

BIODIVERSITY AND ANTIBACTERIAL ACTIVITIES OF ENDOPHYTIC FUNGI ASSOCIATED WITH *UNCARIA GAMBIE* (HUNTER) ROXB. VAR. UDANG FROM JASINGA, BOGOR, INDONESIA

[*Keanekaragaman dan aktivitas antibakteri jamur endofit yang berasosiasi dengan Uncaria gambier Roxb. var. udang from Jasinga, Bogor, Indonesia*]

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ABSTRACT

Endophytic fungi living in medicinal plant tissues are potential bioactive metabolite sources for drug discovery and development. However, the research regarding endophytic fungi associated with Indonesian medicinal plants is still limited. This study aimed to explore the biodiversity of endophytic fungi associated with the medicinal plant *Uncaria gambier* (Hunter) Roxb. var. udang and their antibacterial activities. Twenty-seven endophytic filamentous fungi were isolated from the surface-sterilized leaves, stems, and roots of *Uncaria gambier* (Hunter) Roxb. var. udang collected in Jasinga, Bogor, West Java, Indonesia. The observation based on the morphological characters both macroscopically and microscopically, as well as chemical profiling using thin layer chromatography (TLC), revealed that some of the isolated endophytic fungi were identical. Overall, the isolated endophytic fungi consist of six Coelomycetes, three Dematiaceae, four *Pestalotiopsis* sp., two *Penicillium* sp., one *Aspergillus terreus* and two fungi were unidentified. Antibacterial activities of the ethyl acetate extract from the isolated endophytic fungi associated with *Uncaria gambier* (Hunter) Roxb. var. udang were observed against *E. coli*, *B. subtilis*, *S. aureus*, and *M. luteus*. Twelve types of isolated endophytic fungi from *Uncaria gambier* (Hunter) Roxb. var. udang exhibited antibacterial activities.

Keywords: antibacterial activity, fungal endophytes, thin layer chromatography, *Uncaria gambier* (Hunter) Roxb.

ABSTRAK

Jamur endofit yang berasosiasi dengan tanaman obat merupakan sumber metabolit aktif potensial yang bermanfaat bagi penemuan dan pengembangan obat. Namun demikian, penelitian mengenai jamur endofit yang berasosiasi dengan tanaman obat Indonesia hingga saat ini masih terbatas. Penelitian ini bertujuan untuk mengeksplorasi biodiversitas dan aktivitas antibakteri dari jamur endofit yang berasosiasi dengan tanaman obat *Uncaria gambier* (Hunter) Roxb. var. udang yang dikoleksi di Jasinga, Bogor, Jawa Barat. Sebanyak 27 isolat jamur endofit berfilamen telah diisolasi dari daun, batang dan akar *Uncaria gambier* (Hunter) Roxb. var. udang yang telah disterilkan permukaannya. Pengamatan terhadap karakter morfologi secara makroskopis dan mikroskopis serta kajian kemotaksonomi menggunakan kromatografi lapis tipis (KLT) memperlihatkan bahwa dari ke-27 isolat jamur tersebut terdapat beberapa jamur yang identik. Secara keseluruhan, jamur endofit yang diisolasi dari *Uncaria gambier* (Hunter) Roxb. var. udang ini terdiri dari enam jenis Coelomycetes, tiga jenis Dematiaceae, empat jenis *Pestalotiopsis* sp., dua jenis *Penicillium* sp., satu *Aspergillus terreus* dan dua jenis isolat yang tidak dapat teridentifikasi. Aktivitas antibakteri dari ekstrak etil asetat kultur jamur endofit tersebut juga dievaluasi menggunakan menggunakan empat jenis bakteri yaitu *E. coli*, *B. subtilis*, *S. aureus*, and *M. luteus*. Dua belas jenis jamur endofit yang diisolasi dari *Uncaria gambier* (Hunter) Roxb. var. udang menunjukkan aktivitas antibakteri.

Kata Kunci: antibakteri, jamur endofit, kromatografi lapis tipis, *Uncaria gambier* (Hunter) Roxb.

