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Research Article

NOVEL ANTIBACTERIAL EFFECTS OF ALUMINA NANOPARTICLES ON *BACILLUS CEREUS* AND *BACILLUS SUBTILIS* IN COMPARISON WITH ANTIBIOTICS

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ABSTRACT

Alumina nanoparticles were synthesized through ball milling method, typically characterized and the antimicrobial activity was analysed against four major pathogenic strains such as *Bacillus cereus MTCC 441, Bacillus subtilis MTCC 430, Klebsiella pneumoniea MTCC 109 and vibrio cholerae.* Upon characterization, the X-Ray Diffraction pattern defines the size distribution of the particle corresponding to the characteriestic peaks obtained as 66.5 nm, 50.15 nm, 50.94 nm, 51.96 nm, 34.9 nm, 35.93 nm, 54.41 nm, 36.91 nm, 56.62 nm, 39.55 nm and 39.95 nm, respectively. The formation of spherical shaped nanoparticles was confirmed by Scanning Electron Microscopy. Also, the FTIR spectrum indicates the presence of significant functional groups such as O-H stretching, N-H amine group, a strong N-O nitro group, an alkene =C-H bending and a strong alkyl halide C-I stretch, respectively. In this investigation, alumina showed a characteristic inhbition zone of 12 mm diameter against *Bacillus subtilis* and 23 mm diameter against *Bacillus cereus* for 100 µg MIC (Minimum Inhibitory Concentration) of nanoparticles where as, no inhibitory effect was observed against Klebsiella pneumoniea and Vibrio cholerae. Also, the antimicrobial effects of alumina nanoparticles were compared with reference antibiotics like amoxicillin, chloremphenicol, erythromycin and rifamycin for MIC value of 100 µg.

Keywords: Alumina nanoparticles, Antibiotics, Antimicrobial activity, Pathogenic strains

INTRODUCTION

Aluminium oxide is an electrical insulator posing relatively high thermal conductivity (30 $Wm^{-1}K^{-1}$) l. The most commonly available form was crystalline in nature, namely corundum or α-aluminium oxide. The hardness of the material makes it suitable for using as an abrasive. Aluminium oxide offers resistance to metallic aluminium, when exposed to weathering.

When metallic aluminum comes in contact with atmospheric oxygen, it becomes very reactive. A thin layer of alumina (4 nm thickness) forms in about 100 picoseconds on any exposed aluminium surface, which acts a protective covering for further oxidation.

It was well known that metal oxide nanoparticles exhibit antimicrobial properties. But, free radical scavenging property of the alumina particles may cause lowered antimicrobial activity and as a result even at higher concentration, the particles exhibit mild toxicity toward micro organisms[1]. The mechanism of inducing the oxidative stress makes the propensity for the nanoparticles to cross the cell barriers, enter the cells and interact with the sub cellular structures [2].

The influence of four different preparation methods of C_{60} fullerene (stirred C_{60}, THF-C_{60}, toluene-C_{60}, and PVP-C_{60}) on Bacillus subtilis exhibited relatively strong antibacterial activity ranging from 0.09 ± 0.01 mg/L- 0.7 ± 0.3 mg/L [3]. Smaller aggregates offered greater antibacterial activity, and the increase in toxicity was disproportionately higher than the associated increase in the surface area.

ROS-independent oxidative stress created by (nC_{60}) fullerene favours changes in cell membrane potential accompanied by protein oxidation and thereby interfere with cellular respiration[4] which was different from the previously reported nanomaterial based antibacterial mechanisms that involves ROS generation (metal oxides)[5].

Ions like Ag^{+}, Cu^{2+}, Fe^{3+}, Zn^{2+} and Nb^{5+} dispersed in different matrices such as zeolites, apatite, phosphates, clays, titanium oxides and glasses have been employed in many applications[6]. Also, micro patterns fabricated with antimicrobial enzymes and alumina nanoparticles offering antimicrobial activity and abrasions resistant property were reported [7]. With respect to alumina nanoparticles, surface characteristics of the particles play a significant role in phytotoxicity[8]. The volume of surface that comes in contact with microorganism is directly proportional to the amount of antimicrobial activity exhibited by the nanoparticle.

