CHAPTER 2

EXPERIMENTAL

Materials

1. The lime used in the experiment were *Citrus aurantifilia, var.* They were purchased from the markets in Hatyai district, Songkhla province, Thailand. The seeds of *Citrus* fruits were collected and cleaned with water, and dried in a hot air oven (Kanseri®, Thailand) at 105°C for 12 hours. Then the dried seeds were ground to obtain a powder with a particle size of 0.125 mm, and kept in a dessicator with phosphorous pentoxide.
2. The water was doubled-distilled de-ionized water, purchased from the Songklanakarin Hospital, Faculty of Medicine, Prince of Songkla University.
3. Male mice of Swiss albino were obtained from Southern Laboratory Animal Facility, Faculty of Sciences, Prince of Songkla University. All animals were maintained at a controlled temperature (23$\pm$1°C) and under the guidelines for care and use of animals specified by the Faculty of Sciences, Prince of Songkla University.
4. Acetic acid was analytical grade of J.T.Baker®, USA. Cat. No. UN2789, Batch No. 518735 017603241
5. Acetone was analytical grade of Mercks®, Damstad, Germany. Cat. No. 100014, Batch No. 2914 11 00
6. Acetonitrile was HPLC grade of Labscan®, Ireland. Cat. No. C2502U, Batch No. 04 10 0064
7. Ammonium chloride solution was purchased from Biochrome®, Berlin, Germany.

8. Benzene was analytical grade of Mercks®, Damstad, Germany. Cat. No. 101783, Batch No. 2902 20 90
9. C18 cartridge, 6.0 ml of Supelco®, Bellefonte, USA. Model LC-18 SPE, Code No. 57054, Lot No. SP2760A

10. Chloroform was analytical grade of Mercks®, Damstad, Germany. Cat. No. 102445, Batch No. 903K11297563
11. Ethanol was analytical grade of Mercks®, Damstad, Germany. Cat. No. 100983, Batch No. 2207 10 00
12. Ethylenediaminetetraacetic acid disodium salt was purchased from Carlo Erba®, Italy. Code No. 405491, Lot No. 2987D100
13. FACSFlow optimized sheath fluid for use on flowcytometer instrumentation, Becton Dickinson, USA. Cat. No. 342003
14. Filter flask, pyrex, USA. Code No. 5340
15. Filter holder of Filtrationsvorsatz, Sartorius AG®, USA. Model 16514-E, 13 mm, Lot No. 16514 10/03
16. Filter polyamid of Sartolon 0.2 μm, Sartorius AG®, USA. Model 25007, 13 mm, Lot No. 0703 25007 0340073
17. Filter polyamid of Sartolon 0.45 μm, Sartorius AG®, USA. Model 25007, 47 mm, Lot No. 0802 25007 0240483
18. Fluoresbrite plain YG 2.0 μ, Polysciences®, Narrington, USA. Cat. No. 18338, Lot No. 509135
19. Glycerin was lab grade of Vidtayasom®, Thailand.
20. Hank Balanced Salt Solutions (HBSS) was purchased from Biochrome KG®, Berlin, Germany. Cat. No. L201-01, Lot No. 373A
21. Heparin sodium solution USP, 5000 unit/ml, Kamada®, Israel. Reg. No. 1C52/44, Lot No. H5186A

22. Isopropanol was analytical grade of Mercks®, Damstad, Germany. Cat. No.
23. Methanol was analytical grade of Labscan®, Ireland. Cat. No. Labscan®, Ireland. Cat. No. A3513J, Batch No. 04 03 1114
24. Methylene chloride was analytical grade of Labscan®, Ireland. Cat. No. A3508L, Batch No. 2K030113
25. N-2-hydroxy-ethyl-piperazine-N'-2-ethane sulfonic acid (HEPES) was purchased from Biochrome KG®, Berlin, Germany. Cat. No. L1603, Lot No. B32501
26. *para* dimethylaminobenzaldehyde was analytical grade of Mercks®, Damstad, Germany. Cat. No. 103058, Batch No. 2922 30 00
27. Petroleum ether was analytical grade of Labscan®, Ireland. Cat. No. NA3814U, Batch No. 0101 0156
28. Phosphate Buffer Saline (PBS) was purchased from Biochrome KG®, Berlin, Germany. Cat. No. L182-01, Lot No. 401B
29. Phosphorous pentoxide was analytical grade of Mercks®, Damstad, Germany. Cat. No. 100570, Batch No. 2809 10 00
30. Sodium chloride, BP. 1993, was industrial and pharmaceutical grade of Merck®, Germany. Cat. No. K26229600
31. Standard limonin used in the quantitative and qualitative analysis was purchased from Sigma®, St.Louis, USA. Cat. No. L-9647, Lot No. 120K4833
32. Trypan blue 0.4% was purchased from Gibco® BRL, USA. Cat. No. 15250-061, Lot No. 1076476
33. EDTA-PBS was PBS containing 3 mM ethylenediaminetetraacetic acid disodium salt.
34. HEPES Hank Buffer Solutions (HBSS-HEPES) was HBSS containing 25 mM HEPES

