Chapter 3

Methodology

1. Materials

1.1 Plant materials

The parts of plants, which were reported to be used against fever, cold and anti-allergy by folk doctors in Thailand, were purchased from several regions of Thailand, China, India, Indonesia and Australia. The place of collection, plant parts and voucher specimens are shown in Table 3.1. The voucher specimens were deposited at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand.

Table 3.1

Plants and part of plants component in Prasaprohyai preparation

Species (Family)	Places for specimen collection	Voucher specimen number	Thai name	Part used
Amomum testaceum Ridl. (Zingiberaceae)	Chanthaburi	SKP206011101	Krawan	fr
Anethum graveolens L. (Umbelliferae)	India	SKP199010701	Thian ta takkataen	fr
Angelica dahurica Benth. (Umbelliferae)	China	SKP199010401	Kot so	r
Angelica sinensis (Oliv.) Diels (Umbelliferae)	China	SKP199010901	Kot chiang	r
Artemisia annua L. (Compositae)	China	SKP051010101	Kot chula lampha	a

Table 3.1 (Continued)

Species (Family)	Places for specimen collection	Voucher specimen number	Thai name	Part used
Atractylodes lancea (Thunb.) DC. (Compositae)	China	SKP051011201	Kot kamao	rh
Cuminum cyminum L. (Umbelliferae)	India	SKP199030301	Thian khao	fr
Dracaena loureiri Gagnep. (Dracaenaceae)	Ratchaburi, Kanchanaburi	SKP065041201	Chan daeng	st
Foeniculum vulgare Mill. var. *dulce* (Mill.) Thell. (Umbelliferae)	India	SKP199062201	Thian khao plueak	fr
Kaempferia galanga L. (Zingiberaceae)	Ratchaburi, Kanchanaburi	SKP206110701	Proh hom	rh
Lepidium sativum L. (Cruciferae)	India	SKP057121901	Thian daeng	se
Ligusticum sinense Oliv. cv. Chuanxiong (Umbelliferae)	China	SKP199121901	Kot hua bua	rh
Mammea siamensis Kosterm. (Guttiferae)	Ratchaburi, Kanchanaburi	SKP083131901	Saraphi	fl
Mesua ferrea L. (Guttiferae)	Ratchaburi, Kanchanaburi	SKP083130601	Bunnak	fl
Mimusops elengi L. (Sapotaceae)	Ratchaburi, Kanchanaburi	SKP171130501	Phikul	fl
Myristica fragrans Houtt. (Myristicaceae)	Australia	SKP121130601	Chan thet	st

Table 3.1 (Continued)

Species (Family)	Places for specimen collection	Voucher specimen number	Thai name	Part used
Myristica fragrans Houtt. (Myristicaceae)	Suratthani	SKP121130601	Mace	ar
Myristica fragrans Houtt. (Myristicaceae)	Suratthani	SKP121130601	Nutmeg	se
Nelumbo nucifera Gaertn. (Nelumbonaceae)	Ratchaburi, Kanchanaburi	SKP125141401	Kasorn bua luang	p
Nigella sativa L. (Ranunculaceae)	India	SKP160141901	Thian dam	se
Syzygium aromaticum (L.) Merr. et Perry (Myrtaceae)	Indonesia	SKP123190101	Kan phlu	fl

Part used: a=all part, ar=aril, fl=flower, fr=fruit, p=pollen, rh=rhizome, r=root, se=seed, st=stem

1.2 Animal cell lines

1.2.1 Anti-inflammatory activity

RAW 264.7 murine macrophage leukemia cell line was established and kindly provided by Assoc. Prof. Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. RAW 264.7 cells were cultured in RPMI 1640 medium supplemented with 10% heated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were maintained at 37°C in 5% CO_2 atmosphere with 95% humidity and changed cultured medium three times a week.

1.2.2 Anti-allergic activity

RBL-2H3 rat basophilic leukemia cell line was established and kindly provided by Assoc. Prof. Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. RBL-2H3 cells were cultured in MEM (Minimum essential medium eagle) medium supplemented with 10% heated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were maintained at 37°C in 5% CO_2 atmosphere with 95% humidity and changed cultured medium three times a week.

