ORIGINAL ARTICLE

Bioactivity-Guided Extraction and Identification of Antibacterial Compound from Marine Actinomycetes Strains Isolated from Costal Soil Samples of Rameswaram and Dhanushkodi, Tamil Nadu, India

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Abstract

Aim: To isolate and identify marine actinomycetes with potential antibacterial activity. **Materials and Methods:** Marine actinomycetes were isolated from soil samples collected at the Rameswaram and Dhanushkodi coast of Tamil Nadu, India. The isolated strains were screened for antibacterial activity by cross-streaking method against *Escherichia coli* and *Bacillus cereus*. Actinomycetes isolates demonstrated potential antibacterial activity were mass cultured and secondary metabolites were extracted using chloroform. The crude extract prepared was analyzed for antibacterial activity by well diffusion method (100 µg/0.1 ml) and also subjected to chemical characterization using gas chromatography-mass spectrometry (GC-MS) JEOL (GCMATE II GC-MS, Agilent Technologies 6890N Network GC system for GC). **Results:** Among the five different isolation media used, composed Starch Casein Agar (composed) proved to be most effective for isolation of actinomycetes from Rameswaram and Dhanushkodi coastal soil samples. Among the 100 isolated strains, 10 isolates (LGK001 to LGK010) demonstrated a significant antibacterial activity with the zone of inhibition ranging from 8 to 12 mm against *Escherichia coli* and *B. cereus*. Based on the GC-MS analysis, all 10 isolates produced either gancidin-W (and/or) cyclo-pro-phe as major secondary metabolites in their crude extracts. Both these molecules are previously reported antibiotic compounds. **Conclusion:** The results of this study suggest that Rameswaram and Dhanushkodi coastal soil samples are the rich sources for antibacterial bioactive compounds (gancidin-W and cyclo-pro-phe) producing actinomycetes.

Key words: Antibacterial activity, cyclo (-phe--pro), gancidin-W, gas chromatography-mass spectrometry, marine actinomycetes

INTRODUCTION

Gulf of Mannar is the largest bioreserve in India, covering an area of 10,500 km^2 containing 21 islands of varying area and it is unique in several aspects.[1] Rameswaram is located on top of Indian Peninsula and is well known for its largest coral reef and rich microbial flora. Isolation of potential actinomycetes from Rameswaram was reported by several researchers. Many novel microbial strains isolated from Rameswaram coastal soil was explored many biological applications in the recent past. Radhakrishnan *et al*., 2010, have isolated marine actinomycetes from less explored ecosystems in Tamil Nadu, including Rameswaram and have studied their antibacterial

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and antimycobacterial activity. Many of the isolated strains showed good antibacterial and antimycobacterial activity.[2] Radhakrishnan *et al*., 2011, have isolated marine actinomycetes from the Tamil Nadu coastal line, including Rameswaram and have studied their antituberculosis activity. 41 isolates demonstrated a significant antituberculosis activity against tested *Mycobacterium tuberculosis* strains.[3] Kumar *et al*., 2013, have reported the isolation of marine *Streptomyces* sp. NMR2 from Rameswaram. Ethyl acetate extract of this *Streptomyces* sp. NMR2 isolate demonstrated a significant antifungal activity against *Candida albicans*, *Drechslera oryzae*, *Colletotrichum gloeoporioide,* and *Curvularia lunata.*[4] Thirumalairaj *et al*., 2014, have isolated marine actinomycetes from various regions of Tamil Nadu, including Rameswaram and have identified a potential isolate, designated as MSU5, that demonstrated a significant antileptospiral activity against *Leptospira serovar* Autumnalis.[5] Wahaab *et al*., 2015, have isolated marine actinomycetes from Rameswaram and have identified the strain RAM24C2 with the highest antibacterial activity against ocular pathogen *Pseudomonas aeruginosa.*[6] Rameswaram is one of the major hotspots for exploration of novel actinomycetes species and extraction of bioactive secondary metabolites.

In this study, an attempt was made to identify the alkaloid secondary metabolites produced by marine actinomycetes isolated from Rameswaram soil samples. Alkaloids are nitrogen-containing heterocyclic compounds, with strong physiological activity. Alkaloids are used as a drug for various applications and are well known for its high bioactivity. Many bioactive alkaloids have been identified from marine actinomycetes. Maloney *et al*., 2009, have reported an alkaloid lodopyridone produced by marine actinomycetes *Saccharomonospora* sp.[7] Jiao *et al*., 2013, have reported an alkaloid xinghaiamine A produced by marine actinomycetes *Streptomyces xinghaiensis* NRRL B24674(T). This compound exhibited significant antibacterial and also antitumor activity.[8] Kadarin *et al*., 2013, have identified a novel aporphine alkaloid SSV, produced by *Streptomyces* sp. KS1908. It demonstrated significant antitumor and antibacterial activity.[9] Abdelfattah, 2013, has reported a novel antitumor alkaloid maroxazinone along with several known molecules, produced by marine *Streptomyces* sp., e.g., 25.[10] In the present study, we report the isolation of two alkaloid molecules from actinomycetes isolates and their antibacterial activity against bacterial pathogens.

