

A detailed scanning electron micrograph (SEM) of various bacterial structures, including long, rod-shaped bacilli and smaller, more complex forms, set against a textured, brownish background.

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# Journal of Microbiology and Antimicrobials

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## ARTICLES

**Antimicrobial activity of endophytic fungi isolated and identified from salt marsh plant in Vellar Estuary**

Indira Kalyanasundaram, Jayaprabha Nagamuthu and Srinivasan Muthukumaraswamy

Full Length Research Paper

## Antimicrobial activity of endophytic fungi isolated and identified from salt marsh plant in Vellar Estuary

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The purpose of this work was to evaluate the antimicrobial potential of endophytic fungi isolated from leaves and stems of *Suaeda maritima* and *Suaeda monoica*. Many endophytes were isolated by using potato dextrose agar (PDA) medium. All the endophytic isolates were identified by using standard taxonomic keys and monographs. From a total of 16 isolates, nine potent strains were taken for further study. The fungal culture was extracted with ethyl acetate and used as a crude extract for checking antimicrobial activities by well diffusion method. The crude extract showed different inhibitory activity against all pathogens, the zones of inhibition obtained was between 2 and 12 mm. SM-EF 3 crude extract showed high zone of inhibition of  $11.6 \pm 0.57$  mm against *Salmonella typhi*,  $8.3 \pm 1.52$  mm against *Trichophyton rubrum*, respectively. The present findings concludes that SM-EF 3, SM-EF 7 and SM-EF 9 showed comparatively higher antimicrobial activity against all the human pathogens. From the present work, it is possible to conclude that these microorganisms could be promising source of bioactive compounds and warrant further study.

**Key words:** Endophytic fungi, antimicrobial activity, salt marsh plant, secondary metabolite.

### INTRODUCTION

Endophytic microorganisms colonize living, internal tissues of the plants without causing any immediate, overt negative effects (Bacon and White, 2000; Wasser, 2002). Endophytes have proved to be the promising sources of biologically active products which are of interest for specific medicinal applications (Strobel, 2002). Recent investigations have been intensified by the potentialities of endophytic fungal strains in the production of pigments, bioactive metabolites, immune-

suppressants, anticancer compounds and biocontrol agents (Wang et al., 2002; Stinson et al., 2003; Strobel and Bryn, 2003; Selvin et al., 2004; Strobel et al., 2004). Presently, research groups have identified more than hundreds of endophytic isolates from South Indian medicinal plants that showed promising activity against anti-tumour and antimicrobial agents (Gangadevi and Muthumary, 2007, 2009).

The development of drug resistance in human patho-

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genic bacteria has prompted a search for more and better antibiotics, especially as diseases caused by pathogenic microorganisms now represent a clear and growing threat to world health (Raviglione et al., 1995; Pablosmendez et al., 1997). The increase of microbial resistance to antibiotics threatens public health on a global scale as it reduces the effectiveness of treatments and increases morbidity, mortality and health care costs (Coast et al., 1996). Evolution of highly resistant bacterial strains has compromised the use of newer generations of antibiotics (Levy, 2002; Levy and Marshall, 2004). Although, the active constituents may occur in lower concentrations, endophytic fungal pigments may be a better source of antimicrobial compounds than synthetic drugs. Therefore, the investigations of the antimicrobial activity of natural products have opened new ways for drug development in the control of antibiotic resistant pathogens. The researchers are currently paying more attention to the drug development from the endophytic fungi isolated from medicinal plants (Tan and Zou, 2001). The purpose of the present study was to extract, explore and characterize antimicrobial activity produced by the endophytic fungi isolated from salt marsh plant leaves and stems of *Suaeda maritima* and *Suaeda monoica*.

## MATERIALS AND METHODS

### Sampling

Healthy (showing no visual disease symptom) and mature plants were carefully chosen for sampling. Leaves and stem of each plant were randomly collected from Vellar estuary in Porto Novo (also known as Parangipettai Lat. 11° 29' N; Long. 79° 47' E). The plant material was brought to the laboratory in sterile bags and processed within a few hours after sampling. Fresh plant materials were used for isolation work to reduce the chance of contamination.

