ORIGINAL ARTICLE

Studies on larvicidal potential of actinomycetes isolated from various regions of Chennai

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Abstract

Background: Actinomycetes are organisms with characteristics common to both bacteria and fungi. It acts against Gram-positive bacteria and fungi. It is non-pathogenic. It is also able to produce different compounds such as anticancer, larvicidal compounds, pigments, and secondary metabolites. **Objective:** The objective of this study was to isolate and identify active actinomycetes from terrestrial soil sample for larvicidal activity. **Methodology:** The larvicidal crude compounds were extracted from the actinomycetes by solvent extraction method. The identification and characterization of active strain by using cultural characteristics and scanning electron microscopy image. The purified active larvicidal compounds were identified by using UV –spectrophotometry, Fourier transform infrared (FT-IR) and Gas chromatography–mass spectrometry (GC–MS) analysis. **Results and Discussion:** Active crude compound showed that LC_{50} value was found to be 62.5 µl. The identification of bioactive compound ranges from 254 nm to 265 nm was done by UV spectrum. FT-IR analysis was showed that functional groups such as alcohol, alkane, carboxylic acid, aromatics, alkene, and aliphatic amines were present, of which alkane group was predominant. GC–MS analysis report was showed that 1-Eicosanol has significant larvicidal potential of actinomycetes. **Conclusion:** The purpose of this study is to explore the larvicidal activity of natural microbial isolates of actinomycetes species which are isolated from soil against the life-threatening diseases caused by mosquitoes.

Key words: Actinomyces, larvicidal activity, terrestrial soil

INTRODUCTION

Actinomycetes are Gram-positive free-living, soil-borne, and saprophytic bacteria which comprise a group of branching unicellular microorganisms. They produce branching mycelium which may be of two different kinds (substrate mycelium and aerial mycelium).[1,2] The genus *Streptomyces* is an important group of actinomycetes due to its ability to produce many types of primary and secondary metabolites produced by these organisms which are most potent biologically active and remains a powerful source for pharmaceutical discovery of many compounds. The antibiotics are the best known products of actinomycetes.

Actinomycetes were produced many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. These researches have been extraordinarily successful, and approximately two-thirds of naturally occurring antibiotics, including many of medically importance, have been isolated from actinomycetes.[3] Intensive screening program carried out over the past several decades resulted in the production of actinomycetes which are more abundant in terrestrial soil than in marine sediments.[4]

Insect transmitted diseases remain a major source of illness and death worldwide. Mosquitos are one of the major vectors responsible for the transmission of diseases to more than 700 million people annually.[5] Although mosquito-borne

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diseases currently represent a greater health problem in tropical and subtropical countries, no part of the world is immune to this risk.[6]

Mosquito vectors are mostly responsible for transmitting diseases, such as malaria, chikungunya, dengue, Japanese encephalitis, yellow fever, and lymphatic filariasis.[7] Malaria is an important cause of death and illness in adults and children, especially in tropical countries. Malaria is caused by a parasite that is transmitted from one human to another by the bite of infected *Anopheles stephensi*. Each year almost 250 million cases occur, causing 860,000 deaths.[8] Approximately 3.5 billion people live in dengue-endemic countries which are located in the tropical and subtropical regions of the world.[9] Lymphatic filariasis commonly known as elephantiasis and also neglected tropical disease. The infection occurs when filarial worms are transmitted to human through *Culex quinquefasciatus*. More than 1.3 billion people in 81 countries worldwide are threatened by lymphatic filariasis.[10]

Mosquitoes are the oldest human enemy and are the most medically important arthropod vectors of disease. Keeping these points in view, the present study has been undertaken to isolate and screen the larvicidal compounds producing actinomycetes from the soil of the Chennai, Tamil Nadu, India, and also an attempt has been made to characterize the different isolates by analyzing the larvicidal activity of actinomycetes.[11]

To accomplish this goal, the present investigation has been designed to find out the potentiality of the production of larvicidal compounds by actinomycetes isolates possessing significant larvicidal property.

