World J Microbiol Biotechnol (2011) 27:3005–3008
DOI 10.1007/s11274-011-0765-8

SHORT COMMUNICATION

Antimicrobial activity of crude extracts from mangrove fungal endophytes

Jirayu Buatong · Souwalak Phongpaichit · Vatcharin Rukachaisirikul · Jariya Sakayaroj

Received: 19 February 2011 / Accepted: 21 April 2011 / Published online: 6 May 2011

Abstract The aim of this work was to select endophytic fungi from mangrove plants that produced antimicrobial substances. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) or minimal fungicidal concentrations (MFC) of crude extracts from 150 isolates were determined against potential human pathogens by a colorimetric microdilution method. Ninety-two isolates (61.3%) produced inhibitory compounds. Most of the extracts (28–32%) inhibited *Staphylococcus aureus* (MIC/MBC 4–200/64–200 μg ml^{-1}). Only two extracts inhibited *Pseudomonas aeruginosa* (MIC/MBC 200/ >200 μg ml^{-1}). 25.5 and 11.7% inhibited *Microsporum gypseum* and *Cryptococcus neoformans* (MIC/MFC 4–200/8–200 μg ml^{-1} and 8–200/8–200 μg ml^{-1}, respectively), while 7.5% were active against *Candida albicans* (MIC/MFC 32–200/32–200 μg ml^{-1}). None of the extracts inhibited *Escherichia coli*. The most active fungal extracts were from six genera, *Acremonium*, *Diaporthe*, *Hypoxylon*, *Pestalotiopsis*, *Phomopsis*, and *Xylaria* as identified using morphological and molecular methods. *Phomopsis* sp. MA194 (GU592007, GU592018) isolated from *Rhizophora apiculata* showed the broadest antimicrobial spectrum with low MIC values of 8–32 μg ml^{-1}against Gram-positive bacteria, yeasts and *M. gypseum*. It was concluded that endophytic fungi from mangrove plants are diverse, many produce compounds with antimicrobial activity and could be suitable sources of new antimicrobial natural products.

Keywords Antimicrobial agents · Endophytic fungi · Mangrove plants · ITS rRNA · LSU rRNA · Molecular identification

Introduction

Drug resistance problems are increasing worldwide. There is a need to find novel sources of antimicrobial drugs**.** At present, natural products still remain the most important resources for discovering new drugs. Endophytic fungi have been shown to produce a wide range of biologically active metabolites (Aly et al. 2010). Natural products from mangrove fungal endophytes with biological activity have been reported (Chen et al. 2007; Huang et al. 2008). However antimicrobial activity from endophytic fungi isolated from mangroves in Thailand has been rarely studied. Recently, (Chareprasert et al. 2010) reported that an endophytic fungus *Xylaria* sp.1 isolated from *Acanthus ilicifolius* from Thailand had antibacterial properties against both Gram-positive and Gram-negative bacteria. However, only the agar diffusion test method was used in their study. This study aimed to screen for fungal

Electronic supplementary material The online version of this article (doi:10.1007/s11274-011-0765-8) contains supplementary material, which is available to authorized users.

J. Buatong · S. Phongpaichit (✉)
Natural Products Research Center and Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
e-mail: souwalak.p@psu.ac.th

V. Rukachaisirikul
Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

J. Sakayaroj
Phylogenetics Laboratory, National Center for Genetic Engineering and Biotechnology, Thailand Science Park, Pathum Thani 12120, Thailand

endophytes from mangrove plants in the south of Thailand that produced antimicrobial metabolites against potential human pathogens. Minimal inhibitory concentrations (MIC), minimal bactericidal concentrations (MBC) or minimal fungicidal concentrations (MFC) of crude extracts from their culture media and hyphae were determined.

Materials and methods

Sampling of mangrove plants

Leaves and branches from 54 healthy plants of 12 mangrove species were collected from mangrove areas in the south of Thailand in Satun, Songkhla, Surat Thani and Trang provinces. The twelve mangrove species were *Aegiceras corniculatum* ($n = 1$), *Avicennia alba* ($n = 4$), *Avicennia officinalis* ($n = 5$), *Bruguiera gymnorrhiza* ($n = 1$), *Bruguiera parviflora* ($n = 5$), *Lumnitzera littorea* ($n = 2$), *Rhizophora apiculata* ($n = 12$), *Rhizophora mucronata* ($n = 9$), *Sonneratia caseolaris* ($n = 4$), *Scyphiphora hydrophyllacea* ($n = 3$), *Xylocarpus granatum* ($n = 4$), and *Xylocarpus moluccensis* ($n = 4$). Plant specimens were compared with the voucher specimens at Prince of Songkla University Herbarium.

