CHAPTER 4

RESULTS AND DISCUSSION

4.1 Equipment, materials and chemicals

General experimental procedures

Optical rotations were measured using a JASCO DIP-370 polarimeter. ^{1}H (300 or 500 MHz), ^{13}C (75 or 125 MHz), and 2D NMR spectra were recorded on Varian Mercury 300 and Varian Unity 500 spectrometers. High resolution EIMS were recorded on a Fison/VG Autospec-TOF-oa mass spectrometer (70 eV). High resolution ESIMS (for MH^{+}) were obtained with a Micromass Q tof 2 mass spectrometer using a cone voltage of 30 V and polyethyleneglycol (PEG) as an internal reference. TLC was performed on aluminium-backed Merck 60 GF_{254} silica gel and bands were detected by UV light (λ 254 nm) or by staining with Dragendroff's reagent and ammonium molybdate reagent. Column chromatography was performed using Merck GF_{254} flash silica gel (40-63 μm).

4.2 Collection of plant materials

Two different *Stemona* spp. i.e. *Stemona curtisii* Hook F., collected from Trang and Petchaboon Provinces and *Stemona aphylla* Craib. collected from Lampang Province were identified by Mr. James F. Maxwell from the Department of Biology, Chiang Mai University.

***Stemona curtisii* Hook F. (Trang Province)**

The roots of *S. curtisii* were collected at Tumbol Kaunmao, Amphur Rasda, in the North of Trang province, Thailand, in November 2008. A voucher specimen was deposited at the Herbarium (number 17581) of the Department of Biology, Chiang Mai University.

***Stemona curtisii* Hook F. (Petchaboon Province)**

This plant was growing naturally at Tumbol Bo Thai, Amphur Nong Phai, Petchaboon province, Thailand, in May 2010. A voucher specimen was deposited at the Herbarium (number SC-1) of the Department of Biology, Chiang Mai University.

***Stemona aphylla* Craib. (Lampang Province)**

This plant was growing naturally in Amphur Chae Hom, Lampang province, Thailand, in March 2009. A voucher specimen was deposited at the Herbarium (number 09-111) of the Department of Biology, Chiang Mai University.

(a) (b) (c)

Figure 4.1 Root, flower and stem of *Stemona* species.

(a) *S. curtisii*, Petchaboon Province.

(b) *S. curtisii*, Trang Province.

(c) *S. aphylla*, Lampang Province.

4.3 Extraction and isolation

The chemical structures of each pure compound isolated from two different *Stemona* spp. i.e. *S. curtisii* from Trang Province, *S. curtisii* from Petchaboon Province and *S. aphylla* from Lampang Province, Thailand were established and elucidated from interpretation of their spectroscopic data compared with those of known compounds.

The dried root samples of *S. curtisii* from Trang Province, Petchaboon Province and *S. aphylla* from Lampang Province were extracted with 95% ethanol and the extracts were then evaporated and dried. The ethanolic crude extract of *S. curtisii* from Petchaboon Province represented about 10.40 % of the dry weight, followed by *S. curtisii* from Trang Province (8.43 % of dry weight) and *S. aphylla* from Lampang Province (7.92 % of dry weight) (Table 4.1).

Table 4.1 Ethanolic crude extract of *Stemona* species from different origins

plant material	fresh weight (kg)	dry weight (kg)	crude extract (g)	% of dry weight
S. curtisii - Trang Province	6.10	1.00	84.34	8.43
S. curtisii - Petchaboon Province	17.50	2.50	259.89	10.40
S. aphylla - Lampang Province	6.80	0.98	78.00	7.92

4.3.1 *S. curtisii* extraction and isolation (Trang Province)

The dried roots of *S. curtisii* were extracted with 95% ethanol for 4 days and the combined extracts were evaporated using a rotary evaporator to obtain the crude ethanol extract. The extract resembled a dark brown gum. The ethanolic crude extract was subsequently partitioned between water, petroleum spirit, chloroform, ethyl

acetate, 3% hydrochloric acid solution and dichloromethane. The aqueous solution was basified with aqueous ammonia and extracted with dichloromethane. Successive purifications of this crude residue by column chromatography and preparative TLC gave pure samples of one new benzofuran, stemofuran L (**1**), and six known compounds namely, stemofuran K (**2**), stemofuran J (**3**), stemofuran F (**4**), stemocurtisinol (**5**), dehydro-γ- tocopherol (**6**) and stigmasterol (**7**).

4.3.2 *S. curtisii* extraction and isolation (Petchaboon Province)

The dried roots of *S. curtisii* were extracted with 95% ethanol for 4 days and the combined extracts were evaporated using a rotary evaporator to obtain the crude ethanol extract. The extract was a dark brown gum. The ethanolic crude extract was partitioned with dichloromethane. Successive purifications of this crude residue by column chromatography and preparative TLC gave pure samples of one new benzofuran, stemofuran S (**8**), and three known alkaloids namely oxystemokerrin (**9**), oxystemokerrin-*N*-oxide (**10**) and oxyprotostemonine (**11**).

4.3.3 *S. aphylla* extraction and isolation (Lampang Province)

The dried roots of *S. aphylla* were extracted with 95% ethanol for 4 days and the combined extracts were evaporated using a rotary evaporator to obtain the crude ethanol extract. The extract was a dark sticky residue. The ethanolic crude extract was partitioned between water, petroleum spirit, 5% hydrochloric acid solution and dichloromethane. The aqueous solution was basified with aqueous ammonia and extracted with dichloromethane. Successive purifications of this crude residue by column chromatography and preparative TLC gave pure samples of three known

compounds namely, dehydro-δ- tocopherol (**12**), stigmasterol (**7**) and stemofuran J (**3**) and one new benzofuran, stemofuran L (**1**), corresponding to that isolated from *S. curtisii* in Trang Province.

4.4 Structure elucidation

The structural elucidation of the isolated compounds were established by analysis of their spectroscopic data and comparison of the spectral data with those published previously in literature. The details of the structural elucidation of each compounds are as follows.

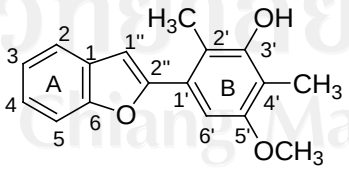
Stemofuran L (1)

Stemofuran L **(1)** was isolated as a light brown gum; HRMS analysis (EI, *m*/*z* [$M^{+\bullet}$] 268.1095, calcd 268.1099) indicated that this compound had the molecular formular $C_{17}H_{16}O_3$. This benzofuran compound was characterized by two independent aromatic systems separated by the furan ring of the benzofuran moiety. The connectivities of the directly coupled protons were determined using H/H-COSY experiments, and the positions of methyl and methoxy groups in ring B were elucidated by NOESY experiments. The ^{1}H NMR showed resonances for five benzofuran protons at 7.62 (d, 1H, *J* = 7.5 Hz, H-2), 7.50 (d, 1H, *J* = 8.0, H-5), 7.30 (ddd, *J* = 8.0, 8.0, 1.5 Hz, 1H, H-4), 7.28 (ddd, *J* = 8.0, 8.0, 1.5 Hz, 1H, H-3) and 6.63 (s, 1H, H-1″) ppm. The substituted phenyl group showed a singlet aromatic proton signal at 6.51 (s, 1H, H-6′) ppm, a hydroxyl resonance at 5.20 (s, 1H, 3′-OH) ppm and resonances for a methoxy group at 3.78 (s, 3H, 5′-OCH_3) ppm and two aromatic

methyl groups 2.01 (s, 3H, 2′-CH_3) and 1.98 (s, 3H, 4′-CH_3) ppm. The positions of these substituents were established from NOESY and HMBC NMR experiments (summarized in Table 4.2). In particular the diagnostic NOESY correlations were between, H-1′′ and the 2′-CH_3, H-6′ and the 5′-OMe and 4′-CH_3 and the 3′-OH and the 5′-OCH_3. From a search of SciFinder scholar this compound was a new natural product.

Table 4.2 ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectroscopic data of stemofuran L (**1**) in $CDCl_3$ solution

position	δ_C	DEPT	δ_H	gHMBC
1	128.7	C	-	H-1", H-3, H-5
2	120.8	CH	7.62 (d, J = 7.5 Hz)	H-4
3	122.6	CH	7.28 (ddd, J = 8.0, 8.0, 1.5 Hz)	H-5
4	123.8	CH	7.30 (ddd, J = 8.0, 8.0, 1.5 Hz)	H-2
5	111.2	CH	7.50 (d, J = 8.0 Hz)	H-3
6	154.5	C	-	H-2, H-4, H-1"
1′	132.3	C	-	H-2′
2′	115.5	C	-	H-2′
3′	152.2	C	-	H-2′
4′	119.5	C	-	H-4′
5′	156.2	C	-	H-5′
6′	101.1	CH	6.51 (s)	-
1"	106.3	CH	6.63 (s)	H-2
2"	154.7	C	-	H-4, H-1"
2′-Me	12.9	CH_3	2.01 (s)	H-4′
3′-OH	-	OH	5.20 (s)	-
4′-Me	12.6	CH_3	1.98 (s)	H-4′
5′-OMe	55.7	OCH_3	3.78 (s)	H-6′



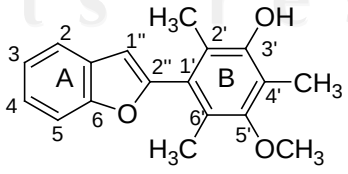
Stemofuran L **(1)**

Stemofuran K (2)

Stemofuran K (**2**) was isolated as a yellow gum; HRMS analysis (EI, *m*/*z* [$M^{+\bullet}$] 282.1254, calcd 282.1256) indicated that this compound had the molecular formular $C_{18}H_{18}O_3$. Stemofuran K was characterized by one methoxy group at position 5′, three methyl groups and an additional hydroxyl group at position 3′ in ring B. The structure of stemofuran K was established from a comparison of the ^{1}H NMR spectroscopic data of stemofuran K reported in the literature (Pacher *et al.*, 2002).

Table 4.3 ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectroscopic data of stemofuran K (**2**) in acetone-d_6 solution

position	δ_C	DEPT	δ_H in acetone-d_6	δ_H Literature in acetone-d_6 (Pacher *et al.*, 2002).
1	129.7	C	-	-
2	121.7	CH	7.52 (d, J = 8.0 Hz)	7.52 (dd, J = 7.6, 1.5 Hz)
3	123.5	CH	7.26 (dd, J = 7.5, 7.0 Hz)	7.27 (ddd, J = 7.6, 7.6, 1.5 Hz)
4	124.6	CH	7.30 (ddd, J = 7.5, 7.0, 0.5 Hz)	7.31 (ddd, J = 7.6, 7.6, 1.5 Hz)
5	111.7	CH	7.66 (d, J = 7.5 Hz)	7.67 (ddd, J = 7.6, 1.5, 0.8 Hz)
6	152.7	C	-	-
1′	130.3	C	-	-
2′	123.0	C	-	-
3′	155.6	C	-	-
4′	120.0	C	-	-
5′	156.2	C	-	-
6′	121.3	C	-	-
1"	107.0	CH	6.75 (br s)	6.76 (d, J = 0.8 Hz)
2"	155.9	C	-	-
2′-Me	14.0	CH_3	2.07 (s)	2.07 (s)
3′-OH	-	OH	7.34 (br s)	7.36 (br s)
4′-Me	9.9	CH_3	2.25 (s)	2.25 (s)
5′-OMe	60.2	OCH_3	3.69 (s)	3.69 (s)
6′-Me	13.5	CH_3	2.07 (s)	2.05 (s)



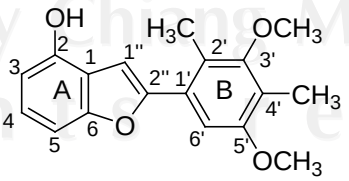
Stemofuran K (**2**)

Stemofuran J (3)

Stemofuran J (**3**) was isolated as a dark blue gum; HRMS analysis (EI, *m*/*z* [$M^{+\bullet}$] 298.1205, calcd 298.1273) indicated that this compound had the molecular formular $C_{18}H_{18}O_4$. Stemofuran J was characterized by methoxy groups at positions 3′ and 5′ and additional methyl groups at positions 2′ and 4′ in ring B. The structure of stemofuran J was established from a comparison of the ^{1}H NMR spectroscopic data of stemofuran J reported in the literature (Pacher *et al.*, 2002).

