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Evaluation of antibacterial and antioxidant activity of extracts of endophytic fungi isolated from Indonesian Zingiberaceous plants

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Abstract. *Praptiwi, Palupi KD, Fathoni A, Wulansari D, Ilyas M, Agusta A. 2016. Evaluation of antibacterial and antioxidant activity of extracts of endophytic fungi isolated from Indonesian Zingiberaceous plants. Nusantara Bioscience 8: 306-311.* Thirty two endophytic fungi isolated from rhizome of six species of Zingiberaceae from Karanganyar, Central Java, Indonesia. Based on their morphological characters, all of endophytic isolates were classified into 10 genera. All of endophytic fungi isolates were then cultivated into 200 mL of Potato Dextrose Broth and incubated at room temperature. After three weeks, each of the fungi culture were extracted with appropriate amount of ethyl acetate, and dried under reduced pressure. The extracts, then evaluated for DPPH antioxidant activity and antibacterial activities against *Escherichia coli* Ina-CC B5 and *Staphylococcus aureus* Ina-CC B4 based on non-eluted TLC bioautography assays. The results showed there are 19 extracts possessing antioxidant activity, 23 extracts inhibit the growth of *E. coli* Ina-CC B5, and 19 extracts inhibit the growth of *S. aureus* Ina-CC B4. The minimum inhibitory concentration (MIC) value of active extracts against *S. aureus* were in the range of 80-1280 ug/mL, while MIC against *E. coli* were in the range of 80->1280 ug/mL. The IC_{50} of active extracts were in the range of 50.97-2031.26 ppm. Further analysis by the eluted TLC-bioautography assay of active extracts showed that several fungal metabolites on active extract responsible for its/their bioactivity. It can be concluded that endophytic fungi isolated from Zingiberaceae can be used as new antibacterial and antioxidant resources.

Keywords: Antibacterial, antioxidant, endophytic fungi, bioautography, Zingiberaceae

INTRODUCTION

Zingiberaceae (commonly called as ginger plant) is a plant family that has been widely known and used in various aspects of life, including used as traditional medicine for curing several diseases, spices and for ritual purposes (Hartanto et al. 2014). Zingiberaceous plants contain essential oil (Chen et al. 2008), which have been reported for their biological activities such as antifungal, antioxidant, insecticidal, anti-inflammatory and antitumor (Julie and Ernest 2012). In addition to essential oil, Zingiberaceous plant produce many complex compounds that have been used as herbal medicines, spices, flavoring, seasoning, cosmetics and medicinal industries as antioxidant and antimicrobial agents (Chen et al. 2008).

Plants are host of one or more endophytic microbes (Yadav et al. 2012) which resides inside the plant tissue without causing any symptoms of disease to the host plant (Tan and Zou 2001), and have capability to synthesize chemical compounds that similar to the host plant (Zhao et al. 2011). Endophytic fungi produce a number of substances such as antioxidants, novel antibiotics, antimycotics, immunosuppresants and anticancer compounds, and rich source of biologically active metabolites (Strobel and Daisy 2003; Bhagopaty and Joshi 2011). Some of the compounds produced by the endophytic fungi have been proven to be useful as leads for novel drug discovery (Tan et al. 2001; Yu et al. 2010; Yadav et al. 2014).

The search for new antimicrobial and antioxidant sources are important due to the growing number of antibiotic resistance of the infectious agent. Recently, the search of natural antioxidant also has much attention. Antioxidant can protect the human body from various diseases caused by free radicals (Yadav et al. 2012). The natural antioxidants are very effective to prevent the destructive processes caused by oxidative stress (Shah 2015).

Present study was carried out to evaluate the antibacterial and antioxidant activity of endophytic fungal extracts associated with six species of Indonesian Zingiberaceous plant by TLC-bioautographic method as well as the MIC values of active extracts.

