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Isolation and molecular characterization of bacteria found in soils of Rahat region, and their toxicity to the vector of Dengue fever (*Aedes aegypti* larvae)

Naif Mohammed Alotaibi[1,2*], Saber Abdelkader Saidi[1], Mohamed Mohamed Roushdy[1], Turki M. Alsheikh[1], Naser AL-Kenani1[2] , Rashad M. Saleh[3] and Saleh M. Al-Maaqar[2,4]

[1]Department of Biology, College of Sciences and Arts-Khulis, University of Jeddah, Jeddah, **Saudi Arabia**
[2]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, P.O. Box 80203, Jeddah 21589, **Saudi Arabia**
[3]Medical Microbiology Department, Faculty of science, IBB University, **Yemen**
[4]Department of Biology, Faculty of Education, Al-Baydha University, Al-Baydha, **Yemen**

***Correspondence:** naifoo654@gmail.com **Received 17-06-2022, Revised: 15-07-2022, Accepted: 16-07-2022 e-Published: 17-07-2022**

Since ancient times, many medically significant pathogens and parasites, such as viruses, bacteria, and protozoans, are transmitted by mosquitos and caused serious illnesses such as dengue fever, malaria, encephalitis, yellow fever, and filariasis. This suggest the need for continuous development of new and appropriate control measures of mosquito-borne diseases in Saudi Arabia and elsewhere. This study investigated the potential of local bacteria isolated from the soil of Rahat region of Makkah, Saudi Arabia for the biological control of the mosquito, *Aedes aegypti* larvae, a major cause of dengue fever. The bacteria were identified using morphological and molecular characteristics. Bioassays were used to determine the pathogenicity of various strains against *Aedes aegypti* larvae. A total of 66 different bacteria were isolated. Overall, two (3.03%) of the 66 bacteria tested caused mortality in the *A. aegypti* larvae, and only two (*Bacillus velezensis,* and *Priestia megaterium*) of these occasioned 100% mortality in 24 h. The findings of this study demonstrated that local isolates found in the soils of the Rahat region of Makkah, Saudi Arabia have larvicidal activity against the larvae of *Aedes aegypti*. Further research is needed to better understand and identify other mechanisms involved in the production of larvicidal toxins in these isolates.

Keywords: Larvicidal activity*, Aedes aegypti, Bacillus velezensis, Priestia megaterium, Saudi.*

INTRODUCTION

Saudi Arabia is a country in the Middle East. Every year, millions of Muslims go to Makkah in the Kingdom of Saudi Arabia (KSA) for a religious pilgrimage known as the "Hajj." Pilgrims suffer significant health concerns during the Hajj, due to the enormous number of people present at this time, which increases the danger of getting infectious diseases (Ahmed et al. 2006; Aldossari *et al.* 2019). In truth, this religious gathering draws attention to some of the world's most challenging public health and disease management issues. The possibility for infectious disease and other health problems to spread during this time is a major concern for Saudi public health officials. Furthermore, the potential for new infectious diseases to become epidemics is a serious worry. The Saudi authorities are constantly refining and upgrading their disease prevention procedures for the administration of Hajj ahead of each Hajj season. In Saudi Arabia, a variety of mosquito species transmit many sorts of life-threatening mosquito-borne illnesses. *Aedes* (Stegomyia) *aegypti* is the primary vector in the global resurgence of dengue fever epidemic, as well as a variety of other arboviruses that impact human populations worldwide, such as Zika and Chikungunya (dos Santos Lobo et al. 2018). Dengue fever is a serious public health problem in Saudi Arabia and many other regions, and it is the world's second most common arbovirosis based on total number of people infected (WHO,2013). Dengue fever, which is spread by the *Aedes aegypti* mosquito (Charrel et al. 2001; Madani, 2005; Ghramh *et al.* 2018), was reported in Makkah and Jeddah in 2001. Rift Valley fever, which is spread by the mosquito *Aedes caspius*, is common in KSA's southern and eastern areas (Ahmad, 2000; Alhaj et al. 2019). Approximately 300–500 million clinical cases of malaria, transmitted by the genus *Anopheles*, occur globally each year, with up to 3 million deaths, the majority of which occur in children under the age of five (Harbach, 1994). As a result, KSA is affected by a number of life-threatening mosquito-borne illnesses, and the Saudi government is working hard to combat these diseases by reducing mosquito vectors.

