# BIOSYNTHESIS, CHARACTERIZATION AND ANTIBACTERIAL PROPERTIES OF ZNO NANOPARTICLES OF COLD TOLERANT *MICROBACTERIUM* SP.

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## ABSTRACT

Nanotechnology has attracted a great interest in recent years due to its unexpected impact on many areas of science and life, especially in biology and biomedicine. Nanoparticles due to the unique physical, chemical, optical, electronical, and magnetics properties have led to an increasing interest in their synthesis. They have been synthesized by various physical and chemical processes; but most of these methods require vast amount of energy or cannot avoid the formation of toxic byproducts in their synthesis process. Therefore, there is an urgent need to develop green processes for nanoparticle synthesis. Such as biological synthesis of nanoparticles by bacteria have been considered. In this study zinc oxide nanoparticles were biosynthesis by using microbial culture supernatant from the cold-tolerant strain of *Microbacterium* sp. OSNP13. The Biosynthesized nanoparticles were characterized by UV-Vis, DLS and XRD analysis. The results indicated that the average size of zinc oxide nanoparticles were 59.16 nm. The antibacterial activity of the produced nanoparticles was then evaluated. The MIC of zinc oxide nanoparticles for *E. coli* and *S. aureus* was also calculated to be 500 µg/ml. The zinc oxide nanoparticles produced in this study have shown good antimicrobial properties and can be suitable candidates for use as antimicrobial agents.

**Keywords:** Cold-tolerant *Microbacterium* sp, ZnONPs, Antibacterial activity.

## 1. Introduction

Nanotechnology is a field of technology in which humans can design different types of compounds, alloys, devices and tools

and in general, various systems and structures or factors in atomic and molecular scale and nanometer dimensions and bring them to the manufacturing stage. Antibiotics are widely used in health care as a result, pathogens are turning into antibiotic-resistant microbes and becoming a serious disease for human health. In the last decades, scientists all over the world have been looking for new antimicrobial agents with increased ability (Hu et al., 2010; Li et al., 2015). To meet the ever-increasing demand for more effective antibacterial agents, several nanomaterials have been developed. However, many antimicrobial nanomaterials that have been reported so far are associated with concerns about toxicity, environmental pollution, biological incompatibility and complex synthesis methods (Musee et al., 2011). As a result, there is still a challenge to produce efficient, new and environmentally friendly antibacterial nanomaterials. Zinc oxide is a crystalline semiconductor oxide with a direct energy gap of 3.37 eV. The important crystalline structure of zinc oxide is its wurtzite type. Zinc oxide is one of the main metal nanoparticles due to its special properties and high energy gap which has various applications in industry and environmental sciences. The immunity of Zinc oxide and its compatibility with human beings skin makes it a suitable additive for textiles and surfaces that come into contact with the human body (Liu et al., 2014). Zinc oxide nanoparticles show antimicrobial effects on Gram-positive and Gram-negative bacteria as well as spores that are resistant to high and low temperatures (Azam et al., 2012). The antimicrobial activities of Zinc oxide nanoparticles have been improved compared to micro particles due to the increase in the surface of nanoparticles, so the smaller the size of the particles has the greater antimicrobial properties (Padmavathy & Vijayarghavan, 2008). In general, Zinc oxide reduces the ability of bacteria; However, the mechanism of its antimicrobial activity is not well known yet. It has been suggested that the main factor of antimicrobial activities can be the production of hydrogen peroxide. The accumulation of particles on the surface of bacteria due to electrostatic force can be another mechanism of the antimicrobial effect of Zinc oxide nanoparticles (Zhang et al., 2008). However, interacting types of the produced oxygen on the surface of the particles, releasing of Zinc ion, disturbance in membrane and membrane processes, as well as the internal nanoparticles can be the possible causes of the cell damage (Hajipour et al., 2012). Considering the efficient metal resistance mechanisms and the high potential of Psychotropic microorganisms in the synthesis of nanoparticles and the very less studies that have been done regarding the synthesis of metal nanoparticles with these microorganisms, therefore, in the present research, the ability of new strains of cold-resistant bacteria isolated from the Zagros highlands located

in Lorestan province of Iran and some strains of *Streptomyces* isolated from the Sea of Oman to synthesize and determine the structure of Zinc oxide nanoparticles and its inherent properties as antibacterial agents have been studied.

