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Bioactive compounds isolation from efficient Marine *Streptomyces MW09-1(RW2-3)* by GC-MS analysis

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Abstract
The Objective was to analysis the secondary metabolites of marine *Streptomycetes MW9-1 (RW2-3)*, isolated from Bay of Bengal, Adirampattinam, Thanjavur district. The secondary metabolites were extracted by solvent extraction and Presences of active components are confirmed by GC-MS analysis. The identification of bioactive chemical compounds is based on the peak area, retention time, molecular weight and molecular formula. The GC-MS analysis of *Streptomycetes MW9-1 (RW2-3)* revealed the presence of molybdenum complex, Spiro compounds, iodo compounds, Chlorocompounds, Diethyl phthalate, Butyl phthalate, Phthalic acid mono ester, Phthalic acid diester, Acetyl benzoic acid were the major compounds present in fermented extracts of *Streptomyces MW09-1 (RW2-3).*

Keywords: secondary metabolite, ethyl acetate, diethyl phthalate, acetyl benzoic acid

Introduction
Marine actinomycetes have become increasingly important source for new bioactive natural components. Secondary metabolites are bioactive compounds produced during stationary phase when there is a nutrient depletion in the nutrient medium [1]. Secondary metabolites are non-essential for the growth and reproduction but have a defence mechanism to the producer organism. Marine actinomycetes have known to be dominant source of bioactive compound producer. These bioactive compounds have a therapeutic and industrial value [2].

Members of actinomycetes especially *streptomyces sp*. Have major role in bioactive compounds production with commercial value and are able to produce variety of antibiotics and extracellular enzymes. In fact 80% of bioactive compounds are produced from *streptomyces sp* [3]. *Streptomyces* from marine samples have rarely undergone for screening of secondary metabolites, and there is evidence that *strptomyces* usually make up only small portion of the bacterial flora of marine habitat with absolute number of *streptomyces* much lesser in terrestrial habitats. The marine *strepomyces* are unique for bioactive compounds production compared to other sources due to variations in physical, chemical and biological factors.

In the past few years, Gas chromatography- mass spectrometry is used as one of the technical platform for finger print analysis of secondary metabolite from both plant and microorganisms. GCMS is the best technique to identify long chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids, amino and nitro compounds etc. Taking into consideration GCMS analysis was carried out to detect the bioactive compounds present in the actinomycetes crude and ethyl acetate extracts. GCMS is a highly effective and versatile analytical technique that combines the separation process of gas chromatography, with detection features of mass spectrometry to identify different compounds with in a test sample [4, 5]. Using these modern techniques we can detect the bioactive compounds easily with less duration.

The present study was carried out to screen the bioactive compounds from marine Streptomyces *MW09-1(RW2-3)* strain by using Gas chromatography- Mass spectrometry techniques.

Materials methods
Isolation of *Streptomycetes*
In total 50 sea water samples are collected from Adirampattinam, Thanjavur district, Tamil nadu. The samples were subjected to physical and chemical treatment to felicitate *Streptomyces* isolation. *Streptomyces* isolation was carried out by spread plate method using 50% sea water in the starch casein agar medium. The pure colonies were selected isolated and maintained in ISP4 agar slants [6].

Extraction of bioactive components
Antimicrobial substance productions from *Streptomyces MW09-1 (RW2-3)* were done on M14 medium. This medium was selected based on optimization study. M14 medium was prepared by adding 2% maltose and 1.5% beef extract. pH of the medium was adjusted to 7 and sterilized at 121°C for 15 minutes. About 2% of inoculums from seed culture was added and incubated at 27±2°C for 7 days. The broth was centrifuged at 5000rpm for 10 minutes with equal volume of ethyl acetate (1:1) in a separation funnel to extract the compounds and the antibacterial study was carried out by agar well diffusion method. 100µl of the supernatant were loaded in the well using micropipette. The zone of inhibition was measured as a total diameter was subtracted from the total diameter [7].

Identification of Bioactive components
The presences of active compounds were identified by Gas chromatography- Mass spectrometry Technique. GC - MS analysis was performed using an Agilent GC – MS 5973 assembly equipped with a HP -5 cross – linked fused silica capillary column (25m/0.32mm/0.25µm), The GC-MS instrument made is of Thermo scientific [8]. Helium was

used as carrier gas at 38 cm/s. The column total flow rate was 1ml/min. General temperature conditions were: split/split less injector at 2800C, transfer line at 2800C, source 2300C, and column temperature program of 800C D 3100C at 100^0C / min. Mass detection limits were 50D-700Da. Samples were reacted with BSTFA – pyridine (1:1 v/v) at room temperature for 30 minutes before analysis.

Results and Discussion

Isolation of *Steptomycetes*

A total of 13 isolates were isolated from marine samples based on their colony morphology and colour variation on starch casein agar medium. The majority of these isolates were assigned to the genus streptomyces on the basis of their morphological, physiological, biochemical properties [9].

