CHAPTER III

EXPERIMENT

3.1 PLANT MATERIALS

The plant materials of *C. oblongifolius* Roxb. used in this study were collected from Amphoe Maung, Prachaubkhirikhan province, Thailand, in August 1999. The plant specimen was compared against voucher specimen NO. BKF 084729 deposited in herbarium of the Royal Department of Thailand.

3.2 INSTRUMENTS AND EQUIPMENTS

3.2.1 Mass spectrometer (MS)

The mass spectra were obtained with a Fison Instruments Mass Spectrometer model Trio 2000 at 70 ev.

3.2.2 Ultraviolet-Visible Spectrophotometer (UV-VIS)

The UV-VIS spectra were recorded on a Hewlett Packard 8452 A diode array spectrophotometer in chloroform.

3.2.3 Fourier Transform-Infrared spectrophotometer (FT-IR)

The IR spectra were obtained on Nicolet impact 410 Spectrophotometer. Spectra of solid sample were recorded as KBr pellets and liquid samples were recorded as thin film on NaCl cell.

3.2.4 ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H and ^{13}C Nuclear spectra were recorded at 200.13 and 50.32 MHz respectively, on a Bruker Model AC-F200 spectrometer, and at 500.00 and 125.65 MHz on JEOL JNM-A500 spectrometer in $CDCl_3$. Chemical shifts are given in parts per million using residual protonated solvent as a reference COSY, NOESY, HMBC and HMQC experiments were performed on the JEOL JNM-A500 spectrometer.

3.2.5 Optical Rotation

The optical rotation spectra were recorded on a Perkin-Elmener 341 polarimeter.

3.2.6 Elemental analysis (EA)

The EA were measured on a Perkin Elmer PE 2400 SERIES II (CHN/O ANALYSER)

3.3 CHEMICALS

All commercial grade solvents were distilled prior to use. Silica gel (Merck Kiesel gel 60) and silica gel TLC plate (silica gel 60F254) were purchased from Merck Company.

