Isolation of endophytic fungi of marine macro algae from the Southeast coast of India and investigation of their anti-microbial potential.

P.Keerthana[a], A.K. Kathireshan[a,*] and Abiramavalli Moorthy[b]

[a]- Research scholar, Department of Microbiology, Vels Institute of Science Technology & Advanced Studies (VISTAS) Chennai.

[a*]- Corresponding author, Department of Microbiology, Vels Institute of Science Technology & Advanced Studies (VISTAS) Chennai.

[b]- Assistant Professor, Dhanalakshmi Srinivasan Agriculture College (affiliated to TNAU), Perambalur-621212

Abstract

Background: Marine fungal endophytes are an important repository of novel bioactive compounds and secondary metabolites which could be potential drug targets. Among all the marine organisms, macro algae (seaweeds) is the paramount sources of fungal endophytes. Endophytic fungi have a strong mutualistic association with their host by secreting bioactive compounds that aids the host in stress resistance and tolerance.

Objectives: The present study aims at isolation of endophytic fungi from two seaweeds and to observe the antimicrobial activity of the fungal extracts.

Methods: In this study, two seaweeds *Haloplegma* and *Halymenia* were collected from Mandapam coast, Ramnad district, Tamil Nadu, India. The endophytic fungi were isolated from the seaweed samples following standard procedure and the pure culture of the isolates were characterized based on the spore morphology. The crude extracts of the fungal isolates were extracted using hexane and screened for its anti-microbial activity through well-diffusion assay.

Results: A total of four fungal endophytes from isolated from the two seaweeds. They were morphologically identified as *Alternaria sp., Mucor sp., Cladosporium sp.,* and *Aspergillus sp.* The fungal extracts at varying concentration ranging from 100 µg-1000µg showed varying degrees of antimicrobial activity against four different pathogens viz., *Penicillium* sp., *Aspergillus flavus, Escherichia coli,* and *Staphylococcus aureus*. The Minimum inhibitory concentrations (MICs) were determined, that ranged from 0.1-1 mg of fungal extracts against the test pathogens.

Conclusions: Our study is the first report to isolate endophytic fungi from *Haloplegma duperreyyi* seaweed. Our observations suggest that these fungal endophytes could be promising sources for metabolites with anti-microbial potential. Nonetheless, further detailed studies on other bioactive potentials of these fungal extracts would pave way for discovery of new antibiotics for a wide spectrum of infections.

Keywords: Endophytic fungi, Macro algae, Secondary metabolites, Anti-microbial activity, Pathogens.

1. INTRODUCTION:

In the modern era of medicine, natural products derived from plants, microbes and marine creatures aid in treatment and management of several infections and diseases [1]. Especially, as antibiotic resistance among bacterial and fungal pathogens is alarmingly high in the recent times, there is an increasing demand for identification of novel antibacterial and antifungal compounds derived from natural sources [2].

Marine environment is considered as one of the significant sources of natural products with bioactive potentials [3]. The biodiversity of marine organisms is very high with almost 60 out of 76 phyla of *Eukariota* belong to the marine environment [4]. Correspondingly, natural products derived from microbes exemplify an extensive zone for novel therapeutic compounds hunt [5]. The secondary metabolite compounds secreted by the microorganisms such as bacteria, and fungi deed as targets for discovery of novel drugs for bacterial, viral and fungal infections and cancers [6, 7].

Marine microbes have been proven as an endowed natural source for secretion of novel bioactive compounds and metabolites that could potentially be drug candidates [8]. In the recent times, several bioactive compounds from marine bacteria and fungi have been proven to have antimicrobial, antifungal, anticancer, and antiviral activities [9, 10, 11].

Besides, marine organisms exhibit tolerance to extremities such as salinity, chemical pollution, acidification and climate change through their mutualistic association with endophytic microbes. Endophytic microbes reside inside the host organism without affecting the host and in turn the host regulates the metabolism of these endophytes to secrete molecules that are involved in protective functions of both [12]. Lately, endophytic fungi have gained importance owing to their ability to produce a myriad of medicinally significant metabolites [9].

The marine macro algae or sea weed has been well-demonstrated as a tremendous source of endophytic fungi [13]. Fungal endophytes isolated from the seaweeds exhibited enormous medicinal potential such as antimicrobial, antifungal and anticancer properties [14, 15]. The southern coastal area of India embodies a sundry source of seaweed derived fungal endophytes. Though the endophytes of East and West coast of India have been investigated for drug mining, the southern part has not yet well explored [16]. Thus, the present study has intended to evaluate the antimicrobial potential of the endophytic fungi associated with two important marine seaweeds *Haloplegma duperreyi montagne* and *Halymenia dilatata zanadarini* found in South coast of India (Tamil Nadu).