The Saudi Ministry of Health has designed and

implemented comprehensive policies to reduce the risk of mosquito-borne infections, particularly during the Hajj season. So, insect control was carried out mainly through the use of chemical insecticides, but due to their lack of specialization, the resistance of insects to them, and their negative impact on the environment and human health (Al-Sarar, 2010), in addition to the fact that some types are toxic or carcinogenic, as well as the high cost of their production, it was necessary to turn attention to research about alternatives, such as biological control. Biological control is an integrated strategy used to control pests and reduce their population by using natural enemies. The desirable characteristics of organisms used in biological control are that they are specialized and safe to use so as not to harm humans, animals, or crops, that control is carried out quickly to avoid widespread damage, and that the production process is inexpensive. Biological control of mosquito vectors has not been widely applied in Saudi Arabia, to our knowledge.

Therefore, as a continuation to our interest in the control of mosquito-borne diseases in Saudi Arabia and elsewhere (El-Kersh et al. 2012; El-Kersh et al. 2014). This study will investigate the potential of local bacterial isolates obtained from the Rahat region of Makkah, Saudi Arabia for the biological control of *Aedes aegypti* larvae.

MATERIALS AND METHODS

2.1 Sample collection

For the current study, Soil samples were collected in February, 2021 in sterile plastic bags from different sites of Makkah Region, Saudi Arabia (22°06'02.0"N 40°01'36.6"E and 22°05'48.7"N 40°01'32.6"E), At the collection site, Temperature, pH range, and other physical properties were noted during the sampling. A pH meter was used to confirm the pH further in the laboratory. A total of six samples were collected and labelled as samples A to F. Each sample was air dried by spreading it on paper sheets at room temperature followed by sieving using a sieve with 0.2 mm size. One gram of the sieved sample was added to a tube containing 9 ml of sterile distilled water for serial dilution. An aliquot 0.1 ml of the suspension was transferred to the surface of Nutrient Agar (NA) plates and spread with the aid of a spreader. The plates were incubated at 37°C for 4 days. Same procedure was repeated for each of the sample in duplicate.

2.2 Purification and preservation of bacterial isolates

All collected samples were serially diluted, the plates were incubated for 48 h at 35 ± 2 ^{0}C; The cultivated bacterial colonies were counted as CFU/g and a single colony was streaked on Nutrient agar until pure colonies were obtained. The purified single colony of bacteria was cultured in Nutrient broth overnight and stored at −80 °C in NB broth containing 16% glycerol for preservation. The bacteria were kept in the department of biological sciences until required for use. The bacteria were revived from the glycerol stock for use by culturing in Nutrient-Broth (NB) medium. A sterile loop was utilized to transfer culture to a glass tube containing 10 mL of NB broth a day before the experiment. It was then cultured on a Nutrient agar (NA) plate.

2.3 Identification of bacteria

2.3.1 Biochemical characterization of bacterial isolates

2.3.1.1 Gram staining

To differentiate between Gram-positive and Gram-negative bacteria, the Gram staining technique was used following the method described by Vincent and Humphrey (1970). A loopful of a bacterial strain was put on a glass slide and covered with a single drop of crystal violet. After 1 min, it was rinsed with sterilized distilled water. Then, iodine solution was added for 1 min, followed by washing with sterilized distilled water. After that, ethanol solution (90%) was used for 5 to 10 secs and was washed with added sterilized distilled water. Finally, safranin was added for 45 secs and washed off with sterilized distilled water. The slide was then observed using a light microscope.

2..3.1.2 Oxidase test

In order to determine the presence of cytochrome oxidase, bacterial culture from a solid medium was smeared on a filter paper that was impregnated with freshly made 1% aqueous solution of N-N-N-tetramethyl-P-phenylene diamine dihydrochloride followed by the method as described by Shekhawat (Shekhawat *et al.* (1992). The appearance of dark purple color within 10 s was considered a positive reaction.

2.3.1.3 Catalase test

In order to examine the catalase activity in the bacteria, a loopful of each bacterial culture, grown on NA plates at 37 ºC for 24 h, was mixed with a drop of hydrogen peroxide (H_2O_2) on a clean glass slide. The production of gas bubbles indicates a positive reaction (Hayward *et al.* 1960).

