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by Hary Widjajanti

Submission date: 15-Jun-2023 12:12PM (UTC+0700)
Submission ID: 2116425202
File name: BIODIVERSITAS_ELFITA_DKK_SEPT_2022.pdf (1.06M)
Word count: 7520
Character count: 41797

BIODIVERSITAS
Volume 23, Number 9, September 2022
Pages: 4783-4792

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230946

Antibacterial and antioxidant activity of endophytic fungi isolated from *Peronema canescens* leaves

ELFITA[1,♥], RIAN OKTIANSYAH[2,3], MARDIYANTO[4], HARY WIDJAJANTI[5], ARUM SETIAWAN[5]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya. Jl. Raya Palembang-Prabumulih Km 32, Indralaya, Ogan Ilir 30662, South Sumatera, Indonesia. Tel.: +62-711-580269, ♥email: elfita.elfita.69@gmail.com
[2]Graduate School of Sciences, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya. Jl. Padang Selasa No. 524, Palembang 30129, South Sumatra, Indonesia
[3]Universitas Islam Negeri Raden Fatah Palembang. Jl. Pangeran Ratu, 5 Ulu, Kecamatan Seberang Ulu I, Palembang 30267, South Sumatra, Indonesia
[4]Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya. Indralaya, Ogan Ilir 30662, South Sumatra, Indonesia
[5]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya. Jl. Raya Palembang-Prabumulih Km 32, Indralaya, Ogan Ilir 30662, South Sumatra, Indonesia

Manuscript received: 8 August 2022. Revision accepted: 12 September 2022.

Abstract. *Elfita, Oktiansyah R, Mardiyanto, Widjajanti H, Setiawan A. 2022. Antibacterial and antioxidant activity of endophytic fungi isolated from* Peronema canescens *leaves. Biodiversitas 23: 4783-4792. Peronema canescens* Jack. or *sungkai* is a plant found in tropical rainforests. Also known as *Peronema heterophyllum* Miq., it belongs to the Verbenaceae family and grows widely in Thailand, Malaysia, and Indonesia. The local people believe that the antibacterial and antioxidant properties of its leaves increase immunity. Bioactivities of endophytic fungi associated with medicinal plants are currently of great interest due to their potential for development and use in the pharmaceutical, agricultural, and industrial fields. In this study, we investigated the antibacterial and antioxidant properties of an extract of leaves from *P. canescens* host plant and compared them with those of extracts from an endophytic fungus. Antibacterial and antioxidant compounds derived from active extracts of endophytic fungi have the capability to be developed as a source of antibacterial and antioxidant compounds equivalent to those of their host. In this study, endophytic fungi were isolated from the fresh tissue of *P. canescens* leaves and identified morphologically. Their antibacterial activity was tested using the Kirby-Bauer method, and their antioxidant activity was tested using the DPPH method. The potential antibacterial and antioxidant activities of the endophytic fungi extracts were identified molecularly, and the compounds were isolated using column chromatography. A structural determination of the antibacterial and antioxidant compounds was conducted by spectroscopy involving NMR 1D and 2D. Twelve of the endophytic fungi were gained from the *P. canescens* leaves, namely RD1-RD12. The RD6 isolate showed the greatest potential for antibacterial and antioxidant activity, and its molecular identification was expressed as *Penicillium oxalicum*. The pure compound produced was a yellowish white solid with strong antibacterial and antioxidant activity. Based on an analysis of spectroscopy NMR 1D and 2D, compound I was identified as 3-(2,6-dihydroxyphenyl)-2-hydroxyacrylic acid. This study demonstrated that endophytic fungi associated with medicinal plants and functioning as antibacterial and antioxidant agents have the potential to produce compounds as strong as those of the host plant. These compounds represent a potential new source for antibacterials and antioxidants.

Keywords: Antibacterial, antioxidant, endophytic fungi, *Peronema canescens*

INTRODUCTION

The outbreak of the Covid-19 pandemic caused great concern among the Indonesian people. One approach to prevention was to maintain and increase immunity, thereby making it possible for the body to fight bacterial and viral infections including the Covid-19 virus. The immune system plays an important role in enabling the body to overcome various diseases and assaults caused by viruses or bacteria. During the Covid-19 pandemic, people consumed a stew made from *Peronema canescens* Jack. or *sungkai* leaf as a means of increasing their immunity. The antioxidant and antibacterial compounds contained in these leaves work as natural immune-modulators, which can increase the leukocytes that are part of the immune system. The chemical structures contained in the leaves of *P. canescens* include peronemin, sitosterol, isopropanol, phytol, diterpenoids, and flavonoids. These function as antioxidants and antibacterials and probably create a natural immunostimulant (Kitagawa et al. 1994; Marshall et al. 2018; Maigoda et al. 2022).

The natural antioxidant is very effective in preventing the destructive process caused by free radicals. Free radicals have been implicated in the pathology of many diseases, including cancer, coronary artery disease, hypertension, diabetes and neurodegenerative disorders, in addition for aging (Sharifi-Rad et al. 2020; Dewage et al. 2022; Michalak 2022). Many synthetic antioxidant components have been shown to have toxic and/or mutagenic effects, and these findings have prompted research on the properties of natural antioxidants (Lourenço et al. 2019; Stoia and Oancea 2022). On the other hand, bacterial resistance for available synthetic and semi-synthetic antibacterial agents is growing rapidly. The available antibiotics also cause various adverse drug reactions, such as hypersensitivity and immunosuppression (Maker et al. 2019; Pancu et al. 2021). As these negative effects, and the constant development of bacterial

resistance, in the pharmaceutical industry there is an urgent need for the development of new antimicrobial agents which are effective against microorganisms and are less harmful to the host (Dadgostar 2019; Terreni et al. 2021). One of the most significant natural resources of antimicrobial agents is medicinal plants (Ilonga et al. 2018). Therefore, it is very important to find new sources of antioxidants and antimicrobial compounds that are safe and inexpensive from natural sources.

