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Original Article

Serratia marcescens (Enterobacteriaceae): An alternate biocontrol agent for mosquito vectors *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae)

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ABSTRACT

Background: The insecticides of microbial origin may serve as suitable alternative biocontrol agents. The present study aimed to identify the major chemical constituents and test the larvicidal toxicity of *Serratia marcescens* against *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes. **Materials and Methods:** The mosquito bioassay test of four different concentrations (100, 200, 300 and 400 ppm/ml) of bacterial extracts was performed as per standard protocols. The chemical compounds and its functional groups were identified by GC-MS and FT-IR analysis, respectively. **Results:** The larval bioassay results observed better effect with the varied levels of LC_{50} and LC_{90} values. Six major compounds identified by Gas Chromatography Mass Spectrophotometer (GC-MS) and named as follows: 1-o-monoacetyl-2, 3-o-dibenzoyl-d-ribofuranose (20.255%), 1-butanol, 3-methyl-, benzoate (19.380%), Bentiamine (18.855%), Benzoic acid 2-bromoethyl ester (18.415%), and Cyclotrisiloxane hexamethyl- (2.658%). Fourier transform infrared spectroscopy (FT-IR) spectral analysis reflect the functional groups of (aromatic, alkynes and amines) the biomolecules. **Conclusion:** We found the increased level of larval mortality based on extract concentrations and exposure time.

Key words: *Aedes aegypti, Culex quinquefasciatus, Serratia marcescens,* Spectral study, Larvicidal bioassay

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INTRODUCTION

Mosquitoes are the principal vector of several serious diseases, including malaria, yellow fever, dengue and encephalitis. In India, mosquito borne diseases are still the most important cause of morbidity and mortality of three million new cases rising every year.[1] *Aedes aegypti*, a vector of dengue, yellow fever and chikungunya that carries the arbovirus responsible for these diseases, widely distributed in the tropical and subtropical zones.[2] *Culex quinquefasciatus* is a vector of *Wuchereria* species causing lymphatic filariasis, found in tropical regions with around 120 million people infected and 44 million people under clinical manifestation.[3] In general, the chemical control is carried out by the indoor residual spraying of insecticides such as dichloro diphenyl trichloro ethane, benzene hexa chloride, synthetic pyrethroid and melathion. Of late, development of resistance against these chemicals in various mosquito populations was being reported and believed that any widely used chemical may similarly select for resistant populations, ultimately lead to a systemic failure in mosquito control.[4] Synthetic organic insecticides, although still extensively used, are being progressively replaced by microbial controlling agents from certain strains of bacteria, like *Bacillus thuringenesis var. israelensis* and *B. sphaericus,* which are highly effective against mosquito larvae at very low dosage and safe to non-target organisms.[5] Various bacteria such as *Serratia*, *Pseudomonas* and *Streptomyces*[6,7] possess an ability to produce biopigments. With the brief background, the present study was aimed to assess the mosquito larval toxicity effect of bacterial metabolite (pigment) produced by an indigenous *Serratia marcescens* isolated from soil.

MATERIALS AND METHODS

General

Soil collection and isolation of *S. marcescens*

The soil sample was collected from the Karuppur village, Salem District, Tamilnadu, India. (Latitude 11°43'31' N, Longitude 78°05'57' E). The soil sediments were collected through scuba diving to the depth of 1–100cm. The sample was collected in sterile plastic bags and immediately transported to the laboratory. The collected sample was processed using different methods. A tenfold serial dilution was made and plated in triplicates on Nutrient agar media. The plates were incubated at Bacteriological Incubator (37°C) for 24 hours and monitored periodically over three months for *S. marcescens* growth. The pure isolate was stored on a Nutrient agar slant at 4°C for future use.

