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Green Approaches in Synthesizing Zinc Oxide Nanoparticles (ZnO NPs) of Pomelo (Citrus Maxima) Extract for Anti-Biofilm and Anti-Bacterial Agent

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Abstract:

Introduction: This study focuses on synthesizing (Zno-NPs) using a green synthesis method with Citrus Maxima extract and investigating their antibacterial and anti-biofilm properties. Nanoparticles, including ZnO-NPs, possess unique characteristics such as shape and size dependency, and Citrus fruits, being globally popular, contain various phytochemicals.

Methods: This study aimed to synthesize ZnO-NPs using pomelo extract and evaluate their antibacterial and anti-biofilm properties. The chemical components were analyzed using GC-MS, while techniques like UV-Vis spectroscopy, FTIR, SEM-EDX, and XRD were used to characterize the size and morphology of the nanoparticles. Antibacterial activity was tested against *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus. aureus* using agar well diffusion method. Additionally, anti-biofilm activity was assessed against biofilm-forming clinical isolates.

Results: The study characterized Citrus maxima extract using FT-IR and GC-MS, confirming its high phytochemical content. The synthesis of (ZnO-NPs) involves reducing Zn^{+2} to Zn^{0}, confirmed by a color change to brownish. UV-Vis spectroscopy confirms ZnoNP formation with SPR at 372 nm, and FESEM, EDX, and XRD show that the nanoparticles have a spherical and crystalline morphology with a size of 30-90 nm and an average size of 57 nm. The study revealed substantial antibacterial activity in both extract and ZnoNPs, with MIC experiments showing effective antibacterial activity at low doses. Furthermore, the anti-biofilm study demonstrated ZnoNPs' ability to suppress biofilm development across several bacterial strains.

Discussion: This study demonstrates how ZnO-NPs made with green chemistry to battle bacterial infections and biofilm-associated disorders, advancing both environmental health and nanomedicine.

Keywords: Citrus maxima, ZnoNPs, anti-bacterial activity, anti-biofilm, GC-MS

Introduction:

There is increased concern about reducing the environmental effects of waste production on water, soil, air pollution, and overall environmental contamination (1). In the past few decades, new technologies have been created to improve the use and efficiency of existing environmental treatments. Metallic particles of specified shapes and sizes, as well as composites with various materials, were synthesized at nanometer scales (2,3). These nanoscale materials have a wide range of applications, including energy, the environment, electronics, optoelectronics, magnetics, cosmetics, biomedicine, pharmaceuticals, and catalysis (4,5). Nanoparticles (NPs) exhibit numerous unique features like shape and size dependency (6,7). Metal

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oxide nanoparticles (MO NPs) have aroused a great deal of interest in catalysts due to their numerous unique and demanding features. Nanoparticles play a significant role in the removal of these contaminants(8). Zinc oxide nanoparticles (ZnO-NPs) are a valuable and versatile inorganic substance with unique physical and chemical properties (9). They have strong chemical stability, a wide radiation absorption spectrum, a high electrical coupling coefficient, and great photostability due to their molecular formula ZnO (10). ZnO-NPs have been widely made and used in a variety of commercial and industrial goods, including lubricants, adhesives, sealants, pigments, batteries, cosmetics, and sunscreens, as well as a zinc nutrient source in foods(4). Nano-sized ZnO particles exhibit significant antibacterial properties because of their tiny dimensions; they can stimulate distinct bactericidal processes once inside the cell of the bacteria, including the bacteria's outer layer or bacterial core, generate ROS (reactive oxygen species), emit Zn2+, and even be endocytosed by cells(11,12). Citrus fruits are the most popular fruit crops worldwide(13). Pomelo and grapefruit are grouped due to their similar appearances (14). Citrus maxima contains a variety of phytochemicals, including polyphenols, flavonoids, carotenoids, Coumarins, and alkaloids (15). Pomelos are high in Vitamin C. They are typically consumed as fruit (16). In traditional medicine, it is used as a sedative to treat neurological disorders, convulsive cough, hemorrhagic infections, and epilepsy (17,18). The ethanolic extract of leaves showed strong antibacterial activity against *Pseudomonas aeruginosa* and *Escherichia coli* using the disc diffusion method (19). The presence of phyto-constituents such as flavonoids, alkaloids, tannins, and saponins in the leaves demonstrated antibacterial activity (20). Crude ethanolic extract of Citrus maxima fruit peel exhibits anti-inflammatory activity; the peel extract exhibited CNS depressive, mild analgesic, and anti-inflammatory effects. Leaves were extracted with acetone, ethanol, and water (21).

Although there is a lot of research that works on ZNO-NPs, the research gap in the previous studies is that there is little research on the anti-biofilm activity of ZNO-NPs. So, the aim of this study is to synthesize ZNO-NPs by the green synthesis method by using Citrus Maxima extract and study their anti-bacterial and anti-biofilm activity.

Material and methods:

Citrus Maxima collection and extraction:

Citrus Maxima were collected from a local market in Kirkuk and cleaned twice with distilled water to remove all contaminants and insect traces. The fruit peels were collected, dried, and then ground to obtain a precipitate of fruit peels. 50 grams of Citrus Maxima peels were dissolved in 250 ml of 99% ethanol. The mixture was put on 30 C^0 heating for 24 hours using an ultrasonic apparatus to extract the active compounds in the Citrus Maxima (22). The extract was then cooled and filtered by filter paper and then using a rotary evaporator to condense the extract. The extract is kept for use in the coming work.

Biosynthesis of zinc oxide nanoparticles:

By using the method (23) with a slight modification, Zinc oxide nanoparticles were synthesized by mixing 0.1 M zinc nitrate salt with 200 mg/ml ethanolic extract of Citrus Maxima extract to reduce Zn^{+2} to Zn^0 and coated with the bioactive compounds appears in the Citrus Maxima extract. The conditions were optimized utilizing various parameters (time, temperature, pH, $Zn(NO_3)_2$ concentration, and ethanolic extract concentration). The conditions were determined to be 0.1m $Zn(NO_3)_2$,200 mg/ml extract, 5pH, and 2 hours.

