BIOASSAY OF SECONDARY METABOLITESFROM ENDOPHYTIC FUNGI ISOLATED FROM MEDICINAL PLANTS

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ABSTRACT

Endophytes are micro-organisms that reside in the internal tissues of living plants without causing any overt symptoms. Generally, the relationship between the plant and its endophytes is one of a symbiotic nature whereby the endophyte colonizes the internal tissue of the plant. There is growing interest in endophytes and their origin, their biodiversity, endophyte-host interactions, their role in ecology and the characterization of their secondary metabolites. Only a handful of plants, mainly grass species, have been completely studied in relation to their endophytic biology. Endophytic fungi are being explored by both pharmaceutical and agricultural industries as they represent an untapped pool of secondary metabolites. In the present study endophytic fungi was isolated from four different medicinal plants. Then the extraction of secondary metabolites from the fungi was carried out. Anti-microbial assay was carried out and the fungi from Hugonia mystax showed maximum activity against Streptococcus pneumonia, MRSA and Vibrio cholera. The cytotoxic effect of fungal endophyte isolated from Hugonia mystax and Morinda pubscens was tested by the MTT assay. The fungal extract from Morinda pubescens showed 52% cyto-toxicity while that of Hugonia mystax exhibited 40% cyto-toxicity which is nominal when compared to the metabolite from Morinda pubescens.

Keywords: Antimicrobial assay, anti-tumour assay, Endophyticfungi, MTT assay.

1. INTRODUCTION

Endophytic microorganisms are to be found in virtually every plant on earth. By definition, endophytes are those microbes colonising healthy tissues of plants and can stimulate plant growth,

increase disease resistance, improve the plants ability to withstand environmental stress and recycle nutrients (Strutz *et al*.2000; Strobel 2002). These organisms reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic. Endophytes may contribute to their host plant by producing a plethora of substances that provide protection and ultimately survival value to the plant. Ultimately, these compounds, once isolated and characterized, may also have potential for use in modern medicine, agriculture, and industry. Novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of what has been found after the isolation and culturing of individual endophytes followed by purification and characterization of some of their natural products. The prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great. Other applications in industry and agriculture may also be discovered among the novel products produced by endophytic microbes (Strobel *et al*, 2004).

Endophytes were mentioned for the first time by Bray in 19th century (Azevedo *et al*. 1998). It has been found in nearly all plant families (Sieber *et al*. 1998). It was estimated that there may be atleast one million species of endophytic fungi alone (Dryfuss and Chapera, 1994). Endophytic fungi have been described as fungi that asymptomatically colonize healthy plant tissue, even though they may after incubation or a latency period cause disease (Petrini 1991; Stone *et al*. 2000). Diverse association with host plants have been reported, ranging from mutualistic relationships (Schultz *et al*.2002) and cryptic commensalism (Deckert *et al*. 2001) to latent and quiescent pathogen (Sinclair and Cerkauskas 1996). They frequently demonstrate single host specificity at the plant species level but this specificity could be influenced by environmental conditions (Cohen, 2004). Recently, they are viewed as outstanding source of secondary metabolites and bioactive anti-microbial natural products (Jalgaonwala *et al*. 2010).

Medicinal plants are known to harbour endophytic fungi that are believed to be associated with the production of pharmaceutical products (Zhang *et al*.2006). Increasing levels of antibiotic resistance in both pathogenic and` non-pathogenic bacteria have spurred the search for new antibiotics to control diseases. Realising the capability of microorganisms to produce diverse bioactive molecules and the existence of unexplored microbial diversity, research is underway to isolate and screen microbes from diverse habitats and unique environment for discovery of novel metabolites. In recent years the search for new metabolites has been directed towards microbes residing inside plant tissues called endophytes.

