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Eco-friendly synthesis of zinc oxide nanoparticles using *Bacillus Subtilis*, characterization and antibacterial potential against *Staphylococcus aureus* associated with cardiac catheterization

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ABSTRACT

Zinc Oxide nanoparticles (ZnONPs), which have well-known antimicrobial properties, are used extensively in various medical and general applications. In this analysis, 70-gram positive bacterial isolates were obtained from 100 patients using cardiac catheterization, with 54 *Staphylococcus aureus* and 16 other positive pathogenic bacteria. Accordingly, morphological, cultural and biochemical testes confirmed the results by VITEK 2 System. The synthesis of Zinc Oxide nanoparticles (ZnO NPs) was done using eco-friendly biological methods by *Bacillus Subtilis* filtrate which was identified and characterized by UV–Vis Spectrophotometer, SEM, AFM and FTIR, the pH value for the various of ZnONPS is about 7.1 and temperature 37 °C. Furthermore, the antibacterial efficacy of biological synthesized ZnO NPs against this isolated *Staphylococcus aureus* was determined. The results of SEM illustrated the morphology and sizes of ZnO NPs which are spherical and ovoid with the size range of 20-70 nm. The UV-Vis spectrum indicated the absorption bands of ZnO NPs at 378 nm. Antimicrobial susceptibility test was conducted for 54 isolates against 10 commonly-used antimicrobial agents using Kirby-Bauer disk diffusion method. The results of this study showed the highest rate of resistance against Amoxcillin/Clavulanic acid, Methicillin, tetracycline, Erythromycin and Azithromycin, and moderate resistance to Chloramphenicol. The synergistic effect of antibiotics (Amoxcillin / Clavulanic acid, Methicillin, tetracycline, Erythromycin, Azithromycin, Amikacine, penicillin G, Ampecilline, Trimethoprim\ sulphamethazole and Chloramphenicol) against *Staphylococcus aureus* was significantly increased in presence of ZnONPs compared to antibiotics only. Conclusion: ZnO NPs demonstrate a good synergistic effect with antibiotics, which can open avenues for a future combination therapy against pathogenic bacteria.

Keywords: *Zinc Oxide NPs, Bcillus subtilis, SEM, Antibacterial Activity*

1. Introduction

Nanobiotechnology has arisen as a combination of nanotechnology and biotechnology for producing biosynthetic and environmentally friendly nanomaterial synthesis. It has advanced quickly, and nanoparticles are now used in all fields of research including chemistry, physics, medicine, and biology [1]. Normally, nanoparticles range from1-100 nm. Any change in the dimensions of the materials to the atomic level alters their properties [2]. Nanotechnology has sparked worldwide interest due to the unique features of nanoparticles (NP) compared to their bulky counterparts. Industrially, Ag, CuO, and ZnO NP are utilized for a variety of applications including (1) textile and cosmetics modifications, sprays, plastics, and paints (2). Antimicrobial activity is a common property of these three NPs. (3) Nanoparticles have a quantum size effect [3].

Nanoparticles as antimicrobial agents have better efficiency against resistant bacteria, less toxicity, and heat resistance, and among metal oxide Nanoparticles, ZnO Nanoparticles have many important characteristics such as chemical and physical stability, high catalysis activity, and effective antibacterial activity [4]. ZnO NPs have been shown to be antibacterial activities against a variety of microorganisms [5].

Nanoparticle can also act as carriers for antibiotics. Therefore, because of biocidal activities of metal nanoparticles against microorganisms, they can be

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useful for designing novel antibiotics [6]. The synergistic activity of Cu [7], Ag [8], Au [9], Zn [10] have been observed with different antibiotics.

The mechanism of toxicity varies in different media as the type of soluble zinc may change according to the components of the media as well as the physical and chemical properties of nanoparticles [11]. The Antibacterial activity of nanoparticles are very important as they can reduce the severity of infection and skin infections [12], Zinc nanoparticles are used to inhibit the growth of Escherichia coli by breaking down the cell membrane and increasing membrane permeability [13]. Some research indicates that bacterial inhibition causes the production of hydrogen peroxide and its penetration into the bacterial cell membrane and its irregularity upon contact with zinc nanoparticles[14].

Intravascular catheters are primarily used to control fluids, medicines, parenteral nourishment, and blood products, as well as to monitor hemodynamic state and to do chronic outpatient hemodialysis. During vascular catheterization, two barriers are breached: the skin and the vein wall, allowing significantly contaminated blood to penetrate the surrounding area[15].

