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RESEARCH ARTICLE

# Screening of antimicrobial and antioxidant activities of endophytic actinomycetes isolated from *Rhizophora mucronata* and *Sonneratia caseolaris*

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## ABSTRACT

The aim of the present study was to evaluate the antimicrobial and antioxidant activity of crude extract or secondary metabolites of Endophytic actinomycetes species isolated from two mangrove plants collected from Cortalim, Goa, India. The plant parts were surface sterilized and plated on Oat meal agar supplemented with 10% sea water and actinomycetes isolates were obtained. Antimicrobial activity was studied by cross streak method and agar diffusion method by using six bacteria and four fungi. The potent isolates were subjected for Fermentation and the extracellular metabolites were extracted by using the ethyl acetate solvent and were subjected for antimicrobial screening. Isolate RO 7, RO 11 showed more inhibition against all the tested bacteria and Isolate RO 9 showed both ntibacterial and antifungal activity. Antioxidant activity was done by DPPH radical scavenging assay. The results showed dose dependant antioxidant activity. The ethyl acetate extract RO 7 showed 94.11% inhibition at 100µg/ml followed by RO 11 and RO 9 showing 64.70% and 61.17% inhibition at 100µg/ml and the inhibition was compared with standard antioxidant ascorbic acid which showed 100% inhibition at 100µg/ml. The findings of the present study show that endophytic actinomycetes could be potential natural source of antioxidants and antibiotics.

**Keywords:** Antimicrobial activity, Antioxidant activity, Endophytic actinomycetes, *Rhizophora mucronata*, *Sonneratia caseolaris*

## INTRODUCTION

Actinomycetes remain major sources of novel bioactive compounds and therapeutically relevant natural products [1-3]. Actinomycetes are the filamentous gram positive microorganisms with high G+C content primarily saprophytic and contribute in breakdown of complex biopolymers such as hemicelluloses, pectin, lignocelluloses, keratin and chitin [4]. Actinomycetes are the main source of clinically important antibiotics and find applications as anti-infective, anticancer agents and other pharmaceutically useful compounds [5]. Members of actinomycetes which live in marine environment are poorly understood and only few reports are available pertaining to actinomycetes from mangroves [6-8]. Mangrove ecosystem is the most productive ecosystem diversified with variety of microbes [9]. Unexplored natural habitats and neglected habitats are proving to be good source of novel actinomycetes and bio active compounds [10]. Several studies reported that reactive oxygen species (ROS), such as hydroxyl (OH), superoxide ($O_2$) nitric oxide (NO) radicals and hydrogen peroxide ($H_2O_2$), are damaging for most biomolecules and cause many diseases, and these compounds should be scavenged. The agents that scavenge or inhibit the formation of these radicals are called antioxidants [11]. Antioxidants play an important role in inhibiting and scavenging free radicals and provide protection to humans against various infections and degenerative diseases [12]. Antioxidants deficiency in nutrition leads to oxidative stress conditions. Free radical scavenging activity of the samples has widely been investigated by DPPH scavenging method [11]. The objective of the present study is to evaluate the antimicrobial and antioxidant potential of endophytic actinomycetes isolated from mangrove plants *Rhizophora mucronata* and *Sonneratia caseolaris* from Cortalim Goa.

## MATERIALS AND METHODS

### Collection of Mangrove plants

The Mangrove plants were collected from Cortalim, Goa, India during January 2017. The plant parts included leaves, stem, bark, roots. The Mangrove plants were authenticated by Department of Botany, Sahyadri Science College (Autonomous), Shimoga. The plants were collected in sterilized polythene bags and were stored in the Laboratory at 4° C until use.