Identification of *S. marcescens*

The organism was cultured in the Muller Hinton Agar (MHA) where it grows as red colored pigmented colonies. The identification of pure culture was done by performing routine biochemical tests and refereed with Bergey's manual of systemic bacteriology.[8]

Culture of organism and extraction

For extraction of metabolites from *S. marcescens*, loopful of 24-h old culture of *S. marcescens* inoculated in the Muller Hinton Broth medium containing mannitol and incubated for 2 days at 30±2°C with Benchtop Incubator cum orbital Shaker (OSI-262) for120 rpm. After 48 h, the broth was mixed with 1% acidified ethanol and cells were separated by centrifugation (Remi Centrifuge) at 5,000 rpm for 10 min. The supernatant was extracted with water/methanol (1:1); the methanol layer was separated and concentrated by evaporation.

Test mosquitoes

For the laboratory trial, different larvae stages (second, third and fourth-instar) of *Ae. aegypti* and *Cx. quinquefasciatus* were obtained from National Centre for Disease Control (NCDC), Mettupalayam, Tamilnadu, India. The larvae were kept in plastic enamel trays containing dechlorinated tap water and they were maintained and reared in the laboratory as per the slightly modified method of Kamaraj *et al*.[9] All the experiments were carried out at 28 ± 2°C and 75-85% relative humidity under 14:10 light and dark cycles. Larvae were fed with a diet of finely ground brewer's yeast and dog biscuits (3:1).

Larvicidal bioassay

Twenty milligrams of dried crude extract were dissolved in 20 ml of 10% Dimethyl sulphoxide (DMSO), which was used for extraction (1mg/ml stock solution). From the stock solution, each sample (1,000 ppm/ml) was prepared with dechlorinated tap water. The larvae toxicity effect was assessed as per WHO protocol.[10] The *Ae. aegypti* and *Cx. quinquefasciatus* larvae were taken in four batches of ten in 99 ml of water and1.0 ml of the crude extracted pigment. The control was set up with similarly desired concentrations (10 % DMSO). The numbers of dead larvae were counted after 48 h of exposure, and the percentage mortality was recorded for the average of five replicates. The control mortalities were corrected using Abbott's formula.[11] The LC_{50} and LC_{90} were calculated from toxicity data using probit analysis.[12]

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment} - \text{Observed mortality in control}}{100 - \text{Control mortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

Dose–response bioassay

From the stock solutions, different concentrations (ranged from 100 to 400 ppm/ml) were prepared modified as per the method of Rahuman *et al*.[13] Based on the preliminary screening results, bacterial metabolite in 10% DMSO was subjected to dose–response bioassay for larvicidal activity against *Ae. aegypti* and *Cx. quinquefasciatus* different instar larvae. One hundred milliliters of each test solution was placed in glass beaker (250 ml) along with 20 individuals of different stages of larvae. Each experiment was conducted with five replicates and a concurrent control group. Control groups consists of 1.0 mL of 10% DMSO and 99.0 mL of distilled water and an untreated one, contained only 100 mL of distilled water. No food was provided during the experiment. The numbers of dead larvae were counted after 24h exposure. The time-dependent effect of extracts was observed upto 48 h against fourth-instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus*.

UV-Visible spectrophotometry and Fourier transform infrared spectroscopy (FT-IR) analysis

The extracted metabolite was identified by UV Visible Spectrophotometry (Systronics 2203) in the range of 350-700nm in 95% ethanol. A FT-IR spectrum of the pigment was recorded with mid IR region of (400-4000 cm^{-1}) a (Brucker Co., Germany) FT-IR spectrophotometer. The sample (500 mg) was mixed with 300 mg of KBr powder and IR spectra was recorded using Attenuated Total Reflectance (ATR) technique beach measurement at resolution of $1cm^{-1}$.[14] 2010). The FT-IR analysis was done by Department of Physics, School of Sciences, Periyar University, Salem, Tamilnadu. India.

