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Toxicity of Bioactive Compound from Endophytic Fungi Isolated from Red Ginger (*Zingiber officinale* var. *rubrum*) Utilizing Brine Shrimp Lethality Assay

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ABSTRACT

Red ginger (*Zingiber officinale* var. *rubrum*) has been proven to show anticancer activity. Direct use bioactive compound from red ginger has many obstacles such as large amount of red ginger's rhizome needed, limitation of planting area, and very long time of harvesting. Utilization of endophytic fungi from red ginger's rhizome could be an alternative to the problems. The aims of this study were to determine bioactive compound produced by endophytic fungi and toxicity activity based on LC_{50}. Endophytic fungi were isolated from red ginger and were identified macroscopically and microscopically. The bioactive compounds were extracted using ethanol 96%. Flavonoid test was done qualitatively, bioactive compounds were analyzed by Thin Layer Chromatography (TLC), and the toxicity test was done using Brine Shrimp Lethality Assay (BSLA). The present research found two endophytic fungi isolated from red ginger rhizome. Isolate 1 was similar to *Mucor* sp. and isolate 2 was similar to *Trichoderma* sp. Phytochemical test revealed bioactive compound extracted from the isolates were contained flavonoid. TLC analysis did not detect quercetin from the bioactive compound extracted from the isolates. LC_{50} values of the bioactive compound from the isolates were 2.300 and 1.747 µg/ml, respectively. The toxicological results suggest that both isolates produce non-toxic compound to *Artemia salina*.

Keywords: brine shrimp lethality assay; endophytic fungi; red ginger; toxicity

INTRODUCTION

Red ginger (*Zingiber officinale* var. *rubrum*) contains bioactive compounds that have capabilities as anticancer, such as flavonoid, gingerol, and shogaol (Danciu *et al*., 2015; Semwal *et al*., 2015). Usage of the bioactive compounds directly from ginger faces many obstacles, i.e. large biomass needed, land use limitation, and length of harvesting time (Ginting, 2013). One of the alternatives to solve the problems is to utilize endophytic fungi from red ginger.

Endophytic fungi are a group of microorganism that all or parts of their lives are found in the plant tissues (Kumara *et al*., 2014). Endophytic fungi produce useful bioactive substances such as antimicrobial, fungicide, antivirus, and antioxidant (Lakshmi & Selvi, 2013). Usage of endophytic fungi as sources of bioactive substances is believed to give benefits, i.e. substances produced are homogenous, fast production, ease to be scaled up, and the amount of productions can be multiplied (Kumara *et al*., 2012).

Brine Shrimp Lethality Assay (BSLA) is the initial screening method for anticancer substances (Kolbeck & Tintjer, 2016). BSLA is done based on acute toxicity of an extract to *Artemia salina* larvae by LC50 value after 24 hours treatment. If LC50 < 1000 µg/ml, the sample extract is considered to have high toxic effect and is hoped to have cytotoxic effect that becomes a prerequisite for an anticancer substance (Kolbeck & Tintjer, 2016; Bücker *et al*., 2013).

Kaitu *et al*. (2013) reported that endophytic fungi found from red ginger rhizome was able to inhibit *Escherichia coli* and *Streptococcus pyogenes*. Endophytic fungi isolated from red ginger rhizome were also able to inhibit pathogenic fungi Fusarium oxysporum (Ginting, 2013). However, the bioactive substances produced by the endophytic fungi and their toxicity have never been reported.

The objectives of the research are to determine bioactive substances produced by endophyte fungi isolated from red ginger qualitatively, toxicity level of the bioactive substances utilizing BSLA method, and LC_{50} value of the bioactive substances.

MATERIALS AND METHODS

Red ginger rhizomes were purchased from cultivation field at Rajek Lor village, Mlati subdistrict, Sleman district, Yogyakarta Special Province. Other materials used were as follows eggs of *Artemia salina* from Balai Besar Pengembangan Budidaya Air Jepara, Central of Java.

PDA and PDB media preparations. PDA medium was made by diluting 39 g PDA powder in 1 L aquadest and adding it with chloramphenicol antibiotic 0.5 g/L, stirred and boiled to dilute. Afterward it was sterilized in the autoclave for 15 min at 121^{o}C and 1.5 atm (Sigma Aldrich, 2017). PDB medium was made by slicing 300 g potato, then add with 1000 ml aquadest. The medium was boiled and then filtered. As much as 500 ml of the potato extract was put into a bottle and add with 10 g dextrose and NH_4NO_3 1%. The media were sterilized with autoclave at 121^{o}C for 15 min (Sigma Aldrich, 2017).