Extraction of bioactive components

Out of thirteen selected and identified actinobacteria, *Streptomyces* sp., *MW09-1(RW2-3)* showed significant antimicrobial activity against multidrug resistant UTI pathogens. Five pathogenic strains isolated from cases of UTI infection were used as a test organism for antagonistic study. Among the actinobacteria tested, *Streptomyces* sp., *MW09-1 (RW2-3)* strain produced the best activity against all the test organisms at 100µl / disc concentrations against MDR urinary isolates. The results indicated that all the actinobacterial strains showed good antibacterial activity. *Streptomyces* sp., *MW09-1 (RW2-3)* strain produced a 16.6±2.08 mm zone of inhibition against E51 and E44 strains of UPEC. Best antimicrobial activity was exhibited by MW09-1(RW2-3) strains (Figure I). This strain was considered as a *Streptomyces* sp [10].

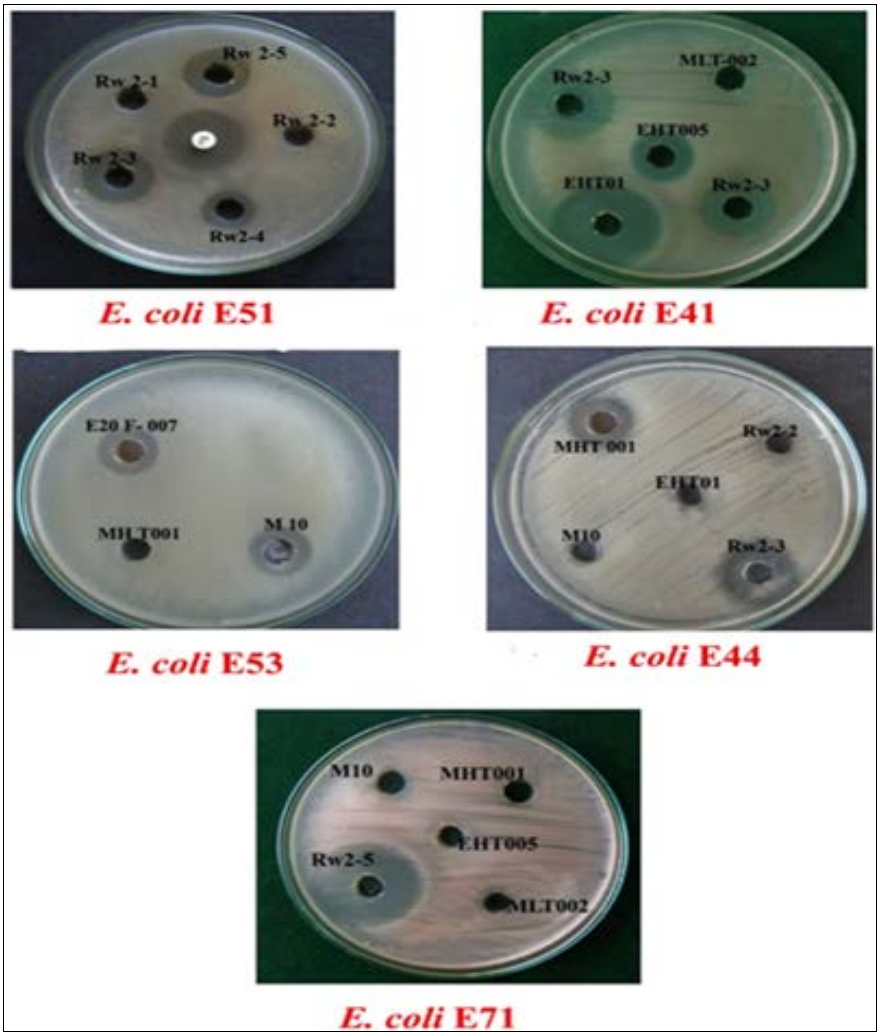

Fig 1: Antimicrobial Activity of Antagonestic Actinobacteria

Identification of Bioactive components

Crude extract obtained from *Streptomyces MW09-1 (RW2-3)* fermentation was analysed by GC-MS method. The GC-MS spectra are given in (Figure 2). The major constituents in the crude extract were obtained at a retention time of 20 - 22.9 mins. The mass of important constituents are in the range m/z=50-250. GC-Ms data analysis revealed the presence of 72 different compounds in the crude extract. At retention time 3.918 min, dichloronitro methane is available as a major chemical constituent (Table 1).

