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Antibacterial activity and synergistic antibacterial potential of chemically synthesized zinc nanoparticles on pathogenic bacteria species

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Abstract
In present work the antibacterial activity of chemically synthesized Zinc oxide nanoparticles and their synergistic effect with antibiotics on six pathogenic bacteria was analyzed. The nanoparticles were characterized by high resolution SEM (Scanning Electron Microscopy), FTIR (Fourier Transform Infra-Red spectroscopy) and UV-VIS analytical techniques to probe their structure, surface morphology and physical characteristics. When ZnO NPs were applied alone at various concentrations in *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella typhimurium*, *Streptococcus spp.*, *Klebsiella spp*. and *E. coli* cultures, it was observed that minimum zone of inhibition in *E.coli* i.e. 0.4 cm and maximum growth inhibition in *Klebsiella spp*. i.e. 3.4 cm. Thereafter, antibiotic effect was monitored with three antibiotics, namely, gentamicin, ciprofloxacin and ampicillin. Among all the six bacteria, *Klebsiella spp*. was highly sensitive to all the three antibiotics and *Streptococcus spp* was most sensitive to ampicillin. When the synergistic effect of ZnO NPs in combination with three antibiotics was observed it revealed the maximum growth inhibition in *Pseudomonas aeruginosa* with combination of ZnO NPs and chlorofloxacin, followed by combination of ZnO NPs and gentamicin in *E.coli*, combination of ZnO NPs and ampicillin in *E.coli*. All the bacterial species exhibited more than 300 per cent augmentation in growth inhibition.

Keywords: nanoparticles: zinc oxide, antibacterial activity, nanoparticles characterization

Introduction
Metal oxide nanoparticles are widely used in the field of research and development and have diverse applications in industries such as diagnostic techniques, drug delivery, sunscreens, antimicrobial bandages, disinfectant, diagnostic techniques and an alternative energy production [1-3]. The widespread applications of metal nanoparticles (particles less than 100 nm) is due to their unique properties [5]. An important additional distinction should be made between nano structured thin films or other fixed nanometer-scale objects (such as the circuits within computer microprocessors) and free nanoparticles [4]. Different chemical and physical methods are used for the synthesis of metal nanoparticles. Among various metal nanoparticles, ZnO nanoparticles have received a great attention in the recent past due to their unique properties such as high chemical stability, high electrochemical coupling coefficient, broad range of radiation absorption and high photostability and wide applications such as solar cells, gas sensors, ceramics, catalysts, cosmetics [6-8]. Different methods are used for the synthesis ZnO nanoparticles such as sol-gel method, thermal decomposition, homogeneous precipitation, solvothermal method, reverse micelles, direct precipitation, hydrothermal, thermal decomposition, sono chemical method and microwave irradiation [9]. The basic properties of metal nanoparticles are mostly characterized by their size, composition, crystallinity, and morphology. Reducing the size to nanoscale can modify their chemical, mechanical, electrical, structural, morphological, and optical properties. These modified features allow the nanoparticles to interact in a unique manner with cell biomolecules and thus facilitate the physical transfer of NPs into the inner cellular structures [10]. ZnO is currently being investigated as an antibacterial agent in both microscale and nanoscale formulations. ZnO when reduced in nanometer range to form ZnO nanoparticles, these interact with bacterial surface to enter inside the cell and exhibit distinct bactericidal mechanisms that can be used for antibacterial mechanisms [11, 21-22]. Many researchers explained antibacterial acitivity of ZnO nanoparticles over a wide range of bacterial species [12-17]. In this research work, ZnO nanoparticles were synthesized by the wet chemical method and were characterized using UV-Vis spectrophotometer, Scanning Electron Microscopy (SEM) and Fourier Transform Infra-Red spectroscopy (FTIR). The antibacterial activity of ZnO nanoparticles was analyzed using different bacterial strains and antibiotics.

