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SYNERGISTIC EFFECT OF *CITRUS SINENSIS* (ORANGE) AND *ALOE BARBADENSIS* (ALOE VERA) EXTRACTS ON BIOSYNTHESIS AND ANTIMICROBIAL ACTIVITIES OF ZINC OXIDE NANOPARTICLES

MUHAMMAD ASJAD KHAN[1], MATIULLAH KHAN[1*], REHAN NAEEM[2], MARIA HAFEEZ[3], UME-NASARIA MANZOOR[1], YOUSRA ANWAR[1] AND SADIA[1]

[1]*Department of Biological Sciences, Virtual University of Pakistan, Lahore campus, Pakistan*
[2]*Department of Biotechnology & Genetic Engineering Kohat University of Science and Technology (KUST), Pakistan*
[3]*Industrial Biotechnology Division, National Institute of Biotechnology & Genetic Engineering, Constituent College of PIEAS, Pakistan*
**Corresponding author's email: matiullah916@gmail.com*

Abstract

The synthesis of nanoparticles through the green route has become a straightforward, protected, long-lasting, consistent, and ecologically sustainable process. Bacteria are increasing its resistance against antibacterial solutions which is becoming a very critical issue nowadays. Nanoparticles can help prevent the distribution of resistance in bacteria. Metallic nanoparticles like Zinc oxide nanoparticles (ZnONPs) are among the most promising agents because they showed strong antibacterial activity without any scarcity of resistance development inside the microbes. Synthesis of ZnONPs from *Citrus sinensis* and *Aloe vera* leaf extract was conducted in this study. This was performed with the concept that the medicinal effectiveness of NPs relies on their antibacterial features. Fourier Transform Infrared Spectroscopy (FTIR) analysis has revealed that availability of aromatic compounds, amides, phenolics, and alkaloids in the stabilization, capping, and bio-reduction of ZnONPs. Furthermore, X-Ray Diffraction Analysis estimated the crystalline structure of ZnONPs to be hexagonal, and the size was found to be 15.6 nm. Transmission Electron Microscopy (TEM) determined the shape of the nanoparticle as a mixed spherical-triangular shape. All these techniques were utilized to obtain the morphology and crystalline nature of nanoparticles. ZnONPs derived from green synthesis, demonstrated promising anti-bacterial activity against *E. coli, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella*, Methicillin-resistant *Staphylococcus aureus*. Green synthesized ZnONPs exhibited a maximum inhibition zone against *Salmonella typhi* (9.5 mm) and Methicillin-resistant *Staphylococcus aureus* (MRSA) (19.3 mm) and a minimum inhibition zone against *E. coli* (16 mm).

Key words: Zinc Oxide nanoparticles, Zone of inhibition, Antibacterial.

Introduction

Metallic nanoparticles can now be produced in an eco-friendly manner that is secure, practical, easy, and quick. (Parveen *et al*., 2016). Green synthesis of Nps appeared as a safe and effective method because plants are non-hazardous. Numerous types of research about the Nanoparticles produced by the use of different plant extracts have been published (Parveen *et al*., 2016; Gour & Jain, 2019). Phytochemicals are present in abundance in plants that play a vital role in NP stabilization. These phytochemicals include flavonoids, alkaloids, vitamins, and polysaccharides. (Jadoun *et al*., 2021). The production of NPs by plant extract consists of 03 phases. By interaction with NaOH, zinc acetate salt is altered into zinc hydroxide in the first step. (Khan *et al*., 2021) Following reduction, zinc hydroxide yields ZnO2−2 nuclei. It further rises to form zinc oxide nanoparticles. These nanoparticles are stabilized by phytochemicals present in plants (Hussain *et al*., 2016).

Different kinds of metal oxide and metallic nanoparticles can be synthesized using eco-friendly methods. These NPs include zinc oxide, silver, iron, copper, and copper oxide (Singh *et al*., 2018). There are many research studies about the green production of ZnONPs; however, the combined usage of *Citrus sinensis* and *Aloe vera* extract for the formation of ZnONPs has never been documented before. *Citrus sinensis* (orange) and *Aloe vera* both are evergreen plants. Aloe vera is a medicinal plant that is well-known for its antibacterial, antioxidant, reducing constipation, reducing dental plaque, and lowering blood sugar levels. (Barcroft & Myskja, 2003). Glucomannans and other carbohydrate polymers, as well as several other inorganic and organic compounds, have been traced in *Aloe barbadensis* through chemical analysis (Mukherjee *et al.*, 2013).

