

Larvicidal and Genotoxic Activity of Silver Nanoparticle Synthesized from *Bacillus megaterium* against *Aedes aegypti* (Diptera: Culicidae)

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Abstract: Mosquitoes are one of the most important insect pests that affect the health and wellbeing of humans and domestic animals worldwide. They can cause a variety of health problems due to their ability to transfer (vector) viruses and other disease-causing pathogens. Female mosquitoes require a blood meal for egg production, and they can produce a painful bite as they feed. While feeding they can transmit to humans and other animals – brain inflammation (encephalitis), dengue fever, yellow fever, malaria and filariasis. The most effective strategy for mosquito management in communities in general is prevention. The best way to prevent mosquito-borne diseases is to minimize the number of mosquitoes and to prevent them from breeding in the first place. Therefore, the exploration of more –effective and eco-friendly techniques to control mosquitoes seems to be very promising. There are many serious drawbacks in the use of synthetic insecticides for vector control. This factor created the need for environmentally safe, degradable and target specific insecticides against mosquitoes. Nanotechnology gives the promising report on wide range of applications especially in vector control. The main focus of the present study is to obtain effective and efficient synthesis of silver nanoparticles (AgNPs) from *Bacillus megatarium* and their applicability on larvicidal activity and genotoxicity against *Aedes aegypti*. In conclusion, our findings showed that AgNPs of *Bacillus megatarium* can be developed as ecofriendly larvicides and have the potential to be used as an ecofriendly approach for the control of the *Aedes aegypti*.

Index terms: Silver nanoparticles, *Bacillus megaterium*, mosquito control, larvicidal activity, dengue.

I. INTRODUCTION

Mosquitoes are one of the most life-threatening vectors which are responsible for transmitting diseases such as malaria, dengue, chikungunya, Japanese encephalitis, and lymphatic filariasis (Najitha Banu et al., 2014). In this verad, *Aedes aegypti* is the principal vector for flaviviruses which causes yellow fever and dengue fever (Barrett and Higgs, 2007; Halstead, 2008) and alphavirus which causes chikungunya (Ligon, 2006). Several mosquitoes of the *Aedes species* have developed resistance towards various mosquito control methods for example, have developed tolerant to temephos, an organophosphate larvicide (Boyer et al., 2006) *Aedes albopictus* larvae also has the ability to increase their tolerance to benzothiazole and pentachlorophenol (Suwanchaichinda and Brattsen, 2001, 2002). Therefore, a novel and efficient way to control the mosquitoes is essential. An ideal way to prevent the vector-borne diseases is to prevent them from emergence (Najitha Banu et al., 2014). *Bacillus megaterium*, a Gram-positive bacterium, is capable of effectively binding with metals than Gram negative bacteria (Beveridge and Fyfe, 1985). The silver nanoparticles (AgNPs) synthesized from *Bacillus megaterium* act as an effective antimicrobial agent against bacteria such as *Staphylococcus aureus*, *Escherichia-coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris* (Dahikar and Bhutada, 2013). Nanoparticles act as versatile compounds and can be used in pesticides, insecticides, and insect repellants (Owolade et al., 2008). Biological synthesis of AgNPs using microorganisms, enzymes, and plant extracts is suggested as an eco-friendly alternative to chemical methods (Ahmad et al,2004). In this study, the larvicidal and genotoxic activities of AgNPs synthesized from *Bacillus megaterium* against *Aedes aegypti* were determined. Comet assay SCGE (Single Cell Gel Electrophoresis) is widely used to identify the DNA damage of prokaryotic and eukaryotic organisms. This assay proves to be a rapid, sensitive, and economical method to identify the damage of DNA strands in an individual eukaryotic cell (Chaudhry et al, 2006). Comet assay has been applied in many areas such as environmental monitoring (Cavallo et al., 2002), *in-vivo* and *in-vitro* genotoxicity (Godard et al., 1999), epidemiological and bio-monitoring studies in humans who are exposed to the toxic environment (Valverde and Rojas, 2009). Only a few studies have been reported on DNA damage in insects which are exposed to toxic environment including *D.melanogaster* (Rahman et al., 2002). This technique has been recommended by the Committee of Mutagenicity Guidelines of the UK Department of Health (COM) for determining *in vitro* mutagenicity of chemicals (Mutagenicity of chemicals in food, consumer products and the environment).

