CHAPTER III
RESULT AND DISCUSSION

The main feature of this research was to search for insecticidal compounds from Thai plants against adult brown planthoppers. This chapter could be divided into 4 parts: 1) preliminary insecticidal activity screening test against adult brown planthoppers by contact poison from fourteen Thai plants belonging to family Amaranthaceae, Cleomaceae, Euphorbiaceae, Leguminosae, Piperaceae, Rubiaceae and Zingiberaceae, 2) the extraction and isolation of bioactive compounds from the fruits of *Piper sarmentosum* 3) the insecticidal activity test of isolated compounds and 4) study on the acetylcholinesterase inhibiting activity of selected compounds using computational molecular docking method.

3.1 The extraction for preliminary screening test

The air-dried samples were milled to coarse powder and extracted with 95% ethanol followed the procedure described in Chapter II. The results of extraction are accumulated as shown in Table 3.1.

Table 3.1 The extraction for preliminary screening test

Family/Plant	Plant part	Dry weight (g)	Ethanolic extract (g)	(%wt/wt)
Amaranthaceae				
Achyranthes aspera L.	stem	100	18.16	18.16
Cleomaceae				
Cleoma viscosa L.	leave	100	4.80	4.80
Euphorbiaceae				
Trigonostemon riediodes (Kurz) Craib	leave	150	7.09	4.72
Capparaceae				
Senna surattensis (Burm.f.)	stem	150	14.29	9.52
Piperaceae				
Piper aurantuacum Miq	seed	150	16.26	10.84
Piper chaba Hunt.	fruit	100	37.24	37.24
Piper cubeba L.f.	fruit	200	5.77	2.88
Piper longum L.	fruit	150	16.29	10.86
Piper nigrum L.	seed	100	39.33	39.33
Piper sarmentosum Roxb.	fruit	247	14.28	5.78
Rubiaceae				
Mitragyna speciosa Roxb.	leave	100	3.36	3.36
Paederia foetida Hockf.	leave	100	14.86	14.86
Zingiberaceae				
Zingiber montanum (Koenig) Link ex Dietr.	rhizome	100	3.81	3.81
Zingiber zerumbet(L.)Sm	rhizome	150	17.94	11.96

According to the results presented in Table 3.1, it was revealed that *P. nigrum* (seed) and *P. chaba* (fruit) provided the highest yield of ethanolic extract around 37-39%. All extracts were further investigated for insecticidal activity against adult brown planthoppers by Topical application method.

3.2 The results of preliminary screening test for insecticidal activity of ethanolic extract by Topical application method

Each ethanolic extract was preliminarily screened for its insecticidal activity against adult brown planthoppers comparing with standard insecticide (etofenprox) as described in Chapter II. The bioassay results are presented in Table 3.2.

Table 3.2 The insecticidal activity test of the ethanolic extract at 5% (wt by v) against adult brown planthoppers by Topical application method

Plant	Plant part	%Mortality at 24 h
Achyranthes aspera L.	stem	15.0
Cleome viscosa L.	leave	10.0
Trigonostemon reidioides (Kurz) Craib	leave	5.0
Senna surattensis (Burm.f.)	stem	0
Piper aurantuacum Miq.	seed	25.0
Piper chaba L.f.	fruit	20.0
Piper cubeba L.	fruit	40.0
Piper longum L.	fruit	0
Piper nigrum L.	seed	60.0 (10.0)*
Piper sarmentosum Roxb.	fruit	80.0 (80.0)*
Mitragyna speciosa Roxb.	leave	0
Paederia linearis Hock.f.	leave	0
Zingiber montanum (Koenig) Link ex Dietr	rhizome	75.0 (20.0)*
Zingiber zerumbet (L.)Sm	rhizome	15.0
etofenprox	-	60.0

See appendix A Table 1 exposed for 24 h * exposed for 6 h

According to the above-mentioned table, three ethanolic extracts at concentration of 5% wt/v: *P. nigrum* (seed), *Z. montanum* (rhizome) and *P. sarmentosum* (fruit), displayed the highest insecticidal activity against adult brown planthoppers. On the other hand, those derived from *S. surattensis* (stem), *P. longum* (fruit) and *P. linearis* (leave) did not exhibit the insecticidal activity for this particular target insect. Regarding the activity results attained at 6 h, the ethanolic extract of the

fruits of *P. sarmentosum* displayed the highest insecticidal activity of 80% mortality comparable with etofenprox commercial grade whereas those of *P. nigrum* and *Z. montanum* showed only 10-20% mortality during that interval. It was thus reasonable to select the fruits of *P. sarmentosum* for further investigation on its chemical constituents and looking for the active ingredients as bioinsecticide.

3.3 The effects of the concentration of the ethanolic extract of the fruits of *P. sarmentosum* against brown planthoppers

In this section, the experiments were conducted using 2 methods. The first one was Topical application method that used to observe the activity test against adult brown planthoppers by contact poison. This test would be focused on two main parameters: insect bodies and insecticide. The second one was Parafilm method that was normally used for investigation of the systemic poison effect against adult brown planthoppers. The results are presented in Table 3.3 and appendix A, Tables 2-4.

Table 3.3 Lethal dose (LC_{50}) of the ethanolic extract of the fruits of *P. sarmentosum* against brown planthoppers

Method	Stage	LC_{50} (ppm) at 24 h
Topical application	adult	3,981
Parafilm	nymp	5,715
	adult	5,462

Regarding to Table 3.3, the activity of the ethanolic extract of the fruits of *P. sarmentosum* against adult brown planthoppers by Topical application revealed LC_{50} 3,981 ppm. Using Parafilm method, the observed activity against nymp fifth star brown planthoppers exposed LC_{50} 5,715 ppm while that against adult brown planthoppers exhibited LC_{50} 5,462 ppm.