MATERIALS AND METHODS

Plant materials

Rhizomes of six species of Zingiberaceous plants which were: ginger (*Zingiber officinale* Rosc.), turmeric (*Curcuma domestica* Val.), round-rooted galangal (*Kaempferia rotunda* L.), temulawak (*Curcuma xanthorrhiza* Roxb.), mango ginger/wild turmeric (*Curcuma mangga* Val.) and round zedoary (*Curcuma zedoaria* (Christm.) Rosc.) collected from Sambirejo Village, Jumantono Sub-District, Karanganyar District, Central Java, Indonesia in 2014.

Procedures

Isolation and cultivation of endophytic fungi

Fresh rhizomes collected from the field were kept on low temperature. After arriving in the laboratory, the rhizomes were washed under tap water and cut about 2 cm length. The rhizomes were then surface sterilized in the laminar air flow by successive soaking in 70% alcohol for 2 minutes, transferred to 5.3% hypochloride solution for 5 minutes and alcohol 70 % for 30 seconds and washed with sterile distilled water. After surface sterilization, rhizomes were split aseptically with sterile blade. The inner part placed onto Corn Meal Malt Agar (CMMA) containing 0.5% chloramphenicol and incubated for 1 week at room temperature. The emerging fungi were isolated and sub-cultured on Potato Dextrose Agar (PDA) several times to obtain pure culture (Agusta 2005).

Mycelial agar plugs from fungal pure culture were cultivated into 500 mL culture flask containing 200 mL Potato Dextrose Broth (PDB). The culture was incubated at still condition at room temperature under dark condition for 3 weeks.

Identification of endophytic fungi

Identification of fungal endophytes associated with rhizome of Zingiberaceous plant were done based on their morphological characters (macroscopically and microscopically) that grown on PDA at room temperature (Kobayashi 1970; Ellis 1971; Domsch 1980; Sutton 1980; Webster 1980; Samson et al. 1995; and Barnett et al. 1998). The macroscopic characteristics observed: color and surface colonies (granular, such as flour, mounting, slippery), texture, zonation, growth area, the lines of radial and concentric, reverse color, and exudate drops.

Bacterial isolates

Bacteria isolates used for antibacterial assay were provided from Indonesia Culture Collection (Ina-CC), Research Center for Biology, Indonesian Institute of Sciences, Cibinong, Bogor, West Java, Indonesia which were *Escherichia coli* Ina-CC B4 and *Staphylococcus* Ina-CC B5.

Extraction of endophytic fungal culture

After incubation period, biomass and culture media were extracted with ethyl acetate. The ethyl acetate fraction was evaporated under reduced pressure by rotary evaporator.

TLC-base approach-Antibacterial assay

Screening of antibacterial activity of fungal extracts were done against *Escherichia coli* Ina-CC B4 (gram negative bacterium) and *Staphylococcus aureus* Ina-CC B5 (gram positive bacterium). One hundred micrograms of extract (10 µg/µL) were transferred on silica TLC plate (Silica gel $GF_{254,}$ Merck). After air dried, the plate was dipped in bacteria suspension (10^6 cfu/mL) and placed on petri dish, the humidity was kept by put sterile wet cotton, incubate for 18 hours at 37°C. After incubation, the plate was sprayed with iodonitrotetrazolium p-violet (INT, Sigma). Clear zone or white area indicated inhibition growth of bacteria by fungal extract.

Minimum inhibitory concentration of active the extracts

The minimum inhibitory concentration (MIC) of active extract was done by serial microdilution method in 96 well microplate by 2-fold microdilution. The extracts were dissolved in 10% DMSO. Streptomycin and chloramphenicol were used as positive controls, while broth medium and DMSO were used as negative controls. On the first row, 100 µL of 2-fold MHB medium were dispensed into each well, and on the 2nd to 8th rows 1-fold MHB medium. In column 1-3, dispense 100 µL of stock solution of extract (5120 µg/mg) for sample 1, and column 4-6 for sample 2 and so on. Test was performed in triplicate. The microplate was inoculated with bacterial suspension (100 µL/well) which contained 1 x 10^6 cfu/mL of test bacteria and incubated at 37°C for 18 hours. After incubation period, 10 µL of *p*-iodonitrotetrazolium was added to each well. Growth inhibition was determined by clear area. MIC value is the lowest concentration showing no color change and exhibited complete growth inhibition (Perumal et al. 2012).

