

CHAPTER IV



RESULTS AND DISCUSSION

Part 1. Evaluation of skin whitening efficacy of aqueous extract of *Artocarpus lakoocha* heartwood (PuagHaad), niacinamide, lactic acid, and their combinations in guinea pigs

1.1 Effect of UVB induced hyperpigmentation in guinea pigs

To accelerate the skin darkening, the back of each guinea pig was irradiated under two fluorescent UVB lamps 290-320nm (model TL 20w/12, Phillips), at 0.3 mW/cm² for 50 min/day (total energy, 900 mJ/cm²/day) for 3 consecutive days. This procedure was suggested by Imokawa and co-worker [42] that the daily exposure of brownish-black guinea pigs skin to UVB irradiation at this energy for 3 successive days induced clearly visible black pigmentation within 3 days and the maximum effect being reached about 21 days after irradiation without particular irritation.

The data illustrated in Table 7 coincide with that of Imogawa [42]. It can be seen that the absolute melanin (M) value went upward from week -3 to week 0 in all groups. For example, the mean M value increased from 689.9 to 756.4 in group A, from 681.2 to 738.0 in group B, from 730.6 to 760.5 in group C, from 721.9 to 775.1 in group D, and from 686.5 to 744.2 in group E. Also, the absolute erythema (E) values slightly changed after irradiation; for example, the mean E value of group A changed from 576.2 to 579.9 (Table 8). However, the extent of change was much smaller than the M values in all groups and was thus considered negligible. The E values even slightly decreased after UVB exposure as seen in group D and group E, indicating that the irradiation process was non-irritating.

Thereafter, the test solutions were applied on the back of guinea pigs since the melanin values had reached the peak level at 18 days after the last UVB exposure. The M and E values were measured immediately before the first application (= week 0) and again at a weekly interval until 4 weeks.

Table 7. The absolute melanin values (mean \pm SD) in guinea pigs treated with different whitening agents. The initial values before UVB-induced pigmentation (week -3) are also given for comparison. The values at week 0 = the values taken 18 days after the last UVB exposure and before application of the substances.

Treatment group	Absolute melanin values (M)						
		Before UVB	Week 0	Week 1	Week 2	Week 3	Week 4
A (n = 6) 0.25% Puag-Haad	PG-control	689.9 \pm 24.65	756.4 \pm 15.32	737.9 \pm 23.11	728.0 \pm 18.72	718.4 \pm 22.30	709.7 \pm 21.80
	A	689.9 \pm 24.65	750.4 \pm 14.27	732.1 \pm 23.83	714.1 \pm 17.54	695.0 \pm 19.33	690.8 \pm 19.34
	P-value	-	0.116084	0.159617	0.004235*	0.000107*	0.00033*
B (n = 6) 1% Niacinamide	PG-control	681.2 \pm 50.19	738.0 \pm 18.17	728.2 \pm 6.93	721.8 \pm 4.58	699.1 \pm 10.06	697.1 \pm 10.91
	B	681.2 \pm 50.19	736.0 \pm 12.84	707.7 \pm 12.41	694.3 \pm 14.00	676.6 \pm 11.35	673.6 \pm 12.26
	P-value	-	0.596277	0.029059*	0.010037*	0.001395*	0.009568*
C (n = 6) 1% Lactic acid	PG-control	730.6 \pm 38.54	760.5 \pm 41.54	738.9 \pm 44.33	727.0 \pm 32.29	711.1 \pm 36.25	710.8 \pm 38.85
	C	728.1 \pm 34.05	752.2 \pm 35.12	729.3 \pm 49.89	717.8 \pm 47.21	694.8 \pm 34.15	687.1 \pm 34.13
	P-value	-	0.278017	0.062316	0.381005	0.002288*	0.010719*

Treatment group	Absolute melanin values (M)						
		Before UVB	Week 0	Week 1	Week 2	Week 3	Week 4
D (n = 6) 0.25% Puag-Haad +1%Niacinamide	PG-control	721.9 ± 43.11	775.1 ± 30.02	764.1 ± 26.88	759.7 ± 23.91	752.9 ± 31.01	753.5 ± 27.44
	D	720.4 ± 42.68	771.4 ± 24.65	753.2 ± 23.04	740.8 ± 24.79	726.3 ± 28.32	719.2 ± 20.99
	P-value	0.572628	0.303376	0.028036*	0.012020*	0.000034*	0.001891*
E (n = 5) 0.25% Puag-Haad +1%Niacinamide+ 1% Lactic acid	PG-control	686.5 ± 22.49	744.2 ± 13.26	738.3 ± 35.55	744.0 ± 42.29	732.9 ± 45.94	731.4 ± 48.45
	E	688.4 ± 22.96	746.9 ± 16.53	733.2 ± 33.98	721.3 ± 38.26	703.5 ± 45.42	702.7 ± 40.15
	P-value	0.547298	0.482950	0.242597	0.013018*	0.002411*	0.011251*

*denotes significant difference between the treated and control areas by paired student's t-test.

Table 8. The absolute erythema values (mean \pm SD) in guinea pigs treated with different whitening agents. The initial values before UVB-induced pigmentation (week -3) are also given for comparison. The values at week 0 = the values taken 18 days after the last UVB exposure and before application of the substances.

Treatment group	Absolute erythema values (E)						
		Before UVB	Week 0	Week 1	Week 2	Week 3	Week 4
A (n = 6) 0.25% Puag-Haad	PG-control	576.2 \pm 4.52	579.9 \pm 13.56	577.8 \pm 9.78	581.0 \pm 11.89	579.2 \pm 8.95	594.0 \pm 5.95
	A	576.2 \pm 4.52	579.9 \pm 13.45	579.0 \pm 11.07	582.1 \pm 14.87	581.3 \pm 8.71	592.5 \pm 8.43
	P-value	-	0.99047	0.374702	0.721607	0.116183	0.588059
B (n = 6) 1% Niacinamide	PG-control	573.1 \pm 10.54	573.7 \pm 14.17	579.6 \pm 7.71	584.2 \pm 11.79	577.2 \pm 8.59	590.6 \pm 9.36
	B	573.1 \pm 10.54	570.1 \pm 12.91	578.4 \pm 8.33	577.8 \pm 10.22	575.4 \pm 14.60	585.6 \pm 8.16
	P-value	-	0.074932	0.460486	0.010495*	0.724731	0.224649
C (n = 6) 1% Lactic acid	PG-control	571.6 \pm 9.86	591.2 \pm 13.39	592.3 \pm 9.99	589.2 \pm 6.65	586.8 \pm 13.09	597.4 \pm 15.40
	C	570.9 \pm 9.13	589.4 \pm 13.18	591.3 \pm 10.17	590.2 \pm 5.41	587.1 \pm 8.09	596.0 \pm 16.51
	P-value	-	0.206303	0.689472	0.311018	0.944474	0.518613

Treatment group	Absolute erythema values (E)						
		Before UVB	Week 0	Week 1	Week 2	Week 3	Week 4
D (n = 6) 0.25% Puag-Haad + 1% Niacinamide	PG-control	582.8 ± 8.42	576.3 ± 4.89	584.3 ± 11.46	578.7 ± 11.59	579.3 ± 8.81	582.5 ± 13.81
	D	583.1 ± 11.94	575.5 ± 10.40	582.7 ± 10.27	578.8 ± 8.01	569.5 ± 12.97	577.0 ± 13.22
	P-value	-	0.88344	0.105731	0.980037	0.027680*	0.225127
E (n = 5) 0.25% Puag-Haad + 1% Niacinamide + 1% Lactic acid	PG-control	579.3 ± 8.27	570.6 ± 16.65	584.7 ± 7.85	580.6 ± 10.05	574.2 ± 15.49	580.8 ± 20.54
	E	583.5 ± 10.52	572.7 ± 14.53	581.1 ± 12.96	581.5 ± 10.47	573.6 ± 21.91	581.4 ± 19.16
	P-value	-	0.224649	0.224649	0.224649	0.224649	0.224649

*denotes significant difference between the treated and control areas by paired student's t-test.

1.2 Determination of melanin and erythema values in guinea pigs

After the guinea pigs in each group had been applied with propylene glycol (control) on their left flank and the test solution on their right flank, they were measured with Mexameter (5 readings on each area) at every week. The absolute M and E values of the individual guinea pigs (mean \pm SD of 5 readings) in each group are shown in Tables 1 and 2 of Appendix I whereas the group averages are shown in Tables 7 and 8. The *P*-values of the paired t-test at 5% significance level are also provided in these tables to facilitate comparison between the control and the treated areas within in each animal at each week. The melanin reduction was obtained by subtracting the M values of the control area with that of the treated area ($X_c - X_{tr}$). The change in erythema (E) was also obtained using the same formula $E_c - E_{tr}$.

The data in Tables 7 and 8 showed that the initial absolute M and E values at week 0 between the control and the treated areas did not significantly differ in all groups ($p > 0.05$), indicating that the two areas (flanks) of each animal had the same starting values. After application with the test solutions on the right flank and propylene glycol (control) on the left flank, it can be observed that the absolute M value gradually declined from the initial period in both areas of the animals. The decrease in the M value observed with the control area might result from the natural keratinization and desquamation of the skin. Nevertheless, the side applied with the control solvent seemed to whiten slower than the treated side. For example, the average M values of 0.25% Puag-Haad treated-area (group A in Table 7) reduced from 750.4 at week 0 to 732.1 at week 1, 714.1 at week 2, 695.0 at week 3, and down to 690.8 at week 4. On the other hand, the control area of this group receiving only propylene glycol merely decreased from 756.4 at week 0 to 709.7 at week 4. Significant difference between the two areas in this group was noticed at week 2 and afterwards until week 4 ($P < 0.05$).

The mean M values of 1% niacinamide treated-area (group B) reduced from 736.0 at week 0 to 707.7 at week 1, 694.3 at week 2, 676.6 at week 3, and down to 673.6 at the 4th week. The statistical difference between the two areas had been detected since the 1st week until week 4. In group C, the mean M values of 1% lactic acid treated-area reduced from 752.2 at week 0 to 729.3 at week 1, 717.8 at week 2,

694.8 at week 3, and down to 687.1 at week 4. The statistical difference had been detected since the 3rd week. For the combination groups, the area treated with 0.25% Puag-Haad and 1% niacinamide (group D) initially declined from 771.4 at week 0 to 753.2, 740.8, 726.3 and 719.2 at week 1, 2, 3 and 4, respectively. The double combination had demonstrated statistically significant difference between the control and the treated areas since week 1 similar to group B ($P < 0.05$). On the other hand, animals treated with triple combination containing 0.25% Puag-Haad, 1% niacinamide, and 1% lactic acid exhibited a decline in the M values from 746.9 at week 0 to 733.2 at week 1, 721.3 at week 2, 703.5 at week 3, and down to 702.7 at week 4. Statistical difference between the control and the treated areas was detected at 2nd week and afterwards similar to group A.

Thus, based on the current observations, the test solutions containing niacinamide alone (B) and in combination with Puag-Haad (D) appeared to show the fastest whitening activity, with significant effect over their self-controls detected as early as 1 week after application. Group A (Puag-Haad) and group E (Puag-Haad + niacinamide + lactic acid) were the next fastest group, showing significant whitening at week 2 and onward. Group C (lactic acid alone), however, gave the slowest onset of significant activity observed at week 3 after application.

Niacinamide or vitamin B3 was reported to have vasodilating effect as well as tyrosinase inhibitory activity. Recently, it has been reported that it may reduce melanin content in the skin by inhibiting the transfer of melanosomes from the melanocyte to keratinocyte [34]. Clearly, niacinamide was the most rapid whitening substance observed in this study. Its combination with Puag-Haad (group D) might have accelerated the onset of pure Puag-Haad (group A) from 2 weeks to 1 week. On the other hand, lactic acid (group C) possessed whitening effect via the stimulation of keratinization or epidermal turnover, which normally takes about 2 weeks. Thus, the effect may have taken a little longer than other whitening agents, resulting in a slightly slower onset observed at week 3. The triple combination (group E), however, was somewhat more difficult to explain. It contained all three ingredients, i.e., Puag-Haad, niacinamide and lactic acid. Since the double combination of Puag-Haad and lactic acid was not tested in this study, the reasons as to the intermediate onset of whitening activity (2 weeks) observed with group E were not clearly known. It is possible,

however, that the high acidic pH value of the solution E due to the presence of lactic acid may not be suitable for the whitening activity of Puag-Haad and niacinamide, which have mechanisms of action different from lactic acid.

