ORIGINAL ARTICLE

Evaluation of the stability and antibacterial activity of crude extracts of hydro-endophytic fungi

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ABSTRACT

The production and screening of secondary metabolites of four hydro-endophytes isolated from lotus, and the stability of bioactive compounds was evaluated. Surface-sterilized technique was used to isolate the endophytic fungi (EF) on potato dextrose agar and identified by using morphological and molecular techniques. The extracts were tested for anti-microbial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) DMST20651, *Streptococcus mutans* (SM) DMST18777, *Staphylococcus epidermidis* (SE) ATCC12228, *Pseudomonas aeruginosa* (PA) TISTR1467, and *Propionibacterium acnes* (PN) DMST14916. The bacteriostatic and bactericidal activities were determined. Finally, thermal and ultraviolet (UV) stability was evaluated. Four endophyte isolates (EF 14, EF36, EF53, EF58, and EF60) produced secondary metabolites and showed activity against MRSA, SM, SE, PA, and PN, respectively. The crude ethyl acetate (EtOAc) and methanol (MeOH) extract of EF14 showed activity against MRSA with the inhibition zone of 9.00 ± 0.00 and 7.50 ± 0.50 mm, and minimum inhibitory concentration was 4.80 and 4.90 mg/mL, respectively. The minimum bactericidal concentration was 9.60 mg/mL. Whereas, the crude EtOAc and MeOH extract EF60, which were extracted by EtOAc and MeOH, showed inhibition zone of SE as 12.33 ± 0.57 and 12.33 ± 0.57 mm, respectively. Crude EtOAC extracts of EF14 showed highest thermal stability at 55°C–121°C, and UV stability with MRSA and SE, respectively. The results showed that the EtOAc extracts of EF could be potential antibacterial pathogens and displayed UV-thermal stability. This information is beneficial for future investigations, since some bioactive compounds have potential as anti-resistant strains of some bacterial pathogens.

Key words: Antimicrobial activity, bioactive compound, hydro-endophytic fungi

INTRODUCTION

The fungal endophytic fungi (EF) are normally found in the intracellular tissue of plants. They have been reported as the producers of important biologically bioactive molecules.[1] In general, EF originates in plant tissues without eliciting disease symptoms, and consequently forms a symbiotic relationship. They can produce bioactive molecules for defense against pathogens. In addition, its bioactive molecules were applied for new drug development processes,[2-4] including antifungal, antiviral, anti-malarial, anticancer,[5,6] and especially antibacterial pathogens.[7] However, the EF contained in different plants may have different biosynthetic properties, leads to the production

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of special bioactive compounds with varied biological activities. Many EF colonize inside hydro-plants, which frequently possess unique morphological and physiological properties. These appearances are probably responsible for hydro-plants' specific natural atmosphere for the growth of potential extraordinary endophytes.[8]

Therefore, in this research, we report the characterization of lotus endophytes and stability tests of bioactive compounds. In addition, antibacterial activities from solvent extracts and isolate of EF submerged culture were also investigated.

MATERIALS AND METHODS

Plants collection

Healthy plants Bua Khao Mongkol, Bua Chalong Khuan, Bua Luang, and Bua Chompoo Mamaew were collected from Rajamangala University Thanyaburi, Pathum Thani, Thailand. The identity of the hydro-plant specimens was confirmed by a specialist from the Lotus museum at Rajamangala University Thanyaburi.

Isolation of endophytic fungi

Plant samples were placed in sterile plastic bags for storage at the room temperature. All part of plants, including leaves, vein, inter vein, petal, stems, stolon, and roots were surface sterilized.[9] The tissue segments were allowed to air-dry before placed on potato dextrose agar (PDA) supplemented with ampicillin 200 mg/L in sterile culture plates and sealed with parafilm, incubated at the room temperature for 5 days. Fungal colonies tips were transferred aseptically to new PDA. The diversity analysis was conducted based on both colonization and isolation rate.[10]

Mycelial growth rate

The mycelial of endophytes was cut from colonies and grown in PDA, incubated at 30°C for 7 days. The mycelial diameter was measured daily.[11]