Table 4.4 ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectroscopic data of stemofuran J (**3**) in acetone-d_6 solution

position	δ_C	DEPT	δ_H in acetone-d_6	δ_H Literature in acetone-d_6 (Pacher *et al.*, 2002).
1	119.2	C	-	-
2	156.6	OH	8.87 (br s)	8.90 (br s)
3	108.5	CH	6.71 (d, J = 7.5 Hz)	6.71 (dd, J = 8.1, 0.9 Hz)
4	125.9	CH	7.15 (t, J = 8.5 Hz)	7.15 (dd, J = 8.1, 8.1 Hz)
5	103.3	CH	7.07 (d, J = 8.0 Hz)	7.07 (ddd, J = 8.1, 0.9, 0.9 Hz)
6	151.8	C	-	-
1′	129.5	C	-	-
2′	121.7	C	-	-
3′	158.8	C	-	-
4′	120.6	C	-	-
5′	157.3	C	-	-
6′	106.4	CH	7.19 (br s)	7.19 (s)
1"	103.2	CH	7.10 (br s)	7.10 (d, J = 0.9 Hz)
2"	154.6	C	-	-
2′-Me	13.6	CH_3	2.43 (s)	2.43 (s)
3′- OMe	60.2	OCH_3	3.73 (s)	3.73 (s)
4′-Me	9.3	CH_3	2.17 (s)	2.17 (s)
5′-OMe	55.8	OCH_3	3.91 (s)	3.91 (s)



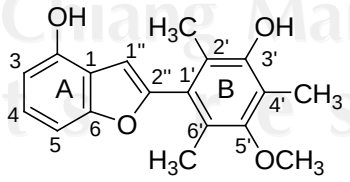
Stemofuran J **(3)**

Stemofuran F (4)

Stemofuran F (**4**) was isolated as a dark brown gum; MS analysis (EI, *m*/*z* [$M^{+\bullet}$] 298.1305) indicated that this compound had the molecular formular $C_{18}H_{18}O_4$. Stemofuran F was characterized by one methoxy group at position 5′, methyl groups at positions 2′, 4′ and 6′ and an additional hydroxyl group at position 3′ in ring B. The structure of stemofuran F was established from a comparison of the ^{1}H NMR spectroscopic data of stemofuran F reported in the literature (Pacher *et al.*, 2002).

Table 4.5 ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectroscopic data of stemofuran F (**4**) in acetone-d_6 solution

position	δ_C	DEPT	δ_H in acetone-d_6	δ_H Literature in acetone-d_6 (Pacher *et al.*, 2002).
1	119.0	C	-	-
2	156.4	C	-	-
3	108.6	CH	6.71 (d, J = 8.0 Hz)	6.71 (dd, J = 7.9, 1.0 Hz)
4	125.5	CH	7.12 (t, J = 8.5 Hz)	7.12 (dd, J = 7.9, 7.9 Hz)
5	103.6	CH	7.03 (d, J = 8.0 Hz)	7.03 (ddd, J = 7.9, 1.0, 0.9 Hz)
6	151.8	C	-	-
1′	130.3	C	-	-
2′	123.2	C	-	-
3′	152.8	C	-	-
4′	120.3	C	-	-
5′	157.3	C	-	-
6′	121.4	C	-	-
1"	104.2	CH	6.79 (br s)	6.80 (d, J = 0.9 Hz)
2"	154.0	C	-	-
2′-Me	14.1	CH_3	2.07 (s)	2.08 (s)
3′-OH	-	OH	-	7.30 (br s)
4′-Me	9.9	CH_3	2.24 (br s)	2.25 (s)
6′-Me	13.5	CH_3	2.05-2.04 (m)	2.06 (s)
5′-OMe	60.2	OCH_3	3.68 (s)	3.69 (s)



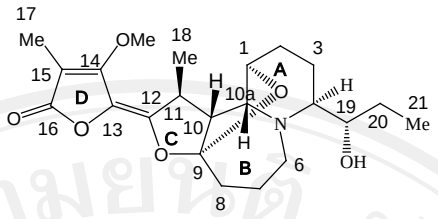
Stemofuran F (**4**)

Stemocurtisinol (**5**)

Stemocurtisinol (**5**) was isolated as a yellow brown gum; $[\alpha]^{25}_{D}$ +247° (*c* 0.15, $CHCl_3$); lit. (Sastraruji, 2006). $[\alpha]^{25}_{D}$ +233° (*c* 0.33, $CHCl_3$). HRMS analysis (EI, *m/z* $[M^{+\cdot}]$ 405.2103, calcd 405.2151) indicated that this compound had the molecular formular $C_{22}H_{31}NO_6$. The structure of stemocurtisinol was established from a comparison of its ^1H spectroscopic data with those of stemocurtisinol reported in the literature (Sastraruji, 2006). The NMR chemical shifts were in agreement with those previously reported as summarized in Table 4.6.

Table 4.6 ^{13}C NMR (100 MHz) and ^1H NMR (400 MHz) spectroscopic data of stemocurtisinol (**5**) in $CDCl_3$ solution

position	Stemocurtisinol	Literature in $CDCl_3$ solution (Sastraruji, 2006) ^{13}C NMR (125 MHz) and ^1H NMR (500 MHz)		
	δ_H	δ_C	DEPT	δ_H
1	4.04 (s)	75.4	CH	4.05 (s)
2a	1.94 (m)	22.4	CH_2	1.95 (m)
2b	1.73 (m)			1.73 (dd, J = 12.3, 5.8 Hz)
3a	1.94 (m)	18.4	CH_2	1.96 (m)
3b	1.34 (m)			1.37 (m)
4	2.53 (m)	65.5	CH	2.53 (m)
6a	3.48 (m)	54.8	CH_2	3.48 (m)
6b	2.92 (dd, J = 15.6, 4.0 Hz)			2.92 (dd, J = 15.5, 4.5 Hz)
7a	2.00 (m)	25.8	CH_2	2.00 (m)
7b	1.65 (m)			1.65 (m)
8a	2.37 (dd, J = 13.2, 3.2 Hz)	33.5	CH_2	2.37 (dd, J = 13.0, 4.1 Hz)
8b	1.77 (dd, J = 13.6, 5.6 Hz)			1.76 (dd, J = 13.0, 5.8 Hz)
9	-	120.1	C	-
10	2.70 (d, J = 4.8 Hz)	56.9	CH	2.70 (d, J = 4.7 Hz)
10a	3.40 (s)	57.5	CH	3.40 (s)
11	3.04 (m)	39.3	CH	3.06 (quin, J = 6.1 Hz)
12	-	146.8	C	-
13	-	125.0	C	-
14	-	162.7	C	-
15	-	97.5	C	-
16	-	169.7	C	-
17	2.06 (s)	9.2	CH_3	2.07 (s)
18	1.37 (d, J = 6.8 Hz)	22.6	CH_3	1.38 (d, J = 7.0 Hz)
19	3.48 (m)	67.9	CH	3.50 (m)
20a	1.60 (m)	26.4	CH_2	1.61 (m)
20b	1.24 (m)	75.4		1.25 (m)



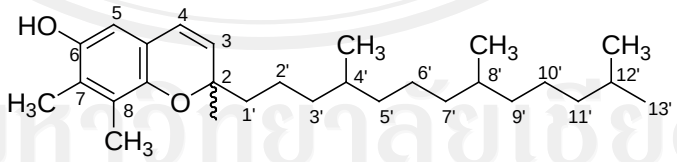
Stemocurtisinol (**5**)

Dehydro-γ-tocopherol (6)

Dehydro-γ-tocopherol **(6)** was isolated as a dark brown gum; $[\alpha]^{25}_{D}$ +14.85° (*c* 1.0, EtOH); lit. (Brem *et al.*, 2004) $[\alpha]^{25}_{D}$ +11.0° (*c* 0.2, EtOH). MS analysis (EI, *m/z* [$M^{+\bullet}$] 414.3570) indicated that this compound had the molecular formular $C_{28}H_{46}O_2$. The structure of dehydro-γ-tocopherol was characterized by an olefinic AB system at 6.22 (dd, *J* = 10.0, 3.0 Hz, H-4) ppm and 5.54 (dd, *J* = 9.5, 3.5 Hz, H-3) ppm. Additionally, two aromatic methyl group resonances appeared at 2.13 and 2.11 (s, 6H, 7-CH_3, 8-CH_3), respectively. A phenolic OH resonance was observed at 4.32 (br s, 1H, H-6) ppm.

Table 4.7 ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectroscopic data of dehydro-γ-tocopherol **(6)** in $CDCl_3$ solution

position	Dehydro-γ-tocopherol in $CDCl_3$ solution		Literature in acetone-d_6 (Brem *et al.*, 2004)	
	δ_C	δ_H	δ_C	δ_H
2	77.82	-	77.82 s	-
3	130.25	5.54 (dd, J = 9.5, 3.5 Hz)	130.25/130.27 d	5.54 (d, J = 9.8 Hz)
4	122.62	6.22 (dd, J = 10.0, 3.0 Hz)	122.61 d	6.22 (d, J = 9.8 Hz)
4a	118.88	-	118.90 s	-
5	109.77	6.30 (s)	109.76 d	6.31 (s)
6	146.83	4.32 (br s)	146.84 s	4.22 (br s)
7	123.47	-	123.45 s	-
8	125.25	-	125.24 s	-
8a	144.70	-	144.74 s	-
2-Me	25.64	1.33 (s)	25.66 q	1.34 (s)
7-Me	12.09	2.13 (s)	12.08 q	2.14 (s)
8-Me	11.66	2.12 (s)	11.65 q	2.12 (s)
1′	40.92/40.87	1.62 (m)	40.95/40.91 t	1.61 (m)
2′	21.33	1.05-1.41 (m)	21.33/21.31 t	1.0-1.4 (m)
3′, 5′, 7′, 9′[a]	37.43, 37.33, 37.28, 37.20	1.05-1.41 (m)	37.45, 37.43, 37.35, 37.26 t	1.0-1.4 (m)
4′	32.68	1.05-1.41 (m)	32.68 d	1.0-1.4 (m)
6′	24.44	1.05-1.41 (m)	24.44 t	1.0-1.4 (m)
8′	32.79	1.05-1.41 (m)	32.79 d	1.0-1.4 (m)
10′	24.78	1.05-1.41 (m)	24.79 d	1.0-1.4 (m)
11′	39.36	1.05-1.41 (m)	39.37 t	1.0-1.4 (m)
12′	27.97	1.52 (m)	27.98 d	1.52 (nonett, J = 6.6 Hz)
4′-Me	19.68/19.63	0.83 (d, J = 6.5 Hz)	19.68/19.65 q	0.83 (d, J = 6.6 Hz)
8′-Me	19.73	0.83 (d, J = 6.5 Hz)	19.74 q	0.83 (d, J = 6.6 Hz)
12′-Me	22.71	0.85 (d, J = 6.0 Hz)	22.71 q	0.86 (d, J = 6.6 Hz)
13′	22.61	0.85 (d, J = 6.0 Hz)	22.62 q	0.86 (d, J = 6.6 Hz)



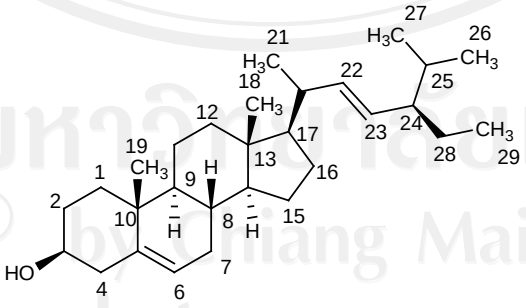
Dehydro-γ-tocopherol **(6)**

Stigmasterol (7)

Stigmasterol **(7)** was isolated as a white solid; $[\alpha]^{25}_{D}$ -45.35° (*c* 1.0, $CHCl_3$), mp 152-154 °C; lit. (Chaiyadej *et al*., 2004) mp 135-136 °C. MS analysis (EI, *m/z* $[M^{+\bullet}]$ 412.270) indicated that this compound had the molecular formular $C_{27}H_{44}O_2$. The ^1H NMR spectrum showed a peak of the H-6 olefinic proton at 5.35 ppm. Another two olefinic protons resonances at 5.15 and 5.02 ppm (dd, *J* = 15.0, 8.4 Hz) were identified as those from H-22 and H-23 on the side-chain, respectively. A one proton multiplet appeared at 3.52 ppm which was assigned to the H-3 methine proton. Moreover, six methyl group were observed at 0.70, 1.01, 1.02, 0.85-0.83, 0.80 and 0.79 ppm. The ^{13}C NMR spectroscopic data of stigmasterol was also quite similar to the data reported in the literature (Forgo and Kover, 2004).