1.3 Comparison of the melanin content in brownish black guinea pigs

To compare the inhibitory effect of each substance more clearly, the melanin reduction values were used. The mean values of melanin reduction ($X_c - X_{tr}$) of each whitening agent are shown in Table 9 and graphically represented in Figure 18. One-way ANOVA was applied to compare the whitening efficacy ($X_c - X_{tr}$) among the different groups at 5% significance level. Only the groups, which had previously demonstrated significant whitening activity over their self-control by paired t-test were tested. Thus ANOVA was applied on the data of the first week comparing only group B and D. At week 2, groups A, B, D and E were compared whereas all the five groups (A, B, C, D and E) were compared at weeks 3 and 4.

ANOVA results did not show any significant differences among the different treatment groups at all weeks ($P > 0.05$). This suggested that, once effective, all the test substances whether alone or in combination did not significantly differ in their whitening activities. However, a closer look at the data revealed subtle differences in the whitening activity among different groups. For example, at week 4 the double combination (group D) gave the highest whitening efficacy, with the mean melanin reduction of 34.33. The relative ranking of $X_c - X_{tr}$, in a decreasing order, was D (34.33) > E (28.72) > C (23.68) ~ B (23.5) > A (18.87). Thus, the combination of two or three whitening agents might have a tendency to produce a slightly greater whitening effect than a single component although significance was not detected in this study.

Table 9. The melanin reduction after application of the test substances

	0 week	1st week	2nd week	3rd week	4th week
Puag-Haad 0.25% (A)	6.02 ±7.76	5.8 ±8.6	13.9* ±6.85	23.43* ±5.2	18.87* ±5.3
Niacinamide 1% (B)	1.98 ±8.82	20.5* ±16.56	27.47* ±16.7	22.5* ±8.63	23.5* ±14.11
Lactic acid 1% (C)	8.35 ±16.81	9.62 ±9.85	9.17 ±23.38	16.28* ±6.97	23.68* ±14.64
Puag-Haad 0.25% + Niacinamide 1% (D)	3.65 ±7.79	10.9* ±8.71	18.95* ±12.06	26.62* ±4.65	34.33* ±14.09
Puag-Haad 0.25% + Niacinamide 1% + Lactic acid 1% (E)	-2.78 ±7.28	5.12 ±7.76	22.72* ±14.12	29.4* ±14.77	28.72* ±17.43
p-value	-	0.2377	0.3100	0.5570	0.3250
		nonsig	nonsig	nonsig	nonsig

** denotes significant difference between treated and control by paired student's t-test
p-value obtained from one-way ANOVA comparing only groups which had shown significance after paired t-test.

Values = mean ± SD (n = 5-6 guinea pigs/group).

Table 10. The erythema change after application of the test substances

	0 week	1st week	2nd week	3rd week	4th week
Puag-Haad 0.25% (A)	0.05 ±9.74	-1.2 ±3.01	-1.03 ±6.71	-2.07 ±2.66	1.5 ±6.35
Niacinamide 1% (B)	3.58 ±3.91	1.17 ±3.57	6.4* ±3.93	1.8 ±11.83	5.03 ±8.9
Lactic acid 1% (C)	1.83 ±3.09	1 ±5.78	-1.03 ±2.24	-0.3 ±10.03	1.38 ±4.88
Puag-Haad 0.25% + Niacinamide 1% (D)	0.78 ±12.43	1.6 ±1.98	-0.07 ±6.2	9.82* ±7.82	5.52 ±9.76
Puag-Haad 0.25% + Niacinamide 1% + Lactic acid 1% (E)	-2.18 ±4.54	3.58 ±5.91	-0.88 ±3.36	0.56 ±6.55	-0.6 ±4.38
p-value	-	-	-	-	-

*denotes significant difference between the treated and the control areas by paired student's t-test.

p-value obtained from one-way ANOVA comparing only groups which had shown significance after paired t-test.

Values = mean ± SD (n = 5-6 guinea pigs/group).

It is interesting to note that the double combination group D (0.25% Puag-haad and 1% niacinamide) gave higher whitening efficacy than the triple combination group E (0.25% Puag-haad + 1% niacinamide + 1% lactic acid). This behavior had been previously observed with the onset time, where group D was also found to give faster effect than group E. As previously discussed, the presence of lactic acid may have reduced the pH of the solution E such that the whitening activity of niacinamide and Puag-Haad were somewhat compromised. However, more studies are needed to clarify this observation.

The results for niacinamide and its double combination appeared to agree with that of Barratt et al. [33]. They reported the evidence that combination of 1% niacinamide and sunscreen product (1.25% parsol MCX, 0.4% parsol 1789) could lead to the lightening of the basal skin color [33]. Similarly, Hakozaiki et al. [33] found that niacinamide plus sunscreen produced superior skin whitening efficacy than the sunscreen alone. Although niacinamide did not show much inhibition of melanogenic activity via the tyrosinase-limiting enzyme [34], it may inhibit melanogenesis by interfering with the melanosome transfer [33]. Since the melanin reduction value at week 4 of niacinamide (23.5) was higher than Puag-Haad (18.87), it was probable that the inhibition of melanogenesis via the melanosome transfer might be more potent than tyrosinase inhibition. However, more experiments are needed to prove this hypothesis as the concentration of niacinamide used in this study (1%) was much higher than 0.25% Puag-Haad.

Also, it should be noticed that 1% lactic acid, although appeared to give the slowest onset of whitening activity, was able to produce substantial whitening effect at week 4 (23.68), which was very close to that of 1% niacinamide (23.5). This result suggested that lactic acid was also a very effective whitening agent. Its major mechanism is by stimulation of the epidermal turnover, thereby accelerating the formation of new layers of epidermis with less melanin pigmentation. Since the process usually takes some time, this may have resulted in the somewhat slower onset of activity as previously observed.

However, it should be pointed out that the concentration of lactic acid used in this study was relatively low. Only 1% was employed whereas in the commercial

preparations, concentration of 5% or more is usually present. At higher concentration, the effect of lactic acid on the skin will definitely intensify. In particular, the exfoliative action will increase, which could result in rapid sloughing of the surface epithelium. In the preliminary study, 5% lactic was first employed for application to guinea pigs. However, the animals experienced extensive skin exfoliation, suggesting that 5% solution was too caustic to their skin. Some of the animals died within one month of application. Their death might have been associated with the high concentration of lactic acid though the specific causes were not known. Thus, the concentration was reduced to 1% in this study. It is possible that using a slightly higher concentration of lactic acid (e.g. 2 - 2.5 %) may have resulted in a further increase in the rate and extent of whitening efficacy, which could be better than 1% niacinamide. Indeed, Rojanadilog [46], who studied the efficacy of several commercially available brands of skin whitening lotions, found that the product containing lactic acid (8%) apparently gave greater whitening effect than the one containing niacinamide (1%).

0.25% Puag-Haad was also found to be an effective skin whitening agent. Its mechanism was by inhibition of the tyrosinase enzyme [3,27]. In this study, 0.25% Puag-Haad was able to produce significant whitening effect only after 2 week-application, with the mean melanin reduction of 13.9. This result was also in agreement with Pengrungruangwong [27], who reported that 0.5% Puag-Haad solution in propylene glycol produced significant whitening effect in guinea pigs after the same period of application. Addition of niacinamide to Puag-Haad was found to cause further increase in the whitening activity at all weeks (Table 9). However, the effect was not greater than niacinamide alone, particularly at week 1 and 2. In fact, the values of the combination (group D) were even less than that of niacinamide alone (group B). No explanation could be offered at present. Nevertheless, at week 3 and 4 the value of group D became greater than group B, suggesting an increase in whitening efficacy after combination of the two substances.

In summary, all the five whitening agents were effective in significantly reducing the melanin content when compared to their self-control. Despite non-significance in the extent of melanin reduction among the five groups at week 3 and 4,

subtle differences in the rate and extent of whitening appeared to exist. This could be a result of differences in their mechanisms of action. 1% Niacinamide, which acts as a vasodilator, weak tyrosinase inhibitor, and probably inhibitor of melanosome transfer [33], appeared to produce the fastest onset of whitening, followed by 1% lactic acid, which is a skin rejuvenating agent [15], and 0.25% Puag-Haad, a potent tyrosinase inhibitor [3]. However, the above ranking could be greatly influenced by the concentration of the individual agents. Combination of these agents produced a further increase in whitening extent at the end of 4-week study but the increase was not significant.

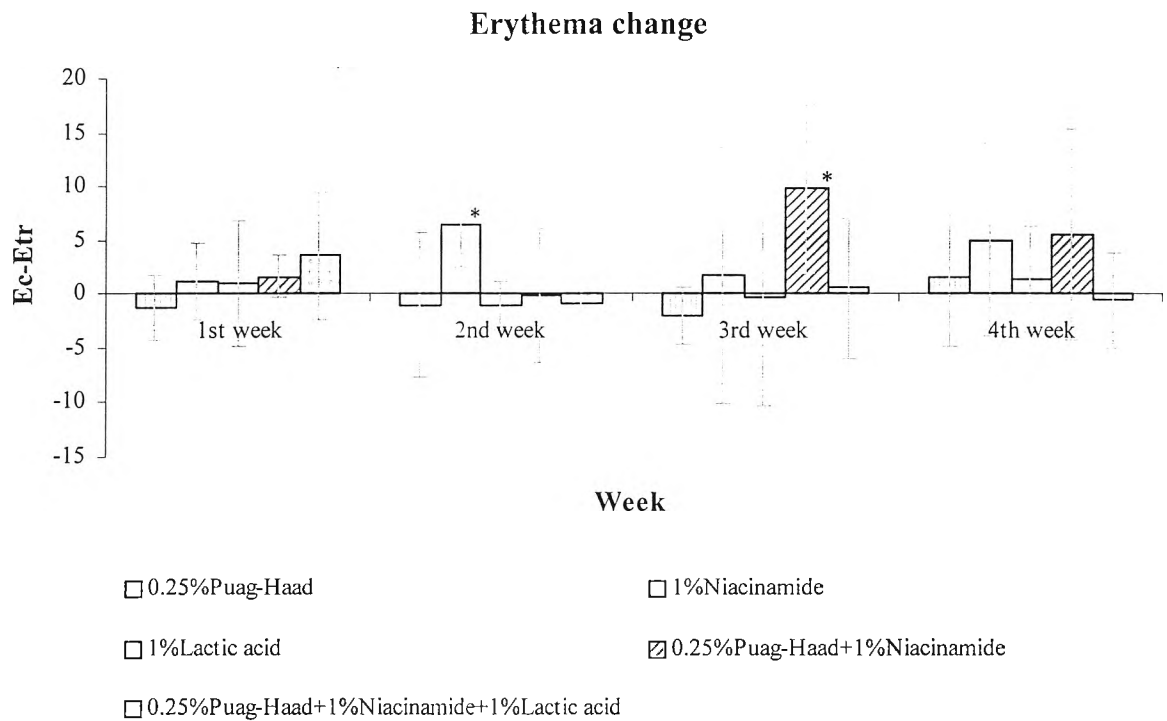


Figure 19. Comparison of erythema change (Ec-Etr) of each test substance at each week. Data = mean \pm SD (n = 5-6 guinea pigs/group)

*denotes significant difference between the treated and the control areas by paired student's t-test.

1.4 Comparison of the skin erythema in black guinea pigs

From the data in Table 8, for assessment of the erythema change, statistical significance between the control and treated areas was found in only week 2 of 1% niacinamide (group B) and week 3 of 1% niacinamide + 0.25% Puag-Haad (group D). The absolute erythema (E) value reduced from 584.2 to 577.8 and from 579.3 to 569.5, respectively. However, most of the data did not show any significant differences in the E values between the control and the treated areas regardless of the test substance and the week of application. This observation suggested that all the five treatments and their concentrations employed in this study were well tolerated by the guinea pigs. No serious signs of skin disorders were visually observed such as contact dermatitis and skin lesions. A few guinea pigs developed minor scaling of skin, especially in groups C and E. This could be due to the mild exfoliative action of 1% lactic acid. However, the scaling diminished within a week and did not affect the melanin and erythema measurements.

Part 2 Formulation of the skin whitening lotion

2.1 Determination of physical appearance of lotion base

The physical appearance of each formulation is described in table 11.