2.3.2 Molecular identification of bacterial isolates

2.3.2 .1 Nucleic Acid Isolation

The nucleic acid (DNA) from each of the bacteria were isolated by Zymo research “Quick-DNA™ Fungal/Bacterial Miniprep “ kit (cat# D6005) according to the manufacturer’s procedure. The quality and quantity of DNA measurements were evaluated using Denovix DS-11 Spectrophotometer.

2..3.2 .2 Polymerase chain reaction

PCR was done by DreamTaq Green PCR Master Mix (2X) (cat# K1081) and the universal primers used are 27

Forward (5`AGAGTTTGATCMTGGCTCAG3`) and 1492 Reverse (5`TACGGYTACCTTGTTACGACTT 3`). The process is performed according to manufacturer's insstructions with a total volume of 25 ul. The PCR amplified the target DNA using Verti™ Thermal Cyclers.

2.3.2.3 Gel Electrophoresis

Agarose gel electrophoresis was used to confirm the PCR product size and quality prior to sequencing. The gel was prepared at 2% using Ultrapure Agarose cleaver scientific and 1X . TBE buffer. It was stained with syber safe DNA stain (Invitrogen, California USA) before loading. To each well, 4µl of PCR product was loaded. A 100-1000 DNA ladder was used and run alongside the samples at 100 mV for 30 minutes. The size of the DNA fragments was visualized using a Gel Doc system imager system with UV Trans-illuminator.

2.3.2.4 Sanger Sequencing

2.3.2 .4.1 PCR Purification

Amplicon purification was carried out using ExoSAP-IT™. According to the manufacturer's procedure, 5 µL of the PCR reaction product was mixed with 2 µL of ExoSAPIT™. It was incubated at 37°C for 15 minutes to degrade the remaining primers and nucleotides then incubated at 80°C for 15 minutes to inactivate the ExoSAP-IT™ reagent.

2.3.2 .4 .2 Cycle sequencing PCR

The cycle sequencing PCR reaction was done using BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, USA). The concentration of purified PCR product was measured using nanodrop and the used concentration depended on the product size of the PCR. The mixture for cycle sequencing reaction was prepared for each sample. Then, the tube was vortexed for 2-3 sec and spun it down.

2.3.2 .4 .3 Cycle sequencing PCR

The cycle sequencing purification was performed using BigDye XTerminator (Applied Biosystem, USA). Forty-five µl of SAM solution was added to the cycle sequencing PCR reaction. Then, 10 µl of XTerminator (beads) was added after mixing the mixture well and the plate was sealed and vortex at 3500 rpm for 45 minutes, followed by centrifugation at 14,000 rpm for 2 minutes. Then, 30 µl of the upper layer was carefully transferred into wells of the optical 96-well plate. After that, the plate was sealed by Septa 96 well and spun down.

2.3.2 .5 Sequencing Platform

Sequencing was performed using SeqStudio (Life Technologies, USA) and run following a long module.

2.3.2 .6 Sequence Alignment

Sequence analysis was done by SNapGene version 6.0.2 to extract fasta format and alignment through an online tool, the NCBI blast

2.4 Larvicidal activity

2.4.1 Obtaining and growing larvae in the laboratory

The larvae of *A. aegypti* mosquitoes have been grown. In the Department of Biology at King Abdulaziz University, where the larvae were placed in a separate breeding room in which the appropriate environmental conditions for the life of mosquitoes were taken into account, such that the temperature was 26 ± 2 °C and the relative humidity rate was 10%, with average light (12:12) (Ali *et al.* 2002).

2.4.2 Blood feeding of *A. aegypti* mosquitoes

After growing the larvae and reaching the adult stage, *A. aegypti* females need blood meals in order to produce eggs, pigeons were used to feed the larvae with blood, thus obtaining eggs.

2.4.3 Susceptibility bioassays

Bacteria isolated from the soil samples were selected and evaluated for toxicity to the third-instar larvae of *A. aegypti*. The susceptibility bioassays against the larvae was carried out under controlled conditions. The larvae were obtained from a colony maintained at King Abdulaziz University, at a mean temperature of 26 ± 2◦C, and 12 h photophase period (Consoli et al. 1994; Lobo et al. 2018). Bacterial suspensions were prepared for each isolate by growing the bacteria in nutrient broth at 28◦C for five days until sporulation. All the bacterial content was then transferred to new tubes. The tissue culture dish was used for the bioassay test in triplicates. For each tissue culture dish, 10 third-instar larvae of A. aegypti, and 2 ml of the bacterial suspension were added in each well of the plates. Negative control well contain no bacterial suspension but rather sterile distilled water. The plates were maintained at mean temperature and larval mortality was observed after 24 h and 48 h of the addition of the bacterial suspension. Mortality as a result of the suspension was confirmed by counting the living and dead larvae in each well. According to Dulmage et al. (1990) , a larvae is considered dead when the larvae did not move when touched with a sterile stick.

