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BIOACTIVITY ASSESSMENT OF ENDOPHYTIC FUNGI ASSOCIATED WITH THE ETHNOMEDICINAL PLANT *POTENTILLA FULGENS*

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ABSTRACT

Potentilla fulgens L. is an ethno-medicinal plant used for the cure of various ailments by the ethnic tribes of Northeast India. Considering its excessive exploitation for medicinal usage, the present investigation was aimed at screening the plant for associated endophytic fungi to assess their bioactivity like antioxidant and anti-inflammatory activity. The isolates were also screened for presence of phytochemicals. A total of six fungi namely, *Bulgaria* sp., *Paraconiothyrium* sp., *Penicillium* sp., *Chaetomium elatum*, *Aspergillus* sp., *Sirococcus conigenus* were characterized using molecular approaches. The antioxidant activity was measured using the scavenging activity of DPPH radical and reducing power assay. The ethanol extract of *Aspergillus* sp. and *Sirococcus conigenus* possessed strong scavenging activity of DPPH and high reducing potential. *In vitro* anti-inflammatory activity was evaluated using membrane stabilization assay i.e human red blood cell membrane stabilisation (HRBC) method. The isolates having high antioxidant potential also showed a potent anti-inflammatory activity. Phytochemical analysis revealed the presence of cardiac glycosides, steroids, flavonoids, terpenoids, phenols and saponins. The findings indicated that the endophytic fungi offer scope for bioactive properties which otherwise is being done using the host plant.

KEYWORDS: Anti-inflammatory, Antioxidant, Phytochemicals, *Potentilla fulgens*.

INTRODUCTION

Potentilla fulgens L., commonly found at higher altitudes of Eastern Himalayan range, has been used by the ethnic tribal population for the treatment of various ailments. Several reports on the presence of important chemical constituents such as polyphenols, flavonoids and triterpenoids of therapeutic and commercial importance have been reported in *Potentilla fulgens.*[1] The use of this plant for its anti-hyperglycemic, hypoglycemic, anti-hyperlipidemic, antimicrobial, antitumor, antioxidant, anti-inflammatory and antiulcerogenic properties in ethnotherapeutic use is well extablished.[2] Owing to its diverse ethnomedicinal usage, the plant is facing rapid exploitation and destruction. Plants are known to possess endophytic organisms which invade the plants tissues throughout or part of their lifecycle with a asymptomatic infection and are known to be chemical synthesizers inside plants.[3] Most of them produces bioactive compounds which are capable antimicrobials and can be useful in novel drug discovery.[4] The search for new antimicrobial compound is important as bacterial and fungal infection remains the main cause for morbidity and mortality worldwide because of the microbial resistance against the limited antimicrobial agent present commercially.[5]

The present investigation was aimed at screening the phytochemicals and bioactivity like antioxidant and anti-inflammatory activity of fungal endophytes associated with *Potentilla fulgens* considering the ethno-medicinal uses of this plant and to infer if the fungi can offer an alternative to the host plant for medicinal usages.

MATERIALS AND METHODS

Collection of samples

Healthy plant samples were collected randomly from Shillong Peak in Meghalaya, Northeast India, placed in sterile polybags and transported to the laboratory within 12h and stored at 4°C until isolation procedure was completed.

Isolation of endophytic fungi

Samples were washed thoroughly in running tap water and then air dried properly before being processed. The samples were then surface sterilized by immersing them sequentially in 70% ethanol for 1min followed by 4% NaOCl for 3min and again in 70% ethanol for 1min and then rinsed thoroughly with sterile distilled water. The excess water was dried under laminar airflow chamber. The surface sterilized explants were then inoculated into the Petri dishes containing potato dextrose agar (PDA) (Himedia, India) supplemented with 100

µg/mL of streptomycin sulphate to suppress bacterial growth. The plates were incubated at 25±2°C until fungal growth appeared. The plant segments were observed once a day for the growth of endophytic fungi. Hypal tips growing out from the explants were transferred into PDA slant and maintained at 4°C.

Morphological features

The endophytic fungal isolates were presumptively identified microscopically based on their hyphal feature, arrangement of spores and reproductive structures.

