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Foreword

The tri-fold functions of academic institutions include instruction, research and community extension. Among these three, research stands as the very foundation of knowledge which directs what the teacher needs to provide his community. Without research therefore, instruction is void of development while community extension is void of adaptability and sustainability. It cannot therefore be overemphasized how vital the research process is for the continued development of a teacher and of his institution. Realizing this fact, the faculty members of the Allied Medical Professions of Lyceum of the Philippines University - Batangas remains committed to the research endeavors of the school.

The research journal of the College is one venue on how the research outputs of every faculty members and students can be disseminated and utilized. It is the hope and prayer of the College that the culture of research continues to grow in the hearts of every faculty member as wells as with their students. We are sowing the seeds of this culture through this research journal and we need to stand guard to its development and nurture it to a full bloom.

Antibacterial property of chemically reduced silver nanoparticles against Klebsiella pneumoniae and its synergistic effect in combination with antibiotics

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Abstract

Due to the alarming cases of some of the microorganisms, especially bacteria, that are becoming resistant to many antibacterial agents, previous studies suggest the use of silver nanoparticles (AgNPs). The purpose of this study was to determine if chemically reduced AgNPs exhibit antibacterial property against *Klebsiella pneumoniae*. Silver nanoparticles were prepared by chemical reduction method. Ascorbic acid, acting as stabilizing agent, was used in the synthesis of silver nanoparticle. The resulting AgNPs antibacterial activity was tested against *Klebsiella pneumoniae.* The synergistic antibacterial activity of AgNPs with three CLSI recommended antibiotics was also determined by the fractional inhibitory concentration index (FICI) using the checkerboard microdilution method. The AgNPs exhibited antibacterial activity against *Klebsiella pneumoniae* with a Minimum Inhibitory Concentration (MIC) of 1.25 µg/ml and Minimum Bactericidal Concentration (MBC) of 39.91 µg/ml. Moreover, the combination of AgNPs and antibiotics such as ceftazidime with FICI of 0.375 and levofloxacin with FICI of 0.250 showed a synergistic effect, while piperacillin-tazobactam with FICI of 3 showed no synergistic effect. This concludes that AgNPs exhibited inhibitory and bactericidal activities against *Klebsiella pneumoniae* and the combination of AgNPs with ceftazidime and levofloxacin shows inhibitory actions against *Klebsiella pneumoniae.*

Keywords:, Antibacterial, Antibiotics, Multidrug-resistant, Silver nanoparticles

INTRODUCTION

Multidrug-resistant (MDR) bacterial pathogens widespread rise is the most significant public health challenge worldwide (World Health Organization, 2014). MDR organisms associated infections correlates with high mortality rate, increased healthcare costs, and longer hospital stays. (Lambert et al, 2011; Neidell et al, 2012; Martin-Loeches et al, 2015). Because of misuse many antibiotics loses their power to fight and cure disease all over the world. Klebsiella pneumoniae is one of the most potent pathogens that is widely associated with hospital and community acquired infections. According to the 2017 report of DOH from Antimicrobial Resistance Surveillance Program, a total of 12,591 isolates of Klebsiella pneumoniae was isolated and majority was from respiratory samples having 57% while others were isolated from urine, wound, and blood specimens.

Encouraging results of alternative therapies such as bacteriophage therapy, phytochemicals, probiotics, metal salts were found in cases of infections caused with multidrug resistant strains (Chhibber et al., 2017). The top priority of the public health and the biggest challenge to pharmaceutical industry is the effective prevention and treatment for an increasing rate of infection due to multidrug resistant viruses, bacteria, fungi, and parasites (CDC, 2013).

Nanotechnology is referred to as the manipulation of matter at the atomic molecular level. The word ‘nano’ is derived from the Greek word nanos which means dwarf. Nanotechnology involves the investigation, use of nanomaterials, and devices typically with dimensions smaller than 100nm (Nikolelis, 2018). AgNPs have received attention because of its broad antibacterial property against bacteria, and antibiotic resistant bacterial strains as well (Pelgrift & Friedman, 2013). Based on the study of Prabhu and Poulose (2012), silver nanoparticles have the property which help in molecular diagnostics and therapies. Its major application in the medical field include diagnostic applications. Because of its increased surface area to volume ratio, it shows an antimicrobial efficacy against a wide spectrum of bacterial species. Its microbial property is being used in therapeutic applications. There are many uses of silver nanoparticles but the most important is its antimicrobial activity and anti-inflammatory capacity.

