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A Novel Mosquitocidal Bacterium as a Biocontrol Agent in Saudi Arabia: I - A Promising Larvicide Against *Aedes caspius* Mosquito

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Abstract.- In this study, laboratory assessment was made with a water-dispersible toxin solution formulation of *Pseudomonas frederiksbergiensis* bacterium. The study was conducted to determine the activity of an isolated extract formulation of *P. frederiksbergiensis* toxin against 3rd stage larvae of the Rift Valley Fever vector, *Aedes caspius*, mosquito in Saudi Arabia for the first time. This bacterium was isolated from the contaminated Saudi soil and proved to be safe to living organisms and environment. This *P. frederiksbergiensis* extract, identified as glycolipid, has afforded a potent mosquito larvicidal activity against 3rd instar larvae of *Ae. caspius*. Larvicidal bioassay showed that the LC_{50} and LC_{90} against larvae were 253.91 or 441µl/l respectively at 24 hours post-treatment. Cessation of feeding was noticed starting from 6h post-treatment with the LC_{50}. Moreover, the histological studies with the light microscope showed destructive effects for the *P. frederiksbergiensis* extract on the midgut epithelial layer of treated larvae. Epithelial cells appeared degenerated with degenerated nuclei in treated larvae compared to those of control ones. Transmission electron microscopy showed degenerated nuclei, mitochondria and microvelii in treated larvae compared to control ones. These gut epithelial destructions may have caused septicemia which may have resulted in death. This might be the reason of stopping feeding by larvae at 6 hours post-treatment prior to death that started from 12 h post-treatment. Based on these data, *P. frederiksbergiensis* bacterial extract could be suggested as a suitable bio-larvicide for use in the battle against *Ae. caspius* in Saudi Arabia. This bio-pesticide may help in reducing the spread of this serious vector and that, may limit mosquito-borne diseases in the country.

Keywords: *Pseudomonas frederiksbergiensis*, biocontrol, *Aedes caspius*, histological effects, bacterial extract, larvicide.

INTRODUCTION

Mosquitoes constitute one of the most important group of insects that are not only the cause of nuisance by their bites but also transmit deadly diseases like malaria, filariasis, yellow fever, dengue and Japanese encephalitis (Service, 1995). Subsequently, these diseases contribute significantly to poverty and social debility in tropical countries, causing millions of death every year (Christophers, 1960; Knight and Stone, 1977). These vector-borne diseases affect two-thirds of the world's population and kill millions annually (Nasci and Miller, 1996; Gubler, 1998).

Mosquitoes have a cosmotropical distribution between 30ºN and 20ºS (Christophers, 1960; Knight and Stone, 1977) and exhibit a distinct preference for human habitats, including artificial oviposition sites, *e.g.,* tires, flower vases and water storage containers (Tabachnick, 1991). In the Kingdom of Saudi Arabia, different types of mosquito vectors spread all over the country (Mattingly and Knigh, 1956; Büttiker, 1981; Abdoon and Ibrahim, 2005; Al-Khreji, 2005; Ahmed *et al.*, 2011). These vectors transmit common mosquito-borne diseases including dengue fever (Fakeeh and Zaki, 2003; Ayyub *et al.,* 2006; Khan *et al.,* 2008), filaria (Hawking 1973), malaria (Warrel, 1993; Abdoon and Alsharani, 2003), and Rift Valley Fever (Jupp *et al.,* 2002; Al-Hazmi *et al.,* 2003; Balkhy and Memish, 2003; Madani, 2005). Three filarial cases were reported from Saudi residences in Armed Forces Hospital, Riyadh in 2002 (Haleem *et al.,* 2002). Omar (1996) reported that local *Culex pipiens* mosquitoes might act as a potential vector of introduced Bancroftian filariasis in Saudi Arabia. Thus, the Saudi Ministry of Health developed and implemented strict plans to prevent the appearance of mosquito-borne diseases specially in Hajj

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(pilgrimage) time. Hence, and in support of these governmental plans, we propose this study here as a support of establishing effective mosquito control strategies in the country.

