

CHAPTER 2

MATERIALS AND METHODS

The study is divided into four parts.

Part I Preparation of Thai medicinal plant extracts

Part II *In vitro* assay for the antibacterial activity of the plant extracts

Part III *In vitro* assay for the cytotoxic activity of the studied plant extracts on gingival connective tissue fibroblasts

Part IV *In vitro* assay for the anti-inflammatory activity of the studied plant extracts

Part I Preparation of Thai medicinal plant extracts

Materials

1. Five Thai medicinal plants of which seven parts were obtained: leaves and bark of *Ao*, dried-fruit of *Tb*, leaves and bark of *Sc*, pericarp of *Pn*, and leaves of *Rn*.
2. Ethanol
3. Dimethyl sulfoxide (DMSO) (MERCK®)
4. Distilled water (DW)

Equipment

1. Incubator
2. Balance
3. Rotary evaporation machine
4. Water bath (Memmert)
5. Freeze dried machine

Method

Seven parts of the medicinal plants were studied. *Ao* leaves and bark, *Sc* leaves and bark, *Pn* pericarp, and leaves of *Rn* were collected from plants of the same age, size, and place in Songkhla, Southern Thailand. *Tb* dried-fruit was purchased from a Thai traditional medicine shop in Hatyai, Songkhla. Botanical identification of the studied plants was acquired by Thai herbalists at the Faculty of Pharmaceutical Sciences, Prince of Songkla University.

The studied plants were cleaned, weighed and recorded, shade dried in the incubator at 50°C for 1 day, then re-weighed and recorded. The plants were then cut into small pieces, and then weighed and recorded. The next step, dried ground plant material was macerated with pure ethanol for 3 days and then filtrated and evaporated solvent from the first extract. This process was repeated for 3 times, thus obtaining 3 sets of measurements. Because the studied extracts were sticky and wet (except *Sc* and *Rn* leaves), the wet studied plant extracts were freeze dried, weighed and stored in 2-8°C. The weights of the studied plant extracts are shown in Table 2-1.

Each sample of plant extracts was then dissolved with DMSO and mixed well. From pilot studies *Ao* leaves, *Sc* leaves and bark, *Pn* pericarp, and *Rn* leaves extracts were more easily dissolved than *Ao* bark and *Tb* dried-fruit extracts. So selected *Ao* leaves, *Sc* leaves and bark, *Pn* pericarp, and *Rn* leaves extracts 1,000 mg were prepared by DMSO 2.5 ml and distilled water 7.5 ml to get the concentration of 100 mg/ml and *Ao* bark and *Tb* dried-fruit extracts 5,000 mg prepared by DMSO 5 ml and DW 5 ml to get the concentration of 500 mg/ml (Table 2-2). The next step we centrifuged each plant extract solution at 4,500 rounds per minute (rpm), for 30 minutes and stored in 2-8°C.

Table 2-1 Weights of the studied plant extracts after drying, volume of used ethanol, and the weight of the studied plant extracts after filtration, evaporation of solvent and freeze drying in Part I experiment (Note; *, not freeze dried)

Step \ Plant extracts	<i>Ao</i>		<i>Tb</i> dried-fruit	<i>Sc</i>		<i>Pn</i> pericarp	<i>Rn</i> leaves
	leaves	bark		leaves	bark		
Weight after dried (g)	220	390	540	566.15	170	523.96	80
Volume of ethanol for evaporated (ml)	1,400	1,350	700	1,800	700	800	500
Weight of the plant extracts after freeze drying (g)	17.87	19.29	37.4	49	4.96*	68.88	4.12*
Wet part (g)	7.14	7.62	-	-	-	-	-

Table 2-2 Concentration of each plant extracts prepared

Plant extracts	Weight of plant extracts (g)	DMSO/distilled water (ml/ml) (%DMSO)	Beginning concentration of plant extracts (mg/ml)
<i>Ao</i> leaves	1	2.5/7.5 (25)	100
<i>Ao</i> bark	5	5/5 (50)	500
<i>Tb</i> dried-fruit	5	5/5 (50)	500
<i>Sc</i> leaves	1	2.5/7.5 (25)	100
<i>Sc</i> bark	1	2.5/7.5 (25)	100
<i>Pn</i> pericarp	1	2.5/7.5 (25)	100
<i>Rn</i> leaves	1	2.5/7.5 (25)	100

