CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

All chemicals and materials used in this study are listed in Appendix A.

2.2 Plant material

Dried longan (*Euphoria longana* Lam.) cultivar Biewkiew was purchased from local markets in Chiang Mai, Thailand.

2.3 Preparation of plant extract

Dried fruits of *Euphoria longana* Lam. were separated into two different parts (pulp and seed) and crushed into small pieces using a pestle and mortar or a blender. Each part of dried longan (pulp and seed) was accurately weighed, 100 g, blended with 1000 ml of 80% aqueous acetone. The samples were stirred at room temperature overnight. The acetone extract was filtered through Whatman filter paper No. 1. The filtrate was evaporated in a rotary evaporator under reduced pressure at 45-50 ^{o}C. The liquid residue was fractionated by solvent partition using ethyl acetate twice (ratio 1: 1). The ethyl acetate-soluble fraction was evaporated in a rotary evaporator under reduced pressure at 45-50 ^{o}C. The yields of ethyl acetate-soluble fraction from the dried longan pulp and seed were 0.5% and 2.8%, respectively. A schematic diagram of the plant extraction protocol is shown in Figure 2.1.

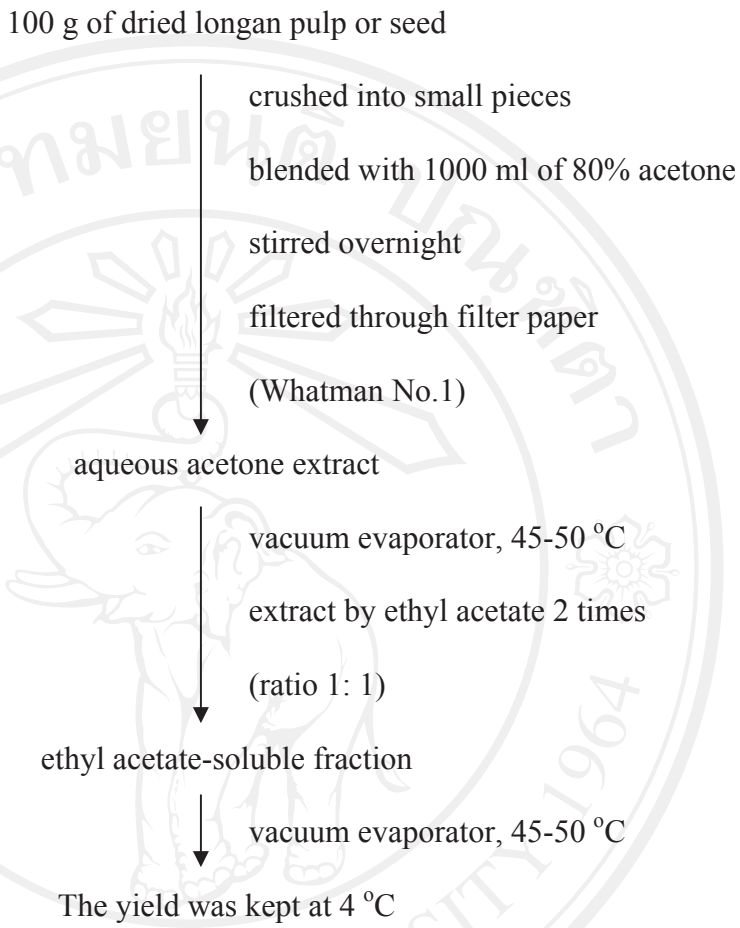


Figure 2.1 Schematic protocol for preparing dried longan pulp or seed extract.

2.4 Determination of total phenolic compounds

The total phenolic content in crude acetone extract and ethyl acetate-soluble fraction of dried longan pulp or seed were determined by using the Folin-Ciocalteu assay. An aliquot (125 µl) of extract or standard solution of gallic acid (25, 50, 100, 150, 200 mg/l) was added to test tube, containing 500 µl of deionised distilled water (DI). The reagent blank of extract and standard were DMSO and DI, respectively. Folin-Ciocalteu's phenol reagent (125 µl) was added to the mixture and shaken. After 6 min, 1.25 ml of 7 % Na_2CO_3 solution were added to the mixture. The solution was diluted to a volume (3 ml) with DI and mixed. After incubation for 15 min at 45 ^oC, the absorbance against prepared reagent blank was determined at 765 nm with an UV-Vis Spectrophotometer. Total phenolic content of extracts was expressed as mg gallic acid equivalents (GAE)/1 g sample. A schematic protocol for determination of total phenolic compounds is shown in Figure 2.2.

