Chemical and antimicrobial evaluations of a culture of *Phoma sp.*, an endophytic fungus isolated from *Rhizophora racemosa.*

Olusegun S. Ajala*[1], Afolabi S. Lamina[1], Titilope A. Adelekan[1] and Akinsinde K. Adewale[2]
[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Lagos, CMUL Campus, PMB 12003, Surulere, Lagos.
[2]Clinical Science Division, Nigerian Institute of Medical Research, 6,Edmund Crescent, Off Murtala Mohammed way, P.M.B. 2013, Yaba, Lagos.

ABSTRACT
Inner tissues of the aerial roots of the mangrove medicinal plant *Rhizophora racemosa* were subjected to standard fungal isolation techniques, leading to the isolation and subsequent large-batch culturing of an endophytic coelomycetous fungal morphotype, identified as *Phoma sp*. Extracts of the culture were obtained after a 14-day fermentation period, subjected to chemical evaluation using standard phytochemical methods and antimicrobial evaluation by agar diffusion techniques. Extracts of the fungal culture demonstrated broad spectrum antibacterial activities against Gram positive and Gram negative organisms and were found to be rich in diverse bioactive chemical groups including alkaloids, tannins, saponins and cardiac glycosides. A *Phoma sp.* is thus, herein, reported as the first from the endophytic community of *Rhizophora racemosa* and its culture recommended as a veritable lead source for the discovery of new broad spectrum antibacterial drugs and other therapeutic agents.

Keywords: Endophytic fungi culture, Antimicrobial activity, Natural products, *Rhizophora racemosa.*

INTRODUCTION

Emergence of new diseases, re-emergence of long-eradicated ones, rapid rate of development of resistant strains by deadly pathogens and poor toxicity profiles of many rather effective drugs are major therapeutic problems necessitating the adoption of less common approaches for discovering novel drugs with high therapeutic indices. One such approach is the isolation and biological screenings of secondary metabolites of endophytes.

Endophytes are microorganisms (mostly bacteria, actinomycetes and fungi) dwelling in plant tissues in diverse symbiotic associations, without any immediate symptomatic overt negative impacts on the plant (Petrini, 1991; Bacon and White, 2000). They represent a huge diversity of microbial adaptations developed in special sequestered environments (Ravindran et. al., 2012). The long term interaction that endophytes have with the defence mechanisms of the plant hosts is a potential selection pressure for the development of novel metabolic pathways in them (Tan and Zou, 2000). Endophytes are thus unique microorganisms with unique secondary metabolic pathways leading to diverse and novel chemical structures that could contribute in no small ways to providing solutions to the rather diverse health problems facing man today (Schulz et. al., 2002, Gunatilaka, 2006, Joseph and Priya, 2011).

Endophytic fungi, being essentially eukaryotes, are expected to have a more complex secondary metabolism than their actinomycetal and bacterial counterparts. In addition, fungi are generally more cultivable. These and other remote factors may be responsible for the greater focus on fungi in endophytic biology researches. Notwithstanding, there is yet a great work to be done in this area, given the fact that less than 1% of about 1million endophytic fungi estimated to be in existence have actually been isolated (Dreyfuss and Chapela, 1994; Strobel et. al., 2005). There is therefore a great chance of isolating new endophytic fungi and, hence, new fungi-derived secondary metabolites, especially if the plant hosts are carefully selected to ensure dereplication.

One method of dereplication-ensuring host plant selection involves looking for plants dwelling in uncommon environments like the mangroves (Strobel et. al., 2005), where plants and other organisms alike are subjected to harsh (high salinity) and changing environmental conditions (Ong and Gong, 2013) capable of exerting selection pressures that could induce novel and diverse secondary metabolic pathways as biochemical adaptation measures against the uncommon external stress (Rodriguez et. al., 2004; Thatoi et. al., 2013; Sivakumar, 2013).

