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Original Article

ASSESSMENT OF SECONDARY METABOLITES WITH RELATION TO THEIR ANTIOXIDANT ACTIVITY OF FUNGAL ENDOPHYTES ISOLATED FROM MEDICINAL PLANTS

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ABSTRACT

Objective: The detection of fungal endophytes and assessment of their antioxidant activity isolated from two medicinally important plants, *Calotropis procera* (L.) R. Br. and *Catharanthus roseus* (L.) G. Don. from parts of Burdwan district of West Bengal, India.

Methods: Isolation of fungal endophytes was carried out following the standard methods in potato dextrose agar (PDA) media. Identification, determination of colonization frequency, fermentation and extraction was done as stated in the standard protocol. Qualitative assay of the bioactive compound has investigated by standard protocol specifically for them. Quantitative estimation of total phenol was done by spectrophotometric method using gallic acid as standard. The antioxidant activity of the cell-free extract was determined by phosphomolybdenum assay and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method.

Results: Three dominant endophytic fungi each from C. *procera* and *C. roseus* with high colonization frequency in *Penicillium singorense* 25% and *Alternaria alternata* 21.87 % respectively has isolated and identified. Qualitative detection of secondary metabolites revealed that *Penicillium singorense* isolated from both plants and *Aspergillus neoflaviceps* (from C. *procera*) were found to be able to produce all the functional metabolites so far tested. It is evident that highest phenol 27.65±1.2 mg/g and flavonoid 2.06±0.1 mg/g content was shown by *Curvularia geniculata.* Highest antioxidant activity was exhibited by C. *geniculata* in both phosphomolybdenum assay i.e., total antioxidant capacity 2.46±0.11 and % inhibition of DPPH radical 94.55±0.015%.

Conclusion: Present study, therefore highlights the growing concept that the bioactive compounds produced by the endophytes not only established host endophyte relationship but also have an immense chance of an application in the field of medicine, agriculture and industry.

Keywords: Medicinal plants, Fungal endophytes, Secondary metabolites, Antioxidant, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

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INTRODUCTION

Fungal endophytes are those organisms which establish mutuality with the tissues of actively growing plants. It has been thought that all plants in natural ecosystems bear endophytes as their symbionts [1]. Endophytes play an important role in plants in overcoming stress condition and better living under variable environment. Fungal endophytes are capable to produce several bioactive compounds (phenol, steroids, saponins, flavonoids, etc.) which help the host plants to defend against pathogenic attack. Endophytes performs antioxidant activity against reactive oxygen species (ROS) like superoxide, hydroxyl radical and hydrogen peroxide produced in the metabolic process within the living cells of all organisms. The free radicals play a beneficial role in cell signaling at low or moderate concentration, become harmful when produced in excess causing oxidative stress leading to damage of DNA, RNA, proteins and lipids. In human body excess production of free radicals enhances the risk of cardiovascular disease, cancer, autism, etc. To scavenge such free radical endogenous production of an antioxidant has been found insufficient enough and therefore exogenous supply of antioxidants is earnestly needed to make a balance between the level of free radicals and antioxidants. Fungal endophytes may act as an alternative easily available low cost source of antioxidant.

Calotropis procera (L.) R. Br. belonging to the family Asclepiadaceae is a common wild shrub grown in tropical countries like India, Bangladesh and Pakistan. This medicinal plant is well known for its role in curing disease like arthritis, skin diseases, bronchial problems, muscular sprains and joint pain etc.

Catharanthus roseus (L.) G. Don. belonging to the family Apocynacae is a commonly cultivated ornamental herb in tropical countries. It is well-known for its anticancer and antidiabetic properties.

