CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

Seventy five fungal isolates were isolated from healthy leaves of *Croton sublyratus* from 4 sources. All endophytic fungi isolates were selected for further study, as shown in Table 4.1

Table 4.1 Number and isolates of endophytic fungi

Source	Number of endophytic fungi (Isolates)	Endophytic fungi isolates
Bangkok	16	CsBkk01-16
Chachengsao Province	10	CsCh01-10
Prachuab Khiri Khan Province	40	CsPr01-40
Patum Thani Province	9	CsPt01-09
Total	**75**	**75**

4.2 Charecterization of endophytic fungi

Each fungus isolates was grown on MEA media, for 2-4 weeks at room temperature. Colonial morphology of 8 fungal isolates is shown in Figures 4.1-4.8, as examples. Because the lack of sporulation so, identification by conventional morphological-based methods could not be made. However, each isolates showed unique colonial morphology allowing them to be considered distinctive fungi. Thus an isolate code besed on the scientific name of the host plant and followed with the source of plant collected.

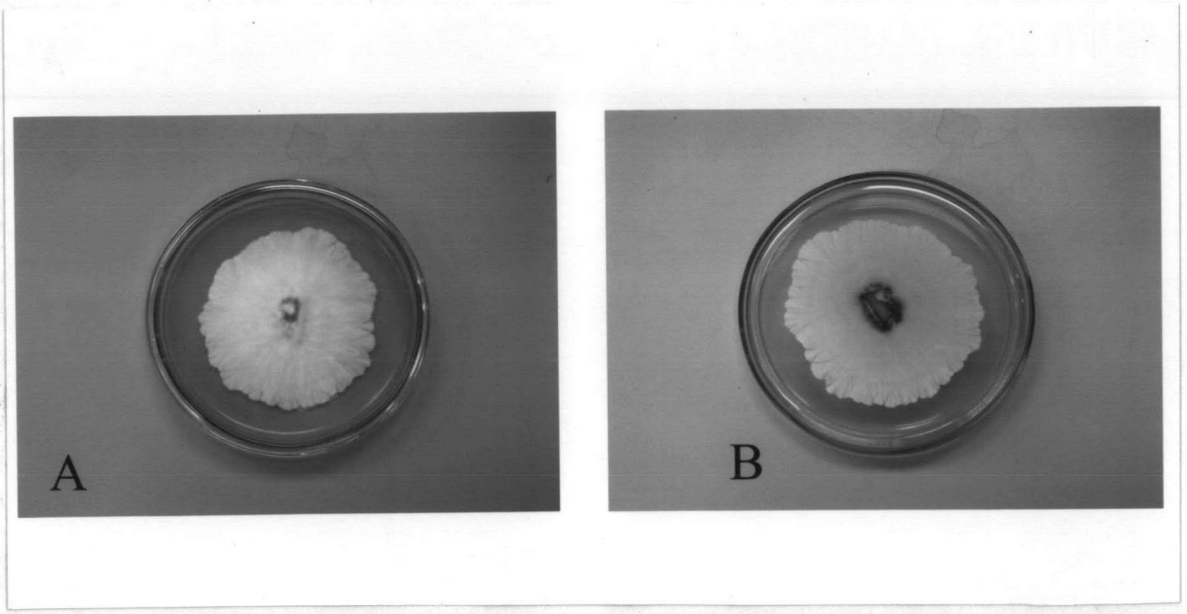


Figure 4.1 Colonial morphology of endophytic fungus isolate,CsPr02, on MEA media after cultivation for 2 weeks at room temperature. Appearance on top side (A), and on bottom side (B).

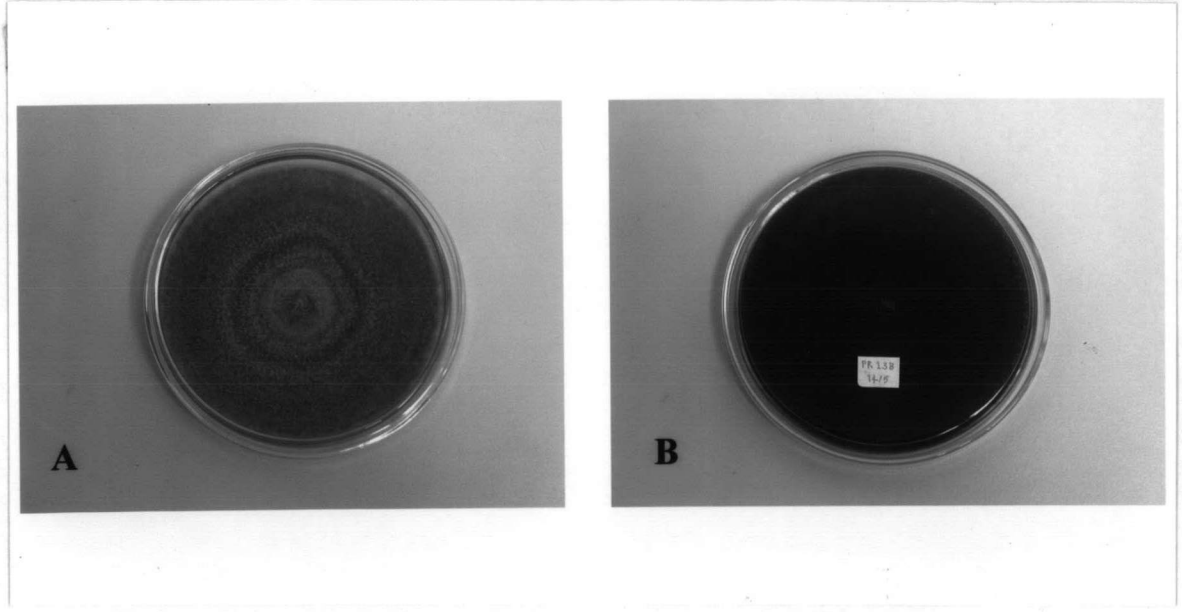


Figure 4.2 Colonial morphology of endophytic fungus isolate,CsPr03, on MEA media after cultivation for 2 weeks at room temperature. Appearance on top side (A), and on bottom side (B).

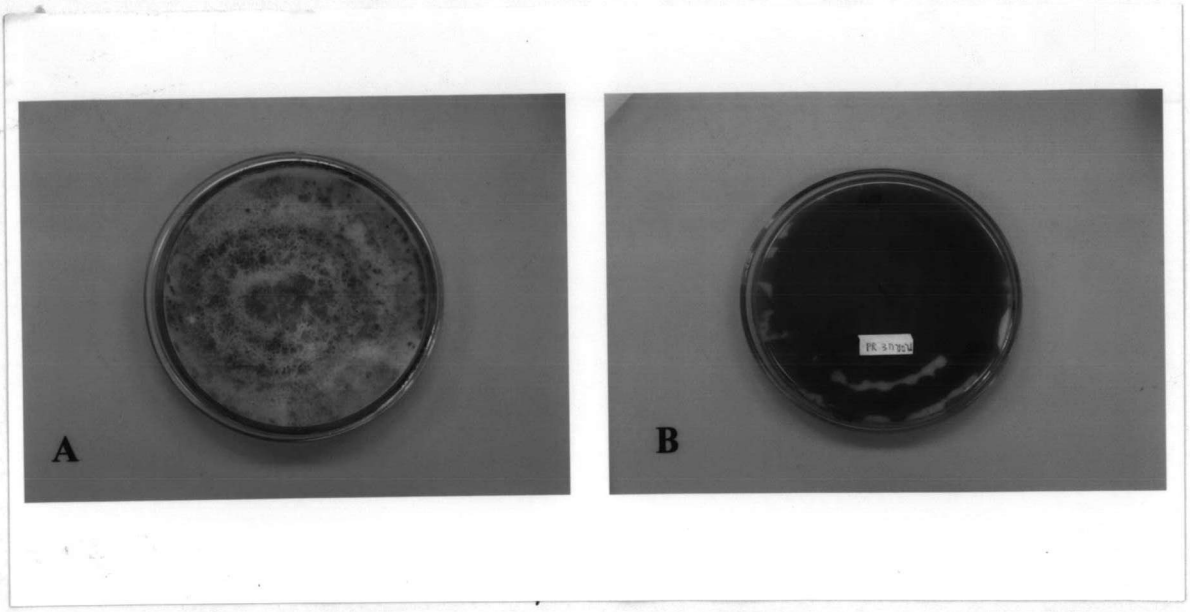


Figure 4.3 Colonial morphology of endophytic fungus isolate,CsPr08, on MEA media after cultivation for 2 weeks at room temperature. Appearance on top side (A), and on bottom side (B).

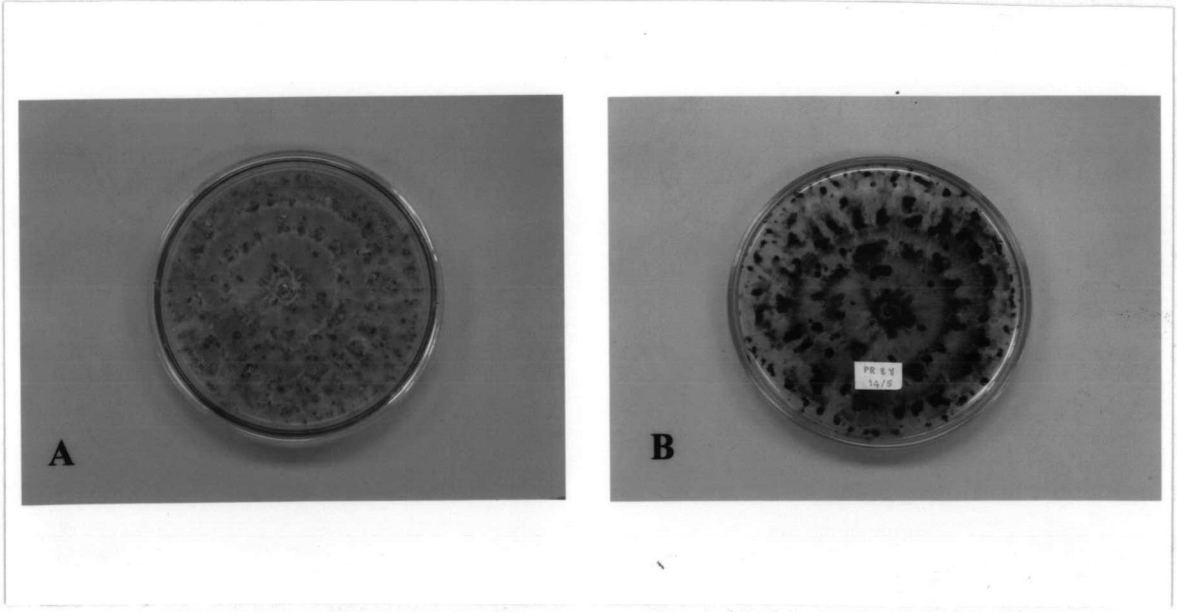


Figure 4.4 Colonial morphology of endophytic fungus isolate,CsPr24, on MEA media after cultivation for 2 weeks at room temperature. Appearance on top side (A), and on bottom side (B).