Isolation and identification of endophytic fungi

The isolation methods for fungi were as previously described (Phongpaichit et al. 2006). Briefly, leaves and branches were cut into small segments and surface-sterilized by sequential washes in 95% ethanol (30 s), 5% sodium hypochlorite (5 min), 95% ethanol (30 s) and rinsed with sterile water. The sample segments were then placed on corn meal agar medium supplemented with antibiotics (penicillin plus streptomycin sulphate 50 mg l^{-1}) to restrict bacterial growth. Plates were incubated at 25°C for 1 week. Fungal growth was observed every day. Pure cultures were obtained by hyphal tip isolation and stored in 15% glycerol at −80°C. A total of 619 fungal isolates were obtained and 150 were selected for antimicrobial assays based on their morphotypes and host plant source.

Endophytic fungi that showed good antimicrobial activity were identified based on morphology and the analyses of the DNA sequences of the large subunit (LSU) and the internal transcribed spacer (ITS1-5.8S-ITS2), ITS regions of their ribosomal RNA gene using fungal universal primers (White et al. 1990; Bunyard et al. 1994; Landvik 1996). The LSU and ITS sequences of active endophytic fungi have been submitted to GenBank for retrieving their accession numbers.

Fermentation and extraction of endophytic fungi

Endophytic fungal cultures were grown on potato dextrose agar and incubated at 25°C for 3–5 days. Six mycelial plugs (1×1 cm^2) were inoculated into 500 ml Erlenmeyer flasks containing 300 ml potato dextrose broth and incubated for 3 weeks at 25°C under a stationary condition (Phongpaichit et al. 2006). The culture broth was filtered to separate the filtrate and mycelium. The filtrate was extracted with ethyl acetate (EtOAc) and this was evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to give the BE extract. The fungal mycelia were extracted with methanol (MeOH) for 2 days. The aqueous MeOH layer was concentrated under reduced pressure. Distilled water (50 ml) was added to the extract and the mixture was then mixed with hexane (100 ml). The aqueous layer was then extracted with an equal volume of EtOAc. The hexane extract and the combined EtOAc extracts were evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to give CH and CE extracts respectively.

Antibacterial assay

The dried fungal extracts were dissolved in dimethyl sulfoxide and stored at −4°C until used. They were tested against *Staphylococcus aureus* ATCC25923, a clinical isolate of methicillin-resistant *S. aureus* (MRSA) SK1, *Escherichia coli* ATCC25922, and *Pseudomonas aeruginosa* ATCC27853 by a microdilution method according to a modification of Clinical and Laboratory Standards Institute (CLSI) M7-A4 (CLSI 2000). The lowest concentration of extract that inhibited visible growth was recorded as the MIC. Concentrations of crude extract less dilute than the MIC and the MIC were streaked onto a nutrient agar plate and incubated under appropriate conditions. The lowest concentration of extract that showed no growth was recorded as the MBC. Vancomycin and gentamicin were used as standard antibacterial agents for positive inhibitory controls.

Antifungal assay

The MICs of fungal crude extracts were determined by a modification of the microbroth dilution CLSI M27-A2 (CLSI 2002a) against yeasts (*Candida albicans* ATCC90028, *Cryptococcus neoformans* ATCC90112) and a modification of the microbroth dilution CLSI M38-A (CLSI 2002b) against a clinical isolate of *Microsporum gypseum* MU-SH4. Microtiter plates were incubated at 35°C for 24 h for *C. albicans*, 48 h at room temperature for *C. neoformans*, and 7 days at room temperature for *M. gypseum*. The MFCs of the active extracts were

determined by the streaking method on Sabouraud's dextrose agar. Amphotericin B was used as a positive inhibitory control for yeasts and miconazole for *M. gypseum*.

Results and discussion

Antimicrobial activity

A total of 385 extracts from 150 fungal endophytes were evaluated in antimicrobial screening tests. The results showed that 47% of extracts from 92 isolates (61.3%) inhibited at least one test microorganism. Of these, the endophytic fungi that produced secondary metabolites had most activity against Gram-positive bacteria (*S. aureus* ATCC25923 and MRSA-SK1) with 65 and 70 isolates, followed by *M. gypseum* (59 isolates), *C. neoformans* (35 isolates) and *C. albicans* (21 isolates). Only two endophytic fungal extracts inhibited *P. aeruginosa* and none inhibited *E. coli* (Table 1). It is interesting to note that both strains of *S. aureus* tested gave very similar results. *S. aureus* ATCC25923 is a susceptible strain whereas MRSA is resistant to methicillin. MRSA has the *mecA* gene encoding for an altered penicillin binding protein gene (PBP2a) which has low affinity to beta-lactam antibiotics (Barber 1963). It is well known that MRSA are multi-drug resistant strains (Kwong et al. 2008). This may indicate that different resistance mechanisms are involved in the resistance of MRSA to different classes of antibiotics. Active fungal crude extracts in this study may act on different or new targets.