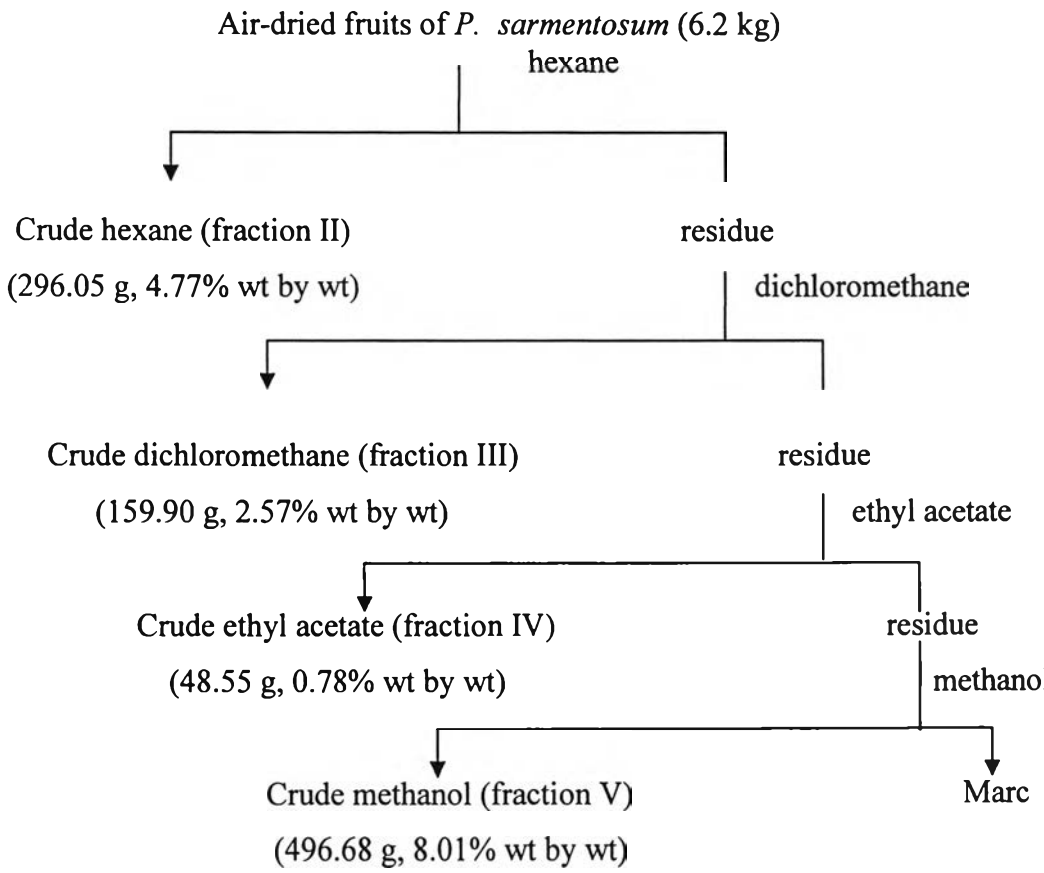
In adult stage, the LC_{50} derived by Topical application method was obviously higher than Parafilm method. This could be explained *via* the pathway that ethanolic extract was taken directly into the insect body better than Parafilm method. In addition, the ethanolic extract of the fruits of *P. sarmentosum* was found to affect on nymp fifth star higher than adult brown planthoppers. This was possibly because

nymp fifth star could suck plant sap faster than adult. This observed phenomenon similar to that addressed by Preecha, 2545.

3.4 Extraction and insecticidal activity of the fruits of *P. sarmentosum*

3.4.1 Extraction of the fruits of *P. sarmentosum*

As the results presented in Table 3.2, it was clearly seen that the ethanolic extract of the fruits of *P. sarmentosum* showed the best insecticidal activity against adult brown planthoppers by Topical application method. Thus, 6.2 kg of the fruits of *P. sarmentosum* were extracted by soxhlet using four solvents with diverse increasing polarity. Each extract was evaporated by rotatory evaporator and then subjected to the insecticidal activity test. The results of extraction are shown in Scheme 3.1 and those of insecticidal activity test are presented in Table 3.4.



Scheme 3.1 The extraction procedure

The results displayed in Scheme 3.1 indicated that the major constituents of the fruits of *P. sarmentosum* are present in polar part (methanol extract). The other parts: hexane, CH_2Cl_2 and EtOAc extracts could be less than methanol extract.

3.4.2 Insecticidal activity of fractions II – V

The preliminary insecticidal activity test of fractions II-V: hexane, CH_2Cl_2, EtOAc and MeOH extracts of the fruits of *P. sarmentosum* were performed by Topical application at 0.50% (w/v). The results are presented in Table 3.4 and appendix A Table A5.

Table 3.4 The results of insecticidal activity of fractions II – V

Fraction	Crude extract	%Mortality at 24 h
II	hexane	60.0
III	CH_2Cl_2	20.0
IV	EtOAc	0
V	MeOH	0

Regarding to the preliminary results in Table 3.4, fraction II (hexane extract) showed the highest activity against adult brown planthoppers by Topical application method, while EtOAc and MeOH extracts did not display any insecticidal activity. This result clearly shows a higher potency of hexane extract over the others. The hexane extract was thus chosen for further investigation to search for bioactive constituents.

3.5 Chemical constituents of the fruits of *P. sarmentosum*

3.5.1 Separation and insecticidal activity of fraction II

With reference to the results from section 3.4.2, the hexane extract (fraction II) revealed the highest insecticidal activity by Topical application method. Thus, 150 g of dark brown oil of fraction II was separated by quick column chromatography. The column was initially eluted by hexane, then increasing polarity of solvent to dichloromethane and methanol, respectively. The results of the separation of fraction II by quick column chromatography and insecticidal activity are revealed in Table 3.5 and appendix A Table A6.

Table 3.5 The separation and insecticidal activity of fraction II

Eluents	Fraction No. (250 mL)	Remarks	Weight (g)	% Mortality
100% C_6H_{14}	1-8	white oil (H_1)	14.70	20.0
20% CH_2Cl_2–C_6H_{14}	9-16	yellow oil (H_2)	18.06	10.0
40% CH_2Cl_2 – C_6H_{14}	17-24	dark brown oil (H_3)	9.06	10.0
60% CH_2Cl_2 – C_6H_{14}	25-32	dark brown oil (H_4)	22.24	60.0
80% CH_2Cl_2 – C_6H_{14}	33-40			
100% CH_2Cl_2	41-48	dark brown oil (H_5)	19.44	0
5%MeOH- CH_2Cl_2	49-56			
20% MeOH- CH_2Cl_2	57-64	dark brown oil (H_6)	56.34	0

According to Table 3.5, fraction H_4 showed strong insecticidal activity against adult brown planthoppers while fractions H_5-H_6 did not display insecticidal activity. In the cited literature, *Piper spp.* significantly showed the insecticidal activity against *Callosobruchus chinesis* and *Aedes aegyptii.* Moreover, some reports by Likhitwitayawuid and Ruangrungsi, 1987 and Rukachaisirikul *et al.*, 2004 implied that the bioactive compounds of hexane extract of the fruit of *P. sarmentosum* was as alkaloid constituents. Thus, all separated fractions from the hexane extract were subjected to Dragendroff's reagent. Only H_4, H_5 and H_6 fractions were clearly shown the positive test which indicated that those fractions composed of alkaloid constituents. Due to the highest %mortality of fraction H_4 and its positive test to Dragendroff's reagent, only fraction H_4 was interesting for further investigation to search for chemical constituents.