2.5. Phenotypic antimicrobial susceptibility

In compliance with the Clinical and Laboratory Standards Institute instructions, Kirby-Bauer disk diffusion method was used to determine the antimicrobial profile of bacterial tested (Oxoid, Basingstoke, UK) (Wayne *et al.* 2011). Following antibiotics were used: Amikacin (AK) (30µg), Penicillin G(PG) 10 Unit, Piperacillin (PRL) (100µg), Imipenem (IMI) (10µg), Cephalothin (KF) (30µg), Ciprofloxacin (CIP) (5µg), Erythromycin(E)(15µg), Ampicillin (AP)(10µg), Clinamycin(CD)(2µg),

Cotrimoxazole (TS)(25µg), Ceftazidime (CAZ)(30µg), Aztronam (ATM)(30µg).

RESULTS

3.1 Physiochemical parameters of collected soil samples

The soil samples collected from different sites were subjected to different Physiochemical parameters. The collected soil samples had a pH in the range of 6.5 to 8.1 (Table .3.1).

The lowest pH recorded from the soil samples from Rahat region is 6.44 ± 0.05. In contrast, the sample from other place has the highest pH of 7.54 ± 0.05. The average and permissible pH value for soil samples from Rahat region is 6.99± 0.05. Among the other physiochemical parameters, such as the temperature of all the samples was in between 27 ^{o}C to 32 ^{o}C. However, the sample from R-A1 had the lowest temperature of 27 ^{o}C compared to all the samples, while the temperature of the sample collected from R-F1 the highest. In six samples, the soil's colors were due to a combination of various chemicals. The physical characteristics of the soils vary according to the blending of multiple forms of compounds derived from different environmental conditions.

3.2 Isolation and purification of the Isolates isolated

Six soil samples from 2 areas of the Rahat region were analyzed and 66 bacterial colonies were isolated from selected locations and purified by repeated streak culture on NA (Figure3.1), of which, 14 (21.2%)were selected to complete this study to their bioactivities after the preliminary test. The 66 isolates were designated as (R-A1- R-A10), (R-B1- R-B12), (R-C1- R-C13), (R-D1- R-D6), (R-E1- R-E17), and (R-F1- R-F8).

Table 3.1 : Physiochemical parameters of the collected soil samples

S. No.	Code	GPS Coordinates	pH	Temp.	Source of soil	Date of collection
1.	**R-A1**	22°06'02.0"N 40°01'36.6"E	7.02 ± 0.04	32 ^{o}C	Rahat region	2/ 2021
2.	**R-B1**	22°06'02.0"N 40°01'36.6"E	6.44 ± 0.05	32 ^{o}C	Rahat region	2/ 2021
3.	**R-C1**	22°06'02.0"N 40°01'36.6"E	7.54 ± 0.05	30 ^{o}C	Rahat region	2/ 2021
4.	**R-D1**	22°05'48.7"N 40°01'32.6"E	6.58 ± 0.05	29 ^{o}C	Rahat region	2/ 2021
5.	**R-E1**	22°05'48.7"N 40°01'32.6"E	6.90 ± 0.05	30 ^{o}C	Rahat region	2/ 2021
6.	**R-F1**	22°05'48.7"N 40°01'32.6"E	6.58 ± 0.05	27 ^{o}C	Rahat region	2/ 2021

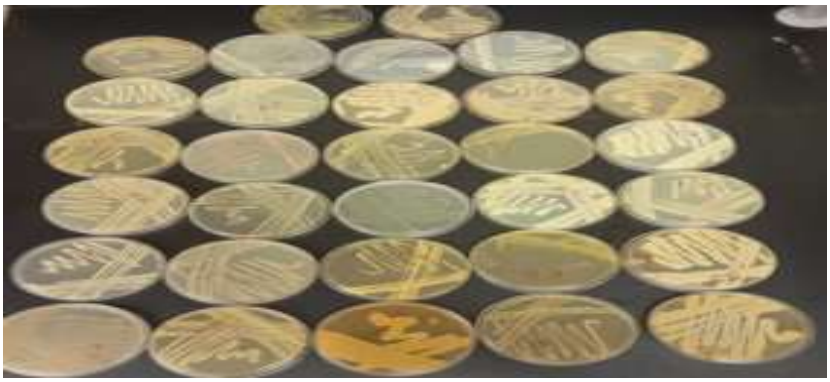

Figure 3.1: Pure Culture(Single colony) of bacterial strains.

