CHAPTER III

MATERIALS AND METHODS

3.1 Instruments and equipments

3.1.1 Ultraviolet-visible Spectrophotometer (UV-VIS)

The UV-VIS spectra were recorded on a Shimadzu UV-vis 2001s and a Varian CARY 1E UV-visible spectrophotometer.

3.1.2 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on Perkin Elmer Spectrum One and a Bruker VECTOR 22 spectrometer. Solid samples were generally examined by incorporating the sample with Universal Attenuted Total Reflectance (UATR)

3.1.3 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, NOESY, HMQC, and HMBC spectra were recorded on a Bruker AM-400 (400 MHz), operating at 400 MHz for protons and 100 MHz for carbons. Deuterated solvents; chloroform-d_1 ($CDCl_3$), deuterium oxide (D_2O), acetone-d_6 (CD_3COCD_3) and dimethylsulfoxide (DMSO-d_6) were used in NMR experiments.

3.1.4 Mass spectrometer

The mass spectra were obtained from Finnigan Mat GCQ mass spectrometer.

3.1.5 Melting point

The melting points were examined using a Büchi 535 melting point apparatus.

3.1.6 Optical rotations

Optical rotations were measured either in MeOH with sodium D line (589 nm) JASCO DIP-370 digital polarimeter.

3.2 Chemical reagents

3.2.1 Solvents

All commercial grade solvents, used in this research such as hexane, dichloromethane, ethyl acetate, acetone and methanol, were purified by distillation prior to use. The reagent grade solvents were used for re-crystallization.

3.2.2 Other chemicals

1. Sephadex LH-20 (No. 17-009-01)
2. Merck's silica gel 60 Art. 1.07734.1000 (70-230 mesh ASTM) was used as adsorbents for column chromatography.
3. Merck's TLC aluminum sheets, silica gel 60 PF_{254} precoated 25 sheets, 20x20 cm^2, layer thickness 0.2 mm were used for thin layer chromatography.

3.3 Culture media

Potatoes Dextrose Agar (PDA) was used for the endophytic fungi isolation. Malt Czapek Agar (MCzA), Malt Extract Agar (MEA), PDA, Sabouraud's Dextrose Agar (SDA), Yeast Czapek Agar (YCz), and Yeast Extract Sucrose Agar (YES) were used for observation morphology and determination antimicrobial activities of isolated endophytic fungi.

The media's formula are shown in the Appendix A.

3.4 Collection of plant samples

Healthy leaves and twigs of *Hydnocarpus anthelminthicus* Peirre ex. were gathered from the Central Botanical Garden (Pukae), Saraburi Province, Thailand. The fresh-cut ends of plant samples were wrapped with a parafin film and they were placed in zip-lock plastic bags and stored in a refrigerator prior to isolation of endophytic fungi. The plant samples were used for the fungal isolation within 72 h after collection.

3.5 Isolation of endophytic fungi

Samples were cleaned under running tap water and then air-dried. Before surface sterilization, the cleaned stems were cut into small pieces of ca. 5 cm long leaves and limb fragments were sterilized in 70% ethanol for 1 min, 5% sodium hypochlorite solution for 5 min, sterile distilled water for 1 min two times and air-dried in a laminar flow chamber. The surface-sterilized leaves and stems were cut into small pieces using a sterile blade and transfer to sterile water agar plates for incubation at 30°C. The hyphal tip of endophytic fungus growing out from the plant tissue was cut by a sterile pasture pipette and transferred to a sterile potato dextrose agar (PDA) plate. Afer incubation at 30°C for 7-14 days, culture purity was determined from colony morphology (Strobel and Torczynski, 1997; Helander *et al.*, 1994).

3.6 Preservation of endophytic fungi

The isolated fungal endophyte were kept at 4°C by storage as agar slants under liquid paraffin or as agar blocks in sterile water (Smith and Onions, 1994).

3.6.1 Storage under liquid paraffin

The isolated fungal endophyte were grown on corn meal malt extract agar slant at 25°C for 10 days. The mature cultures were then covered up to 10 mm

height with sterile liquid paraffin and sealed. The liquid paraffin was steriled and by autoclaving twice at 121°C for 15 min.

3.6.2 Storage in water

Six pieces (6×6 mm^2) of the grown culture cut from the plate were put in vials containing 1 mL sterile distilled water, sealed, and kept at 4°C.

3.7 Screening of endophytic fungi for anti-*Candida albicans* activity

3.7.1 Preparation of endophytic fungi for anti-*C. albicans* activity

Each the isolated fungal endophyte were cultivated on six media: Malt Czapek Agar (MCzA), Malt Extract Agar (MEA), Potatoes Dextrose Agar (PDA), Sabouraud's Dextrose Agar (SDA), Yeast Czapek Agar (YCz), and Yeast Extract Sucrose agar (YES) at room temperature (25-30°C) for 14 days. Then the agar cultures of each fungal endophyte isolate that grew on each medium were photographed, cut into a piece, and put on the disk with a flamed 7 mm cork borer and were removed from cork borer hole by a sterile needle.