## 2. Materials and Methods

### 2.1. The investigated strains

In this research, first, the 44 cold-resistant isolated strains from Zagros highlands located in Lorestan province and some *Streptomyces* strains from Oman Sea were purified. Then, their ability to produce Zinc oxide nanoparticles was investigated.

### 2.2. The determination of the Maximum Tolerance Concentration (MTC)

First, solid culture medium (TSB) 200ml with different concentrations (2.5, 5, 10, 25 and 50 Mm/l) of $ZnSO_4.4H_2O$, $Zn(NO_3)_2.5H_2O$ salts were prepared in separate jars, then the contents of each of the jars were transferred into steriled microbial plates and were placed at $30^oC$ for 24 hours. Then, all the plates were divided into different parts and the plates without salt (Zinc) were considered as the control for the growth of bacteria. Then, 5 µL of the suspension of the studied strains (24-hour culture) with a 0.5 McFarland turbidity was inoculated on each of the plates. All the plates were placed in a greenhouse at 20°C and 30°C for 48 hours. A high concentration of metal in which bacteria cannot grow was introduced as MTC, and in this way, resistant and efficient strains were identified in the reduction of zinc cations (Shakya et al., 2012).

### 2.3. Synthesis, characterization of zinc oxide nanoparticles and molecular identification of the producing strain

The selected strains were inoculated in 400 ml of TSB liquid culture medium and were kept in a greenhouse for 48 hours in a shaker incubator at 20 °C at 150 rpm. After the growth and proliferation of the cells, the resulting supernatant was separated by centrifugation at a speed of 1000 rpm for 15 minutes. The resulting supernatant was added with nitrate and sulfate salts with a concentration of 0.01 Molar in a ratio of 1:1. All the samples were placed in an autoclave with a temperature of 121°C for 15 minutes and after 24 hours of incubation at room temperature, the produced nanoparticles were separated by centrifugation and after washing three times with deionized water and ethanol, they were dried at a temperature of 80°C. In order to remove the biological agents on the surface of the nanoparticles, the produced nanoparticles were placed in an oven at 470°C for 4 hours (Ghanbari et al., 2018). Then, the strain with the highest amount of sediment formation and color change of the supernatant was

selected for purification and characterization of the synthesized zinc oxide nanoparticles.

The purified nanoparticles were analyzed to measure the optical absorption by UV-vis spectrophotometer in the range of 200-400 nm. And some of the obtained sediment was used for X-ray diffraction (XRD) and dynamic light scattering (DLS) analysis. Molecular identification of the selected strain was done by amplification and sequencing of the 16srRNA gene.

### 2.4. Measuring the antibacterial activity of nanoparticles

To study the antibacterial properties of zinc oxide nanoparticles, a stock of 2000 µg/ml of the nanoparticles was dispersed in ammonium citrate at a ratio of 2:1 and 40% glycerol. *E. coli* and *S. aureus* bacteria were inoculated in MHB sterile medium and kept in a greenhouse at 37 °C for 18 hours. And finally, by using physiological serum (0.9% sodium chloride), the turbidity of bacteria was adjusted in half McFarland (absorbance 0.08-0.13 at 630 nm wavelength), which in this case, the number of bacteria is equal to 1.5 x $10^8$ CFU/ml. The antibacterial activity of nanoparticles in different concentrations (4000 to 125 micrograms/ml) was used using the micro-dilution method (Andrews, 2001).