Moreover, ZnONPs show inhibition of different bacterial species which can cause lethal infectious diseases (Gudkov *et al*., 2021). The prevalence of bacterial infections that are multi-drug resistant necessitates alternative treatment options. Due to their unique outstanding therapeutic capabilities, ZnONPs are becoming more and more prominent as a treatment option (Reddy *et al*., 2014). The higher antibacterial activity of nanoparticles depends upon surface area to volume ratio that permits a high number of ligands to bind with bacterial receptors on their surface (Agarwal *et al*., 2018). Drug resistance is a critical challenge worldwide, and ZnONPs synthesized through the green route can offer harmless and competent treatment against drug-resistant bacterial species. Several researchers reported the antimicrobial property of ZnONPs against different food-spoiling and infection-causing microbial strains that include *Streptococcus aureus, and Bacillus subtilis* (Baek & An, 2011). Hence, ZnONPs are therefore serving as a substitute antibacterial agent. In this study, green synthesized ZnONPs are tested against Gram-positive bacteria and Gram-negative bacteria.

ZnONPs exhibit anticancerous properties as well by destroying cancerous cells (Hanley *et al*., 2008). The toxicity of other cancer therapies, such as chemotherapy, and radiation, is greater for rapidly dividing cells (Khashan *et al*., 2020). Hence, there is a necessity to use substitute anticancerous agents that act upon cancer cells rather than normal body cells. In a recent study, (Farrugia *et al.*, 2019) reported the effectiveness of *Aloe vera* in cancer treatment. Radiation therapy is necessary in many cancer carrying patients, and it has severe side effects that include itching, erythema, and pain. *Aloe vera* has been reflected as an anti-inflammatory and antioxidant agent. Moreover, it is very effective when used as a monotherapy to treat severe skin reactions.

For any of these uses to be therapeutically effective, NPs must be administered to humans; therefore, safety is of utmost importance. Due to this reason, safer and more biocompatible NPs produced through green synthesis, should be the first choice for biomedical applications (Kalpana *et al.*, 2018).

Antibacterial agents are important in water disinfection, textiles, food packaging and medicines. Utilizing organic chemicals for disinfection has some drawbacks, such as human body toxicity, allergic reaction, skin irritation etc. This has led to an increase in the usage of inorganic disinfectants that include nanoparticles. The antibacterial properties of nanoparticles make them more beneficial (Hajipour *et al*., 2012). In this research study, we reported the synthesis of ZnONPs by using leaf extracts of *Citrus sinensis* and *Aloe Barbadensis* and assess their antimicrobial/anti-bacterial activity.

Material and Methods

Preparation of *Citrus sinensis* and *Aloe barbadensis*: *Citrus sinensis* and *Aloe barbadensis* leaves were freshly collected from Bagh Jinnah, Lahore. Firstly, 10g weight of *Citrus sinensis* and *Aloe barbadensis* leaves were taken and washed under running tap water and then distilled. Small pieces of *Aloe barbadensis* leaves were obtained by cutting them into small pieces. After washing, 300 ml of distilled water was used to boil the leaves until only 100 ml of solution remained. Whatman's filter paper was used to filter the mixture twice. Plant leaf extract was stored at 04°C to facilitate the synthesis of more ZnONPs.

Green synthesis of the ZnONPs: ZnONPs were prepared using the green approach (Khan *et al*., 2021) with slight alterations. 500 µl of *Citrus sinensis* and 500 µl of *Aloe barbadensis* leaf extracts were combined with 100 ml of a 0.02 M zinc acetate solution, and 2 M NaOH was gradually added till the pH reached 12. A magnetic stirrer was used to mix the fluid continuously for two hours. The appearance of the white colloidal solution indicated the presence of ZnONPs in the solution. After 2 h stirring, the mixture of ZnONPs was then centrifuged at 6000 rpm for 20 min. Once discarding the supernatant, the pallet was washed by using deionized water. Pallets of prepared ZnONPs were dried out in an incubator at 37°C for an entire night after completing the process three times.