Gas Chromatography - Mass Spectrometry (GC–MS) analysis

GC–MS analysis of sample (Perkin Elmer, International, Boesch, Huenenberg, Switzerland) was carried out using a system (Clarus 680) equipped with a HP-5MS fused silica capillary column (30 m×0.25 mm i.d.; film thickness, 0.25 µm). For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 ml/min. Injector and mass transfer line temperatures were set at 250 and 300°C, respectively. The solution (1 µL) in methanol was injected and analyzed with the column held initially at 60°C for 2 min and then increased to 300°C with a 10°C/min heating ramp and subsequently kept at 300°C for 6 min. The major components the extract was identified by co-injection with standards (wherever possible), confirmed with Turbo Mass ver 5.4.2 using the NIST (V.2008) and National Institute of Standards and Technology V.5.4.2 GC–MS library. The relative concentration of each compound in the extract was expressed as percentage by peak area normalization.[15]

Statistical Analysis

The lethal concentrations (LC_{50} and LC_{90}), and the 95% confidence intervals of LC_{50} and LC_{90} (UCL-upper confidence limit and LCL-lower confidence limit) were calculated. Results with $p<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The isolated bacterial colonies were identified as *S. marcescens* based on the colony morphology, gram staining and biochemical characteristics. Morphological and microscopic characteristics of the isolate revealed that the circular or mucoid colonies which is convex with entire margins and umbonate elevation (Figure 1). It was a Gram-negative, rod shaped, facultative anaerobe soil bacterium, spirillum produces a non-diffusible red pigment. Biochemical characteristics of isolate showed negative results in Methyl red, oxidase, indole production, raffinose and L-arabinose whereas Voges-Proskauer and catalase tests were positive (data not shown).

The results of larvicidal activity of the present study shows the fourth instar larvae of *Cx. quinquefasciatus* found to be highly susceptible compared to *Ae. aegypti* larvae with the LC_{50} of 10.802 ppm/ml and LC_{90} of 103.697 ppm/ml after 48 h (Figures 2a&b). Similarly, Rajesh *et al.* [16] reported that extracellular secondary metabolites from actinobacterial isolates showed larvicidal activity against *Aedes* sp, *Anopheles* sp and *Cx. pipiens*. Likewise, Dua *et al.* [17] obtained best lethal concentration (LC_{50}) of the *Azadirachta indica* formulation for *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. The effect on larval mortality and the survival rate was purely depends on the doses of the bacterial metabolites (Figures 3; Table 1 and 2). The chemical constituents of medicinal plants showed an excellent toxic effect on *An. subpictus* and *Cx. tritaeniorhynchus* after 24 h of exposure at 1000 mg/l.[18] The microbial metabolites from *Bacillus sphaericus*,[19-21] *Lagenidium giganteum*[22] reported as strongest larvicidal activity against the selected mosquito populations. *B. sphaericus*, a spore-forming, entamopathogenic bacterium, has been showed a potent larvicidal activity against Chikungunya vector *Ae. aegypti* mosquito larvae.[23] The FT-IR spectrum results (Figure 4 and Table 3) reflect the red pigment was determined by very strong and sharp bands at 3,448.84 cm^{-1} evident that stretching of aromatic (O-H) and 2,360 cm^{-1} are due to carboxylic acids (O-H) prodigiosin exhibits similar range absorptions in -C= at 2,130 cm^{-1} and 1,643.45 cm^{-1} are due to primary amine (N-H). Likewise, Arsia Tarnam *et al.*[25] reported that similar functional groups were obtained in the leaf extract of *Clausena anisata*. Gas chromatography–mass spectrophotometry (GC–MS) study illustrated the methanolic extract of *S. marcescens* (Figure 5) shown six peaks which represents 6

Table 1: Larvicidal activity of *S. marcescens* extract against *Ae. aegypti*

Test insect	Larvae stages	Concentrations (ppm/ml)	a %Mortality ±SD	LC_{50} LCL-UCL	LC_{90} LCL-UCL	χ 2 (df=3)
Ae. aegypti	II	100	66.66±1.0	61.954	277.745	6.494
		200	82.22±2.0	(31.767-86.672)	(226.352-385.210)	
		300	92.22±1.5			
		400	94.44±1.1			
		Control	0.0±0.0			
	III	100	76.66±1.0	44.772	140.934	17.764
		200	87.77±0.5	(18.127-66.404)	(111.369-173.881)	
		300	92.22±1.5			
		400	94.44±1.1			
		Control	0.0±0.0			
	IV	100	90.0±0.0	10.802	103.697	7.191
		200	96.66±1.0	(0.002-37.329)	(16.916-159.084)	
		300	97.77±1.1			
		400	98.88±0.5			
		Control	0.0±0.0			

Control (deionized water with DMSO) - Nil mortality

LC_{50} – Lethal concentration that kills 50% of the exposed larvae, LC_{90} – Lethal concentration that kills 90% of the exposed larvae, LCL = Lower Confidence Limit, UCL = Upper Confidence Limit, df degree of freedom, χ^2 – Chi-square values are significant at P<0.05 levels.

