RESEARCH PAPER | Botany | Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

Isolation characterization and antioxidant potential of endophytic fungi of *Ocimum sanctum* Linn. (Lamiaceae)

KEYWORDS *Ocimum sanctum. Endophytic fungi . Antioxidant activity . ITS sequencing . Correlation*

Robin Sharma
Research Scholar, Department of Biosciences, Sri Sathya Sai Institute of Higher Learning, Prasanthi Nilayam Campus, Puttaparthi, Anantapur Dist., A.P., India.

B.S. Vijaya Kumar
Associate Professor, Department of Biosciences, Sri Sathya Sai Institute of Higher Learning, Prasanthi Nilayam Campus, Puttaparthi, Anantapur Dist., A.P., India.

ABSTRACT *Endophytic fungi of medicinal plants have gained tremendous importance with emerging evidence of their immense ecological and biotechnological relevance. In the present study an attempt has been made to isolate, characterize and screen endophytes associated with leaves of Ocimum sanctum Linn. (Lamiaceae) for their antioxidant potential. A total of 147 endophytic fungal strains, with high colonization rate of 73.5% were isolated from the host plant. Using multiple assay system ethyl acetate extracts of representative endophytes grown in improved Czapex Dox broth were subjected to free radical scavenging activities against 2,2-Diphenyl-1-picrylhydrazyl (DPPH), reducing power assay (RP) and ferric reducing antioxidant power (FRAP). Total phenolic content (TPC) and total flavonoid content (TFC) were also evaluated. The total phenolic and flavonoid content ranged from 1.6±0.13 to 18.13±0.09 mgGAE/100mL culture and, 1.7±0.03 to 5.33±0.21 mgRE/100mL culture respectively. Endophytic fungi, Mycelia sterilia was found to be the most potent free radical scavenger with highest phenolics and flavonoid content, and was therefore subjected to molecular characterization based on rDNA sequencing of internal transcriber sequence (ITS) region. The results obtained indicate a significant positive correlation between antioxidant capacity and total phenolic and flavonoid content thereby suggesting that the antioxidant activity of these endophytes may be attributed to its high phenolic and flavonoid content. Therefore, fungal endophytes isolated from O. sanctum represent a potential antioxidant resource.*

Introduction

Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radicals and hydrogen peroxide, are chemically reactive molecules derived from oxygen. They are generated in living organisms as by-products through many metabolic pathways. ROS can readily react with and oxidize most biomolecules including carbohydrates, proteins, lipids and DNA (Zhao et al. 1, 2006).These reactive oxidants when accumulated in large quantities lead to a condition generally referred to as oxidative stress. Implications of oxidative stress on various human diseases viz., Alzheimer's, Parkinson's, rheumatoid arthritis, cardiovascular diseases, etc. (Nunomora et al. 2,2006; Wood-Kaczmar et al.3, 2006; Hitchon et al.4, 2004; Van Gaal et al.5, 2006) are well documented. Antioxidants are molecules that have the capacity to neutralize or scavenge these reactive species. Naturally occurring antioxidants are present in natural diet whereas enzymatic antioxidants are synthesized within biological systems. Currently, scientists are trying to expedite the search of these natural antioxidants and the organisms that produce them. Though poorly investigated, recent research findings have christened endophytic fungi associated with medicinal plants as a "promising source" of antioxidant and various other bioactive metabolites.

Endophytes are ecological group of fungi that colonize living, internal tissue of plants without any discernible features of their presence (Gehlot and Soytong 6, 2008; Hyde et al.7, 2008).They are ubiquitous, share symbiotic relationships with their hosts (Tejesvi et al.8, 2005) and are found in all plant species examined to date (Naik et al.9, 2008; Stone et al.10, 2000). In mutualistic or symbiotic associations, infected plants benefit by exhibiting increased resistance to herbivore grazing through the production of various alkaloids (Naik et al. 9, 2008; Owen and Hundley 11, 2004) improved growth and competitive ability by increasing the mineral uptake potential, plant phenotypic traits, temperature and drought tolerance, leaf chemistry, tolerance to heavy metals in soils, propensity for vegetative reproduction (Naik et al.9, 2008; Malinowski et al.12, 2002; Redman et al.,13,2002; Suryanarayanan et al.14, 2009) and defence against microbial pathogens (Tejesvi et al.8, 2005; Naik et al.9, 2008; Rubini et al.15, 2005; Arnold et al.16, 2003). In addition to their ecological value, endophytic fungi are also probably a major potential source for various natural products that possess a unique structure and bioactivity (Xing et al.17, 2010). Interestingly, endophytic fungi are reported to be metabolically more innovative than soil fungi (Suryanarayanan et al.14, 2009; Schulz et al.18, 2002) or fungi associated with algae (Suryanarayanan et al.14, 2009; Schulz et al.19, 2008).Their production of unique bioactive metabolites, such as, alkaloids, benzopyranones, benzoquinones, flavonoids, phenols, steroids, terpenoids, tetralones, xanthones , and others are well known (Suryanarayanan et al.14, 2009; Huang et al.20, 2007; Mitchell et al.21, 2008; Tan and Zou 22, 2001; Stadler and Keller 23, 2008). Endophytes have been extensively screened for antibacterial, cytotoxic, as antioxidants, antidiabetic and anti-immunosuppressive compounds (Shukla et al.24, 2012).

