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Research Article

Isolation and bioassay screening of medicinal plant Endophytes from Western Ghats forests, Goa, India.

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Abstract

Eighty nine distinct fungal endophytes were isolated from 21 traditionally used medicinal plants from Western Ghats, forests in Goa, India. All the plants were found colonized with endophytic fungi. This is the first report of endophytes from the 21 medicinal host plants. Five hundred endophytes were screened for qualitative enzyme assays i.e amylase, cellulase and pectinase, from which two hundred and eighty-four showed positive activity showing highest cellulose activity (45.07%) followed by amylase activity(36.62%) and the least was pectinase (18.31%). One hundred and forty-five crude extracts of medicinal plant endophytes were screened for antibacterial and fungal activity. With regards to antibacterial activity 53 extracts were found to be active against *Bacillus subtilis* and 26 against *Escherichia coli*. With regards to antifungal activity 11 extracts were active against *Alternaria brassicola* while 04 were active against *Aspergillus niger*. Eighteen extracts were found to be active against *Candida albicans*. Overall, 41 extracts showed activity index (AI) greater than 0.4 are considered 'promising candidates' of novel antibiotics. The study reinforced the assumption that endophytes of ethno medicinal plants could be a promising source of antimicrobial substances.

Keywords: Endophyte; enzyme assays; antibacterial; antifungal; bioassay screening; medicinal plants.

Introduction

The search for new antibiotics to overcome the growing human problems of drugs resistance in microorganisms and appearance of new diseases has been rapidly increasing around the world. Realizing the capability of microorganisms to produce diverse bioactive molecules and the existence of unexplored microbial diversity, research is underway to isolate and screen microbes of diverse habitat and unique environment for discovery of novel metabolites. One such unexplored and less studied microorganism is the endophytic fungi, which are defined as those microbes that colonize healthy tissues of plants, at least for a part of their life cycle, without causing apparent disease symptoms in their host (Petrini 1991; Wilson 1995). These endophytes are also recognized as rich sources of bioactive metabolites of multifold importance (Strobel and Daisy 2003).

From ages, medicinal plants are known to possess curative properties and hence different plant parts have been traditionally used by indigenous communities in different ways for the treatment of various diseases. Western Ghats of India are known to harbor a wide variety of medicinal plant species. Studies on endophytes concentrated on medicinal plants have shown that the curative property of the medicinal plant is not only because of the chemicals present in the plant but also because of the endophytes that reside within the plant (Strobel et al., 2004; Huang et al., 2008; Verma, 2011; Suryanarayanan, 2013).

Additionally, there are compounds produced by the associated endophytic fungi that live inside medicinal plants as discussed in the review by Kaul et al. (2012). Thus there is a need to isolate more number of fungi

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and subject them to screening to determine endophytes that are capable of producing useful metabolites. Very little is known about endophytes from indigenously used Goan medicinal plants and perhaps this is the first such report. With this knowledge, the present investigation was carried out to isolate endophytes from 21 traditionally used Goan medicinal plants from the Western Ghats forests in India and to determine their potential to produce bioactive compounds against five disease causing organisms like *Alternaria brassicola*, *Aspergillus niger*, *Candida albicans*, *Bacillus subtilis*, and *Escherichia coli*.

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2. Materials and Methods

2.1. Location and study Area

Western Ghats in India is a rich biodiversity hotspot representing a great aesthetic treasure as well as a grand repository of biological wealth. A total of 21 medicinal plants belonging to sixteen families from ten sites along the forests of Western Ghats, Goa, India, Asia were scanned for endophytic fungi. Plants were collected from Mandrem, Arambol, Donapaula, Molem, Cotigao, Amboli, Porvorim, Bondla, Miramar and Usgao between August 2011 and April 2012 (Table 1).

2.2. Collection of plant parts

For each plant species, plant parts which have been traditionally used as medicine such as fresh disease free root, stem, leaves, bark, fruits, leaf petiole, flowers and corm were collected were placed separately in sealed polythene bags after removing excess moisture. The samples were transported to the laboratory within 12h and stored at 4°C until isolation procedures were completed.

2.3. Isolation and Identification of Endophytic fungi

The isolation of endophytes was carried out using 'Three step surface sterilization' (Petrini 1986). The collected samples were washed thoroughly with sterile distilled water to remove any dust particles and air dried before they are processed. The samples were then surface sterilized by immersing them sequentially in 70% ethanol for 1min and 4% sodium hypochlorite for 3min, 70% ethanol again for 3 secs and finally washed in sterile distilled water to remove the traces of alcohol. The excess water was dried under laminar airflow chamber. Then, with a sterile scalpel, the samples were cut into pieces (approximately 2 mm^2) and transferred onto MEA media plates. Two percent Malt Extract Agar (MEA) at pH 5.5 was the basic medium that was used for isolation of fungi in pure cultures. The isolation medium was incorporated with a cocktail of antibiotics (0.02 g/l each of Neomycin sulphate, Penicillin G, Streptomycin sulphate, Tetracycline and Bacitracin) to suppress bacterial growth. After inoculating the plates were incubated at 28°C for 3-7 days to observe the emerging mycelial growth. After the incubation period, the hyphal tips of the fungi were removed and transferred to MEA slants (maintained at 4^oC) and also on MEA plates. The fungal isolates were grown in MEA plates for 5-7 days and were encouraged to sporulate. Examination of the samples, to locate and subsequently to isolate the fungi, was done using a Lynx NSZ 606 Stereomicroscope. Both microscopic and cultural characters of the fungal isolate were recorded in addition to details of collections. Observation aimed at understanding the micro-morphology of the fungi was done using a Magnus MLX-Bi compound microscope. The fungal isolates were identified based on their morphological and reproductive characters using standard identification manuals, taxonomic keys, monographs, research papers and fungal taxonomy related websites (Ellis, 1971; Ellis, 1976; Sutton, 1980; Matsushima, 1971; Matsushima, 1975; Seifert et al., 2011). The fungal cultures that failed to sporulate were categorized as non sporulating mycelia. All the isolates are maintained in Malt Extract Agar slant in the department of Botany, Dhempe College of Arts & Science, Miramar.

