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Bioactive Compound from the Endophytic Fungus *Paecilomyces variotii* Isolated from the Stems of *Physalis angulata* L.

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Abstract

Physalis angulata L., also referred to as "ciplukan" locally, is well-known for its use in traditional medicine due to the presence of bioactive compounds. Considered to be promising sources of bioactive chemicals, endophytic fungi living symbiotically within *P. angulata* tissues warrant investigation. The purpose of this study is to identify and investigate the characteristics of extracts and bioactive compound of endophytic fungus isolated from stem of *P. angulata*. The morphological identification was the first step in the study. For 4 weeks, the fungi were grown in PDB medium at room temperature in static circumstances. After dividing the liquid medium with ethyl acetate and evaporating it to produce concentrated extracts, it extracts' antibacterial and antioxidant properties were examined. The disc diffusion method was used to assess antibacterial activity, while the *1,1-diphenyl-2-picrylhydrazyl* (DPPH) assay was used to investigate the antioxidant potential. From the chosen active extracts, bioactive molecules were separated using column chromatography method, and spectroscopic analysis was used to identify the chemical structures of these compounds. Additionally, molecular identification of the endophytic fungus isolates was carried out. Five endophytic fungi in all were identified, numbered CB1, CB2, CB3, CB4 and CB5. Morphological identification indicated that the fungi belonged to 3 genera: *Papulaspora* sp. (CB1, CB2, and CB4), *Verticillium* sp. (CB3), and *Paecilomyces* sp. (CB5). The antibacterial activity of all the fungal extracts was found to be moderate to weak, with isolate CB5 exhibiting the highest action against *Staphylococcus aureus, Salmonella typhi, Escherichia coli,* and *Bacillus subtilis*. Molecular analysis identified the CB5 isolate as *Paecilomyces variotii*. The pure compound isolated from this fungus was identified as 4,5,6-trihydroxy-2',3',5',6-tetramethyl-[3,4'-bipyranylidene]-2-one, which demonstrated significant antioxidant activity (IC_{50} 23.82 μg/mL) and potent antibacterial effects (MIC < 100 μg/mL). These results demonstrate this endophytic fungus's potential as a useful source of bioactive chemicals for the treatment of bacterial infections and oxidative diseases associated with stress.

Keywords: Antibacterial, Antioxidant, Bioactive compound, *Paecilomyces variotii, Physalis angulata* L.

Introduction

The importance of health is increasingly recognized by people worldwide as modern society continues to evolve. Rapid advancements in science and technology have brought positive effects, such as easier access to healthcare services, medicines, and food. However, these developments also have a downside, including rising air pollution and waste accumulation, which can lead to increased free radicals and the emergence of various bacterial infections [1]. Today, society faces numerous challenges for survival due to the emergence of new diseases, such as degenerative conditions, pathogenic bacterial infections, drug resistance, and ecosystem imbalances. Endophytic fungi hold great potential for producing pharmaceutically valuable compounds, including anticancer, antioxidant, antimicrobial, and antidiabetic agents, which could serve as the basis for new therapies. These fungi are regarded as a treasure trove for bioprospecting, offering solutions to various complications in medical treatment [2].

The investigation of microorganisms as potential reservoirs of bioactive compounds commenced with the identification of the anticancer agent "Taxol" derived from *Taxomyces andreanae* in the early 1990s, as well as the antibiotic Penicillin isolated from *Penicillium notatum* by Alexander Fleming in 1928. Both of these pharmacological agents were extracted from fungal organisms. Taxol is derived from *Taxus brevifolia* and *Taxus wallinchiana*, which have endophytic fungi *Taxomyces andreanae* and *Pestalotiopsis microspora*. Both synthesize Taxol. The identification of these anticancer and antibiotic compounds from fungal microorganisms has paved the way for the exploration of novel pharmaceuticals derived from endophytic fungi. Numerous antimicrobial agents produced by endophytic fungi have demonstrated significant efficacy against pathogens that have acquired resistance to conventional antibiotics
[3].

Traditionally, *P. angulata* has been used to treat diabetes, inflammation, jaundice, and high cholesterol, as well as to support bone health and immune function. The active compounds found in *P. angulata* include steroids, flavonoids, terpenoids, alkaloids, saponins, withanolides, glycosides, physalins, and tannins, particularly the C28 steroidal lactone series [4,5]. The active compounds found in the stems of *Physalis angulata* include terpenoids, saponins, tannins, flavonoids, quercetin-3-O-glycoside, phenolic acids, and kaempferol-3,7-di-O-glycoside. The stem extract of *P. angulata*, with a total phenolic content of 15.43 mg GAE/g and total flavonoid content of 9.04 mg QE/g, exhibits antioxidant and antibacterial activity against *Streptococcus mutans* [5,6]. Physalin has been shown to enhance the activity of enzymes such as *glutathione peroksidase*, *catalase*, and *superoxide dismutase*, which function as antioxidants in inflammation and help prevent organ damage [6].

Based on several studies, *P. angulata* shows the potential to act as a repository of new antioxidant and antibacterial compounds. It is certain that its endophytic fungi will contain compounds or have the same biological activity as its host. The results of this study provide a strong theoretical basis that the endophytic fungus *Paecilomyces variotii* from the stem of *P. angulata* can accompany or replace the function of its host in producing bioactive compounds to enrich drug preparations.

Material and methods

Research plants

Samples were collected from Timbangan, North Indralaya, Ogan Ilir, South Sumatra, Indonesia (Latitude: −3.220330; Longitude: 104.649159). Plant identification was conducted at the Indonesian Biology Generation (Genbinesia) under determination number 08.115/Genbinesia/IX/2023. The *P. angulata* stems were cut 5 cm above the ground and measured 20 cm in length, with only healthy, dark green stems selected.

Isolation of endophytic fungi

The stems of *P. angulata* were meticulously cleansed using a continuous flow of water. The procedure for surface sterilization of the specimens was executed in 3 distinct phases. Initially, the specimens were submerged in a 70 % ethanol solution for an approximate duration of 2 min, subsequently treated with a 3 % sodium hypochlorite (NaOCl) solution for 1 min. Lastly, the specimens were thoroughly rinsed with distilled water for about 1 min and subsequently placed on sterile filter paper to dry [4]. The specimens were cut using a sterile knife and then transferred to sterile PDA media with chloramphenicol (0.2 g/L). The specimens

underwent incubation at 30 °C for a duration of 2 to 7 days. Fungi exhibiting distinctive morphological characteristics were individually inoculated onto PDA medium to procure pure isolates [12,13].