Table 4.8 ^{13}C NMR (75 MHz) and ^{1}H NMR (300 MHz) spectroscopic data of stigmasterol **(7)** in $CDCl_3$ solution

	Stigmasterol in $CDCl_3$ solution		**Literature in $CDCl_3$ solution (Forgo and Kover, 2004)**	
position	**δ_C**	**δ_H**	**δ_C**	**δ_H**
1	37.3	1.86 (m)	37.6	1.84 (m)
2	31.6	1.82 (m)	31.9	1.83 (m)
3	71.8	3.52 (m)	72.0	3.51 (m)
4	42.3	2.22-2.33 (m)	42.5	2.23-2.30 (m)
5	140.7	-	140.8	-
6	121.7	5.35 (br d, J = 5.4 Hz)	121.8	5.34 (m)
7	31.9	1.95-2.08 (m)	32.1	1.97 (m)
8	32.0	1.47 (m)	32.2	1.46 (m)
9	50.2	0.93 (m)	50.5	0.94 (m)
10	36.5	-	36.5	-
11	21.2	1.51 (m)	21.2	1.50 (m)
12	39.7	1.18-2.0 (m)	40.0	1.18-2.0 (m)
13	42.2	-	42.2	-
14	56.9	1.09 (m)	57.1	1.01 (m)
15	24.3	1.57 (m)	24.5	1.56 (m)
16	28.9	1.69 (m)	28.9	1.72 (m)
17	55.9	1.15 (m)	56.3	1.15 (q)
18	12.0	0.70 (s)	12.2	0.70 (s)
19	19.4	1.01 (s)	19.5	1.01 (s)
20	40.5	2.08 (m)	40.4	2.06 (m)
21	21.1	1.02 (d, J = 6.6 Hz)	21.4	1.03 (d, J = 6.2 Hz)
22	138.3	5.15 (dd, J = 15.3, 8.7 Hz)	138.3	5.17 (dd, J = 15.2, 8.6 Hz)
23	129.3	5.02 (dd, J = 15.3, 8.7 Hz)	129.7	5.04 (dd, J = 15.2, 8.6 Hz)
24	51.3	1.53 (m)	51.5	1.54 (m)
25	32.0	1.57 (m)	32.2	1.55 (m)
26	21.2	0.86 (br s)	21.2	0.85 (d, J = 6.4 Hz)
27	19.0	0.84 (br s)	19.2	0.80 (d, J = 6.4 Hz)
28	25.4	1.43 (m)	25.4	1.43 (m)
		1.16 (m)		1.18 (m)
29	12.2	0.79 (br m)	12.2	0.81 (t, J = 7.3 Hz)



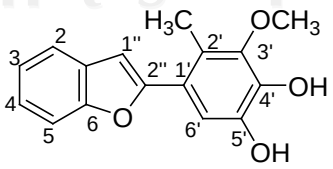
Stigmasterol **(7)**

Stemofuran S (8)

Stemofuran S **(8)** was isolated as a dark brown amorphous powder; HRMS analysis (EI, *m/z* $[M-H]^+$ 270.0870, calcd 270.0892) indicated that this compound had the molecular formular $C_{16}H_{14}O_4$. The ^1H NMR showed resonances for five benzofuran protons at 7.62 (d, 1H, *J* = 5.0 Hz, H-2), 7.52 (d, 1H, *J* = 5.0, H-5), 7.28 (m, 1H, H-4), 7.22 (m, 1H, H-3) and 6.90 (s, 1H, H-1′′) ppm. The substituted phenyl group showed a singlet aromatic proton signal at 7.18 (s, 1H, H-6′) ppm and a singlet resonance for a methoxy group at 3.81 (s, 3H, 3′-OCH_3) ppm. Additionally, an aromatic methyl group resonance was observed at 2.39 (s, 3H, 2′-CH_3) ppm and OH correspond to the C-3′ position of stemofuran L. From a search of SciFinder scholar this compound was a new natural product.

Table 4.9 ^{13}C NMR (125 MHz) and ^1H NMR (500 MHz) spectroscopic data of stemofuran S (**8**) in acetone-d_6 solution

position	δ_C	DEPT	δ_H	gHMBC	NOESY
1	121.7	C	-	-	-
2	121.6	CH	7.62 (d, J = 5.0 Hz)	H-6, H-4	-
3	123.6	CH	7.22 (m)	H-1′, H-5	-
4	124.7	CH	7.28 (m)	H-6, H-4	-
5	111.4	CH	7.52 (d, J = 5.0 Hz)	H-4	-
6	154.9	C	-	-	-
1′	130.3	C	-	-	-
2′	122.0	C	-	-	-
3′	147.8	C	-	-	-
4′	144.7	OH	-	-	-
5′	140.0	OH	-	-	-
6′	111.7	CH	7.18 (s)	H-2", H-4′, H-5′, H-2′	H-1"
1"	104.7	CH	6.90 (s)	H-2", H-6, H-1′	H-2, H-3, H-2′CH_3
2"	156.8	C	-	-	-
2′-Me	13.6	CH_3	2.39 (s)	H-3′, H-2′	H-1", H-3′OCH_3
3′-OMe	60.5	OCH_3	3.81 (s)	H-3′	H-2′CH_3



Stemofuran S **(8)**

Oxystemokerrin (9)

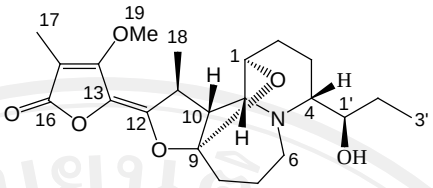
Oxystemokerrin **(9)** was isolated as a yellow brown gum; $[\alpha]^{25}_{D}$ +79.26° (*c* 1.5, $CHCl_3$); lit. (Kaltenegger *et al*., 2003) $[\alpha]^{20}_{D}$ +289° (*c* 0.40, MeOH). HRMS analysis (EI, *m/z* $[M^{+\bullet}]$ 405.2220, calcd 405.2230) indicated that this compound had the molecular formular $C_{22}H_{32}NO_6$.

Table 4.10 ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectroscopic data of oxystemokerrin (**9**) in $CDCl_3$ solution

position	Oxystemokerrin in $CDCl_3$			Literature in $CDCl_3$ (Kaltenegger *et al*., 2003)		
	δ_C	DEPT	δ_H	δ_C	DEPT	δ_H
1	74.9	CH	4.04 (br s)	75.0	CH	4.02 (m)
2a	26.9	CH_2	1.75-1.62 (m)	**	CH_2	2.23 (m)
b			1.36-1.27 (m)			2.0-1.3 (m)
3a	29.7	CH_2	1.28-1.25 (m)	**	CH_2	2.0-1.3 (m)
b			1.24-1.22 (m)			2.0-1.3 (m)
4	65.8	CH	2.48 (m)	65.8	CH	2.43 (ddd, 12.0, 9.0, 2.0)
6a	42.9	CH_2	3.21 (br m)	42.8	CH_2	3.20 (br m)
b						2.94 (br t, ~15, ~12)
7a	20.5*	CH_2	1.41 (m)	**	CH_2	2.0-1.3 (m)
b						2.0-1.3 (m)
8a	37.2*	CH_2	1.28 (m)	**	CH_2	2.28 (m)
b						2.0-1.3 (m)
9	117.22*	C	-	119.9	C	-
10	56.4	CH	2.72 (s)	56.5	CH	2.70 (d, 5.0)
10a	65.03*	CH	3.42 (br s)	**	CH	3.39 (br s)
11	39.7	CH	3.03 (br m)	39.7	CH	3.03 (br m)
12	152.9*	C	-	**	C	-
13	120.0*	C	-	**	C	-
14	162.8	C	-	162.9	C	-
15	97.5	C	-	97.5	C	-
16	169.9	C	-	169.9	C	-
17	9.1	CH_3	2.07 (s)	9.1	CH_3	2.08 (s)
18	22.5	CH_3	1.40 (d, 5.0)	22.5	CH_3	1.40 (d, 7.0)
19	70.6	CH	3.52 (br s)	70.5	CH	3.53 (br m)
20a	26.9	CH_2	1.83-1.60 (m)	26.9	CH_2	2.0-1.3 (m)
b			1.35-1.20 (m)			2.0-1.3 (m)
21	9.6	CH_3	1.01 (br s)	9.6	CH_3	1.02 (t, 7.4)
OMe	58.9	CH_3	4.13 (s)	58.9	CH_3	4.15 (s)

* = signals detection from HMBC spectrum

** = signals for these carbon atoms too weak for detection in literature



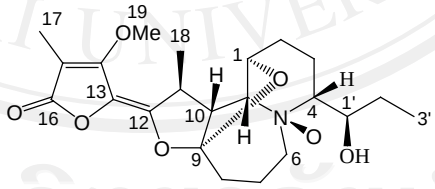
Oxystemokerrin (**9**)

Oxystemokerrin-*N*-oxide (10)

Oxystemokerrin-*N*-oxide **(10)** was isolated as a yellow brown gum; $[\alpha]^{25}_{D}$ +41.3° (*c* 1.5, $CHCl_3$); lit. (Kaltenegger *et al*., 2003). $[\alpha]^{20}_{D}$ +247° (*c* 0.30, MeOH). HRMS analysis (EI, *m/z* $[M^{+\cdot}]$ 421.2171, calcd 421.2179) indicated that this compound had the molecular formular $C_{22}H_{32}NO_7$. The structure of oxystemokerrin-*N*-oxide was established from a comparison of the ^{1}H and ^{13}C NMR spectroscopic data of oxystemokerrin-*N*-oxide with that in the literature (Kaltenegger *et al*., 2003). The NMR chemical shifts were in agreement with those previously reported as summarized in Table 4.11.