2.4. Screening the isolated fungi for their biological activity

2.4.1. Enzyme analysis

Qualitative enzyme analysis was carried of 500 fungal isolates from a total of 2621 isolates of endophytic fungi recovered from 4130 plant parts (segments) of the 21 medicinal plant species. These isolates were screened for enzymes such as amylase, cellulose and pectinase.

2.4.2. Amylolytic activity (Carder, 1986)

The ability to decompose starch was used as a criterion for analysis of amylolytic activity of fungi.

The composition of the 'starch agar' medium was as follows: malt extract 5g/l, agar 20g/l, soluble starch 0.2%. The mineral salts solution contained per litre: (NH4)2SO4, 2g; KH2PO4, 4g; Na2HPO4, 6g; FeSO4.7H2O, 0.2g; CaCl2, 1mg; H3BO3, 10 µg; MnSO4, 10 µg; ZnSO4, 70 µg; CuSO4, 50 µg; MoO3, 10 µg; pH 6. The test fungus was point-inoculated in the centre of the medium and allowed to grow for 7 days. Amylolytic activity was observed by flooding the plates with a 1% iodine solution. A clear yellow zone developed around the colony indicated the production of amylase in an otherwise dark blue medium.

2.4.3. Cellulolytic Activity (Hankin & Anagnostakis, 1975)

The composition of test medium was as follows: agar 20g/l, carboxymethylcellulose 10g/l, the mineral salts solution contained per litre: (NH4)2SO4, 2g; KH2PO4, 4g; Na2HPO4, 6g; FeSO4.7H2O, 0.2g; CaCl2, 1mg; H3BO3, 10 µg; MnSO4, 10 µg; ZnSO4, 70 µg; CuSO4, 50 µg; MoO3, 10 µg; pH 6. After 7 days incubation, the plates were flooded with 1% Congo red solution. Cellulase production was indicated by a clear zone in positive colonies. The plates were subsequently flooded with 20 ml of 1 N NaCl to allow the clearance zone to remain for a longer duration.

2.4.4. Pectinolytic activity (Hankin & Anagnostakis, 1975)

To detect the production of pectate lyase, pectin agar medium was used. The composition was as under: agar 15g/l, yeast extract 1.0g, pectin 5g. The mineral salts solution contained per 500ml: (NH4)2SO4, 1g; KH2PO4, 2g; Na2HPO4, 3g; FeSO4.7H2O, 0.1g; CaCl2, 0.5mg; H3BO3, 5 µg; MnSO4, 5µg; ZnSO4, 35µg; CuSO4, 25µg; MoO3, 5µg; pH 7. The plates were incubated for 5-7 days and later flooded with Centrimide (Cetyltrimethyl-ammoniumbromide). The centrimide precipitates the intact pectin in the medium and pectin utilized was revealed as clear zones around active colonies in an otherwise opaque medium.

2.5. Preliminary antimicrobial assay

Eventually, when an endophyte was acquired in pure culture, it was tested for its ability to produce metabolites and is subjected to assays. The screening was carried out by developing extracts of the fungal isolates using Agar Disc Diffusion method. The isolates were screened for their antimicrobial activity against test organisms spread on specific medium plates for antibacterial assay and antifungal assay. The diameter of the inhibition zone will be measured and the average of three repeated agar disks will be taken to assess the strength of antimicrobial activity.

2.5.1. Preparation of culture extracts (Wiyakrutta et al., 2004)

One hundred and forty five fungal isolates were selected randomly from the total isolates from the twenty-one medicinal plants. These five fungal cultures were grown on Malt Extract Agar plates for 7 days and then agar blocks of actively growing pure culture (3mm in diameter) were inoculated in 50 ml sterile YES broth taken in 250 ml Erlenmeyer flasks. The flasks were incubated at 25±1°C on an orbital shaker (Orbitek, Scigenics Biotech Pvt. Ltd, Chennai) at 200 rpm for 10-15 days. After the incubation period, the cultures were taken out and the fungal cultures (mycelium together with the broth) were pulverized in a sonicator (Bandelin Electronics, Berlin, Germany). The pounded culture extract was extracted in methanol for 2 days and filtered. The methanolic extract was vacuum-dried, lyophilized and stored at 4 ºC until further use.

2.5.2. Procuring the Standard cultures from IMTECH, Chandigarh

Five standard cultures were used to evaluate the antimicrobial activity of endophytic crude extracts. These test pathogens included two filamentous fungi *Alternaria brassicola* (MTCC2102) and *Aspergillus niger* (MTCC8652)*;* one yeast *Candida albicans* (MTCC227) and two bacteria *Bacillus subtilis*- (MTCC441- Gram-positive) and *Escherichia coli* (MTCC1687 Gram-negative) were procured from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India (IMTECH). Fungi and yeast cultures were grown on MEA plates

for 4-7 days at 25ºC while the bacterial cultures were grown on nutrient agar (NA) at 37ºC for 24-48 hours. In order to use for screening a spore/cell suspension of the cultures was made in sterile distilled water for

fungi (10^3 cfu/ml) and sterile saline for bacteria (10^6 cfu/ml).

2.5.3. Testing biological activity (Pieckova and Roeijmans, 1999)

Biological activity of the extracts was tested in triplicate using the 'Agar-well diffusion' method with dimethlysulfoxide (DMSO) as solvent. To check antifungal and antibacterial activity, MEA and NA plates were seeded with 0.1ml of the spore/cell suspension and spread evenly using a spreader. Wells of size 6 mm were bored on to these plates. The culture extracts to be tested were dissolved in (DMSO) at a concentration of 100mg/ml. 25 µl of the extract was dispensed in the wells bored on the seeded plates. The experimental plates were incubated at 25 ºC for 7 days for fungi and yeast and at 37 ºC for 24-48 hours for bacteria. Positive control was maintained by using standard antibiotics, viz Streptomycin (antibacterial) and Fluconazole (antifungal) at a concentration of 1 mg/ml. Negative control was also maintained using DMSO.