In this modern world, advances in nanotechnology paved the way to diagnose, treat, and prevent diseases in all aspects of human life. Among various metallic nanoparticles used in

biomedical, silver nanoparticles are one of the most vital and fascinating nanomaterials. Silver nanoparticles play an important role in nanoscience and nanotechnology, particularly in nanomedicine (Zhang, 2017). Current researches are getting attention in the antimicrobial activity of nanoparticles, primarily against drug-resistant bacteria with more than four-fold increase in activity using nanoparticles (Qayyum & Khan, 2016).

In view of the fact that chemically synthesized nanoparticles shows antimicrobial activity, the study seeks to synthesize and use silver nanoparticles to validate its synergistic effect with the antibiotics to obliterate *Klebsiella pneumoniae.* It would be limited to synthesis of silver nanoparticles and the susceptibility of *Klebsiella pneumoniae* to the silver nanoparticles combined with existing antibiotics. The study will help our healthcare system to obliterate the

MATERIALS AND METHODS

Chemical reduction of silver nanoparticles

The chemical reduction of AgNPs was based on the method done by El-Kheshen and Gad El-Rab (2012). A 40 ml of 0.074 M AgNO3 was placed in a beaker using ascorbic acid as the reducing agent then a 0.25 ml of ascorbic acid was added drop by drop with continuous stirring until the solution turns to grayish-black. The solution was covered and incubated at room temperature for 30 minutes. The silver nanoparticles were purified by centrifugation at 3,500 *g* for 5 minutes. The supernatant was separated from the precipitate and was used for the characterization study and antimicrobial susceptibility testing. The solution was characterized using UV-Vis spectra and transmission electron microscopy. It was then dissolved in a 500 mL of deionized water.

Test microorganism

The bacteria that was used in the study was *Klebsiella pneumoniae* ATCC 13881 which was acquired from the Department of Medical Microbiology, UP Manila College of Public Health

Antibacterial Susceptibility Assay

Based on the procedure described by Chikezie (2017), 14 test tubes were used wherein 1 ml of Mueller-Hinton broth was dispersed on tubes 1-12 while only 1 mL of the broth was placed in tube 13, which became the negative control. For the positive control,

1 mL of ceftazidime added in tube 14 without the broth. The AgNPs were then added to tube 1 and a two- fold serial dilution up to tube 12 with Mueller- Hinton Agar was performed. The preparation of the bacterial suspension was done by inoculating *Klebsiella pneumoniae* from the Nutrient Agar and was suspended in a 0.9% NSS comparing turbidity of which to 0.5 MacFarland Standard. A 1 mL of the microbial suspension was dispensed to each tube and was incubated at 37°C for 24 hours. After incubation, it was then read for the determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC). To confirm the MBC, 0.01 mL of sample from tube 1- 12, was subcultured in Mueller-Hinton Agar. The agar plates were also incubated at 37°C for 18 to 24 hours and the plate with no growth was recorded as the Maximum Bactericidal Concentration.

Evaluation of synergistic effects between AgNPs and antibiotics by broth microdilution checkerboard method

The degree of synergy between antibacterial drugs was expressed in terms of the fractional inhibitory concentration (FIC). The FIC is the minimum inhibitory concentration (MIC) of the drug in combination divided by the MIC of drugs acting alone. The antibiotics levofloxacin, piperacillin-tazobactam and ceftazidime were used to examine their combined synergistic effects with prepared AgNPs against *Klebsiella pneumoniae*. Based from the study of Sopirala et al. (2010), stock solutions of these agents were prepared in sterile Millipore water to a concentration from 0.5 to 128 µg/mL and refrigerated at 2-4 °C. A checkerboard microdilution technique was used to examine the synergism between the antibiotics and AgNPs against test organisms.