In this study we investigated, the antimicrobial properties of alumina nanoparticles synthesized by ball milling method using toluene as a solvent, rendered maximum inhibitory effects against four major pathogenic strains *Bacillus cereus, Bacillus subtilis, Vibrio cholerae and Klebsiella pneumoniae.*

MATERIALS AND METHODS

Synthesis and characterization of alumina nanoparticles

Ball milling method

The zirconium balls were washed thoroughly using acetone and water. The washed balls were dried completely. Materials and balls were taken in the ratio of 1:10 (w/w). About 10 ml of Toluene (solvent) was added to the mixture. The mixture was set in a vial and ball milling was started fixing the speed at 200 rpm. Time was set for two hours and was continued for 8 hours and at every interval of 2 hours toluene was added. After 8 hours the balls and materials were separated. The material was subjected to drying in the oven at 60 ^{0} C overnight. After drying, the material was placed in the furnace to sinter the material at 400^{o}C.

The synthesized alumina nanoparticles were characterized by XRD analysis for predicting size and structure, SEM analysis to determine the shape and FTIR analysis for finding specific groups.

Antimicrobial Test

Test organisms used for the analysis

Clinical isolates of *Bacillus cereus, Bacillus subtilis, Klebsiella pneumoniea, Vibrio cholerae* were collected from KMCH, Coimbatore (India). Each test strain was inoculated in Mueller Hinton liquid medium (broth) and incubated in a temperature controlled shaker (120 rpm) at 30°C overnight. Antibiotics (amoxicillin, chloremphenicol, erythromycin and rifamycin) used for the analysis were purchased from Sigma Aldrich, Bangalore, India and Merck Limited, Mumbai, India.

Well Diffusion assay

16 petriplates containing Nutrient agar were prepared. 24 hours growing culture (*Bacillus subtilis MTCC 441, Bacillus cereus MTCC*

430, Vibrio cholerae and *Klebsiella pneumoniae MTCC 109*) were swabbed on it. Four plates for each organism were inoculated. Four wells (10mm diameter) were made by using cork borer. First well was loaded with 100µl of distilled water. Second well was loaded with 100µl of 1mg/ml concentration of alumina nanoparticles. Third well was loaded with 100µl of 1mg/ml of reference antibiotics (Amoxicillin, Chloremphenicol, Erythromycin and Rifamycin). The fourth well was loaded with 100µl final volume with equal amounts of alumina nanoparticle (0.5mg/ml) and antibiotic (0.5mg/ml) altogether. The plates were then incubated for 24 hours at 37 º C. The zone of inhibition (in diameter) was measured.

RESULTS

X-Ray diffraction analysis

The scintillation detector present in the instrument moves through the required angle at specific counts and scans the sample with a start angle at 10^0 and a stop angle at 70^0.The output was obtained in the form of a graph with 2θ on x- axis and intensity on y-axis. The obtained graph containing different peaks corresponding to different planes of the crystal was compared with the standard data in JCPDS tool. From the result obtained, the average size of the nanoparticle was calculated using Scherer's formula,

$D = 0.9\lambda / \beta \cos\theta$

Where,

λ is wavelength of copper Kα line (1.5406 Å),θ is diffraction angle, β is full width at half maximum of peak (FWHM), and D is the average particle size. Depending upon the characteristic peaks obtained in Fig.1, the X-RD pattern defines the distribution of the size of the particle as 66.5 nm, 50.15 nm, 50.94 nm, 51.96 nm, 34.9 nm, 35.93 nm, 54.41 nm, 36.91 nm, 56.62 nm, 39.55 nm and 39.95 nm, respectively.

Scanning Electron Microscopy Analysis

The scanning electron microscopy results clearly indicate the formation of spherical alumina nanoparticles with its size ranging between 100 nm to 200 nm is shown in the Fig.2.