2.5.4. Determining the activity index and creativity index

The culture extracts showing positive activity against any of the test organisms was determined by the extent of diameter of 'zone of inhibition' (IZ) formed around them using the formula:

Inhibition zone (IZ) = Diameter of total inhibition zone – Diameter of the well (6mm)
Inhibition zone (IZ) = > 12 highly active; 8-12 moderately active; < 8 slightly active

Based on mean inhibition zone of positive controls, activity index (AI) of each positive extract was calculated by the formula below. Extracts having Activity index (AI) greater than 0.4 are considered 'promising candidates' of novel antibiotics.

$$\text{Activity index (AI)} = \frac{\text{Mean inhibition zone for test sample}}{\text{Mean inhibition zone for std. antibiotic}}$$

$$\text{Creativity index (CI)} = \frac{\text{Number of cultures showing +ve activity}}{\text{Total no. of cultures screened}}$$

3. Results

All the plant species were found colonized with endophytic fungi. The endophytes were isolated using malt extract agar (MEA). In many instances this media were commonly used for isolation of endophytes (Mahesh et al., 2005; Tejesvi et al., 2005). To the author's knowledge there are no published reports of endophytes from eight of the 12 traditionally used medicinal plants used for this study. The endophytes listed in this study represent the first such list for those plants and highlight the lack of data on endophytic fungi from medicinal plants. A total of 2621 isolates of endophytic fungi from 4130 plant parts (segments) overall resulting in a colonization frequency of 63.46% (Table 1). The overall isolation frequency was 63.46% (Table 2). There was a great deal of variation in the colonization frequency amongst the host medicinal plants ranging from a low of 30.83 % for *Holarrhena antidysentrica* to a high of 116.66 % for *Saraca asoca* although the total number of fungal species/morphotypes isolated from twenty-one plant species varied greatly from 05 species/morphotypes in *Jatropha curcas* to 45 species/morphotypes in *Hemidesmus indicus* (Table 2). Host re-occurrence and tissue specificity has also been observed in our present study in the twenty-one medicinal plants. Some of the fungi were ubiquitous whereas some were host specific. *Alternaria alternata*, *Aspergillus niger*, *Cladosporium oxysporum*, *Fusarium monoliforme*, *Nigrospora oryzae*, *Nodulisporium gregarium*, *Penicillium chrysogenum*, *Penicillium griseofulvum*, *Vermiculariopsiella parva*, *Vermiculariopsiella* sp.1, *Phoma* sp. and one unidentified pycnidial fungus were ubiquitous in occurrence whereas some were specific to a particular plant species.

Fungal endophytes have been recognized as repository of novel secondary metabolites for potential therapeutic use (Tan and Zou, 2001). Further, Strobel and Daisy (2003) necessitated that medicinal and endemic plants should use for endophytic studies as they are expected to harbor rare and interesting endophytes with novel bioactive metabolites. This has lead to the discovery of several bioactive compounds from fungal endophytes and wealth of literature on antimicrobial activity of endophytic fungi isolated

Table 1. Sampling sites, sampling date of twenty-one medicinal studied:

No	Place	Date	Plant species (Abbrv.)	Family	Geographical Coodinates
1.	Mandrem	13.08.2011	*Plectranthus amboinicus* (Lour.) Spreng (Pa)	Lamiaceae	15°39'48.39"N 73°44'29.92"E
2.	Arambol	23.08.2011 23.08.2011	*Costus speciosus* (J. Konig) Sm.(Cs) *Jatropha curcas* Linn. (Jc)	Costaceae Euphorbiaceae	15°41'14.15"N 73°43'16.36"E
3.	Donapaula	12.09.2011 12.09.2011 13.09.2011	*Ocimum tenuiflorum* Linn. (Ot) *Cymbopogon citratus* (DC.) Stapf. (Cc) *Justicia adhatoda* Linn. (Ja)	Lamiaceae Poaceae Acanthaceae	15°27'39.92"N 73°48'34.14"E
4.	Molem	03.10.2011 03.10.2011	*Strychnos nux-vomica* Linn. (Sn) *Alstonia scholaris* Linn. R. Br. (As)	Loganiaceae Apocynaceae	15°20'19.07"N 74°17'20.79"E
5.	Cotigao	11.10.2011 11.10.2011	*Hydnocarpus laurifolia* (Dennst.) Sleumer (Hl) *Chlorophytum* sp. (Csp)	Flacourtiaceae Asparagaceae	14°59078.85"N 74°12'12.26"E
6.	Amboli	03.11.2011	*Nothapodythes nimmonia* (Graham)Mabb. (Nn)	Icacinaceae	15°57'54.13"N 74°00'12.86"E
7.	Porvorim	14.12.2011 14.12.2011 14.12.2011	*Piper nigrum* Linn. (Pn) *Garcinia indica* Choisy (Gi) *Myristica fragrans* Houtt. (Mf)	Piperaceae Clusiaceae Myristicaceae	15°31'55.95"N 73°49'43.06"E
8.	Bondla	14.01.2012 14.01.2012	*Saraca asoca* (Roxb.) Wilde (Sa) *Hemidesmus indicus* L.R.Br. (Hi)	Caesalpiniaceae Apocynaceae	15°26'24.60"N 74°06'22.89"E
9.	Miramar	09.03.2012 09.03.2012	*Andrographis paniculata* (Burm.f.)Wall.ex Nees (Ap) *Boerhaavia diffusa* L.(Bd)	Acanthaceae Nyctaginaceae	15°28'56.66"N 73°48'39.53"E
10.	Usgao	10.04.2012 10.04.2012 10.04.2012	*Holarrhena antidysentrica* Wall.(Ha) *Rauwolfia serpentina* (L.) Benth. ex Kurz (Rs) *Solanum xanthocarpum* Schrad. & Wendl. (Sx)	Apocynaceae Apocynaceae Solanaceae	15°26'17.00"N 74°04'15.44"E

Table 2. Total number of fungal endophytes isolated from leaf segments of 21 medicinal plants.

Plant species	Total leaf segments plated	Segments with endophytes isolated	Distinct fungal species/morphotypes	Isolation frequency (%) isolates
Plectranthus amboinicus	170	68	18	40
Costus speciosus	140	75	5	75
Jatropha curcas	140	54	14	38.57
Ocimum tenuiflorum	170	99	15	58.23
Cymbopogon citratus	120	52	17	43.33
Justicia adhatoda	160	74	20	46.25
Strychnos nux-vomica	150	80	18	53.33
Alstonia scholaris	160	99	18	61.87
Hydnocarpus laurifolia	140	130	9	92.85
Chlorophytum sp.	120	55	18	45.83
Nothapodythes nimmonia	280	192	24	68.57
Piper nigrum	220	170	33	77.27
Garcinia indica	210	169	19	80.47
Myristica fragrans	210	189	24	90
Saraca asoca	270	315	26	116.66
Hemidesmus indicus	290	314	45	108.27
Andrographis paniculata	240	151	31	62.91
Boerhaavia diffusa	210	88	20	41.9
Holarrhena antidysentrica	240	74	21	30.83
Rauwolfia serpentina	270	50	22	18.51
Solanum xanthocarpum	220	123	33	55.9
	4130	**2621**	**450**	**63.46**

Table 3. Total number of fungal endophytes belonging to different groups of fungi.