INTRODUCTION

In nature, it is estimated that around 5.1 million fungal species exist globally (Blackwell, 2011), however, a more recent study suggested that the predicted number of fungal species is approximately 2.2–3.8 million (Hawksworth and Lücking, 2017). The fungal species that live in association with a plant are called endophytic fungi. An endophytic fungus can form a mutualistic symbiosis with the host plant where both of them are benefited from the relationship (Jia *et al.*, 2016). Endophytic fungi associated with the host plant can induce plant growth and protect the host plant from abiotic as well as biotic stresses, for

instance, extreme temperatures, high heavy metal and saline level, drought and phytopathogens (Khare *et al.*, 2018).

Endophytic fungi can synthesize secondary metabolites to guard their host plant and mimic the biosynthetic pathway of the secondary metabolites produced by their host plant (Khare *et al.*, 2018; Rai *et al.*, 2021). Hence, endophytic fungi are rich origin for active secondary metabolites (Patil *et al.*, 2016). Some endophytic fungi can produce anti-cancer agents such as taxol-producing *Aspergillus aculeatinus* Tax-6 from *Taxus chinensis* var. *maire*, (Qiao *et al.*, 2017) and camptothecin-producing endophytic fungus isolated

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from *Miquelia dentata* Bedd. (Shweta *et al.*, 2013). In addition, many endophytic fungi can synthesize active secondary metabolites with various potential bioactivities such as *Fusarium solani* isolated from *Glycyrrhiza glabra*, which produced Fusarubin that active as antitubercular agent (Shah *et al.*, 2017), *Diaporthe* sp., *Colletotrichum* sp. and *Arthrinium* sp. associated with *Aquilaria subintegra* which produced sesquiterpenoids with strong antioxidant activities (Monggoot *et al.*, 2017) and *Nemania* sp. UM10M isolated from *Torreya taxifolia* leaf, which produced Cytochalasins with potential antiplasmodial activity (Kumarihamy *et al.*, 2019).

For these reasons, endophytic fungi associated with medicinal plants from Indonesia are a promising resource to be developed as active secondary metabolite producers. One of the medicinal plants that have been traditionally used in Indonesia is *Uncaria gambier* (Hunter) Roxb. *Uncaria gambier* (Hunter) Roxb. has been utilized to cure headaches, diarrhea, sore skin and also used as gargles (Rauf *et al.*, 2015). However, the information about endophytic fungi from this plant is still limited. In this study, the biodiversity and antibacterial activities of endophytic fungi isolated from *Uncaria gambier* (Hunter) Roxb. var. udang collected in Jasinga, Bogor, West Java, Indonesia are explored and reported.

MATERIALS AND METHODS

Plant materials.

Plant parts of *Uncaria gambier* (Hunter) Roxb. var. udang (UGvU) was collected from Jasinga, Bogor, West Java, Indonesia. The plant was identified at Herbarium Bogoriense, Research Center for Biology, National Research and Innovation Agency.

Isolation of endophytic fungi.

Young plant parts of UGvU were washed with running water and surface sterilized by immersing the plant parts in 75% ethanol for 2 minutes, followed by immersion in 5.3% sodium hypochlorite for 5 minutes and again in 75% ethanol for 30 seconds. The sterilized plant parts were cut into fragments and placed on corn-meal malt agar (CMMA) medium supplemented with 2% UGvU juice and chloramphenicol 0.05 mg/mL. After 3–7 days of incubation at room temperature, the fungi colonies were transferred to potato dextrose agar (PDA) until single isolates were obtained (Agusta *et al.*, 2006). For preservation, endophytic fungi isolates were incubated in 10% glycerin at -4 °C for an hour and further stored at -80 °C.

Morphological characteristic observation.

The isolated endophytic fungi grown on PDA at room temperature were identified by observing their morphological characteristics, both macroscopically and microscopically. The macroscopic characters, including the colony surface color, topography, texture, radial lines, concentric circles, colony reverse color and exudate drops were observed. The microscopic features were analyzed under the Eclipse 80i microscope (Nikon). Septate, hyphae pigmentation, clamp connection, and reproductive structures were observed (Ilyas *et al.*, 2019).

Secondary metabolites production.

