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RESEARCH ARTICLE

Sustainable mosquito control: A tool in the fight against *Aedes aegypti* using *Flemingia wightiana*

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Abstract

Mosquito-borne diseases, particularly those transmitted by *Aedes aegypti,* have been proven to be a global health challenge. *A. aegypti*, a major vector of Zika virus, Dengue virus, Chikungunya, is traditionally controlled through synthetic insecticides. However, the factor of environmental issues and rising insecticide resistant breeds have prompted the exploration of eco-friendly and sustainable alternatives. Here, we attempt to use the leaf extract of *Flemingia wightiana* to produce silver nanoparticles (FWAgNP). The construct of AgNPs was first indicated by UV-Vis spectroscopy, with a peak at 461 nm. NP was then characterized by SEM, EDX and functional groups were analyzed using FTIR spectroscopy. Safety assessments of synthesized NP were carried out on *Oreochromis niloticus*. Percentage mortality was studied on *A. Aegypti* with both test samples, FWAgNP and FWME. FWAgNP were found to be effective; the lowest percentage mortality of 70 % was recorded for forth instar larvae and 100 % mortality was observed in the first and second instar larvae. Oxidative stress assays such as AChe, SOD, CAT, GSH and GST were carried out. SOD, CAT and GSH showed significant elevated levels. GST and AChe levels reduced as the concentration increased, indicating the role of test samples in oxidative stress. Antiviral assay was conducted to check the effect of AgNPs in inhibiting the growth and infection of Zika virus (ZIKV) on Vero cells. The percentage inhibition property of AgNP was found to be 25 %. In conclusion, the developed FWAgNPs have significant potential in the control of vectors and a limited inhibitory activity on Zika virus.

Keywords: larvicide; nanoparticles; oxidative stress; Zika virus

Introduction

Mosquito-borne diseases present a significant public health challenge on a global scale. Zika virus, dengue, chikungunya and yellow fever are accountable for causing 300,000 deaths in tropical and subtropical regions (1). The primary vector behind the transmission of these diseases is *Aedes aegypti*, a species of mosquito that is very well adapted to urban conditions. Vector control has been a challenge for the governing bodies as well as for individuals in the community. During tropical monsoon seasons where rainfall is plenty, a rise in vector-borne diseases is always observed. Collection of rainwater in derelict places can be a breeding ground for the mosquito (2). Spread of the viral infections can be drastic and fatal if left uncontrolled. Hence, the need to eradicate the larval population from the water bodies before their hatching to adults is a sustainable way to control mosquitoes. Though several biological and nonbiological deterrents are available in the market, its impact on human health, environment and insecticide resistance pose a concern for its prolonged application (3). Thus, an urgency to seek an alternative and a better sustainable method for eradicating mosquito larvae exists. Nanoparticles have emerged as a promising alternative to the commercially marketed pesticides and insecticides.

Vector control efforts are majorly relied on chemical repellents as no potential prophylactic vaccines are available. Chemical components of mosquito repellents are recorded for causing discomfort in the respiratory tract and causing allergies, oxidative stress and dermatitis (4). Pyrethroids and organophosphates are common mosquito repellents in use. Incidences of insecticide resistance among the mosquito vectors have emerged on a larger scale (5, 6). The overall vector control project is majorly dependent on protecting from mosquito bites, formulation of natural eco-friendly repellents, limiting undesirable impact and can exhibit prolonged protection from mosquito vectors. Plant-mediated nanoparticles of size range 1 - 100 nm have been a good base material that can be considered to replace traditional pesticides (7, 8). Several studies have shown the potency of green synthesized silver nanoparticles for vector control. *Annona squamosa* mediated silver nanoparticles proved to be effective in eradication of the vectors of malaria and dengue. For *A. aegypti* and *Culex quinquefasciatus* an IC_{50} value was 0.30 and 0.41 ppm respectively (9). *Azadiracta indica* leaves which were investigated for their larvicidal potential exhibited an IC_{50} of 0.006 for *A. aegypti* larvae (10). Silver nanoparticles synthesized from *Achyranthes aspera* leaf extract were effective in controlling the growth of *Aedes albopictus* larvae with 100 % mortality obtained at 6 ppm (11).

Flemingia wightiana is a long shrub found mostly in Asia in which the phytochemical and pharmacognostic properties are very poorly surveyed (12). Plant extracts and green synthesized nanoparticles were exploited for the examination of the larvicidal property of *F. wightiana* therefore bringing these understudied species to light. This research targets the efficiency of *F. wightiana* leaf extract in controlling and eradication of *A. aegypti* and the potency to inhibit the infection rate of causative organism Zika virus.

Materials and Methods

Collection of plant samples

Leaves of *F. wightiana* were collected from Shivaji University, Kolhapur. Collected samples were identified as *F. wightiana* following the key features such as triangular hairy stems, unifolitate/trifoliate leaves, flower consisting of 2 stipules and grooved/winged petioles. The inflorescences are solitary, germinate or axillary and terminal racemes with 2 - 3 flowers (12). Leaf samples were washed using tap water and distilled water and were shade-dried for 7 days after which these were ground into fine powder.

Synthesis of methanolic extract

Ground powder was used for methanol extraction using maceration. 10 g of leaf was mixed with 100 mL of methanol and was kept overnight in a shaker incubator. After incubation, the mixture was transferred into a pre-weighed beaker and was stored at room temperature for 6 hr. The dried sample was then weighed and an equal volume of methanol was added and mixed well and was stored at 4 °C for later use (13).

Synthesis of silver nanoparticles

Silver nanoparticles were synthesized from the aqueous extract of powdered leaf samples. 10 g of leaf and 100 mL of distilled water was mixed and incubated on a magnetic plate at 60 °C with an rpm of 1000 for 1 hr. The mixture was filtered using Whatman No.1 filter paper. 10 mL of extract was added to 90 mL of 1mM silver nitrate solution and was incubated in an orbital shaker in the dark for 24 hr. After 24 hr, the color changed from light brown to dark brown color indicating the formation of silver nanoparticles (14, 15).

Characterization of samples

Characterization of plant leaf extract

The methanolic extract was subjected to both qualitative and quantitative phytochemicals assessments:

Alkaloid estimation: Total alkaloids were examined using a modified protocol of Shafay (16). A reaction mixture was prepared containing 70 % ethanol and glacial acetic acid at 4:1 ratio and 1 gram of dried plant extract powder was added and mixed thoroughly. The mixture was allowed to settle for 6 hr at room temperature and was filtered. Using concentrated ammonia solution, the alkaloids were precipitated and filtered through a pre-weighed Whatman filter paper. The filter paper was dried in an oven until constant weight was obtained.

Phenol estimation: Total phenolic content was estimated using modified protocol of Mole & Waterman (17). 0.2 mL of 0.2 N FC reagent was added to 1 mL of extract. The solution was left to settle for 5 min. 2 mL of 7.5 % sodium carbonate solution was added and the solution was kept in dark for 2 hr and absorbance readings were measured at 700 nm. Standard graph was made using gallic acid.

Flavonoid estimation: Total flavonoid was estimated using modified protocol of Mohoy El-Din & El-Ahwany (18). 0.1 mL of 10 % aluminum chloride was mixed with 1 mL of plant extract and 0.1 mL of 1 M potassium acetate was added and mixed well. To this mixture, 2.8 mL of methanol was added and the solution was allowed to settle for 30 min. The absorbance reading was measured at 415 nm.

Characterization of synthesized AgNPs

Synthesis of silver nanoparticles from the plant extract was verified by UV-visible spectroscopy (200 - 800 nm) (Shimadzu UV-1800 ENG240V UV Spectrophotometer). SEM was used to study the surface morphology, shape and size of the NPs and the elemental composition profile was assessed using EDX analysis (19).

Antioxidant activity

DPPH Assay: Antioxidant activity for both the methanolic leaf extract and NPs was performed by DPPH assay. 0.1 mM 2,2 diphenylpicrylhydrazyl (DPPH) and 2 mL of ethanol were mixed in dark. Silver nanoparticles and methanolic extract of concentrations 500, 1000 µg/L for both were added and mixed separately and were incubated in dark for 30 min. Ascorbic acid was used as standard and the absorbance was measured at 517 nm (20).

Ferrous reducing antioxidant capacity assay (FRAP/FRAC): FRAP assay was conducted using a modified protocol of Gomaa (21). 1 mL of each sample was taken and mixed with 1 mL of 0.2 M potassium phosphate buffer (pH 6.6) and 1 mL of 1 % potassium ferricyanide. This mixture was incubated for 20 min at 50 °C. Further, 1 mL of 10% trichloroacetic acid was added and mixed well. The mixture was centrifuged for 10 min at 3000 rpm. 1 mL of the topmost layer was taken and added to a mixture containing 0.5 mL of 0.1 % $FeCl_3.6H_2O$ and 1 mL of distilled water. Absorbance was measured at 700 nm.

Total antioxidant capacity (TAC): A reaction mixture was made with 28 mM sodium phosphate, 0.6 M sulphuric acid, 4 mM ammonium molybdate and 0.3 mL of sample concentration 1.0 mg/mL. The mixture was incubated at 95 ° C for 90 min. The samples were cooled to room temperature and the absorbance was taken at 695 nm. Standard curve was generated using ascorbic acid (AAE) (22).

Rearing of larvae

Larvae eggs (*A. aegypti*) collected from National Centre for Disease Control (NCDC), India were used for the studies. Collected eggs were introduced into plastic trays containing tap water and were kept at room temperature of 27 °C. Sufficient nutrients including 140 mg of calcium, 1.4 mg vitamin E and 5 % fat and protein were incorporated along with grounded yeast. Once hatched, larvae were transferred to separate trays based on the stages identified by the enlargement of body size, antenna brush developed in the head and thickening of the external cuticle (23).

