

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.708.133>

Antimicrobial Activity of the Crude Extract Derived from *Streptomyces* spp. Associated with Sponges

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ABSTRACT

The objective of the present investigation was to isolate and identify the antimicrobial activity showing actinomycetes associated with sponges. Sponges such as *Acanthella klethra*, *Theonella cylendrica*, *Heliconia* sp., *Auletta* sp., and *Callyspongia* sp. were collected at Sangumal, in Rameswaram island, Ramanathapuram District, Tamil Nadu, India. Isolates of 22 actinomycetes were isolated from sponges. Isolates No. 16 showed high antibacterial and antifungal activity and the isolate was identified as *Streptomyces* sp. Antibacterial and antifungal activity was determined by agar diffusion method and Minimum Inhibitory Concentration (MIC) of the extract was determined by two fold dilution method using microstate plate. The study showed that fungal pathogens were more susceptible to the extract than bacteria. Among bacterial pathogens, *Escherichia coli* was more susceptible to the extract, followed by *P. aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* and their growth were inhibited at the concentration of 2 mg/ml. Other bacterial pathogens were resistant to the crude extract derived from culture broth extract of *Streptomyces* sp. Fungal pathogens such as *Candida albicans*, *Zygomonas* sp., *Aspergillus niger* and *A. rutilus* were susceptible to the extract and the MIC for the fungal pathogens ranged between 0.015 mg/ml and 0.025 mg/ml. *A. rutilus* was susceptible to the extract and its growth was inhibited at the concentration less than 0.1 µg/ml. Since the inhibitory concentrations against the fungal pathogens were too less, its purified form may be the potential source for controlling fungal pathogens.

Keywords

Sponges,
Actinomycetes,
Human pathogens,
Crude extract,
Antibacterial
activity, Antifungal
activity, Minimum
inhibitory
concentration

Article Info

Accepted:
08 July 2018
Available Online:
10 August 2018

Introduction

Sponges are sessile invertebrates. Hence they were unable to hide or flee from predators and fouling organisms. Sessile organisms depend on chemical defense mechanisms against predator animals and also against pathogens (Gorajana *et al.*, 2005). They protect themselves by secreting chemical substances as secondary metabolite such as antibiotics,

and antifouling compounds. Sponges are said to be an excellent source for production of variety of substances. Different kinds of microbes are associated with sponges and are species specific (Imhoff, 2004). Associated microbes are responsible for these production of variety of secondary metabolites secreted by the host organisms. Microorganisms associated with host organisms contribute the integrity of their

host and defense by secreting biologically active compound like polypeptides, and alkaloids (Proksch *et al.*, 2002). Actinomycetes are one group of bacteria associated with sponges. They gram positive, filamentous and are potential source for the production of bioactive substances that are medically and industrially important. *Nocardia brasiliensis* produces antibacterial substances against the fish pathogens *Vibrio damsela* (Takahashi and Omura, 2002). *Streptomyces* species are greater source for producing antibiotics, antitumor, immunosuppressive agent and enzymes. *Streptomyces chibaensis* produces quinine related antibiotics, 1-Hydroxy-1-norvesistomycin which acts cytotoxic against the cell line Vi2, HMO2-gastric adenocarcinoma1. Many researchers reported the association of actinomycetes with sponges like Great Barrier Reef sponge *Rhopalocides odorabile* (Webster *et al.*, 2001). Mediterranean sponges, like *Aplysina aerophoba* and *Theonella swinhoehi* (Hentschel *et al.*, 2002). Sponges associated actinomycetes like *Streptomyces* sp., and *Altermonas* sp., produce antibiotic and therapeutically important compounds with diverse biological activities. Actinomycetes associated with the sponge *Hyrtios altum* produce antibacterial compounds. Trisisindolins inhibited the growth of *E. coli* at the concentration of 10µg/disc. *Streptomyces* sp. associated with *Dendrilla nigra*, produce compounds which, inhibited the growth of *Micrococcus leuteus* at the concentration of 44µl/ml. Based on the above information it was understood that sponges and sponges associated actinomycetes are the potential source for the production of bioactive compounds that show different biological activities especially antibacterial and antifungal activity. The purpose of the present investigation is to isolate and identify high antibacterial activity showing actinomycetes associated with marine

sponges and to determine their antibacterial and antifungal activity against human bacterial and fungal pathogens.

Materials and Methods

Sponges were collected from Sangumal at Rameswaram island, Ramanathapuram District, Tamil Nadu state India during May, 2018. Sponges were collected at the depth of 10 meters by the fishermen and identified by experts, using specimen available at Central Marine Fisheries Research Institute, Mandapam, Ramanathapuram district, Tamil Nadu, India. They are identified as *Acanthella klethra*, *Theonella cylindrica*, *Heliconia* sp, *Auletta* sp, and *Callyspongia* sp. by Dr. Gerold Wilson, Assistant Professor of Marine Sciences, Kings Institute.

