CHAPTER 3

MATERIALS AND METHODS

Part I Acquisition of bioactive compounds from TD plant

1. Materials

Plant material *Tabernaemontana divaricata*, (Thailand)

Solvent Deuterated dichloromethane (AR grade), Ethanol (AR grade), Methanol (AR grade), Merck (Darmstadt, Germany)

Ethyl acetate (AR grade), Labscan (Dublin, Ireland)

Dichloromethane (AR grade), Methanol (HPLC grade), Fisher Chemicals (Loughborough, UK)

Reagent 5,5'-dithiobis-(2-nitrobenzoic acid), Acetylthiocholine iodide, Bismuth nitrate, Potassium iodide, Sigma-Aldrich, (St. Louis, MO, USA)

Buffering agents Disodium hydrogen phosphate, Sodium acetate, Sodium bicarbonate, Fisher Chemicals (Loughborough, UK)

Tris(hydroxymethyl)aminomethane hydrochloride, Sigma-Aldrich (St. Louis, MO, USA)

Acid Glacial acetic acid, Hydrochloric acid, Orthophosphoric acid, Merck (Darmstadt, Germany)

Base Ammonia solution, Merck (Darmstadt, Germany)

Enzyme Acetylcholinesterase from *Electrophorus electricus* (electric eel) E.C. 3.1.1.7, Sigma-Aldrich (St. Louis, MO, USA)

2. Methods

2.1 Identification of TD plant

TD was be identified in accordance with four typical characteristics including evergreen shrub forms shaped like symmetrical mounds 6-feet high, horizontal branches having the appearance of an attractive, almost horizontal shrub (the species name, *divaricata*, means an obtuse angle), large, shiny, deep 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 (29).

Voucher specimen collection of TD in the herbarium was done in order to increases the security of the initial identification and the specimen can be identified by comparison with other herbarium specimens (53).

2.2 Extraction and isolation of TD plant

There were two steps of TD extractions including maceration to obtain TD crude extract and acid-base extraction to obtain the alkaloidal extract.

2.2.1 Maceration

The dried powder of TD stems was macerated with 95% ethanol for 3 days. After filtration, the filtrate was collected and the residue was macerated again with 95% ethanol for 3 days. Finally, total filtrate was evaporated under reduced pressure until dryness to give TD crude extract.

2.2.2 Acid-base extraction

The TD crude extract was dissolved in acetate buffer (pH 3) and washed with ethyl acetate. The aqueous part was basidified with sodium bicarbonate solution until pH 10 and be extracted with ethyl acetate. The organic layer was dried under reduced pressure to give the alkaloidal extract.

2.3 Quantitative and fingerprint analyses of TD alkaloidal extract

In recent years, significant efforts have been made to devise methods for the quality control of plant materials by utilizing quantitative methods and/or qualitative fingerprinting technologies (54). The quality of TD alkaloidal extract can be assessed by the content of multiple compounds. TLC and HPLC were used as fingerprinting methods for the quality evaluation (55). Moreover, the relative retention time and relative peak area of the characteristic peak were also constructed for quantitative measurement of the TD alkaloidal extract.

2.3.1 Fingerprint analysis

2.3.1.1 TLC

Because of variations in the composition of extracts, as well as differences in culturing methods and climate, individual TD may differ in their bioactivity. Therefore, TLC accompanied with Ellman's reagent was used to confirm its composition and bioactivity (56). After the TLC plate was developed in developing solvent which were dichloromethane : methanol (9:1), it was observed in daylight, UV at 254 nm and UV at 366 nm. After that it was sprayed with Dragendorff's

reagent and then observed in daylight. The positive result which referred to the alkaloid was seen as an orange spot on the yellow background. To determine the bioactivity, after the TLC plate was developed with dichloromethane : methanol (9:1), it was sprayed with 30 mM acetylthiocholine iodide (ATCI), 8 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and AChE, respectively. The plate was then observed in daylight. The positive result which referred to the inhibitor was observed as a white spot on the yellow background.

2.3.1.2 HPLC

Briefly, HPLC analyses were performed using an HP1100 system with a thermostatically controlled column oven and a UV detector set at 295 nm (Hewlett-Packard, Palo Alto, CA). A reversed phase column Inertsil® ODS-2 (250 × 4.6 mm i.d., 5 µm, GL Sciences Inc., Japan) was connected with a guard column (125 × 4.6 mm i.d., 5 µm, Algilent, USA). A mixture of methanol and phosphate buffer solution (pH 7.4) in the ratio of 86:14 was used to elute samples at ambient temperature at a flow rate of 1 mL/min and 20 µL of sample was injected. Samples and mobile phases were filtrated through a 0.45 µm Millipore filter, type GV (Millipore, Bedford, MA) prior to HPLC injection.

