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Screening, production, and characterization of biologically active secondary metabolite(s) from marine *Streptomyces* sp. PA9 for antimicrobial, antioxidant, and mosquito larvicidal activity

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Bioprospecting of actinobacteria from understudied ecosystems is a promising source for extracting novel bioactive metabolites**.** A study was undertaken to characterize and analyze the bio-efficacy of actinobacterial extract for antimicrobial, larvicidal, and antioxidant activities. Seven morphologically different actinobacterial cultures isolated from mangrove rhizosphere sediment near Parangipettai, South India, were tested for antimicrobial activity. Bioactive metabolites from one potential strain PA9 were produced by submerged fermentation. The selected *Streptomyces* sp. PA9 was subjected to the production of crude extract for antimicrobial, larvicidal, and antioxidant activity. The actinobacterial compound was characterized by Fourier transform infrared spectroscopy (FTIR), high-performance liquid chromatography (HPLC), and gas chromatography–mass spectrometry (GC–MS). The PA9 actinobacterial crude extract showed best antimicrobial activity against clinical bacteria, *Salmonella typhi* (21.6 ± 0.88 mm) and fungi, *Candida albicans* (26.6 ± 0.88 mm). The PA9 extract showed significant larvicidal activity against *Culex quinquefasciatus* with LC_{50}=173.21 µg/ml and r^2=0.841. The PA9 extract also exhibited antioxidant activity from DPPH (72%) and nitric oxide free radicals (85%). The characterization of the PA9 extract by FTIR analysis showed the presence of possible functional groups. Active compounds were isolated by HPLC and GC–MS with major and minor peaks observed on the basis of retention time. The bio-efficacy of PA9 has warranted further studies to develop a baseline for the drug development.

[Keyword: Actinobacteria; Clinical pathogens; *Culex quinquefasciatus;* FTIR; HPLC; GC–MS]

Introduction

In the last 20 years, more than 30 new diseases have emerged, which together threaten the health of hundreds of millions of people. Some of the major diseases such as cholera, malaria, and tuberculosis are making a deadly comeback in many parts of the world, despite being preventable or treatable[1]. Meanwhile, antibiotics used against many diseases are rapidly losing their efficiency because of resistance by microbes. Thus, the quest for better antibiotics is an unceasing process in the medical world[2].

Biologically active metabolites are produced by some organisms, such as bacteria, fungi, plants, and actinobacteria. Among the different groups of organisms that have the ability to produce such metabolites, the actinobacteria occupy a prominent place[3]. Actinobacteria are a diverse group of filamentous Gram-positive and aerobic bacteria having high G+C content (more than 50%) and they play a major role in the medical field; they are producers of biologically active secondary metabolites, such as antibiotics, enzymes, and vitamins[4,5]. About 70% of naturally occurring bioactive compounds have been separated from different genera of actinobacteria[6]. *Streptomyces* is the largest genus known for the production of many secondary metabolites[7], which have various biological activities, such as antibacterial, antifungal, antiparasitic, antitumor, anticancer, and immunosuppressive actions[8,9].

It is well known that actinobacteria are potential sources of antibiotics, which could profitably be developed in the pharmaceutical industries and the best-known example is the products of *Streptomyces*. There is an increasing demand for new types of antibiotics to control mosquitoes and infectious diseases. With this perspective, the present study is aimed at producing potential secondary metabolites from actinobacteria for various biomedical applications.

Materials and Methods

Preparation of medium for culture

The actinobacterial strains used in this study were isolated from marine sediment samples collected from

mangrove rhizosphere region, Parangipettai (Lat: 11.5085°N; Long: 79.7568°E), Tamil Nadu, India, by the standard spread plate method using starch casein nitrate agar. The cultures were revived in yeast extract malt extract (YEME) agar (g/L: yeast extract 4; malt extract 10; glucose 4; NaCl 5; Agar 18; pH 7±0.2) medium.