*Address correspondence to: olusegunajala@yahoo.co.uk, olajala@unilag.edu.ng Tel. +234 (0) 803 562 2151

Another selection method involves selecting plants of special medicinal interests, as there are now growing speculations that plant medicinal principles may, after all, not have come from plant secondary metabolism but from the secondary metabolisms of the endophytes community they harbour (Strobel et. al., 2005). In the light of the afore-mentioned selection principles, therefore, we subjected the inner tissues of the aerial roots of the mangrove medicinal plant *Rhizophora racemosa* (Bandaranayake , 2002; Ejike et. al., 2014) to standard fungal isolation procedures for the ultimate end of obtaining large-batch cultures of any fungi so isolated therefrom, and extracting such cultures with organic solvents to, hopefully, obtain bioactive secondary metabolites with potential structural and functional novelties.

MATERIALS AND METHODS

Plant Collection and Identification

Healthy aerial roots of *Rhizophora racemosa* were carefully collected around the swampy region of the lagoon front of the University of Lagos in the morning hours of the 20th of July, 2015. The plant was identified and authenticated at the Department of Botany, University of Lagos, where a herbarium sample was also deposited with the voucher number LUH6641.

Isolation of endophytic fungus

The plant materials were processed not later than the fifth day after collection. The collected roots were rinsed gently in running water to remove dust and debris. The root samples were cut into small segments of about 0.2 - 0.3cm length to expose their interior surface. The segmented roots were subjected to surface sterilization procedure. Surface sterilization was done by rinsing the segmented roots with 70% ethanol for thirty seconds, followed by 0.5% sodium hypochlorite for two minutes, and then rinsing again in 70% ethanol for ninety seconds, and finally with sterile distilled water three times. The segments were dried under aseptic conditions. In each petri dish (four in total), four segments of the sterilized roots were placed on Sabouraud Dextrose Agar (SDA) supplemented with 1% w/v streptomycin. The petri dishes were sealed with parafilm and incubated at 27°C for seven days. The plates were observed for visual growth daily, noting incubation period from the plating date.

Purification and preservation of endophytic fungus

Isolation from the master plates was done by the transfer of the hyphal tips to fresh SDA plates. The plates were incubated at 27°C for four days. The endophytic fungus in the pure culture was transferred to a slant agar and preserved in the refrigerator at about 8°C.

Identification of isolated endophytic fungus

Fungal identification was based on the comparison of cultural and morphological characteristics of plate cultures of the isolate with those of reported fungi. Morphological studies were aided by mycelial microscopy, performed by transferring a small portion of the mycelium from the pure fungal culture into a drop of lactophenol cotton blue stain placed on a clean slide (with the aid of a moistened sterile mounting needle) followed by mounting and observing with x10 and x40 objective lenses under the microscope.

Cultivation of isolated endophytic fungus

Into each of ten 1Litre flasks was placed 500 ml of freshly prepared sabouraud dextrose broth and autoclaved at 121°C for fifteen minutes. The sterile broth was allowed to cool. Isolated fungal strain was inoculated aseptically into all the flasks. The flasks were kept at 27°C for fourteen days for growth of the fungus as well as production of secondary metabolites. The flasks were examined periodically for any contamination.

Extraction of bioactive metabolites

Mycelia were harvested from the broth culture by filtration at the end of the 14-day incubation period. The harvested mycelia were extracted by cold maceration and subsequent trituration in methanol. The aqueous culture broth (i.e., the filtrate) was extracted by partitioning into dichloromethane and ethyl acetate in succession. Each of the mycelia methanolic extract and the broth dichloromethane and ethylacetate extracts was evaporated to dryness under reduced pressure at 40°C using a rotary evaporator.

Chemical evaluation of extracts

Each of the three extracts from the fungal culture was chemically evaluated for possible presence of pharmacologically important bioactive chemical groups such as alkaloids, anthraquinones, phenols, flavonoids, saponins, cardiac glycosides, and tannins by standard phytochemical procedures (Trease and Evans, 2002).

Preparation of standard references (ofloxacin and clotrimazole) solutions

Four working concentrations (50, 25, 12.5 and 6.25µg/ml) of aqueous ofloxacin were prepared by serial dilution from a 5mg/ml stock. In the same vein, four working concentrations (160, 80, 40, and 20µg/ml) of clotrimazole solution in ethanol were prepared from a 16mg/ml stock.

Preparation of Test Samples solutions

Guided by yield and empirical antimicrobial evaluations, 6.25mg/ml of each of the three extracts was prepared in DMSO, followed by a 1 in 2 dilution to give two working concentrations, 6.25mg/ml and 3.125mg/ml, per extract.

