CHAPTER IV

RESULTS AND DISCUSSION

4.1 Cytotoxicity preliminary screening

Preliminary screening test for cytotoxicity against cancer cell lines of selected plants used as anticancer in Thai traditional medicine were performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method. The most interesting plant with strong anticancer activity will be selected to extract and identify the active compounds.

Table 7 Cytotoxicity activity of ethanol crude extract of selected plants from preliminary screening procedure.

Species		% Survival				
	common name	BT 474	CHAGO	Hep-G2	KATO-3	SW 620
Croton oblongifolius Roxb.	เปล้าใหญ่	19	6	10	11	5
Hydnophytum formicar Jack.	หัวร้อยรู	71	120	84	97	111
Cuscuta chinensis	ฝอยทอง	51	118	77	46	9
Curcuma caesia *Curcuma Zedoaria* Rose.	ขมิ้นอ้อย	19	7	9	9	5
Nelambo nucifera	บัวหลวง	83	126	108	129	101
Livisticum officnale	โกฐเชียง	19	7	8	10	6
Acanthus ebracteatus Vahl.	เหงือกปลาหมอ	61	121	72	54	97
Mucuna collettii	กวาวดำ	56	110	78	40	78
Kaempferia parviflora	กระชายดำ	17	7	8	10	4
Curcuma spp.	เอ็นเหลือง	19	8	8	11	5
Zingiber cassumunar	ไพล	19	8	7	11	5
Zingiber ottensii Val.	ไพลดำ	19	12	43	11	7

Table 7 continued

Species		% Survival				
	common name	BT 474	CHAGO	Hep-G2	KATO-3	SW 620
Curcuma domestica Val. ; *Curcuma longa*	ขมิ้นชัน	25	11	14	15	5
Orthosiphon aristatus (Blume) Mig.	หญ้าหนวดแมว	19	12	27	14	5
Gelonium multiflorum Juss	ขันทองพยาบาท	43	77	22	14	8
Salacia chinensis	กำแพงเจ็ดชั้น	33	59	29	15	10
Rhinacanthus nasutus Kurz. (*Rhinacanthus communis* Nees.)	ทองพันชั่ง	20	28	15	12	6
Garcinia cowa Roxb.	ชมวง	27	102	29	20	9
Zingiber rubens Roxb.	ขิงแห้ง	17	7	12	12	5
Euphorbia lacei	สลัดได	22	7	9	13	6
Rauvolfia serpentina (L.) Benth. Ex Kurz	ระย่อม	27	63	45	16	15
Curcuma spp.	ม้าเหลือง	13	47	7	9	3
Artemisia pallens	โกฐจุฬาลำพา	22	21	23	21	6
Phyllanthus emblica Linn.	มะขามป้อม	20	113	11	10	7
Murdannia loriformis (Hassk.) Rolla Rao et Kammathy	หญ้าปักกิ่ง	31	68	45	13	24

Ref: HS27 (fibroblast); KATO-3 (gastric); BT 474 (breast); CHAGO (lung); SW 620(colon); Hep-G2 (hepatoma)

Data of screening test for cytotoxicity against cancer cell lines of selected plants used as anticancer agents in Thai traditional medicine showed that the ethanol crude extract from the stem barks of *Croton oblongifolius* Roxb. from Amphur Vicheinburi, Petchaboon Province had better anticancer activity when compared to other selected plants. For that reason, this study decided to select *Croton oblongifolius* Roxb. for further isolation and purification of their active compounds.

4.2 Antioxidant activity preliminary screening

This study also investigated the antioxidant activity of samples by DPPH method which used to investigate the potential of samples to reduce DPPH radicals (radical 2,2-diphenylpicryhydrazyl).

Table 8 Antioxidant activity of ethanol crude extract of selected plants (α-tocopherol was used as positive control and it gave high antioxidant activity:IC_{50} 32 μg).

Species	Common name	IC_{50} (μg)	Activity level
Croton oblongifolius Roxb.	เปล้าใหญ่	258	Low
Hydnophytum formicar Jack.	หัวร้อยรู	28.5	High
Cuscuta chinensis	ฝอยทอง	110	Medium
Curcuma caesia *Curcuma Zedoaria* Rose.	ขมิ้นอ้อย	321	Low
Nelambo nucifera	บัวหลวง	127	Medium
Livisticum officnale	โกฐเชียง	>400	Very low or inactive
Acanthus ebracteatus Vahl.	เหงือกปลาหมอ	>400	very low or inactive
Mucuna collettii	กวาวดำ	356	Low
Kaempferia parviflora	กระชายดำ	>400	very low or inactive
Curcuma spp.	เอ็นเหลือง	>400	very low or inactive
Zingiber cassumunar	ไพล	325	Low
Zingiber ottensii Val.	ไพลดำ	>400	very low or inactive
Curcuma domestica Val. ; *Curcuma longa*	ขมิ้นชัน	137	Medium
Orthosiphon aristatus (Blume) Mig.	หญ้าหนวดแมว	182	Medium
Gelonium multiflorum Juss	ขันทองพยาบาท	360	Low
Salacia chinensis	กำแพงเจ็ดชั้น	37.5	High
Rhinacanthus nasutus Kurz. (*Rhinacanthus communis* Nees.)	ทองพันชั่ง	>400	very low or inactive

Table 8 continued

Species	Common name	IC_{50} (µg)	Activity level
Garcinia cowa Roxb.	ชมวง	>400	very low or inactive
Zingiber rubens Roxb.	ขิงแห้ง	>400	very low or inactive
Euphorbia lacei	สลัดได	270	Low
Rauvolfia serpentina (L.) Benth. ex Kurz	ระย่อม	345	Low
Curcuma spp.	ม้าเหลือง	>400	very low or inactive
Artemisia pallens	โกฐจุฬาลำพา	>400	very low or inactive
Phyllanthus emblica Linn.	มะขามป้อม	254	Low
Murdannia loriformis (Hassk.) Rolla Rao et Kammathy .	หญ้าปักกิ่ง	>400	very low or inactive

The antioxidant activity could be classified as high, medium low and very low activity or inactive by 50<, 51-199, 200-399 and >400 µg respectively.

Extraction and purification of active compounds from *Croton oblongifolius* Roxb.

The hexane crude extract (50.0 g, 0.50 % wt by fresh wt) and the ethyl acetate crude extract (15.0 g, 0.15% wt by fresh wt) were separated using column chromatography. The separation results are shown in Table 9.

Table 9 The results of separation of hexane crude extracts and ethyl acetate crude extracts by column chromatography.

Compounds	Physical appearance	% wt by fresh wt	Part
1	Colorless monoclinic crystals	6.14×10^{-2}	Hexane
2	Colorless triclinic crystals	0.93×10^{-2}	Hexane
3	A viscous transparent oil	0.65×10^{-2}	Hexane
4	White solid	2.38×10^{-2}	Hexane
5	A viscous transparent oil	0.24×10^{-2}	Hexane
6	White solid	1.03×10^{-2}	EtoAC

4.3 Purification and properties of the compounds eluted from column chromatography of hexane crude extract.

Purification and properties of Compound 1

Compound 1 was eluted with 5% ethyl acetate in hexane on silica gel column chromatography. Similar fractions were combined and the solvents were removed by rotary evaporation. Compound 1 was recrystallized from ethyl acetate to give colorless monoclinic crystals. It is soluble in chloroform, hot ethyl acetate, ethanol, methanol and slightly soluble in hexane.

Compound 1 is a colorless monoclinic crystals (1.6750 g, 6.14 x 10^{-2} % wt by fresh wt) with melting point 109-110°C. R_f ; 0.26 (10% ethyl acetate in hexane), $[\alpha]_D^{20}$ +1.65 ($CHCl_3$, c 0.50), UV λ_{max} ($CHCl_3$) 248sh (logε 3.36)

FT-IR spectrum (KBr) (Fig.11) ν_{max} (cm^{-1}) :2400-3500(br), 2962 and 2885(s), 1682(s), 1635(m)

^{1}H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.12) δ (ppm): 6.03(1H,d), 6.00 (1H,t), 5.90(1H,dd), 5.09(1H,t), 2.70(2H,q), 2.39(4H,m), 2.32(1H,m), 2.28(2H,m), 2.15(4H,m), 1.73(3H,d), 1.54(3H,s), 1.03(6H,d)

^{13}C-NMR spectrum ($CDCl_3$, 200MHz) (Fig.13) δ (ppm) : 173.9(s), 146.8(s), 146.3(d), 135.2(s), 134.0(s), 130.9(s), 125.7(d), 121.6(d), 118.7(d), 39.2(t), 38.6(t), 33.8(d), 33.6(t), 28.7(t), 26.4(t), 25.1(t), 22.1(q), 22.1(q), 17.0(q), 15.8(q)

m/z (EI) (rel int.) (Fig.15) : 302[M^+](20), 152(36), 136(63), 121(100), 93(56)

Purification and properties of Compound 2

Compound 2 was eluted with 5% ethyl acetate in hexane. Similar fraction were combined and evaporated to about 20 ml. Compound 2 was crystallized from 20% ethyl acetate in hexane and recrystallized in ethyl acetate respectively to give colorless triclinic crystals. It is soluble in chloroform, hot ethyl acetate, ethanol, methanol and slightly soluble in hexane.

Compound 2 is colorless triclinic crystals (0.2534g, 0.93 x 10^{-2} % wt by fresh wt) with melting point 128-129°C, R_f ; 0.26 (10% ethyl acetate in hexane), $[\alpha]_D^{20}$ –1.07 ($CHCl_3$, c 0.50) ,UV λ_{max} ($CHCl_3$) 248 (logε 3.84)

FT-IR spectrum (KBr) (Fig.16) ν_{max} (cm^{-1}) : 2400-3500(br), 2958 and 2922 (m), 2871(m), 1680(s), 1639(w)

^{1}H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.17) δ (ppm): 6.89(1H,t), 6.04 (1H,d), 5.94(1H,d), 5.14(1H,t), 2.18-2.42(13H,m), 1.71(3H,s), 1.69(3H,s), 1.07(6H,d)

^{13}C-NMR spectrum ($CDCl_3$, 200MHz) (Fig.18) δ (ppm) : 173.6(s), 146.5(s), 145.7(d), 135.6(s), 134.8(s), 132.1(s), 127.8(d), 119.9(d), 118.6(d), 38.5(t), 37.7(t), 34.5(d), 30.6(t), 29.1(t), 26.7(t), 24.7(t), 22.1(q), 22.1(q), 18.0(q), 17.4(q)

m/z (EI) (rel int.) (Fig.20): 302[M^+](37), 152(13), 136(83), 121(100), 93(87)

Purification and properties of Compound 3

Compound 3 was obtained from 10% ethyl acetate in hexane. Similar fractions were combined, evaporated and then further purified by column chromatography (Merck's silica gel Art. 1.09385.1000). This compound is soluble in chloroform, ethyl acetate, ethanol, methanol.