Safety assessment with *Oreochromis niloticus*

Safety assessment of synthesized samples was carried out on *O. niloticus* as a non-target species. Young samples were obtained and maintained in common tanks for a period of 2 months. Young fishes were maintained in optimum conditions (28.74 ± 5.54 °C) with regular feeding with commercially available fish feed. Sufficient aeration in the tanks was ensured by utilizing blowers attached to air pumps. Concentrations of 0.1 mg L^{-1}, 0.5 mg L^{-1} and 1 mg L^{-1} were mixed into the fish tanks. Fishes of weights 20 ± 2.33 g were separated and deployed into the tanks with regular feeding (24).

Larvicidal activity

Following the protocols and conditions set by the World Health Organization (WHO) in 2009, larvicidal activity was performed on *A. aegypti* larvae with *F. wightiana* synthesized silver nanoparticles as well as methanolic leaf extract. Four test concentrations of 0.5, 1.0, 2.0 and 4.0 mg L^{-1} were prepared for both the samples. A total of 50 larvae were transferred to separate beakers containing 125 mL distilled water, one consisting of silver nanoparticles and the other, methanolic extract. Ovicidal, Larval mortality and emergence were calculated using the formula: (23)

$$\text{Total mean mortality percentage} = (Ld/Lt) \times 100$$

where, Ld = Dead larvae and Lt = Total larvae treated

$$\text{Total emergence percentage} = (La/Lb) \times 100$$

where, La = emerged larvae and Lb = treated larvae

$$\text{Total ovicidal percentage} = (Lx/Ly) \times 100$$

where, Lx = hatched larvae and Ly = treated eggs

Enzymatic assay

Acetylcholinesterase assay

Five mosquito larvae were homogenized in 1 mL of Triton X-100, 38.03 mg of ethylene glycol tetra-acetic acid (EGTA) and 10 mM Tris buffer at pH 7. After filtration, 100 µL of the homogenate was mixed with 1 mL of 0.1 M Tris, 100 µL of DNTB prepared in 0.001 M Tris buffer and 100 µL of acetylthiocholine. Acetylcholinesterase (AChE) activity was then measured at 412 nm (25).

Superoxide dismutase (SOD) assay

0.5 mL of 96 uM NBT, 0.1 mL of 0.6 % Triton X-100 and 1.2 mL of 50 mM sodium carbonate were mixed together to form the reaction mixture and were incubated for 10 min at 37 °C. 0.1 mL of 20 mM hydroxylamine hydrochloric acid was added to start the reaction. Continuous readings for 3 min were taken at 560 nm at 1 min intervals. After adding hydroxylamine HCl, PMS was added to the mixture and the absorbance was taken to analyze the SOD activity (26).

Catalase (CAT) assay

Reaction mixture with 0.0067 M phosphate buffer (pH 7) and 0.01 mL of sample was prepared. 2.9 mL of 12.5 mM H_20_2 was added and absorbance at 240 nm was taken every 30 sec for 3 min (27).

Glutathione (GSH) assay

0.1 mL of samples was mixed with 2 mL of 0.2M phosphate buffer, 1 mM of DTNB prepared in 1 % potassium citrate. Centrifuged for 15 min at an rpm of 3000, the supernatant was taken and the absorbance was read at 412 nm (28).

Glutathione-S-Transferase (GST) assay

A reaction mixture of 0.8 mL DDW, 0.1 mL of 20 mM CDNB which was made in 95 % ethanol and 1 mL 0.2M phosphate buffer of pH 7 was prepared. 0.1 mL of sample was added and incubated for 5 minutes. Furthermore, 0.1 mL of 20 mM GSH was added and readings were recorded immediately every 30 sec at 340 nm for 5 minutes. Reaction mixture without samples was kept as blank (29).

Histology

Larvae that were exposed to test samples were subjected to dissection in saline solution. Larvae were transferred to a fixative solution for 12 hr. The samples were transferred into a series of ethanol solutions (70, 80, 90 and 95 %) for dehydration. The samples were engulfed in historesin JB-4 and sectioned at a thickness of 3 µm in a microtome using a glass knife. Prepared sections were then observed under the Lyca Optical microscope (30).

Antiviral assay

The viral plaque assay was evaluated to test antiviral activity of silver nanoparticles on viral Vero cells. Vero cells were cultured on 96-well plate with Dulbecco's Modified Eagle Medium (DMEM)(10 % FBS) (Fig. 1). 30000 cells/well plate was incubated overnight at 37 °C. 30 µL of virus (MOI 0.01) and 10 µL of the test item was added to the infection medium. After 1 hr of incubation at 37 °C, DMEM was removed and the plate was incubated for 3 days. The cells were fixed with 4 % formaldehyde, flooded with 0.05 % crystal violet followed by washing to visualize the plaques. PFU/mL was calculated and percentage reductions in viral load was determined (31).

Results

Flemingia wightiana leaf methanolic extract characterization

The preliminary qualitative analysis for phytochemicals for *F. wightiana* methanolic leaf extract was conducted and alkaloids were seen at the highest amount with 22.45 mg/g. Total phenolics and flavonoids were found in amounts of 15.51mg/g and 10.28 mg/g respectively (Table 1).

Nanoparticle characterization

Synthesis of silver nanoparticles was indicated by color change to dark brown of the extract solution. The first confirmation was done by UV- spectrum ranging from 200 - 800 nm. The peak absorption was at 461 nm (Fig. 2) being in between the suggested range of 380 nm and 460 nm. SEM and EDX analysis of FW-AgNP was conducted for its elemental composition through Scanning Electron microscopy. Results indicated the presence of spherical

Table 1. Quantitative estimation of methanolic leaf extract of *Flemingia wightiana*

No	Test	Amount (mg/g of leaf samples)
1	Total phenolic content	15.51 ± 1.2 mg/g
2	Total flavonoid content	10.28 ± 0.88 mg/g
3	Total alkaloids content	22.45 ± 0.34 mg/g

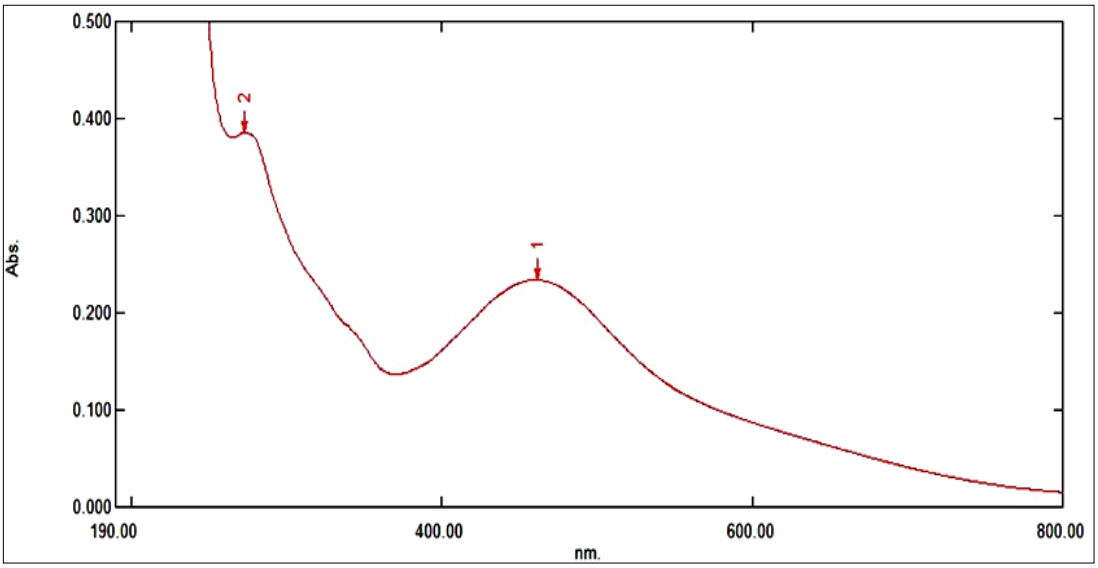

Fig. 1. UV-Visible spectroscopy (Peak 1 - 461 nm) indicating the synthesis of silver nanoparticles from plant extract.

FW-AgNP with average size of 62 nm in diameter (Fig. 3 A & B). EDX data (Fig. 4C) showed different elements present being silver (90 %), Carbon (55 %), oxygen (18 %). In FTIR analysis, the synthesized silver nanoparticles showed the peaks at 3278, 2920, 1637, 1543, 1370, 1243, 1034 and 525cm^{-1} (Fig. 5).

Antioxidant activity

The study found that in comparison, FW-AgNps had better antioxidant properties when compared to methanolic leaf extract of the plant. In DPPH assay (Table 2), 500 µg L^{-1} concentration NPs showed 12 % scavenging activity and 1000 µg L^{-1} having 20 % activity. DPPH scavenging activity for methanolic extract showed 47 % inhibition for 1 mg mL^{-1}. FRAP assay for NPs showed 62.75 µg AAE (Ascorbic acid equivalent) activity for 500 µg L^{-1} and 137 ug AAE activity for 1000 µg L^{-1}. TAC analysis showed lowest activity with 3.74 µg AAE for 500 µg L^{-1} and 6.04 µg AAE for 1000 µg L^{-1}. The results here proved that the synthesized nanoparticles as well as plant extracts possessed significant antioxidant properties.