There are three principal ways for microorganisms to reach and contaminate catheters: 1) migration of skin microorganisms from the insertion site into the cutaneous catheter tract and along the surface of the catheter tip. 2) direct contamination of the catheter or catheter hub by contact with hands or contaminated fluids or devices. 3) bacterial contamination spread by blood from another focus of infection. Staph. aureus is a commensal bacterium, asymptomatically colonizing in 30% of humans [16]. This bacteria is one of the most successful opportunistic bacteria to humans because of its adaptability to survive in a new environment. Staph. aureus infections range in severity from mild skin infections to severe necrotizing pneumonia. It is also the leading cause of bacteremia and infective endocarditis (IE), and can cause osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections [17].

As a serious issue, bacterial resistance occurs because of the modification of metabolic trails, reduced accumulation, and change or inactivation [18]. Nanoparticles act as antimicrobial agents against drug resistant bacteria., Antimicrobial activity of the metals and metal oxide nanoparticles [19] are the following:

(1) Easy penetration into the cell wall,

(2) DNA replication inhibition,

(3) Disruption of the cell wall,

(4) Electron transport disruption

(5) Protein denaturation

This study aimed to investigate multi-resistance of Staph.aureus to antibiotics that causes many diseases, and to assess the influence of Nanoparticles on bacteria, which is used as an alternative treatment.

2. Experimental

2.1. Isolation of Staphylococcus aurous isolates patients

A total number of 100 patients with cardiac catheterization attending to Cardiac Centre of AL- Najaf province were included in the study. The diagnosis was based on clinical examinations under the supervision of physicians. The participants were informed of the aims of the study and provided verbal consent before taking samples.

2.2. Catheter Tip Culture and identification of pathogenic bacteria

The pathogenic bacteria were taken from a cardiac catheter kit (catheter, sheath, cord and needle**),** under aseptic conditions**.** The, end of each catheter from the 100 patients with cardiac catheterization were cut about 15 cm from the tip of the upper catheter immediately. They were then divided into 3 pieces, (5 cm by sterile scissors), and each piece was placed in the media of the brain heart infusion broth. Afterwards, they were, transferred to the laboratory and incubated under aerobic and anaerobic conditions with the availability of the CO_2 at the temperature of 37 °C for 24-48 hours. After the end of the incubation period, the tubes were examined for turbidity and cultured on the enriched and differential media. The, pathogenic bacterial isolates were examined according to the standard methods recommended by [20] as. confirmed by VITEK 2 System (Version 5.01 BioMerieux).

2.3. Biosynthesis of ZnO NPs

The procedures in this section are similar to the previous section [21]. *Bacillus subtilis* inoculum (obtained from Department of Microbiology, College of science, Baghdad University) was inoculated in a flask containing a nutrient broth and incubated at 37 ℃ for 24 h. Then, 25 mL of this culture was taken and diluted 4 times in the nutrient broth (75 ml) and again incubated for 24 h. After the incubation, the pH of the culture broth was adjusted to 7 using 0.4M NaOH. Then, 0.1M $Zn(NO_3)_2.6H_2O$ was added to the culture solution and heated on a water bath in the range of 70- 80 °C for 5-10 min. The bacterial culture medium without a salt solution as positive control and saline solution of zinc nitrate without bacteria culture medium as negative

control were tested at identical conditions. The flasks were extracted from the water bath and kept in a shaker incubator at 37 °C and 130 rpm for 24 h until all the particles settled down at the bottom of the flask. The precipitate was then collected by centrifugation at 3400 rpm and washed with deionized water three times, and then dried at 40 °C in an oven.

2.4. Characterization of ZnO nanoparticles

The biosynthesized ZnO NPs were characterized through UV–Vis Spectrophotometer, Scanning Electron Microscopy (SEM), Atomic force microscope (AFM) and Fourier-transform infrared spectroscopy (FTIR) described by [22].

2.4.1. UV-Visible Spectroscopy

For UV-Visible spectroscopy, ZnO NPs concentration (5 mg/20 ml) was prepared by diluting in the de-ionized water, and spectrum scans were performed in a wavelength range 300-700nm using HACH DR5000 spectrophotometer to find a wavelength for maximum absorbance **[**23**].**

2.4.2. Scanning Electron Microscopy (SEM)

SEM analysis was performed with a scanning electron microscope (JEOL JSM-6480). It is used to examine topology of the surface. For SEM images, the dried particles were mounted on an aluminum stub and coated with gold to obtain an enhanced contrast [24].

