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Antibacterial activity of ethyl acetate extracts of fungal endophytes isolated from leaf gambir leaves (*Uncaria gambir* (Hunter) Roxb)

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ABSTRACT

Gambir (*Uncaria gambir* (Hunter) Roxb) is a Sumatran medicinal plant that has various bioactivities, including antibacterial. This study aims to isolate endophytic fungi from gambier leaves and test the antibacterial activity of ethyl acetate extracts of fungal endophytes from gambir leaves. The fungal endophytes were isolated by inoculating the fungus obtained from gambir leaves on potato dextrose agar (PDA) media. The fungal endophytes were identified microscopically and macroscopically. Furthermore, the fungal endophytes were cultivated using rice media : aquades (100 : 110). The cultivated fungus was macerated using ethyl acetate solvent and tested using a screening test. The antibacterial activity of the ethyl acetate extract of endophytic fungi was conducted using the agar diffusion method against Gram-positive bacteria (Bacillus subtilis ATCC 6633 and Staphylococcus aureus ATCC 25923) and Gram-negative bacteria (Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853). This study has revealed five fungal isolates from gambir leaves and identified the isolates as Penicillium sp 1 (0.39 g), Penicillium sp 2 (0.26 g), Neopestalotiopsis sp (0.97 g), Colletotrichum capsici (0.46 g), and Aspergillus sp (0.25 g). The ethyl acetate extracts of each fungal endophyte show the presence of phenolic compounds and have inhibition against Gram-positive and Gram-negative bacteria. The highest antibacterial activity is shown by ethyl acetate extracts of the fungal endophytes of Neopestalotiopsis sp at a concentration of 7.5% on P. aeruginosa ATCC 27853 23±3.9 mm and S. aureus ATCC 25923 14±2.5 mm. Tests on B. subtilis ATCC 6633 and E. coli ATCC 25922 with the highest inhibition zones were indicated by the ethyl acetate extracts of the fungus Aspergillus sp of 15.3±4 mm and 14±1.9 mm, respectively.

1. Introduction

Antibiotic resistance is an antibiotic inability to inhibit or kill bacteria with a normal dose. The main cause of resistance is the wide use of antibiotics without a precise indication (Ventola, 2015). Antibiotic resistance would result in a critical condition and nonfulfillment of medical needs; such a condition requires an investigation to discover and develop new classes of antibacterial compounds (WHO, 2020).

Fungal endophytes are biota associated with the living tissue of plants without causing disease symptoms to the plants' hosts. Fungal endophytes are a source of bioactive metabolites potentially used in medicine and biotechnology (Rana et al., 2019). Several new compounds have been isolated

from fungal endophytes, which are structurally classified as alkaloids, lactones, phenols, quinine, terpenoids, steroids, and lignans. Based on a literary study, bioactive metabolites that are isolated from the endophytic fungus have various bioactivities, such as antimicrobial, insecticidal, cytotoxic, and anticancer (Zhao et al., 2012).

One of the potential medicinal plants of Sumatra is gambir (*Uncaria gambir* (Hunter) Roxb). The main chemical constituent of gambir is catechin. Catechine has an antibacterial effect by damaging the membrane or cell wall of bacteria so that they can interfere with the permeability of the bacterial cell. Since the permeability is disrupted, the cell cannot perform life activities so that its growth is stunted or even dies (Pambayun et al., 2008).

Research on gambir extracts as an antibacterial substance has been investigated by (Kresnawaty & Zainuddin, 2020), who have discovered that gambir extracts at a concentration of 200 ppm can inhibit the growth of E. coli and S. aureus bacteria. Meanwhile, (Voravuthikunchai et al., 2004) state that gambir extracts on the minimum inhibitory concentration of 6.25 µg/ml can inhibit E. coli bacteria. Gambir extracts on a concentration of 2.5 mg/disc can inhibit Helicobacter pylori bacteria. (Voravuthikunchai & Mitchell, 2008).