The complexity of the metabolites contained in medicinal crops results in a lower yield of the bioactive compounds, making it difficult to source traditional medicines from plants. In addition, in Indonesia, the cultivation of medicinal plants faces many obstacles, including unprofessional approaches to cultivation, the inability of farmers to maintain plant quality, and a lack of attention from industry to scientific results directed at product development (Lu et al. 2020; Adeleke and Babalola 2021; Aini et al. 2022).

Due to the fresh discoveries and expansion of knowledge about life processes at the molecular, cellular, and genetic levels, the biotechnological revolution is currently of major interest to researchers and industry. Endophytic fungi are considered important to biotechnology because they produce large yields of bioactive compounds and can be reproduced in a short time (±4 weeks). As microorganisms living in plant tissue, endophytic fungi are able to form colonies without harming their host. Several endophytic fungi generate the same compounds as their host plants. This possibly results from a mechanism of genetic transfer from the host to the endophytic fungal (El-Hawary et al. 2020; Habisukan et al. 2021). Natural products derived from endophytic fungi are considered as one of the most relevant sources of discovery for new drugs. Several studies have shown that most of the secondary metabolites obtained from endophytic fungi have a unique chemical structure (Rodrigo et al. 2022; Wen et al. 2022). They are capable of producing metabolites similar to those produced by their host or different novel compounds.

In this research, we wanted to explore the antibacterial as well as antioxidant bioactive compounds of the various endophytic fungi that can be isolated from *P. canescens* leaves. Furthermore, we wanted to compare the biological activity and chemical structure of these compounds, extracted from the endophytic fungi, with those extracted from their host. In doing this, we wanted to open opportunities for the future development of immunomodulatory products.

MATERIALS AND METHODS

Plant materials

Sungkai (*Peronema canescens*) leaves were collected from Palembang. Then, plant was identified in the Laboratory of Biosystematic, University of Sriwijaya, number 302/UN9.1.7/4/EP/2021. A fresh sample was collected in May 2022. The endophytic fungi were isolated using old leaf tissue. The leaves used were dark green and were positioned second from the base of the branch.

Isolation of endophytic fungi

Isolation began with the sterilization and disinfection of the *P. canescens* leaf surfaces. The leaves were rinsed with running water for five minutes, and then soaked in 70% alcohol for three minutes. Next, they were flushed with sterilized-distilled water (±1 minute) and soaked in 3% (w/v) of sodium hypochlorite for one minute. The samples were cut aseptically into ±2 cm strips. The samples were then inoculated in plates containing potato dextrose agar (PDA) and chloramphenicol (100 µg/mL) and incubated during 3-14 days at room condition. Inspection was conducted daily until mycelia appeared. The fungal colonies that grew around the leaves had different morphological characteristics (color, size, and texture). The colonies were transferred into the plates containing new PDA and chloramphenicol to be purified, with single spore isolation, and then incubated it at room temperature for 2×24 hours. Fungal colonies purified were then moved to a culture medium (Habisukan et al. 2021; Aini et al. 2022).

Morphological identification of endophytic fungi

Phenotypical characteristics, both macroscopic and microscopic, were used to identify the endophytic fungi. The colony characteristics observation included surface and reverse side color, texture of colony, appearance of exudate drops, radial lines, and concentric circles. The microscopic characterization used the slide culture method to observe hyphae, spores, color, and other specific properties up to 1000X magnification. Both the macroscopic and microscopic characteristics were compared to relevant references to fungal identification (Pitt and Hocking 2013; Watanabe 2010; Walsh et al. 2018).

Molecular identification of endophytic fungi

The most potential isolate of endophytic fungi was identified molecularly based on ITS DNA (rDNA) area. ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')- and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used as primer for amplification. Reverse and forward primer of DNA sequence assembly was compiled by using Bioedit program. The sequences were submitted to the BLAST in http://blast.ncbi.nlm.nih.gov/Blast.cgi. Furthermore, the sample and database sequences were aligned by using the Clustal W in MEGA11 program. The tree of phylogenetic was constructed by using *Neighbor-joining* with 1000 of bootstrap (Tamura et al. 2021).

Cultivation and extraction

Every endophytic fungus isolate was cultured by setting six blocks of pure-cultured agar (±6 mm) in 300 ml of potato dextrose broth (PDB, composition consisting of 20 g dextrose monohydrate, 200 g potatoes, and 1000 mL aquadest) and cultured in three culture bottles of 300 mL. The cultures were incubated under static conditions for four weeks at room temperature. The medium and biomass were separated using filter paper. The medium was extracted by partition in eth (three times in repetition). The ethyl acetate extract was evaporated by using rotary evaporator (Gustianingtyas et al. 2020; Aini et al. 2022). The extract

was then concentrated in the oven at 45°C. The concentrated extract was weighed with an analytical-balance.