A few investigations on both *Calotropis procera* (L.) R. Br. and *Catharanthus roseus* (L.) G. Don has been carried out in relation to their endophytic inheritance. It is, however clear that the endophytes and their metabolic activity vary greatly in the same plants depending on the ecological factors like geographical locations [2, 3], differences in site [4], microclimate [5] etc. Studies of endophytic inheritance and formation of silver nanoparticles on both *Calotropis procera* and *Catharanthus roseus* have been carried out by the present research group [6, 7].

Moreover, the lack of information on bioactive compounds, including antioxidant activities of fungal endophytes in those two plants from Eastern India has led the investigators to undertake the present studies.

MATERIALS AND METHODS

Collection of plant materials

Leaves and stem bits of healthy *Calotropis procera* and *Catharanthus roseus* was collected from different parts of Burdwan district (latitude and longitude: 23.4595 ° N, 87.6186 ° E) of West Bengal, India, in the month of July to August. The plant material was brought to the laboratory in sterile polyethene bags and processed within a few hours after collection. Fresh plant materials were used for isolation of endophytic fungi to reduce the chance of contamination. Both the plant material was authenticated at Department of Botany, Visva-Bharati, Santiniketan, West Bengal where two herbarium voucher specimen was deposited (Accession No. 1037 and 1038).

Chemicals and reagents

All ingredients for media, chemicals, reagents, the substrates used in this study are of the highest purity, i.e. AR, Extra pure grade and

purchased from a reputed manufacturer like Merck, SRL and Hi-Media.

Isolation of endophytic fungi

Isolation and pure culture development of endophytic Fungi from *Calotropis procera* and *Catharanthus roseus* were carried out using the standard protocol with little modification [8].

The leaf of two medicinal plants was washed under running tap water for 1 h followed by sterile double distilled water (ddH_2O). The leaves were cut into small pieces with the help of sterilized cork borer. The samples were then immersed in ethanol (70%) for 1 min, followed by sodium hypochlorite (4%) for 2 min and then rinsed with sterile distilled water (2-3 times). The excess moisture was blotted with the help of sterile filter paper (Whatman No. 1). The surface sterilized 4 leaf segments were placed in petri plates containing potato dextrose agar (PDA) media supplemented with streptomycin 100 units/ml concentration. The petri plates were sealed using parafilm and incubated at 29±1 °C for 18 d. The petri plates were monitored regularly to check the growth of endophytic fungal colonies from the segments. The isolated hyphal tips of the organism were then subcultured in PDA slants to obtain a pure culture of the endophytic fungi.

Identification of endophytic fungi

The isolated fungi were identified based on cultural characteristics and available reproductive structures following mainly the protocol of Burnett and Hunter [9] Gilman [10], Whatnabe [11].

Calculation of colonization frequency

Colonization Frequency (CF %) of endophytic fungi was calculated by using the following formula [12, 6].

$$\text{Colonizing frequency \%} = \frac{\text{Number of segment colonized by fungi}}{\text{Total number of segments analysed}} \text{x100}$$

Fermentation and extraction

Fungal inoculums obtained from pure culture were inoculated into 250 ml Erlenmeyer flask containing 100 ml potato dextrose broth and incubated for 18 d at 29±1 °C at 120 rpm/min with constant shaking. The fungal culture was filtered; the filtrate was extracted with ethyl acetate (1:1 ratio) for three times. The organic phase was evaporated to dryness and stored at 4 °C for further use. The crude extract was dissolved in dimethyl sulphoxide (DMSO) to obtain different a concentration.

Qualitative detection of bioactive compounds

The ethyl acetate extract of the dominant endophytic fungi was used to detect the presence of the secondary metabolites such as alkaloids, phenols, flavonoids, saponins, cardiac glycosides, terpenoids and tannins following standard procedures.

