

Antimicrobial activities and detection of crude extract derived from *Magnolia garrettii* inhabiting fungi

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ABSTRACT

One hundred and fifty-three isolates of endophytic and saprobic fungi from healthy-living and decayed-fallen branches of *Magnolia garrettii* were examined in order to find their antimicrobial potentials. Fungal isolates were grown in five different culture media (F1, F3, F4, F5 and PDB). The antimicrobial activity of their culture filtrate was determined using paper disc diffusion assay against *Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus* and *Pectobacterium* sp. Nineteen culture filtrates (17 endophytes and two saprobes) exhibited antimicrobial activity towards at least one of the test organisms, producing a 7-18 mm clear zone. No culture filtrate was found to suppress *Pectobacterium* sp. Endophytic isolate MG5/2.4-1 and saprobic isolate 1MG9001 (*Dichotomopilus* sp.) were grown in PDB and extracted with ethyl acetate. Chemical constituents of the culture filtrate extracts were determined by thin layer chromatography and found to contain flavonoids, steroids, terpenoids, aldehydes, ketones, unsaturated compounds and alcohols. Mycelial crude extracts were made largely of terpenoids, unsaturated compounds and alcohols. Culture filtrate extracts showed higher antimicrobial efficiency against *B. cereus*, *E. coli* and *S. aureus* than did the mycelial extracts and, in the case of *S. aureus*, even greater than chloramphenicol. Morphological and molecular data confirmed the identification of isolate 1MG9001 as *Dichotomopilus ramosissimum*. Based on molecular data, isolate MG5/2.4-1 is a species belongs to the genus *Diaporthe*.

Key words: bioactive compounds, endophytes, saprobes, taxonomy

INTRODUCTION

Fungi are widely accepted and used in several fields and applications for welfare of mankind. In Thailand, research relating to fungal diversity and utilization of fungi is increasing (e.g. Supaphon et al. 2013, Kaewnarin et al. 2016, Sangdee et al. 2017, Suwannarach et al. 2017) and many new fungi and fungal bioactive compounds have been described and reported (Mahidol et al. 2014, Pudhom et al. 2014). However, it is evident that many Thai fungi are still awaiting discovery and utilization.

There is an increasing need for new bioactive compounds in order to overcome various medical-related problems i.e. drug resistance of pathogens,

emergence of new pathogenic microorganisms, reoccurrence of disease in organ-transplant patient, human-fungal infection and food shortage crisis (Moore & Chiu 2001, Peláez & Genilloud 2003, Peláez 2004, 2006, Strobel et al. 2004, Monciardini et al. 2014). Fungi are a potential solution to these problems as they can be used as food and source of new bioactive compounds. However, in order to find potential candidates, numerous fungal strains are needed for screening purposes.

Fungal isolates obtained from plants, especially those in tropical region where there is high diversity, have proven to be a rich source of fungal bioactive compounds (Arnold et al. 2000, Hyde 2000). Previous reports have shown that endophytic fungi living in medicinal plants are a good source of new bioactive compounds, as they may instigate biosynthesis of bioactive compounds from their host plants (Fisher et al. 1984, Bills & Polishook 1992, Bruner & Petrini 1992). For example, the endophytic fungus *Taxomyces andreanae* isolated from Pacific yew (*Taxus brevifolia* Nutt.), was able to produce paclitaxel (taxol; terpenoid group) (Stierle et al. 1993, Pulici et al. 1996a, b). More examples are mentioned in Zhao et al. (2010) who reviewed and discussed endophytic fungi that are able to produce paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin and diosgenin, bioactives that were also produced by the host plants.

Magnoliaceae are a family of medicinal plants widely used in China, Vietnam and Japan (Chalermklin 2002, Watanabe et al 2002). Plants in this family, especially the genus *Magnolia*, have been used as a sedative drug, anticonvulsant, skeletal muscle relaxant and relief of gastrointestinal problems caused by mental stress (Watanabe et al. 2002), lungs and bronchial systems diseases, abdominal pain, cough and dyspnea (Toriizuka 2002). This plant family comprises mainly ancient plants species, which possess less ability in adaptation in comparison with other plant families, causing extinction risk (Chalermklin 2002). According to the ancient age of the host plants, the fungi living in these plants are expected to be diverse, and since this plant family is vulnerable to extinction so study on its fungi is urgently needed. Wiyakrutta et al. (2004) found that some endophytic fungi from a magnoliaceous plant *Manglietia garrettii* (\equiv *Magnolia garrettii*) provided anti-*Mycobacterium tuberculosis* activity. The present study aimed to evaluate the antimicrobial activity of endophytic fungi inhabiting *M. garrettii* against *Bacillus cereus*, *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus* and *Pectobacterium* sp., to identify bioactive compounds produced by these fungi and to identify the active fungal isolates to various taxonomic levels.

