*Tropical Biomedicine* 35(2): 392–407 (2018)

# Silver nanoparticles enhance the larvicidal toxicity of *Photorhabdus* and *Xenorhabdus* bacterial toxins: an approach to control the filarial vector, *Culex pipiens*

El-Sadawy, H.A.[1], El Namaky, A.H.[1*], Hafez, E.E.[2], Baiome, B.A.[1], Ahmed, A.M.[3,4], Ashry, H.M.[1] and Ayaad, T.H.[5]

[1]Department of Parasitology and Animal Diseases, National Research Centre, El Bohouth Street, Dokki, P.O. Box 12622, Giza, Egypt

[2]City for Scientific Research and Technology Applications (MuCSAT), Arid Lands and Cultivation Research Institute, Plant Molecular Pathology, New Borg El-Arab City 21934 Alexandria, Egypt

[3]Zoology Department, College of Science, King Saud University, Saudi Arabia

[4]Zoology Department, College of Science, Minia University, El Minia, Egypt

[5]Entomology Department, College of Science, Cairo University, Giza, Egypt

[*]Corresponding author e-mail: amiraelnamaky@gmail.com.

Received 7 November 2017; received in revised form 6 January 2018; accepted 9 January 2018

**Abstract.** Mosquito-control is still based mostly on chemical insecticides which are toxic and cause environmental deprivation. This study investigates synthesizing silver bio-nanoparticles (AgNPs) from nematode-symbiotic bacterial toxin complexes as an alternative larvicidal bioinsecticide agent against *Culex pipiens* larvae. Five species/strains of nematode-symbiotic bacteria, *Xenorhabdus indica*, *Xenorhabdus* spp., *Photorhabdus luminescens laumondii* HP88, *Photorhabdus luminescens akhurstii* HRM1 and *Photorhabdus luminescens akhurstii* HS1 were used. AgNPs were characterized by scanning electron microscopy and x-ray diffraction analysis. Larvae were initially exposed to descending concentrations (300, 150, 75, 37.5 and 18.75 µg/ml) of each of the five bacterial toxins (as positive controls) or to the bio-AgNPs synthesized from the same bacterial toxins (200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.5 µg/ml) for 48 hours. Results of toxicity bioassays showed that mortality of treated larvae was concentration-dependent, toxins from *X. indica*, *P. luminescens laumondii* HP88 and *P. luminescens akhurstii* HS1 showed $LC_{50}$ of 29, 28 and 2002 µg/ml, respectively. While, toxins from *P. luminescens akhurstii* HRM1 and *Xenorhabdus* sp. showed $LC_{50}$ of 199, 318 µg/ml, respectively. Bio-AgNPs synthesized from, *X. indica* or *Xenorhabdus* sp. toxins have significantly increased their larvicidal activities ($LC_{50}$ of 1.6, 3.7 µg/ml ) at 48h post-treatment. Moreover, bio-AgNPs synthesized from *P. luminescens laumondii* HP88, *P. luminescens akhurstii* HRM1 or *P. luminescens akhurstii* HS1 toxins significantly increased their larvicidal activities ($LC_{50}$ of 2.1, 1.5, 13.9 µg/ml, respectively) at 48h post treatment. In conclusion, the highest larval toxicity was observed when larvae were treated with bio-AgNPs synthesized from *P. luminescens akhurstii* HRM1 and *X. indica*, followed by *P. luminescens laumondii* HP88 and *Xenorhabdus* sp. Subsequently, data of the present study suggest these bio-AgNPs toxin complexes as potentially effective bio-control candidates in the battle against mosquito. However, testing other types of bio-synthesized nanomaterials, and their synergistic combinations against different mosquito species still under investigation.

## INTRODUCTION

Some genera of mosquito species are considered major vectors of many diseases affecting humans and animals especially in tropical areas (Rozendaal, 1997). *Anopheles stephensi* Liston, for instance, is the primary vector of malaria (Murray *et al.*, 2012). *Culex. quinquefasciatus* Say and *Cx. pipiens* Linnaeus is the principal vector of bancroftian filariasis including *Wucheria bancrofti*, *Brugia malayi* (Khalil, 1930; Gad *et al.*, 1996; Kuppusamy & Murugan, 2008) Rift Vally fever virus (Darwish & Hoogstraal, 1981) and

Western Nile virus (Pelah *et al*., 2002). These pathogens affect two-thirds of the world's population and kill millions annually (Gubler, 1998). In this aspect, different mosquito species such *Culex* and *Aedes* are spreading all over Egypt (Meagan *et al*., 1980) and Kingdom of Saudi Arabia (Al-Khreji, 2005; Ahmed *et al*., 2011; Al-Ahmed, 2012). These mosquitos are the main vectors of dengue fever (Khan *et al*., 2008), *Filariasis* (Hawking, 1973), malaria (Madani, 2005) and Rift Valley fever (Al-Hazmi *et al*., 2003).

Currently the using of broad-spectrum chemical insecticides have introduced resistant mosquito to the environement (Al-Sarar, 2010). Also, some synthetic mosquito repellents have produced encephalopathy of children (Briassoulis, 2001). Biological control, as an alternative approach to chemical insecticides, comes from the sustainability development concept of the growing awareness of environmental pollution, especially in food, which has produced resistance strains of vector-mosquito including *Cx. pipiens* populations in many parts of the world (Osta *et al.*, 2012; Alwafi *et al.*, 2013; Aziz *et al.*, 2014). Regarding environment friendly methods, integrated pest management (IPM) depending on insect predators are involved in mosquito control. Microbial insecticides based on endo-toxins of *Bacillus* spp. have also achieved considerable IPM program goals (Rydzanicz *et al*., 2009). Additionally, the entomopathogenic nematodes, *Steinernema* and *Heterorhabditis* together with their symbiotic bacteria *Xenorhabdus* and *Photorhabdus*, respectively, upon reaching the host insect haemocoel produce a variety of secondary metabolites that enable their colonization and reproduction (Webster *et al*., 2002). The host insect can be destroyed by these toxic metabolites (Ffrench-Constant *et al*., 2007). Rodou *et al.* (2010) have characterized and classified these toxins into four major groups. The toxin complexes (Tcs) is one of these four groups which attracted attention from the fact that some of their complexes showed high toxicity towards insects suggesting potentiality as insecticides.

In the present decade, nanotechnology as a promising field of research is expected to give major impulses to technical innovations in a variety of industrial sectors. Nanoparticles of noble metals including silver and gold have exhibited remarkable physical, chemical and biological properties from their bulk counter parts (Priya & Santhi, 2014). Benelli (2016) has investigated the biosynthesis of AgNPs for mosquito control. And also, Govendarajan *et al.* (2016) have recently synthesized AgNPs using aqueous leaf extract of *Bauhinia variegate* and confirmed their potentiality for mosquito control. In the present investigation, an approach towards the synthesis of five species of symbiotic crude bacterial-toxin complexes, *X. indica*, *Xenorhabdus* sp., *P. luminescens laumondii* HP88, two types of *P. luminescens akhurstii* HRM1 and *P. akhurstii luminescens* HS1 toxins. And the characterization of these bio- AgNPs using scanning electron microscopy, x-ray diffraction analysis as well as the confirmation of their significant role as bio-insecticide agents towards the 3rd larval instar of *Cx. pipiens* has been explored.

## MATERIALS AND METHODS

**Rearing experimental mosquitoes**

A susceptible strain of *Cx. pipiens*, was reared at Zoology Department, King University, for several generations prior to conducting this study according to Ahmed *et al.* (1999). Briefly, adults at 26 ± 1°C and :12 h (light:dark) and provided with 10% glucose solution. Females were fed on CD mice blood to lay eggs and hatched larvae were fed on 'Liquifry' (Interpet Ltd, Dorking, U.K.) for two days, then provided with ground 'TetraMin' flake food (Tetra Werke, Melle, Germany) until pupation. Third-instar larvae were subjected to bioassay tests.