Quantitative determination of total phenol content

The concentration of phenolics in fungal extracts was determined using spectrophotometric methods [13]. A methanolic solution of the extract (1 mg/ml) was used for the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic extract with 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent (prepared with water), and 2.5 ml of 7.5% of $NaHCO_3$. The samples were then incubated in a thermostat at 37 °C for 45 min. The absorbance was checked using UV-VIS spectrophotometer at a wavelength of 765 nm. The samples were prepared in triplicate for each analysis to obtain the mean value of absorbance. The same procedure was followed for the standard curve of gallic acid. The phenol content in the fungal extracts was derived from the standard curve; the results expressed as mg of gallic acid eq. per g dry weight of the extract. The values were expressed as mean±SD.

Quantitative determination of total flavonoid content

Total flavonoid content was measured the standard protocol of aluminium chloride assay [14]. Sample (1 ml) was mixed with 4 ml of distilled water and 0.3 ml of sodium nitrite solution (5% w/v), allowed to stand for 5 min. Aluminum chloride solution (10%, 0.3 ml) was added to the sample mix followed by the addition of 0.2 ml of NaOH (1 M) after 1 min. The volume was made up to 10 ml with distilled water and mixed thoroughly. The absorbance was measured at a wavelength of 510 nm in UV-VIS spectrophotometer. Different concentration of quercetin (100-1000 µg/ml) was used for preparing the standard curve. The experiment performed in triplicates and the standard curve was then plotted using the optical density O.D values obtained for quercetin. The total flavonoid content was calculated from the standard curve, the result expressed as mg quercetin equivalent per g dry weight of the fungal extract. The values were expressed as mean±SD.

Antioxidant assay

(a) Determination of antioxidant activity of phospho-molybdenum (total antioxidant capacity) assay

The antioxidant activity of endophytic fungi was determined by a colourimetric method using ascorbic acid as standard [15]. An unknown sample (1 ml) was mixed with ammonium molybdate (4 mmol, 1 ml) followed by disodium hydrogen phosphate (28 mmol, 1 ml). Then the mixture was incubated at 37 °C for 30 min after the addition of 2 ml of sulfuric acid (0.6 M). A standard set of ascorbic acid was prepared by taking a concentration of 20 µg-500 µg. A standard curve was used to calculate the antioxidant concentration in the fungal extract.

$$\text{Total antioxidant capacity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

(b) DPPH free radical scavenging assay

Various concentrations of fungal extracts (20-100 µg/ml, 2.5 ml) were mixed with a methanolic solution of DPPH radicals (0.1 mm, 0.5 ml). The mixture was shaken vigorously and allowed to stand in the dark for 30 min. The reduction in the DPPH radical concentration was determined by measuring the absorbance at a wavelength of 517 nm. Methanol and DPPH solution without the extracts was taken as blank and control respectively [16]. The inhibition percentage of DPPH free radical activity was calculated using the equation:

$$\text{Percent inhibition} = \frac{Ac - As}{Ac} \times 100$$

Where "Ac" is the absorbance of control, and "As" is the absorbance of a solution containing sample extracts.

Statistical analysis

All assays were carried out in triplicate and the results were expressed as a mean % value±standard deviation (SD).

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

All the isolated endophytic fungi were identified on the basis of cultural characteristics, reproductive structure and molecular basis. Three dominant endophytic fungi out of ten (higher CF %) were selected from C. *roseus* for further studies. Among eight isolates of C. *procera* three dominant endophytic fungi (higher CF %) were selected for further studies. Endophytic fungi selected were *Penicillium singorense* Visagie, Seifert and Samson; *Aspergillus neoflavipes* Hubka, Novakova, Kolarik and Peterson; *Curvularia geniculata* (Tray and Earle) Boedijn; *Alternaria alternata* and *Nigrospora* sp. *DRC4 MH021686 Alternaria alternata roseus,* which were subjected to plate culture showed growth of fungal endophytes in most of the sample. A total of eight fungal endophytes from C. *procera* and ten fungal endophytes from C. *roseus* were obtained. Out of eight fungal isolates of C. *procera* three (all from leaf discs) isolates namely *Penicillium singorense* (CF%=25%), *Curvularia geniculata* (CF% =12.5%) and *Aspergillus neoflavipes* (CF%=21.87%) were found to be dominant. Out of ten fungal isolates of C. *roseus* three (from leaf discs) namely *Alternaria alternata* (CF%=21.87%), *Nigrospora* sp (CF%=12.5%) and *Penicillium singorense*

