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RAPID SCREENING OF ANTIBACTERIAL AND ANTIOXIDANT METABOLITES FROM ENDOPHYTIC FUNGI ISOLATED FROM *Papuacedrus papuana* BY TLC-BIOAUTOGRAPHY

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ABSTRACT

RAPID SCREENING OF ANTIBACTERIAL AND ANTIOXIDANT METABOLITES FROM ENDOPHYTIC FUNGI ISOLATED FROM *Papuacedrus papuana*. *Papuacedrus papuana* is a plant species that grows in the highland of Papua. The aims of the study were to determine in vitro antibacterial activity and antioxidant activity of 17 endophytic fungal extracts isolated from *P. papuana* stem by the TLC-bioautography method. The antibacterial activity was assessed against *Staphylococcus aureus* InaCC B4 and *Escherichia coli* InaCC B5. The antioxidant activity was assessed by DPPH radical scavenging assay. All of 17 endophytic fungi were grouped into 7 taxa based on their morphological traits. The results showed that 11 fungal extracts were active against *S. aureus* InaCC B4, 15 fungal extracts were active against *E. coli* InaCC B5, and 6 extracts had antioxidant activity. Further analysis of active extracts by eluted TLC-bioautography showed several compounds responsible for antibacterial or antioxidant activity in one extract. The results showed a diversity of endophytic fungi inhabited *P. papuana* stem, and these endophytic fungi might be used as a good source of novel antibacterial or antioxidants.

Keywords: *Papuacedrus papuana*, endophytic fungi, antibacterial, antioxidant.

ABSTRACT

SKRINING METABOLIT ANTIBAKTERI DAN ANTIOKSIDAN JAMUR ENDOFIT YANG DIISOLASI DARI Papuacedrus papuana. *Papuacedrus papuana adalah salah satu tanaman langka yang tumbuh di dataran tinggi di Papua. Tujuan penelitian adalah untuk mengetahui aktivitas antibakteri secara in-vitro dan aktivitas antioksidan dari 17 ekstrak jamur endofit yang diisolasi dari batang P. papuana dengan metode Kromatografi Lapis Tipis (KLT)-bioautografi. Aktivitas antibakteri dievaluasi terhadap bakteri Staphylococcus aureus InaCC B4 dan Eschericia coli InaCC B5. Aktivitas antioksidan dievaluasi dengan uji radikal bebas DPPH. Berdasarkan ciri-ciri morfologinya, maka 17 jamur endofit tersebut dikelompokkan menjadi 7 genus. Hasil penelitian menunjukkan bahwa 11 ekstrak aktif menghambat pertumbuhan S. aureus InaCC B4, 15 ekstrak aktif menghambat pertumbuhan E. coli InaCC B5 dan 6 ekstrak memiliki aktivitas antioksidan. Analisis lebih lanjut dari ekstrak aktif yang dielusi menunjukkan bahwa ada beberapa senyawa yang menunjukkan aktivitas antibakteri atau antioksidan. Hasil penelitian menunjukkan bahwa ada keragaman jamur endofit pada batang P. papuana yang kemungkinan dapat dimanfaatkan sebagai sumber yang potensial sebagai antibakteri atau antioksidan baru.*

Kata kunci: *Papuacedrus papuana*, *jamur endofit*, *antibakteri*, *antioksidan.*

INTRODUCTION

Papuacedrus papuana (syn. *Libocedrus papuana*) is one of the plant species that belongs to Cupressaceae and the only species in the genus of *Papuacedrus*. According to Johns (1995), *P. papuana* is native to New Guinea and Maluku. The timber of *P. papuana* has been used as a building material, while the stem bark is used for roof material. Some other

species from the conifer family (Cupressaceae) have been investigated for their bioactivity. According to Perry and Foster (1994), the extract of *Libocedrus pulmosa, L. bidwillii,* and *Cupressus macrocarpa* are active against leukemia cells P-388.

