DIVERSITY OF ENDOPHYTIC FUNGI IN PHYLLANTHUS AMARUS (SCHUM AND THONN) AND THEIR ANTIBACTERIAL ACTIVITY

Article *in* Asian Journal of Microbiology Biotechnology and Environmental Sciences · August 2015

CITATIONS
0

READS
706

2 authors, including:

Kalyanaraman Rajagopal
Ramakrishna Mission Vivekananda College, Mylapore, Chennai-600004
118 PUBLICATIONS **2,076** CITATIONS

Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 17, No. (4) : 2015 : 999-1003

ISSN-0972-3005

DIVERSITY OF ENDOPHYTIC FUNGI IN *PHYLLANTHUS AMARUS* (SCHUM AND THONN) AND THEIR ANTIBACTERIAL ACTIVITY

KALYANARAMAN RAJAGOPAL., SOWPARTHANI* K., KATHIRAVAN G., P. ARUMUGAM, JAMITH BASHA, N., MEENAMBIGA, M., DURGA, A., ASHWINI, S. AND RADHI, R.

Department of Biotechnology, Vels Institute of Science, Technology and Advanced Studies (VISTAS), VELS University, Pallavaram, Chennai, Tamilnadu, India.
*Department of Biotechnology, Valliammal College, University of Madras, Chennai, Tamilnadu, India.

(Received 13 June, 2015; accepted 20 Agust, 2015)

Key words : *Phyllanthus amarus, Endophytic fungi, Gleosporium quercinum, Ethylacetate extract, Antibacterial activity.*

Abstract –Phyllanthus amarus endophyte assemblage and their bioactivity against bacteria was studied. The initial studies on screening the endophytic fungi from *Phyllanthus amarus* indicate their potential generation of bioactive compounds for new antibiotic discovery. Further research is in progress to determine bioactive compounds of other endophytic fungi and which fractions of the *Gleosporium quercinum* extract was responsible for the biological activity. Work is ongoing to elucidate the chemical structure of the metabolites. Hence, to our knowledge for the first time *Phyllanthus amarus* endophyte assemblage and their bioactivity against bacteria was studied.

INTRODUCTION

Endophytic Fungi colonize almost all plants and have been isolated from all plant parts such as roots, stems, leaves, barks, floral organs and even seeds. There could be more than one type of endophytic fungi found within one plant (Petrini *et al.*, 1992) .Endophytic Fungi are relatively unexplored producers of metabolites useful to pharmaceutical and agricultural industries. Filamentous fungi produce a diverse array of secondary metabolites – small molecules that are not necessary for normal growth or development. Secondary metabolites have a tremendous impact on society; some are exploited for their antibiotic and pharmaceutical activities, others are involved in disease interactions with plants or animals. (Pavithra *et al.*, 2012; Rezwana Khan *et al.*, 2007)

The emergence of antibiotic- resistant micro-organisms calls for inventive research and development strategies. Inhibition of these pathogenic micro-organisms may be a promising therapeutic approach. The screening of antimicrobial compounds from endophytes is a promising way to meet the increasing threat of drug- resistant strains of human and plant pathogens. (Gangadevi *et al.*, 2008).

Phyllanthus amarus is the largest genus in the flowering plant family *Euphorbiaceae*. Fresh leaf paste has wound healing capacity and used to cure white spots on skin and jaundice. In India, *Phyllanthus amarus* is widely distributed as a weed in cultivated and waste lands. It has many valuable compounds such as lignans, flavonoids, hydrolysable tannins (ellagitannins), polyphenols, triterpenes, sterols and alkaloids. The compounds isolated from *Phyllanthus amarus* show a wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, anti-inflamatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, nephroprotective and diurectic properties. Fresh leaf paste has wound healing capacity and used to cure white spots on skin and jaundice. (Itoro *et al*., 2013; Patel *et al.*, 2011; Saranraj *et al*., 2012; Sonia Verma *et al.*, 2014).

