CHAPTER 3

MATERIALS AND METHODS

This chapter gives details about the plant materials as component in Ridsriduanmahakarn preparation, parted use and to the methods for investigating. Details about animal cell lines are also given, they are RAW 264.7, LS174T and SW480. Brief description about chemical constituents are given (for more detail see Appendix). Then methodology for testing biological activities are described. Finally calculation of statistical from results is given.

3.1 Materials

3.1.1 Plant materials

Plant materials were collected from several parts of Thailand. Then parts of plants were prepared by drying and deriving powders from the plants. Each powder ingredient was macerated with 95% ethanol three times for 3 days. Rotary evaporator was used to reduce the pressure of the extracts. After that, we calculated the percentage of yield of the extracts and dissolve in dimethyl sulfoxide (DMSO) before bioassay. The place of collection, plant parts and voucher specimens are shown in Table 3-1. The voucher specimens are 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 Ridsidaungmahakan preparation

Species (Family)	Places for specimen collection	Voucher specimen number	Thai name	Part used
Anacyclus pyrethrum (L.) DC. (Compositae)	China	SKP051011601	Kot kakra	st
Anethum graveolens Linn. (Umbelliferae)	India	SKP199010701	Thian Ta Takkataen	fr
Angelica sylvestris Linn. (Umbelliferae)	China	SKP199011901	Kot so	r
Artemisia vulgaris Linn. (Compositae)	China	SKP051012201	Kot Jula lumpa	arp
Cinnamomum bejolghota (Buch.-Ham.) Sweet (Lauraceae)	Kanjanaburi, Thailand	SKP096030201	Samun vang	b
Cinnamomum zeylanicum Linn. (Lauraceae)	Kanjanaburi, Thailand	SKP096032601	Ob chuey Tet	b
Commiphora abyssinica (Berg.) Engel (Burseraceae)	India	SKP031030101	Mod yob	m
Cuminum cyminum Linn. (Umbelliferae)	India	SKP199030301	Thian khow	fr
Foeniculum vulgare Mill. (Umbelliferae)	India	SKP199062201	Thian Khow pluek	fr
Lepidium sativum Linn. (Asclepiadaceae)	India	SKP019121901	Thian dang	s
Myristica fragrans Linn. (Myristiceae)	Surat Thani, Thailand	SKP121130601	Luk jan	s
Myristica fragrans Linn. (Myristiceae)	Surat Thani, Thailand	SKP121130601	Dok jan	ar

Table 3-1 (continued)

Species (Family)	Places for specimen collection	Voucher specimen number	Thai name	Part used
Nigella sativa Linn. (Ranuncukaceae)	India	SKP160141901	Thian dum	s
Picrorrhiza kurroa Royle ex Benth	China	SKP177161101	Kot Kan Prow	rh
Piper chaba Hunt (Piperaceae)	Kanjanaburi, Thailand	SKP146160301	De plee	fr
Piper nigrum Linn. (Piperaceae)	Lopburi, Thailand	SKP146161401	Prik tai	s
Piper ribesioides Wall. (Piperaceae)	Lampoon, Thailand	SKP146161801	Sa kan	st
Platycladus orientalis (L.) Franco (Cupressaceae)	India	SKP0581161501	Son tet	st
Pouzolzia pentandra J.J. Bennett (Urticaceae)	Pathumtani, Thailand	SKP200161601	Khob cha nang khow	arp
Pouzolzia pentandra J.J. Bennett (Urticaceae)	Angthong, Thailand	SKP200161602	Khob cha nang dang	arp
Terminalia chebula Retz gall (Asclepiadaceae)	China	SKP019200301	Kot pung pla	g
Zingiber officinale Roscoe. (Zingiberaceae)	Angthong, Thailand	SKP206261501	Khing	rh

Part used: arp=aerial part, ar=aril, b=bark, fr=fruit, g=gall, m=myrrh, rh=rhizome, r=root, s=seed, st=stem

