CHAPTER III

MATERIALS AND METHODS

The experiment were divided into five parts:

4. Development, validation of HPLC method and determination of oxyresveratrol content in *Artocarpus lakoocha* heartwood extract (Puag-Haad)
5. Stability evaluation of different aqueous solutions of Puag-Haad
6. Evaluation of anti-wrinkle efficacy of Puag-Haad solutions in human volunteers
7. Formulation of anti-wrinkle lotions containing Puag-Haad
8. Evaluation of anti-wrinkle efficacy of lotions containing Puag-Haad in human volunteers

Crude Drug

1. Dried aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) (80% w/w content of oxyresveratrol as assayed by HPLC), E.A.R. drugstore, Chiangmai, Thailand (Lot no. 25/10/04)

Materials

1. 3-tert-butyl-4-hydroxyanisole (BHA), Fluka, Switzerland
2. Citric acid anhydrous, Ajax Finechem., Australia
3. Dimethicone oil, Namsiang Co., Ltd., Thailand
4. Disodium edetate (EDTA), E. Merck, Germany
5. Disodium hydrogen orthophosphate, E. Merck, Germany
6. Emulgade 1000 NI (Cetearyl alcohol/ceteareth-20), Cognis, Germany
7. Eumugin B2 (Ceteareth-20), Cognis, Germany
8. Finsolve TN (C12-15 alkly benzoate), Finetex, Inc., USA
9. Glyceryl monostearate, Namsiang Co., Ltd., Thailand
10. Green tea extract powder (70% EGCG), Specialty Natural Product Co., Ltd., Thailand

11. Methanol HPLC grade, Labscan Asia Co., Ltd., Ireland
12. Myristol 318 (Caprylic/capric triglycride), Cognis, Germany
13. Oxyresveratrol (244 g/mol), purified from Puag-Haad (purity > 95%) (Wachiranuntasin, 2005)
14. Propyl gallate, Fluka, Switzerland
15. Propylene glycol, Srichand United Dispensary Co., Ltd., Thailand
16. Puresyn No.2 (Hydrogenated polydecene), ExxonMobil Corp., USA
17. Sepicide HB (Phenoxyethanol + combined paraben), Seppic, France
18. Sodium citrate dihydrate, E.Merck, Germany
19. Sodium dihydrogen phosphate dihydrate, E. Merck, Germany
20. Sodium metabisulfite, Ajax Finechem., Australia
21. STAY-C®50 (50% l-ascorbic acid), E.Merck, Germany

Instruments

1. Analytical balance, AG 258, Mettler Toledo, Switzerland
2. Corneometer® (CM 825, Courage + Khazaka electronic GmbH, Germany)
3. Cutometer® (MPA 580, Courage + Khazaka electronic GmbH, Germany)
4. High Performance Liquid Chromatography (LC-10 AD, Shimadzu, Japan)
    - Autosampler (SIL-10A, Shimadzu, Japan)
    - Communications Bus Module (CBM-10A, Shimadzu, Japan)
    - UV detector (SPD-10A, Shimadzu, Japan)
    - Pumps (LC-10 A, Shimadzu, Japan)
5. International Rheology Viscometer (RI:H:I2, Rheology (International) Shannon Ltd., Ireland)
6. Mexameter® (MX 18, Courage + Khazaka electronic GmbH, Germany)
7. Micropipette, Gilson, France
8. pH meter, model 420A, Orion, USA
9. Visioscan® (VC 98, Courage + Khazaka electronic GmbH, Germany)

Methods

Part 1. Development, Validation of HPLC Method and Determination of Oxyresveratrol Content in *Artocarpus lakoocha* Heartwood Extract (Puag-Haad)

The determination of active constituent (oxyresveratrol) was performed by HPLC method due to its specificity and high sensitivity.

