Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(4): 87-92
Received: 21-05-2018
Accepted: 25-06-2018

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Isolation, molecular identification, phytochemical screening and *in vitro* anti-oxidant activity of endophytic fungi from *Achilea millefolium* Linn

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Abstract
Endophytic fungi isolated from a medicinal plant of western Himalayas (*Achillea millefolium*) were explored and investigated for its antioxidant potential and also phytochemical screening. Fungal endophytes were subjected to molecular identification based on ITS sequence analysis. Four endophytes namely *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus* and *Rhizopus oryzae* were isolated from the medicinal plant. Out of four three endophytes are evaluated for their activity. It was noted from DPPH assay that highest inhibition percentage was recorded by *Aspergillus niger* (88.53%), followed by *Aspergillus flavus* and (82.35%) and *Aspergillus terreus* sp (70.1%). In FRAP assay, it was noted that *Aspergillus niger* sp. recorded high reduction percentage followed by *Aspergillus flavus* and *Aspergillus terreus* sp. Endophytic fungi isolated from *Achillea millefolium* (L) were tested for various phytochemicals. *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus* yielded the flavonoids, tepenoids, phenol and saponins from ethanol extract. Antioxidant compounds like total phenol and flavonoid were also determined. The ethanol extracts of *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus* showed potent antioxidant activity against FRAP and DPPH. The total amount of phenol and flavonoid quantified were of 12.30, 10.42 and 9.24 gallic acid equivalent per gram of three endophytic fungi, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus terreus* respectively *and* 6.45, 5.15 and 3.35 µg/mg of quercetin equivalent respectively. This investigation suggests that the ethanolic extract of endophytes, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus* of *Achillea millefolium* possess potential antioxidant properties and also sheds some phytochemical constituents.

Keywords: endophytes, *Achillea millefolium* L., molecular identification, ethanolic extracts, antioxidants, DPPH, FRAP assay

Introduction
Endophytes are group of microorganism that resides asymptomatically inside plant tissues without causing any apparent harm to their host. These endosymbiotic fungi are found to synthesize bioactive secondary metabolites offer enormous potential for exploitation for medicinal, agricultural and industrial purposes [1]. Almost all classes of vascular plants examined to date are found to host endophytic organisms [2]. It has been estimated that there may be over a million different fungal species on this earth, of which only a small fraction, approximately 5% have been identified so far. A number of bio active metabolites have been isolated from endophytes possessing various types of bioactivities such as antibacterial, antifungal, antiviral, antioxidant, insecticidal, anti diabetic and immunosuppressive action [3, 4]. Free radical reactive oxygen species and reactive nitrogen species are generated by our body by various endogenous system, exposure to different physiochemical conditions or pathological states, which damage cellular components and even lead to cell death. Imbalance between free radicals and antioxidants contributes degenerative conditions such as hypertension, muscular dystrophy, neurodegenerative disorder, arthrosclerosis, cancer, inflammation, liver cirrhosis, nephrotoxicity etc., [5]. Free radicals are atoms, ions or molecules that contain unpaired electron. The unpaired electron makes them unstable and highly reactive. The antioxidants scavenge free radicals and prevent them from causing damages [6]. The literature survey reveals that the presence of flavonoids and phenolic compounds have proved potent antioxidant and free radical scavengers [7].

Achillea millefolium Linn. or yarrow is a flowering plant in the family Asteraceae. The herb is purported to be a antispasmodic [8], anti-inflammatory [9]. It contains isovaleric acid, salicylic acid, sterols, flavonoids, bitters, tannins and coumarines [10]. The plant also has a long history as a powerful healing herb used topically for wound, cuts and abrasions [11].

In the present study we focus on to examine the antioxidant and also to identify the phytochemical constituents in the extracts of endophytic fungi from *Achillea millefolium*.

Materials and Methods

Healthy and mature plant was collected from Pahalgam, Jammu and Kashmir District, Anantnag, and Kashmir. The samples were washed with sterile distilled water to remove the soil particles, and tightly sealed in polythene bags under humid conditions and kept at room temperature. The plant was taxonomically identified and authenticated. The authenticated specimens of the collected under specimen voucher No. 2356 –KASH, were deposited in the herbarium, Centre for Biodiversity and Taxonomy, University of Kashmir, India.

