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# Fabrication of green synthesis zinc nanoparticles using (pomegranate), antibacterial and antibiofilm evaluation against human and animals MDR E.coli

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***Abstract*---**Multidrug-resistant bacteria (MDR bacteria) cause a significant rise in mortality, morbidity, and treatment costs. To combat the problem of MDR bacterium emergence, scientists are turning to nanoparticles in general and green zinc oxide Nano particle (ZnO-NPs) in particular. Hence, UV spectroscopy, Fourier-Transform Infrared (FTIR) Spectroscopy, Scanning Electron Microscope (SEM), and Energy Dispersive Spectroscopy (EDS) were used to evaluate ZnO-NPs synthesized by a green synthesis technique using pomegranate leaves. The antibacterial undertaking of the synthesized green ZnO-NPs had been examined in opposition to Multidrug Resistant (MDR) microorganism of scientific importance (E.coli) by using ability of Agar well diffusion, it used to be decided that green ZnO-NPs has a synergetic effect in opposition to E.coli, which makes it a promising choice for use in drug products.

***Keywords*---**zinc oxide nanoparticle, biosynthesis, antibacterial activity, XRD, TEM.

## Introduction

The emergence of pathogenic organisms resistant to antibiotics is one of the greatest concerns facing the health system today. MDR infections are infections caused by multidrug-resistant bacteria (MDR) Bacteria are becoming more


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abundant and pose a severe threat. This is a public health issue (Vivas *et al.,* 2018). Originally, these species were reintroduced. They used to be restricted to the medical setting, but today they can be used anywhere. Among the gram-negative bacteria, Escherichia coli is the most common. (Bassetti and Garau, 2021; Bratanis *et al.,* 2020).

Biofilm-forming *E.coli* is an important multidrug-resistant (MDR) pathogen affecting humans and a major source for hospital infections associated with high morbidity due to limited treatment options. It has been reported that biofilm formation is a means for a bacterium to resist hostile environmental influences such as antibiotics and antimicrobial agents. Biofilm is recalcitrant to antibiotic therapy and a major cause of persistent and recurrent infections by clinically important pathogens worldwide (Verderosa et al., 2019).

Antimicrobial resistance in these strains is genetically determined, and is most typically transmitted through horizontal gene transfer of extra-chromosomal genetic components. Bacteria use a variety of strategies to resist anti-biotic toxicity, including low permeability of the outer membrane in gram-negative bacteria, efflux pumps, the development of degrading enzymes, and the alteration of targets. Globalization, excessive antibiotic usage in animal husbandry and aquaculture, the use of several broad-spectrum drugs, and a lack of adequate antimicrobial stewardship are all factors that have contributed to the development of antimicrobial resistant species (Patini e*t al.,* 2019).

Some authors have also suggested using nanotechnology to combat MDR strains. Green Zinc oxide NPs are among the potential NPs. "Nanomaterials have unique properties, and they are more effective than traditional medications due to numerous bactericidal processes." Furthermore, Zaidi *et al.* assert that NPs have the potential to address the current problem of antibiotic resistance and "to change the diagnosis and treatment of bacterial infections (Zaidi *et al.,* 2017).

Nanotechnology: has revolutionized almost all the aspects of human life, and their potential applications in industries such as agriculture, medicine, cosmetics, electronics, and textiles have recently gained momentum. Due to nanoparticles large surface to volume ratio, their surface reactivity is greatly enhanced resulting in extraordinary antimicrobial, antifungal, catalytic, and wound healing properties which are generally not observed in their bulk counterpart (Siddiqi *et al.,* 2018; Marimuthu *et al.,* 2020). Generally, nanomaterials can be synthesized either through physical, chemical, or green synthesis methods. The physical methods, via., inert gas condensation, physical vapor deposition, laser and flame spray pyrolysis, electro spraying techniques, and melt mixing are costly and require high throughput equipment, while the chemical methods, via., sol-gel synthesis, micro emulsion technique, hydrothermal synthesis, polyol synthesis, and plasma enriched vapor deposition utilizes chemicals that produce toxic by-products that could be harmful to the body or the ecosystem. However, the green synthesis route offers an eco-friendly, cost-effective, and time-efficient mode of nanomaterial synthesis, which is being intensively investigated as an alternative to the conventional mode of synthesis (Shivani *et al.,* 2020).

