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Diversity of endophytic fungi isolated from tree spinach (*Cnidoscolus aconitifolius*) (Euphorbiaceae) and their bioactivities

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Abstract. *Praptiwi, Palupi KD, Ilyas M, Marlina L, Fathoni A. 2023. Diversity of endophytic fungi isolated from tree spinach* Cnidoscolus aconitifolius *(Euphorbiaceae) and their bioactivities. Biodiversitas 24: 6229-6239.* Endophytic fungi associated with medicinal plants have been known as a reservoir for therapeutic compounds. *Cnidoscolus aconitifolius* I.M. Johns (Euphorbiaceae), which possesses several local names, including papaya jepang in Indonesia, has been historically used as traditional medicine. However, research on the bioactivity of endophytic fungi associated with it has yet to be conducted. The purposes of the study were to isolate endophytic fungi from *C. aconitifolius* and determine their bioactivity, i.e., antibacterial activity against *Escherichia coli* and *S. aureus* and free radical scavenging activity representing antioxidant activity. Endophytic fungi were identified based on their morphological characteristics, and the potential endophyte was further identified using molecular identification. Total phenolic and flavonoid contents were determined by the spectrophotometric method. The qualitative antibacterial and antioxidant bioactivity screening was performed using the TLC-bioautography method. The quantitative analysis of antibacterial and antioxidant activity was conducted by microdilution to obtain IC_{50} and MIC values. In total, 14 endophytic fungal isolates were successfully isolated. Antioxidant analysis showed that one isolate had strong antioxidant activity, and one had moderate growth inhibition activity against *E. coli*. The total phenolic content strongly correlates with the AAI value as an indicator of antioxidant activity. The findings of this study reveal that *Phomopsis* PjBg-1, an endophytic fungus from *C. aconitifolius, is* identified as *Phomopsis* sp. LH243 possesses strong antioxidant activity and moderate antibacterial activity against *E. coli*. Endophytic fungi from *C. aconitifolius* have the prospect of being an alternative to antioxidants and antibacterials. The isolation of compounds responsible for bioactivities needs to be further studied.

Keywords: Antioxidant, bioactivities, *Cnidoscolus aconitifolius*, endophytic fungi, papaya jepang

INTRODUCTION

Antimicrobial resistance (AMR) is increasing at an alarming rate daily. AMR may result in prolonged hospital stays, high medical costs, and mortality. Antimicrobial-resistant bacteria could reduce antibiotic effectiveness, increasing therapeutic failure and mortality (Kovač et al. 2015). The overuse and misuse of antibiotics primarily cause antimicrobial-resistant bacteria, which are estimated to cause 10 million deaths yearly (Sugden et al. 2016). Therefore, identifying new antimicrobials from natural sources that could overcome AMR is urgently needed.

Another serious health problem is oxidative stress due to altered lifestyles (Santra and Banerjee 2022). Oxidative stress occurs due to the imbalance between the production and accumulation of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNC) in cells and tissues and the ability of cells to detoxify these by-products (Olufunmilayo et al. 2015). Reactive substances such as ROS and RNS are produced continuously in the human body as by-products of human activities and are known as endogenous free radicals. However, due to lifestyle changes, there is excessive production of reactive substances, resulting in oxidative stress. Oxidative stress plays a role in the development or progression of several diseases, such as cancer, diabetes, atherosclerosis, and cardiovascular diseases (Pizzino et al. 2017). The human body has a defense mechanism to combat the effects of free radicals and oxidative stress; however, this is insufficient to scavenge ROS produced by mitochondria (Glasauer and Chandel 2014), especially in the excessive production of free radicals. It leads to the emergence of several diseases in the long term (Losada-Barreiro et al. 2017).

The excessive free radicals and reactive oxygen species (ROS) in the body could be neutralized by antioxidants. There is an increasing demand for natural antioxidants due to the possible adverse effects and increasing safety concerns of synthetic antioxidant consumption (Lourenço et al. 2019). One potential source of natural antioxidants could be endophytic fungi. Deshmukh et al. (2015) stated that endophytic fungi are a significant source of therapeutically effective chemicals.

Cnidocolus aconitifolius has several local names. i.e., tree spinach, chaya, and pepaya jepang (Indonesia). It belongs to Euphorbiaceae and has been used for several purposes in daily life, such as a green leafy vegetable, living fence, decoration, and traditional medicine. It contains terpenoids, alkaloids, flavonoids, and phenolics (Panghal et al. 2021). The uses of *C. aconitifolius* in traditional medicine were for treating snake bites, insect

bites, skin irritation, and wounds (Bautista-Robles et al. 2020). Previous studies reported on the bioactivities of *C. aconitifolius*. The extract of *C. aconitifolius* inhibited the growth of several bacterial and fungal isolates (Ogbu et al. 2019). The aqueous extract of *C. aconitifolius* could ameliorate liver damage in CCl_4-induced hepatotoxicity in rats (Akachukwu et al. 2014). Ethyl acetate and methanol extracts possess antioxidant and free radical scavenging activity against DPPH reagents (Hamid et al. 2016).

Many studies have been conducted on endophytic fungi from healthy plant tissue and evaluated their bioactivities. Endophytic fungi are microorganisms that inhabit internal plant tissues without causing pathogenic symptoms to the host plant (Song et al. 2017). It is estimated that there may be more than 3 million species, but only about 150,000 species of fungi have been named and classified to date (Bhunjun et al. 2023).

Recently, fungal endophytes have become increasingly recognized for their significant contribution as a valuable reservoir of bioactive substances possessing distinct chemical properties and fascinating biological effects (Gakuubi et al. 2021). Endophytic fungi also establish host defense mechanisms through secondary metabolites to enhance the host's capacity to adapt to a wide range of biogeographical ecosystems. Endophytic fungi can also produce metabolites that mimic the host plant (Rai et al. 2021). They are called the "factories" of multipotent metabolites with various bioactivities advantageous to the modern pharmaceutical business (Santra and Banerjee 2022). For instance, three endophytic fungi from *Ocimum basilicum* were reported as promising antimicrobial and antioxidant sources (Sharaf et al. 2022). Due to the ability of endophytic fungi to mimic plant-based metabolites and results from initial studies on the endophytic fungi from tree spinach, this study was performed to isolate and identify the endophytic fungi from tree spinach (*C. aconitifolius*) and evaluate their potential, especially as alternative sources of antibacterials and antioxidants. The potential endophytic fungi from *C. aconitifolius* could be used as alternative sources of antioxidant and antibacterial.

