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Anti–diabetic effect of *Daldinia eschscholtzii* and *Phomopsis mangiferae* isolated from *Bryophyllum pinnatum*

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Abstract

The aim of the present study was to isolate endophytic fungi and examine their anti-diabetic as well antioxidant potential. Twenty-nine endophytic fungi were isolated from the stem and root parts of *Bryophyllum pinnatum*. Based on the presence of phytoconstituents, 16 endophytic isolates were tested for the inhibition of α-amylase enzyme and free radicals. Thirteen isolates showed significant inhibition of α-amylase enzyme (18.8 ± 0.8 to 62.5 ± 0.4%) and free radicals (15.3 ± 0.2 to 87.4 ± 0.8%). Good inhibitions were found in six isolates, which was further tested to validates its anti-diabetic activity by glucose uptake in yeast cell and glucose diffusion inhibition studies. The most active isolates were identified as *Daldinia eschscholtzii* (BPS17) and *Phomopsis mangiferae* (BPK9) by microscopic and molecular identification method. Identified fungal isolates were tested for glucose uptake using 3T3 cell lines (100 µg/mL) and the result showed effective glucose utilization. These finding provides evidence that *D. eschscholtzii* and *P. mangiferae* are potent endophytic fungi having anti-diabetic activity.

Introduction

Diabetes mellitus is considered as a chronic metabolic disorder characterized by hyperglycemia which results in causing various chronic vascular complications. One therapeutic strategy to control hyperglycemia is by inhibiting α-amylase, an enzyme involves in the metabolism of carbohydrates where starch will hydrolyze into simple sugars which then enters the bloodstream. Therefore, the level of postprandial blood glucose will be reduced. Commercially available inhibitors are acarbose and miglitol, which competitively and reversibly inhibit these enzymes present in the intestine and pancreas, but the use of synthetic drugs results in causing several adverse effects (Pavithra et al., 2014).

Despite the treatment options available today, many herbal based products are suggested for treating diabetes. Plants such as *Branchylaena elliptica* (Sagbo et al., 2018), *Calpurnia aurea* (Belayneh and Birru, 2018), *Kandelia candel* (Shettar and Vedamurthy, 2017), *Momardica charantia* (Joseph and Jini, 2013), *Punica granatum* (Pottathil et al., 2020), *Rhizophora apiculata* (Shettar and Vedamurthy, 2017), *Iphiona aucheri* (Shah et al., 2020), *Sorghum halepense* (Shah et al., 2019), *Treculia africana* (Ogbonnia et al., 2008) show anti-diabetic activity.

Plants used as traditional medicine that produce strains of endophytes have become an excellent source for finding new bioactive metabolites since their beneficial properties for plants can be a result of metabolites

produced by the endophytes (Venkateswarulu et al., 2018). Anti-diabetic effect of the endophytic fungi like *Schizophyllum commune*, *Alternaria sp.*, *Aspergillus sp.* and *Talaromyces sp.* (Kaur, 2018; Rustamova et al., 2020; Tejaswini et al., 2021), *Stemphylium globuliferum* (Pavithra et al., 2014), *Penicillium brevicaule* alba Thom. (Gulyamova et al., 2020), *Curvularia lunata* (Jayant and Vijayakumar, 2021), *Cochliobolus sp.* (Shoba and Sathiavelu, 2018) have been reported.

Bryophyllum pinnatum (Lam.) Kurz (Syn. *Kalanchoe pinnata*) belongs to the Crassulaceae family and common names include love plants, miracle leaves and Cathedral bells. This plants *B. pinnatum* has anti-diabetic (Ojewole, 2005; Ogbonnia et al., 2008; Aransiola et al., 2014; Ezeagu et al., 2017; Ojo et al., 2018) and antioxidant effects (Gupta et al., 2016; Mbachu et al., 2019; Daniel et al., 2020). The anti-diabetic activity was reported in *B. crenata* (Effah-Yeboah et al., 2021) and *B. laciniata* (Fernandes et al., 2019).

Three biologically active phenolic compounds namely bryophillor, bryophorone and bryopholenone were isolated from *B. pinnatum* and these biologically active molecules have antioxidant properties (Gupta et al., 2016). Alpha glycosidase activity was exhibited by two flavonoid glycosides [quercetin 3-O-α-L-rhamnoside and quercetin-3-O-α-L-arabinopyranosyl (1 → 2)α-L-rhamnopyranoside] isolated from leaves ethyl acetate extract of *B. pinnatum* (Sofa et al., 2018).

The endophytes isolated from the plant *B. pinnatum* were *Aspergillus brunneoviolaceus* (Okonkwo-Uzor et al., 2022), *Diaporthe phaseolorum* (Sowemimo et al., 2008, Ali et al., 2023).

Cytochalasin D and (3R,4R)-3,4,5-trihydroxy-1-tetralone isolated from *Diaporthe phaseolorum* of B. pinnatum leaf had shown cytotoxic activity (Sowemimo et al., 2008, Ali et al., 2023). Anti-diabetic and antioxidant activity were exhibited by unknown endophytic fungi isolated from the genus *Bryophyllum* (Kaur, 2018). Therefore, the current study focused on the isolation and identification

of endophytic fungi from stem and root parts of *B. pinnatum* and examines their anti-diabetic and antioxidant effects.

Materials and Methods

Collection of plant samples

The plant samples were collected from the Vellore Institute of Technology, Vellore, Tamil Nadu, India. Plant materials were transferred to the laboratory in a sterilized bag and processed within 24 hours of sampling (Bhardwaj et al., 2015).

Isolation of endophytic fungi

Healthy stem and root samples are thoroughly washed in running tap water to remove dust and debris and then surfaces sterilized according to the method described elsewhere with slight modification. Soak the plant segments in 95% ethanol for 30 sec, 4% sodium hypochlorite for 3 min, and 95% ethanol for 30 sec and then washed 3 times in sterilized distilled water and dried with sterilized blotting paper in sterile condition. After drying, cut each segments into small pieces and place them on potato dextrose agar plate and incubated at 27 ± 2°C for 1 week. Monitor the growth of endophytic colonies every day. The fungi grown from the samples were then transferred to fresh potato dextrose agar plate plates to isolate pure colonies and maintained by subsequent sub-culturing method (Bhardwaj et al., 2015).

Secondary metabolite production

Mycelium from the edge of the growing colony (agar block) was inoculated in potato dextrose broth and incubated for 21 days at 27 ± 2°C for fungal metabolite production by the fermentation method. After incubation period, each culture was filtered by using Whatman No. 1 filter paper to separate fungal filtrate and mycelium mat. A solvent extraction procedure was carried out for the fungal filtrate using dichrome-

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Box 1: Antioxidant Activity by DPPH Assay

Principle

1,1 Diphenyl 2-picryl hydrayzl are dark purple, long-lived organic nitrogen radicals. When DPPH solution is mixed with an antioxidant, it changes color from purple to yellow from the corresponding hydrazine.

Requirements

Ascorbic acid, DPPH (1,1-diphenyl-2-picryhydrazyl), Fungal crude extracts, Methanol

Procedure

Step 1: DPPH was prepared by dissolving in methanol for

making stock solution

Step 2: Add 2 mL of DPPH solution and different concentration (50, 100, and 150 µg/mL) of crude extracts in test tube. Incubate for 30 min under complete darkness

Step 3: Absorbance was taken at 517 nm.