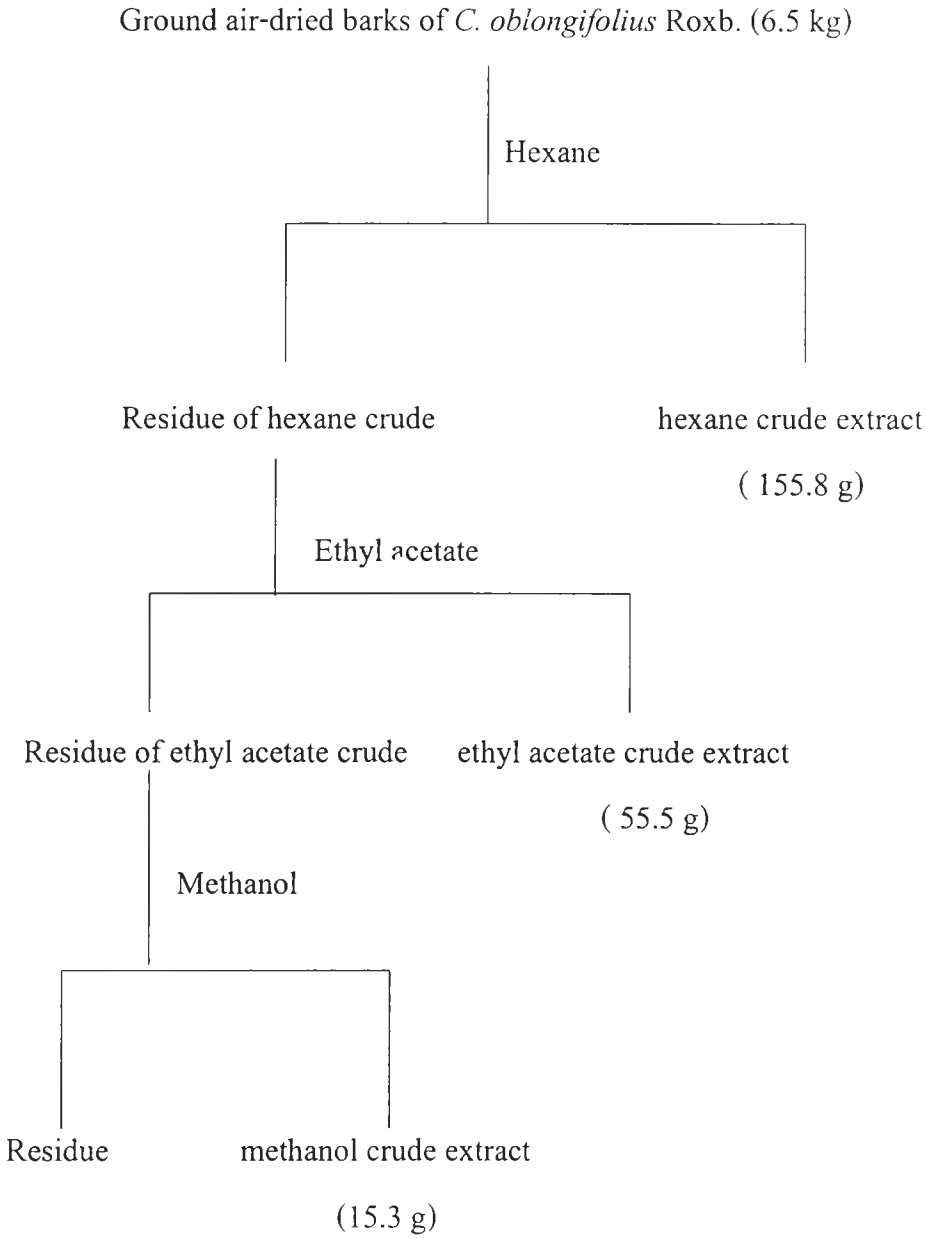
3.4 EXTRACTION AND ISOLATION

The powdered, sun-dried stem barks (6.5 kg) of *C. oblongifolius* Roxb. were soaked with hexane (3 x 10 liters), ethyl acetate (3 x 10 liters) and methanol (2 x 10 liters), respectively. The first two extracts were evaporated under reduced pressure until dry, yielding hexane crude extracts as yellow green oil (155.8 g) and ethyl acetate crude extracts as yellow oil (55.5 g). The methanolic extract also evaporated under reduced pressure to obtain dark-red gummy residue (15.3 g). They are shown in Table 4.

Table 4. **The solvent extracts of the stem barks of *Croton oblongifolius* Roxb.**

Solvent extract	Appearance	Weight(g)	%wt by wt of the dried stem barks
Hexane	Yellowish green oil	155.8	2.40
Ethyl acetate	Yellow oil	55.5	0.85
Methanol	Dark-red gummy	15.3	0.24

The procedure and results of extraction are shown in Scheme 1.



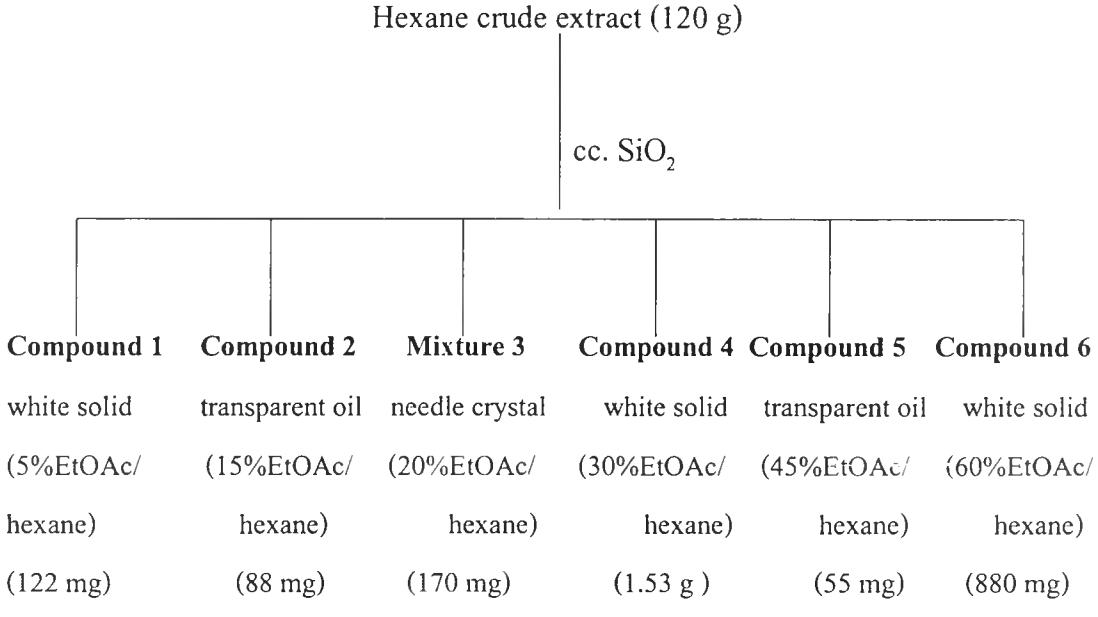
Scheme 1 The procedure of extraction of the stem barks of *C. oblongifolius* Roxb.

