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Combined effect of zinc oxide nano particle incorporated chitosan for better antimicrobial activity towards wound healing

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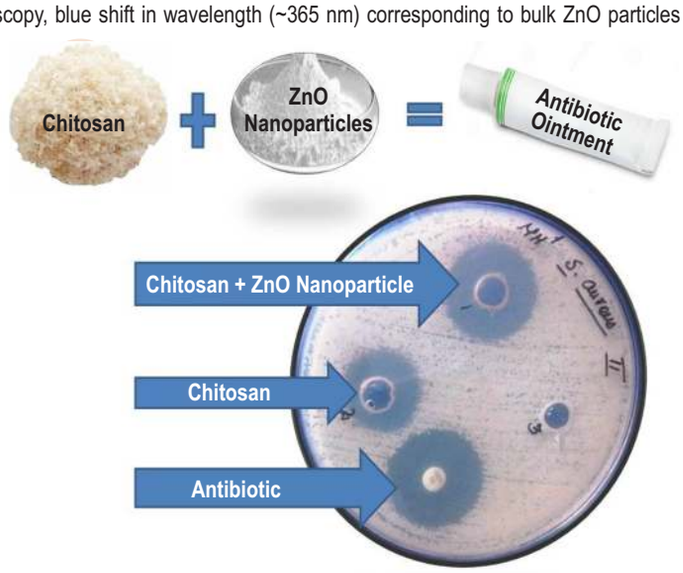
Abstract

Aim : The aim of the present study was to characterize the zinc oxide nano particle incorporated Chitosan (ZnO-NP-CS) and its antimicrobial activity.

Methodology : Zinc oxide nanoparticles (ZnO-NP) were prepared by sol-gel method and Minimum Inhibitory Concentration (MIC), Minimum bactericidal Concentration (MBC) and agar well diffusion method was used for the assessment of antibacterial activity of ZnO-NP and ZnO-NP-CS as well.

Results : In UV–spectroscopy, blue shift in wavelength (~365 nm) corresponding to bulk ZnO particles (~385 nm) indicates the nano size. In SEM image, ZnO-NP appeared as nano flake shape and ZnO-NP treated Methicillin resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* (PA) bacteria illustrates leakage of intracellular content, fusion and shrinkage of bacteria, respectively. The MIC of ZnO-NP for most of food pathogens were between 0.01 to 0.1mg. Lower MIC was observed for *Vibrio cholerae* and *Listeria monocytogenes;* higher MIC was observed for *Bacillus cereus* and *Pseudomonas aeruginosa.* In antibiogram assay, the zone of inhibition of ZnO-NP-CS was equal to commercial antibiotics against Multiple Drug Resistant bacteria.



Interpretation : The combined effect of ZnO-NP and chitosan is better than the individual component, *i.e.,* around 5–15 mm wider zone of inhibition than chitosan. ZnO-NP-CS can be a suitable alternative for the treatment of wound infected by multiple drug resistant bacteria.

Key words: Antibiogram, Chitosan, MRSA, Zinc oxide nanoparticles

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Introduction

Chitosan is a glucosamine polymer commercially obtained by deacetylation of chitin from exoskeletons of crustaceans and a variety of insects, worms, fungi and mushrooms (Muzzarelli *et al.,* 2012). The process of deacetylation (DA) involves removal of acetyl groups from molecular chain of chitin. Activity of chitosan is mainly dependent upon the degree of deacetylation (Baxter *et al.,* 1992). Hence, higher the degree of deacetylation leads to higher antimicrobial activity. Commercially chitosan is available with > 85% deacetylated units (Goy *et al.,* 2009). Attraction of positively charged chitosan molecules with negatively charged microbial cell membranes leads to disturbance of internal osmotic balances and increased membrane permeability leading to cell death. Even though chitosan possess a broad spectrum of activity; several *in vitro* experiments indicate that Gram-negative bacteria appear to be more sensitive to chitosan (Goy *et al.,* 2009). Coma *et al.* (2002) reported that chitosan has higher bacteriostatic effect than bactericidal effect. Commercially, chitosan is being consumed as a drug to reduce the body weight *i.e.,* it is considered as an anti-fat products (Cherniack *et al.,* 2008). Owing to rapid haemostatic active and better healing ability, it is widely used in bandages (Zhang *et al.,* 2010). It is highly efficient to treat defects of joint in bones (Kumar *et al.,* 2017).