MATERIALS AND METHODS

Isolation of actinomycetes from marine soil sample

Marine soil samples were collected from Rameswaram and Dhanushkodi coastal line (10 cm below surface) in a sterile polythene bag and transferred to the laboratory in a thermal container. The samples were pretreated in hot air oven at 70°C for 15 min. After which, 1 g of soil is serially diluted up to 10^{-4} dilution and was plated on to the following isolation agar using spread plate technique:[11,12] Starch Casein Agar (SCA) - HiMedia, actinomycetes isolation agar - HiMedia, SCA - composed media (g/L: Starch: 10 g, casein: 0.3 g, KNO3: 2 g, NaCl: 2 g, K2HPO4: 2 g, MgSO4.7H2O: 0.05 g, CaCO3: 0.02 g, FeSO4.7H2O: 0.01 g, and agar: 18 g).[13] Humic acid vitamin agar - Composed media (Humic acid: 1 g, KCl: 1.7 g, Na2HPO4: 0.5 g, MgSO4: 0.5 g, CaCO3: 0.02 g, FeSO4: 0.01 g, VB stock solution: 1 ml, agar: 10 g, distilled water: 1000 mL, pH: 7.2 [VB stock solution: 50 mg VB1, VB2, niacin, VB6, D-calcium pantothenate, inositol, and PABA (para amino acid), 25 mg biotin, and 100 ml distilled water])[14] and soil extracts agar - Composed media (peptone: 5 g, beef extract: 3 g, soil extracts: 1000 mL, agar: 10 g, and pH: 7.2).[14]

Cross-streaking method

The pure actinomycetes isolates were streaked on nutrient agar (NA) - Modified (50% NA and 50% SCA) as a straight line across the Petri plates and incubated at room temperature for 5 days. On the 6th day, the test pathogens (*E. coli* - MTCC1687 and *Bacillus cereus* - MTCC430) were streaked across the Petri plate at right angle to the actinomycetes streak. The actinomycetes isolates that suppressed the growth of the test pathogens at their proximity were further tested for their antibacterial activity using well diffusion method.[15]

Secondary metabolites crude extraction

International *Streptomyces* Project No. 1 (ISP No.1) broth (800 ml) was inoculated with the selected isolates and incubated at 37°C for 7 days in a rotary shaker at 110 rpm. After incubation, the broth was filtered using Whatman No.1 filter paper and the filtrate was mixed with equal volume of chloroform (1:1). The mixture was shaken vigorously for 1 h. The organic phase was separated using separation flask. The chloroform is then evaporated to dryness using rotary-vacuum evaporator.[16]

Assay of antibacterial activity

Fresh microbial lawn cultures of the test pathogens (*E. coli* and *B. cereus*) were prepared on Mueller-Hinton agar medium. Wells were made on the agar medium using sterile cork borer. The crude extract prepared from actinomycetes secondary metabolites was diluted to a final concentration of 100 µg/100 µl in dimethyl sulfoxide. 100 µl of the final dilution of this crude extract was added to each well. The plates were incubated at 37°C overnight, and the zone of inhibition was measured.[17,18]

Gas chromatography-mass spectrometry (GC-MS) analysis of crude extracts

The crude extracts prepared from marine actinomycete isolates were analyzed in GC-MS JEOL (GCMATE II GC-MS, Agilent Technologies 6890N Network GC system for GC). The column (HP5) was fused silica 50 m × 0.25 mm I.D. Analysis conditions were 20 min at 100°C, 3 min at 235°C for column temperature, 240°C for injector temperature, Helium was the carrier gas, and split ratio was 5:4. The sample (1 µl) was evaporated in a splitless injector at 300°C. Run time was 30 min. The compounds were identified by GC coupled with MS. The molecular weight and structure of the compounds were ascertained by matching with reference compounds available in the National Institute Standard and Technology (NIST).[19]

RESULTS

Isolation of actinomycetes

A total of 100 actinomycetes colonies were isolated from marine soil samples. Among the five different growth media used, SCA composed media was found to the excellent for the growth of actinomycetes colonies [Table 1]. SCA composed media yielded 88 ± 5 actinomycetes colonies at 10^{-1} dilution [Table 2]. The growth of actinomycetes colonies and other microbial strains in three different media is shown in Figure 1. From the obtained results, it is evident that SCA composed media is best suitable media for isolation of actinomycetes colonies from marine soil samples of Rameswaram and Dhanushkodi. Some of the morphologically different actinomycetes strains obtained from the Gulf-of-Mannar marine soil sample are shown in Figure 2.

