CHAPTER IV
RESULTS AND DISCUSSION

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

1. Inhibitory effect of Puag-haad and Haadnun on melanogenesis in the back skin of guinea pigs

1.1 Effect of UVB irradiation on pigmented guinea pigs

Each guinea pig was irradiated by the UVB light (290-320 nm) for 3 consecutive days with the total energy of 2.7 J/cm^2. The irradiation technique was modified from that of Imokawa et al. (1986), and Jang et al (1997). The purpose of pretreatment of the back of each guinea pig with UVB was to accelerate the skin darkening process by stimulation of melanogenesis. After 3-day exposure to UVB the melanin values of each animal was found to steadily increase and reach the optimum level 18 days following the third day of irradiation (Imokawa et al., 1986; Shimizu et al., 1998). Then, the test substances (or control) were daily applied on the guinea pigs' back skin for 4 weeks (Jang et al., 1997) and their melanin and erythema values were monitored at a two-week interval, i.e., at week 0 (immediately before application), week 2 and week 4 using Mexameter MX 16.

As seen from data in Table 3, the average melanin values substantially increased in all groups of guinea pigs. The normal pretreatment values (before UVB irradiation) range from 627.67 to 637.88 and increased to 654.00 and 667.53 on the eighteenth day following three-day exposure, which was the starting day of substance application (week 0). The values agreed with that of Imokawa et al. (1986), who observed similar increase in melanin content at the same period after UVB exposure.

Brown or black guinea pigs were chosen as models to test the whitening effect of many substances due to their similarity in skin color to human subjects, particularly those of the Mongolian race (Imokawa et al., 1986; Robinson and Huxtable, 1988). In this study, guinea pigs were carefully selected for inclusion based on the color of the *shaved* skin, which must be uniformly black although the covering hair color might vary from animal to animal. Data from Table 3 indicate that daily exposure of the guinea pig's skin to UVB radiation (290-320 nm) at 0.9 J/cm^2/day for 3 consecutive days was sufficient to induce clearly visible black pigmentation on the irradiated areas. Black pigmentation appeared on the 3rd day following UVB exposure and reached its maximum after about 10-15 days. This time course of pigmentation was similar to that observed when a Mongoloid skin is exposed to UVB radiation (Imokawa et al.).

The appearance of UV induced pigmentation depended on the extent of the original pigmentation of the guinea pig's skin as well as on the dose of UVB administration. Thus, in guinea pig's skin with less pigment, the pigmentation tended to be weak or absent. Imokawa et al. (1986) reported that a daily dose of 0.9 J/cm^2 of UVB repeated for 3 days induced more marked pigmentation than a single exposure of 2.7 J/cm^2 and also did not cause any distinct inflammatory response. Our data thus agreed with them in that the measured erythema value, which was used as an indicator of initial inflammatory reaction, was relatively similar before and after UVB irradiation (Table 3). Therefore, the irradiation dose and regimen employed in this study was sufficiently safe and effective to stimulate pigmentation in the guinea pig model without causing distinct erythema.

Table 3 The absolute melanin and erythema values (mean ± SD) in guinea pigs treated with different substances for 4 weeks. The pretreatment values (before UVB irradiation) are also provided. (n = 6-7 guinea pigs per group).

Substance	Melanin values (M)				Erythema values (E)			
	Before UVB	Week0*	Week 2	Week 4	Before UVB	Week0*	Week 2	Week 4
Negative control (Propylene glycol)	637.88 ± 6.57	654.00 ± 8.90	647.93 ± 8.74	632.58 ± 2.09	564.27 ± 6.40	556.60 ± 6.45	552.10 ± 10.18	558.82 ± 6.45
Positive control (Kojic acid)	627.07 ± 16.14	664.37 ± 8.77	632.13 ± 6.66	628.57 ± 8.40	566.30 ± 7.89	554.13 ± 4.47	550.83 ± 7.57	556.33 ± 11.00
3% Haadnun root extract	632.62 ± 12.51	659.05 ± 5.00	638.95 ± 19.90	624.38 ± 14.83	559.80 ± 9.75	554.40 ± 6.71	547.62 ± 11.49	558.68 ± 18.05
5% Haadnun root extract	632.62 ± 12.51	659.05 ± 5.00	639.20 ± 10.97	624.07 ± 9.88	559.80 ± 9.75	554.40 ± 6.71	553.50 ± 15.41	555.73 ± 11.88
0.5% Puag-Haad	637.40 ± 10.47	667.53 ± 5.34	635.30 ± 12.40	616.84 ± 7.84	558.80 ± 12.46	552.79 ± 4.87	551.50 ± 5.77	559.94 ± 11.79
1.0% Puag-Haad	637.40 ± 10.47	667.53 ± 5.34	647.51 ± 12.16	625.39 ± 10.45	558.80 ± 12.46	552.79 ± 4.87	559.83 ± 13.50	563.64 ± 16.07

* The value at week 0 = initial value measured immediately before application of the test or control substances. Application was started on the 18th day following 3-day UVB exposure.

1.2 Evaluation of skin whitening efficacy in guinea pigs

The average melanin (M) and erythema (E) values measured after application of the test substances are also provided in Table 3. These are the absolute values, i.e., the non-transformed data obtained at particular predetermined time-points (week 0, week 2 and week 4). The absolute M and E values for the individual guinea pigs are also provided in Appendix I

From this table, it can be seen that the absolute M values decreased with time even in the negative control (propylene glycol-treated) group. This was not surprising since all groups of guinea pigs had been pretreated with UVB irradiation to stimulate melanogenesis, thereby resulting in hyperpigmentation. Thus, when pigmentation reached its maximum (about 15-18 days after UVB exposure), the skin will restore itself to normal skin condition leading to the lower M values at later time. The stratum corneum naturally would shed itself with the formation of new layers having decreasing extent of melanocytes. However, the groups treated with the positive control (kojic acid) and the test substances (Puag-Haad and Haadnun extract) were found to accelerate this pigment normalization process. Their absolute M values decreased at a faster rate than the negative control group. For example, the mean absolute M value of the kojic acid-treated group decreased from 664.37 at week 0 to 628.57 at week 4, equivalent to about 5.38% whitening, whereas in the propylene glycol-treated group, the mean absolute M value decreased from 654.00 at week 0 to 632.58 at week 4, which was equivalent to only 3.26% whitening activity.

Thus, in order to compare the inhibitory effect on melanogenesis more effectively, the value of % whitening was used instead of the untransformed, absolute M value. As previously stated in Chapter III, the % whitening was defined as the percentage decrease in absolute M value from the initial time (week 0) or $= [(X_0 - X_t)/X_0] \times 100\%$. Thus, comparison between treatment groups was always made on changes in the M values relative to the initial values. The data on the average % whitening are

given in Tables 4 – 9 for different groups of guinea pigs whereas the values for the individual animals are provided in Appendix I.

Comparison was first made on the values of % whitening among the test and control substances after 2-week application using one-way ANOVA at 5% significance level. Since the Haadnun and Puag-Haad extracts were applied at two different concentrations on the same guinea pigs, only the lower concentration of each test substance was chosen for the preliminary ANOVA comparison, i.e, 3% for Haadnun and 0.5% for Puag-Haad. Thus, comparison was made on 4 independent treatment groups, which were negative control (propylene glycol), positive control (3% kojic acid), 3% Haadnun and 0.5% Puag-Haad. These concentrations were calculated based on the values of IC_{50} (concentration showing 50% inhibition on tyrosinase enzyme activity *in vitro*) by Sritularak (1998). Thus, 3% kojic acid, 3% Haadnun (containing norartocarpetin as active component) and 0.5% Puag-Haad (containing $2,4,3',5'$-tetrahydroxystilbene as active component) were supposed to possess equivalent *in vitro* tyrosinase inhibitory activity and may have exhibited equivalent *in vivo* skin whitening effect in animals.

As seen from Table 4, application of propylene glycol for 2 weeks resulted in minimal whitening effect of only 0.90 % whereas application of 3% kojic acid in the same solvent yielded about 4.84% whitening efficacy (Table 5). Similarly, the two extracts in propylene glycol, even at lower concentrations, were also capable of whitening the guinea pig's skin, with the mean % whitening at 2 weeks of 3.05% and 4.82% for 3% Haadnun (Table 6) and 0.5% Puag-Haad (Table 8), respectively. ANOVA results showed that there was significant difference in the values of % whitening at 2 weeks among the four groups ($P < 0.05$). Subsequent Duncan's new multiple range test was further applied at the same level to rank the whitening activity with the following results in an increasing order:

Table 4 Percentage whitening after application of propylene glycol (negative control) to guinea pigs

Guinea pig	% Whitening	
	After 2 weeks	After 4 weeks
1	-2.44	1.41
2	1.29	3.45
3	4.72	4.58
4	0.09	2.46
5	1.50	3.69
6	0.26	3.99
Mean ± SD	0.90 ± 2.34	3.26 ± 1.15

Table 5 Percentage whitening after application of 3 %w/v kojic acid (positive control) to guinea pigs

Guinea pig	% Whitening	
	After 2 weeks	After 4 weeks
1	6.98	5.38
2	4.57	4.19
3	6.57	6.27
4	2.31	2.93
5	4.12	6.54
6	4.47	6.95
Mean ± SD	4.84 ± 1.71	5.38 ± 1.55

Table 6 Percentage whitening after application of 3 % w/v Haadnun extract to guinea pigs

Guinea pig	% Whitening	
	After 2 weeks	After 4 weeks
1	5.04	7.70
2	3.83	3.99
3	7.18	7.00
4	0.42	2.99
5	2.46	4.63
6	-0.63	5.29
Mean ± SD	3.05 ± 2.91	5.27 ± 1.80

Table 7 Percentage whitening after application of 5 % w/v Haadnun extract to guinea pigs

Guinea pig	% Whitening	
	After 2 weeks	After 4 weeks
1	3.23	5.40
2	4.38	5.22
3	4.33	6.39
4	1.10	3.45
5	3.06	5.86
6	1.99	5.54
Mean ± SD	3.01 ± 1.29	5.31 ± 1.00

Table 8 Percentage whitening after application of 0.5 % w/v Puag-Haad to guinea pigs

Guinea pig	% Whitening	
	After 2 weeks	After 4 weeks
1	3.91	7.74
2	6.02	5.60
3	7.06	7.06
4	6.68	10.22
5	5.34	7.64
6	1.75	6.95
7	3.00	7.90
Mean ± SD	4.82 ± 1.99	7.59 ± 1.40

Table 9 Percentage whitening after application of 1.0 % w/v   Puag-Haad   to guinea pigs

Guinea pig	% Whitening	
	After 2 weeks	After 4 weeks
1	2.16	5.46
2	3.66	5.66
3	3.70	4.41
4	6.56	8.14
5	2.27	7.11
6	1.33	6.06
7	1.29	7.36
Mean ± SD	3.00 ± 1.85	6.31 ± 1.28

Propylene glycol < 3% Haadnun < 0.5% Puag-Haad < 3% Kojic acid

% Whitening   0.90   3.05   4.82   4.84

According to the Duncan's test results, the four substances could be classified into two groups with overlapping whitening activities. The first group consisted of 3% Haadnun, 0.5% Puag-Haad and 3% kojic acid. They all possessed equivalent whitening activity because they were joined by the same underline. This implied that there was no significant difference among the three treatments with respect to their % whitening values (P > 0.05). The second group, which had lower whitening activity than the first, consisted of the negative control (propylene glycol) and 3% Haadnun. The line joining the two substances also implied that they had equivalent % whitening values (P > 0.05).

From the Duncan's test, 3% Haadnun appeared to have an intermediate whitening effect since it could be classified in both groups as shown by the overlapping underlines. However, 0.5% Puag-Haad and 3% kojic acid demonstrated significantly higher % whitening values than the negative control group (P < 0.05) as there was no line joining Puag-Haad and kojic acid with propylene glycol. Thus, the preliminary results obtained after application of the substances for only 2 weeks showed that 0.5% Puag-Haad was significantly more effective in facilitating skin whitening process in guinea pigs than the solvent and its effect was also similar to 3% kojic acid used as a reference positive control.

Further application of the test substances for another two weeks resulted in a further increase in % whitening values in all guinea pigs even with the group treated with propylene glycol, though at a much slower rate. For example, the average % whitening in guinea pigs treated with propylene glycol increased from 0.90% at 2 weeks to 3.26% at 4 weeks (Table 4) whereas the values for 3% kojic acid increased from 4.84% at 2 weeks to 5.38% at 4 weeks (Table 5). The two test extracts also exhibited a

marked increase in % whitening values after 4-week application, i.e., from 3.05% to 5.27% for 3% Haadnun extract and from 4.82% to 7.59% for 0.5% Puag-Haad (Tables 6 and 8).

ANOVA was then applied on the % whitening at 4 weeks. Significant difference was found among the four treatments ($P < 0.05$) and subsequent Duncan's test gave the following ranking result:

Propylene glycol < 3% Haadnun < 3% Kojic acid < 0.5% Puag-Haad

% Whitening   3.26   5.27   5.38   7.59

The ranking after 4 weeks was somewhat different from that at 2 weeks. The propylene glycol-treated guinea pigs appeared to have better natural skin whitening than previously observed at 2 weeks, with a mean value of 3.26%. Application of 3% Haadnun and 3% Kojic acid also resulted in further increase in skin whitening after 4 weeks. However, the increase was not substantial enough to be significantly greater than the propylene glycol-treated animals since the three substances were all on the same line ($P > 0.05$). Thus, only 0.5% Puag-Haad was shown to be the most effective skin whitening substance after 4 week-application as it was not joined by any line ($P < 0.05$). The % whitening at 2 and 4 weeks are also plotted in Figure 8 for visual comparison.

