CHAPTER III

MATERIALS AND METHODS

The experiments were divided into four parts:

1. Effect of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and its active constituent (oxyresveratrol) on normal human dermal fibroblast proliferation and cytotoxicity.
2. Effect of *Artocarpus lakoocha* heartwood extract and oxyresveratrol in decreasing of cell damage induced by oxidative stress.
3. Effect of *Artocarpus lakoocha* heartwood extract and oxyresveratrol in decreasing of DNA damage in cells exposed to UV A.
4. Evaluation of *Artocarpus lakoocha* heartwood extract and oxyresveratrol for anti-collagenase activity.

Crude Drugs and oxyresveratrol

1. Dried aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad), E.A.R. drugstore, Chiangmai, Thailand, lot no. 01/03 contained 80% w/w oxyresveratrol.
2. Active constituent (oxyresveratrol) from aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) as purified by Wachiranuntasin (2005), % purified = 98.64% by HPLC.

Cell culture

1. Primary human normal dermal fibroblast (NHDF) cells from biopsies of normal human skin (coded SiF 49), Siriraj Hospital, Mahidol University, Bangkok, under kind provision of Dr. Adisak Wongkajornsilp.
2. Human normal dermal fibroblast (HNDF) cell line (coded CC2511), Cambrex Corp.

Materials

1. Dulbecco's Modified Eagle Medium
2. Fetal bovine serum
3. Antibiotic-Antimycotic (penicillin G, streptomycin sulphate, amphotericin B)
4. Sodium bicarbonate
5. Trypsin-EDTA solution cell culture
6. MTT
7. Trypan blue
8. Dimethyl sulfoxide
9. Sodium chloride
10. Hydrogen peroxide solution (30%)
11. Ribonuclease A Type I-AS Bovine Pan Form
12. Propidium iodide 95-98%
13. Potassium chloride
14. Disodium hydrogen phosphate
15. Potassium dihydrogen phosphate

Reference Antioxidants

1. (-)-Epigallocatechin gallate (EGCG), Sigma-Aldrich, Inc., USA
2. 6-Hydroxy-2, 5, 7, 8,-tetramethylchroman-2-carboxylic acid (Trolox®), EMD Biosciences, Inc., USA
3. L-ascorbic acid, DSM Nutritional Product Co., Ltd., Switzerland
4. Pine bark extract, Human Province Resources Products Imp & Exp, Corp., China

Apparatus

1. Analytical balance, AG 285, Mettler Toledo, Switzerland
2. Centrifuge, Centrifugette 4206, ALC, USA
3. UVA lamp
4. Micropipette, Gilson, France
5. Pipette gun
6. Microplate reader, Model 450, Bio-rad, USA

7. Microplate reader, Victor$^{3®}$ multilable counter, Perkin Elmer Ltd., USA.
8. Multi-channel micropipette, Gilson, France
9. pH meter, Model 420A, Orion, USA
10. Radiometer (UVA probe), IL 1700, International light, Inc., USA
11. Sonicator (Transonic digitals), Elma, Germany
12. Vacuum pump, CB 169 Vacuum System, Buchi, Switzerland
13. Vortex mixer, Vortex Genie-2, Scientific Industries, Inc., USA
14. Hot air oven (UL, Memmert, Germany)
15. Light microscope (CKX31 SF, Olympus, Japan)
16. Water bath
17. Autoclave (HA-3D, Harayama Manufacturing Corporation, Japan)
18. Humidified carbon dioxide incubator (Model 3164, Forma Scientific, USA)
19. Laminar air flow (HBB 24485, Holten, Denmark)
20. Flow cytometer (Becton Dickinson, San Jose, CA, USA)

Others

1. 6-well microplates
2. 96-well TC plate
3. 15 mL Centrifuge tube
4. 50 mL Centrifuge tube
5. 75 Sq.cm. TC flasks Vent Cap
6. Hematocytocrit
7. Appendorf 1.75 mL
8. EnzCheck® Gelatinase/ collagenase Assay kit (E-12055) (Molecular probes, USA)

Methods

Part 1. Effect of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and its active constituent (oxyresveratrol) on normal human dermal fibroblast proliferation and cytotoxicity.