3.7.2 Preparation of *C. albicans* tested inoculum

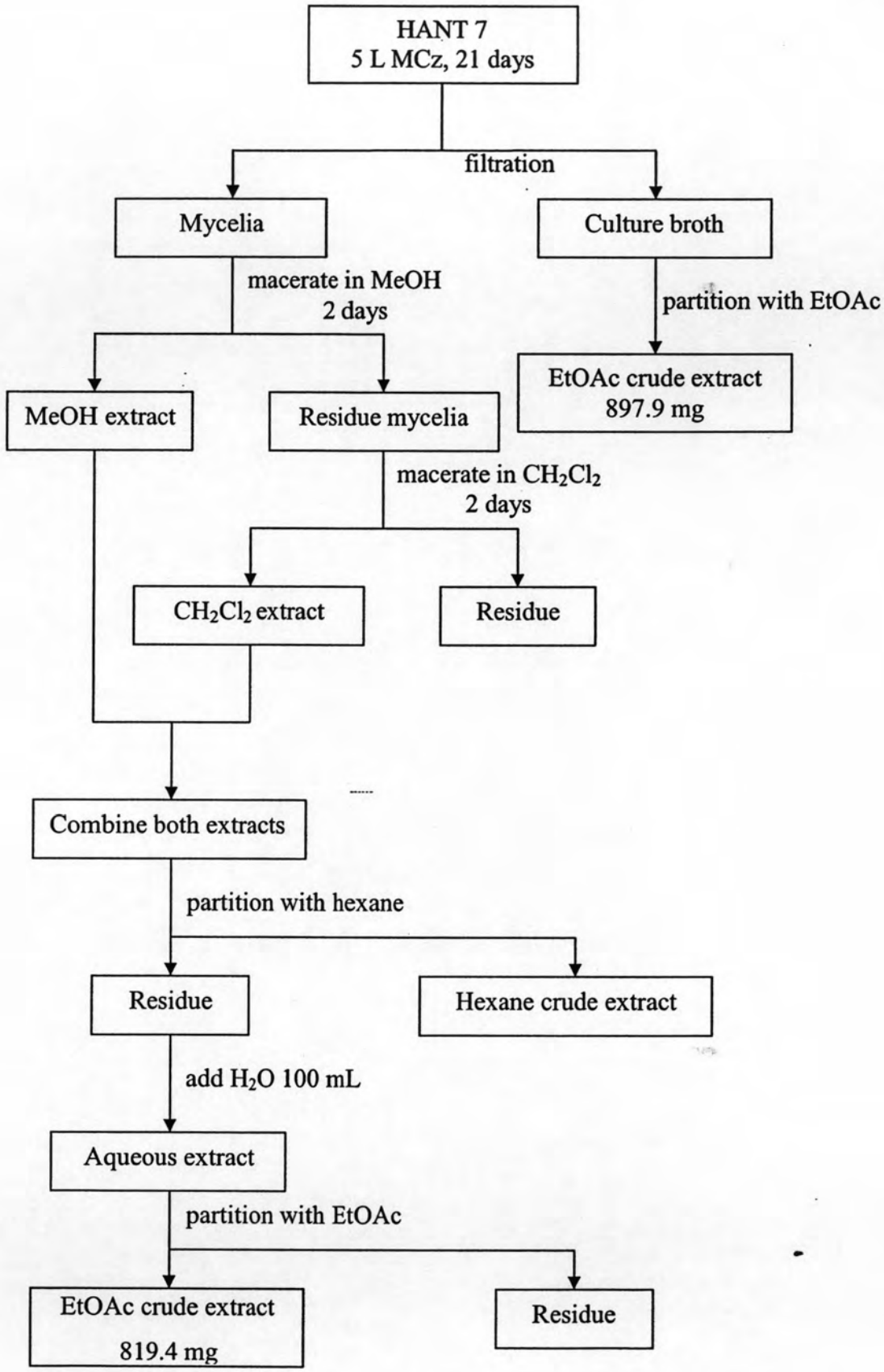
C. albicans was grown on Sabouraud's Dextrose Agar (SDA) for 24-48 h at room temperature (25-30°C). Inocula should be prepared by the spectrophotometric method. The inoculum suspension is prepared by picking four or five colonies, each at least 1 mm in diameter, with a sterile wire loop and suspension the material in a tube containing 5 mL of sterile normal saline solution (0.85% $NaCl_2$). The turbidity of the cell suspension was adjusted with normal saline solution to match the turbidity of 0.5 McFarland Standard (OD 0.1 at 625 nm).

3.7.3 Testing for anti- *C. albicans* activity

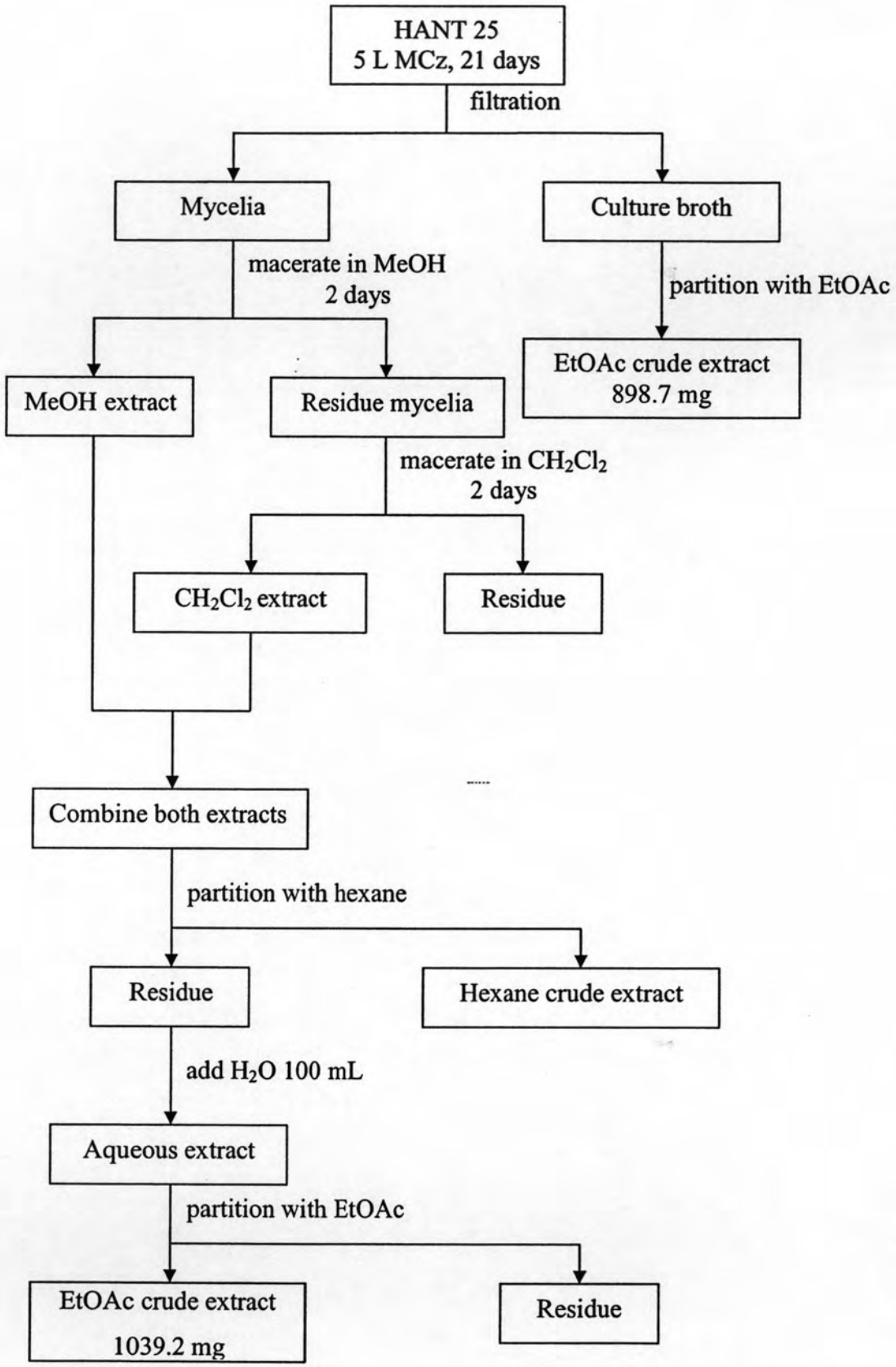
A sterile cotton swab was dipped into the *C. albicans* suspension and presses lightly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab was then streaked over the entire surface of the agar plate three times, with the plate rotated approximately 60° each time to ensure an even distribution of the inoculum. The agar block, from the section 3.6.1, were distributed evenly on the surface. Cellulose disk impregnated with the 100 μg ketoconazole and 25.6 μg nystatin were placed onto the center of agar plate. The plates were incubated at 37°C for 24 h. The inhibition zones around the either agar block or combination of drug and agar block were measured in mm with a ruler.