Table 3-2

Plants component in Ridsidaungmahakan preparation

 *Anacyclus pyrethrum* (L.) DC.	 *Anethum graveolens* Linn.
 *Angelica sylvestris* Linn.	 *Artemisia vulgaris* Linn.
 *Cinnamomum bejolghota* (Buch.-Ham.) Sweet	 *Cinnamomum zeylanicum* Linn.
 *Commiphora abyssinica (Berg.) Engel* or *C. molmol Engler*	 *Cuminum cyminum Linn.*

Table 3-2 (Continued)

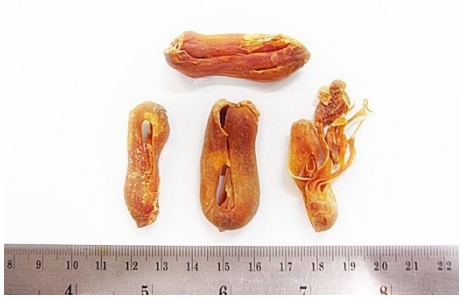
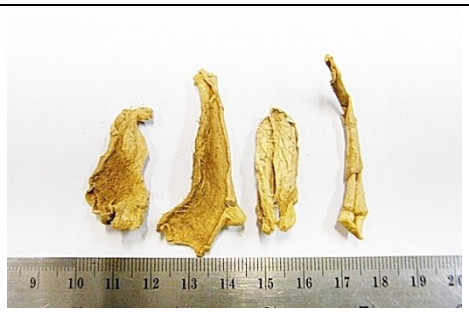
 *Foeniculum vulgare* Mill.	 *Lepidium sativum* Linn.
 *Myristica fragrans* Linn. (seed)	 *Myristica fragrans* Linn. **(aril)**
 *Nigella sativa Linn.*	 *Picrorrhiza kurroa Royle. Ex Benth.*
 *Piper chaba Hunt or P. retrofractum Vahl.*	 *Piper nigrum Linn.*

Table 3-2 (Continued)

<table>
<tr><td> 
Piper ribesioides Wall.</td><td> 
Platycladus orientalis (L.) Franco</td></tr>
<tr><td colspan="2"> 
Pouzolzia pentandra J.J. Bennett (red and white)</td></tr>
<tr><td> 
Terminalia chebula Retz gall</td><td> 
Zingiber officinale Roscoe.</td></tr>
</table>

3.1.2 Ridsriduangmahakan formula

Ridsiduangmahakan formular consists of A. pyrethrum 6.67%,*A. graveolens* 1.33%, *A. sylvestris* 6.67%, *A. vulgaris* 6.67%, *C. bejolghota* 6.67%, *C. zeylanicum* 6.67%, *C. abyssinica* 6.67%, *C. cyminum* 1.33%, *F. vulgare* 1.33%, *L. sativum* 1.33%, *M. fragrans* (aril) 6.67%, *M. fragrans* (seed) 6.67%, *N. sativa* 1.33%, *P. kurroa* 6.67%, *P. chaba* 2.20%, *P. nigrum* 2.20%, *P. ribesioides* 6.67%, *P. orientalis* 6.67%, *P. pentandra* (red) 3.33%, *P. pentandra* (white) 3.33%,*T. chebula* 6.67% and *Z. officinale* 2.20%.

3.2 Methods

3.2.1 Preparation of plant extracts

Each part used of the plant materials as shown in Table 3-1 were cleaned, sliced slab, dried at temperature of 50^{o}C in an oven and made to be powder. Then these ingredients were weighed to be formula of Ridsriduangmahakan preparation (RSD) showed above and mixed planted powder together. This preparation was extracted by maceration extraction method. It was macerated by 95% ethanol 3 days filtrated and concentrated to dryness under pressure. The marc was macerated 2 times and dried by evaporator. All extract of 3 times were combined and calculated percentage of yield. In the same patthern, each plant (300 g of each plant material) as the ingredients of Ridsriduangmahakan was also macerated by 95% ethanol 3 times and dried by evaporator All extracts of each plant was also combined and calculated percentages of yield. Then the crude extracts were kept in freezer (-20°C) until use.