Compound 3 was a viscous transparent oil (0.1770 g, 0.65 x 10^{-2} % wt by fresh wt), R_f ; 0.41 (15 % ethyl acetate in hexane), $[\alpha]_D^{20}$ +13.31 ($CHCl_3$, c 0.50), UV λ_{max} ($CHCl_3$) 244 (logε 1.92)

FT-IR spectrum (KBr plate) (Fig.21) ν_{max} (cm^{-1}) : 3376(br), 3200-2700 (br), 2956, 2918 and 2861(br), 1679(s), 1417(s), 1260(s), 1024(m)

^{1}H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.22) δ (ppm) : 5.39 (1H,br, t), 5.24 (1H, br s), 4.13(2H,d), 2.16-1.03(15H,m), 1.66(3H,br s, m), 1.56(3H,m), 1.01(3H,s), 0.78(3H,d), 0.73(3H,s),

^{13}C-NMR spectrum ($CDCl_3$, 200 MHz) (Fig.23) δ (ppm) : 141.0(s), 139.9(s), 123.1(d), 122.8(d), 59.4(t), 44.6(d), 40.0(s),37.8(t), 37.3(q), 36.7(s), 36.4(d), 33.1(q), 32.7(t), 28.8(t), 24.0(t), 19.8(q), 17.7(t), 17.3(q), 16.5(q), 15.9(q)

m/z (EI) (rel int.) (Fig.25): 290[M^+](7), 272[M-H_2O](5), 257(4), 189(63), 175 (19), 161(18), 135(23), 121(51), 107(100), 95(89), 81(28), 69(20), 55(22)

Purification and properties of Compound 4

Compound 4 was eluted with 15% ethyl acetate in hexane. The solvent was removed by rotary evaporation and the residue was purified by column chromatography (Merck's silica gel Art. 1.09385.1000). It is soluble in chloroform, ethyl acetate, ethanol and methanol.

Compound 4 is a white solid (0.6484g, 2.38 x 10^{-2} % wt by fresh wt), with melting point 102-103°C, R_f ; 0.31 (20 % ethyl acetate in hexane), $[\alpha]_D^{20}$ –122.72 ($CHCl_3$, c 0.50), UV λ_{max} ($CHCl_3$) 242sh (logε 1.14)

FT-IR spectrum (KBr) (Fig.26) ν_{max} (cm^{-1}): 2300-3600(br), 2960 and 2925(s) 2868(s), 1682(s), 1626(m)

^{1}H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.27) δ (ppm) : 7.33(1H,m), 7.18 (1H,s), 6.85(1H,t), 6.24(1H,m), 2.44(1H,m), 2.37(1H.m), 2.30(1H,m), 2.24(1H,m), 2.18(2H,m), 2.03 (2H,m), 1.66(2H,m), 1.63(1H,m), 1.58(1H,d), 1.52(1H,m), 1.39 (3H,s), 1.24(1H,s), 1.03(3H,s), 0.75(3H,s)

^{13}C-NMR spectrum ($CDCl_3$, 200MHz) (Fig.28) δ (ppm) : 173.0(s), 142.7(d), 141.5(s), 140.4(d), 138.4(d), 125.6(s), 111.0(d), 46.7(d), 38.8(s),38.7(t), 37.6(s), 36.2 (d), 35.8(t), 27.5(t), 27.3(t), 20.5(q), 18.3(q), 18.2(t), 17.4(t), 16.0(q)

m/z (EI) (rel int.) (Fig.30) : 316[M^+](4), 299(9), 283(7), 221(41), 203(33), 175(11), 151(15), 137(32), 125(100), 105(15), 96(46), 81(47)

Purification and properties of Compound 5

Compound 5 was eluted with 20% ethyl acetate in hexane. The solvent was removed by rotary evaporation and the residue was purified by column chromatography (Merck's silica gel Art. 1.09385.1000). It is soluble in chloroform, ethyl acetate and methanol.

Compound 5 is a viscous transparent oil (0.0653g, 0.24 x 10^{-2} % wt by fresh wt), R_f ; 0.43 (30 % ethyl acetate in hexane), $[\alpha]_D^{20}$ –79.86 ($CHCl_3$, c 0.50), UV λ_{max} ($CHCl_3$) 242sh (logε 1.22)

FT-IR spectrum (KBr plate) (Fig.31) ν_{max} (cm^{-1}): 3600-3100(br), 2933(s), 1715(s), 1683(s), 1632(m), 1272(s)

^{1}H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.32) δ (ppm): 8.01(1H,d), 7.55 (1H,dd), 7.47(2H,dd), 7.34(1H,d), 7.24(1H,s), 6.90(1H,s), 6.26(1H,s), 4.52(1H,d), 4.34(1H,d), 2.53-2.19(5H,m), 2.03(1H,m), 1.98-1.55(7H,m), 1.30(3H,s), 1.24(1H,m), 1.01(3H,d)

^{13}C-NMR spectrum ($CDCl_3$, 200MHz) (Fig.33) δ (ppm): 172.3(s), 166.8(s), 142.9(d), 140.9(s), 140.6(d), 138.5(d), 132.9(d), 130.3(s), 129.5(d), 128.5(d), 125.2 (s), 111.0(d), 67.8(t), 47.4(d), 42.3(s), 37.6(s), 36.3(d), 36.0(t), 32.4(t), 28.1(t), 27.2 (t), 20.2(q), 19.2(t), 17.9(t), 17.0(q)

m/z (EI) (rel int.) (Fig.35): 436[m^+](2), 341 [M^+- $C_6H_7O^+$, (10)], 314(14), 219 (13), 125(17), 105(100), 95(73), 81(43), 77 [Ph^+, (37)].

4.4 Purification and properties of the compounds eluted from column chromatography of ethyl acetate crude extract.

Purification and properties of Compound 6

Compound 6 was eluted with 70% ethyl acetate in hexane on silica gel column chromatography. Similar fractions were combined and the solvents were removed by rotary evaporation. It is soluble in hot ethyl acetate, DMSO, ethanol, methanol.

Compound 6 is a white solid (0.2823g, 1.03 x 10^{-2} % wt by fresh wt) with melting point 161-163°C, R_f ; 0.29 (75 % ethyl acetate in hexane), $[\alpha]_D^{20}$ +7.2 (MeOH, c 0.50), UV λ_{max} (EtOH) 280sh (logε 4.76)

FT-IR spectrum (KBr) (Fig.36) ν_{max} (cm^{-1}) : 3400-2900(br), 2926(w), 2849 (w), 1626(s), 1608(s), 1286(m)

^{1}H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.37) δ (ppm) : 6.70(d), 6.60(d), 6.56 (dd), 5.87(d), 5.68(d), 4.89(d), 4.46(d), 3.80(m), 2.68(ax,dd), 2.33(eq,dd)

^{13}C-NMR spectrum ($CDCl_3$, 200MHz) (Fig.38) δ (ppm): 156.4(s), 156.1(s), 155.3(s), 144.8(2s), 130.5(s), 118.4(d), 115.0(d), 114.4(d), 99.0(s), 95.0(d), 93.8(d), 80.9(d), 66.2(d), 27.8(t)

m/z (EI) (rel int.) (Fig.40): 290[M^+](68), 272[M-H_2O](12), 152(B-ring, 72), 139(A-ring, 100), 123(A-ring, 31), 110(7), 77(19), 69(26), 44(24)

4.5 Structural elucidation of the isolated compounds from the stem bark of *Croton oblongifolius Roxb.*

1. Structure elucidation of Compound 1

The IR spectrum of compound 1 is shown in Figure 11 and the important absorption peaks were assigned as in Table 10.

Table 10 The IR absorption bands assignment of compound 1.

Wavenumber (cm^{-1})	Intensity	Tentative assignment
2400-3500	Broad	O-H stretching vibration of carboxylic acid
2962, 2885	Stong	C-H stretching vibration of $-CH_2$,-CH_3
1682	Strong	C=O stretching vibration of carbonyl group
1635	Medium	C =C stretching vibration of olefin

The ^{1}H-NMR spectrum (Fig.12, Table 11) showed that compound1 possessed an isopropyl group which showed doublet signals of two methyl groups attaching to the saturated methine carbon (C-15) presented at δ1.03, (6H,d). In addition, it showed one olefinic methyl groups attached to the double bond (δ1.54,3H,s and 1.73,3H,d) and four olefinic protons(δ5.09,1H,t); (5.90,1H,dd); (6.00,1H,t); (6.03,1H,d,m)

The ^{13}C-NMR data (Fig.13, Table 12) suggested the presense of olefinic carbons according to the signals at 146.8(s), 146.3(d), 135.2(s), 134.0(s) 130.9(s), 125.7(d), 121.6(d), 118.7(d) ppm. The signal at 173.9(s) ppm. should be the carboxylic acid. There were 11sp^3 carbon signals at 39.2(t), 38.6(t), 33.8(d), 33.6(t), 28.7(t), 26.4(t), 25.1(t), 22.1(2q), 17.0(q) and 15.8(q) ppm.

The DEPT-90 and DEPT-135 ^{13}C-NMR (Fig.14) indicated that this compound possesed twenty carbon atoms and thirty protons. Assuming the compound may contain only carbon, proton and oxygen atoms. Thus, its molecular formula was established as $C_{20}H_{30}O_2$ which was confirmed by observing molecular ion at *m/z* 302 (Fig.15) and indicated the double bond equivalent of six.

The spectroscopic data of compound 1 were consistent with crotocembraneic acid (m.p. 109-111°C) which was previously isolated in 1999 (Singtothong, 1999). The ^{1}H-NMR and ^{13}C-NMR agreed well with those reported for crotocembraneic acid in Table 11 and 12, respectively.