The phytochemicals present in the plant extract can act as reducing agents for converting the metal precursors to metal nanoparticles. Phytochemicals are antioxidants and toxic-free chemicals; hence they can act as both reducing and stabilizing agents. The essential phytochemicals such as Terpenoids, Flavonoids, Phenolic compounds, Aldehydes, and Alkaloids have contributed to the reducing process. These phytochemical reducing agents vary at different concentrations in different types of plant extracts. So the leaf extract composition has a significant effect on nanoparticle synthesis. The factors such as pH, temperature, contact time, metal salt concentration, and phytochemical profile of plant extract affect the nanoparticle synthesis, nanoparticle stabilization, and the quantity of nanoparticle produced (Khezerlou *et al.,* 2018).

Various types of nanomaterials are known to have specific properties that can be exploited in several industries. Metallic nanoparticles such as zinc oxide nanoparticles are currently being explored due to their properties such as large binding energy and high piezoelectric properties. Further, they are deemed to be biocompatible and non-toxic increasing its industrial potential (Cavallo *et al.,* 2021).

Green ZnO nanoparticles facilitate better interaction with microbes thus possess an excellent antibacterial property (Raghunath and Perumal, 2017). The antimicrobial action of green ZnO is on the cell membrane, production of strong oxidizing agents related to either disruption such as hydrogen peroxide (H2O2) which are lethal and can penetrate bacterial cells, or the generation of reactive oxygen species (ROS) (Naseer *et al.,* 2020). On the other hand, the high photocatalytic performance and high photosensitive activity of green ZnO nanoparticles owing to its large bandgap, make them an excellent choice to be used for photo catalysis, which has lately emerged as a clean and economical alternate for the industrial treatment of polluted water and different types of organic matter (Balcha *et al.,* 2016; Vinayagam *et al.,* 2020). Considering this perspective, we attempted to Synthesize green ZnO nanoparticles from the pomegranate leaf extract and examine their antimicrobial against E.coli.

## Materials and Methods

### Experimental Materials

All the chemicals were obtained from Al-Qasim Green University Laboratories in Babylon, Iraq. Zinc acetate, deionized water, were purchased from Al-Ghadeer Company, Hillah City, Babylon, Iraq. Pomegranate leaves were purchased from the local garden in Hillah City, Babylon, Iraq.

### Preparation of plant extract

The *(Punica granatum*) leaves preparation were washed first with tap water, followed by deionized water to remove the external dust particles and allowed to dry in the room air. 10 g of the dried leaves was taken in a glass beaker along with 100 mL of deionized water and boiled at 60 °C for 2hr at stirring. The color of the aqueous solution changed from green to brown. The prepared extract was cooled at room temperature and was filtered using Whatman filter paper. The extract was stored at 4 °C for further use.

| | | | |
|---|---|---|---|
|  |  |  |  |
| Ground dried pomegranate leaf | 100 ml deionized water is added to 10 g powdered leaf and boiled | Filtration of leaf extract | Pomegranate leaf extract |

Fig 1: shows the preparation of pomegranate extract

**Green Synthesis of the ZnO-NPs**

The 1.0 M Zinc acetate solution was prepared by dissolving 6.58 g in 300 mL double-distilled water. Ten milliliters of the aqueous leaf of pomegranate extracts were each slowly added drop wise into the solution under magnetic stirring at 60 ◦C for roughly 2 h to obtain complex formation. The complex formed after stirring was collected and centrifuged at 10,000 rpm for 10 min. The separated pellets were dried in an oven at 60 ◦C for 24 h and preserved in airtight bottles for further studies.

**Characterization of green ZnO-NPs**

Detection of green Zinc oxide Nanoparticles using UV-Vis Spectrophotometer. The surface resonance Plasmon absorption transition of green ZnO_NPs was performed using the spectrophotometer (UV-260, Shimadzu Corp. Tokyo, Japan) and the absorbance was read in the wavelength of 197–800 nm.

**Fourier Transform Infrared (FTIR) Spectroscopy**

Studies on characterization of pomegranate extract with green ZnO-NPs were carried out at UGC-DAE-CSR Indoor India. FT-IR spectral analyses were performed using VERTEX-70, Bruker spectroscopy (Optik GmbHEttlingen, Germany). The different functional groups present in ZnNPs pellet was checked by treating with KBr and scanning at 600–4000 $cm^{-1}$.