The test of antioxidant activity

Antioxidant activity was determined using DPPH method. The ethyl acetate extracts of the endophytic fungi were dissolved in methanol to concentrations of 1000, 500, 250, 125, 62.5, 31.25, and 15.625 μg/mL (three repetitions). To 0.2 mL of each concentration, 3.8 mL of 0.5 mM DPPH solution was added. The mixture was homogenized and left in a dark tube for 30 minutes. Absorption was measured using a UV-Vis spectrophotometer at a maximum of 517 nm. Ascorbic acid was used as the antioxidant standard. Antioxidant activity was calculated by the percentage inhibition of DPPH absorption and the IC_{50} value (Metasari et al. 2020; Abbas et al. 2021).

$$\% \text{ Inhibition} = \frac{A_k - A_s}{A_s}$$

Where:
A_k = Absorbance of control
A_s = Absorbance of samples

The test of antibacterial activity

The Antibacterial activity was analyzed using the Kirby-Bauer method with the MHA (Muller Hinton Agar) medium. Two types of gram-negative bacteria (*Escherichia coli* InaCCB5 and *Salmonella typhi* ATCC1048) and two types of gram-positive bacteria (*Staphylococcus aureus* InaCCB4 and *Bacillus subtilis* InaCCB1204) were used in this study. A disc paper was dripped with the endophytic fungi extract at 400 μg/disc in concentration. Extracts were diluted by dimethyl sulfoxide (DMSO). The positive control used was tetracycline at a concentration of 30 μg /disc. The disc paper was placed on a MHA (Muller Hinton Agar) medium, which had been inoculated with bacteria. The plate was incubated for 1 × 24 hours in the incubator at 37°C, and then the inhibition zone was observed. The diameter of the inhibition zone was measured with a caliper. Antibacterial activity and the inhibition zone diameter were determined with the formula (Elfita et al. 2019):

Strong ($\frac{A}{B}$x100% > 70 %); Moderate (50 % <$\frac{A}{B}$x100% < 70 %); Weak ($\frac{A}{B}$x100% <50 %)

Where:
A: The sample inhibition zone
B: The antibiotic standard inhibition zone

Isolation and identification of secondary metabolite compound

The selected sample extracts were prepared by preabsorption. They were then placed evenly into a chromatographic column, and eluted using an eluent with graded polarization. The eluate was collected every 10 ml in a vial, and each eluate was evaluated using TLC (Thin-Layer Chromatography) and grouped into column-fractions. Each column fraction was concentrated, separated, and purified by technique of chromatographic to gain pure compounds. The chemical structure was then identified by spectroscopic analysis.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

Based on the isolation of endophytic fungi on *P. canescens* leaves, 12 isolates were found (code RD1 to RD12). Colonies of 12 isolates of endophytic fungi revealed various macroscopic characteristics (shape and color) and microscopic characteristics (Figure 1). The color of the colonies that appeared on the *P. canescens* leaf samples was dominated by white, gray, and black. The observation of macroscopic and microscopic the endophytic fungi results characteristics which can be seen in Tables 1 and 2.

Tables 1 and 2 described the morphological characters of endophytic fungi colonies isolated from *P. canescens* leaves on each isolate. There were 7 genera of endophytic fungi found in *P. canescens* leaves, specifically Cylindrocarpon (4 isolates: RD3, RD5, RD8, RD10), *Phytium* (2 isolates: RD1, RD2), *Trichoderma* (2 isolates: RD11, RD12), 1 isolate for the Genus *Lasiodiplodia* (RD4), *Penicillium* (RD6), *Plectospira* (RD7), and *Phialophora* (RD9). Based on the morphological characteristics (macroscopic and microscopic) that emerged, 12 isolates of *P. canescens* leaf endophytic fungi were identified.

Table 1. The characteristics of endophytic fungi colonies isolated from *Peronema canescens* leaves

Code	Surface colony	Reverse colony	Structure	Elevation	Pattern	Radial line	Exudate drops	Concentric circle
RD1	Broken white	Broken white	Velvety	Verrucose	Flowery	√	-	-
RD2	Broken white	Broken white	Velvety	Verrucose	Flowery	√	-	-
RD3	White	Milky white	Cottony	Umbonate	Zonate	-	-	√
RD4	White	White	Cottony	Umbonate	Radiate	√	-	-
RD5	Milky white	Milky white	Cottony	Umbonate	Zonate	-	-	√
RD6	Green	Green	Velvety	Umbonate	Zonate	-	-	√
RD7	White	White	Velvety	Rugose	Radiate	√	-	-
RD8	White	White	Cottony	Umbonate	Zonate	-	-	√
RD9	Gray	Black	Cottony	Rugose	Radiate	√	-	-
RD10	White	Yellowish white	Cottony	Umbonate	Zonate	-	-	√
RD11	White	Brownish white	Cottony	Rugose	Radiate	√	-	-
RD12	White	Brownish white	Cottony	Rugose	Radiate	√	-	-

Table 2. Microscopic characteristics of endophytic fungi isolated from *Peronema canescens* leaves

Code	Spore	Shape of spore	Hypha	Characteristic	Identification result
RD1	Sporangia	Subglobose	Septate	Subglobose, hyphal swelling terminal.	*Pythium elongatum*
RD2	Conidia	Globose	Coenocytic	Sporangia hypha-like, chlamydospore-like hypal swellings terminal, globose.	*Pythium afertile*
RD3	Conidia	Cylindrical	Septate	Conidiophores branched, conidia phialosporous	*Cylindrocarpon janthothele*
RD4	Conidia	Globose	Septate	Conidiophores hyaline, simple, inflated globosely, pale brown to yellowish brown.	*Lasiodiplodia theobromae*
RD5	Conidia	Cylindrical	Septate	Conidiophores branched, conidia phialosporous	*Cylindrocarpon janthothele*
RD6	Sporangia	Globose	Septate	Conidia pale green, dark brown in mass, subglobose, minutely echinulate on the surface	*Penicillium oxalicum*
RD7	Conidia	Globose	Coenocytic	Sporangia simple, curved, branched	*Plectospira* sp.
RD8	Conidia	Cylindrical	Septate	Conidiophores branched, conidia phialosporous, brown	*Cylindrocarpon* sp.
RD9	Conidia	Globose	Coenocytic	Conidiophores pale brown, erect, conidia hyaline	*Phialophora fastigiata*
RD10	Conidia	Cylindrical	Septate	Conidiophores pale brown, erect, conidia hyaline	*Cylindrocarpon* sp.
RD11	Sporangia	Subglobose	Septate	Hyalin conidiophore, phialides verticillate, clamidospores light brown, subglobose.	*Trichoderma* sp.
RD12	Sporangia	Subglobose	Septate	Hyalin conidiophore, phialides verticillate, clamidospores light brown, subglobose.	*Trichoderma* sp.

