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Evaluation of *In Vitro* Antioxidant Activity and *L-Asparaginase* Enzyme Production of Four Endophytic Fungi Isolated from *Acanthus Ilicifolius*

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ABSTRACT

The need for novel and useful bioactive secondary metabolites to help and relieve people from all aspects of human conditions is constantly expanding. Every plant tissue has a variety of endophytic fungi, which are thought to be highly effective producers of natural products. In this work, the determination of total phenolics, antioxidants, and *L-asparaginase* enzyme activity in four fungal endophytes associated with the mangrove plant *Acanthus ilicifolius* was evaluated. The study indicated that total phenolic content (1633±8.7 μg equivalent to gallic acid) and reducing power (0.96) were the highest for methanolic extracts of the isolate *Aspergillus terreus* while scavenging activity was highest for the isolate *Colletotrichum xishuangbannaense* (78.2±4.5 %). The enzyme activity of *L-asparaginase* was expressed predominantly by all the isolates except *Colletotrichum xishuangbannaense*. Maximum enzyme activities of 50.1 *U/mL*, 48.1 *U/mL*, and 47.7 *U/mL* were observed in *Aspergillus terreus*, *Colletotrichum cobbiţense*, and *Fusarium multiceps* respectively. The current research demonstrated that mangrove-associated fungi have a high potential for producing bioactive molecules and *L-asparaginase*, which can be used as a possible source for the creation of anticancer drugs.

Keywords: *Acanthus ilicifolius*; Antioxidant activity; Endophytic fungi; *L-asparaginase*

1. INTRODUCTION

Mangrove ecosystems are dynamic, and different mangrove species have different adaptations to deal with these conditions. In-depth research of the mangrove environment is being done in the hope of locating novel chemical compounds with the potential for use in medicine.[1] The mangroves have produced several physiologically active substances with varying degrees of activity, including anticancer, antiulcer, antioxidant, antidiabetic, and antibacterial characteristics.[2] Mangroves are biodiversity "hotspots" for marine fungi that have been tailored to the high saline and challenging habitat. In the coastal regions, microbial communities would be able to withstand many abiotic stresses, like salinity, humidity, temperature, and soil composition, and would also develop novel metabolites with bioactive qualities.[3] Among the various microbial communities in the coastal ecosystems, endophytic fungi associated with mangroves can prove to be potential producers of bioactive compounds. Recently, there has been an increase in interest evinced in the natural compounds of endophytic fungi and their role to treat oxidative tissue damage.[4]

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Endophytic fungi are symbiotic microorganisms that colonize plant interior tissues and coexist in harmony with their hosts.[5] Endophytic microorganisms release biologically active substances that help host plants grow under challenging circumstances. They can defend plants from predatory organisms and pathogens. These endosymbionts consist of rich bioactive compounds with potential applications in pharmaceutical industries.[6] Recently, there has been an increase in interest in how natural compounds from endophytic fungi can treat oxidative tissue damage.

Endophytic fungi from several mangrove plants have demonstrated promising antibacterial and antioxidant activity in several recent research.[7] *Acanthus ilicifolius* is one of the mangrove plants that has been selected to screen as it contains various bioactives, hence endophytes associated with this plant can produce commercially important secondary metabolites with good antioxidant properties. The study focuses on screening antioxidant compounds and checking the production of potent enzymes from their associated fungi. One of the most effective anti-cancer drugs is the *L-asparaginase* enzyme. By converting free asparagine from the blood into aspartic acid and ammonia, this enzyme renders asparagine inaccessible to

malignant cells.[8] Currently, the *L-asparaginase* market is dominated by *Escherichia coli* and *Erwinia* species, however, commercially available *L-asparaginase* from bacterial sources has several limitations. Due to its limited specificity to asparagine, the enzyme from the bacteria results in severe allergic reactions. The toxicity and suppression induced by this *L-asparaginase* moved attention to alternate microorganisms like fungi to increase the specificity and reduce hypersensitivity.[9,10] Fungal endophytes are potential but less explored source of *L-asparaginase*, so they become an important candidate in the study.[11] Since there is a need to explore more resources to produce *L-asparaginase*. In consideration of the above, the present research is designed to study the enzyme production by mangrove-derived endophytic fungi.