## 3. Results and discussion

The resistance level of 44 cold-resistant and Streptomyces strains to two salts, zinc sulfate and zinc nitrate at concentrations (2.5, 5, 10, 25 and 50 mM) were evaluated. The results showed that all strains have the ability to resist at concentrations more than 5 mM. According to the initial culture results, two cold-resistant strains and two *Streptomyces* strains were selected and also considering the results of the MTC test, the resistance of these strains to the zinc sulfate salt was more than that of the zinc nitrate. Therefore, concentrations of 10 mmol/L zinc sulfate were used to produce nanoparticles. In the following, *Streptomyces* strains were excluded from further analysis due to their slow growth. Of these, the OSNP13 strain with the highest amount of sediment formation and color change of the supernatant along by heating in the autoclave was selected for the purification and analysis of zinc oxide nanoparticles (figure 1).

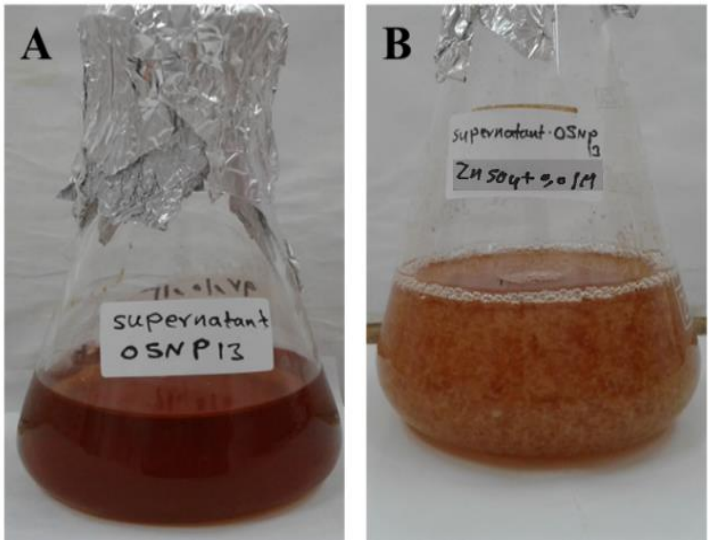


Figure 1: Production of oxide nanoparticles using the microbial culture supernatant method with autoclave heat. A and B, respectively, the supernatant of the OSNP13 strain, the supernatant after adding zinc sulfate salt and placing in the autoclave.

In this method, production of nanoparticles is in autoclave conditions or high pressure and temperature, the metal ions in the used salts are mostly in contact with the functional groups and secondary metabolites in the microbial culture supernatant and the reaction of producing nanoparticles or nucleation is done at a faster rate. In many studies has been mentioned that the NADH dependent enzymes have an important role in electron transfers during the biological production of nanoparticles. Therefore, there is no report about the production of zinc oxide nanoparticles by cold-resistant strains with this method. Ghanbari, Vaghari, Sayyar, Adibpour & Jafarizade 2017, succeeded in producing of silver nanoparticles by using *Aspergillus fumigatus* supernatant in autoclave conditions.

The results of UV-vis spectroscopy in Figure 2 show a distinct peak between 200 and 400 nm with maximum absorption at 269 nm, which indicates the presence of zinc oxide nanoparticles in the interaction solution and is caused by the superficial Plasmon resonance characteristic of these nanoparticles.