1.3 Chemicals, reagents, instruments, plastics and glasswares

The chemicals, materials and instruments employed in the present studies are summarized in Table 3.2 and 3.3.

Table 3.2

List of chemicals and reagents used in the studies

Name	Source
Absolute ethanol	Merck, Germany
Albumin bovine fraction V powder	Sigma, USA
Anti-dinitrophenylated bovine albumin (DNP-BSA)	Sigma, USA
Anti-DNP IgE (Monoclonal Anti-DNP)	Sigma, USA
Butylated hydroxytoluene (BHT)	Fluka, Germany
Calcium chloride dehydrate	Merck, Germany
Citric acid monohydrate	Merck, Germany
D-(+)-glucose	Sigma, USA
Dimethyl sulfoxide (DMSO)	Fluka, Germany
1,1-Diphenyl-2-picrylhydrazyl (DPPH)	Fluka, Germany
Distilled water (Milli-Q, ≥ 18 Mega Ohm)	Milford, USA
Ethanol 95% (commercial grade)	Sasol, South Africa

Table 3.2 (Continued)

Name	Source
Hydrochloric acid	Merck, Germany
Ketotifen fumarate	Sigma, USA
Lipopolysaccharide from *E. coli* O55:B5 (LPS)	Sigma, USA
Magnesium chloride $6H_2O$	Merck, Germany
Minimum Essential Medium (MEM)	Gibco, USA
N-(1-Naphthyl)ethylenediamine dihydrochloride	Sigma, USA
4-Nitrophenyl *N*-acetyl-β-D-glucosaminide (PNAG)	Sigma, USA
Penicillin-Streptomycin (P/S)	Gibco, USA
Phosphate buffer saline (PBS)	Amresco, USA
Phosphoric acid solution	Sigma, USA
PIPES	Amresco, USA
Potassium chloride	Merck, Germany
RPMI medium 1640	Gibco, USA
Sodium bicarbonate	BHD, England
Sodium carbonate	Merck, Germany
Sodium chloride	Univar, Australia
Sodium hydroxide (analytical grade)	Univar, Australia
Sulfanylamide	Sigma, USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma, USA
Trisodium citrate dihydrate	Merck, Germany
Trypan blue	Gibco, USA
Trypsin-EDTA	Gibco, USA

Table 3.3

List of equipments, plastics and glasswares used in the studies

Name	Source
24-well plate flat, bottom	Costar Corning, USA
96-well plate flat, bottom with lid	Costar Corning, USA
96-well plate flat, bottom without lid	Corning, USA
Autoclave	Hirayama, Japan
Cell culture flask, canted neck 25, 75 cm^3	Corning, USA
Centrifuge tube15, 50 ml	Corning, USA
Centrifuge machine	Boeco, Germany
CO_2 humidified incubator	Forma, USA
Cryogenic tube 2 ml	Corning, USA
Disposable pipette 2, 5, 10, 25 ml	Corning, USA
Eppendrof	Costar Corning, USA
Glass bottles	Schott Duran, Germany
Glasswares	Schott Duran, Germany
	Pyrex, USA
Hematocytometer	Boeco, Germany
Hot air oven	Memmert, Germany
Hot plate	Thermolyne, USA
Inverted microscope	Nikon, Japan
Laminar air flow	Faster, Italy
Lyophilizer	Telster, Spain
Micropipettes	Eppendorf, Germany
Microplate reader	Bio Tek, USA
Multi-channels pipette	Costar Corning, USA
pH meter	WTW inolab, Germany
Pipette boy	Brand, USA
Quantikine mouse TNF-α ELISA test kit	R&D Systems, USA

Table 3.3 (Continued)

Name	Source
Refrigerator (-20°C)	Sanyo, Japan
Rotary evaporater	Buchi, Japan
Sonicator	Elma, Germany
Vortex	Scientific industries, USA
Water bath	Lauda, Germany

2. Methods

2.1 Preparation of plant extracts

The parts of these plants (Table 3.1) were washed, sliced thinly, dried in an oven at 50°C and powdered. These extracts were obtained by two methods, namely maceration and decoction methods. The Prasaprohyai preparation was also extracted in the same procedure (Figure 3.1).