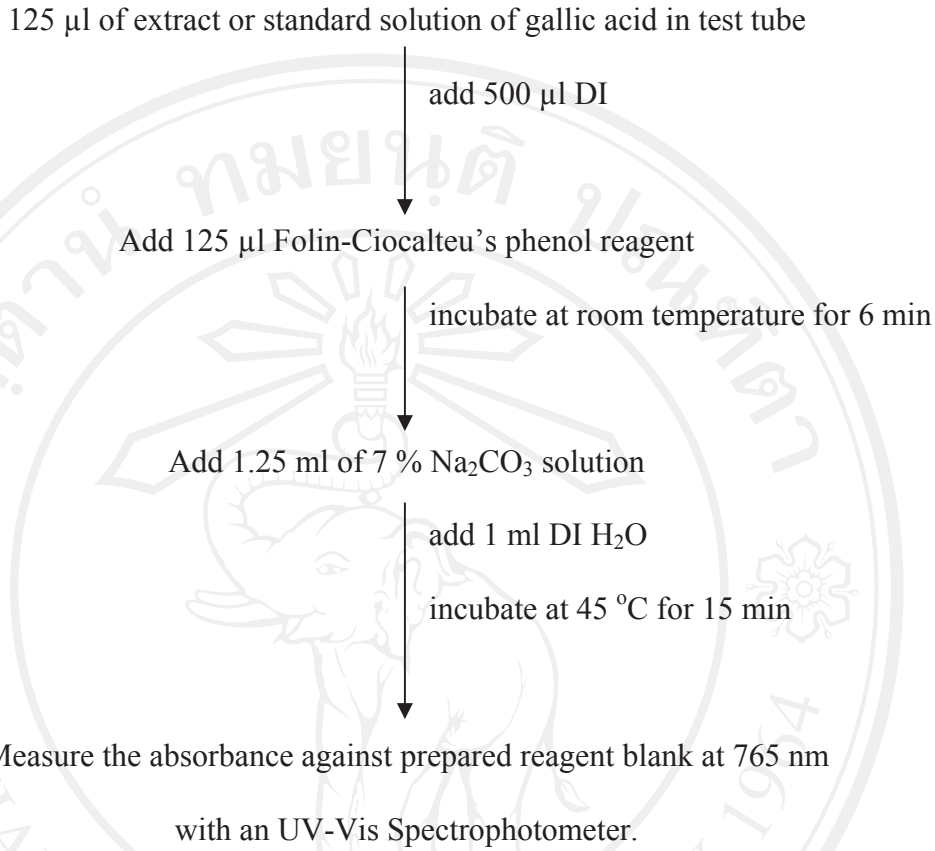


Figure 2.2 A schematic protocol for determination of total phenolic compounds.

2.5 Determination of total flavonoid

Total flavonoid content was measured by the aluminum chloride colorimetric assay. An aliquot (250 µl) of extracts or standard solution of catechin (25, 50, 100, 150, 200 mg/l) was added to test tube containing 1.25 ml of DI and 75 µl of 5% $NaNO_2$. The reaction mixture was incubated at room temperature for 6 min before adding 150 µl of 10% $AlCl_3$. The mixture was incubated at room temperature for 5 min. Five hundred µl of 1 M NaOH and 275 µl of DI were added. The solution was mixed well and the absorbance was measured at 510 nm against the reagent blank. Total flavonoid content of extracts was expressed as mg catechin equivalents (CE)/1 g sample. A schematic protocol for determination of total flavonoid is shown in Figure 2.3.

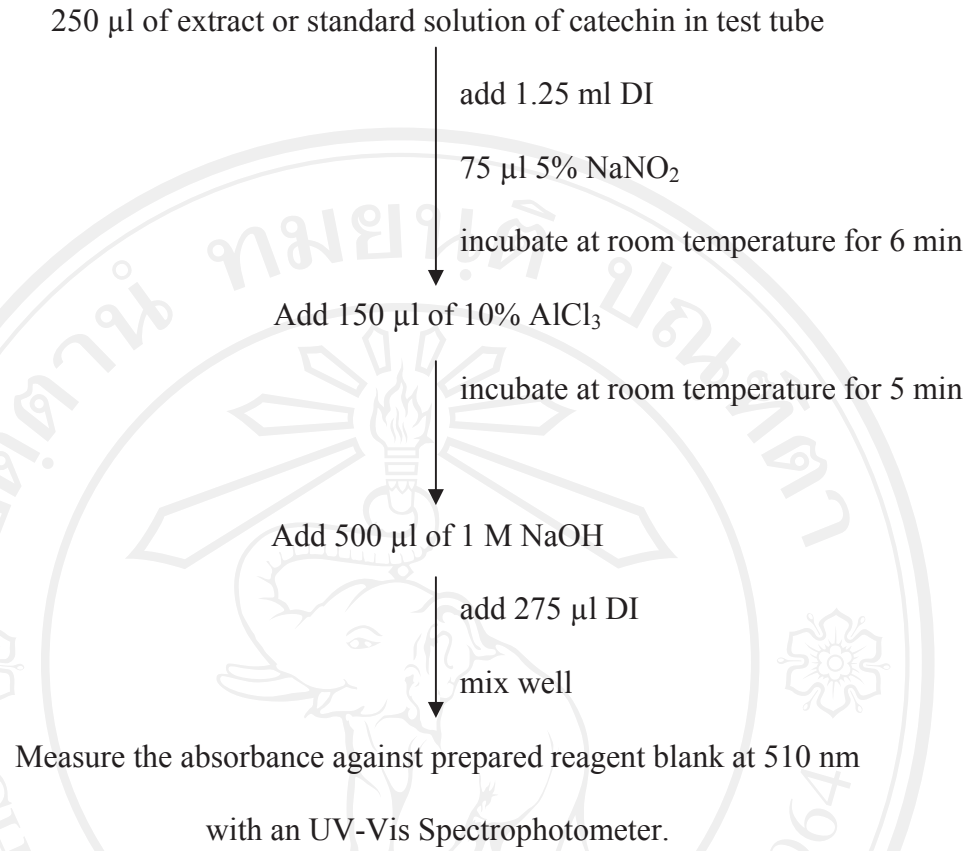


Figure 2.3 A schematic protocol for determination of total flavonoid.