### Isolation of endophytic fungi

Endophytic fungi were isolated from the leaves and stems of *S. maritima* and *S. monoica*. Isolation of endophytic fungi was carried out according to the method described by Suryanarayanan et al. (2003). The plant samples were rinsed gently in running water to remove dust and debris. After proper washing, stems and leaves were cut into pieces 0.5-1 cm long (150 bits per tissue/season), under aseptic conditions. Surface sterilization was done by 1-13% Sodium hypochlorite according to the type of tissues (for example higher concentration was used for leaf samples). Each set of plant material was treated with 75% ethanol for 1 min followed by immersion in sodium hypochlorite and again in 75% ethanol for 30 s. Later, the segments were rinsed three times with sterile distilled water. The plant pieces were blotted on sterile blotting paper. In each Petri dish, 8-10 segments were placed on potato dextrose agar (PDA) amended with chloramphenicol 150 mg/l. The dishes were sealed with parafilm and incubated at  $27 \pm 2^\circ\text{C}$  for four to six weeks in dark room. The Petri dishes were monitored frequently to check the growth of endophytic fungal colonies.

### Identification of endophytic fungi

For characterisation of the morphology of fungal isolates, slides

prepared from cultures were stained with lactophenol cotton blue reagent and examined with a bright-field and phase-contrast microscope. The taxa were assigned to genera following Barnett and Hunter (1998).

### Cultivation for screening and isolation of secondary metabolites

For small scale fermentation, each fungal strain was inoculated into a 1000 ml Erlenmeyer flask containing 300 ml of liquid PDA medium. For this purpose, a strain that nearly covered the surface of a Petri dish (after one to two weeks growth on PDA medium) was cut into small pieces and these were transferred to an Erlenmeyer flask containing the sterilised medium. Cultivation was performed at room temperature under static conditions and daylight. Depending on the fungal growth, cultures on liquid medium were incubated for three to four weeks. The fermentation was brought to an end by adding 250 ml EtOAc to the culture flask and standing closed for at least 24 h.

### Extraction of fungal liquid cultures

#### Total extraction of culture media and mycelia

250 ml EtOAc were added to each Erlenmeyer flask containing 300 ml culture medium and left overnight to stop cell growth. Culture media and mycelia were then extracted in the Ultraturrax for 10 min for cell destruction and filtered under vacuum using a Buchner funnel. The mycelium was discarded and the culture filtrate transferred to a separation funnel. The EtOAc and H<sub>2</sub>O phases were separated and the aqueous phase extracted two more times with 300 ml EtOAc each. All obtained extracts were taken to dryness under reduced pressure at 40°C. After evaporating the solvent, the residue was mixed with the same solvent, these crude extracts were subjected to antimicrobial assays.

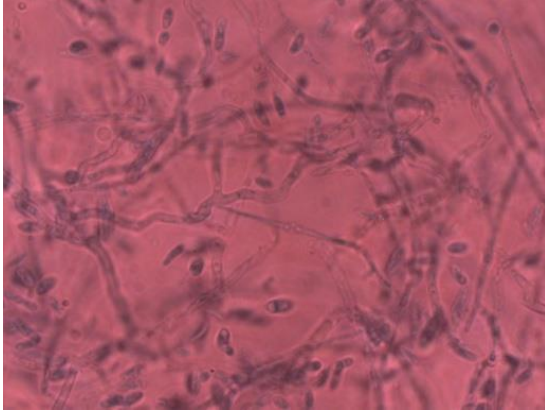
### Pathogens used for antimicrobial activity

Totally eight human bacterial pathogens used were *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhi*, *Salmonella paratyphi*, *Vibrio cholera*, *Klebsiella oxytoca*, *Klebsiella pneumonia* and *Staphylococcus aureus*. The stock culture was maintained on nutrient agar medium at 4°C. Five human fungal pathogens namely *Candida albicans*, *Epidermophyton floccosum*, *Microsporium canis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* were also used. The stock culture was maintained on PDA medium at 4°C. These bacterial and fungal strains were isolated and obtained from the Department of Microbiology, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar, Tamil Nadu, India.