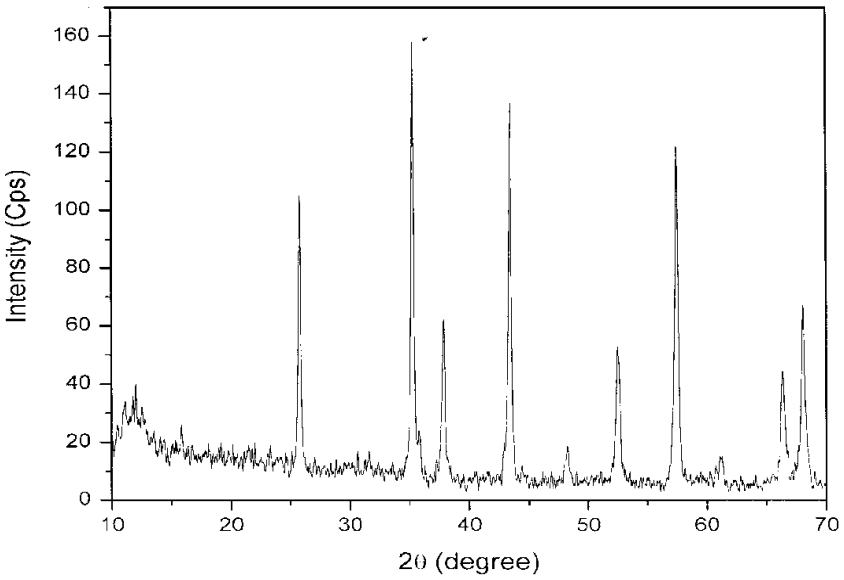

Fig.1: X-Ray Diffraction (X-RD)analysis pattern for alumina nanoparticles

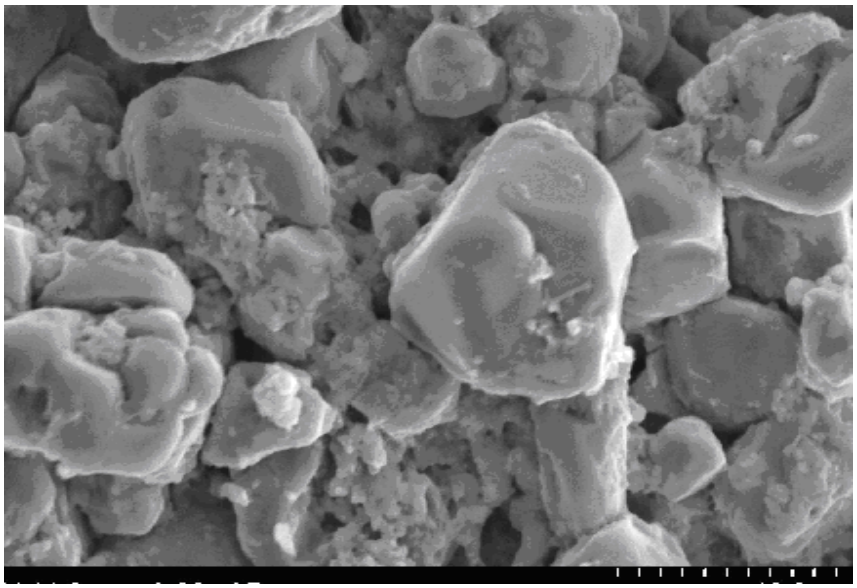

Fig. 2: Scanning Electron Microscopy (SEM) pattern depicting spherical shaped alumina nanoparticles

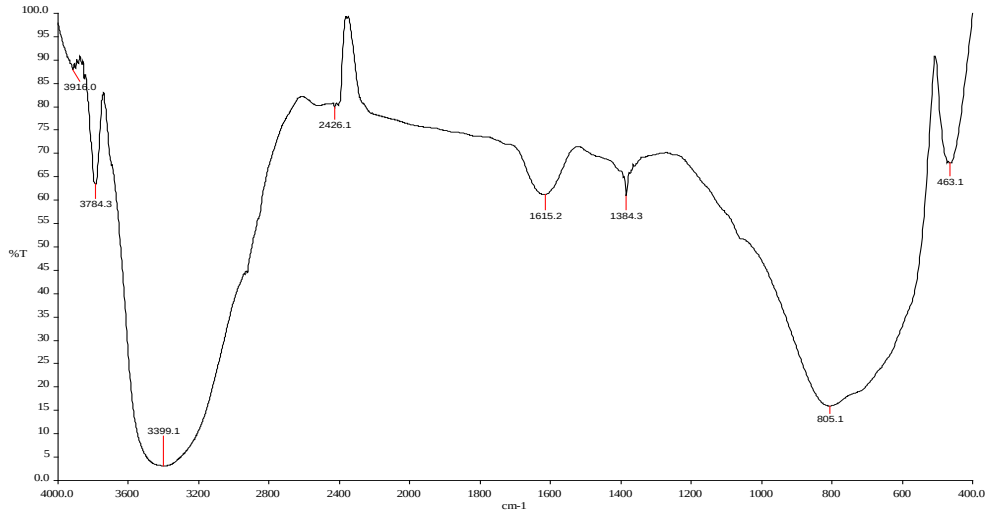

Fig. 3: Fourier Transform Infra-red Spectroscopy (FTIR) analysis of alumina nanoparticles showing the presence of characteristic functional groups

Fourier Transform Infra Red Spectroscopy Analysis

The FTIR spectrum of Al_2O_3 nanoparticles is shown in Fig.3. The higher energy region peaks at 3784 cm^{-1} and 2426cm^{-1} are assigned to O-H stretching of Al_2O_3 nanoparticles. Also a stretch of N-H amine group was observed at 3399 cm^{-1} and 1615 cm^{-1} respectively. A strong stretch of N-O nitro group was observed at 1384 cm^{-1}. An alkene =C-H bending was observed at 805 cm^{-1}. Also a strong alkyl halide C-I stretch was observed at 463cm^{-1} .Upon verification with the present database, the spectrum matches with that of Al_2O_3.