Materials and Methods
Chemical synthesis of zinc oxide nanoparticles (CH-ZnO NPs)
Zinc nitrate, soluble starch and sodium hydroxide of analytical grade used for chemical synthesis of zinc oxide nanoparticles were obtained from Sigma. 1% soluble starch was prepared by dissolving 0.5gm in 500 ml of luke warm distilled water. Zinc nitrate 14.874gm (0.1mol) was added in above solution followed by constant stirring for one hour using magnetic stirrer to completely dissolve the zinc nitrate. After its

complete dissolution, 0.2 M sodium hydroxide was added drop wise under constant stirring. The reaction was allowed to proceed for two hours and after completion of reaction, the solution was kept overnight. The supernatant was discarded carefully and the rest of the solution was centrifuged at 10,000 rpm for 10 min, after centrifugation the supernatant was discarded again. Thus, the nanoparticles obtained were washed twice using distilled water to remove by-product and excessive starch bound with nano particles. After washing, the nanoparticles were dried at 80^{o}C overnight. During drying, $Zn(OH)_2$ converted into ZnO. The samples were used for its antibacterial activities and were compared with available antibiotic available as antibiotic discs.

Collection of bacterial sample

The toxic effects of ZnO nanoparticles were observed against six bacterial species and its toxicity was compared with three antibiotics gentamicin, ciprofloxacin and ampicillin. The six bacterial species *Pseudomonas aeruginosa* (MTCC-10462), *Bacillus subtilis* (MTCC-441), *Salmonella typhimurium* (MTCC-0968), *Streptococcus* spp. (MTCC-01927), *Klebsiella* spp. (MTCC-07407) and *E. coli* (MTCC-1687) were obtained from IMTECH Chandigarh.

Characterization of CH-ZnO NPs

The ZnO nanoparticle was characterized in a JASCO-V-530, UV-Vis spectrophotometer, to know the kinetic behavior of zinc oxide nanoparticles. The scanning range for the samples was 280-700 nm at a scan speed of 400 nm/min. Base line correction of the spectrophotometer was carried out by using a blank reference. The UV-Vis absorption spectra of ZnO NPs of the samples was recorded and numerical data was plotted.

FTIR studies were carried out in order to ascertain the purity and nature of the metal nanoparticles. Metal oxides generally give absorption bands in fingerprint region i.e. below 1500 cm-1 arising from inter-atomic vibrations and SEM analysis was also done for confirmed the formation of single phase nanoparticles of pure ZnO and agglomerations of particles.

Antibacterial activity

Antibacterial activities of CH-ZnO NPs were observed against six bacteria in two different sets of experiment. In first set of experiment, antibacterial activities of CH-ZnO NPs alone was studied while in next set of experiment, antibacterial activities of CH-ZnO NPs in combination with three antibiotics were studied. Antibacterial activities were determined, using the disc diffusion method. Approximately 20 ml of autoclaved nutrient broth agar was poured in sterilized petri dishes. The petri dishes were left overnight at room temperature to check for any contamination to appear. The freshly grown overnight cultures of inoculum (100µl) were spread on each agar plates. Sterile paper disc of whatman filter paper, 5mm diameter that was earlier dipped in 50mg/l CH-ZnO NPs along with three standard antibiotic containing discs were placedin each petri dish. The plates containing the test organism and CH-ZnO NPs were incubated at 37°C for 24 - 48 h. The petri dishes were examined for evidence of zone of inhibition, which appear as a clear area around the disc. The diameter of such zones of inhibition was measured using a meter ruler and the mean value for each organism was recorded and expressed in centimeter. The interaction of ZnO NPs with the antibiotics was checked by dipping antibiotic disc in ZnO NPs solution (0.3M) for overnight. The effect of ZnO NPs in combination with the antibiotic was checked against the six bacterial species by disc diffusion method as described earlier.

Results and Discussion

Zinc oxide nanoparticles (ZnO NPs) were synthesized by chemical method, characterized, their antibacterial activity was checked, compared with commercially available antibiotics such as Gentamicin, Ciprofloxacin and Ampicillin and their syngerstic effect with commercially available antibiotics on bacteria was examined.

Chemical synthesis of ZnO nanoparticles

Zinc oxide nanoparticles have some excellent properties like exceptional mechanical strength and good antistatic, antibacterial and UV absorption properties. The zinc oxide nanoparticles were synthesized using wet chemical method (Figure 1).

Fig 1: Chemical synthesis of zinc oxide nanoparticles

Characterization *of* CH-ZnO NPs

The UV-Visible (UV-Vis) spectrum of ZnO NPs was recorded. The absorption spectrum of ZnO NPs was in the range of 390- 420 nm with absorption maxima at 390 nm (Fig. 2). The spectrum of interference pattern by FTIR analysis in the wavelength range of 500 - 4000 cm^{-1} clearly shows that the absorption band of ZnO nearer to 1383 cm^{-1}. The peak observed at 3250 and 3510 cm-1 are may be due to O-H stretching and deformation, respectively assigned to the water adsorption on the metal surface. The peaks at 1383.00, 932.93 cm-1 are corresponding to Zn-O stretching and deformation vibration, respectively. The metal-oxygen frequencies observed for the respective metal oxides are in accordance with literature values. Kumar H. and Rani R. (2013) [19] reported similar FTIR spectra observed of zinc oxide nanoparticles in their investigation [19], (Fig 3). SEM micrographs of ZnO NPs are, revealed the presence of variable sized and some agglomerated NPs at some regions (Fig. 4).

