

Full Length Research Paper

Isolation of fungal endophytes from *Garcinia mangostana* and their antibacterial activity

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The objective of this study is to screen the antibacterial activity of endophytic fungi isolated from surface sterilized leaves and small branches of *Garcinia mangostana* plant found in Indonesia. The crude extracts of ethyl acetate (EtOAc) of the 24 fermentation broths from 24 endophytic fungi were tested for their antibacterial activity against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 14028 and *Micrococcus luteus* (ATCC 10240), by the agar diffusion method. The result showed that 10 of the 24 isolates (41.6%) exhibited antibacterial activity against at least one pathogenic microorganism. Isolate RGM-02 displayed the strongest antibacterial activity against gram-positive bacteria. The minimum inhibitory concentration (MIC) of the crude ethyl acetate extracts of isolate RGM-02, inhibited *S. aureus* (MIC 25 µg/ml), *B. subtilis* (MIC 50 µg/ml), *M. luteus* (MIC 25 µg/ml), *E. coli* (MIC 200 µg/ml), *S. typhi* (MIC 200 µg/ml) and *P. aeruginosa* (MIC 100 µg/ml), respectively. The molecular identification revealed that the isolate RGM-02 represented *Microdiplodia hawaiiensis* CZ315. This study shows that endophytes of ethno medicinal plants could be a good source of antibacterial substances.

Key words: Endophytic fungi, antibacterial agents, *Garcinia mangostana*, molecular identification.

INTRODUCTION

Endophytes are microorganisms that are present in living tissues of various plants, establishing mutual relationship without apparently any symptom of diseases (Strobel and Daisy, 2003). It has been known that endophytic fungi are important sources of bioactive compounds (Schulz et al., 2002; Strobel, 2003; Pan et al., 2008). A number of new bioactive compounds from endophytes have been recognized as potential sources of antimicrobial substances

(Strobel, 2003; Corrado and Rodrigues, 2004; Ezra et al., 2004; Kim et al., 2004; Liu et al., 2004; Wiyakrutta et al., 2004; Atmosukarto et al., 2005; Chomchoen et al., 2005; Li et al., 2005; Phongpaichit et al., 2006).

Garcinia mangostana belong to the family Clusiaceae which is native to Asia, Australia, Africa and Polynesia and has been used as herbal medicine to treat infections (Phongpaichit et al., 2006). In Indonesia, the indigenous communities have been using *G. mangostana* plant in different ways for the treatment of various infectious diseases (Kasaha and Henmi, 1986). However, increasing consumption of *G. mangostana* in recent years, over-exploitation and over-cutting of original plants in Indonesia has caused depletion of the natural resources.

It has been reported that fungal endophytes residing within part of plants could also produce metabolites similar to or with more activity than that of their respective hosts (Strobel, 2002). Therefore, it is believed that search for novel compounds should be directed towards plants

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Abbreviations: PDA, Potato dextrose agar; PDB, potato dextrose broth; EtOAc, ethyl acetate; DMSO, dimethyl sulfoxide; MHA, Mueller-Hinton agar; MICs, minimum inhibitory concentrations; PCR, polymerase chain reaction; BLAST, basic local alignment search tool.

that commonly serve indigenous populations for medicinal purposes as they are expected to harbor novel endophytes that may produce unique metabolites with diversified applications (Strobel and Daisy, 2003).

In Indonesia, however, endophytic fungi in medicinal plants have not been well-studied. In this study, therefore, we isolated and identified fungal endophytes from leaves and small branches of *G. mangostana* to study their diversity and evaluate their antimicrobial activity against human pathogens, since these parts of plants have been used as traditional medicine. The endophytic fungi displayed the strongest antimicrobial activity, and were identified based on the gene encoding for 18 S rRNA.

