Extraction of Secondary metabolites from isolatedmarine *streptomyces sp*and their antibacterial activity against *Methicillin*-Resistant *Staphylococcus aureus*

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Abstract

The dominance of MRSA has been linked to infectious diseases that has increased morbidities and mortality in patients. The goal of the present study was assessing the isolation and identification of marine actinomycetes were screened clinical bacterial pathogens.In this study the potential strain KA8 were isolate from Keelkatalai soil sample. The isolated marine actinomycetes were screened for their antibacterial activity by using the Cross-streak method against MRSA, *Escherichia coli, Streptococcus pyogenes* and *Streptococcus pneumoniae.*In light microscopy, KA8 strain results showed gram-positive, filamentous rod shape and short chains of spores. The KA8 strain identified as *Streptomyces sp*by genus level identification. Finally the ethyl acetate extract showed the maximum zone of inhibition observed in MRSA (28±1.00) at 100µl concentration.The scavenging activity shows the 25.92µg/g at 100 (µg/ml) concentration.The major compounds are Monoethylmalonate monoamide,Propanal, 2,3-dihydroxy ether,dl-Aspartic acid,N-Hydroxymethylacetamide and N,1-Dimethylhexylamine were found in the ethyl acetate extract of KA8 strain detected by GC-MS analysis. Hence this identified compound will be screened for drug synthesis after purified compound.

Keywords: Marine actinomycetes, Monoethylmalonate monoamide, Drug synthesis and GC-MS.

INTRODUCTION

The gram positive MDR bacterial pathogen *staphylococcus aureus* is one of the most prevalent spreading bacteria. The trending concern is the possible development of multi-resistant bacteria to conventional antibiotics by this organism. This makes treatment of *S. aureus* caused diseases such as septicaemia, pneumonia, wound sepsis, septic arthritis and post-surgical toxic shock syndrome. WHO recognized each year infections are caused by MDR bacteria cause high mortality. Thus, bioactive compounds used for treat the MDR pathogens *staphylococcus aureus, Escherichia coli,streptococcus pyogenes* and *streptococcus pneumoniae.*[1]

Methicillin Resistant *Staphylococcus aureus* (MRSA) are strains of *Staphylococcus aureus* that are not susceptible to the methicillin and related beta-lactam antibiotics. The dominance of MRSA has been linked to infectious diseases that has increased morbidities and mortality in patients. The dominance of MRSA has been linked to infectious diseases that has increased morbidities and mortality in patients. Failed attempts in the use of most antibiotics have initiated the search of suitable drugs that are effective, low cost, and with lesser side effect[2]. To treat numerous infectious diseases caused by MDR pathogenic microbes, new medicines or antibiotics are urgently needed to fight pathogens, it is necessary to develop drug synthesize[3].

S. aureus strains resistant to the Beta-lactam antibiotics such as methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin are referred to Methicillin resistant *Staphylococcus aureus* (MRSA). The hospital, Community and the Environment can be potential reservoir for MRSA.

Marine habitats are one of the important sources for secondary metabolites and the production of medicinal products[4].Marine sediments are the major sources of marine actinomycetes population. Actinomycetes are free-living microorganisms commonly found in soil, sediments and marine water. Actinomycetes are filamentous gram-positive bacteria that come under the phylum of Actinomycetales well defined by aerial mycelium.They are produced lot of new bioactive molecules. In world, 80% of antibiotics derived from actinomycetes particularly marine actinomycetes [5]. The prolonged usage of chemical-based antibiotics having side effects compare then natural derived bioactive compounds from actinomycetes. The development of novel drugs is necessity for treating variety of bacterial diseases [6].

MATERIALS AND METHODS

Sample Collection

For this study, the soil samples were collected from Keelkatalai (12.9564° N, 80.1895° E), Chennai, India. Sample were collected at 10Cm depth in the soil surface and stored in sterile zip- lock cover for further use.

Isolation of marine actinomycetes

The collected soil suspension was shaken vigorously under room temperature (25 ± 2^{o}C) on an orbital shaker at 200 rpm for 1hr and serially diluted (10^{-2}, 10^{-3} and 10^{-4}) 0.1 ml of each soil suspension were pipettedand plated in Starch Casein Agar media using spread plate techniques and incubated at 28 °C. The plates were incubated at 30^{o}C for 1 - 2 weeks and Colony forming unit (CFU) was determined. The emerging actinomycetes were picked for further subculturing and pure cultures were also maintained on slants (4 °C) using glycerol stocks (-20 °C) for further analysis.