Table 4.11 ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectroscopic data of oxystemokerrin-*N*-oxide (**10**) in $CDCl_3$ solution

position	Oxystemokerrin-*N*-oxide in $CDCl_3$			Literature in $CDCl_3$ (Kaltenegger *et al.*, 2003)		
	δ_C	DEPT	δ_H	δ_C	DEPT	δ_H
1	75.1	CH	4.33 (br s)	75.0	CH	4.31 (br s)
2a	22.5	CH_2	2.20-1.87 (m)	22.2	CH_2	2.20-1.80 (m)
b			2.20-1.87 (m)			2.20-1.80 (m)
3a	22.3	CH_2	2.20-1.87 (m)	22.0	CH_2	2.20-1.80 (m)
b			2.20-1.87 (m)			2.20-1.80 (m)
4	77.9	CH	3.14 (m)	77.9	CH	3.13 (ddd, 11.6, 8.8, 2.8)
6a	61.0	CH_2	3.91 (m)	60.4	CH_2	3.90 (ddd, 12.4, 11.5, <1)
b			3.59 (m)			3.59 (ddd, 12.4, 6.5, <1)
7a	18.6	CH_2	2.69 (br s)	18.6	CH_2	2.69 (m)
b			2.20-1.87 (m)			2.20-1.80 (m)
8a	31.8	CH_2	2.20-1.87 (m)	31.7	CH_2	2.20-1.80 (m)
b			2.20-1.87 (m)			2.20-1.80 (m)
9	124.7	C	-	155.6	C	-
10	51.0	CH	3.48 (br s)	50.9	CH	3.48 (d, 3.6)
10a	84.8	CH	3.75 (br s)	84.8	CH	3.72 (br s)
11	39.2	CH	2.93 (m)	39.1	CH	2.92 (dq, 4.1, 7.1)
12	146.9	C	-	146.7	C	-
13	119.3	C	-	119.2	C	-
14	162.7	C	-	162.6	C	-
15	97.9	C	-	97.9	C	-
16	169.7	C	-	169.6	C	-
17	9.2	CH_3	2.07 (s)	9.1	CH_3	2.08 (s)
18	22.1	CH_3	1.42 (m)	22.5	CH_3	1.42 (d, 7.1)
19	72.6	CH	3.98 (m)	72.5	CH	3.98 (ddd, 9.6, 7.1, 2.8)
20a	28.1	CH_2	1.63 (m)	28.0	CH_2	1.63 (m)
b			1.42 (m)			1.41 (m)
21	8.6	CH_3	1.02 (t, 5)	8.5	CH_3	1.01 (t, 7.3)
OMe	59.1	CH_3	4.15 (s)	59.0	CH_3	4.15 (s)



Oxystemokerrin-*N*-oxide (**10**)

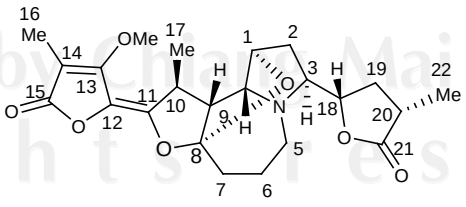
Oxyprotostemonine (**11**)

Oxyprotostemonine (**11**) was isolated as a yellow brown gum; $[\alpha]^{25}_D$ +80.21° (*c* 1.5, $CHCl_3$); lit. (Mungkornasawakul *et al.*, 2004) $[\alpha]^{25}_D$ +328° (*c* 0.17, $CHCl_3$). HRMS analysis (EI, *m/z* $[M^{+\bullet}]$ 431.2025, calcd 431.2022) indicated that this

compound had the molecular formular $C_{23}H_{30}NO_7$. The ^1H and ^{13}C NMR spectroscopic data of oxyprotostemonine was very similar to the data reported in the literature (Mungkornasawakul *et al.*, 2004).

Table 4.12 ^{13}C NMR (125 MHz) and ^1H NMR (500 MHz) spectroscopic data of oxyprotostemonine (**11**) in $CDCl_3$ solution

position	Oxyprotostemonine in $CDCl_3$			Literature in $CDCl_3$ (Mungkornasawakul *et al.*, 2004)		
	δ_C	DEPT	δ_H	δ_C	DEPT	δ_H
1	87.8	CH	4.76 (s)	87.8	CH	4.67 (s)
2a	33.0	CH_2	2.32 (m)	33.0	CH_2	2.25 (m)
b			1.74 (m)			1.74 (m)
3	66.3	CH	3.33 (s)	66.3	CH	3.31 (br s)
5a	50.7	CH_2	3.10 (m)	50.8	CH_2	3.08 (m)
b			2.98 (m)			2.97 (m)
6a	20.7	CH_2	1.74 (m)	20.9	CH_2	1.74 (m)
b			1.45 (m)			1.45 (m)
7a	32.3	CH_2	2.24 (m)	32.4	CH_2	2.25 (m)
b			1.74 (m)			1.74 (m)
8	120.7	CH	-	120.6	CH	-
9	56.9	CH	2.57 (s)	57.0	CH	2.56 (br s)
9a	69.8	CH	3.62 (s)	69.8	CH	3.60 (br s)
10	39.6	CH	3.10 (m)	39.6	CH	3.08 (m)
11	146.8	C	-	146.5	C	-
12	125.7	C	-	125.6	C	-
13	162.9	C	-	162.7	C	-
14	97.5	C	-	97.5	C	-
15	170.0	C	-	169.8	C	-
16	9.1	CH_3	2.04 (m)	9.2	CH_3	2.08 (s)
17	22.0	CH_3	1.37 (d, J = 7.5 Hz)	22.2	CH_3	1.38 (d, J = 6.5 Hz)
18	82.2	CH	4.26 (m)	82.2	CH	4.23 (t, J = 4.5 Hz)
19a	34.0	CH_2	2.24 (m)	34.1	CH_2	2.27 (m)
b			1.70 (m)			1.80 (m)
20	35.8	CH	2.69 (m)	35.9	CH	2.68 (m)
21	179.3	C	-	179.1	C	-
22	14.9	CH_3	1.28 (m)	15.1	CH_3	1.30 (d, J = 7.0 Hz)
OMe	59.0	CH_3	4.16 (s)	58.9	CH_3	4.15 (s)



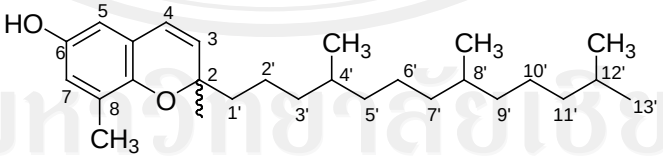
Oxyprotostemonine (**11**)

Dehydro-δ-tocopherol (12)

Dehydro-δ-tocopherol (**12**) was isolated as a yellow brown gum; $[\alpha]^{25}_{D}$ +8.56° (*c* 1.0, EtOH); lit. (Brem *et al.*, 2004) $[\alpha]^{25}_{D}$ +11.0° (*c* 1.0, EtOH). HRMS analysis (EI, *m/z* [$M^{+\cdot}$] 401.3405, calcd 401.3396) indicated that this compound had the molecular formular $C_{27}H_{44}O_2$. The structure of dehydro-δ-tocopherol was characterized by an olefinic AB system at 6.23 and 5.58 ppm (d, *J* = 10.0 Hz, H-4, H-3). Additionally, a singlet aromatic methyl group resonance appeared at 2.13 (s, H-8, CH_3) ppm. An aromatic AB system resonated at 6.47 and 6.32 (d, *J* = 2.5 Hz, H-7, H-5) ppm, characteristic of meta coupled aromatic protons. A hydroxyl group resonance was observed at 4.35 (br s, 1H, H-6) ppm. All other protons were aliphatic, consisting of a methyl group at 1.36 (t, *J* = 4.05 Hz, 2-CH_3) ppm and a saturated terpenoid side chain characterized by four methyl groups resonated at 1.62 (t, *J* = 8.0 Hz, H-1′) and 1.52 (t, *J* = 4.05 Hz, H-12′) ppm with two methyl groups and one methylene group as neighbours. The remaining aliphatic protons resonated in the range of 1.0-1.4 ppm. The ^{13}C NMR spectroscopic data of dehydro-δ-tocopherol was also quite similar with the data reported in the literature (Brem *et al.*, 2004).

Table 4.13 ^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) spectroscopic data of dehydro-δ-tocopherol **(12)** in $CDCl_3$ solution

position	Dehydro-δ-tocopherol in $CDCl_3$		Literature in acetone-d_6 (Brem *et al.*, 2004)	
	δ_C	δ_H	δ_C	δ_H
2	77.96	-	77.97 s	-
3	131.09	5.58 (d, J = 10.0 Hz)	131.09/131.11 d	5.59 (d, J = 9.7 Hz)
4	122.69	6.23 (d, J = 10.0 Hz)	122.68 d	6.24 (d, J = 9.7 Hz)
4a	121.46	-	121.46 s	-
5	110.22	6.32 (d, J = 2.5 Hz)	110.21 d	6.32 (d, J = 2.9 Hz)
6	148.48	4.35 (br s)	148.47 s	4.32 (br s)
7	116.98	6.47 (d, J = 2.5 Hz)	116.97 d	6.47 (d, J = 2.9 Hz)
8	126.41	-	126.42 s	-
8a	144.92	-	144.95 s	-
2-Me	25.73	1.34 (s)	25.75 q	1.35 (s)
8-Me	15.48	2.13 (s)	15.47 q	2.13 (s)
1′	40.98/40.94	1.62 (t, J = 8.0 Hz)	41.00/40.96 t	1.62 (m)
2′	21.29	1.22-1.11 (m)	21.29/21.28 t	1.22-1.11 (m)
3′, 5′, 7′, 9′[a]	37.43, 37.31, 37.28, 37.23	1.22-1.11 (m)	37.42, 37.34, 37.33, 37.27 t	1.22-1.11 (m)
4′	32.67	1.22-1.11 (m)	32.67 d	1.22-1.11 (m)
6′	24.44	1.22-1.11 (m)	24.43 t	1.22-1.11 (m)
8′	32.79	1.22-1.11 (m)	32.79 d	1.22-1.11 (m)
10′	24.79	1.26 (m)	24.78 d	1.22-1.11 (m)
11′	39.36	1.22-1.11 (m)	39.37 t	1.22-1.11 (m)
12′	27.97	1.52 (m)	27.97 d	1.53 (nonett, J = 6.6 Hz)
4′-Me	19.67/19.63	0.83 (s)	19.67/19.62 q	0.839/0.833 (d, J = 6.6 Hz)
8′-Me	19.74	0.83 (s)	19.74 q	0.839 (d, J = 6.6 Hz)
12′-Me	22.72	0.86 (s)	22.71 q	0.87 (d, J = 6.6 Hz)
13′	22.63	0.87 (s)	22.62 q	0.87 (d, J = 6.6 Hz)



Dehydro-δ-tocopherol **(12)**

The chemical diversity of *Stemona* spp. have been investigated. From these results indicated that there are quite variable in alkaloids component among two different samples of *S. curtisii* collected from Trang Province and Petchaboon Province. The extract of *S. curtisii* which was collected from Trang Province,

Thailand contained stemocurtisine as a major component with trace amounts of others alkaloids (Mungkornasawakul *et al*., 2004). Whereas, oxystemokerrin, oxystemokerrin-*N*-oxide and oxyprotostemonine were isolated from the extract of *S. curtisii* in Petcaboon Province. This was in contrast to previous investigations that the presence of stemofoline was reported as the main component in *S. curtisii* which was collected from the Satun Province (HG 865), Thailand (Kaltenegger *et al*., 2003). These differences may be due to the fact that these plants were harvested from different geographical regions and genetic factors. Another possibility is environmental factors, such as differences in age and seasons of collection.

