CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and instruments

Chemicals

1. Absolute ethanol (Analytical grade, Labscan, Ireland)
2. Acetone (Analytical grade, Labscan, Ireland)
3. Acetonitrile (HPLC grade, Fisher, Italy)
4. Ammonium hydroxide (Analytical grade, J.T. Baker, USA)
5. Dimethylsulfoxide (Analytical grade, Labscan, Ireland)
6. Disodiumhydrogenphosphate anhydrous (Analytical grade, Labscan, Ireland)
7. Ethylacetate (Analytical grade, Labscan, Ireland)
8. Ethylphenylpropiolate (EPP) (Fluka, U.S.A)
9. Glacial acetic acid (Analytical grade, Labscan, Ireland)
10. n-Hexane (Analytical grade, Labscan, Ireland)
11. Methanol (HPLC grade, Fisher, Italy)
12. Ortho-phosphoric acid (Analytical grade, Merck, Germany)

Instruments

1. Analytical balance
   Sartorius AC210 Sartorius, Germany
2. Preparative HPLC
   Hewlett Packard series 1100, Agilent Technologies, U.S.A
3. Ultraviolet-Visible Spectrophotometer
   UV-2450, Shimadzu, Japan

4. Differential Scanning Calorimeter (DSC)
   7 series/UNIX DSC7, Parkin Elmer, U.S.A
5. Microplate UV/VIS Spectrophotometer
   Model 680, BIO RAD, Japan
6. High pressure homogenizer
   C3, Avestin, Cannada
7. pH meter
   pH Level 2, Inolab, Germany
8. Photon Correlation Spectrophorometer
   Zetasizer ZS, Malvern Instruments, UK
9. Polytron
   PT-MR 3000, Kinematica AG, Switzerland
10. Centrifugation machine
    Avanti30, Beckman, U.S.A
11. Brookfield viscometer
    Rheometer R/S-CPS (Plate&Plate), Scientific Promotion Co., Ltd., UK
12. Scanning Electron Microscope
    JEOL JSM-5410LV, Japan
13. Sonicator
    Elma, Transsonic T460/H, Germany
14. Milli-Q water filtered system
    (Milli-Q®, U.S.A)

3.2 Plant material

The stem of *Tabernaemontana divaricata* (Linn.) R. Br. was collected from Chiang Mai province, the northern of Thailand. The voucher specimen of this plant (No. 0010115) has been deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.

3.3 Extract preparation

T. divaricata (TD) stem was cut into small thin pieces and dried at 50 °C for 3 days. The dried stem was ground into fine powder. There are two processes for

extract preparation. One uses the method of maceration with 95% ethanol to obtained crude ethanolic extract. The other use the manner maceration with solvents in order increasing of polarity to obtained fractionated extract. Briefly, to obtain the crude ethanolic extract, 100 g of TD stem dried powder was macerated in 500 ml of 95% ethanol for 7 days (4 cycles) at 27 °C. The filtrates were pooled together and concentrated by using a rotary evaporator at 40 °C. The obtained crude ethanolic extract from maceration was termed TDE. TDE was divided into two parts. The first part was stored at 4 °C until further used, while the second part was clean up by eletrocoagulation technique [55]. By using the eletrocoagulation technique, 30 g of TDE was dissolved in 1.5 l of 95% ethanol in 2000 ml-breaker. A pair aluminium plates (20 x 6 cm) was immersed 18 cm under the solution where the space between these two plates was 1.5 cm. Then 0.2% w/v sodium chloride was added as a supporting electrolyte. Direct current in the range of 2.4-3.0 A and 25-30 V was passed through the solution *via* the two electrodes for 3 h. After that, the solution was filtrated and the solvent was removed by a rotary evaporator until dryness. The remained salt in the residue was extracted with ethanol. The ethanolic solution was further dried to give a crude alkaloid which was designated TDC. To obtain the fractionated extract, 100 g of TD dried powder was macerated in 500 ml of hexane for 7 days (4 cycles) at 27 °C. After the fourth extraction, the residue was dried at 27 °C for 1 day to ensure that hexane was completely removed. The dried residue was further macerated with ethyl acetate and 95% ethanol, respectively. The filtrate of each solvent was pooled and removed under vacuum at 40 °C by using a rotary evaporator. The obtained fractionated extracts were termed HF, EAF and EF, respectively. The extracts from each solvent were kept at 4 °C after preparation.