Figure.1 *Haloplegma duperreyi montagne*

Figure.2 *Halymenia dilatata zanadarini*

Halymenia dilatate zanadarini belongs to the family *Halymeniaceae* and is well distributed in the coastal regions of Asia, Pacific Islands, Africa, Australia and New Zealand.

The seaweed appears luminous, supple, slippery and slimy clusters of sheets, with 10-20cm long thallus. Based on the geographical location where it is distributed, the color of the thallus may vary ranging from brownish-orange, dark pink to reddish [17].

Haloplegma duperreyi montagne, belong to the family *Wrangeliaceae* and is commonly found in the coastal areas of Indian Ocean viz., Aldabra Islands, Cargados Carajos, India, Kenya, Maldives, Mauritius, Réunion, Seychelles, South Africa, Sri Lanka [18].

2. MATERIALS AND METHODS:

2.1 Collection of seaweed samples:

The 2 seaweed species were collected from Mandapam, Ramanathapuram district - Coordinates: 9.28°N 79.12°E. The seaweeds were differentiated and collected based on their morphology and colour. The samples collected, washed immediately with sterile water to eliminate exogenous microbes present if any before isolation of endophytes and were transferred to polythene zip-lock covers and kept in ice until transported to the laboratory.

2.2 Isolation of endophytic fungi from seaweed samples:

Both the seaweed samples were cut into small pieces and surface sterilized with 70% ethanol and sterile water. The two samples were then placed aseptically on to individual Zobell marine agar medium plates using sterile forceps. The plates were sealed and stored at 25°C for two weeks [19]. Different fungal growth were observed from the two plates. The distinct fungal growth was secluded by taking away the hyphal tips growing out of the cut seaweed pieces using a sterile loop onto a fresh Zobell marine agar medium plate.

2.3 Morphological Identification of fungal endophytes isolated:

The isolated fungal endophytes were observed under a microscope to identify the genus of the fungi by comparing it with standard articles. They were macroscopically differentiated from each other by their external characteristics [20]. All the pure isolates were recognized and maintained in Potato dextrose agar (PDA) slants for future use.

2.4 Collection of crude extracts from the fungal endophytes isolated:

Mass multiplication of the isolates were performed from pure culture plates using Zobell marine broth [21]. The cultures were incubated for 3 days followed by liquid-liquid extraction using a suitable non-polar solvent (100ml of hexane) [22]. The extracts were then concentrated at their boiling points using rotary evaporator.

2.5 Screening of crude extracts for anti-fungal activity:

Agar well diffusion assay was performed to evaluate the antifungal activity of the endophytic cultures against the two fungal pathogens viz., *Penicillium sp.* and *Aspergillus flavus*. Swabs moistened with the pathogenic suspensions containing each culture were used to spread onto the Czapek Dox Agar plates. 8mm diameter wells were made on the medium. The crude hexane extracts of the fungal endophytes were dissolved in DMSO (10mg/ml stock). The extracts were added to the wells in varying concentrations (100-1000 µg/well). A well holding DMSO was considered as negative control and standard drug fluconazole (30 µg/ml stock) was used as the positive control. The plates were kept at room temperature for 2 hours for diffusion. Then the plates were incubated at 20-25℃ for 72 hrs. The diameters of zones of inhibition were calculated in millimetres after incubation [22].

2.6 Screening of crude extracts for anti-bacterial activity:

Agar well diffusion assay was performed to evaluate the antibacterial activity of the endophytic cultures against the two bacterial pathogens viz., *Escherichia coli*, and *Staphylococcus aureus*. Swabs moistened with the pathogenic suspensions containing 106 cfu/ml of each culture were used to spread onto the Nutrient Agar plates. 8mm diameter wells were made on the medium. The crude hexane extracts of the fungal endophytes were dissolved in DMSO (10mg/ml stock). The extracts were added to the wells in varying concentrations (100-1000 µg/well). A well holding DMSO was considered as negative control and standard drug Ampicillin (30 µg/ml stock) was used as the positive control. The plates were kept at room temperature for 2 hours for diffusion. Then the plates were incubated at 37℃ for 24 hrs. The diameters of zones of inhibition were calculated in millimetres after incubation [22].