In this study, endophytic fungi form *P. canescens* leaf were found 12 isolates included to 7 different genera, specifically *Phytium*, *Cylindrocarpon*, *Lasiodiplodia*, *Penicillium*, *Plectospira*, *Phialophora*, and *Trichoderma*. This finding concordanced to several researchers who also found *Penicillium*, *Cylindrocarpon*, *Alternaria*, *Trichoderma*, *Cylindrocarpon*, *Phialophora*, and *Trichoderma* in Verbenaceae family (De Siqueira et al. 2011; Gong et al. 2015; Singh et al. 2017; Talita et al. 2017; Vaz et al. 2018; Tomy and Rakhra 2021). Some genera, such as *Trichoderma*, are specific for the roots and stems of the host plants (Habisukan et al. 2021; Pescador et al. 2022; Taylor et al. 2022). Nonetheless, genera of *Trichoderma* in this study were found in leaf tissue of *P. canescens*. The existence of this discovery indicated that endophytic fungi had capability to survive in different anatomical structures and physiological conditions of the host plant.

Antibacterial and antioxidant activity of endophytic fungi extracts

Endophytic fungi were extracted from *P. canescens* leaves using ethyl acetate as a solvent to investigate their potential as antibacterial and antioxidant agents. Endophytic fungi were extracted from *P. canescens* leaves using ethyl acetate as a solvent to investigate their potential as antibacterial and antioxidant agents (Table 3). The extracts tested revealed antimicrobial activity against *Salmonella thypi*, *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus aureus*. Five of the endophytic fungi extracts showed strong antibacterial activity against *Escherichia coli*, and seven of the endophytic fungi extracts exhibited strong antibacterial properties against *Streptococcus aureus*. Most of the endophytic-fungal extracts revealed strong antibacterial properties against the *Salmonella typhi* and *Bacillus subtilis* bacteria. The endophytic fungi extract used also showed very strong antioxidant activity (IC_{50} < 20).

Table 3 shows the extracts of endophytic fungi isolated from *P. canescens* leaves, which inhibited the growth of the bacteria by forming antibacterial inhibition zones. The methanol extract from the *P. canescens* leaves showed strong antibacterial activity. Isolate codes RD1, RD2, RD6, and RD8 provided strong antibacterial activity against the four bacteria tested. However, some extracts presented only moderate antibacterial activity. In addition to antibacterial activity, the extracts of endophytic fungal were shown to have antioxidant properties. All the extracts of endophytic fungi exhibited very strong and strong antioxidant activity, similar to that of the host plant. Compared to the IC_{50} value of ascorbic acid, the IC_{50} value of the endophytic fungi extract was lower. Nevertheless, the IC_{50} value of the RD6 endophytic fungal isolate almost reached the IC_{50} value of ascorbic acid, which was 11.415 μg/mL. Therefore, RD6 was selected for molecular identification.

RD6 was isolated from the *P. canescens* leaf for molecular identification. The antibacterial and antioxidant activities of the RD6 extract were shown to be better than those of the other extracts. The number of extracts produced by RD6 was also better than the other isolates, indicating that RD6 has potential for development as a raw material for drug use. The results of its molecular identification can be seen in Figure 2 in the form of a phylogenetic tree. The sequence of the rDNA isolate RD6 was as follows:

CCGGTTAGGTGACTGCGGAGGACATTATCGAGTTACC
GCTGCTTATAAGCCTTTGTGATCATACCCCAAACGTTGC
CTCGGCGGGGCTTCGGAGCCTAACTCCCTCGCCGGGAAC
CCCCCTCGCACCACGCCCCTTCCGGCGGCAGACCACCAA
ACTCTATTTAAACAACGTCTCTCCTGAGTGGCTTTCACC
AATGATCAAAACTTTTAACAGCGGATCTCTTGGTTCTGG
CATCGATGAATAACGCATGTGAATGCGATAAGTAATGTG
AATTGCAAAATTTTGTGAATCATCGAATCTTTGAACGCA
TATTGGGCCCATGCCCGTTCTAGCGGGCATGCCTGTTCG
AGCGTCATTTTTGCCCTTTGGCTCCGCTTGCCGTTGGGG
CCATACGGCTTCCGTAGGCCCCGAAATACATGGGCGGAC
CCTCCCGGAGCCTCCTTTGCGTAGTAACATAACAACTCC
CAGTGGGATCCCGAGGGACTCCTGCCGTAAAACCCCCTC
ATTTTCACCACGTAGACCGCGGATAAAGTAGGAAAACCC
GCTGATCTTAAAAATATCAGGAGCCGGAAGGAAA.