MATERIALS AND METHODS

Soil sample collection

Sediment soil samples were collected in sterile vials from the five different places in Chennai (Sholinganallur, Tambaram East, Thiruvanmiyur, Palavakkam, and Kottivakkam) from the depth of 5 cm after removing the 1 cm of upper soil surface by scrapping with the help of spatula in sterile plastic bags, transfer to the laboratory and stored at the 4°C until use for the isolation process.[12]

Isolation and purification of actinomycetes

The sediment samples collected were air-dried for 1 week and kept at 45°C for 1 h to minimize the bacterial contaminants. About 1 g of samples was transferred to an Erlenmeyer flask containing 99 ml of distilled water. The sediment suspension was further diluted up to 10^{-5} level. One milliliter of the diluted suspension was spread over the surface of the Starch Casein Agar medium[13] for the isolation of actinomycetes.[14] The pH of the selected media used was adjusted to 7.4. To prevent the fungal and bacterial contaminates, cycloheximide (100 mg/L) and nalidixic acid (20 mg/L) were added to the medium. The Petri plates were then incubated at 37°C and the colonies were observed from the 5th day onward for 1 month. Based on the colony morphology, the isolates were picked and subcultured on International Streptomyces Project-2 (ISP-2) medium[15] and incubated at 37°C for 1 week and stored at 4°C.[16]

Extraction of secondary metabolites

The selected isolates were inoculated into 250 ml conical flask containing 100 ml of ISP-2 broth culture and kept in a shaker incubator at 28 ± 2°C and 200 rpm for 7 days. The cell-free culture filtrates were separated by centrifugation and the supernatant was added with an equal amount of ethyl acetate by solvent extraction method[17] and shaken well for 20–30 min using separating funnel[18] and separated the upper layer and collected in the tubes used for larvicidal activity.

Mosquito larval collection and identification

Mosquito larvae were collected in large numbers from stagnant water in and around Chennai cities and stored in plastic bottles for the experiment. A part of the mosquito larvae collected was given for species identification in the Indian Council of Medical Research (ICMR), National Institute for Malaria Research at Ayapakkam, Chennai. The larvae were collected examined under the microscope for its morphological features.

Screening of larvicidal activity

The bioassays were performed according to the standard procedures recommended by the World Health Organization with some modifications.[10] The seven vials were taken and each vial was filled with 1 ml of sterilized tap water without any chlorine and then different concentrations of culture filtrate of active isolate 15.6 µl, 31.2 µl, 62.5 µl, 125 µl, 250 µl, and 500 µl were added and six mosquito larvae were transformed to each tube. Tubes were kept under controlled conditions for 24 h. The number of live larvae was counted and the percentage of mortality was calculated after 24 h.[19] The following formula was used to calculate the % mortality,

$$\text{Larval mortality \%} = Y/X*100$$

Where,
X = Number of tested larvae
Y = Number of dead larvae.

Identification of active actinomycetes

Cultural and morphological characteristics, including the presence of aerial mycelia, spore mass color, distinctive

reverse colony color, color of diffusible pigments, and sporophore and spore chain morphology, were used as identification characters. All morphological characters were observed on ISP-2 agar and the criteria used for classification and differentiation.[13] The mass color of mature sporulating aerial mycelium was observed the following growth on ISP-2 agar plates. The aerial mass was classified according to Bergey's Manual of Systematic Bacteriology in the following color series gray, white, red, yellow, green, blue, and violet.

Identification of active compounds

Active band compounds were screened using ultraviolet (UV) spectrophotometer in the range from 200 nm to 400 nm. The spectral data are consistent with those obtained by Bystrykh *et al*.[20] Fourier transform infrared (FT-IR) spectrometer is a MultiRAM, standalone model. It is used to determine the groups of compound. The spectral range is 50–4000 cm^{-1}. The laser source is Nd: YAG 1064 nm. Gas chromatography–mass spectrometry (GC–MS) techniques were to identify different compounds within a test sample.

RESULTS AND DISCUSSION

Isolation and purification of actinomycetes

A total of 10 soil sediment samples were collected from five different places. Twenty isolates were isolated using Starch Casein Agar medium and subcultured using ISP-2 medium. The study of Nabar and Lokegaonkar[21] found 124 isolates, in which 21 isolates were found to be effective against the mosquito larvae *Culex* and *Aedes* species.

Extraction of crude compounds

Of 20 isolates, only one active strain was selected for larvicidal activity based on purity and stability of growth parameters and that active isolates used for the extraction of crude compounds by solvent extraction method. Then, the collected crude compounds were tested for larvicidal activity.

Mosquito larval collection and identification

The collected test mosquito larvae from in and around Chennai were identified as *C. quinquefasciatus* in species identification center in ICMR, National Institute for Malaria Research at Ayapakkam, Chennai.

Larvicidal activity

The number of live larvae was counted and the percentage of mortality was calculated after 24 h.[18]

The LC_{50} value was found to be 62.5 µl [Table 1]. The study of Janaki[22] reported that the seven test tubes each with 10 ml sterilized tap water without any chlorine were taken and different concentrations of culture filtrate of isolate M20 – 10 µl, 25 µl, 75 µl, 125 µl, 250 µl, and 500 µl were added and six larvae of *Aedes* were transferred into each tube. The number of live larvae was counted and the percentage of mortality was calculated after 24–96 h and the LC_{50} value found to be 75 µl.