**Isolation of nematode symbiotic bacteria**

The bacterial symbionts, *P. luminescens laumondii* HP88 as well as *P. luminescens akhurstii* HRM1 and *P. luminescens akhurstii* HS1 were isolated from the ento-

mopathogenic nematodes *H. bacteriophora* HP88, *H. indica* RM1 and *Heterorhabditis* sp. S1, respectively (El-Sadawy *et al.*, 2016). Also, *X. indica* species and *Xenorhabdus* sp. were isolated from the entomopathogenic nematodes *Steinernema abbasi* and *Steinernema* spp. respectively. *Photorhabdus* and *Xenorhabdus* bacteria have been isolated from their symbiotic nematodes according to Goetsch *et al.* (2006). For each subculture, the phase status was determined by culturing on NBTA agar (2.3% nutrient agar [Difco], 0.0025% bromothymol blue "Merck", 0.004% 2, 3, 5- triphenyltetrazolium "Merck"). Phase I colonies are blue on NBTA while phase II colonies are red. Twenty nematode infective juveniles were surface sterilized for 1 min in 1% sodium hypochlorite, washed 3 times in sterile distilled water, transferred to a Petri dish containing 50 µl of the nematodes suspension (about 100 IJs) was placed in a sterile 5 ml Petri dish, followed by 100 µl of sterile Luria-Bertani Miller (LB) broth (Difco). Sterile nematode (5–7 IJs) was picked and submerged in a sterile 100 µl of LB broth supplemented with 2.5% ampicillin. Using a sterile pestle, the nematodes were ground for 1 min on ice. NBTA media plates were incubated at 29°C, and single bacterial colonies were subculture onto new NBTA plates until no contamination was detected. A single pure colony was subculture into LB broth at 29°C for 24 h in a shaking incubator at 200 rpm. The isolated bacteria were maintained on LB broth monthly and kept at 10°C until used.

**Extraction of bacterial cell-free crude toxins**

200 ml of *Photorhabdus* or *Xenorhabdus* culture was grown for 48h in 2% Proteose Peptone no. 3 (PP3) plus 0.5% Tween 60 at 29°C, centrifuged at 9,000 rpm for 30 min. A sample of the supernatant fluid of each culture was dialyzed for protein concentration using poly ethylene glycol for molecular biology, average MW 8,000 (Sigma-Aldrich) according to Stair *et al.* (1972). Protein content of concentrated cell-free toxin proteins was estimated according to Lowry *et al.* (1951). Then, concentrated cell-free toxin proteins were stored at -80°C until used for larvicidal bioassay.

**Synthesis of bio- silver nanoparticles (bio-AgNPs)**

Colonies of bacterial symbionts were cultured overnight in 2 ml Luria-Bertani Miller (LB) broth "Difco" selected media. Bacteria were then inoculated in fresh 2–3 ml of LB broth in 250 ml flask and further incubated for 24 h in a shaking incubator at 29°C and 200 rpm. Bacterial culture was then calibrated on electro-photometer to 0.48 on the 600-nm scale to representing $4 \times 10^7$ cells/ml. Then, 1 ml was added to 300 ml of 2% Proteose Peptone no. 3 (PP3) plus 0.5% Tween 60. The culture was then incubated in rotary shaker incubator at 29°C and 200 rpm for 48h. For the AgNPs bio-production, the bacterial filtrate was used to reduce the $AgNO_3$ into AgNPs. In 500 ml Erlenmeyer flask, 200 ml of the bacterial filtrate was added to one mM $AgNO_3$/10 ml bacterial filtrate. The flasks were then incubated in a rotary shaker incubator at 200 rpm in the dark at 29°C. The filtrate was centrifuged at 10,000 rpm for 30 min. The precipitate was collected, washed with sterile $dH_2O$ for 3 times and then dried in oven at 50°C for 24h, then weighed and kept in dark until used. One mM of $AgNO_3$/10 ml suspended in 20 ml deionized water was used as control (Birla *et al.*, 2013).

**Characterization of the bio-AgNPs**

The synthesis of AgNP bacterial toxins (the reaction mixture) was subjected to centrifugation at 15,000 rpm for 20 min, resulting pellet was dissolved in deionized water and filtered through Millipore filter (0.45 ml). An aliquot of this filtrate containing AgNP was used for SEM and XRD analysis (Stuart, 2002). The structure and composition of freeze-dried purified AgNP was analyzed by using a 10 kV ultra-high-resolution scanning electron microscope with 25µL of sample was sputter coated on copper stub and the images of AgNP were studied using a FEI QUANTA-200 SEM (Murugan *et al.*, 2015).

**Preliminary mosquito bioassay**

Initially, a high concentration of each of the crude toxin proteins or the synthesized bio-AgNPs were used in parallel with that of control in preliminary screenings of larvicidal activity. Concentrations of either material were adjusted in 1 ml sterile $H_2O$ prior to each experiment. For preliminary bioassays, groups of ten 3rd instar larvae were transferred into 30 ml plastic cups containing 20 ml of $H_2O$ using a Pasteur pipette. To detect larvicidal activity, the crude toxins or bio-AgNPs was then added to experimental larvae and mortality was scored at 3,6,12 and 48h post-treatment compared to that of the control larval group (with no treatments). Out of the tested materials, those exhibiting significant mosquito larvicidal activity, were selected for further bioassay assessment.

**Main larvicidal bioassays**

**i) Using crude bacterial toxins**

Based on the screening results of the preliminary bioassays, each of the potentially effective toxins were adjusted to 5 descending concentrations (300, 150, 75, 37.5 and 18.75 µg/ml), and applied to the experimental larvae, while control replicates were treated with distilled water only. Larval mortality was recorded at 48h post-treatment, and five replicates were performed (n = 5) in each experiment as recommended by WHO (2005) with some modifications. Briefly, 10 larvae (3rd instar) were placed in each well of a sterile standard 12-wells tissue culture test plate (Nunclone Delta Surface, Thermo-Fischer Scientific, Denmark) with 2 ml $dH_2O$ mixed with the assigned concentration of bacterial toxin proteins or $dH_2O$ for the negative control larval group. Each bioassay was run for 48h, during which larvae were fed to avoid mortality caused by starvation. A lack of larvae reaction to gentle prodding with a glass pipette was considered as mortality according to Brown *et al.* (1998). After 48h, the percentage of larval mortality was calculated for each concentration using Abbott's formula (Abbott, 1925). Bioassays comprising one replicate of each concentration were replicated five times for every experimental tested. The $LC_{50}$ and $LC_{95}$ of each concentration were estimated using Probit Analysis.

**ii) Using bio-AgNPs**

The bio-AgNPs powder synthesized from each of the bacterial symbiont toxins were suspended in sterile $dH_2O$ to the concentration 100 mg/ml in tween 80 (as emulsifier), then sonicated for 2–3 min prior to each experiment. Based on the results of the preliminary bioassay test, the potentially effective concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.5 µg/ml) were applied to the experimental larvae. Toxin application was carried out in 5 replicates as detailed above. Negative control larvae were treated with $dH_2O$, and positive control was considered using $AgNO_3$ in deionized water. All experimental larvae were kept for 48h, and mortalities were recorded at 3, 6, 12, 24 and 28h post-treatments, and subsequently, $LC_{50}$ and $LC_{95}$ were calculated as described above.

**Data analysis**

Mean mortality of each toxin concentrations was calculated using MINITAB software (MINITAB, State College, PA, v: 13.1, 2016). Then, data of mosquito mortality were analyzed by Probit Analysis for calculating $LC_{50}$ and $LC_{95}$ (lethal concentration (µg/ml) that kills 50 and 95% of larvae, respectively), slopes, and standard error values of each treatment (5 replicates) were calculated according to Finney (1971). Relevant treatments were considered as not significantly different in their toxicity if confidential limits (95%) of $LC_{50}$ were overlapped (Litchfield and Wilcoxin, 1949).

## RESULTS

**Characterization of silver nanoparticles (AgNPs)**

The AgNPs were characterized by scanning electron microscopy (SEM) and X-ray diffraction analysis, SEM micrographs of the synthesized AgNPs of the five tested bacterial toxins magnified at x50,000 are represented in (Fig. 1 A-E). The triangular and spherical

structures are indicated by arrows. XRD analysis (Fig. 2) showed five intense peaks of AgNPs of the five tested bacterial toxins (*X. indica*, *Xenorhabdus* sp., *P. laumondii* HP88, *P. akhurstii* HRM1 and *P. akhurstii* HS1) in the whole spectrum of 2 theta (θ) values ranging from 20–220. The corresponding intense peaks were observed at values of 111, 200, 220, 220, and 311, respectively (Fig. 2). The present investigation using X-ray diffraction of the synthesized AgNPs bacterial toxins revealed crystalline morphological features.