MATERIALS AND METHODS

Collection and surface sterilization of *Cnidoscolus aconitifolius*

Endophytic fungi were isolated from *C. aconitifolius* (Mill.) I.M. Johns (chaya, tree spinach, or papaya jepang) grown in Cibinong (S: 6°29'30.4" E: 106°51'23.9" 140 m alt.), West Java Province, Indonesia. Plant samples were collected from fresh material and healthy living tissue consisting of stems, lamina, petioles, peduncles, and flowers. All fresh samples were packed carefully in plastic bags and then transferred to the laboratory for isolation purposes for less than 24 hours. Fresh samples were cleaned under running tap water, followed by surface sterilization.

Isolation of endophytic fungi

Plant samples were immersed in 70% ethanol for 1 minute and then sterilized with 1% sodium hypochlorite (NaOCl) solution for 2 minutes. Samples were rinsed twice in sterile distilled water and put into sterile paper towels to remove water from the surface. Afterward, samples were aseptically cut into small segments of about 5 mm^2 and then placed onto 90-mm Petri dishes containing corn meal malt agar (CMMA) media. Three replicates were made for each plant part and placed on CMMA. The Petri dishes were then incubated at room temperature for about one week. The mycelia of endophytic fungi that emerged from samples were isolated and purified by being transferred onto a 60-mm Petri dish containing potato dextrose agar (PDA). Backup collections were kept by freezing at -80°C using 10% (v/v) glycerol and 5% (g/v) trehalose as a cryoprotectant (Kanti et al. 2018)

Identification of endophytic fungi from *C. aconitifolius*

Pure endophytic fungi were transferred, grown on PDA plates, and then incubated at 27°C for 5-10 days. Initial fungi identification was carried out based on morphological characteristics. Morphological identification was conducted by observing both macroscopic and microscopic properties. Macroscopic characterizations include observations on color, colony shape, surface, texture, exudate drop, and reverse color. Microscopic slides of each selected strain were prepared using lactophenol as a mounting medium. Microscopic characterizations were performed with a light microscope by observing hyphae, hyphae pigmentation, septate, clamp connection, conidia, spores, and other reproductive structures.

The potential strains were then selected for further molecular identification. The molecular identification analysis of the DNA sequence of an internal transcribed spacer (ITS) of rDNA regions includes the 5.8S rRNA. The total fungal genomic DNA was isolated according to the manufacturer`s instructions using Nucleon PhytoPure, plant, and fungal DNA extraction kits (GE Healthcare). DNA amplification of the ITS rRNA gene region was performed by polymerase chain reaction (PCR). PCR amplification was performed in 25 µL reaction mixtures containing 10 µL distilled water, 12,5 µL GoTaq Green Master Mix (Promega), 0.5 µL DMSO, 0,5 µL each primer (10 pmol), and 1 µL (5 to 10 ng) extracted genomic DNA as a template. The primer set of ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) was used to amplify approximately 550 nucleotides from ITS1 and ITS 2, including 5.8S rDNA (Fathoni et al. 2022a). Amplification was performed in a TaKaRa PCR Thermal Cycler P650 (TAKARA BIO Inc.), programmed under the following conditions: initial denaturation at 95°C for 3 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were then subjected to purification and sequence analysis. The sequencing reaction was carried out using Sanger sequencing methods provided by Macrogen Inc., Korea.

Initial phylogenetic tree construction of selected strains was conducted by editing the raw sequence data using

ChromasPro (http://www.technelysium.com.au/ChromasPro.html). The assembled sequences were submitted to GenBank and then aligned with those downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nlh.gov/) using the Muscle (http://www.ebi.ac.uk/Tools/msa/muscle). The phylogenetic analyses of sequence data were done based on the neighbor-joining (NJ) method using the Molecular Evolutionary Genetics Analysis (MEGA) version 7 program (Kumar et al. 2016). The reliability of each branch was evaluated by bootstrapping with 1000 resampling.

Extraction and analysis of secondary metabolites produced by endophytic fungi

Extraction of secondary metabolites produced by endophytic fungi was carried out by extraction using ethyl acetate as a solvent thrice. The extract was concentrated using a rotary evaporator. Part of the concentrated extract was made as the stock solution at 10 mg/mL. Ten µL of stock solution was spotted on the TLC plate (Silica F_{254}, Merck). After finishing spotting, the TLC plate was eluted using an eluent of dichloromethane: methanol (10:1). The chromatogram of the eluted plate was observed under UV light at 254 nm and 366 nm. The eluted plate was also sprayed with cerium sulfate and vanillin sulfate.

Antibacterial assay by TLC-bioautography (Praptiwi et al. 2020)

Antibacterial assay of the endophytic fungal extract was performed against *Escherichia coli* and *Staphylococcus aureus*. Ten µL of extract stock solution was spotted on the TLC plate and then eluted using dichloromethane: methanol (10:1). The plate was dried, dipped into the bacterial suspension, and incubated at 37°C for 18 hours under humid conditions. After incubation, the microplate was sprayed with iodonitrotetrazolium solution (4 mg/mL) and incubated again for 5 min. The formation of white spots or bands indicated the chemical compounds with antibacterial activity.

Assay of DPPH antioxidant activity by TLC-bioautography (Praptiwi et al. 2020)

Ten µL of extract stock solution were spotted on the TLC plate and then eluted using dichloromethane: methanol (10:1). The plate was dried and sprayed with DPPH methanolic solution (0.2%) followed by incubation at room temperature in darkness for 30 min. Compounds with DPPH antioxidant activity were indicated by yellowish-white spots or bands on a purple background.