Preliminary screening for antimicrobial activity

Morphologically different actinobacterial strains were selected and screened for antimicrobial activity by the agar plug method. All the actinobacterial strains were grown on YEME agar plates (20 ml/plate) and incubated at 28 °C for 7 to10 days. After removing the mycelia growth, an agar plug of 5 mm diameter was cut from the YEME agar plates. The test organisms were inoculated in Muller Hinton Agar (MHA) plates using a sterile cotton swab. The agar plugs were placed over the MHA with test bacterial strains, such as *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. The zone of inhibition was measured after 24 h of incubation at 37 °C and its diameter expressed in millimeters[10].

Production and extraction of bioactive metabolites

Bioactive metabolites from the selected strains PA6, PA9, PA12, and PA13 were produced by agar surface fermentation using YEME agar[11]. After five days of incubation at 28 °C, the metabolite produced by the strains in the agar medium was extracted using ethyl acetate as organic solvent. The solvent extracts were collected and concentrated using a rotary vacuum evaporator. Further, the crude extracts were tested against a group of bacterial and fungal pathogens.

Antimicrobial activity

The solvent extracts were tested for antibacterial and antifungal activities using bacterial pathogens, namely, *S. aureus, Corynebacterium diphtheriae, Proteus vulgaris, Salmonella typhi, K. pneumoniae*, and the fungal strain, *Candida albicans*. The bacterial and fungal cultures were swabbed in the MHA plates and 5 mm diameter wells were cut and about 40 µl of actinobacterial crude bioactive compounds (1 µg/1 µl) dissolved in 10% dimethyl sulfoxide (DMSO) was poured into each well of all the plates and then they were incubated at 37 °C for 24 h. After incubation, the zone of inhibition was measured[12].

Larvicidal activity

For larvicidal activity, larvae of *Culex quinquefasciatus* (vectors of lymphatic filariasis) were acquired from the National Center for Disease Control Research Institute, Coonoor, India, and maintained in plastic trays containing dechlorinated tap water[13].

The larvae were exposed to a wide range of concentrations (PA9 extract) with control. A total of 25 fourth-instar healthy larvae were transferred into a chamber with 250 ml tap water. Different concentrations (100, 300, 500, 700, and 900 µg/ml) of the actinobacterial solvent extract were added to 250 ml water to obtain the desired target dosage. Larval mortality was observed at 48 h and larvae mortality percentage was calculated using the formula with triplicate larvicidal data[14].

Mortality (%) =X − Y/X × 100, where X = survival in the untreated control, Y = survival in the treated sample.

Antioxidant activity

DPPH radical scavenging activity: DPPH (1,1-Diphenyl-2-picrylhydrazyl) was dissolved in 3.3 ml of methanol contained in test tubes. The test tubes were covered with aluminium foil to protect them from light. Then about 150 µl of DPPH solution was added to 3 ml of methanol and the absorbance was recorded immediately at 517 nm for control reading. About 100, 200, 300, 400, and 500 µl concentration of actinobacterial solvent fractions (stock concentration, i.e., 1 mg/1 ml), as well as the standard compound (ascorbic acid), were taken. Then added 3 ml of methanol and 150 µl of DPPH into all the reaction tubes. Absorbance was taken after 15 min at 517 nm using methanol as blank on the UV–Vis spectrometer, Cyber lab Model[15]. The DPPH free radical scavenging activity was calculated using the formula:

Scavenging percentage $= (T_0 - T)/T_0 \times 100$,

where T_0 *= absorbance of control, T = absorbance of the test sample at 517 nm.*