Table 11 ^{1}H-NMR spectral data of compound 1 and crotocembraneic acid (Singtothong, P.,1999).

Protons No.	Crotocembraneic acid (500 MHz)	Compound 1 (200 MHz)
H-1	-	-
H-2	6.03 (1H, d, J = 11.0 Hz)	6.03 (1H,d,m)
H-3	5.90 (1H, dd, J = 11.0 , 0.9 Hz)	5.90 (1H,dd, J=11.15, 0.67 Hz)
H-4	-	-
H-5	2.15 (2H, m)	2.15 (2H,m)
H-6	2.20 (2H, m)	2.28 (2H,m)
H-7	5.10 (1H,dt, J = 6.4, 1.2 Hz)	5.09 (1H,t, J= 6.22 Hz)
H-8	-	-
H-9	2.15 (2H, m)	2.15 (2H,m)
H-10	2.70 (2H, m)	2.70 (2H,q, J= 6.49 Hz)
H-11	6.01 (1H, t, J = 6.5 Hz)	6.00 (1H,t, J= 3.33 Hz)
H-12	-	-
H-13	2.41 (2H,m)	2.39 (2H,m)
H-14	2.41 (2H,m)	2.39 (2H,m)
H-15	2.34 (1H,m)	2.32 (1H,m)
H-16	1.04 (3H,d ,J = 6.7 Hz)	1.03 (3H,d, J= 6.78 Hz)
H-17	1.04 (3H,d ,J = 6.7 Hz)	1.03 (3H,d, J= 6.78 Hz)
H-18	1.73 (3H,d ,J = 0.9 Hz)	1.73 (3H,d, J= 0.69 Hz)
H-19	1.54 (3H, br s)	1.54 (3H,s)
COOH	-	-

Table 12 ^{13}C-NMR spectral data of compound 1 and crotocembraneic acid (Singtothong, P.,1999).

Carbon No.	Crotocembraneic acid (125 MHz)	Compound 1 (50 MHz)
1	146.9 s	146.9 s
2	118.7 d	118.7 d
3	121.6 d	121.6 d
4	135.2 s	135.2 s
5	39.2 t	39.2 t
6	25.1 t	25.1 t
7	125.7 d	125.7 d
8	134.0 s	134.0 s
9	38.6 t	38.6 t
10	26.4 t	26.4 t
11	146.3 d	146.3 d
12	130.9 s	130.9 s
13	33.6 t	33.6 t
14	28.7 t	28.7 t
15	33.8 d	33.8 d
16	22.1 q	22.1 q
17	22.1 q	22.1 q
18	17.0 q	17.0 q
19	15.8 q	15.8 q
20-COOH	174.1 s	173.9 s

Compound1 was recrystallized from ethyl acetate to give white monoclinic crystals for single crystal x-ray diffraction analysis. Data from single crystal x-ray diffraction method were shown in Table 13, 14, 15, 16 and 17.

Table 13 Crystal data and structure refinement for Compound 1

Empirical formula	$C_{20}H_{31}O_2$
Formula weight	303.45
Temperature	293(2) K
Wavelength	0.71073 A
Crystal system, space group	monoclinic, P21/a
Unit cell dimensions	a = 9.8513(5) A alpha = 90 deg.
	b = 10.5630(10) A beta = 102.136(2) deg.
	c = 18.5873(11) A gamma = 90 deg.
Volume	1891.0(2) A^3
Z, Calculated density	4, 1.066b Mg/m^3
Absorption coefficient	0.066 mm^{-1}
F(000)	668
Theta range for data collection	2.23 to 30.51 deg.
Limiting indices	-13<=h<=13, -14<=k<=12, -25<=1<=22
Reflections collected / unique	13443 / 5391 [R(int) = 0.0455]
Completeness to theta = 30.51	93.4%
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	5391 / 0 / 278
Goodness-of-fit on F^2	1.036
Final R indices [I>2sigma (I)]	R1 = 0.0854, wR = 0.1690
R indices (all data)	R1 = 0.1883, wR2 = 0.2160
Largest diff. Peak and hole	0.167 and –0.222 $e.A^{-3}$

Table 14 Atomic coordinates($x10^4$) and equivalent isotropic displacement parameters ($A^2 \times 10^3$) for Compound 1.

U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

	X	Y	Z	U(eq)
C(1)	3905(3)	322(2)	3059(1)	58(1)
C(2)	3789(3)	212(3)	2330(2)	65(1)
C(3)	3954(3)	1202(3)	1814(1)	65(1)
C(4)	3808(3)	1075(3)	1084(2)	77(1)
C(5)	4083(5)	2134(4)	587(2)	104(1)
C(6)	4587(4)	3388(4)	917(2)	95(1)
C(7)	3499(4)	4164(4)	1193(2)	75(1)
C(8)	3657(3)	5266(3)	1526(2)	75(1)
C(9)	2513(3)	5916(3)	1792(2)	73(1)
C(10)	2642(4)	5764(3)	2629(2)	75(1)
C(11)	2524(3)	4401(3)	2825(2)	61(1)
C(12)	3043(3)	3746(2)	3428(1)	54(1)
C(13)	2837(3)	2339(2)	3451(2)	57(1)
C(14)	4171(3)	1580(2)	3443(1)	51(1)
C(15)	3778(4)	-834(3)	3527(2)	90(1)
C(16)	2601(7)	-733(5)	3936(4)	127(2)
C(17)	5147(5)	-1128(3)	4066(2)	118(1)
C(18)	3383(4)	-159(4)	693(2)	113(1)
C(19)	5057(4)	5928(4)	1690(3)	143(2)
C(20)	3888(3)	4327(2)	4106(1)	61(1)
O(1)	4466(3)	3622(2)	4612(1)	95(1)
O(2)	3989(3)	5527(2)	4158(1)	89(1)

Table 15 Bond lengths [A] and angles [deg] for compound 1

C(1)-C(2)	1.341(4)
C(1)-C(14)	1.504(3)
C(1)-C(15)	1.520(4)
C(2)-C(3)	1.452(4)
C(3)-C(4)	1.340(4)
C(4)-C(18)	1.508(4)
C(4)-C(5)	1.510(5)
C(5)-C(6)	1.500(6)
C(6)-C(7)	1.521(5)
C(7)-C(8)	1.313(4)
C(8)-C(9)	1.491(4)
C(8)-C(19)	1.518(5)
C(9)-C(10)	1.543(4)
C(10)-C(11)	1.495(4)
C(11)-C(12)	1.324(3)
C(12)-C(20)	1.490(3)
C(12)-C(13)	1.502(3)
C(13)-C(14)	1.542(3)
C(15)-C(16)	1.518(6)
C(15)-C(17)	1.533(5)
C(20)-O(1)	1.239(3)
C(20)-O(2)	1.273(3)
C(2)-C(1)-C(14)	121.7(2)
C(2)-C(1)-C(15)	120.6(2)
C(14)-C(1)-C(15)	117.7(2)
C(1)-C(2)-C(3)	127.4(3)
C(4)-C(3)-C(2)	126.4(3)
C(3)-C(4)-C(18)	122.3(3)

Table 15 continued

C(3)-C(4)-C(5)	123.3(3)
C(18)-C(4)-C(5)	114.3(3)
C(6)-C(5)-C(4)	119.4(3)
C(5)-C(6)-C(7)	114.7(4)
C(8)-C(7)-C(6)	127.9(4)
C(7)-C(8)-C(9)	122.8(3)
C(7)-C(8)-C(19)	121.0(3)
C(9)-C(8)-C(19)	116.1(3)
C(8)-C(9)-C(10)	112.3(3)
C(11)-C(10)-C(9)	110.6(2)
C(12)-C(11)-C(10)	132.3(3)
C(11)-C(12)-C(20)	123.2(2)
C(11)-C(12)-C(13)	120.7(2)
C(20)-C(12)-C(13)	116.0(2)
C(12)-C(13)-C(14)	113.2(2)
C(1)-C(14)-C(13)	113.6(2)
C(16)-C(15)-C(1)	113.2(3)
C(16)-C(15)-C(17)	110.3(4)
C(1)-C(15)-C(17)	111.7(3)
O(1)-C(20)-O(2)	121.7(2)
O(1)-C(20)-C(12)	118.7(2)
O(2)-C(20)-C(12)	119.6(2)

Symmetry transformations used to generate equivalent atoms:

Table 16 Anisotropic displacement parameters ($A^2 \times 10^3$) for compound 1.

The anisotropic displacement factor exponent takes the form:

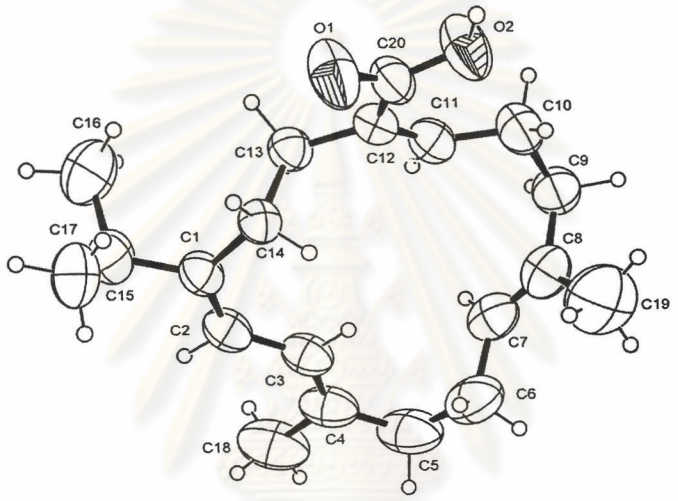
$-2\ pi^2\ [h^2\ a^{*2}\ U11 + \ldots + 2hka^*b^*U12]$

	U11	U22	U33	U23	U13	U12
C(1)	62(2)	49(1)	64(2)	-10(1)	11(1)	-4(1)
C(2)	66(2)	59(2)	66(2)	-19(1)	7(1)	0(1)
C(3)	65(2)	71(2)	56(2)	-12(1)	4(1)	13(1)
C(4)	71(2)	103(2)	53(2)	-13(2)	6(1)	25(2)
C(5)	121(3)	138(4)	57(2)	2()	27(2)	46(3)
C(6)	97(3)	123(3)	73(2)	24(2)	34(2)	20(2)
C(7)	66(2)	97(2)	59(2)	18(2)	10(1)	10(2)
C(8)	71(2)	88(2)	63(2)	13(2)	5(1)	-4(2)
C(10)	88(2)	60(2)	73(2)	-1(1)	12(2)	6(2)
C(11)	60(2)	58(2)	64(2)	-9(1)	8(1)	0(1)
C(12)	54(1)	52(1)	54(1)	-6(1)	12(1)	4(1)
C(13)	58(2)	53(2)	61(2)	-7(1)	14(1)	-4(1)
C(14)	56(2)	49(1)	47(1)	-5(1)	5(1)	-4(1)
C(15)	134(3)	50(2)	94(2)	-11(2)	42(2)	-18(2)
C(16)	130(4)	103(4)	168(5)	17(4)	74(4)	-30(3)
C(17)	156(4)	92(3)	113(3)	48(2)	48(3)	36(2)
C(18)	114(3)	144(3)	73(2)	-49(2)	3(2)	18(2)
C(19)	87(3)	159(4)	185(5)	-27(4)	32(3)	-37(3)
C(20)	80(2)	45(2)	56(2)	-5(1)	10(1)	5(1)
O(1)	156(2)	56(1)	57(1)	-3(1)	-17(1)	1(1)
O(2)	129(2)	52(1)	72(1)	-9(1)	-11(1)	1(1)

Table 17 Hydrogen bonds for compound 1 [A and deg.].