Antimicrobial Activity

The antibacterial activity of alumina nanoparticles were tested against four major pathogenic strains *Bacillus cereus MTCC 441, Bacillus subtilis MTCC 430, Klebsiella pneumoniea MTCC 109 and vibrio cholerae* and the zone of inhibition produced was illustrated in the Table.1, given below. Also the antimicrobial activities of nanoparticles were compared with the antimicrobial activity of antibiotics for the minimum inhibitory concentration value of 100µg. The combined effects of nanoparticles and antibiotics were also analysed by well diffusion method.

Alumina showed a characteristic inhibition zone of 23 mm diameter against *Bacillus cereus* (Fig.4) and 12 mm diameter against *Bacillus subtilis* (Fig.5) for 100 µg MIC (Minimum Inhibitory Concentration) of nanoparticles. However, Alumina nanoparticles showed a mild growth-inhibitory effect on *E. coli*, only at very high concentration of 1000 µg [1].This focuses the sensitivity of the nanoparticles towards different organisms and strains. Though the alumina nanoparticles exhibited a pronounced antibacterial effect over *Bacillus subtilis and Bacillus cereus*, no inhibitory effect was observed against *Klebsiella pneumoniea and Vibrio cholerae*. It was inferred that both nanoparticles and bulk counterparts (metal particle more than nanosize) were seems to be toxic in inhibiting the growth of microbes, especially as stated in the case of nematodes. It was noticed from the literature that the toxicity of the nanoparticle and the oxide solubility influences the toxicity of alumina nanoparticles[9].

Also, the antimicrobial effects of alumina nanoparticles were compared with reference antibiotics like amoxicillin, chloremphenicol, erythromycin and rifamycin for MIC value of 100 µg. Against *Bacillus subtilis*, all the four broad spectrum antibiotics showed typical zone of inhibition whose diameter was double the time when compared with alumina nanoparticles (Fig.5). With respect to *Bacillus cereus*, antibiotics expressed sensitivity nearly equal to that of alumina nanoparticles. Inspite of which no effect was observed for alumina nanoparticles against *Klebsiella pneumoniea and Vibrio Cholerae*.

No characteristic enhancement in inhibitory zone area was observed for the assay, when alumina nanoparticles and antibiotics are given together. Similar type of analysis was performed with silver nanoparticles combined with and without antibiotics. This analysis inferred a characteristic increase in the fold area over the inhibitory zone when silver nanoparticles and antibiotics are given together against test organisms like *Staphylococcus aureus and Escherichia coli*[10].

Table 1: Zone of inhibition produced by alumina nanoparticles, reference antibiotics and nanoparticle with antibiotics

Organisms	Antibiotics used	Control ± 2mm (diameter)	Alumina NP (100 µg) ±2 mm (diameter)	Antibiotics (100 µg) ± 2mm (diameter)	Alumina NP + Antibiotics (100 µg) ± 2mm (diameter)
Bacillus subtilis	Amoxicillin	-	12	21	22
	Chloramphenicol	-	12	26	26
	Erythromycin	-	11	18	19
	Rifamycin	-	-	21	23
Bacillus cereus	Amoxicillin	-	24	38	40
	Chloramphenicol	-	20	33	33
	Erythromycin	-	23	35	35
	Rifamycin	-	23	40	41
Vibrio cholerae	Amoxicillin	-	-	28	28
	Chloramphenicol	-	-	26	27
	Erythromycin	-	-	-	-
	Rifamycin	-	-	20	20
Klebsiella pneumoniae	Amoxicillin	-	-	18	18
	Chloramphenicol	-	-	22	24
	Erythromycin	-	-	13	13
	Rifamycin	-	-	23	22