Calculation

The ability of the fungal crude extracts to scavenge DPPH free radicals was calculated by using the following equation,

Percentage inhibition of free radicals = Absorbance of control-Absorbance of sample/Absorbance of control×100

References

Xiao et al., 2020; Baliyan et al., 2022

thane, ethyl acetate and butanol and mycelium mat in methanol for the extraction of fungal crude. An equal amount of solvents were added to the fungal filtrate in separatory funnel and shaken vigorously for 10 min and kept aside until two transparent immiscible layers were formed. The solvent containing the extracted compounds were collected and evaporated to yield dry crude. The obtained fungal crudes were used to carry out the further studies (Jayant and Vijayakumar, 2021).

Morphological and molecular identification of endophytic fungi

Lactophenol cotton blue method was used to identify the morphological observation of endophytic fungi by the method reported elsewhere (Kumari and Silva, 2017). For molecular identification, about 100 mg of fungal mycelium was taken for DNA isolation. DNA was isolated according to the standard method described elsewhere (Ferdous et al., 2022; Hamzah et al., 2018). Nuclear ribosomal internal transcribed spacer (ITS) of the fungal isolate was amplified with a forward primer, ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and reverse primer, ITS4 (5-TCCTCCGCTTATTGATATGC-3) (White et al., 1990). The final volume of the reaction mixture 25 µL (1.5 µL of forward and reverse primer, 5 µL of deionized water and 12 µL of Taq Master Mix) was added to 1.5 µL of DNA isolates. For negative control, DNA was replaced with distilled water to ensure that there was no contamination. PCR was programmed and performed by thermal cycling conditions. The millipore mounting kit was used to remove unincorporated PCR primers and dNTP from the PCR product. Sequencing was carried out for the primers obtained from PCR products and reaction was performed using ABI PRISM® BigDye™ terminator cycle sequencing kit and AmpliTaq™ DNA polymerase. The resulting endophytic fungi sequence was then exposed to the nucleotide BLAST search and similar regions of query sequence were compared with biological sequence deposited in NCBI database. To understand evolutionary relationships, phylogenetic tree was constructed for each isolate strain using the MUSCLE 3.7 program. Phylogenetic tree was built using PhyML software (Ferdous et al., 2022).

Qualitative phytochemical analysis

Qualitative phytochemical screening was performed for the fungal extracts by following the procedure described elsewhere (Bhardwaj et al., 2015). The fungal crude extracts were analysed for the presence of phenol, flavonoids, alkaloids, terpenoids, steroids, saponin and tannin.

In vitro anti-diabetic activities

Alpha-amylase inhibition assay

About 1 mg of fungal crude extracts was prepared by dissolving in solvents. Alpha amylase enzyme was slowly dissolved in 0.1 M of phosphate buffer. In each test tube, add 100 µL of solution (enzyme+ buffer) and 100 µL of samples of varying concentrations (100, 200 and 300 µg/mL) followed by addition of 100 µL of 1% starch solution and incubated at 37°C for 10 min. After the incubation time, add 200 µL of DNSA (3,5-dinitrosalicylic acid) solution and kept in boiling water bath for 10 min. Cool down to room temperature, add 200 µL of 40% Rochelle salt to the mixture and diluted with distilled water, absorbance was taken at 540 nm. Acarbose was used as the standard (Tiwari et al., 2017). The percentage inhibition of alpha-amylase was calculated using the formula:

% Inhibition of α-amylase = (Abs of control - Abs of sample/ Abs of control) × 100

Glucose uptake by yeast cell study

The commercially available baker's yeast was dissolved in distilled water and kept for overnight incubation. After the incubation period, the tubes were centrifuged (3,000 × g, 5 min) until a clear supernatant was obtained. In the same way, 10% (v/v) yeast suspension was prepared. Different concentrations of crude extracts (100-500 µg/mL) were added in each test tube containing 1 mL glucose solution (10 and 25 mM) and the mixture was incubated at 37°C for 10 min. After the incubation period, the reaction was initiated by adding 100 µL of yeast suspension, and vortex and incubated at 37°C for 60 min. After the incubation period, the tubes were centrifuged to evaluate the glucose concentration present in the supernatant by taking the absorbance in UV-Vis spectrophotometer at 520 nm (Paul and Majumdar, 2020). The percentage of glucose uptake by yeast cells was calculated by the following formula:

%Increase in glucose uptake = (Abs of control - Abs of sample/Abs of control) × 100

Glucose diffusion inhibition assay

The extracts (1.1 mL) were taken in the dialysis membrane (12000 MW, Hi Media Laboratories, India) with a glucose solution (0.22 mL in 0.15 M NaCl₂). The edges of the membrane were tied and then immersed in a beaker containing 40 mL of 0.15 M sodium chloride solution and 10 mL distilled water. The beakers were placed in a magnetic stirrer and the entire process was carried out in room temperature. Pipette out 4.5 mL of the solution for every 30 min up to 150 min of observation period. Each time the volume was compensated by adding new solution. The absorbance was taken at 248 nm in a UV spectrophotometer. Control tube was also carried out in a same way by replacing sample with distilled water (Jayamol et al., 2020).

Glucose uptake assay by 3T3 cell lines

Cell culture

The 3T3 cell lines were purchased from National Centre for Cell Sciences, Pune, India. Cells were maintained with DMEM containing 10% FBS (fetal bovine serum),

penicillin (100 U/mL) and antibiotics (100 µg/mL) in a humidified environment. 3T3 cell lines was cultured with DMEM in 24-well plates at a final volume of 1000 µL with a cell thickness of 6×10^4 cells/wells and incubated for 48 hours until the cells intersected.

Preparation of sample

In a six well plate add 5 mL of solution (cells+ medium) and 1 mL of fungal crude extracts (100 µg/mL) and standard (1 IU/mL) and incubated for 24 hours. After the incubation period, the entire medium was taken off from the 6-well plate and washed with 2 mL of 10× PBS. Then add 150 µL of 10% SDS and shaken well. With the cell scraper, the cells were transferred to the eppendorf tube and named accordingly. Then the tubes were centrifuged and the supernatant was transferred to the marked eppendorf and stored at 4°C for further process.

Determination of glucose concentration by DNS assay

In each test tube add 200, 400, 600, 800, and 1000 µL of glucose stock solution. From each supernatant sample, 100 µL was added and a volume of 2 mL was prepared with distilled water. Distilled water serves as the blank. Add 1 mL of DNS reagent to all the test tubes and cover it with aluminium foil and kept in a boiling water bath for 5 min at 100°C to observe the discoloration. The test tubes were allowed to cool down and absorbance was taken at 540 nm in different time intervals. The graph was plotted with the amount of glucose (mg/mL) on the X-axis and the absorbance value of the sample on the Y-axis. Measure the absorbance of the sample and the unknown concentration was detected from the standard (Kumar et al., 2021).

GC-MS analysis

The extract was analysed with Perkin Elmer Clarus 600 mass spectrometer (EI) with an Elite-5MS capillary column (30, 0.5 mm ID, 259 m df), the device was stored for 6 min at an initial temperature of 55° C in the oven for 3 min and the ramp program for 10 min^{-1} to 300°C at a constant flow rate of 1 mL/min, helium was used as a carrier gas, the base temperature and mass transfer line was set at 240°C. Turbo software version 5.4.2 was used for spectral analysis. Individual components recognized in the general mass spectrum by the National Institute of Standards and Technology (NIST-LIB 0.5) included in the GC-MS system software (Wiley GC-MS-2007) and literature data. Metabolites present in the fungal crude extract are separated by a gas chromatography column. Each compound separated by GC enters the MS and get ionized. The MS ionization spectrum was recorded and compared with the mass spectrum of compounds known in the NIST library. The MS spectrum accurately maps the molecular weight of each molecule (Shankar et al., 2018).

