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APPENDICES

APPENDIX A

CHEMICAL AGENTS AND INSTRUMENTS

A. Laboratory supplies

Aerosol resistance pipette tip: 10,200, and 1000 µl (Oxygen, USA)

Aluminum foils (M FOIL®, USA)

Autoclave tape (3M, USA)

Beakers: 50 ml, 1,000 ml (Pyrex®, USA)

Bottle top filter 0.22 µM (Costar®, USA)

Coplin staining jars

Cryotube (Sorenson™, USA)

Cylinders (Pyrex®, England)

Disposable gloves (Latex, Thailand)

Eppendorf tube

Glass pipettes: 5 and 10 ml (Witeg, Germany)

Humidified chamber

Microscope glass cover slips (Chance, England)

96 multiwell plates (Nunc, USA)

Parafilm (American National Can™, USA)

Pipette boy (Falcon®, USA)

Plastic cover slips (ApopTag®)

Reagent bottles: 250 ml, 500 ml, 1000 ml (Duran®, Germany)

Slide (Soilbrand, China)

Slide box

Slide film (Eritchrome 400, Kodak)

Slotted microscope slide staining dish

Sterile membrane filters (Whatman®, Japan)

Sterile millipore 0.22 µM (Millex®-GP, USA)

Sterile polypropylene centrifuge tube: 15 ml, 50 ml. (CellStar®, USA)

T 25 Tissue Culture flasks (CellStar®, USA)

T 75 Tissue Culture flasks (CellStar®, USA)

Tube rack

B. Equipments

Autoclave (HICLAVE™, HIRAYAMA)

Autopipette (Gilson, France)

Balance (Precisa, Switzerland)

CO_2- Incubator (REVCOULTIMA)

Differential counter

ELISA Microplate Reader (Multiskan EX)

Freezer – 20° C (SANYO, Japan)

Freezer – 80° C (SANYO, Japan)

Hemocytometer (Boeco, Germany)

Hot Plate Stirrer (HL Instrument, Thailand)

Incubator (Heraeus)

Light microscope (Olympus, Japan)

Low- speed centrifuge (Beckman)

pH meter (ECOMET, Korea)

Refrigerator 4°C (SANYO, Japan)

Spectrophotometer (BIO-RAD, USA)

Timer

Thermometer

Ultrasonicator (Virtis, USA)

Vacuum pump

Vortex (Labnet, USA)

C. General Reagents

Absolute ethanol (Merck, Germany)

Acetic acid (Merck, Germany)

Ascorbic acid (Sigma, Germany)

Bovine Serum Albumin (Gibco®, USA)

Clorox (Clorox, USA)

DMSO (Euroclone®, Italy)

Dulbecco's modified Eagle's medium (Hyclone, Germany)

Fetal Bovine Serum (Hyclone, Germany)

Glucose

HEPES (Hyclone, Germany)

Hydrochloric acid: (Hyclone, Germany)

Kojic acid (Sigma, Germany)

L- Tyrosine (Sigma, Germany)

L- Glutamine (Gibco BRL)

Paraformaldehyde powder (Sigma, Germany)

Penicillin-Streptomycin (Hyclone, Germany)

Potassium chloride (BDH)

Potassium hydrogen phosphate (Baker, USA)

Sodium chloride (BDH)

Sodium hydroxide (Merck, Germany)

Tyrosinase enzyme (Sigma, Germany)

di- Sodium hydrogen phosphate monobasic (BDH)

Sodium bicarbonate (Baker, USA)

0.4% Trypan blue dye (Sigma, USA)

APPENDIX B

CELL COUNTIING BY HEMOCYTOMETER AND TRYPAN BLUE DYE EXCLUSION TEST

The concentration of a cell suspension can be determined by using a hemocytometer slide.

Protocol

1. Clean the surface of the slide with 70% alcohol, taking care not to scratch the semisilvered surface. Clean the coverslip, and, wetting the edges very slightly, press it down over the grooves and semisilvered counting area (Figure 13).

