CHAPTER III

RESEARCH METHODOLOGY

Materials

1. Lipoid® S75 (75% phosphatidylcoline from soybean, Lipoid, Darmstadt, Germany)

2. 99% Cholesterol (A.R. grade, Lot No. 123K0968, Sigma-Aldrich Co., St.Louis, Missouri, USA)

3. Tartaric acid (A.R. grade, Lot No. S27001-215, Sigma-Aldrich Co., St.Louis, Missouri, USA)

4. Chloroform (A.R. grade, Batch No. 07623008, LabScan Asia Co. Ltd., Bangkok, Thailand)

5. Diethylether (A.R. grade, Batch No. 04090204, LabScan Asia Co. Ltd., Bangkok, Thailand)

6. Chitosan derived from crab with molecular weight in range of 100,000-1,000,000 Dalton and more than 90% degree of deacetylation (Aqua Premier Co., Ltd., Chonburi, Thailand)

7. Chitosan derived from shrimp with molecular weight in range of 100,000-1,000,000 Dalton and more than 90% degree of deacetylation (Aqua Premier Co., Ltd., Chonburi, Thailand)

8. Chitosan derived from squid with molecular weight in range of 100,000-1,000,000 Dalton and more than 90% degree of deacetylation (Aqua Premier Co., Ltd., Chonburi, Thailand)

9. HaCaT human keratinicyte primary cell (passage 34, Lot No. 300493-524, Cell Lines Service, Eppelheim, Germany)

10. MML-1 human melanocyte cell line (passage 17, Lot No. 300288-23, Cell Lines Service, Eppelheim, Germany)

11. Dimethylsulfoxide; DMSO (A.R. grade, Lot No. 0320064, Sigma-Aldrich Co., St.Louis, Missouri, USA)

12. Dulbecco's Modified Eagle's Medium; DMEM (A.R. grade, Lot No. 054K8302, Sigma-Aldrich Co., St.Louis, Missouri, USA)

13. Fetal bovine serum;FBS (A.R. grade, Lot No. 41F7063K, GIBCO, UK)

14. Kojic acid (A.R. grade, Lot No. 0832534, Sigma-Aldrich, Germany)

15. Tyrpsin EDTA (A.R. grade, Lot No. 1212385, GIBCO, California, USA)

16. Trypan blue solution (R&D grade, Lot No. 55K2342, Sigma-Aldrich Co., St.Louis, Missouri, USA)

Apparatus

1. Freeze Dryer (model VP190D, s/n VP95C03, Trivac, New York, USA)
2. Rotary Evaporator (model R153, Buchi, Switzerland)
3. Sonicator (model transonic TP690, s/n V901056124, Elma, Germany)
4. Vortex mixer (model G560E, s/n 2-64563, Scientific Industries Inc., USA)
5. LipoFast™-100 extruder (Avestin, Canada)
6. Ultracentrifuge (model XL-80, s/n COX94611, Beckman, USA)
7. Transmission electron microscope (TEM)
8. High performance liquid chromatography HPLC (model ClassVP, Shimadzu, Japan)
9. Incubator (model 311,ThermoForma, USA)
10. Inverted microscope (model TS100, Nikon Eclipse, Japan)
11. Microplate reader (model CeresUV900C, Bio-Tek Instrument, USA)
12. Microplate spectrophotometer (model Spectra Count®, Perkin Elmer, USA)
13. Vacuum system B-169 (NESLAB Instruments Inc., USA)
14. Vertical larminar air flow cabinet (model BHG2004S, Faster s.r.l., Italy)
15. Water bath (model LWB-211A, Daihom LabTech Co. Ltd., Korea)

Experimental Methods

1. Preparation of tamarind fruit pulp's extract

One kilogram of fresh tamarind fruit pulps with a brownish red color and an acid flavor without seed was extracted with 4.5 L water overnight at room temperature. The resultant paste was then filtered through a cloth in order to remove rubbish. The aqueous solution of the fruit pulp was to lyophilized using a freeze dryer. The brown crude extract powder was kept in a tight container at 4^0C. (Viyoch et al., 2003)

2. Quantification of tartaric acid in the extract

2.1 HPLC Condition

The content of tartaric acid in the extract powder was determined by using a high pressure liquid chromatography (HPLC). In isocratic condition, the C8 column (Alltech Econosphere C8 5u Column 4.6x250 mm) was eluted with 0.05 M potassium dihydrogen phosphate (pH 3). Quantification of tartaric acid was based on the peak area at 215 nm. All experiment was performed in triplicate (Viyoch et al., 2003). The chromatographic conditions are as follows:

Flow rate : 1.0 mL/min
Injection volume : 10 μL
Pressure : 1000 Psi
Run time : 10 min
UV absorption : 215 nm

2.2 Calibration curve

The standard solution obtained from dissolving the tartaric acid standard in distilled water to a concentration of 10 ug/mL. The stock solution was then diluted to the concentrations of 0.5, 1, 2, 5 and 10 ug/mL. The calibration curves were performed by plotting the mean peak areas versus the concentrations after run in triplicate.