4.5 Screening bioactive compounds

Brine shrimp assays

The toxic activities of the pure compounds from *Stemona* spp. on *Artemia salina* Leach (brine shrimp) were determined. The brine shrimp were treated with pure samples of stemofuran L (**1**), stemofuran K (**2**), stemofuran J (**3**), stemofuran F (**4**), stemocurtisinol (**5**), dehydro-γ-tocopherol (**6**), stigmasterol (**7**), stemofuran S (**8**) oxystemokerrin (**9**), oxystemokerrin-*N*-oxide (**10**), oxyprotostemonine (**11**) and dehydro-δ-tocopherol (**12**). The ratio of dead/alive nauplii was analysed to determine the percentage of mortalities and the LC_{50} values were culculated by Probit analysis. The results indicated that stemofuran J (**3**) had the highest activity against brine shrimp with the LC_{50} value less than 1.0 ppm, followed by stemofuran S (**8**), stemofuran L (**1**), stemofuran K (**2**), stemofuran F (**4**), oxystemokerrin (**9**), oxyprotostemonine (**11**), stemocurtisinol (**5**), dehydro-δ-tocopherol (**12**), dehydro-γ-tocopherol (**6**), oxystemokerrin-*N*-oxide (**10**) and stigmasterol (**7**) with LC_{50} values of

2.83, 4.96, 7.22, 10.66, 13.45, 21.71, 56.14, 82.62, 89.52, 211.25 and 285.69 ppm, respectively (Table 4.14). Concerning the percentage mortalities it was found that stemofuran J (**3**) had the highest mortality rates of 100% at the concentration less than 1.0 ppm followed by stemofuran S (**8**) at the concentration 10.0 ppm, stemofuran L (**1**), stemofuran K (**2**), stemofuran F (**4**), oxystemokerrin (**9**) and oxyprotostemonine (**11**) at the concentration 50.0 ppm, stemocurtisinol (**5**) at the concentration 100.0 ppm, dehydro-δ-tocopherol (**12**) and dehydro-γ-tocopherol (**6**) at the concentration 500.0 ppm and stigmasterol (**7**) and oxystemokerrin-*N*-oxide (**10**) at the concentration more than 500.0 ppm. (Table 4.15-4.26). The percentage mortalities could be correlated with LC_{50} values.

A wide variety of biological active compounds, in particular cytotoxic agents, are toxic to brine shrimp. Especially, the stilbenes and stilbenoids play important roles in plants such as inducible defense mechanisms, growth inhibition and in dormancy. Some of them are toxic to insects and other organisms, and have antifeedant and nematicidal properties (Gorham *et al*., 1995; Croteau *et al*., 2000). In this study it would seem that the LC_{50} values were different for each compound possibly due to the high sensitivity of the brine shrimp. However, while the brine shrimp assay is rather inadequate regarding the elucidation of the mechanism of action, it is very useful to assess the toxicity.

Table 4.14 The mean LC_{50} values of isolated compounds from *Stemona* species on *Artemia salina* Leach (brine shrimp)

Isolated compounds from *Stemona* spp.	LC_{50} ± S.D. (ppm)[a]
Stemofuran L (**1**)	4.96 ± 0.13
Stemofuran K (**2**)	7.22 ± 0.12
Stemofuran J (**3**)	< 1 ± 0.61
Stemofuran F (**4**)	10.67 ± 0.46
Stemocurtisinol (**5**)	56.14 ± 0.10
Dehydro-γ-tocopherol (**6**)	89.52 ± 0.57
Stigmasterol (**7**)	285.69 ± 0.28
Stemofuran S (**8**)	2.83 ± 0.70
Oxystemokerrin (**9**)	13.45 ± 0.39
Oxystemokerrin-*N*-oxide (**10**)	211.25 ± 0.21
Oxyprotostemonine (**11**)	21.71 ± 0.36
Dehydro-δ-tocopherol (**12**)	82.62 ± 0.98
Solvent control (ethanol)	> 500

[a] = 95% confidence intervals.

Table 4.15 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of stemofuran L (**1**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	30	0	99	0	30/30	100
100	2.0000	30	0	69	0	30/30	100
50	1.6989	30	0	39	0	30/30	100
1	0	9	21	9	21	9/30	30.0

Estimated LC_{50} = 4.96 ppm.

Table 4.16 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of stemofuran K (**2**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	30	0	97	0	30/30	100
100	2.0000	30	0	67	0	30/30	100
50	1.6989	30	0	37	0	30/30	100
1	0	7	23	7	23	7/30	23.3

Estimated LC_{50} = 7.22 ppm.

Table 4.17 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of stemofuran J (**3**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	30	0	120	0	30/30	100
100	2.0000	30	0	90	0	30/30	100
50	1.6989	30	0	60	0	30/30	100
1	0	30	0	30	0	30/30	100

Estimated LC_{50} < 1.00 ppm.

Table 4.18 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of stemofuran F (**4**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	30	0	94	0	30/30	100
100	2.0000	30	0	64	0	30/30	100
50	1.6989	30	0	34	0	30/30	100
1	0	4	26	4	26	4/30	13.3

Estimated LC_{50} = 10.67 ppm.

Table 4.19 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of stemocurtisinol (**5**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	30	0	69	0	30/30	100
100	2.0000	30	0	39	0	30/30	100
50	1.6989	9	21	9	21	9/30	30.0
1	0	0	30	0	51	0/30	0

Estimated LC_{50} = 56.14 ppm.

Table 4.20 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of dehydro-γ-tocopherol (**6**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
500	2.6989	30	0	52	0	30/30	100
100	2.0000	19	11	22	11	19/30	63.3
50	1.6989	3	27	3	38	3/30	10.0
1	0	0	30	0	68	0/30	0

Estimated LC_{50} = 89.52 ppm.

Table 4.21 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of stigmasterol (**7**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
500	2.6989	24	6	29	6	24/30	80.0
100	2.0000	3	27	5	33	3/30	10.0
50	1.6989	2	28	2	61	2/30	6.7
10	1	0	30	0	91	0/30	0.0
1	0	0	30	0	121	0/30	0.0

Estimated LC_{50} = 285.69 ppm.

Table 4.22 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of stemofuran S (**8**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	30	0	125	0	30/30	100
100	2.0000	30	0	95	0	30/30	100
50	1.6989	30	0	65	0	30/30	100
10	1	30	0	35	0	30/30	100
1	0	5	25	5	25	5/30	16.7

Estimated LC_{50} = 2.83 ppm.

Table 4.23 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of oxystemokerrin (**9**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	30	0	94	0	30/30	100
100	2.0000	30	0	64	0	30/30	100
50	1.6989	30	0	34	0	30/30	100
10	1	4	26	4	26	4/30	13.3
1	0	0	30	0	56	0/30	0

Estimated LC_{50} = 13.45 ppm.

Table 4.24 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of oxystemokerrin-*N*-oxide (**10**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	19	11	29	11	19/30	63.3
100	2.0000	4	26	10	37	4/30	13.3
50	1.6989	4	26	6	63	4/30	13.3
10	1	2	28	2	91	2/30	6.7
1	0	0	30	0	121	0/30	0

Estimated LC_{50} = 211.25 ppm.

Table 4.25 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of oxyprotostemonine (**11**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
250	2.3979	30	0	94	0	30/30	100
100	2.0000	30	0	64	0	30/30	100
50	1.6989	30	0	34	0	30/30	100
10	1	3	27	4	27	3/30	10.0
1	0	1	29	1	56	0/30	0

Estimated LC_{50} = 21.71 ppm.

Table 4.26 Mortality of the brine shrimp larvae after 24 hr of exposure to various concentrations of dehydro-δ-tocopherol (**12**)

Dose (ppm)	Dosage (log dose)	Dead	Alive	Accumulated dead	Accumulated alive	Ratio Dead:Total	Mortality (%)
500	2.6989	30	0	55	0	30/30	100
100	2.0000	24	6	25	6	24/30	80.0
50	1.6989	1	29	1	35	1/30	3.3
10	1	0	30	0	65	0/30	0.0
1	0	0	30	0	95	0/30	0.0

Estimated LC_{50} = 82.62 ppm.

4.6 Determination of efficiency on acetylcholinesterase inhibitory activity by TLC bioautographic assay

A study of the acetylcholinesterase inhibitory activity of the isolated compounds from *Stemona* species was performed by TLC bioautographic assay in which AChE inhibition appeared as white spots on a purple background of the chromatogram (Fig. 4.2). This bioassay is easy to apply test samples and convenient for screening various samples at the same time. The result showed that stemofuran S (**8**), stemofuran K (**2**), oxystemokerrin-*N*-oxide (**10**), oxystemokerrin (**9**) and stemocurtisinol (**5**) were able to inhibit the acetylcholinesterase activity with a minimum inhibit requirement (MIR) of 100-1,000 ng as shown in (Table 4.27). Two of them, stemofuran S (**8**) and oxystemokerrin-*N*-oxide (**10**) were found to be the most active compounds with a MIR of 100 ng. In this case stemofuran S (**8**) showed a more intense inhibition zone indicating that it may be more active than oxystemokerrin-*N*-oxide (**10**). However, both compounds were not as active as the positive control, eserine and galanthamine (MIR values of 1 and 5 ng, respectively). On the other hand, stigmasterol (**7**), dehydro-δ-tocopherol (**12**), dehydro-γ-tocopherol (**6**), stemofuran F (**4**), stemofuran J (**3**), stemofuran L (**1**) and oxyprotostemonine (**11**) had no acetylcholinesterase inhibitory activity at 1,000 ng. Morever, stemofuran J (**3**), stemofuran F (**4**), dehydro-δ-tocopherol (**12**) and dehydro-γ-tocopherol (**6**) changed colour to reddish brown on the TLC plate because of their chemical instability to the reagents or the silica gel.

Table 4.27 Minimum inhibitory concentrations of samples required to inhibit AChE

compounds	Minimum inhibitory requirement	
	ng	nmol
Stemofuran L (**1**)	> 1,000	> 3.73
Stemofuran K (**2**)	500	0.56
Stemofuran J (**3**)	> 1,000	> 3.35
Stemofuran F (**4**)	> 1,000	> 3.35
Stemocurtisinol (**5**)	1,000	2.47
Dehydro-γ-tocopherol (**6**)	> 1,000	> 2.42
Stigmasterol (**7**)	> 1,000	> 2.43
Stemofuran S (**8**)	100	0.37
Oxystemokerrin (**9**)	1,000	2.47
Oxystemokerrin-*N*-oxide (**10**)	100	0.24
Oxyprotostemonine (**11**)	> 1,000	> 2.32
Dehydro-δ-tocopherol (**12**)	> 1,000	> 2.50
Eserine	1	0.003
Galanthamine	5	0.017

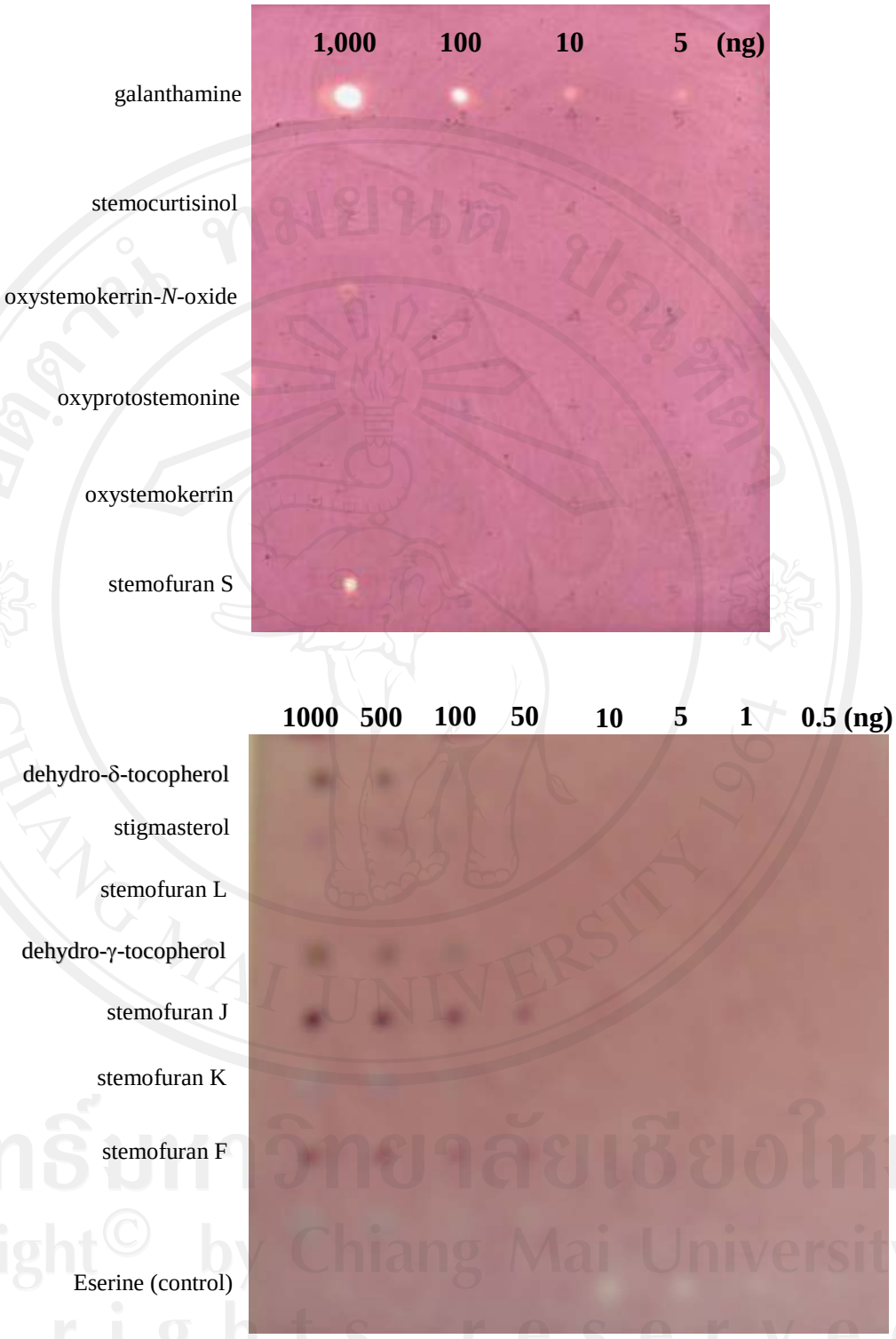


Figure 4.2 Bioautographic thin layer chromatography showing the acetylcholinesterase inhibition of the *Stemona* compounds and standards (galanthamine, eserine).