MATERIALS AND METHODS

Source of endophytic fungi

Plant materials of *G. mangostana* were collected in March 2008 from Bogor Botanical Gardens, Indonesia. Mature healthy plant leaves and small branches were collected from different parts of the *G. mangostana* tree. Samples were transported in plastic bags in a cooler, stored overnight at 4°C and processed on the following day. Plant samples were identified in Center for Plant Conservation, Bogor Botanical Gardens, and the voucher specimens were deposited in the herbarium at the Department of Pharmacy, Faculty of Mathematics and Sciences, University of Indonesia.

Isolation of endophytic fungi

The isolation of endophytic fungi from *G. mangostana* was carried out as described by Strobel et al. (1996) with minor modifications. Briefly, plant samples, which included leaves and small branches were washed under running tap water for 10 min, and then air-dried. Before surface sterilization, leaves and branches were cut into small fragments using sterile surgical blades into 1 cm long segments. Sample fragments were successively surface sterilized by immersion in 70% ethanol for 1 min, 5.25% sodium hypochlorite solution for 5 min, 70% ethanol for 30 s and sterile distilled water for 3 to 5 s. The cut surfaces of the segments were placed on petri dishes containing potato dextrose agar (PDA) (oxid) supplemented with chloramphenicol (50 µg/ml, Merck) and streptomycin sulphate (250 µg/ml, Sigma) to suppress bacterial growth and incubated at 28°C until the outgrowth of endophytic fungi was discerned. Pure cultures were then transferred to PDA plates free of antibiotics and cultivated for 14 days on PDA plates at 28°C.

Extraction and isolation of crude ethyl acetate extracts from fungal fermentation broths

Each of the pure cultures was re-cultivated on PDA at 28°C for 7 to 14 days. Three pieces (0.5 × 0.5 cm²) of mycelia agar plugs were inoculated into 500 ml Erlenmeyer flasks containing 300 ml potato dextrose broth (PDB) and incubated at room temperature for four weeks under stationary conditions. The broth culture was filtered to separate the filtrate and mycelia. The filtrate was extracted three times by shaking with an equal volume of ethyl acetate (EtOAc). The culture broths were pooled and dried in a rotary evaporator (Buchi, Switzerland). The extract residue was dissolved in dimethyl sulfoxide (DMSO), Sigma) and stored at 4°C as stock solution for antimicrobial bioassay.

Antibacterial assay

The crude ethyl acetate extracts of the 24 endophytic fungi were tested for their antibacterial activity against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 14028 and *Micrococcus luteus* (ATCC 10240). Antibacterial activity was determined using the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS), 2003. Pre-warmed Mueller-Hinton agar (MHA) (oxid) plates were seeded with 10⁶ CFU suspension of test bacteria. Endophytic extracts dissolved in DMSO (1 mg/ml) were pipetted (20 µl) onto sterile paper discs (6 mm diameter, oxid) and placed onto the surface of inoculated agar plates. Plates were incubated at 37°C for 24 h. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts. DMSO was used as negative control.

Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) of crude ethyl acetate extracts which displayed the strongest antibacterial activity was determined by agar micro dilution according to NCCLS (National Committee for Clinical Laboratory Standards, 2004), with slight modifications. The MIC assay was determined by the 2-fold micro titer broth dilution method in sterile 96-well plate. Overnight culture of each test organisms (approximately 10⁶ CFU) was seeded into the wells and the crude metabolites was tested at concentration from 400 to 3.125 µg/ml. The plates were incubated for 24 h at 37°C. MIC was determined as the least concentration of the crude metabolites that inhibited the growth of the test organisms. Amoxicillin (Brataco Chemical) was used as positive control.