2.3 Sample preparation

The stock of sample solutions was prepared by dissolving the extracts in distilled water to a concentration of 10 mg/mL then filtering through a 0.45 μm of Millipore before injection.

3. Liposome preparation

3.1 Liposome loaded tamarind fruit pulp's extract

Reverse phase evaporation (REV) method (Weiner et al., 1989) was used to prepare liposome loaded tamarind pulp's extract. To optimize the condition for developing liposome formulation, various ratios of lipoid® and cholesterol were dissolved in organic solvent (chloroform:diethylether mixture, 1:1 v/v). Tamarind pulp's extract which had exactly amount of tartaric acid was dissolved in distilled water. The aqueous solution was dropped to the lipid solution to form a W/O emulsion. The volume ratio between the two phases was varied. The solution mixture was ultrasonicated by a sonicator until appearance of white W/O emulsion. This emulsion had to be stable or unseparated within 30 min. Then, the emulsion was evaporated by a rotary evaporator under vacuum at 45°C until it appeared as gel film around the bottom of the evaporating flask. The gel film was shaked by a vortex until obtaining the free- flow liposome suspension. The various parameters in each batch are shown in table 4.

In order to obtain unilamellar liposomes with homogeneous size, some batches of liposome loaded tamarind pulp's extract was extruded through an extruder device LipoFast™-100 equipped couple with a 100 nm pore size polycarbonate membrane.

Table 4 Compositions of liposomes containing tamarind pulp's extract

Batch	Lipid molar ratio	Lipoid (mg)	Cholesterol (mg)	W/O ratio
1-3	1:1	58.09	41.9	1:5
4-6	1:1	73.49	26.51	2:5
7-9	1:1	82.91	17.09	3:5
10-12	1:1	86.18	13.82	4:5
13-15	2:1	87.39	12.61	1:5
16-18	3:1	58.09	41.9	1:5
19-21	4:1	58.09	41.9	1:5
22-24	5:1	58.09	41.9	1:5
25-27	extrude			

Remark: Molecular weight of lipoid = 532, cholesterol = 386.65

3.2 Chitosan coated liposome

By fix amount of prepared liposome at 1 ml, three selected independent parameters were source of chitosan, chitosan concentration and total amount of chitosan. They were taken at three levels; low, medium and high, which were represented by the transformed values of -1, 0 and 1, respectively. The values of these selected parameters at different levels are shown in table 5. Based on factorial design, twenty-seven batches of chitosan coated liposome (Table 6) were prepared by the method of Perugini with some modification (Perugini et al., 2000). Chitosan was dissolved in an acid solution pH 5. The prepared liposome was coated with chitosan by dropping the vesicle suspension into the chitosan solution under continuous magnetic stirring. Then, they were incubated at room temperature for 2 hr before further analysis.

Table 5 Units of 3^3 Factorial design for preparation of chitosan coated liposome.

Parameters	Levels		
	Low	Medium	High
X1 (source of chitosan)	Crab	Squid	Shrimp
X2 (chitosan concentration)	0.1%	0.5%	1%
X3 (amount of chitosan solution)	1 ml	2 ml	3 ml
Transformed values	-1	0	1

Table 6 Different parameters of each batch

Batch number	x1	x2	x3	Chitosan		
				Source	Concentration (%)	Amount of solution (ml)
28	-1	-1	-1	crab	0.1	1
29	0	-1	-1	squid	0.1	1
30	1	-1	-1	shrimp	0.1	1
31	-1	0	-1	crab	0.5	1
32	0	0	-1	squid	0.5	1
33	1	0	-1	shrimp	0.5	1
34	-1	1	-1	crab	1	1
35	0	1	-1	squid	1	1
36	1	1	-1	shrimp	1	1
37	-1	-1	0	crab	0.1	2
38	0	-1	0	squid	0.1	2
39	1	-1	0	shrimp	0.1	2
40	-1	0	0	crab	0.5	2
41	0	0	0	squid	0.5	2
42	1	0	0	shrimp	0.5	2
43	-1	1	0	crab	1	2
44	0	1	0	squid	1	2
45	1	1	0	shrimp	1	2
46	-1	-1	1	crab	0.1	3
47	0	-1	1	squid	0.1	3
48	1	-1	1	shrimp	0.1	3
49	-1	0	1	crab	0.5	3
50	0	0	1	squid	0.5	3
51	1	0	1	shrimp	0.5	3

Table 6 (cont.)

