Journal of Applied Pharmaceutical Science Vol. 8(08), pp 069-074, August, 2018
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2018.8811
ISSN 2231-3354

Antibacterial and antioxidant activities of endophytic fungi extracts of medicinal plants from Central Sulawesi

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ARTICLE INFO

Article history:
Received on: 09/12/2017
Accepted on: 08/02/2018
Available online: 31/08/2018

Key words:
antibacterial, antioxidant, endophytic fungi, bioautography.

ABSTRACT

Endophytic fungi are a group of fungi which grow inside the plant tissues without causing negative symptoms to the host plant and are able to produce biologically active substances. This research was carried out to evaluate the antibacterial and antioxidant activity of 40 endophytic fungi isolated from 10 species of medicinal plants collected from Palolo, Central Sulawesi. Thin layer chromatography (TLC) bioautography guided screenings were done to evaluate antibacterial and antioxidant activities. The antibacterial activity was done against *Staphylococcus aureus* InaCC-B5 and *Escherichia coli* InaCC-B4, while antioxidant activity was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Minimum inhibitory concentrations (MICs) of active extracts were further evaluated against these bacteria, while half-maximal inhibitory concentration (IC_{50}) of active extracts was determined by the microdilution broth method. The results of TLC bioautography screening showed 30 extracts inhibited the growth of *S. aureus*, 29 extracts inhibited the growth of *E. coli*, 27 extracts inhibited both *S. aureus* and *E. coli*, and 23 extracts posessed antioxidant activity. There were six extracts with MIC value of <100 µg/ml against *S. aureus* and nine extracts with MIC value of <100 µg/ml against *E. coli*. Six extracts indicated very strong antioxidant activity.

INTRODUCTION

Endophytic fungi are groups of fungi with very specific ecosystem inside plant tissues and produces varieties of secondary metabolites (Agusta, 2009). Secondary metabolites from endophytic fungi show important biological activities such as antioxidant, anticancer, immunomodulatory, antivirus, antituberculosis, anti-parasite and insecticides (Hussain *et al*., 2014). Endophytic fungi produce secondary metabolites similar to the host plant; therefore, endophytic fungi can be used as a source of producing active metabolites and leads in drug developments (Strobel, 2003; Owen and Hundley, 2004). The study on the isolation and evaluation of bioactivities of endophytic fungi from medicinal plants are increasing lately.

On the other hand, antimicrobial resistance has been a major concern in the health care system globally (Ferri *et al*., 2017). Discovery of novel and active metabolites against pathogenic microbes as well as to overcome antimicrobial resistance become very important. In addition to health problems with increasing resistance, there is also a growing tendency to search natural antioxidants to overcome degenerative disease problems. Reactive oxygen species (ROS) are by-products of biological reactions that cause oxidative damage to biomolecules and play vital roles in programmed cell death (Cui *et al*., 2015). To overcome the negative effect of excessive ROS in human body, exogenous antioxidant is required. The main characteristic of antioxidant compounds is the ability to capture and stabilize free radicals (Prakash *et al*., 2011), inhibit or delay the occurrence of free radical reactions due to the presence of relative oxygen; these properties become important in the prevention of various diseases, such as cancer and coronary heart disease (Leong and Shui, 2002).

Medicinal plants are reported as host of some endophytic fungi that are involved in the co-production of active metabolites (Alvin *et al*., 2014). The study conducted by Ilyas (2009); Praptiwi *et al*. (2010); Praptiwi *et al*. (2015) showed that endophytic fungi isolated from medicinal plants such as gambier (*Uncaria gambier*), cinnamon (*Cinnamomum burmannii*) dan Zingiberaceous plants have antioxidant and/or antibacterial activity.

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Khiralla *et al*. (2015) has identified and classified 21 endophytic fungi from five medicinal plants of Sudan origin and some contain phenol compounds that have the potential as antioxidant natural sources. The present study aims to isolate and evaluate the antibacterial and antioxidant activity of endophytic fungi from ten species of medicinal plants originating from Palolo.

