Preliminary Screening of Antagonistic Fungal Endophytes from Zingiberaceae

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Abstract: Efforts to elaborate the role endophytes have been intensively studied from a different view point for the last two decades. One of the most encouraging works is to find potential sources for novel medicinal compounds. Preliminary screening of fungal endophytes possessing antagonistic activity from rhizomes of Zingiberaceae has been conducted. The aim of this study is to collect potential antagonistic fungal strains against representative microbial pathogens: *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. Antagonisms assay was performed by agar plug method in dual culture plate assay. The study found thirty-nine (39) fungal strains collected from rhizomes of five species of Zingiberaceae namely *Alpinia* sp*., Amomum centrocephalum, Elettaria* sp., *Etlingera* sp. and *Hedychium coronarium*. Among all strains tested, 20 of them were antagonists of *S.aureus,* 3 of *E.coli* and 1 of *C.albicans*. All antagonist strains showed different degree of inhibitory activities which indicated the different nature of fungal endophytes and their chemical properties.

1 INTRODUCTION

Zingiberaceae is a family of herbs that grow abundantly in tropical to subtropical region with center of divergence located in Southeast Asia. The medicinal herbs covers about 1400 species around the region, mainly from Peninsular Malaysia, Indonesia, Brunei, Singapore, Philippines and New Guinea (Pandey, 2001). In North Sumatera itself, about 47 species of Zingiberaceae have been reported while most of them were known to inhabit Hutan Sibayak (Siregar, 2008). Notable species like ginger and turmeric, has long been known as potential therapy towards some illness and diseases such as, digestive disorders, fever, cold, cough, arthritis and muscle cramps (Ma, 2012). In scientific reports, biological activities from the Zingiberaceae compounds are proven to act as anti-inflammation, anti-tumor, anti-apoptosis and antimicrobials (Karuppiah, 2012).

On the other hand, microbial resistance is a big challenge on medicinal view point. These ways of finding may be achieved through the exploitation of fungal endophytes to synthesize novel antibiotics, especially the modified or somewhat similar with Zingiberaceae compounds. The first step to study the potential fungal endophytes is by isolating some antagonistic strains from healthy and non-symptomatic plant parts. Successful isolation of fungal endophytes have been reported by several researchers. Antagonistic *Penicillium* sp. has been isolated from *Curcuma longa*, exhibiting inhibitory activity against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Rathod, 2013). Chemical compounds produced by culture of *Pestalotiopsis vaccinii* were known to be a newly discovered natural products, solely synthesized by the strain itself (Wang, 2014; Wang, 2017). As far literatures has been surveyed, the study on the study on exploitation of fungal endophytes from Zingiberaceae is still limited. In this study, we reported that rhizomes of representative species from Zingiberaceae in North Sumatera, were harbored by several strains of fungal endophytes. The results of antagonistic activity towards tested pathogens were distinct among fungal strains, indicating different capability of strains in producing antimicrobial compounds.

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2 MATERIALS AND METHODS

2.1 Plant Materials

Samples of wild Zingiberaceae were collected during exploration in Hutan Sibayak, located in Deli serdang district, North Sumatera. Sampling were conducted incidentally without considering any climate and spatial factors. Plants anchoring to soils were dug up and cut to separate its shoots and roots. The root parts or rhizome were wrapped with paper and stored in plastic bags. Duplicate samples were collected separately to be authenticated by Herbarium Medanese, Universitas Sumatera Utara for identification. In laboratory, rhizomes were later cut into smaller segments and composites were made by pooling segments into one bulk sample for each species of Zingiberaceae. The samples were then used in isolation step.

2.2 Isolation of Fungal Endophytes

Step in isolating fungal endophytes were based on previous report (Yurnaliza, 2014). Bulk samples from each species of Zingiberaceae were washed with tap water to remove remaining soil and dirt. The samples were surface-sterilized by dipping in 75% ethanol for 2 min, 5.3% NaOCl for 5 min and 75% ethanol for 30 secs. The pieces were again washed several times with sterile distilled water to remove remaining solutions. Samples were dried on Whatman filter paper and cut into 1–2 smaller pieces. The pieces were placed on top of Potato Dextrose Agar (Oxoid™) supplemented with chloramphenicol. Plates were incubated in ambient condition for 3 days. Any visible fungal growth from each pieces were then sub-cultured onto new medium to preserve the strains. Each fungal strains were differentiated from their colony appearances.

2.3 Antagonisms Assay of Fungal Endophytes

Pathogenic strains used in this study were: *Staphylococcus aureus* ATCC® 29213™, *Escherichia coli* ATCC® 25922™ and clinical strain of *Candida albicans*. Both *S. aureus* and *E. coli* were first grown in Nutrient Agar (NA) while *C. albicans* in Potato Dextrose Agar (PDA) prior antagonisms assay. Antagonisms assay were performed based on agar plug method in dual culture plate assay (Balouiri, 2016).