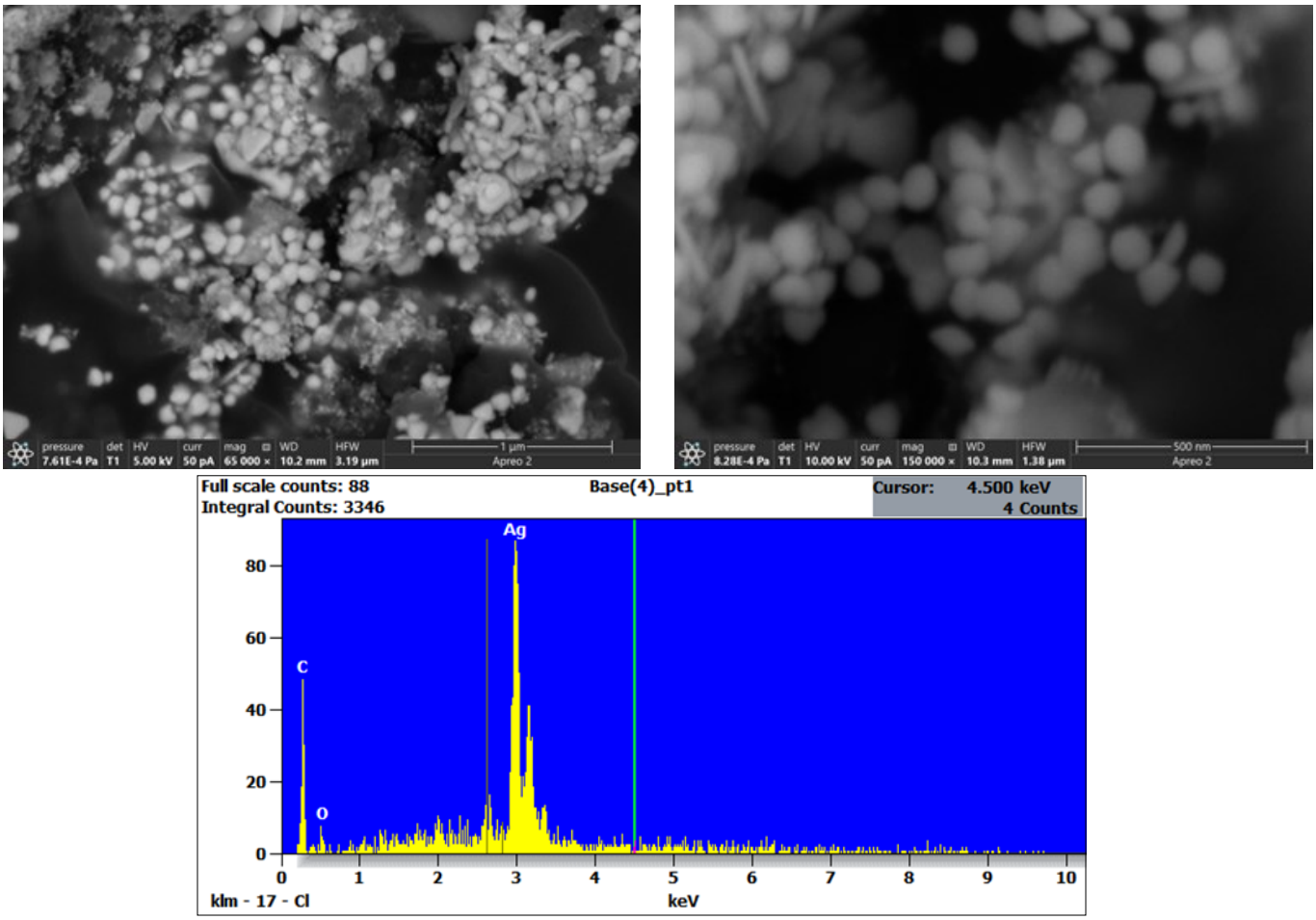

Fig. 2. SEM images of synthesized nanoparticles showing the (A) 65 000x and (B)150 000x magnification and EDX analysis of the synthesized nanoparticles.

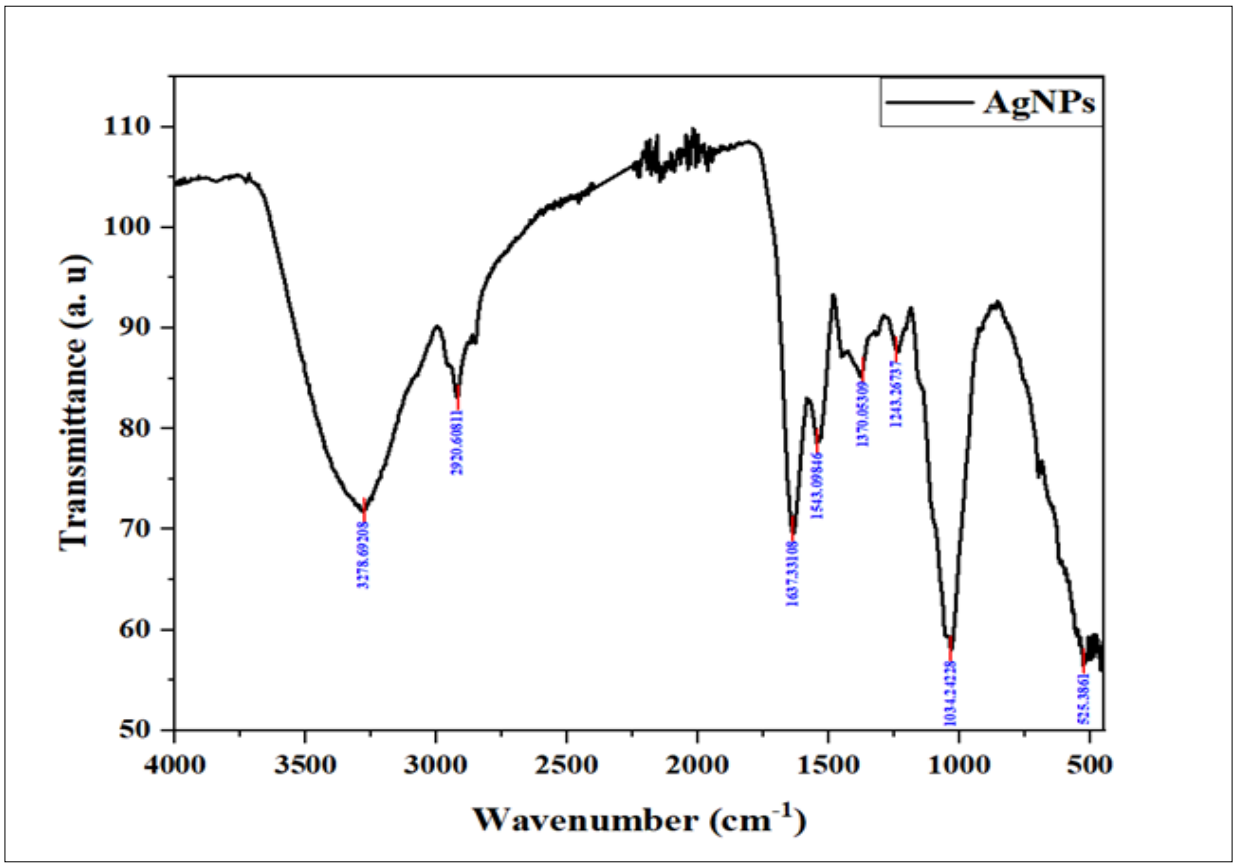

Fig. 3. FTIR spectrum of FW-AgNP.

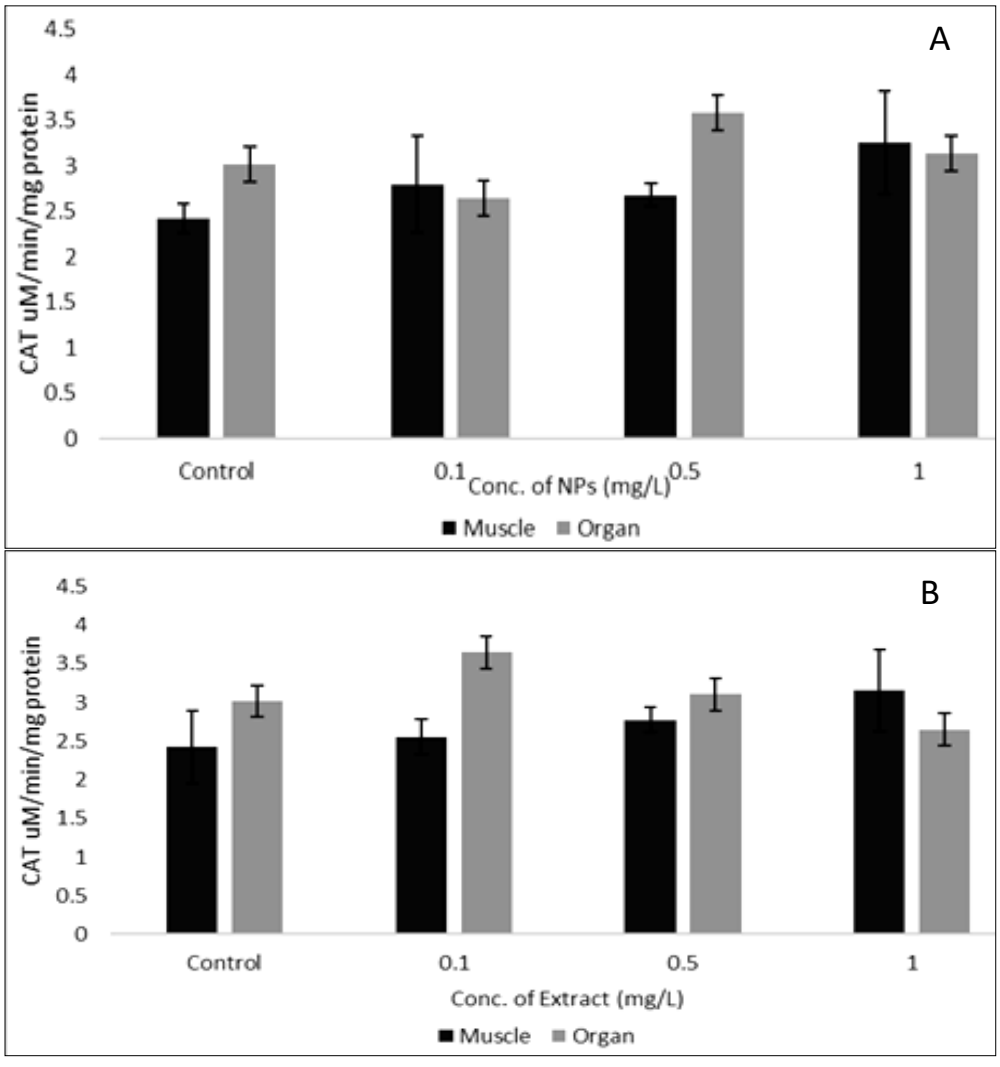

Fig. 4. Catalase assay of *Oreochromis niloticus* exposed to (A) silver nanoparticles and (B) methanol plant extract.