### Isolation of Endophytic Actinomycetes

The plant parts such as leaves, stem roots and bark were separated from plant, washed thoroughly in water to

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remove adhered epiphytes, soil debris on the surface. The explants were surface sterilized with 0.1% Mercuric chloride for 1 min followed by 70% alcohol for 1-2 min and then with Distilled water. The explants were cut into small fragments and were inoculated on Oat meal agar media (Himedia laboratories, India). The media was supplemented with griseofulvin and fluconazole to prevent fungal contamination. The inoculated plates were incubated at 30ºC for 14 to 21 days. After the incubation period, individual colonies with characteristic actinomycete morphology emerging out from the plant tissue were isolated. The pure cultures of the isolates were obtained by streaking on Starch Casein Nitrate agar media plates and slants and were preserved in refrigerator at 4ºC.

**Characterization of the actinomycetes isolates**

The isolates were subjected for characterization by morphological (colour of aerial and substrate mycelium, pigment production), biochemical tests (Starch hydrolysis, Casein hydrolysis, KOH solubility test, Citrate Utilization test), microscopic (cover slip method) and staining (Gram's staining) [13-14].

**Primary Screening of Actinomycetes for Antimicrobial activity**

The primary screening was carried out by cross streak method for actinomycetes isolates. The isolates were inoculated on SCN agar plates by a single line streak in the centre of the petriplate and were incubated for 4-5 days at 30±2°C. After 4-5 days the plates were then inoculated with the test organisms (*Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis, Vibrio cholera, Escherichia coli, and Klebsiella pneumoniae*) perpendicular to the growth of actinomycetes isolates. The molds (*Curvularia sp, Fusarium oxysporum, Helminthosporium sp, Alternaria solani*) were inoculated by point inoculation. The plates were incubated at 37ºC for 24 hours for bacteria and 28ºC for 48-72 hours for fungi respectively. The SCN agar media was supplemented with peptone and beef extract (to facilitate bacterial growth) and dextrose (to facilitate fungal growth). The absence of growth or a less dense growth of test organism near the actinomycete isolate was considered positive for production and secretion of antimicrobial metabolite by the isolates [15].

**Fermentation and Solvent Extraction**

The potent strains were inoculated into Starch casein nitrate broth in a 250 ml Erlenmeyer flask and kept for incubation at 30º C for 7 -10 days. The flasks were observed constantly for any possible contamination. After incubation the broth culture was filtered aseptically through Whatmann No 1 filter paper. The crude extracts were subjected for solvent extraction in separating funnel. Culture filtrate and ethyl acetate solvent were taken in 1:1 (v/v) proportion and mixed thoroughly. The separating funnel was allowed to stand for about 30 minutes for separation of solvent and filtrate layer. Further, the solvent layer was separated, dried and was used for further assays.

**Secondary Screening of Actinomycetes for Antimicrobial activity**

Agar well diffusion assay was carried out for secondary screening. The test bacterial pathogens (24 hrs old nutrient broth culture of *Staphylococcus epidermidis, Staphylococcus aureus, Bacillus subtilis, Vibrio cholera, Escherichia coli, Klebsiella pneumoniae*) and molds (48 hours old potato dextrose broth cultures of *Curvularia sp, Fusarium oxysporum, Helminthosporium sp, Alternaria solani*) were swab inoculated uniformly over the surface of sterile Nutrient agar plates and Potato dextrose agar plates respectively using sterile cotton swabs to get lawn growth of the pathogens. Well was made on inoculated plates by using sterile cork borer (6mm diameter) at equidistance in the plates. About 100µL of crude ethyl acetate extract of the potent isolates at a concentration of 10mg/ml was carefully dispensed in each well using micropipette. Plates were allowed to diffuse for about 15min before incubating. Standard antibiotic discs of Streptomycin, was also placed. All the plates were incubated at $37^0$ C for 24 hours for bacteria and 48-72 hours at 28ºC for fungi. The plates were observed for zone of inhibition formed around the well and measured in millimeter.

**Antioxidant Activity of solvent extracts of Actinomycetes**

The solvent extracts showing promising antimicrobial activity were subjected to antioxidant activity by DPPH (1, 1- biphenyl – 2- picrylhydrazyl) assay.