TLC Base Approach-DPPH Antioxidant activity

Screening of antioxidant activity of endophytic fungal extracts were done by TLC-bioautography. An aliquot of 10 µL extract was deposited as spot onto TLC plate (Silica gel GF254, Merck) as well as 10 µL of positive control (+)-catechin), and negative control (culture media). After air dry, the plate was sprayed with 0.2% methanolic DPPH solution. The spot that turned yellow at 30 minutes after spraying indicated positive result. The active extracts were then spotted onto TLC plate. TLC plate was developed with mobile phase dichloromethane: methanol (10: 1) until about 1 cm from the top of the plate, and allow to dry. The plate was sprayed with 0.2% methanolic DPPH solution. The yellow bands indicated active compounds responsible for antioxidant activity in the extract.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

Isolation of endophytic fungi from six species of Zingiberaceous rhizome from Solo (Central Java) in total gained 33 isolates. Based on morphological characteristics, endophytic fungi from Zingiberaceous rhizomes are classified into 10 genera (Table 1).

Antibacterial activity

Totally of 32 extracts of endophytic fungal extracts associated with 6 species of Indonesian Zingiberaceous plants were tested against *E. coli* Ina-CC B4 and *S. aureus* Ina-CC B5 (Figure 1, Table 2).

Results of antibacterial screening indicated 19 extracts inhibited the growth of *S. aureus* Ina-CC B5 and 23 extracts inhibited the growth of *E. coli* Ina-CC B4. Some endophytic fungi inhibit the growth of *E. coli* and *S. aureus* such as JES-01, KPS-03, KPS-04, KS-04, TMS-02, TMS-03 and TPS-07.

Table 1. Endophytic fungi from six species of Zingiberaceous rhizomes collected from Sambirejo Village, Central Java, Indonesia

Isolate	Substrate (rhizome)	Taxa
JES-01	*Z. officinale*	*Phomopsis* sp.
JES-02	*Z. officinale*	*Colletatrichum* sp.
JES-03	*Z. officinale*	Dematiaceae
JES-04	*Z. officinale*	Dematiaceae
JES-05	*Z. officinale*	Hypomycetes
JES-06	*Z. officinale*	Xylariaceae
JES-07	*Z. officinale*	Hypomycetes
KS-01	*C. domestica*	Hypomycetes
KS-02	*C. domestica*	Dematiaceae
KS-03	*C. domestica*	Dematiaceae
KS-04	*C. domestica*	Dematiaceae
KPS-01	*C. alba*	*Fusarium* cf. *oxysporum*
KPS-02	*C. alba*	*Fusarium* cf. *oxysporum*
KPS-03	*C. alba*	*Fusarium* cf. *solani*
KPS-04	*C. alba*	*Fusarium* cf. *solani*
KPS-05	*C. alba*	Dematiaceae
KPS-06	*C. alba*	Dematiaceae
TLS-01	*C. xanthorrhiza*	*Fusarium* cf. *oxysporum*
TLS-02	*C. xanthorrhiza*	*Fusarium* cf. *solani*
TLS-03	*C. xanthorrhiza*	*Fusarium* cf. *oxysporum*
TLS-04	*C. xanthorrhiza*	*Eupenicilium* sp.
TMS-01	*C. mangga*	Dematiaceae
TMS-02	*C. mangga*	Dematiaceae
TMS-03	*C. mangga*	Dematiaceae
TMS-04	*C. mangga*	Dematiaceae
TMS-05	*C. mangga*	Dematiaceae
TPS-01	*C. zedoaria*	*Cladosporium* sp.
TPS-02	*C. zedoaria*	*Mucor* sp.
TPS-03	*C. zedoaria*	Dematiaceae
TPS-04	*C. zedoaria*	Coleomycetes
TPS-05	*C. zedoaria*	Dematiaceae
TPS-06	*C. zedoaria*	*Eupenicilium* sp.
TPS-07	*C. zedoaria*	*Mucor* sp