MATERIALS AND METHODS

Collection of samples

The *Phyllanthus amarus* leaf samples were collected from Anna Herbal Garden, Tamilnadu. The samples

were transported in closed sterile polythene bags and processed within 24 hrs collection

Surface Sterilization

The fresh leaf samples were surface sterilized by following the modified (Dobranic *et al*., 1995).

Isolation of fungal endophytes from collected samples

The leaves were washed in running tap water. Approximately 0.5 cm^2 on leaf segments were cut from the healthy leaves. They were surface sterilized by 70% ethanol for 5 seconds, 4% Sodium hypochloride for 90 seconds and rinsed by sterile distilled water for 10 seconds. After sterilization these leaf segments were evenly placed in Petri dishes containing Potato dextrose agar (PDA), Containing Potato 200, dextrose 20 and agar 20 medium amended with 10mg of Chloromphenicol. The petri dishes incubated at 26 ± 1°C in a light chamber (Bills and Polishook, 1992) and monitored every day for the growth of endophytic fungi. The hyphal tips, which grew out from leaf segments were isolated and brought into pure culture (Rajagopal, 1999). The isolated endophytic fungi were identified using standard manuals and monographs.

Extraction of bioactive compounds

The endophytic fungi *Gleosporium quercinum* was grown in Czapek's broth and incubated for 21 days at 120 rpm. The crude extract was separated using Whatman's No 1 filter paper. Equal volume of ethyl acetate was added in culture filtrate and organic layer was concentrated in rotary vacuum evaporator. The dry semi solid residue was redissolved in ethylacetate for the subsequent study using (Suthep *et al*., 2004)

Qualitative test for chemical compounds

The different qualitative tests for various types of chemical compounds are given below (Krohn, *et al.,* 2007; Langenheim, 1994; Dennis, 1995).

Test for Steroids

About 2mg/mL of the endophytic fungal extracts were added to the concentrated sulphuric acid in alcohol heated gently if necessary, green colour shows the presence of steroids.

Test for Alkaloids

2 mg/mL of the endophytic fungal extracts is treated with 2 drops of dragendroff reagent (Bismuth nitrate and tartaric acid – solution A and potassium Iodide – Solution B). Solution A and B mixed together in equal volume) red or orange precipitate indicates the presence of alkaloids.

Test for Flavonoids

10% $NaoCl_3$ in alcohol is treated with 2 mg/mL of the endophytic fungal extracts and heated at 150°C. Blue colour shows the presence of flavonoids.

Test for Terpenoids

2 mg/mL of the endophytic fungal extracts is treated with Puncal reagent (ammonium heptamolybedate, Ceric sulphate in Concentrated sulphuric acid) and heated at 150^0C. Blue colour shows the presence of terpenoids.

Test for Tannins

2 mg/mL of the endophytic fungal extracts is treated with saturated solution of ferric chloride; blue colour indicates the presence of Tannins.

***In vitro* antibacterial activity**

The *Gleosporium quercinum* crude extract was tested against both gram positive and negative bacteria using a disc diffusion method (Yadav *et al.,* 2010). 25µl of crude extract was added on to a sterile disc with a size of 6mm and allowed to dry for 10 min. The disc containing extract was placed on the medium. The experiment was carried out in triplicates. Chloremphenicol was used as control. The plates were incubated as 28°C to 35°C ±1 for 24 - 48 hrs. The diameter of inhibition zone around the disc was measured by using ruler (Pavithra *et al.,* 2012).

RESULTS AND DISCUSSION

Endophytic fungi are one of the most unexplored and diverse group of organisms that make symbiotic associations with higher plants and may synthesis more compounds for host (Wester, 1981; Shiomi *et al.,* 2006; Maheshwari and Rajagopal, 2011). Endophytic fungi have been widely explored as source secondary metabolites which are biologically active Sowparthini and Rajagopal, (2011). Several herbs, shrubs and trees have been intensively studied for endophytic fungi in the tropics (Suryanarayanan and Thennarasan, 2004; Strobel *et al.,* 2002). Therefore, the present work was initiated to find out endophytic flora of *Phyllanthus amarus*

and any endophytic fungi would produce such compounds (Secondary metabolites).

