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Antibacterial Activity of various ZnO Nanostructures on Pathogenic Bacteria found in Selaiyur Lake, Tamil Nadu, India

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ABSTRACT

Water is the elixir of life, and globally, the imperative need of the hour is uncontaminated water. Water has a great impact on every aspect of human life. With increasing environmental pollution and rapid growth of population, the need for developing technologies to remove pathogenic bacteria from the water bodies has become inevitable. This research was aimed at studying the effect of synthesized ZnO nanostructures on a few very common gram-positive and gram-negative bacteria - Klebsiella, Staphylococcus, Proteus and Bacillus, found in Selaiyur Lake, Tambaram, Tamil Nadu, India. Zinc oxide nanoparticles used for the anti-bacterial study were synthesized by dry mechano-chemical and sol-gel methods. The SEM images of the samples have shown the formation of spherical, flower-shaped, nut-shaped and hexagonal disc-shaped nanostructures. The XRD analyses have shown the formation of pure hexagonal wurtzite crystalline structures. The particle sizes of the synthesized samples were found to be in the range of 15-90 nm. The lake water samples taken for studies have undergone serial dilution using saline solution under sterile conditions. By the method of spread plate, the bacterial colonies were observed. The experimental results have shown that the synthesized zinc oxide nanoparticles have appreciable antibacterial properties. Using the gram stain test, both gram-positive and gram-negative bacteria were found in the sample. The nanoparticles were found to be resistant to both types of bacteria. The spherical nanostructures with ZnO nanoparticles of 16-19 nm size, synthesized by dry mechano-chemical method were found to be more effective against all the four types of bacteria studied. The size and surface morphology effect of ZnO nanostructures on bacterial growth were also studied in comparison with commercial bulk ZnO nanopowder.

Keywords: Dry mechano-chemical; Pathogenic bacteria; Sol-gel; ZnO nanostructures.

1. INTRODUCTION

In this present era of technology, nanoscience has taken centre stage in modern material science. It is a branch of science capable of providing innovative applications in almost every walk of life by exploring the uniqueness of materials at the nanoscale level (1-100 nm) (Jones *et al.* 2008). The most important aspect of nanomaterial is that these nano-sized materials bring about a new quantum mechanical effect because of their vast increase in surface area to volume ratio, which results in higher catalytic activity. Changes in the size, composition and morphologies of metal oxide nanoparticles like zinc oxide (ZnO), silver, titanium oxide (TiO), MgO and CuO can modify their chemical, mechanical, structural, electrical and optical properties. Among these metal oxides, ZnO, taken in the current study, is one of the vastly studied and used metal oxide, because of its bio-compatible, eco-safe, simpler and cost-effective nature (Yanping Xie *et al.* 2011).

Zinc oxide (ZnO) has become the epitome of recent metal oxide research, with its variety of morphologies and sizes giving way to many applications. It is an inorganic compound used in many materials or end products like plastics, ceramics and glass in powdery form. It has a strong ionic bond and crystallizes into three crystal forms, namely: the hexagonal wurtzite, the cubic zinc blende and the cubic rock salt (Gertrude Neumark and Kuskovsky, 2007; Ozgur *et al.* 2005). Zinc oxide has a wide bandgap of 3.3 eV with a high exciting binding energy (60 meV) (Fab abd Ky, 2005). Zinc oxide, among all other metal oxides or materials, has the richest nanostructures and properties, exhibiting a large range of different morphologies like nano-sheets, nano-tubes, nano-rods, and nano-combs, which are implemented on a variety of applications (George *et al.* 2009; Yahya *et al.* 2010) like anticancer studies (Selvakumari *et al.* 2015) and cosmetics. The properties and versatility of ZnO pave the way to synthesize ZnO nanoparticles using various methods like Sol-gel, Dry mechano-chemical, Hydrothermal, Metal-catalyzed, Vapor emulsion precipitation, Liquid-solid growth and Precipitation methods (Janotti and Van de Walle, 2009). By controlling the synthesis parameters such as the temperature, reactant ratios, pH and other environmental parameters, a better yield of the desired nanoparticles can be achieved. Optimization of geometry, structure, morphology and the electronic, mechanical and optical properties of the nano-size systems are of fundamental importance for the design of nanostructures (Wang, 2004; Song *et al.* 2011; Padmavathy and Vijayaraghavan, 2008).