Table 11. The physical appearance of lotion base formulations

Formulation	Appearance	After 2 weeks
Guide formulation	White, thick, greasy cream	White, thick, greasy cream
1	White, fluid, smooth lotion	White, more fluid, unstable lotion
2	White, fluid, smooth lotion	White, more fluid, unstable lotion
3	White, fluid, smooth lotion	White, more fluid, stable lotion
4	White, thick, greasy lotion	White, thick, greasy lotion
5	White, thick, greasy lotion	White, thick, greasy lotion
6	White, fluid, smooth lotion	White, more fluid, unstable lotion
7	White, fluid, smooth lotion	White, more fluid, unstable lotion
8	White, fluid, smooth lotion	White, fluid, smooth lotion
9	White, fluid, smooth lotion	White, fluid, smooth lotion
10	White, fluid, smooth lotion	White, fluid, smooth lotion
11	White, fluid, greasy lotion	White, thick, greasy lotion

2.2 Selection of the skin whitening lotion

Formulation 8 was selected as the best lotion base for incorporation of the test whitening agents. The finished formulations are presented in Table 12.

Table 12. Composition of the test whitening lotions (% w/w)

		Common Ingredient	A	B	C	D	E
A	Finsolve TN	3					
	Lanette O (cetearyl alcohol)	0.8					
	Cutina KD 16 (Glyceryl monostearae SE)	1					
	Cetereth-20	0.5					
	Emulgrad NI 1000	2.3					
	Myristol 318	3					
	Dimethicone	1					
	Jjoba oil	0.5					
	Vit-E acetate	0.5					
B	Deionized water	81.64	81.64	80.64	79.14	80.64	79.14
	Glycerin	2					
	Propyleneglycol	3	0.5			0.5	0.5
	Sodium EDTA	0.1					
	Glydant plus	0.2					
	Niacinamide			1		1	
	Tranexamic acid				2.5		2.5
C	BHA	0.01					
	Sodium metabisulfite	0.15					
	Perfume	0.3					
	Puag-Haad extract		0.25			0.25	0.25

A = Puag-Haad 0.25% lotion

B = Niacinamide 1% lotion

C = Tranexamic acid 2.5% lotion

D = Puag-Haad 0.25% + Niacinamide 1%

E = Puag-Haad 0.25% + Tranexamic acid 2.5%

All formulations had pH 5.5 and were freshly prepared every 2 weeks with each substance according to the procedure previously described in Chapter III.

Part 3 Evaluation of skin whitening efficacy of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad), niacinamide, tranexamic acid, and their combinations in human volunteers

3.1 Determination of melanin (M) and erythema (E) values in human volunteers

Eighty-four subjects participated in this study to confirm the efficacy and safety of Puag-Haad and its combination with other whitening agents using the same study design (self-control) as in the guinea pigs. The outer areas of both upper arms of each subject were separately applied with 0.2 ml of either lotion base (control) or one of the lotions. The assignment of the treatments (control or test lotion containing whitening agents) to the right or left arms was carefully balanced to reduce the variation from different sides of upper arms as previously described.

The volunteers were divided into 6 groups of 14 and obtained different test lotions. Group A received lotion containing only 0.25% Puag-Haad where as group B and C respectively received lotion containing 1% niacinamide and 2.5% tranexamic acid. Group D received double combination lotion containing 0.25% Puag-Haad + 1% niacinamide where as group E received lotion containing 0.25% Puag-Haad + 2.5% tranexamic acid. The last group (F) received a commercial whitening lotion. Details of active ingredients of the commercial product F are given in Appendix II

Since addition of lactic acid to the mixture of Puag-Haad and niacinamide failed to produce an additive whitening effect in the guinea pigs study, it was excluded from the study in human volunteers. Alternatively, tranexamic acid was chosen in place of lactic acid for evaluation of its potential whitening effect. Tranexamic acid is one of the prostaglandin suppressors. Maeda and Naganuma (1998) reported that it reduced the melanocyte tyrosinase activity by suppressing the UV-induced production of prostaglandins, and thus melanogenesis [38,39].

The first seven subjects in each group applied lotion base as a control on their left upper arms and the test lotion on their right upper arms. An opposite order of application was used on the rest. Subsequently, they were measured with Mexameter (5 readings per arm) at a 2-week interval until 12 weeks. The mean absolute M and E values of the six treatment groups are provided in Tables 13 and 14 whereas the individual values (mean \pm SD of 5 readings in each volunteer) are given in Appendix II.

At week 0, all groups of subjects started with the same skin color on both left and right upper arms as there was no statistical difference in the absolute M values after paired t-test ($P > 0.05$). Surprisingly, paired t-test also yielded the same non-significance between the control and the treated arms at every week up to week 10 in all groups. Closer examination of the data in Table 13 revealed that the absolute M values hardly changed with time during the first 10 weeks in all of these groups, be it the control or the treated arms. For instance, the average M values of the arms treated with 0.25% Puag-Haad (group A) moderately changed from 506.34 at zero week to 504.31 at 4th week, 502.47 at 8th week, 502.27 at 10th week before slightly dropping down to 498.66 at week 12. Similarly, the M value of the arms treated with 1% niacinamide (group B), 2.5% tranexamic acid (group C), 0.25% Puag-Haad + 1% niacinamide (group D), and commercial product (group F) also remained the same over the first 10 weeks. Also, a slight but marked drop in the M value was observed during the last week (week 12) in all of these groups.

For example, the absolute M value of 1% niacinamide treated-arms (group B) hardly showed any changes during the first 10 weeks, with the mean value of 499.67, 499.80, 502.07, and 500.69 at 0, 4th, 8th and 10th week, respectively, before dropping down to 495.13 at 12th week. Similarly, the M value in the treated arms of group C stayed relative unchanged (512.06 - 511.89) from week 0 - 8, slightly dropped to 508.92 at week 10 and then further decreased to 505.77 at week 12. The M value of group D also remained constant during the first 10 weeks (503.50 - 501.64) before slightly dropped to 498.49 at week 12. The subjects treated with commercial product (group F) also showed minimal changes in the M value during the first 10 weeks (507.6 - 505.35) before slightly dropping down to 502.25 at week 12.

Plots of average absolute M values as a function of time are shown in Figures 20-25 to compare the melanin profiles between the control and the test lotions of each group. As with the data in Table 13, these Figures reveal that all the six treatments (A, B, C, D, E and F) failed to produce significant whitening over their self-controls during the first 10 weeks ($P > 0.05$). Significance was observed only at week 12 and only for groups A, B, C, D and F. Group E (0.25% Puag-Haad + 2.5% tranexamic acid), unexpectedly failed to show significant whitening effect at all periods. Its M value marginally decreased from 512.41 at zero week to 508.68 at 8th week and remained constant afterwards until 12 week (506.18), which was not significantly different from the self-control (506.49) ($P > 0.05$).

Table 13. The absolute melanin values (mean \pm SD) in the upper arms of human volunteers treated with different substance for 12 weeks (n= 13-14/treatment group)

Treatment group	Absolute melanin values (M)							
		Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
A (n = 14) 0.25% Puag-Haad	Lotion base	505.63 \pm 24.39	504.48 \pm 22.36	505.33 \pm 23.42	501.79 \pm 21.78	503.59 \pm 21.83	504.34 \pm 20.98	503.54 \pm 20.94
	A	506.34 \pm 24.63	503.99 \pm 21.40	504.31 \pm 23.28	503.06 \pm 23.73	502.47 \pm 21.56	502.27 \pm 19.94	498.66 \pm 18.44
	P-value	0.592984	0.739240	0.432105	0.362180	0.236228	0.190571	0.004802*
B (n = 14) 1% Niacinamide	Lotion base	499.97 \pm 16.78	500.73 \pm 19.63	499.13 \pm 15.83	500.36 \pm 20.19	501.69 \pm 19.64	502.47 \pm 19.51	499.57 \pm 17.72
	B	499.67 \pm 17.33	501.17 \pm 14.88	499.80 \pm 15.34	498.99 \pm 18.38	502.07 \pm 17.70	500.69 \pm 16.98	495.13 \pm 15.75
	P-value	0.766317	0.333123	0.617107	0.476470	0.833688	0.256857	0.022698*
C (n = 13) 2.5% Tranexamic Acid	Lotion base	519.04 \pm 29.61	519.74 \pm 31.16	509.67 \pm 22.49	510.2 \pm 24.31	514.48 \pm 26.98	511.58 \pm 23.72	510.68 \pm 21.27
	C	512.06 \pm 19.57	511.74 \pm 20.29	510.26 \pm 19.78	509.18 \pm 21.68	511.89 \pm 35.40	508.92 \pm 20.26	505.77 \pm 20.76
	P-value	0.354945	0.294631	0.703466	0.515434	0.287883	0.155249	0.000667*

Treatment group	Absolute melanin values (M)							
		Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
D (n = 14) 0.25% Puag-Haad + 1% Niacinamide	Lotion base	506.31 ± 15.44	503.64 ± 16.22	503.96 ± 15.57	503.41 ± 15.72	504.36 ± 16.23	503.09 ± 13.81	503.41 ± 15.89
	D	503.50 ± 15.57	503.59 ± 15.87	504.39 ± 14.98	502.23 ± 19.47	501.78 ± 16.85	501.64 ± 15.92	498.49 ± 15.97
	P-value	0.055050	0.974686	0.739954	0.523414	0.075907	0.396776	0.001965*
E (n = 13) 0.25% Puag-Haad + 2.5% Tranexamic acid	Lotion base	511.57 ± 24.92	510.74 ± 24.52	517.94 ± 30.77	506.83 ± 24.14	506.60 ± 22.21	506.12 ± 23.08	506.49 ± 22.57
	E	512.41 ± 26.21	512.97 ± 25.37	511.44 ± 24.20	507.42 ± 24.72	508.68 ± 24.29	506.29 ± 22.72	506.18 ± 22.18
	P-value	0.583633	0.053196	0.342417	0.657221	0.144088	0.865266	0.706048
F (n = 13) Commercial product	Lotion base	507.44 ± 20.35	507.42 ± 18.27	506.82 ± 18.28	507.55 ± 19.90	507.42 ± 16.02	506.54 ± 16.74	506.6 ± 15.38
	F	507.60 ± 17.63	508.66 ± 12.81	507.32 ± 12.78	505.72 ± 15.35	505.62 ± 13.06	505.35 ± 12.67	502.25 ± 12.07
	P-value	0.925883	0.566651	0.802364	0.473625	0.339611	0.564561	0.022569*

*denotes significant difference between the treated and control arms by paired student's t-test.

Table 14. The absolute erythema values (mean \pm SD) in the upper arms of human volunteers treated with different substance for 12 weeks (n=13-14/treatment group)

Treatment group	Absolute erythema values (E)							
		Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
A (n = 14) 0.25% Puag-Haad	Lotion base	596.19 \pm 23.25	592.51 \pm 23.60	592.64 \pm 25.13	593.40 \pm 19.43	594.47 \pm 24.98	589.43 \pm 21.83	590.61 \pm 20.81
	A	599.60 \pm 21.55	593.22 \pm 20.14	593.47 \pm 23.51	597.16 \pm 20.95	594.96 \pm 21.41	589.36 \pm 20.83	584.37 \pm 16.54
	P-value	0.108093	0.737751	0.731911	0.162295	0.806122	0.975554	0.039516*
B (n = 14) 1% Niacinamide	Lotion base	603.90 \pm 22.68	601.53 \pm 20.89	593.14 \pm 15.65	594.61 \pm 22.53	598.99 \pm 16.77	588.66 \pm 19.04	591.76 \pm 17.50
	B	597.11 \pm 17.86	596.19 \pm 16.87	595.31 \pm 14.12	594.04 \pm 19.89	596.99 \pm 15.45	588.97 \pm 16.09	587.96 \pm 18.84
	P-value	0.044417*	0.050007	0.140686	0.821115	0.226384	0.869483	0.022698*
C (n = 13) 2.5% Tranexamic Acid	Lotion base	603.76 \pm 16.75	600.60 \pm 16.61	596.30 \pm 17.14	600.43 \pm 19.81	602.28 \pm 19.50	595.08 \pm 16.57	598.28 \pm 15.80
	C	606.43 \pm 16.05	599.21 \pm 15.83	597.50 \pm 16.02	600.91 \pm 16.13	604.02 \pm 17.62	593.94 \pm 14.29	593.37 \pm 16.60
	P-value	0.113516	0.363667	0.512474	0.811282	0.545946	0.556189	0.027991*

Treatment group	Absolute erythema values (E)							
		Week 0	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
D (n = 14) 0.25% Puag-Haad + 1% Niacinamide	Lotion base	604.70 ± 20.67	600.59 ± 28.72	594.71 ± 18.93	594.77 ± 16.31	595.87 ± 20.02	589.11 ± 16.81	589.03 ± 20.14
	D	601.30 ± 14.48	591.40 ± 16.42	596.56 ± 18.08	592.89 ± 18.04	593.59 ± 20.75	589.37 ± 18.76	582.39 ± 18.26
	P-value	0.235302	0.231554	0.226532	0.494132	0.149634	0.927056	0.004197*
E (n = 13) 0.25% Puag-Haad + 2.5%Tranexamic acid	Lotion base	604.19± 21.47	596.91 ± 21.08	595.81 ± 21.06	595.17 ± 21.31	599.14 ± 17.86	591.05 ± 20.26	592.08 ± 18.67
	E	603.70 ± 22.64	598.11 ± 21.33	596.76 ± 21.97	594.14 ± 21.64	595.65 ± 18.85	589.32 ± 20.39	591.94 ± 21.52
	P-value	0.789681	0.537743	0.545182	0.728799	0.233774	0.524823	0.961589
F (n = 13) Commercial product	Lotion base	602.50± 18.85	595.85 ± 15.44	596.18 ±18.42	597.86 ± 18.04	598.18 ± 15.56	590.77 ± 17.37	591.66 ± 20.16
	F	602.49 ± 17.26	593.11 ± 25.50	605.91 ± 32.04	596.46 ± 16.06	598.20 ± 12.29	590.15 ± 15.99	590.75 ± 18.63
	P-value	0.995526	0.679683	0.271044	0.612159	0.995365	0.829862	0.522476

*denotes significant difference between the treated and control arms by paired student's t-test.