3. RESULTS:

3.1 Samples collected:

A total of 2 seaweeds *Haloplegma duperreyi montagne* and *Halymenia dilatata* were collected from Mandapam, Ramanathapuram coast, Gulf of Mannar, India. Coordinates - 9.28°N 79.12°E were shown in (Table 1), (Figure 1 &2).

Table 1. Seaweeds collected from Mandapam, Ramanathapuram district.

S.NO	Location	Seaweed name
1	Mandapam, Ramanathapuram Dt.	*Haloplegma duperreyi montagne*
2	Mandapam, Ramanathapuram Dt.	*Halymenia dilatate zanadarini*

3.2 Fungal endophytes pure culture isolation from collected seaweeds:

About 4 marine endophytic fungi (HP1, HP2, HP3, and HD1) were isolated from the two collected samples using standard procedure [19]. The pure culture of the fungal isolates were maintained in PDA (Figure.3).

3.3 Morphological characterization of the fungal endophytic pure cultures:

Microscopic observation of the spores of the isolates using LPCB assay identified and confirmed the genus of the isolates (Figure.4). Morphological characterization revealed the various genus of the pure isolates of fungal endophytes (Table 2).

Figure.3. Macroscopic view of the spores of the pure fungal endophytic cultures.

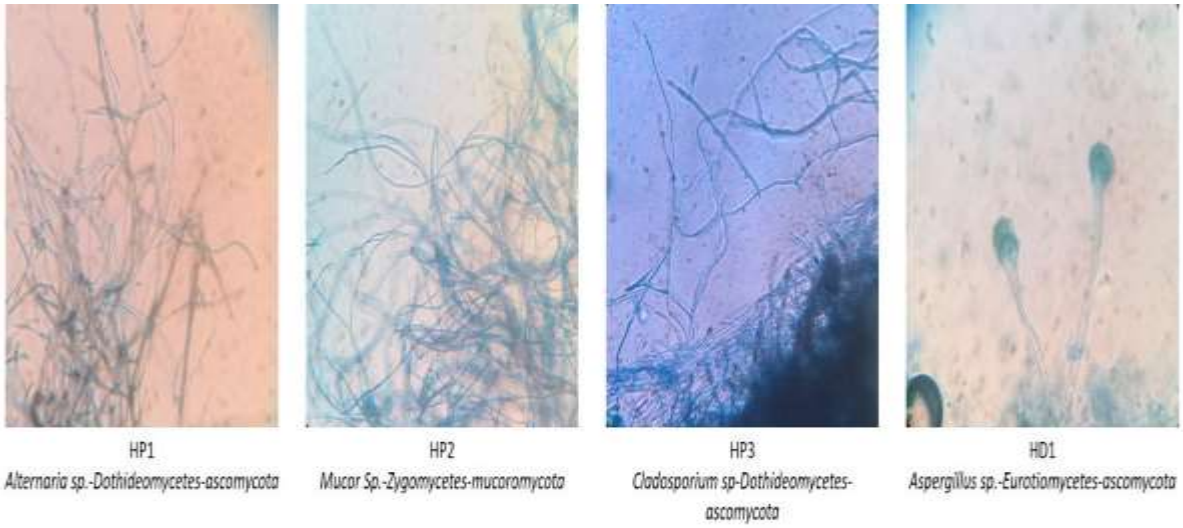

Figure 4. Microscopic view of the isolated fungal endophytes from *Haloplegma duperreyi* (HP1, HP2, HP3) and *Halymenia dilatate zanadarini* (HD1)

Table 2. Morphological Identification of marine fungal endophytes isolated from collected seaweeds.

S.NO	Collected seaweeds	Fungal endophytes identified as:
1	*Haloplegma duperreyi montagne*	HP1- *Alternaria sp.* HP2- *Mucor sp.* HP3- *Cladosporium sp.*
2.	*Halymenia dilatate zanadarini*	HD1- *Aspergillus sp.*

3.4 Extraction of metabolites from fungal endophytes:

The pure isolates of fungal endophytes isolated from this study were subjected to crude extraction using hexane solvent. Hexane was considered as a suitable solvent for this study based on the previous reports [22]. The concentrated crude extracts were stored at -20°C for further analysis.