**Scanning Electron Microscope**

The size and shape of the green ZnO-NPs were observed using scanning electron microscopic studies (JSM 5600, JEOL, Tokyo, Japan) by placing the green Zn0-NPs on copper grid coated with carbon and measured at 80 kV. To obtain the TEM images, green ZnO-NPs were placed on copper grid coated (200-mesh) with carbon and observed using Tecnai G2 20 microscope (FEI, Amsterdam, Netherlands).

**X-ray diffraction (XRD)**

The structure and composition of green ZnO nanoparticles were characterized by x-ray diffraction (XRD). An X'Pert Pro X-ray diffractometer (PAN analytical BV, The Netherlands) set with voltage maintained at 40 kV and current kept at 30 mA with Cu Kα radiation in θ- 2θ configurations was used. Based on the width of the XRD peaks, the Scherrer formula was used to determine the crystallite domain size with the assumption that they are free from non-uniform strains, the formula:

$$D=0.94\ \lambda\beta\ \cos\theta D=0.94\ \lambda\beta\ \cos\theta$$

**Transmission electron microscopy (TEM)**

Green ZnO nanoparticles were used for transmission electron microscopy (Tecnai G220 (FEI) S-Twin 200kv) (TEM) analysis. Around 10 µl of sample solution was added onto a carbon-coated copper TEM grid. The grid was dried overnight before TEM analysis. The crystalline nature of green ZnO nanoparticles was determined through the selected area electron diffraction (SAED) pattern. The dried green ZnO nanoparticles were added onto a double-sided tape and placed directly into the SEM machine (Leica Stereoscan-440 SEM instrument equipped with a Phoenix EDAX attachment) for EDAX measurements based on the images of the nanoparticles obtained.

**Multidrug Resistant (MDR) clinical isolates of E.coli-**

Fifteen Multidrug resistant MDR E.coli were collected from UTI patients of Al-Hillah Teaching Hospital, and then, in the laboratory, the identification and purification of each isolate was confirmed following standard microbiological methods and additionally confirmed by VITEK 2 to obtain the very last diagnostic. By Kirby-Bauer disc method, the susceptibility was checked. The strain that was resistant to three or more antibiotic class was considered as a MDR strain.

**Antibacterial Assay of green ZnO-NPs**

The antibacterial activity of the green ZnO-NPs was detected using agar well diffusion method

**Agar Well Diffusion method**

The antibacterial activity of green ZnO-NPs was tested using Agar Well Diffusion. The inoculums of MDR E.coli isolates were checked to be equal to the standard McFarland tube (1.5X $10^8$ cells.ml-1), the sterile swab was cast off to the suspension of the bacteria, and then inoculum was streaked on the Muller Hinton Agar plate then left-hand to dry. Green ZnO-NPs (100, 70, 50, 25, µg.ml-1) were inserted into every well that made by corn borer at 8mm and permitted to position at room temperature for one hour to disperse to medium before incubation at 37 oC for 24 hours, zones of inhibition were assessed by a ruler or caliper and matched to the inhibition zones identified to determine the tolerance or sensitivity of the organism to green ZnO-NPs.

## Results and Discussion
### Visual observation

In agreement with previous researches, the color of the reaction change from colorless to yellow confirmed the formation of green ZnO-NPs as shown in Figure 2