**Larvicidal activities**

***Crude bacterial toxin complexes***

Based on the preliminary bioassay screening for larvicidal activity under laboratory conditions, all of the tested bacterial toxins showed variable larvicidal activities (data not shown). Subsequently, toxins were further subjected to quantitative $LC_{50}$ and $LC_{95}$ determination which revealed that, at 48h post-treatment, mortality of treated larvae with each of the 5 tested toxins was concentration dependent. Both *X. indica*

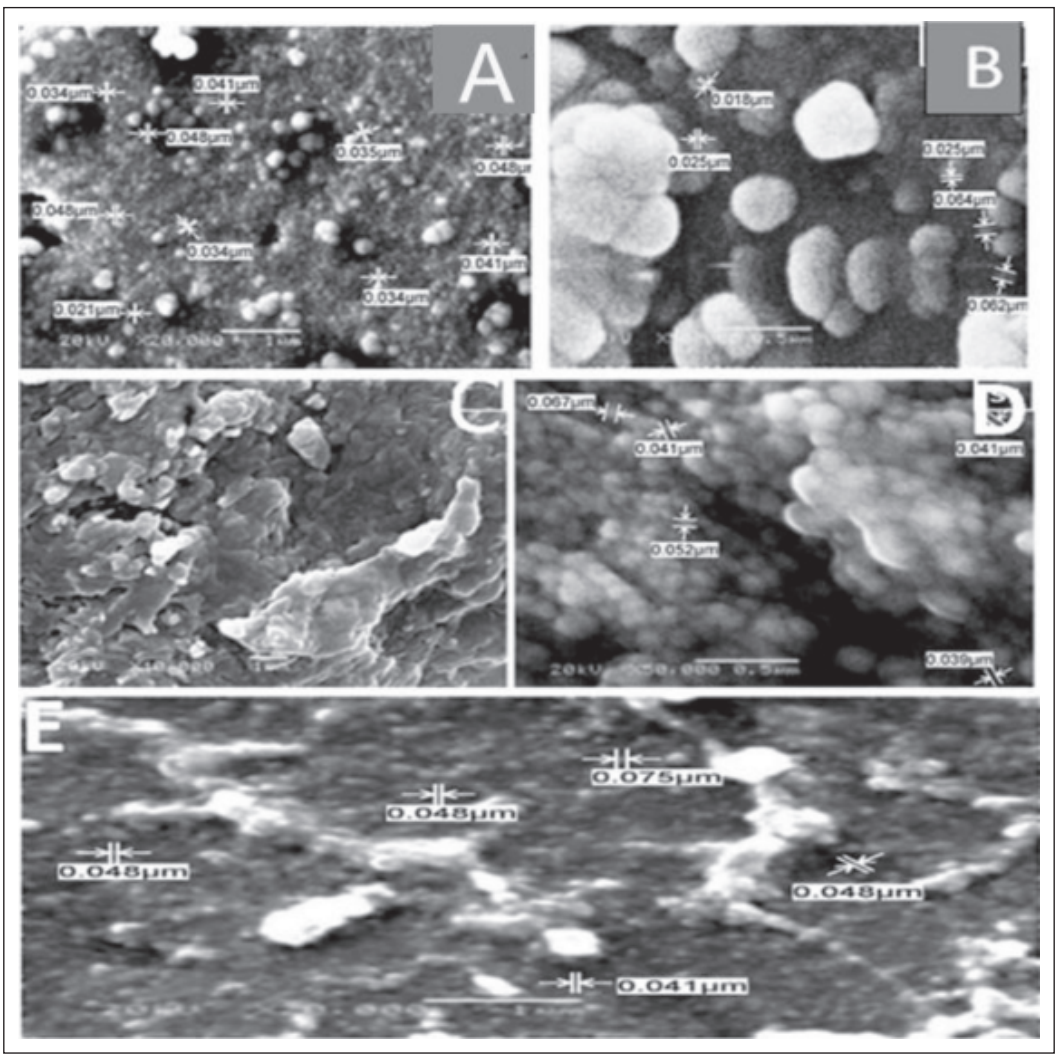


Figure 1. Scanning electron microscopy (SEM) micrograph showing the morphological characteristics of silver nanoparticles synthesized using different bacterial strains; **A)** *Xenorhabdus indica*, **B)** *Photorhabdus luminescens laumondii* HP88 **C)** *P. luminescens akhurstii* HRM1, **D)** *P. luminescens akhurstii* HS1 and **E)** *Xenorhabdus* sp. The triangular and spherical structures are indicated by arrows.

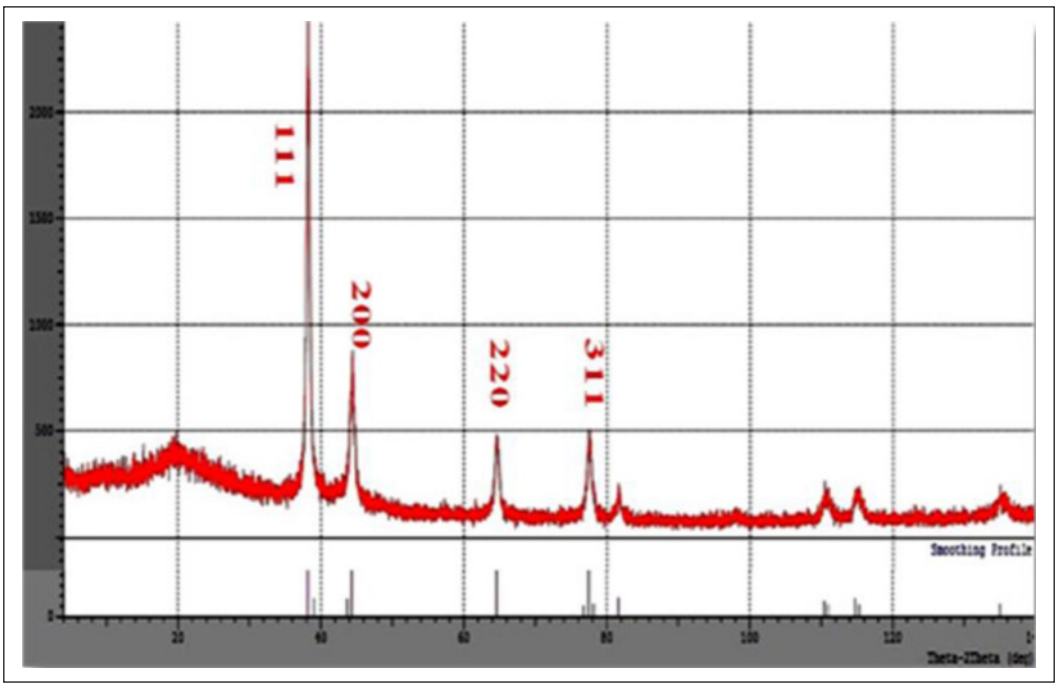


Figure 2. X-ray diffraction patterns of AgNPs biosynthesized from the five tested bacterial toxins. 111 for *Xenorhabdus indica*, 200 for *Xenorhabdus* sp., 220 for *Photorhabdus luminescens laumondii* HP88, 311 for *P. luminescens akhurstii* HRM1 and *P. luminescens akhurstii* HS1.

and *P. laumondii* HP88 showed significant higher larvicidal toxicity comparing to *P. akhurstii* HRM1, *Xenorhabdus* sp. and *P. akhurstii* HS1 ($LC_{50}$=29 *v* 199, 318 and 2002 µg/ml respectively), which is confirmed by the slope values (Table 1). On the other hand, toxicity of *P. akhurstii* HS1 was significantly the lowest compared with those of the other ones ($LC_{50}$ = 2002 µg/ml) and slope values confirm these observations. While, $LC_{50}$ of toxins from bacterial type *P. akhurstii* HRM1 and *Xenorhabdus* sp. showed moderate toxicity ($LC_{50}$ = 199 and 318 µg/ml, respectively (Table 1).