2.6 Scavenging activity on DPPH radical

DPPH radical scavenging assay was used to determine the free radical scavenging activity of the extracts. The crude acetone extract and the ethyl acetate-soluble fraction of dried longan pulp and seed were allowed to react with a stable free radical, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The reduced DPPH was determined using a spectrophotometer. Briefly, 75 µl of each sample or 100% ethanol (negative control) or ascorbic acid (positive control) were allowed to react with 75 µl of 0.3 mg/ml DPPH ethanolic solution in a 96-well microplate. The plate was then incubated at room temperature for 30 min. The scavenging activity on DPPH radical was determined by measuring the absorbance at 517 nm. The antioxidant activity was expressed as a percentage of scavenging activity on DPPH radical: SC% = [1-(absorbance of sample/absorbance of negative control)] x 100%. The results expressed as the concentration of the samples which scavenged free radicals by 50% (SC_{50}). A schematic protocol for DPPH assay is shown in Figure 2.4.

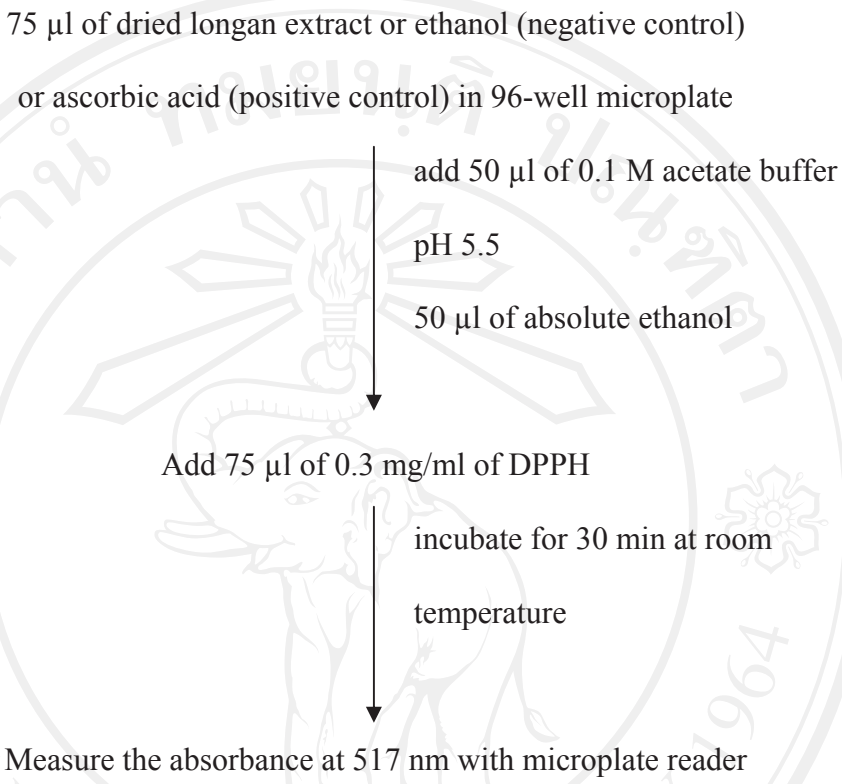


Figure 2.4 A schematic protocol for DPPH assay

2.7 Effect of dried longan extract on the growth of colon cancer cell lines

2.7.1 Cell lines and culture conditions

The human colon cancer cell lines HCT-15 and RKO (from American Type Culture Collection, Rockville, USA) were used as models. HCT-15 and RKO were grown at 37 ^{o}C in a fully humidified atmosphere containing 5% CO_2. HCT-15 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate. RKO cells were maintained in minimal essential medium containing 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate. The number of viable cells was determined using a hemocytometer, based upon their exclusion of 0.2% trypan blue dye. Cells were plated at a density of $1x10^6$ cells/well in 6-well culture plates. One day after seeding, cells were treated with the extract at various concentrations. An equivalent amount of the solvent (medium) was added to the control cells.

2.7.2 Cell proliferation assays

The viability of cultured cells was determined by assaying for the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, which colorimetrically measures a purple formazan compound produced by viable cells. Cells were cultured in 96-well plates at a density of 1 x 10^4 cells per well. The cells were then treated with varying concentrations of crude acetone extract and the ethyl acetate-soluble fraction of dried longan pulp and seed. After 24, 48 and 72 h, 20 µl of MTT solution (5 mg/ml of PBS) were added to each well. Plates were

incubated at 37 ^{o}C for 4 h. After incubation, culture media were discarded and dimethyl sulfoxide DMSO (100 µl) was added to dissolve the formazan crystals. Absorbance was measured at 540 nm with microplate reader. A summary of the procedure of cell proliferation determination is shown in Figure 2.5.