Isolation of fungal endophytes from gambir twig has been reported and obtained two fungal endophytes: Pestalotiopsis sp. and Fusarium sp. (Jamal et al., 2008). However, fungal endophytes on gambir leaves have never been investigated. The content of tannins calculated as the catechins in gambir leaves is 90% or greater than that of other parts of the gambir plant (Kemenkes, 2017). Therefore, this research isolated fungal endophytes from gambir leaves and tested the antibacterial activity of ethyl acetate extracts of the fungal endophytes on Gram-positive bacteria (Bacillus subtilis ATCC 6633 and Staphylococcus aureus ATCC 25923) and Gram-negative bacteria (Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853).

2. Materials and Methods

2.1. Tools

The tools of this research were the autoclave (Hiramaya®), oven (Memmert®), incubator (Memmert®), Laminar airflow (LAF) (Innotech-Biobase®), rotary evaporator (IKA®), hot plate (Velp Scientifica®), analytical balance (Kern®), Erlenmeyer (Iwaki®), test tube (Iwaki®), and petri dish (Normax®).

2.2. Materials

The materials of this research were gambir leaves (Uncaria gambir (Hunter) Roxb), potato dextrose agar (PDA) media (Merck®), nutrient agar (NA) (Merck®), sodium hypochlorite 10-14 % (Bratacem®), ethanol 70 % (Bratacem®), paper disc, rice, and aquadest (Bratacem®).

2.3. Sample Collection

The gambir plants were taken from the Medicinal Plants Garden of Universitas Andalas, Padang. The samples were identified at the herbarium of Andalas University with the Identification Letter No. 096/K-ID/YOU/III/2020.

2.4. Isolation and Characterization of a Fungal Endophytes on Gambir Leaves (Uncaria gambir (Hunter) Roxb.)

The isolation of fungal endophytes adopted a method developed by Hormazabal and Piontelli (2009) with several modifications. Fresh gambir leaves (1 x 1 cm) were sterilized by soaking them in 70% ethanol for one minute and then soaking them in the sodium hypochlorite 10-14% for seven minutes. Afterward, the leaves were rinsed with sterile aquadest three times. Pieces of sterile gambir leaves were planted on the PDA media and incubated at a temperature of 37ºC for 5-7 days. To control the growth, final aquadest rinses of the leaves inoculated on the PDA media were applied. The growing fungal endophytes were purified to a new PDA media by observing different isolate shapes macroscopically to obtain pure isolates (single). The fungal endophytes were characterized and identified by the Center for Veterinary of Bukittinggi with macroscopic and microscopic morphological observation (29008/PK.310/F48.1/11/2019).

2.5. Cultivation and Extraction Procedures of Fungal Endophytes

Fungal endophytes were cultivated using the media of rice : aquadest (100 : 110). The rice was sterilized by autoclaving for 15 minutes at a temperature of 121ºC and a pressure of 2 atm. Furthermore, the fungal inoculum was put in the media of sterile rice. The fungus cultivation was incubated at room temperature for four weeks. Then, the cultivated fungi were macerated with 200 ml of solvent ethyl acetate for three days to produce an optimal ethyl acetate extract. Furthermore, the ethyl acetate extract was evaporated with a rotary evaporator to get a thick extract of ethyl acetate that would be used to test antibacterial activity (Kjer et al., 2010).

2.6. Phytochemical Test

The secondary metabolite content, such as alkaloids, flavonoids, phenolics, terpenoids, steroids, and saponins, of ethyl acetate extract of each fungal endophyte was determined using the phytochemical test developed in previous research (Efendi et al., 2020).

2.7. Profiles of Thin Layer Chromatography (TLC) of Ethyl Acetate Extracts of Fungal Endophytes

TLC patterns of the ethyl acetate extract of the fungal endophytes were determined using a TLC plate of silica gel 60 F254. The mobile phase used was n-hexane : ethyl acetate (1 : 9). The observation employed 254 and 365 nm UV lamps. The Rf values of visible stains were determined using the following formula.

$$Rf = \frac{Distance\ traveled\ by\ compounds}{Distance\ traveled\ by\ solvents}$$