MATERIALS AND METHODS

Isolation of endophytic and saprobic fungi from *M. garrettii*

Endophytic and saprobic fungal isolates used in this study were obtained from a previous study (Kodsueb 2012). Briefly, endophytic fungi were isolated from healthy branches of *Magnolia garrettii* (Craib) V.S. Kumar (synonym: *Manglietia garrettii* Craib) via triple surface sterilization method on half strength potato dextrose agar (½ PDA) following the method of Wiyakrutta et al. (2004). Saprobian fungi were directly isolated from wood specimens using single spore isolation technique on water agar (WA), modified from Choi et al. (1996). Fungal isolates were preserved in mineral oil for further use.

Test organism strains

Bacillus cereus, *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* were obtained from Microbiology Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok, Thailand. *Pectobacterium* sp. was isolated from diseased tissues of sugarcane collected from the field and its typical characteristics were confirmed by biochemical tests.

Antimicrobial activity screening

Endophytic and saprobic fungi were revived from stock cultures and grown on PDA for a few days in order to examine their viability and purity. Five-day old pure cultures of each isolate were then inoculated into 22 ml test tubes containing 10 ml of each culture media (PDB; Himedia, F1, F3, F4 and F5: Cheeptham et al. 1999) and incubated at 28±2 °C for 6 days on a rotary shaker at 130 rotations per minute (rpm). The culture filtrates were then centrifuged at 11,000 g for 5 mins to obtain a supernatant for antimicrobial assay. Agar disc diffusion assay was carried out and modified following the protocol of Bauer et al. (1966). Firstly, bacteria and yeast were cultured in nutrient broth (NB) and yeast malt broth (YMB; Himedia) at 37 °C for 24 hours prior to adjustment of final concentration of the inoculum to approximately 10⁸ CFU/ml. The prepared inoculum was then swabbed on petri dishes containing Mueller Hinton agar (MHA; Himedia). Six mm diameter paper discs were placed on the seeded plate and loaded with 10 µl of culture filtrate. The plates were incubated at 37 °C for 24 hours. The antimicrobial activity of culture filtrates was determined in terms of zone of inhibition (ZOI). The diameter of inhibition zone was measured and photographed. Chloramphenicol and sterile distilled water were used as positive and negative controls, respectively.

Ethyl acetate extraction of fungal mycelia and culture filtrate

Two fungal isolates that actively inhibited the test organisms, representing an endophyte (MG5/2.4-1) and a saprobe (1MG9001) were selected for identification of bioactive compounds. They were inoculated into 1000 ml Erlenmeyer flasks containing 500 ml PDB, and incubated at 28 ± 2 °C on a rotary shaker at 130 rpm. After 6 days, the medium containing the fungi was mixed thoroughly and filtered through cotton cheesecloth and Whatman no. 1 filter paper to separate fungal mycelia from the culture filtrate. The culture filtrates were extracted three times with ethyl acetate (250 ml) at 28 ± 2 °C by partitioning in a separating funnel. The fungal mycelium was extracted by adding 150 ml of ethyl acetate to 500 ml Erlenmeyer flasks containing the mycelium and shaking at 160 rpm at 28 ± 2 °C for 3 days before finally filtering through cotton cheesecloth. The ethyl acetate extracts from each fungal mycelium and culture filtrate was evaporated in order to eliminate remaining solvent on a rotary evaporator at 40 °C (Büchi Rotavapor R-124, Switzerland). One hundred µl of crude extracts was used in TLC test, and the remaining was kept at 4 °C until further use. The dried crude extract was later dissolved with DMSO and used for testing antimicrobial activity via paper disc diffusion assay method.

Antimicrobial activity evaluation of crude extracts via paper disc diffusion assay against *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*

Antimicrobial activity of crude extracts dissolved with DMSO was examined as mentioned above. In short, 6 mm diam. paper discs were placed on seeded plates. Crude extract (10 µl, concentration 10 mg/ml) was applied to each disc. The plates were incubated at 37 °C for 24 hours before antimicrobial activity was determined by measuring the inhibition zone. Chloramphenicol (concentration 12.5 mg/ml) and sterile distilled water were used as positive and negative control, respectively. All treatments were done with four replicates. The results were statistically analyzed by analysis of variance (ANOVA) and the significance of the difference between the mean of the control and treated groups was determined at $P < 0.05$.