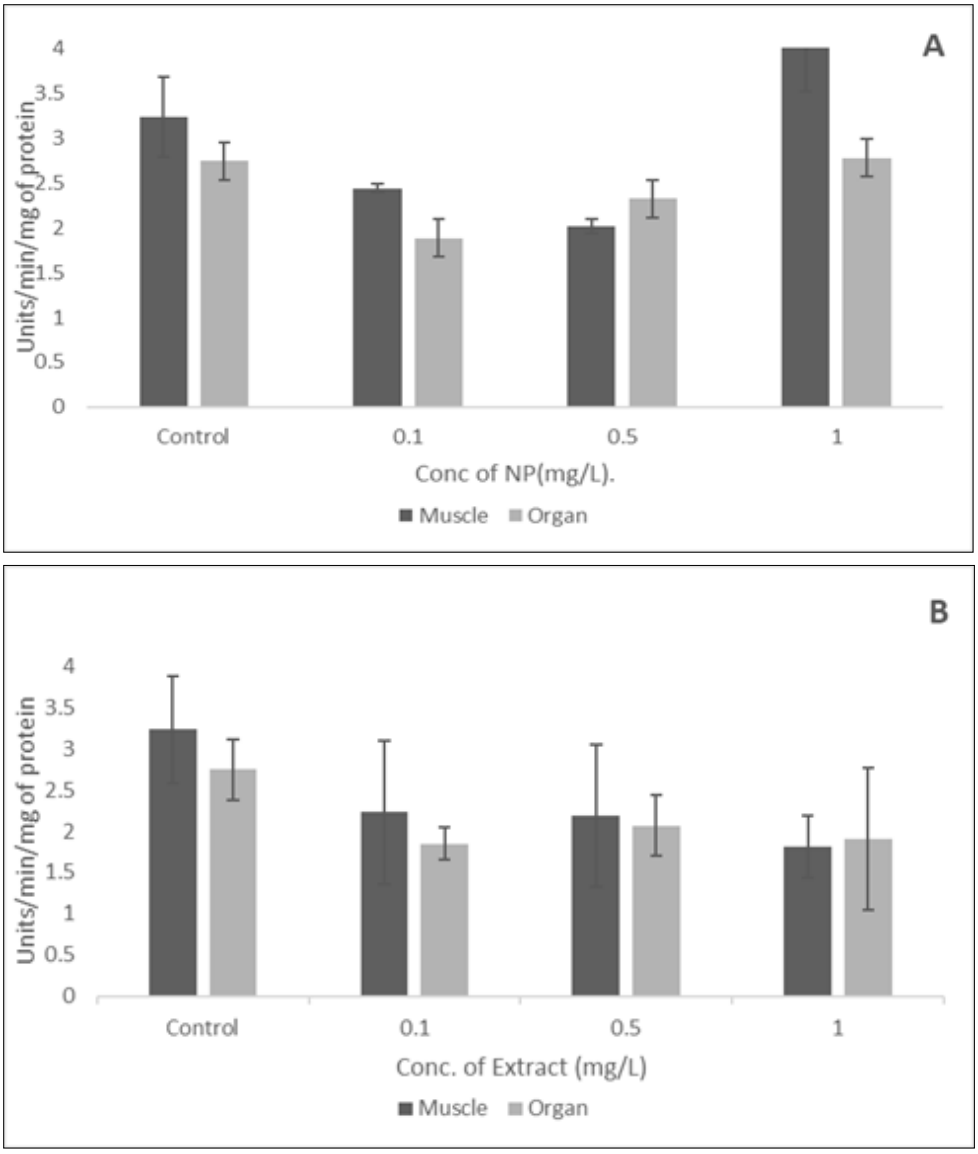

Fig. 5. Glutathione S-transferase assay of *Oreochromis niloticus* exposed to (A) silver nanoparticles and (B) methanol plant extract.

Table 2. Antioxidant property of synthesized silver nanoparticles and methanolic leaf extract

Assay	Nanoparticle		Methanol extract
	500 µµg/L	1000 µg/L	1 mg/mL
DPPH	12 ± 0.85 %	20 ± 0.64 %	47 ± 0.76 %
FRAP	62.75 ± 1.22 µg AAE	137 ± 0.56 µg AAE	250 ± 0.25 mg AAE
TAC	3.74 ± 0.06 µg AAE	6.04 ± 0.09 µg AAE	74 ± 0.2 mg AAE

Safety assessment on *O. niloticus*

Safety assessments of both the test samples were conducted to study any non-target toxicity effects. After the incubation period, no mortality was observed in any of the fishes in control as well as the fishes subjected to test samples. Stress assays, CAT and GST were performed on both muscles and viscera of fishes (Fig. 3, 6). Results indicated that no significant difference was found between fishes in control from fishes in test samples (Fig. 7).

Larvicidal activity

Larvicidal activity was conducted for a period of 24 hr. The number of deceased and alive larvae was documented and the larvae were investigated for physical damages as well as oxidative stress. First and second instar larvae exhibited 100 % mortality at 4 mg L^{-1} (Fig. 8). Compared to the primitive stages of the larvae, third and fourth instar larvae had more resilience as percentage mortality was higher (Fig. 9). LC_{50} values of the first instar larvae were determined to be 1.28 mg L^{-1} whereas the instar, (C) instar and 4th instar larvae exhibited an LC_{50} value of 1.43, 0.91 and 1.87 mg L^{-1} respectively. Ovicidal activity was estimated by analysing the hatched eggs from the unhatched. Ovicidal activity for 0.5 mg L^{-1} was observed to be 0 % for both AgNP and PE. At 1 mg L^{-1}, ovicidal activity was estimated to be 10 ± 5 % for AgNP and 0 % for PE. For conc. 2 mg L^{-1}, the activity was 35 ± 5 % for AgNP and 0 % for PE. At 4 mg L^{-1}, ovicidal activity was found to be 54 ± 5 % for AgNP and 26 ± 5 % for PE. Emergence rate was also quantified, with control 100 % emergence from all instar stages. For emergence to second instar (0.5 mg L^{-1}) was found to be 80 ± 2 % for AgNP and 100 % for PE. At 1 mg L^{-1}, emergence was 54 ± 3 % for AgNP and 80 ± 3 % for PE. In 2 mg L^{-1}, emergence was found to be 30 % ± 4 for AgNP and 76 % ± 3 for PE. At higher concentration of 4 mg L^{-1}, emergence percentage was 0 % for AgNP and 65 ± 1 % for PE. From second to third instar emergence, at 0.5 mg L^{-1} the percentage was 60 ± 1 for AgNP and 100 % for PE. At 1 mg L^{-1}, emergence was 50 ±4 % for AgNP and 80 ± 1 % for PE. In 2 mg L^{-1}, emergence was found to be 30 ± 2 % for AgNP

Fig. 6. Fourth instar larvae of *Aedes aegypti* treated with silver nanoparticles at highest test concentration of 4 mgL^{-1} exhibiting (A) abdominal segments disintegrated, siphon and anal segments non-observable, (B) thorax disfigured, (C) degenerated thorax, lateral hairs damaged. (D) Larvae kept under control condition showing nominal morphology.