Part II *In vitro* assay for the antibacterial activity of the plant extracts

Materials

1. Seven prepared plant extracts from part I
2. Dimethyl sulfoxide (DMSO) (MERCK[®])
3. Distilled water
4. Brain heart infusion (BHI) (MERCK[®])
5. Yeast extract
6. Bacto agar (Nunton[®])
7. Blood
8. Vitamin K (Menadione of Sigma[®])
9. Hemin (Sigma[®])
10. Brain heart broth (BHB) (MERCK[®])
11. Ministart[®]
12. 96 wells plate (NUNCTONTM)
13. Loop
14. Cup (diameter 6 mm)
15. Forceps
16. Sterile Petri dishes
17. Vernier caliper
18. Magnetic bar
19. Test tubes
20. Glass pipette 1 ml, 5 ml, 10 ml, 20 ml
21. Vortex mixer (Vortex Genie 2)
22. Automatic pipette 1-10 μ l, 10-100 μ l, 100-1,000 μ l (Drummond, Drummond scientific co. Broomall PA, USA)
23. Micropipette tips
24. 0.5 McFarland concentration (1.5×10^8 CFU/ml) standard
25. Flask 125 ml, 250 ml, 500 ml, 1,000 ml

26. Black paper sheet
27. Glass bottles
28. Beaker 50 ml, 100 ml, 500 ml, 1,000 ml

Equipment

1. Anaerobic Incubator (Thermo-Forma, USA)
2. Adventure™ Pro (Ohaus corporation, Pine Brook, NJ, USA)
3. Autoclave (Hiclave™ HV-50, Hirayama co.)
4. Water bath (Mettler)
5. Hot air oven (Mettler)
6. UV/Visible spectro-photometer (Ultraspec 3300/Pharmacia of Amersham Biosciences)

Microorganism

1. *Porphyromonas gingivalis* (*Pg*) (ATCC 33277)
2. *Aggregatibacter actinomycetemcomitans* (*Aa*) (Clinical isolate)
3. *Prevotella intermedia* (*Pi*) (Clinical isolate)

Standard antibiotic

Metronidazole as a control for *Pg*

This part consists of 3 experiments. Antibacterial experiments were modified from Koneman, *et al.*, 1988.⁶¹

1. Screening the antibacterial activity against *Porphyromonas gingivalis* (*Pg*), *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Prevotella intermedia* (*Pi*) by agar diffusion method

2. Conducting the minimal inhibitory concentration (MIC) of the extracts by broth microdilution method
3. Evaluating the minimal bactericidal concentration (MBC) using blood agar plate

Method

1. Screening the antibacterial activity against *Porphyromonas gingivalis* (*Pg*), *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Prevotella intermedia* (*Pi*) by agar diffusion method

Each of *Pg*, *Aa* and *Pi* 800 µl were mixed well with Brain Heart Infusion (BHI) agar 25 ml. The bacteria mixture was poured into sterile petri dishes containing 3 or 4 cups (6 mm diameter) and allowed to set. Each plant extracts, metronidazole 0.1 mg/ml, 25%, 50% DMSO and DW was added 100 µl/well. The plates were placed in an anaerobic condition containing N₂:CO₂:H₂= 80:10:10 at 37 ° C for 48 hours.

The antibacterial activity was recorded if the inhibition zone was greater than 7 mm (Figure 2-1). The experiments were repeated 3 times.

