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SYNTHESIS OF SILVER NANOPARTICLES BY GUT OF SILKWORM, *BOMBYX MORI* (L.) (LEPIDOPTERA: BOMBYCIDAE) USING BACTERIAL PATHOGENS AND THEIR ANTIBACTERIAL ACTIVITY

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ABSTRACT

Objective: Silver nanoparticles are nanoparticles of silver precursor (or) silver oxides like silver nitrate, silver citrate and silver acetate. Silver nanoparticles sizes ranges between 1 nm and 100 nm. This study was undertaken to analyze the antibacterial activity of green synthesized silver nanoparticles against silkworm bacterial pathogens. Methods: Antimicrobial activity studies were adopted by the methods of *in vitro* and *in vivo* studies. In the *in vitro* study, using the disc diffusion and agar well diffusion methods. The bacterial strains were isolated from gut of the V instar of diseased silkworm, *Bombyx mori* larvae. Results: The zone of inhibition of silver nanoparticles against the gram negative *Bacilli*-I (8 mm and 10 mm), gram negative *Bacilli*-II (9 mm and 11 mm) and gram positive *Cocci* (8 mm and 9 mm) in disc and agar well diffusion (zone of inhibition) methods respectively. The antibacterial activity was found to be positive in all the three bacteria. In the *in vivo* study, the bacterial strains were isolated from the gut region of control and silver nanoparticles treated MR_2 mulberry leaves fed V instar of diseased *Bombyx mori* larvae. The counting of gram negative *Bacilli*-I (3850 and 225 colonies), gram negative *Bacilli*-II (2270 and 176 colonies) and gram positive *Cocci* (3680 and 210 colonies) for control and silver nanoparticles treated MR_2 mulberry leaves fed group respectively. Conclusion: In this two studies were indicating the all three bacterial strains were not resistant to the silver nanoparticles and also the silver nanoparticles were recorded the positive antibacterial activity against silkworm bacterial pathogens.

Keywords: *Bombyx mori*, Silver nanoparticles, Antibacterial activity, Bacterial pathogens .

1.INTRODUCTION

Antibacterial properties of silver are documented since 1000 BC, when silver vessels were used to preserve water. The first scientific papers describing the medical use of silver report the prevention of eye infection in neonates in 1881 and internal antisepsis in 1901. After this, silver nitrate and silver sulfadiazine have been widely used for the treatment of superficial and deep dermal burns of wounds and for the removal of warts (Rai et al., 2009). Silver's mode of action is presumed to be dependent on Ag^+ ions, which strongly inhibit bacterial growth through suppression of respiratory enzymes and electron transport components and through interference with DNA functions(Li et al., 2006).

Silver in a nanometric scale (less than 100 nm) has different catalytical properties compared with those attributed to the bulk form of the noble metal, like surface Plasmon resonance, large effective scattering cross section of individual silver nanoparticles, and strong toxicity to a wide range of microorganisms (Elechiguerra et al., 2005). The antibacterial activity of silver has been well known since ancient times (Holt and Bard, 2005; Shrivastava et al., 2007) and it has been demonstrated that, in low concentrations, silver is non-toxic to human cells (Zhang et al., 2003; Pal et al., 2007). The actual bactericide mechanism of silver nanoparticles is not well known. Some researcher support the idea that silver

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species release Ag^+ ions and they interact with the thiol groups in bacteria proteins, affecting the replication of DNA(Marini et al., 2007). It has also been reported that Ag^+ ions uncouple the respiratory chain from oxidative phosphorylation or collapse the proton-motive force across the cytoplasmic membrane (Holt and Bard, 2005). Silver nanoparticles interactions with bacteria are dependent on the size and shape of the nanoparticles(Pal et al., 2007; Panacek et al., 2006; Morones et al., 2005).

