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Antifungal Activity of Endophyte Bacterial Isolates From torch Ginger (*Etlingera elicitor*(Jack.) RM Smith)) Root to Some Pathogenic Fungal Isolates

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Abstract : A study on assay of antifungal activity of endophytic bacterial isolates from root of torch ginger (*Etlingera elicitor*(Jack.) RM Smith), known as kecombrang by natives, to some plant pathogenic fungi has been conducted. Bacterial characterization was carried out by microscope observation and simple biochemical tests. Antagonist assaywas carried out toplant pathogenic fungi such as *Fusarium oxysprorum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Rigidoporus microporus*, and *Culvularia* sp. and of fish pathogenic fungi such as *Saprolegnia* sp. using paper disc method in potato dextrose agar.To extract antimicrobial compounds from selected endophytic bacterial isolates, organic solvents such as methanol, ethyl acetate, and n-hexane were used, followed by preliminary chemical compound test of bacterial cell extract. Bacterial cell extracts were subjected to antifungal assay.Eleven bacterial isolates consisted of seven Gram-negative and four Gram-positive were found from the root. Antifungal assay showed that the bacterial isolates varied in inhibiting the fungal growth. Three isolates, IAK3, IAK9, and IAK11 were chosen for further study based on their higher ability to inhibit the tested fungi. Ethylacetate extract of IAK9 cells showed more effective to inhibit *R. solani*, while n-hexane extract of IAK11 showed to inhibit more on *R. microporus*. Preliminary chemical test of the bacterial cell extracts showed that methanol extract of the three isolates contained alkaloids, terpenes/steroids and saponins, while ethyl acetate extract contained alkaloids and terpenes/steroids, and n-hexane extract contained terpenes/steroids.

Keywords : antifungal activity, Culvularia sp., Etlingera elicitor, Fusarium oxysprorum, Rhizoctonia solani, Rigidoporus microporus, Saprolegnia sp., and Sclerotium rolfsii.

Introduction

Torch ginger (*Etlingera elatior* (Jack.) RM Smith), locally known askecombrang belongs to ginger family Zingiberaceae is a herbaceous perennial plant native to South East Asia. This plant has been widely cultivated and traditionally and commercially used as food, condiment, medicine, and ornamentals[1].All parts of the plants are strongly scented indicated the presence of biologically active volatile constituents.

Many studies reported mainly on secondary metabolites from torch ginger with antimicrobial, antioxidant, cytotoxic, and antitumor activity[1,2,3,4], but not from its endophytic bacteria. n-hexane extract of *E. elatior* flower bud demonstrated high inhibitory activity of *Colletotrichum gloeosporioides* mycelial growth[5]. methanol extract of torch ginger flower was active against bacteria such as *Staphylococcus aureus*, *Bacillus thuringiensis*, *B. subtilis*, *Salmonella* sp, and *Proteus mirabilis*, and fungi such as *Candida albicans* and *Aspergillus niger*, but was weak to *Escherichia coli* and *Micrococcus* sp.[2].Extract of its relative, *E. brevilabrum*

inhibited Gram-positive of *Staphylococcus aureus*, methicillin resistant *S. aureus*, *S. epidermidis*, *Bacillus thuringiensis* and Gram-negative of *Vibrio paraehaemolyticus*, and *Aeromonas hydrophila*[6]. Ginger rhizomes methanol and hexane extract showed to inbihit*S. epidermidis*, *S.aureu*s, *Enterococcus* sp., *Proteus* sp*., E. coli, Pseudomonas fluorescent*, and *C. albicans*[7].

It has been speculated that endophytes and their secondary metabolites associated with plant that they reside through specific interaction and communication with the plant host. Endophytic bacteria attracted increasing attention as they are efficient producers of sources of novel bioactive substances of commercial interest, since they seem to have unique genetic and biological system[8]. Endophytic bacteria promote plant growth and health and beneficial effects by metabolic interactions and competition with pathogenic microbes[9,10,11,12]. They enter plant through root, stomata, or other open tissues[13], and may live asymptomatically within plant tissues[8]. More than one endophytes of fungi and bacteria inhabit many plant species[10,14], and one plant tissue may harbor more than one species of endophytes[15].

