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Antioxidant Profiling of Fungal Endophytes Isolated from a Critically Endangered Endemic Medicinal Plant, *Artemisia amygdalina* Decne. of Kashmir Himalayas

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ABSTRACT: Micro-organisms living within a plant that causes asymptomatic infections entirely within the plant tissues are called as "endophytes." These can be bacteria as well as fungi and can be isolated from different parts of plants. They have been observed to be saprophytic and are cultivable on agar. Medicinal plants had been used to isolate and characterize directly the bioactive metabolites. However, the discovery of fungal endophytes inside these plants with capacity to produce the same compounds shifted the focus of new drug sources from plants to fungi. This work is being conducted to test the hypothesis that endophytic fungi produce bioactive compounds which might have some pharmaceutical potential. This hypothesis is based on the rationale that endophytes are capable of protecting their host in adverse and diverse conditions by the production of many novel bioactive metabolites which can be a lead for the development of novel pharmaceutical agents against many diseases. When working with endophytic fungi, the choice of host plant is of critical importance. Endophytic fungi have been isolated from an endemic species (*A. amygdalina*) of genus *Artemisia.* Stems, leaves and roots of this plant were sampled for the investigation of endophytic fungal communities. Healthy and mature host plants were collected from different areas in Kashmir valley. Fresh plant material was used for isolation work using surface-sterilization technique. The fungi have been identified on the basis of their morphological and cultural characteristics using standard taxonomic keys and monographs. The fungi were cultured in appropriate media for the production of secondary metabolites. A total of twenty-seven (27) fungal endophytes were isolated from this medicinal plant found belonging to twenty-four (24) different species. Almost all the endophytic fungal metabolites tested possessed some degree of antioxidant activity.

Keywords: Endophytic fungi, Kashmir, *A. amygdalina*.

INTRODUCTION

The literal meaning of the word endophyte means 'in the plant' (endon Gr., within; phyton, plant). The usage of this term is as broad as its literal meaning and has a broad tspectrum of potential plant hosts and inhabitants, including bacteria (Kobayashi and Palumbo, 2000), fungi (Stone *et al.,* 2000), algae (Peters, 1991), and insects (Feller, 1995). By definition, an endophytic fungus lives in mycelial form in biological association with living plant at least for some time. Therefore, the minimal requirement before a fungus to be termed as an endophyte should be the demonstration of its hyphae in the living tissue (Kaul *et al.,* 2012). Endophytes appear to be ubiquitous; indeed, no study has yet shown the existence of a plant species without endophytes (Nisa *et al.* 2015). The genus *Artemisia* L. (Asteraceae) containing 500 species is the largest genus in the tribe Anthemideae, and one of the largest genera in the family (Watson *et al*. 2002). Several *Artemisia* species have medicinal importance and are used in traditional medicine for the treatment of a variety of diseases and complaints (Demirci *et al*. 2004). This endemic medicinal plant belongs to the Asteraceae family and grows in the subalpine region of Kashmir Himalaya and is also found in the North-West Frontier Province of Pakistan (Dar *et al.,* 2006). The extracted plants are locally used for the treatment of a number of diseases like epilepsy, piles, nervous disorders, cough, cold, fever and pain (Rasool *et al*., 2012). Thus this important medicinal plant of Kashmir valley was chosen for the present study because of its pharmacological and therapeutic importance in the folkloric medicines.

METHODOLOGY

A. Collection of Plant samples

Twenty (20) symptoms-less whole plant samples were collected from different regions of Kashmir valley, J&K. The samples were collected in clean paper bags and brought to laboratory where they were further processed within 24 hours after collection.

B. Isolation of Endophytic Fungi

The method most commonly used to detect and quantify endophytic fungi is isolation from surface-sterilised host tissue (Stone *et al*. 2004). Surface-sterilization of plant material usually entails treating the plant material with a strong oxidant or a general disinfectant for a brief period, followed by a sterile rinse to remove residual sterilant (Stone *et al*. 2004). The plant material was rinsed gently in running water to remove dust and debris. After proper washing, stem and root samples were cut into small pieces, and leaves were selected for further processing under aseptic condition. Highly sterile condition was maintained for the isolation of endophytes. All the work was performed in the laminar air hood. Sterile glassware and mechanical things, such as scissor, forceps, scalpel and blades were used in all experiments. The isolation of Endophytic fungi was done according to the method described by Petrini, (1986).