3.8 Fermentation and extraction

Endophytic fungal isolates HANT 7 and HANT 25 were grown on PDA plate at 30°C for 7 days. Six pieces (6×6 mm^2) of the grown culture cut from the plate were incubated into a 1000 ml Erlenmeyer flask containing 200 mL of malt Czapek (MCz) broth or yeast extract sucrose (YES) broth (Paterson & Bridge 1994). After incubation at 25°C for 21 days under stationary condition, the fungal culture was filtered to remove mycelium. The filtrate broth was partitioned with an equal volume of EtOAc for three times to obtain broth crude extract. The mycelia were sequentially macerated in methanol and dichloromethane, respectively, at room temperature each for 2 days. Both solutions were evaporated under reduced pressure and partitioned with an equal volume of hexane for three times. Then, the residue was added with 100 ml H_2O and partitioned with EtOAc to obtain mycelia crude extract (Scheme 3.1 and Scheme 3.2).



Scheme 3.1 Extraction of culture broth and mycelia of endophytic fungal isolates HANT 7.



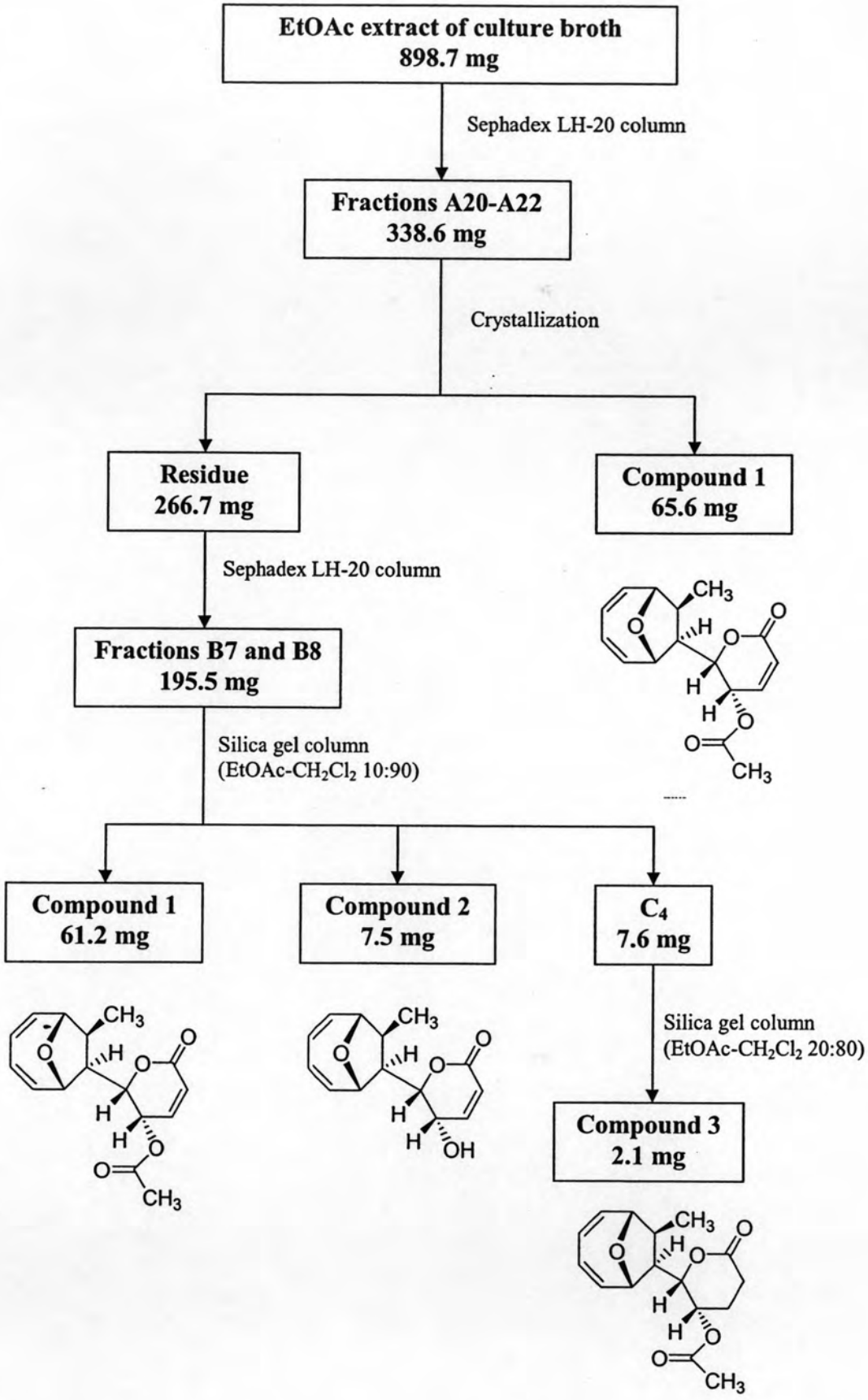
Scheme 3.2 Extraction of culture broth and mycelia of endophytic fungal isolates HANT 25.

3.9 Separation of fungal secondary metabolites

3.9.1 Separation of broth extract of endophytic fungal isolate HANT 25

The broth extract of endophytic fungal isolate HANT 25 (898.7 mg) was chromatographed on Sephadex LH-20 column, eluted with 100% methanol. Each fraction was analyzed by TLC and ^{1}H-NMR spectrum. Fractions A20-A22 were combined (338.6 mg) and re-crystallization in methanol to obtain compound **1** (65.6 mg, 7.3% yield) as a colorless needle. Then, the mother liquor (266.7 mg) was subsequently chromatographed on Sephadex LH-20 column, eluted with 100% methanol. Fractions B_7 and B_8 were combined and subjected to further column chromatography over silica gel, eluting with 10:90 mixture of EtOAc/CH_2Cl_2, to obtain compound **1** (61.2 mg, 6.8% yield) and compound **2** (7.5 mg, 0.8% yield) as a white feather-like crystal. Faction C4 was repeatedly chromatographed on silica gel, eluting with 20:80 mixture of EtOAc/CH_2Cl_2, to obtain compound **3** (2.1 mg, 0.2% yield) as a colorless needle.

The isolation of the broth crude of the endophytic fungal isolate HANT 25 is briefly summarized in Scheme 3.3.