II. RESEARCH METHODOLOGY

2.1 Bacteria

Bacillus megaterium (MTCC 428) culture were procured from the Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

2.2 Biosynthesis of silver nanoparticles

The characterized isolate was inoculated into sterile nutrient broth and 2g of wet biomass was harvested at different points of time. The biomass obtained was washed with phosphate buffer (pH 7.0) thrice and collected in a 500-ml Erlenmeyer flask. Using deionized water, 1mM solution of AgNO₃ was prepared, and 100 ml of the solution was added to the biomass harvested at each point of time. The Erlenmeyer flasks were incubated at 37°C under agitation (200rpm) for 24 h.

2.3 Sonication of samples

The cells from each Erlenmeyer flask were washed twice with 50-mM phosphate buffer (pH 7.0) and re-suspended in 1 ml of the same buffer. Ultrasonic disruption of cells was carried out with an ultrasonic processor over three 45-s periods, and with an interval of 45 s between periods. The sonicated samples were centrifuged at 15,000 rpm for 30min at 4°C to remove cell debris. The supernatants were then used for the characterization of silver nanoparticles.

2.4 Characterization of AgNPS

After 48 hours of incubation, the preliminary detection of Ag-NPs was carried out by visual observation of color change of the cell filtrate. These samples were later subjected to optical measurements, which were carried out using a UV-Vis spectrophotometer (Shimadzu 1650 PC, include manufacturer details) at 430 nm with a resolution of 1 nm. For Fourier transform infrared (FTIR) analysis, the FTIR spectrum of the dried sample was recorded on a Shimadzu 8400s instrument in the range 750 to 4000 cm⁻¹ at a resolution of 1 cm⁻¹.

2.5 Determination of the larvicidal activity of silver nanoparticles

For each test, 25 late fourth instar of mosquito larvae was kept in different beakers containing 250 ml of dechlorinated water. Nanoparticles were added to the beakers at different concentration. For each concentration, five replicates were performed. Each test included a set of control groups. Larval mortality was recorded after 24hrs of exposure.

2.5 morphological variations of mosquito larvae

The morphological variations in mosquito larvae treated with various concentrations of silver nanoparticles and control alone were observed using inverted phase contrast microscope (RADICAL).

2.6 Apoptosis analysis acridine orange staining

To investigate the role of apoptosis by AgNPs toxicity, acridine orange staining of nanoparticle-treated mosquito larvae was performed. Mosquito larvae treated with various concentrations of silver nanoparticles were gently placed in the cavity slides. 10 μ L of acridine orange stain from the stock of 5 μ g/mL was added to the mosquito larvae for 20 minutes at room temperature. Mosquito larvae were washed in phosphate buffer saline (1X PBS, pH 7.4). Stained samples were observed under inverted fluorescence microscope (RADICAL).

2.7 Single-cell gel electrophoresis (Comet assay)

The assay was performed to determine the DNA damage (Singh et al, 1988). 25 late fourth instar of mosquito larvae was collected 24 hours after treatment from the control and LC50 value and were pooled for a comet assay. Treated organisms were placed in 1mL of phosphate-buffered saline (PBS) containing 20-mM ethylene diamine tetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO) and disintegrated mechanically by mincing. The cell suspension was precipitated by vortexing and then immediately mixed with 100 μ L of 1% low-melting agarose (LMA) at 39°C and spread on a fully frosted microscopic slide pre-coated with 200 μ L of 1% normal melting agarose. After the solidification of agarose, slides were covered with another 75 μ L of 0.5% LMA and then immersed in lysis solution (2.5M NaCl, 10mM Na-EDTA, 10mM Tris, 1% Trion X100, and 10% DMSO, pH 10) for 1 hour at 4°C. The slides were then placed in a gel electrophoresis apparatus (containing 300mM NaOH and 10mM Na-EDTA, pH 13) for 40 minutes to allow DNA unwinding and alkali labile damage. Then an electrical field (3000mA, 25V) was applied for 20min at 4°C to draw the negatively charged DNA toward an anode. After electrophoresis, slides were washed thrice for 5 min at 4°C in a neutralizing buffer (0.4M Tris, pH 7.5), followed by staining with 75 μ L of propidium iodide (40 μ g/mL) and then the slides were observed using fluorescence microscopy.