2.3.2 Quantitative analysis

2.3.2.1 Acquisition of HPLC marker

Quantitative analysis aims to separate and identify the marker from TD alkaloidal extract and then use it as indicators or standards to assess the quantity.

2.3.2.1.1 Purification of marker

Gel filtration (Sephadex®) column with 2.0 cm diameter and 150 cm length was used to isolate pure marker when the mobile phase is 100% methanol. Each fraction collected from the column was evaporated on the 50°C water bath. The purity of isolated marker was checked by TLC accompanied with Ellman's reagent and HPLC.

2.3.2.1.2 Structure elucidation

The ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, NOESY, HMQC and HMBC NMR experiments were carried out using a Bruker Avance 400 NMR spectrometer (Bruker, Germany), operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The electrospray ionization time-of-flight mass spectrometry (ESITOFMS) spectra were obtained using a Micromass LCT mass spectrometer (Water, USA).

3'-*R/S*-Hydroxyvoacamine - $C_{43}H_{52}N_4O_6$; $[M-H]^-$ 719; uv λ max (EtOH) nm 200, 224, 295; ms *m/z* (%) 719 (10), 704 (99), 660 (29), 637 (23), 394 (90), 353 (100), 337 (98), 321 (37), 307 (39), 280 (42); ^{1}H and ^{13}C nmr see **Table 6**.

2.3.2.2 Validation of HPLC analysis

Evaluation of the suitability of HPLC analytical procedure with UV/VIS detection was done in the terms of interday-precision, intraday-precision, accuracy, LOD, LOQ and linearity of calibration curves.

2.3.2.2.1 LOD and LOQ

LOD and LOQ of purified alkaloid were investigated using the signal to noise ratio (S/N) of the chromatogram. The purified alkaloid solution was dilute until the S/N is 3:1 and LOD was established whereas LOQ was established when S/N were 10:1.

2.3.2.2.2 Calibration curve

For the calibration curve of purified alkaloid, six solutions in different concentrations were prepared where the smallest concentration is the concentration of LOQ. All solutions were injected twice. Three calibration curves were done and the average area under the curve (AUC) of each concentration was plotted.

2.3.2.2.3 Intraday precision

For the intraday precision, six concentrations of 2.5, 5, 10, 20, 40 and 80 µg/mL were prepared and each concentration was prepared in triplicate. Therefore, there are totally 18 samples. Each sample was injected twice on the same day. % R.S.D. was then calculated by the following equation; % R.S.D. = $(S.D./\bar{x}) \times 100$, when S.D.is standard deviation and $\bar{x}$ is mean of the measured AUC.

2.3.2.2.4 Intermediate precision

For the intermediate precision, purified alkaloid was injected on three different days. A fresh solution was prepared every day with exact concentrations, each on a separate day. Every solution was injected six times. % R.S.D. was then calculated by

the following equation; % R.S.D. = (S.D./$\bar{x}$) × 100, when S.D.is standard deviation and $\bar{x}$ is mean of the measured AUC.

2.3.2.2.5 Accuracy

For the accuracy, a recovery experiment was performed. The actual amount of purified alkaloid was added to TD alkaloidal extract. The solutions were prepared in triplicate and each solution was injected twice. Moreover, the TD alkaloidal extract alone was analyzed using the same method. % Recovery was then calculated by the following equation; % Recovery = (quantity measured/quantity expected) × 100.

<u>Part II</u> Anticholinesterase activity determination

1. **Materials**

1.1 *In vitro* assay

Enzyme Acetylcholinesterase from *Electrophorus electricus* (electric eel) E.C. 3.1.1.7, Butyrylcholinesterase from equine serum E.C. 3.1.1.8, Sigma-Aldrich (St. Louis, MO, USA)

Chemicals Galantamine hydrobromide, Sigma-Aldrich (St. Louis, MO, USA)

Reagent 5,5'-Dithiobis(2-nitrobenzoic acid), Acetylthiocholine iodide, Butyrylthiocholine iodide, Sigma-Aldrich (St. Louis, MO, USA)

Buffering agents Tris(hydroxymethyl)aminomethane hydrochloride, Sigma-Aldrich (St. Louis, MO, USA)

Acid Hydrocholric acid, Merck (Darmstadt, Germany)

