska D, Adam V. Zinc, zinc nanoparticles and plants. Journal of hazardous materials. 2018 May 5;349:101-10.
3. FDA U. Select Committee on GRAS Substances (SCOGS) Opinion: Tannic acid (hydrolyzable gallotannins). GRAS substances (SCOGS) database. 2015.
4. Pulit-Prociak J, Chwastowski J, Kucharski A, Banach M. Functionalization of textiles with silver and zinc oxide nanoparticles. Applied Surface Science. 2016 Nov 1;385:543-53.
5. Sirelkhatim A, Mahmud S, Seeni A, Kaus NH, Ann LC, Bakhori SK, Hasan H, Mohamad D. Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism. Nano-micro letters. 2015 Jul;7(3):219-42.
6. Jayaseelan C, Rahuman AA, Kirthi AV, Marimuthu S, Santhoshkumar T, Bagavan A, Gaurav K, Karthik L, Rao KB. Novel microbial route to synthesize ZnO nanoparticles using Aeromonas hydrophila and their activity against pathogenic bacteria and fungi. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2012 May 1;90:78-84.

7. Yusof HM, Mohamad R, Zaidan UH. Microbial synthesis of zinc oxide nanoparticles and their potential application as an antimicrobial agent and a feed supplement in animal industry: a review. Journal of animal science and biotechnology. 2019 Dec;10(1):1-22.
8. MARTÍNEZ-CARMONA Marina, GUN'KO Yurii, VALLET-REGÍ María ZnO nanostructures for drug delivery and theranostic applications. *Nanomaterials*, 2018, 8.4: 268.
9. CHANDRASEKARAN Murugesan, PANDURANGAN Muthuraman. In vitro selective anti-proliferative effect of zinc oxide nanoparticles against co-cultured C2C12 myoblastoma cancer and 3T3-L1 normal cells. Biological trace element research, 2016, 172.1: 148-154.
10. Niveditha S, Pramodhini S, Umadevi S, Kumar S, Stephen S. The isolation and the biofilm formation of uropathogens in the patients with catheter associated urinary tract infections (UTIs). Journal of clinical and diagnostic research: JCDR. 2012 Nov;6(9):1478.
11. BABAPOUR Ebrahim. Biofilm formation in clinical isolates of nosocomial Acinetobacter baumannii and its relationship with multidrug resistance. *Asian Pacific Journal of Tropical Biomedicine*, 2016, 6.6: 528-533.
12. DANESHVAR N. Preparation and investigation of photocatalytic properties of ZnO nanocrystals: effect of operational parameters and kinetic study. *Evaluation*, 2008, 900.6.
13. HU Yi, CHEN Hung-Jiun. Preparation and characterization of nanocrystalline ZnO particles from a hydrothermal process. *Journal of Nanoparticle Research*, 2008, 10.3: 401-407.
14. Baltzar BK. Minimal Inhibitory Concentration (MIC). 14 Marc 2017.
15. Hasan ZH. Inhibition of Biofilm formation by silver nanoparticles biosynthesized by pathogenic Escherichia coli. MSC Thesis.Collage of Scinence, Mustansyriah University. 2016.
16. ALI Omar Abd Ulkareem. Prevention of Proteus mirabilis biofilm by surfactant solution. *Egyptian Academic Journal of Biological Sciences, G. Microbiology*, 2012, 4.1: 1-8.
17. NAMASIVAYAM S, Karthick Raja. Anti-biofilm effect of biogenic silver nanoparticles coated medical devices against biofilm of clinical isolate of Staphylococcus aureus. *Global Journal of Medical Research*, 2013, 13.3: 1-7.
18. Saadi Al-Baer A, Hussein AA. Isolation and Identification of Escherichia coli Producing Cytosine Deaminase from Iraqi patients. Int. J. Adv. Res. Biol. Sci. 2017;4(11):1-6.
19. Antony AC, Mini KP, R Silvester, PA Aneesa, K Suresh, PS Divya, S Paul, PA Fathima and Abdulla. Comparative Evaluation of EMB Agar and Hicrome E. coli Agar for Differentiation of Green Metallic Sheen Producing Non E. coli and Typical E. coli Colonies from Food and Environmental Sample. *J Pure Appl Microbio* 2016. 10(4).
20. DUARTE Andreia. Clinical isolates of Acinetobacter baumannii from a Portuguese hospital: PFGE characterization, antibiotic susceptibility and biofilm-forming ability. *Comparative immunology, microbiology and infectious diseases*, 2016, 45: 29-33.
21. KARIGOUDAR Rashmi M. Detection of biofilm among uropathogenic Escherichia coli and its correlation with antibiotic resistance pattern. *Journal of laboratory physicians*, 2019, 11.1: 17.
22. MURUGAN N. Unraveling genomic and phenotypic nature of multidrug-resistant (MDR) Pseudomonas aeruginosa VRFPA04 isolated from keratitis patient. *Microbiological research*, 2016, 193: 140-149.
23. NIRWATI, Hera. Biofilm formation and antibiotic resistance of Klebsiella pneumoniae isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. In: *BMC proceedings*. BioMed Central, 2019. p. 1-8.
24. KHAN, Mujeebur R., et al. Metal nanoparticle–microbe interactions: synthesis and antimicrobial effects. *Particle & Particle Systems Characterization*, 2020, 37.5: 1900419.
25. IFEANYICHUKWU, Ugochi Lydia; FAYEMI, Omolola Esther; ATEBA, Collins Njie. Green synthesis of zinc oxide nanoparticles from pomegranate (Punica granatum) extracts and characterization of their antibacterial activity. *Molecules*, 2020, 25.19: 4521.
26. MUHAMMAD Wali. Optical, morphological and biological analysis of zinc oxide nanoparticles (ZnO NPs) using Papaver somniferum L. *RSC advances*, 2019, 9.51: 29541-29548.

27. SAPUTRA IS, YULIZAR Yoki. Biosynthesis and characterization of ZnO nanoparticles using the aqueous leaf extract of Imperata cylindrica L. In: *IOP Conference Series: Materials Science and Engineering*. IOP Publishing, 2017. p. 012004.
28. Peletiri C, Matur BM, Ihongbe JC, Okoye M. The effect of Azadirachta indica (Neem Tree) on human plasmodiasis: the laboratory perspective. *J Med Sci* 2012. 2 (1), 013-017.
29. Alaskaree AA. Biosynthesis of Zinc Oxide nanoparticles (ZnO NPs) by probiotic bacteria and their effect on bacterial skin infections. (PhD Thesis). Collage of Science, Mustansyriah University. 2018.
30. THAKUR, Shaila; NEOGI, Sudarsan; RAY, Ajay K. Morphology-Controlled Synthesis of ZnO Nanostructures for Caffeine Degradation and Escherichia coli Inactivation in Water. *Catalysts*, 2021, 11.1: 63.
31. MIYAUE, Saki, et al. Bacterial memory of persisters: bacterial persister cells can retain their phenotype for days or weeks after withdrawal from colony–biofilm culture. *Frontiers in microbiology*, 2018, 9: 1396.