Equipment

1. Centrifuge system for 15,000 rpm of Hermle®, Germany. Model Z323K, Serial No. 43030012
2. Centrifuge system for 4,000 rpm of Hettich®, Germany. Model universal 16 R, Cat. No. D78532, Serial No. 5561-02
3. Differential Scanning Calorimeter System, Perkin Elmer®, USA. Model 1020 DSC7, Serial No. 139553
4. Flowcytometer of FACScalibur®, Becton-Dickinson, U.S.A. Serial No. E1367
5. FT-IR Spectrometer, Bruker®, Germany. Model EQUINOX55, Serial No. NN36-99
6. Hood for killing mice was modified by the Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The hood was cleaned by UV-sterilization and by 70%v/v alcohol spraying.
7. Hot air oven, Kanseri®, Sataporn Engineering, Thailand. Type STP, Serial No. B01
8. HPLC column of Water®, Japan, 5 µm, C-18, 250 x 4.6 mm, Part No. PSS839540, Serial No. 0126300821
9. HPLC system of Shimadzu®, Japan. Consisted of system controller, Model SCL-10 AVp, Cat. No. 228-34350-38, Serial No. C2103908951CD; UV-Vis detector, Model SPD-10 AVp, Cat. No. 228-40000-38, Serial No. C20993907373LP; Autoinjector, Model SIL-10ADVp, Cat. No. 228-39005-38, Serial No. C21054007398SA; Column oven, Model CTO-10ASVp, Serial No. C21043600898KL; Liquid chromatogram, Model LC-10ADVp, Cat. No. 228-38000-38, Serial No. C20963810020LP and Model FCV-10ALVp, Cat. No. 228-

34700-39, Serial No. C21083903068KL; Degasser, Model DGU-14A, Cat. No. 228-35350-36, Serial No. C20923906127CN

10. Incubator of Memmert®, Western Germany. For 80°C was model UL80, Serial No. 850 063; for 70°C was model BE600, Serial No. 600 0237; for 45°C was model B50, Serial No. 820 219;
11. Inverted microscope of Olympus®, Japan. Model CK2, Serial No. T2-6M08521
12. Laboratory CO2 Incubator of Biosafe Plus Integra Bioscience, USA. Model INW-5000-T-7C-VBA70020, Serial No. 012F-291649-0F
13. MS Spectrometer, ThermoFinnigan®, Germany. Model MAT 95 XL, Serial No. 2803
14. NMR spectrometer, Varian®, Germany. Model unity INOVA 500 MHz, Serial No. C009013
15. pH-meter, Denver Instrument®, USA. Model 50, Serial No. C0022680.
16. Polarimeter of Atago®, USA. Model polax-L, Serial No. 971716
17. The soxhlet apparatus was modified by the Department of Food Technology, Faculty of Agro-industry, Prince of Songkla University.
18. Vacuum evaporator of Napco®, Japan. Model 5861 E-series, Cat. No. 5861-220, Serial No. 9609-001
19. Vacuum manifold processing station, Agilent Technologies, USA.
20. Vacuum pump of KnF Neuberger®, USA. Model UN0353TTP, Serial No. 373204

Methodology and Analysis

1. Isolation of Limonin

1.1 Extraction and Purification

The method of extraction used in the study was as described by Pifferi *et al*. (1993). The lemon seeds (1 Kg) were ground to obtain a powder after drying these seeds at 105°C for 12 hours. The powder was then placed in a soxhlet extractor and defatted with 3 L of petroleum ether for 8 hours. It was then extracted using 3 L of acetone for 12 hours. The acetonic extract was then concentrated to about 100 ml under vacuum and at a temperature no higher than 30°C in order to avoid browning reactions. Three volumes (~300 ml) of petroleum ether were added to the concentrate stirred at 20°C in order to cause the limonin to precipitate, the yellow-colored solution thus obtained, mainly containing carotenoids, fats and essential oils were discarded. The precipitate, made up of a crystalline white phase (consisting of limonoids) and an amorphous yellow one (consisting of flavonoids, phospholipids, traces of fats and gummy components), were washed three times with 20 ml of 95% aqueous ethanol. The raw limonin thus obtained was then purified by dissolving in 25 ml of methylene chloride at 25°C, and recrystallized by dilution in 50 ml of isopropanol which was added drop by drop with stirring. The resulting crystals were filtered through a buchner funnel. Some limonin crystals were also added to the mother liquors and cooled to 4°C, after 12 hours the remaining limonin was precipitated, separated by filtering and dried at about 40°C overnight.