3.5 SEPARATION OF CRUDE EXTRACT OF *C. oblongifolius* Roxb.

3.5.1 Separation of hexane crude extract

The hexane crude extract was obtained as a yellowish green oil (155.8 g). The crude extract (120 g) was fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 100% ethyl acetate. The volume of each fraction was approximately 500 ml and each was evaporated to about 30 ml. The similar fractions were combined together according to the TLC profile. The isolation of all compounds from hexane extracts was briefly summarized in Scheme 2 and the result of separation was shown in Table 5.

Isolation procedure of hexane crude extract



Scheme 2 : Isolation procedure of hexane crude extract

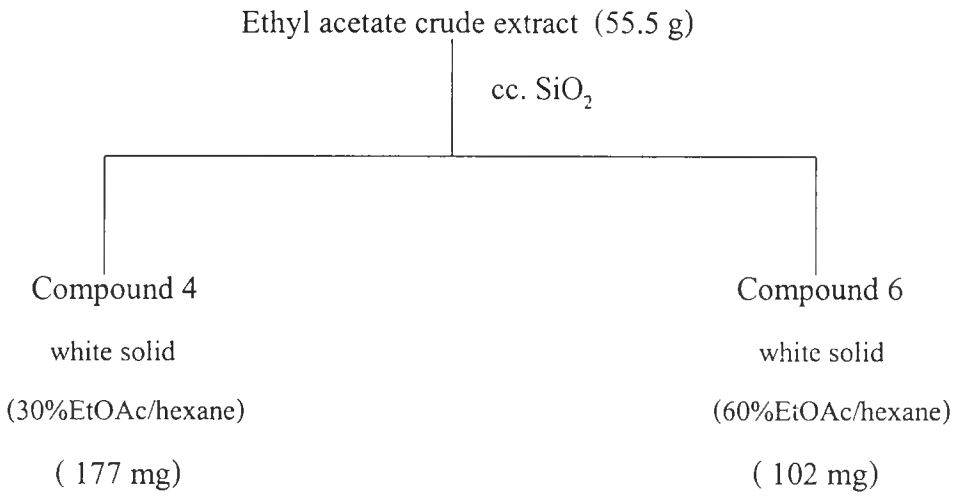
Table 5. The result from column chromatography of hexane crude extract.

Fraction no.	Appearance of solution	Combined fraction	Compounds
1-3	Clear	F_1	-
4-9	Light yellowish	F_2	**Compound 1**
10-15	Yellowish	F_3	**Compound2**
16-22	Yellowish	F_4	Yellowish oil
23-29	Light yellowish	F_5	**Mixture 3**
30-35	Light yellowish	F_6	Light yellowish oil
36-46	Light yellowish	F_7	**Compound 4**
47-51	Light green	F_8	Light yellowish oil
52-58	Dark green	F_9	Dark green oil
59-64	Light yellowish	F_{10}	**Compound 5**
65-70	Light yellowish	F_{11}	Light yellowish oil
71-74	Light green	F_{12}	Light green oil
75-84	Light green	F_{13}	**Compound 6**
85-92	Dark green	F_{14}	Dark green oil
93-102	Light brown	F_{15}	Light brown oil
103-110	Dark brown	F_{16}	Dark brown oil
111-120	Light yellowish	F_{17}	Light yellowish oil