Ocimum sanctum Linn. (Tulsi) a medicinal herb, renowned as sacred basil for its religious and spiritual sanctity has been used for thousands of years in Ayurveda for its diverse healing properties (Luthra 25, 2010. The pharmacological property of the plant is credited to the synergistic interactions of many different active phytochemicals; eugenol, urosolic acid, carvacrol, linaleol, methyl carvicol, sitosterol etc., including saponins, flavonoids, triterpenoids, and tannins all of which have various biological activity. In addition, many phenolics have been identified, which also exhibit antioxidant and anti-inflammatory activities (Kelm et al.26, 2000; Shishodia et al.27, 2003; Jaggi et al.28, 2003). However, there is only one report on antioxidant properties of endophytic fungi from *O. sanctum* (Shukla et al., 24, 2012). Hence, based on the prior literature that endophytes of medicinal plants are the potential source of bioactive compounds and possess the same biological activity, it was deemed worthwhile to isolate endophytic fungi from *O. sanctum* and investigate the metabolites for their antioxidant potential. Therefore, we investigated four strains of endophytic fungi viz; *Aspergillus* sp., *Aspergillus terreus, Aspergillus versicolor* and Mycelia sterilia for their antioxidant capacity.

Materials and methods

Sample collection

Leaf samples of *O. sanctum* (Family: Lamiaceae) were collected from the *Prashanti* Nilayam campus garden of Sri Sathya Sai Institute of Higher Learning (14°9'N,77°48'E), Prasanthi Nilayam, Puttaparthi, Anantapur Dist., A.P., India during March 2012. Plant parts were washed with tap water and processed for isolation of endophytic fungi immediately after the collection.

Media preparation

Potato Dextrose Agar (PDA) was used for isolation and purification of endophytic fungi. Antibiotic, streptomycin (100 mg L-1) was added to the media to suppress bacterial growth. The media and antibiotics were purchased from Highmedia, India.

Isolation of endophytic fungi

Endophytic fungi were isolated from the healthy plants of *O. sanctum* as per the protocol described by (Gond et al.29, 2007). To eliminate epiphytic microorganisms, all the samples were surface sterilized by dipping in ethanol (70%) for 1-3 min, followed by a solution of sodium hypochlorite (4% available chlorine) for 3-5 min and then rinsed in ethanol (70%) for nearly 2-5 s, before a final rinse in sterilized double distilled water. Samples were then allowed to surface dry under sterile conditions (Naik et al. 9, 2008). Tissue segments were placed on a 9 cm Petri plate containing PDA medium.

The effectiveness of sterilization was confirmed by following the leaf imprint method (Sánchez Márquez et al.30, 2007; Schulz et al.31, 1998). The absence of growth of any on the medium confirmed that the surface sterilization process was effective. The petri dishes were then incubated at 27±2°C. The plates were checked on a routine basis and hyphal tips growing out from the tissues were subsequently transferred onto fresh PDA slants. All isolated and identified endophytic fungi are maintained in the Department of Biosciences, Sri Sathya Sai Institute of Higher Learning, *Prashantinilayam* campus, Puttaparthi India.

Identification of endophytic fungi

Slide preparation

Fungal mycelium was stained in cotton blue and observed under microscope. Four representative fungi were deposited to and identified by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India.

DNA isolation and amplification

The DNA extraction protocol was followed according to (Rashmi et al.32, 2013) with minor modifications. A pinch of 7 day-old mycelia from the surface of Czapex Dox Agar (CDA) plates was transferred to 50 ml Potato Dextrose Broth (PDB) and was subjected to constant agitation for 3 days at 25±1 °C. After incubation, mycelia were separated from medium by centrifugation at 12000 X g for 10 min. The mycelia pellet was ground in liquid nitrogen using a pestle and mortar. DNA was extracted using the DNeasy plant Minikit (Qiagen, Germany) according to manufacturer's instructions. Nuclease free water (Qiagen, Germany) was used to elute and the DNA concentration was estimated by NanoDrop-ND- 1000 spectrophotometer (Thermo Scientific, India). The DNA was stored at -80°C until further use.

The internal transcriber sequence region (ITS) is the most widely sequenced DNA region in fungi because of its higher degree of variation than other genic regions of rDNA (SSU and LSU) and for its advantage in molecular systematics at the species level, and even within species (e.g., to identify geographic races). In the current study, conserved ITS 1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 2 (5' GCTGCGTTCTTCATCGATGC 3') primer sequences (White et al.,33, 1990), custom synthesized from Eurofins, Bangalore, India were employed for PCR amplification of ITS. All PCRs were performed in Master Cycler-Pro thermal cycler (Eppendorf, Germany) in a 30 µl reaction mix containing 50 ng template DNA of the endophytic fungi, 1X PCR buffer (with 2 mM MgCl2), 200 µM each dNTP, 1µM ITS 1 and ITS 2 primers, and 1 unit Taq polymerase (Fermentas, New Delhi, India). The PCR cycling conditions included an initial denaturation at 94 °C for 4 min, followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min with a final extension of 72 °C for 8 min. The PCR products were electrophoresed on 1.2 % agarose gel stained with ethidium bromide and visualized under UV transilluminator (G-Box, Syngene, India).

To obtain DNA sequence from the amplicons, single bands were cut from a 1% agarose gel and purified using a Genelute gel extraction kit (Sigma, Bangalore, India). Purified PCR products were ligated into pTZ57RT TA cloning vectors (Fermentas, Bangalore, India) and transformed into calcium chloride competent E.coli DH5α cells following the protocol from Sambrook et. al.,34, (2001). Transformants were selected by Blue-white screening after overnight incubation of the LB plates inoculated with transformation mix. White colonies identified as containing a plasmid with an insert of the same size as the initial RAPD-PCR product were picked and grown overnight at 37°C with shaking in 2 to 4 ml of LB broth supplemented with Ampicillin. Plasmid DNA was purified from overnight cultures using a QIAprep spin miniprep kit (Qiagen) and was sequenced by Eurofins, Bangalore, India. Sequence trimming and contigs were formed from forward and reverse sequences and were analyzed using BLAST against the GenBank subject databases. BLAST homologs were considered significant at an expectation value (e value) of≤10-3. Sequences from the most significant similarities were retrieved from NCBI nucleotide database and multiple sequence alignment was performed by ClustalW tool. Cluster analysis of the pairwise similarity values was performed by using the UPGMA (unweighted pair group method using averages) algorithm in Mega5 software. The rDNA sequence of Mycelia sterilia was submitted to NCBI Gene Bank database for accession number.