MATERIAL AND METHODS

Material

10 species of plants belonging to seven families which are Urticaceae (*Villebrunea rebescens* (Blume), *Poikilospermum suaveolens* (Blume) Merr.), Euphorbiaceae (*Euphorbia heterophylla* L., *Acalypha caturus* Blume), Asteraceae (*Blumea balsamifera* (L.) DC.), Zingiberaceae, Piperaceae (*Piper peltatum* L.), Lamiaceae (*Plectranthus scutellarioides* (L.) R. Br), and Verbenaceae (*Cleodendron fragrans* Wild.) were collected from Palolo, Central Sulawesi. Identification of the plant specimens were done at Herbarium Bogoriense, Research Center for Biology-Indonesian Institute of Sciences.

Isolation of endophytic fungi

Leaves, stems, and rhizomes collected from the field were stored at low temperature. After arriving in the laboratory, these samples were cleaned under tap water and immersed in 70% ethanol for 1 minutes, then immersed in 5.3% Na-hypochlorite for 5 minutes and finally immersed in 70% ethanol for 30 seconds. Samples were dried under aseptic conditions. The sterilized samples were cut aseptically into small pieces (1 × 1 cm^2), and then, placed on top of the Corn Meal Malt Agar (CMMA) growth medium added with chloramphenicol 0.05 mg/ml, and incubated at room temperature for 1 week. The emerging colonies were subcultured several times on Potato dextrose agar (PDA) to obtain pure isolates.

Secondary metabolites extraction from endophytic fungi

Pure isolate of endophytic fungi was cultured on broth medium [Potato dextrose broth (PDB)] (200 ml) and incubated in dark condition, at room temperature for 3 weeks. After incubation period is completed, growth media and endophytic fungi biomass were extracted three times with ethyl acetate. The extract was evaporated by rotary evaporation and the concentrated extract was stored in the glass vial.

Chemical compounds analysis by Thin Layer Chromatography (TLC)

The analysis of chemical compounds of endophytic fungi extracts were performed on silica gel thin layer chromatography (TLC) plates (silica gel GF_{254}, Merck). The dried extract was prepared in 10 mg/ml. 10 µl of extract was transferred on TLC plate and developed in CH_2Cl_2:MeOH (10:1). Separated chemical compounds were visualized under 254 nm and 366 nm ultraviolet (UV) light followed by spraying with spray reagent 1% $Ce(SO_4)_2$ and 1% vanillin sulphuric acid.

Detection of antibacterial activity by TLC-bioautography

TLC-bioautography guided screening was performed to evaluate the antibacterial potency of endophytic fungal extracts. 10 µl of extract was transferred on TLC plate and dried. Plate was then dipped into bacterial suspension, followed by incubating the plate under humid condition for 18 hours at 37˚C. After incubation was completed, plates were sprayed with iodonitrotetrazolium p-violet (INT, Sigma). Growth inhibition of bacteria was observed by clear zone formation around the extract. The active extracts were further analyzed by developing the extract with mobile phase CH_2Cl_2:MeOH (10:1). The plate was dried and sprayed with iodonitrotetrazolium p-violet (INT, Sigma).

Detection of antioxidant activity by TLC-bioautography

10 µl of extract was transferred on TLC plate and catechin used as positive control was also transferred on the TLC plate. The plate was dried and sprayed with 0.02% DPPH in methanol. Yellow spot on purple background indicated the antioxidant activity. The active extract was developed with CH_2Cl_2:MeOH (10:1). After drying, plate was sprayed with 0.02% DPPH in MeOH.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of active extracts were determined by serial microdilution in 96-well microplate (Pessini *et al.*, 2003). The wells in column A were filled with 100 µl of Mueller-Hinton Broth (MHB) medium and 100 µl stock solution of extract (1024 µg/ml) and homogenized. Columns B through H were filled with 100 µl of MHB. A serial dilution was carried out with corresponding final concentration (256 µg/ml). The test was done in triplicate. After the dilution process, each well was added with 100 µl of bacterial suspension (10^6 cfu/ml). In similar way, it was done with the positive control of chloramphenicol, growth media as negative control. Microtiter plate sealed with parafilm and incubated at 37˚C for 24 hours. After incubation was completed, each well was added with 10 µl INT 4 mg/ml. The MIC was the lowest concentration showing clear wells that indicate the absence of bacterial growth.