Measurement of the Minimum Inhibitory Concentration (MIC) (Praptiwi et al. 2020)

The minimum inhibitory concentration value was measured using the serial dilution method in a 96-well microplate against *E. coli* and *S. aureus*. The well on the 1st row was filled with 100 µL of double-strength Mueller Hinton Broth and 90 µL of sterile aquadest. Other wells were filled with 100 µL of Mueller Hinton Broth Ten µL extract at 10.240 µg/mL in DMSO (Merck, Germany), added to the well in row A, and homogenized. Serial dilutions were performed by transferring 100 µL to the next row in the same column. In the last row, 100 µL of the was discarded after homogenizing. After diluting, 100 µL of bacterial suspension (5 x 10^5 CFU/mL) was added to each well and then incubated at 37°C for 18-20 hours in humid conditions. It was followed by adding 10 µL of iodonitrotetrazolium chloride (INT) to each well and incubating again for 15-30 minutes. Mixtures in the wells that did not change color to red indicated that the extract inhibited the growth of bacteria. The lowest concentration that did not change color was the value of MIC (Praptiwi et al. 2016).

Determination of IC_{50} value for DPPH radical scavenging activity and Antioxidant Activity Index (AAI) (Praptiwi et al. 2020)

The IC_{50} value for DPPH radical scavenging activity was measured by a serial dilution method in a 96-well microplate. One hundred and ninety-five µL of methanol p.a were transferred to the wells in row A, added with 5 µL extract at 10.240 µg/mL, and homogenized. Wells on other rows were filled with 100 µL of methanol p.a. One hundred µL in row A were pipetted and transferred to row B in the same column. The same procedure was applied to the next row. After adding 100 µL in the last row and homogenized, 100 µL was discarded. After serial dilution, DPPH solution in methanol (61.50 µg/mL) was added to every well, followed by incubating the plate in darkness for 90 min at room temperature. The absorbance of the extract was observed at 517 nm using a microplate reader (Varioscan Flash, Thermo Scientific). Inhibitory concentration was calculated using the following equation:

$$IC\ (\%) = (A_{DPPH}\ 100\% - A_{extract}) * 100 / A_{extract}$$

Where:
IC: Inhibitory concentration
A: Absorbance

Total phenolic content determination

Total phenolic content was measured using a UV-Vis spectrophotometer (Shimadzu, Japan). 0.2 mL of the extract with a concentration of 10 mg/mL in ethanol p.a, 0.2 mL of 50% Folin-Ciocalteu solution was added, and then vortexed for 1 minute, followed by the addition of 4 mL of 2% sodium carbonate (Na_2CO_3) solution. This mixture was kept in a dark room for 30 minutes. The absorbance of the extract solution was read at a wavelength of 750 nm with a UV-Vis spectrophotometer (Shimadzu, Japan). The results were expressed as mg of gallic acid/g extract (Praptiwi et al. 2020).

Total flavonoid content determination

Total flavonoid content was measured using a UV-Vis spectrophotometer (Shimadzu, Japan). As much as 2 mL of aquabidest 0.15 mL of 5% $NaNO_2$ were added to 0.5 mL extract in ethanol p.a with a 10 mg/mL concentration. After 6 minutes, 0.15 mL of 10% $AlCl_3$ was added, and the mixture was vortexed and incubated. After another 6 minutes, 2 mL of 1 M NaOH and aquabidest were added to

a total volume of 5 mL and incubated for 15 minutes in a dark room. The absorbance of the solution was measured at a wavelength of 510 nm (Praptiwi et al. 2020).

Chemical compounds analysis of potential fungal endophyte by Gas Chromatography-Mass Spectrometry

Analysis of GC-MS was performed using a Gas Chromatograph (Agilent 19091S-433UI:93.928) coupled with aMass Spectrophotometer (MS) and equipped with a capillary column of 5% phenyl methyl siloxane, 30 m x 250 µm x 0.25 µm. Helium was used as a mobile phase (carrier gas) with a 1.0 mL/min flow rate) column velocity flow. The injection volume was 1µL. The initial oven temperature was 40°C and increased at 10°C/min until reaching 300°C for 4 min. Compound identification was done by comparing the mass spectrum with the standard mass spectra available in the NIST library.

RESULTS AND DISCUSSION

Fourteen isolates of endophytic fungi were isolated from stems, lamina, petioles, peduncles, and flowers of *C. aconitifolius* collected from Cibinong, West Java Province, Indonesia. Based on the macroscopic and microscopic identification, the fungal isolates belonged to three genera, i.e., *Colletotrichum* sp., *Lasiodiplodia* sp., and *Phomopsis* sp. (Figure 1, Table 1). Identification of potential isolate PjBg-1 based on ITS rRNA gene sequence showed that PjBg-1 was *Phomopsis* sp. LH243 with a similarity of 98.16% (Figure 2, Table 2).

Analysis of secondary metabolites of fungal endophytes by thin-layer chromatography (TLC)

Secondary metabolites produced by fungal endophytes from *C. aconitifolius* were analyzed using TLC (Figure 3). The chromatogram in Figure 3 showed several spots. Every spot represents chemical compounds in the fungal endophyte.

Table 1. Endophytic fungi inhabiting *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst. collected from Cibinong, West Java Province, primary grouping based on morphology

Strain code	Plant part	Fungal taxa
PjBg-1	Flower	*Phomopsis* sp.
PjBg-2	Flower	*Phomopsis* sp.
PjBg-3	Flower	*Phomopsis* sp.
PjBt-1	Stem	*Phomopsis* sp.
PjBt-2	Stem	*Phomopsis* sp.
PjBt-3	Stem	*Phomopsis* sp.
PjTd-1	Petiole	*Phomopsis* sp.
PjTb-1	Peduncle	*Lasiodiplodia* sp.
PjTb-2	Peduncle	*Phomopsis* sp.
PjDn-1	Lamina	*Lasiodiplodia* sp.
PjDn-2	Lamina	*Colletotrichum* sp.
PjDn-3	Lamina	*Phomopsis* sp.
PjDn-4	Lamina	*Phomopsis* sp.
PjDn-5	Lamina	*Lasiodiplodia* sp.

