CHAPTER III
MATERIALS AND METHODS

The experiments were divided into three parts:

1. Evaluation of skin whitening efficacy of aqueous extracts of *Artocarpus lakoocha* heartwood (Puag-Haad) and *A. gomezianus* root (Haadnun) in guinea pigs.
2. Evaluation of skin whitening efficacy of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) in human volunteers.
3. Stability evaluation of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) solutions.

Materials:

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

The root extract of *A. gomezianus*, donate by Department of Pharmacognosy, Faculty of Pharmaceutical Science, Chulalongkorn University.

Tyrosinase (EC 1.14.18.1: from mushroom T-7755), Lot no. 17495594, Sigma Chemicals Co., St. Louis, MO, USA.

L-DOPA (L-3,4-Dihydroxyphenyl-ALANINE), Lot no. 55H0565, Sigma Chemicals Co., St. Louis, MO, USA.

Kojic acid, Lot no. 300015, Nikko Chemicals Co., Ltd., Japan.

Licorice extract PT-40, Lot no. 30208026, Maruzen Pharmaceutical Co., Ltd., Japan.

Disodium Edetate (EDTA), Lot no. R010352, T Chemical Co., Ltd., Thailand.

Butylated Hydroxyanisole (BHA), Lot no. 000760, Nikko Chemicals Co., Ltd., Japan.

Sodium Metabisulfite, Lot no. 000849, VIV Interchem Co., Ltd., Thailand.

Propylene glycol, Lot no. 990807, Srichand United Dispensary Co., Ltd., Thailand.

Equipment:

Microplate reader, Model 450, BIO-RAD, USA.

96-well microplate, Nunc, Denmark.

Micropipet, Gilson, France.

Analytical Balance, Sartorius 1615 MP, Gottingen, Germany.

pH Meter, Model 420A, Orion Research Operation, Boston, MA, USA.

Mexameter MX 16, Courage+Khazaka electronic GmbH, Germany.

UVB lamp (290 – 320 nm), Model TL 20 W/12, Philips.

Hot air oven, Memmert, Germany.

Part 1. Evaluation of skin whitening efficacy of aqueous extracts of *Artocarpus lakoocha* heartwood (Puag-Haad) and A. gomezianus root (Haadnun) in guinea pigs

This preliminary screening test was to evaluate the skin depigmenting (whitening) effect of four natural agents using black-skinned guinea pigs as a model.

1. UV induced pigmentation

Black guinea pigs were used in this study because they have a moderate number of melanocytes and melanosomes, not only in the pilary structures but also in the epidermis, which are similar to the distribution found in human skin. These animals show a good skin response to UV and chemicals (Scott et al., 1969; Curry, 1974; Imokawa et al., 1986). This method was modified from those of Imokawa et al., and Jang et al., 1997.

1.1 Twenty-five black-skinned guinea pigs (weight, 250 - 300 g) were shaved on the back and irradiated by a UVB (290 - 320 nm) lamp at a total energy of 900 mJ/cm^2 per day for 3 consecutive days.

Method of UVB exposure

UVB lamp is fluorescent lamp (290 – 320 nm). The black skinned guinea pig was shaved cleanly by a hair clipper. The back was irradiated with 900 mJ/cm^2 (0.25 mW/cm^2 x 60 minutes) from UVB lamp (Philips TL 20 w/12) daily for 3 consecutive days. The distance between lamp and back of guinea pig was determined by measuring power density by UV meter. The UV meter reading 0.25 mW/cm^2 at 17.5 cm distanced from UVB lamp. The exposed time with lamp, estimated as follows:

$$\text{Exposed time (sec)} = \text{Dosage (mJ)/Power density (mW/cm}^2\text{)} \quad (1)$$