Table 1: GC-MS analysis – Peak analysis at 3.918 min

S. No.	Molecular Weight	Name of the compound
1	129	Dichloronitro- Methane
2	118	Chloroform
3	182	Trichloromethane
4	202	Methane, oxybis(dichloro-
5	162	Propane,3,3-dichloro 1,1,1,2,2pentafluoro-
6	150	Bromodichloromethane
7	152	Ethane, 1,1,2-trichloro-2-fluoro-
8	182	Ethane, 2,2-dichloro-1,1,1-trifluoro-
9	162	Dichlorine heptoxide

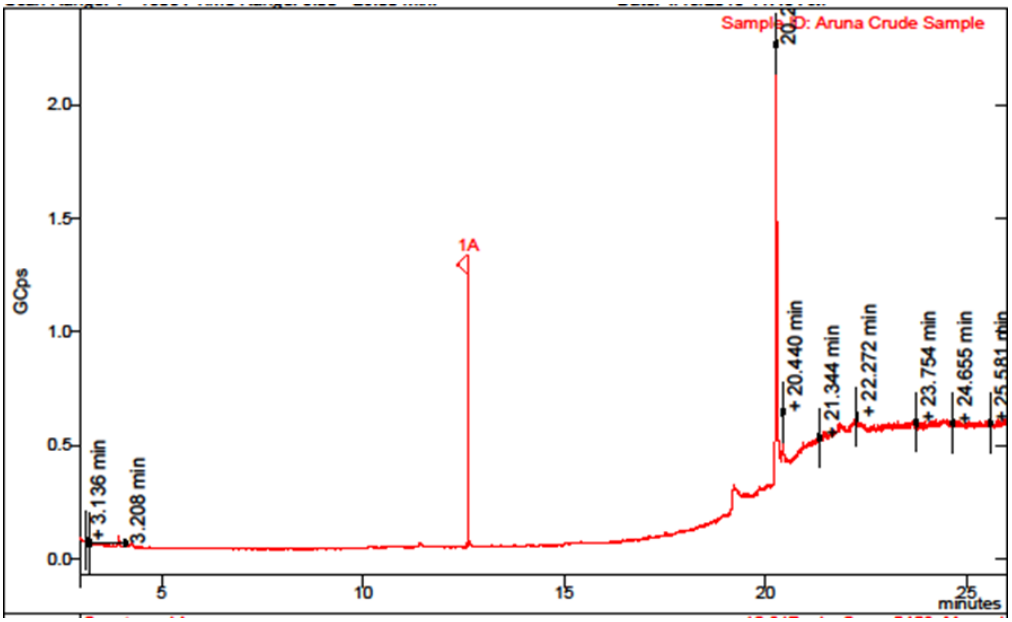

Fig 2: GCMS Patten of Actinobacterial Extract (Streptomyces MW09-1)

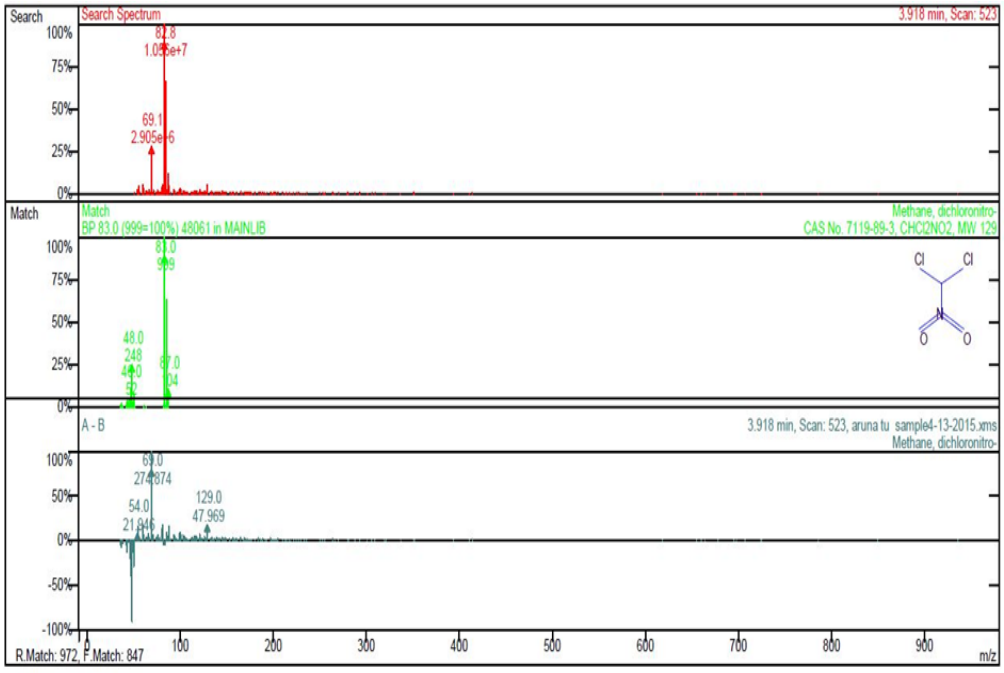

Fig 3: *GC-MS peak analysis at 3.918min*

At retention time 4.018 min the mol.wt of compounds lie in the range of 180-510. Dodecanoic acid esters, 3, 6- diethyl – octone-2-oned-manose and 2- heptadecanol acetate are found to an extent of 30 % (Table 2, Figure 3). Among these chemicals Dodecanoic acid is available as an important chemical moiety, which is an organic compound with flavour ketone. Dodecanoic acts as a non-competitive AMPA receptor antagonist at therapeutically relevant concentrations, in a voltage- and subunit-dependent manner, and this is sufficient to explain its anti- seizure effects [11, 12].