Plant species	Determined/Identified Conidial forms					Undetermined conidial forms	Non-sporulating morphotype
	Anamorphic	Ascomycete	Coelomycete	Zygomycete	Basidiomycete		
Plectranthus amboinicus	05	01	04	00	00	00	08
Costus speciosus	01	00	01	00	00	00	03
Jatropha curcas	03	01	04	00	00	01	05
Ocimum tenuiflorum	01	00	01	00	00	01	12
Cymbopogon citratus	05	00	06	00	00	01	05
Justicia adhatoda	06	00	03	00	00	03	08
Strychnos nux-vomica	07	00	02	00	00	01	08
Alstonia scholaris	04	00	05	00	00	01	08
Hydnocarpus laurifolia	00	00	03	00	00	01	05
Chlorophytum sp.	06	00	01	00	00	00	11
Nothapodythes nimmonia	11	01	03	01	00	01	07
Piper nigrum	09	01	05	00	03	00	15
Garcinia indica	04	01	03	00	04	00	07
Myristica fragrans	08	02	06	00	01	01	06
Saraca asoca	08	00	03	01	00	02	14
Hemidesmus indicus	15	00	08	00	00	00	22
Andrographis paniculata	13	00	04	00	00	01	13
Boerhaavia diffusa	08	02	02	00	00	00	08
Holarrhena antidysentrica	11	00	02	00	00	00	08
Rauwolfia serpentina	16	00	02	00	00	00	04
Solanum xanthocarpum	15	00	01	00	00	00	17
	156	**9**	**69**	**2**	**08**	**14**	**194**

from medicinal plants (Raviraja et al., 2005; Li et al., 2005; Tayung and Jha, 2006; Li et al., 2009). In view of this we have subjected the pure fungal isolates from 21 medicinal plants to both qualitative enzyme assays and antimicrobial activity. The screening of fungal endophytes for qualitative enzyme assays and antimicrobial activity aimed at determining the potential of the isolate which will give more information about their use as potentially promising candidates which have application in industry.

3.1. Qualitative Enzyme studies

Enzyme analysis study showed that from the total of 500 fungal isolates screened for the three enzymes i.e. amylase, cellulose and pectinase, 284 fungal isolates showed positive enzyme activity i.e. 56.8%. In terms of percentages the endophytic fungi scanned for qualitative enzyme assays showed highest cellulose activity (45.07%) followed by amylase activity (36.62%) and the least was pectinase (18.31%). Fig.1. The qualitative enzyme activity was demarcated or marked using the following criteria using 3 different levels i.e. low activity < 0.5 cm, moderate activity between 0.5-1.0 cm and good activity >1.0 cm.. Fungal species showing clearance zones > 1 cm are shown in Table 4 and were categorized as good candidates. Endophytic fungi are said to be the sources of potentially useful enzymes and other metabolites which have application in industry. From this study it is an indication that there are potential endophytes which can be further subjected to quantitative enzyme assays to study their complete enzyme profile.

Fig.1. Endophytic fungi showing enzyme activity (%)

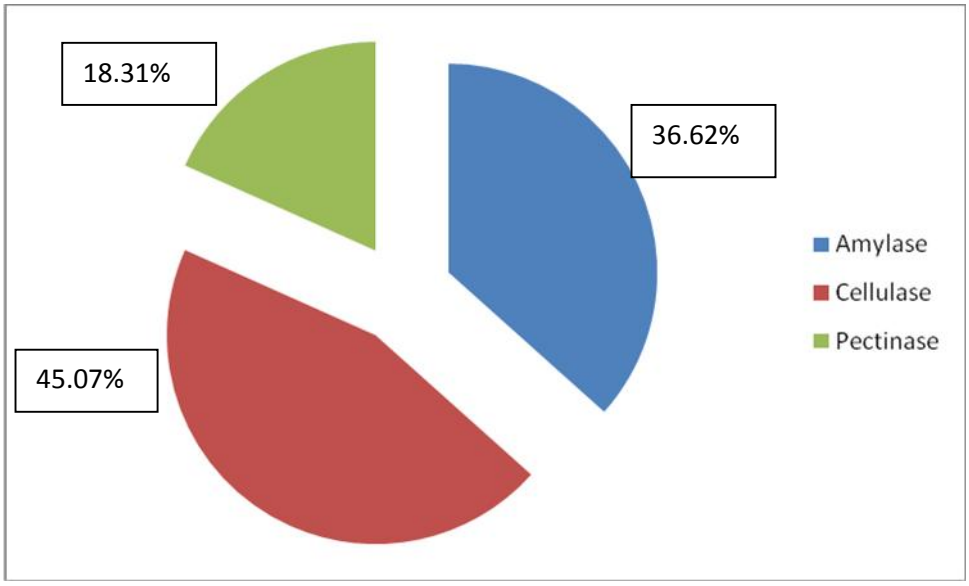

Table 4. Endophytic fungi showing good enzyme activity. i.e zones >1 cm

Amylase	Cellulase	Pectinase
Penicillium chrysogenum	*Monicillium indicum*	*Fusarium monoliforme*
Aspergillus candidus	*Pestalotiopsis* sp.	
Acremonium sp.	*Cladosporium oxysporum*	
Emericella nivea	*Penicillium griseofulvum*	
Unidentified pycnidial fungus 1	*Arthrographis* sp.	

3.2. Antimicrobial assays – Antifungal and Antibacterial

The mutual interactions between the host and the endophytes are beneficial not only to the host, but also to the endophytes by supplying nourishment to it (Tan and Zou, 2001). Endophytic fungi can produce antimicrobial metabolites with the supply of plant nutrients. There are many reports about antimicrobial compounds produced by endophytes in cultures

that were active against plant and human pathogenic microorganisms (Strobel, 2002) but there are no reports pertaining to the endophytic fungi from these twenty-one medicinal plants from Western Ghats and their bioactivity against microbial pathogens.

