CHAPTER 3

METERIALS AND METHODS

3.1 Sample collection

Healthy leaves of *Croton sublyratus* were collected from 4 sources which were the Institute of Biotechnology and Genetic engineering, Chulalongkorn University, Bangkok, Panomsarakam district Chachengsao Province, Muang district Prachuap Khiri Khan Province and Patum Thani Province.

3.2 Fungal isolation and culture methods

Endophytic fungi were isolated using the surface sterilization method by Mekkamol et.al.(1996). The leaf of *Croton sublyratus* was cleaned with runing tap and cut into small pieces (5 x 5 mm) then surface sterilized by immersing in 95 % ethanol for 1 minutes followed by immersing in sodium hypochlorite (5% available chlorine) for 5 minutes and then transferred to 95 % ethanol for 1 minutes. Finally washed 2 times in sterilize water and the sterilized leaf pieces were placed on the surface of malt extract agar plates. Plates were then incubated at room temperature (25–30°C) and examine for fungal germination everyday. Fungal endophytes germinating from the leaf pieces were transferred to fresh MEA medium plate by hyphal tip transfer. They were incubated for 7-14 days at room temperature (25–30°C) and purity was determined by colony morphology. Fungal isolates with different morphology were collected for further study.

3.3 Characteriztion of the endophytic fungi

3.3.1 Morphological identification of isolated fungi

A. Microscopical feature

The microscopic analyses were based on observations by light microscopy on an Olympus CH2 using a 40x dry objective. Specimens for light microscopy preparation were mounted in lectophenol-cotton blue for observation of spores and other characteristic

B. Macroscopic features

Characters such as shape, size, color, type of stroma surface, and others were studied using stereomicroscope on a Leica MZ6.

3.3.2 Molecular identification of isolated fungi

Sequences of internal transcribed spacer (ITS) regions of rDNA (Figure 3.1) from isolated endophytic fungi were sent for identification by molecular techniques at Asian Natural Environmental Science Center, The University of Tokyo, Japan. The procedures were listed as below.

A. DNA extraction

Genomic DNA was prepared from the fresh mycelium by homoginization in 1.5 ml tubes with a FastPrep FP 120 homogenizer (Savant, faxmingdale, NY, USA) and followed by extraction with cetyltrimethylammonium bromide (CTAB) as described in Zhou et al. (1991).Fungal DNA extract was applied in CTAB buffer (2% CTAB,0.1 M Tris-HCl (pH8.0),20mM EDTA (pH8.0), 1.4M NaCl and 0.5% 2-mercaptoethanol) at 65 $^{\circ}$C for 1 hour, extracted with chloroform-isoamyl alcohol (25:24:1,v/v), then extracted with phenolchloroform-isoamyl alcohol mixture (24:1,v/v) twice. Fungal DNA was precipitated with isopropanol and centrifuged at 8000 rpm for 5 min. Fungal DNA was dissolved in 100 µl TE buffer (10mM Tris-HCl (pH8.0) and 1mM EDTA) and kept at -30 $^{\circ}$C for further study.

B. **ITS amplification**

The ITS region of isolated endophytic fungus was amplified with the primers ITS1f (Gardes and Burns,1993), and ITS4 (White et al.,1990). Twenty micrometers of reaction mixture contained 5 ng template DNA, 0.2 mM each dNTP,1xPCR buffer, 1.5mM $Mg2^{+}$, 0.5U Ampli Taq Gold (Ampli Taq Gold kit; Perkin Elmer, Branchburg, NJ, USA), and 0.5 μM of primer pair. The amplification reations were performed in a thermal cycler (TP 3000;Takara Shuzo, Tokyo,Japan). Amplification was started at 94°C for 9 min,followed by 38 cycles of denaturing step at 94°C for 1 min, an annealing step at 51°C for 1 min, and an extension step at 72°C for 1 min, and ended with an additional 5-min extension step at 72°C (Kanchanaprayudh et al.,2003).