Determination of IC_{50} of active extract

The IC_{50} of the extract was determined by serial microdilution in 96-well microplate by Takao *et al*. (2015) with minor modification. The wells in column A were filled with 195 µl MeOH and 5 µl extract (10240 µg/ml) and homogenized. Columns B through H were filled with 100 µl of MeOH. A serial dilution was carried out with corresponding concentration (256 µg/ml). After the dilution process completed, each well was added with 100 µl of DPPH (61.50 µg/ml). Methanol was used as negative control, while catechin was used as positive control. Microplates were incubated in dark condition at room temperature for 90 minutes. The absorbances of the samples were measured at 517 nm. Antioxidant activity index (AAI) was calculated as follows:

$$AAI = \text{final concentration of DPPH in the reaction}/IC_{50}$$

IC_{50}: the concentration of 50% inhibition was calculated by linear regression equation.

RESULT AND DISCUSSION

A total of 40 isolates of endophytic fungi recovered from 10 species of medicinal plants collected from Palolo, Central Sulawesi. The results in Table 1 show that a plant part is colonized

by more than one endophytic fungus and different plant parts might have different composition of endophytic fungi community. This finding is in accordance with Zabalgogeazcoa (2008) that one species of plant inhabited by more than one endophytic fungus. Previous reports by Huang *et al*. (2008) and Ilyas (2009) showed similar results that one species of plant inhabited by various endophytic fungi. The distribution, composition, and population structure of endophytic fungi rely largely on the taxonomy, genetic background, age, and tissues of the host plants, and the types of environments (Sieber, 2007; Jia *et al*., 2016). Older plant parts may be colonized by higher number of endophytes than the younger ones (Zabalgogeazcoa, 2008).

Table 1: MIC of endophytic fungi extracts isolated from plants collected from Palolo, Central Sulawesi.

No	Sample	MIC (ug/ml)		No	Sample	MIC (ug/ml)	
		S. aureus	*E. coli*			*S. aureus*	*E. coli*
1	PAL-01B1	**64**	**64**	21	PAL-09D2	NT	256
2	PAL-01B2	**32**	**32**	22	PAL-10B1	128	256
3	PAL-01D1	NT	NT	23	PAL-10B2	256	128
4	PAL-01D2	NT	256	24	PAL-10B3	128	**8**
5	PAL-02D1	128	256	25	PAL-10D1	256	**64**
6	PAL-02D2	256	NT	26	PAL-10D3	256	128
7	PAL-03B1	**64**	**32**	27	PAL-10D8	NT	NT
8	PAL-03B2	256	128	28	PAL-11B1	NT	256
9	PAL-03B3	128	256	29	PAL-11B2	NT	NT
10	PAL-03D1	256	**64**	30	PAL-11B3	128	NT
11	PAL-03D2	256	256	31	PAL-11D1	**64**	256
12	PAL-04R1	256	256	32	PAL-11D2	256	256
13	PAL-04R2	128	128	33	PAL-14D1	**64**	128
14	PAL-07B1	**8**	**8**	34	PAL-14D2	128	**8**
15	PAL-07B2	128	**64**	35	PAL-14D3	256	**64**
16	PAL-07D1	256	128	36	PAL-15B1	256	128
17	PAL-09B1	256	NT	37	PAL-15B2	NT	NT
18	PAL-09B2	128	256	38	PAL-15D1	NT	256
19	PAL-09B3	NT	NT	39	PAL-15D2	NT	NT
20	PAL-09D1	NT	NT	40	PAL-15D3	NT	NT

Analysis of the chemical compounds of the extract

The chemical compounds of the endophytic fungi were analyzed by TLC in order to separate the chemical compounds within the extract. TLC is an important method for qualitative and quantitative analysis of drugs and has several advantages compared to HPLC and GC methods (Pyka, 2014). The plates were sprayed with color reagents (vanillin reagent and cerium reagent) to detect compounds in extract.

