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Isolation, identification, phytochemical screening, and antibacterial activity of *Aspergillus* sp. MFD-01, an endophytic fungus derived from *Mesua ferrea*

Dwi Hartanti*, Risda Yunita Andestia Sinaga, Asmiyenti Djaliasrin Djalil, Retno Wahyuningrum
Faculty of Pharmacy Universitas Muhammadiyah Purwokerto
Jl. Raya Dukuhwaluh PO Box 202, Kembaran, Banyumas 53182

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ABSTRACT

We have successfully isolated and identified endophytic fungi from medicinal plant *Mesua ferrea* L. grown at Banyumas for the first time. One of those fungi was MFD-01. In this study, we report the isolation, identification, antibacterial activity, and phytochemical screening of *Aspergillus* sp. MFD-01. The endophytic fungus was isolated from the leaves of *M. ferrea* by subsequent inoculation on potato dextrose agar (PDA). The identification was based on its morphology and ITS-DNA sequence. The antibacterial activity was determined by dilution method. The identification of compounds in ethyl acetate extract of it was conducted according to the standart phytochemical screening method. MFD-01 was identified as *Aspergillus* sp. MFD-01. The ethyl acetate extract of *Aspergillus* sp. MFD-01 inhibited the growth of both *Staphylococcus aureus*, and *Escherichia coli*. At concentration of 1000 ppm, its diameter of inhibitory zone against those bacteria was 10.53 and 11.84 mm, respectively. The extract contained flavonoids, alkaloids, saponins, terpenoids, and tannins, which might be responsible for its antibacterial activity against both tested bacteria.

Keywords: Aspergillus, antibacterial activity, endophytic fungi, *Mesua ferrea*, phytochemical screening

Corresponding author:
Dwi Hartanti
Faculty of Pharmacy Universitas Muhammadiyah Purwokerto
Jl. Raya Dukuhwaluh PO Box 202, Kembaran, Banyumas, Jawa Tengah 53182
Email: dwihartanti@ump.ac.id

INTRODUCTION

Endophytes, defined as those inhabit the interior of plant tissues and organs without causing harms to their hosts, have been widely studied for these last two decades (dos Banhos *et al*., 2014). Endophytic fungi have been known to produce various bioactive secondary metabolites, with the profound emphasis on antimicrobial ones (dos Banhos *et al*., 2014; Duan *et al*., 2016; Hartanti *et al*., 2016; Hussain *et al*., 2014; Jadson *et al*., 2015; Katoch *et al*., 2014; Liang *et al*., 2012; Luo *et al*., 2016; Zuo *et al*., 2014). Some of those metabolites are same or similar to those produced by their respective hosts (Kusari *et al*., 2012; Nicoletti and Fiorentino, 2015; Zuo *et al*., 2014). The selection of host plants played an important role in developing new antimicrobial agent from metabolites of endophytic fungi. Hence, the plants possessing an ethnobotanical history and producing bioactive metabolites are prioritized to be studied their endophytes further (Selim *et al*., 2012).

Messua ferrea is traditionally used to treat wound in Java, Indonesia (Suparman *et al*., 2012). It has been reported producing metabolites with antimicrobial activities *(Chanda et al., 2013; Dennis*, 1988; Roy *et al*., 2013; Verotta *et al*., 2004). The preeliminary study has been succeed isolating six endophytic fungi from *M. ferrea* grown in Banyumas, Indonesia (Hartanti, 2015). In our continuation studying endophytes, we are reporting isolation, identification and antimicrobial activity of *Aspergillus* sp. MFD-01, an endophytic fungi isolated from the leaves of *M. ferrea*.

MATERIALS AND METHODS

Plant Material

Fresh *M. ferrea* was collected from Banyumas, Central Java, Indonesia in February 2015. The plant material was determined at the Laboratory of Botany, Universitas Jenderal Soedirman, Purwokerto, Indonesia.

METHODS

Endophytic fungi isolation

Isolation of endophytic fungi was performed according to previously report (Hartanti, 2015). In brief, leaves of *M. ferrea* were surface sterilized with 70% ethanol and rinsed with sterile aquadest. The sterilized stems were cut aseptically and parts of the inner tissues were imprinted onto agar plates containing potato dextrose agar (PDA) medium added with powdered dried plant (15 g/l). Pure strains were obtained by repeated inoculation of growing fungi on agar plates with fresh PDA medium.

Identification of *Aspergillus* sp. MFD-01

Identification of the fungus was conducted by evaluating its morphology and molecular. The morphology identification was based on its macroscopic and microscopic (on light microscope with 100x magnification) characters. Molecular identification was conducted based on its rDNA sequence. The endophytic fungus is cultured in malt extract broth at room temperature for 5 days. DNA isolation was performed with following the instructions of the Nucleospin Plant II kit (Macherey-Nagel, Germany). The fungal ITS4 (5'TCCTCCGCTTATTGATATGC-3') and ITS5 (5'GGAAGTAAAAGTCGTAACAA-3') regions were amplified by PCR with the following reaction program: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 15 sec., 55°C for 30 sec., 72°C for 45 sec., and final extension at 72°C for 7 min (Qadri *et al*., 2013). The amplified products were examined by electrophoresis in 1.5% agarose gels in TAE buffer. Sequencing of the samples was performed at the sequencing facility of the Laboratory of Gene Function in Animal, Nara Institute of Science and Technology (NAIST), using the above mentioned primers. Fungal rDNA-ITS sequences was manually edited and compared with available data from GenBank databases (National Centre for Biotechnology Information website; http://www.ncbi.nlm.nih.gov/) using the BLAST program.

