Artikel Riset

Jurnal Kefarmasian Indonesia
Vol.6 No.1-Feb. 2016:1-7
p-ISSN: 2085-675X
e-ISSN: 2354-8770

Antimicrobial Activity of Fractions of Ethyl Acetate Extract of *Cladosporium oxysporum*, An Endophytic Fungus Derived from *Alyxia reinwardtii*

Aktivitas Antimikroba Fraksi-fraksi Ekstrak Etil Asetat Jamur Endofit *Cladosporium oxysporum* yang Diisolasi dari *Alyxia reinwardtii*

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Received: October 29, 2015 Revised: January 14, 2016 Accepted: February 11, 2016

Abstract

The endophytic fungi *Cladosporium oxysporum* was isolated from medicinal plants *Alyxia reinwardtii*. The antimicrobial activity of fractions of ethyl acetate extract and also antioxidant and antimicrobial activities of fraction 7 of ethyl acetate extract of (AR-7) were determined in this study. Antimicrobial activity was determined by TLC bioautography and disc diffusion methods. Antioxidant activity was determined by TLC-DPPH autography assay. The fractions of ethyl acetate extract of *C. oxysporum* at dose of 100 mg/ml were active as antimicrobial against *S. aureus*, *E. coli* and *C. albicans* in disc diffusion method test. Fraction AR-7 was active as antioxidant in TLC-DPPH autography assay. Fraction AR-7 was active as antimicrobial against *S. aureus*, but was not active against another tested microoorganisms in TLC-bioautography.
Keywords: *Alyxia reinwardtii*; antimicrobial; *Cladosporium oxysporum;* endophytic fungi

Abstrak

Jamur endofit Cladosporium oxysporum telah berhasil diisolasi dari tumbuhan inang pulasari (Alyxia reinwardtii). Penelitian ini bertujuan untuk mengetahui aktivitas antimikroba dari fraksi-fraksi ekstrak etil asetat dan aktivitas antioksidan dan antimikroba dari fraksi 7 ekstrak etil asetat (AR-7). Aktivitas antimikroba fraksi-fraksi ekstrak etil asetat diuji dengan menggunakan metode difusi cawan, sedangkan aktivitas antimikroba dari fraksi AR-7 diuji dengan KLT-bioautografi. Aktivitas antoksidan fraksi AR-7 dianalisis dengan menggunakan metode KLT-DPPH autografi. Hasil penelitian menunjukkan bahwa fraksi-fraksi ekstrak etil asetat C. oxysporum pada dosis 100 mg/ml aktif sebagai antimikroba terhadap S. aureus, E. coli, dan C. albicans. Fraksi 7 ekstrak etil asetat hanya hanya aktif menghambat pertumbuhan S. aureus, tapi tidak menghambat pertumbuhan bakteri uji yang lain. Fraksi AR-7 memiliki aktivitas antioksidan pada model uji KLT-DPPH autografi.
Kata kunci*: Alyxia reinwardtii*; antimikroba; *Cladosporium oxysporum*; jamur endofit

INTRODUCTION

Endophytic fungi are those grow intra or intercelullarly in the tissues of higher plants without causing overt symptoms of disease.[1] Endophytic fungi have been known to produce various bioactive metabolites such as agrochemicals, antibiotics, immunosuppressants, anti-parasitics, antioxidants, anticancer, antiviral, insecticidal and antidiabetic activity.[2,3,4]

Alyxia reinwardtii (Apocynaceae) is widely used in *jamu* (Indonesian traditional herbal medicine.[5,6] It has been reported producing antimicrobial and antioxidant metabolites such as 5-hydroxycoumarin, 8-hydroxycoumarin, pinoresinol, 9-α- hydroxypinoresinol, alyxialactone, pulosarioside and salisifoliol.[7-10] Hence, *A. reinwardtii*meets the rationale for selection of host plant for endophytes isolation.[11] Previously, lecythomycin, a new antifungal metabolite, and other metabolites have been isolated from endophytic *Lecythopora* sp. residing in *A. reinwardtii.*[12,13]

Endophytic fungi were also known for producing antioxidant metabolites. Antioxidant activity screening was performed as an early step to obtain antioxidant metabolites from endophytic fungi.[14-17] To the best of our knowledge, there is no report on antioxidant activity of metabolites produced by endophytic *Cladosporium* sp. to date.