The present study was aimed at the anti-bacterial properties of ZnO nanoparticles against some of the pathogenic bacteria present in lake water. Even though the earth is covered with 70% of water, its high content of salt makes it unfit for human consumption; most of the water bodies like sea, rivers and lakes are highly polluted by dumping of wastes from industries and household, which increases the number of harmful pathogenic bacteria like *Bacillus*, *Proteus* and so on. Bacterial diseases are serious health problems that have drawn public attention worldwide and are a human health threat that extends to economic and social complications. Hence, a case study has been done on various bacteria found in Selaiyur Lake, Tambaram, Tamil Nadu, India, and the experimental work on the antibacterial property of ZnO against such bacteria has been carried out.

The Serial dilution method and Spread plate method were employed for growing the lake water bacteria which was then followed by Well diffusion method incorporating the ZnO nanoparticles into the well smeared by the bacteria; after incubation, the inhibition zones were observed.

2. MATERIALS AND METHODS

2.1 Synthesis of ZnO by Dry Mechano-chemical Method

The Dry mechano-chemical method is one of the simple and less-sophisticated methods adopted to synthesize ZnO 1 and ZnO 1.5. It involves manual grinding of reactants followed by calcination. Zinc Acetate dihydrate and Oxalic acid were taken in measured molar concentration, and using an agate mortar and pestle, the mixture was ground for an hour under room temperature conditions. Then the mixture was annealed at 450 °C for about another hour.

$$Zn(CH_3COO)_2 \pm 2H_2O + H_2C_2O_4 \pm 2H_2O \rightarrow ZnC_2O_3.XH_2O + CH_3COOH + 2H_2O \quad (1)$$

$$ZnC_2O_4 \pm XH_2O \rightarrow ZnO + CO + CO_2 + XH_2O \quad (2)$$

Nomenclature

ZnO 1	ZnO nanoparticles synthesized by Dry mechano-chemical method with1:1 molar ratio of the reactants - Zinc acetate and oxalic acid
ZnO 1.5	ZnO nanoparticles synthesized by Dry mechano- chemical method 1:1.5 molar ratio of the reactants - Zinc acetate and oxalic acid
PEG	Polyethylene glycol
ZnO 2000	ZnO nanoparticles synthesized by Sol-gel method by using PEG 2000 as a surfactant
ZnO 4000	ZnO nanoparticles synthesized by Sol-gel method by using PEG 4000 as a surfactant
DMSO	Dimethyl sulpha oxide
Sample A	100 mg of ZnO 1 in 1 ml of DMSO.
Sample B	100 mg of ZnO 1.5 in 1 ml of DMSO
Sample C	100 mg of ZnO 2000 in 1 ml of DMSO
Sample D	100 mg of ZnO 4000 in 1 ml of DMSO
Sample E	50 mg of ZnO 1 in 1 ml of DMSO
Sample F	50 mg of ZnO 1.5 in 1 ml of DMSO
Sample G	50 mg of ZnO 2000 in 1 ml of DMSO
Sample H	50 mg of ZnO 4000 in 1 ml of DMSO

General reagent grade (Merck) zinc acetate dihydrate, $Zn(CH_3COO)_2 \pm 2H_2O$ and oxalic acid, $H_2C_2O_4 \pm 2H_2O$ were taken in an equal molar ratio of 1:1, *i.e.,* 0 ± 01 M of zinc acetate with 0 ± 01 M of oxalic acid in an agate mortar and continuous grinding was carried out manually for one hour. As the reaction proceeded, the smell of acetic acid was sensed in the first 10 minutes, with a formation of paste-like intermediate product. On further grinding at room temperature for another 50 minutes, dry free-flowing, zinc oxalate powder was obtained. The resulting white powder was annealed in a muffle furnace at 450 ˚C for about an hour. Similarly, ZnO 1.5 was prepared with zinc acetate and oxalic acid in the molar concentration ratio of 1:1.5. After the heat treatment, the obtained nano ZnO powder was cooled to room temperature and taken for XRD and SEM analyses.

2.2 Synthesis of ZnO by Sol-gel method

In this method, zinc acetate was used as a precursor, ethanol as a solvent and PEG as a surfactant. Ammonium hydroxide was added to vary the pH of the solution. 13.17 g of zinc acetate was added to 60 ml of ethanol and stirred using a magnetic stirrer for two hours at room temperature until a clear homogenous solution was obtained. Ammonium hydroxide was added to this solution until the pH of the solution turned 7.18, and the solution was further stirred for two hours at room temperature. A milky white solution was obtained. The obtained solution was divided into two equal parts. PEG 2000 was mixed with 100 ml distilled water to obtain a gel, and this was mixed to one part of the obtained solution. Similarly, the same was done using PEG 4000. These two prepared sol-gels were allowed to age for four days. Then the aged sol gel was annealed at 500 ˚C in a muffle furnace for two hours to obtain the nanostructured zinc oxide powder.