Chemical evaluation of crude extracts via thin layer chromatography (TLC)

Crude extracts (20 µl) was separately applied on 1.5 cm × 5 cm pre-coated chromatographic silica gel 60 F₂₅₄ plates (Merck, Germany). The TLC plates were developed through a glass chamber containing a mixture of n-hexane and ethyl acetate (2.5:1 v/v) as the mobile phase. The plates were removed when the solvent front had moved 4.5 cm from the spot origin

(sample line) and allowed to dry at room temperature. The spots on the developed plates were visualised under visible light (white), short UV (254 nm) and long UV (366 nm) light. For post-derivatization test, the plates were sprayed with a series of chemicals to detect specific compounds, including anisaldehyde for metabolic carbohydrate, permanganate for unsaturated and alcoholic compounds, 2,4-dinitrophenylhydrazine for aldehyde and ketone, aluminium chloride for flavonoid, Dragendorff reagent for alkaloid, and a mixture of p- anisaldehyde/sulfuric acid/acetic acid to detect steroid and terpenoid. The plates were dried at room temperature before visualization. The compound composition of each separating spot of the extract was marked, noted and photographed.

Morphological and molecular identification of isolate MG5/2.4-1 and 1MG9001

The saprobic fungus 1MG9001 was determined morphologically as *Chaetomium* sp. (Kodsueb 2012). The living culture of MG5/2.4-1 and 1MG9001 were examined using 20-day-old colony on PDA, mounted in water and observed using a compound microscope. For molecular identification, genomic DNA was extracted from the living culture following the method of Utashima (2009) with minor modifications. In detail, fungal mycelium from a 7–10-day old living culture was sampled using fine forceps and put in an Eppendorf tube containing 100 µl TE buffer. The tube was incubated at 95 °C for 20 mins before centrifuging at 12,000 g for 5 mins. The collected supernatant was subject to PCR using Tks Gflex™ DNA Polymerase (Takara, Japan) and the primer pairs ITS1/ITS4 (White et al. 1990) for amplifying internal transcribed spacer (ITS) region 1 and 2 and the 5.8S rDNA. The PCR reactions were carried out on a Bio-Rad T100™ thermal cycler, following Kodsueb et al. (2007), with slight modifications as follows: initial denaturation at 94 °C for 3 mins, followed by 30 cycles of denaturation 98 °C for 10 secs, annealing 52 °C for 15 secs and extension 72 °C for 1 min, and final extension at 72 °C for 3 mins. PCR products were purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) before sequencing by FASMAC (Japan). The newly generated sequences were searched against GenBank non-redundancy nucleotide database using BLASTn (Altschul et al. 1997) to find the most similar sequences. The sequences generated in this study were deposited in GenBank nucleotide archive under accession numbers MF538625, MF538626.

RESULTS

Antimicrobial activity of culture filtrates detected from paper disc diffusion assay

A total of 153 isolates of selected fungi were grown in five different culture media and tested for their antimicrobial production against five test organisms. Culture filtrates of 19 isolates were active towards at least one of the test organisms. However, none of the culture filtrates was able to inhibit the growth of *Pectobacterium* sp. (Table 1). Of 19 active isolates, 17 isolates were endophytic fungi and two isolates were saprobic fungi (1MG9001 and 5MG1002). The culture filtrates of 11 isolates were active against the growth of *B. cereus* producing a 9-18 mm clear zone. Culture filtrates of 2 isolates inhibited the growth of *C. albicans* (10-15 mm clear zone), 3 isolates inhibited *E. coli* (7-12 mm clear zone) and 3 isolates inhibited *S. aureus* (9-18 mm clear zone) (Table 1).

Ethyl acetate extraction of culture filtrate and fungal mycelia

Ethyl acetate was used to extract bioactive compounds in the culture filtrates of MG5/2.4-1 and 1MG9001. The ethyl acetate layer was dark brown with some precipitation, which turned into a dark and brownish sticky sludge after evaporation. In the case of fungal mycelia extraction, MG5/2.4-1 ethyl acetate layer was pale brown, while the dried crude extract was precipitated and changed to brown after evaporation. Isolate 1MG9001 ethyl acetate layer was yellow, however, the crude extract became dark brown and sticky after evaporation of the solvent (Table 2). The dry weight of crude extracts of culture filtrate and fungal mycelia of MG5/2.4-1 and 1MG9001 were 0.41, 0.40, ≤ 0.01 and 0.04 g, respectively (Table 2).