Solvent Methanol (AR grade), Merck (Darmstadt, Germany)

1.2 *In vivo* assay

Animals Male ICR mice, the National Laboratory Animal Center (Nakorn Pathom, Thailand)

Chemicals Galantamine hydrobromide, Scopolamine hydrobromide, Sigma-Aldrich (St. Louis, MO, USA)

Solvent Propylene glycol, Sigma-Aldrich (St. Louis, MO, USA)

Method

2.1 *In vitro* assay

Ellman's method with slight modification was used to evaluate the ChE activity (5, 57). Two types of cholinesterase, electric eel AChE and horse serum BChE enzymes, were used whereas ATCI and butyrylthiocholine iodide (BTCI) were employed as enzyme substrates of the reactions, respectively. Briefly, 50 µL of 50 mM Tris-HCl buffer, 25 µL of 1.5 mM ATCI, 125 µL of 3 mM DTNB, pH 8.0, and 25 µL of sample dissolved in buffer containing not more than 10% methanol were added to each well of the microplate and followed by 25 µL of 0.25 U/mL AChE. The microplate was then read at 415 nm every 7 s for 2 min after 1 min of moderate shaking. To evaluate the activity of BChE, 0.91 U/mL BChE was used as an enzyme whereas BTCI was used as a substrate. Galantamine hydrobromide was used as a standard inhibitor. The mean velocity of reactions was recorded. All of the experiments were done in triplicate. The cholinesterase inhibitory activity was then calculated using the following equation; $E = 1-(Vs/Vb)$, where *Vs* is the mean reaction rate in the presence of a certain concentration of the extract and *Vb* is the mean reaction rate in absence of the extract. IC_{50} values were statistically evaluated using the graphpad/prism program. The inhibitory effects of the samples on AChE and BChE were presented as % inhibition and IC_{50} values.

2.2 *In vivo* assay

For *in vivo* determination of the learning ability, rodents were used as animal model under MWM test since the swimming ability is related to learning rate (58).

2.2.1 Animals

Three-week old male ICR mice were obtained from the National Laboratory Animal Center, Nakorn Pathom. The mice were housed in groups of 6 in a controlled room (25±2°C, a 12h dark-light cycle) and allowed food and water *ad libitum*. They were acclimatized at least 1 week before starting the experiments. All experiments reported herein were reviewed and approved by Animal Ethics Committees, Faculty of Medicine, Chiang Mai University, Thailand.

2.2.2 Study design

The male ICR mice employed in this study were divided into 4 groups (n = 6) and received either TD alkaloidal extract in the dose of 250, 500 or 1000 mg/kg per day by transdermal application or 1 mg/kg galantamine hydrobromide (Sigma-Aldrich, USA) by oral administration for 7 days prior to the start of training. While the control group (n = 11) received no treatment. All treatments were also administered 1 hr prior to training session and probe test. During the training phase and probe test, the mice received an intraperitoneal (i.p.) injection of 1.0 mg/kg scopolamine 30 min prior to the swimming session (59).

2.2.3 MWM test

The water maze apparatus consisted of a circular pool (1 m diameter, 45 cm high) filled with water rendered opaque by additional of the powder. A platform (10 cm in diameter) constructed from white plastic was submerged 1.0 cm below the water surface in order to hide from the mice during the training phase.

2.2.3.1 Training phase

The training procedure has been carried out as described previously by Hirst *et al*. (60). Each trial started with the mice were placed facing the wall of the maze and the mouse was allowed to explore the maze to find the hidden platform. The time taken to find the platform, with a 120 s criterion period, was defined as the escape latency time. In case of failing to locate the platform within the criterion period, the mice were placed on it for 15 s. Animals were trained two trials a day for 4 consecutive days.

2.2.3.2 Probe test

Recall of the platform position was assessed by a probe test, at 1 and 3 days following the final training session, in which the platform was removed and animals were allowed to explore the maze for 60 s. The time spent in target quadrant, where the platform used to be located, was recorded and used to compare recall of the platform position by each treatment group.