2.3 Antibacterial Study

Serial dilution of the collected water samples was the first step, wherein using a sterile conical flask 1 ml of the water sample was diluted in 9 ml of saline solution in a test tube to make a total solution of 10 ml. Further, 1 ml of this sample was diluted by another 9 ml of saline solution; the procedure was carried for dilution for up to 10^{-6} concentration of the sample. Later by Spread plate method, 0.1 ml of the sample was swabbed using a sterile glass rod in a nutrient agar medium and incubated at 37 ˚C for 24 hours. The same procedure was followed for various concentrations of the solution. After 24 hours, the formations of distinguished colonies of bacteria were found. These colonies of bacteria were isolated, and by various biochemical tests, the colonies were identified. The first test involved checking whether the bacteria were gram-positive or gram-negative. After the biochemical tests, the bacteria were identified as *Staphylococcus, Proteus, Bacillus and Klebsiella*. The final step was the use of Well diffusion method, wherein the nutrient agar was set into a sterilized petri dish and using a good cutter, wells were cut into the nutrient agar medium. Nano ZnO powder dissolved in dimethyl sulpha oxide of different concentrations was diffused into these wells and were incubated at 37 ˚C overnight. The zone of inhibition observed in the wells have shown the antibacterial effect of the nano ZnO against the bacteria.

3. RESULTS AND DISCUSSION

The crystal structure and surface morphology of the synthesized ZnO nanoparticles were characterized using XRD and SEM analysis. The Fig. 1 (a) and (b) show the XRD pattern of the standard ZnO (JCPDS35-1451) along with the diffraction pattern of the samples ZnO 1, ZnO 1.5, ZnO 2000 and ZnO 4000. All peak positions and relative peak intensities of the ZnO product agree well with those of the standard XRD pattern, and no characteristic peaks of impurities, such as ZnC_2O_4, were observed, indicating that the ZnO nanoparticle product is of high purity. The phase structure of ZnO nanoparticles belongs to a wurtzite structure. The average crystal sizes of the particles were calculated by Scherrer's equation,

$$D = k\left(\frac{\lambda}{\beta cos\theta}\right) \tag{3}$$

where, D – mean size of crystalline domains in nm; k - shape factor = 0.9, λ - X-ray wavelength; β - line broadening at half the maximum intensity (FWHM) and θ – Bragg's angle.

Table 1. Average crystal size of ZnO 1

Peak	FWHM	2ϴ	Size (D in nm)	h	k	l
1	0.37379	31.72366	22	1	0	0
2	0.41197	34.39124	20	0	0	2
3	0.38432	36.4432	22	1	0	1
4	10.62528	47.5376	18	1	0	2

Table 2. Average crystal size of ZnO 1.5

Peak	FWHM	2ϴ	Size (D in nm)	h	k	l
1	0.53569	31.87414	15	1	0	0
2	0.52681	34.5554	16	0	0	2
3	0.57177	36.36114	14	1	0	1
4	0.68784	47.61969	12	1	0	2

Table 3. Average crystal size of ZnO 2000

Peak	FWHM	2θ	Size (D in nm)	h	k	l
1	0.244	31.665	34	1	0	0
2	0.264	34.432	31	0	0	2
3	0.309	36.292	28	1	0	1
4	0.330	47.674	27	1	0	2

Table 4. Average crystal size of ZnO 4000

Peak	FWHM	2θ	Size (D in nm)	h	k	l
1	0.244	31.965	35	1	0	0
2	0.264	34.445	32	0	0	2
3	0.251	36.306	34	1	0	1
4	0.309	47.450	29	1	0	2

