SCREENING OF MARINE ENDOPHYTIC FUNGI ISOLATED FROM SOME SEAGRASSES LEAVES AND THEIR ANTIBACTERIAL ACTIVITIES ON *MICROCOCCUS LUTEUS* NITE83297

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Abstract

The isolation of endophytic fungi and antibacterial activities of some seagrasses species such as *Halophila ovalis* (R.Brown) Hooker.f, *Thalassia hemprichii* (Ehrenberg) Ascherson and *Syringodium isoetifolium* (Ascherson) Danty were used for the investigation. The samples of seagrasses were collected from (Lat 17˚04'20.36" N and Long 94˚27'08.01"E), Magyi coastal area, Shwe Thaung Yan Sub-township which is located at lower Rakhine Coast. The experimented seagrass samples were stored as herbarium sheets in Marine Science Department, Pathein University. Screening of marine endophytic fungi from seagrass species was carried out by Washing Method . A total of 12 marine endophytic fungi were isolated in this study, 4 different fungi from *Halophila ovalis*, 5 different fungi from *Thalassia hemprichii* and 3 from *Syringodium isoetifolium*. Paper disc diffusion assay method was carried out in the investigation of antibacterial activities. Among the isolation of endophytic fungi, 7 endophytic fungi named KF-01, KF-03, KF-06, KF-07, KF-08, KF-10 and KF-11 showed the antibacterial activities on *Micrococcus luteus* NITE83297.

Key words: antibacterial activities, endophytic fungi, *Halophila ovalis, Micrococcus luteus, Syringodium isoetifolium, Thalassia hemprichii*.

Introduction

Endophytes are microorganisms that can produce the chemical inside the plants (Owen and Hundley 2004). Many of them are capable of synthesizing bioactive compounds that can be used as potential sources of pharmaceuticals leads. Endophytic fungi have been proven useful for novel drug discovery as suggested by the chemical diversity of their secondary

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metabolites. Many endophytic fungi have been reported to produce novel antibacterial, antifungal, antiviral, anti-inflammatory, antitumor, and other compounds (Guo et *al*., 2008, Yu et *al*., 2010). Endophytes are universally present in the world as higher plants, so it was reasoned that plants might support certain endophytic microorganisms that could synthesize important phytochemicals of medicinal plants as well as the plant itself (Strobel,2003).

Plants can be considered as a new isolation source of microorganisms. Among the plants species, seagrasses are also a suitable host for a variety of endophytes. Seagrasses are angiosperms which grow in shallow saline water in tropical marine coastal areas. There are 13 genera and 58 species available all over the world. However, fewer studies have been done on endophytes in seagrasses as difficult to assess and available for seagrasses (Ravikumar et *al*., 2005).

In Myanmar, seagrasses as a relatively small group of flowering plants distributes along the shallow saline coastal regions particular in Rakhine coast and southern Myeik Archipelago. There are 10 species of seagrasses were recorded in Myanmar, according to U Soe Tun, 2016. Seagrasses are also a rich source of secondary metabolites. It is now well documented that endophytic fungi are a good source of bioactive natural products. Most of the studied endophytic fungi have been isolated from terrestrial plants. Bioactive natural products from endophytic fungi from marine plants in particular from seagrasses have been rarely studied (Preuttipon et *al*., 2014).Therefore, the isolation of endophytic fungi from *Halophila ovalis* (R.Brown) Hooker.f, *Thalassia hemprichii* (Ehrenberg) Ascherson and *Syringodium isoetifolium* (Ascherson) Danty from lower Rakhine Coast has been carried out and antibacterial activity of those endophytic fungi were also studied.

Materials and Methods

Sample Collection

Seagrass species such as *Halophila ovalis* (R.Brown) Hooker.f, *Thalassia hemprichii* (Ehrenberg) Ascherson and *Syringodium isoetifolium* (Ascherson) Danty were collected from Magyi coast (Lat 17˚04′ N and Long 94˚27′ E)**,** Shwe-Thaung-Yan Sub-Township, Pathein Township, Ayeyarwady Region. The seagrasses were collected at low tide time of spring tide period as the plants are mostly growing at the subtidal region. At low tide, the plant samples were collected by hand and carried by ice box with natural seawater. In the laboratory, the collected samples were washed thoroughly by using seawater to remove epiphyte, sand, mud and debris for the observation and identification. The samples were preserved with 4% formalin and prepared for herbarium and wet-stacked specimens. These specimens are deposited at the Herbarium of Department of Marine Science, Pathein University and identify based on the external morphologies of vegetative features as compared with other related voucher materials also housed in the same Herbarium. The external morphologies of these specimens were photographed by using a digital camera and the fresh samples were then used to isolate the microorganisms in the laboratory to get superior endophytes.