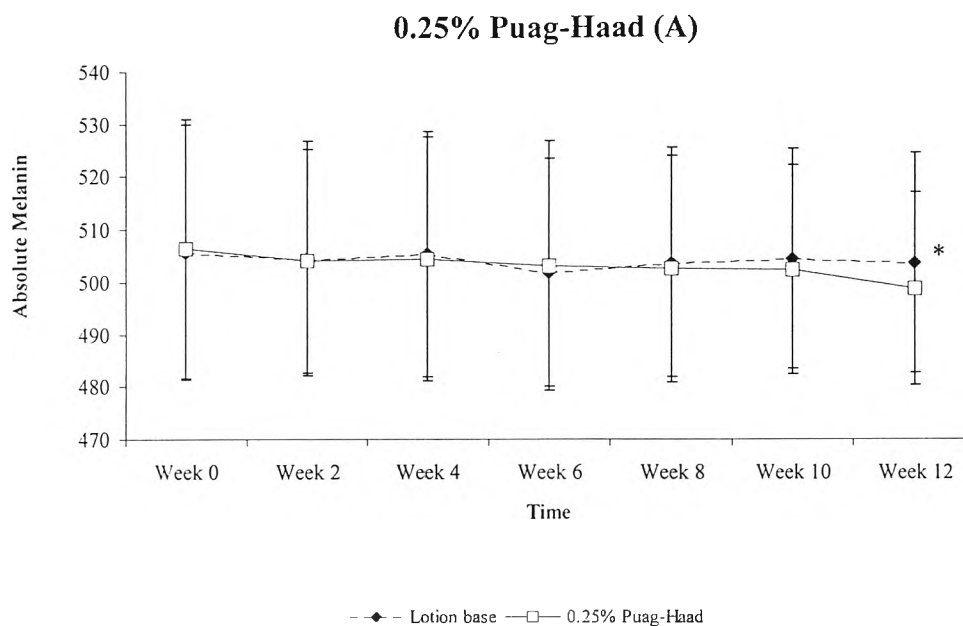


Figure 20. Mean of absolute melanin value after applying lotion base as a control and 0.25% Puag-Haad as a treated substance. Each point represents mean \pm SD (n = 14)

* values are significantly different ($p < 0.05$)

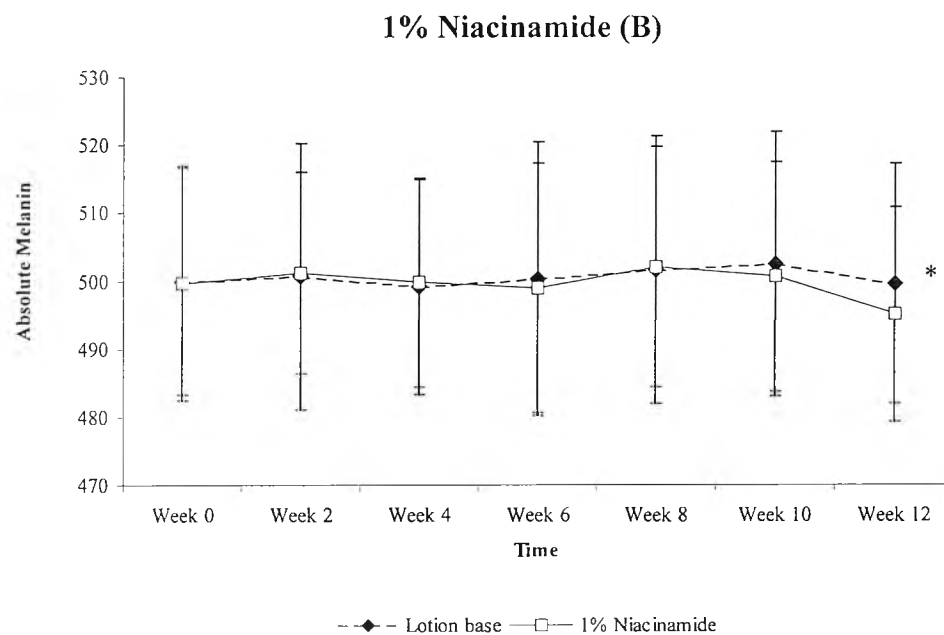


Figure 21. Mean of absolute melanin value after applying lotion base as a control and 1% Niacinamide as a treated substance. Each point represents mean \pm SD (n = 14)

* values are significantly different ($p < 0.05$)

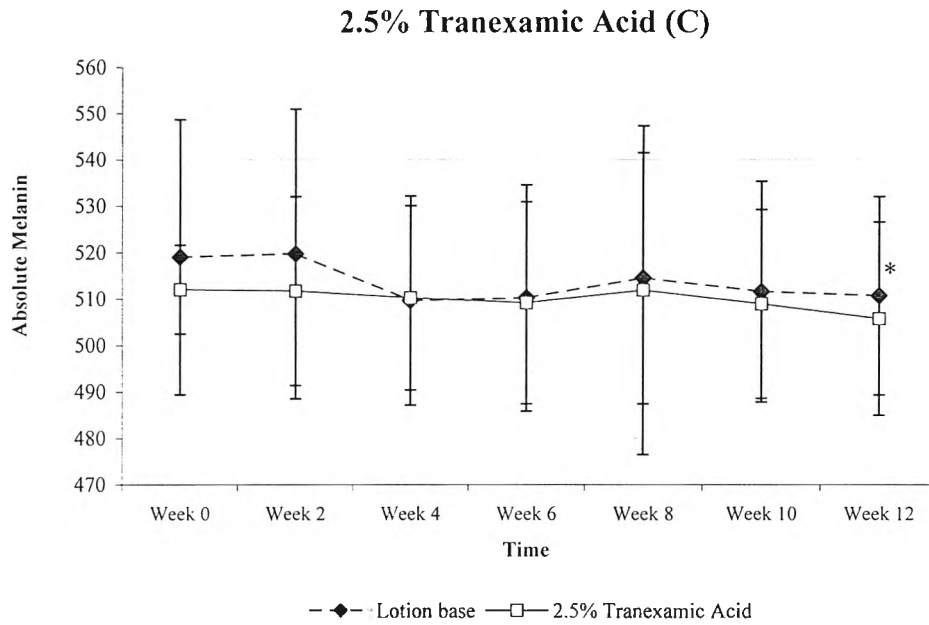


Figure 22. Mean of absolute melanin value after applying lotion base as a control and 2.5% Tranexamic acid as a treated substance. Each point represents mean ± SD (n = 13)

* values are significantly different (p<0.05)

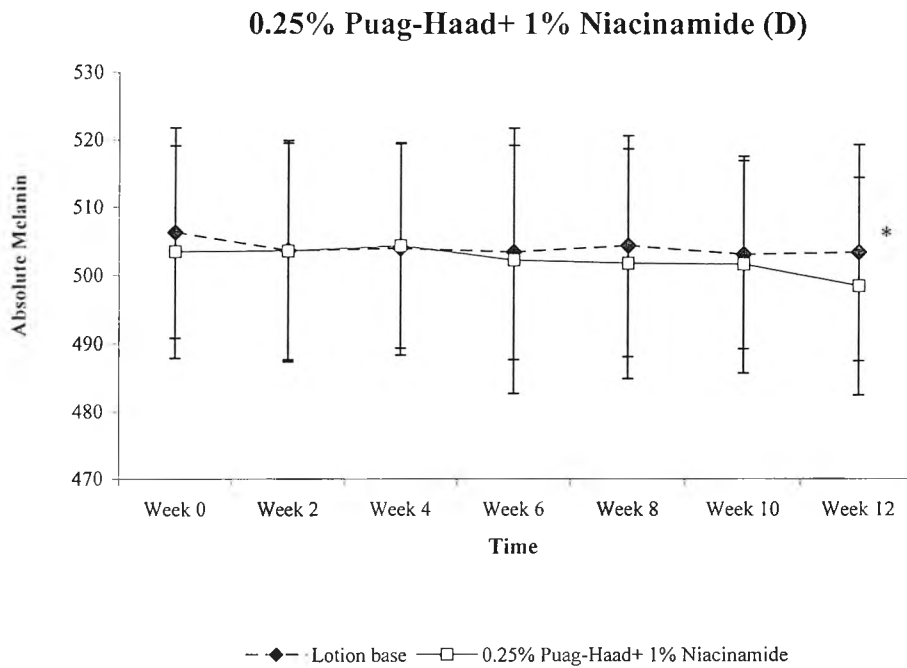


Figure 23. Mean of absolute melanin value after applying lotion base as a control and 0.25% Puag-Haad +1%Niacinamide as a treated substance. Each point represents mean ±SD (n = 14)

*values are significantly different (p<0.05)

0.25% Puag-Haad+ 2.5%Tranexamic acid (E)

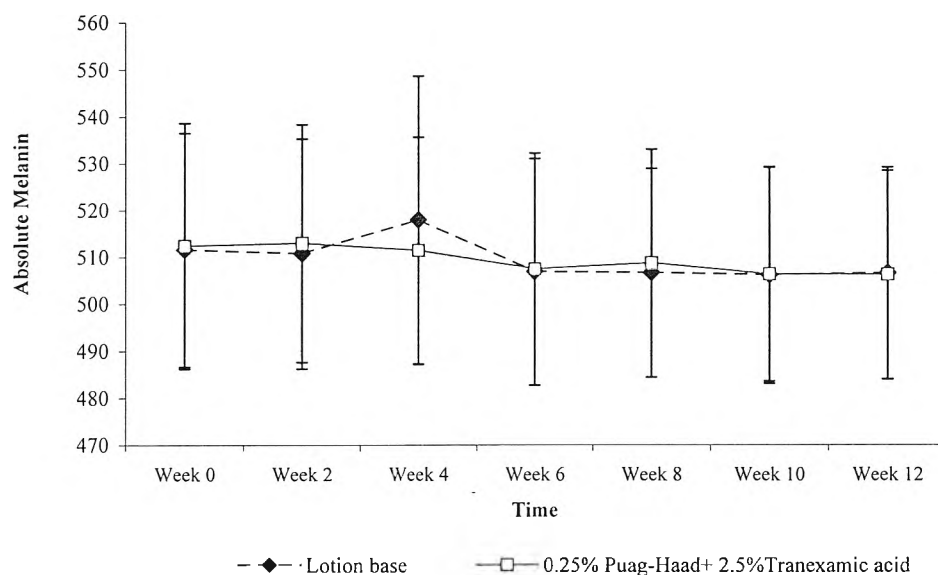


Figure 24. Mean of absolute melanin value after applying lotion base as a control and 0.25% Puag-Haad +2.5% Tranexamic acid as a treated substance. Each point represents mean \pm SD (n = 13)

*values are significantly different ($p < 0.05$)

Commercial product (F)

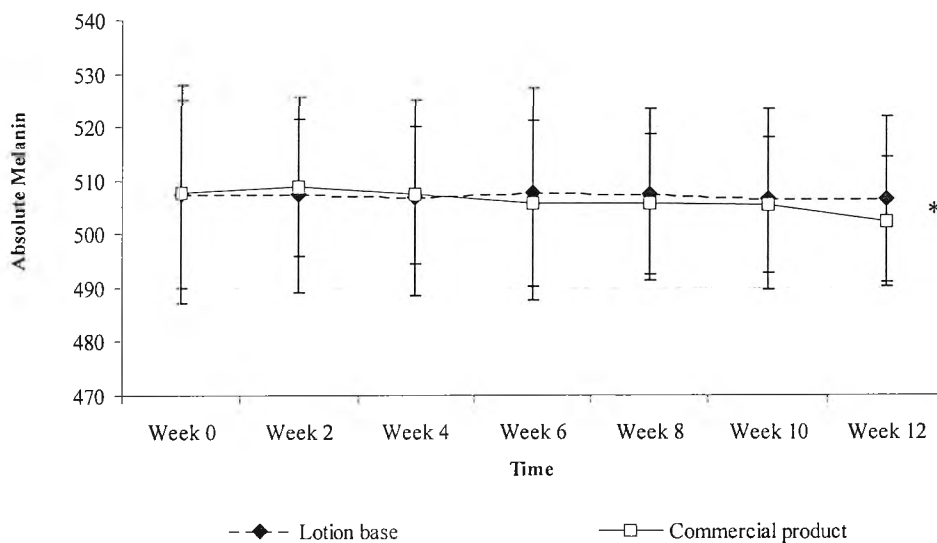


Figure 25. Mean of absolute melanin value after applying lotion base as a control and Commercial product as a treated substance. Each point represents mean \pm SD (n = 13)

* values are significantly different ($p < 0.05$)

3.2 Comparison of the melanin reduction in human volunteers

To examine the melanin content more accurately, the value of melanin reduction ($X_c - X_{tr}$) was used for analysis. These values are given in Table 15 and the individual melanin reduction and erythema change are also shown in Appendix II.