Pure culture of endophyte fungi. Red ginger rhizomes were cleansed with running water. The rhizomes were sterilized by soaking into ethanol 70% for 1 minute and then were rinsed three times with sterile water. The rhizomes were soaked again into hypochlorite solution 0.5% for 5 min and were rinsed for six times with sterile water. Red ginger rhizomes were cut into smaller sizes, i.e. 2 x 2 x 2 cm^3. The pieces of rhizome were put on the surface of PDA medium and were incubated at 28^{o}C. Observations were done everyday to determine the growth of endophytic fungi after isolation (Ginting, 2013; Sadrati *et al*., 2013). Colonies of endophytic fungi growth were purified in new PDA medium and incubated for 5 days. If there were different colonies growth at the PDA medium, thus it will be re-isolated until pure culture was obtained. The pure culture of endophytic fungi was grown on agar slant to be identified (Noverita *et al*., 2009).

Endophytic fungi identification. Identification was done macroscopically by observing the morphology, textures, and colors of the colonies. Microscopic identification was done by observing the types of mycelia, spores, and conidia. Microscopic identification was done using lactophenol cotton blue dyes. The identification was done based on Rodriguez *et al*., (2009); Ginting *et al*. (2013).

Cultivation and production of secondary metabolites. Endophytic fungi were rejuvenated in PDA slant media and were incubated at 28^{o}C for 5 days. Cultivation of the endophytic fungi were done by transferring the isolates into 100 ml PDB media. PDB medium was incubated for 7 days at 30^{o}C into shaker incubator at 120 rpm (Sadrati *et al*., 2013; Lakshmi & Selvi, 2013; Li *et al*., 2015). Fermentation medium was separated from the mycelia by filtration and was rinsed with ethanol 96%. Extraction process was done by adding ethanol 96% into the fermentation medium (1:1). The solvent which contains bioactive substances was evaporated using rotary evaporator and put into the oven to get the viscous extract (Nath *et al*., 2012).

Qualitative flavonoid test. The test was done by adding 5 ml ethanol to 50 mg extract and was boiled for 5 minutes. The solution was added with 5 drops of concentrated HCl and 1 mg powder. Positive results which determine the existence of flavonoid were shown by the appearance of yellow, pink or purple colors (Ali *et al*., 2018; Sangi *et al*., 2008).

Bioactive substance analysis. Bioactive substance extracts were weighed as much as 5 mg, diluted into 5 ml methanol, and filtered. Quercetin as the standard substance was weighed as much as 0.01 gram, diluted into 10 ml methanol. Silica gel plate 60 GF_{254} sizes 9 cm x 5 cm was used. Sample substance and

quercetin were spotted at the distance of 1.5 cm from the lower part of the silica plate. The plate was put into the jar that contains mobile phase composed of toluene:ethyl acetate:methanol (5:3:2). The plate was put at the borderline and was observed by UV lamp at the wavelength 254 nm, and the R_f values were then measured (Males *et al*., 2013; Preethi *et al*., 2017; Rao & Ahmed, 2013).

Hatching of *Artemia*. Two g of *Artemia* eggs were hatched in the aquarium filled with 500 ml seawater and equipped with aerator. Seawater temperatures were set at 25-30^{o}C and supported with LED lamps. After 48 hours, *Artemia* eggs will be hatched into larvae, the latter were pipetted and ready to be used in the toxicity test (Kolbeck & Tintjer, 2016; Eimanifar, 2014).

Preliminary toxicity test. Preliminary toxicity test was done to determine the critical concentration ranges to be applied at the determinative toxicity test (Bücker *et al*., 2013). At the preliminary test, every ten larvae of *A. salina* were put into small bottle with extract solutions at different concentrations, namely 0, 10, 100, 1000 µg/ml. The toxicity tests were done in five replications. After 24 hours, the percentage of death larvae was counted (Naidu *et al*., 2014; Hiola & Bahri, 2010).