Table 2. Bioactivity of endophytic fungal extracts of 6 species of Zingiberaceous plant collected from Sambirejo Village, Central Java, Indonesia

Isolate	Growth inhibition (mm)		Antioxidant activity
	S. aureus	*E. coli*	
JES-01	+++	+++	+
JES-02	+	+	+
JES-03	+	+	++
JES-04	-	+	++
JES-05	-	-	-
JES-06	+	-	-
JES-07	-	-	-
KS-01	-	++	++
KS-02	+	+	-
KS-04	++	++	+
KPS-01	+	+	+
KPS-02	-	-	-
KPS-03	++	++	+
KPS-04	+	++	-
KPS-05	-	-	++
KPS-06	-	-	-
TLS-01	-	+	-
TLS-02	+	+	-
TLS-03	+	+	-
TLS-04	-	-	++
TMS-01	+	+	+
TMS-02	++	++	+
TMS-03	++	++	+
TMS-04	-	+	-
TMS-05	-	+	+
TPS-01	+	+	-
TPS-02	+	+	++
TPS-03	+	+	+
TPS-04	-	-	+
TPS-05	+	+	-
TPS-06	-	-	++
TPS-07	+++	+++	+

The active extracts which have good clear zone were analyzed for their minimum inhibitory concentration (MIC) by serial microdilution. The results are presented on Table 3.

The MIC values of active extracts of endophytic fungi against *S. aureus are* in the range of 80-1280 µg/mg. On the other hand, the MIC value against *E. coli* that has a value ≤ 1280 ug/mL only 2 extracts.

In order to know the chemical compounds in the active extract that responsible for antimicrobial activity, the extract was developed/eluted with mobile phase of dichloromethane: methanol (10: 1). The chromatogram of active extracts was performed on Figure 2.

Figure 3 showed that in one active extracts contain more than one active antioxidant compounds that indicated by yellow band. Yellow spot or yellow band indicated the active chemical compounds responsible for antioxidant activity. Further analysis of active extract showed that IC_{50} of active extracts were in the range of 50. 97-2031.36 ppm, only two extracts have IC_{50} < 100 ppm (Table 4).

Antioxidant activity

Antioxidant activity was done based on TLC-bioautography on DPPH radical scavenging activity.

Table 3. Minimum inhibitory concentration (MIC) for antimicrobial of endophytic fungal extracts from 6 species of Zingiberaceae rhizomes against *S. aureus* and *E. coli*

Endophytic extracts	MIC (µg/mL)	
	S. aureus	*E. coli*
JES-01	1280	>1280
KPS-01	320	>1280
KPS-03	320	>1280
KPS-04	160	80
KS-02	1280	>1280
KS-04	320	320
TLS-02	160	>1280
TMS-02	160	>1280
TMS-03	80	>1280
TPS-03	1280	>1280
TPS-07	640	>1280
Streptomycin	2	32
Chloramphenicol	4	16
Vancomycin	0.5	>32