Molecular characterization of the endophytic fungi

SSU rDNA region of the endophytic fungal genomes were amplified by using the specific primers nu-SSU-0817-5 and nu-SSU-1536-3.[6] A total of 50 µL PCR reaction mixture comprising 10 µL fungal DNA, 5 µL 10× PCR buffer, 1.5 µL of 50 mM MgCl2, 1 µL of 10 mM dNTP, 0.25 µL Taq polymerase, 40 pM each of the forward and the reverse primers. Thirty five cycles of PCR were then performed at 94°C for 0 sec, 56°C for 10 sec, and 72°C for 30 sec, followed by 72°C for 2 min. PCR was performed in a GeneAmp® 9700 ThermalCycler (Applied Biosystems, USA). Aliquots (10 µL) of each amplified product were electrophoretically separated on a 2% agarose gel in 1× TAE buffer and visualised using ethidium bromide under UV illumination in an Kodak gel logic 100 gel documentation system (Kodak, USA). DNA molecular weight ladder of 100-bp (Bangalore Genei, India) was used in each run. The PCR amplicons were excised and purified using a QIA Quick Gel Extraction Kit (Qiagen, Germany). The amplicons were sequenced in Applied Biosystems 3700 Genetic Analyser (Applied Biosystems, USA) with Big Dye Terminator ver. 3.1. Sequences obtained were then searched for similarity with other deposited sequences in GenBank. Alignments and phylogenetic analysis were performed using MEGA 4.0 software.[7]

Fungal metabolite extraction

Fungi were cultivated on Potato dextrose broth (Himedia, India) by placing agar blocks of actively growing pure culture in Erlenmeyer flask of 250 ml containing 100ml of broth medium. The flasks were incubated at 25±1°C for 3 weeks with periodical shaking at 150 rpm. After the incubation period, the fermentation broth were filtered through sterile cheese cloth to remove the mycelia mats. The culture filtrate was filtered with three equal volumes of solvent 70 % ethanol. The filtrate obtained was concentrated under reduced pressure (45°C) in a rotary evaporator to obtain the crude extract. The dry solid residue was

redissolved in 70 % ethanol (5mg/ml) and the crude extract was evaluated for the stated biological activities.[8]

Antioxidant assays

Reducing power assay (RPA)

The reducing power of the fungal extract was determined according the method of Chang et al.[9] An aliquot of 0.5 ml extract was added to 0.1 ml of 1% potassium ferricyanide. After incubating the above mixture at 50°C for 30min, ferricyanide was reduced to ferrocyanide, which was then supplemented with 0.1ml of 1% trichloroacetic acid and 0.1% FeCl3, and left for 20min. Absorbance was read at 700nm to determine the amount of ferric ferrocyanide (Prussian blue) formed. Higher absorbance of the reaction mixture indicated higher reducing power of the extract.

Radical scavenging activity using DPPH method

Different concentrations of the sample extracts were taken in separate test tubes and the volume was adjusted to100µl with ethanol. Five ml of 0.1mM ethanolic solution was added to these tubes. The mixture was then shaken vigorously and left to stand for 30min in the dark, and the absorbance was then measured at 517nm against a blank.[10] The percentage inhibition of DPPH radical by the samples was calculated using the following equation:

DPPH scavenging effect (%) = [(A0-A1/A0) × 100],

where A0 was the absorbance of the control reaction and A1 the absorbance in the presence of the sample.

***In vitro* anti-inflammatory activity**

Human red blood cell (HRBC) membrane stabilization method was used for this study.[11] Blood was collected from healthy volunteers and was mixed with equal volume of sterilized Alsevers solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% NaCl) and centrifuged at 3,000rpm. The packed cells were washed with isosaline solution and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property. Different concentrations of extract were separately mixed with 1mL of phosphate buffer, 2mL of hyposaline and 0.5mL of HRBC suspension. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3000rpm. The hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560nm. The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

Phytochemicals screening

Phytochemicals screening of the ethanolic extract of endophytic fungi was done according to the method described by Bandoni et al.[12]

Saponins

1ml aliquot of the endophytic fungal extracts were mixed with 5ml water at 60°C and shaken for 2min. The volume of froth produced in this experiments was observed because saponins are known to possess frothing activity and recorded every 10min for a period of 30min.

Phenolic compounds

1ml of fungal extract test solution was treated with 10% ethanolic ferric chloride. A change of colour to blue green were considered positive for phenols.

