

RESEARCH ARTICLE

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Antagonistic Activity of Endophytic Fungi Isolated from *Globba patens* Miq. Rhizome against Human Pathogenic Bacteria

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Abstract

The emergence of bacterial infections caused by resistant strains poses a threat to the development of new antibiotics. The majority of antibiotics being produced has been accelerated through the finding of newly reported natural products, especially those originated and produced by biological sources. Endophytic fungi residing in medicinal plants may be regarded as potential sources and encourage the exploration of more plant species for their antimicrobial activity. Our current study reports on the assemblage of endophytic fungi that colonize the rhizomes, using *Globba patens* a representative of Zingiberaceae species from North Sumatra. Twenty-six fungal morphotypes were obtained and differentiated by their morphological features. Each isolate was tested against human pathogenic bacteria namely *Staphylococcus aureus* ATCC[®] 29213[™], Methicillin-resistant *S. aureus* (MRSA) ATCC[®] 43300[™], *Escherichia coli* ATCC[®] 25922[™], and Enteropathogenic *E. coli* (EPEC) K11 in a dual culture assay. The results revealed that the majority of fungal isolates were strong antagonists against *S. aureus* and *E. coli* but not against MRSA and EPEC. Isolate Gp07 was the most potential fungus with a wide range of antibacterial activities and was subjected to further species-level identification based on its morphological characteristics and DNA sequence in the ITS-rDNA region. The isolate Gp07 was identified as *Colletotrichum siamense*, yet the presence of *C. siamense* in the rhizome of *G. patens* is not fully understood while possibly being characterized as the antibiotics-producing agent in the future.

Keywords: Antibiotic resistance, Biocontrol, *Colletotrichum siamense*, Endophytic fungi, ITS-rDNA

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INTRODUCTION

The emergence and rapidly increasing incidence of bacterial resistance limited the discovery and development of antibiotics for treating infections. During the past two decades, there have been very numerous reports of untreatable infections caused by multidrug-resistant bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and its resistant strains as also of serious infections by diarrheagenic *Escherichia coli* strains^{1,2}. Ninety-one years after the discovery of Penicillin in 1929, scientists from each region of the world continue to explore and investigate the many sources of new natural products. Natural products are significant sources that deserve investigation as alternative sources of antibiotics and are commonly derived or synthesized by plants, bacteria, and fungi. One study of bioactive compounds discovered between 1981 and 2010 reported that half of the molecules discovered originated from natural sources³.

In most cases, fungi are known as notable producers of natural products. Furthermore, half of the novel natural products derived from fungal sources was from endophytic origins, with the most studies on their antimicrobials in specific to antibacterial activities⁴. Endophytic fungi are new sources for the discovery of bioactive compounds with potential development in biotechnology, although their true potential may not be fully investigated due respect of their unknown and unlimited host species^{5,6}. Interaction of endophytic fungi with medicinal plants as their hosts has revealed some beneficial features such as promotion of plant growth, increase of stress resistances, and increased bioactive accumulation of molecules by the plants⁷. The medicinal properties of directly consumed or consumption of processed medicinal plants may be studied thoroughly, particularly their endophytic fungal colonization. This may broaden the scale of the discovery of natural products, especially in the tropical regions that are rich with many plant species.

Zingiberaceae is one of the most studied families of pharmacological plants growing in the tropical region including Indonesia for its bioactive compounds and some of its endophytic associates. *Globba* spp. are examples and the third-largest genus of Zingiberaceae distributed in Southeast

Asia. It comprises about 100 species in that region⁸. Members of the genus *Globba* have been used in many ethnobotanical practices by the local communities of India, Malaysia, and Indonesia⁹. Although most ginger species are known to exhibit antimicrobial activities, the reports in this respect on the species of *Globba* are still limited¹⁰. Furthermore, information regarding the microbial associates, especially endophytic fungi colonizing the rhizomes of *Globba* species is even more limited and, therefore, merit investigation.