3.4 *In vitro* anti-inflammatory procedures

The anti-inflammatory action of all extracts was done by *in vitro* enzymatic assay using colorimetric COX inhibitor assay kit (Cayman, Ann Arbor, USA) [35]. This assay was employed to select the extract that showed the highest anti-inflammatory activity. This process measured the peroxidase component of COXs. The peroxides activity was colorimetrically assayed by monitoring the appearance of oxidized N, N, N', N'-tetramethyl-*p*-phenylenediamine (TMPD) at 595 nm. All

samples were dissolved in DMSO at a concentration of 4.00 mg/ml. To measure the anti-inflammatory activity, there need to be three wells including background well, COX-2 100% initial activity well and COX-2 inhibitor well. The detail of each well was described as follow;

1. Background well

The background well was added with 160 µl of assay buffer, 10 µl of heme and 10 µl of solvent (Figure 4A).

2. COX-2 100% initial activity well

The 100% initial activity well contained 150 µl of assay buffer, 10 µl of heme, 10 µl of COX-2 enzyme and 10 µl of solvent (Figure 4B).

3. COX-2 inhibitor well

The inhibitor well was added the same composition as 100% initial activity well plus an aliquot of 10 µl of sample tests was added (Figure 4C).

After all the substances were added in each well, the plate was carefully shaken and incubated for 5 min at 37 °C. All wells were added with 20 µl of colorimetric substance solution and 20 µl of arachidonic acid, respectively. After that it was carefully shaken and incubated for 5 min at 37 °C. The absorbance was read by a microtitre plate reader (Model 680, BIO RAD, Japan) at 595 nm. All measurements were performed in duplicate. Aspirin was used as a positive control.

The decreased absorbance was compared with 100% initial activity well and the percentage of inhibition was calculated. The extract which showed the highest anti-inflammatory activity was selected for the further study.

$$A_{\text{COX-2 enzyme}} = A_{\text{100 \% initial COX-2 activity well}} - A_{\text{background well}}$$

$$A_{\text{COX-2 inhibitor}} = A_{\text{COX-2 inhibitor well}} - A_{\text{background well}}$$

$$\% \text{ Inhibition} = \frac{(A_{\text{COX-2 enzyme}} - A_{\text{COX-2 inhibitor}})}{A_{\text{COX-2 enzyme}}} \times 100$$

Where; A = absorbance of substances at 595 nm.