Table 1 Antimicrobial activity in paper disc diffusion assay

Isolate	Occurrence of clear zone on test organisms (mm)																								
	<i>Bacillus cereus</i>					<i>Candida albicans</i>					<i>Pectobacterium sp.</i>					<i>Escherichia coli</i>					<i>Staphylococcus aureus</i>				
	PDB	F1	F3	F4	F5	PDB	F1	F3	F4	F5	PDB	F1	F3	F4	F5	PDB	F1	F3	F4	F5	PDB	F1	F3	F4	F5
MG6/1.4-2	-	-	-	-	-	-	-	14	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-
MG5/2.4-1	-	-	-	-	14	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-
MG1/6.5-1	-	-	-	-	18	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-
MG1/2.1-1	-	-	-	-	15	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-
WMG1/5.3-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	10	-	-	-	-
MG4/2.2-1	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	9	8	9	10	8	-	-	-	-	-
1MG9001	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	8	10	7	12	-	-	-	-	-	-
MG7/1.3-1	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	10	8	9	9	8	-	-	-	-	-
WMG5/1.3-1	-	-	-	-	-	15	10	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-
MG2/6.4-2	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	11	-	-
MG6/2.2-2	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	9	-	18	-	12
MG2/4.2-1	-	-	15	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-
5MG1002	-	-	-	10	15	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-
MG7/1.2-1	-	12	10	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-
MG6/3.3-1	-	-	-	-	10	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-
MG2/6.1-3	10	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-
MG6/1.5-1	-	-	-	10	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-
MG3/2.4-2	-	-	-	10	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-
MG 1/6.1-4	-	-	-	9	-	-	-	-	-	-	nd	nd	nd	nd	nd	-	-	-	-	-	-	-	-	-	-

Note: - = no inhibition, nd = not determined

Table 2 Appearance and mass of crude extract obtained from culture filtrate and fungal mycelia.

Isolate no.	Crude obtained	Solvent	Crude appearance	Weight (g)
MG5/2.4-1	Culture filtrate	Ethyl acetate	sticky, dark brown	0.41
MG5/2.4-1	Mycelia	Ethyl acetate	precipitate, brown	≤0.01
1MG9001	Culture filtrate	Ethyl acetate	sticky, dark brown	0.4
1MG9001	Mycelia	Ethyl acetate	sticky, dark brown	0.04

Antimicrobial activity of crude extracts detected from paper disc diffusion assay

Re-examination of antimicrobial activity of ethyl acetate crude extracts against *B. cereus*, *E. coli* and *S. aureus* revealed that four extracts possess promising antibacterial properties against three bacteria (Figure 1). The crude extracts from the culture filtrate (sample no. 1 and 2) inhibited growth of the test organisms better than the crude extracts from the fungal mycelia (sample no. 3 and 4). Comparison of antimicrobial activity between crude extracts and chloramphenicol showed that in the case of *B. cereus* and *E. coli* chloramphenicol was much more active against the growth of these two bacteria. On the other hand, culture filtrate crude extract of both isolates provided greater inhibition against *S. aureus* than chloramphenicol (Table 3).

Statistical analyses using one-way ANOVA and Duncan's multiple comparisons were tested. In the case of *B. cereus*, inhibition could be divided into three significantly different groups. Group 1 is composed of culture filtrate crude extracts 1 and 2 and group 2 is composed of mycelial crude extracts 3 and 4 while group 3 was chloramphenicol assigned as the positive control (Table 3). Inhibition activity against *E. coli* showed that crude extract 1 provided the same inhibition activity compared to chloramphenicol with no significance. Crude extracts 2, 3 and 4 provided significantly less inhibition activity compared to chloramphenicol (Table 3). In the case of *S. aureus*, inhibition activity of fungal filtrate crude extracts 1 and 2 were significantly different from mycelial crude extract 4 but they were not significantly different from chloramphenicol and mycelial crude extract 3.

Table 3 Zone of inhibition of crude extracts against the growth of *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*

Sample	Clear zone on test organisms (mm)		
	<i>B. cereus</i>	<i>E. coli</i>	<i>S. aureus</i>
Chloramphenicol	13.54 ± 0.37 ^a	14.18 ± 0.13 ^a	20.42 ± 2.36 ^{ab}
MG5/2.4-1, filtrate	9.64 ± 1.17 ^b	11.25 ± 1.63 ^{ab}	22.66 ± 1.74 ^a
1MG9001, filtrate	8.58 ± 0.78 ^b	9.88 ± 0.72 ^b	23.27 ± 1.21 ^a
1MG9001, mycelia	6.16 ± 0.64 ^c	9.42 ± 2.64 ^b	17.67 ± 1.04 ^{bc}
MG5/2.4-1, mycelia	6.62 ± 1.04 ^c	8.37 ± 0.58 ^b	16.62 ± 1.16 ^c

* Mean ± SD of four replications. Different letters in the same column indicate significant differences according to Duncan's test ($p \leq 0.05$).