Nitric oxide (NO) free radical scavenging activity: Different concentration (100, 200, 300, 400, and 500 µl) of the actinobacterial crude extract (1 mg/1 ml) dissolved in DMSO as well as ascorbic acid (standard) were taken in separate tubes; in each tube, 2.0 ml of sodium nitroprusside in phosphate buffer saline was added. The solution was incubated at room temperature for 150 min. After incubation, 5 ml of Griess reagent was added in each tube, including the

control. Methanol was used as a blank. The absorbance was measured at 546 nm on the UV–Vis spectrometer[15]. The NO free radical scavenging activity was calculated using the formula:

Scavenging percentage = $(T_0 - T)/T_0 \times 100$
where T_0 = *absorbance of control, T = absorbance of the test sample at 546 nm.*

Fourier transform infrared (FTIR) spectral analysis

The infrared (IR) spectrum of the compound PA9 was determined by EXI-Spectrum FTIR Model. The spectrum was obtained using potassium bromide (KBr) pellets in the range of 4000 to 400 cm^{-1} at a resolution of 1.0 cm^{-1}. Before the analysis, potassium bromide (AR grade) was dried under vacuum at 100 °C and 100 mg of KBr with 1 mg of sample was used to prepare KBr pellets. The spectrum was plotted as intensity versus wave number[16].

High-performance liquid chromatography (HPLC) analysis

The metabolite profile of the actinobacterial extract was determined using analytical HPLC (Shimadzu, Japan). The chromatographic separation of the actinobacterial extract was carried out by LC-10 AT VP model HPLC using 15 cm × 4.6 mm CLC-ODS column (C-18). The mobile phase consisted of methanol (HPLC grade) and water (HPLC grade) at 9:1 ratio. The UV–Vis (SPD-10 AVP) detector was set at 254 nm with 1 ml/min flow rate. The sample was mixed with the solvent in the ratio of 50:50 and filtered using a Millipore filter. About 20 µL of the sample filtrate was injected into the column. The sample was allowed to run for 10 min and the crude compounds present in the extract of strain PA9 were identified on the basis of retention time and quantified[17].

Gas chromatography–mass spectrometry (GC–MS) analysis

The GC–MS analysis of actinobacterial extract was done using BRUKER GC - Trace Ultra Ver: 6.0, Thermo MS dsq II, fitted with a silica capillary column (ZB5 - ms capillary standard nonpolar column – 30 × 0.25 mm I.D., Film thickness 0.25 µm). Hundred microliters of actinobacterial extract was injected into the chromatographic column. The process temperature was maintained at 70-260 °C at a rate of 6 °C/min for 2 min. The scan ranged from 50 to 650 m/z. Compounds were identified based on comparison of their mass spectra with those of Wiley and NIST libraries[18].

Statistical analysis

All the experiments were carried out in triplicate and subjected to statistical analysis carried out by variance value calculated using SPSS v.16 software. Results with $P < 0.05$ were measured to be statistically significant[19].

Results and Discussion

During primary screening of the agar plug method, the strain PA9 showed maximum antibacterial activity on *S. aureus* (14 mm) and *E. coli* (16 mm) (Fig. 1). Furthermore, crude bioactive compounds from the strains PA9, PA12, PA6, and PA13 were extracted by agar-based solid-state fermentation using ethyl acetate as the solvent system. The available literature shows that agar-supported surface fermentation has comprehensive interest in the field of microbial secondary metabolites production due to notable advantages in the extraction and concentration of highly diluted compounds[20]. In the present study, secondary screening using crude extracts showed that the PA9 extract had maximum inhibition against test bacterial pathogen *S. typhi* (21.6 ± 0.88 mm) and fungal pathogen *C. albicans* (26.6 ± 0.88 mm) (Fig. 2 and Table 1). Duddu and Guntuku[21] have reported *in vitro* antimicrobial activity of actinomycetes (strain M3) isolated from the mangrove ecosystem which

Fig. 1 — Antagonistic activity by the agar plug method (1-PA9, 2-PA2, 3-PA8, 4-PA12, 5-PA7, 6-PA6, and 7-PA 13)