Table 2: Important chemical constituents in GC-MS at 4.018min.

S. No.	Molecular Weight	Name of the compound
1	358	Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester, 2-(acetyloxy)-1((acetyloxy)methyl)ethyl ester
2	438	(2S,2'S)-2,2'-Bis(1,4,7,10,13-pentaoxacyclopentadecane)
3	510	3-(2,5,8,11,14-Pentaoxacyclohexadecyl)-1,5,8,11,14,17-hexooxacy
4	438	(1S,17S)-3,6,9,12,15,18,21,24,27,30-Decaoxabicyclo(15.13.0)tria
5	180	d-Mannose
6	350	(1S,14S)-Bicyclo(12.10.0)-3,6,9,12,15,18,21,24-octaoxatetracosa
7	180	1,3-Dihydroxyacetone dimer
8	156	Octan-2-one, 3,6-dimethyl-
9	298	2-Heptadecanol, acetate

1-octanol 2, 7 - dimethyl has been detected in actinobacterial extract. Molybdenum, bis ((1, 2, 3, 4, 5, 6 – η) - methylbenzene) with molecular formula $C_{14}H_{16}Mo$ was detected from the extract, which is one of the newly detected compound (Table 3, Figure 3). This compound may be new and responsible for enhanced antimicrobial activity. The identification of the molybdenum complex throws light on the ability of soil microbes to synthesise inorganic complex from the metals in the soil; this result thus opens a new path for the synthesis of chemical complexes in the chemistry laboratory by the use of actinomycetes which can be prepared in the broth in a biochemical laboratory [13].

Table 3: GC-MS peak analysis at 4.235 min

S. No.	Molecular Weight	Name of the compound
1	92	1,3,5-Cycloheptatriene,Toluene,Cyclobutene,2-propenylidene-1,6-Heptadien-3-yne,Spiro(2,4)hepta-4,6-diene
2	532	Molybdenum,di-.mu.-chlorobis(1,2,3,4,5,6-.eta.) methylbenzene
3	254	Methyl 2-O-benzyl-d-arabinofuranoside
4	274	2-Benzyloxy-4-bromobutane-1,3-diol
5	220	Tricyclo (3.2.2.0 (2,4))non-8-ene-6,6,7,7-tetracarbonitrile

The GC-MS analysis revealed that at this retention time the compounds are as found in the crude extract except tricyclo (3.2.2.0(2, 4) non-8-ene-6, 67, 7- tetra carbonitrile) which may be the cause of antimicrobial activity. In the 4.243 min of GC-MS spectrum search revealed the presence of 2, 4-dichlorophenl ethylamine m/z=189 which is a highly potent antimicrobial agent. It is quite abundant in the crude extract (Table 4, Figure 3). All the compounds identified and presented in (Figure 3a) were new and novel chemicals. It is interesting to identify a molybdenum complex of molecular weight 532and several organic di ester toluene of identical molecular weight of 92 and (Figure 3b) illustrated the structure of new molybdenum compound [14].

Table 4: Chemical constituents identified by GC-MS analysis at 4.243 min.

S. No.	Molecular Weight	Name of the compound
1	92	Spiro(3.3) hepta-1,5-diene,1,3,5-Cycloheptatriene, Cyclobutene, 2-propenylidene-,Toluene
2	532	Molybdenum, di-.mu.-chlorobis ((1,2,3,4,5,6-.eta.)-methyl benzene
3	254	Methyl 2-O-benzyl-d-arabinofuranoside
4	178	Benzyl isopentyl ether
5	274	2-Benzyloxy-4-bromobutane-1,3-diol