Direct colony suspensions from each pathogenic strains were made by swabbing colonies into sterile physiological saline solution (0.95% NaCl) to obtain OD_{600} = 0.5. One mililitre of cell suspensions were mixed with 15 mL molten PDA (45^{o}C) medium, supplemented with 1% (w/v) yeast extracts for bacteria and 1% peptone (w/v) for *C. albicans*. Molten agar medium were then plated to obtain microbial lawns. Three plugs of aerial mycelium from each fungal endophytes were placed on top of medium. Plates were incubated for 2 days in ambient condition. Clear zones around mycelial plugs indicating antagonisms were measured using standard caliper in millimetre unit (mm).

3 RESULTS AND DISCUSSIONS

The species list of Zingiberaceae found in this study along with its fungal endophytic associates is presented in Table 1. Previous report revealed that there were eight genera of Zingiberaceae in Hutan Sibayak that were: *Amomum, Etlingera, Geocharis, Geostachys, Globba, Hedychium, Hornstedtia* and *Zingiber* (Siregar, 2008). Although we just managed to collect five genera in this study, here we reported two new genera, *Alpinia* and *Elettaria*.

From the results, it can be seen that isolated fungal endophytes were distinct to each species as shown in Figure 1. Although the isolates were seemed to be different among others, morphological characters alone are not enough to identify species level. Further molecular characterization is needed to ensure the species identification.

In this study, we successfully isolated 39 fungal strains with 30% of isolates were from *H. coronarium* (13 isolates). Rhizome as being part of plants' food reserve is thought to be harbored by various endophytes. Previous study showed that between parts like leaf, petiole, stem, root, adventitious root and rhizome used in isolation of endophytic fungi from *H. coronarium*, the rhizome showed the greatest diversity in number of culturable fungal strains. Although some exceptions can be found in other species of Zingiberaceae, that was *Zingiber officinale* and *Amomum siamense* with most recovered isolates were from petiole and pseudostem, respectively, rather than from their rhizomes (Bussaban, 2001; Uzma, 2016).

While several authors have reported the number of fungal isolates from species of Zingiberaceae, mostof their reports are about culturable actinomycetes from genera *Alpinia, Curcuma, Hedychium* and *Zingiber* (Taechowisan, 2003; Taechowisan 2003; Taechowisan, 2008; Krishnapura, 2015). Based on our knowing, this is

the first report on succesful isolation of fungal endophytes from *Elettaria.* Molecular identification is needed to confirm the possibility of finding novel fungal strains from this species and is our current concerns. Results will be published elsewhere.

Table 1: Species list of Zingiberaceae and their fungal endophytic associates

Species	Isolate Code	Number of Isolates
Alpinia sp.	JRT 1A, JRT 1B, JRT 2A, JRT 2B, JRT 2C, JRT 3A	6
Amomum centrocephalum	JRL 1A, JRL 1B, JRL 2A, JRL 2B, JRL 2C, JRL 2D, JRL 3A, JRL 3B , JRL 4A	9
Etlingera sp.	JRN 1A, JRN 1B, JRN 1C, JRN 2A, JRN 3A, JRN 4A, JRN 4B	7
Elettaria sp.	JRS 1A, JRS 1B, JRS 1C, JRS 2A, JRS 2B	5
Hedycium coronarium	JRD 1A, JRD 2A, JRD 2B, JRD 2C, JRD 2D, JRD 3A, JRE 1A, JRE 1B, JRE 2A, JRE 2B, JRE 4 A, JRE 4B	13
Total		39

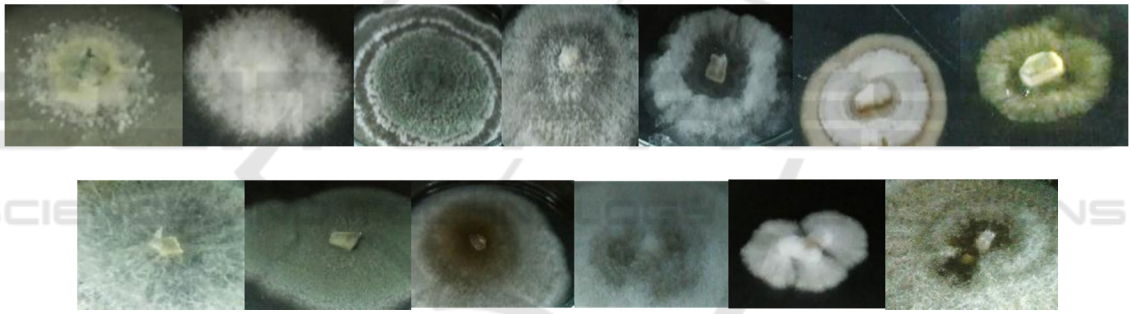

Figure 1: Representative colony images of isolated fungal endophytes from Zingiberaceae