-H... A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
(2) –H(20)... O(1) #1	1.12(6)	1.51(6)	2.626(3)	175(5)

Symmetry transformations used to generate equivalent atoms: 1 –x+1, -y+1, -z+1



Figture 3 ORTEP drawing of Compound 1

The crystal structure of crotocembraneic acid was reported for the first time . From the data above, it can be concluded that compound 1 was Crotocembraneic acid. and the structure of compound 1 is shown below.

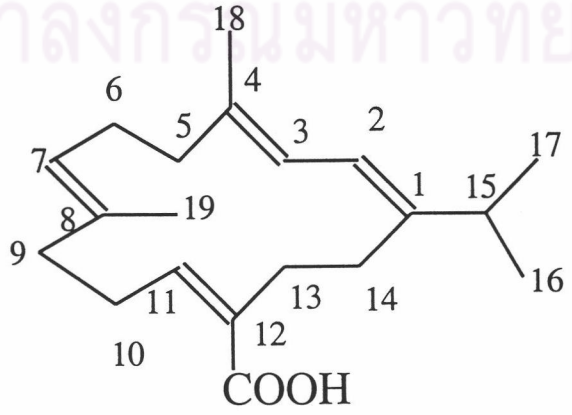


Figure 4 The structure of compound 1

2. Structure elucidation of Compound 2

The IR spectrum of compound 2 is shown in Figure 16 and the absorption peaks were assigned as in Table 18.

Table 18 The IR absorption band assignment of compound 2.

Wavenumber (cm^{-1})	Intensity	Tentative assignment
2400-3500	Broad	O-H stretching vibration of carboxylic acid
2958, 2922, 2871	Medium	C-H stretching vibration of $-CH_2$,$-CH_3$
1680	Strong	C=O stretching vibration of carbonyl group
1639	Weak	C =C stretching vibration of olefin

The ^{1}H-NMR spectrum (Fig.17, Table 20) indicated that compound 2 possessed an isopropyl group which showed doublet signals of two methyl groups attaching to saturated methine carbon (C-15) presented at δ1.07, 6H, d. In addition, it showed one olefinic methyl groups attached to double bonds (δ1.71, 3H, s and 1.69, 3H, s) and four olefinic proton (δ6.89,1H, t); (6.04, 1H, d); (5.94, 1H, d); and (5.14, 1H, t).

The ^{13}C-NMR spectrum (Fig.18, Table 20) suggested the presence of olefinic carbons according to the signal at 146.5(s), 145.7(d), 135.6(s), 134.8(s), 132.1(s), 127.8(d), 119.9(d) and 118.6(d) ppm. The signal at 173.6 ppm. should be the carbonyl group of carboxylic acid. There were 11 sp^3 carbon signals at 38.5(t), 37.7 (t), 34.5(d), 30.6(t), 29.1(t), 26.7(t), 24.7(t), 22.1(2q), 18.0(q), 17.4(q) ppm.

From DEPT-90 and DEPT-135 (Fig.19) indicated this compound possesses twenty carbon atoms and twenty-nine protons. Assuming this compound may contain only carbon, hydrogen and oxygen atoms. For that reason, Its molecular formula was established as $C_{20}H_{30}O_2$, which was confirmed by observing molecular ion at *m/z* 302 (Fig.20). and indicated the double bond equivalent of six.

To confirm the structure of this compound, the ^{1}H-NMR and ^{13}C-NMR chemical shift were compared with literature suggested that this compound might consist of a cembranoid structure, 14-membered-ring diterpene skeleton. The structure of neocrotocembraneic acid a seemed to fit all the number and type of bonds and presented in compound 2 (Table 19 and 20, respectively).

Table 19 ^{1}H-NMR spectral data of compound 2 and neocrotocembraneic acid (Singtothong, P.,1999).

Protons No.	Neocrotocembraneic acid (500 MHz)	Compound 2 (200MHz)
H-1	-	-
H-2	6.01 (1H, d, J = 11.0 Hz)	6.04(1H,d, J= 11.01 Hz)
H-3	5.91 (1H, dd, J = 11.0 , 0.9 Hz)	5.94(1H,d, J= 10.98 Hz)
H-4	-	-
H-5	2.15 (2H, m)	2.18-2.24 (2H,m)
H-6	2.23 (2H, m)	2.18-2.24 (2H,m)
H-7	5.14 (1H,dt, J = 8.0, 2.2 Hz)	5.14(1H,t, J= 5.70 Hz)
H-8	-	-
H-9	2.20 (2H, m)	2.18-2.24 (2H,m)
H-10	2.38 (2H, m)	2.18-2.24 (2H,m)
H-11	6.89 (1H, t, J = 8.0 Hz)	6.89 (1H,t, J= 7.82 Hz)
H-12	-	-
H-13	2.36 (2H, m)	2.18-2.24 (2H,m)
H-14	2.26 (2H, m)	2.18-2.24 (2H,m)
H-15	2.39 (1H, m)	2.18-2.24 (1H,m)
H-16	1.05 (3H,d , J = 7.0 Hz)	1.07 (3H,d, J= 6.79 Hz)
H-17	1.05 (3H,d , J = 7.0 Hz)	1.07 (3H,d, J= 6.79 Hz)
H-18	1.71 (3H,s)	1.71 (3H,s)
H-19	1.68 (3H, s)	1.69 (3H,s)
COOH	-	-

Table 20 ^{13}C-NMR spectral data of compound 2 and neocrotocembraneic acid (Singtothong, P.,1999).

Carbon No.	Neocrotocembraneic acid (125 MHz)	Compound 2 (50 MHz)
1	146.5 s	146.5 s
2	118.6 d	118.6 d
3	120.0 d	119.9 d
4	135.6 s	135.6 s
5	37.7 t	37.7 t
6	24.7 t	24.7 t
7	127.8 d	127.8 d
8	134.8 s	134.8 s
9	38.5 t	38.5 t
10	30.5 t	30.6 t
11	145.7 d	145.7 d
12	132.1 s	132.1 s
13	26.7 t	26.7 t
14	29.1 t	29.1 t
15	34.6 d	34.5 d
16	22.1 q	22.1 q
17	22.1 q	22.1 q
18	18.0 q	18.0 q
19	17.4 q	17.4 q
20	173.5 s	173.6 s

Compound 2 was crystallized from 20% ethyl acetate in hexane and recrystallized in ethyl acetate respectively to give colorless triclinic crystals then used single crystal x-ray diffraction method to determined it's structure. Data from single crystal x-ray diffraction method were shown in Table 21, 22, 23, 24 and 25.

Table 21 Crystal data and structure refinement for Compound 2

Empirical formula	$C_{20}H_{30}O_2$	
Formula weight	302.44	
Temperature	293(2) K	
Wavelength	0.71073 A	
Crystal system, space group	triclinic, P(-1)	
Unit cell dimensions	a = 7.64120(10) A	alpha = 95.39 deg.
	b = 9.7269(2) A	beta = 98.2220(10) deg.
	c = 13.11200(10) A	gamma = 98.8990(10) deg.
Volume	946.19(2) A^3	
Z, Calculated density	2, 1.062 Mg/m^3	
Absorption coefficient	0.066 mm^{-1}	
F(000)	332	
Theta range for data collection	1.58 to 30.49 deg.	
Limiting indices	-10<=h<=10, -13<=k<=9, -18<=1<=17	
Reflections collected / unique	7069 / 5158 [R(int) = 0.0144]	
Completeness to theta = 30.51	89.6%	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	5158 / 0 / 271	
Goodness-of-fit on F^2	1.070	
Final R indices [I>2sigma (I)]	R1 = 0.0646, wR2 = 0.1754	
R indices (all data)	R1 = 0.0928, wR2 = 0.1991	
Largest diff. Peak and hole	0.247 and –0.193 $e.A^{-3}$	

Table 22 Atomic coordinates($x10^4$) and equivalent isotropic displacement parameters ($A^2 \times 10^3$) for Compound 2.