1.2 Evaluation and Statistical Analysis

The yield of limonin was calculated and the purity was established in triplicate. Data was given as mean ± S.D.

2. Identification of Limonin

2.1 Chemical Analyses

The limonoid extracts were analyzed as follows:-

2.1.1 TLC analysis using limonin standard prepared to approximately 1 g/L in acetonitrile, then 20 µL of limonin in acetonitrile was spotted onto silica gel TLC plates and

developed in a solvent consisting of benzene, ethanol, water and acetic acid (200:47:15:1 v/v/v/v, upper phase), or in a solvent consisting of benzene, ethanol, water, acetic acid, and isopropanol (185:47:30:1:15 v/v/v/v/v, upper phase). The plate was then carefully dried and sprayed with Ehrlich's reagent (5% *para* dimethylaminobenzaldehyde in ethanol), and then exposing to hydrogen chloride gas in order to detect the limonin as reddish orange spot.

2.1.2 NMR analysis was determined by obtaining a ^{1}H NMR spectrum using NMR spectrometer, Varian®, Germany.

2.1.3 IR analysis was determined by using a FT-IR Spectrometer , Bruker®, Germany, Model EQUINOX55, using a frequency range of 4000 to 400 cm^{-1}.

2.1.4 Mass spectrum was determined by using MS Spectrometer, ThermoFinnigan®, Germany, Model MAT 95 XL using a low resolution electron ionization mode.

2.1.5 Optical rotation of 1% limonin in acetone was determined by using polarimeter of Atago®, USA. Model polax-L

2.2 Physical Analyses

Some useful properties of the limonin were studied to access the stability and biological predictive data, as following:-

2.2.1 Taste and color were simple tested by sensation.

2.2.2 Melting point was determined by Differential Scanning Calorimeter System of Perkin Elmer®, USA. Model 1020DSC7.

2.2.3 Acidity was determined by pH-meter of Denver®, USA. Model 50

2.2.4 Molecular weight was determined by mass spectrometry using Spectrometer of ThermoFinnigan®, Germany, Model

MAT 95 XL, using a low resolution electron ionization mode.

2.2.5 Solubility was determined by weighting 5 mg. of limonin mixed incrementally with 1 ml of tested solvent, sonicated at room temperature for 2-3 min. The solubility was observed. If limonin dissolved in any solvent, some 5 mg. of limonin was added to the solution, and the solubility observed. But if limonin could not dissolve in any solvent, 1 ml. of solvent was added until limonin dissolved or till 10 ml. The tested solvents were as follows: water, ethyl alcohol, acetone, acetonitrile, chloroform and glycerin.

2.3 Statistical analysis.

The necessary data was given as mean ± S.D.

3. Quantitative Analysis of Limonin

3.1 HPLC Analysis.

A calibration curve of standard limonin was constructed and determined by injection of 50 μL of five standards containing different concentration of limonin in the solvent mobile phase, ranging from 5.0 to 25.0 ppm.(5, 10, 15, 20, and 25 ppm.) The conditions of HPLC system analysis was as follows:- column-Water 5 μm C-18, 250 x 4.6 mm; solvent of acetonitrile and water, 40:60 v/v; isocratic elution; flow rate of 1.0 ml/min at 30°C, with detection at 207 nm, injection volume of 50 μL. The standard limonin content in acetonitrile and water, 40:60 v/v was measured by HPLC analysis after filtration through a 0.2 μm membrane filter.

3.2 Statistical Analysis

All data was given as mean ± S.D. The calibration curve of standard limonin was done both intra-day and inter-day for 4 times a day and for consecutive 4 days respectively.

4. Stability Study of Limonin from Lime Seeds in Aqueous Solution

Stability of limonin from lime seeds was evaluated according to the experiment of Garrett and Seyda (1983) and Pifferi *et al*. (1993).

4.1 Acid Hydrolysis

Aliquot of acetonic solutions of limonin (1,000 ppm) was prepared. One ml of the acetonic aliquot solution of limonin was mixed with 39 ml of standardized phosphate buffer pH of 1.0 to 6.0 (Table 3). Two ml. of buffered sample was put in tightly closed 10 ml vial. The buffered samples in tightly closed vials were placed in 80, 70, and 45°C thermostat hot air oven with 70% humidity, three samples for each pH and three samples for each temperature as well.