2.8 Statistical analysis

Mortality data was subjected to probit analysis to predict the LC50 and LC90 values by using SPSS 10.0. Percentage mortality was also calculated for the mortality data using Microsoft Excel 2007.

III. RESULTS

3.1 Biosynthesis of silver nanoparticles

In this research study, the formations of AgNPs were determined by analyzing the color change from yellowish to brown (Fig. 1). UV-Vis spectroscopy is a preliminary and important step in the confirmation of AgNPs formation in aqueous solution. The surface Plasmon resonance absorption band reflects the intensity and the band width which are influenced by the structure of the nanoparticles, and the dielectric constant of the solution. The optical absorption band peak is observed at 470nm (Fig. 2). The AgNPs were further characterized using FTIR (Fig. 3), and the absorbance bands observed in the region of 400-4000cm were 2923-NH Stretching, 1609-C=O stretching, 1384-CN Stretching, 1051 absorbent band are known to be associated with C-O groups. The AgNPs were spherical in shape and found to be 2nm in size. The *B. megaterium* AgNPs also produced a characteristic peak during the bio-reduction of Ag^+ to Ag^0 (Fig. 4). The SEM images of samples (Fig. 5) serve as the morphological evidence of the AgNPs. The efficacy (LC_{50} and LC_{90}) of *B. megaterium* AgNPs is shown in Table 1. The treated *A. aegypti* larvae suffered higher mortality rate when treated with *B. megaterium* AgNPs. The LC_{50} and LC_{90} values of the tested concentration are 6.28 and 17.42 ppm with respect to 2, 5, 10, 15, and 20 ppm of *B. megaterium* AgNPs. The percent mortality of the tested concentration is 08.34, 09.00, 10.67, 16.33, and 19.67 with respect to the above ppm concentration at 24 hrs. The mortality rate is directly proportional to the concentration.

The death of both the control and treated larvae by AgNPs were further confirmed by morphological analysis using acridine orange staining. In the treated larvae, AgNPs were found in siphon region, tail, thorax, and gut regions, and in most of the body regions after 24 hrs when given at 2 and 5 ppm, 10 and 15 ppm, and 20 ppm, respectively. Whereas in case of control larvae, no significant stains of AgNPs were found. Furthermore, the DNA damage study of *A. aegypti* larvae treated with AgNPs and control was performed. This is the first report of an analysis of DNA damage by comet assay. The study observation showed tail damage of lengths 87 μ m and 40 μ m at LC_{90} and LC_{50} , respectively, for 24-hrs treatment and extent of the tail DNA damage was 3.18% and 31.70% at LC_{50} and LC_{90} , respectively; the control larvae showed no damage.



Fig. 3.1. Synthesis of silver nanoparticles.

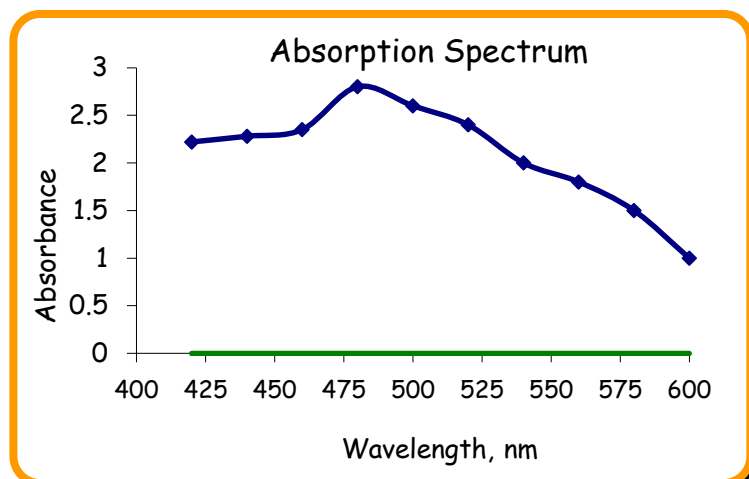


Fig. 3.2. UV-Visible Spectroscopic spectrum showing the presence of AgNPs at 470nm range.

