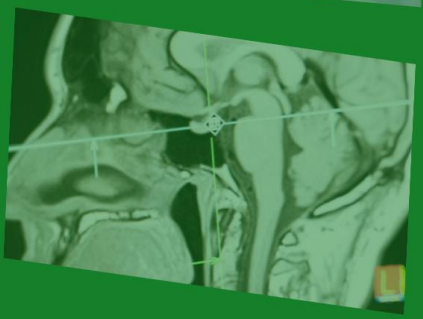
ISSN: 2094-5906

THE STETH

THE OFFICIAL PUBLICATION OF COLLEGE OF ALLIED MEDICAL PROFESSIONS

OF LYCEUM OF THE PHILIPPINES UNIVERSITY-BATANGAS

VOLUME 15, 2021| PART 2



THE STETH
ISSN 2094-5906
Published by the College of Allied Medical Professions
Lyceum of the Philippines University
Batangas City, Philippines

The Steth

Volume 15, 2021 | Part 2

The Official Research Publication of the
College of Allied Medical Professions
Lyceum of the Philippines University-Batangas

The Effect of Bio Reduced Culture Extract of Silver Nanoparticles against ESBL-Producing Escherichia Coli and Klebsiella Pneumoniae

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Abstract

Extended-spectrum beta-lactamase-producing organisms such *as Klebsiella pneumoniae and Escherichia coli* are notoriously obtained in clinical isolates of patients with urinary tract infection. It is primarily caused by invasive pathogens that are continuously increasing due to ineffective treatment. It results in a recurring multi-drug resistant microorganism. Silver nanoparticle is a scientific innovation developed to substitute antibiotic therapy and reduce the exponentiation of MDR-ESBL infections. Bio reduction of silver nanoparticles limits the exposure on carcinogenic reagents and imposes a decreased risk of chemical hazards. The characterized supernatant of *K. pneumoniae and E. coli* synthesized with silver nanoparticle is used to test the inhibitory and bactericidal susceptibility of ESBL-producing bacteria. Piperitone accelerates the nitro reduction of silver nanoparticles which enhances its ability to inhibit the rapid growth of the organism. This study aims to validate the significance of *K.pneumoniae & E.coli* supernatant as a sub-inhibitory factor against clinically isolated ESBL producing *K. pnuemoniae & E.coli.* Recommended action of silver nanoparticles as an effective antimicrobial includes the ability of silver ions to penetrate and interfere with the cell processes resulting in cell destruction using scanning electron microscope and FTIR for confirmation. Clinically, the effective antimicrobial inhibition of the pathogen reduces the probable use of antibiotics. It results in the decrease of the development of multidrug resistant microbes.

Keywords: *Biosynthesis, E. coli, K. pneumoniae, piperitone, silver nanoparticles ESBL*

INTRODUCTION

Extended-spectrum beta-lactamases (ESBLs) is a type of enzyme or chemical produced by several bacteria which enhances its ability to resist antibiotics. Cephalosporin and penicillin, which are often used against ESBL-producing bacteria, can become useless when there is an infection (Shahverdi, Minaeian, Shahverdi, Jamalifar, & Nohi, 2007a). The worldwide emergence of antimicrobial-resistant microbes was commonly caused by the indiscriminate and inadvertent use of antibiotics which continuously spread leading to increase cases of multidrug-resistant pathogens. In contrast, the emergence of ESBL-producing *K. pneumoniae* persists as a crucial part in the development of therapy against multidrug-resistant strains. A more recent report of the Antimicrobial Resistance Surveillance Program (ARSP) of the Department of Health for 2016 presented the prevalence of ESBL-producing *K. pneumoniae* suspects around 40% which was relatively higher than the previous year. Surveillance studies have also shown that presence of ESBL in several countries is increasing with varying frequencies (Lota & Latorre, 2014). Nanotechnology deals with the development of structured particles or equipment at least one dimension within 1 to 100 nanometers. It is a study of atomic and molecular material manipulation (Nathisuwan, Burgess, & Lewis, 2001). Applications of biogenic synthesization of silver nanoparticles include bio labeling and as antimicrobials (Shahverdi et al., 2007a). Silver nanoparticles enhance the antimicrobial activity of nanoparticles, caused by the presence of high surface area which is often compared to bulk atoms (Kalpana & Lee, 2013). The synthesization of silver nanoparticles involving chemical and physical procedures present major disadvantages such as the use of toxic, hazardous chemicals, which will potentially harm our environment and its costly reproduction. Biogenic wastes are often utilized in the production of silver nanoparticles. Biological methods are used to synthesize a stable and potent product that does not cause deterioration of ecological resources and its complex conditions (Gudikandula & Charya Maringanti, 2016). Scientists are currently attempting to synthesize metallic nanoparticles such as cadmium sulfide, silver, and gold from variant strains of microorganisms (Fayaz et al., 2010). Various studies about the effectivity of rapidly reduced silver nanoparticles extracted from fungal, bacterial, and plant culture were published (Naqvi et al., 2013). However, there are limited discussions regarding the characterized and synthesized *K. pneumoniae* and *E. coli* against any variant strain. Silver nanoparticles were reported to possess antibacterial activity which enhances the inhibitory property of the cell-free supernatant isolated

