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

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
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.\textsuperscript{[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 Chomphoo 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.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[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 \textit{Staphylococcus aureus} (MRSA), \textit{Streptococcus mutans} (SM), \textit{Staphylococcus epidermidis} (SE), \textit{Pseudomonas aeruginosa} (PA), and \textit{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 (TCCGTAAGGTGACCCACG and ITS4 (TCCGCCTATTTGATATGC). 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.\textsuperscript{[12-14]}

The fermentation of endophytic fungi
The selected EF were tested for anti-bacterial activity by paper disc-diffusion assay.\textsuperscript{[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.\textsuperscript{[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 1:2.5 (v/v) and evaporated under reduced pressure at 45°C.\textsuperscript{[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\textsuperscript{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.\textsuperscript{[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.\textsuperscript{[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  |
|-------|----------|------------------|-----|
|       |          | Vn | IV | Pn | Sc | Sl | Rn | Vn | IV | Pn | Sc | Sl | Rn |
| 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|

*Vein, †Inter-vein, petals, sStolon, 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 | PN | SE | SM | PA | MRSA          | Plants origin |
|------------|----|----|----|----|----------------|---------------|
| 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/BCM          |
| EF58       | -   | 7.33±0.57 | -  | -  | 7.50±0.35 P/BCM |
| EF60       | 9.00±0.00  | 9.00±0.00 | -  | -  | -              |

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
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

---

**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)
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 |    |
| EF 36          |                      |    |      |
| Hexane         | -                    | -  | -    |
| Ethyl acetate  | -                    | -  | -    |
| Methanol       | -                    | 9.00±0.00 | 9.00±0.00 |
| EF 53          |                      |    |      |
| Hexane         | -                    | -  | -    |
| Ethyl acetate  | -                    | -  | -    |
| Methanol       | -                    | -  | -    |
| EF 60          |                      |    |      |
| Hexane         | -                    | -  | -    |
| Ethyl acetate  | -                    | -  | -    |
| Methanol       | -                    | 12.33±0.57 | 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) (mg/mL) | MIC | MBC |
|----------|-------------------------------------|-----|-----|
|          |                                     |     |     |
| EF 14    |                                     |     |     |
| Ethyl acetate | 4.80                  | 9.60 |     |
| Methanol  | 4.90                               | -   | -   |
| EF 60    |                                     |     |     |
| Methanol  | 303.80                             | -   | -   |

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

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 EtOAc 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.

Acknowledgment

This research was funded by the NRCT, Thailand. We would also thank RMUTT for all facilities and would also like to thank Dr. Natnaree Siriwon for proof-reading this article.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 2003;67:491-502.
2. Selim KA, El-Beih AA, AbdEl-Rahman TM, El-Diwany AI. Biology of endophytic fungi. Curr Res Environ Appl Mycol 2012;2:33-82.
3. Mitchell AM, Strobel GA, Hess WM, Vargas PN, Ezra D. Muscodor crispans, a novel endophyte from Ananas anannassoides in the Bilivian Amazon. Fung Divers 2008;31:37-43.
4. Stadler M, Keller NP. Paradigm shifts in fungal secondary metabolism research. Mycol Res 2008;112:127-30.
5. Strobel GA. Endophytes as sources of bioactive products. Microbes Infect 2003;5:535-44.
6. Nicoletti R, Fiorentino A. Plant bioactive metabolites and drugs produced by endophytic fungi of dermatophyta. Agricul 2015;5:918-70.
7. Jadulco R, Brauers G, Edrada RA, Ebel R, Wray V, Sudarsono S, et al. New metabolites from sponge-derived fungi Curvularia lunata and Cladosporium herbarum. J Nat Prod 2002;65:730-3.
8. Dissanayake RK, Ratnaweera PB, Williams DE, Wijayarathne CD, Wijesundera RLC, Andersen RJ, et al. Antimicrobial activities of endophytic fungi of the Sri Lankan aquatic plant Nymphaea nouchali and chaetoglobosin A and C, produced by the endophytic fungus Chaetomium globosum. Mycology 2016;7:1-8.
9. Marcellano JP, Collanto AS, Fuentes RG. Antibacterial activity of endophytic fungi isolated from the bark of Cinnamomum mercedai. Pharmacogn J 2017;9:405-9.
10. Se-Al RA. Department of Zoology, University of Oxford. Oxford OX1 4JD UK; 1996.
11. Swofford DL, PAUP: Phylogenetic Analysis Using Parsimony, Version 4.0b10. Sunderland, Massachusetts: Sinauer Associates; 2002.
12. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through
sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-80.

13. Trisuwak K, Rukachaisirikul V, Sukpondma Y, Preedanon S, Phongpaichit S, Rungjindamai N, et al. Epoxydrons and a pyrone from the marine-derived fungus Nigrospora sp. PSU-F5. J Nat Prod 2008;71:1323-6.

14. Durairaj S, Srinivasan S, Lakshmanaperumalsamy P. In vitro antibacterial activity and stability of garlic extract at different pH and temperature. Ej Bio 2009;5:5-10.

15. Pan F, Liu ZQ, Chen Q, Xu YW, Hou K, Wu W. Endophytic fungus strain 28 isolated from Houttuynia cordata possesses wide-spectrum antifungal activity. Braz J Microbiol 2016;47:480-8.

16. Manandhar S, Luitel S, Dahal RK. In vitro antimicrobial activity of some medicinal plants against human pathogenic bacteria. J Trop Med 2019;2019:1895340:1-6.

17. Handayani D, Ananda N, Artasasta A, Ruslan R, Fdriyanti O, Tallei TE. Antimicrobial activity screening of endophytic fungi extracts isolated from brown algae Padina sp. J Appl Pharm Sci 2019;9:9-13.

18. Wang L, Ren L, Li C, Gao C, Liu X, Wang M, et al. Effects of endophytic fungi diversity in different coniferous species on the colonization of Sirex noctilio (Hymenoptera: Siricidae). Sci Rep 2019;9:5077.

19. Bhushan S, Lee WH, Han SK, Sung JM. Observations on some of the mycelial growth and pigment characteristics of Cordyceps militaris isolates. Mycobiol 2006;34:83-91.

20. Du W, Yao Z, Li J, Sun C, Xia J, Wang B, et al. Diversity and antimicrobial activity of endophytic fungi isolated from Securinega suffruticosa in the yellow river delta. PLoS One 2020;15:e0229589.

21. Dos Santos IP, da Silva LC, da Silva MV, de Araújo JM, Cavalcanti Mda S, Lima VL. Antibacterial activity of endophytic fungi from leaves of Indigofera suffruticosa Miller (Fabaceae). Front Microbiol 2015;6:350.

22. Lappa IK, Kizis D, Natskoulis PI, Panagou EZ. Comparative study of growth responses and screening of inter-specific OTA production kinetics by A. carbonarius isolated from grapes. Front Microbiol 2015;6:502.