### Screening for antimicrobial activity

#### Antagonistic assay for endophytic fungal extract against bacterial pathogens

Antagonistic assay was done by an agar-well diffusion method in aerobic condition. Isolated endophytic fungal extract were tested for the antibacterial activity. Bacterial pathogens were spread on MHA plates. Then wells were made and 50 µL crude extract of each strain was inoculated into a separate well. Antagonistic activity was detected after an incubation of 24 to 48 h at 35°C. The presence of zone clearance on agar plates was used as an indicator for the antibacterial activity. The strains which showed the maximum zone of clearance was chosen for further study. The presence of zone of



**Figure 1.** Light microscopic observation of fungi. *Acremonium* sp.

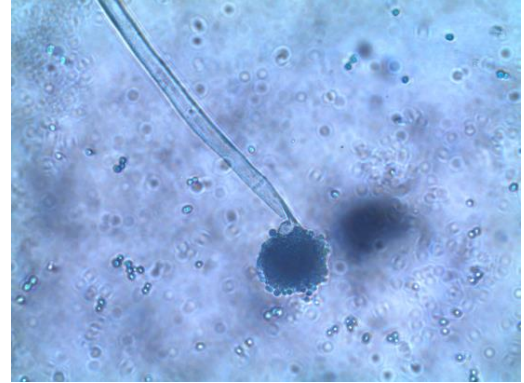


**Figure 2.** Light microscopic observation of fungi. *Alternaria alternate*.

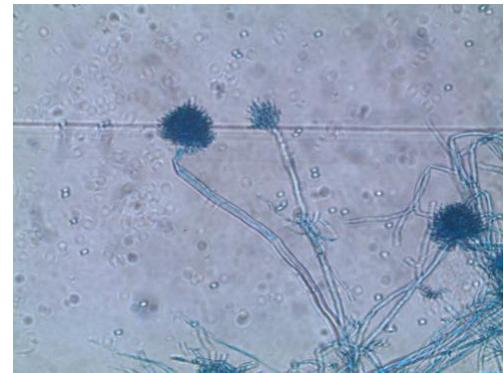


**Figure 3.** Light microscopic observation of fungi. *Alternaria* sp.

clearance on agar plates was used as an indicator of bioactive potential of the strain (Portrait et al., 1999).



**Figure 4.** Light microscopic observation of fungi. *Aspergillus terreus*.



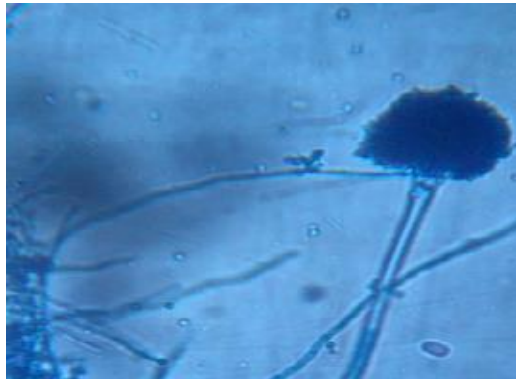
**Figure 5.** Light microscopic observation of fungi. *Aspergillus flavus*

#### **Antagonistic assay for endophytic fungal extracts against fungal pathogens**

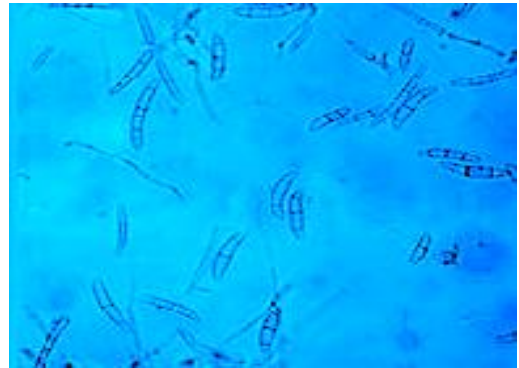
Antagonistic activities of dominant endophytic fungal extracts were tested for their antifungal activity (Geels and Schippers, 1983) against selected human fungal pathogens. Initial screening for *in vitro* antagonistic activity was tested against fungal strains on PDA agar plates. Wells were made and 50  $\mu$ L of each fungal extracts was inoculated into a separate well. Antagonistic activity was detected after an incubation of 24-48 h at 35°C. The presence of zone of clearance on agar plates was used as an indicator of bioactive nature of the strain.