Observations under 254 nm showed different chemical compounds in each extract which emitted green and dark-colored compounds. A substance having a maximum wavelength (λ_{max}) of 250–260 nm may contain aromatic groups such as aromatic amino acid, simple phenol, and purines or pyrimidines (Harborne, 1973). Observation under 366 nm showed that TLC plates as background emitted purple and spot dark-colored chemical compounds. Substances having λ_{max} 200–400 nm indicated the presence of compounds has an aromatic group or a conjugated double bond (Fried and Sherma, 1999). TLC plates sprayed with vanillin and cerium showed the presence of different chemical compounds in extract that was characterized by stain spots with multiple colors. Crude extract of endophyic fungi (Figure 1) contained several chemical compounds indicated by several spots with different R_f. These chemical compounds might have biological activities and mixture of chemical compounds in crude extract may increase the potential of the active component to produce additive or synergistic effects, while others may be neutral or inhibit (Dhankhar *et al*., 2012).

Antibacterial activity detection by TLC

Antibacterial screening of endophytic fungi extracts was done by TLC method. TLC method is finest, rapid, efficient, and uncomplicated method (Masoko and Ellof, 2006; Shahverdi *et al*., 2007), requires small amount of test sample and simple interpretation of results (Valle Jr. *et al*., 2016). TLC dot-blot direct bioautography of antibacterial activity screening shown in Figure 2. The results of antibacterial screening of 40 endophytic fungi extracts showed that 30 extracts inhibited the growth of *S. aureus,* 29 extracts inhibited the growth of *E. coli* and 27 extracts were able to inhibit *S. aureus* as well as *E. coli.* The growth inhibition of bacteria was indicated by clear zone formation on TLC against a purple background (Das *et al*., 2010). The purple colour on TLC plate after spraying with INT was resulted from the conversion of INT to intensely colored formazan by the dehydrogenases enzyme of living microorganisms (Silva *et al*., 2005). Shahverdi *et al.* (2007) also stated that INT interacted with viable microorganisms caused a colour change of INT to red-purple one. Screening antibacterial activity by TLC dot-blot is simple and time saving method; however, the component mixtures in the crude extract

can have synergistic or antagonistic effects (Choma and Jesionek, 2015). The active extracts developed with mobile phase to separate the bioactive compounds in the extract (Figure 3). Separated bioactive compounds with antibacterial properties indicated by white band formation.

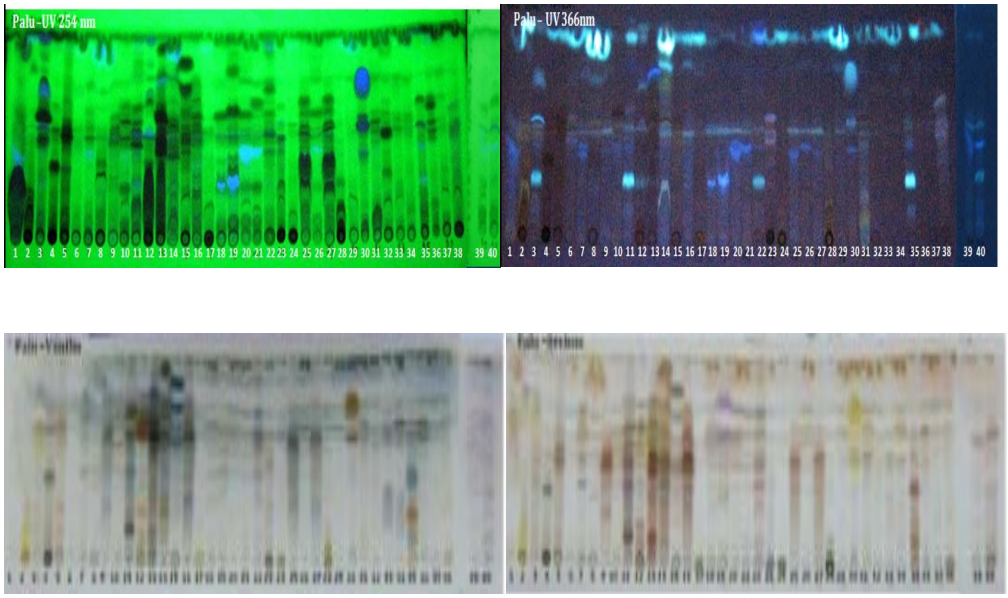

Fig. 1: Chromatograms of endophytic fungal extracts developed in dichloromethane-methanol (10:1 v/v), (a) viewed under 254 nm wavelength, (b) viewed under 366 nm wavelength, (c) sprayed with vanillin reagent, (d) sprayed with cerium reagent.