2. METHODOLOGY

2.1 Isolation of Endophytic Fungi

Plant tissues of *Acanthus ilicifolius* devoid of diseases were collected from two sampling stations (19.1545 °N, 72.9581 °E, and 19.108492 °N, 72.920539 °E) from Mumbai, India. Samples were collected in triplicates between October and November 2019. The tissue samples were processed in the laboratory in the span of 24 h of collection. The explants of each tissue of size 1 *cm* X 1 *cm* were surface sterilised by treating it with 80 % ethanol for 10 seconds and with 4.5 % sodium hypochlorite for 60 seconds. Finally, the samples were cleaned in 2 sets of Sterile Distilled Water.[12] All the treated explants were inoculated on St. PDA (Potato Dextrose Agar) medium, with chloramphenicol (150 *µg/mL*) under aseptic conditions which were then incubated in the dark at 28 °C for a week. The fungal growth was continuously monitored and actively growing hyphal tips of morphologically different endophytic fungi were transferred to fresh PDA medium for purification and the purified isolates were stored in PDA medium at 4 °C for further analysis.

2.2 Identification Studies

Morphologically fungi were assessed as per their features like colony features arrangement of spores and sporulating bodies as per the standard methods. Genus-level identification of four isolates was carried out by the 18S rRNA sequencing method. Extraction of Genomic DNA (gDNA) was performed using the phenol/chloroform method.[13] Further, amplification of ITS regions using universal primers, *viz.* ITS1 and ITS4 were done using polymerase chain reaction (PCR). Purification by polyethylene glycol-sodium chloride precipitation of PCR product was carried out. The purified samples were then sequenced using ABI® 3730XL automated DNA sequencer. A Laser gene package was used for the assembly and the assembled sequences were subjected to the BLAST program for identification.[14] An accession number was assigned to the ITS sequence once it was submitted to GenBank in the NCBI database.

2.3 Culturing of Fungi and Preparation of Crude Extracts

All four organisms were grown in St. PDB medium for two weeks at 28°±1 °C at static conditions. After the fermentation, the spent medium of individual strains was separated from mycelia and it was extracted twice (v/v, 1:1) with ethyl acetate and methanol separately and concentrated under reduced pressure. The extracts were diluted to 1 *mg/mL* in Dimethyl Sulfoxide (DMSO) and were used for assessing the activity.

2.4 Assessment of Total Phenolic Content (TPC)

The TPC of the sample extracts was estimated following the standard method.[15] For this, 0.5 *mL* extract, 2 *mL* of 1:10 diluted Folin-Ciocalteau's reagent was added, followed by 4 *mL* of 7.5 % Na2CO3. The assay tubes were placed in the dark for 30min, and the color change was measured at 765 *nm* using a spectrophotometer. TPC in the samples was reported as equivalents of gallic acid in *µg/mL* as it was used to generate the standard curve.

2.5 Determination of Antioxidant Activity

Antioxidant properties of four endophytic fungi isolated from *Acanthus ilicifolius* were assessed by determining reduction potential and free radical scavenging activity (DPPH assay).

2.5.1 Assessment of Reduction Potential

The reducing property of the extracts was quantified by the protocol described by Zeng, *et al.*[16] The reaction system was set up using 1 *mL* of extract and 2.5 *mL* of buffer (phosphate buffer saline pH 6.6) along with 2.7 *mL* of 0.9 % K3Fe (CN).[6] Assay tubes were kept at 50 °C for 20 mins. 2.6 *mL* of 10 % TCA was put in to stop the reaction and it was subjected to centrifugation for 10 mins at 3000 rpm. This was followed by the addition of 3 *mL* supernatant, 3 *mL* D/W, and 0.5 *mL* $FeCl_3$. Reducing power capacity was interpreted based on an increase in absorbance by the sample at 700 nm by comparing it with the blank solution.

2.5.2 Assessment of Free Radical Scavenging (FRS) Activity by DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay

The FRS activity was determined using the protocol described by Zeng, *et al.*[16] An extract of 0.1 *mL* was added to 2.9 *mL* of 0.1 mM DPPH. The mixture was incubated for 30 min in dark. UV/Visible spectrophotometer at 517 *nm* was used to measure the absorbance. DPPH and pure methanol solutions were used as control and blank respectively.

The equation was used to determine the scavenging capacity for DPPH radicals

% Scavenging Activity (% SCA) = [(A1 - A2) / A1] x 100

A1 = Absorbance of the control A2 = Absorbance of sample extracts.