GC-MS analysis:

Two milliliters of Citrus Maxima extract were centrifuged and filtered. The extract was extracted using a separating funnel filled with ethyl acetate (2 x 10). The EtAc fraction was recovered and concentrated using vacuum evaporation at 55°C using a rotating evaporator. The GC-MS analysis was carried out using a Shimadzu QP2010Ultra (Kyoto, Japan). The sample was separated using a capillary column (30 m x 0.25 mm x 0.25 µm) and stratified by temperature. Column oven temperature: 70°C, injection temperature: 240°C. Injection Mode: Split-less, Sampling Time: 1 minute, Pressure: 100 kPa. Total flow rate is 200 ml/min, with a column flow of 1.54 ml/min and a linear velocity of 454. Purge flow rate: 3mL/min, split ratio: 10. The GC Programmed, [GCMS], QP2010 Ultraj, ion Source temperature: 201^0. Interface temperature: 245 degrees Celsius, solvent cut time: 3 minutes, ionization mode. SEI detector gain is 1.10 kV + 0.01 kV in relative mode. Start time: 3.50 minutes, end time: 27 minutes, ACQ mode: Scan, event time: 0.30 seconds, scan speed: m/z. End 700.00 and interpretation of mass spectrum. GC-MS analysis was performed using the National Institute of Standards and Technology (NIST) database. The unknown component's spectra were compared to recognized components kept in the NIST library. The name, chemical structure, and weight of test materials were verified (22).

Characterization of ZnO NPs:

UV-Vis spectra analysis:

UV-Vis spectral technique was performed utilizing a spectrophotometer from PG Instruments Limited, T 80, Germany. Reduction of ZnO NPs was confirmed by UV-vis spectroscopy at constant intervals between 400 and 1000 nm. 3 ml of the substance was pipetted into a test tube and inspected at the temperature of the room. De-ionized water is used to create a blank. The wavelength of absorption of the ZnO NPs solution for surface Plasmon resonance ought to fall between 300 and 400 nm.

FTIR spectra analysis:

After accommodating ZnoNPs, the nanoparticles were centrifuged at about 6000 rpm for 11 minutes. The treatment was repeated 4-6 times. The precipitate should then be dried in an oven set to 40 degrees Celsius for 5 hours. The Citrus Maxima powder was washed multiple times with distilled water to remove dust and pollutants before being oven-dried at 45°C. To compare, dried NPs and Citrus Maxima peel powder were analyzed using an FTIR-Shimadzu-8400S spectrophotometer. The wave ranged from 500-4000 cm-1(24).

FESEM analysis:

SEM was utilized to determine the morphology and form of biologically synthesized ZnO NPs (SEM-Angstrom Advanced Inc.-AIS2300C). An INCA EDX probe is used to do energy-dispersive X-ray microanalysis.

X-ray differentiation (XRD) analysis:

The University of Kashan recorded the X-ray diffraction (XRD) pattern of zinc oxide nanoparticles using a Bruker D8 Advance X-ray diffractometer using CuKα radiation (λ = 1.541 Å), 40 kV-40mA, and 2θ/θ scanning mode. Data was obtained with a step of 0.0202 degrees, covering the 2θ range of 30 to 80 degrees. Peak sites were compared to standard data to determine the crystalline phases.

Anti-bacterial activity:

The Citrus Maxima ethanolic extract and ZnO NPs were tested for antibacterial activity against *Escherichia coli*, Klebsiella, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* using the standard agar well diffusion method. Citrus Maxima extract and ZnoNPs extract (5µl) have been added to the wells. The plates were incubated at 37°C for 24 hours. Experiments were performed in triplicate, and the zone of inhibition was assessed (24). Double serial dilutions (1-1024 µg/ml) of Citrus Maxima extract and ZnoNPs were made from a stock (10mg/1ml) in a microtiter plate utilizing Mueller-Hinton broth as diluent. Except for the negative control wells, all wells received 20µl of a bacterial suspension similar to McFarland standard no.0.5 (1.5×108CFU/ml). (25).

Anti-biofilm activity:

Biofilm formation

In the current study, 20 clinical isolates of gram-*positive and negative bacteria* obtained from fresh agar plates were tested for biofilm formation using a microtiter plate. They were then injected in 5 mL of brain heart infusion (BHI) with 2% sucrose and incubated at 37ºC for 24 hours. To inoculate microtiter wells with 180 µl of Brain Heart Infusion Broth, 20 microlitres of each isolate's bacterial suspension (equal to 0.5 McFarland standard) were added. The microplate was then covered and incubated at 37°C for 24 hours. After the incubation process, the plate was washed off multiple times with normal saline to remove any non-adherent cells. 200 µl of 99% ethanol was added to each well for 15 minutes. The plate was dried for thirty minutes at room temperature. 200 µl of 1% crystal violet solution was added for 15 minutes. After removing the solution of dye and washing it with sterile distilled water, the attached dye was solubilized with 96% ethanol, and the optical density of the solution was measured in a micro-titer plate reader at 630. nm (26,27) (The biofilm formation was evaluated as described in Table 1.)

Determination of citrus maxima extract and ZnO NPs MIC:

Duplicate series of dilutions (1-1024 µg/ml) of citrus maxima extract and ZnoNPs were made using a stock (10mg/1ml) on a microtitration plate utilizing Mueller-Hinton broth as diluent.

Except for the negative control wells, all wells received 20µl of a bacterial suspension similar to McFarland grade no.0.5 (1.5×108CFU/mL). Microlitre plates have been incubated at 37°C for 18–20 hours. After incubation, 20 µl of a substance called dye was added to each well and incubated for 2 hours to detect any color changes. The Sub-MIC. The concentrations were measured optically in broth micro diluted forms as the lowest levels at which the color altered from blue to pink during the resazurin broth test (4). Figure 1 shows the concentration of MIC and sub-MIC

Table 1: Evaluation of biofilm formation by microtiter plate method.

Optical density	Adherence
Optical D ≤ Optical Dc	Non–adherent
2 Optical Dc > Optical D > Optical Dc	Weak
4 Optical Dc > Optical D > 2ODc	Moderate
Optical D >4 O Optical Dc	Strong
Cut off value (Optical Dc) = average optical density of negative control + (3 *Standard Deviation).	