The array of alkaloids and other chemicals synthesised by plants endow the plant with more resistance to nematodes, insect herbivores and livestock (Schardl *et al*., 2004). Many workers have demonstrated that the endophytes isolated from medicinal plants are excellent producers of strong fungicidal, bactericidal and cyto-toxic metabolites (Radu and Kqueen, 2002; Wang *et al*., 2007).

Hugonia mystax Cav. is scrambling shrub with spreading branches, set with numerous short stiff woody tendrils (modified peduncles, sometimes bearing flowers), belonging to the family Linaceae. *Morinda pubescens* Sm. belongs to the family Rubiaceae, grows wildly and is distributed throughout Southeast Asia. *Tinospora cordifolia* (Willd.) Miers is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae. It is distributed throughout tropical Indian subcontinent and China (Anonymous, Wealth of India, 1976). *Toddalia* is a monotypic genusof the citrus family containing the single species *Toddalia asiatica* L. It is native to many countries in Africa and Asia.

2. MATERIALS AND METHODS

2.1 COLLECTION OF PLANT MATERIAL:

To acquire endophytes, healthy medicinal plants were collected from the scrub vegetation of Tambaram, Chennai, India. The sample were brought to laboratory and processed within 24hrs according to (Fisher and Petrini, 1987). The soil is red laterite earth and at numerous places there are outcrops of rocks. The depth of the soil varies considerably and in some areas there is much gravel.

2.2 STERILIZATION OF GLASSWARES:

Sterilization of glasswares is essential to prevent the interference of other microbes present on them. The glasswares can be sterilized by using two methods:

1. Wet sterilization
2. Dry sterilization

2.3 STERILIZATION OF INCUBATION CHAMBER

The laminar air flow chamber consists of HEPA filter that sterilizes the air and also prevent the entry of microbes into the chamber. The chamber was wiped with ethanol and UV light was switched on for 30 minutes whose radiation is germicidal.

2.4 ISOLATION OF ENDOPHYTIC FUNGUS:

Endophytic fungi were isolated from the leaves of *Hugonia mystax, Morinda pubescens,Tinospora cordifolia* and *Toddalia asiatica.* The leaves were removed from the plants and the surface tissue was sterilized by washing it with laboline for 15minutes. After rinsing it with sterile water it was washed in mercuric chloride for two minutes. This was followed by rinsing the leaves in sterile distilled water for seven times. 0.1ml of the last (7^{th}) wash of sterile water was used for spread plating in both nutrient agar (NA) and potato dextrose agar (PDA) media. The tissues were aseptically

cut into small pieces, placed on to the prepared NA and PDA plates and incubated. The NA plates were incubated at 37˚C for 24-48hours and the PDA plates were incubated at 25-26˚C for upto 3-7 days to allow for hyphal growth from within the plant tissue. The hyphal material was transferred to PDA plates and incubated at 30˚C for 7 days to allow the growth of mycelium.

2.5 EXTRACTION OF SECONDARY METABOLITES**:**

An agar block containing fungi from each subcultured plate was taken and inoculated in conical flasks containing minimal media. They were kept for incubation in shaker for 15 days. After incubation, the media were transferred to centrifuge tubes and were centrifuged at 5,000 rpm for 10 minutes. The supernatant contains the secondary metabolites of the particular fungi. It was taken in separating funnel (Plate 3; Fig. 3.1). Ethyl acetate (organic solvent) was added in the ratio 1:1 to the supernatant. The mixture was shaken vigorously and left overnight. The organic phase (Plate 3; Fig. 3.2) was collected in petriplate and solvent was allowed to vaporize in the room temperature leaving the secondary metabolite behind. The dried extract was scrapped from the petriplate using scalpel and transferred into glass vials.

2.6 ANTIMICROBIAL ASSAY:

Mueller Hinton Agar (MHA) was prepared according to the manufacturer's instructions (HiMedia). Molten media was dispensed into petriplates to a uniform thickness of 4mm, under strict aseptic conditions in Laminar Air Flow chamber and the medium was allowed to solidify.