The surface sterilization was done by sodium hypochlorite (NaOCl) and 75% ethanol. The time of treatment and concentration of sodium hypochlorite was changed according to the type of tissues. The concentration of NaOCl used was 1-13% and time of sterilization 3-10 minutes. Each set of plant material was treated with 75% ethanol for 1 minute followed by immersion in sodium hypochlorite and again in 75% ethanol for 30 seconds. Lastly, the segments were rinsed three times with sterile (autoclaved) distilled water. The plant pieces were blotted on sterile blotting paper. The efficiency of surface sterilization procedure was ascertained for every batch of plant tissue following the imprint method of Schulz *et al*. (1993). In each petri dish 5-6 segments were placed on medium supplemented with antibiotics, penicillin G 100 units/ml and streptomycin 100µg/ml concentrations. The dishes were sealed with parafilm and incubated at 27°C ± 2°C for 4 – 7days. Most of the fungal growth was initiated within four days of inoculation. The incubation period for each fungus was recorded. It was almost similar for the same species of plants used. Isolation from the master plates was done by the transfer of hyphal tips to fresh Potato Dextrose Agar (PDA) plates without addition of antibiotics to obtain pure cultures for identification.

Table 1: Fungal Endophytes isolated from different species of *Artemisia.*

Name of plant	Common Name (English)	Common/Local Name	Parts used
Artemisia amygdalina	Almond Wormwood,	Veeri tethwan	Root, Stem, and Leaves

C. Colonizing Frequency (CF)

The colonizing frequency of each endophytic fungus was calculated as according to Suryanarayanan *et al.* (2003). Briefly, proper time of incubation was given for CF counting. Colonization frequency (%) of an endophyte species was obtained by the following formula.

$$CF(\%) = \frac{Number\ of\ plant\ segments\ colonised\ by\ a\ single\ fungi}{Total\ number\ of\ plant\ segments\ observed} \times 100$$

Isolation Rate. Isolation Rate is a measure of fungal richness in a given sample of plant tissue, *i.e.,* the incidence of multiple infections per segment or piece. It is calculated as according to the formula given by Photita *et al*. (2001).

$$Isolation\ Rate = \frac{No.\ of\ isolates\ obtained\ from\ segments}{Total\ no.\ of\ segments} \times 100$$

D. Preservation of endophytic fungi

The purified fungal isolates were transferred separately to PDA slants with proper labelling and kept at 4ºC.

E. Identification of Fungi

The fungi have been identified on the basis of their morphological and cultural characteristics.

Fungi were grown on specified media at specified culture condition for identification. All the endophytic isolates were identified and placed in appropriate genera and species of fungi using standard taxonomic keys and monographs. Authoritative monographs were referred for identification of endophytes (Domsch *et al*., 1980; Ellis 1971; Sutton 1980).

Point inoculation method. A point inoculation on 100×15 mm or 90×15 mm Petri dish, an accepted standard technique for cultivation and morphological identification of *Penicillium*, *Aspergillus* and other related genera was followed. A point inoculation was done by using glass needles, with very low quantities of conidia on to the glass Petri dishes. Petri dishes were incubated at 27°C ± 2°C upside down for 7 days to prevent spread of conidia all over the plate and growth of the colonies.

F. Slide culture technique

The desired agar medium (10 ml) was poured into a 60mm petri dish, allowed to solidify and cut with a sterile stainless steel spatula into blocks approximately 5 to 8 mm. One block was aseptically removed and placed on the cover glass. Inoculation of the agar block on one or more sides with fungal hyphae or conidia was followed by placement of a second sterile cover glass on top of it. After this petri dish lid was replaced, the completed modified slide culture was incubated at the desired temperature until adequate growth and conidiogenesis had occurred. Each cover glass was used to prepare a semi-permanent mount on a standard microscopic slide. The top cover glass was lifted off with forceps and wetted on the specimen side with a drop of ethanol (70 to 90%). One drop of fungus mounting medium (lactophenol cotton blue) was applied to the specimen, and the cover glass was lowered gently onto the slide, specimen side down. The bottom cover glass was lifted and similarly mounted on a second slide (Riddel, 1950; Harris, 1986). The slide was then mounted and observed with 10X, 40X and 100X objective lenses respectively.

G. Culturing of Endophytic Fungi for the Production of Metabolites

This procedure was carried out as described by Ghisalberti (2002). The fungi were cultured in appropriate media for the production of secondary metabolites. Small scale cultivation was carried out primarily to perform bioassays for the detection of active metabolites. Both liquid and solid phase fermentation were performed. In most cases fungi were cultured on Potato Dextrose Agar (PDA) for primary screening. Sabouraud's Dextrose Agar (SDA), Rice Extract Agar, Potato Dextrose Broth (PDB), Czapek's Solution Agar, Malt Extract Agar and other media were also used for evaluation of the growth rate. The growth of the organisms was observed in different media and then suitable media was selected for the cultivation of fungi.