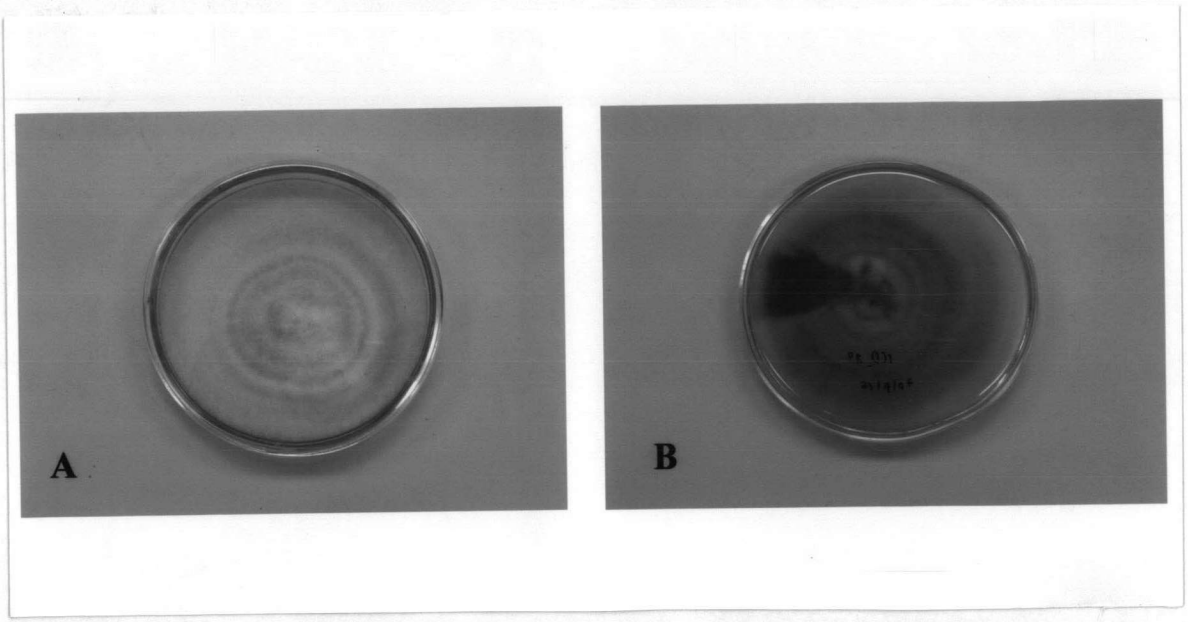


Figure 4.5 Colonial morphology of endophytic fungus isolate,CsPr33, on MEA media after cultivation for 2 weeks at room temperature. Appearance on top side (A), and on bottom side (B).

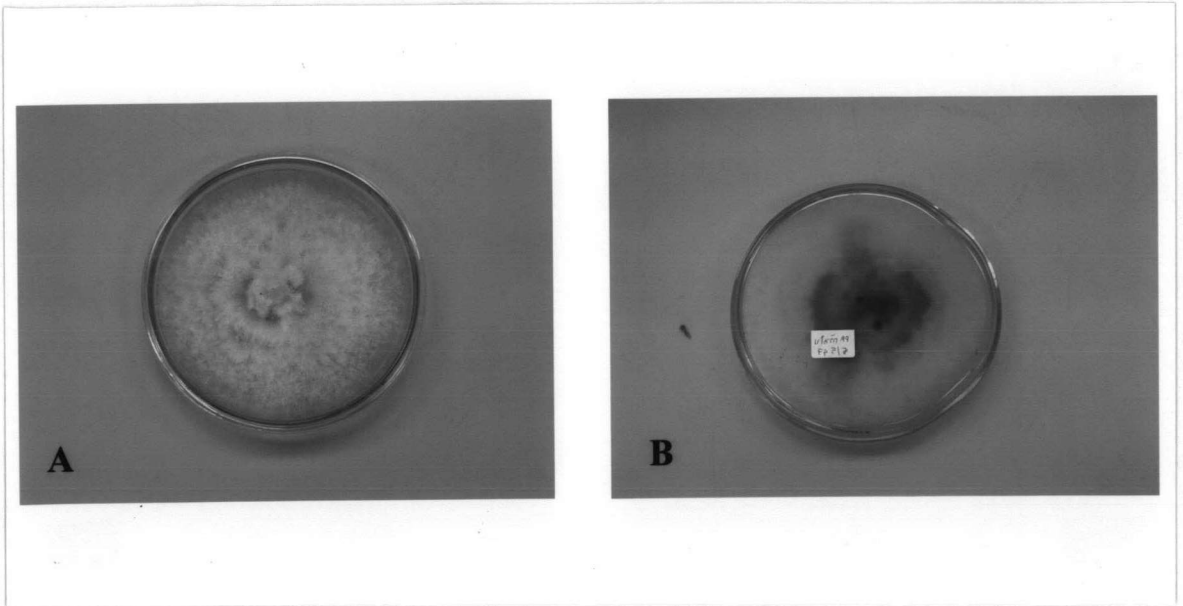


Figure 4.6 Colonial morphology of endophytic fungus isolate,CsPr36, on MEA media after cultivation for 2 weeks at room temperature. Appearance on top side (A), and on bottom side (B).

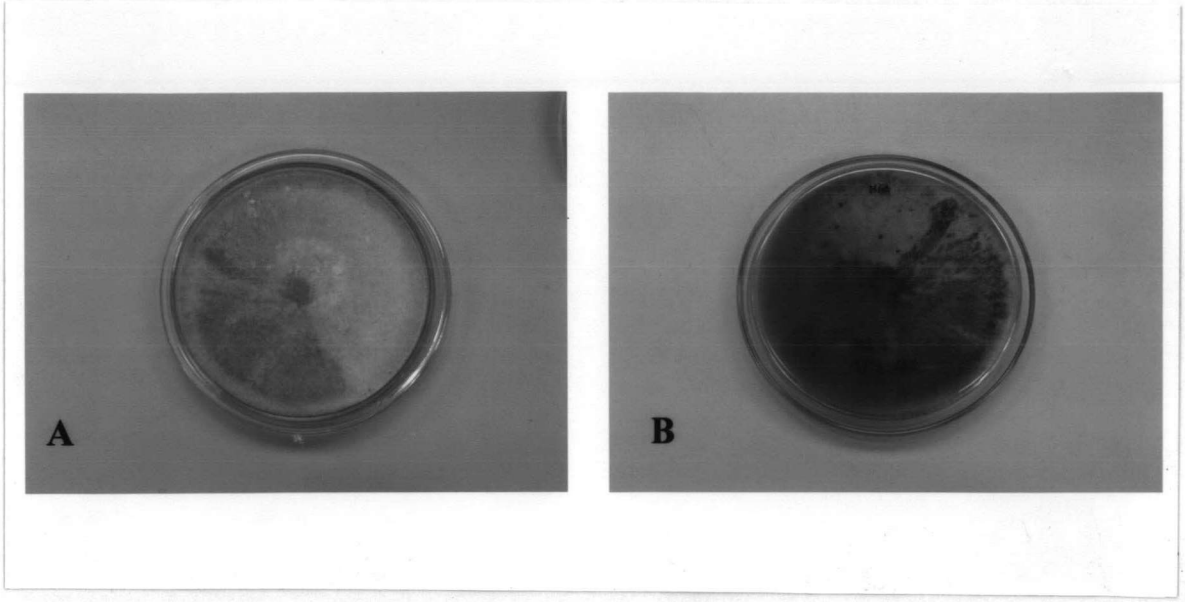


Figure 4.7 Colonial morphology of endophytic fungus isolate,CsBkk05, on MEA media after cultivation for 2 weeks at room temperature. Appearance on top side (A), and on bottom side (B).

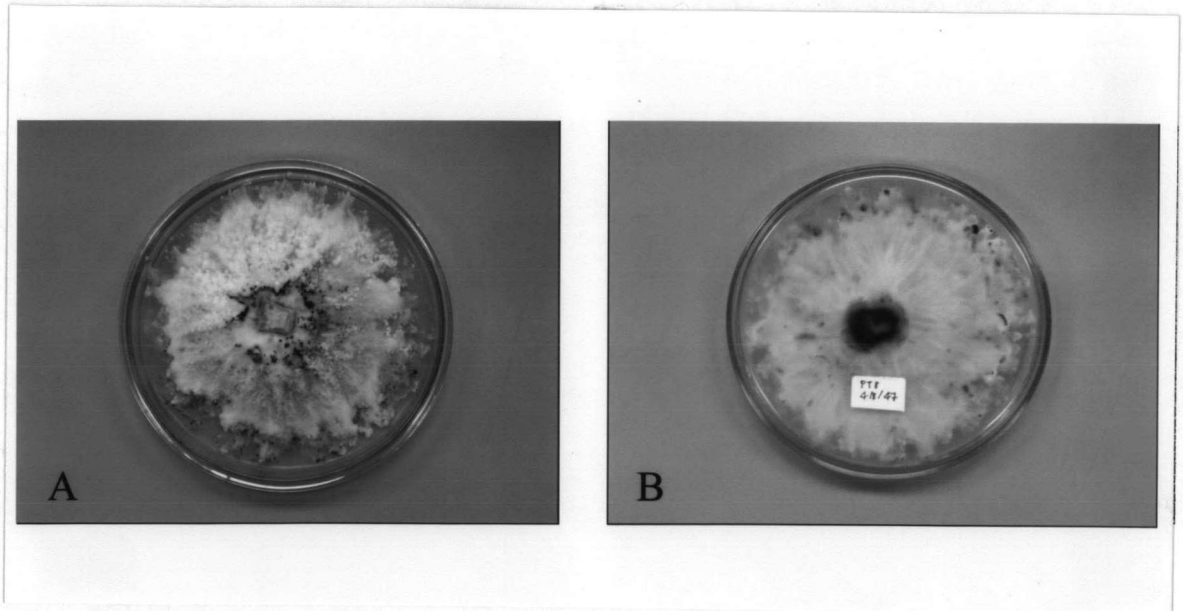


Figure 4.8 Colonial morphology of endophytic fungus isolate,CsPt08, on MEA media after cultivation for 2 weeks at room temperature. Appearance on top side (A), and on bottom side (B).