Identification of active actinomycetes

The active strain was identified as *Streptomyces* sps. based on colony morphology on ISP-2 medium that showed gray color colonies [Figure 1] and scanning electron microscope image was showed that [Figure 2] the active strain appeared as slender and linear chains of spores and the surface of spores appeared as smooth.

Identification of active compounds

The crude extracts from active strain were screened using UV spectrophotometer in the range from 200 nm to

Figure 1: Colonies on International Streptomyces Project-2 medium

Table 1: Larval mortality % (LC_{50})

Parameter	Control (water)	Culture filtrate at different concentrations					
		15.6 µl	31.2 µl	62.5 µl	125 µl	250 µl	500 µl
Larval mortality %	-	16.7%	33.3%	50%	66.6%	83.3%	100%

400 nm and it was showed that the peak in the ranges from 254 nm to 265 nm [Figure 3 and Table 2]. The spectral data are consistent with those obtained by the World Health Organization[10] and reported that the maximum absorbance peaks of UV spectral analysis data ranged between 215 and 270 nm of actinomycetes isolate from the soil samples of Southeastern Serbia.

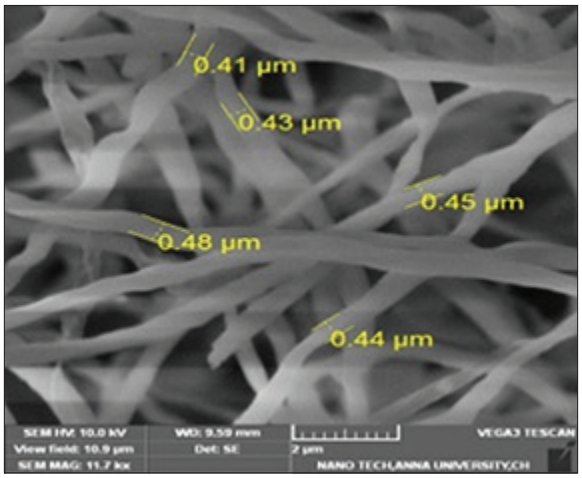

Figure 2: Scanning electron microscope image of *Streptomyces* sps.

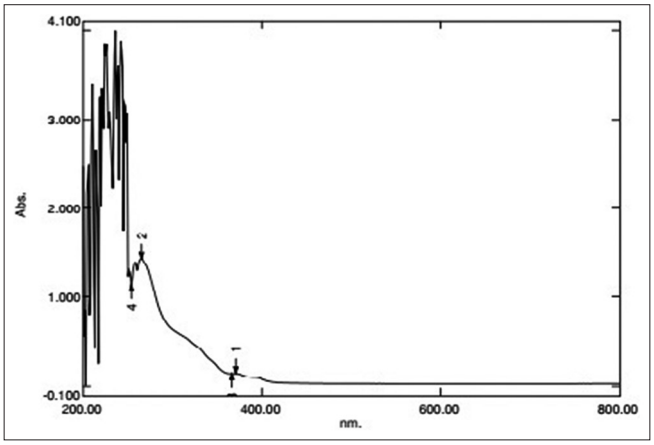

Figure 3: Ultraviolet–visible spectrophotometer analysis for crude compounds from active strain

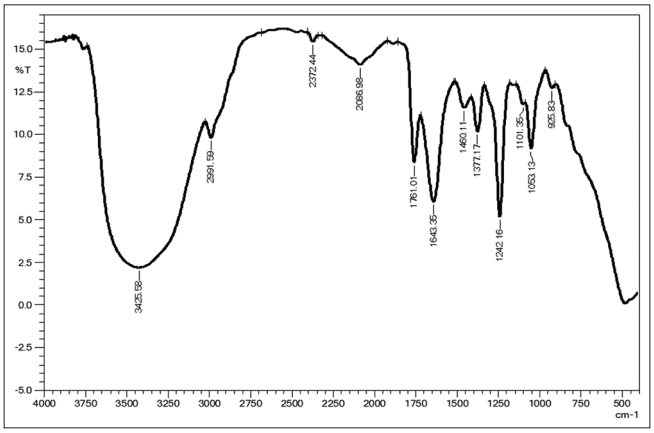

Figure 4: Fourier-transform infrared spectroscopy analysis of active crude compounds

FT-IR spectrometer is a MultiRAM, standalone model. It is used to determine the groups of compound.

The characteristic peak of active crude compounds is observed in Table 3 and Figure 4 and their corresponding groups were showed.

The GC–MS analysis report is shown in Figure 5 and Table 4.