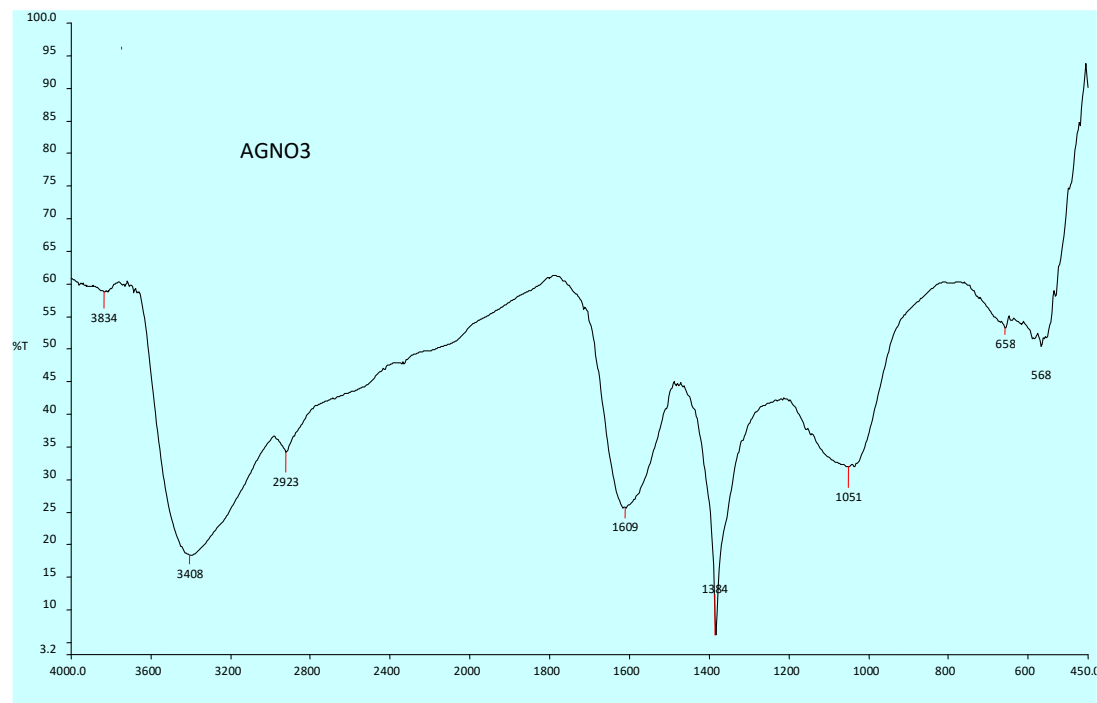


Fig. 3.3. FTIR spectrum of synthesized silver nanoparticles.

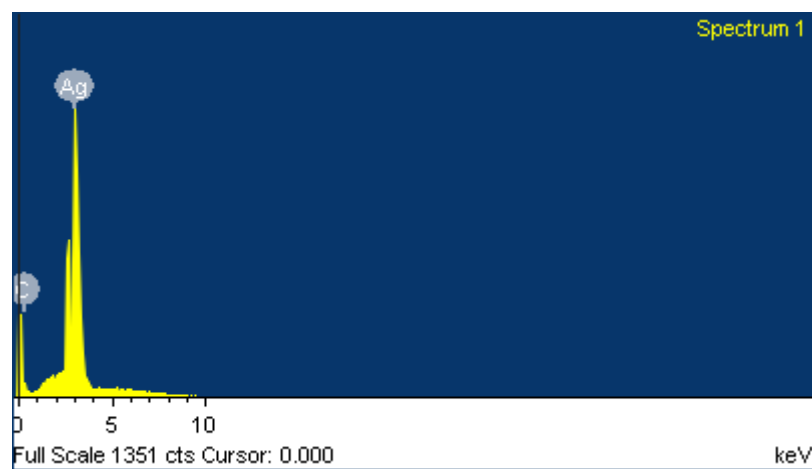


Fig. 3.4. Energy Dispersive X-ray (EDX) Spectroscopic spectrum of nanoparticles.