Characterization of green synthesized ZnONPs

UV-Visible spectroscopy: Using UV spectroscopy, the progress of the synthesis of ZnONPs was observed throughout a two-hour reaction. Using a spectrophotometer T80+, the UV spectra of the ZnONPs reaction mixture were obtained over the wavelength range of around 300 to 800 nm.

Fourier transform infrared spectroscopy (FTIR): Functional groups and other phytochemical components that elaborate the reduction and stability of the produced ZnONPs were identified using FTIR. FTIR was performed using a Jasco FTIR 4100 spectrophotometer at reduced total reflectance mode (Japan). The measurements were made between 4500 and 500 cm^{-1}.

Transmission electron microscopy (TEM): It is a very effective technique to analyze and determine the crystalline shape of ZnONPs synthesized through the green route. Images from a Philips EM208 (Holland) TEM with an increasing voltage of 150 kV were obtained.

X-ray diffraction analysis: The crystalline form of green synthesized ZnONPs was determined through XRD. To determine the existence of ZnONPs, their size and structure, the powdered sample was exposed to X-Ray radiation (=1.5406A) operating at 40 kV and 30 mA. Moreover, the size of the green synthesized ZnONPs was calculated using the Scherrer equation (Fakhari *et al*., 2019).

Antibacterial activity of the green synthesized ZnONPs: The antimicrobial activity of the synthesized ZnONPs tested against *pseudomonas aeruginosa*, *Klebsiella*, *E. coli*, *Salmonella typhi*, and *Methicillin-resistant Staphylococcus aureus* (MRSA) utilizing the well-diffusion method (Thong *et al.*, 2011). On nutrient agar, stock cultures of Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Klebsiella*, *E. coli*, and *Salmonella typhi* were first revived by streaking. To make NA, 150 mL of distilled water was used to dissolve a total of 4.2 g of nutrient agar powder. NA was then autoclaved under normal circumstances. NA was autoclaved before being poured into Petri dishes that had already been sterilized. Solidified *pseudomonas aeruginosa*, Klebsiella, *Salmonella typhi*, Methicillin-resistant *Staphylococcus aureus*, and E. *coli* were then streaked to create a pure culture, which was then incubated for an entire night at 37°C in an incubator. By dissolving 4.5 g of Mueller Hinton agar (MHA) in 150 mL of distilled water and autoclaving it, MHA was created. Salmonella typhi, E. coli, MRSA, Klebsiella, and *Pseudomonas aeruginosa* colonies were taken from Nutrient Agar A and carefully swabbed on (Muller Hinton Agar) to determine the antibacterial properties. 04 wells on agar plate were constructed with sterile borer (6 mm), and wells were filled with 15 µL plant extract,0.02 M zinc acetate, antibiotic disc (ampicillin 10 µg/disc), and 10 mg ZnONPs were placed on MHA plates. Then these samples were placed for 24 hours at 37ºC in an incubation chamber. On a millimeter scale, the zone of inhibition was assessed.

Results

Characterization of ZnONPs

UV-Visible Spectroscopy: Spectra of UV visible around 300 to 800 nm in wavelength were used to measure the green synthesis of ZnONPs. Prepared ZnONPs's reaction mixture displayed strong peaks around 330-360nm which can be due to its high room temperature excitation binding energy. ZnONPs exhibit maximum Surface Plasmon Resonance (SPR) peak around the range of 330-360 nm wavelength. The following graph in (Fig. 1) shows the absorption spectral scan of ZnONPs. The findings indicate an absorption spectrum peak at 355 nm wavelength with an absorbance of 4.5 for ZnONPs which points to the intrinsic band gap of zinc oxide absorption. The prominent absorption peak in the UV visible spectrum confirmed the presence of ZnONPs (Vijayakumar *et al*., 2018). Results of this study showed a resemblance with reports of Khan *etal* (Khan *et al*., 2021) in which ZnONPs prepared from *Casuarina equisetifolia* presented a peak around 350 nm. Related to the present study results, ZnONPs synthesized through leaf extract of *Laurus nobilis* presented an absorption peak around 350 nm (Fakhari *et al*., 2019). In the present study, green synthesized ZnONPs showed a peak at 355nm.