Screening of bioactive molecules

EF was cultivated on potato dextrose broth (PDB) in 250 mL Erlenmeyer flasks and incubated at the room temperature for 2 weeks at 150 rpm. After fermentation, the fungus was filtered by filtrate paper (Whatman No. 1) and tested for antibacterial activities against Methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus mutans* (SM), *Staphylococcus epidermidis* (SE), *Pseudomonas aeruginosa* (PA), and *Propionibacterium acnes* (PN), respectively. After incubation for 24 h, the inhibition zone was measured. Erythromycin (15 µg/disc) and 10% dimethyl sulfoxide (DMSO) were used as controls.

Identification of endophytic fungi

The morphological characteristics, macroscopic and microscopic were determined. For genotypic identification, the DNA sequence of the ITS, SSU, and LSU region of the mRNA gene were analyzed. The fungal DNA was extracted using previously reported protocols. The ITS segment of genomic DNA was amplified by the polymerase chain reaction (PCR) using a pair of universal primers, ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). Purification and sequencing of PCR products were carried out by Macrogen Inc. (Rep. of Korea). The sequences have been deposited in National Center for Biotechnology Information (NCBI) via Basic Local Alignment Search Tool (BLAST) searches.[12-14]

The fermentation of endophytic fungi

The selected EF were tested for anti-bacterial activity by paper disc-diffusion assay.[15] Initial culture was prepared in 250 mL Erlenmeyer flasks by adding 5 plugs of growing mycelia cultured in 50 mL PDB, incubated at the room temperature under continuous shaking at 150 rpm for 2 weeks and scale up to 500 mL as the same conditions. Finally, fungal fermentation was filtered.[16]

The extraction of bioactive molecules of endophytes

The fungal supernatant was extracted by sequential extraction procedure using hexane, ethyl acetate (EtOAc), and MeOH. Each solvent extraction was done in the ratio of 1:25 (v/v) and evaporated under reduced pressure at 45°C.[16]

Bacteriostatic and bactericidal determination

The broth micro-dilution technique was used to determine the bacteriostatic activity. Two-fold dilutions of fungal extracts were added directly in a test-tube containing nutrient broth to obtain difference concentrations. The starter was added to get a final concentration of 5×10^5 CFU/mL in each tube and incubated for 24 h at 37°C. The minimum inhibitory concentration (MIC) value was considered as the lowest concentration of the extracts that completely inhibits the bacterial growth. Erythromycin (10 µg/disc) and 10% DMSO were used as controls. The minimum bactericidal concentration (MBC) was determined from the MIC result by transferred into a fresh medium and incubated at 37°C for 24 h. The lowest concentration of fungal crude extract that showed no visible growth of bacterial pathogen was considered the MBC value.[17]

The stability of cured extracts of fungal endophytes

The suitable MIC concentration of crude extracts was used to determine the various temperature and then tested for antibacterial activity. Two milliliters of crude extracts were added to test tubes, which were then heated in water bath for 15 min at varying temperatures: 55°C, 70°C, and 12°C. For ultraviolet (UV) stability, the extracts were exposed to UV radiation for 15 min. After the designated incubation period, the antibacterial activity was evaluated.[17]

RESULTS AND DISCUSSION

Isolation of endophytic fungi

Seventy distinct EFs were isolated in this study. The petal fragments of BKM analyzed showing the highest percentage colonization rate (80%), while vein and stem fragments of BL were 60% [Table 1]. The result showed that EF were more prevalent in the leaves of BL than other parts of the lotus. Similar results were obtained from previous reports, which found that eight from twenty fungal endophytes were obtained from *Nymphaea nouchali*.[18] All fungal isolates were screened to test anti-bacterial activity.

Screening of bioactive molecules

The supernatant of all EF were tested for their antibacterial activity against MRSA, SM, SE, PA, and PN. It was found that five of 70 EF were active against at least two bacterium tested [Table 2]. Interestingly, the supernatant from cell-free cultured of fungi were found to exhibit growth inhibitory activity against PN, SE and MRSA, but other bacterial strains were not inhibited.