In our present study, one hundred and forty five crude methanolic extracts of medicinal plant endophytes were screened for antibacterial and antifungal activity and out of these eighty one fungal isolates showed positive activity against a panel of human pathogenic microorganisms, with overall percentage of 55.86%. With regards to antibacterial activity 53 extracts (36.55%) were found to be active against *Bacillus subtilis* and 26 (17.93%) against *Escherichia coli*. With regards to antifungal activity, 11 extracts (7.59%) were active against *Alternaria brassicola* while 04 (2.76%) were active against *Aspergillus niger*. Eighteen extracts (12.41%) were found to be active against *Candida albicans* (Table 5). The methanol extract of most of the host plants studied showed highest activity against *B. subtilis* among the microorganisms tested. According to literature, methanol is known to be a far more consistent extraction of antimicrobial substances from compared to other solvents such as water, ethyl acetate, dichloromethane, butanol, etc. (Homan 2002, Neysa et al., 2013), hence methanol was used as a solvent for the present study.

The activity, measured as diameter of clear zones (mm), was different for all the isolates. The endophytic fungi exhibited significant inhibitory activity at least against one test microorganism, with diameters of inhibition zones ranging from 3 to 34 mm for the test bacteria. However, only moderate antifungal activities were observed for methanolic extracts, with diameter of clear zone less than 16 mm on disc diffusion assay for all the endophytic isolates. Three isolates, namely *Acremonium* sp.2, *Penicillium chrysogenum* and Unidentified pycnidial fungus 1 demonstrated the most significant antimicrobial activity on the test organisms i.e the maximum zone of inhibition was 34 mm. Among these, the maximum anti-yeast activity was observed from crude extracts of *Acremonium* sp.2 and *Penicllium chryosgenum* found to occur in *Andrographis paniculata* whereas only Acremonium sp.2. also found to occur on the same plant showed maximum antibacterial activity against gram negative bacteria only i.e. *E. coli.* Only one isolate Unidentified pycnidial fungus 1 from *Saraca asoca* exhibited antifungal activity against *Alternaria brassicola* . They are potential candidates for further investigations. (Table 5).

Several preliminary studies have been reported on screening of endophytic fungi from medicinal plants for production of antimicrobial activities found that more than 50% of isolates displayed antimicrobial activity against at least one tested microorganisms (Wang et al., 2007). Gong and Guo (2009) reported that 56% of endophytic fungi from *Dracaena cambodiana* (Agavaceae) inhibited growth of at least one of the test organisms and 8% showed broad spectrum inhibition. Crude extracts of nine endophytic fungi (75%) isolated from *Adenocalymma alliaceum* Miers. showed antibacterial potential against one or more clinical human pathogen (Kharwar et al., 2011).

Siqueira et al., (2011) reported that 16 out of 203 endophytic isolates showed antimicrobial activity with a wider action spectrum inhibiting Gram-positive and Gram-negative and fungi. Chareprasert et al., (2006) reported an antimicrobial activity exhibited by endophytic fungi isolated from teak and rain trees and these fungi were found to produce some metabolites active against bacteria and yeast. Most of the bioactive metabolite compounds from endophytic fungi reported were more effective against Gram-positive bacteria than Gram-negative bacteria and pathogenic fungi (Chareprasert et al., 2006). In the present study, it has been observed that Gram-positive bacteria were found to be more susceptible as compared to the Gram-negative bacteria. These findings were in agreement with observations by Cos et al., 2006; Borkotoky et al., 2013, that the Gram-negative bacteria are much more resistant than Gram-positive bacteria. It has been stated that the possible mechanism behind the sensitivity of Gram-positive bacteria may lie in the significant differences in the outer layers of Gram-negative and Gram-positive bacteria. Gram-negative bacteria possess an outer membrane and a unique periplasmic space (Duffy and Power, 2001) whereas the Gram-positive bacteria found to be much more susceptible since they have only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt, 1971). The presence of some metabolic toxins or broad-spectrum antibiotic compounds might confer for its antibacterial activity (Raktim, 2013)

Table 5. Activity of crude extracts showing significant inhibition to *Escherichia coli, Bacillus subtilis, Bacillus subtilis, Aspergillus niger, Alternaria brassicola* and *Candida albicans* obtained from endophytic fungi from medicinal plants from Western Ghats, Goa, India.

Sr. No.	Endophyte	Fungus Code	Plant species	Ec	Bs	Asp	Alt	Ca
			(Abbr.)	(Inhibition zone in mm)				
1	*Acremonium* sp. 1	D-820	Ha	17.3				
2	*Acremonium* sp. 1	D-800	Bd		9			14
3	*Acremonium* sp.1	D-882	Rs		20.67			
4	*Acremonium* sp.2	D-86	Ap	34	27.34			34
5	*Alternaria alternate*	D-698	Hi					
6	*Alternaria alternate*	D-889	*Rs*		15.67			
7	*Alternaria* sp.	D-894	Sx	4				
8	*Alternaria* state of *Pleospora infectoria*	D-884	Rs	3	14			
9	*Alternaria* state of *Pleospora infectoria*	D-840	Sx		22.34			
10	*Aspergillus alutaceus*	D-28	Pa		22.33			3.34
11	*Aspergillus alutaceus*	D-654	Hi					
12	*Aspergillus candidus*	D-780	Bd	5				
13	*Aspergillus candidus*	D-754	Ap					
14	*Aspergillus niger*	D-334	As		8			3
15	*Aspergillus niger*	D-596	Mf		6.34			9.67
16	*Aspergillus niger*	D-06	Pa		9			
17	*Aspergillus niger*	D-895	Sx	21				
18	*Aspergillus niger*	D-874	Ha					
19	*Aspergillus sclerotiorum*	D-529	Pn					
20	*Aspergillus sclerotiorum*	D-141	Cc		5.67			
21	*Aspergillus sydowii*	D-686	Hi					
22	*Aspergillus sydowii*	D-687	Hi					
23	*Aspergillus terreus*	D-410	Csp					
24	*Aspergillus terreus*	D-837	Rs					
25	*Chrysosporium merdarium*	D-34	Pa		13.34			
26	*Cladosporium oxysporum*	D-663	Hi				6	
27	*Cladosporium oxysporum*	D-611	Sa			9.34		
28	*Cladosporium oxysporum*	D-788	Ap		12			
29	*Colletotrichum dematium*	D-718	Ap	8.66				
30	*Colletotrichum dematium*	D-772	Bd		9			
31	*Corynespora cambrensis*	D-431	Nn					
32	*Corynespora proliferate*	D-715	Ap	24	22.34			
33	*Corynespora proliferate*	D-824	Rs					
34	*Corynespora proliferate*	D-657	Hi					
35	*Corynespora* sp.	D-580	Mf		19			
36	*Curvularia lunata*	D-595	Mf	16				
37	*Curvularia lunata*	D-671	Hi		14.34			6.34
38	*Curvularia lunata*	D-859	Sx	24				
39	*Curvularia lunata*	D-610	Sa					
40	*Curvularia trifolii*	D-429	Nn	24				
41	*Cylindrocladium* state of	D-68	Cs	19				