1.1. HPLC condition

The HPLC conditions as adapted from Wachiranuntasin (2005) for the analysis of oxyresveratrol in *Artocarpus lakoocha* extract were as folllows :

Column	:	Luna Phenomenex® C18 (2) (5 µm, 250 x 4.6 mm)
Precolumn	:	µBondapack C18, 10µm 125 A°
Mobile phase	:	methanol : water (40:60)
Injection volume	:	20 µl
Flow rate	:	1 ml/min
Detector	:	UV detector 329 nm
Temperature	:	ambient
Run time	:	22 min
Internal Standard	:	Furazolidone

The mobile phase was prepared by using methanol and water with the ratio of 40:60 % v/v. The mixture solution was thoroughly mixed, filtered through 0.45 µm membrane filter and then degassed by sonication for 30 minutes prior to use.

1.2. Standard solutions of HPLC Method

Furazolidone was used as an internal standard due to its appropiate retention time and potential optimal resolution from oxyresveratrol peak. A stock solution of furazolidone was prepared by accurately weighing 50 mg of furazolidone into a 100 ml volumetric flask. A 40 ml of acetonitrile was added to dissolve and mobile phase was added to adjust to the final volume. The final concentration of furazolidone was 0.5 mg/ml.

A stock solution of oxyresveratrol was prepared by accurately weighing 10 mg of oxyresveratrol into 10 ml volumetric flask. Mobile phase was added to dissolved and adjust to the final volume. This stock solution had a concentration of 1 mg/ml. Standard solutions of oxyresveratrol were prepared by pipeting 250, 500, 750, 1000, 1250 and 1500 µl of the above solution into a respective 5-ml volumetric flask. Then, 1 ml of furazolidone stock solution was added into each of these volumetric flasks. The solutions were adjusted to volume with mobile phase so that the concentrations of oxyresveratrol were 50, 100, 150, 200, 250 and 300 µg/ml, respectively and 0.1 mg/ml furazolidone. These standard solutions were prepared for each HPLC run. As a result, the standard curve of oxyresveratrol between concentration and peak area ratio were plotted.

1.3. Preparation of sample solution

The sample stock solution was prepared by accurately weighing 10 mg of Puag Haad extract into a 10 ml volumetric flask. Methanol was added to dissolve the extract and mobile phase was added to adjust to the final volume. This stock solution had a concentration of 1000 µg/ml. Then 1.0 ml of stock solution and 1.0 ml of furazolidone stock solution were transferred into a 5 ml volumetric flask. The solutions was adjusted to volume with mobile phase to give the final concentration of Puag Haad and furazolidone of 200 µg/ml and 100 µg /ml, respectively.

1.4. Validation of HPLC method

The analytical parameter used in the assay validation for the HPLC method were specificity, linearity, precision and accuracy.

1.4.1 Specificity

Under the chromatographic conditions used, the peak of oxyresveratrol must be completely seperated from and not be interfered by the peaks of other components in the sample.

1.4.2 Linearity

Three sets of six standard solutions were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

1.4.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of analytical recovery. Five sets of three concentrations at 60, 180 and 240 µg/ml were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration multiplied by 100.

1.4.4 Precision

a) Within-run precision

The within-run precision was determined by analyzing five sets of three concentrations at 60, 180 and 240 µg/ml in the same day. Peak area ratios of oxyresveratrol to furazolidone were calculated and the percent coefficient of variation (%CV) at each concentration was determined.

b) Between-run precision

The between-run precision was determined by analyzing three concentration at 60, 180 and 240 µg/ml on five different days. The coefficient of variation (%CV) at each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of analytical recovery should be within 98.0-102.0 % of each nominal concentration, whereas the percent coefficient of variation for both within-run precision and between-run precision should be less than 2 %.

Part 2. Stability Evaluation of Different Aqueous Solutions of Puag-Haad

The objective of the present study is to investigate the physical and chemical stability of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) in solutions. Propylene glycol in water (20% v/v) was selected as the solvent since it was shown to provide adequate solubility for Puag-Haad and other reference substances (Pengrungruangwong, 2001). This study was divided into two parts.

