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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF BIOLOGICALLY ACTIVE ENDOPHYTIC FUNGI ISOLATED FROM *DILLENIA INDICA* L.

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Abstract

This study reported the broad spectrum endophytic variety from the elephant apple *Dillenia indica* L. Endophytes are microorganisms lying within the plant interior tissues, lasting as the whole or part of their life cycle without causing any conspicuous symptoms of infection to host plants. Surface sterilization of leaves and stems was the basic doing work to isolate endophytic fungi. 16 isolates were identified and grouped into 7 based on of morphological characteristics. Through morphological colony, all the isolated strains were identified up to genus level. Isolated seven fungi, 6 from the leaves part and 1 from the bark part were subjected to sequence analysis of internal transcribed spacer (ITS) gene. Finally, seven well-known species named *Colletotrichum Siamense, Phomopsis liquidambaris, Diaporthe perseae, Fusarium incarnatum, Colletotrichum falcatum*, and *Lasiodiplodia theobromae* were identified compared with the Basic Local Alignment Search Tool (BLAST) results analysis. This study provides the broad theory of the interrelation of morphological and molecular homologies for the identification of prospective bioactive fungi for further study and experiment so that those fungus acts as a catalyst for novel thinking and the discovery of drug molecules for the welfare of mankind.

Introduction

Endophytes play an important role in plant growth and can produce bioactive compounds which contribute an enormous application in biotechnology, pharmaceutical and agrochemical industries. About 80% of people in developing countries use medicines derived from medicinal plants. They are a rich source of natural products and are extremely valuable for the prevention of diseases and ailments (Yirga *et al*., 2011; Pan *et al*., 2013). Medicinal plants have different compounds that have been utilized as an essential resource of medicinal products and can be used in the pharmaceutical industry in anticancer agents, contraceptives, analgesics, antibiotics, diuretics, laxatives, etc.

Dillenia indica (Elephant apple) belongs to the family Dilleniaceae. It is a large, knobby fruit with acidic flavored. Recently, scientists have given attention to this plant for its various biological activities including anti-cancer and anti-diabetic properties. The leaf, bark, and fruit of the plant are used in the indigenous system of medicine. It relieves abdominal pain and regulates the heat in the body. The fungal endophytes from this plant also play an important role in treating various diseases.

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The actual number of isolated fungi is still unknown. Maheswari and Komalavalli (2013) recommended only 5-13 % of the overall evaluated worldwide. Blackwell (2011) focused, the isolation, identification and characterization of fungi from different environmental sources are still much needed for the viewing and recognizing of more species, editing scientific classification, evaluating their effects in nature and supplying strains for ecological remediation, biological control and industrial aspects.

Landeweert (2003) represents molecular identification techniques based on total fungal DNA extraction provide a unique barcode for the determination and identification of different fungal isolates up to a species level. Molecular identification using this barcode has turned into a vital tool for mycologists studying fungal taxonomy, molecular evolution, population genetics or fungus-plant interactions (Moller *et al.*, 1992). The identification of fungi using molecular techniques is carried out by the sequencing of PCR amplified part of rRNA genes with universal primers to fungal species (Monod *et al*., 2006).

Materials and Methods

Collection of plant sample

The plant samples were collected from the Vanga Upazila, Faridpur district during autumn and winter seasons between October, 2019 and February, 2020 when the tree filled with new leaves and fruits. The fungal endophytes were isolated from the leaves, bark and fruit parts through a surface sterilization method described by Qadri *et al*. (2013). The study was carried out in Pharmaceutical Sciences and Research Division (PSRD), located at Bangladesh Council of Science and Industrial Research (BCSIR) laboratories, Dhaka, Bangladesh. All the research work was done under aseptic conditions.

Media preparation

Water Agar Medium (HiMedia Laboratories Pvt. Ltd) was used for the inoculation and Potato Dextrose Agar (PDA) (Titan Biotech Ltd) was used for the isolation of endophytic fungi and prepared them as per the manufacturer's instruction written on the jar.

Isolation of endophytes:

Sample preparation: Fungal isolation followed the method of Hallman et al. (2007), with modifications. Leaf, bark, and root samples were washed with tap water (Qadri *et al.*, 2013), sterilized using 70% ethanol, 1.3M sodium hypochlorite, and 70% ethanol, then rinsed with distilled water and dried on sterile filter paper. The sterilized samples were inoculated onto water agar containing streptomycin, using four sections per plate, and incubated at 28 ± 2°C in darkness for 4–6 weeks. Emerging mycelia were transferred to Potato Dextrose Agar (PDA) for endophyte isolation and compared to exophytes from unsterilized samples incubated under identical conditions (Abraham *et al.*, 2015).