C. **DNA sequencing**

$ITS_{1f\text{-}4}$ regions were amplified from the representative sample of isolated endophytic fungus. Amplified $ITS_{1f\text{-}4}$ fragments were cloned using pT7 Blue vectors (Novagen, Madison, WI, USA) and transformed into *Escherichia coli* strain XL1-Blue MFR. Legation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones and sequenced with a Thermo Sequence Pre-mixed Cycle Sequencing kit (Hitachi) using the T7 and M13 forward primers labeled with Taxas Red (Hitachi) in and SQ-5500E sequencer (Kanchanaprayudh et al.,2003).

$ITS_{1f\text{-}4}$ sequences were automatically aligned with fungi ITS sequences obtained from Genbank DNA database (http:// www.ddbj.nig.ac.jp)

Primers for amplification and sequencing of ITS region and ITS2 sequence of rRNA gene.

ITS1f CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATGC

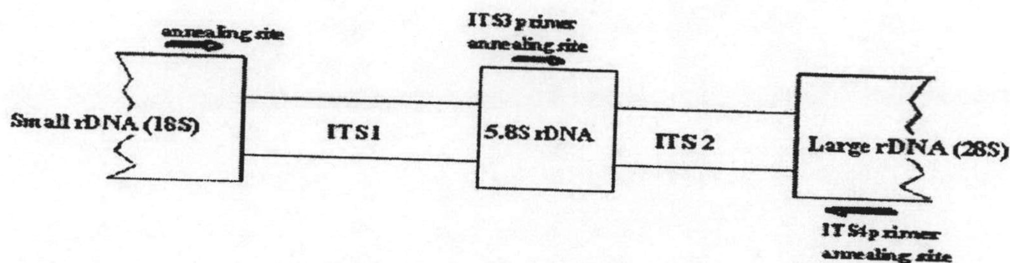


Figure 3.1 ITS regions of rDNA (Kanchanaprayudth et al., 2003)

3.4 Fungal cultivation

Each fungal endophyte isolate was grown on MEA for 1-2 weeks'depanding on the individual fungal growth rate. After that ,the agar culture was cut with flamed cork borer(diameter 8 mm.). Five pieces of agar culture were transferred aseptically into 250 ml. Erlenmeyer flasks containing 100 ml of malt extract broth. The flasks were incubated at room temperature (25 – 30°C) under static conditons for 4-6 weeks.

3.5 Evaluation of the antimicrobial activity

3.5.1 Test microorganisms

Microorganisms used for antimicrobial activity assay were two Gram-positive bacteria (*Bacillus subtilis* ATTC 6633, *Staphylococcus aureus* ATTC 25923),two Gram-negative bacteria (*Pseudomonas aeruginosa* ATTC 27853, *Esherichia coli* ATTC 25922), Fungus,yeast form (*Candida albicans* ATTC10231) and yeast (*Saccharomyces cerevisiae* TISTR 5169)

3.5.2 Procedures

A. Preparation of medium

Nutrient agar plates were poured into dishes of 90 mm internal diameter. If the plates were not required for immediate use they were stored in refrigerator and protected from desiccation.

B. Preparation of inoculum

From a pure culture of the pathogen, four or five colonies were taken with a wire loop and transferred onto 5 ml of nutrient broth. Incubation of the inoculum at 37°C for 6-8 hours. Turbidity was adjusted with nutrient broth to match the turbidity of Standard Mc.Farland No. 0.5 (OD 0.1 at 625 nm,for bacteria and at 620 nm for yeast)

C. Inoculation of the test plate

The test plates were inoculate by streaking the swab across the entire surface. This was repeated twice, turning the plate 60 degree between each streaking. The surface of the medium was allowed to dry for 3-5 minutes.

D. Application of antimicrobial by dual culture agar diffusion technique

Cultures ager were removing disk cut with flamed cork hole borer (8 mm diameter) then placed on test plate . All plates were incubated at 37°C overnight. Inhibition zones were measured in mm using a ruler.