2.8. Testing Antibacterial Activity Using the Agar Diffusion Method

The antibacterial activity was tested by the agar diffusion method on Gram-positive bacteria (Bacillus subtilis ATCC 6633 and Staphylococcus aureus ATCC 25923) and Gram-negative bacteria (Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853). The tested bacteria was inoculated and taken with sterile ose wire. Then, it was resuspended in a test tube containing 5 ml of NaCl 0.9% to obtain a suspension with a 25% transmittance at λ = 580 nm or equivalent to a concentration of 1 x 108 CFU/mL of bacteria (of 0.5 Mc Farland). The same treatment was done in any kind of bacteria test. The extract was weighed as much as 75 mg and dissolved in 1 ml DMSO. Thus, this study obtained the 7.5% test concentration and sterilized discs or paper discs that were spilled with 10 µl extract and then put on the surface of the nutrient agar media. This media had been previously swabbed with the suspension of the tested bacteria (1 x 10Eight CFU/mL). Then Petri was incubated in the incubator for 18 hours at a temperature of 37ºC. Antibacterial activity was observed by measuring the inhibition diameter of the discs using a caliper. The positive control used in this research was 30 µg/disc chloramphenicol. Meanwhile, the negative control used in this research was the DMSO.

3. Results and Discussion

This research has discovered five fungal endophyte isolates isolated from samples of gambir leaves (Uncaria gambir (Hunter) Roxb). The fungal endophytes were isolated by planting sterile leaf samples in the fungal growth medium (PDA media). 70% ethanol and sodium hypochlorite (10-14% w/v) solutions were used to rinse the leaves and kill the fungus that lives on the surface of the leaf samples. Sodium hypochlorite is a solution that has antimicrobial activity and extensive spectrums that work by deactivating the enzyme. The fungal endophytes were characterized in macroscopic levels by visually observing the shapes and colors of hyphae. The spore's shapes and reproduction (sexual and asexual) were observed microscopically at a magnification of 40 and 100 times. The results of characterizing fungal endophytes on gambir leaves are presented in Table 1 and Figure 1.

Fungal endophytes were cultivated using the growth media of the rice to double the number of fungal endophytes. The optimal cultivation time occurs when the entire surface of the rice media is overgrown by the fungus of which the color has changed. Generally, the cultivation process lasts for 4-6 weeks. The cultivated fungal endophytes were then macerated using the ethyl acetate solvent. Ethyl acetate is a semi-polar solvent with low toxicity and can attract antibacterial compounds. In

addition, ethyl acetate wets and reduces the number of spores that would be carried into the air when opening a cultivation container (Kjer et al., 2010).

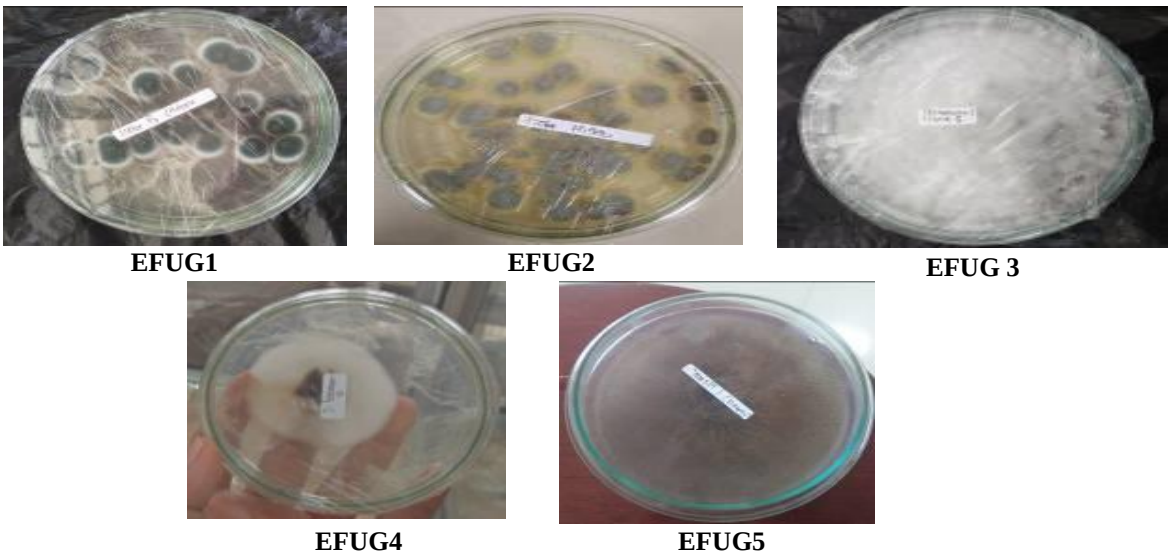

Fig. 1. Fungal endophytes of gambir leaves (Uncaria gambir (Hunter) Roxb.)