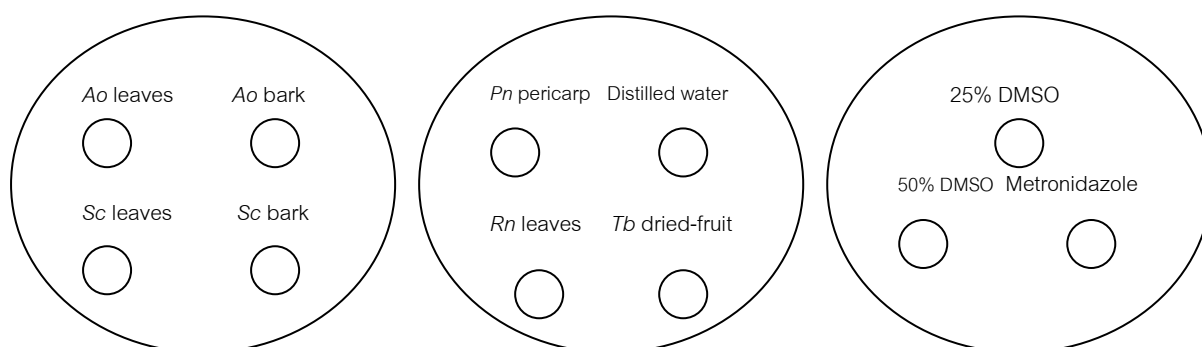


Figure 2-1 Location of each tested cups in plates of the antibacterial activity screening. (Cups of *Ao* leaves, *Ao* bark, *Sc* leaves and *Sc* bark extracts were tested in the same plate, cups of *Pn* pericarp, distilled water, *Rn* leaves and *Tb* dried-fruit extracts were tested in the same plate, and cups of 25% DMSO, 50% DMSO, and metronidazole 0.1mg/ml were tested in the same plate)

2. Determination of Minimal Inhibitory Concentration (MIC) of the extracts

MIC was determined by using the broth microdilution method. Using a 96-well plate, each well was filled with 100 μ l of BHI and serial broth microdilution was done by adding 100 μ l of each beginning concentration of plant extract (100 mg/ml of *Ao*, *Sc*, *Rn* leave, *Sc* bark, and *Pn* pericarp and 500 mg/ml of *Ao* bark and *Tb* dried-fruit) into the first well, mixed well and pipetted 100 μ l to the next right well until finished at the well number 11. The 12th well was used for intra-experiment control (Table 2-3).

Controls consisted of a) positive control: metronidazole for *Pg* b) negative control: broth and microorganism c) intra-experimental controls: pure broth, broth and plant extracts without microorganism, 25% and 50%DMSO and broth with microorganism.

Pg, *Aa* and *Pi* 0.5 MacFarland concentration (1.5×10^8 CFU/ml) 100 μ l were then added to the prepared wells.

All plates were incubated in an anaerobic condition for 48 hours. The MIC was recorded if the solution did not appear turbid of bacterial accumulation when compared with the negative control. The experiments were repeated 5 times.

The 96 well plate consisted of 8 rows (row A-H) and 12 columns (1st -12th column). In this experiment (Figure 2-2 and final concentration presented in Table 2-3)

Row A represented tested *Ao* leave extracts at the MIC in 1st -11th columns.

Row B represented tested *Rn* leave extracts at the MIC in 1st -11th columns.

Row C represented tested *Sc* leave extracts at the MIC in 1st -11th columns.

Row D represented tested *Sc* bark extracts at the MIC in 1st -11th columns.

Row E represented tested *Pn* pericarp extracts at the MIC in 1st -11th columns.

Row F represented tested *Ao* bark extracts at the MIC in 1st -11th columns.

Row G represented tested *Tb* dried-fruit extracts at 5.95 mg/ml in 1st -11th columns.

Row H represented tested metronidazole at the MIC in 1st -6th columns (positive control).

12th column (row A-G wells) were intra-experiment control (broth+plant extracts of each row, not added microorganism).

7th column of row H was intra-experiment control of metronidazole.

8th-10th column of row H were intra-experiment control of 10, 25, and 50% DMSO (broth+DMSO+microorganism).

11th column of row H was negative control (broth+microorganism).

12th column of row H was intra-experiment control (pure broth).