The objective of the present study was to investigate the possible proliferative and cytotoxic effects of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and its active constituent (oxyresveratrol) on fibroblasts using the MTT assay. Dulbecco's Modified Eagle's Medium (DMEM) with 15%v/v fetal bovine serum (FBS) was used as medium due to their ability to provide suitable conditions for maintaining fibroblast cells. Initial concentrations of 10, 25 and 50 μg/mL of the samples were investigated. Cytotoxicity of the test samples at higher concentrations was also determined using the same test.

Cell culture experiments

Fibroblast cultures

Primary cultured fibroblasts were obtained from biopsies of normal human skin coded SiF 49. Cells were maintained in 25-cm^2 tissue culture flasks (T-flask) in 5 mL Dulbecco's Modified Eagle's Medium (DMEM) containing 15%v/v fetal bovine serum (FBS) at 37 °C in a 5 % CO_2 humidified atmosphere. This study used between the 3^{rd} – 5^{th} passages of fibroblasts.

Subculture for each passage

Upon confluency, the medium was removed and washed with 2 mL PBS. Cells were exposed briefly to 1 mL of 4 °C 0.05 % w/v trypsin-EDTA solution and incubated 2 to 5 min at room temperature while examining periodically under the inverted phase-contrast microscope. As soon as fibroblasts were rounded up, added 1 mL of DMEM containing 15%v/v FBS to inactivate trypsin and disperse the cells. Then the cells were harvested by gentle pipetting. Fibroblast suspensions were collected into a 15-mL polypropylene centrifuge tube and centrifuged for 5 min at 1200 rpm. The supernatant was aspirated. The pellet was tapped to dissociate the cells, and

resuspended in 100 to 200 μL of fresh DMEM containing 15%v/v FBS. Sample of cell suspension (10 to 20 μL) was mixed with an equal volume of 0.4% trypan blue and PBS, then the total and viable cells were counted under a microscope using a hemacytometer. Viable cells (3-10 x 10^4) were plated in 5 mL of fresh DMEM containing 15%v/v FBS in a 25-cm^2 tissue culture flask. Medium was changed every 3 to 4 days until the culture became confluent.

Preparation of serum containing medium

One sachet of DMEM powder was dissolved with constant stirring in 800 mL of ultrapure water and stirred until powder was completely dissolved. After completely dissolved, 3.7 g of sodium bicarbonate was added and stirred until a clear solution was obtained. The pH of the medium was adjusted to 7.4 with 5N NaOH, and water was added to achieve a final volume of 1000 mL. The medium was sterilized by filtration through a 0.2 μm filter in laminar air flow. Finally, FBS was added into the DMEM at a final concentration of 15%v/v. Antibiotic-antimycotic was added into DMEM containing 15%v/v FBS at a final concentration of 1%v/v. Completely prepared medium was stored at 4°C in the dark.

MTT assay

MTT assay was measurement of cell viability and proliferation forms the basis of numerous *in vitro* assays in determining a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2) -2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized by the addition of a detergent and quantified by spectrophotometric means. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation. The MTT system is therefore considered a sensitive test to evaluate the extent of proliferation and/or cell viability.

1.1 Proliferation assay

1.1.1 Instrument

Microplate reader, Model 450, Bio-rad, USA,and monitor was set to plate shaker for 10 minutes and read immediately at 595 nm.