2.4.3. Atomic force microscope (AFM)

The shape of the surfaces and the dimensions of the prepared zinc oxide particles were studied by means of this microscope. It produced Dimethyl Sulfoxide of 17 ml, a cloud of the substance to be tested and dissolved in 7.1 for a 27-minute ultrasound. After that, the analysis was performed according to standard instructions [25].

2.4.4. Fourier transforms infrared spectroscopy

The substance was completely dissolved and then mixed with less potassium bromide powder and then turned into pellets. A hydraulic press is inserted into the machine. The aim of using High pressure due to use The device is to analyze the groups present in the prepared material [26].

2.5. Screening of ZnO NPs and antibiotics as antibacterial activity

In order to examine the antibacterial activity of the ZnO nanoparticles on these microorganisms, ZnO nanoparticles were suspended in sterile normal saline and constantly stirred until a uniform colloidal suspension was formed to yield a powder concentration of 1000 mg/ml. To assess the toxicity range of ZnO nanoparticles against *S. aureus*, an appropriate volume of test bacteria were inoculated in nutrient broth medium supplemented with serially diluted ZnO nanoparticles and bulk suspensions [27].

The agar well diffusion technique has been used in producing the antibacterial activity of (ZnO) Nano particles. Each preparation of ZnO-NPs was inoculated in different wells on Mueller-Hinton agar plates dispersed in advance by 100 µl of 24 h old bacterial inoculate. Incubation occurred at 37 °C for 24 hours to grow the bacteria. Antibacterial activity has been documented through the measurement of the inhibition zone diameter (mm) [28].

Susceptibility of all the isolates to different antibiotics were determined by the disc diffusion method as mentioned by the Clinical and Laboratory Standards Institute CLSI (16). The antibiotic discs used in this study were Amikacin (AK\30 µg) Amoxcillin / Clavulanic acid (AX/25 µg), Erythromycin(E/15 µg), Chloramphenicol (C/30 µg),. Trimethoprim\ sulphamethazole(SXT\25 µg), Methicillin(ME\5 µg) Azithromycine (AZM\15 µg), Ampicillin(AMP\10 U),Tetracycline(TE\30 µg) and Penicillin G(p\10). Each antibiotic concentration was applied on the surface of Muller -Hinton agar plates after being inoculated with *Staph.aureus* isolates and incubated at 37 °C for 24 h. *Staph.aureus* ATCC 25923 was used as control.

2.6. Synergistic activity of ZnO NPs with antibiotics

To study possible synergistic antimicrobial activity and thus diminish potential toxicity and resistance problems, mixtures of ZnO nanoparticles were combined with antibiotics to remove the strain *Staph.aureus*. The Agar well diffusion method was employed. As mentioned above, to screen the synergistic effect of ZnONPs and antibiotics, Erythromycin, Azithromycin, Amoxcillin / Clavulanic acid, Methicillin, tetracycline Chloramphenicol Pencillin G, Trimethoprim sulphamethazole, Amikacine and Ampicillin were selected in this study. Each well of the petriplate was labelled with specific bacterial strain and filled with mixed standard antibiotic solution and ZnO NPs solution (30 µl/well). All the plates were left to diffuse the sample and kept in an incubator at 37 °C for 24 hrs. At the end of incubation, mean inhibition zone diameters were measured [29].

3. Results and Discussion

Fig. 1 shows that from the total 100 catheterization patients, 54 (54.0%) *Staph.aureus* were isolated based on morphological characteristics, biochemical tests and Viteksystem 2.