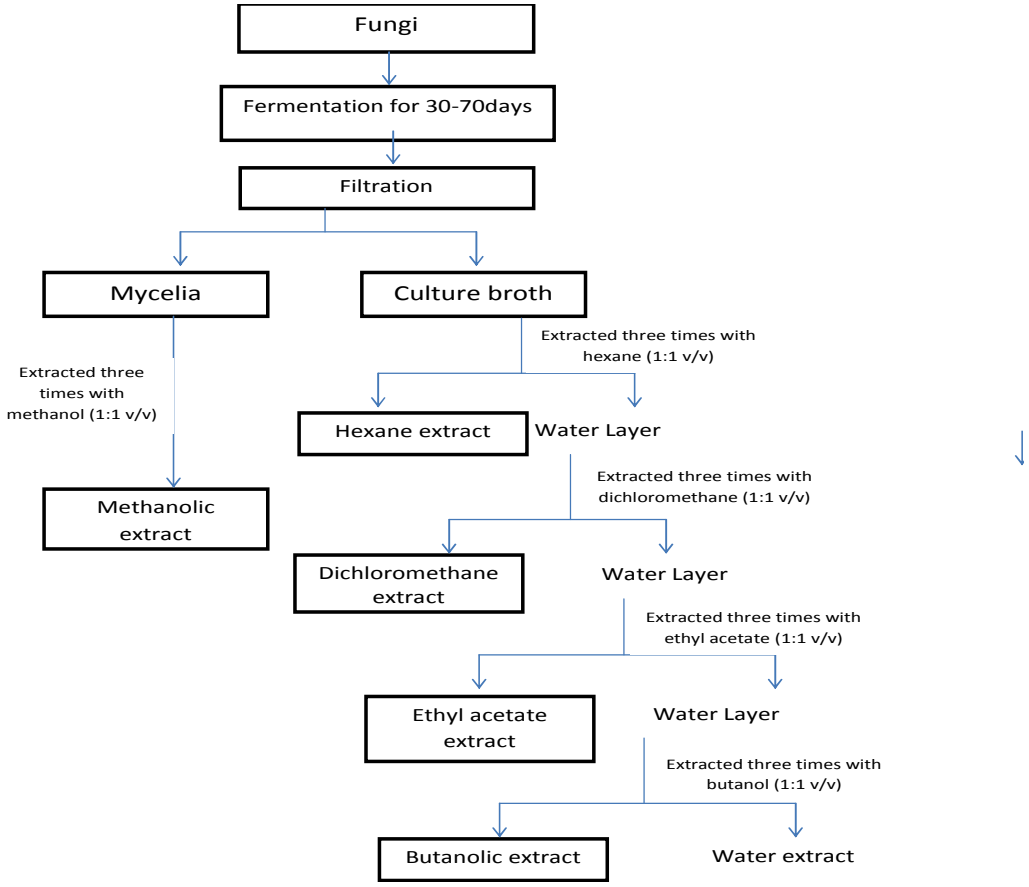
Small Scale Cultivation of Fungi. In most of the cases liquid state fermentation was carried out for the production of secondary metabolites.

H. Fermentation in Liquid Media

The biological screening of fungal secondary metabolites was carried out on small scale (500-1000ml). Appropriate media were prepared for the specific fungi. The prepared media was distributed among the flasks of 1L capacity (300 ml in each) and autoclaved at 121°C for 15 minutes. Different samples of 8 days fresh mycelia of different fungi, grown on PDA in a Petri dish at 27°C, were inoculated aseptically into all flasks. The flasks were kept on shaker at 27°C with 120rpm for 30 to 70 days for growth (Lin *et al*., 2002). The flasks were examined periodically for any contamination.

I. Extraction of Metabolites from the Liquid Media

Extraction of metabolites from liquid media was done as described by Choudhary *et al*. (2004) and outlined in **Scheme-I**. Briefly, the culture media and the mycelium were separated by filtration. The mycelium was then soaked in methanol. The methanolic extract of mycelia was collected after 7-10 days of soaking. The filter was extracted with organic solvents; hexane, dichloromethane, ethyl acetate and butanol sequentially. Liquid-liquid extraction was carried out three to four times for each solvent. These organic extracts were evaporated under reduced pressures to obtain solid residues.



Scheme I: Extraction of fungal metabolites from broth culture (Choudhary *et al.*, 2004).

J. Screening of Antioxidant Activity

Antioxidant activity of all extracts of endophytic fungi was determined by the method described by Lee *et al*., 1998, with minor modification. DPPH was prepared in ethanol (316µM solution). Test samples were prepared by dissolving in dimethyl sulfoxide (DMSO) and two different concentrations of test samples were used; 200mg/ml and 400mg/ml. Reaction mixtures were prepared in test tubes, containing 100µl of test samples (separately from both the concentrations) and 900µl of DPPH (final concentration of test samples was 200mg/ml).Test tubes were incubated at 37°C for 30 minutes. Absorbance was measured at 515nm using spectrophotometer. Ascorbic acid was used as positive control. 10% aqueous DMSO was used as negative control. Percentage inhibition of radicals by treatment of test samples was determined by comparison with a DMSO treated control group.

$$\% \text{ Inhibition} = \frac{(Absorbance)Control - (Absorbance)Sample}{(Absorbance)Control} \times 100$$

RESULTS

This is the first report regarding the isolation and identification of endophytic fungi of *A. amygdalina* an endemic species of Kashmir valley belonging to the genus *Artemisia*. A total of 65 plant segments; including 14 root parts, 28 leaf parts and 23 stem parts were investigated for this purpose. Screening of the different parts of *A. amygdalina* resulted in the isolation of twenty-seven (27) different fungal endophyte isolates belonging to 24 different species.