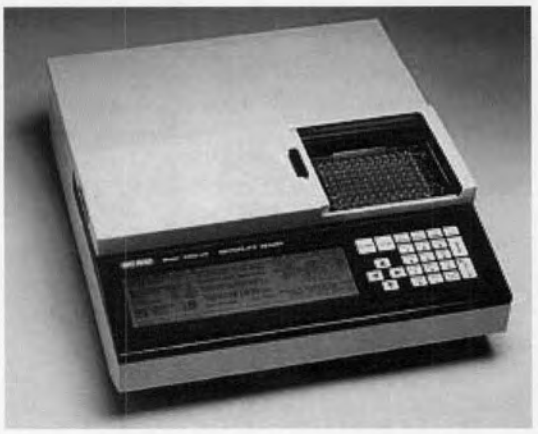

Figure 8 Microplate reader, Model 450

1.1.2 Reagents

1.1.2.1 MTT (3- (4, 5-dimethylthiazolyl-2) -2, 5-diphenyltetrazolium bromide)

1.1.2.2 Dimethyl sulfoxide (DMSO)

1.1.2.3 Test solution

1.1.3 Preparation of the reaction mixture

1.1.3.1 Preparation of MTT solution (0.2 mg/mL)

One mg of MTT was dissolved in 5 mL of DMEM with 15%v/v fetal bovine serum and the solution was stirred until a clear solution was obtained.

1.1.3.2 Preparation of the test solution

The test samples (Puag-Haad and oxyresveratrol) and reference anti-oxidants (EGCG, pine bark extract, L-ascorbic acid, Trolox®) were

separately prepared in DMEM with 15%v/v FBS to give three concentrations of 10, 25 and 50 μg/mL. Assays were carried out in triplicate.

1.1.4 Assay of fibroblast proliferation

An assay of fibroblast proliferation was performed by placing 4 x 10^3 freshly trypsinized fibroblasts, contained in 100 μL of DMEM with supplemented 15% FBS, into 96-well multi-chamber plates and incubated for 24 hr at 37 °C in 5% CO_2 atmosphere to permit adherence to well bottoms. After adherence, the medium was removed and replaced with 200 μL of fresh DMEM with 15%v/v FBS with different concentrations of Puag-Haad and reference anti-oxidants. Then, the cultures were incubated for an additional 72 hr. After 72 hr incubation, fibroblasts were used for the MTT assay.

MTT assay was based on colorimetric method. The 50 μL of 0.2 mg/mL of MTT was added to each well. The plates were incubated for an additional half an hour at 37 °C and then all supernatant was removed. After that, 150 μL of DMSO was added to each well. Plates were placed on a plate shaker for 10 minutes and read immediately at 595 nm using multi-microplate reader. Results were given as the absorbance of viable cells in compound-treated wells and the absorbance of viable cells in non-treated wells.

1.1.5 Statistic analysis

Statistical comparison of the viable cells and % relative OD values was analyzed using ANOVA and Dunnett's test at $\alpha = 0.05$, where appropriate.

1.2 Cytotoxicity assay

1.2.1 Preparation of the test solution

The test samples (Puag-Haad and oxyresveratrol) and reference anti-oxidants (EGCG, pine bark extract, L-ascorbic acid, Trolox®) were separately prepared in DMEM with 15%v/v FBS to give three concentrations of 25, 100 and 250 μg/mL. Assays were carried out in triplicate.

1.2.2 Assay of fibroblast cytotoxicity

An assay of fibroblast cytotoxicity was performed by placing 4 x 10^3 freshly trypsinized fibroblasts, contained in 100 μL of DMEM with supplemented 15%v/v FBS, into 96-well multi-chamber plates and incubated for 24 hr at 37 °C in 5% CO_2 atmosphere to permit adherence to well bottoms. After adherence, the medium was removed and replaced with 200 μL of fresh DMEM with 15%v/v FBS with different concentrations of Puag-Haad and reference anti-oxidants. Then the cultures were incubated for an additional 72 hr. After 72 hr incubation, fibroblasts were used for the MTT assay, which was similar to the previous proliferation test.

1.2.3 Statistic analysis

Statistical comparison of the viable cells and % relative OD values was analyzed using ANOVA and Dunnett's test at $\alpha = 0.05$, where appropriate.

Part 2. Effect of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and oxyresveratrol in decreasing of cell damage induced by oxidative stress.

