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RESEARCH ARTICLE

FUNGAL ENDOPHYTES FROM THE CULINARY HERBS AND THEIR ANTIOXIDANT ACTIVITY

[1]Amitha, V., [2]Shylaja, M. D. and [3*]Nalini, M. S.

[1]Department of Microbiology, Maharani's Science College, JLB Road, Mysore- 570 001, Karnataka, India
[2]Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore-570 012, Karnataka, India
[3]Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore–570 006, Karnataka, India

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ABSTRACT

Endophytic microbes are often associated with the internal parts of plant tissues. Endophytic fungi were isolated from the surface sterilized root fragments of herbaceous culinary leafy vegetable and spices viz., *Coriandrum sativum, Anetham graveolens, Spinacea oleracea* and *Trigonella foenum-graecum*. 533 isolates were obtained from the plating of root fragments. Important fungi were identified as *Myrothecium roridum*, *Chaetomium fumicola*, *Heterosporium alli*, *Fusarium oxysporum* and *Aspergillus tereus*. The dominant endophyte documented was *M. roridum* (29.85%) from the roots of *T. foenum-graecum*, and was evaluated for the antioxidant activity. The fungus was inoculated onto potato dextrose broth (PDB) and cultivated for two weeks and mycelia was harvested and dried into fine powder. Both the endophyte and the host powder were extracted in water and methanol and the extracts tested for their total phenolic content and radical scavenging potentials. Results indicate that the water extracts of both host and endophyte showed good phenolic content (2.8 mg/g and 2.5 mg/g GAE) as well as radical scavenging ability (72% and 59%). Similarly, a decrease in the phenolic content and the scavenging potentials of endophyte with respect to its host were observed in the methanolic extracts. Results indicate the antioxidative potentials of fungi from herbaceous species.

INTRODUCTION

Herbaceous green leafy vegetables are considered to be a part of diet since ancient days and their inclusion in daily diet is essential for the maintenance of good health. Many epidemiological studies suggest that an increased consumption of medicinal plants rich in antioxidants protect against DNA damage and carcinogenesis, and do exhibit a wide range of pharmacological properties such as anti-inflammatory, anti-bacterial, and anti-fungal (Rasineni *et al*., 2008). The wellness derived from the leafy vegetables is often attributed to different antioxidant compounds in fruits and vegetables such as ascorbic acid, vitamin E, carotenoids, lycopenes, polyphenols and other phytochemicals (Prior and Cao, 2000; Gupta and Prakash, 2009). Green leafy vegetables are known to be rich source of these compounds and their consumption reduces the risk of several serious diseases. In recent years, the presence of microbes called the 'endophytes' residing within the tissues are well documented (Bacon and White, 2000). The role of endophytes in herbaceous plant species are well known, as they help plants to tolerate stressful environments, obtain nutrients (nitrogen fixation and phosphate), protect plants from pathogens and promote plant growth (Strobel and Daisy, 2003). Thus, one can attribute the medicinal value of plants to the endophytic microorganisms too, which are known to produce secondary metabolites of immense medicinal value.

Natural anti-oxidants have been characterized from the fungal compounds (Sun *et al*., 2004). Endophytic fungi such as *Pestalotiopsis* (Strobel *et al*., 2002; Harper *et al*., 2002; Phongpaichit *et al*., 2007), *Chaetomium* sp. (Huang *et al*., 2007), *Alternaria alternata* (Fernandes *et al*., 2009) *Phyllosticta* sp. (Srinivasan *et al*., 2010), *Phomopsis sp.* (Jayanthi *et al*., 2011) from various medicinal plants have been evaluated for their anti-oxidative potential. Herbs are regularly used in Indian traditional food preparations and garnishing due to their dietary importance as well as health protecting benefits. *Coriandrum sativum* L. (coriander) and *Anetham graveolans* (dill) are annual herbs of the family Apiaceae. *Spinacia oleracea* L. referred to as spinach is an edible herbaceous leafy vegetable of the family Amaranthaceae. *Trigonella foenum-graecum* L., commonly referred to as fenugreek is an annual herb of the family Fabaceae. Studies on the fungal endophytes from four commonly used edible culinary herbs have not been reported so far. The current investigation was carried out to

Corresponding author: Nalini, M. S.
Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore–570 006, Karnataka, India.

enumerate the endophytic fungi from the roots of *Coriandrum sativum*, *Anethum graveoleus*, *Trigonella foenum-graecum*, *Spinacia oleracea* and evaluate their antioxidant capacities.