from clinical *E. coli* and *K. pneumoniae (Subashini, Gopiesh Khanna, & Kannabiran, 2014).* Synthesis and characterization of silver nanoparticles are to be validated by Hitachi 5300 UV-vis spectrophotometer. It aims to signify the inhibitory capacity of characterized culture supernatant of *K. pneumoniae* and *E. coli* incorporated with silver nanoparticles. The enhancement of the inhibitory and bactericidal susceptibility is associated with the synergistic interaction of piperitone and isolated culture supernatant leading to the determination of the sub-inhibitory property of piperitone. It also aims to synthesize silver nanoparticles *using E. coli* and *K. pneumoniae* against ESBL-producing *K. pneumoniae* and *E. coli*, characterization of the nanoparticles for its size and appearance using Transmission Electron Microscopy (TEM), screening the synthesized nanoparticles by agar-well diffusion, and determination of Minimum Inhibitory Concentration (MIC) by using a two-fold dilution of silver nanoparticles and Minimum Antibacterial Concentration (MAC).

ARTICLE SELECTION AND SEARCH CRITERIA

The references of this study were based on NCBI, ScienceDirect, and Google Scholar. Search strategy included combinations of the following key words: extended spectrum beta lactamase, silver nanoparticles, *Klebsiella pneumoniae, Escherichia coli*, ESBL and Piperitone. Initial screening of journals was conducted through the analysis of the abstract. Studies included are those that focused on the use of silver nanoparticles as a substitute of antibiotics which also possess inhibitory property against the bacteria. Articles that passed the inclusion criteria were further screened by checking the references for relevant citations. From the total number of studies screened, only 71 were considered relevant for this review article.

EXTENDED-SPECTRUM BETA-LACTAMASE

Nosocomial and community-acquired infections are commonly caused by Extended-Spectrum Beta-Lactamase (ESBL) pathogens. It was originally from the mutation in TEM-1, TEM-2, or SHV-1 genes which are generally seen in the *Enterobacteriaceae* family (Khanfar, Bindayna, Senok, & Botta, 2009). The progression of its activity is inhibited by clavulanic acid. ESBL-producing pathogens were reported to exhibit plasmid-mediated resistance to broad-spectrum Beta-lactam antibiotics by inactivating oxyimino-cephalosporins (Subashini et al., 2014). ESBL is

detected through a double-disc synergy method as per CLSI guidelines. The confirmation of the empirical therapy regimen is complicated and difficult to treat.

Beta-lactam antibiotics are the largest and most common group of antimicrobial agents used worldwide. It is often distinguished by the presence of a predominant chemical structure such as the beta-lactam ring. The beta-lactam antibiotics kill the bacteria through targeting transpeptidase enzymes that synthesize the bacterial cell wall forming a lethal covalent penicilloyl-enzyme complex that blocks the normal transpeptidation reaction. The cross-linked peptidoglycan is weak which makes it susceptible to cell lysis and death (Manikprabhu & Lingappa, 2014). Depressed production of AmpC beta-lactam has previously been documented as a prevalent mechanism of beta-lactam resistance (Tzelepi et al., 2000). The indiscriminate use of antibiotics is the leading cause of multidrug-resistant ESBL-producing microbes. The utilization of alternatives for the control of hospital-acquired infections sparked the interest of researchers (Kar et al., 2016).