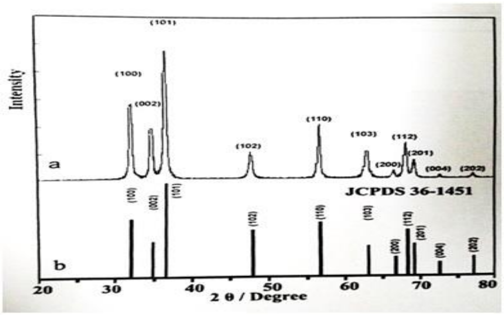
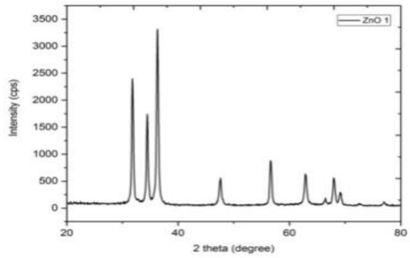
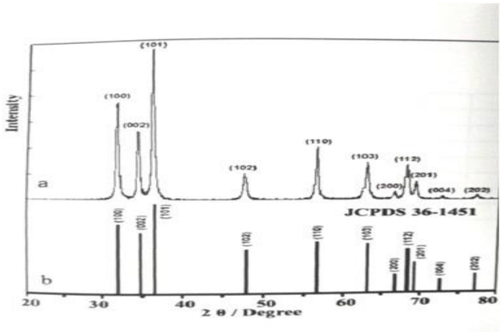
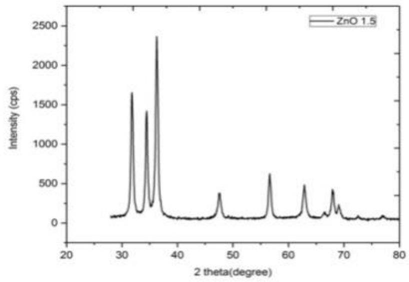
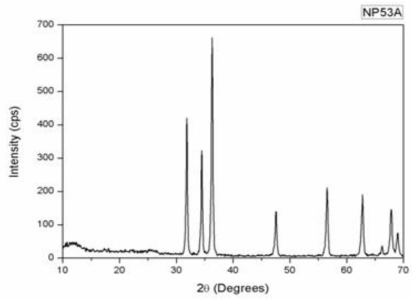
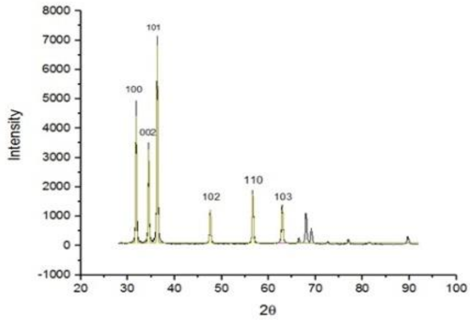
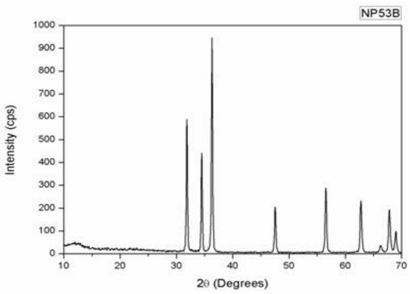
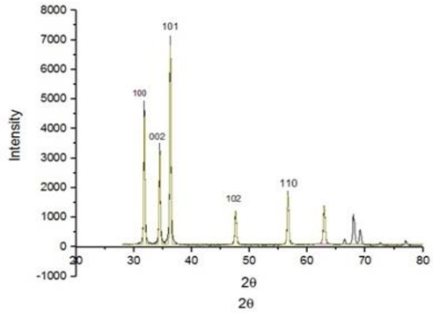
Sample	XRD (Trial 1)	XRD (Trial 2)
ZnO 1		
ZnO 1.5		
ZnO 2000		
ZnO 4000		

Fig. 1: XRD patterns of samples in: (a) Trial 1 and (b) Trial 2

The SEM images of all the four ZnO nanoparticles (for both trials) are shown in Fig. 2 (a) and (b). The pictures indicate that ZnO 1 nanoparticles have small spherical-shaped morphology, whereas ZnO 1.5 nanoparticles have very geometrically proportional nano-rods grown in a flower pattern. The variations in the molar ratio of the reactants (zinc acetate di hydrate and oxalic acid) seem to significantly affect the morphology of the nanoparticle. It is also observed that the distribution of sizes or morphology of the nanoparticles was very uniform. The repeatability of size and morphology of the nanoparticles were also tested in this method of synthesis.

