APPENDICES

APPENDIX A

Acquisition of bioactive compounds from TD plant

1. Voucher specimen collection

The voucher specimen collection of TD kept in the herbarium of Faculty of Pharmacy, Chiang Mai University, Thailand as shown in **Figure 63** shows an obtuse angle of branches which is an origin of the species name, *divaricata*. Large green leaves, 6 or more inches in length and 2 inches wide and waxy blossoms with white, five-petal pinwheels, gathered in small clusters on the stem tips can be seen from the voucher specimen.

Figure 63 Voucher specimen of TD at herbarium of Faculty of Pharmacy, Chiang Mai University, Thailand (no. 0010115).

2. Extraction and isolation of TD plant

Weight and % yield of TD crude extract which was obtained from the maceration and TD alkaloidal extract which was obtained from the acid-base extraction are shown in **Table 18**.

Table 18 Weight and %yield of TD crude extract and TD alkaloidal extract.

Materials	Weight (g)	% Yield
Fresh stem of TD	7558.38	-
Dry stem of TD	3361.67	-
TD crude extract	123.30	3.67
TD alkaloidal extract	27.43	0.82

APPENDIX B

Structure elucidation of 3'-*R/S*-hydroxyvoacamine

Chemical structure of 3'-*R/S*-hydroxyvoacamine was elucidated by means of ^{1}H, ^{13}C, DEPT 135, ^{1}H-^{1}H COSY, NOESY, HMQC and HMBC NMR. The NMR spectra of ^{1}H, ^{13}C, DEPT 135, ^{1}H-^{1}H COSY, NOESY, HMQC and HMBC are shown in **Figure 64, 65, 66, 67, 68, 69** and **70**, respectively.

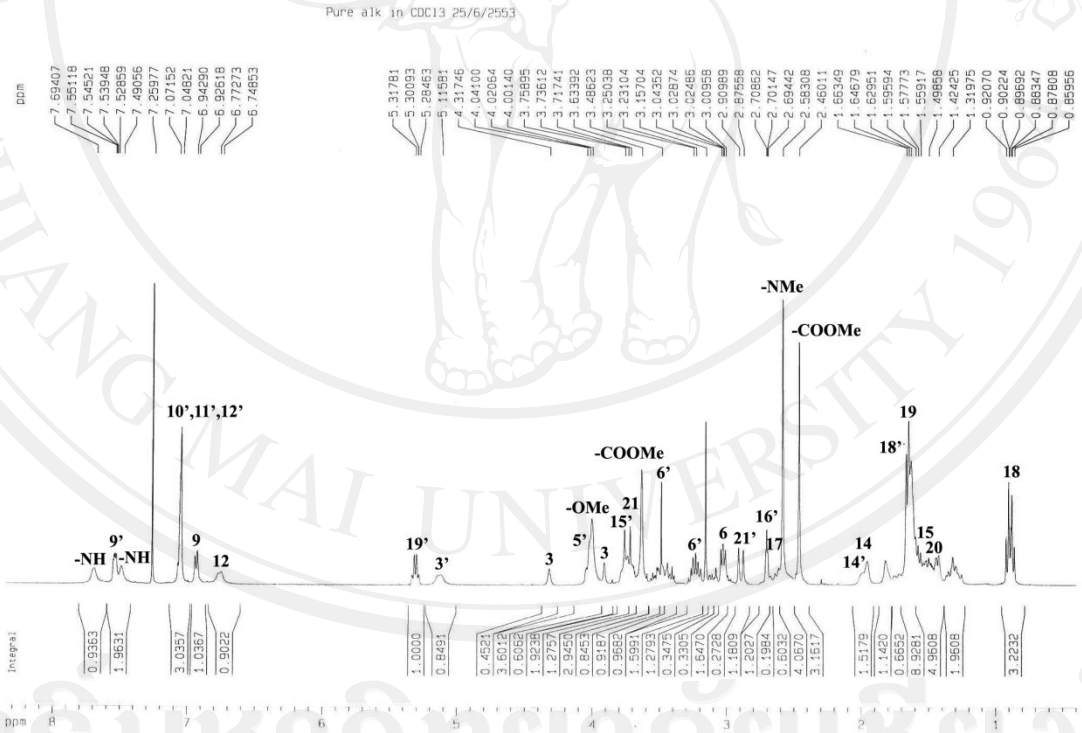


Figure 64 ^{1}H NMR spectra of 3'-*R/S*-hydroxyvoacamine.