Exposure to multidrug-resistant (MDR) Enterobacteriaceae has become a widespread concern in the past years. Nosocomial and community-acquired infections are commonly caused by the Enterobacteriaceae family (Rodriguez-Baño, Gutiérrez-Gutiérrez, Machuca, & Pascual, 2018). This harbor ESBL which is a group of enzymes encoded by genes (Sharma, Pathak, & Srivastava, 2013) and often manifest resistance to antimicrobial drugs (Stürenburg & Mack, 2003). ESBL-producing organisms were first discovered in Germany in 1983 whereas the first isolates in the United States were reported in 1989 (Shanthi & Sekar, 2010). The most common ESBL-producing organisms are *Escherichia coli* and *Klebsiella pneumoniae* (Jeong et al., 2004). Their rapid proliferation has contributed to the several records of infection throughout the world. If not treated adequately, ESBL infected patients are at risk of having a fatal outcome (Stürenburg & Mack, 2003).

Beta-lactamases are grouped according to two schemes namely: Ambler molecular classification which was discovered in 1980, and the Bush-Jacoby-Medeiros functional classification in 1995. The Ambler scheme is classified into four groups according to the enzymes' protein homology. Class A, C, and D enzymes are part of the serine β-lactamase while class B enzymes are included in Metallo-β-lactamases (Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015). On the other hand, Bush's classification is categorized depending on the substrate and inhibitor profiles and physical characteristics (e.g. molecular weight, isoelectric point). There are two subgroups namely:

2be primarily for TEM- and SHV-derived ESBLs, and 2d for OXA-derived ESBLs (Stürenburg & Mack, 2003).

There are more than 300 different ESBL variants and these have been clustered into nine different structural and evolutionary families based on amino acid sequences. Most ESBLs are mutants of temoneira (TEM) and sulfhydryl variable (SHV) class A enzyme, which possess one or more amino acid substitutions around the active site (Sharma et al., 2013). These are often plasmid-mediated (Jeong et al., 2004). The L-lactamases conform to the broad-spectrum prototype, TEM-1 and SHV-1 (Yagi, Kurokawa, Shibata, Shibayama, & Arakawa, 2000). These can hydrolyze ceftazidime, cefotaxime and aztreonam, which are stable to classic TEM and SHV enzymes (Jeong et al., 2004). Although the major ESBL types are TEM and SHV, CTX-M type is more prevalent in other countries (Sharma et al., 2013)

Due to the low incidence of cytotoxicity of beta-lactam antibiotics, it is used traditionally in the hospital setting. Beta-lactamases have major resistance mechanism to beta-lactam antibiotics in gram-negative bacteria (Ishii, Alba, Kimura, Shiroto, & Yamaguchi, 2005). These enzymes resist the bacterial host by hydrolyzing the amide bond of the beta-lactam ring. The spread of beta-lactamase genes is enhanced by plasmids, which is responsible for the transfer of genetic material between microbes. Integrons also take part in the multi-drug resistance mechanism not only for beta-lactams but also for other antibiotics such as aminoglycosides, macrolides, sulphonamides, and chloramphenicol (Wilke, Lovering, & Strynadka, 2005).

The National Committee for Clinical Laboratory Standards (NCCLS) detects the MIC of these antibiotics through the agar-dilution method. Isolates are thought to be ESBL producers when it shows resistance either to cefotaxime, ceftazidime, or ceftriaxone. These strains are kept for further testing through the double-disc synergy method. Kirby-Bauer disks containing the said antibiotics are used to confirm ESBL production (Yagi et al., 2000).

SILVER NANOPARTICLES

Nanoparticles were introduced and evaluated for their antimicrobial and anti-virulence capability. Nanoparticles are monodispersed, crystalline, and successfully employed for drug delivery (Subashini et al., 2014). Nanoparticles vary in sizes which presents a higher surface area to volume ratio. Specific surface is relevant for catalytic activity and other related properties such as antimicrobial activity of AgNPs (Jeevan, Ramya, & Rena, 2012). Uncoupling of the respiratory chain is caused by silver ions. It causes

oxidative phosphorylation or collapse of the proton-motive force across the membrane of the cytoplasm (Gurunathan et al., 2009).

Silver nanoparticles have a size ranging from 1 to 100 nm and vary in shape such as spherical, oval, triangular, hexagonal, cubed, and rod-shaped (Korbekandi, Iravani, & Abbasi, 2009b). It has profound qualities such as high surface-to-volume ratio, low melting point, high superconductivity, transition temperature, low structure stability, specific optical property, and high reactivity (Javaid, Oloketuyi, Khan, & Khan, 2018). Silver nanoparticles are harmless at low concentrations inside the body. Studies have shown excellent bactericidal properties against a wide range of microorganisms and act as an antimicrobial agent even in a solid state (Ahamed, AlSalhi, & Siddiqui, 2010). Aside from its anti-microbial property, these particles can be used as selective coatings for solar energy absorption, material for electrical batteries, optical receptors, catalysts in chemical reactions, bio labels, formula of dental resin composites and coat of medical devices.