3.5.2 Separation of ethyl acetate crude extract

The ethyl acetate crude extract (55.5 g) was separated on a silica gel column chromatography. The column was eluted with hexane, hexane-ethyl acetate, ethyl acetate and ethyl acetate-methanol, respectively. From ^{1}H and ^{13}C NMR spectra and TLC analysis it indicated ethyl acetate crude extract contained similar compounds found in the hexane crude extract.

Isolation procedure of ethyl acetate crude extract



Scheme 3 : Isolation procedure of ethyl acetate crude extract

3.5.3 Separation of methanol crude extract

The methanol crude extract was obtained as a gummy residue (15.3 g). It was not soluble in all solvents, thus, crude methanol could not be purified.

3.6 PURIFICATION AND PHYSICAL PROPERTIES OF COMPOUNDS FROM HEXANE CRUDE EXTRACT.

3.6.1 Purification and Physical property of Compound 1

The compound 1 was eluted with 5% ethyl acetate in hexane on silica gel chromatography. This compound was a white solid powder (122 mg, 0.003% wt by wt) having a melting point of 128-130 °C. It was soluble in various solvents such as chloroform, ethyl acetate, methanol and ethanol.

Compound 1 is a white solid (122 mg), $[\alpha]_D^{20}$ −74.5° ($CHCl_3$; *c*1.0)
This compound has no UV absorption.

FT-IR spectrum (KBr), υ_{max} (cm^{-1}): 3300-2400(br), 2924 and 2848 (s), 1695 (s) , **(Fig 18)**

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 0.58(1H,dt,*J*=2.4,7.9), 0.78(1H,dd,*J*=4.0,13.1),0.82(1H,dd,*J*=3.3,7.9),0.88(3H,s),0.99 (1H,ddd,*J*=4.3,13.4,13.4), 1.0(1H,dd,*J*=2.8,11.0), 1.08(1H,m), 1.14(3H,s), 1.19 (1H,m),1.21(3H,s),1.23(1H,d,*J*=11.3),1.32(1H,dt,*J*=4.9,13.1),1.35(1H,m), 1.39 (1H,d,*J*=11.3),1.45(1H,dt,*J*=3.4,6.7),1.67(1H,ddd,*J*=2.4,7.3,14.7),1.72 (1H,ddd,*J*=1.2,9.2,12.4), 1.75(1Hdt,*J*=2.7,6.1), 1.84(1H,ddd,*J*=6.4,10.1,13.7), 1.87(1H,ddd,*J*=3.1,3.1,11.4), 2.05(1H,d,*J*=11.6) and2.12(1H,ddd,*J*=3.4,3.4,7.3) , **(Fig 19)**

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm): 12.4(q), 18.7(t), 19.7(t), 20.5 (d), 20.5(q), 21.7(t). 22.4(s), 24.3(d), 28.8(q), 33.1(t), 37.8(t), 38.9(s), 39.2(t), 39.4(t), 40.7(s), 43.7(s), 50.3(t), 52.8(d), 57.0(d) and 184.5(s), **(Fig 20 and 21)**

EI-MS spectrum m/z (70 ev); 302 [M^+(100)], 287[M^+- CH_3, (41)], 269[287-H_2O,(2)], 260(11), 247(10), 246(82), 231(30), 220(8), 201(13), 187(18), 175 (20), 159(22), 147(27), 134(33), 119(55), 105(61), 95(23), 93(59), 91(25), 81 (22) and 79(20) **(Fig 22)**

3.6.2 Purification and Physical property of Compound 2

The compound 2 was isolated as transparent oil (88 mg, 1.35 x 10^{-3} %wt by wt). It was obtained after eluting it with 15% ethyl acetate in hexane and removing the solvent by rotary evaporation. It was found to be soluble in hexane, chloroform and ethyl acetate.