Identification of endophytic fungi

Uncontaminated endophytic fungal isolates were characterized through both microscopic and macroscopic examinations. Macroscopic characteristics were determined based on growth patterns, texture, colony color, topography, margin pattern, surface color, and other relevant attributes. For microscopic identification, the slide culture method was employed, and observations were made under a microscope (Hirox MXB-2500REZ), focusing on characteristics such as the shape of hyphae and spores. The data from both microscopic and macroscopic observations were then compared with references from books and several journals related to endophytic fungal identification [7-11].

Cultivation and extraction of endophytic fungi

The fungus was cultivated by inoculating 6 agar plates from uncontaminated isolates into 300 mL PDB. The cultures underwent incubation for a duration of 3 - 4 weeks or until a discernible color alteration was noted. Subsequently, the mycelium and liquid culture were isolated employing filter paper. The mycelium (biomass) was desiccated at 60 °C and preserved in a dehydrated form. Liquid culture was extracted with ethyl acetate at a ratio of 1:1 (1L:1L) for 3 days, accompanied by daily agitation. Using a separator funnel, the ethyl acetate extract was separated from the liquid culture and then evaporated at 40 °C in a rotary evaporator. The resultant concentrated extract was quantified using an analytical balance [18,19].

Antioxidant activity test

Test results from the samples ranged from 1,000 to 15.63 µg/mL. A DPPH solution (62.5 µM) volume of 3 mL was mixed with 1 mL of each endophytic fungus extract. A spectrophotometer was used to measure the absorbance at 517 nm after 30 min of incubation. Applying ascorbic acid served as positive [20]. The following formula was used to get the inhibition percentage [13]:

$$\text{Inhibition (\%)} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} \times 100$$

The inhibition percentage data is used to create a linear regression graph. The IC50 value is obtained from the linear regression equation $y = ax + b$.

Antibacterial activity test

The Kirby-Bauer method was used to test the antibacterial activity. The test bacteria included *Salmonella typhi* (IPCCCB.11.669), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), and *Escherichia coli* (ATCC 25922). Endophytic fungal extracts were applied at a concentration of 400 µg/disk. Positive control disks were treated with 30 µg of tetracycline, and negative control disks contained 10 µL of DMSO. The test microorganisms were added to Petri dishes, paper disks were placed on top of them, and the plates were then incubated for 24 h at 37 °C. The inhibitory zones' diameter was measured with a caliper. The inhibitory zone diameter was assessed using predetermined standards. [22]:

Weak : $\frac{A}{B}$ x 100 % < 50 %

Medium : 50 % < $\frac{A}{B}$ x 100 % < 70 %

Strong : $\frac{A}{B}$ x 100 % > 70 %

Note:
A: Zone of inhibition for sample (mm)
B: Zone of inhibition for antibiotics (mm)

The antibacterial activity test was further extended to determine the Minimum Inhibitory Concentration (MIC) for isolates exhibiting strong antibacterial activity, as well as their purified active compounds. The MIC of the purified compounds was tested at concentrations 256 to 1 µg/mL. The MIC value was determined by measuring the size of the inhibition zone, which was more than 9 mm and indicated the presence of antibacterial activity of the chemical at the corresponding concentration [23,24].

Molecular identification of endophytic jamur strain

The ITS region of rDNA was used to molecularly identify the most promising endophytic fungus isolates. Primers ITS5 5'(GGA AGT AAA AGT CGT AAC AAG G)3' and ITS45'(TCC TCC GCT TAT TGA TAT GC)3' were employed for DNA amplification. The acquired sequences were aligned with reference sequences from the database using Clustal W in the MEGA11 program, and they were then sent to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison. The DNA sequence's forward and reverse primers were put together using the BioEdit program. Using 1,000 bootstrap repetitions and the neighbor-joining method, a phylogenetic tree was created [25,26].

Isolation of pure compounds from selected endophytic jamur

The CB5 extract (2 g) was analyzed using TLC on silica gel G-60 F254 plates with various solvent systems to observe the spot patterns. Using silica gel as the stationary phase in column chromatography, the extract was pre-adsorbed, separated, and eluted using a gradient solvent system. The eluates were separated into column fraction groups based on spot patterns by *thin layer chromatography* (TLC) analysis after being collected in vials at intervals of 10 milliliters. Spot detection was carried out using reagent spraying and UV light at λ 254 nm. Each fraction was evaporated, weighed, transferred to vials, sealed, and stored in a refrigerator. Fractions with multiple spots were subjected to further separation by column chromatography using appropriate solvents. Fractions with the same TLC retention factor were combined, evaporated, weighed, transferred to vials, sealed, and stored in the refrigerator. This process was repeated until a fraction with a single spot on TLC was obtained. The pure fraction was then recrystallized to isolate the pure compound, which was analyzed by spectroscopy, including 1D and 2D NMR [17].

Result and discussion

Endophytic fungi isolated from stem of *P. angulata*

Five isolates, designated CB1, CB2, CB3, CB4, and CB5, were obtained from the stems of *P. angulata* through the isolation of endophytic fungi. The colony colors of these fungi varied, reflecting the distinctive characteristics observable from both the front and reverse sides of the colonies (**Figure 2**). Most of the front colonies were white and gray, while the reverse colonies exhibited colors ranging from white to cream, white to orange, and white to black.

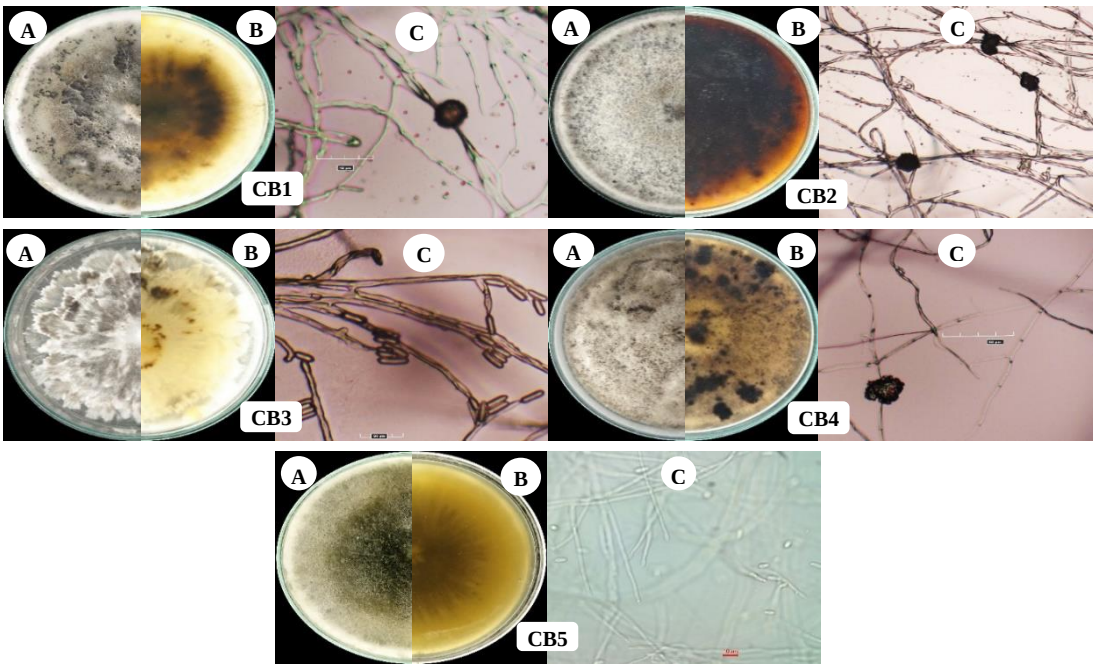

Figure 1 Characteristics of endophytic fungi from *P. angulata* stem. Macroscopic view (A): Front, (B): Reverse and (C) Microscopic view.