Isolation and identification of Endophytic Fungi

The plant surfaces sterilized was followed as described by Tartte Vijaya with minor modification, to remove all microbial epiphyte by immersion for 1 minute in 75% ethanol then 1 minutes in 0.1% sodium hypochlorite and then again 75% ethanol for 30 seconds. The plant segments were cut in to 0.5 - 1.0 cm in length. Later the segments were rinsed three times with sterile distilled water. The segments were blotted on sterile blotting paper. In each petri plate 2-3 segments were placed on potato dextrose agar medium supplemented with antibiotic to eliminating the growth of bacteria's. The plates were sealed and incubated 28 °C ± 2 °C for 2 weeks. After several days, fungi were observed; hyphal tips from the master plates were transferred to new potato dextrose agar plates without antibiotics. Fungal entities were differentiated on the base of the morphology of fungal culture, the characteristic of spores and the mechanism of spore production [12].

Molecular Identification of fungal endophytes

Genomic DNA from the five fungal strains (AR1, AMS6, AML4, HCL and AL) was isolated using the HiPuraTM Fungal DNA isolation kit method (Hi Media). Identification of the fungal species is based on mycelia i.e. color, size and shape and morphological characteristics such as conidia size and conidiophores. But merely based on morphology, identification is not accurate. Also, di- and pleomorphic life cycles obstruct accurate morphological identification to species level [13]. Hence, the need for molecular methods was realized and now DNA barcoding is widely accepted and appreciated. The ITS region located between the 18S and 28S rRNA genes, offer distinct advantages over other molecular targets including sensitivity. The intraspecific variability is limited and clearly separated from interspecific variability (Nilsson *et al*., 2009). Sequence of the ITS region has been proposed to be a primary fungal barcode marker by the Consortium for the Barcode of Life [14]. This approximately 650-bp region can be obtained in a single round of Sanger DNA sequencing.

In the present study, ITS1 (5'TCCGTAGGTGAACCTG CGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') primers obtained from Eurofins Genomics were used for amplification of Internal Transcribed Spacer (ITS) region. PCR conditions followed: 95 °C-5min, 95 °C-30s, 55 °C-30s, 72 °C-1: 30s, 72 °C-7min. Agarose gel electrophoresis was performed to confirm the amplification of ITS gene region.

Purification of amplified products

The left over primers and unbound nucleotides were removed by purifying the PCR products using standardized PEG-NaCl precipitation protocol [15].

Sequencing and phylogenetic studies

Applied Biosystems Genetic Analyzer (3500xL) was used to perform DNA sequencing based on the principle of chain termination. The Chromas Lite software was used to analyze the sequences obtained in chromatogram format. Once the sequence was known, similarity search was done by NCBI-BLAST analysis [16]. The strain showing maximum homology was reported.

Phylogenetic tree construction

The five fungal strains along with few Aspergillus sp., and Rhizopus sp., were selected to construct phylogenetic tree using MEGA6 software [17].

Cultivation and extraction of metabolites

The fungal endophytes were cultivated on PDA by placed mycelia disks (10 mm diameter) aseptically from the fresh culture to the flask containing 200 ml solid media and placed on incubator at 27°C without agitation for 2 weeks. After the incubation period, 200 ml of ethyl acetate were added to the flask and mixed properly. The culture was filtered under vacuum using a Buchner funnel to separate culture media and mycelia mates. The mycelium was discarded and the culture filtrate transferred to a separation funnel. The filtrate was treated with ethanol and ethanol phase were separated and concentrated under reduced pressure. Mates were oven dried, crushed and mixed with distilled water and ethanol and then filter. After evaporating the solvent, the residue was utilized for phytochemical screening and antioxidant activity by dissolved in ethanol at 1mg/ml [18].

Phytochemical screening

Preliminary phytochemical screening of ethanolic extract of endophytic fungi for the presences or absences of important constituents such as tannins [6], phenolic compounds, anthraquionines, steroids, cardiac glycosides and saponins using ethanolic extract [19].

