CHAPTER III

MATERIALS AND METHODS

Materials

1. Media

- Agar (Merck, Germany)
- Corn meal agar (Difco, USA)
- Czapek solution agar (Difco, USA)
- Malt extract (Difco, USA)
- Mueller-Hinton agar (Difco, USA)
- Nutrient agar (Difco, USA)
- Nutrient broth (Difco, USA)
- Potato dextrose agar (Merck, Germany)
- Sabouraud's dextrose agar (Difco, USA)
- Tryptic soy broth (Difco, USA)
- Yeast extract (Difco, USA)

2. Chemicals

- Dichloromethane (Lab-Scan, Thailand)
- Dimethylsulfoxide (DMSO) (Merck, USA)
- D-Glucose (Serva, USA)
- Sodium Chloride (Merck, Germany)
- Sodium hypochlorite (Kao Industrial, Thailand)
- Sucrose (Commercial grade)
- Tween 80 (Acros, Belgium)

3. Instrument

- Analytical balance (Sartorious, USA)
- Autoclave (Hirayama, Japan)
- Centrifuge (Clay-Adams Inc, USA)

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- Deep freezer –85 ^{0}C (Forma Scientific, USA)
- Freezer –20 ^{0}C (Tropical Ariston, Italy)
- Freeze dryer (FTS, USA)
- Hot air oven (Precision, USA)
- Incubator (Mammert, Germany)
- Light microscope (Olympus, Japan)
- Magnetic stirrer (Thomas scientific, USA)
- Micropipette (Gilson, France)
- Stereomicroscope (Olympus, Japan)

4. Glassware

- Beaker (Pyrex, USA)
- Erlenmeyer flask (Pyrex, USA)
- Measuring cylinders (Pyrex, USA)
- Measuring pipette (HBG, Germany)
- Pasteur pipette (Brand, Germany)
- Petri dishes (Anumba, Germany)
- Seperating funnel (Pyrex, USA)
- Test tubes (Pyrex, USA)

5. Others

- Aluminium foils (Diamond foil, USA)
- Disposable gloves (Imperial, Thailand)
- Membrane filter (Gelman Sciences, USA)
- Microtiter plate (Nunclon, Denmark)
- Sensitivity disc
    - Bacitracin (B-10) (Oxoid, England)
    - Chloramphenicol (C-30) (Oxoid, England)
    - Erythromycin (E-15) (Oxoid, England)
    - Kanamycin (K-30) (Oxoid, England)
    - Penicillin (P-10) (Oxoid, England)
    - Polymyxin B (PB-300) (Oxoid, England)

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- Tetracycline (Te-30) (Oxoid, England)

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Methods

1. Collection of Plant Samples

Healthy leaves and limbs were collected from 10 species of Thai medicinal plants in the botanical garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand on March 17, 1999.

2. Isolation of Endophytic Fungi

After cleaning by the running tap water, the plant samples were surface sterilized as previously described (31). Cleaned leaves and limbs were submerged consecutively in 70% ethanol for 1 minute, sodium hypochlorite solution (6%) for 5 minutes followed by 1 minute wash in sterile distilled water for 2 times. Aseptically, the surface-sterilized leaves and limbs were cut into small pieces and placed on water agar. The water agar plates were incubated at 30 °C. The hyphal tip of growth fungus emerging from the plant tissue was isolated by cutting and picking with a sterile pasture pipette and was transferred to grow on half-strength potato dextrose agar (PDA). Purity of the isolated culture was determined from the homogeneity of colony morphology.

3. Preservation of Endophytic Fungus Isolates (32)

3.1 Storage under Liquid Paraffin

Endophytic fungi were grown on corn meal malt extract agar slant (CMMA) at room temperature for 7 days or until they grew cover the agar slant. This was done in duplicate. After that, the slant culture was added with sterile liquid paraffin to cover the surface and kept at 4 °C.

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3.2 Storage in 15% Glycerol

Endophytic fungi grown on PDA were cut into 5×5 mm^2. The agar blocks were put into cryotube containing 800 μl of sterile 15% glycerol and preserved at –70 °C.