Usually Zinc salt are prescribed by medical practitioners for zinc deficient person (Saldamli *et al.,* 1996). Zinc gluconate is famous form of zinc recommended as a supplement for the zinc deficiency. In Ayurveda, metal-based preparations, that is, bhasmas, are indicated for the treatment of several diseases. Standard textbooks of Ayurveda recommend Jasada bhasma (zinc based bhasma) as the treatment of choice for diabetes (Umrani *et al.,* 2014). Reports states that zinc supplement has beneficial effect on type 2 diabetes by elevating the serum level resulting in better glycemic control. Zinc oxide nano particles (ZnO-NP) also encompass broad spectrum of antibacterial activity similar to chitosan. ZnO-NPs are much more active than its macro size particles (bulk ZnO) due to higher surface availability (Sirelkhatim *et al.,* 2015). The properties of ZnO-NP are strongly dependent on structure, morphology, size, orientation and density of crystal. Hence, development of a controllable synthesis of ZnO-NP with specific morphology is important to explore their potential applications and exploitation. Until now, a variety of ZnO nanostructures such as nano-wires, nano-rods, nano-tubes, nano-belts, nano-flakes and nano-flowers have been synthesized using various techniques such as sol–gel, anodization, sono-chemical, chemical bath deposition, hydrothermal synthesis, gas phase process method and chemical vapor deposition method (Xiong *et al.,* 2014).

Numerous researches have been carried about individual antimicrobial activity of chitosan and ZnO-NP; but its combined effect has never been studied in such detail. In the present study, zinc oxide Nanoparticle incorporated chitosan (ZnO-NP-CS) was characterized in detail and its antimicrobial activity was estimated for further applications.

Materials and Methods

Preparation of ZnO-NP: ZnO-NPs were prepared as per Sangeetha *et al.* (2011) with minor modification. One molar zinc nitrate was added drop wise into 1M NaOH solution with constant stirring at 70°C in equal quantity and kept for 16 -24 hrs undisturbed for settlement. Further, the settlement which contained the nanoparticles was centrifuged at 5000 rpm for 5 min and ZnO-NP was washed repeatedly and kept in vacuum oven overnight at 70°C. This dried ZnO-NP was further used for characterization studies.

Chitosan: Chitosan (#GRM 9385, Hi-Media, Mumbai) from shrimp shell with ≥ 75 deacetylation degree was used in the present study. One percent chitosan was prepared by dissolving in 1% glacial acetic acid for further biochemical and microbiological analysis.

UV-Visible spectroscopy: ZnO-NP were analyzed using ultraviolet visible (UV-Vis) spectroscopy in the range of 250–700 nm at room temperature in a quartz cuvette having a path length of 1 cm. Similarly, chitosan and ZnO-NP-CS were analyzed in the range of 200–700nm wave length.

Scanning Electron Microscopy: 16 mM ZnO-NP was directly added into 1ml of freshly grown MRSA culture and allowed to react for 20 min. The culture was then centrifuged three times at 5000 rpm for 5 min and pellet was washed with PBS. A 2.5% of glutaraldehyde in phosphate buffer saline (PBS) was added over the pellets and allowed to react for overnight; then washed with PBS. Finally, to remove water the entire pellet was washed with ascending grade of ethanol, *i.e.,* 20, 40, 60, 80 and 100% and examined in SEM at 2000X to 20000X.