Fermentation and extraction of metabolites

The endophytes were transferred to fresh PDA plates and allowed to grow at 25°C±1 °C for 7-14 days. Some colonized plugs of PDA (5 mm in diameter) were transferred into 250 mL Erlenmeyer flask containing 100 mL improved Czapex Dox broth (CDB). Flasks were put on a shaker in an incubator (orbital shaking incubator Orbitex Scigenics Biotech) at 120 rpm for 14-21 days at 25°C±1. Metabolites were extracted thrice with ethyl acetate (with equal volume) at room temperature and concentrated in a rotary vacuum evaporator (Heidolph, Germany) to obtain the residue dry (crude) extract prior to antioxidant assays.

Antioxidant activity

DPPH radical scavenging assay

Various concentrations of the ethyl acetate extracts of endophytic fungi (20-100 µg/ml, 2.5 ml) were mixed with methanolic solution containing DPPH radicals (0.1 mM, 0.5 ml). The mixture was shaken vigorously and left to stand in dark for 30 min. The reduction in the DPPH radical concentration was determined by measuring the absorbance at 517 nm (Hitachi spectrophotometer, U-2000). Methanol was taken as blank and DPPH solution without the extracts was taken as control (Dandamudi and Rao 35, 2011). The percentage of DPPH scavenged was calculated using the equation: % Scavenged = [(Ac-As)/Ac] x 100, where Ac is the absorbance of control, and As is the absorbance of solution containing sample extracts. TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-arboxylic acid) was used as standard.

Reducing power assay

The reducing power was measured by the method of (Oyaizu 36, 1986). Ethyl acetate extracts of endophytic fungi (20-100 µg/ml, 2.5 ml) were mixed with sodium phosphate buffer (0.2 M, pH 6.5, 2.5 ml) and potassium ferricyanide [$K_3Fe(CN)_6$]

(1%, 2.5 ml). The mixture was then incubated at 50°C for 20 min. Trichloroacetic acid (10% w/v, 2.5 ml) was then added and the mixture was centrifuged at 3000 rpm for 10 min. To the supernatant layer (2.5 ml), 2.5 ml of deionised water and ferric chloride (0.1%, 0.5ml) were added, and the absorbance was measured at 700 nm (Hitachi spectrophotometer, U-2000). Higher absorbance indicated better reducing power. TROLOX was used as standard.

Ferric reducing antioxidant power assay

In FRAP method, the complex formed when ferric tripyridyl triazine (Fe^{3+} TPTZ) complex was reduced to the ferrous (Fe^{2+}) ion is determined using UV-Vis Spectrophometer (Hitachi spectrophotometer, U-2000). The oxidant in the FRAP assay was prepared by mixing TPTZ (10 mM in 40 mM HCl, 2.5 ml), acetate buffer (0.3 M pH 3.6, 25 ml), and 2.5 ml of $FeCl_3.6H_2O$ (20 mM). To 3600 µl of freshly prepared FRAP reagent, 360 µl of water and 120 µl of endophytic fungal extracts (40-200 µg/ml) were added. The mixture was then incubated at 37°C for 30 min. The absorbance was measured spectrophotometrically at 595 nm. Higher absorbance indicates better ferric reducing ability of the extracts. BHT (Butylated hydroxytoluene) was used as standard (Benzie 37, 1996).

Determination of total phenolic content

The amount of total phenolic was determined using the Folin-Ciocalteu reagent (Djeridane et al.38, 2006). 1 ml sample was dissolved in 1.5 ml distilled water and 0.5 ml Folin-Ciocalteu's reagent. After 1 min, 1 ml 20% sodium carbonate solution was added. The final mixture was shaken and incubated at 25°C for 2 h in the dark. The absorbance of the mixture was measured at 760nm. A standard graph was first plotted using gallic acid (1-20 µg/ml) as a standard, giving an equation as:Absorbance = 0.041 gallic acid (µg/ml) + 0.044 (R^2 = 0.993). All tests were carried out in triplicate and the results were expressed as gallic acid equivalents (mg GAE/100ml broth).

Determination of total flavonoid content

The flavonoid content was determined based on the formation of flavonoid-aluminium (Djeridane et al. 38, 2006). 1 ml sample was mixed with 1 ml 2% aluminium chloride solution. After incubation for 15 min at room temperature, the absorbance of the reaction mixture was measured at 430 nm. Rutin was used as standard to make the calibration curve, giving an equation as: Absorbance = 0.033 rutin (µg/ml) + 0.005 (R^2 = 0. 996). The amount of flavonoids was expressed as rutin equivalents (mg RE/100ml broth). All tests were carried out in triplicate.

Statistical analysis

The relative frequency (%CF) of colonization of endophytic fungi was calculated using the formula given by (Hata and Futai 39, 1995). %CF = (N_{col}/N_t) X 100; where, N_{col} = the number of segments colonized by each fungus, and N_t = the total number of segments. The dominance of endophytes was calculated using the formula as per Kumaresan and Suryanarayanan 40, (2002). Dominance = %CF of a given endophyte/sum of %CF of all endophytes X 100.