The data obtained from UV-Visible graphs, FTIR analysis and SEM images confirmed the structure, surface morphology and physical characteristics of zinc oxide nanoparticles. These characterization studies revealed the successful synthesis of pure ZnO NPs without any impurities and unreacted excessive precursor.

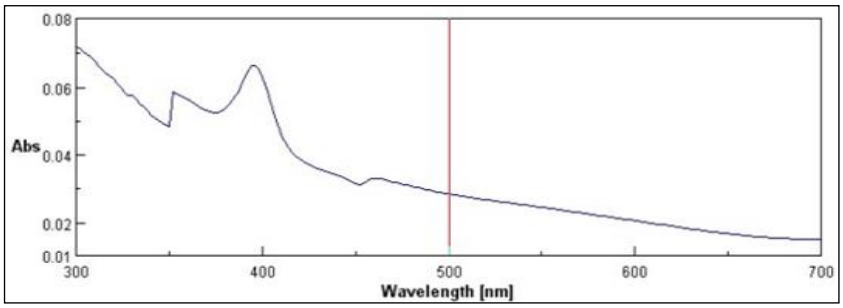

Fig 2: UV-Vis absorption spectra for chemical synthesized ZnO NPs

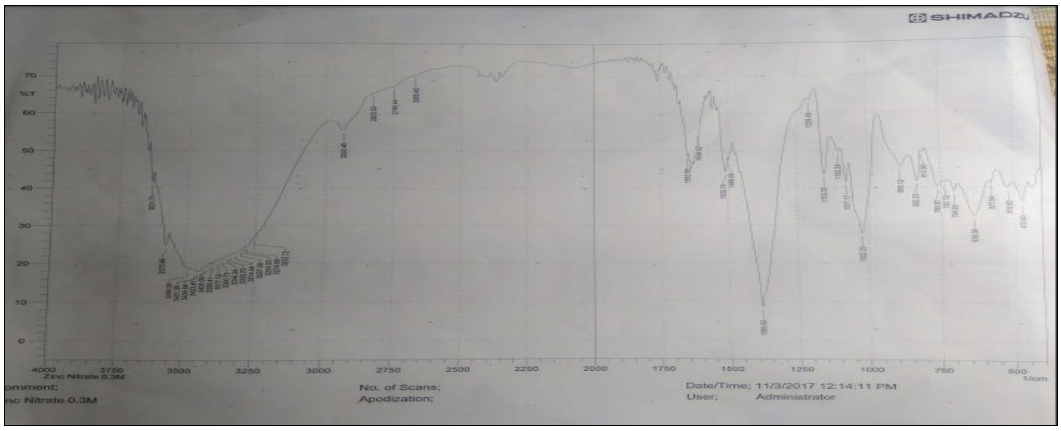

Fig 3: FTIR analysis of chemical synthesized ZnO NPs

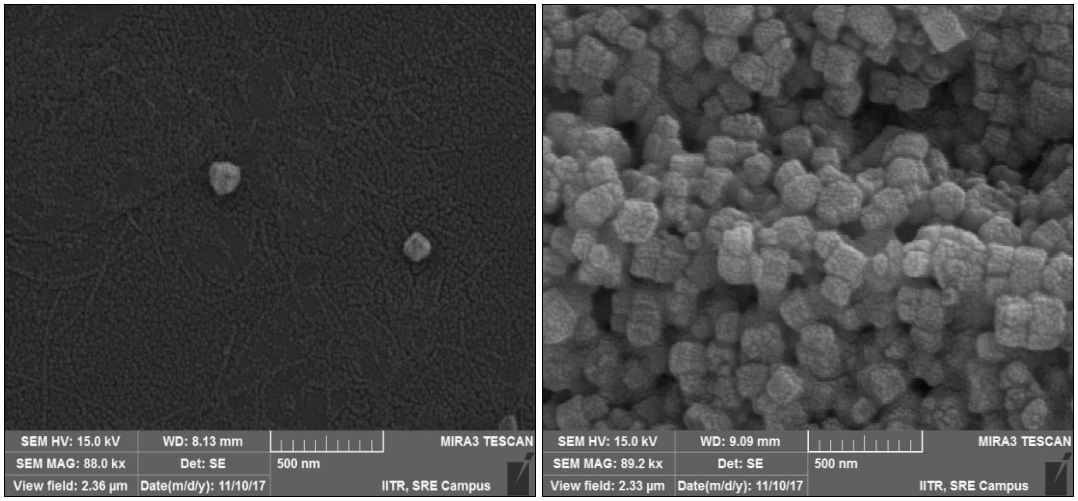

Fig 4: SEM images of ZnO NPs

***Antibacterial activity of zinc oxide nanoparticles* (ZnO NPs)**

The effect of different concentration of ZnO NPs on six bacteria (*Pseudomonas aeruginosa, Bacillus subtilis, Salmonella typhimurium, Streptococcus spp., Klebsiella spp.