U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

	X	y	z	U(eq)
C(1)	6933(2)	-1161(2)	7108(1)	47(1)
C(2)	7542(2)	-1981(2)	7790(2)	51(1)
C(3)	6816(3)	-3437(2)	7870(2)	53(1)
C(4)	7234(3)	-4121(2)	8682(2)	60(1)
C(5)	6511(4)	-5646(3)	8718(2)	77(1)
C(6)	5261(4)	-6439(2)	7772(2)	77(1)
C(7)	3447(3)	-6005(2)	7604(2)	67(1)
C(8)	2458(3)	-5847(2)	6723(2)	65(1)
C(9)	678(3)	-5346(2)	6704(2)	71(1)
C(10)	820(3)	-3770(2)	6622(2)	63(1)
C(11)	1872(2)	-2900(2)	7573(1)	53(1)
C(12)	3056(2)	-1725(2)	7626(1)	46(1)
C(13)	3602(2)	-1072(2)	6699(1)	50(1)
C(14)	5236(3)	-1603(2)	6334(1)	50(1)
C(15)	7890(3)	325(2)	7076(2)	62(1)
C(16)	9142(4)	979(3)	8062(2)	91(1)
C(17)	8918(4)	358(3)	6148(2)	92(1)
C(18)	8453(4)	-3419(3)	9657(2)	90(1)
C(19)	3031(4)	-6082(3)	5678(2)	100(1)
C(20)	3889(2)	-974(2)	8662(1)	49(1)
O(1)	3665(2)	-1671(2)	9453(1)	76(1)
O(2)	4712(2)	230(1)	8764(1)	62(1)

Table 23 Bond lengths [A] and angles [deg] for compound 2

C(1)-C(2)	1.334(2)
C(1)-C(14)	1.505(2)
C(1)-C(15)	1.523(2)
C(2)-C(3)	1.458(3)
C(3)-C(4)	1.334(3)
C(4)-C(18)	1.506(3)
C(4)-C(5)	1.509(3)
C(5)-C(6)	1.517(4)
C(6)-C(7)	1.503(3)
C(7)-C(8)	1.321(3)
C(8)-C(19)	1.506(3)
C(8)-C(9)	1.512(3)
C(9)-C(10)	1.535(3)
C(10)-C(11)	1.491(3)
C(11)-C(12)	1.333(2)
C(12)-C(20)	1.485(2)
C(12)-C(13)	1.505(2)
C(13)-C(14)	1.542(2)
C(15)-C(16)	1.515(3)
C(15)-C(17)	1.540(3)
C(20)-O(2)	1.226(2)
C(20)-O(1)	1.308(2)
C(2)-C(1)-C(14)	123.33(16)
C(2)-C(1)-C(15)	122.07(17)
C(14)-C(1)-C(15)	114.58(16)
C(1)-C(2)-C(3)	127.69(18)
C(4)-C(3)-C(2)	125.11(19)
C(3)-C(4)-C(18)	122.4(2)

Table 23 continued

C(3)-C(4)-C(5)	123.5(2)
C(18)-C(4)-C(5)	114.05(19)
C(4)-C(5)-C(6)	117.78(19)
C(7)-C(6)-C(5)	114.0(2)
C(8)-C(7)-C(6)	128.8(2)
C(7)-C(8)-C(19)	123.5(2)
C(7)-C(8)-C(9)	121.6(2)
C(19)-C(8)-C(9)	114.8(2)
C(8)-C(9)-C(10)	112.91(17)
C(11)-C(10)-C(9)	112.29(19)
C(12)-C(11)-C(10)	127.54(18)
C(11)-C(12)-C(20)	118.90(16)
C(11)-C(12)-C(13)	124.52(16)
C(20)-C(12)-C(13)	116.57(15)
C(12)-C(13)-C(14)	112.81(14)
C(1)-C(14)-C(13)	113.05(14)
C(16)-C(15)-C(1)	115.02(18)
C(16)-C(15)-C(17)	109.9(2)
C(1)-C(15)-C(17)	109.68(17)
O(1)-C(20)-O(1)	122.46(16)
O(2)-C(20)-C(12)	121.73(15)
O(1)-C(20)-C(12)	115.81(15)

Symmetry transformations used to generate equivalent atoms:

Table 24 Anisotropic displacement parameters ($A^2 \times 10^3$) for compound 2.
The anisotropic displacement factor exponent takes the form:
$-2\ pi^2\ [h^2\ a^{*2}\ U11 + \ldots + 2hka^*b^*U12]$

	U11	U22	U33	U23	U13	U12
C(1)	50(1)	43(1)	51(1)	4(1)	18(1)	10(1)
C(2)	46(1)	51(1)	58(1)	6(1)	11(1)	9(1)
C(3)	53(1)	51(1)	57(1)	10(1)	11(1)	14(1)
C(4)	59(1)	66(1)	63(1)	19(1)	16(1)	19(1)
C(5)	86(2)	67(1)	87(2)	36(1)	17(1)	26(1)
C(6)	96(2)	47(1)	95(2)	18(1)	27(1)	21(1)
C(7)	82(1)	48(1)	72(1)	6(1)	28(1)	3(1)
C(8)	70(1)	50(1)	68(1)	-12(1)	20(1)	-5(1)
C(9)	60(1)	69(1)	74(1)	-14(1)	14(1)	-12(1)
C(10)	53(1)	71(1)	59(1)	-9(1)	6(1)	4(1)
C(11)	49(1)	60(1)	50(1)	-4(1)	12(1)	7(1)
C(12)	45(1)	50(1)	44(1)	-1(1)	7(1)	13(1)
C(13)	52(1)	54(1)	44(1)	7(1)	1(1)	14(1)
C(14)	63(1)	49(1)	39(1)	4(1)	12(1)	13(1)
C(15)	62(1)	47(1)	81(1)	11(1)	23(1)	9(1)
C(16)	94(2)	63(1)	103(2)	-3(1)	14(2)	-18(1)
C(17)	100(2)	81(2)	105(2)	30(1)	51(2)	2(1)
C(18)	92(2)	110(2)	66(1)	29(1)	0(1)	9(2)
C(19)	101(2)	124(2)	69(2)	-26(2)	18(1)	24(2)
C(20)	53(1)	48(1)	45(1)	0(1)	13(1)	10(1)
O(1)	111(1)	62(1)	43(1)	2(1)	10(1)	-16(1)
O(2)	87(1)	47(1)	48(1)	-1(1)	11(1)	0(1)

Table 25 Hydrogen bonds for compound 2 [A and deg.].

-H... A	D(D-H)	d(H...A)	d(D...A)	<(DHA)
(1) –H(10)... O(2) #1	0.93(3)	1.72(3)	2.6433(19)	176(3)

Symmetry transformations used to generate equivalent atoms: #1 –x+1, -y, -z+2

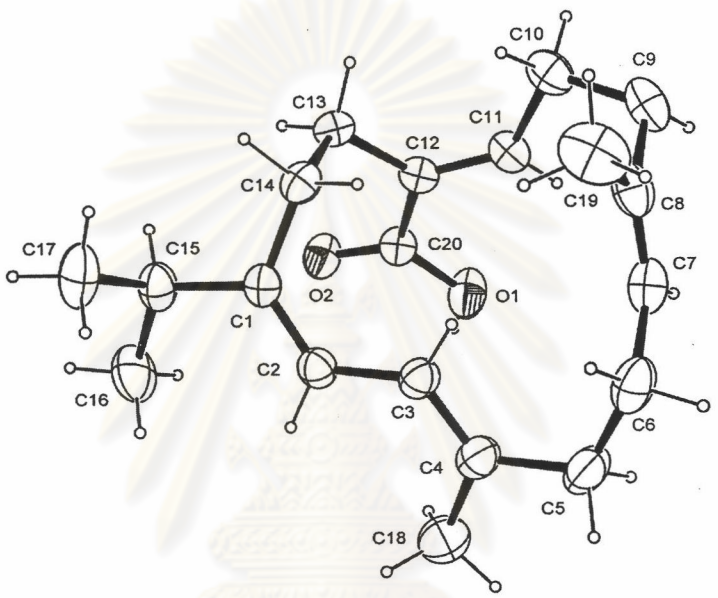


Figure 5 ORTEP drawing of Compound 2

The crystal structure of neocrotocembraneic acid was reported for the first time. From the Data above, it can be concluded that compound 2 was neocrotocembraneic acid and the structure of compound 2 can be shown below.

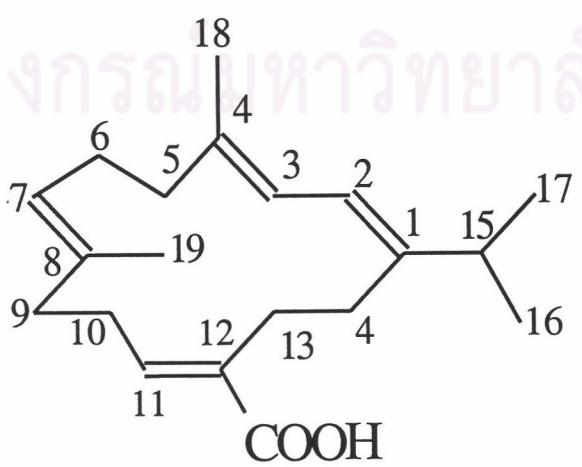


Figure 6 The structure of compound 2

4. Structure elucidation of Compound 3

The IR spectrum of compound 3 (Fig.21) and the absorption peaks were assigned as in Table 26.

Table 26 The IR absorption bands assignment of compound 3.

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3200-2700	Broad	O-H stretching vibration of acid
2956, 2918, 2861	Strong	C-H stretching vibration of $-CH_3$, $-CH_2$
1679	Strong	C=C stretching vibration of alkene

The ^{1}H-NMR spectrum (Fig.22, Table 27) indicated that compound 3 possessed five methyl group attaching to quaternary carbons at δ_H 0.73, 0.78, 1.01, 1.56 and 1.66 ppm, three olefinic proton at δ_H 5.38, 5.18 and 4.14 ppm.

The ^{13}C-NMR spectrum (Fig.23, Table 28) show twenty signals. Four signals of olefinic carbons appeared at δ141.03(s), 139.87(s), 123.11(d), 138.4(d), 122.79(d) ppm. There were thirteen sp^3 carbon signals at δ59.44(t), 44.59(d), 40.06(s), 37.77(t), 37.34(d), 36.66(s), 36.45(t), 33.08(q), 32.67(t), 28.77(t), 24.05(t), 19.76(q), 17.26(q), 16.52(q) and 15.94(q) ppm.

The DEPT-90 and DEPT-135 ^{13}C-NMR (Fig.24) indicated this compound possesses twenty carbon atoms and thirty-four protons. Assuming the compound may contain only carbon, proton and oxygen atoms, thus, its molecular formula was established as $C_{20}H_{34}O$ and indicated the double bond equivalent of six. This formula was confirmed by observing molecular ion at 290 *m/z* (Fig.25), and a signal at 272 corresponding to loss of water from the parent ion.

The ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 3 and kolavenol are shown in Table 27 and 28, respectively.