In our continuing studies on endophytic fungi, we now describe the bioactivity of another fungal species that we have isolated from the same host plant which was identified as *Cladosporium oxysporum*.The present study is aimed to determine the antimicrobial activity of the crude ethyl acetate extracts and the antioxidant and antimicrobial activities of fraction 7 of ethyl acetate extracts (fraction AR-7) of the *C.oxysporum* isolated from *A. reinwardtii*. AR-7 was the most abundant fraction and possessed relatively high antimicrobial activity among other fractions, so it was chosen to be studied further as early step of bioactive-guided isolation in order to obtain potent and save antioxidant and antimicrobial metabolites from the fungus.

METHODS

General

This is a laboratory experimental study, conducted at Microbiology Laboratory of Assessment Service Unit, Faculty of Pharmacy, Airlangga University on July – August 2014.

Plant materials

A. reinwardtii was collectedfrom Purwodadi Botanical Garden, East Java, Indonesia. The plant material was identified by Dr. Irawati at Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia (voucher no 710/IPH.1.02.If.8/ 2003)

Isolation and Identification of endophytic fungi

Isolation of endophytic fungi was conducted as previously described.[12] The isolated fungus was identified by Dr. Arnulf Diesel (Heinrich - Heine - Universität Düsseldorf, Germany) as *Cladosporium oxysporum* based on its ITS-DNA sequences.

Culture of *C. oxysporum*

A small part of *C. oxysporum* was transferred under sterile conditions to the MEB medium (250 mL/flask), with pH of 5,6. The fungus was grown under static conditions at room temperature (approx. 30±3^{o}C) for four weeks.

Preparation of crude extract and fraction

The culture broth and mycelia were extracted with ethyl acetate. The organic layers were collected, and concentrated *in vacuo* to obtain a dried extract (5.49 g). The dried extract (3.0 g) was subjected to column chromatography on silica gel 60, and eluated with gradient mixtures of *n*-

hexane, ethyl acetate and methanol. The eluates were collected and grouped based on their Thin Layer Chromatography (TLC) profile to yield 16 fractions, AR-1 to AR-16.

Determination of antioxidant activity

Thin Layer Chromatography2,2-diphenyl-1-picrylhydrazyl (TLC-DPPH) autography was conducted as previously described.[18] In brief, AR-7 was diluted in methanol and applied on silica gel F_{254} 60 plates. Vitamin C (1000 ppm, 2 µL) was used as positive control. The plates were developed in presaturated solvent chamber with chloroform:methanol (9:1) as mobile phase and then sprayed with 0.04% DPPH solution in methanol.

Determination of antimicrobial activity

Disc diffusion method to determine antimicrobial activity of the fractions of ethyl acetate extract was conducted as previously described.[19] Microorganisms used in this assay were obtained from the American typeculture collection (ATCC). They were *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Candida albicans*ATCC 10231. All microorganisms were stocked in appropriate conditions and regenerated before used. The tested microorganisms were cultured for 24 hours before the tests. Microbial suspensions in normal saline with 25%T were prepared. Ten µL of microbial suspension was added into 15 mL sterile agar media (fungi were grown on Saboroud dextrose agar and bacteria were grown on nutrient agar) and then homogenized and poured into steril petri dish. Sterile filter paper discs (diameter 6 mm) impregnated with 20µl of fractions at concentrations of 100000 ppm were applied over each of the culture plates previously seeded with the 10^6 cfu/ml cultures of bacteria and fungi respectively. Twenty µl of ketoconazole (2000 ppm) was used as positive control. Bacterial cultures and those of *Candida albicans* were then incubated at at 37 ^oC for 24 h. After incubation the diameter of the inhibition zone was measured in millimeter. The experiment was carried out in triplicates.

TLC-bioautography to evaluate antimicrobial activity of fraction AR-7 was conducted as previously described.[20] Sixteen microbial strains were obtained from the American typeculture collection (ATCC), Food and Nutrition Culture Collection (*FNCC*) as well as patient's isolates culture collection of Institute of Tropical Disease, Airlangga University and Balai Besar Laboratorium Kesehatan, Surabaya, Indonesia. They were *E. coli* ATCC 8739, *E. coli* FNCC 0091, *Pseudomonas aeruginosa* ATCC 9027, *Vibrio cholerae, Salmonella typhimurium, Salmonella* sp., *S. enterica typhimurium* ATCC 14028, *S. aureus*ATCC 6538, *S. aureus* FNCC 0047, *Bacillus subtilis* ATCC 6633, *C. albicans* ATCC 10231, *C. albicans, C. glabrata, C. tropicalis* and *Aspergilus braziliensis* ATCC 16404. Majority of the tested microorganisms were Gram negative ones, since the result of the previously performed disc diffusion method showed that Gram negative bacteria were more sensitive to AR-7 than Gram positive ones. AR-7 was diluted in methanol. Tetracyclin HCl (2 µg) were used as positive control. AR-7 and positive control were applied on silica gel F_{254} 60 TLC plate, and then developed in presaturated solvent chamber with chloroform-methanol (9:1). The plates were overlayed on medium containing tested microorganism. Petri dishes were kept on temperature 0-8^oC for 2 hours and then the TLC plates were removed aseptically. Petri dishes were then incubated at 37^oC for 24 hours. After incubation the diameter of the inhibition zone was measured in millimeter. The experiment was carried out in triplicates.