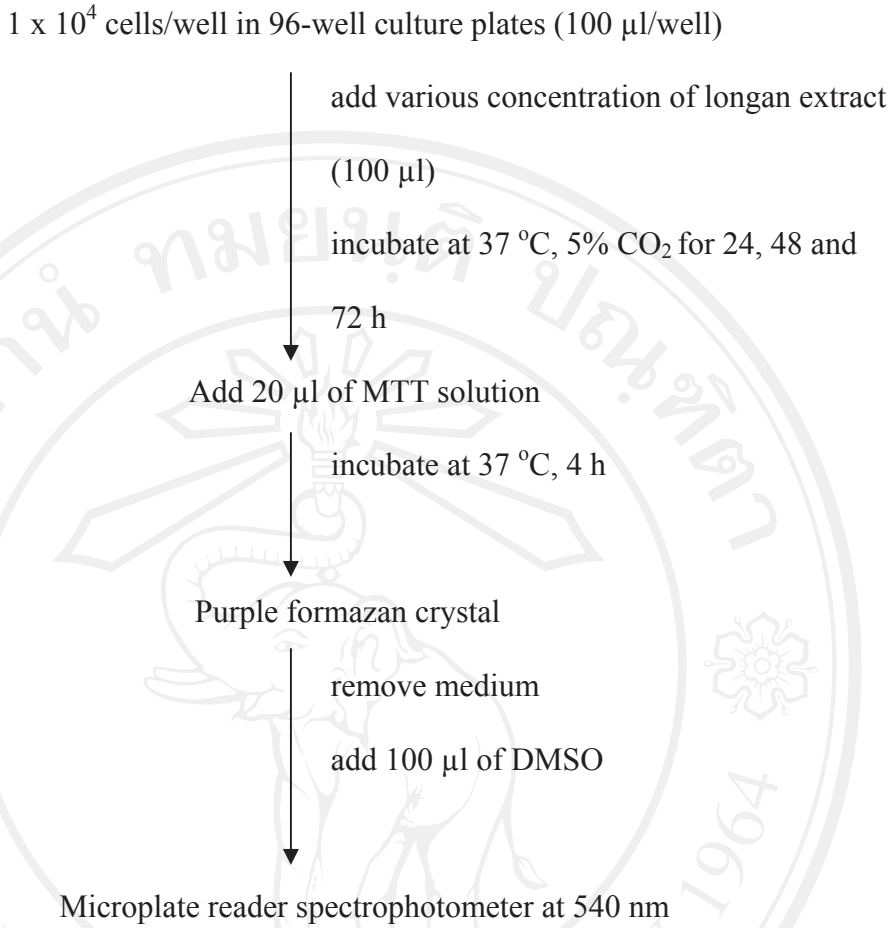
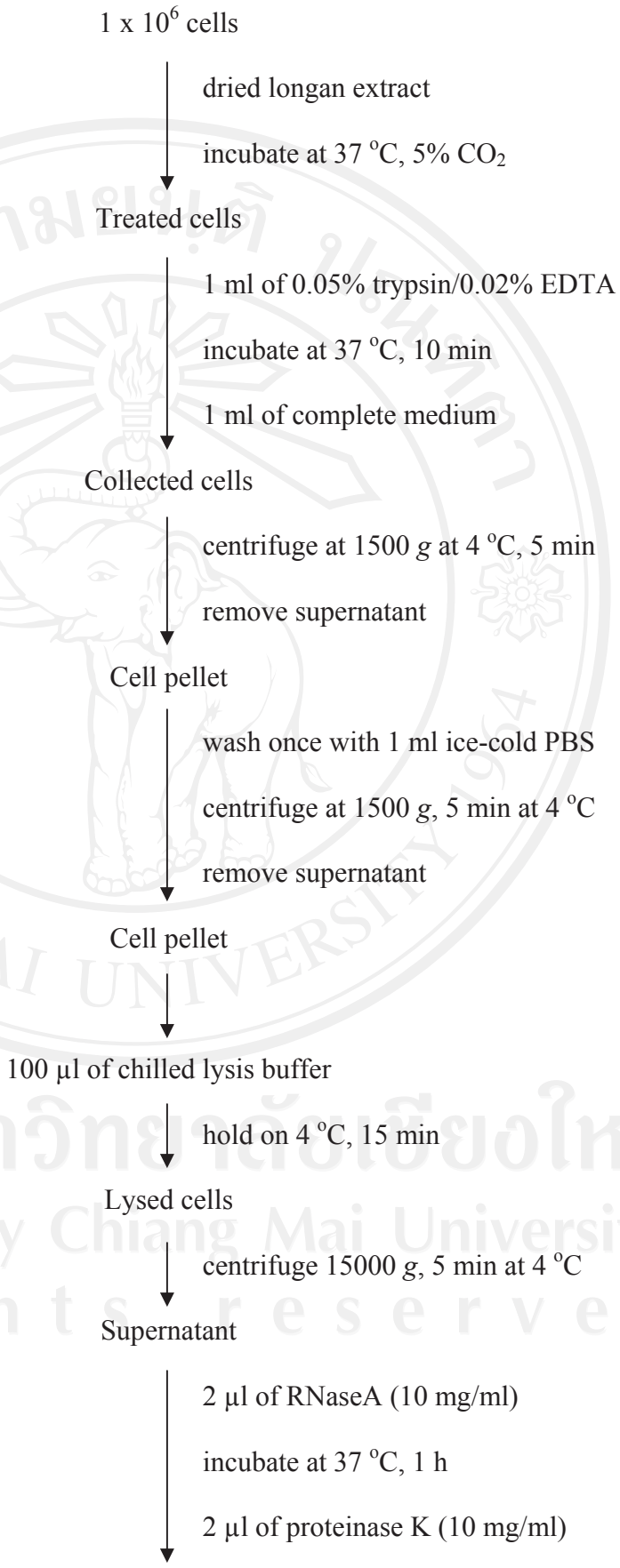


Figure 2.5 A schematic protocol for cell proliferation assay.

2.7.3 DNA fragmentation analysis

DNA fragmentation in cells treated with the ethyl acetate-soluble fraction of dried longan pulp (EFLP) and seed (EFLS) was analyzed by procedure of Ohyama and Shimokawa (Ohyama and Shimokawa, 1999) with slight modifications. Briefly, various concentrations of extract were added to the $1x10^6$ cells and incubated for 48 h. After incubation, cells were collected and lysed in 100 µl of chilled lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, and 1% Triton X-100 for 15 min at 4 ^oC. Lysates were harvested by centrifugation at 15000 *g* for 5 min, and supernatants were collected and incubated with 2 µl of RNaseA (10 mg/ml) at 37 ^oC for 1 h. Then, 2 µl of Proteinase K were added and incubated at 50 ^oC for 30 min. DNA was precipitated with isopropyl alcohol and 5M NaCl at -20 ^oC overnight. DNA was collected, washed with ice-cold 70% ethanol once and dried. Then, the DNA was dissolved in TE buffer. DNA sample was separated by electrophoresis on a 2% agarose gel containing 0.5 µg/ml of ethidium bromide. DNA fragments were visualized under UV light. A summary of the procedure of DNA fragmentation analysis is shown in Figure 2.6.