4.7 Determination of antimicrobial activities

Antimicrobial activities of the isolated compounds from *Stemona* species against the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae* and the Gram-positive bacteria *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureu*s (MRSA), and *Streptococcus pyogenes* and the antifungal activities against *Candida albicans* and *Cryptococcus neoformans* were determined. Two antibiotic agents, gentamycin and amphotericin B, were used as the positive controls for the antibacterial activity and antifungal activity, respectively. The results showed that all of the tested compounds were less active than the positive control. It was found that stemofuran J (**3**) could effectively inhibit *Cr. neoformans* and MRSA at the MIC of 7.8 and 15.6 μg/mL, respectively. Whereas, stigmasterol (**7**), dehydro-δ-tocopherol (**12**), dehydro-γ-tocopherol (**6**), stemofuran F (**4**), stemofuran K (**2**), stemofuran L (**1**), stemofuran S (**8**), stemocurtisinol (**5**), oxystemokerrin (**9**), oxystemokerrin-*N*-oxide (**10**) and oxyprotostemonine (**11**) displayed weak inhibitory activity with MIC values ranging from 62.5 to 125 μg/mL. The previous study showed that a variety of stilbenoids has been shown to inhibit fungal growth or spore germination (Gorham, 1995). Meanwhile, several stilbenoids from the roots of *S. sessilifolia* exhibited antibacterial activities against *S. aureus* and *S. epidermidis* (Yang *et al*., 2006). In addition, Adams *et al*. (2005) has studied fifteen stilbenoids from *S. collinsae, S. tuberosa*, and *S. pierrei*. The stilbenoids, dihydropinosylvin, stilbostemin A, B, D, F, G, stemofuran B, C, D, G, J, and stemanthrene A, B, C, D showed structure-dependent activities with IC_{50} values ranging from 6.7 to >50 μM.

According to Pacher *et al.* (2002), stemofuran B showed the highest antifungal activity against the four parasitic fungi, *Alternaria citri*, *Fusarium avenaceum*,

Pyricularia grisea and *Botrytis cinerea* with MIC_{50} values ranging from 50-200 µg/mL but only weak effects against *Cladosporium herbarum*. In contrast, weaker activities were also determined for stilbostemin A and the dihydrophenanthrene racemosol, showing EC_{50} values of more than 200 µg/mL against *F. avenaceum* and *C. herbarum*. Furthermore, dihydrostilbene displayed strong antimicrobial activitiy against *Bacillus pumilus* with MIC_{50} values ranging from 12.5–25 µg/mL Meanwhile, this compound exhibited moderate activity against *Klebsiella pneumoniae* with a MIC_{50} value of 50 µg/mL (Lin *et al*., 2008). From these results, it could be concluded that the antimicrobial activities of these stilbenoids is dependent upon the types and position of substituents.

For the microbicidal activity, all compounds were effective against the test organisms with the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values from 31.3 to 250 µg/mL as shown in Table 4.29. All of them were antifugicidal against *Cr. neoformans* at a concentration of 31.3 µg/mL. While dehydro-δ-tocopherol possessed the least activity against MRSA with a MBC value of 500 µg/mL.

Table 4.28 The minimum inhibitory concentrations (MIC) values of isolated compounds from *Stemona* species

Isolated compounds	Minimum inhibitory concentrations (MIC) μg/ml						
	E. coli	***K. pneumoniae***	***S. aureus***	**MRSA**	***Str. pyogenes***	***C. albicans***	***Cr. neoformans***
Stemofuran L (**1**)	125	62.5	62.5	62.5	62.5	31.3	31.3
Stemofuran K (**2**)	125	62.5	62.5	62.5	62.5	31.3	31.3
Stemofuran J (**3**)	62.5	62.5	62.5	15.6	62.5	31.3	7.8
Stemofuran F (**4**)	125	62.5	62.5	62.5	62.5	62.5	31.3
Stemocurtisinol (**5**)	125	62.5	62.5	62.5	62.5	31.3	31.3
Dehydro- γ - tocopherol (**6**)	125	62.5	62.5	62.5	62.5	31.3	31.3
Stigmasterol (**7**)	125	62.5	62.5	62.5	62.5	31.3	31.3
Stemofuran S (**8**)	62.5	62.5	62.5	62.5	-	15.6	15.6
Oxystemokerrin (**9**)	62.5	125	125	62.5	-	31.1	15.6
Oxystemokerrin-*N*-oxide (**10**)	62.5	62.5	62.5	62.5	-	31.1	15.6
Oxyprotostemonine (**11**)	62.5	62.5	125	62.5	-	31.1	15.6
Dehydro - δ - tocopherol (**12**)	125	62.5	62.5	62.5	62.5	31.3	31.3
gentamicin	11.3	11.3	22.5	45	5.6	-	-
amphotericin B	-	-	-	-	-	15.6	3.9

Table 4.29 The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values of isolated compounds from *Stemona* species

Isolated compounds	Minimum bactericidal concentration (MBC) (µg/ml)					Minimum fungicidal concentration (MFC) (µg/ml)	
	E. coli	*K. pneumoniae*	*S. aureus*	MRSA	*Str. pyogenes*	*C. albicans*	*Cr. neoformans*
Stemofuran L (**1**)	250	250	125	125	62.5	31.3	31.3
Stemofuran K (**2**)	250	250	125	125	62.5	31.3	31.3
Stemofuran J (**3**)	250	250	250	125	62.5	31.3	31.3
Stemofuran F (**4**)	250	250	125	125	62.5	62.5	31.3
Stemocurtisinol (**5**)	250	250	125	125	62.5	31.3	31.3
Dehydro- γ - tocopherol (**6**)	250	250	125	125	62.5	62.5	31.3
Stigmasterol (**7**)	250	250	125	125	125	62.5	31.3
Stemofuran S (**8**)	250	250	62.5	250	-	31.3	31.3
Oxystemokerrin (**9**)	250	250	125	250	-	125	31.3
Oxystemokerrin-*N*-oxide (**10**)	250	250	62.5	250	-	125	31.3
Oxyprotostemonine (**11**)	250	250	125	250	-	125	31.3
Dehydro - δ - tocopherol (**12**)	250	250	250	500	62.5	62.5	31.3
gentamicin	45	22.5	22.5	>90	22.5	-	-
amphotericin B	-	-	-	-	-	15.6	3.9

4.8 Determination of insecticidal properties

Leaf disk assays were carried out in the laboratory with third instar larvae of *Spodoptera littoralis* Boisduval (*S. littoralis*). The insecticidal activities of the crude extracts from two different *Stemona* species i.e. *S. curtisii* and *S. aphylla* were determined. The results are presented in Tables 4.30. The ethanolic crude extracts from *S. curtisii* displayed antifeedant activity at the concentration of 0.1 % while weaker activities were found in *S. aphylla*, in comparison with the commercial insecticide methomyl which showed antifeedant inhibition at the concentration of 0.01 %. In contrast, only the crude extracts of *S. curtisii* demonstrated repellent activity against larvae of *S. littoralis*. While the crude extracts of *S. aphylla* had no repellent activity. There was no mortality from the control solvent.

The higher antifeedant activity of the crude alkaloid extracts of *S. curtisii* and *S. aphylla* were responsible for the activity due to the chemicals contained in this fraction. These effects would expectedly result in larvae being unable to feed on the treated disks, paralysis and softening of the larval body causing death of the insect. A diverse range of secondary plant compounds such as alkaloids have a wide variety of insecticidal effects. For example, Brem *et al*. (2002) reported the potent insecticidal activity of stemofoline was assumed to be caused by neurotoxic interactions resulting in uncontrolled hyperactivity of larvae. In addition, the extracts from *S. collinsae* displayed strong antifeedant activity, whereas *S. tuberosa* showed repellent activity. In another report, the extracts from *S. curtisii* exhibited strong antifeedant properties at a concentration of 25 ppm, while the pure compounds from *S. curtisii* showed that oxyprotostemonine was the most active compound; it displayed strong antifeedant properties at a concentration of 1 ppm (Mungkornasawakul, 2004). According to

Jiwajinda *et al*. (2001) they also found antifeedant activities of *Stemona* alkaloids such as 16,17-didehydro-16(*E*)-stemofoline and its isomer at C-4, 16-17-didehydro-4(*E*)-16(*E*)-stemofoline against the diamondback moth larvae. Furthermore, methanol extracts of *S. sessilifolia* exhibited moderate feeding-deterrent activity against the two stored-grain insects, *Sitophilus zeamais* and *Tribolium castaneum* (Liu *et al*., 2007).

However, the crude extracts are usually complex mixtures of various substances and less active than the isolated pure compounds. This reason should be taken into consideration due to synergistic effects (Kaltenegger *et al*., 2003).

Table 4.30 Antifeeding activities of the ethanolic crude extracts, alkaloid crude extracts and non-alkaloid crude extracts from *S. curtisii* from Petchaboon Province and *S. aphylla* from Lampang Province

Sample	**Concentration of the total formulation (%w/v)**					
	5.0	**1.0**	**0.5**	**0.1**	**0.05**	**0.01**
S. curtisii Petchaboon Province						
- Crude ethanol	R	A	A	a	-	-
- Crude alkaloid	R	A	A	a	a	-
- Crude non-alkaloid	R	A	A	a	a	-
S. aphylla Lampang Province						
- Crude ethanol	a	a	-	-	-	-
- Crude alkaloid	A	A	A	a	a	-
- Crude non-alkaloid	A	A	a	-	-	-
Methomyl (commercial insecticide)	R	R	R	A	a	a
Control solvent	-	-	-	-	-	-

R = repellent activity, feeding inhibition without testing treated leaf disk.

A = strong antifeedant activity, less than 5% of total area of treated leaf disks in each Petri dish was consumed.

a = antifeedant activity, 5-20% of total area of treated leaf disks in each Petri dish was consumed.

- = inactive.