Table 3 Buffer used in the experiments (Citrate-phosphate-borate/ hydrochloride universal buffer)

pH	Stock Solution A N/3 Citric cid solution 100 ml N/3 Phosphoric acid solution 100 ml 1N Sodium hydroxide solution 343 ml Orthoboric acid crystal 3.54 g. Water to male 1,000 ml (ml)	Stock Solution B Hydrochloric acid 0.1 N (ml)	Made up to (ml)
2.0	74.4	20	100
3.0	56.9	20	100
4.0	50.7	20	100
5.0	45.4	20	100
6.0	39.7	20	100
7.0	32.9	20	100
8.0	28.1	20	100
9.0	24.0	20	100

10.0	18.1	20	100
11.0	14.7	20	100
12.0	1.3	20	100

Source: Diem and Lentner (1973) pp.280-282.

The vials were withdrawn periodically, every 30 min for 5 hours (30, 60, 90, 120, 150, 180…..min), and every day for 5 days, ice-cooled to stop the reaction, The buffered reaction mixture was passed through a 6.0 ml Supelco® C18 cartridge, treated with 2 ml of acetonitrile and then with 5.0 ml of distilled water. After filtration of the sample, the column was washed three times with 2 ml distilled water and then with 2 ml acetonitrile. After concentration in vacuum at 30°C, the residue was taken up with 2 ml of the solvent of acetonitrile and water, 40:60 v/v, and analysis was performed by injection of a 50 µL sample. The limonin content in 2 ml of acetonitrile and water, 40:60 v/v was analyzed after filtration through a 0.2 µm membrane filter and the concentration of extracted limonin in all the above sample were measured by HPLC analysis (see section 3.1)

4.2 Alkaline Hydrolysis

The experiments were taken in the same way as the study of acid hydrolysis, but aliquots of acetonic solution of limonin (1,000 ppm) were prepared and diluted to 25 ppm in standardized phosphate buffer pH of 7.0 to 12.0 (table 3). The same conditions and methods were used, three samples for each pH and three samples for each temperature as well.

4.3 Reaction Rate and Reaction Order

A plot of the percentage of remaining limonin both in normal value and natural logarithm value as Y-axis against time at various pH and temperature was made, and the reaction order was predicted. The reaction rate was calculated and the plot of pH-rate profile was made, as well as the activation energy was calculated.

4.4 Statistical Analysis

The data were obtained in triplicate. All data were given as mean ± S.D. Statistical difference between data was determined by univariate analysis of variance (UNIANOVA) using Scheffe's Method and significant values was represented by $P<0.05$

5. Stability Study of Limonin from Lime Seeds in Solid Phase

Limonin (5 mg.) was weighed in tightly closed 1 ml vials and placed in 80, 70, and 45°C, three samples for each temperature. The vials were withdrawn periodically, every 10 days for 3 months (10, 20, 30, 40, 50, 60…..days) and every month for a year, ice-cooled to stop the reaction, and the processed limonin was dissolved in 5 ml of acetonitrile. Then the limonin solution was diluted with a solvent of acetonitrile and water, 40:60 v/v, to 25 ppm. The diluted sample was then passed through a 6.0 ml Supelco® C18 cartridge, treated with 2 ml of acetonitrile and then with 5.0 ml of distilled water. After filtration of the sample, the column was washed three times with 2 ml distilled water and then with 2 ml acetonitrile. After concentration in vacuum at 30°C, the residue was taken up with 2 ml of the solvent of acetonitrile and water, 40:60 v/v, and a 50 µL sample injected into the HPLC column (see section 3.1) to measure the concentration of limonin.

The data were triplicate done. All data were given as mean ± S.D. Statistical difference between data was determined by univariate analysis of variance (UNIANOVA) using Scheffe's Method and significant values was represented by $P<0.05$

Semilogarithic plots of limonin degraded concentration against time at various temperatures was made (Y-axis was concentration in log scale, X-axis was time with normal scale), and the stability was discussed and predicted

6. The Immunological Evaluation of Limonin from Lime Seeds

6.1 Macrophage-Stimulation Activity of Limonin

Macrophage-stimulation activity of limonin from lime seeds was evaluated by counting the number of exudated cells in the peritoneal cavity after stimulation by limonin orally administered to the mice. The phagocytic activity was indicated by the number of microparticles taken up by peritoneal exudated cell (PEC).