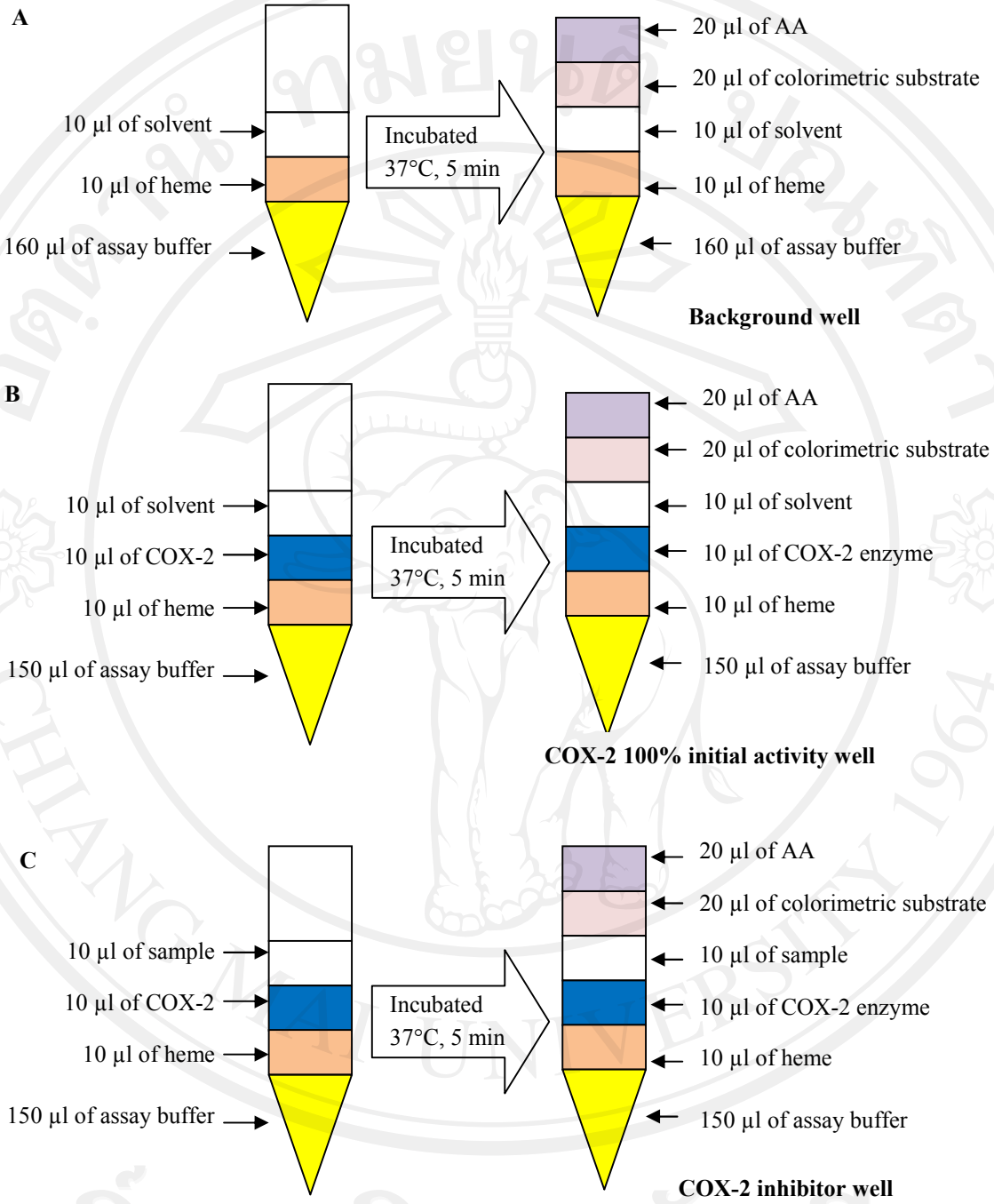

Figure 4 Diagram illustration the procedure for preparing the background well (A), COX-2 100% initial activity well (B) and COX-2 inhibitor well (C)

3.5 Study of physicochemical properties of the extract

3.5.1 Appearance of the extract

Color, odor and physical status of the extract were observed by organoleptic test.

3.5.2 Solubility of the extract

A 1.0 mg of the extract was weighed accurately. Then the solvent was gradually added until it could be completely dissolved. The amount of solvent added was recorded and the solubility was calculated.

3.5.3 Determination of physicochemical properties of the extract using DSC and PXRD

The thermal behavior and crystalline characteristic of the extract was determined by DSC and PXRD, respectively. The conditions used are shown in Table 3.

Table 3 DSC and PXRD conditions for physicochemical properties study of the extract

Operating parameter	Conditions
DSC	
Rate	10 °C/min
Temperature	50-200 °C
Pan size	50 µl
Cover pan size	0.1 µl
Weight of sample	2.00-3.00 mg
X-ray diffractometer	
Bragg angle	2θ

3.5.4 Study of factors influencing characteristic of the extract

Factors influencing the physicochemical properties of the extract such as temperature (LT, RT and HT) light, oxidizing-reducing agent and pH were investigated as a pre-formulation study. A concentration of 1.0% w/v extract in an appropriate solvent was mixed with acidic-basic solutions and oxidizing-reducing solutions in a volume ration of 1:1. The obtained color and precipitation were observed by organoleptic test.

3.5.5 Primary active compounds screening test of the extract

Primary phytochemical screening related to chemical constituents including alkaloids, glycosides, phenolics and tannins were studied [56, 57].

3.5.5.1 Alkaloid testing

The extract was placed in an evaporation dish. A 0.5 ml of 2 N HCl was added into the extract and then stirred for 10 min. After that, 0.5 mg of NaCl was added and stirred until it was completely dissolved. An acidic extract solution was filtered for the further use.