Determinative toxicity test. BSLA was done by putting every ten *A. salina* larvae aged 48 hours into bottle filled with 5 ml extract solution with the concentration determined from preliminary test and was added with 5 ml seawater. The control treatment was seawater with ten *A. salina* larvae without extract. The bottles were incubated for 24 hours with LED lamps. After 24 hours, the numbers of life larvae were counted (Kolbeck & Tintjer, 2016; Naidu *et al*., 2014). The criterion of life larvae was larvae that still able to move for few seconds during observations (Rajabi *et al*., 2015).

Data Analysis. Data were analyzed using probit to determine LC_{50} value. Extracts were considered as toxic if the LC_{50} values are lower than 1000 µg/ml (Kolbeck & Tintjer, 2016).

RESULT AND DISCUSSION

Isolation of Endophytic Fungi. There were two isolates of endophytic fungi isolated from red ginger rhizome (Figure 1).

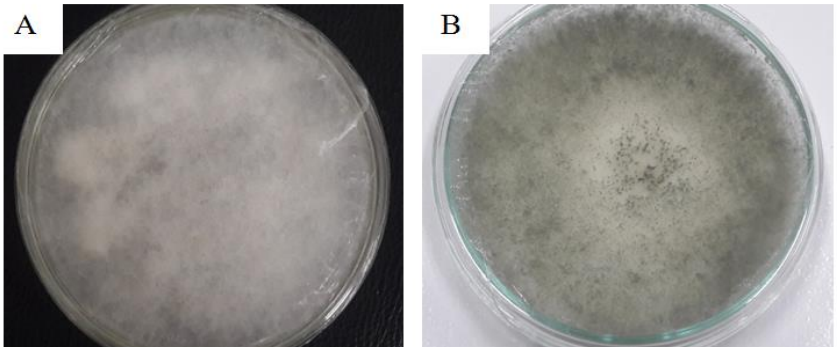


Figure 1. Pure culture of endophytic fungi isolated from red ginger rhizome: A. Isolate 1 with white coloration as cotton; B. Isolate 2 with black green colour

Macroscopic characteristics of the isolate 1 were white color, smooth as cotton, and colony's back color was yellowish white; while the microscopic characters observed were the types of sporangiophore, sporangium, columella, and sporangiospore (Figure 2). Macroscopic and microscopic characteristics showed that isolate 1 was very similar to *Mucor* sp. and according to Rodriguez *et al*. (2009) the species that has white colony color and can be changed into greyish brown was identified as *Mucor racemosus*, while colony with back color yellowish white was identified as *Mucor hiemalis*, and species with thick walled and branched sporangiospores was identified as *Mucor plumbeus*. The isolate 1 was also similar to *Rhizopus* sp., but differ in that *Rhizopus* sp. has rhizoid, while *Mucor* sp. does not have rhizoid (Purwantisari & Hastuti, 2009; Rodriguez *et al*., 2009).

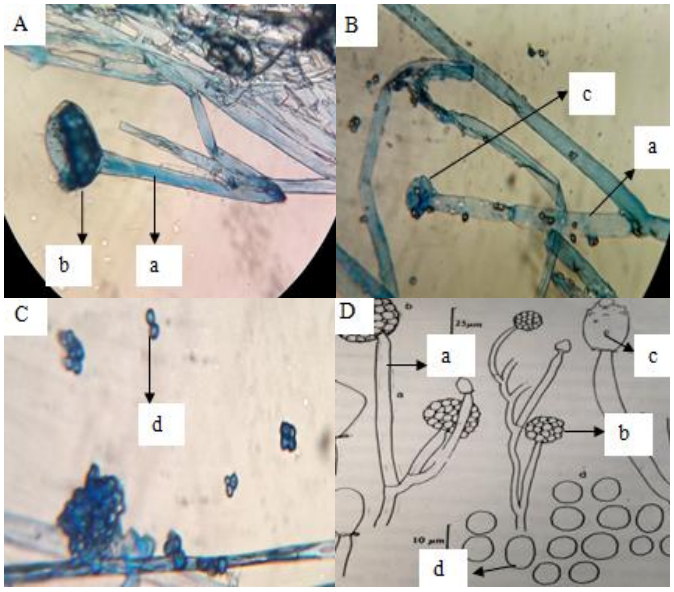


Figure 2. A-C. Morphology of isolate 1 by lactophenol cotton blue dyes and observed under light microscope at 450 x; D. Morphology of one of the species of *Mucor* sp., i.e. *Mucor plumbeus* (Purwantisari & Hastuti, 2009); a. Sporangiophore; b. Sporangium; c. columella; d. round shape sporangiospore