The objective of the present study was to investigate the ability of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and its active constituent (oxyresveratrol) to reduce the cellular damages induced by oxidative stresses such as hydrogen peroxide (H_2O_2) and UV-A. Dulbecco's Modified Eagle's Medium (DMEM) containing 15%v/v fetal bovine serum (FBS) was also used as the medium. Initial concentrations of 25, 50 and 100 μg/mL of the samples were also used. The cytotoxicity of the test and reference samples were evaluated by both the MTT and LDH (Lactate dehydrogenase) assays.

2.1 Hydrogen peroxide (H_2O_2)-induced cell damage test

2.1.1 Instrument

Microplate reader :As in 1.1.1

2.1.2 Reagents

2.1.2.1 MTT (3- (4, 5-dimethylthiazolyl-2) -2, 5-diphenyltetrazolium bromide)

2.1.2.2 Dimethyl sulfoxide (DMSO)

2.1.2.3 Hydrogen peroxide (H_2O_2)

2.1.2.4 Test solutions

2.1.3 Preparation of the reaction mixture

2.1.3.1 Preparation of MTT solution (0.2 mg/mL) : As in 1.1.3.1

2.1.3.2 Preparation of hydrogen peroxide (H_2O_2) 2 mM solution

Prepare a 100 mM stock solution of the H_2O_2 (30%) by adding 100 μL of H_2O_2 (30%) directly to 9.9 mL of fresh DMEM with 15%v/v FBS with different concentrations of Puag-Haad or reference anti-oxidants. And then, prepare a 2 mM working solution by adding 400 μL of this stock solution to 19.6 mL

of fresh DMEM with 15%v/v FBS with different concentrations of Puag-Haad or reference anti-oxidants to yield a final concentration of 2 mM.

2.1.3.3 Preparation of the test solution

The test samples (Puag-Haad and oxyresveratrol) and reference anti-oxidants (EGCG, pine bark extract, L-ascorbic acid, Trolox®) were separately prepared in DMEM with 15%v/v FBS to give three concentrations of 25, 50 and 100 μg/mL. Assays were carried out in triplicate.

2.1.4 Assay of fibroblast proliferation

An assay of fibroblast proliferation was performed by placing 1 x 10^4 freshly trypsinized fibroblasts, contained in 100 μL of DMEM with supplemented 15%v/v FBS, into 96-well multi-chamber plates and incubated for 24 hr at 37 °C in 5% CO_2 atmosphere to permit adherence to the well bottoms. After adherence, the medium was removed and replaced with 200 μL of fresh DMEM with 15%v/v FBS with different concentrations of Puag-Haad and reference anti-oxidants. Then the cultures were incubated for an additional 24 hr. After 24 hr incubation, the medium was removed and replaced with 100 μL of fresh DMEM with 15%v/v FBS with different concentrations of Puag-Haad or reference anti-oxidants with 2 mM H_2O_2 for 2 hr. After exposure to Puag-Haad and reference anti-oxidants with 2 mM H_2O_2 for 2 hr, the fibroblasts were used for the MTT assay as previously described.

2.1.5 Statistic analysis

Statistical comparison of the viable cells and % relative OD values was analyzed using ANOVA and Dunnett's test at $\alpha = 0.05$, where appropriate.

2.2 Ultraviolet A-induced cell damage test

2.2.1 Instrument

Microplate reader : As in 1.1.1

2.2.2 Reagents

2.2.2.1 MTT (3- (4, 5-dimethylthiazolyl-2) -2, 5-diphenyltetrazolium bromide)

2.2.2.2 Dimethyl sulfoxide (DMSO)

2.2.2.3 Ultraviolet A lamp

2.2.2.4 Test solutions

2.2.3 Preparation of the reaction mixture

2.2.3.1 Preparation of MTT solution (0.2 mg/mL) : As in 1.1.3.1

2.2.3.2 Preparation of the test solutions

The test samples (Puag-Haad and oxyresveratrol) and reference anti-oxidants (EGCG, pine bark extract, L-ascorbic acid, Trolox®) were separately prepared in DMEM with 15%v/v FBS to give three concentrations of 25, 50 and 100 μg/mL. Assays were carried out in triplicate.