Fourier transform infrared spectroscopy (FTIR): From the leaf extract of *Citrus sinensis* and *Aloe Barbadensis*, FTIR analysis assisted in the identification of numerous phytochemicals that may have contributed to the stabilizing and reduction of NPs. Plant phytochemicals are accountable for the reduction of ZnO nanoparticles determined by FTIR. Different peaks were observed in the range of 500-4500 cm^{-1} which showed the presence of various functional groups in ZnONPs. For ZnONPs, different peaks at 671.2 cm^{-1}, 1157cm^{-1}, 1639.4 cm^{-1}, 2200 cm^{-1}, 2343 cm^{-1} and 3410.3 cm^{-1} were observed (Fig. 2). FTIR peaks showed the presence of corresponding bonds of C-O, O-H (oxygen groups), C=C– stretch (alkenes), –C≡C– stretch (alkynes), C=N stretch (nitriles), C–N stretch (aromatic amines), and N-H (1˚, 2˚ amines, amides) which correspond to phenolic, alkaloids and amide group. (Ramesh *et al*., 2015) The peak observed at 1639 cm^{-1} indicates C=O according to the literature (Rastogi & Arunachalam, 2011). The peak at 671 cm^{-1} is conforming to the hexagonal ZnO symmetric bending. The presence of O-H stretching of the carboxylic acid is indicated by absorption peaks in the range of 3300–2500 cm1, which also showed resemblance to the phenomenon shown in cited research (Fahimmunisha *et al*., 2020).

Transmission electron microscopy (TEM): The crystalline characteristics and shape of the green synthesized ZnONPs were determined by TEM analysis. TEM analysis at mentioned magnifications was carried out (200nm, 100nm, and 50nm) as showen in figures A, B, and C which indicated that the synthesized ZnONPs have mixed triangular and spherical shapes as described by (Srivastava *et al*., 2013).

X-Ray diffraction analysis: The crystalline property of synthesized ZnONPs was analyzed by XRD. XRD diffractogram observed at 2θ showed numerous peaks including low, medium, and high-intensity peaks. Three intense peaks confirmed that ZnONPs are crystalline. The peaks in the range of 20-70º were at positions 31.75, 34.4, 36.2, 47.5, 56.6, 62.9, and 68.05 degrees which correspond to the (100), (002), (101), (102), (110), (103) and (112) in the (Fig. 3 A, B & C) reflection planes and hexagonal structure of ZnONPs. The results approved the crystalline nature of ZnONPs as stated by (Abbasi *et al*., 2017) Using the Scherrer equation, the particles' size was calculated to be 15.6 nm, which is comparable to the values reported by (Ramesh *et al*., 2015; Gawade *et al*., 2017; Khan *et al*., 2021). Synthesized ZnONPs were of small size, and as a result, adroitly reduced ZnO into ZnONPs. Since the physicochemical properties of NPs strongly influence their uses, smaller ZnONPs are believed to be more biocompatible than bigger ones especially those larger than 100 nm (Jiang *et al*., 2018).