Morphological identification of endophytic fungi

The endophytic fungi isolated from the stems of *P. angulata* were identified both macroscopically and microscopically. Macroscopic characteristics were observed without magnification, focusing on criteria such as surface colony, reverse colony, exudate drops, concentric circles, topography, texture, pattern and radial lines. Using a Hirox MXB-2500REZ binocular compound microscope, microscopic features were seen, and 400x magnification images were captured. The observations included spore type, spore forms, hyphae, and specific features. These characteristics were compared with fungal identification books and related journals. The identification results are presented in **Tables 1** and **2**.

Table 1 Colony characteristics of endophytic jamur from the stem of *P. angulate*.

Code	Surface colony	Reverse colony	Texture	Topography	Pattern	Exudate drops	Radial line	Concentric cyrcle
CB1	Black-grey	Dark brown to pale cream	Cottony	flat	Spread	-	-	-
CB2	Black-grey	Dark brown to deep orange	Cottony	flat	Spread	-	-	-
CB3	Black-White	Cream to White	Cottony	flat	Flowery	-	-	-
CB4	Black-grey	Black and pale cream	Cottony	flat	Spread	-	-	-
CB5	Grey-cream	yellow	Cottony	flat	Spread	-	-	-

Isolates CB1, CB2, and CB4 share similar macroscopic characteristics. The surface colonies of all 3 fungi are black-grey, while their reverse colonies differ in color (**Figure 2**). They exhibit a cottony texture, flat topography, and a spread pattern. None of the 3 fungi displayed exudate drops, radial lines, or concentric circles (**Table 1**). The 3 isolates are comparable in microscopical aspects as well, such as the type of papulospores that form on agar, the gloobose spore forms, the septate hyphae, and the feature of the papulospores being fluffy and appearing smoky brown to dark brown. The hyphae have a hyaline or yellowish-green color, which subsequently turns brown. They are frequently spherical in shape and include granules (**Table 2**). These macroscopic and microscopic characteristics indicate that the 3 fungi are *Papulaspora* sp.

Isolate CB3 has a macroscopic surface colony color of black-white and a reverse colony color ranging from cream to white (**Figure 2**). It has a cottony texture, flat topography, and a flowery pattern, with no exudate drops, radial lines, or concentric circles (**Table 1**). Microscopically, it features conidia spore type, cylindrical spore forms, and septate hyphae. Conidiophores that are upright, hyaline, and primarily branching with verticillate phialides carrying terminal spore masses are a crucial feature. Phialides have a subtle collarette and taper progressively toward the points. The cylindrical, hyaline conidia. Based on these features, isolate CB3 was identified as *Verticillium* sp.

Isolate CB5 has a macroscopic surface colony of grey-cream and a reverse colony of yellow (**Figure 2**). The texture is cottony, with a flat topography and a flowery pattern. No exudate drops, radial lines, or concentric circles are present (**Table 1**). Microscopically, it displays a conidia spore type, obovate spore forms, and septate hyphae. The conidiophores (phialides) are easily recognized by their simple or infrequently branching erect, 1-septate basally, hyaline, tapering from base to apex, and bearing more than 10 catenulate conidia apically. The conidia have one cell, are ovate, phialosporous, hyaline, and somewhat apiculate at one end. Based on these characteristics, isolate CB5 was identified as *Paecilomyces* sp.

Table 2 Microscopic characteristics of endophytic jamur from the stem of *P. angulate*.

Isolates	Spora tyype	Spore forms	Hyphae	Specific characteristic	Genus
CB1	Papulospores	Globose	septate	Papulospores with a smoky brown or dark brown color that appeared puffy were developed on agar. Hyphae hyaline, or greenish-yellow in color, turning brown with time, frequently spherical in form, with granules inside	*Papulaspora* sp.
CB2	Papulospores	Globose	septate	Papulospores that appeared puffy and had a dark brown or smoky brown color on agar were developed. Hyphae hyaline, or yellowish-green colored, are frequently spherical in shape, have granules inside of them, and eventually turn brown.	*Papulaspora* sp.
CB3	Conidia	Cylindrical	septate	Hyaline, upright conidiophores that are primarily branching and have terminal spore masses borne by verticillate phialides that taper progressively toward the tips and have an inconspicuous collarette. cylindrical, hyaline conidia.	*Verticillium* sp.
CB4	Papulospores	Globose	septate	Papulospores that appeared puffy and had a dark brown or smoky brown color on agar were developed. Hyphae hyaline, or yellowish-green colored, are frequently spherical in shape, have granules inside of them, and eventually turn brown.	*Papulaspora* sp.
CB5	Conidia	Ovate	septate	Simple or infrequently branching, erect, hyaline, tapering from base to apex, 1-septate basally and bearing more than 10 catenulate conidia apically are the characteristics of conidiophores (phialides). Conidia hyaline, ovoid, one-celled, with a small apiculate tip at one end, phialosporous.	*Paecilomyces* sp.

Antioxidant activity of endophytic fungi

The endophytic fungi that were isolated from *P. angulata* stems have moderate to low levels of antioxidant activity. The IC_{50} values, from 125 to 950 µg/mL, demonstrate on **Table 3**. Four of the endophytic fungal extracts, namely CB1, CB2, CB4, and CB5, demonstrated moderate antioxidant activity, while only one extract, CB3, showed weak activity with an IC_{50} of 949.95 µg/mL.

The genus of endophytic fungi isolated from the stems of *P. angulata* has also been found in other medicinal plant organs, exhibiting varying bioactivities. *Papulaspora* sp., for example, has also been identified in other plants such as *Taxus chinensis* [19]. The endophytic fungus *Papulaspora nishigaharanus*, isolated from *Syzygium polyanthum*, exhibits strong antioxidant activity, with an IC_{50} of 25.42 µg/mL [20]. The endophytic fungus *Verticillium dahliae*, when inoculated into tomato plants treated with 100 and 50 mmol/L NaCl, was able to stimulate an increase in the production of antioxidants (proline, anthocyanin) and antioxidant enzymes (catalase, peroxidase) [21].