4.9 Determination of antioxidant activities

The antioxidant activity was assessed by measurement of the scavenging ability of each *Stemona* spp. crude extracts and some pure compounds on the free radical, 2, 2′-diphenyl-1-picryldrazyl (DPPH). When the DPPH radical was scavenged by an antioxidant through the donation of $H^{\bullet}$ to form the reduced DPPH-H, its color changes from purple to yellow. The results are shown in Tables 4.31 and Fig. 4.3-4.12. The non-alkaloid crude extracts of *S. aphylla* and *S. curtisii* showed ability to scavenge DPPH radical with IC_{50} values of 113.80 and 148.70 μg/mL, respectively followed by the ethanolic crude extract and the alkaloid crude extract of *S. aphylla* (IC_{50} = 539.32 and 671.55 μg/mL). It appeared that their potencies were about five folds less than that of the non-alkaloid crude extracts of *S. aphylla* and *S. curtisii.*

Antioxidant activity of the non-alkaloid crude extracts (e.g. stilbenoids, phenolic compounds) was correlated to their chemical structures, which free radical scavenging mainly depends on the presence of number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules (Rice-Evans

et al., 1996; Nakatani, 2000; Zheng and Wang, 2001). These results assumed that the non-alkaloid crude extracts contains substances capable of quenching free radicals.

While the alkaloid crude extract and ethanolic crude extract of *S. curtisii* showed lower antioxidant capacity with IC_{50} values of 760.04 and 1,017.34 μg/mL, respectively. In contrast, the pure compound (stemofuran S) displayed the highest antioxidant capacity with an IC_{50} value of 30.19 μg/mL whereas, BHA was the most potent antioxidant compared to the other 2 standard antioxidants (trolox and α-tocopherol). From a structural point of view, the reason of the antioxidant activity of stemofuran S may be the hydroxyl groups at the position 4′ and 5′ which has the ability to quench the absorption free radicals. Morever, it can release hydrogen atoms to combine with the radicals produced from auto-oxidation and breaking the chain reaction. The other stilbene from natural sources was also observed for antioxidant activity. For example, pieceid-2"-*O*-gallate from the roots of *Pleuropterus ciliinervis* had an inhibitory scavenging effect on DPPH with a IC_{50} value of 16.5 μM (Lee *et al*., 2003). In addition, four prenylated arylbenzofurans, (moracins R–U) displayed potent of antioxidant activity with EC_{50} values of 7.17, 5.06, 4.12 and 6.08 μg/mL, respectively (Kapche *et al*., 2009). The antioxidant assay based on scavenging of DPPH radical is dependent on the solubility of the test compound, sensitivity of DPPH to light and pH (Sharma and Bhat, 2009). Whereas, other studies on *Stemona* species found that dehydrotocopherols also showed high antioxidant capacities (Brem *et al*., 2004).

Table 4.31 The scavenging activity of *Stemona* spp. crude extracts and some pure compounds on DPPH free radical

Sample	IC_{50} ± S.D. (μg/mL)
S. curtisii Petchaboon Province	
- Crude ethanol	1017.34 ± 11.71
- Crude alkaloid	760.04 ± 11.17
- Crude non-alkaloid	148.70 ± 0.23
S. aphylla Lampang Province	
- Crude ethanol	539.32 ± 4.08
- Crude alkaloid	671.55 ± 19.76
- Crude non-alkaloid	113.80 ± 1.24
Stemofuran S	30.19 ± 0.15
Standard	
- α-tocopherol (vitamin E)	24.62 ± 0.47
- BHA	6.67 ± 0.07
- Trolox	12.77 ± 0.08

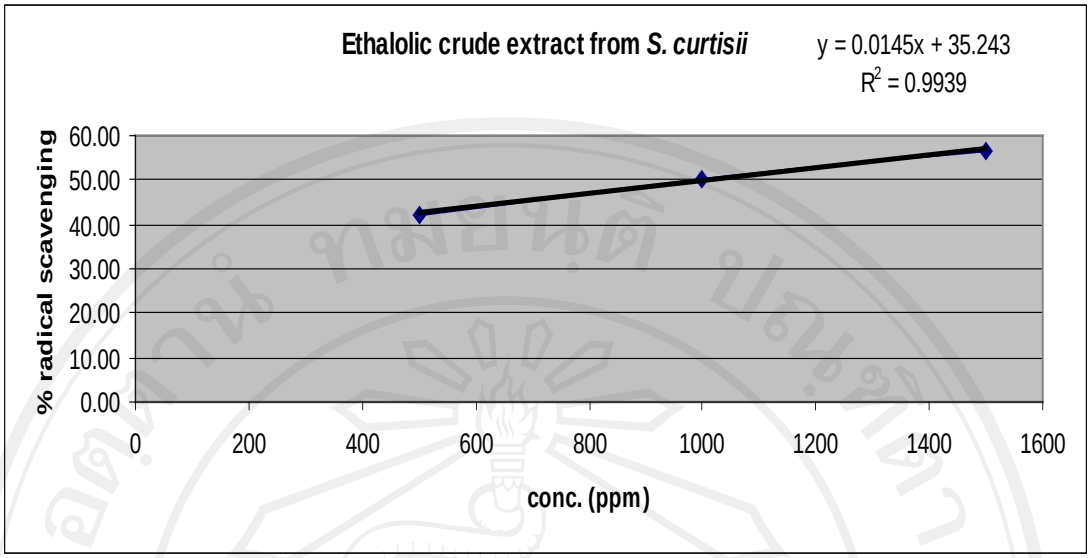


Figure 4.3 Radical scavenging curve of the ethanolic crude extract from *S. curtisii*.

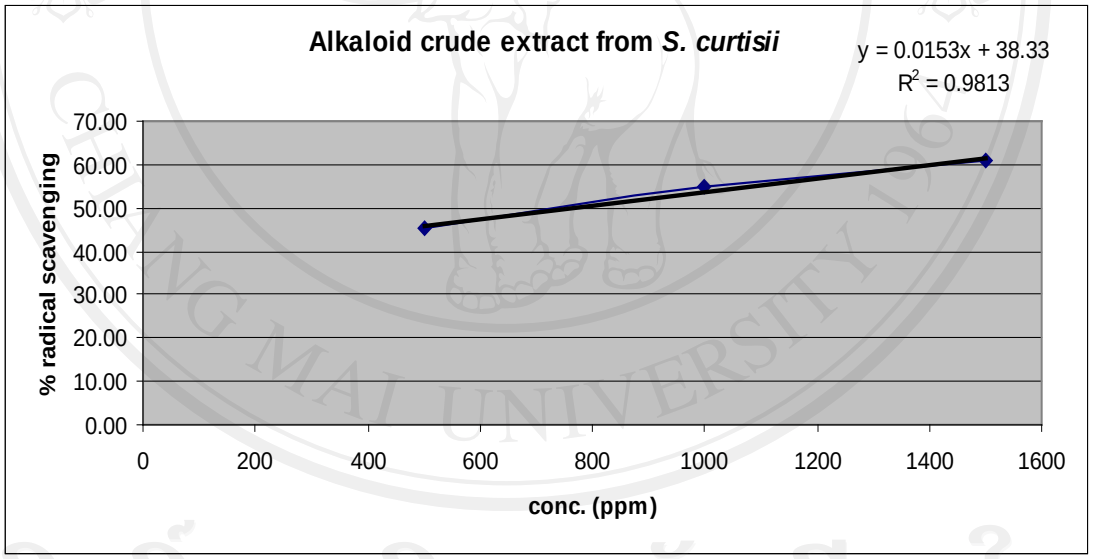


Figure 4.4 Radical scavenging curve of the alkaloid crude extract from *S. curtisii*.

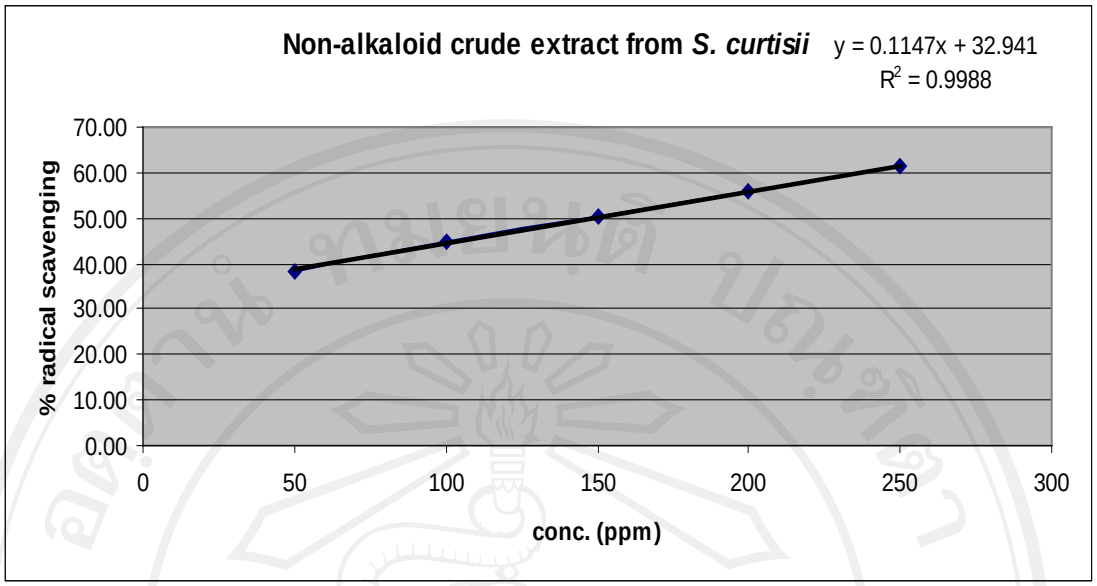


Figure 4.5 Radical scavenging curve of the non-alkaloid crude extract from *S. curtisii*.

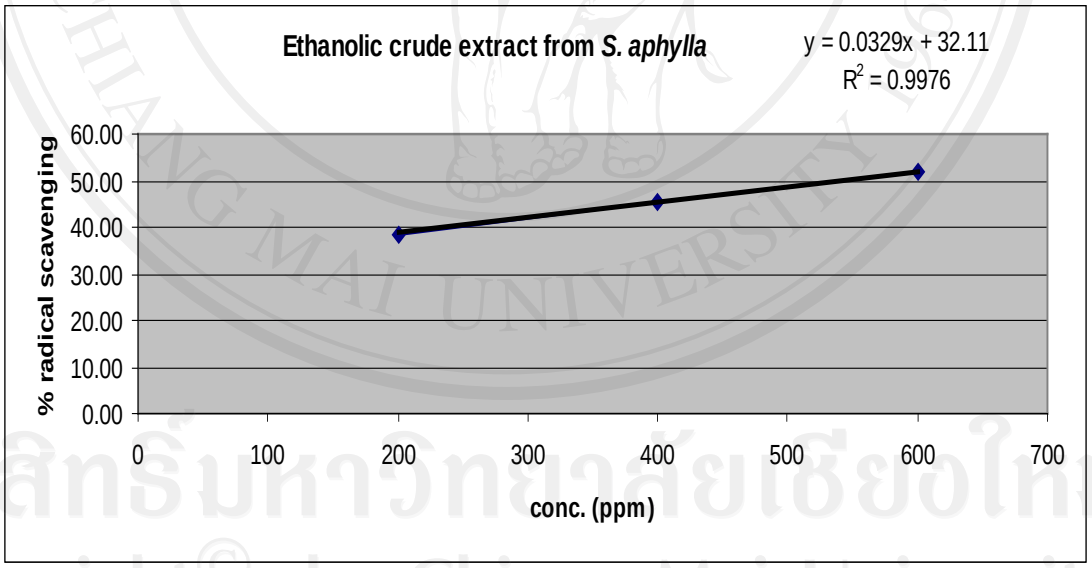


Figure 4.6 Radical scavenging activity curve of the ethanolic crude extract from *S. aphylla*.