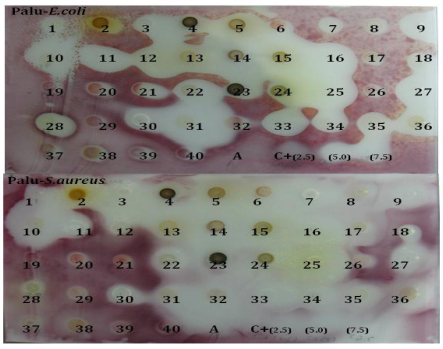

Fig. 2: TLC dot-blot assay for antibacterial activity of endophytic fungi against *E. coli* (top) and *S. aureus* (bottom).

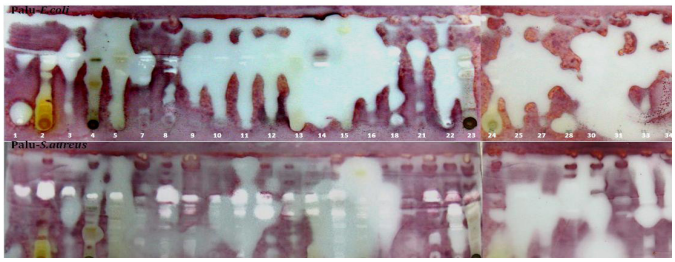

Fig. 3: Bioautograms of endophytic fungi against *E. coli* (top) and *S. aureus* (bottom). The TLC plates were developed in dichloromethane:methanol (10:1 v/v). Clear bands indicated antibacterial activity.

The minimum inhibitory concentration (MIC) of active extracts was assessed against *E. coli* and *S. aureus*. The result in Table 1 showed that MIC values of PAL endophytic fungi extracts ranging from 8 to 256 µg/ml. MIC in the range of 100–1000 µg/ml could be classified as antimicrobial (Borges *et al*., 2012). The MIC of several endophytic fungi were <100 µg/ml. According to Pessini *et al*. (2003), the MIC value of extract <100 µg/ml was classsified as good antibacterial activity, while extracts with MIC value ranging from 100 µg/ml to 500 µg/ml classified as moderate activity. Among 40 endophytic fungi tested for antibacterial activity, endophytic fungus PAL-07B1 derived from *Piper peltatum,* has good antibacterial activity against *S. aureus* and *E. coli*, with equivalent MIC for both isolates (8 µg/ml). Several previous studies reported the antibacterial activity of ethyl acetate extracts of endophytic fungi isolated from *Piper* (Orlandelli *et al*., 2012; Astuti *et al*., 2014). This result suggested the endophytic fungus PAL-07-B1 contains potential bioactive compounds as antibacterial.

Antioxidant activity by TLC-bioautography

The antioxidant activity of extracts was done by DPPH free radical scavenging activity. DPPH radical when receiving an electron from antioxidant compound would be reduced to DPPH. The violet color of DPPH radical turned into yellow (Pavithra and Vadivukkarasi, 2015). Screening for antioxidant activity by dot-blot TLC (Figure 4) showed 23 extracts had antioxidant activity. Antioxidant activity is indicated by the color change to yellow against a purple background (Dewanjee *et al*., 2015). The intensity of yellow color indicates the antioxidant capacity. Further analysis of active extract was shown in Figure 5.

The developed TLC-bioautography of endophytic fungi extracts (Figure 5) showed several compounds possess antioxidant activity within extract. This is indicated by the formation of

yellowish white bands. Further analysis of active antioxidant extracts is to determine its IC_{50} value (Table 2).