2.6 Primary Screening and Assessment of *L-Asparaginase*

The enzyme activity was qualitatively screened on Modified Czapek Dox's agar (Hi media laboratories, India) supplemented with 1 % L-asparagine. Fungal mycelia or surface spores were spotted on the surface of the medium and incubated for 3-5 days at 28 °C.[17] Change in color because of the reduction of pH of the medium, around the colonies was the indicator for positive enzyme activity.

Modified Czapek Dox's broth media (sterile) containing 1 % L-asparagine was used as a substrate. For enzyme production, the broth was inoculated with a suspension of fungal spores followed by incubation for 15 days at 28 °C. The fermented medium was filtered, and the spent medium served as the crude source of an enzyme to assess enzyme activity by Wriston's method.[18] Substrate was prepared by adding 189 *mM L-asparagine* to Tris buffer (pH 8.6). To the 0.1 *mL* of a crude enzyme, an equal amount of substrate was added. The enzyme and substrate reaction were carried out at 37 °C for 30 min. 0.1 *mL* of 15 % TCA was added to the tubes after incubation to stop the reaction and to collect the supernatant tubes were spun at 3000 *rpm* for 5 min 0.5 *mL* of Nessler's reagent and 4.3 *mL* of distilled water were mixed with 0.2 *mL* of supernatant and the tubes were incubated for 10 min at 28 °C. the liberated ammonia was determined by a spectroscopic method where Ammonium sulfate was used as standard.

A. Statistical Analysis:

Assays were performed in triplicates, and they were shown as mean + SD. The findings were put to the test using one-way analysis of variance (ANOVA), which was utilized to identify differences that are significant at $p < 0.05$.

3. RESULTS

3.1 Identification of Endophytic Fungi

From the leaf, root, and stem of *Acanthus ilicifolius*, four endophytic fungi with distinct morphologies were effectively isolated and purified. To confirm the identification of the isolates all four isolates were subjected to ITS sequence analysis. All the identified isolates belonged to different taxa: *Aspergillus terreus* ATCC 1012, *Fusarium multiceps* NRRL 43639, *Colletotrichum cobbitense* BRIP 66219, and *Colletotrichum xishuangbannaense* strain MFLUCC 190107. Table 1 lists the fungal isolates that were obtained from this study along with the best BLAST results. The characteristics of spores of four isolates are shown in Fig. 1

3.2 Assessment of Phenolic Content Production

To determine the phenolic content of the isolated endophytic fungus in ethyl acetate and methanol extracts. The Folin-Ciocalteau method was used. Comparing methanolic extracts to ethyl acetate extracts, a higher phenol concentration was detected. Overall, the methanolic extracts of all four isolates indicated the presence of high phenolic content when compared to ethyl acetate. The maximum phenolic content was found in methanolic extracts of *Aspergillus terreus* 1633.3±8.7 *µg* and *Fusarium multiceps* 737.3+6.8 *µg* equivalent to gallic acid, and methanolic extract of *Colletotrichum xishuangbannaense* and *Colletotrichum cobbitense* showed a phenolic content of 718.7±4.2 and 289.3+3.1 *µg* equivalent to gallic acid respectively (Fig. 2)

Table 1. BLAST analysis of ITS sequences from fungi isolated from *Acanthus ilicifolius*

Gene bank accession number	Isolate code	Identified species	% similarity
ON668122	BPAL2	*Aspergillus terreus* ATCC 1012	100
ON668123	GAIS9	*Fusarium multiceps* NRRL 43639	99.81
ON668124	GAIS10	*Colletotrichum cobbitense* BRIP 66219	99.03
ON668125	BPAL4	*Colletotrichum xishuangbannaense strain* MFLUCC 190107	99.37

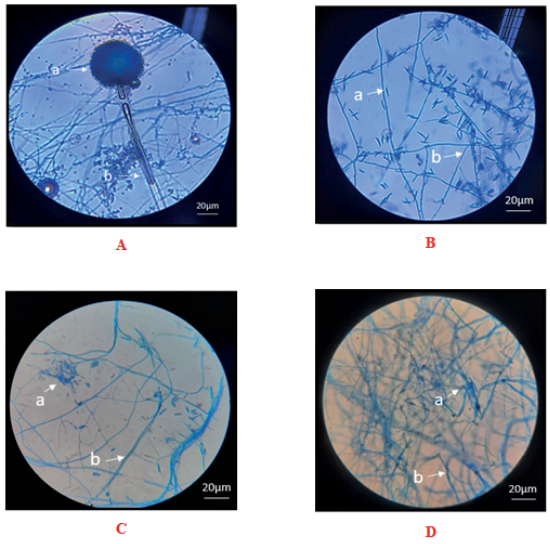

Figure 1. Microscopic examination of isolated fungal endophytes.