4.3 Enumeration of tested microorganisms

Viable counts of bacteria and yeast were performed for standardized inocula whose turbidity matched a 0.5 McFarland standard. The CFU/ml values in Table 4.2.

Table 4.2 Ouantity of standardized inocula

Test microorganisms	Quantity
Bacillus subtilis ATCC 6633	9×10^7 CFU/ml
Staphylococcus aureus ATCC 25923	3.8×10^7 CFU/ml
Escherichia coli ATCC 25922	1.32×10^7 CFU/ml
Psudomonas aeruginosa ATCC 27853	1.27×10^7 CFU/ml
Candida albicans ATCC 10231	4.1×10^7 CFU /ml
Saccharomyces cerevisiae TISTR 5169	3.2×10^7 CFU /ml

4.4 Determination of antimicrobial activities

Antimicrobial activities of endophytic fungi against test microorganisms were determined using dual culture ager diffusion technique. The antimicrobial activities of the various isolates are shown in Figure 4.9, Table 4.3

The results showed that 29 isolates inhibition at least one test microorganism. for these result can divided the group of active isolate into 4 groups were group one; inhibited one test microorgamism, group two; inhibited two test microorganisms, group three; inhitbited three test microorganisms and group four; inhibited four test microorganisms the result summerized in Table 4.4 and the percentage of each group were shown in Figure 4.10

Twelve isolates of endohytic fungi were against *B. subtilis* ATCC 6633. Six isolates were against *S. aureus* ATCC 25923.One isolate was againts *E. coli* ATCC 25922. No fungal isolate inhibited *P. aeruginosa* ATCC 27853.

Seven isolates of endophytic fungi were against *C. albicans* ATCC 10231 and fifteen isolates were against *S. cerevisiae* TISTR 5196. The results showed in histrogram in Figure 4.11

Table 4.3 Antimicrobial activities of endophytic fungi isolated from *C. sublyratus* leaves.

Isolates	Test microorganisms					
	B.subtilis	*S.aureues*	*E.coli*	*P.aereuginosa*	*C.albicans*	*S.cerevisiae*
CsBkk01	-	-	-	-	-	-
CsBkk02	-	-	-	-	-	-
CsBkk03	-	-	-	-	-	+
CsBkk04	-	-	-	-	-	-
CsBkk05	-	-	-	-	-	-
CsBkk06	-	-	-	-	-	++
CsBkk07	-	-	-	-	-	-
CsBkk08	-	-	-	-	-	-
CsBkk09	-	-	-	-	-	-
CsBkk10	-	-	-	-	-	-
CsBkk11	-	-	-	-	-	-
CsBkk12	-	-	-	-	-	-
CsBkk13	-	-	-	-	-	-
CsBkk14	++	-	-	-	-	-
CsBkk15	-	-	-	-	-	-
CsBkk16	-	-	-	-	-	++
CsCh01	-	-	-	-	-	-
CsCh02	+	-	-	-	-	-
CsCh03	-	-	-	-	-	++
CsCh04	-	-	-	-	-	++
CsCh05	-	-	-	-	-	-
CsCh06	-	-	-	-	-	-
CsCh07	-	-	-	-	-	-
CsCh08	-	-	-	-	-	-
CsCh09	-	+	-	-	-	-
CsCh10	-	-	-	-	-	-
CsPt01	-	-	-	-	-	-

Table 4.3 (continued)

Isolates	Test microorganisms					
	B.subtilis	*S.aureues*	*E.coli*	*P.aereuginosa*	*C.albicans*	*S.cerevisiae*
CsPt02	+	-	-	-	-	-
CsPt03	-	-	-	-	++	+
CsPt04	-	-	-	-	-	+
CsPt05	-	-	-	-	-	-
CsPt06	-	-	-	-	-	-
CsPt07	-	-	-	-	-	-
CsPt08	-	-	-	-	-	-
CsPt09	+	-	-	-	-	-
CsPr01	-	-	-	-	-	++
CsPr02	-	-	-	-	-	-
CsPr03	++	+++	++	-	-	++
CsPr04	-	-	-	-	-	-
CsPr05	-	-	-	-	-	-
CsPr06	+	-	-	-	-	-
CsPr07	-	-	-	-	-	-
CsPr08	-	-	-	-	-	+
CsPr09	-	-	-	-	-	-
CsPr10	-	-	-	-	-	+
CsPr11	-	-	-	-	-	-
CsPr12	-	-	-	-	-	-
CsPr13	-	-	-	-	-	-
CsPr14	+	-	-	-	++	++
CsPr15	-	-	-	-	-	-
CsPr16	-	-	-	-	-	-
CsPr17	-	-	-	-	+	-
CsPr18	++	++	-	-	-	++
CsPr19	-	-	-	-	+	-

Table 4.3 (continued)

Isolates	Test microorganisms					
	B.subtilis	*S.aureues*	*E.coli*	*P.aereuginosa*	*C.albicans*	*S.cerevisiae*
CsPr20	-	-	-	-	-	-
CsPr21	-	-	-	-	-	-
CsPr22	-	-	-	-	+	-
CsPr23	-	-	-	-	+	-
CsPr24	++	++	-	-	+	+
CsPr25	-	-	-	-	-	-
CsPr26	-	-	-	-	-	-
CsPr27	-	-	-	-	-	-
CsPr28	-	-	-	-	-	-
CsPr29	+	-	-	-	-	-
CsPr30	-	-	-	-	-	-
CsPr31	+	-	-	-	-	-
CsPr32	-	-	-	-	-	-
CsPr33	-	-	-	-	++	++
CsPr34	-	++	-	-	-	-
CsPr35	-	-	-	-	-	-
CsPr36	-	-	-	-	-	-
CsPr37	-	-	-	-	-	-
CsPr38	-	-	-	-	-	-
CsPr39	-	-	-	-	-	-
CsPr40	-	-	-	-	-	+++

Activities were classified according to the diameter of the point of application of the sample

+++ = Inhibition zone more than 20 –29 mm

++ = Inhibition zone more than 10 –19 mm

+ = Inhibiton zone more than 8 mm

- = No inhibition

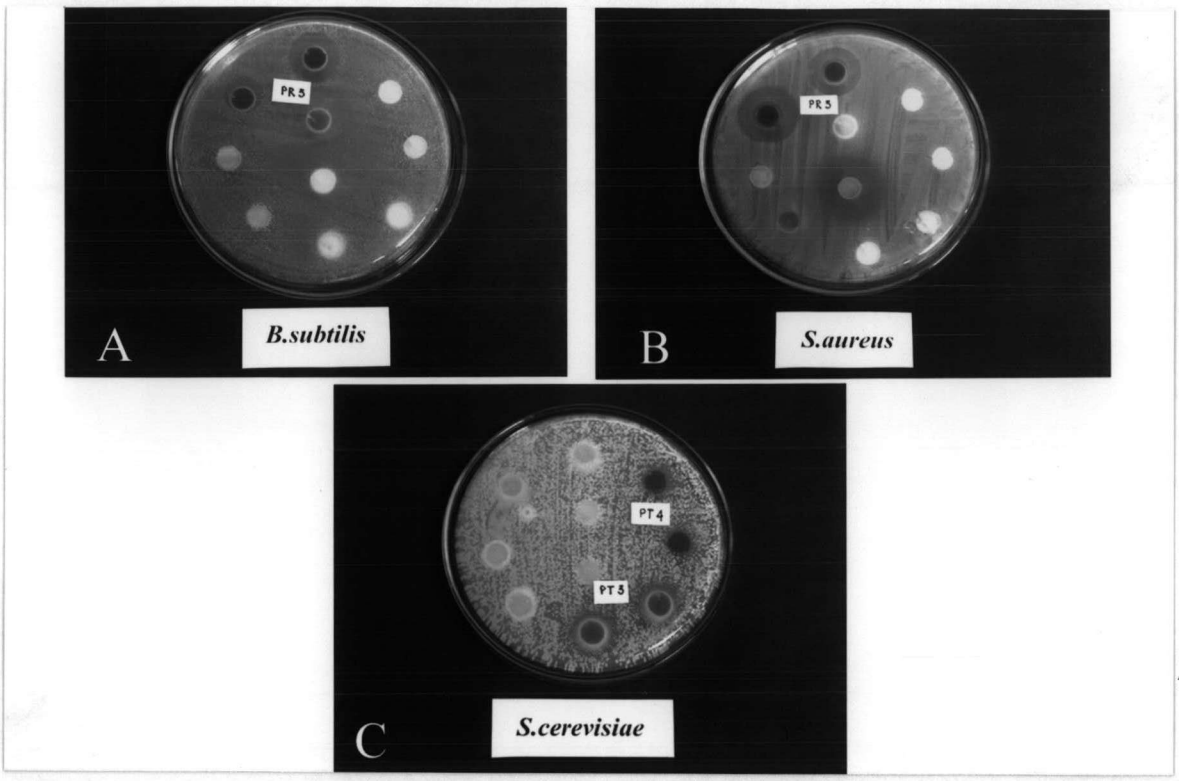


Figure 4.9 Dual culture agar diffusion technique for antimicrobial activities. A. against *B. Subtilis* ATCC 6633. B. against *S.aureus* ATCC 25923. C. against *S. cerevisiae* TISTR 5169

Table 4.4 The number of endophytic fungi isolates in each inhibition groups.