Macroscopic characteristics of isolate 2 (Figure 1B) were black green in color, rough surface, and colony's back color were white, while its microscopic characteristics (Figure 3) showed the existence of conidiophore, round shape conidium, and phyalides or cells that formed conidium. The characters were very similar to *Trichoderma* sp. Rodriguez *et al*., (2009) stated that *Trichoderma* sp. colony has no color at the beginning but will change to greenish white and turned into dull green; has no color for the colony's back. Change of colony color was due to the age of the colony. Colony of young *Trichoderma* will have white color, but later it will turn into green and finally into black green (Suparno *et al*., 2016). Macroscopically it has the same color as *Penicillium* sp., i.e. from greyish green (*Penicillium griseofulvum*) to dark green (*Penicillium purpurogenum*). The difference was seen microscopically i.e. *Penicillium* sp. has metulae that is the apical branch of conidiophore that bring phyalides, while at *Trichoderma* sp. metulae does not exist at all (Rodriguez *et al*., 2009).

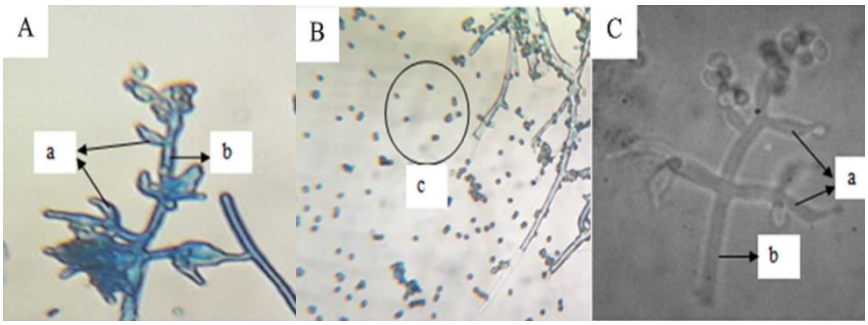


Figure 3. A. Morphology of isolate 2 with lactophenol cotton blue dyes observed under light microscope at 450 x; B. Morphology of conidia from isolate 2 with lactophenol cotton blue dyes at 450 x; C. Morphology of *Trichoderma* sp. at 1000 x (Purwantisari & Hastuti, 2009); a. fialid or cells that formed conidia; b. conidiophore; c. semicircle conidia shape

Kaitu *et al*. (2013) found an isolate that was similar to *Mucor* sp. from red ginger rhizome. Ginting (2013) isolated *Mucor* sp. and *Trichoderma* sp., but was not from red ginger rhizome. The existence of endophytic fungi in plant tissues were influenced by climate and geographical position as well as culture medium used; since culture medium gave

similar conditions to natural habitat of endophytic fungi. The mechanism adaptation of endophytic fungi to physiological and microecological conditions of the plant's hosts were well noted (Costa *et al*., 2012; Fernandes *et al*., 2015; Noverita *et al*., 2009).

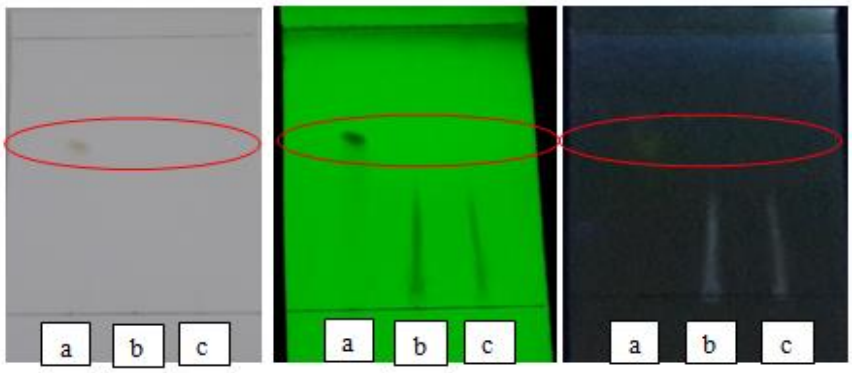
Qualitative Flavonoid Test. The result of flavonoid qualitative test showed that both isolates were able to produce flavonoid (Table 1). Yellow color appeared due to reduction reactions between Mg, HCl, and the samples (Danciu *et al*., 2015). Endophytic fungi were able to produce flavonoid through shikimate pathway that is started with condensation process between phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E-4-P) (Tohge *et al*., 2013). Shikimate pathway produces chorismate that will become the precursor for phenylalanine and the latter will become the precursor for flavonoid (Tohge *et al*, 2013).