2.2.4 Assay of fibroblast proliferation

An assay of fibroblast proliferation was performed by placing 1 x 10^4 freshly trypsinized fibroblasts, contained in 100 μL of DMEM with supplemented 15%v/v FBS, into 96-well multi-chamber plates and incubated for 24 hr at 37 °C in 5% CO_2 atmosphere to permit adherence to the well bottoms. After adherence, the medium was removed and replaced with 200 μL of fresh DMEM with 15%v/v FBS with different concentrations of Puag-Haad and reference anti-oxidants. Then the cultures were incubated for an additional 24 hr. After 24 hr incubation, cells were exposed to UV-A (20 J/cm^2). Then, the viability of the cells was evaluated with MTT assay as previously described.

MTT assay was based on colorimetric method. The 50 μL of 0.2 mg/mL of MTT was added to each well. The plates were incubated for an additional half an hour at 37 °C and then all supernatant was removed. After that, 150 μL of DMSO was added to each well. Plates were placed on a plate shaker for 10 minutes and read immediately at 595 nm using multi-microplate reader. Results were given as the absorbent of viable cell in compound-treated wells and the absorbent of viable cell in non treated wells.

2.2.5 Statistic analysis

Statistical comparison of the viable cells and % relative OD values was analyzed using ANOVA and Dunnett's test at $\alpha = 0.05$, where appropriate.

2.3 LDH assay

Lactate dehydrogenase or LDH is a cytoplasmic enzyme. Normally, it is not secreted outside the cell but upon damage of cell membrane, LDH leaks out. With the LDH test, it is possible to measure the release of LDH from cells based on a colorimetric quantitation after an enzymatic reaction (Vihola et al., 2005). The assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of tetrazolium dye (Figure 9). LDH leakage was measured from human normal dermal fibroblast cells using TOX-7 Cytotoxicity Assay-kit. This method was modified from that of Issa et al. (2004).

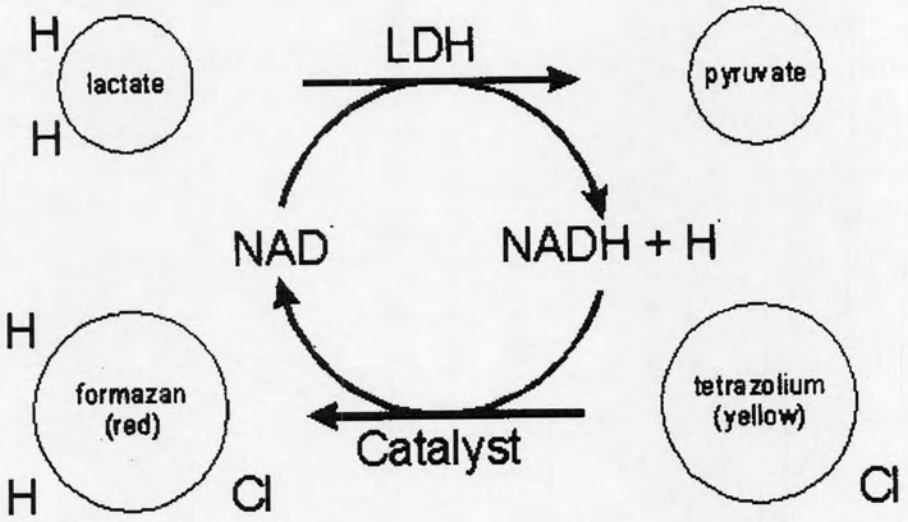

Figure 9 The principle of the cytotoxicity detection kit (LDH)

2.3.1 Instrument

Microplate reader, Victor$^{3®}$ multilable counter, Perkin Elmer Ltd., USA.