The isolated endophytic fungi were inoculated to 10 mL potato dextrose broth (PDB) in a 100 mL Erlenmeyer flask. After three weeks of incubation at room temperature, the culture medium and fungi biomass were extracted with ethyl acetate. The solvent was evaporated using a rotary evaporator and further dried using nitrogen gas before further analysis. Extracts (10 mg/mL) then were loaded onto silica gel GF₂₅₄ TLC aluminum plate (Merck, Darmstadt, Germany) and developed using dichloromethane and methanol (20:1). Spots were visualized using 1% Ce(SO₄)₂ in 10% H₂SO₄ spray reagent (Praptiwi *et al.*, 2018).

Antibacterial activity determination.

The antibacterial activity test was performed using the disk diffusion method (Razmavar *et al.*, 2014). The dried extract was dissolved in 1 mL acetone and a sterile paper disk was immerse into the extract solution and let dry for 30 minutes at room temperature inside the laminar airflow to remove the solvent. The paper disk loaded with extract was placed on top of the Mueller-Hinton agar (MHA) inoculated with *Escherichia coli* (NBRC 14237), *Staphylococcus aureus* (NBRC 14276), *Bacillus subtilis* (NBRC 3134) and *Micrococcus luteus* (NBRC 14218). After 24–48 hours of incubation, antibacterial activity was observed as a clear zone around the paper disk.

Data analysis.

The chemical profile of the extracts was analyzed using thin layer chromatography (TLC) using 1% Ce(SO₄)₂ in 10% H₂SO₄ spray reagent. The antibacterial activity was determined qualitatively (in triplicates) by observing clear zone around the paper disk on the agar plates which previously inoculated with bacteria.

RESULTS

Characterization of endophytic fungi associated with UGvU

Eleven endophytic fungi (UGD-ex1 - UGD-ex11) were successfully isolated from leaves of UGvU. Other eleven endophytic fungi (UGB-ex1 - UGB-ex11) were also obtained from the stems of *Uncaria gambier* ROXB. var. *udang*, but only five endophytic fungi were successfully

isolated from the roots (UGA-ex1 - UGA-ex5). The list of isolated endophytic fungi are displayed in Table 1 and some of the macroscopic and microscopic morphological characteristics of some isolated endophytic fungi are displayed in Figure. 1.