Table 2: Fungal Endophytes isolated from different species of *Artemisia.*

Host plant	Plant parts	Number of samples	Number of fungal endophytes isolated
Artemisia amygdalina	Roots	14	7
	Leaves	28	7
	Stems	23	13
	Total number of isolates		**27**

Seven (7) endophytic fungal species were isolated each from the root parts and leaf parts and a maximum of 13 endophytic fungal species from stem parts. All the isolated endophytic fungi were found to be culturable on artificial media and were maintained as pure cultures. Distinct macro-morphology and micro-morphology were exhibited by all these endophytes which could be used to differentiate them. Most of them belonged to ascomycota and also basidiomycota and also fungi imperfecti. The isolated fungal endophytes included; *Phoma glomerata* (Corda) Wollenw. & Hochapt., *Fusarium ciliatum* Link, *Aspergillus terreus* Thom., *Fusarium tricinctum*, *Mycocentrospora* Deighton, *Acremonium* sp., *Pythium ultimum* Trow, *Paraphoma* sp., *Fusarium solani* (Mart.) Sacc., *Phomopsis* (Sacc.) Bubak, *Aspergillus niger* van Teigh., *Acremonium* sp., *Rhizoctonia* sp., *Fusarium ciliatum* Link, *Dictyuchus* sp., *Fusarium monoliforme* (Sheldon) Snyder & Hansen, *Curvularia brachyspora* Boedijn, *Alternaria alternata* (Fr.) Keissler, *Humicola* sp., *Penicillium corylophilum* Dierck, *Alternaria* sp., *Aspergillus flavus* Link: Fr, *Alternaria* sp., *Aspergillus niger* van Teigh., *Fusarium solani* (Mart.) Sacc., *Achaetomium globosa* Rai, Tiwari & Mukerji, *Chalara theilavioides* Peyron (Table 3).

Table 3: Genera of isolated fungal endophytes from cultures *Artemisia amygdalina.*

S. No.	Culture Code	Identified fungi
1.	AG01R	*Phoma glomerata* (Corda) Wollenw. & Hochapf.
2.	AG02R	*Fusarium ciliatum* Link
3.	AG03R	*Aspergillus terreus* Thom.
4.	AG04R	*Fusarium tricinctum*
5.	AG05R	*Mycocentrospora* sp. Deighton
6.	AG06R	*Acremonium* sp.
7.	AG07R	*Pythium ultimum* Trow
8.	AG01L	*Paraphoma* sp.
9.	AG02L	*Fusarium solani* (Mart.) Sacc.
10.	AG03L	*Phomopsis* spp. (Sacc.) Bubak
11.	AG04L	*Aspergillus niger* van Tiegh.
12.	AG05L	*Acremonium* sp.
13.	AG06L	*Rhizoctonia* sp.
14.	AG07L	*Fusarium ciliatum* Link.
15.	AG01S	*Dictyuchus* sp.
16.	AG02S	*Fusarium monoliforme* (Sheldon) emend. Snyder & Hansen
17.	AG03S	*Curvularia brachyspora* Boedijn
18.	AG04S	*Alternaria alternata* (Fr.) Keissler
19.	AG05S	*Humicola* sp.
20.	AG06S	*Penicillium corylophilum* Dierckx
21.	AG07S	*Alternaria* sp.
22.	AG08S	*Aspergillus flavus* Link: Fr
23.	AG09S	*Alternaria* sp.
24.	AG10S	*Aspergillus niger* van Tiegh.
25.	AG11S	*Fusarium solani* (Mart.) Sacc.
26.	AG12S	*Achaetomium globosa* Rai, Tiwari & Mukerji
27.	AG13S	*Chalara thielavioides* Peyron.

A. Morphological Identification

The fungal endophytes were identified on the basis of morphological observation and culture characteristics, using differential culture media like Potato Dextrose Agar (PDA), Potato dextrose Broth (PDB), Nutrient Agar, Nutrient Broth, Czapek Yeast Agar (CYA), Czapek Dox Agar (CZ), Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA), Sabouraud Dextrose Broth (SDB), Rose Bengal Agar (RBA) and classical slide culture technique. Table 4 shows the characteristic features of colony morphology of few of these isolated fungal endophytes on differential media.

Table 4: Macromorphological features of some of the fungal endophytes on differential media.