Table 3 *P. angulata* stem-isolated endophytic fungal extract's antioxidant properties (IC_{50} value).

Samples	Extract	Genus	Antioxidant activity IC_{50} (µg/mL)
Endophytic fungi	CB 1	*Papulaspora* sp.	285.44**
	CB 2	*Papulaspora* sp.	125.10**
	CB 3	*Verticillium* sp.	949.95*
	CB 4	*Papulaspora* sp.	239.26**
	CB 5	*Paecilomyces* sp.	151.26**
Positive control	Ascorbic acid		10.72****

Note: Antioxidant activity IC_{50} (µg/mL): ****very strong < 20 µg/mL; ***strong 20 - 100 µg/mL; **moderate 100 - 500 µg/mL; * weak > 500 µg/mL [18].

The endophytic fungus *Paecilomyces tenuis* is capable of producing the active compound alkaloid and Huperzine A (HupA), similar to its host *Huperzia serrata*. Huperzine A is commonly used in Alzheimer's therapy. IC_{50} of 1.27 ± 0.04 mg/mL indicates antioxidant activity in the *Paecilomyces tenuis* extract. Fungi from the genus *Paecilomyces* also function as biocontrol agents against fungal and bacterial phytopathogens, as well as saprophytes in soil. They are found in various organic substrates such as soil, bark, dead plants, and insect bodies [31]. These results imply that the endophytic fungi that were separated from *P. angulata* stems may one day be developed into natural antioxidants.

Antibacterial activity of endophytic fungi

Endophytic fungal extracts obtained from *P. angulata* stems showed a variety of responses in their antibacterial activity, from weak to strong (**Table 4**). Among the extracts, CB5 showed the most promising antibacterial activity, with strong activity observed against all 4 test bacteria, while its antioxidant activity remained moderate. However, compared to the other extracts, CB5's antioxidant activity was among the best, with an IC_{50} value of 151.26 µg/mL. The antibacterial activity of isolates CB1, CB2, and CB4 varied across the test bacteria, ranging from weak to strong. Isolate CB3 demonstrated moderate antibacterial activity against all bacterial strain

Table 4 Comparison of the percentage of antibacterial activity of endophytic jamur from stem of *P. angulate*.

Samples	Extract	Genus	Ethyl acetate extract weight (g)	Antibacterial activity (%)			
				S. typhi	***E. coli***	***B. subtilis***	***S. aureus***
Endophytic fungi	CB 1	*Papulaspora* sp.	0.87	61.54 ± 1.41**	80.00 ± 0.71***	86.96 ± 0.71***	66.67 ± 0.71**
	CB 2	*Papulaspora* sp.	0.58	61.54 ± 0.00**	70.00 ± 0.32***	69.57 ± 0.00**	50.00 ± 0.71*
	CB 3	*Verticillium* sp.	0.62	53.85 ± 0.71**	65.00 ± 0.00**	56.52 ± 0.00**	58.33 ± 0.50**
	CB 4	*Papulaspora* sp.	0.48	50.00 ± 0.00*	60.00 ± 0.21**	60.87 ± 0.00**	54.17 ± 0.04**
	CB 5	*Paecilomyces* sp.	0.98	73.08 ± 0.00***	85.00 ± 0.00***	78.26 ± 0.00***	70.83 ± 0.71***
Positive control	Tetracycline			100 ± 0.00***	100 ± 0.00***	100 ± 0.00***	100 ± 0.00***

Note: (***) strong > 70 %; (**) moderate 50 - 70 %; (*) weak < 50 %;

The fungus *Papulaspora immersa*, isolated from *Smallanthus sonchifolius*, exhibits antibacterial effects with a MIC of 90 µg/mL against *P. aeruginosa*, 240 µg/mL against *S. aureus*, and 220 µg/mL against *K. rhizophila*. The antibacterial compounds produced by *Papulaspora immersa* include diketopiperazines, taxol, and the enzyme amylase [23]. *Verticillium* sp. fungus has compounds from the *acremonidin* and

acremoxanthone groups. Acremonidin shows antibacterial activity against gram-positive bacteria (*Bacillus cereus and Enterococcus faecium*) with MIC of 0.39 - 6.25 and 1.56 - 25.0 µg/mL, respectively, while acremoxanthone shows antibacterial activity against Bacillus cereus with MIC of 6.25 - 25.0 µg/mL [24].

Isolated from the medicinal plant *Cornulaca monacantha*, the endophytic fungus *Paecilomyces* sp. (AUMC 15510) demonstrates strong antibacterial activity against *Pseudomonas aeruginosa* ATCC 90274 and *Bacillus subtilis* ATCC 6633 with MBC of 15.6 µg/mL and MIC of 3.9 µg/mL [25]. Additionally, *Paecilomyces* sp., isolated from *Panax ginseng*, produces the active compound falcarinol. The causal agent of rice neck rot, *Pyricularia oryzae*, was found to be inhibited in growth by an ether extract of *Paecilomyces* sp. at 7.8 µg/mL [26]. This suggests that the endophytic fungi isolated from *P. angulata* stems may be used to create novel therapeutic agents to fight pathogenic microorganisms because they have the capacity to manufacture antimicrobial chemicals.

Molecular identification of selected endophytic jamur

The endophytic fungal isolate CB5 was selected for biomolecular identification and secondary metabolite isolation due to its superior antibacterial activity compared to all other endophytic fungal isolates from the stems of *Physalis angulata* L. The antioxidant activity of isolate CB5 was classified as moderate, slightly weaker than the moderate activity of isolate CB3. A bootstrap consensus tree with 1,000 replications, the Neighbor-joining method, MEGA 11.0.13 was used to evaluate the molecular data, and the P-distance method was used to compute evolutionary distances. The result was a phylogenetic tree (**Figure 2**). Fourteen 100 % identical fungal DNA sequences were used in the phylogenetic study. To identify the fungus species, the ITS rDNA sequence from the molecular tests was sent to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Gene Bank. The ITS rDNA sequence is as follows:

