International Journal of Phytomedicine 6 (2014) 523-528
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Antimicrobial and anti oxidant activities of an Endophytic fungi Isolated from an endemic medicinal plant Pterocarpus santalinus

Nagam Venkateswarlu[1], Netala Vasudeva reddy[1], Tartte Vijaya[1]* Krishna kanth shama[2]

***Corresponding author:**

Tartte Vijaya

[1]Department of Botany, Sri Venkateswara University, Tirupati-517 502, A.P, India.
[2]Department of Microbiology, Sri Venkateswara institute of medical science, Tirupati-517 502, A.P, India.

Abstract

Endophytic fungi are intriguing microorganisms live inside the healthy plant tissues. They are quite diverse in nature and have enormous potential for production of important secondary metabolites of biomedical, pharmaceutical and clinical importance. In the present work we have isolated endophytic fungi from the leaves of *Pterocarpus santalinus* an endemic medicinal plant of Eastern ghats, Tirumala hills, India. Based on the molecular characterization by 18S rRNA analysis the fungi was identified as *Xylaria* spp. ENT2 (Accession No. KF493856.1). the crude extract of *Xylaria* sp. was evaluated for antioxidant and antimicrobial activities. Among the other extracts tested methanolic extract showed highest activity against all the bacterial and fungal pathogens with a minimum bactericidal concentration (MBC) of about 30µg/mL against *Staphylococcus aureus* and a Minimum Fungicidal Concentration (MFC) of about 50µg/mL against *Candida albicans*. Similarly methanolic extract proved to be potential natural antioxidant with 56.26±0.08 radical scavenging activity (RSA).

Keywords: Endophytic fungi, molecular characterization, antimicrobial activity, antioxidant activity.

Introduction

Plants and microorganisms are the chief source of natural compounds. In recent years endophytic fungi have attracted researchers around the world as they produce a wide variety of bioactive secondary metabolites of therapeutical importance [1-4]. The present study has been made to isolate fungal endophytes from *Pterocarpus santalinus* L. an endemic medicinal plant of Tirumala hills in Eastern ghats, India. It is a semi evergreen tree belongs to the family Fabaceae. It has been used in almost all the traditional system of medicine, ayurveda, unani, and sidha from the ancient time. It has been used as a cooling agent, antipyretic, anti-inflammatory, anthelmintic, tonic, hemorrhage, dysentery, aphrodisiac, and ulcers [5]. The wood in combination with other drugs is also prescribed for snake-bites and scorpion-stings [6]. Despite its multiple medicinal values and also being an endemic and red listed species, it is remained unexplored for endophytic fungal communities associated with this plant.

Medicinal plants are known to harbour endophytic fungi that are believed to be associated with the production of pharmaceutical products [4]. Endophytic fungi asymptomatically inhabit plant tissues and have been isolated from many species of woody plants and grasses [7]. Endophytes may contribute to their host plant by producing a plethora of compounds that provide protection and survival value to the plant [8, 9]. Ultimately, these compounds, once isolated and characterized, may also have potential use in modern medicine. Novel antibiotics, antimycotics, immunosuppressants, and anti-cancer compounds are only a few examples of compounds produced by endophytes [9]. Plants from unique environmental settings, with an ethnobotanical history or which are endemic are likely to house novel endophytic microorganisms as a source of novel bioactive products [8]. The discovery of novel antimicrobial metabolites from endophytes is an important and alternative to overcome the increasing levels of drug resistance by plant and human pathogens [10, 11].

Endophytic fungi represent an important and quantifiable component of fungal diversity, with an estimate of at least 1 million species [12, 13]. They are found in nearly all plant families and have been investigated to be a rich source of novel biological active secondary metabolites of high significance [14]. These compounds have potential for use in modern medicine, agriculture, and industry. Thus endophytic fungi are considered to be rich and reliable source of genetic diversity with novel and undescribed species and their metabolites [15].

Hence the aim of the present study was to isolate endophytic fungi from an endemic medicinal plant *Pterocarpus santalinus* from Tirumala hills of Eastern ghats and to evaluate antimicrobial and antioxidant activities of the extract obtained with different organic solvent extracts.

Materials and Methods

Collection of Plant Materials

The plant samples were collected from Tirumala hills of Eastern ghats, Andhra Pradesh, India “Fig. 1”. The plant material was identified by the taxonomist, the voucher specimen was deposited

in Department of Botany. Healthy leaves were cut into segments with a sterile scalpel and stored at 4ºC in a sterile polythene bag prior to use.