**DPPH free Radical Scavenging Activity**

The DPPH (1, 1- biphenyl – 2- picrylhydrazyl) assay was done to evaluate free radical scavenging activity of the metabolite. 1ml of different concentrations of ethyl acetate extract (0-400µg/ml of methanol) was added to 3ml of 0.004% DPPH (in methanol). The tubes were incubated at room temperature in dark for about 30 minutes followed by measuring the absorbance at 517nm spectrophotometrically. Ascorbic acid was used as standard. The absorbance of DPPH without extract/ standard was noted. The scavenging activity (%) of each concentration was calculated using the formula,

Scavenging activity (%) = [(A-B)/A] x 100

Where A refers to absorbance of DPPH control and B refers to absorbance of extract/ standard respectively [16-17].

**RESULTS**

**Selection of plants**



The two mangrove plants selected for the study are *Rhizophora mucronata* belonging to the family *Rhizophoraceae* and *Sonneratia caseolaris* belonging to the family *Lythraceae.*

## Isolation of endophytic actinomycetes

A total of 11endophytic actinomycetes were obtained from both plants. 7 isolates were obtained from *Rhizophora mucronata* (5 from stem, 1 from bark and 1 from leaf). 4 isolates were obtained from *Sonneratia caseolaris* (3 from stem, 1 from leaf and no isolates were obtained from bark). All the isolates were subcultured on Starch Casein Nitrate Agar media and were preserved in refrigerator at 4 $^0$ C. The number of endophytic actinomycetes isolated is represented in Table no 1.

**Table1: Number of endophytic actinomycetes isolated from plant samples**

| Number of Isolates | *Rhizophora mucronata* | | | *Sonneratia caseolaris* | | | Oat meal agar media |
|---|---|---|---|---|---|---|---|
| Part of plants | Stem | Leaf | Bark | Stem | Leaf | Bark | |
| RO -1 | - | - | - | ✓ | - | - | ✓ |
| RO-2 | - | - | ✓ | - | - | - | ✓ |
| RO -3 | - | ✓ | - | - | - | - | ✓ |
| RO -4 | - | - | - | ✓ | - | - | ✓ |
| RO -5 | - | - | - | - | ✓ | - | ✓ |
| RO -6 | - | - | - | ✓ | - | - | ✓ |
| RO -7 | ✓ | - | - | - | - | - | ✓ |
| RO -8 | ✓ | - | - | - | - | - | ✓ |
| RO -9 | ✓ | - | - | - | - | - | ✓ |
| RO -10 | ✓ | - | - | - | - | - | ✓ |
| RO -11 | ✓ | - | - | - | - | - | ✓ |

**Fig 1: Representative Forms of actinomycetes isolates**

RO 7 RO 9 RO 11

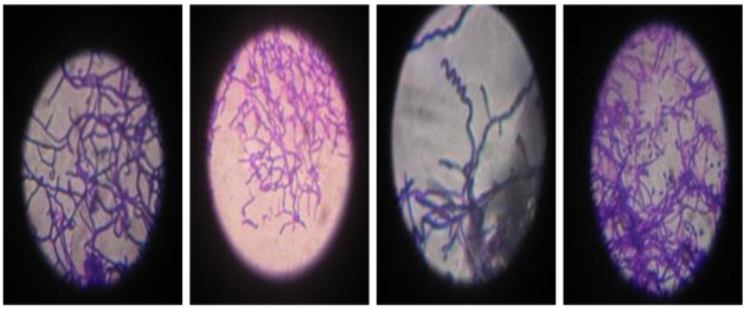
## Characterization of the actinomycetes isolates

**Morphological characteristics:** The morphological characteristics of isolates revealed a wide range of aerial and substrate mycelia colours such as white, grey, light grey, creamy white. None of the isolates produced diffusible pigments. The spore arrangement studies revealed a diverse morphological characters with varied spore colour, different arrays of spore arrangement that varied from *Flexibilis*, *Retinaculum aparatum* – open loop, hooks, simple spira and compact spira. The morphological and microscopic characterization of endophytic actinomycetes is represented in Table no. 2.