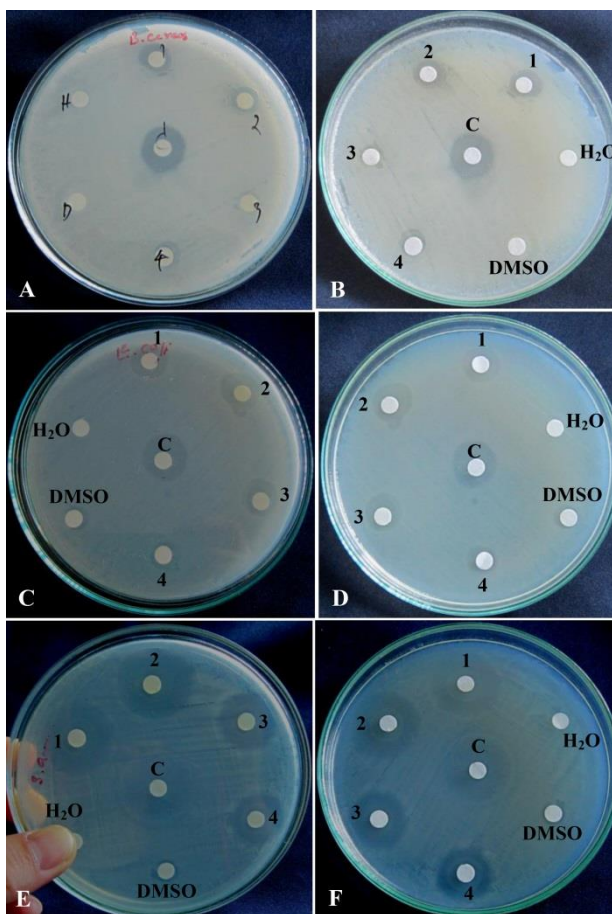


Figure 1 Zone of inhibition of crude extracts against the growth of test organisms. A-B. against *B. cereus*. C-D. against *E. coli*. E-F. against *S. aureus*. Notes: 1 = crude extract of culture filtrate of MG5/2.4-1, 2 = crude extract of culture filtrate of 1MG9001, 3 = crude extract of fungal mycelia of 1MG9001, 4 = crude extract of fungal mycelia of MG5/2.4-1, C = chloramphenicol (positive control).

Chemical evaluation of crude extracts obtained from TLC

Preliminary investigation on the composition of crude extracts by TLC suggested that the crude extracts of both fungal isolates were composed of different compound groups, including those that illuminated under UV light at 254 nm and 366 nm and those provided different colors when reacted with anisaldehyde. Previous observation, prior to the tests with specific reagents, showed that compositions of the crude extracts obtained from both culture filtrates (crude extracts 1 and 2) were not significantly different. It is likely that the culture filtrate crude extracts were composed of ≥ 5 compounds. The crude extracts obtained from the fungal mycelia (crude extracts 3 and 4) exhibited totally different composition in comparison with the culture filtrate crude extracts. The TLC plate detecting with anisaldehyde showed variations in color spots from each crude extract. Further examination of the culture filtrate crude extracts with aluminium chloride gave an orange spot, indicating the presence of flavonoids. On the other hand, there was no flavonoid in fungal mycelial crude extract as no orange spot was seen on the TLC plate.

Post derivatization with 2,4 DNP for aldehyde/ketone detection showed an orange to reddish orange spot occurred on the plate of culture filtrate crude extract, suggesting presence of aldehyde/ketone (which carbonyl functional group included) in the crude extract. On the other hand, there was no orange/reddish orange band when the fungal mycelia crude extracts were tested. We found post derivatization with permanganate returning an orange spot in all of the crude extracts. This suggested all of the tested crude extracts contained unsaturated compound/alcohol. The TLC plates detected with p-anisaldehyde/sulfuric acid/acetic acid (steroid and terpenoid detecting reagent; steroid = red, terpenoid = green/blue) exhibited both red and green bands on TLC plate of culture filtrate crude extract whereas only blue bands on TLC plate were visualized when the fungal mycelial crude extracts were introduced. These indicated the presence of steroids and terpenoids in the culture filtrates, but only terpenoids in the mycelial crude extracts. The treatment of the culture filtrates and the mycelial crude extracts with Dragendorff showed no orange spot in all cases, suggesting that there was no alkaloid in either extract. According to results from all post derivatization detected using specific reagents, we concluded that the culture filtrate crude extracts contained flavonoids, steroids, terpenoids, aldehydes, ketones, unsaturated compounds and alcohols, but the fungal mycelial crude extracts contained only terpenoids, unsaturated compounds and alcohols (Table 4).

Table 4 Chemical evaluation of crude extracts.

Crude extract	Alkaloids	Flavonoids	Steroids	Terpenoids	Aldehydes and ketones	Unsaturated compounds and alcohols
MG5/2.4-1, filtrate	-	+	+	+	+	+
1MG9001, filtrate	-	+	+	+	+	+
1MG9001, mycelia	-	-	-	+	-	+
MG5/2.4-1, mycelia	-	-	-	+	-	+

Note: + = detected - = not detected

Identification of isolate MG5/2.4-1 and 1MG9001

The morphological characteristics of MG5/2.4-1 and 1MG9001 are illustrated (Figure 2). Mycelia of the living culture of 1MG9001 was yellowish cream, reverse side pale brown, perithecia produced in culture when old. Perithecia superficial, solitary, ostiolate, hairy, dark brown to black, with dichotomously branched terminal ascomatal hairs. Lateral hairs seta-like, tapering, septate; asci not observed; ascospores limoniform, slightly apiculate at the ends, hyaline when young, pale bluish grey to dark brown when mature, (6-) 6.5 (-7.5) × (4.5-) 5 (-6) μm. In the case of isolate MG5/2.4-1, entire colony whitish cream on surface, fluffy, little bit zonation with sparse and crenate margin, reverse center brown. No fruiting bodies or spores produced.