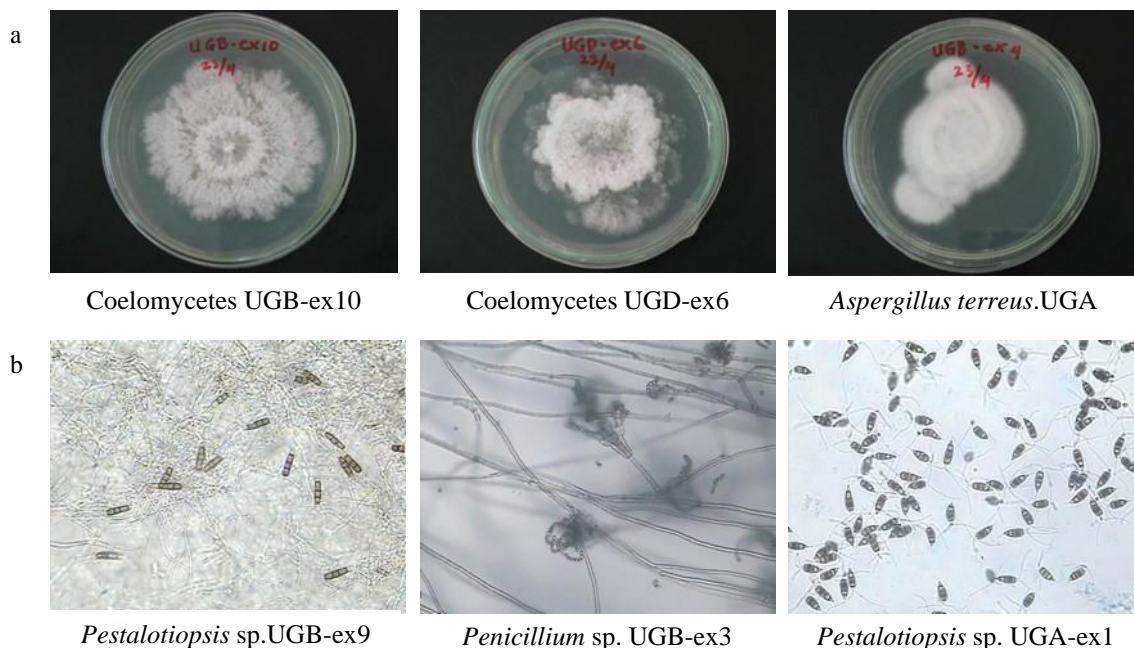


Figure. 1. The macroscopic (a) and microscopic (b) view of several isolated endophytic fungi associated with UGvU collected in Jasinga, Bogor, West Java, Indonesia. (*Penampakan makroskopik (a) dan mikroskopik (b) dari beberapa jamur endofit yang berasosiasi dengan UGvU yang dikoleksi di Jasinga, Bogor, Jawa Barat, Indonesia*).

Characterization of secondary metabolite produced by the isolated endophytic fungi was also done using a chromatographic profiling analysis. Thin layer chromatography (TLC) was used. Various secondary metabolites were produced by the isolated endophytic fungi displayed by various

bands with different retention factor (Rf) and color. However, some of the isolated endophytic fungi exhibited similar TLC profile as displayed in Figure 2.

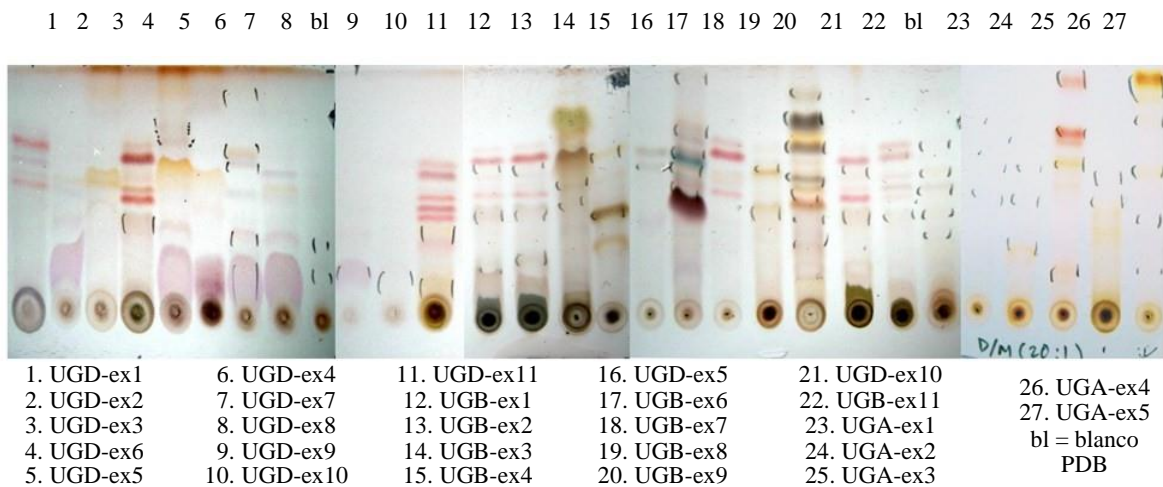


Figure. 2. TLC profiles of the ethyl acetate extracts from endophytic fungi associated with UGvU. TLC plate was developed using CH₂Cl₂:MeOH (20:1) (*Profil KLT ekstrak etil asetat dari jamur endofit yang berasosiasi dengan UGvU. Plat KLT dielusi menggunakan CH₂Cl₂:MeOH (20:1)*).

Table 1. Isolated endophytic fungi associated with UGvU and their antibacterial activities. (*Jamur endofit yang berasosiasi dengan UGvU dan aktivitas antibakterinya*).