Figure 1. Map showing the location of sample collecting area

Screening of Marine Endophytic Fungi From Seagrasses

Seagrasses *Halophila ovalis* (R.Brown) Hooker.f, *Thalassia hemprichii* (Ehrenberg) Ascherson and *Syringodium isoetifolium* (Ascherson) Danty were employed for the isolation of endophytic fungi. The isolation was carried out by washing method (Inaba and Ando, 2002). Firstly, the leaves, rhizomes and roots of seagrasses were rinsed with tap water for 15 minutes in the laboratory. The plant parts (leaves and rhizome) were then cut into pieces and then again rinsed by 95% ethanol and 1% sodium hypochloride solution for 5 minutes. It was then washed by 95%ethanol for 15 seconds. Finally, the plants were then stirred and washed with sterile water on vortex mixer. Then, the samples were dried on sterilized filter paper. The dried samples were then cut at the edge on glass plate and placed onto the agar medium. The agar plates were incubated for 3-7 days at 27˚C according to the range of fungi, they start growing on LCA medium. The endophytic fungi were stored in slant culture using potato dextrose agar medium (PDA). After autoclaving chloramphenicol and Penicillin-G were added to the medium.

LCA medium (Ando, 2004)	
Glucose	1.0 g
Sucrose	0.5 g
Yeast Extract	1.0 g
K_2HPO_4	0.2 g
$MgSO_4.7H_2O$	0.05 g
KNO_3	0.1 g
KCl	0.05 g
Agar	1.8 g
DW	100 mL
pH	6.5

PDA medium (Ando, 2004)	
Potato	20 g
Dextrose	1.5g
Agar	1.8 g
DW	100 mL

Antibacterial Activity by Paper Disc Diffusion Assay

Screening (or) Preliminary study for antimicrobial activities was carried out by paper disc diffusion assay (Tomita, 1988). Paper disc having eight millimeter diameter were used for antimicrobial assays. The isolated fungi were grown on GSY medium for 7 days at 25°C for sporulation. Then isolated fungi were inoculated on seed medium and incubated at 25°C for 3 days. Then, 20 mL of seed culture was transferred again into the fermentation medium and fermentation was undertaken at 25°C for 7 days. After that, fermented broth (20µl) was put onto the paper discs (8.0 mm) and allowed to dry. The dry paper discs were placed on assay plates containing test organisms for 24 hours. The assay medium was used for the antimicrobial activity test and one percent of test organism was added to assay medium, then poured into plates. After solidification, paper discs impregnated with samples (fermented broth) were applied on the agar plates and the plates were incubated for 24-36 hours at 28 to 30°C. The clear zones (inhibitory zones) surrounding the test discs can be seen in the next day indicating the presence of bioactive metabolites which inhibit the growth of test organisms.

Seed medium	
BR-BDC-Screening Media (2004)	
Glucose	2.0 g
Polypepton	0.3 g
KNO_3	0.1 g
K_2HPO_4	0.01 g
DW	100 mL
pH	6.5

Fermentation medium	
BR-BDC-Screening Media (2004)	
Glucose	2.0 g
Yeast Extract	0.8 g
K_2HPO_4	0.001 g
$MgSO_4$	0.001 g
$CaCO_3$	0.1 g
DW	100 mL
pH	6.5

Assay medium	
BR-BDC-Screening Media (2004)	
Glucose	1.0 g
Polypepton	0.3 g
KNO_3	0.1 g
Agar	1.8 g
DW	100 mL
pH	6.5

Results

Identification key to the species of seagrasses from Myanmar (U Soe Htun, 2016)

1a. Leaf blade cylindrical----------1. *Syringodium isoetifolium*
1b. Leaf blade flat----------2
2a. Leaves strap-shaped----------3
2b. Leaves paddle-shaped----------8
3a. Leaves with ligula----------4
3b. Leaves without ligula----------7
4a. Leaf tip without serration----------2. *Cymodocea rotundata*
4b. Leaf tip with serration----------5
5a. Leaves greater than 3mm wide----------3. *C.serrulata*
5b. Leaves less than 3mm wide----------6
6a. Leaf tip tridentate without secondary teeth; leaf blade more than 1mm wide----------4. *Halodule uninervis*
6b. Leaf tip with many secondary teeth; leaf blade less than 1mm wide----------5. *H. pinifolia*
7a. Leaves 10-15mm wide----------6. *Enhalus acoroide*
7b. Leaves 4-10 mm wide----------7. *Thalassia hemprichii*
8a. Leaves linear to lanceolate, 1-3 mm wide with 1-3 paralleled veins----------8. *Halophila beccarii*
8b. Leaves lanceolate to oblong, 3-10mm wide with 6-17 cross- veins----------9
9a. Leaf margin serrulate----------9. *H.decipiens*
9b. Leaf margin entire----------10. *H.ovalis*