3.5.2 Separation and insecticidal activity of fraction H_4

TLC of fraction H_4 (hexane-EtOAc 8 : 2) revealed a major and two minor spots. The concentrated extract of fraction H_4 22.24 g as dark brown oil was further separated by silica gel column chromatography. The column was initially eluted by hexane following by increasing polarity of solvent. The similar fractions based on TLC were combined and subjected to insecticidal activity. The results of the

separation and insecticidal activity of fraction H_4 are revealed in Table 3.6 appendix A Table 7.

Table 3.6 The separation and insecticidal activity of fraction H_4

Eluents	Fraction No. (50 mL)	Remarks	Weight (g)	% Mortality
100% C_6H_{14}	1-15	white liquid ($H_{4.1}$)	0.07	18.4
30%CH_2Cl_2- C_6H_{14}	16-30	yellow liquid ($H_{4.2}$)	1.27	55.3
35%CH_2Cl_2- C_6H_{14}	31-38			
40%CH_2Cl_2- C_6H_{14}	39-45	yellow liquid ($H_{4.3}$)	4.81	18.4
50%CH_2Cl_2- C_6H_{14}	46-65			
60%CH_2Cl_2- C_6H_{14}	66-84	dark brown oil ($H_{4.4}$)	12.69	86.8
70%CH_2Cl_2- C_6H_{14}	85-92			
80%CH_2Cl_2- C_6H_{14}	93-106			

Regarding to the results in Table 3.6, fraction $H_{4.4}$ exhibited the best insecticidal activity. All fractions, $H_{4.1}$ - $H_{4.4}$ were also tested to Dragendroff's reagent. Only $H_{4.3}$ and $H_{4.4}$ gave positive results to this reagent. Developing of fraction $H_{4.4}$ by TLC using hexane: EtOAc 8:2 revealed two main spots on the plate. The top spot on TLC could absorb ultraviolet light at λ 254 nm and the below one could absorb ultraviolet light at λ 365 nm. Therefore, fraction $H_{4.4}$ was needed for further fractionation by silica gel column chromatography.

3.5.3 Separation of fraction $H_{4.4}$

Fraction $H_{4.4}$ as dark brown oil, 12.69 g was separated by column chromatography. The column was initially eluted by 20%EtOAc-C_6H_{14} followed by increasing polarity of solvent. The results of separation and fractionation are presented in Table 3.7.

Table 3.7 The separation of fraction $H_{4.4}$

Eluent	Fraction No. (50 mL)	Remarks	Weight (g)
100%C_6H_{14}	1-45	yellow liquid	0.05
10% EtOAc- C_6H_{14}	46-54	white crystal in yellow liquid (compound **1**)	3.74
20% EtOAc- C_6H_{14}	58-69	white crystal in yellow liquid (compound **2**)	2.67

Purification of compound **1**

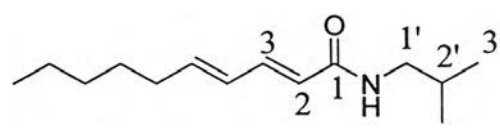
Compound **1** as the major component of fraction $H_{4.4}$ was obtained as white crystal in yellow liquid from fraction No.46-54 (Table 3.7) eluted by 10%EtOAc-C_6H_{14}. After recrystallization with hexane for several times, compound **1** was obtained as white crystal 1,120 mg. (8.82% w/w of dried fruits) and displayed melting range of 69-72°C. This compound revealed a single spot and gave an orange spot on TLC after dipping in Dragendroff's reagent suggested that presence of alkaloid nucleus.

The ^{1}H-and ^{13}C-NMR spectra of compound **1** were similar to those reported for pellitorine. (Likhitwitayawuid and Ruangrungsi, 1987 and Jacobson, 1948) The comparative assignments of ^{1}H and ^{13}C-NMR spectra of those two compounds are presented as shown in Table 3.8.

Table 3.8 The tentative assignment of ^{1}H- and ^{13}C-NMR spectra data of Compound **1** compared pellitorine (Likhitwitayawuid and Ruangrungsi, 1987 and Jacobson, 1948)

Position	^{1}H-NMR		^{13}C-NMR	
	Compound **1**	Pellitorine	Compound **1**	Pellitorine
1	-	-	166.4	166.4
2	5.78 (1H, d, J=14.8)	5.76 (d, J=15.0)	121.7	121.8
3	7.22 (1H, dd, J=10.0,15.2)	7.19 (dd, J =15.3,10.0)	141.4	143.2
4	6.13 (2H, m)	6.10(dd,13.1,10.0)	128.2	128.2
5		6.12 (dt,13.1,7.0)	143.1	141.3
6	2.19 (2H, m)	2.14 (dd,6.8,7.3)	32.9	32.9
7	1.45 (quint)	1.42 (quint,7.1)	28.5	28.5
8	1.31 (m)	1.30 (m)	31.4	31.4
9	1.32 (m)	1.30 (m)	22.5	22.5
10		0.89 (t,6.9)	14.0	14.0
1′	3.20 (2H, t, J = 7.2)	3.16 (t, 6.5)	46.9	46.9
2′	1.83 (1H, m)	1.80	28.6	28.6
3′	0.96 (6H, d, J= 6.8)	0.92 (d,6.7)	20.1	20.1
NH	5.58 (1H, brs)	5.60 (brs)		

The information attained from ^{1}H- and ^{13}C-NMR spectra was closed to those reported by Likhitwitayawuid and Ruangrungsi, 1987 and Jacobson, 1948. Hence, it was obvious to conclude that compound **1** was pellitorine. The structure of this compound is shown below.