Antibiogram: Antibiogram was carried as per CLSI (2012, 2014) standard agar well diffusion technique using Muller Hinton Agar. Test bacterial culture was inoculated into Brain Heart Infusion Broth and incubated at 37°C for 2–4 hr. The grown cultures were adjusted with sterile normal saline solution until 0.5 McFarland standard turbidity appeared. Sterile cotton swab was immersed into test culture and spread over preset MHA plates, 5–6 mm diameter well was formed using cork borer (#LA 373, Himedia) and the bottom of agar was sealed with sterile molten agar to avoid leakage. The prepared chitosan (100 µl containing 1mg ZnO-NP in 1mg chitosan) was added to the wells and the plates were incubated at 37°C. After 24hr, halo zone was measured using standard antibiotic zone scale (PW 096, Hi Media).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): MIC for ZnO-NP was carried out using sterile 96 well micro titre plates as per EUCAST (2003). A 100µl of Muller Hinton Broth was added to the wells followed by 100 µl mM of ZnO-NP in the first well and mixed firmly. This was followed by serial dilutions (2 fold dilution) upto 11th well. Finally, 10 µl containing test culture of 3 - 7x10^4 CFU was added to all wells in a row, *i.e.,* final concentration of 5x10^5 CFU m l^{-1}. After 10-16 hr, the

highest dilution showing no visible growth in 96 well plates was considered as MIC. Similarly, the highest dilution showing no bacterial growth was considered as MBC.

Results and Discussion

Nanoparticles can destroy bacteria by disturbing the membranes potential and integrity and also by production of reactive oxygen species (ROS). Similarly, chitosan also damage microbial cell membranes by disturbing the internal osmotic balance and by increasing membrane permeability. Since ZnO-NP and chitosan destroy the bacteria by mechanical damage, the possibility of bacteria to develop resistance against chitosan and ZnO-NP is restricted (Beyth *et al.,* 2015). Complete physico-chemical characterization of chitosan and ZnO-NP is not possible without using spectroscopic techniques (Kumirska *et al.,* 2010). In this study, the λ_{max} value of ZnO-NP was around 365 nm (Fig. 1). Similarly, the λ_{max} value for bulk ZnO particles was around 385 nm (Fig.1) and λ_{max} for chitosan and ZnO-NP-CS was around 228 nm and 233 nm (Fig. 1), respectively. A clear indication of blue shift due to smaller size of nanoparticles was observed. Gupta *et al.* (2015) and Dobrucka and Dugaszewska (2016) reported that absorption edge systematically shifted to lower wavelength or higher energy with the decreasing size of nanoparticles. Koch *et al.* (1985) also reported that blue shift in ZnO nanostructures, comparing with bulk ZnO (380 nm) was due to size quantization effect. Similarly, Nawaz *et al.* (2011) prepared nano and bulk ZnO particles which exhibited λ_{max} value around 370 and 388 nm, respectively.

As per the studies of Kumirska *et al.* (2010) and Pedroni *et al.* (2003), UV spectra of mixtures of N-acetyl-glucosamine and glucosamine hydrochloride were quite similar to the spectra of chitosan, and the λ_{max} value was 201 nm in 0.1 M HCl solution. In