In ancient medicine, silver has always been on top choice in treating microorganisms causing infections which led to this time silver-based antimicrobial (Ahamed et al., 2010). Synthesizing silver ions into minute molecule exhibits virulent activity against pathogens even those that are multidrug resistant caused by biofilms (Javaid et al., 2018), thus, inventing silver nanoparticles (Naqvi et al., 2013). Some strongly concluded that silver nanoparticles are size dependent (Naqvi et al., 2013) for it determines the in vivo distribution, biological fate, toxicity and the targeting ability of nanoparticle systems (Lara, Garza-Treviño, Ixtepan-Turrent, & Singh, 2011).

Some researchers proposed that silver nanoparticles dissolved in aqueous solutions prevents the entrapment of an antimicrobial agents to nanoparticle matrix making it a candidate as an microbicidal. It is less cytotoxic and requires a small dosage at low concentration (Lara et al., 2011). The popular and frequently method used is the addition of bacterial supernatant to AgNO3 (Henkel et al., 2014). If silver nanoparticles are combined with antimicrobial agents, it demonstrates a synergistic effect due to the improvement of the potency of antibiotics and may increase the local concentration of the bactericidal effect at the site of action (Dakal, Kumar, Majumdar, & Yadav, 2016).

Mechanism by which silver nanoparticles act as a microbicidal agents are still unknown. However, some researchers argue that silver ions have high affinity to phosphorus and sulfide contents found at the cell membrane and also the interior structure of microorganism (Siddique et al., 2020). DNA, a phosphorus containing compound

which silver nanoparticles targets to attach to, blocks its function to contaminate or even to destruct (Lara et al., 2011).

Scanning transmission electron microscope analysis shows that the nanoparticles are found all throughout the cell which binds with the membrane and invade the inside (Morones et al., 2005). Fourier-transform Infrared (FTIR) spectrum also shows loss of numerous functional groups (present in polysaccharides and protein) that were present in the cellular envelope (Siddique et al., 2020). The changes in morphology presented in the membrane of the bacteria as well as the possible damage caused by the nanoparticles reacting with the DNA (Morones et al., 2005), respiratory chain enzymes and/or interferes through covering permeability to phosphate and protons, and cell division is essential evidence that silver nanoparticles are effective to combat pathogens (Roy, Bulut, Some, Mandal, & Yilmaz, 2019). Reports on Sondi and Salopek-Sondi (2004) studies mentioned that on gram-negative bacteria (e.g *E.coli*), the susceptibility of silver nanoparticles depends on its concentration against pathogens. The cell membrane morphology changes and demonstrate pits formation due to the accumulation of the silver nanoparticles inside the microorganisms causing cell death. Amro et al. (2000) strongly opposed the former study and argues that the depletion of silver ions causes the pits formation of the outer membrane leading to the secretion of lipopolysaccharide molecules and membrane proteins. Growth of yeast and *E.coli* is effectively inhibited whereas mild inhibition is exhibited in *S. aureus* (Morones et al., 2005). These results suggest that the antimicrobial effects of Ag nanoparticles may be associated with characteristics of certain bacterial species. Gram-positive and gram-negative bacteria have differences in their membrane structure, the most distinctive of which is the thickness of the peptidoglycan layer (Kim et al., 2007).

Antimicrobial or antibiotics substances are assessed by the inhibitory and disrupting potential against microorganisms. Evaluating its capability to combat unto pathogens are necessary for the treatment of microbial infections (Chikezie, 2017). One method used to measure the susceptibility of pathogens to various antimicrobials is through MIC which employs tube dilution where antimicrobial agents are added to broths containing inoculated microorganisms and incubated for 18 to 24 hours. Thereafter, testing for bacterial viability is done by sub culturing on agar media prepared without the antibiotic (Whiteway et al., 1998). MIC is determined with the least concentration in which the pathogen is inhibited. Distinction between growth and no growth is examined, and the concentration of inhibitor in the well with no growth is termed as MIC (Chikezie, 2017).