Apart from the skin whitening effect, ANOVA was also performed on the absolute erythema (E) values to determine if any of the four substances could induce any kind of skin irritation. The results showed that there were no significant differences in the E values among the four treatments, either at 2 or 4 week-application ($P > 0.05$). This may indicate that, at the concentrations employed, none of the substances possess any serious skin irritation potential since their E values were not different from the negative control group.
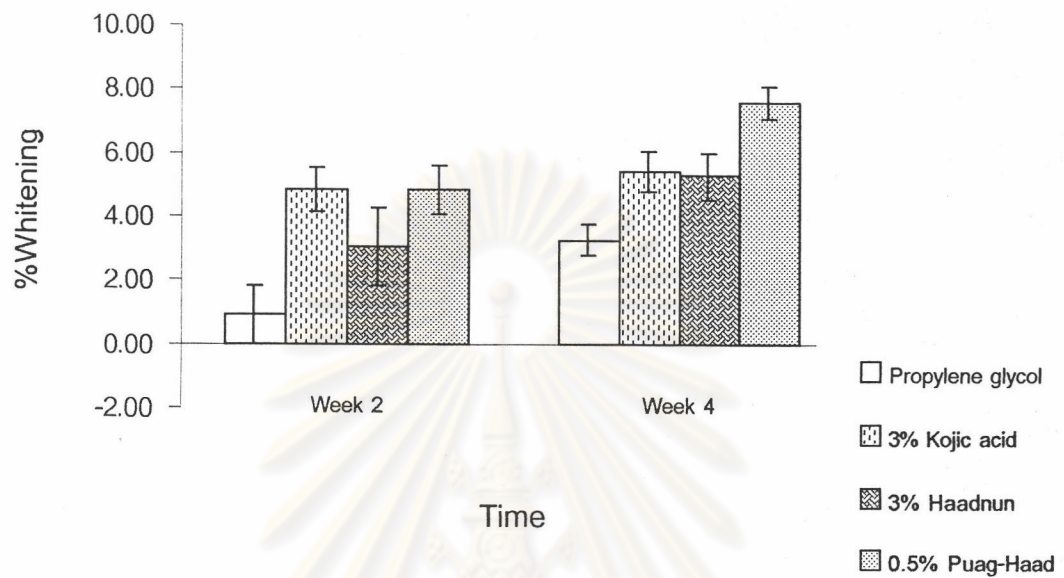
Figure 8 Histogram comparing % whitening of four substances after 2 and 4 weeks application in guinea pigs. Data = mean ± SEM   (n = 6-7 guinea pigs/group)

However, the E value was merely an index of acute or early inflammatory responses such as skin rashes. There are many other types of skin irritation that can occur after a more frequent or prolonged contact and may not be detected simply by measuring only the E values, which are based on the concentration of red blood cells or hemoglobin in the application area. Therefore, a more prolonged study (longer than 4 weeks) must be conducted especially in human volunteers in order to draw a more concrete conclusion about the skin irritation potential of the test substances (See next part). With the human study, additional parameters about skin irritation could be obtained by visual observation of the affected areas and interviews.

The data so far have indicated that the extract of 0.5% *Artocarpus lakoocha* heartwood (Puag-Haad) exhibited the most effective skin whitening action in the guinea pig model, especially following 4-week application. Its effect was significantly better than 3% kojic acid and 3% *A. gomezianus* root extract (Haadnun). The greater *in vivo* whitening efficacy of Puag-Haad could be related to its more potent *in vitro* inhibitory activity on mushroom tyrosinase enzyme (Sritularak, 1998). The major active component of Puag-Haad is 2,4,3′,5′-tetrahydroxystilbene (oxyresveratrol), which has an IC_{50} (concentration of 50% tyrosinase inhibition) of only 1.5 μM. This value is much smaller than the IC_{50} for norartocarpetin, the active component of Haadnun root extract (19.44 μM) and that of kojic acid (26.8 μM) (Likhitwitayawuid, 2000).

To determine if the higher concentrations of the two extracts might give better whitening effect, guinea pigs also received daily application of Puag-Haad at 1.0% w/v and Haadnun at 5.0% w/v concentration. The values of % whitening were then compared with the lower concentrations using paired student's t-test at 5% significance level. Paired student's t-test was used instead of unpaired t-test because both the low and high concentrations of each extract were applied on the same animal as previously described in Chapter III.

Comparison of the data in Tables 6 and 7 showed that increasing the concentration of Haadnun extract from 3% to 5% did not result in increased % whitening

as there was no significant difference in these values after paired t-test regardless of the application period ($P > 0.05$). For example, the average % whitening after 4-week application of 3% Haadnun was 5.27%, which was essentially similar to the value for 5% concentration (5.31%). This observation could be due to the possible saturable inhibition of the tyrosinase enzyme by Haadnun at these concentrations. The data is graphically represented in Figure 9.

On the other hand, the result for Puag-Haad was quite different from Haadnun. Increasing the concentration of Puag-Haad from 0.5% to 1.0% resulted in significantly decreased whitening effect at both periods ($P < 0.05$) (Tables 8 and 9). For example, the mean % whitening after 4-week application of 1.0% Puag-Haad was only 6.31% compared to 7.59% obtained after application of the lower concentration (0.5% Puag-Haad). Similarly, the value after 2-week application of 1.0% (3.0% whitening) was much smaller than that of 0.5% concentration (4.82% whitening). The data is graphically shown in Figure 10. The reasons as to this observation were not known at present. However, it is possible that 2,4,3′,5′-tetrahydroxystilbene may have additional mechanism(s) in promoting skin whitening which might have a feed-back inhibition at higher concentration. Recently, L'Oreal has received a US patent claiming that 2,4,3′,5′-tetrahydroxystilbene has a stimulating effect on fibroblast proliferation and collagen synthesis (US patent no. 6,147,121; 2000). More information about the various activities and different mechanisms of tetrahydroxystilbene and its derivatives is still unknown and needs further research. Nevertheless, the data obtained in guinea pigs strongly suggested the potential of Puag-Haad as an effective and probably safe whitening agent. The next step was thus to confirm its *in vivo* efficacy and safety in human volunteers.
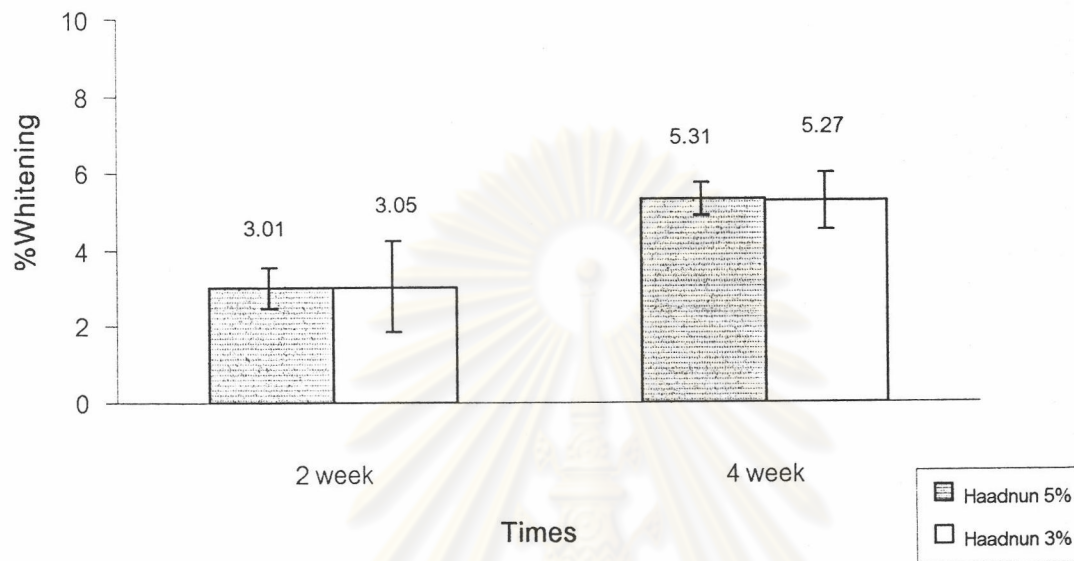
Figure 9 Comparison of % whitening of Haadnun 3% and 5%. Data = mean ± SEM (n = 6 guinea pigs/group)
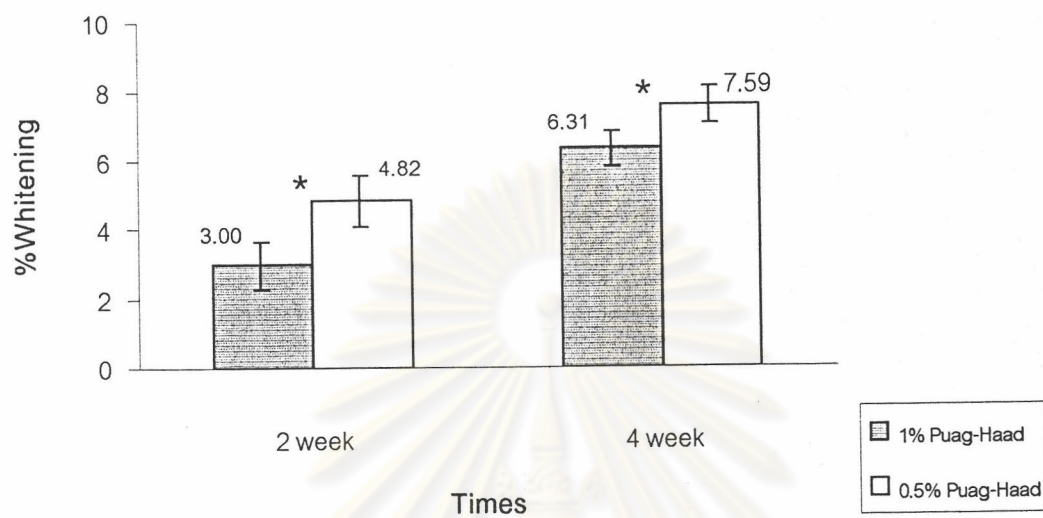
ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Figure 10 Comparison of % whitening of Puag-Haad 0.5% and 1.0%. Data = mean $\pm$ SEM (n = 7 guinea pigs/group)

*Values are significantly different, $P < 0.05$

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

1. Inhibitory effect of Puag-haad on melanogenesis in the upper arm of human skin in comparison with other commonly used whitening agents (licorice extract and kojic acid)

The purpose of this part was to confirm the *in vivo* efficacy and safety of the test extract in human volunteers. Since Puag-Haad had been shown to be a more potent whitening agent than Haadnun in the guinea pig model, it was chosen for further study in human subjects. Two concentrations of Puag-Haad were arbitrarily chosen for testing in this study, i.e., 0.5 and 0.25% w/v solutions in propylene glycol. The decision for selecting these two concentrations were based on the initial data obtained from the guinea pig study, in which 0.5% was shown to give better whitening activity than 1.0%, although the reasons behind this observation were not clearly understood. However, using a lower concentration of Puag-Haad did have some advantages, at least in terms of appearance and cost. The lower the concentration of Puag-Haad, the solution would become less yellow in color, thereby giving better appearance. Furthermore, the extent of increased coloring with time upon standing was also less pronounced with the lower concentration. The stability of the Puag-Haad extract was discussed in more detail in Part III of this chapter.

As the reference whitening agents (positive control), kojic acid and licorice extract were included in this part of study. Both kojic acid and licorice extract are widely used as whitening agents in many cosmetic products. For example, kojic acid has been used in Brightess white[©] and White natural Moisture cream[©] (Kose, Japan) at concentration of 1 - 4 %, whereas licorice extract is available in many lotions and creams such as Nivea whitening body lotion[©] at concentration of 0.5 %. Thus, the concentration of kojic acid used in this part was chosen to be 3% (as in the guinea pig study) and that of licorice extract (PT-40 high purity grade) was fixed at 0.25%.

Eighty female volunteers participated in this parallel study with self-control. They were allocated into four groups of 20 subjects each. The subject allocation was such that there was equal distribution of subjects with high, intermediate and low melanin values in all groups. Then each group was randomly assigned to the treatments, either A, B, C or D. The group assigned to treatment A received 0.50% Puag-Haad (or simply called group A). Group B received 0.25% Puag-Haad whereas groups C and D received 0.25% licorice extract and 3% kojic acid, respectively. As previously mentioned, all treatment solutions were prepared using propylene glycol as solvent. Thus, all the subjects in each group also received pure propylene glycol as the self-control on their remaining arms.

Before application of the treatments, each subject was monitored for their baseline melanin for 2 weeks to observe for any fluctuation. The average baseline values are shown in Table 10. Their individual data on age, baseline melanin values and identification number are also provided in Appendix II.

As seen from Table 10, the average baseline melanin (M) values of each group were similar at two weeks (week -2) prior to the start of experiment. One-way ANOVA on either the left or right arms revealed that there was no significant difference in the mean M values at this period among the four groups ($P > 0.05$), indicating that the subjects were equally distributed within each treatment group such that the baseline group means were similar. One-way ANOVA was also applied on the M values at the start of experiment (week 0) and yielded similar results, i.e., no significant difference among the four groups was detected ($P > 0.05$). However, when randomized block ANOVA was applied to test for the effect of time on the baseline values *within* each group, significant difference was found among the values at weeks-2, -1 and 0 ($P < 0.05$) in all groups. The results indicated that the baseline melanin values tended to change with time, or more specifically, decrease with time. For example, the mean baseline M values in the left arms of subjects in group A slightly decreased from 515.87 at week -2 to 516.31 at week -1 and then to 511.91 at week 0. Similar behavior was also seen with the right

Table 10 The average baseline melanin values at 2 and 1 week before application of treatments (week -2 and week -1). The values at the start of the experiment (week 0) are also shown. Data = mean $\pm$ SD (n = 20 subjects per group).

Subject group	Left arm				Right arm			
	Week -2	Week -1	Week 0	P-value	Week -2	Week -1	Week 0	P-value
A	515.87 $\pm$ 19.01	516.31 $\pm$ 18.18	511.87 $\pm$ 18.13	0.0120 *	514.30 $\pm$ 18.09	514.94 $\pm$ 17.77	509.71 $\pm$ 19.29	0.0043 *
B	511.14 $\pm$ 16.80	512.37 $\pm$ 14.70	509.28 $\pm$ 18.54	0.0171 *	514.92 $\pm$ 17.55	516.34 $\pm$ 17.43	510.91 $\pm$ 18.56	0.0004 *
C	510.21 $\pm$ 17.36	512.22 $\pm$ 16.49	507.11 $\pm$ 16.70	0.0004 *	511.57 $\pm$ 16.93	513.51 $\pm$ 15.55	510.27 $\pm$ 17.92	0.0249 *
D	514.86 $\pm$ 15.29	516.37 $\pm$ 14.68	511.71 $\pm$ 15.52	0.0031 *	514.96 $\pm$ 15.37	517.36 $\pm$ 14.66	513.19 $\pm$ 16.21	0.0058 *

* Significant time effect on the baseline M values ($P < 0.05$) after randomized block ANOVA.

arms. In fact, the baseline values slightly but significantly decreased in both arms of every group.