Yoon *et al*.,(2008) have defined the antibacterial activity of silver nanoparticles in four types of Gram negative bacteria: *Escherichia coli, Vibrio cholera, Pseudomonas aeruginosa, and Salmonella tiphy* and suggested that silver nanoparticles attach to the surface of the cell membrane and disturb its function, penetrate bacteria, and release silver ions. Other groups determined a similar antibacterial activity in Gram positive bacteria, such as *Bacillus subtilis* (Yoon et al., 2008), *S. aureus* (Shrivastava et al., 2007) and *Enterococcus faecalis* (Panacek et al., 2006). Silver nanoparticles have also been found to exert antibacterial activity against some drug-resistant bacteria (Birla et al., 2009; Inoue et al., 2009). In order to have bactericidal effect silver must be released into the solution. The efficacy of the solution is dependent on the concentration and shape of silver ions present. Additionally, silver interacts with structural proteins and preferentially binds with DNA bases to inhibit replication (Silver, 2003; Silver et al., 2006). Furthermore, bactericidal effect of silver has also been attributed to inactivation of the enzyme phosphomannose isomerase. A novel and unique biological properties of metal nanoparticles of gold, silver and platinum. These nanoparticles have the properties to interact specifically with selected proteins and inhibit their activities. Since many diseases such as cancer, arthritis, macular degeneration, etc, are dependent on angiogenesis, these discoveries open new possibilities to inactivate the function of "disease inducing" protein by metal nanoparticles and surface modified metal nanoparticles (Bhattacharya and Mukherjee, 2008).

2. MATERIALS AND METHODS

Isolation of bacteria from the gut of diseased Bombyx mori 5th instar larvae

All the procedures followed for the isolation of the bacteria from the gut of the diseased *Bombyx mori* larvae was strictly done with aseptic precautionary methods. Gut of the larvae were carefully separated and macerated. The macerated content was diluted with 10 ml of sterile distilled water. A loop full of the macerated gut content was inoculated on to Brain heart infusion agar (BHIA), incubated at 37 °C for 24 h then the growth of the bacteria was examined for the presence of different types of bacterial colony. Based on the size, shape, colour, the bacterial colonies were differentiated. Over all three different types of bacterial colonies (common bacteria present in all larvae) had been selected and included in the antibacterial assay (Bhattacharya and Mukherjee, 2008).

Bacterial inoculum and Testing Agent

Three different bacterial species, isolated from the gut of the diseased *Bombyx mori* larvae, and three different bacterial species of clinical source was selected to perform the antimicrobial activity of the silver nanoparticles. Two different gram negative bacteria and one gram positive *Cocci* (species not identified) isolated from the gut was used in this study. All the bacterial strains were sub cultured from the stock culture and 24 h grown fresh cultures had been mixed with normal saline (2 loop full culture + 1ml saline) and it was adjusted to Mac Farland opacity no 0.5 (10^8 cells/ml), and was used in the antibacterial assay. Silver nanoparticles were the selected agent to be tested against the bacterial isolates included in this study. (Oxaciline (+C) and distilled water (−C) was used as positive and negative control respectively).

In vivo and in vitro antibacterial assay of silver nanoparticles

Totally 30 diseased V instar *Bombyx mori* larvae had been chosen for this test. Among 30 V instar diseased larvae, 15 larvae were fed with control mulberry leaves and for 15 larvae fed silver nanoparticles treated MR_2 mulberry leaves for five days. On 6th day, all the *Bombyx mori* larvae were dissected and their gut was removed and finely macerated, and used for the antibacterial assay (Nataraju et al., 2005). The macerated gut material was serially diluted and inoculated on Brain heart infusion agar (BHIA). The inoculated plates were incubated at 37 °C for 24 h, and then the bacterial counts had been read and recorded. Nutrient agar (NA) was used in this assay. The sterile filter paper (Whatman filter paper no.1) of 6 mm was charged with silver nanoparticles solution and placed on nutrient agar (NA) medium inoculated with bacterial species. The same way 100 µl of silver nanoparticles solution was dispensed in wells made in Nutrient Agar inoculated with the bacterial isolates. The above said plates were incubated at 37 °C for 24 h. After 24 h, antibacterial activity was read and recorded. The *in vivo* antibacterial activity of the silver nanoparticles was performed in view of analyze and to compare the *in vitro* antibacterial activity of the silver nanoparticles by counting the bacterial colonies after the oral administration of the silver nanoparticles to the diseased V instar *Bombyx mori* larvae (Nataraju et al., 2005).