BIOSYNTHESIS

An important area of research in nanotechnology deals with the synthesis of nanoparticles of different chemical compositions and monodispersed sizes (Sastry, Ahmad, Islam Khan, & Kumar, 2003). Chemically, several reports prevailed in literature are toxic to the environment and expensive. Thus, there is a growing need to develop eco-friendly processes, which do not use toxic chemicals in the synthesis protocols. Biotechnological applications such as bioremediation of toxic metals have been employed for a long time. However, the possibility of using such microorganisms in the deliberate synthesis of nanoparticles is a relatively new procedure (Korbekandi et al., 2009b).

The synthesis of nanoparticles usually employs a handful of different methods like particulate processing in a liquid medium or a vacuum, atomistic, and molecular (Klaus-Joerger, Joerger, Olsson, & Granqvist, 2001). There are several physical and chemical methods for the synthesis of metallic nanoparticles (Bhainsa & D'Souza, 2006). Increased prevalence of reports in the literature shows that chemical synthesis of silver nanoparticles is expensive and most of the techniques are environmentally unfriendly (Korbekandi, Iravani, & Abbasi, 2009a). Fulfilling the aim to develop a non-toxic eco-friendly and simple method for the synthesis of silver nanoparticles, researchers embark to use biological system procedures (Bhainsa & D'Souza, 2006). The application of microorganisms ease the expenses for synthesis and has developed great success in the formation of nanoparticles (Korbekandi et al., 2009a). The study has shown that biosynthetic methods employing either biological microorganisms or plant extracts have emerged as a simple and viable alternative to chemical synthetic procedures and physical methods (Sastry et al., 2003).

The researchers found out that microorganisms have the capability of reducing ions to nanoparticles by the conjugation between electron shuttles or other reducing agents such as hydroquinones (Saifuddin, Wong, & Yasumira, 2009). The disadvantage of biologically synthesized nanoparticles is that it has a longer reaction compared to other methods and give rise to nanoparticles at a slower rate (Saifuddin et al., 2009). It has been reported that in the rapid biosynthesis of metallic nanoparticles, the synthetic process was quite fast and silver nanoparticles were formed within 5 minutes of silver ion coming in contact with the cell filtrate (Shahverdi, Minaeian, Shahverdi, Jamalifar, & Nohi, 2007b).

PIPERITONE

NfsA is the major oxygen-insensitive nitro reductase of *Enterobacteriaceae.* It has a low level of flavin reductase activity In the past studies, nitroreduction activity of Enterobacteriaceae was inhibited by plant-based natural products such as piperitone (A. R. Shahverdi, Rafii, Fazeli, & Jamalifar, 2004). Nitroreductases are enzymes capable of accelerating the reduction of nitro aromatic compounds. The end products are biologically inactive (Whiteway et al., 1998). NfsA found in *Escherichia coli* is classified as flavoprotein that can catalyze the reduction of nitro groups under aerobic conditions into different nitro aromatic compounds (Whiteway et al., 1998). In the study of Shahverdi et al. (2004), the reduction of silver occurs when electron shuttles is conjugated with nitro reductases. Natural resources like piperitone has been introduced. Piperitone produces a synergistic effect with nitrofurazone, it constitutes a sub-inhibitory property in the antimicrobial effect towards the elimination of resistant strains. The higher concentration of piperitone showed an enhanced intrinsic antibacterial activity against the strain when inoculated with an antibiotic. To investigate the reduction of silver, piperitone was incorporated with the culture supernatants of *Klebsiella pneumoniae* and other different strains of *Enterobacteriaceae*. The study shows that piperitone can partly impede the reduction of Ag+ to silver nanoparticles (Shahverdi, Rafii, Fazeli, et al., 2004).

KLEBSIELLA PNEUMONIA

Klebsiella pneumoniae is a gram- negative opportunistic microorganism which belongs to the *Enterobacteriaceae* family. It causes serious diseases such as septicemia, pneumonia, urinary tract infections, chronic lung disorders, and nosocomial infections in immunocompromised patients (Vasaikar, Obi, Morobe, & Bisi-Johnson, 2017). The development of therapies against bacterial infection deems *K. pneumoniae,* a critical pathogen to cure. These strains are resistant to ESBLs antibiotics, aminoglycosides, and fluoroquinolones. In a retrospect study on ESBL bacteria, it was shown that there was an increase in the prevalence of ESBL-producing *K. pneumoniae* ranging 10–42% from 1999 to 2013 in the Philippines (Lota & Latorre, 2014). The colonization of *Klebsiella pneumoniae* in both the community and hospital setting are caused by additional virulence factors which varies in multinational setting. It essentially developed into a clinically significant organism subjected as a public health concern.