3.3 Selection of isolates according to their activity

Overall, two (3.03%) of the 66 bacteria tested caused mortality in the *A. aegypti* larvae, and only four (R-A4 and RF-5) of these occasioned 100% mortality in 24 h).

3.4 Identification of bacteria

3.4 1 Biochemical characteristics

Gram reaction test showed that among bacteria, four isolates were gram-positive and only two isolates were gram-negative bacteria (Table 3.2). Of these isolates, a total of three isolates exhibited oxidase and three isolates showed catalase activity (Table 3.2).

3.4 2 Molecular characteristics

The BLAST search at the NCBI database showed varying degrees (98.73 – 99.56%) of 16s rRNA gene sequence similarity of the isolated bacteria to a wide array of species belonging to *Bacillus velezensis* as shown in Table 3.3.

3.5 Antibiotic Susceptibility Test

The resistant profile of bacterial isolates has been shown in (Table3.4). In this study, results indicate that both bacteria are 100%resistant to antibiotics Ceftazidime (CAZ)(30µg), and Aztronam (ATM)(30µg) (Fig 3.2). and both strains have sensitivity to Amikacin (AK) (30µg), Piperacillin (PRL) (100µg), Imipenem (IMI) (10µg), Cephalothin (KF) (30µg), Ciprofloxacin(CIP) (5µg), Ampicillin (AP)(10µg), Cotrimoxazole (TS)(25µg), Clindamycin (CD)(2µg), Penicillin G(PG) 10 Unit, Erythromycin(E)(15µg) (Table 3.4).

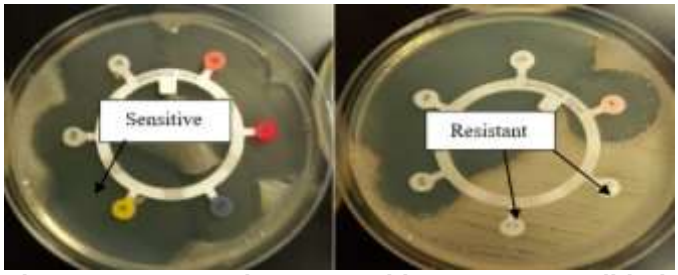

Figure 3.2 : Bacteria are sensitive to some antibiotics and resistant to CAZ and ATM antibiotics.

Table 3.2: Biochemical features of the isolated bacteria.

No	Code. No	Scientific Name	Gram reaction	Oxidase	Catalase
1	N1- R-A4	*Bacillus velezensis*	Positive	Positive	Positive
2	N5- RF-5	*Priestia megaterium*	Positive	Negative	Positive

Table 3.3: 16s rDNA gene sequences identification and accession number of the isolated bacteria

No	Code. No	Scientific Name	Query Cover	Per.Ident	Accession
1	N1- R-A4	*Bacillus velezensis*	99%	99.56%	NR_075005.2
2	N5- RF-5	*Priestia megaterium*	99%	98.73%	NR_112636.1

Table 3.4: Phenotypic antimicrobial susceptibility profile of the tested bacteria

Antibiotics	Bacteria	
	Bacillus velezensis	*Priestia megaterium*
Amikacin	32 ± 0.57	29 ± 0.5
Penicillin	28 ± 0.57	24 ± 0.5
Piperacillin	25 ± 0.5	26 ± 1
Imipenem	29 ± 1	17± 0.57
Cephalothin	29 ± 1	26 ± 1
Ciprofloxacin	35 ± 0.5	28± 2
Erythromycin	22 ± 0.5	24 ± 1
Ampicillin	33 ± 0.5	20± 0.5
Clindamycin	28± 1	21± 2
Cotrimoxazole	30 ± 1	22± 2
Ceftazidime	R	R
Aztronam	R	R

Antimicrobial; Amikacin (AK) (30µg), Penicillin G(PG) 10 Unit, Piperacillin (PRL) (100µg), Imipenem (IMI) (10µg), Cephalothin (KF) (30µg), Ciprofloxacin(CIP) (5µg), Erythromycin(E)(15µg), Ampicillin (AP)(10µg), Clindamycin(CD)(2µg), Cotrimoxazole (TS)(25µg), Ceftazidime (CAZ)(30µg), Aztronam (ATM)(30µg)**, S;** sensitive**, R**; Resistant.