MATERIALS AND METHODS

Collection of herbs

Mature and healthy plant materials of culinary herbs such as *Coriandrum sativum* L., (coriander), *Anethum graveoleus* L. (dill), *Trigonella foenum-graecum* L. (methi/fenugreek) and *Spinacia oleracea* L. (spinach) were collected from the local vendors of Devaraja Market, Mysore. The root parts were thoroughly cleaned with tap water to remove soil and debris. The roots of herbs were excised and placed in polyethylene zip lock covers and brought to the laboratory and processed immediately for the surface sterilization.

Isolation and identification of endophytes

The endophytic fungi were isolated by surface sterilization of root samples by the modified procedure of Petrini *et al.* (1992). All the root samples of plant species were first washed thoroughly under running tap water to remove dust and debris. The surface sterilization was carried out in a clean airflow bench system. The samples were immersed in 70% ethanol (v/v) for one min followed by 3.5% sodium hypochlorite (NaOCl) (v/v) for three min. The samples were rinsed three times in changes of sterile distilled water and dried on sterile blotters. The root samples were cut into 1.0 cm x 0.1cm using sterile scalpel. The root segments were placed equidistantly on the water agar medium (2% Agar/L) supplemented with the antibiotic streptomycin. The petriplates inoculated with the root segments were incubated at 28°±2 for 4 to 6 weeks. Pure cultures were then transferred to potato dextrose agar (PDA) slants and cultivated for 14 days at 28°C.

Identification of endophytic fungi

The endophytic fungi were observed morphologically and microscopically. Morphological identification was done according to the standard taxonomic key based on the cultural and conidial characteristics (Domsch *et al*., 1980; Barnett and Hunter, 1998; Leslie and Summerell, 2006) using stereo-binocular as well as light microscope. For this purpose, a small bit of fungal mycelia was isolated from pure culture grown on PDA and stained with lactophenol cotton blue for visual observation.

Data analysis

Colonization rate (CR) was calculated according to Huang *et al*. (2008) using the following formula:

$$CR\ (\%) = \frac{\text{Total no. of segments colonized by endophytic fungi} \times 100}{\text{Total no. of segments plated}}$$

Colonization frequency (CF) was calculated according to Tejesvi *et al*. (2006) using the following formula:

$$CF\ (\%) = \frac{\text{No. of segments colonized by a single fungus} \times 100}{\text{Total no. of segments plated}}$$

Percentage dominance was calculated according to Photita *et al*. (2001)

$$D\ (\%) = \frac{\%\ CF}{\text{Sum of percentage all endophytes}}$$

Evaluation of antioxidant activity

Preparation of extracts (Plant extract): Fresh methi/fenugreek leaves were washed and shade dried overnight. They were ground to fine powder in a mixer. The contents were transferred to pre-weighed polyethene zip-lock covers and stored at 4^{o}C until further use. 0.1 g of dry powder was extracted in 10 ml of water and methanol separately and designated as aqueous and methanolic extracts respectively.

Fungal mycelial extract

Cultivation of the fungus: The dominant endophytic fungus was cultivated in 500 ml of potato dextrose broth contained in Erlenmeyer flasks and incubated for three weeks. The mycelia was filtered and transferred to a glass petriplate and dried overnight in a hot air oven at 40°C. The content of the dry mycelia were powdered using sterilized mortar and pestle. The contents were transferred to pre-weighed polyethene zip-lock covers and stored at 4^{o}C. 0.1 g of dry powder was extracted in 10 ml of water and methanol separately and designated as aqueous and methanolic extracts respectively.

