Eksakta : Berkala Ilmiah Bidang MIPA
VOLUME 22 NO 02 2021, pp 137-144
ISSN : Print 1411-3724 — Online 2549-7464
DOI : https://doi.org/10.24036//eksakta/vol21-iss2/266

Eksakta
Berkala Ilmiah Bidang MIPA
http://www.eksakta.ppj.unp.ac.id/index.php/eksakta

Article

Study of The Antibacterial Activity of Endophytic Fungus That Colonize With The Twig of *Andrographis paniculata*

Article Info

Article history :

Received March 15, 2021
Revised May 02, 2021
Accepted May 05, 2021
Published June 27, 2021

Keywords :

Andrographis paniculata, antibacterial, endophytic fungus, twig

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Abstract. Reports of the chemical constituents of *Andrographis paniculata* showed that this plant produced various secondary metabolites with antibacterial activity. Further exploration of bioactive compounds from *A. paniculata* can also be conducted by analyzing its endophytic fungi. Isolation of endophytic fungi from the twig of *A. paniculata* obtained three isolates of endophytic fungi. One of the isolates, RS-2, was fermented on rice media and extracted with ethyl acetate to give the EtOAc extract. The EtOAc extract from fungus RS-2 was analyzed for their antibacterial and phytochemical screening. The results exhibiting the EtOAc extract of fungus RS-2 has activity to inhibite bacterial growth. Overall, the study of the antibacterial activity of endophytic fungus obtained from the twig of *A. paniculata* was firstly carried out in this study.

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1. Introduction

Endophytic fungi are microorganism which reside in tissues of living plants. In general, every plant is the potential host for the growth of endophytic fungi [1]–[3]. Fungi and their host plants have a mutually positive relationship. Plants provide nutrients for fungal growth, while endophytic fungi will produce bioactive compounds to protect their host plants [4]–[6]. Several groups of compounds such as alkaloids, steroids, terpenoids, and phenolic compounds have been reported from endophytic fungi [7]–[10]. These compounds showed various biological activities including antibacterial [11], [12]. *A. paniculata* (sambiloto) as a traditional medicinal plant is a potential host for the development of endophytic fungi.

A. paniculata is a plant species in the family Acanthaceae. This plant can can be found on subtropical areas such as South Asia, China, Europe, and Indonesia [13]. Previous phytochemical studies showing a diverse group of secondary metabolites have been isolated from *A. paniculata*. These compounds have the ability of biological activities, including antibacterial [14]. Further exploration of antibacterial compounds from *A. paniculata* can also be conducted by advanced technologies such as developing culture of its endophytic fungi.

Research on the chemical constituents of endophytic fungi derived from *A. paniculata* have been previously reported. The results of these studies indicate that organic extracts and secondary metabolites obtained from endophytic fungi obtained from the leaves of *A. paniculata* showed antibacterial, cytotoxic, and antioxidant activities [6], [15]. To the best of our knowledge, this is the first report on chemical investigation of endophytic fungus derived from the twig of *A. paniculata*. In this research, the antibacterial activity and phytochemical screening of the EtOAc extract of endophytic fungus isolated from the twig of *A. paniculata* were investigated.

Figure 1. *Andrographis paniculata* and map of Banda Gadang, Naggalo, Padang (www.google.com)

2. Method

2.1 Collection of Plant Material

The twig of *A. paniculate* was colleted from Kelurahan Tabing Banda Gadang, Kecamatan Nanggalo, Padang. The twigs were processed within a few hours after sampling reducing the chances of contamination.