Identification of isolated fungal endophytes

Morphological identification: The fungus was identified according to their colony morphology, filamentous structure and spore characteristics. Through morphological identification the selected fungus was identified as their genus level. All the microscopic study was done under the method of Lactophenol Cotton Blue staining method (Shamly *et al*., 2014).

Molecular identification: *DNA extraction and PCR (Polymerase Chain Reaction) amplification:* Genomic DNA was extracted from one-week-old PDA fungal cultures using the DNeasy Plant Mini Kit (QIAGEN, USA). Species-level identification was performed using PCR

amplification of ribosomal internal transcribed spacer (ITS) regions with primers ITS4 and ITS5. The PCR products were purified using the QIAquick PCR Purification Kit (Bao *et al.*, 2012).

*Sequence and analysis:*The obtained PCR products were prepared for sequencing and then the sequences were compared with the other related sequences using BLAST search in Gen Bank (NCBI) (Landeweert *et al*., 2003).

Preliminary Chemical Screening: TLC Method

Thin layer chromatography (TLC) was performed using pre-coated silica gel plates (Macherey-Nagel, Germany) and a solvent mixture of 20% ethyl acetate in toluene. Extracts (1% solution) were applied, and spots were visualized under UV light at 254 and 365 nm, followed by staining with 1% vanillin-sulfuric acid and heating at 110°C (Sohrab *et al.*, 2004).

Biological Assay of Isolated Fungus

Antimicrobial screening: The antimicrobial potential of fungal extracts was evaluated using the disc diffusion method (Bauer, 1966) against four pathogenic bacteria (*Escherichia coli*, *Bacillus megaterium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*) and two fungi (*Aspergillus niger* and *Aspergillus flavus*). Bacterial suspensions (~10^8 CFU/ml) and fungal strains were cultured on Nutrient Agar (NA) and Potato Dextrose Agar (PDA), respectively, at room temperature for 24 hours. Zones of inhibition were measured after 24 hours of incubation at 37°C, using kanamycin (30 µg/disc) and ketoconazole (30 µg/disc) as positive controls and solvent discs as negative controls.

Antioxidant Activity

The antioxidant activity of fungal extracts was determined using DPPH free radical scavenging (Brand-Williams *et al*., 1995). Extracts were serially diluted (0.78–200 µg/ml) in methanol and mixed with DPPH solution (20 µg/ml). The reduction of violet DPPH to yellow diphenylpicryl hydrazine was measured at 517 nm. IC50 values were calculated using regression analysis. Butylated hydroxyanisole (BHA), ascorbic acid, and Trolox served as positive controls, with methanol as the negative control.

Results and Discussion

Identification of endophytic fungi

A total of 7 endophytes were isolated from the bark and leaf part of *Dillenia indica* plant (Fig. 1). The endophytes isolated from the leaves were named as DILE-1, DILE-2, DILE-3, DILE-4, DILE-5, DILE-6 and the endophyte isolated from the bark was named as DIBE-1. All the isolated endophytes were identified according to their morphological and molecular characteristics. From morphological identification, the fungus was identified at the genus level and from molecular identification the fungus was identified at the species level.

Morphological identification:

Based on obtained morphological characteristics from 3, 6, 9 and 12 days observation of the fungal growth on PDA media and the fungus was characterised according the Fig. 2.

All the endophytic fungi were identified according to their genus level (Table 1) such as the strains DILE-1, DILE-2 and DILE-6 were identified as *Colletrotrichum* sp., DILE-3 as *Phomopsis* sp., DILE-4 as *Diaporthe* sp., DILE-5 as *Fusarium* sp. and DIBE identified as *Lasiodiplodia* sp. respectively. Both the macroscopic and microscopic views of all the endophytic fungi are described Table 1.