Batch number	x1	x2	x3	Chitosan		
				Source	Concentration (%)	Amount of solution (ml)
52	-1	1	1	crab	1	3
53	0	1	1	squid	1	3
54	1	1	1	shrimp	1	3

4. Liposome characterization

4.1 Morphology

Shape and appearance of liposome vesicle were observed by an optical microscope after the preparation.

Transmission electron microscope (TEM) was used to investigate the differentiation between chitosan coated and non-coated liposome. A drop of liposome suspension or chitosan coated liposome were applied to carbon coated grids. Two minutes later, the excess was drawn off with filter paper. A satured uranyl acetate aqueous solution was used as a staining agent. After air dried, the sample was analyzed by TEM at 120 kV.

4.2 Zeta potential and particle size analysis

The zeta potential and mean particle size were analyzed by photon correlation spectroscopy (PCS) employing a Zetasizer (Model Nano Zs 90, Malvern, England).

To analyze the zeta potential, liposome formulation was placed in a specific cuvette and then measured by the Zetasizer using laser Doppler electrophoresis. Electrophoresis is a movement of charged particle relative to the liquid that it is suspended in under the influence of an applied electric field. The velocity of a particle

measured in a unit field is referred to as the electrophoretic mobility that is then inverted to zeta potential using a simplification of Henry's equation.

To analyze the mean particle size, an aliquot of liposome formulation were resuspended in sodium chloride solution. Measurements were performed at a fixed angle of 90° to the incident light and data were collected over a period of 3 min.

5. Entrapment efficiency

Each liposome formulation was diluted by distilled water in an exact volume. Then, centrifugation was performed by an ultracentrifuge at 42,000 g for 30 min at 4^{0}C. The supernatant of unencapsulated crude extract was analyzed for the tartaric acid content by HPLC technique as described above. Entrapment efficiency was then calculated as differences between the total amount of the tartaric acid added to the preparation and the amount of tartaric acid in the supernatant.

6. Determination of chitosan reacted with liposome

The same condition of centrifugation technique as described above was used to determine the amount of chitosan reacted with liposome in liposome modified by chitosan formulation. The obtained supernatant was analyzed by colorimetric assay with an anionic reactive dye (congo red). Before analyzing by UV spectrometer at 575 nm, the calibration curve was drawn for chitosan at the concentrations of 10, 20, 30, 40 and 50 ug/ml reacted with fix amount of congo red. The amount of chitosan react with liposome was calculated as differences between the total amount of the chitosan added to the preparation and the amount of chitosan in the supernatant.

7. *In vitro* release of tartaric acid

A dialysis technique was used to analyze an *in vitro* release of liposome formulation. Briefly, 1 ml of liposome formulation was placed in the dialysis sack (Cellu Sep F3, MWCO 12,000-14,000), suspended in 10 ml phosphate buffer (pH 5.5) at 30^0C and agitated at 50 rpm. At schedule time intervals, agitation was stopped; the dissolution medium was collected, filtered and analyzed for tartaric acid content by HPLC. The dissolution medium was then placed in a fresh medium. All dissolution tests were run in triplicate.

8. *In vitro* efficacy of tamarind's AHAs on keratinocyte proliferations

Determination of keratinocyte cell proliferation refers to the cytotoxicity test of tamarind's AHAs in various formulations on skin simulation. HaCaT human keratinocyte primary cells were purchased from Cell Line Services (CLS). The following primary cell descriptions are given as follow:

Designation:	HaCaT
Depositor:	DKFZ, Heidelberg
Organism:	homo sapiens (human)
Ethnicity:	caucasian
Age/Stage:	62 years
Gender:	male
Tissue:	skin
Celltype:	keratinocyte
Growth Properties:	monolayer
Description:	in vitro spontaneously transformed keratinocytes from histologically normal skin.
Culture Medium:	DMEM medium (high glucose) supplemented with 2 mM L-glutamine and 10% fetal calf serum.

Subculturing:	Removed medium, rinsed with 0.05% EDTA, added 0.05% EDTA solution and incubate for 10 to 15 min at 37°C. Took off EDTA, add fresh 0.05% trypsin/0.025% EDTA solution (final concentrations) and let culture sit at 37°C until the cells detach (approx. 2 to 5 minutes). Added fresh medium, aspirated and dispensed into new flasks.
Split Ratio:	A ratio of 1:5 to 1:10 was recommended
Fluid Renewal:	2 times weekly
Freeze Medium:	CM-1, Cell Lines Service
Sterility:	tests for mycoplasma, bacteria and fungi were negative
Biosafety Level:	1
Tumorigenic:	no
Karyotype:	aneuploid (hypotetraploid)

8.1 Treatment of cells

Before being tested, the cell suspension was transferred from 25 cm^2 flask into a 96-well plate ($1x10^4$cells/well) and kept in the incubator (37°C, 5% CO_2) for overnight to complete adhering of the cells on the plate. After 24 hr of cultivation, the old medium was replaced with 200 uL of new DMEM medium containing the various concentrations of tamarind's crude extract solution, liposomes loaded tamarind's crude extract and chitosan coated liposomes loaded tamarind's crude extract. The control cells were cultured in DMEM medium without the extract. Then, the microplate was incubated for 24 hr before proliferation assay.