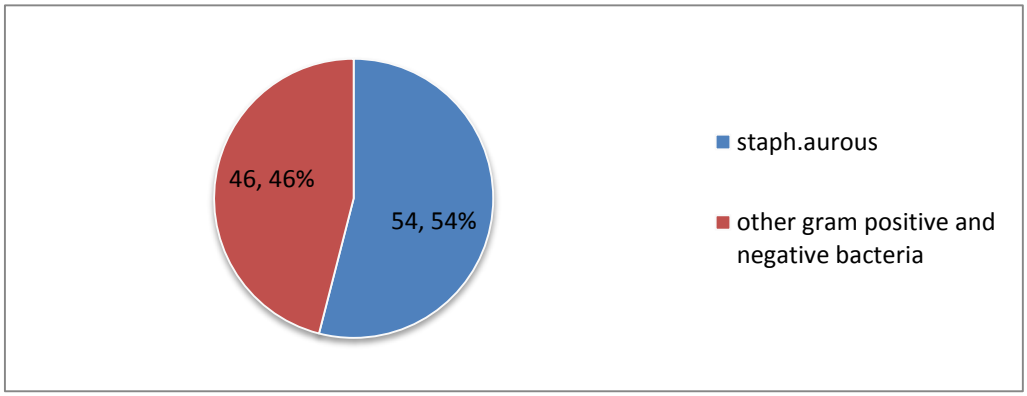

Fig. 1. The proportions and percentage of positive bacterial growth in cardiac catheterization

The findings revealed that most frequently isolate belonged to the genera *Staphylococcus* spp. It was consistent with the reports from other studies [30] that cardiac infection with *S.aureus* is most likely associated with endogenous source which is a member of the skin and nasal flora. It is also associated with an exogenous source with contamination from environment, surgical instruments or from the hands of health workers.

The presence of these bacteria in large numbers in cardiac catheterization patients indicates that the patient may be previously infected with these species, so occasionally it is possible to continue or recurrent bacteria due to the ability of these bacteria to resisted antibiotics and harsh extreme environment [31].

Bacterial infection may play a role in atherosclerosis, coronary artery disease, and rheumatic heart diseas [32]. They demonstrate that veins peripheral catheterization indicator of the risk of bacteremia measured by catheter tip methods, this method was considered as a factor and proof of diagnosis infections related with catheterization and confirmed by [33] who explained that cardiac catheterization using a catheter tip is one of the risk factors for pathogens.

Table 1 demonstrates antibiotic sensitivity for 54 *Staph.aureus* isolates. The results of this test showed that isolates have resistance to most commonly antibiotics used in hospitals.The highest rate of resistance is seen with Amoxcillin / Clavulanic acid 52/54 (96.2%), Methicillin and tetracycline (79.6%,81.4%), Erythromycin (77.7%) and. Azithromycin (68.5%) and moderate resistance to Chloramphenicol (62.9%), Pencillin G (53.7%), Trimethoprim sulphamethazole (53.7%), and relatively lower resistance toward Amikacine (44.4%) and Ampicillin (16.6&%).

The reason for multidrug resistance might be the unregulated over-the-counter sale of antimicrobials, mainly for the self-treatment of suspected infection in humans without prescription, which would inevitably lead to the emergence and rapid dissemination of resistant strains. In addition, the availability of cheaper generic drugs (like amoxicillin) of variable quality in the market for the treatment of bacterial infections may also contribute to the increased level of resistance[34].

The mechanism of this resistance is mostly due to either production of β-lactamase that hydrolyzes β-lactam ring which is controlled by plasmid or chromosomal regulation, or lack of penicillins receptors on cell wall and/or alteration in their permeability to β-lactam antibiotics preventing their uptake. This can be attributed to the fact that antibiotics may have revolutionized the treatment of common bacterial infections and some isolates have virulence factors more than other isolates, also differences in source of samples, conditions of tests used and type of techniques. All these factors may lead to differences in resistance levels [35].

Characterization of ZnO Nanoparticles

UV-visible Spectroscopy

The spectroscopy device is used to find out the optical properties for nano-sized particles. A nanoparticles size was considered as significant factor in changing the entire properties for materials., Absorption spectra of pure zinc oxide NPs are shown in **Fig. 2**. This spectrum indicates that absorption peak is strong in the wavelength (378) nm, due to the band-gap absorption for zinc oxide because of the removed electrons from the valence band to the conduction band [36]. Furthermore, because ZnO particles are in nano-size and the particle size distribution is narrow, a sharp absorption peak appears. Zinc oxide nanoparticles have good absorption in UV region (200-400) nm, which makes them convenient to medical applications like sunscreen protectors or antiseptic ointments [37].

