Synthesis of zinc oxide nanocomposite films and its antibacterial activity against ESBL-producing bacteria

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Abstract

Extended spectrum ß-lactamases (ESBLs) represent a major group of lactamases mostly produced by gram-negative bacteria. The antibacterial activity of zinc oxide nanoparticles (ZnO-NPS) has received significant interest worldwide particularly by the implementation of nanotechnology to synthesize particles in the nanometer region. The purpose of this study is to identify the antibacterial effect of zinc oxide nanocomposite films against ESBL-producing bacteria. Using *Klebsiella pneumoniae* and *Escherichia coli* that exhibit Extended spectrum ß-lactamases (ESBLs), this research tends to prove the potency of zinc oxide nanoparticles against them to prevent further growing population of ESBL positive patients here in the Philippines. Zinc oxide nanocomposite films were synthesized and its composition and topographical features were observed using Fourier-transform infrared spectroscopy (FTIR) and Scanning Electron Microscope (SEM). Its antibacterial property was tested against *Escherichia coli* and *Klebsiella pneumoniae* using disk diffusion, liquid culture and spectrophotometer by measuring its absorbance after 16, 24, and 48 hours of incubation. Results indicate that the synthesized zinc oxide nanoparticles have antibacterial effect against ESBL positive *Escherichia coli* and *Klebsiella pneumoniae* but the polystyrene film, which served as a matrix, inhibited the release of nanoparticles.

Keywords: Zinc oxide nanoparticles, polystyrene, ESBL producing bacteria, antibacterial

INTRODUCTION

The high incidence of bacterial infection and the increasing rate of bacterial resistance to antibiotics led to the innovation of scientific methods to be able to compensate such needs.

Extended-spectrum beta-lactamases (ESBLs) are enzymes produced by bacteria that impose a threat to the effectivity of antibiotics to kill bacteria. Such group breaks down antibiotics which gives an increasing number of antibiotic-resistant bacteria. The common isolates of ESBLs usually cause several community-acquired diseases (Huizen, 2017). Improvision of new techniques is being applied due to the high prevalence of antibiotic-resistant isolates.

Nanotechnology is a modern technique capable of providing a wide range of usage in food processing, agricultural production and it has also high demands in the field of medicine. It is used to explore and characterize molecules in nanometers from 1 to 100 nm, which in this level its properties can be observed and defined (Ann et al., 2015). Its focus is due to the growing number of drug-resistant bacteria which employs giving higher doses of antibiotics that could overcome the major clinical problem all around the globe (Manohar, 2017).

One of the major health problems is resistance of numerous microorganisms from certain antibiotics; thus, necessity for the development of alternative antimicrobial agents is highly in demand. Knowledge in nanotechnology is rapidly growing. The study of these nano-sized particles presents a good antimicrobial activity which can serve as one of the promising alternative for controlling microbial infections (Mohanraj, 2017). Nanoparticles such as silver, gold, iron oxide and zinc oxide exhibit a wide spectrum against pathogenic microorganism (Al-Khedhairy & Khan, 2017).

Zinc is an essential trace element for humans. Zinc oxide nanoparticles (ZnO-NPs) were found to have a strong synergistic effect with antibiotics used for treating bacterial infections. As the rate of bacterial mutation and antimicrobial resistance increases, innovation for a modern medicine using nano-based particles is highly in demand (Ann et al., 2015). Zinc oxide nanoparticles' properties are believed to have a great antimicrobial activity which may be used to augment or replace antibiotics.

This study aims to develop materials for health prevention purposes, to lower the risk of disease transmission and spread of infection, serve as antimicrobial prophylaxis in various food borne diseases. The purpose of this study was to test the

antibacterial activity of zinc oxide nanoparticles against ESBL - producing bacteria. The specific objectives include synthesis of zinc oxide nanocomposite film and the determination of effectivity of zinc oxide nanoparticles as an alternative nanoantibiotic by measuring the zone of inhibition and comparison of the bacterial growth at different incubation time through its absorbance.

MATERIALS AND METHODS

Zinc oxide preparation

The chemicals used were purchased from Batangas Medical Trading.

A 12.6 g of zinc acetate dihydrate was dissolved in 400 ml of distilled water stirred continuously at 65°C. Additional 600 ml of ethanol was added in the solution. About 6 ml of H_2O_2 (47%) was added dropwise to the vessel and mixed to get a clear solution. The solution was stored inside a laminar flow hood for 24 hours and dried at 80°C for several hours to obtain white colored zinc oxide (Ali et al., 2015).