In fact, repeated use of synthetic insecticides for mosquito control has fostered several environmental and human health concerns, including disruption of natural-biological control systems, resurgences in mosquito populations, widespread development of resistance, and undesirable effects on non-target organisms (WHO, 1971). These problems have highlighted the need of new alternative strategies for mosquito control. Thus, the urgent need for a clean and safe environment has forced the majority of scientists to focus on the utilization of environmentally safe biocontrol agents to manage mosquito vectors and to keep their numbers down the threading level. Researchers have pointed at the use of entomopathogenic microorganisms to control insects since long time ago (Becker and Margalit, 1993). Microbial biopesticides such as fungi, *Bacillus thuringiensis* and *Bacillus sphaericus*, are widely used to control mosquitoes (Margalith and Ben-Dov, 2000; Kay *et al*., 2002; Lord, 2005; Federici, 2005). In this context, *B. thuringiensis* was found to induce cellular and oxidative stress prior to mosquito death (Ahmed, 2012, 2013).

The current study proposed *Pseudomonas frederiksbergiensis* bacterium, a decomposer of organic matter in soil, water and food products, (Palleroni, 1993) in support of eco-friendly control measure. This bacterium is used as a biological control of fungal diseases in plants (Nielsen *et al*., 1998). Abdel-Megeed *et al.* (2006) found that the surface active agents produced by these bacteria could have biotechnological application for insect control. Thus, the current study is conducted to evaluate the efficiency of a bacterial extract, the extracellular glycolipids of *P. frederiksbergiensis*, as an environmentally safe biocontrol agent, against the larval stages of *Ae. caspius* mosquito, the Rift Valley Fever vector in Saudi Arabia (Ahmad, 2000; Al-Hazmi *et al*., 2003) in the lab. This could be a first step towards utilization in the biocontrol measure against mosquitoes at a large scale in open fields.

MATERIALS AND METHODS

Field collection of mosquito

Mosquito larvae were collected from different breeding locations in AL-Ahsaa district, the Eastern region of Saudi Arabia using nets consisting of an iron ring (20 cm in diameter) to which a muslin sleeve (30 cm long) was attached (Ahmed *et al.*, 2011). Collected larvae from different locations were separately labeled and moved to the rearing insectary in Zoology Department, Faculty of Science, King Saud University for experimental purposes as detailed below.

Ae. caspius *identification and rearing*

Field collected larvae were morphologically identified according to the classification keys of Mattingly and Knight (1956) for selecting *Ae. caspius*. Morphological identification of collected samples was confirmed by the Natural History Museum (London, UK). Larvae of *Ae. caspius*, collected from different locations were separately reared until adult emergence under standard insectarium conditions (26ºC, 12h/12h light/dark period and 80–82% humidity) (Ahmed *et al.,* 1999). Adults emerging within a 24h period were maintained in rearing cages (30 × 30 × 30 cm each) with continuous access to a 10% glucose solution (w/v). After adult emergence, mosquitoes of the same age were used for the relevant experiments in the current study. To maintain a stock of mosquito colony, they were kept accessing 10% glucose until being allowed to feed on CD mouse blood for triggering vitellogenesis as detailed in Ahmed *et al.* (1999). Mosquitoes were reared in the lab for more than 25 generations prior to conducting the current study.

Bacterial isolation and culturing

The bacterium *P. frederiksbergiensis* were isolated from contaminated soil in Riyadh, Kingdom of Saudi Arabia according to Abdel Megeed and Mueller (2009). The bacteria were grown aerobically in a mineral salt medium containing 0.5% Na $HPO_4.12H_2O$, 0.1 %KH_2PO_4, 0.05% $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, pH 7.0 in Erlenmeyer flasks with a working volume of 1 liter. The

enrichment of these strains is carried out using Luria Bertani (LB) media. One mM docosane is used as a sole source of carbon and energy. Bacteria-seeded medium was incubated for 24h at 36°C until visible colonies are formed. A sample of bacteria was moved onto a suitable nutrient solid agar medium then incubated for 24 h at 36°C. Resulting colonies, observed as identical in morphology, size and colour, were used for extracting their toxins.