Molecular identification of endophytic fungus RGM-02

Molecular identification of isolate RGM-02 was conducted by analyzing the endophytic fungus 18S ribosome RNA sequence using polymerase chain reaction (PCR) cloning technology. Genomic DNA was extracted from fungal mycelia using PrepMAN Ultra Sample Preparation Reagent kit according to the manufacturer's recommendation (Applied Biosystem). A pair of primer NS1 (5'GTAGTCATATGCTTGCTCTC 3') [Qiagen] and NS4 (5'CTTCCG-TCAATTCCTTTAAG 3') [Qiagen] were used to amplify the highly specific for endophytic fungi targeting the gene encoding for 18S rRNA (Wu et al., 2002). PCR was carried out in a programmable thermal controller (MJ Mini Biorad). PCR reaction mixture (2.5 µl) contained 10 µl template DNA, 12.5 µl PCR Master Mix (PCR buffer, 4 mM MgCl₂, 0.4 mM of each dNTP, 0.05U/µl Taq polymerase) [Fermentas], 1 µl of each primer (NS1 and NS4) and ddH₂O to make up the volume. The amplification was performed for 30 cycles with 1 min at 94°C, 1 min at 45°C and 2 min at 72°C. After the final cycle, the amplification was extended for 10 at 72°C. The amplified DNA fragment (approximately 1100 bp) was purified and was sequenced by Genetic Analyzer [AB3130]. The analysis and comparison of the sequence were performed with nucleotide Basic Local Alignment Search Tool (BLAST) of GenBank (<http://www.ncbi.nlm.nih.gov>). The phylogenetic tree was produced using BLAST pair wise alignments.

RESULTS AND DISCUSSION

Screening of endophytic fungi for antibacterial activity

During this study, 24 fungal isolates were recovered from *G.*

Table 1. The inhibition zone (diameter, mm) of the crude ethyl acetate extracts of 24 endophytic fungi from *G. mangostana*.

Code of isolate**	Diameter of inhibition zone (mm)*					
	<i>E. coli</i>	<i>S. typhi</i>	<i>P. aeruginosa</i>	<i>M. luteus</i>	<i>S. aureus</i>	<i>B. subtilis</i>
RGM-01	0	0	0	0	0	0
RGM-02	6.6 ± 0.5	6.5 ± 0.2	7.2 ± 0.4	13.5 ± 0.5	12.9 ± 0.7	14.7 ± 1.5
RGM-03	0	0	0	0	0	0
RGM-04	0	0	0	0	0	0
RGM-05	0	0	0	0	6.5 ± 0.1	7.2 ± 0.6
RGM-06	0	0	6.5 ± 0.4	6.5 ± 0.6	0	0
RGM-07	6.5 ± 0.2	0	0	0	0	0
RGM-08	0	0	0	0	0	0
RGM-09	0	0	0	0	0	0
RGM-10	0	0	0	0	6.5 ± 0.6	6.5 ± 0.4
RGM-11	0	0	0	0	0	0
RGM-12	0	0	0	0	0	0
RGM-13	0	6.7 ± 0.2	0	0	0	6.5 ± 0.1
RGM-14	0	0	0	0	0	0
RGM-15	0	0	0	0	0	0
RGM-16	0	0	0	0	0	0
RGM-17	0	0	0	0	0	0
RGM-18	0	0	0	0	0	6.6 ± 0.3
RGM-19	0	0	6.5 ± 0.5	6.7 ± 0.3	6.5 ± 0.4	6.5 ± 0.2
DGM-01	0	0	0	0	0	0
DGM-02	0	0	0	6.8 ± 0.1	6.5 ± 0.1	0
DGM-03	0	0	0	0	6.5 ± 0.6	6.5 ± 0.1
DGM-04	0	0	0	0	0	0
DGM-05	6.5 ± 0.3	0	6.5 ± 0.1	7.2 ± 0.1	7.6 ± 0.2	0
DMSO	0	0	0	0	0	0

* Values are means of three replicates ± standard deviation; ** RGM = small branches of *G. mangostana*; DGM = leaves of *G. mangostana*; DMSO was used as negative control.

Table 2. The minimum inhibitory concentration (MIC, µg/ml) of the crude ethyl acetate metabolites produced by isolate RGM-02 which displayed the stronger antibacterial activity.