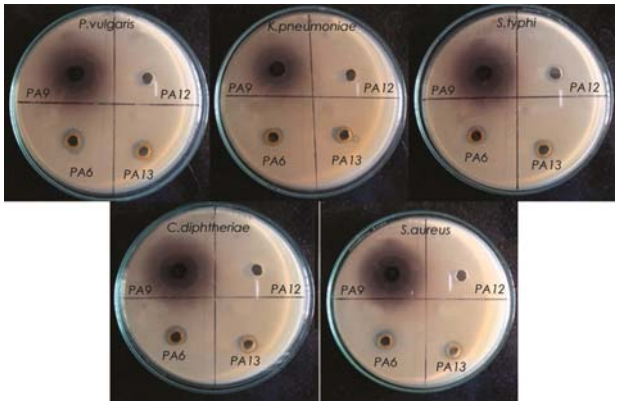

Fig. 2 — Antibacterial study: zone of inhibition by clinical pathogens against actinobacterial solvent extract

showed maximum zone of inhibition on *S. aureus* (19.5 mm) and *C. albicans* (22.6 mm). *Streptomyces parvus* bioactive compounds were active against pathogenic bacteria; the maximum inhibition was recorded on *P. aeruginosa* (20 mm) and *E. coli* (20 mm)[22]. Zothanpuia *et al.*[23] have reported that *Streptomyces cyaneofuscatus* strain produced broad spectrum antimicrobial activities against bacterial and fungal pathogens. The maximum zone inhibition was obtained from *E. coli MTCC 739* (15 mm) and *C. albicans* MTCC 3017 (12.6 mm). In the current study, the PA9 ethyl acetate extract showed promising activity against clinical pathogens and it was further used for larvicidal and antioxidant activity.

The larvicidal effect of ethyl acetate extract of actinobacterial strain PA9 showed maximum larval mortality rate on *C. quinquefasciatus* (LC_{50}=173.21 µg/ml and r^2=0.841) (Fig. 3 and Table 2). Similarly, the strain KA13 showed 100% mortality at 1000 ppm concentration and the strain KA25-A showed a maximum of 90% mortality at 24 h incubation[24]. Vijayakumar *et al.*[25] have reported the larvicidal activity of *Streptomyces* sp. crude extract against *C. quinquefasciatus* as LC_{50}=255.12 mg/L. Ganesan *et al.*[26] reported that crude extracts of secondary metabolites of the actinomycete showed larvicidal activity against *C. quinquefasciatus* with LC_{50}=170.55 mg/l.

In the present study, significant scavenging activity was recorded (72% to 85%) using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and NO free radical scavenging assay, respectively (Fig. 3). Priya *et al.*[27] have reported that the ethyl acetate extract of *Streptomyces* showed 83% of scavenging activity on DPPH assay. Vanmathi *et al.*[28] isolated antioxidant compound from marine actinobacterium, which showed the maximum percentage of scavenging (40.24%) during NO radical scavenging assay. Similarly in the present study, PA9 extract also exhibited good scavenging of DPPH and NO free radical and it was near that of the standard. In an earlier investigation, antioxidant potential of ethyl acetate solvent extract from *Streptomyces* sp. VITMK1 was studied using DPPH radical and NO radical assay and the compound of VITMK1 strain showed strong DPPH radical scavenging activity (72.48 ± 0.32% at 500 µg/ml) and NO radical scavenging activity (73.03 ± 1.02% at 500 µg/ml)[29].