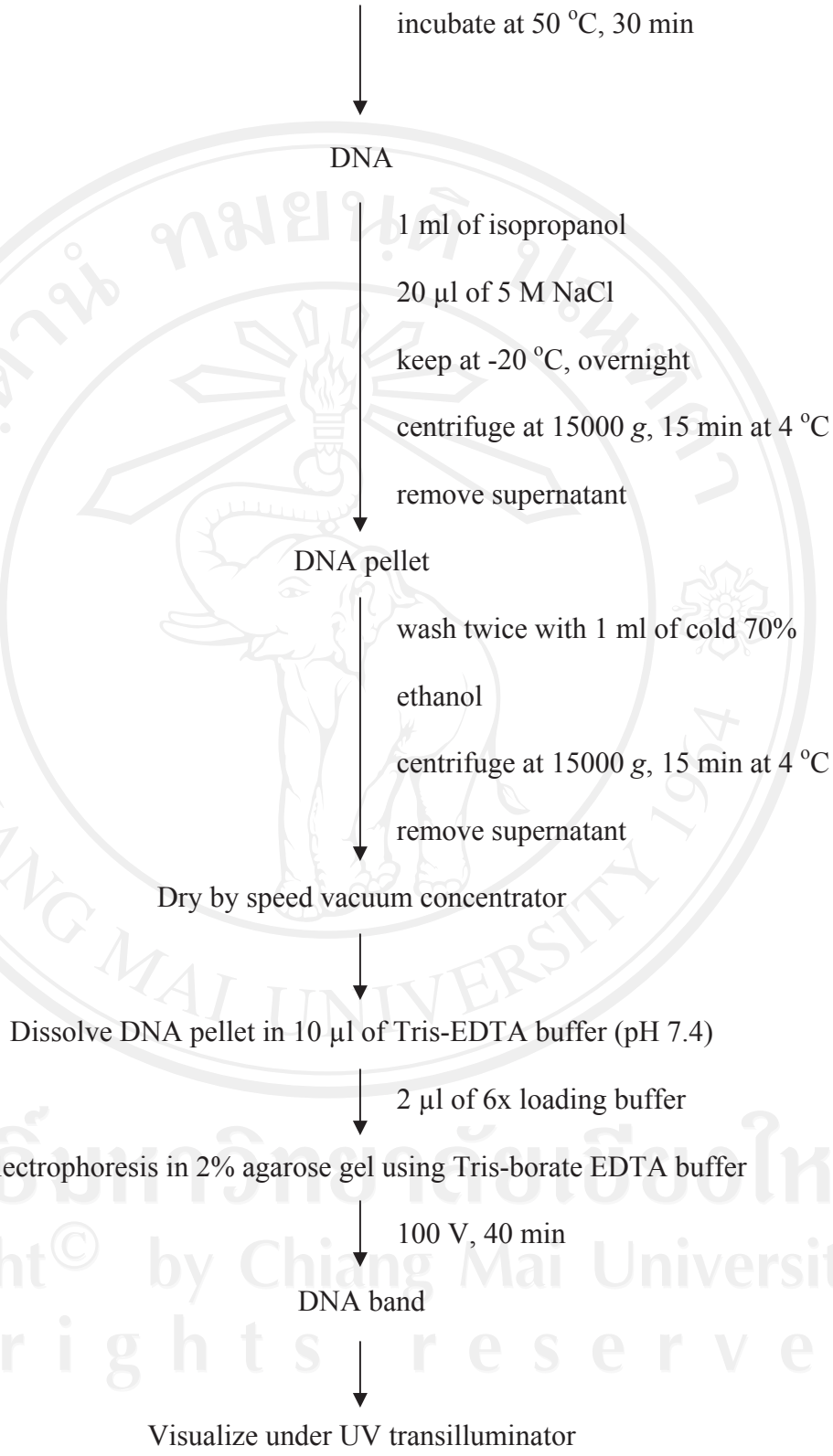


Figure 2.6 Schematic protocol for DNA fragmentation analysis.

2.7.4 Caspase-3 activity assay

1 x 10^6 of HCT-15 or RKO cells were incubated with various concentration of the ethyl acetate-soluble fraction of dried longan seed (EFLS) for 48 h. After incubation, cells were harvested by centrifugation at 1500 *g* for 5 min at 4 ^{o}C. The cell pellets were washed twice with ice-cold PBS and resuspended with 200 µl of hypotonic cell lysis buffer containing protease inhibitor. The cells were lysed by subjecting them to four cycles of freezing and thawing. The lysates were then centrifuged at 16000 *g* for 20 minutes at 4 ^{o}C. Supernatants were collected and frozen at -20 ^{o}C. Protein concentration was determined using BCA^{TM} Protein Assay Kit. Total proteins were determined for caspase-3 activity. Caspase-3 activity assay (Promega, USA) was performed following the manufacturer's instructions. 100 µg of total protein were pre-incubated with caspase assay buffer, 100 mM DTT and DMSO (or 2.5 mM caspase-3 inhibitor for negative control) in 96-well plates at 37 ^{o}C for 30 min. After incubation, 2 µl of 2.5 mM CPP32 substrate were added and further incubated at 37 ^{o}C for 60 min. The fluorescence of the reaction was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Deionized water was used as blank. A summary of the procedure of protein extraction and caspase-3 activity assay are shown in Figure 2.7 and 2.8, respectively.