***Bio-AgNPs***

The preliminary larvicidal bioassay screening of the bio-AgNPs, synthesized from the targeted 5 toxins of the nematode-bacterial symbionts, confirmed variable mortalities (data not shown). Subsequently, $LC_{50}$ and $LC_{95}$ of the bio-AgNPs synthesized from each of the 5 individual bacterial toxins were determined at 3,6, 12, 24 and 48h post-treatment. Bio-AgNPs synthesized from both the toxins of *Xenorhabdus* bacterial species showed time-dependent high toxicity against treated larvae which was clearly noticeable commencing from 3h post-treatment, and reached their highest larvicidal activities by 48h post-treatment (Tables 2 & 3). Upon treatment with bio-AgNPs, the lethal doses were significantly declined from 29; 318; 28; 199 and 2002 µg/ ml to 1.6; 3.7; 2.1; 1.5 and 13.9 µg/ml for *X. indica*, *Xenorhabdus* sp., *P. laumondii* HP88, *P. akhurstii* HRM1 and *P. akhurstii* HS1, respectively (Tables 1–6). This indicates that *X. indica* synthesized AgNPs showed a significant 18.1 higher toxicity folds of that obtained using *X. indica* toxin alone ($LC_{50}$ = 1.6 *v* 29 µg/ml, respectively) 48h post treatment in each case (Tables 1 and 2). While *Xenorhabdus* sp. AgNPs showed significant 85.9 higher toxicity folds of that shown by *Xenorhabdus* sp. toxin alone ($LC_{50}$ = 318.0 *v* 3.7 µg/ml) 48h post-treatments (Tables 1 and 3, slope values confirm these observations).

Also, bio-AgNPs synthesized from toxins of the three *Photorhabdus* bacteria showed significant increase in larvicidal activities against treated larvae. Larvicidal activities

Table 1. Probit analysis for the toxicity cell free toxin proteins of *Photorhabdus* and *Xenorhabdus* spp. against 3rd instar *Cx. pipiens* larvae 48 hours post-treatment

| Parameter | Bacterial species | | | | |
|---|---|---|---|---|---|
| | *X. indica* | *Xenorhabdus* sp. | *P. laumondii* HP88 | *P. akhurstii* HRM1 | *P. akhurstii* HS1 |
| $LC_{50}$ (µg/ml) (lower to upper) | 29 (15.3–42.9)[a] | 318 (241.3–493.5)[b] | 28 (15.8–46.9)[a] | 199 (161.2–230.42) | 2002 (811–16228)[c] |
| $LC_{95}$ (µg/ml) (lower to upper) | 1401 (605.8–7894)[d] | 2198 (1136–7100)[e] | 1410 (615–7898)[d] | 790 (592.1–1241.2) | 44276 (7477–30890)[f] |
| Slope ± SE | 0.76 ± 0.13 | 1.52 ± 0.22 | 0.76 ± 0.14 | 2.3 ± 0.03 | 0.95 ± 0.20 |
| n | 5 | 5 | 5 | 5 | 5 |

$LC_{50}$: Lethal concentration (concentration to kill 50% of test larvae), $LC_{95}$: Lethal concentration (concentration to kill 95% of test larvae), SE: Standard Error of means (n=5). Values with different letters (a, b, c, d, e and f) are significantly different within treatments (based on the non-overlapping confidence limits) according to Litchfield and Wilcoxin (1949). No mortality was observed within the control groups.

Table 2. Probit analysis for the toxicity of *X. indica* – synthesized silver nanoparticles against 3rd larval instar of *Cx. pipiens* after different times post-treatment

| Parameters | Times post-treatment | | | |
|---|---|---|---|---|
| | 3h | 12h | 24h | 48h |
| $LC_{50}$ (µg/ml) (lower to upper) | 8.3 (5.4–12.5)[a] | 3.5 (3.2–3.9)[b] | 2.5 (2.3–2.8)[c] | 1.6 (1.5–1.8)[d] |
| $LC_{95}$ (µg/ml) (lower to upper) | 24 (19–47)[e] | 7.3 (6.4–8.5)[a] | 4.3 (3.9–5.0)[a] | 2.1 (1.9–3.2)[c] |
| Slope ± SE | 2.81 ± 0.18 | 4.14 ± 0.33 | 5.51 ± 0.51 | 10.23 ± 2.74 |
| n | 5 | 5 | 5 | 5 |

$LC_{50}$: Lethal concentration (concentration to kill 50% of test larvae), $LC_{95}$: Lethal concentration (concentration to kill 95% of test larvae), SE: Standard Error of means (n=5), Values with different letters (a, b, c, d and e) are significantly different within treatments (based on the non-overlapping confidence limits) according to Litchfield and Wilcoxin (1949). No mortality was observed within the control groups.

Table 3. Probit analysis for the toxicity of *Xenorhabdus* sp. synthesized silver nanoparticles against 3rd instar *Cx. pipiens* larvae after different times post-treatment

| Parameter | Times post-treatment | | | |
|---|---|---|---|---|
| | 3h | 12h | 24h | 48h |
| $LC_{50}$ (µg/ml) (lower to upper) | 8.0 (5.2–12.4)[a] | 6.2 (3.3–9.4)[a] | 4.9 (2.8–8.4)[a] | 3.7 (2.0–6.4)[a] |
| $LC_{95}$ (µg/ml) (lower to upper) | 58.0 (46.2–75.9)[b] | 22.0 (12.8–52)[c] | 10.9 (9.9–35.0)[a,c] | 8.9 (7.9–38.1)[a,c] |
| Slope ± SE | 2.90 ± 0.20 | 1.32 ± 0.15 | 3.72 ± 0.27 | 3.41 ± 0.27 |
| n | 5 | 5 | 5 | 5 |

$LC_{50}$: Lethal concentration (concentration to kill 50% of test larvae), $LC_{95}$: Lethal concentration (concentration to kill 95% of test larvae), SE: Standard Error of means (n=5), Values with different letters (a, b and c) are significantly different within treatments (based on the non-overlapping confidence limits) according to Litchfield and Wilcoxin (1949). No mortality was observed within the control groups.

Table 4. Probit analysis for the toxicity of *P. laumondii* HP88 – synthesized silver nanoparticles against 3rd larval instar of *Cx. pipiens* after different times post-treatment

| Parameter | Times post-treatment | | | |
|---|---|---|---|---|
| | 3h | 12h | 24h | 48h |
| $LC_{50}$ (µg/ml) (lower to upper) | 264.3 (155.6–605.7)[a] | 28.5 (22.1–37.4)[b] | 5.1 (4.1–6.36)[c] | 2.1 (1.5–2.7)[d] |
| $LC_{95}$ (µg/ml) (lower to upper) | 18861.2 (4921–181184.1)[e] | 909.3 (503.0–2040.0)[e] | 57.0 (44.1–78.0)[f] | 7.1 (13.5–23.1)[b] |
| Slope ± SE | 0.69 ± 9.17 | 0.85 ± 7.20 | 1.23 ± 0.08 | 1.41 ± 0.12 |
| n | 5 | 5 | 5 | 5 |

$LC_{50}$: Lethal concentration (concentration to kill 50% of test larvae), $LC_{95}$: Lethal concentration (concentration to kill 95% of test larvae), SE: Standard Error of means (n=5), Values with different letters (a, b, c, d and e) are significantly different within treatments (based on the non-overlapping confidence limits) according to Litchfield and Wilcoxin (1949). No mortality was observed within the control groups.

Table 5. Probit analysis for the toxicity of *P. akhurstii* HRM1– synthesized silver nanoparticles against 3rd larval instar of *Cx. pipiens* after different times post-treatment

| Parameter | Times post-treatment | | | |
|---|---|---|---|---|
| | 3h | 12h | 24h | 48h |
| $LC_{50}$ (µg/ml) (lower to upper) | 14.5 (10.8–18.7)[a] | 4.5 (2.4–6.7)[b] | 2.0 (0.9–2.9)[c] | 1.5 (0.6–2.0)[d] |
| $LC_{95}$ (µg/ml) (lower to upper) | 409.5 (242.2–864.8)[e] | 31.0 (21.5–63.4)[f] | 14.5 (9.8–28.8)[a] | 5.4 (4.1–12.9)[a] |
| Slope ± SE | 0.88 ± 8.68 | 1 .52 ± 9.52 | 1.49 ± 0.11 | 2.38 ± 0.26 |
| n | 5 | 5 | 5 | 5 |

$LC_{50}$: Lethal concentration (concentration to kill 50% of test larvae), $LC_{95}$: Lethal concentration (concentration to kill 95% of test larvae), SE: Standard Error of means (n=5), Values with different letters (a, b, c, d, e and f) are significantly different within treatments (based on the non-overlapping confidence limits) according to Litchfield and Wilcoxin (1949). Control mosquitoes showed no mortality.