CCCTTGAGGGGGAACTGCGGGAAGATCATTAC
CGAGTGAGGGTCCCTCGGGGCCCAACCTCCCA
TCCGTGTTGTCCTGACACCTGTTGCTTCGGCGG
GCCCGCCGTGGTTCACGCCCCGGCCGCCGGGG
GGTTCACGCCCCCGGGCCCGCGCCCGCCGAAG
ACCCCTGGAACGCTGCCTGGAAGGTTGCCGTC
TGAGTATACAATCAATCAATTAAAACTTTCAA
CAACGGATCTCTTGGTTCCGGCATCGATGAAG
AACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCCGTGAATCATCGAATCTTTGAAC
GCACATTGCGCCCCCTGGCATTCCGGGGGGCA
TGCCTGTCCGAGCGTCATTGCTAACCCTCCAGC
CCGGCTGGTGTGTTGGGCCGCCGTCCCCCCTCC
CCGGGGGACGGGCCCGAAAGGCAGCGGCGGC
GTCGCGTCCGGTCCTCGAGCGTATGGGGCTCT
GTCACACGCTTCAGTAGAACCGGCCGGCTTGC
TGGCCATCACCTATATTTTTCTCTTAGGTTGAC
CTCGGATCAGGTAGGGATACCCGCTGAACTTA
AGCATATCAATAAGCCGGAGGAAACGTCATGA
GAGTGGGTGCCGCTCGAACTCGGTGGTCCAAA
CTTTTTGGGGGGAGCCGTCTAATGTGAGACAC
GTGACTGGGACTAAATCTATAAAAAGCAACCC
AAGTTCCATCCGTTTCGCAGAGCAGGCCCGGA
AGAAGGGGCTTATCGATGGGGAGTCAGCCGTA
GGCCCTGCACCCATATCCAACCCGAGGTTGAA
GGATCCCCTTCGACTTGTATGAGTGCCGCCACC
CCGGACCACTCAGGGGAAAGTAATAGAATTAC
AAAAATGGGCAAGTATGGTGGTTATCTTAAAT
CTGGATAAATTCCGGTAGTCGTGCGTACCTTAT
CCGAATTCGTTGGTAGAAGTGTAGAGATTCTC
GGGGCTAGTTATGTGGAGTGGCATAGCTATTG
CCCCGTCTTGGGAAGTTGGGAAATTACATAAC
AACTAGTGCTACCCAGGCGACAATCGTAGGTA
GGGCTTGCGGGGATGGGCAGAGTCGCCGGTGC
GTGTAGTCGTCGGAAAATGGGGGGGGTCCGGT
CTAGAATGAAAGCAGTCACCCGCTGGACAGAT
GACATCGCTGCACTTGTCAGAAATGGAGGTTC
CTAATATAAGGGGTGAGAGGATTTGTGGTCAC
TCAGACTCGAGCGTGCCCGATGCTCATCGCGG
ATATTCGGAGTCGTTGGGGATCTTACTGATCCG
AGGTCACCTAAGAGAAAAATATAGGTGATGGC
CAGCAAGCCGGCCGGTTCTACTGAAGCGTGTG
ACAGAGCCCCATACGCTCGAGGACCGGACGCG
ACGCCGCCGCTGCCTTTCGGGCCCGTCCCCCG
GGGAGGGGGGACGGCGGCCCAACACACCAGC
CGGGCTGGAGGGTTAGCAATGACGCTCGGACA
GGCATGCCCCCCGGAATGCCAGGGGGCGCAAT
GTGCGTTCAAAGATTCGATGATTCACGGAATT
CTGCAATTCACATTACTTATCGCATTTCGCTGC
GTTCTTCATCGATGCCGGAACCAAGAGATCCG
TTGTTGAAAGTTTTAATTGATTGATTGTATACT
CAGACGGCAACCTTCCAGGCAGCGTTCCAGGG

GTCTTCGGCGGGCGCGGGCCCGGGGGCGTGAA
CCCCCCGGCGGCCGGGGCGTGAACCACGGCGG
GCCCGCCGAAGCAACAGGTGTCAGGACAACAC
GGATGGGAGGTTGGGCCCCGAGGGACCCTCAC
TCGGTAATGATCCTTCCGCAGGTTCACCTACGG
AAACCTTGTTACGACTTTTACTTCCAGAGAATT
GATACACACCAACTCACTGGACGGATCCTTGC
GTCGATCTGTAATGGCAGGGACTGCCTGGATC
CAACGAATGGAGCATCCAAATTTCCGCCCATT
GCCAAACTATTCCGGTATATGGGGCTACTCAT
GCGCTTACTGAGGGCTCGGCACTCTCACTACC
AACGCAAGTTGTGATGGTTCACGTTCACCTTG
AGTTGTCAAGCCCCCCGGCTTCGATCTGCTGAC
AGTCTAAGATTACTAAAAAAGTCAAGTCGTGA
TTGGAGTTCTAAGACTCGGAAAATTCGTGCGT
GGGTAACATCACTAATATAAAAGGATGAGGAC
GATTTGATGGGGGGTACGTAGACGGTGTTATT
TTGCTCCCTGTTGGGAATGGTTTACAGCTGCGA
ATCATTCATACCAACGGAATTTAATGTGAATG
TGGCCGTGTGAGTGTTGTCGTTGAGGTCCACG
GAAACAGGGGGGACGGTACAGGCGATCGTGA
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TTCACGAAATAAGAAATAAGAGATTCTGACTT
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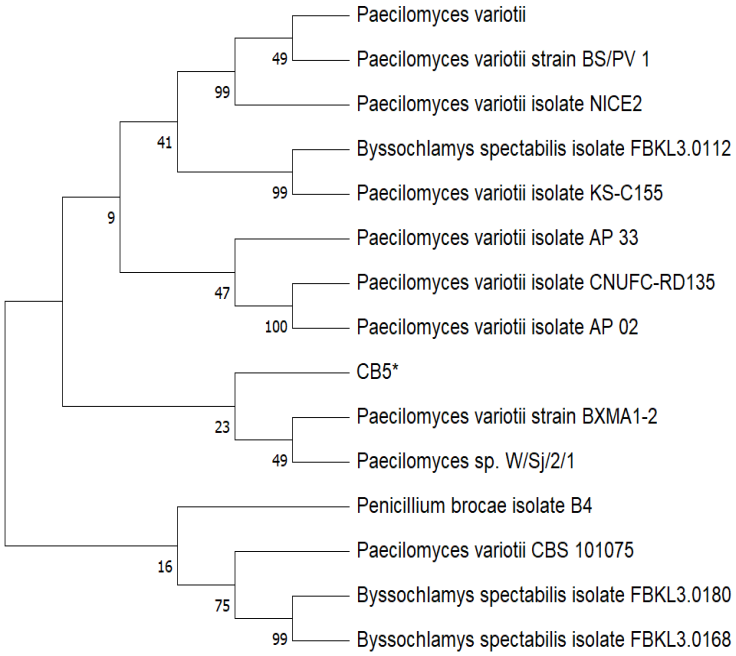

Figure 2 With a bootstrap value of 1,000, the phylogenetic tree of the CB5 isolates (signed*) was reconstructed using the neighbor-joining method.