3.2.2 Assay for quality control of Ridsiduangmahakan preparation and its plant ingredients

The quality control method of plant materials or crude drugs which were components of RSD preparation, were used and followed by Thai Herbal Pharmacopoeia used. The physical standardized were used in this investigation such as loss on drying (moisture content), total ash and acid insoluble ash for inorganic

contaminate , extractive value for quality of plant material and toxic heavy metals (As, Pb, Cd) were also determined.

3.2.2.1 Loss on drying

Moisture content is one of the most important factor for material quality and storability. It can be determined either by air oven or moisture meter. In this work, we used electronic Moisture analyzer for analysis of loss on drying. For this purpose, we placed sampled in the analyzer and put 5 grams of ingredient powder on samples for 30 minutes. After the operation, we took samples using forceps then we analyzed the recorded data.

3.2.2.2 Total ash and acid insoluble ash

For analysis of quantity of total ash and acid insoluble ash, we prepared crucible by cleaning and put in hot air oven (at temperature 105 ^{o}C) for 5 hours or until crucible's weight is stable. Then we put 2 grams of powder in prepared crucible and burned crucible using muffle furnace until the powder changed to grey color of ash. After that we put crucible into muffle furnace (450 ^{o}C) for 9 hours and placed crucible in desiccators to cool down. Then we weighed ash and then put crucible into muffle furnace at 450 ^{o}C for 5 hours and placed crucible in desiccators to cool down. Then we weighed ash again. We did this until crucible's weight is stable then we calculated total ash using the following formula:

$$\% \text{ total ash} = \frac{\text{Stable weight after burning(gram)}}{\text{Weight before burning (gram)}} \times 100$$

After we calculated total quantity of ash, we also calculated acid insoluble ash from following procedure: First we put total ash in beaker and pour 10% hydrochloric acid and then we boiled on hot plate for 5 minutes. After that we filtered boiled ash using Whatman paper no.42 and washed sample by distilled water until pH = 7. Then we put the filtered paper in crucible and burned in muffle furnace at 500 ^{o}C until the weight is stable. Finally we calculated acid insoluble ash using the following formula:

$$\% \text{ acid insoluble ash} = \frac{\text{Stable weight after burning(gram)}}{\text{Weight before burning (gram)}} \times 100$$

3.2.2.3 Extractive value

In method for analysis of quantity of extractive value, we separated into two procedures which corresponded to two solubles: ethanol and water. For analysis ethanol soluble extractive value is as follow:

Ethanol soluble extractive value

Macerate 5 g of the air-dried drug, coarsely powdered and accurately weighted, with 100.0 ml of ethanol of the specific strength in a closed flask for 24 hours. Shaking frequently during the first 6 hours and then allowing to stand for 18 hours. Filter rapidly and taking precautions against loss of ethanol then evaporate 20.0 ml of the filtrate to dryness in a tared, flat-bottomed, shallow dish and dry at 105 ^{o}C to constant weight. Finally, the percentage of ethanol-soluble extractive with reference to the air-dried drug were calculated using the formula:

$$\text{ethanol extractive value} = \frac{\text{Weight of the extracts(gram)}}{\text{Weight of powder before the process (gram)}} \times 100$$

Water soluble extractive value

Proceed as directed in Ethanol-soluble Extractive but using chloroform water instead of ethanol.

3.2.2.4 Determination for the presence of heavy metals by inductively coupled plasma-mass spectrometry (ICP-MS)

Method validation

Method validation is a process for calibration of linearity, accuracy, recovery and precision of digestion. In this research, we used Model 7500c ICP-MS spectrometer (Agilent Technologies Inc., Palo Alto, CA, USA) for analyzing and determination of 11 heavy metals, namely, Aluminium (Al), Cromium (Cr), Manganese (Mn), Iron (Fe), Nicle (Ni), Copper (Cu), Zinc (Zn), Arsenic (As),

Cadmium (Cd), Mercury (Hg) and Lead (Pb). Method validation is summarized as follow:

Linearity is a method to determine concentration of heavy metals in standard soluble. This method will determine concentration in 2 ranges. The first range is 5, 10, 20, 30, 40, 50 and 100 ppb. The other is 50, 100, 200, 300, 400, 500 and 1,000 ppb. (These series dilution use 5% nitric acid as solvent. Concentration of heavy metals were analyzed by ICP-MS. The analysis was repeated 3 times (n = 3) and then averaged concentrations of 11 heavy metals. Then we plotted average concentrations versus count and fitted the graph with linear equation $y = mx + c$. We found that r2 from calibration curve is greater than 0.99.