6.1.1 Animal Preparation

A total of 96 male mice (7-10 weeks-old, 20-26 grams weight) were divided into 8 groups.

Group I (12 mice) were fed with basal diet with normal water treatment and untreated with limonin or vehicle.

Group II (12 mice) were treated orally per animal per day with vehicle (PBS) 0.5 ml. Four mice were fed for 2 days. Another 4 mice were fed for 4 days. And 4 mice were fed for 6 days.

Group III (12 mice) were treated orally per animal per day with 0.5 ml of 5 ppm of extracted limonin suspended in PBS. Four mice were fed for 2 days. Another 4 mice were fed for 4 days. And 4 mice were fed for 6 days.

Group IV (12 mice) were treated orally per animal per day with 0.5 ml of 10 ppm of extracted limonin suspended in PBS. Four mice were fed for 2 days. Another 4 mice were fed for 4 days. And 4 mice were fed for 6 days.

Group V (12 mice) were treated orally per animal per day with 0.5 ml of 20 ppm of extracted limonin suspended in PBS. Four mice were fed for 2 days. Another 4 mice were fed for 4 days. And 4 mice were fed for 6 days.

Group VI (12 mice) were treated orally per animal per day with 0.5 ml of 50 ppm of extracted limonin suspended in PBS. Four mice were fed for 2 days. Another 4 mice were fed for 4 days. And 4 mice were fed for 6 days.

Group VII (12 mice) were treated orally per animal per day with 0.5 ml of 100 ppm of extracted limonin suspended in PBS. Four mice were fed for 2 days. Another 4 mice were fed for 4 days. And 4 mice were fed for 6 days.

Group VIII (12 mice) were treated orally per animal per day with 0.5 ml of 200 ppm of extracted limonin suspended in PBS. Four mice were fed for 2 days. Another 4 mice were fed for 4 days. And 4 mice were fed for 6 days.

On the designated days after the administration, the mice were killed by cervical dislocation, and peritoneal exudated cells (PEC) were isolated from each mouse to evaluate the macrophage activities.

6.1.2 PEC Preparation

PEC was isolated from the peritoneal cavity of each mouse by lavaging with 5 ml of HBSS according to the method of Yoshizawa *et al*. (1996). After centrifugation at 4,000 rpm for 20 min, 4°C, the cell pellet was washed twice with HBSS and resuspended in 1 ml of HBSS-HEPES. This experiment was carried out in the antiseptic environmental modified hood (UV-sterilization and spraying with alcohol 70%v/v).

6.1.3 Peritoneal Cell-Inducing Activity

PEC was counted by a hemacytometer and according to the procedure of Brousseau *et al*. (1995). The hemacytometer and the cover-glass was cleaned with ethanol and rinsed with distilled water and dried completely. The

cover-glass was laid across the two counting areas and pressed on the slides. The 0.1 ml of PEC suspension was gently mixed with 0.1 ml of the 0.4% trypan blue solution using a pipette in a disposable tube. The colored PEC fluid was incubated at 37°C for 10 min. and then dropped to the edge of the cover glass and on the slanted loading platform. The fluid was then released until it reached the level of the cover-glass and began to seep over the counting area. The pipette was then removed, allowing the counting chamber to fill by capillary action. If bubbles formed under the cover glass, an insufficient amount of fluid addition was indicated, on the other hand, if fluid was overflowing into the moats, the procedure needed repeating on the unused side of the chamber. The cell was allowed to settle for about 3 min, and the hemacytometer placed on the microscope stage, and moved so that the optical field was filled by the central area.

The hemacytometer consisted of 25 large squares enclosed by triple lines, and each of them was divided into 16 smaller squares. The cells in five large squares, the four corners and one of the central large squares were counted. Counting was within 5 to 15 min after introduction of the cell suspension onto the counting area under the cover-glass. If the total was less than 30 cells, the number of cells in the total counting area (25 large squares) was counted. The two counting areas of the hemacytometer for the same sample were counted and a mean value was calculated. A maximum different of 10% between the two counts was acceptable.

The concentration of the cell suspension was calculated using the following equation

$$[\ ] \text{ cells/ml} = X\, Y \times 10^4$$

Where X = total number of cells in 25 squares

Y = dilution factor

For example, calculating the cell concentration per ml, a dilution is 1:200 and total of 500 cells were counted in five large squares. So the total number of cells counted in five large squares was adjusted to 1 mm^2 by multiplying 5, multiplied by the dilution, and then by 10^4 (x10 to adjust for 1 mm^3 since the chamber depth is 0.1 mm and x10^3 to adjust for 1 cm^3). The cells/ml was calculated as: 500 x 5 x 200 x 10^4 = 5 x 10^9 cells/ml.