Another approach to improve the number of isolated endophytes from plant parts, is by varying the medium composition. Endophytic bacteria isolated from rhizomes of *Curcuma zedoaria* were succesfully recovered through the use of four isolation medium, mainly modified Nutrient Agar (NA) and Water Yeast Extract Agar (WYEA). The study also incorporated turmeric extracts itself into the medium to induce growth of certain endophytic strains (Krishnapura, 2014).

The results of antagonism assay of fungal endophytes against three pathogens are presented in Table 2. Twenty isolates or more than 50% inhibit *S. aureus* with various zone measuring from 8.46 to 30.75 mm. Three isolates inhibit *E. coli* and one inhibit *C. albicans.* Among all, JRN 4B enable to inhibit all tested pathogens. From the results it is indicated that fungal endophytes from Zingiberaceae mostly synthesized compounds effectively against gram positive bacteria. The analyses of compounds produced by isolates are now under investigation.

Although majority of tested fungal strains did not show any inhibition zones in this study, especially to *E. coli* and *C. albicans*, most of them were observed to grow on top of microbial lawns. We assumed that this might be some type of interaction or synergisms between two competing microbes. Since we were using agar plug method to exhibit inhibitory effect towards pathogen, it is also possible that the fungal strains secreted antimicrobial metabolites into its agar medium. Based on our observation, future study on evaluating antimicrobial activity of fungal strains whether from the extracts and culture filtrates, may support our assumption.

4 CONCLUSIONS

Five species of Zingiberaceae sampled from Hutan Sibayak, North Sumatera are known to be inhabited by fungal endophytes. Thirty nine culturable fungal strains were succesfully isolated from rhizomes of *Alpinia*, *Amomum*, *Etlingera*, *Elettaria* and *Hedychium*. The study also reported the first successful attempt on isolating fungal endophytes from genus *Elettaria*. Each fungal strains showed different degree of antagonisms against *S. aureus* while most of them did not show any inhibition against *E. coli* and *C. albicans*. Confirmation on antimicrobial metabolites secreted by fungal strains will be considered in future study to evaluate possible novel antimicrobial compounds.

Table 2: Diameter of inhibition zones among tested fungal isolates

Code Isolate		Diameter of Inhibition Zone (mm)		
		S. aureus	*E. coli*	*C. albicans*
JRS	1A	30,75	-	-
	1B	-	-	-
	1C	11,35	-	-
	2A	-	-	-
	2B	-	-	-
JRD	1A	24,65	-	-
	2A	-	-	-
	2B	8,46	-	-
	2C	19,55	-	-
	2D	23,61	-	-
	3A	23,85	-	-
JRE	1A	-	-	-
	1B	9,41	-	-
	2A	-	-	-
	2B	-	-	-
	4A	-	-	-
	4B	18,23	-	-
JRT	1A	20,2	-	-
	1B	-	-	-
	2A	10,28	-	-
	2B	16,13	13,3	-
	2C	13,91	-	-
	3A	-	-	-
JRL	1A	25,2	-	-
	1B	13,5	-	-
	2A	-	-	-
	2B	-	-	-
	2C	20,11	19,23	-
	2D	9,51	-	-
	3A	-	-	-
	3B	13,2	-	-
	4A	-	-	-
JRN	1A	-	-	-
	1B	-	-	-
	1C	-	-	-
	2A	13,06	-	-
	3A	-	-	-
	4A	-	-	-
	4B	23,36	14,86	15,77

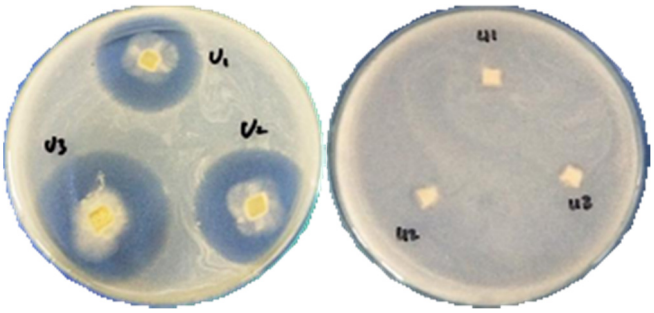

Figure 2: Representative images of antagonistic (Left) and non-antagonistic (Right)

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