CHAPTER III

MATERIAL AND METHODS

Materials

1. Chemicals and Reagents

The dried aqueous extract of *Artocarpus Lakoocha* heartwood (Puag-Haad powder), E.A.R. drug store, Chiangmai province, Thailand

Tranexamic acid (Batch no. EY200, Daiichi pharmaceutical Co., Ltd, Japan)

Niacinamide USP (Vita Co.,Ltd, Thailand)

Lactic acid (South city supplier Ltd., Part, Thailand)

Myristol 318 (Batch no. Z313516869, Cognis, Thailand)

Eumulgrade 1000 NI T (Batch no. Z312224304 supplied by GeTeCe Co., Ltd., Thailand)

Glydant plus (Water doctor Co., Ltd., Thailand)

Dimethicone oil (Namsiang Co., Ltd., Thailand)

Jojoba oil (Chemico supply Co., Ltd., Thailand)

Cutina KD 16 (Batch no. CD02990008 supplied by GeTeCe Co., Ltd., Thailand)

Lanette O (Batch no. CF027766917 supplied by GeTeCe Co., Ltd., Thailand)

Eumulgin B2 (Batch no. 303012 supplied by GeTeCe Co., Ltd., Thailand)

Glycerin 99% (Batch no.0343/GLY/XI/01, Sinar oleochemical., Thailand)

Propylene glycol (Batch no. PG23945921, Dow, USA.)

Vitamin E acetate (Lot no. 37-5266, BASF, Germany)

Disodium Edetate (EDTA) (Batch no. K90926921, Merck, Germany)

Butylated Hydroxyanisole (BHA) (Lot no. 99-18 supplied by Namsiang Co., Ltd., Thailand)

Sodium metabisulfite (Batch no.001276 01, Interchem, BASF, Germany)

Thiopental sodium (Batch no. 67-091-B1, Abbott Laboratories Ltd., Thailand)

Normal saline

Alcohol

2. Equipment

Analytical balance (Mettler Toledo, Switzerland)

Mexameter MX16® (Courage+Khazaka electronic GmbH, Germany)

UV B lamps (Model TL 20w/12, Phillips)

Insulin syringe 100 unit

Disposable syringe 5 ml (Nissho Nipro Co., Ltd)

Homogenizer (serial no. 51700906, National*SS*motor, Matsushita electronic industrial Co., Ltd.)

pH meter (Model 420A, Orion Research Operation, Boston, USA.)

Volumetric flask (25ml, 50ml, 100ml)

Beaker (50ml, 100ml, 250ml)

3. Animals

Brownish black guinea pigs (Nudee pet shop, Jatujak, Bangkok, Thailand)

Methods

Part 1 Evaluation of skin whitening efficacy of aqueous extracts of *Artocarpus lakoocha* heartwood (Puag-Haad), niacinamide, lactic acid, and their combinations in guinea pigs

In vivo guinea pig depigmentation study. The procedure follows that of Imokawa et al. [42] with slight modifications.

1.1 Animal selection and experimental design

Thirty brownish black guinea pigs weighing 150-200 g were used in the experiments (Figure 14). All guinea pigs were maintained at room temperature with circulated airflow. Two or three guinea pigs per cage were allowed to acclimatize for 2 weeks before UVB irradiation was induced (Figure 15). They received commercial pellet diet and vitamin C-supplemented water once daily. (One tablet of vitamin C was added to the typical 12-ounce water bottle). Bedding materials and the cages were changed every three days to prevent ammonia and bacterial grow-up.

The guinea pigs were randomly divided into 5 groups (n=6/group) as described below.

- group A received 0.25% Puag-Haad solution in propylene glycol only
- group B received 1% niacinamide (vitamin B_3) solution in propylene glycol only
- group C received 1% lactic acid solution in propylene glycol only
- group D received 0.25% Puag-Haad + 1% niacinamide solution in propylene glycol
- group E received 0.25% Puag-Haad + 1% niacinamide + 1% lactic acid solution in propylene glycol