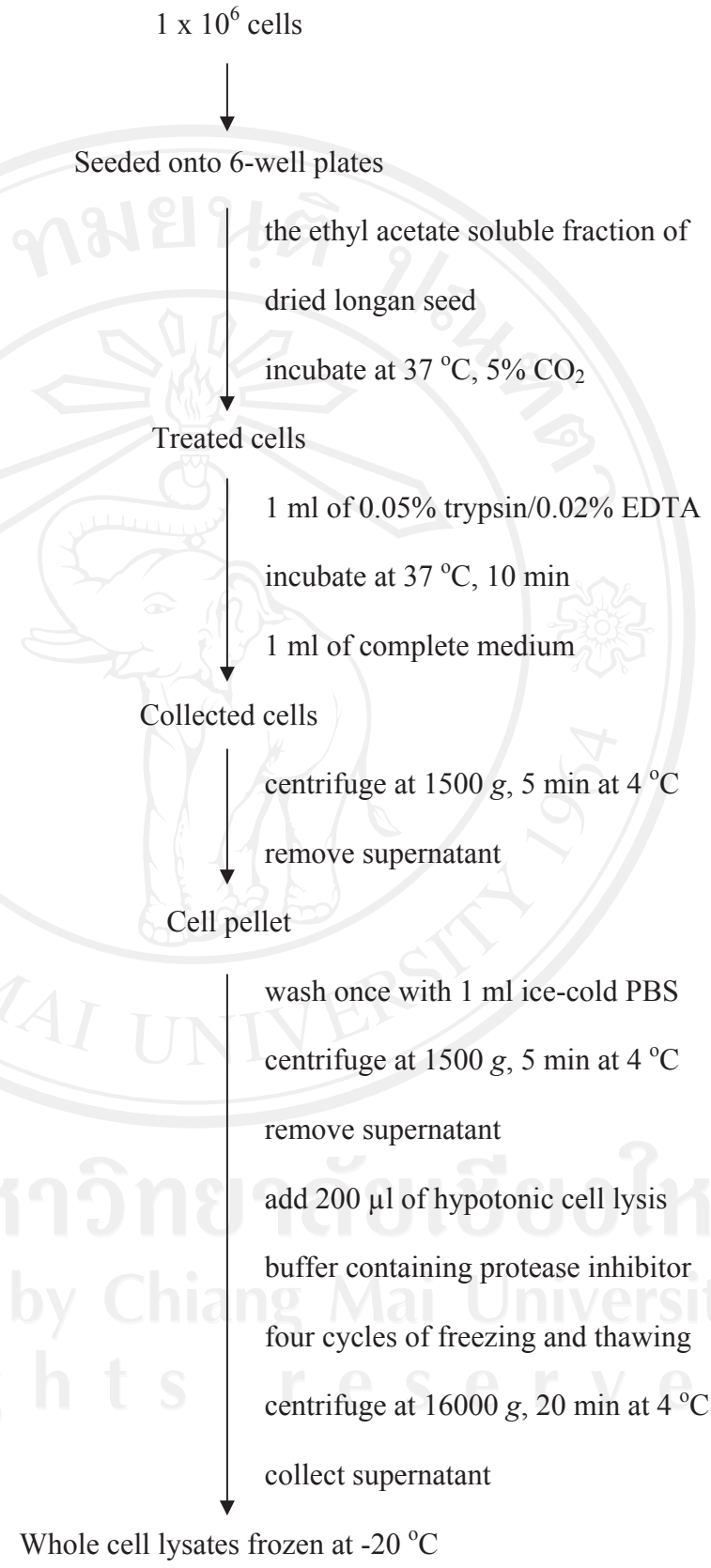


Figure 2.7 A schematic protocol for protein extraction.