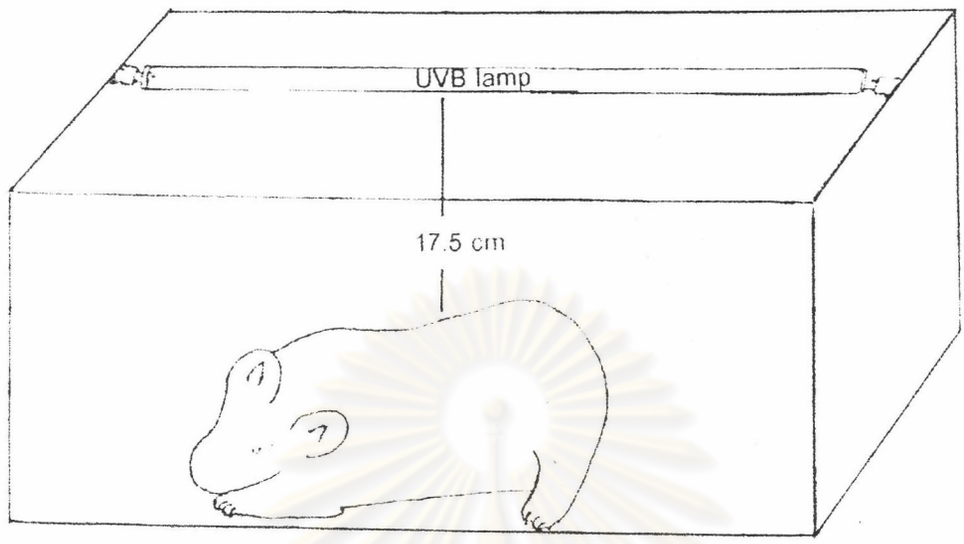


Figure 6 Diagram depicting the irradiation process in a guinea pig by a UVB lamp. Each animal was sedated by ketamine dose 4.4 mg/100 g, i.m. (Wallach and Boever, 1983) prior to irradiation.

1.2 Before irradiation the melanin value (M) and the erythema value (E) of each guinea pig were measured by Mexameter MX 16 to determine the initial, nomal values.

1.3 Eighteen days after the third UVB exposure the back of the guinea pig had darkened. The values of M and E were again measured with Mexameter. (Each animal was shaved each time before the measurements.)

2. Preparing of the test solutions

The heartwood aqueous extract of *Artocarpus lakoocha* (Puag-Haad) were prepared at 0.5 % and 1 % w/v concentration by dissolving the Puag-Haad powder in propylene glycol. The root extracts of *A. gomezianus* or Haadnun (3 % and 5 % w/v concentration) and kojic acid (3 % w/v) were similarly prepared by dissolving the dried substances in the same solvent. All the test solutions were freshly prepared every 3 days.

3. Application of test preparations on the guinea pig skin

After UVB irradiation the guinea pigs were divided into four groups (6 guinea pigs per group). The study was a parallel design with separate and independent negative and positive control groups. The first group was a negative control, which received only the solvent propylene glycol. The second group served as a positive control and was applied with 3% kojic acid in propylene glycol. Kojic acid was selected as a positive control group due to its strong skin whitening activity (Matsuda et al., 1994; Iida, 1995; Jang, 1997; Sritularak et al., 1998; Shimizu et al., 1998; Likhitwitayawuid et al., 2000) and common availability in the market at a relatively economical price. The third and the fourth groups were respectively applied with the test solutions of Haadnun and Puag-Haad in propylene glycol. For the negative and positive control groups, the designated application area was a single square-shaped area (3 x 3 cm^2) located on the center of the pre-shaved back of each animal (Figure 7).

For the third and fourth groups, however, two square-shaped areas were assigned for each animal, each square having the same 3 x 3 cm^2 area as in the control groups. This type of experimental design was necessary in order to allow for the concomitant application of two different concentrations of the test solution on the same animal. The first square or the "front area" was located on the back close to the neck whereas the second square (the "hind area") was located toward the distal (tail) end of the animal. The two squares were separated from each other by a distance of 1 cm (Figure 7). Thus, three of the guinea pigs in the third group would receive the lower concentration of Haadnun (3%) on the front area and the higher concentration (5%) on the hind area. Vice versa, the remaining three guinea pigs in this group would receive 5% Haadnun on the front area and 3% on the hind area. The equal distribution of the test concentrations was to balance out any possible interference due to difference in the application areas, if it did exist. Likewise, the guinea pigs in the fourth group was applied with both 0.5% and 1% Puag-Haad solutions in the similar manner.