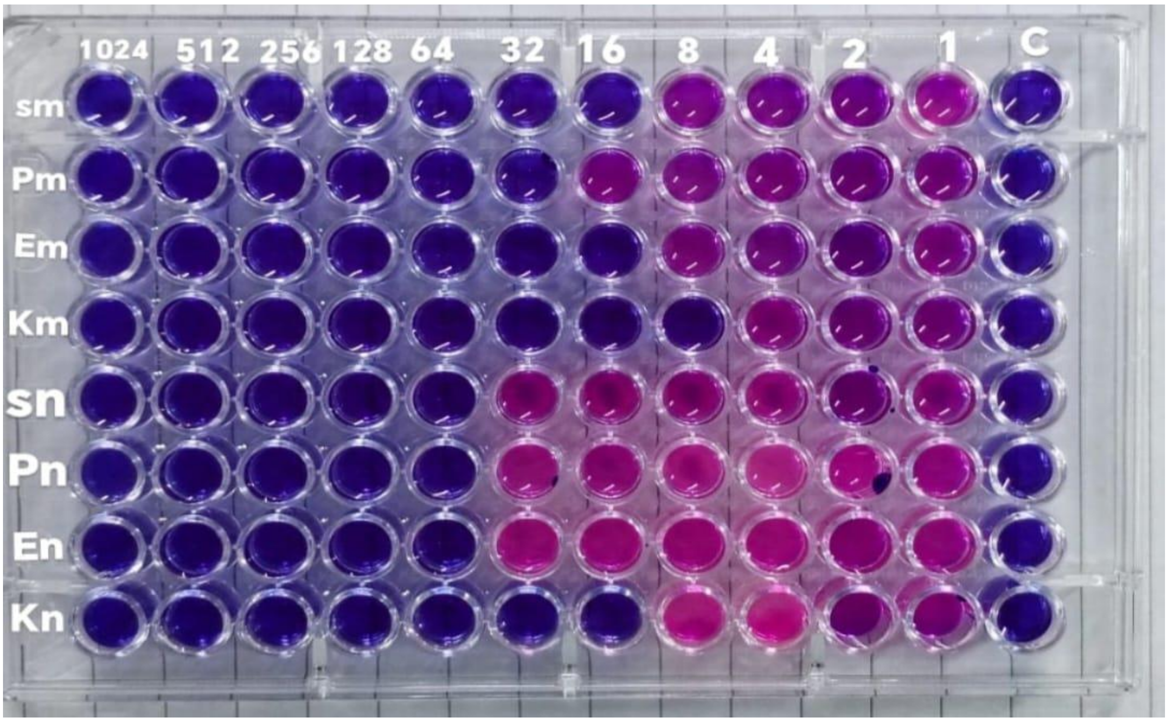

Figure 1: MIC and sub-MIC for citrus maxima extract and ZnO NPs

Results:

GC-MS analysis for Citrus Maxima:

A GC-MS study has been done to estimate the active components of Citrus Maxima extract. The results of the GC-MS study are shown in Figure 2.

GC-MS found 94 volatile compounds in the extracts. This extract contains a variety of chemicals, the majority of which are esters like (1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl), and Phthalic acid, di(2-propyl pentyl)), carboxylic acids like (n-Hexadecanoic acid, 9,12-Octadecadienoic acid), alcoholic compounds like (2-Furanmethanol, 5-ethenyltetrahyd, 2-Furanmethanol, 5-ethenyltetrahyd), cyclic compounds like (Cyclohexene, 4-(4-ethylcyclohexyl), 9,19-Cyclolanost-24-en-3-ol, aceta), and phenolic compounds like (2,4-Di-tert-butylphenol, Phenol, 2,4-bis(1,1-dimethylethyl)). Most of these compounds may operate as antioxidants and antibacterial agents, as well as participate in oxidation-reduction reactions that aid in the synthesis of ZnO NPs.

FT-IR analysis for citrus maxima:

An FT-IR study has been done to evaluate the functional groups of Citrus Maxima extract to detect the compounds in the extract. The results of the FT-IR study are shown in Figure 3.

FTIR examination of ethanolic citrus maxima leaf extracts identified important functional groups such as protein, oil, lipids, phenolic chemicals, flavonoids, saponins, tannins, and carbohydrates. In bioreduction, absorbance bands at 400-4000 cm-1 include 1517, 1608.63, 2927.94, and 3494.72 cm-1. Major peaks at 2927 cm-1 correspond to C-H stretching vibrations of methyl, methylene, and methoxy groups. The crude ethanolic extract of citrus maxima shows peaks at 3394, indicating the existence of O-H alcohol, and 1608, 1517, suggesting the C=O bond.

Green synthesis of ZnO NPs:

The Green synthesis of ZnO NPs has been done by reducing Zn ions in $Zn(NO3)_2$ salt by using the ethanolic extract of citrus maxima as a reducing agent due to it is high content of phytochemicals.

The change of color observed in the formation of ZnoNPs happened in the reaction media, where there is a change from a fine, transparent solution light brown to a dark gray, and a precipitate of zinc nanoparticles formed that ensuring the formation of ZnoNPs. The equation that depicts the reduction of the Zinc oxide solution to ZnO NPs is.

$Zn(NO3)_2$ + citrus maxima ethanolic extract → Zn0 (nanoparticle)

Characterization of ZnO NPs:

UV-Vis spectra analysis:

In this study, the greatest wavelength was 315 nm, and it is pointed out that ZnO NPs were synthesized by reference to appropriate surface Plasmon resonance (SPR) with good band intensities and peaks under the visible spectrum. SPR attitude of nanoparticles synthesized by ethanolic extract appeared by absorption at different wavelengths. The figure shows the UV-Vis chart for ZnO NPs and the extract. The SPR of ZnoNPs is shown in Figure 4.

SEM and EDX Analysis:

SEM was used to characterize the ZnoNPs like the form and size of the ZnO NPs. The source of the asymmetrical particle sizes, surface morphology, and the expanded size dispersion of ZnoNPs produced using the ethanolic extract of Citrus maxima are shown in Figure 5.

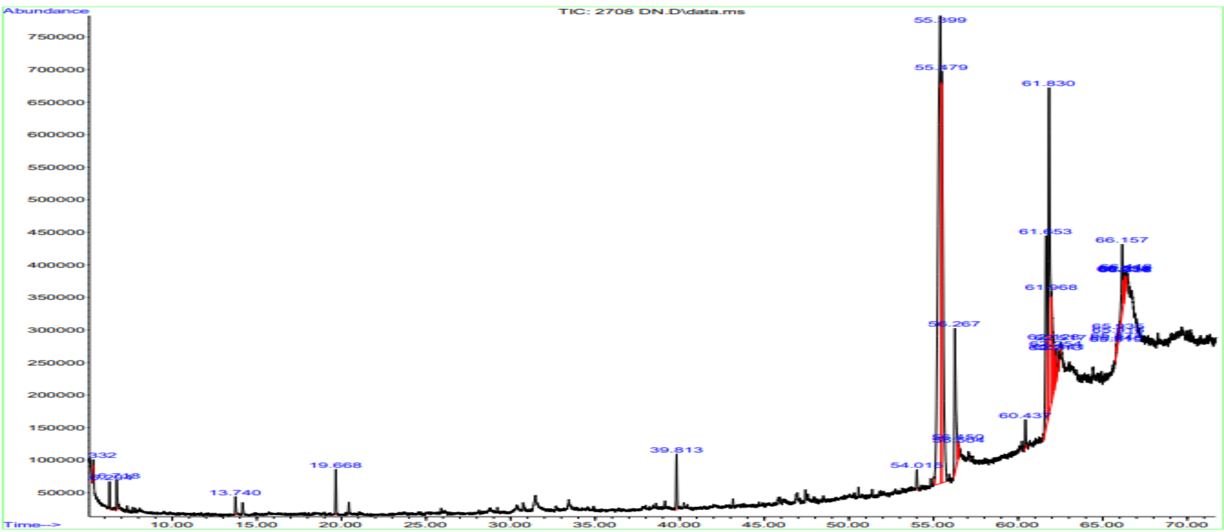

Figure 2 : GC-Mass analysis for citrus maxima .