Screening for potential isolates

Among the 100 actinomycetes isolates, 10 isolates demonstrated antibacterial activity against *B. cereus* and *E. coli*. These 10 isolates were designated as LGK001 to LGK010. The antibacterial activity of 10 isolates was confirmed by primary screening (cross-streaking method) followed by secondary screening (well diffusion method). The crude extract (100 µg) showed a moderate zone of inhibition against the bacterial pathogens tested. The zone of inhibition exhibited byLGK001 and LGK002 against *B. cereus* is shown in Figure 3. Table 3 summarizes the zone of inhibition showed by crude extracts (100 µg) prepared from LGK001 - LGK010 against *B. cereus* and *E. coli*.

GC-MS analysis of crude extracts

GC-MS analysis of the crude extracts demonstrated that all the 10 isolates had a major peak at 22 min and/or 18 min retention time (RT). The GC-MS chromatograms of crude extract LGK001 to LGK010 are shown in Figure 4. Presence and absence of the major peaks at 22 min and/or 18 min in the 10 crude extracts is summarized in Table 4. LGK001 and LGK002 contain major peak at 18 min, whereas LGK003 - LGK010 contains major peaks both at 18 and 22 min. NIST library matching of all the peaks in chromatogram revealed that more than 50% of the entire crude extracts consist of pyrollo compounds. The majority of the peaks had more than 85% spectrum similarity (reverse spectrum and forward spectrum) to either pyrrolo[1,2-A] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) and/or pyrrolo[1,2-A]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl). The molecular weight of compounds so had an exact match with these molecules.

Table 1: Suitable growth media for actinomycetes from Rameswaram marine soil samples

Media	Growth
AIA (Hi-Media)	Good
SCA (Hi-Media)	Good
SCA (Composed)	Excellent
Soil leach extract agar (Composed)	Very poor
HVA (Composed)	Very poor

AIA: Actinomycetes isolation agar, SCA: Starch Casein Agar, HVA: Humic acid vitamin agar

Table 2: Colony forming units of actinomycetes on different media

Sample dilution	SCA (Composed)	SCA (Hi-Media)	AIA (Hi-Media)
10^{-1}	88±5	Overgrowth	Overgrowth
10^{-2}	40±3	28±5	32±6
10^{-3}	17±1	12±3	15±3
10^{-4}	4±1	2±1	1±1

AIA: Actinomycetes isolation agar, SCA: Starch Casein Agar

Table 3: Antibacterial activity of crude extracts prepared from 10 actinomycetes isolates

Isolate	*E. coli*	*B. cereus*
LGK001	12 mm	11 mm
LGK002	10 mm	10 mm
LGK003	11 mm	13 mm
LGK004	8 mm	9 mm
LGK005	10 mm	10 mm
LGK006	-	8 mm
LGK007	10 mm	12 mm
LGK008	-	9 mm
LGK009	8 mm	10 mm
LGK010	9 mm	10mm

E. coli: *Escherichia coli*, *B. cereus*: *Bacillus cereus*

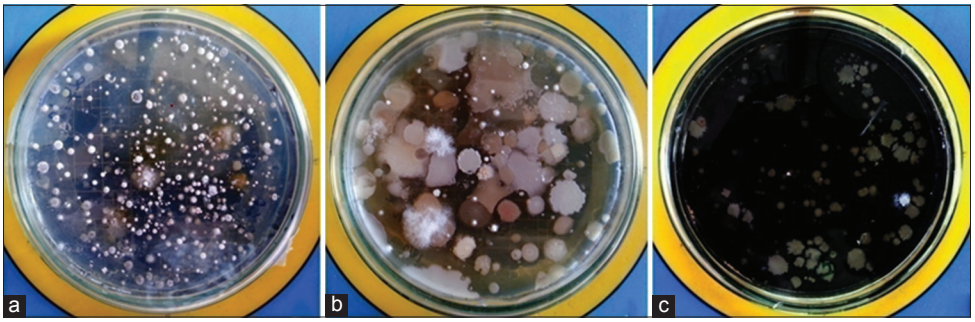

Figure 1: Actinomycetes colonies cultured on different media, (a) Composed Starch Casein Agar, (b) Hi-Media actinomycetes isolation agar, (c) Composed Humic acid vitamin agar