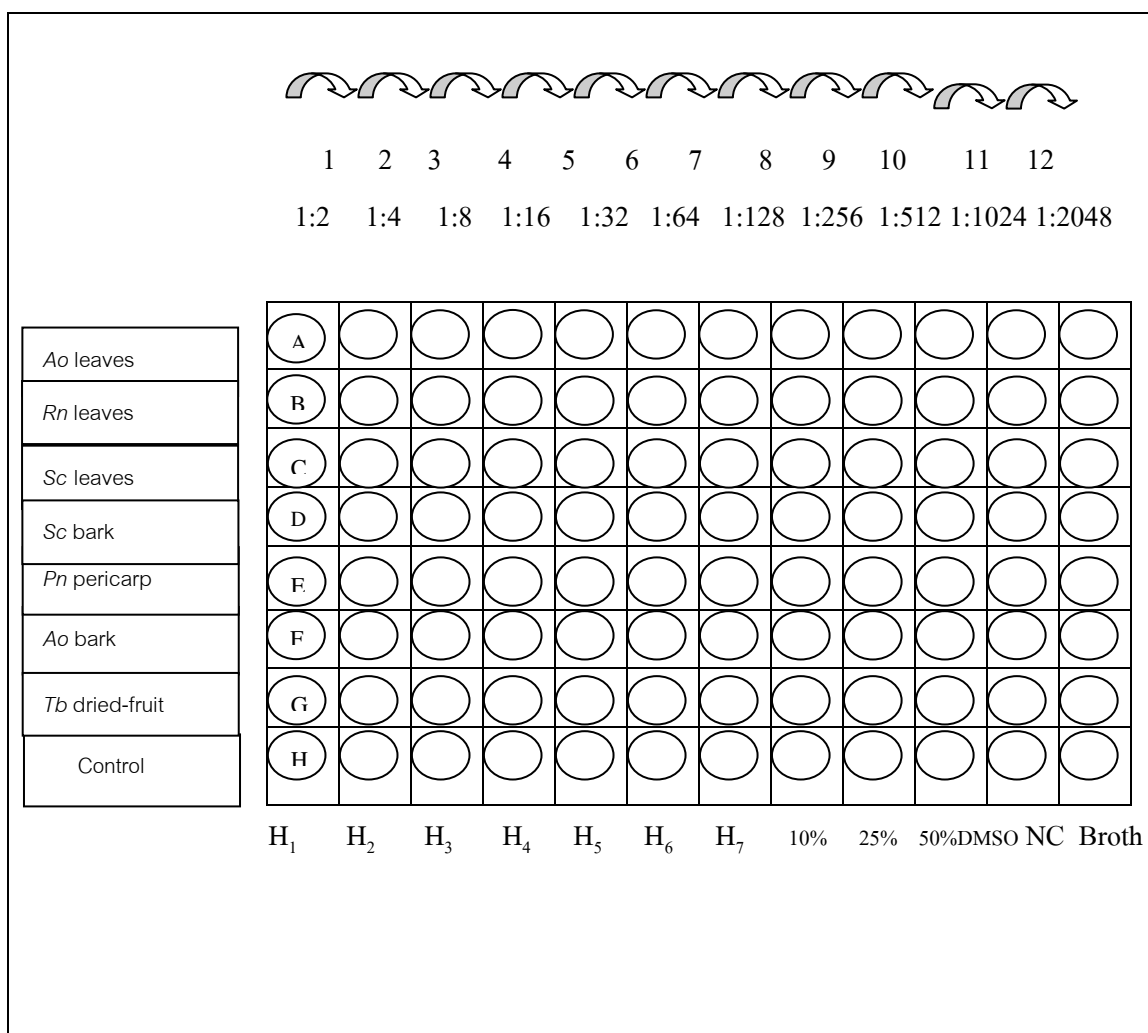


Figure 2-2 Method of broth microdilution for exploring the Minimal Inhibitory Concentration (MIC). (Note; NC= Negative control)

Table 2-3 Concentration of each studied plant extract and control groups in each well of 96-well plate of the broth microdilution method for exploring the Minimal Inhibitory Concentration (MIC) (Note; IC= Intra-experiment control, micro= Microorganism)

Well no. (mg/ml)	1	2	3	4	5	6	7	8	9	10	11	12
<i>Ao</i> leaves (Row A)	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.097	0.048	0.024	IC
<i>Rn</i> leaves (Row B)	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.097	0.048	0.024	IC
<i>Sc</i> leaves (Row C)	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.097	0.048	0.024	IC
<i>Sc</i> bark (Row D)	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.097	0.048	0.024	IC
<i>Pn</i> Pericarp (Row E)	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.097	0.048	0.024	IC
<i>Ao</i> bark (Row F)	125	62.5	31.25	15.62	7.8	3.9	1.95	0.97	0.48	0.24	0.12	IC
<i>Tb</i> dried- fruit (Row G)	125	62.5	31.25	15.62	7.8	3.9	1.95	0.97	0.48	0.24	0.12	IC
Metroni dazole (Row H)	0.025	0.0125	0.0062	0.0031	0.0016	0.0008	Metro +broth	10% DMSO +broth +micro	25% DMSO +broth +micro	50% DMSO +broth +micro	broth+ micro (positive control)	Only broth

3. Evaluating the Minimal Bactericidal Concentration (MBC) using blood agar plate.

After completing the broth microdilution method for exploring the MIC of studied plant extracts, we now confirm the results of this experiment by evaluating the MBC using a blood agar plate. The solution from each well was dropped onto blood agar plates and incubated under anaerobic conditions for 2 days. The last concentration was recorded as the MBC if there was no evidence of *Pg*, *Aa* and *Pi* colony formation. The experiments were repeated 5 times.