[a]Mean value of five replicates

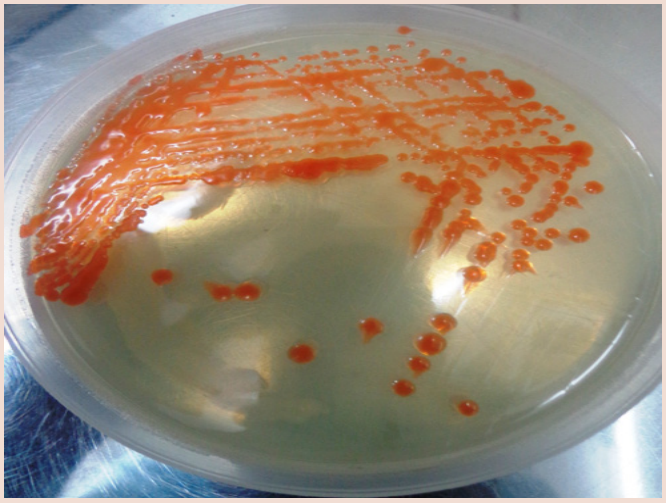


Figure 1: *Serratia marcescens* colony on Nutrient agar (after 24 h plate culture)

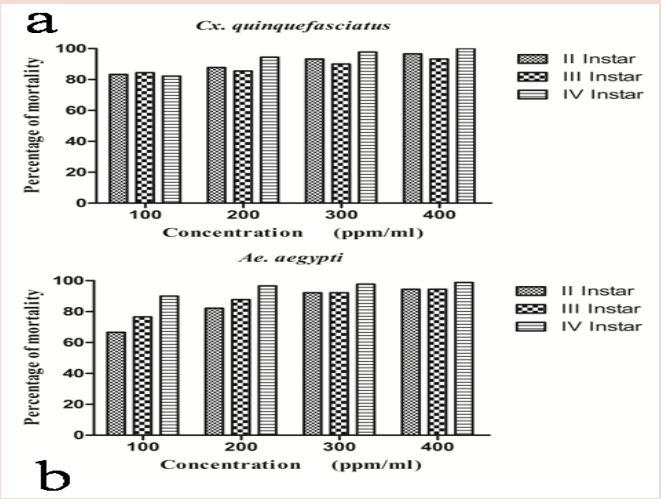


Figure 2 a & b: Percentage of mortality of mosquito vector a *Cx. quinquefasciatus* **b** *Ae. aegypti.*

Table 2: Larvicidal activity of *S. marcescens* extract against *Cx. quinquefasciatus*

Test insect	Larvae stages	Concentrations (ppm/ml)	a %Mortality ±SD	LC_{50} LCL-UCL	LC_{90} LCL-UCL	χ 2 (df=3)
Cx. quinquefasciatus	II	100	83.33±1.0	19.355	194.708	4.432
		200	87.77±0.5	(44.7-48.126)	(130.234-301.942)	
		300	93.33±1.0			
		400	96.66±1.0			
		Control	0.0±0.0			
	III	100	84.44±4.7	105.154	269.579	10.154

	200	85.55±1.5	(18.427-155.336)	(200.428-435.658)	
	300	90.00±1.0			
	400	93.33±1.0			
	Control	0.0±0.0			
IV	100	82.22±4.5	44.772	140.934	17.764
	200	94.44±2.0	(18.127-66.404)	(111.369-173.881)	
	300	97.77±0.5			
	400	100			
	Control	0.0±0.0			

Control (deionized water with DMSO) - Nil mortality

LC_{50} – Lethal concentration that kills 50% of the exposed larvae, LC_{90} – Lethal concentration that kills 90% of the exposed larvae, LCL = Lower Confidence Limit, UCL = Upper Confidence Limit, df degree of freedom, χ^2– Chi-square values are significant at P<0.05 levels.