Accuracy is method for checking accuracy of concentration analysis. For this method, we determined concentration of 11 heavy metals used ICP-MS, 3 times (n = 3) and find % recovery (will discuss later). We found that % recovery is between 90.33% to 116.87% and standard deviation (SD) < 2.97.

Recovery test is method for indication accuracy of analysis, this method compares between experimental values and true values. Results from recovery test are represented as % recovery. Recovery test can perform by add appropriate concentration of spike in sample. Concentration range of spike is between 50% and 150% of expected quantity found in sample. Percentage recovery (% recovery) is calculated by (spike + sample) – sample / spike.

International Conference on Harmonoization recommends to investigate at least 3 different concentrations and each concentration should repeat 3 times. Our analysis has % recovery in the range 94.58% - 118.47% and SD < 3.93%. This result indicates that our analysis is accurate.

Repeatability indicates precision of analysis. For this method, researcher must repeat experiment with same method both intra-day and inter-day. In this work, we repeated 10 times of same experiment intra-day and inter-day (3 day interval). We calculated SD and get SD < 3.83 which indicates good reproducibility.

Limit of detection (LOD) is the least concentration that can be detected. Limit of quantification (LOQ) is the least concentration that can be quantitative analysis. LOD and LOQ can be found by analyzed concentration of standard dilution 10 ppb 10 times and calculated SD. LOD equals to 3 times of SD and LOQ equals to

10 times of SD. Our analysis found that LOD of Aluminium (Al), Cromium (Cr), Manganese (Mn), Iron (Fe), Nicle (Ni), Copper (Cu), Zinc (Zn), Cadmium (Cd), Mercury (Hg), Lead (Pb) and Arsenic (As) are 2.64, 0.94, 1.23, 4.95, 1.50, 2.55, 2.43, 1.74, 3.15, 4.89 and 4.29 μ/L respectively. LOQ of Aluminium (Al), Cromium (Cr), Manganese (Mn), Iron (Fe), Nicle (Ni), Copper (Cu), Zinc (Zn), Cadmium (Cd), Mercury (Hg), Lead (Pb) and Arsenic (As) are 8.80, 3.14, 4.08, 16.50, 5.00, 8.50, 8.10, 5.80, 10.50, 16.30 and 14.30 μ/L respectively.

3.2.2.5 Analysis of chemical fingerprint of Ridsiduangmahakan preparation

Analysis of chemical composition of Ridsiduangmahakan preparation and its ingredient using Gas Chromatography-Mass Spectrometry (GC-MS).

3.2.3 *In vitro* assay for anti-inflammatory activity

3.2.3.1 Inhibitory effects on LPS-induced Nitric Oxide release from RAW 264.7 (Tewtrakul and Itharat, 2007; Tewtrakul and Subhadhirasakul, 2008)

Animal Cell lines

RAW 264.7 murine macrophage leukemia cell line has been 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 which supplemented with 10% heated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Then the cells were incubated at temperature of 37°C, 95% humidity in 5% CO2 atmosphere. Cultured medium were changed three times a week.