Antagonistic activity

The isolated marine actinomycetes were screened for their antibacterial activity by using the Cross-streak method against MRSA, *Escherichia coli, Streptococcus pyogenes* and *Streptococcus pneumoniae*. The actinomycetes were streaked (4-6 mm in diameter) on the centre region of the Starch Casein Agar plate and incubated for 7 days. After the incubation, the bacterial pathogens were streaked on the sides of the plates and incubatedat 37ºC for 24 h. Based on the presence or absence of an inhibition zone indicates antibacterial activity. The potential halophilic actinomycetes were taken for further study[7].

Secondary screening of supernatant

The potential actinomycetes strain was inoculated into Starch casein broth and incubated at 28°C in a shaker at (120 rpm) for 7 days. After incubation, the broths were filtered through Whatman No. 1 filter paper. Then the filtrates were centrifuged separately at 10000 rpm for 10 min to extract the supernatant. It was transferred aseptically into screw-capped bottles and stored at 4°C for further assay. The activity of cell-free supernatant was assessed against pathogenic bacteriaby well diffusion method.

Identification of actinomycetes

The identification by morphological, physiological and biochemical analysis of the isolate was performed according to the guidelines described in Bergey's Manual of Systemic Bacteriology. Cultures were identified by their characteristic appearance on the media, Gram's staining reaction, by the pattern of biochemical profiles using standard procedures. Biochemical tests such as IMViC tests, TSI, Urease test, Nitrate reduction test, Oxidase test, Catalase tests, and carbohydrate tests [8].

Extraction of secondary metabolites

The most prominent secondary metabolites production strains were scaled up for extraction. Each culture was inoculated into 25ml culture SCA broth at optimum salt concentration and grown in rotary shaker incubator at 140rpm and 28°C for 7 days. This media was scaled upto to 1-2 liters with inoculum and incubated for 2 weeks. After incubation the fermented culture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected. Organic solvent extraction was carried out with organic solvent ethyl acetate in a volume ratio of 1:1 (culture supernatant: organic solvent). The organic layer was evaporated to dryness using a rotary vacuum evaporator (EQUITRON® Roteva) at 45°C and the resultant residue was then dissolved in 1 ml of the respective organic solvent and then checked for its bioactivity against pathogens [9].

Antibacterial activity

Crude extracts were tested against bacterial pathogens. For this, bacterial pathogens were inoculated in nutrient broth and incubated for 12 h before antibacterial assay.All the bacterial strains were individually spread on the Muller Hinton agar plates. Wells were made in the plates at 6mm by using cork borer.The different concentrations of crude extracts addedto the wells and incubated for 24 hours.Assay was carried out in triplicates. The zone of inhibition was measured in mm after the completion of the incubation period[10]

Antioxidant activity

The antioxidant activity of crude ethyl acetate extract was determined by DPPH scavenging assay. Various concentration (0.1, 0.5, 1.0 & 5.0 mg/ml) of crude extract were taken in separate tubes. α-tocophenol was used as reference compound (0.2, 0.4, 0.8.1.0 & 5.0 mg/ml). A freshly prepared solution of 0.002% DPPH (1, 1, diphenyl-2-picryl hydrazyl) was added in freshly prepared in methanol. DPPH (2 ml) was added to each tube containing different concentrations of extract (2 ml) and of standard solution (2 ml). The samples were incubated in dark place at 37 °c for 20 min and read at 515 nm containing 100 µl of methanol in the DPPH solution was prepared as blank sample and its absorbance was measured (AB) all the experiment was carried out in triplicate. The data were expressed as the percent decrease in the absorbance compared to the control. Percentage inhibition of radical scavenging activity was calculated using formula

$$AA\% = \frac{(\text{Absorbance of control}) - (\text{absorbance of sample})}{\text{Absorbance of control}} \times 100$$

Characterization of crude extract

Thin layer chromatography

TLC done to determine the composition of the crude extract. Optimization of mobile phase - ethyl acetate: hexane in various ratios (0.1:4.9, 0.3:4.7, 0.5:4.5) by using 5cm TLC sheet solvent system was prepared and kept for saturation (7 minutes). Simultaneously, on the TLC sheet, condensed extract to be placed and dried in hot air oven for 10 – 30 seconds.

Then the TLC sheet kept in solvent system and left to run till 3/4th of the sheet without disturbing the set-up. Later remove the TLC sheet to air dry and observe under UV illuminator for various bands spots on the plates were visualized in an iodine chamber[11].

GC-MS Studies

GC-MS analysis of crude extract was carried out on Shimadzu 2010 plus comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column RTX 5Ms (Column diameter is 0.32mm, column length is 30m, column thickness 0.50µm), operating in electron impact mode at 70eV; Helium gas (99%) was used as carrier gas at a constant flow of 1.73 ml /min and an injection volume of 0.5 µI was employed (split ratio of 10:1) injector temperature 200ºC. Total GC running time is 51.25 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Turbo Mass Ver 5.2.0. Interpretation on GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained[12].