Among the active crude extracts, hexane extracts from fungal mycelia had the highest inhibitory activity in the screening tests with 51%, followed by ethyl acetate extracts from the mycelia (46.5%) and ethyl acetate extracts from the fermentation broths (44.8%). This result indicated that most of the active materials were present in the fungal mycelia. Active extracts may contain cell-bound components and low polarity substances.

A total of 181 active extracts inhibited 1–5 strains of the test microorganisms (Fig. 1). Most of them (35.4%) inhibited 2 strains. Only 7.2% had a broad antimicrobial spectrum that inhibited 5 strains of test microorganisms that included *S. aureus*, MRSA-SK1, *C. albicans*, *C. neoformans* and *M. gypseum*. In this study, we found six endophytic fungi that produced extracts with low MIC values against test microorganisms (Table S1, see Electronic Supplementary Material). These isolates were MA12, MA34, MA96, MA148, MA156 and MA194. The MIC values of their extracts were comparable to the MIC values of standard drugs. MA194 isolated from *Rhizophora apiculata* showed the broadest antimicrobial spectrum against Gram-positive bacteria, yeasts and *M. gypseum* (MIC 8–32 μg ml^{-1}).

Table 1 Selected endophytic fungi from various mangrove species and their antimicrobial activity against pathogenic microorganisms

	No. of endophytic fungi from mangrove species												
	Ac	Aa	Ao	Bg	Bp	Ll	Ra	Rm	Sc	Sh	Xg	Xm	Total
No. of selected fungi	2	14	9	1	11	7	64	17	3	8	9	5	150
No. of active fungal isolates from each mangrove species (%)	1 (50.0)	8 (57.1)	8 (88.9)	0 (0.0)	8 (72.7)	6 (85.7)	34 (53.1)	9 (52.9)	3 (100)	6 (75)	5 (55.6)	4 (80)	92 (61.3)
% of active fungi (92 isolates)	1.1	8.7	8.7	0.0	8.7	6.5	37.0	9.8	3.3	6.52	5.4	4.4	100
Anti-*S. aureus* ATCC25923	1	3	5	0	7	6	24	6	3	4	3	3	65
Anti-MRSA-SK1	1	5	7	0	6	4	26	7	3	4	5	2	70
Anti-*E. coli* ATCC25922	0	0	0	0	0	0	0	0	0	0	0	0	0
Anti-*P. aeruginosa* ATCC27853	0	1	0	0	0	0	0	0	0	0	1	0	2
Anti-*C. albicans* ATCC90028	0	2	4	0	0	1	10	0	0	1	2	1	21
Anti-*C. neoformans* ATCC90112	1	5	5	0	0	2	13	2	1	4	1	1	35
Anti-*M. gypseum* MU-SH4	1	7	5	0	4	5	21	5	1	4		3	59

Ac, *Aegiceras corniculatum*; Aa, *Avicennia alba*; Ao, *Avicennia officinalis*; Bg, *Bruguiera gymnorrhiza*; Bp, *Bruguiera parviflora*; Ll, *Lumnitzera littorea*; Ra, *Rhizophora apiculata*; Rm, *Rhizophora mucronata*; Sc, *Sonneratia caseolaris*; Sh, *Scyphiphora hydrophyllacea*; Xg, *Xylocarpus granatum*; Xm, *Xylocarpus moluccensis*; MRSA, methicillin-resistant *Staphylococcus aureus*

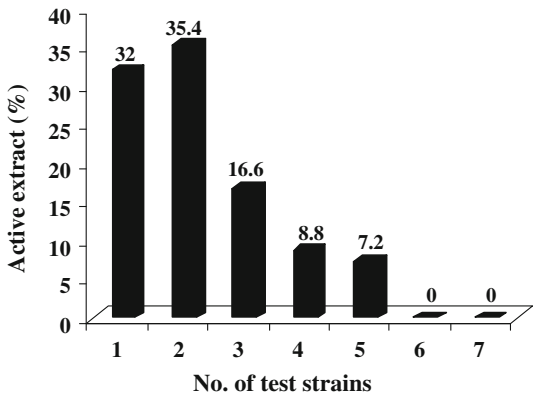

Fig. 1 Number of test strains inhibited by mangrove endophytic fungal crude extracts