2. Mix the cell suspension thoroughly pipetting to disperse any clumps.

3. Transfer the cell suspension immediately to the edge of the hemocytometer chamber, and let the suspension run out of the pipette and be drawn under the coverslip by capillarity. Do not overfill or underfill the chamber, or else its dimensions may change, due to alterations in the surface tension; the fluid should run only to the edges of the grooves.

4. Transfer the slide to the microscope stage.

5. Select a 10 x objective, and focus on the grid lines in the chamber (Figure 13). Move the slide so that the field is the central area of the grid and is the largest area that bounded by three parallel lines. This area is 1 mm^2.

6. Count the cells lying within this 1 mm^2 area, using the subdivisions (also bounded by three parallel lines) and single grid lines as an aid for counting. Count cells that lie on the top and left-hand lines of each square, but not those on the bottom or right-hand lines, in order to avoid counting the same cell twice.

7. Move to the second chamber and do a second count.

Analysis

Calculate the average of the two counts, and derive the concentration of sample using the formula:

$$c = n/v$$

c = cell concentration (cells/ml)

n = number of cells counted

v = volume counted (ml)

For the Improved Neubauer slide, the depth of the chamber is 0.1 mm (Figure 32 a), and, assuming that only the central 1 mm^2 (Figure 32 c) is used, v is 0.1 mm^3, or $1x10^{-4}$ ml. The formula then becomes

$$c = n \times 10^4$$

Only the 5 diagonal squares within the central 1 mm^2 (Figure 32 d) were counted this equation becomes.

$$c = n \times 5 \times 10^4$$

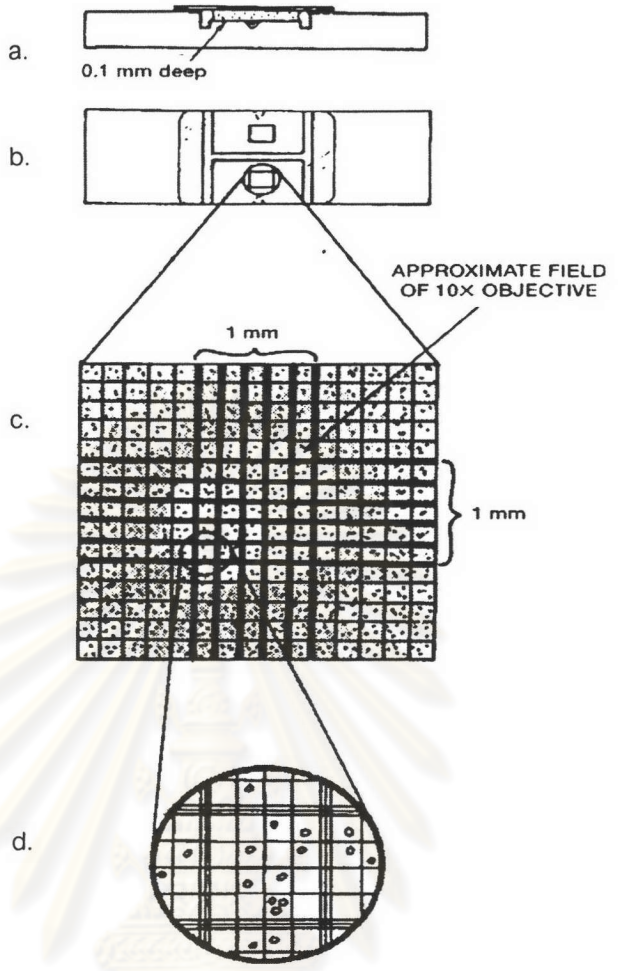


Figure 13. Hemocytometer slide. a) Longitudinal section of the slide, showing the position of the cell sample in a 0.1-mm-deep chamber. **b)** Top view of the slide. **c)** Magnified view of the total area of the grid. The light central area is that area which would be convered by the average 10 x objective. This area covers approximately the central 1 mm^2 of the grid. **d)** Magnified view of one of the 25 smaller squares, bounded by the triple parallel lines that make up the 1- mm^2 central area. This view is subdivided by single grid lines into the 16 smallest squares to aid counting.