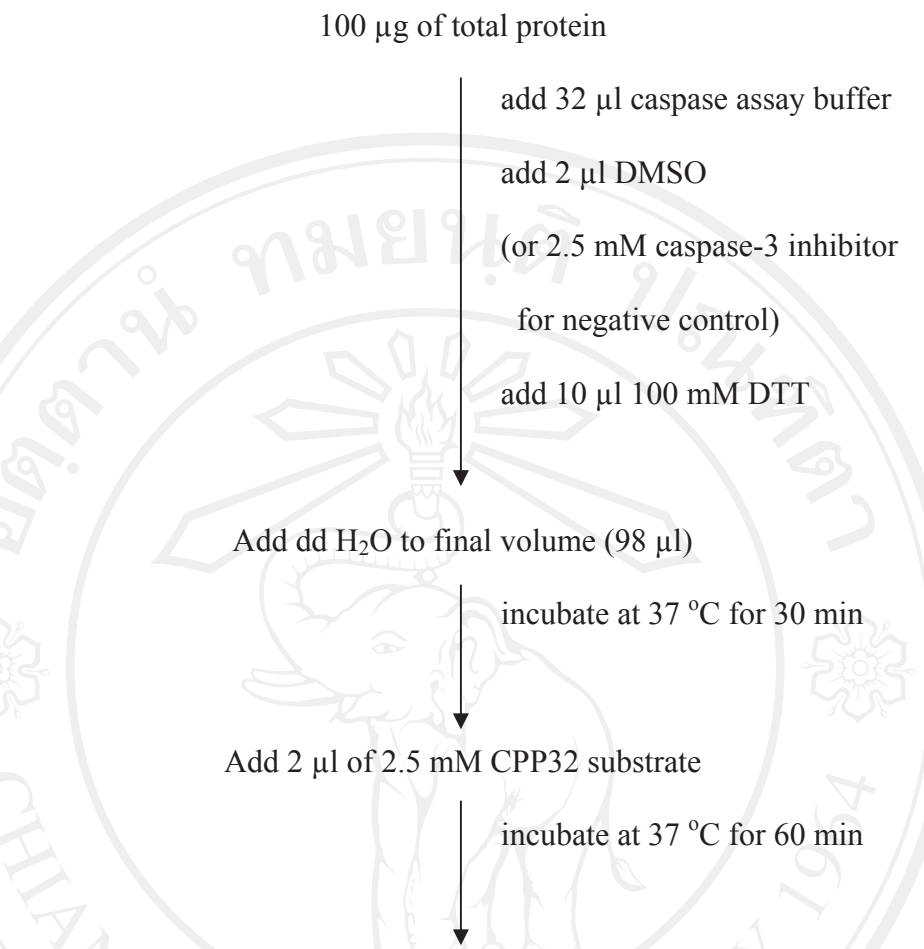


Figure 2.8 A schematic protocol for caspase-3 activity assay.

2.7.5 Western blot analysis

1 x 10^6 of HCT-15 or RKO cells were incubated with various concentrations of the ethyl acetate-soluble fraction of dried longan seed (EFLS) for 48 h. After incubation, whole cell lysates were prepared as summarized in figure 2.6. Protein concentration was determined using BCA™ Protein Assay Kit. Samples were subjected to 12% SDS-polyacrylamide gel electrophoresis with 150 V for 90 min and transferred to nitrocellulose membranes and run at 20 V for 90 min. The membranes were blocked in TTBS containing 5% skim milk for 1 h and probed overnight with a primary antibody (rabbit anti-Bax, rabbit anti-Bcl-2, rabbit anti-Akt, rabbit anti-p-Akt or rabbit anti-actin) at 4 ^{o}C. Primary antibody binding was detected with a goat anti-rabbit IgG conjugated with horseradish peroxidase (1:2000) and visualized by an enhanced chemiluminescence method. A summary of the procedures of Western blot analysis are shown in Figure 2.9.