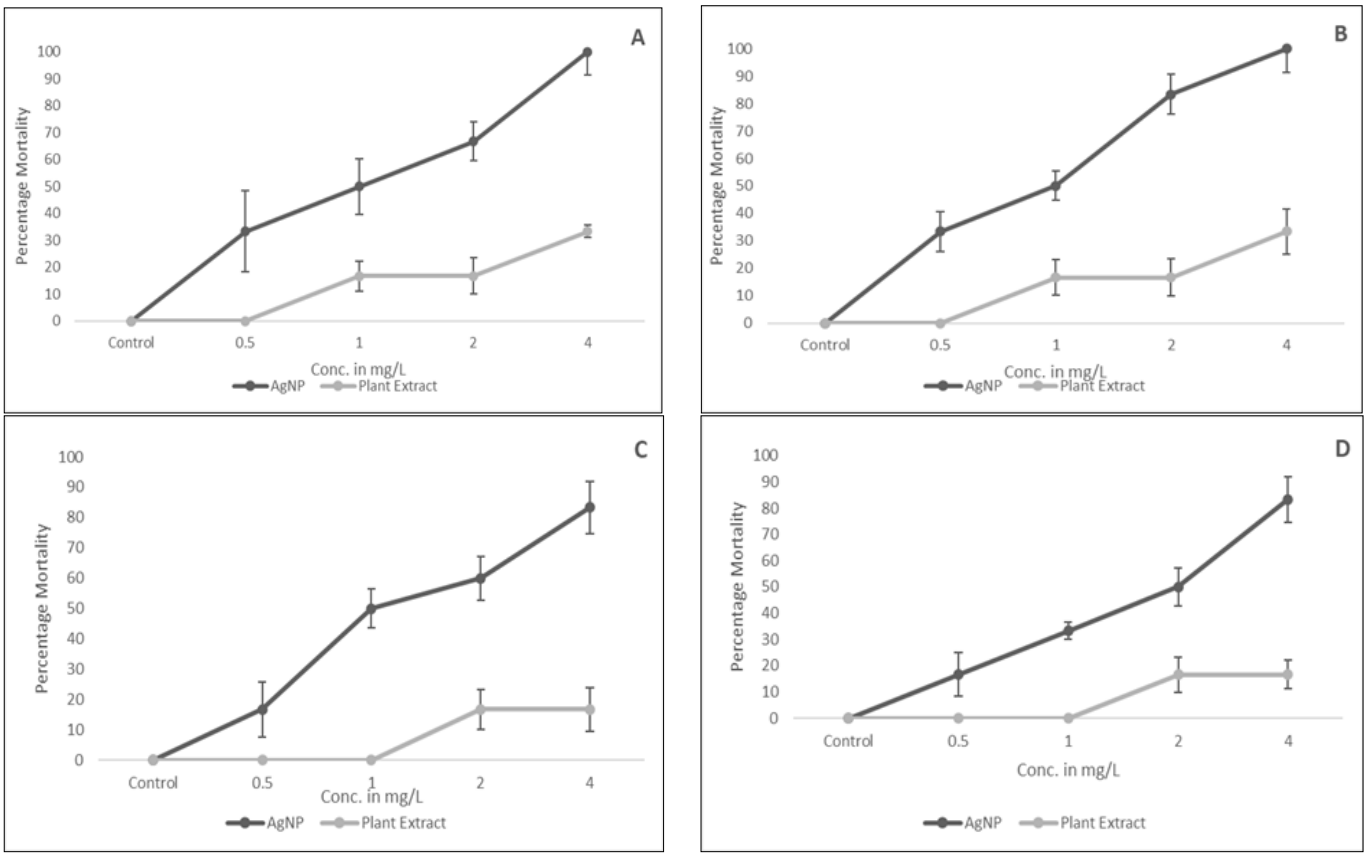

Fig. 7. Percentage mortality of *Aedes aegypti* larvae treated with silver nanoparticles and methanolic leaf extract(A) 1st instar, (B) 2nd instar, (C) 3rd instar and (D) 4th instar larvae.

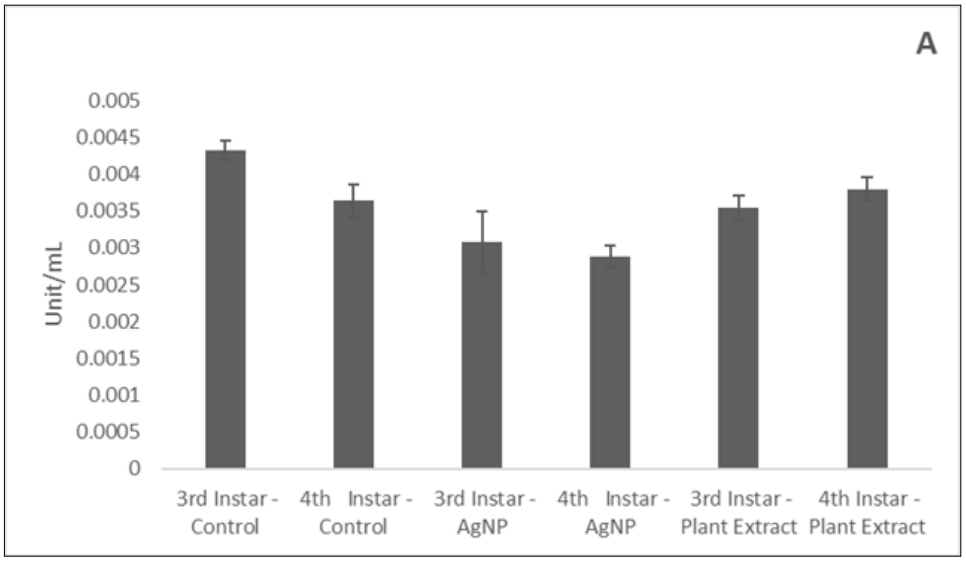

Fig. 8. Acetylcholinesterase activity of larvae treated with silver nanoparticles and plant extract.

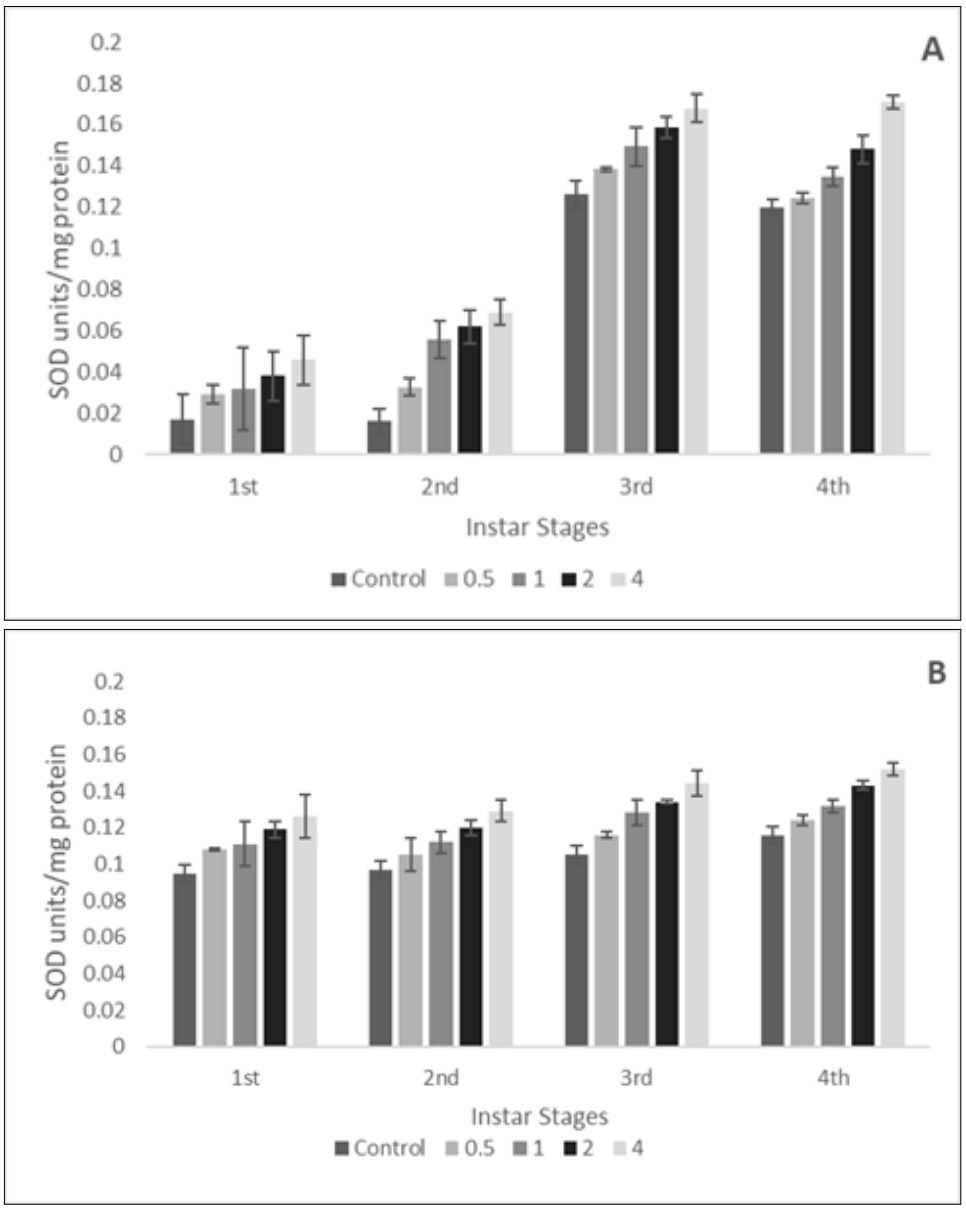

Fig. 9. Superoxide dismutase activity of *Aedes aegypti* treated with (A) silver nanoparticles and (B) methanol plant extract.

and 70 ± 4% for PE. At higher concentration of 4 mg L^{-1}, Emergence percentage was 0 % for AgNP and 60 ± 1 % for PE. From second to third instar emergence, at 0.5 mg L^{-1} the percentage was 80 ± 3 % for AgNP and 100 % for PE. At 1 mg L^{-1}, emergence was 40 ± 3 % for AgNP and 100 % for PE. In 2 mg L^{-1}, emergence was found to be 30 ± 5 % for AgNP and 80 ± 2 % for PE. At higher concentration of 4 mg L^{-1}, the emergence percentage was 20 ± 2 % for AgNP and 80 ± 5 % for PE. From fourth instar to pupaeat 0.5 mg L^{-1} the percentage was 80 ± 3 % for AgNP and 100 % for PE. At 1 mg L^{-1}, emergence was 72 ± 1 % for AgNP and 100 % for PE. In 2 mg L^{-1}, emergence was found to be 30 ± 4 % for AgNP and 82 ± 3 % for PE. At higher concentration of 4 mg L^{-1}, the emergence percentage was 0% for AgNP and 65 ± 5 % for PE.

Oxidative stress assay

Compared to control for both third and fourth instar larvae, the larvae treated with AgNP presented a significant reduction in the AChe activity (Fig. 10). Subjection to plant extract at lower concentrations did not yield a statistically significant difference from respective control. Superoxide dismutase assay showed that the effect of AgNP and methanolic plant extract on *A. aegypti* larvae had significant differences in the stress hormone levels. SOD levels were found to be the highest in the treated samples than the control (Fig. 11) and the higher SOD was observed in the maximum studied concentration of 4 mg L^{-1}. GSH was observed to elevate as the concentration increased in all larval stages (Fig. 12). CAT assay was conducted and rate of reaction was more in samples treated with AgNP (Fig. 13). Total amount of GST was reduced for the treated samples. Highest value was observed in control samples, whereas the amount of GST decreased as the concentration of test samples decreased.

Histology

Histology studies of the 4th instar larvae treated with highest concentration (4 mg L^{-1}) of FWAgNPs revealed the internal structures of the larval body were disrupted as a result of exposure to the test sample. Histology sections of the larval body revealed that the internal cell lining was damaged. In the midgut region, the lining of the gut consisting of epithelial cells (EP) had been broken (Fig. 14).

Antiviral assay

An antiviral assay for silver nanoparticles was conducted to check its effectiveness in controlling the infection rate of Zika Virus. Positive control Adenine analog (NITD008) has shown a significant inhibitory activity at IC_{50} of 0.5 µM (Fig. 15 and 16). Test sample showed limited inhibitory activity than the control though lowest concentrations didn't show any inhibitory effect on the Zika virus. Highest test concentration of 3 mg/L showed inhibition of 25 % (Fig. 16).