<u>Part III</u> Preformulation study

1. **Materials**

Solvents	Dimethyl sulfoxide (AR grade), Ethanol (AR grade), Methanol (AR grade), Merck (Darmstadt, Germany)
	Acetone (AR grade), Acetonitrile (AR grade), Ethyl acetate (AR grade), Hexane (AR grade), Labscan (Dublin, Ireland)
	Butanol (AR grade), Chloroform (AR grade), Dichloromethane (AR grade), Diethyl ether (AR grade), Isopropanol (AR grade), Octanol (AR grade), Fisher Chemicals (Loughborough, UK)
Cosolvent	Glycerin, BP/USP (Malaysia)
	Polyethylene glycol 200, Polyethylene glycol 300, Polyethylene glycol 400, Polyethylene glycol 600, USP (Wilhelmshaven, Germany)
	Propylene glycol, Sigma–Aldrich (St. Louis, MO, USA)
Surfactants	Span 80, Tween 20, Tween 80, Acros Organics (New Jersey, USA)
Fats and oils	Cetyl alcohol, USP (Tokyo, Japan)
	Isopropyl myristate, USP (Jurong Island, Singapore)
	Liquid paraffin, BP/USP (Mumbai, India)
	Stearyl alcohol, BP/USP (Tokyo, Japan)
Biologicals	Fetal bovine serum (FBS), Biochrom AG (Berlin, Germany), Human blood, Human serum, (volunteers in Chiangmai, Thailand)

Biologicals	Ficoll-paque plus, Lymphoprep™ Axis-Shield PoC AS, (Oslo, Norway)
Buffering agents	Disodium hydrogen phosphate, Dipotassium hydrogen phosphate, Potassium chloride, Sodium chloride, Fisher Chemicals (Loughborough, UK)
Reagents	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), Sigma–Aldrich (St. Louis, MO, USA)
Media	RPMI 1640, GIBCO™ Invitrogen (Grand Island, NY, USA)
Chemicals	Penicillin, Streptomycin, Trypan blue, GIBCO™ Invitrogen (Grand Island, NY, USA)

2. Method

2.1 Solubility study

The solubility of TD alkaloidal extract in various solvents was done. Each solvent was added into the exact amount of the extract and stir until the extract was completely dissolved. The amount of solvent was recorded and the solubility was finally calculated by the following equation. The solubility definition was then given according to **Table 3**.

$$\text{solubility} = \frac{\text{weight of solvent (g)}}{\text{weight of TD alkaloidal extract (g)}}$$

Table 3. Solubility definition

Descriptive term	**Solubility (weight of solvent per 1 g of TD alkaloidal extract)**
Very Soluble	Less than 1
Freely Soluble	1-10
Soluble	10-30
Sparingly Soluble	30-100
Slightly Soluble	100-1000
Very Slightly Soluble	1000-10,000
Practically Insoluble	More than 10,000

2.2 Partition coefficient study

Octanol-water, in the ratio of 1:9 (v/v), were mixed and allowed to presaturate for 48 hr at room temperature. An accurately weighed aliquot of the TD alkaloidal extract was added to the presaturated octanol-water mixture and allowed to equilibrate for 48 hr at room temperature with shaking. After equilibration, samples was carefully drawn from the aqueous phase and analyzed by HPLC. The concentration of the TD alkaloidal extract in the octanol phase was calculated assuming all drug partitioned into either the water or octanol phase after equilibration. The partition coefficient was finally calculated according to the following equation (61).

$$\log P_{\text{octanol/water}} = \log\left(\frac{\text{concentration of TD alkaloidal extract in octanol}}{\text{concentration of TD alkaloidal extract in water}}\right)$$

2.3 Thermal analysis

2.3.1 TGA

TA Instruments Hi-Res TGA 2950 thermogravimetric analyser was used and calibrated for weight and temperature (alumel and nickel). Nitrogen was used as a purge gas at a flow rate of 100 mL/min. The 3-6 mg samples were heated in open aluminium pans to 300°C and data was analyzed using Universal Analysis 2000 software.

2.3.2 DSC

DSC was carried out to analyze thermal behavior of TD alkaloidal extract. Accurately weighed aliquots of the extract were placed in standard aluminum DSC

sample pans, which were sealed by crimping, with a lid. Pans containing the samples were placed in the sample cell of the DSC, and similarly sealed empty pans were placed in the reference cell of the DSC. DSC thermograms was obtained by heating the samples from 10°C to 300°C at a linear scanning rate of 10°C/min under a constant nitrogen purge (61).

2.4 Stability study

The TD alkaloidal extract was stored in closed containers at various conditions including exposure to light, exposure to oxygen or normal condition in the temperatures of 4°C, 30°C, and 45°C for 0, 7, 30, 60, 120 and 180 days. Changes in physical appearances such as physical status and color were investigated by visualization and the amount of the extract was determined using HPLC. The inhibitory activities against AChE and BChE were also investigated by Ellman's assay as previous described. **Table 4** shows the factors involved in the stability study.