Maceration: Dried plant material (300 g) was macerated by 95% ethanol for 3 days, 2 times per day, filtered and dried using an evaporator. All extracts of each plant were combined and calculated percentage of yield.

The residue from maceration were continued to be boiled in water or decoction: Dried plant material from residue of maceration (100 g) was boiled in distilled water at boiling point for 30 min, filtered and dried using a lyophilizer or freeze dryer. The percentages of yield for all extracts of each plant were calculated.

Decoction: Dried plant materials (100 g) were boiled in distilled water at boiling point for 30 min, filtered and dried by freeze dryer. The percentages of yield for all extracts of each plant were calculated.

Prasaprohyai preparation consists of twenty parts of proh hom and one part of each plant remainder.

The crude extracts were then kept in freezer (-20°C) until required.

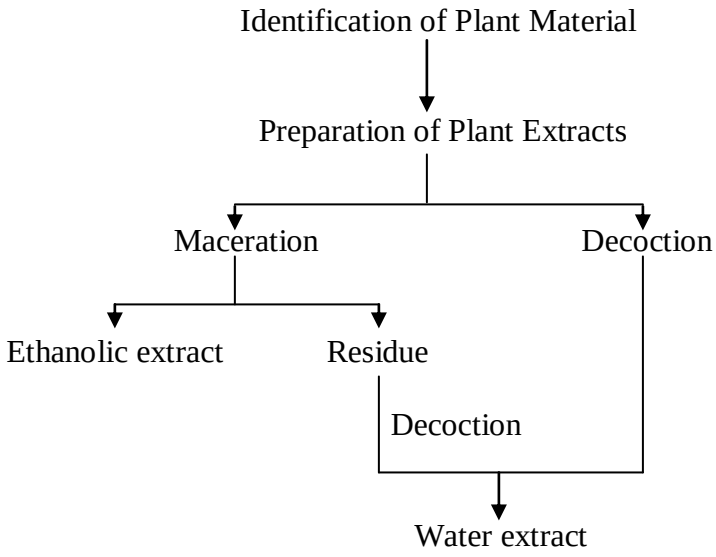

Figure 3.1

Extraction of plant materials

2.2 Assay for antioxidant activity

2.2.1 DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The antioxidant activity of these plant extractions was evaluated by DPPH radical scavenging assay. The assay was modified from those described by Yamasaki (1994). Samples for testing were dissolved in absolute ethanol or distilled water to obtain the highest concentration of 200 μg/ml. Each sample was further diluted for at least 4 concentrations (two-fold dilutions). Each concentration was tested in triplicate. A portion of the sample solution (100 μl) was mixed with an equal volume of 6×10^{-5} M DPPH (in absolute ethanol) and allowed to stand at room temperature for 30 min. The absorbance was then measured at 520 nm. BHT (butylated hydroxytoluene), a well known synthetic antioxidant, was tested in the same system as a positive standard. The scavenging activity of the samples corresponded to the reduction in the intensity of DPPH. Inhibition (%) was calculated using the following equation and EC_{50} values (effective concentration of sample required to scavenge DPPH radical by 50%) was calculated from the Prism program.

$$\% \text{ Inhibition} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100$$

2.3 *In vitro* assay for anti-inflammatory activity

2.3.1 Assay for NO inhibitory effect (Tewtrakul & Itharat, 2007; Tewtrakul & Subhadhirasakul, 2008)

For the assay, cells were washed with phosphate buffer saline (PBS) free of magnesium and calcium. The PBS was decanted and cells were harvested with 0.25% trypsin-EDTA and fresh medium was added. The cell pellet, was obtained by centrifugation (1000 rpm, 6 min), and was resuspended in 10 ml of medium to make a single cell suspension. The viable cells were counted by trypan blue exclusion in hematocytometer and diluted with medium to give a final concentration of 1×10^6 cells/ml for RAW 264.7. One hundred microlitres per well of these cells suspension were seeded in each 96-well microplates with 1×10^5 cells/well and allowed to adhere for 1 h at 37°C in 5% CO_2. After that, the medium was replaced with fresh medium containing 5 µg/ml of LPS (Lipopolysaccharide) together with test samples at various concentration levels and then incubated for 48 h. Each extract was initially dissolved in DMSO for ethanolic extracts, or dissolved in sterile distilled water, for water extracts. The extracts were diluted in medium to produce required concentrations. A hundred microlitres of each concentration was added to each well of plates to obtain final concentrations of 1-100 µg/ml. The final dilution used for treating the cells contained not more than 1% of the initial solvent; this concentration was used in the solvent control wells. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric method. Briefly, after 48 h incubation with test samples, MTT solution (10 µl, 5 mg/ml in PBS) was added to the wells. After 2 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of