In North Sumatra, four species of *Globba* are found, namely, *Globba aurantiaca*, *G. paniculata*, *G. patens*, and *G. pendula*¹¹. Our preliminary investigation on endophytic fungi began with *G. pendula* rhizome and some of the fungal morphotypes found in it exhibited potential antibacterial activities against *S. aureus* ATCC 29213¹². Our study was undertaken to isolate endophytic fungi from the rhizomes of *Globba patens* and use the dual culture assay to test them for their antagonistic activities against human pathogenic bacteria. Screening of endophytic fungi from *G. patens* for antibacterial activities will provide the basis for the discovery and development of new antibiotic agents.

MATERIALS AND METHODS

Plant materials

Globba patens Miq. was collected during an exploration in Sibolangit Forest located in Deli Serdang District, North Sumatra, Indonesia. The asymptomatic *G. patens* plants were uprooted from the intact soil and stored in sterile plastic bags, then transported to the laboratory at Universitas Sumatera Utara (Fig. 1). The plant samples were kept in cold temperature until further analysis. Duplicate samples were submitted to Herbarium Medanese for species authentication. Fungal isolation was performed within 48 h of sample collection.

Isolation of endophytic fungi

The procedure of isolating endophytic fungi was based on a modified surface sterilization method¹². Rhizomes were gently cleaned under running tap water and cut into segments 10 cm long. The segments were surface sterilized in 75% EtOH for 2 min, 5.3% NaOCl for 5 min, and 75% EtOH for 1 min followed by three washings in sterile distilled water, each for about 1 min.

The sterilized rhizome segments were cut into 1–2-cm fragments and placed on Petri dishes (90 mm) containing Potato Dextrose Agar (PDA) supplemented with Chloramphenicol (0.01%, w/v). Plates were incubated at $25^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and monitored daily for any fungal colonies emerging from the rhizome fragments. Individual fungal colonies were picked from the edge of growth and transferred to new PDA plates. Stock cultures were made by sub-culturing of fungal colonies for maintenance and further analysis. Each isolate was differentiated based on the features of their colonies such as form, elevation, margin, upper-lower color, texture, concentric rings (+/-), and radial line (+/-). Each isolate was assigned a code and maintained for further analysis.

Antagonistic activity of endophytic fungi against human pathogenic bacteria

Each isolate was tested against four representative human pathogenic bacteria, namely, *Staphylococcus aureus* ATCC[®] 29213[™], Methicillin-resistant *S. aureus* ATCC[®] 43300[™], *Escherichia coli* ATCC[®] 25922[™], and Enteropathogenic *E. coli* K11 using a modified dual culture method^{12,14}. Three 6-mm mycelial plugs were cut from active-growing fungal cultures and placed on top of PDA + 1% yeast extract (w/v) that were previously seeded with $1\text{--}2 \times 10^8$ CFU/mL of bacterial suspensions. Plates were incubated at 37°C for 2 days. Clear zones surrounding mycelial plugs indicated the antagonistic activities and measured in millimeters (mm) using digital calipers.

Molecular identification of the antagonistic fungus

The potential antagonistic fungal isolate,

Gp07 was identified to the genus level based on the sequence of the internal transcribed spacer (ITS) rDNA. For extracting DNA, the manufacturer's prescribed Protocol provided with Wizard[®] Genomic DNA Purification Kit (United States) was followed. Polymerase chain reaction (PCR) was performed using universal primers for fungi i.e ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3')¹⁵ and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')¹⁶. Each reaction was performed in a 40 μL final volume containing 12 μL nuclease-free water (NFW), 20 μL GoTaq DNA Polymerase solution, 2 μL of each primer, and 4 μL DNA template solution in an Eppendorf tube. The PCR was conditioned as 95°C for 3 min, followed by 35 cycles of 95°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec, and a final extension at 72°C for 7 min. The PCR products were visualized on 1% agarose gels and delivered for sequencing (Macrogen, Inc., Singapore). Multiple sequence alignments were generated from MUSCLE feature provided in MEGA X¹⁷. Phylogenetic analysis of the sequences retrieved from BLAST results (<http://blast.ncbi.nlm.nih.gov/>) and the Gp07 sequence was constructed into a dendrogram using a Neighbor-Joining (NJ) clustering method.