Table 4. The stability conditions.

Conditions	Light	Oxygen	Temperature
Control	No	No	Room temperature
Light protected condition	No	Yes	Room temperature
No oxygen condition	Yes	No	Room temperature
Cold condition	No	No	4°C
Warm condition	No	No	45°C

2.5 Cytotoxicity

The effect of TD alkaloidal extract on cell viability of peripheral blood mononuclear cell (PBMC) was determined by using a colorimetric technique, which was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (62).

2.5.1 PBMC isolation

Blood (20-25 mL) was taken from same donor throughout the research by using the 25 mL syringe. The blood sample was diluted with the same volume of phosphate buffer saline (PBS). After that, the diluted blood sample was carefully layered on Ficoll-Paque Plus. Then the mixture was centrifuged under at 5000 × g for 30 min at 18-20°C. The undisturbed lymphocyte layer was carefully transferred out. The lymphocyte was washed and pelleted down with three volumes of PBS for twice and resuspended RPMI-1640 media with 100 IU/mL of penicillin, 100 µg/mL of streptomycin, 10% v/v fetal bovine serum (FBS). Cell counting was performed to determine the PBMC cell number with equal volume of trypan blue.

2.5.2 Cell viability assay

The effect of the TD alkaloidal extract on cell viability of PBMC was determined by using a colorimetric technique (MTT assay). Briefly, 100 µL of PBMC with cell concentration at 10^5cells/mL was added into all wells in the 96-well plate and incubate in 37°C, 5% CO_2 and 90% humidity incubator for 24 hr. Then 100 µL of various concentrations of the extract was added to the cells compared with untreated cells and incubate again in the same condition for 48 hr. After the corresponding period, 100 µL of media was removed from each well and 25 µL of MTT at 5 mg/mL

was added into each well and incubated again for 4 hr. All the media was removed by turning the 96-well plate upside down. Then 200 µL of dimethyl sulfoxide (DMSO) was added to each well to extract and solubilize the formazan crystal. Finally, the plate was read at 540 nm by using microplate reader. All the experiment will be done at least twice.

<u>Part IV</u> Development of microemulsions containing TD alkaloidal extract

1. Materials

Plant materials *Centella asiatica*, *Polyscias fruticosa*, *Eupatorium odoratum*, *Cymbopogon citratus*, *Ocimum sanctum*, *Ocimum canum*, *Ocimum gratissimum*, *Melissa officinalis*, *Ocimum basilicum*, *Cinnamomum bejolghota*, *Piper sarmentosum*, *Polygonum odoratum Lour.*, *Citrus hystrix*, *Citrus aurantifolia*, *Citrus maxima*, *Citrus reticulata* Blanco cv. Shogun, *Citrus reticulata* var. Fremont, *Alpinia galangal*, *Zingiber officinale*, *Zingiber cassumunar*, (Thailand)

Solvent Methanol (AR grade), Merck (Darmstadt, Germany)

Surfactants Brij 97, Sigma–Aldrich (St.Louis,USA)

Tween 20, Tween 85, Acros Organics (New Jersey, USA)

Triton X-114, Sigma–Aldrich (St. Louis, MO, USA)

Co-surfactant Ethanol (AR grade), Merck (Darmstadt, Germany)

n-Propanol, Isopropanol, Butanol, Hexanol, Fisher Chemicals (Loughborough, UK)

Cetyl alcohol, USP (Tokyo, Japan)

Polyethylene glycol 400, USP (Wilhelmshaven, Germany)

Glycerin, BP/USP (Malaysia)

Propylene glycol, Sigma–Aldrich (St. Louis, MO, USA)

Chemicals Anhydrous sodium sulphate, Fisher Chemicals (Loughborough, UK)

Method

2.1 Oil phase selection

2.1.1 Essential oil extraction

Plant samples of *Centella asiatica*, *Polyscias fruticosa*, *Eupatorium odoratum*, *Cymbopogon citratus*, *Ocimum sanctum*, *Ocimum canum*, *Ocimum gratissimum*, *Melissa officinalis*, *Ocimum basilicum*, *Cinnamomum bejolghota*, *Piper sarmentosum*, *Polygonum odoratum Lour.*, *Citrus hystrix*, *Citrus aurantifolia*, *Citrus maxima*, *Citrus reticulata* Blanco cv. Shogun, *Citrus reticulata* var. Fremont, *Alpinia galangal*, *Zingiber officinale*, and *Zingiber cassumunar* were separately cut into small pieces and subjected to hydrodistillation for 3 hr using a clevenger type apparatus. The essential oils obtained were dried over anhydrous sodium sulphate and stored in a refrigerator and protected from light until further use. The yield of each essential oil was recorded and density was analyzed by using pycnometer.