We performed nucleotide BLASTn against NCBI's GenBank non-redundancy nucleotide database and found that, for isolate 1MG9001, the closest hit was *Chaetomium ramosissimum* (≡ *Dichotomopilus ramosissimum*) (GenBank KC109759 and GU563371, Identities = 546/546 (100%), Gaps = 0/546 (0%), deposited from China), while isolate MG5/2.4-1 returned *Diaporthe* sp. G25 (GenBank EF432257, Identities = 568/583 (97%), Gaps = 4/583 (0%), deposited from USA) for best hit.

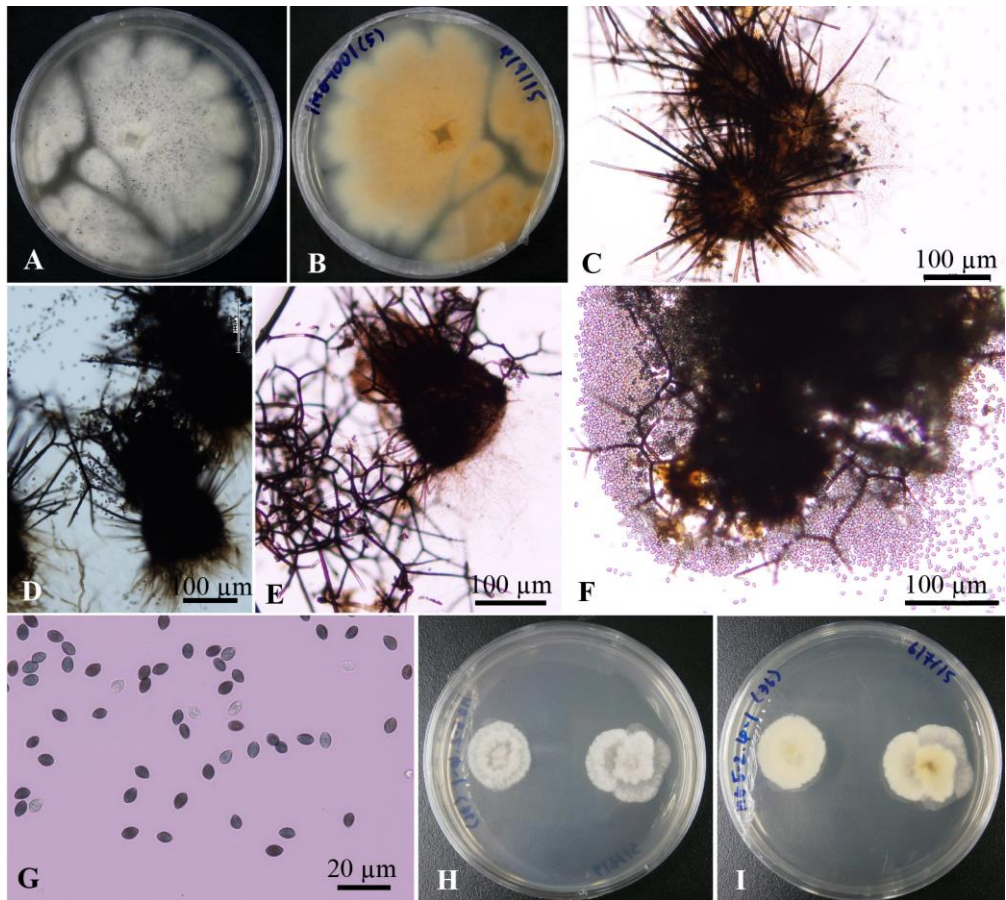


Figure 2 Morphological characters of 1MG9001 and MG5/2.4-1. A-G. *Dichotomopilus ramosissimum* 1MG9001. A-B. colony morphology on PDA from above (day 20). B. colony morphology on PDA from below (day 20). C-F. perithecia with dichotomously branched terminal ascomatal hairs and setae lateral hairs. G. ascospores. H-I. *Diaporthe* sp. MG5/2.4-1. H. colony morphology on PDA from above (day 3). I. colony morphology on PDA from below (day 3).