RESULTS

Twenty-six fungal isolates were successfully recovered from the rhizomes of *G. patens* and differentiated based on the characteristics of their colonies (Table 1). The number of isolates found is higher than the 16 (sixteen) fungal isolates derived in the previous study of *G. pendula*¹². Also, the number of isolates was more than in the cases of other genera that



Fig. 1. (a) Leaf and (b) inflorescence part of *Globba patens* Miq. collected from Sibolangit forest, North Sumatra

have been studied, such as *Amomum*, *Elettaria*, *Etlingera*, and *Hedychium* from North Sumatra¹⁸⁻²¹. The results indicated that the possible habit of endophytic fungi colonizing the root parts may be heterotrophic eukaryons. The roots or rhizomes of Zingiberaceae play an important role as reservoirs of metabolites and nutrition for initiating and promoting the metabolism of endophytic fungi. The great diversity of endophytic fungi in a plant species may be explained by the differences in spatio-temporal distribution of host plants across regions or the wide range of colonization niches occupied by certain rhizospheric fungi²². Also, plants of a species at different stages of life-cycle may harbor distinct endophytic fungal communities over time. Younger parts of plants tend to accumulate a higher concentration of bioactive and anti-herbivory compounds which may reduce the opportunity for colonization by endophytic fungi. On the contrary, an endophytic

study of *Dioscorea zingiberensis* showed that the number of endophytic fungal isolates may be accompanied by the higher concentration of saponins accumulated in the rhizome²³. Another study on *Alpinia officinarum* using a metagenomic approach revealed that there was a connection between the colonization by endophytic fungal communities and two types of accumulated chemicals, namely, volatile oils and galangin which constructed the whole plant-microbe interaction in the rhizome²⁴. However, no such link has so far been discussed for other Zingiberaceous species, including *Globba spp.*, which may open the possibility of further investigation in the future due to the metabolite-rich rhizomes and their connection to endophytic fungi colonization.

The endophytic fungal isolates were subjected to antagonistic test against human pathogenic bacteria using dual culture assay. The majority of isolates were antagonists of *S. aureus*

Table 1. Morphological features of 26 endophytic fungal isolates from *Globba patens* Miq

Isolate code	Form	Elevation	Margin	Color		Texture	Concentric ring (+/-)	Radial line (+/-)
				Upper	Lower			
Gp01	Circular	Flat	Entire	Black	Black	Smooth	+	-
Gp02	Irregular	Flat	Undulate	Grey	Black	Rough	-	-
Gp03	Filamentous	Flat	Filiform	Black	Black	Rough	-	-
Gp04	Circular	Raised	Undulate	White	White	Dull	-	-
Gp05	Circular	Raised	Filiform	Brown	Dark Brown	Cottony	-	-
Gp06	Irregular	Raised	Undulate	White	White	Powdery	-	-
Gp07	Filamentous	Raised	Entire	Greyish Brown	Light Brown	Cottony	+	-
Gp08	Circular	Raised	Filiform	White	White	Cottony	-	-
Gp09	Irregular	Flat	Undulate	White	White	Cottony	-	-
Gp10	Irregular	Raised	Entire	White	White	Smooth	-	-
Gp11	Circular	Raised	Filiform	White	White	Cottony	-	+
Gp12	Circular	Raised	Entire	White	White	Dull	-	-
Gp13	Irregular	Flat	Undulate	Tangerine	Brownish Grey	Smooth	-	-
Gp14	Rhizoid	Flat	Lobate	Black	Black	Dull	-	-
Gp15	Filamentous	Raised	Filiform	Grey	Black	Rough	-	-
Gp16	Irregular	Flat	Filiform	Black	Grey	Powdery	+	-
Gp17	Filamentous	Umbo-nate	Filiform	Green	Brownish Red	Smooth	+	+
Gp18	Irregular	Flat	Filiform	Pinkish White	Brownish Red	Smooth	+	-
Gp19	Irregular	Flat	Filiform	Grey	Grey	Smooth	+	-
Gp20	Filamentous	Flat	Filiform	White	White	Cottony	-	-
Gp21	Irregular	Flat	Filiform	Green	Green	Powdery	-	-
Gp22	Filamentous	Curvy	Filiform	Black	Black	Dull	-	-
Gp23	Irregular	Flat	Filiform	White	White	Cottony	+	-
Gp24	Filamentous	Flat	Filiform	Purple	Purple	Smooth	-	-
Gp25	Filamentous	Flat	Filiform	White	White	Smooth	-	-
Gp26	Filamentous	Curvy	Filiform	Pinkish White	Brown	Smooth	+	-