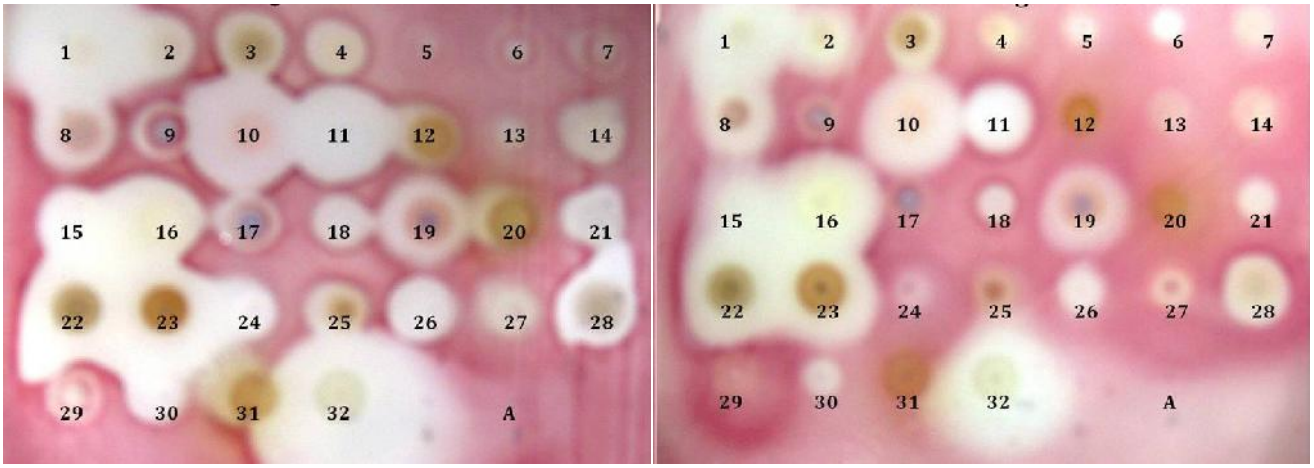

Figure 1. Bioautogram of endophytic fungal extracts of 6 species Indonesian Zingiberaceous plant against *E. coli* Ina-CC B4 (left) and *S. aureus* Ina-CC B5 (right). Clear zone indicated growth inhibition.