Many valuable secondary metabolites have been isolated from Zingiberaceae rhizome but the physiological processes in these tissues and the functional role of associated microorganisms remain totally unexplored[16].In this study, extraction of cell of endophytic bacterial isolate from torch ginger root was done using solvent such as methanol, ethyl acetate, and hexane to know their metabolic antifungal potential. To our knowledge, this is the first study of endophytic bacterial isolates and their cell extract of torch ginger to inhibit important plant pathogenic fungi such as *Fusarium oxysprorum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Rigidoporus microporus*, and *Culvularia* sp. and of fish pathogenic fungi such as *Saprolegnia* sp.

Materials and Methods

Endophytic Bacterial Isolation from Torch Ginger Root

Root samples were washed with water to remove soil and debris. Sterilization of root surface was done by soaking it in subsequent solution: 75% ethanol for 2 minutes, 5.3% sodium hypochlorite for 5 minutes, and 75% ethanol for 30 seconds. The roots then rinsed with sterile distilled water and dried with sterile tissue paper. Both root ends were cut ± 1 cm each. The remain were cut longitudinally, putthe cut position faced to nutrient agar added with0.3 g/100 ml ketoconazole, and incubated at ambient temperature for 5 days. Growing endophytic bacterial colony around the root were subcultured onto new media.

EndophyticBacterial Characterization

To characterize bacterial isolates, direct and microscope observation, and simple biochemical tests were performanced. Microscope observation included bacterial cell shape and arrangement, Gram staining, and spores. To know biochemical characterization utilization of glucose, sucrose, lactose, gelatin and citric acid, cell motility, production katalase production were conducted.

Antifungal Assay of Endophytic Bacterial Isolates again Some Pathogenic Fungi

Antagonistic assay was conducted to know endophytic bacterial ability to inhibit growth of plant pathogenic fungi such as *Fusarium oxysprorum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Rigidoporus microporus*, and *Culvularia* sp. and of fish pathogenic fungi such as *Saprolegnia* sp. Fungal growing mycelium was taken with a cork borer and inoculated in the center of petridish of potato dextrose agar added with 1% yeast extract. A 10 µl of endophytic bacterial isolates of ≈ 10^8cfu/ml in paper disc (Oxoid) was put on two sideswith a distance of 3.5 cm from fungal culture. Cultures were incubated at ambient temperature for 5-6 days. Potential isolates was indicated by high fungal growth inhibition around bacterial paper disc.

Endophytic Bacterial Cell Extraction

Three isolates IAK3, IAK9, and IAK11were chosen for further study based on their higher ability in inhibiting fungal growth in petridish. To extract antimicrobial compounds from selected bacterial isolates, organic solvents such as methanol, ethyl acetate, and n-hexane were used. Extraction of secondary metabolites of endophytic bacterial isolates was carried out as described[17]. Bacterial isolates were cultured by spreading it on nutrient agar. Cultures was incubated at ambient temperature for 5-6 days. Medium with bacterial culture was cut into small pieces and soaked in selected solvent for 72 hours in flask. The flask was wrapped with

aluminum foil to avoid light. Maserate was filter-collected and subjected to concentrate using rotary evaporator at ≈50°C to getsemi solid extract.

Antifungal Assay of Endophytic Bacterial Cell Extract

Similar to that of antifungal assay of endophytic bacterial, antifungal assay of the extract was conducted in potato dextrose agar added with 1% yeast extract to all pathogenic fungi. Fungal growing mycelium cut with core borer was put on the center of petridish of PDA. Paper disc was dipped into each extract of concentration 40, 60, 80, and 100% and was air-dried. Dimethylsulfooxide(0.3%) was used as extract solvent. Cultures were incubated at ambient temperature for 2 days.

Preliminary Test of Endophytic Bacterial Cell Extract Chemical Compounds

Chemical compounds from the extracts was detected using methods as described previously[18], includingalkaloid, flavonoid/tannin, terpenoid/steroid, and saponin.

Results and Discussion

Endophytic Bacterial Characterization

Endophytic bacteria have been found in most plant species. Isolation of endophytes have been carried out from root, stem, leaf, and seed[15,19]. Endophytic bacteria contribute in increasing plant performance and protecting plant against plant disease[10,15] through their producing of plant growth hormon and antifungal substances[9,10,11,12]by antibiosis, competition for niches and nutrients, interference with pathogen signaling or by stimulation of host plant defenses[20].