4. Screening of Endophytic Fungus Isolates for their Antimicrobial Activities by Dual-Culture Agar Diffusion Assay (31)

4.1 Test Microorganisms

Bacterial strains

- *Staphylococcus aureus* ATCC 29213
- *Enterococcus faecalis* ATCC 29212
- *Bacillus subtilis* ATCC 6633
- *Escherichia coli* ATCC 25922
- *Pseudomonas aeruginosa* ATCC 27953

Fungal strains

- *Candida albicans* ATCC 10231
- *Saccharomyces cerevisiae* ATCC 9763
- *Trichophyton mentagrophytes* (Clinical isolate)

4.2 Cultivation of Endophytic Fungus Isolates

Endophytic fungus isolates were grown on Czapek yeast autolysate agar (CzYA), malt Czapek agar (MCzA), malt extract agar (MEA), Sabouraud's dextrose agar (SDA), and yeast extract sucrose agar (YES) at room temperature for 14 days. The agar culture was cut into small cube of 1×1 cm^2.

4.3 Preparation of Inoculum

Test bacteria were grown in tryptic soy broth (TSB) at 37 °C for 2-3 h. The bacterial cultures were adjusted with sterile normal saline to give turbidity matched with that of 0.5 McFarland standard.

C. albicans, *S. cerevisiae* and *T. mentagrophytes* were grown on SDA slant at 30 °C for 24 h, 24 h and 5 days, respectively. *C. albicans* and *S. cerevisiae* were suspended in 0.85% NaCl. *T. mentagrophyte* conidia were suspended in 0.05% Tween 80 solution. The turbidity of each fungal suspension was adjusted with respective solution to match that of 0.5 McFarland standard.

4.4 Testing for Antimicrobial Activity

A sterile cotton swab was dipped into the microbial suspension and pressed lightly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab was then streaked over the entire surface of the agar plate three times, with the plate rotated approximately 60 ° each time to ensure an even distribution of the inoculum. The agar blocks, from section 4.2, were distributed evenly on the surface. The plates were left at room temperature for 1 h. Bacterial inoculated plates were incubated at 37 °C for 18-24 h. Fungal inoculated plates were incubated at 30 °C for 24 and 72 h for yeast and mold, respectively. The inhibition zone diameter was measured with a vernier caliper.

5. Determination of Stability of Antimicrobial Phenotype

All active endophytic fungus isolates in the stock culture were retrieved and grown on PDA. After incubation at 30 °C for 7-10 days, the pure cultures were transferred to grow on respective medium that provided the highest activity against *S. aureus*, *E. coli*, *C. albicans*, or *S. cerevisiae.* After cultivation for 14 days at 30 °C, the fungal cultures were tested for antimicrobial activities by dual-culture agar diffusion assay as described previously in section 4.

6. Selection of Low Susceptible Microbial Strains

Antibiotics used for selection of low susceptible *S. aureus* were bacitracin, cephalosporin C, chloramphenicol, D-cycloserine, erythromycin, kanamycin, lincomycin, penicillin G, rifampin, and tetracycline. In selection of low susceptible *E. coli*, cephalosporin C, chloramphenicol, D-cycloserine, kanamycin, polymyxin B, rifampin, and tetracycline were used. For *C. albicans* and *S. cerevisiae*, strains with low susceptibility to nystatin were selected.

6.1 Determination of Minimum Inhibitory Concentrations (MICs) of Antibiotics for Reference Strains

MICs of antibiotics described above for *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *C. albicans* ATCC 10231, and *S. cerevisiae* ATCC 9763 were determined by broth microdilution method (33).

6.1.1 Preparation of Diluted Antibiotics

Each antibiotic was solubilized in a final concentration of 1.024 mg/ml in Mueller-Hinton broth (MHB) except that cephalosprorin C and nystatin were solubilized in dimethylsulfoxide (DMSO) in a final concetration of 102.4 mg/ml. Cephalosporin C and nystatin solution were further diluted 1:100 in MHB and RPMI 1640 (+2% glucose), respectively. Then, each antibiotic solution was diluted in serial two-fold dilutions in the respective medium to give the concentrations as shown in Table 1. A 50-μl volume of each dilution except nystatin solution was dispensed into the corresponding wells of each microtitre plate (96 well, flat shape). Nystatin solution was dispensed in a volume of 100 μl. A 50-μl volume of MHB containing no antibiotic was dispensed into one well for a growth control of each antibiotic except cephalosporin and nystatin. A growth control for cephalosporin C was a 50-μl volume of MHB containing 1% of DMSO. A 100-μl volume of RPMI 1640 (+2% glucose) containing 1% of DMSO was dispensed into a growth control well for nystatin. For a sterility control well, 100 μl of respective medium was dispensed. These experiments were done in duplicate.

6.1.2 Preparation of Inoculum

The final bacterial inoculum concentration was 5×10^5 CFU/ml. Each bacterial strain was grown in TSB at 37 °C for 2-3 h. Turbidity of the bacterial culture was adjusted with normal saline to match that of 0.5 McFarland standard. The adjusted bacterial suspension was further diluted in MHB to obtain 10^6 CFU/ml.