Species Name	On CYA (Czapek Yeast Agar)				
	Colony colour	**Reverse colour**	**Colony surface morphology**	**Colony margin**	**Diameter (mm)**
A. niger	White to olivaceous green	White to cream, pale brown, yellow	Thick mat of white mycelia beneath the colonies	Forming radiating furrows	50-60
P. corylophilum	Grey green with sterile margin	Brown	Velvety	With concentric zones and slightly raised margin	33-35
A. alternata	Olivaceous green with white border	cream	Cottony with concentric rings	Round	40-50
P. glomerata	Olivaceous buff to dull green	Dirty yellow	Producing little aerial mycelium and abundant pycnidia	Irregular and round	50-65
A. terreus	Green	Yellow	Cottony growth	Round	18-20
C. brachyspora	Blackish-brown	Black	Suede-like, downy	Irregular	45-55
A. flavus	Yellow in the centre with white to creamish mycelia at the edges	Straw yellow to brown	Raised at the centre, thin merging margin	Round with radial grooves	55-60
On CZ (Czapek Dox Agar)					
A. niger	Olivaceous buff changing to brown with age, basal felt covered by a dense layer of dark-brown or black conidial heads, exudates present	Greyish to straw	Producing black conidia at the centre and white mycelia towards the edge	Floccose	50-65
P. corylophilum	White and slightly pink	Light pink	Crateriform, radially folded	Regular, round	20-25
A. terreus	Cinnamon-buff to sand-brown in colour	Yellow to deep dirty-brown reverse	Colony is typically suede-like	Floccose margins	
A. flavus	Yellow to green variations, or dark green; Exudates transparent to red-brown droplets in heavily sclerotial strain	Reverse hyaline, yellow to cream	Granular, flat colonies, floccose texture	Often with radial grooves	35-40
A. alternata	Dark green	White to creamish-yellow	Suede-like, coating of spore stalks	Wavy but almost round	25-35
On PDA (Potato Dextrose Agar)					
F. solani	Aerial mycelium white to cream became bluish-brown when sporodochia were present	Yellow to dark dirty-yellow	Floccose	Round, smooth	45-50
Acremonium sp.	Greyish-white	Dirty white	Powdery, floccose	Round	20-30
Paraphoma sp.	White with centre creamish	Yellowish	Cottony	Round with irregular margin	20-32

The morphological identification of the fungal species was based on the observation of some macro-morphological features like conidium (front) colour, reverse colour of colonies, colony margins, colony diameter and some micro-morphological features like conidia length, conidia width, conidia shape, conidia ornamentation, stipe length, stipe width, stipe ornamentation, phialide shape and branching. Different colour of colonies like white, grey, brown, yellow, orange, green, pink, red were observed, varied with the type of media and type of fungal endophytes as well. Some of the fungi showed cottony, velvety, powdery, crateriform, radially grooved and suede-like colony surface morphology (Table 4). The colony diameter shown by different species ranged between 18 – 65mm. However, there were also nine (9) species which could not be identified up to the species level.

B. Isolation Rate

The isolation rate was calculated as the number of isolates obtained from tissue segments, divided by the total number of segments and expressed as percentages. Isolation rate, a measure of the richness of fungal endophytes colonizing the different parts of the selected medicinal plant showed that the stem of *A. amygdalina* was very rich with an isolation rate of 0.56% (Table 5).

C. Colonizing Frequency

From *A. amygdalina* the total number of fungal endophytes isolated was twenty-seven (27) with a maximum number of isolates (13) from stem and seven (7) each from the root and leaf parts. *A. alternata* was the most frequent colonizer in the stem of *A. amygdalina* with a colonizing frequency of 43.47% followed by *F. monoliforme* (21.73%), *P. corylophilum* (17.39%), *C. brachyspora* (13.04%), *Dictyuchus* sp. and *Alternaria* sp. (8.7% each).

Table 5: Isolation Rate of fungal endophytes from different plant parts.

Plant species	Site of Isolation	Isolation Rate (%)
Artemisia amygdalina	Roots	0.50
	Leaves	0.25
	Stems	0.56

Table 6: Colonizing frequency of fungal endophytes in different parts of *A. amygdalina.*

S. No.	Fungal Endophytes	Number of cases of Isolation (NCI)	Colonizing Frequency (%)		
			Root	Leaf	Stem
1.	*Phoma glomerata*	2	14.28	-	-
2.	*Fusarium ciliatum*	1	7.14	-	-
3.	*Aspergillus terreus*	1	7.14	-	-
4.	*F. tricinctum*	1	7.14	-	-
5.	*Mycocentrospora* sp.	1	7.14	-	-
6.	*Acremonium* sp.	1	7.14	-	-
7.	*Pythium ultimum*	1	7.14	-	-
8.	*Paraphoma* sp.	5	-	17.85	-
9.	*F. solani*	4	-	14.28	-
10.	*Phomopsis* sp.	3	-	10.71	-
11.	*A. niger*	3	-	10.71	-
12.	*Acremonium* sp.	2	-	7.14	-
13.	*Rhizoctonia* sp.	1	-	3.57	-
14.	*F. ciliatum*	3	-	10.71	-
15.	*Dictyuchus* sp.	2	-	-	8.70
16.	*F. monoliforme*	5	-	-	21.73
17.	*Curvularia brachyspora*	3	-	-	13.04
18.	*Alternaria alternata*	10	-	-	43.47
19.	*Humicola* sp.	1	-	-	4.34
20.	*Penicillium corylophilum*	4	-	-	17.39
21.	*Alternaria* sp.	1	-	-	4.34
22.	*A. flavus*	1	-	-	4.34
23.	*Alternaria* sp.	2	-	-	8.70
24.	*A. niger*	1	-	-	4.34
25.	*F. solani*	1	-	-	4.34
26.	*Achaetomium* sp.	1	-	-	4.34
27.	*Chalara thielavioides*	1	-	-	4.34