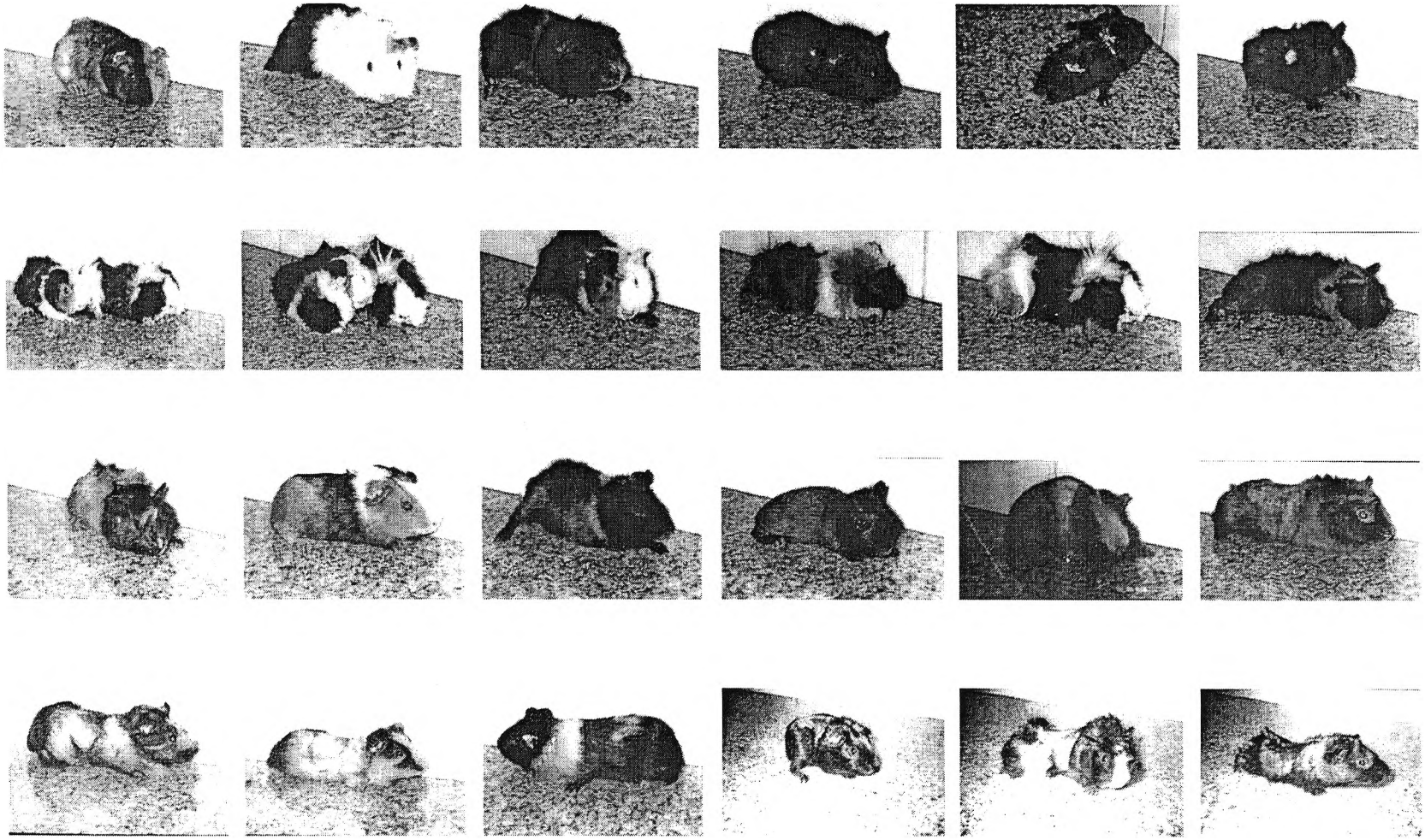

Figure 14. Picture of some brownish black guinea pigs (weighing 150-200 g) were used in the experiments

Figure 15. Picture showing the room and shelf for keeping the guinea pigs (2-3 guinea pigs per cage). The animals were allowed to acclimatize for 2 weeks before UVB-induction.

1.2 Anesthetization

All animals were weighed to adjust dose before being anesthetized with intraperitonial injection of thiopental sodium (40mg/kg) [43]. Before irradiation, the melanin value (M) and erythema value (E) of each guinea pig were measured using Mexameter MX16®. Their backs were shaved with a hair clipper 24 hr before UVB exposure and before every melanin/erythema measurements.