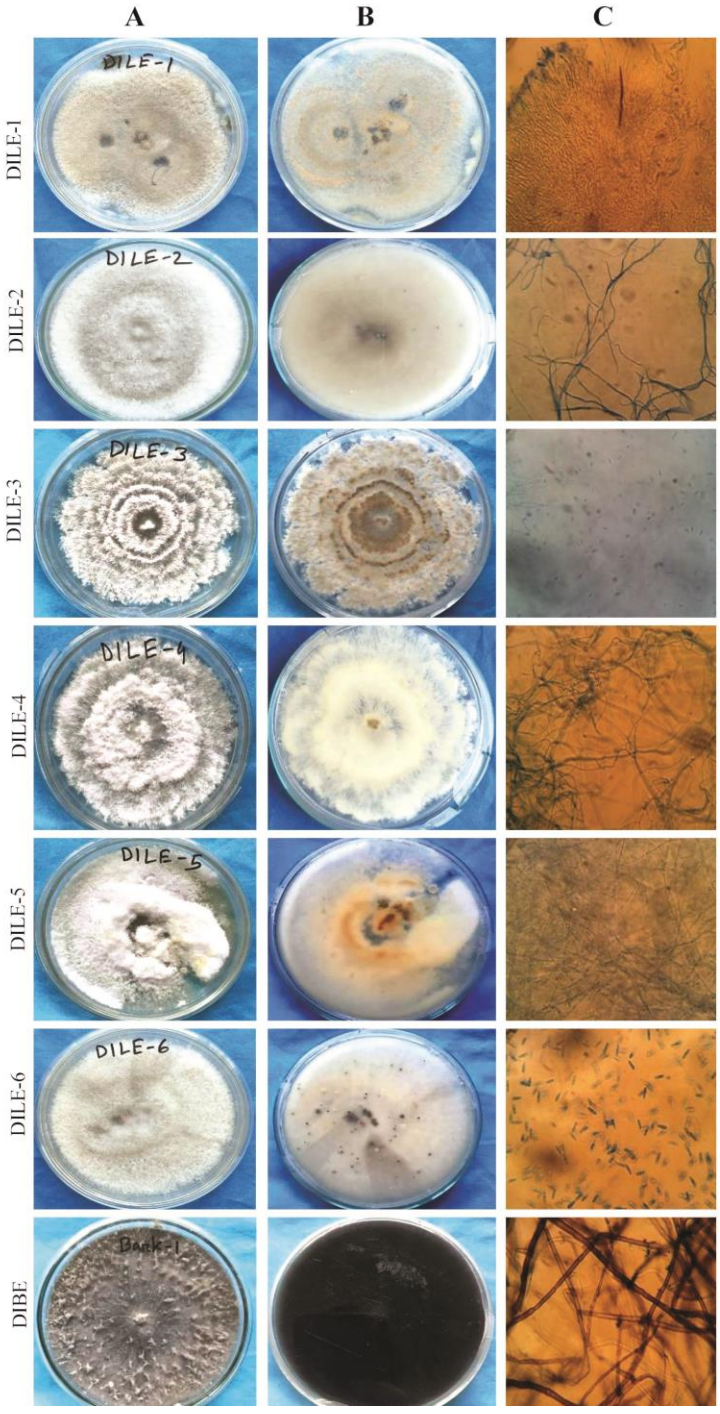

Fig. 1. Isolated endophytic fungi from *Dillenia indica*. a) Front view b) Back view c) Microscopic image.