3.6 Cultivation and metabolite extraction of endophytic fungus isolate CsPr03

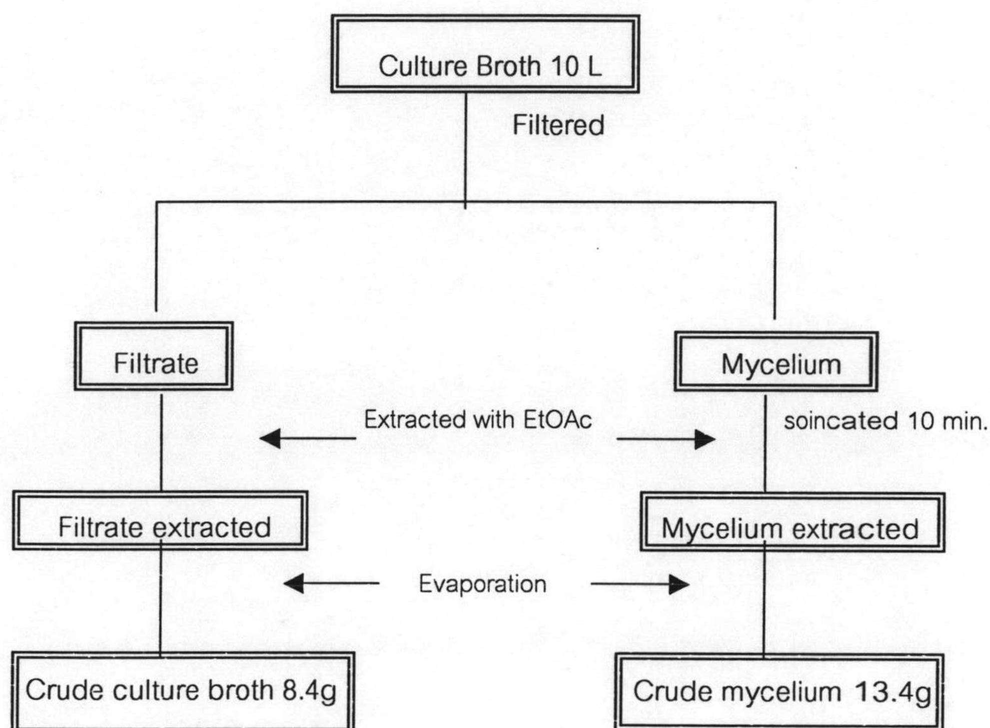
3.6.1 Cultivation

Fungal endophyte isolate CsPr03 was cultivated on MEA agar for 1 week. The agar culture was cut with flamed cork borer(diameter 8 mm). Five pieces of agar culture were transferred aseptically into 250 ml. Erlenmeyer flasks containing 100 ml of malt extract broth. The flasks were incubated at room temperature (25-30°C) for 14 days.

3.6.2 Metabolite extraction of endophytic fungus isolate CsPr03

The culture broth (10 L) was filtered through 4 layer of cotton gauze and exhaustively pressed. The filtrate were extracted with an equal volume of ethyl acetate (EtOAc) 6 times. The EtoAc layers were collected and dehydrated with anhydrous sodium sulfate then concentrated under reduced pressure at 40°C to yield 8.4 g of EtoAc extract (dark brown oily liquid). For the mycelial were blended and extracted with

EtoAc(500ml x 15) in ultrasonic bath to yield of 13.9 g (red brown oily liquid). The extraction of the culture broth and mycelium of the endophytic fungus isolate CsPr03 shown in Scheme 3.1



Scheme 3.1 Extraction of culture broth and mycelium of endophytic fungus isolate CsPr03

3.7 Application of crude culture broth extracted and mycelium extracted

Wells were made in the agar by removing disk cut (8 mm of diameter) cut with a flamed cork hole borer. One hundred µl of culture broth extracts and mycelium extracts were pipetted into the agar wells. This was absorbed by the media surrounding the walls. The test microorganisms were incubated at 37°C and room temperature for 24 hours. Inhibition zones around the wells were measured in mm with ruler.