#### **RESULTS**

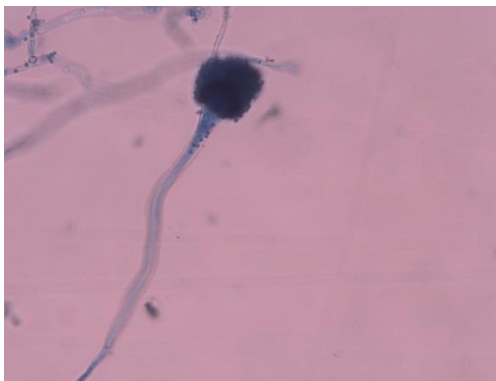
*S. maritima* and *S. monoica* were collected from Vellar estuary in Southeast coast of India. Totally, 1200 segments (150 leaves, 150 stem per season) were screened for isolating endophytic fungi. From 1200 tissue segments, 433 isolates were produced in culture of *S. maritima* grouped into 15 taxa. 422 isolates were produced in the culture of *S. monoica* grouped into 14 taxa. All the endophytic isolates were identified by using standard taxonomic keys and monographs and are shown in Figures 1 to 16. In the present investigation, a



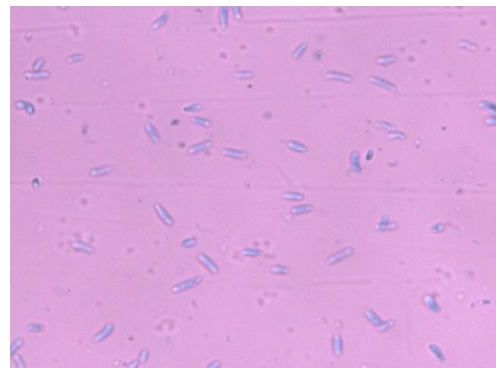
**Figure 6.** Light microscopic observation of fungi. *Aspergillus niger*.



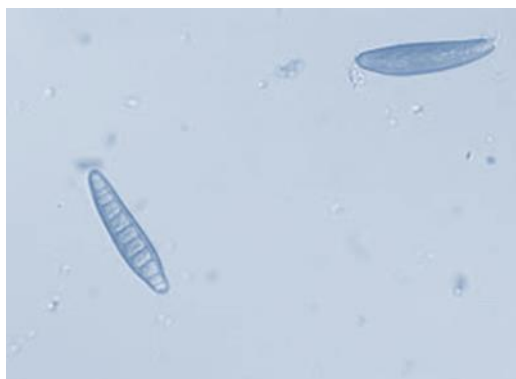
**Figure 9.** Light microscopic observation of fungi. *Fusarium* sp. 1.



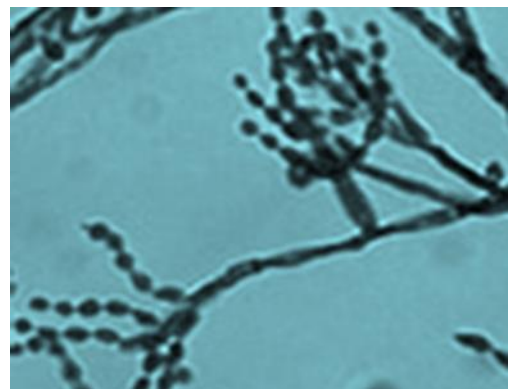
**Figure 7.** Light microscopic observation of fungi. *Aspergillus nidulans*



**Figure 10.** Light microscopic observation of fungi. *Fusarium* sp. 2



**Figure 8.** Light microscopic observation of fungi. *Bipolaris* sp.



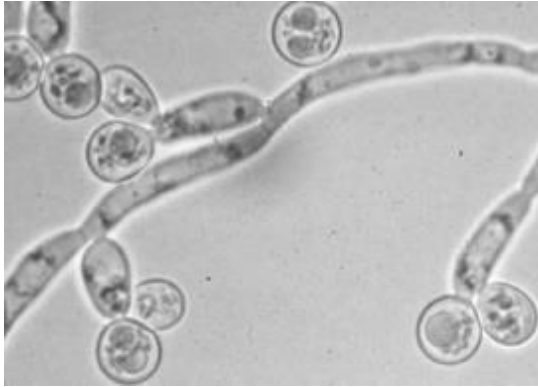
**Figure 11.** Light microscopic observation of fungi. *Cladosporium* sp.

total of nine endophytic fungi were selected for screening of antimicrobial activity against human pathogens. The selected endophytic fungi are shown in Table 1.