Primary testing

An acidic extract solution at a volume of 0.5 ml was divided into five tubes. A few drops of the specific reagent such as Dragendroff's reagent, Mayer's reagent, Wagner's reagent, Marme's reagent and Kraut's reagent were added to each test tube, respectively. The turbidity and precipitation were observed for the positive results.

Confirm testing

The 10.0 ml of the acidic extract solution was placed in the separating funnel and then 10.0 ml of ether was added. This separating funnel was slowly rotated and stood until separated. The ether layer was removed. The aqueous layer was added with NH_3 solution until the color of litmus paper changed from red to blue. Then, 10 ml of ether was again added and slightly rotated. Then it was left standing until the separation of ether layer and aqueous layer was observed. The ether layer was collected. This extract solution was extracted with ether twice. The ether layers were pooled and let the solvent evaporated on the water bath to give the mass. For alkaloid

testing, the obtained mass was dissolved with 2.5 ml of 2 N HCl. This solution was filtered and placed in five test tubes. The first tube was tested with Wagner's reagent while the second one was tested with Mayer's reagent. If the precipitation was observed, it could be concluded that the extract contained the primary, secondary or tertiary alkaloid as the chemical constituents. The others were added with a few drops of the specific reagent such as Dragendroff's reagent, Marme's reagent and Kraut's reagent, respectively. The turbidity and precipitation were observed to confirm the positive results.

3.5.5.2 Glycoside testing

The extract was dissolved in 95% ethanol at a concentration of 1.0 mg/ml. Then 25.0 ml of the extract solution was added with 15.0 ml of 10% HCl and refluxed for 30 min. The obtained solution was cooled at ambient temperature (27-30 °C) and extracted in separating funnel with 10.0-20.0 ml of ether for 3 times. The ether layers were collected and pooled. Then anhydrous Na_2SO_4 was added to absorb remaining water from the ether layer. The obtained ether and aqueous acidic layer were separately investigated.

Anthracene/ Anthraquinone glycoside testing

The 4.0 ml of the ether layer was evaporated until the volume was reduced to approximately 2.0 ml. Then, it was added with 1.0-2.0 ml of 25.0% ammonia solution and shaken. The red color in the ammonia layer was observed as a positive result.

Coumarin testing

The 5.0 ml of the ether layer was evaporated to obtain dry powder. The dry powder was dissolved in 1.0-2.0 ml of hot DI water and divided into 2 tubes. One tube was dissolved with 2.5 ml of 10.0% the ammonia solution. The other tube was used as a control. The fluorescence of solution was detected under ultraviolet source. The blue or green fluorescence was detected as a positive result.

Sterol glycoside/ Triterpene glycoside testing

The sterol glycoside/ triterpene glycoside testing were done by Lieberman Burchard's reaction. Briefly, 10.0 ml of the ether layer was completely evaporated. The obtained residue was dissolved with 0.5 ml of acetic anhydride and 0.5 ml of $CHCl_3$. This solution was poured into the dry and clean tube. An approximate 1.0-2.0 ml of the concentrated sulfuric acid was slowly added into the tube using a dropper. The color formed between both layers was observed at 5, 15 and 30 min. The positive result of sterol testing showed when the color was red/reddish brown, violet and green/blue-green at 5, 15 and 30 min, respectively.

Cardiac glycoside testing (Kadde's test)

The 4.0 ml of the ether layer was completely an evaporated in evaporating dish. The obtained residue was dissolved in 1.0-2.0 ml of methanol and 1.0-2.0 ml of potassium hydroxide solution in 1 N ethanol was added. After that, 3-4 drops of 1.0% 3, 5-dinitrobenzoic acid in ethanol were added and boiled on the water bath. The violet color was observed as a positive result.

Saponin glycoside testing

The 4.0 ml of the ether layer was completely evaporated in an evaporating dish. The obtained residue was dissolved in DI water and then placed into the tube. If saponin glycoside was existed in the extract, an aqueous solution would be froth when shaken.