2.1 Stability evaluation of *Artocarpus lakoocha* heartwood extract in three buffer systems

Two buffer systems, i.e., citrate and phosphate buffers were selected for testing due to its popularity and compatibility with the skin. Two pH values, 5.5 and 7.0, were chosen because the former value is closed to the human skin pH and the latter is neutral pH value. The buffer system that provided the best physical stability and maintained the highest content of active constituent (oxyresveratrol) was selected for further study. Test samples were kept at accelerated temperature (45°c) for 4 weeks. At initial time (week 0) and at week 2 and 4 the physical appearances and the pH values of the samples were evaluated. Chemical stability of the test samples at various times was also determined by HPLC method.

2.1.1 Sample preparation

Solutions of Puag-Haad at 0.1% w/v concentration dissolved in 20% v/v propylene glycol in water or in 20% v/v propylene glycol and 80% v/v various buffer systems were prepared. The freshly prepared Puag-Haad solution was also used as a control sample in every period. The test samples were as follows:

P = 0.10%w/v Puag-Haad

P+ A1 = 0.10%w/v Puag-Haad in citrate buffer pH 5.5* (50 mM)

P+ A2 = 0.10%w/v Puag-Haad in phosphate buffer pH 5.5 (50 mM)

P+ A3 = 0.10%w/v Puag-Haad in phosphate buffer pH 7 (50 mM)

* Citrate buffer could not be prepared at pH 7.0.

The test samples were kept at accelerated temperature (45°C) in tightly closed glass vials and protected from light for 4 weeks. At the start and every 2 weeks of the study period, their physical properties (color/clarity and pH values) and chemical property (content of active constituent) were investigated.

2.1.2 Physical stability test

For the physical stability test the samples were investigated for pH value, clarity, and color of the solution. The color of each vial was visually compared with the freshly prepared solution of 0.1% Puag-Haad. The degree of discoloration was measured using the following simple numerical scoring system:

Score	Degree of discoloration
0	Normal (pale yellow), no change
+1	Slightly (light yellow) changed
+2	Noticeably (light brown) changed
+3	Markedly (brown) changed
+4	Seriously deteriorated (dark brown)
+5	Almost or completely deteriorated (intense deep brown)

2.1.3 Chemical stability test

For chemical stability test the sample were investigated for content of active constituent (oxyresveratrol) by HPLC method at initial time and every 2 weeks (week 2 and 4).

2.2 Stability evaluation of aqueous solutions of *Artocarpus lakoocha* heartwood extract in the presence of various antioxidants

Solutions of Puag-Haad at 0.10% w/v concentration dissolved in 20% v/v propylene glycol in appropriate buffer (selected from 2.1) were chosen for stability test. Degradation of Puag-Haad solution occurs probably via oxidation, which results in brownish color and can be stabilized by proper use of antioxidant (Pengrungruangwong, 2001). The antioxidants (0.10% w/v sodium metabisulfite, 0.02% butylated hydroxyanisole (BHA), 0.1% propyl gallate (PG) and 0.05%

ethylenediamine tetraacetic acid (EDTA) were chosen as representative antioxidants for inclusion in the samples due to their different mechanisms of action.

2.2.1 Sample preparation

The test samples consisted of 0.10% w/v Puag-Haad, with and without the four antioxidants as well as their combination, dissolved in 20% v/v propylene glycol and 80% v/v selected buffer solution as a solvent. The freshly prepared Puag-Haad solution was also used as control sample in every period. The test samples were as follows:

Sample No.	In selected buffer	0.10% sodium metabisulfite	0.02% BHA	0.10% PG	0.05% EDTA
1*	-	-	-	-	-
2	√	-	-	-	-
3	-	√ (0.15%)**	-	-	-
4	-	√	-	-	-
5	√	√ (0.15%)**	-	-	-
6	√	√	-	-	-
7	√	-	√	-	-
8	√	-	-	√	-
9	√	-	-	-	√
10	√	√	√	-	-
11	√	√	-	√	-
12	√	√	-	-	√
13	√	-	√	√	-
14	√	-	√	-	√
15	√	-	-	√	√
16	√	√	√	√	-
17	√	√	√	-	√
18	√	√	-	√	√
19	√	-	√	√	√
20	√	√	√	√	√

* Sample No. 1 or 0.1% w/v Puag-Haad solution in 20% propylene glycol in water (without buffer) served as control sample.