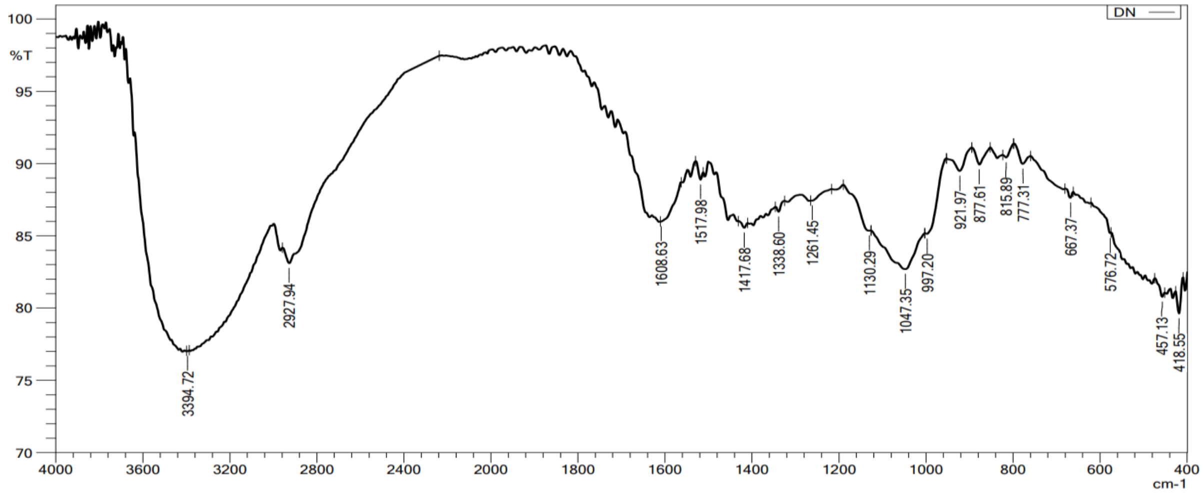

Figure 3 : FT-IR analysis for citrus maxima.

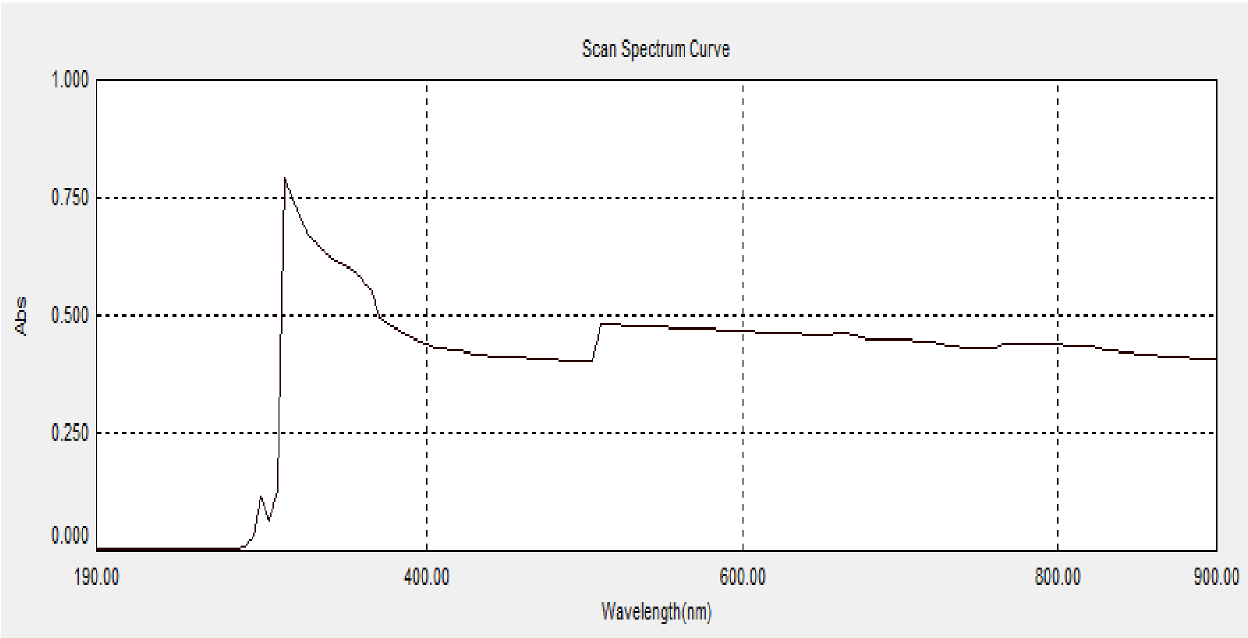

Figure 4: UV-vis for ZnO NPs with SPR and citrus maxima.