Mycelial growth rate determination

The average colony diameters of all five EF after 7 days incubation are shown in Figure 1. Radial growth of the colonies was more rapid in PDA agar plates on day 5. The growth rates of EF36 and EF60 were higher than EF14, EF53, and EF58 with diameter of 7.00 ± 0.00 and 7.33 ± 0.28 cm. This indicated that different fungal species have different growth rates under the same condition. Recent research has suggested that nutrition-rich media promotes abundant mycelial density and radial growth rate.[19]

Identification of endophytic fungi

This study successfully amplified the ITS gene by using ITS1 and ITS4 primer from the four isolated EF. The nucleotide sequences of the four isolated EF were BLAST on the NCBI database. Result found that EF from four isolates were matched to the ITS gene of the EF and showed >99% identical with formerly recorded microbial species in the NCBI Genbank, and therefore, their identities were confirmed [Table 3].

Antibacterial activity of fungal crude extracts

The crude extracts from different solvents (hexane, EtoAc, and MeOH) were tested for antibacterial activity against

Table 1: The percentage of colonization rate and isolation rate of endophytic fungi with different part of lotus

Lotus	Isolates	Percentage of CR						IR					
		V[e]	IV[f]	P[g]	St[h]	Sl[i]	R[j]	V[e]	IV[f]	P[g]	St[h]	Sl[i]	R[j]
BL	35	60	40	50	60	20	10	0.6	0.5	0.8	0.3	0.2	1
BCK	15	30	20	50	40	0	0	0.2	0.5	0.4	0	0	0.3
BKM	10	10	0	80	0	0	0	0	0.8	0	0	0	0.1
BCM	10	20	0	40	0	0	0	0	0.7	0	0	0	0.2

[e]Vein, [f]Inter-vein, [g]Petal, [h]Stem, [i]Stolon, [j]Roots. BKM: Bua Khao Mongkol, BCK: Bua Chalong Khuan, BL: Bua Luang, BCM: Bua Chompoo Mamaew, IR: Isolation rate, CR: Colonization rate

Table 2: Antibacterial activity of supernatant of endophytic fungi

Endophytes	Clear zone of bacterial pathogens (mm)					Plants origin
	PN	SE	SM	PA	MRSA	
EF14	10.33±0.57	10.00±0.00	-	-	10.33±0.57	L/BL
EF36	7.17±0.28	-	-	-	9.00±0.00	P/BL
EF53	7.00±0.00	-	-	-		P/BCK
EF58	-	7.33±0.57	-	-		P/BKM
EF60	9.00±0.00	9.00±0.00	-	-	7.50±0.35	P/BKM

L: Leaf, P: Petal, BKM: Bua Khao Mongkol, BCK: Bua Chalong Khuan, BL: Bua Luang, BCM: Bua Chompoo Mamaew, EF: Endophytic fungi, PN: *Propionibacterium acnes*, SE: *Staphylococcus epidermidis*, SM: *Streptococcus mutans*, PA: *Pseudomonas aeruginosa*, MRSA: Methicillin resistant *Staphylococcus aureus*

Table 3: Identities of ITS sequences of endophytic fungi with their closest GenBank sequences (according to BLAST searches)

Samples	Description	Percentage of identity	Genes	Reference of accession number
EF36	*Preussia minima*	99.56	ITS	MG022134.1
EF60	*Alternaria tenuissima*	100	ITS	MF405157.1
EF58	*Curvularia* spp.	100	ITS	MG661740.1
EF53	*Exserohilum rostratum*	100	ITS	EU571210.1
EF4	*Alternaria alternata*	100	ITS	KV441469.1