Group of inhibition	Endophytic fungi isolates	Sum	%
Inhibited one test microorganism	CsBkk03,CsBkk06,CsBkk14,CsBkk16, CsCh02,CsCh03,CsCh04,CsCh09, CsPt02,CsPt04,CsPt09 CsPr01,CsPr06,CsPr08,CsPr10,CsPr17, CsPr19,CsPr22,CsPr23,CsPr29,CsPr31,CsPr34	23	79.31
Inhibited two test microorganism	CsPt03,CsPr33	2	6.90
Inhibited three test microorganism	CsPr14,CsPr18	2	6.90
Inhibited four test microorganism	CsPr03,CsPr24	2	6.90

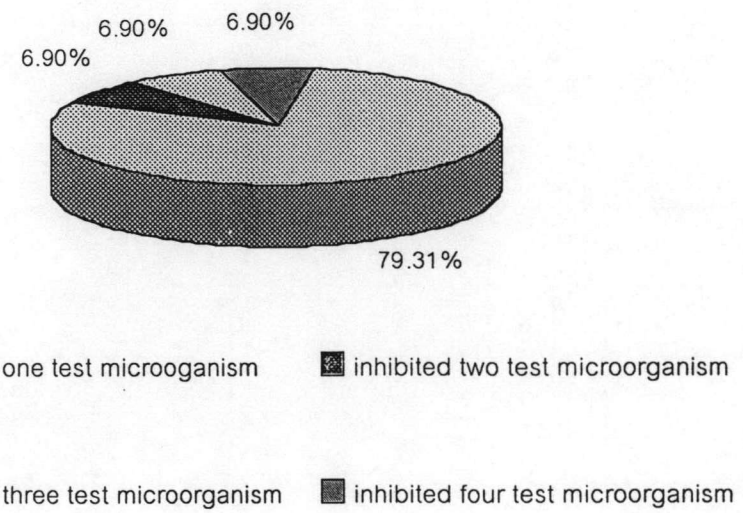


Figure 4.10 Percentage of each groups inhibiton

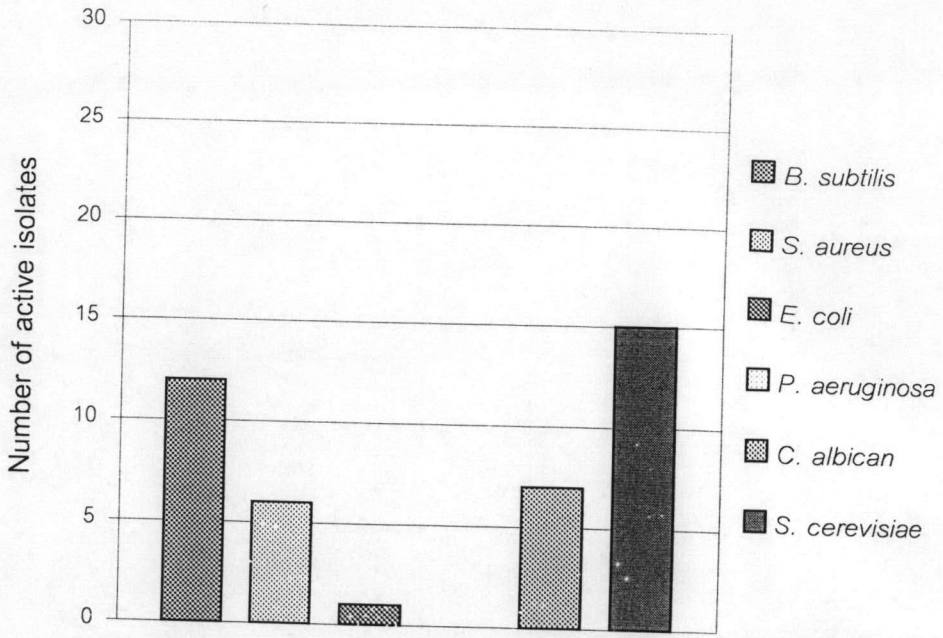


Figure 4.11 A summary of the dual culture agar diffusion technique assay results for the antimicrobial activities of endophytic fungi

4.5 Identification of fungal endophyte CsPr03

Fungal isolate CsPr03 was chosen for further study for bioactive compounds. Because these fungal can against a large number of test microorganism as *B.subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E.coli* ATCC 25922, and *S.cerevisiae* TISTR 5169.

4.5.1 Morphology identification

Fungal isolate CsPr03 was identified as *Bipolaris* sp. Descriptions of the genus Bipolaris is described in section 4.5.2. The fungus was grown on Malt extract agar (MEA) ,for 14 days at room temperature. Colony morphology and slide culture of isolate CsPr03 is shown in Figures 4.12

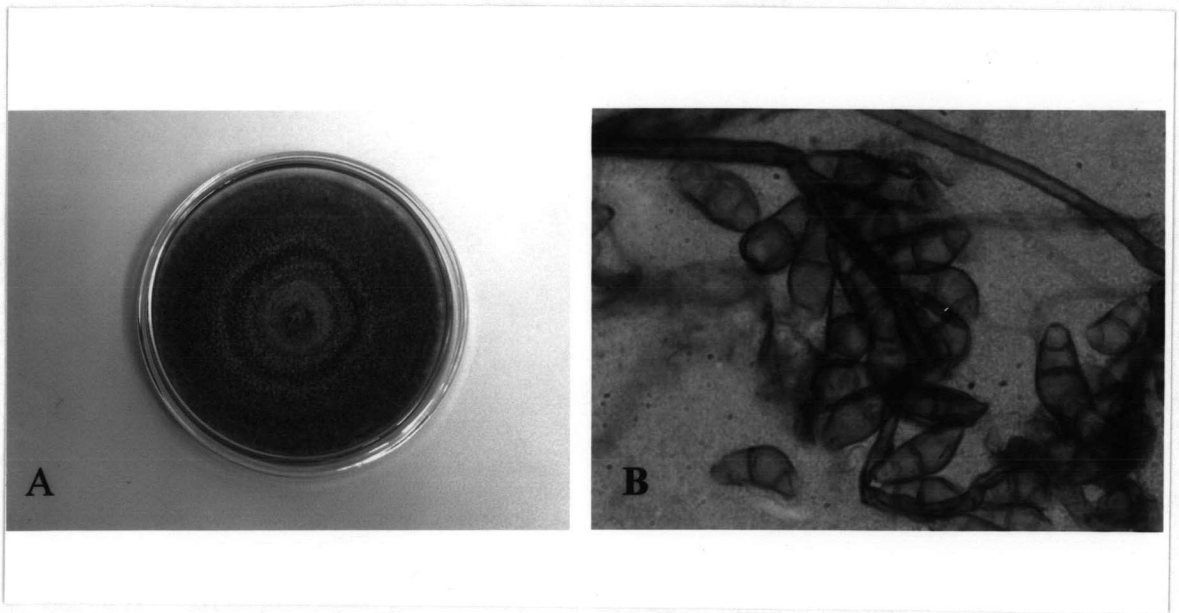


Figure 4.12 Charecteristics of endophytic fungal isolate CsPr03. A: Culture on MEA. B: Conidia (X100)

4.5.2 Taxonomy of Biporalis

Description of genus BIPOLARIS (Shoemaker,1959)

Mycelium brown,gray or black. Conidiophores straight or flexuous, multiseptate, usually simple, smooth, macronematous, mononematous, often geniculate, sometimes nodoes, cylindrical. Conidiogenous cells cylindrical, integrated, terminal or intercalary,proliferating sympodially, cicatrized. Conidia acropleurogenous, fusoid, obpyriform, navicular, oblong, cylindrical, obclavate, clavate, ovoid, solitary, curved to straight, mostly smooth, rarely echinulate to rought-walled, 2-or more distoseptate, septa sometimes thickened and dark, pale brown, olivaceous brown, reddish brown or dark brown, germinating principally from one or both polar cells with the basal germ tube originating close to hilum and growing semiaxially, hilum asscociated with a slightly protruding, truncate section of the wall, and often visible as two dark lenticular spots in optical section section arranged close together and seperated by a small obscure narrow canal, or rarely protuberant, first conidial septum median to submedian, second septum delimiting the basal cells, the third septum distal, conidiogenous nodes rough to smooth.

4.5.3 Molecular identification of endophytic fungi isolate CsPr03

The rDNA ITS region of isolate CsPr03 was amplified with the conserved fungal primer ITS_1 and ITS_4. Isolate CsPr03 produced a single ITS band .