On the other hand, in the last two decades, many studies focus on the bioactivity of microbial endophytes. Endophytes usually colonized the healthy tissue plants (Aly *et al*. 2010) without causing any harm to the host plant (Idris, Al-tahir, and Idris 2013). Endophytes may have the ability to produce bioactive compounds that are the same or similar to bioactive compounds derived from the host plant so that endophytic fungi can serve as an alternative source of plant metabolites (Tikole, Tarate, and Shelar 2018). According to Sturz and Nowak (2000), there are several roles of endophytic fungi in the host plant such as stimulate plant growth, increase disease resistance, improve the ability of the plant to environmental stresses and recycle nutrients. A wide variety of natural products have been obtained from endophytes. Kusari, Lamshöft, and Spiteller (2009) stated that endophytes usually produce the same bioactive compounds as the host plant. Endophytic fungi can be used as a source of secondary metabolites that are useful for novel drug discovery (Yan, Sikora, and Zheng 2011; Guo *et al*. 2008). The endophytic fungi from several species of Cupressaceae have been investigated for their antimicrobial activity.

The search for novel antibacterial and natural antioxidants has become important. This is due to increasing resistance by pathogenic bacteria to commercial drugs (Costelloe *et al*. 2010). At the same time, the use of natural antioxidants was also increased lately. This is due to the use of synthetic antioxidants is suspected to be carcinogenic (Govindarajan *et al*. 2003).

To the best of our knowledge, there is still no report about the diversity of endophytic fungi from *P. papuana*, and also the study of their activities, especially for the antimicrobial and antioxidant activity. Therefore, this study aimed to determine the most potential endophytic fungi associated with *P. papuana* as a source of antibacterial and antioxidant metabolites.

MATERIALS AND METHODS

Materials

Plant Source

Young healthy stems of *P. papuana* were collected from Habema, Papua. The plant voucher was deposited and identified in the Botany Division, Research Center for Biology-Indonesian Institute of Sciences (LIPI).

Chemicals

Ethanol (70%), NaOCl, ethyl acetate (Merck), Thin Layer Chromatography (TLC) plates (silica gel GF254, Merck), Dichloromethane (Merck), MeOH (Merck), $Ce(SO_4)_2$ (Merck), Iodonitrotetrazolium *p*-violet (INT, Sigma), H_2SO_4 (Merck), chloramphenicol (Merck), Mueller-Hinton Broth (MHB, Merck), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma), and (+)-catechin (Sigma).

Methods

Endophytic fungi isolation and cultivation

The fresh young healthy stems of *P. papuana* were thoroughly washed under tap water. Stems were cut about 2 cm in length, then sterilized by dipping in 70% alcohol for 2 minutes, transferred into NaOCl 5.3% solution for 5 minutes, then transferred into 70% alcohol for 1 minute, and washed with distilled water. Sterilization is performed aseptically in laminar airflow. The samples that had been surface-sterilized were split with a sterile blade, and the inner part was placed on Corn Meal Malt Agar (CMMA) media that has been added with chloramphenicol 0.05 mg/mL in a petri dish. Petri dishes were incubated for 7 days at room temperature. The emerging fungi were isolated and subcultured on Potato Dextrose Agar (PDA) media several times to obtain single isolates (Agusta *et al*. 2005).

Identification of endophytic fungal isolates

Identification of endophytic fungi isolated from *P. papuana* was based on fungal morphological characters (Domsch, Gams, and Anderson 2008; Webster and Weber 2007; Ellis 1965). Morphological traits and characteristics macroscopically and microscopically were observed from fungal colonies grown on PDA at room temperature. Color and surface colonies (granular, such as flour, mounting, slippery), texture, zonation, growth area, the lines of radial and concentric, reverse color, and exudate drops were amongst of macroscopic characters that observed.

Endophytic fungi cultivation and extraction

A single isolate of endophytic fungi was inoculated into 200 ml Potato Dextrose Broth (PDB) and incubated for 3 weeks in a static condition at room temperature under dark conditions. After an incubation period, the biomass and culture broth was extracted with ethyl acetate. The organic phase was collected and concentrated with a rotary evaporator.