* and *E. coli*) was studied. The undiluted ZnO NPs was considered as 100% and 70%, 50% and 20% diluted ZnO NPs was prepared by addition of deionized water. It was observed that the high concentration (100%) of ZnO NPs is effective against all bacterial species. The zone of inhibition using 100% concentration of ZnO NPs was observed to be minimum (0.4 cm) in *E.coli* and maximum (3.4 cm) in *Klebsiella* spp. (Table 1) (fig 5 & 6).

Table 1: Relationship between concentration of ZnO NPs and zone of inhibition (cm) of different bacteria

Concentration of ZnO NPs	*Bacillus subtilis*	*Klebsiella spp*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*E.coli*	*Streptococcus spp*
20%	0.1	2	0.7	0.6	0.2	0.8
50%	0.3	2.4	1.3	0.9	0.2	0.9
70%	0.9	2.3	1.5	1.0	0.3	1.3
100%	1.1	3.4	1.7	1.1	0.4	1.5

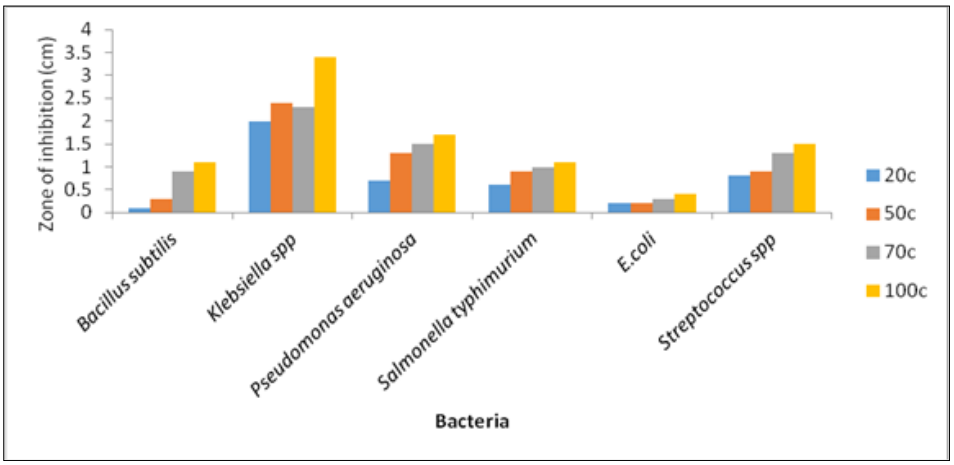

Fig 5: Diameter of zone of inhibition (cm) produced by ZnO NPs on different pathogenic bacteria using well diffusion assay.