Flavonoid glycoside testing (Shibata's reaction)

The 5.0 ml of the ether layer was completely evaporated in evaporating dish. The obtained residue was dissolved in 1.0-2.0 ml of 50.0% methanol on the water bath. Then, one small thin piece of magnesium metal was put and the 5-6 drops of the concentrated HCl were added. The color of the solution would be red when the flavanol compound was existed in the extract. On the other hand, the color of the solution would be orange when the flavonone compound was existed.

Anthocyanine testing

An aqueous acidic solution was added with 25% ammonia solution to adjust this solution to be a neutral and finally basic. The positive result was detected when the test solution was red in acidic solution and changed to violet in neutral solution and finally turned to green/blue in basic solution.

3.5.5.3 Phenolic and Tannin testing

The extract was completely evaporated on the water bath. After that, 25.0 ml of heated DI water was added and then stirred until cooled. Then, few drops of 10.0% NaCl solution was added to salt out the other compounds. The mixture was filtered using a buchner funnel. The obtained clear solution was divided into 4 tests which contained 2.0 ml in each tube. The first tube was added with the 4-5 drops of 1.0% gelatin solution. The precipitation was detected as a positive result. The second tube was added with the 4-5 drops of 1% gelatin solution and 10.0% NaCl solution. The precipitation was observed as a positive result. The third tube was added with 3-4 drops of 1.0% ferric chloride solution. Finally, the last one was used as a control test tube. For the result interpretation, if there was no reaction with ferric chloride solution, the phenolic or tannin compounds were not existed in the extract. In another case, the sample solution was a blue-green or dark green to almost black after adding the ferric chloride solution associated with the precipitation of gelatin in saline solution, it could be concluded that catechol, subgroup of tannin, was existed in the extract. Whereas, pyrogallol or gallic tannin, subgroup of tannin, was existed when the sample solution was a dark blue after adding the ferric chloride solution associated with the precipitation of gelatin in saline solution. Finally, if the result indicated that the sample solution reacted with ferric chloride solution to give a green or blue solution, it could be concluded that the other phenolic compound was existing in the extract.

3.5.6 Study of finger print of the extract using HPLC

The standardized alkaloidal markers of the extract were kindly provided by Assoc. Prof. Dr. Kornkanok Ingkaninan, Naresuan University, Thailand. Two alkaloidal substances found in the extract, vobasine and 19, 20 dehydroervatamine,

were used as markers for quality and quantitative analysis by a high performance liquid chromatography (HPLC). The HPLC condition is shown in Table 4.

Table 4 HPLC condition for physicochemical properties study of the extract

Operating parameter	**Conditions**
Stationary phase (column)	Pinnacle II C18 (Restek, 250 mm × 4.6 mm, 5.0 µm)
Guard column	Trident™ Integal HPLC guard column system (Restek, 10 × 4.0 mm Guard Cartridge Pack of 3)
Mobile phase	Acetonitrile + 500 µM phosphate buffer pH 7.4 (40:60)
Detector	UV-Visible
Wavelength	280 nm
Injection volume	40 µl
Solvent	Methanol

3.6 Development of nanocream formulations

3.6.1 Nanocream base development using high pressure homogenizer

The ingredients in lipid phase and water phase were accurately weighed. The lipid phase was heated to 75 °C when the water phase was heated to 80 °C. Then, the water phase was gradually poured into the lipid phase under high speed stirring (Polytron) at 7000 rpm for 30 min. The obtained emulsion was passed through the high pressure homogenizer at 1500 psi. The samples were collected before and after passing the high pressure homogenizer (0, 3 and 6 cycles). The particle size diameter, polydispersity index and zeta potential were determined by a photon correlation spectrophotometer (PCS). The viscosity and rheological behavior were studied by a Brookfield viscometer. Their physicochemical properties were studied at 4, 27 and 45 °C for 90 days. The compositions of all formulations were shown in Table 5.