Table 1. Antibiogram of 54 *Staph.aureus* isolates

Antibiotics	Symbol	S		I		R	
		NO.	(%)	NO.	(%)	NO.	(%)
Amikacin	AK	30	55.5	0	0	24	44.4
Penicillin G	P	22	40.7	3	5.5	29	53.7
Amoxicillin / Clavulanic acid	AMC	2	3.7	0	0	52	96.2
Erythromycin	E	12	22.2	0	0	42	77.7
Chloramphenicol	C	20	37.03	0	0	34	62.9
Trimethoprim sulphamethazole	SXT	22	40.7	3	5.5	29	53.7
Methicillin	ME	11	20.3	0	0	43	79.6
Azithromycin	AZM	17	31.4	0	0	37	68.5
Ampicillin	AMP	40	74.04	5	9.2	9	16.6
Tetracycline	TE	10	18.5	0	0	44	81.4

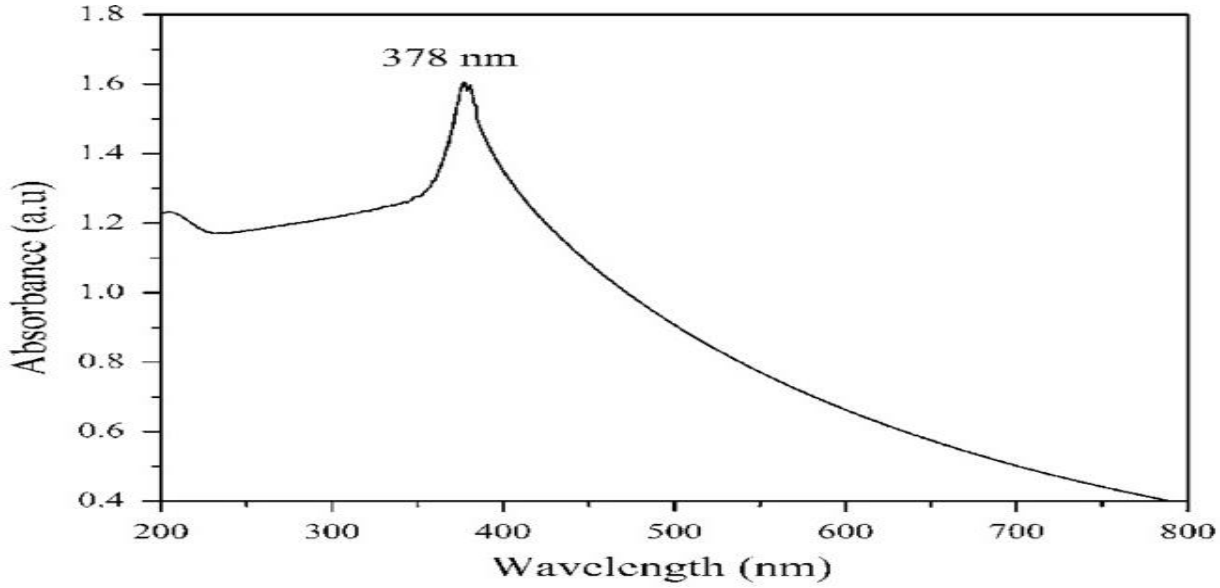

Fig. 2. Visible spectrum curve of zinc oxide nanoparticles by spectrophotometer

Atomic Force Microscope (AFM)

AFM analysis was used to study the topography of surface and the crystalline structure of the thin film. AFM images illustrate the roughness and morphology of the surface for the zinc oxide nanoparticles biosynthesized by filtrate *Bacillus subtilis*., Images were taken with a binary atomic force microscope and holographic drop-coated offline. Results showed the variance in phenotypic traits for zinc oxide nanoparticles, in addition to calculating the size of nanoparticles, which ranges between 20-100 nm, **Fig. 3** shows a 3D image of the film, where a regularity in the composition of film is observed and the grains have a vertical structure on the crystal axis and are approximately equal. This means that the distribution was uniform and homogeneity was satisfactory within the scanning area [38].

Fourier transform infrared spectroscopy (FTIR)

The FTIR Spectra for pure ZnO-nanoparticles in range of 4000 – 400 cm^{-1} are shown in **Fig. 4**. The ZnO-NPs synthesized by *Bacillus subtilis* were subjected to FTIR spectroscopy analysis to identify the biomolecules involved in stabilizing the nanoparticles in solution and detect the active groups present in the filtrate of

bacterium that participated in the process of reducing zinc oxide nanoparticles. spectrum recorded in Figure (4). shows the nanoparticles under investigation.

