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Bioactive Secondary Metabolites of Endophytic fungus *Alternaria brassicae* (KUMBMDBT-29) from *Phoenix sylvestris*

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ABSTRACT
The Phoenix sylvestris plant, also called the Indian date palm, has been found to be useful in the treatment of a number of different ailments, according to traditional medicine. Despite the fact that the fungi known as "endophytes" are completely harmless to the plants they inhabit, there has been a growing interest in them recently due to the fact that they can create bioactive compounds with potential medical and industrial uses. Nucleotide sequencing of the ITS region of the 18S rRNA gene confirmed that an endophytic fungus strain isolated from a Phoenix sylvestris leaf segment was indeed Alternaria brassicae - KUMBMDBT-29. (NCBI accession number MW007917). Analysis of secondary compounds in an ethyl acetate extract of submerged-fermented Alternaria brassicae. The GC-MS results for the extract show that it contains 41 bioactive molecules of secondary metabolites. Alternaria brassicae, an endophytic fungus isolated from Phoenix sylvestris, is, in conclusion, a potential source for bioactive compounds.
Keywords: Phoenix sylvestris, Alternaria brassicae, Fungus extract,GC-MS analysis, Secondary metabolites

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INTRODUCTION

Medicinal plants comprise a country valuable natural wealth and contribute significantly to its health economy. They serve as essential therapeutic agents in the production of traditional medicines and play a crucial role in providing primary health care services to rural areas. In Bangladesh, *Phoenix sylvestris* is locally known as 'Khejur' and has a variety of medicinal applications[1]. *Phoenix* L. is a Phoenicoid palm of the Arecaceae family with 17 species found in a variety of habitats, including marshes, deserts, and mangrove shorelines. The majority of *Phoenix* species are native to semi-arid regions, but they are typically found near rivers or springs. The genus *Phoenix* is the only member of the subfamily Coryphoideae to possess pinnate compound leaves[2, 3].In India and Indonesia, *P. sylvestris* sugars are recognized as more nutritious than cane sugar. *P. sylvestris* has been considered to be a traditional treatment for a variety of ailments, including abdominal complaints, fevers, and loss of consciousness, constipation, and heart problems. The laxative, nutritive, and cooling fluid of the plant is used to treat gonorrhea, while the tender core of the plant is used to treat gonorrhea. The plant root is effective for treating neuralgia, nervous debility, and helminthiasis [4].

Endophytic fungi are found all over the world and associate with their host plant [5, 6, 7, 8, 9, 10, and 11].A majority of species of plants investigated to date consist of endophytic fungi in the asymptomatic aerial tissues of terrestrial plant communities [12, 13, 14, 15, 16, and 17]. Endophytic fungi are a prospective source of secondary metabolites, enzymes, peptides, and health-promoting substances utilized in medicine, pharmaceuticals, and plant growth enhancement [5, 18, and 19]. In addition to heavy secondary metabolite molecules such as alkaloids, flavonoids, and peptides, endophytic fungi yield volatile compounds such as organic acids, organic ester, and alcohols that can be measured using GC-MS apparatus [20].

Endophytic fungi extract has been isolated for GC-MS study of secondary metabolites in several studies. Antimicrobial, antioxidant, anticancer, antiviral, and antimalarial bioactive compounds are found in fungal endophytes. These activities are ascribed to effective secondary metabolites like alkaloids, phenols, steroids, terpenoids, saponins, glycosides, tannins, and flavonoids. Antimicrobial, antioxidant, antiviral,

anticancer, and phytotoxic properties are found in endophytic *Alternaria* spp[21, 22, 23, 24, 25, 26, 27].Endophytic *Alternaria alternata* was isolated from *Ziziphusspina-christi*, and GC–MS analysis of its ethyl acetate crude extract revealed many bioactive substances [28].Endophytic *Alternaria* spp. are antibacterial, antioxidant, antiviral, anticancer, and phytotoxic activities [26, 27]. The endophytic fungus *Trichoderma atroviride* from healthy *Colquhouniacoccineavar* flowers had strong antimicrobial action [29]. The purpose of this research was to obtain a pure culture of the endophytic fungus *Alternaria brassicae* -KUMBMDBT-29 from a *Phoenix sylvestris* leaf. This fungus is a member of the order Ascomycota and the class Dothideomycetes. GC-MS research has identified secondary bioactive metabolites in the ethyl acetate extract of the fungus.