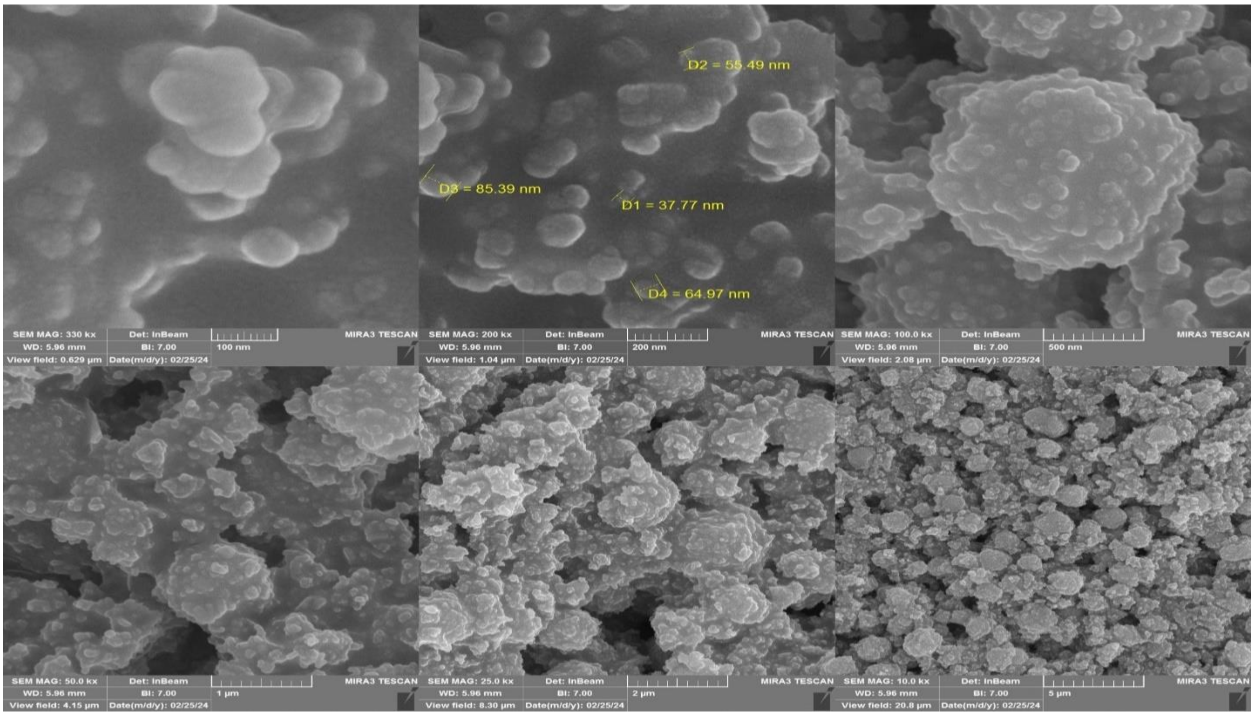

Figure 5: SEM analysis for ZnoNPs in 6 magnifications (100nm, 200nm, 500nm, 1μm, 2μm and 5 μm).

The figure above shows the results gained from SEM analysis, the figures taken at 100nm, 200nm, 500nm, 1μm, 2μm and 5 μm respectively, clarify The mean size of the synthesized ZnoNPs was in an area of 30_90 nm, and the results of the SEM study revealed that the synthesized ZnoNPs had a spherical shape and crystalline morphology, the figures of ZnoNPs particles shows some aggregation in synthesized particles because of the time of storage and the presence of plant traces. The total size and morphology of the synthesized particles show that ZnoNPs were prepared in a good state.

ZnoNPs are confirmed to be present by the EDX analysis, which reveals that the ZnoNPs are composed of Zn, C, and O components. The binding energies of Znkα are represented by the peaks about 2.20, 8.70, and 9.6 KeV, respectively, whereas the carbon-related peak is shown near 0.4 KeV. The EDX holding grid and the carbon peak match. Over the whole binding energy scanning range, the oxygen-related peak is shown near 0.8 keV. No clear impurity peak is found. The outcome shows that high-purity ZnNPs make up the product as it was synthesized. Figure 6 shows the EDX graph for ZnO NPs. Figure 5 shows the EDX graph for ZnO NPs.

XRD analysis:

X-ray examination of diffraction for the XRD examination, the zinc oxide nanoparticles' dry powders were utilized. At two theta angles, the scattered intensities ranged from 20◦ to 80◦. Figure 7 shows the XRD graph for ZnO NPs.

Zinc oxide nanoparticles show substantial line stretching in their X-ray diffraction pattern, indicating a nanoscale particle size. Peaks at 31.5, 34.4, 36.2, 47.5, 56.4, 62.8, and 67.9° suggest a spherical to hexagonal phase of ZnO with high crystallinity. All of the different peaks were discovered to be ZnO-NPs, which are not found in synthesized ZnO-NPs. The circumference of zinc oxide crystallites was calculated using the Debye-Scherrer formula. Based on θ (Bragg's diffract angle) and β (full breadth at half-maximum (FWHM)) of more powerful peaks corresponding to 101 planes centered at 36.2°, the crystallite size is approximately 38 nm, with an average crystallite size of 57.2 nm.

Antibacterial activity :

Antibacterial study results collected by calculating the inhibition diameter appears on the dish, citrus maxima extract and ZnoNPs show a good ability to work as an antibacterial agent ndthat the minimum inhibition concentration for each sample (citrus maxima extract and ZnoNPs) evaluated by calculating the diameter for each dilution and find the minimum concentration for giving an antibacterial activity, the results recorded and shown in the table 2.

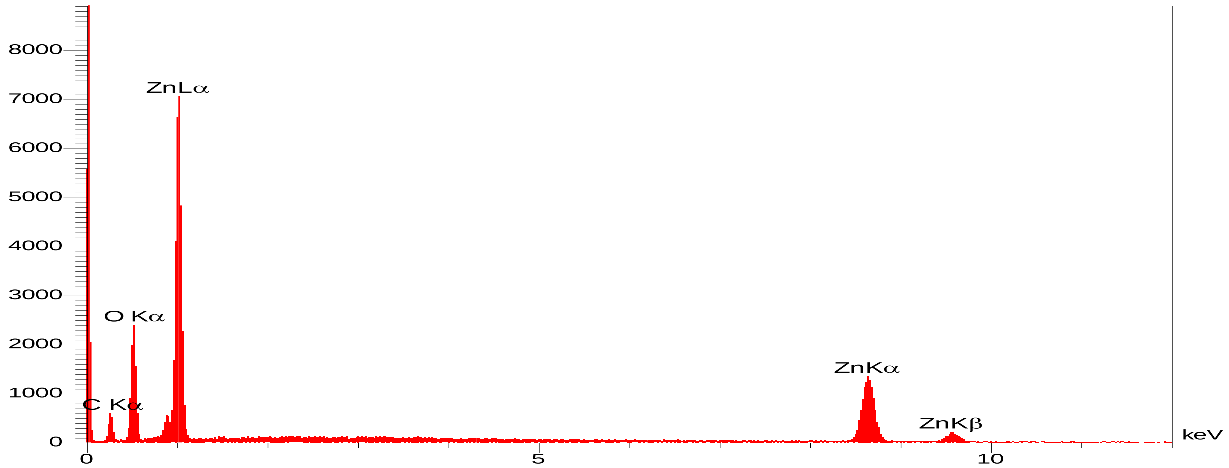

Figure 6: EDX graph for ZnoNPs.