Figure 10 The Victor$^{3®}$ multilable plate reader

2.3.2 Reagents

2.3.2.1 LDH assay substrate solution

2.3.2.2 LDH assay cofactor preparation

2.3.2.3 LDH assay dye solution

2.3.2.4 LDH assay lysis solution

2.3.2.5 1N HCl

2.3.3 Preparation of the test solution

The test samples (Puag-Haad and oxyresveratrol) and reference anti-oxidants (EGCG, pine bark extract, L-ascorbic acid, Trolox®) were separately prepared in DMEM (no FBS) to give three concentrations of 25, 50 and 100 μg/mL. Assays were carried out in triplicate.

2.3.4 Preparation of the reaction mixture

2.3.4.1 Preparation of LDH assay cofactor

Preparation was made by adding 25 mL of DMEM to a bottle of lyophilized cofactors. The reconstituted cofactor preparation was stored at 0 °C.

2.3.4.2 Preparation of LDH assay mixture

Equal volumes of LDH assay substrate, cofactor, and dye solution were mixed together at time of use.

2.3.5 LDH assay

Normal dermal human fibroblast cells were seeded in 96-well plates at $5x10^3$ cells/well in 200 μL DMEM and incubated for 24 hours.

a) Total LDH (100% LDH release, no test extract added)

After 24 hr seeding, the cultures were removed from the incubator into laminar air flow hood and 20 μL of LDH assay lysis solution was added to each well. The plate was shaken and returned to incubator for 45 min to allow for precipitation. The supernatant was used for analysis (n = 30 wells).

b) LDH release

After 24 hr incubation, the medium was removed and replaced with 200 μL various of concentrations of the test samples (Puag-Haad and oxyresveratrol) and the reference anti-oxidants (EGCG, pine bark extract, L-ascorbic acid, Trolox®) and incubated for 24 hr, then after 24 hr incubation, cells were exposed

to UV-A (20 J/cm^2), then the cultures were removed from the incubator into laminar air flow hood and the supernatant was used for analysis (n = 3 wells/concentration).

c) Enzymatic assay

One hundred µL supernatant of each well was removed to a new 96-well plate and 50 µL of LDH assay mixture added. The plates were then kept in the dark at room temperature. After 30 min, the reaction was determined by the addition of 15 µL of 1N HCl to each well and absorbance recorded at a wavelength of 490 nm. When 1N HCl was added, the color of the reaction mixture would change from yellow to red. Cytotoxic effects, in term of membrane initiation extent, were calculated as a percentage of average reading of single treatment groups compared to that of total LDH.

2.3.6 Calculation of percentage loss of membrane integrity

The percentage loss of membrane integrity, as seen from the extent of LDH release, was calculated using the following equation.

$$\% \text{ LDH release} = \frac{A - B}{C - D} * 100$$

Where A: The absorbance for LDH release outside the cells that were treated with test solutions

B: The absorbance of test solution without cells (blank of A)

C: The absorbance for maximum LDH release in lysised cells

D: The absorbance of medium without cells (blank of C)

2.3.6 Data evaluation

All experiments were carried out in triplicate (n = 3). The data were expressed as mean $\pm$ SD. Statistical tests at were applied to the data $\alpha = 0.05$ where appropriate.

Part 3. Effect of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and oxyresveratrol in decreasing of DNA damage in cells exposed to UV-A.

Flow cytometry is a laser-based technology that is used to measure characteristics of biological particles. Flow cytometers scan single particle or cell as it flows in a liquid medium past an excitation light source. The underlying principle of flow cytometry is that light is scattered and fluorescence is emitted as light from the excitation source strikes the moving particles.

3.1 Instrument

Flow cytometer (Becton Dickinson, San Jose, CA, USA)

Figure 11 Becton and Dickinson FACS calibur flow cytometer

3.2 Reagents

Fixative reagent : 70% ethanol

3.3 Preparation of test solution

The test samples (Puag-Haad and oxyresveratrol) were separately prepared in DMEM containing 15%v/v of fetal bovine serum to give two concentrations of 25 and 50 μg/mL. Assays were carried out in triplicate.

3.4 Preparation of mixture

3.4.1 Phosphate-buffered saline (PBS)

Eight grams of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 of KH_2PO_4 were dissolved in 800 mL of deionized water and adjusted pH to pH 7.4 with 1N HCl, then adjusted volume to 1,000 mL, the solution was stirred until a clear solution was obtained.