Test organisms

The microorganisms used were obtained from the stock cultures in Pharmaceutical Microbiology Laboratory of the department of Pharmaceutics and

Pharmaceutical Technology, Faculty of Pharmacy, University of Lagos. A total of five bacterial strains (*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella sp, Pseudomonas aeruginosa)* and two fungal strains (*Saccharomyces caevisiae, Candida albicans*) were used for the assays.

Antibacterial activity assay

The antibacterial activities of the extracts were evaluated by agar well diffusion method (Ramakrishnan et al, 2011, Jahangirian et al, 2013) using Mueller Hinton agar medium. The medium was autoclaved at 121°C, cooled and transferred to each of petri plates containing one test organism, and allowed to set at room temperature before 5 holes were bored in it with a 10mm sterile cork borer. Each of the two working concentrations (0.2ml) of an extract solution made in DMSO was aseptically transferred into two of the holes while same volume of DMSO was transferred to the remaining hole. Similarly, 5 holes were bored in each of another set of organism-seeded plates, transferring into each hole one of four concentrations of an aqueous solution of ofloxacin or distilled water. All plates were incubated at 37°C for 24 hours at the end of which each plate was observed for inhibition zones measured in millimetres.

Antifungal activity assay

The above-described procedure for the antibacterial assay was essentially followed using Saboraud Dextrose Agar as medium, fungi as test organisms, clotrimazole as standard reference and incubation done at 27°C for 24 hours.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) were determined for extracts that produced zones of inhibition in the agar diffusion experiments by the broth micro-dilution method described by the European Committee for Antibacterial Susceptibility Testing (EUCAST), 2003, using Mueller Hinton broth as medium.

RESULTS

Morphological and microscopic examination of fungal isolate.

One fungal morphotype was identified from the master plate culture and subcultured as such. Figures 1a and b are photographs of 7-day and 14-day old pure plate cultures of the isolate.

Figure 1: (a) 7-day old colonies and (b) 14-day old colonies of *Phoma. Sp* isolated from *Rhizophora racemosa*.

The results of the mycelium lactophenol cotton blue staining microscopy at x40 magnification is as shown in Figure 2.

Figure 2: Microscopic view (x40) of the lactophenol cotton blue stained mycelium of *Phoma sp*. isolated from *Rhizophora racemosa.*

Chemical Evaluation

Screening of the extracts for major bioactive chemical groups showed abundance of reducing sugars, alkaloids, and flavonoids in the ethyl acetate extract, moderate presence of cardiac glycosides, saponins, anthraquinones, and tannins, in both the dichloromethane and ethyl acetate extracts. With the exception of a trace presence of saponins, the tested chemical groups were observed to be conspicuously absent in the methanolic mycelia extract (Table 1).

Table 1: Relative abundance of pharmacologically important bioactive chemical groups in the methanolic mycelia extract and broth dichloromethane and ethyl acetate extracts of a 14-day old broth culture of *Phoma sp*.from *Rhizophora racemosa.*

Chemical group	Methanol extract	Dichloromethane extract	Ethyl acetate extract
Tannins	-	++	++
Anthraquinones	-	++	++
Alkaloids	-	++	+++
Flavonoids	-	++	+++
Saponins	+	++	++
Cardiac glycosides	-	++	++
Reducing sugars	-	+	+++

Key: +++ =Abundantly present, ++ =Moderately present, + = slightly present, - =Absent

Antimicrobial assays

The antimicrobial assays results are as presented in **Tables 2-4** and **Figure 3**: The relative zones of inhibitions produced by the standards and the active extracts are depicted in Tables 2 and 3, while the comparison of the relative scopes and potencies of the active dichloromethane and ethyl acetate extracts of the culture broth are depicted in Figure 3 and in the MICs (Table 4).