It can be observed from this table that the melanin reduction of all groups, except the combination of Puag-Haad and tranexamic acid (group E), began to reveal significant difference over control at the same period (12th week). In contrast, group E did not give any statistical significance. The extent of the melanin reduction over control at 12th week was 4.89 for 0.25% Puag-Haad (A), 4.44 for 1% niacinamide (B), 4.56 for 2.5%, tranexamic acid (C), 4.93 for 0.25% Puag-Haad +1% niacinamide (D), and 4.04 for the commercial product (F). These values are not much different and one-way ANOVA yielded non-significance among the five groups ($P > 0.05$), suggesting that the five treatments were similarly effective. On the other hand, the melanin reduction value of 0.25% Puag-Haad + 2.5% tranexamic acid (E) showed a minimal value of only 0.29 despite significant reduction observed with the single component (Puag-Haad or tranexamic acid alone). The melanin reduction data is also graphically represented in Figure 26.

Table 15. The melanin reduction after application of the test substances

	0 week	2nd week	4th week	6th week	8th week	10th week	12th week
Puag-Haad 0.25% (A)	-0.71 ±4.87	0.48 ±5.30	1.01 ±4.68	-1.27 ±5.04	1.11 ±3.36	2.07 ±5.61	4.89* ±5.39
Niacinamide 1% (B)	0.30 ±3.70	-0.44 ±7.67	-0.67 ±4.90	1.37 ±7.00	-0.39 ±6.74	1.79 ±5.63	4.44* ±6.43
Tranexamic acid 2.5% (C)	-0.16 ±4.34	0.86 ±6.40	-0.59 ±5.63	0.97 ±5.34	2.40 ±6.99	3.11 ±5.04	4.56* ±3.96
Puag-Haad 0.25%+Niacinamide 1% (D)	2.81 ±5.00	0.06 ±6.60	-0.43 ±4.73	0.76 ±6.06	2.58 ±5.01	1.44 ±6.16	4.93* ±4.78
Puag-Haad 0.25% +Tranexamic acid 2.5% (E)	-0.84 ±5.61	-2.23 ±3.92	-0.64 ±4.16	-0.54 ±4.45	-1.93 ±4.64	-0.16 ±3.38	0.29 ±2.76
Commercial product (F)	-0.16 ±6.20	-1.16 ±7.33	-0.47 ±6.87	1.70 ±8.58	1.67 ±6.28	1.31 ±6.75	4.04* ±5.88
<i>p</i> -value	-	-	-	-	-	-	0.9974
	-	-	-	-	-	-	nonsig

*denotes significant difference between the treated and the control arms by paired student's t-test

p-value obtained from one-way ANOVA comparing only groups showing significant paired t-test.

Table 16. The erythema change after application of the test substances

	0 week	2nd week	4th week	6th week	8th week	10th week	12th week
Puag-Haad 0.25% (A)	-3.41 ±7.40	-0.71 ±7.73	-0.83 ±8.86	-3.76 ±9.49	-0.49 ±7.25	0.07 ±8.56	6.24* ±10.20
Niacinamide 1% (B)	6.79* ±11.41	5.34 ±9.25	-2.17 ±5.17	0.57 ±9.26	2.00 ±5.89	-0.31 ±7.01	3.80* ±9.35
Tranexamic acid 2.5% (C)	-2.67 ±5.89	1.41 ±5.45	-1.20 ±6.66	-0.44 ±6.76	-1.61 ±9.70	1.06 ±6.52	4.56* ±6.92
Puag-Haad 0.25% + Niacinamide 1% (D)	3.40 ±10.22	2.04 ±11.87	-1.84 ±5.43	1.89 ±10.02	2.28 ±5.57	-0.26 ±10.30	6.64* ±7.17
Puag-Haad 0.25% + Tranexamic acid 2.5% (E)	0.49 ±6.67	-1.20 ±7.09	-0.94 ±5.67	0.96 ±10.06	3.24 ±9.69	1.60 ±9.12	0.13 ±9.75
Commercial product (F)	0.01 ±9.34	-3.16 ±9.38	-1.89 ±5.38	1.30 ±9.32	-0.01 ±8.98	0.57 ±9.70	0.84 ±4.77
<i>p</i> -value	-	-	-	-	-	-	-

* denotes significant difference between the treated and the control arms by paired student's t-test

Thus, the data obtained from the human study revealed that only five of the six lotions (A, B, C, D, and F) were effective in significantly reducing the M values from the control lotion base. Nevertheless, the extent of melanin reduction was extremely small and significance was observed at a very late period, i.e., the last week of the 12-week study. This was in contrast to the work of Rojanadilog [46], who reported that twice daily application of the commercial whitening lotions on the lower forearms of subjects yielded significant whitening after only a few weeks. The difference in the results could be ascribed to two major factors. The first was the difference in the experimental methodology. Although using the same type of volunteers (young females with the same skin type), Rojanadilog's study used a Latin-square design, in which each subject received *all* the test lotions at the same time but on different areas of her forearms. Latin square ANOVA was used to analyze the data which separated variability of the data into many sources, thereby considerably increasing the precision of the test in detecting significant difference. In the present study, the design was parallel with self-control in each group. Each subject received *only* one test lotion and a common lotion base as a control. Thus, only paired t-test could be used in analyzing such data within each group.

The second factor was the subject compliance. In Rojanadilog's experiment, only 12 subjects were used and thus monitoring their compliance was much easier than the present studies, which involved 84 subjects. These subjects were factory workers who commuted between their workplace and home and thus were not confined in the same ward as in a well controlled clinical trial. Despite the best effort to instruct them to strictly comply with the application protocol and total avoidance of sunlight exposure, there was absolutely no way to control their behavior, particularly beyond the office hours.

It should be noted that after assessing the M values for 10 weeks and found that the values hardly changed, the subjects were warned of being excluded from the study (and thus would not receive the full wages). The M values of the last measurements (week 12) suddenly dropped as seen in Figures 20 – 25, suggesting that poor compliance was the major factor responsible for the slow melanin reduction.

The results from the present human study were also different from the guinea pig study. In guinea pigs, 1% niacinamide and 1% niacinamide + 0.25% Puag-Haad exhibited the fastest onset (1st week) whereas combination of niacinamide, Puag-Haad and lactic acid as well as Puag-Haad alone exhibited the statistical significance at 2nd week. The slowest reaction was the group applying with lactic acid. It is possible that there had been many variations in the human study more than the animal study despite the human experiment was carefully designed to reduce such variation. This included balancing the order of application in each group as well as controlling the hole of dropper to obtain equal volume of lotion.

Also, it was surprising to observe that the combination between Puag-Haad and tranexamic acid (group E) did not result in any significant melanin reduction despite the significant effect observed with either Puag-Haad or tranexamic acid alone. For some unknown mechanisms, the two substances, when combined together, appeared to act against or inhibit each other's activity such that no whitening effect could be produced.

As previously been discussed, the most probable source of variation in the human data derived from the subjects' poor compliance. The manner in which each person applied the substances as well as their adherence to the detailed protocol could be drastically different among the individual subjects and extremely difficult to control. Adherence to the protocol was mainly based on the honor-system, i.e., the subjects were believed to have followed the protocol to their fullest capacity throughout the 12-week study period.

3.3 Comparison of the skin erythema change in human volunteers

Tables 14 and 16 show that only at week 12, there was a statistical difference in the absolute erythema (E) values between the control and the treated areas in groups A (0.25% Puag-Haad), B (1% niacinamide), C (2.5% tranexamic acid), and D (0.25% Puag-Haad + 1% niacinamide). Focusing on the four significant groups mentioned above, the E values of the treated arms tended to be smaller than their corresponding control, especially at week 12, resulting in the positive Ec-Etr values. This observation was surprising because the substance did not seem to induce

any erythema but instead tended to decrease its values. The reason as to the erythema-reducing property of the test lotions A, B, C and D are not presently known. However, similar observations were observed with the solutions of 0.25% Puag-Haad, 3% kojic acid, and 0.25% licorice extract in propylene glycol [27]. The author attributed the erythema-reducing effect of these whitening agents to many possible mechanisms such as anti-inflammatory, vasoconstriction and UV-protective effects. It is probable that Puag-Haad, niacinamide and tranexamic acid may exert some of these mechanisms. However, more studies are needed to clarify this observation since some conflicting data still existed. For example, combination of Puag-Haad and tranexamic acid (group E) failed to give significant erythema-reducing effect despite the ability of the individual components (Puag-Haad or tranexamic acid alone) to reduce erythema. The inexplicable behavior of group E had been noticed before with the melanin data. Moreover, the initial erythema values between the treated and the control areas of group B were not similar, with $E_c - E_{tr} = 6.79$ ($P < 0.05$). This also had interfered with the interpretation of the erythema-reducing effect of group B (niacinamide) at week 12 since the starting value for the treated side was already lower than the control. The commercial product F also failed to give significant erythema-reducing effect despite the inclusion of chemical sunscreens in its formulation, which should have provided protection against UV-induced erythema.

Other types of skin irritation may also exist and may not be detected by the Mexameter. These include skin inflammation, lesions, contact dermatitis etc. Mexameter merely measured the absorbance of hemoglobin available in the area and hence the absolute erythema values may not be a good indicator of skin irritation. Other tests such as the primary human skin irritation test, which follows the CTFA guidelines (patches testing), or the eye irritation test should be further studied to confirm the safety of these whitening agents.

Since the E value was not the sole parameter of skin irritation potential, visual observation of the individual subjects during the experiment was also made at each visit. Skin rashes and itching were detected in two subjects (A2, C14). The volunteer A2 who applied Puag-Haad lotion on her right arm and the lotion base on her left arm developed skin rashes and itching on the left arm after 2-week application. However, the symptom vanished within a few days and she continued to participate in the study.

This could be due to the sensitizing action of the preservative in the base formulation. Another subject (C14) noticed the itching on both arms after applying the lotions for 1 day. She withdrew from the study and was treated with calamine lotion. Two more subjects (E12, F13) were excluded from the experiment due to changes in their work without any visible side effects. The 81 remaining subjects continued until the end of the experiment without skin irritation.

In brief, the data obtained from 81 human volunteers show that the test lotions A, B, C, D, and F were all effective in reducing the melanin content over the self-control. Lotions A, B, C and D also exhibited small but significant erythema-reducing effect via some unknown mechanisms. However, the onset of both effects was extremely slow with significant paired t-test observed at the last week (week 12). No serious skin reactions were observed in all cases. The rather slow onset was attributed to the poor compliance of the subjects. In contrast, the test lotion E (0.25% Puag-Haad + 2.5% tranexamic acid) did not produce any significant whitening nor erythema-reducing effects despite the lotions containing either Puag-Haad or tranexamic acid alone exhibited both effects at 12th week. In order to obtain a more accurate and explainable results, another set of human study was conducted at Nakorn Ratchasima facility. The subjects were female volunteers who were under custody at the Baan Metta House, which belongs to the Public Welfare Department, Ministry of Labour and Public Welfare. Each subject was explained of the nature of the experiments and had given consent form to participate in this new study. The protocol was exactly the same as in the previous human study, with the exception that application of the test (and control) lotions was performed by licensed nurses. This would ensure absolute subject's compliance and full adherence to the protocol.