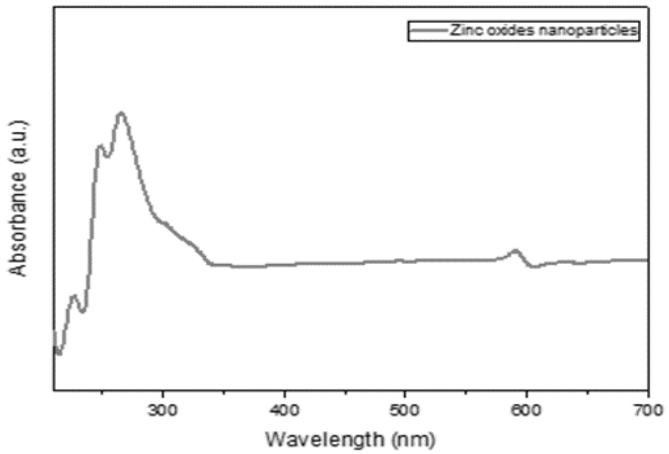


Figure 2: Uv-vis spectroscopy diagram for zinc oxide nanoparticles

The observed peak for zinc oxide nanoparticles is correspondent with the studies of Fooladsaz, Negahdary, Rahimi, Habibi-Tamijani, Parsania & Akbari-dastjerdi 2012 and Patil & Taranath 2016. The results of the nanoparticle size distribution diagram showed that the synthesized zinc oxide nanoparticles by *Microbacterium* sp. OSNP13 has an average size of 59.16 nm. As shown in Figure 3, the size distribution curve of the nanoparticles is almost bell-shaped, which indicates the distribution of the uniform proportion of the synthesized zinc oxide nanoparticles. The polydispersity index (PDI) of nanoparticles was recorded as 0.19, which indicates the high uniformity of the colloidal solution of nanoparticles synthesized in this study.

Based on reliable references, it has been reported that a uniform colloid sample of nanoparticles in which the monodispersity of the particles is high, has a PDI between 0.01 to 0.7. Non-uniform
and polydisperse samples have a PDI higher than 0.7 to 1, which represents an inappropriate sample (Honary et al., 2013).

In Ghorbani, Mehr, Pazoki & Rahmani study in 2015, according to the results of the DLS technique, the average size of oxide nanoparticles produced by co-precipitation method was reported to be 30 nm. The difference in the average size of nanoparticles with the present study is due to the different method of nanoparticles production in these two studies.

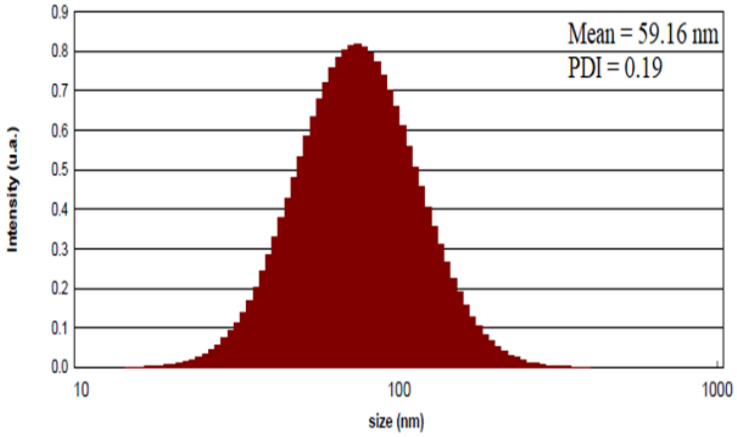


Figure 3: The size distribution pattern of synthesized zinc oxide nanoparticles.

In order to determine the network type and prove the crystalline structure of the zinc oxide nanoparticles, XRD analysis was used at 2θ angle and scanning range from 20 to 80 degrees which has specific peaks at the levels of 100, 002, 101, 102, 110, 103, 200, 112, 201 and 004 that shows the monoclinic structure of zinc oxide nanoparticles and is completely consistent with standard pattern (JCPDS File) 0002-043 (figure 4) (Shatnawi et al., 2016).

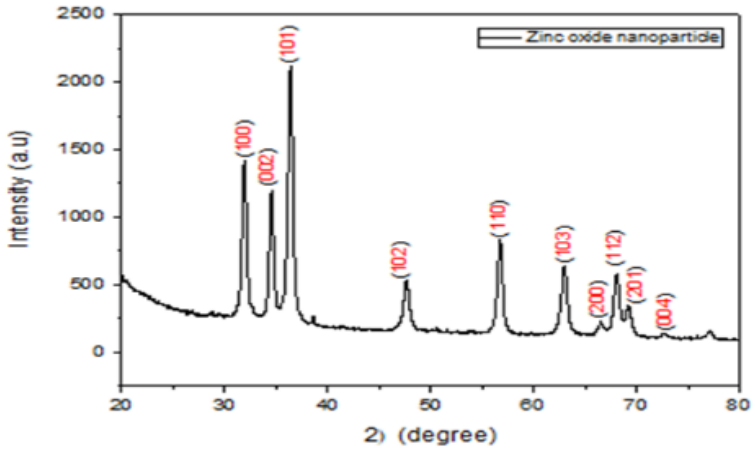


Figure 4: X-ray refraction analysis of zinc oxide nanoparticles.