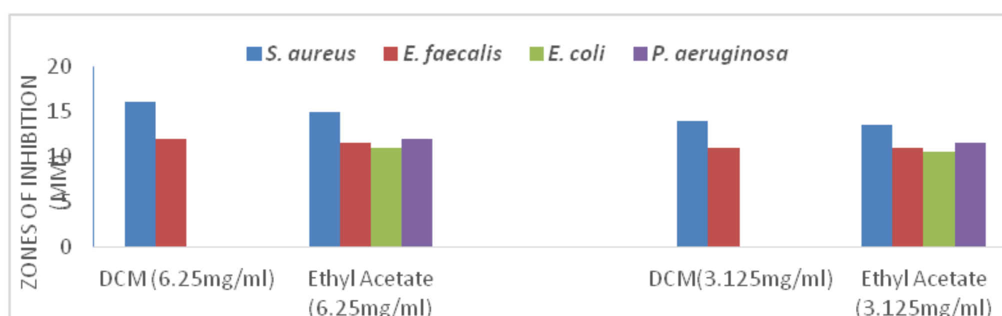

Figure 3: Comparison of the antibacterial activities of Dichloromethane (DCM) and Ethyl acetate culture extracts of the broth of a 14-day old culture of *Phoma sp*.

Table 2: Zones of inhibition (mm) produced by ofloxacin and clotrimazole in the plate cultures of bacterial and fungal test organisms respectively.

OFLOXACIN

Test organisms	50(µg/ml)	25(µg/ml)	12.5(µg/ml)	6.25(µg/ml)
Staphylococcus aureus	23.5	21.5	18.0	15.0
Enterococcus faecalis	20.0	16.5	13.0	12.0
Escherichia coli	19.5	17	15.5	14.0
Klebsiella sp	15	13.0	12.0	10.5
Pseudomonas aeruginosa	18	16	13	11
Distilled water (Control)	0.0	0.0	0.0	0.0

CLOTRIMAZOLE

Test organisms	160(µg/ml)	80(µg/ml)	40(µg/ml)	20(µg/ml)
Saccharomyces caevisiae	18.0	16.0	14.5	13.0
Candida albicans	25.0	23.0	21.0	19.0
5% Ethanol (Control)	0.0	0.0	0.0	0.0

Table 3: Zones of inhibition (mm) produced by culture extracts in plate cultures of (I) bacterial and (II) fungal test organisms.

I. Antibacteria assay

Test organisms	Methanol extract		Dichloromethane extract		Ethyl acetate extract		DMSO (Control)
	6.25 (mg/ml)	3.125 (mg/ml)	6.25 (mg/ml)	3.125 (mg/ml)	6.25 (mg/ml)	3.125 (mg/ml)	
Staphylococcus aureus	0.0	0.0	16.0	14.0	15.0	13.5	0.0
Enterococcus faecalis	0.0	0.0	12.0	11.0	11.5	11.0	0.0
Escherichia coli	0.0	0.0	0.0	0.0	11.0	0.0	0.0
Klebsiella sp	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Pseudomonas aeruginosa	0.0	0.0	0.0	0.0	12.0	11.5	0.0

II. ANTIFUNGAL ASSAY

Test organisms	Methanolic Extract		Dichloromethane Extract		Ethyl acetate extract		DMSO (Control)
	6.25 (mg/ml)	3.125 (mg/ml)	6.25 (mg/ml)	3.125 (mg/ml)	6.25 (mg/ml)	3.125 (mg/ml)	
Saccharomyces caevisiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Candida albicans	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 4: Minimum Inhibitory Concentrations (MIC) of the Dichloromethane and ethyl acetate extracts of the broth of a 14-day old culture of *Phoma sp.* isolated from *Rhizophora racemosa*.

Test organisms	Working concentrations of extracts (mg/ml)										MIC Values
	A 0.025	B 0.05	C 0.1	D 0.2	E 0.4	F 0.8	G 1.6	H 3.2	I 6.4	J 12.8	
Dichloromethane Extract											
S. aureus	+	+	+	+	-	-	-	-	-	-	0.4mg/ml
E. faecalis	+	+	+	+	+	+	-	-	-	-	1.6mg/ml
Ethyl acetate Extract											
S. aureus	+	+	+	+	+	+	-	-	-	-	1.6mg/ml
E. faecalis	+	+	+	+	+	+	+	+	-	-	6.4mg/ml
E. coli	+	+	+	+	+	+	+	+	+	-	12.8mg/ml
P aeruginosa	+	+	+	+	+	+	+	+	+	-	12.8mg/ml

Key: (+) Growth (-) No growth

DISCUSSION

Inadvertent isolation of epiphytes could preclude endophytes isolation or at the very least contaminate the cultures of isolated endophytes, thereby interfering with identification. Ruling out the chances of isolating epiphytes using standard surface sterilization techniques was therefore a crucial step in the isolation procedure (Caruso et. al., 2000, Kjer et. al., 2010).