Extracting bacterial toxins

Extraction and purification of the *P. frederiksbergiensis* toxic biosurfactants was carried out according to Abdel-Megeed and Majhdi (2009). Briefly, 200 ml liquid bacterial culture were centrifuged at 10,000 rpm for 10 min at 4°C. The floating materials resulting from the treatment of the supernatant by 40% ammonium sulphate were collected by centrifugation and then dissolved in a small fraction of water. Chilled acetone was added to the solution to remove the protein and acetone-insoluble materials. This step was critical in the purification procedure because the biosurfactant is soluble in acetone at high concentration. Any decane that may have remained in the acetone fraction was removed by extracting it three times with hexane. For sugar fatty acid esters separation, the mixture was subjected to extraction with warm hexane (50°C) and filtration. This step resulted in fatty acids in the organic phase and sugar esters, enzymes and sugars onto the filter.

Determination of the hydrophilic moiety was carried out according to dinitrosalicyclic assay that depends mainly on reducing ends of carbohydrates. Reagent composition was 1 % of 3,5-dinitrosalicylic acid, 30 % of sodium potassium tartarate, and 0.4 M NaOH. Equal volumes of the sample and the previously mentioned reagent were mixed and heated in a boiling water bath for 10 min. After rapid cooling to RT and diluting with 10 volumes of water, the absorbance at 570 nm was measured. Sucrose was used as a reference for the calibration curve. For further identification of the sugar moiety, the analysis was carried out by HPLC. Methyl esters of fatty acids will be prepared as previously described by Morrison and Smith (1964). Equal amounts from the sample and 1M of methanolic NaOH are mixed well in a glass vial. The mixture was incubated in a water bath at 60°C for 20 min in order to cleave off fatty acids. Equal amount of BF_3/methanol was then added after cooling on ice for 1 min in order to create methyl esters. After incubation at 60°C for 10 min, 200 µl of saturated NaCl is added and mixed well. The sample was transferred to a 1.5 ml Eppendorf tube, then 300 µl hexane was added and finally centrifuged at 13,000 rpm for 10 min. The hexane layer was separated. The extract was dried over anhydrous Na_2SO_4 before analysis with GC-MS. The supernatant was transferred to a fresh tube. The water layer was extracted for second time and the hexane layer was combined with the previous extract, then subjected to GC-MS. The system parameters: inlet temperature was 250°C and detection temperatures was 280°C, a temperature program with an initial oven temperature 160°C for 1 min, final temperature 240°C and heating rate 10°C/min to 240°C with Helium as carrier gas at P = 0.37 atm.

Larvicidal activity bioassay

Bioassay for mosquito-larvicidal activity was performed using different concentrations of the *P. frederiksbergiensis* extract. Fractions were dissolved in hexane and made up to different concentrations in serial dilutions from the stock solution of the extracts. A preliminary bioassay was performed using a wide range of nine ascending concentrations ranging from 3.5–1000 µl/l. Based on the preliminary results, a narrower range of the lethal concentrations ranging from 31.25 – 1000 µl/l in five replicates each (n = 5) was used for carrying the bioassay experiment in rearing plastic trays (30 × 15 × 10 cm). Each tray was containing 100 third-instar larvae in 1000 ml dH_2O infused with various concentrations of the bacterial extract according to Rey *et al.* (1999) in the presence of food (ground Tetramin flakes). In parallel, control larvae were treated in the same manner with hexane, the solvent of the bacterial toxic extract. The medium was not subsequently replaced, and food was added as normal, so that larvae were fed normally until feeding cessation was noticed starting from 6h post-treatment. Larvae were monitored 3 h for observing mortality until 24 h had occurred. The number of larvae surviving was counted at 24 h. Death or lack of reaction to gentle prodding with a glass

pipette was the measured mortality according to (Brown *et al*., 1998). Larval mortality percentages were calculated until 24 h post-treatment in 5 replicates (n=5) for each concentration according to the WHO guide lines (2005) using the Abbott's formula (Abbott, 1925) as following:

$$\text{Mortality (\%)} = [(X - Y)/X]100$$

Where X = % survival in control,
and Y = % survival in treated mosquitoes.