The results of FTIR spectroscopic analyses of PA9 crude extract from the present study are given in Figure 4 and Table 3, which shows the presence of various functional groups of bioactive metabolites in the form of peaks. The results confirmed the presence of alcohol with a peak ratio at 3769.15 and 1183.48 cm^{-1} which corresponded to O-H and C-O stretching frequency, respectively. The peak at 3429.11 and 2078.37 cm^{-1} assigned to the –C=C- and -C-H- stretching indicates the presence of some alkyne compounds. The peak at 2926.62 and 1454.88 cm^{-1} confirms alkane and the peak at 1655.94 cm^{-1} confirms alkene. The FTIR functional groups were

Table 1 — Antimicrobial activity of the actinobacterial extracts against clinically important pathogens.
The results are presented as mean ± (SD) (n = 3)

S. No	Bacterial and fungal Isolates	Zone of inhibition (in mm)			
		PA9	PA12	PA6	PA13
	Bacteria				
1	*Staphylococcus aureus*	15 ± 0.57	–	8.6 ± 0.88	–
2	*Cornybacterium diptheriae*	15.6 ± 0.66	–	–	7.3 ± 0.88
3	*Klebsiella pneumoniae*	12.6 ± 1.20	–	–	–
4	*Proteus vulgaris*	14.3 ± 0.88	–	8.3 ± 1.85	–
5	*Salmonella typhi*	21.6 ± 0.88	–	–	–
	Fungi				
6	*Candida albicans*	26.6 ± 0.88	–	–	–

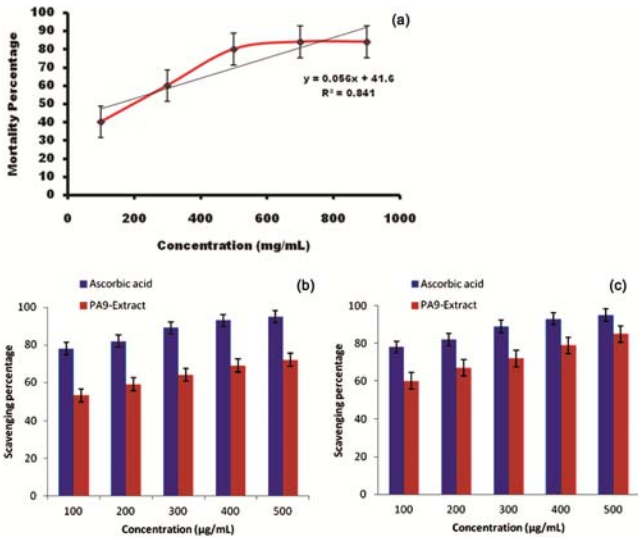

Fig. 3 — (a), Larvicidal activity of the actinobacterial extract against *Culex quinquefasciatus*. (b) and (c), DPPH and nitric oxide scavenging activity of PA9 extract

Table 2 — Larvicidal activity of the actinobacterial (PA9) extract against *Culex quinquefasciatus*

Sample	Concentrations (mg/l)	*Percent mortality ± SD	LC_{50} (µg/ml) (LCL–UCL)	r^2	Regression equation
PA9 extract	100	40 ± 0.57			
	300	60 ± 1.0			
	500	80 ± 1.15	173.21 (80.91-370.78)	0.841	$Y = 0.056X+41.6$
	700	84 ± 1.52			
	900	84 ± 1.52			
Control (DMSO)	900	–			

*Significant at $P<0.05$ level, LC_{50} lethal concentration that kills 50 % of the exposed larvae, UCL upper confidence limit, LCL lower confidence limit, r^2 regression

Table 3 — FTIR peaks and their respective assigned functional groups (PA9)

S.NO	FTIR peak (cm^{-1})	Stretching and bending vibration	Assigned functional group
1	3769.15	O–H (Stretch)	Hydroxyl (or) Alcohol
2	3429.11	–C–H– (Stretch)	Alkyne
3	2926.62	C–H (Stretch)	Alkane
4	2078.37	–C=C– (Stretch)	Alkyne
5	1655.94	C=C (Stretch)	Alkene
6	1454.88	–C–H– (Bending)	Alkane
7	1183.48	C–O (Stretch)	Alcohol