Table 1. Morphological and biochemical characterization of endophyticbaterial isolates from torch ginger root

Isolates	Gram staining	Cell shape and arragement	Biochemical tests*						
			Glucose	Sucrose	Lactose	Katalase	Gelatin	Motility	Citric acid
IAK1	-	diplococcus	m	um	um	p	um	ut	um
IAK2	-	diplococcus	um	um	um	p	um	ut	um
IAK3	+	diplococcus	m	um	um	up	m	t	m
IAK4	-	diplococcus	um	um	um	p	um	ut	um
IAK5	+	streptobacillus	m	um	um	p	m	t	um
IAK6	-	diplococcus	m	um	um	up	um	t	um
IAK7	+	streptobacillus	um	um	um	up	m	t	m
IAK8	-	staphylococcus	um	um	um	p	um	t	um
IAK9	-	diplococcus	m	m	m	p	um	t	um
IAK10	-	staphylococcus	um	um	um	p	um	ut	m
IAK11	+	streptobacillus	m	um	um	up	m	t	um

∗m: metabolized, um: unmetabolized, p: produce, up: unproduce; t: motile, ut: unmotile

To investigate endophytic bacteria isolates as sources of bioactive secondary compounds, isolation of endophytic bacteria was undertaken. Endophytic bacterial isolation from torch ginger root tissue found 11 isolates. Most of them were with different morphological and biochemical characterization (Tabel 1.). Seven isolates belonged to Gram-negatives and 4 isolates were Gram-positives.Various endophytic bacteria were isolated from ginger rhizome[17].Isolation of endophytic bacteria from *Alpinia galanga* found at least 82 isolates *Streptomyces* sp., 11 isolates of *Nocardia* sp., 3 isolates *Microbispora*, and 2 isolates of Micromonospora[21].

Antifungal Assay of Bacterial Isolates to Some Pathogenic Fungi

Antifungal assay of endophytic bacterial isolates showed that six out of eleven isolates have antifungal activity. Bacterial ability to inhibit fungal growth varied to some extent (Table 2.) shown as clear zone with no hyphal growth surround bacterial colony (Figure 1.).

Table 2. Fungal inhibition growth by endophytic bacterial isolates of torch ginger root

	Inhibition zone of fungal growth in mm					
Bacterial isolates	***F. oxysporum***	***Saprolegnia*sp.**	***R. solani***	***S. rolfsii***	***R. microporus***	***Curvularia*sp.**
IAK1	8,9	32	21	14,6	30,8	12,2
IAK2	7,5	30,4	19,7	14,9	27,8	16
IAK3	11	29,8	22,6	14	30,9	14,1
IAK4	5	28,9	14,5	12,2	30,9	12,7
IAK5	7,3	26,1	11,8	12,6	31,2	4,5
IAK6	8,8	20,5	17,9	13,6	31,3	7,6
IAK7	3,4	8,6	5,9	14,9	19,3	30
IAK8	9	28,4	12,3	14,9	7,7	12,4
IAK9	14,1	31,1	11,8	21,6	38,4	13,9
IAK10	7,5	28,4	11,5	17	28,1	9
IAK11	19,1	29,7	20,5	24,5	34,8	16

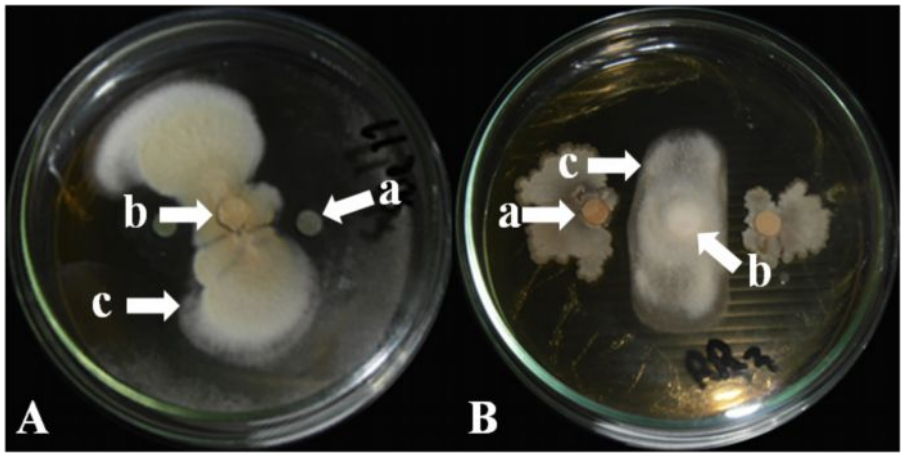

Figure 1. Fungal growth inhibition ofA. *R. solani* and B. *S. rolfsii* by endophytic bacterial isolate a. endopytic bacterial isolate, b. fungal isolate, and c. mycelium inhibition