The length of corresponding fragment was 604 bp.,containing a part of the 18S, ITS_1, 5.85 and 28 rDNA., as shown in Figure 4.13

```
1
   5'CTTGGTCATT        TAGAGGAAGT        AAAAGTCGTA        ACAAGGTCTC
     CGTAGTGAAC        CTGCGGAGGG        ATCATTACAC        AATACAATAT
     GAAGGCTGTC        CGCAGCTGGA        GTATTTTATT        ACCCTTGTCT
     TTTGCGCACT        TGTTGTTTCC        TGGGCGGGTT        CGCTCGCCAC
     CAGGACCACC        AAATAAACCT        TTTTTATGCA        GTTGCAATCA
     GCGTCAGTAC        AAACAATGTA        AATCATTTAC        AACTTTCAAC
     AACGGATCTC        TTGGTTCTGG        CATCGATGAA        GAACGCAGCG
     AAATGCGATA        CGTAGTGTGA        ATTGCAGAAT        TCAGTGAATC
     ATCGAATCTT        TGAACGCACA        TTGCGCCCTT        TGGTATTCCA
     AAGGGCATGC        CTGTTCGAGC        GTCATTTGTA        CCCTCAAGCT
     TTGCTTGGTG        TTGGGCGTTT        TTGTCTTTGG        TCGCCCAAAG
     ACTCGCCTTA        AAGTGATTGG        CAGCCGGCCT        TTCTGGTTTC
     GCAGCGCAGC        ACATTTTTGC        GCTTGCCATC        AGCAAAACGG
     CAATCCATCA        AGCCTCCTTC        TCACGTTTGA        CCTCGGATCA
     GGTAGGGATA        CCCGCTGAAC        TTAAGCATAT        CAATAAGCGG
     AGGA 3'
        604
```

Figure 4.13 Nucleotide sequences of partial 18S region, complete ITS region of the isolate CsPr03

```
>gi|30027133|gb|AY253918.1| Bipolaris spicifera 18S ribosomal RNA
gene, partial sequence;
          internal transcribed spacer 1, 5.8S ribosomal RNA gene
          and internal transcribed spacer 2, complete sequence;
          and 28S ribosomal RNA gene, partial sequence
          Length = 559

            10        20        30        40        50        60
GGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTAG-TGAACCTGCGGAGGGAT
                                 :::::::::::: ::::::::::::::::
                                AGGTCTCCGTAGGTGAACCTGCGGAGGGAT
                                        10        20        30

          70        80        90       100       110       120
CATTACACAATACAATATGAAGGCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTT
::::::::::::::::::::::::: :: ::: :::::: ::::: ::::::::::::::
CATTACACAATAAAATACGAAGGCCGTTCGCGGCTGGACTATTT-ATTACCCTTGTCTTT
          40        50        60        70        80        90


         130       140       150       160       170       180
TGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCGCCACCAGGACCACCAAATAAACCTTT
:::::::::::::::::::::::::::::::::::::::::::::::: : ::::::::::
TGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCGCCACCAGGACCACAATATAAACCTTT
         100       110       120       130       140       150

         190       200       210       220       230       240
TTTATGCAGTTGCAATCAGCGTCAGTACAAACAATGTAAATCATTTACAACTTTCAACAA
:::::::::::::::::::::::::: ::::::::::::::::::::::::::::::::
TTTATGCAGTTGCAATCAGCGTCAGTATAACAAATGTAAATCATTTACAACTTTCAACAA
         160       170       180       190       200       210

         250       260       270       280       290       300
CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAAT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAAT
         220       230       240       250       260       270
```

Figure 4.14 Alignment data of ITS region of isolate CsPr03 and 1 reference taxa.

```
       310        320        330        340        350        360
TGCAGAATTCAGTGAATCATCGAATATTTGAACGCACATTGCGCCCTTTGGTATTCCAAA
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TGCAGAATTCAGTGAATCATCGAATATTTGAACGCACATTGCGCCCTTTGGTATTCCAAA
       280        290        300        310        320        330

       370        380        390        400        410        420
GGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTT
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTT
       340        350        360        370        380        390

       430        440        450        460        470        480
GTCTTTGGTCGCCCAAAGACTCGCCTTAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGC
:::::::: : : ::::::::::::::::: ::::::::::::::: : ::::::::::
GTCTTTGGCCCCGCAAAGACTCGCCTTAAAATGATTGGCAGCCGGCC-TACTGGTTTCGC
       400        410        420        430        440        450

       490        500        510        520        530        540
AGCGCAGCACATTTTTGCGCTTGCCATCAGCAAA-----ACGGCAATCCATCAAGCCTCC
:::::::::::::::::::::::::::::::::     ::::::::::::::: ::::
AGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAAGAGGACGGCAATCCATCAAGACTCC
       460        470        480        490        500        510

       550        560        570        580
TTCTCACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC
:::::::::::::::::::::::::::::::::::::::::::::::::
TTCTCACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC
       520        530        540        550
```

Figure 4.14 (continued)

Classical identification of fungi is based on observed characteristics. Assignment of morphological spicies can be based on colony texture, hyphal pigments, exdates, margin shapes, growth rates, and sporulating sturctures (Rodlin and Carris,1985). Fungal isolate CsPr03 was identified as genera *Bipolaris*.

Molecular methods of identification was also performed. The nucleotide sequence of the ITS region of rDNA is conserved. It can be used to delineate spicies relationships and seperated taxonomy from class to spicies (Mitchell et al., 1995). The nucleotide sequence of ITS region of fungal isolate CsPr03 was similar to 95.349% identify of *Bipolaris spicifera* reported by Buzina et al., 2003

4.6 Cultivation and extraction

Fungal isolate PcBr20 was cultivated in malt extract broth totalling 10 L to yield 8.4 g of crude EtOAC from culture broth, and 13.4 g of crude EtOAc from mycelium.

4.7 Isolation and purification of bioactive compounds in crude culture broth and crude mycelium

Two compounds were isolated from the culture broth and crude mycelium of endophytic fungus isolated CsPr03. Compound 1 (112 mg, 8.47% yield of fraction BE01 and BE06) from fraction BE0121 and BE0602 were obtained from crystallization as colorless needle Crystal and compound 2 (10.5 mg, 8.61% yield of fraction ME04) from fraction ME0405 as the orange amorphous solid. Chemical structure of these compounds were determined by analyes of spectroscopic data. Including IR,UV,NMR and Mass spectra, as well as by comparison their spectral data with those of published values.

4.8 Structure elucidation of the pure compounds from endophytic fungus isolate CsPr03

4.8.1 Structure elucidation of compound 1

Compound 1 was a colorless needle crystals, m.p.147-148 ^{o}C The structure of compound 1 was elucidated by using spectroscopic techniques.

The IR spectra of compound 1 is shown in Appendix B Figure 2 and the absorption peaks were assigned as table 4.5 and indicated important absorption band at 3300-3600 cm^{-1} (O-H streching vibration of alcohol), and the strong absorption band at 1703 cm^{-1} (C=O vibration of carbonyl group) due to the carbonyl streching. The IR spectral data of compound 1 are summerized in Table 4.5

Table 4.5 The IR absorption band assigment of compound 1

Wave number (cm^{-1})	Intensity	Vibration
3300-3600	Broad	O-H streching vibration of alcohol
2968,2832	Weak	C-H streching vibration of $-CH_3$, $-CH_2$
1703	Medium	C=O streching vibration of carbonyl group
1481	Weak	C=C streching vibration of aromatic ring
1264	Strong	C-O streching vibration
1022	Medium	C-O streching vibration

The ^{1}H-NMR spectrum (Figure 3 in Appendix B) of compound **1** possessed two methyl groups at 1.26 ppm, the other attached with the carbonyl group at 2.53 ppm, and two methylene groups at 3.73, 4.12 ppm, and two olefinic protons of aromatic groups at 6.22, 6.31 ppm.

The ^{13}C-NMR spectrum (Figure 4 in Appendix B) of compound **1** showed 12 signals, which the carbonyl group of ester corresponded to the signal at 172 ppm. Six signals of olefinic carbons appeared at δ 160.5, 159.8, 136.4, 110.8, and 101.5 ppm.

The LC-MS mass spectrum (Figure 9 in Appendix B) showed the $[M+Na]^+$ ion peak at *m/z* 261.04, $[M+H]^+$ ion peak at 239.06 and $[M-OAc]^+$ ion peak at 192.99. The mass spectrum indicated that it possesses the molecular weight (238). If it is assumed that this compound contains only carbons, protons and oxygens, then the molecular formula of $C_{12}H_{14}O_5$ can be established. The molecular formular,$C_{12}H_{14}O_5$, of this compound indicated six degree of unsaturation, therfore, compound **1** must consist of one aromatice ring and two carbonyl groups.

The information from 2D-NMR techniques, including HSQC correlation (Table 4.6 , Figure 5 in Appendix B), HMBC corrlation (Table 4.7, Figure 4.15 and Figure 6 in Appexdix B),COSY correlation (Table 4.7, Figure 4.16 and Figure 7 in Appendix B) and NOESY correlation (Figure 4.17 and Figure 8 in Appendix B) were used to assist the interpretation of compound **1** structure.

Table 4.6 The HSQC spectral data of compound **1**

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
136.4 (s)	-
119.5 (s)	-
159.8 (s)	-
101.5 (d)	6.31 (1H, d, *J* =2)
160.5 (s)	-
110.8 (d)	6.22 (1H, d, *J* = 2)
39.7 (t)	3.73 (2H, s)
172.4 (s)	-
60.6 (t)	4.12 (2H, q, *J* =6.8)
13.1 (q)	1.26 (3H, t, *J* =6.8)
204.5 (s)	-
31.2 (q)	2.53 (3H,s)

Table 4.7 The HSQC,HMBC and COSY spectral data of compound **1**

Position	δ_C	δ_H	HMBC	COSY
1	136.4 (s)	-	-	-
2	119.5 (s)	-	-	-
3	159.8 (s)	-	-	-
4	101.5 (d)	6.31 (1H,d, *J* =2)	C-3 ,C-2 ,C-6	-
5	160.5 (s)	-	-	-
6	110.8 (d)	6.22 (1H, d, *J* =2)	C-5 ,C-2 ,C-4 ,C-1′	-
1′	39.7 (t)	3.73 (2H, s)	C-2′ ,C-1 ,C-2 ,C-6	-
2′	172.4 (s)	-	-	-
3′	60.6 (t)	4.12 (2H, q, *J* =6.8)	C-2′ ,C-4′	H-4′ (1.26)
4′	13.1 (q)	1.26 (3H, t, *J* =6.8)	C-3′	H-3′ (4.12)
5′	204.5 (s)	-	-	-
6′	31.2 (q)	2.53 (3H, s)	C-5′ ,C-2	-