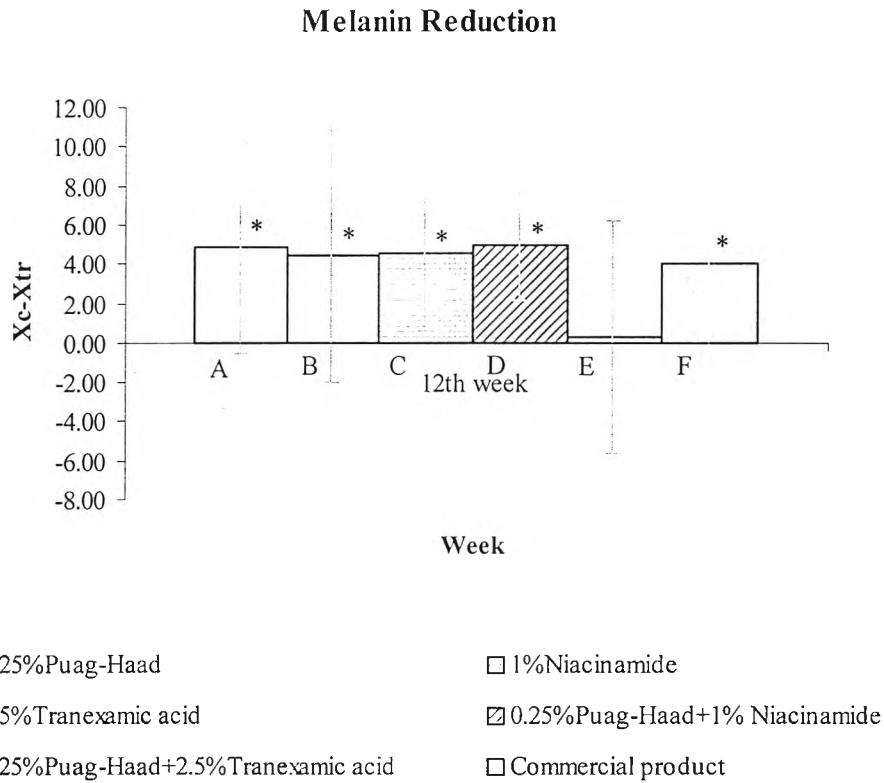


Figure 26. Comparison of melanin reduction (Xc-Xtr) of each test substance at week 12. Data = mean ± SD (n = 13-14/group)

*denotes significant difference between the treated and the control arms by paired student's t-test

Erythema change

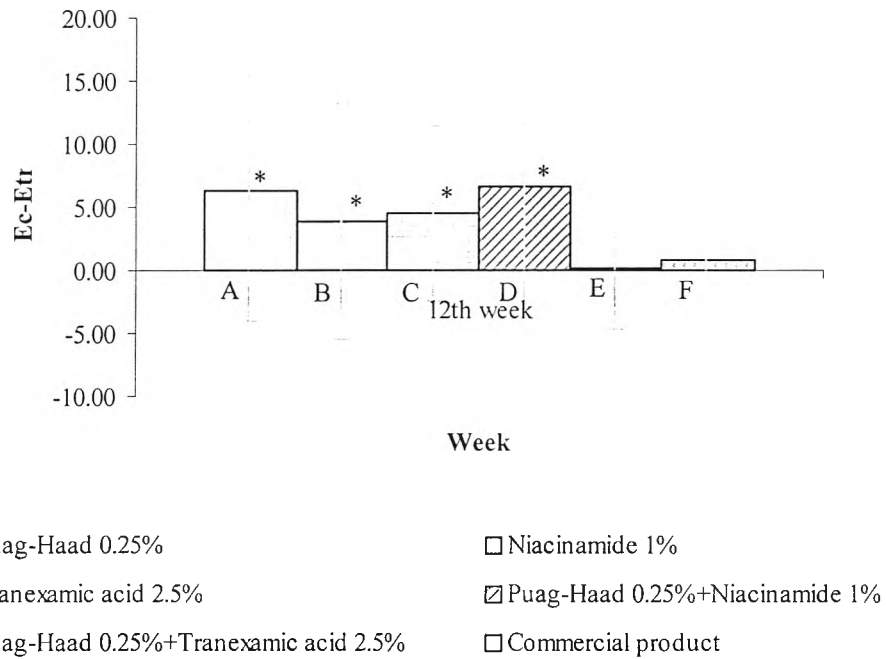


Figure 27. Comparison of erythema change (Ec-Etr) of each test substance at week 12. Data = mean ± SD (n = 13-14/group)

*denotes significant difference between the treated and the control arms by paired student's t-test

Part 4 Evaluation of skin whitening efficacy of aqueous extract of *Artocarpus lakoocha* heartwood (Puag-Haad), niacinamide, tranexamic acid, and their combinations in human volunteers - The supplemental experiment conducted at Nakornratchasima (Korat)

4.1 Determination of melanin content and erythema in human volunteers

Additional human study was conducted at Nakornratchasima province using the same study design as in the previous study. However, the protocol was under a very strict control to obtain more reliable data. Forty-two subjects were carefully selected and divided into six groups of 7 each. Their upper arms were applied with the control and the test lotions twice a day in a manner similar to the previous study. However, instead of self-application professional nurses were asked to perform the task. Since all the subjects resided in the same place, control of their behavior and strict adherence to the application protocol were easily achieved. Subsequently, their arms were measured with Mexameter every 2 weeks until 8 weeks. The mean absolute M and E values are respectively shown in Tables 17 and 18. The individual values are also provided in Appendix III.

The melanin data in Table 17 shows that the values of the treated arms in group A (0.25% Puag-Haad) declined from 568.89 at week 0 to 551.31 at week 6 and 544.89 at week 8. The melanin value of group B (1% niacinamide) reduced from 563 at week 0 to 555.37 and 546.33 at week 6 and 8, respectively. The downward trend was also observed in group C (2.5% tranexamic acid), D (0.25% Puag-Haad + 1% niacinamide), E (0.25% Puag-Haad + 2.5% tranexamic acid), and F (commercial product). The melanin of group C dropped from 552.94 at week 0 to 543.26 and 541.29 at week 6 and 8, respectively. In group D, the value dropped from 578.6 at zero week to 564.4 at 6th week and 548.5 at 8th week. For group E, the value reduced from 539 at zero week to 526.37 at 6th week and 518.54 at 8th week. Finally, the melanin of group F reduced from 539.91 at week 0 to 522.46 at 6th week and 513.17 at 8th week.

When paired t-test was applied on the absolute melanin data at each week comparing between the treated and the control arms, significance was first detected at week 8 for group A and B. Group C, D and F had a shorter onset, starting to give significant whitening effect at week 6. On the contrary group E failed to show significant whitening at all weeks (week 2 – 8). Although the starting values between the control and the treated areas of group E were not the same, the non-significance observed at all weeks agreed with the previous humans study, which also failed to show significant melanin reduction during the 12-week period. Thus, combination of Puag-Haad and tranexamic acid in group E may not have a beneficial effect in providing adequate skin whitening despite the significant activity observed with the separate compounds. More studies are thus needed to verify if Puag-Haad and tranexamic acid could interfere with each other's whitening activity, and if true, what are the responsible mechanisms.

Table 17. The absolute melanin values (mean \pm SD) in the upper arms of human volunteers treated with different substance for 8 weeks at Nakornratchasima (n= 6-7/treatment group)

Treatment group	Absolute melanin values (M)					
		Week 0	Week 2	Week 4	Week 6	Week 8
A (n = 7) 0.25% Puag-Haad	Lotion base	559.86 \pm 69.13	560.26 \pm 36.75	557.74 \pm 35.45	559.09 \pm 37.52	558.86 \pm 37.07
	A	568.89 \pm 42.87	559.51 \pm 31.26	555.29 \pm 30.69	551.31 \pm 31.60	544.89 \pm 29.78
	P-value	0.540766	0.867403	0.466828	0.076862	0.011786*
B (n = 6) 1% Niacinamide	Lotion base	559.7 \pm 16.68	556.57 \pm 19.77	562.87 \pm 22.36	558.5 \pm 22.62	562.6 \pm 14.38
	B	563 \pm 17.68	560.10 \pm 23.80	562.03 \pm 22.98	555.37 \pm 20.60	546.33 \pm 22.67
	P-value	0.253045	0.333123	0.855412	0.248597	0.030313*
C (n = 7) 2% Tranexamic acid	Lotion base	556.86 \pm 25.86	551.78 \pm 26.32	551.6 \pm 21.78	550.57 \pm 24.49	551.57 \pm 25.42
	C	552.94 \pm 25.72	548.11 \pm 20.14	545.11 \pm 20.73	543.26 \pm 21.96	541.29 \pm 21.16
	P-value	0.286243	0.467917	0.089382	0.020891*	0.033684*

Treatment group	Absolute melanin values (M)					
		Week 0	Week 2	Week 4	Week 6	Week 8
D (n = 6) 0.25% Puag-Haad +1% Niacinamide	Lotion base	581.33 ± 20.66	574.33± 20.15	570.97 ± 21.3	573.1 ± 20.53	572 ± 19.35
	D	578.6 ± 22.29	571.77 ± 21.27	566.7 ± 17.8	564.4 ± 15.84	548.5 ± 23.44
	P-value	0.174377	0.154579	0.113066	0.029394*	0.036380*
E (n = 7) 0.25% Puag-Haad + 2.5%Tranexamic acid	Lotion base	530.40 ± 11.11	529.23 ± 8.46	528.14 ± 10.90	527.80 ± 8.23	522.63 ± 7.27
	E	539.00 ± 13.69	531.74 ± 11.30	524.54 ± 9.94	526.37 ± 13.60	518.54 ± 5.79
	P-value	0.032721*	0.534698	0.361994	0.698670	0.143565
F (n = 7) Commercial product	Lotion base	538.74 ± 20.56	534.20 ± 17.94	525.26 ± 17.79	530.23 ± 18.81	525.37 ± 17.10
	F	539.91 ± 22.38	533.43 ± 17.92	524.03 ± 16.21	522.46 ± 17.48	513.17 ± 16.02
	P-value	0.801337	0.683493	0.630711	0.046053*	0.00706*

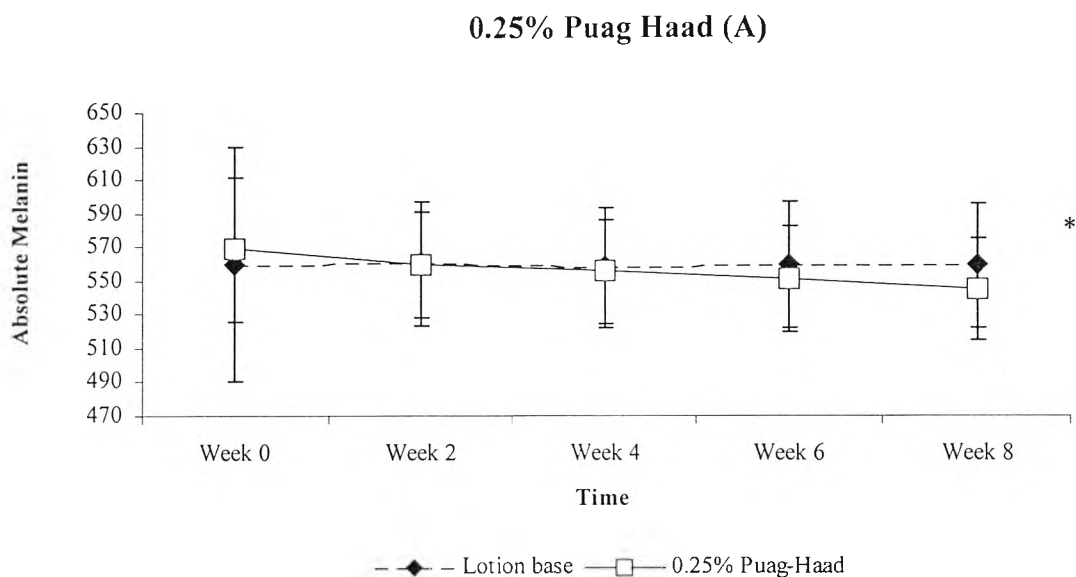
*denotes significant difference between the treated and the control arms by paired student's t-test.