Samples of sponges were kept in the pre sterilized sampling bottles containing sterile seawater and kept in the ice box. Collected samples were brought to the laboratory and analyzed within 4hr after collection. One gm of sponge was taken from each sponge and rinsed with sterile distilled water thrice and ground with nine ml of phosphate buffer (pH 7.0) and serially diluted. Dilution of each sponge extract was used for the isolation of sponges associated actinomycetes using Starch Casein Agar (pH 7.2). The medium was supplemented with Nalidixic acid 20µg/ml, Nystatin 25µg/ml and Cycloheximide 100µg/ml. 0.1ml of diluted sample was spread over the starch casein agar medium using sterile 'L' rod. The plates were incubated at 28°C for 7 days. To screen the high efficiency antagonistic activity showing actinomycetes, cross streak method was followed, using modified nutrient agar medium (Peptone 0.57%, Glucose 0.5%, Beef extract 0.3%, NaCl 0.5%, pH 7.2 ± 0.2). Antagonistic activity showing actinomycetes were identified based on their morphological, physiological and biochemical tests.

Identified organisms were used to evaluate the antibacterial, antifungal activity and to determine their MIC value for bacterial and fungal pathogens.

Antagonistic activity showing actinomycetes were grown in yeast extract malt extract broth (pH 6.2 ± 0.2). Broth inoculated with actinomycetes was incubated at 28°C for 3-5 days. After incubation period, 20ml cultures were transferred to 1000ml of Glycerol – Asparagines broth (Glycerol 1% Asparagine 0.1%, KHPO 0.1%, NaCl 0.5% and 1ml of each trace element solution of FeSO 0.1% and MgSO7HO, 0.1%) containing. Flask was incubated at room temperature under continuous shaking for 7 days.

After incubation period broth was adjusted to pH 5 with 1N HCl and centrifuged at 8000 rpm for 10 minutes to remove the mycelial mats and further filtered through membrane filter (0.45µm) to get cell free extract. The supernatant was collected and kept in the separatory funnel. Equal volume of ethyl acetate was added to the supernatant and shake well and the ethyl acetate layer was separated which contained bioactive compounds. This process was repeated thrice to completely extract the bioactive compounds present in the culture broth. Ethyl acetate extract was concentrated in a vacuum evaporator at 40°C. As a result a yellowish brown residue was obtained. 20mg of crude extract dissolved in 1 ml of 20% DMSO, which served as a stock solution. This crude extract solution was used further, to determine antibacterial activity. 10µl of the crude extract solutions were added with sterile paper discs (6mm diameter, Himedia Company, Chennai) aseptically and air dried.

Antibacterial activity of the extract by agar diffusion method

Antibacterial activity of the extract derived from culture broth of *Streptomyces* sp. was

determined by using the method of agar diffusion described by Bauer *et al.*, (1966). After complete drying, crude extract was used for evaluating anti-bacterial activity. Bacterial pathogens were cultured individually and aseptically in nutrient broth at 37°C for 24 hr. 0.1ml of each culture (corresponding to 10 CFU/ml) was placed on Muller-Hinton agar plates and spread throughout the plates by spread plate method. Disc containing the extract was kept on the petriplate containing respective pathogen culture and the plate was incubated at 37°C for 24h. Diameter of the inhibition zone formed around the disc was measured in mm. Disc containing 10µl of 20% DMSO solution used as negative control and chloramphenicol antibiotic disc (30mcg/disc) used as positive control.

Antifungal activity of the crude extract of *Streptomyces* spp. was determined by agar diffusion method (Barry and Thornsberry, 1991)

An agar diffusion method (Barry and Thornsberry, 1991) was used for evaluating the antifungal activity. Potato Dextrose Agar medium was used for testing the antifungal activity of the extract. The test fungi were cultured in Potato Dextrose broth at room temperature for 24h. After incubation period, a small amount of fungal culture was transferred into 2.0 ml sterile 0.9% (w/v) NaCl. The turbidity of the fungal suspensions was measured at 625 nm with a spectrophotometer (UV-Visible Spectrophotometer, Pharmacia LKB Biochrom 4060 Uppsala, Sweden). The suspensions were diluted to reach an absorbance of 0.1 at 625 nm.

Two hundred fifty microliters of the suspensions were spread evenly on the Petri dishes. Sterile, standardized Whatmann filter papers (Ø = 6 mm) containing 200µl, 50 mg/ml extracts, and the antibiotics were placed equidistantly on the Petri dishes. Prior

to incubation in the dark at +35 °C, the Petri dishes were kept at +4 °C for 1 h to promote the diffusion of the extract into the agar. All the species were incubated at room temperature for 24 and 48 h. The diameter of the clear inhibition zones that had formed around the filter papers was measured. Methanol was used as a negative control. All the tests were made in triplicate.