[a]Mean value of five replicates

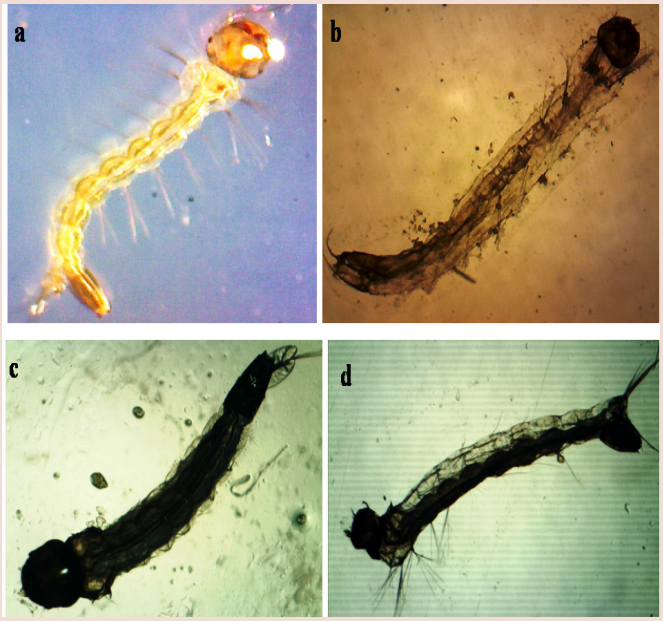


Figure 3: Growth disruption effects mediated by *Serratia marcescens* extract at 48 h exposure. a *Cx. quinquefasciatus* control larva. b *Cx. quinquefasciatus* morphological alterations and fourth instar larvae dead after 24 h treatment with 400 ppm/ml. c *Ae. aegypti* control larva. d. *Ae. aegypti* morphological alterations and fourth instar larvae dead after 24 h treatment with 400 ppm/ml (visualization of the changes in larval morphology and after treatment with extract was monitored using light microscopy at 25× magnification.

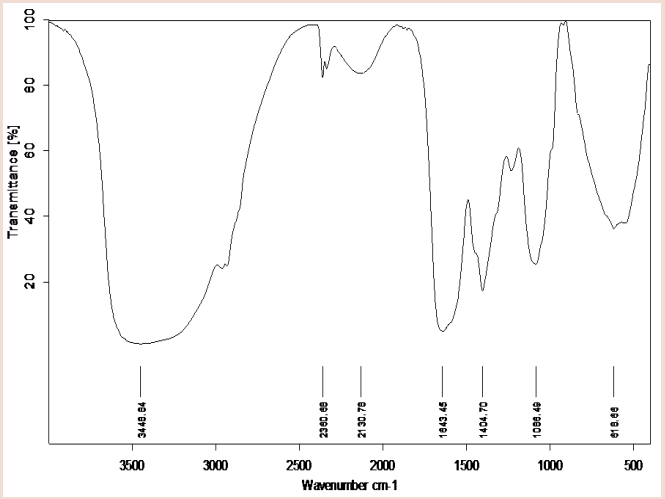
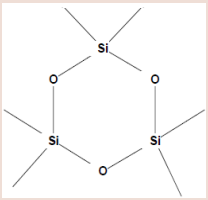
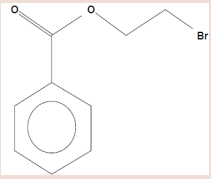
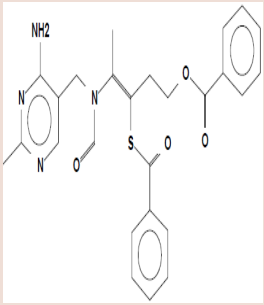
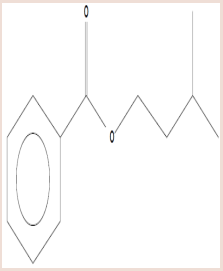
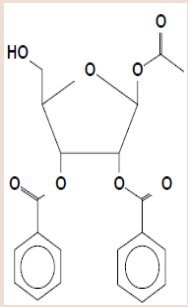