The murine macrophage cells (RAW 264.7) cells were cultured in medium above and seeded 96-well plates with 1×10^5 cells/well at 37 °C in 5% CO_2 unless otherwise stated. The extracts were prepared at a concentration of 10 mg/ml in DMSO as stock solution and diluted as concentration 3, 10, 30 and 100 μg/ml with complete media. The cells were stimulated with 5 μg/ml Lipopolysaccharide (LPS, Sigma) together with test samples at various concentration for 48 hours. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using

the Griess reagent (0.1% naphthalene diamine dihydrochloride, 1% sulfanilamide in 5% H2SO4). Cytotoxicity testing were also tested to determine that nitric oxide production were not produced by destroying the cell membrane. This testing were performed using MTT assay or the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) colorimetric method. The absorbance were determined at 540 nm. The IC_{50} calculated in percentage by the Prism program using the equation:

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

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

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

Quantikine mouse TNF- α ELISA test kit (R&D Systems) provides inhibitory effects on the release of TNF- α from RAW 264.7 cells. The following process were followed: 1×10^5 cells were added into 96-well plates per well, incubate them at temperature of 37°C, 95% humidity in 5% CO_2 atmosphere for 1 hour. Then replace the medium with test samples in fresh medium containing 100 μg/ml of LPS at various concentrations. After that incubated them for 48 hours. In order to determine TNF- α concentrations, transfer supernatant 50 μl into 96-well plate. Then the inhibition of TNF-α were calculated using the Prism program governed by the equation:

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

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

3.2.3.3 Inhibitory effects on LPS- stimulated PGE_2 release from RAW 264.7 cells (Tewtrakul and Itharat, 2007; Tewtrakul and Subhadhirasakul, 2008 ; Hong et al., 2002)

The RAW 264.7 cells were cultured in 24-well culture plates (2.5×10^5 cells/ml) with 1 µg/ml Lipopolysaccharide stimulated macrophage (LPS, Sigma), incubate them at temperature of 37°C, 95% humidity in 5% CO_2 atmosphere for 24 hour. After the incubation period, the culture supernatant were collected, and the amount of PGE_2 were determined with a PGE_2 Enzyme Immuno-Assay Kit according to the manufacturer's instructions (Cayman Chemical Company). The productivity of PGE_2 were measured in relative productivity to that of the control treatment.

3.2.4 *In vitro* assay for cytotoxic activity by SRB assay (Skehan et al., 1990; Freshney, 1994)

Human Cancer Cell lines

Two types of human colon cancerous cell lines were used for testing cytotoxic activity . SW480 (ATCC CCL-228) as human colon cancerous cell line as colon, colorectal adenocarcinoma type was established from NCI and LS174T (ECACC 87060401) cell line as type B adenocarcinoma of colon were established from Europe. LS174T was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO™) containing 10% foetal bovine serum and 1% of 10,000 U penicillin and 10 mg/ml streptomycin. SW480 were cultured in RPMI1640 medium (GIBCO™) supplement with 10% heated foetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. (Itharat et al., 2003). The cells were maintained at 37°C in an incubator with 5% CO_2 and 95% humidity and the culture medium were changed twice a week.

Cytotoxic assay

The antiproliferative assay, SRB (sulphorhodamine B) assay, were performed according to the method of Skehan et al. (1990). This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. The principle of SRB, which is a bright pink aminoxanthene dye, is that it is an anionic protein stain containing two sulphonic groups, which bind electrostatically to

basic amino acid residues of cellular protein under mildly acidic condition. The protein-bound dye is extracted from cells and solubilized for spectrophotometry by weak bases. This colorimetric assay can be used to estimate cell number indirectly only for monolayer by providing a sensitive index of total cellular protein content which is linearly related to cell density. This assay was found to give good results over both high and low cell densities.