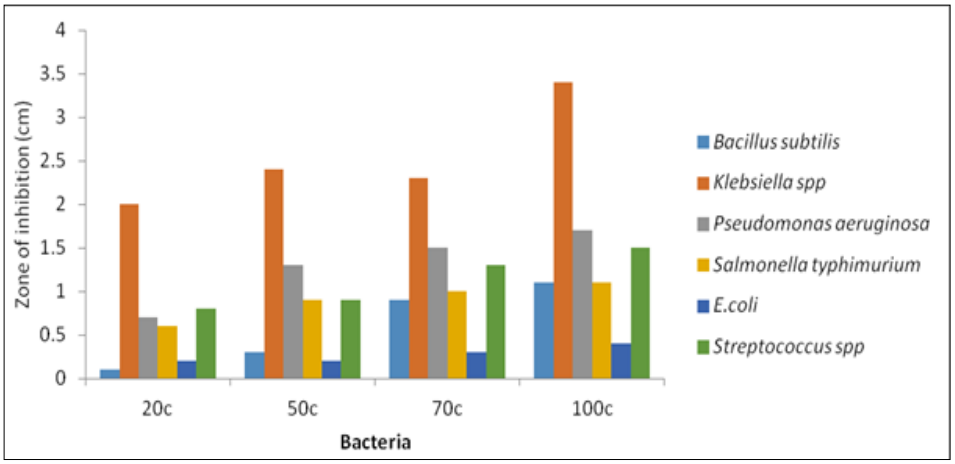

Fig 6: Relationship between zone of inhibition (cm) of bacteria and different concentration of ZnO NPs

Antibacterial activity of antibiotics against pathogens

The effect of three antibiotics was checked on six bacterial species (Fig. 7). Among all the three antibiotics, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptococcus spp* exhibited maximum zone of inhibition with ampicillin, namely 2.3, 2.8 and 4.0 cm respectively whereas in

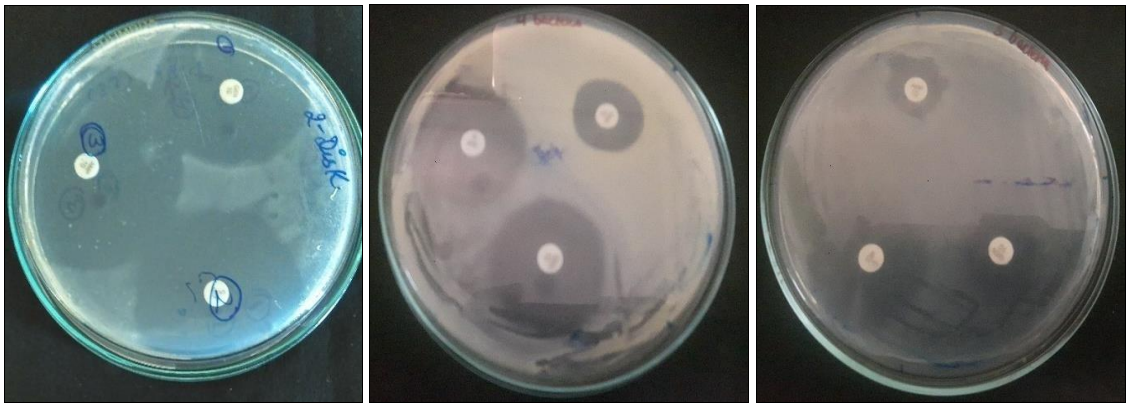

Fig 7 Relationship between pathogenic bacteria and zone of inhibition (cm) of antibiotics

case of *Klebsiella spp*. *Salmonella typhimurium* and *E.coli*, zone of inhibition was maximum with chlorofloxacin namely 5.0cm, 1.3 and 1.1 respectively.

Gentamycin and ampicillin has resulted in almost same zone of inhibition in *Bacillus subtilis* (2.0 and 2.3 cm), in *Klebsiella spp* (4.3cm and 4.4cm) and in *Pseudomonas aeruginosa* (2.7 and 2.8cm) (Table 2, fig 8).

The minimum zone of inhibition of 0.1 cm was produced by ciprofloxacin on *Pseudomonas aeruginosa*. Among all the six bacteria, *Klebsiella spp*. was highly sensitive to all the three antibiotics and *Streptococcus spp* was most sensitive to ampicillin (Table 2,fig 9).

Table 2: Relationship between pathogenic bacteria and zone of inhibition (cm) for different antibiotics

Antibiotics	*Bacillus subtilis*	*Klebsiella spp*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*E.coli*	*Streptococcus spp*
Gentamicin	2	4.3	2.7	1.1	0.6	1.5
Ampicillin	2.3	4.4	2.8	0.7	0.5	4
Chlorofloxacin	1.5	5	0.1	1.3	1.1	1