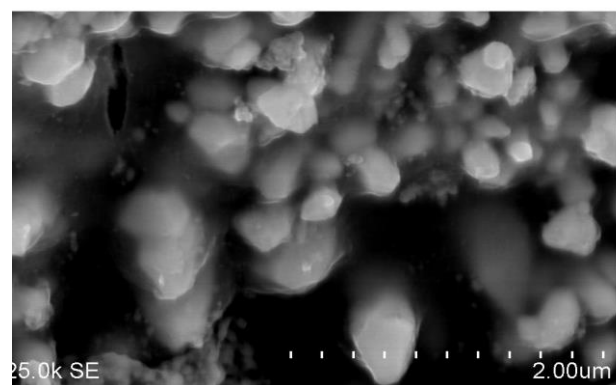


Fig. 3.5. SEM image of the silver nanoparticles.

DETERMINATION OF THE LARVICIDAL ACTIVITY OF SILVER NANOPARTICLES:

Table 3.2 Larval mortality in silver nanoparticles.

S.No	Time (Hrs)	Number of Larvae	Concentration (ppm)	Mortality
1	24	20	2	08.34 ± 0.05
2	24	20	5	09.00 ± 0.07
3	24	20	10	10.67 ± 0.24
4	24	20	15	16.33 ± 0.21
5	24	20	20	19.67 ± 0.15

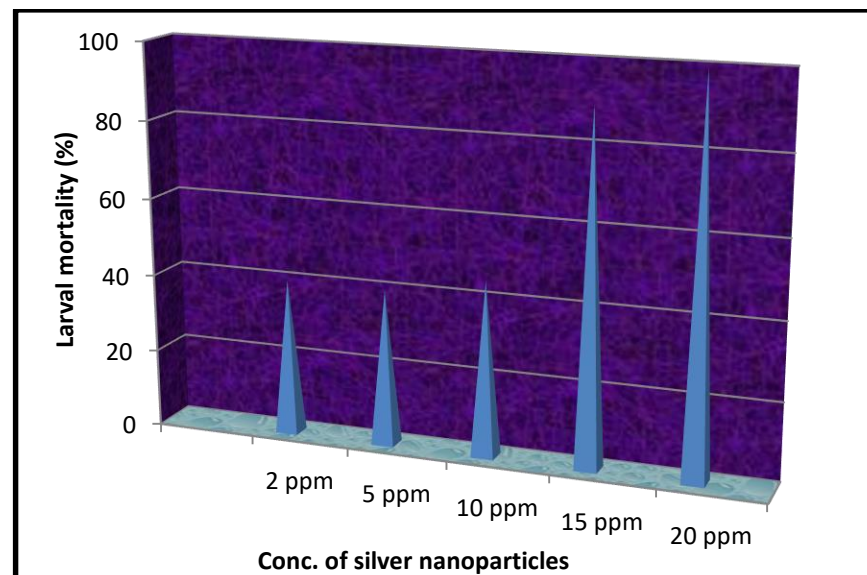


Fig. 3.6 Percentage larval mortality in silver nanoparticles. LC50 = 6.28 ppm; LC90 = 17.42 ppm.