3.4.2 Propidium iodide staining solution (PI)

Five milligrams of PI powder was dissolved in 1 mL of deionized water and the solution was stirred until a clear solution was obtained.

3.4.3 Ribonuclease A (RNase A)

One milligram of RNase A was dissolved in 1 mL of deionized water and the solution was stirred until a clear solution was obtained.

3.5 Flow cytometric analysis

3.5.1 Preparing cell suspension for fixation

Fibroblast cells were seeded in 6-well plate at a density of 2 x 10^5 cells/mL. The cells were further cultivated for 24 hr and treated with Puag-Haad and oxyresveratrol at same concentrations (25 and 50 μg/mL) for 24 hr, then after 24 hr incubation cells were exposed to UV-A (20 J/cm^2), then the cell were further cultivated for 24 hr and treated cells were harvested by exposed to 0.5 mL of 0.05% w/v trypsin-EDTA solution and incubated 2 to 5 min at room temperature, trypsinized cells were floated in the medium into 12 x 75-mm centrifuge tubes. The cell suspension was centrifuge 5 min at 1200 rpm, remove the supernatant and resuspend cells in 0.5 mL of PBS.

3.5.2 Fixing cell in ethanol

Prepare for fixation by adding 4.5 mL of 70% ethanol to each centrifuge tubes, keep on ice. Cell suspensions, prepared as in 3.5.1, 0.5 mL was transferred in to the tubes containing the cold 70% ethanol fixative and keep cells in fixative $\geq$ 2 hr on ice.

3.5.3 Staining cells with PI

Suspended cells in the ethanol were centrifuge 5 min at 1200 rpm, decant ethanol thoroughly. Wash time with PBS by resuspending the pellet of cells in 2 mL of PBS, then centrifuge again and remove the supernatant. After washing with PBS, the cells were resuspended in PBS containing RNase A 100 µg/mL and incubated at 37 °C for 30 min. PI solution was added to a final concentration of 50 µg/mL, and the mixture was then incubated at room temperature for 30 min while protected from light.

3.5.4 Measuring cell fluorescence by flow cytometry

Samples were analyzed for DNA content by a FACSort flow cytometer, excitation was done at 488 nm and emission filter was at 600 nm. CellQuest™ Pro software (Becton Dickinson), was used for detected sub G_0/G_1 phase for DNA distribution in apoptosis process. Fifteen thousand cells in each sample were analyzed and expressed as percentage of total cells.

3.6 Data evaluation

All experiments were carried out in triplicate (n = 3). The data were expressed as mean ± SD. Statistical tests were applied to the data at $\alpha = 0.05$where appropriate by using ANOVA.

Part 4. Evaluation of *Artocarpus lakoocha* heartwood extract (Puag-Haad) and oxyresveratrol for anti-collagenase enzyme activity.

In this study, collagenase inhibitory activity was performed in a 96-well microplate reader (VICTOR®) using EnzCheck® Gelatinase/Collagenase Assay Kit (E-12055) (Molecular probes, product information, 2001) that has been previously used in the study of Chaudhuri, Hwang, Puccetti (2004). DQ™ gelatin (fluorescein conjugate gelatin) was used as a substrate which is gelatin that has been labeled by fluorescein to such a degree that the fluorescence is quenched. The substrates typically exhibit less than 3% of the fluorescence of the corresponding free dye. This substrate is efficiently digested by most gelatinases and collagenases to yield highly fluorescent peptides. Collgenase Type IV (from *Clostridium histolyticum*) was used as a control enzyme. The increase in fluorescence is proportional to proteolytic activity and can be monitored with a fluorescence microplate reader. Therefore, to assay for gelatinase/collagenase inhibitors, observation of the decrease in fluorescence compared with the enzyme activity alone was performed. Fluorescence was measured using a fluorescence microplate reader at 485 nm excitation wavelength and 535 nm emission wavelength (Molecular Probes, product information, 2001).