3.5 Screening of endophytic extracts for anti-microbial activity using well diffusion assay:

Anti-microbial activity (anti-fungal and anti-bacterial) tests revealed that zones of inhibition of varied concentration (100-1000 µg) of the crude extracts of the isolates (HP1, HP2, HP3, and HD1) against the test pathogens *Penicillium sp*., *Aspergillus flavus*, *Escherichia coli*, and *Staphylococcus aureus* were varying from 11-20mm (Figure.5 & Figure.6). The fungal endophytic extracts demonstrated maximum zones of inhibition against the pathogens at 1mg (Table 3). While, the minimum concentrations for forming inhibitory zone were wide-ranging (0.1mg-1mg) for different pathogens (Table 4). Based on this, the MICs were determined for each extract against the test pathogens (Table 5).

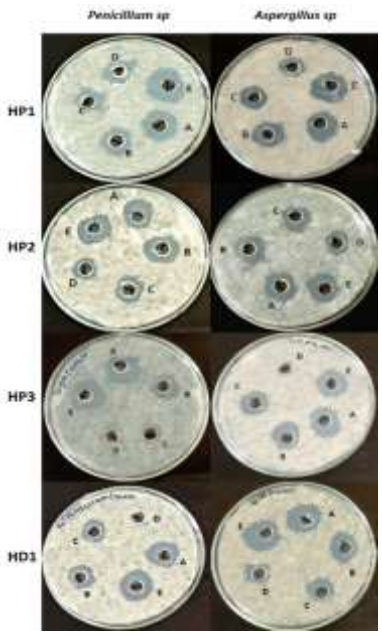

Figure.5. Well diffusion assay of endophytic fungal extracts illustrating the zone of inhibition in diameters against the test fungal pathogens.

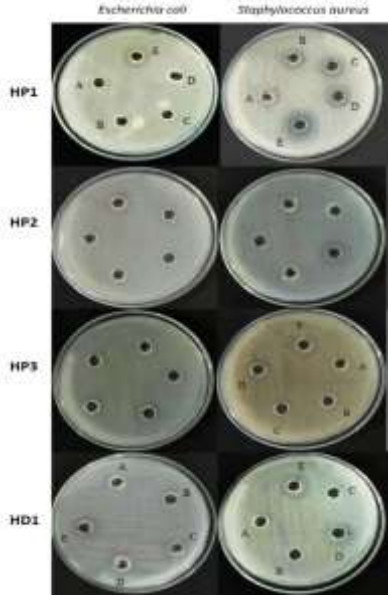

Figure.6. Well diffusion assay of endophytic fungal extracts showing the zone of inhibition in diameters against the test bacterial pathogens.

Table 3. Anti-microbial activity of fungal endophytes crude hexane extracts at their maximum inhibition zones diameter.

	Zone of inhibition in mm at 1000µg				**Positive control**	**Negative control**
Fungal pathogens	**HP1 extract**	**HP2 extract**	**HP3 extract**	**HD1 extract**	**Fluconazole**	**DMSO**
Penicillium sp.	17	20	16	19	19-23	0
A.flavus	19	16	19	17	19-21	0

Bacterial pathogens	**HP1 extract**	**HP2 extract**	**HP3 extract**	**HD1 extract**	**Ampicillin**	**DMSO**
E.coli	-	14	11	12	17-19	0
S.aureus	19	13	14	14	19-22	0

Table 4. Inhibitory concentrations of the endophytic fungal extracts against the test pathogens

Extract concentration	**HP1** ***Alternaria sp.***				**HP2** ***Mucor sp.***				**HP3** ***Cladosporium sp.***				**HD1** ***Aspergillus sp.***			
	P	***A***	***E***	***S***	***P***	***A***	***E***	***S***	***P***	***A***	***E***	***S***	***P***	***A***	***E***	***S***
100 µg	+	+	-	-	+	+	-	-	-	-	-	-	+	-	-	-
250 µg	+	+	-	-	+	+	-	-	+	-	-	-	+	+	-	-
500 µg	+	+	-	+	+	+	-	-	+	+	-	+	+	+	-	-
1000 µg	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+

Test pathogens: ***P*** **–***Penicillium sp.*, ***A*****-** *A.flavus*, ***E*****-***E.coli*, ***S*****-***S.aureus*

\+ Zone of inhibition present**, –** Zone of inhibition absent

Table 5. Minimum Inhibitory Concentrations (MICs) of fungal extracts

4. DISCUSSION:

A total of 4 fungal endophytes were isolated in this study, from 2 macro algal species belonging to 2 different algal families sampled from the same location in Tamil Nadu, India (Table 1). Collection sites and seaweed species were chosen randomly. All the isolated endophytes were screened for their anti-microbial potential against a variety of bacterial and fungal pathogens (two bacterial and two fungal). Morphological characterization based on the macroscopic and microscopic view of the isolates, revealed that HP1, HP2, HP3, and HD1 as *Alternaria sp., Mucor sp., Cladosporium sp.* and *Aspergillus sp.* respectively (Table 2).