The antioxidant activities of the extracts were evaluated by total phenolics and radical scavenging assays as follows:

Estimation of total phenolic content

Total phenolic content of fungal mycelia were determined by Folin-Ciocalteau (FC) method employing Gallic acid as standard (1mg/ml) as per the procedure of Volluri *et al*. (2011) with some modifications. Different concentrations of standard as well as the water and methanolic extracts (5-20 µg/ml) were taken and one ml of FC reagent (1:1 dilution) was added, 3-5min later 2.0 ml of sodium carbonate (20%, w/v) was added and the mixture was allowed to stand for 45 min under dark condition. After the specified incubation period, the absorbance of standard and samples were read at 765 nm using Spectrophotometer. The concentration of total phenolics was expressed in terms of mg/g GAE (Gallic acid equivalents).

DPPH radical scavenging assay

Different aliquots of aqueous and methanolic extracts of plant sources (5 – 20µg) were taken and the total volume was made up to 250 µL with water and methanol respectively. One ml of DPPH (4 mg/ 100 ml) was added and the tubes were kept in dark for incubation at room temperature for 20 min. The absorbance was checked against the blank at 517 nm. Per cent free radical scavenging was calculated based on the extent of reduction in the colour (Pannangpetch *et al*., 2007). The per cent radical scavenging was calculated as follows:

$$\% \text{ radical scavenging} = \frac{A_c - A_s \quad X\ 100}{A_c}$$

Where A_c = absorbance of control and A_s = absorbance of test sample.

RESULTS

Isolation and identification of endophytic fungi

The root segments of four important herbaceous species yielded 533 isolates of endophytic fungi. The relative isolation rate differed among the plant species, of which *C. sativum* had high percent isolations (51.4%), Among the 533 isolates obtained, *Anethum graveolens* (dill) accounted for maximum isolates (47.5%), whereas from coriander 9.38% of isolates were recorded (Fig. 1).

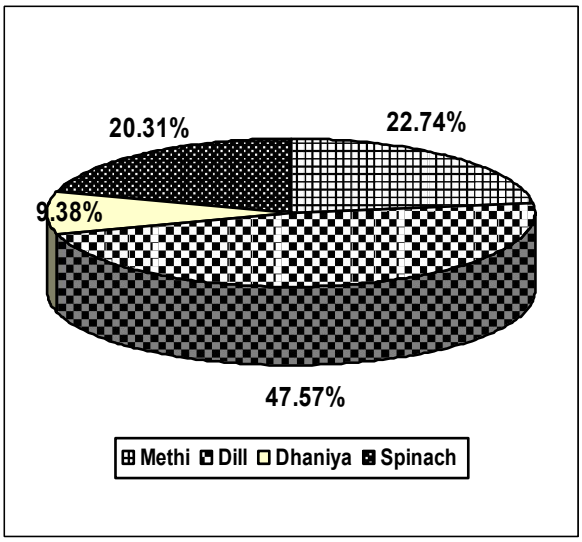

Fig. 1. Relative per cent endophytic fungal isolations from four plant species Fungal endophytes were isolated from four medicinal plants. The number of isolates from each species was counted and relative percentage of fungal isolations were calculated for each plant species and represented.

Some of the important fungal genera were identified as *Myrothecium roridum*, *Chaetomium fumicola*, *Heterosporium alli*, *Fusarium oxysporum* and *Aspergillus tereus*. The colonization on root segments and the spore photographs are depicted in Fig. 2. The frequency of fungal colonization was expressed in terms of percentage is represented in Table 1. Host specificity was observed for fungal endophytes. *M. roridum* was isolated from methi (*T. foenum graceum*) and dill (*A. graveolens*). *C. fumicola* from spinach (*S. oleracea*) and dill and *Trichoderma* sp. from coriander (*C. sativum*) and dill only. Endophytes such as *C. crispatum*, *H. alli*, *F. oxysporum*, *A. terreus* and *Verticillium* were isolated from individual plant species (Table 1). *M. roridum* was isolated as the dominant endophyte (29.8%). Other dominant endophytes include *C. fumicola* and *Trichoderma* sp.

Determination of anti-oxidant activity in fungal and host extracts

The host plant (fenugreek/methi) as well as its dominant endophyte, *M. roridum* was tested for antioxidant activity by determining the total phenolic content as well as the radical scavenging ability and expressed with gallic acid as standard in terms of gallic acid equivalents (mg/g).