***Halophila ovalis* (R.Brown) Hooker f.**

Division Anthophyta
Class Monocotyledoneae
Order Helobiae
Family Hydrocharitaceae
Genus *Halophila* Du Petit-Thouars
Species *Halophila ovalis* (R.Brown) Hooker f.
Common name- Paddle weed, spoon grass or dugong grass.

Description - Plants small; rhizomes less than 1mm in diameter, internodes 1.8-2.4cm long; erect shoot at each node, bearing a pair of petiolated leaves; leaf blades lanceolate to obovate or elliptic;1.5-2.2cm long 7-10mm wide, margin entire, apex obtuse, base rounded, petiole 2,2-3.0cm long, midrib prominent with 14-17 cross-veins.

***Thalassia hemprichii* (Ehrenberg) Ascherson**

Family Hydrocharitaceae

Genus *Thalassia* Banks and Solander ex Konig

Species *Thalassia hemprichii* (Ehrenberg) Ascherson

Common name- Turtle grass

Description - Plants moderate in size; intervals of internode 1.9-9.0 cm long; rhizome creeping, leaf blade linear, 1.5-15.0 cm long, 1.5-2.0 mm wide, leaf-tip obtuse, sometime serrulate,2-6 leaves, a thick rhizome prominently marked by several shoot scars between erect shoots.

***Syringodium isoetifolium* (Ascherson) Danty**

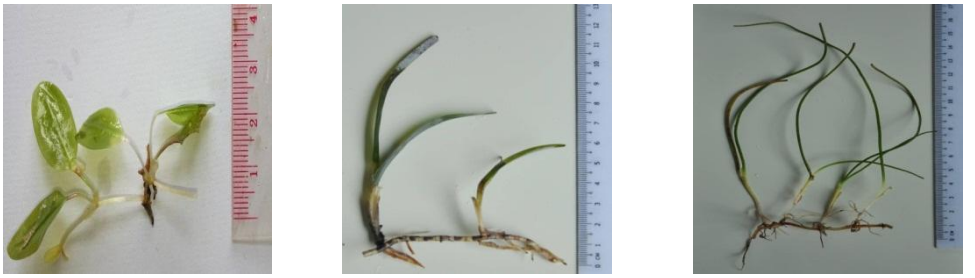
Family Cymodoceaceae

Genus *Syringodium* Kutzing

Species *Syringodium isoetifolium* (Ascherson) Danty

Common name- Noodle grass

Description - Plants erect; rhizome 1mm thick, with internodes, 1.4-2.5 cm long; each node giving a shoot with 1-3 leaves; leaves terete, tapering to the tip, 5.5-12.5 cm in length, 1mm wide, base covered by leaf sheath, 1-3 cm long.



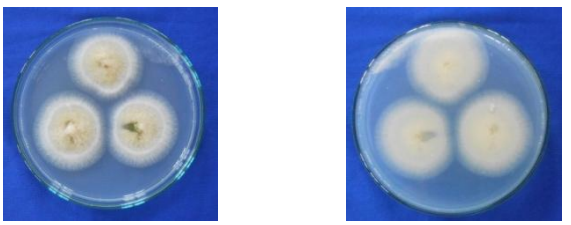
(A) (B) (C)

Figure 2. Morphologies of collected seagrasses ; **(A)** *Halophila ovalis* (R.Brown) Hooker f. **(B)** *Thalassia hemprichii* (Ehrenberg) Ascherson and **(C)** *Syringodium isoetifolium* (Ascherson) Danty

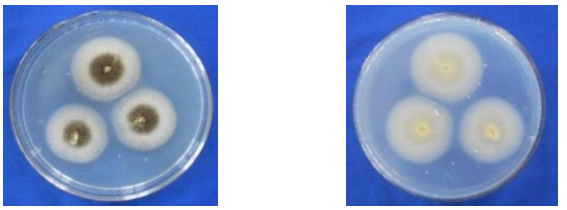
Screening of Marine Endophytic Fungi from Seagrasses

In the study of isolation of endophytic fungi, a total of 12 different fungi were isolated from seagrasses, 4 different fungi namely KF-01, KF-02, KF-03, KF-04 from *H.ovalis*, 5 different fungi from *T.hemprichii* namely KF-05, KF-06, KF-07, KF-08, KF-09 and 3 different fungi namely KF-10, KF-11, KF-12 from *S.isoetifolium*.