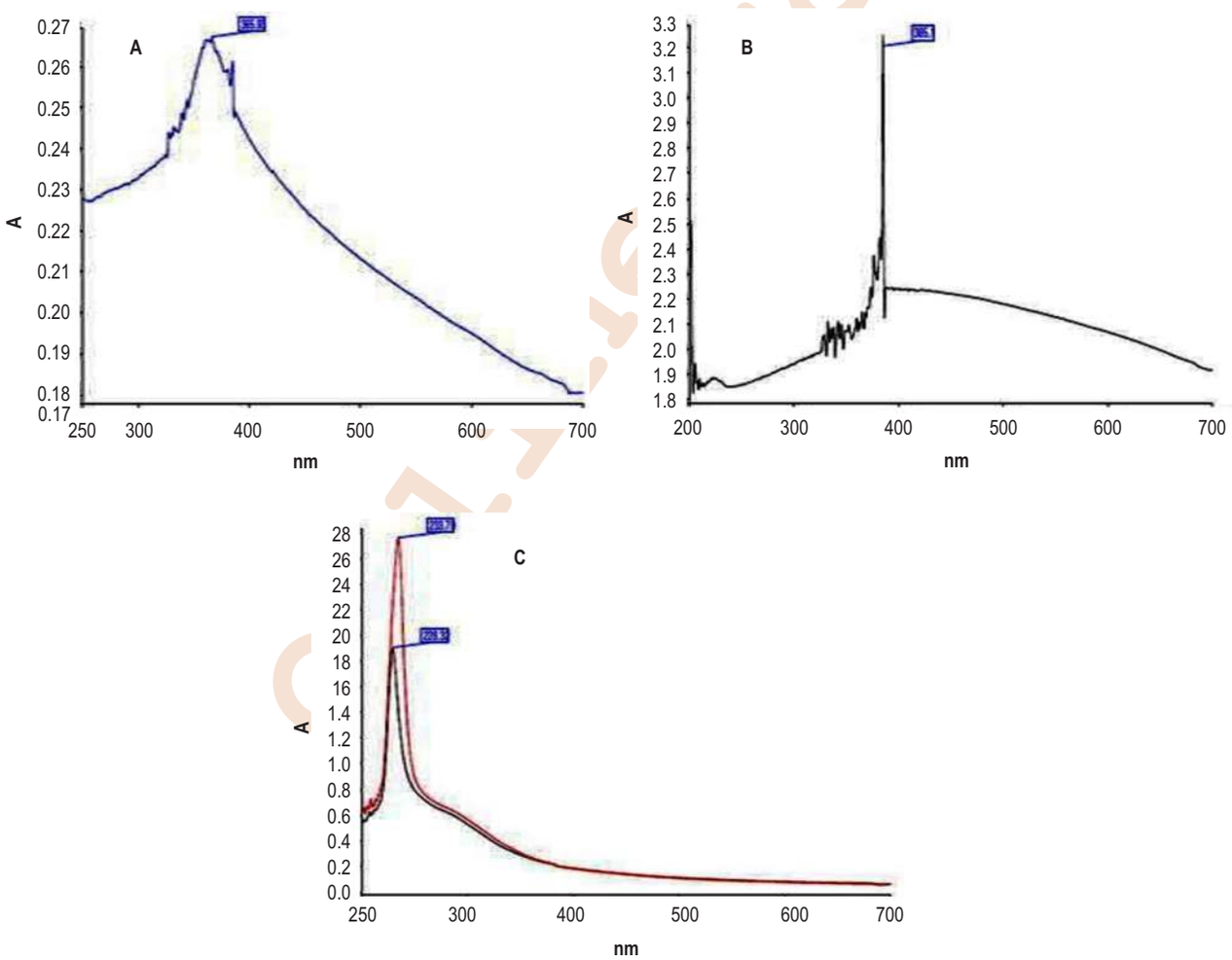

Fig. 1: (A) UV absorption spectra for zinc oxide nano particles; (B) UV absorption spectra for zinc oxide bulk particles; (C) UV absorption spectra for Chitosan and ZnO-NP-CS.