** To verify whether increasing percentage of sodium metabisulfite contributed to change in color.

The test samples were kept at accelerated temperature (45°C) and ambient temperature (30 °C) in tightly closed glass vials encased and protected from light for 12 weeks and 24 weeks, respectively. At the start and every 4 weeks of the study

peroid, their physical properties (color/clarity and pH values) and chemical property (content of active constituent) were investigated.

2.2.2 Physical stability test

The procedure was the same as those of 2.1.2

2.2.3 Chemical stability test

For chemical stability test the samples were investigated for content of active constituent (oxyresveratrol) by HPLC method at initial time and every 4 weeks.

Part 3 Evaluation of Anti-Wrinkle Efficacy of Puag-Haad Solutions in Human Volunteers

The primary propose of this part was to demonstrate the *in vivo* anti-wrinkle efficacy of *Artocarpus lakoocha* extract or Puag-Haads solution at two different concentrations in healthy volunteers in comparison with 20%v/v propylene glycol which served as control. The anti-wrinkle efficacy of Puag-Haad solutions was also compared with that of two anti-wrinkle agents commonly used in commercial preparations, namely, epigallocatechin gallate (EGCG) and vitamin C. The optimum concentration of Puag-Haad solutions that could provided the highest anti-wrinkle efficacy would be selected to prepare lotions for the next part.

3.1 Study design and subject selection

Ninety healthy female volunteers with age ranging from 30-61 years participated in this single-blind parallel study. The protocol was approved by an independent Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. All of them had given written informed consents prior to enrollment. They were allowed to drop out from the study at any time and were closely monitored for any unwanted side effects such as skin rash or other inflammatory responses throughout the study period. A questionnaire was also given to each volunteer to fill out personal statistic and relevant information as shown in Appendix C.

Subject selection

Inclusion criteria

- Healthy female volunteer
- Aged 30-65 years old
- Permission to write her informed consent before participating in the study.
- Passed the patch test procedure

Exclusion criteria

- Allergic and/or hypersensitivity to cosmetics.
- Allergic to the test solution.
- History of skin disease such as eczema and psoriasis on their faces.

- Receiving systemic or topical drug which could interfere with the evaluation.

They were divided into 4 groups. Each group thus separately received different treatment sample solutions. All of them stopped using any cosmetics on the application areas for at least one week before the start of the experiments. The application areas were the middle areas of both the left and right cheeks . The roughness values were monitored for 4 weeks before the start of the experiment to observe for any fluctuation in the baseline using Visioscan® VC 98 .

3.2 **Patch test** (Anita, Stephen, and McEwen, 1991)

Small amount of test solutions and control were dropped on the 1 x 1 cm gauze pad, secured to the inner side of the arm with Tegaderm®. The right side was the test solution and the left side was a control solution. The patches remained in place for about 24 hours (if any of the patches caused severe itching or pain, it was immediately removed). Then the patches were removed and the skin was flooded with water to remove any residual test substance, then dried with cotton. Visual reading were taken 30 minutes later. The tested area was inspected visually according to the following grading:

0	No erythema
+1	Mild erythema
+2	Severe erythema
+3	Erythema and papules
+4	Erythema, papules and vesiculation

3.3 **Preparation of test samples**

Puag-Haad solution at 0.25% and 0.1% w/v were prepared in 20% v/v propylene glycol in water and were assigned as treatment A and B, respectively. Similarly, solution of 0.1% EGCG and 0.1% vitamin C were prepared in the same solvent and respectively assigned as treatment C and D.

3.4 Application of the test samples on the volunteers' skin

Subjects were divided into four groups. Each group was then ramdomly assigned to the treatment, which was either A,B,C or D. Although the study was of a parallel design, each group of subjects had its own control, i.e., apart from receiving the treatment sample (A, B, C or D) on the right cheeks , the individual subject also received a negative control sample (20%v/v propylene glycol in water) on the left cheeks. The application amount was always 0.05 ml for both the treatment and negative control samples.