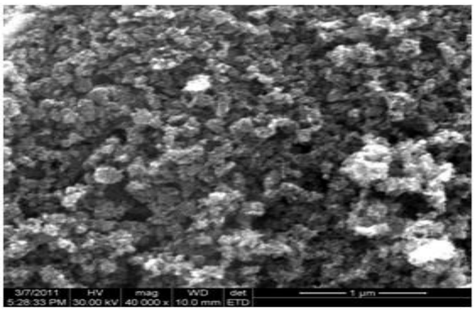
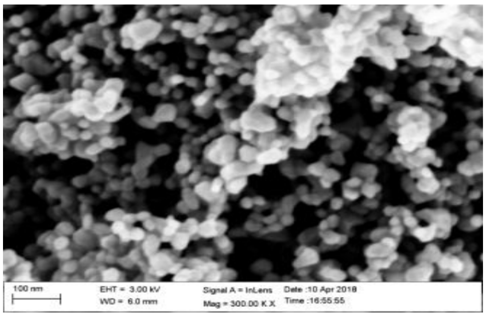
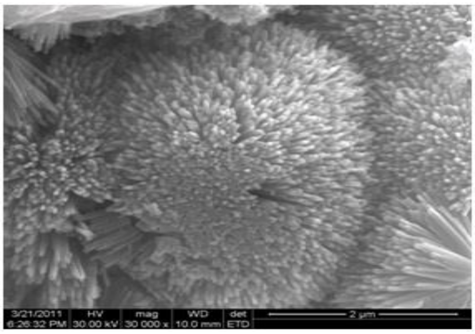
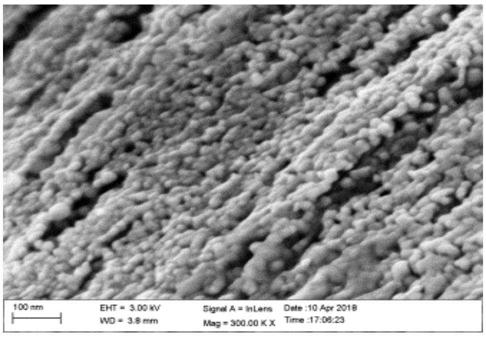
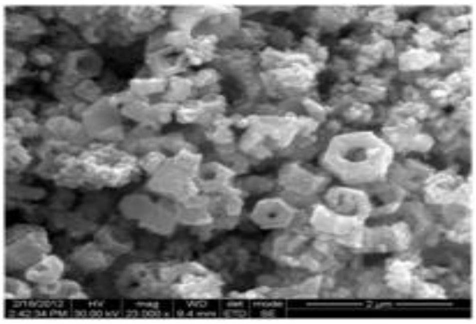
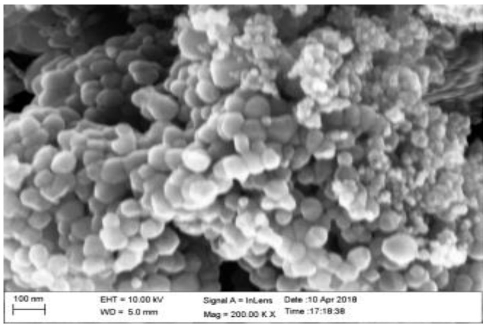
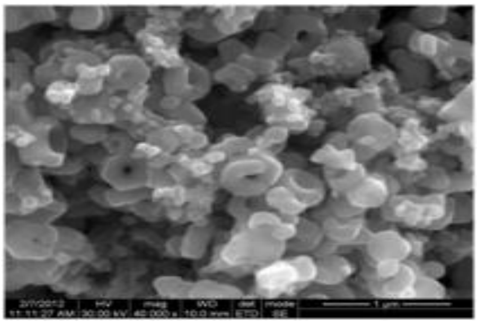
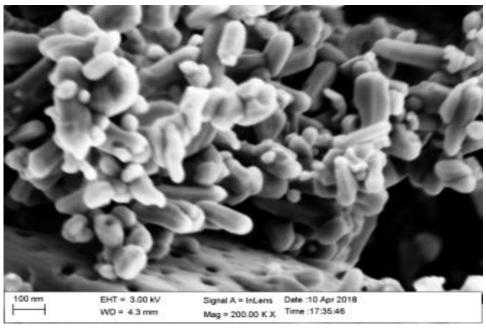
Sample	SEM (Trial 1)	SEM (Trial 2)
ZnO 1		
ZnO 1.5		
ZnO 2000		
ZnO 4000		

Fig. 2: SEM Images of ZnO samples in: (a) Trial 1 and (b) Trial 2.