This finding was not surprising, though, because during this baseline (pre-study) period, all the subjects were asked to cover their upper arms with appropriate clothes and to minimize their outdoor activities. It was thus expected that their previously sun-tanned skin might gradually and naturally become somewhat lighter than at the beginning of the baseline period.

Thus, although there appeared to be a slight decrease in average baseline melanin with time, this would not interfere with the data analysis during the application period. Fluctuation in the baseline melanin was taken care of by applying a self-control solvent (propylene glycol) to each subject throughout the 12 week-application period. In addition, the values of % whitening relative to the initial values (week 0) were used in statistical comparison instead of the absolute M values to account for the time effect.

Furthermore, the initial M values at week 0 (X_0) of the left and right arms were also compared within each subject of each group using paired student's t-test. There were no significant differences among the initial M values ($P > 0.05$) within subjects of group A, B and D, with the average X_0 (left vs right arms) values of 511.91 vs 509.71, 509.28 vs 510.91, and 511.71 vs 513.19, respectively. A small but significant difference was observed, however, between the left and right arms of subjects in group C (mean X_0 of left vs right arms = 507.11 vs 510.27; $P = 0.0437$). Although the starting values for group C may be different between the left and the right arms, this did not impose any problems on data evaluation. The study had been designed such that there was equal distribution of treatments among subjects within each group, i.e. the first half of subjects (10) received propylene glycol on their left arms and the substance solution on the right arms while the latter half (10) received the opposite order. Thus, the effect of initial difference in the baseline M values, if existed, was balanced out in each group. In addition, statistical comparison was always made on the values of % whitening, in which the value at week 0 (X_0) had been taken into account.

Table 11 shows the average absolute melanin values for each treatment groups. As seen from this table, the values in all groups further decreased after treatment with Puag-Haad, licorice extract and kojic acid when compared to their respective self-controls. For example, the average absolute M value for 0.25% Puag-Haad treated subjects at the start of the study (week 0) was 509.45 and decreased to 504.52, 499.07, and 495.12 after 4, 8, and 12 weeks of application, respectively. On the other hand, the M values of the propylene glycol-treated upper arms of the subjects in this same group showed only a minimal decrease, i.e., from 510.74 at week 0 to 509.77, 507.25, and 507.31 at week 4, 8, and 12, respectively.

As previously discussed, % whitening relative to the initial values was a preferred parameter for statistical comparison of the whitening activity within each treatment group. They were calculated from the absolute values using the same formula as in the guinea pig study. The average % whitening data are shown in Table 12 whereas the values of the individual subjects are provided in Appendix II. The data were also plotted as a function of time for visual comparison as shown in Figures 11 -14.

Paired student's t-test was applied every two weeks to compare the values of % whitening between the substance-treated and the solvent-treated arms of the same subjects within each treatment group at 5% significance level. The results are also shown in Table 12 where significant difference ($P < 0.05$) is denoted by an asterisk. It can be seen from this table that subjects treated with 0.25% Puag-Haad (group B) gave the fastest whitening effect, showing significant % whitening over their solvent-treated arms after application for only 4 weeks. Thereafter, the whitening effect continued to be significantly greater than the self-control at all weeks until the end of the study (12 weeks).

Table 12 also showed that kojic acid (group D) was the second most effective whitening agent after 0.25% Puag-Haad. 3% kojic acid started to give significant whitening result over its self-control after 8 week-application. The time to reach significant whitening effect (effective onset time) for 3% kojic acid was thus 4 weeks or approximately one month slower than 0.25% Puag-Haad. On the other hand, 0.5%

Table 11 The absolute melanin values (mean ± SD) in the upper arms of human volunteers treated with different substances for 12 weeks.

(n = 17 – 20 subjects per treatment group).

Treatment group		Melanin values (M)						
		Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
A (n = 17)	PG-control	512.80 ± 18.60	511.45 ± 18.50	510.67 ± 18.12	509.15 ± 17.03	510.06 ± 17.31	511.14 ± 16.58	509.84 ± 16.50
0.50% Puag-Haad	A	510.40 ± 18.00	510.09 ± 17.05	507.82 ± 16.55	504.16 ± 16.99	505.27 ± 16.68	503.72 ± 14.12	501.29 ± 14.49
B (n = 20)	PG-control	510.74 ± 19.01	510.66 ± 17.97	509.77 ± 17.11	507.16 ± 16.74	507.25 ± 17.37	508.10 ± 16.10	507.31 ± 15.72
0.25% Puag-Haad	B	509.45 ± 18.09	508.47 ± 16.95	504.52 ± 16.25	500.56 ± 15.42	499.07 ± 15.38	497.47 ± 13.62	495.12 ± 14.23
C (n = 20)	PG-control	508.34 ± 17.63	506.96 ± 16.11	504.70 ± 15.50	503.11 ± 14.38	504.18 ± 15.44	507.23 ± 15.64	506.73 ± 15.23
0.25% Licorice	C	509.04 ± 17.15	506.55 ± 16.86	505.04 ± 15.43	501.45 ± 14.88	502.06 ± 15.64	501.75 ± 14.62	500.26 ± 14.88
D (n = 19)	PG-control	510.86 ± 14.35	510.76 ± 14.34	508.58 ± 13.74	507.94 ± 14.46	508.23 ± 12.65	509.88 ± 13.33	509.27 ± 13.35
3.0% Kojic acid	D	512.17 ± 16.98	511.05 ± 17.25	508.62 ± 14.92	504.26 ± 14.65	504.78 ± 14.82	503.00 ± 13.04	500.58 ± 12.30

PG control = propylene glycol self-control within each treatment group

Table 12 The average % whitening values (mean ± SD) in the upper arms of human volunteers treated with different substances for 12 weeks. (n = 17 – 20 subjects per treatment group).

Treatment group		%Whitening					
		Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
A (n = 17) 0.50%Puag-Haad	PG-Control	0.26 ± 1.12	0.40 ± 1.27	0.69 ± 1.48	0.52 ± 1.22	0.30 ± 1.39	0.55 ± 1.53
	A	0.05 ± 1.00	0.49 ± 0.98	1.21 ± 1.08	0.99 ± 1.54	1.28 ± 1.49	1.75 ± 1.56
	P-value	0.4808	0.7333	0.1384	0.2139	0.0097*	0.0059*
B (n = 20) 0.25%Puag-Haad	PG-Control	0.00 ± 0.97	0.17 ± 1.05	0.68 ± 1.19	0.67 ± 1.01	0.49 ± 1.18	0.65 ± 1.16
	B	0.18 ± 0.72	0.95 ± 1.04	1.73 ± 0.91	2.02 ± 1.17	2.32 ± 1.47	2.78 ± 1.47
	P-value	0.3496	0.0008*	0.0001*	6.00×10^{-6}*	0.0097*	0.0059*
C (n = 20) 0.25%Licorie extract	PG-Control	0.26 ± 1.18	0.70 ± 1.30	1.00 ± 1.44	0.80 ± 1.31	0.20 ± 1.37	0.29 ± 1.36
	C	0.48 ± 1.02	0.77 ± 1.10	1.47 ± 1.44	1.35 ± 1.41	1.41 ± 1.37	1.70 ± 1.42
	P-value	0.4160	0.7047	0.1456	0.0552	0.0003*	3.64×10^{-5}*
D (n = 19) 3.0%Kojic acid	PG-Control	0.02 ± 0.81	0.44 ± 1.34	0.57 ± 1.12	0.50 ± 0.99	0.18 ± 1.18	0.30 ± 1.19
	D	0.21 ± 1.18	0.68 ± 1.09	1.53 ± 0.97	1.42 ± 1.28	1.76 ± 1.40	2.23 ± 1.35
	P-value	0.4550	0.3431	0.0562	8.50×10^{-4}*	1.30×10^{-4}*	9.77×10^{-6}*

PG control = propylene glycol self-control within each treatment group

* denotes significant difference between PG-control and treatment within each group by paired student's t-test (P < 0.05).
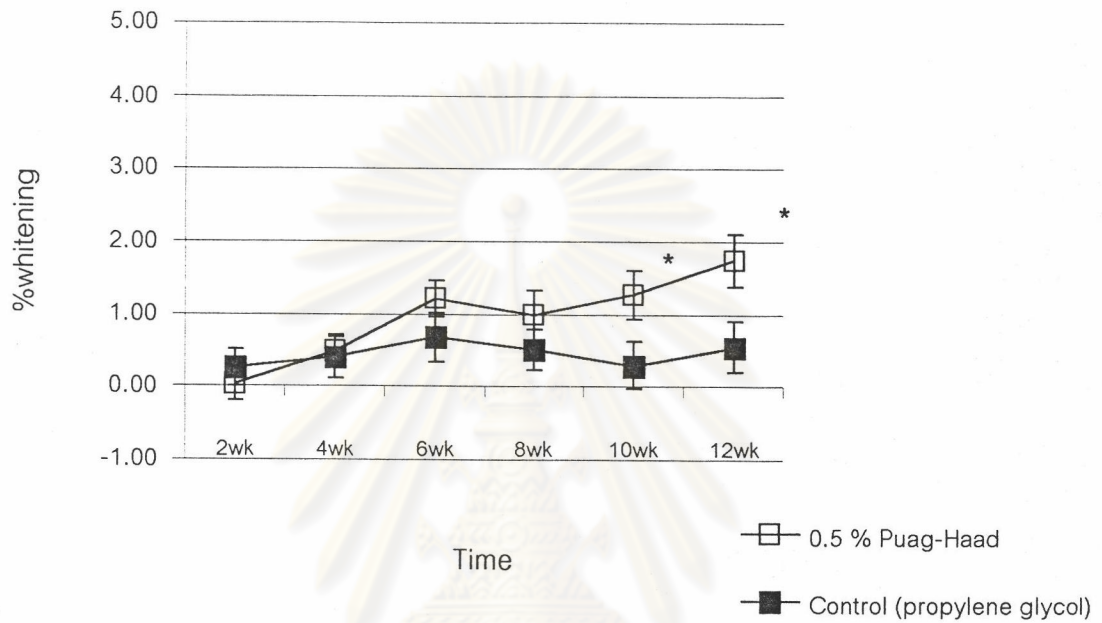
Figure 11 Percent whitening after applying 0.5 % Puag-Haad and propylene glycol for different times. Each point represents mean ± SEM (n = 17).
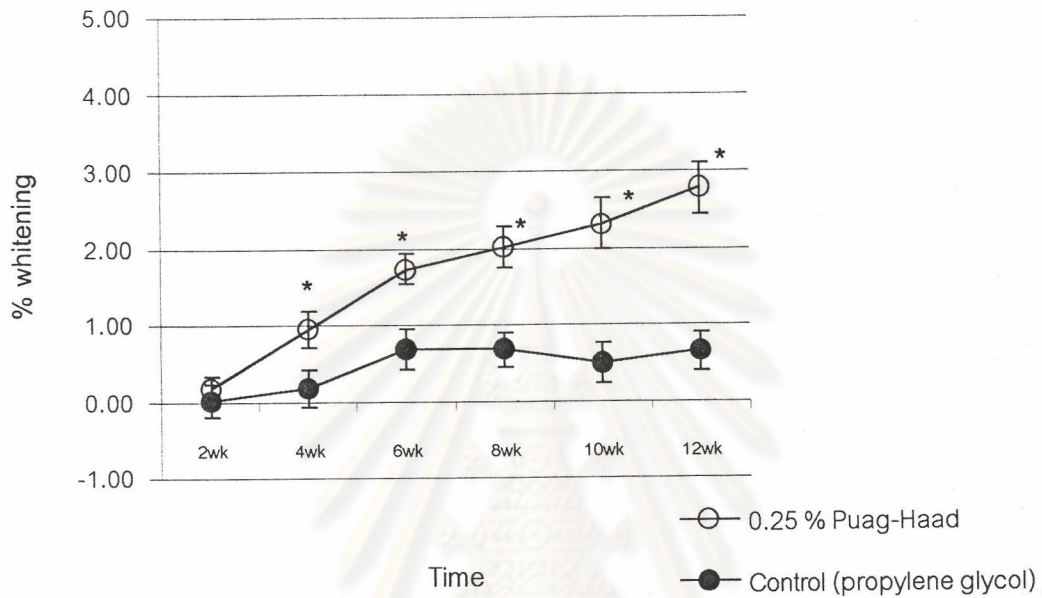* Values are significantly different (p < 0.05).

Figure 12 Percent whitening after applying 0.25 % Puag-Haad and propylene glycol for different times. Each point represents mean ± SEM (n = 20).

* Values are significantly different (p < 0.05).