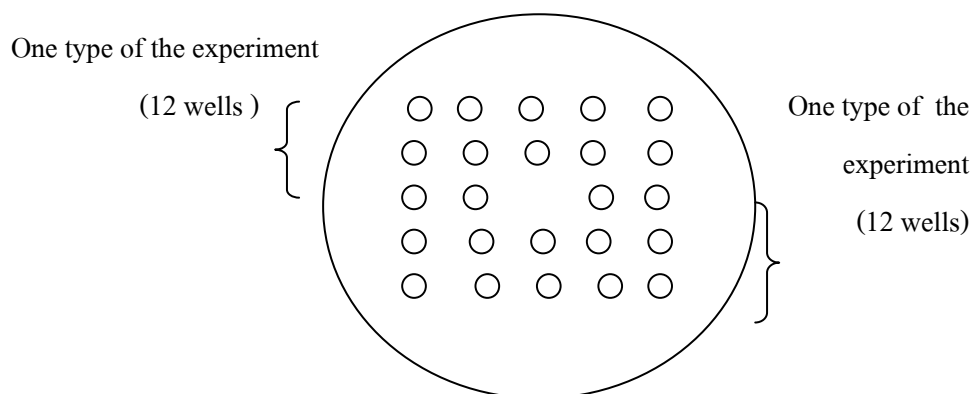


Figure 2-3 Example of the minimal bactericidal concentration (MBC) method.

Statistical analysis

Descriptive statistics are presented.

Part III *In vitro* assay for the cytotoxic activity of the studied plant extracts on gingival connective tissue fibroblasts (from Part II)

Materials

1. Seven prepared plant extracts from part I
2. Dimethyl sulfoxide (DMSO) (MERCK®)
3. Distilled water
4. 96 wells plate (NUNCTON™)
5. Forceps
6. Glass pipette 1 ml, 2 ml, 5 ml, 10 ml, 20 ml
7. Fetal bovine serum (FBS)
8. Magnetic bar
9. Dulbecco-MEM (DMEM)
10. Vortex mixer (Vortex Genie 2)
11. Automatic pipette 1-10 µl, 10-100 µl, 100-1,000 µl (Drummond, Drummond scientific co. Broomall PA, USA)
12. Micropipette tips 200 µl, 1,000 µl
13. Flask 125 ml, 250 ml, 500 ml, 1,000 ml
14. Beaker 50 ml, 100 ml, 500 ml, 1,000 ml
15. Phosphate buffered saline (PBS)
16. MTT agent (Sigma, USA)
17. Culture plate
18. Tissue Culture flask 25, 75 cm²
19. Trypsin/EDTA solution
20. Penicillin/Streptomycin
21. Gentamycin
22. Fungizone
23. Centrifuge tube 15, 50 ml
24. Ministart 0.45 µl 26 ml

Equipment

1. AdventureTM Pro (Ohaus corporation, Pine Brook, NJ, USA)
2. Autoclave (HiclaveTM HV-50, Hirayama co.)
3. Water bath (Mettler)
4. Hot air oven (Mettler)
5. UV/Visible spectro-photometer (Ultraspec 3300/Pharmacia of Amersham Biosciences)
6. Microflow Laminar (Biohazard Laminar Air Flow Class II, Astec Microflow, UK)
7. Liquid Nitrogen Tank (Taylor-Wharton)
8. Nikon Antimould (Hollywood international co. , LTD, Japan)
9. BiotrakTM visible plate reader (Amersham Biosciences, USA)
10. Hot plate stirrer (Corning)
11. pH meter (Precisa pH900)
12. Eppendorf centrifuge 5417c (Pharmed and science)
13. Spectromicroscope
14. Refrigerated centrifuge (Labofuge[®])
15. Inverted Microscope (Nikon Eclipse, TE2000-U)

Method

1. Cell culture

Human gingival fibroblast (HGF) derived from healthy gingival tissue under the Prince of Songkla University Ethic committee regulation. Cells were cultured in Dulbecco-MEM (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 µg/ml of penicillin, 100 µg/ml of streptomycin), glutamine and non-essential amino acid at 37° C in humidified atmosphere of 95% air and 5% CO₂.