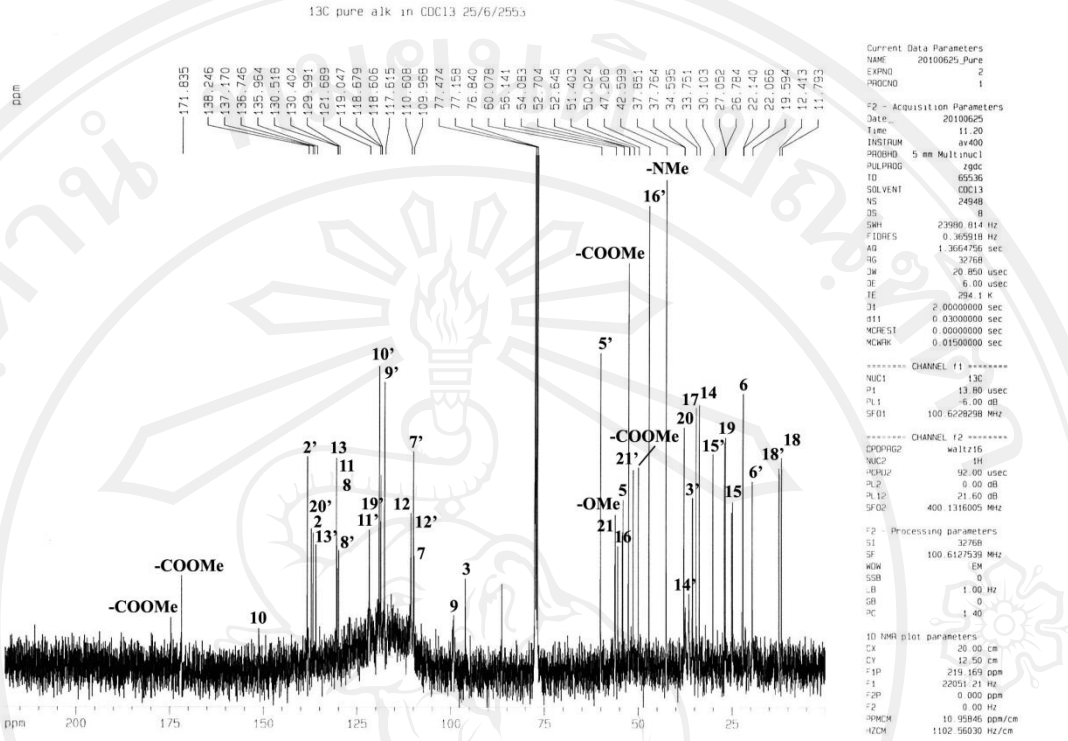


Figure 65 ^{13}C spectra of 3'-*R/S*-hydroxyvoacamine.

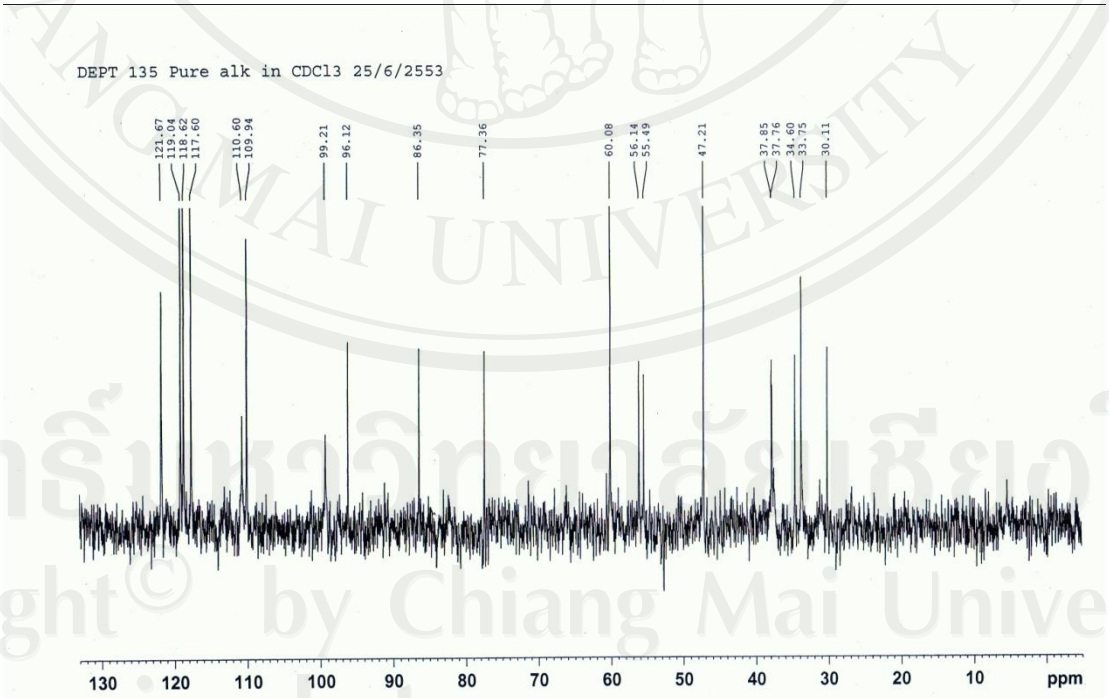


Figure 66 DEPT 135 spectra of 3'-*R/S*-hydroxyvoacamine.

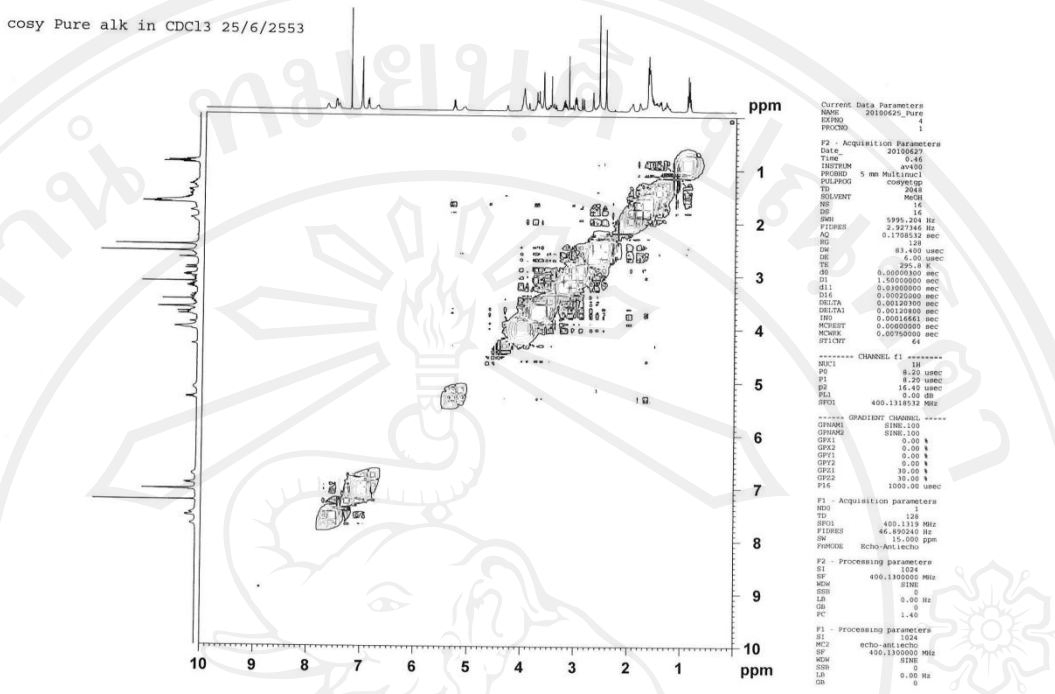


Figure 67 ^{1}H-^{1}H COSY spectra of 3'-*R/S*-hydroxyvoacamine.