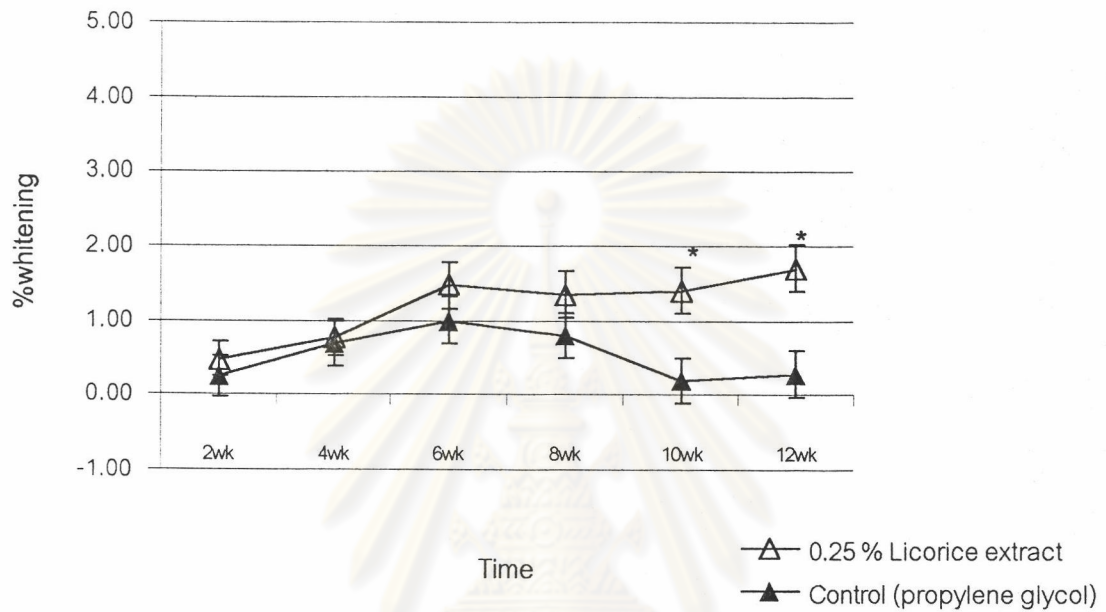Figure 13 Percent whitening after applying 0.25 % licorice extract and propylene glycol for different times. Each point represents mean ± SEM (n = 20).
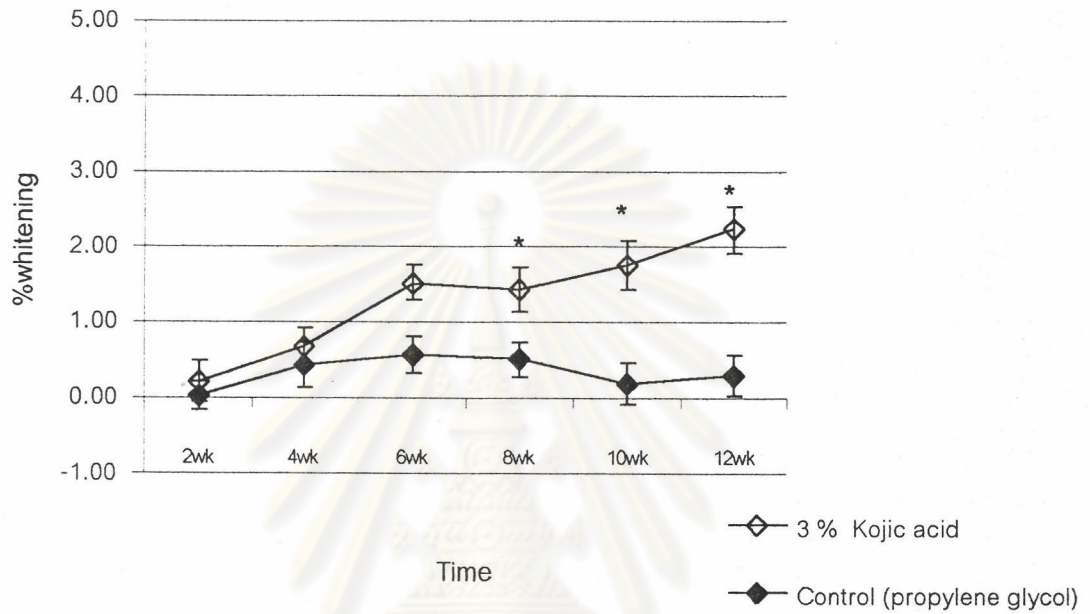
* Values are significantly different (p < 0.05).

Figure 14 Percent whitening after applying 3.00 % kojic acid and propylene glycol for different times. Each point represents mean ± SEM (n = 19).

* Values are significantly different (p < 0.05).

Puag-Haad (group A) and 0.25% licorice extract (group C) gave similar effective onset time, showing significant % whitening over their self-controls after 10-week application. Thus, these two treatments were considered to be less effective than B and D in terms of the *rate* of whitening.

As stated in Chapter III (Materials and Methods), comparison of the whitening *extent* among the four groups would be tested only when all four groups showed significant whitening effect over their self-controls. Thus, one-way ANOVA was applied on the data only at weeks 10 and 12, which were the times when all four treatments became effective. Furthermore, comparison was made on the *corrected* % whitening, in which the % whitening value of the individual subject's upper arm treated with either compound A, B, C or D had been subtracted by the value of the self-control (propylene glycol), which was applied on the remaining arm of the same subject. This corrected value was referred in Chapter III as the skin whitening extent or % whitening efficacy. The individual data for each group are shown in Tables 13 - 16, respectively. The average values are also plotted in Figure 15 for visual comparison.

From these figures, Puag-Haad at 0.25% gave the highest whitening efficacy with the average values after 10- and 12-week application of 1.82 and 2.14%, respectively. This was followed by 3% kojic acid, which gave % whitening efficacy of 1.58 and 1.93% at 10 and 12 weeks, respectively. 0.25% licorice extract was next, giving the whitening extent of 1.21 and 1.41% whereas 0.5% Puag-Haad gave the lowest % whitening efficacy of 0.98 and 1.20% at 10 and 12 weeks, respectively.

Table 13 % Whitening efficacy over corresponding control (difference in % whitening between product treated forearm and propylene glycol treated forearm) of 0.5 % Puag-Haad in propylene glycol (n = 17)

Volunteer no.	% Whitening efficacy over corresponding control	
	Week 10	Week 12
A1	-0.46	-0.28
A2	0.68	0.80
A3	1.31	1.63
A4	0.62	0.70
A5	-0.47	-1.08
A6	1.86	1.82
A7	3.96	4.26
A8	0.97	1.31
A10	-1.52	-1.31
A11	1.00	1.15
A12	3.16	3.80
A13	1.00	0.51
A14	1.61	2.44
A15	2.13	2.59
A16	0.19	2.18
A17	-0.63	-0.16
A19	1.17	0.02
Mean ± SD	0.98 ± 1.37	1.20 ± 1.56

Table 14 % Whitening efficacy over corresponding control (difference in % whitening between product treated forearm and propylene glycol treated forearm) of 0.25 % Puag-Haad in propylene glycol (n = 20)

Volunteer no.	% Whitening efficacy over corresponding control	
	Week 10	Week 12
B1	1.12	1.37
B2	0.17	0.93
B3	1.33	1.97
B4	0.90	0.47
B5	1.33	1.14
B6	3.26	3.44
B7	0.57	0.54
B8	2.60	3.18
B9	0.82	1.66
B10	1.32	0.84
B11	2.35	3.35
B12	1.78	1.64
B13	1.98	3.02
B14	0.12	2.11
B15	1.59	1.44
B16	2.57	2.18
B17	1.51	1.70
B18	2.75	2.40
B19	5.35	5.51
B20	3.07	3.83
Mean ± SD	1.82 ± 1.23	2.14 ± 1.27

Table 15 % Whitening efficacy over corresponding control (difference in % whitening between product treated forearm and propylene glycol treated forearm) of 0.25 % Licorice extract in propylene glycol (n = 20)

Volunteer no.	% Whitening efficacy over corresponding control	
	Week 10	Week 12
C1	2.09	2.51
C2	2.37	2.17
C3	0.75	1.11
C4	1.23	1.77
C5	3.30	3.29
C6	3.06	2.80
C7	2.39	3.02
C8	1.11	1.56
C9	2.12	2.28
C10	0.32	0.93
C11	-0.46	0.18
C12	1.54	1.65
C13	0.16	0.27
C14	0.26	1.00
C15	1.07	2.07
C16	1.63	1.32
C17	-0.56	-0.47
C18	0.56	-0.51
C19	-1.13	-0.67
C20	2.44	1.92
Mean ± SD	1.21 ± 1.23	1.41 ± 1.18

Table 16 % Whitening efficacy over corresponding control (difference in % whitening between product treated forearm and propylene glycol treated forearm) of 3.00 % Kojic acid in propylene glycol (n = 19)

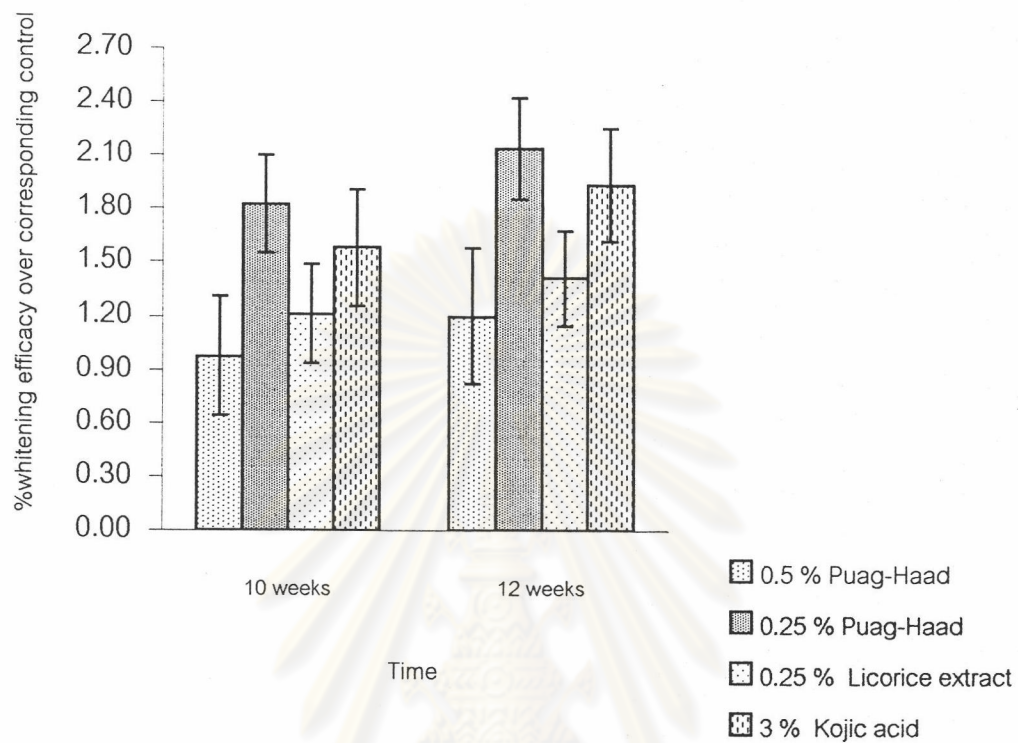| Volunteer no.	% Whitening efficacy over corresponding control	
	Week 10	Week 12
D1	0.33	0.17
D2	0.77	1.46
D3	0.28	0.56
D4	3.37	2.75
D5	1.50	1.99
D6	4.50	4.76
D7	0.39	0.03
D8	-0.64	0.96
D9	3.19	3.77
D10	1.43	1.96
D11	2.48	1.39
D12	3.00	3.12
D13	2.34	3.04
D14	1.91	3.86
D16	0.95	1.44
D17	2.66	2.74
D18	0.32	0.44
D19	-0.67	0.19
D20	1.92	2.07
Mean ± SD	1.58 ± 1.42	1.93 ± 1.39

Figure 15 Histogram comparing % whitening efficacy (difference from corresponding control) of four products after 10 and 12 week-application in female volunteers. Data = mean ± SEM (n = 17 –20 subjects per group).

However, when one-way ANOVA was applied on these values, no significant differences were detected among the four treatment groups either at 10 or 12 weeks (P > 0.05). The statistical results thus suggested that by the time when all the four products demonstrated effective whitening activity (as observed from significant paired t-test over their corresponding self-controls), the extent of whitening effect was similar. Nevertheless, 0.25% Puag-Haad demonstrated the fastest efficacy with the shortest onset time (about 4 weeks). Furthermore, it has been previously observed that the whitening extent of 0.25% Puag-Haad was highest of the four groups at both 10 and 12 week-application despite the non-significance in ANOVA. It is probable that prolonged application of 0.25% Puag-Haad for more than 12 weeks may result in a significantly greater whitening efficacy than kojic acid and licorice extract. However, more studies need to be conducted in the future to confirm this postulation.

It is also interesting to note that while 0.25% Puag-Haad gave the best whitening activity in terms of both the rate and extent, results for 0.5% Puag-Haad were quite opposite. Again, the human study showed the same observation as in the previous guinea pig study in that increasing the Puag-Haad concentration was found to cause an unexpected decrease in the whitening effect. In the guinea pig study, % whitening of 1.0% extract in propylene glycol was significantly smaller than 0.5% concentration. In this human study, the whitening extent of 0.5% extract in the same solvent was obviously smaller (though not significant) than 0.25% extract. As previously discussed, the reasons as to this observation are not presently known. However, the data seem to point out the importance of Puag-Haad concentration on its whitening activity and that there might exist an 'optimal' concentration range for this plant extract. Future experiments are thus needed to establish the most effective concentration for Puag-Haad in humans.

The high whitening activity of 0.25% Puag-Haad also reflects the potent activity of its major component 2,4,3',5'-tetrahydroxystilbene. The current human study results firmly supported the previous findings obtained in guinea pigs that Puag-Haad was as a more potent whitening agent than other established substances like kojic acid and

licorice extract, which are also highly expensive and have to be imported from abroad. In this human study Puag-Haad was more effective with respect to the shorter onset time than 3% kojic acid, which in turn, was more effective than 0.25% licorice extract. This could be partly due to the difference in its *in vitro* antityrosinase activity. Licorice extract was reported to have a weak tyrosinase inhibitory activity. Its concentration for 50% *in vitro* inhibition (IC_{50}) was about 12.88 μg/ml whereas the value for kojic acid was 5.82 μg/ml, which was 2.2-fold more potent than licorice extract (Kim and Lee, 1998). On the other hand, Puag-Haad showed the lowest IC_{50} value of only 1.5 μM, equivalent to 0.37 μg/ml (Sritularak, 1998).