4.1 Instrument

Microplate reader, Victor$^{3®}$ multilable counter, Perkin Elmer Ltd., USA (As in 2.3.1).

4.2 Reagents

4.2.1 EnzCheck® Gelatinase/Collagenase Assay kit (E-12055)

a) 10X Reaction buffer, 50 mL of 0.5M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM sodium azide, pH 7.6

b) DQ gelatin from pig skin, fluorescein conjugate

c) Collagenase, Type IV from (*Clostridium histolyticum*)

4.2.2 Test samples

4.3 Preparation of the Reaction Mixture

4.3.1 Preparation of 1X reaction buffer 100 mL

10X reaction buffer 10 mL was diluted 90 mL deionized water

4.3.2 Preparation of a 1.0 mg/mL stock solution of the DQ gelatin

A DQ gelatin (1.0 mg/mL) stock solution was prepared by adding 1.0 mL of deionized H_2O directly to one of the vials containing the lyophilized substrate. And then, prepare a 125 µg/mL working solution by adding 1.0 mL of this stock solution to 7 mL of 1X reaction buffer prepared in step 4.3.1 to yield a final substrate concentration of 125 µg/mL. Use 40 µL of 125 µg/mL working solution for assay. (final volume of the reaction mixture = 200 µL per microplate well)

4.3.3 Preparation of a 1000 U/mL stock solution of the *Clostridium* collagenase

A 1000 U/mL stock solution of the *Clostridium* collagenase was prepared by dissolving the contents of the vial in 0.5 mL deionized H_2O. And then, A 0.25 U/mL working solution was prepared by diluting the stock solution with the 1X reaction buffer prepared in step 4.3.1 to yield a final collagenase concentration of 0.25 U/mL, use 40 µL of 0.25 U/mL working solution was used for the assay (final volume of the reaction mixture = 200 µL per microplate well).

4.3.4 Preparation of the test samples

The test compound (6.25 mg) was dissolved in 5 mL of 1X reaction buffer to prepare 1.25 mg/mL stock solution. The stock solution was further diluted with 1X reaction buffer until a suitable range of concentrations (µg/mL) was obtained. For each well, 80 µL of the test solution was added to the reaction mixture to finish the total volume of 200 µL. The final concentration was calculated by the formula below.

$$C_1V_1 = C_2V_2$$

C_1 = Beginning concentration (mg/mL)
V_1 = Beginning volume (μL)
C_2 = Final concentration (mg/mL)
V_2 = Final volume (μL)

For example,

Final concentration of sample solution = 1.25mg/mL * 80μL/200μL
(beginning conc. = 1.25 mg/mL) = 0.5 mg/mL

4.4 Measurement of Activity

The absorbance of the reaction mixtured in four wells (A, B, c and D). In each well, the substance was added in the order of mixing (final volume = 200 μL) as follows:

A (control) 120 μL of 1X reaction buffer (pH 7.6)
40 μL of collagenase, type IV solution (0.25 U/mL)
40 μL of DQ™ gelatin 125 μg/mL

B (blank of A) 160 μL of 1X reaction buffer (pH 7.6)
40 μL of DQ™ gelatin 125 μg/mL

C (sample) 40 μL of 1X reaction buffer (pH 7.6)
80 μL of sample solution in 1X reaction buffer (pH 7.6)
40 μL of collagenase, type IV solution (0.25 U/mL)
40 μL of DQ™ gelatin 125 μg/mL

D (blank of C) 80 μL of 1X reaction buffer (pH 7.6)
80 μL of sample solution in 1X reaction buffer (pH 7.6)
40 μL of DQ™ gelatin 125 μg/mL

After each well was mixed, the mixture was incubated at room temperature for 90 minutes. The fluorescence intensity of each well was measured in a fluorescence microplate reader. Digested products from the DQ™ gelatin were

detected at 485 nm of excitation and at 535 nm of fluorescence emission. EGCG, Pine bark extract, L-ascorbic acid and Trolox® were used as reference anti-collagenase. The assay mixture of each concentration was performed and measured in triplicate.