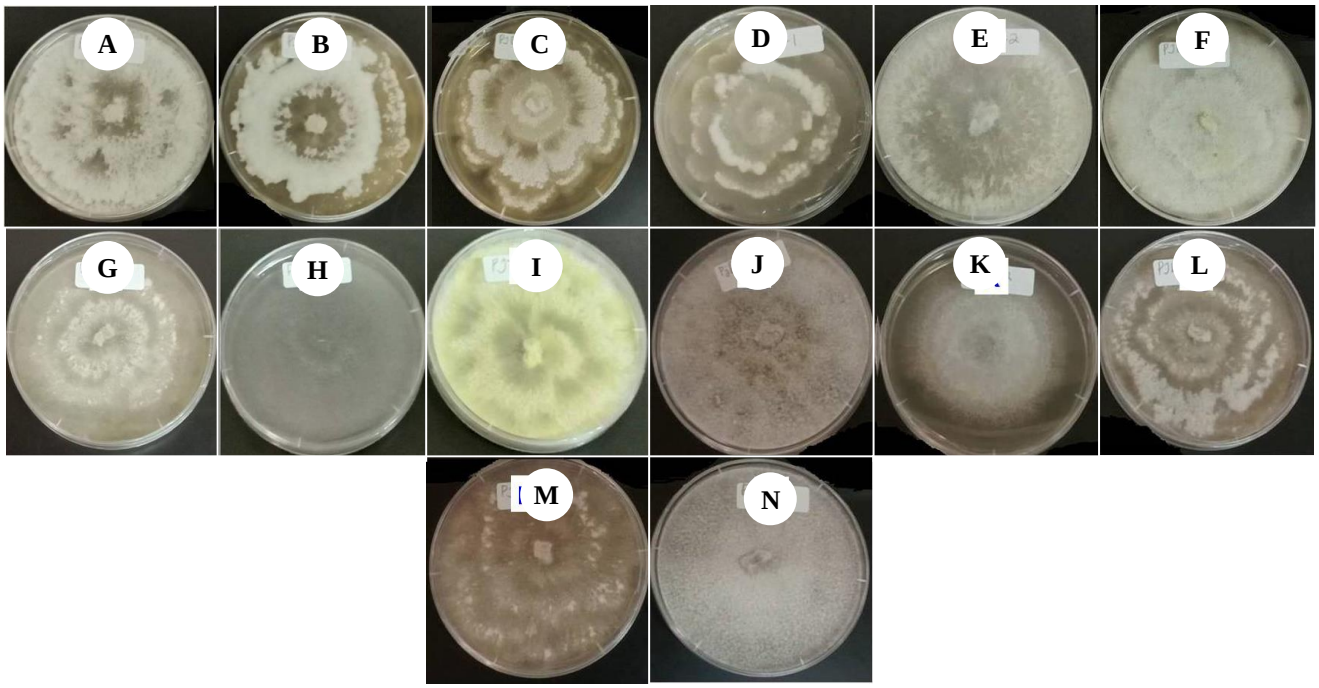

Figure 1. The macroscopic view of 14 selected strains of fungal endophyte inhabiting *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst. from Cibinong, West Java Province on PDA, 7-10 days incubation at T 27°C: A. *Phomopsis* PJBg-1, B. *Phomopsis* PJBg-2, C. *Phomopsis* PJBg-3, D. *Phomopsis* PJBt-1, E. *Phomopsis* PJBt-2, F. *Phomopsis* PJBt-3, G. *Phomopsis* PJTD-1, H. *Lasiodiplodia* PJTB-1, I. *Phomopsis* PJTB-2, J. *Lasiodiplodia* PJDn-1, K. *Colletotrichum* PJDn-2, L. *Phomopsis* PJDn-3, M. *Phomopsis* PJDn-4, and, N. *Phomopsis* PJDn-5

Qualitative and quantitative antioxidant activity of fungal endophytes from *C. aconitifolius*

TLC-bioautography analyzed the qualitative antioxidant activity of fungal endophytes by DPPH-free radical scavenging activity. The dot-blot method was conducted to determine the antioxidant activity of the extract (Figure 4A), while Figure 4B shows the chemical compounds with antioxidant activity indicated by yellowish spots or bands.

The quantitative assay results for antioxidant activity (Table 3) showed that one fungal endophyte (PjBg-1, *Phomopsis* sp.) had strong antioxidant activity.

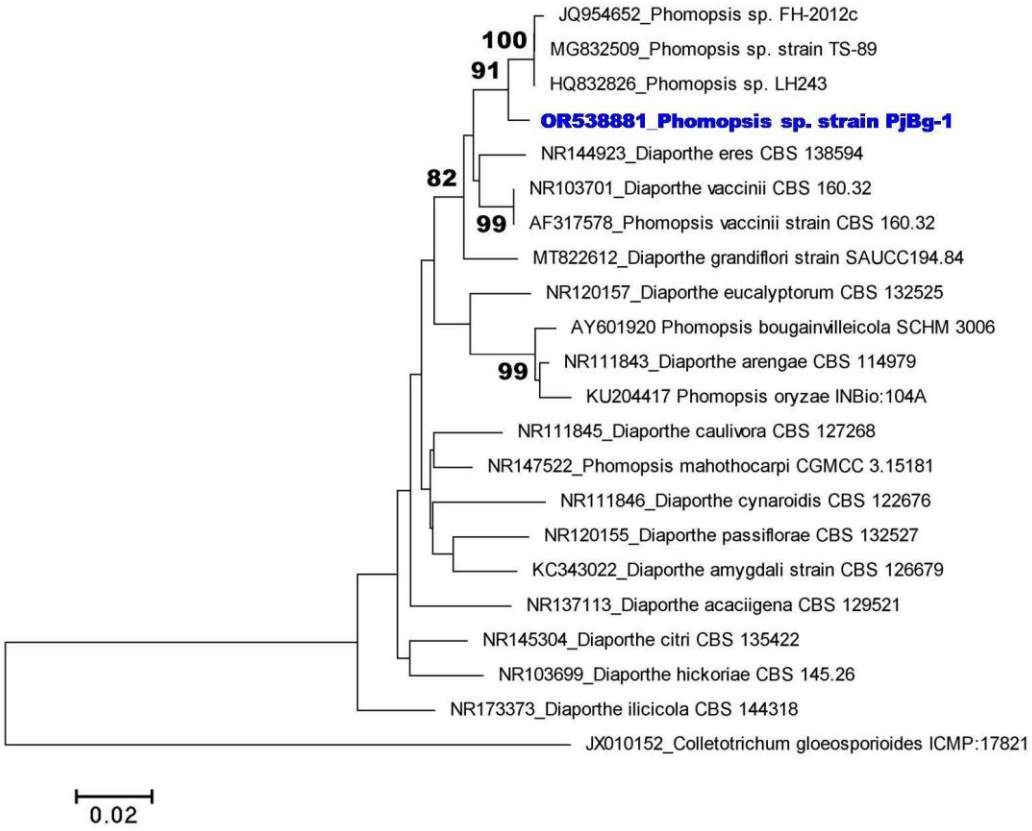

Figure 2. Neighbor-joining tree of fungal endophytes strain PjBg-1 based on ITS rRNA gene sequence and *Colletotrichum gloeosporioides* as outgroup. Only bootstrap values above 70 are shown.