The obtained nano zinc oxide powder was washed with triple distilled water and centrifuged 4 times at 4000 rpm for 6 minutes and dried at 80°C in drying oven to further complete its conversion process (Syväjärvi & Tiwari, 2015).

Zinc oxide nanocomposite film preparation

Polystyrene was procured from Batangas Medical Trading. In order to treat polystyrene films with zinc oxide, 2 g of polystyrene was dissolved in 50 ml of toluene for an hour. The prepared gel of zinc oxide nanoparticle was mixed into 30 ml methanol and then into the polystyrene-toluene solution. Three concentrations (1, 2.5, and 5 wt %) of zinc oxide in polystyrene was prepared. The solution was placed in a glass plate and was heated at 80°C to remove solvents in the polymer. It was further evaporated at room temperature using a dust free chamber. After evaporation, polystyrene/zinc oxide nanocomposite films were obtained. For contrasting basis, a neat polystyrene without zinc oxide (ZnO) was also prepared (Ali et al., 2016).

Fourier-transform infrared spectroscopy (FTIR)

The prepared zinc oxide nanoparticle was analyzed and compared with standard zinc oxide nanoparticle which was purchased from Batangas Medical Trading. Through the use of FTIR at Lyceum of the Philippines University - Batangas, the

researchers confirmed that the product produced was a zinc oxide nanoparticle.

Visual Analysis of Zinc oxide Concentration

The topographical features of prepared zinc oxide polystyrene film and plain polystyrene film were analyzed using scanning electron microscope (Dual Beam Helios Nanolab 600i). It assured the researchers about the present concentration of zinc oxide in polystyrene films. This procedure was done at Department of Science and Technology - Taguig City.

Bacterial cultivation

ESBL producing bacteria NCTC 13351 *Escherichia coli* and ATCC 700603 *Klebsiella pneumoniae* were obtained from Microbiologics. The strains were cultivated on MacConkey Agar at 37 °C and were incubated for 18 hours until use (Ali et al., 2016).

Turbidity standard for inoculum preparation

Bacterial suspensions were prepared by emulsifying colony from pure cultivated bacteria in 3 ml of 0.85% sterile saline solution. Turbidimetry was compared and adjusted with the use of 0.5 MacFarland standard in order to come up with 1.5 x 10^8 colony forming units (CFU/ml) bacterial suspension. A 0.5 MacFarland standard served as the reference to achieve a standard suspension for antimicrobial testing (Ali et al., 2016).

Antimicrobial Susceptibility Testing - Disk Diffusion Method

Kirby Bauer Disk Diffusion method was applied in this study. An inoculum from prepared bacterial suspension specifically 1.5 x 10^8 CFU/ml of *E. coli* and *K. pneumoniae* was spread in Mueller Hinton agar separately.

The prepared zinc oxide polystyrene films were cut into a disc measuring 6 mm using an autoclaved metal hole puncher. Each film was placed on the inoculated Mueller Hinton for each bacterial strain and incubated at 37 °C for 18-24
hours. Plain polystyrene films were used as control in this study. The microorganisms were tested against zinc oxide films under varying concentrations (1, 2.5 and 5 wt %). Zone of inhibitions were measured to determine the antimicrobial activity of each zinc oxide polystyrene films (Ali et al., 2016).

For confirmatory purposes, synthesized zinc oxide nanoparticles were tested for its antibacterial activity against *K. pneumoniae* and *E. coli*. A 100 mg of zinc oxide nanoparticle

were dissolved in 1 ml of 70% ethanol. Whatman No. 1 filter paper was cut into a 6 mm filter paper discs using autoclaved metal hole puncher and were immersed in zinc oxide nanoparticle solution for 24 hours. Paper discs immersed in 1 ml of 70% ethanol were also prepared which serve as a negative control. The discs were allowed to dry before plating on Mueller Hinton Agar for each bacterial strain using sterile forceps. The paper discs were pressed slightly to make complete contact with the surface's medium. The plates were incubated at 37 °C for 18-24 hours. The zone of inhibition around each discs were measured in millimeters (Abraham et al., 2014).

Antibacterial Testing - Liquid culture

The prepared nanocomposite films containing different concentrations of zinc oxide nanoparticle and plain polystyrene film were cut into 1x1 cm and inserted in each tryptic soy broth. A 1 ml of 1.5 x 108 CFU/ml bacterial suspension of *E. coli* and *K. pneumoniae* was transferred in 7ml broth each containing zinc oxide nanocomposite films. The broth which does not contain nanocomposite film was prepared acting as the negative control. Each film were incubated at 37°C and the turbidity reading was measured after 16, 24, and 48 hours. The turbidity was analyzed with the use of spectrophotometer by reading the absorbance or optical density at 630 nm wavelength. For confirmatory, spectrophotometric analysis was parallel with the density of bacterial colony growth. Each treated broth cultures and the negative control was streaked on MacConkey Agar. Plates were incubated at 37°C for 18-24 hours and the bacterial growth was observed (Carotenuto & Nicolais, 2014).