3.3 Determination of Reducing Power

A molecule's antioxidant properties and reducing power are closely related, and it was determined by measuring the reduction of Fe^{3+} to Fe^{2+}. The amount of reduction was estimated by measuring the amount of formation of Fe^{2+} ions by spectrometric analysis. An increase in absorbance indicated higher reducing power of extracts. It was found that overall, methanol extracts have a higher reducing property as compared to ethyl acetate extracts (Fig. 3). The maximum reducing potential activity was exhibited by methanol extracts of *Aspergillus terreus* followed by that of *Fusarium multiceps*.

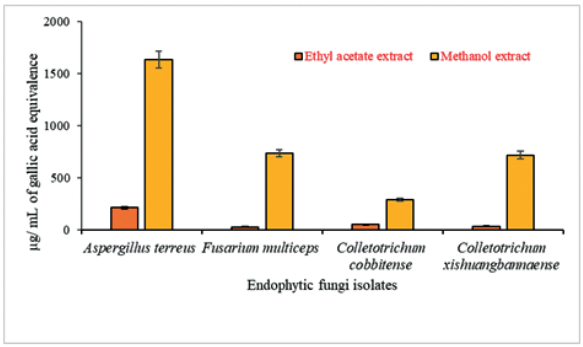

Figure 2. Total phenolic content values of ethyl acetate and methanol extracts. The values are represented as Mean ± SD. Statistical analysis was performed using one- way ANOVA. p<0.05.

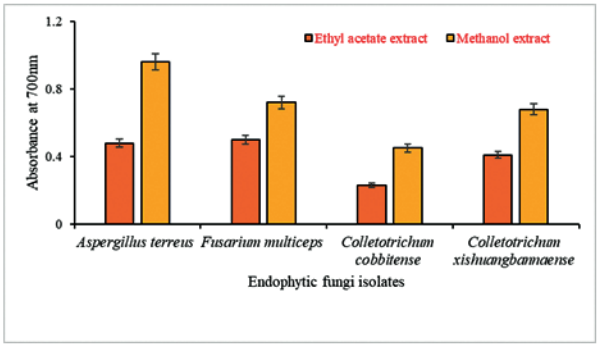

Figure 3. Reducing power activity of ethyl acetate and methanol extracts. The values are represented as Mean ± SD. Statistical analysis was performed using one-way ANOVA. p<0.05.

3.4 Free Radical Scavenging Assay (DPPH ASSAY)

The DPPH method is one of the most preferred methods to determine the free radical scavenging activity.[19] The highest percentage scavenging activity (% SCA) of 78.2±4.5 % was found in the methanolic extract of isolate *Colletotrichum xishuangbannaense* followed by ethyl acetate extract of *Fusarium multiceps* of 54.36±3.8 %. methanol extracts showed a higher % SCA compared to ethyl acetate extracts in most of the cases. Ascorbic acid as a standard showed 95.4 % of scavenging activity. Table 2 summarises the % SCA of both extracts of all four isolates.

3.5 Primary Screening and Estimation of *L-asparaginase* Activity

Primary screening of *L-asparaginase*-producing fungi could be distinguished by the pink zone that developed around the colony. The media's pH increased due to the production of ammonia due to the breakdown of *L-asparagine* to ammonia by the enzyme, and phenol red, a pH indicator, was used to measure this change. *Aspergillus terreus, Fusarium multiceps, and Colletotrichum cobbitense* were found positive for *L-asparaginase* production while *Colletotrichum xishuangbannaense* showed no enzyme production (supplementary data). The enzyme activity was determined by Wriston's method, and the activity was estimated using a linear equation based on the calibration curve of ammonium sulphate. *Aspergillus terreus* showed the highest enzyme activity of 50.1 *U/mL*, followed by *Colletotrichum cobbitense* (48.1 *U/mL*) and *Fusarium multiceps* (47.7 *U/mL*).