Trypan blue dye exclusion test

Trypan blue is one of the several stains recommended to use for viable cell counting. The method is based on the principle that viable cells do not take up trypan blue, where as dead cells do. The procedures are as follows:

1. Take a clean hemocytometer slide and fix the coverslip in place.

2. Aliquot 20 µl of the suspended cell culture into an eppendorf test tube. Add 50 µl of 0.4 % trypan blue solution and 30 µl of DMEM free serum.

3. Leave the mixture for 5 minute. (Do not leave them for a longer period of time, or else viable cells will deteriorate and take up the dye.)

4. Pipette an aliquot of the stained culture on the hemocytometer; place the slide on the microscope. The number of unstained (viable) and stained (dead) cells were counted separately.

5. Calculate the percentage of cell viability and the concentration, which gave 50% inhibition of cell growth (IC_{50}), was calculated.

Cells/ml = the average count per square x dilution factor x 10^4

Total cells = Cells/ml x the original volume of fluid from which cell sample was taken.

$$\% \text{ cell viability} = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained and unstained)}} \times 100$$

APPENDIX C

RT-PCR AND SPECIFIC BUFFER

1. Preparation of the reaction mix for cDNA synthesis

Before starting the experiment thaw all reagents except of the polymerase, mix them thorughly and centrifuge briefly. Use a sterile microcentrifuge tube to add the reagents to the reaction mix in a fixed order as outlined in the table.

Table 3 Preparation of the reaction mix for cDNA synthesis

RT Reacion	Volume/1 sample (µl)	Volume/2 sample (µl)	Volume/3 sample (µl)	Volume/4 sample (µl)
10X Reaction Buffer	2	4	6	8
25mM MgCl2	4	8	12	16
dNTP	2	4	6	8
Random Primer	2	4	6	8
RNase Inhiitor	1	2	3	4
AMV Reverstranscriptase	1	2	3	4
Master Mix	12	24	36	48
Master Mix Aliquot	12	2x12	3x12	4x12
2 ug of RNA (Xi)	Xi	2xXi	3xXi	4xXi
RNase Free Water (20-12-Xi)	(20-12-Xi)	(20-12-Xi)	(20-12-Xi)	(20-12-Xi)
Total Volume	20	2x20	3x20	4x20

i = the number labeled in each sample.

2. Preparation of the reaction mix for PCR

Before starting the experiment thaw all reagents except of the polymerase, mix them thorughly and centrifuge briefly. Use a sterile microcentrifuge tube to add the reagents to the reaction mix in a fixed order as outlined in the table.

Table 4 Preparation of the reaction mix for PCR

PCR Reacion	Volume/1 sample (μl)	Volume/2 sample (μl)	Volume/3 sample (μl)	Volume/4 sample (μl)
10X Reaction Buffer	2.5	5	7.5	10
25mM MgCl2	1.5	3	4.5	6
dNTP	0.5	1	1.5	2
Taq	0.2	0.4	0.6	0.8
Forward Primer	0.7	1.4	2.1	2.8
Reward Primer	0.7	1.4	2.1	2.8
Master Mixt	6.1	12.2	18.3	24.4
Master Mix Aliquot	6.1	2x6.1	3x6.1	4x6.1
cDNA	5	2x5	3x5	4x5
RNase Free Water (25-6.1-5)	13.9	2x13.9	3x13.9	4x13.9
Total Volume/sample	25	2x25	3x25	4x25

3. Buffers prepations for RT-PCR

10x Ficoll loading buffer 10 ml

Ficoll	25	g
Bromphenol blue	0.025	g
0.5 M EDTA (pH 8.0)	0.2	ml

Adjust volume to 10 ml with dH_2O, Store at -20°c.

1.5% Agarose gel (w/v) 100 ml

Agarose	1.5	g
1x TBE	100	ml

Dissolve by heating and occational mixing until no granules of agarose are visible. Add ethidium bromide (stock 10 mg/ml) 10 μl (final concentration 1 μg/ml)

10x TBE buffer (pH 8.0) 1 liter

Tris base	108	g
EDTA $2H_2O$ (pH 8.0)	40	ml
dH_20	800	ml
Slowly add the boric acid, anhydrous	55	g