Total phenolic content

The host plant as well as the endophyte was compared for their total phenolic content in polar solvent system such as water and methanol. The phenolic content of plant and fungal mycelia extracted with water were 2.8 mg/g and 2.5 mg/g respectively (Fig. 3); whereas a significant decrease in the total phenolic content of the host (0.22 mg/g) to its endophyte (0.35 mg/g) in the methanolic extract was observed (Fig. 4).

DPPH radical scavenging activity

The radical scavenging activity of water extracts of fungal mycelia and its host revealed 72% activity of host extract against its endophyte (59%) (Fig. 5). Similarly, decrease in the scavenging potential of endophyte (26.6%,) with respect to its host (63.8%) was observed (Fig. 6)

DISCUSSION

Endophytic fungi are microbes that colonize the living internal tissues of hosts without causing any obvious symptoms of disease. They are a part of microbial diversity and estimates suggest their diverse species richness in plants of tropics to temperate regions. Bioprospecting of fungal endophytes over the past two decades have suggested that they are valuable source of bioactives, with manifold applications in therapeutics (Strobel and Daisy, 2003). Fungal endophytes have been frequently isolated from shrubs and tree species; and to mention, a few herbaceous plants such as *Catharanthus roseus* (Krishnamurthy *et al*., 2008; Huang *et al*., 2008), *Ocimum* sp., *Tridax procumbens, Leucas aspera* and *Coleus aromaticus* (Rajagopal *et al.*, 2010) have been evaluated for the isolation of endophytes. In majority of plant species leaf samples were considered for analysis. Less frequently, the stem and roots were considered for isolations. Therefore, in the current investigation, we report the isolation of endophytic fungi from the surface-sterilized root segments of four herbaceous leafy vegetables that form a part of our daily, regular diet from food sources, such as spinach, coriander, methi and dill. The above ground parts of these plant species are known to possess good nutritional values (USDA Nutrition Database). Owing to the soft texture and dissected morphology of leaves, they were not considered in the study, as surface sterilization would render them unacceptable for plating as well as enumeration of endophytic fungi. Hence, as an alternative, roots were selected. A total of 600 fragments yielded 533 isolates, some of the endophytes like *M. roridum*, *C. crispatum*, *C. fumicola*, *Trichoderma* sp., *A. terreus*, *F. oxysporum* are frequently reported as endophytes of host plants (Tejesvi *et al*., 2006; Krishnamurthy *et al*., 2008; Nalini *et al*., 2014) and host-specificity have been noted in our observations since fungi such as *C. crispatum*, *Chaetomium* sp., *Verticillium* and *A. terreus* were specific to a host and were exclusively isolated.