The graph of the FTIR infrared spectrum analysis showed two peaks, which are 445 cm^{-1} and 484 cm^{-1}. This indicates the transfer of bonds between the oxygen molecule and the zinc molecule to two types of vibration. Also, there is a weak peak and severely 1519 cm^{-1} with a range (1500-1600 cm^{-1}) which indicates the presence of a carbon-carbon group (the quinine group or an aromatic ring). These results are in agreement with earlier findings [39]. FTIR results indicate that zinc oxide nanoparticles were pure.

Scanning electron microscopy(SEM)

The surface phenotype of the synthesized zinc oxide nanoparticles was explored by SEM. **Fig. 5** explains SEM Infiltration of *Bacillus subtilis* by scanning electron microscope. These particles are under different amplification force, as they are irregular and multi-shaped, because the crystal shapes varied. These particles have rectangular, spherical, radial and cylindrical shapes with the medium size between 28-43 nanometer.

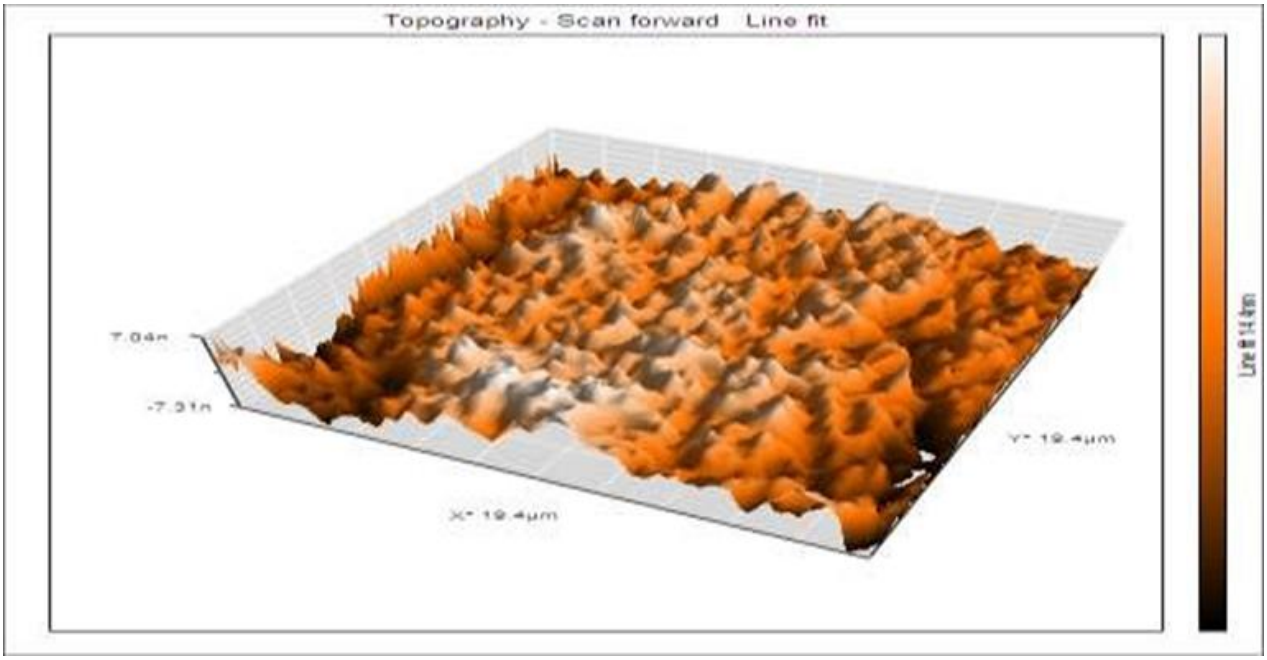

Fig. 3.Topography of three-dimensional zinc oxide nanoparticles by AFM

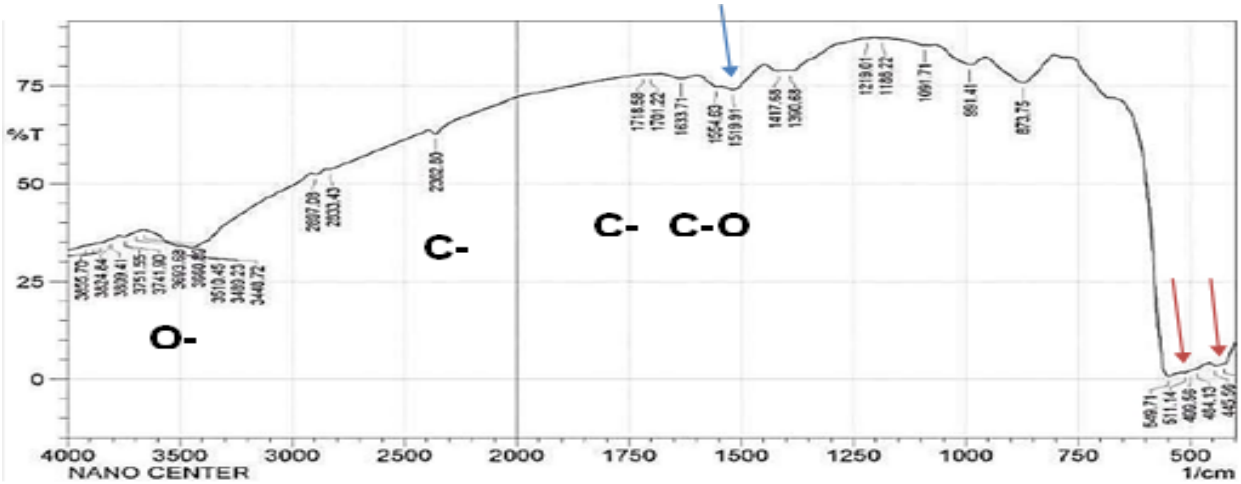

Fig. 4. FTIR spectrum for Biosynthetic Nano particles 445 cm^{-1} and 484 cm^{-1}.

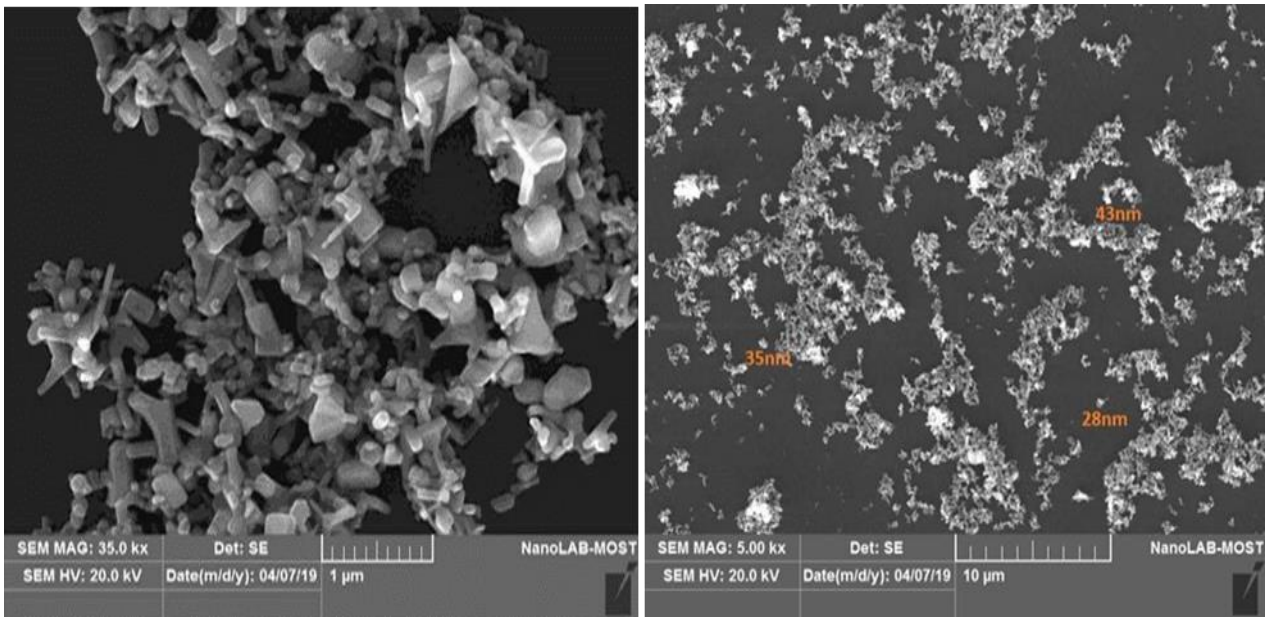

Fig. 5. Diversity in shapes and sizes obtained from the biosynthesis of zinc particles Biosynthetic nanoparticles with measured force 10 µm (right) and 1 µm (left).