Table 27 ^{1}H-NMR spectral data of compound 3 and kolavenol (Lu, T. et al., 1993).

Protons	Kolavenol (400 MHz)	Compound 3 (200 MHz)
H-1	-	-
H-2	-	-
H-3	5.18 (br s)	5.24 (br s)
H-4	-	-
H-5	-	-
H-6	-	-
H-7	-	-
H-8	-	-
H-9	-	-
H-10	-	-
H-11	-	-
H-12	-	-
H-13	-	-
H-14	5.38 (br t, J = 6.8 Hz)	5.39 (br t, J = 5.89 Hz)
H-15	4.14 (d, J = 6.8 Hz)	4.13 (d, J = 6.93 Hz)
H-16	1.64 (s)	1.66 (br s)
H-17	0.78 (d, J = 6.0 Hz)	0.78 (m)
H-18	0.71 (s)	0.73 (s)
H-19	0.99 (s)	1.01 (s)
H-20	1.58 (d, J = 1.6 Hz)	1.56 (m)

Table 28 ^{13}C-NMR spectral data of compound 3 and kolavenol (Lu, T. et al., 1993).

Carbon No.	Kolavenol (125 MHz)	Compound 3 (50 MHz)
1	36.7 t	37.77 t
2	26.9 t	24.05 t
3	120.4 d	122.79 d
4	144.5 s	141.03 s
5	38.2 s	36.66 s
6	36.8 t	36.45 t
7	27.5 t	28.77 t
8	36.2 d	37.34 d
9	38.6 s	40.06 s
10	46.4 d	44.59 d
11	18.2 t	17.70 t
12	32.8 t	32.67 t
13	140.9 s	139.87 s
14	122.8 d	123.11 d
15	59.4 t	59.44 t
16	16.5 q	16.52 q
17	16.0 q	15.94 q
18	18.3 q	17.26 q
19	19.9 q	19.76 q
20	18.0 q	33.08 q

From the Data above, it can be suggested that all data of compound 3 was very similar to kolavenol which is shown in Figure 7 except chemical shift value of carbon number twenty which was significantly difference. Thus, it can be concluded that the structure of compound 3 should be similar to kolavenol, however in this study there was not enough data to complete its structure elucidation, so this compound was left for the further work.

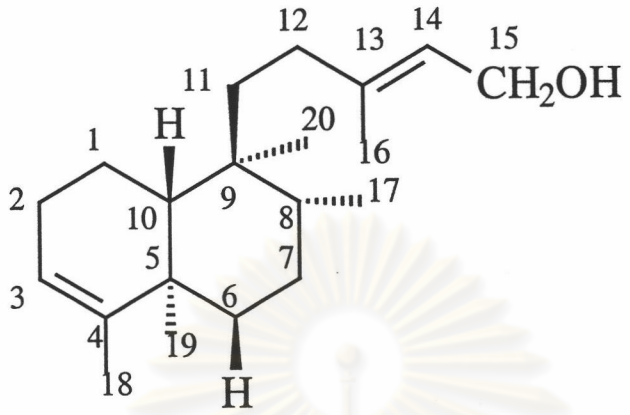


Figure 7 The structure of kolavenol.

Kolavenol or 3,13-Clerodadien-15-ol with variant: (ent-13*E*)-from was previously described as a constituent of *Hardwickia pinnata* (Misra, R,. Pandey, R.C. and Dev, S., 1968) and *Solidago elongata* (Anthonsen, T. and McCrindle, R. C., 1969).

4. Structure elucidation of compound 4

The IR spectrum of compound 4 (Fig.26) and the absorption peaks were assigned as in Table 29.

Table 29 The IR absorption bands assignment of compound 4.

Wavenumber (cm^{-1})	Intensity	Tentative assignment
2300-3600	Broad	O-H stretching vibration of acid
2960, 2925, 2868	Strong	C-H stretching vibration of $-CH_3$, $-CH_2$
1682	Strong	C=O stretching vibration of carbonyl group
1626	Medium	C=C stretching vibration of alkene

The ^{1}H-NMR spectrum of compound 4 (Fig.27, Table 30) indicated that it posses three methyl groups at δ_H 0.75, 1.03, 1.39 ppm, three olefinic protons of furanoid group at δ_H 7.33, 7.19 and 6.24 ppm and one vinylic at δ_H 6.85 ppm.

The ^{13}C-NMR, DEPT-90 and DEPT-135 spectrum (Fig.29, Table 31) showed twenty signals. Six signals of olefinic carbons appeared at δ142.7(d), 141.5(s), 140.4 (d), 138.4(d), 125.6(s), and 110.0(d) ppm. The signal at 173.1(s) should be the carbonyl of carboxylic acid. There were thirteen sp^3 carbon signals at δ46.7(d), 38.8 (s), 38.7(t), 37.6(s), 36.2(d), 35.8(t), 27.5(t), 27.3(t), 20.5(q), 18.3(q), 18.2(t), 17.4(t) and 16.0(q) ppm. Assuming the compound may contain only carbon, proton and oxygen atoms, thus its molecular formula was established as $C_{20}H_{28}O_3$ which was confirmed by observing molecular ion at 316 *m/z* (Fig.30). The molecular formula, $C_{20}H_{28}O_3$, of compound 4 defined the double bond equivalent of seven, therefore, compound 4 must consist of one ring of furan (DBE =3) in addition to one double bond, two ring and one carbonyl group of carboxylic acid.

The spectroscopic data of compound 4 were consistent with (–)-Hardwikiic acid which was isolated from *Croton californicus* (Luzbeta, D.J. et al., 1978) *Hardwickia pinnata* (Misra, R., Pandey, R.C and Dev, S., 1968), *Solidago arguta* (Henderson ,M.S. and Murray, R.D.H.,1973) , *Crangea maderaspatana* (Pandey, C.C. et al.,1984), *Baccharis macraei* (Gambaro, V. et al.,1986) and *Clerodendrum neriifolium* (Misra, R., Pandey, R.C and Dev, S., 1979)

The ^{1}H-NMR chemical shifts of Compound 4 is shown in Table 30 and a comparison of the ^{13}C-NMR chemical shifts of Compound 4 with (–)- hardwikiic acid is shown in Table 31.

Table 30 ^{1}H-NMR spectral data of compound 4.

Protons No.	Compound 4 (200 MHz)
H-1	1.66 (1H,m) 2.03 (1H,m)
H-2	2.18 (1H,m) 2.30 (1H,m)
H-3	6.85 (1H,t)
H-4	-
H-5	-
H-6	1.24 (1H,s) 2.44 (1H,m)
H-7	1.52 (1H,m) 1.63 (1H,m)
H-8	1.66 (1H,m)
H-9	-
H-10	1.58 (1H,d)
H-11	2.03 (1H,m) 2.18 (1H,m)
H-12	2.24 (1H,m) 2.37 (1H,m)
H-13	-
H-14	6.24 (1H,m)
H-15	7.33 (1H,m)
H-16	7.18 (1H,s)
H-17	1.03 (3H,s)
H-18	-
H-19	1.39 (3H,s)
H-20	0.75 (3H,s)

Table 31 ^{13}C-NMR spectral data of compound 4 and (–)-Hardwikiic acid (Aiyar, V. N. and Seshadri, T. R., 1971).

Carbon No.	(-)-Hardwikiic acid (125 MHz)	Compound 4 (50 MHz)
1	35.8 t	35.8 t
2	18.2 t	18.2 t
3	140.3 d	140.4 d
4	141.5 s	141.5 s
5	37.6 s	37.6 s
6	38.7 t	38.7 t
7	27.3 t	27.3 t
8	36.3 d	36.2 d
9	38.8 s	38.8 s
10	46.7 d	46.7 d
11	17.5 t	17.4 t
12	27.5 t	27.5 t
13	125.6 s	125.6 s
14	110.0 d	111.0 d
15	142.7 d	142.7 d
16	138.4 d	138.4 d
17	15.9 q	16.0 q
18	172.6 s	173.0 s
19	20.5 q	20.5 q
20	18.3 q	18.3 q

From the Data above, it can be concluded that compound 4 was (–)-Hardwikiic acid and the structure of compound 4 is shown in Figure 8.

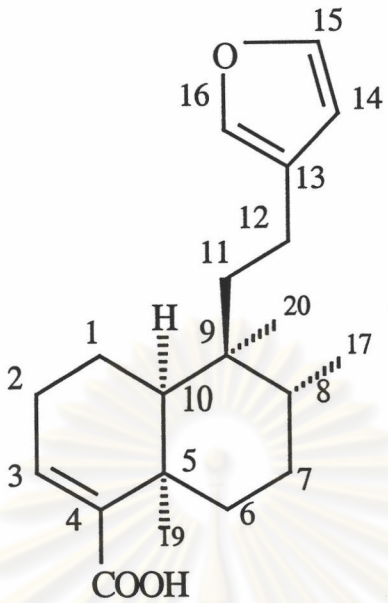


Figure 8 The structure of compound 4

5. Structure elucidation of compound 5

The IR spectrum of compound 5 (Fig.31) revealed the presence of carboxylic group according to the broad absorption band between 3600 to 3100 cm^{-1} and the strong absorption band at 1715 cm^{-1} due to the carboxylic acid carbonyl stretching.

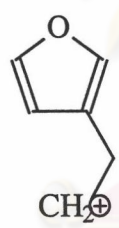
Table 32 The IR absorption bands assignment of compound 5.

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3600-3100	Broad	O-H stretching vibration of acid
2933	Strong	C-H stretching vibration of -CH3, -CH2
1715, 1683	Strong	C=O stretching vibration of acid
1632	Medium	C=C stretching vibration
1272	Strong	C-O stretching vibration

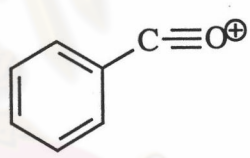
The ^{1}H-NMR spectrum (Fig.32, Table 33) and ^{13}C-NMR data of Compound 5 were similar to those of Compound 4 except for the downfield position of C-20 (δ_C

67.8 ppm.) when compared to that of Compound 4 (δ_C 18.2 ppm.). Its ^{1}H-NMR spectrum (Fig.32) showed two doublet signals (δ_H 4.34 and δ_H 4.50 ppm.) of 2H-20.