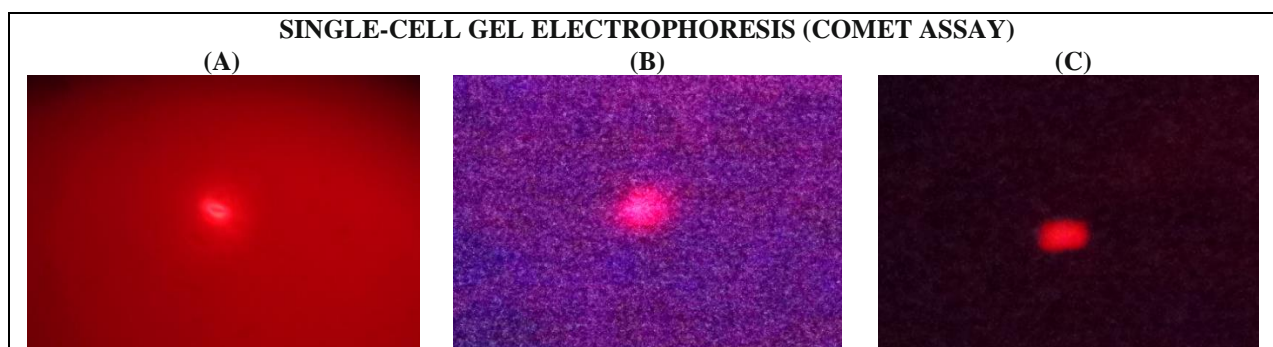
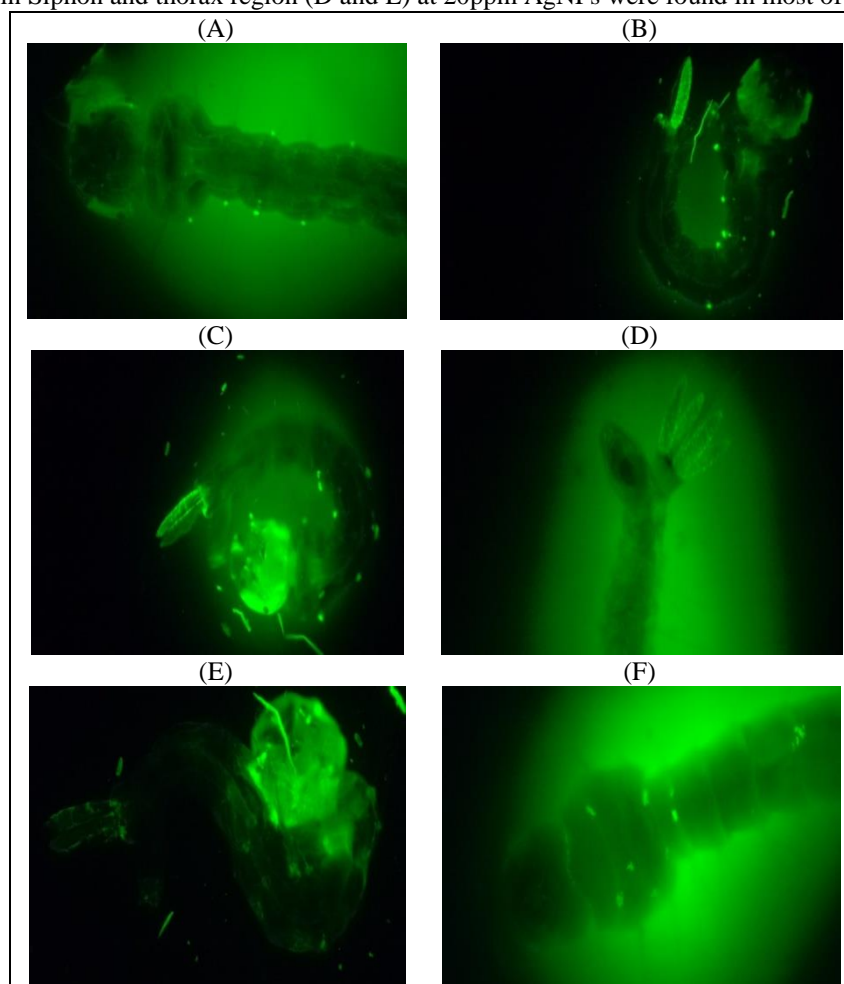
Fig. 3.7 Fluorescence microscope images (comets) of the propidium iodide-stained DNA of *Aedes aegypti* larvae cells exposed to 20ppm of AgNPs (c), minimum concentration of 2ppm of AgNPs (b), and as compared with the control (a) (40x magnification).

Table 3.2 Comet assay parameters using CASP software.

COMET ASSAY PARAMETER	CONTROL	LC ₅₀ VALUE	MAXIMUM CONCENTRATION
Length of head	113	141	221
Length of the tail	20	40	87
Length of the COMET	133	181	308
Head DNA %	99.2666	96.8111	68.2926
Tail DNA %	0.733394	3.18888	31.7074
Tail movement	0.146679	1.27555	27.5855
Olive tail movement	0.461868	2.87841	32.336

Fig. 3.8 Control (A), concentration of 2 and 5ppm AgNPs found in thorax and Siphon regions (B and C), at 10 and 15ppm nanoparticles were found in Siphon and thorax region (D and E) at 20ppm AgNPs were found in most of the body regions (F).