Results and discussion

Isolation of endophytic fungi

A total of 147 isolates of endophytic fungi belonging to six genera were isolated from 200 leaf segments of *O. sanctum*. The present study revealed a high colonization rate of 73.50 % (Table 1). The composition included 93.20 % hyphomycetes and 6.80 % mycelia sterilia. No instance of members from ascomycotina, basidiomycotina or zygomycotina occured. Hyphomycetes of deuteromycotina are reported as common fungal endophytes among plants inhabiting temperate, tropical and rainforest vegetations (Bacon and White 41, 1994; Gond et al. 29, 2007). Cultures lacking reproductive structures or distinctive features were grouped under mycelia sterilia. This group of fungi is considerably prevalent in endophyte studies (Lacap et al. 42, 2003; Huang et al. 43, 2008). Most of the taxa were common endophytic fungi, some of which were consistent with many previous studies. The dominant genera observed were *Aspergillus* (60.54%) and *Fusarium* (25.85%). *Aspergillus* is cosmopolitan and usually epiphytic, but may also occur endophytically (Gond et al.29, 2007; Schulthess and Faeth 44, 1998). The result in terms of occurrence of dominant endophytic fungi in this study is not in agreement with previous reports on endophytic fungi of *O. sanctum*. In contrast, others have observed *Phoma* sp., *Sporormiella minima* and *Talaromyces* sp. as the dominant endophytic fungi in *O. sanctum* (Rajagopal et al.45, 2010). *Hymenula* sp., *Tricoderma, Aureobasidium* sp. and *Tubercularia* sp. (Banerjee et al.46, 2009) have also been recorded existing as endophytes in *O. sanctum*, but non of these organisms appeared as endophytes in this study. Recently, Bhagat et al.47, (2012) reported *Alternaria tenuissima, Cladosporium cladosporoides, Aspergillus fumigatus* and Alternaria sp. from *O. sanctum*. However, the present work reports *Curvularia trifolli, Drechslera halodes* and *Penicillium chrysogenum*, not reported earlier as endophytes from *O. sanctum*. The isolation of *Penicillium* sp. from *O. sanctum* in dry season was in agreement (Chareprasert et al. 48, 2006) because of the ability of spores of *Penicillium* sp. to survive and grow in lower water environment or in dry conditions categorizing it as a xerophilic type of fungus.

Table 1 Endophytic fungi isolated from leaves of O. sanctum.
The data presented are on the basis of 200 segments plotted.

Endophytic fungi	No. of endophytes	CF (%) [200 segments]	Dominance
Hyphomycetes			
Alternaria humicola	03	01.50	02.04
Aspergillus candidus	20	10.00	13.61
A. deflectus	01	00.50	00.68
A. itaconicus	51	25.50	34.70
A. japonicus	13	06.50	08.84
A. species	01	00.50	00.68
A. terreus	01	00.50	00.68
A. versicolor	02	01.00	01.36
Curvularia trifolli	04	02.00	02.72
Drechslera halodes	02	01.00	01.36
Fusarium avenaceum	01	00.50	00.68
F. chlamydosporum	37	18.50	25.17
Penicillium chrysogenum	01	00.50	00.68
Sterile fungi			
Sterile mycelium (with chlamydospores)	01	00.50	00.68
Sterile mycelium (dark brown)	05	02.50	03.40
Sterile mycelium (brown)	01	00.50	00.69
Sterile mycelium (branched mycelium)	01	00.50	00.69
Sterile mycelium (hyaline)	02	01.00	01.36
No. of Isolates	147	73.50	-

Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

The genus *Aspergillus* includes many important fungi that are sources of various natural products. Reports indicate that these fungi isolated as endophytes may be a potential source of novel secondary metabolites that could possibly have tremendous pharmaceutical and biotechnological implications. Recently, a new sesquiterpene, albican-1,14-diol (Liu et al.49, 2012) and new compounds Asperversin A and 9 –O-2(2,3,-dimethylbut-3-enyl) brevianamide Q (Miao et al. 50, 2012) were isolated from endophytic fungi *A. versicolor.* Also, Qiao et al.51, (2010) reported two new indoloditerpene derivatives asporyzin A and asporyzin B and one new indoloditerpene asporyzin C from *A. oryzae* that showed antimicrobial and insecticidal activity. Ravindran et al. 52, (2012) reported *A. flavus* a dominant endophyte from mangrove species as having antioxidant potential. Methanolic extract of an endophyte, A. species isolated from *Lobelia nicotianifolia* was reported by Murthy et al.53, (2011) to have significant antioxidant potential. Mycelia sterilia have been isolated as endophytes from a wide range of host plants (Naik et al. 9, 2008). They have been reported to produce novel potent immunosuppressants, mycestericins that suppressed the proliferation of lymphocytes in the mouse allogenic mixed lymphocyte reaction, with potency similar to that of myriocin (Sasaki et al.54, 1994). Brunner et al.55, (2004) reported metabolites such as; myriocin, mycestericins and the sphingofungins from Mycelia sterilia that structurally resemble the sphingosines, that are important components of cell membranes and were reported to have remarkable in vitro immunosuppressive activity. There are reports on Mycelia sterilia as source of antioxidant compounds. Moon et al.56, (2006) described three new phenolic glycoside antioxidants; Glyscavins A, B and C from Mycelia sterilia F020054.

Identification of endophytic fungi

The four representative endophytes selected for antioxidant capacity screening in this study were identified by NFCCI, Agharkar Research Institute, Pune, India, as *Aspergillus* sp., *A. terreus*, *A. versicolor* and Mycelia sterilia dematiaceous form with NFCCI accession number; 3001, 3002 and 2998 respectively. The endophytic fungi, Mycelia sterilia was also identified by rDNA sequencing of ITS region with NCBI Gene Bank accession number KC560013. This fungus had a maximum percentage similarity of 59% with Sporormiella dakotensis. Phylogenetic relationship of this fungus with the related fungi is shown in Fig 1.