Table 18. The absolute erythema values (mean \pm SD) in the upper arms of human volunteers treated with different substance for 8 weeks at Nakornratchasima (n=6-7/treatment group)

Treatment group	Absolute erythema values (E)					
		Week 0	Week 2	Week 4	Week 6	Week 8
A (n = 7) 0.25% Puag- Haad	Lotion base	642.91 \pm 22.15	637.51 \pm 17.69	633.74 \pm 19.25	648.2 \pm 21.25	648.91 \pm 18.02
	A	642.09 \pm 16.5	640.94 \pm 7.69	640.94 \pm 7.69	643.94 \pm 13.33	641.31 \pm 14.71
	P-value	0.779260	0.427975	0.204683	0.495639	0.130214
B (n = 6) 1% Niacinamide	Lotion base	647.33 \pm 17.75	642.7 \pm 17.98	647.57 \pm 13.47	649.53 \pm 19.76	651.07 \pm 13.88
	B	650.2 \pm 14.69	643.2 \pm 18.18	646.63 \pm 15.44	646.83 \pm 15.30	647.97 \pm 11.17
	P-value	0.537942	0.877317	0.698242	0.355290	0.184574
C (n = 7) 2% Tranexamic acid	Lotion base	645.11 \pm 21.62	634.29 \pm 22.55	636.74 \pm 23.75	642.83 \pm 19.97	629.17 \pm 37.14
	C	641.6 \pm 17.91	632.14 \pm 17.83	632.8 \pm 22.7	640.66 \pm 20.94	644.89 \pm 19.31
	P-value	0.263042	0.613662	0.121629	0.326201	0.446039

Treatment group	Absolute erythema values (E)					
		Week 0	Week 2	Week 4	Week 6	Week 8
D (n = 6) 0.25% Puag- Haad + 1% Niacinamide	Lotion base	649.43 ± 12.62	641.07 ± 11.28	642.17 ± 13.92	651.07 ± 13.41	651.87 ± 12.42
	D	645.2 ± 19.62	644.03 ± 15.73	632.87 ± 12.75	645.1 ± 8.6	645.27 ± 9.89
	P-value	0.323768	0.431001	0.001598*	0.185165	0.070587
E (n = 7) 0.25% Puag- Haad + 2.5% Tranexamic acid	Lotion base	628.00 ± 30.96	628.66 ± 17.74	634.57 ± 27.24	637.31 ± 16.12	639.31 ± 18.62
	E	633.03 ± 13.36	627.69 ± 16.3	636.57 ± 24.44	631.54 ± 12.24	631.8 ± 11.18
	P-value	0.614848	0.766995	0.644518	0.273331	0.217475
F (n = 7) Commercial product	Lotion base	635.86 ± 16.75	632.74 ± 13.01	630.69 ± 13.97	631.74 ± 10.96	633.97 ± 14.56
	F	634.8 ± 14.3	632.31 ± 17.82	629.17 ± 10.07	627.31 ± 10.98	631.94 ± 12.44
	P-value	0.865285	0.940021	0.730958	0.168304	0.664944

*denotes significant difference between the treated and the control arms by paired student's t-test.



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and 0.25% Puag-Haad as a treated substance. Each point represents mean \pm SD (n = 7)

* values are significantly different ($p < 0.05$)

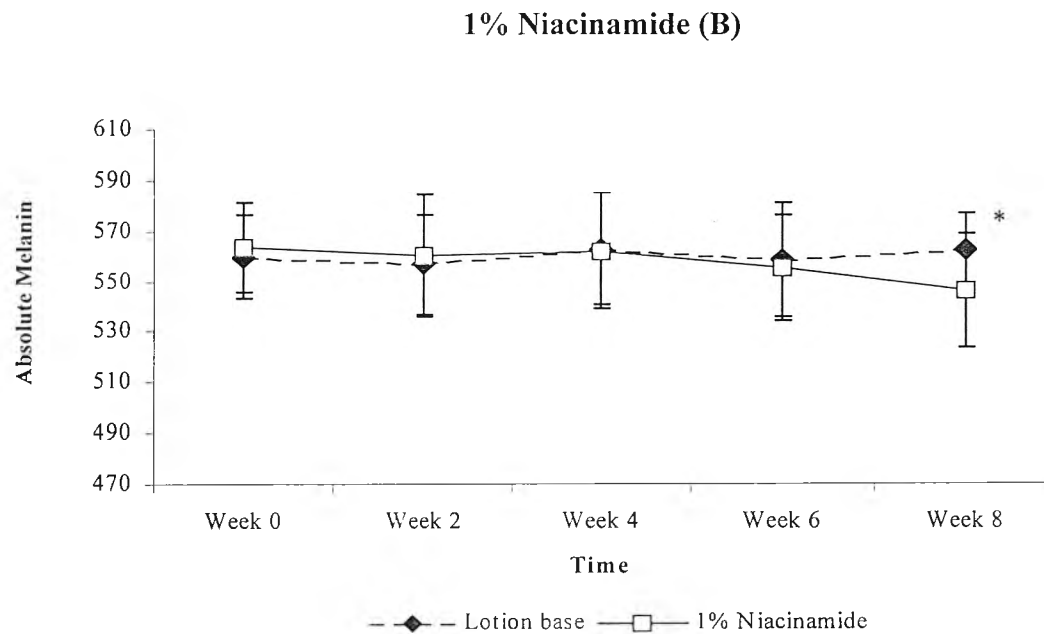


Figure 29. Mean of absolute melanin value after applying lotion base as a control and 1%Niacinamide as a treated substance. Each point represents mean \pm SD (n = 6)

* values are significantly different ($p < 0.05$)

2.5% Tranexamic Acid (C)

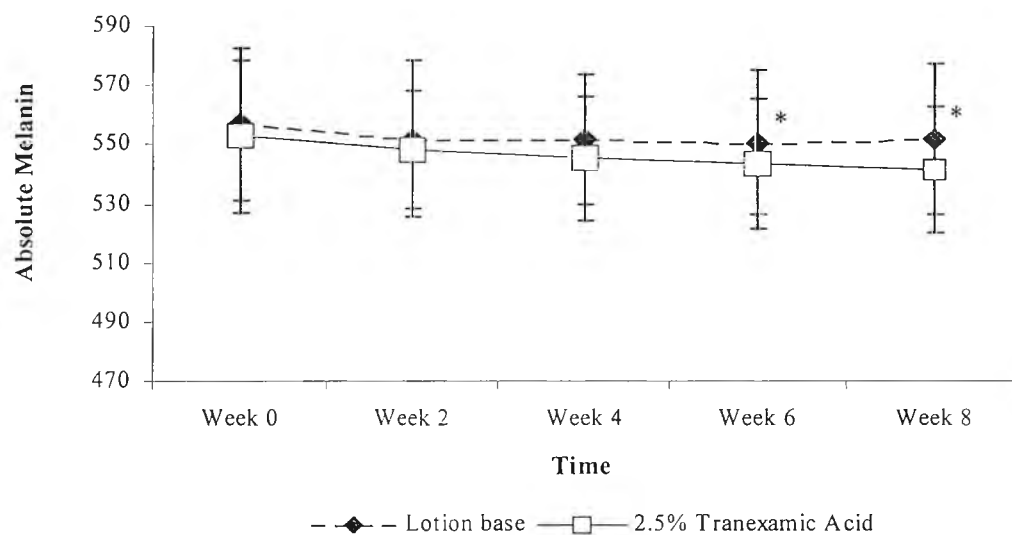


Figure 30. Mean of absolute melanin value after applying lotion base as a control and 2.5% Tranexamic acid as a treated substance. Each point represents mean \pm SD (n = 7)

* values are significantly different ($p < 0.05$)

0.25% Puag-Haad + 1% Niacinamide (D)

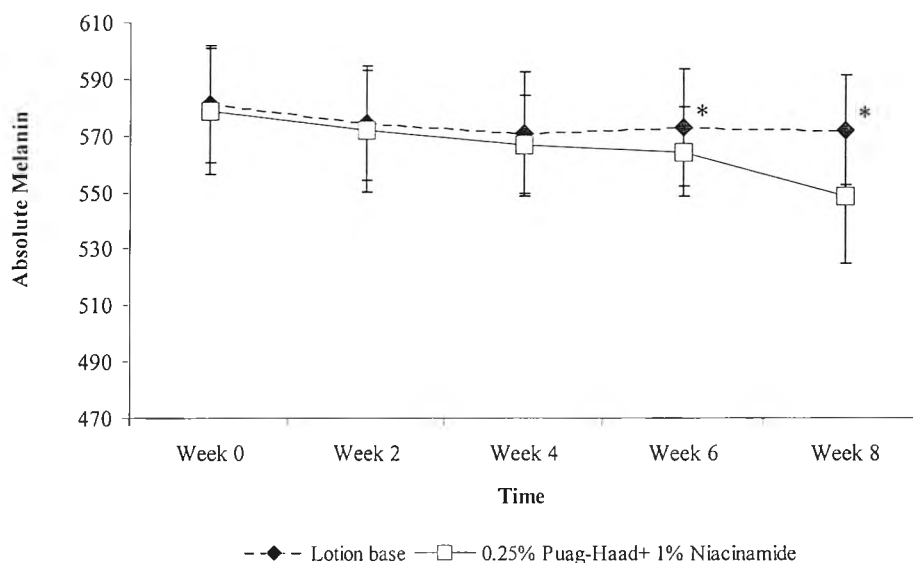


Figure 31. Mean of absolute melanin value after applying lotion base as a control and 0.25% Puag-Haad + 1% Niacinamide as a treated substance. Each point represents mean \pm SD (n = 6)

* values are significantly different ($p < 0.05$)

0.25% Puag-Haad + 2.5% Tranexamic acid (E)

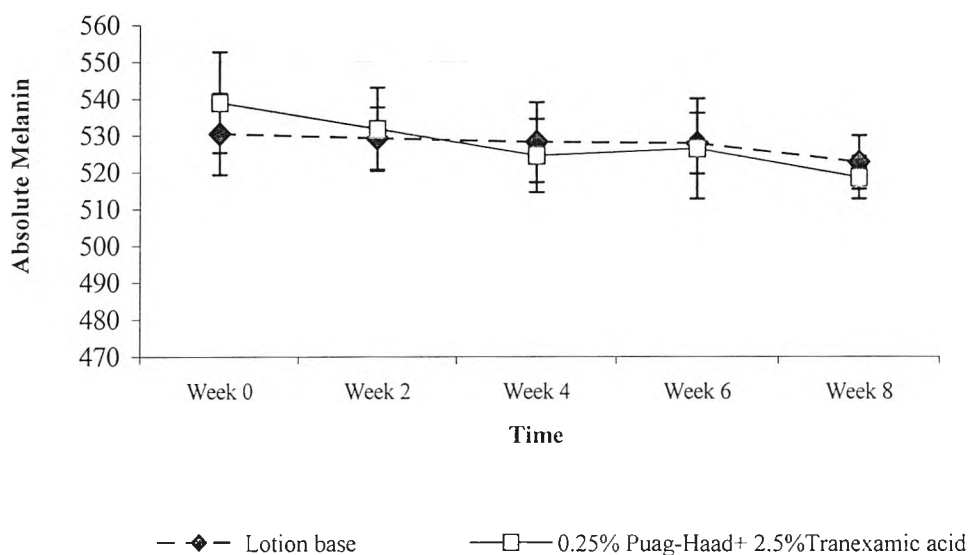


Figure 32. Mean of absolute melanin value after applying lotion base as a control and 0.25% Puag-Haad +2.5% Tranexamic acid as a treated substance. Each point represents mean \pm SD (n = 7)

*values are significantly different ($p < 0.05$)

Commercial product (F)

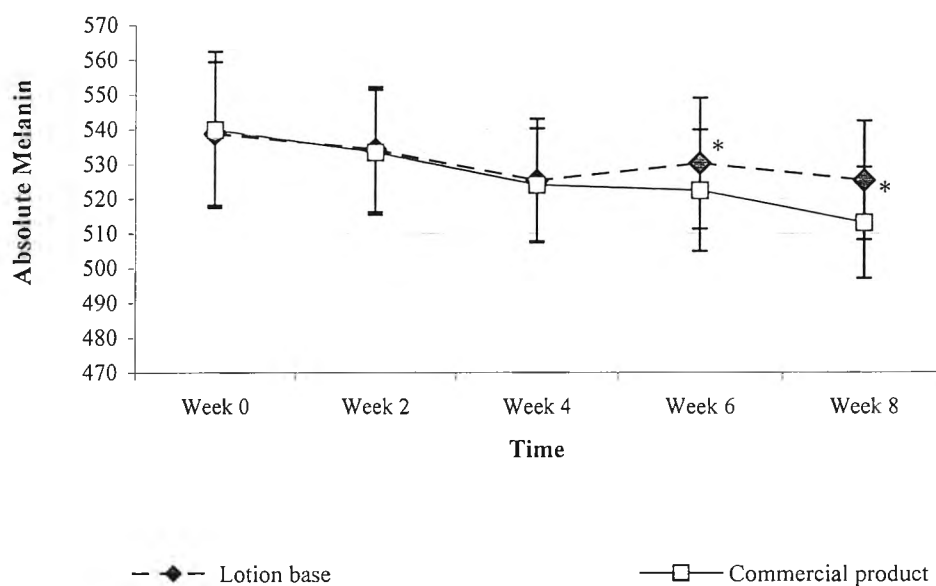


Figure 33. Mean of absolute melanin value after applying lotion base as a control and Commercial product as a treated substance. Each point represents mean \pm SD (n = 7)

* values are significantly different ($p < 0.05$)

4.2 Comparison of the melanin content in human volunteers

To facilitate melanin data analysis, Xc-Xtr or melanin reduction value was used. The average values together with the data on erythema change are given in Tables 19 and 20 whereas the individual results are provided in Appendix III. Plots of the absolute melanin values as a function of time are also provided in Figures 28 – 33 for visual comparison of the melanin profiles between the control and the treated areas within each group.