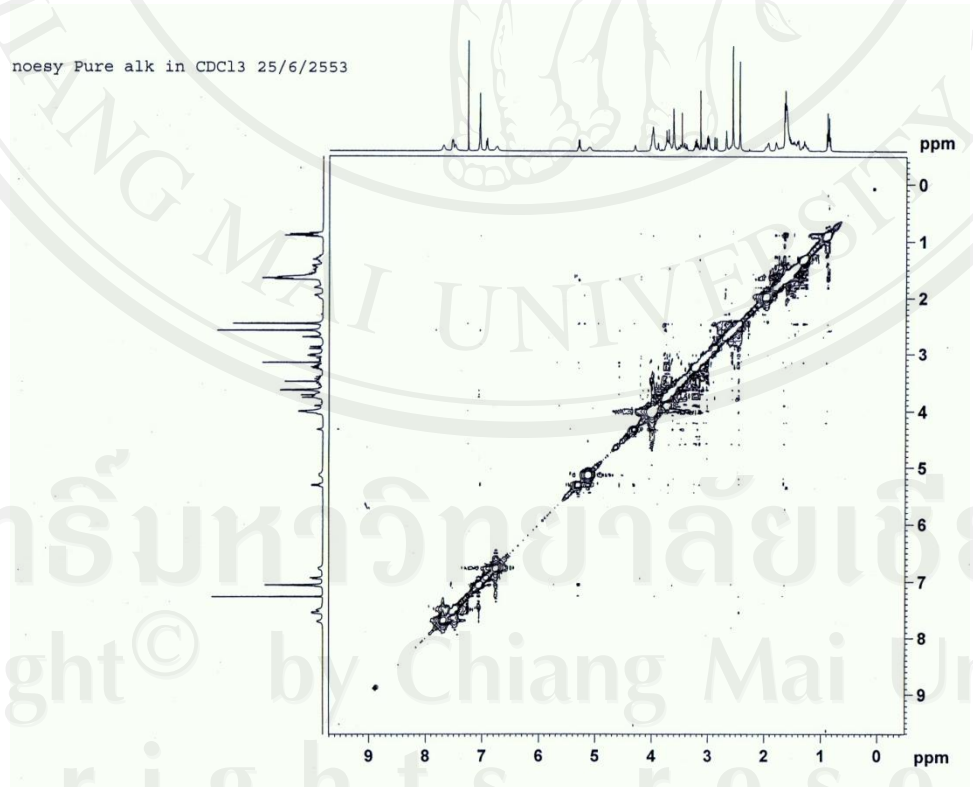


Figure 68 NOESY spectra of 3'-*R/S*-hydroxyvoacamine.

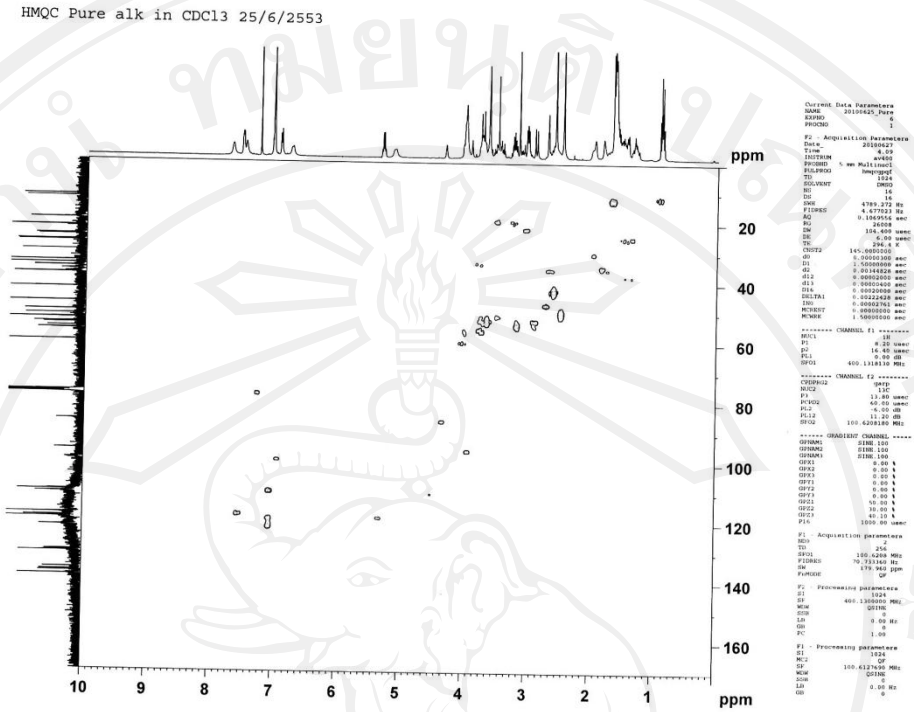


Figure 69 HMQC spectra of 3'-*R/S*-hydroxyvoacamine.