Microbial cultures obtained from the lab were used for detection of anti-microbial activity, 4 different bacterial cultures: *Aeromonas hydrophila, Streptococcus pneumoniae,* MRSA and *Vibrio cholera* were used. MHA plates were swabbed with the above mentioned cultures individually in each plate using sterile cotton swabs. One of the well was inoculated with 5µl of Ciprofloxin which was used as a positive control in the antimicrobial assays. The plates were incubated at 37˚C for 24 hours and diameter of inhibition zone was recorded.

2.7 ANTI-TUMOUR ASSAY

The anticancer activity of the secondary metabolites extracted was tested against human colon cancer cell line Colo320 was obtained. In brief, approximately 5x10³cells/well (cell line) were seeded into 96 well plate, 100µl of RPMI 1640 medium was added and incubated at 37ºC. After 24hours, the medium was discarded and fresh medium was added with different concentration of plant extract (Hexane, ethyl acetate, methanol) such as 125, 250, 500(100-2000µg/ml). The plates were incubated for 1-3 days at 37ºC in a CO_2 incubator. Then the absorbance was read in a spectrophotometer at 630nm and cell survival was calculated by using the following formula.

Viability % = Test OD/Control OD X 100

Cytotoxocity% = 100 – viability%

The absorbency was compared with co-assayed positive control, Cyclo90, a known cancer drug. The cyto-toxicity of 50% or more was defined as positive for anti-tumoural activity.

3. RESULTS AND DISCUSSION

3.1 ANTIMICROBIAL ASSAY

The endophytic fungi were isolated from the cultured leaves of the four medicinal plants (Plate 2). The crude metabolite was extracted from the fungus by solvent extraction procedure. The metabolite exhibited strong to moderate antimicrobial activity against the test pathogens. (Table 1). To assess the magnitude of antimicrobial action, the metabolites were co-assayed with the reference antibiotic Ciprofloxin. As the fungal isolate from *Toddalia asiatica* produced only less amount of secondary metabolite, it was not taken for further investigation. The metabolites were assessed for their anti- microbial activity against 4 different bacterial cultures: *Aeromonas hydrophila* (AERO) (Plate 4; Fig. 4.1), *Streptococcus pneumonia* (STREPTO) (Plate 4; Fig. 4.2), Methicillin-resistant *Staphylococcus aureus* (MRSA) (Plate 5; Fig. 5.1) and *Vibrio cholera* (VIBRIO) (Plate 5; Fig. 5.2). It was observed that none of them exhibited antimicrobial activity against *Aeromonas hydrophila.* The diameter of the inhibition zones formed by the antimicrobial activity was measured and recorded.

TABLE-1

The inhibition zone (diameter, mm) of the crude ethyl acetate extracts

Endophytes from:	**AERO**	**STREPTO**	**MRSA**	**VIBRIO**
Control (Ciprofloxin)	35mm	38 mm	40 mm	40 mm
Hugonia mystax	-	22 mm	20 mm	20 mm
Morinda pubescens	-	15 mm	16 mm	18 mm
Tinospora cordifolia	-	14 mm	11 mm	19 mm

Among the endophytes isolated, the fungi from *Hugonia mystax* showed maximum activity against *Streptococcus pneumonia*, MRSA and *Vibrio cholera* with an inhibition zone of 22 mm, 20 mm and 20 mm respectively.

3.2 ANTI-TUMOUR ASSAY

The cytotoxic effect of fungal endophyte isolated from *Hugonia mystax* and *Morinda pubscens* was tested by the MTT assay, which showed the effect of its secondary metabolites on the cell viability in Colo320, human colon cancer cell line. The cells treated with the fungal extracts of concentration ranging between 125µg/ml and 500µg/ml showed a significant decrease in the cell viability (Table 2 & 3). The fungal extract from *Morinda pubescens* showed 52% cyto-toxicity while that of *Hugonia mystax* (Plate 6; Fig. 6.1)exhibited 40% cyto-toxicity which is nominal when compared to the metabolite from *Morinda pubescens* (Plate 6; Fig. 6.2).