Purification of compound **2**

Compound **2** the minor compound was separated from fraction $H_{4.4}$ which was eluted by 20%EtOAc-C_6H_{14} from fraction No. 58-69 (Table 3.7) as white crystal in yellow liquid. After recrystallization with hexane for several times, white crystal 580 mg (4.57% w/w of dried fruits) was obtained with melting range of 130-132°C. This compound exhibited a single spot on TLC and absorb ultraviolet light at λ 365 nm. This compound gave a positive test with Dragendroff's reagent, indicating the presence of alkaloid nucleus in this compound.

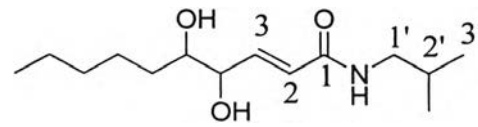
The identification of Compound **2** was conducted by direct comparison of the ^{1}H- and ^{13}C-NMR data with those reported by Banerji and Sudhir, 1983 as shown in Table 3.9.

Table 3.9 The tentative assignment of ^{1}H- and ^{13}C-NMR spectra data of compound **2** and sylvamine (Banerji and Sudhir, 1983)

Position	^{1}H-NMR		^{13}C-NMR	
	Compound **2**[1]	Sylvamine[2]	Compound **2**[1]	Sylvamine[3]
1	-	-	166.0	165.0
2	6.14 (1H, d, *J*=15.2)	6.06 (1H, d, *J*=15.4)	124.7	124.0
3	6.85 (1H, dd, *J*=15.2)	6.76 (1H, dd,*J*=15.4, 5.2)	141.6	143.0
4	2.10 (2H,brs)	4.02 (1H, m)	74.1	74.0
5		3.52 (1H, m)	74.4	73.0
6	1.45 (2H,m)		31.7	32.6
7	1.23 (6H, m)	1.0-1.5 (8H, m)	25.6	24.9
8			32.0	31.5
9			22.6	22.1
10	0.88 (3H, t, *J*= 6.4)	0.83 (3H, d, *J*= 6.6)	14.0	13.9
1′	3.16 (2H, t, *J*= 6.4)	2.99 (2H, d, *J*=6.4)	47.1	46.1
2′	1.81 (1H, s, *J*= 6.8)	1.60 (1H, m)	28.7	28.1
3′	0.93 (5H, d, *J*= 6.4)	0.83 (6H, d, *J*= 6.6)	20.1	20.1
OH-4	3.76 (1H, brs)	4.02 (1H, m)	20.1	20.1
OH-5	4.32 (1H, brs)	3.52 (1H, m)		
NH	5.58 (1H, brs)	7.14 (brs)		

[1] $CDCl_3$, [2] *d*-$_6$ Me_2CO, [3] d_6- DMSO

Comparing the ^{1}H- and ^{13}C-NMR spectroscopic data of Compound **1** (Table 3.8) with that of Compound **2** (Table 3.9), it was found that the proton and carbon signals were in good agreement with those reported by Banerji and Sudhir, 1983. From these above data, the structure of compound **2** was sylvamine. To our best knowledge, this is the first report of sylvamine as the chemical constituent of fruits of *P. sarmentosum*. The structure of this compound is shown below.



3.5.4 Separation of fraction $H_{4.3}$

Fraction $H_{4.3}$, 4.81 g was separated by silica gel column chromatography. The column was initially eluted by 100%*n*-hexane and then 10% and 20%EtOAc in n-hexane. Eluting solvent was collected for each fraction approximately 50 mL. The results of separation and combination are tabulated in Table 3.10.

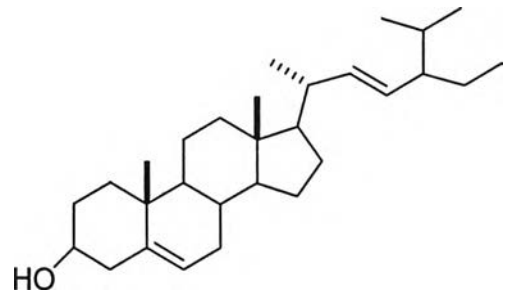
Table 3.10 The separation fraction of $H_{4.3}$

Eluents	Fraction No.(50 mL)	Remarks	Weight (g)
100%C_6H_{14}	1-33	white liquid	0.19
10% EtOAc- C_6H_{14}	34-39	white crystal in yellow liquid (compound **3**)	3.67
20% EtOAc- C_6H_{14}	40-48	yellow liquid	0.07

Purification of compound **3**

Compound **3** was obtained as white crystal in yellow liquid from fraction No.34-39 (Table 3.10) eluted by 10%EtOAc-*n*-hexane. After recrystallization by C_6H_{14} for several times, white crystal 189 mg (0.12% w/w of dried fruits) with melting range 100-106°C was obtained. This compound showed a single spot on TLC.

The ^{1}H-NMR spectrum ($CDCl_3$) (Appendix B, Figure 5) displayed the signals of $-CH_3$, $-CH_2-$ and CH of steroid at δ 0.50-2.50 and a hydroxyl group at δ 3.50. The multiplex signal at δ 5.07 was assigned for disubstituted vinyl protons (CH=CH). The last signal at δ 5.33 was the signal of trisubstituted vinyl proton (-CH=C-). The information from ^{1}H- NMR spectrum was close that of stigmasterol addressed by Rukachaisirikul *et al.*, 2004. All of these results indicated that Compound **3** was stigmasterol. The structure of this compound was shown below.



3.5.5 Separation of fraction $H_{4.2}$

Fraction $H_{4.2}$ as yellow liquid 1.27 g was separated by silica gel column chromatography. The column was initially eluted by 100%*n*-hexane and then 10% and 20%EtOAc in *n*-hexane. Eluting solvent was collected for each fraction approximately 50 mL. The equivalent fractions were combined. The results of the separation of this fraction are shown in Table 3.11.

Table 3.11 The separation of fraction $H_{4.2}$

Eluents	Fraction No.(50 mL)	Remarks	Weight (g)
100%C_6H_{14}	1-9	white liquid	0.05
10% EtOAc- C_6H_{14}	10-24	white crystal in yellow liquid (compound **3**)	0.62
20% EtOAc- C_6H_{14}	88-99	yellow liquid	0.46
	107-127	yellow liquid	0.02

3.5.6 Separation of fraction H_2

Fraction H_2 as yellow liquid 18.06 g was separated by silica gel column chromatography. The column was initially eluted by 100%*n*-hexane and increased polarity of solvent to CH_2Cl_2 and MeOH respectively. The results of the separation of fraction H_2 are presented in Table 3.12.