For the determination of the fractional inhibitory concentration (FIC), the microdilution checkerboard method was applied in microwell-containing plates. In this method, minimum inhibitory concentration (MIC) was determined, for both antibiotics and AgNPs alone and in their paired combinations (Isenberg, 2007). For antibiotics and AgNPs, the test range was 0.5–128 µg/ml. The sterility of prepared microwell plates were checked by incubation at 37 °C for 24 hours. Each microwell was inoculated with 100 µl of bacterial inoculum which was prepared from an 18–24 hours incubation of the test organism grown on Muller-Hinton broth (MHB). The inoculated plates were incubated at 37 °C for 16 - 18 hours. The lowest concentration at which no visible growth occurred was recorded to be the MIC value of the individual and combined test agents. The FIC was calculated using the Chou-Talalay Method

wherein the MIC of the test agent A and the MIC of the test agent A in combination with test agent B. The result of the FIC A and FIC B was added to give the ΣFIC index.

The calculated FIC index (FICI) was used to detect the nature of interaction between the two test agents and the interaction either synergism or indifference or antagonism type. The values pu blished by the American Society of Microbiology (2010) was used to decide the nature of the interaction. Synergy was defined by a FIC value of 0.5, no synergism is defined by an FICI value of 0.5-4.0 and antagonism was defined by an FICI value of 4 and above.

RESULTS AND DISCUSSION

Chemical reduction of silver nanoparticles

The silver nitrate combined with the ascorbic acid shows a yellow-colored solution. As shown in Figure 1, a grayish-yellow colored solution was produced during the chemical reduction. As the synthesis proceeded, a change in color was observed from being a colorless solution to grayish-black as ascorbic acid was added drop by drop. After centrifugation, the color of the solution changed from grayish black to yellowish solution. The uncentrifuged solution showing a grayish black color was due to the production of aggregated AgNPs wherein the electrons near each particle are delocalized causing aggregation. Separation of the aggregated AgNPs and unaggregated AgNPs can be done by using centrifugal force; thus, causing a yellow solution.

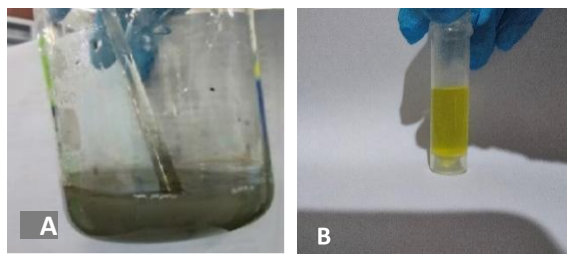

Figure 1. Chemically reduced Ag-NPs before and after centrifugation A. before centrifugation B. after centrifugation

The formation of yellowish color therefore indicates the presence of silver nanoparticles as also revealed in the study of

Suriati et al. (2014). It states that the yellow solution after incubation and centrifugation reveals that silver nanoparticles were synthesized.

UV- Vis Spectroscopy

The presence of silver nanoparticles in the yellow solution was determined by using UV-Vis Spectroscopy. One mL aliquot of the AgNPs solution was tested. Spectra was measured at the range of 300 to 600 nm having a resulting peak of 407 nm (Figure2) that clearly shows the presence of silver nanoparticles in the solution.

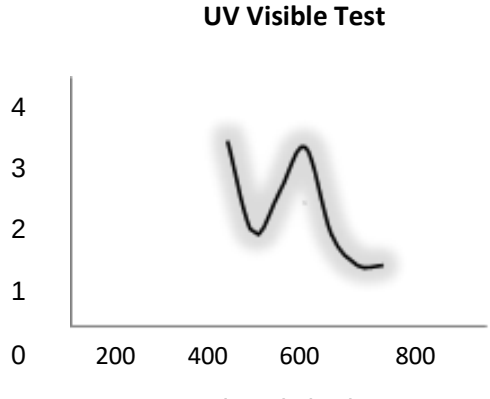

Figure 2. UV-Vis Spectra showing a peak at 407

UV-Vis spectroscopy is one of the most commonly used techniques in determining properties of nanoparticles. The suggestive visible spectra of AgNPs based on the study by Saeb et al. (2014) is 400-500. The study of Guzman et al. (2009) and Badi'ah (2005) also showed a peak absorbance of silver nanoparticles at 418 nm and 406 nm, respectively.