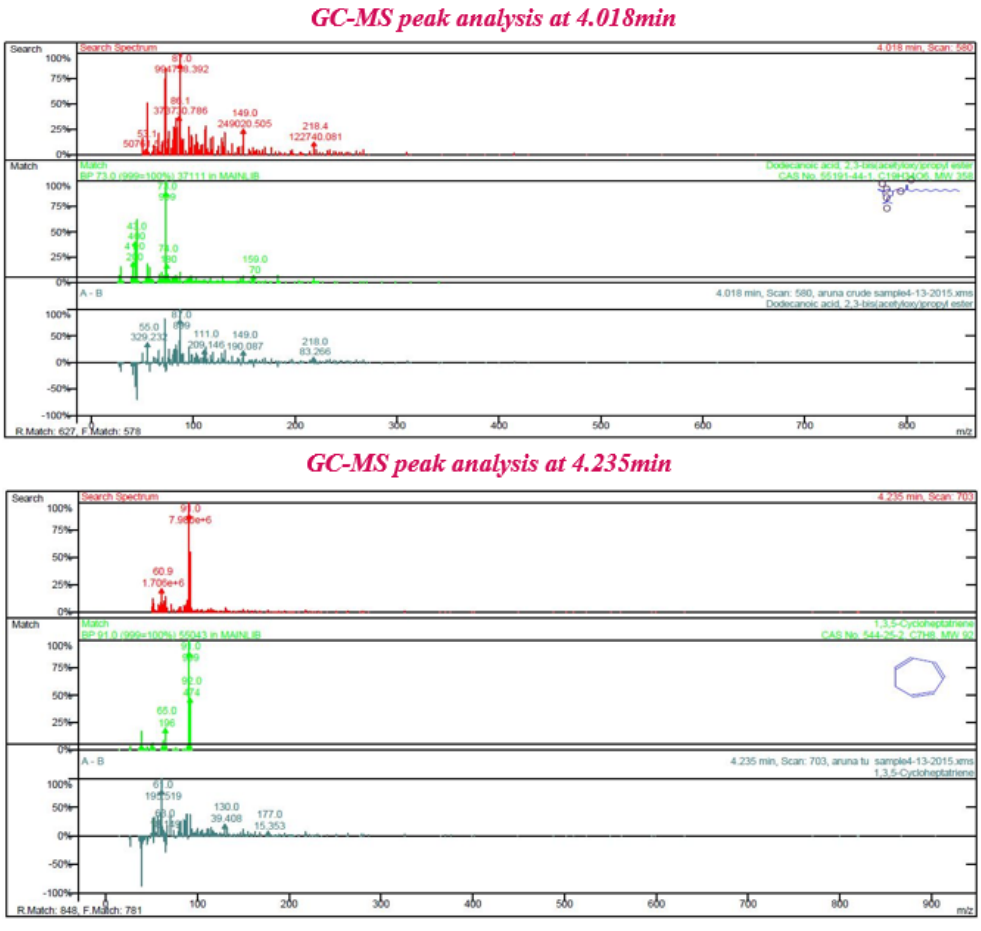

Fig 4: GC-MS analysis of Actinobacterial extract

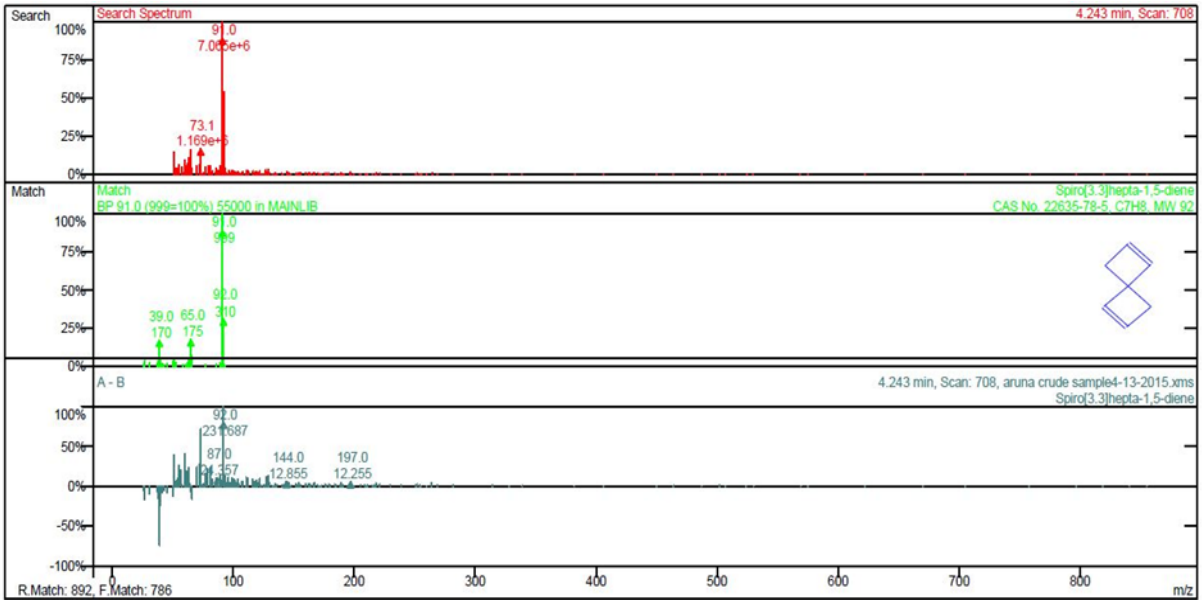

Fig 5: GC-MS peak analysis at 4.243 min

At 11.45 minutes, the molecular weight of the various chemical constituents is in the range of 114 - 484. The various compounds identified are found (not reported elsewhere) to an extent of 30% among the various chemical constituents o-decyl hydroxylamine of molecular weight 173 and (E) isomer of 2-octen-1-ol of molecular weight 128 can produce appreciable antimicrobial activity. 2-Myristynoyl pantetheine is an organic compound which is responsible for all kinds of biological activities (Table 5) [15].