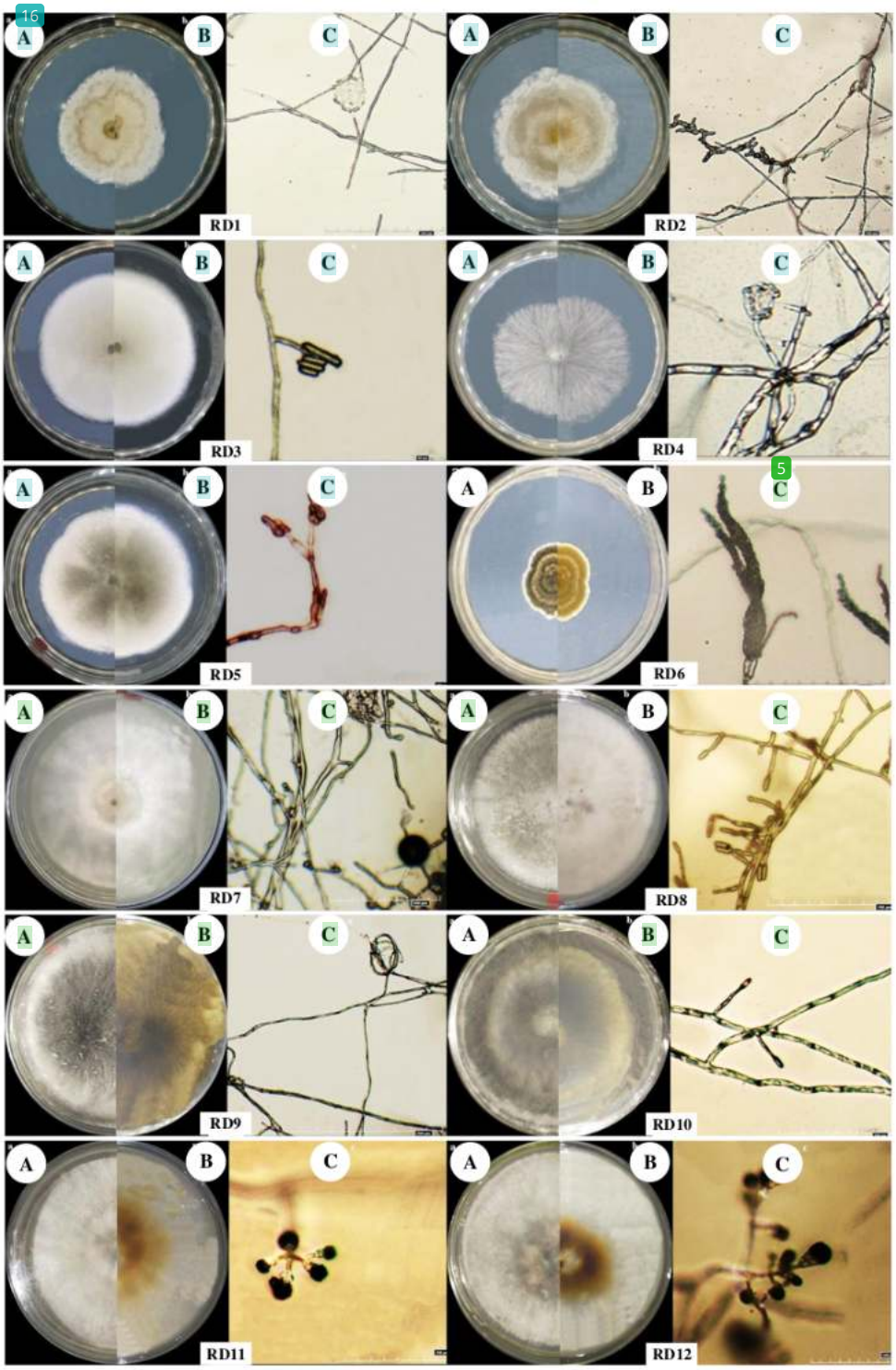

Figure 1. Morphological characters of endophytic fungi colonies from *Peronema canescens* leaves. A. Macroscopic characters (front view; B. Reverse view; C. Microscopic characters

Table 3. The percentage of antibacterial properties of endophytic fungal extract from *Peronema canescens* leaves compared to tetracycline and- antioxidant activity compared to ascorbic acid as an antioxidant standard

Sample	Extract	Ethyl acetate extract weight (g)	% Antibacterial activity				Antioxidant activity IC50 (µg/mL)
			E. coli	*S. aureus*	*S. thypi*	*B. subtilis*	
Host plant	Methanol of *P. canescens* leaves		76.86±0.73 ***	80.15±0.41 ***	74.99±0.68 ***	79.6±0.44 ***	19.366 ****
Endophytic fungi	RD1 (*Pythium elongatum*)	1.5	72.1+0.48 ***	77.1+0.94 ***	87.9+0.77 ***	80.9+0.22 ***	47.829 ***
	RD2 (*Pythium afertile*)	1.6	74.8±0.18 ***	85.5+1.62 ***	90.2+0.35 ***	89.4+1.58 ***	15.395 ****
	RD3 (*Cylindrocarpon janthothele*)	1.8	71.4+1.11 ***	65.3+0.39 **	75.2+ 0.05 ***	74.7+0.31 ***	17.388 ****
	RD4 (*Lasiodiplodia theobromae*)	1.2	68.1+0.72 **	62.1+0.84 **	90.5+ 0.75 ***	78.6+2.11 ***	12.058 ****
	RD5 (*Cylindrocarpon janthothele*)	1.5	68.3+0.47 **	72.4+1.07 ***	87.8+ 0.40 ***	81.5+ 0.83 ***	42.755 ***
	RD6 (*Penicillium oxalicum*)	2.3	75.5+0.69 ***	89.9+2.09 ***	98.3+1.68 ***	89.2+2.46 ***	11.415 ****
	RD7 (*Plectospira* sp.)	2.2	61.3+0.37 **	73.0+0.86 ***	79.5+0.83 ***	81.3+ 0.81 ***	25.307 ***
	RD8 (*Cylindrocarpon* sp.)	1.5	74.8+0.24 ***	78.8+0.78 ***	83.7+0.92 ***	88.7+ 1.68 ***	13.751 ****
	RD9 (*Phialophora fastigiate*)	1.8	67.3+0.11 **	60.0+ 0.55 **	72.9+ 0.51 ***	72.1+ 0.74 ***	30.877 ***
	RD10 (*Cylindrocarpon sp*)	1.8	61.9+0.47 **	58.4+ 0.39 **	78.4+ 0.17 ***	69.2+0.15 **	22.747 ***
	RD11 (*Trichoderma* sp.)	1.5	63.3+0.16 **	62.7+ 0.63 **	81.1+ 0.38 ***	88.0+0.90 ***	19.674 ****
	RD12 (*Trichoderma* sp.)	1.5	61.6+0.19 **	75.7+0.64 ***	87+0.38 ***	78.1+0.44 ***	26.618 ***
Positive control			Tetracycline 100 ***	Tetracycline 100 ***	Tetracycline 100 ***	Tetracycline 100 ***	Ascorbic acid 10.083 ****