DISCUSSION

Controlling *Aedes aegypti* mosquitoes, which transmit diseases including dengue, Zika, yellow fever, and chikungunya, is a good strategy to keep pathogens from spreading to humans. Current evidence reveals that pesticide resistance is posing a persistent threat to our ability to introduce new biological and chemical insecticides.

Soil samples from two places in Makkah were collected and investigated in order to know more about the biodiversity of the soils and to find new entomopathogenic strains of bacteria with larvicidal activity against *Aedes aegypti*.

A natural soil sample from the Rahat area of Makkah, Saudi Arabia, is a good habitat for several microorganisms capable of producing secondary metabolites used in biocontrol activities. On this basis, natural and harmless microbe were isolated from the soil sample for use in a larval control program in a laboratory setting. In the screening assay, sixty-six bacterial cultures were tested for larvicidal activity against the *Aedes aegypti* mosquito, with two isolates proving to be effective. The effective isolates were identified as *Bacillus velezensis,* and *Priestia megaterium,* based on their biochemical and molecular Features.

Therefore, soils have been shown to be a good source of bacteria that can be investigated for their biocontrol capabilities (Panneerselvam et al. 2012). *Bacillus* sp., *Penicillium* sp., *Streptomyces* sp., and *Trichoderma* sp. are being investigated as biocontrol agents that could be used instead of chemical compounds (Radhika *et al.* 2011). Due to their known metabolic diversity, *Bacillus* species and related genera are promising candidates for discovering novel strains of bacteria and larvicidal metabolites. There are several studies demonstrated that the toxicity of the wild-type *Bacillus* sp. was determined towards *Aedes aegypti* larvae, such as *B. thuringiensis* and *Bacillus israelensis* (Lahkim et al. 1983), *Bacillus sphaericus* (Subramaniam et al. 2012)..

In the present study, the isolate N1- R-A4 was identified as *Bacillus velezensis* having the highest activity against *Aedes aegypti* mosquito larvae among the isolates tested. Falqueto (2021), with the goal of evidencing and identifying multiple insecticidal components. He found that the crude lipopeptide extracts (CLEs) extracted from *Bacillus velezensis* B15 and *Bacillus velezensis* B64a killed the larvae of *Aedes aegypti* (Falqueto et al. 2021). In this regard, Roh (2009), discovered *Bacillus velezensis* strain as a Plant Disease and Insect Pest Control Agent, he demonstrated that the *Bacillus velezensis* strain has insecticidal activity and antifungal activity (Roh et al. 2009).

In the present study, the isolate N7- RC-8 was identified as *Priestia megaterium* has larvicidal activity against *Aedes aegypti* mosquito larvae. This is the first report of *Priestia megaterium* as a mosquito pathogenic bacteria with considerable toxicity to *Aedes aegypti* larvae. It should be noted that Shwed (2021), provides full genome sequences and plasmid arrays of high quality of the bacterium (Shwed et al. 2021). The Gram-positive bacterium *Priestia megaterium* (formerly known as *Bacillus megaterium*) was systematically developed for biotechnological purposes, according to (Biedendieck et al. 2021). However, there have been no previous reports of its mosquito larvicidal action.

CONCLUSION

We isolated bacterial strains that exhibit larvicidal activity against A. aegypti. The data are promising for potentially developing novel bioinsecticides for the control of mosquitoes of medical importance. Further research is needed to better understand and identify other mechanisms involved in the production of larvicidal toxins in these isolates.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NMO and SMA performed all the experiments and wrote the manuscript draft. NA conceived the Idea and supervised the work. SAS, MMR designed the experiment, plan of action for the study. NMO and TMA analysed data from the experiments. MMR, SAS and TMA reviewed the manuscript. NA RMS and MMR are involved in administrative and financial activities. All authors read and approved the final version before submission.

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