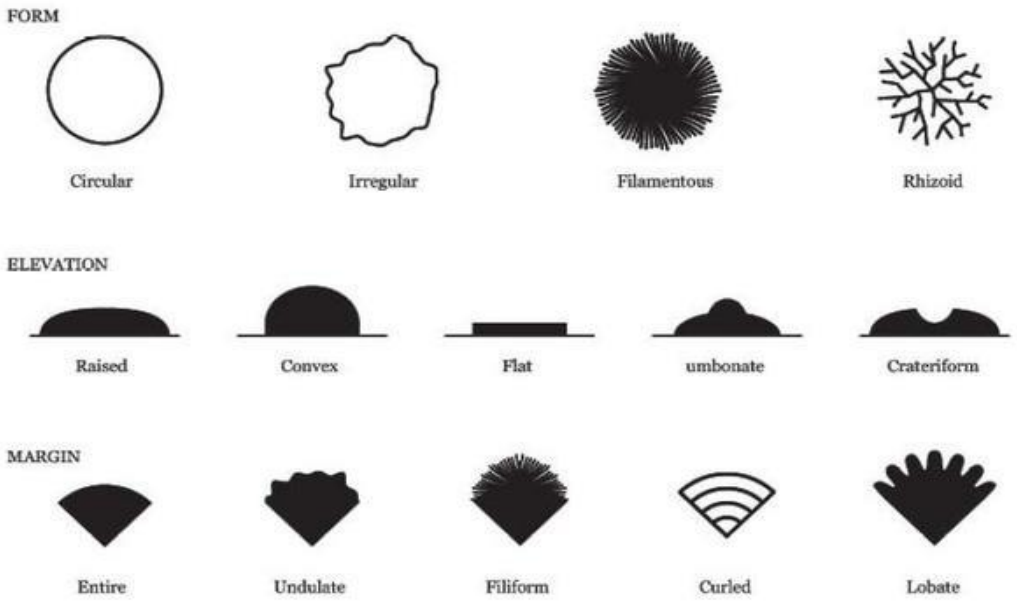

Fig. 2. Identified fungus characterized according to form, elevation and margin.

Table 1. Morphological characteristics of identified fungus.

Strain	Morphological characteristics	Identified genus
DILE-1 DILE-2 DILE-6	Macroscopic view- upper view: white with wooly texture, bottom color: same as top color, hyphae: surficial, growth rate: Moderate and morphology of colony: irregular Microscopic view-hyaline cylindrical conidia	*Colletrotrichum* sp.
DILE-3	Macroscopic view- upper view: pure white with wooly texture, bottom color: same as top color. hyphae: surficial, growth rate: slow and morphology of colony: Filamentous Microscopic view- filiform conidia or slightly curved at one end.	*Phomopsis* sp.
DILE-4	Macroscopic view- upper view: pure white with wooly texture, bottom color: same as top color. hyphae: surficial, growth rate: slow and morphology of colony: Entire Microscopic view- conidial morphology alpha or beta.	*Diaporthe* sp.
DILE-5	Macroscopic view-upper view: yellowish color with wooly texture, bottom color: same as top color. hyphae: surficial, growth rate: moderate and morphology of the colony: circular Microscopic view-hook shaped macroconidia	*Fusarium* sp.
DIBE	Macroscopic view-upper view:Ash color with wooly texture, bottom view: black color, hyphae: surficial, growth rate: rapid and morphology of colony: irregular Microscopic view-initially the conidia was hyaline and aseptate and became brown and one septate with age.	*Lasiodiplodia* sp.

Molecular identification

A total of 7 fungi isolated from the plant *Dillinea indica* were identified at their species level (Table 2) through molecular identification which includes DNA sequencing and NCBI Gene Bank database.

Table 2**.** Blast result analysi**s showing matched sequences with coverage and maximum identity assay.**

Fungal Internal strain no.	Morphological Identification	One of top BLAST match sequences		
		References accession no.	Coverage	Maxident
DILE-1	*Colletotrichum* sp.	*Colletotrichum Siamense* MT434660.1	100%	99.66%
DILE-2	*Colletotrichum* sp.	*Colletotrichum Siamense* MT450691.1	98%	98.12%
DILE-3	*Phomopsis* sp.	*Phomopsis liquidambaris* FJ478124.1	99%	98.44%
DILE-4	*Diaporthe* sp	*Diaporthe perseae* KC343173.1	99%	99.48%
DILE-5	*Fusarium* sp.	*Fusarium incarnatum* MN882828.1	99%	99.45%
DILE-6	*Colletotrichum* sp.	*Colletotrichum falcatum* MW301214.1	95%	99.82%
DIBE	*Lasiodeplodia* sp	*Lasiodiplodia theobromae* Mk929514.1	98%	99.29%

TLC screening

Prior to initial screening using the Thin Layer Chromatography (TLC) method, each isolated fungal extract was progressively arranged onto a TLC plate by placing a single spot on it. Figure 3 displays all the obtained results. Following solvent treatment, each extract shows distinct colored spots in different places. TLC spots of fungal crude extracts showed the presence of secondary metabolites like sterols, terpenoids, flavonoids, isocoumarins, anthocyanins, anthraquinones, and naphthoquinones or their derivatives (Sohrab *et al*., 2004; Krohn *et al*., 2004; Khan *et al*., 2018; Mahmud *et al*., 2020). All extracts were screened visually, under UV light (254 and 365 nm), and after being sprayed with a vanillin-H2SO4 spray reagent (Table 3).