The endophytic flora, both numbers and types, differ in their host and depends on host geographical position (Gange *et al.,* 2007; Arnold and Herre, 2003). Morphological characters of the endophytes were used to identify the isolated endophytic fungal taxa with the assistance of contemporary mycological literature. All the endophytic fungi isolated and identified were maintained in PDA slants with (15% v/v). All the cultures were deposited in the Department of Biotechnology Vels University, Fungal Culture Collection (VUFCC 141-150). The colonization frequency of (%CF) of endophytic fungi isolated from *Phyllanthus amarus* was calculated as the number of plant segments colonized by a single endophyte divided by total number of segments observed x 100 (%CF = $N_{col}/N_{t)}$ x 100; Where N_{col} is the number segments colonized by each fungus, and N_t the total number of segments.

In the present investigation the study showed that *Phyllanthus amarus* supports diverse population of endophytic fungi (Table 1). A total of 10 fungal endophytes were obtained from *Phyllanthus amarus* leaf. Out of 10 endophytic fungi isolated 7 species belonging to Hypomycetes and 3 species belonging to Coelomycets (Table 1).

Table 1. Occurence of endophytic fungi in the leaf of *Phyllanthus amarus*

S. No.	Name of the Endophyte	Cf%	Standard Deviation
1.	*Dracheslera australiensis*	2.5	8.33 ± 0.1
2.	*Chaetomium indicum*	5.0	16.77 ± 0.03
3.	*Botrytis sp*	4.9	16.12 ± 0.04
4.	*Emericella nidulans*	5.0	16.7 ± 0.3
5.	*Chaetomium globosum*	5.3	17.9 ± 0.2
6.	*Nigrospora oryzae*	3.0	9.25 ± 0.1
7.	*Curvularia lunata*	2.8	8.51 ± 0.2
8.	*Gleosporium quercinum*	6.8	19.58± 0.4
9.	*Pestalotiopsis microspora*	5.0	16.77 ± 0.2
10.	*Phyllosticta sp*	2.4	8.31 ± 0.3

P ≥ 0.5 level (Triplicates were maintained)

Table 2. Phytochemical analysis of crude extract of *Gleosporium quercinum.*

S. No.	Name of the Phytochemical Test	*Gleosporium quercinum.*
1	Steroid	+
2	Terpenoids	+
3	Tannins	+
4	Alkaloids	+
5	Flavonoids	+

Hypomycetes were the dominant endophytes followed by Coelomycetes. Although 10 different taxa were present in leaf (Table 1), only 5 endophytic fungi showed appreciable densities of colonization (above 5%). The dominant endophytic fungi recored was *Gleosporium quercinum* (6.8%), *Chaetomium indicum* (5.0), *Emericella nidulans* (5.0), *Pestalotiopsis microspora*. Other endophytic fungi isolated were showed less than 5% of CF. Thus, the leaf tissues harbour rich biodiversities in their endophytic communities and this result corroborates those of a previous study (Rajagopal and Suryanarayanan, 1998); Suryanarayanan *et al.,* 1998; Caroll, 1995; Ravindra *et al.,* 2008). According to Petrini (1986), Suryanarayanan, (1998) and Rajagopal, (1998) only one or few endophytic taxa dominate a single most species, but in this study few endophytes distributed considerably. Petrini (1986) grouped endophytic fungi into xylariaceous, coprophilous, epiphytic and true endophytic forms. In the present study, representatives from epiphytic and endophytic were present to constitute the endophyte assemblage of the *Phyllanthus amarus* leaf.

An understanding of the physiology of endophytic fungi of leaves is expected to provide information about host endophyte association. Endophytic fungi have been shown to produce extracellular enzymes, growth regulators (Carroll and Petrini, 1983; Pugh, 1973) bioactive compounds which are active against insect and bacteria (Findlay *et al.,* 1997). *Phyllanthus amarus* have numerous phytocompounds such as alkaloids, flavonoids, tannis, lignins, polyphenolic compounds and tetracyclic compounds (Sonia Varma *et al*., 2014). The medicinal properties of the plant could be attributed to their endophytic fungi (Rezwana Khan *et al.,* 2007).