RESULTS AND DISCUSSION

Isolation of marine actinomycetes

Marine actinomycetes lead the important role for novel secondary metabolites. The numerous new actinomycetes isolates from marine soil sample from all over Indian coastal regions. Many researchers proved that marine actinomycetes metabolites has more pharmacological properties.The strain streptomyces VITMK1 produced the potential antibacterial compound. A total of 235 marine actinomycetes were isolated from soil samples in Philippines University based on microbiological characterization.[13] A total of 10 actinomycetes were isolated from Keelkatalai marine region located in Chennai, tamilnadu. The isolated colonies were purified and used for further analysis. The strains names are encoded with sample code (KA-1 to KA10). The sample code KA refer Keelalatalai actinomycetes

Antagonistic activity

Hamed evaluated 16 actinomycetes were screened for antagonistic activity by cross-streaking method. Among the 16 isolates, the *streptomyces sp* exhibited antibacterial activity against *Aeromonas hydrophila, Vibrio damsel*, *Staphylococcus aureus* and *Salmonella typhi* [14].In this research totally ten actinomycetes strains were screened for preliminary antibacterial activity by using cross- streak methodagainst MRSA, *Escherichia coli*, *Streptococcus pneumoniae* and *streptococcus pyogenes*. In this primary screening KA1, KA2, KA8, KA9 and KA10 showed maximum linear inhibition against all the bacterial pathogens.

Table.1. Antagonistic properties of marine actinomycetes

S.No	Isolates	MRSA	*E.coli*	*S. pneumoniae*	*S. pyogenes*
1	KA1	+	++	++	+
2	KA2	+	++	++	++
3	KA3	+	-	-	+
4	KA4	-	-	+	+
5	KA5	-	-	-	-
6	KA6	+	-	-	-
7	KA7	+	+	-	-

8	KA8	++	+	++	+
9	KA9	+	++	+	++
10	KA10	+	++	+	+

-: No inhibition, +: moderate inhibition, ++: high inhibition

Secondary Screening

Streptomyces sp strains cell free supernatant was checked for their antibacterial activity against selected bacterial pathogens [15]. The potential strains KA1, KA2, KA8, KA9 and KA10 supernatants screened for antibacterial activity. The antibacterial activity results noted the strain KA8 only showed highest zone of inhibition against all the bacterial pathogens.The strain KA8 showed maximum zone of inhibition observed in MRSA (20±0.58) at 100µl concentration.

Table. 2. Secondary screening of cell-free supernatant of strains against bacterial pathogens

S. No	Isolates	Zone of Inhibition (mm)			
		MRSA	***E.coli***	***S. pneumoniae***	***S. pyogenes***
1	KA1	10±0.58	14±1.00	12±1.00	16±1.00
2	KA2	15±0.58	14±1.00	13±1.00	12±1.00
3	KA8	**20±0.58**	10±1.00	9±1.53	16±1.00
4	KA9	11±1.00	11±1.00	12±1.00	15±1.53
5	KA10	8±0.58	16±1.00	10±0.58	9±1.53

Value represents mean ±SD;n=3,- No zone

Identification of potential strain KA8

The identification of potential actinomycetes were done by gram-staining, morphological characteristics, microscopical characteristics and biochemical tests for genus level. Molecular profiling of potential strain determined by 16S rRNA gene sequencing method. In 16S rRNA gene sequences are terminated to removal of low level of DNA.Based on the primary and secondary screening of antibacterial activity results, KA8 strainwere selected for physiological, cultural and Biochemical tests performed and tabulated. In light microscopy, KA8 strain results showed gram-positive, filamentous rod shape and short chains of spores.

Fig.1. Pure culture of KA8 strain

Table.3. Genus level identification of KA8 strain

S.No	Test	KA8
1	Gram staining	+
2	Motility	-
3	Colony form	Circular
4	Spores arrangement	Short chain of spores
5	Aerial mycelium and colour of the spore	Whitish grey

Table.4. Biochemical Characterization of KA8strain

S.No	Biochemical Test	KA8	Tentative genera
1.	Indole	Positive	*Streptomyces sp*
2	Methyl- Red	positive	*Streptomyces sp*
3	Voges-Proskauer	positive	*Streptomyces sp*
4	Triple Sugar Iron	positive	*Streptomyces sp*
5	Urease	Negative	*Streptomyces sp*
6	Nitrate	positive	*Streptomyces sp*
7	Catalase	positive	*Streptomyces sp*
8	Oxidase	positive	*Streptomyces sp*

Extraction and antibacterial activity of crude extract

Secondary metabolites extracted from *Streptomyces sp*. AS11 in 500 ml of ISP4 supplemented starch casein broth. After the 7th day of incubation, the cell free extract was allowed to extraction with ethyl acetate ratio (1:1) and bioactive compound obtained through evaporating organic layer [15].The potential strain KA8 were subjected to extraction of crude extract by using organic solvent extraction method. The ethyl acetate solvent used for extraction of crude metabolite. The collected crude metabolite screened for antibacterial activity by agar well diffusion method. The maximum zone of inhibition observed in MRSA (28±1.00) at 100µl concentration.