Table 2

	Hugonia			**Control**	**PC**
	500µg	**250µg**	**125µg**		
Viability	59.16156	60.41667	62.3265	100	25.7644
Cytotoxicity	40.8384	39.5833	37.6735	0	74.2356

Table 3

	Morinda			**Control**	**PC**
	500µg	**250µg**	**125µg**		
Viability	47.16024	52.28495	49.27239	100	25.7644
Cytotoxicity	52.8398	47.7151	50.7276	0	74.2356

4. CONCLUSIONS

There is an increasing need for new bioactive compounds that can be used in medicine, industry and agriculture. The need for new antimicrobial agents in general comes from the increasing rates of resistance to antimicrobial agents such as antibiotics. In addition, the community desire for products that are organic has increased interest in natural methods of pathogen control. Thus there is growing need for new environment friendly antimicrobial agents that may be used safely in agriculture to control plant pathogens and spoilage organisms post-harvest. While plants have been a major source of new lead compounds for drug discovery, attention has more recently turned to endophytes as these

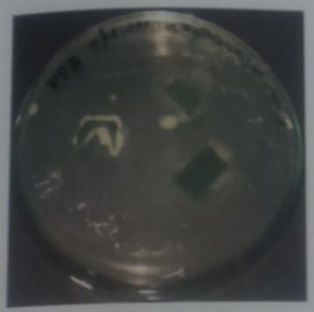
microorganisms are seen as having great potential as sources for new bioactive compounds (Strobel, 2003). While much of the interest in endophyte bioactive compounds has been in drugs for medicinal use, compounds that may have industrial or agricultural applications are also gaining attention. (Tran *et al.,* 2010).

Endophytes are also recognized as rich sources of bioactive metabolites of multifold importance (Tan and Zou, 2001; Strobel and Daisy, 2003). In this study, the crude extracts from the endophytic fungi displayed anti-bacterial and anti- tumour activity. The fungal extracts from *Hugonia mystax* has good antibacterial potency compared to other extracts and this isolate could be a good candidiate for further studies of their anti-bacterial bioactive compounds. The extract from the endophytic fungal culture of *Morinda pubescens* showed positive activity for anti-tumour in the MTT assay. Other endophytic fungal extracts which showed low anti-bacterial or anti-tumour activity may have active compounds but probably in smaller amounts and they could yield more potent compounds once they had undergone some purification (Fabry *et al.,* 1998).

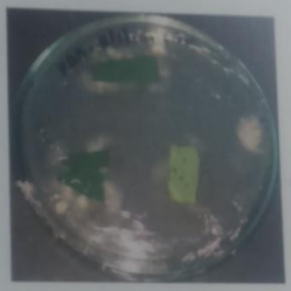
Therefore, any research on endophyte-plant symbiosis, such as in this study is of value, especially taking into account the positive biological activity as anti-microbial and anti-tumour agents. Effective extracts could provide potential leads towards the development of novel and eco friendly biologically agents (Radu and Kqueen, 2001). Thus, biological controls to prevent diseases offer an attractive alternative to disease management without the negative impact of chemical control. This study reinforced the assumption that endophytes of medicinal plants play an important role in the search for anti-tumoral compounds and also could be a promising source of antimicrobial substances. The observations from our study encourage further investigation on these plants.