Many bacteria were found associated as endophytes within medical plants. *Streptomyces aureofaciens* CMUAc130 from ginger root tissue showed an antagonist to *Colletotrichum musae* and *Fusarium oxysporum*, the causative agents of anthracnose of banana and wilt of wheat, respectively[22].In another study, Taechowisan et al. (2008) showed that *Streptomyces* sp TC052 strongly inhibited *S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis*, *C. albicans*, and *C. musae*. *Bacillus amyloliquefaciens* isolated from some Thai medical plants showed antimicrobial activity to four pathogenic bacteria and one fungus[23].One isolate of endophytic bacterial isolation of ginger rhizome, *Pseudomonas* sp. ZoB2 which is identified as was found to have the ability to produce growth promoting substances such as indole 3 acetic acid, 1-aminocyclopropane-1-carboxylic acid deaminase,

and siderophore[17].Sixty seven endophytic bacterial isolates were isolated from of ethnovarieties of cassava cultivated by Brazilian Amazon Indian tribes, one isolate *Bacillus pumilus*MAIIIM4awas shown to have antifungal activity against *R. solani*, *Pythium aphanidermatum*, and *S. rolfsii*[24].

Antifungal Assay of Endophytic Bacterial Cell Extract to Some Pathogenic Fungi

To know secondary metabolite activity of endophytic bacterial cells of torch ginger rhizome against several plant pathogenic fungi, cell extraction of bacterial isolates was conducted. All cell extracts of endophytic bacteria of torch ginger root inhibited test pathogenic fungi to some extent. *F. oxysporum* and *S. rolfsii* were inhibited more by n-hexane extract of IAK3, while *R. solani*, *R. microporus*, *Culvularia* sp., and *Saprolegnia* sp. were inhibited more by ethyl acetate extract of IAK9 (Table 3).

Table 3. Fungal inhibition growth by endophytic bacterial cell extract

		Inhibition of extract of bacterial cell (mm)								
		Methanol			**Ethyl acetate**			**n-hexane**		
	[extract]	IAK3	IAK9	IAK11	IAK3	IAK9	IAK11	IAK3	IAK9	IAK11
F. oxysporum	40	2,5	2,0	4,5	4,2	4,9	2,5	5,1	3,9	2,5
	60	4,6	2,3	6,3	5,4	3,9	1,6	5,1	4,0	3,8
	80	5,4	5,5	5,3	5,5	7,8	3,1	5,0	4,5	6,5
	100	4,3	6,3	5,5	5,5	1,7	5,6	6,5	4,6	6,0
Saprolegnia sp.	40	4,3	1,2	6,4	5,6	13,0	3,5	4,5	6,2	4,3
	60	4,5	2,1	4,8	4,5	12,4	3,8	2,5	6,4	5,5
	80	4,8	4,1	4,2	4,0	12,0	5,0	10,1	3,4	5,0
	100	5,3	6,5	1,8	8,8	9,6	8,5	9,9	4,8	8,1
R. solani	40	3,3	1,5	5,8	9,1	10,0	11,0	17,0	3,5	13,5
	60	2,0	2,0	2,3	8,3	7,0	11,9	17,0	10,6	15.75
	80	2,3	3,8	1,8	10,1	11,0	11,0	11,5	12,5	10,5
	100	4,0	4,4	4,2	13,0	17,3	16,6	14,5	12,9	11,0
S. rolfsii	40	6,9	7,2	8,4	3,1	2,8	3,4	10,3	5,0	4,5
	60	6,9	8,4	6,3	4,6	2.50	2,7	12,4	5,0	6,5
	80	10,0	7,8	7,1	4,2	3,9	3,2	8,9	10,8	5,5
	100	11,5	8,1	6,6	5,4	4,1	4,7	14,1	4,7	9,3
R .microporus	40	12,5	8,8	7,0	7,0	9,9	9,1	13,8	10,9	13,5
	60	11,0	7,4	10.01	4,6	8,5	9,9	5,5	10,1	10,1
	80	11,8	10,5	7,1	11,3	11,5	9,5	10,6	12,3	13,5
	100	9,5	13,5	13,6	11,9	13,5	10,0	12,4	18,5	14,6
*Culvularia*sp.	40	2,4	6,5	6,3	10,5	5,8	7,3	8,8	3,2	5,8
	60	4,4	5,5	6,5	6,8	3,5	7,3	7,9	8,4	5,5
	80	4,4	6,8	6,4	4,0	5,3	4,8	7,5	5,8	8,8
	100	5,5	7,3	7,6	6,9	12,5	6,0	8,0	10,3	10,0