6.1.4 Evaluation of the Phagocytic Activity

The phagocytic activity of PEC was evaluated by an *in vitro* culture with fluorescent microparticles according to the method of Yoshizawa *et al.* (1996). A PEC portion (density of suspension is 1 x 10^5/ml) was centrifuged at 4,000 rpm for 20 min, 4°C, and resuspended to 100 µL of HBSS-HEPES. Twenty milliliters of fluoresbrite carboxylate microsphere (2.0 µm) diluted 100-fold with HBSS-HEPES was added to PEC 1 x 10^5/ml in HBSS-HEPES, and the suspension was incubated at 37°C for 1 hour. After stopping the reaction by adding 2 ml of cold EDTA-PBS and collecting a cell pellet by centrifugation at 4,000 rpm for 20 min, 4°C, the pellet was suspended in 300 µL of EDTA-PBS and was measured for phagocytic activity with a flow-cytometer of Facalibur®, Becton-Dickinson, USA.

The flow-cytometer was used to measure the number of particles per cell according to the method of Ichinose *et al.* (1994) and Tachavanich *et al.* (1996). Flow-cytometric analysis was performed using argon laser light source equipped with 488 nm excitation wavelength. Fluorescence

parameters from single cells was collected using a logarithmic amplifier after gating on the combination of the forward light scatter (FSC) and side light scatter (SSC). The green fluorescence (FL1) from fluoresbrite carboxylate microsphere was collected through a 530 nm bandpass filter. Analysis was performed on 10,000 macrophages per tube. Data was acquired in the list mode and process using CellQuest® software (Becton Dickinson) with an attached computer. A histogram of phagocytosis contents (X-axis, FL1) *versus* counts (Y-axis) was displayed. The percentage of phagocytosis cell and the phagocytosis index was calculated.

The percentage of phagocytosis cell (PP) defined as the percentage of macrophages with one or more ingested particles within the total macrophage population (10,000 cells).

The phagocytosis index (PI) defined the average number of ingested particles per macrophage. So PI could be calculated as follows:

$$PI = \frac{\text{The number of total ingested beads}}{\text{The number of total macrophages analyzed (10,000 cells)}}$$

The total number of ingested beads was obtained by summing (number of one-bead-ingested cells) x 1, (number of two-bead-ingested cells) x 2, (number of three-bead-ingested cells) x 3, (number of four-bead-ingested cells) x 4, and (number of more than four-bead-ingested cells) x (mean number of ingested beads in more than four-bead-ingested cells population).

Number of cells and means of ingested beads was obtained as histogram statistics by the computer system connected to the flow-cytometer.

6.2 Determination of the Effect of Limonin on the Hematological Parameters

The hematological parameter concerned in the study was total white blood cell (WBC), which was counted according to the method of Raphael and Kuttan (2003). Eight groups of male mice (7-10 weeks-old, 20-26 grams weight) n = 6 per group were used in the experiment.

Group I was kept as untreated control.

Group II was treated orally per animal per day with vehicle (PBS) 0.5 ml.

Group III was treated orally per animal per day with 0.5 ml of 5 ppm of extracted limonin suspended in PBS.

Group IV was treated orally per animal per day with 0.5 ml of 10 ppm of extracted limonin suspended in PBS.

Group V was treated orally per animal per day with 0.5 ml of 20 ppm of extracted limonin suspended in PBS.

Group VI was treated orally per animal per day with 0.5 ml of 50 ppm of extracted limonin suspended in PBS.

Group VII was treated orally per animal per day with 0.5 ml of 100 ppm of extracted limonin suspended in PBS.

Group VIII was treated orally per animal per day with 0.5 ml of 200 ppm of extracted limonin suspended in PBS.

All animal were fed for 1 month. The change in the body weight was recorded. Total white blood cell count (total WBC count) was recorded every third day for one month. Blood was collected from the tail vein and 100

μl of blood was mixed with 3 ml of 0.15 M NH_4Cl and incubated for 10 min at room temperature. Then white blood cells could be obtained by centrifugation at 4,000 rpm at 4°C for 20 min. The supernatant was removed and discarded. The cell pellet was washed by adding 5 ml of PBS and centrifuging at 4,000 rpm at 4°C for 20 min. The supernatant was discarded and the cell pellet re-suspended in 100 μl of PBS. The WBC was strained by adding 100 μl of 0.2% trypan blue in PBS, and incubating at 37°C for 10 min

WBC was counted by a hemacytometer according to the procedure of Brousseau *et al*. (1995). The counting chamber was filled as described in section 6.1.3. Counting and calculating was conducted using the same procedure as described in section 6.1.3 as well..