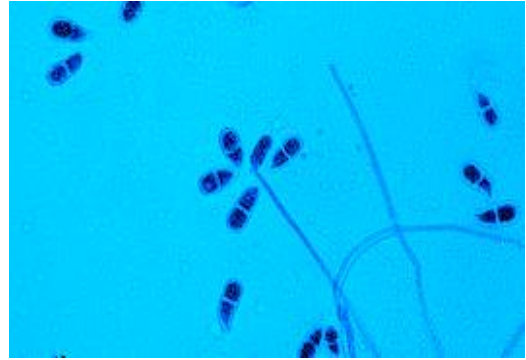
Endophytic fungal crude extracts were tested against the bacterial and fungal pathogens by well diffusion

method. Totally, 13 microorganisms which consisted of 8 bacteria and five fungi were tested. The ethyl acetate extracts were assayed against the test organisms, the zones of inhibition obtained was between 2 and 12 mm. The results of preliminary screening tests are summa-

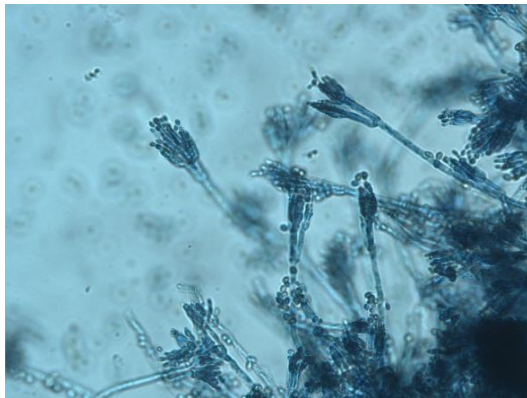




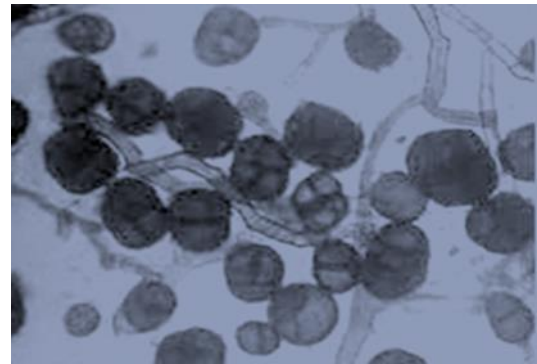
**Figure 12.** Light microscopic observation of fungi. *Meyerozyma* sp.



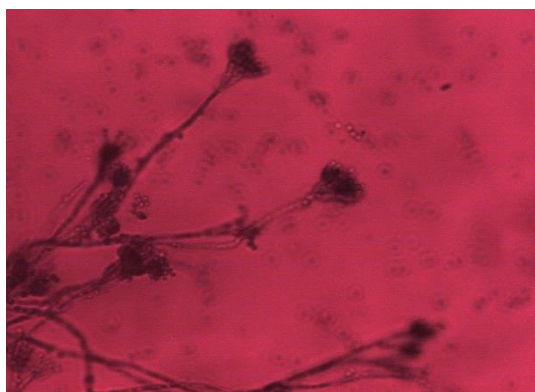
**Figure 15.** Light microscopic observation of fungi. *Phoma* sp.



**Figure 13.** Light microscopic observation of fungi. *Penicillium* sp. 1.



**Figure 16.** Light microscopic observation of fungi. *Ulocladium* sp.



**Figure 14.** Light microscopic observation of fungi. *Penicillium* sp. 2.

**Table 1.** The selected endophytic fungi.

Strain code	Strain name
SM-EF 1	<i>Alternaria alternate</i>
SM-EF 2	<i>Aspergillus flavus</i>
SM-EF 3	<i>A. terreus</i>
SM-EF 4	<i>A. niger</i>
SM-EF 5	<i>Cladosporium</i> sp.
SM-EF 6	<i>Fusarium</i> sp.
SM-EF 7	<i>Penicillium</i> sp.
SM-EF 8	Sterile mycelium
SM-EF 9	<i>Meyerozyma</i> sp.

rized in Tables 2 and 3, which revealed that endophytic fungi species possessed antibacterial and antifungal activity.