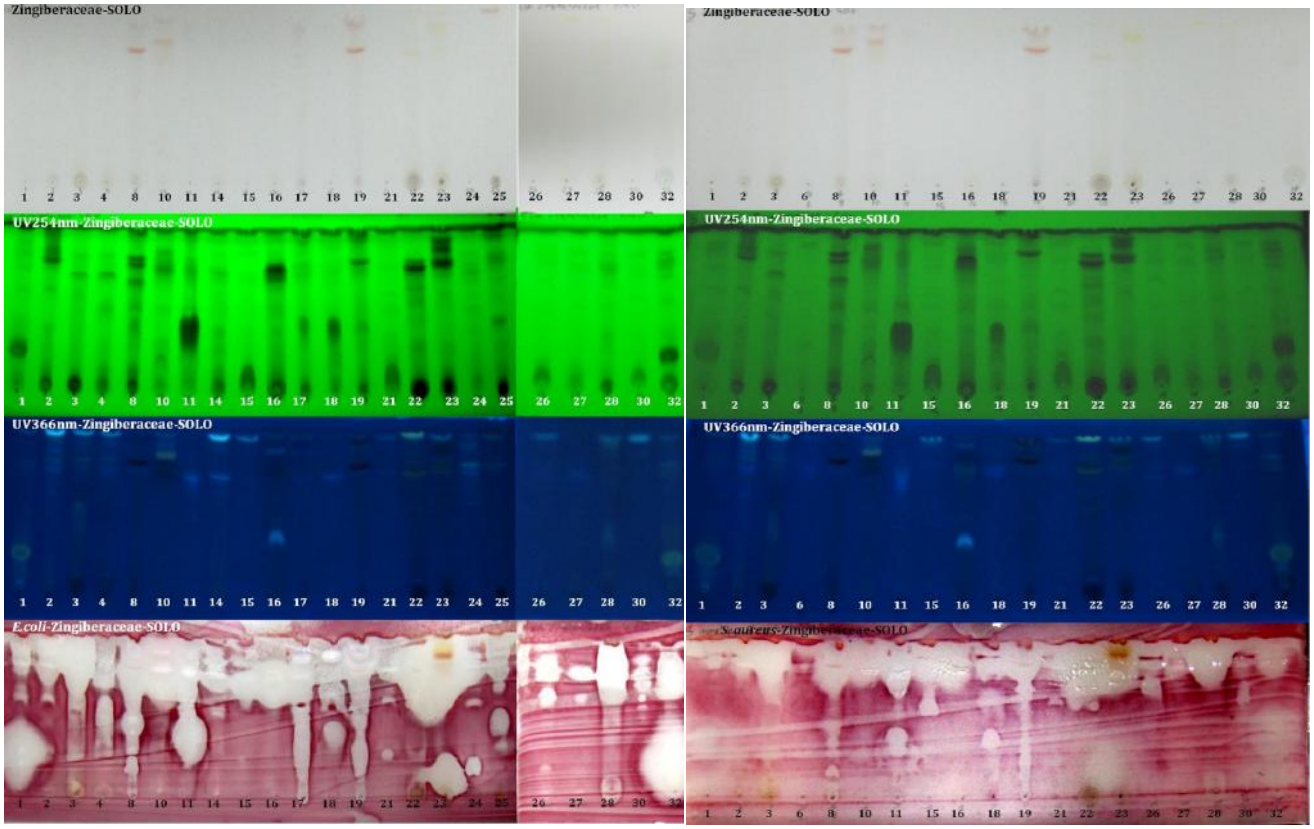

Figure 2. Bioautograms of active antibacterial compounds of endophytic fungi from Zingiberaceae against *E. coli* (left) and *S.* aureus (right). TLC plates were developed in dichloromethane: methanol (10: 1) and sprayed with INT. Clear bands indicated inhibition of bacterial growth

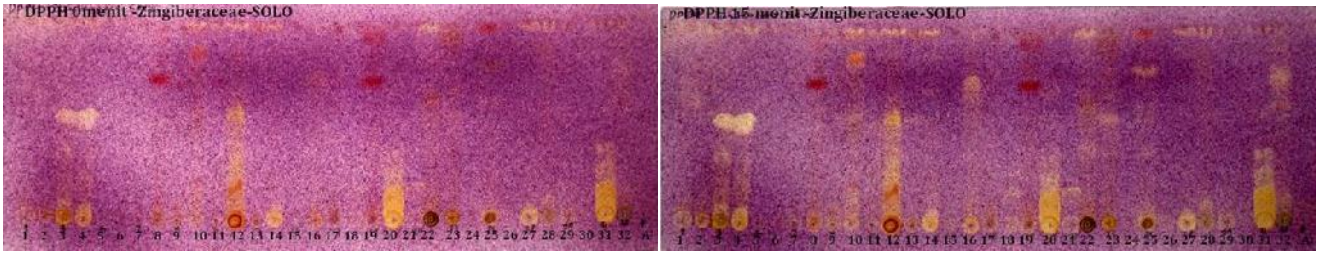

Figure 3. Bio-autographic screening of active antioxidant compounds of endophytic fungal extracts of 6 species Zingiberaceae plant. Chromatogram developed with dichloromethan: methanol (10: 1) and sprayed with DPPH. Yellow whitish bands indicated separated antioxidant compounds. A. 0 minute, B. 10 minutes

Table 4. IC50 of active extracts of Indonesian Zingiberaceous plants

Sample	IC50 (ppm)
JES-03	238.44
JES-04	253.29
KPS-05	2031.26
KS-01	50.97
TLS-04	61.34
TPS-02	763.06
TPS-06	858.73
Catechin (positive control)	8.04

Discussion

The rhizomes from six species of Zingiberaceous plants used for sources of endophytic fungal isolation. All the rhizomes collected from domesticated Zingiberaceous plants. All of them have been used as condiments or herb. The isolation of endophytic fungi totally gained 33 endophytic fungi, in which 7 endophytic fungi associated with ginger (*Zingiber officinale*), 4 isolates from turmeric (*Curcuma domestica*), 6 isolates from round-rooted galangal (*Kaempferia rotunda*), 4 isolates from temulawak (*Curcuma xanthorrhiza*), 5 isolates from mango ginger/wild turmeric (*Curcuma mangga*), and 7 isolates from round zedoary (*Curcuma zedoaria*). This is indicated that the rhizome of domesticated Zingiberaceous plant harbors many endophytic fungi. Based on their morphological characters these endophytic fungi were classified into 10 genera. The dominant genera seem to be Dematiaceae. Dematiaceae contain melanin in their cell-wall and have a dark color and occasionally infect humans (Medical Dictionary 2009). Besides Dematiaceae, Fusarium is the second highest number of endophytic fungi associated with Zingiberaceous rhizome collected from Solo. According to Ilyas et al. (2009) most member of Fusarium are plant pathogenic. While *Fusarium oxysporum*, a non-pathogenic fungus associate with *Cucumis sativus*, can maintain the host survival under *Phytium ultimum* infection (Rubini et al. 2005).