Generally, endophytic microbes isolated from marine plants might be highly diverse. Number of endophytes and genus/species variations are owing to several natural extremities such as salinity, temperature, sunlight, water pollution, geographical location and also the genus of the marine algae [23, 24]. Correspondingly our study has demonstrated that though the two seaweeds were from the same location they were inhabited by varying number of endophytic fungi (Table 2). This supports the fact that environmental factors are involved in the diversity and abundance of endophytes.

The four isolated endophytic fungi *Alternaria sp., Cladosporium sp., Aspergillus sp.* and *Mucor sp.*, belong to three different classes viz., *Dothideomycetes, Eurotiomycetes* and *Zygomycetes*. The classes *Dothideomycetes, Eurotiomycetes* belong to *Ascomycota* division and *Zygomycetes* belong to *Mucoromycota* division. The *Alternaria sp., Mucor sp., Cladosporium sp.* were isolated from *Haloplegma duperreyi montagne* whereas *Aspergillus sp.* was isolated from *Halymenia dilatata zanadarini.*

In an earlier report from Japan, has recorded the presence of endophytic fungi *Fusarium sp.* and *Acremonium sp.* from *Halymenia dilatate zanadarini.* They have also isolated an

antimicrobial substance halymecins from those fungal endophytic cultures [25]. This is the only report on isolation of endophytic fungi from *Halymenia dilatate zanadarini* whereas there are no such research records available for *Haloplegma duperreyi montagne.* Our study is the first to isolate endophytic fungi from *Haloplegma duperreyi montagne* and to isolate *Aspergillus sp.* from *Halymenia dilatata zanadarini.*

Lately, substantial attention has been given to isolation marine endophytic fungi owing to their bioactive potential and this has led to numerous research works involving isolation of endophytic fungi from plants, and macro algal species. In addition, owing to the significance of marine derived metabolites, the exploration of bioactive potential of endophytic fungi has been emphasized recently [26-29].

As per our observations from the present study, extracts of all the fungal endophytes studied (HP1, HP2, HP3, and HD1) have well-demonstrated anti-microbial (both bacterial and fungal) activities at 1mg concentration (Table 3). Extract of *Alternaria sp.* has shown a strong anti-fungal potential at all the concentrations tested (0.1-1mg) though it does not show anti-bacterial activity against one of the test pathogens. Similarly, the extract of *Mucor sp.* (HP2) has exhibited a good anti-fungal potential although it demonstrated anti-bacterial property at 1mg. The extract of *Cladosporium sp.* (HP3) has shown a better anti-fungal activity at 0.25-0.5 mg whereas the antibacterial activity was demonstrated at 1 mg (Table 4). Similarly the extract of *Aspergillus sp.* (HD1) has exhibited an excellent anti-fungal ability at 0.1-0.25 mg while it showed antibacterial activity at 1 mg. Based on these observations, the MICs of these extracts were determined (Table 5).

Our results suggest that the fungal endophytes isolated in this study possess compounds which could be rich sources of anti-fungal drugs. Nevertheless, these fungal extracts also have anti-bacterial activity at a concentration of 1 mg or more against the two common bacterial human pathogen. Thus, further research on isolation of individual compounds from these extracts and studying their effects would aid in discovery of new anti-microbial drugs for fungal and bacterial infections.

5. CONCLUSION:

Our study has unveiled the presence of different fungal endophytes from the two seaweeds *Haloplegma duperreyi montagne* and *Halymenia dilatata zanadarini* found in South coast of India (Tamil Nadu). This is the first report on isolation and analysis of endophytic fungi from Haloplegma. Our observations suggest that these fungal endophytes could be promising sources for metabolites with anti-microbial potential. Nonetheless, further detailed studies on other bioactive potentials of these fungal extracts and isolation of individual metabolites from these extracts would pave way for discovery of new antibiotics for a wide spectrum of infections.

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7. AUTHOR CONTRIBUTIONS :

All authors made substantial contribution to conception and design acquisition of data, and interpretation of data.

8. FUNDING :

There is no funding to report.

9. CONFLICTS OF INTEREST :

The authors declare that there is no conflict of interest.

10. ETHICAL APPROVALS:

This study does not involve experiments on animals or human subjects.

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