Transmission Electron Microscopy (TEM)

The characterization of AgNPs present in the synthesized solution was analyzed at the electron microscopy laboratory of Research Institute of Tropical Medicine, using model JEM 1220. The resulting size range of the AgNPs was 35.6 to 109 nm while the average size was 56.794 nm as shown in Figure 3. The said size is within the range where it can be classified as a nanoparticle, based on the study conducted by Paulkumar (2017) where they

characterized AgNPs at the size range of 10-60 nm. According to Zhang et al. (2016), the enhancement of
antibacterial action was observed with the particles having a size of 59 nm compared to 83 nm. This suggests that the synthesized AgNPsin this study fits the definition for nanoparticles in terms of size.

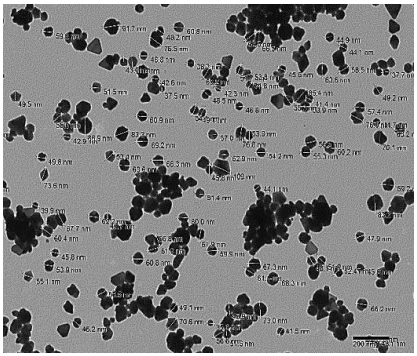

Figure 3. TEM micrograph of AgNPs showing an average of 56.794 nm

Antibacterial Susceptibility Assay

The effect of AgNPs as an antibacterial agent was demonstrated by the clearing of solution from Tube 6 to Tube 11. From trials 1 to 3, Tube 1 to 5 can be seen with a hazy white solution that looked like the solution in Tube 13 which was the negative control of the test that contained Klebsiella pneumoniae only. Tubes number 6 to 11 which shows a clear yellow solution were found to be comparable with the positive control containing the bacteria and ceftazidime as also observed in Tube 14 found in Figure 4. Clearing of the sample that started from Tube 6 indicates the Minimum Bactericidal Concentration (MBC) of the test while the last clear tube (Tube 11) indicates the Minimum Inhibitory Concentration (MIC) of the test (Table 1).

The MIC value of silver nanoparticles that indicates antimicrobial property against Klebsiella pneumoniae was observed at 1.25 µg/ml (Table 2). It shows that a low concentration of the AgNPs exhibits antimicrobial properties unlike using a high concentration of AgNPs. The inhibition rate of the AgNPs was greatly affected by the increase in particle size due to its absorption of proteins present in the media (MHB) that causes the nanoparticles to

Table 1
Minimum Inhibitory Concentration and Maximum Bactericidal Concentration Results

Tube	Concentration (ug/ml)	Trial 1	Trial 2	Trial 3
1	1277.16	Turbid	Turbid	Turbid
2	638.58	Turbid	Turbid	Turbid
3	319.29	Turbid	Turbid	Turbid
4	159.64	Turbid	Turbid	Turbid
5	79.82	Turbid	Turbid	Turbid
6	39.91	Clear	Clear	Clear
7	19.96	Clear	Clear	Clear
8	9.98	Clear	Clear	Clear
9	5.99	Clear	Clear	Clear
10	3.49	Clear	Clear	Clear
11	1.25	Clear	Clear	Clear
12	0.62	Turbid	Turbid	Turbid
13	Negative Control	Turbid	Turbid	Turbid
14	Positive Control	Clear	Clear	Clear

Figure 4. Macrodilution Tube Method for the MIC and MBC of AgNPs

aggregate in higher concentration. The aggregation was also related to the zeta potential property of the nanoparticle wherein the high dilution shifts the zeta potential to a less negative values (>-30 mV) that reduced the antimicrobial property of the nanoparticle according to the study of Tantra et al. (2010).