DEPT-90 and DEPT-135 ^{13}C-NMR spectra (Fig.34) indicate that there were twenty-seven carbon atoms and thirty-two protons. This compound probably contained carbon, hydrogen and oxygen atoms. The molecular formula, $C_{27}H_{32}O_5$, was determined from its mass spectrum (Fig.35) which showed the molecular ion at 436 *m/z* and indicated the double bond equivalent of twelve. Besides, the prominent ion at m/z 341 [M^+-$C_6H_7O^+$], 175 [219-COO^+] and 105 [$PhCO^+$] indicated that compound 4 probably contained a furano-ethyl side chain, carboxylic group and benzoyl group, respectively. Therefore, compound 4 should be consisted of one ring of furan (DBE=3) in addition to one double bond (DBE=1), two rings (DBE=2), one carbonyl of carboxylic acid (DBE=1) and one benzoyl group (DBE=5).



A furano-ethyl side chain



A benzoyl group

Comparison of spectral data including ^{1}H-NMR, ^{13}C-NMR, DEPT-90 and DEPT-135 of this compound with that of compound 4 demonstrated that compound 5 differed from compound 4 only in having a benzoly ester group attached to C-20. These data indicated that compound 5 was (–)-20-Benzyloxyhardwikiic acid which firstly found in *Croton oblongifolius* Roxb., from Udonthani province (Baingern, S., 1999).

The ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 5 and (–)-20-Benzyloxyhardwikiic acid (Baingern, S., 1999) are presented in Table 33 and 34, respectively.

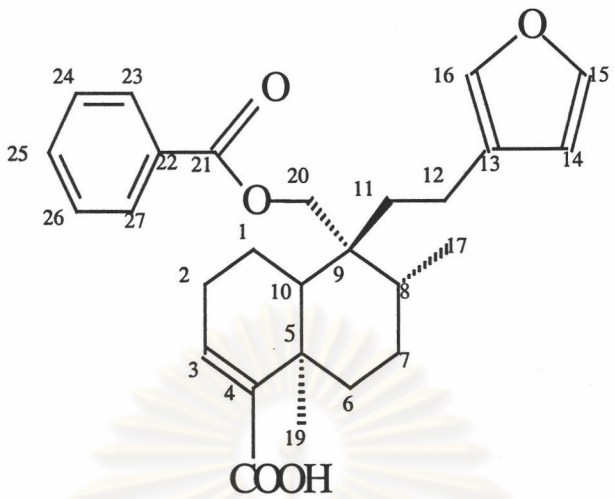
Table 33 ^{1}H-NMR spectral data of compound 5 and (–)-20-Benzyloxyhardwikiic acid (Baingern, S., 1999).

Protons	(-)-20-Benzyloxyhardwikiic acid (500 MHz)	Compound 5 (200 MHz)
H-1	1.72 (1H,m) 1.95 (1H,m)	1.98-1.55 (1H,m) 1.98-1.55 (1H,m)
H-2	2.20 (1H,m) 2.35 (1H,m)	2.53-2.19 (1H,m) 2.53-2.19 (1H,m)
H-3	6.92 (1H,dd, (*J*=2.45,4.58 Hz)	6.90 (1H,s)
H-4	-	-
H-5	-	-
H-6	1.24 (1H,m) 2.53 (1H,ddd, J=3.05, 3.05, 12.82 Hz)	1.24 (1H,m) 2.53-2.19 (1H,m)
H-7	1.53 (1H,m) 1.65 (1H,m)	1.98-1.55 (1H,m) 1.98-1.55 (1H,m)
H-8	1.78 (1H,m)	1.98-1.55 (1H,m)
H-9	-	-
H-10	1.58 (1H,d, *J*=12.51Hz)	1.98-1.55 (1H,m)
H-11	1.93 (1H,m) 2.08 (1H,m)	1.98-1.55 (1H,m) 2.03 (1H,m)
H-12	2.25 (1H,m) 2.40 (1H,m)	2.53-2.19 (1H,m) 2.53-2.19 (1H,m)
H-13	-	-
H-14	6.28 (1H,d, *J*=1.53 Hz)	6.26 (1H,s)
H-15	7.35 (1H,d, *J*=1.53 Hz)	7.34 (1H, d, *J* = 1.22 Hz)
H-16	7.24 (1H,s)	7.24 (1H,s)
H-17	1.02 (3H,d, *J*=6.71Hz)	1.01 (3H,d, *J* = 6.12 Hz)
H-18	-	-
H-19	1.32 (3H,s)	1.30 (3H,s)
H-20	4.30 (1H,d, *J*=11.9 Hz) 4.50 (1H,d, J=11.9 Hz)	4.34 (1H,d, *J* = 11.67 Hz) 4.52 (1H,d, *J* = 11.72 Hz)
H-21	-	-
H-22	-	-
H-23	8.01 (1H,d, *J*=1.22 Hz)	8.01 (1H, d, J=1.63Hz)
H-24	7.45 (1H,dd, *J*=7.63, 7.63 Hz)	7.45 (1H, dd, *J*=7.56, 7.56Hz)
H-25	7.55 (1H,dd, *J*=7.63, 7.63 Hz)	7.55 (1H, dd, *J*=7.56, 7.56Hz)
H-26	7.45 (1H,dd, *J*=7.63, 7.63 Hz)	7.45 (1H, dd, *J*=7.56, 7.56Hz)
H-27	8.01 (1H,d, *J*=1.22 Hz)	8.01 (1H, d, *J*=1.63Hz)

Table 34 ^{13}C-NMR spectral data of compound 5 and (–)-20-Benzyloxyhardwikiic acid (Baingern, S., 1999).

Carbon No.	(-)-20-Benzyloxyhardwikiic acid (125 MHz)	Compound 5 (50 MHz)
1	19.2 t	19.2 t
2	28.1 t	28.1 t
3	140.5 d	140.6 d
4	140.9 s	140.9 s
5	37.7 s	37.6 s
6	36.0 t	36.0 t
7	27.2 t	27.2 t
8	36.3 d	36.3 d
9	42.3 s	42.3 s
10	47.4 d	47.4 d
11	32.4 t	32.4 t
12	17.9 t	17.9 t
13	125.1 s	125.2 s
14	110.9 d	110.9 d
15	142.9 d	142.9 d
16	138.5 d	138.5 d
17	16.9 q	17.0 q
18	172.0 s	172.3 s
19	20.2 q	20.2 q
20	67.7 t	67.8 t
21	166.8 s	166.8 s
22	130.4 s	130.3 s
23	129.5 d	129.5 d
24	128.5 d	128.5 d
25	132.9 d	132.9 d
26	128.5 d	128.5 d
27	129.5 d	29.5 d

From the Data above, it can be concluded that compound 5 was (–)-20-Benzyloxyhardwikiic acid and the structure of compound 5 is shown in Figure 9.



Figture 9 The structure of Compound 5

5. Structure elucidation of compound 6

The IR spectrum of compound 6 is shown in Figure 36 and the important absorption peaks were assigned as shown in Table 35.

Table 35 The IR absorption bands assignment of compound 6.

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3400-2900	Broad	O-H stretching vibration
2926, 2849	Weak	C-H stretching vibration of $-CH_2$, $-CH_3$
1626, 1608	Strong	C=C stretching vibration
1286	Medium	C-O stretching vibration

The ^{1}H-NMR spectrum (Fig.37, Table 36) of compound 5 indicated four hydroxy groups attaching to quaternary carbons at δ_H 8.86, 8.89, 8.94 and 9.22 ppm, one hydroxy groups attaching to methine carbon at δ_H 3.80 ppm. There were five olefinic proton attaching to quaternary carbons at δ_H 5.68, 5.87, 6.56, 6.60 and 6.70 ppm. There were two protons of a propanyl group showed at δ_H 2.33 and 2.68 ppm.

From ^{13}C-NMR spectrum (Fig.38, Table 37), there were 15 carbon signals, which the signals of aromatics carbons appeared at δ 156.4(s), 156.1(s), 155.3(s), 144.8(2s), 130.5(s), 118.4(d), 115.0(d), 114.4(d), 99.0(s), 95.0(d), 93.8(d) ppm. There were two sp^3 carbon signals at 66.24(d) and 27.81(t) ppm, one signal of alcohol carbon at 80.93(d) ppm.

DEPT-90 and DEPT-135 ^{13}C-NMR spectra (Fig.39) indicated fifteen carbon atoms and protons. This compound probably contained fifteen carbon, fourteen hydrogen and six oxygen atoms. The molecular formula, $C_{15}H_{14}O_6$, was determined from its mass spectrum (Fig.40) which showed the molecular ion at 290 *m/z* and a signal at 272 corresponding to loss of water from the parent ion. In addition, its signal at 152 indicated the B-ring while at 139 and 123 indicated the A-ring. Moreover, its also indicated the double bond equivalent of nine.

The spectroscopic data of compound 6 were consistent with (+)-catechin or 3,3′,4′,5′,7-pentahydroxyflavan with variant:(2R,3S)-form. (+)-catechin was widespread in plant and firstly isolated in 1832 from *Nauclea gambir* (common name: Gambir-cate chu) which mostly isolated as a mixture of (+)-catechin and (–)-epicatechin.

The ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 6 and (+)-catechin (Chein-Chang, S., Yuan-Shiun, C., Li-Kang, H., 1993) were compared in Table 36 and 37, respectively.

Table 36 ^{1}H-NMR spectral data of compound 6 and (+)-catechin (Chein-Chang, S., Yuan-Shiun, C., Li-Kang, H., 1993).

Protons	(+)-Catechin (300 MHz)	Compound 6 (200 MHz)
H-1	-	-
H-2	4.51 (d, J = 7.3 Hz)	4.46 (d, J = 7.45 Hz)
H-3	3.84 (m)	3.80 (m)
H-4	2.38 (ax, dd, J = 16.0, 7.9 Hz) 2.68 (eq, dd, J = 16.0, 5.3 Hz	2.33 (eq, dd, J = 16.02, 7.96 Hz) 2.68 (ax, dd, J = 16.00, 5.32 Hz)
H-5	-	-
H-6	5.90 (d, J = 2.2 Hz)	5.87 (d, J = 2.24 Hz)
H-7	-	-
H-8	5.72 (d, J = 2.2 Hz)	5.68 (d, J = 2.20 Hz)
H-2′	6.74 (d, J = 1.9 Hz)	6.70 (d, J = 2.15 Hz)
H-3′	-	-
H-4′	-	-
H-5′	6.70(d, J = 8.0 Hz)	6.60 (d, J = 8.16 Hz)
H-6′	6.61 (dd, J = 8.0, 1.9 Hz)	6.56 (dd, J = 8.19, 1.79 Hz)
OH-3	4.75 (d, J = 4.7 Hz)	4.89 (d, J = 5.09 Hz)

Table 37 ^{13}C-NMR spectral data of compound 6 and (+)-catechin (Chein-Chang, S., Yuan-Shiun, C., Li-Kang, H., 1993).