After the baseline (no treatment) measurements of skin surface roughness for 4 weeks, each volunteer would self-apply the test and the control sample twice daily, in the morning and at night time for 8 consecutive weeks. They were monitored for any changes in roughness parameters at a two-week interval using Visioscan® VC 98. Each subject was supplied with a pair of similar amber glass bottles capped with a glass dropper on each bottle. The content of each bottle was about 10 ml, which was sufficient for daily application up to two weeks before the next visit. They were all blind regarding which bottle was a control or treatment. The label on each bottle merely stated the coded letter and number of subject and whether the individual bottle was intended to use on which cheek. For example, at each visit subject no. A1 received two bottles, one with a label "A1 left" and the other with a label "A1 right". Only the investigator who prepared all the solutions knew the exact content within each bottle. All the treatment solutions were freshly prepared every two weeks. Upon the next visit, the individual subjects were again measured for the roughness parameters and received another pair of bottles for further application.

To achieve uniform application procedures, each subject was instructed to dispense 1 drop (equivalent to 0.05 ml) of the sample on the finger tips before gently apply with a whirling motion on the cheek.

3.5 Measurement of roughness of the skin

Skin roughness is measured by Visioscan® VC 98 (Figure 7). The video camera was placed at the middle areas of both the left and right cheeks. The roughness parameter was read three times at 3 adjacent spots and the average value was calculated and further used in statistical analysis.

Figure 7. Visioscan® VC 98

3.6 Statistical analysis

The data of average baseline roughness values (week -4 to week 0) among the four groups were analyzed by using one way ANOVA. Paired student's t-test was also applied to test for any differences in roughness values between the left and the right cheeks at every period of the study. Randomized block ANOVA was applied to test for effect of time on the baseline roughness values and application roughness values (week 0 to week 8) within each group, when the significant difference ($P < 0.05$) was indicated, post-ANOVA Dunnett's test was subsequently applied to test the difference in values between the starting time and the subsequent application time. The statistical package for the social sciences (SPSS) program version 11.5 was used in this study.

Part 4 Formulation of the Skin Anti-wrinkle Lotion Containing Puag-Haad

4.1 Development of skin anti-wrinkle lotion

The lotion was formulated by development from a guide formulation which was originally developed by Pheansri (2001), as demonstrated in Table 5. The further modified formulation are shown in Table 6.

Table 5. The overview of the ingredients and compositions of the guide formulation (Pheansri, 2001)

Part	Ingredients	% by weight
A	Petrolatum	3
	Finsolve TN (C12-15 Alkyl benzoate)	1
	Lanette O (Cetostearyl alcohol)	1.4
	Cutina KD 16 (Glyceryl monostearate SE)	2
	Ceteareth-20	1
	Emulgrade NI 1000 (Cetearyl alcohol/Ceteareth-20)	3
	Myristol 318 (Caprylic/capric triglyceride)	3
	Dimethicone	1
	Jojoba oil	0.5
	Vitamin E acetate	0.5
B	Deionized water	77.84
	Glycerine	2
	Propylene glycol	3
	Sodium EDTA	0.1
	Glydant plus (DMDM Hydantoin)	0.2
C	Butylated hydroxyanisole (BHA)	0.01
	Sodium metabisulfite	0.15
	Perfume	0.3