In vitro antioxidant assay

The ability of the fungal crude extracts to inhibit DPPH free radicals was assessed using standard methods. Crude extracts were taken in a test tube at different concentrations (50, 100, and 150 µg/mL) and 2 mL of DPPH was added. The tubes were incubated for 30 min at room temperature under dark condition and after the incubation time, the absorbance was taken at 517 nm in UV-Vis spectrophotometer. Ascorbic acid was used as the standard (Gurnani et al., 2016). The percentage inhibition of free radicals was calculated using the following formula:

% Inhibition of free radicals = [Abs of control-Abs of sample/ Abs of control] × 100

Statistical analysis

The experiments were conducted in triplicates and the results are expressed in mean ± SEM. Graph pad prism software was used for statistical calculation.

Results

Isolation of endophytic fungi and extraction of secondary metabolites

Twenty-nine pure endophytic fungi were successfully isolated from the sterile stem and root segments of *B. pinnatum* (Figure 1). All the fungal endophytes were sub-cultured on new agar plates and stored in various ways, such as agar plates, and agar slants. Twenty-nine endophytic fungi were grown separately and on a large scale in potato dextrose broth to extract secondary metabolites by using different polarities of solvent such as dichloromethane, ethyl acetate, butanol and fungal mat in methanol by the solvent extraction process. The obtained fungal extracts were dried and stored at 4°C for further studies.

Qualitative phytochemical analysis of fungal crude extracts

Qualitative phytochemical analyses were performed for ethyl acetate and methanol fractions of twenty-nine endophytic fungi isolates. The presences of phytoconstituents in endophytic fungi are responsible for the various biological activities shown by the crude extracts and the use of this plant as traditional medicine practices since ancient times. Based on the presence of phytoconstituents, 16 endophytic fungi were further selected to examine antioxidant and anti-diabetic potential (data not shown).

Anti-diabetic activity

Alpha amylase inhibition assay

Alpha amylase inhibition assay was performed to determine the anti-diabetic activity of fungal crude extracts. Thirteen endophytic fungi isolate showed inhibition of alpha amylase which was compared with the standard drug acarbose. The tested extracts showed significant inhibition of about 18.7 ± 0.7%- 62.5 ± 0.3%
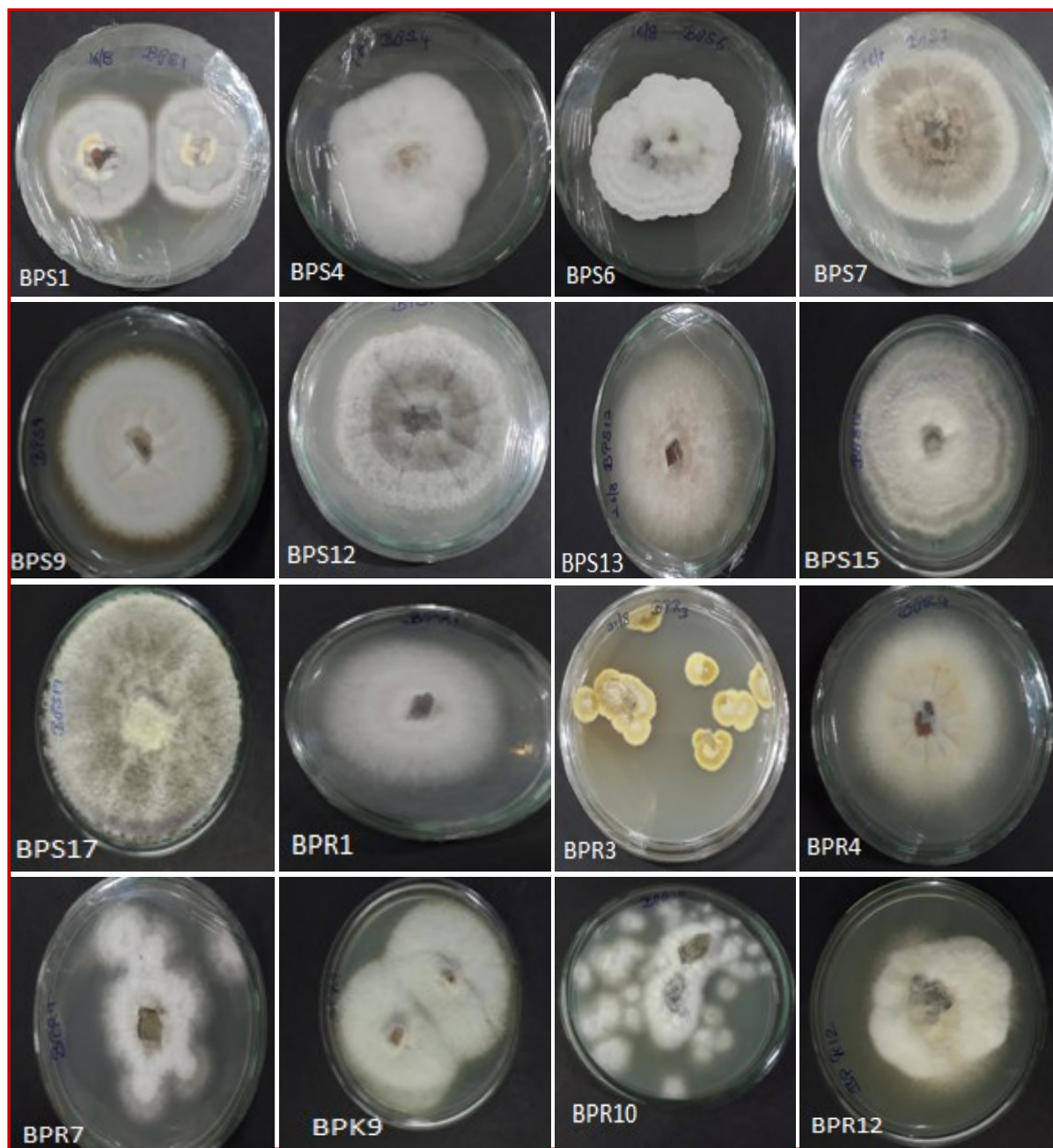
Figure 1: Colony morphology of endophytic fungi isolated from stem and root parts of *Bryophyllum pinnatum*

and the standard (acarbose) showed 70.5 ± 0.5% (Table I). The maximum inhibition was showed by BPS17 and BPK9 of about 61.7 ± 1.7% in dichloromethane extract and 62.5 ± 0.3% in ethyl acetate extract respectively. The least inhibition was exhibited by BPR1 isolate of about 18.7 ± 0.7% in 300 μg/mL concentration. Based on the result obtained in antioxidant and α-amylase inhibition studies six isolates namely BPS4, BPS7, BPS17, BPR7, BPK9 and BPR10 were further chosen to validate its anti-diabetic activity by glucose uptake by yeast cell and glucose diffusion inhibition studies.

Glucose uptake by yeast cell study

The fungal crude extracts (dichloromethane, ethyl acetate, butanol and methanol) obtained from six endophytic fungi were taken at different concentrations to determine the glucose uptake by yeast cell study. After treating yeast cells with fungal crude extracts, dose-dependent glucose uptake was observed in both 10 and 25 mM glucose concentration (Figure 2). As the percentage of glucose concentration increases, the uptake of glucose by yeast cells also increased. The maximum glucose uptake was exhibited by BPS17 and BPK9 of about 70.1 ± 1.8% and 72.3 ± 1.7% respectively in the presence of 25 mM glucose concentration at 500 μg/mL concentration of crude extracts. The standard drug (metformin) had shown 75.1 ± 1.8% of glucose uptake in the presence of 25 mM glucose concentration where-

as the glucose uptake was found to be comparatively low in 10 mM glucose concentration of about 63.1 ± 1.9% in 500 µg/mL. The results revealed that the rate of increase in glucose absorption by yeast cells is directly proportional to the concentration of glucose.