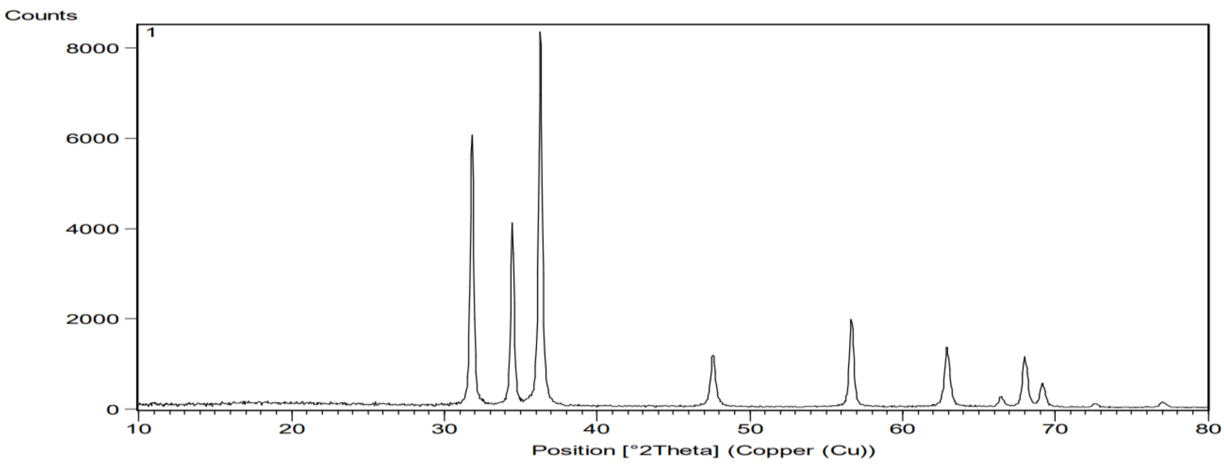

Figure 7: XRD graph for ZnoNPs

Table 2: The inhibition zone (mm) for citrus maxima extract and ZnoNPs against different Bacteria.

	Extract		**Extract**		**Nano**		**Nano**	
	Conc. of MIC in µg/ml	Mic mm	Conc. of sub-MIC in µg/ml	Sub mm	Conc. of MIC in µg/ml	Mic mm	Conc. of sub-MIC in µg/ml	Sub Mm
E.coli	64	28	32	25	16	30	8	28
P.aeroginosa	64	25	32	23	32	30	16	25
K.pneumoniae	32	25	16	22	8	29	4	25
Staph.aureus	64	26	32	24	16	31	8	26

The results in the tables show that each one of the extracts and ZnoNPs could work as an antibacterial, but nanoparticles show a higher ability to inhibit the studied bacteria in lower concentrations, as compared with the extract, and have a higher impact on bacterial growth. The results recorded in the table above are calculated from the dishes in Figure 8.

Anti-biofilm activity:

Biofilm formation:

The study of anti-biofilm activity was done, and the results show the ability of the chosen bacterial strains to make a biofilm. The study shows that all chosen bacteria made biofilm with a difference in the biofilm strength; 30% of bacteria showed weak biofilm activity, and 50% of bacteria showed moderate biofilm activity. while 20% of bacteria show a strong biofilm activity. Table 3 and Figure 9 show the number of isolates for each bacterium and biofilm activity strength.

Determination of citrus maxima extract and ZnO NPs MIC:

After the formation of biofilm and calculating the strength of biofilm for each bacteria and the percentage for each one, MIC where determined for the extract and nanoparticles.

The results show that both citrus maxima and ZnoNPs have a high ability to work as anti-biofilm agents, and nanoparticles used have a higher ability to work as anti-biofilm agents with higher inhibition ability in lower concentrations. ZnoNPs. The results of MIC activity are shown in Table 4.

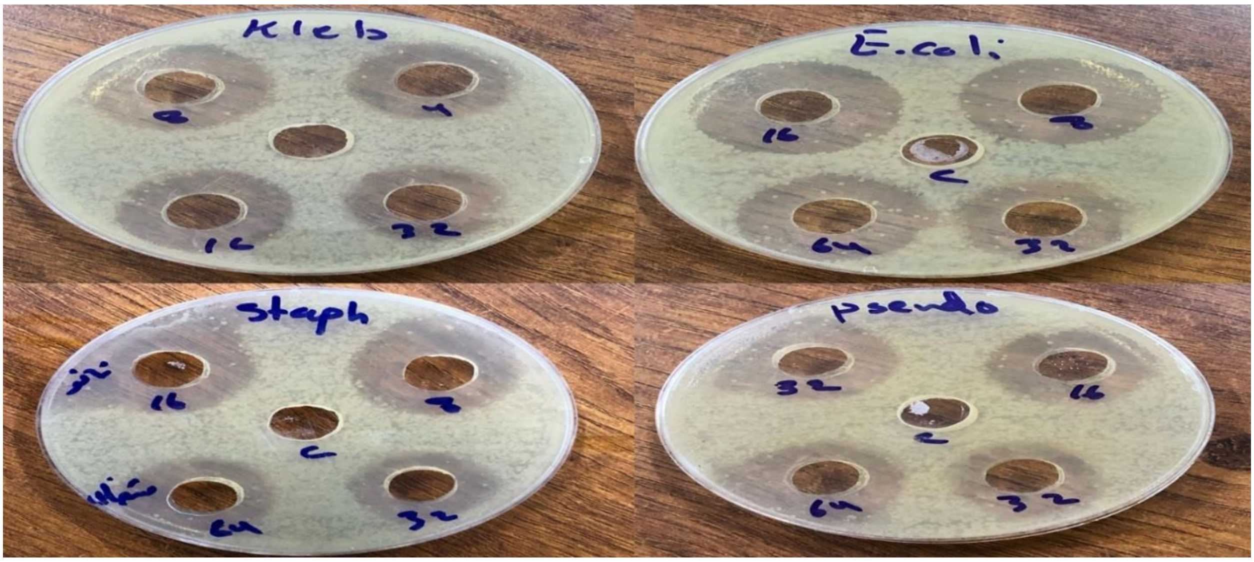

Figure 8: MIC and sub MIC for citrus maxima extract and ZnoNPs against the four types of bacteria.

Table 3: number of isolates and biofilm activity of bacteria.

Intensity	OD630 Limits the number of isolates	Number of isolates	Number of isolates	percentage) %
Non-biofilm producer	< 0.0632	0	0	0
Weak	0.0632-0.126	6	Staph.aureus (3) P.aeroginosa.(0) E.coli (2) Kleb.pneumoniae. (1)	30%
Moderate	0.126- 0.252	10	Staph.aureus.(1) P.aeroginosa.(4) E.coli (2) Kleb.pneumoniae (3)	50%
Strong	≥ 0.252	4	Staph.aureus.(1) P. aeroginosa.(1) E.coli (1) Kleb.pneumoniae.(1)	20%

cutoff value = 0.0629 (defined as the Mean of control OD630 plus 3 Standard deviation).