Figure 2: Morphologically different isolates obtained from Gulf of Mannar marine soil samples

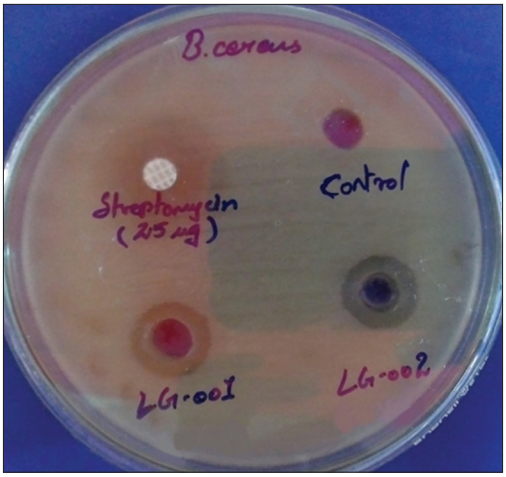

Figure 3: Antibacterial activity of actinomycetes isolates LGK001 and LGK002 against bacterial pathogen *Bacillus cereus*

The major compound-1 at the RT 18 min is commonly known as gancidin-W, also with the chemical names 3-(2-methylpropyl)-octahydropyrrolo[1,2-a]piperazine-1,4-dione and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl). The structure of this major compound-1 is represented in Figure 5. The major compound-2 at the RT 22 min is commonly known as cyclo(pro-phe) and also with the chemical names 3-benzyl-octahydropyrrolo[1,2-a]piperazine-1,4-dione and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl). The structure of this major compound-2 is represented in Figure 6.

Table 4: Actinomycetes crude extracts with major peaks at RT of 18.1 and 22.1 min

Actinomycetes isolates	RT 18.1 min	RT 22.1 min
LGK001	✓	--
LGK002	✓	--
LGK003	✓	✓
LGK004	✓	✓
LGK005	✓	✓
LGK006	✓	✓
LGK007	✓	✓
LGK008	✓	✓
LGK009	✓	✓
LGK010	✓	✓

RT: Retention time

DISCUSSION

The key objective of this study was to identify alkaloid secondary metabolites produced by marine actinomycetes isolated from Rameswaram coastal soil sample. Among the 100 isolated strains, 10 isolates showed moderate antibacterial activity and mass spectra analysis of chloroform extract revealed the presence of pyrrole compounds, as the major compound in all isolates. The spectral similarity of more than 85% (reverse and forward spectrum) toward gancidin-W (18 min RT) and for cyclo(-phe-pro) (22 min RT). The molecular weight of gancidin-W and cyclo(-phe-pro) was matched with the reference compounds available in the NIST database. Even though all isolates are morphologically different, it is evident from the results that the antibacterial activity exhibited by all 10 isolates against the tested bacterial pathogens may be due to the presence of gancidin-W and cyclo(-phe-pro) compounds.