**Table no. 2: Characterization of actinomycetes**

| Isolates | Aerial mycelium | Substrate mycelium | Spore arrangement | Tentative genera |
|---|---|---|---|---|
| **RO 1** | Creamy white | Light brown | Micromonospora | *Micromonospor a* |
| **RO 2** | Grey | Grey | Flexibilis | *Streptomyces Sp* |
| **RO 3** | Cream | Brown | Flexibilis | *Streptomyces Sp* |
| **RO 4** | White | White | Retinacullum aparatum- Hook | *Streptomyces Sp* |
| **RO 5** | Greyish white | Brown | Flexibilis | *Streptomyces Sp* |
| **RO 6** | Peach | Light brown | Flexibilis | *Streptomyces Sp* |
| **RO 7** | Grey | Creamy white | Spira | *Streptomyces Sp* |
| **RO 8** | Grey | Creamy | Open spiral | *Streptomyces Sp* |
| **RO 9** | Grey | Creamy white | Retinacullum aparatum- spiral | *Streptomyces Sp* |
| **RO 10** | Dark grey | White | Hook | *Streptomyces Sp* |
| **RO 11** | Light grey | Creamy white | Spiral | *Streptomyces Sp* |

**Fig 2: Representative forms of spore morphology of endophytic actinomycetes**



Hook Flexibilis Spiral Micromonospora

## Staining characteristics and Biochemical Characteristics:

All the 11(100%) isolates were found to be Gram positive and were negative for KOH solubility test and positive for Casein hydrolysis. 7 (63.63%) isolates were positive for starch hydrolysis, 6 (54.54%) isolates were positive for citrate utilization test. The staining and biochemical characteristics of the isolates are represented in Table no. 3**.**

**Table No 3: Staining and Biochemical Characteristics**

| Isolate No | Grams Staining | KOH solubility Test | Casein Hydrolysis | Citrate utilization Test | Starch Hydrolysis Test |
|---|---|---|---|---|---|
| **RO-1** | + | - | + | - | - |
| **RO-2** | + | - | ++ | - | - |
| **RO-3** | + | - | + | - | - |
| **RO-4** | + | - | +++ | + | +++ |
| **RO-5** | + | - | +++ | +++ | - |
| **RO-6** | + | - | + | - | +++ |
| **RO-7** | + | - | ++ | +++ | + |
| **RO-8** | + | - | + | +++ | + |
| **RO-9** | + | - | + | +++ | + |
| **RO-10** | + | - | +++ | - | + |
| **RO-11** | + | - | ++ | +++ | + |

## ANTIMICROBIAL ACTIVITY

### Antibacterial Activity:

### Primary Screening of actinomycetes for antibacterial activity:

The primary screening of isolates showed antagonistic activity against test organisms to varied level. The antibiosis was seen as a clear zone around the endophytic actinomycetes isolate inhibiting the growth of test organisms. Majority of isolates caused inhibition of one or more test organisms. Out of 6 test pathogens *Vibrio cholerae* was inhibited by 8 isolates (72.72%), *Escherichia coli* and *Bacillus subtilis* were inhibited by 5 organisms (45.45%), S*taphylococcus epidermidis* and *Klebsiella pneumoniae* were inhibited by 9 isolates (81.81%). Isolates 9 and 11 inhibited all the test organisms followed by isolates 7 and 8. Isolates 2 and 3 were ineffective against all the test pathogens. The result of primary screening is represented in Table no. 4.