MATERIAL AND METHODS

Isolation and Identification of the endophytic fungus

The medicinal plant *Phoenix sylvestris* was collected in the Chitradurga district of Karnataka, INDIA. [30]Detailed surface sterilization of collected samples. To remove adhered detritus, the plant sample was thoroughly washed in running water for five minutes. After sterilizing the surface with 70% ethanol for 60 seconds, sodium hypochlorite for 3 minutes, and lastly 75% ethanol for 30 seconds, autoclaved distilled water was used to rinse the surface sterilized sample three times before drying in laminar airflow. Plant leaf were cut into small pieces and put in Petri dishes with potato dextrose agar. (PDA, Himedia Pvt. Ltd. INDIA). Isolated endophytic fungus was subcultured on potato dextrose agar plates (PDA) and incubated at 28°C for 4 to 7 days to produce pure culture. Following isolation, the fungus culture was recognized to the genus level using microscopic and cultural characteristics. This culture availability was then refrigerated at 4°C for future use.

Molecular characterization of the endophytic fungus

Genomic DNA was isolated using the Chromous Biotech gDNAminispin kit and CTAB method[31]. Fungal ITS regions were sequenced. ITS1-5′-CCGTAGGTGAACCTGCGG-3′ (forward primer) and ITS4-5′-TCCTCCGCTTATTGATATGC-3′ (reverse primer) amplified the endophytic fungus genome ITS region. A CG palm cycler was used to amplify 50 ng of DNA template, 1.5 mM MgCl2, 0.4 U Taq DNA polymerase, 200 M dNTP, and 20 pmol/L of each primer. The amplification cycles were changed to five minutes at 94 °C for early denaturation, thirty cycles for synthesis, one minute at 55 °C, one minute at 72 °C, and seven minutes for final extension. Ethidium bromide was used to strain the amplified product on 1.2 % w/v Agarose gel electrophoresis to confirm the band. Gel documentation and gel extraction purified the PCR result. BLAST was used to analyze the ITS sequences.

Biomass preparation of endophytic fungus

Submerged fermentation was used to cultivate the endophytic fungus *Alternaria brassicae*. To prepare media, we modified the method outlined by Barkhade [32]. A complete colony of fungus was inoculated into 100 ml of potato dextrose broth for 7 to 14 days, and the container was incubated at 28 degrees Celsius. Following collection, filtrate was collected by filtering it through Whatman No. 1 filter paper. With an equal quantity of ethyl acetate, the metabolites in the culture filtrate were extracted. To derive the extract, the resulting residue was dried in a vacuum evaporator[33]. The extract was evaluated using GC-MS.

Gas-Chromatography mass spectrometry (GC-MS)

Gas-chromatography mass spectrometry detected secondary metabolites of endophytic fungus *Alternaria brassicae*. RtxR-5 was a 30 m column with a 0.25 mm inner diameter and film finish. The Whatman filter No.1 (0.2 m) filtered the sample. Split mode used helium gas (99.999%) at 1 ml/min. 1 µL of endophytic fungi *Alternaria brassicae* extract was injected to column at 280°C. After 2 min at 70°C, the oven was heated at 7°C/min to 320°C. Ion sources were 250°C. The detector scans 30–500 Da atomic units. 22.5 minutes, including a 3-minute solvent delay. The endophytic fungus *Alternaria brassicae* extract spectra were compared to the NIST05 library database[33].

RESULTS AND DISCUSSION

Isolation, identification and molecular characterization of endophytic fungus

The endophytic fungus was isolated from medicinal *Phoenix sylvestris*(Fig. 1, a, b). The purified endophytic fungus was whites color and had spore chain cell morphology. (Fig. c and d,). Fungus characterized through PCR amplification of the 18s rRNA gene using the ITS1 primer and ITS4 primer, then identified by Sangers dideoxy nucleotide sequence of the amplified ITS region. Blastn analysis showed substantial multiple sequence alignment and pairwise results that showed identity with *Alternaria brassicae*-KUMBMDBT-29 and was deposited in NCBI GenBank with isolate accession number (MW007917). MEGA-Version 7.0.14 can build a phylogenetic tree using the neighbor-joining (NJ) method with nucleotide pairwise variation adjustments from ABI sequencing files (Fig.2). Similar to our report,

Colletotrichum gloeosporioides (strain JGC-9) was isolated from medicinal plant *Justiciagendarussa* and tested for taxol production [34]. *Penicillium cinnamopurpureum* from medicinal *Curculigoorchioides*. Morphology and genetic analysis identified this fugus [35]. Endophytic fungus isolated from the ethnomedicinal plant *G. superba* L. *A.solani* and *P. funiculosum* using ITS sequences. The ITS section of fungal rDNA was amplified and sequenced, obtaining nucleotide sequences of 564 base pair. The obtained nucleotide sequences were submitted in NCBI GenBank. (Accession Number: HQ600978 and JN040730).The sequences closest homologues were chosen, and numerous sequence alignments were performed with the MEGA 5 software ClustalW program. The neighbor merging method was used to build the phylogenetic tree [36].