Depending on the group assignment, each guinea pig was daily applied with either the test or control solutions every morning for 4 weeks. The values of the melanin (M) and erythema (E) extent were then measured at a two-week interval, i.e., at week 0 (immediately before application) and at 2 and 4 weeks following topical application. The volume of 0.5 ml was always applied on each application area regardless of the group assignment or the concentrations employed.

% Whitening in each group was calculated according to the following formula:

$$\% \text{ Whitening} = [(X_0 - X_t)/X_0] \times 100\% \qquad (2)$$

Where X_0 = melanin value (M) at the start of study (week 0 or immediately before sample application)

X_t = melanin value (M) after daily application of sample for 2 or 4 weeks.

The values of calculated % whitening were then compared among the 4 groups using one-way analysis of variance (ANOVA) at 5% significance level. If significance was found, post-ANOVA test (Duncan's new multiple range test) was further applied to rank their whitening activities at the same significant level.

For assessment of the erythema extent, the raw erythema values (E) were directly compared among different groups using one-way ANOVA at each measurement period (i.e., at week 0, 2 and 4) without data transformation. If significant difference were detected, similar Duncan's test would be applied at the same 5% level.

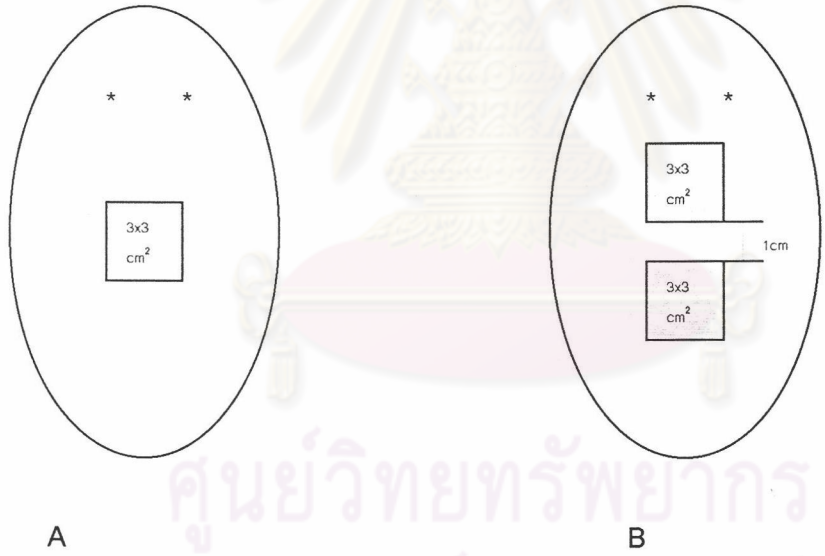


Figure 7 Single square-shape area (A) design application of propylene glycol and kojic acid, Two square-shape areas (B) design application of Haadnun and Puag-haad

Part 2. Evaluation of skin whitening efficacy of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) in human volunteers

The primary purpose of this part was thus to demonstrate the *in vivo* skin whitening efficacy of Puag-Haad solution at two different concentrations in healthy volunteers in comparison with the well-established whitening agents and the pure solvent propylene glycol.

The detailed experiment in this part was largely based on the results obtained from the preliminary study in the guinea pig model. Since the animal study had shown that Puag-Haad gave better skin whitening activity than Haadnun, it was chosen for further investigation in humans. The preliminary animal data are provided in Chapter IV (Results and Discussion). The whitening activity of Puag-Haad was also compared with that of two natural whitening agents commonly used in commercial preparations, namely, kojic acid and licorice extract. Licorice extract was included as an additional positive control reference agent in this part due to its popularity and low skin irritation incidence (Lee et al., 1997; Lee and Choi, 1999; Tabibian, 2000).