Note: Antibacterial activity percentage: * ≥ 70% (strong), **50-70% (moderate), and *< 50% (weak). antioxidant activity IC50 (µg/mL): ****very strong < 20 µg/mL ***strong < 100 µg/mL; **moderat 100-500 µg/mL; * weak > 500 µg/mL.

Based on its molecular identification, RD6 showed 100% similarity and is in the same clade as *Penicillium oxalicum*.

Isolation and identification of chemical compounds

The ethyl acetate extract (2.0 g) was separated by column chromatography using silica gel 60 (70-230 mesh, 40 g) and it eluted using n-hexane:ethyl acetate (10:0 to 0:10) and ethyl acetate: methanol (10: 0 to 5: 5) as a gradient solvent system to obtain five fractions A1-A5. The F4 fraction separation was followed by eluent n-hexane: ethyl acetate (6: 4 to 0: 10) to obtain two subfractions A4.1 and A4.2. The A4.2 subfraction was purified to obtain compound 1 in white solid form (49.3 mg). Pure compound was tested for antioxidant and antibacterial activity and the molecular structure were identified by spectroscopic analysis includes NMR 1D (^{1}H-NMR, ^{13}C-NMR) and NMR 2D (HMQC, HMBC).

The ^{1}H-NMR spectrum of compound 1 (Figure 3) revealed the existence of three signals of proton including two aromatic dublet signals in different integrations and the same ortho-capling constant (J = 8.5 Hz) at δ_H 6.48 (1H, d, J = 8.5 Hz) and 7.23 ppm(1H, d, J= 8.5 Hz). This indicated that compound 1 is an aromatic compound which had three protons, namely two identical protons next to a proton in the ortho position. Hence, it was admitted that the compound 1 was a trisubstituted of aromatic compound, where the two substituents were identical groups. A subsequent proton signal appeared singlet at δ_H 7.10 ppm (1H, s) which was a proton outside the aromatic ring.

The ^{13}C-NMR spectra of compound 1 (Figure 4.A) revealed the presence of 5 signals of carbon, all of which were at δC > 100 ppm. This indicated that all the carbons of compound 1 were sp^2. The HMQC spectrum (Figure 4.B) showed three ^{1}H-^{13}C correlations through one bond. There was a high intensity methine carbon signal at δ_C 109.1 ppm indicated the presence of a pair of aromatic methine carbon equivalents. Two other methine carbon signals of half intensity appeared at δ_C 119.1 (vinyl methine carbon) and 135.4 ppm (aromatic methine carbon). A quaternary aromatic carbon at δ_C 158.1 ppm appeared with high intensity indicated the presence of an identical pair of oxyaryl carbons. Two other signals arised with low intensity as quaternary carbon, namely at δ_C 121.8 and 128.7 ppm. Another carbon signal in the lowest field at δ_C 182.8 ppm was a carbonyl carboxylate carbon atom. Thus, compound 1 was an aromatic compound substituted for two equivalent hydroxyl groups and a carboxylic acid group.

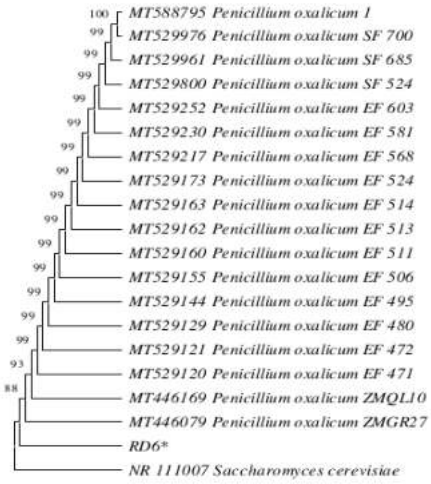

Figure 2. Phylogenetic tree of RD6* constructed to Neighbor-Joining (bootstrap value = 1000)

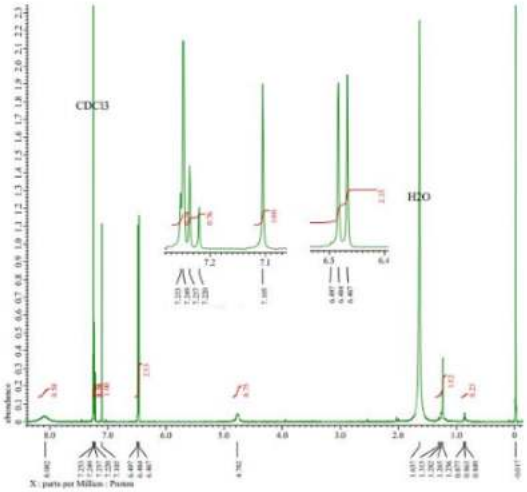

Figure 3. The ^{1}H-NMR spectra of compound 1 (^{1}H-500 MHz in $CDCl_3$)