Table 2. DPPH scavenging activity of ethyl acetate and methanol extracts

Isolate	Ethyl Acetate Extract (% SCA)	Methanol Extract (% SCA)
Aspergillus terreus	11.15 +1.48	23.8 + 0.33
Fusarium multiceps	54.36 + 3.8	26.83 + 0.95
Colletotrichum cobbitense	3.21 + 0.68	21.54 + 1.08
Colletotrichum xishuangbannaense	42.90 + 0.56	78.2 + 4.5

4. DISCUSSION

Endophytic organisms associated with mangrove vegetation contribute to the medical world in the form of sources of antibiotics, anti-bacterial, inhibitors of iNOS activity, and anti-cancer drugs across the world.[20–22] These studies covered the mangrove flora of *Acanthus ilicifolius*, *Sonnertia, Rhizophora*, and *Avicinnea* species. The endophytic bacteria and endophytic fungi of mangroves' phytal population indicated their overall activity against *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa*.[21,22] The endophytic fungi that expressed *L-asparaginase* activity include *Aspergillus sp.* from *Avicennia germinans sp.*[20] Similar results were observed in another study conducted on *Rhizophora mucronata*-associated *Lysinibacillus fusiformis* B27.[20] However, the *L-asparaginase* activity of the endophytic fungi was studied qualitatively and required further studies.

Acanthus ilicifolius is traditionally used for treating human ailments, as it contains important bioactive compounds like triterpenoids, phenolic compounds, terpenoids, etc.[2,3,23] Studies on the occurrence of endophytes are conducted on temperate-zone plants rather than tropical-zone plants. and an attempt has been made in the tropical zones of Maharashtra, India. Mangrove plants usually harbor endophytes which are reservoirs of biologically active compounds.

Four endophytic fungal sp. isolated from *Acanthus ilicifolius* indicated antioxidant activity and extracellular production of the medically important enzyme *L-asparaginase* during the current study. *Aspergillus terreus* and *Fusarium multiceps* showed good antioxidant activity (Fig. 3) and the methanolic extract of *Colletotrichum xishuangbannaense* showed the highest DPPH activity (Table 2) among the four isolates, *Aspergillus terreus* had highest phenolic content and maximum antioxidant property which is in the line of the fact that phenolic content is directly proportional to antioxidant property. The antioxidant assays of methanol extracts showed significantly higher

activities ($p < 0.05$) as compared to ethyl acetate extracts (Fig. 2 and 3) indicating polar solvents as ideal solvent systems for the maximal extraction of bioactive compounds from the fermented broth.[24] Fungal isolates from different plant tissues showed a significant difference in their antioxidant activities ($p < 0.05$) indicating that isolates from plant parts are the reservoir of biologically active metabolites and underline their potential possibility of other antioxidant activities.

Previous studies indicated two endophytic fungal species *Aspergillus sp.* from *Avicennia germinans sp*.[25] and an isolate from *Rhizophora mucronata*[26] that expressed *L-asparaginase* activity qualitatively. *Aspergillus terreus*, *Fusarium multiceps, Colletotrichum cobbitense* and *Colletotrichum xishuangbannaense* from *Acanthus ilicifolius* indicate these endophytes as important candidates and good alternative sources of enzyme production[17]. A high enzyme activity of 50.1 *U/mL* was noted in the crude extract of *Aspergillus terreus* and, employing better methods of purification would further increase the efficiency of this fungal enzyme.

5. CONCLUSIONS

Endophytic fungal isolates associated with tissues of *Acanthus ilicifolius* differed significantly in their morphological and microscopic characteristics, and their enzymatic potential. According to the current investigation, methanolic crude extracts of these isolate-growing media had higher antioxidant activity than those made of ethyl acetate. *Aspergillus terreus* possessed the maximum *L-asparaginase* activity *viz*., 50.1 (*U/mL*). The antioxidant activity and *L-asparaginase* production were differently expressed by the endophytic fungi in all four isolates indicating the importance of the diversity of composition of these fungi in *Acanthus ilicifolius*. Due to the limited availability of potent *L-asparaginase*-producing microbes, the mangrove-associated fungal endophyte isolates like *Aspergillus terreus*, *Colletotrichum cobbitense,* and *Fusarium multiceps* can be considered promising sources of biologically active compounds.

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In the current investigation, he was involved in the conceptualization of the work, literature search, experimentation, data acquisition and manuscript writing.

Dr Jayaprada Rao Chunduri is an Assistant professor and HoD, Biotechnology at SVKM's Mithibai College, Mumbai. She is a university recognised PhD guide for Biotechnology. In the present study, she has contributed to the design of the work, data analysis and manuscript editing.