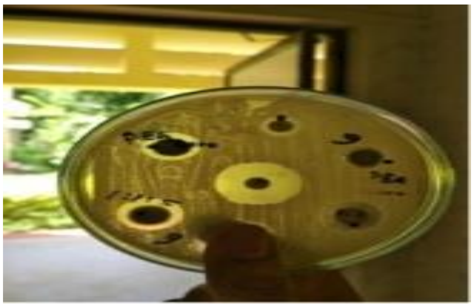
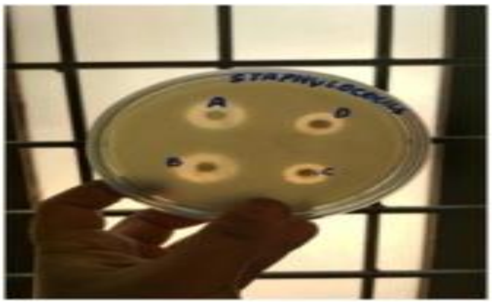
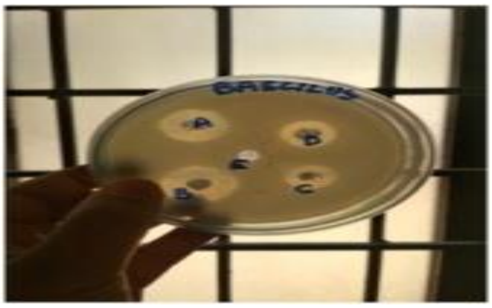
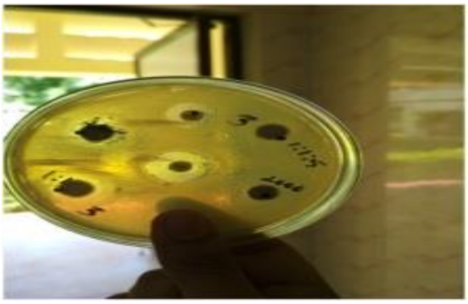
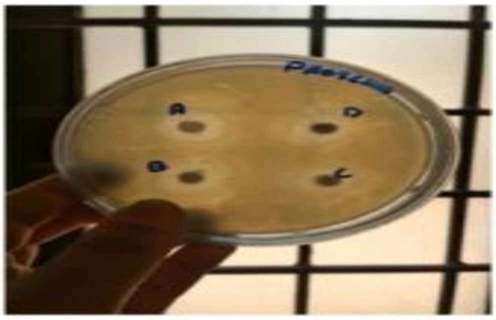
Bacteria	Zone of Clearance (Trial 1)	Zone of Clearance(Trial 2)
Staphylococcus		
Bacillus		
Proteus		
Klebsiella		

Fig. 3: Antibacterial activity studies - (a) Trial 1 and (b) Trial 2

Table 5. Diameter of zone of clearance observed from Fig. 2 (a) and Fig. 2 (b)

Bacteria	Diameter of Zone of inhibition, in cm								Bulk ZnO
	ZnO 1		ZnO 1.5		ZnO 2000		ZnO 4000		
	Trial 1	Trial 2	Trial 1	Trial 2	Trail 1	Trail 2	Trail 1	Trail 2	
Staphylococcus	1.6	2.0	1.6	1.9	1.4	1.5	1.3	1.3	1.1
Bacillus	2.0	2.3	2.0	1.9	2	1.7	1.6	1.3	1.1
Klebsiella	1.5	1.5	1.3	1.3	1.5	1.4	1.2	1.2	1.1
Proteus	2.3	2.0	1.7	1.3	2.3	1.6	1.7	1.7	1.1