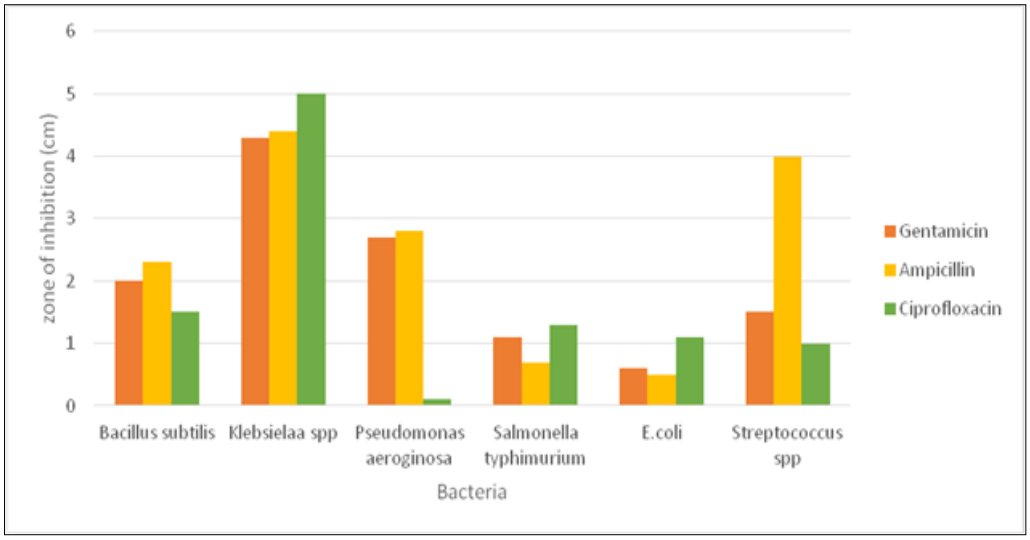

Fig 8: Relationship between zone of inhibition (cm) of bacteria and different antibiotics.

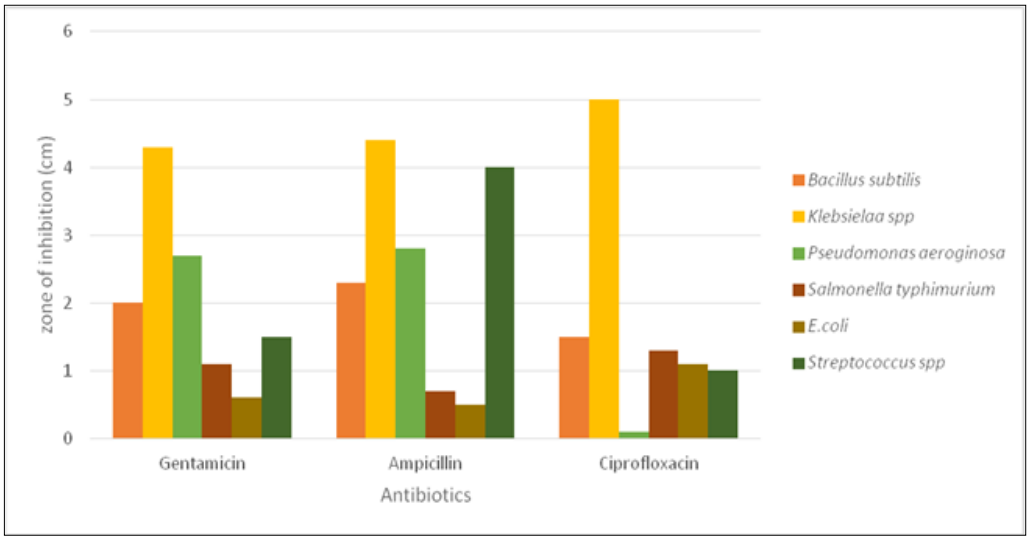

Fig 9: Relationship between antibiotics and zone of inhibition (cm) of different bacteria

Table 3: Relationship between pathogenic bacteria and zone of inhibition (cm) for different concentrations of ZnO NPs + antibiotics.

Concentration	*Bacillus subtilis*			*Klebsiella spp*			*Pseudomonas aeruginosa*			*Salmonella typhimurium*			*E.coli*			*Streptococcus spp*		
	Gentamicin	Ampicillin	Chloroflox acin	Gentamicin	Ampicillin	Chloroflox acin	Gentamicin	Ampicillin	Chloroflox acin	Gentamicin	Ampicillin	Chloroflox acin	Gentamicin	Ampicillin	Chloroflox acin	Gentamicin	Ampicillin	Chloroflox acin
20c	0.5	0.4	0.3	1.1	0.8	1.5	0.3	0.4	0.8	0.9	0.4	0.5	0.3	0.4	0.7	0.6	1	0.8
50c	1.2	1.1	2	1.9	2.0	3.9	2.2	0.7	2.5	2	0.9	1.8	0.8	0.9	1.6	1.4	1.7	1.5
70c	2.2	1.7	2.6	4.1	3	4.7	2.8	1.5	3	2.9	1.5	2.5	1.5	1.2	2.6	2	3.2	2
100c	2.6	2.4	2.9	4.6	4.4	5.2	3.2	2.9	3.4	3.5	2	3	2.5	2	3.2	3	3.8	2.8