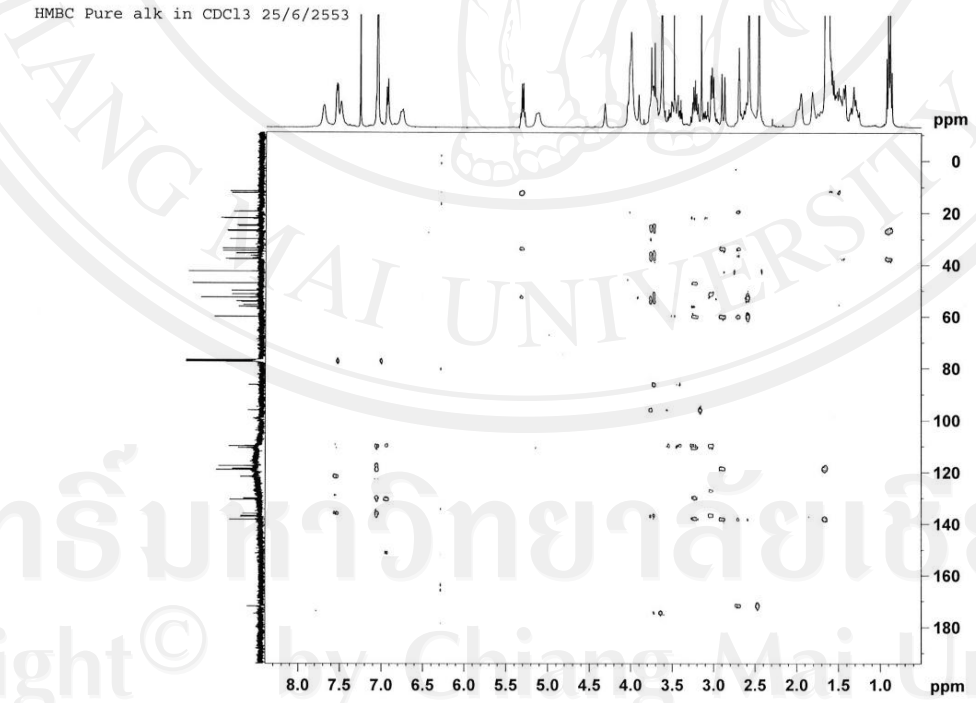


Figure 70 HMBC spectra of 3'-*R/S*-hydroxyvoacamine.

APPENDIX C

In vivo anticholinesterase activity determination

1. Morris water maze test

The male ICR mice were divided into several groups (n = 6) and received either galantamine hydrobromide by oral administration as shown in **Figure 71a** or TD alkaloidal extract by transdermal application as shown in **Figure 71b** for 7 days prior to the start of training. During the training phase, a mouse was allowed to swim in a water maze apparatus to find the hidden platform within 120 s. In case of failing to locate the platform within the criterion period, the mouse was placed on it for 15 s as shown in **Figure 71c.**

(a) (b) (c)

Figure 71 Galantamine hydrobromide in PG (1 mg/kg) was feeding to ICR mouse by oral administration (a), a mouse received TD alkaloidal extract via transdermal application can find the hidden platform within a 120 s (b) and a mouse was placed on a hidden platform for 15 s in case of failing to locate the platform within the criterion period (c).

2. Weight of the animals

Weight of each mouse was recorded during the time of the experiments as shown in **Figure 72.**

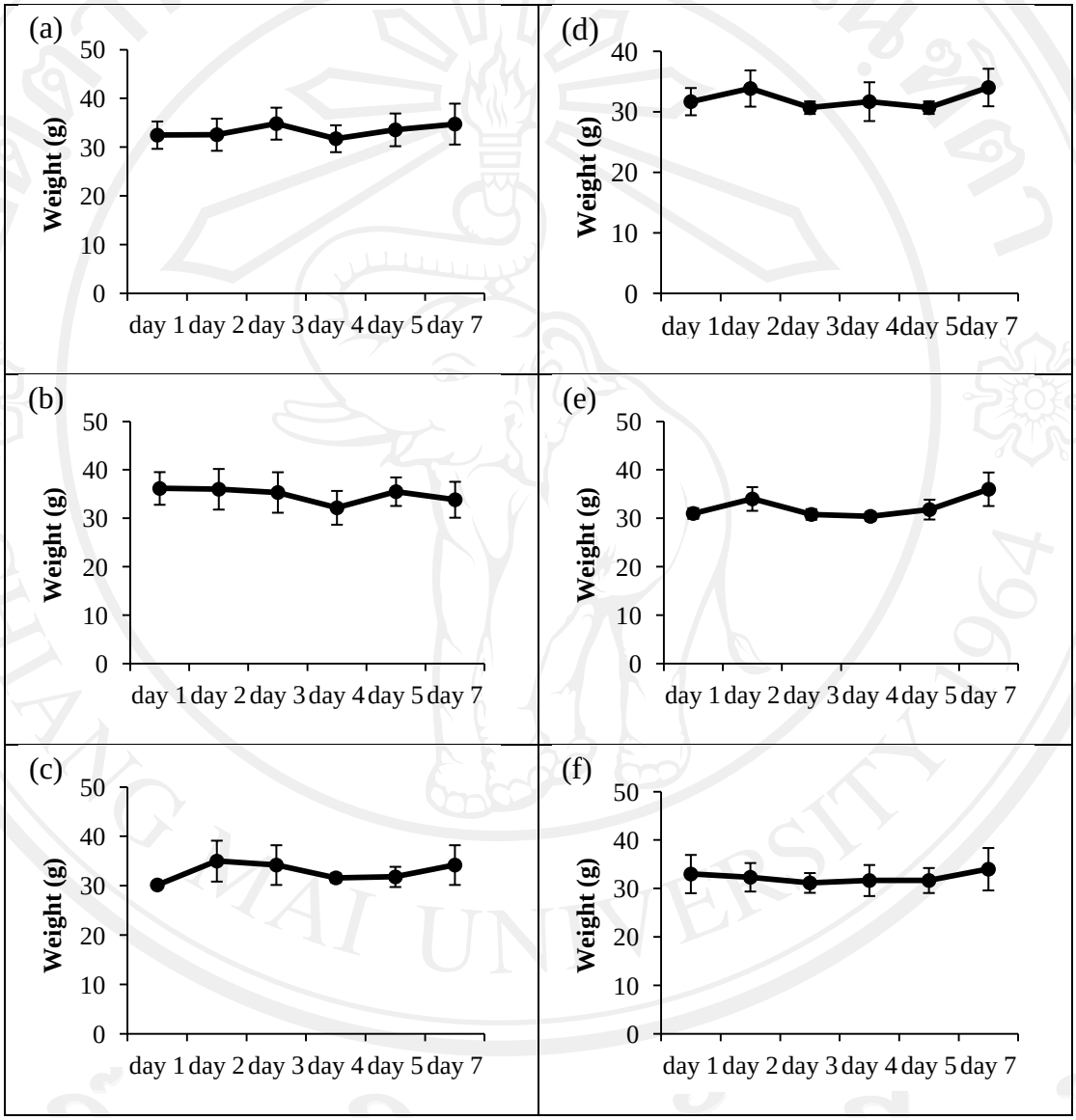


Figure 72 Weight of male ICR mice which received no treatment (a), 1 mg/kg scopolamine hydrobromide with no treatment (b), treatment of 1 mg/kg galantamine hydrobromide (c), treatment of TD alkaloidal extract in the dose of 250 mg/kg (d), 500 mg/kg (e) or 1000 mg/kg (f) during the time of training phase and probe test.