Endophyte produces several compounds in culture. The crude culture filtrate of *Gleosporium quercinum* was analysed for various chemical groups.The results are presented in (Table 2). Production of alkaloid, steroids and flavonoids already been reported for endophytic fungi (Schulz, 2002; Tan and Zou, 2001). Our study with *Gleosporium quercinum.* crude extract showed the presence of different compounds such as Steroid, Terpenoids, Tannins, Alkaloids, and Flavonoids (Table 2).

Table 3. Invitro antibacterial activity of endophytic fungi *Gleosporium quercinum.*

Endophytic fungi	Zone of inhibition by the extract of endophytic fungi (mm)				
	Mean ± SD, n=3 Test organism				
	1	2	3	4	Control
Gleosporium quercinum	*St* 11.2±0.4	*E.coli* 21.0±0.1	*Sa* 13.9±0.5	*Kp* 19.1±0.2	21.1±0.1

St. Salmonella typhi, E. coli, Staphylococus aureus, Klebseilla pneumonia.

The crude extract from the endophytic fungi *Gleosporium quercinum* was tested for anti bacterial activity against gram positive and gram negative bacterium. The cultures were obtained from MTCC (Microbial Type Culture Collection, Chandigarh India). The antibacterial activity was tested using disc diffusion method (Pavithra, *et al*., 2012; Mabrouk, *et al*., 2008 and Yadav *et al*., 2010). Twenty five micro litre of crude extract was added on to a sterile 6mm disc. (Hi-media) using a micro pipette and allowed to dry for 10 min. The experiments were carried out in triplicates. Chloromphenicol (5mg/ml) was used as control. The petriplates were incubated at 27°C ± 1°C for 24-48 hrs for growth. The zone of inhibition around the disc was measured by using ruler. The results are tabulated in Table 3.

The crude extract of *Gleosporium quercinum* has significantly inhibited the gram positive and gram negative bacteria (Table 3). The zone of inhibition by the endophytic fungi was very much comparable to the standard antibiotic. Among the tested organisms *E. coli* was susceptible for the compounds extracted. The zone of inhibition was 21mm which is equivalent to standard antibiotic used chloremphenicol (21.1). *S.typhi* was least inhibited 11.2mm just 50% when compare to the control. The differences in inhibition would be due to compounds present in the crude extract was not as effective as control antibiotic or due to the solvent used. Hence, use of other organic solvents for extraction would provide answer to this. Similar studies carried out by (Radji *et al*., 2011; Zhang, *et al*., 2009).

REFERENCES

Arnold, A.E. and Herre, E.A. 2003. Canopy cover and leaf age affect colonization by tropical fungal endophytes: Ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycologia* 95 : 388-398.

Bills, G. F. and Polishook, J. D. 1992. A new species of *Mycoleptodiscus* from living foliage of *Chamaecyparis thyoides. Mycotaxon.* 43 **:** 453-460.

Bills, G.F and Polishook J.D. 1992. Recovery of endophytic fungi from *chamaecyparis thyoides, Sydowia*. 44 : 1-12.

Carroll and Petrini, O. 1983. Patterns of substrate utilization by some fungal endophytes from coniferous foliaee *Mycologia.* 75 : 53-63.

Carroll, G.C. 1995. Forest Endophytes: Pattern and process. *Canadian Journal of Botany* 73 : 1316-1324.

Dennis, W. 1995. Fungal endophytes which invade insect galls; insect pathogen, beningn saprophytes or fungal inquilines. *Ecologia*. 103 : 255-260.

Dobranic, J.K., Johnson, J.A. and Alikhan, Q.R. 1995. Isolation of endophytic fungi from eastern larch (*Larix laricina*) leaves from New Brunswick, Canada. *Canadian Journal of Microbiology.* 41 : 194-198.