Effect of fungal crude extracts on glucose diffusion inhibition study

In this study, dialysis membrane containing glucose and extracts soaked in sodium chloride solution was

Table I

Alpha-amylase inhibition activity of fungal extracts obtained from stem and root endophytic fungi

| Fungal extracts (µg/mL)		%Inhibition of α-amylase			
		Dichloromethane	Ethyl acetate	*n*-Butanol	Methanol
BPS4	100	15.3 ± 1.0	23.2 ± 0.9	21.2 ± 0.7	28.2 ± 0.6
	200	39.1 ± 0.6	46.8 ± 0.8	28.6 ± 0.6	26.2 ± 0.3
	300	46.8 ± 0.8	57.2 ± 0.4	32.26 ± 0.6	31.8 ± 0.7
BPS6	100	23.9 ± 0.5	25.9 ± 0.9	18.82 ± 0.7	17.3 ± 1.0
	200	27.2 ± 0.8	35.1 ± 0.7	26.63 ± 0.4	23.9 ± 0.5
	300	32.6 ± 0.8	38.6 ± 0.6	29.25 ± 0.7	28.9 ± 0.7
BPS7	100	28.2 ± 1.2	29.2 ± 0.5	25.6 ± 0.2	27.7 ± 0.6
	200	33.8 ± 1.1	45.6 ± 0.7	28.3 ± 0.4	35.2 ± 0.8
	300	37.2 ± 1.6	52.3 ± 0.1	33.8 ± 0.6	38.5 ± 0.6
BPS9	100	11.6 ± 0.2	19.5 ± 0.8	17.8 ± 0.3	13.3 ± 0.8
	200	17.3 ± 0.6	35.4 ± 0.3	22.5 ± 0.8	20.7 ± 0.3
	300	23.8 ± 0.7	42.4 ± 0.2	25.2 ± 0.9	27.9 ± 0.7
BPS12	100	12.3 ± 0.6	17.2 ± 0.4	15.15 ± 0.6	17.3 ± 0.8
	200	16.2 ± 0.7	21.7 ± 0.6	26.5 ± 0.6	23.6 ± 0.6
	300	22.7 ± 0.8	28.8 ± 0.7	31.3 ± 0.7	28.8 ± 0.7
BPS13	100	20.6 ± 0.3	29.0 ± 0.8	27.1 ± 0.8	18.7 ± 0.2
	200	27.5 ± 0.5	36.3 ± 0.2	38.2 ± 0.7	25.1 ± 0.7
	300	33.3 ± 0.7	42.2 ± 0.3	58.2 ± 0.3	28.5 ± 0.3
BPS17	100	31.1 ± 1.0	29.8 ± 0.5	27.2 ± 0.7	25.7 ± 0.4
	200	47.4 ± 1.3	37.6 ± 0.4	36.3 ± 0.6	33.4 ± 0.8
	300	61.8 ± 1.7	45.2 ± 0.2	43.6 ± 0.3	41.4 ± 0.8
BPR1	100	10.2 ± 0.5	20.3 ± 0.3	16.8 ± 0.6	10.8 ± 0.3
	200	13.3 ± 0.3	23.6 ± 0.1	18.5 ± 0.3	13.7 ± 0.2
	300	18.8 ± 0.8	27.8 ± 0.3	20.4 ± 0.5	19.9 ± 0.9
BPR3	100	10.2 ± 1.8	21.5 ± 0.2	12.7 ± 0.7	27.9 ± 0.4
	200	15.7 ± 0.5	32.2 ± 0.6	17.9 ± 0.6	39.3 ± 0.1
	300	23.7 ± 0.6	39.5 ± 0.6	25.2 ± 0.2	59.6 ± 0.4
BPR7	100	31.3 ± 0.1	32.8 ± 0.7	28.2 ± 0.5	23.7 ± 0.2
	200	45.5 ± 0.7	47.2 ± 0.6	33.6 ± 0.6	35.6 ± 0.2
	300	51.7 ± 0.8	53.3 ± 0.3	41.4 ± 0.2	38.3 ± 0.4
BPK9	100	30.2 ± 0.7	31.0 ± 0.5	24.8 ± 0.4	18.9 ± 0.6
	200	39.3 ± 0.2	48.1 ± 0.2	32.2 ± 0.8	26.8 ± 0.2
	300	46.3 ± 1.3	62.5 ± 0.4	39.8 ± 0.3	34.7 ± 0.3
BPR10	100	29.9 ± 0.7	30.2 ± 0.7	26.2 ± 0.3	23.9 ± 0.1
	200	42.7 ± 0.3	43.5 ± 0.3	35.8 ± 0.5	37.3 ± 0.5
	300	47.6 ± 0.3	49.7 ± 0.1	42.7 ± 0.5	46.9 ± 0.6
BPR12	100	12.9 ± 0.5	28.4 ± 0.3	13.7 ± 0.2	11.7 ± 0.3
	200	18.7 ± 0.7	31.8 ± 0.5	17.9 ± 0.8	16.3 ± 0.4
	300	25.2 ± 0.7	38.5 ± 0.3	29.6 ± 0.7	24.5 ± 0.5
Standard (acarbose)	100	33.5 ± 0.2			
	200	50.3 ± 0.2			
	300	70.5 ± 0.5			
Data are mean ± SEM; n = 3					
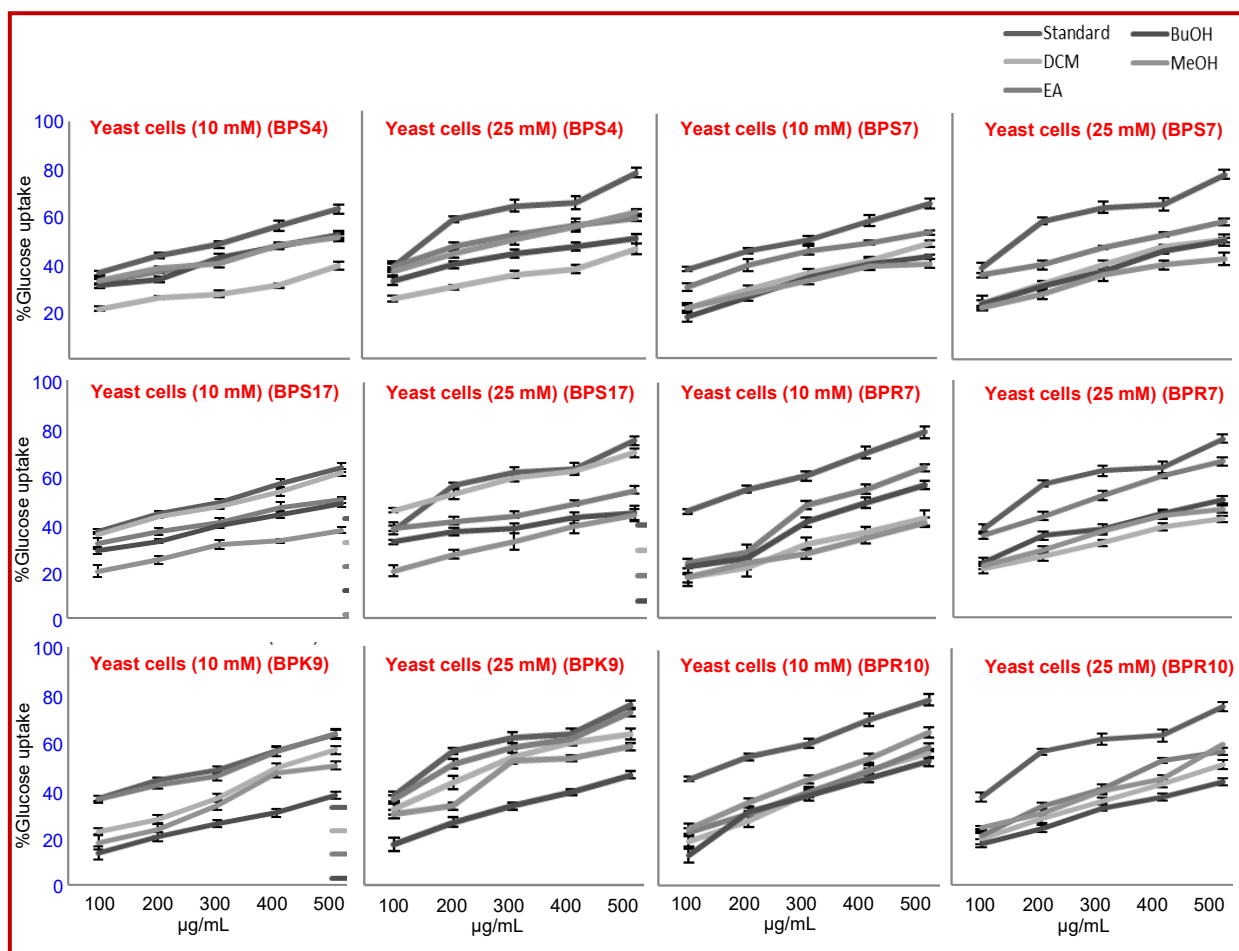
Figure 2: Effect of fungal crude extracts on glucose uptake by yeast cells. Data are mean ± SEM; n = 3