2.1.2 ChE activity determination of essential oil

The inhibitory activities against AChE and BChE of essential oils were determined by using Ellman's method as previously described. Each essential oil was dissolved in methanol which was not allowed to exceed 10% of the total concentration. Two types of cholinesterase, electric eel AChE and horse serum BChE enzymes, were used whereas ATCI and BTCI were employed as enzyme substrates of the reactions, respectively. The enzyme inhibition was done by means of Ellman's method with slightly modifications (5, 57). The mean velocity of reactions was recorded. All of the experiments were done in triplicate. The cholinesterase inhibitory activity was then calculated using the following equation; $E = 1-(Vs/Vb)$, where Vs is

the mean reaction rate in the presence of a certain concentration of the oils and *Vb* is the mean reaction rate in absence of the oils. IC_{50} values were statistically evaluated using the graphpad/prism program. The inhibitory effects of the samples on AChE and BChE were represented as % inhibition. The essential oils possess high anti-ChE activities with high %yield were selected for further studies.

2.1.3 GC-MS

The isolated essential oil was analyzed for their compositions by GC-MS. The GC-MS analysis was performed on Agilent 6890 gas chromatography (Agilent Technologies, CA, USA) coupled to electron impact (EI, 70 eV) with HP 5973 mass selective detector (Hewlett Packard, CA, USA) and fitted with a fused silica capillary column (HP-5MS) supplied by Hewlett Packard, USA (30.0 m × 250 mm, i.d. 0.25 mm film thickness). The analytical conditions were; carrier gas: helium (ca. 1.0 mL/min), injector temperature: 260°C, oven temperature: 3 min isothermal at 100°C (No peaks before 100°C after first injection), then at 3°C/min to 188°C and then at 20°C/min to 280°C (3 min isothermal), and detector temperature: 280°C. The identification of individual compounds was based on their retention times relative to those of authentic samples and matching spectral peaks available with Wiley, NIST and NBS mass spectral libraries.

2.2 Construction of phase diagrams of blank microemulsion

Pseudoternary phase diagrams were constructed using a water titration method (61). Various surfactants, co-surfactant, pH and ionic strength of aqueous phase were

involved in the study as the factors affecting microemulsion area existed in the phase diagram.

2.2.1 Effect of oil type

Essential oil was selected to be an oil phase in the microemulsion for transdermal application since terpenes, the major constituents, acted as a penetration enhancer (35). The essential oil from overground part of *C. citratus* and rhizomes of *Z. cassumunar* were employed as the oil phase in microemulsion system when the other components are constant.

2.2.2 Effect of surfactant type

Various nonionic surfactants including Tween 20, Tween 85, Brij 97 and Triton X-114 were employed as the surfactant in microemulsion system when the other components are constant.

2.2.3 Effect of co-surfactant type

Alkyl alcohol with various chain length including ethanol, n-propanol, isopropanol, hexanol and cetyl alcohol as well as propylene glycol (PG), polyethylene glycol 400 (PEG 400) and glycerin were employed as the co-surfactant in microemulsion system when other conditions are constant.

2.2.4 Effect of surfactant to co-surfactant ratio

Various surfactants to co-surfactant ratios including 2:1, 1:1 and 1:2 were studied when other conditions are constant.

2.2.5 Effect of pH of aqueous phase

Buffer solutions with pH of 4.0, 6.0 and 8.0 were used instead of water when other conditions are constant.

2.2.6 Effect of ionic strength of aqueous phase

Monovalent and divalent salt solution with various ionic strengths which were 0.1, 0.5 and 1.0 mM were used instead of water when other conditions are constant. Sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) or calcium chloride ($CaCl_2$) was used as the representative of monovalent and divalent salts, respectively.

2.3 Characterization of selected blank microemulsion

2.3.1 Particle size / size distribution

Particle size analysis was carried out using photon correlation spectroscopy (Zetasizer® version 5.00, Malvern Instruments Ltd., Malvern, UK). The sizing measurements were carried out at a fixed angle of 90°. The reported results are the mean and S.D. of at least ten measurements on the sample.

2.3.2 Conductivity determination

Electrical conductivity of the microemulsions was measured using 100 mM NaCl solution as aqueous phase. The conductivity was measured by Cyberscan CON 11: hand-held conductivity meter (Eutech Instruments, Singapore) using conductivity/TDS electrode cell. The experiment was performed at 25±1.0°C by

dipping the electrode into the test sample until equilibrium was reached and reading became stable. The measurements were done in triplicate.