Description: EFUG1 = Penicillium sp 1, EFUG2 = Penicillium sp 2, EFUG3 = Neopestalotiopsis sp, EFUG4 = Colletotrichum capsici, EFUG5 = Aspergillus sp

Table 1. Results of macroscopic and microscopic observations on fungal endophytes

Fungi codes	Fungal endophytes	Biomass (grams)
EFUG1	*Penicillium* sp 1	0.39
EFUG2	*Penicillium* sp 2	0.26
EFUG3	*Neopestalotiopsis* sp	0.97
EFUG4	*Colletotricum capsici*	0.46
EFUG5	*Aspergillus* sp	0.25

The results of maceration were evaporated using a rotary evaporator to gain acetate ethyl extracts. The yield of ethyl acetate extracts of the five fungal isolates is presented in Table 1. Ethyl acetate extracts were obtained by characterizing the thin layer chromatography (TLC) method and phytochemical test on the five fungal endophyte extracts. The separation using the TLC method aims to determine compound patterns in the extracts. Figure 2 indicates that each ethyl acetate extract of the fungal endophytes shows different compound patterns. The phytochemical screening on all ethyl acetate extracts shows positive results of phenol in FeCl3 10% reagent. Meanwhile, the tests on alkaloids, flavonoids, and terpenoids do not show positive results on all extracts.

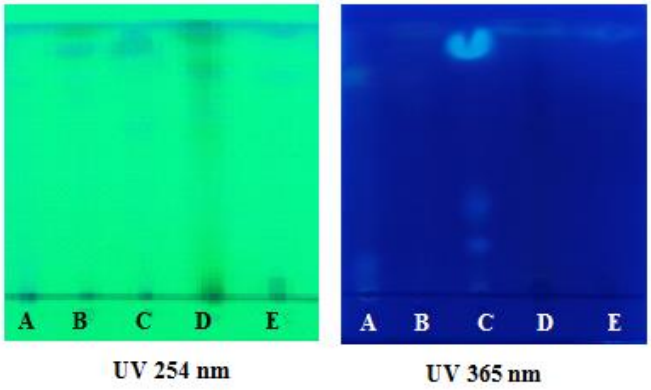

Fig. 2. Profile of TLC eluent n-hexane : ethyl acetate (1 : 9) at 254 and 365 nm UV lamp

Description: A = Neopestalotiopsis sp fungus extracts, B= Aspergillus sp fungus extracts, C= Penicillium sp 2 fungus extracts, D= Penicillium sp 1 fungus extracts, E= Colletotricum capsici fungus extracts.

Table 2. Results of the phytochemical test on ethyl acetate extracts of Fungal endophytes on gambir leaves

Ethyl Acetate Extract Fungal Endophytes	Phytochemical Test					
	Alkaloids	**Phenolics**	**Flavonoids**	**Terpenoids**	**Steroids**	**Saponins**
Penicillium sp 1	-	+	-	-	-	-
Penicillium sp 2	-	+	-	-	-	-
Neopestalotiopsis sp	-	+	-	-	-	-
Colletotricum capsici	-	+	-	-	-	-
Aspergillus sp	-	+	-	-	-	-