Cardiac glycosides

1ml of sample solution was mixed with 1ml of glacial acetic acid and then was treated with one drop of 5% ethanolic ferric chloride solution. 1ml of concentrated sulphuric acid was carefully poured down the sides of test tube. The appearance of a brownish ring between the two layers with lower acidic layer turning blue green upon standing indicated the presence of cardiac glycosides.

Tests for steroids

To 2ml of fungal extract, 2ml chloroform and 2ml conc. H_2SO_4 were added and shaken. Chloroform layer appeared red and acid layer showed greenish yellow florescence.

Ferric chloride test for flavonoids

About 5mg of the concentrated fungal extract was dissolved in ethanol (2ml). A few drops of 10% ferric chloride solution were added. Presence of phenolic hydroxyl group was indicated by green-blue coloration.

Test for terpenoids (Salkowski test)

Fungal extract (0.5gm) was added to 2ml of chloroform. Concentrated H_2S0_4 (3ml) was carefully added to the above mixture to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

RESULTS

Isolation and characterization of endophytic fungi

A total of six isolates were isolated from different parts (stem, root and leaves) of the host plant based on morphology, colour and genomic characterization based on BLAST search of ribosomal RNA gene sequence. The rDNA sequence of 18S ribosomal RNA gene was then assembled and submitted to the NCBI GenBank with accession numbers JN408711, JN408712, JN408713, JN613573, JN613574 and JN613580. Based on BLAST search of ribosomal RNA gene sequence, the endophytic fungi were found to be closest homolog of *Bulgaria* sp., *Paraconiothyrium* sp., *Penicillium* sp., *Chaetomium elatum, Aspergillus* sp., *Sirococcus conigenus* respectively (Fig 1).