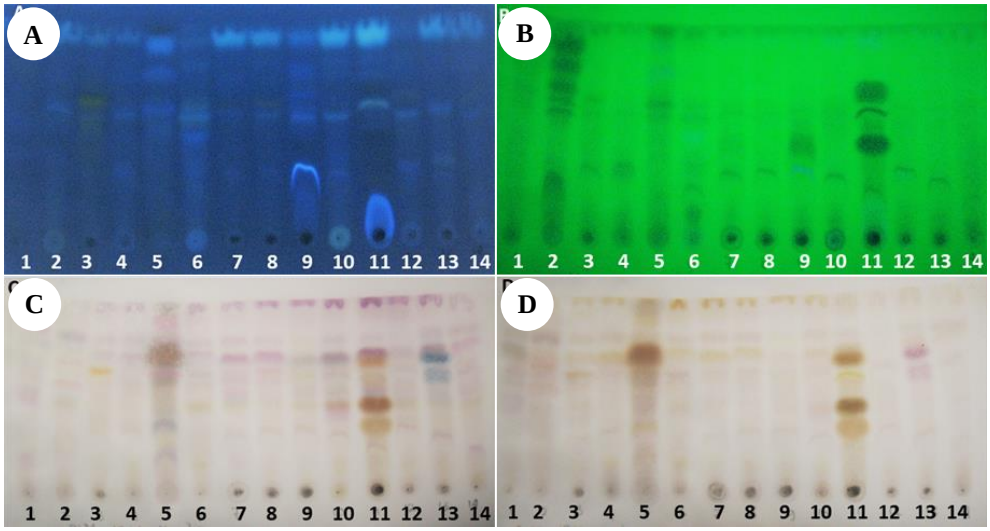

Figure 3. Chromatogram profile of secondary metabolites of fungal endophytes from *C. aconitifolius* eluted using a mobile phase of dichloromethane: methanol (10:1). Observed under 366 nm (A), 254 nm (B), sprayed with vanillin sulfate (0.25% in 10% sulfuric acid-ethanol p.a. (C), sprayed with cerium sulfate (1% Ce2(SO4)3 in 10% sulfuric acid-methanol (D)

Qualitative and quantitative assay for antibacterial

The results of the quantitative assay of antibacterial activity showed that fourteen isolates of fungal endophytes from *C. aconitifolius* possessed weak antibacterial activity against *S. aureus*, and one fungal endophyte possessed moderate antibacterial activity against *E. coli*, which is PjBg-1, *Phomopsis* sp. (Figure 5, Table 4)

Quantitative determination of total phenolic and total flavonoid content

The total phenolic content (TPC) and Total flavonoid content (TFC) of fungal endophytes varied widely. The highest phenolic content was obtained in isolate PjBg-1 (*Phomopsis* sp.), and the highest flavonoid content was obtained in isolate PjTb-1 (*Lasiodiplodia* sp.) (Table 5).

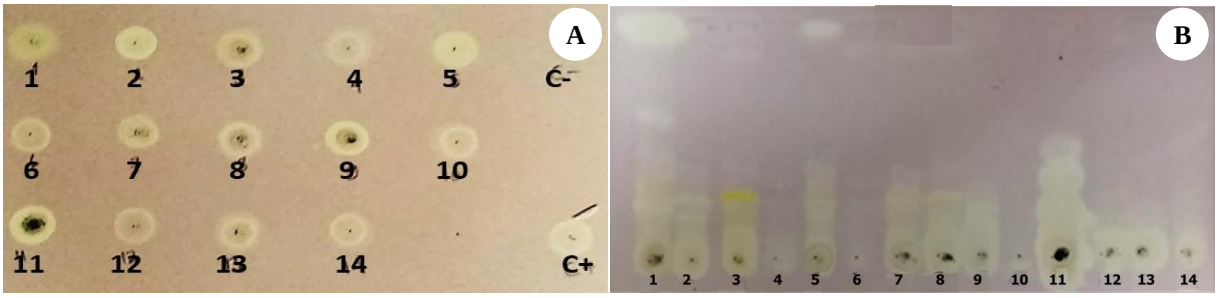

Figure 4. Bioautogram of antioxidant activity of fungal endophytes by Dot-Blot method (A), eluted with dichloromethane: methanol (10:1) (B) after sprayed with DPPH solution in methanol. A yellowish-white spot or band indicates the antioxidant active compound. The number is according to Table 3

Table 2. The BLAST result of the potential fungal endophyte strain PjBg-1 based on ITS rRNA gene sequence according to NCBI BLAST (https://blast.ncbi.nlm.nih.gov/)

Fungal strain	GenBank acc. number(s)	1st-3rd closest taxa on NCBI BLAST (https://blast.ncbi.nlm.nih.gov/)
PjBg-1	OR538881	*Phomopsis* sp. LH243 (Accession no: HQ832826) [Similarity: 98.16%; Max score: 1042; Total score: 1042; Query coverage: 99%; E-value: 0.0; Max identities: 587/598 (98%); Gaps: 2/598 (0%)] *Phomopsis* sp. FH-2012c (Accession no: JQ954652) [Similarity: 97.99%; Max score: 1031; Total score: 1031; Query coverage: 100%; E-value: 0.0; Max identities: 585/598 (98%); Gaps: 2/598 (0%)] *Phomopsis* sp. strain TS-89 (Accession no: MG832509) [Similarity: 97.83%; Max score: 1014; Total score: 1014; Query coverage: 100%; E-value: 0.0; Max identities: 585/598 (98%); Gaps: 2/598 (0%)]