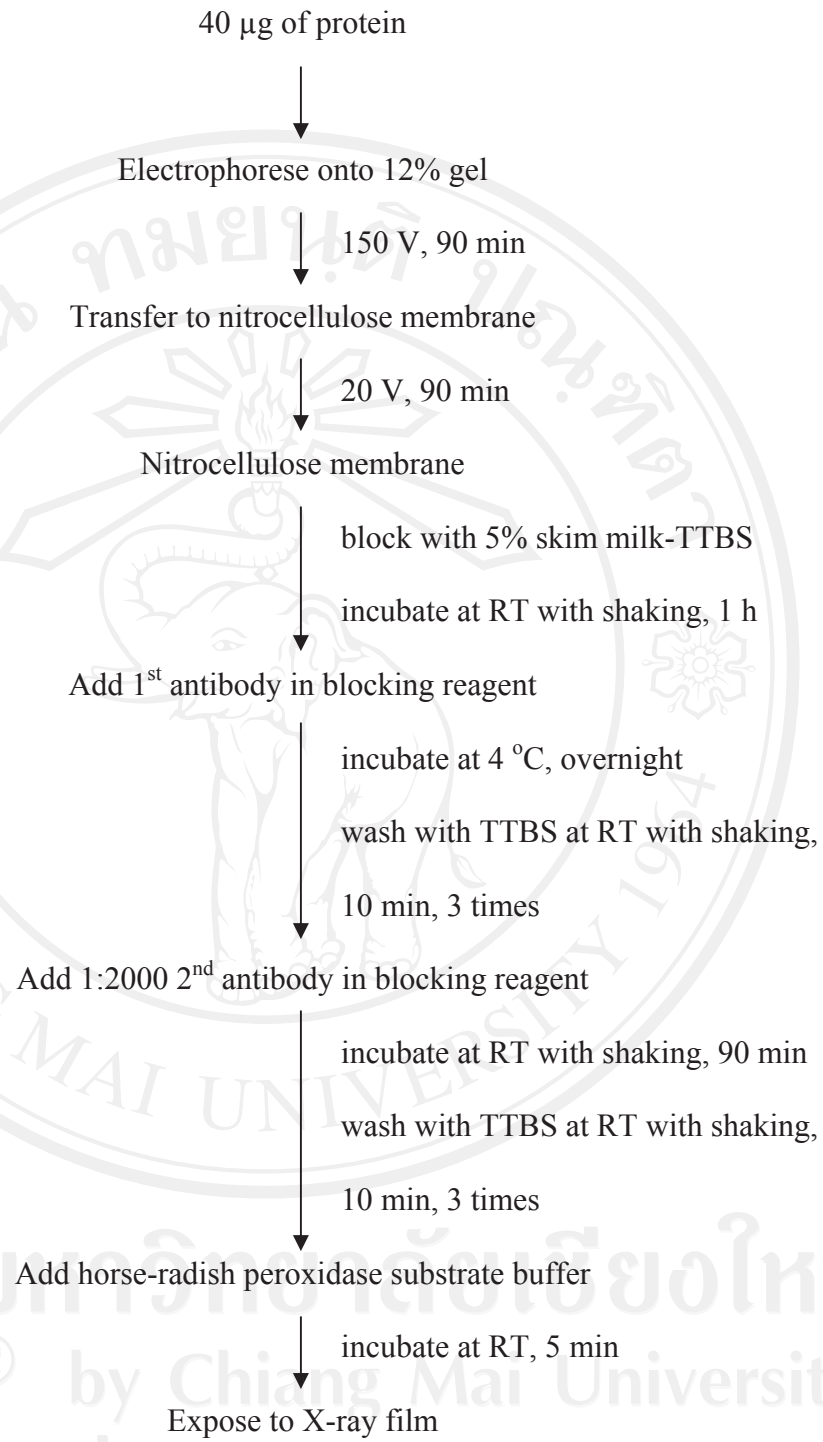
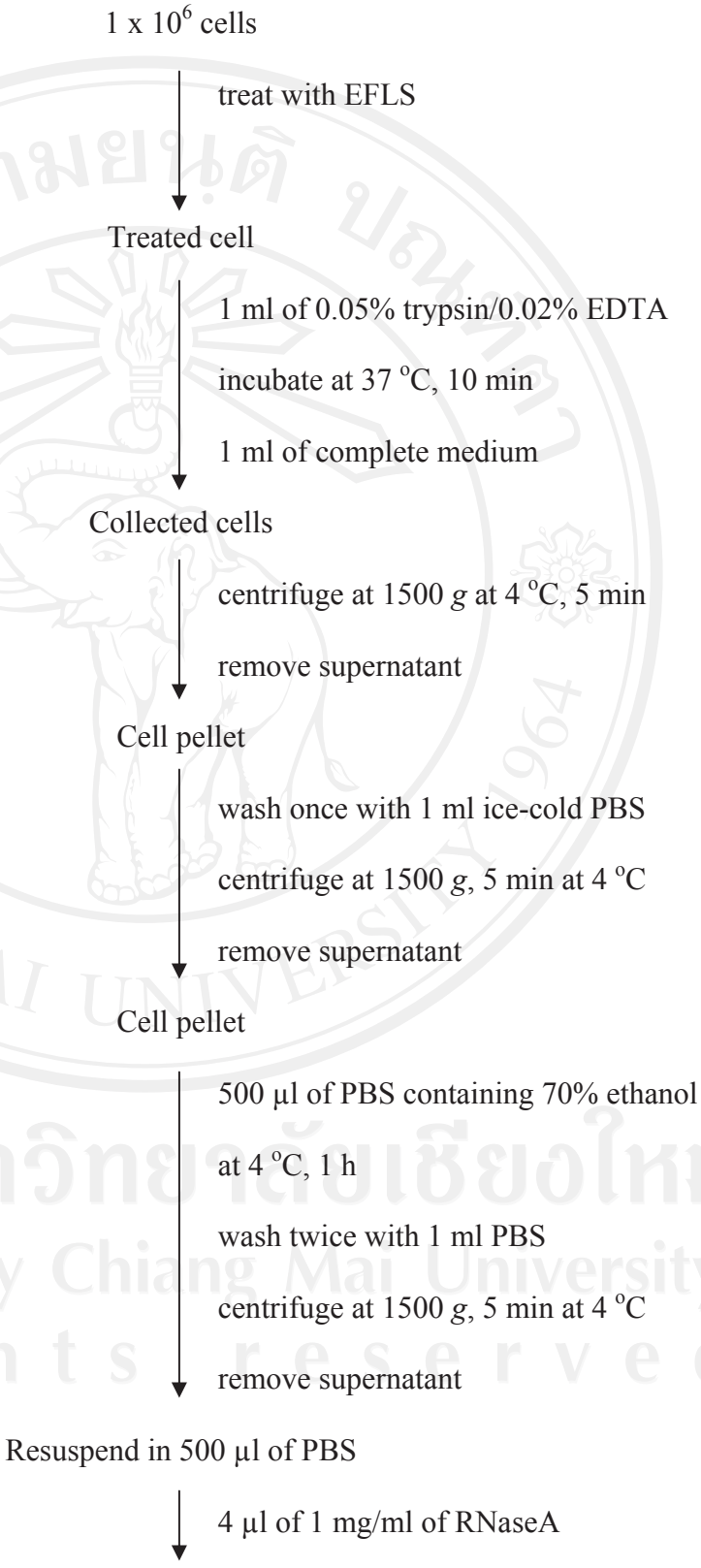


Figure 2.9 A Schematic protocol for Western blot analysis

2.7.6 Flow cytometric analysis of cell cycle

To examine cell cycle arrest, culture cells were treated with EFLS for 24 and 48 h, then resuspended cells in 500 µl PBS containing 70% ethanol at 4 ^{o}C for 1 h. Then, cells were washed 2 times with PBS and incubated with 1 ml of 50 µg/ml propidium iodide and 4 µl of RNaseA (1 mg/ml) for 30 min at 37 ^{o}C. Then, cells were detected fluorescence with a flow cytometer. Data acquired was analyzed using BD FACS Diva^{TM} software v. 6.1. A summary of the procedure of cell cycle is shown in Figure 2.10.



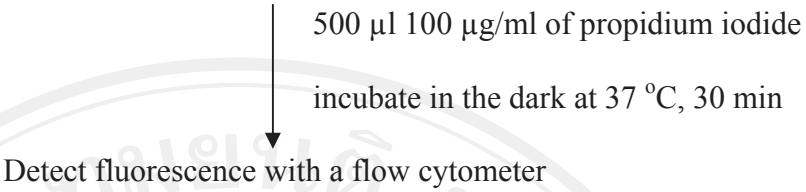


Figure 2.10 Scheme of cell cycle distribution analysis

2.8 Statistical analysis

All the comparisons were done using Student's *t*-test. All values were expressed as mean ± SD. A level of $p < 0.05$ was considered significant.