Of the root colonizing endophytes the only dominant species was *P. glomerata* with a colonizing frequency of 14.28% followed by the rest of the six species with a colonizing frequency of 7.14% each. However, a prominent trend was observed in the colonizing frequency of the leaf endophytes with a highest colonizing frequency of 17.85% for *Paraphoma* sp., 14.28% for *F. solani*, 10.71% each for *Phomopsis* sp., *A. niger* and *F. ciliatum*, 7.14% for *Acremonium* sp. and a lowest of 3.57% for *Rhizoctonia* sp (Table 6).

D. Evaluation of antioxidant activity of different extracts of fungal endophytes isolated from A. amygdalina

Emergence of resistance to a plethora of synthetic drugs in human and animal pathogens as well as the undesirable side effects of a number of such drugs and antibiotics has triggered immense interest in the search for new drug sources and drugs of biological origin. It is with this interest that the antioxidant activity of seven of the isolated fungal endophytes from host plant, *A. amygdalina* was carried out using a bench-top assay. The antioxidant activity of six crude extracts (hexane, dichloromethane, butanol, ethyl acetate, methanol and aqueous) as measured by the ability to scavenge DPPH free radicals was compared with the standards ascorbic acid as positive control against a DMSO treated control group. The replicated experimental trials of DPPH assay with a fixed concentration of 200mg/ml and 400mg/ml revealed that the antioxidant activity of the extracts was concentration dependent i.e., higher the concentration of the extract in the reaction mixture higher is the scavenging potential. Some of the endophytic fungi out of seven (7) endophytic fungi of *A. amygdalina* subjected to antioxidant assay, hexane and dichloromethane extracts were less effective in scavenging of the free radicals. Not only they were less effective in their antioxidant potential, they were absolutely ineffective in certain cases. The methanolic extract of *A. alternata* exhibited highest %age inhibition of 82% at higher (400mg/ml) concentration of extract in the reaction mixture and the overall activity of the ethyl acetate, methanol, butanol and aqueous extracts was quite comparable vis-à-vis the inhibition percentage of the free readicals (Table 7).

Table 7: Antioxidant activity of various extracts of different fungal endophytes isolated from *A. amygdalina*

Fungal Endophytes	Concentration of extract (mg/ml)	Hexane	DCM	Ethyl acetate	Butanol	Methanol	Aqueous
P. glomerata	200	23.5±6.3	22.8±9.6	42.4±4.8	46.3±5.8	40.2±7.4	36.8±7.0
	400	38.8±6.5	29.5±7.3	52.6±5.8	58.5±2.8	51.6±3.0	44.3±5.0
Paraphoma sp.	200	15.5±3.8	32.8±6.0	59.1±6.8	58±7.6	54.5±8.7	32.8±2.2
	400	18.7±8.4	42.5±4.3	72.6±6.6	60±4.1	70.3±8.5	50.8±3.9
F. solani	200	NA	NA	59.2±9.0	44.1±5.0	30.3±6.5	45.8±5.2
	400	NA	NA	62.7±5.9	55±5.1	57.8±8.0	53.9±3.8
F. monoliforme	200	28.6±2.8	NA	47.5±8.1	28.3±9.7	50.7±8.6	16.8±5.0
	400	40.8±7.4	NA	50.9±6.4	40±6.1	48.9±6.7	29.4±5.6
C. brachyspora	200	NA	NA	61.3±4.7	41±4.0	47±7.6	17±5.5
	400	NA	NA	65±5.0	65.5±9.2	58.2±5.5	23.7±4.0
A. alternata	200	33.1±7.5	44.6±5.5	68.8±5.0	48.5±2.3	56±9.7	44.5±3.6
	400	59±4.9	63.8±5.0	80.2±2.6	68±8.1	81.9±9.8	56.8±8.5
P. corylophilum	200	NA	NA	58.9±3.7	38.8±5.0	27±7.3	NA
	400	NA	NA	68.6±3.2	59.7±9.5	49.3±7.0	NA