Multidrug resistant organisms contain antibiotic-resistant genes which are commonly seen and described first in Klebsiella

species. It is also known to spread on other gram-negative bacteria (Okuyama, Nakaso, & Chan, 2002). Acute infections were induced by increasingly frequent resistance of *K. pneumoniae* against the last-line antibiotics. The sudden rise in number is due to the acquisition of multiplying MDR strains (Okuyama et al., 2002). The emergence of hypervirulent strains is relatively connected to the additional genetic virulence. Effective treatments against the strain has elevated scarcity (Paczosa & Mecsas, 2016). According to European Centre for Disease Prevention and Control, more than two thirds of the isolates were resistant to at least one antimicrobial group (Okuyama et al., 2002).

Klebsiella spp. has a high resistance to a variety of beta-lactam antibiotics. It is mainly caused by the ineffective treatment on hospital acquired infections (Pinto et al., 2010). ESBL positive *Klebsiella pneumoniae* are isolated in Intensive Care Units (ICUs) worldwide. It has become the leading cause of nosocomial infections and sepsis. It was among the 2017 list of World Health Organization as one of the most dangerous superbugs. All clinical isolates were resistant to aminopicillins and third-generation cephalosporins but were all sensitive to meropenem, amikacin and ciprofloxacin (Khaertynov et al., 2018). Carbapanems, a Beta-lactam antibiotic is usually the treatment of choice for ESBL-producing *Klebsiella pneumoniae*. It is considered as the final course of action for the management of this life-threatening condition (Pinto et al., 2010).

ESCHERICHIA COLI

Escherichia coli is a gram-negative bacillus measuring 1 um long and 0.5 um wide, which varies among different strains. It is a facultative anaerobic microorganism which can endure oxygenation at different levels. The increased availability of oxygen will cause a micro aerobic state wherein both fermentative and respiratory pathways are active intermediately (Henkel et al., 2014). *E. coli* and *K. pneumoniae* are capable of hydrolyzing penicillin, broad spectrum due to its predominant enzyme production. One of the major causes of increased mortality and morbidity rate is associated with therapeutic failure and poor infection control program against MDR ESBL-producing *E.coli* (Kar et al., 2016) . They are virulent enteropathogens. Most are gut commensals that cause harm if they reach other body sites such as the urinary tract where *E. coli* is the most common pathogen (Day et al., 2019). Extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-*E coli*) are the largest group of multidrug-resistant pathogens from bacteremia in the UK (Day et al., 2019). These are capable of hydrolyzing penicillin (e.g.

ampicillin and piperacillin), cephalosporins of the first, second, third and fourth generations (Perez, Endimiani, Hujer, & Bonomo, 2007).

CONCLUSION

Emergence of antimicrobial agents against multi-drug resistant pathogens including ESBL-producing *K. pneumoniae* and *E. coli* has led the researchers to conduct and promote an eco-friendly, less toxic for humans and stronger antimicrobial agents. It was found out that silver nanoparticles which exhibit wide-range reactivity in inhibiting and destroying microbes, has the potential to combat these microorganisms. It directly interacts with the membrane and interior of the cell, by synthesizing silver into minute molecules and increasing the susceptibility of microbes thus, decreasing the MIC of *E. coli* and *K. pneumoniae* with silver nanoparticles solution. Silver nanoparticles react effectively against gram-negative bacteria proposing that these microorganisms have a thin layer of peptidoglycan as its cell wall.

Low-cost approach for the reduction of silver nitrate solution were made to form nanoparticles and the use of toxic chemicals were limited. The enzymes secreted by *E. coli* and *K. pneumoniae* in synthesizing the nanoparticles which inhibit ESBL produced by the same microorganism imitate the vaccine's mechanism of action.

Piperitone induces a sub-inhibitory result magnifying the antibacterial activity of the bioreduced AgNP. Incorporation of bacterial supernatants exhibit a higher biocidal efficacy due to reactive oxygen produced by silver nanoparticles. It affects the growth inhibition of ESBL-producing microorganisms making it a reliable source of antibacterial agent incorporated in susceptibility testing and a disinfectant for medical devices which causes prevalent nosocomial infection.

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