	Calonectria kyotoensis							
42	*Dactylaria fusiformis*	D-530	Pn		9			9.67
43	*Drechslera australiensis*	D-447	Nn	15	29.34			14
44	*Drechslera australiensis*	D-761	Bd					
45	*Emericella nivea*	D-467	Pn					4
46	*Emericella nivea*	D-801		22.34		20.67		
47	*Fusariella* sp.	D-87		8				
48	*Fusarium dimerum*	D-452	Nn	9.66				
49	*Fusarium monoliforme*	D-302	Sn					
50	*Fusarium monoliforme*	D-226	Ot					
51	*Fusarium monoliforme*	D-756	Ap					
52	*Fusarium monoliforme*	D-775	Bd		4.34			
53	*Fusarium monoliforme*	D-506	Pn		26.67			
54	*Fusarium monoliforme*	D-606	Sa		19			
55	*Fusarium monoliforme*	D-861	Sx	14				
56	*Fusarium oxysporum*	D-929	Ha	12.67				
57	*Fusarium solani*	D-351	Sn				3.33	
58	*Fusarium* sp. 2	D-672	Hi		19			
59	*Fusarium* sp. 2	D-612	Sa					
60	*Gliomastix novae-zelandiae*	D-881	Rs					
61	*Humicola grisea*	D-862	Sx		26.34			
62	*Idriella* sp.	D-560	Gi	4				
63	*Kumbhamaya* sp.	D-152	Ja					
64	*Lasidiploidia theobromae*	D-91	Cs					
65	*Leptodontidium elatius*	D-246	Cc		10			
66	*Marielliottia biseptata*	D-826	Rs					
67	*Marielliottia biseptata*	D-827	Rs		4			
68	*Marielliottia biseptata*	D-928	Ha			13.34	21.67	
69	*Monocillium indicum*	D-286	Cc	3.66	10			
70	*Mucor hiemalis* f. *silvaticus*	D-435	Nn					
71	*Myrothecium inundatum*	D-885	Rs		17.34			
72	*Nigrospora oryzae*	D-676	Hi					
73	*Nigrospora oryzae*	D-639	Sa		8.34			
74	*Nigrospora oryzae*	D-887	Rs					
75	*Nodulisporium gregarium*	D-541	Gi					
76	*Nodulisporium gregarium*	D-711	Hi					
77	*Nodulisporium* sp.	D-812	Ha					
78	*Non sporulating mycelium*	D-45	Jc					
79	*Paecilomyces carneus*	D-457	Nn		19	12.67		
80	*Paecilomyces fumosoroseus*	D-753	Ap					
81	*Paecilomyces variotii*	D-878	Ha					
82	*Penicillium chrysogenum*	D-828	Rs					
83	*Penicillium chrysogenum*	D-745	Ap	24	20.64			34
84	*Penicillium chrysogenum*	D-614	Sa					
85	*Penicillium chrysogenum*	D-876	Ha					
86	*Penicillium chrysogenum*	D-863	Sx	12.34	19			
87	*Penicillium frequentans*	D-262	Ja					
88	*Penicill ium frequentans*	D-920	Sx					

89	*Penicillium griseofulvum*	D-323	Sn					
90	*Penicillium griseofulvum*	D-475	Pn					
91	*Penicillium griseofulvum*	D-556	Gi					
92	*Penicillium griseofulvum*	D-407	Csp					
93	*Penicillium griseofulvum*	D-608	Sa	3	20.67		24	3
94	*Penicillium griseofulvum*	D-873	Ha		19			
95	*Penicillium griseofulvum*	D-696	Hi					
96	*Penicillium griseofulvum*	D-879	Rs		26.67			
97	*Pestalotiopsis* sp.	D-609	Sa		14			
98	*Pestalotiopsis* sp.	D-710	Hi					
99	*Phialophora cinerescens*	D-458	Nn		15.67			
100	*Phialophora* sp.	D-926	Rs					
101	*Phoma* sp.	D-682	Hi					
102	*Pithomyces graminicola*	D-593	Mf					
103	*Raperia* sp.	D-865	Ap					
104	*Sodaria* sp.	D-39	Pa		10.67			
105	*Sphaerulina rubi*	D-67	Cs		13.33			
106	*Stachybotrys kampalensis*	D-880	Rs					
107	*Thozetella* sp.	D-306	Sn		15			
108	Unidentified pycnidial fungus 1	D-325	As	5.33				12.34
109	Unidentified pycnidial fungus 1	D-88	Cs					3
110	Unidentified pycnidial fungus 1	D-623	Sa	4			34	
111	Unidentified pycnidial fungus 1	D-379	Hl	21			9	
112	Unidentified pycnidial fungus 1	D-804	Ha					
113	Unidentified pycnidial fungus 1	D-584	Mf				6.34	
114	Unidentified pycnidial fungus 1	D-485	Pn	24.67				
115	Unidentified pycnidial fungus 1	D-426	Nn					
116	Unidentified pycnidial fungus 1	D-548	Gi					
117	Unidentified pycnidial fungus 1	D-717	Ap					
118	Unidentified sp.1.	D-322	Sn					
119	Unidentified sp.2	D-72	Cs					
120	*Vermiculariopsiella immersa*	D-331	As	6.67				
121	*Vermiculariopsiella immersa*	D-675	Hi	6.67	9.34			
122	*Vermiculariopsiella parva*	D-255	Ja				3.33	4.66
123	*Vermiculariopsiella parva*	D-128	Cc		3			
124	*Vermiculariopsiella parva*	D-52	Jc					
125	*Vermiculariopsiella parva*	D-09	Pa		15.34			
126	*Vermiculariopsiella parva*	D-655	Hi					