Table 3. IC_{50} value and AAI of fungal endophytes from *C. aconitifolius*

No	Extract code	IC_{50} (µg/mL)	AAI	Category of antioxidant activity
1	PjDn-1	81.28 ± 9.06	0.38 ± 0.04	Weak
2	PjDn-2	125.50 ± 2.50	0.25 ± 0.00	Weak
3	PjDn-3	128 ± 0.00	0.24 ± 0.00	Weak
4	PjDn-4	128 ± 0.00	0.24 ± 0.00	Weak
5	PjDn-5	128 ± 0.00	0.24 ± 0.00	Weak
6	PjTb-1	128 ± 0.00	0.24 ± 0.00	Weak
7	PjTb-2	128 ± 0.00	0.24 ± 0.00	Weak
8	PjBt-1	128 ± 0.00	0.24 ± 0.00	Weak
9	PjBt-2	128 ± 0.00	0.24 ± 0.00	Weak
10	PjBt-3	128 ± 0.00	0.24 ± 0.00	Weak
11	**PjBg-1**	**18.54[a] ± 1.87**	**1.69 ± 1.17**	**Strong**
12	PjBg-2	128 ± 0.00	0.24 ± 0.00	Weak
13	PjBg-3	128 ± 0.00	0.24 ± 0.00	Weak
14	PjTd-1	116 ± 3.01	0.35 ± 0.37	Weak

Table 4. Minimum Inhibitory Concentration (MIC) of fungal endophytes from *C. aconitifolius* against *E. coli* and *S. aureus*

No	Sample	MIC (µg/mL)	Category	MIC (µg/mL)	Category
		S. aureus		*E. coli*	
1	PjDn-1	>256	Weak	>256	Weak
2	PjDn-2	>256	Weak	>256	Weak
3	PjDn-3	>256	Weak	>256	Weak
4	PjDn-4	>256	Weak	>256	Weak
5	PjDn-5	>256	Weak	>256	Weak
6	PjTb-1	>256	Weak	>256	Weak
7	PjTb-2	>256	Weak	>256	Weak
8	PjBt-1	>256	Weak	>256	Weak
9	PjBt-2	>256	Weak	>256	Weak
10	PjBt-3	>256	Weak	>256	Weak
11	**PjBg-1**	**128**	**weak**	**64**	**Moderate**
12	PjBg-2	>256	Weak	>256	Weak
13	PjBg-3	>256	Weak	>256	Weak
14	PjTd-1	>256	Weak	>256	Weak

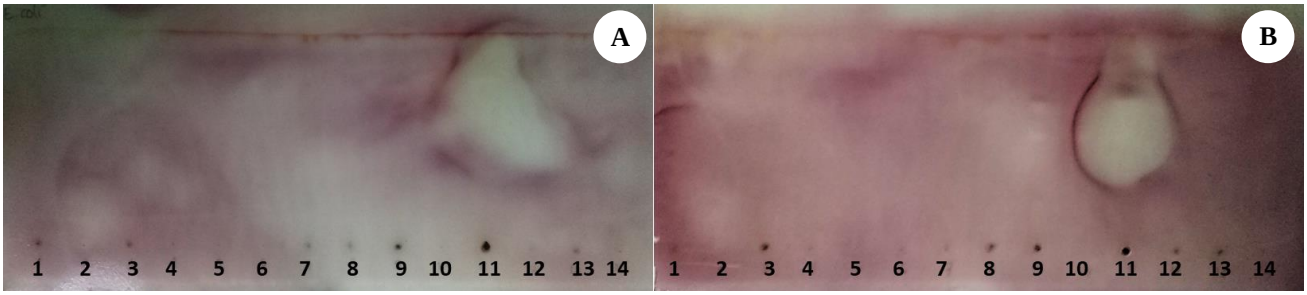

Figure 5. Bioautogram of antibacterial activity of fungal endophytes from *C. aconitifolius* against *E. coli* (A) and *S. aureus* (B). White bands indicate antibacterial compound(s)

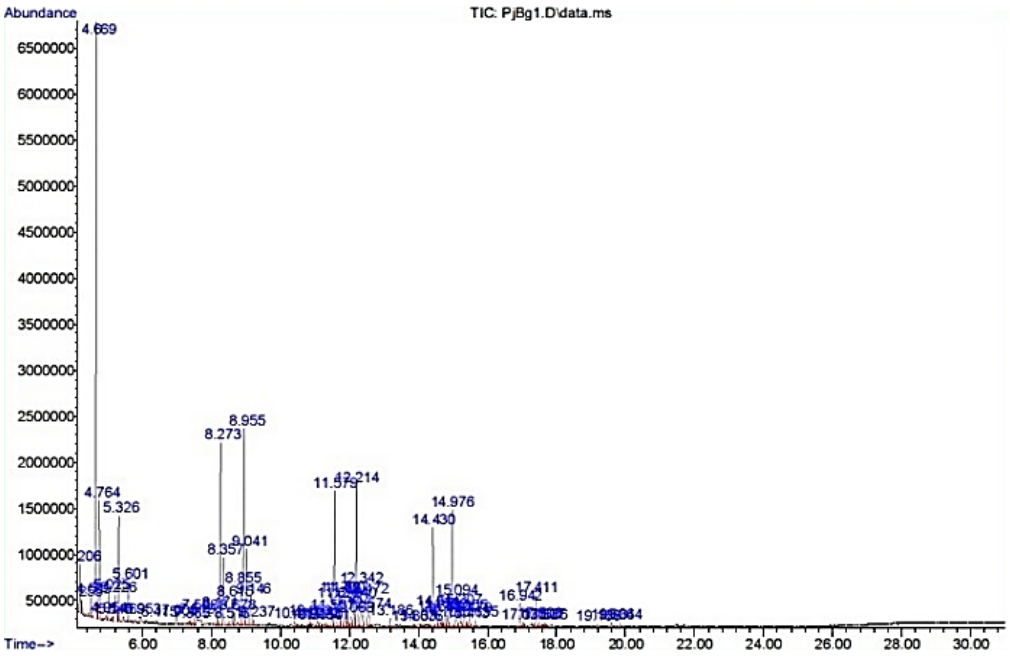

Figure 6. Chromatogram of PjBg-1 ethyl acetate extract

Table 5. Total phenolic and total flavonoid content of fungal endophytes from *C. aconitifolius*