3. RESULTS

In vitro antimicrobial activity of silver nanoparticles by disc diffusion method

Table 1 shows that the *in vitro* antibacterial activity of silver nanoparticles against the gram negative *Bacilli*-I, gram negative *Bacilli*-II and gram positive *Cocci*. Among this, as par disc diffusion test, it was obtained. The antibacterial activity was found to be positive in all the three bacteria. The zone of inhibition was recorded in gram negative *Bacilli*-I (8mm), gram negative *Bacilli*-II (9mm) and gram positive *Cocci* (8mm) respectively. All the three bacterial isolates were not resistant to the silver nanoparticles in the disc diffusion method and the silver nanoparticles recorded as positive for the antibacterial activity against gut bacterial strains of diseased *Bombyx mori* V instar larvae. The other testing agents control positive C+ (Oxaciline) zone of inhibition was recorded in gram negative *Bacilli*-I (15mm), gram negative *Bacilli*-II (13mm) and gram positive *Cocci* (12mm) respectively. The control negative C- (Distilled water), ware not form the zone of inhibition against all the three bacterial strains.

In vitro antimicrobial activity of silver nanoparticles by agar well diffusion method

Table 1 shows that the *in vitro* antibacterial activity of silver nanoparticles against the gram negative *Bacilli*-I, gram negative *Bacilli*-II and gram positive *Cocci*. Among this, as par agar well diffusion test, it was obtained. In this three bacterial strains, the zone of inhibition was recorded in gram negative *Bacilli*-I (10mm), gram negative *Bacilli*-II (11mm) and gram positive *Cocci* (9mm) respectively. All the three bacterial isolates were not resistant to the silver nanoparticles in the agar well diffusion method and the silver nanoparticles recorded as positive for the antibacterial activity against gut bacterial strains of diseased *Bombyx mori* V instar larvae. The other testing agents control positive C+ (Oxaciline) zone of inhibition was recorded in gram negative *Bacilli*-I (18mm), gram negative *Bacilli*-II (15mm) and gram positive *Cocci* (18mm) respectively. The control negative C- (Distilled water), ware not form the zone of inhibition against all the three bacterial strains.

In vivo antibacterial activity of silver nanoparticles by counting of bacterial colonies

Table 2 shows that the *in vivo* antibacterial activity of silver nanoparticles against the gram negative *Bacilli*-I, gram negative *Bacilli*-II and gram positive *Cocci* bacterial strains were isolated from the gut of diseased *Bombyx mori* V instar larvae fed by control MR_2 mulberry leaves and silver nanoparticles treated MR_2 mulberry leaves fed larvae. Among this, the bacterial colonies obtained test group, Control MR_2 mulberry leaved fed larval gut isolated *Bacilli*-I obtained 3850 colonies, gram negative *Bacilli*-II obtained 2270 colonies and gram positive *Cocci* obtained 3680 colonies. Silver nanoparticles treated MR_2 mulberry leaves fed larvae gut isolated bacterial strain *Bacilli*-I were obtained (225 colonies), gram negative *Bacilli*-II were obtained (176 colonies) and gram positive *Cocci* were obtained (210 colonies). The silver nanoparticles treated MR_2 mulberry leaves fed diseased *Bombyx mori* V instar larvae gut were consist of minimum counting of pathogenic bacteria compare to control MR_2 mulberry leaves fed diseased *Bombyx mori* V instar larvae, in this reason silver nanoparticles have been antibacterial activity and also control the pathogenic bacterial growth of gut region of *Bombyx mori* larvae.