The observed pattern for zinc oxide nanoparticles in Raoufi study in 2013 and Anvekar, Chari & Kadam in 2017 is completely consistent with the pattern observed in this study. According to the calculations based on the Debye-Scherer equation, the average size of the nanocrystals was calculated to be 19.25 nm for zinc oxide nanoparticles at an angle of 36.21 with the 101 crystal plane. The results of this section are consistent with the results of DLS

analysis and indicates the smaller size of the produced zinc oxide nanoparticles.
Regarding the molecular identification of the strain that produces zinc oxide nanoparticles, after determining the sequence of the 16SrRNA gene and matching the results with EZTaxon and NCBI databases, the strain of *Microbacterium* sp. OSNP13 belongs to the genus *Microbacterium* with 100% similarity.

In the continuation of the antibacterial effect of ZnO nanoparticles produced by *Microbacterium* sp. SNP13 on Gram-negative *E. coli* and gram-positive *S. aureus* bacteria were investigated as indicators of gram-negative and gram-positive pathogens, respectively, in the concentrations range of 4000 to 125 µg/ml by broth microdilution method. (MIC) and (MBC) values of zinc oxide nanoparticles for two bacteria *E. coli* and *S. aureus* were calculated as 500 and 1000 µg/ml, respectively (Table 1).

Table 1: MIC and MBC values of zinc oxide nanoparticles.

| Nanoparticles | Bacteria | MIC(µg/ml) | MBC(µg/ml) |
|---|---|---|---|
| ZnO NPs | *E. coli* | 500 | 1000 |
| | *S. aurous* | 500 | 1000 |

With the increase in the concentrations of nanoparticles, a greater decrease is observed in the growth of bacteria compared to the control sample, and this increased inhibition depends on the concentration of nanoparticles. At concentrations lower than the MIC, nanoparticles inhibit the growth of part of the microbial population and are unable to inhibit the growth of the entire microbial population (Figure 5).

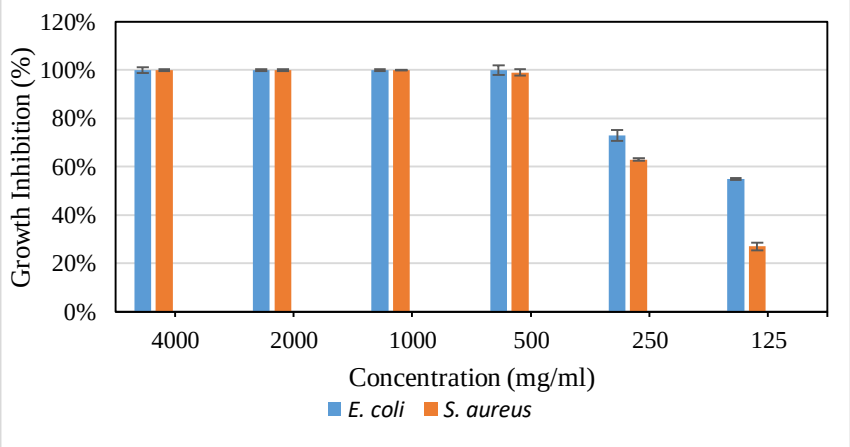


Figure 5: The inhibition percentage of the growth of pathogenic bacteria in different concentrations of zinc oxide nanoparticles

The results showed that the lethal concentration of nanoparticles used in this research had the same effects on Gram-positive and Gram-negative bacteria (Figure 6).