The greater *in vivo* activity of Puag-Haad than kojic acid observed in this work was highly encouraging. Kojic acid is already a widely used whitening agent. Its efficacy has been extensively studied and well proven. There was reduction in skin darkness after 6 week and 8 week application of 1 % kojic acid (Majmudar et al., 1998; Zuidhoff et al., 2001). However, some study reported a longer onset time. For example, Masuda et al. (1996) found that a cream containing 1 % kojic acid was able to improve almost all pigmentary disorders in subjects after a 14-month clinical trial. It is thus promising that application of Puag-Haad, particularly at low concentrations, may give better skin-whitening performance than kojic acid and licorice extract in a larger clinical trial. This is strongly supported by its greater *in vitro* tyrosinase inhibitory activity and the faster *in vivo* whitening effect observed in this study.

2. Effect of Puag-Haad on skin erythema and other skin reactions in comparison with other commonly used whitening agents (licorice extract and kojic acid)

The average erythema values of subjects in each group are given in Table 17 together with the paired t-test results. The individual data are provided in Appendix II. From this table, paired t-tests revealed that during weeks 0 – 4 there was no significant difference in the E values between the substance-treated and propylene glycol-treated arms of the subjects within each group. However, after 6-week application, subjects in groups A, B and D started to show significant difference in the E values between the substance-treated and propylene glycol-treated arms ($P < 0.05$). On the other hand, subjects in group C started to show significant difference between the two arms after 10 weeks.

Interestingly, the significant difference so observed was always in the direction that the mean E values of the substance-treated arms were *smaller* than that of propylene glycol. Moreover, the difference seemed to slowly widen with increasing application time. This indicated that all the four substances (0.50% Puag-Haad, 0.25% Puag-Haad, 0.25% licorice extract and 3% kojic acid) appeared to have some erythema-reducing effect compared to their self-control propylene glycol. The reasons as to this type of behavior are not presently known since it was somewhat unexpected. If the substances were irritating to the skin, the results should have been opposite, i.e., application of either A, B, C or D would have induced some degrees of erythema on the treated skin, leading to the measured E values which would have been greater than the propylene glycol-treated arms.

It is thus postulated here that all the four substances may possess some protective effect against erythema. Their continuous application as employed in this study may have resulted in improved skin resistance to certain irritants such as UV light and soaps, which are known to cause skin erythema upon exposure. Furthermore, it has been reported that certain tysorinase inhibitors like licorice extract possess some anti-inflammatory activities (Lee et al., 1997). Also, many polyphenolic compounds were shown to have anti-inflammatory effects (Lee and Choi, 1999). It is thus possible that

2,4,3′,5′-tetrahydroxystilbene, which is a polyphenolic compound, may exert similar activities. Further studies are thus needed to find out more about many possible actions of Puag-Haad and other whitening substances. These unknown mechanisms might be responsible for the observed erythema-reducing effect.

On the other hand, measurements of the erythema (E) values may not provide adequate information on the skin irritation potential of each whitening agent. As discussed previously, Mexameter merely measures the absorbance of hemoglobin (and hence the concentration of red blood cells) available in the area of interest. Vasodilatation of the microcirculation in the affected skin area is not the sole manifestation of skin irritation. Therefore, visual observation of the skin texture and subject interviews were also performed as additional means to investigate the substances' detrimental effects on skin.

After each skin measurement by Mexameter, the individual subjects were visually observed for any skin disorders and interviewed if there was any itching or discomfort upon application of the substances. Only a few subjects in each group complained about the feeling of dried skin, which could be visually detected by light scaling of the epidermis. However, this symptom of dried skin disappeared within 3 to 4 days after they were instructed to apply a few drops of mineral oil on the affected area half an hour following each application of the test solutions. The observation of skin dryness was most likely due to propylene glycol since the scaling occurred in both upper arms. Propylene glycol is highly hygroscopic and can absorb a good deal of moisture. It is often used as a humectant in many cosmetic products such as creams and lotions (Smolinske, 1992). It is likely that prolonged application of propylene glycol may have resulted in the dehydration of the outer skin layer. Nevertheless, this was considered a minor skin disturbance and occurred in only 10% of the subjects. In some subjects the symptom even disappeared within a few days without application of mineral oil.

Other reported skin disorders include itching, which could occur with or without visible skin rash. Only one subject in group A (No. A18) and one in group D (No. D15)

developed visible skin rash in the substance-treated arms after 4 and 6 weeks, respectively, and thus voluntarily withdrew from the study. These represented only 2.5% overall dropout (2 of 80 subjects) and 5% dropout each in groups A and D (1 of 20). They were treated with aqueous calamine cream and their skin returned to normal conditions within a few days. Two more subjects (No. A9 and A20) dropped out of the study after 6 weeks due to change of job and not from any side effects, leaving the total number of 17 subjects in group A for statistical comparison. The rest of the subjects in all groups remained in the study until completion without any visible skin disorders. All substances were well tolerated by the remaining 76 subjects.

Hence, the results obtained from the human study have clearly indicated the efficacy and safety of Puag-Haad as a promising skin whitening agent, particularly at the concentration of 0.25% w/v. Its rapid onset of whitening action, coupled with the observed erythema-protective effect and low irritation potential (0% dropout in group B), made it a highly attractive choice as a novel whitening agent from natural sources. In addition, its low cost (only 400 baht/kg compared to 8,000 baht/kg for kojic acid, 5,800 bath/kg for licorice extract and 600,000 baht/kg for licorice extract PT-40) further substantiate its potential use in cosmetic industry.

Table 17 The average erythema values (mean ± SD) in the upper arms of human volunteers treated with different substances for 12 weeks. (n = 17 – 20 subjects per treatment group).

Treatment group		Erythema values (E)						
		Week0	Week2	Week4	Week6	Week8	Week10	Week12
A (n = 17) 0.50%Puag-Haad	PG-Control	604.99 ± 16.48	595.13 ± 16.15	591.84 ± 17.19	600.02 ± 16.27	591.55 ± 12.57	588.34 ± 15.33	590.59 ± 14.74
	A	600.52 ± 17.32	596.22 ± 14.63	591.79 ± 14.56	593.16 ±16.57	585.21 ± 13.69	580.93 ± 13.67	583.38 ± 14.50
	P-value	0.0555	0.6264	0.9838	0.0014*	0.0001*	0.0013*	0.0049*
B (n = 20) 0.25%Puag-Haad	PG-Control	600.97 ± 18.70	597.34 ± 19.50	594.71 ± 18.60	595.55 ± 15.64	590.39 ± 17.35	593.26 ± 20.84	591.13 ± 14.74
	B	602.46 ± 20.77	594.69 ± 17.22	592.81 ± 18.33	591.13 ±16.43	582.60 ± 16.81	583.50 ± 14.94	581.73 ± 15.31
	P-value	0.4681	0.2708	0.3262	0.0201*	0.0006*	0.0025*	0.0003*
C (n = 20) 0.25%Licorie extract	PG-Control	597.91 ± 18.92	592.76 ± 17.25	588.50 ± 16.86	592.80 ± 14.58	588.87 ± 15.07	588.53 ± 16.25	589.58 ± 16.20
	C	597.74 ± 20.32	588.96 ± 18.26	588.49 ± 17.86	591.62 ±14.76	585.76 ± 14.75	582.30 ± 15.57	582.67 ± 14.51
	P-value	0.9443	0.1063	0.9956	0.3886	0.1554	0.0036*	0.0016*
D (n = 19) 3.0%Kojic acid	PG-Control	604.06 ± 18.00	599.85 ± 19.23	594.45 ± 15.56	597.23 ± 14.47	591.23 ± 15.90	593.08 16.30	593.46 ± 17.00
	D	605.54 ± 18.30	600.47 ± 19.03	592.45 ± 15.29	593.51 ±13.88	586.19 ± 15.20	589.72 ± 14.95	584.35 ± 15.87
	P-value	0.3715	0.7441	0.2458	0.0309*	0.0199*	0.2005	0.0043*

PG-control = propylene glycol self-control within each treatment group

* denotes significant difference between PG-control and treatment within each group by paired student's t-test (P < 0.05).

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

The purpose of this part was to evaluate the physical and biochemical stability of Puag-Haad solutions upon storage at room (~ 27 $^{\circ}$C ambient) temperature and at 45 $^{\circ}$C. Physical stability was evaluated with respect to clarity, color and pH whereas biochemical stability was determined through measurements of its *in vitro* tyrosinase inhibitory activity.

Solutions of Puag-Haad were prepared in 20% propylene glycol – 80% water instead of pure propylene glycol as previously used in the guinea pig and human studies. This was to establish the stability of Puag-Haad in water. Propylene glycol was added as a cosolvent at a minimal concentration of 20%. Thus, the data obtained would provide basic preformulation information necessary for subsequent development of Puag-Haad into lotions or creams, in which water is the major component of these dosage forms. Concentration of Puag-Haad was fixed at 0.25% in this part because this concentration was found to give the most promising whitening effect with good safety profile.

Since the active component of Puag-Haad (2,4,3′,5′-tetrahydroxystilbene) is a polyphenol derivative, it was thus expected to be degraded by oxidation (Tiptabiankarn, 1967). Solutions of 0.25% Puag-Haad in various types of antioxidants were prepared in the same solvent (20% propylene glycol in water) and coded as follows:

P   = 0.25% Puag-Haad

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

P+A2 = 0.25% Puag-Haad + 0.10% butylated hydroxy anisole (BHA)

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

P+A4 = 0.25% Puag-Haad + 0.15% sodium metabisulfite + 0.10% BHA

       = P + A1 + A2

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

$$= P + A1 + A3$$

P+A6 $\quad = 0.25\%$ Puag-Haad + 0.10% BHA + 0.05% EDTA

$$= P + A2 + A3$$

P+A7 $\quad = 0.25\%$ Puag-Haad + 0.15% sodium metabisulfite + 0.10% BHA + 0.05%EDTA

$$= P + A1 + A2 + A3$$

Each of the above solutions was prepared and assayed in triplicate (3 vials per solution). Sodium metabisulfite was chosen as a representative antioxidant of a reducing agent-type at a concentration of 0.15%. BHA at 0.10% was used as a representative of true antioxidant whereas 0.05% EDTA as an auxiliary antioxidant (chelating agent). Hence, antioxidants with different mechanisms were added to Puag-Haad solution both separately and in combination to see if there was any enhancement in the protective effect against oxidation.

1. Physical stability

Tables 18 and 19 show changes in coloring of Puag-Haad samples upon storage at room temperature and 45 $^\circ$C for 24 weeks (6 months). The initial color of 0.25% Puag-Haad solution was of pale yellow (graded by number 0). Upon storage at room temperature, the solution of pure Puag-Haad (solution P) gradually darkened to dark brown (graded as +4) after 24 weeks. The color of all the stored Puag-Haad solutions was always compared with that of the freshly prepared 0.25% pure Puag-Haad. Since the addition of antioxidants did not cause any changes in the initial color of Puag-Haad solutions, only one freshly prepared solution of pure Puag-Haad was used as a common reference for color comparison.

However, addition of antioxidants, particularly 0.15% sodium metabisulfite was able to pretect 0.25% Puag-Haad from increased coloration after prolonged storage. As seen from the data in Tables 18 and 19, solutions P+A1, P+A4, P+A5, P+A7 did not increase in color after 24-week storage at room temperature or only slightly increased at 45 $^\circ$C. All these solutions contained 0.15% sodium metabisulfite as a common

antioxidant. On the other hand, 0.10% BHA and 0.05% EDTA, either single or in combination (solutions P+A2, P+A3, P+A6) hardly protected Puag-Haad from discoloration at both temperatures. After 24 week-storage, these solutions became brown (+3) or dark brown (+4) in color at room temperature whereas the effect was worsened at 45 °C, with the color became intense deep brown (+5). Photographs of different Puag-Haad solutions taken after storage for 6, 12 and 24 weeks are also provided for visual comparison in Figures 16 - 18. Therefore, sodium metabisulfite and its combination appeared to give the best protection against Puag-Haad discoloration.

The pH of each solution was also measured in triplicate and the data at room temperature and 45 °C are respectively given in Tables 20 – 21. Pure Puag-Haad solution showed only minor decrease in pH upon storage at room temperature, from 5.00 at 0 week to 4.51 at 12 weeks, which was equivalent to a 0.49 unit change in pH. Addition of 0.10% BHA (P+A2) also resulted in a similar decrease in pH, from an initial value of 5.05 to 4.62, equivalent to 0.43 unit change in pH. On the other hand, addition of 0.05% EDTA (P+A3) was able to stabilize the pH of Puag-Haad solution (initial pH = 4.38 vs 4.35 after 12 weeks). Solution containing EDTA and BHA (P+A6) also stabilized the pH of Puag-Haad solution (initial pH = 4.35 vs 4.33 after 12 weeks). Thus, the stabilization observed with solution P+A6 was likely due to the effect of EDTA rather than BHA, since 0.10% BHA (P+A2) did not have any protective effect against pH decrease. Similar pattern was also observed at 45 °C (Table 21).

In contrast, addition of 0.15% sodium metabisulfite was found to show further decrease in pH upon storage. As seen in Table 20, all solutions containing sodium metabisulfite, either alone (P+A1) or in combination (P+A4, P+A5, P+A7), showed a marked decrease in pH values with changes in pH ranging from 0.78 (P+A1) to 1.52 (P+A4) after 12 weeks at room temperature. Similar results were also observed at 45 °C (Table 21), at which solutions P+A1, P+A4, P+A5, and P+A7 showed an overall decrease in pH in the range of 1.37 (P+A4) to 2.44 (P+A1) after 12 weeks.