6.3 Determination of the Effect of Limonin on Production of a Specific Antibody

The production of specific antibody induced by limonin was determined according to the experiment of Raphael and Kuttan (2003).

6.3.1 Animal Preparation

Fifty-four male mice (7-10 weeks-old, 20-26 grams weight) were divided into 9 groups of n = 6 per group. All animal were fed for 1 month.

Groups I and II were kept as untreated control.

Group III was treated orally per animal per day with 0.5 ml of vehicle (PBS).

Group IV was treated orally per animal per day with 0.5 ml of 5 ppm of extracted limonin suspended in PBS.

Group V was treated orally per animal per day with 0.5 ml of 10 ppm of extracted limonin suspended in PBS.

Group VI was treated orally per animal per day with 0.5 ml of 20 ppm of extracted limonin suspended in PBS.

Group VII was treated orally per animal per day with 0.5 ml of 50 ppm of extracted limonin suspended in PBS.

Group VIII was treated orally per animal per day with 0.5 ml of 100 ppm of extracted limonin suspended in PBS.

Group IX was treated orally per animal per day with 0.5 ml of 200 ppm of extracted limonin suspended in PBS.

6.3.2 Animal Immunization

All the animals except group I were immunized with 0.2 ml of 20% sheep red blood cells (SRBC) per mouse per day, subcutaneous route on the back leg of the mice, along

with the first dose of limonin administration. Immunization was conducted every day for 5 days. After the fifth immunization, the animals were used to collect the antiserum.

6.3.3 Preparation of Specific Antiserum

After the fifth immunization, blood was collected from the tail vein and continuously collected every third day for one month. Serum was separated by centrifugation at 15,000 rpm at 4°C for 10 min and heat-inactivated at 56°C for 1 hour.

6.3.4 Assay for Antibody Titer

Antibody titer was determined by agglutination assay using 2% SRBC as antigen according to the method of Garvey *et al*. (1977).

A 96-wells-microplate was used, twelve wells for a set of a test. The wells were numbered automatically both the row and the column. The 100 μl of Phosphate Buffer Solution pH 7.2 was pipette into each of the wells of the plate. Then 100 μl of antiserum was put into well number 1 of the row, and mixed thoroughly by gently pulling the fluid up and down in the pipette, but avoiding bubbles. Using a clean pipette, 100 μl of the mixture from well number 1 of the row was transferred to well number 2 of the row, and mixed thoroughly as previous described. The same pipette was used to transfer 100 μl from well number 2 of the row to well number 3 of the row. This two fold serial dilution procedure was continued through well number 11. Then 100 μl was discarded from well number 11, so that all eleven wells were consecutive declined concentration of antiserum from ½, ¼, 1/8, 1/16 and so on. Well number 12 of the row

now served as the antigen control and therefore contained no antiserum. The 100 µl of 2% SRBC was added to each of the wells, and the contents mixed by shaking the microplate vigorously three or four times. The plate was then placed in an incubator at 56°C for 1 day, and then the titer of the antiserum was read.

6.4 Statistical Analysis

All data were given as mean ± S.D.

6.4.1 The data of macrophage-stimulation activity of limonin was determined the difference between groups by multivariate analysis of variance (MANOVA) using Scheffe's contrast method and significant values was represented by $P<0.05$

6.4.2 The data of effect of limonin on total white blood cell count was determined the difference between groups by univariate analysis of variance (UNIANOVA) using Scheffe's contrast method and significant values was represented by $P<0.05$

6.4.3 The data of effect of limonin on specific antibody titer was determined the difference between groups by univariate analysis of variance (UNIANOVA) using Scheffe's contrast method and significant values was represented by $P<0.05$

7. The Effect of Processing Technique on Limonin Content in Lime Juices

7.1 Lime Juices Preparation

Lime was purchased from the markets in Hatyai district, Songkhla province, which is in the southern part of Thailand. The surface of the lime was cleaned, cut into two parts with a sterilized knife. The first half was expressed by hand to make lime juice of 300 ml; the second half was expressed through a mechanical machine to make lime juice of 300 ml. Then

100 ml lime juice expressed by hand was heated to 100°C for 1 hour. Another 100 ml lime juice expressed by hand was frozen to 0°C. And 100 ml lime juice expressed by hand was untreated. One hundred milliliters aliquot of lime juice expressed mechanically was treated as previous described too.