Cultivation of *Aspergillus* sp. MFD-01

A small part of endophytic fungi *Aspergillus* sp. MFD-01 was transferred under sterile conditions to the potato dextrose broth medium (250 mL/flask). The fungus was grown under static conditions at room temperature (approx. 30±3^{o}C) for 28 days. The biomass of fungi was dried and powdered in room terperature afterward (Hartanti *et al*., 2016).

Preparation of extract of *Aspergillus* sp. MFD-01

The ethyl acetate extract of fungi was prepared as a method previously reported (Hartanti *et al*., 2017). In brief, the powdered endophytic fungi (34 g) were extracted with ethyl acetate (350 ml) using remaceration method. Each extraction was conducted for 24 hours, and the process was repeated for three times. The filtrates were collected, and concentrated in vacuo to obtain a dried extract.

Antibacterial Activity Assay of *Aspergillus* sp. MFD-01

Ethyl acetate extract of *Aspergillus* sp. MFD-01 was tested as previously described (dos Santos *et al*., 2015). The tested bacteria used in this study were *Eschericia coli* and *Staphylococcus aureus*, both were cultured by Laboratory of Microbiology and Genetics, Universitas Muhammadiyah Purwokerto. Ethyl acetate extract of *Aspergillus* sp. MFD-01 was prepared into solution in 10% DMSO, with series of concentration of 15, 31, 62, 125, 250, 500, and 1000 ppm. 10% DMSO and ciprofloxacin (2000 ppm) were used as negative and positive control, respectively.

Phytochemical Screening of Extract of *Aspergillus* sp. MFD-01

The group of secondary metabolites (flavonoids, alkaloids, saponins, terpenoids, and tannins) in ethyl acecate extract of leaves of *M. ferrea* was analyzed with standard phytochemical screening reported elsewhere (Dior *et al*., 2017; Gama *et al*., 2014).

Data Analysis

Means separation of diameter of inhibitory zone of each concentration of extracts of endophytic fungi against tested bacteria was accomplished by Kruskal Wallis' multiple range tests. Significance was evaluated at $p < 0.05$. Statistical analysis was conducted by the general procedures of SPSS Statistics v.13 (SPSS Inc.).

RESULT AND DISCUSSION

MFD-01 on PDA exhibited a black with white perifer colony. The surface of the colony was powdery. Zonation of the fungus was observed on day-14 while there was no radial line observed. The microscopic observation of MFD-01 showed globose conidial heads. Conidiophores are smooth-walled. Conidial heads are biseriate with the phialides. Conidia are globse. Hyphae were septa divided and branched (Figure 1).

The data obtained from morphology of the fungi alone was not sufficient to identify it. The morphological approaches to fungal identification may not always perform well for lower-level classifications due to some limitations. A more powerful identification using DNA barcoding is suggested (Raja *et al*., 2017). Hence, we performed sequence-based identification of fungi using the ITS region of rDNA to ensure the identity of MFD-01. Identification was performed using BLASTN 2.8.0 (query ID: lcl|Query_187207). The analysis of the nucleotides of ITS sequences indicated that MFD-01 possessing similarity with those of some *Aspergillus* spp. (Table I). Hence, MFD-01 was identified as *Aspergillus* sp. MFD-01.

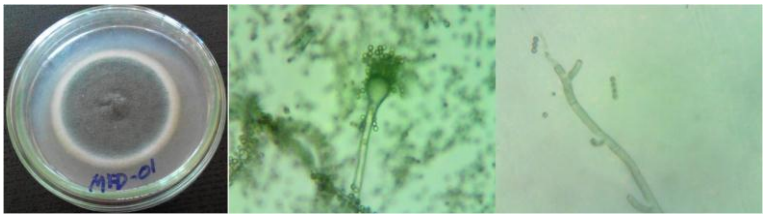

Figure 1. Morphology of MFD-01

Table I. Fungi with the most similar sequences from Genbank based on BLASTN search

Fungi name	GenBank accession numbers	Query coverage (%)	Identity (%)
Aspergillus flavus clone ISOJ25	MH270610.1	89	94
A. versicolor clone ISOJ24	MF164542.1	89	94
A. sydowii strain ND104	MF164494.1	89	94
Aspergillus sp. isolate SLS01	MF169502.1	89	94
A. sydowii strain DJ515-2	MF359934.1	89	94