Compound 2 is a transparent oil (88 mg), $[\alpha]_D^{20}$ –132.1°C ($CHCl_3$; *c*1.0)

FT-IR spectrum, (KBr), υ_{max} (cm^{-1}): 3400-2400 (br), 2950(s), 1697(s), 1640 and 1620 (s), **(Fig 23)**

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 0.80(3H,d,*J*=6.5), 0.84 (3H,d,*J*=6.5), 1.5-2.5(11H,m), 1.66(3H,s),1.82(3H,s), 3.05(1H,m), 5.21(1H,m), 5.56(1H,m) and 6.04(1H,m) **(Fig 24)**

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 14.4(q), 19.3(q), 19.9(q), 20.9(t), 25.8(t), 16.2(t), 28.9(t), 32.1(t), 32.7(d), 38.8(t), 37.8(d), 125.7(d), 127.9(d), 128.8(d), 130.4(d), 130.9(s), 131.3(d), 135.1(s), 147.6(d) and 173.3 (s), **(Fig 25 and 26)**

EI-MS spectrum m/z (70 ev); 302 [M^+(100)], 287[M^+-CH_3, (66)], 279(11), 260(6), 259(23), 246(32), 241(16), 231(12), 213(7), 185(11), 175(13), 171(16), 167(13), 159(17), 131(14), 119(23), 111(20), 105(84), 99(14), 93(79), 91(45), 85(20), 81(22), 80(25) and 79(7) **(Fig 27)**

3.6.3 Purification and Purification of Mixture 3

Mixture 3 was obtained as a white needle like solid, which was eluted with 20% ethyl acetate in hexane. It was purified by crystallization from hot hexane to afford this compound as colorless needles (170 mg, 2.62 x 10^{-3} %wt by wt) with a melting point of 140-142 °C. This compound is soluble in chloroform, slightly soluble in hexane, ethyl acetate and insoluble in methanol.

Mixture 3 is a colorless needle (170 mg)

FT-IR spectrum, (KBr), υ_{max} (cm^{-1}): 3695-3048 (br), 2959(s) and 2937(s), 1500(s), 1400(s) and 1100(s) **(Fig 28)**

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 0.66(3H,s), 0.80(3H,s), 0.95 (6H,s), 1.30(3H,s), 1.75(3H,s), 1.85-2.3(m), 3.53(1H,m), 4.90-5.10(4H,m) and 5.34(1H,m)**(Fig 29)**

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 11.9(q), 11.9(d), 18.8(q), 19.1(d), 19.4(d), 19.8(q), 21.1(q), 21.1(t), 24.3(t), 26.2(t), 28.2(t), 29.3(q), 31.8 (t), 32.0(d), 32.0(t), 34.0(d), 36.2(d), 36.5(s), 37.4(t), 39.4(t), 42.2(s), 42.4(t), 50.3(d), 50.3(q),56.2(d), 56.8(d), 71.6(d), 121.8(d) and 140.9(s) **(Fig 30)**

EI-MS spectrum m/z (70 ev); 414 [M+, 28], 412(7), 396[M^{+}-H_2O, (11)], 381 [396-CH_3, (8)], 367(2), 354(4), 329(15), 314(7), 303(16), 273(13), 255(30), 231(18), 229(9), 213(25), 199(11), 187(9), 173(16), 159(29), 145(48), 133(39), 119(44), 107(71), 95(70), 81(73), 69(57), 55(88), and 43(100) **(Fig 31)**

3.6.4 Purification and Physical properties of Compound 4

Compound 4 was obtained from the crude hexane extract eluting with 30% ethyl acetate in hexane. This compound was a white solid (1.53 g, 0.024 % wt by wt) which melted at 72-73°C. This compound is soluble in various solvents such as chloroform, ethyl acetate, methanol and ethanol.