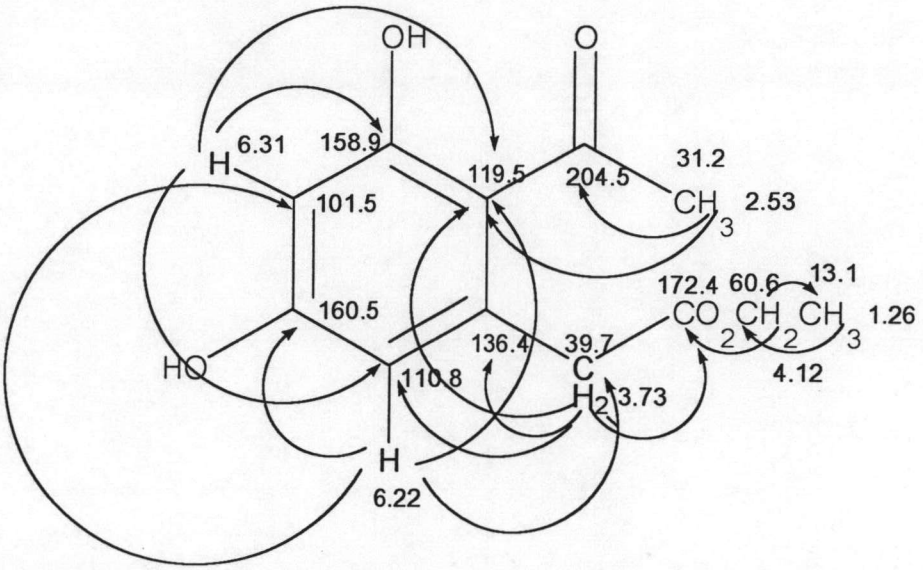


Figure 4.15 HMBC correlation of compound 1

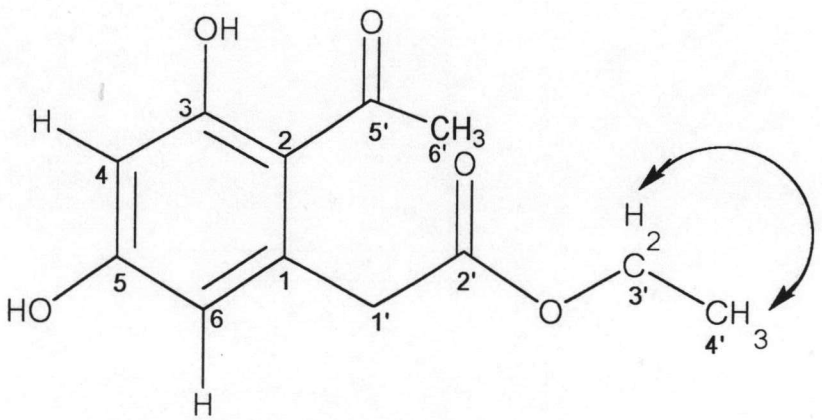


Figure 4.16 COSY correlation of compound 1

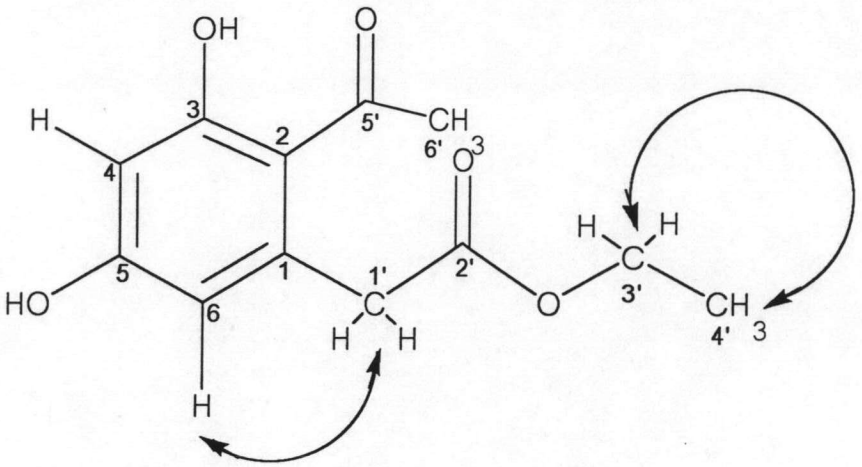


Figure 4.17 NOESY correlation of compound **1**

Compound 1 showed spectral data identical to that of curvulin, (2-acethyl-3,5-dihydroxy-phynyl-)-acetic acid ethyl ester, which was reported in the literature (Kenfield et al., 1989) The ^{1}H-NMR and ^{13}C-NMR singnal of compound 1 and curvulin are presented in Table 4.8 and 4.9 as follows.

Table 4.8 The ^{1}H-NMR spectral data of compound 1 (in CD_3OD) and Curvulin (in Acetone- d_6)

Position	δ_H(ppm)	
	Compound 1	Curvulin
1	-	-
2	-	-
3	-	-
4	6.31 (1H, d, *J* = 2)	6.55 (1H, d, *J* = 2.4)
5	-	-
6	6.22 (1H, d, *J* = 2)	6.49 (1H, d, *J* = 2.4)
1′	3.73 (2H, s)	3.95 (2H, s)
2′	-	-
3′	4.12 (2H, q, *J* = 6.8)	4.27(2H, q, *J* = 6.8)
4′	1.26 (3H, t, *J* = 6.8)	1.38(3H, t, *J* = 6.8)
5′	-	-
6′	2.53 (3H,s)	2.69 (3H,s)

Table 4.9 The ^{13}C-NMR spectral data of compound **1** (in CD_3OD) and curvulin (in Acetone-d_6)

Position	δ_c(ppm)	
	Compound 1	Curvulin
1	136.4	138.1
2	119.5	*
3	159.8	*
4	101.5	102.8
5	160.5	161.5
6	110.8	112.4
1'	39.7	40.8
2'	172.4	171.7
3'	60.6	61.2
4'	13.1	14.6
5'	204.5	205.7
6'	31.2	32.3

* = No Reported

After elucidation of compound **1** by 2D NMR technique, the chemical shift on ^{1}H-NMR and ^{13}C-NMR spectrum of compound **1** and curvulin were compared signal by signal. This result indicated that the structure of compound **1** is identical to curvulin. Thus, it could be concluded that compound **1** was a curvulin. The structure was presented in Figure 4.18

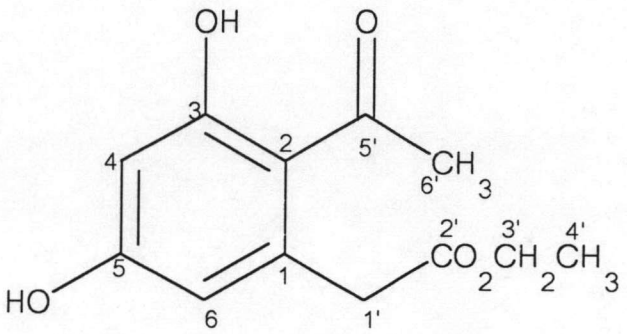


Figure 4.18 The structure of compound **1**

4.8.2 Structure elucidation of compound 2

Compound 2 was an orange needle crystals, m.p. 261-262 ^{o}C. The structure of compound was elucidated by using spectroscopic techniques.

The IR spectra of compound 2 is shown in Appendix B Figure 10 and the absorption peaks were assigned as table 4.10 and indicated important absorption band at 3200-3500 cm^{-1} (O-H streching vibration of alcohol), and the medium vibration at 1738 cm^{-1}(C=O vibration of carbonyl group) due to the carbonyl streching. The IR spectral data of compound 2 are summerized in Table 4.10

Table 4.10 The IR absorption band assignment of compound 2

Wave number (cm^{-1})	Intensity	Vibration
3200-3500	Broad	O-H streching vibration of alcohol
2925	Strong	C-H streching vibration of -CH_3
2844	Medium	C-H streching vibration of -CH
1738	Medium	C=O streching vibration of carbonyl group
1614,1470	Weak	C=C streching vibration of aromatic ring
1264,1209,1042	Weak	C-O streching vibration

The ^{1}H-NMR spectrum (Figure 11 in Appendix B) of compound 2 possessed a methyl proton attached to aromatic group (Ar-C*H*$_3$) at 2.52 ppm, four methine proton attached to aromatic group (Ar-H) at 6.30, 7.15, 7.21, 7.54 ppm. The hydroxy protons was exchanged at 12.06 and 12.13 ppm.

The ^{13}C-NMR spectrum (Figure 12 in Appendix B) of compound 2 showed 15 signals, which two carbonyl carbon at 191.9, 182.0 ppm.12 methine carbons of aromatic carbon signal at 166.0, 164.9, 161.9, 148.8, 135.7, 133.4, 124.7, 121.0,113.9,109.2, 108.5, ppm a methyl carbon at 22.0 ppm.

The maldy TOF mass spectra (Figure 15 Appendix B) showed the [MH^+] ion peak at *m/z* 273.32. If it is assumed that this compound contains only carbons, proton, and oxygen, then the molecular formular of $C_{15}H_{10}O_5$ can be estabished. The molecular formular, $C_{15}H_{10}O_5$, of this compound indicated 11 degree of unsaturation, therefore, compound **2** must consist of one ring, two aromatic rings and two carbonyl group.

The information from 2D-NMR technique; HSQC correlation (Table 4.11, Figure 13 in Appendix B), HMBC correlation (Table 4.12, Figure 14 in Appendix B) were used to assist the interpretation of the structure of compound **2**.