According to their growth profiles, the optimal plating densities of the cell line SW480 and LS174T were determined to be 3.0×10^3, 1.0×10^3 cells/well, respectively to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay (Skehan, et al., 1990). Cells growing as monolayer in a 25 cm^3 flask were washed with magnesium and calcium free phosphate buffer saline (PBS) pH 7.4. PBS was decanted and cells detached with 0.025% trypsin-EDTA to make a single cell suspension. The viable cells were counted by trypan blue exclusion in haemocytometer (Freshney, 1994) and diluted with medium to give a final concentration of $1x10^4$, $3x10^4$ cells/ml for SW480 and LS174T, respectively. 100 µl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24h the cells were treated with the extracts or pure compounds. Each extract or pure compounds were initially dissolved in a quantity of DMSO. According to National Cancer Institute guidelines (Boyd, 1997) extracts with IC_{50} values < 20 µg /ml were considered active. The extracts were diluted in medium to produce the required concentrations. 100 µl/well of each concentration was added to the plates to obtain final concentrations of 1, 10, 50, 100 µg/ml for the extract and 0.1, 1, 10, 50 µM for pure compound, the final mixture used for treating the cell contained not more than 1% of the solvent, the same as in solvent control wells. The plates were incubated for selected exposure time of 72 hours. At the end of each exposure time, the medium was removed. The wells were then washed with medium, and 200 µl of fresh medium were added to each well. The plates were incubated for a recovery period for 72 hours. On the seventh day of culture period, cells were fixed by 100 µl of ice-cold 40% trichloroacetic acid (TCA) per well, incubated at 4°C for 1 hour in the refrigerator and washed 5 times with tap

water to wash non viable cells, so viable cells were fixed as monolayer in each well. 50μl of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and left in contact with the cells for 30 min; then the plates were washed 4 times with 1% acetic acid until only dye adhering to the cells was left. The plates were dried and 100 μl of 10 mM Tris base (tris (hydroxy methyl) aminomethane, pH 10.5) was added to each well to solubilize the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (4 replicate) was read on Microplate reader at 492 nm as an indication of cell number. Cell survival was measured as the percentage absorbance compared with the control (non-treated cells). The IC_{50} values were calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine.

3.2.5 Bioassay-guided fractionation

Bioassay-guided fractionation is a procedure used for finding active compounds or markers or the preparation. In this research, we use a method called vacuum liquid chromatography (VLC). Ethanolic extracts from the preparation were separated using hexane (4×500 ml) (RSD1), hexane:chloroform (1:1) (4×500 ml) (RSD2), chloroform (4×500 ml) (RSD3), chloroform:methanol (1:1) (4×500 ml) (RSD4 and RSD5) and methanol (4×500 ml) (RSD6). After that, each fraction was dried and evaporated. Then we selected the highest activity for anti-inflammation fraction for isolated anti-inflammation compounds. This compound was used for stability study.

3.2.6 Isolation of chemical constituents from Ridsiduangmahakan preparation

Aliquot of RSD4 fraction (6.0 g) which showed the highest activity on NO inhibitory was separated by CC [silica gel with single solvent ;-chloroform] Twenty ml fractions were collected for eluting solvent and fraction combined, followed TLC examination [silica gel/ hexane:ethyl acetate (8:2)] and detection with acidic anisaldehyde spray. The fraction 13^{th} (from 34 fractions) 315 mg from RSD4 was separated by column chromatography [silica gel with hexane:ethyl acetate (8:2)]. Fifteen ml fractions were collected for eluting solvent and fraction combined.

Compound 1 (48.2 mg or 0.80%) was isolated as yellow crystals from fraction 44-66 and recrystalized in MeOH.

The structure of isolates (Figure 3-1) was identified as piperine by comparison on TLC and HPLC chromatography with authentic sample purchased from Merck and was a major product isolated. We tested pure compounds for anti-inflammatory activity: NO inhibitory effect (see also Section 3.2.3.1).