Plant part	Endophytic fungi isolate	Fungal taxa	Antibacterial activity			
			<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>M. luteus</i>
Leaf	UGD-ex1					
	UGD-ex6	Coelomycetes UGD-A	+	+	+	-
	UGD-ex11					
	UGD-ex4	Coelomycetes UGD-B	-	-	-	-
	UGD-ex2					
	UGD-ex5	<i>Phoma</i> sp. UGD	+	-	-	-
	UGD-ex8					
	UGD-ex7	Dematiaceae UGD-A	-	-	+	-
	UGD-ex10	Dematiaceae UGD-B	-	-	-	-
	UGD-ex3	<i>Pestalotiopsis</i> sp. UGD	-	-	-	-
UGD-ex9	NI	-	-	-	-	
Stem	UGB-ex1					
	UGB-ex2					
	UGB-ex7	Coelomycetes UGB-A	+	-	+	-
	UGB-ex10					
	UGB-ex4	Coelomycetes UGB-B	-	-	-	-
	UGB-ex8	Coelomycetes UGB-C	-	-	-	-

Plant part	Endophytic fungi isolate	Fungal taxa	Antibacterial activity			
			<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>M. luteus</i>
	UGB-ex11	Coelomycetes UGB-D	-	-	+	-
	UGB-ex5	Dematiaceae UGD-A	-	-	-	-
	UGB-ex3	<i>Penicillium</i> sp. UGB	+	+	+	-
	UGB-ex9	<i>Pestalotiopsis</i> sp. UGB	+	-	-	-
	UGB-ex6	NI	-	-	+	-
Root	UGA-ex1	<i>Pestalotiopsis</i> sp. UGA-A	-	-	-	+
	UGA-ex2		-	-	-	-
	UGA-ex3	<i>Pestalotiopsis</i> sp. UGA-B	+	+	+	-
	UGA-ex4	<i>Aspergillus terreus</i> UGA	+	-	+	-
	UGA-ex5	<i>Penicillium</i> sp. UGA	-	+	+	-

Antibacterial activities of the ethyl acetate extract from the endophytic fungi cultures.

The antibacterial activities of the endophytic fungi ethyl acetate extracts were also observed in this study. Only three from seven types of endophytic fungi isolated from the leaf of *Uncaria gambier* ROXB. var. udang exhibited antibacterial activities. Coelomycetes UGD-A had antibacterial activities against *E. coli*, *S. aureus* and *B. subtilis*, while *Phoma* sp. UGD and Dematiaceae UGD-A only active against *E. coli* and *B. subtilis*, respectively (Table 1). For the endophytic fungi that originated from the stems, five out of eight types exhibited antibacterial activities. While for the endophytic fungi from the roots of *Uncaria gambier* ROXB. var. udang, three types had antibacterial activity. Overall, 12 types of endophytic fungi displayed antibacterial activities, including one extract from an unidentified endophytic fungus from the stem. Three extracts from the cultures of Coelomycetes UGD-A, *Penicillium* sp. UGB and *Pestalotiopsis* UGA-B displayed a wide spectrum of antibacterial activities against *E. coli*, *S. aureus* and *B. subtilis*. Interestingly, only *Pestalotiopsis* sp. UGA-A had antibacterial activity against *M. luteus*.

DISCUSSION

Characterization of endophytic fungi associated with UGvU

Morphological characters observation of the 11 isolated filamentous endophytic fungi from the leaf of *Uncaria gambier* ROXB. var. udang showed that three isolates (UGD-ex1, UGD-ex6 and UGD-ex11) had identical characters (Table 1). However, these three isolates did not display any form of the specific reproductive organ, both sexual

or asexual, that can be used as a distinct characteristic to identify the fungi until genus level. On PDA medium, three weeks old cultures of these isolates formed black sclerotium characterized as Coelomycetes. Furthermore, the TLC analysis of the ethyl acetate extracts from UGD-ex1, UGD-ex6 and UGD-ex11 (Fig. 2) exhibited a similar chromatographic pattern indicating these three isolates produced the same secondary metabolites. Thus, these chemotaxonomy data supported the morphological identification result that showed they were the same endophytic fungi and identified as Coelomycetes UGD-A. On the other hand, UGD-Ex-4 that also identified as Coelomycetes formed brown mycelium, unlike the Coelomycetes UGD-A that had white mycelium. This difference was confirmed by the fact that UGD-ex4 had a different chromatogram profile; hence, UGD-ex4 was identified as Coelomycetes UGD-B.