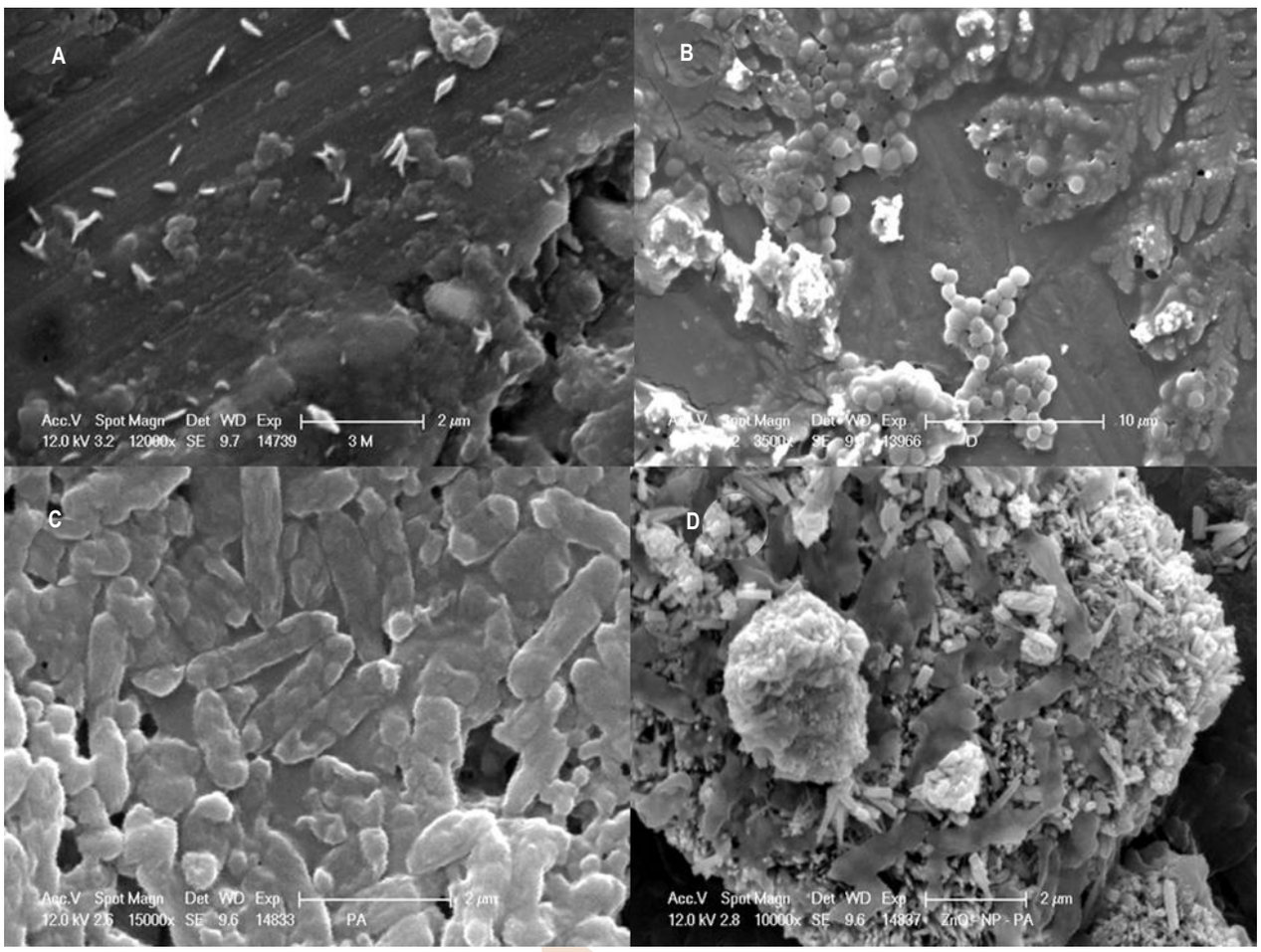

Fig. 2: SEM Images of (A) ZnO-NP; (B) ZnO-NP treated *S. aureus;* (C) Untreated *Pseudomonas aeruginosa* and (D) ZnO-NP treated *Pseudomonas aeruginosa.*

the present study, chitosan was diluted in the acetic acid which might be the reason for λ_{max} being 228 nm (Fig. 1). Previous studies clearly mention that the structure of nanoparticles (NP), including its morphology, size, orientation and density of the crystal plays a vital role in biological activity (Shinde *et al.,* 2010). Controllable synthesis of ZnO nanomaterials having specific morphology is important to explore their potential application as smart and functional materials. In SEM analysis, it was found that ZnO-NP was nanoflake (NF) shaped with an average width of 50 - 70 nm and length of 100-200 nm (Fig. 2). Based on the morphology, it is assumed that the flake size morphology with sharp edges has the ability to penetrate the bacterial surface, ultimately damaging the bacterial cell walls.