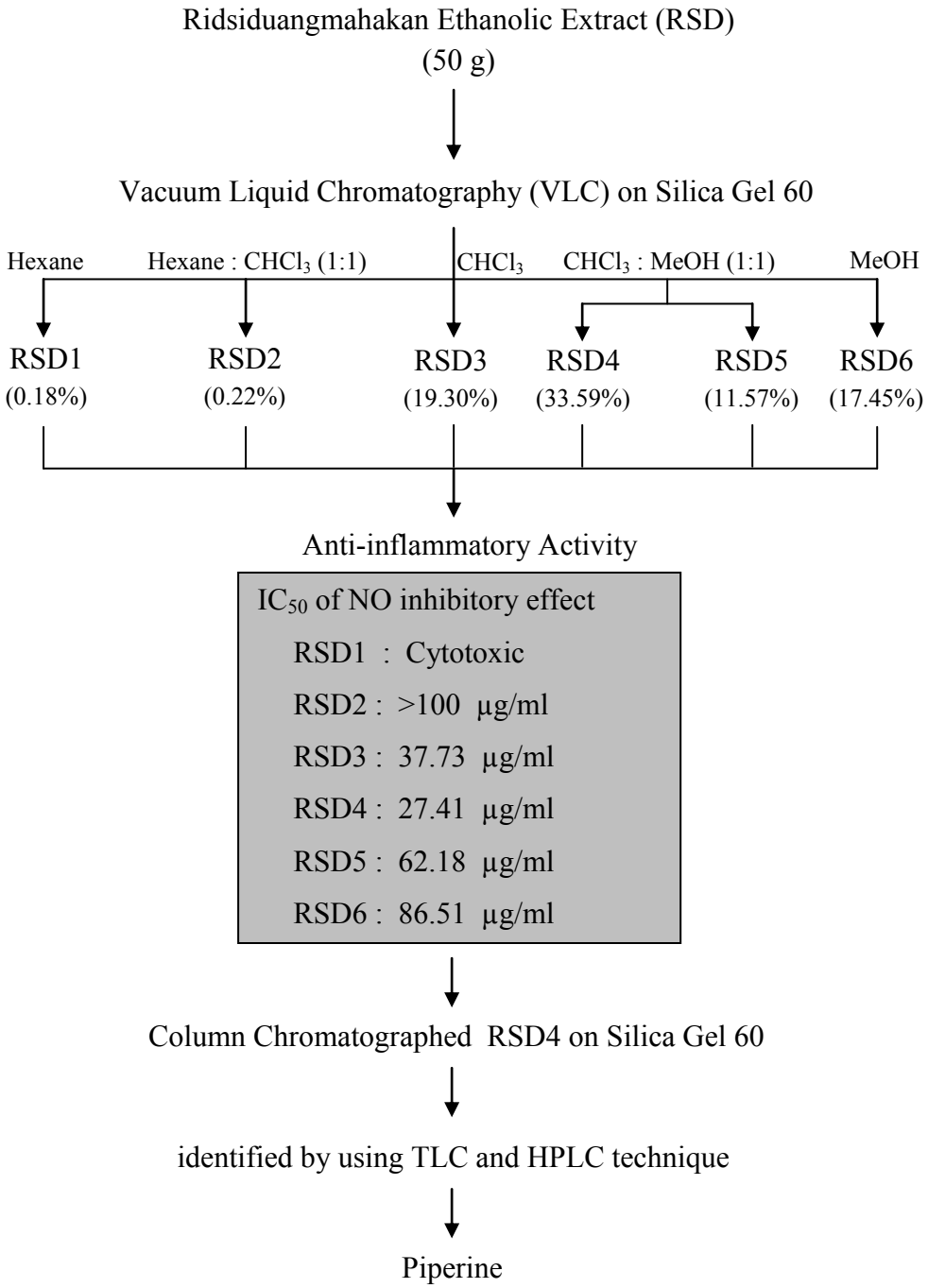
Figure 3-1 Structure of Piperine

3.2.7 The stability study on RSD extract

Stability testing was done using transparent vials. We exposed all samples under 45±2°C with 75±5% RH as accelerated testing (Koseywattana, 2002) for a period of 4 months. The apparatus utilized for these tests were the desiccators and incubators. We tested the samples on day 0, 15, 30, 60, 90 and 120. The content of marker compounds was evaluated by using Gas Chromatography-Mass Spectrometry and high performance liquid chromatography.

3.2.8 Statistical analysis

For statistical analysis, the values were expressed as mean ± SEM of three determinations. The IC_{50} values were calculated using the Prism program.



Schemes 3-1

Bioassay-guided fractionation of the ethanolic extract of Ridsiduangmahakan preparation