EF: Endophytic fungi

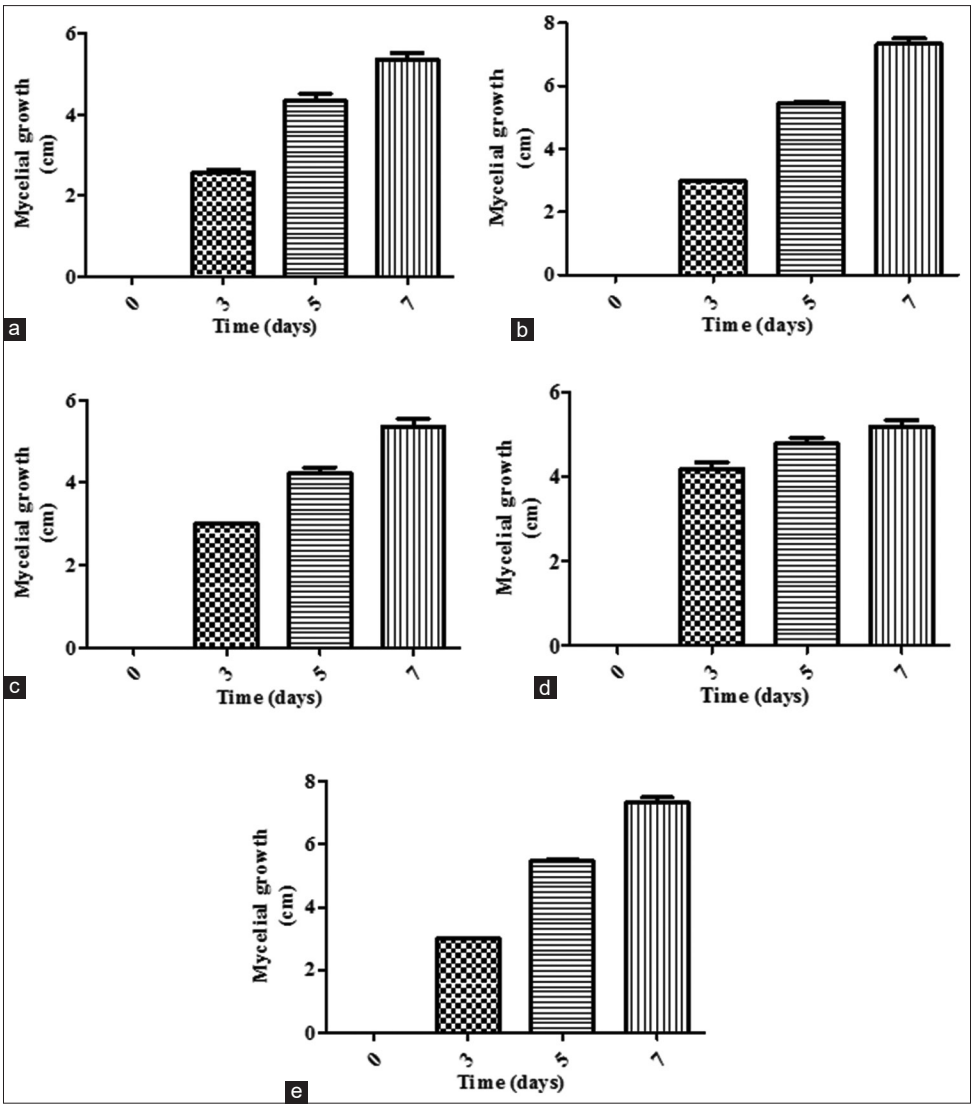

Figure 1: The mycelial growth of endophytic fungi at room temperature for 7 days of different endophytic fungi: (a) (endophytic fungi 14), (b) (endophytic fungi 36), (c) (endophytic fungi 53), (d) (endophytic fungi 58), (e) (endophytic fungi 60)

MRSA and SE Table 4 showed inhibitory activity on the tested bacteria in different crude extracts. It was shown that EtOAc crude extracts of EF14 displayed more potential against both bacterial pathogens MRSA and SE which gave the zone of inhibition of 9.00 ± 0.00 and 12.67 ± 0.57 mm, whereas methanol (MeOH) extracts showed weak inhibition against MRSA. In addition, the fraction of MeOH extract of EF14 showed active activity against SE at clearing zone of 10.00 ± 0.00 mm. For fungal isolate EF60, both crude extracts of EtOAc and MeOH showed best inhibition against MRSA with inhibition zone of 12.33 ± 0.57 mm.