Table 6. Probit analysis for the toxicity of *P. akhurstii* HS1 – synthesized silver nanoparticles against 3rd larval instar of *Cx. pipiens* after different times post-treatment

| Parameter | Times post-treatment | | | |
|---|---|---|---|---|
| | 3h | 12h | 24h | 48h |
| $LC_{50}$ (µg/ml) (lower to upper) | 0.0 | 115.4 (73.6–480.7)[a] | 27.7 (14.1–56.7)[b] | 13.9 (7.2–25.5)[c] |
| $LC_{95}$ (µg/ml) (lower to upper) | 0.0 | 2752.4 (2694.7–98373.6)[d] | 326.8 (264.3–1786.3)[a] | 143.7 (104.2–474.2)[a] |
| Slope ± SE | 0.0 | 0.93 ± 9.39 | 1.19 ± 9.28 | 1.263 ± 0.08 |
| n | 5 | 5 | 5 | 5 |

$LC_{50}$: Lethal concentration (concentration to kill 50% of test larvae), $LC_{95}$: Lethal concentration (concentration to kill 95% of test larvae), SE: Standard Error of means (n=5), Values with different letters (a, b, c and d) are significantly different within treatments (based on the non-overlapping confidence limits) according to Litchfield and Wilcoxin (1949). Control larvae showed no mortality.

of these toxicities were also time-dependent as they were clearly reached 48h post-treatment (Tables 4, 5 & 6). Bio-AgNPs synthesized from *P. laumondii* HP88 toxin showed significant 13.1 higher toxicity folds of that obtained using bacterial toxin alone ($LC_{50}$ = 2.1 *v* 28 µg/ml) 48h post-treatments (Tables 1 and 4). Moreover, *P. akhurstii* HRM1–synthesized AgNPs showed a significant 132.6 higher toxicity folds of that shown by the crude toxin (1.5 *v* 199 µg/ml) 48h post-treatments (Tables 1 and 5). Finally, *P. akhurstii* HS1 synthesized AgNPs showed time-dependent larvicidal activity commencing from 12h post-treatment, its toxicity was significant higher by 144.0 folds compared to that shown by the crude bacterial toxin ($LC_{50}$ = 13.9 *v* 2002 µg/ml) 48h post-treatments (Tables 1 and 6).

## DISCUSSION

Mosquito-borne diseases are considered as a global fatal health threat for millions of people every year (WHO, 2016). The presented study herein was conducted as a further step in support of the bio-control measures against *Cx. pipiens*, the most common vectors of filariasis, across the Middle East including Saudi Arabia (Al-Khuriji, 2007; Al-Ghamdi *et al.*, 2008; Ahmed *et al.*, 2011; Al-Ahmed, 2012). This is because we believe that the recent outbreaks of mosquito-borne diseases, such as Dengue virus in Saudi Arabia (Aziz *et al.*, 2014) and Zika virus (Benelli & Mehlhorn, 2016), has, in fact, highlighted the urgent needs of effective mosquito control managements, bearing in mind that the management of mosquito vector is challenging due to four main reasons. First, the spread of mosquito vectors worldwide (Mehlhorn, 2015). Second, the rise of new dangerous arboviruses for public health, (Benelli & Mehlhorn, 2016). Third, the rapid development of mosquito resistance to synthetic chemicals used as pesticides (Al-Sarar, 2010; Naqqash *et al.*, 2016). Fourth; chemical insecticides are known to leave both soil and groundwater contaminated with hazardous chemicals, and affect target organisms (Patel *et al.*, 1992). Therefore, we believe that effective alternative agents to the conventional pesticides is a must nowadays. In this context, it is important to clarify three points; a) we have successfully isolated local mosquitocidal *B. thuringiensis* that could be suggestive as potential effective candidate in mosquito control measures in Saudi Arabia (Ahmed *et al.*, 2017), b) and also isolated natural toxins from bacterial symbionts of locally isolated entomopathogenic nematodes (El Sadawy *et al.*, 2016) that significantly showed moderate mosquito larvicidal activities against *Cx. pipiens* (Ahmed *et al.*, 2017) and c) in the current study, we have explored on a larger scale the enhancing efficacy of these bio-AgNPs, on mosquito control.

Silva *et al.* (2013) reported that entomopathogenic bacteria *Photorhabdus luminescens* TTO DSM15139 (Fischer-le Saux, 1999) and *Xenorhabdus nematophilus* ATCC 19061 (Thomas & Poinar, 1979) suspensions were highly toxic to the 3rd instar larvae of *Ae. aegypti*. Similarly, the present investigation showed variable larvicidal toxicity of the five tested bacterial toxins. Out of them, the 100% mortality (at high concentrations) and the 6.8, 10.9 and 69.03 folds higher larvicidal toxicity of both *X. indica* and *P. laumondii* HP88 compared to *P. luminescens akhurstii* HRM1, *Xenorhabdus* sp. and *P. luminescens akhurstii* HS1 could be suggested as good candidates to be utilized in mosquito bio-control measures. In addition, Vani & Lalithambika (2014) have recorded a maximum mortality of (93.32%) against 3rd instar larvae of *An. gambiae* when exposed to protein isolated from *Xenorhabdus* strains. However, earlier study reported that protein toxins isolated from *X. nematophilus* strain A24 showed high toxic effect when injected to *Galleria mellonella* and *Helicoverpa armigra* (Khandelwal & Bhatanagar, 2003; Brown *et al.*, 2004). Further, Park (2015) have reported that *X. nematophilus* and *P. luminescens* bacteria enhanced *Bacillus thuringiensis* Cry4Ba toxin against *Ae. aegypti* (L) as it enhanced the larval mortality up to 95% at 48h post-treatment. These findings, in fact, could be

the reason behind the assumption of Hinchliffe *et al*. (2010) that these toxin complexes might be a major candidate for replacement of the *Bacillus thuringiensis* bacteria in the future. Taking these findings into consideration, the current investigation confirmed the potential enhancive effect of AgNPs on the mosquito larvicidal activity of these bacterial toxins. This, on one hand, is based on the discovery of nano-pesticides as a candidate to fight arthropod vectors (Govindarajan *et al*., 2016), and on the other hand, the development of reliable safe process for the synthesis of AgNPs using entomopathogenic bacterial toxins as suitable alternative which has been considered not only less eco-hazard than synthetic chemical insecticides but also enhance the bacterial toxicity against mosquito larvae (Priya & Santhi, 2014; Hajra & Mondal, 2015; Murugan *et al*., 2016).

In recent years, important efforts have been conducted to propose bio-synthesized nanoparticles as a valuable alternative to synthetic insecticides. However, the mosquitocidal potential of the bio-nanoparticles synthesized from bacteria toxins has been scarcely investigated. Thus, the current study focused on investigating the enhancive effect of the bio-AgNPs synthesized from five entomopathogenic bacterial toxins proved that, the bio-AgNPs synthesized from *X. indica* showed a significant 18.1 higher toxicity folds of that showed by *X. indica* toxin alone 48h post-treatment in each case. Moreover, *Xenorhabdus* sp.-synthesized AgNPs showed significant 85.9 higher toxicity folds compared to the toxin alone. In addition, bio-AgNPs synthesized from *P. laumondii* HP88 and *P. akhurstii* HRM1 toxins showed significant 13.1 and 132.6 higher toxicity folds of that shown by each of the individual bacterial toxin only. Taking all together, these data indicate that all tested bacterial toxins are mosquito larvicidal in a time-dependent manner. In addition, *X. indica* and *P. laumondii* HP88 showed significant higher larvicidal activities compared to the rest of the five tested bacterial toxins 48h post-treatment. In addition, the bio-AgNPs synthesized from each individual toxin clearly enhanced the larvicidal toxicity, which reached a significant increase by 144.0 folds in case of *P. akhurstii* HS1. Further, the highest larvicidal activity recorded was shown by the *P. akhurstii* HRM1-synthesized AgNPs ($LC_{50}$ = 1.5 µg/ml) 48h post-treatment, which was 132.6 folds higher than that of the toxin alone. These results may indicate that AgNPs have significantly enhanced the mosquito larvicidal activities of the five-targeted nematode-bacterial symbiont toxins. Another supporting result revealed that the larvicidal activity of AgNPs synthesized using *Bacillus thuringiensis* (Bt) was highly toxic against *Ae. aegypti* (Banu *et al*., 2014). These observations are comparable and support the current approach in the present investigation of using microbial toxins for bio-synthesizing of nanoparticle as it is a rapid, low-cost-method for biosynthesis process (Murugan *et al*., 2015; Benelli, 2016; Murugan *et al*., 2016).