Statistical Analysis

Mean and percentage was used to analyze the growth of bacteria. Descriptive statistical data was used for all observations.

RESULTS AND DISCUSSIONS

Fourier-transform infrared spectroscopy (FTIR)

Fourier-transform infrared spectroscopy is a method that is effective in revealing products' composition. Figure 1 shows the FTIR spectrum of both prepared and standard zinc oxide nanoparticles. The synthesized zinc oxide nanoparticle peaks at 650, 1400, 1550 and 3400. This is similar to the standard zinc

oxide used that peaks at 650, 1350, 1500 and 3400. Both standard and sample have an anchor at 2300.

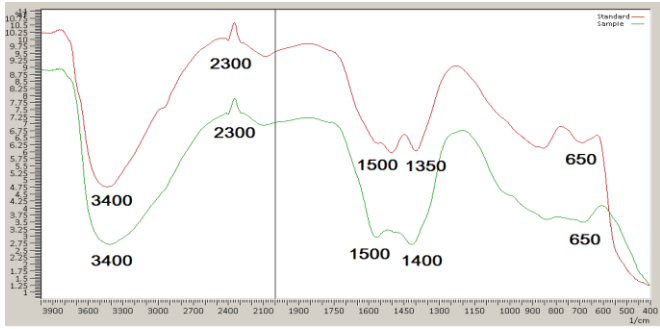

Figure 1. FTIR pattern of prepared zinc oxide nanoparticle in comparison to standard zinc oxide nanoparticles

The gathered data was also similar with the results observed by Manyasree, Kiranmayi, and Venkata et al., (2018) where they tested the synthesized zinc oxide nanoparticles to FTIR. The observed peaks at 450 and 603 is due to the zinc oxide stretching vibration while the peaks at 1638 and 3405 are due to the bending and stretching vibration mode of water molecule. Another study showing similar results was obtained by Hultman et al. (2014), stating that it was observed from 1500 to 1650 and 3200 and 3650.

Overall, the peaks between the two products are almost similar which shows that the product produced was a pure zinc oxide nanoparticle.

Scanning Electron Microscope

The Scanning Electron Microscope (SEM) images of the visual topography of films and distribution of zinc oxide concentration is seen in Figure 2. The films were observed up to 50,000 magnification. In plain polystyrene film, the surface is free of nanoparticles unlike the other treated films where nanoparticles are dispersed and increasing in number as the concentration of nanoparticle increases. The particles' sizes that were measured ranges from 17.06 to 83.11 um.

Disk Diffusion Method

The antibacterial activity of zinc oxide nanocomposite film was tested against NCTC 13351 *Escherichia coli* and ATCC 700603 *Klebsiella pneumoniae*. Figures 3 and 4 display the results of the antibacterial testing. It clearly shows that there was

no zone of inhibition observed around the plain and treated polystyrene disks. Same results were also observed by Jin, Su, Sue, and Sun,)2008).

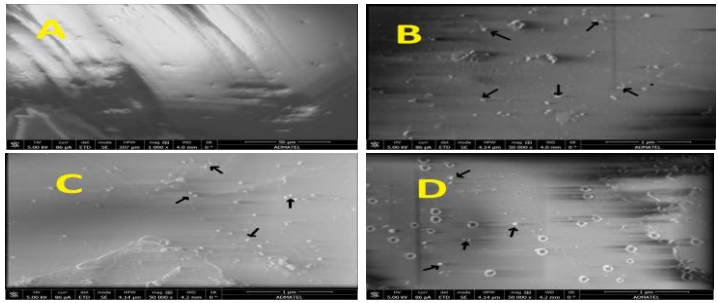

Figure 2. Synthesized nanocomposite films under scanning electron microscope. A) plain polystyrene film B) 1 % zinc oxide nanocomposite film C) 2.5 % zinc oxide nanocomposite film D) 5% zinc oxide nanocomposite film

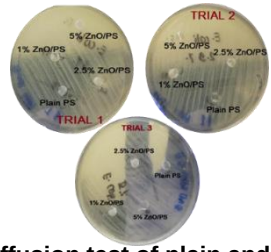

Figure 3. Disk Diffusion test of plain and treated films against NCTC 13351 *Escherichia coli* showing no inhibitions on all films.