RESULTS AND DISCUSSIONS

One of the endophytic fungi isolated from host medicinal plant *A. reinwardtii* was identified as *C. oxysporum* based on its ITS-DNA gene sequences. The occurence of isolation of *C. oxysporum* as endophyte is limited to date. The isolation of this species was reported previously from host *Pinus densiflora* and *P. rigida* growing in Daejeon, Korea and *Euphorbia bupleuroides* subsp. *Luteola*.[21,22]

The previous study showed that ethyl acetate extract of C. oxysporum possessed the highest antimicrobial activity against *S. aureus*, *S. thypi*, *E. coli*, *P. aeruginosa*, *B.subtilis* and *C. albicans,* compared to methanolic and dichloromethane extracts.[23] Hence, we separated ethyl acetate extract of *C. oxysporum.*. The fractionation of the crude ethyl acetate of *C. oxysporum* yielded 16 fractions. The antimicrobial activity of the fractions against *S. aureus*, *E. coli* and *C. albicans* was determined by disc diffusion method. The diameter of inhibition zone of the fractions at dose of 2 mg/ disc is shown in table 1. Based on the amount and antimicrobial activity of the obtained fractions, fraction AR-7 was studied further for antioxidant and antimicrobial activities.

TLC-DPPH autography is a test based on scavenging the stable free radical DPPH. It is one of the techniques commonly applied for screening plant extracts for the presence of antiradical compounds. It is proved as a powerful tool for screening antioxidant compound in bioactive-guided isolation.[24]

Fraction AR-7 was active as antioxidant in TLC-DPPH autography assay, shown by the bleached spots developed after spraying TLC plate with DPPH solution. Chromatogram of TLC plate after derivatization was shown in figure 1. We have not determined the metabolites responsible for antioxidant activity of AR-7 yet. In general, antioxidant phytochemicals are phenolic compounds such as flavonoids, catechines and carotenoids.[25]Endophytic fungi had been reported as the source of antioxidant metabolites. For example, graphislactone A is a well known antioxidant metabolite produced by *Cephalosporium* sp. IFB-E001 residing in *Trachelospermum jasminoides* (LINDL) LEM.[17]

Table 1. The antimicrobial activity of fractions of ethyl acetate extract of *C. oxysporum* determined by disc diffusion method

Fractions	Means of diameters of inhibition zone (mm)		
	E. coli	***S. aureus***	***C. albicans***
1	-	-	-
2	-	-	-
3	13,83 ± 1,56	8,15 ± 0,18	14,00 ± 0,61
4	-	-	-
5	17,80 ± 0,39	16,08 ± 1,00	19,18 ± 1,23
6	21,93 ± 1,65	22,08 ± 0,62	25,25 ± 1,00
7	17,47 ± 0,56	16,08 ± 1,00	17,80 ± 0,71
8	19,93 ± 0,73	19,25 ± 1,20	22,13 ± 1,77
9	24,48 ± 0,50	22,10 ± 1,01	24,70 ± 0,61
10	20,48 ± 0,56	21,83 ± 0,33	26,12 ± 0,88
11	12,82 ± 0,41	15,78 ± 1,27	20,20 ± 1,11
12	11,50 ± 0,44	12,05 ± 1,02	13,16 ± 0,82
13	14,85 ± 0,67	12,72 ± 0,62	12,28 ± 0,20
14	-	-	-
15	-	-	-
16	-	-	-
Positive control	18,85 ± 1,00	19,68 ± 0,55	15,90 ± 0,78

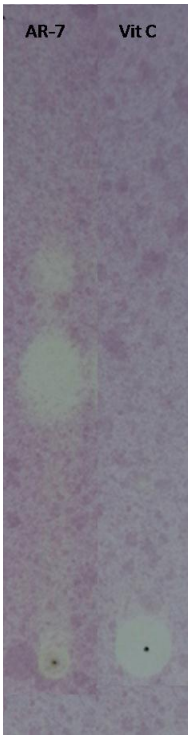

Figure 1. Chromatogram of TLC after derivatization. Vitamin C was used as positive control. Solution of 0.04% DPPH was used as derivatization reagent. Separation was conducted on silica gel F_{254} 60 using $CHCl_3$:MeOH (9:1) as mobile phase. Antioxidant activity was shown by bleached spots on purple background after derivatization.