the sample-treated group was less than 70-80% of that in the control (vehicle-treated) group. Indomethacin was used as positive controls. Inhibition (%) was calculated using the following equation and IC_{50} values were calculated from the Prism program.

$$\% \text{ Inhibition} = \frac{A - B}{A - C} \times 100$$

A – C: NO_2^- concentration (µM) [A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)]

2.3.2 Inhibitory effects on LPS-induced TNF-α release from RAW 264.7 cells (Tewtrakul & Itharat, 2007; Tewtrakul & Subhadhirasakul, 2008)

Inhibitory effects on the release of TNF-α from RAW 264.7 cells were evaluated using Quantikine mouse TNF-α ELISA test kit. Briefly, the cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing 5 µg/ml of LPS together with test samples at various concentration levels and incubated for 48 h. The supernatant (50 µl) was then transferred into 96-well ELISA plate and TNF-α concentrations were determined. The inhibition of TNF-α production was calculated by the following equation and IC_{50} values were calculated from the Prism program.

$$\% \text{ Inhibition} = \frac{A - B}{A - C} \times 100$$

A – C: TNF-α concentration (pg/ml) [A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)]

2.4 *In vitro* assay for anti-allergic activity

2.4.1 Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells (Tewtrakul & Subhadhirasakul, 2007)

Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells were evaluated by the following method (Tewtrakul & Subhadhirasakul, 2007). Briefly, cells were washed with phosphate buffer saline (PBS). The PBS was decanted and cells were harvested with 0.05% trypsin-EDTA and fresh medium was added. The cell pellet, obtained by centrifugation (1000 rpm, 6 min), were resuspended in 10 ml of medium to make a single cell suspension. The viable cells were counted by trypan blue exclusion in hematocytometer and diluted with medium to give a final concentration of 5×10^5 cells/ml for RBL-2H3. Volumes 400 µl/well of these cell suspensions were seeded in each 24-well microplates with 2×10^5 cells/well and allowed to adhere for 1 h at 37°C in 5% CO_2. After that RBL-2H3 cells cultured in 24-well microplates in culture media MEM were sensitized with anti-DNP IgE (anti-dinitrophenyl-immunoglobulin E) (0.45 µg/ml), then incubated 24 h at 37°C in 5% CO_2. The cells were washed with 400 µl of Siraganian buffer (buffer A) (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albumin (BSA) and 40 mM NaOH, pH 7.2) and then incubated in 160 µl of Siraganian buffer (buffer A) for an additional 10 min at 37°C. After that, 20 µl of test sample solution was added to each well and incubated for 10 min, followed by addition of 20 µl of antigen (DNP-BSA, final concentration is 10 µg/ml) at 37°C for 20 min to stimulate the cells to degranulate. The supernatant was transferred into 96-well microplates and incubated with 40 µl of substrate (PNAG) (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1.5 h. The reaction was stopped by adding 200 µl of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. Each extract was initially dissolved in DMSO for ethanolic extracts, or dissolved in sterile distilled water, for water extracts. The test sample solution was diluted in Siraganian buffer (final solution 0.1 mg/ml) to produce required concentrations. The inhibition (%) of

the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were calculated from the Prism program.

$$\% \text{ Inhibition} = \left(\frac{1 - (T - B - N)}{(C - N)} \right) \times 100$$

Control (C): DNP-BSA (+), test sample (-)

Test (T): DNP-BSA (+), test sample (+)

Blank (B): DNP-BSA (+), test sample (+)

Normal (N): DNP-BSA (-), test sample (-)

2.5 Statistical analysis

For statistical analysis, the values were expressed as mean ± SEM of three determinations. The EC_{50} and IC_{50} values were calculated using the Prism program. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.