8.2 XTT assay

Sodium 3,3'-{1-[(phenylamino) carbonyl]-3,4-tetrazolium}-bis (4-metoxy-6-nitro) benzene sulfonic acid hydrate (XTT) was used for cell proliferation assay. The assay is based on the ability of metabolic active cells to reduce the tetrazolium salt XTT to orange compounds of formazan. The dye formed is water soluble and the dye intensity can be read at a given wavelength with a spectrophotometer. The greater the number of

active cells in the well, the greater the activity of mitochondria enzymes, and the higher the concentration of the dye formed, which can then be measured and quantitated. The intensity of the dye is proportional to the number of metabolic active cells. During incubation orange color is formed, of which the intensity can be measured with a spectrophotometer, in this instance with an ELISA reader.

Following incubation, 50 ul XTT labeling mixture was added to each well. The microplate was further incubated for 4 hr. The fraction of viable cells was calculated by subtracting the optical density fraction of treated cells from the untreated cells. Each arrangement had a minimum of three measurements.

9. *In vitro* efficacy of tamarind's AHAs on melanogenesis inhibition

Determination of melanin content in melanocyte cell refers to the potential of AHAs on melanogenesis inhibition. MML-1 human melanoma cells were purchased from Cell Line Services (CLS). The following cell line descriptions are given by:

Designation:	MML-1
Depositor:	CLS
Organism:	Homo sapiens (human)
Tissue:	malignant melanoma; skin
Morphology:	Epithelial
Growth Properties:	monolayer
Culture Medium:	RPMI 1640 medium, 90%; fetal bovine serum, 10%
Subculturing:	Removed medium, added fresh 0.025% trypsin solution for 2 to 3 minutes, removed trypsin, added fresh medium, aspirated and dispensed into new flasks. Subcultured every 6 to 8 days.
Split Ratio:	A ratio of 1:2 to 1:5 is recommended
Fluid Renewal:	2 to 3 times weekly
Freeze Medium:	CM-1, Cell Lines Service
Sterility:	Tests for Mycoplasma, bacteria and fungi were negative

Biosafety Level:	1
Tumorigenic:	yes, in nude mice
Reverse Transcriptase:	Negative

9.1 Cultivation of cells

MML-1 melanocyte cell line were initially cultured in 25 cm^2 flask ($2x10^6$ cells/mL) with RPMI 1640 supplement with 10% FBS at air containing 5% CO_2 and temperature of 37°C. The medium was changed every 2 days. The passage numbers of 3 was used in this study.

9.2 Study of MML-1 growth curve

MML-1 melanocyte $1x10^5$ cells/ml were passage to a 24-well plate, cultured with RPMI 1640 supplement with 10% FBS at air containing 5% CO_2 and temperature of 37°C. The cell morphological was observed under a light microscope and the cells were counted everyday by Trypan blue technique within 7 days. Cell number versus time (day) was plotted to elucidate the graph pattern of cells without the interfering of the samples and then to select the optimum harvesting time for the sample testing.

9.3 Treatment of cells

Before being tested, the cell suspension was transferred from 25 cm^2 flask into a 24-well plate ($1x10^5$cells/well) and kept in incubator for overnight to complete adhering of the cells on the plate. After 24 hours of cultivation, the old medium was replaced with 1.0 mL of new RPMI 1640 medium containing the various concentrations of tamarind's crude extract solution, liposomes loaded tamarind's crude extract and chitosan coated liposomes loaded tamarind's crude extract. The control cells were cultured with RPMI medium without the extract. Kojic acid was used as a positive control.

9.4 Melanin content assay

Melanin content assay was performed by using the modified method (Mun, et al., 2004.) and with triplicate run.

After incubation for 3 days, the treated cells were harvested by using trypsinization and washed twice with phosphate buffer saline (PBS). The samples were air-dried and dissolved in 200 μL of 1 N NaOH. The samples were heated at 80 °C for 1 hr and then cooled down at room temperature. The absorbance of melanin was measured at wavelength of 490 nm. The melanin content per cell was calculated by comparing to the absorbance of the control adjusted to 100%.

9.5 Statistic analysis

All experimental data were analyzed using analysis of variance (ANOVA) and significant difference among means from triplicate analysis at $p<0.05$ were determined by Post Hoc's multiple comparison tests using SPSS 10.0 for window (SPSS Inc., Illinois, USA).