Figure 4: FT-IR analysis of metabolite from *S. marcescens*

Table 3: FTIR analysis of methanolic extract of *S. marcescens*

S. No	Frequency numbers (cm^{-1})	Functional groups	Vibration mode
1	3448.84	O-H Stretching	Strong, Sharp
2	2360.68	O-H Carboxylic acids	Medium
3	2130.78	-C= Stretching, Alkynes	Weak
4	1643.45	N-H Bending, Primary amine	Medium
5	1404.70	C-C Stretch, Aromatics	Medium
6	1086.49	C-N Stretch, Aliphatic amines	Medium
7	618.66	C- Br Alkyl halides	Medium

Table 4: Chemical composition of *S. marcescens* methanolic extract

Name of the compounds	Cyclotrisiloxane, hexamethyl-	Benzoic acid 2-bromoethyl ester	Bentiamine	1-butanol, 3-methyl-, benzoate	1-o-monoacetyl-2,3-o-dibenzoyl-d-ribofuranose
Structure					
Molecular formula	$C_6H_{18}O_3Si_3$	$C_9H_9O_2Br$	$C_{26}H_{26}O_4N_4S$	$C_{12}H_{16}O_2$	$C_{21}H_{20}O_8$
Molecular weight	222	228	490	192	400
RT	2.658, 26.878	18.415	18.885	19.380, 19.675	20.255
Peak area %	2.583, 20.028	14.981	5.006	17.692, 11.942	4.349
Applications	Antioxidant property, Antibacterial activity, Larvicidal	Antimicrobial activity Food preservatives	Analgesic and anti-inflammatory activity NSAID drugs	Responsible for the aroma in fruit juice	Antioxidant property
References	Alok Prakash and Suneetha[26]; Priyanka,[27] Venkatesh *et al.*[28]	Azzena *et al*[29]	Mohammad Asif[30]; Gauthier[31]	Luis Zea *et al.*[32]	Ayaz Ali Memon,[33]