Table.5. Antibacterial activity of ethyl acetate extract from KA8 strain

Bacterial pathogens	Zone of inhibition mm					
	25µl	50 µl	75 µl	100 µl	Positive	Negative
MRSA	18±0.58	22±0.58	25±1.00	**28±1.00**	30±1.00	-
Escherchia coli	17±1.53	19±1.53	20±1.00	26±0.58	25±1.00	-
S. pneumoniae	20±1.00	21±1.00	25±1.00	25±0.58	23±1.15	-
S. pyogenes	10±1.00	12±1.00	18±0.58	23±1.15	22±0.58	-

Value represents mean ±SD;n=3,- No zone

Antioxidant activity

The active crude extract from KA8 strain were checked inhibition of DPPH radical at different concentrations (20- 100 µg/ml) and α-tocophenol used as Standard. The scavenging activity shows the 25.92µg/g at 100 (µg/ml) concentration.

Table.6. DPPH activity assay

s.no	Test Concentration	AA% OF KA8 crude extract	AA% ofα-Tocophenol
1.	Control	0	0
2.	20	96.29	55
3.	40	62.96	50
4.	60	53.70	47
5.	80	46.29	35
6.	100	**25.92**	18

Thin layer chromatography

The Partial purification of secondary metabolites was done by TLC method. Rf value was calculated for KA8 crude, ethyl acetate: hexane (0.5: 4.5) ratio showed the ideal bands on the TLC sheet. Maximum Rf value obtained by UV- long wave length with stock solution of 10µg/ml stock solution of crude KA8 extract.The Retention factor ofproduce maximum value in UV and IODINE is 0.400 (0.5:4.5 ratios).Thin layer chromatography sheets to obtain a single spot.

Table.7. TLC profiling

S.NO	RATIO	Rf VALUE		
		UV-strong	UV-long	Iodine
1.	0.5:4.5	0.100	0.100	0.100
		0.225	0.225	0.225
			0.400	
2.	0.3:4.7	0.150	0.150	0.150
		0.225	0.225	0.225
			0.350	
3.	0.1:4.9	0.100	0.100	0.100

GC-MS analysis

All the compounds were summarized with theirretention time, compound names and biological activities of compounds.The compounds with more area of peak were further focused in isolation studies. The area of a peak is directly proportional to the amount of the compound that is found in a given mixture. Totally 15 compounds found in the ethyl acetate extract from KA8 strain. The major compounds are Monoethylmalonate monoamide,Propanal, 2,3-dihydroxy ether,dl-Aspartic acid,N-Hydroxymethylacetamide and N,1-Dimethylhexylamine were found in the ethyl acetate extract of KA8 strain.

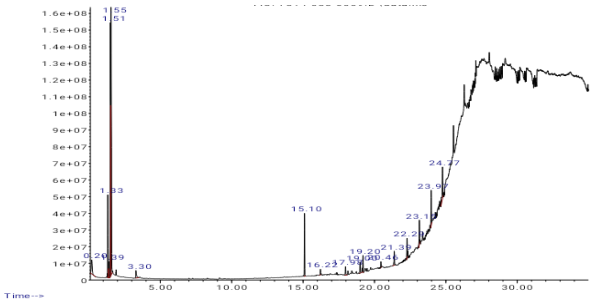

Fig.2. GC-MS spectrum

Table.8. GC-MS profiling of KA8 ethylacetate extract

S.No	Retention time	Name of the compounds	Biological activities
1.	1.55	Monoethylmalonate monoamide	Analgestic activity
2.	1.51	Propanal, 2,3-dihydroxy ether	Antiviral activity & Antiproliferative activity
3.	1.83	dl-Aspartic acid	Antibacterial activity
4.	15.10	N-Hydroxymethylacetamide	Anti-inflammatory
5.	1.33	N,1-Dimethylhexylamine	Anticancer

CONCLUSION

The research study confirmed the marine actinomycetes particularly *streptomyces* sp having great potential biological activities. The secondary metabolites monoethylmalonate monomide,Propanal,2,3-dihydroxy ether, dl-aspartic acid,N-hydroxymethylacetamide and N-1-Dimethylhexylamine showed the better antibacterial activity against Multi drug resistant *staphylococcus aureus* and antioxidant activity. Hence this identified compound will be screened for drug synthesis after purified compound.

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