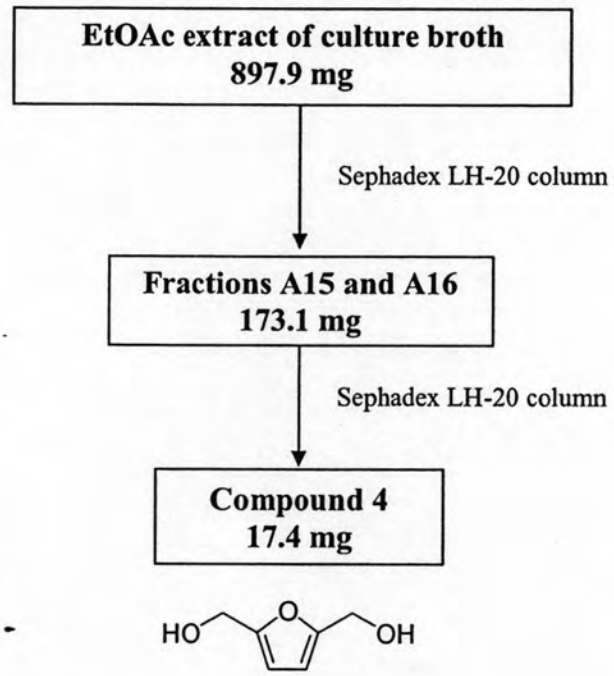


Scheme 3.3 The isolation procedure of a broth extract of the endophytic fungal isolate HANT 25.

3.9.2 Separation of a broth extract of the endophytic fungal isolate HANT 7

The broth extract of the endophytic fungal isolate HANT 7 (897.9 mg) was chromatographed on Sephadex LH-20 column, eluted with 100% methanol. Factions A15 and A16 were combined and repeatedly chromatographed on Sephadex LH-20 column, eluted with 100% methanol, to obtain compound **4** (17.4 mg, 1.9% yield) as a brown oil.

The isolation of the broth extract of the endophytic fungal isolate HANT 7 is briefly summarized in Scheme 3.4.



Scheme 3.4 The isolation procedure of a broth extract of the endophytic fungal isolate HANT 7.

3.10 Physico-chemical properties of the isolated compounds from endophytic fungal isolates HANT 7 and HANT 25 from *Hydnocarpus anthelminthicus* Pierre.

3.10.1 Physico-chemical properties of compound 1

Compound **1** was obtained as a colorless needle (126.8 mg, 14.1% yield); mp. 189-190°C; $[\alpha]_D^{29}$ +303 (*c* 0.310, MeOH); UV (MeOH) λ_{max} (log ε) 257.5 (3.70), 221.3 (3.66).

ESI-TOFMS *m/z*: Figure C1 in appendix C

291.1232 $[M+H]^+$ (calcd 291. 1232 for $C_{16}H_{19}O_5$)

IR (UATR-solid) ν_{max} cm^{-1}: Figure C2 in appendix C, Table 4.2

1738, 1719, 1366, 1231, 1022, 734, 691

^{1}H-NMR δ ppm, 400 MHz, $CDCl_3$: Figures C3 and C4 in appendix C, Table 4.3

7.04 (1H, dd, *J* = 9.7 and 6.0 Hz, H-3), 6.23 (1H, d, *J* = 9.7 Hz, H-2), 6.10 (1H, m, H-11), 6.03 (1H, m, H-8), 5.92 (1H, m, H-10), 5.90 (1H, m, H-9), 5.09 (1H, dd, *J* = 6.0 and 2.4 Hz, H-4), 4.52 (1H, t, *J* = 5.8 Hz, H-7), 4.51 (1H, dd, *J* = 11.0 and 2.4 Hz, H-5), 4.29 (1H, d, *J* = 4.5 Hz, H-12), 3.05 (1H, m, H-6), 3.01 (1H, m, H-13), 2.03 (3H, s, H-16), and 1.14 (3H, d, *J* = 6.8 Hz, H-14).

^{13}C- NMR δ ppm, 100 MHz, $CDCl_3$: Figure C5 in appendix C, Table 4.3

170.0 (C-15), 162.20 (C-1), 140.2 (C-3), 137.4 (C-11), 136.9 (C-8), 126.2 (C-9), 124.9 (C-10), 124.4 (C-2), 86.3 (C-12), 77.6 (C-5), 75.8 (C-7), 63.1 (C-4), 52.6 (C-13), 50.0 (C-6), 20.6 (C-16), and 14.1 (C-14).

3.10.2 Physico-chemical properties of compound 2

Compound **2** was obtained as a white feather-like crystal (7.5 mg, 0.8% yield); $[\alpha]_D^{30}$ +80 (*c* 0.595, MeOH); UV (MeOH) λ_{max} (log ε) 264 (3.60), 222 (3.52).

ESI-TOFMS *m/z*: Figure C16 in appendix C

249.1117 $[M+H]^+$ (calcd 249.1127 for $C_{14}H_{17}O_4$)

IR (UATR-solid) ν_{max} cm^{-1}: Figure C17 in appendix C, Table 4.5

3351, 1714, 1380, 1259, 1018, 691

^{1}H-NMR δ ppm, 400 MHz, $CDCl_3$: Figures C18 and C19 in appendix C, Table 4.6

7.05 (1H, dd, *J* = 9.7 and 6.0 Hz, H-3), 6.26 (1H, m, H-8), 6.14 (1H, d, *J* = 9.7 Hz, H-2), 6.13 (1H, m, H-11), 5.97 (1H, m, H-9), 5.92 (1H, m, H-10), 4.44 (1H, t, *J* = 5.6 Hz, H-7), 4.35 (1H, dd, *J* = 10.6 and 2.4 Hz, H-5), 4.33 (1H, bd, *J* = 2.5 Hz, H-12), 4.09 (1H, dd, *J* = 6.1 and 2.3 Hz, H-4), 3.06 (1H, ddd, *J* = 10.6, 7.0, and 5.6 Hz, H-6), 2.98 (1H, qn d, *J* = 6.7 and 1.3 Hz, H-13), and 1.14 (3H, d, *J* = 7.0 Hz, H-14).

^{13}C-NMR δ ppm, 100 MHz, $CDCl_3$: Figure C20, Table 4.6

163.1 (C-1), 144.1 (C-3), 137.3 (C-11), 137.2 (C-8), 126.3 (C-9), 124.8 (C-10), 123.1 (C-2), 86.1(C-12), 79.3 (C-5), 76.8 (C-7), 62.0 (C-4), 51.7 (C-6), 51.6 (C-13), and 14.2 (C-14).

3.10.3 Physico-chemical properties of compound 3

Compound **3** was obtained as a colorless needle (2.1 mg, 0.2% yield); $[\alpha]_D^{22}$ +21 (*c* 0.180, MeOH); UV (MeOH) λ_{max} (log ε) 258 (3.52).