1.3 UVB irradiation

As soon as the animals were anesthetized, their eyes were covered with eye pads to protect them from UVB ray. Then, their backs were irradiated under UVB lamps having wavelengths of 290-320 nm (model TL 20w/12, Phillips, Austria), at an intensity of 0.3 mW/cm^2 for 50 min per day (total energy = 900 mJ/cm^2 per day) for 3 consecutive days (Figure 16). The exposure level was calculated by using the following formula:

Dosage (milli-Joules)/Power density (mW/cm^2) = Exposure time in seconds

Three weeks after the first day of irradiation, the back of each guinea pig was darkened. The melanin and erythema values were measured again with Mexameter MX16®.

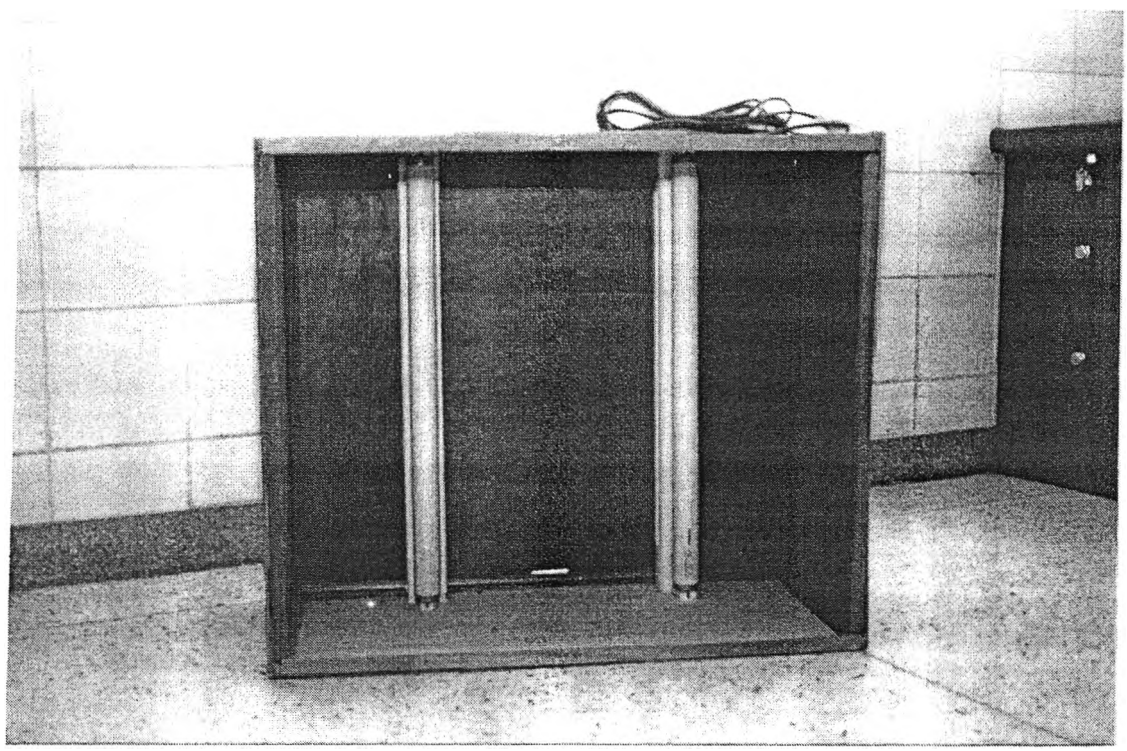

Figure 16. The UVB fluorescent lamp, 290-320nm (model TL 20w/12, Phillips)

1.4 Preparation of the test solutions

The dried aqueous extract of *Artocarpus Lakoocha* (Puag-Haad powder, niacinamide and lactic acid were separately weighed and dissolved in propylene glycol to make 0.25% Puag-Haad (solution A), 1% niacinamide (solution B) and 1% lactic acid (solution C) as well as their combinations (solutions D and E). All test solutions were freshly prepared every two days.

1.5 Application of the test solutions on the back skin of guinea pigs

The back of each guinea pig was divided into 2 areas via the dorsal line along the body. The left-hand side was to be applied with propylene glycol (control) and the right- hand side with the test solution. Next, the test sample and propylene glycol (PG) were separately applied (0.5 ml each) on the designated areas of each guinea pig (3×3 cm^2) every morning for 4 weeks (Figure 17).