127	*Vermiculariopsiella parva*	D-740	Ap					
128	*Vermiculariopsiella parva*	D-446	Nn	7.34	24			
129	*Vermiculariopsiella parva*	D-389	Hl				9	
130	*Vermiculariopsiella parva*	D-324	As				9	
131	*Vermiculariopsiella parva*	D-540	Gi		10.67			
132	*Vermiculariopsiella parva*	D-500	Pn					
133	*Vermiculariopsiella* sp.1.	D-90	Cs					
134	*Vermiculariopsiella* sp.1.	D-326	As					
135	*Vermiculariopsiella* sp.1.	D-02	Pa					
136	*Vermiculariopsiella* sp.1.	D-653	Hi					
137	*Vermiculariopsiella* sp.1.	D-716	Ap					
138	*Vermiculariopsiella* sp.1	D-343	As			12		
139	*Vermiculariopsiella* sp.2	D-565	Mf					3
140	*Vermiculariopsiella* sp.2	D-673	Hi				17.34	
141	*Vermiculariopsiella* sp.2	D-818	Ha		4			
142	*Vermiculariopsiella* sp.3	D-677	Hi					
143	*Veronae botryose*	D-284	Cc		14.33			
144	*Verticillium* sp.	D-757	Ap					
145	*Xylaria* sp. 2	D-455	Nn		12.34			1.67

Note: The values in the table are Inhibition zones IZ (mm) mean of 3 replicates.

The culture extracts were also made of endophytic fungi which were found to occur in more number of host plants to test their antimicrobial activity in the various plants. i.e whether they behave differently in different host plants or not. The extracts of fungi i.e. unidentified pycnidial fungus 1, *Penicillium griseofulvum, Cladosporium oxysporum, Vermiculariopsiella parva* and *Fusarium monoliforme* showing more than 50% association with the host plants are shown in Table 5. It is evident from the results that some fungi irrespective of the host it belonged to showed same antimicrobial activity eg. *Penicillium griseofulvum* showed consistently an inhibition zone of 20.67 in 3 host plants *Saraca asoca, Andrographis paniculata* and *Rauwolfia serpentine* whereas the other fungi vary considerably with respect to their antimicrobial activity.

Forty six fungal cultures showed inhibition zone (IZ) > 12 and were considered as highly active; 19 isolates showed 8-12 and were moderately active and 15 isolates showed inhibition zone < 8 slightly active. Forty one extracts showed activity index (AI) greater than 0.4 are considered 'promising candidates' of novel antibiotics (Table 5). In addition the creativity index (CI) was also calculated and was 0.6 indicates a significant antimicrobial activity.

4. Justification of research

The study has enabled to document the endophytic microfungi associated with medicinal plants and screening them to antimicrobial assays which has given us a clue which endophytes can be potent producers of useful metabolites with biotechnological potential.

5. Discussion

There is a dearth of reported data relating to endophytes from traditionally used medicinal plants and the potential for the production of bioactive compounds with significant activity from the endophytes isolated from these plants. Information on habitat, ecology and taxonomic identification of endophytic fungi isolated from 21 medicinal plants from the Western Ghat forest of this region hitherto unavailable is now available. This study found out that twenty-one traditionally used medicinal plants occurring in the Western Ghat forests in Goa were able to habour diverse species of endophytic fungi and produce antibiotic or antifungal compounds. In addition the study has also demonstrated that the endophytes producing these antibiotic or antifungal compounds exhibited significant inhibitory activity against some selected human pathogenic microorganisms.

It has been estimated by the World Health Organization (WHO) that approximately 80% of the world's population from developing countries rely mainly on traditional medicines (mostly derived from plants) for their primary health care. And at least 119 chemical compounds, derived from 90 plant species, are important drugs currently in use in one or more countries (Balick et al., 1996). However, due to over exploitation of these genetic resources and other biotic interferences, many plants used as medicines have become critically endangered or are in verge of extinction. Since, it is believed that these plant species may harbor quite distinct and potential fungal endophytes that might produce novel metabolites with multifold applications, research priority should be directed to study them because disappearance of any of these plant species will also lead to disappearance of the entire suite of associated endophytes. The use of endophytes as producers of bioactive agents will help in conservation of medicinal plants and maintenance of environmental biodiversity. Studies of plant-associated endophytes could therefore provide the best possible way of acquiring novel metabolites. The present study thus, reinforced the assumption that endophytes of ethno medicinal plants could be a promising source of antimicrobial substances and our data supports the general scientific opinion that endophytic microorganisms of medicinal plants are potential sources of bioactive compounds.

Further investigations on isolation of these antimicrobial compounds are crucial as an approach to search for novel natural products and in order to determine the structure of the specific compounds for the observed activity.

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References

Balick, M.J., Elisabetsky, E. and Laird, S.A. 1996. Medicinal Resources of the Tropical Forest: Biodiversity and its Importance to Human Health. Columbia University Press, New York.

Borkotoky, R., KalitaBarooah, M., Bora, S.S., Goswami, C. 2013. Evaluation and Screening of Antimicrobial activity of some important medicinal plants of Assam. Int. J. of Advancements in Res. and Tech. 2(4):132-139.

Carder, J. 1986. Detection and Quantitation of Cellulase by Congo Red Staining of Substrates in a Cup-Plate Diffusion Assay. Anal Biochem 153: 75.

Chareprasert, S., Piapukiew, J., Thienhirun, S., Whalley, A. and Sihanonth, P. 2006. Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr. World J. Microbiol. Biotechnol. 22: 481–486.

Cos, P., Vlietinck, A.J., Vanden, B.D. and Maes, L. 2006. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of concept'. J. of Ethnopharmacology 106: 290-302.

Duffy, C.F. and Power, R.F. 2001. Antioxidant and antimicrobial properties of some Chinese plant extracts. Int. J. Antimicrob. Agents 17: 527-529.