Carbon No.	(+)-Catechin (75 MHz)	Compound 6 (50 MHz)
1	81.0 d	80.93 d
2	66.4 d	66.24 d
3	27.7 t	27.81 t
4	156.1 s	156.39 s
5	95.3 d	95.05 d
6	156.4 s	156.12 s
7	94.0 d	93.79 d
8	155.3 s	155.30 s
9	99.2 s	99.01 s
10	130.7 s	130.52 s
11	114.5 d	114.45 d
12	144.8 s	144.79 s
13	144.8 s	144.79 s
14	115.1 d	115.02 d
15	118.4 d	118.39 d

From the Data above, it can be concluded that compound 6 was (+)-catechin and the structure of compound 6 is shown in Figure 10.

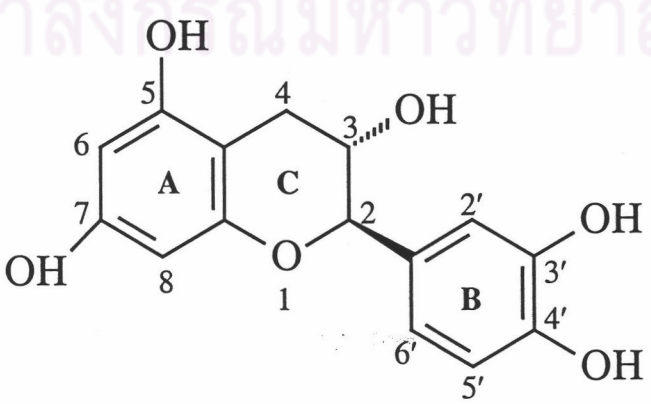


Figure 10 The structure of Compound 6

From the literature, (+)-catechin is shown to possesses antiulcer properties and formerly used in the treatment of hepatic disorders. In term of it's toxicity, it severe and occasionally fatal haemolytic anaemia reported when used therapeutically with LD_{50} (mus,ipr)1000 mg/kg. Beside, it was investigated for the inhibitory effect on the growth of four selected human tumor cell lines consist of F-7 breast carcinoma, HT-29 colon carcinoma, A-427 lung carcinoma, and UACC-375 melanoma. From that study, it is found that this compound does not have a significant effect to inhibit growth of tumor lines (Valcic, S., et al, 1996).

4.6 Result of biological activity test

Cytotoxic activity against cell lines

Compound 1-6 (10 μg/ml) were tested the *in vitro* for the cytotoxic activity against 6 cell lines such as fibroblast (HS27), gastric carcinoma (KATO-3), breast carcinoma (BT474), lung carcinoma (CHAGO), colon carcinoma (SW 620), hepato carcinoma (HEP-G2).

The cytotoxic activity of all compounds from *Croton oblongifolius* Roxb. against 6 cell lines were reported in Table 38.

Table 38 Cytotoxic activity against 6 cancer cell line of compounds 1-6 from *Croton oblongifolius* Roxb.

Compound	% Survival of cell line				
	BT 474	CHAGO	Hep-G2	KATO-3	SW 620
1	83	59	70	36	78
2	35	22	31	13	26
3	38	7	30	10	5
4	77	56	43	47	59
5	72	36	39	26	51
6	124	111	154	62	106

compounds 1 - 5 showed cytotoxic activity against 6 cell lines, but compound 6 was inactive against all cell lines. In addition, compound 2 and 3 showed remarkable cytotoxicity against all cell lines tested. The cytotoxicity data of compound 1 – 5 are shown in Table 39.

Table 39 Cytotoxicity data of compound 1- 5

Compound	IC_{50} (μg/ml) for cell lines				
	BT 474 (breast)	CHAGO (lung)	Hep-G2 (hepatoma)	KATO (gastric)	SW 620 (colon)
1	5.9	>10	6.0	5.7	>10
2	3.8	5.4	1.6	4.4	6.1
3	2.7	4.3	0.4	4.3	5.0
4	4.6	>10	0.8	5.9	7.8
5	7.7	5.6	1.2	6.5	8.8

From Table 38 and Table 39, compound 2, 3 and 5 showed moderated cytotoxic activity against 6 cell lines while compound 1 exhibited cytotoxic activity against the breast carcinoma (BT474), hepato carcinoma (HEP-G2), gastric carcinoma (KATO-3). Compound 4 exhibited cytotoxic activity against the breast carcinoma (BT474), hepato carcinoma (HEP-G2), gastric carcinoma (KATO) and colon carcinoma (SW 620) *in vitro*.

Antioxidant activity

The antioxidant assay of sample by DPPH method was used to investigate potential of sample for reducing DPPH radicals (radical 2,2-diphenylpicryhydrazyl). The antioxidant activity was reported in term of IC_{50} (μg) and are shown in Table 40. The results could be classified as high, moderate low and very low activity or inactive by 50<, 51-199, 200-399 and >400 μg, respectively.

Table 40 Antioxidant activity of compound 1-6 and Vitamin E (positive control).

Compound	Activity level	IC_{50} (μg)	IC_{50} (μM)
1 (MW 302)	Very low or inactive	>400	-
2 (MW 302)	Very low or inactive	>400	-
3 (MW 290)	Very low or inactive	>400	-
4 (MW 316)	Very low or inactive	>400	-
5 (MW 436)	Very low or inactive	>400	-
6 (MW 290)	High	10.5	0.036
Vitamin E (MW430)	High	32	0.074

From Table 40, compound 6 showed high antioxidant activity with IC_{50} 0.036 μM while compound 1-5 showed very low antioxidant activity or inactive.

Cembrane : crotocembraneic acid and neocrotocembraneic acid

Crotocembraneic acid and neocrotocembraneic acid, were found for the first time in 1998 (Roengsumran, S., 1998). They were obtained from *Croton oblongifolius*, from Petchaboon province. According to literature reviews, cembranoid diterpene compounds have been found in soft coral and marine organisms. It was firstly found in tobacco *Nicotina tabacum* L.(Rowland, R.L., et al.,1963). Other cembranoid diterpenes were isolated from this plant again (Berh, D., et al., 1978; Wahlberg, I., et al., 1982; Wahlberg, I., et al., 1985). Moreover, they have been found in other plants such as pine tree (*Haploxylon sp.)* (Dauben, W.G., Thiessen, W.E. and Resnick, P.R., 1965), frankincense *(Boswellia carteri)* (Corsano, S. and Nicoletti, R.,1967), Termite soilder (*Isoptera termitidae)* Wiemer, D.F. and Meinwald, J.,1979), *Cleome viscosa* Kosela, S., et al.,1985), leather hat *(Echinodorus grandiflorus)* (Tanaka, C.M.A., et al.,1997) and *Croton poilanei* (Sato, A., et al., 1981).

(-)-Hardwikiic acid

Hardwikiic acid was firstly found from *Croton oblongifolius*, from India (Aijar,V.N. and Seshadri, T.R., 1972) and found again in Loei province Thailand (Kutiyanuwat, N., 1999). According to literature reviews, isolation of this compound from other plants have been reported for several time.

From literature reviews in biological activity of (-)-hardwikiic acid it is found that this compound has been widely studied for its biological activity such as antimicrobial activity, insecticidal activity and anti-tumor activity. The bioactive properties of (-)-hardwikiic acid are shown below.

In 1987, B.M. Ratnayake Bandara nad coworkwers isolated (-)-hardwikiic acid from the root of *Croton aromaticus* and also modified its derivatives. (-)-hardwikiic acid showed insecticidal activity against *Aphis craccivora* (Ratnayake Bandara, B.M., Wimalasiri, W.R., Premaratne Bandara, K.A.N., 1987).

In 1991, James D. McChesney and Alice M. Clark reported that (-)-hardwikiic acid which was isolated from *Croton sonderianus* showed significant qualitative antibacterial activity against the Gram-positive bacteria (*B. subtillis, St. auerus*) and *M. smegmatis* (McChesney, J.D., Clark, A.M., 1991)

In 1994, Zheng-Ping Chen and coworkers isolated (-)-harkwikiic acid from the sap of *Croton lechleri*. In that studied, it was investigated the cytotoxicity against human oral epidermoid carcinomar showed slightly inhibitory activity (IC_{50} = 21.90± 3.50 μg/ml) (Chen, Z.P., Cai, Y., Phillipson, D., 1994).

(-)-Benzyloxyhardwikiic acid.

Benzyloxyhardwikiic acid was firstly found from *Croton oblongifolius*, from Udonthani province (Baingern, S., 1999). It was found again from *Croton oblongifolius*, Chachoengsao province (Bunyamanee, P., 2000). Beside, it is suggested that Benzyloxyhardwikiic acid may be found together with the hardwikiic acid (Baingern, S., 1999; Bunyamanee, P., 2000)

(+)-Catechin

(+)-Catechin or 3,3',4',5',7-Pentahydroxyflavan with variant:(2R,3S)-form. (+)-catechin was widespread in plant and firstly isolated in 1832 from *Nauclea gambir* (common name: Gambir-cate chu) which mostly isolated a mixture of (+)-catechin and (-)-epicatechin. From the literature, it is found that (+)-catechin possesses antiulcer properties and formerly used in the treatment of hepatic disorders. In term of it's toxicity, it severe and occasionally fatal haemolytic anaemia reported when used therapeutically with LD_{50} (mus,ipr)1000 mg/kg. Beside, it was investigated for the inhibitory effect on the growth of four selected human tumor lines consist of F-7 breast carcinoma, HT-29 colon carcinoma, A-427 lung carcinoma, and UACC-375 melanoma. From that study found that this compound does not have a significant effect to inhibit growth of tumor lines (Valcic, S., et al, 1996).