ESI-TOFMS *m/z*: Figure C30 in appendix C

293.1389 $[M+H]^+$ (calcd 293.1389 for $C_{16}H_{21}O_5$)

IR (UATR-solid) ν_{max} cm^{-1}: Figure C31 in appendix C, Table 4.8

2921, 1733, 1457, 1437, 1373, 1235, 1041, 694

^{1}H-NMR δ ppm, 400 MHz, $CDCl_3$: Figures C32 and C33 in appendix C, Table 4.9

6.07 (1H, m, H-11), 6.06 (1H, m, H-8), 5.90 (1H, m, H-10), 5.88 (1H, m, H-9), 5.05 (1H, m, H-4), 4.38 (1H, dd, *J* = 11.0 and 1.4 Hz, H-5), 4.30 (1H, d, *J* = 4.4 Hz, H-12), 4.26 (1H, t, *J* = 6.0 Hz, H-7), 2.96 (1H, qn, *J* =7.0 Hz, H-13), 2.91 (1H, dt, *J* = 11.0 and 6.7 Hz, H-6), 2.59 (2H, dd, *J* = 6.0 and 2.8 Hz, H-2), 2.18 (2H, m, H-3), 2.04 (3H, s, H-16), and 1.12 (3H, d, *J* = 6.9 Hz, H-14).

^{13}C- NMR δ ppm, 100 MHz, $CDCl_3$: Figure C34 in appendix C, Table 4.9

170.1 (C-15), 169.4 (C-1), 137.5 (C-11), 137.3 (C-8), 126.1 (C-9), 124.4 (C-10), 86.4 (C-12), 79.5 (C-5), 75.9 (C-7), 66.7 (C-4), 52.6 (C-13), 51.2 (C-6), 25.2 (C-2), 25.1 (C-3), 21.0 (C-16), and 14.1 (C-14).

3.10.4 Physico-chemical properties of compound 4

Compound **4** was obtained as a brown oil (17.4 mg, 1.9% yield).

GC-MS *m/z*: Figure C46 in appendix C

128 [M] (calcd 128.0473 for $C_6H_8O_3$)

^{1}H-NMR δ ppm, 500 MHz, acetone-d6: Figure C47 in appendix C, Table 4.11

6.18 (H, s, H-6 and H-7), 4.47 (2H, s, H-3 and H-4)

^{13}C- NMR δ ppm, 100 MHz, acetone-d6: Figure C48 in appendix C, Table 4.11

155.0 (C-2 and C-5), 107.4(C-3 and C-4), and 56.4 (C-6 and C-7)

3.11 Classification of the endophytic fungal isolates HANT 7 and HANT 25

3.11.1 Conventional method

3.11.1.1 Macroscopic morphology

Both HANT 7 and HANT 25 isolates were grown on six different media, including Czapek yeast autolysate agar (YCz), yeast extract sucrose agar (YES), malt extract agar (MEA), potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA), and yeast extract sucrose agar (YEA). After cultivation for 14 days at room temperature they were photographed. Colony morphology of specimens such as shape, size, color, margin, pigment, and others were examined.

3.11.1.2 Microscopic morphology

Both HANT 7 and HANT 25 isolates were grown on water agar and small pieces of sterilized banana leaves at room temperature for 2 months. Fungal spores and fruiting bodies appearing on the banana leaf fragments were examined by light microscopy.

3.11.2 Molecular method

3.11.2.1 DNA extraction

Both HANT 7 and HANT 25 isolates were grown on potato dextrose broth at 25°C for 7 days. The mycelia were harvested by centrifugation and washed 3 times with sterile distilled water. The pellets were lyophilized and then ground into fine powder using a mortar and pestle. The ground powder would be further subjected to DNA extraction.

The ground mycelium was filled up to one third of a 1.5 mL microfuge tube and subjected to DNA extraction according to Lee and Taylor (1990). A 400-μL volume of lysis buffer (Appendix A) was added and the mixture was mixed with vortex until being homogeneous. The tube was then incubated at 65 °C for 1 h. A 400-μL volume of chloroform: phenol (Appendix A) was added to the mixture and

the tube was inverted several times. The mixture was centrifuged at 10,000 rpm (Sigma 202 MC) for 15 min at room temperature. The top phase containing the DNA was transferred to a new tube. Then, 10 μL of 3M sodium acetate was added to the aqueous phase followed by 0.54 volume of cold isopropanol. The tube was inverted gently and DNA precipitate was spun down at room temperature as previously for 2 min. The pellet was washed once with cold 70% ethanol before leaving dry. The DNA pellet was resuspended in100 μL Tris-EDTA (10 mM Tris HCI pH 8.0, 0.1 mM EDTA) buffer.

3.11.2.2 Polymerase chain reaction (PCR) amplification

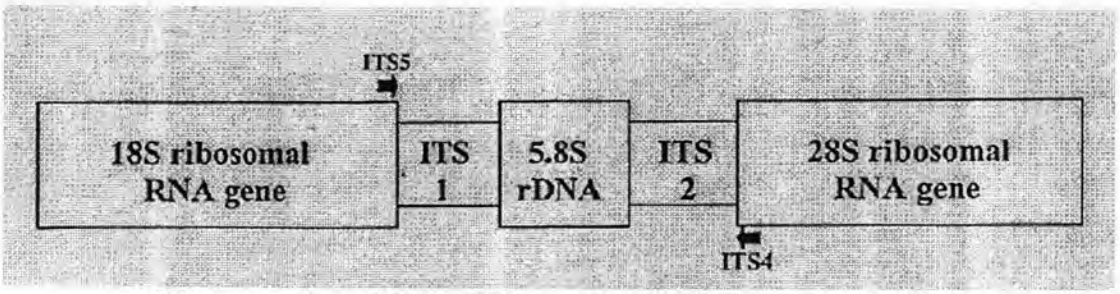
ITS1-5.8-ITS2 regions of ribosomal DNA (rDNA) (Figure 3.1) according to White *et al,* (1990). The primer sequences are shown in Table 3.1. Oligonucleotide primers were synthesized using ABI PRISM^{TM}, DNA/RNA synthesizer model 392, Perkin Elmer, by the Bioservice Unit (BSU) at the National Center for Genetic Engineering and Biotechnology (BIOTEC). The reaction mixture was prepared on ice. The amplification reaction was performed in the total volume of 50 μL:2 ng/μL of template DNA, 0.5 mM of each primer, 0.2 mM of individual dNTP, 3 mM of $MgCl_2$, 50 mM KCl, 10 mM of Tris-HCl at pH 8.8 and 1.0 U of *Taq* DNA polymerase (Appendix B). For each test, a primer negative control was included without template DNA. Ice-cold PCR reaction tubes were transferred to an Eppendrof Mastercycler Gradient PCR machine.

The thermal cycling program was as follow: 3 min initial denaturation at 95°C, followed by 30 cycles of 50s denaturation at 95°C, 40s primer annealing at 48°C, 40s extension at 72°C, and a final 10 min extension at 72°C.