Gangadevi, V. and Muthumary, J. 2007. Preliminary studies on cytotoxic effect of fungal taxol on cancer cell lines. *African Journal of Biotechnology*. 6 (12) : 1382-86.

Gangadevi, V., Yogeswari, S., Kamalraj, S., Rani, G. and Muthumary, J. 2008. The Antibacterial activity of *Acalypha in-dica L. Indian Journal of Science and Technology*. 1 (6): Page

Itoro, E., Ukana, D. and Ekaete, D. 2013. Phytochemical screening and nutrient analysis of *Phyllanthus amarus*. *Asian Journal of Plant Science and Research*. 3 **:** 116-122.

John, A., Findlay, Guoqiang Li, and John, A. 1997. Bioactive compounds from an endophytic fungus from eastern larch (*Larix laricina*) needles. Johnson *Canadian Journal of Chemistry*. 75 (6) : 716-719.

Krohn, K., Ullah, Z., Hussain, H., Flörke, U., Schulz, B., Draeger; S., Pescitelli, G., Salvadori, P., Antus, S., and Kurtan, T. 2007. Massarilactones E-G; New metabolites from the endophytic fungus *Coniothyrium*. sp., associated with the Plant *Artimisia maritime*. 19 : 464–470.

Langenheim, J.H. 1994. Higher plant terpenoids: a phytocentric overview of their ecological roles. *Journal of Chemical Ecology*. 20 : 1223–1279.

Mabrouk, A.M., Kheiralla, Z.H., Hamed, E.R, Youssry, A.A. and Aty, A.E 2008. Production of some biologically active secondary metabolites from marine-derived fungus *Varicosporina ramulosa*. *Malaysian Journal of Microbiology* 4 : 14-24.

Maheswari, S. and Rajagopal, K. 2011. Biodiversity of endophytic fungi associated with *Ficus religiosa* and *F. benghalensis, Mycologia balcanica.* 8 : 169–172.

Patel, J.R., Tripathi, P., Sharma, V., Chauhan, N.S., and Dixit, V.K. 2011. *Phyllanthus amarus*: ethnomedicinal uses, phytochemistry and pharmacology: a review, *Journal of Ethnopharmacology*. 138 (2) : 286-313.

Pavithra, N., Sathish, L., Ananda, K. 2012. Antimicrobial and enzyme activity of endophytic fungi isolated from Tulsi, *Journal of Pharmaceutical and Biomedical Sciences*. 16 (12) : 2230-7885.

Pavithra, P.S., Janani, V.S., Charumathi, K.H., Indhumathy, R., Sirisha Potala, Rama and Sonia Verma, 2012. Antibacterial activity of plants used in Indian herbal medicine. *International Journal of Green Pharmacy*. 4 **:** 23-28.

Petrini, O., Sieber, T.N., Toti and Viret, O. 1992. Ecology; metabolite production; and substrate utilization in endophytic fungi. *Natural Toxins* 1 : 185-196.

Petrini, O. 1986. Taxonomy of endophytic fungi of aerial plant tissues. In: *Microbiology of the Phyllopshere* (eds. N.J. Fokkema and lVan. den *Heuvel*). Cambridge University Press, Cambridge, UK: 175-187

Pugh, G.J.F. 1972. Saprophytic fungi and seeds. In *Seed Ecology* (ed. W. Heydecker), 337-345. Butterworth. London.

Radji, M., Sumiati, A., Rachmayan, R., Elya, B. 2011. Isolation of fungal endophytes from *Garcinia mangostana* and their antibacterial activity. *African. Journal of Biotechnology.* 10 : 103-107.

Ravindra, N., Kharwar., Vijay, C., Verma., Gary Strobel., and David Ezra. 2008. The Endophytic fungal complex of *Catharanthus roseus* (L) G. Don. *Current Science*. 95 (2): 25.

Rezwana Khan, Saleem Shahzad, M., Iqbal Choudhary., Shakeel, A., Khan and Aqeel Ahmad 2007. Biodiversity of the Endophytic fungi isolated from *Calotropis procera* (AIT.) R. BR. *Pakistan Journal of. Botany*. 39 (6): 2233-2239.