Moreover, the use of green techniques for the synthesis of nanoparticles from silver as a new and rapidly evolving research field of nanotechnology, our findings, are in support of the successful work of Rajakumar *et al*. (2011) who have synthesized AgNPs using aqueous extract of *Ecliptapro stratato* against 3rd instar larvae of *Cx. quinquefasciatus* and *An. subpictus*. They observed that the maximum efficacy in crude aqueous and synthesized AgNPs against *Cx. quinquefasciatus* ($LC_{50}$=27.49 and 4.56 mg/L; $LC_{90}$=70.38 and 13.14 mg/L), and against *An. Subpictus* ($LC_{50}$=27.85 and 5.14 mg/L; $LC_{90}$=71.45 and25.68 mg/L). And also, Gnanadesigan *et al*. (2011) have evaluated the efficacy of synthesized AgNPs from *Rhizophora mucronata* leaf extract towards larvae of *Aedes aegypti* and *Cx. quinquefasciatus*. They found that the $LC_{50}$ and $LC_{90}$ values of the synthesized AgNPs were 0.585 mg/L and 2.615mg/L for *A. aegypti* and 0.891 mg/L and 6.291 mg/L for *Cx. quinquefasciatus*, respectively. These findings, as well as those of Borase *et al*. (2013) towards 2nd and 4th instars larvae of *Ae. aegypti* and *An. stephensi* revealed suggestive larvicidal potentiality of AgNPs with comparable $LC_{50}$ values. In addition, Dhanasekaran & Thangaraj (2013) have

evaluated the larvicidal activity of biogenic nanoparticles of *Agaricus bisporus*, *E. coli*, *Pencillium* sp. and *Vibrio* sp. against *Culex* mosquito vector. The mortality rate of *Culex* larvae, using *Agaricus bisporus* biogenic nanoparticles were 100% (5 mg/L), 81% (2.5 mg/L), 62% (1.25 mg/L), 28% (0.625 mg/L) and 11% (0.312 mg/L). A supportive evidence for this was also provided by Naik *et al.* (2014) as they tested AgNPs with the extract of *Pongami apinnata* leaves against mosquito vector. They found that plant extracts exhibited moderate larvicidal effects but the synthesized AgNPs was found to be toxic to mosquito larvae at $LC_{50}$ = 0.25. All these findings summarized the suggestion that synthesized bio-AgNPs have the potential to be used as a new candidate for controlling mosquito vectors.

There are some evidences that highlighted the interaction of the nanoparticles with mosquito larvae and their mode of action. Bannoth *et al.* (2014) proposed that the cause of larval death could be *via* binding the nanoparticles to proteins containing sulphur in the intracellular space or phosphorus in the DNA, which leads to enzyme and organelle degradation. Basically, cell death is mainly caused by decreased membrane permeability and disturbed proton motive force which leads to cellular function loss. Bowen *et al.* (1998), Owuama (2000) and Sheets *et al.* (2011) discussed the pathogenicity of these toxin complexes upon releasing into the host haemolymph to cause histopathological lesions and septicaemia leading to host death. Moreover, Borase *et al.* (2013) attributed the high larvicidal activity of AgNPs to their very miniature particle size which increases the surface area to volume ratio, and thus, increases its action against larvae. In addition, Miao *et al.* (2010) suggested that silver engineered nanoparticles can be taken in and accumulate inside cells, where they exerted their toxic effects. Similar results of the effect of particle size and shape on antibacterial application was recorded by Sosenkova & Egorova (2011). The present SEM investigation of the synthesized toxin AgNPs revealed its crystalline morphological nature. A similar crystalline nature was observed by Murugan *et al.* (2016) where AgNPs are synthesized using *Centrocerus clavulatum* extract. Also, AgNPs fabricated using seaweed extract of *Ulva lactuca* were predominantly cubical in shape ranging from 20 to 35 nm (Murugan *et al.*, 2016). Mahyoub *et al.* (2017) found that the *Halodule uninervis* synthesized AgNPs were predominantly spherical or cubic in shape. Additionally, the XRD peaks corresponding to 111, 200, 220, and 311 planes of face centered-cubic lattic of silver crystal in the present work are comparable to that observed by Murugan *et al.* (2016 ). Also, the unassigned peaks were identified due to the presence of phytochemicals from extracts that may capping on the surface of AgNPs (Singh *et al.*, 2008).

In conclusion, the significant enhancive effect the bio-AgNPs synthesized from the five-tested species of symbiotic crude bacterial toxins complexes in the current study could be suggestively used as a suitable effective candidate in the biocontrol battle against mosquito vectors. Finally, testing these nanoparticles combination against larvae of the dengue fever vector, *Ae. aegypti* is now under investigation in our lab. Moreover, the synergistic effect of different types of bio-synthesized nanoparticles are still further under investigation on other mosquito vectors.

*Acknowledgment.* This project was funded by the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology Kingdom of Saudi Arabia, Award number (11-MED-1848-02).

REFERNCES

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* **18**: 265-7.

Ahmed, A.M., Taylor, P., Maingon, R. & Hurd, H. (1999). The effect of *Plasmodium yoeliinigeriensis* on the reproductive fitness of *An. gambiae*. *Invertebrate Reproduction and Development* **36**: 217-222.

Ahmed, A.M., Shaalan, E.A., Aboul-Soud, M.A.M. & Al-Khedhairy, A.A. (2011). Mosquito vectors survey in AL-Ahsaa district eastern region Kingdom of Saudi Arabia. *Insect Science* **1**: 176.

Ahmed, A.M., Hussein, H., El-Kersh, T.A., Al-Sheikh, Y.A., Ayaad, T.H., El-Sadawy, H., Al-Mekhlafi, F.A., Ibrahim, M.S., Al-Tamimi, J. & Nasr, F.A. (2017). Larvicidal activities of indigenous *Bacillus thuringiensis* isolates and nematode symbiotic bacterial toxins against the mosquito vector, *Culex pipiens* (Diptera: Culicidae). *Journal of Arthrobode-Borne Diseases* **11**(2): 250-267.

Al-Ahmed, A.M. (2012). Mosquito fauna (Diptera: Culicidae) of the Eastern region of Saudi Arabia and their seasonal abundance. *Journal of King Saud University Science* **24**(1): 55-62.

Al-Ghamdi, K., Alikhan, M., Mahayoub, J. & Afifi , Z.I. (2008). Studies on identification and population dynamics of *Anopheline* mosquito from Jeddah, Saudi Arabia. *Bioscience Biotechnology Research Communication* **1**: 19-24.

Al-Hazmi, M., Ayoola, E.A., Abdurahman, M., Banzal, S., Ashraf, J., El-Bushra, A., Hazmi, A., Abdullah, M., Elamin, A., Al-Sammani, E., Gadour, M., Menon, C., Hamza, M., Rahim, I., Hafez, M., Jambavalikar, M., Arishi, H. & Aqee, A. (2003). Epidemic Rift Valley Fever in Saudi Arabia: A Clinical study of severe illness in humans. *Clinical Infectious Disease* **36**(3): 245-252.

Al-Khreji, M.A. (2005). Survey and distribution of mosquito species (Diptera: Culicidae) and description of its habitat in Riyadh district, Kingdom of Saudi Arabia. M.Sc. Thesis, King Saud University, Kingdom of Saudi Arabia.