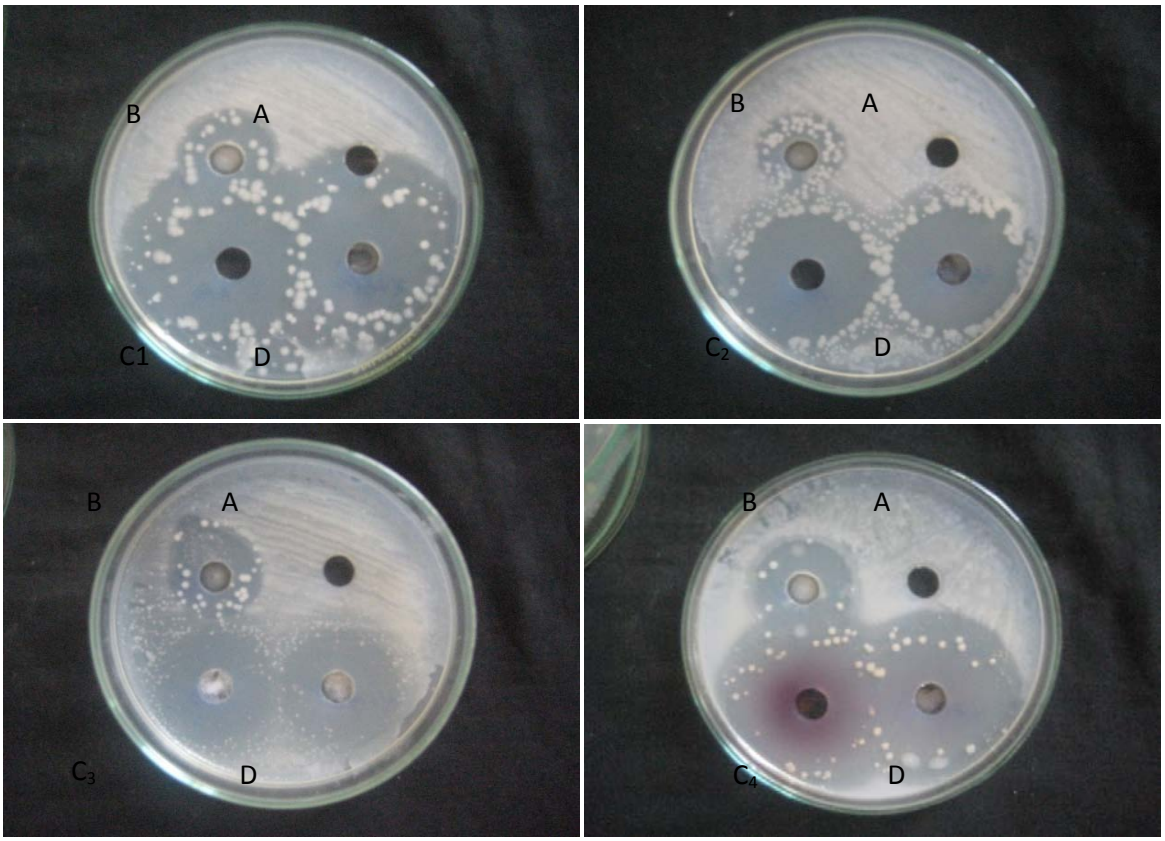

Fig. 4: zone of inhibition formed against *Bacillus cereus*

A - Control; B - Nanoparticle; C_1 - Amoxicillin; C_2- Chloremphenicol; C_3- Erythromycin; C4 – Rifamycin; D - Nanoparticles + Antibiotics