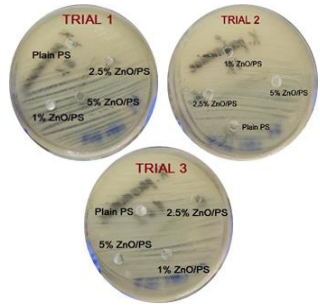

Figure 4. Disk Diffusion test of plain and treated films against ATCC 700603 *Klebsiella pneumoniae* showing no inhibitions on all films.

They tested the antimicrobial efficacy of zinc oxide quantum dots against *L. monocytogenes, S. enteritidis and E. coli* O157:H7. There was no zone of inhibition observed around the films. The nanoparticles did not diffuse from the disks as it is bound within the polystyrene film resulting to failure in observing zone of inhibition. It implicates that zinc oxide nanocomposite film is not effective in suppressing the growth of ESBL producing bacteria.

To ensure that it is the polystyrene matrix that is inhibiting the activity of the zinc oxide nanoparticle, the antibacterial property of zinc oxide nanoparticles were also tested against each bacterial strain. The results were shown in Figures 5 and 6. An 8 mm and 10 mm zone of inhibition were observed around the disc containing zinc oxide nanoparticle against *E. coli* and *K. pneumoniae* respectively. There was no zone of inhibition observed around the disc immersed in 70% ethanol.

This same result was also observed by Banoee et al., (2010). They tested the antimicrobial activity of zinc oxide nanoparticle enhance with ciprofloxacin against *E.coli* and *S. aureus*. They observed zone of inhibition around the disc containing 500 µg of zinc oxide nanoparticle while a greater zone of inhibition were measured around the disc containing zinc oxide and ciprofloxacin. The antibacterial effectivity of zinc oxide nanoparticles was also observed by Abraham et al., (2014). There were zones of inhibition observed around discs containing different concentrations of zinc oxide which were tested against *S. aureus, E. faecalis, E. coli, K. peumoniae,* and *P. aeruginosa.* The antibacterial activity of zinc oxide increases as the zinc oxide concentration increases. Same observations were seen by Alikhani, et al., (2013). They tested the zinc oxide nanoparticles against four bacterial strains *(E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, as gram negative bacteria, *S. epidermidis* PTCC 1114 and *S. aureus* ATCC 25923 as gram positive bacteria). They concluded that as the concentration of ZnO nanoparticles increases, the antibacterial activity also increased for all strains. Among the studied bacteria, ZnO nanoparticles showed maximum activity against *P. aeroginosa* at lower concentrations with 13.01 ± 1.04 mm D (156.25 lg/mL) and the minimum activity was against *S. aureus* with 16.75 ± 0.6 mm D (1,250 lg/mL).

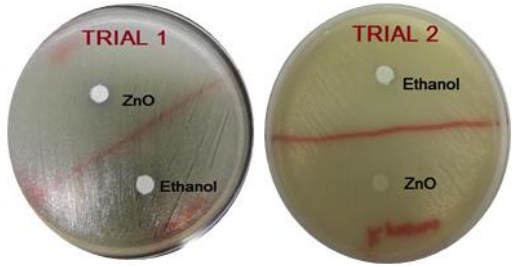

Figure 5. Disk Diffusion test of zinc oxide nanoparticles embedded on filter paper discs against ATCC 700603 *Klebsiella pneumonia.*

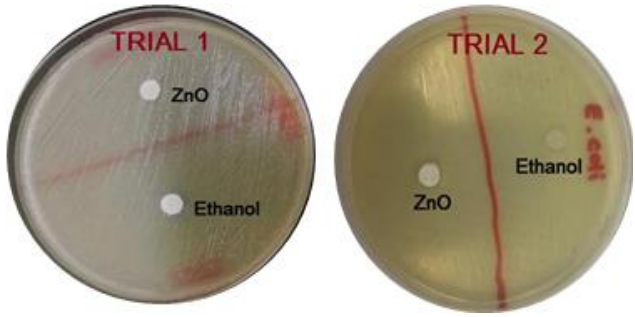

Figure 6. Disk Diffusion test of zinc oxide nanoparticles embedded on filter paper discs against NCTC 13351 *Escherichia coli.*

Liquid Culture

The antimicrobial action of the nanocomposite films was confirmed by measuring the absorbance or optical density of each liquid culture containing treated and untreated films. The absorbance reading of ATCC 700603 *K. pneumoniae* is shown in Figure 7 wherein the absorbance increases as the incubation period increases except for the 5% Zno/PS where its absorbance decreases by .044 after 24 hours incubation but it increases by .031 after 48 hours incubation. This result was in contrast with the research conducted by Kim, Mishra, Shin, Wahab, and Yun (2010) where the *Klebsiella pneumoniae* was inhibited using 5 µg/ml of the nanoparticles solution during the logarithmic phase between 7 and 12 h. This proved that the absorbance of the samples was affected by the length of incubation.