Adjust the pH to 8.0 with conc.HCl

Adjust the volume to 1 liter with dH_2O

1x TBE buffer 1 liter

10x TBE buffer	100	ml
dH2O	900	ml

Adjust the pH to 7.4 with conc. HCl

Adjust the volume to 1 liter with dH_2O

APPENDIX D

SPECIFIC BUFFER FOR WESTERN BLOT

Buffers preparations for western blot

1.5 M Tris base (pH 8.8) 100 ml

Tris base	18.171	g
dH_2O	80	ml

Adjust the pH to 8.8 with conc. HCl and conc.NaOH

Adjust the volume to 100 ml with dH_2O

1 M Tris base (pH6.8) 100 ml

Tris base	12.14	g
dH_2O	80	ml

Adjust the pH to 6.8 with conc. HCl and conc.NaOH

Adjust the volume to 100 ml with dH_2O

0.5 M Tris-HCl 100 ml

Tris base	6	g
dH_2O	40	ml

Adjust the pH to 6.8 with conc. HCl

Adjust the volume to 100 ml with dH_2O

10% SDS 100 ml

SDS	10	g

Adjust the volume to 100 ml with dH_2O

1x Triton lysis buffer 100 ml

25 mM Tris-HCl (pH 8.0)	0.4	g
150 mM NaCl	0.88	g
0.5% Triton X-100	0.5	ml
5 mM EDTA	0.186	g
dH_2O	100	ml

10x Laemmli running buffer (pH 8.3) 1 liter

Tris base	30.3	g
Glycine	144.2	g
SDS	10	g
dH_2O	900	ml
Adjust the pH to 8.3 with conc. HCl and conc.NaOH		
Adjust the volume to 1 liter with dH_2O		

1x Transfer buffer 1 liter

Tris base	5.8	g
Glycine	2.9	g
SDS	0.37	g
Adjust the volume to 800 ml with dH_2O		
100% Methanol	200	ml

Blocking buffer

5% non fat dry milk in TBS

10x TBS washing buffer (pH 7.4) 1 liter

Tris base	30	g
NaCl	80	g
KCl	2	g
dH_2O	800	ml
Adjust the pH to 7.4 with conc. HCl		
Adjust the volume to 1 liter with dH_2O		

Stain 0.5% coomassei blue 500 ml

Methanol	200	ml
Acetic acid	50	ml
coomassei blue	2.5	g
Adjust the volume to 500 ml with dH_2O		

Destain 500 ml

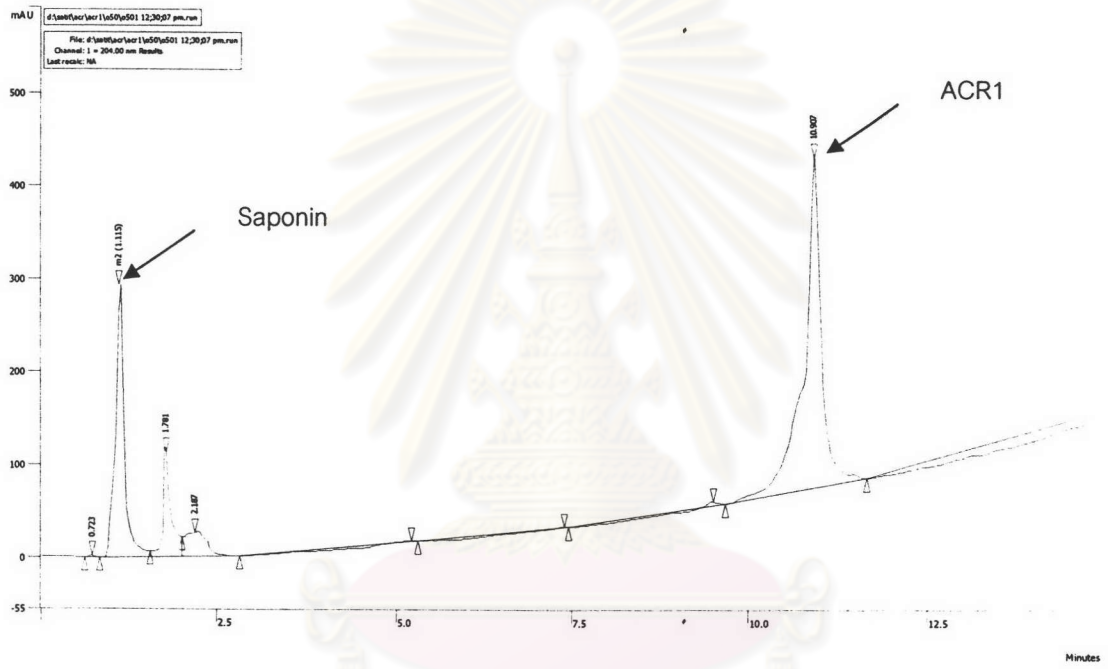
Methanol	150	ml
Acetic acid	50	ml
Adjust the volume to 500 ml with dH_2O		