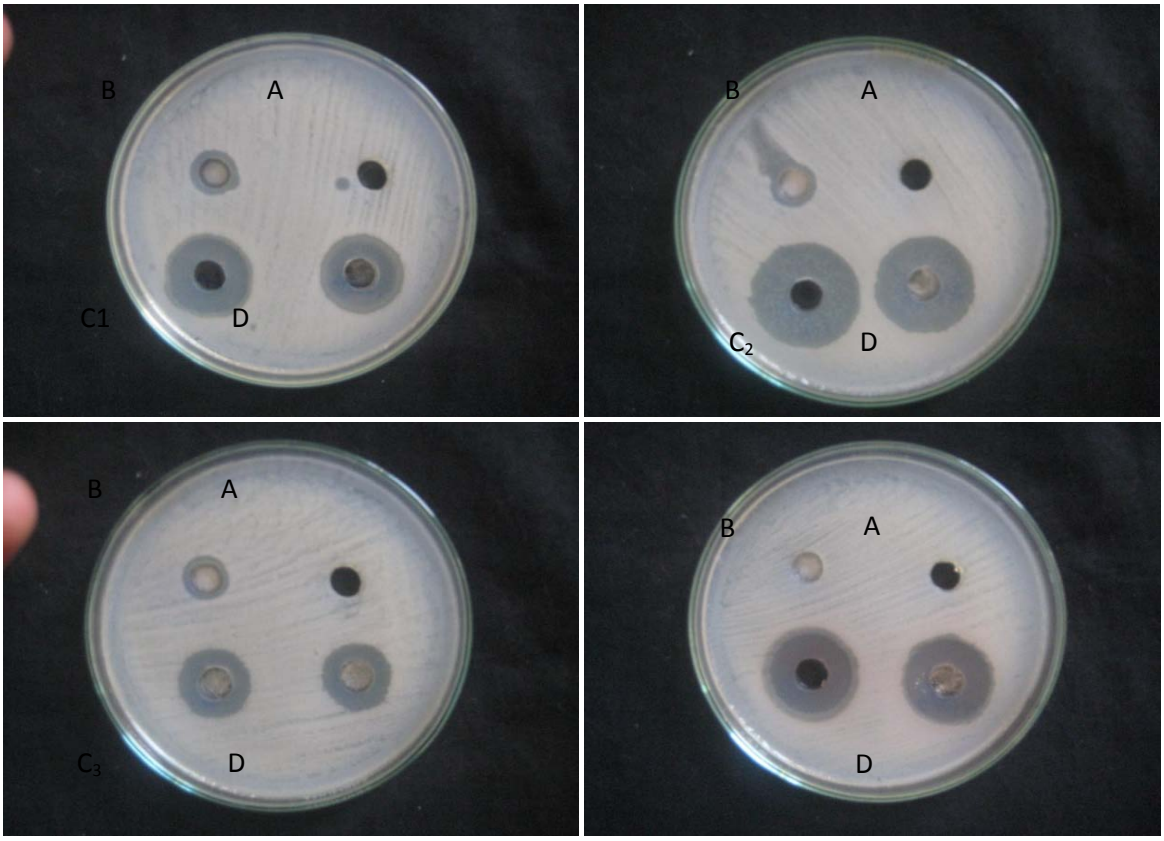

Fig. 5: zone of inhibition formed against *Bacillus subtilis*

A - Control; B - Nanoparticle; C_1 - Amoxicillin; C_2- Chloremphenicol; C_3- Erythromycin; C_4 – Rifamycin; D - Nanoparticles + Antibiotics

DISCUSSION

From the above experimental analysis, it was concluded that, the alumina nanoparticles synthesized through ball milling method conferred uniform size distribution ranging between 50 nm to 60 nm, which was evident from the X-RD results. Besides, the spherical shaped alumina nanoparticles analysed through scanning electron microscopy (SEM) inculcates the significance of the surface morphology of the nanoparticle participating in the cellular interaction i.e, the number of active sites coming in contact with the cells which render cytotoxic effect[8]. The presence of high energy bands and stretches strenghthening the formation of nanoparticles was confirmed by FTIR analysis. With respect to the context of antimicrobial activity, alumina nanoparticles produced 13 mm and 23mm diameter of inhibition zone (Table 1) against *Bacillus cereus and Bacillus subtilis*, respectively. This infers the sensitivity of the microbial species towards the nanoparticles for 100µg MIC (Minimum Inhibitory Concentration). But, no inhibitory effect was found with *Vibrio cholerae* and *Klebsiella pneumoniea.*

Not only the antimicrobial property of alumina nanoparticles were analysed, but also the inhibitory effects of reference antibiotics were analysed. The formation inhibition zone infers the antibacterial properties of the particles used. Similar types of activity were reported on antibacterial effects of *Withania somnifera L. Euphorbia hirta L. and Trianthema decandra L.*[11, 12]. Antibiotics offered typical antimicrobial effect for all the four pathogens (Table 1).Further, any enhancement in zone area was tested for the combination of nanoparticles and antibiotics. No enhancement in inhibition zone was observed.

CONCLUSION

Based on these results obtained, three major points were predicted as follows,

1. Nanoparticles, metals and metal oxides are not showing the same sensitivity to all the microbial species and strains (Table 1).

2. The solubility of metal oxides influences the toxicity of nanoparticles. The concentration of the particle plays another significant role in the determination of antimicrobial activity.

3. The volume (or) the surface area of the nanoparticles that comes in contact with cells is directly proportional to the amount of antimicrobial activity offered by the particle.

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