3. Escape latency of control group

Raw data of the escape latency (s) which were investigated in the control group (n = 11) are shown in **Table 19.**

Table 19 Raw data of the escape latency of control group (n = 11).

N	Escape latency(s)			
	Day 1	**Day 2**	**Day 3**	**Day 4**
1.	120	120	120	120
2.	88	32	6	4
3.	83	29	42	33
4.	120	32	7	34
5.	120	55	41	16
6.	120	30	6	7
7.	120	120	120	120
8.	50	42	12	12
9.	120	72	55	10
10.	120	120	120	120
11.	56	27	16	60
Mean	101.59	61.84	49.52	48.70
S.D.	27.63	39.60	48.16	48.51

4. Time in target quadrant of control group

Raw data of the time in target quadrant (s) which was investigated in the mice received no treatment (control), scopolamine and galantamine are shown in **Table 20.**

Table 20 Raw data of the time in target quadrant of control, scopolamine and galantamine group.

N	Time in target quadrant (s)					
	Control		Scopolamine		Galantamine	
	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3
1.	30	25	29	25	56	39
2.	41	43	25	31	40	25
3.	34	30	35	18	30	35
4.	42	33	31	29	35	33
5.	32	30	32	23	36	41
6.	33	17	33	25		
7.	4	15				
8.	33	32				
9.	49	43				
10.	27	30				
11.	34	23				
Mean	32.69	29.37	30.85	25.04	39.53	34.55
S.D.	11.45	9.04	3.38	4.64	9.96	6.24

Raw data of the time in target quadrant (s) which was investigated in the mice received TD alkaloidal extractat the dose of 250, 500 and 1,000 mg/kg are shown in **Table 21.**

Table 21 Raw data of the time in target quadrant of the mice receiving various doses of TD alkaloidal extract.

N	Time in target quadrant (s)					
	TD Alkaloidal extract (mg/kg)					
	250		500		1,000	
	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3
1.	44	37	44	37	38	32
2.	51	33	40	30	35	36
3.	43	30	36	33	32	24
4.	43	33	35	31	29	27
5.	38	25	38	28	25	40
6.	30	25			33	28
Mean	41.29	30.52	38.38	31.82	31.90	31.40
S.D.	6.93	4.88	3.79	3.11	4.66	5.89

APPENDIX D

Essential oils

1. Density

The voucher specimen number of each plant and density of the essential oils are list in **Table 22**.

Table 22 Voucher specimen number and density of essential oils.

Species	Voucher number	Plant part analyzed	Density (g/mL)
Centella asiatica	22107	Whole plant	0.92
Polyscias fruticosa	22108	Leaf	0.81
Eupatorium odoratum	22109	Whole plant	0.90
Cymbopogon citratus	22110	Stem	0.89
Ocimum sanctum	22111	Leaf	0.94
Ocimum canum	22112	Whole plant	0.81
Ocimum gratissimum	22113	Leaf	0.83
Melissa officinalis	22114	Leaf	0.88
Ocimum basilicum	22115	Leaf	0.91
Cinnamomum bejolghota	22116	Leaf	0.80
Piper sarmentosum	22117	Leaf	0.81
Polygonum odoratum Lour .	22118	Whole plant	0.76

Table 22 (Cont.)

Species	Voucher number	Plant part analyzed	Density (g/mL)
Citrus hystrix	22119	Leaf	0.82
		Fruit peel	0.86
Citrus aurantifolia	221120	Leaf	0.81
		Fruit peel	0.84
Citrus maxima	22121	Leaf	0.83
		Fruit peel	0.84
Citrus reticulata Blanco cv. Shogun	22122	Leaf	0.82
		Fruit peel	0.83
Citrus reticulata var. Fremont	22123	Leaf	0.82
		Fruit peel	0.85
Alpinia galanga	22124	Rhizome	0.90
Zingiber officinale	22125	Rhizome	0.86
Zingiber cassumunar	22126	Rhizome	0.90

2. Anticholinesterase activity of essential oils

The dose response curve of *C. citratus* oil and *Z. cassumunar* oil against AChE and BChE are shown in **Figure 73**. IC_{50} value of *C. citratus* oil against AChE was 19.05±0.18 µg/mL while that of BChE was 3.03±0.07 µg/mL. The IC_{50} value of *Z. cassumunar* oil against AChE was 61.91±1.97 µg/mL whereas that of BChE was 2.42±0.55 µg/mL.