ATCC 29213 (96%), followed by *E. coli* ATCC 25922 (73%), Enteropathogenic *E. coli* K11 (15%), and Methicilin resistant *S. aureus* ATCC 43300 (3%). Gp07 stood out as the most potential isolate that exhibits a wide range of activities antagonistic to all the tested pathogenic bacteria based on the diameter of the clear zone (mm). Also, the isolate Gp07 was the only isolate which exhibited inhibitory activities towards the growth of MRSA, which was the only drug-resistant strain used in this study. The antibacterial activity demonstrated by each fungal isolate was attributable to certain metabolites diffusible in agar plugs and not the mycoparasitism of the isolates. These results may lead to the identification of the anti-MRSA compound and the significance of Gp07 for its production through fermentation. Meanwhile, the discovery of antibacterial compounds, particularly those with special potential as anti-MRSA, has

been reported for endophytic fungi from other sources. *Dothideomycete* sp. isolated from a Thai medicinal plant species, *Tiliacora triandra* showed potential antibacterial activities against both *S. aureus* ATCC 25923 and MRSA ATCC 33591 by producing Dothideomycetide A with minimum inhibitory concentration (MIC) from 128 to 256 µg/mL²⁵. *Pestalotia* sp. isolated from a mangrove medicinal plant species in Bangladesh, *Heritiera fomes*, was reported to produce oxysporone and xylitol that have the anti-MRSA properties with MIC from 32 to 128 µg/mL²⁶. The major portion of antibacterial components produced by Gp07 may be identified in the future for the possibility of finding new compounds to combat bacterial infections.

The identification of isolate Gp07 was based on its macroscopic and microscopic morphological features confirmed with a

Table 2. Antagonistic activity of endophytic fungi isolated from *Globba patens* Miq. rhizome

Isolate Code	Antagonistic activity (mm ± S.D)			
	<i>S. aureus</i> ATCC 29213	MRSA ATCC 43300	<i>E. coli</i> ATCC 25922	Enteropathogenic <i>E. coli</i> K11
Gp01	15.93 ± 2.19	n.d.	27.57 ± 1.72	15.09 ± 2.07
Gp02	16.67 ± 2.03	n.d.	13.63 ± 0.4	n.d.
Gp03	24.30 ± 0.9	n.d.	14.6 ± 1.93	n.d.
Gp04	19.20 ± 2.78	n.d.	14.57 ± 3.19	n.d.
Gp05	20.73 ± 2.73	n.d.	n.d.	n.d.
Gp06	13.40 ± 2.52	n.d.	18.53 ± 0.25	n.d.
Gp07	34 ± 1.22	12.83 ± 1.87	21.1 ± 0.85	18.87 ± 0.92
Gp08	24.97 ± 1.69	n.d.	13.93 ± 0.81	n.d.
Gp09	21.63 ± 0.99	n.d.	12.4 ± 3.63	n.d.
Gp10	18.43 ± 0.91	n.d.	19.6 ± 0.61	n.d.
Gp11	24.8 ± 2.72	n.d.	21.73 ± 0.65	n.d.
Gp12	23.03 ± 5.96	n.d.	17.77 ± 3.41	18.1 ± 0.0
Gp13	18.67 ± 3.19	n.d.	14.07 ± 0.93	n.d.
Gp14	25.77 ± 4.31	n.d.	n.d.	n.d.
Gp15	22.83 ± 5.82	n.d.	15.53 ± 3.19	n.d.
Gp16	22.8 ± 2.72	n.d.	12.73 ± 1.47	n.d.
Gp17	16.53 ± 4.22	n.d.	10.4 ± 1.11	n.d.
Gp18	29.77 ± 0.49	n.d.	9.73 ± 0.5	n.d.
Gp19	31.47 ± 0.55	n.d.	17.77 ± 0.59	n.d.
Gp20	28.6 ± 2.6	n.d.	18.4 ± 1.31	14 ± 2.77
Gp21	22.17 ± 2.99	n.d.	n.d.	n.d.
Gp22	26.8 ± 3.54	n.d.	n.d.	n.d.
Gp23	27.8 ± 0.56	n.d.	14.33 ± 3.07	n.d.
Gp24	31.23 ± 4.7	n.d.	n.d.	n.d.
Gp25	19.67 ± 5.48	n.d.	n.d.	n.d.
Gp26	n.d.	n.d.	n.d.	n.d.