Morphological characteristics (from mycelia microscopy) revealing various globosses and subglobosses of pycnidial conidiomata in a mesh of hypae (Figure 2) are unequivocally diagnostic of the coelomycetous *Phoma* genus or its closely related ally, *Phyllosticta* (Boerema, 2004; Aveskamp, 2008; Irinyi et. al., 2009;Wikee et. al. 201). Cultural studies, however, revealing rapidly growing, largely medium-submerged colonies, initially velvet white but gradually turning olive grey and dark brown with time (Figure 1) (Irinyi et. al., 2009), and the fact that the conidiomata-bearing substrate/host in question is a root and not leaf (Wikee et. al. 2011), skew the balance in favour of *Phoma*, and hence the identification of the isolate as *Phoma sp.* Nevertheless, conidial ontogeny and/or molecular phylogenetic analysis would be needed to provide confirmatory data and for further (i.e., species) classification.

It is worthy of note that this report is not only the first for the isolation of an endophytic fungus from *Rhizophora racemosa,* it is actually the first for the isolation of an endophyte, at all, from the plant. However, the fact that only one endophytic fungal morphotype could be isolated does not, in any way, suggest that the plant harbours only one endophytic fungus. Most likely, there are more endophytic fungi in the plant tissues, but their growth could not be supported by common growth media like the one used in this study. Media and /or media components variation could be employed in further endophytic fungi-related studies on *Rhizophora racemosa* to increase the chances of isolating more endophytic fungi from the plant.

The dichloromethane extract showed a modest possession of a number of the bioactive chemical groups screened for, while the presence of these secondary metabolites was seen to be in copious amounts in the ethyl acetate fraction (Table 1). Most of the chemical groups identified in the culture of the isolated fungus have also been isolated from the aerial parts of the host plant, *Rhizophora racemosa* (Bandaranayake , 2002; Ejike et. al., 2014). It is therefore difficult to say with any sense of exactitude which of these chemical groups actually originated from the plant's secondary metabolism and which from the harboured endophytes. Nevertheless, the novelty and diversity of the endophyte culture-derived secondary metabolites are expected to be greater due to the uniqueness of the secondary metabolic pathway of the latter and the proneness of its genome to mutation.
The similarity of the chemical nature of the ethylacetate and dichloromethane extracts implies presence of compounds of similar chemical groups

but varying polarities. The fact that most of the chemical groups are present in more copious amounts in ethyl acetate than in the dichloromethane extracts confirms that the compounds involved are different molecules present at differing concentrations within the milieu.

One symbiotic benefit that plants derive from harbouring endophytes is protection against fungal and bacterial pathogens (Gimenez et. al., 2007; Hadoim et. al., 2008; Pravabathy and Nachiyar, 2011). It is therefore almost natural to think that secondary metabolites isolated from cultures of endophytic fungi could demonstrate impressive antimicrobial activities against human bacterial and fungal pathogens. This informed our screening the various extracts obtained from the endophytic fungal isolate from *Rhiophora racemosa* for their inhibitory activities against the human bacterial and fungal pathogens tested.

The lack of both antibacterial and antifungal activities in the methanolic mycelia extract could be correlated to the paucity of secondary metabolites in it (Table 1) giving credence to the fact that secondary metabolites are mostly secreted into the growth medium for adaptation and ecological purposes and rarely concentrated in the tissues as primary metabolites are. The MIC values, 0.4mg/ml and 1.6mg/ml, of the dichloromethane (DCM) extract against the Gram positive bacteria *staphylococcus aureaus* and *Enteroccocal faecalis* respectively are particularly remarkable and indicative of the great susceptibility of these and possibly other gram positive organisms to the antibacterial principles in the extract. The lack of activity of the extract against the three Gram negative organisms tested, however, is a good index of the general insensitivity of Gram negative organisms in to the antibacterial components of this extract.