Antibacterial activity of the green synthesized ZnONPs: NPs act as substitute medications to treat bacteria that are becoming more and more resistant to antibiotics. The negatively charged bacterial membrane can interact strongly with NPs, changing the permeability of the membrane and causing oxidative stress, that inhibits bacterial cells (Tayel *et al*., 2011). ZnONPs are found to be the most effective among all nanoparticles and are safe for human use as reported in various studies (Padmavathy & Vijayaraghavan, 2008; Souza *et al*., 2019). For that reason, we assessed the antibacterial activity of ZnONPs prepared through the green route against *Pseudomonas aeruginosa*, *Klebsiella*, *E. coli*, *Methicillin-resistant Staphylococcus aureus* (MRSA), and *Salmonella typhi.* This study exposed that ZnONPs synthesized through the green route exhibited strong antibacterial properties against *Salmonella typhi* even in a greater inhibition zone than a standard antibiotic disc (9.5 mm) as shown in Figure 4 (Table 1) *Salmonella typhi* is bacteria responsible for typhoid fever. This study found that synthesized ZnONPs exhibited beneficial antimicrobial activity against *Methicillin-resistant Staphylococcus aureus* (MRSA) and formed an inhibition zone (19.3 mm) as shown in Figure 4 (Table 1). P. *aeruginosa* is a Gram-negative bacterium and in nosocomial infection, it can be harmful, especially to severely ill and immunosuppressed patients. Plant-mediated ZnONPs have become new and powerful antibacterial agents to stop this dangerous pathogen and combat the problem of drug resistance that is now becoming a problem. This study found that synthesized ZnONPs exhibited a noteworthy inhibition zone against P. *aeruginosa* which is (14.6 mm) as shown in Figure 4 (Table 1) In this study, ZnONPs also showed significant antibacterial activity against, *Klebsiella*, a gram-negative non-motile rod-shaped bacterium that causes severe health issues including bloodstream infection and wound or surgical site infection. ZnONPs formed a (17 mm) inhibition zone against *Klebsiella* and (16 mm) against a Gram-negative rod-shaped bacteria *Escherichia Coli,* as shown in (Fig. 4 & Table 1).

Table 1. Zone of inhibition of green-synthesized ZnONPs against different bacterial strains.

Organism	ZnONPs	Antibiotic disc	Plant extracts (Negative control)	Zinc acetate (Positive control)
MRSA	19.3 ± 0.09	26 ± 0.08	5.8 ± 0.06	5.6 ± 0.04
Pseudomonas	14.6 ± 0.04	16.6 ± 0.09	6.1 ± 0.08	5.6 ± 0.04
Klebsiella	17 ± 0.08	20 ± 0.08	6 ± 0.08	5.8 ± 0.11
E. coli	16 ± 0.08	23 ± 0.28	6±0.08	5.6 ± 0.04
S. typhi	9.5 ± 0.23	8 ± 0.56	2 ± 0.12	6.5 ± 0.17

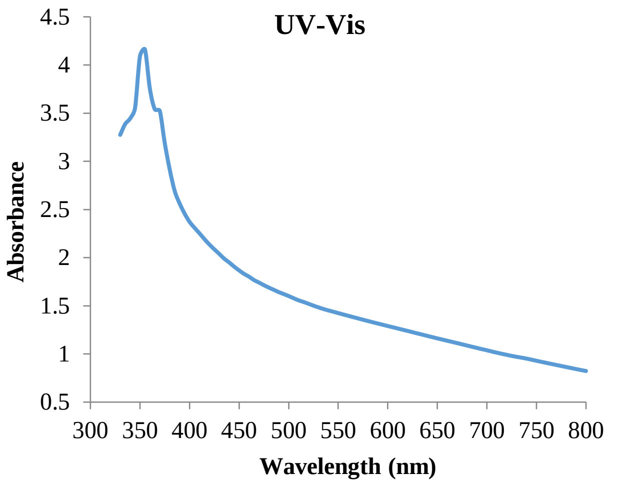

Fig. 1. Absorption spectrum of ZnONPs showing a sharp peak at 355nm wavelength.

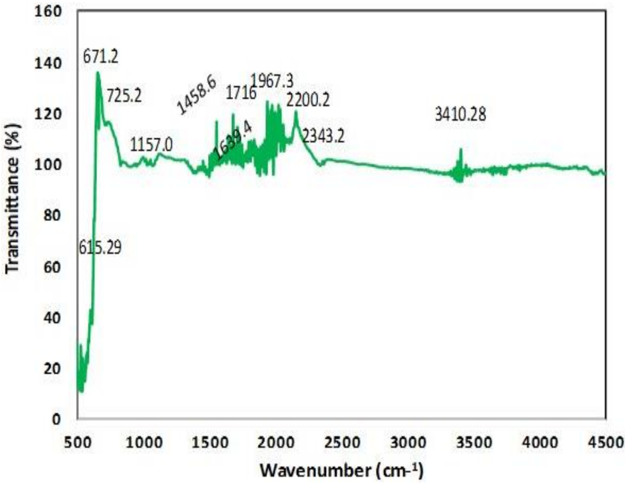

Fig. 2. FTIR analysis of ZnONPs.