KF-01



KF-02



KF-03

KF-04

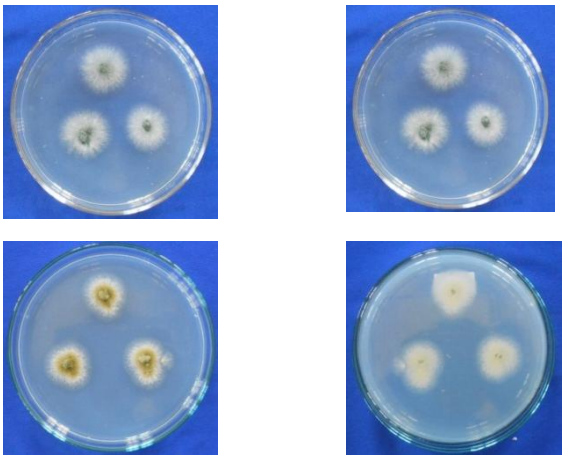
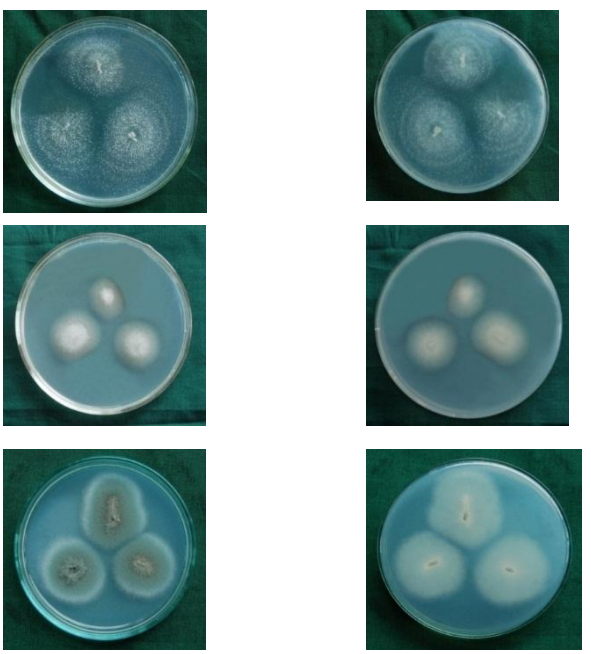


Figure 3. Morphologies of front view and back view of endophytic fungi from *H.ovalis* (KF-01, KF-02, KF-03 and KF-04)

KF-05

KF-06

KF-07

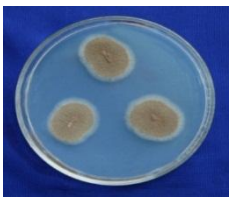


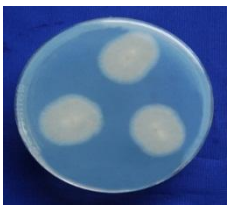
KF-08

KF-09

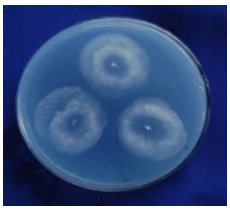
Figure 4. Morphologies of front view and back view of endophytic fungi from *T.hemprichii* (KF-05, KF-06, KF-07, KF-08 and KF-09)

KF-10





KF-11



KF-12

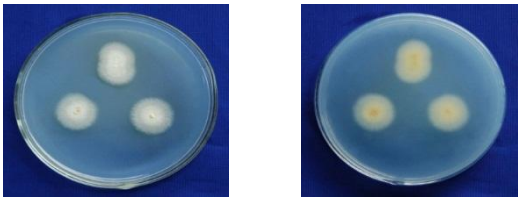


Figure 5. Morphologies of front view and back view of endophytic fungi from *S.isoetifolium* (KF-10, KF-11 and KF-12)

Antibacterial Activities by Paper Disc Diffusion Assay

In the investigation into antimicrobial activities of isolated fungi, KF-01, KF-03, KF-06, KF-07, KF-08, KF-10 and KF-11 showed the activities *Micrococcus luteus* NITE 83297. KF-01 (18.42 mm), KF-03 (21.06 mm), KF-06 (14.57 mm) , KF-07 (21.96 mm), KF-08 (21.72 mm), KF-10 (15.48 mm) and KF-11 (22.53 mm) against respectively.