The MIC values (Table 4) show the ethyl acetate extract to be less potent against Gram positive organisms compared to the dichloromethane extract. Its activity against virtually all the organisms tested (Gram positive and Gram negative), however, shows the broad spectrum of activity of its antibacterial principles. (Table 3, Figure 3). The potency of the active principles of the ethyl acetate extract components could be enhanced by structural modification after isolation to exploit them as broad spectrum antibacterial agents. The active extracts demonstrated dose-dependent antibacterial activities which could be considered significant compared to the negative control (DMSO) which showed no zone of inhibition at all to the growth of any of the tested organisms (Tables 3). There was however no basis for comparing the "mg/ml" working concentration scale of the extracts to the "µg/ml" scale of the reference standard, ofloxacin, as the latter is a pure compound while the extracts are mere crude mixtures of antibacterial compounds and other bioactive compounds that are nothing, in this context, better than impurities. Moreover, in addition to being present in small concentrations, the constituent antibacterial compounds of the extracts may be in some chemical and/or intermolecular association with themselves and/or other non-antibacterial molecules in the milieu, thereby reducing available antibacterial molecules below their lethal concentrations. Isolation of the antibacterial constituents of the active extracts is therefore recommended. It is also worthy of note that the fact that the antimicrobial spectrum of action of the two extracts differ considerably is a clear indication the two extracts are made up of different components, though their group chemical test results are similar.

The cultures and a number of secondary metabolites isolated from *Phoma spp*. from other sources have also been reported to show remarkable antibacterial activities (Hoffman et. al., 2008), showing the possible common metabolic/genomic characteristics and hence possible similar ecological roles that endophytic *Phoma spp*. from other plant sources may be sharing with the *Rhizophora racemosa*-derived, herein reported.

CONCLUSION

This study demonstrated the presence of a diverse range of bioactive molecules in the culture broth of the endophytic fungus, *Phoma sp*., isolated from the mangrove, *Rhizophora racemosa*. While this range portends a diversity of potential pharmacological and/or therapeutic applicability of the culture of this fungus, its broad spectrum antibacterial was unequivocally established. activities, the presence of antibacterial components was established. Endophytic *Phoma sp*. from *Rhizophora racemosa* is therefore recommended as a veritable source of antibacterial and other drugs, or at the very least, their templates.

ACKNOWLEDGEMENT

All technical assistance received from Mrs. Ijeoma Olanipekun, head of microbiology department of GOZ Pharmaceuticals, Ogba, Lagos, and Mr. A. Usman, head of microbiology laboratory unit of the department of Pharmaceutics and Pharmaceutical Technology, faculty of pharmacy, University of Lagos, are hereby thankfully acknowledged.

REFERENCES

Aveskamp MM, De Gruyter J, Crous PW (2008). Biology and recent developments in the systematics of Phoma, a complex genus of major quarantine significance. *Fungal Diversity* 31:1-8

Bacon CW, White Jn. JF (2000). Microbial endophytes. Marcel Dekker, New York; p 487

Bandaranayake WM (2002). Bioactivities, bioactive compounds and chemical constituents of mangrove plants. *Wetlands Ecology and Mnagement* 10:421-452

Boerema GH,Gruyter J, Noordeloos ME and Hamers MEC(2004). Phoma identification manual. CABI Publishing, CAB International, Wallingford, Oxfordshire, UK, p. 470

Caruso M, Colombo AL, Fedeli L, Pavesi A, Quaroni S, Saracchi M and Ventrella G (2000). Isolation of endophytic fungi and actinomycetes taxane producers. *Annals of microbiology* 50(1): 3-14

Dreyfuss MM and Chapela IH (1994). Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticcals. In: Gulls VP (ed), The discovery of natural products with therapeutic potential. Butterworth-Heiniemann, Boston, pp.49-80

Ejike CM, Ezeanu CS and Eboatu AN(2014). Determination of physical and phytochemical constituents of some tropical timbers indigenous to Niger delta area of Nigera. *European Scientific Journal* 10(18):247-270

European Committee for Antibacterial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (2003). Determination of minimum inhibitory concentration (MIC) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection* 9(8):1-7