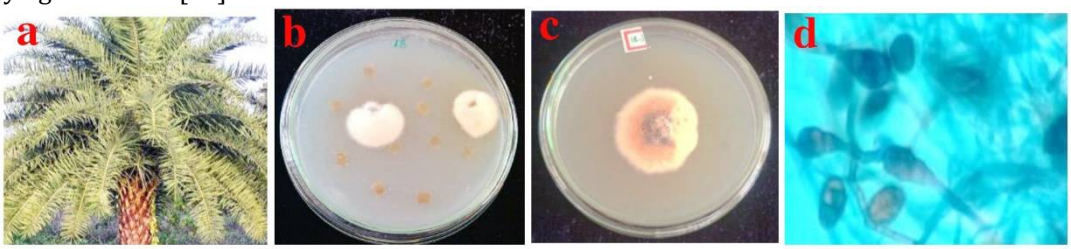

Fig.1. (a)*Phoenix sylvestris* (b) Endophytic fungus grown from surface sterilized leaf segment of *Phoenix sylvestris* on PDA media after 4 to 7 days (c) Colony morphology of *C. cladosporioides*,(d) Microscopic view of endophytic fungus *Alternaria brassicae*,

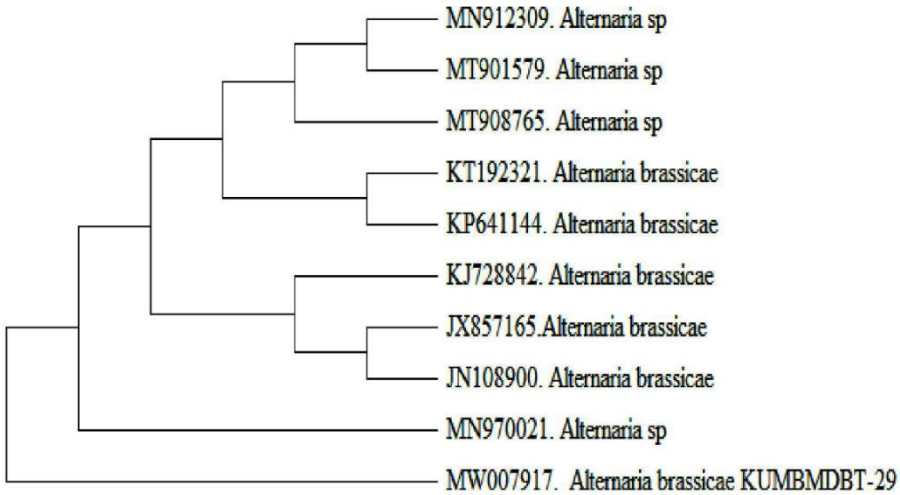

Fig.2. Phylogenetic analysis of *Alternaria brassicae* strains with ITS sequences of closely related fungal strains.

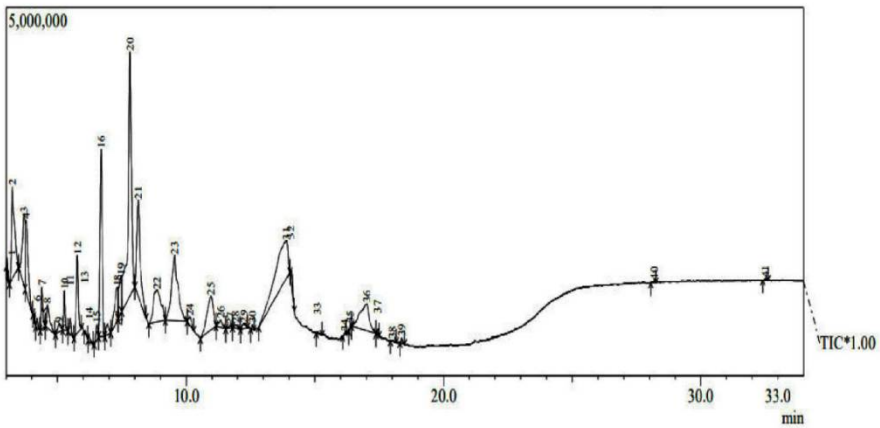

Fig. 3. GC-MS Chromatogram of ethyl acetate extract of endophytic fungus *Alternaria brassicae*.