For yeast, the test organisms were grown on Sabouraud's dextrose agar at 30 °C for 24 h. The inoculum suspension was prepared by picking 5 colonies of 24 h culture and suspending in 5 ml of RPMI 1640 (+2% glucose). The turbidity of the cell suspension was adjusted with RPMI 1640 (+2% glucose) to match that of 0.5 McFarland standard. The adjusted suspension was then diluted 1:1,000 in RPMI 1640 (+2% glucose) to provide 1×10^3-5×10^3 CFU/ml.

Table 1. Ranges of antibiotic concentrations used in determination of MICs for reference and low susceptible strains

Antibiotics	Concentrations (μg/ml)	
	Reference strains	Low susceptible strains
Bacitracin	1,024-2	32,768-64
Cephalosporin C	1,024-2	32,768-64
Chloramphenicol	1,024-2	2,048-4
D-cycloserine	1,024-2	2,048-4
Erythromycin	256-0.5	32,768-64
Kanamycin	1,024-2	1,024-2
Lincomycin	1,024-2	2,048-4
Nystatin	256-0.5	256-0.5
Penicillin G	256-0.5	1,024-2
Polymyxin B	1,024-2	1,024-2
Rifampin	2-0.0039 (for testing *S. aureus*) 1,024-2 (for testing *E. coli*)	32,768-64
Tetracycline	128-0.25	1,024-2

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6.1.3 Inoculation of Broth in Microdilution Tray

For tray containing antibacterial antibiotics, each well was inoculated with 50 μl of bacterial inoculum except the sterility control well. The microdilution plates were incubated at 37 °C for 18 h. Then, 20 μl of 0.5 mg/ml p-iodonitrotetrazolium violet (INT) solution was added into each well. The microdilution plates were further incubated for 1 h. The violet color of formazan developed indicates the microbial growth. The lowest concentration of antibiotic that inhibited the visible growth of test microorganism was regarded as the MIC. For

nystatin tray, the amount of yeast inoculum dispensed into each well except the sterility control well was 100 µl. After incubation of the inoculated plate at 30 °C for 24 h, INT solution (0.5 mg/ml) was added into each well in a volume of 20 µl. The microdilution plate was further incubated overnight before being read.

6.2 Selection of Low Susceptible Strains by Gradient Plate Technique (10)

Plate containing gradient concentration of antibiotic was prepared with two layers of agar. The lower layer consisted of 10 ml of plain nutrient agar for bacteria and 10 ml of Sabouraud's dextrose agar (SDA) for yeasts. After placing the dish in the normal horizontal position, another 10 ml of agar containing antibiotic in a concentration of 3×MIC was added. The heavy suspension of microbial strain was spread over the surface of the agar. After incubation at 37 °C for 24 h, the colony grown beyond the boundary of confluent growth was selected. Then, the low susceptible microbial strain was resuspended in broth and spreaded on another gradient plate that contained upper agar layer with antibiotic in higher concentration than 3×MIC. This was done consecutively until the low susceptible colony could be selected from plate containing antibiotic in concentration of 10×MIC or higher, as shown in Table 2. The selected microbial strains were kept in 15% glycerol at –70 °C.

7. Determination of Susceptibility of Selected Mutants

Susceptibility of mutants selected in section 6 was tested by broth microdilution method and agar diffusion assay (32).

7.1 Broth Microdilution Method

Susceptibility test of selected mutants by broth microdilution method was done as previously described in section 6.1, except that each selected mutant was grown in 5 ml of medium containing respective antibiotic in the concentration that was used in final selection of mutant in section 6. After incubation at 37 °C for 2-3 h, the bacterial culture was centrifuged at 3,000 rpm for 10 min. Then, the cell pellet was washed and resuspended in MHB to give cell suspension with turbidity matched with that of 0.5 McFarland standard. The adjusted suspension was further diluted and

used as described in section 6.1.2 and 6.1.3. Concentrations of antibiotics used in this experiment were shown in Table 1.

Table 2 Final concentrations of antibiotics (×MIC) used in selection of low susceptible mutants*

Antibiotics	Final concentrations (×MIC)	
	S. aureus	*E. coli*
Bacitracin	10	ND**
Cephalosporin C	10	14
Chloramphenicol	10	10
D-cycloserine	10	10
Erythromycin	10	ND**
Kanamycin	14	14
Lincomycin	10	ND**
Penicillin G	16	ND**
Polymyxin B	ND**	14
Rifampin	10	10
Tetracycline	20	10

* For selection of low susceptible mutants of *C. albicans* and *S. cerevisiae*, the final concentration was 10×MIC.