Based on the resulting mortality percentages, the suitable concentrations used in the subsequent toxicity experiment of the current study were assessed within the 6 ascending lethal concentrations (from 31.25 – 1000.00 µl/l) of 5 replicates each (n=5). The relationship between concentrations and mean mortality % was plotted *via* a Regression Plot using MINITAB software (MINITAB, Stat College, PA, version 13.1, 2001), and the resulting Linear Formula was used for determining the fifty percent and ninety percent lethal concentrations (LC_{50} and LC_{90}, respectively). LC_{50} was found to be 253.91µl/l, (see results) and used in the subsequent experiments of the current study.

Histological examination of midgut epithelium

After treatment with LC_{50} dose (253.91µl/l) of *P. frederiksbergiensis* extract, alive, but sluggish, 3rd instar *Ae. caspius* larvae were used for investigating histological alterations at 24h post-treatment under light microscopy according to Ahmed *et al.* (2010) and Villalon *et al.* (2003). Briefly, control and treated midguts were fixed overnight in cold 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) and post-fixed for 1 h at room temperature in 1% OsO4 in the 100 mM phosphate buffer. Tissues were then dehydrated through ascending ethanol series, treated with propylene oxide and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Thin 10 µm sections were mounted on slides, stained with hematoxylin and eosin (Sigma-Aldrich) and mounted with Paramount (Fisher) and examined by light microscopy using a Zeiss Axioskop 50 compound microscope (Carl Zeiss, Inc., Thornwood, NY). Resulting images from control or treated larval preparations were imported into Adobe Illustrator Cs, 2003 Software and adjusted for contrast and suitable qualities.

Ultrastructural examination of larval midgut epithelium

Transmission electron microscopy (TEM) was used for studying the impact of bacterial extract on the ultrastructure of gut epithelial cells. Control or treated midguts were dissected at 24 h post-treatment just prior to death. Dissected midguts were fixed overnight in cold 0.8% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate, (pH 7.0) and postfixed for 4 h at 4°C in 1% OsO4 in 0.1 M sodium cacodylate (pH 7.0). Midguts were then dehydrated through ascending ethanol series, treated with propylene oxide and embedded in Epon-Araldite resin (1:1). Ultrathin 4 µm sections were mounted on slides, stained with 2% uranyl acetate for 30 min and then incubated in lead citrate for 10 min. Samples were then analyzed with a transmission electron microscope (Jeol Ltd., model JEM-100CX II) at 80 kV.

Statistical analysis

All statistical analyses were undertaken using MINITAB software (MINITAB, State College, PA, v: 13.1, 2001). Data pertaining to the mortality % were used for basic statistical analysis for assessing means and standard errors. Five replicates (five different mosquito groups: n = 5) were carried out for better statistical analysis.

RESULTS

Morphological identification

Larvae of *Ae. caspius* mosquitoes were morphologically identified from amongst the collected larvae from different breeding sites in the Eastern region of Saudi Arabia. This mosquito species was found to be the most abundant within the other collected species**.** These *Ae. caspius* mosquitoes were recorded in water localities exhibited high salinity levels of around 12800 ppm and pH levels of around 7.4 (Ahmed *et al*., 2011). However, this mosquito species was found to be able to live in distilled H_2O water in the lab and

complete its lifecycle normally. *Ae. caspius* mosquitoes were reared in the lab using tap water for experimental purposes of the present study as detailed in Ahmed *et al*., 1999).

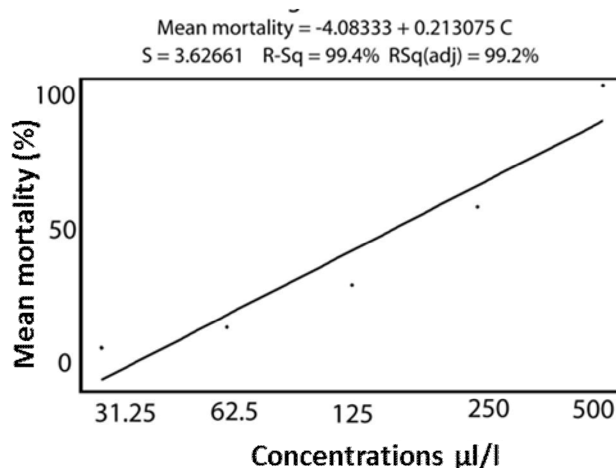

Fig. 1. A regression plot showing the linear relationship between the ascending lethal concentrations of *P. frederiksbergiensis* extract, and means of mortality percentages (n = 5) of 3rd larval instar of *Ae. caspius* at 24h post-treatment. The resulting regression formula was used for calculating both LC_{50} and LC_{90}.