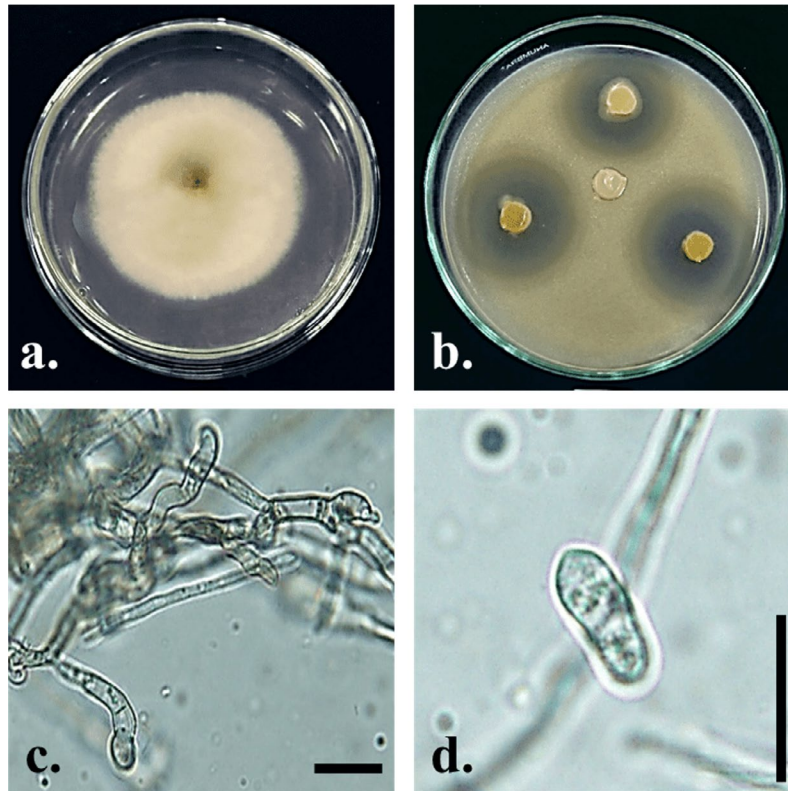


Fig. 2. *Colletotrichum siamense* Gp07. Upper (a) side of culture on PDA 7 days after inoculation; (b) antagonistic activity against *S. aureus* ATCC 29213 on PDA + 1% yeast extract (w/v) 2 days after incubation; (c) conidiogenous cell; (d) conidia; Bars = 10 μ m.