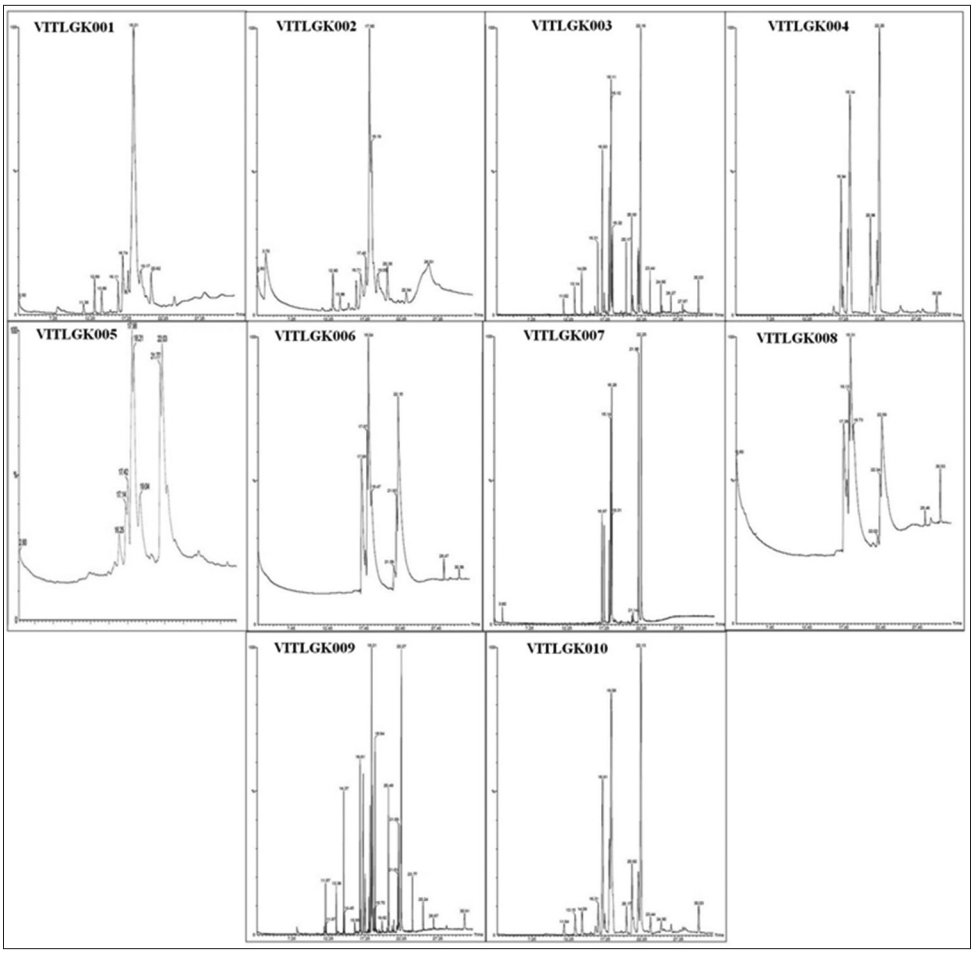

Figure 4: Gas chromatography-mass spectrometry, spectrum of crude extracts prepared from actinomycetes isolates LGK001-LGK010

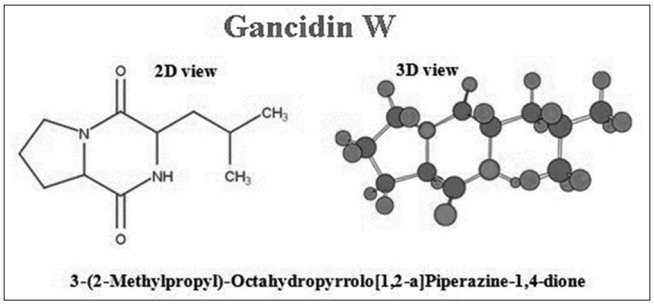

Figure 5: The structure of the major compound gancidin-W present in crude extracts of actinomycetes isolates LGK001-LGK010

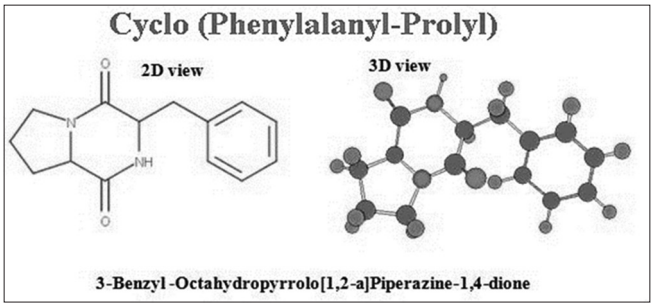

Figure 6: The structure of the major compound cyclo (phenylalanyl-prolyl) present in crude extracts of actinomycetes isolates LGK001-LGK010

Gancidin-W is a well-known antibiotic produced by *Streptomyces* sp., No. AAK-84.[20] Gancidin-W also extracted from *Corollospora pulchella* was reported to possess antibacterial activity.[21] They referred this compound as cyclodipeptide molecule containing leucine and proline moieties.[20]

Wang *et al*.[22] reported the production of antibacterial secondary metabolite cyclo(-pro-phe) by marine bacteria *Bacillus subtilis*. Extraction of cyclo(-pro-phe) from natural source was first reported by Wang *et al*.[22] The compound exhibited strong antibacterial activity against *B. subtilis* (terrestrial), *Staphylococcus aureus*, and *E. coli*. The authors referred this compound as diketopiperazines and also it is a dipeptide molecule consisting of proline and phenylalanine.[22]

From the results of previous reports, it is evident that the antibacterial activity demonstrated by all the 10 marine actinomycetes isolates obtained from Rameswaram coastal soil is due to their ability to produce antibacterial compounds gancidin-W and cyclo(-pro-phe). Moreover, gancidin-W and

cyclo(-pro-phe) are cyclodipeptide molecules formed by fusion two aminoacids.

CONCLUSION

Based on the results, it could be concluded that Rameswaram and Dhanushkodi costal soil samples are the rich sources for isolation of bioactive actinomycetes capable of producing antibacterial compounds (gancidin-W and cyclo-pro-phe).

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