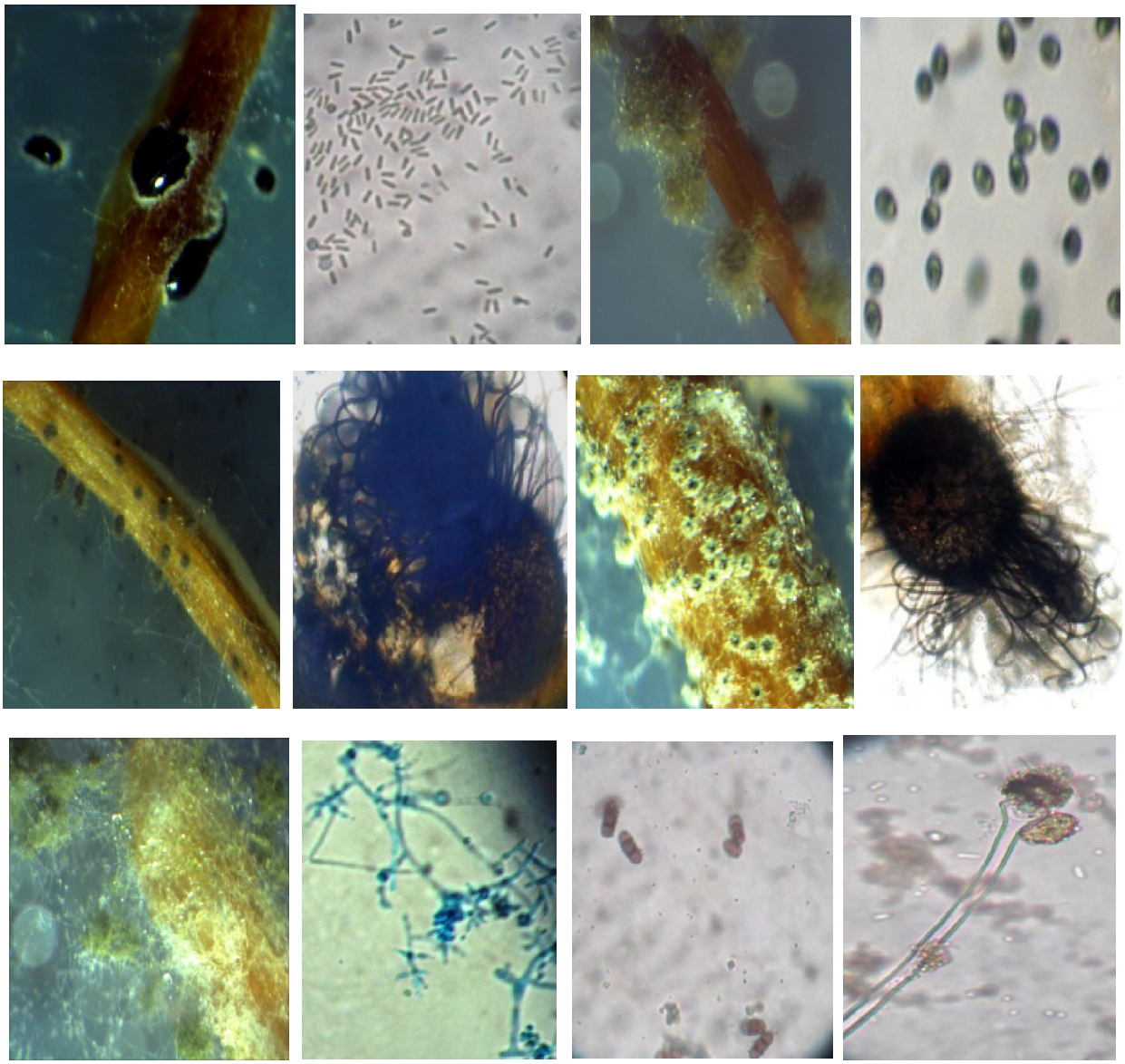

Fig. 2. Morphology of endophytes on root segments and their spore characteristics. A-*Myrothecium roridum* on root of *T. foenum-graecum*, B- Microscopic view of *M. roridum* spores, C-*Chaetomium* species on root of *A. graveolens* D-Microscopic view of *Chaetomium* spores, E- *Chaetomium* sp., on root of *Spinacea oleracea* F- Microscopic view of *Chaetomium* spores., G-*C. fumicola* on root of *A. graveolens*, H- Close up I- *Trichoderma* species on root of *A. graveolens,* J- Microscopic view of *Trichoderma* sp., K- Microscopic view of *Heterosporium alli* spores, *L*- Microscopic view of conidial heads of *Aspergillus terreus*

Table 1. Colonization frequency of endophytic fungi isolated from the root fragments of herbaceous species

Endophytic fungi/ host plant	TF	CS	SO	AG	Per cent Dominance	No. of isolates
Myrothecium roridum	94 (62.6)			78 (52.0%)	29.85	172
Heterosporium alli				32 (21.3%)	5.54	32
Chaetomium crispatum	36 (24%)				6.24	36
Chaetomium fumicola			38 (26.3%)	78 (52.0%)	20.24	116
Chaetomium sp.			36 (24%)		6.24	36
Trichoderma sp.		44 (29.3%)		45 (30%)	15.43	89
Fusarium oxysporum		10 (6.6%)			1.73	10
Verticillium sp.	1 (0.6%)				0.12	1
Aspergillus terreus				41 (27.3%)	7.10	41
Total	131	54	74	274	-	533