Table 18 Changes in color of Puag-Haad samples at initial of the study and upon storage at room temperature

No.	Samples	Time (week)					
		0	4	8	12	16	24
0	Fresh P	0	0	0	0	0	0
1	P	0	+1	+2	+3	+3	+4
2	P+A1	0	0	0	0	0	0
3	P+A2	0	+1	+2	+3	+3	+4
4	P+A3	0	+1	+1	+2	+2	+3
5	P+A4	0	0	0	0	0	0
6	P+A5	0	0	0	0	0	0
7	P+A6	0	+1	+1	+2	+2	+3
8	P+A7	0	0	0	0	0	0

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

0 = normal (pale yellow); no change, +1 = light brown, +2 = light brown, +3 = brown, +4 dark brown, +5 = intense deep brown

Table 19 Changes in color of Puag-Haad samples at initial of the study and upon storage at 45 °C

No.	Samples	Time (week)								
		0	2	4	6	8	10	12	16	24
1	P	0	+1	+2	+3	+3	+4	+4	+4	+5
2	P+A1	0	0	0	0	0	+1	+1	+1	+2
3	P+A2	0	+1	+2	+3	+3	+3	+4	+4	+5
4	P+A3	0	+1	+2	+3	+3	+3	+4	+4	+5
5	P+A4	0	0	0	0	0	+1	+1	+1	+1
6	P+A5	0	0	0	0	0	+1	+1	+1	+1
7	P+A6	0	+1	+2	+3	+3	+4	+4	+4	+5
8	P+A7	0	0	0	0	0	+1	+1	+1	+1

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

0 = normal (pale yellow); no change, +1 = light brown, +2 = light brown, +3 = brown, +4 dark brown, +5 = intense deep brown
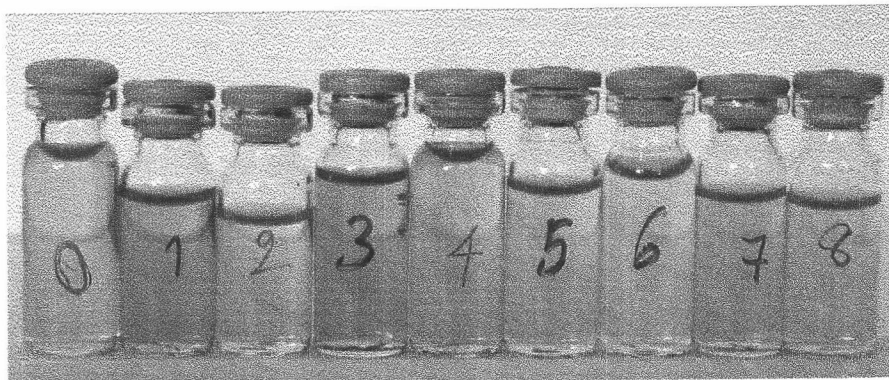
Room temperature

45 °C

Figure 16 Physical appearances of Puag-Haad samples upon storage at room temperature and 45 °C for 6 weeks.

0 = Fresh 0.25 % Puag-Haad (P), 1 = Pure P, 2 = P + 0.15% sodium metabisulfite (A1), 3 = P+0.10% BHA (A2), 4 = P+0.05% EDTA (A3), 5 = P+A1+A2, 6 = P+A1+A3, 7 = P+A2+A3, 8 = P+A1+A2+A3

Room temperature

45 °C

Figure 17 Physical appearances of Puag-Haad samples upon storage at room temperature and 45 °C for 12 weeks.

0 = Fresh 0.25 % Puag-Haad (P), 1 = Pure P, 2 = P + 0.15% sodium metabisulfite (A1), 3 = P+0.10% BHA (A2), 4 = P+0.05% EDTA (A3), 5 = P+A1+A2, 6 = P+A1+A3, 7 = P+A2+A3, 8 = P+A1+A2+A3
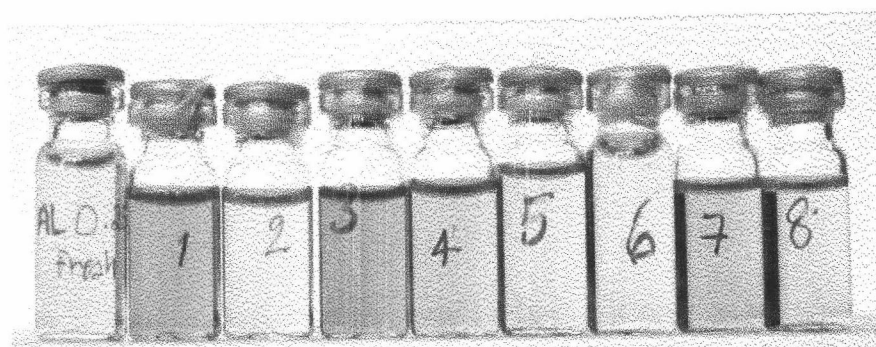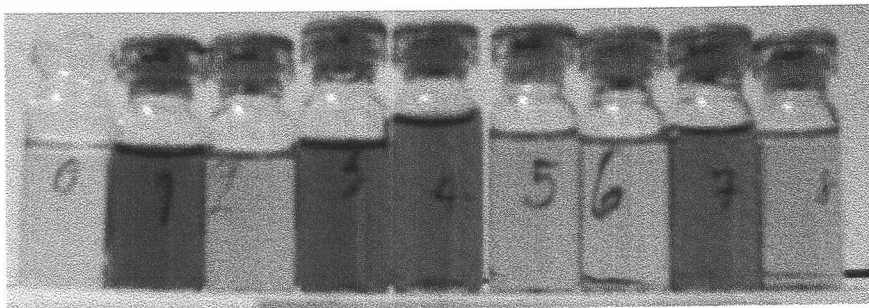
Room temperature

45 °C

Figure 18 Physical appearances of Puag-Haad samples upon storage at room temperature and 45 °C for 24 weeks.

0 = Fresh 0.25 % Puag-Haad (P), 1 = Pure P, 2 = P + 0.15% sodium metabisulfite (A1), 3 = P+0.10% BHA (A2), 4 = P+0.05% EDTA (A3), 5 = P+A1+A2, 6 = P+A1+A3, 7 = P+A2+A3, 8 = P+A1+A2+A3

To determine if the change in pH was due to direct interaction of the antioxidant (s) with the active component or due to degradation of the antioxidant(s) itself, separate sets of antioxidant solutions without Puag-Haad (A1 to A7) were prepared in the same solvent and kept concomitantly for pH determination. The results are shown in Table 20/1 (room temperature) and Table 21/1 (45 °C). All the pure antioxidant solutions remained clear and colorless throughout the entire storage period. However, solutions A1, A4, A5 and A7 all demonstrated substantial drop in pH, with the changes ranging from 0.68 (A5) to 2.08 (A1) after 12-week storage at room temperature, and from 1.38 (A4) to 3.08 (A1) at 45 °C. On the other hand, the remaining antioxidants, namely BHA (A2), EDTA (A3) and their combination (A6) demonstrated negligible drop in pH after 12-week storage at both temperatures, indicating that they were quite stable in the solutions.

Hence, the drop in pH observed with sodium metabisulfite was mainly a result of its self-degradation. It is known that sodium metabisulfite is gradually changed to sodium sulfate thereby giving hydronium ions upon degradation. (Boylan et al., 1896). This reaction had obviously led to a drop in pH of the solution regardless of whether Puag-Haad was present or not. On the other hand, the pH-stabilizing effect of EDTA (disosium ethylenediamine tetraacetate salt) observed here could be due to its chelating action on Puag-Haad solution since EDTA is known to possess form stable water soluble complexes (chelates) with alkaline earth and heavy metal ions (Capter, 1975). Nevertheless, the exact mechanism(s) by which EDTA could stabilize the pH of Puag-Haad is still not known. More studies are needed to elucidate the degradation pathways of 2,4,3′,5′-tetrahydroxystilbene in Puag-Haad solution.

Table 20 Changes in pH values of Puag-Haad samples at initial of the study and upon storage at room temperature

Puag-Haad samples	Time (week)				ΔpH (0 -12 week)
	0	4	8	12	
P	5.02 5.00 4.97	4.93 4.93 4.94	4.56 4.60 4.60	4.50 4.51 4.51	0.49
Mean ± SD	5.00 ± 0.03	4.93 ± 0.01	4.59 ± 0.02	4.51 ± 0.01	
P+A1	5.34 5.36 5.34	4.63 4.62 4.63	4.60 4.59 4.60	4.57 4.57 4.58	0.78
Mean ± SD	5.35 ± 0.01	4.63 ± 0.01	4.60 ± 0.01	4.57 ± 0.01	
P+A2	5.06 5.05 5.04	4.81 4.82 4.82	4.77 4.73 4.73	4.62 4.63 4.62	0.43
Mean ± SD	5.05 ± 0.01	4.82 ± 0.01	4.74 ± 0.02	4.62 ± 0.01	
P+A3	4.38 4.38 4.37	4.39 4.40 4.42	4.39 4.37 4.37	4.35 4.36 4.35	0.03
Mean ± SD	4.38 ± 0.01	4.40 ± 0.02	4.38 ± 0.01	4.35 ± 0.01	
P+A4	5.36 5.38 5.39	4.45 4.46 4.48	4.30 4.31 4.31	3.86 3.87 3.85	1.52
Mean ± SD	5.38 ± 0.02	4.46 ± 0.02	4.31 ± 0.01	3.86 ± 0.01	
P+A5	5.21 5.20 5.20	4.48 4.48 4.51	4.34 4.29 4.27	4.18 4.17 4.17	1.03
Mean ± SD	5.20 ± 0.01	4.49 ± 0.02	4.30 ± 0.04	4.17 ± 0.01	
P+A6	4.35 4.36 4.35	4.43 4.44 4.46	4.38 4.39 4.38	4.33 4.33 4.32	0.02
Mean ± SD	4.35 ± 0.01	4.44 ± 0.02	4.38 ± 0.01	4.33 ± 0.01	
P+A7	5.35 5.36 5.36	4.37 4.36 4.36	4.19 4.19 4.21	4.07 4.08 4.08	1.28
Mean ± SD	5.36 ± 0.01	4.36 ± 0.01	4.20 ± 0.01	4.08 ± 0.01	

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

Table 20/1 Changes in pH values of antioxidants at initial of the study and upon storage at room temperature

Antioxidants	Time (week)				ΔpH
	0	4	8	12	(0–12 week)
A1 Mean ± SD	5.40 5.40 5.42 5.41 ± 0.01	4.67 4.67 4.64 4.66 ± 0.02	4.25 4.25 4.23 4.24 ± 0.01	3.34 3.34 3.32 3.33 ± 0.01	2.08
A2 Mean ± SD	4.70 4.71 4.70 4.70 ± 0.01	4.65 4.66 4.65 4.65 ± 0.01	4.61 4.63 4.60 4.61 ± 0.02	4.58 4.60 4.59 4.59 ± 0.01	0.11
A3 Mean ± SD	4.33 4.33 4.33 4.33 ± 0.00	4.32 4.33 4.33 4.33 ± 0.01	4.33 4.31 4.31 4.32 ± 0.01	4.32 4.32 4.33 4.32 ± 0.01	0.01
A4 Mean ± SD	5.46 5.47 5.46 5.46 ± 0.01	4.75 4.75 4.74 4.75 ± 0.01	4.32 4.36 4.36 4.35 ± 0.02	4.21 4.22 4.22 4.22 ± 0.01	1.24
A5 Mean ± SD	5.70 5.70 5.71 5.70 ± 0.01	5.24 5.28 5.28 5.27 ± 0.02	5.19 5.20 5.20 5.20 ± 0.01	4.95 5.07 5.05 5.02 ± 0.06	0.68
A6 Mean ± SD	4.51 4.51 4.51 4.51 ± 0.00	4.54 4.51 4.49 4.51 ± 0.03	4.50 4.50 4.50 4.50 ± 0.00	4.48 4.49 4.50 4.49 ± 0.01	0.02
A7 Mean ± SD	5.51 5.54 5.54 5.53 ± 0.02	5.01 5.01 5.02 5.01 ± 0.01	4.85 4.86 4.86 4.86 ± 0.01	4.61 4.62 4.63 4.62 ± 0.01	0.91

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

Table 21 Changes in pH values of Puag-Haad samples at initial of the study and upon storage at 45 °C

Puag-Haad samples	Time (week)							ΔpH (0-12 week)
	0	2	4	6	8	10	12	
P	5.02 5.00 4.97	4.31 4.32 4.31	4.26 4.27 4.26	4.15 4.16 4.13	4.08 4.09 4.08	3.95 3.93 3.91	3.75 3.74 3.73	
Mean ± SD	5.00 ± 0.03	4.31 ± 0.01	4.26 ± 0.01	4.15 ± 0.02	4.08 ± 0.01	3.93 ± 0.02	3.74 ± 0.01	1.26
P+A1	5.34 5.36 5.34	4.13 4.15 4.13	3.95 3.96 3.95	3.97 3.82 3.91	3.51 3.52 3.53	3.07 3.11 3.08	2.94 2.90 2.88	
Mean ± SD	5.35 ± 0.01	4.14 ± 0.01	3.95 ± 0.01	3.90 ± 0.08	3.52 ± 0.01	3.09 ± 0.02	2.91 ± 0.03	2.44
P+A2	5.06 5.05 5.04	4.43 4.41 4.41	4.33 4.27 4.32	4.23 4.22 4.23	4.19 4.21 4.20	4.02 4.01 4.05	3.82 3.82 3.81	
Mean ± SD	5.05 ± 0.01	4.42 ± 0.01	4.31 ± 0.03	4.23 ± 0.01	4.20 ± 0.01	4.03 ± 0.02	3.82 ± 0.01	1.23
P+A3	4.38 4.38 4.37	4.39 4.42 4.40	4.39 4.38 4.39	4.34 4.35 4.30	4.33 4.34 4.30	4.30 4.31 4.30	4.13 4.12 4.08	
Mean ± SD	4.38 ± 0.01	4.40 ± 0.02	4.39 ± 0.01	4.33 ± 0.03	4.32 ± 0.02	4.30 ± 0.01	4.11 ± 0.03	0.27
P+A4	5.36 5.38 5.39	5.15 5.14 5.13	4.75 4.87 4.93	4.62 4.63 4.63	4.08 4.10 4.11	4.00 4.02 4.05	4.00 4.02 4.00	
Mean ± SD	5.38 ± 0.02	5.14 ± 0.01	4.85 ± 0.09	4.63 ± 0.01	4.10 ± 0.02	4.02 ± 0.03	4.01 ± 0.01	1.37
P+A5	5.21 5.20 5.20	4.55 4.55 4.57	4.46 4.50 4.48	4.46 4.47 4.45	4.25 4.24 4.26	3.83 4.00 3.91	3.37 3.36 3.35	
Mean ± SD	5.20 ± 0.01	4.56 ± 0.01	4.48 ± 0.02	4.46 ± 0.01	4.25 ± 0.01	3.91 ± 0.01	3.36 ± 0.01	1.84