Table 4.11 The HSQC spectral data of compound **2**

^{13}C-NMR	^{1}H-NMR chemical shifts (ppm)
190.9	-
181.9	-
166.0	-
164.9	-
161.9	-
148.8	-
135.7	-
133.4	-
124.7	7.21 (1H, d, *J* =2.5)
121.0	7.54 (1H, br,s)
114.0	-
110.5	-
109.2	7.15 (1H,br,s)
108.5	6.63 (1H,d, *J* = 2.5)
22.0	2.52 (3H,s,Ar-CH_3)

Table 4.12 The HSQC and HMBC spectral data of compound **2**

Position	δ_C	δ_H	HMBC
1	164.4	-	-
2	108.5	6.63 (1H,d, J = 2.5)	-
3	166.0	-	-
4	109.2	7.15 (1H,br,s)	C-10,C-2
4a	135.7	-	-
5	124.7	7.21 (1H, d, J =2.5)	C-7,C-9a
6	148.8	-	-
7	121.0	7.54 (1H, br,s)	C-5, C-8a
8	161.9	-	-
8a	113.9	-	-
9	190.9	-	-
9a	110.5	-	-
10	181.9	-	-
10a	133.4	-	-
11	22.0	2.44 (3H,s, Ar-CH_3)	C-8, C-7, C-6, C-5

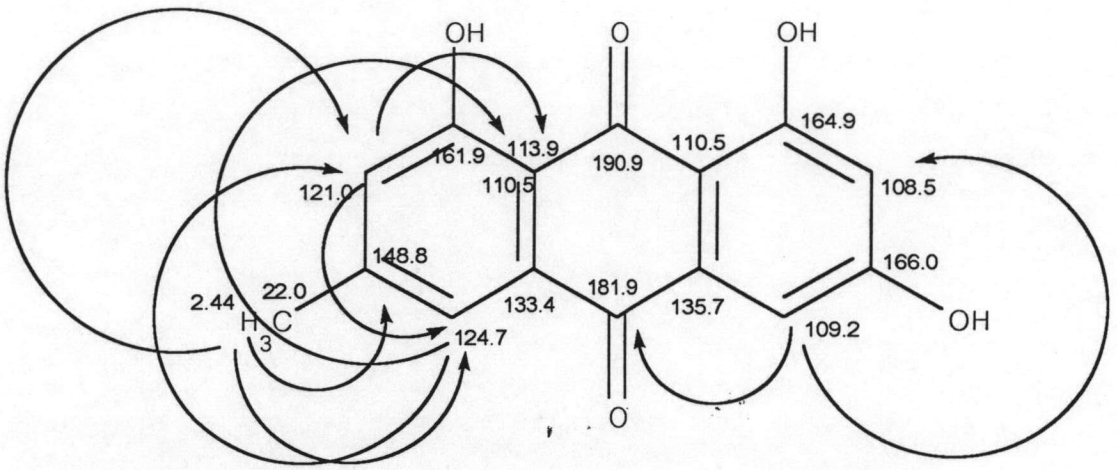


Figure 4.19 HMBC correlation of compound 2

Compound **2** showed spectral data identical to that of Emodin, (1,3,8-trihydroxy-6-methylanthraquinone), which was reported in the literature (Cheol et al., 2003) The ^{1}H-NMR and ^{13}C-NMR singnal of compound 2 and emodin are presented in Table 4.13 and 4.14 as follows.

Table 4.13 The ^{1}H-NMR spectral data of compound **2** (in DMSO-d_6) and Emodin Acetone-d_6)

Position	δ_H (ppm)	
	Compound 1	Emodin
1	7.15 (1H,s)	7.12(1H,s)
2	6.63 (1H, d *J* =2.5)	6.65 (1H, d, *J* =2.5)
3	-	-
4	7.21 (1H,d, *J* =2.4)	7.24 (1H,d, *J* =2.5)
4a	-	-
5	7.54 (1H,s)	7.55 (1H,s)
6	-	-
7	-	-
8	-	-
8a	-	-
9	-	-
9a	-	-
10	-	-
10a	-	-
11	2.44 (3H,s)	2.46 (3H,s)

Table 4.14 The ^{13}C-NMR spectral data of compound **2** (in DMSO-d_6) and emodin (in Acetone-d_6)

Position	δ_C (ppm)	
	Compound 2	Emodin
1	164.9	165.9
2	108.5	108.6
3	166.0	165.9
4	109.2	109.7
4a	135.7	136.6
5	124.3	124.8
6	148.8	149.3
7	121.0	121.2
8	161.9	161.9
8a	113.9	114.4
9	190.9	191.9
9a	110.5	110.0
10	181.9	182.2
10a	133.4	134.0
11	22.0	21.8

The chemical shift on ^{1}H-NMR and ^{13}C-NMR spectra of compound **2** and emodin were compared signal by signal. This result indicated that the structure of compound **2** is identical to emodin. Thus, it could be concluded that compound **2** was emodin. The structure was presented in Figure 4.20.

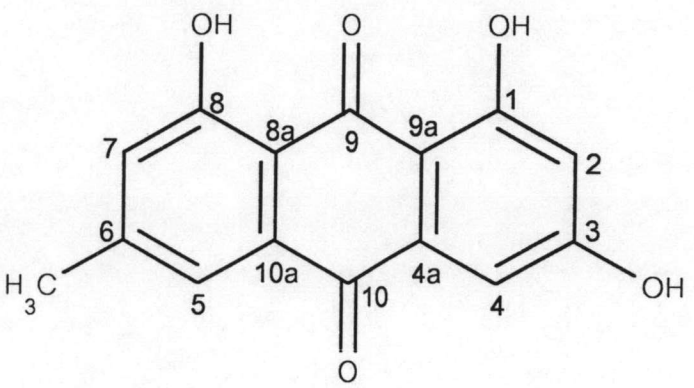
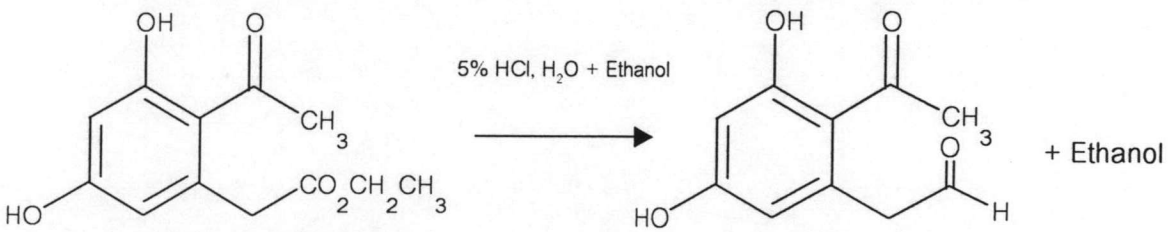


Figure 4.20 The structure of compound **2**

4.9 Hydrolysis of compound 1



Compound **1** (10 mg) Compound **3** (8.4 mg)

Sheme 4.1 Hydrolysis reaction of compound **1**

From the chemical shift on ^{1}H-NMR of compound **1** and compound **3** (figure 16 in Appendix B) compared signal by signal. This result indicated that the structure of compound 3 as showed in sheme 4.1.

4.10 Biological activities

4.10.1 Antimicrobial activity of the culture broth of endophytic fungus isolate CsPr03

The antimicrobial activity of the culture broth of endophytic fungus isolates CsPr03 was evaluated by the agar well diffusion method. Aliquote of 100µl of culture filtrate was pipetted into the agar wells. The antimicrobial activity was calculated from the inhibition zones (mm) of test microorganism. Results indicated that culture broth was active against *B. subtilis* ATCC 6633, *S.aureus* ATCC 25932, *E. coli* ATCC 25922 and *S. cerevisiae* TISTR 5169.

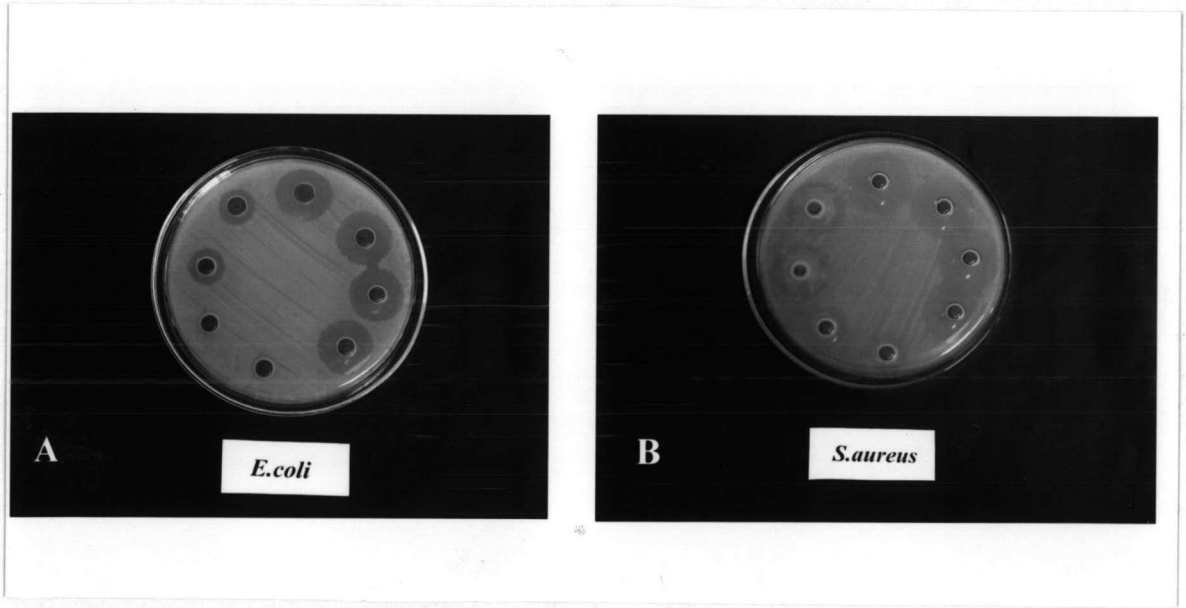


Figure 4.21 Antimicrobial activty of culture broth of endophytic fungus isolate CsPr03 A: Against *B. subtilis* ATCC 6633. B: Againts *S. aureus* ATCC 25923

4.10.2 Antimicrobial activity of the crude extracts from endophytic fungus isolate CsPr03

The antimicrobial activity of the crude extracts from endophytic fungus isolate CsPr03 were evaluated by the agar well diffusion method. The fractions were examined at a concentration of 5 and 10 mg/ml (1mg /well; 8 mm diameter). The antimicrobial activities were calculated from the inhibition zones (mm) of test microorganisms ,including the bacterial strains *B.subtilis* ATCC 6633, *S.aureus* ATCC 25923, *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853, and fungi, yeast form strains *C.albicans* ATCC 10231. and *S.cerevisiae* TISTR 5169. Antimicrobial activity of each fraction from crude extract is shown in Table 4.15

Table 4.15 Antimicrobial activities of the crude extracts from endophytic fungus isolate CsPr03

Test microorganisms	Crude extract			
	Culture broth		Mycelium	
	5 mg/ml	10 mg/ml	5 mg/ml	10 mg/ml
B.subtilis	++	++	++	+++
S.aureus	++	++	-	-
E.coli	++	++	-	-
P.aureuginosa	-	-	-	-
C.albicans	-	-	-	-
S.cerevisiae	-	-	-	-

Activities were classified according to the diameter of the point of application of the sample

+++ = Inhibition zone more than 20 –29 mm

++ = Inhibition zone more than 10 –19 mm

\+ = more than 8 mm

\- = No inhibition

4.10.3 Antimicrobial activities of the fractions from crude extracts

The antimicrobial activities of the each fractionated crude extract was evaluated by the agar well diffusion method. The fractions examined at a concentration of 1000 μg/ml (0.1 mg /well; 8 mm diameter). The antimicrobial activities were calculated from the inhibition zones (mm) of test microorganism including, the bacterial strains *B.subtilis* ATCC 6633, *S.aureus* ATCC 25923, *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853, and fungi, yeast form *C.albicans* ATCC 10231. and *S.cerevisiae* TISTR 5169. Antimicrobial activities of each fraction from crude extract is shown in Table 4.16 and Table 4.17.