Table 5: GC-MS analysis at 11.45 min

S. No.	Molecular Weight	Name of the compound
1	484	2-Myristynoyl pantetheine
2	118	1-Pentanol, 5-methoxy-
3	142	1,2-Epoxynonane
4	128	2-Octen-1-ol
5	173	Hydroxylamine, O-decyl-
6	240	Tetradecane, 2,6,10-trimethyl-
7	286	Methoxyacetic acid, 2-tetradecyl ester
8	128	2-Octen-1-ol, (E)-
9	114	Heptane, 2,3-epoxy-

Table 6: GC-MS analysis at 11.430 min

S. No.	Molecular Weight	Name of the compound
1	272	Methoxyacetic acid, 2-tridecyl ester
2	414	Heptacosane, 1-chloro-
3	352	1-Octadecanesulphonyl chloride
4	604	Tritetracontane
5	234	Threitol, 2-O-octyl-
6	302	Nonadecane, 1-chloro-
7	286	Methoxyacetic acid, 2-tetradecyl ester
8	240	3-(Prop-2-enoyloxy)dodecane
9	998	Nonahexacontanoic acid
10	274	Diethylene glycol monododecyl ether

The new compounds found in the ethyl acetate extract are listed in table 6. Methoxy acetic acid 2-tridecyl ester is identified as the important antimicrobial compounds in the actinobacteria [16].

The novel isolates at this retention time 12.621 minutes are 1-iodo-2-methyl undecane (m/z=296), 1-iodo tetradecane (m/z=324) and sulfurous acid ester (m/z = 292). This analysis creates a new path for producing iodo substituted organic compounds which are important in food and chemical industries. The antimicrobial processing activity may also be due to these chemical constituents (Table 7).

Table 7: GC-MS analysis at 12.621 min

S. No.	Molecular Weight	Name of the compound
1	212	Pentadecane
2	268	Heptadecane, 2,6-dimethyl-
3	296	1-Iodo-2-methylundecane
4	324	Tetradecane, 1-iodo-
5	292	Sulfurous acid, butyl undecyl ester
6	282	Nonadecane, 2-methyl-
7	226	Hexadecane
8	156	Octane, 2,4,6-trimethyl-
9	376	Sulfurous acid, hexyl pentadecyl ester
10	184	Dodecane, 2-methyl-

At 12.622 min only hydrocarbons are found (Table 8). At 17.11 min several cyclic compounds are identified. At 20.428 min aliphatic, aromatic amines heterocyclic compounds are present along with phthalates reported by earlier workers [17].

Table 8: List of compounds at 12.622 min

S. No.	Molecular Weight	Name of the compound
1	156	1,3,5-Triazine-2,4(1H,3H)-dione, 6-(ethyl amino)-
2	432	Pregnan-20-one, 3-(acetyloxy)-5,6-epoxy-6-methyl-, cyclic 20-(1
3	242	N,N-Diethyl-N'-(1-naphthyl)ethylenediamine, Cyclo acetyl cuparene
4	634	N-Acetyl-D-glucosamine, tetrakis(trifluoroacetate), methyloxime
5	213	Acetic acid, 2-diacetylamino-1-methyl-1-propenyl ester
6	343	beta.-D-Glucopyranose, 1-thio-, 1-(N-hydroxy benzene propanimida
7	296	5-Heptenoic acid, 6-methyl-4-((4-methylphenyl) sulfonyl)

8	227	1,4-Dioxaspiro(4.7) dodecane, 6,10-epoxy-8-formamido-
9	156	Tricyclo(8.2.0.0(2,5))dodeca-3,6,8,11-tetraene

At 20.428 min aliphatic, aromatic amines heterocyclic compounds are present along with phthalates (Table 9, figure 4). These compounds are reported by earlier workers

Table 9: GC- MS List of compounds at 20.428 min

S. No.	Molecular Weight	Name of the compound
1	222	Diethyl Phthalate
2	278	Di-N-butyl phthalate
3	264	Phthalic acid, ethyl pentyl ester
4	194	Phthalic acid, monoethyl ester
5	238	2-((2-Ethoxyethoxy)carbonyl)benzoic acid
6	206	2-(Allyloxycarbonyl)benzoic acid
7	372	Phthalic acid, ethyl tridec-2-yn-1-yl ester
8	264	Phthalic acid, ethyl 2-methylbutyl ester
9	178	Benzoic acid, 2-(1-oxopropyl)-

Diethyl phthalate, butyl phthalate, phthalic acid mono ester, phthalic acid diester, acetyl benzoic acid were the major compounds detected in 20.44 minutes of GC-MS spectrum. Diethyl phthalate are considered as a good antibacterial and anti-insecticidal agent (Table 9, figure 4) [18].