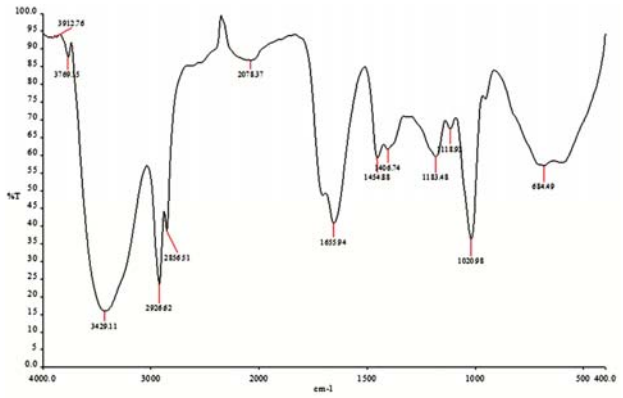

Fig. 4 — FTIR analysis of ethyl acetate extract of potential strain PA9

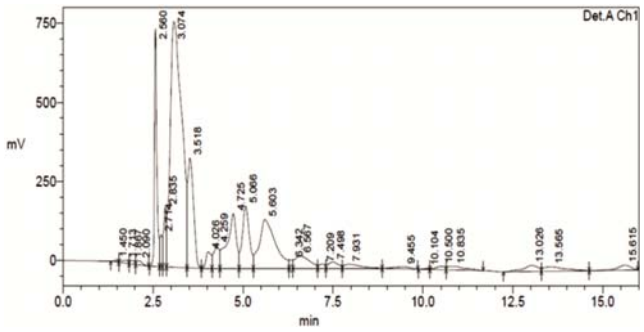

Fig. 5 — HPLC chromatogram of potent bioactive crude compound

easily identified through standard IR functional data report. FTIR spectrum of ethyl acetate extract of AS9 showed absorption at 3380 cm^{-1} peak, which indicates the N-H amino group, 2510 cm^{-1}, 1798 cm^{-1}, and 1618 cm^{-1} peaks indicate C=C alkene group, 1423 cm^{-1} indicates C=C aromatic group, 1140 cm^{-1} peak indicates CF fluorine group, 998 cm^{-1}, 874 cm^{-1}, 711 cm^{-1}, 675 cm^{-1} peaks confirm C-CI, 656 cm^{-1} peak shows C-CI, and 599 cm^{-1} peak confirms CBr-bromine group[30].

Faja *et al.*[31] subjected crude compounds from potential actinobacterium to HPLC (HPLC-SPD-20AShimadzu-Japan). Major peak value was recorded in the retention time of 5.36 min. In the present study, the crude extract of PA9 isolate showed many peaks in HPLC graph, with retention times (Fig. 5). Further purification and structure elucidation of bioactive compound is under progress.

Different compounds such as 1-butene, 3-methyl-3-(methoxy ethoxy), pyrrolidine,1-(1-oxo-2, 5-octadecadienoyl), 3,4-dichlorophenethylamine, 3-trifluoroacetoxypentadecane, octaethylene glycol mono dodecyl ether, 3-(Prop-2-enoyloxy) dodecane, 1,4,7,10,13,16-hexaoxacyclo-octadecane, heptaethylene glycol, and ethanol, 2-[2-(2-ethoxyethoxy) ethoxy] were present in the sample (Fig. 6 and Tables 4 and 5). The GC–MS characterization of actinobacteria SMS_SU21 showed the presence of four major compounds, vinylbital, 2,6-triazolo[1,5-a] pyrimidine, 5,7-dimethyl1-2-phenyl, and digitoxigenin. All the major compounds

Table 4 — The major components found during the GC–MS analysis of the ethyl acetate extract of *Streptomyces* PA9