Analysis of endophytic fungi metabolites

The chemical compounds of ethyl acetate extract were analyzed by separation with TLC. The plate was developed using mobile phase

dichloromethane: methanol (10:1). The chromatogram was viewed under UV light at wavelengths of 254 and 366 nm then sprayed with a staining reagent: 1% $Ce(SO_4)_2$/10% H_2SO_4, and 1% vanillin/H_2SO_4.

TLC-Bioautography: Antibacterial Assay

Endophytic fungal extracts were screened for their antibacterial activity against two bacteria (*E. coli* InaCC-B5 and *S. aureus* InaCC-B4). One hundred µg of endophytic fungal extract (10 mg/mL) were transferred on a TLC plate, then dipped into a bacteria suspension (10^6 CFU/mL). The plate was placed on a sterile petri dish and incubated at 37^{O}C for 18 hours. After an incubation period, the plate was sprayed with INT. A clear zone indicated the inhibition zone against a purple background on the TLC plate.

The active extracts were then observed for the active compounds responsible for antibacterial activity. One hundred µg of endophytic fungi extract (10 mg/mL) was transferred on a TLC plate, and the compounds were separated with the mobile phase of dichloromethane : methanol (10:1) and then dipped into bacteria suspension. The plate was placed on a sterile petri dish and incubated at 37^{o}C for 18 hours. After an incubation period, the plate was sprayed with INT. The spots that are responsible for antibacterial activity were indicated by white spot formation.

TLC-Bioautography: Antioxidant potential by DPPH radical scavenging assay

Ten µl of endophytic fungi extract (10 mg/mL) were transferred to the TLC plate, with (+)-catechin as positive control and culture media as negative control were also transferred to the TLC plate. The plate was then allowed to air dry, followed by spraying with 1 mM DPPH in methanol solution. The antioxidant potential was indicated by the formation of a white-yellow spot against a purple background. The active extract (100 µg) was transferred on a TLC plate, then eluted with the mobile phase of dichloromethane-methanol (10:1). After air drying, the plate was sprayed with 1 mM DPPH in methanol solution.

RESULTS AND DISCUSSION

Endophytic fungi isolation and identification

The potential of the endophytic fungi associated with *P. papuana* has not been had much attention yet. Totally 17 filamentous fungi isolates were obtained from the young stem of *P. papuana* (Table 1). Based on their morphological characters, including macroscopic (Figure 1) and microscopic characters, these fungi were classified into 5 taxa, which are *Phomopsis* sp. (1 isolate), Dematiaceae (9 isolates), Coelomycetes (3 isolates), Hyphomycetes (2 isolates), and Sordariomycetes (2 isolates). The genus *Phomopsis* is a good source of bioactive compounds which have remarkable value in medical application (Xu *et al.* 2021).

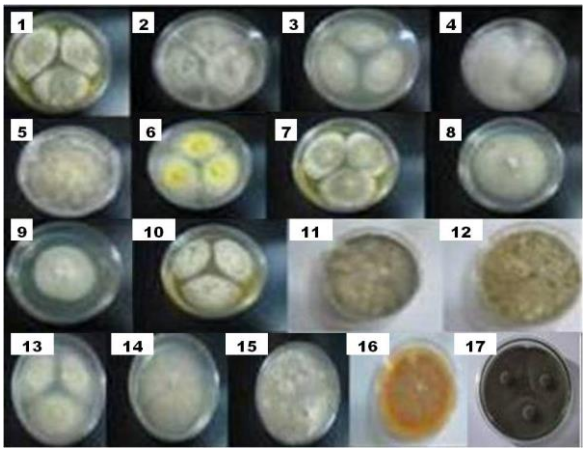

Figure 1. Endophytic fungi associated with *P. papuana* stem. The fungal taxa PC-1 – PC-20 are referring to Table 1.