Minimum Inhibitory Concentration (MIC) determination

MIC value for bacterial and fungal pathogens was determined by two fold dilution method using 96 well microtitre plate (Eloff *et al.*, 1988). From the stock solution a twofold serial dilution was made in 96 well containing microtitre plate. Two hundred microlitre of crude extract solution was added to well A. 100µl of sterile distilled was added to well B to well H. 100µl of the extract taken from well A and added to well B and mix thoroughly. 100µl of diluted extract was taken from the well B and added to the well C and mixed well. This process was continued till the well H. After through mixing of the content in the well H, 100µl was taken and discarded. This brought the final volume in each well to 100µl, i.e., the first of the two fold dilution. This procedure was followed in all the wells in the remaining rows. Column from 1 to 7 was used to determine the MIC value of the extract for different pathogens. Well 10 used for positive control (culture plus chloramphenicol), well 11 used as negative control (culture plus 20% DMSO solution) and well 12 used as growth control (bacterial/fungal culture). 100µl of bacterial culture (24h old)/ fungal spore suspension was added from the well A to H. This brings volume of each well to 200µl, i.e., second of two fold dilution. Microtitre plate was covered with sterile aluminium foil and incubated at 37°C for 24h. After incubation period, 50µl of 0.2% Idonitro tetrazolium chloride solution (INT) was added to each

well and the plate was incubated further 30 minutes at 37°C. Growth was indicated by red colour change of the solution and read visually. The MIC was determined as the minimum concentration at which growth was inhibited, i.e., no colour change was visible.

Results and Discussion

Out of 22 actinomycete isolates, isolate no. 16, showing antagonistic activity against bacterial pathogens, was identified by morphological, physiological tests and Gram's staining. Based on this, isolate no. 16 was identified as *Streptomyces* sp. The results of the present investigation showed that the crude extract of *Streptomyces* sp actively inhibited growth of both gram positive bacteria and gram negative bacteria. Among gram negative bacteria *E. coli* and *P. aeruginosa* were susceptible to the crude extract of *Streptomyces* sp. *E. coli* was highly sensitive to the extract and the diameter of inhibition zone was 25 mm. Inhibition zone produced against *P. aerogeniosa*, *S. aureus* and *B. subtilis* was 20mm (Table 1). *K. pneumoniae*, *P. mirabilis*, *E. aerogens*, *Citrobacter* sp., *S. typhi*, and *S. paratyphi* B were resistant to the extract (Table 1). MIC value for both the bacterial and fungal pathogens were determined by microtitre plate. The results of the MIC value determination showed that fungal pathogens were more susceptible than bacteria tested. The MIC of the crude extracts against the fungal pathogens ranged from 0.015mg to 0.025mg/µl, whereas for bacterial pathogens it was 2mg/ml (Table 2 and 3). The crude extract derived from *Streptomyces* sp, inhibited the growth of *C. albicans*, *Zynomonas* sp., *A. niger* and *A. rutilus*, whereas *A. flavus* and *A. fumigatus* were resistant to the extract. Among the fungal pathogens *A. rutilus* was more susceptible and its growth was inhibited at the concentration of less than 1µg/µl. The extract inhibited the

growth of *A. niger* and *Zynomonas* sp at the concentration of 0.062mg/ml and it is followed by *C. albicans* whose growth was inhibited at the concentration of 0.025mg/ml.

Table 1 Antibacterial activity of ethyl acetate extract of the marine sponges associated *Streptomyces* sp.

Name of the bacteria	Inhibition zone diameter in mm	
	Positive control Chloramphenicol (30mcg/disc)	Crude ethylacetate extract (10µl/disc) Mean ± SD
<i>E. coli</i>	14	25 ± 0.6
<i>K. pneumonia</i>	9	ND
<i>P. mirabilis</i>	15	ND
<i>P. aeruginosa</i>	11	20 ± 0.4
<i>S. typhi</i>	11	ND
<i>S. paratyphi B</i>	10	ND
<i>E. aerogens</i>	10	ND
<i>Citrobacter</i> sp	11	ND
<i>B. subtilis</i>	12	20 ± 0.5
<i>S. aureus</i>	10	20 ± 0.6

Table.2 Minimum Inhibitory Concentration of (MIC) crude extract of *Streptomyces* sp. on bacterial pathogens

Name of the bacteria	MIC concentration (mg/ml)
<i>E. coli</i>	0.2 mg/ml
<i>P. aeruginosa</i>	0.2 mg/ml
<i>B. subtilis</i>	0.2 mg/ml
<i>S. aureus</i>	0.2 mg/ml

Table.3 Antifungal activity of crude extract of *Streptomyces* sp. on fungal pathogens