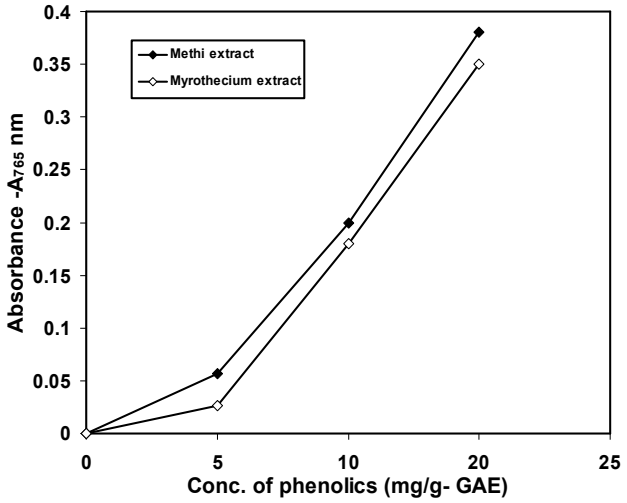

Fig. 3. Comparison of total phenolic content in the aqueous extracts of host plant and endophyte

M. roridum **was isolated as the dominant endophyte from the host plant methi (Fenugreek). The fungus was cultivated on potato dextrose broth for three weeks, mycelia harvested and dried. The powdered materials of both host (-■-) and fungal mycelia (-□-) were extracted with water. Total phenolic contents were determined with Gallic acid as standard and expressed in terms of mg/g Gallic acid equivalents (GAE).**

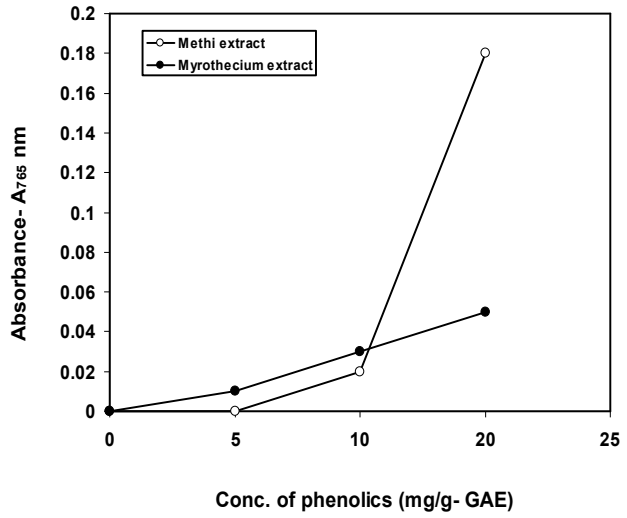

Fig. 4. Comparison of total phenolic content in methanol extracts of host plant and endophyte

M. roridum **was isolated as the dominant endophyte from the host plant methi (Fenugreek). The fungus was cultivated on potato dextrose broth for three weeks, mycelia harvested and dried. The powdered materials of both host (-○-) and fungal mycelia (-●-) were extracted with methanol. The total phenolic contents were determined with gallic acid as standard and expressed in terms of mg/g Gallic acid equivalents (GAE).**

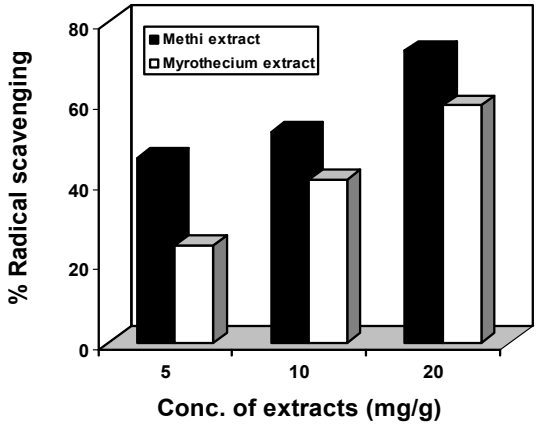

Fig. 5. Radical scavenging activity in aqueous extracts of host plant and endophyte

M. roridum **was isolated as the dominant endophyte from the host plant methi (Fenugreek). The fungus was cultivated on potato dextrose broth for three weeks, mycelia harvested and dried. The powdered materials of both host and fungal mycelia were extracted with water and assayed for the radical scavenging potentials by DPPH assay. Per cent radical scavenging was calculated and represented**.