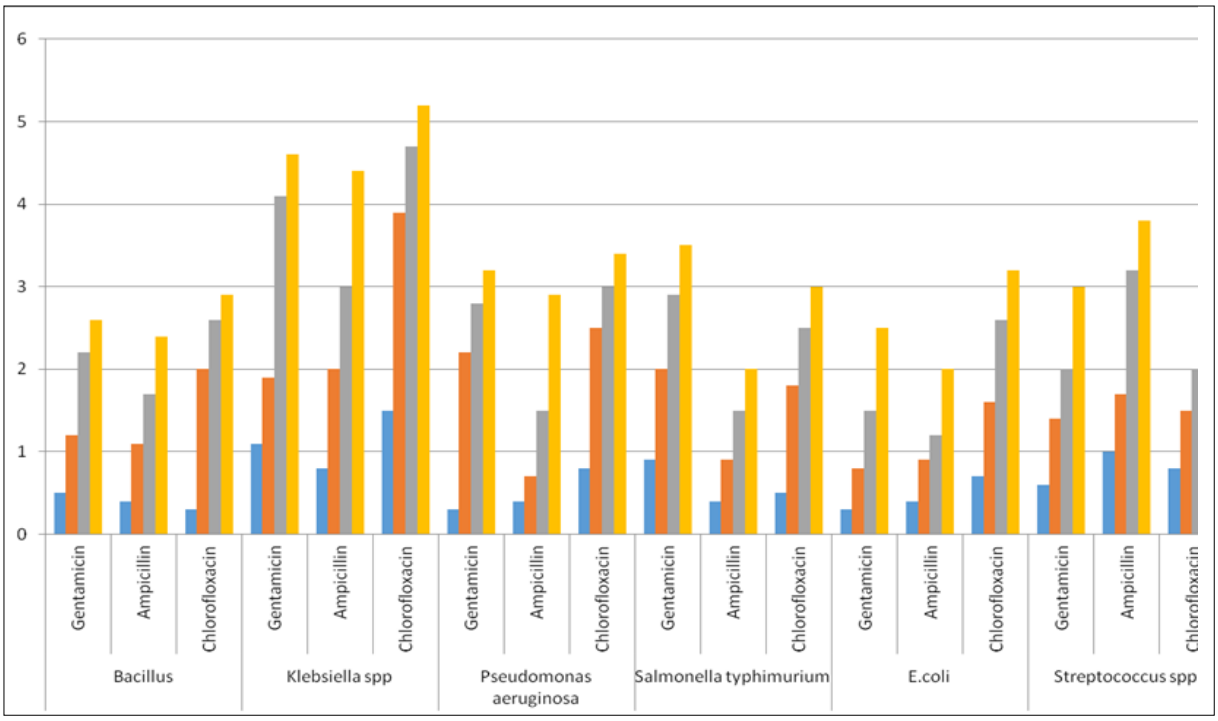

Fig 10: Relationship between zone of inhibition (cm) of bacteria and different concentration of ZnO NPs + antibiotics.

Synergistic effect of ZnO NPs and antibiotics on pathogens

The effect of different concentration of ZnO NPs in combination with the three antibiotics was observed for the six bacteria by disc diffusion method on nutrient agar plate (Fig. 6). The zone of inhibition was found to be more for combination of the antibiotics with the maximum concentration of ZnO NPs than antibiotics alone in all the bacteria. The highest percentage increase in zone of inhibition was exhibited in Pseudomonas aeruginosa with combination of ZnO NPs and chlorofloxacin, followed by combination of ZnO NPs and gentamicin in E.coli, combination of ZnONP and ampicillin in E.coli. All of these showed more than 300% increase in zone of inhibition (Table 3 & fig 10).