Several studies have been conducted to prepare ZnO-NP of varying dimensions. Vabbina *et al.* (2015) prepared ZnO-Nanoflakes of 20 nm thickness and average lateral dimensions of 5µm x 5µm. Lee *et al.* (2002) synthesized ZnO nano-wire with average length of 13 mm and typical diameter of 50 nm. Kashif *et al.* (2012) prepared ZnO nanoflakes of 80–100 nm and a diameter of 300–500 nm. Kaneti *et al.* (2013) prepared ZnO Nanoflakes with the edge lengths 200 to 400 nm and thickness ranging from 20 to 35 nm. MRSA is an emerging multidrug resistant bacteria responsible for most skin infection, frequently isolated from the hospitals, humans, animal and fish (Visnuvinayagam *et al.,* 2015; Sivaraman *et al.,* 2017). SEM images of ZnO-NP treated MRSA revealed damage in MRSA membrane along with fusion and shrinkage of cocci (Fig. 2). Similarly, an intra-cellular leakage of the content caused flattening of *P. aeruginosa* (Fig. 2). Scanning electron microscopic studies revealed that the ZnO-NPs were not able to destroy the membrane of *P. aeruginosa,* it may be the reason for the resistance of *P. aeruginosa* against ZnO-NP, thus leading to higher MIC and MBC values (Table 1). So, based on the SEM analysis, the susceptibility of bacteria against ZnO-NP was assessed. Xie *et al.* (2011) analyzed the antimicrobial property of ZnO-NP with average size of 30 nm against *Campylobacter jejuni*

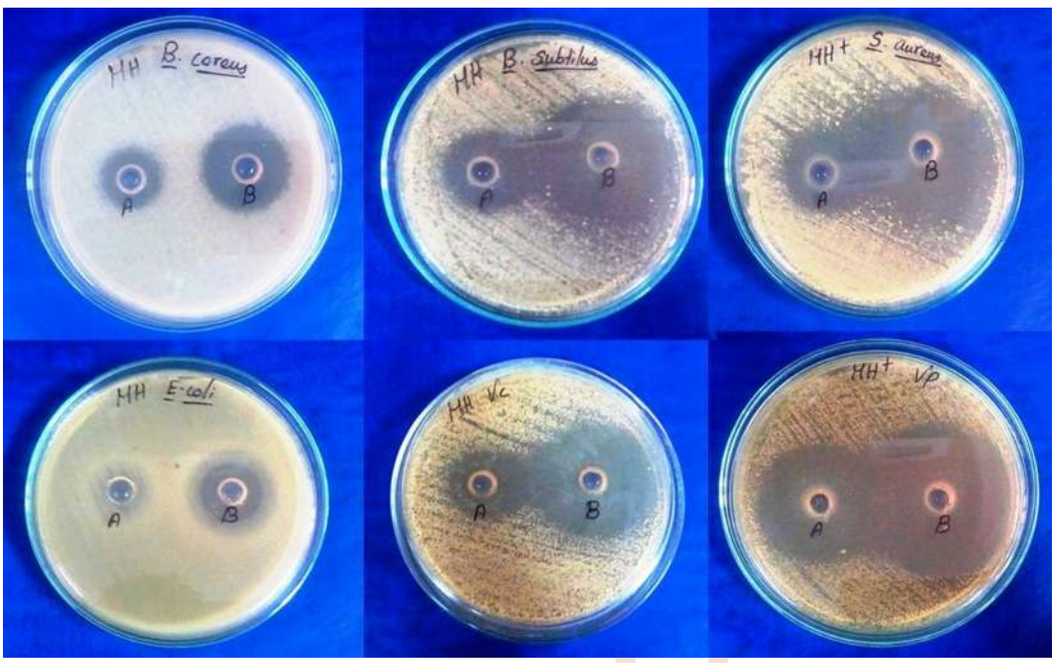

Fig. 3: Antibiogram against food borne pathogens (A) Chitosan and (B) ZnO-NP-CS.