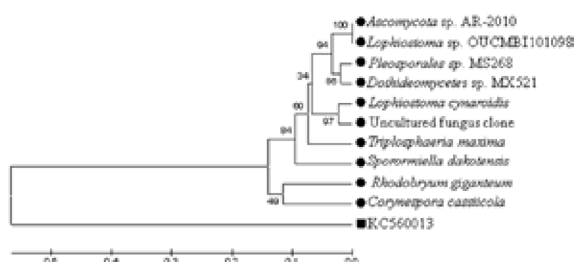

Fig. 1 Cluster analysis of the pairwise similarity values performed by using the UPGMA (unweighted pair group method using averages)

Total phenolics and flavonoid contents of endophytic fungi and their correlation with antioxidant activity

Reports of the past have suggested that phenolic and flavonoid compounds play an important role in stabilizing lipid oxidation and are thus credited for antioxidant activity as emphasized by several reports (Liu et al. 57, 2007; Chandra and Arora 58, 2012). Therefore, in this work we determined the total phenolics and flavonoid contents of the ethyl acetate extract of four endophytic fungi. The results of total phenolic and flavonoid content are shown in Table 2.

Table 2 Total phenolic and flavonoid contents of ethyl acetate extracts of various endophytic fungi from Ocimum sanctum. Each value is expressed as mean ± standard deviation (n=3)

	Aspergillus versicolor	*Aspergillus species*	*Aspergillus terreus*	Mycelia sterilia
Total phenolic (mg GAE/100 ml culture)	1.6±0.13	3.25±0.16	14.96±0.07	18.13±0.09
Total flavonoid (mg RE/100 ml culture)	1.7±0.03	1.85±0.04	1.91±0.24	5.33±0.21

The highest levels of total phenolic and flavonoid contents were found in ethyl acetate extract of Mycelia sterilia (18.13 ± 0.09 mg GAE/100 ml culture broth for phenolics and 5.33 ± 0.21 mg RE/100 ml culture broth for flavonoids) followed by A. terreus (14.96 ± 0.07 mg GAE/100 ml culture broth for phenolics and 1.91 ± 0.24 mg RE/100 ml culture broth for flavonoids), while total phenolic and flavonoid contents of A. versicolor were lowest, 1.6 ± 0.13 mg GAE/100 ml culture broth for phenolics and 1.70 ± 0.03 mg RE/100 ml culture broth for flavonoids respectively. The results revealed that the ethyl acetate extract of endophytic fungi Mycelia sterilia contains significant phenolics and flavonoids than those reported in the previous studies. Huang et al.20, (2007) have reported a total phenolics content in the range of 0.52 ± 0.02 mgGAE/100 ml – 13.95 ± 0.11 mgGAE/100ml in endophytic fungi of *Nerium oleander*.

With further correlation analysis for Mycelia sterilia, it was found that there was positive correlation between the phenolic contents of the extracts for various antioxidant activities (DPPH; R^2 = 0.977; RP; R^2 = 0.992; FRAP; R^2 = 0.990) and flavonoid contents (DPPH; R^2 = 0.988; RP; R^2 = 0.925; FRAP;R^2 = 0.975) respectively. Reports suggest a wide variation between different phenolics compounds in their effectiveness as antioxidant (Liu et al.57, 2007), which could possibly be argued on the basis that the antioxidant activity may not only depend on the concentration, but also on the chemistry of the molecules and the nature of interaction. In this study we assume that the antioxidant activity may have resulted from synergistic action of phenolics and flavonoid-type compounds.

Antioxidant activity

Free radical-scavenging capacity of the ethyl acetate extract using DPPH analysis

The ethyl acetate extracts of four endophytic isolates showed increasing scavenging effect with increased concentration. The activity was encouraging (53.12%) even at 100 µg/ml (Fig. 2) in comparison to the activity of the extract of an endophytic fungi Mycelia sterilia isolated from *Withania somnifera* which showed a mild activity of 20.35 % at similar concentration (Madki et al.59, 2010).

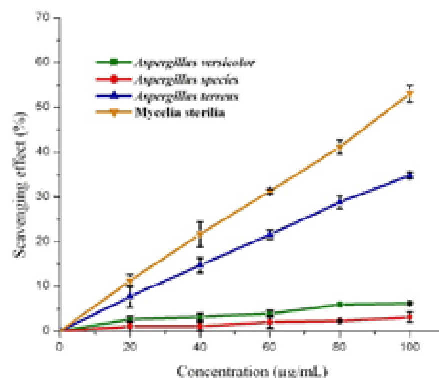

Fig.2 Effects of fungal extracts on scavenging DPPH radicals. Each value is expressed as mean ± standard deviation (n=3)

Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

However, the scavenging effect of TROLOX at 10µg/ml was 90.61 %. Of the four species studied, Mycelia sterilia exhibited maximum scavenging activity (53.12%) followed by *Aspergillus terreus* (34.83%) against DPPH radical by neutralizing the free radical, either by transfer of electron or hydrogen atom thus, revealing the hydrogen donating property of the fungal extracts (Shon et al.60, 2003).

Antioxidant capacity using Reducing power assay

Reducing power of the ethyl acetate extract of four endophytic isolates was moderate (Fig. 3). At100 µg/ml the reducing power was in range of 0.163-0.349. However, the reducing power of TROLOX at 60µg/ml was 0.468. Results show that *Aspergillus terreus* exhibited maximum reducing power (0.349), followed by Mycelia sterilia (0.263). *Aspergillus species* (0.163) exhibited least reducing power.

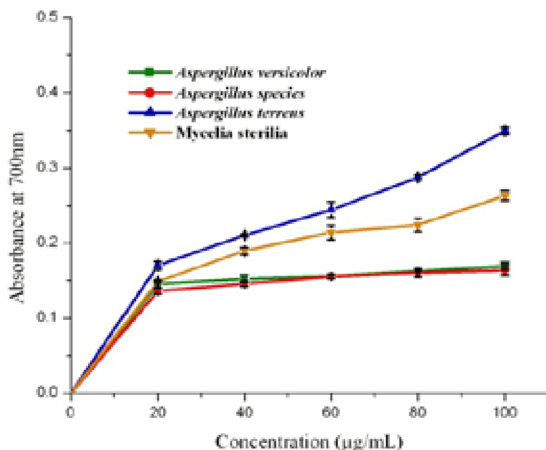

Fig. 3 Reducing power of fungal extracts. Each value is expressed as mean ± standard deviation (n=3)

Though, Zeng et al.61, (2011) reported endophytic fungi from liverwort *Scapania verrucosa* to have moderately and similar activity to the endophytes in this study, their study also demonstrated *Chaetomium globosum* exhibiting excellent reducing potential (0.7). The maximum reducing potential of *Aspergillus terreus* may be attributed to the potential of the extracts to act as reductones that inhibit lipid peroxidation by donating a hydrogen atom thereby terminating the free radical chain reaction. Moreover, this reducing potential may be due to the di or monohydroxy substitution in the aromatic rings that possess potent hydrogen-donating ability (Zhao et al.1, 2006).