Compound 4 is a white solid (1.53 g), $[\alpha]_D^{20}$ –24.3°C ($CHCl_3$; *c*1.0)

UV λ_{max} (nm), CH_3Cl (log ε) = 244sh (4.03)

FT-IR spectrum, (KBr), υ_{max} (cm^{-1}): 3420 (br), 2951(s), 2925(s) and 2879(s), 1465(br) and 1090(br), **(Fig 33)**

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 0.77(3H,s), 0.85(3H,s), 1.00 (1H,dd,*J*=2.1,12.5),1.10(3H,s),1.26(1H,dd,*J*=12.5,12.8),1.37(2H,m),1.54 (2H,m), 1.73(3H,s), 1.81(1H,ddd,*J*=2.1,4.6,12.8), 2.15 (1H,dt,*J*=6.1,6.1,16.2), 2.35(1H,dt,*J*=6.1,6.1,16.2),3.48(1H,dd,*J*=4.6,11.6),2.82(2H,m),5.02 (1H,d,*J*=17.4),4.87(2H,d,*J*=11.0),5.52(1H,t,*J*=6.7)and 6.29(1H,dd,*J*=11.0,17.4) **(Fig 34)**

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 11.8(q), 15.6(q) , 17.9(q), 18.5(t) , 21.6(q), 23.5(t), 27.6(t), 33.2(s), 33.5(q), 39.2(s), 39.8(t), 41.6(t), 53.6 (d), 60.2(d), 78.1(s), 80.3(d), 110.6(t), 132.7(s), 135.5(d) and 141.5(d) **(Fig 35 and 36)**

EI-MS spectrum m/z (70 ev); 306 [M^+, (5)], 288[M^+-H_2O, (50)], 270[288-H_2O, (21)], 255[270-CH_3, (11)], 245(6), 233(5), 220(7), 208(7), 207(20), 205 (15), 193(14), 189(21), 177(68), 164(35), 150(100), 135(41), 123(65), 109 (52), 93(52), 81(59), 69(49) and 55(57) **(Fig 37)**

3.6.5 Purification and Physical property of Compound 5

Compound 5 was obtained as transparent oil (55 mg, 8.46 x 10^{-4} %wt by wt) from crude hexane eluting with 45% ethyl acetate in hexane on silica gel column. This compound was soluble in various solvents such as chloroform, ethyl acetate, methanol and ethanol.

Compound 5 is a transparent oil (55 mg) $[\alpha]_D^{20}$ –17.4°C ($CHCl_3$; *c*1.0)

UV λ_{max} (nm), CH_3Cl (log ε) = 244sh (3.82)

FT-IR spectrum, (KBr), υ_{max} (cm^{-1}): 3250(br), 2960(s), 1691(s), 1200(s) and 1160(s), **(Fig 38)**

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 0.87(3H,s), 0.98 (3H,s), 1.15 (3H,s), 1.28(2H,m), 1.33(1H,m), 1.35(2H,s), 1.45(2H,m), 1.74(3H,s), 2.12 (3H,s),2.17(1H,ddd,*J*=5.7,11.3,16.1),2.37(1H,ddd,*J*=6.1,12.8,15.7),3.37 (1H,d,*J*=9.4), 4.92(1H,dd,*J*=9.1,10.6), 5.05(1H,d,*J*=10.6), 5.15(1H,d,*J*=16.9), 5.52(1H,dd,*J*=7.0,14.1), and 6.33(1H,dd,*J*=10.5, 17.2) **(Fig 39)**

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 11.8(q), 16.7(q), 18.1(t), 19.5 (q), 21.8(q), 22.1(t), 23.6(t), 33.3(s), 35.9(q), 39.3(d), 39.7(t), 43.4(t), 56.5(d), 59.0(d), 73.2(d), 76.7(s), 83.6(d), 110.4(t), 132.6(s), 135.5(d), 141.5(d) and 171.8(s) **(Fig 40)**

EI-MS spectrum m/z (70 ev); 364 [M^+, (1)], 346[M^+-H_2O, (2)], 331[346-CH_3, (1)], 309(1), 286(14), 271(8), 257(5), 249(3), 243(7), 231(5), 220(5), 205 (81),191(13), 189(28), 177(38), 161(36), 150(45), 147(49), 137(75), 121(64), 109(59), 95(59), 81(100), 69(44) and 55(4) **(Fig 42)**

3.6.6 Purification and Physical property of Compound 6

From the crude hexane extract, compound 6 was obtained eluting with 60% ethyl acetate in hexane on silica gel column. It was recrystalized from 55% ethyl acetate in hexane solvent to provide 880 mg of white solid (0.014% wt by wt). It had melting point at 82-83°C. It was soluble in chloroform, ethyl acetate, methanol and ethanol.