S. No	RT	Name of the compounds	Mol. wt	Mol. form
1	5.39	1-Butene, 3-methyl-3-(1 ethoxyethoxy)	158	$C_9H_{18}O_2$
2	7.82	Pyrrolidine, 1-(1-oxo-2,5-octadecadienyl)	333	$C_{22}H_{39}NO$
3	12.73	3,4-Dichlorophenethylamine	189	$C_8H_9CL_2N$
4	13.173	3-Trifluoroacetoxypentadecane	324	$C_{17}H_{31}F_3O_2$
5	15.453	Octaethylene glycol monododecyl ether	538	$C_{28}H_{58}O_9$
6	17.530	3-(Prop-2-enoyloxy)dodecane	240	$C_{15}H_{28}O_2$
7	19.554	1,4,7,10,13,16-Hexaoxacyclooctadecane	264	$C_{12}H_{24}O_6$
8	20.947	Heptaethylene glycol	326	$C_{14}H_{30}O_8$
9	23.133	Ethanol, 2-[2-(2-ethoxyethoxy)ethoxy]	178	$C_8H_{18}O_4$

Table 5 — Properties of major components found in ethyl acetate extracts of *Streptomyces* PA9

S.NO	Name of the compounds	Applications of the compounds
1	1-Butene, 3-methyl-3-(1 ethoxyethoxy)	Antibacterial activity
2	Pyrrolidine, 1-(1-oxo-2,5-octadecadienyl)	No activity reported
3	3,4-Dichlorophenethylamine	Cytotoxic activity Anticancer activity
4	3-Trifluoroacetoxypentadecane	Used as an nephro-protective Possesses antioxidant activities Antimicrobial activities
5	Octaethylene glycol monododecyl ether	No activity reported
6	3-(Prop-2-enoyloxy)dodecane	No activity reported
7	1,4,7,10,13,16-Hexaoxacyclooctadecane	Synthesis of 2-cyanoethyl-*O*-β-cholesterol ether 2
8	Heptaethylene glycol	Antioxidant activity Anticancer activity
9	Ethanol, 2-[2-(2-ethoxyethoxy)ethoxy]	No activity reported

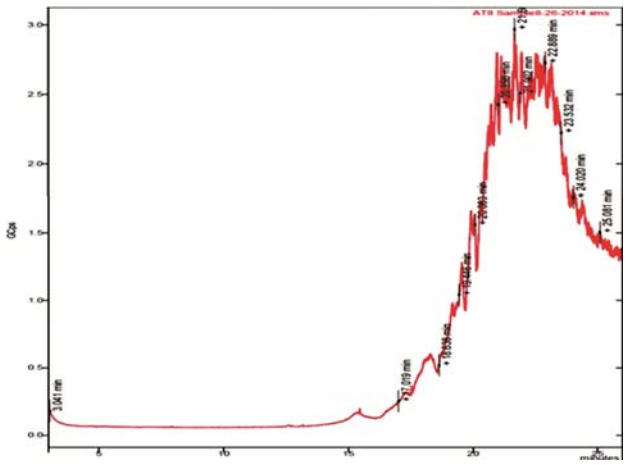

Fig. 6 — GC–MS analysis of metabolites synthesized by actinobacterial strain PA9

are related to antimicrobial, antioxidant, and cytotoxic properties[32]. Khattab *et al.*[33] have reported that GC–MS analysis of *Streptomyces* exhibited eight compounds gained from the PS 1 isolate and 11 compounds gained from the PS 28 isolate. The crude compound of the isolate TS 1010 was characterized by GC–MS. The GC–MS showed a list of 13 bioactive molecules and among them three were found to have antimicrobial, anticancer, and antibacterial activities[34].

Conclusion

In the present study, an actinobacteria, *Streptomyces* sp (PA9) was isolated from mangrove soil and screened for its ability to produce bioactive compounds against antimicrobial, larvicidal, and antioxidant activity. Further, the compound was characterized by FTIR, HPLC, and GC–MS. The extracted bioactive compound has shown good antimicrobial, larvicidal, and antioxidant properties.

Further research is in progress for compound identification, structure elucidation, and production of the bioactive compound in large quantities to make it industrially important.

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