Table 3.12 The separation of fraction H_2

Eluents	Fraction No. (50 mL)	Remarks	Weight (g.)
100%C_6H_{14}	1-8	colorless liquid ($H_{2.1}$)	0.05
10% CH_2Cl_2- C_6H_{14}	9-16		
20% CH_2Cl_2- C_6H_{14}	17-24	colorless liquid ($H_{2.2}$) (compound **4**)	3.51
40% CH_2Cl_2- C_6H_{14}	25-29		
50% CH_2Cl_2- C_6H_{14}	30-36	yellow liquid ($H_{2.3}$)	4.02
70% CH_2Cl_2- C_6H_{14}	37-43	yellow liquid ($H_{2.4}$)	3.71
90% CH_2Cl_2- C_6H_{14}	44-52	yellow liquid ($H_{2.5}$)	3.25
100%CH_2Cl_2	53-55	yellow liquid ($H_{2.6}$)	3.57
5%MeOH- CH_2Cl_2	56-58	yellow liquid ($H_{2.7}$)	2.52
20%MeOH- CH_2Cl_2	59-72	orange solid ($H_{2.8}$)	1.19

Purification of compound **4**

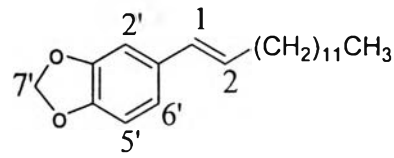
Compound **4** as colorless crystal 30 mg (2.36% w/w of dried fruits) was obtained from fraction No. 17-24 (Table 3.12), which was eluted by 20%EtOAc-*n*-hexane and showed melting range of 35-37°C. This compound showed a single spot on TLC and did not give a positive test to Dragendroff's reagent.

The ^{1}H- and ^{13}C-NMR spectra of Compound **4** were comparable of those reported for 1-(3, 4- methylenedioxyphenyl)-1*E*- tetradecene (Likhitwitayawuid and Ruangrungsi, 1987). The data of ^{1}H- and ^{13}C-NMR are presented in Table 3.13.

Table 3.13 The tentative assignment of ^{1}H- and ^{13}C-NMR spectral data of Compound **4** compared 1-(3,4-methylenedioxyphenyl)-1*E*-tetradecene (Likhitwitayawuid and Ruangrungsi, 1987)

Position	^{1}H-NMR		^{13}C-NMR	
	Compound **4**	1-(3,4 methylenedioxyphenyl)-1*E*-tetradecane	Compound **4**	1-(3,4-methylenedioxyphenyl)-1*E*- tetradecene
1	6.20 (1H, d, *J*=15.6)	6.29 (1H, d, *J*=15.6)	129.4	129.5
2	6.05 (1H, m)	6.06 (1H, dt, *J*=15.6,6.5)	129.2	129.2
3	2.17 (2H, m,)	2.17 (2H, dt, *J*=7.2, 7.0)	32.9	32.9
4	1.45 (2H, m)	1.45 (2H, m)	31.9	31.9
5	1.28-1.30 (18H, m)	1.28 (18H, m)	29.7	29.6
6			29.5	29.6
7				
8				
9				
10				
11			29.4	29.3
12			29.3	29.2
13			22.7	22.6
14	0.91 (t, *J*=6.8)	0.89 (t, *J*=6.4)	14.2	14.2
1′			132.5	132.5
2′	6.92 (1H, s)	6.91 (1H, s)	105.3	105.3
3′			147.9	147.9
4′			146.5	146.5
5′	6.75 (2H, m)	6.75 (2H, m)	108.1	108.2
6′			120.1	120.1
7′	5.93 (2H, s)	5.94 (2H, s)	100.8	100.9

From all of the above information, Compound **4** was concluded to be 1-(3, 4-methylenedioxyphenyl)-1*E*- tetradecene. Its structure is shown below.



3.5.7 Separation of fraction H_3

Fraction H_3 as yellow oil 9.06 g was separated by column chromatography. The column was initially eluted by 100% *n*-hexane and increased polarity of solvent to CH_2Cl_2 and MeOH. The equivalent fractions were combined. The results of the separation of fraction H_3 are presented in Table 3.14.

Table 3.14 The separation of fraction H_3

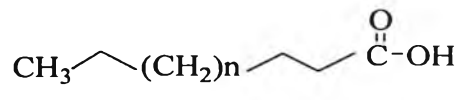
Eluents	Fraction No.(50 mL)	Remarks	Weight (g.)
100%C_6H_{14}	1-3	yellow oil ($H_{3.1}$)	0.22
	4-10	yellow oil ($H_{3.2}$)	0.42
10% CH_2Cl_2- C_6H_{14}	11-13	yellow oil ($H_{3.3}$)	1.46
20% CH_2Cl_2- C_6H_{14}	14-15		
40% CH_2Cl_2- C_6H_{14}	16-22	white solid ($H_{3.4}$)	3.71
50% CH_2Cl_2- C_6H_{14}	23-30	(compound **5**)	
70% CH_2Cl_2- C_6H_{14}	31-33	brown liquid ($H_{3.5}$)	0.63
90% CH_2Cl_2- C_6H_{14}	34-40	brown liquid ($H_{3.6}$)	2.46
5%MeOH- CH_2Cl_2	41-49	brown liquid ($H_{3.7}$)	0.04
20%MeOH- CH_2Cl_2	50-57	brown liquid ($H_{3.8}$)	0.56

Purification of compound **5**

Compound **5** was white solid in yellow liquid from fraction No. 14-30 (Table 3.14), which was eluted by 20% - 50%EtOAc-C_6H_{14}. After recrystallization by hexane for several times to afford white crystal 25 mg (2.75% w/w of dried fruits). This compound displayed melting range 61-62°C and showed a single spot on TLC.

The ^{1}H-NMR spectrum ($CDCl_3$) (appendix B, Figure 8) displayed the signals of methyl at δ 0.92 indicating the presence of a methyl group and the signal of $(CH_2)_n$

at δ 1.45 – 2.45. The information from ^{1}H-NMR spectrum was confirmed and compared with that reported by Mingvanish, 1994. According to the above data, it was obviously concluded that compound **5** was long chain carboxylic acid. The structure of this compound is displayed below.



3.5.8 Separation of fraction H_5

Fraction H_5 as dark brown oil 19.44 g was separated by silica gel column chromatography. The column was initially eluted by 100%*n*-hexane and increased polarity by gradient mixing of CH_2Cl_2 and MeOH. The equivalent fractions were combined. The results of the separation fraction H_5 are presented in Table 3.15.