Based on molecular analysis, isolate CB5 was identified as *Paecilomyces variotii*, also known as *Byssochlamys spectabilis*. Isolate CB5 closely resembled *P. variotii* strain BXMA1-2, with a frequency value of 27, and has been registered in the NCBI GenBank. The ascomycete fungus *P. variotii* is well-known for its capacity to generate secondary metabolites. This fungus also acts as a biocontrol agent (BCA) against nematodes and phytopathogenic fungi, such as *Fusarium moniliforme*, *Pyricularia oryzae*, *Fusarium graminearum*, and *Magnaporthe oryzae* [36,37].

The endophytic fungus *Paecilomyces farinosus* HF599 produces maleimide and farinomalein, which exhibit toxicity against the pathogen *Phytophthora sojae* at a concentration of 5 µg/disk [29]. Furthermore, the pathogenic fungus *Rhizoctonia solani*'s growth is inhibited by *Paecilomyces* sp., which was isolated from *Moringa oleifera*, with an inhibition rate of 76.25 %. Gas chromatography analysis of *Paecilomyces* sp. revealed compounds such as cis-13-Octadecenoic acid, methyl ester, 1-Heptacosanol, 1-Nonadecene, Cyclotetracosane [30].

Leucinostatin, a compound found in *Paecilomyces lilacinus*, exhibits antimicrobial activity against both

bacteria and fungi. After being separated from *P. lilacinus*, phomagpherine C had a MIC of 250 µg/mL against *Acinetobacter baumanii* [31]. *P. lilacinus* also demonstrates antimicrobial activity with inhibition zone of 17 mm (*Staphylococcus aureus*), 18 mm (*Escherichia coli*), and 18 mm (*Candida albicans*). The ethyl acetate extract of *P. lilacinus* shows strong antioxidant activity with a capacity of 76.77 ± 7.06 mg AAE/g dry extract. Active compounds in *P. lilacinus* include methyl ester, 10,13-octadecadienoic acid and hexadecanoic acid [32].

Paecilomyces cicadae contains a nucleoside called N6-(2-hydroxyethyl)-adenosine (HEA), which functions as an antioxidant by preventing H_2O_2-induced cell damage and lowering ROS generation to preserve or restore mitochondrial function [42,43]. The antioxidant activity of *P. cicadae* is further supported by its polysaccharide content, which enhances the performance of Glutathione peroxidase, superoxide dismutase and catalase enzymes and exhibits hepatoprotective properties [44]. *P. cicadae* contains crude protein under 10 kDa, with a MIC of 0.050 mg/mL against *Escherichia coli*. This crude protein disrupts the cell membrane structure and alters whole-cell or membrane protein content. Additionally, it can interact effectively with bacterial DNA [35].

The studies mentioned above provide crucial information indicating that *Paecilomyces* is an endophytic fungus with significant potential for development as a new therapeutic agent. Further research could be conducted to explore the potential of *Paecilomyces* as a biocontrol agent, in medicinal applications, and for environmental protection.

Isolation and Identification of bioactive compound

Using column chromatography and silica gel as the stationary phase, the 2 g EtOAc extract of the endophytic fungus *P. variotii* was separated. It was then eluted in a gradient with n-hexane (10:0 - 0:10) and EtOAc (10:1 - 5:5), yielding 7 subfractions (F1 - F7). Three subfractions (F4.1 - F4.3) were obtained from a further chromatography of subfraction F4 (602.7 mg) on silica gel with n-hexane as the eluent. After subfraction F4.2 was refined, 40 mg of a yellowish pure chemical were obtained.

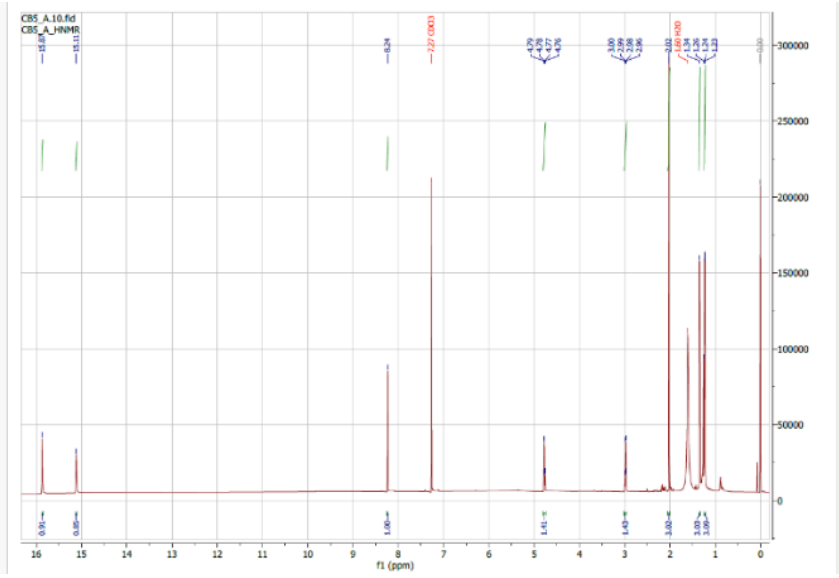

Figure 3 The 1H-NMR spectra of compound 1.

The ^{1}H-NMR spectrum (**Figure 3**) shows that compound 1 has 9 proton signals, consisting of 2 hydroxyl proton signals in the low field at δ_H 15.87 (1H, s) and 15.11 ppm (1H, s), and an oxygenated vinylic proton at δ_H 8.23 ppm (1H, s). Furthermore, singlet multiplicity signals for 4 methyl proton signals are detected at δ_H 2.02 (3H, s) and 1.25 ppm (3H, s), and doublet multiplicity at δ_H 1.34 (3H, d, J = 7 Hz) and 1.21 ppm (3H, d, J = 7 Hz).

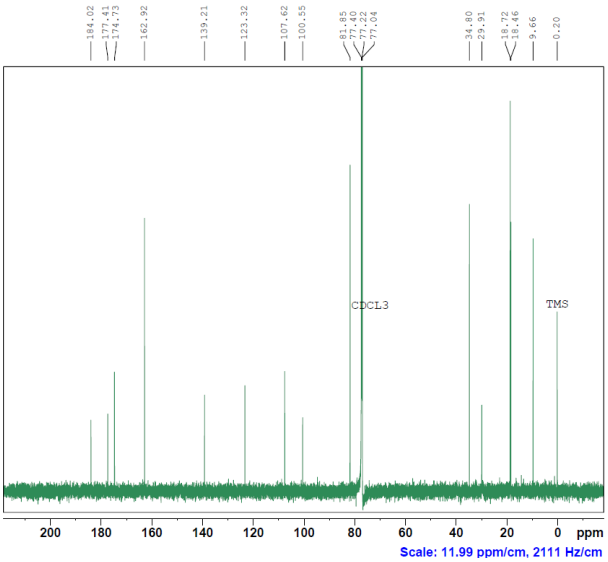

Figure 4 The ^{13}C-NMR spectra of compound 1.