Determination of antioxidant activity

DPPH radical scavenging assay

The method of Lee and his co-workers 1998 was used for the determination of scavenging activity of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical with minor modifications.1 ml of 0.2mM of DPPH prepared in methanol was mixed with 1 ml methanolic extract ranging from 0.1 to 20 mg/ml. Add 2 ml methanol to make up 4 ml. The mixture was vortexed thoroughly and left to stand for 30 min in the dark art room temperature. The absorbance was measured at 517 nm against blank. EC50 value (mg/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and the value was obtained by interpolation from linear regression analysis. BHT was used as reference. Percentage DPPH radicals scavenging activity was calculated by the following equation.

$$\text{DPPH scavenging effect (\%)} = [A_0 - A1/ A_0] \times 100$$

Whereas A_0 is the absorbance of the control reaction and A1 the absorbance in the presence of the sample [20].

FRAP assay

The antioxidant capacity of the sample was estimated spectrophotometrically. At low pH, reduction of ferric

tripyridyl triazine (Fe^{3+} TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593nm. The FRAP reagent was prepared by mixing 25 ml acetate buffer (300 mm, pH 3.6), 2.5 ml TPTZ in 40 mM HCl and 20 Mm $FeCl^3$ water solution. 4.5 ml freshly prepared working FRAP reagent was added each sample (150 µl) (0.5 mg/ml) and mixed thoroughly. After 5 minutes absorbance was measured at 593 nm, using FRAP as blank. The calibration curve of FeSO4 were in turn plotted against concentration of standard antioxidant tecopherol. The results are expressed in micromoler Fe(II)/g dry mass and compared with ascorbic acid [21].

Determination of total phenolic content

Total Phenolic Content (TPC) in endophytic extracts was determined using Folin-Ciocalteu's reagent following a slight modified method of Taga et al. Gallic acid was used as a reference standard for plotting calibration curve. A volume of 5 ml of extract (100 ug/ml) was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) was added to 2 ml of 2% aqueous sodium carbonate. The mixture was allowed to stand for a further 2 min in the dark, and absorbance was measured at 750 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent/g dry weight [22].

Flavonoid determination

The total flavonoid content was followed by slight modified method of Barros et al. The fungal extract 50 µl of crude extract (1 mg/ml ethanol) were made up to 1 ml with methanol, mixed with 4 ml of distilled water and then $NaNO_2$ solution (0.3 ml of 5%); 0.3 ml of 10% $AlCl^3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. After 6 min, 2 ml of 1 M sodium hydroxide was added to the mixture and the final volume of the mixture was brought to 3.3 ml with double-distilled water. The solution was mixed well and the intensity of the pink color was measured at 510 nm against blank. Catechin was used as standard for the calibration curve. Total flavonoids content of the extract was expressed as mg catechin equivalents per gram of sample (mg/g) [6].

Results and Discussion

Three different fungi species *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus* were identified in different parts of *Achillea Millefolium* (Table 1). ITS1 and ITS4 primers were used for amplification of Internal Transcribed Spacer region. Agarose gel electrophoresis of the amplified products was carried out and the presence of bands at about 600-700bp indicated that ITS region was successfully amplified (Fig.1). The amplified products were subjected to purification and further sequencing was performed using Applied Biosystems (3500xL) Genetic Analyzer. From NCBI-BLAST, the strains AMS6 and AR1 were identified as *Aspergillus niger*. The strains AL, HCL and AML4 were identified as *Aspergillus flavus*, *Rhizopus oryzae* and *Aspergillus terreus*, respectively (Table 2). The evolutionary phylogenetic analysis was performed using MEGA6 software. The evolutionary history was inferred using the Neighbor-Joining method and the distances were computed using Jukes-Cantor method. The strains AMS6 and AR1 showed maximum homology to *Aspergillus niger*. The strains AL, HCL and AML4 were found to show closest homology to *Aspergillus flavus*, *Rhizopus oryzae* and *Aspergillus terreus* respectively (Fig. 2). The endophytic fungi are present in almost all parts of the plant (Table.3). From the phytochemical analysis of ethanolic extracts of endophytes reveals the presence of saponins, phenolic compounds, cardio glycosides, anthraquionines, flavonoids, steroids and tannins. Endophyte *Aspergillus niger* has showed the presence of all phytochemical constituents (saponins, phenolic compounds) where Aspergillus flavus have all the phytochemical constituents except tannins and anthraquionines and *Aspergillus terreus* was deficient of cardio glycosides and anthraquionines (Table 4). The anthraquionines was observed in only the endophyte, *Aspergillus niger.*