Four microlitres of PCR products from each PCR reaction were examined by electrophoresis at 100V (4 V cm^{-1}) for 2 h in a 2% (w/v) agarose gel in Tris-acetate- EDTA (TEA) buffer (Appendix B) and visualized with UV light after staining with ethidium bromide (0.5 μg/mL).

3.11.2.3 DNA sequencing

PCR products were purified using minicolumns (Wizard® PCR Preps DNA Purification System, Promega) according to the manufacture's protocol. Primers ITS5 and ITS4 were used in the sequencing reactions. Both DNA strands were sequenced. Purified PCR products were sequenced using dye terminator cycle sequencing and reactions were resolved on the ABI Prism 3100 Genetic Analyzer (AME Bioscience). This sequencing process was done at the Bioservice Unit (BSU), the National Center for Genetic Engineering and Biotechnology (BIOTEC).



[Diagram adapted from: White et al. 1990 PCR protocols: 316]

Figure 3.1 Location on nuclear rDNAs of primers ITS5 and ITS4. The arrow heads represent the 3' end of each primer.

Table 3.1 Primers for amplification of ribosomal RNA genes of fungal isolate HANT 7 and isolate HANT 25.

rRNA	Gene Primer[a]	Product Size (bp)[b]	Tm (°C)[c]
Nuclear, ITS			
ITS5	GGAAGTAAAAGTCGTAACAAGG	620	65
ITS4	TCCTCCGCTTATTGATATGC		58

[a] Primer ITS5 is forward primer; ITS4 is reward primer.

[b] Product sizes are approximated on the basis of the rRNA genes of *Saccharomyces cerevisiae*; the side of the region amplified is the product size minus the primers.

[c] Tm's were calculated by the method of Meinkoth and Wahl (1988).

3.11.2.4 Phylogenetic Analysis

ITS1-5.8S-ITS2 DNA sequence was used as query sequence to search for similar sequence from GenBank using BLASTN 2.2.10 (Altschul et *al.*, 1997). The similar reference sequences with query sequences were obtained and used for subsequent phylogenetic analyses. DNA sequence alignment and identity were performed and determined, respectively, using ClustalW (1.82) multiple sequence alignment program (Thompson et *al.* 1994). The alignment results were adjusted manually where necessary to maximize alignment using BioEdit. The alignment data were subsequently used for maximum-parsimony analysis in which searches for most parsimonious trees were conducted with the heuristic search algorithms with tree-bisection- reconnection (TBR) branch swapping in PAUP® (v 4.0b10) (Swofford, 2003). For each search, 10 replicates of random stepwise sequence addition were performed and 100 trees were saved per replicate. Gaps were treated as missing data. Character states were treated as unordered. Statistical support for the internal branches was estimated by bootstrap analysis with 1000 replications.

3.12 Determination of biological activities

Determination of biological activities were performed by the Bioassay Research Facility (BRF), the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathumthani, Thailand and Chulabhorn Research Institute (CRI), Vibhavadee-Rangsit Highway, Laksi, Bangkok, Thailand. Brief methods of each assay were shown below.

3.12.1 Cytotoxicity assay

Cytotoxic assay was performed using the method as previously described. Briefly, cell lines suspended in RPMI 1640 containing 10% FBS were seeded at 1×10^4 cells (100 μL) per well in 96-well plate, and incubated in humidified atmosphere, 95% air, 5% CO_2 at 37°C. After 24 h, additional medium (100 μL) containing the test compound and vehicle was added to a final concentration of 50 μg/mL, 0.2% DMSO, and further incubated for 3 days. Cells were subsequently

fixed with 95% EtOH, stained with crystal violet solution, and lysed with a solution of 0.1N HCl in MeOH, after which absorbance was measured at 550 nm. The number of surviving cells was determined from the absorbance. Results were expressed as percent survival compared with control. Etoposide was used as the reference compound.

3.12.2 Antifungal assay

The antifungal activity was assessed employing a colorimetric method (Scudiero *et al.,* 1988; Plumb *et al.,* 1989). *Candida albicans* (ATCC 90028) was grown on a potato dextrose agar (PDA) plate at 30°C for 3 days. Three to five single colonies were then suspended in RPMI640 and cultured in a shaking flask until cell density reaches 2 x 10^6 CFU/mL. One hundred μL of the culture was added to each well of 96 well plate containing 100 μL of test sample and incubated at 37°C for 4 h. Fifty μL of 0.5 mg/mL MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-dipheny tetrazolium bromide; thiazolyl blue) in RPMI 1640 was added to each well and incubated at 37°C for an additional 4 h. After incubation period, the microplates were spinned down at 200xg for 5 min. MTT was then removed from the wells and the formazan crystals were dissolved in 200 μL of 100% DMSO and 25 μL of Sorensen' glycine buffer. Subsequently absorbance at 570 nm was determined using the multilabel counter Victor^{3}V. Amphotericin B and 10% DMSO were used as a positive and a negative control, respectively. In our system, the IC_{50} value of the standard drug, amphotericin B, was 0.04±0.01 μg/mL (n=3).

3.12.3 Anti-Mycobacterium assay

Activity against *Mycobacterium tuberculosis* H37Rv was assessed using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). M. *tuberculosis* H37Rv was growth in 100 mL of 7H9GC containing 0.005% Tween 80. Culture was incubated in 500 mL plastic flask on a rotary shaker at 200 rpm and 37°C until they reached an optical density of 0.4-0.5 at 550 nm. Bacteria were washed and suspended in 20 mL of phosphate buffered saline and passed through an 8-μm-poresize filter to eliminate clumps. The filtrates were aliquot, stored at -80°C.

Antimicrobial susceptibility testing was performed in 96-well microplates. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial screened sample dilutions were prepared in either DMSO or distilled deionized water. The dissolved-screened samples were then diluted by Middlebrook 7H9 media containing 0.2% v/v glycerol and 1.0 g/L casitone (7H9GC), and subsequent two-fold dilutions were performed in 0.1 ml of 7H9GC in the microplates. Frozen inocula were diluted 1:100 in 7H9GC. Addition of 0.1 mL to the well resulted in final bacterial titers of about $5x10^4$ CFU/mL. Wells containing sample only were used to determine whether the tested samples themselves could reduce the dye or not. Additional control wells consisting of bacteria (B) or medium (M) were included. Plates were incubated at 37°C. Starting at day 6 of incubation, 20 μL of Alamar Blue solution and 12.5 μL of 20% Tween 80 were added to B and M wells, and plates were re-incubated at 37°C. Wells were observed at 24 h for a colour change from blue to pink. If the B wells became pink by 24 h, Alamar Blue solution was add to all testing plates. However, if a colour (blue) of M and B wells did not change, both wells were tested daily until a colour of B wells change from blue to pink. After the change of B well colour, Alamar Blue solution was subsequently added to all remaining wells. Plates were then incubated at 37°C for 24 h, and the results were recorded with a fluorescence multi-well reader (CytoFluor, Series 4000) at the excitation and emission wavelengths of 530 and 590 nm, respectively. The standard drugs, isoniazid and kanamycin sulfate, showed respective MIC values of 0.040-0.090 and 2.0- 5.0 μg/mL.