5. PLATES

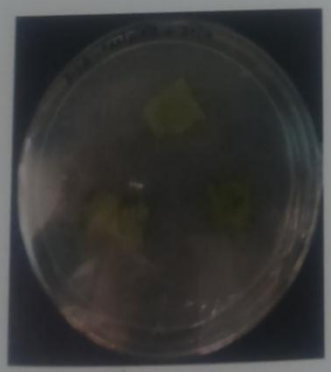
Plate 2
Culture of endophytes



(Fig. 2.1)
Hugonia mystax



(Fig. 2.2)
Morinda pubescens



(Fig. 2.3)
Tinospora cordifolia

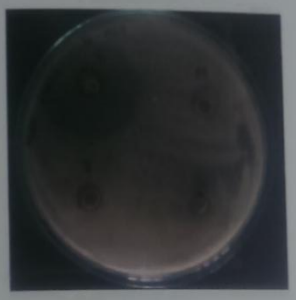
(Fig. 2.4)
Toddalia asiatica

Plate 3
Secondary Metabolite Extraction

(Fig. 3.1)

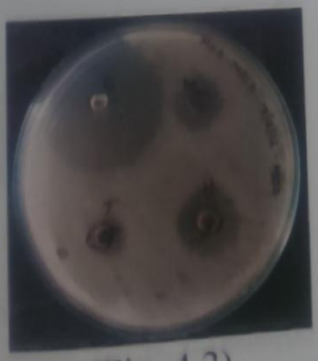
(Fig. 3.2)

Plate 4
Antimicrobial Activity



(Fig. 4.1)
Aeromonas hydrophila

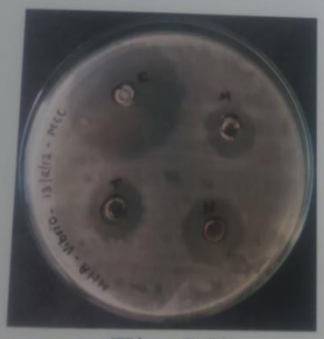
H - *Hugonia mystax*
M - *Morinda pubescens*
T - *Tinospora cordifolia*



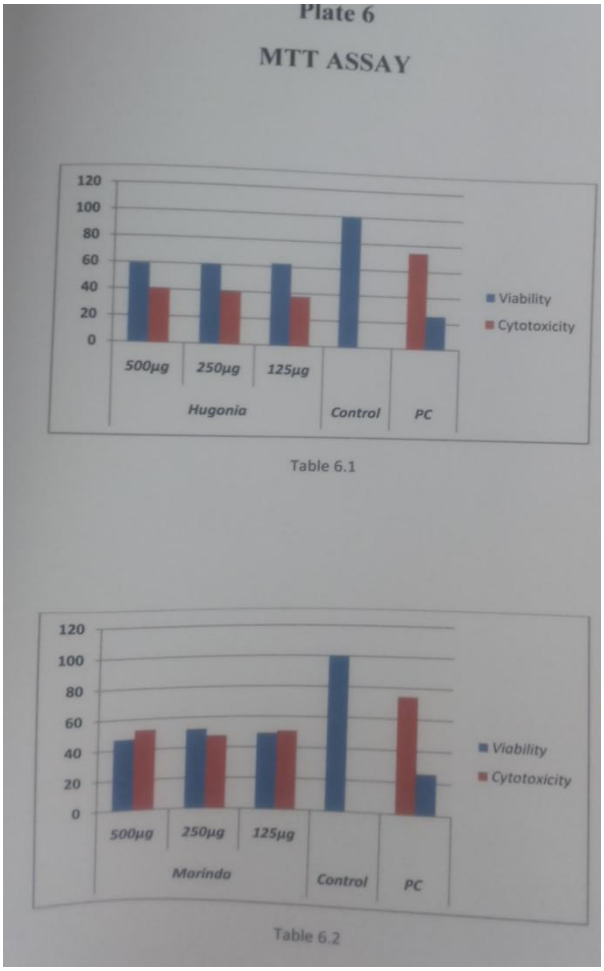
(Fig. 4.2)
MRSA

Plate 5
Antimicrobial activity

(Fig. 5.1)
Streptococcus pneumoniae
H - *Hugonia mystax*
M - *Morinda pubescens*
T - *Tinospora cordifolia*



(Fig. 5.2)
Vibrio cholera



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