Table 2
Minimum inhibitory concentration (MIC) of Silver Nanoparticle against K.pneumonia

Trial Number	MIC Value (µg/ml)
1	1.25
2	1.25
3	1.25

Table 3
Minimal bactericidal concentration (MBC) of Silver Nanoparticle against *K. pneumoniae*

Trial Number	MBC Value (µg/ml)
1	39.91
2	39.91
3	39.91

Table 3 presents the MBC of silver nanoparticles against *Klebsiella pneumoniae*. The growth of this test organism at the concentration of 39.91µg/ml revealed that AgNPs can eradicate the growth of the said organism (Figure 5). The antibacterial property of AgNPs become more stable due to the presence of proteins in the media used. The AgNPs was said to absorb proteins present in the media causing a success in the inhibition of the microorganism by AgNPs (Noriega-Treviño et al., 2011).

Inhibition with *Klebsiella pneumoniae* by the silver nanoparticles can be supported by the work of Sondi and Salopek-Sondi (2004) where treated *E. coli* cells showed formation of “pits” in the cell wall of the bacteria exhibiting cellular damage while the AgNPs were found to accumulate in the bacterial membrane. An increase in membrane permeability caused by the production of reactive oxygen species and interruption in the replication of the bacterial nucleic acids upon the release of the AgNPs,results in the death of the cell according to the study of Yin et al. (2020). The variation in particle size is an important factor that affects the antibacterial activity of AgNPs in which one study shows that 20±5 nm to 50±5 nm has an average MBC of 76.67 µg/ml against E. coli MTCC 443 (Agnihotri et al., 2014).

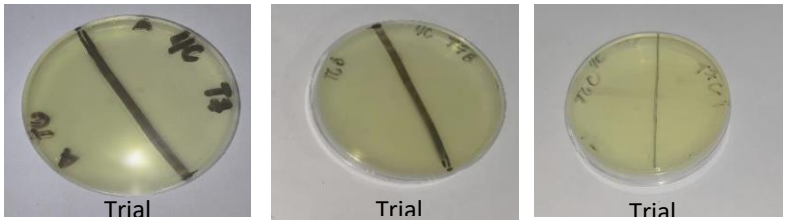

Figure 5. Bactericidal Concentration subculture on Mueller Hinton Agar showing no growth form the subcultured inoculum of Tube 6 and 7

Fractional Inhibitory Concentration (FIC) and Fractional Inhibitory Concentration Index (FICI)

The FIC of the antibiotics and antibiotics combined with AgNPs are the obtained MIC from the microwell dilution. The result of combining the AgNPs with levofloxacin, piperacillin-tazobactam and ceftazidime were 0.5µg/ml, 8µg/ml and 1µg/ml, respectively; while the results of using the commercial antibiotics such as levofloxacin, piperacillin-tazobactam and ceftazidime were 4µg/ml, 8µg/ml and 8µg/ml, respectively (Table 4).

The computed FIC A and FIC B values of the combined AgNPs with levofloxacin were 0.125, while the combination of AgNPs with piperacillin- tazobactam and ceftazidime produced a FIC A of 2 and 0.25 and a FIC B of 1 and 0.125, respectively. The FIC A and FIC B were used to give the value for the fraction inhibitory concentration index that indicates which combination are synergistic or not. The FIC A of each combination was computed from the value of the MIC of the combined antibiotics and AgNPs divided by the MIC of the AgNPs while the FIC B was computed from the MIC of combined antibiotics and AgNPs divided by the MIC of the antibiotics alone according to the study of Isenberg (2007).

The MIC of the AgNPs increased in the microdilution that has a value of 4 µg/ml while it was 1.25 µg/ml in the macrodilution which was a result of two different methods used were the incubation time differs from one another. The increase in MIC value of AgNPs in microdilution method from macrodilution was the effect of the incubation time used wherein the microdilution has a shorter incubation time than the macrodilution test of the AgNPs. In the

study of Bedi et al. (2009), it was observed that the shorter incubation time increases MIC a 2 to 3 dilutions from the MIC of the longer incubation time, but this has no to little effect in the precision of the test for the antimicrobial property of nanoparticles.