3.8 Isolation of metabolites of crude culture broth from endophytic fungus isolate CsPr03

The crude culture broth of isolate CsPr03 (8.4 g) was purified by gel filtration chromatography using silica gel (Quick column chromatography 500 ml), and eluted by increasing polarity from hexane, hexane-EtoAc, EtoAc, EtoAc-MeoH, MeoH. Fractions (100 ml each) were collected and examined. Fraction combination was by TLC on silica gel plates with hexane, hexane and ethyl acetate mixture, ethyl acetate, ethyl acetate and methanol mixtures, and methanol as the developing solvent. Fraction with the same TLC pattern were pooled and dried. The results from the isolation of crude broth extracts were presented in table 3.1. The biological activity of each pool fraction was examined and described in Chapter 4.

Table 3.1 The results from separation of crude culture broth of endophytic fungus isolate CsPr03

Fraction code	Eluents	Fraction No.	Apperance	Weight (mg)
BE01	Hex: EtoAc 100:0	1-5	White solid and Red brown viscous liquid	840.5
BE02	100:0, 90:10	6-11	Red brown viscous liquid	41.7
BE03	90:10, 80:20	12-19	Brown viscous liquid and white solid	186.9
BE04	80:20	20-25	Brown viscous liquid	130.7
BE05	80:20,70:30	26-30	Brown viscous liquid	139.0
BE06	70:30	31-38	Crystal in Brown viscous liquid	481.3
BE07	70:30,60:40	39-50	Brown viscous liquid	1094.4
BE08	60:40,50:50	51-57	Brown viscous liquid	652.5
BE09	50:50,40:60	58-70	Brown viscous liquid	1111.9

Table 3.1 (continued)

Fraction code	Eluents	Fraction No.	Apperance	Weight (mg)
BE10	40:60	71-80	Brown viscous liquid	202.9
BE11	30:70,20:80	81-90	Brown viscous liquid	187.6
BE12	20:80,100:0	91-110	Brown viscous liquid	536.8
BE13	EtoAc:MeOH 90:10	111-120	Brown viscous liquid	200.1
BE14	80:20	121-135	Brown viscous liquid	104.5
BE 15	70:30	136-155	Brown viscous liquid	218.2
BE16	50:50	156-170	Brown viscous liquid	204.1
BE17	40:30	171-183	Brown viscous liquid	189.3
BE18	20:80	183-200	Brown viscous liquid	175.6
BE19	0:100	200-221	Brown viscous liquid	198.5

3.8.1 Isolation of crude culture broth of endophytic fungus isolate CsPr03 form fraction code BE 01

The crude culture broth of fraction code BE01 (840.5 mg) was purified by column chormatography (silica gel, 33 g) using eluents of increasing polarity from hexane to MeoH. Fraction (20 ml each) were collected and the same TLC pattern were pooled and dried. The results from the separation of fraction code BE01 were presented in table 3.2

Table 3.2 The results of crude culture broth of endophytic fungus isolate CsPr03 from fraction code BE01

Fraction code	Eluents	Fraction No.	Apperance	Weight (mg)
BE 0101	Hex : EtoAc 100:0 , 98:2	1-25	Red viscous liquid and solid	22.3
BE 0102	98:2	26-32	Red viscous liquid and solid	12
BE 0103	98:2	33-48	Red viscous liquid and solid	15.6
BE 0104	98:2 , 90:10	49-52	Orange viscous liquid	3.5
BE 0105	90:10	53-62	Orange viscous liquid	13.4
BE 0106	90:10	63-66	Orange viscous liquid	2

Table 3.2(continued)