(CF%=18.75%) were dominant (fig. 1). The occurrence of *Aspergillus neoflavipes*, *Penicillium singorense* and *Curvularia geniculata* as fungal endophytes in C. *procera* has been reported by several researchers [17-20]. The occurrence of *Alternaria alternata*, *Penicillium singorense* and *Nigrospora* sp as fungal endophytes has also been investigated [17, 19, 21, 22].

Qualitative detection of secondary metabolites

It has been revealed that *Penicillium singorense* isolated from both plants and *A. neoflavipes* (isolated from *C. procera)* were found to be able to produce all the functional metabolites so far tested (table 1). *Curvularia geniculata* (from *C. procera)* and *Alternaria alternata* (from *C. roseus*) produced all the secondary metabolites except saponins. The inability for the production of saponin as well as terpenoids was also observed in *Nigrospora* sp (isolated from *C. roseus*). Although all the isolates showed more or less efficient (as observed from intensity of color) for the production of alkaloids, flavonoids and tannins, terpenoids were found to be produced in highest extent in *P. singorense* and A. *alternata*, saponins (highest extent of frothing) by *P. singorense* and phenols (dark bluish green coloration) by *P. singorense*, *Curvularia geniculata* and *Nigrospora* sp. Ability to a produced bioactive compound of the fungal extract has also been reported in recent times [20, 23-25].

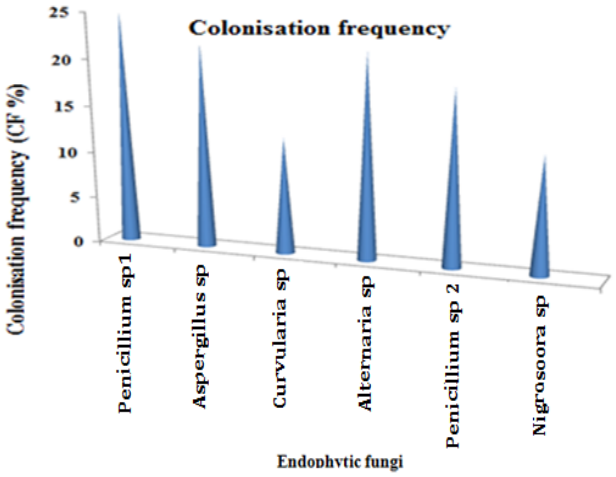

Fig. 1: Graphical representation of colonization frequency of isolated endophytic fungi from the leaf of *Calotropis procera* and *Catharanthus roseus*

Table 1: Qualitative detection of secondary metabolites from six endophytic fungal extract

Endophytic fungi	Phenol	Flavonoids	Saponins	Terpenoids	Alkaloids	Tannins
Penicillium singorense[1]	++	+	++	++	+	+
Aspergillus neoflavipes	+	+	+	+	+	+
Curvularia geniculata	++	+	_	+	+	+
Alternaria alternata	+	+	_	++	+	+
Nigrospora sp	++	+	_	_	+	+
Penicillium singorense[2]	++	+	++	++	+	+

"+"=present "-"=absent "++"=present in high conc.