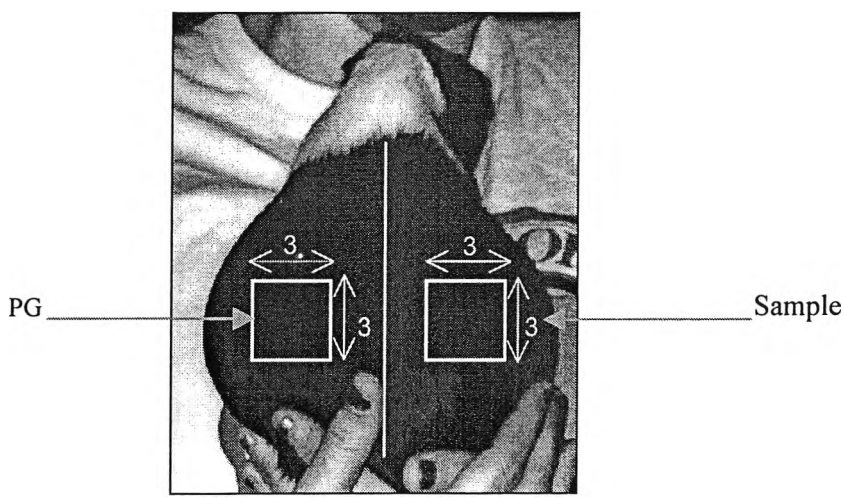

Figure 17. Application areas on the back of each guinea pig. Left = control propylene glycol (PG) and right = test sample.

1.6 Determination of absolute melanin (M) and erythema (E) values and data analysis

To evaluate the effect of skin whitening agents, Mexameter MX16® was used to measure changes in skin color and erythema (5 readings per area per animal). The measurement was continued every week until the end of week 4. The absolute M and E values between the treated (right) and the control (left) areas were compared within in each group every week using paired Student's t-test at 5% significance level. The purpose of this test was to determine the week at which the M values of the treatment began to be significantly lower than the control. The earlier detection of significance suggested the better efficacy of that specific treatment in terms of the skin whitening rate. When all of the five treatments became significantly effective over their self-controls, further one-way ANOVA and Duncan's test would be applied at the same significance level to compare the whitening efficacy among different treatments. The whitening efficacy or melanin reduction was defined as the difference in the absolute melanin value between the control and the treated area in each subject.

The melanin reduction = Xc – Xtr

Where Xc = absolute melanin value (M) of the control area

Xtr = absolute melanin value (M) of the treated area

Similarly, the absolute erythema (E) values were analyzed within each group using paired t-test to see if any of the test solutions gave significant deviation in the E values from its self-control at 5% level.

The erythema change = Ec-Etr

Where Ec = absolute erythema value (E) of the control area

Etr = absolute erythema value (E) of the treated area

Part 2 Formulation of the skin lightening lotion

2.1 Development of skin whitening base formulation

The lotion base was formulated by development of guide formulation as demonstrated in Table 4. The further generated formulation was shown in Table 5 and 6.

Table 4. The overview of the ingredients and compositions of guide formulation

Part	Ingredients	% by weight
A	Petrolatum	3
	Finsolve TN	1
	Lanette O (cetostearyl alcohol)	1.4
	Cutina KD 16 (Glyceryl monostearate SE)	2
	Cetereth-20	1
	Emulgrad NI 1000	3
	Myristol 318	3
	Dimethicone	1
	Jojoba oil	0.5
	Vitamin E acetate	0.5
B	Deionized water	77.84
	Glycerin	2
	Propylene glycol	3
	Sodium EDTA	0.1
	Glydant plus	0.2
C	Butylated hydroxyanisole (BHA)	0.01
	Sodium metabisulfite	0.15
	Perfume	0.3

Procedure

1. Each ingredient was weighed precisely and added by order of mixing
2. Ingredients 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 is stirred with homogenizer for 20 minutes until the emulsion cool down to room temperature.
5. The pH was adjusted to 5.5.