Table 4.16 Antimicrobial activities of each fractioned of culture broth extracts

Fraction	Test microorgamisms					
code	*B. subtilis*	*S.aureus*	*E.coli*	*P.aeruginosa*	*C. albicans*	*S. cerevisiae*
BE01	-	-	-	-	-	-
BE02	-	-	-	-	-	-
BE03	++	-	-	-	-	++
BE04	++	-	-	-	-	+
BE05	-	-	-	-	-	-
BE06	-	-	-	-	-	-
BE07	-	-	-	-	-	-
BE08	-	-	-	-	-	-
BE09	++	-	-	-	-	-
BE10	-	-	-	-	-	-
BE11	-	-	-	-	-	-
BE12	-	-	-	-	-	-

Activities were classified according to the diameter of the point of application of the sample

+++ = Inhibition zone more than 20 –29 mm

++ = Inhibition zone more than 10 –19 mm

\+ = Inhibiton more than 8 mm

\- = No inhibition

Table 4.17 Antimicrobial activities of each fractionated of mycelium extracts

Fraction	Test microorgamisms					
code	*B. subtilis*	*S.aureus*	*E.coli*	*P.aeruginosa*	*C. albicans*	*S. cerevisiae*
ME01	+	-	-	-	-	-
ME02	++	-	-	-	-	-
ME03	+	-	-	-	-	-
ME04	++	-	-	-	-	-
ME05	++	-	-	-	-	+
ME06	-	-	-	-	-	-
ME07	-	-	-	++	-	-
ME08	-	-	-	-	-	-
ME09	+	-	-	++	-	-

Activities were classified according to the diameter of the point of application of the sample

+++ = Inhibition zone more than 20 –29 mm

++ = Inhibition zone more than 10 –19 mm

+ = Inhibiton more than 8 mm

- = No inhibition

4.10.4 Antimicrobial activities of pure compounds

The antimicrobial activities of pure compounds was evaluated by the antimicrobial susceptibility test, broth microdilution method. The pure compound was examined at concentration of 0.48-1,000 μg/ml (two-fold dilution). Antimicrobial activities tests were performed against *B.subtilis* ATCC 6633, *S.aureus* ATCC 25923, *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853, *C. albicans* ATTC 10231, *S.cerevisiae* TISTR 5169. The lowest concentration of pure compoud showing complete inhibition of growth is recorded as the minimal inhibitory concentration (MIC). Antimicrobial activities of pure compounds is shown in Table 4.18.

Table. 4.18 Broth microdilution method for antimicrobial activities of pure compounds

Compound	Test microorganisms and MIC (μg/ ml)					
	Gram positive bacteria		Gram negative bacteria		Yeasts	
	B. subtils ATCC6633	*S. aureus* ATCC 25923	*E.coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. cerevisiae* TISTR 5169	*C. albicans* ATCC 10231
Compound **1**	15.62	-	-	-	500	-
Compound **2**	-	-	-	-	-	500
Compound **3**	-	125	-	-	-	-
Tetracycline HCl	31.25	0.77	0.96	ND	ND	ND
Amoxicillin	-	0.77	0.96	ND	ND	ND

- = inactive

ND = Not determined

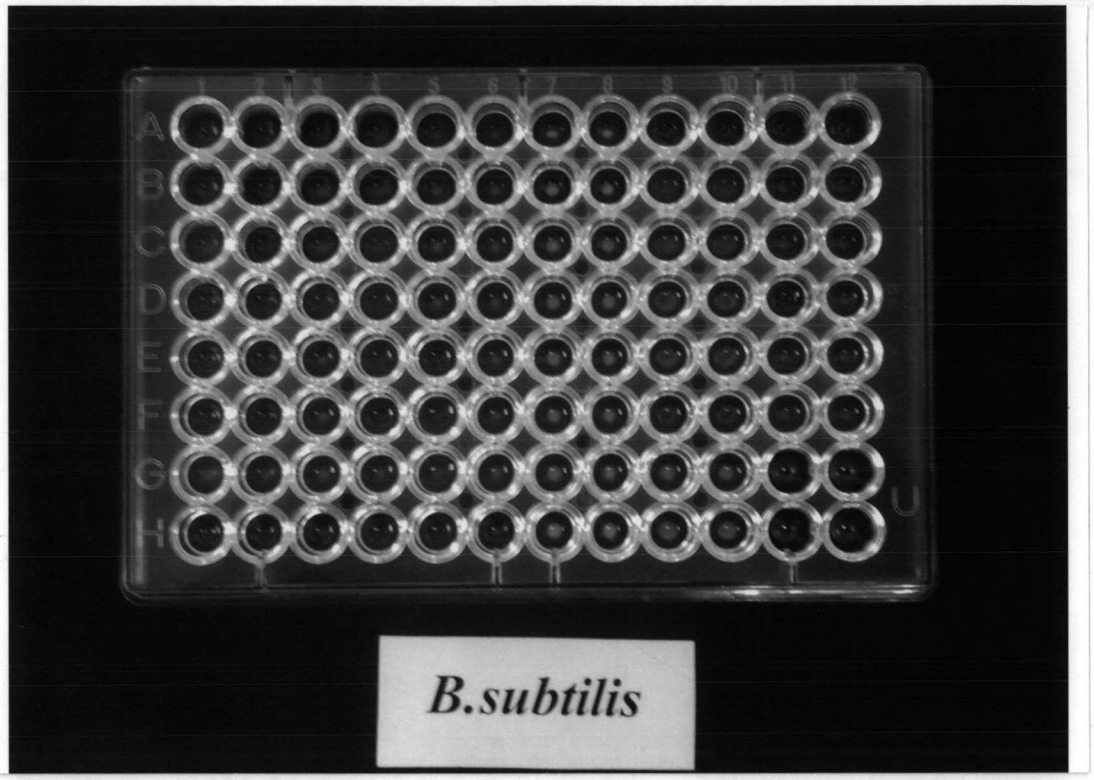


Figure 4.22 Broth microdilution methods for antimicrobial activity of compound **1** against *B. subtilis* ATCC 6633. Clear or colorless wells indicate growth inhibition.

4.8.5 Cytotoxic activity

The *in vitro* activity of pure compounds from fungal isolate CsPr03 was tested against 5 cell lines including, HEP-G2 (hepatoma), SW620 (colon),CHAGO (lung), KATO-3 (gestric), BT474(breast) and is reported in Table 4.19

Table 4.19 Cytotoxic activities against cell line of pure compound from endophytic fungus isolate CsPr03

Compounds	IC_{50} (μg/ml)				
	HEP-G2 (hepatoma)	SW620 (colon)	CHAGO (lung)	KATO-3 (gestric)	BT474 (breast)
Compound 1	>10	>10	>10	>10	>10

IC_{50} was the minimum concentration of 50% inhibitory activity

From the data in Table 4.19, the compound 1 showed no activity on cytotoxic against 5 tumor cell lines.

Presently, endophytic fungi are realized as a potential producer of novel secodary metabolites. From the comprehensive study of endophytic fungi has indicated that 51% of biologically active substance isolated from endophytic fungi were previously unknown (Shutz,2001). In this reserch, endophytic fungi were isolated from *Croton sublyratus* leaves which is a Thai folk medicinal plant. Two compounds were isolated from culture broth and mycelium extracted of endophytic fungi isolate CsPr03, compound 1 was curvulin and compound 2 was emodin. The pure compounds showed antimicrobial activities. Curvulin exhibited inhibiton activity against gram positive bacteria *B. subtilis* ATCC 6633 and yeast *S. cerevisiae* TISTR 5169., Emodin exhibited againts inhibition activity *C. albicans* ATCC 10231.

Curvulin has been reported as a phytotoxin activity of purslane and spiny amaranth. (Kenfield et al.,1989). Phytotoxin may prove useful directly as herbicides or analogs for the development of selective, nonpersistent herbicides (Strobel et al.,1987).

For emodin, it is an anthraquinones produced by various fungi, including such ubiquitous spicies as *Cladosporium fulvum* Cooke.(Agosti et al., 1962), *Aspergillus ochraceus* (Wehner et al.,1979) and *Aspergillus wentii* (Wells et al..,1975). It is regarded as a precursor of many of the naturally ocuuring fungal antraquinones. It has been reported as a framshift mutagen for *Salmonella typhimurium* strain TA 1537 (Wehner et al., 1979). In addition, emodin is an important component of Chinese herbal called Dewhung. In 2003 Yang et al., reported that emodin isolated from the seed of *Cassie obtusifolia* showed larvicidal activity against three Mosquito spicies which were *Aedes aegypti*, *Aedes togoi* and *Culex pipeiens pallens*.

From this result, it indicated that endophytic fungi should be a good source for isolation of active compounds without destruction of the forest (Strobel and Long,1998).