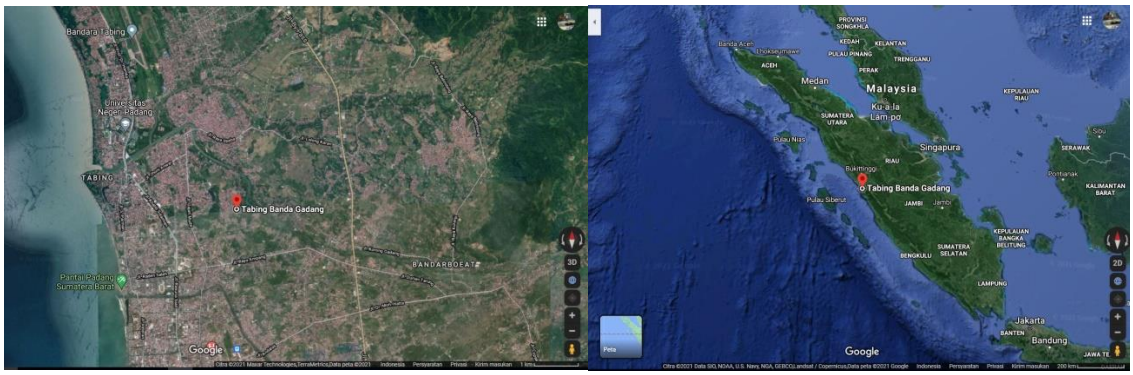

Figure 2. Map of Kelurahan Tabing Banda Gadang, Kecamatan Nanggalo, Padang (www.google.com)

2.2 Isolation of Endophytic Fungus from Twig of *A. paniculata*

The twig of the *A. paniculata* with a size of 2x2 cm was washed with running tap water. The twig was surface-sterilised following the reported procedure [1], [16]. The clean part of the twig was sterilized using ethanol 70% (45 seconds) and NaOCl 3.5% (30 seconds). The sterile twig was placed on the PDA media as a negative control. Then, the twig (1x1 cm) was inoculated on the PDA media. After the incubation process at 28 ^{0}C for 7 days, the endophytic fungi were transferred to the new media to obtain the single strains of endophytic fungi. All equipments were sterilized by autoclave and all steps processed under aseptic conditions.

2.3 Fermentation and Extraction of Endophytic Fungus Isolated from Twig of *A. paniculata*

A single strain of endophytic fungus (2x2 cm) on agar media was transferred to 250 mL Erlenmeyer flsaks containing rice media. Endophytic fungus was fermented for one, two, three, and four weeks under stationary conditions [12]. Then, endophytic fungi were extracted with EtOAc three times and then evaporated to give the crude extract. Endophytic fungus with potential cultivation time was analyzed for their antibacterial activity and phytochemical screening.

2.4 Phytochemical Screening of The EtOAc Extract of Endophytic Fungus

2.4.1 Terpenoid and Steroid Screening

The EtOAc extract was put into a different test tube and added with ammonia-chloroform and H_2SO_4 2 N. This mixture was shaken to form two layers. The bottom layer was transferred to the drip plate. After the solvent has evaporated, anhydrous acetic acid and H_2SO_4 *p.a* were added. The green-blue color represented the presence of steroids, while the red color showed the presence of terpenoids.

2.4.2 Alkaloid Screening

The top layer in the above test was transferred into three test tubes, then each of which was added with Dragendorf reagent, Mayer reagent and Wagne4 reagent. Alkaloid positive results for the three reagents gave a brown precipitate, a white precipitate and an orange precipitate, respectively.

2.4.3 Phenolic Compound Screening

The EtOAc extract was transferred to a drop plate and $FeCl_3$ 1% solution was added. The presence of phenolic compounds will give a pink color.

2.5 Antibacterial Assay of The EtOAc Extract of Endophytic Fungus

Antibacterial activity of the EtOAc extract was carried out following the disc diffusion technique [12], [17], [18] against three bacteria (*Escherichia coli, Staphylococcus aureus, and Streptococcus pyogenes*). The 20 µL extract with the series concentrations (1%, 3%, and 5%), positive control and negative control were applied on disc paper which was on the agar medium containing the tested bacteria with and moxicillin as the positive control.

2.6 Statistical Analysis

Antibacterial activity was established by quantifying the diameter of the inhibition zones in mm. All tests were performed in triplicate. The data resulted were analysed statistically. Data are presented as mean ± standard deviation.