The absorbance readings of liquid culture containing plain and treated films against NCTC 13351 *E. coli* are shown in Figure 8. The absorbance reading increases after 16 and 24

hours of incubation but after 48 hours of incubation, the absorbance readings of all the samples in every trial decreased.

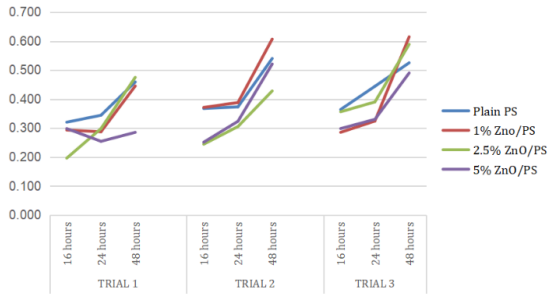

Figure 7. Absorbance reading of liquid culture containing plain and treated films against ATCC 700603 *K. pneumoniae* read after every incubation.

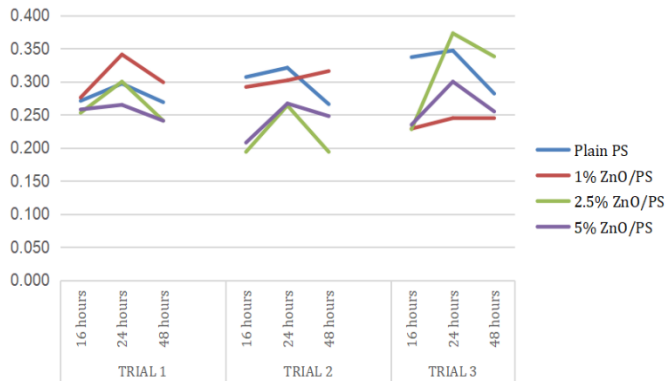

Figure 8. Absorbance reading of liquid culture containing plain and treated films against NCTC 13351 *E. coli* read after every incubation.

According to Hong et al. (2009), when the surface is modified, the UV absorption of the modified zinc oxide was reduced. The reduction of photocatalytic activity of zinc oxide nanoparticles could be explained to some extent. According to the result of Aanand et al. (2017), it indicates that the nanoparticles of zinc oxide can be absorbed in UV and in the visible region of the solar light, suggesting that the nanoparticles of zinc oxide could be used as a visible light photocatalyst.

Upon reading the absorbance, the samples were streaked on MacConkey agar to observe the bacterial growth. It was observed that there were heavy growth of *E. coli* and *K. pneumoniae* in all trials of both treated and untreated films which

is parallel to the result of increasing absorbance. This idea was supported by the study of Borobio, et al., (2001). The principle of spectrophotometer is based on the measurement of light absorbed from the bacterial suspension. Thus, the increasing absorbance is directly related to the increasing growth of bacteria.

In comparison with the spectrophotometric readings, the absorbance gathered correlates well with the bacterial growth of *K. pneumoniae* but it is in contrast with the results obtained from *E. coli* during the 48th hour incubation.

The zinc oxide nanocomposite films present an inefficient antimicrobial activity in liquid culture. Thus, the results signify the inability of nanoparticle to diffuse from the matrix.

According to An and Baek (2011), The effects of ZnO NPs on microbial growth were determined to be species-specific. When exposed to zinc oxide nanoparticles, it shows that *S.aureus* is more sensitive than *E. coli*. The biosorption of metal oxide NPs by bacteria was related to the species sensitivity to different NPs. Interpreting the sensitivity of the species in terms of bacterial classification (Gram + and Gram −) is not currently feasible.

CONCLUSION

Zinc oxide nanocomposite films were successfully synthesized. There were no zones of inhibition produced via disk diffusion method and the bacterial growth was not suppressed in liquid culture using nanocomposite films as compared when using zinc oxide nanoparticles embedded on paper discs. Therefore, the synthesized zinc oxide nanoparticle shows an effective antibacterial activity but its effect was inhibited by the polystyrene since it did not diffuse from the matrix.

RECOMMENDATION

Considering the result of the study, the researchers recommend the following: modification of methods for the antimicrobial activity, time and temperature for the synthesis of nanocomposite film must be properly supervised, and to further investigate the permeability and equal distribution of zinc oxide nanoparticles. Hence, use of more reliable matrix or polymer that would easily diffuse the particles and enhance the ability of the nanocomposite film to inhibit the growth of microorganisms is recommended.

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