In antibacterial activity, SM-EF 3 crude extract showed high zone of inhibition of 11 mm against *S. typhi*, 10 mm against *S. aureus* and *V. cholera*, 9 mm against *E. coli* and *K. pneumonia*, 8 mm against *S. paratyphi*, low activity of 5 mm against *K. oxytoca*. SM-EF 7 crude extract showed a zone of inhibition of 11 mm against *S. typhi*, 10 mm against *V. cholera*, 9 mm against *E. coli*, 8

**Table 2.** Zone of inhibition produced by endophytic fungal strains on human bacterial pathogens.

Endophytic fungi	Zone of inhibition (mm)							
	<i>E. coli</i>	<i>P. mirabilis</i>	<i>S. typhi</i>	<i>S. paratyphi</i>	<i>V. cholera</i>	<i>K. oxytoca</i>	<i>K. pneumonia</i>	<i>S. aureus</i>
SM-EF 1	5.6±0.57	-	-	-	6±1	3±1	5.3±0.57	-
SM-EF 2	-	5.3±0.57	4.3±0.57	7±1	-	-	-	5.6±0.57
SM-EF 3	9.3±1.52	-	11.6±0.57	8.6±1.15	10±1	5.6±0.57	9±1	10.3±0.57
SM-EF 4	4±1	-	-	4±0	5.3±0.57	-	4.6±1.52	3.6±0.57
SM-EF 5	6.6±0.57	7.3±0.57	-	-	-	4.3±0.57	5±1	-
SM-EF 6	4.3±0.57	-	7±1	4±1	6.3±1.15	-	-	5.3±0.57
SM-EF 7	9.6±0.57	-	11.6±1.15	7.6±1.15	10.6±0.57	8.3±0.57	5.3±0.57	-
SM-EF 8	-	-	-	4.6±1.52	6.3±0.57	-	-	5.3±0.57
SM-EF 9	11.3±0.57	8.6±1.15	7.6±1.15	10.3±0.57	7±1	-	6.6±0.57	8.3±0.57

Values are average of three replicates ± SE.

**Table 3.** Zone of inhibition produced by endophytic fungal strains on human fungal pathogens.

Endophytic fungi	Zone of inhibition (mm)				
	<i>C. albicans</i>	<i>E. floccosum</i>	<i>M. canis</i>	<i>T. mentagrophyte</i>	<i>T. rubrum</i>
SM-EF 1	-	3.6±0.57	-	3.3±0.57	-
SM-EF 2	2.3±0.57	5.6±0.57	-	-	-
SM-EF 3	4.6±0.57	-	6.3±0.57	3.6±0.57	8.3±1.52
SM-EF 4	-	2.3±0.57	-	-	-
SM-EF 5	-	-	-	5±1	2.3±0.57
SM-EF 6	-	-	3.3±0.57	2±0	-
SM-EF 7	5.3±1.15	4.6±0.57	6.6±0.57	-	3.6±0.57
SM-EF 8	3.3±0.57	-	-	-	-
SM-EF 9	-	6.3±1.15	7.6±1.15	4.3±0.57	6.6±0.57

Values are average of three replicates ± SE.

mm against *K. oxytoca*, 7 mm against *S. paratyphi*, 5 mm against *K. pneumonia*. SM-EF 9 crude extract showed a zone of inhibition of 11 mm against *E. coli*, 10 mm against *S. paratyphi*, 8 mm against *P. mirabilis* and *S. aureus*, 7 mm against *S. typhi* and *V. cholera* and 6 mm against *K. pneumonia* (Table 2).

In antifungal activity, SM-EF 3 crude extract showed a zone of inhibition of 8 mm against *T. rubrum*, 6 mm against *M. canis*, 4 mm against *C. albicans*, 3 mm against *T. mentagrophyte*. SM-EF 7 crude extract showed only a zone of inhibition of 6 mm against *M. canis*, 5 mm against *C. albicans*, 4 mm against *E. floccosum* and 3 mm against *T. rubrum*. SM-EF 9 crude extract showed only a zone of inhibition of 7 mm against *M. canis*, 6 mm against *E. floccosum* and *T. rubrum*, 4 mm against *T. mentagrophyte* (Table 3).