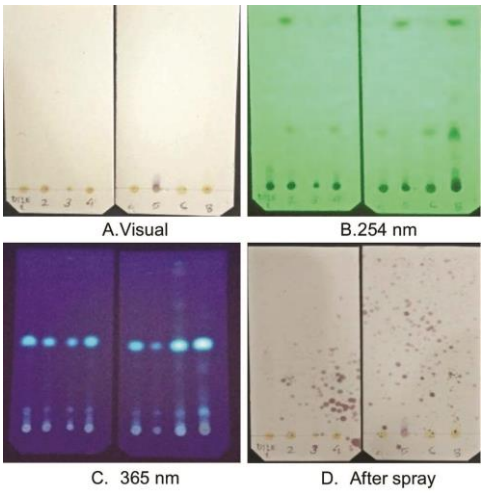

Fig. 3. TLC Screening of the fungal extracts (1=DILE-1, 2=DILE-2, 3=DILE-3, 4=DILE=4, 5=DIlE-5, 6=DILE-6 and B=DIBE) by A) visual observation, B) under UV at 254 nm, C) under UV at 365 nm, D) After spray.

Table 3. Chemical Screening of fungal extract by Thin Layer Chromatography.

Internal strain no.	Identified fungus	Visual obser-vation	Visibility under UV light (254 nm)	Visibility under UV light (365 nm)	Visibility after spray	Prospective compounds
DILE-1	*Colletotrichum Siamense*		Dark quenching	Blue Greenish yellow	Dark purple	Steroids, Terpenoids, Coumarin, Isocoumarin or their derivatives
DILE-2	*Colletotrichum Siamense*		Dark quenching Blue quenching	Greenish yellow	Pink purple	Steroids, flavonoids, Coumarin, Isocoumarin or their derivatives
DILE-3	*Phomopsis liquidambaris*		Light quenching Blue quenching	Blue Sky blue	Dark purple	Coumarin, Isocoumarin Steroids, Terpenoids,
DILE-4	*Diaporthe perseae*		Dark quenching	Blue	Dark purple	Terpenoids, Steroids
DILE-5	*Fusarium incarnatum*		Light quenching Blue quenching	Sky Blue Red Light Purple	Dark purple Magenta	Coumarins Anthocyanins Terpenoids Steroids
DILE-6	*Colletotrichum falcatum*		Dark quenching	Blue	Dark Purple	Coumarins Anthocyanins Terpenoids Steroids
DIBE	*Lasiodiplodia theobromae*	Light yellow	Dark quenching Blue quenching	Blue Sky blue Purple	Dark purple Bluish purple Pink purple	Terpenoids, Steroid, Anthocyanins Coumarin, Isocoumarin or their derivatives

Bioactivity screening

Evaluation of antimicrobial activity

In determining antimicrobial activity, among 7 fungal endophytes, 3 fungal strains like DILE-4 (*Diaporthe perseae*), DILE-5 (*Fusarium incarnatum*) and DILE-6 (*Colletotrichum falcatum)* were showed moderate inhibitory effect on four pathogenic bacteria (Table 4). On the other hand, the fungal strain DIBE showed the highest inhibitory activity against four pathogenic bacteria like *S. typhi* (16mm*), S.aurius* (15mm), *E.coli* (18mm) and *B. megaterium* (16mm). In case of the activity against fungus, all seven fungal strains showed lowest activity (zones<8 mm).

Table 4. Antimicrobial activity of fungal strains.

Bacterial/fungal strain	Diameter of zone of inhibition (mm)								
	DILE-1 100 µg/disc	DILE-2 100 µg/disc	DILE-3 100 µg/disc	DILE-4 100 µg/disc	DILE-5 100 µg/disc	DILE-6 100 µg/disc	DIBE 100 µg/disc	Kanamycin (30µg/disc)	Ketoconazole (30µg/disc)
Gram-positive bacteria									
Staphylococcus aureus	11	**7**	8	12	13	11	15	30	nd
Bacillus megaterium	8	8	7	12	12	15	16	28	nd
Gram-negative bacteria									
Escherichia coli	11	9	12	13	15	12	18	30	nd
Pseudomonas aeruginosa	9	8	8	15	14	15	16	30	nd
Fungal strain									
A.flavus	---	---	---	---	---	---	---	nd	40
A.niger	---	---	---	---	---	---	---	nd	35

'---' Indicates no sensitivity, 'nd' Not done.