**Table no. 4: Preliminary screening of antibacterial activity test**

| Isolates | B1 | B2 | B3 | B4 | B5 | B6 |
|---|---|---|---|---|---|---|
| RO -1 | ++ | - | - | + | + | ++ |
| RO -2 | - | - | - | - | - | - |
| RO -3 | - | - | - | - | - | - |
| RO -4 | + | - | - | + | + | - |
| RO -5 | +++ | - | - | +++ | ++ | - |
| RO -6 | ++ | ++ | ++ | +++ | ++ | - |
| RO -7 | +++ | ++ | ++ | ++ | ++ | + |
| RO -8 | +++ | + | + | ++ | ++ | + |
| RO -9 | +++ | ++ | ++ | ++ | ++ | ++ |
| RO -10 | - | - | - | ++ | ++ | - |
| RO -11 | +++ | +++ | +++ | ++ | ++ | ++ |

**+++ - very good, ++ -good, +positive, -negative,B1: *V.cholera***

**B2: *E.coli*, B3: *S.aureus*, B4: *S. epidermidis*, B5: *K.pneumonia*, B6: *B.subtilis***

### Secondary screening of actinomycetes for antibacterial activity:

The extracts showed marked inhibition against all the 6 test bacteria. Isolate RO-7 showed activity with zone of inhibition ranging from 17 to 23mm, Isolate RO-9 showed zone of inhibition ranging from 19 to 24 mm and Isolate RO-11 showed zone of inhibition ranging from 18-23mm diameter. Standard antibiotics Streptomycin inhibited the test bacteria with zone of inhibition ranging from 16-32mm diameter respectively.

**Table no. 5: Secondary screening of antibacterial activity test**

| Isolates | B-1 | B-2 | B-3 | B-4 | B-5 | B-6 |
|---|---|---|---|---|---|---|
| RO 7 | 22mm | 23 mm | 17 mm | 22 mm | 19 mm | 20 mm |
| RO 9 | 19mm | 24 mm | 19 mm | 22 mm | 19 mm | 22 mm |
| RO 11 | 22mm | 22 mm | 18 mm | 23 mm | 19 mm | 22 mm |
| Strepto mycin | 22mm | 19mm | 29mm | 32mm | 22mm | 16mm |

**Where '-' No inhibition**

### Antifungal activity

### Primary screening of Actinomycetes for antifungal activity:

The preliminary screening of isolates showed activity against to a varied level. The antibiosis is seen as inhibition of growth of fungi .Majority of isolates caused the inhibition of one or more test phytopathogens. Out of 4 test phytopathogens 36.36% inhibited *Curvularia sp*, 72.72% inhibited *Helminthosporium sp*, 24.24% inhibited *Alternaria solani*, and 36.36% inhibited *Fusarium oxysporum.* The antifungal activity of endophytic actinomycetes are represented in Table no. 6

**Table no.6: Preliminary screening of antifungal activity**

| Isolates | F-1 | F-2 | F-3 | F-4 |
|---|---|---|---|---|
| RO-1 | - | + | - | - |
| RO -2 | - | + | - | - |
| RO -3 | + | + | - | - |
| RO -4 | - | + | - | + |
| RO -5 | - | + | + | - |
| RO -6 | - | - | - | - |
| RO -7 | - | - | - | - |
| RO -8 | - | - | - | +++ |
| RO- 9 | +++ | + | +++ | ++ |
| RO -10 | +++ | + | +++ | + |
| RO -11 | +++ | ++ | - | - |