Mosquito-larvicidal bioassay

This part of study was conducted to determine the LC_{50} and LC_{90} of *P. frederiksbergiensis* toxic extract against the 3rd larval instar of *Ae. aegypti* mosquito at 24 h post-treatment. Larvae were treated with wide range of ascending concentrations ranging from 3.5–1000 µl/l (Table I). Concentration below 31.25 µl/l showed no mortality and concentrations of 500 µl/l and above showed 100% mortality before 24h post-treatment. Thus, ascending concentrations ranging from 31.25–500.00 µl/l was assessed for determining LC_{50} and LC_{90} at 24h post-treatment (Table I). The mean larval mortality percentage (calculated by Abbott's formula) was increased with increasing concentrations. Furthermore, cessation of feeding was noticed in treated larvae starting from 6h post-treatment. A linear relationship between concentrations and mean mortality percentages was made *via* a regression plot (Fig. 1) using Minitab statistical program. The following resulting linear formula from the plot was used to calculate both LC_{50} and LC_{90}:

$$M = -4.08333 + 0.2130C$$

where M is mean larval mortality (%), and C is concentration of bacterial extract. The concentration (C) was calculated from the formula in case of M = 50 or 90 (LC_{50} or LC_{90}, respectively), which was equal to 253.91 or 441µl/l, respectively. The LC_{50} (187.5 µl/l) was used for the subsequent experimental purposes of the current study.

Histopathological effect on larval midgut

Third instar larvae of *P. frederiksbergiensis* were histologically examined 24 h post-treatment both by light and transmission electron microscope. Figure 3 shows effect of LC_{50} (253.91µl/l) of *P. frederiksbergiensis* extract on the internal structure of the midgut of *Ae. caspius* 3rd instar larvae. The epithelial cells was disintegrated and nuclei of the epithellial cells were fragmented 24 h post-treatment (Fig. 2D). Although gut was still filled with nutritional contents, the peritrophic membrane and microvilli were degraded by bacterial toxins, whereas those of the control retained their structural integrity (Fig. 2C, D). Hence, this may indicate a high toxicity of *P. frederiksbergiensis* extract on the internal structural integrity of treated larval midguts.

In order to examine the impact of the LC_{50} (253.91µl/l) of the larvicidal bacterial extract on sub-cellular organelles, TEC revealed that the treatment with this dose of larvicidal bacterial extract is associated with disrupted microvilli (Fig. 3B) disintegrated nuclei (Fig. 3D) and mitochrondria (Fig. 3F).

DISCUSSION

P. frederiksbergiensis is rod-shaped Gram-negative phenanthrene-degrading bacterium which acts as an important decomposer of organic matter in soil, water and food products (Palleroni, 1993; Anderson *et al.*, 2000). Its biotechnological importance is partly due to their potential plant growth-promoting effects and application in biological control of fungal diseases in plants (Nielsen *et al.*, 1998). It has been used as biocontrol