Several studies on endophytic bacterial isolate cell extract have been conducted, and its metabolite potential of endophytic bacteria has been reviewed[25]. Diethyl ether and chloroform extract of UD25 were effective in the inhibition of *S. aureus.* Diethyl ether and ethylacetate extract of *B. pumilus*MAIIIM4a of cassava inhibited *E. coli* and *B. cereus* growth[24]. Most extract of endophytic bacteria isolated from the root of selected indigenous Kenyan plants showed to have antimicrobial activity against bacteria (*B. subtilis*, *E. coli*, *P. aeruginosa*, *S.aureus)* and fungus (*C.albicans*)[26].

Endophytic Bacterial Cell Extract Chemical Compounds

Several studies have been conducted to know bioactive compounds of cell of endophytic bacteria lives in plants[24,26].This study was conducted only to know chemical compound groups of the cell extracts. Secondary metabolites of endophytic bacterial isolates were extracted using methanol, ethyl acetate, and hexane. The result showed that methanol extracts of the bacterial cell contained terpene/steroid, but no flavanoid/tanin was detected in all extracts. High terpene/steroid concentration was observed in ethyl acetate extract of IAK3 and IAK11. No saponin was detected in ethyl acetate and n-hexane extract but methanol. Alkaloid was undetected in n-hexane extract(Table 4.).

Table 4. Chemical compounds of endophyticbaterial cell extracts

		Chemical compounds			
Solvents	**Bacterial isolates**	**Alkaloid**	**Flavonoid/ Tanin**	**Terpene/ Steroid**	**Saponin**
Methanol	IAK3	++++	-	+	+++
	IAK9	++++	-	+	++
	IAK11	+++	-	+	+++
Ethyl acetate	IAK3	-	-	++	-
	IAK11	-	-	+++	-
	IAK9	+++	-	+	-
n-hexane	IAK3	-	-	+	-
	IAK9	-	-	+	-
	IAK11	-	-	+	-

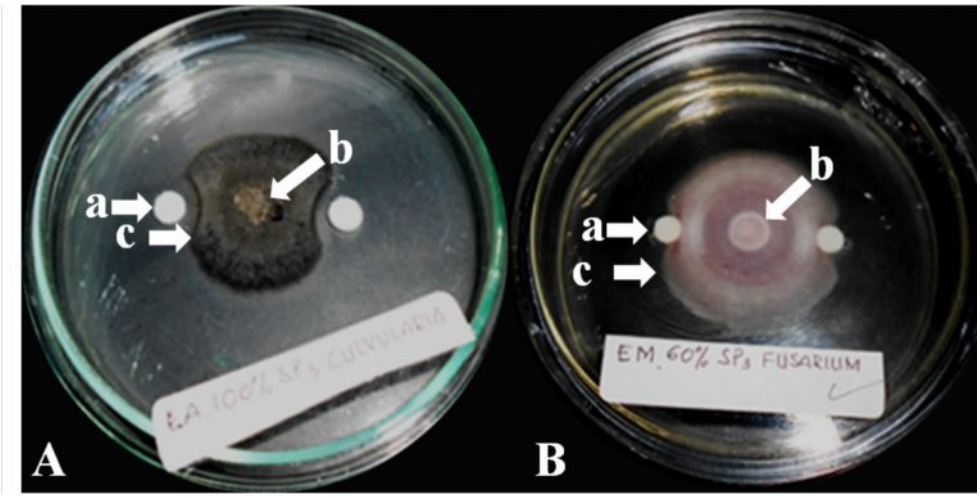

Figure 1. Fungal growth inhibition of A. *Culvularia* sp. and B. *F. oxysporum* by endophytic bacterial cell extract: a. endopytic bacterial cell extract, b. fungal isolate, and c. mycelium inhibition

In general cell wall consist of (1,3)-ß and (1,6)-ß glucan, chitin, and manoprotein. Polymerization of (1,3)-ß glucan in cell wall was catalized by (1,3)-ß glucan synthase, in which the enzyme work is disrupted by alkaloid[27]. Saponinmay play a role in disruption of sterol in cell membrane to increase its permeability and to denature protein in cell membrane. Terpene was reported to have a wide range of antifungal activity by disrupting membrane integrity of bacteria and fungi[28].

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