Pterocarpus santalinus

Isolation of Endophytic Fungi

Isolation of Endophytic fungi from plant samples was carried out as described by Wang *et al.* [16] with little modifications. Leaves were washed under running tap water for 10 minutes followed by immersion in 75% ethanol for 1 minute and in 2.5% sodium hypochlorite for 5 minutes. Finally, leaves were rinsed with sterile distilled water. Leaves wer cut into small segments of 0.8x1.0cm aseptically and placed on petriplates containing potato dextrose agar (PDA) media supplemented with streptomycin sulphate (50 µg/mL). The plates were sealed using Parafilm and kept under UV light for 15 mins for spore induction. Then the plates were incubated at 27 ±2 C in a light chamber with 12 hours of light followed by 12 hours of dark cycles [17].

The petriplates were visually checked every day to observe the growth of endophytic fungal colonies from the plant segments. As the hyphal tips emerged out from plant segments they were isolated, sub-cultured and brought to pure culture by serial sub-culturing. Pure cultures were transferred to fresh PDA media and incubated.

Identification of Endophytic Fungi

Preliminary identification of fungal endophytes was carried out based on their microscopic and macroscopic characteristics. Lactophenol cotton blue staining method was used for staining the fungal cultures and visualized under Trinacular microscope (Olympus). Colonies were analyzed with respect to their average diameter, coloration of the mycelium, sporulation and production of acervuli and coloration of the conidia. Further identification was confirmed based on molecular characterization. The genomic DNA was extracted from the mycelium and the ITS regions including the intervening 5.8s rDNA were amplified using universal primers ITS 1 (5´GGAAGTAAAAGTAACAAGG3´) and ITS 4 (5´TCCTCCGCTTATTGATATGC3´) [18]. The nucleotide sequence data was submitted to NCBI Genbank. Based on the homology, pairwise and multiple sequence alignment the fungal strain was identified.

Preparation of fungal crude extracts

100ml of Potato Dextrose Broth (PDB) was taken into 250 mL erlenmeyer flask and autoclaved at 121^0C for 15 min and then inoculated with 7days old active fungal culture. The flasks were incubated in a shaker at 27 C ± 2 C with 130 rpm under normal lighting condition for a period of 10 days. After fermentation the fungal biomass was separated from the broth by filtration using Whatmann No.1 filter paper. The culture filtrate was successively extracted with Hexane, Ethyl acetate and Methanol. The successive extracts were concentrated to dryness using a rotary vacuum evaporator (Superfit, India) under reduced pressure. The resultant extract was then lyophilized and stored at 4 C for further use.

Test Microorganisms

The test microorganisms used in this study included three bacterial strains [*Staphylococcus aureus* (ATCC 5923), *Bacillas suptilis,* (ATCC 6633) *Escherichia coli* (ATCC 5922) and *Pseudomonas aeruginosa* (ATCC 7853)], three fungal strains [*Aspergillus niger* (ATCC 6404), *A. flavus* (ATCC 6498),] and *Candida albicans* (ATCC 9548). The bacterial cultures were sub-cultured every two weeks on fresh nutrient agar (NA) slants and incubated at 37 C whereas the yeast and fungal cultures were sub-cultured every four weeks on the fresh PDA slants and incubated at 28 C and 25 C respectively.

Antimicrobial Activity of crude extracts

Antimicrobial activity was determined using the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS), 2003. Pre-warmed Mueller-Hinton agar (MHA), PDA and NA (oxoid) plates were seeded with suspension of tested inoculums. Endophytic extracts dissolved in DMSO (1 mg/ml) were pipetted (20 µl) onto sterile paper discs (6 mm diameter) and placed onto the surface of inoculated agar plates. Plates were incubated at 37 C for bacterial cultures and 27ºC for fungal cultures in BOD incubator. Antimicrobial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts. Streptomycin and Fluconazole were co-assayed as positive antimicrobial references with DMSO as negative control.

Minimum inhibitory concentrations (MICs) of crude extracts which displayed the strongest antimicrobial activity were determined by agar micro dilution method with slight modifications. Overnight

culture of each test organisms (approximately 10^6 CFU) was inoculated into the wells and the crude metabolites was tested at concentration from 400 to 10 µg/ml. The plates were incubated for 24 h at 37 C. MIC was determined as the least concentration of the crude metabolites that inhibited the growth of the test organisms.