**+++ - very good, ++ -good, +positive, -negative**

**F-1:- *Curvularia* sp; F-2:- *Helminthosporium* sp; F-3:- *Alternaria solani*; F-4:- *Fusarium oxysporum***

### Secondary screening of endophytic actinomycetes for antifungal activity:

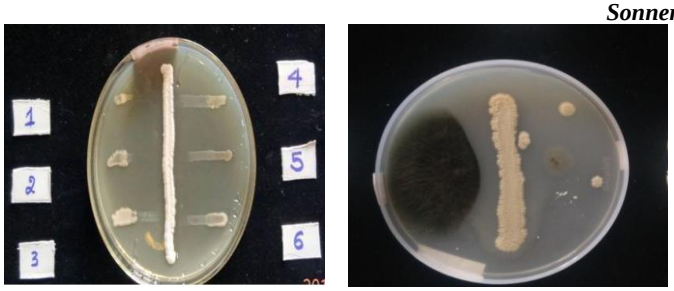
Isolate RO-9 showed marked inhibition against all the 4 test phytopathogens. It showed 19mm inhibition against *Helminthosporium species*, 16mm against *Fusarium oxysporum*, 15mm against *Alternaria solani* and 15mm against *Curvularia species*. Isolate RO-10 was less effective showing 9mm inhibition against *Fusarium oxysporum*, 8mm inhibition against *Curvularia* species, 5mm inhibition against *Alternaria solani* and was ineffective against *Helminthosporium* species. Standard antibiotics Gresiofulvin and Flucanozole have inhibited all the test pathogens, 22 and 21 mm against *Helminthosporium species*, 16 and 15mm against *Curvularia species*, 18 and 17mm against *Alternaria solani* and 19 and 18mm against *Fusarium oxysporum.*

**Table no. 7: Secondary screening of antifungal activity**

| TEST ORGANISMS | ISOLATES | | ANTIBIOTICS | |
|---|---|---|---|---|
| | RO 9 | RO 10 | GRISEOFULVIN | FLUCONAZOLE |
| *Curvularia sp* | 13mm | 8mm | 16mm | 15mm |
| *Alternaria solani* | 15mm | 5mm | 18mm | 17mm |
| *Helminthosporium sp* | 19mm | - | 22mm | 21mm |
| *Fusarium oxysporum* | 16mm | 9mm | 19mm | 18mm |

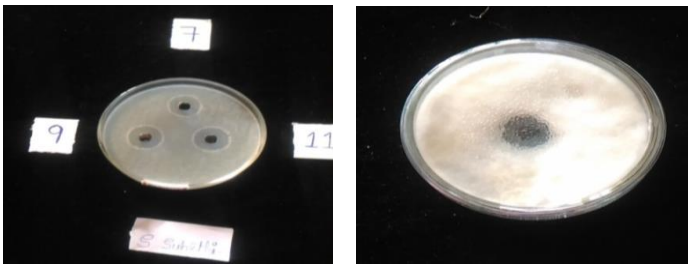
**Fig 4: Primary screening of actinomycetes by Cross streak method.**




**Isolate RO 9 showing marked inhibition against all the test bacteria and fungi**

**Fig 5: Secondary screening of actinomycetes by Agar well diffusion method.**



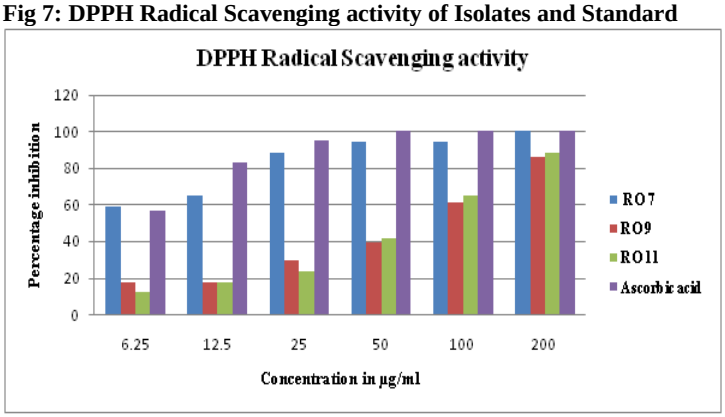

**Plates showing inhibition of test bacteria and fungi**

## Antioxidant activity:

## DPPH Radical Scavenging Assay.

The DPPH free radical scavenging activity (%) of different concentrations of ethyl acetate extracts of culture and reference standard is shown in Fig 7. The degree of stable DPPH decolorization to DPPHH (reduced form) yellow showed the scavenging ability of the extract. Ascorbic acid exhibited marked antioxidant activity of 100% in 100µg/ml as compared with ethyl acetate extracts. Both ethyl acetate extracts and the standard exhibited dose dependant activity.