Table 6. Summary of modified Puag-Haad formulations used in this study

Ingredient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Phase A															
Finsolve TN*	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Puresyn No.2**	-	3	3	3	3	3	3	3	3	3	3	3	5	3	3
Cetearyl alcohol	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.6	0.3	0.6	-	0.8	0.8	0.8	0.8
Glyceryl monosterate	1	1	1	1	1	0.8	0.5	1	1	0.5	0.5	1	1	0.8	1
Ceteareth-20	0.5	0.5	0.8	1	0.8	0.5	0.5	0.5	0.5	0.5	0.5	0.8	0.8	0.5	0.5
Emulgade NI1000 ***	2.3	2.3	2	1.8	1.6	1.6	1.6	1.6	1.6	1.6	1.6	2.3	2.3	1.6	2.3
Myristol 318 ****	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Dimethicone	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Phase B															
Glycerine	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Propylene glycol	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Xanthan gum	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	0.8
DI water	81.6	81.6	81.3	81.6	82	82.3	81.6	82.3	82.9	82.9	82.4	81.2	79.2	80.4	79.6
Phase C															
Puag-Haad	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
BHA	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Buffer	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28
Sepicide HB *****	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

* = C12-15 Alkyl benzoate, ** = Hydrogenated polydecene, *** = Cetearyl alcohol/Ceteareth-20, **** = Caprylic/capric triglyceride,

***** = Phenoxyethanol + combined paraben

Procedure

1. Each ingredient was weighed precisely and added by order of mixing.
2. Ingredient of part A and B were separately heated to 70°C with constant stirring.
3. Part B was slowly poured into part A with continuous stirring until the emulsion was formed and then allowed to cool down to 50°C
4. Part C was added to the emulsion and the mixture was stirred with homogenizer for 20 minutes until the emulsion cooled down to room temperature.

4.2 Determination of physicochemical appearance

Physicochemical properties of the lotions were determined as the followings:

4.2.1 Determination of physical appearances

Physical appearances of the prepared lotions including color and phase separation were recorded before and after 6 heating-cooling cycles (1 cycle = 4 °C for 24 hours and 45 °C for 24 hours) .

4.2.2 Viscosity measurement

The lotion viscosity was determined using International Rheology Viscometer®. The measurement of all samples was performed in triplicate before and after 6 heating-cooling cycles .

4.2.3 pH measurement

The lotion pH was measured using the Orion® pH-meter. An electrode was immersed into the lotion sample. The pH value was read when it appeared constant in triplicate before and after 6 heating-cooling cycles.

Part 5 Evaluation of Anti-wrinkle Efficacy of Lotion Containing Puag-Haad in Human Volunteers

The purpose of this part was to demonstrate the *in vivo* anti-wrinkle efficacy of *Artocarpus lakoocha* or Puag-Haad lotion at a selected concentration in healthy volunteers. The anti-wrinkle activity of Puag-Haad lotion was also compared with that of one anti-wrinkle agent selected from Part 3.

5.1 Study design and subject selection

Fourty-four female healthy volunteers (selected from the same group as in Part 3), aged ranging from 30 – 55 years were recruited by the same criteria as those in 3.1

They were divided into 2 groups of 22 subjects each. Each group thus separately received different treatment sample lotion. All of them stopped using any cosmetics on the application areas for at least one week before the start of the experiments. The application areas were the whole area of both the left and the right face.

5.2 Preparation of test samples

Puag-Haad at the optimum concentration selected from Part 3 was prepared in lotion base and was assigned as treatment A. Similarly, lotion of a reference antioxidant, also selected from Part 3, was prepared in the same lotion base and assigned as treatment B.

5.3 Application of the test samples on the volunteers' skin

Subjects were divided into two groups, each group was then ramdomly assigned to the treatments, which was either A or B. Although the study was of a parallel design, each group of subjects had its own control, i.e., apart from receiving the treatment sample (A or B) on the one side of the face, the individual subject also received a negative control lotion base on the other side of the face. The application amount was always 0.2 g for both the treatment and negative control samples.

The application areas on which the samples were to be applied were also balanced in each group. For example, the first eleven subjects (subjects no. A1 to A11) of group A were applied with 0.2 g of lotion base (control) on the left side of

their faces and 0.2 g of Puag-Haad lotion (A) on the right side of their faces. Vice versa, the remaining eleven subjects of Group A (Subject no. A12-A22) received 0.2 g of A on their left faces and 0.2 g of lotion base on their right countenances.