Antioxidant activity by DPPH assay

The scavenging effects of samples for DPPH radicals were monitored according to the method described by Duan, Zhang, Li, and Wang [18]. Hundred microliters of various concentration of the each solvent extract 25, 50, 75,100 µg were mixed with DPPH solution (4 mg of DPPH 100 ml of 70% methanol) in methanol and incubated in dark at 37ºC for 30 min. The absorbance was recorded at 517 nm. Inhibition of free radical by DPPH in percentage (I %) was calculated with the following equation:

$I\% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100;$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of the extract from fruiting bodies of Xylaria spp.

Ascorbic acid (vitamin C) was used as positive controls, and all tests were carried out in triplicate.

Results

The endophytic fungi isolated from the leaves of the endemic medicinal plant *Pterocarpus santalinus* was identified as *Xylaria spp.* (Fig. 2) based on its morphological and molecular characteristics. The nucleotide sequence obtained by ITS sequencing was submitted to NCBI with accession number KF493856.1. Phylogenetic tree was constructed using Mega 4.0 and boot strap analyses was done using Nucleotide-Joining (NJ) method. Boot strap values were indicated at the nodes of the tree.

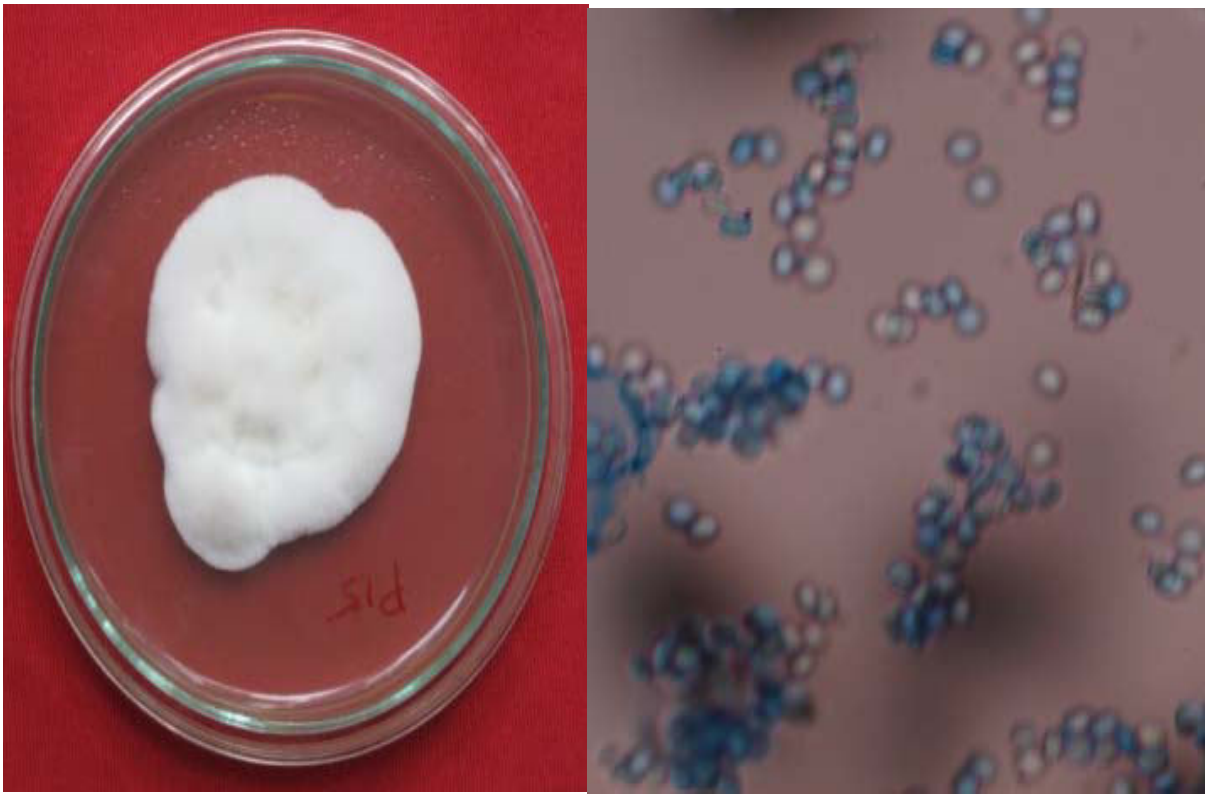

Figure 2. Colony morphology and Spore image of *Xylaria* spp.