Table 1. *In vitro* antibacterial activity of silver nanoparticles by disc diffusion and agar well diffusion methods against diseased *Bombyx mori* V instar larvae gut bacterial strains

S.No	Bacterial strains	Zone of inhibition (mm) Disc diffusion method (diameter in mm) TS	C+	C–	Agar well diffusion method (diameter in mm) TS	C+	C–
1	Gram negative *Bacilli* – I	8mm	15mm	0mm	10mm	18mm	0mm
2	Gram negative *Bacilli* –II	9mm	13mm	0mm	11mm	15mm	0mm
3	Gram positive *Cocci*	8mm	12mm	0mm	9mm	18mm	0mm

Test Sample (TS) – silver nanoparticles (100µl), Positive Control (C+) – Oxaciline (100µl), Negative Control (C–) – Distilled water (100µl)

Table 2. *In vivo* antibacterial activity of silver nanoparticles against diseased *Bombyx mori* V instar larvae gut bacterial strains

S. No	Bacteria Strains	(serial dilution method) Control	Treated
1	Gram negative *Bacilli – I*	3850 colonies	225 colonies
2	Gram negative *Bacilli – II*	2270 colonies	176 colonies
3	Gram positive *Cocci*	3680 colonies	210 colonies

4. DISCUSSION

A study of the fundamental questions concerning the mechanism of antibacterial activity of silver nanoparticles is in the initial stage of its development. One can find diametrically opposing points of view on the mechanism of the interaction of nanoparticles with bacteria in publications (Panacek et al., 2006; Raffi et al., 2008). On the basis of the results of previous research (Morones et al., 2005) one can define three stages of interactions between nanoparticles and the cell; (1) The binding of nanoparticles with the membrane and the considerable increase in its permeability and the respiratory depression of bacteria, (2) Penetration through the membrane and interaction with sulfur and phosphorus containing groups of substances (components of cytoplasm space) and (3) Further penetration inside the cell, the interaction with nucleic acids, and the loss of the ability to replicate and divide. 7 nm silver nanoparticles present the best antibacterial activity against *E. coli* and *S. aureus.* Because of their size, 7 nm silver nanoparticles can easily reach the nuclear content of bacteria and they present the greatest surface area; therefore the contact with bacteria is the greatest (Lok et al., 2006). The bactericidal properties of silver nanoparticles are size dependent and that the only nanoparticles that present a direct interaction with the bacteria or virus preferentially have a diameter of 1–10 nm[3, 10]. A smaller size implies the ability to reach structures that otherwise is not available for bigger nanoparticles. Silver compounds are not specific and have several targets that can be present in both eukaryotic and bacterial cells. However, bacteria have a larger surface area to volume ratio than eukaryotic cells, which allows for rapid uptake and intracellular distribution of nutrients and excretion of wastes. This characteristic is achieved by having a rigid cell wall composed of peptidoglycan (Murray et al., 2005).

Silver based nanoparticles of approximately 10 nm inhibit Methicillin resistant *Staphylococcus aureus* (MRSA) growth *in vitro* at noncytotoxic concentrations, supporting their potential use as antibacterial agents with a wide number of biomedical and therapeutic applications. Since drug resistance does not interfere with the bactericidal effect of nanosilver, they may prove useful in manufacturing pharmaceutical products and medical devices that may help to prevent the transmission of drug resistant pathogens, but toxicological limitations for eukaryotic cells should be taken in account since nano-silver is not a target specific antibacterial agent (Ayala-Nunez et al., 2009). The data presented here are novel in that they prove that silver nanoparticles are effective bactericidal agents against Methicillin resistant *Staphylococcus aureus* regardless of the resistance

mechanisms that confer importance to these bacteria as an emergent pathogen. Besides, it is the first time that the efficacy and safety of nano-silver in different sizes is determined for Methicillin resistant *Staphylococcus aureus* and human cells *in vitro* (Ayala-Nunez et al., 2009).

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