Table. 1. List of identified major bioactive compounds of an ethyl acetate extract of the endophytic fungus *Alternaria brassicae*

Sl. No.	Name of the compound	Molecular Formula	Molecular Weight	Retention Time	Peak	Area %
01	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126g/mol	7.815	20	17.32
02	3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$	162g/mol	13.886	31	11.40
03	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144g/mol	6.699	16	8.68
04	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	$C_{12}H_{14}O_3$	206g/mol	9.546	23	8.57
05	1,2,3-Propanetriol, monoacetate	$C_5H_{10}O_4$	134g/mol	8.133	21	7.02
06	Propane, 1-(1-methylethoxy)-	$C_6H_{14}O$	102g/mol	3.243	2	5.99
07	Glucitol	$C_6H_{12}O_5$	164g/mol	17.011	36	5.18
08	Propanoic acid, 3-(acetyloxy)-2-(hydroxymethyl)-, ethyl ester, (+)	$C_8H_{14}O_5$	190g/mol	8.862	22	5.03
09	2-Formyl-9-[.beta.-d-ribofuranosyl]hypoxanthine	$C_{11}H_{12}N_4O_6$	296g/mol	10.962	25	4.34
10	2-Cyclopenten-1-one, 2-hydroxy	$C_5H_6O_2$	98g/mol	3.779	4	4.11
11	(S)-2-Hydroxypropanoic acid	$C_3H_6O_3$	90.08g/mol	3.696	3	4.08
12	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$	144.12g/mol	4.399	7	1.34
13	2H-Pyran-2,6(3H)-dione	$C_5H_4O_3$	112.08g/mol	4.604	8	1.39

Gas-Chromatography and mass spectroscopy (GC-MS)

Gas chromatograph with mass spectrometer is an effective instrument for analyzing natural phytochemical compounds due to its stability, sensitivity, and movement [37].The biologically active compounds in the extract were analyzed and identified using GC-MS. The compounds were identified by comparing their retention times to the NIST05s data the database. Based on their retention time, the obtained results show the presence of 41 compounds in the chromatogram (Fig. 3 and Table. 1). The major compounds, Peak 20 (RT7.815, Area percent 17.32) was 2-Furancarboxaldehyde, 5-(hydroxymethyl), peak 31 (RT13.886, Area percent 11.40) was 3-Deoxy-d-mannoic lactone, peak 16 (RT6.699, Area percent 8.68) was 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl. Similar to our study, GC-MS evaluation of crude ethyl acetate extract of endophytic *C. gloeosporioides* isolated from *Lanneacorammendalica* showed 9-octadecenamide, hexadecanamide, Diethyl pythalate, 2-methyl-3-methyl-3-hexene, 3-ethyl-2,4-dimethyi-pentane, and antimicrobial activity [38].GC-MS research on crude extract from endophytic fungi isolated from medicinal plant *Phlogacanthus thyrsiflorus* revealed volatile compounds. Phenol, 2,4-bis (1,1-dimethylethyl), 1-Hexadecene, 1-Hexadecanol, octadecanoic acid methyl ester, and 1-Nonadecene were the main components [36]. *Alternaria alternata* bioactive compounds from *Picrorhizakurroa* showed similar results [39].Plants also produced bioactive compounds. *Symplocosracemosa* GC-MS study revealed 57 phytochemicals with distinct pharmacological activities [40]. Pattnaik et al., [41] investigated the antibacterial and antifungal effects of *Calotropisprocera* and *Calotropis gigantea* crude extracts.Bioactive compounds responsible for the antimicrobial action of endophytic fungi isolated from *Dilleniaindica* L. [42].

CONCLUSION

In the present investigation, a promising endophytic strain of *Alternaria brassicae* (KUMBMDBT-29) was isolated from the leaf of *Phoenix sylvestris* and deposited in the gene bank under the accession number MW007917. The endophytic fungus *Alternaria brassicae* is easily cultured in the laboratory for the fermentation-based production of secondary metabolites. The results of this study confirmed the presence of 41 biologically active compounds in ethyl acetate extract as determined by GC-MS analysis. Therefore, this study is beneficial to the pharmaceutical industry for the production of biologically active compounds with therapeutic value and for the discovery of novel drugs to treat diseases.

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