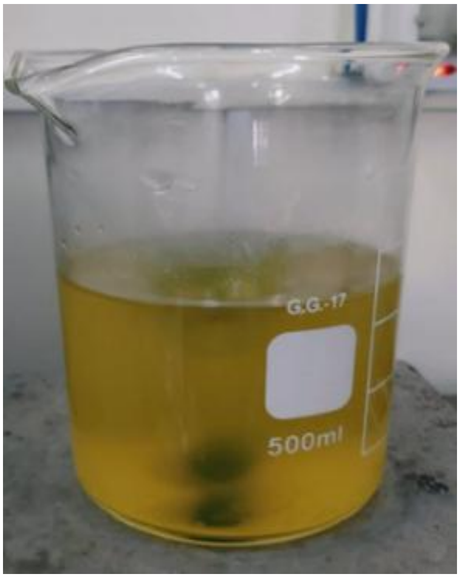


Fig 2: Yellowish green synthesized zinc oxide nanoparticle

### UV-visible spectroscopy

The *(Punica granatum)* extract was added to the aqueous zinc acetate solution which changes color from colorless to yellow indicating the formation of ZnNPs. The UV-visible absorption spectrum of the green ZnO€NPs and the leaf extract of *(Punica granatum*) are shown in Fig 3. The UV-visible absorption spectrum of the green ZnO NPs showed a maximum absorption peak (λ) at 310 nm. The higher absorbance at (239 nm) and (197 nm) of the leaves is attributed to the extract were observed at 3660 and 3700 cm−1, which fall within the range of 4000–3600 cm−1. This absorption range confirms the presence of the O-H stretch bond of free hydroxyl groups, which signifies that the pomegranate leaf contains flavonoids, polyphenols and alcohol functional groups that have different hydrogen bonds. Every bond type observed in FTIR analysis has a different frequency of vibration. Absorbance peaks were also obtained at 1539.20 cm−1 , indicating the O-H stretch bond of carboxylic acid functional groups, and the -C-H stretch of alkanes. A less pronounced peak at 1411.89 cm−1 represents the -C≡C stretch of alkynes and C≡N stretch of nitriles. Peaks in the 1346.31 cm−1 range confirm the presence of C=O carbonyl groups in the molecule, the C=O stretch of ketones, aldehydes, saturated aliphatic and the stretch of α, β unsaturated esters. A less prominent absorption peak of 1026.13 cm−1 is indicative of C-C stretching of aromatics and N-H bending of primary amines. Other absorption peaks obtained at 671.23 cm−1 are representative of the C-H rock of alkanes and N=O symmetric stretching of nitro compounds. Peaks at

613.23 cm−1- represent the C-O stretch of alcohols, carboxylic acids, esters and ethers. A 563.21 cm−1 peak represents the C-N stretch of aliphatic amines.

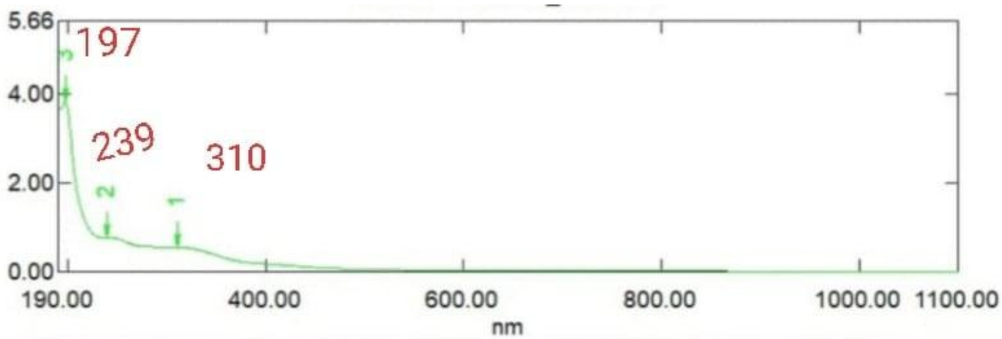


Figure 3. UV–visible spectra of zinc oxide nanoparticles synthesized from pomegranate leaf extracts