Antioxidant property of the ethanol extract was evaluated by the ability to scavenge DPPH free radicals, was compared with the standards Butylated Hydroxy Toluene (BHT). The ethanolic extract of the *Aspergillus niger*, *Aspergillus flavus* showed potent antioxidant activity than that of *Aspergillus terreus*. At a concentration of 0.20 mg/ml, the scavenging activity of ethanol extracts of the *Aspergillus niger* (88.53%), *Aspergillus flavus* (82.35%), and *Aspergillus terreus* (70.1%). The study showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants (Figure 3).

Phenolic contents were the major constituents of the endophytes and possessing antioxidant activities [23]. The ethanolic extract of an endophyte *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus terreus*, isolated from the medicinal plant *Achillea millefolium* exhibited strong antioxidant capacity due to the presence of "phenolic" and "flavonoid" constituents. Phenolic constitute the main class of natural antioxidants and possess a wide range of therapeutic uses that are usually quantified employing Folin's reagent [24]. Antioxidant activities of the endophytic fungal cultures were correlated with their total phenolic contents, indicates that phenolic compounds are the main constituents of the endophytes that are responsible for possessing the antioxidant activity [25]. The antioxidant activity and total phenolic contents of endophytic fungi present in the *Achillea millefolium* were evaluated for the first time and the study showed positive correlation between the phenol content of the extracts with their antioxidant. Phytochemical analysis of the fungal extracts showed the presence of various secondary metabolites including flavonoids. These phytochemicals may be acting as antioxidants. The reducing ability revealed that the endophytic extracts have good FRAP activity (888.30-1026.0.04 µm Fe (II)/mg) (Table 5). The antioxidant potentials of the ethanol extract of *Aspergillus* niger, *Aspergillus flavus*, and *Aspergillus terreus* were estimated for their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). Among the three extracts, the *Aspergillus niger* has highest activity followed by *Aspergillus flavus* and *Aspergillus terreus*. According to recent reports, Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses and plays an important role in stabilizing lipid oxidation related to antioxidant and free radical scavenging activities [21]. Total phenol found to be in *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus terreus* of 12.30, 10.42, and 9.24 mg/GAE/g dry weight and flavonoid content of *Aspergillus niger* and *Aspergillus flavus* and *Aspergillus terreus* of 6.45,5.15 and 3.35 µg/mg equivalent respectively (Table 6). It is widely accepted that phenolic and flavonoids compounds may significantly contribute to overall antioxidant activities. The ethanol extract of *Aspergillus*

terreus and *Aspergillus flavus* contains phenolic and flavonoid contents are previously reported and similar results are obtained from the endophytic fungi. Results of our findings confirmed the use *Aspergillus niger* and *Aspergillus flavus* extract can be as traditional medicine. We found strong antioxidants activities specifically in the ethanol extract of *Aspergillus niger* and *Aspergillus terreus* [6, 26]. The Phenolic compounds especially the flavonoids possess a wide range of biological effects including antibacterial, anti- inflammatory, anti-allergic and antiviral actions [27]. They endophytic fungi secrete some important bioactive components like alkaloids, flavonoids, phenolic acids, quinones, steroids, terpenoids which are directly correlated with antioxidant activities [28]. Presence of phytochemicals like saponins and phenolic possess strong antimicrobial and antioxidant activity [19]. The antioxidant activity was comparable with standard ascorbic acid and BHT. The results of this study demonstrate that the endophytic fungal have medicinal uses and indicate a promising potential for the development of an antioxidant agents. These selective endophytic fungi by *in vitro* results appear as interesting and promising and may be effective as potential sources of novel antioxidant drugs.