3.6.2 Development of the extract loaded solid lipid nanoparticles (SLN)

SLN consisted of 0.25 and 0.50% w/w of the extract based on lipid contents and the extract-free SLN were produced by hot melt high pressure homogenizer using the method of Müller et al. [43, 44] with some modification. Briefly, the lipid phase containing the extract was melted to 70 °C and the aqueous solution phase containing mixtures of surfactants was heated at same temperature. Then, the aqueous phase was dispersed in the lipid phase. A pre-emulsion was obtained after stirring by high speed stirrer, Polytron, at 8000 rpm for 5 min. A hot pre-emulsion was then rapidly passed through high pressure homogenizer at 1000 bars (85 °C). After homogenization, the o/w nanoemulsion was cooled down at 4 °C until the internal oil phase was solidified. The compositions of all formulations were shown in Table 6. The samples were collected before and after passing the high pressure homogenizer (0, 3 and 6 cycles). This study was done to investigate the factors, such as the number of homogenization cycle and the amount of ingredients in the SLN formulation, on physicochemical properties of the extract loaded SLN.

3.6.2.1 Effect of surfactant and number of homogenization cycles

The preparations of the extract loaded SLN were described in 3.6.2. The lipid phase was composed of 5.0% w/w cetyl palmitate as a solid lipid component and 0.25% w/w extract as an active compound. The aqueous phase was composed of the mixture of Tween 80 and Span 80 at various weigh ratios of 10, 15, and 20% w/w. Homogenization was performed at 1000 bars for 3 and 6 homogenization cycles. The effect of surfactant and number of homogenization cycles on characteristics of SLN formulations such as particle size diameter, polydispersity index and zeta potential were determined by using PCS. The optimum concentration of surfactant and homogenization cycle will be used for further production of extract loaded SLN in nanocream.

3.6.2.2 Effect of lipid

The preparations of the extract loaded SLN were described in 3.6.2. The lipid phase was composed of various amounts of cetyl palmitate at in the range of 2.5-7.5% w/w while the amount of the extract was maintained at 0.25% w/w. The 10.0%

w/w of the mixture of surfactants was used in this experiment. The effect of lipid on characteristics of SLN formulations such as particle size diameter, polydispersity index and zeta potential were determined by using PCS. The optimum concentration of solid lipid will be used for further production of extract loaded SLN in nanocream.

3.6.2.3 Effect of extract

The preparations of the extract loaded SLN were described in 3.6.2. The lipid phase was composed of various amounts of extract, at concentrations of 0.25 and 0.50% w/w, and solid lipid, at in the range of 2.5-7.5% w/w. The 10.0% w/w of the mixture of surfactants was used in this experiment. The effect of extract on characteristics of SLN formulations such as particle size diameter, polydispersity index and zeta potential were determined by using PCS and entrapment efficiency (EE) was analyzed by using HPLC.

3.6.3 Preparation of the extract loaded SLN nanocream

The extract loaded SLN was incorporated into the nanocream base at a weight ratio of 1:1 by high speed stirrer and then passed through the high pressure homogenizer.

3.7 Characterization of the formulations

3.7.1 Organoleptic parameters

Appearances, colors and odors of the formulations were evaluated organoleptic test.

3.7.2 Shape of nanoparticles

The shape of the nanoparticles in the nanocream formulation was viewed by using scanning electron microscope at 10 kV (20,000×).

3.7.3 Particle size diameter, polydispersity index and zeta potential

The measurement of particle size diameter, polydispersity index and zeta potential of SLN was performed by using PCS. All formulations were diluted with

milli Q water to an appropriate concentration. The polydispersity index indicated the size distribution of the nanoparticles population. Each reported value was an average of at least three measurements.

3.7.4 Rheological property

The measurement of viscosity and rheological behavior of the formulations was done by using Brookfield viscometer (Cone & Plate).

3.7.5 Percentage of entrapment efficiency (% EE)

The amount of the extract which was encapsulated in the nanoparticles was determined using indirect method and analyzed by using HPLC. Briefly, 1.00 g of extract loaded SLN formulations was accurately weighed. The extract loaded SLN was dispersed in 4.0 ml of methanol. Then it was centrifuged at 15,000 rpm for 30 min (4 °C). The supernatant was used to determine the amount of the extract which was not entrapped in the nanoparticles. The percentage of entrapment efficiency (% EE) was calculated using the following equation.

$$\% \text{ EE} = \frac{\text{Mass of the extract used in the formulation} - \text{Mass of free extract}}{\text{Mass of the extract used in the formulation}} \times 100$$

3.7.6 Analysis of the extract in SLN nanocream

One gram of extract loaded SLN nanocream was dissolved in 4.0 ml of methanol and sonicated for 1 h. Then, it was centrifuged at 15000 rpm for 30 min (4 °C). The supernatant was collected for the determination of extract in the SLN nanocream.