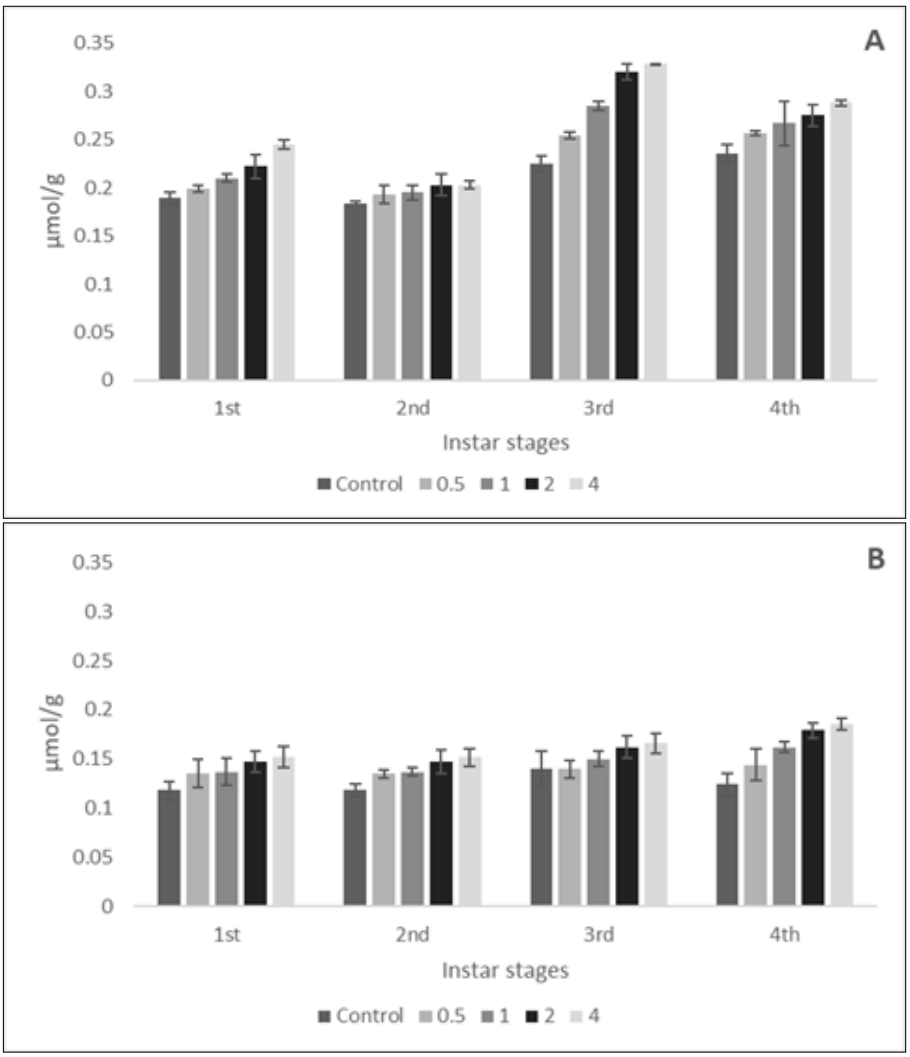

Fig. 10. Reduced glutathione activity of *A. aegypti* treated with (A) silver nanoparticles and (B) methanol plant extract.

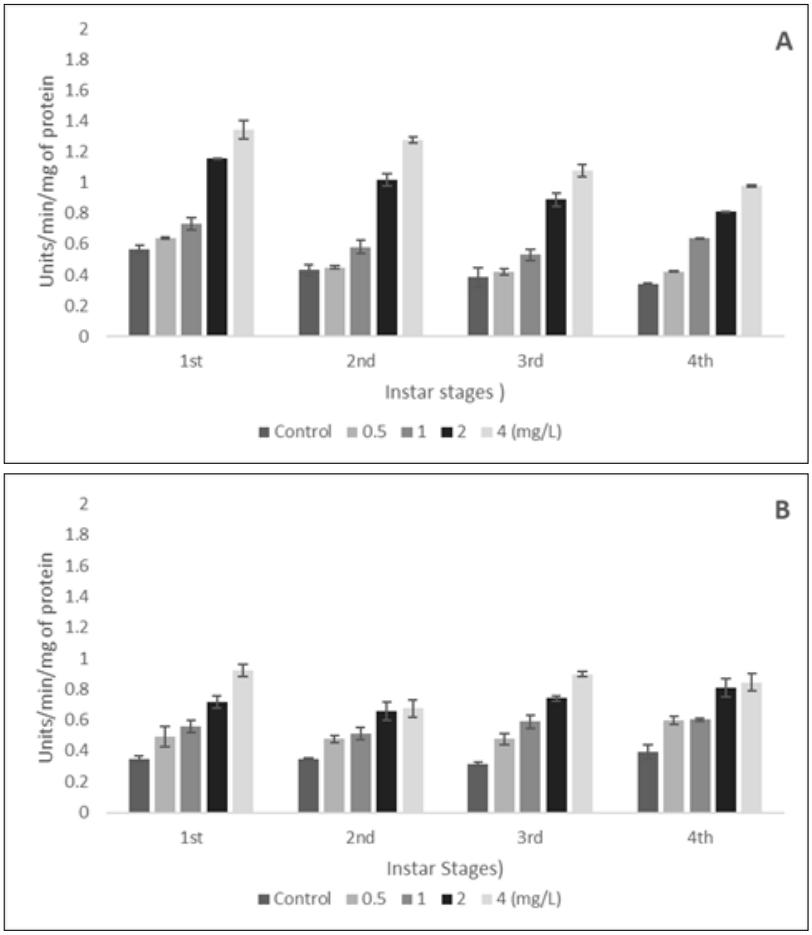

Fig. 11. CAT activity of *Aedes aegypti* treated with (A) silver nanoparticles and (B) methanol plant extract.

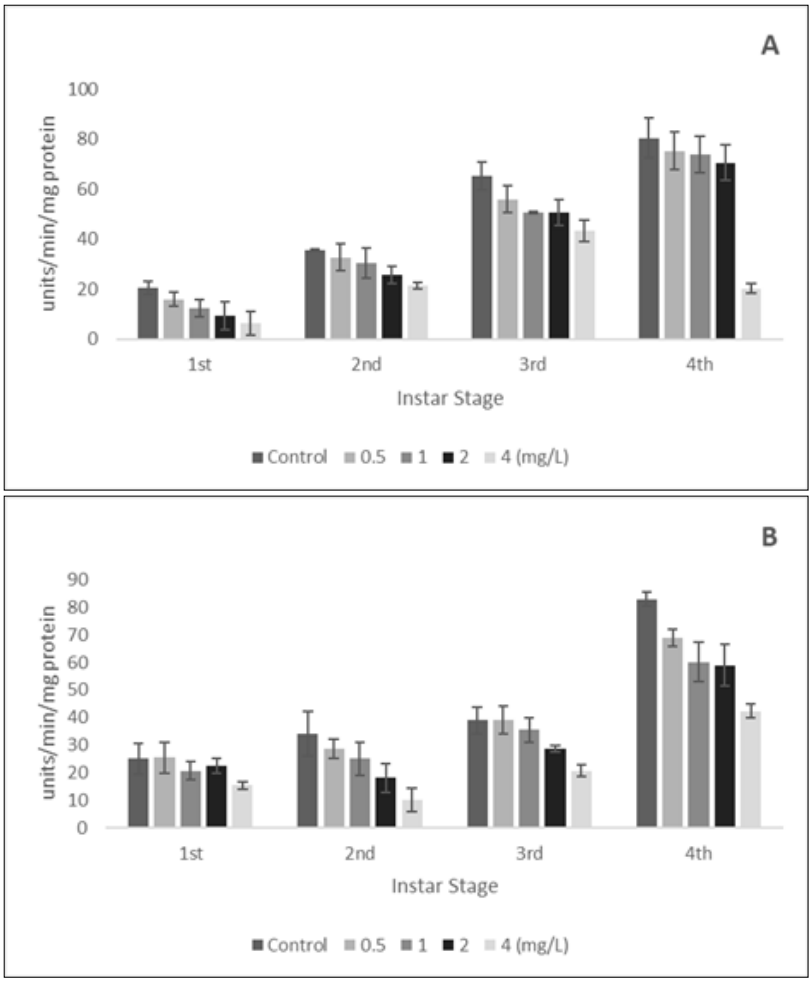

Fig. 12. GST activity of *Aedes aegypti* treated with (A) silver nanoparticles and (B) methanol plant extract.

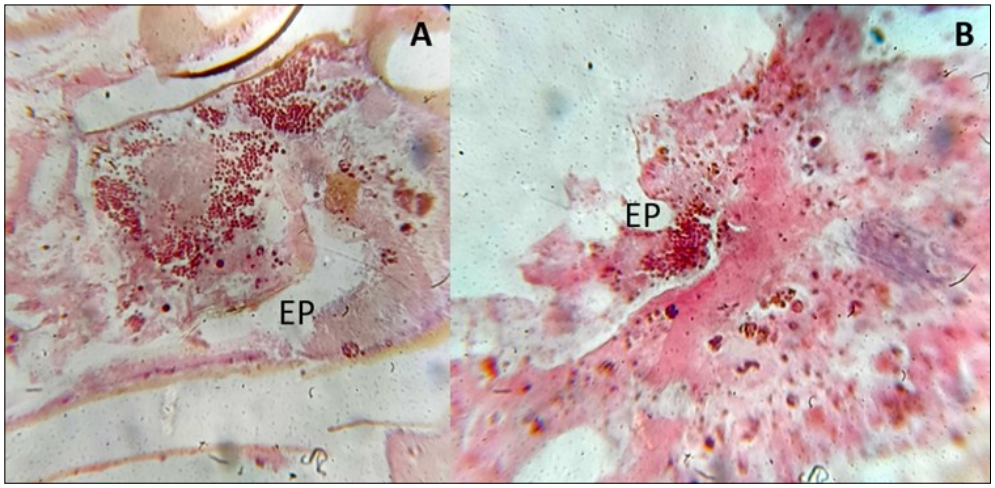

Fig. 13. Histology of larval midgut showing the damaged epithelial cells of *A. aegypti* larvae treated to higher concentrations of FWAgNPs.