Endophytic fungi, in general, has a wide range of host. They could be isolated from the different plants from different taxa and grow under different ecological and geographical conditions. However, some endophytic fungi have only one specific host genera. The result indicated that *P. papuana* stem harbored diverse taxa of fungal endophytes. Qadri *et al*. (2013) stated that conifers possessed a broad range of fungal endophytes. Carroll and Carroll (1978) observed some specificity concerning the location of endophyte species within the petiole of conifers in the Pacific Northwest.

Table 1. List of endophytic fungi isolated from *P. papuana* (F. Muell.) H.L.Li. stem.

No.	Fungal Isolates	Fungal taxa	Extract Production (mg/200 ml medium)
1	PC-01	Dematiaceae	227.4
2	PC-02	Coelomycetes	687.5
3	PC-03	Sordariomycetes	66.6
4	PC-04	Coelomycetes	314.5
5	PC-05	*Phomopsis* sp.	127.8
6	PC-06	Hyphomycetes	141.5
7	PC-07	Dematiaceae	212.2
8	PC-08	Dematiaceae	2090.0
9	PC-11	Dematiaceae	68.0
10	PC-13	Coelomycetes	814.1
11	PC-14	Dematiaceae	368.2
12	PC-15	Dematiaceae	145.9
13	PC-16	Dematiaceae	122.6
14	PC-17	Sordariomycetes	236.4
15	PC-18	Hyphomycetes	95.9
16	PC-19	Dematiaceae	114.3
17	PC-20	Dematiaceae	83.8

TLC-Bioautography: Antibacterial Activity

The antibacterial and antioxidant assay was done by TLC-bioautography because this method was a rapid approach (Rajauria and Abu-Ghannam 2013). The result from TLC-bioautography for antibacterial (Figure 2) showed that 11 extracts (64.71%) inhibit the growth of *S. aureus*, 15 extracts (88.24%) inhibit the growth of *E. coli* and 6 extracts (35.29%) have antioxidant activity (Table 2.). Further analysis by eluted TLC-bioautography was used to determine the active spots (metabolites) as clear zones surrounding the purple background as shown in Figure 3.

Table 2. The Antibacterial activity and antioxidant potential of endophytic fungi extract isolated from the young stem of *P. papuana*.

No	Isolate	Antibacterial activity		Antioxidant potential
		S. aureus	*E. coli*	
1	PC-01	-	-	-
2	PC-02	+	+++	-
3	PC-03	-	+	-
4	PC-04	+	+	+++
5	PC-05	+	+	-
6	PC-06	-	+	-
7	PC-07	+	+	++
8	PC-08	-	-	-
9	PC-11	++	++	+
10	PC-13	+	+	-
11	PC-14	-	+	+
12	PC-15	+	+	+
13	PC-16	+	+	+
14	PC-17	+	+	-
15	PC-18	+	+	-
16	PC-19	-	+	-
17	PC-20	++	++	-

Note: +++ : strong activity, ++ moderate activity, + weak activity, - : no activity

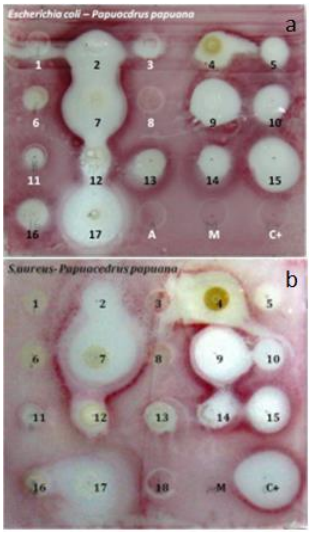

Figure 2. Bioautogram of endophytic fungal extracts of *P. papuana* against *E. coli* (a), and *S. aureus* (b). The white area showed the inhibition growth of bacteria. Picture information refers to Table 1. A: acetone, M: methanol, and C+: chloramphenicol.