Table 3.15 The separation of fraction H_5

Eluents	Fraction No. (50 mL)	Remarks	Weight (g.)
100%C_6H_{14}	1-7	pale yellow liquid ($H_{5.1}$)	0.21
	8-13	pale yellow liquid ($H_{5.2}$)	0.50
10% CH_2Cl_2- C_6H_{14}	14-18	pale yellow liquid ($H_{5.3}$)	0.21
20% CH_2Cl_2- C_6H_{14}	19-22	yellow liquid ($H_{5.4}$)	3.85
40% CH_2Cl_2- C_6H_{14}	23-25	yellow liquid ($H_{5.5}$)	4.35
	26-29	yellow liquid ($H_{5.6}$)	9.04
50% CH_2Cl_2- C_6H_{14}	30-35	white solid ($H_{5.7}$)	0.30
	36-41	white solid ($H_{5.8}$)	0.21
70% CH_2Cl_2- C_6H_{14}	42-45	white solid ($H_{5.9}$)	0.40
	46-49	white solid ($H_{5.10}$)	0.11
90% CH_2Cl_2- C_6H_{14}	50-54	dark brown oil ($H_{5.11}$)	0.33
100%CH_2Cl_2	55-56	dark brown oil ($H_{5.12}$)	0.55
5%MeOH- CH_2Cl_2	57-64	dark brown oil ($H_{5.13}$)	0.40
20%MeOH- CH_2Cl_2	64-74	dark brown oil ($H_{5.14}$)	0.18

The separated fractions were tested with Dragendroff's reagent. Only fractions $H_{5.5}$ and $H_{5.6}$ gave a positive test. Thus, fractions $H_{5.5}$ and $H_{5.6}$ were reseparated by column chromatography.

3.5.9 Separation of fraction $H_{5.5}$

Fraction $H_{5.5}$ as yellow liquid 4.35 g was separated by silica gel column chromatography. The column was initially eluted by 100%*n*-hexane and increasing the polarity by gradient mixing of EtOAc. The results of separation and fractionation are displayed in Table 3.16.

Table 3.16 The separation of fraction $H_{5.5}$

Eluents	Fraction No. (50 mL)	Remarks	Weight (g)
100%C_6H_{14}	1-12	white liquid	0.14
10% EtOAc - C_6H_{14}	13-17	white solid	0.07
	18-26	white solid (compound **5**)	0.58
20% EtOAc - C_6H_{14}	27-28	pale yellow liquid	0.30
	29-30	pale yellow liquid	2.11
	31-38	pale yellow crystal	0.12
	39-40	pale yellow crystal	0.13

3.5.10 Separation of fraction $H_{5.6}$

Fraction $H_{5.6}$ as yellow liquid 9.04 g was separated by silica gel column chromatography. The column was eluted by 100%*n*-hexane and increasing the polarity by gradient mixing of EtOAc. The equivalent fractions were combined. The results of the separation are showed in Table 3.17.

Table 3.17 The separation of fraction $H_{5.6}$

Eluents	Fraction No. (50 mL)	Remarks	Weight (g.)
100%C_6H_{14}	1-7	white liquid	0.05
10% EtOAc - C_6H_{14}	8-19	pale yellow liquid	0.28
	20-26	pale yellow liquid	0.40
20% EtOAc - C_6H_{14}	27-32	white crystal in yellow liquid (compound **6**)	1.32
	39-43	dark brown liquid	1.38

Purification of compound **6**

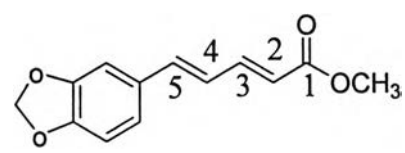
Compound **6** was obtained as white crystal in yellow liquid from fraction No. 27-32 (Table 3.17), which was eluted by 10%EtOAc- C_6H_{14}. After recrystallization by *n*-hexane for several times, Compound **6** as white crystal 1,320 mg. (14%w/w of dried fruits) with melting range of 141-143°C was obtained. This compound gave a single spot on TLC.

The ^{1}H-NMR spectrum of compound **6** was comparable to those reported by Kijjoa *et al.*, 1989. The comparable ^{1}H-NMR data of this compound and methyl piperate is displayed in Table 3.18.

Table 3.18 The tentative assignment of ^{1}H- NMR spectral data of Compound **6** compared methyl piperate (Kijjoa *et al.*, 1989)

Position	^{1}H-NMR	
	Compound **6**	Methyl piperate
1	-	-
2	6.76 (1H, d, *J*=16.5)	6.75 (1H, d, *J*=17)
3	7.42 (1H, dd, *J*=16.8, 10.9)	7.40 (1H, d, *J*= 17, 11)
4	6.76 (1H, dd, *J*= 10.3, 17.5)	6.46 (1H, dd, *J*=11, 17)
5	6.81 (1H, d, *J*=18.2)	6.80 (1H, d, *J*=17)
6	-	-
7	7.03 (1H, d, *J*=1.9)	6.97 (1H, d, *J*=2)
8	-	-
9	-	-
10	6.78 (1H, d, *J*=7.6)	6.76 (1H, d, *J*=8)
11	6.92 (1H, dd, *J*=1.9, 7.8)	6.89 (1H, dd, *J*= 2, 8)
OMe	3.76 (3H, s)	3.85 (3H, s)
OCH_2O	5.98 (2H, s)	5.95 (2H, s)

According to ^{1}H-NMR spectral data addressed by Kijjoa *et al.*, 1989, Compound **6** could be concluded as methyl piperate. The structure of this compound is displayed below.



3.6 Study on insecticidal activity test of isolated compounds

Chemical investigation on the interaction between organisms have led to the isolation and identification of biologically active natural products which have served as leads to the discovery and development of commercialized agrochemicals. Thus, in this research study, the insecticidal activity would be performed and confirmed by 2 different methods. The first one was the study on insecticidal activitiy test of isolated bioactive compounds against adult brown planthoppers *via* Topical application

method and the second one was to study on the acetylcholinesterase inhibiting activity of selected compounds *via* computational molecular docking method.