used to evaluate the inhibition of glucose transfer. Glucose diffuse into the sodium chloride (external) solution was measured at 30, 60, 90, 120 and 150 min and the effect of fungal extracts on glucose diffusion inhibition has been summarized in Table II. All the extracts showed an inhibitory effect whereas dichloromethane extract of BPS17 and ethyl acetate extract of BPK9 maintained the maximum inhibition of glucose diffusion of about 64.2 ± 0.3% and 66.5 ± 0.7% respectively, close to the standard drug (acarbose) of about 71.5 ± 1.2%.

Morphological and molecular identification of endophytic fungi

Based on the *in vitro* antioxidant and anti-diabetic studies, BPS17 and BPK9 are considered as a potent strain and it was identified as *Daldinia eschscholtzii* and *Phomopsis mangiferae* respectively based on the microscopic observation by LPCB method (Figure 3A, B, C) and 18s rRNA gene sequencing. The sequence was submitted to GeneBank and the accession number was obtained OP740989 and OP741081 respectively. The phylogenentic relationship was established through homologous nucleotide sequences and evolutionary

tree was constructed (Figure 3D, E).

Glucose uptake in 3T3 cell lines

D. eschscholtzii and *P. mangiferae* are considered as the potent endophytic strains, therefore dichloromethane and ethyl acetate extracts were chosen to study the effect of glucose uptake in 3T3 cell lines. The highest glucose uptake activity was found in *P. mangiferae* as the concentration of glucose decreases from the 0th hour to 24th hour of about 2.5 ± 0.0 to 1.9 ± 0.0 μg, whereas the activity was slightly lower in *D. eschscholtzii* and the results were compared with the standard anti-diabetic drug, insulin showed the glucose uptake by 3T3 cell lines from 2.3 ± 0.1 to 1.8 ± 0.0 μg of glucose concentration (Figure 4). The study concludes that the uptake of glucose in 3T3 cell lines was found in both *P. maniferae* and *D. eschscholtzii*.

GC-MS analysis of fungal crude extracts

D. eschscholtzii and *P. mangiferae* were chosen as potent endophytic isolates. Therefore, this analysis was carried out to find out the biologically active compounds present in the dichloromethane extract of BPS17 and ethyl acetate extract of BPK9 using gas chromatography

Table II						
Effect of fungal crude extracts on glucose diffusion through dialysis membrane						
Fungal crude extracts (1 mg/mL)		%Glucose diffusion inhibition at every 30 min				
		30 min	60 min	90 min	120 min	150 min
BPS4	Dichloromethane	41.6 ± 0.5	43.9 ± 1.0	44.4 ± 0.6	50.0 ± 0.4	53.3 ± 0.2
	Ethyl acetate	42.4 ± 0.9	43.8 ± 0.8	47.2 ± 1.0	50.9 ± 0.6	54.1 ± 0.6
	n-Butanol	25.5 ± 0.2	26.0 ± 0.3	31.3 ± 0.6	35.3 ± 0.8	37.8 ± 0.7
	Methanol	32.5 ± 0.5	36.0 ± 0.6	38.6 ± 0.8	40.6 ± 0.9	46.9 ± 1.1
BPS7	Dichloromethane	22.5 ± 0.5	25.9 ± 0.6	26.4 ± 0.7	32.6 ± 0.6	36.9 ± 1.0
	Ethyl acetate	32.5 ± 1.0	36.4 ± 0.8	38.2 ± 0.9	43.6 ± 0.2	45.0 ± 0.5
	n-Butanol	16.0 ± 0.8	17.3 ± 1.3	21.1 ± 0.6	25.6 ± 0.3	29.9 ± 0.8
	Methanol	7.4 ± 0.6	9.6 ± 0.7	15.0 ± 0.6	19.8 ± 0.4	21.6 ± 1.3
BPS17	Dichloromethane	46.8 ± 0.2	51.0 ± 0.9	55.3 ± 0.8	60.9 ± 0.1	64.2 ± 0.4
	Ethyl acetate	37.7 ± 0.8	41.8 ± 0.3	45.1 ± 0.4	49.6 ± 1.5	52.8 ± 0.5
	n-Butanol	24.2 ± 0.9	28.0 ± 0.1	32.5 ± 0.8	37.2 ± 1.1	41.7 ± 0.6
	Methanol	29.4 ± 0.6	33.5 ± 0.6	37.0 ± 0.5	41.5 ± 0.2	46.1 ± 0.7
BPR7	Dichloromethane	14.3 ± 0.2	18.4 ± 0.2	22.0 ± 0.9	26.7 ± 0.4	30.6 ± 1.0
	Ethyl acetate	19.5 ± 0.4	23.0 ± 0.8	26.0 ± 0.4	29.2 ± 0.2	33.9 ± 0.4
	n-Butanol	7.1 ± 0.3	12.6 ± 1.3	15.5 ± 0.3	16.0 ± 0.2	16.8 ± 1.5
	Methanol	6.1 ± 0.6	10.5 ± 0.3	13.8 ± 0.6	14.2 ± 0.7	14.7 ± 0.6
BPK9	Dichloromethane	40.7 ± 0.2	43.9 ± 0.9	47.6 ± 0.9	52.7 ± 0.6	56.5 ± 0.4
	Ethyl acetate	45.9 ± 0.2	49.8 ± 0.3	52.8 ± 0.7	56.6 ± 0.3	66.5 ± 0.8
	n-Butanol	34.6 ± 1.8	37.7 ± 0.6	39.8 ± 0.6	43.4 ± 0.9	45.1 ± 1.2
	Methanol	29.9 ± 0.3	34.3 ± 0.7	38.2 ± 0.6	42.3 ± 0.7	43.1 ± 0.6
BPR10	Dichloromethane	19.5 ± 0.3	23.8 ± 0.8	26.4 ± 0.8	30.6 ± 0.2	31.0 ± 0.2
	Ethyl acetate	32.0 ± 0.8	36.0 ± 0.5	39.0 ± 0.7	41.6 ± 0.7	45.9 ± 0.9
	n-Butanol	7.8 ± 1.1	8.9 ± 0.6	12.8 ± 1.0	17.4 ± 0.8	22.5 ± 0.6
	Methanol	16.5 ± 0.7	19.7 ± 0.7	22.8 ± 1.3	27.8 ± 0.6	31.7 ± 0.2
	Standard (Acarbose)	48.9 ± 1.2	54.4 ± 1.0	60.6 ± 0.7	67.8 ± 0.8	71.6 ± 1.3
Data are mean ± SEM; n = 3						