The screenings of antibacterial and antioxidant activities of endophytic fungal extracts were done by TLC-bioautography. This method was a quick-approach (Rajauria and Abu-Ghannam 2013), fast, cheap and better bioassay-directed fractionation of bioactive compounds (Hamburger and Cordell 1987) and enabled rapid progress for quick detection of new antimicrobial compounds (Suleimana et al. 2010). The growth inhibition of bacteria tested was performed by the presence of clear zone around the extract against the purple background on TLC plate. The purple background was due to the ability of growing microorganism to reduce INT to a purple-red color (Begue and Klein 1972).

The analysis of the chemical compounds of the extracts revealed several chemical compounds in the extract. After spraying with INT, it also shown that in one extract there are several active chemical compounds that inhibit bacteria growth. The appearance of white bands indicated the active compounds in the extracts that responsible for antibacterial activity. White bands indicate the reduction of INT to the colored formazan did not take place because of the presence of antibacterial compounds that inhibit the growth of bacterial tested (Suleimana et al. 2010).

The minimum inhibitory concentration (MIC) of 11 active extracts against *S. aureus* Ina-CC B4 were in the range of 80-1280 µg/mL, while the MIC against *E. coli* Ina-CC B5 only 2 extracts had MIC value less than 1280 µg/mL while 9 extracts had MIC value > 1280 µg/mL. The MIC values of extracts are greater than that of positive control chloramphenicol or streptomycin. This might be caused by the various mixture compounds in the crude extracts in which not all of the chemical compounds have bioactivity. The activity of the extract can also be caused by synergism or additive action (Ahmad and Aqil 2007) or neutralize or inhibit (Yadav et al. 2014) of the active compounds in the extract, While, chloramphenicol and streptomycin are pure compounds. Therefore, it is necessary to isolate and purify the chemical compounds that responsible for antibacterial activity. Based on the MIC values of the active extracts, it can be concluded that *S. aureus* is more sensitive than *E. coli* toward endophytic fungal extracts from Zingiberaceae. This is in accordance with Shan et al. (2007) that Gram positive bacteria were more sensitive than Gram negative bacteria. This might be due to the cell wall structure of Gram negative bacteria which is less permeable to antimicrobial compounds (Hodges 2012).

Alligianis et al. (2001) proposed a classification for plant material based on the MIC results as follow: strong inhibitors (MIC≤ 500 ug/mL), moderate inhibitors (MIC 600-1500 ug/mL), weak inhibitors (MIC≥1600 ug/mL). The endophytic fungal extracts isolated from Zingiberaceous plant can be classified as moderate inhibitors against *S. aureus* Ina-CC B4.

Bioautographic screening of antioxidant compounds from endophytic fungal extracts was done by DPPH assay. The DPPH assay is a common, rapid, simple and low cost method to measure the antioxidant capacity (Burits and Bucar 2000). Mahlo et al. (2013) stated that DPPH in methanol produces purple color and is reduced to a yellow color product, diphenylpicril hydrazine. DPPH acts as hydrogen donors and free radical scavengers (Anis et al. 2012). Kumar et al. (2012) stated that the degree of discoloration indicates the antioxidant capacity of the extract. There are four Dematiaceae extracts and two *Fusarium* extracts that possess antioxidant activity. The result of the study from Li et al. (2011) indicated that *Fusarium oxysporum* Dzf17 as an endophytic fungus isolated from the rhizomes of *Dioscorea zingiberensis* possess in-vitro antioxidant activity. The value of IC_{50} indicated that KS-01 and TLS-04 have strong antioxidant activity in which the IC_{50} value of these extracts were between 50-100 ug/mL (Blois 1958).

The result of this study indicate that rhizome of various Zingiberaceae harbors many endophytic fungi.

Some of these endophytic fungi possess antibacterial and/or antioxidant activity. Further study to obtain pure compound with good bioactivities is still needed.

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