3.12.4 The *In vitro* Drug Sensitivity Test of Antimalarial Activity

Human erythrocytes (type O) infected with *P. falciparum* 94 strain, (Chloroquine resistant) was maintained in continuous culture, according to the method described by Trager (Trager, W and Jensen, J.B., 1976). RPMI 1640 culture medium (Gibco, USA) supplemented with 25 mM of HEPES (sigma, USA), 40 mg/L gentamicin sulfate (Government Pharmaceutical Organization, Thailand) and 10 mL of human serum was used in continuous culture. Before starting the experiment, *P. falciparum* culture was synchronized by using sorbitol induced hemolysis according to the method of Lambros (Lambros C. and Vanderberg J.P., 1979) to obtain only ring-infected cells and then incubated for 48 hours prior to the drug testing to avoid effect of sorbitol. The experiments were started with synchronized suspension of

0.5%-1% infected red blood cell during ring stage. Parasites were suspended with culture medium supplemented with 15% human serum to obtain 10% cell suspension. The parasite suspension was put into 96-well microculture plate; 50 μL in each well and then add 50 μL of various test drug concentrations. These parasite suspensions were incubated for 48 hours in the atmosphere of 5% CO_2 at 37°C. The percents parasitemia of control and drug-treated groups were examined by microscopic technique using methanol-fixed Giemsa stained of thin smear blood preparation.

3.12.5 Antiviral assay

The colorimetric method previously described by Skehan and Coworkers (1990) was employed for antiviral assay. Herpes simplex virus type 1 (HSV-1) was maintained in the Vero cell line (kidney fibroblast of an African green monkey), which was cultured in the Eagle's minimum essential medium (MEM) with the addition of heat inactivated fetal bovine serum (FBS) (10%) and antibiotics. The test samples were put into wells of a microtiter plate at the final concentrations ranging from 20 to 50 μg/mL. The viral HSV-1 (30 PFU) was added into 96-well plate, followed by plating of Vero cells ($1x10^5$ cells/mL); the final volume was 200 μL. After incubation at 37°C for 72 h, under 5% of CO_2 atmosphere, cells were fixed and stained, and optical density was measured at 510 nm. Under the screening conditions, the reference compound, Acyclovir, typically exhibited the antiviral HSV-1 with the IC_{50} of 2-5 μg/mL.

3.12.6 Cancer chemoprevention assay

The samples consisting of extracts of natural product were tested for their cancer chemoprevention potential. The activity to scavenge reactive 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was detected by DPPH assay. Inhibition of TPA-induced superoxide anion radical formation in differentiated human promyelocytic leukemia cells (HL-60) was analysed by cytochrome c reduction. The capacity to scavenge superoxide anion radicals generated chemically or enzymatically was tested by reduction of XTT. Peroxyl radical-scavenging ability was measured in a modified oxygen radical absorbance capacity (ORAC) assay. Inhibition of 5-

lipoxygenase was performed by measuring leukotriene metabolite. Aromatase inhibition assay was monitored via dealkylation of fluorometric substrate DBF.

3.12.6.1 Diphenyl-picryl-hydrazyl (DPPH) Assay

The DPPH assay was performed according to van Amsterdam et al. with some modifications (I). Samples were reacted with 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free radical generating formazan form which was measured photometrically. For the reaction, 195 μL of 100 μM DPPH solution was pipetted in 96-well plate. 5 μL of sample or DMSO as blank or 10 mM Vitamin C as positive control was mixed. After 37°C incubation for 30 min the optical density (OD) at 515 nm wavelength was measured by a microplate reader. Percentage of scavenging of DPPH radical (% scavenging) was calculated using below equation. The samples which contain scavenging activity higher than 50% were further analyzed the value of 50% inhibitory concentration (IC_{50}) which was calculated using the Microsoft Excel

$$\% \text{ Scavenging} = 100 - \frac{(\text{OD } \textit{sample})}{(\text{OD } \textit{control})} \times 100$$

3.12.6.2 Inhibition of superoxide anion radical formation by xanthine/xanthine oxidase (NXO or XXO)

The activity to scavenge superoxide anion was performed by following the formazan product of XTT. The procedure was done as described by Ukeda et al. with some modifications (IV). The premix containing 110 μL of 50 mM $NaHCO_3$ buffer, pH 9.4, 20 μL of 0.5 mM hypoxanthine, 20 μL of EDTA, 20 μL of 0.25 mM XTT and 10 μL of sample or 10 μL of pure DMSO as negative control or 2 mM Allopurinol as positive control was pipetted in a 96-well plate. The reaction was initiated with 20 μL of 150 mU/mL xanthine oxidase or buffer as background. The kinetic measurement was immediately performed by microplate reader at 480 nm for

5 min, every 20 seconds. Percentage of scavenging of superoxide anion (% scavenging) was calculated from the following equation:

$$\% \text{ Scavenging} = 100 - \frac{(\text{Vmax } \textit{of sample})}{(\text{Vmax } \textit{of DMSO})} \times 100$$

3.12.6.3 HL-60 Antioxidant by Reduction of Cytochrome C

3.12.6.3.1 Differentiation of HL-60

The differentiation of human promyelocytic leukemic cells (HL-60) to granulocyte was performed following the method established by Takeuchi-T et al. with minor modifications (II). 2.5 x 10^5 cells/mL of HL-60 were differentiated with culture medium containing 1.3% DMSO and incubated in 37°C CO_2 incubator for 4 days. The differentiated cells were harvested by centrifugation and washed twice with Hank's balanced salt solution, pH 7.8 containing 30 mM Hepes and resuspended at concentration 1 x 10^6 cells/mL.