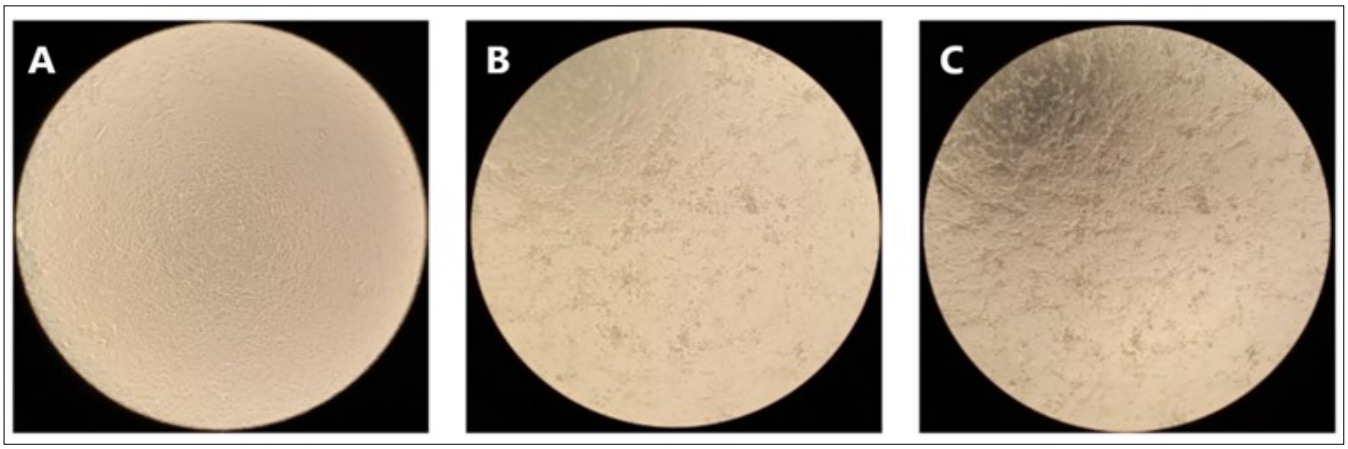

Fig. 14. Vero cell monolayer grown in 96 well plates; A: cell only, B and C: monolayer showing infection (rounded cells) with Zika virus at 24 hr and 48 hr post-infection.

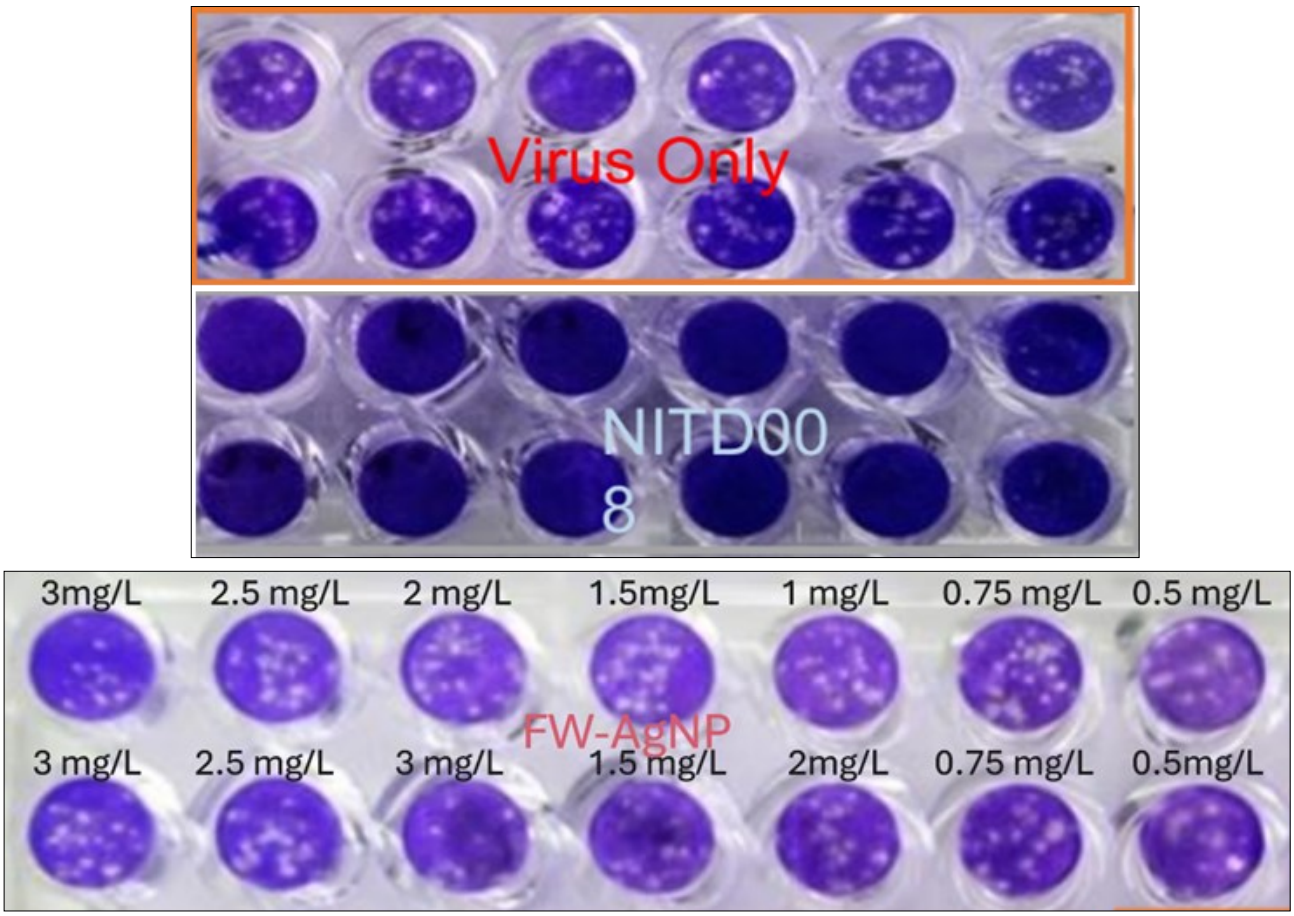

Fig. 15. Plaques observed in the 96 well plate after development and treatment with NITD008 - control and FW-AgNP.

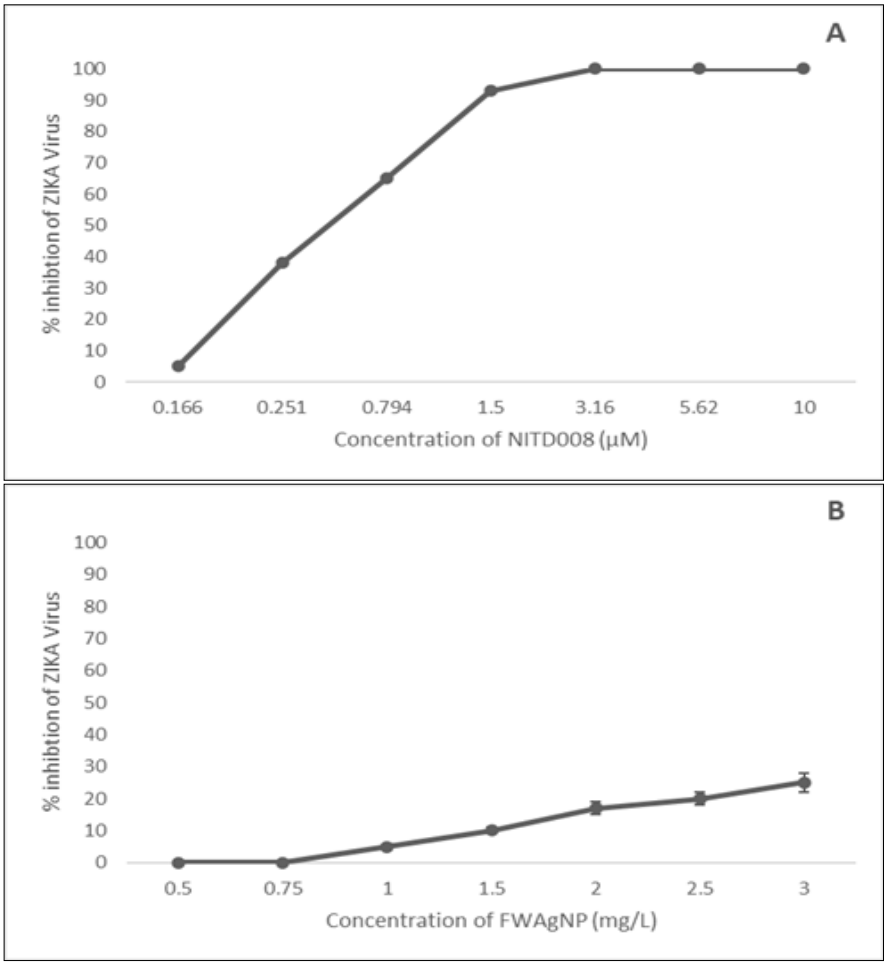

Fig. 16. Dose-response curve of (A) control and (B) silver nanoparticles against Zika virus.

Discussion

Characterization

Total flavonoids in the FW ethanolic extract was 18.175 ± 0.117 mg QE/g dry weight of extract and FW ethanolic extract was 92.468 mg QE/g dry weight of extract (32). As a significant quantity of phenolics as well as flavonoids are found in the FW methanol extract, it can be utilized for biomedical applications and phytoremediation techniques.