mass spectrometer. Chromatogram obtained from GC-MS analysis (Figure 5). The major compounds found in *D. eschscholtzii* were indano[2,1-D]1,3-dioxane (M.W - 176), and 1(2H)-naphthalenone, 3,4-dihydro-8-methyl-(M.W - 160) present in the retention time of 15.8 and 15.9 with area percentage of 20.7% and 24.5% respectively. *P. mangiferae* as shown 3-nonanone, 2-methyl-(M.W - 156) and 1,6-dimethyl-3,7,9-trioxabicyclo(4,2,1) nonane (M.W- 158) as the major compounds with the retention time of 13.9 and 17.1 along with area percentage of 41.5% and 34.1% respectively.

Antioxidant activity

The reaction is visible when the color changes from purple to yellow. The antioxidant activity was performed for the dichloromethane, ethyl acetate, butanol and methanol extracts of 16 endophytic fungi based on the presence of phytochemical constituents. The results indicate that inhibition percentage increases as the concentration of crude extracts increases. The extracts obtained from thirteen endophytic fungi showed significant inhibition of free radicals (Figure 6), whereas BPS17 and BPK9 had the highest inhibition percentage of free radical of about 87.3 ± 0.8% in dichloromethane

extract and 85.1 ± 0.8% in ethyl acetate extract respectively and the unknown sp. (BPR1) showed least antioxidant activity of about 15.3 ± 0.2%. The standard ascorbic acid had 92 ± 0.2% of antioxidant activity.

Discussion

D. eschscholtzii and *P. mangiferae* are considered as the potent endophytic strains shown good inhibition of alpha amylase enzyme, glucose uptake in yeast cell and 3T3 cell lines, glucose diffusion inhibition study and also in inhibition of free radicals. Twenty-nine endophytic fungi were isolated from stem and root parts of *B. Pinnatum,* in which BPS17 and BPK9 showed maximum anti-diabetic and antioxidant effects and identified as *D. eschscholtzii* and *P. mangiferae* respectively.

A study showed 22 endophytic fungi isolated from *Momordica charantia* and *Trigonella foenum-graceum.* Among these, 9 extracts exhibited α-amylase and α-glycosidase inhibition activity. The fungal isolate which exhibited maximum inhibition activity was identified as *Trichoderma atroviride* and *Stemphylium globuliferum* (Pavithra et al., 2014).
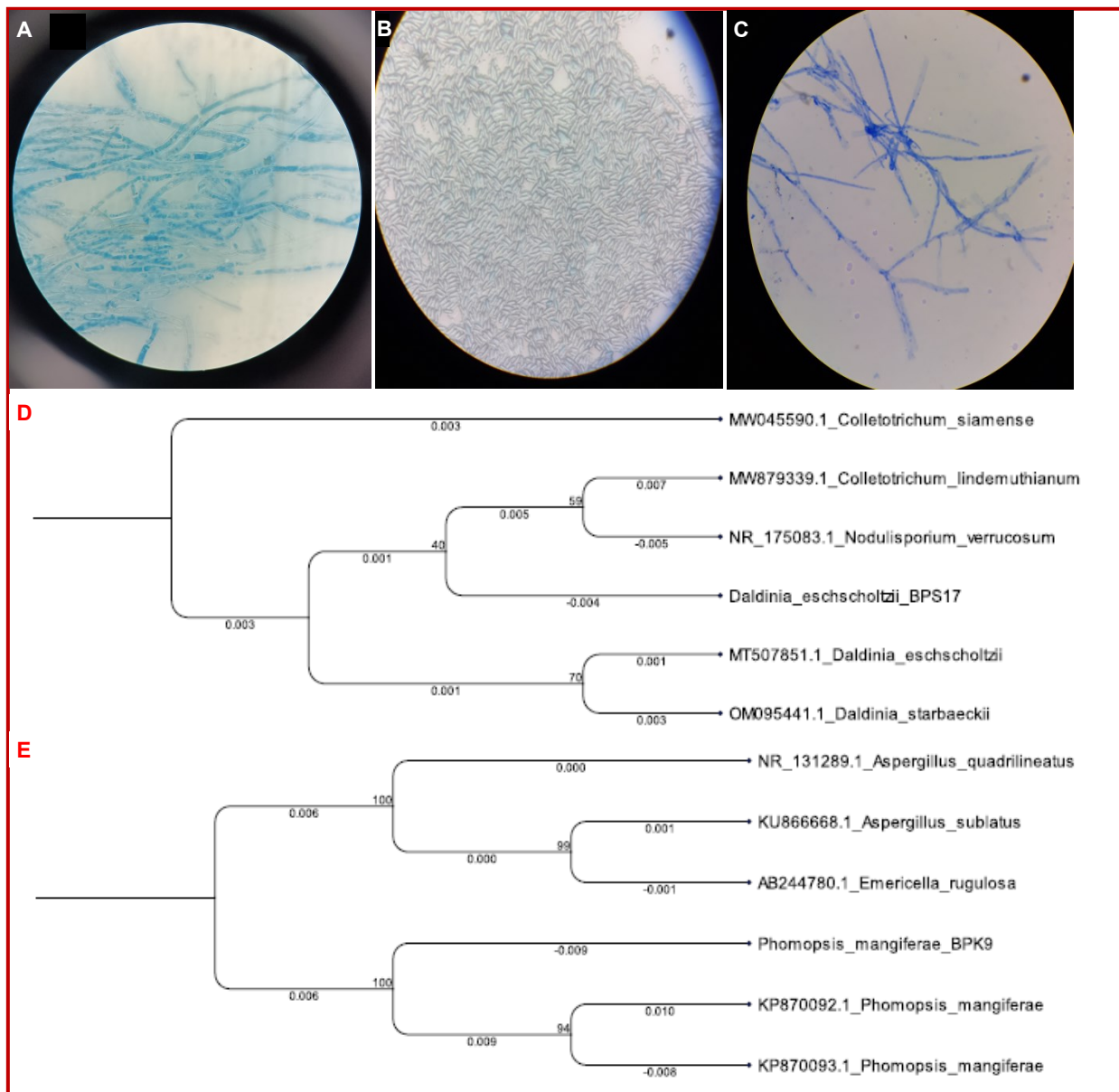
Figure 3: Microphotograph of LCB stain at 100x view of hyphae, conidiophores, conidia of BPS17 (A, B) and BPK9 (C) and phylogenetic tree (D, E) of fungal isolates