Fraction code	Eluents	Fraction No.	Apperance	Weight (mg)
BE 0107	90:10	67-82	Brown viscous liquid	11.7
BE 0108	90:10	83-91	Brown viscous liquid	30.2
BE 0109	90:10	92-106	Brown viscous liquid	18.7
BE 0110	90:10 , 85:25	107-116	Brown viscous liquid	50.3
BE 0111	85:25	117-121	Brown viscous liquid	6.5
BE 0112	85:25	122-128	Brown viscous liquid	10
BE 0113	85:25 , 80:20	129-147	Yellow viscous liquid	6.3
BE 0114	80:20	149-157	Yellow viscous liquid	0.8
BE 0115	80:20	158-164	Yellow viscous liquid	0.6
BE 0116	80:20 , 70:30	165-174	Red Brown viscous liquid liquid	11.9
BE 0117	70:30	175-181	Red Brown viscous liquid	15.3
BE 0118	70:30	182-185	Yellow viscous liquid	3.4
BE 0119	70:30	186-195	Yellow brown viscous liquid	9.4
BE 0120	70:30	196-199	Yellow brown viscous liquid	9.6
BE 0121	70:30,50:50	200-205	Crystal with yellow viscous liquid	51.3
BE 0122	50:50	206-209	White solid with yellow viscous liquid	39.7
BE 0123	50:50	210-225	Yellow brown viscous liquid	12.7
BE 0124	50:50	226-229	Yellow brown viscous liquid	10.4
BE 0125	50:50,30:70	230-240	Yellow brown viscous liquid	27.9
BE 0126	30:70,100:0	241-260	Brown viscous liquid	7.6
BE 0127	EtOAc : MeOH 100:0	261-270	Brown viscous liquid	8.9
BE 0128	100:0,10:90	271-279	Brown viscous liquid	10.3

3.8.2 Isolation of crude culture broth of endophytic fungus isolated CsPr03 form fraction code BE 06

The crude culture broth from fraction code BE06 (481.3mg) was purified by column chormatography (silica gel, 30 g) using eluents of increasing polarity from hexane to MeoH. Fraction (20 ml each) were collected and the same TLC pattern were

pooled and dried. The results from the separation of fraction code BE06 were presented in table 3.3

Table 3.3 The results of crude culture broth of endophytic fungi isolate CsPr03 from fraction code BE06

Fraction code	Eluents	Fraction No.	Apperance	Weight (mg)
BE0601	Hex: EtoAc 40:60	1-29	Yellow viscous liquid	20.5
BE0602	40:50 , 50:50	30-35	Crystal with yellow viscous liquid	80.3
BE0603	50:50 , 60:40	36-42	Yellow viscous liquid	104.3
BE0604	70:30	43-50	Yellow viscous liquid	55.3
BE0605	70:30 ,80:20	51-72	Yellow viscous liquid	45.8
BE0606	80:20, 0:100	73-96	Yellow viscous liquid	89.3
BE0607	EtOAc : MeOH 90:10	97-121	Brown viscous liquid	83.3

3.9 Isolation of metabolites of crude mycelium from endophytic fungus isolate CsPr03

The crude mycelium (13.4 g) of isolate CsPr03 was purified by gel filtration chromatography using silica gel (Quick column chromatography 500 ml), and eluted by increasing polarity from hexane, hexane-EtoAc, EtoAc, EtoAc-MeoH, MeoH. Fraction (100 ml each) were collected and examined. Fraction combination was by TLC on Silica gel plates with hexane, hexane and ethyl acetate mixture, ethyl acetate, ethyl acetate and methanol mixtures, and methanol as the developing solvent. Fraction with the same TLC pattern were pooled and dried. The results from the isolation of crude mycelium extracts were presented in table 3.4. The biological activity of each pool fraction was examined and described in Chapter 4.

Table 3.4 The results from separation of crude mycelium of endophytic fungus isolate CsPr03

Fraction code	Eluents	Fraction No.	Apperance	Weight (mg)
ME01	Hex : EtOAc 100:0	1-5	Orange viscous liquid	485.3
ME02	100:0 , 95:5	6-22	Orange viscous liquid with solid	28.5
ME03	95:5 , 90:10	23-26	Orange brown viscous liquid and white solid	34.9
ME04	90:10 , 80:20	27-34	Red viscous liquid with solid	122
ME05	80:20 , 70:30	35-45	Red viscous liquid with solid	210.3
ME06	70:30	46-51	Brown viscous liquid	538.9
ME07	70:30,50:50	52-66	Brown viscous liquid with solid	5202.3
ME08	50:50 , 40:60	67-81	Brown viscous liquid	780.1
ME09	40:60 , 20:80	82-102	Brown viscous liquid	492.0
ME10	20:80, 0:100	103-128	Brown viscous liquid	201.3
ME11	EtOAc : MeOH 100:0, 90:10	129-141	Brown viscous liquid	189.2
ME12	90:10,60:40	142-155	Brown viscous liquid	132.8
ME13	50:50	155-159	Brown viscous liquid	50.5