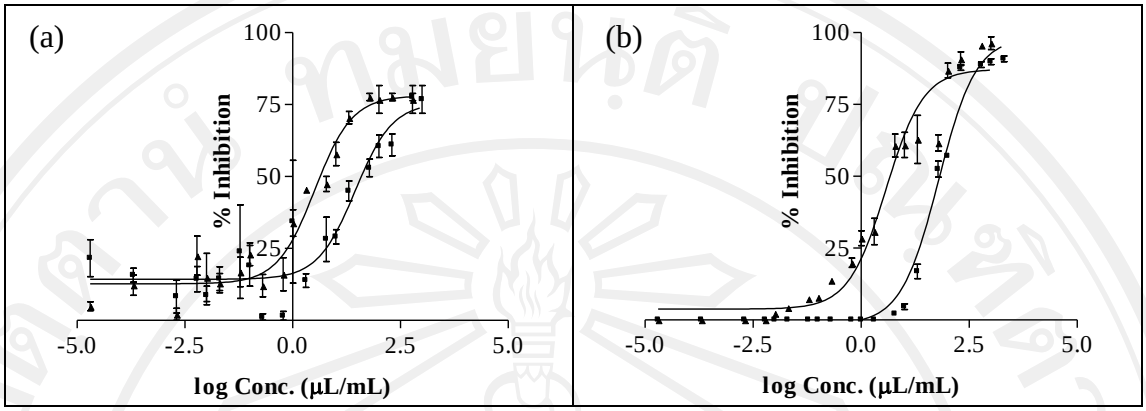
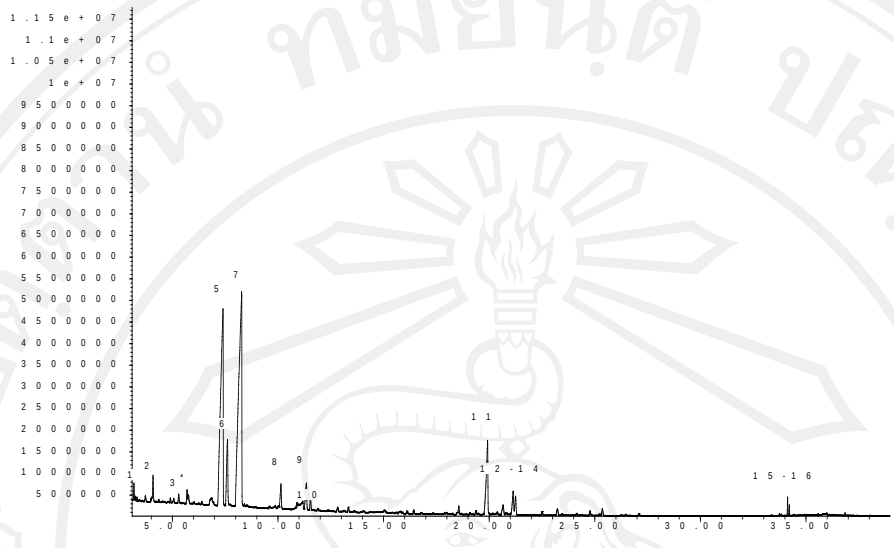


Figure 73 Dose response curve of *C. citratus* oil (a) and *Z. cassumunar* oil (b) against AChE (■) and BChE (▲).

3. GC of essential oils

The composition of the essential oils from *C. citratus* was examined by GC-MS. Twelve components were identified accounting for 89.53% of the total yields. The main components were geranial (42.01%) and neral (32.07%) which can be seen in **Figure 74** as a peak number 7 and 5, respectively. The results were in a good agreement with the previous reports that 75% (GC analysis) of *C. citratus* oil was composed of citral (3,7-dimethyl-2,6-octadienal) which is the name given to a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial (*trans*-citral, citral A) and neral (*cis*-citral, citral B) (166-168). On the other hand, the GC chromatogram of *Z. cassumunar* oil is shown in **Figure 75**. Fourteen components were identified accounting for 98.49% of the total yields. Terpinen-4-ol (55.88%) and gamma-terpinene (11.05%) which can be seen as peak number 8 and 2 were major constituents. The results were in a good agreement with the previous reports (169-170)

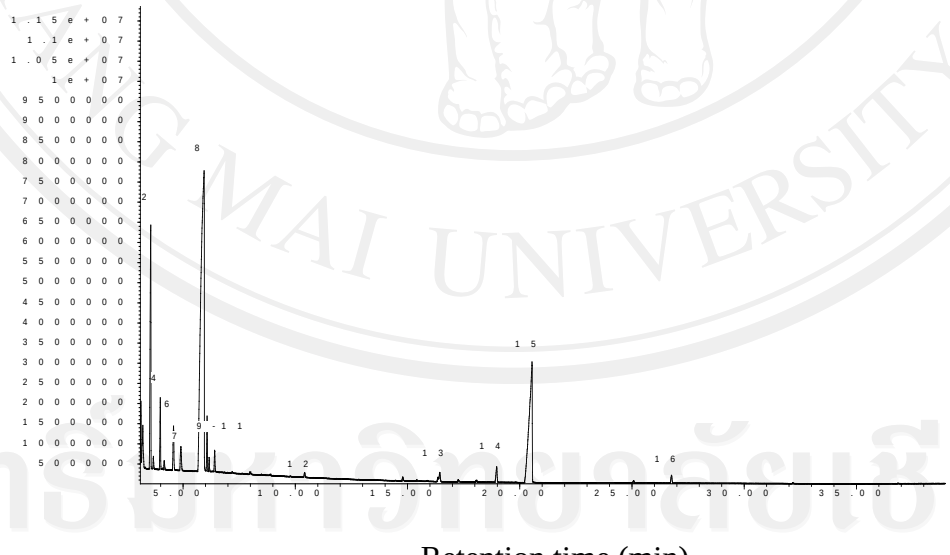
Relative abundance



Retention time (min)

Figure 74 GC chromatograms of *C. citratus* oil.

Relative abundance



Retention time (min)

Figure 75 GC chromatograms of *Z. cassumunar* oil.

APPENDIX E

Microemulsion characterizations

The chart summarizing the characterizations of TD alkaloidal extract loaded microemulsions along dilution line A and B are shown in **Figure 76** and **Figure 77**, respectively. The characterizations include physical appearance, PLM, viscosity, DSC, conductivity and FF-TEM.