Gimenez C, Cabrera R, Reina M and Gonzalez-Coloma A (2007). Fungal endophytes and their role in plant protection. *Current Organic Chemistry* 11: 707-720

Gunatilaka AAL (2006). Natural products from plant-associated microorganisms; distribution, structural diversity, bioactivity and implications of their occurrence. *J Nat Prod* 69: 509-526

Hardoim PR, Van Overbeek LS, Elsas JD (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in microbiology* 16(10): 463-471

Hoffman AM, Mayer SG, Strobel GA, Hess WM, Sovocool GW, Grange AH, Harper JK, Arif AM, Grant, DM and Kelley-Swift EG (2008). Purification, identification and activity of phomodione, a furandione from an endophytic Phoma species. *Phytochemistry* 69(4):1049-1056

Irinyi L, Gade AK, Ingle AP, Kovics GJ, Rai MK and Sandor E (2009). Morphology and molecular biology of Phoma. In Gherbawy Y, March RL, and Rai M (eds.) Current advances in Molecular Mycology. Nova Science Publishers inc., New York. Pp 171-203.

Jahangirian H, Haron MJ, Shay MH, Abdollai Y, Rezayi M and Vafaei N, (2013). Well diffusion method for evaluation of antibacterial activity of copper phenyl fatty hydroxamate synthezised from canola and palm kernel oils. *Digest Journal of Nanomaterials & Biostructures* (DJNB) 8(3):1263-1270

Joseph B, and Priya RM (2011). Bioactive compounds from endophytes and their potentioal in pharmaceutical effect: a review. *Am J Biochem Biotechnol* 1: 291-309

Kjer J, Debbab A, Aly AH and Proksch P (2010). Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. *Nature protocols 5*(3): 479-490

Ong JE and Gong WK (2013). Structure, function and management of mangrove ecosystems. ISME Mangrove educational book series. No 2. International Society for Mangrove Ecosystem (ISME), Okinawa, Japan, and Internatioal Tropical Timber Organization (ITTO), Yokohama, Japan, pp. 7-24.

Petrini O (1991). Fungal endophytes of tree leaves. In: Andrews JH and Hirana SS (eds). Microbial ecology of leaves, Springer-Verlag, New York, pp179-197

Pravabathy D and Nachiyar CV (2011). Screening and Characterization of antimicrobial compound from endophytic *Aspergillus sp.* isolated from *Ficus carica. Journal of Pharmacy Research* 4(6): 34-38

Ramakrishnan G, Kothai R, Jaykar B and Rathnakumar TV (2011). In vitro Antibacterial Activity of different extracts of Leaves of *Coldenia procumbens*. *International Journal of Pharm Tech Research* 3:1000-1004

Ravindran C, Naveenan T, Varatharajan GR, Rajasabapathy R and Meena RM (2012). Antioxidants in mangrove plants and endophytic fungal associations. *Botanica Marina* 55: 269-279

Rodriguez RJ, Redman RS and Henson JM (2004). The role of fungal symbioses in the adaptations of plants to high stress environments. *Mitigation and Adaptation Strategies for Global Change* 9:261-272

Schulz B, Boyle C, Draeger S, Römmert AK and Krohn K (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological research* 106: 996–1004

Sivakumar T (2013). A review on biodiversity of marine and mangrove fungi. *International Journal of Current Research and Academic Review* 1(1):26-44

Strobel G, Daisy B and Dastillo U (2005). Novel natural products from rainforest endopnytes. In: Zhang L and Demain AL (eds) Natural product drug discovery and therapeutic medicine. Humana Press, Totowa, New Jersey, pp 329-351

Thatoi H, Behera BC and Mishra RR (2013). Ecological role and biotechnological potential of mangrove fungi: a review. Mycology: *An International Journal of Fungal Biology* 4(1):54-71

Trease GE and Evans WC (2002). Pharmacognosy. 15th Ed. London, Saunders Publishers, pp. 42–44, 221-229

Wikee S, Udayanga D, Crous PW, Chukeatirote E, McKenzie EHC, Bahkali AH, Dai D and Hyde KD (2011).Phyllosticta – a current view of species recognition. *Fungal Diversity*, 51(1):43-61