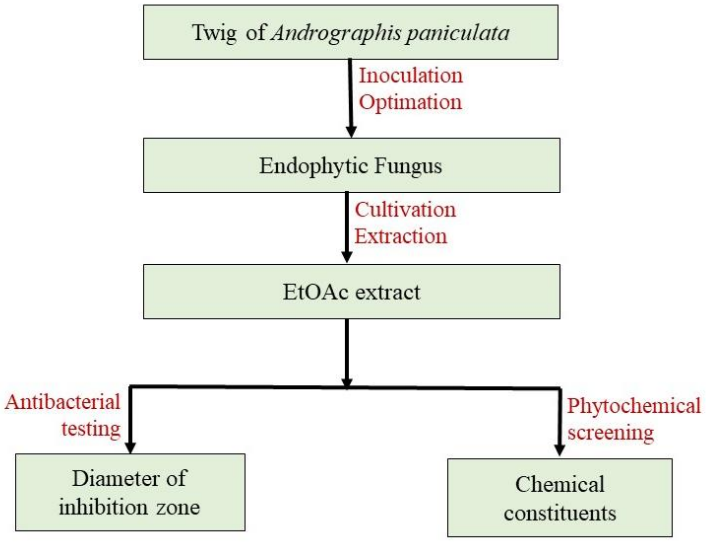

Figure 3. Flow chart of research

3. Results and Discussion

The first step of this research was the isolation of endophytic fungus from twig of *A. paniculata*. A twig with a size of 2x2 cm was drained with clean water to remove the impurities on its surface. Then, the surface of this twig was sterilized using ethanol 70% for 45 seconds and NaOCl 3.5% for 30 seconds. This process aims to eliminate the epiphytic microbes that live on the surface of the twig. The sterile twig was inoculated on the PDA media. After incubation for seven days at 28 ^{0}C, three endophytic fungus grew on the surface of the PDA media. Single strains of endophytic fungus were obtained by subculturing the endophytic fungus. Based on morphological observations [19], an endophytic fungi with code RS-2 (Figure 1) was selected for their chemical and biological activity. The macroscopic morphology of the fungus RS-2 was observed for their shape and color of the colony. The color of fungus RS2 is white with fibrous shape and forms a central colony. The surface of the fungus RS-2 is smooth and spreads to all parts of the solid media.

Figure 4. Morphology of endophytic fungus (RS-1, RS-2, and RS-3)

The fungus RS-2 isolated from twig of *A. paniculata* was optimized to determine the time for the fungus to produce secondary metabolites. Optimization was carried out by analyzing the mass of the extract (Figure 2) on the first, second, third and fourth week of the EtOAc extract. The results showed that the second week is the optimum cultivation time for fungus RS-2. This data indicated that the second week is the stationary phase for the fungus RS-2. The stationary phase is the balance phase between cell division and cell death. In this phase, the amount of nutrients contained in the growth media has started to deplete, which causes the enzymes responsible for the production of secondary metabolites to accumulate. It will make the production of secondary metabolites increasing significantly [19], [20].

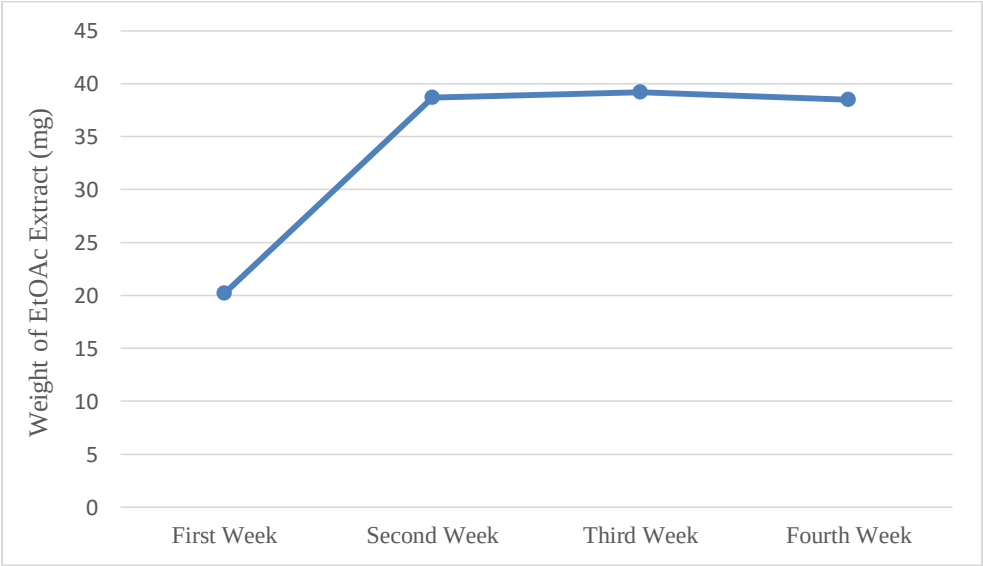

Figure 5. Optimization of the cultivation time of endophytic fungi based on weight of the EtOAc extract