Rezwana Khan., Saleem Shahzad., Iqbal Choudhary, M., Shakeel A. Khan and Aqeel Ahmad. 2007. Biodiversity of the Endophytic fungi isolated from *Calotropis Procera* (AIT.) R. BR. *Pakisthan Journal of Botany*. 39 (6): 2233-2239.

Saranraj, P and Sivasakthivelan, P. 2012. Screening of antibacterial activity of the medicinal plant *Phyllanthus amarus* against urinary tract infection causing bacterial pathogens. *Applied Journal of Hygiene*. 1 (3): 19-24.

Schulz, B., Boyle, C., Draeger, S., Rommert, A. and Krohn, K. 2002. Endophytic Fungi: a source of novel biologically active secondary metabolites. *Journal of Mycological Society*. 106 : 996-1004.

Shiomi, H.F., HSA Silva., IS De Melo., Nunes, F.V., and Bettiol, W. 2006. Bioprospecting endophytic bacteria for biological control of coffee leaf rust. *Sci. Agric*., 63 (1): 32-39.

Sonia Verma., Hitender Sharma., Munish Garg. 2014. *Phyllanthus amarus*: A Review, *Journal of Pharmacognosy and Phytochemistry*. 3 (2): 18-22.

Sonia Verma., Hitender Sharma., Munish Garg. 2014. *Journal of Pharmacognosy and Phytochemistry* 3 (2) **:** 18-22.

Sowparthani, P. and Rajagopal, K. 2011. Assemblage, Bioactivity and Chemical profile (ABC) of endophytic fungi associated with *Ocimum sanctum* L. — In: Proceedings *of Asian Mycological Congress*, Pp 278.1. Incheon, Korea.

Strobel, G., Ford, E., Worapong, J., Grant, D.M. and P.C. Fung. 2002. Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. *Phytochemistry*. 60 : 179-183.

Suryanarayanan, T.S. and Thennarasan, S. 2004. Temporal variation in endophyte assemblages of *Plumeria rubra* leaves. — *Fungal Diversity* 15 : 197–204.

Suryanarayanan, T.S., Rajagopal, K. and Ramanan, B.V. 1998. Occurrence of *Acremonium* fungal endophytes in Tropical forage grasses. — *Forage-Research.* 24 (2) : 87–89.

Suryanarayanan,T.S., Kumaresan, V. and Johnson, A. 1998. Foliar fungal endophytes from two species of the mangrove *Rhizophora. Canadian Journal of Microbiology.* 44 : 1003-1006.

Suthep, W., Nongluksna, S., Wattana, P., Nuntawan, T., Kannawat, D., Nijsiri, R. and Vithaya, M. 2004. Endophytic fungi with antimicrobial, anti-cancer and anti- malarial activities isolated from Thai medicinal plants, *World Journal of Microbiology and Biotechnology*. 20 : 65-272.

Tan, R.X. and Zou, W.X. 2001. Endophytes: A rich source of functional metabolites. *National Proceedings Report.* 18: 448-59.

Yadav, R., Nagendra, S., Chauhan, Amit, S., Chouhan, Soni, V.K., Omray, L. 2010. Antimicrobial screening of various extracts of *Aphanmixis polystachya* stems bark. *International Journal of Advances in Pharmaceutical Sciences*. 1 : 147-50.

Yadav, V., Kumar, M., Deep, D.K., Kumar, H., Sharma,R., Tripathi, T., Tuteja, N., Saxena, A.K. and Johri, J.K., 2010. A Phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. *Journal of Biological Chemistry*. 285 : 26532-26544.

Zhang, Yi, Mu, J., Feng, Y., Kang, Y., Zhang, J., Gu, P.J., Wang, Y., Ma, L.F. and Zhu, Y.H. 2009. Broad spectrum antimicrobial epiphytic and endophytic fungi from marine organisms: Isolation, Bioassay and Taxonomy. *Marine Drugs*. 7: 97-112.