Table 21 Changes in pH values of Puag-Haad samples at initial of the study and upon storage at 45 °C

Puag-Haad samples	Time (week)							ΔpH (0-12 week)
	0	2	4	6	8	10	12	
P+A6	4.35 4.36 4.35	4.42 4.46 4.45	4.38 4.40 4.38	4.34 4.36 4.34	4.34 4.35 4.34	4.32 4.32 4.33	4.13 4.12 4.11	
Mean ± SD	4.35 ± 0.01	4.44 ± 0.02	4.39 ± 0.01	4.35 ± 0.01	4.34 ± 0.01	4.32 ± 0.01	4.12 ± 0.01	0.23
P+A7	5.35 5.36 5.36	4.35 4.36 4.40	4.17 4.16 4.15	4.01 4.05 4.00	3.84 3.85 3.84	3.49 3.50 3.52	3.45 3.49 3.46	
Mean ± SD	5.36 ± 0.01	4.37 ± 0.03	4.16 ± 0.01	4.02 ± 0.03	3.84 ± 0.01	3.50 ± 0.02	3.47 ± 0.02	1.89

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

Table 21/1 Changes in pH values of antioxidants at initial of the study and upon storage at 45 °C

Antioxidants	Time (week)							ΔpH (0-12 week)
	0	2	4	6	8	10	12	
A1	5.40 5.40 5.42	4.32 4.33 4.33	2.41 2.40 2.37	2.33 2.34 2.33	2.33 2.33 2.33	2.33 2.34 2.32	2.33 2.33 2.34	3.08
Mean ± SD	5.41 ± 0.01	4.33 ± 0.01	2.39 ± 0.02	2.33 ± 0.01	2.33 ± 0.00	2.33 ± 0.01	2.33 ± 0.01	
A2	4.70 4.71 4.70	4.64 4.66 4.64	4.57 4.55 4.56	4.38 4.39 4.40	4.28 4.29 4.29	4.13 4.15 4.13	4.02 4.01 4.01	0.69
Mean ± SD	4.70 ± 0.01	4.65 ± 0.01	4.56 ± 0.01	4.39 ± 0.01	4.29 ± 0.01	4.14 ± 0.01	4.01 ± 0.01	
A3	4.33 4.33 4.33	4.30 4.28 4.29	4.33 4.31 4.32	4.21 4.22 4.23	4.18 4.21 4.21	4.22 4.19 4.21	4.15 4.15 4.14	0.18
Mean ± SD	4.33 ± 0.00	4.29 ± 0.01	4.32 ± 0.01	4.22 ± 0.01	4.20 ± 0.02	4.21 ± 0.02	4.15 ± 0.01	
A4	5.46 5.47 5.46	4.22 4.25 4.24	4.21 4.20 4.21	4.15 4.19 4.16	4.1 4.1 4.1	4.07 4.09 4.06	4.1 4.1 4.1	1.38
Mean ± SD	5.46 ± 0.01	4.24 ± 0.02	4.21 ± 0.01	4.17 ± 0.02	4.08 ± 0.01	4.07 ± 0.02	4.08 ± 0.01	
A5	5.70 5.70 5.71	5.55 5.51 5.51	5.46 5.46 5.44	5.31 5.32 5.31	5.09 5.07 5.07	4.81 4.82 4.81	4.12 4.13 4.14	1.57
Mean ± SD	5.70 ± 0.01	5.52 ± 0.02	5.45 ± 0.01	5.31 ± 0.01	5.08 ± 0.01	4.81 ± 0.01	4.13 ± 0.01	

Table 21/1 Changes in pH values of antioxidants at initial of the study and upon storage at 45 °C

Antioxidants	Time (week)							ΔpH (0-12 week)
	0	2	4	6	8	10	12	
A6	4.51 4.51 4.51	4.52 4.51 4.51	4.44 4.42 4.43	4.41 4.40 4.42	4.39 4.37 4.37	4.34 4.35 4.34	4.21 4.20 4.21	0.30
Mean ± SD	4.51 ± 0.00	4.51 ± 0.01	4.43 ± 0.01	4.41 ± 0.01	4.38 ± 0.01	4.34 ± 0.01	4.21 ± 0.01	
A7	5.51 5.54 5.54	4.74 4.77 4.75	4.65 4.66 4.66	4.47 4.48 4.48	4.44 4.44 4.43	4.38 4.37 4.37	4.08 4.07 4.06	1.46
Mean ± SD	5.53 ± 0.02	4.75 ± 0.02	4.66 ± 0.01	4.48 ± 0.01	4.44 ± 0.01	4.37 ± 0.01	4.07 ± 0.01	

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

2. Biochemical stability

The biochemical stability of Puag-Haad solutions were determined by quantitating their *in vitro* anti-tyrosinase activities upon storage. 3% freshly prepared kojic acid was used as a reference solution to validate for accuracy and precision of the enzymatic assay. It was assayed at the same intervals as the Puag-Haad solutions. In addition, preliminary anti-tyrosinase activity testing of Puag-Haad (0.5 and 0.25%), 0.25% licorice extract and 3% kojic acid was also conducted to check for their short-term stability after 2 week-storage at room temperature.

Table 22 shows the individual and average values (mean, SD and %CV) of % tyrosinase inhibition of freshly prepared 3% kojic acid. The values of tyrosinase inhibitory activity were consistent both within each run and between different runs, giving the overall mean of 49.65% inhibition. The within-run coefficient of variation (CV) was in the range of 1.23 – 6.01% and the between-run CV was 2.38%. These values were much smaller than the 15%-limit generally allowed for biological assay, indicating that the method had good within-run and between-run precision.

Table 23 also indicated that solutions of Puag-Haad (0.50 and 0.25%) and 0.25% licorice extract in 20% propylene glycol/80% water were stable for at least two-weeks without any antioxidants. The initial values for 0.5 and 0.25% Puag-Haad were comparable, with average % inhibition of 74.09 and 72.93%, respectively. This may imply that 0.25% concentration of Puag-Haad may already have saturated the tyrosinase enzyme, resulting in the maximum inhibition of about 73% under this particular testing condition. After two-week storage, the values dropped by only 2-3% (71.34 and 69.79%), which was considered negligible. The value for 0.25% licorice also remained stable during this period (52.68 vs 51.38%). These preliminary results formed the basis for the decision to freshly prepare the test solutions every two weeks for the human study. On the other hand, 3% kojic acid showed somewhat greater drop in % inhibition from 48.86% to 39.90% after two weeks. Nevertheless, kojic acid solution was still prepared at similar two-week intervals for the human study because the type of solvent was different. In the human study, pure propylene glycol was used in preparing all the

solutions. It was probable that biochemical stability of kojic acid, Puag-Haad and licorice would improve when the water was removed from the preparations, at least during the two-week interval.

Table 22 Precision of the enzymatic method used in determining tyrosinase inhibitory activity of whitening agents. 3% freshly prepared kojic acid solution was used as a reference standard.

Week of assay	% Inhibition					
	Vial 1	Vial 2	Vial 3	Mean	SD	Within-run %CV
0	52.30	47.87	47.18	49.12	2.78	5.66
2	49.78	48.48	51.54	49.93	1.54	3.08
4	54.07	52.81	48.17	51.68	3.11	6.01
6	50.73	49.45	49.10	49.76	0.86	1.72
8	51.51	48.48	50.09	50.03	1.52	3.03
10	48.20	49.40	48.80	48.80	0.60	1.23
12	48.65	50.36	47.44	48.82	1.47	3.01
16	46.47	46.95	50.00	47.81	1.91	4.00
24	52.23	50.93	49.66	50.94	1.29	2.52
Overall	Between run % CV = 2.38			49.65	1.18	-

Table 23 Stability at room temperature of Puag-Haad, Licorice extract and Kojic acid in 20% propylene glycol / 80% water with respect to % tyrosinase inhibitory activity

Samples	0 week (initial)		2 week	
	Vial 1	Vial 2	Vial 1	Vial 2
0.50% Puag-Haad	74.58	73.60	72.18	70.50
Mean	74.09		71.34	
0.25% Puag-Haad	72.75	73.11	70.24	69.33
Mean	72.93		69.79	
0.25% Licorcie extract	54.03	51.33	50.00	52.76
Mean	52.68		51.38	
3.00% Kojic acid	49.06	48.66	40.58	39.22
Mean	48.86		39.90	

Following preliminary studies, 0.25% Puag-Haad solutions, with and without antioxidants, were assayed for their tyrosinase inhibitory activity at various times after storing at room temperature and at 45 °C. Table 24 shows the average % inhibition (mean ± SD of triplicate determinations) of various Puag-Haad solutions kept at room temperature. From this table, it can be seen that addition of different types of antioxidants (A1 – A3) and their combinations (A4 to A6) to 0.25% Puag-Haad (P) did not cause any noticeable increase in tyrosinase inhibitory activity, with the mean % inhibition in the range of 65.42 – 73.38%. This indicated that the individual antioxidants (A1, A2, A3) might not possess significant anti-tyrosinase activity, which could positively contribute to the overall activity of Puag-Haad. In fact, the overall activity seemed to slightly reduce in the presence of BHA and/or EDTA (A2, A3, and A6). Only the triple combination of antioxidants (A7) gave an increase in overall anti-tyrosinase with the average % inhibition of 82.21%. The mechanism(s) by which the triple combination could slightly enhance the initial activity of Puag-Haad solution was not clearly known, as with the small negative effect of BHA, EDTA and their double combination on Puag-Haad's activity.

Actually, even the detailed mechanism of 2,4,3′,5′-tetrahydroxystilbene (oxyresveratrol) in inhibiting tyrosinase enzyme is not clearly understood and several reports showed conflicting data. For example, Shimizu, Kondo and Sakai (2000) reported that oxyresveratrol inhibited tyrosinase in a competitive manner, with IC50 somewhat similar to kojic acid (20.8 vs 17.2 μM). On the other hand, Shin et al. (1998) and Kim et al. (2002) reported the tyrosinase inhibitory mechanism of oxyresveratrol to be non competitive, with IC much smaller than kojic acid. More research on multi-faceted mechanisms and activities of tyrosinase is still going on which should reveal better understanding in the future. Nevertheless, it could be assumed from the above observations that the interference from the individual antioxidants and their double combinations in the determination of Puag-Haad's anti-tyrosinase activity was negligible in this study.

Table 24 also indicates that 0.25% Puag-Haad solution without any antioxidant (solution P) showed a steady decline in anti-tyrosinase activity upon storage even at room temperature. The mean value decreased from 72.93% at the start of study to 47.62% and 36.71% after 12- and 24- week storage, respectively. This was equivalent to about 50% loss in activity after 6 months. Addition of different antioxidants was found to stabilize Puag-Haad solution to a varying degree. To facilitate comparison among different solutions, the absolute percent inhibition was normalized to percent inhibition relative to the initial value as shown in Table 25.

From this table, all antioxidants and their combinations (A1 – A7) were capable of stabilizing Puag-Haad solution, giving the remaining activity well above 80% after 12 weeks (range = 81.62% to 94.33% as opposed to 65.30% without antioxidant). Closer examination of the data at this period revealed that the three best solutions, which still gave % inhibition higher than 87% were P+A1 (87.81%), P+A4 (94.33%), and P+A7 (87.85%). All of these solutions had 0.15% sodium metabisulfite as a common antioxidant. The high biochemical stability was also enhanced by good coloring appearance, in which solutions P+A1, P+A4, and P+A7 were found to give negligible change in color. Although solutions P+A2 (84.04%), P+A3 (81.62%), and P+A6 (82.49%) still gave fairly good relative anti-tyrosinase activity at this period, their

coloring, however, had become visibly darkened by this time and hence may not give an attractive appearance to potential users. Solution P+A5, which contained sodium metabisulfite and EDTA, showed % inhibition of 82.64% comparable to P+A2, P+A3 and P+A6. However, its color was unchanged which was due to the presence of sodium metabisulfite.

After 24 week-storage at room temperature, relative anti-tyrosinase activity further declined in all groups. Only solution P+A4 still gave remaining % relative inhibition above 80% (80.78%), whereas the second highest activity was observed with P+A6 (75.68%). However, the color of P+A6 (BHA + EDTA) became unpleasantly brown. The rest of the solutions gave relative tyrosinase inhibitory activity less than 70%, with the average value ranging from 58.40% to 69.74%. Besides the substantial drop in relative activity, solutions P+A2 (68.48%) and P+A3 (58.40%) also turned brown to dark brown, which was an undesirable characteristic. On the other hand, despite the marked decrease in activity, the color of solutions P+A1 (62.87%), P+A5 (69.74%) and P+A7 (69.14%) was still unchanged, thanks to the stabilizing effect of sodium metabisulfite as previously discussed.

The result at room temperature so far had indicated that combination of antioxidants 0.15% sodium metabisulfite and 0.10% BHA (P+A4) provided the best protection against loss of anti-tyrosinase activity. In addition, change in color of a solution was not always associated with a large drop in activity. For example, the uninviting brown solution of P+A6 (0.10% BHA + 0.05% EDTA) still provided good protection against loss of activity even after 24 week-storage (75.68%).

Stability data at 45 $^{\circ}$C also demonstrated similar observations, although the drop in activity was naturally more severe. For example, data in Table 26 reveal that % tyrosinase inhibitory activity of pure Puag-Haad solution decreased dramatically at elevated temperature, from 72.93% initially to only 28.16% at 24 weeks, equivalent to about 61% loss in activity. Addition of antioxidants was able to slow down this process as seen in this table and the normalized data in Table 27. From this table, solution P+A4

gave the best protection at both 12 and 24 weeks, with the remaining relative % inhibition values of 80.14% and 59.35%, respectively. The data on relative % inhibition were also plotted as a function of time for visual observation in Figures 19 and 20.

Thus, based on the color observation and relative % inhibitory activity, combination of 0.15% sodium metabisulfite and 0.10% BHA appeared to have synergistic effect in stabilizing 0.25% Puag-Haad solution. Further addition of 0.05% EDTA to make a triple combination (solution P+A7), however, did not result in increased protection against loss of anti-tyrosinase activity. The reasons as to this observation were not understood since the specific chemical interplay among 2,4,3′,5′-tetrahydroxystilbene and different antioxidants are not known and need to be thoroughly investigated.