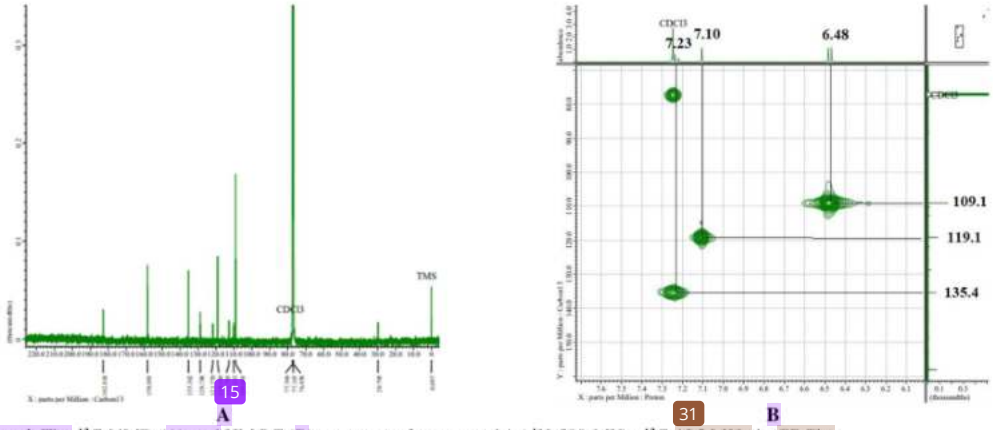

Figure 4. The ^{13}C-NMR (A) and HMQC (B) spectrum of compound 1 (^{1}H-500 MHz; ^{13}C-125 MHz in $CDCl_3$)

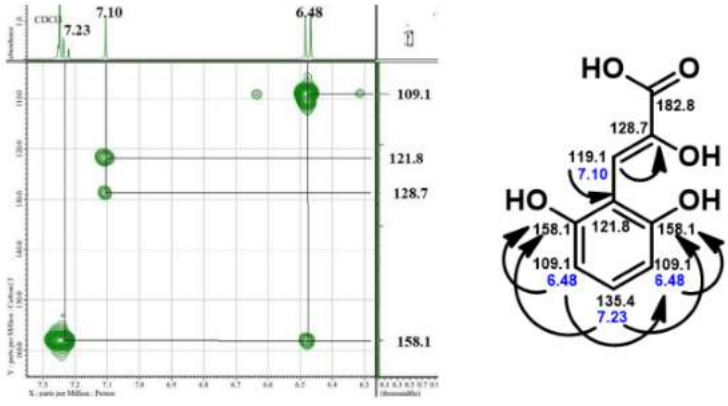

Figure 5. The HMBC spectrum of compound 1

Tabel 4. The NMR data of compound 1, documented at ^{1}H-500 MHz; ^{13}C-125 MHz in $CDCl_3$

No. C	δ_C ppm	Type of C	δ_H ppm (ΣH. multiplicity (Hz))	HMBC
1	182.8	C		
2	128.7	C		
3	119.1	CH	7.10 (1H, s)	121.8; 128.7
1'	121.8	C		
2'	158.1	C		
3'	109.1	CH	6.48 (1H, d, J= 8.5 Hz)	158.1; 109.1
4'	135.4	CH	7.23 (1H, d, J= 8.5 Hz)	158.1
5'	109.1	CH	6.48 (1H, d, J= 8.5 Hz)	158.1; 109.1
6'	158.1	C		

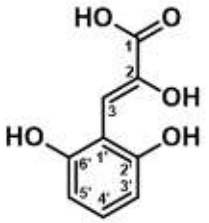

Figure 4. The structure of compound 1 as 3-(2,6-dihydroxyphenyl)-2-hydroxyacrylic acid

The HMBC spectra (Figure 5) exhibited the correlation of ^{1}H-^{13}C through two or three bonds. The equivalent signal of aromatic proton at δ_H 6.48 ppm correlated to two atoms of aromatic carbon, specifically at δ_C 109.1 and 158.1 ppm, including the equivalent carbon atom. The aromatic proton at δ_H 7.23 ppm correlated to the equivalent oxyaryl carbon atom at δ_C 158.1 ppm. The vinylic methine proton at δ_H 7.10 ppm correlated with aromatic carbon at 121.8 ppm and oxyvinic carbon at δ_C 128.7 ppm. This correlation revealed that the three aromatic protons were in the meta and para positions of the carboxylate substituent, while the oxyaryl carbon was in the ortho position. The carboxylate substituent was a hydroxyacrylic acid group. The spectral data of 1D and 2D NMR for compound 1 were demonstrated in Table 4.

According to the spectrum analysis of ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC, it can be described that compound 1 had the trisubstituted benzene ring by two hydroxyl groups and a hydroxyacrylic acid group. The three aromatic protons were in the meta and para positions of the hydroxyacrylic acid substituent group, while the two hydroxyl groups are in the ortho position. Therefore, the expected chemical structure of compound 1 was 3-(2,6-dihydroxyphenyl)-2-hydroxyacrylic acid as shown in Figure 4.

The methanol extraction of *P. canescens* leaves revealed strong antibacterial activity against the four tested bacteria and strong antioxidant activity, equal to that of ascorbic acid as the antioxidant standard (Table 3). The strong biological activity of the host plant extract is closely related to its chemical content. Dillasamola et al. (2021) report that *P. canescens* leaves contain flavonoids, alkaloids, steroids, fenolic, tannin, and saponin. *P. canescens* leaves have also been shown to contain sitosterol, isopropanol, phytol, β amyrin, peronemin, betulinic acid, and stigmasterol (Muharni et al. 2021). These function as antibacterial and antioxidant agents.