1. Study design and subject selection

Eighty female healthy volunteers, aged ranging from 20-48, participated in this single-blind parallel study. All of them had the initial melanin values (M) in the range of 477 – 552, which fell within the skin type V (dark skin, 450 – 550) as measured by Mexameter MX 16 (Zuidhoff and Rijsbergen, 2001). The protocol was approved by an independent Ethics Committee of the Faculty of Pharmaceutical Sciences of 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. They were divided into 4 groups of 20 subjects each. The selection of subjects into each of the four groups was carefully conducted such that there was equal distribution of subjects with light skin color (M values between

476 - 500), intermediate skin color (M values between 501 - 525) and darker skin color (M values between 526 - 550) within each group.

Each group thus separately received different treatment sample solution. All of them stopped using any cosmetics on the application areas for at least two weeks before the start of the experiments. The application areas were the outside areas of both the left and right upper arms. During this "control" period, the subjects were instructed to cover their upper arms at all time by wearing proper attires during the day (no sleeveless shirt). The melanin (M) and erythema (E) values were monitored at a weekly interval to observe for any fluctuation in the baseline. They were also instructed to continue covering both upper arms and avoiding direct exposure to sunlight throughout the entire study period. The data on the baseline M and E values during the two-week prestudy (control) period, as well as the age profile of the individual subjects in each group are provided in Table 1 – 4 of Appendix II.

2. Preparation of the test samples

Puag-Haad solutions at 0.5% and 0.25% w/v were prepared in propylene glycol and were assigned as treatments A and B, respectively. Similarly, solutions of 0.25% licorice extract and 3% w/v kojic acid were prepared in the same solvent and respectively assigned as treatments C and D respectively. The reason for choosing these concentrations are given in Chapter IV (Results and Discussion).

3. Application of the test samples on the volunteers' skin.

After careful selection of subjects into the four groups, each group was then randomly assigned to the treatments, 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 one arm, the individual subjects also received a negative control sample (pure propylene glycol solvent) on the other arm. The application amount was always 0.2 ml for both the treatment and negative control samples.

The areas of the upper arms on which the samples were to be applied were also balanced in each group. For example, the first ten subjects (subjects no. A1 to A10) of the group previously assigned to receive 0.5% Puag-Haad solution (called Group A) were applied with 0.2 ml of propylene glycol (control) on their upper left arms and 0.2 ml of 0.5% Puag-Haad in propylene glycol (called treatment A) on their upper right arms. Vice versa, the remaining subjects of Group A (subjects no. A11-A20) received 0.2 ml of A on their upper left arms and 0.2 ml solvent on their upper right arms.

Similarly, subjects no. B1 to B10 of group B received only propylene glycol on the left arms and 0.25% Puag-Haad solution (treatment B) on the right arms. Subjects B11 to B20 then received B and control solvent in an opposite manner. For the group receiving licorice extract (Group C), subjects C1 – C10 similarly received control solvent on the left arms and 0.25% licorice extract (treatment C) on the right arms whereas the rest of the group (C11 – C20) received the opposite sequence. Likewise, the group, which was randomly assigned to receive 3% kojic acid solution (Group D), would have the first 10 subjects (D1 – D10) receive the control sample on the left arms and 3% kojic acid (treatment D) on the right arms. The remaining subjects (D11 – D20) thus received D and control samples on the left and right arms, respectively.

Each volunteer would self-apply the test and the control samples twice a day, in the morning after taking a shower and at nighttime for 12 consecutive weeks. They were monitored for any changes in the melanin and erythema values at a two-week interval using Mexameter MX16. Each subject was supplied with a pair of similar amber glass bottles capped with a glass dropper on each bottle. The content in each bottle was about 8 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 bottles was intended to be used on which arm. 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 M and E values and received another pair of bottles for further application.