The ethyl acetate extract of of *Aspergillus* sp. MFD-01 exhibited the antibacterial activity against both tested bacteria (Table II). The extract at higher concentrations (250 ppm and above) inhibited the growth of *E. coli*, shown by a significant difference between those samples and negative control (DMSO) in Kruskal Wallis' test. The activity of the extract was dose dependent that in concentration of 1000 ppm gave the highest inhibition with the diameter of 10.53 mm. Somehow the activity of the extract was not comparable to that of Ciprofloxacin, the positive control used in this study. The antibacterial activity of ethyl acetate extract of *Aspergillus* sp. MFD-01 against *S. aureus* was similar to that against *E. coli*. It started to show bioactivity at concentration of 250 ppm, and indicated a dose dependant manner, with the maximum diameter of inhibitory zone 11.84 mm, achieved by extract at the concentration of 1000 ppm. Its activity was also lower than that of Ciprofloxacin. Hence, *Aspergillus* sp. MFD-01 inhibited the growth of both Gram negative and Gram positive bacteria with the similar efficacy.

Table II. Diameter of inhibitory zones of ethyl acetate extract of *Aspergillus* sp. MFD-01 against *E. coli* and *S. aureus*

Treatment	Diameter of inhibitory zone (mm) against	
	E. coli	***S. aureus***
MFD-01 15 ppm	0	0
MFD-01 31 ppm	0	0
MFD-01 62 ppm	0	0
MFD-01 125 ppm	0	0
MFD-01 250 ppm	$3.75 \pm 0.70^{*\Delta}$	$4.82 \pm 0.45^{*\Delta}$
MFD-01 500 ppm	$5.87 \pm 0.23^{*\Delta}$	$6.99 \pm 0.35^{*\Delta}$
MFD-01 1000 ppm	$10.53 \pm 0.73^{*\Delta}$	$11.84 \pm 0.30^{*\Delta}$
Ciprofloxacin	$18.86 \pm 1.10^{*}$	$19.61 \pm 1.23^{*}$
DMSO	0	0

Note: * indicated that mean value of diameter or inhibitory zone (n=3) was significantly different from that of negative control; $^{\Delta}$ indicated that mean value of diameter or inhibitory zone (n=3) was significantly different from that of positive control; analysis was conducted at level of confidence of 0.95

The antimicrobial activity of endophytic *Aspergillus* sp. has been recognized and reported elsewhere. For example, *Aspergillus* sp. xy02 derived from a Thai mangrove *Xylocarpus moluccensis* produced 12 phenolic bisabolane sesquiterpenoids, which 7 of them displayed moderate inhibitory activities against *Staphylococcus aureus* ATCC 25923 (Wang *et al*., 2018). Another study reported *Aspergillus* sp. IFB-YXS, an endophytic fungus residing in the apparently healthy leave of *Ginkgo biloba* L., also producing xanthoascin that was significantly inhibited the growth of the *Clavibacter michiganense* subsp. *Sepedonicus* (Zhang *et al*., 2015). The antimicrobial activity was also exhibited by *Aspergillus* sp. isolated from *Justicia adathoda* (Prabavathy and Nachiya, 2012), *Aspergillus* sp. of *Eucommia ulmoides* (Hongchi Zhang *et al*., 2014), *Aspergillus fumigatus* D derived from *Edgeworthia chrysantha* (Huawei Zhang *et al*., 2014), and *Aspergillus terreus*-F7, an endophytic fungus from *Hyptis suaveolens* (L.) Poit (da Silva *et al*., 2017)

The phytochemical screening test showed that the ethyl acetate extract of *Aspergillus* sp. MFD-01 contained flavonoids, terpenoids, alkaloids, tannins, and saponins (Table III). Those compounds might be responsible for the antibacterial activity of *Aspergillus* sp. MFD-01 against *E. coli* and *S. aureus*. Flavonoids and tannins as phenolic compounds have been known for their antimicrobial activities (Sari *et al*., 2015; Yakhlef *et al*., 2018). Phenolic bisabolane sesquiterpenoids produced by *Aspergillus* sp. xy02 are terpenoids, and some of them possessing antibacterial activity against *S. aureus* with IC_{50} values ranging from 31.5 to 41.9 μM (Wang *et al*., 2018).

Table III. The results of phytochemical screening

Constituents	Reagents	Positive results	Observed results	Conclusions
Flavonoids	Mg-HCl	formation of orange color	orange hue was observed	positive
Terpenoids	H_2SO_4	formation of red color in organic layer	red hue in organic layer was observed	positive
Alkaloids	Mayer	formation of white precipitate	white precipitate was formed	positive
Tannins	$FeCL_3$	the color was changed	the color was changed from yellow to reddish orange	positive
Saponins	-	formation of stable foam	stable foam was formed	positive

CONCLUSION

Aspergillus sp. MFD-01 has successfully isolated from leaves of M. ferrea. Its identity was established by morphology characteristic and ITS4 rDNA sequences. The ethyl acetate extract of *Aspergillus* sp. MFD-01 inhibited the growth of both *Staphylococcus aureus* and *Escherichia coli*. It contained flavonoids, alkaloids, saponins, terpenoids, and tannins that might be responsible for its antibacterial activity.

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