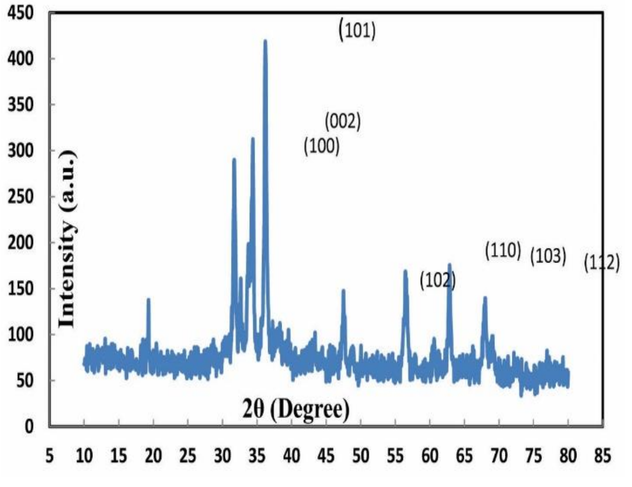

Fig. 3 D. XRD pattern indicating the presence of ZnONPs peaks

To determine the level of antibacterial activity, the zone of inhibition (mm) was assessed. *Citrus sinensis* and *Aloe barbadensis* leaf extracts served as a negative control, and zinc acetate served as a positive control, causing a large zone of inhibition to appear in all MHA plates. Moreover, all zones of inhibitions are in millimeters.

Discussion

NPs act as substitute medication to combat bacteria developing antibiotic resistance. The negatively charged bacterial membrane can strongly interact with NPs, which changes membrane permeability, causes oxidative stress, and inhibits bacterial cells. ZnONPs exhibit antibacterial properties with less toxic effects than any other nanoparticles. Just due to this reason, they are promising antibacterial agents in biomedical directions. ZnONPs show inhibition of bacterial species, antioxidant and antifungal properties. (Gudkov *et al*., 2021) Different approaches have been optimized to prepare nanomaterials, specifically metallic NPs. There are four methods for the synthesis of these nanomaterials which are divided into Physical, Chemical, and biological methods. Whereas, Physical and chemical methods have high production costs, toxic by-products, very high energy consumption, and some complications in scaling up their manufacturing. To avoid all these issues, scientists have developed an eco-friendly method by using living organisms. These can be plants, fungi, or bacteria. (Parveen *et al*., 2016) In this study, Plant extracts of *Citrus sinensis* and *Aloe vera* were prepared to synthesize ZnONPs. Research reported that they prepared ZnONPs using *Casuarina equisetifolia*. (Khan *et al*., 2021) and synthesized NPs were further analyzed by using different techniques. (Ramesh *et al*., 2015) In another study, zinc oxide nanoparticles were synthesized from green and chemical methods and their properties were determined. (El-Arab, 2018). UV-visible spectroscopy was used to confirm the synthesis of ZnONPs and analyze optical properties. After two hours of constant stirring, the solution of nanoparticles was analyzed on a spectrophotometer and the absorbance value was taken. The spectral scan from 330-800 nm showed a visible peak at 360 nm, which confirmed the synthesis of ZnONPs. This visible peak was also determined by (Moballegh *et al*., 2007), and (Chen *et al*., 2008). The size of the ZnONPs was determined by XRD analysis (Fig. 03D) using the Sherrer equation. The size of the ZnONP calculated through the Sherrer equation was 15.6nm. If the size is less than 100nm, it is considered to be very small. Smaller the size of the NP, the more efficient properties it possesses. The size of the ZnONPs through XRD was determined by two other studies (Etacheri *et al*., 2012; Khan *et al*., 2021) XRD result peaks confirmed the crystalline nature of ZnONPs. Numerous peaks were observed including low, medium, and high-intensity peaks. Three intense peaks confirmed that ZnONPs are crystalline. It is similar to as reported by Khan *et al.* and Ramesh *et al.* (Ramesh *et al*., 2015; Khan *et al*., 2021) Results of FTIR explained the presence of phytochemicals and functional groups inside the plant and synthesized ZnONPs. Research revealed the availability of ZnONPs of sizes ranging from 43 nm to 73 nm in diameter through FTIR. In their study, different functional groups that act as capping agents were also determined through FTIR. (Xiong, *et al*., 2006). Davar *et al* also determined the functional groups and capping agents of ZnONPs through FTIR. (Davar *et al*., 2015) Results of the Transmission electron microscope (TEM) was used to determine the crystalline shape of green synthesized ZnONPs. Transmission electron microscope images have indicated that the synthesized ZnONPs have mixed triangular and spherical shapes with an average size of 15.6 nm similar to those reported by (Srivastava *et al.*, 2013). Antimicrobial activity of prepared ZnONPs was determined through the agar well diffusion method against *Pseudomonas aeruginosa, Klebsiella,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *E. coli, Salmonella typhi* bacterial strains. Prepared ZnONPs exhibited maximum zone of inhibition against *Salmonella typhi* and MRSA. ZnONPs exhibited a noteworthy zone of inhibition against *Pseudomonas aeruginosa* (14.6 mm) similar to those reported by (Khan *et al*., 2021). In this study, ZnONPs exhibited a maximum zone of inhibition against *E. coli* similar to as reported by (Dobrucka & Długaszewska, 2016). Results showed a notable zone of inhibition against *Klebsiella.* Another study revealed that ZnONPs exhibited a significant zone of inhibition (Hameed *et al*., 2016).