KF-01

KF-03

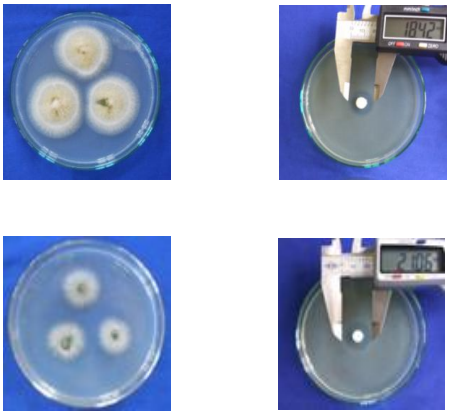


Figure 6. Morphologies and antibacterial activities of isolated endophytic fungi KF-01 and KF-03 from *H.ovalis* against on *M. luteus* NITE 83297

KF-06

KF-07

KF-08

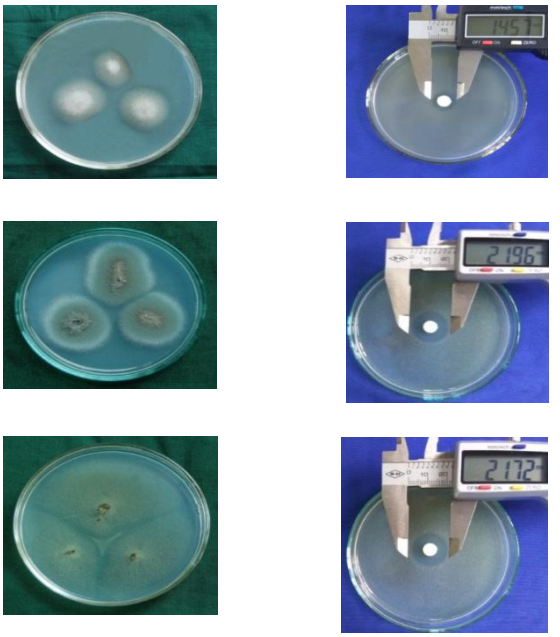


Figure 7. Morphologies and antibacterial activities of isolated endophytic fungi KF-06 ,KF-07 and KF-08 from *T.hemprichii* against on *M. luteus* NITE 83297

KF-10

KF-11

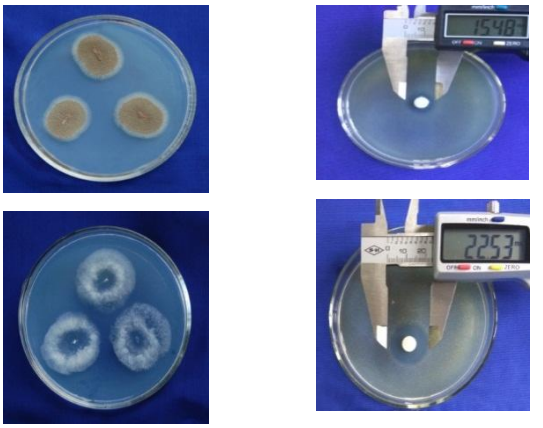


Figure 8. Morphologies and antibacterial activities of isolated endophytic fungi KF-10 and KF-11 from *S.isoetifolium* against on *M.luteus* NITE 83297

Discussion

A variety of medicines and chemical are also prepared from seagrasses and their associates. However, endophytic fungi from seagrasses have been rarely studied. Devarajan et *al*., 2002 stated that some endophytes have been isolated from the leaves of *Halophila ovalis*, *Zostera marina*, *Z. japonica*, *Thalassia testudinum* and *Posidonia oceanica* and from the rhizomes/roots of *H. ovalis* and *Halodule wrightii*. Preuttiporn et *al*.,2014 also reported that seagrasses have been used in traditional medicine in India: roots of *Enhalus acorides* have been applied as a remedy against stings from different kinds of rays and scorpions, *Cymodocea* spp has been used as a tranquillizer for babies, or for soothing help during pregnancy and against coughs and even malaria and some *Halophila* spp produce a strong traditional preparation that can act against malaria, skin diseases and the early stages of leprosy. Ravikumar, S. et *al*., 2010 showed that a new trend in drug discovery from natural sources (like marine seagrasses) emphasize on the investigation of marine ecosystem to explore numerous complex and novel chemical entities. In this study twelve different endophytic fungi remarked were isolated from seagrasses *Halophila ovalis* (R.Brown) Hooker.f, *Thalassia hemprichii* (Ehrenberg) Ascherson and *Syringodium isoetifolium* (Ascherson) Danty and in the investigation of antibacterial activities on Micrococcus luteus NITE83297, seven endophytic fungi showed the activities.