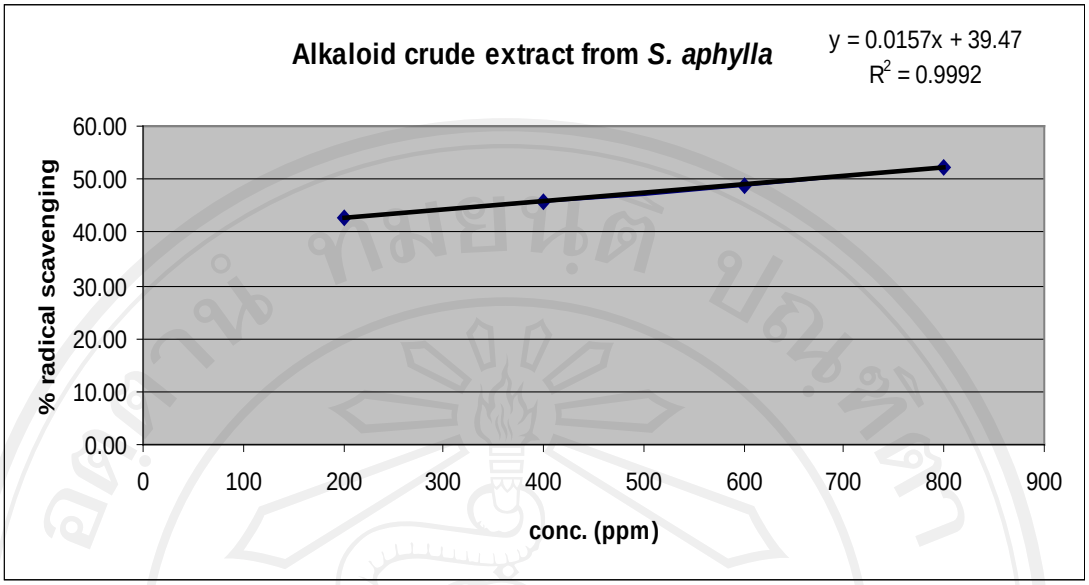


Figure 4.7 Radical scavenging curve of the alkaloid crude extract from *S. aphylla*.

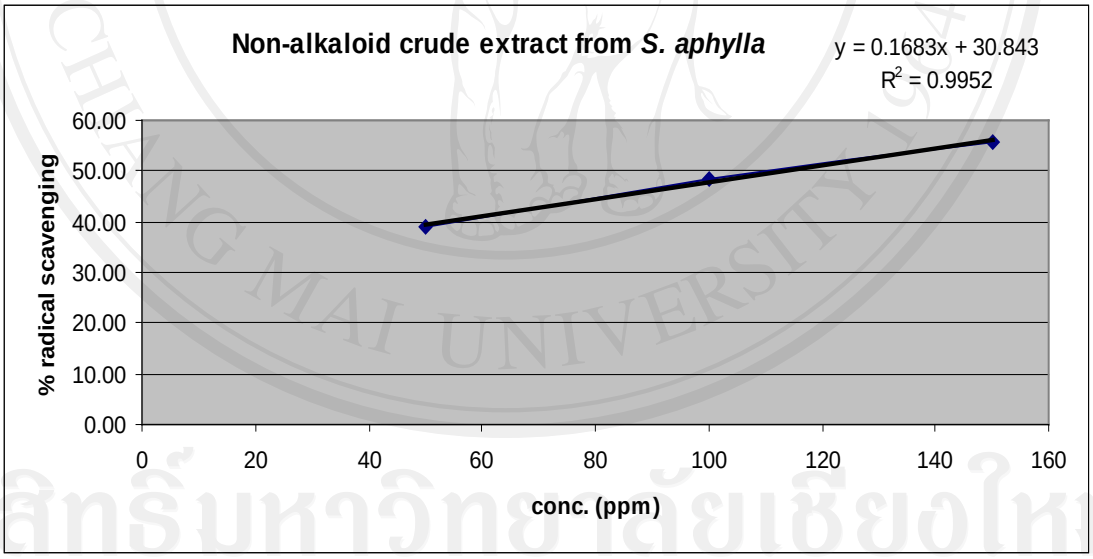


Figure 4.8 Radical scavenging curve of the non-alkaloid crude extract from *S. aphylla*.

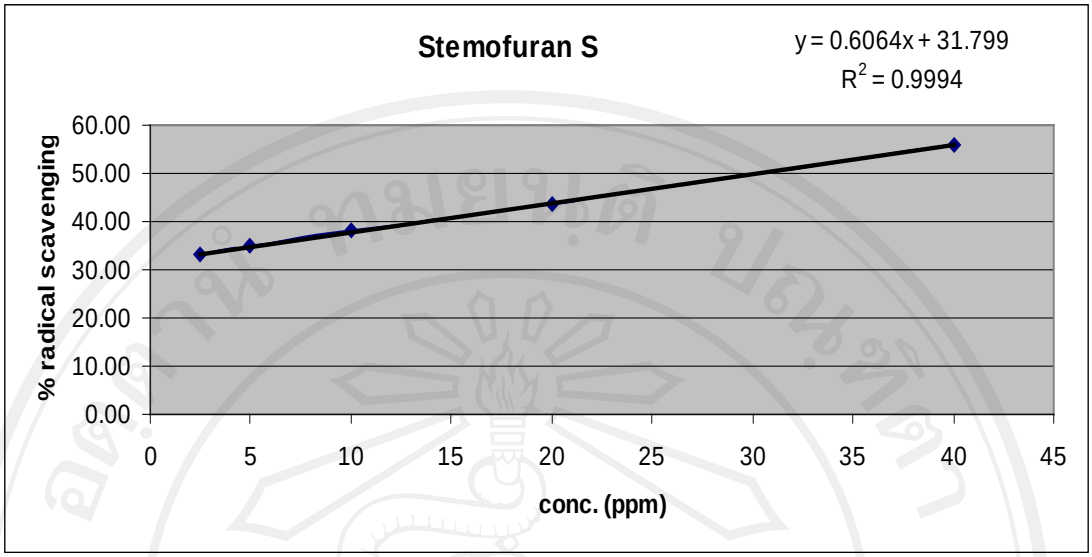


Figure 4.9 Radical scavenging curve for stemofuran S.

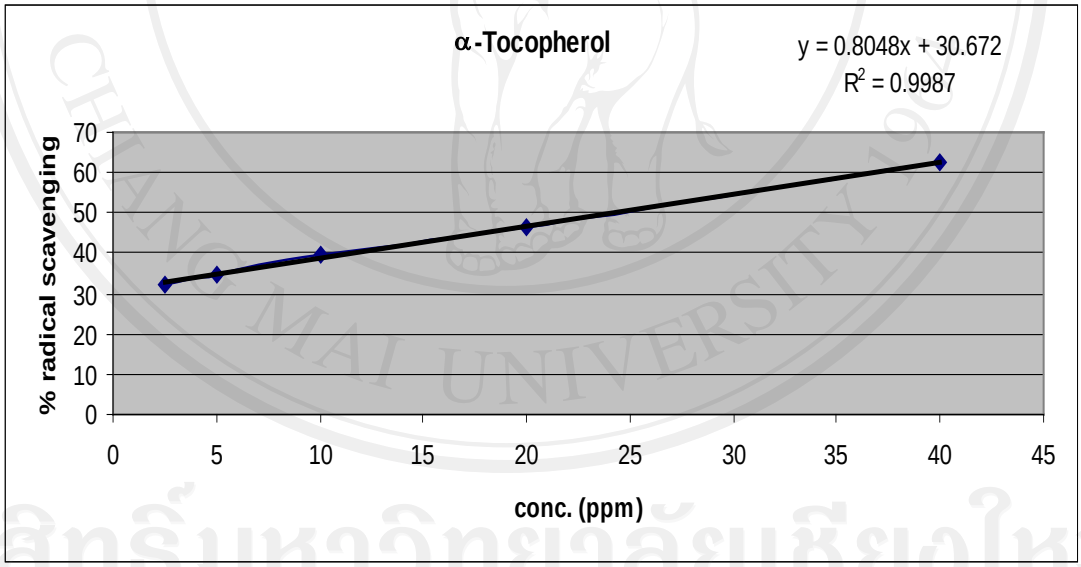


Figure 4.10 Radical scavenging curve for α-tocopherol.

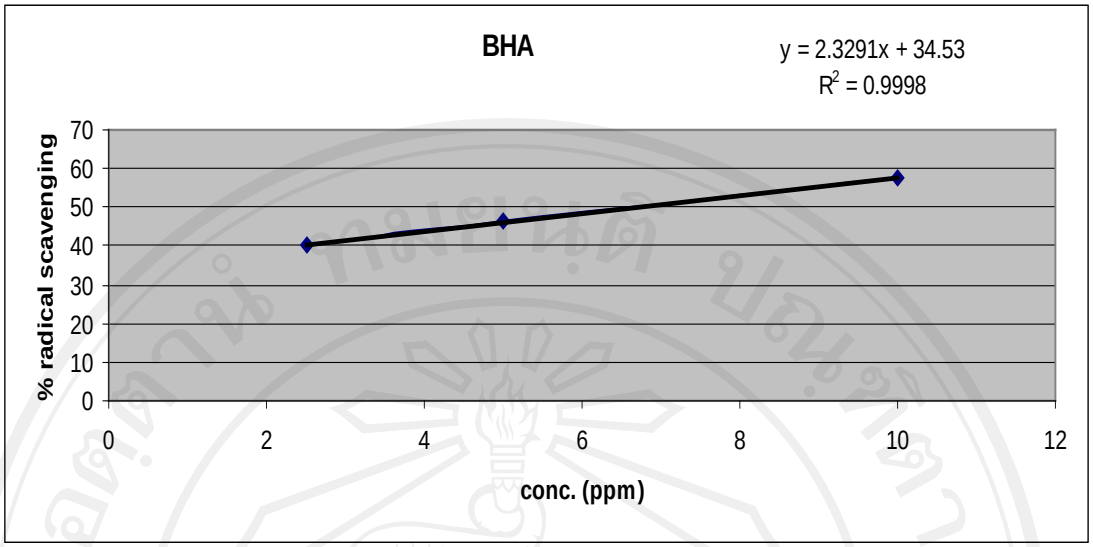


Figure 4.11 Radical scavenging curve for BHA.

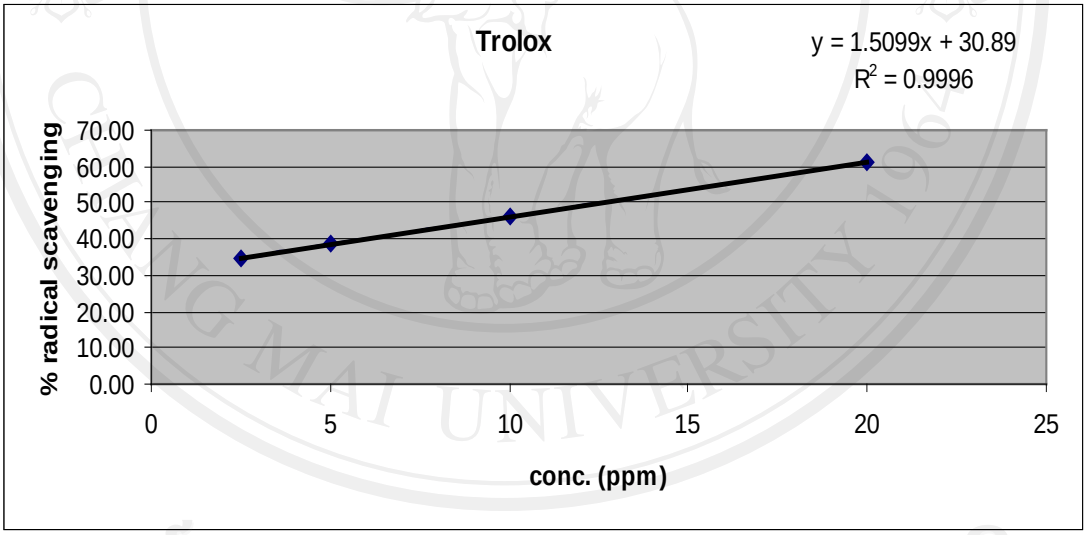


Figure 4.12 Radical scavenging curve for trolox.