Bacteriostatic and bactericidal

Fungal crude extracts with antibacterial activity were further examined for their MIC and MBC by the agar disc-diffusion assay[20] against MRSA [Table 5]. EtOAc and MeOH extracts of EF14 had MIC value of 4.80 and 4.90 mg/mL, respectively, though only EtOAc extracts displayed MBC at the concentration of 9.60 mg/mL. The MeOH extract of EF36 showed MIC value of 303.80 mg/mL. Our findings corroborated with previous results from Dos Santos *et al*.[21] that indicated the MIC value of MeOH and EtOAc extracts of EF isolated from *Indigofera suffruticosa* to be 1.56 and 0.39 mg/mL, respectively.

The stability of cured extracts of endophytic fungi

Investigating the thermal stability of EtOAc and MeOH extracts is essential to prevent thermal degradation of bioactive compounds. The thermal stability of the crude extracts was tested by immersing the samples in a water bath at different temperatures of 55 and 70°C and autoclaved at 121°C for 15 min. The anti-bacterial activity of the EtOAc extract showed inhibition zone of MRSA with 10.00 ± 0.00 mm at all temperature conditions, whereas SE was 8.00 ± 0.00 mm. Regarding UV radiation stability, the antibacterial activity of the crude EtOAc extracts showed

Table 4: Anti-bacterial activity of crude extracts of endophytic fungi

Crude extracts	Inhibition zone (mm)	
	SE	MRSA
EF 14		
Hexane	-	-
Ethyl acetate	9.00±0.00	12.67±0.57
Methanol	-	10.00±0.00
EF36		
Hexane	-	-
Ethyl acetate	-	-
Methanol	7.00±0.00	9.00±0.00
EF53		
Hexane	-	-
Ethyl acetate	-	-
Methanol	-	-
EF58	-	
Hexane	-	-
Ethyl acetate	-	-
Methanol	-	-
EF60	-	
Hexane	-	-
Ethyl acetate	-	12.33±0.57
Methanol	-	12.33±0.57
Erythromycin	20.00±0.00	20.00±0.00
10% DMSO	-	-

EF: Endophytic fungi, SE: *Staphylococcus epidermidis*, MRSA: Methicillin resistant *Staphylococcus aureus*, DMSO: Dimethyl sulfoxide

Table 5: The minimum inhibition concentration and minimum bactericidal concentration of endophytic fungi

Extracts	*Staphylococcus aureus* (MRSA)	
	MIC (mg/mL)	MBC (mg/mL)
EF 14		
Ethyl acetate	4.80	9.60
Methanol	4.90	-
EF60		
Methanol	303.80	-

MRSA: Methicillin resistant *Staphylococcus aureus*, EF: Endophytic fungi, MIC: Minimum inhibition concentration, MBC: Minimum bactericidal concentration

the higher inhibition zone against SE compared to MRSA of 8.67 ± 0.57 and 7.50 ± 0.50 mm, respectively. On the contrary, the EF60 expressed less activity compared to fungal strain EF14. Hence, there is justification in that the crude EtOAc extracts of EF14 is thermal stable and UV stable between 55°C and 121°C for 15 min, indicating that it was maintained the biological activity. The EF has been confirmed as a bioactive molecules sources of novel bioactive agents against various microbial pathogens, and the crude extract was stable at temperatures up to 80°C.[22]

CONCLUSIONS

A total of 70 EF isolated from lotuses were screened for antibacterial activity. The results showed that EF from BL has the highest colonization rate. Three isolates from BL (EF14, EF36, and EF53), one isolate from BCK (EF58) and BKM (EF60) produced bioactive molecule against PA, SE, and MRSA and were also selected for further investigation. The EtAOc extracts of EF14 showed the best potential antibacterial activity against MRSA with MIC and MBC value of 4.8 and 9.6 mg/mL and showed thermal stability and UV stability at temperatures between 55°C and 121°C for 15 min of exposure. To our knowledge, lotus is one of the potential sources of EF that produces secondary metabolites. In future, the optimization and structure characterization of EtOAc crude extracts of fungal isolate EF14 will be conducted.

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Conflicts of interest

There are no conflicts of interest.

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