Conclusion

In the screening of endophytic fungi, 12 different fungi namely KF-01, KF-02, KF-03, KF-04, KF-05, KF-06, KF-07, KF-08, KF-09, KF-10, KF-11 and KF-12 were isolated from seagrasses *Halophila ovalis* (R.Brown) Hooker.f, *Thalassia hemprichii* (Ehrenberg) Ascherson and *Syringodium isoetifolium* (Ascherson) Danty, collected from from Magyi Coast, Shwe-Thaung-Yan Sub-Township, Pathein Township, Ayeyarwady Region. In the investigation of antibacterial activities of isolated fungi, fungus KF-01, KF-

03, KF-06, KF-07, KF-08, KF-10 and KF-11 showed antibacterial activity against on *Micrococcus luteus*NITE83297. The endophytic fungi KF-01 and KF-03 were isolated from *H.ovalis*, KF-06, KF-07 and KF-08 were isolated from *T.hemprichii* and KF-10 and KF-11 were isolated from *S.isoetifolium*. The isolated endophytic fungi KF-01showed (18.42 mm), KF-03 showed (21.06 mm), KF-06 showed (14.57 mm) , KF-07 showed (21.96 mm), KF-08 showed (21.72 mm), KF-10 showed (15.48 mm) and KF-11showed (22.53 mm) against respectively.

Acknowledgements

The author greatly appreciates Rector and Pro-rectors of Pathien University for their kind permission to do this research work. Special thanks goes to Dr.Cherry Aung, Professor and Head of Marine Science Department, Pathein University for her supporting and giving valuable suggestions. The author would like to express her sincere thanks to Dr. Zaw Linn Aung, Lecturer of Botany Department, Pathein University for his valuable suggestions, guidance and literature provided. Also sincere thanks goes to U Min Oo, Lecture of Marine Science Department of Pathein University for his suggestions and necessary helps.

References

Ando, K. (2004), **Isolation and identification of fungi**, Workshop , BRBDC- Pathein University.

Dawes, J. W. (1981), **Marine Botany**. Wiley Interscience, 629 pp

Devarajan: et *al*., (2002), **Endophytic fungi associated with the tropical seagrass *Halophila ovalis* (Hydrocharitaceae),***J.Indian, vol **31**(1),pp. 73-74*

Guo B, Wang Y, Sun X, Tang K. (2008), **Bioactive Natural Products from Endophytes:** A Review1**.** Applied Biochemistry and Microbiology 44, 136–142.Harayama, T. and K.

Inaba, S and K. Ando. (2004): **Washing method for the isolation of endophytes**.

Owen NL, Hundley N. (2004), **Endophytes-the chemical synthesizers inside plants.** Science Progress 87, 79–99.

Preuttiporn, S. , S.Phongpaichit V.Rukachaisirikul and J.akayaroj , (2014), **Diversity and antimicrobial activity of endophytic fungi isolated from the seagrass *Enhalus acoroides*.**Indian journal of Geo-Marine Sciences Vol.43(5), pp 785-797.

Ravikumar S, S. Nazar, A. Nuralshiefa and S. Abideen: (2005), **Antibacterial activity of traditional therapeutic coastal medicinal plants against some pathogens.** J. Environ. Biol., 26, 383-386.

Ravikumar, S. et *al*., (2010), **Bioactive potential of seagrass bacteria against human bacterial pathogens ,** *Journal of Environmental Biology,**31** ;pp.387-389*

Soe Htun U, (2016), **Seagrass Conservation and Monitoring in Myanmar Coastal Zones**, Training Course, Department of Marine Science, Pathein University.

Strobel, G. (2003), **Endophytes as sources of bioactive products.** Microbes and Infection , 535–544.

Tomita, F. (1988), **Fermentation method and paper chromatography**, Hokkaido University, Japan.

Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, Sun P, Qin L. (2010) **– Recent developments and future prospects of antimicrobial metabolites produced by endophytes.** Microbiological Research 165, 437–449.