Sample code	TPC (mg GAE g^{-1} extract)	TFC (mg QE g^{-1} extract)
PjDn-1	$47.08^{c} \pm 0.05$	$205.58^{d} \pm 0.91$
PjDn-2	$6.74^{b} \pm 0.06$	$167.44^{f} \pm 3.75$
PjDn-3	$15.89^{g} \pm 0.03$	$120.67^{h} \pm 1.39$
PjDn-4	$3.21^{l} \pm 0.02$	$103.64^{i} \pm 2.13$
PjDn-5	$36.32^{d} \pm 0.00$	$88.72^{j} \pm 0.83$
PjTb-1	$8.23^{j} \pm 0.01$	**$239.11^{a} \pm 0.91$**
PjTb-2	$21.81^{f} \pm 0.03$	$142.06^{g} \pm 0.24$
PjBt-1	$22.68^{e} \pm 0.01$	$215.72^{c} \pm 2.20$
PjBt-2	$14.77^{h} \pm 0.81$	$92.97^{j} \pm 1.12$
PjBt-3	$9.51^{i} \pm 0.02$	$42.36^{k} \pm 7.40$
PjBg-1	**$79.08^{a} \pm 0.16$**	$227.03^{b} \pm 1.62$
PjBg-2	$0.72^{m} \pm 0.02$	$204.53^{d} \pm 1.13$
PjBg-3	$8.16^{j} \pm 0.07$	$189.25^{e} \pm 1.54$
PjTd-1	$5.97^{k} \pm 0.05$	$214.44^{c} \pm 4.66$

Identified compounds of potential fungal endophyte PjBg-1 by GC-Ms

Based on the results of the GC-MS chromatogram (Figure 6, Table 6), the ethyl acetate extract of PJBg-1 had 73 peaks, which means it may contain 73 chemical compounds. However, several chemical compounds showed <90% similarity. Chemical compounds that had ≥90% similarity were 2,4-dimethyl-heptane, 4-methyl-octane, tetradecane, hexadecane, octacosane, nonadecane, tetracosane, and 2-bromo dodecane (Table 7).

Discussion

Fourteen isolates of fungal endophytes from *C. aconitifolius* were dominated by the genus *Phomopsis*. *Phomopsis* is a fast-growing endophyte that can inhibit the growth of slow-growing endophytes (Singh et al. 2017). Several species from the genus *Phomopsis* have been known to possess biological activity. Chemical compounds of the acetonitrile fraction of *Phomopsis* sp. from *Senna spectabilis* showed antifungal, inhibitory acetylcholinesterase enzyme (AChE), and reactive oxygen species (ROS) (Chapla et al. 2014). Another study revealed that *Phomopsis* sp. isolated from *Tinospora crispa* contained (+)-epoxydone and possessed antibacterial activity (Fathoni et al. 2022b).

Table 6. Identified compounds of PjBg-1 ethyl acetate extract by GC-MS analysis

Peak	RT (min)	Name of compound	Similarity (%)
1	4.2007	Isobutyl acetate	64
2	4.2511	Butanoic acid, 2-methyl-, methyl ester	43
3	4.5032	Malic Acid	59
4	4.667	Acetic acid, butyl ester	78
5	**4.7678**	**Heptane, 2,4-dimethyl-**	**95**
6	4.9569	(3-Methyl-oxirane-2-yl)-methanol	37
7	5.0199	2,4-Dimethyl-1-heptene	76
8	5.1459	(3-Methyloxiran-2-yl)-methanol	47
9	5.2215	Oxalic acid, isohexyl pentyl ester	72
10	**5.3224**	**Octane, 4-methyl-**	**91**
11	5.4736	Butanoic acid, 3-cyano-3-hydroxy-, ethyl ester	42
12	5.5996	n-Butyl ether	72
13	5.9525	Ethanol, 2-butoxy-	43
14	6.4188	Amphetamine	27
15	6.9986	Butanal, 3-hydroxy-	35
16	7.364	N-Hydroxycarbamic acid,2-(isopropoxycarbonylamino)ethyl ester	28
17	7.4145	1-Pentanol, 2-methyl-	27
18	7.5153	Decane, 4-methyl-	47
19	7.5909	.alpha.-Pinene	60
20	8.1707	Decane, 3-methyl-	53
21	8.2715	Hexadecane	78
22	8.3597	Ether, hexyl pentyl	83
23	8.5109	Butanal, 3-hydroxy-	37
24	8.6118	1-Octene, 3,7-dimethyl-	50
25	8.6748	1-Octene, 3,7-dimethyl-	43
26	8.8512	Undecane, 4,7-dimethyl-	72
27	8.952	Decane, 3,7-dimethyl-	72
28	9.0403	Undecane, 4,7-dimethyl-	86
29	9.1411	Undecane, 4-methyl-	81
30	9.2419	N-Ethyl-N'-nitroguanidine	28
31	10.414	Decane, 2,4,6-trimethyl-	43
32	10.8551	3-Hexyn-1-ol	53
33	10.9055	Glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1,4-dideoxy-4-(D-2-(2-(methylamino)acetamido) hydracrylamido)-, .beta.-D-	32
34	11.0694	Oxalic acid, isobutyl nonyl ester	43
35	11.145	Decane, 2,4,6-trimethyl-	43
36	11.3466	1,3-Propanediamine, N-methyl-	38
37	11.5105	Dodecane, 1-iodo-	58
38	**11.5735**	**Hexadecane**	**90**
39	**11.6995**	**Hexadecane**	**90**
40	11.7751	Decane, 2,3,5,8-tetramethyl-	78
41	**11.8885**	**Hexadecane**	**90**
42	11.9515	Octane, 4-methyl-	43
43	12.065	Octane, 4-methyl-	43
44	12.1406	Dodecane, 1-iodo-	86
45	**12.2162**	**Hexadecane**	**90**
46	**12.3423**	**Hexadecane**	**90**
47	12.4683	Decane, 2,3,5,8-tetramethyl-	86
48	12.5691	Hexadecane	64
49	13.1867	Tetradecane	81
50	13.8672	Ether, hexyl pentyl	38
51	14.0815	1-Iodo-2-methylnonane	38
52	**14.4344**	**Tetradecane**	**90**
53	14.5478	Hexadecane	72
54	14.6234	Dodecane, 1-iodo-	62
55	14.699	Nonadecane	52
56	14.7746	Nonyl tetradecyl ether	64
57	14.8503	Nonadecane	72
58	**14.9763**	**Octacosane**	**90**
59	**15.0897**	**Hexadecane**	**91**
60	**15.2031**	**Nonadecane**	**90**
61	15.3544	Hexadecane	80
62	15.4174	Sulfurous acid, pentadecyl 2-propyl ester	49