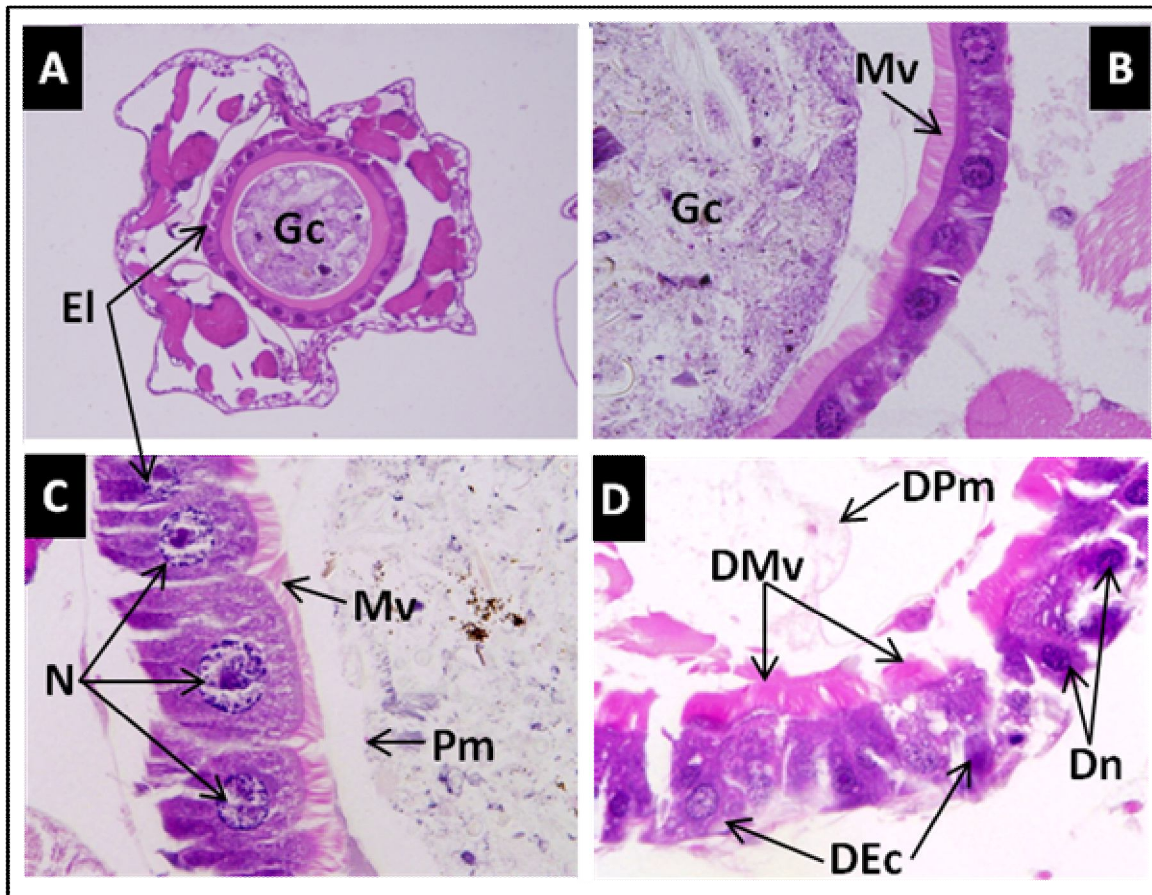

Fig. 2. Effect of *P. frederiksbergiensis* extract on histological structure of midgut epithelial tissue of *Ae. caspius* larvae 24 h post-treatment. A and B (100 and 600×, respectively): 10 μM thin cross-section of untreated control larvae showing normal gut epithelial layer (El), microvellii (Mv), and nutritional gut contents (Gc) filling the gut lumen. C, gut epithelium of control larva (1000×) showing normal gut epithelial cells with prominent nuclei (N) microvilli (Mv) and peritrophic membrane (Pm). D, showing gut epithelium of treated larva (1000×) with clear degeneration in epithelial cells with vacuoles (DEc), nuclei (Dn), microvilli (DMv) and peritrophic membrane (DPm).

agent against plant pathogens (Haas and Defago, 2005). Prabakaran *et al.* (2003) has preliminarily tested it against some culicine and anopheline mosquito showing promising effects. Furthermore, Abdel-Megeed and Majhady (2009) found that the toxins produced by this bacterium could have biotechnological application for insect control. Hence, the toxic surface-active extract from this bacterium was tested against *Aedes caspius* mosquito larvae in the current study.