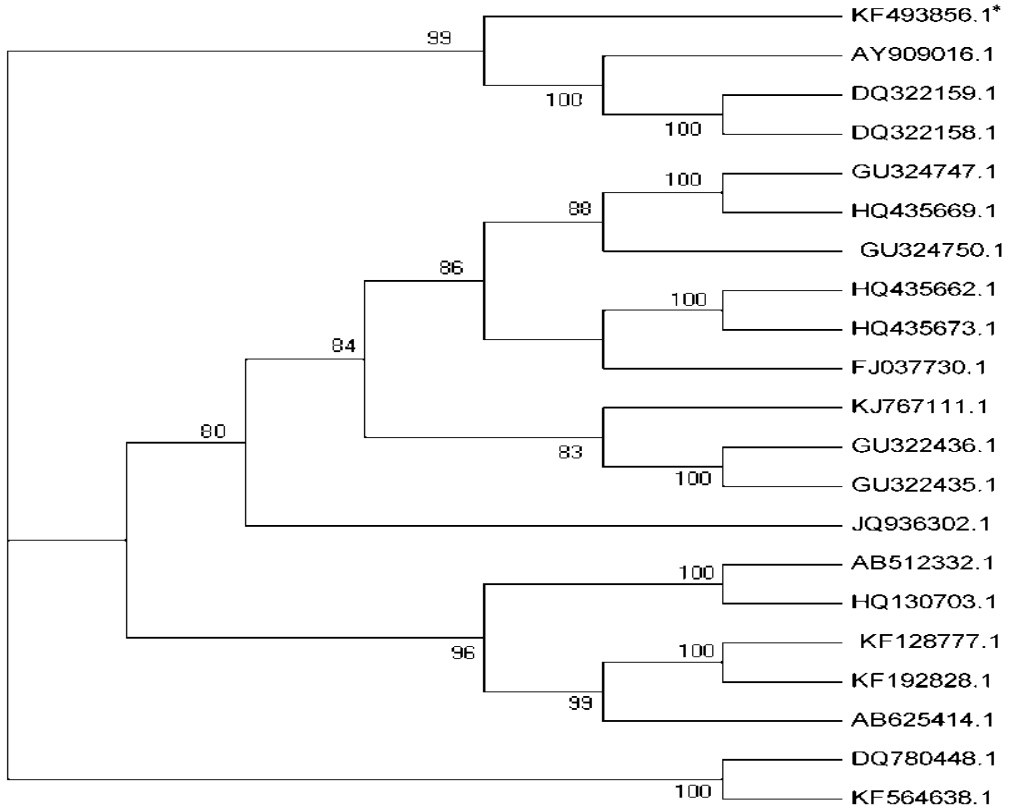

Figure: phylogenitic relationships between Xylaria sp. KF493856.1 and related taxa based on ITS regions. The values at nodes are boot strap values for 1000 replicates.

Antimicrobial Activity of Different solvent Extracts.

The fungal biomass of the *Xylaria spp.* extracted using three different solvents such as methanol, ethyl acetate and hexane have exhibited quite good antimicrobial activity against the selected human pathogens indicating the efficiency of compounds having biological activities. (Table-1) The highest antimicrobial activity was found in the methanol extract and the lowest in hexane. The maximum zone of inhibition was observed in *S. aureus, followed E. coli, B. subtilis , C. albicans , P. aeruginosa , A. flavus, and the least activity was found in A. niger.*

Different concentrations of crude methanol extract ranging from 10 – 100 µg were assessed against the test microorganisms. The crude extract of *Xylaria* was most effective against the gram positive bacteria *S. aureus* with a minimum bactericidal concentration (MBC) of 30 µg/mL. Whereas MBC for other test microorganisms was found to be 80 µg/mL. For *C. albicans*, minimum fungicidal concentration (MFC) was about 50 µg/mL and MFC for other test 100 µg/mL.