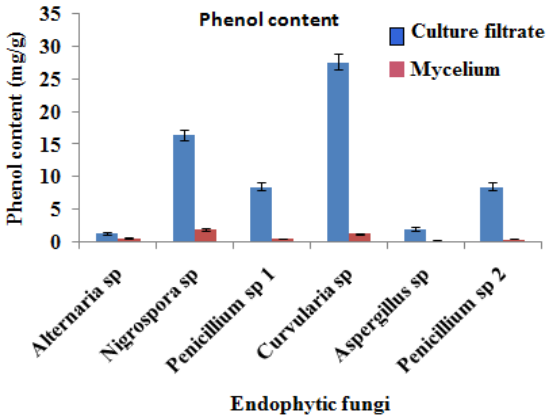

Fig. 2: Graphical representation of the phenol content of the culture filtrate and mycelium, all assays carried out in triplicate; mean data were plotted to the graph. "I" on top of the bar denote the value of standard deviation (±SD)

Total phenol and flavonoid content

Total phenol content was estimated using Folin-Ciocaltaeu reagent; it is a simple, convenient and reproducible method. It is routinely used for the estimation of phenolic compounds [26]. Phenol content of fungal endophytes was estimated both in culture filtrate and mycelium. It is evident that the highest phenol content (fig. 2) was observed in C. *geniculata* (27.65±1.2 mg/g) followed by P. *Singorense* [1] (8.54±0.61 mg/g) and A. *neoflavipes* (2.03±0.25 mg/g) isolated from C. *procera.* While *Nigrospora* sp *(*16.44±0.8 mg/g) proved to be the best in phenol production among the dominant fungi isolated from C. *roseus* followed by P. *singorense* [2] (8.52±0.58 mg/g) and A. *Alternata* (1.38±0.25 mg/g). Moreover, it is evident that the phenol content in the culture filtrate of all the fungi was higher than that of in the mycelium. Considering all the fungal isolates both from C. *procera* and C. *roseus* it is confirmed that C. *geniculata* is the best producer of phenol followed by *Nigrospora* sp.

It is evident that the flavonoid content was also found to be higher (fig. 3) in the culture filtrate of the fungal endophyte than that of in the mycelium. Highest extent of flavonoid content in the culture filtrate was observed in C. *geniculata* (2.06±0.1 mg/g) isolated from C. *procera* followed by *Nigrospora* sp (1.55±0.28 mg/g) isolated from C. *roseus.*

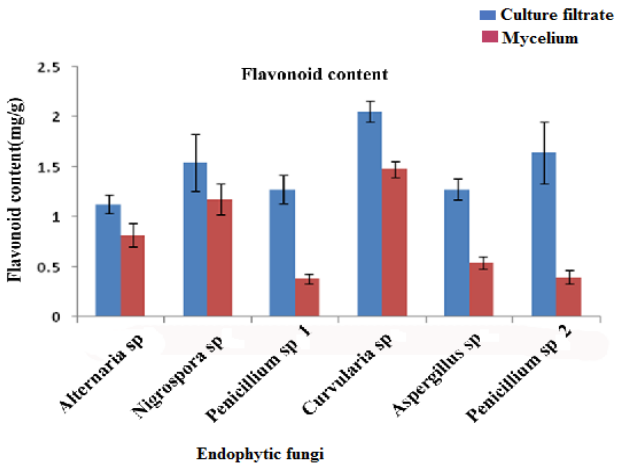

Fig. 3: Graphical representation of the flavonoid content of the fungal culture filtrate and mycelium, all assays carried out in triplicate; mean data were plotted to the graph." I" on top of the bar denote the value of standard deviation (±SD)