** Not determined

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7.2 Agar Diffusion Assay

7.2.1 Test Bacteria

S. aureus ATCC 29213, *E. coli* ATCC 25922, and low susceptible strains selected in section 5.2 were used.

7.2.2 Preparation of Inoculum

The inocula were prepared as described previously in section 4.3 for reference strains and section 7.1 for low susceptible mutants.

7.2.3 Antibiotic Disks

Commercially available antibiotic disks used in susceptibility tests of *S. aureus* ATCC 29213 and the low susceptible strains were bacitracin (B-10), chloramphenicol (C-30), erythromycin (E-15), kanamycin (K-30), penicillin G (P-10), and tetracycline (Te-30). For *E. coli* ATCC 25922 and the low susceptible strains, commercially available chloramphenicol (C-30), kanamycin (K-30), polymyxin B (PB-300) and tetracycline (Te-30) disks were used.

For cephalosporin C, D-cycloserine and lincomycin, each antibiotic solution in a volume of 20 μl were applied directly on sterile blank disk (diameter 9 mm.) placed on inoculated agar to yield 100, 100 and 10 μg per disk, respectively. For rifampin, disks containing 0.75 μg/disk and 75 μg/disk were prepared. Cephalosporin C, D-cycloserine, lincomycin and rifampin (0.75 μg) were used in susceptibility test for *S. aureus* ATCC 29213 and the low susceptible strains. For *E. coli* ATCC 25922 and the low susceptible strains, cephalosporin C, D-cycloserine and rifampin (75 μg) were used in susceptibility test.

7.2.4 Susceptibility Test

Fifteen ml of MHA was poured into a plastic petri dish and was allowed to cool to room temperature. Streaking method as described previously in section 4.4 was used to inoculate the bacterial culture onto the agar surface. The commercially available antibiotic disk and blank disk were distributed evenly on the agar surface. Antibiotic solution as mentioned in 7.2.3 was applied to the blank disk.

Each experiment was done in duplicate. After incubation at 37 °C for 16-18 h. The inhibition zone diameters were examined by a vernier caliper

8. Determination of Antimicrobial Activities of Endophytic Fungi against Low Susceptible Strains

Endophytic fungi exhibiting stable antimicrobial activities against reference strains, as determined in section 5, were grown on the medium that provided the highest activity as shown in Table 9 in Appendix. After incubating at 30 °C for 14 days, they were tested for their activities against the low susceptible test strains by dual-culture agar diffusion assay as previously described in section 4.

9. Determination of MICs of Crude Extracts Obtained from Culture Broths of Selected Endophytic Fungus Isolates

The media YES, MEB, MCzB, MEB and MCzB were used in cultivation of isolates *Bore* 04, *Ccoc* 08A, *Cfis* 01A, *Line* 13 and *Oind* 05A. Each fungus isolate that had activity against low susceptible strains were grown in 400 ml of respective broth contained in 2-L Erlenmeyer flask under standstill condition at 30 °C for 3 weeks. The fungal broth culture was filtered and subsequently extracted three times with equal volume of dichloromethane. The organic phase was taken and evaporated by using rotary evaporator. The residue was dissolved in DMSO. MICs of the crude extract for reference strains and the low susceptible strains were determined by broth microdilution assay as described in section 6.1 and 7.1, respectively.

10. Identification of Selected Endophytic Fungus Isolates

Endophytic fungus isolates *Bore* 04, *Ccoc* 08, *Cfis* 01A, *Line* 13 and *Oind* 05A that showed activities against the low susceptible strains were identified by conventional method based on morphology. The selected fungi were grown on PDA, banana leaf agar and banana leaf agar with 1% vitamin B complex at 30 °C for 2 months under the condition of 12 h darkness / 12 h black light (near UV light). The fungal culture was examined for conidia or spore production. Slide cultures was done to observed morphology of endophytic fungus isolates producing conidia (34). A small block of PDA (about $8\times8\times4$ mm^3) was placed in the center of a sterile slide and each of four sides was inoculated with the endophytic fungus isolate. A sterile

coverglass was placed on the top of the block and 6 ml sterile water was added in petri dish. Slide culture set was incubated in room temperature until the endophytic fungus isolate grew on the coverglass and on the slide. Slide and coverglass with growing endophytic fungus isolate was mounted with lactophenol cotton blue. The microscopic morphology of endophytic fungus isolate was observed under light microscope.

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