63	15.4804	Hexadecane	72
64	15.6569	Hexadecane	46
65	**16.9424**	**Tetracosane**	**90**
66	17.0306	Pentacosane	53
67	**17.4087**	**2-Bromo dodecane**	**90**
68	17.5095	Heneicosane, 11-(1-ethylpropyl)-	59
69	17.6103	Heptadecane, 2,6,10,15-tetramethyl-	52
70	17.6607	Pentadecane	43
71	19.1857	Pentacosane	47
72	19.6016	Tetratetracontane	53
73	19.841	Decanoic acid, ethyl ester	47

Table 7. Selected compounds of PjBg-1 ethyl acetate extract with high similarity (≥90%)

Peak	R.T (min)	Name of compound	Ref. No	CAS No.	Library
5	4.7678	Heptane, 2,4-dimethyl-	17174	006915-15-7	NIST20
10	5.3224	Octane, 4-methyl-	14533	002216-34-4	NIST20
38-39	11.5735-1.6995	Hexadecane	107217	000544-76-3	NIST20
41	11.8885	Hexadecane	107217	000544-76-3	NIST20
45-46	12.2162-2.3423	Hexadecane	107217	000544-76-3	NIST20
52	14.4344	Tetradecane	74005	000629-59-4	NIST20
58	14.9763	Octacosane	298427	000630-02-4	NIST20
59	15.0897	Hexadecane	107217	000544-76-3	NIST20
60	15.2031	Nonadecane	158600	000629-92-5	NIST20
65	16.9424	Tetracosane	246481	000646-31-1	NIST20
67	17.4087	2-Bromo dodecane	132769	013187-99-0	NIST20

The chemical compounds of endophytic fungal extracts were analyzed by thin-layer chromatography (TLC) due to their quick results, cost-effectiveness, and ease of use. TLC can also separate and identify the chemical compounds. The chemical compounds of the extracts were observed under long UV light (366 nm) and short UV light (254 nm) and visualized using a staining reagent to detect chemical compounds. After spraying with vanillin-sulfuric acid reagent, blue-colored spots were identified as saponin. Alkaloids are yellowish-brown, and flavonoids are orange after spraying with Cerium (IV)-sulfate.

The chemical compounds of PjBg-1 extracts which had ≥90% similarity were 2,4-dimethyl-heptane, 4-methyl-octane, tetradecane, hexadecane, octacosane, nonadecane, tetracosane, and 2-bromo dodecane. Besides, octacosane possesses anti-inflammatory, antimicrobial, and antioxidant properties (Khatua et al. 2016), and tetracosane has antioxidant activity (Paudel et al. 2019).

The qualitative antibacterial activity of the endophytic fungal extract was performed using TLC-bioautography against *E. coli* and *S. aureus. Escherichia coli* and *S. aureus* represent Gram-negative and Gram-positive bacteria, respectively. A TLC-bioautography method was used to analyze antibacterial activity. The TLC-bioautography method enables the rapid detection of antibacterial compounds in the extract. It is a high throughput method enabling analyses of many samples in parallel and comparing their activity (Choma and Jesionek 2015). Visualization of bacterial growth inhibition by extracting a compound was performed by spraying the TLC plate with a tetrazolium salt solution. The growth inhibition appeared as white spots against a purple background (Choma and Jesionek 2015). The purple background was due to the dehydrogenase enzymes of living microorganisms converting tetrazolium salt to purple formazan (Bhujpal et al. 2022).

The quantitative antibacterial assay showed that all fungal endophyte extracts possessed weak antibacterial activity against *S. aureus,* but one isolate (*Phomopsis* PjBg1) had moderate antibacterial activity against *E. coli.* The difference in antibacterial activity against Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*) might be due to differences in cell membranes between *S. aureus* and *E. coli.* The antibacterial activity of the fungal endophyte PjBG1 might also be related to the total phenolic content, in which the extract PjBg1 contained the highest TPC. Phenolic compounds could act as antibacterials by damaging bacterial cell membranes, suppressing the formation of bacterial biofilm, and inhibiting virulence factors, such as toxins and enzymes (Miklasi´nska-Majdanik et al. 2018), destabilizing the plasma membrane (Górniak et al. 2019). Phenolic compounds, widely found secondary plant metabolites, exhibit diverse biological effects and are acknowledged for their ability to modify the structure and characteristics of proteins through interaction, including antibacterial activity (Shahidi and Dissanayaka 2023). The assessment of the antioxidant activity of fungal endophyte extracts was performed by TLC-bioautography using the DPPH method. The qualitative test results showed several extracts possessed antioxidant activity (Figure 4A). In the extract, several compounds indicated antioxidant activity by yellowish-white color formation after spraying with methanolic DPPH solution. Free radical DPPH can react

with compounds that can donate hydrogen (Praptiwi et al. 2022). The yellowish-white color indicated antioxidant activity due to the reduced form of DPPH to diphenyl picryl hydrazine, which has a yellowish-white color (Afsar et al. 2018). The color intensity might indicate the potential for antioxidant activity.

The quantitative antioxidant activity showed that the fungal extract of *Phomopsis* PjBg1 possessed strong antioxidant activity. The Pearson correlation analysis showed that the fungal endophyte's antioxidant activity strongly correlated with its total phenolic content. The antioxidant activity of the phenolic compound could be delivered through several mechanisms, for instance, by transferring a hydrogen atom, a single electron, or by chelation of transition metal (Zeb 2020). The presence and number of hydroxyl groups in phenolic compounds strongly influence their antioxidant properties (Platzer et al. 2020) and their arrangement (Olszowy 2019). The strong antioxidant activity of PjBg-1 extract might be attributed to several compounds, such as hexadecane, octacosane, and tetracosane.

In conclusion, the results of this study confirm that the fungal endophyte *Phomopsis* PjBg1, identified as *Phomopsis* sp. LH243 could act as a promising antibacterial and antioxidant source. Further study is needed to determine the fungal isolate and isolating active compounds responsible for the antibacterial and antioxidant.

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