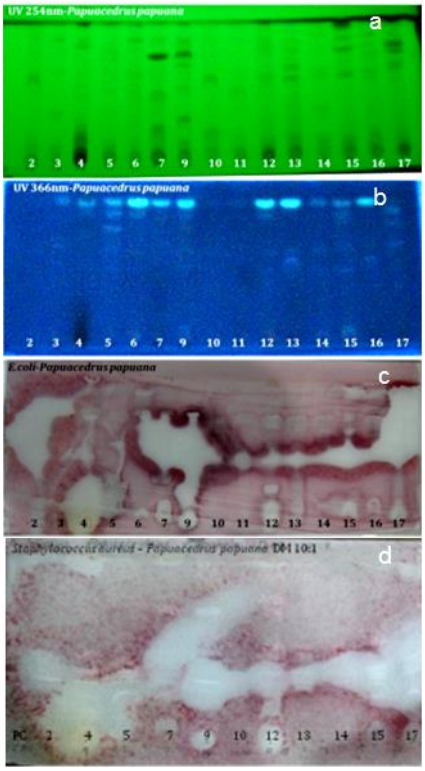

Figure 3. Bioautogram of active extracts developed with dichloromethane : methanol (10:1), (a) viewed under 254 nm, (b) viewed under 366 nm, (c) against *E. coli*, and (d) *S. aureus*. White bands indicated the active chemical compounds in the extracts. Picture information refers to Table 1.

Table 3. The MIC (antibacterial) and IC_{50} value (antioxidant) of endophytic fungi extract from *P. papuana*

No	Isolate	MIC (µg/mL)		IC_{50} (ppm)
		S. aureus	*E. coli*	
1	PC-02	256	256	>1000
2	PC-04	>256	>256	345
3	PC-07	256	256	710
4	PC-11	>256	>256	>1000
5	PC-18	>256	>256	>1000
6	PC-20	256	256	>1000

Further analysis of active extracts to determine their MIC values. The MIC values of active extracts were in the range of 256 µg/mL or more (Table 3). It showed that the antibacterial activity of endophytic fungi extracts from *P. papuana* were not strong antibacterial. Extracts are considered to have significant antimicrobial activity if the MIC values are lower than 100 µg/mL (Ríos and Recio 2005; Gibbons 2004).

Results of the present study showed that endophytic fungal extracts of *P. papuana* inhibited the growth of bacteria. The growth inhibition is indicated by clear zone formation around the extract or the chemical compounds responsible for antibacterial activity. Clear zone

formation indicated the reduction of INT into a colored formazan that inhibits the growth of bacteria (Suleiman *et al*. 2010). In several previous studies, the crude extract of culture broth of endophytic fungi showed various bioactivity such as antibacterial, antifungal, anti-inflammatory, antiviral, and anti-tumor activity (Silva *et al*. 2006).

The result from the study also showed that endophytic fungi associated with *P. papuana* stem are dominated by Dematiaceae such as PC-7, PC-11, PC-14, PC-15, and PC-16. The result showed that these fungi isolates have antibacterial activity and antioxidant activity. This result is in harmony with Tsuge *et al*. (2013), and Bräse *et al*. (2009) that *Alternaria fungi* belong to Dematiaceae produce metabolites with a variety of biological activities such as phytotoxic, cytotoxic, and antimicrobial properties. Tenuazonic acid produced by *Alternaria alternata* was found to be active against *Mycobacterium tuberculosis* H37Rv (Logrieco, Moretti, and Solfrizzo 2009). The result also showed that PC-05 (*Phomopsis* sp.) was active against *S. aureus* and *E. coli*. Previous studies showed that the genus *Phomopsis* is a rich source of bioactive compounds as antimalarial and antitubercular phomoxanthones (Isaka *et al*. 2001), antifungal phomoxanthone-A *(Elsässer et al*. 2005), and antibacterial chromones (Ahmed *et al*. 2011).

TLC-Bioautography: Antioxidant potential by DPPH radical scavenging assay

The antioxidant activity of the endophytic fungi extracts associated with *P. papuana* was assessed by TLC-bioautography with DPPH radical scavenging assay. The result showed 6 isolates have antioxidant activity (PC-04, PC-07, PC-11, PC-14, PC-15, and PC-16), which is indicated by white-yellowish area formation on the TLC plate (Figure 4).