The ^{13}C-NMR spectrum (**Figure 4**) indicates that compound 1 contains 14 carbon signals, including 7 sp^2 carbons and 7 sp^3 carbons. The carbon signal at δ_C 184.0 ppm suggests the presence of an ester carbonyl. The presence of oxygenated vinyl carbons is indicated by 3 additional low-field carbon signals at δ_C 162.9, 174.7, and 177.4 ppm. Three other sp^2 carbon signals appear as quaternary carbons. The sp^3 carbon signals at δ_C 81.8 and 100.6 ppm suggest that compound 1 contains mono-oxygenated and di-oxygenated carbons. The remaining sp^3 carbons appear as 3 methyl carbons and one methine carbon.

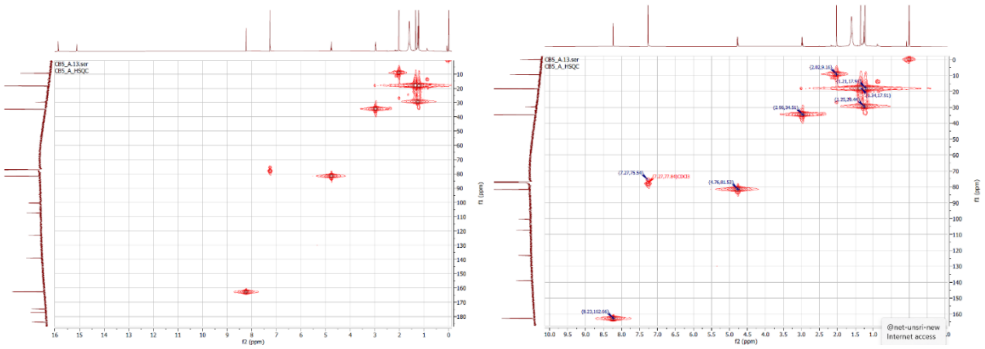

Figure 5 The HSQC spectral of compound 1.

The direct correlation between carbon and proton atoms in compound 1 can be observed in the HSQC (Heteronuclear Single Quantum Correlation) spectrum, as shown in **Figure 5**. The spectrum reveals 7 proton-to-carbon correlations. A vinylic proton signal at δ_H 8.23 ppm (1H, s) correlates with an sp^2 carbon at δ_C 162.9. An oxygenated carbon at δ_C 81.8 ppm is observed to correspond with the proton signal at δ_H 4.76 ppm (1H, q, J = 7 Hz). There is a correlation between a carbon at δ_C 34.8 ppm and another quartet proton at δ_H 2.96 ppm (1H, q, J = 7 Hz). Additionally, 4 methyl proton signals at δ_H 1.25 (3H, s), 1.21 (3H, d, J = 7 Hz), 1.34 (3H, d, J = 7 Hz), and 2.02 ppm (3H, s) each correlate with carbons at δ_C 29.9, 18.7, 18.5, and 9.7 ppm, respectively. Moreover, 2 low-field proton signals were observed without carbon correlations, and 7 carbon signals did not show proton correlations. Thus, the HSQC spectrum indicates that compound 1 contains one vinylic proton, one oxygenated sp^3 methine proton, one sp^3 methine proton, and 4 methyl protons. Additionally, compound 1 has 2 hydroxyl groups involved in hydrogen bonding, one hydroxyl group attached to a di-oxygenated carbon (δ_C 100.6 ppm), and 7 quaternary carbon atoms.

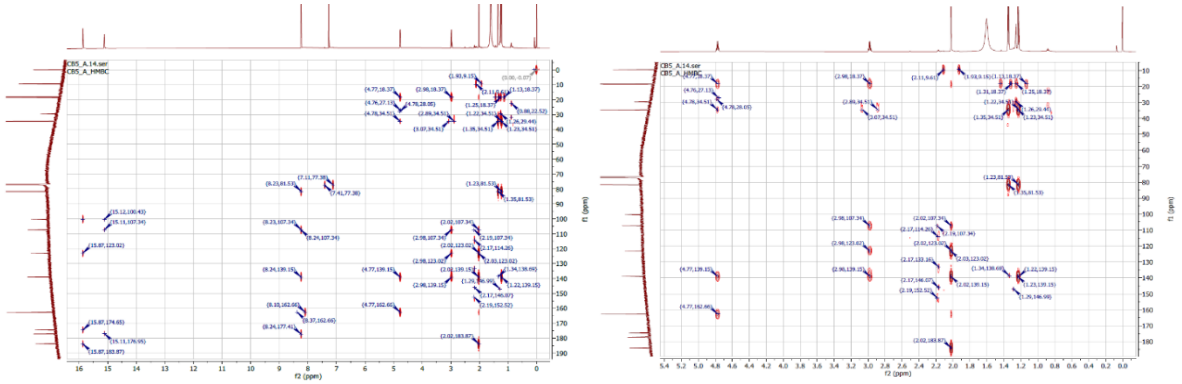

Figure 6 The HMBC Spectral of Compound 1.

The HMBC spectrum was utilized to ascertain the correlation between protons and adjacent carbons throughout a span of 2 to 3 bonds, as illustrated in **Figure 6**. The spectrum reveals that 2 protons, an sp^3 methine proton at δ_H 2.96 ppm (1H, q, J = 7 Hz) and an oxymethine proton at δ_H 4.76 ppm (1H, q, J = 7 Hz), correlate with 2 common carbons at δ_C 18.5 and 139.2 ppm. Additionally, the oxymethine proton correlates via 3 bonds with a carbon at δ_C 162.9 ppm, while the methine proton correlates via 3 bonds with carbons at δ_C 107.6 and 123.3 ppm. This suggests that the 2 protons are adjacent and positioned on a pyran ring. The vinylic proton at δ_H 8.23 ppm (1H, s) correlates over 2 and 3 bonds with carbons at δ_C 81.8, 107.6, and 139.2 ppm. In addition to showing long-range coupling with carbon at δ_C 123.3 ppm, the methyl proton at δ_C 2.02 ppm (3H, s) correlates via 3 bonds with carbons at δ_C 107.6, 139.2. This suggests the presence of a double bond within the pyran ring, involving a methine carbon and a quaternary carbon that also binds a methyl group. The 2 methyl protons at δ_H 1.21 ppm (3H, d, J = 7 Hz) and 1.34 ppm (3H, d, J = 7 Hz) both correlate over 2 and 3 bonds with the same carbons at δ_C 34.8 and 81.8 ppm. Additionally, the methine proton at δ_H 1.21 ppm further correlates with a carbon at δ_C 139.2 ppm. These findings confirm that the pyran ring in compound 1 is attached to 2 additional methyl groups.