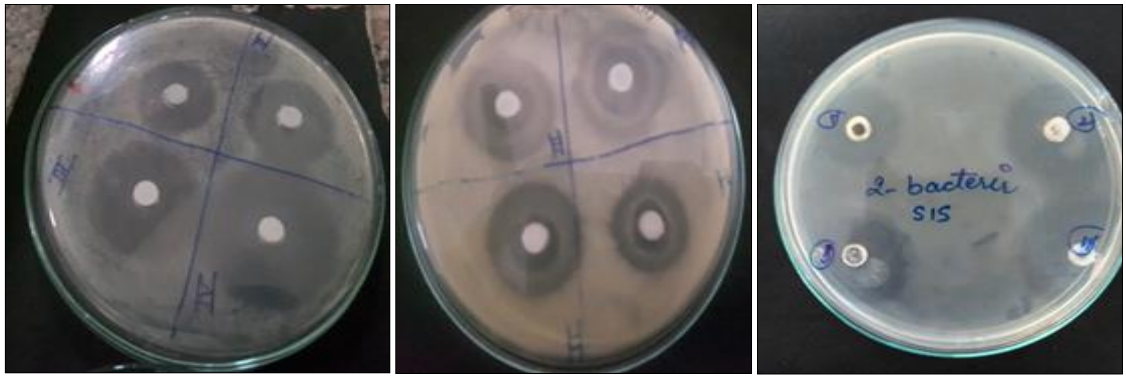

Fig 6: Zone of inhibition of bacteria using antibiotic disc along with ZnO NPs\

Table 4: Comparison of antibacterial activity of ZnONP, antibiotics and synergistic effect of ZnO NPs and antibiotics. (The zone of inhibition is given in cm)

	Bacillus subtilis			***Klebsiella spp***			***Pseudomonas aeruginosa***			***Salmonella typhimurium***			***E.coli***			***Streptococcus spp***		
	Gentamicin	**Ampicillin**	**Chlorofloxacin**	**Gentamicin**	**Ampicillin**	**Chlorofloxacin**	**Gentamicin**	**Ampicillin**	**Chlorofloxacin**	**Gentamicin**	**Ampicillin**	**Chlorofloxacin**	**Gentamicin**	**Ampicillin**	**Chlorofloxacin**	**Gentamicin**	**Ampicillin**	**Chloroloxacin**
ZnONP 100c	1.1	1.1	1.1	3.4	3.4	3.4	1.7	1.7	1.7	1.1	1.1	1.1	0.4	0.4	0.4	1.5	1.5	1.5
Antibiotic	2	2.3	1.5	4.3	4.4	5	2.7	2.8	0.1	1.1	0.7	1.3	0.6	0.5	1.1	1.5	4	1
Antibiotic + ZnONP (100c)	2.6	2.4	2.9	4.6	4.4	5.2	3.2	2.9	3.4	3.5	2	3	2.5	2	3.2	3	3.8	2.8

The antimicrobial activity of gentamycin improved by 7%-316%, ampicillin improved by 0%-300%, chlorofloxacin improved by 4%-3300% (Table 4). Strong synergistic effect of metal nanoparticles on the antimicrobial activities of commercial antibiotics has been reported earlier. Antimicrobial activity of tetracycline improves by 286%–346% and 0%–28% when being tested in the presence of 250 ppm of silver and copper nanoparticles, respectively. For kanamycin, the improvement is 154%–289% for silver and 3%–20% for copper nanoparticles. Irrespective of the antibiotics and tested organisms [18].

Conclusion

Antibiotics from pharmaceutical industries and poultry are released in environment which accumulates and interact with metal nanoparticles, extensively used as biocidal agent in domestic products. Interaction of antibiotics and metal nanoparticles with microorganisms has a potential to alter the ecosystem of the earth. In this work, interaction of Zinc Oxide nanoparticles with three antibiotics i.e. Gentamicin, Chlorofloxacin and Ampicillin and their effect on the six bacteria was studied. Zinc Oxide nanoparticles were synthesized using soluble starch, sodium hydroxide and zinc nitrate. The chemically synthesized zinc oxide nanoparticles were characterized using UV-Vis spectroscopy, FTIR and SEM analytical techniques. The absorption spectrum of ZnO NPs in the range of 390- 420 nm with absorption maxima at 390 nm and the spectrum of interference pattern by FTIR analysis in the wavelength range of 500 - 4000 cm^{-1} showed that the absorption band of ZnO nearer to 1383 cm^{-1}. The antibacterial activity performance of ZnO nanoparticles was done by using disc diffusion method. From the above study it was assumed that the zone of inhibition was observed against different pathogenic bacteria for zinc oxide nanoparticles. In the presence on ZnO NPs antimicrobial activity of gentamicin, ampicillin and chloro floxacin improved.

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