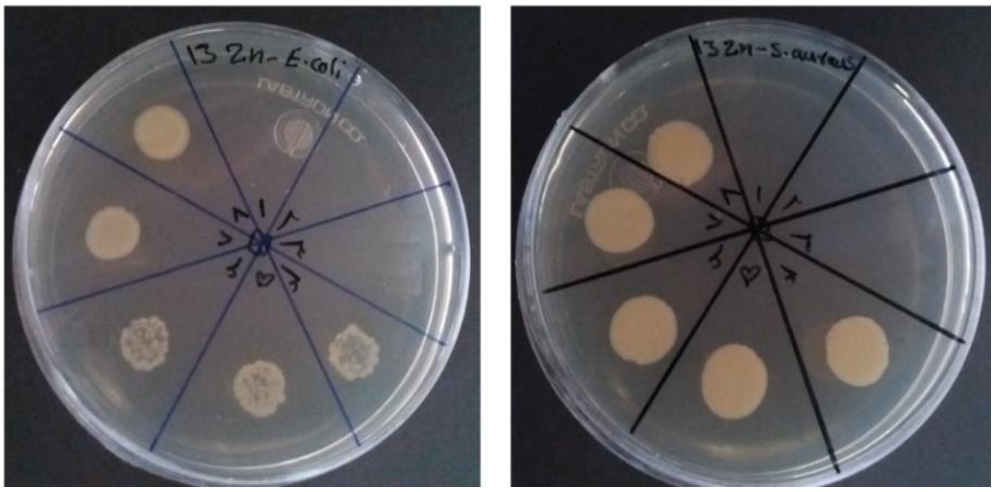


Figure 6: MBC test results for zinc oxide nanoparticles after 18 hours of greenhouse storage

In 2017 Şahin, Musevi & Aslani. reported the MIC value of zinc oxide nanoparticles produced in their studies on E. coli ATCC 25922 as 512 µg/ml. which is higher than the values calculated for the nanoparticles of this research. In 2013, Siddique, Shah, Shahid & Yasmin.. reported the MIC values of zinc oxide nanoparticles for E. coli and S. aureus as 21 and 8 mM, respectively (each milli molar was equivalent to 72 micrograms/ml), compared to the results of this research, the produced nanoparticles have shown less antimicrobial properties. Of course, in terms of comparing the nanoparticles used in this research, it has shown a suitable inhibitory and lethal effect compared to previous studies.

## 4. Conclusion

In this research, the ability of cold-resistant bacteria was used in the biosynthesis of oxide nanoparticles. The production of zinc oxide nanoparticles by the selected strain of *Microbacterium* sp.OSNP13 was evaluated using the microbial culture supernatant method along with heating in autoclave conditions. The structure and characteristics of the produced nanoparticles were characterized by using Uv-Vis, XRD and DLS analyses. The characterization results have shown the accuracy of the production of these nanoparticles. According to the DLS analysis, the average size of the produced zinc oxide is 59.16 nm. Also, the polydispersity index (PDI) was calculated 0.19 for zinc oxide nanoparticles at physiological pH, which indicates the relatively appropriate

uniformity of these nanoparticles. The size of nanocrystals was also calculated by using the Debye-Scherer equation and it was 19.25 nm. The results are consistent with the results of DLS analysis and indicate the smaller size of the produced zinc oxide nanoparticles. Examining the antimicrobial effect of nanoparticles on *E. coli* and *S. aureus* bacteria showed that, in general, the produced nanoparticles have significant antibacterial properties, that can be used as an antimicrobial agent in many different fields, including Medical equipment, antibacterial surfaces, etc.

## 5. References

1. Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of antimicrobial Chemotherapy*, *48*(suppl_1), 5-16.

2 .Anvekar, T. S., Chari, V. R., & Kadam, H. (2017). Green synthesis of ZnO nanoparticles, its characterization and application. *Material Science Research India*, *14*(2), 153-157.

3 .Azam, A., Ahmed, A. S., Oves, M., Khan, M. S., Habib, S. S., & Memic, A. (2012). Antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria: a comparative study. *International journal of nanomedicine*, *7*, 6003.

4. Fooladsaz, K., Negahdary, M., Rahimi, G., Habibi-Tamijani, A., Parsania, S., Akbari-dastjerdi,H., ... & Asadi, A. (2012). Dopamine determination with a biosensor based on catalase and modified carbon paste electrode with zinc oxide nanoparticles. *Int. J. Electrochem. Sci*, *7*, 9892-9908.