3.9.1 Isolation of crude mycelium of endophytic fungus isolate CsPr03 form fraction code ME 04

The crude mycelium from fraction code ME04 (122 mg) was purified by column chormatography (silica gel, 30 g) using eluents of increasing polarity from hexane to MeoH. Fraction (20 ml each) were collected and the same TLC pattern were pooled and dried. The results from the separation of fraction code ME04 were presented in Table 3.5

Table 3.5 The results from the separation of fraction code ME04 of endophytic fungus isolate CsPr03

Fraction code	Eluents	Fraction No.	Apperance	Weight (mg)
ME0401	Hex : EtOAc 100:0	1-35	Red amorphous	2.1
ME0402	100:0 , 95:5	36-52	Red amorphous	2.4
ME0403	92:8	53-79	Red viscous liquid	3.3
ME0404	92:8	80-110	Red viscous liquid	12.7
ME0405	90:10 , 80 20	111-145	Yellow viscous liqiud with orange amorphous	10.2
ME0406	80:20, 70:30	146-175	Yellow viscous liquid	9.8
ME0407	70:30,50 :50	176-192	Yellow viscous liquid	8.2
ME0408	50:50 , 40:60	193-224	Yellow viscous liquid	7.1
ME0409	40:60 , 30:70	225-259	Brown viscous liquid	6.9
ME0410	30:70 , 20:80	260-300	Brown viscous liquid	11.2
ME0411	EtOAC : HeX, EtOAC :MeOH 20:80, 90:10	301-340	Brown viscous liquid	10.7
ME0412	90:10, 0:100	341-360	Brown viscous liquid	10.5

3.10 Purification and properties of pure compounds from endophytic fungus isolate CsPR03

3.10.1 Purification and properties of compound 1 from fraction BE0121

The yellow solids of the fraction BE0121 isolated from column chromatography using Hex:EtOAc (70:30 → 50:50) as eluents. The solids were further washed with hexane and ether mixtures to afford compound 1 as colorless needle crystals (30.5mg). Compound 1 has m.p.147-148 ^{o}C and showed a single spot at the R_f value 0.25 on TLC plate using 45% EtOAc in hexane as the mobile phase. TLC spot were visualized with UV lamp (254 nm) and with iodine vapour. Compound 1 is soluble in EtOAc, MeOH, DMSO, H_2O.

Compound 1 is a colorless needle crystals (122 mg), $[\alpha]_D^{20}$ +2.4 (C 0.1,MeOH), UV λ_{max} (nm), MeOH (ε) : 270 (1286) nm., (Figure 1 in Appendix B)

FT-IR spectrum (KBr), ν_{max}(cm^{-1}) : 3300-3600 (br), 1703 (m), 1571 (m), 1481 (w), 1264 (s), 1166 (w), 1022 (w), 859 (w), 773(w). (Figure 2 in Appendix B)

^{1}H-NMR spectrum (CD_3CO,400 MHz.) δ (ppm) : 1.3 (3H, t, J = 6.8), 2.8 (3H, S), 3.7 (2H, S), 4.2 (2H, q ,J = 6.8), 6.2 (1H, d, J =2), 6.3 (1H, d ,J = 2).(Figure 3 in Appendix B)

^{13}C-NMR spectrum (CD_3CO,100 MHz) δ (ppm): 13.1(q), 31.2(q), 39.7(t), 60.6(t), 101.5(d), 110.8 (d), 119.5(s), 136.4 (s), 159.8(s), 160.5(s), 172.4(s), 204.5(s). (Figure 4 in Appendix B)

LC-MS spectrum (*m/z*) 238(20) and 191(100) (Figure 9 in Appendix B)

3.10.2 Purification and properties of compound 2 from fraction ME0405

The yellow viscous with orange needle crystals of the fraction ME0405 isolated from column chromatography using Hex:EtOAc (90:10 → 80:20) as eluents. The orange needle crystals were further washed with hexane to afford compound 2 (10.5mg). Compound 2 has m.p. 261-262°C and showed a single spot at the R_f value 0.25 on TLC plate using 20:80 EtOAc in hexane as the mobile phase. TLC spot were visualized with UV lamp (254 nm) and with iodine vapour. Compound 2 is soluble in EtOAc, MeOH, DMSO.