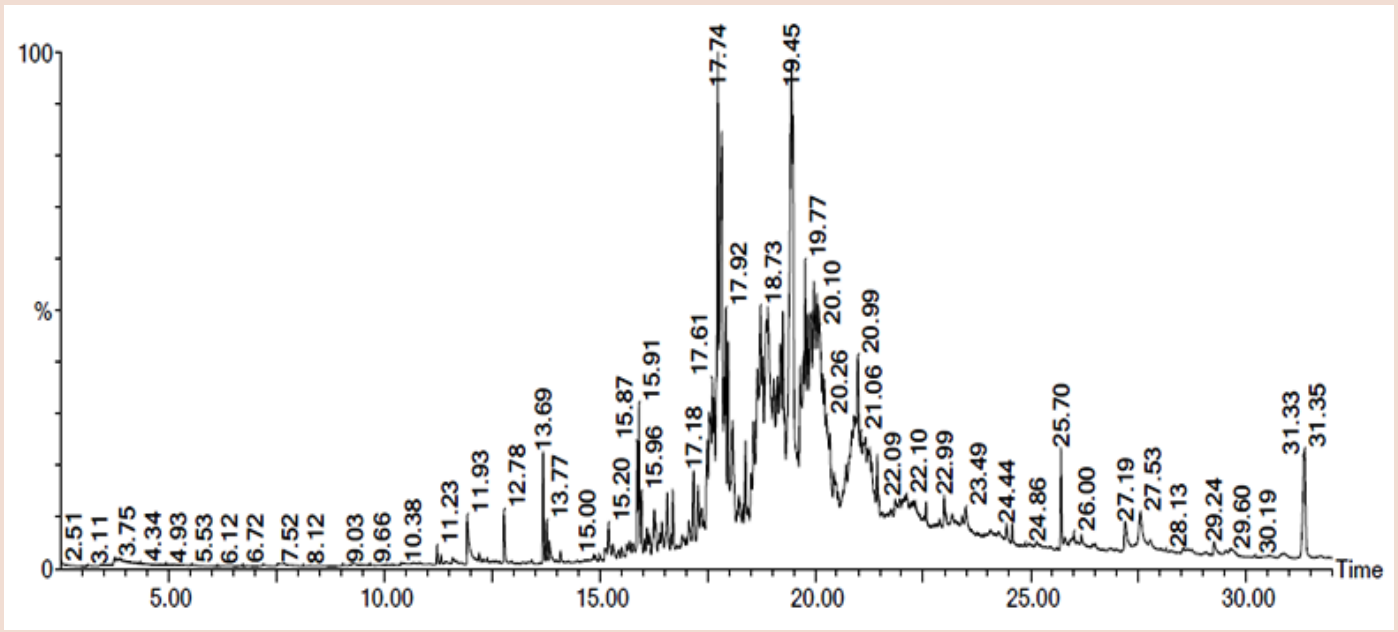


Figure 5: GC-MS chromatogram of metabolite from *S. marcescens*

chemical constituents (Table 4) namely 1-o-monoacetyl-2, 3-o-dibenzoyl-d-ribofuranose (20.255%), 1-butanol, 3-methyl-, benzoate (19.380%), Bentiamine (18.855%), hexamethyl-Benzoic acid 2-bromoethyl ester (18.415%), and Cyclotrisiloxane (2.658%) were identified based on the mass spectra of the constituents with the NIST library. Recently, Shailajan *et al.*[24] analysed the chemical nature of *Mangifera indica* using validated GC-MS technique and evaluate its bioactivity.

In a nutshell, the present study highlights the chemical constituents and larvicidal activity of *S. marcescens* shows better bio-control agents for *Ae. aegypti* and *Cx. quinquefasciatus* mosquito vectors. This study also encourages further investigation will be needed for isolating novel, more selective, biodegradable, ecofriendly larvicidal compounds in the near

future.

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CONFLICT OF INTEREST

None

ABBREVIATIONS USED

FT-IR: Fourier transform infrared spectroscopy; GC-MS: Gas Chromatography-Mass Spectrometry; NCDC: National Centre for Disease Control; DMSO: Dimethyl sulphoxide; WHO: World Health Organization; ATR: Attenuated Total Reflectance; NIST: National Institute of Standards and Technology; LC_{50}: lethal concentrations; LC_{90}: lethal concentrations; UCL: upper confidence limit; LCL: lower confidence limit.

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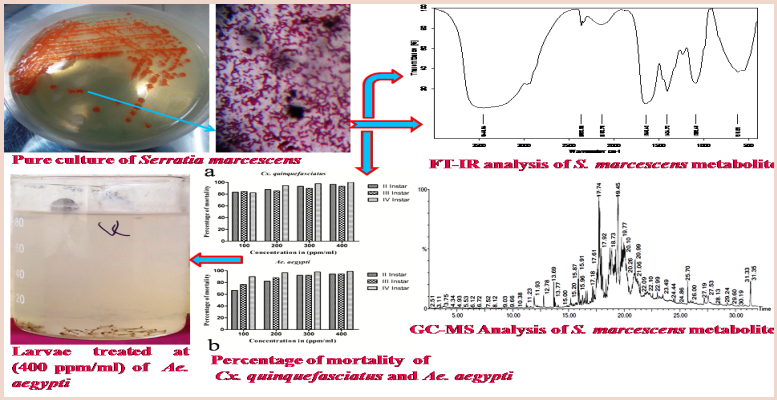
SUMMARY

- We observed Serratia marcescens as circular or mucoid colonies, convex with have entire margins, umbonate elevation, gram-negative and rod shaped.
- We found 4th instar larvae of Culex quinquefasciatus to be highly susceptible compared to Aedes aegypti larvae with an LC50 of 10.802 ppm/ml and an LC90 of 103.697 ppm/ml after 48 h.
- We identify 1-o-monoacetyl-2,3-o-dibenzoyl-d-ribofuranose is a major compound from S. marcescens metabolites by GC-MS Spectrum.

PICTORIAL ABSTRACT



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