2.3.3 Rheology study

Viscosity of the microemulsions was measured using a Brookfield DVIII rheometer (Brookfield Engineering Laboratories, Stroughton, MA) fitted with a bob spindle. Brookfield Rheocalc operating software was used to control the measurement. A sample volume of 70 mL was used. The measurements were performed in triplicate at 25°C.

2.3.4 PLM

Microemulsions were evaluated by a polarizing light microscope (Nikon Optiphot PFX, Tokyo, Japan) to identify if they were microemulsions (non-birefringent) or liquid crystalline structures (birefringent). A digital camera (Nikon CoolPix 990, Japan) was attached to the microscope for capturing images of the different structures.

2.3.5 DSC

DSC measurements were performed with a DSC TA Q100 instrument equipped with a refrigerated cooling system (TA Instruments, New Castle, DE). Nitrogen with a flow rate of 50 mL/min was used as purge gas. Approximately 2-4 mg of sample was weighed precisely into hermetically sealable aluminium pans. An empty hermetically sealed pan was used as reference. Measurements were carried out in a heating mode heating the sample up from -50 to 115°C at a heating rate of

10°C/min. Sample thermograms were analysed using the TA Universal Analysis software 4.0 C.

2.4 Characterization of TD alkaloidal extract loaded microemulsion

2.4.1 Preparation of TD alkaloidal extract loaded microemulsion

The blank microemulsions which showed a good characteristic were selected from the phase diagram to be incorporated with the TD alkaloidal extract. The TD alkaloidal extract was dissolved in the co-surfactant first. Then it was mixed with surfactant and the essential oil, respectively. Finally, the water phase was added. Along the mixing procedure, each component was mixed up by vortex mixture. The resulting microemulsions were evaluated as followed.

2.4.2 Particle size / size distribution

Particle size analysis was carried out using photon correlation spectroscopy (Zetasizer® version 5.00, Malvern Instruments Ltd., Malvern, UK). The sizing measurements were carried out at a fixed angle of 173°. The reported results are the mean and S.D. of at least ten measurements on the sample.

2.4.3 Conductivity determination

Conductivity measurements were carried out for the microemulsion samples using a Riac CM/100 conductivity meter fitted with an YSI 3481 electrode having a cell constant of 0.11 cm^{-1} (Yellow Springs Instruments Inc., Yellow Springs, OH). Electrical conductivity was measured of microemulsions with addition of 100 mM

NaCl solution since NaCl did not affect the phase behaviour of the systems investigated. Measurements were carried out in triplicate at 25±1.0°C by dipping the electrode into the test sample until equilibrium was reached and reading became stable.

2.4.4 Rheology study

Apparent viscosity of each sample was measured at 20±1°C using a plate and double cone configuration (RheoStress1, Gebrüder Haake GmbH, Karlsruhe, Germany) surrounded by a circulating water bath. Shear stress was measured as a function of decreasing shear rate from 1000 to 10 s^{-1} (within 60 s). The rheological measurements were made in triplicate.

2.4.5 PLM

Microemulsions were evaluated by a polarizing light microscope (Nikon Optiphot PFX, Tokyo, Japan) to identify if they were microemulsions (non-birefringent) or liquid crystalline structures (birefringent). A digital camera (Nikon CoolPix 990, Japan) was attached to the microscope for capturing images of the different structures.

2.4.6 DSC

DSC measurements were performed with a DSC TA Q100 instrument equipped with a refrigerated cooling system (TA Instruments, New Castle, DE). Nitrogen with a flow rate of 50 mL/min was used as purge gas. Approximately 2-4 mg of sample was weighed precisely into hermetically sealable aluminium pans. An

empty hermetically sealed pan was used as reference. Measurements were carried out in a heating mode heating the sample up from -50 to 115°C at a heating rate of 10°C/min. Sample thermograms were analysed using the TA Universal Analysis software 4.0 C.