Using UV-Vis spectroscopy the synthesized product was confirmed as silver nanoparticles. Free electrons present within the metal component of the nanoparticle oscillate with respect to its particle arrangement in the presence of visible light (14). The peak range for EDX between 2- 4 keV for silver ions indicates formation of AgNP. Using SEM analysis, the size and shape of the silver nanoparticles were found to be 20 nm and spherical in shape (33). The FTIR peak near 3278 cm^{-1} corresponds to the phenols or flavonoids from the plant extract which interacted with the silver nitrate to form the nanoparticles. The peak indicates the hydroxyl group from either of the aforementioned compounds. C-H stretching of methyl compounds is indicated by the peak at 2920 cm^{-1}. Proteins are indicated by the peaks at 1637 and 1543 cm^{-1} as the secondary amides present within the protein interact with silver ions during synthesis. Aromatic hydrocarbons found in polyphenols are indicated by peaks at 1370 and 1243 cm^{-1}. 1034 cm^{-1} peak shows the carboxylic compounds of polypeptides in proteins. Peak at 525 cm^{-1} indicates the presence of metal oxide (Ag+ to AgO) (34, 35).

Antioxidant activity

The antioxidant properties of both FW-AgNP and FW-ME were determined. Antioxidant activity is crucial for evaluating the therapeutic property and the health benefits of products. Plants, a rich reservoir of natural antioxidants, serve as a necessary aspect of counteracting oxidative stress and reducing free radical damage. Analysis of antioxidant properties gives the preliminary idea whether the synthesized NPs can be used for medicinal or other beneficial application (36).

Similar results were indicated by comparing AgNPs and plant extract of fabaceae member *Parkia speciosa* (37). *Medicago sativa* (Fabaceae) mediated NPs also showed higher free radical activity of 80 % at 43 µg mL^{-1} compared to its plant extract of 30 % at 5 mg mL^{-1}. When concentration of the FW-AgNP was doubled, the activity also increased two-fold indicating an excellent free radical scavenging potential of NP. Several factors can lead to oxidative stress in a biological system and it is important to remediate the same as synthetic antioxidants can have a drastic impact on the functioning of a biological system (38, 39).

Safety assessment on *O. niloticus*

Oxidative stress responses of fishes have been used as a biomarker for the stress induced from certain biotic and abiotic factors (40). As stress levels in the organism elevate, the responsive enzymes that aid the organism in counteracting the stress increase. Thus, a direct link is present between oxidative damage and the enzymatic

activity like SOD, CAT and GSH. Various types of factors like aquatic environment, heavy metals, parasitic infections, change in chemical constituents and temperature of water can influence the stress levels of fishes therefore indicating oxidative damage to organs. The levels of enzymatic activity can be correlated to the amount of oxidative stress levels in a biological system (41).

Larvicidal activity

Against *A. aegypti* and *Anopheles stephensis* mosquito, *Pergularia daemia* mediated NPs were found to be effective in eradication of the larval group (42). Studies on *A. aegypti* and *C. quinquefasciatus* for the effect of *Rhizphora mucronata* mediated nanoparticles were effective larvicidal activity as it limits the survivability of the larvae with an LC_{50} of 0.05 mg L^{-1} (43). Studies on larvicidal activity of AgNPs and plant extract of *Ambrosia arborescens* proved that the former had a very decisive role in the lethality of larvae (44). LC_{50} for AgNP was found to be 0.28 ppm and LC_{50} of plant extract was high at 1844.23 ppm. The effect of AgNP also had a substantial effect in delaying the emergence of larvae into the next instar stage. Silver nanoparticles from aqueous extract of the plants have been proven to have an effective mechanism for eradicating larvae and can be incorporated into main components to replace traditional compounds present in modern day larvicides and pesticides. AgNP as a whole does cause disruption in a biological system by limiting the availability of copper as it is a significant element needed in several functionalities such as connective tissues, immune system and iron absorption (45). In drosophila, a similar copper starvation was reported by Armstrong (46). Research has also proven that when NPs are made from plant sources, they act as antifeedants, fumigants, repellent and reproductive inhibitors for many pests (47).

Oxidative stress assay

Breakdown of acetylcholine into choline for various neurological and other biological pathways is mediated by Acetylcholinesterase (AChe). The decrease in AChe indicates that the organism is undergoing a relatively stressed environment condition. A reduction in the AChe activity has proven to cause more stress in larvae species and therefore induce better larvicidal potential in *Culex pipens* larvae (48). Metals such as Ag are known to induce ROS in the biological body, which can lead to oxidative stress. When larvae are subjected to a foreign particles, it is proven to increase the SOD and glutathione peroxidase levels in response to the foreign particle (49). When the amount of stress in the system exceeds the threshold value, the production of defense enzymes declines. This leads to a reduction in the survivorship for the organism. As the organism evolves, so do its defensive capabilities against external disruptions. Higher oxidative stress levels can cause a spike in the SOD, glutathione and CAT (50). Studies have proven that high levels of SOD can be a cause of hydrogen peroxide toxicity. Excess H_20_2 arises due to the high levels of stress in the larval body (51). As a result of subjecting the *A. aegypti* larvae to FW-AgNPs, it was observed that the treated samples showed higher levels of enzymatic activity as compared to control. It indicates that the test samples have a direct impact on the bioactivities within the body. As the concentration of AgNP increased, so did the enzymatic levels as a result of coping with the increased amounts of the test sample.

Histology

By microscopic analysis of larvae sections, the internal parts showed disruptions in the cell layers. The ability of NPs to penetrate the cuticle and disrupt the cellular membranes leads to cell lysis. Nanoparticles have emerged to be good agents for mosquito control with studies highlighting damage to the cells of mosquito larvae. Subjecting mosquitoes to certain concentrations of silver nanoparticles induces reactive oxygen species (ROS) which can disrupt cellular membranes by impairing metabolic functioning. This leads to oxidative stress and these disruptions ultimately hinders the survivability of the larvae. The treated larvae showed several lesions in the cell lining and breakage lumen layer (52). The minute size of the NPs grants it the ability to closely interact with the cellular structures and cause oxidative damage to the cell components. The cell lysis happens as a result of reaction of cell membrane to the AgNps thus leading to disruption and leakage of intracellular contents (53, 54).

Antiviral assay

Plaque reduction in positive control was effective as adenine analog is a potent inhibitor for the infection of Zika virus (55). Metal nanoparticles encapsulated with synthetic macromolecules like glycodendrimers have been proven to be effective viral inhibitors (56). It proved the potency of nanoparticle formulations as a medium for drug delivery. Curcumin encapsulated with poly(lactic-co-glycolic acid) nanoparticles to produce the combined PLGA-CUR NPs. These synthetic NPs have a significantly higher viral RNA synthesis reduction and protein expression than the solitary components (57).

Apart from acting as direct inhibitors of viral growth, NPs have shown alternative properties like vaccines and biosensors. Utilizing the hybrid compounds such as silsesquioxane, gold nanoparticles which were synthesized proved to be an effective tool for the diagnosis of Zika virus in tested human serum (58). Nanoparticles have also shown potential as a vaccine against Zika virus. Ferritin mediated nanoparticles exhibited the potential to induce an immune response. This response was observed to have similar effects as neutralizing antibodies which help in protection of the tested animals against Zika Virus infections (59). Diferuloylmethane (commonly known as curcumin) is a proven compound that has significant antimicrobial and wound-healing properties. Curcumin was encapsulated in mesoporous silver nanoparticles which were temperature sensitive and when the host body temperature increased (high fever), the drug release occurred and Zika virus infection was inhibited (60). Nanoparticles show significant potential as biosensors for various diseases and infections. Gold nanostructures which in conjugation with carbon electrodes and ruthenium ions, act as a biosensor which can detect the presence of Zika virus from patient samples (61). This paves for an easier, more efficient detection for the presence of Zika virus in patients. The potential of

nanoparticles as means for vaccinations have been discussed for many years. Murine studies on rats injected synthesised zEDIII-rHF nanoparticle made with ZIKV envelope protein domain III (zEDIII) and human heavy chain ferritin (rHF) did provide protection from lethal Zika virus infection and eradicated the symptoms from the mice brains (62). Nanoparticles have a myriad of applications in the field of virology itself like vaccines, sensors and direct infection treatment thus paving the way for innovative advancements in combating viral diseases.

Conclusion

Silver nanoparticles and methanolic leaf extract was synthesized from *Flemingia wightiana* leaves. Methanolic extract was found to be having a significant amount of phenols, flavonoids and alkaloids. FW-AgNP were synthesized from aqueous extract. Characterization of synthesized nanoparticles revealed that the size to be 20 nm in diameter and spherical in shape and FTIR spectrum analysis indicated the presence of aromatic hydrocarbons, proteins and hydroxyl group.

The FW-AgNPs and FWME showed antioxidant activity against DPPH, TAC and FRAP assay. Safety assessment in non-target species (Nile tilapia) didn't exhibit damage or elevated stress levels. Silver nanoparticles were found to have 100 % mortality effect on first and second instar mosquito larvae and 80 % and 70 % for third and fourth instars respectively. Studying the oxidative stress levels of mosquito larvae subjected to test samples revealed the elevated stress levels exhibited by larvae exposed to test samples. Increased levels of SOD, CAT and GSH showed that the larvae do experience oxidative damage. GST and AChe had declined levels indicating that the stress is related to elevated ROS. Antiviral assay of FW-AgNP against Zika Virus showed that high concentrations of AgNP showed a 20 % inhibition of infection on the vero cell line. The study demonstrated the potential of FW-AgNP as an effective method in eradicating mosquito larvae and showed promising antiviral activity.

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Authors' contributions

AG and KAP both designed the work. AG collected the samples, prepared the samples, performed each assay, writing and editing. AG and KAP jointly interpreted the results and drafted the original manuscript. AG prepared all necessary charts and tables depicted in the paper which illustrates the effect of the samples on larval development. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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