To achieve uniform application procedures, each subject was instructed to use dropper and dispense 5 drops (equivalent to 0.2 ml) of the sample on the finger tips before gently apply with a whirling motion on the outside surface of the upper arm. Caution was given to them not to over apply the solution (e.g. using more than 5 drops or overspread the area). Five drops had been previously found to be sufficient to give a thin film of solution over the application surface of about 40 to 50 cm^2.

4. Measurements of melanin and erythema extents of the skin

The Mexameter MX 16 model was chosen as an instrument of choice for measuring skin color due to ease of use and its ability to directly quantitate the melanin content present in the skin. Moreover, it can measure both the melanin and erythema extent at the same time by comparing the reflected lights of different wavelengths (Zuidhoff and Rijsbergen, 2001). The measurement is based on the absorption principles (Wiechers and Wortel, 1998). The special probe of the Mexameter MX 16 emits light of three known wavelengths (568 nm: green, 660 nm: red and 880 nm: infrared). A receiver located in the same probe measures the light that is reflected by the skin. The positions of emitter and receiver guarantee that only the diffuse and scattered light is measured. As the quantity of the emitted light is defined, the quantity of the light absorbed by the skin (melanin or hemoglobin) can be calculated. The melanin is thus measured by two wavelengths. These wavelengths (660 and 880) have been chosen in order to achieve different absorption rates by the melanin pigments.

Measurement of erythema extent was achieved by a similar technique. Two different wavelengths (568 and 660 nm) are used to measure the absorption capacity of the skin. The melanin and erythema values are calculated as follows (Yoshimara et al., 2000):

Melanin value = 500/ log 5 x (log infrared–reflection / red-reflection + log 5) **(3)**

Erythema value = 500/ log 5 x (log red–reflection / green-reflection + log 5) **(4)**

One of these wavelengths (660 nm) corresponds to the spectral absorption peak of hemoglobin. The other wavelength (568 nm) has been chosen to avoid influences from other colors (e.g. bilirubin). The achieved results are shown on a clear digital display (E for the erythema values and M for the melanin values).

Measurements by Mexameter were achieved via a special probe, which was always put on the same skin area during each visit. The probe was gently pressed for about 1 second against the skin surface over the predetermined application area. The M and E values will be automatically shown on the display with the accuracy of $\pm$ 5%. Five readings (replicates) were taken for each area of the individual subjects and the average value and standard deviation were calculated (Zuidhoff and Rijsbergen, 2000). The instrument was always calibrated against known color standards before each use (i.e., every two weeks) to ensure reproducibility between different measurement periods.

5. Evaluation of melanin and erythema data

Calculation of % whitening

%Whitening values were calculated for both the left and right arms of each subject using the same formula as in the guinea pig study:

$$\% \text{ Whitening} = [(X_0 - X_t)/X_0] \times 100\%$$

Where X_0 = melanin value (M) at the start of study (week 0 or immediately before sample application)

X_t = melanin value (M) after daily application of sample for 2, 4, 6, 8, 10 or 12 weeks.

However, the % whitening was first compared *within* each group using a paired t-test at 5% α-level to see if there was any significant difference between the treatment and the negative control (pure propylene glycol) in the same subject. The paired t-test

was applied on the % whitening data within each group at 0, 2, 4, 6, 8, 10 and 12 weeks. The purpose of this test was to find out the week at which the whitening effect of the treatment became significantly greater than the negative control. Thus, the earlier detection of such difference would be indicative of the better efficacy of that particular treatment in terms of the skin-whitening *rate*. ANOVA was to be applied only when *all* of the four treatment groups demonstrated significant whitening effect over their self-negative controls. If significant difference were found, post-ANOVA Duncan's test would be applied to rank the relative skin whitening *extent* among the four treatments.