and found that ZnO-NP was able to destroy *C. jejuni* at 0.05 mg ml^{-1}, *i.e.,* MIC value was 0.05 mg ml^{-1}. Jones *et al.* (2008) reported an MIC of 1 mM for ZnO-NP against *Staphylococcus aureus.* But, in the present study MIC for *S. aureus* was 0.03 mg 100 μl^{-1}, which may due to size variation of ZnO-NP. Similarly, Padmavathy and Vijayaraghavan (2008) prepared different size of nanoparticles and proved that nanoparticle sizes were inversely related to the antibacterial activity in terms of both MIC and MBC. In this study, antibiogram was carried out for chitosan alone and compared with nanoparticle incorporated chitosan too. At constant pH of 3.5, all food borne pathogenic organisms exhibited a zone of inhibition in the range of 5-15 mm, with higher zone size in ZnO-NP-CS compared to chitosan alone (Table 1) (Fig. 3). It clearly indicated that antibacterial activity of chitosan was drastically increased

Fig. 4: (A) Antibiogram against MRSA 1. ZnO-NP-CS, 2. Chitosan, 3. ZnO-NP, 4. Gentamicin; (B) Antibiogram against *P. aeruginosa* 1. Chitosan, 2. ZnO-NP-CS, 3. Gentamicin, 4. ZnO-NP.

Table 1: MIC and antibiogram result of various food borne and MDR bacteria

Bacteria	MIC (mg)	MBC (mg)	Chitosan zone of Inhibition (mm)	ZnO-NP-CS zone of Inhibition (mm)	Difference in zone of Inhibition (mm)
S. aureus	0.03	1.25	29	40	11
Methicillin Resistant *Staphylococcus aureus* (MRSA)	0.06	1.25	24	37	13
L. monocytogenes	0.03	0.16	22	36	14
Bacillus cereus	3.75	10.00	22	29	5
B. subtilis	0.05	0.16	27	40	13
Vibrio cholera	0.01	0.23	25	40	15
E. coli	0.05	1.56	17	22	5
Salmonella	0.10	0.94	15	26	11
P. aeruginosa	15.00	20.00	21	32	12

Values are mean of triplicate

after incorporating the ZnO-NP with chitosan. Synergistic effect of ZnO-NP-CS against *S. aureus* was reported by Sathiya *et al.* (2018) and they concluded that production of reactive oxygen species (ROS) would be a major reason for antibacterial activity. Similarly, Al-Naamani *et al.* (2018) reported that higher reduction in bacterial and fungal was observed in ZnO-NP-CS coated package film thus extended the shelf life of Okra (*Abelmoschus esculentus*) for four more days. It has been reported that chitosan was more active against gram negative bacteria than gram positive bacteria (Goy *et al.,* 2009). But, ZnO-NP-CS was active against both gram negative and gram positive bacteria. Since, the chitosan and its derivatives are being used in various biomedical applications (Martins *et al.,* 2011); ZnO-NP-CS can be applied for improved activity antimicrobial and healing purpose. In the present study, more emphasis was given to the ZnO-NP-CS as an ointment against pathogenic bacteria causing delayed wound healing.

Antibiogram was also carried out against multiple drug resistant bacteria *viz.,* MRSA and *Pseudomonas aeruginosa,* which are responsible for various major surface wound infections. In antibiogram against MDR bacteria, ZnO-NP-CS zone of inhibition was found to be equal to commercial antibiotics (Fig. 4). Hence, 100 µl of ZnO-NP-CS (1 mg of ZnO-NP in 1 mg of chitosan) was found equally effective to commercial antibiotics (10 µg of Gentamicin). The present study has revealed the suitability of zinc oxide nano particle incorporated chitosan (ZnO-NP-CS) as an effective alternative to commercial antibiotics for treatment of wound infections affected by multiple drug resistant bacteria.

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