Table-1. Antimicrobial activity of *Xylaria* spp. extracted using different solvents

Test Micro-organism	Methanol extract	Ethyl Acetate extract	Hexane extract	Streptomycin	Fluconazole
	Zone of inhibition in mm				
P. aeriginosa	12.4 ±0.18	11.1 ±0.15	10.2 ±0.19	15.5 ±0.14	-
B. subtilis	12.7±0.14	12.5±0.18	11.8±0.14	14.6±0.14	-
S. aureus	13.5±0.15	13.0 ±0.21	12.2 ±0.13	14.0 ±0.12	-
E. coli	13.2 ±0.12	12.3 ±0.17	11.7 ±0.15	14.0 ±0.12	-
C. albicans	12.6± 0.20	12.6 ±0.24	9.1 ±0.21	-	15.0 ±0.14
A. niger	11.4 ±0.13	10.8 ±0.18	10.5 ±0.15	-	14.5 ±0.16
A. flavus	11.7 ±0.15	9.7 ±0.14	10.9 ±0.14	-	14.2 ±0.12

Free radical-scavenging capacity of the extracts using DPPH analysis

DPPH method is based on the reduction of DPPH in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H [19]. The DPPH radical-scavenging activities of the methanol extract from cultivated fruiting bodies of *Xylaria* spp. and of references (Ascorbic acid) are presented in Table 2. It was observed that the crude extracts from *Xylaria* spp. notably reduced the concentration of DPPH free radical. It is clearly demonstrated that free radical scavenging increases with increasing extract concentration. Inhibition values in the concentrations of 25, 50, 75 and 100, µg/ml were 22.10%, 30.60%, 45.42%, and 56.26%, respectively. The highest antioxidant activity was noticed in the methanolic extract followed by ethyl acetate and hexane. Ascorbic acid was observed to have the highest activity in DPPH assay, which is in agreement with previous study [18].

Table-2: Free radical-scavenging capacity of different solvent extracts from *Xylaria* spp. with different concentrations, in DPPH assay

extract	25 µg	50 µg	75 µg	100µg
Hexane	16.68±0.08	26.42±0.16	36.74±0.12	48.15±0.18
Ethyl acetate	15.80±0.18	28.20±0.08	39.46±0.16	54.20±0.15
Methanol	22.10±0.04	30.60±0.12	45.42±0.14	56.26±0.08
Control (Ascorbic acid)	25.42	45.18	60.84	78.62

Discussion

Endophytic fungi have been recognized as a repository of novel secondary metabolites, possessing beneficial biological activities [20]. This is the first report on the antimicrobial and antioxidant activity of endophytic fungus *Xylaria* spp. isolated from leaves of the endemic medicinal plant *Pterocarpus santalinus* collected from Tirumala hills of Eastern ghats.

From the results it was evident that this fungus has the potential for extraction of novel metabolites possessing antimicrobial activity against a broad range of pathogens. Generally utilization of fermentation broths has been reported for extraction of antimicrobial compounds. Here we are reporting the extraction of bioactive compounds from the fungal biomass using three different organic solvents and comparing the efficacy of individual extract for their antimicrobial and antioxidant potentials. The MIC for the selected pathogenic bacterial strains was 30 to 80 µg/mL, where as 100 µg/mL for fungal pathogen and 50 µg/mL for yeast was obtained. Similar minimum inhibition concentrations for *S. aureus* ATCC 25923 was reported by Powthong et al [21] with the crude extract of fungal endophyte of *Sesbania grandiflora* (L.).

Rios and Recio [22], in the search for substances of natural origin with antimicrobial activity, those that present concentrations higher than 1 mg/mL for extract and 0.1 mg/mL for isolated compounds should be avoided. However, the evaluation of activity is very interesting in case of concentration below 100µg/mL for extracts and 1 µg/mL for isolated compound. Our observation reveals that the endophytic fungi of *Xylaria* spp. possess a potential for isolation of antimicrobial agents against human pathogens which are prone to develop resistance against the commercially available antibiotics [23]. Thus this work would provide a scope to study the various biological activities of the endophytic fungal isolates.

DPPH is a stable free radical, which has been widely used for studying the free radical scavenging activities of natural antioxidants. The DPPH radical-scavenging activities of the different organic solvent extracts of the *Xylaria* spp was assayed. The DPPH radical-scavenging activity was very high in methanolic extract when compared to ethylacetae and hexane extracts. It is concluded that *Xylaria* sp. was a potential source of natural antioxidants In addition, the characteristics of the phytochemicals and the antioxidant mechanisms of the extract should be further studied, to gain more understanding of their antioxidant activity.

Conflict Of Interest

All the authors declare that there is no conflict of interest.

Acknowledgement

We are very much thankful to DBT, New delhi for providing financial support.

References

[1]. Xiaoli Liu, Mingsheng Dong, Xiaohong Chen, Mei Jiang, Xin Lv, Guijun Yan. Antioxidant activity and phenolics of an endophytic Xylaria sp. from *Ginkgo biloba*. Food Chemistry 2007;105: 548–554.