Test organism	MIC (µg/ml)	
	Metabolite produce by isolate RGM-02	Amoxicillin
<i>E. coli</i>	200	50
<i>S. typhi</i>	200	25
<i>P. aeruginosa</i>	100	12.50
<i>M. luteus</i>	25	12.50
<i>S. aureus</i>	25	6.25
<i>B. subtilis</i>	50	6.25

mangostana plant. Out of 24 isolates, 10 isolates (41.6%) could inhibit some tested human pathogenic bacteria used in this study. Each of them displayed antimicrobial activity against at least one test microorganism with inhibition zones that ranged from 6.5 to 14.7 mm, as shown in Table 1. More than half of the active isolates inhibited strains of gram-positive bacteria better than gram-negative

bacteria. Only 8.3% inhibited *S. typhi* and 12.5% inhibited *E. coli* and *P. aeruginosa*. 70% of the active isolates were isolated from leaves and 30% were from small branches, and the stronger antibacterial activity against selected bacteria was displayed by isolate RGM-02. The inhibition zones against *B. subtilis*, *S. aureus*, and *M. luteus* were 14.7, 12.9 and 13.5 mm, respectively (Table 1). Determination of the MIC of fungal crude ethyl acetate extract from isolate RGM-02 that showed inhibitory activity in the preliminary screening test was assayed for their MIC by dilution methods. The MIC values of crude ethyl acetate extract from isolate RGM-02 is shown in Table 2. The result showed that fungal crude ethyl acetate extract inhibited gram-positive than gram-negatives bacteria.

The antibacterial activity of bioactive compound produced by isolates RGM-02 is comparable with amoxicillin as standard antibiotic. Interestingly, the ethyl acetate extract from isolate RGM-02 had MIC values of 25 µg/ml against *M. luteus* and 25 µg/ml against *S. aureus*, which was only two and four times higher than that of amoxicillin (MIC 12.5 and 6.25 µg/ml), respectively (Table 2). This RGM-02 isolate could be good candidates for further