Evaluation of antioxidant activity

All the fungal strains showed different free radical scavenging activity compared to the standard as shown the Fig. 4. In comparison with standard, fungal strains DILE-4 and DILE-5 showed the most prominent activity as 12.27 µg/ml and 15.64 µg/ml respectively. On the other hand, DIBE and DILE-6 also showed moderate antioxidant activity in comparison with the standard.

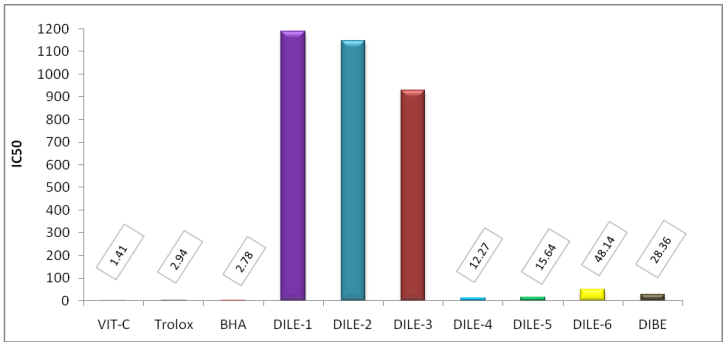

Fig. 4. Free radical scavenging activity of isolated fungal strains of *Dillenia indica*.

Discussion

This investigation was carried out to identify the fungi isolated from the leaves and bark sections of *Dillenia indica* using a variety of morphological and molecular evaluation techniques. Total of seven fungal strains were isolated from the plant parts like *Colletotrichum Siamense, Phomopsis liquidambaris, Diaporthe perseae, Fusarium incarnatum, Colletotrichum falcatum*, *Lasiodiplodia theobromae.* All the fungal strains were isolated and identified their genus level through morphological views as their growth pattern, colony appearance, texture, diameter etc. and molecular view using DNA sequencing analysis and blast search results. After identification, all the fungal strains were selected for preliminary bioactivity studies through small scale cultivation in PDA medium. All of the fungal strains initially represent the potentially intriguing spots on the TLC plate in the Thin Layer Chromatography procedure. The spots in various positions suggest that the fungal strains may contain substances such as anthraquinones, naphthoquinones, anthocyanins (Khan *et al*., 2018), terpenoids, steroids, flavonoids (Sohrab *et al*., 2004), isocoumarins (Krohn *et al*., 2004) and their derivatives (Mahmud *et al*., 2020). As per TLC analysis, the fungal strain DIBE (*Lasiodiplodia theobromae*) may be prioritized over all other fungal strains for further research since it may contain unique chemical compounds.

Ketoconazole and kanamycin were employed as standards in the assessment of antimicrobial research to inhibit the proliferation of bacteria and fungi, respectively. DILE-4, DILE-5, and DILE-6 demonstrated a slight inhibitory effect against four pathogenic bacteria out of all the fungal strains. Conversely, the fungal strain DIBE, isolated from *Dillenia indica* bark, revealed increased restrictive activities. In DPPH free radical scavenging activity, DILE-4 and DILE-5 exhibited most prominent activity and the fungal strains DILE-6 and DIBE shown moderate activity in comparison with the standard. From the bioactivity study of the above fungal strain, it can be mentioned that these strains can be a huge resources for antimicrobial and antioxidant products and plays important role for further research.

Huge spreading of world population leads to increase in health problems of humans, animals, and plants and increased resistance of pathogens toward drugs. Transmittable diseases are worldwide health challenges because of drug resistance to pathogens. Nowaday's researchers focus on inventing new or novel compounds from natural resources. Because of the huge chance

of getting new compounds, endophytic fungi are attractive topics for pharmacists, scientists and researchers. Endophytic fungi also have the ability to provide beneficial contribution to human by production of bioactive compounds application in pharmacy. In a literature survey we found that as a plant, *Dillenia indica* was a precious medicinal plant and several kinds of compound isolated from the plant which plays an important role in treating different diseases. In this research study initially we found seven characteristics fungal strains from the plant *Dillenia indica*. These also play a major role in the recovery of infectious, inflammatory and also certain kinds of certain known or unknown diseases. So, mass research is essential in the area of endophytic fungi isolation and then compound isolation and characterization of the compounds from *Dillenia indica*.

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