5 .Ghanbari, S., Vaghari, H., Sayyar, Z., Adibpour, M., & Jafarizadeh-Malmiri, H. (2018). Autoclave assisted green synthesis of silver nanoparticles using A. fumigatus mycelia extract and the evaluation of their physico-chemical properties and antibacterial activity. *Green Processing and Synthesis*, *7*(3), 217-224.

6 . Ghorbani, H. R., Mehr, F. P., Pazoki, H., & Rahmani, B. M. (2015). Synthesis of ZnO nanoparticles by precipitation method. *Orient. J. Chem*, *31*(2), 1219-1221.

7. Hajipour, M. J., Fromm, K. M., Ashkarran, A. A., de Aberasturi, D. J., de Larramendi, I. R., Rojo, T., ... & Mahmoudi, M. (2012). Antibacterial properties of nanoparticles. *Trends in biotechnology*, *30*(10), 499-511.

8. Honary, S., Barabadi, H., Gharaei-Fathabad, E., & Naghibi, F. (2013). Green synthesis of silver nanoparticles induced by the fungus Penicillium citrinum. *Tropical Journal of Pharmaceutical Research*, *12*(1), 7-11.

9. Hu, W., Peng, C., Luo, W., Lv, M., Li, X., Li, D., & Fan, C. (2010). Graphene-based antibacterial paper. ACS Nano 4 (7): 4317–4323.

10. Li, Z., Zhang, F., Meng, A., Xie, C., & Xing, J. (2015). ZnO/Ag micro/nanospheres with enhanced photocatalytic and antibacterial properties synthesized by a novel continuous synthesis method. *RSC Advances*, *5*(1), 612-620.

11. Liu, Q., Zhang, M., Fang, Z. X., & Rong, X. H. (2014). Effects of ZnO nanoparticles and microwave heating on the sterilization and product quality of vacuum-packaged Caixin. *Journal of the Science of Food and Agriculture*, *94*(12), 2547-2554.

12. Musee, N., Thwala, M., & Nota, N. (2011). The antibacterial effects of engineered nanomaterials: implications for wastewater treatment plants. *Journal of Environmental Monitoring*, *13*(5), 1164-1183.

13. Padmavathy, N., & Vijayaraghavan, R. (2008). Enhanced bioactivity of ZnO nanoparticles—an antimicrobial study. *Science and technology of advanced materials*.

14. Patil, B. N., & Taranath, T. C. (2016). Limonia acidissima L. leaf mediated synthesis of zinc oxide nanoparticles: A potent tool against Mycobacterium tuberculosis. *International journal of mycobacteriology*, *5*(2), 197-204

15. Raoufi, D. (2013). Synthesis and microstructural properties of ZnO nanoparticles prepared by precipitation method. *Renewable Energy*, *50*, 932-937.

16. Şahin E, Musevi SJ, Aslani AJAJoC. (2017). Antibacterial activity against Escherichia coli and characterization of ZnO and ZnO–Al2O3 mixed oxide nanoparticles, 10, S230-S5.

17. Shakya, S., Pradhan, B., Smith, L., Shrestha, J., & Tuladhar, S. (2012). Isolation and characterization of aerobic culturable arsenic-resistant bacteria from surfacewater and groundwater of Rautahat District, Nepal. *Journal of Environmental Management*, *95*, S250-S255.

18. Shatnawi, M., Alsmadi, A. M., Bsoul, I., Salameh, B., Mathai, M., Alnawashi, G., ... & Bawa'aneh, M. S. (2016). Influence of Mn doping on the magnetic and optical properties of ZnO nanocrystalline particles. *Results in Physics*, *6*, 1064-1071.

19. Siddique, S., Hussain, Z., Shahid, S., & Yasmin, F. (2013). Preparation, characterization and antibacterial activity of ZnO nanoparticles on broad spectrum of microorganisms.

20. Zhang, L., Ding, Y., Povey, M., & York, D. (2008). ZnO nanofluids–A potential antibacterial agent. *Progress in Natural Science*, *18*(8), 939-944.