Al-Khuriji, A.M., Alahmed, M.A. & Kheir, S.M. (2007). Distribution and seasonal activity of mosquitoes (Diptera: Culicidae) in Riyadh Region, Saudi Arabia. *Journal of King Saud University Agriculture Science* **19**(2): 39-55.

Al-Sarar, A.S. (2010). Insecticide resistance of *C.pipiens* populations (Diptera: Culicidae) from Riyadh city, Saudi Arabia: Status and overcome. *Saudi Journal of Biological Science* **17**(2): 95-100.

Alwafi, O.M., Scott, J.M., Ziad, A.M. & Abdullah, A. (2013). Dengue fever in Makkah, Kingdom of Saudi Arabia, 2008–2012. *American Journal of Research Communication* **10**.

Aziz, A.T., Al-Shami, S.A., Mahyoub, J.A., Hatabbi, M., Ahmad, A.H. & Rawi, C.S. (2014). An update on the incidence of dengue gaining strength in Saudi Arabia and current control approaches for its vector mosquito. *Parasites and Vectors* **7**: 258.

Bannoth, R.N., Singh, Y., Swarnagowreswari, G., Satyavathi, R. & Ramachandra, R.P. (2014). Silver nanoparticle synthesis from leaf extract of *chloroxylonswietenia* as an effective larvicide on dengue vector *Ae. albopictus* (Skuse) (insecta: diptera: culicidae). *International Journal of Science and research* **5**(3): 580-584.

Banu, A.N., Balasubramanian, C. & Moorthi, P.V. (2014). Biosynthesis of silver nanoparticles using *Bacillus thuringiensis* against dengue vector, *Ae. aegypti* (Diptera: Culicidae). *Parasitology Research* **113**(1): 311-316.

Benelli, G. (2016). Plant-synthesized nanoparticles: an eco-friendly tool against mosquito vectors. Nanoparticles in the fight against parasites. Part of the Parasitology Research Monographs book series, vol. 8 pp 155-172.

Benelli, G. & Mehlhorn, H. (2016). Declining malaria, rising dengue and Zika virus: insights for mosquito vector control. *Parasitology Research* **115**: 1747–54.

Birla, S.S., Gaikwad, S.C., Gade, A.K. & Rai, M.K. (2013). Rapid synthesis of silver nanoparticles from *Fusarium oxysporumby* optimizing physico-cultural conditions. *The Scientific World Journal* **2013**: 1-12.

Borase, H.P., Patil, C.D., Salunkhe, R.B., Narkhede, C.P., Salunke, B.K. & Patil, S.V. (2013). Phyto-synthesized silver nanoparticles: A potent mosquito biolarvicidal agent. *Journal of Nanomedicine and Biotherapeutic Discovery* **3**(1): 111.

Bowen, D., Rocheleau, T.A., Blackburn, M., Andreev, O., Golubeva, E., Bhartia, R. & ffrench-Constant, R.H. (1998). Insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science* **280**: 2129-2132.

Briassoulis, G. (2001). Toxic encephalopathy associated with use of DEET insect repellents: a case analysis of its toxicity in children. *Human and Experimental Toxicology* **20**: 8-14.

Brown, M.D., Thomas, D., Watson, K. & Kay, B.H. (1998). Laboratory and field evaluation of efficacy of vectobac 12AS against *Culex sitiens* (Diptera: Culicidae) larvae. *Journal of the American Mosquito Control Association* **14**: 183-185.

Brown, S.E., Cao, A.T., Hines, E.R., Akhurst, R.J. & East, D. (2004). A novel secreted protein toxin from the insect pathogenic bacterium *Xenorhabdus nematophila*. *Journal of Biological Chemistry* **279**(15): 14595-14601.

Darwish, M. & Hoogstraal, H. (1981). Arboviruses infesting human and lower animals in Egypt. A review of thirty years of research. *Journal of the Egyptian Public Health Association* **56**: 110-112.

Dhanasekaran, D. & Thangaraj, R. (2013). Evaluation of larvicidal activity of biogenic nanoparticles against filariasis causing Culex mosquito vector. *Asian Pacific Journal of Tropical Diseases* **3**(3): 174-179.

El-Sadawy, H.A., Forst, S., Abouelhag, H.A., Ahmed, A.M., Alajmi, R.A. & Ayaad, T.H. (2016). Molecular and phenotypic characterization of two bacteria, *Photorhabdus luminescens* subsp. akhurstii HRM1 and HS1 isolated from two entomopathogenic nematodes, *Heterorhabditis indica* RM1 and *Heterorhabditis* spp. S1. *Pakistan Journal of Zoology* **48**(1): 51-58.

Ffrench-Constant, R.H., Dowling, A. & Water Field, N.R. (2007). Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. *Toxicon* **49**: 436-451.

Finney, D.J. (1971). Probit analysis, 3rd ed. Cambridge University Press, Cambridge, Vol. 551 pp 68-72.

Fischer-Le Saux, M., Viallard, V., Brunel, B., Normand, P. & Boemare, N.E. (1999). Polyphasic classification of the genus Photorhabdus and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. Luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. *International Journal of Systematic Bacteriology* **49**: 1645–1656.

Gad, M., Hammad, R.E. & Farid, H.A. (1996). Uptake and development of *Wucheria bancrofti* in *C. pipiens* L. and *Ae. caspius* Pallas. *Journal of Egyptian Society of Parasitology* **26**: 305-314.

Gnanadesigan, M., Anand, M., Ravikumar, S., Maruthupandy, M., Vijayakumar, V., Selvam, S., Dhineshkumar, M. & Kumaraguru, A.K. (2011). Biosynthesis of silver nanoparticles by using mangrove plant extract and their potential mosquito larvicidal property. *Asian Pacific Journal of Tropical Medicine* 799-803.

Goetsch, M., Owen, H., Goldman, B. & Forst, S. (2006). Analysis of the PixA inclusion body protein of *Xenorhabdus nematophila*. *Journal of Bacteriology* **188**: 2706-2710.

Govindarajan, M., Rajeswary, M. & Veerakumar, K. (2016). Novel synthesis of silver nanoparticles using *Bauhinia variegata*: a recent eco-friendly approach for mosquito control. *Parasitology Research* **115**(2): 723-33.

Gubler, D.J. (1998). Dengue and dengue hemorrhagic fever. *Clinical Microbiology Reviews* **11**: 480-496.

Hajra, A. & Mondal, N.K. (2015). Silver nanoparticles: An eco-friendly approach for mosquito control. *International Journal of Science Research in Environmental Science* **3**(2): 0047-0061.

Hawking, F. (1973). The distribution of human Filariases throughout the world. *Mimeograph WHO/FIL* **73**: 114.

Hinchliffe, S.J., Hares, M.C., Dowling, A.J. & ffrench-Constant, R.H. 2010. Insecticidal toxins from the *Photorhabdus* and *Xenorhabdus* bacteria. *The Open Toxicology Journal* **3**: 101-118.

Khalil, M., Malawani, A. & Hilmi, I.S. (1930). The transmission of *Bancroftian filariasis* in Egypt. *The Journal of the Egyptian Medical Association* **15**: 315-332.

Khandelwal, P. & Bhatnagar, N.B. (2003). Insecticidal activity associated with the outer membrane vesicles of *Xenorhabdus nematophilus*. *Journal of Applied and Environmental Microbiology* **69**: 2032-2037.

Khan, N.A., Azhar, E.I., El-Fiky, S., Madani, H.H., Abuljadial, M.A., Ashshi, A.M., Turkistani, A.M. & Hamouh, E.A. (2008). Clinical profile and outcome of hospitalized patients during first outbreak of dengue in Makkah, Saudi Arab. *Acta Tropica* **105**(1): 39-44.

Kuppusamy, C. & Murugan, K. (2008). Mosquitocidal effect of *Euphorbia heterophylla* Linn against the bancroftian filariasis vector *C. quinquefasciatus* Say (Diptera: Culicidae). *International Journal of Integrative Biology* **4**(1): 34-39.

Litchfield, J.T. & Wilcoxin, F.A. (1949). Simplified method of evaluating dose-effect experiments. *Journal of Pharmacology and Experimental Therapeutics* **96**: 99-103.

Lowry, O.H., Rosebrough, N.J., Farr, A.B. & Randall, R.J. (1951). Protein measurement with the folin-phenol reagent. *Journal of Biological Chemistry* **193**: 265-275.