3.6.1 Effect of concentration on insecticidal activity test against adult brown planthoppers by Topical application method

In this experiment, carbosulfan was selected as the standard active ingredient. The main reason to use this standard compound that it is commonly used in Thai agriculture and could perform its activity quite well against a number of insect pests, furthermore, mode of action of this compound was typically well-known.

Table 3.19 Effect of concentration of insecticidal activity test against adult brown planthoppers by Topical application method

Compounds	LC_{50} (ppm)	LD_{50} (μg/g)	Fiduncial limit (ppm)	Slope	SE slope
carbosulfan	2,859	178	2,108 – 4,052	0.00036	±0.09784
compound **1**	3,843	247	3,034 - 5,281	0.00030	±0.10302
compound **2**	2,827	160	1,992 – 4,457	0.00026	±0.25949

3.6.1.1 Effect of concentration on carbosulfan against adult brown planthoppers

Regarding to this experiment from Table 3.19 and appendix A Table 8, LD_{50} of the carbosulfan exhibited LD_{50} 178 μg/g 95% fiduncial limit 2,108 – 4,052 ppm slope 0.00036 SE slope ±0.09784 for adult brown planthopper from Chinat province. This could be suggested that carbosulfan display as non-toxic against adult brown planthoppers by Topical application. The comparison of data obtained in this present work and those obtained by Wantana *et al.*, 1990 suggested that the brown planthoppers species tested here were more tolerant to the insecticide susceptibility among brown planthoppers from 17 provinces of Thailand and brown planthopper collected on the Chinat, Khonkhaen, Pichit and Pattalung could tolerate and resist to insecticides (resistant strain) in the class of carbamate, organophosphate and synthetic pyrethroid while those from Phae could not resist to insecticides. With reference to Chinat in 1990 and 2003, it was revealed that brown planthopper collected from Chinat (1990) displayed LD_{50} values of carbosulfan 95 μg/g 95% fiduncial limit 66.0-143.0 ppm whereas brown planthopper collected from Chinat (2003) revealed LD_{50}

values of carbosulfan 178 μg/g 95%fiduncial limit 2,108 – 4,052 ppm This could be suggested that brown planthopper species could develop its resistant to the test insecticidal carbamate compounds from winter to winter over a year. However, the attained results showed a large difference in LD_{50} for carbamate test (carbosulfan) as revealed in Table 3.19.

3.6.1.2 Effect of concentration of compound 1 against adult brown planthoppers by Topical application method

According to Table 3.19 and appendix A Table 9, Compound **1** displayed strong activity against adult brown planthoppers with LC_{50} 3,843 ppm (95%CI) 3,034-5,281 ppm and displayed non-toxicity against adult brown planthoppers. LC_{50} of compound **1** was nearly the same value as carbosulfan. From above data, it could be concluded that compound **1** was potential insecticide.

3.6.1.3 Effect of concentration of compound 2 against adult brown planthoppers by Topical application method

As summarized in Table 3.19 and appendix A Table 10, compound **2** a minor compound displayed LC_{50} 2,827 ppm (95%CI) 1,992-4,557 ppm and displayed non-toxicity against adult brown planthoppers. Compared LC_{50} with carbosulfan, it could be concluded that compound **2** was also potential insecticides.

3.6.1.4 Dosage-mortality studies

The dose-mortality relationship between carbosulfan (left), compound **1** (right) and compound **2** (below) is displayed in Figure 3.1.

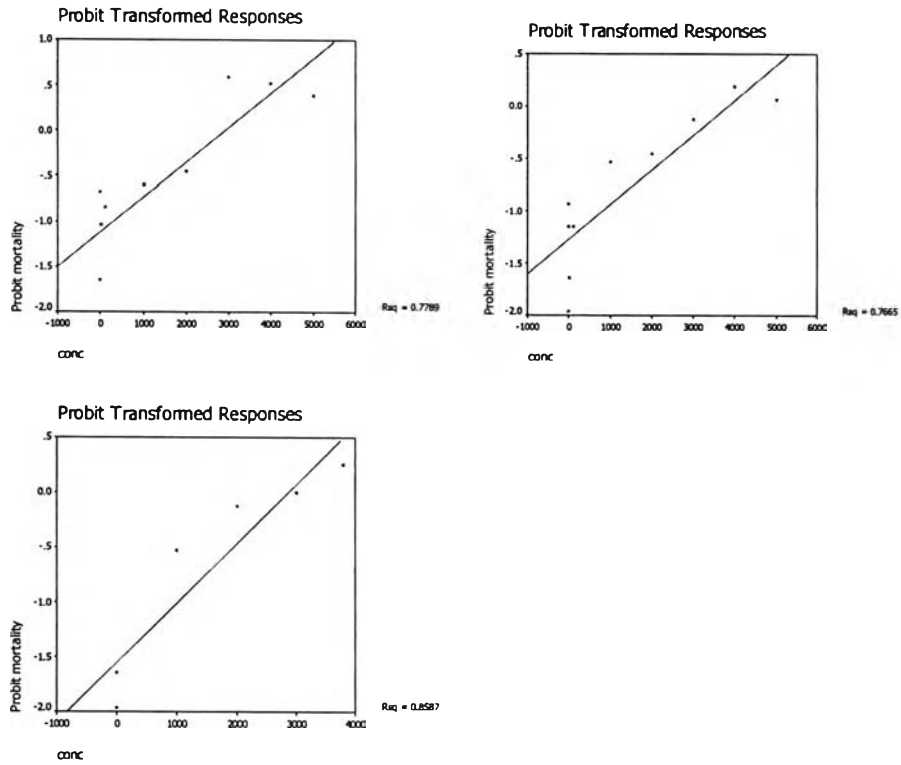


Figure 3.1 Dose-mortality relationship between carbosulfan (left) compound **1** (right) and compound **2** (below) against adult brown planthoppers

The dose-mortality relationship between compound **1** (see Figure 3.1 right) and compound **2** (Figure 3.1 below) as compared with carbosulfan (Figure 3.1 left) displayed the slopes of the dose-response lines were correlation as carbosulfan. The slope of compound **2** could be much less susceptible illustrated by the increasing in the LC_{50} values when compared with carbosulfan.

Compound **1** (pellitorine) and compound **2** (sylvamine) displayed the same trend of insecticidal activity as carbosulfan. It could be therefore concluded that compounds **1** and **2** were active compounds of the fruits of *P. sarmentosum*. In addition, compound **2** showed the highest insecticidal activity against adult brown planthoppers. This is the first report concerning compound **2** to display the highest insecticidal activity against adult brown planthoppers.