**Values represented as Mean±SD; NA= No activity; DCM= Dichloromethane; 10 % Aq. Ascorbic acid used as positive control showed 94.9% inhibition*

DISCUSSION

The emergence of new dangerous diseases, re-emergence of old ones, the development of resistance in pathogenic strains, side-effects and allergic reactions of some of the currently available drugs including their toxicity, are a few major problems which need immediate attention to combat these diseases with drugs of high therapeutic value. Likewise, the agriculture sector is also in dire need of effective drugs and agro-chemicals because of the fact that it is facing huge economic losses due to the development of resistant plant pathogens and enormous crop destruction by pests. Natural products are usually derived from micro-organisms, plants and animals. Plants continued to remain a rich source of many important therapeutic substances since time immemorial. As in also today, a major portion of new drugs are obtained from natural products or their derivatives (Newman *et al*., 2003). Endophytic micro-organisms are fungi and bacteria that colonize inter or intracellular spaces of plant tissues during at least one phase of their life cycle. (Azevedo *et al*., 2000; Kaneko *et al*., 2010). Endophytic fungi appear to be ubiquitous; indeed, no study has yet shown the existence of a plant species without endophytes (Nisa *et al*., 2015). As discussed earlier, *Artemisia* L. of the family Asteraceae is a genus with enormous economic importance. It is the largest and diverse genus of the tribe Anthemideae (Martin *et al*., 2001; Watson *et al*., 2002; Martin *et al*., 2003). We have undertaken this present study with the objective of analysing the fungi present inside the plants which belong to this genus, particularly one of the species endemic to the Valley of Kashmir, namely *Artemisia amygdalina*. Thus, *A. amygdalina* was worked upon for the first time for its endophytic fungal biodiversity. The method most commonly used to detect and quantify endophytic fungi is isolation from surface-sterilized host tissue. Detection of organisms from natural substrata and their identification are influenced by the sampling procedures, isolation methods, composition of the culture media, and physiological adaptations of the fungi. In some cases, such problems can be resolved by comparing cultures obtained from tissue isolations with those from sporulating states on the host (Bills and Pelaez 1996). Host species, host-endophyte interactions, interspecific and intraspecific interactions of endophytes, tissue types and ages, geographic and habitat distributions, types of fungal colonization, culture conditions, surface sterilants, and selective media all influence the efficiency of a sampling strategy for detection and enumeration of endophytic fungi.

The plant tissues are studied at length for their distribution pattern of endophytic fungi. Bills and Polishook, (1991) previously, examined the endophytes in inner bark and roots. Many researchers then extended the studies of previous authors with correspondence to foliar endophytes. Then later on many researchers confirmed the endophytic flora mainly from leaves. Thus, our findings were based on endophytic fungal investigation of four (4) medicinal plants which were isolated from above-ground and under-ground parts of the host plants. A wide range of plant species surveyed composed of endophytic fungal symbionts in foliar tissues (Stone *et al*. 2000). All the isolated endophytic fungi have been given in Table 3. Some fungal isolates could not be identified to the species level. This is a common problem concerning the identification of endophytes (Gamboa and Bayman, 2001; Promputtha *et al*., 2005; Huang *et al*., 2008). Morphologically similar and phylogenetically different endophytes were isolated from same host plant (Clay and Schardl, 2002; Suryanarayanan and Thennarasan, 2004; Jeewon *et al*., 2004; Seymour *et al*., 2004; Gange *et al*., 2007). This genetic diversity may be due to their isolation from distinct geographical regions (Yan, 2001). In case of *A. amygdalina* this type of study was done for the first time as it is the species belonging to genus *Artemisia* which is endemic to this part of the world. Thence, a maximum (27) number of endophytic fungi were isolated from different parts of this plant identified as; *Phoma glomerata*, *Fusarium ciliatum*, *Aspergillus terreus*, *F. tricinctum*, *Mycocentrospora* sp. *Acremonium* sp., *Pythium* sp., *Paraphoma* sp., *F. solani*, *Phomopsis* sp., *A. niger*, *Acremonium* sp., *Rhizoctonia* sp., *Dictyuchus* sp., *F. monoliforme*, *Curvularia brachyspora*, *Alternaria alternata*, *Humicola* sp., *P. corylophilum*, *Alternaria* sp., *A. niger*, *Achaetomium globosa*, *Chalara thielavioides*. This outcome also collates with the work done by earlier researchers. According to Hoffman and Arnold, (2008) the native plants are known to harbour greater endophytic fungal diversity than the non-native species.