Madani, T.A. (2005). Alkhumra virus infection, a new viral hemorrhagic fever in Saudi Arabia. *Journal of Infection* **51**: 91-97.

Mahyoub, J.A., Aziz, A., Panneerselvam, C., Murugan, K., Roni, M., Trivedi, S., Nicoletti, M., Hawas, U.W., Shaher, F.M., Bamakhrama, M.A., Canale, A. & Benelli, G. (2017). Seagrasses as sources of mosquito nano-larvicides? toxicity and uptake of halodule uninervis-biofabricated silver nanoparticles in Dengue and Zika virus vector *Aedes aegypti*. *Journal of Cluster Science* **28**: 565-580.

Meagan, J.M., Khalil, G.M., Hoogstraal, H. & Adham, F.K. (1980). Experimental transmission and field isolation studies implicating *C. pipiens* as a vector of rift valley virus in Egypt. *American Journal of Tropical Medicine and Hygiene* **80**: 1405-1410.

Mehlhorn, H. (2015). Encyclopedia of parasitology, 4th edn. Springer, New York, pp 893

Miao, A.J., Luo, Z., Chen, C.S., Chin, W.C., Santschi, P.H. & Quigg, A. (2010). Intracellular uptake: a possible mechanism for silver engineered nanoparticle toxicity to a fresh water alga *Ochromonas danica*. *Public Library of Science* **1**(5): 15196.

Murugan, K., Dinesh, D., Paulpandi, M., Dakhellah Meqbel Althbyani, A., Subramaniam, J., Madhiyazhagan, P., Wang, L., Suresh, U., Mahesh Kumar, P., Mohan, J., Rajaganesh, R., Wei, H., Kalimuthu, K., Parajulee, M.N., Mehlhorn, H. & Benelli, G. (2015). Nanoparticles in the fight against mosquito-borne diseases: bioactivity of *Bruguiera cylindrica*-synthesized nanoparticles against dengue virus DEN-2 (*in vitro*) and its mosquito vector *Aedes aegypti* (Diptera: Culicidae). *Parasitology Research* **114**: 4349-4361.

Murugan, K., Nataraj, D., Madhiyazhagan, P., Sujitha, V., *et al.* (2016). Carbon and silver nanoparticles in the fight against the filariasis vector *Culex quinquefasciatus*: genotoxicity and impact on behavioral traits of non-target aquatic organisms. *Parasitology Research* **115**: 1071-1083.

Murray, C.J., Rosenfeld, L.C., Lim, S.S., Andrews, K.G., Foreman, K.J., Haring, D., Fullman, N., Naghavi, M., Lozano, R. & Lopez, A.D. (2012). Global malaria mortality between 1980 and 2010: a systematic analysis. *Lancet* **379**(9814): 413-31.

Naik, B.R., Gowreeswari, G.S., Singh, Y., Satyavathi, R., Daravath, S.S. & Reddy, P.R. (2014). Bio-Synthesis of Silver Nanoparticles from Leaf Extract of *Pongamia pinnata* as an effective larvicide on gengue vector *Aedes albopictus* (Skuse) (Diptera: Culicidae). *Advances in Entomology* **2**(2) Article ID: 45433, 9, DOI: 10.4236/ae. 2014.22016.

Naqqash, M.N., Gökçe, A., Bakhsh, A. & Salim, M. (2016). Insecticide resistance and its molecular basis in urban insect pests. *Parasitology Research* **115**: 1363-1373.

Osta, M.A., Zeinab, J.R., Pierrick, L., Mylène, W. & Khouzama, K. (2012). Insecticide resistance to organophosphates in *C. pipiens* complex from Lebanon. *Parasites and Vectors* 5132-137.

Owuama, C.I. (2000). Entomopathogenic symbiotic bacteria *Xenorhabdus* and *Photorhabdus*of nematodes. *World Journal of Microbiology and Biotechnology* **17**: 505-515.

Park, Y. (2015). Entomopathogenic bacterium, *Xenorhabdus nematophila* and *Photorhabdus luminescens*, enhances *Bacillus thuringiensis* Cry4Ba toxicity against yellow fever mosquito, *Ae. aegypti* (Diptera: Culicidae). *Journal of Asia-Pacific Entomology* **18**(3): 459-463.

Patel, C.C., Mehta, D.M., Patel, J.R. & Patel, N.M. (1992). Resistance of gram pod borer *Helicoverpa* 909 *armigera* (Hubner) to insecticides in Gujarat. *Indian Journal of Agriculture Science* **62**: 421-423

Pelah, D., Abramovich, Z., Markus, A. & Wiesman, Z. (2002). The use of commercial saponin from *Quilleja saponaria* bark as a natural larvicidal agent against *Ae. aegypti* and *C. pipiens*. *Journal of Ethnopharmacology* **81**: 407-409.

Priya, S. & Santhi, S. (2014). A Review on nanoparticles in mosquito control – A green revolution in future. *International Journal for Research in Applied Science and Engineering Technology* **2**(XII): 378-387.

Rajakumar, G. & Abdul Rahuman, A. (2011). Larvicidal activity of synthesized silver nanoparticles using *Ecliptaprostrata* leaf extract against filariasis and malaria vectors. *Acta Trop Journal* **118**: 196-203.

Rodou, A., Ankrah, D.O. & Stathopoulos, C. (2010). Toxins and secretion systems of *Photorhabdus luminescens*. *Toxins* **2**: 1250-1264.

Rozendaal, J.A. (1997). Vector control: Methods for use by individuals and communities. World Health Organization, Geneva.

Rydzanicz, K., Lonc, E. & Becker, N. (2009). Current procedures of the integrated urban vector mosquito control as an example in Cotonou (Benin, West Africa) and Wroclaw area (Poland). *Wiadomości Parazytologiczne* **55**(4): 335-340.

Sheets, J.J., Hey, T.D., Fencil, K.J., Burton, S.L., Ni, W.T., Lang, A.E., Benz, R. & Aktories, K. (2011). Insecticidal toxin complex proteins from *Xenorhabdus nematophilus*. Structure and pore formation. *Journal of Biological Chemistry* **286**: 22742-22749.

Silva, O.S., Prado, G.R., Silva, J.L.R., Silva, C.E., Costa, M. & Herrmann, R. (2013). Oral toxicity of *Photorhabdus luminescens* and *Xenorhabdus Nematophila* (Enterobacteriaceae) against *Ae. aegypti* (Diptera: Culicidae). *Parasitology Research* **112**: 2891-2896.

Singh, M., Singh, S., Prasad, S. & Gambhir, I.S. (2008). Nanotechnology in medicine and antibacterial effect of silver nanoparticles. *Digest Journal of Nanomaterials and Bio Structures* **3**(3): 115-122.

Sosenkova, L.S. & Egorova, E.M. (2011). The effect of particle size on the toxic action of silver nanoparticles. III Nanotechnology International Forum. *Journal of Physics: Conference Series* **291**: 012027.

Stair, E.L., Rhodes, M.B., White, R.G. & Mebus, C.A. (1972). Neonatal calf diarrhea: purification and electron microscopy of a coronavirus-like agent. *American Journal of Veterinary Research* **33**: 1147-1156.

Stuart, B.H. (2002). Polymer analysis. Wiley, Hoboken.

Thomas, G.M. & Poinar, G.O. (1979). *Xenorhabdus* gen. Nov., a genus of entomopathogenic nematophilic bacteria of the family Enterobacteriaceae. *International Journal of Systematic Bacteriology* **29**: 352-360.

Vani, C. & Lalithambika, B. (2014). Effect of outer membrane vesicle proteins of *Xenorhabdus* bacteria against malarial vectors. *International Journal of Pharma and Bio Science* **5**(4): (B) 1072-1080.

Webster, J.M., Chen, G. & Hu, K.L.J. (2002). Bacterial metabolites. Entomopathogenic nematology. In R. Gaugler, ed. CAB International, Wallingford, UK, pp. 99-114.

WHO (2005). Guidelines for laboratory and field testing of mosquito larvicides. WHO/CDS/WHOPES/GCDPP/2005.13 http://www.who.int/whopes/gcdpp/publications/en/index1.html.

WHO (2016). Vector-borne diseases. http://www.who.int/mediacentre/factsheets/fs387/en/