In addition, the stabilizing effect of sodium metabisulfite could be enhanced if the process of self-degradation to sulfate is delayed since solution P+A4 showed a drop in pH after 12 weeks similar to that of A4 (antioxidants alone). P+A4 was still found to exhibit high anti-tyrosinase activity despite the loss of sodium metabisulfite and concomitant drop in pH.

Table 24 Stability of Puag Haad with and without antioxidants as determined from % tyrosinase inhibitory activity at room temperature. Data = mean ± SD (n = 3)

Time (week)	% Tyrosinase inhibitoty activity							
	P	P+A1	P+A2	P+A3	P+A4	P+A5	P+A6	P+A7
0	72.93	73.24	66.50	69.05	70.25	73.38	65.42	82.21
(SD)	(1.92)	(1.29)	(1.50)	(2.32)	(1.27)	(1.14)	(3.45)	(2.43)
4	60.44	70.69	63.80	63.96	68.39	75.67	64.45	82.26
	(2.42)	(3.01)	(4.27)	(2.32)	(1.58)	(2.96)	(1.45)	(3.22)
8	52.08	67.80	61.21	61.70	67.69	63.82	56.09	76.59
	(2.29)	(1.21)	(2.57)	(4.15)	(1.28)	(3.42)	(0.99)	(1.31)
12	47.62	64.31	55.88	56.36	66.27	60.64	53.97	72.22
	(3.78)	(1.55)	(1.69)	(2.20)	(0.62)	(2.34)	(2.02)	(3.43)
16	50.25	57.24	56.40	57.24	64.11	61.34	55.66	66.90
	(1.41)	(1.45)	(3.03)	(3.18)	(0.85)	(1.71)	(1.93)	(0.50)
24	36.71	46.05	45.53	40.33	56.75	51.18	49.52	56.85
	(2.09)	(0.31)	(1.63)	(1.83)	(0.44)	(1.45)	(0.93)	(0.73)

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

Table 25 Stability of Puag-Haad with and without antioxidants as determined from % tyrosinase inhibitory activity relative to initial value at room temperature. Data = mean ± SD (n = 3)

Time (week)	% Relative tyrosinase inhibitory avtivity							
	P	P+A1	P+A2	P+A3	P+A4	P+A5	P+A6	P+A7
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
4	82.88	96.51	95.95	92.62	97.35	103.11	98.51	100.05
(SD)	(3.32)	(4.11)	(6.42)	(3.37)	(2.25)	(4.03)	(2.21)	(3.92)
8	71.41	92.57	92.05	89.35	96.35	86.97	85.74	93.16
	(3.14)	(1.66)	(3.86)	(6.02)	(1.83)	(4.66)	(1.52)	(1.59)
12	65.30	87.81	84.04	81.62	94.33	82.64	82.49	87.85
	(5.18)	(2.12)	(2.55)	(3.18)	(0.88)	(3.18)	(3.08)	(4.17)
16	68.90	78.15	84.82	82.89	91.25	83.58	85.07	81.38
	(1.94)	(1.98)	(4.55)	(4.61)	(1.21)	(2.33)	(2.96)	(0.61)
24	50.34	62.87	68.46	58.40	80.78	69.74	75.68	69.14
	(2.87)	(0.42)	(2.46)	(2.65)	(0.62)	(1.98)	(1.42)	(0.89)

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

Table 26 Stability of Puag Haad with and without antioxidants as determined from %
tyrosinase inhibitory activity at 45 $^{\circ}$C. Data = mean $\pm$ SD (n = 3)

Time	% Tyrosinase inhibitoty activity							
(week)	P	P+A1	P+A2	P+A3	P+A4	P+A5	P+A6	P+A7
0	72.93	73.24	66.50	69.05	70.25	73.38	65.42	82.21
(SD)	(1.92)	(1.29)	(1.50)	(2.32)	(1.27)	(1.14)	(3.45)	(2.43)
2	63.47	60.71	60.50	62.77	68.13	70.85	61.83	81.29
	(0.74)	(2.07)	(1.36)	(2.01)	(0.98)	(1.83)	(3.83)	(2.19)
4	53.70	58.36	60.45	64.69	68.43	68.32	61.72	80.08
	(2.31)	(1.53)	(1.90)	(2.88)	(3.64)	(6.29)	(5.84)	(1.63)
6	52.66	57.59	57.94	63.33	64.31	69.21	62.53	72.02
	(1.97)	(2.10)	(2.15)	(2.94)	(0.63)	(1.46)	(1.24)	(3.72)
8	52.69	47.35	50.03	56.55	58.69	57.09	59.44	60.35
	(0.98)	(2.22)	(2.25)	(0.74)	(1.03)	(3.52)	(1.90)	(0.37)
10	56.00	48.50	45.21	51.85	54.60	53.38	49.04	55.41
	(0.98)	(0.78)	(2.21)	(2.29)	(1.50)	(1.46)	(1.96)	(2.63)
12	42.95	48.25	45.34	45.91	56.30	49.59	43.33	51.07
	(1.91)	(2.78)	(0.13)	(1.87)	(0.56)	(1.23)	(0.21)	(0.89)
16	45.39	50.67	48.36	49.06	54.42	50.32	45.64	50.63
	(0.69)	(0.49)	(5.35)	(2.53)	(1.81)	(2.50)	(1.96)	(2.03)
24	28.16	37.40	36.54	35.81	41.70	41.52	35.65	41.89
	(0.22)	(1.20)	(1.71)	(0.28)	(1.22)	(0.95)	(1.03)	(2.26)

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05%
EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3

Table 27 Stability of Puag-Haad with and without antioxidants as determined from % tyrosinase inhibitory activity relative to initial value at 45 $^{\circ}$C. Data = mean $\pm$ SD (n= 3)

Time (week)	% Relative tyrosinase inhibitory avtivity							
	P	P+A1	P+A2	P+A3	P+A4	P+A5	P+A6	P+A7
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2	87.03	82.89	90.99	90.90	96.98	96.55	94.50	98.87
(SD)	(1.02)	(2.83)	(2.05)	(2.92)	(1.40)	(2.49)	(5.85)	(2.66)
4	73.63	79.68	90.91	93.67	97.40	93.10	94.33	97.40
	(3.17)	(2.09)	(2.86)	(4.17)	(5.17)	(8.57)	(8.92)	(1.98)
6	72.21	78.63	87.13	91.71	91.54	94.31	95.58	87.61
	(2.70)	(2.87)	(3.24)	(4.26)	(0.90)	(1.99)	(1.90)	(4.53)
8	72.25	64.65	75.23	81.90	83.55	77.80	90.85	73.40
	(1.34)	(3.04)	(3.39)	(1.07)	(1.47)	(4.80)	(2.91)	(0.45)
10	76.79	66.22	67.99	75.09	77.72	72.75	74.96	67.40
	(1.35)	(1.07)	(3.32)	(3.31)	(2.13)	(1.99)	(3.00)	(3.19)
12	58.90	65.87	68.18	66.48	80.14	67.58	66.23	62.12
	(2.62)	(3.79)	(0.19)	(2.71)	(0.80)	(1.68)	(0.33)	(1.08)
16	62.23	69.17	72.73	71.04	77.47	68.57	69.76	61.59
	(0.94)	(0.67)	(8.04)	(3.66)	(2.58)	(3.41)	(2.99)	(2.47)
24	38.61	51.06	54.95	51.86	59.35	56.58	54.49	50.95
	(0.30)	(1.63)	(2.57)	(0.41)	(1.74)	(1.29)	(1.58)	(2.74)

P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3
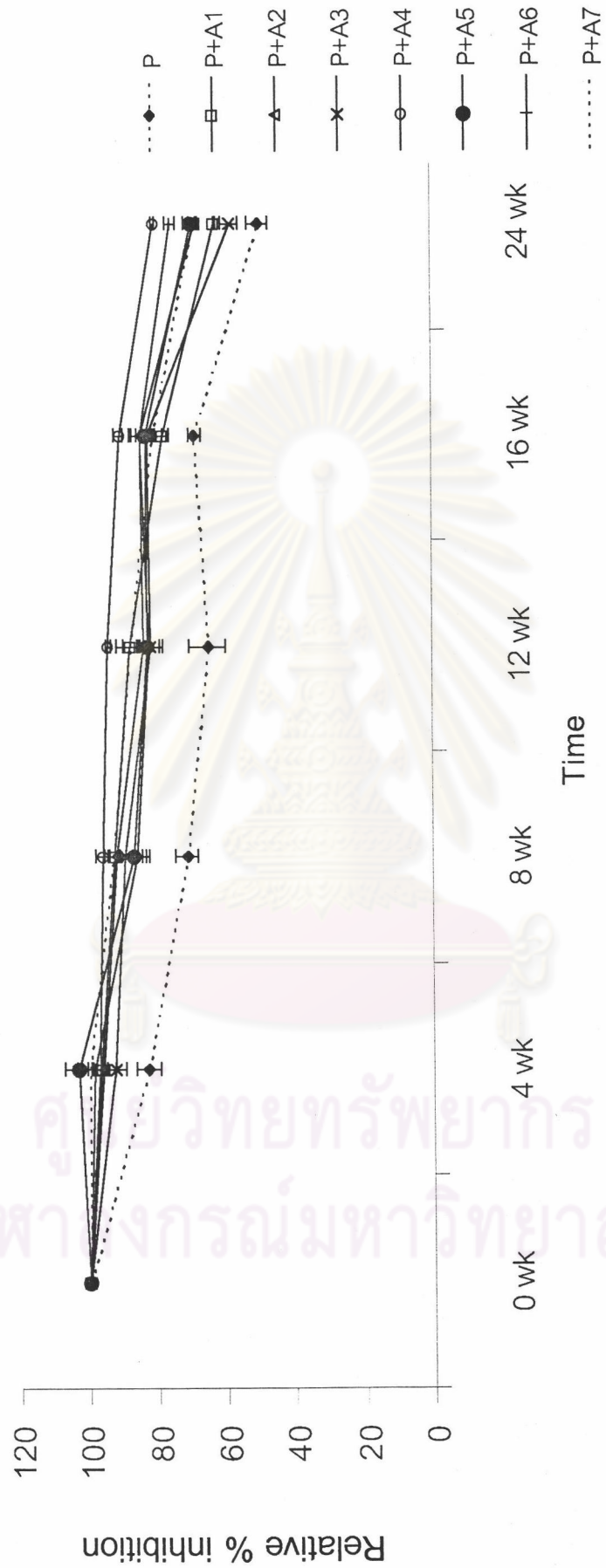
Figure 19 Plots of % tyrosinase inhibitory activity (relative to initial value) remaining after storage at room temperature up to 24 weeks. Each point represents mean ± SD (n = 3). P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3
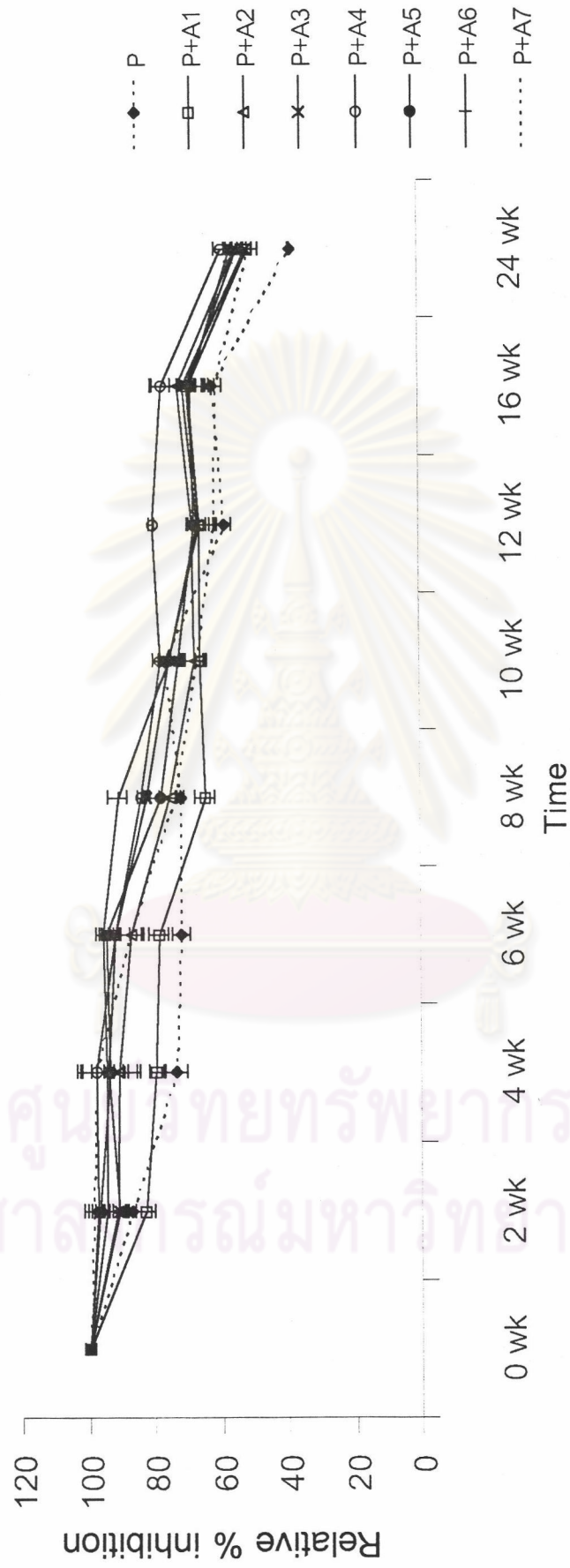
Figure 20 Plots of % tyrosinase inhibitory activity (relative to initial value) remaining after storage at 45 °C up to 24 weeks. Each point represents mean ± SD (n = 3). P = 0.25% Puag-Haad, A1 = 0.15% sodium metabisulfite, A2 = 0.10% BHA, A3 = 0.05% EDTA, A4 = A1+A2, A5 = A1+A3, A6 = A2+A3, A7 = A1+A2+A3