In summary, this research presents the chemical constituents of bioactive compounds from the fruits of *P. sarmentosum* and insecticidal activity against adult brown planthoppers. Futhermore, compound **1** (pellitorine) and compound **2** (sylvamine) were selected to study on the acetylcholinesterase inhibition activity.

3.6.2 Molecular Docking Studies

Generally, a drug must have suitable structure, both in terms of steric and electrostatic properties, to properly bind with a receptor in order to mediate its activity. Therefore, information on ligand-enzyme interaction at molecular level can be used to assist a drug development process. In this study, molecular docking technique was employed to figure out why compounds **1** and **2** are active while the other compounds are inactive. For this purpose, docking calculations between acetylcholinesterase (see Figure 3.2) and inhibitors were carried out. Moreover, carbosulfan, a general pesticide used in Thai agriculture, was additionally included as reference drug for comparison of the mode of action between this compound and our inhibitors.

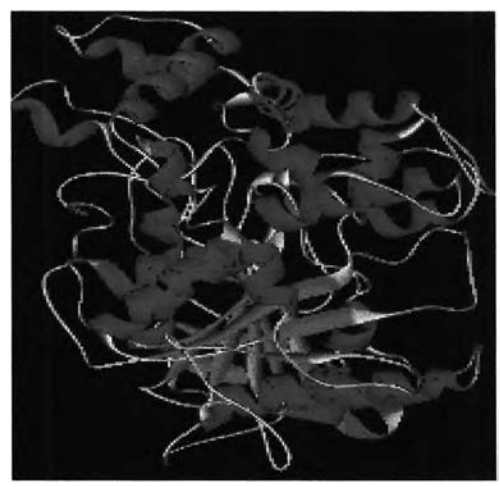


Figure 3.2 Three dimensional structures of acetylcholinesterase

3.6.2.1 Validation of the docking method

In order to validate the method used for our docking calculations, the binding configuration between AChE and tarcine was predicted and subsequently it was superimposed to its X-ray complex structure, taken from the Protein Data Bank (code 1QON). The results were displayed in Figure 3.3 and Table 11 in Appendix A.

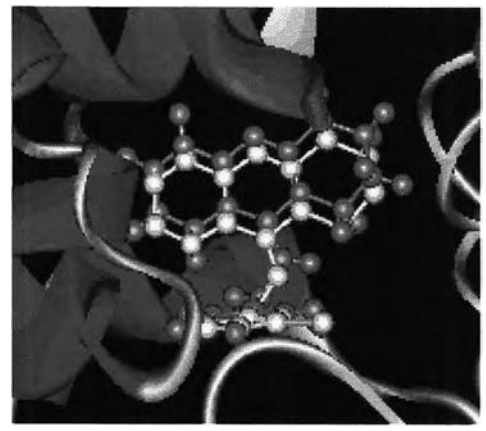


Figure 3.3 Superposition between the docked (violet color) and the X-ray (yellow color) complex structure between AChE and tarcine.

From the docking calculations, the obtained complex structure is very similar to the experiment one, indicating reliability of the method used for our docking calculations.

3.6.2.2 Docking results of carbosulfan and our inhibitors

The docking results between AChE and carbosulfan as well as our inhibitors are presented in Figure 3.4 and Tables 12-14 in Appendix A. Carbosulfan and compounds 1 and 2 can bind to the active site of AChE (see Figure 3.4) while the other alkaloid, *i.e.* guineensine, brachystamide B, brachyamide B and sarmentine, which are inactive compounds, cannot enter inside the active site (data not shown) due to their large molecular size and structural difference, *i.e.* number of hydrocarbon, position of double bond and number of double bond.

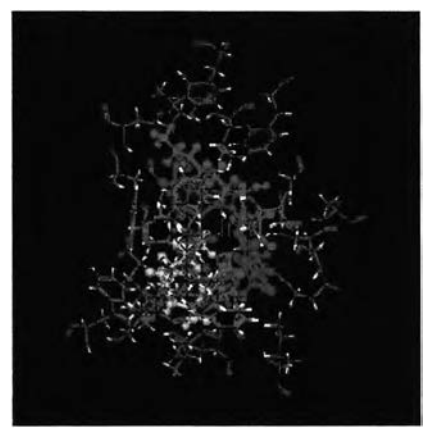


Figure 3.4 Active-site in AChE red-carbosulfan, yellow-pellitorine and green-sylvamine

For carbosulfan, the O2 atom of this compound forms hydrogen bond with the H2 atom of Gly117 with a distance of 1.95 Å. The H2 atom in aromatic compound is located near the O2 atom of Tyr129 with the H2-O2 distance of 1.79 Å. In the pellitorine-AChE complex, the pellitorine was stacked against Trp-83. Pellitorine points its N2 and NH atoms toward HE1 and NE1 atoms of Trp83, respectively, with distances of 2.44 and 2.27 Å. The structure of sylvamine was fitted into the active site of acetylcholineterase. Four aromatic residues, Tyr71, Gly118, Ser205 and Tyr337, in the active site of AChE have interaction with sylvamine. The side chains of these residues were all moved to accommodate the binding of inhibitor. Four hydrogen bonds were found, namely Try71-O2 atom (distance = 2.47 Å), Gly118-N2 atom (distance = 2.18 Å), Ser205-N2 atom (distance = 2.37 Å) and Tyr337-NH atom (distance = 2.13 Å). Both pellitorine and sylvamine have different binding mode from carbosulfan.

Interestingly, pellitorine and sylvamine could be fitted into the active site of AChE and the aromatic side chain of amino acid could form hydrogen bond interaction with pellitorine and sylvamine. The prediction of binding configuration by molecular docking method is reliable as indicated by a comparison with experimentally observed structure. These obtained complex configurations give detail information about drug-receptor interaction at molecular level, which is very helpful to drug development process.

In these studies, possible interactions between carbosulfan-AChE, pellitorine-AChE and sylvamine-AChE were investigated and the docking results indicate that pellitorine and sylvamine could possibly be used as insecticides due to their binding characters with AChE receptor similar to that of carbosulfan, which is a standard insecticide. This study is an example of how the introduction of properly technique to predict drug-receptor complex which can be used to describe a mode of action of insecticides, *i.e.* by blocking the catalytic pocket of the AChE. In conclusion, the docking method could be used to explain why compounds 1 and 2 are active while the others are inactive.