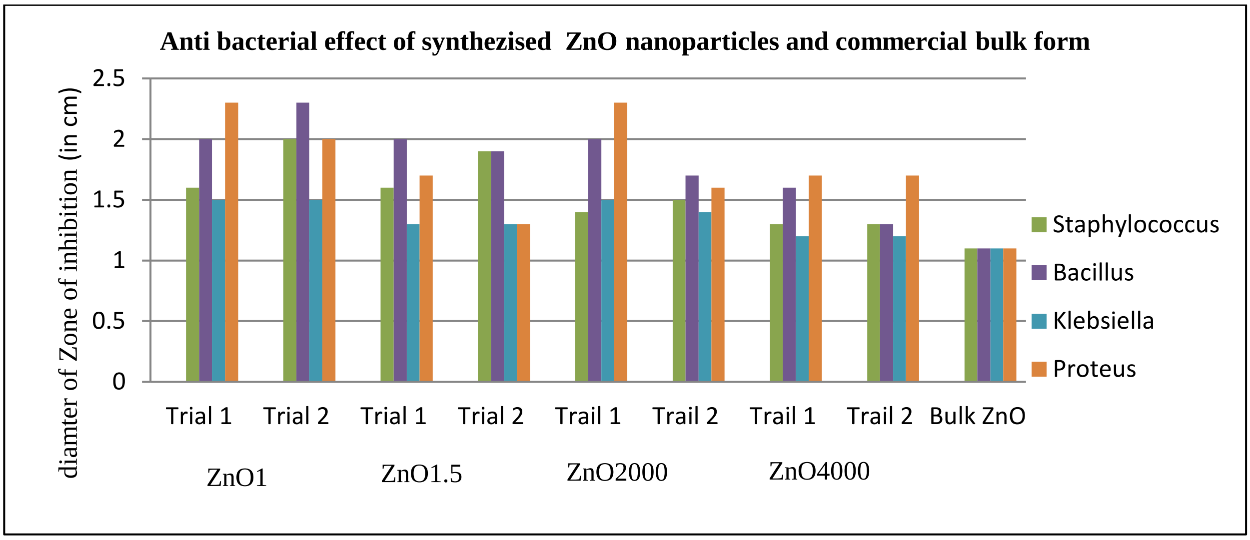


Fig. 4: Bar graph showing the antibacterial activity of various ZnO nanoparticles of Trial 1 and Trial 2 and Bulk ZnO

The SEM images of ZnO 2000 have a bolt and small cylindrical shaped morphology in Trial 1 and Trial 2, respectively. The SEM images of ZnO synthesized in the presence of PEG of varying molecular weights at different magnifications are shown. The variation in the molecular weight of PEG did not seem to affect the morphology to a greater extent. The shape of ZnO with PEG 2000 was well-defined when compared to ZnO with PEG 4000. The added water molecules in the case of PEG 4000 may be the reason for the bolt shape of ZnO. Water in glycols plays an important role in the formation of the characteristic structures, and by controlling the volume ratio of PEG to water, one can vary the morphology of ZnO nanoparticles. Higher water content in PEG resulted in hollow spheres (bolt), and the cavity size increased with decreasing PEG to water ratio. The distribution of ZnO nanoparticles improved and the particles aggregated less, as the molecular weight of the surfactant decreased.

From the measurement of the diameter of the zone of clearance (Table 5), it can be observed that all the four samples of ZnO nanostructures exhibit a noticeable antibacterial effect than the commercially obtained Bulk ZnO sample. It is also evident that the ZnO 1 sample with spherical morphology had a greater antibacterial effect on almost all four kinds of bacteria studied in both the trails. The antibacterial effect of ZnO 1 sample, consisting of spherical nanostructure with the average grain size 12-16 nm was significantly higher in both the trials compared to the other nanostructures.

Table 6. Diameter of zone of clearance (cm) based on two concentrations (mg/ml)

Bacteria	Concentration, in mg/ml							
	ZnO 1		ZnO 1.5		ZnO 2000		ZnO 4000	
	100 mg/ml	50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml	50 mg/ml
Staphylococcus	1.9	1.4	1.7	1.3	1.3	1.1	1.2	1.1
Bacillus	2.0	1.6	1.8	1.5	1.3	1.0	1.3	1.2
Klebsiella	1.5	1.2	1.3	1.1	1.4	1.2	1.6	1.2
Proteus	1.5	1.3	1.3	1.1	1.2	1.0	1.4	1.3