Table 4
Fractional inhibitory concentration

Antibiotics	FIC	FIC A	FIC B
Levofloxacin	4 µg/ml	-	-
Piperacillin- tazobactam	8 µg/ml	-	-
Ceftazidime	8 µg/ml	-	-
Silver nanoparticle	4 µg/ml	-	-
Silver nanoparticle + Levofloxacin	0.5 µg/ml	0.125	0.125
Silver nanoparticle + Piperacillin- tazobactam	8 µg/ml	2	1
Silver nanoparticle + Ceftazidime	1 µg/ml	0.25	0.125

FIC A = MIC of A combined with AgNPs/ MIC of A alone
FIC B = MIC of A combined with AgNPs/ MIC of AgNPs

The computed FIC A and FIC B values of combined AgNPs with levofloxacin were 0.125 while the combination of AgNPs with piperacillin- tazobactam and ceftazidime has an FIC A of 2 and 0.25 and an FIC B of 1 and 0.125 respectively. The FIC A and FIC B were used to give the value for the fraction inhibitory concentration index that indicates which combination are synergistic or not. The FIC A of each combination was computed from the value of the MIC of the combined antibiotics and AgNPs divided by the MIC of the AgNPs while the FIC B was computed from the MIC of combined antibiotics and AgNPs divided by the MIC of the antibiotics alone according to the study of Isenberg (2007).

Table 5 presents that combining AgNPs with levofloxacin, ceftazidime, and piperacillin-tazobactam shows an FICI of 0.250, 0.375, and 3 respectively. The synergy falling below the value interpreted as synergistic which is less than 0.5 based on the published values of the American Society of Microbiology. From the three used antibiotics with Klebsiella pneumoniae synergistic effect is noted with levofloxacin and ceftazidime only. Piperacillin-tazobactam on the other hand, exhibits no synergism with AgNPs.

in which it is within the range value of 0.5 to 4.0 that denotes no synergism effect.

Table 5
Fractional inhibitory concentration index

Sample	FICI	Interpretation
Silver nanoparticle + Levofloxacin	0.250	Synergism
Silver nanoparticle + Piperacillin- Tazobactam	3	No Synergism
Silver nanoparticle + Ceftazidime	0.375	Synergism

Synergism = <0.5; **No Synergism** = 0.5 – 4.0; **Antagonism** = > 4.0

Antibiotic synergism occurs when the effects of a combination of antibiotics was greater than the sum of the effects of the individual antibiotics (Sopirala et al., 2010). Another study of synergism of antibiotics and AgNPs against multidrug resistant bacteria shows an increase in surface area to volume ratio due to increase entry of silver atoms into cell. Therefore, more silver atoms were in contact with the solution which actively participate in cellular damage and inhibiting cell division (Malawong et al., 2021). The absence of synergistic effect of piperacillin-tazobactam with AgNPs according to the study of Maioli (2008) was due to the interference with the cell wall synthesis contradicting the mechanism of AgNPs to adhere, pit and accumulate in the membrane of the target organism.

CONCLUSION

Silver nanoparticles (AgNPs) were successfully obtained from the chemical reduction of silver nitrate using ascorbic acid acting as the reducing agent. The result of the test clearly shows that the AgNPs exhibit inhibitory and bactericidal activity against Klebsiella pneumoniae. In the Fractional Inhibitory Concentration (FIC), the combination of CLSI recommended antibiotics with the chemically synthesized AgNPs presents a synergistic effect with two antibiotics levofloxacin and ceftazidime and demonstrates no synergistic effect with piperacillin-tazobactam. The combination of AgNPs with ceftazidime and levofloxacin shows inhibitory actions against *Klebsiella pneumoniae.*

RECOMMENDATION

We recommend further study in the synthesis of silver nanoparticles using different methods. Moreover, an analysis in the synergistic effect of silver nanoparticles using other antibiotics against other multi-drug resistant bacteria can also be done. It is also suggested to conduct an *in vivo* toxicity test to determine the effective dose and toxic dose of silver nanoparticles.

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