Many mosquito species have been identified from the Eastern region of Saudi Arabia (Ahmed *et al.*, 2011). *Ae. caspius* was found to be the most abundant mosquito species in this region that was collected from salty water in the borders of this region but could not be found in the fresh-water (Ahmed *et al*., 2011). However, it has been found to live normally in fresh water in the lab, which may indicate that it has the ability to live in both fresh and salty water. In this context, marine-like elements are widespread in nature in a wide variety of insects (Robertson and Macleod, 1993) including mosquitoes (Zakharkin *et al*., 2004). Virtually all marin-like elements found in natural populations are non-functional pseudogenes, containing stop signals, deletions, frameshifts, or missense mutations that either disrupt the open reading frame or produce an inactive transposase (Robertson and

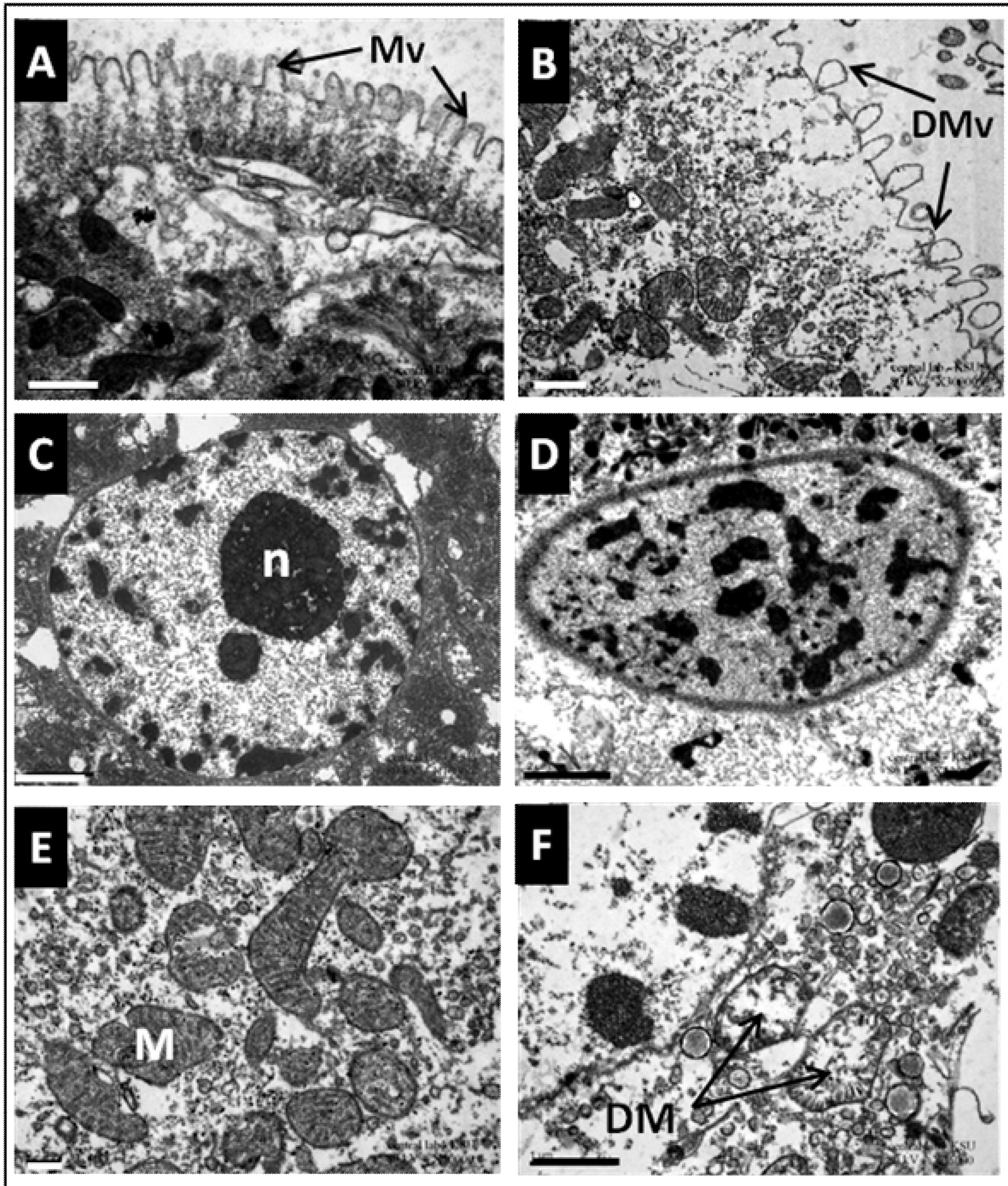

Fig. 3. Transmission electron microscopic micrographs showing cytological effects of *P. frederiksbergiensis* extract on the ultrastructure of midgut epithelial tissue of *Ae. caspius* 3rd instar larvae at 24 h post treatment. A: normal microvilli (Mv) in control larvae. B: degenerated microvilli (DMv) with bubbling and stretching in treated larvae. C: normal nucleus with clear normal nucleolus (n) and chromatin contents in control epithelial cell. D: degenerating nucleus and contents of an epithelial cell of treated gut. E: normal mitochondrial structures in control larvae (M). F: damaged mitochondria (DM) in treated midgut cells showing deformation and loss of cristae and matrix. Ultrathin 4 µm sections were analyzed with transmission electron microscope model JEOL JEM-100CX II at 80 kV. Scale bar = 1µm.

Table I.- A preliminary experiment showing mortality percentages of 3rd larval instar of *Ae. caspius* at 24h post-treatment effect of different concentrations of *P. frederiksbergiensis* extract at. Control larvae showed no mortality during the experiment. Means and standard errors were statistically calculated from 5 replicates for each concentration (n=5).

Concentrations (µl/l)	% Mortality
3.9	0
7.8	0
15.6	0
31.25	0
62.5	8±0.06
125	24±3.11
250	54±6.42
500	100
1000	100

Macleod, 1993). This may indicate that *Ae. caspius* has the ability to switch on and off the mariner-like gene element properly and appropriately based on the nature of its aquatic environment.