In the present study, the maximum anti-diabetic activity was reported in dichloromethane extract of *D. eschscholtzii* isolated from *B. pinnatum* whereas, the same effect was found in ethyl acetate extract of *D. eschscholtzii* isolated from *Cinnmomum bejolghota* (Wutthiwong et al., 2021). Five compounds namely (+)-regiolone (isosclerone), (+)-3,4-dihydro-3,4,8-trihydroxy -naphthalenone, (+)-mellein, daldiniaeschsone A and daldiniaeschsone B were isolated from *D. eschscholtzii* of *Cinnamomum bejolghota*. And all these compounds exhibited anti-diabetic activity with an IC_{50} value of 0.16 -0.85 mM (Wutthiwong et al., 2021). In this study, the major compound found in dichloromethane extract of *D. eschscholtzii* are indano[2,1-D]1,3-dioxane, 1(2H)-naphthalenone,3,-dihydro-8-methyl- and O-metyliso-
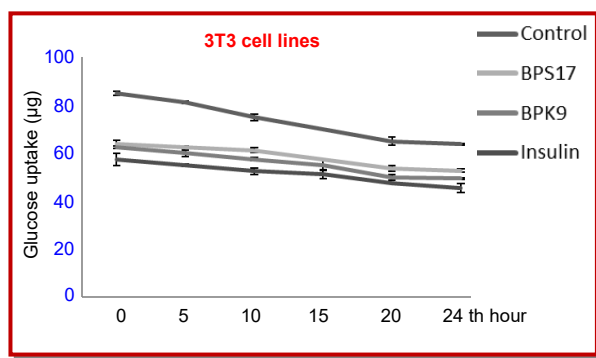

Figure 4: Effect of glucose uptake in 3T3 cell lines of BPS17 (dichloromethane) and BPK9 (ethyl acetate) crude extracts. Data are mean ± SEM; n=3
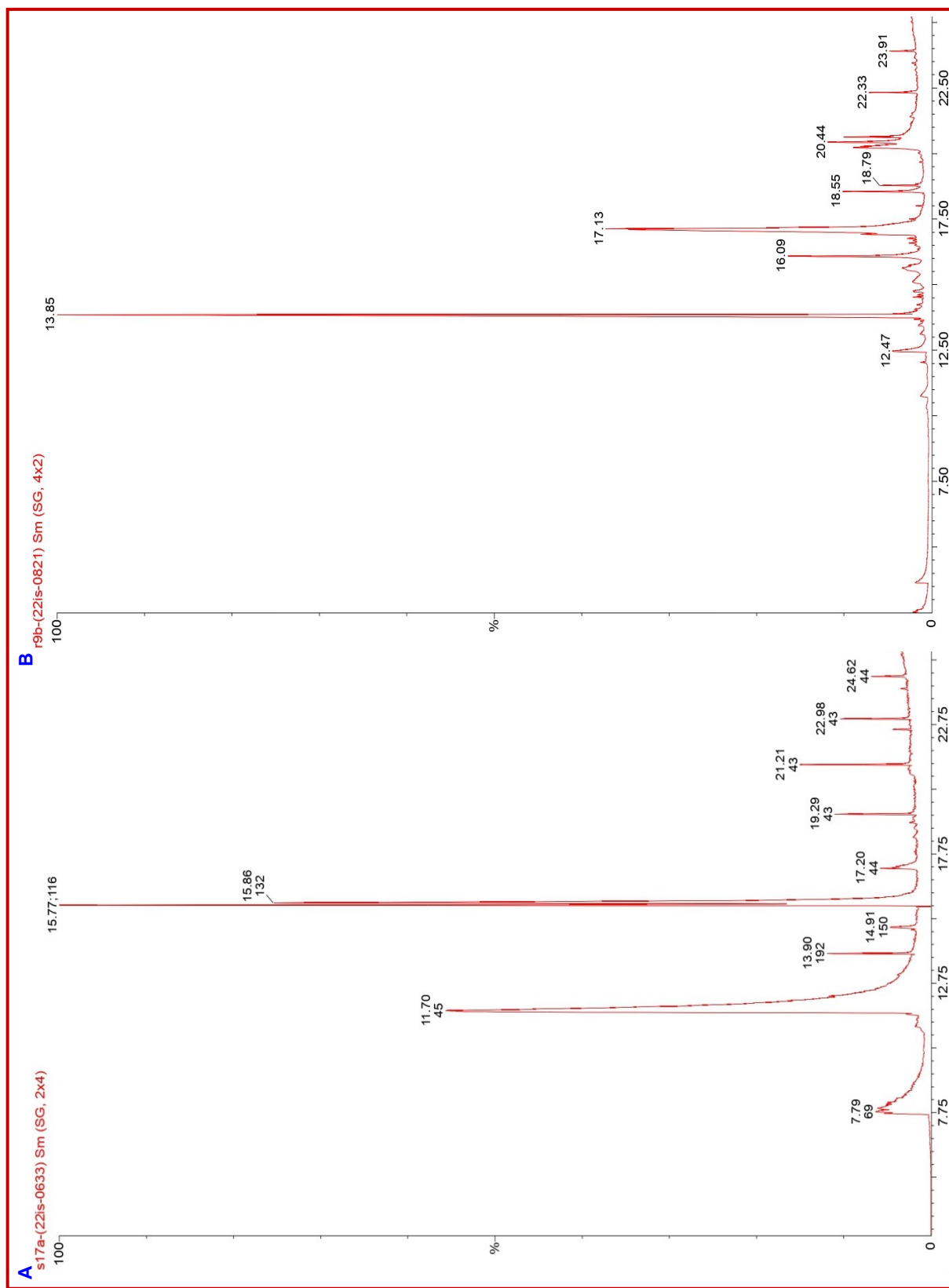
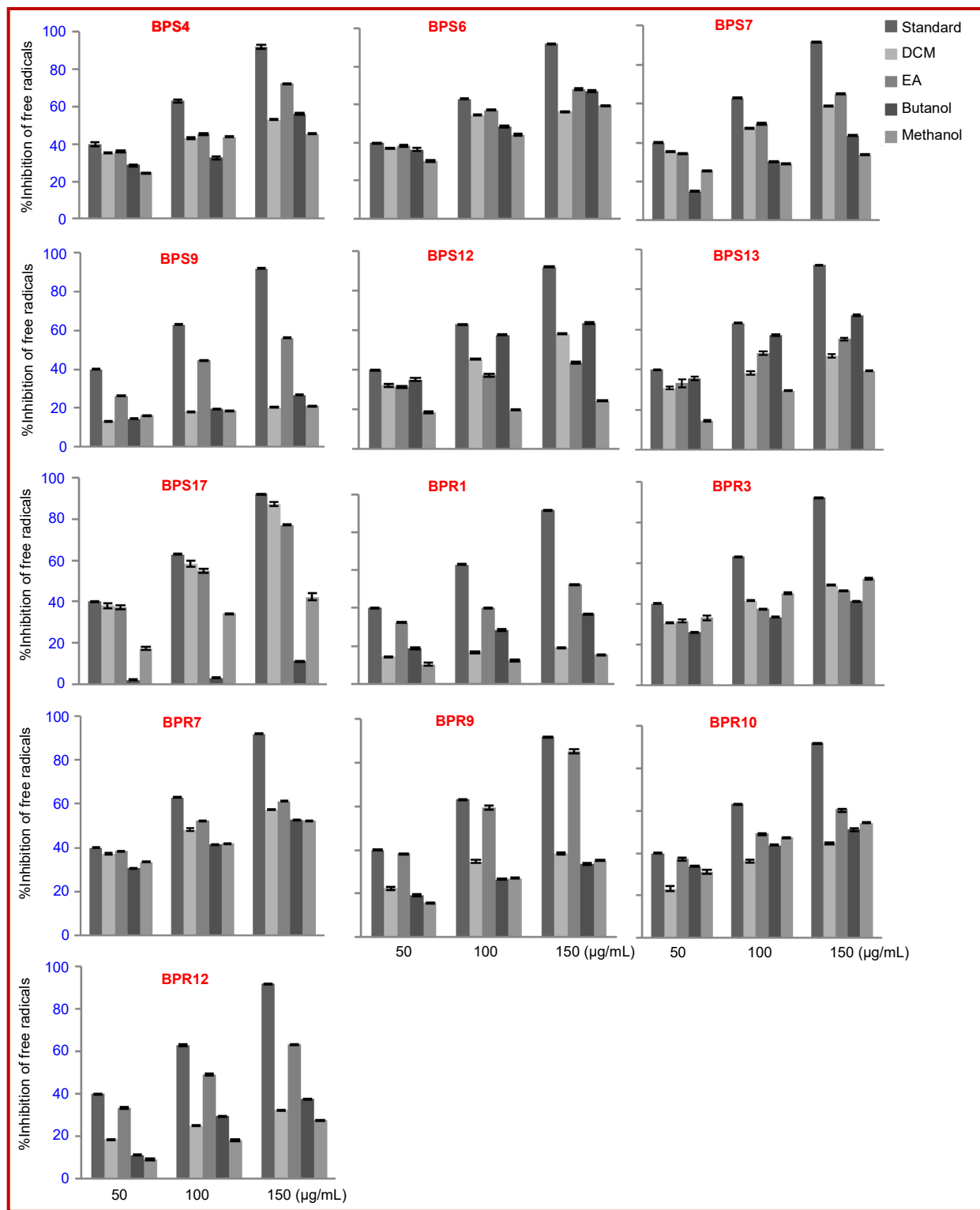Figure 5: GC-MS chromatogram of fungal crude extracts *D. eschscholtzii* (BPS17) (A) and *P. mangiferae* (BPK9) (B)