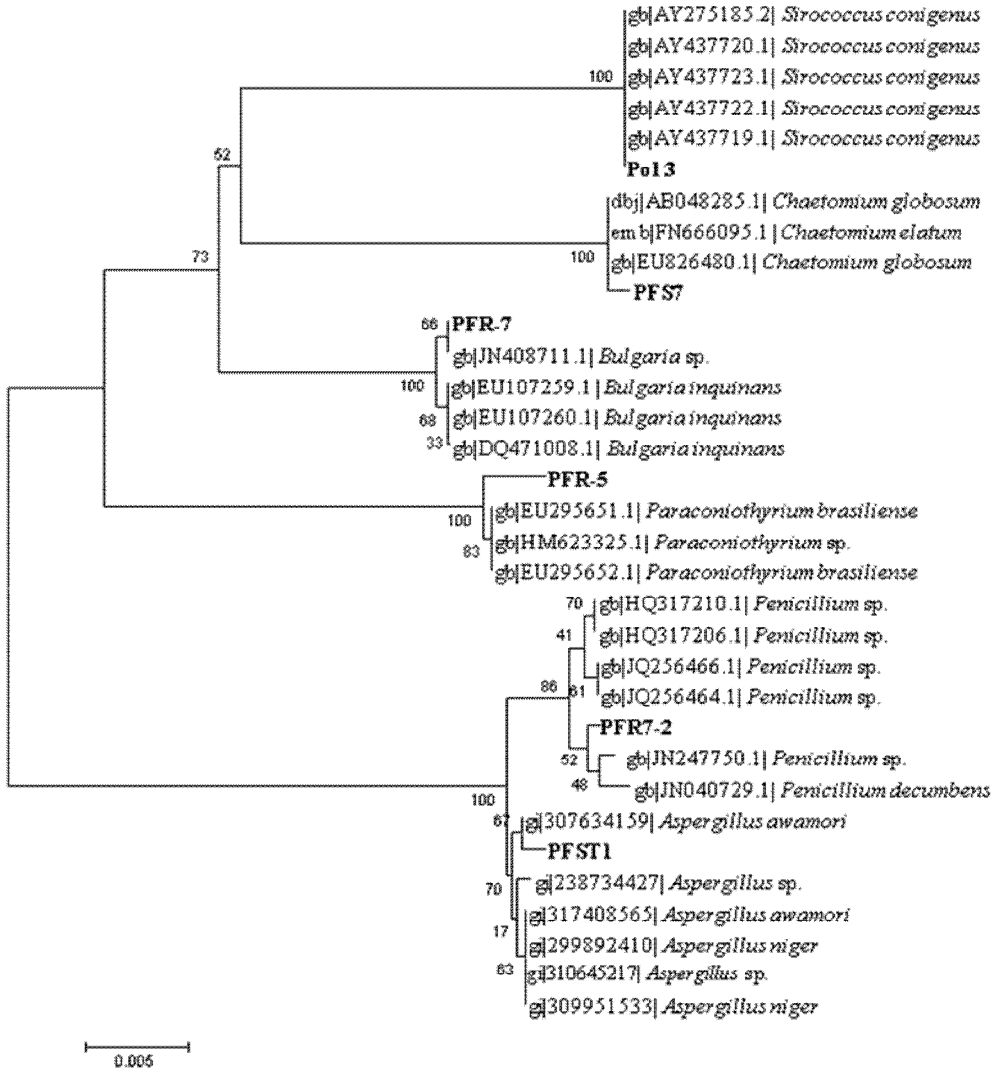

Fig 1. Phylogenetic tree of the endophytic fungal isolates constructed with MEGA 4 software using the neighbor joining method.

Reducing power assay

The reducing capacity of compounds could serve as indicator of potential antioxidant property. Among all the isolates, *Aspergillus* sp. showed a potent reducing activity which was closer to ascorbic acid standard and showed activity in the order of *Aspergillus* sp.> *Sirococcus conigenus* > *Penicillium* sp.> *Chaetomium elatum* > *Bulgaria* sp.> *Paraconiothyrium* sp. (Table 1).

Table 1. Reducing Power Assay and phytochemicals (qualitative) analysed from the fungal extracts.

Endophytic Fungal Isolates	Reducing Power Assay	Phytochemicals					
		Saponins	Phenolic compounds	Cardiac glycosides	Steroids	Flavonoids	Terpenoids
Aspergillus sp. (PFST1)	1.65±0.27	++	+++	+	++	+++	++
Sirococcus conigenus (POL3)	1.32±0.62	+	+++	++	+	++	+++
Penicillium sp. (PFR7-2)	1.01±0.33	_	++	_	_	+	+
Chaetomium elatum (PFS7)	0.72±0.09	_	+	+	_	+	_
Bulgaria sp. (PFR7)	0.63± 0.63	+	++	_	_	+	+
Paraconiothyrium sp. (PFR5)	0.42± 0.81	_	+	_	_	+	_
Ascorbic acid	1.78±0.46	NA					

+ indicates less intense ; ++ indicates moderately intense; +++ indicates highly intense

DPPH radical scavenging activity (RSA) assay

Aspergillus sp. showed promising free radical scavenging effect of DPPH in a concentration dependent manner. The free radical scavenging activities were found to in the order of the isolates *Aspergillus* sp.> *Sirococcus conigenus*> *Bulgaria* sp.> *Penicillium* sp.> *Paraconiothyrium* sp. > *Chaetomium elatum.* Ascorbic acid were used as the reference standards (Fig 2).

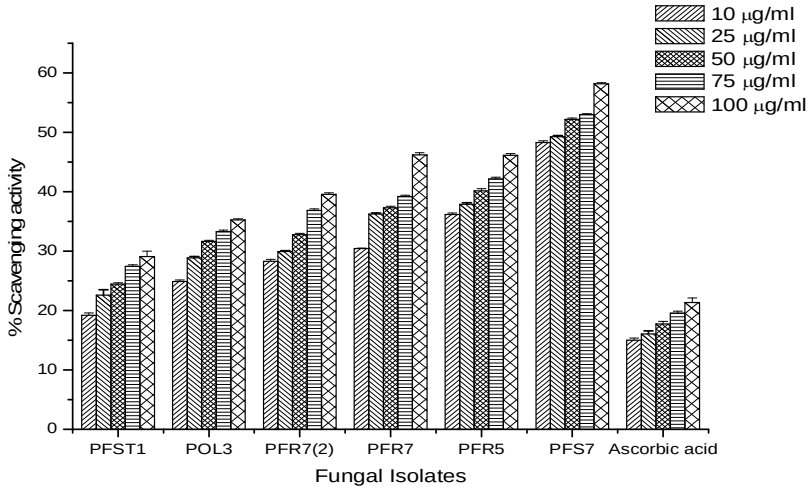

Fig 2. Scavenging activity (%) on DPPH radicals of ethanolic extract of different endophytic fungi at different concentration (µg/ml).