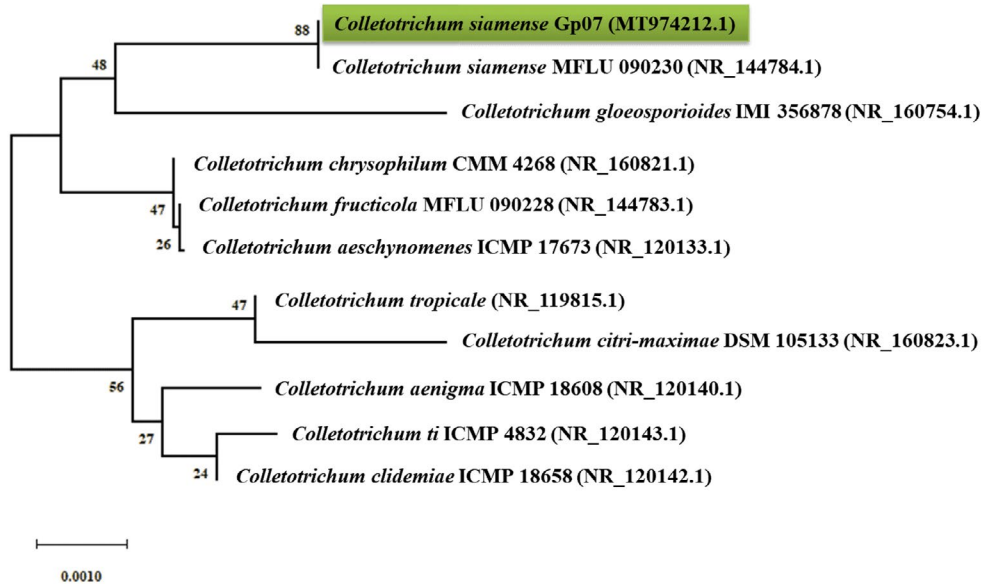


Fig. 3. Neighbor-joining (NJ) phylogram showing phylogenetic relationships between *Colletotrichum siamense* Gp07 and selected species of *Colletotrichum* based on ITS-rDNA sequences retrieved from GenBank. Phylogenetic tree was constructed based on the genetic distance using the Jukes-Cantor method with pairwise deletion of ambiguous positions and 1000 \times bootstrapping.

molecular identification in the region of ITS-rDNA (Fig. 2, 3). Based on the preliminary identification, the isolate Gp07 was preliminarily assigned as the member of *Colletotrichum*, for having the unique morphological characteristics, i.e, colony color, absence of setae, and cylindrical conidia²⁷. However, some discrepancies in morphological features may exist due to the variation of the species and variations across the range of the host species. A phylogenetic tree was then constructed to reveal the species-level identification which assigned the Gp07 position as *C. siamense*, a fungal species described firstly from Thailand. However, no information is available so far about this species, especially the one extracted from Zingiberaceous species. Also, this is the first report from Indonesia on finding *C. siamense* as an endophytic fungus from *G. patens*. The ITS sequence of *C. siamense* Gp07 was then submitted to GenBank and allotted the accession number of MT974212.1. The majority of recent reports on this species are regarding its being found in new host species, mainly as a newly reported phytopathogenic fungus causing anthracnose on various horticultural plants grown in the Southeast Asia region^{28,29}. Antibacterial or bioactive compound-producing *Colletotrichum* strains derived from a range of hosts and origins as endophytes have been documented. Colletotric acid produced by *C. gloeosporioides* from *Artemisia mongolica* in China was reported to inhibit a wide range of human pathogenic bacteria with MIC from 25 to 50 µg/mL³⁰. Two novel compounds, apigenin-8-C-β-D-glucopyranoside, and 2-(hydroxymethylthio) ethanol were identified as produced by an endophytic *Colletotrichum* sp. from Chinese *Ginkgo biloba* through fermentation³¹. However, the presence of *C. siamense* in the rhizome of *G. patens* is not fully understood though it is, possibly, an antibiotics-producing agent of the future.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AL: Concept of the main idea, Developed the supporting theory, Performed the experiment, Writing of manuscript. EM: Verified the analytical methods, Supervised the project. YY: Developed the theoretical formalism, Providing the laboratory necessity. MB: Supervised the findings of this project.

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DATA AVAILABILITY

All datasets generated and analyses are provided in this manuscript. The raw data may be requested upon acceptance by the authors.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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