The skin whitening extent was defined as the difference between the observed % whitening of the treatment and the negative control within each subject. Therefore, it served as an indicator of the efficacy of that particular treatment over its corresponding self-control. It was calculated for each subject by the formula:

Skin whitening extent (or % whitening efficacy)

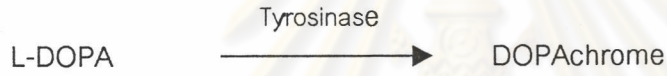
= % whitening of treatment sample - % whitening of self-control sample (5)

For assessment of the effect on skin erythema, the raw erythema data (E values) were directly compared among different groups using one-way ANOVA at each measurement period (at week 0, 2, 4, 6, 8, 10 and 12) without prior transformation. If significant difference were detected, similar Duncan's test would be applied at the same 5% level.

Part 3. Stability evaluation of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad) solutions

1. Determination of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was determined by the DOPAchrome method using L-DOPA as the substrate (Iida et al., 1995). DOPAchrome is one of the intermediate substances in the melanin biosynthesis. The red color of DOPAchrome can be detected by visible light. In this experiment a microplate reader (BIO-RAD, model 450) with 492 nm interference filter was used for detection. The potential tyrosinase inhibitor would show minimal DOPAchrome absorption. This method was modified from the methods of Sritularak (1998) and Shin et al. (1998).



1.1 Preparation of the reaction mixtures

1.1.1 Preparation of 20 mM phosphate buffer (pH 6.8)

Solution A : $NaH_2PO_4.2H_2O$ (312 mg) was dissolved with 100 ml of H_2O.

Solution B : Na_2HPO_4 (284 mg) was dissolved with 100 ml of H_2O.

Then, solution A and B were mixed until pH 6.8 was reached.

1.1.2 Preparation of 0.85 mM L-DOPA

L-DOPA (0.8 mg) was dissolved with 5 ml of 20 mM phosphate buffer (pH 6.8).

1.1.3 Preparation of tyrosinase solution

Tyrosinase enzyme (1 mg) was dissolved with 5 ml of 20 mM phosphate buffer (pH 6.8).

1.1.4 Preparation of test sample

All the test samples for stability evaluation were prepared by dissolving in solvent (20 % v/v propylene glycol in water and kept under controlled temperatures until testing time). Each test sample was diluted 250 times with the same solvent prior to analysis for tyrosinase inhibitory activity at different storage times.

1.2 Measurement of activity

The reaction mixture (total volume of 200 µl each) was measured in four wells designated as A, B, C and D. In each well, the substance was added in the order of mixing, as follows:

A (control)	40 µl of mushroom tyrosinase solution (480 unit/ml)
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of solvent
B (blank of A)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of solvent
C (test)	40 µl of mushroom tyrosinase solution (480 unit/ml)
	80 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test sample in solvent
D (blank of C)	120 µl of 20 mM phosphate buffer (pH 6.8)
	40 µl of test sample solvent

After each well was mixed and pre-incubated at 25 ^{o}C for 10 minutes, 40 µl of 0.85 mM L-DOPA was added and incubated at 25 ^{o}C for 20 minutes. The absorbance of each well was measured at 492 nm with the microplate reader after incubation.

1.3 Calculation of the percent inhibition of tyrosinase enzyme

The percent inhibition of tyrosinase reaction was calculated as follows:

$$\% \text{ tyrosinase inhibition} = [(A-B) - (C-D)/ (A-B)] \times 100 \quad (6)$$

Where A : The difference in optical density before and after incubation at 492 nm without test sample (only enzyme and substrate)

B : The difference in optical density before and after incubation at 492 nm without test sample and enzyme (blank of A)

C : The difference in optical density before and after incubation at 492 nm with test sample (enzyme plus substrate and test substance)

D : The difference in optical density before and after incubation at 492 nm with test sample, but without enzyme (blank of C)

2. Stability test

The objective of the present study was to investigate both the physical and biochemical stability of the test samples. Test samples were kept at 45 ^{o}C and at room temperature for 24 weeks (Pope, 1980). At initial time (week o) and every 2 weeks (or 4 weeks for study at room temperature), the physical appearances and the pH values of the samples were investigated. Biochemical stability of the test samples at various times at the two different temperatures was also determined by tyrosinase inhibitory activity.