DISCUSSION

Several fungi are known to produce a plethora of secondary metabolites. In medicine, many fungal metabolites have been widely used as antibiotics to save thousands of lives from deadly diseases. Nevertheless, misuse of antibiotics has progressively caused bacterial pathogens to be less sensitive to the antibiotics. Recently, multiple drug resistance has become a global problem, leading to an urgent need for new antibiotics.

In this study, fungal endophytes and saprobes were isolated from the medicinal plant *M. garrettii* and were examined for their production of antimicrobial compounds. Although morphology was used to identify the isolated saprobes, it was not possible to use the method to identify all of the obtained endophytes as many did not produce spores. Mycelia sterilia are the frequently isolated group of fungal endophytes (Shankar & Shashikala 2010). Due to a lack of unique morphological features, molecular tools are thus required for species identification of sterile fungi (Ghimire & Hyde 2004).

We found that the culture filtrates from 19 out of 153 isolates could suppress the test organisms (ZOI less than 20 mm). This indicated the potentials of bioprospecting in fungal microbes associated with *M. garrettii*. In fact, medicinal plants are known to accommodate a great number of fungi producing antimicrobial compounds (Selim et al. 2012). Antibacterial activity of the endophytic fungi from the medicinal herb, *Macleaya cordata* was reported by Shan et al. (2012) where crude extracts of most selected endophytic fungi provided strong inhibition against *Agrobacterium tumefaciens*, *B. subtilis*, *E. coli*, *Pseudomonas lachrymans*, *S. aureus*, *S. haemolyticus*, *Salmonella typhimurium* and *Xanthomonas vesicatoria*. Likewise, Zhang et al. (2013) found that *Phoma* sp. PG23 isolated from the leaves of a Chinese medicinal plant *Taraxacum mongolicum* Hand.-Mazz. displayed antagonism against some pathogenic bacteria with strong antimicrobial activity, while Sharma et al. (2016) demonstrated that crude extract from leaves of *Cupressus torulosa* was able to eradicate *B. subtilis*, *S. aureus*, *E. coli* and *S. typhimurium*. These discoveries suggest great potential for novel antimicrobial discoveries from medicinal plants.

Filamentous fungi generally produce many secondary metabolites, such as polyketides, terpenoids, non-ribosomal peptides, and alkaloids or mixtures of these. Fungal secondary metabolites could also derive from shikimate or nucleotides (Frisvad 2012). In this study, terpenoids were present in both culture filtrates and mycelial crude extracts. Reports from previous studies showed that *Diaporthe* (anamorph: *Phomopsis*) could produce a vast array of terpenoids throughout their growth and development (Filho et al. 2016), some of these terpenoid species could be pharmaceutically relevant (de Souza et al. 2011, Chandra 2012, Barra et al. 2014). Besides terpenoid, antimicrobial metabolites known from the genus *Diaporthe* are diverse. These include cytoskyrin, cytosporones, diaporthalasin, phomochromones and several xanthonones, which possess good antibacterial activity (Chepkirui & Stadler 2017). *Chaetomium* is also a fungal genus that produced terpenoids (Zhang et al. 2012, Fatima et al. 2016). Generally, it produces chemically diverse metabolites as reported in previous studies including chaetoglobusins, anthraquinones, xanthonones, depsidones (Fatima et al. 2016), prenisatin and cochliodinols (Wang et al. 2016). Some groups of

those metabolites were also found in this study i.e. terpenoids and steroids. Apart from terpenoids and steroids, flavonoids (derivative of phenolic compound) are the major bioactive constituents of fungal cultures (Huang et al. 2007, Liu et al. 2007) and this is also in agreement with the results from this study. Culture filtrates of only two saprobic fungi were found to suppress growth of test organisms whereas the remaining bioactive fungi were endophytic. Previous reports have shown similar results with endophytic fungi exhibiting great potential to produce bioactive compounds against several pathogens (Pereira et al. 2015, Santos et al. 2015). This is possibly explained due to differences in lifestyles and niches. Saprobian fungi growing on plant litter may require only limited sets of chemical compounds, whereas endophytes always encounter a series of metabolites either from hosts or fungal competitors.

Accumulating evidence suggests that growth media and incubation conditions may play a crucial role in metabolite production in fungi (Fridsvad 2012, Hegge et al. 2015). In this study, we found similar results in which inhibition efficiency of such culture filtrates derived from each fungus varied among the culture media and the test organisms. It has been presumed that a nutritional requirement for optimal metabolite production is specific in each fungal species (Worapong 2009, Frisvad 2012, VanderMolen et al. 2013). Characteristics of fungi together with additional environmental factors i.e., pH, availability of carbon and nitrogen sources were found to contribute to diversity of fungal metabolites within a fungal isolate (Calvo et al. 2002). However, further investigation is needed before an optimal culture medium for metabolite production can be found. We found that crude extracts of some isolates did not inhibit growth of the test organisms. Since several factors are involved in secondary metabolite production, it is noteworthy to consider all of the mentioned factors, especially culture conditions, which may not induce or promote secondary metabolite production, before reaching a conclusion. Generally, antimicrobial compounds from culture filtrates of endophytic fungi need to be isolated by chromatography (TLC, HPLC) and finally identified by NMR and GC-MS (Zhang et al. 2013, Sharma et al. 2016). Due to limitations in this study, TLC was the only tool available. It is difficult to conclude either a key metabolite or chemical compound acting as antibacterial substance. Further studies on isolation and identification of active chemical metabolites are therefore needed.