Compound 2 is an orange needle crystals (10.5 mg)

FT-IR spectrum (KBr), ν_{max}(cm^{-1}) : 3200-3500(br), 2925(s), 2844(m),1738 (m),1614(m),1470 (w), 1376 (w),1267(w),1042 (w) (Figure 10 in Appendix B)

^{1}H-NMR spectrum (DMSO-d_6 ,400 MHz.) δ (ppm) : 2.52 (3H,s,Ar-CH_3), 6.63 (1H,d, J = 2.5), 7.15 (1H,br,s), 7.21 (1H,d, J =2.5), 7.54 (1H,br,s) (Figure 11 in Appendix B)

^{13}C-NMR spectrum (DMSO-d_6,100 MHz) δ (ppm) : 22.0(q),108.5(d),109.2(d), 110.5 (d), 113.9(d),121.0(d),124.7(d), 133.4(d), 135.7(d), 148.8(d), 161.9(d), 164.9(d), 166.0(d), 181.9(d), 190.9(d) (Figure 12 in Appendix B)

Maldy TOF-MS spectrum (*m/z*) 273 (100) (Figure 15 in Appendix B)

3.11 Biological activities test

3.11.1 Antimicrobial activities test were determined on crude extracts and pure compounds as follow

3.11.1.1 Antimicrobial activities for the crude extracts and pool fractions

Evaluation of the antimicrobial activities of the fractions was determined by the agar well diffusion method (Weaver,Angel and Botlomley, 1994) in the same manner as described in section 3.5.2. Antimicrobial activities was performed agianst *B. subtilis* ATCC 6633, *S.aureus* ATCC 25923, *E.coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 10231 and *S.cerevisiae* TISTR 5169.

3.11.1.2 Antimicrobial activity of pure compounds

Evaluation of the antimicrobial activities of pure compounds was determined by the antimicrobial susceptibility test microdilution method (Wood and Washington,1995). Antimicrobial activities were performed agianst *B. subtilis* ATCC 6633, *S.aureus* ATCC 25923, *E.coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 10231 and *S.cerevisiae* TISTR 5169.

A. Preparation of pure compounds

Four mg of pure compounds and antibiotic drug standards were dissolved in 1 ml of 10% DMSO in sterile distilled water. Antibacterial (Tetracycline HCl, Amoxillin) compound were used as positive controls.

B. Preparation of bacterial tested inoculum

A bacterial inoculum was prepared in the same manner as described in section 3.5.2. The final inculum was diluted with NB to obtain a cell suspension containing approximately 10^6 CFU/ml.

C. Preparation of yeast tested inoculum

A yeast inoculum was performed in the same manner as described in section 3.5.2. The final inoculum was approximately 10^5 CFU/ml.

D. Assay procedure

Solutions of pure compounds were diluted with Mueller-Hinton Broth (MHB) and YEB for assays of anbacterial and antifungal (yeast form) activity respectively. A 50 μl of pure compound was dispensed into each well in sterile microtiter plates (96-well bottom well). Fifty μl of the final adjusted microbial suspension was inoculated into each well (Final inoculum size of bacterial and yeast was approximately 2.5×10^5 and 2.5×10^4 CFU/ml, respectively). 100 μl of medium only was as the sterility control. A100 μl volume of medium and microbial inoculum mixture acted as the growth control. Microbial microliter plates were incubated at 37°C and room temperature for bacterial and yeast, respectively.

E. Reading of microtiter plates assays

Antibacterial and antifungal (yeast form) activites were determined by measuring the turbidity each well in micotiter plates by using the Sunrise microplate reader (TECAN,AUSTRIA) before and after incubation. The lowest concentration of pure compound showing complete inhibition of growth was recorded as minimal inhibitory concentration (MIC).