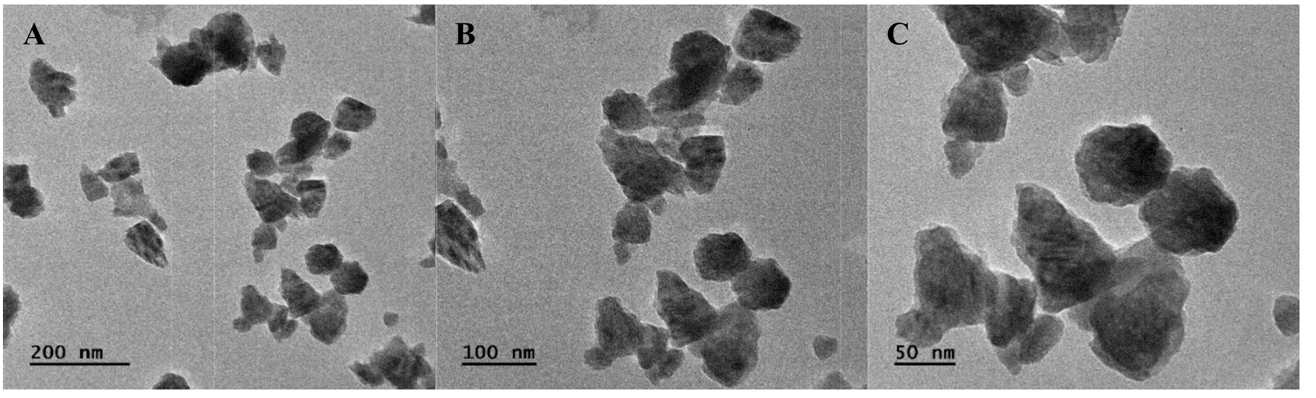

Fig. 3. A, B, and C. TEM images at different magnifications.