2.4.7 FF-TEM

Two copper support disks and a TEM grid (400 mesh) were used. The TEM grid was immersed into the microemulsion and sandwiched between two flat copper disks. The sandwich was then snap-frozen by immersion in liquid propane at -180°C and afterwards loaded into a double-replica device immersed in the liquid nitrogen at -196°C. For fracturing, the double replica device was mounted onto the rotary sample stage of a Balzers BAF 300 freeze etch device (Balzers, Lichtenstein) which was cooled to -110°C. Fracturing was carried out at a vacuum of 6.5×10^{-4} Pa. The fractured surfaces were shadowed with platinum (45°) and then immediately with carbon (90°). The replicas were washed in ethanol and finally distilled water. The replicas were then mounted onto copper grids and viewed with a Philips 410 LS transmission electron microscope (Philips, Eindhoven, Netherlands) operating at an accelerating voltage of 80 kV.

2.4.8 *In vitro* anti-ChE assay

Ellman's method was used to evaluate the anticholinesterase activity (5, 56). Briefly, 125 µL of 3 mM DTNB, 25 µL of 1.5 mM ATCI, 50 µL of 50 mM Tris-HCl buffer, pH 8.0, and 25 µL of microemulsion dissolved in buffer containing not more than 10% methanol were added to each well of the 96-well microplate and followed

by 25 µL of AChE. The microplate was finally read at 415 nm every 7 s for 2 min after 1 min of moderate shaking. To evaluate the activity of BChE, BTCI will be used as a substrate instead of ATCI.

2.4.9 Stability study

The microemulsion loaded with TD alkaloidal extract was stored in closed containers at various temperatures of 4°C, 30°C, and 45°C for 180 days. Changes in physical appearances will be investigated and the amount of TD alkaloidal extract was determined using Ellman's assay as previous described.

2.4.10 *In vitro* permeation experiments

2.4.10.1 Skin Preparation

Full-thickness skin from the flank area of stillborn piglets was used for skin permeation studies. Stillborn piglets (Sus scrofa, Duroc male × large white female) were obtained fresh from a local farm. The hair was trimmed off with electrical clippers and the skin pieces from the flank area were carefully dissected with a surgical blade. After washing in PBS, the skin was wrapped in tin foil and then stored at -20°C for up to 1 month (63). Prior to the skin permeability study, skin was defrosted and hydrated in PBS overnight at room temperature. The subcutaneous fat layer was carefully trimmed off by using surgical scissors before placing the skin onto the diffusion cells. Prior to skin permeability analysis, skin integrity was checked by measuring electrical resistance, using an Ag/AgCl electrode system. Briefly, after a 1-hr equilibration of skin at 37°C mounted on Franz diffusion cells, the direct current

was measured by immersing the Ag electrode into the donor compartment and the AgCl electrode into the receptor compartment, both filled with PBS, and applying an electrical potential of 0.3 V. The electrical resistance was calculated by using the current data. Only skin samples having a resistance higher than 10 $k\Omega cm^2$ were used for skin permeability measurements.

2.4.10.2 Skin permeability

Franz diffusion cells (Hanson Research, Chatsworth, CA, USA) with a diffusional area of 1.77 cm^2 were used for the *in vitro* skin permeation studies (63). The receptor media contained 7 mL of degassed 4% bovine serum albumin (BSA) in PBS (pH 7.4) thermostated at 37°C and was stirred constantly with a magnetic stirrer at 100 rpm. To investigate skin permeability, 500 µL of the microemulsions containing 5 mg/mL alkaloidal extract were applied onto the skin surface. PG was used as a control in which the same concentration of alkaloidal extract as the microemulsion systems was dissolved. The aliquots of 800 µL of the receptor media were withdrawn at specific time intervals.

2.4.10.3 Skin retention

To determine the amount of alkaloidal extract retained in the skin, 24 hr after application of the formulation, the skin was rinsed with tap water and homogenized in a methanol : PBS 1:1 mixture (v/v). To allow the complete extraction of the TD alkaloidal extract from the skin cells, the homogenate was stored in a refrigerator for at least 12 hr prior to analysis. All formulations were tested in triplicate, using skin samples from different animals.

2.4.10.4 HPLC

Briefly, HPLC analyses were performed using an HP1100 system with a thermostatically controlled column oven and a UV detector set at 295 nm (Hewlett-Packard, Palo Alto, CA). A reversed phase column Inertsil® ODS-2 (250 × 4.6 mm i.d., 5 µm, GL Sciences Inc., Japan) was connected with a guard column (125 × 4.6 mm i.d., 5 µm, Algilent, USA). A mixture of acetonitrile and PBS (pH 7.4) in the ratio of 50:50 was used to elute samples at ambient temperature at a flow rate of 1 mL/min and 20 µL of sample was injected. Samples and mobile phases were filtrated through a 0.45 µm Millipore filter, type GV (Millipore, Bedford, MA) prior to HPLC injection.