In *A. amygdalina* the most isolated genus was *Fusarium* with many species being isolated from its roots, leaves and stem parts. The isolated species of *Fusarium* included *F. ciliatum*, *F. tricinctum*, *F. solani*, *F. monoliforme*. Also *F. ciliatum*, *Acremonium* sp. and *Alternaria* sp. were the endophytes commonly isolated in the roots, leaves and stem segments of this plant. For species with rare occurrence in *A. amygdalina* were present cosmopolitan species such as *Phoma* sp., *Mycocentrospora* sp., *Acremonium* sp., *Pythium* sp., *Paraphoma* sp., *Phomopsis* sp., *Rhizoctonia* sp., *Dictyuchus* sp., *Humicola* sp., *Chalara thielavioides* and such endophytic fungi have been isolated from a huge number of host plants (Kumaresan and Suryanarayanan 2001; Cannon and Simmons 2002).

Similar such results have been recorded in a study of Rakotoniriana *et al*., (2007). In our study host-specificity was observed for few of the endophytic fungi isolated from this host plant, *A. amygdalina*. A number of endophytic fungi were found only in *A. amygdalina* viz., *P. glomerata*, *A. terreus*, *F. tricinctum*, *Mycocentrospora* sp., *Acremonium* sp., *Pythium* sp., *Paraphoma* sp., *Phomopsis* sp., *Dictyuchus* sp., *F. monoliforme*, *C. brachyspora*, *Humicola* sp., *A. flavus*, and *Chalara thielavioides*. Of the myriad of ecosystems on earth, those having the greatest biodiversity seem to be the ones also having endophytes with the greatest number and the most bio-diverse microorganisms (Nisa *et al*., 2015). Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land's surface, yet they harbour more than 60% of the world's terrestrial biodiversity (Mittermeier *et al*., 1999).

The history of medicine includes many ludicrous therapies. Pharmaceutical and scientific communities have received the attention of the medicinal plants and the endophytic fungi residing inside them, as various publications have documented the therapeutic worth of the natural products from such microorganisms which validates the claims of their biological activities. Attention has been drawn to the antioxidant properties of endophytic fungi and their metabolites due to the growing incidences of drug resistant pathogens of both clinical and agricultural importance, side-effects of modern drugs, emergence of new diseases where no medicines are available. All these factors have stimulated renewed interest in plants and their endophytic fungi as significant sources of new medicines. Innumerable research work and studies have been concentrated on endophytic fungal isolation and evaluation of their antibiotic activities. But our study has taken this type of research work to another level; where we have isolated endophytic fungi from an endemic and critically endangered species of host plant belonging to genus, *Artemisia* inhabiting varied habitats of a particular geographical area, then we have cultivated some of these endophytes on small-scale to get their metabolites in different extracts, and finally evaluating their antioxidant activity. This study is first of its kind for *Artemisia* plants, in this particular area.

Reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, iron–oxygen complexes, hydrogen peroxide and lipid peroxides are generated by several oxidative reactions. Although ROS can help the immune system to clear out extrusive microorganisms, excessive ROS can also react with biological molecules such as DNA, proteins and phospholipids, and eventually cause oxidative damage in tissues and free radical related diseases such as inflammation, heart disease, diabetes, gout, cancer, etc. For aerobic organisms, the major system of defense against oxidative damage is the use of 'antioxidants' to convert excessive ROS into non toxic compounds. An imbalance between the amount of ROS and antioxidant enzymes is a problem for our health. This is why the daily intake of foods with antioxidant activity is necessary (Lee and Lee, 2007). 1,1–Diphenyl 2–Picryl Hydrazyl (DPPH) is a relatively stable radical. Its assay is based on the measurement of the scavenging ability of antioxidants towards DPPH, a nitrogen-centred radical, which reacts with suitable reducing agents. The electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up (Blois, 2001). The DPPH free radical scavenging activity is due to the neutralization of DPPH free radical by the endophytic fungal extracts, either by transfer of hydrogen or of an electron (Shimada *et al*., 1992). From *A. amygdalina*, the endemic species of genus *Artemisia*, found only in Kashmir valley, seven (7) endophytic fungi were analysed in which the methanolic extract of *A. alternata* exhibited highest %age inhibition of 82% at higher (400mg/ml) concentration of extract in the reaction mixture. It was also recorded that the hexane and DCM extracts showed minimum activity. Overall ethyl acetate, methanol, butanol and aqueous extracts showed comparable antioxidant potential. This plant specie was studied for the first time for this type of research.

CONCLUSIONS

The data produced in the present study has provided a valuable insight and firsthand information on the diversity of endophytic mycoflora of an important medicinal plant species belonging to the genus *Artemisia* of Kashmir valley, *A. amygdalina* which is a critically endangered endemic plant of this region. It was observed that the endophytic fungal isolates collected in this study provided a good source of bioactive compounds. As this study reveals that the endophytic fungi, *A. alternata* isolated from this host plant showed the presence of potent antioxidant compounds in its extracts. Thus, this fungal endophyte could be a potent source of bioactive compounds which can be a prolific source of many important drugs. However, further purification, characterization and structural elucidation is required to predict the accurate mass of these molecules with therapeutic applications. This study reinforced the assumption that endophytes could be a promising source of antioxidant substances. However, these results can be re-confirmed by molecular techniques.

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