Figure 6: DPPH free radical scavenging activity of fungal crude extracts. Data are mean ± SEM; n = 3

urea hydrogen sulphate whereas the ethyl acetate extract of *P. mangiferae* contains major compounds such as 3-nonanone,2-methyl- and 1,6-dimethyl-3,7,9-trioxa-bicyclo(4,2,1)nonane. So, the presences of these compounds are responsible for the maximum anti-diabetic

activity showed by endophytic strains of D. *eschscholtzii* and *P. mangiferae*. The previous studies as isolated peniorcinols A-C, penialidins A, penialidin F, myxo-trichin C, riboflavin, indole-3-acetic acid and 2-(4-hydroxy-2-methoxy-6-methylphenyl) acetic acid from

endophytic fungi, in which peniorcinols C and indole-3-acetic acid has shown good anti-diabetic (Bai et al., 2021). *Aspergillus awamori* isolated from *A. nilotica* showed 81% and 80% inhibition of α-amylase and α-glycosidase respectively (Singh and Kaur, 2016). The previous findings reported anti-diabetic activity was exhibited by ethyl acetate extract of *Penicillium citrinum*, isolated from *Gymnema sylvestre* (Vidyasagar et al., 2021) and *P. lanosum* and *P. radiatolobatum* (Naveen et al., 2023). The current findings reported that ethyl acetate and dichloromethane extracts of *P. mangiferae and D. eschscholtzii* respectively have showed good inhibition of α-amylase enzyme.

The cell lines are considered as a good models for glucose absorption study, since they are widely used to explain the mechanism of glucose absorption in muscles having a complete insulin signaling pathway and show insulin-sensitive GLUT4. The main glucose transporter presented in skeletal muscle and adipose tissue is GLUT4, which is transferred from the intracellular membrane storage site to the plasma membrane (Das and Devi, 2015). The mechanism of glucose transport through yeast cell membranes has attracted attention and also considered to be an important *in vitro* screening method for the hypoglycemic activity of crude extracts. The current study revealed that the extracts obtained from BPS4, BPS7, BPS17, BPR7, BPK9 and BPR10 showed facilitate glucose transport through yeast cells. The amount of glucose remaining in the medium after a certain period of time acts as a measure of glucose uptake by yeast cells. Slowing down the production and/or absorption of glucose is considered as an important strategy for managing diabetes.

The medicinal plants improves glucose uptake by translocation of GLUT4, which is confirmed by *in vitro* glucose models. The study reported that L6 and 3T3 cell lines are the most typical sources of cell models for studying glucose uptake and GLUT4 translocation (Das and Devi, 2015). Therefore, the current study used 3T3 cell lines to determine the glucose absorption activity of dichloromethane and ethyl acetate extracts of BPS17 and BPK9 respectively. The result revealed decreases in glucose concentration from 0th hour to 24th hour of observation period. The presence of bioactive molecules in the endophytic fungal isolates as the ability to induce the absorption of glucose at such low concentrations suggested that it could be a good scaffold as a starting point for the drug development (Jayant and Vijayakumar, 2021). It is evident that dichloromethane (*D. eschscholtzii*) and ethyl acetate (*P. mangiferae*) extracts were recognized as potent inhibitors of glucose diffusion, showing the highest inhibition percentage of glucose diffusion. Cytotoxic activity was exhibited by ethyl acetate extract of *D. eschscholtzii* against lung adinocarcinoma (A549) and mouse fibroblast (NIH3T3) cell lines. Cytotoxic effect ranges from 25.4 ± 0.3 to 92.7 ± 0.6% and 0.8 ± 0.1 to 6.9 ± 0.3% against A549 and

NIH3T3 cell line respectively (Chutulo and Chalannavar, 2020). These findings on anti-diabetic activity of known and unknown species of endophytic fungi are in agreement with the previous study reports. In addition to the usual anti-diabetic drugs, endophytic fungi isolated from *B. pinnatum* are considered as an adjuvant in the treatment of diabetes.

Phenolic compounds, such as flavonoids and terpenoids, are the main antioxidant substances that eliminate free radicals and thus neutralize reactive oxygen species. Flavonoids use different mechanisms, such as inhibition of enzymes involved in the production of free radicals, thus preventing the development of oxidative stress (Shah et al., 2019). Complications of diabetes are predicted as a result of oxidative stress due to the formation of free radicals during the oxidation of glucose. Therefore, in order to avoid such complications, it is often recommended to use antioxidants along with anti-diabetic drugs (Jayant and Vijayakumar, 2021). In this study, the potent strains of endophytes, BPS17 and BPK9 showed the presence of flavonoids and terpenoids and these phytochemicals are responsible for the antioxidant activity shown by the crude extracts obtained, so this study supports the previous study reports.

The previous study reported that antioxidant activity was exhibited by *D. eschscholtzii* and *Daldinia placentiformis* isolated from *Tridax procumbens* (Misra et al., 2019). In this findings, 87.3 ± 0.8% inhibition of free radicals was exhibited by *D. eschscholtzii*. The anti-diabetic activity shown by ethanol extract of *P. hababianum* may be related to its powerful antioxidant activity and therefore it is effective in preventing oxidative and cellular damage of β-pancreatic cells. The endophyic fungi which showed good anti-diabetic activity also exhibited good antioxidant activity. (Ganesan et al., 2016). These findings are in direct correlation with the previous findings. Therefore, the endophytic fungi isolated from *B. pinnatum* acts as an anti-diabetic as well as antioxidant agents.

Bioprospecting of endophytic fungi for biologically active metabolites is beneficial because once isolated, they can be reproduce, safe and helps to preserve biodiversity. This is the first report on isolation of *D. eschscholtzii* and *P. mangiferae* from stem and root parts of *B. pinnatum*. Thus, endophytic *D. eschscholtzii* and *P. mangiferae* are considered as a potential source in anti-diabetic drug development process.

Conclusion

The dichloromethane and ethyl acetate extracts of *D. eschscholtzii* and *P. mangiferae* are considered as the potent strains of anti-diabetic and antioxidant effects.

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Ethical Issue

The development, acquisition, authentication, cryopreservation, and transfer of cell lines between laboratories were followed according to the guidelines published in British Journal of Cancer, 2014.

Conflict of Interest

Authors declare no conflict of interest

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