Table 5. The overview of the ingredients and compositions

Part	Ingredients	Basic ingred.	1	2	3	4	5
A	Finsolve TN	3					
	Lanette O (cetearyl alcohol)	0.5					
	Cutina KD 16 (Glyceryl monostearate SE)		0.5	0.8	1	1.5	2
	Cetereth-20	0.5					
	Emulgrad NI 1000	2					
	Myristol 318	3					
	Dimethicone	1					
	Jojoba oil	0.5					
	Vit-E acetate	0.5					
B	Deionized water		82.84	82.44	82.24	81.74	81.24
	Glycerin	2					
	Propylene glycol	3					
	Sodium EDTA	0.1					
	Glydant plus	0.2					
C	BHA	0.01					
	Sodium metabisulfite	0.15					
	Perfume	0.3					

Table 6. The overview of the ingredients and compositions (continued)

Part	Ingredients	Basic ingred.	6	7	8	9	10	11
A	Finsolve TN	3						
	Lanette O (cetostearyl alcohol)		0.6	0.7	0.8	0.9	1	1.2
	Cutina KD 16 (Glyceryl monostearae SE)	1						
	Cetereth-20	0.5						
	Emulgrad NI 1000	2					2.3	2.3
	Myristol 318	3						
	Dimethicone	1						
	Jojoba oil	0.5						
	Vit-E acetate	0.5						
B	Deionized water		82.14	82.04	81.94	81.84	81.44	81.34
	Glycerin	2						
	Propyleneglycol	3						
	Sodium EDTA	0.1						
	Glydant plus	0.2						
C	BHA	0.01						
	Sodium metabisulfite	0.15						
	Perfume	0.3						

Preparatory procedure was similar in all formulations.

2.2 Determination of physical appearance

The physical appearance of each formulation was recorded at room temperature immediately after preparation and at 2 weeks. Then, the best formulation was chosen as the common lotion base for addition of the whitening agent(s) pre-selected for the clinical test.

Part 3 Evaluation of the whitening efficacy of aqueous extracts of Artocarpus lakoocha heartwood (Puag-Haad), niacinamide, tranexamic acid, and their combinations in human volunteers (Clinical test)

3.1 Subject selection and study design

Eighty-four Asian females (age 25-45), with medium to dark skin were enlisted in this single-blinded parallel study with self-control. All volunteers had the starting melanin values in the range of 450-550 indicating the skin type V (dark skin) as shown below [13]. Subjects, who had used within the last 6 months retin A, chemical peels, hydroquinone products, benzoyl peroxide, or any whitening products were excluded. The protocol was approved by the ethics committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. Each subject also had given a written informed consent. The melanin (M) and erythema (E) values of each subject were measured with Mexameter MX16® before applying the lotion and at 2 week-interval after daily application for 12 weeks. The skin type of subjects was graded by Mexameter as follows:

Type	**Appearance**	**melanin value**
I (Celtic)	very fair skin, red hair, freckles	400-470
II (Caucasian white)	fair skin, blond, blue eyes	410-490
III (Mixed type)	blond-brown hair, brown eyes	420-510
IV (Dark skin)	dark skin	450-550
VI (Black skin)	black skin	520-700

Then, subjects were divided into 6 groups (n = 14 in each group)

- group A received lotion containing 0.25% Puag-Haad only
- group B received lotion containing 1% niacinamide only
- group C received lotion containing 2.5% tranexamic acid only
- group D received lotion containing 0.25% Puag-Haad + 1% niacinamide
- group E received lotion containing 0.25% Puag-Haad + 2.5% tranexamic acid
- group F received a commercial whitening lotion

The test lotions (A, B, C, D, and E) had the same common lotion base with initial pH of 5.5 except for the commercial lotion F, of which the lotion base composition was not known. However, the base formulated in this study was used as the self-control for group F. The active ingredients of the commercial lotion are shown in Appendix II.

The upper arms of each subject were applied separately with 0.2 ml of the lotion base (control) and one of the test lotions. The assignment of the lotion (control or test) to the right or left arms was carefully balanced in each group of subjects. Each subject applied the lotion twice a day for 12 weeks. During the experiment, subjects were instructed to cover their upper arms by wearing suitable attires especially in the daytime to protect them from sun exposure.

3.2 Preparation of the test lotions

The control lotion base and the test lotions containing Puag-Haad, niacinamide, tranexamic acid and their combinations were freshly prepared every 2 weeks using the best formulation of the 11 trial bases as described in Tables 5 and 6.