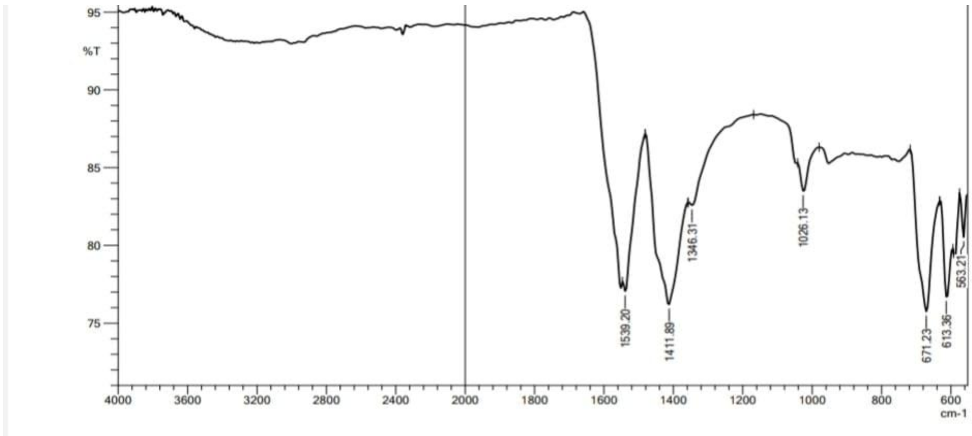


Fig 4: FTIR spectra of ZnO-NPs and *Punica granatum* leaf extract

**X-ray Diffraction (XRD) Analysis-**

Characterization of green zinc oxide nanoparticles by pomegranate leaf extracts was analyzed by X-ray diffraction. This was done to ascertain the crystalline with a scanning speed of 6°/min.

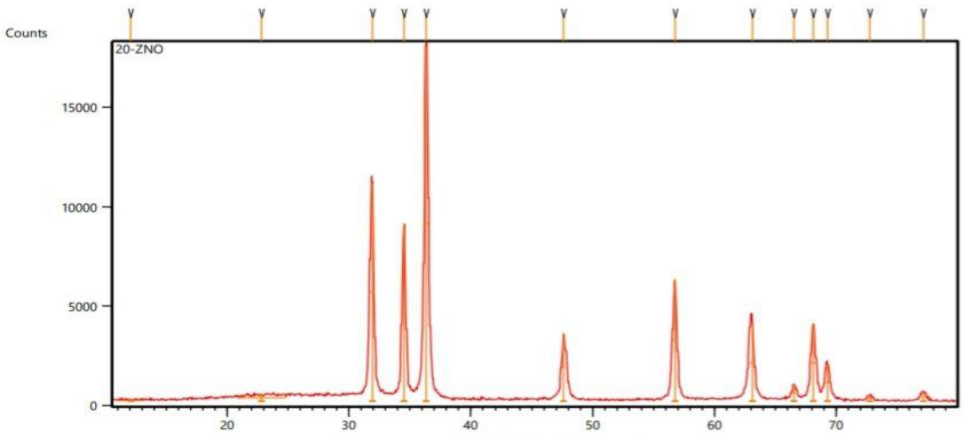


Fig 5: XRD of green synthesized ZnO NPs mediated by Punica granatum leaf extract

**Scanning electron microscopy (SEM)**

Figure 6 represents the SEM pictures of green ZnO nanoparticles at different magnifications. These pictures confirm the formation of green ZnO nanoparticles. These pictures substantiate the approximate spherical shape to the nanoparticles, and most of the particles exhibit some faceting. From the pictures, it also can be seen that the size of the nanoparticle is less than 10 nm which was in good agreement with the particle sizes (36 nm) calculated from the Debye-Scherer formula.