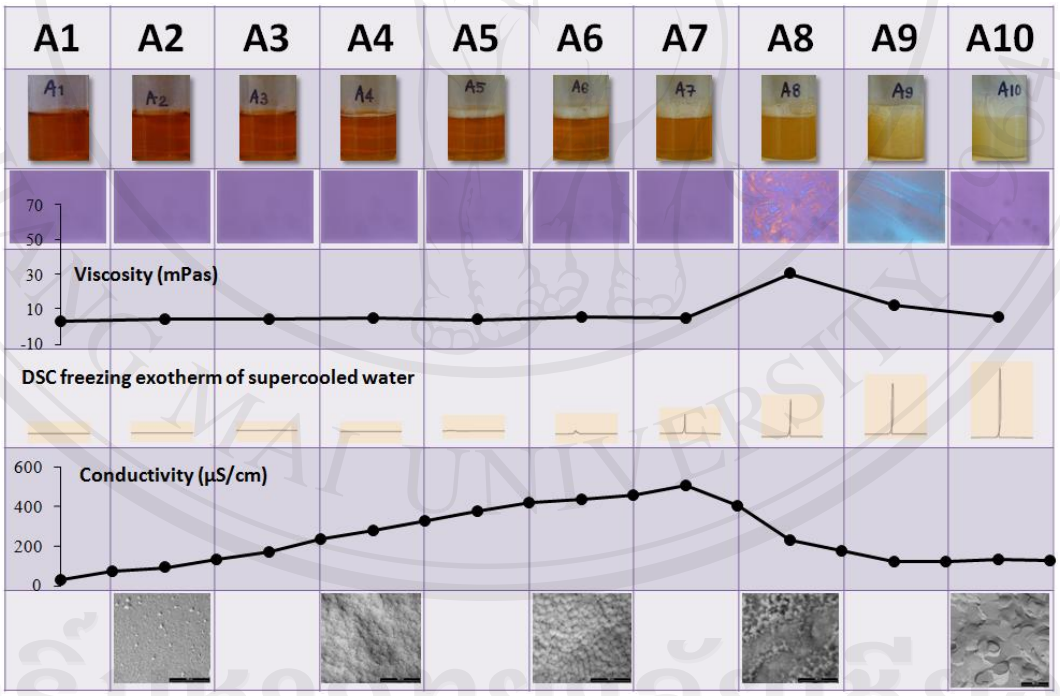


Figure 76 Summary chart of characterizations of TD alkaloidal extract loaded microemulsions along dilution line A.

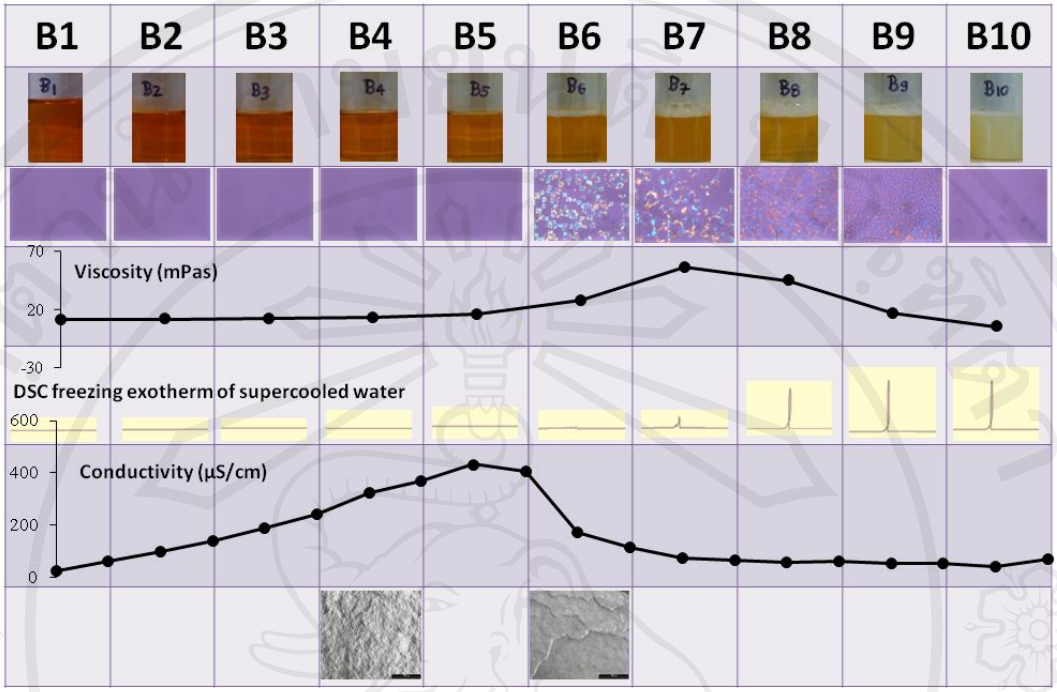


Figure 77 Summary chart of characterizations of TD alkaloidal extract loaded microemulsions along dilution line B.

APPENDIX F

Buffer and reagent preparation

1. Preparation of acetate buffer pH 3.0

1. Prepared 0.1 M of glacial acetic acid (CH_3COOH, MW = 60.05) by dissolving 6.00 g of glacial acetic acid in 1,000 mL DI water

2. Prepared 0.1 M of sodium acetate anhydrous (CH_2COONa, MW = 82.03) by dissolved 8.20 g of CH_2COONa in 1,000 mL DI water

3. Mixed 982.3 mL of 0.1 M of glacial acetic acid with 17.7 mL of 0.1 M sodium acetate

1. Preparation of 0.5 mM PBS pH 7.4 (for HPLC)

1. Dissolved 0.07 g of disodium hydrogen phosphate anhydrous (Na_2HPO_4, MW = 141.96) in 800 mL DI water

2. Adjusted pH by orthophosphoric acid to 2.5

3. Adjusted pH by ammonia solution to 7.4

4. Adjusted volume by DI water to 1,000 mL

2. Preparation of PBS pH 7.4 (for cell experiment)

Dissolved 0.20 g of potassium chloride (KCl, MW = 74.55), 8.00 g of sodium chloride (NaCl, MW = 58.44), 1.44 g of disodium hydrogen phosphate (Na_2HPO_4,

MW = 141.96) and 0.24 g of dipotassium hydrogen phosphate (K_2HPO_4, MW = 174.18) in 1,000 mL of DI water

3. Preparation of 50mM Tris-HCl buffer pH 8.0

1. Dissolved 7.88 g of Tris(hydroxymethyl)aminomethane hydrochloride ($C_4H_{11}NO_3$.HCl, MW = 157.6) in 800 mL milli-Q water

2. Adjusted pH by hydrochloric acid to 8.0

3. Adjusted volume by milli-Q water to 1,000 mL

4. Preparation of Ellman's reagent (3.0 mM 5,5′-Dithiobis(2-nitrobenzoic acid))

Dissolved 1.19 g of 5,5′-Dithiobis(2-nitrobenzoic acid) ($C_{14}H_9N_2O_8S_2$, MW = 396.36) in 1,000 mL of 50mM Tris-HCl buffer pH 8.0