Distribution and identification of endophytic fungi with antimicrobial activity

Among the antimicrobial active isolates listed in Table S1 only two isolates (MA34 and MA99) can be identified based on their morphology as *Xylaria cubensis* and *Pestalotiopsis* sp., respectively. The rest did not sporulate in culture and were identified as sterilia mycelia. Based on their morphology and LSU and ITS sequence analyses, our active mangrove endophytic fungal isolates can be classified into six genera, *Acremonium*, *Diaporthe*, *Hypoxylon*, *Pestalotiopsis*, *Phomopsis*, and *Xylaria*. The genera *Diaporthe, Phomopsis*, *Pestalotiopsis* and *Xylaria* have been reported to be the most common endophytes isolated from mangrove plants (Cheng et al. 2008; Huang et al. 2008; Pang et al. 2008; Chareprasert et al. 2010). Many mangrove endophytes have been reported to produce antimicrobial substances such as *Colletotrichum* sp., *Xylaria* sp., *Pestalotiopsis* sp., *Paecilomyces* sp., *Phomopsis* sp. and *Phoma* sp. (Pang et al. 2008; Schulz et al. 2008). Moreover, *Hypoxylon* spp. found in this study have never been previously reported as mangrove fungal endophytes. (Chareprasert et al. 2010) reported that *Cladosporium* sp. from *Thespesia populneoides* and *Xylaria* sp. 1 from *Acanthus ilicifolius* caused considerable inhibition to Gram-positive and Gram-negative bacteria. They collected mangrove plants from the west coast area in Chanthaburi Province and the upper southern part of Thailand, Prachuap Khiri Khan and Ranong Provinces whereas our collecting sites were from the lower southern part. Results from these studies have indicated that endophytic fungi from mangrove plants from Thailand could be a good source of antimicrobial natural products.

Acknowledgments This work was supported by the Thailand Research Fund and National Center for Genetic Engineering and Biotechnology, under the BRT's Bioresources Utilization Program grant (grant number BRN 001 G-52). J. Buatong thanks the Center for Innovation in Chemistry (PERCH-CIC) for a scholarship and Prince of Songkla University for partial support. Authors are also grateful to Dr. Boonsom Bussaban for the identification of *Xylaria cubensis*. Finally, Dr. Brian Hodgson is gratefully acknowledged for the review of English in this paper.

References

Aly AH, Debbab A, Kjer J, Proksch P (2010) Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers 41:1–16

Barber M (1963) Methicillin-resistant staphylococci. J Clin Pathol 1:308–311

Bunyard BA, Nicholson MS, Royse DJ (1994) A systematic assessment of *Morchella* using RFLP analysis of the 28S ribosomal RNA gene. Mycologia 86:762–772

Chareprasert S, Piapukiew J, Whalley AJS, Sihanonth P (2010) Endophytic fungi from mangrove plant species of Thailand: their antimicrobial and anticancer potentials. Bot Mar 53:555–564

Chen G, Zhu Y, Wang HZ, Wang SJ, Zhang RQ (2007) The metabolites of a mangrove endophytic fungus, *Penicillium thomi*. J Asian Nat Prod Res 9:159–164

Cheng Z, Tang W, Su Z, Cai Y, Sun S, Chen Q, Wang F, Lin Y, She Z, Vrijmoed LLP (2008) Identification of mangrove endophytic fungus 1403 (*Fusarium proliferatum*) based on morphological and molecular evidence. J Forest Res 19:219–224

Clinical and Laboratory Standards Institute (CLSI) (2000) Reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard M7–A4. CLSI, Wayne

Clinical and Laboratory Standards Institute (CLSI) (2002a) Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard-second edition. CLSI documents M27–A2. CLSI, Wayne

Clinical and Laboratory Standards Institute (CLSI) (2002b) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: approved standard. CLSI documents M38-A. CLSI, Wayne

Huang Z, Cai X, Shao C, She Z, Xia X, Chen Y, Yang J, Zhou S, Lin Y (2008) Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. Phytochemistry 69:1604–1608

Kwong SM, Lim R, Lebard RJ, Skurray RA, Firth N (2008) Analysis of the pSK1 replicon, a prototype from the staphylococcal multiresistance plasmid family. Microbiology 154:3084–3094

Landvik S (1996) *Neolecta*, a fruit-body-producing genus of the basal ascomycetes, as shown by SSU and LSU rDNA sequences. Mycol Res 100:199–202

Pang KL, Vrijmoed LLP, Goh TK, Plaingam N, Jones EBG (2008) Fungal endophytes associated with *Kandelia candel* (Rhizophoraceae) in Mai Po Nature Reserve, Hong Kong. Bot Mar 51:171–178

Phongpaichit S, Rungjindamai N, Rukachaisirikul V, Sakayaroj J (2006) Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia* species. FEMS Immunol Med Microbiol 48:367–372

Schulz B, Draeger S, dela Cruz TE, Rheinheimer J, Siems K, Loesgen S, Bitzer J, Schloerke O, Zeeck A, Kock I, Hussain H, Dai J, Krohn K (2008) Screening strategies for obtaining novel, biologically active, fungal secondary metabolites from marine habitats. Bot Mar 51:219–234

White TF, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky FS, White TT (eds) PCR Protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322