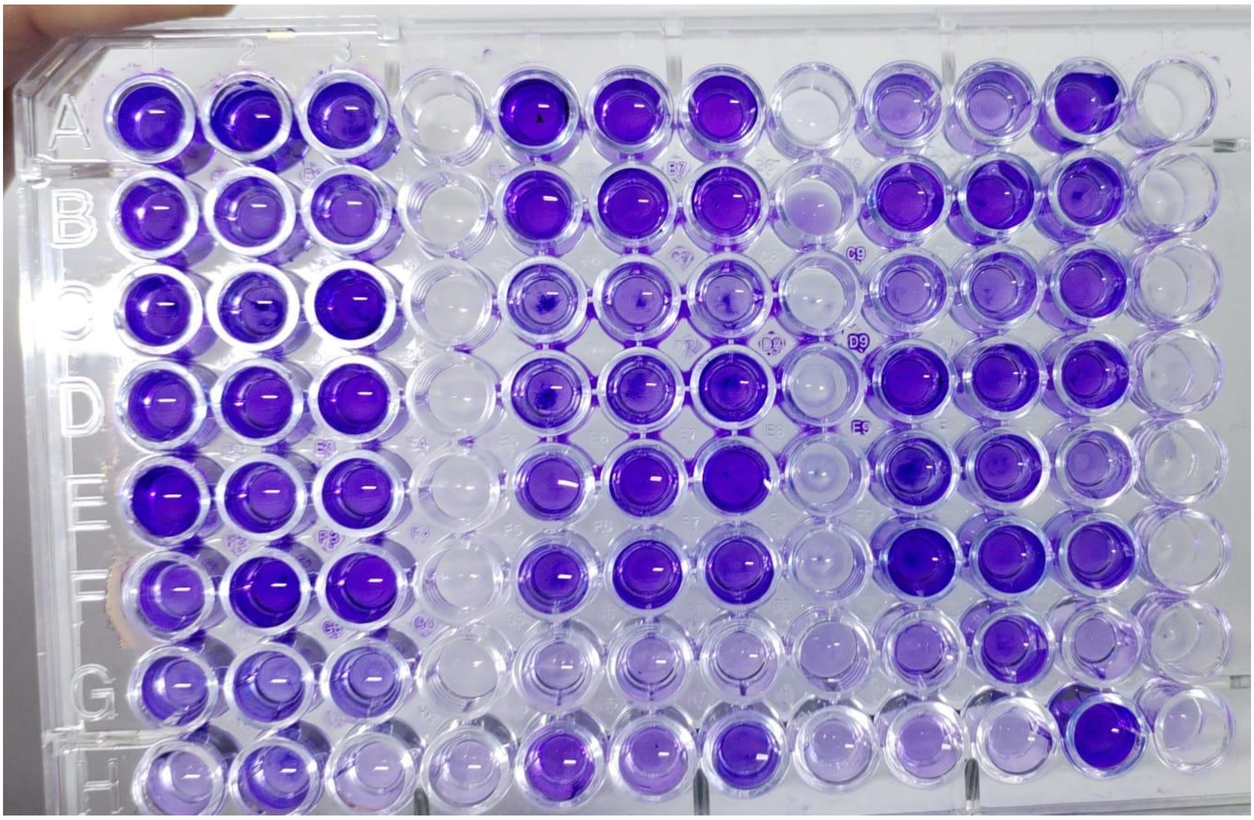

Figure 9. shows biofilm activity strength.

Table 4 : MIC for citrus maxima and ZnoNPs.

Isolates	absorbance Before treatment	ZnoNPs MIC (µg/ml)	Absorbance After treatment with ZnoNPs		Citrus maxima extract MIC (µg/ml)	absorbance After treatment with citrus maxima	
Staph.aureus	0.541-0.494-0.563	16	0.092-0.101-0.125	80% inhibition	64	0.252-0.174-0.205	60.5% inhibition
P. aeruginosa	0.618-0.721-0.631	32	0.084-0.097-0.095	85.9% inhibition	64	0.198-0.260-0.234	64.3% inhibition
E.coli	0.554-0.418-0.463	16	0.156-0.093-0.145	71.6% inhibition	64	0.325-0.221-0.223	46.4% inhibition
Kleb. pneumoniae	0.699-0.775-0.578	8	0.121-0.091-0.088	85.3% inhibition	16	0.362-0.341-0.289	51.6% inhibition

Discussion:

Nanotechnology has been used in various fields, including medicine and pharmacology, for many years now. Zinc oxide nanoparticles have been an important research topic because of their unique physical and chemical properties and viability in optoelectronics, chemical sensing, biosensing, and photo catalysis, although they have limited optical capabilities. The characterization of citrus maxima extract done by GC-MS and FT-IR shows that this plant contains a wide variety of components that could have an antioxidant and antibacterial activity and could be used in the pharmaceutical industry. These results are in agreement with other studies, like Khan, who concluded that citrus maxima may have antioxidant properties (28). The plant extract's possession of phytochemicals like flavonoids, terpenoids, and alkaloids. Additionally, citrus maximum demonstrated antimicrobial action against several types of microorganisms (28) They found that C. maxima is contributing significantly to both biodiversity and long-term sustainable use. The underappreciated C. maxima may be useful in inflammation and degenerative disorders mediated by free radicals (28). The extract of citrus maxima is used to reduce Zinc nitrate salt to zinc oxide nanoparticles and to study its anti-bacterial and anti-biofilm activity. Mixing plant extract with zinc nitrate shows a change in color to brown, and that ensures the formation of ZnO NPs by changing Zn^{+2} to Zn^{0}. This method is used by other researchers (29,30). The synthesis of nanoparticles in a plant extract is closely connected to the presence of capping and reduction agents. (31). As reaction time increases, fewer covering substances become accessible, resulting in proper nanoparticle stabilization and capping.

The mixed solution formed and most containing ZnoNPs were examined by UV-VIS spectroscopy to identify the SPR of ZnoNPs, the SPR was revealed at 315 nm due to the nanoparticles' surface Plasmon resonance excitation. The free electrons in metallic nanoparticles and their mutual fluctuation in resonance with the incident light wavelength cause the SPR absorption band. A strong absorption peak appears as a result of the nano-sized ZnO particles and the restricted particle size distribution. Because zinc oxide nanoparticles absorb well in the UV range of 200–400 nm, they are useful for medical applications such as antiseptic ointments and sunscreen protectors (32).