Three other isolates, UGD-ex2, UGD-ex5 and UGD-ex8, were also morphologically identical to one another. UGD-ex2, UGD-ex5 and UGD-ex8 had spore characters from genus *Phoma* and these three isolates identified as *Phoma* sp. UGD. The identification of these isolates was also supported by the TLC analysis results, which were indistinguishable (Fig. 2). The UGD-ex7 and UGD-ex10 isolates, even though both were characterized as fungi from the Dematiaceae family, displayed distinctive mycelium. The UGD-ex7 isolate had a black powdery cotton-like colony, while UGD-ex10 had a black colony with immersed mycelium and mucilaginous texture. The distinctions between UGD-ex7 and UGD-ex10 were confirmed with the different TLC profiles of the ethyl acetate extracts. Thus, UGD-ex7 was identified as Dematiaceae UGD-A, while

UGD-ex10 was identified as Dematiaceae UGD-B. On the other hand, UGD-ex3 exhibited spore characters specific to *Pestalotiopsis* and characterized as *Pestalotiopsis* sp. UGD. One isolate, UGD-ex9, could not be identified because, after three weeks of incubation on PDA medium, UGD-ex9 did not form either sexual or asexual reproductive structures that could be observed clearly macroscopically or microscopically.

For the eleven endophytic fungi isolated from the stem of *Uncaria gambier* ROXB. var. udang, four isolates UGB-ex1, UGB-ex2, UGB-ex7 and UGB-ex10 displayed identical morphological character and classified as Coelomycetes (Table 1). They had a white cotton-like colony with black sclerotium and also had the same TLC profiles. However, they were different from Coelomycetes UGD-A and characterized as Coelomycetes UGB-A. From the stem, three more isolates, UGB-ex4, UGB-ex8 and UGB-ex11 were identified as Coelomycetes. However, the chromatogram profile displayed distinctive patterns from one another and for this reason, UGB-ex4, UGB-ex8 and UGB-ex11 were identified as Coelomycetes UGB-B, Coelomycetes UGB-C and Coelomycetes UGB-D, respectively.

One of the endophytic fungi isolated from the stem, UGB-ex5 had morphological characters identical to Dematiaceae UGD-A. Even though the chromatogram profiles did not give a clear similarity, UGB-ex5 was still characterized as Dematiaceae UGD-A. Two other isolates, UGB-ex3 and UGB-ex9, were identified as *Penicillium* sp. UGB and *Pestalotiopsis* sp. UGB, respectively. The morphological characters of *Pestalotiopsis* sp. UGB were similar to *Pestalotiopsis* sp. UGD but the TLC profiles were very different; thus, these two isolates were classified as different fungi. UGB-ex6, on the other hand, could not be identified using morphological characters or chemotaxonomy.

From the root of *Uncaria gambier* ROXB. var. udang, only five endophytic fungi were successfully isolated (Table 1). UGA-ex1,

UGA-ex2 and UGA-ex3 were morphologically characterized as fungi from the *Pestalotiopsis* genus. UGA-ex1 and UGA-ex2 had identical characters, while the UGA-ex3 isolate had different characters. The TLC profiles also confirmed the morphological characterization results. UGA-ex1 and UGA-ex2 then were identified as *Pestalotiopsis* UGA-A and UGA-ex3 was identified as *Pestalotiopsis* UGA-B. Two other isolates,

UGA-ex4 and UGA-ex5, were identified as *Aspergillus terreus* UGA and *Penicillium* sp. UGA.

Morphological characterization and metabolite profiling using TLC were used to identify and classify the isolated endophytic fungi in this study.

The metabolites profiling was used for comparative purposes and to confirm the results from morphological characterization. For comparative purposes, secondary metabolite profiling has to be performed using fungi cultures that were grown at the same condition, including the same media, environmental conditions, as well as the same age of cultures (Frisvad, Andersen and Thrane, 2008). This is because filamentous endophytic fungi have a large number of genes that are typically arranged in clusters, encoding a wide array of secondary metabolites (Keller, 2019). Nutritional input from the media and environmental factors can trigger the induction or repression of these genes and, therefore, affect secondary metabolites' production (Keller, 2019). In this study, however, morphological characterization and metabolites profiling using TLC were insufficient to identify and classify the isolated endophytic fungi down to species level. A different possible approach, for instance, using genotypic identification is still needed for further classification of the isolated endophytic fungi to the species level (Frisvad, 2011).