Name of the fungi	Inhibitory zone diameter (in mm)
<i>Candida albicans</i>	21
<i>Zynomonas</i> sp	20
<i>Aspergillus niger</i>	23
<i>Aspergillus rutilus</i>	25

Table.4 Minimum inhibiting concentration (MIC) of crude extract of *Streptomyces* sp. on fungal pathogens

Name of the fungi	MIC value (mg/ml)
<i>Candida albicans</i>	0.025 mg/ml
<i>Zynomonas</i> sp	0.06 mg/ml
<i>Aspergillus niger</i>	0.06 mg/ml
<i>Aspergillus rutilus</i>	0.015 mg/ml

In the present investigation 22 actinomycete isolates were isolated from sponges. Many researchers have isolated actinomycetes from sponges. Schippers *et al.*, (2002), isolated *Nocardiosis alkaliphila* and *Streptomyces microflavus* from alkaline environment. Actinomycetes were isolated from sponges such as *Craniella australiensis* (Li *et al.*, 2006), *Rhapaloeides oderabile*, *Pseudocentina clavata* and *Candida spongia flabellate* (Kim *et al.*, 2004) and from water and sediment (Devi *et al.*, 2006). In the present study it was observed that the crude extract inhibited the growth of both gram positive and gram negative bacteria. Similarly Basilio *et al.*, (2003), and Rath *et al.*, (2005), also reported the inhibitory effect of ethyl acetate extract of *Streptomyces* sp. on both gram positive and gram negative bacteria. In the present study it was observed that *Streptomyces* sp. exhibited higher antifungal activity than antibacterial activity. Similarly Devi *et al.*, (2006), also reported that actinomycetes isolated from water and sediments showed higher antifungal activity than antibacterial activity. Buchanan *et al.*, (2005), reported Saline spore strain associated with sponges showed both antibacterial and antifungal activity. In the present study also even though crude extract derived from *Streptomyces* sp. inhibited the growth of both gram positive and gram negative bacteria it highly inhibited the growth of fungal pathogens.

The results of the present study showed that *K. pneumoniae*, *P. mirabilis*, *E.aerogens*, *Citrobacter* sp, *S. typhi* and *S. paratyphi* B were resistant to the crude extract of *Streptomyces* sp. Normally it was observed that *P. aeruginosa*, *Klebsiella* sp. and *S. typhi* were able to grow in some antibiotics and their resistance to more antibiotics has also been of medical concern (Tortora *et al.*, 2002). The results of the present study revealed that *Streptomyces* sp secretes antibiotic substance that inhibited the growth

of human fungal pathogens (Table 3). Crude extract derived from eighty three percent of actinomycetes isolated from Sagamy Bay were found to be antifungal (Okami and Okazaki, 1972). Many microorganisms isolated from marine environment exhibited antifungal activity against *Aspergillus niger* but not against *Candida albicans*. In contrast, the present study showed that *Streptomyces* sp inhibited not only *A. niger* but also *Candida albicans*, *Zygomonas* sp. *A. rutus* with the MIC value ranging from 0.015µg/ml to 0.025µg/ml (Table 4). The present investigation was well coinciding with study of Nagai *et al.*, (2002), and Kokare *et al.*, (2004). They reported that *Streptomyces* sp associated with sponges and isolated from marine sediments inhibited the growth of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* with the MIC ranging from 2µg to 16µg/ml. Among microorganisms, especially *Streptomyces* sp is proved to be as fruitful as their counter parts isolated from terrestrial habitat (Pisano *et al.*, 1989). From the present study, it was observed that marine actinomycetes particularly *Streptomyces* sp. is a useful and sustainable source of new bioactive natural products.

The bioactive compounds produced by the *Streptomyces* sp. is a potential source for controlling the growth of Multidrug resistant *E. coli*, *P. aeruginosa*, *S. aureus.*, *B. subtilis*, *C. albicans*, *A. niger*, *A. rutus* and *Zygomonas* sp, *Aspergillus* sp. are common contaminants and highly effective antibiotics are required to control the contaminants. *Streptomyces* sp. isolated from sponges may be useful for controlling such common contaminants.

From this study it was concluded that marine actinomycetes are the potential source for new antibiotics. A novel drug could be synthesized or derived from *Streptomyces* sp, which could help to control the multidrug

resistant *E. coli*, *S. aureus* and aflatoxin producing common contaminant of *Aspergillus* sp.

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How to cite this article:

Parimala, S., M. Kandhasamy and Chinnasamy, P.S. 2018. Antimicrobial Activity of the Crude Extract Derived from *Streptomyces* spp. Associated with Sponges. *Int.J.Curr.Microbiol.App.Sci.* 7(08): 1187-1194. doi: <https://doi.org/10.20546/ijcmas.2018.708.133>