Similarly, subjects no. B1 to B11 of Group B received only lotion base on the left side of their faces and reference antioxidant lotion (treatment B) on the right side of their faces. Subjects B12 to B22 then received treatment B and lotion base in an opposite manner.

Each volunteer would self-apply the test and the control sample twice daily, in the morning and at night time for 8 consecutive weeks. They were monitored for any changes in skin roughness parameters and skin elasticity at week 0 and every two weeks interval using Visioscan® VC 98 and Cutometer® MPA 580, respectively. The melanin value and skin hydration extent were also monitored for any changes at week 0 and every four weeks using Mexameter® MX18 and Corneometer® CM 825, respectively. Each subject was supplied with a pair of similar plastic pumped bottles. The content of each bottle was about 30 g, which was sufficient for 8 - week application. They were all blind regarding which bottle was a control or treatment. The label on each bottle merely stated the coded letter and number of subject and whether the individual bottle was intended to be used on which side of the face. For example, at each visit subject no. A1 received two bottles, one with a label "A1 left" and the other with a label "A1 right". Only the investigator who prepared all the lotions knew the exact content within each bottle.

To achieve uniform application procedures, each subject was instructed to dispense 1 drop (equivalent to 0.2 g) of the sample on the finger tips before gently apply with a whirling motion on the face.

5.4 Measurement of roughness of the skin

Skin roughness was measured by Visioscan® VC 98. The video camera was placed at the left and then at the right cheeks. The roughness parameter was read three times from 3 adjacent spots and the average value was calculated and further used in statistical analysis.

5.5 Measurement of elasticity of the skin

Skin elasticity was measured by Cutometer® MPA 580. The probe was placed at the left and then at the right cheeks. The elasticity parameter (R2) was read three

times from 3 adjacent spots and the average value was calculated and further used in statistical analysis.

5.6 Measurement of melanin

The probe of the Mexameter MX® 18 was placed at the left and also at the right cheek. The melanin level were read three times from 3 adjacent spots and the average values were calculated and further used in statistical analysis.

5.7 Measurement of skin hydration

The probe of the Corneometer® CM 825 was placed at the left as well as at the right cheeks. The skin capacitance was read three times from 3 adjacent spots and the average value was calculated and further used in statistical analysis.

5.8 Statistical analysis

Paired student's t-test was also applied to test for any differences in roughness values, elasticity parameter (R2), melanin value and skin capacitance between the control and the treatment cheeks at every period of the study. Randomized block ANOVA was applied to test for effect of time of all parameters above within each group, when the significant difference ($P < 0.05$) was indicated, post-ANOVA Dunnett's test was subsequently applied to test the difference in values between the starting time and the subsequent application time.

5.9 Sensory evaluation by panelists

The panelists evaluated the ease of applying, speed of absorption, stickiness and overall perception after applying the test solutions. They also evaluated some changes in their skin after the experiment. These included skin roughness, elasticity, whitening and softening. The ease of applying, absorption rate, stickiness and overall perception were graded and recorded as 1, 2 and 3 according to the degree of satisfaction. Their details were as follows:

Over all liking of samples characteristic	Score		
	3	2	1
Ease of application	easy	moderate	hard
Speed of absorption	fast	moderate	slow
Stickiness	least sticky	moderate sticky	most sticky
Overall perception	most satisfied	moderate satisfied	least satisfied

The skin roughness, elasticity, whitening, softening were graded as follows:

Skin roughness	a)	roughness reduces more on the left side
	b)	roughness reduces more on the right side
	c)	roughness equally reduces on both left and right side
	d)	no change on both left and right side
Skin elasticity	a)	elasticity increases more on the left side
	b)	elasticity increases more on the right side
	c)	elasticity equally increases on both left and right side
	d)	no change on both left and right side
Skin whitening	a)	more whitening on the left side
	b)	more whitening on the right side
	c)	more whitening on both left and right side
	d)	no change on both left and right side
Skin softening	a)	more softening on the left side
	b)	more softening on the right side
	c)	more softening on both left and right side
	d)	no change on both left and right side