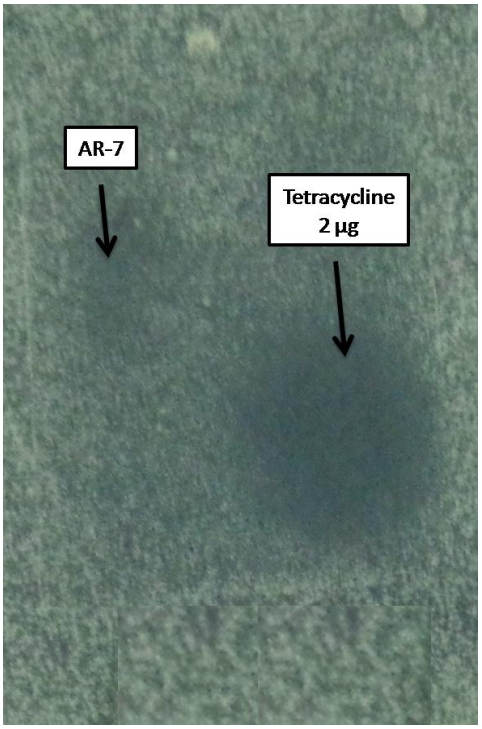

Figure 2. Bioautogram of AR-7 against *S. aureus* FNCC 0047. Tetracycline HCl was used as positive control. Separation was conducted on silica gel F_{254} 60 using $CHCl_3$:MeOH (9:1) as mobile phase. Antimicrobial activity was shown by clear zones developed after incubation.

The antimicrobial activity of fraction AR-7 was determined by TLC-bioautography. The result showed that AR-7 was active as antimicrobial against *S. aureus* FNCC 0047 and was not active against the rest of the tested microorganisms. The diameter of inhibition zone of AR-7 was 3.45 mm, much lower than that of tetracycline HCl (18.25 mm) (figure 2). The antimicrobial activity of AR-7 determined by TLC bioautography was different from that of disc diffusion method previously performed. This difference activity was caused by the different amount of sample applied on each test. Two mg of AR-7 was used in disc diffusion method, while in TLC-bioautography only used 0.2 mg of AR-7. TLC-bioautography was excellent to determine antimicrobial activity of polar compounds with strong efficacy. Hence, the antimicrobial metabolites in fraction AR-7 might be non polar substances with a relative moderate activity.

The main advantage of TLC-bioautography was its capability to analyze antimicrobial activity of each components in a mixture.[26] The result showed that there was one clear spot, indicating there was one compound that active as antibacterial agent. This spot needed to be separated and identified further.

Several antimicrobial metabolites had been isolated from endophytic *Cladosporium* sp. previously. Sumiki's acid and acetylated Sumiki's acid isolated from *Cladosporium herbarum* obtained from marine sponge *Callyspongia aerizusa* were active against *B. subtilis* and *S. aureus.*[27] Sporiolide A isolated from endophytic *Cladosporium* sp. residing in

brown alga *Actinotrichia fragilis* was active against *C. neoformans* and *Neospora crassa.*[28] Brefeldin A isolated from *Cladosporium* sp. residing in *Quercus viriabilis* was reported active as antimicrobial against *C. albicans, Trichophyton rubrum, A. niger, Microsporum canis* and *Epidermophyton floccosum*.[29]

CONCLUSIONS

The result of our study suggested that some fractions of ethyl acetate extract of *C. oxysporum* possessing antimicrobial activity, while fraction AR-7 at dose of 100 mg/ml having antimicrobial activity in disc diffusion method and moderate antioxidant activity.

AKNOWLEGMENT

We thank the Directorate General of Higher Education, Ministry of National Education, Indonesia for financial support, and Dr. Arnulf Diesel (Heinrich –Heine-Universität Düsseldorf, Germany) for identifying the fungus.

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