Table 10: GC- MS Chemical constituents identified at 20.44 min

S. No.	Molecular Weight	Name of the compound
1	222	Diethyl Phthalate
2	278	Di-N-butyl phthalate
3	264	Phthalic acid, ethyl pentyl ester
4	194	Phthalic acid, monoethyl ester
5	330	Phthalic acid, di-(1-hexen-5-yl) ester
6	250	1,2-Benzenedicarboxylic acid, dipropyl ester
7	164	2-Acetylbenzoic acid
8	222	2-(sec-Butoxycarbonyl)benzoic acid

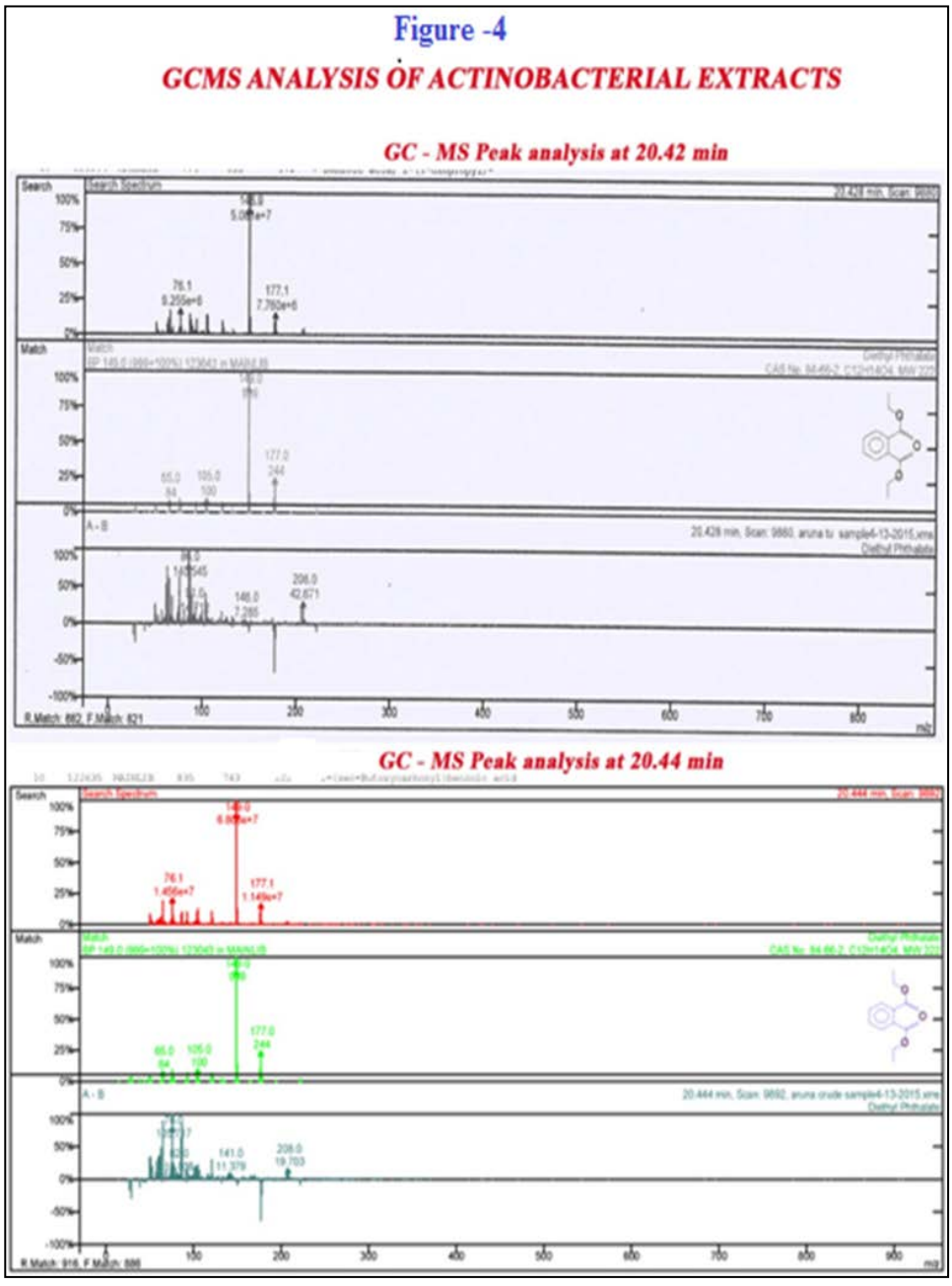

Fig 6

Table 11: The GC-MS analysis – Predicted chemical compounds

S. No.	Molecular Weight	Name of the compound
1	4.018	Fatty acid ester ketone 3,6-dimethyl octon-2-0ne
2	4.243	Molybdenum complex, cyclic trienes and spiro compounds. Diols, esters.
3	11.454	2-octen-1-01esters,o-decyl hydroxyl amine
4	12.621	Iodo compounds, sulphurous acid ester, chloro comounds-2,4-dichloro henol ethyl amine
5	20.444	Phthalates esters substituted benzoic acid

Overall GC-MS analysis revealed the presence of molybdenum complex, spiro compounds, iodo compounds, chlorocompounds, Diethyl phthalate, butyl phthalate, phthalic acid mono ester, phthalic acid diester, acetyl benzoic acid were the major compounds present in fermented extracts of *Streptomycis MW09-1 (RW2-3)* (Table 11) [19].