In addition, **Figure 6** shows 2 signals of hydroxyl proton in the low field (δ_H 15.11 and 15.87 ppm), suggesting that chemical 1 has 2 nearby hydroxyl groups that form hydrogen bonds. The existence of 2 low-field carbon signals at δ_C 174.7 and 177.4 ppm lends support to this. The 2 hydroxyl protons show long-range coupling from the hydroxyl proton at δ_H 15.87 ppm and correspond with the same carbon at δ_C 100.6 ppm via 3 bonds from the hydroxyl proton at δ_H 15.11. While the hydroxyl proton at δ_H 15.87 ppm correlates over 3 bonds with the carbons at δ_C 123.3 and 174.7 ppm and exhibits long-range coupling with the lactone carbonyl carbon at δ_C 184.0 ppm, the hydroxyl proton at δ_H 15.11 further correlates with the carbon at δ_C 177.4 ppm. Because of its linkage to 2 oxygen atoms—one from the lactone and one from a hydroxyl group—as well as a methyl group (δ_H 1.25 ppm), the carbon at δ_C 100.6 ppm is a quaternary sp^3 carbon in the low field.

Table 5 NMR 1D and 2D spectral data of compound 1.

Number of C	δ_C ppm 1	δ_H ppm (ΣH. Multiplicity, Hz) 1	HMBC 1
2	184.0		
3	123.3		
4	177.4		
5	174.7		
6	100.6		
6-CH_3	29.9	1.25 (3H, s)	
2’	81.8	4.76 (1H, q, J = 7 Hz)	18.5; 139.2; 162.9

Number of C	δ_C ppm 1	δ_H ppm (ΣH. Multiplicity, Hz) 1	HMBC 1
2'-CH_3	18.5	1.34 (3H, d, J = 7 Hz)	34.8; 81.8
3'	34.8	2.96 (1H, q, J = 7 Hz)	18.5; 107.6; 123.3; 139.2
3'-CH_3	18.7	1.21 (3H, d, J = 7 Hz)	34.8; 81.8; 139.2
4'	139.2		
5'	107.6		
5'-CH_3	9.7	2.02 (3H, s)	107.6; 123.3; 139.2
6'	162.9	8.23 (1H, s)	81.8; 107.6; 139.2
4-OH		15.87 (1H, s)	100.6; 123.3; 174.7; 184.0
5-OH		15.11 (1H, s)	100.6; 177.4

Based on the analysis of the ^{1}H-NMR, ^{13}C-NMR, HSQC, and HMBC spectra, it was determined that compound 1 possesses a bipyranylidene framework, consisting of 2 pyran rings (A and B), with ring A being a lactone. Compound 1 contains 3 hydroxyl groups attached to ring A, 4 methyl groups distributed on rings A and B, and a vinylic methine group. Therefore, compound 1 is proposed to be 4,5,6-trihydroxy-2',3',5',6-tetramethyl-[3,4'-bipyranylidene]-2-one, with the molecular formula $C_{14}H_{18}O_6$ and a double bond equivalent (DBE) of 6. The 1D and 2D NMR spectra data for chemical 1 are shown in **Table 5**. **Figure 7** shows the molecular structure of compound 1, including the carbon atom numbering, the proton and carbon chemical shifts, and the HMBC correlations.

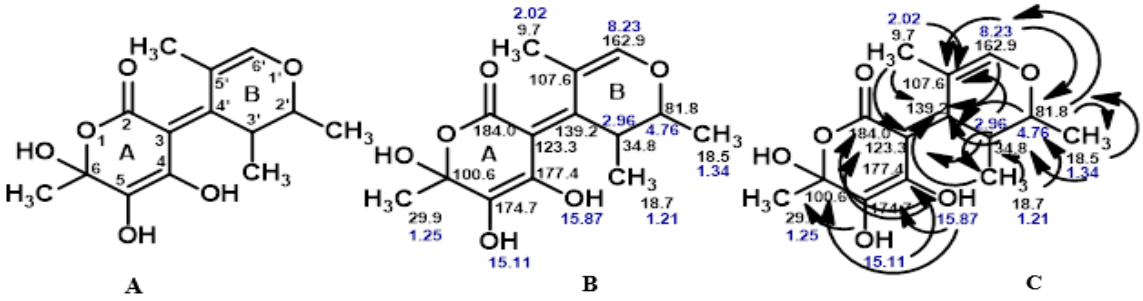

Figure 7 Molecular structure of compound 1 as 4,5,6-trihydroxy-2',3',5',6-tetramethyl-[3,4'-bipyranylidene]-2-one, featuring (A) carbon atom numbering, (B) the placement of proton and carbon chemical shifts, and (C) HMBC correlations.

The pure compound obtained was subjected to Inhibitory Concentration 50 (IC_{50}) and MIC analysis to evaluate its inhibitory effect against test bacteria and DPPH free radicals. The MIC and IC_{50} values of the pure compound are presented in **Table 6**.

Table 6 IC_{50} and MIC values of the pure compound isolated from the ethyl acetate extract of the endophytic fungus *P. variotii*.

Compound	IC_{50} (µg/mL)	MIC Value (µg/mL)			
		S. aureus	***E. coli***	***B. subtilis***	***S. typhi***
Pure compound	23.82	31.25	62.5	62.5	62.5

Based on **Table 6** data, the pure compound's antioxidant activity is categorized as strong, with an IC_{50} value of 23.82 µg/mL. The ethyl acetate extract's antioxidant activity is substantially lower than the pure compound (IC_{50} 151.26 µg/mL). Consequently, by separating its pure components, *P. variotii*'s

development as a source of antioxidant chemicals seems more potential. Furthermore, MIC values of less than 100 µg/mL demonstrate the potent antibacterial activity of the pure chemical against all test microorganisms. Similarly, the ethyl acetate extract produced by the endophytic fungus *P. variotii* exhibits comparable antibacterial potency. Hence, for the development of this endophytic fungus as a source of antibacterial compounds, both the extract and the pure compound demonstrate equally strong potential.

Conclusions

Endophytic fungus *Paecilomyces variotii* isolated from *P. angulata* stem produces 4,5,6-trihydroxy-2',3',5',6-tetramethyl-2',3'-dihydro-[3,4'-bipyranylidene]-2-one compound which shows strong antioxidant and antibacterial activity. This compound is not produced by its host *P. angulata*. This new compound has great potential to be developed into a new antibiotic to overcome the problem of antibiotic resistance and as a source of new bioactive compounds for degenerative diseases. Thus, the results of this study provide a strong scientific basis for making *Paecilomyces variotii* a source of antioxidant and antibacterial bioactive compounds as a companion or substitute for its host *P. angulata* to complement the diversity of new types of drugs in order to overcome global health problems.

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