[2]. Sogra Fathima Musavi and Raj Mohan Balakrishnan A Study on the Antimicrobial Potentials of an Endophytic Fungus Fusarium

oxysporum NFX 06, Journal of Medical and Bioengineering2014; 3 (3):161-166.

[3]. Newman DJ, Cragg MG. "Natural products as source of new drugs over the last 25 years," *J. Nat. Prod.*, 2007;70: 461-477,.

[4]. Demain AL, Sanchez S. "Microbial drug discovery: 80 years of progress," *J. Antibiot.*, 2009;62: 5-16.

[5]. Kirtikar & Basu. Indian Medicinal Plants. IInd Edn., Vol I, 1987; p.826-827.

[6]. Warrier PK, Nambiar VPK, Raman Kutty C. Indian Medicinal Plants, Orient Longman Ltd., 1995; 5: 384-387.

[7]. Petrini O, Fungal endophytes of tree leaves. In: Andrews JH, Hirano SS, eds. Microbial Ecology of Leaves. New York: Spring Verlag: 1991: 179-197.

[8]. Strobel G, Daisy B. Bioprospecting for Microbial Endophytes and Their Natural Products. Microbiol. Mol. Biol. Rev., 2003; 67: 491–502.

[9]. Aly AH, Debbab A, Kjer J, Proksch P. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers. 2010; 41(1):1–16.

[10]. Yu H, Zhang L, Li L. *et al.*, "Recent developments and future prospects of antimicrobial metabolites produced by endophytes," *Microbiological Research*, 2010;165(6): 437-449.

[11]. Song JH. "What's new on the antimicrobial horizon?" *International Journal of Antimicrobial Agents*, 2008;32(4): S207–S213.

[12]. Dreyfuss MM, Chapela IH. "Potential of fungi in the discovery of novel, low molecular weight pharmaceuticals," *The Discovery of Natural Products with Therapeutic Potential*, V. P. Gullo, Ed., Boston: Butterworth- Heinemann, 1993;7:49-80.

[13]. Arnold AE, Meijia LC, Kyllo D, Rojas E I, Maynard Z. *et al.*, "Fungal endophytes limit pathogen damage in a tropical tree," in *Proc. National Academy of Science*, 2003;100 (26): 15649-15654.

[14]. Zhang HH, Song YC, Tan RX. "Biology and chemistry of endophytes," *Nat. Prod. Rep.*, 2006;23: 753-771.

[15]. Narayan CP, Won KK, Sung KW, Myung SP, Seung HY. *Plant Pathol. J.*, 2007;23(4) :287-294.

[16]. Wang F W, Jiao RH, Cheng AB, Tan S H, Song YC. "Antimicrobial potentials of endophytic fungi residing in quercus variabilis and brefeldin a obtained from cladosporium sp.," *World J Microbiol Biotechnol*, 2006; 23: 79-83.

[17]. Gangadevi V, Muthumary J. "Taxol, an anticancer drug produced by an endophytic fungus bartalinia robillardoides Tassi, isolated from a medicinal plant, aegle marmelos correa ex roxb," *World Journal of Microbiology and Biotechnology*, 2008; 24: 717-724.

[18]. Duan XJ, Zhang WW, Li XM, Wang BG. Evaluation of antioxidant property of extract and fractions obtained from a red alga, Polysiphonia urceolata. Food Chemistry, 2006: 95, 37–43.

[19]. Shon MY, Kim TH, Sung NJ. Antioxidants and free radical scavenging activity of Phellinus baumii (Phellinus of Hymenochaetoceae) extracts. Food Chemistry, 2003;82: 593–597.

[20]. Bills GF, Polishook JD. "Microfungi from carpinus caroliniana can," *J. Bot.* vol. 1991;69: 1477-1482.

[21]. Powthong P, Jantrapanukorn B, Thongmee A, Suntornthiticharoen P. "Evaluation of endophytic fungi extracts for their antimicrobial activity from sesbania grandiflora (L.) pers," *Int J Pharm Biomed Res,* 2012; 3(2)P: 132-136.

[22]. Rios JL, Recio MC. "Medicinal plants and antimicrobial activity," *J. Ethnopharmacol*, 2005; 100:80-84.

[23]. Maria GL, Sridhar KR, Raviraja NS. *J Agri Tech*, 2005; 1: 67-80.