Table 1: List of endophytes from different parts of *Achillea Millefolium*

Type of endophyte	Leaves	Stem	Root	Flower
Aspergillus niger	+	+	+	+
Aspergillus flavus	+	+	+	+
Aspergillus terreus	+	+	+	+

Repeated the experiment three times, + = presence

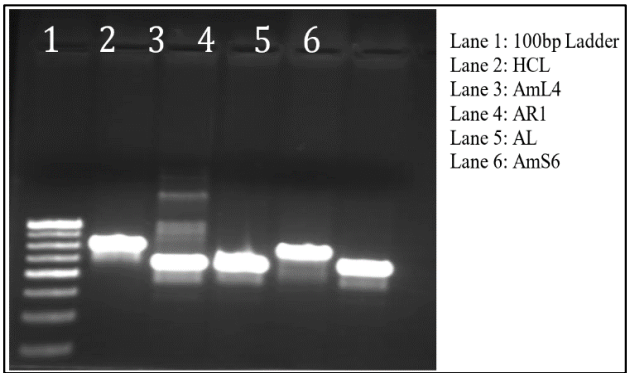

Fig 1: Amplification of ITS region, Agarose gel electrophoresis

Table 2: Isolated and identified endophytes from *Achillea millefolium*, in relation with the genus, and identity percentage found in NCIM (National Collection of Industrial Microorganisms)

Strain	Sequencing Primer	NCBI-BLAST	Identities	Strain identified	Accession number.
AL	ITS4 (482bp)	KJ482648 *Aspergillus flavus*	(100%)	*Aspergillus flavus.*	KU235487
AMS6	ITS4 (363bp)	KJ482656 *Aspergillus niger*	(100%)	*Aspergillus niger.*	KU235488
AR1	ITS4 (404bp)	KR075079 *Aspergillus niger*	(99%)	*Aspergillus niger*	KU235489
HCL	ITS4 (471bp)	HQ844675 *Rhizopus oryzae*	(100%)	*Rhizopus oryzae*	KU235490
AML4	ITS4 (540bp)	KR610363 *Aspergillus terreus*	(99%)	*Aspergillus terreus*	KU235491

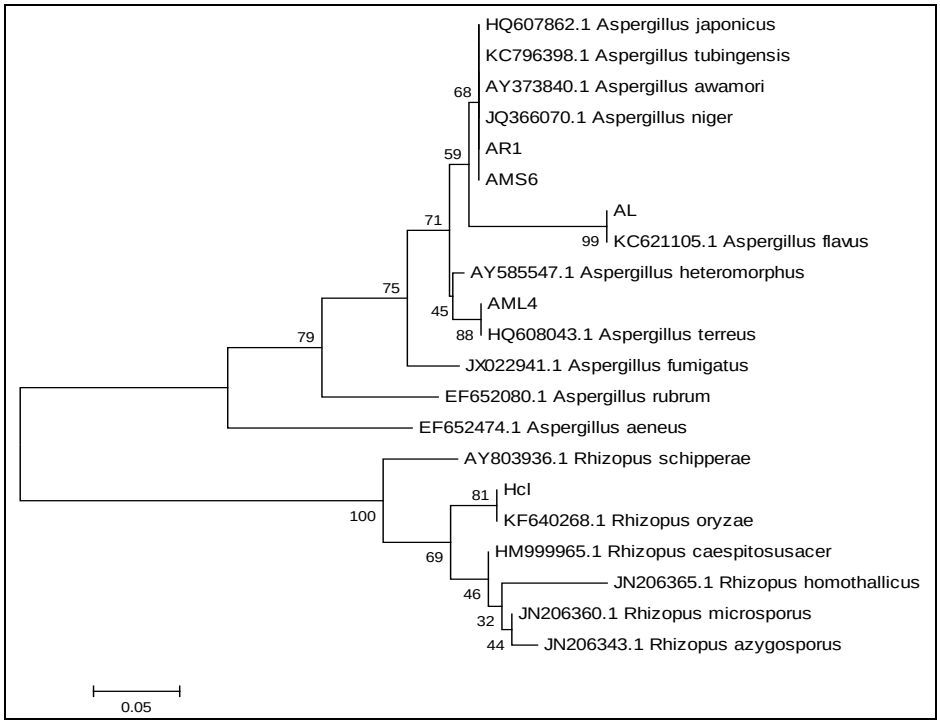

Fig 2: Phylogenetic tree based on neighbor-joining analysis of the rDNA ITS sequences of the endophytic fungal isolates obtained from *Achillea Millefolium*