3.3 Sample application on the volunteer's skin

After volunteers were selected and divided into six groups of 14, each group was randomly assigned to the test lotions A, B, C, D, E or F. The left upper arms of the first 7 subjects in each group were applied with 0.2 ml of the lotion base as a self-control whereas their right upper arms were applied with 0.2 ml of one of the test lotions. Vice versa, the latter 7 subjects in the same group received 0.2 ml of the control lotion base on their right arms and the test lotion on their left arms.

Each volunteer was instructed to apply the lotion base and the test lotion twice a day, in the morning after taking a shower and at bedtime, for 12 consecutive weeks. Each volunteer received a pair of amber glass bottles capped with glass droppers. Each bottle was labeled with the coded letter such that the subject would not know the identity of the bottle contents. Only the investigator knew the identity of the content inside each coded bottle (single-blinded study).

To achieve standard uniform procedure, each subject was instructed to dispense 3 drops of lotion on her fingertips before applying with a soft whirling motion on each of her upper arms, starting with the left arm first and continue with the right arm. Subjects were warned to wash their hands when switching arms. The application area was about 6 x 6 cm^2 located on each of the upper, outer arms. Every 2 weeks, the subjects visited the laboratory to measure the melanin and erythema values and received a new pair of bottles for continuous application.

3.4 Determination of melanin content and erythema extent

At the start of the study and at 2,4,6,8,10, and12 weeks, each subject's upper arms were measured for the melanin (M) and erythema (E) values. The probe of the Mexameter was directly pressed for 1 second against the skin surface. Five readings of the M and E values were taken on each arm. After the measurement had been finished, the Mexameter automatically displayed the M and E values with the accuracy of ± 5%[12]. These values were recorded into a computer. Then, the results between the test and the control-treated arms were compared within each subject at every 2 weeks until the end of the experiment using paired student's t-test.

The Mexameter was calibrated before each use to ensure reproducibility between different evaluation dates. The principle of the mexameter MX16® is based on the absorption. The special probe of the mexameter MX16® emits light of three defined wavelengths (568 nm: green, 660 nm: red, and 880 nm: infrared). A receiver measures the light reflected by the skin. As the quantity of emitted light is defined, the quantity of light absorbed by the skin can be calculated. The melanin is measured by 2 wavelengths (660 and 880 nm). These wavelengths have been chosen in order to achieve different absorption rates by the melanin pigment. Employing the same principle as melanin, two different wavelengths (568 and 660 nm) are used to measure the absorption capacity of the skin in the erythema measurement. One of these wavelengths (660 nm) corresponds to the spectra absorption peak of hemoglobin. The other wavelength (568 nm) has been chosen to avoid other color influences (e.g. bilirubin). The melanin and erythema values were calculated as follows:

Melanin value = 500/log5 *log (Infrared-Reflection/Red-Reflection)+5

Erythema value =500/log5 *log (Red-Reflection/Green-Reflection)+5

The higher the value the more melanin or erythema is detected [44,45].

3.5 Analysis of melanin and erythema data obtained in human volunteers

The volunteers were regularly monitored for any possible skin disorders by a physician throughout the entire period of study. Should any signs of allergic reactions or skin sensitization develop, the subject would be withdrawn from the study and continue to receive proper treatment until full recovery was achieved.

The measurement was conducted every 2 weeks until the end of week 12 (0,2,4,6,8,10,12). Thereafter, the results (absolute M values) between the test and the control areas were compared using a two-tailed paired student's t-test at 5% significance level to indicate a significant difference within each group. The purpose of this test was to determine the week at which the M value of the test lotion became significantly smaller than the control lotion. The earlier significant detection suggested the better efficacy of that specific lotion in terms of the skin whitening rate. Further one-way ANOVA and Duncan's test were then performed on the test lotions, which showed significant whitening effect over their corresponding self-control at the same significance level. This was to compare the relative skin whitening efficacy among different effective lotions. The whitening efficacy or melanin reduction was defined as the difference in the absolute melanin values between the control and the test areas in each subject.

The melanin reduction was calculated according to the following formula:

The melanin reduction = Xc – Xtr

Where Xc = absolute melanin value (M) of the control area

Xtr = absolute melanin value (M) of the test lotion-treated area

For determination of the erythema content, treatment of the data and statistical evaluation were similar to the melanin values.