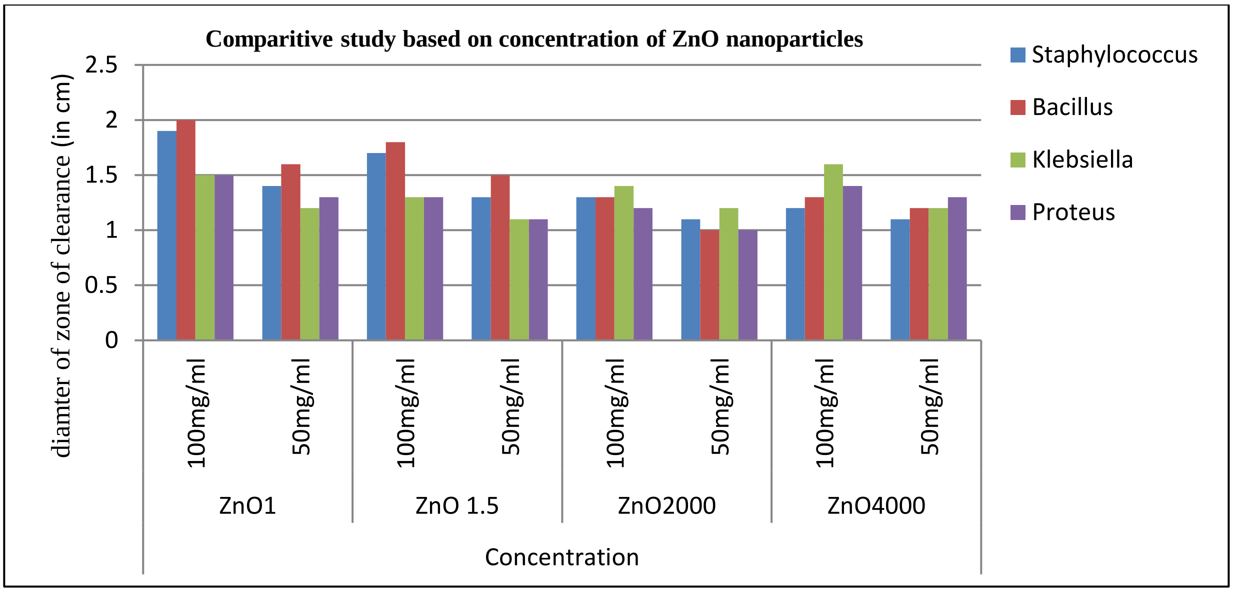


Fig. 5: Antibacterial activity of ZnO in Trial 1 and Trial 2, based on two concentrations

It was observed from Fig. 5, that the antibacterial activity also relied upon the concentration of the nanoparticles. Hence, it was found that more the concentration of ZnO nanoparticles, better the antibacterial effect.

4. CONCLUSION

ZnO nanoparticles are generally considered safe for human consumption. It possesses unique properties and excellent stability with long life compared to other organic-based disinfectants that stimulate their use as antibacterial agents. Compared to other metal oxide nanoparticles such as Fe_2O_3, MgO and AuO, ZnO nanoparticles are less toxic, have a higher antibacterial activity, and can be prepared cost-effectively. Nanoparticles can be synthesized through various methods, but from this study, it is observed that ZnO nanoparticles can be obtained through two simple methods – Dry mechano-chemical and Sol-gel. From the Dry mechano-chemical method, the nanoparticles were found to be highly pure and polycrystalline in nature with considerable small sizes of spherical and flower-shaped morphology. This method was cost-effective and yielded particles with sizes ranging from 12-27 nm. Similarly, Sol-gel method yielded pure and hexagonal-shaped nanoparticles that were distributed uniformly with sizes ranging from 25-35 nm. Sizes and morphology of the ZnO nanoparticles can be altered or varied with the change in the molar ratios of the reactants - zinc acetate and oxalic acid or the surfactants. From the synthesis of ZnO nanoparticles in Trial 1 and Trial 2, it was seen that both yielded particles with almost similar shape and size, revealing the repeatability of the synthesis process. From the XRD analysis, the average grain size of the particles was calculated by Scherrer's formula. It can be seen that Dry mechano-chemical method is an efficient method and produce pure ZnO nano-sized particles.

Furthermore, the study of the anti-bacterial properties of ZnO nanoparticles has shown the effectiveness of the particles on all the four identified bacteria *Bacillus*, *Staphylococcus*, *Klebsiella* and *Proteus*. The concentration of the nanoparticles diffused in the wells has also played a vital role in contributing to the antibacterial property of the nanoparticles. Hence, greater the concentration, better was the effect. The uniformity and size of the ZnO nanoparticles (purity of the particles) also have an effect on the antibacterial activity, as the sizes were comparable to the size of the cell wall and cytoplasm of the bacteria, making ZnO 1 more effective than the other three types of nanoparticles.

Conflict of interest

The authors declare that there are no conflicts of interest. This study was mainly focused on the antibacterial effect of various ZnO nanostructures synthesized in lab on the pathogenic bacteria identified in the Selaiyur Lake in Tambaram, Tamil Nadu, India. Our research was mainly centered upon the synthesis of nanostructures and their applications to environmental problems. The authors haven't received any financial support and they don't have any other relationship with other people or organizations.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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