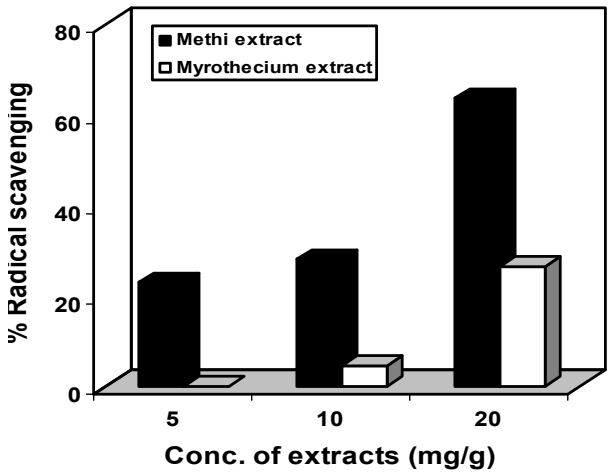

Fig. 6. Radical scavenging activity in methanol extracts of host plant and endophyte

M. roridum **was isolated as the dominant endophyte from the host plant methi (Fenugreek). The fungus was cultivated on potato dextrose broth for three weeks, mycelia harvested and dried. The powdered materials of both host and fungal mycelia were extracted with methanol and assayed for the radical scavenging potentials by DPPH assay. Per cent radical scavenging was calculated and represented.**

The rate of colonization differed among the host species with higher rate of colonization limited to coriander. The frequency of colonization was different for most isolates, with colonization frequency being high for *M. roridum* and thus was considered as the dominant endophyte. The endophytic fungus *M. roridum* with high percent dominance was evaluated for its antioxidant potential with the host plant, *T. foenum graecum*

(methi) as standard. Leafy vegetables from dietary sources are good sources of antioxidants. Plant species with edible value such as green leafy vegetables have been reported as sources of antioxidants (Jacob and Shenbagaraman, 2011; Katerere *et al*., 2012; Lin *et al*., 2013). Plant extracts containing phenolics and water-soluble antioxidants are widely reported from fruits and vegetables; their consumption has been reported with many health benefits.

Our studies have demonstrated that the aqueous extracts (water) of both plant as well as the fungal mycelia showed good phenolic content and radical scavenging activity (Figs. 3 and 5). However, the methanolic extracts from the plant source: methi showed lesser amount of phenolics, but fairly good scavenging potentials. In contrast, the mycelial extract showed lesser amount of phenolics as well as DPPH radical scavenging activity. Our results clearly showed the presence of phenolics in water soluble extracts of plant and in fungal mycelia suggesting that phenolics represent major group of compounds that act as primary free radical scavengers. A strong correlation between total phenolic content and antioxidant activity was observed in our extracts. The phenolic compounds may be responsible for the antioxidant activity of host plant and its isolated endophytic fungus. Some authors disagree with the fact that antioxidant activity of extracts cannot be predicted on the basis of total phenolic content, and the activity of phenolic compounds depend on their chemical structure. The antioxidant activity of phenolics can be interpreted due to ease with which they can act as reducing agents, hydrogen donors or singlet oxygen quenchers (Katerere *et al*., 2012).

Methanolic extracts of samples showed lower phenolic content and scavenging potentials of endophyte, and not the host which suggests that the water extracts may contain water-soluble phenolics, with better antioxidant activity. A study of antioxidant activity with endophytic extracts of *Aspergillus* sp., *Penicillium* sp. and *Phoma* sp. indicated that the acetone extraction yielded good antioxidant activity than water or methanol extraction. This could be possibly due to the low solubility of fungal membranes in the extraction solvent (Dhankar *et al*., 2012). Earlier studies on the screening of endophytic extracts for antioxidants from Chinese medicinal plants by Huang *et al*. (2008) have revealed chemical diversity of phenolic compounds such as phenolic alkaloids, phenolic acids, flavonoids, terpenoids, tannins, quinones and stilbenes from the endophytes. Therefore, bioprospecting of endophytic extracts for biological activity would reveal their potentials as alternatives to plant extracts. Our studies reveal that both host as well as its endophyte are good sources of anti-oxidants. Further, elucidating the profile would reveal the presence of similar compounds both in host and endophyte.

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