Anti-inflammatory activity

Ethanolic extract of the different isolates were studied for *in vitro* anti-inflammatory activity by HRBC membrane stabilization method. Among all the isolates, *Sirococcus conigenus* showed significant anti-inflammatory activity in a concentration dependent manner followed in order of activity by *Aspergillus* sp. > *Penicillium* sp.> *Bulgaria* sp.> *Paraconiothyrium* sp. > *Chaetomium elatum.* The performance of ethanolic extracts of all the endophytic fungi was lower than the standard Diclofenac sodium (Fig 3).

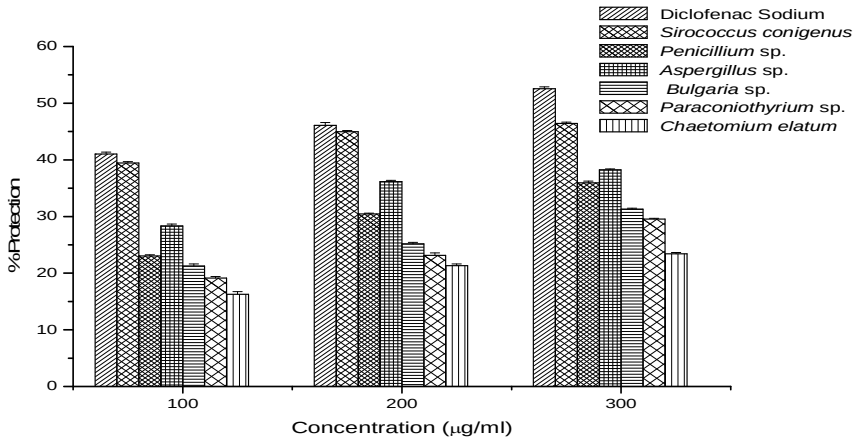

Fig 3. Anti-inflammatory activity of ethanolic extracts of different endophytic fungi by HRBC method.

Phytochemical screening

Phenolic compounds were found to be present in all the isolates. All the assayed phytochemicals were present in both *Aspergillus* sp. and *Sirococcus conigenus.* However, *Paraconiothyrium* sp. showed the presence of very few phytochemicals (phenol and flavonoids) when compared to other isolates (Table 1).

DISCUSSION

Among the diverse group of unexplored organisms, endophytic fungi are having some symbiotic associations with higher life forms which are beneficial for the host.[13] The selective colonization of the endophyte may lead to the production of special compounds within the host plant.[14] The data presented in the study demonstrated that *Aspergillus* sp. and *Sirococcus conigenus* showed excellent reducing power and radical scavenging activity. The DPPH assay has been used as a quick, reliable and reproducible parameter for the *in vitro* estimation of antioxidant activity.[15,16] As the concentration of the extract increases absorbance of the DPPH radical decreases which will be manifested in the rapid discolouration of the purple DPPH, indicating potent antioxidant activity due to its proton donating ability.[17] The activity of reducing power serve as an indicator of potential antioxidant property. The extract showing higher absorbance indicates strong reducing power potential.[18] The presence of phytochemicals within endophytes can be potential source for medicinal and industrial use[19] and the endophytes can be indicator that they can be potential source of precursors in the development of synthetic drugs.[20] The presence of terpenoids in the crude extracts of *Aspergillus* sp. and *Sirococcus conigenus* may be responsible for antioxidant activities.[21] Many researchers have reported that the presence of phenolic contents, flavonoids, terpenoids and saponins tend to possess antioxidant and anti-inflammatory properties.[22-26] which is in agreement with the present study. The erythrocyte membrane is analogous to the lysosomal membrane[27] and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release.[28] The present study showed that ethanolic extract of *Aspergillus* sp. and *Sirococcus conigenus* isolates have potent antiinflammatory activity may be due to the presence of polyphenolic compounds such as flavonoids, terpenoids, phenols and Saponins.[29] It shows that the *Aspergillus* sp. and *Sirococcus conigenus* could be promising agent in scavenging free radicals and treating

diseases related to free radical reactions and also a potent anti-inflammatory agent. These findings provide scientific evidence to support medicinal uses of the endophytic fungi and indicate a promising potential for the development of an antioxidant and anti-inflammatory agent from endophytic fungi isolated from *Potentilla fulgens*.

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