Table 2: Ultraviolet–visible spectrophotometer analysis for crude compounds from active strain

S. No.	Wavelength (nm)	Absorbance
1.	371.00	0.143
2.	265.00	1.434
3.	366.00	0.141
4.	254.00	1.139

Table 3: Fourier transform infrared analysis report of active crude compounds

S. No.	Functional groups	Absorbance/cm
1	Alcohol (O-H)	3425.58
2	Alkane (C-H)	2991.59
3	Carboxylic acid	2372.44
4	C-N(R-N=C=S)	2086.98
5	Aromatics	1761.01
6	Alkene (C=C)	1643.35
7	Alkanes (methyl and methylene)	1460.11
8	Alkanes (isopropyl split)	1377.17
9	Aromatic ethers (C-O)	1242.16
10	-C-O (secondary alcohols)	1101.35
11	C-N (aliphatic amines)	1053.13
12	R-CH=CH2	925.83

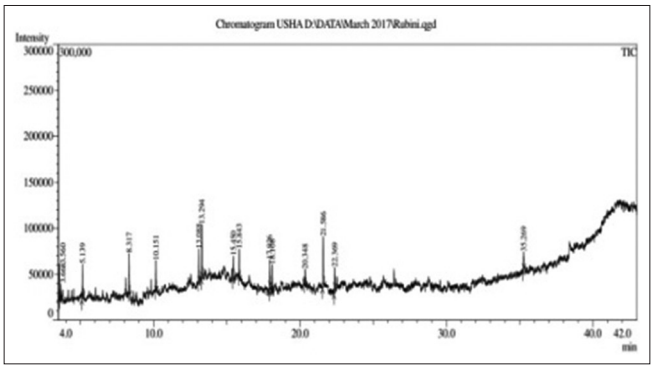

Figure 5: Gas chromatography–mass spectrometry analysis of active crude compounds

Table 4: Gas chromatography–mass spectrometry analysis report for active crude compounds

Peak	R. time	Area	Area (%)	Name
1	3.56	58,753	5.37	Isovaline,3-hydroxy
2	3.668	44,854	4.1	Butanoic acid, 2-Ethyl-
3	5.139	64,573	5.9	1-Undecene,4-methyl-
4	8.317	93,182	8.52	Dodecane
5	10.151	67,699	6.19	Dodecane, 2, 6, 10-trimethyl-
6	13.088	77,472	7.08	1-Hexadecanol
7	13.294	130,265	11.9	Tetradecane
8	15.45	74,677	6.82	Eicosane
9	15.853	58,301	5.33	Phenol, 2, 4-Bis(1, 1-Dimethylethyl)
10	17.26	62,376	5.7	1-Octadecanol
11	18.108	58,580	5.35	Hexadecane
12	20.348	43,387	3.97	Eicosane
13	21.586	153,808	14.06	Tetradecanoic acid
14	22.369	62,094	5.67	1-Eicosanol
15	35.269	44,190	4.04	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)

The characteristic peak of active strain crude compounds was observed and different types of compounds were showed and it may be responsible for a significant larvicidal activity.

CONCLUSION

In the present study, five soil samples were collected from Shoilnganallur, Tambaram East, Thiruvanmiyur, Palavakkam, and Kottivakkam, in Chennai. Based on different morphological color variations, five isolates were selected, purified, and preserved. Among the five isolates, one fast-growing isolate was selected and tested for antilarval activity. The active compounds were extracted from the one active isolate by submerged fermentation followed by ethyl acetate solvent extraction method. The primary screening was done with the extracted crude compounds and the larvicidal activity was observed. The secondary screening was done with one active crude compound and the percentage of mortality of larvae corresponding to the concentration variation was calculated. The LC_{50} value was found to be 62.5 µl. The identification of bioactive compound was done by UV spectrum, and the active band compounds were screened in the ranges from 254 nm to 265 nm. The characteristic peak of active sample was observed in FT-IR analysis and the functional groups such as alcohol, alkane, carboxylic acid, aromatics, alkene, and aliphatic amines were present, of which alkane group was predominant. Further, in our GC–MS analysis, the characteristic peak of active sample was observed and different compounds were reported, of which one compound 1-Eicosanol showed significant larvicidal potential. The present study reveals that soil sample can be potentially used for extracting novel antilarval compounds.

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AUTHORS' CONTRIBUTIONS

Usha Nandhini: Substantial contributions to conception and design actuation of data or analysis and interpretation and final approval of the manuscript version to be published. Sudha Sri Kesavan: Drafting the article and revising it critically for important intellectual content.

CONFLICTS OF INTEREST

We declared no conflicts of interest.

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