3.8 *In vivo* anti-inflammation assay

3.8.1 Experiment animals

Male Sprague Dawley rats weighing 80-100 g were purchased from the National Laboratory Animal center, Mahidol University, Salaya, Nakorn Pathom,

Thailand. All animals were kept in the animal room maintained under environmentally controlled conditions of 24 ± 1 °C and 12 h light–12 h dark cycle. Animals had free access to water and food. They were acclimatized at least one week before starting the experiments. The experiment protocol was reviewed and approved by Animal Ethics Committees, Faculty of Medicine, Chiang Mai University, Thailand (No 36/2553).

3.8.2 Ethylphenyl propiolate (EPP)-induced mouse ear edema

The *in vivo* anti-inflammatory test was done by EPP-induced mouse ear edema [41, 59, 62]. Male rats were divided into 7 groups of 3 animals each.

Group 1	control group, received 20 µl of acetone per ear
Group 2	vehicle control group, received 0.05 g of SLN nanocream base per ear
Group 3	positive control group, received indomethacin (reference drug) dissolved in acetone at concentration of 2.0 mg/20 µl per ear
Group 4-6	treatment groups, received the extract dissolved in acetone at concentrations of 0.125, 0.25 and 0.625 mg /20 µl, respectively, per ear
Group 7-9	treatment groups, received the extract loaded SLN nanocream at concentrations of 0.25, 0.50 and 1.25% w/w (0.05 g/ear), respectively

The ear thickness was measured before and at 15, 30, 60 and 120 min after ear edema induction by a digital vernier caliper. Ear edema was induced by topical application of EPP at a concentration of 50 mg/ml in acetone. Immediately before the irritant, the extract and indomethacin solutions were applied locally at a volume of 20 µl/ear in both inner and outer surface of each ear by an automatic microliter pipette. The extract loaded SLN nanocreams were applied at a concentration of 0.05g/ear to the inner and outer surface of both ears of each rat. EPP, at a concentration of 1 mg/ear, was applied in the same manner with a volume of 20 µl. The ear thickness increased was compared with the control group and percentage of ear edema inhibition was calculated as the following equation.

$$ED_x = ET_x - ET_o$$

$$\% EI = \frac{ED_c - ED_t}{ED_c} \times 100$$

where; ED_x = edema thickness at time x

ET_x = ear thickness (μm) at time x

ET_o = ear thickness (μm) before application of EPP

ED_c = edema thickness of control group at time x

ED_t = edema thickness of sample group at time x

% EDI = percentage of edema inhibition of sample at time x

Table 5 Compositions of ingredients in nanocream base formulations

Ingredients	Formulations	
	1A	**2A**
Cetyl alcohol	2.50%	2.50%
Stearyl alcohol	2.50%	2.50%
Isopropry myristate	10.0%	10.0%
Tween 80	8.00%	9.60%
Span 80	2.00%	2.40%
EDTA	0.01%	0.01%
Sodium metabisulfite	0.01%	0.01%
PEG 400	5.00%	5.00%
Glycerin	5.00%	5.00%
conc. paraben	1.00%	1.00%
Water qs.	100.00%	100.00%
Required HLB	12.83	12.83

Table 6 Compositions of ingredients in the extract loaded SLN formulations

Ingredients	Formulations				
	1	**2**	**3**	**4**	**5**
Cetyl palmitate	5.00%	5.00%	5.00%	2.50%	7.50%
Cetyl alcohol	2.50%	2.50%	2.50%	2.50%	2.50%
Avocado oil	3.00%	3.00%	3.00%	3.00%	3.00%
Tween 80	5.88%	8.62%	11.50%	5.75%	5.67%
Span 80	4.12%	6.38	8.50	4.25%	4.33%
the extract	0.25%	0.25%	0.25%	0.50%	0.50%
conc. paraben	1.00%	1.00%	1.00%	1.00%	1.00%
Mill Q water qs.	100.00%	100.00%	100.00%	100.00%	100.00%
Required HLB	10.59	10.59	10.59	10.45	10.37