SEM is a high-resolution surface imaging technique that uses electron beams to capture minute details of nanostructures and materials(33). SEM images with higher magnification and field depth can reveal the surface topology of nano-objects based on electron density (34). SEM study of the morphological characteristics of the green synthesized ZnoNPs revealed that the ZnoNPs were spherical and poly-dispersed with crystalline nature, the size of particles were from 30-90 nm the size getting bigger because of the agglutination and agglomeration between smaller nanoparticles and that back to the nature of particles, the existence of plant traces in nano, and the electrostatic strength in ZnoNPs (35). The results obtained in this study are in agreement with other studies like Mousavi and Heris, who found that ZnO nanoparticles have an almost spherical shape with an average diameter of 30 nm, which is inclined to aggregation by packing of nanoparticle aggregates (36). And Gur et al said that Zn particles are seen, and these particles are homogeneously distributed in some regions by clustering in some regions (37). The size difference between synthesized ZnO NPs in this study and other studies may go back to the method of synthesis, type of reducing and capping agent, and the conditions of the reaction (38). EDX reveals that the ZnO NPs are composed of Zn, C, and O components with three peaks at 2.20, 8.70, and 9.6 keV to ensure the formation of metallic ZnO NPs with high purity. These results are highly in agreement with (39), which have the same peaks at the same points in their studies to synthesize ZnO NPs (39).

X-ray diffractometer revealed the crystalline character of the synthesized ZnO NPs. The peaks at 31.5, 34.4, 36.2, 47.5, 56.4, 62.8, and 67.9° indicate the spherical to hexagonal phase of ZnO with strong crystallinity. There are no such impurities in synthesized ZnO NPs. These results are in agreement with (40,41), who found the same peaks in the XRD study and ensured the formation of ZnO NPs. The Debye-Scherrer formula gave an average size of 57.2 nm, and that agrees with the results obtained from SEM analysis. All results for the characterization of ZnoNPs gave an evidence that ZnoNPs formed with a nano size between 30-90 nm and with a spherical morphology and crystalline nature with little aggregation and high purity.

Anti-bacterial activity for citrus maxima extract and ZnoNPs have been examined, and the results show good activity for the two compounds against both gram-positive and negative bacteria (42). Mic study shows that ZnoNPs have a higher ability to work as an anti-bacterial agent the effect of gram-positive and negative bacteria looks equal, and it clarifies that plant extract and ZnoNPs have the ability to penetrates the cell wall of gram positive with a thick layer of peptidoglycan (43) and gram negative bacteria with thin wall of peptidoglycan and an outer membrane of lipopolysaccharides (44), citrus maxima's antibacterial ability pointed out in several studies before, Karo et al said that C. maxima peels extract own the capability to work as an antibacterial for bacterial *S.aureus* and *E.coli*, and shows that this ability is back to the high continent of phenolic compounds and vitamins in the plant (45). ZnoNPs antibacterial activity also pointed out in other studies like the study of Ahmadi et al who said that ZnoNPs can work against both gram positive and negative bacteria and describe it is work by The mechanism of ZnO-NPs which involve the reaction of nanoparticles with bacterial cell surfaces, the generation of reactive oxygen species (ROS), and the release of zinc ions. ROS and free ions play essential roles in numerous pathways, including enhanced membrane permeability and cell wall degradation (46).

Anti-biofilm activity for citrus maxima extract and ZnoNPs were examined, and the high activity of ZnoNPs appears with inhibition percentages of (80%,85.9%, 71.6%, and 85.3%) for (*Staph.aureus*, pseudomonas, *E.coli*, and Klebsiella) respectively. While moderate activity for citrus maxima extract appears with inhibition percentages of 60.5%, 64.3%, 46.4%, and 51.6%) for (Staph, pseudo, *E.coli* , and Klebsiella) respectively. For Citrus maxima, there is agreement with Sadeva et al, who discovered that Citrus maxima's ability to inhibit biofilm formation is very significant, and it could serve as a possible pharmacological treatment for MDR Pseudomonas aeruginosa-related infections. Furthermore, the extract can be used with antibiotics to increase their potency as bacteriostatic and/or bactericidal agents (47). Bassig et al show that Citrus fruits contain bioactive chemicals that disrupt biochemical functions and target the biofilm matrix, inhibiting its growth. The extracts contain flavonoids (rutin, naringin, and naringenin), limonoids (limonin), and limonene, which disrupt biofilm processes and prevent biofilm growth (48). ZnoNPs even agreed with other studies like dogan et al, who ensured that ZnoNPs demonstrated significant antibacterial and anti-biofilm properties against the pathogens examined and revealed that eco-friendly and cost-effectively manufactured ZnO NPs might be employed as coating materials and in a variety of industrial applications (49). Jayabalan et al also found, by using the MEP assay, that the green-synthesized ZnO NPs had a very significant action against the investigated microbial biofilms. Furthermore, ZnoNPs have strong antimicrobial action against both gram-positive and gram-negative bacteria (50,51).

Conclusion:

In conclusion, this study reveals the effective manufacture of (ZnoNPs) with citrus maxima extract acting as a reducing and capping agent. The nanoparticles were thoroughly characterized, demonstrating their spherical morphology (sizes ranging from 30-90 nm), crystalline nature, and great purity. UV-VIS spectroscopy, SEM, EDX, and X-ray diffractometry all validated these features, which were consistent with earlier ZnO NPs research. ZnoNPs synthesized demonstrated promising antibacterial and anti-biofilm properties against both gram-positive as well as gram-negative bacteria. Their efficacy was comparable to or greater than that of the citrus maximum extract alone, showing synergistic or increased benefits when coupled with the plant extract. Disruption of cell membranes, the production of reactive oxygen species (ROS), and the release of zinc ions, which increase membrane permeability and break down cell walls, are all hypothesized mechanisms for their antibacterial activity. These findings suggest potential applications of ZnO NPs in pharmaceuticals, particularly in antimicrobial formulations and medical devices. The eco-friendly synthesis route using citrus maxima extract underscores the feasibility of sustainable nanotechnology applications in biomedicine. Further studies should explore the full therapeutic potential and safety profiles of these ZnoNPs to pave the way for their practical utilization in healthcare and other industries.

Institutional Review Board Statement:

Not applicable.

Informed Consent Statement:

Not applicable.

Data Availability Statement:

The raw data used and/or analyzed during the current study will be available from the corresponding author on reasonable request.

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Conflicts of Interest:

The authors declare that there is no conflict of interest.

Declaration of Generative AI and AI-assisted technologies in the writing process:

The authors declare that there is no use of generative AI and AI-assisted technologies in this work.

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