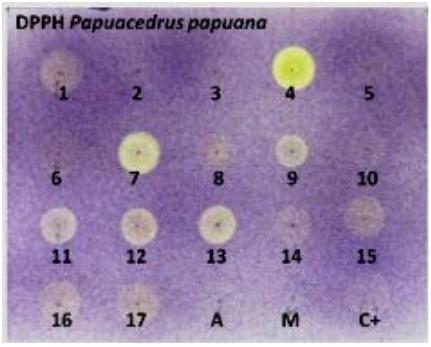

Figure 4. Bioautogram of endophytic fungal extracts of *P. papuana*. TLC was sprayed with 0.2% DPPH in methanol. The white-yellowish area showed the antioxidant activity of the extract. Number 1-17 are extract codes of PC-1 to PC-20 that refers to Table 1. A: acetone, M: methanol, and C+: (+)-catechin.

The active extracts were further assay by separating their chemical compounds on a TLC plate and developed with mobile phase dichloromethane-methanol (10:1). This analysis could be used to determine the active spots (metabolites) as pale yellow spots surrounding the purple background as shown in Figure 5.

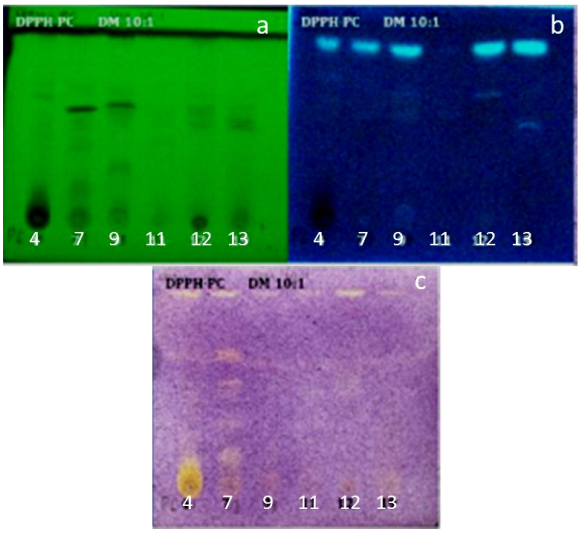

Figure 5. Chromatogram profile of active extracts for antioxidant potential developed with dichloromethane: methanol (10:1), (a) monitored under UV light 254 nm, (b) monitored under UV light 366 nm, (c) sprayed with 0.2% DPPH in methanol. Extracts no. 4-13 refer to Table 1. White yellowish bands indicated the active semipolar compounds in the extract.

The antioxidant activity of the endophytic fungal extracts was done by DPPH radical scavenging activity. DPPH in methanol will produce a purple color, and it reduced to diphenyl picryl hydrazine gave the yellow color because chemical compounds in the extract have antioxidant activity (Mahlo *et al*. 2016). The intensity of the yellow color indicates the potential of free radical scavenging activity by the extract (Kumar and Pandey 2012) and it depends on the amount and nature of radical scavengers in the extract (Qadri *et al*. 2013).

Further analysis of PC-04 and PC-07 by serial microdilution was done to determine the value of IC_{50}. The result showed that the IC_{50} of PC-04 and PC-07 were 345 and 710 ppm respectively (Table 3), indicated that these extracts have weak antioxidant activity (Blois 1958).

CONCLUSION

A total of 17 isolates of endophytic fungi were isolated from *P. papuana* and based on morphological characters they were classified into 5 taxa. Among 17 extracts of endophytic fungi, 11 extracts were active against *S. aureus*

InaCC B4, 15 extracts were active against *E. coli* InaCC B5, and 6 extracts have antioxidant potential. Endophytic fungi from *P. papuana* might use as a novel source for antimicrobial and antioxidant agents which is under further investigation.

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