Extracts of endophytic fungal associated with *P. canescens* leaves also showed strong antibacterial and antioxidant activity, analogous to those of the host plant. RD1, RD2, RD3, and RD8 revealed strong antibacterial agent against the four tested bacterial, which was similar to that of the host plant. Moreover, the extract of *P. canescens* leaves exhibited strong antioxidant properties, as did all extracts of its endophytic fungi. Of all the extracts of the endophytic fungi (12 isolates), RD6 provided the best antioxidant activity and also the most extract weight.

Table 3 shows that a few endophytic fungi extracts (RD2, RD4, RD6, RD8) had IC_{50} values less than the methanol extract of the host plant. This was, presumably, due to the variety of chemical components in endophytic fungi being simpler than those in the host plant extract. This causes the metabolites to be more concentrated in the extract. This differs from the host plant extract, which is known to contain a variety of chemical components, meaning that the bioactive compounds are less concentrated. Gradient fractionation was used to obtain a stronger extract.

The metabolites originated from the endophytic fungi *P. oxalicum* include the phenolic type, which has not yet been found in a host plant. A review of the literature indicates that the chemical compounds produced by endophytic fungi may be the same as those of the host plant, or the compounds may be different. This relates to their role in a mutualistic association with the host. The endophytic fungi play a role in increasing the host's fitness and in assisting it to adapt to environmental and biological stresses. Lately, endophytic fungus has received noticeable attention for their role in protecting the plants from the pathogens, insect pests, and even the domestic herbivores (Khan et al. 2020). In performing these functions, the endophytic fungi produce chemical compounds that are different from those of their host but have good biological activity. This indicates a potential opportunity for endophytic fungi to provide a new source of bioactive compounds that may overcome antibiotic resistance, which is currently a priority. In addition, the new compounds produced by endophytic fungi can increase the diversity of active medicinal ingredients, thereby helping to overcome the surge in the diversity of diseases occurring today.

The pure compounds tested had strong antibacterial activity against all tested bacteria, with MIC 31.25 μg/mL effective against *S. thypi* and 62.5 μg/mL effective against the other test bacteria. Antioxidant activity had an IC_{50} value of 31.33 μg/mL, which is in the strong category. The lower antioxidant activity of the pure compound compared to the extracts was caused by several factors, including the presence of other antioxidant compounds that have not yet been isolated from the endophytic fungi extract. Another factor may be the synergistic effect of several compounds contained in the extracts so that, together, they provide strong antioxidant activity. Thus, if they are to be

developed as sources of medicinal raw materials, extracts with known chemical compositions should be used. The antioxidant activity of compound 1 can be increased if the structure is modified by adding a hydroxyl group to the aromatic ring which forms a chatecol unit. If the hydroxyl proton from the cathecol unit is abstracted by free radicals, a stable free radical will be formed through the distribution and delocalization of free radicals to form a neutral compound with diketo unit. Thus, compound 1 can be used as a precursor for medicinal raw materials.

References that describe the *P. oxalicum* extract say that it contains phenolic compounds, fatty acids, citric acid, and hesperidin, which are its main ingredients (Saleh et al. 2020; Torres-Mendoza et al. 2020; Tang et al. 2021). Citric acid and hesperidin have been reported to have antibacterial properties (El-Sayed et al. 2022). Phenolic compounds reveal a variety of structures, such as the existence, number, and location of substitute the groups of hydroxyl, and the length of the saturated side chain that gives the compound its antibacterial properties (Patra 2012; Miklasińska-Majdanik et al. 2018; Bouarab-Chibane et al. 2019; Kumar and Goel 2019). This is based on the finding that phenolic-acid inhibits the action of ribonucleic-acid-reductase, an enzyme-required for DNA synthesis, thus causing the failure of bacterial DNA synthesis (Makarewicz et al. 2021; Pancu et al. 2021; Bouyahya et al. 2022). The antibacterial agent of citric acid is caused by the physical and chemical properties, which reduce the extracellular aggregates production and the surface bacterial cell walls hydrophobicity. Antibacterial compounds can kill bacteria by working on cell walls of bacterial, plasma membranes, protein synthesis, or the metabolism of nucleic acid (Adamczak et al. 2020; Qiu et al. 2020; Burel et al. 2021).

The fatty acids, phenolic acids, and hesperidin contained in an extract from the endophytic fungus *P. oxalicum* have all been reported to be antioxidant and anti-inflammatory agents (Khan et al. 2020; Toghueo and Boyom 2020). Fatty acids are capable to reduce and resist free radical oxidative-stress through a series of physiological and biochemical reactions (Pizzino et al. 2017; Andrés Juan et al. 2021; Sharifi-Rad et al. 2020). Based on its biological effects, the endophytic fungus *P. oxalicum*, isolated from the leaf of *P. canescens*, has the potential as a promising natural resource of medicinal ingredients in the future. This study provides basic information for researchers to use the active extract as a source of new medicinal raw materials through further research. In addition, pure compounds (compound 1) can be used as star compounds to obtain potential antioxidant compounds through structural modification.

ACKNOWLEDGEMENTS

The authors thank to the *Kementerian Pendidikan, Kebudayaan, Riset, dan Teknologi*, Indonesia which funded this research throughout *Penelitian Dasar Unggulan Perguruan* Tinggi, contract number 142/E5/PG.02.00.PT/2022, derivative contract number 0149.01/UN9.3.1/PL/2022.

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