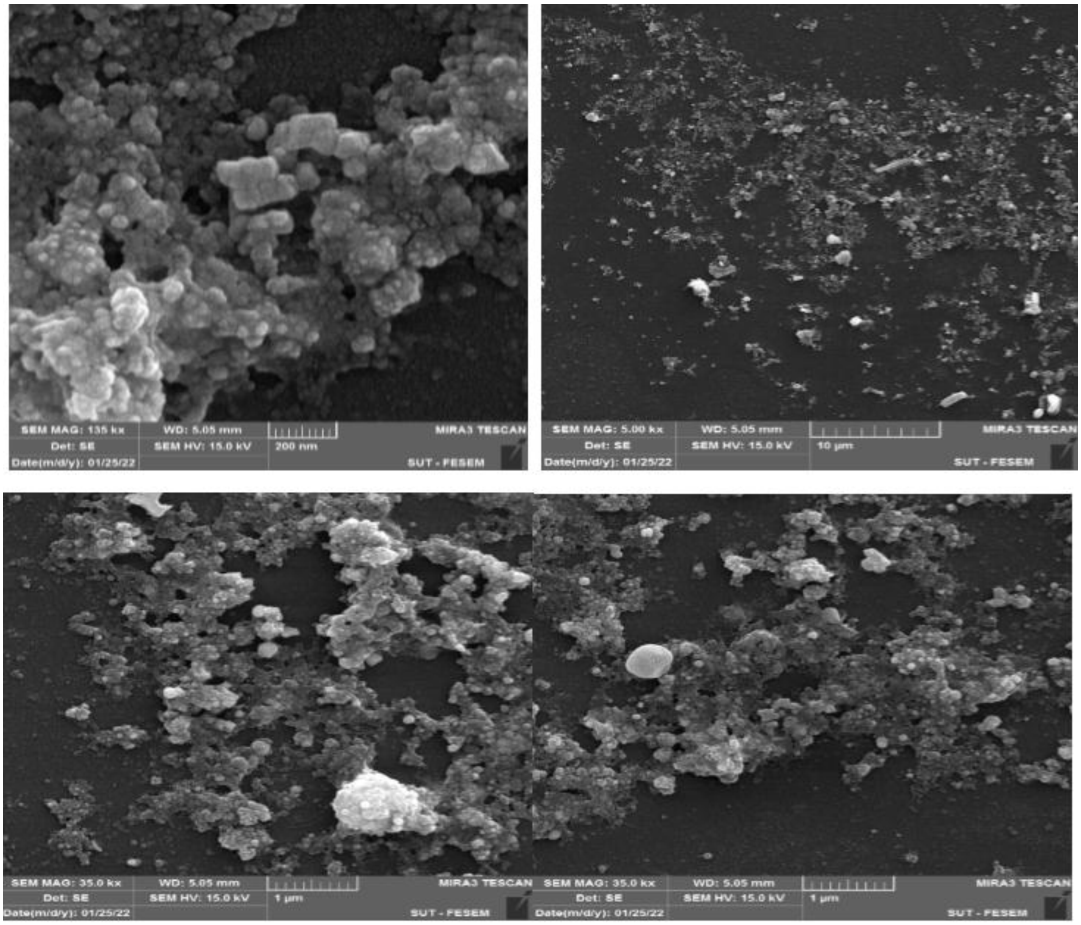


Fig 6: Image of SEM of ZnO-NPs synthesized from pomegranate leave

**Transmission electron microscopy (TEM)**

Figure 7: shows distinct bright rings which confirm the preferential orientation of nanocrystal instead of irregular. Transmission electron microscopic pictures also revealed the green ZnO nanoparticles formation produced at different magnifications by confirming the hexagonal plane to the prepared nanoparticle.