Compound 6 is a white solid (880 mg), $[\alpha]_D^{20}$ −14.8°C ($CHCl_3$; *c*1.0)

UV λ_{max} (nm), CH_3Cl (log ε) = 244sh (3.93)

FT-IR spectrum, (KBr), υ_{max} (cm^{-1}): 3426 (br), 2929(s) and 2842(s), 1639(s), **(Fig 43)**

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 0.87(3H,s), 0.98(3H,s), 1.13 (3H,s), 1.15(3H,s), 1.28(1H,m), 1.35(1H,m), 1.38(1H,m), 1.43(1H,m), 1.45 (1H,m), 1.53(1H,m), 1.54(1H,m), 1.74(3H,s), 2.17(1H,dd,*J*=5.8,11.3,16.2), 2.37(1H,ddd,*J*=6.1,12.8,15.9),3.60(1H,dd,*J*=9.4,10.9),3.37(1H,d,*J*=9.5),4.83 (1H,d,*J*=10.4),5.04(1H,d,*J*=17.4),5.52(1H,t,*J*=7.0,14.0)and6.30 (1H,dd,*J*=10.9,17.3) **(Fig 44 and Fig 48-51)**

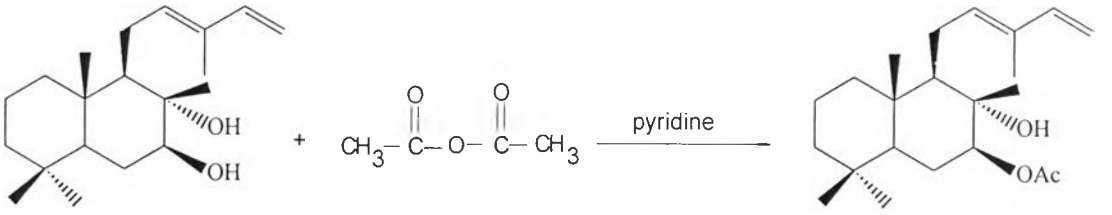
^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 11.8(q), 16.9(q), 18.2(t), 19.4 (q), 22.1(q), 23.7(t), 33.7(s), 36.4(q), 39.1(d), 39.1(t), 43.4(t), 57.3(d), 59.4(d), 71.7(d), 76.9(s), 85.0(d), 110.5(t), 132.6(s), 135.7(d), and 141.4(d) **(Fig 45)**

EI-MS spectrum m/z (70 ev); 323 [M^+H, (4)], 304[M^+H-H_2O,(5)] (29), 289 (11), 286(11), 271(14), 261(13), 257(16), 243(15), 221(9), 205(16), 193(44), 189(27),177(38), 161(31), 151(92),147(54), 134(57), 121(97) and 109(100) **(Fig 47)**

3.6.7 Modification of Compound 4

Acetylation of Compound 4

Compound 4 was acetylated with acetic anhydride in pyridine at room temperature.



200 mg, 0.65 mmole	133.4 mg, 1.30 mmole	155 mg, 0.45 mmole
Compound 4	acetic anhydride	Compound 4a

Figure 5: Acetylation of Compound 4

To the pyridine (5 ml) solution of compound 4 (200 mg, 0.65 mmole) was slowly added acetic anhydride (133.4 mg, 1.30 mmole) and the reaction mixture was stirred overnight. Water was added dropwise and extracted with diethyl ether. The organic layer was washed with 5% HCl and then with 5% $NaHCO_3$. The organic solution was washed with water, dried (Na_2SO_4) and evaporated. The residue was purified by column chromatography eluting with 7% ethyl acetate in hexane to afford the monoacetate of compound 4 (compound 4a, 155 mg, 0.45 mmole, 77.5%yield). The spectral data of this compound was identical to that of authentic sample.