5. Preparation of Dragendorff's reagent

1. Prepared solution A by dissolving 0.17 g of bismuth nitrate ($Bi(NO_3)_3.5H_2O$, MW = 485.11) in 2 mL glacial acetic acid and 8 mL DI water

2. Prepared solution B by dissolving 4.00 g of potassium iodide (KI, MW = 166.00) in 10 mL glacial acetic acid and 20 mL DI water

3. Mixed solution A and solution B

4. Adjusted volume by DI water to 100 mL

CURRICULUM VITAE

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Scholarship	Royal Golden Jubilee Grant 2008

List of publications

Journals

1. **Wantida Chaiyana**, Kiatisak Saeio, Wim E. Hennink, Siriporn Okonogi. Characterization of potent anticholinesterase plant oil based microemulsion. International Journal of Pharmaceutics. 2010;401:32-40.
2. Kiatisak Saeio, **Wantida Chaiyana**, Siriporn Okonogi. Antityrosinase and antioxidant activities of essential oils of edible Thai plants. Drug Discovery & Therapeutics. 2011;5:144-149.
3. **Wantida Chaiyana**, Siriporn Okonogi. Inhibition of cholinesterase by essential oil from food plant. Phytomedicine. 2012;19:836-839.
4. Siriporn Okonogi, **Wantida Chaiyana**. Enhancement of anti-cholinesterase activity of *Zingiber cassumunar* essential oil using a microemulsion technique. Drug discoveries & therapeutics. 2012;6,249-255.

5. **Wantida Chaiyana**, Jan Schripsema, Kornkanok Ingkaninan, Siriporn Okonogi. 3´-*R/S*-Hydroxyvoacamine, a potent acetylcholinesterase inhibitor from *Tabernaemontana divaricata*. Phytomedicine. 2013;20,543-548.
6. **Wantida Chaiyana**, Siriporn Okonogi. Zingiber essential oil microemulsion: Chracterization and anticholinesterase activity. Drug Development and Industrual Pharmacy. (Submitted)
7. **Wantida Chaiyana**, Wirat Niwatananun, Siriporn Okonogi. Inhibition of acetylcholinesterase by alkaloidal extract from *Tabernaemontana divaricata* stem: *in vitro* and *in vivo*. Journal of Ethnopharmacology. (Submitted)
8. **Wantida Chaiyana**, Thomas Rades, Siriporn Okonogi. Characterization and *in-vitro* permeation study of microemulsions loaded with potent anticholinesterase alkaloidal extract from *Tabernaemontana divarivata* stem. (Submitted)

Presentations

1. S. Okonogi, **W. Chaiyana**, K. Saeio, S. Yotsawimonwat, W. Niwatananun. Factors affecting phase behavior of microemulsions comprising *Cymbopogon citratus* oil. XVII International Conference on Bioencapsulation, 24-26 September 2009, Groningen, Netherlands. (Poster).
2. S. Okonogi, K. Saeio, **W. Chaiyana**, S. Yotwimonwat, W. Niwatananun. Encapsulation of sesame oil by microemulsion technique: Study of phase diagram. XVII International Conference on Bioencapsulation, 24-26 September 2009, Groningen, Netherlands. (Poster).
3. **W. Chaiyana**, K. Saeio, K. Inkaninan, W.E. Hennink, S. Okonogi. Encapsulation of potent anticholinesterase essential oil by microemulsion technique: Study of

phase diagram. The Twelfth RGJ-Ph.D. Congress (RGJ-Ph.D. Congress XII), 1-3 April 2011, Jomtien Palm Beach Hotel and Resort, Pattaya, Chonburi, Thailand. (Poster).

4. **W. Chaiyana**, S. Okonogi. Synergistic effect of essential oils from *Citrus* plants on degenerative disease of ageing. 7th Asia Pacific Conference on Clinical Nutrition, 5-9 June 2011, Bangkok, Thailand. (Poster).
5. **W. Chaiyana**, S. Okonogi. Microemulsion of *Zingiber cassumunar* Roxb. oil and its anticholinesterase activity. RGJ Seminar Series LXXXIV - Research and Innovation in Chemistry for Sustainable Development, 2 September 2011, Chiang Mai, Thailand. (Poster with outstanding presentation award).
6. **W. Chaiyana**, T. Radse, S. Okonogi. Formulation of *Zingiber cassumunar* Roxb. essential oil in microemulsions: Study of phase diagram and anticholinesterase activity. 14th Conference on Formulation and Delivery of Bioactives, 16-17 February 2012, Dunedin, New Zealand. (Poster).
7. **W. Chaiyana**, W. Niwatananun, S. Okonogi. Inhibition of acetylcholinesterase by alkaloidal extract from *Tabernaemontana divaricata* stem: *in vitro* and *in vivo*. RGJ Seminar Series LXXXIX - Molecular Mechanisms and Technology Developments in Biomedical Researches, 31 August 2012, Chiangmai, Thailand. (Poster).
8. **W. Chaiyana**, T. Rades, W.E. Hennink, K. Ingkaninan, W. Niwatananun, C. Ampasavate, S. Okonogi. Development of Nanodelivery System of Thai Medicinal Plants for Treatment of Alzheimer's Disease. Research Path: The Path to the Future, 22-23 November 2012, Chiangmai, Thailand. (Poster).

9. **W. Chaiyana**, W. Niwatananun, S. Okonogi. *In vivo* study of memory improvement ability of *Tabernaemontana divaricata* alkaloidal extract and *in vitro* skin permeation study of the extract from various types of microemulsions. The XVII International Congress Phytopharm 2013, 8-10 July 20132, Vienna, Austria. (Oral)
10. S. Okonogi, **W. Chaiyana**. Development of microemulsions containing anticholinesterase extracts from plants. The XVII International Congress Phytopharm 2013, 8-10 July 20132, Vienna, Austria. (Oral)