In this study, sixteen types of endophytic fungi from *Uncaria gambier* ROXB. var. udang collected in Jasinga, Bogor were obtained. However, the isolated endophytic fungi community structure in this study had some differences from a previous study. (ILYAS *et al.*, 2008) showed that besides Coelomycetes, Dematiaceae, *Pestalotiopsis* sp. and *Aspergillus* sp.; *Phomopsis* sp., *Fusarium* sp. and *Cladosporium* sp. were also isolated from *Uncaria gambier* ROXB. var. udang collected in Lembah Harau, West Sumatera, Indonesia. Nevertheless, unlike in this study, this previous study did not isolate any *Fusarium* sp. and only isolated one fungus from the root of *Uncaria gambier* ROXB. var. udang. Some factors such as the age of the host plant, geographical location, rainfall, vegetation surrounding the host plant, and canopy cover in each environment may play major roles in influencing these composition differences (Arnold and Herre, 2003; Gomes *et al.*, 2018; Jia *et al.*, 2016; Miao *et al.*, 2021).

Antibacterial activities of the ethyl acetate extract from the endophytic fungi cultures.

Decades after the discovery of penicillin, the antibiotic resistance problem becomes more prominent. Even though antibiotic resistance happens naturally, misuse of antibiotics both in people and animals is stimulating and accelerating the process (Malik and Bhattacharyya, 2019). For this reason, innovative ways to discover new antibiotic agents are needed and one of them is through endophytic fungi. In this study, 12 types of endophytic fungi from *Uncaria gambier* (Hunter) Roxb. var. udang displayed antibacterial activities.

Endophytic fungi are known to produce secondary metabolites that are pivotal for plant defense mechanisms against pathogenic invasion (Manganyi and Ateba, 2020). Diverse antimicrobial compounds have been isolated from endophytic fungi for the last two decades. Some of them were Phomoxanthones A from *Phomopsis* sp. BCC1323 associated with the leaf of *Tectona grandis* L. which effective against *Mycobacterium tuberculosis* H37Ra (Isaka *et al.*, 2001), Primin from *Botryosphaeria mamane* PSU M-76 isolated from the leaf of *Garcinia mangostana* which active against MRSA SK1 (Pongcharoen *et al.*, 2007), Pachybasin from Coelomycetes associated with yellow moonshed, *Arcangelisia flava* (L.) Merr which effective against *Fusarium oxysporum*, and its MIC value of 16 µg/mL is lower than that of nystatin (32 µg/mL) and equal to that of cabicidin (Wulansari *et al.*, 2014). Six recognized compounds were obtained from an ethyl acetate extract of *Penicillium* sp., an endophytic fungus associated with *Garcinia nobilis* leaves, including penialidin A–C, citromycetin, phydroxyphenylglyoxalaldoxime and brefelfin A.

All of compounds had antibacterial activities against Gram negative multidrug-resistant bacteria (MIC = 0.5–128 µg/mL) (Jouda *et al.*, 2016), Sanguinarine from *Fusarium proliferatum* (strain BLH51) associated with *Macleaya cordata* that active against 15 clinical isolates of *S. aureus* (Wang *et al.*, 2014) and other compounds that have been discussed in detail by Deshmukh *et al.* (2014).

In this study, 12 types of endophytic fungi from *Uncaria gambier* (Hunter) Roxb. var. udang exhibited antibacterial activities. Future studies to isolate and identify the active compounds responsible for the antibacterial activities of these endophytic fungi are needed to further explore the antibacterial potencies of the active fungi.

Secondary metabolites profiling of endophytic fungi using advance and modern methods can also be very useful to effectively find novel compounds for future drug discovery from these endophytic fungi (Kluger *et al.*, 2015).

CONCLUSION

Uncaria gambier (Hunter) Roxb. var. udang collected in Jasinga, Bogor harbored diverse endophytic fungi and many of the isolated endophytic fungi produced metabolites with antibacterial activities. These indicated that endophytic fungi from *Uncaria gambier* (Hunter) Roxb. var. udang are potential to be developed and studied further as potential sources for active metabolites.

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