Compound 4a is a transparent oil (155 mg, 0.45 mmole, 77.5 %yield) $[\alpha]_D^{20}$ –35.8°C ($CHCl_3$; *c*1.0)

UV λ_{max} (nm), CH_3Cl (log ε) = 244sh (3.93)

FT-IR spectrum, υ_{max} (cm^{-1}): 3457 (br), 2929(s) and 2868(s), 1726(s) , 1634 (s), 1654(s) and 1378(s), **(Fig 52)**

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 0.79(3H,s), 0.87(3H,s), 1.15 (3H,s), 1.26(3H,s), 1.3-1.5(4H,m), 1.77(3H,s), 2.09(3H,s), 2.17(1H), 2.37(1H), 4.72(1H), 4.90(2H), 5.04(1H), 5.57(1H) and 6.40(1H) **(Fig 53)**

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) δ(ppm); 11.8(q) , 15.5(q), 17.8(q), 18.3(t), 21.3(q), 21.8(q), 23.4(t), 26.2(t), 33.1(s), 33.3(q), 39.5(s), 39.5(t), 41.5 (t), 53.2(d), 60.9(d), 75.9(s), 81.3(d), 110.1(t), 132.2(s), 135.9(d), 141.2(d)and 171.5(s) **(Fig 54 and 55)**

EI-MS spectrum m/z (70 ev); 348 [M^+, (2)], 330[M^+-H_2O, (29)], 310(5), 302 (2), 293(7), 288(16), 284(24), 271(24), 270(85), 268(7), 255(35), 245(22), 234 (28), 219(15), 205(28), 192(99), 177(41), 163(30), 150(81), 145(41), 133(47), 119(33), 93(34), 84(36), 81(73), 79(78), 69(100), 67(80), 65(50) and 55(50), **(Fig 56)**

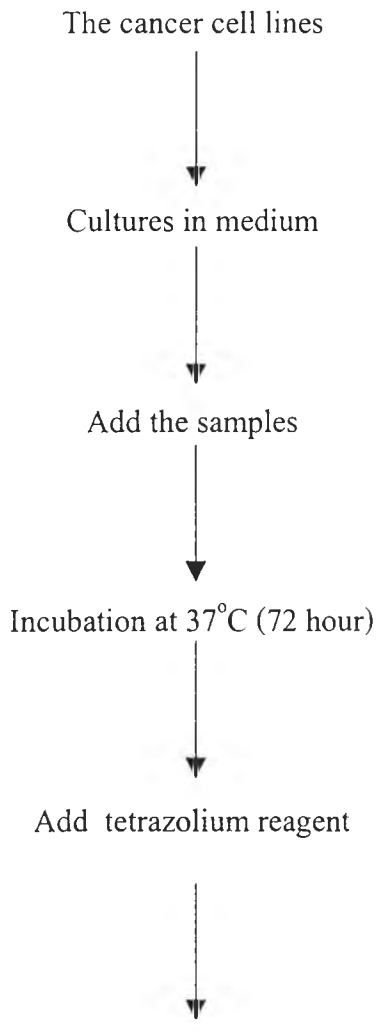
3.7 PURIFICATION OF THE COMPOUNDS FROM ETHYL ACETATE CRUDE EXTRACT BY COLUMN CHROMATOGRAPHIC TECHNIQUE.

The ethyl acetate crude extract (50 g) was subjected to a silica gel column chromatography. The column was initially eluted with 100% hexane and the polarity of eluent was gradually increased from 100% hexane to ethyl acetate in hexane and then 5% methanol in ethyl acetate. Furthermore, fraction with similar composition in hexane crude provided compound 4 (177 mg, 2.72×10^{-3} wt by wt) and compound 6 (102 mg, 1.57×10^{-3} wt by wt).

3.8 BIOLOGICAL EVALUATION

Each compound was tested for cytotoxic activity towards 6 cell lines which consisted of HS 27 (fibroblast), HEP-G2 (hepatoma), SW620 (colon), Chago (lung), Kato-3 (gastric) and BT-474 (breast), *in vitro* was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method [30-32].

The procedure of cytotoxicity test in Scheme 2



Scheme 4 **The procedure of cytotoxicity test**