IV. DISCUSSION

In the present study, AgNPs are biosynthesized from *B. megaterium* and confirmed by the color change observed at 430nm in UV Spectroscopy. The bio-reduction of Ag^+ is being combined with the metabolic process utilizing nitrate (Lenge 2007). Nitrate reductase is an enzyme which plays a vital role in synthesizing AgNPs from the bacteria (Anilkumar et al, 2007; Kalimuthu et al, 2008). The morphological appearance was spherical and the size ranges were at 49nm. Highly reactive silver cations tend to bind strongly to electron donor groups which contains sulfur, O_2 , or N_2 ; therefore, the AgNPs synthesized by biological process will be stable for several weeks (Dahikar et al, 2013). *Aedes aegypti* larvae treated with AgNPs showed an effective mortality rate against LC_{50} at 6.28ppm and LC_{90} at 17.42ppm at 24hrs.

This study also examined the morphological and DNA damage in the larvae treated with AgNPs using acridine orange staining. At concentrations of 2 and 5ppm, traces of AgNPs were found in thorax and siphon region of larvae. At 10 and 15ppm, AgNPs were found in siphon and thorax region, and at 20ppm AgNPs were found in most of the body regions after 24hrs. Whereas in case of control larvae, no significant stains of AgNPs were found.

An organism continuously exposed to environmental stress will undergo DNA damage. In a normal living cell, Reactive Oxygen Species (ROS) will be generated in fewer amounts and were regulated by various metabolic processes such as signal transduction pathway etc. However, if the ROS production increases, it can cause severe damage to DNA, protein and lipids (Vera Garaj-Vrhovac et al, 2010). The extent of DNA damage in the treated larvae showed that the length of the tail damage is 87 μ m and 40 μ m at LC_{90} and LC_{50} at 24hrs treatment, respectively, and the extent of the tail DNA damage was 3.18% and 31.70% at LC_{50} and LC_{90} , respectively; the control larvae showed no damage.

Mosquitoes are responsible for the transmission of several diseases. Biosynthesis of AgNPs from bacteria will prove to be an effective, environment friendly approach to minimize the mosquito population (Priya.S and Santhi 2014). There are several works which evidencing the effective larvicidal activity of silver nanoparticles. (Najiha banu 2014,) reported that AgNPs synthesized from *B.thuringiensis* against *Aedes aegyptii* exhibited high potent larvicidal activity even at low lethal concentration of 0.10ppm. (Dhanasekaran and Thangaraj, 2013) proved that AgNPs synthesized from *E.coli*, *Penicillium sp*, and *Vibrio sp* were effective against the *Culex* larvae. A comparative study of methanol and aqueous extracts and AgNPs biosynthesized from *Nelumbo nucifera* extract by (Santhosh kumar et al, 2011) showed that AgNPs were effective against *A.subpictus* and *Culex sp* with LC_{50} values 8.89, 11.82, and 0.69 and 9.51, 13.65, and 1.10ppm, respectively. Thus, even at low concentrations, AgNPs were lethal against the mosquito larvae. (Borase et al 2013), stated that because the AgNPs are smaller in size (nm), they have increased surface area to volume ratio and, thus, were effective against mosquito larvae.

(Hesham et al, 2010) exposed various concentrations of Cd and Pb in the food of hemocytes of various stages of the Grasshopper, *Schistocera gregaria* and measured the DNA damage by comet assay. (Lalrotlunga et al, 2011) observed DNA damage in *C. longa* treated with plant extract using RAPD which showed increased number of bands in acetone extraction of *M. azedarach* compared with acetone and chloroform extraction. The DNA strand breakage was found to be more in *Culex quinquefasciatus* larvae treated with plant extract. (Virendra et al 2013,) also analyzed the genotoxicity effect of *P. Corylifolia* seed oil on the individual cells of adult *Culex quinquefasciatus* and concluded that the tail length of the DNA damage was proportional to concentration, which confirms the genotoxicity.

Thus, this study attempts for the first time to study the DNA damage in mosquito larvae treated with AgNPs using comet assay.

V. CONCLUSION

In this study, AgNPs were eco-synthesized from *B. megaterium* and their larvicidal and genotoxicity studies were carried out on the mosquito species *Aedes aegypti*. Results showed that AgNPs exhibited efficient larvicidal activity on the 4th instar larvae of *Aedes aegypti* with 100% mortality observed at 17.42ppm at 24hrs. Acridine orange stain was used to study the morphology changes of the larvae. At 20-ppm concentration of AgNPs resulted in the presence of AgNPs in most of the body regions. The study of larval DNA damage by comet assay revealed a 31.70% length of the tail DNA damage at maximum concentrations, thus confirming DNA damage of the treated larvae.

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