Antioxidant activity

The antioxidant activity of culture filtrate and mycelium of the dominant fungal endophyte was determined following two different methods viz. phosphomolybdenum method and DPPH scavenging activity. The phosphomolybdenum method has been routinely used to evaluate the total antioxidant activity of different samples. In the presence of culture filtrate or mycelium extract, the molybdenum Mo (IV) is reduced to Mo (V) and as a result a green coloured phosphomolybdenum–V complex formed which showed maximum absorbance at 695 nm. All the tested fungal endophytes isolated from C. *procera* and C. *roseus* showed their hydrogen donating efficiency and as a result, high inhibition percentage was obtained (fig. 4). It is evident that the total antioxidant activity in the case of the culture filtrate of all the samples was higher than that of the mycelium. Highest antioxidant activity in the culture filtrate was noted in *Curvularia geniculata* (2.46±0.11) isolated from C*.* *procera* followed by A*.* *Neoflavipes* (0.62±0.05) and P. *singorense* (0.39±0.04). The culture filtrate of fungal isolates of C. *roseus*, *Nigrospora* sp (2.03±0.09) showed the highest extent of antioxidant activity followed by A. *alternata* (0.80±0.11 %) and P. *singorense* (0.39±0.04) (fig. 4). It is further evident that the culture filtrate of C*. geniculata* isolated from C*.* *procera* was more potent in exhibiting total antioxidant activity than *Nigrospora* sp from C. *roseus.*

The DPPH assay is considered as a basic and most widely used assay. DPPH free radical scavenging assay is considered a most accurate screening method used to evaluate the antioxidant activity of different samples. The DPPH free radical scavenging potential of both the culture filtrate and mycelium of each fungal endophytes was determined. It is evident that the culture filtrate of fungal isolates in all cases showed higher antioxidant activity than the mycelium extracts. Among the dominant isolates of C*.* *procera* and *C.roseus* highest free radical scavenging activity showed by *Curvularia geniculata* (94.55±0.015 %) followed by *Aspergillus neoflavipes* (30.02±0.02 %) and P. *singorense* (24.29±0.03 %). Considering the isolates from C*.* *roseus*, *Nigrospora* sp proved to be the highest efficient endophyte in free radical scavenging activity (89.48±0.03%)) followed by A. *alternata* (74.21±0.32 %) and P. *singorense* (26.17±0.048 %) (fig. 5). Considering the efficacies of the isolates, C*. geniculata* from *C.procera* proved to be the best, followed closely by Nigrospora sp from C. roseus, followed closely by *Nigrospora* sp. Antioxidants act as free radical scavengers, inhibit lipid peroxidation and other free radical-mediated metabolic processes. In this way antioxidant is able to protect the human body from several diseases attributed to the reaction of radicals such as aging, cancer, neurodegenerative disorders, atherosclerosis and inflammations. Consumption of synthetic antioxidant has been reported to show toxic side effects, thus demanding the search for natural antioxidants and free radical scavengers [26]. DPPH assay is widely accepted in natural product antioxidant studies, because antioxidant donate a proton to this radical to decrease the absorption. The antioxidant effect is proportional to the DPPH free radical conversion to DPPH by anti-oxidant compound [27]. Endophytic fungi isolated from mangrove plants, namely *Phomopsis amygdala*, *Trichoderma* sp and *Alternaria* sp have shown high antioxidant activities against various free radicals which conforms the present study [28-30]. Studies on antioxidant activity, including the production of phenol, flavonoids by endophytic fungi *Penicillium sp* and *Aspergillus sp* from *C. procera* have shown similar result [28]. Antioxidant properties of endophytes from *C. procera* and *C. roseus* have also been investigated and showed that *Aspergillus* sp has a good response in terms of total phenolic content and antioxidant properties [31]. Recently the antiplasmodial activity of endophytic fungi strains namely IP-2 and IP-6isolated from *Artemisia annua* L. been reported [32]. The active metabolites of endophytic fungi, *Aspergillus flavus* isolated from the infected cadavers of butterfly (*Delias eucharis*) have been successfully used as an eco-friendly, reducing agent to generate AgNPs and synthesized nanoparticles showed antimicrobial properties against several human pathogens [32]. Production and anticancer activity of, camptothecin, a novel compound isolated from the Betel vine (*Piper betel* L.) endophyte fungus *Aspergillus niger* against colon cancer cell line has studied very recently [34]. The search and application of bioactive compound from endophytic fungi is gaining momentum, so our study probably might be helpful for further investigation in relation to such compound.