3.12 Cytotoxic test

Cytotoxic test were carried out at the institute of Biotechnology and Genetic Engineering. Chulalongkorn University. Bioassay of cytotoxic activity againts human tumor cell culture in *vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltetrazolium bromide) calorimetric method (Carmichael et al., 1987). In Principle, the viable cell number/well was directly proportional to the production of formazan, followed by solubilization, and could be measured spectrophotometrically.

The human tumor cell line was harvested from exponential-phase maintenance cultures (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed into replicate 96-well culture plates in 100-μl volumes using a repeating pipette. Following a 24-h incubation at 37°C,5% CO_2, 100% relative humidy,100 μl of culture medium, culture medium containing the sample was dispensed into the appropriate wells (control group,N = 6; each sample treatment group, N = 3). Peripheral wells of each plate

(lacking cells) were utilized for sample blank (N = 2) and medium / tetrazolium reagent blank (N = 6) " background" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ml PBS was sterilized and filtered through 0.45-μl filter units. MTT working solutions were prepared just prior to culture application by dilution of MTT stock solution 1:5 (V/V) in prewarmed standard culture medium. MTT working solution (50 μl) was added to each culture well, resulting in 50 μl MTT/250 μl total medium volumes; and cultures were incubated at 37°C for 4 to 24 h depending upon individual cell line requirements. Following incubated cell monolayers and formazan were inspected microscopically. Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μl of DMSO using a pipette. Following formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00).

Cell line growth and growth inhibition were expressed in terms to mean (+/- 1 SD) absorbance units and or percentage of control absorbance (+/-1SD %) following subtraction of mean " background" absorbace.

Sample were also tested for cytotoxic activity towards 5 cell lines, which contain of HEP-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric), and BT474 (breast) following the experimental method of bioassay of cytotoxic activity.

3.13 Chromatographic techniques

Analytical thin-layer chromatography (TLC)

Technique	: one dimension ascending
Absorbent	: silica gel F_{254}coated on aluminum sheet (E. Merck)
Layer thickness	: 250 μm
Distance	: 5 cm
Temperature	: Laboratory temperature (25-30°C)

Detection :1.Visual detection under ultraviolet light at wavelengths of 254 and 365 nm.

:2.Visaul detection in iodine vapour.

:3 Visual detection under daylight after spraying with vanillin reagent (Dissolved 1 g vanillin in 95 ml ethanol and add 4 ml concentrated sulfuric acid) or anisaldehyde reagent (0.5% anisaldehyde reagent,5% sulfuric acid,and 10% glacial acetic acid in MeoH) and heating until the colors developed.

3.14 Spectroscopy

3.14.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were record on Perkin-Elmer Model 1760x Fourier Transform Infrared Spectrophotometer, Solid samples were formally examine by incorporating the sample with potassium bromide(KBr) to form a pellete.

3.14.2 Nuclear Magnetic Resonance Spectrometry (NMR)

^{1}H-NMR, ^{13}C-NMR, gCOSY, gHSQC, gHMBC and NOESY spectra were recorded on Varian Spectrometer opereted at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei. The chemical shift was assigned in ppm unit and internally referenced with the residual protonated chloroform at δ = 7.26 ppm.

3.14.3 Optical rotation

Optical rotation were measured on a Perkin Elmer 341 polarimeter, using a sodium lamp at wavelength 589 nm.

3.14.4 Mass Spectrometry (MS)

The mass spectra were recorded on a Fisons Instrument Mass Spectrometer Model Trio 2000 in EI mode at 70 eV.

3.14.5 Melting point

Melting points were examined using a Stuart scientific melting point SMP1 apparatus.

3.14.6 UV-Vis spectrometry

UV-Vis spectra were recorded on a Perkin Elmer Lambda 25 UV-Vis Spectrophotometry in MeOH.

3.14.7 Solvent

All solvent used in this research as hexane, ethyl acetate (EtOAc) and Methanol (MeOH) were commercial grade and were purified prior to use by distillation. The reagent grades solvents were used for recrystallization.