Antioxidant capacity using Ferric reducing antioxidant power assay

All ethyl acetate extract showed increased FRAP with increasing concentration (Fig. 4). At 200µg/ml concentration, the FRAP values measured were in the range of 0.51 - 0.78. However, the FRAP of BHT at 80µg/ml concentration was 0.885. Results show that Mycelia sterilia exhibited maximum FRAP (0.780) compared to other fungal extracts.

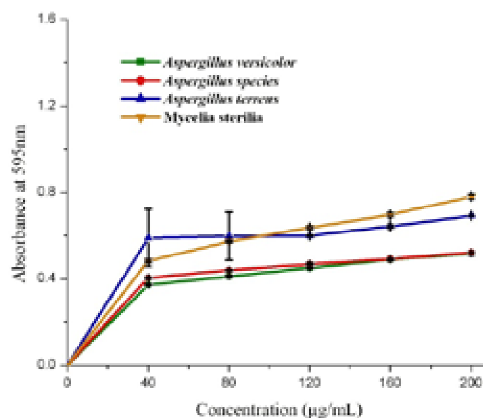

Fig. 4 Ferric reducing antioxidant power (FRAP) of fungal extracts. Each value is expressed as mean ± standard deviation (n=3)

Bhagobaty and Joshi 62, (2012) reported *Mortierella. hyalina* and *Penicillium* sp. isolated from *Osbeckia stellata* and *Schima Khasiana* respectively to produce a high antioxidant activity with a FRAP value of 1.316 and 1.417. The maximum FRAP activity of Mycelia sterilia may be attributed to its reducing potential and also its potential to break the free radical chain by donating a hydrogen atom (Othman A et al. 63, 2007).

Conclusions

In vitro models in this study clearly established the antioxidant potency of endophytic fungi, Mycelia sterilia and *Aspergillus terreus* from *O. sanctum*. This study also illustrates that internal leaf tissue of *O. sanctum* is an interesting biological niche that harbours and act as reservoir of endophytes which may produce important molecules with pharmaceutical relevance. Thus, the study highlights the importance of isolating and screening endophytes of medicinal plants from varied locations and biotopes and further also suggests investigations in relation to purification, characterization and identification of these molecules which would possibly facilitate better understanding on their production and mode of action.

Acknowledgements

Authors dedicate this study with gratitude to the founder Chancellor, Sri Sathya Sai Institute of Higher Learning, Bhagawan Sri Sathya Sai Baba for His constant encouragement and guidance. We thank the University for providing facilities and financial support. Acknowledgements are due to Siva Ramakrishna U., Microbiology division, Defence Food Research Laboratory, Mysore, India for helping in carrying out molecular taxonomy. Authors also acknowledge Prof. Mukesh Doble, Indian Institute of Technology Madras, India, for his timely guidance.

Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

REFERENCE 1. Zhao, G.R., Xiang, Z.J., Ye, T.X., Yuan, Y.J., & Guo, Z.X. (2006). Antioxidant activities of *Salvia miltiorrhiza* and *Panax notoginsen*. Food Chemistry, 99(4),767–774. | 2. Nunomura, A., Castellani, R., Zhu, X., Moreira, P., Perry, G., & Smith, M. (2006). Involvement of oxidative stress in Alzheimer disease. Journal of Neuropathology and Experimental Neurology, 65(7), 631–41. | 3. Wood-Kaczmar, A., Gandhi, S., & Wood, N. (2006). Understanding the molecular causes of Parkinson's disease. Trends in Molecular Medicine, 12(11), 521–8. | 4. Hitchon, C., & El-Gabalawy, H. (2004). Oxidation in rheumatoid arthritis. Arthritis Research & Therapy,6 (6), 265–278. | 5. Van Gaal, L., Mertens, I., & De Block, C. (2006). Mechanisms linking obesity with cardiovascular disease. Nature, 444(7121), 875–80. | 6. Gehlot, P., Bohra, N., & Purohit, D. (2008). Endophytic mycoflora of inner bark of *Prosopis cineraria*—a key stone tree species of Indian desert. American Eurasian Journal of Botany, 1(1), 1-4. | 7. Hyde, K., & Soytong, K. (2008). The fungal endophyte dilemma. Fungal Diversity, 33, 163-173. | 8. Tejesvi, M.V., Mahesh, B., Nalini, M.S., Prakash, H.S., Kini, K.R., Subbiah, V., & Shetty, H.S. (2005). Endophytic fungal assemblages from inner bark and twig of *Terminalia arjuna* W. & A.(Combretaceae). World Journal of Microbiology and Biotechnology, 21(8-9),1535-1540. | 9. Naik S.B., Shashikala, J., & Krishnamurthy, Y. (2008). Diversity of fungal endophytes in shrubby medicinal plants of Malnad region, Western Ghats, Southern India. Fungal Ecology,1 (2-3), 89-93. | 10. Stone, J.K., Bacon, C.W., & White, J.F. (2000). An overview of endophytic microbes: endophytism defined. Microbial Endophytes. Marcel Dekker, New York. | 11. Owen, N.L., & Hundley, N., (2004). Endophytes the chemical synthesizers inside plants. Science Progress, 87(2), 79-99. | 12. Malinowski, D.P., Alloush, G.A., & Belesky, D.P. (2000). Leaf endophyte *Neotyphodium coenophialum* modifies mineral uptake in tall fescue. Plant and Soil.227(1-2),115-126. | 13. Redman, R.S., Sheehan, K.B., Stout, R.G., Rodrigues, R.J., Henson, J.M. (2002). Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis.Science,298, 1581-1581. | 14. Suryanarayanan, T.S., Thirunavukkarasu, N., Govindarajulu, M., Sasse, F., Jansen, R., & Murali, T. (2009). Fungal endophytes and bioprospecting. Fungal Biology Reviews, 23(1-2), 9-19. | 15. Rubini, M.R., Silva-Ribeiro, R.T., Pomella, A.W., Maki, C.S., Araújo, W.L., Dos Santos, D.R., & Azevedo, J.L. (2005). Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis perniciosa*, causal agent of Witches' Broom Disease. International Journal of Biological Sciences,1(1), 24-33. | 16.Arnold, A.E., Mejía, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N., & Herre E.A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences of United States of America, 100(26), 15649-15654. | 17. Xing, X., Guo, S., & Fu, J. (2010). Biodiversity and distribution of endophytic fungi associated with *Panax quinquefolium* L. cultivated in a forest reserve. Symbiosis, 51(2),161-166. | 18. Schulz, B., Boyle, C., Draeger, S., Römmert, A.K., & Krohn, K. (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological Research,106(9), 996-1004. | 19. Schulz, B., Draeger, S., Rheinheimer, J., Siems, K., Loesgen, S., Bitzer, J., Schloerke, O., Zeeck, A., Kock, I., & Hussain, H. (2008). Screening strategies for obtaining novel, biologically active, fungal secondary metabolites from marine habitats. Botanica Marina, 51(3), 219-234. | 20. Huang, W.Y., Cai, Y.Z., Hyde, K.D., Corke, H., & Sun, M. (2007). Endophytic fungi from *Nerium oleander* L (Apocynaceae): main constituents and antioxidant activity. World Journal of Microbiology and Biotechnology,23(9), 1253-1263. | 21. Mitchell, A., Strobel, G., Hess, W., Vargas, P., & Ezra, D. (2008). *Muscodor crispans*, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon. Fungal Diversity, 31,37-43. | 22. Tan, R., & Zou, W. (2001). Endophytes: a rich source of functional metabolites. Natural Product Reports, 18, 448-459. | 23. Stadler, M., & Keller, N.P. (2008). Paradigm shifts in fungal secondary metabolite research. Mycological Research,112(2), 127-130. | 24. Shukla, S.T., Kulkarni, V.H., Habbu, P.V., Jagadeesh, K.S., Patil, B.S., & Smita, D.M. (2012). Hepatoprotectiveand antioxidant activities of crude fractions of endophytic fungi of *Ocimum sanctum* Linn. in rats. Oriental Pharmacy and Experimental Medicine, 12(2),81–91. | 25. Luthra, D. (2010). *Ocimum Sanctum* (Tulsi): A Potent Medicinal Herb.Webmed Central Pharmacology,1(11),1-12. | 26. Kelm, M., Nair, M., Strasburg, G., & DeWitt, D. (2000). Antioxidant and cyclooxygenase inhibitory phenolic compounds from *Ocimum sanctum* Linn. Phytomedicine,7(1), 7-13. | 27. Shishodia, S., Majumdar, S., Banerjee, S., & Aggarwal, B.B. (2003). Urosolic acidinhibits nuclear factor-kappaB activation induced by carcinogenic agents through suppression of IkappaBalpha kinase and p65 phosphorylation: Correlation with down-regulation of cyclooxygenase2,matrix metalloproteinase 9, and cyclin D1. Cancer Research,63, 4375-4383. | 28. Jaggi, R.K., Madaan, R., & Singh, B. (2003). Anticonvulsant potential of holy basil, *Ocimum sanctum* Linn., and its cultures. Indian Journal of Experimental Biology,41(11),1329-1333. | 29. Gond, S., Verma, V., Kumar, A., Kumar, V., & Kharwar, R. (2007). Study of endophytic fungal community from different parts of *Aegle marmelos* Correae (Rutaceae) from Varanasi (India). World Journal of Microbiology and Biotechnology,23, 1371-1375. | 30. Sánchez Márquez, S., Bills, G.F., & Zabalgogeazcoa, I. (2007). The endophytic mycobiota of the grass *Dactylis glomerata*. Fungal Diversity, 27,171-195. | 31. Schulz, B., Guske, S., Dammann, U., & Boyle, C. (1998). Endophyte-host interactions. II. Defining symbiosis of the endophyte-host interaction. Symbiosis,25, 213-227. | 