Table 1. Flavonoid qualitative test

Isolate	Initial Colour	End Colour	Test Result
Isolate 1	White	Yellow	+
Isolate 2	White	Yellow	+

+ = contain flavonoid

Biosynthesis of flavonoid was also done through malonic acid pathway that produced malonyl CoA. Three molecules of malonyl CoA will undergo condensation with one molecule of 4-co chalcone synthase to produce chalcone. Chalcone was isomerized into naringenin that will undergo several reactions to produce flavanone, flavonol, flavanol, and anthocyanin



A B C

Figure 4. TLC results: A. without UV light; B. UV light with wavelength 254 nm; C. UV light with wavelength 365 nm. a. Standard quercetin with R_f value of 0.67; b. Extract of isolate 1; c. Extract of isolate 2

TLC analysis showed R_f value for quercetin standard at 0.67 (Figure 4). Extracts from isolate 1 and 2 were not able to show clear spots at R_f value 0.67. Extracts of bioactive substance from isolate 1 and 2 did not produce quercetin, which was one of the members of flavonoid. Quercetin was not detected from the extracts of both isolates due to very low concentrations. Preethi *et al*., (2017) reported that one of the disadvantages of TLC i.e. it cannot detect low concentration of substances. Other possibility was that the endophytic fungi did not produce quercetin due to ecological factors such as rain falls, climate, and nutrient contents in the soil affected secondary metabolites production (Liu *et al*., 2015).

Figure 4 showed sample substances that were not fully separated by the mobile phase used. Imperfect separation was due to interaction of substances involved, for instance polar substance can form hydrogen bonds; substances bind to immobile phase, and mobile phase did not work optimally. Thus, advance elution needs to be done to utilize mobile phase with different polarity levels (Santosa & Haresmita, 2015).

Toxicity Test. Preliminary toxicity test was done and the result of the death percentage of *A. salina* larvae at concentration 1000 µg/ml

was as high as 30%. The concentration was then needed to be increased in order to reach higher percentage value. For determinative toxicity test the concentrations used were varied, namely 1000, 2000, 3000, 4000, and 5000 µg/ml (Table 2).

Table 2. Toxicity of bioactive substances from isolate 1 and isolate 2

Isolate	Average Death of *A. salina* larvae (%)					
	Concentration (µg/ml)					Control
	1000	2000	3000	4000	5000	
Isolate 1	28	42	52	68	80	4
Isolate 2	38	52	60	70	78	2

LC_{50} of the bioactive compound from the isolates were 2,300 and 1,747 µg/ml, respectively. Bioactive substances from isolate 1 and 2 yielded LC_{50} value > 1000 µg/ml and categorized as not toxic (Kolbeck & Tintjer, 2016). LC_{50} value less than 1000 ppm means that the bioactive substance tested has the potential effect as anticancer, but if the value is higher than 1000 ppm means the bioactive substance has no potency as anticancer (Pasilala *et al*., 2016). Therefore, bioactive substances from isolate 1 and 2 did not show any potency as anticancer.

Flavonoid can kill *A. salina* larvae by disrupting larvae cell walls, inhibiting enzyme activities, disturbing the function of larvae cell membrane, and inhibiting protein synthesis due to the activation of α-subunit of eukaryotic initiation factor 2 (eIF2α). Thus phosphorylation was occurred at eIF2α kinase that caused failure of initiation of mRNA translation (Ginting *et al*., 2013; Roy, 2016). Death of *A. salina* larvae could also cause by flavonoid that gave up its OH^- group to bind to cell membrane integral protein of *A. salina* larvae. The OH^- group that binds to cell membrane integral protein will inhibit active transport of Na^+ and K^+, thus influx of Na^+ to the cells becomes uncontrolled and caused the cell membrane rupture (Chen *et al*., 2014).

CONCLUSION

The present research concluded that Isolate 1 and 2 produce bioactive substances such as flavonoid, but quercetin was not detected in the extracts from the two isolates, not toxic based on BSLA, with LC_{50} value at 2.300 µg/ml (isolate 1), while isolate 2 at 1.747 µg/ml.

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