Ellis, M.B. 1971. Dematiatous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England.

Ellis, M.B. 1976. More dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England.

Gong, L.J. and Gou, S.H. 2009. Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity. Afr. J. Biotechnol. 8(5):731-736.

Hankin, L. and Anagnostakis, S.L. 1975. The use of solid media for detection of enzyme production by fungi. Mycologia 67: 597-607.

Homan, P.G. 2002. Drug Preparation and Extraction, Royal Pharmaceutical Society of Great Britain.

Huang, W.Y., Cai, Y.Z., Hyde, K.D., Corke, H. and Sun, M. 2008. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers. 33: 61–75.

Kaul, S., Gupta, S., Ahmed, M. and Dhar, M. 2012. Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. Phytochem. Rev. 11(4): 487-505.

Kharwar, R.N., Verma, S.K., Mishra, A., Gond, S.K., Sharma, V.K., Afreen, T. and Kumar, A. 2011.

Assessment of diversity, distribution and antibacterial activity of endophtic fungi isolated from a medicinal plant *Adenocalymma alliaceum* Miers. Symbiosis. 55(1):39-46.

Li, H., Qing, C., Zhang, Y. and Zhao, Z. 2005. Screening for endophytic fungi with antitumor and antifungal activities from Chinese medicinal plants. World J. Microbiol. Biotechnol. 27:3005-3008.

Li, Y., Lu, C., Hu, Z., Huang, Y. and Shen, Y. 2009. Secondary metabolites of *Tubercularia* sp. TF5, an endophytic fungal strain of *Taxus mairei*. Nat. Prod. Res. 23(1):70-76.

Mahesh, B., Tejesvi, M.V., Nalini, M.S. and Prakash, H.S. 2005. Endophytic mycoflora of inner bark of *Azadirachta indica* A. Juss. Curr. Sci. 88: 218-219.

Matsushima, T. 1971. Microfungi of Solomon Islands and Papua-New Guinea. Matsushima. Kobe, Japan.

Matsushima, T. 1975. Icones Microfungorum A Matsushima Lectorum. Matsushima. Kobe, Japan.

Neysa, Mai, Teatulohi Matainaho, Prem, P., Rai and Barrows, L.R. 2013. Antimicrobial activity of endophytes in six medicinal plants collected in the Central Province, Papua New Guinea. Pacific Journal of Medical Sciences. 11(2).

Petrini, O. 1991. Fungal endophyte of tree leaves. In: Andrews JH, Hirana SS (eds.) Microbial Ecology of leaves. Springer Verlag, New York.USA, pp. 179-197.

Petrini, O. 1986. Taxonomy of endophytic fungi of aerial plant tissues. In: Microbiology of the Phyllosphere. Fokkema JN, Heuvel Van-Den J. eds Cambridge University Press, Cambridge. 175-187.

Pieckova, E. and Roeijmans, H. 1999. Antibiotic secondary metabolites of *Dichotomomyces cejpii*. Mycopathol. 146: 121-126.

Raviraja, N.S. 2005. Fungal endophytes in five medicinal plants species from Kudremukh Range, Western Ghats of India. J. Basic Microbiol. 45:230-235.

Scherrer, R. and Gerhardt, P. 1971. Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. J. Bacteriol. 107: 718-735.

Seifert, K., Morgan-Jones, G., Gams, W. and Kendrick, B. 2011. The Genera of Hyphomycetes. CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands.

Siqueira, V.M., Conti, R., Araujo, J.M. and Souza-Motta, C.S. 2011. Endophytic fungi from the medicinal plant *Lippiasidoides* Cham and their antimicrobial activity. Symbiosis 53:89-95.

Strobel, G., Daisy, B., Castillo, U. and Harper, J. 2004. Natural products from endophytic microorganisms. J. of Nat. Pro., 67: 257-268.

Strobel, G.A. and Daisy, B. 2003. Bioprospecting for Microbial Endophytes and Their Natural Products. Microbiol Mol Biol Rev., 67: 491-502.

Strobel, G.A. 2002. Rainforest Endophytes and Bioactive Products. Crit. Rev. Biotechnology. 22: 315.

Suryanarayanan, T.S. 2013. Endophyte research: going beyond isolation and metabolite documentation. Fungal Eco. 6: 561-568.

Sutton, B.C. 1980. The Coelomycetes: Fungi Imperfectii with Pycnidia, Acervuli and Stromata. Commonwealth Mycological Institute, U.K.

Tan, R.X. and Zou, W.H. 2001. Endophytes: a rich source of functional metabolites. Nat. Prod. Rep.18: 448-459.

Tayung, K. and Jha, D.K. 2006. Antimicrobial evaluation of some fungal endophytes isolated from bark of Himalayan yew. World J. of Agri.Sci. 2: 489.

Tejesvi, M.V., Mahesh, B., Nalini, M.S. and Prakash, H.S. 2005. Endophytic fungal. assemblages from inner bark and twig of *Terminalia arjuna* W. & A. (Combretaceae). World J. of Microbiol. & Biotech. 2: 1535-1540.

Verma, V.C., Gond, S.K., Kumar, A., Kharwar, R.N., Boulanger, L.A. and Strobel, G.A. 2011. Endophytic fungal flora from roots and fruits of an Indian neem plant *Azadirachta indica* A. Juss and impact of culture media on their isolation. Ind.J.of Microbiol. 51:469-476.

Wang, F.W., Jiao, R.H., Cheng, A.B., Tan, S.H. and Song, Y.C. 2007. Antimicrobial potentials of endophytic fungi residing in *Quercus variabilis* and brefeldin A obtained from *Cladosporium* sp. World J. Microbiol. Biotechnol. 23:79-83.

Wilson, A.D. 1995. Endophyte – the evolution of the term, a clarification of its use and definition. Oikos 73:274-276.

Wiyakrutta, S., Sriubolmas, N., Panphut, W., Thongon, N., Danwiserkanjana, K., Ruangrungsi, N., Meevootisom, V. 2004. Endophytic fungi with anti-microbial, anti-cancer, anti-malarial activities isolated from Thai medicinal plants. World J. of Microbiol.and Biotech. 20: 265-272.

World Health Organization 2009. Medicinal Plants in Papua New Guinea. WHO Western Regional Publications. Western Pacific Series. 1: 16-205.