In this study, a group of zinc oxide nanoparticles were obtained , which were smaller than the nanoparticles obtained by Kulkarni [40] and Pavani [41]. The average size of the nanoparticles referred to in their research ranged between 50-125 nm..

Antimicrobial activity of ZnO NPs

The antimicrobial activity of the zinc oxide nanoparticles was approved on human pathogens *Staphylococcus aurous* by agar well diffusion method (**Table 2**)**.** ZnO NPs showed a clear zone of inhibition against the tested pathogens. The presence of an inhibition zone clearly indicates the mechanism of the biocidal action of nanoparticles in disrupting the membrane. The extent of inhibition depends on the concentration of nanoparticle as well as on the initial bacterial concentration.

Reddy et al. [42] have reported similar results, emphasizing higher susceptibility of gram-positive bacteria in comparison with gram-negative bacteria. In the study conducted by Selahattin [43], it has been proposed that the higher susceptibility of gram-positive bacteria could be related to differences in cell wall structure, cell physiology, metabolism or the extent of contact.

Among metal oxide powders, ZnO reveals substantial growth inhibition of a wide-ranging spectrum of bacteria. The recommended mechanism for the antibacterial activity of ZnO is constructed mainly on the catalysis of formation of the reactive oxygen species (ROS) from water and oxygen that interrupt the integrity of the bacterial membrane, although further mechanisms have also been suggested. Since the catalysis of radical formation occurs on the particle surface, particles with larger surface area have stronger antibacterial activity. Therefore , the size of the ZnO particles decreases with enhanced antibacterial activity[44].

The syneristic effect of ZnO nanoparticles with antibiotics

The synergistic potential of the ZnO NPs together with the standard antibiotics (Amoxcillin / Clavulanic acid, Methicillin. tetracycline, Erythromycin, Azithromycin and Chloramphenicol) were assessed for all 54 pathogenic bacteria in the same conditions of temperature at 37 °C for 24 hr incubation, and the results are summarized in **Table 3**. The antibacterial activity of the combination of different antibiotics was studied against pathogenic bacteria using agar well diffusion method. The antibacterial activity of antibiotics increased in the presence of ZnO NPs against all isolates.

It can be concluded that a close contact between ZnO NPs and microorganism may enhance the transfer of ZnO NPs to the bacterial cell. Bacterial degradation of capping and stabilizing agent helps the release of ZnO NPs. The reaction between antibiotic and ZnO NPs led to synergism [30]. These results were supported by previous reports in which possible interaction of nanoparticles with the cell walls of bacteria was studied by [45].

Table 2. Antibacterial activity of Zn nanoparticles alone against *staph.aurous* isolates

Antibacterial activity of ZnO nanoparticles					
Strain	Doses	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml
		Zone of inhibition (mm)			
	Bulk Zno	0	0	0	0
Staphylococcus *aurous*		12	17	20	23

Table 3. Synergistic effects between antibiotics and zinc oxide nanoparticles on *S. aureus* isolates

Antibiotic only	Zone of inhibition (mm)	Aantibiotic+ZnONPs	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml
			Zone of inhibition (mm)			
Amoxcillin / Clavulanic acid	4	Amoxcillin / Clavulanic acid +ZnONPs	13	17	23	33
Methicillin	7	Methicillin+ZnONPs	13	15	21	30
Tetracyclin	8	Tetracycline+ZnONPs	12	15	20	27
Erythromycin	8	Erythromycin+ZnONPs	15	18	20	32
Azithromycin	6	Azithromycin+ZnONPs	13	20	25	35
Chloramphenico	7	Chloramphenicol+ZnONPs	12	17	22	27
Amikacine	8	Amikacine+ ZnONPs	11	13	18	21
Penicillin G	5	Penicillin G+ ZnONPs	10	12	17	19
Ampicilline	5	Ampicilline + ZnONPs	13	15	19	24
Trimethoprim sulphamethazole	4	Trimethoprim sulphamethazole+ ZnONPs	10	16	20	22

4. Conclusions

It was found, in this study, that the synergism of ZnO NPs with antibiotics rendered excellent antimicrobial activity against the tested bacteria. The diameter of inhibition zone reflects the magnitude of the susceptibility of microbes. The largest increase was observed for Azithromycin., The results proved the efficiency and purity for ZnO NPs, making it suitable for medical and industrial applications.

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