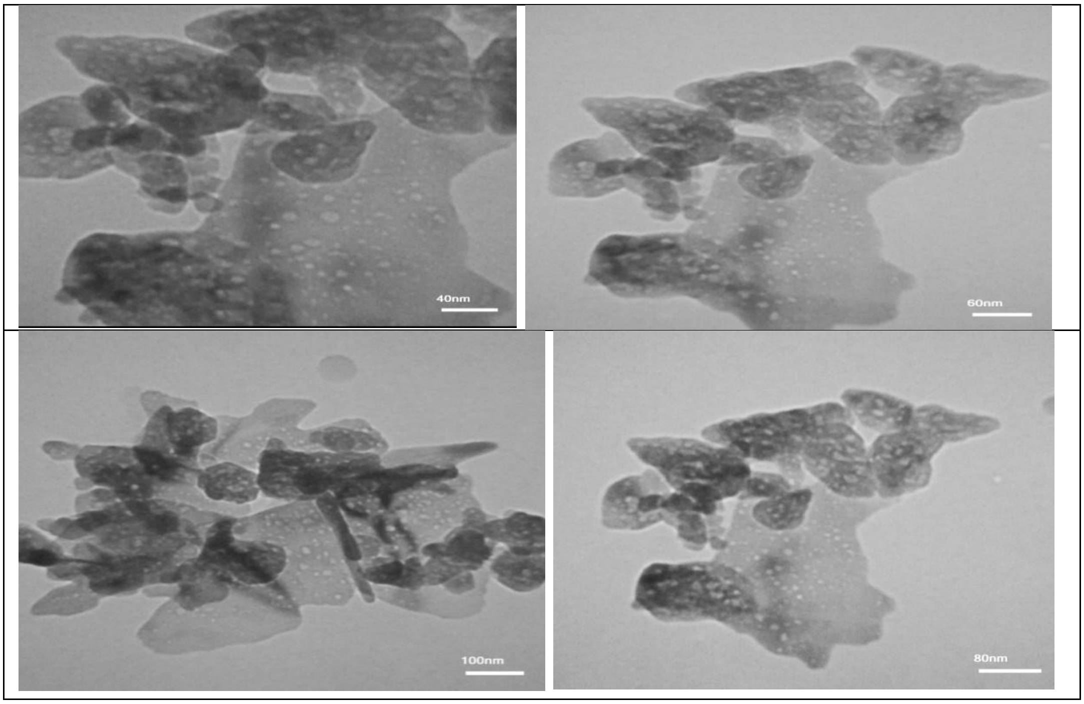


Fig 7: Image of TEM of green ZnO_NPs synthesis from pomegranate leaves.

**Antibacterial activity assay**

The inhibition zone of E.coli growth as a result of antibacterial activity of green ZnO-NPs in this study as shown in table1 and Figure 7. the lowest concentration of 25 µg.ml-1 showed an inhibition zone of (7.16) mm which is the lowest inhibition zone, for the concentration of 50 µg.ml-1 the inhibition zone was (9.33) mm, for the concentration of 75 µg.ml-1 the inhibition zone was (17.33) mm, and for the higher concentration of 100 µg.ml-1 the inhibition zone was mm which is the largest, (22.5) the antibacterial effect of green ZnO-NPs was highly significant difference, $P<0.05$. Excellent antibacterial activity was revealed by the green synthesized Zn0-NPs against bacterial pathogens [Haq *et al.,* 2017]. The negatively charged bacterial cell wall may attach to the green Zn0-NPs and Zinc rupture it, consequently leading to the denaturation of protein and death of the cell [Lopez *et al.,* 2020].

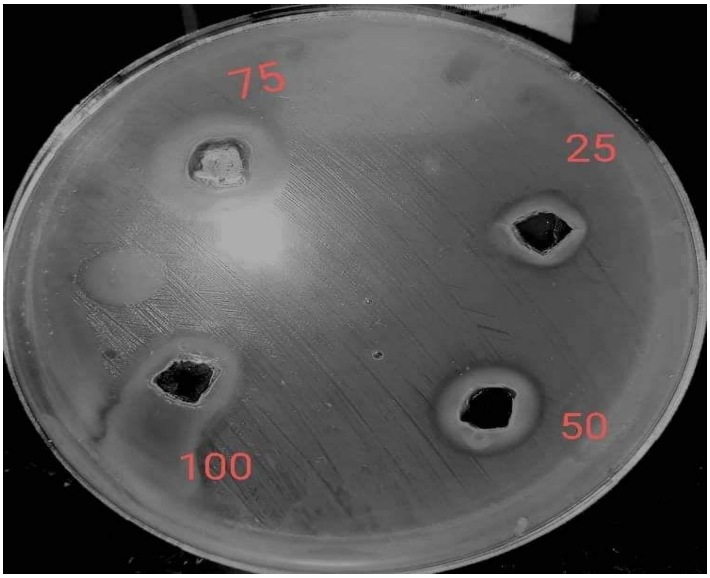


Fig 8: Green ZnO_NPs in different concentration

Table 1
Green ZnO_NPs in different concentration

| ZnNPs µg.ml-1 | inhibition zone diameter in mm for E.coli |
|---|---|
| mean± SE | |
| .28 A±0100 nm | 22.5 |
| 75 nm | 17.33±0.33 B |
| 50 nm | 9.33±0.16 BC |
| 25 nm | 7.16±0.16 D |
| P. value (<0.05) | 0.81 |

**Conclusion**

In this study, green zinc oxide nanoparticles were successfully green synthesized using plant extracts of (Punica granatum). The resultant nanoparticles were characterized using UV – Visible spectroscopy, FTIR, XRD, SEM, TEM. The antibacterial activity of the green ZnO-NPs were examined and it was discovered that zinc oxide(zinc acetate) synthesized by leaves (Punica granatum) had the highest significant antibacterial activity in the bacterial inhibition zone, bacterial growth and biofilm formation which could be attributed to its dominant crystallite small size of 36 nm as compared to others. The inhibitory effects increase as the concentration of green ZnO-NPs increased. Results indicate that green ZnO-NP may distort and damage bacterial cell membrane, resulting in a leakage of intracellular contents and eventually the death of bacterial cells.

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