APPENDIX E

FINGER PRINT OF HERBAL CRUDE EXTRACT

Mallotus Spodocarpus

Bring roots of *Mallotus Spodocarpus* to extract with 95% ethanol (dilution1:10).



Column: C18

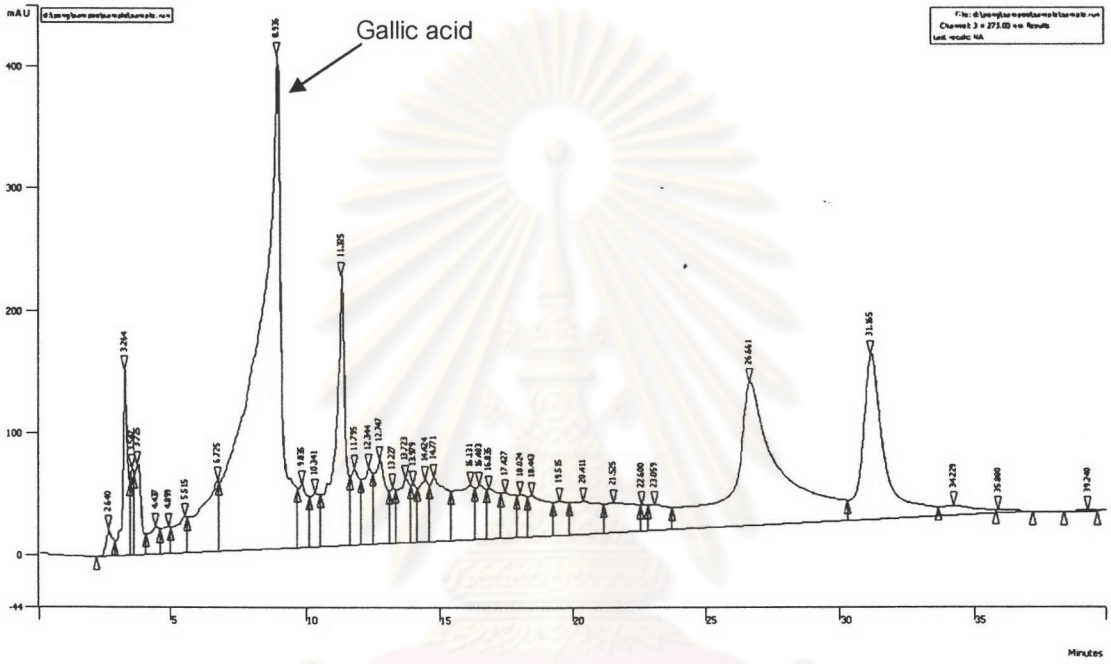
Solvent system: methanol: water (20:80 – 60:40)

Flow rate: 0.8 ml/min

Figure 14. Finger print of *Mallotus Spodocarpus*. Shown that this crude extract has mainly saponin and ACR1 components. (by Dr. Saroj's Research Lab Co., Ltd)

Excoecaria Bicolor

Bring leaves of *Excoecaria Bicolor* to extract with 40% ethanol (dilution 1:10)



Column: C18

Solvent system: 0.01 M Formic acid: Methanol: water (1:1:98-5: 25: 70)

Flow rate: 0.8 ml/min

Figure 15. Finger print of *Excoecaria Bicolor*. Shown that this crude extract has mainly gallic acid component. (by Dr. Saroj's Research Lab Co., Ltd)

BIOGRAPHY

Name	Mr. Ekapot Singsuksawat	**Sex**	Male
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