2. Cytotoxicity of plant extracts

Using 96-well culture plate, HGF (passage 5th-10th) were added to each well at 5×10^3 cells in 200 μ l. At the subconfluent stage, the cells were quiescent in DMEM supplemented with 2% FBS for 8 hours before adding the plant extracts at the concentrations of 100, 50, 20 and 2 times of their MIC. Controls consisted of a) positive control: metronidazole b) negative control: HGF cells only c) Intra-experimental controls: DMEM supplemented with 2% FBS, 25% and 50% DMSO.

After incubation for 24 hours, the subconfluent cells were then treated with plant extracts at concentrations mentioned above at 12, 24, or 48 hours. The control group consisted of a) positive control: HGF cells only b) intra-experimental controls: DMEM supplemented with 2% FBS, DMSO at the concentration of MIC and MBC for *Pg* of each plant extracts, 25% and 50% DMSO, and metronidazole.

The positions of each tested plant extracts and controls consisted of; (Figure 2-4)

Row 1A-C, row 2A-C represent *Ao* leaves extracts 100, 50 and 2 times of MIC.

Row 1E-F, row 2E-F represent *Sc* leaves extracts 50 and 2 times of MIC.

Row 1H, row 2H represent *Rn* leaves extracts 2 time of MIC.

Row 3A-B, row 4A-B represent *Sc* bark extracts 10 and 2 times of MIC.

Row 3C-E, row 4C-E represent *Pn* pericarp extracts 100, 50 and 2 times of MIC.

Row 3G-H, row 4G-H, row 5A, row 6A represent *Ao* bark extracts 100, 50 and 2 times of MIC.

Row 5C-E, row 6C-E represent *Tb* dried-fruit extracts 148.75, 59.5, and 5.95 mg/ml.

Row 5F, row 6F represent control (HGF cells and DMEM supplemented with 2% FBS).

Row 6G-H represent metronidazole 100 times of MIC.

Row 7A-C, row 8A-C represent metronidazole 50, 20, and 2 times of MIC.

Row 7D-G, row 8D-G represent DMSO 600, 300, 100, and 10 μ l/ml.

Row 11A, row 12A represent the blank.

Row 11C, row 12C represent pure DMEM supplemented with 2% FBS.

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	○	○	○	○	○	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	○	○	○	○	○	○	○	○	○	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○

Figure 2-4 Positions of each tested plant extracts and controls

MTT assay

MTT (Sigma, USA) solution was prepared as 5 mg/ml in DMEM just before use and filtered through a 0.22- μ m filter to sterilize and remove a small amount of insoluble residue. The method of MTT assay described by Kasugai *et al.*,⁶² was used with some modification. Briefly, after 12, 24, or 48 hours, 10 μ l of MTT solution was added to each well, and the incubation was continued for 4 hours. Then the entire medium was removed by gently inverting and tapping the plates. After the plates were washed with 100 μ l of PBS twice, the wells were filled with 100 μ l DMSO. The plates were stirred to enhance the dissolution of the MTT formazan. The spectrophotometric absorbance at 562 nm then was measured by an ELISA reader (Ceres UV 900 HDi, Biotrak Instrument, USA) with DMSO as blank. The mitochondrial dehydrogenase activity at each plant extracts concentration was calculated as a percentage of the control activity from the absorbance values. The experiment was repeated 3 times.

Statistical analysis

The comparison of mean cell survival rate between the studied plant extracts was carried out using one way Analysis of variance (ANOVA). SPSS version 10 was used for all statistical analysis. A *p*-value less than 0.05 was considered to be significant.