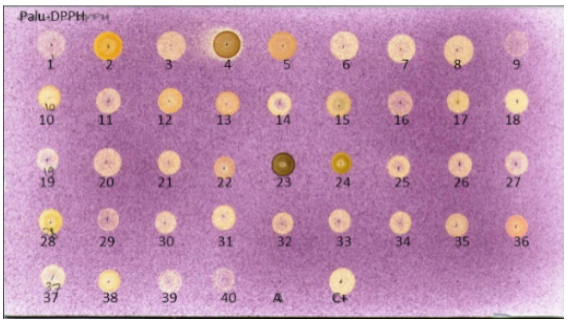

Fig. 4: TLC dot-blot assay for antioxidant activity of endophytic fungi extracts isolated from medicinal plants from Central Sulawesi.

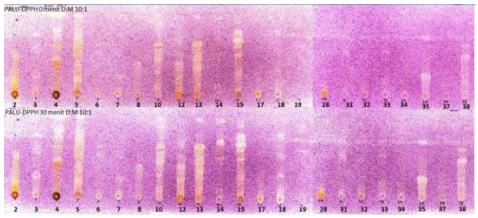

Fig. 5: TLC-bioautogram of antioxidant activity of endophytic fungi extracts. The yellowish white band indicates the compounds with antioxidant activity.

Table 2: IC_{50} and antioxidant activity index (AAI) of endophytic fungi extracts.

No	Sample	IC50 (ug/ml)	AAI value	Criteria of AAI value
1	PAL-01B2	5.26	5.846	**Very strong**
2	PAL-01D2	7.84	3.922	**Very strong**
3	PAL-02D1	26.00	1.183	Strong
4	PAL-03D1	107.51	0.286	Moderate
5	PAL-04R1	52.70	0.583	Moderate
6	PAL-04R2	10.02	3.069	**Very strong**
7	PAL-07B2	77.60	0.396	Moderate
8	PAL-09B1	99.72	0.308	Moderate
9	PAL-11B1	10.03	3.066	**Very strong**
10	PAL-14D3	14.06	2.187	**Very strong**
11	PAL-15D1	43.15	0.713	Moderate
12	Catechin	1.71	17.982	**Very strong**

Based on the criteria of AAI value by Scherer and Godoy (2009), there are five extracts that displayed very strong antioxidant activity (PAL 01-B2, PAL 01-D2 from *Villebrunearubescens* (Urticaceae), PAL 04-R2 (Zingiberaceae), PAL 11-B1 from *Clerodendronfragrans* (Verbenaceae), and PAL 14-D3 from *Acalyphacaturus* (Euphorbiaceae); one extract displayed strong antioxidant activity and five extracts displayed moderate activity. Previous study by Praptiwi *et al*. (2016) showed some endophytic fungi isolated from Zingiberaceae had strong antioxidant activity, while endophytic fungi from other studied plants had no previous reports. The very strong antioxidant activity of extracts was related to many bioactive compounds as antioxidant within extract may act synergistically.

CONCLUSION

This study revealed the presence of bioactive secondary metabolites produced by endophytic fungi from several medicinal plants collected from Central Sulawesi with antibacterial and/or antioxidant activity. Endophytic fungi PAL-01B2 and PAL-07B1 showed good antibacterial activity against *S. aureus* InaCC-B5 and *E. coli* InaCC-B4. Five endophytic fungi showed very strong radical scavenging activity (PAL 01-B2, PAL 01-D2, PAL 04-R2, PAL 11-B1), and PAL 14-D3. Further studies needed to isolate and purify bioactive compounds, which is responsible for antibacterial and antioxidant activity. These findings indicated that endophytic fungi from medicinal plants collected from Palolo, Central Sulawesi could be potential for the development as pharmaceutical agents.

ACKNOWLEDGEMENT

The authors are thankful to Research Center for Biology, Indonesian Institute of Sciences for financial support through DIPA-Pusat PenelitianBiologi.

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How to cite this article:
Praptiwi, Raunsai M, Wulansari D, Fathoni A, Agusta A. Antibacterial and antioxidant activities of endophytic fungi extracts from medicinal plant from Central Sulawesi. J App Pharm Sci, 2018; 8(08): 069-074.