3.9 Release profile experimental

The release study was performed by passive diffusion cellulose membrane. A 50% v/v ethanolic aqueous solution with 1% Tween 80 was employed as a receptor fluid for the release studies because of the low solubility of the extract in water and buffer solution. Cellulose membrane was soaked in receptor fluid for 24 h before the experiment. One gram of 0.25 and 0.50% w/w of the extract loaded SLN nanocream were accurately weighed. The extract which was dissolved in ethanol at concentrations of 0.125 and 0.25 mg/ml were studied for release profile to compare with the extract loaded SLN nanocream. Then, the samples were placed in dialysis membrane and put into test tanks containing the receptor medium controlled temperature at 37 ± 1 °C and magnetically stirred at 400 rpm throughout the experiment. Receptor fluid was collected and replaced by fresh fluid at 0.25-12.0 h for release study. The amount of released extract was determined by HPLC method.

3.10 Stability study

All formulations including nanocream base and the extract loaded SLN nanocream were investigated and placed in transparent glass bottom with a lid that assures good closing for 3 months. Various conditions have been employed to evaluate the stability of the formulation. The conditions and evaluation criteria were described as following:

Conditions

- At room temperature of 27 °C
- At refrigerator of 4 °C
- At oven of 45 °C
- At heating-cooling condition of 4 °C for 24 h and 45 °C for 24 h, for 6 cycles

Evaluation criteria

- Particle size, polydispersity index and zeta potential
- Viscosity and rheological behavior
- Degradation of the extract in SLN nanocream
- % EE

3.11 Skin irritation study

3.11.1 Experimental animals

Male New Zealand white rabbits weighing 1.5-2.5 kg were purchased from the Royal Project Foundation, Chiang Mai, Thailand. All animals were kept in the animal room maintained under environmentally controlled conditions of 24 ± 1 °C and 12 h light–12 h dark cycle. Animals had free access to water and food. They were acclimatized at least one week before starting the experiments. The experiment protocol was reviewed and approved by Animal Ethics Committees, Faculty of Medicine, Chiang Mai University, Thailand (No 36/2553).

3.11.2 Skin irritation test

The rabbit skin irritation test was performed by Draize test [63-65]. The hair on the back of each rabbits was shaved at least 4 h before the experiment. The rabbit's back was divided into 8 marked area (1 × 1 $inch^2$) for the topical application of test substances. Each area received test substances as follows:

Area 1	negative control group, received no substances
Area 2	control group, received 0.5 g of SLN nanocream base
Area 3	vehicle control group, received 0.5 ml of 95% ethanol
Area 4	positive control group, received 0.5 ml of 98% lactic acid
Area 5	sample groups, received 0.5 ml of the extract at concentrations of 0.25% w/v
Area 6	sample groups, received 0.5 g of the extract loaded SLN nanocream at concentrations 0.50% w/w

Each test substance was applied on each marked area, covered with gauze and then wrapped with a non-occlusive bandage. After 4 h, the bandage and gauze were removed and the rabbit's back was then washed by tap water. One hour later, the tested sites for skin irritation were observed at 1, 24, 48 and 72 h after application. The reactions, defined as erythema and edema, were evaluated according to the scoring system with Draize technique for skin reactions as shown in Table 7 and 8.

Table 7 Draize scoring system [63]

Skin reaction	Score
Erythema	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness to eschar formation)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well raised)	2
Moderate edema (raised approx.1mm)	3
Severe edema (raised more than 1mm and extending beyond area of exposure)	4

Table 8 Evaluation of primary skin irritation index

Evaluations	Score
Non irritation	0.00
Irritation barely perceptible	0.04-0.99
Slight irritation	1.00-1.99
Mild irritation	2.00-2.99
Moderate irritation	3.00-5.99
Severe irritation	6.00-8.00

3.12 Statistical analysis

The data from the experiments were expressed as mean ± standard error of mean (S.E.M.). The statistical comparison between groups was analyzed by analysis

of variance (ANOVA), post hoc least-significant difference (LSD). *P*-values less than 0.05 were considered significant.