3.12.6.3.2 HL-60 Antioxidant Assay

Inhibition of TPA-induced superoxide radical formation in differentiated HL-60 by determination of cytochrome c reduction (III). For this assay, 25 μL of a 10-fold diluted sample in H_2O or 10% DMSO as negative control was mixed with 100 μL of 1x10^6 cells/mL cell suspension in 96-well plate. After 37°C incubation for 5 mín, 25 μL of HBSS was added to sample and negative control while added 25 μL of 600 U/mL of SOD for positive control and then 75 μL of 4.17 mg/ml cytochrome c were added. To start the reaction, 25 μL of 0.55 mg/mL TPA was added. The mixture was incubated at 37°C for 30 min. The reaction was stopped by keeping on ice for 15 min. After centrifugation at 2000 rpm for 10 min, 200 μL of the supernatant was aspirated and the optical density (OD) was measured at 550 nm wavelength with a microplate reader. Percentage of inhibition of TPA-induced superoxide radical formation (% inhibition) was calculated using the equation

shown below. The samples which provide inhibition of TPA-induced superoxide radical formation activity more than 50%, were further investigated and IC_{50} was computed using the Microsoft Excel

$$\% \text{ Inhibition} = 100 - \frac{(\text{OD } sample)}{(\text{OD } DMSO)} \times 100$$

3.12.6.3.3 Cell Viability Assay

Since some samples exert high toxicity, cell viability was examined in parallel to avoid false positive results. Cell viability was measured fluorimetrically by enzymatic hydrolysis of the fluorogenic esterase substrate calcein AM. For this assay, the cell pellet after HL-60 Antioxidant assay was collected by centrifugation, washed twice with PBS and then added 50 μL of warm 0.25 μM calcein AM. The fluorescence was continuously measured every 1 min for 10 min using an excitation wavelength of 485 nm and an emission wavelength of 520 nm with cutoff 515 nm using a microplate fluorescence reader. Percentage of cell viability (% cell viability) was calculated from V_{max} by following equation:

$$\% \text{ Cell viability} = \frac{(V_{max} \text{ of } sample)}{(V_{max} \text{ of } DMSO)} \times 100$$

3.12.6.4 Microplate-Oxygen Radical Absorbance Capacity Assay (MORAC)

The oxygen radical absorbance capacity assay (ORAC) was developed by Cao and Prior (VI). The mixture containing 175 μL of 75 mM phosphate buffer, pH 7.0, 10 μL of 7×10^{-5} mM fluorescein and 10 μL of 1/80 dilution sample or DMSO as negative control or 20 μM Trolox as positive control was preincubated at 37°C for 10 min. The reaction was initiated by addition of 15 μL of 255 mM AAPH as ROO$^{\bullet}$ generator. The plate was subjected immediately to the fluorescence microplate reader at 37°C measuring at excitation 485 nm and emission

530 nm with cutoff 530 nm. The signal was read every 2 min for 45 min and the area under curve (AUC) was calculated autometrically.

The final results were expressed as ORAC units, where 1 ORAC unit equals the net protection of fluorescein produced by 1 μM Trolox, a water soluble Vitamin E analog. Scavenging capacity > 1 ORAC unit were considered as positive. The ORAC values were calculated using the equation as below.

$$\text{ORAC (Unit)} = [(AUC_{Sample} - AUC_{Blank})/(AUC_{Trolox} - AUC_{Blank})]$$

3.12.6.5 Inhibition of xanthine oxidase (IXO)

Xanthine oxidase activity was determined by quantifying the amount of uric acid produced from xanthine. The method was followed as described by Nagao et al. with some modifications (V). The premix containing 130 μL of 50 mM $NaHCO_3$ buffer, pH 9.4, 20 μL of 0.5 mM xanthine, 20 μL of EDTA and 10 μL of sample or 10 μL of pure DMSO as negative control or 2 mM Allopurinol as positive control was pipetted in 96-well plate. The reaction was initiated with 20 μL of 150 mU/ml xanthine oxidase and then immediately measured in a kinetic at 295 nm for 5 min, every 30 seconds. Percentage of inhibition of uric acid formation (% inhibition) was quantitated and calculated from the following equation:

$$\% \text{ Inhibition} = 100 - \frac{(\text{Vmax } of\ sample)}{(\text{Vmax } of\ DMSO)} \times 100$$

3.12.6.6 Lipoxygenase Inhibition Assay (LOX)

Lipoxygenase is a dioxygenase that catalyzes conversion of linoleic acid and other polyunsaturated lipids that contain a *cis,cis*-1,4-pentadiene moiety. The reaction can be followed by observing in absorption at 234 nm wavelength. The assay was performed as described by Gleason et al. with some modications (VII). For the assay, 120 μL of 10 mM PBS, pH 7.4, 20 μL of 2500 U/mL

LOX and 20 μL of 1/10 dilution sample or NDGA for positive control or 10% DMSO as negative control were pipetted in 96-well plate for UV measurement. The plate was preincubated at room temperature for 10 min with shaking. The reaction was started with 40 μL of 0.5 mM arachidonic acid (diluted in PBS buffer), further incubated at room temperature for 10 min with shaking. The optical density was measured at wavelength 234 nm. The LOX inhibition was calculated by following equation:

$$\% \text{ Inhibition} = 100 - \frac{(\Delta OD\ sample)}{(\Delta OD\ DMSO)} \times 100$$

ΔOD *sample* = OD (Sample with enzyme) – OD (Sample without enzyme)

ΔOD *DMSO* = OD (DMSO with enzyme) – OD (DMSO without enzyme)

3.12.6.7 Aromatase Inhibition Assay

Aromatase is a membrane-bound enzyme which catalyzes the formation of estrogen, the final step in the estrogen synthesis. Screening of aromatase inhibition was performed following the method reported by Stresser et al. (VIII) with minor modifications. This method was followed the Gentest kit utilizing a recombinant human aromatase (CYP19) and a fluorometric substrate *O*-benzyl fluorescein benzyl ester (DBF). DBF is dealkylated by aromatase and then was hydrolyzed providing the fluorescein product.

For the Aromatase Inhibition Assay, 100 μL of C/SD which contains 78.4 μL of 50 mM PB, pH 7.4, 20 μL of 20x NADPH-generating system (26 mM $NADP^+$, 66 mM G-6-P and 66 mM $MgCl_2$) and 1.6 μL of 100 U/mL G-6-PDH were pipetted in 96-well black plate and then preincubated in 37°C water bath for 10 min. The reaction was initiated by addition of 100 μL of enzyme/substrate (E/S) mix containing 77.3 μL of 50 mM PB, pH 7.4, 12.5 μL of 16 pmol/mL CYP19, 0.2 μL of 0.2 mM DBF, and 10 μL of 1/40 dilution sample or 10% DMSO as negative control or 0.76 mM ketoconazole as positive control. To exclude background fluorescence of sample, E/S was added after the reaction was terminated. After incubation at 37°C for 30 min, the reaction was stopped by addition of 50 μL of 2.2 N NaOH. To develop

adequate signal to background ratio, the plate (with lid) was then incubated for 2 hrs in 37°C air incubator. Fluorescence signal was measured using an excitation wavelength of 490 nm and emission wavelength of 530 nm with cutoff 515 nm. Percentage of inhibition (% inhibition) was calculated as shown below. Samples which have inhibition higher than 50% were determined for dose-response inhibition. IC_{50} of samples were calculated using Microsoft Excel.

$$\% \text{ Inhibition} = 100 - \frac{(\text{Fluorescence } \textit{sample-blank})}{(\text{Fluorescence } \textit{DMSO-blank})} \times 100$$