On GC-MS analysis of the crude extract obtained from *Streptomyces MW09-1 (RW2-3)* fermentation, various compounds were detected. It was found to be fatty acid ester ketone 3, 6-dimethyl octon-2-0ne with the retention time 4.018, Molybdenum complex (4.243), 2-octen-1-01esters, o-decyl hydroxyl amine (11.454), Iodo compounds (12.621) with the retention time of 15.635, 21.606, 16.608. The GCMS spectrum of isolate shows Phthalates esters substituted benzoic acid (20.444), Mono ethyl hexyl phthalate (20.33%) at 15.635, and 21.606 retention times. Amino compounds, heterocyclic compounds, steroids, substituted napthyl amine sulphur compounds; substitute form amide, Among 13 actinobacterial isolates, and MW09-1, (RW2-3) strain inhibited all uropathogenic *E. coli* effectively with zone of inhibition ranges from 8.06±2.08-16.6±2.08mm [20]. Based on secondary screening, MW09-1 (RW2-3) strain was selected and subjected for further antimicrobial studies. Tones phenol chloro sulphuric acid at retention time 17.121. It was also contains 1, 2-Benzenedicarboxylic acid, bis (2-methyl propyl) esters (12.7%) and Isooctyl phthalate (15.29%) with the retention time 15.642, 21.612. The isolate SU 2 contain Di isobutyl phthalate (31.84), Mono ethyl hexyl phthalate (20.33), Di butyl phthalate (5.51) with the retention time of 15.635, 21.606, 16.608. The GCMS spectrum of isolate SU 4 shows Di isobutyl phthalate (31.84%), Mono ethyl hexyl phthalate (20.33%) at 15.635, and 21.606 retention times. The crude extract of isolate SU 13 showed Di isobutyl phthalate (13.26), 1, 2- Benze nedicarboxylic acid, bis (2-ethylhexyl) ester. (14.13%) at retention time 15.643, 21.614 [20, 21].

New and novel chemicals detected from the culture filtrate of *Streptomyces MW09-1 (RW2-3).* It is interesting to indicate that a molybdenum complex of molecular weight 532 and several organic diester toluene of identical mol.wt of 92. The identification of the molybdenum complex throws light on the ability of microbes to synthesise inorganic complex from the metallic compounds in the sea water. This result thus opens a new path for the synthesis of chemical complexes in the chemistry laboratory by the use of actinobacteria which can be prepared in the broth in a biochemical laboratory for the large scale antibiotic production.

The major component obtained from the *Streptomyces MW09-1 (RW2-3)* culture filtrate was found to be phthalate. It could be reported as Dibutyl phthalate and diisobutyl phthalate. They were active against partially four selected immortal cell lines [22]. Similar compounds were also noted in plant extracts [18]. In a similar study, the natural occurrence of 1, 2-Benzenedicarboxylic acid bis (2-ethylhexyl) phthalate has been isolated from a marine alga, *Sargassum weightii*. It is a plasticizing agent. It was also found to have antibacterial effect on a number of bacteria [17]. Bis (ethyl hexyl) phthalate reported from *Streptomyces bangladeshiensis* show antimicrobial activity against gram positive bacteria and some pathogenic fungi [23]. Adipogenesis and glyceroneogenesis activity of Monoethylhexyl Phthalate in human adipocytes was reported by others also [24]. mono- (2-ethylhexyl) phthalate affects the differentiation of human liposarcoma cells (sw 872) [25]. Diisooctyl phthalate isolated from Nigella glandulifera Freyn. They have the ability to inhibiting melanogenesis [15].

Conclusion

The findings of the present study emphasize the role of aquatic actinobacteria in the production of novel antimicrobial compounds. Overall GC-MS analysis revealed the presence of molybdenum complex, spiro compounds, iodo compounds, chloro compounds, Diethyl phthalate, butyl phthalate, phthalic acid mono ester, phthalic acid diester, acetyl benzoic acid were the major compounds present in fermented extracts of *Streptomyces MW09-1 (RW2-3).* Chromium based compounds and phthalates were responsible for antimicrobial activity. To implement the illustrations of this study for the benefit of human beings, it is necessary to carryout clinical trials in future.

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