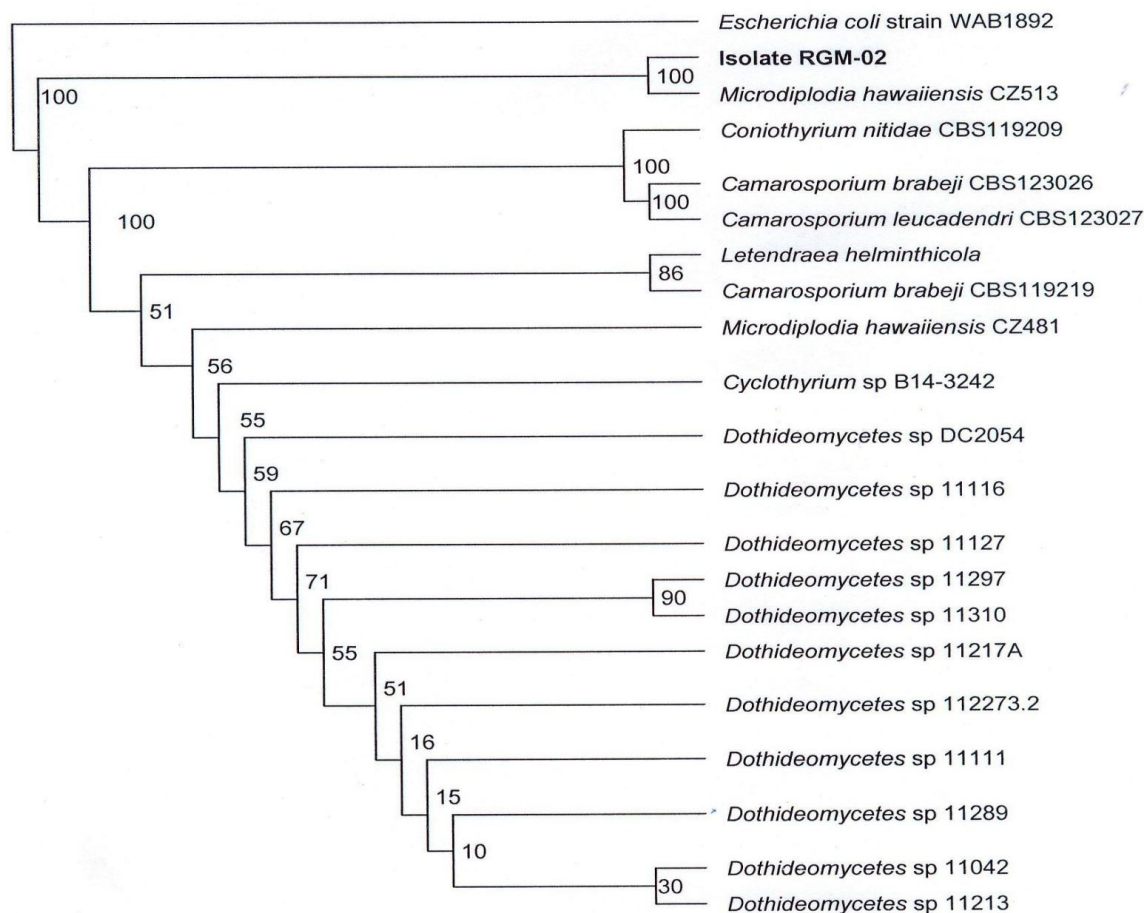


Figure 1. Phylogenetic tree based on 18S rRNA sequence, showing the relationship between fungal endophytic isolate from *G. mangostana* (isolate RGM-02) and reference strains.

studies of their antibacterial bioactive compounds. This result is similar to those of Phongpaichit et al. (2006) in which some endophytic fungi were isolated from *Garcinia* species and 18.6% displayed antimicrobial activity against at least one test microorganism with inhibition zones that ranged from 7 to 19 mm.

Antimicrobial activities of plant endophytic fungi have also been reported by several groups (Rodrigues et al., 2000; Guimaraes et al., 2008; Ghadin et al., 2008; Xu et al., 2008; Hazalin et al., 2009; Li and Guo, 2009). Guimaraes et al. (2008) screened extracts from 39 endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia*, and reported 5.1% active extracts against *S. aureus* and 25.6% active extracts against *E. coli*. An extract of *Streptomyces* sp. (SUK 06) isolated from the stem of a Malaysian plant was found to be as effective as oxacillin against *B. subtilis* (Ghadin et al., 2008).

Identification of endophytic fungus RGM-02

The identification of fungi usually has been based on the characteristic of their macroscopic and microscopic

morphology. However, molecular analysis of fungal rDNA at the sequence level provides a powerful technique for assessing fungal diversity. The use of molecular tools aided in rapid identification of cultured fungi to the genus and strain level. In this study, fungal molecular identification of the most active isolate RGM-02, based on 18S ribosome RNA sequence analysis revealed that isolate RGM-02 was identified as *Microdiplodia hawaiiensis* CZ315. The percentage of identity was found to be 98%. Phylogenetic tree analysis indicated that 18S ribosome RNA sequence of RGM-02 strain is closely related to *M. hawaiiensis* CZ315 (Figure 1). With regard to the results of antibacterial activity of *M. hawaiiensis* CZ315 isolated from *G. mangostana* which displayed the strong antibacterial activity against both gram-positive and negative bacteria, it is clear that *M. hawaiiensis* CZ315 could produce some metabolites which are active against tested bacteria.

Further investigation of bioactive compounds produced by *M. hawaiiensis* CZ315 is important to be characterized since the strain obtained in this study is a potential source for exploring novel bioactive metabolites with a high activity against pathogenic bacteria. Taken together,

this study reinforced the assumption that endophytes of ethno medicinal plants could be a promising source of antimicrobial substances.

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