**Fig 7: DPPH Radical Scavenging activity of Isolates and Standard**




**Percentage inhibition produced by ascorbic acid, Isolates RO 7, RO 9 and RO 11 at six different concentrations.**

## DISCUSSION:

Endophytic actinomycetes are well adapted and are functional members of mangrove environment. They have worldwide distribution and adapt themselves to varied extreme environment. These microbes have huge potential to synthesize numerous novel compounds that can be exploited in pharmaceutical, agricultural and other industries [10]. The antimicrobial resistance leads to ineffectiveness of treatment and increased morbidity, mortality and health care expenditure. High cost, side effects of antibiotics and development of resistance in pathogens against antibiotics stimulated research on finding antimicrobials from natural sources. Natural products can be effectively used as promising alternates to combat drug resistance. The present study was focused on Screening of antimicrobial and antioxidant activities of endophytic actinomycetes isolated from *Rhizophora mucronata* and *Sonneratia caseolaris*. A total of 11 endophytic actinomycetes were obtained from root, bark, stem of mangrove plants *Rhizophora mucronata* and *Sonneratia caseolaris*. Similar studies have shown that diverse group of endophytic actinomycetes are present in mangrove plants and environment i.e rhizosphere soil of mangrove plant *Avicennia marina* [10], medicinal plants [18-23], mangrove forests[24], Ethnomedicinal plants [25]. Most of the actinomycetes were identified as *Streptomyces* species by morphological characteristics, which was consistent with the other reports from different hosts. It has been reported that actinomycetes isolated from mangrove environment exhibit antimicrobial activity. The actinomycetes species from Mangrove *Avicennia marina* [10], mangrove and estuarine sediments [26], Medicinal plants[18-20], wheat [27], *Azardirachta indica*[28], *Citrus aurantifolia*[29], have exhibited antimicrobial activity. The present study was successful in isolating endophytic actinomycetes with broad antibacterial and antifungal potential. The isolates have shown inhibition of both Gram positive bacteria and Gram negative bacteria and phytopathogens. Isolate RO 9 was found to be more potent against all the pathogens tested. There are several methods available to assess the antioxidant potential of isolates among which DPPH assay is easy and rapid assay. After accepting an electron or hydrogen atom from donor (antioxidant), DPPH radical is converted into a non-radical DPPHH and the purple color changes to yellow compound diphenylpicrylhydrazine[17,31]. In the present study, a decrease in the absorption of DPPH radical solution in the presence of various concentrations of ethyl acetate extract was measured at 517 nm. The scavenging activity of ethyl acetate extracts and ascorbic acid was found to be dose dependant. These results are in accordance with the studies carried by[17,32-34]. The percentage of DPPH scavenging activity for the ethyl acetate extract RO 7 was 94.11% at 100µg/ml followed by RO 11 and RO 9 64.70% and 61.17% at 100µg/ml respectively. The results obtained indicate that the ethyl acetate extracts can be used as primary antioxidants.

## CONCLUSION

Endophytic actinomycetes have attracted attention in search of bioactive natural compounds. They can be used as new drugs replacing those against which pathogenic organisms have acquired resistance. Actinomycetes are found to be the strongest antagonists among the microbes and are potential source for producing antibiotics for bacteria and fungi. The present



work reveals that among the different parts tested, stem of *Rhizophora mucronata* plant yielded more isolates and is found to be more potent. Anticancerous property and sequencing studies of the potent isolates can be further exploited.

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