32. Rashmi, R., Ramana, M.V., Shylaja, R., Uppalapati, S., Murali, H., & Batra, H. (2013). Evaluation of a multiplex PCR assay for concurrent detection of four major mycotoxigenic fungi from foods. Journal of Applied Microbiology,114(3), 819-827. | 33. White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. Academic Press, Inc., New York. | 34. Sambrook J, Russel David W. (2001). Molecular cloning: a laboratory manual (3rd ed.).Cold Springer Harbor Laboratory Press, New York. | 35. Dandamudi, R.B., & Rao Nageswara, G. (2011). Antioxidant properties and electrochemical behaviour of cultivated commercial Indian edible mushrooms. Journal of Food Science and Technology,50(2),301-308. | 36. Oyaizu, M. (1986). Studies on products of the browning reaction. Antioxidative activities of browning reaction products prepared from glucosamine. Japanese Journal of Nutrition, 44, 307-315. | 37. Benzie IFF, S.J. (1996).The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power". The FRAP assay. Analytical Biochemistry, 239(1),70–76. | 38. Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P., & Vidal, N. (2006). Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chemistry, 97(4),654-660. | 39. Hata, K., & Futai, K. (1995). Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge, *Thecodiplosis japonensis*. Canadian Journal of Botany,73 (3), 384-390. | 40. Kumaresan, V., & Suryanarayanan, T. (2002). Endophyte assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. Fungal Diversity, 9,81-91. | 41. Bacon, C.W., & White Jr, J.F. (1994).Biotechnology of endophytic fungi of grasses. CRC Press Inc. | 42. Lacap, D., Hyde, K., & Liew, E. (2003). An evaluation of the fungal 'morphotype'concept based on ribosomal DNA sequences. Fungal Diversity, 12, 53-66. | 43. Huang, W., Cai, Y., Hyde, K., Corke, H., & Sun, M. (2008). Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Diversity,33,61-75. | 44. Schulthess, F.M., & Faeth, S.H. (1998). Distribution, abundances, and associations of the endophytic fungal community of Arizona fescue (*Festuca arizonica*). Mycologia, 90(4),569-578. | 45. Rajagopal, K., Kalavathy, S., Kokila, S., Karthikeyan, S., Kathiravan, G., Prasad, R., & Balasubraminan, P. (2010). Diversity of fungal endophytes in few medicinal herbs of south India. Asian Journal of Experimental Biological Science,1(2), 415- 418. | 46. Banerjee, D., Manna, S., Mahapatra, S., & Pati, B.R. (2009). Fungal endophytes in three medicinal plants of Lamiaceae. Acta Microbiologica et Immunologica Hungarica, 56(3), 243–250. | 47. Bhagat, J., Kaur, A., & Sharma, M. (2012). Molecular and functional characterization of endophytic fungi from traditional medicinal plants. World Journal of Microbiology and Biotechnology,28(3), 963–971. | 48. Chareprasert, S., Piapukiew, J., Thienhirun, S., Whalley, A.J., & Sihanonth, P. (2006). Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr. World Journal of Microbiology and Biotechnology, 22(5) 481-486. | 49. Liu, X.H., Miao, F.P., Li, X.D., Yin, X.L., & Ji, N.Y. (2012). A new sesquiterpene from an endophytic *Aspergillus versicolor* strain. Natural Products Communications,7 (7), 819-820. | 50. Miao, F.P., Li, X.D., Liu, X.H., Cichewicz, R.H., & Ji, N.Y. (2012). Secondary metabolites from an algicolous *Aspergillus versicolor* strain. Marine Drugs,10 (1), 131-139. | 51. Qiao, M.F., Ji, N.Y., Liu, X.H., Li, K., Zhu, Q.M., & Xue, Q.Z. (2010). Indoloditerpenes from an algicolous isolate of *Aspergillus oryzae*. Bioinorganic and Medicinal Chemistry Letters,20 (19), 5677-5680. | 52. Ravindran, C., Naveenan, T., Govindaswamy, Varatharajan, R., Rajasabapathy, & R., Meena, R.M. (2012). Antioxidants in mangrove plants and endophytic fungal associations. Botanica Marina,55(3), 269-279. | 53. Murthy, K.N., Pushpalatha, K.C., & Joshi, C.G. (2011). Antioxidant activity and phytochemical analysis of endophytic fungi isolated from *Lobelia nicotianifolia*. Journal of Chemical and Pharmaceutical Research, 3(5), 218-225. | 54. Sasaki, S., Hashimoto, R., Kiuchi, M., Inoue, K., Ikumoto, T., Hirose, R., Chiba, K., Hoshino, Y., Okumoto, & Fujita, T. (1994). Fungal metabolites. Part 14. Novel potent immunosuppressants, mycestericins, produced by Mycelia sterilia. Journal of Antibiotics, 47,420-33. | 55. Brunner, M., & Koskinen, A.M.P. (2004). Biology and chemistry of sphingosine-related metabolites. Current Organic Chemistry, 8(17),1629-1645. | 56. Moon, B.S., Ryoo, I.J., Yun, B.S., Bae, K.S., Lee, K.D., Yoo, I.D., & Kim, J.P. (2006). Glyscavins A, B and C, new phenolic glycoside antioxidants produced by a fungus Mycelia sterilia F020054. Journal of Antibiotics., 59(11), 735–739. | 57. Liu, X., Dong, M., Chen, X., Jiang, M., Lv, X., & Yan, G. (2007). Antioxidant activity and phenolics of an endophytic *Xylaria* sp. from *Ginkgo biloba*. Food Chemistry,105 (2), 554–584. | 58. Chandra, P., & Arora, D.S. (2011). Optimization of antioxidant potential of *Penicillium granulatum* Bainier by statistical approaches. ISRN Microbiology, 2012,10. | 59. Madki, M.A., Manzoor, A.S., Powar, P.V., & Patil, K.S. (2010). Isolation and biological activity of endophytic fungi from *Withania somnifera*. International Journal of Pharmaceutical Sciences,2(3), 848-858. | 60. Shon, M.Y., Kim, T. H., & Sung, N. J. (2003). Antioxidants and free radical scavenging activity of *Phellinus baumii* (Phellinus of Hymenochaetoceae) extracts. Food Chemistry, 82, 593-597. | 61. Zeng, P.Y., Wu, J.G., Liao, L.M., Chen, T.Q., Wu, J.Z., & Wong, K.H. In vitro antioxidant activities of endophytic fungi isolated from the liverwort *Scapania verrucosa*. Genetics and Molecular Research,10(4): 3169-3179. | 62. Bhagobaty, R.K., & Joshi, S.R. (2012). Antimicrobial and antioxidant activity of endophytic fungi isolated from ethnomedicinal plants of the "Sacred forests" of Meghalaya, India. Mikologia Lekarska,19(1), 5-11. | 63. Othman, A., Ismail, A., Abdul Ghani, N., & Adenan, I. (2007). Antioxidant capacity and phenolic content of cocoa beans. Food Chemistry, 100(2007),1523–1530. |