We found the pattern of post-derivation colour of the crude extracts derived from culture media and mycelia was distinctly different. Terpenoids and unsaturated compounds and alcohols are the most common compound in both fungal mycelial and culture filtrate crude extract. On the other hand, flavonoids, steroids, aldehydes and ketones were present in the culture filtrate

crude extracts but were absent in the mycelial crude extracts. This suggests a possibility that the fungi produce a variety of metabolites – both soluble and insoluble. The soluble compounds (glycone) are likely to be secreted extracellularly and accumulated in the culture media, whereas the insoluble ones (aglycone) remain in the mycelia. Previously, it was shown from the cultivation of *Fusarium graminearum* that fusaristatin A was present only in mycelia, but not in the culture filtrate (Hegge et al. 2015). This supported the intracellular and extracellular secretion hypothesis in which some compounds are produced and localized intracellularly. On the other hand, terpenoids, unsaturated compounds and alcohols were groups of metabolites presented in both crude extracts, whereas no alkaloids were detected in crude extracts. This may be due to either the limited species of fungi able to produce this metabolite (Mahmood et al. 2010) or very little amount of alkaloid had been obtained and not be able to detect by TLC. Extraction methodology may also be the possible reason since in this study we did not use acid/alkaline solvent which specific for alkaloid extraction. Further investigation is however needed to confirm the mentioned hypotheses.

Wang et al. (2016) recently proposed a new genus *Dichotomopilus* for those *Chaetomium* having dichotomously branched terminal ascomatal hairs. Based on that typical character, saprobic isolate IMG9001 is fitted in the genus *Dichotomopilus*. Observed microscopic characters showed that isolate IMG9001 is closely related to *D. ramosissimum* (*Chaetomium ramosissimum*; in Wang et al. 2014) with minor variation of ascospore's size. Molecular evidence supported our assumption that isolate IMG9001 is *Dichotomopilus ramosissimum*. Unfortunately, it was not possible to identify the other isolate MG5/2.4-1 below the level of *Diaporthe* despite having molecular data available. In many fungal species complexes such as *Fusarium* (Lin et al. 2014) and *Phoma* (de Gruyter et al. 2009, Rai et al. 2014), similar problem arose when species identification based on a single gene marker was not sufficient for species determination. The use of multiple gene markers i.e., large and small subunit of rDNA, rpb2 and beta-tubulin might be considered together with phylogenetic analysis to solve the genetic identity of such species complexes.

CONCLUSION

A total of 19 out of 153 fungal isolates exhibited inhibition activity against at least one of the test organisms. In detail, 11, 2, 3 and 3 isolates inhibited the growth of *B. cereus*, *C. albicans*, *E. coli* and *S. aureus*, respectively. However, none of the fungal isolates could suppress *Pectobacterium*. Crude extracts derived from the culture filtrate and the fungal mycelia of *Diaporthe* sp. (isolate MG5/2.4-1) and *Dichotomopilus ramosissimum* (isolate IMG9001) were evaluated for their antimicrobial

activity and the results showed that culture filtrate crude extract inhibited the test organisms better than crude extracts derived from fungal mycelia. Analysis of the crude extracts from both culture filtrate and fungal mycelia revealed that the culture filtrate crude extracts were composed of flavonoids, steroids, terpenoids, aldehyde, ketone, unsaturated compound and alcohol while the fungal mycelial crude extracts were made up of terpenoids, unsaturated compound and alcohol.

COMMENTS FOR OTHER STUDY

Several fungal isolates in this study demonstrated the ability for inhibiting growth of the test organisms. It is worthwhile to further elucidate the chemical structures and functions of each active compound. In addition, growth conditions of each potential isolate should be optimized to maximize active compound production. As the crude extracts of culture filtrate and fungal mycelia of *Diaporthe* sp. MG5/2.4-1 and *Dichotomopilus ramosissimum* IMG9001 provided promising results in this study, they should be further evaluated based on bioautographic investigation. We only grew the isolates in PDB so in any further study growth in other media should be attempted before comparing the antimicrobial activity of each crude extract. Other active isolates should be studied for their specific characteristics, which could be used as supporting information for bioactive compound production.

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