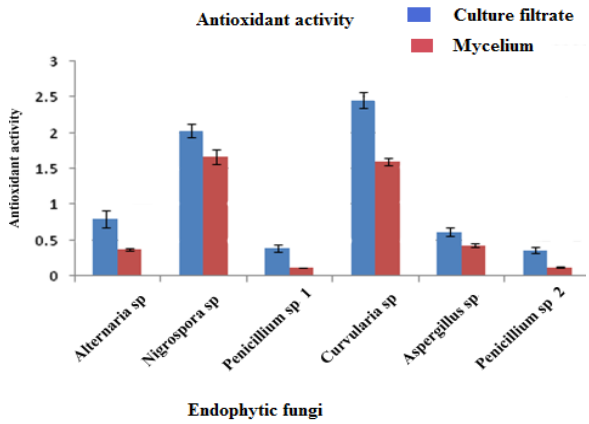

Fig. 4: Graphical representation of antioxidant activity (total antioxidant capacity) of fungal culture filtrate and mycelium by phosphomolybdenum assay, all assays carried out in triplicate, mean data were plotted to the graph. "I" on top of the bar denote the value of standard deviation (±SD)

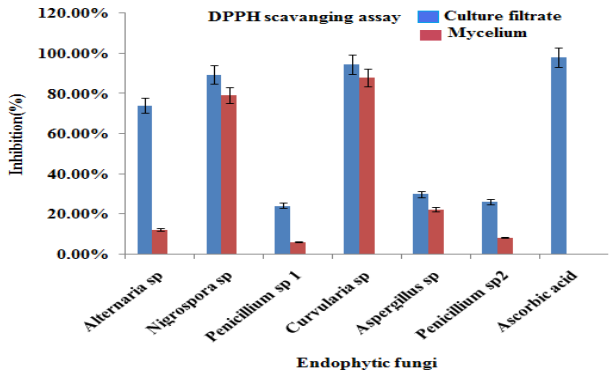

Fig. 5: Graphical representation of % inhibition of DPPH radical activity of fungal culture filtrate and mycelium by free radical scavenging assay, all assays carried out in triplicate, mean data were plotted to the graph. "I" on top of the bar denote the value of standard deviation (±SD)

CONCLUSION

The present study, therefore, highlights the growing concept that the bioactive compounds produced by the endophytes not only established host endophyte relationship but also have an immense chance of an application in the field of medicine, agriculture and industry. Because of their dual role in providing the ability to the host plant to overcome stress conditions and acting as a source of pharmaceutical important secondary metabolites, they are expected to become an important component of fungal biology. Moreover, they have drawn recognition as an important area of natural product research. From the perusal of literature, it is revealed that few or little attention has been made for the study of fungal endophytes of medicinal plant origin especially from the Eastern part of India. Our study anticipating providing an immense focus for further related study in this region and abroad also.

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ABBREVIATION

North-N, East-E, Temperature- °C, Milliliter-ml, Microgram-µg, Microliter-ul, Millimeter-mm, Percentage-%,, Milligram-mg, Gram-g, Milligram-mg, Mole-M, Milimole-mm, Minutes-Mins, Nanometer-nm Sodium Bicarbonate-$NaHCO_3$, Sodium Hydroxide-NaOH, Standard Deviation-SD, Mo (IV)-Molybdenum (IV), Mo (V)-Molybdenum (V), UV-VIS spectrophotometer-Ultraviolet spectrophotometer, OD-Optical Density, SRL-Cisco Research Laboratory, AR-Analytical Grade Reagent.

AUTHORS CONTRIBUTIONS

The experimental part and manuscript were done by Debjani Roy-Chowdhury. Writing, correction and revision of the manuscript were done by Dr. Swapan Kumar Chattopadhyay and Dr. Subhash Kanti Roy.

CONFLICT OF INTERESTS

Authors declares there is no conflict of interest

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