Table 3: Endophytes from different parts of *Achillea millefolium*

Type of endophytes	Root	Leaves	Stem	Flower	Total
Aspergillus niger	3	4	3	2	12
Aspergillus flavus	4	4	2	1	11
Aspergillus terreus	2	2	1	1	6
Total	09	10	06	04	29

Table 4: Phytochemical analysis of ethanol extract of different endophytes

Tests	*Aspergillus niger* (KU235488)	*Aspergillus flavus* (KU235487)	*Aspergillus terreus* (KU235491)
Tannins	+	-	+
Steroids	+	+	+
Cardio glycosides	+	+	-
Flavonoids	+	+	+
Terpenoids	+	+	+
Alkaloids	+	+	+
Phenol	+	+	+
Saponins	+	+	+
Anthraquionines	+	-	-

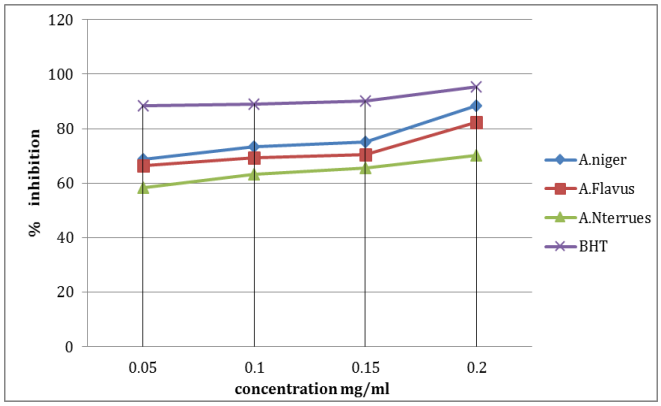

Fig 3: DPPH scavenging activities of the ethanolic extracts of *Achillea millefolium*

Table 5: Antioxidant activities of ethanol endophytic extracts of *Achillea millefolium*

Samples	FRAP
Aspergillus niger (KU235488)	1026.20 ± 0.04
Aspergillus flavus (KU235487)	945.42 ± 0.05
Aspergillus terreus (KU235491)	888.30 ± 0.05
Ascorbic acid	1566.40 ± 0.04
BHT	52.10 ± 0.04

Table 6: Phenolic and flavonoids content from ethanol extract of endophytic of *Achillea millefolium*

Sample	Phenol (mg/g)	Flavonoid (mg/g)
Aspergillus niger (KU235488)	12.30 ± 0.02	6.45 ± 0.04
Aspergillus flavus (KU235487)	10.42 ± 0.02	5.15 ± 0.04
Aspergillus terreus (KU235491)	9.24 ± 0.04	3.35 ± 0.04

Repeated the experiments three times for each replicates, According to Duncan's Multiple Range Test (DMRT), values followed by different subscripts are significantly different at $P \leq 0.05$, SE-standard error of the mean.

Conclusion

Based on the above results, all three endophytes, *Achillea millefolium* yielded medically important phytochemical compounds, may be due to these endophytes possess potent antioxidant potentials of ethanol and water extracts in all *in vitro* study. In addition, both endophytic extracts was found to possess significant amount of total phenolic content. Different constituents of *Achillea millefolium* have been claimed in different studies to possess biological properties related to antioxidant mechanisms. Hence, the significant antioxidant activity of ethanolic extract in the present study may be attributed to these aforementioned potent antioxidant ingredients of three endophytes of *Achillea millefolium*.

Acknowledgements

The authors gratefully acknowledge UGC, MANF for providing financial support for conducting this research. Very thankful to Dr. M.I. Zargar provided invaluable technical assistance also thankful to Prof. Mahesh M. Dharni, NCIM Resource Centre, CSIR-National Chemical Laboratory, Pune, India, for assistance in identifying endophytic fungi.

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