## DISCUSSION

In the present study, endophytic fungi of halophytes were investigated on two plant species (*S. maritima* and *S.*

*monoica*). 433 isolates were produced in culture of *S. maritima* grouped into 15 taxa, 422 isolates were produced in culture of *S. monoica* grouped into 14 taxa. Suryanarayanan and Kumaresan (2000) isolated endophytic fungi of some halophytes from an estuarine mangrove forest. Kumaresan and Suryanarayanan (2001) reported the fact that the species diversity of the endophytes varied in different mangrove hosts indicating that a selection mechanism was operating in constituting the endophyte assemblages.

Discovery of endophytic fungi in plant tissues opened up new possibilities in search for metabolically active compounds. Cuomo et al. (1995) examined a large number of terrestrial and marine fungal isolates and found a higher number of anti-microbially active species among marine isolates. Endophytes or any type of fungus are capable of producing novel secondary metabolites as the reports says many of the endophytes are still unknown and the compound are produced by the respective fungus are still unknown. So with this view, the salt marsh endophytic fungus is taken for testing its production for secondary metabolites.

Antimicrobial activities of compounds biosynthesised by the plant endophytes have been reported only by few researchers (Nishioka et al., 1997; Huang et al., 2001; Strobel et al., 2001; Harper et al., 2003). The bioassay method is very useful for applying the screening for antimicrobial and antifungal activities of secondary metabolites from various natural sources and it is quick and easy method (Jagessar, 2007; Kobayashi et al., 1996).

In the present study, nine crude extracts of endophytic fungal species were tested against the human bacterial and fungal pathogens by well diffusion method. All crude extracts of endophytic fungal species showed activity against human bacterial and fungal pathogens. Guimaraes et al. (2008) screened extracts from 39 endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia*, and reported 5.1% active extracts against *S. aureus* and 25.6% active extracts against *E. coli*. Similarly, several metabolites of the marine isolate, *A. niger* showed anti-bacterial and anti-fungal potential.

In this study, 70% of endophytic fungi showed antibacterial and antifungal activity against at least one of the test human pathogens, which was coincidence with our previous report (Liu et al., 2001). The ethyl acetate extracts were assayed against the test organisms, the zones of inhibition obtained in this study was between 2 and 12 mm. The maximum zone of inhibition was  $11.6 \pm 0.57$  mm against *S. typhi*. Selim et al. (2011) reported ethyl acetate crude extract of 55 endophytic isolates (55.5%) of 99 screened strains, exhibited significant inhibitory activity against a wide range of pathogenic test microorganisms, with diameters of inhibition zones ranging from 9 to 27 mm for the test bacteria, and from 8 to 31 mm for test *Candida* on disc diffusion assay.

Antimicrobial activity of *A. terreus*, *Meyerozyma* sp. and *Penicillium* showed significant effect on different Gram positive and negative bacteria and on different fungi. These endophytes can reduce the growth of the harmful bacteria or fungi by different mode of action. Our results correlated with the findings of other reports (Verma et al., 2009; Wiyakrutta et al., 2004; Corrado and Rodrigues, 2004; Li et al., 2008; Ramasamy et al., 2010) which they reported the antimicrobial activity of endophytes. Similarly, Prabavathy and Valli Nachiyar (2012) studied the crude extract from culture filtrate of the endophytes from *Plumbago zeylanica*, *Aegle marmelos* and *Ficus carica* that showed considerable activities, the mycelial as well as culture filtrate extract of endophyte isolated from *Ficus carica* alone showed appreciable antibacterial activities against *Pseudomonas aeruginosa*.

## Conclusion

Salt marsh plants harbours diverse species of endophytic fungi and some of these isolates exhibited significant

inhibitory activity on human pathogenic microorganisms. The study revealed the presence of good antibacterial activity for the crude extracts SM-EF 3, SM-EF 7 and SM-EF 9 could be a good source for bioactive compounds and the isolated compounds may be further checked in *in vivo* model as antimicrobial agents.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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A microscopic view of various bacteria, including rod-shaped and spiral-shaped organisms, set against a dark, textured background. The bacteria are illuminated with a warm, orange-red light, highlighting their intricate structures and textures.

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