From the point of view of histological impact, results obtained from light and transmission electron microscopy revealed that *P. frederiksbergiensis* extract treatment brought about a massive disintegration to the larval midgut epithelial tissues in *Ae. caspius* larvae as compared to control ones. This disintegrated of midgut epithelial layer might be the main reason of observed cessation in feeding by 6h post-treatment then septicemia and finally death at 24h post-treatment as previously recorded in case of *B. thuringiensis* (Gill *et al.,* 1992). It has been reported that exotoxins of *Pseudomonas* bacteria are capable of translocating a catalytic domain into the host cells and inhibiting protein synthesis by the ADP-ribosylation of cellular elongation factor (Allured *et al*., 1986). The mode of action of *P. frederiksbergiensis* extract could have been occurred in the same manner as described for *B. thuringiensis* (Bravo *et al*., 2007) or toxic plant extracts (Al-Mehmadi and Al-Khalaf, 2010). The dramatic effect of midguts of treated larvae could be as a result of vacuolated and swollen epithelial cells, which finally lost their normal appearance. Also, destruction of the peritrophic membrane was observed indicating severe effect on the midgut wall. The drastic effect on the epithelial ultra-structure was shown as vacuolated mitochondria, degraded nuclei, vacuolated cytoplasm and completely degenerated and vacuolated epithelial cells as previously recorded (Clark *et al*., 2005). This may explain the cytological impact recorded in this study. Furthermore, the severe damage detected by electron microscopy in microvilli, nuclei and mitochondria of the gut epithelial cells as well as cellular damage is an extra evidence of gut destruction by this toxin. This may indicate that *P. frederiksbergiensis* toxic extract causes destruction of the gut epithelium. It has been reported that destruction of the epithelial cells lining the midgut of mosquito larvae is often associated with the midgut paralysis and cessation of feeding as previously recorded in *B. thuringiensis* (Clark *et al.*, 2005; Bravo *et al.*, 2007). The current results show similar symptoms which, in fact, may indicate high toxicity of these agents against mosquitoes and being an effective biocontrol agent in the field of mosquito control since long time ago (Goldberg and Margalit, 1977; Goettel *et al*., 1982).

In conclusion, the current study provides solid evidence that the *P. frederiksbergiensis* is a suitable larvicide for control of *Ae. caspius* mosquito in Saudi Arabia.

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