2.1 Preparation of test sample

The dried aqueous extract of the heartwood of *Artocarpus lakoocha* (Puag-Haad) at 0.25 % w/v concentration dissolved in 20 % propylene glycol and 80 % water was chosen for stability test. The reasons as to selection of this concentration and medium are given in Chapter IV (Results and Discussion). Degradation of Puag-Haad solution occurs probably via oxidation, which results in brownish color and can be stabilized by proper use of antioxidants. Three antioxidants (0.15 % w/v sodium metabisulfite, 0.1 % w/v BHA and 0.05 % w/v EDTA) were chosen as representative antioxidants for their different mechanisms of action. Thus, the test samples consisted

of 0.25 % Puag-Haad, with and without the three antioxidants, as well as their combination using 20 % propylene glycol in water as solvent. 3 % w/v kojic acid freshly prepared was used as the reference standard during each assay to validate the accuracy of the tyrosinase inhibitory activity test. The test samples were as follows:

P = 0.25 % Puag-Haad

P + A1 = 0.25 % Puag-Haad + 0.15 % sodium metabisulfite

P + A2 = 0.25 % Puag-Haad + 0.1 % BHA

P + A3 = 0.25 % Puag-Haad + 0.05 % EDTA

P + A4 = 0.25 % Puag-Haad + 0.15 % sodium metabisulfite + 0.1 % BHA

= P + A1 + A2

P + A5 = 0.25 % Puag-Haad + 0.15 % sodium metabisulfite + 0.05 % EDTA

= P + A1 +A3

P + A6 = 0.25 % Puag-Haad + 0.1 % BHA + 0.05 % EDTA

= P + A2 + A3

P + A7 = 0.25 % Puag-Haad + 0.15 % sodium metabisulfite + 0.1 % BHA + 0.05%EDTA

= P + A1 +A2 + A3

2.2 Stability test of tyrosinase inhibitory activity

2.2.1 Room temperature study: The test samples were kept at room temperature (ambient temperature ~ 27 °C) in tightly closed glass vials encased in a box to protect from light for up to 24 weeks. At the start and after 4, 8, 12, 16 and 24 weeks, their physical appearances (color and clarity), pH values and tyrosinase inhibitory activity were investigated.

2.2.2 Study at 45 °C: The test samples (protected from light) were kept at 45 °C in a controlled temperature oven. At initial and after 2, 4, 6, 8, 10, 12, 16 and 24 weeks, they were investigated using the same method as 2.1.1.

3. Analytical method

3.1 Visual assessment

The color of each vial was visually compared with the freshly prepared solution of each test sample. The degree of color was assessed using the following simple numerical scoring system:

Score	Degree of color
0	Normal (pale yellow), No change
+1	Slightly (light yellow)
+2	Noticeably (light brown)
+3	Markedly (brown)
+4	Seriously deteriorated (dark brown)
+5	Intense deep brown

3.2 Calculation of the % tyrosinase inhibitory activity relative to initial value

The % relative tyrosinase inhibitory activity of each test sample was calculated using the following equation:

$$\frac{\text{\% tyrosinase inhibitory activity at storage testing time (week)}}{\text{\% tyrosinase inhibitory activity at initial time (0 week)}} \times 100 \quad (7)$$

Thus, the percentage of tyrosinase inhibitory activity remaining at any particular time-point was expressed in relation to the initial inhibitory activity at time zero.

Statistical analysis

The mean values and the standard deviation of all data were calculated. Pair Student's t-test and analysis of variance (ANOVA) made statistical evaluations of the data, where appropriate, and by multiple comparison of the means using Duncan's test. Differences between group means were considered significant at p-value < 0.05.