Part IV *In vitro* assay for the anti-inflammatory activity of the studied plant extracts

Materials

1. Five prepared plant extracts from part II
2. Dimethyl sulfoxide (DMSO) (MERCK®)
3. Distilled water
4. 96 wells plate (NUNCTON™)
5. Prostaglandins E₂ (PGE₂) by a commercial specific enzyme-linked immunosorbant assay (ELISA) kit (R&D system, USA)
6. ELISA kit (R&D system, USA)
7. Glass pipette 1 ml, 2 ml, 5 ml, 10 ml, 20 ml
8. Fetal bovine serum (FBS)
9. Magnetic bar
10. Dulbecco-MEM (DMEM)
11. Vortex mixer (Vortex Genie 2)
12. Automatic pipette 1-10 µl, 10-100 µl, 100-1,000 µl (Drummond, Drummond scientific co. Broomall PA, USA)
13. Micropipette tips 200 µl, 1,000 µl
14. Flask 125 ml, 250 ml, 500 ml, 1,000 ml
15. Beaker 50 ml, 100 ml, 500 ml, 1,000 ml
16. Phosphate buffered saline (PBS)
17. MTT agent (Sigma®, USA)
18. Culture plate
19. Tissue Culture flask 25, 75 cm²
20. Trypsin/EDTA solution
21. Penicillin/Streptomycin
22. Gentamycin
23. Fungizone

24. Centrifuge tube 15, 50 ml
25. Ministart 0.45 µl 26 ml
26. LPS of *Escherichia coli* (Sigma[®], USA)

Equipment

1. Adventure[™] Pro (Ohaus corporation, Pine Brook, NJ, USA)
2. Autoclave (Hiclave[™] HV-50, Hirayama co.)
3. Water bath (Mettler)
4. Hot air oven (Mettler)
5. UV/Visible spectro-photometer (Ultraspec 3300/Pharmacia of Amersham Biosciences)
6. CO₂ incubator (Thermo Forma)
7. Microflow Laminar (Biohazard Laminar Air Flow Class II, Astec Microflow, UK)
8. Liquid Nitrogen Tank (Taylor-Wharton)
9. Nikon Antimould (Hollywood international co. , LTD, Japan)
10. Biotrak[™] visible plate reader (Amersham Biosciences, USA)
11. Hot plate stirrer (Corning)
12. pH meter (Precisa pH900)
13. Eppendorf centrifuge 5417c (Pharmed and science)
14. Spectromicroscope
15. Table Top Centrifuge PLC-03 (Centrifuge PLC series, Taiwan)
16. Refrigerated centrifuge (Labofuge[®])
17. Inverted Microscope (Nikon Eclipse, TE2000-U)

Method

This experiment was performed for the evaluation of the anti-inflammation effect of plant extracts on HGF cells. HGF (passage 5th-10th) were added to each well of a 24-well culture plate at 5×10^4 cells in 1,000 µl of DMEM supplemented with 10% FBS, antibiotics (100 µg/ml of penicillin, 100 µg/ml of streptomycin), glutamine and non-essential amino acid. Cells were incubated at 37°C in humidified atmosphere of 95% air and 5% CO₂ for 24 hours.

The HGF cells were then treated with LPS of *E. coli* 1 µg/ml incubated at 37°C in humidified atmosphere of 95% air and 5% CO₂ for 9 hours (peak of PGE₂ release).⁶³ We selected 5 plant extracts that were less cytotoxic from part III and prepared them by 20 and 2 times of MIC plant extracts, except *Tb* dried-fruit (5.95 and 59.5 mg/ml). Controls consisted of a) HGF cells treated with LPS but not treated with plant extracts b) HGF cells not treated with LPS and plant extracts. The subconfluent cells were then treated with prepared plant extracts. Cells were incubated at 37°C in humidified atmosphere of 95% air and 5% CO₂ for 6, 12, 24 hours. At the end of the exposure time, the supernatant was collected. We measured PGE₂ of the cell supernatants by a commercial specific enzyme-linked immunosorbant assay (ELISA) kit (R&D system, USA). The assay was conducted through the manufactures instructions (Table Appendix). The spectrophotometric absorbance at 405 nm was then measured by an ELISA reader (Ceres UV 900 HDi, Biotrak Instrument, USA).

Statistical analysis

Descriptive statistics are presented. Comparison between the studied plant extracts was carried out using the non-parametric Kruskal-wallis test. SPSS version 10 was used for all statistical analysis. A *p*-value less than 0.05 was considered to be significant.