The EtOAc extract was tested for its antibacterial activity following the modified diffusion method [12]. The bacteria tested in this study consisted of two Gram-positive bacteria (*S. aureus* and S. *pygones*) and one Gram-negative bacteria, *E. coli*. In this study, three different concentrations (1%, 3% and 5%) of the EtOAc extract to evaluate the antibacterial activity were used. Amoxicillin was applied as a positive control. The antibacterial activity test was carried out three times (triple) and

the activity value was expressed as the zone of inhibition (mm). The diameter of inhibition zone for various concentrations of the EtOAc extract against the three tested bacteria are shown in Table 1.

Table 1. Inhibition Zone of EtOAc Ectract of Fungus RS-2

Concentration	Diameter of inhibition zone*		
	E. coli	***S. aureus***	***S. pygones***
1%	5.67 ± 0.58	5.67 ± 0.58	6.33 ± 0.58
3%	6.33 ± 0.58	6.33 ± 0.58	7.67 ± 0.58
5%	7.33 ± 0.58	7.67 ± 1.15	8.67 ± 0.58
Control (+)	9.33 ± 1.53	9.33 ± 0.58	9.33 ± 0.58

**All the values are mean ± SD of three parallel measurements*

Table 1 showed that the EtOAc extract of fungus RS-2 associated with the twig of *A. paniculata* has the potential to inhibit the growth of all tested bacteria. The increasing concentration of the EtOAc extract has positive implications for its ability to inhibit bacterial growth. This is due to the increasing composition of bioactive compounds with higher extract concentration. Increasing the composition of bioactive compounds will increase the extract's potential as an antibacterial agent [21].

The activity of the EtOAc extract as an antibacterial agent is related to the composition of secondary metabolites in this extract. Based on this fact, the phytochemical screening of the EtOAc extract was carried out using chemical reagents. The results of the secondary metabolite screening for the EtOAc extract are shown in Table 2. Alkaloids play a role in inhibiting bacterial growth by disrupting the components of peptidoglycan in bacterial cells. The mechanism of antibacterial activity of terpenoid compounds is disrupting the formation of the membrane or cell wall. The cell wall or membrane will not be perfectly formed [22], [23].

Table 2. Phytochemical Screening of EtOAc Extract Fungus RS-2

Phyctochemical Screening	Results
Terpenoid and Steroid	+
Alkaloid	+
Phenolic compound	+

The antibacterial activity of phenolic compounds is influenced by several factors, such as lipophilicity, electronic activity and polyphenol content. Phenolic compounds can inhibit the work of reverse transcription enzymes and DNA topoisomerase which causes bacterial cells not to form [23]–[25]. Some issues such as and secondary metabolites that play a role in antibacterial activity produced by fungus RS-2 and also mechanisms of action need to be further investigated in the future.

4. Conclusions

In this work, three endophytic fungus, RS-1, RS-2 and RS-3, were isolated from the twig of *A. paniculata*. Based on morphology, fungus RS-2 was studied for their antibacterial activity and phytochemical screening. The results of antibacterial assay indicate that EtOAc extract of fungus RS-2 is a potential source of antibacterial agents. The phytochemical screening of EtOAc extract of fungus RS-2 showed the presence of steroid, terpenoid, alkaloid and phenolic compound. Further

investigations of the bioactive compounds produced by fungus RS-2 colonizing with the twig of *A. paniculata* will be performed in the future.

5. Acknowledgment
The authors would like to thank Lembaga Penelitian dan Pegabdian Masyarakat Universitas Negeri Padang for funding this work with a contract number: 866/UN35.13/LT/2021.

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