The next step was testing activities of Gram-positive bacteria (S. aureus ATCC 25923 and B. subtilis ATCC 6633) and Gram-negative bacteria (P. aeruginosa ATCC 27853, and E. coli ATCC 25922). This test aimed to investigate the antibacterial activity from the ethyl acetate extract of fungal endophytes. Moreover, this test employed the agar diffusion method because it is simple, and the measurement results are easily interpreted by measuring the diameter of the stunted growth of bacteria. In addition, the agar diffusion method can also be used to test pathogenic aerobic bacteria and fastidious bacteria, such as the bacterium tested in this study (Schumacher et al., 2018). The positive control used in testing antibacterial activity was 30 µg/disk chloramphenicol. Chloramphenicol is used as a positive control for bacteria because it is included in an antibiotic class with a wide spectrum that can inhibit the growth of Gram-positive bacteria and Gram-negative bacteria (Ritter et al., 2008). The extract concentration of antibacterial testing was 7.5%, which was dissolved in the dimethylsulfoxide solvent (DMSO). The extract activity is estimated based on the classification of inhibition zones; the diameter of less than 8 mm is categorized inactivated, the inhibition zone of 8-12 mm is categorized weak, the inhibition zone of 13-15 mm is categorized medium, and the inhibition zone of 15 mm or more is categorized strong (Becerra et al., 2002).

In testing the antibacterial activity, all ethyl acetate extracts of fungal endophytes at a concentration of 7.5% could inhibit Gram-positive bacteria and Gram-negative bacteria. The results of measuring the diameter of the inhibition zone are summarized in Table 3.

Table 3. Results of testing antibacterial activity of ethyl acetate extracts in fungal endophytes

Fungal Endophytes	Inhibition Diameter (mm) of Bacterial Test			
	Gram-positive		**Gram-negative**	
	S. aureus	***B. subtilis***	***P. aeruginosa***	***E. coli***
Penicilium sp 1	11 ± 2.5	15 ± 4	17 ± 3.9	12.6 ± 1.9
Penicilium sp 2	8.6 ± 2.5	7 ± 4	14 ±3.9	13 ± 1.9
Neopestalotiopsis Sp.	14 ± 2.5	9 ± 4	23 ± 3.9	13.16 ± 1.9
Colletotricum capsici	7.3 ± 2.5	7.6 ± 4	13.5 ± 3.9	9 ± 1.9
Aspergillus sp	10.1 ± 2.5	15.3 ± 4	14.13 ± 3.9	14 ± 1.9
Control (+)	18 ± 3.6	20 ± 0.8	24 ± 1.9	20 ± 2.7
Control (-)	-	-	-	-

Table 3 denotes that the ethyl acetate extract of the Neopestalotiopsis sp fungus at a concentration of 7.5% has the highest diameter of an inhibition zone on P. aeruginosa ATCC 27853 bacteria by 23 + 3.9 mm and S. aureus ATCC 25923 bacteria by 14 + 2.5 mm. A test on B. subtilis ATCC 6633 and E. coli ATCC 25922 with the highest inhibition zone is shown by the ethyl acetate extract of the Aspergillus sp fungus by 15.3 + 4 mm and 14 + 1.9 mm.

This research has revealed that the ethyl acetate extract of the fungal endophytes from gambir leaves has an antibacterial ability in the bacteria test because the extract has secondary metabolite content functioning as an antibacterial substance. This statement is supported by the phytochemical result constituting a phenolic class. Phenol compounds kill microorganisms by denaturing protein cells. A hydrogen bond that is formed between phenols and proteins damages protein structures. Hydrogen bonds will damage the permeability of the cell wall and cytoplasmic membrane because both of them consist of proteins. The disturbed permeability of the cell wall and cytoplasmic membrane can lead to imbalanced macromolecules and ions in the cell; thus, the cell becomes lysis (Nazzaro et al., 2013).

4. Conclusion

Fungal endophytes isolated from gambir leaves (Uncaria gambir (Hunter) Roxb) are Penicillium sp. 1, Penicillium sp. 2, Neopestalotiopsis sp, Colletotrichum capsici, and Aspergillus sp. Ethyl acetate extracts of each fungal endophyte signify the presence of phenolic compounds and the inhibition diameter of Gram-positive bacteria and Gram-negative bacteria.

Author Contributions: M. Rifqi Efendi conceived and designed the study. M. Rifqi Efendi performed all data analyses. M. Rifqi Efendi, Mesa Sukmadani Rusdi and Anita Dinda interpreted the results and revised the paper. M. Rifqi Efendi wrote the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors disclose no conflict.

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