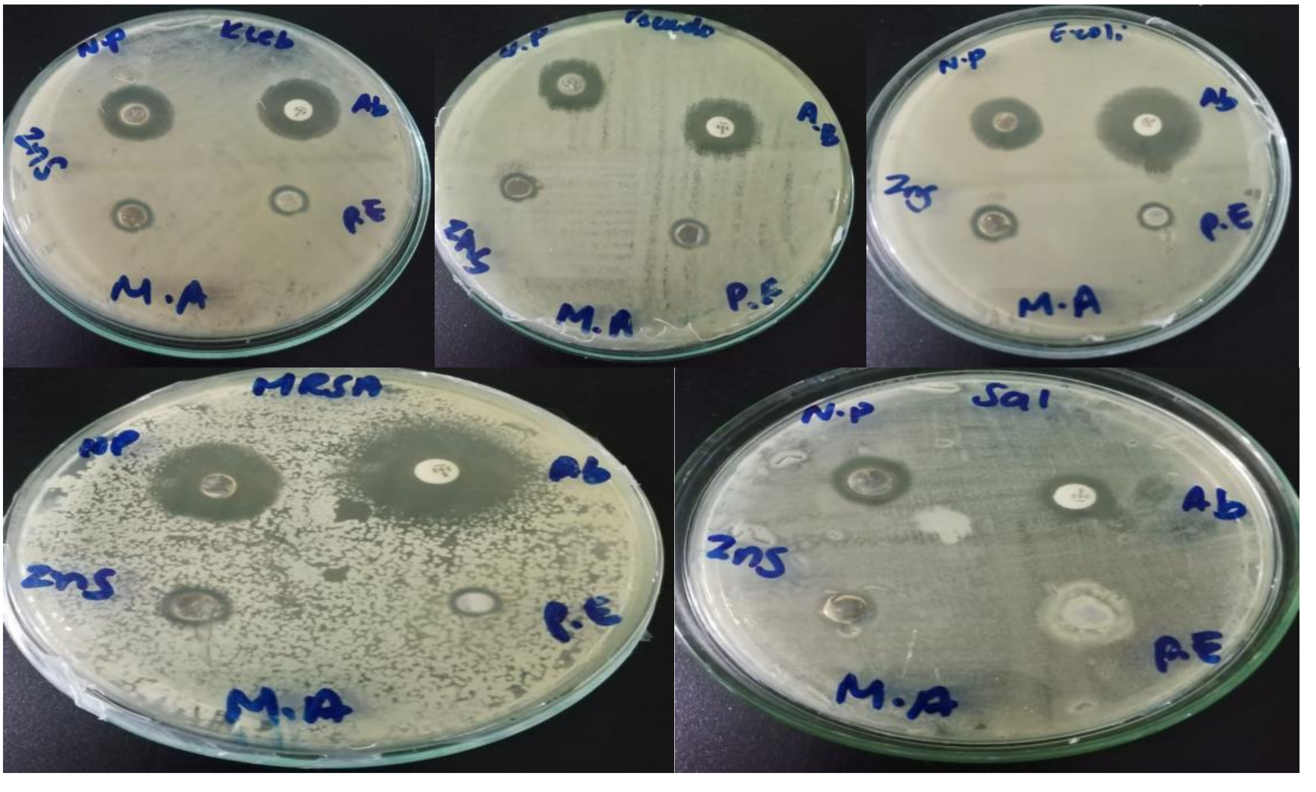

Fig. 4. Antibacterial activity of synthesized ZnONPs against *Pseudomonas aeruginosa, Klebsiella,* (MRSA), *E. coli,* and *Salmonella typhi.*

Conclusion

From the results of this study, it can be concluded that plant mediated ZnONPs exhibited strong antibacterial activity. NPs' small size and shape make them more potent antibacterial agents. ZnONPs were effectively synthesized from leaf extracts of Orange (*Citrus sinensis)* and *Aloe vera* which was confirmed through UV-visible spectroscopy by a typical peak within the range of 330-400 nm. Different functional groups, such as phenolic, alkaloids, and amide groups involved in capping and stabilizing ZnONPs, were detected by FTIR analysis. X-Ray diffraction (XRD) analysis estimated the crystalline structure and size of the synthesized ZnONPs. The small size of the NPs is responsible for their improved antimicrobial activity. Results of the Transmission electron microscope reveal the shape of synthesized ZnONPs which are mixed spherical-triangular shaped NPs and show different properties according to their shape, crystalline nature, and size. Results of antimicrobial activity through the agar well diffusion method presented significant zones of inhibition against different bacterial strains. The results of this study concluded that plant-mediated synthesis called the green synthesis of NPs could be an admirable way to develop useful and environment-friendly biomedical products.

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