7.2 Quantitative Evaluation of Processed Limonin

The lime juice or samples was centrifuged at 4,000 rpm, at room temperature, for 20 min. Then 2 ml of the sample was passed through a 6.0 ml Supelco® C18 cartridge, treated with 2 ml of acetonitrile and then with 5.0 ml of distilled water. After filtration of the sample, the column was washed three times with 2 ml distilled water and then with 2 ml acetonitrile. After concentration in vacuum at 30°C, the residue was made up to 2 ml with a solvent of acetonitrile and water, 40:60 v/v, and analysis was performed by injection of a 50 µL sample. The limonin content was analyzed after filtration through a 0.2 µm membrane filter and the concentration of limonin in all of above sample was measured by HPLC analysis (see section 3.1).

7.3 Immunological Evaluation of Processed limonin

Lime juices from different processes was tested for the effect of limonin on the hematological parameters and the production of specific antibody

7.3.1 Determination of the effect of limonin on the hematological parameters was done using the same techniques as described in 5.2

Seven groups of male mice (7-10 weeks-old, 20-26 grams weight) n = 6 per group were used in the experiment.

Group I was fed orally per animal per day with 0.5 ml. of lime juice expressed by hand

Group II was fed orally per animal per day with 0.5 ml. of lime juice expressed by machine.

Group III was fed orally per animal per day with 0.5 ml. of lime juice expressed by hand and heated 100°C for 1 hour.

Group IV was fed orally per animal per day with 0.5 ml. of lime juice expressed by machine and heated 100°C for 1 hour.

Group V was fed orally per animal per day with 0.5 ml. of lime juice expressed by hand and frozen 0°C for 1 day, then melted before feeding to the animal.

Group VI was fed orally per animal per day with 0.5 ml. of lime juice expressed by machine and frozen 0°C for 1 day, then melted before feeding to the animal.

Group VII was kept as untreated control.

All groups of animals were fed for 1 month. The change in the body weight was recorded. Blood was collected from the tail vein and 0.1 ml of blood was mixed with 3 ml of 0.15 M NH_4Cl and incubated for 10 min at room temperature. Then white blood cells could be obtained by centrifugation at 4,000 rpm at 4°C for 20 min. The supernatant was removed and discarded. PBS was added and centrifuged at 4,000 rpm 4°C for 20 min. The supernatant was discarded and the cell pellet re-suspended in 0.1 ml of PBS. Then 0.1 ml of 2% trypan blue solution was mixed to strain the cell. White blood cells were counted by a hemacytometer according to the procedure of Brousseau *et al*. (1995). The counting chamber was filled as described in section 6.1.3. Total white blood cell count (WBC count) was recorded every third day for one month. Counting and calculating were conducted using the same equation as described in section 6.1.3.

7.3.2 Determination of the effect of the production of specific antibody was done the same techniques as described in 6.3

Eight groups of male mice (7-10 weeks-old, 20-26 grams weight) n = 6 per group were used in the experiment.

Group I was fed orally per animal per day with 0.5 ml. of lime juice expressed by hand

Group II was fed orally per animal per day with 0.5 ml. of lime juice expressed by machine.

Group III was fed orally per animal per day with 0.5 ml. of lime juice expressed by hand and heated 100°C for 1 hour.

Group IV was fed orally per animal per day with 0.5 ml. of lime juice expressed by machine and heated 100°C for 1 hour.

Group V was fed orally per animal per day with 0.5 ml. of lime juice expressed by hand and frozen 0°C for 1 day, then melted before feeding to the animal.

Group VI was fed orally per animal per day with 0.5 ml. of lime juice expressed by machine and frozen 0°C for 1 day, then melted before feeding to the animal.

Group VII and group VIII were kept as untreated control.

All groups of the animals except group VIII were immunized with 0.2 ml of 20% sheep red blood cells (SRBC) per mouse per day, subcutaneous route on the back leg of the mice, along with the first dose of limonin administration. Immunization was conducted every day for 5 days. After the fifth immunization, the animals were used to collect the antiserum. Blood was collected from the tail vein and

continuously collected every third day for one month. Serum was separated by centrifugation at 15,000 rpm at $4^{o}C$ for 10 min and heat-inactivated at $56^{o}C$ for 1 hour. Antibody titer was determined by agglutination assay using 2% SRBC as antigen as described in 6.3.4.

7.3 Statistical Analysis

All data was given as mean ± S.D. Statistical difference between groups was determined by univariate analysis of variance (UNIANOVA) using Scheffe's contrast method and significant values was represented by $p<0.05$