From Table 19, it can be seen that application of 0.25% Puag-Haad (group A) resulted in significant whitening observed at week 8, with the mean melanin reduction of 13.97. Application of 1% niacinamide alone (group B) induced a whitening effect similar to 0.25% Puag-Haad, with significant reduction of 13.94 occurring at the same time (week 8). Application of 2.5% tranexamic acid alone (group C) was found to induce significant decrease in melanin at an earlier period (week 6), with the mean reduction of 7.31, which increased to 10.29 at week 8. Combination of Puag-Haad and niacinamide (group D) resulted in acceleration of the whitening effect, which became significant at earlier period, i.e. at week 6 instead of week 8. The mean melanin reduction was 7.46 at week 6 and increased to 17.29 at week 8. The commercial product (F) also became effective at week 6, with the mean reduction of 7.77 increasing to 12.20 at week 8.

The onset of whitening activity observed here was much different from the previous human study, in which significant whitening were detected only at week 12 in all groups. The difference in the results could be simply explained by a tighter control of experiment exercised in this study. All the subjects at Nakornratchasima stayed in the same place 24 hr a day. Arrangement had been made with the warden of the Metta House to see to it that all the subjects wear proper clothes at all time, which fully covered their upper arms. In addition, application of lotions was performed by professional nurses, who had been trained to dispense and apply the lotions precisely according to the protocol. Therefore, the results obtained were more reliable than the previous study, in which poor compliance was a compromising factor.

It is interesting to note that combination between Puag-Haad and tranexamic acid (group E) again failed to produce significant whitening effect at all weeks despite the high whitening activity of the individual component when applied alone. The reasons were not clearly understood. However, the starting melanin values (week 0) between the control and the treated areas of this group were not the same ($P < 0.05$), with a very high difference of -8.60 (Table 19). The negative sign indicated that the starting melanin value of the arms to be treated with the whitening lotion was greater than that of the control arms (539.0 vs 530.4). If the whitening effect were judged from the absolute melanin data of the treated-arms alone (Table 17), it can be seen that the value slowly decreased with time, from 539.0 at week 0 to 518.54 at week 8. This somehow indicated the ability of the test lotion E to induce a decrease in melanin upon prolonged application. However, due to the difference in the starting value from the control, significance could not be detected. Thus, more studies are needed to verify the whitening efficacy of Lotion E or to prove otherwise that the two compounds could counteract each other's activity. The starting melanin values between the treated and the control arms in all other groups were similar ($P > 0.05$), thereby creating no difficulties in the data analysis.

Also, it should be noted that the onset time of 0.25% Puag-Haad alone observed here (8 weeks) was slower than that previously reported by Pengrungruangwong [27]. Using the same protocol as in this study, the author found that the onset time for 0.25% Puag-Haad was only 4 weeks. The difference in the results may be explained in term of the vehicle used in the study. Pengrungruangwong used pure propylene glycol as the solvent for the human study whereas the complex emulsion-type lotion was utilized here as a vehicle. Since the lotion contained substantial amount of an oil phase, the partition of moderately water-soluble oxyresveratrol, the active constituent of Puag-Haad, from the lipophilic vehicle might be slower than from pure propylene glycol, which is less lipophilic.

The commercial product (F) contained 0.001% each of mulberry root and *Scutellaria baicalensis* extracts in addition to 0.3% capryloyl salicylic acid and 0.7% terephthalylidene dicamphor sulfonic acid (Mexoryl SX). The two plant extracts act as tyrosinase inhibitors whereas capryloyl salicylic acid is a beta-hydroxy acid, which promotes epidermal turnover. Mexoryl SX is a UV filter which acts as a chemical

sunscreen. The combined effect of this formula has been claimed by the manufacturer to provide effective whitening of the skin since each component exerts different whitening mechanism(s). The product F was thus used as a reference in this study.

One-way ANOVA was then applied to test for significant difference in the melanin reduction data among different treatment groups, which had previously demonstrated significant whitening effect by the paired t-test. Results are also given in Table 19, which revealed no significant difference among the effective groups, either at week 6 (comparing between C, D and F) or week 8 (comparing between A, B, C, D and F) ($P > 0.05$). The data suggested that, once effective, the *extent* of whitening did not differ significantly among different types and combinations of the whitening agents. However, a closer examination of the data still revealed subtle differences. At week 8, the last week of this confirmatory study, combination of 0.25% Puag-Haad and 1% niacinamide (group D) tended to give the highest whitening effect (mean melanin reduction = 17.29). This was followed by pure Puag-Haad (A) and niacinamide (B), giving equal melanin reduction of 13.9. Next was the commercial product (F), which gave the mean reduction of 12.20, followed by pure tranexamic acid (C) rendering the mean reduction of 10.29. The data are also graphically represented in Figure34.

Table 19. The melanin reduction after application of the test substances

	0 week	2nd week	4th week	6th week	8th week
Puag-Haad 0.25% (A)	5.26 ±10.01	0.74 ±11.28	2.46 ±8.36	7.77 ±9.63	13.97* ±10.35
Niacinamide 1% (B)	-2.83 ±5.84	-3.03 ±7.49	0.71 ±9.71	2.69 ±5.49	13.94* ±13.61
Tranexamic acid 2.5% (C)	3.91 ±8.84	3.66 ±12.48	6.49 ±8.47	7.31* ±6.22	10.29* ±9.92
Puag-Haad 0.25%+Niacinamide 1% (D)	2.34 ±3.99	2.20 ±3.55	3.66 ±5.22	7.46* ±7.23	17.29* ±14.33
Puag-Haad 0.25% +Tranexamic acid 2.5% (E)	-8.60* ±8.23	-2.51 ±10.10	3.60 ±9.65	1.43 ±9.30	4.09 ±6.42
Commercial product (F)	-1.17 ±11.78	0.77 ±4.76	1.23 ±6.42	7.77* ±8.19	12.20* ±8.05
<i>p</i> -value	-	-	-	0.9410	0.7508
	-	-	-	nonsig	nonsig

*denotes significant difference between the treated and the control arms by paired student's t-test.

p-value obtained from one-way ANOVA comparing only the groups showing significant whitening effect after paired t-test (groups C, D, F at week 6 and groups A, B, C, D and F at week 8).

Table 20. The erythema change after application of the test substances

	0 week	2nd week	4th week	6th week	8th week
Puag-Haad 0.25% (A)	0.83 ±7.47	-3.43 ±10.67	-2.26 ±9.41	4.26 ±15.53	7.60 ±11.47
Niacinamide 1% (B)	-2.46 ±9.76	-0.43 ±6.88	0.80 ±5.09	2.31 ±6.01	2.66 ±4.65
Tranexamic acid 2.5% (C)	3.51 ±7.52	2.14 ±10.65	3.94 ±5.78	2.17 ±5.37	-15.71 ±50.99
Puag-Haad 0.25%+Niacinamide 1% (D)	3.63 ±8.79	-2.54 ±7.82	7.97* ±4.85	5.11 ±8.97	5.66 ±6.90
Puag-Haad 0.25% +Tranexamic acid 2.5% (E)	-5.03 ±25.08	0.97 ±8.28	-2.00 ±10.89	5.77 ±12.66	7.51 ±14.43
Commercial product (F)	1.06 ±15.79	0.43 ±14.45	1.51 ±11.11	4.43 ±7.48	2.03 ±11.78
<i>p</i> -value	-	-	-	-	-

*denotes significant difference between the treated and the control arms by paired student's t-test.

4.3 Comparison of erythema change in human volunteers

Significant erythema reduction was only found at the 4th week after application of 0.25% Puag-Haad + 1% niacinamide (group D). However, the absolute erythema values, despite some fluctuation, became slightly lower than the control at weeks 6-8 in all groups except group C (Table 18). This tendency of erythema-reducing effect had been observed in the previous study for lotions A, B, C and D. Nevertheless, the non-significance observed in this confirmatory study suggested that the erythema-reducing effect of these lotions, if existed, was negligible. The average change in the erythema data are given in Table 20 and graphically represented in Figure 35.

Visual observation of the skin conditions was also made. Visible skin irritation was observed in 2 subjects (B7, D1). The volunteer B7 who applied niacinamide lotion at the right arm and the lotion base on the left arm developed skin rashes and itching on the right arm after 4 weeks. Another subject (D1) experienced the itching on both arms after applying the lotions for 4 weeks. Both volunteers withdrew from the study and were treated with calamine lotion. It was obvious that the rashes observed in subject B7 were caused by niacinamide, which has been reported to produce skin irritation in many cosmetic products [47]. On the other hand, the side effect experienced by subject D1 might be due to certain components of the lotion base since the symptom occurred in both arms. Nevertheless, the remaining 40 subjects completed the 8-week study without skin irritation.

It can be concluded at this point that all the test lotions (except lotion E) were effective in significantly reducing the melanin value in the subject's upper arms. Combination of 0.25% Puag-Haad with 1% niacinamide (lotion D) produced the highest whitening effect after 8-week application and also gave the most rapid onset observed at 6th week. The data also agreed with the results previously obtained in guinea pigs. The incidence of skin irritation was low and most subjects well tolerated the test preparations. Thus, it is possible to formulate Puag-Haad in an emulsion-type lotion base, either alone or in combination with other whitening agents, to produce a safe and effective whitening product for cosmetic and medical applications.

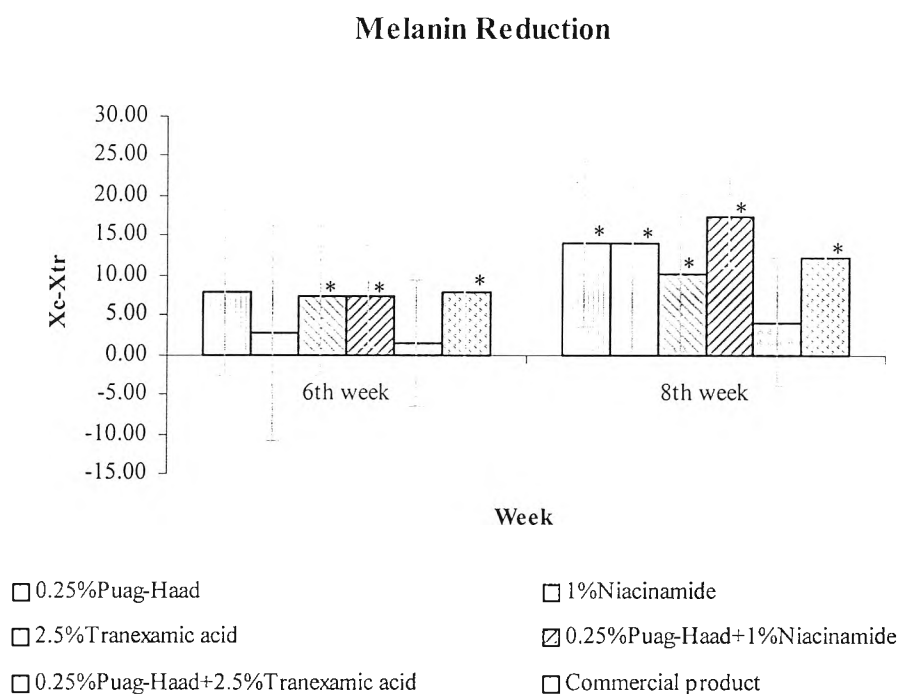


Figure 34. Comparison of melanin reduction ($X_c - X_{tr}$) of each test substance at each week. Data = mean \pm SD (n = 6-7 /group)

*denotes significant difference between the treated and the control arms by paired student's t-test.

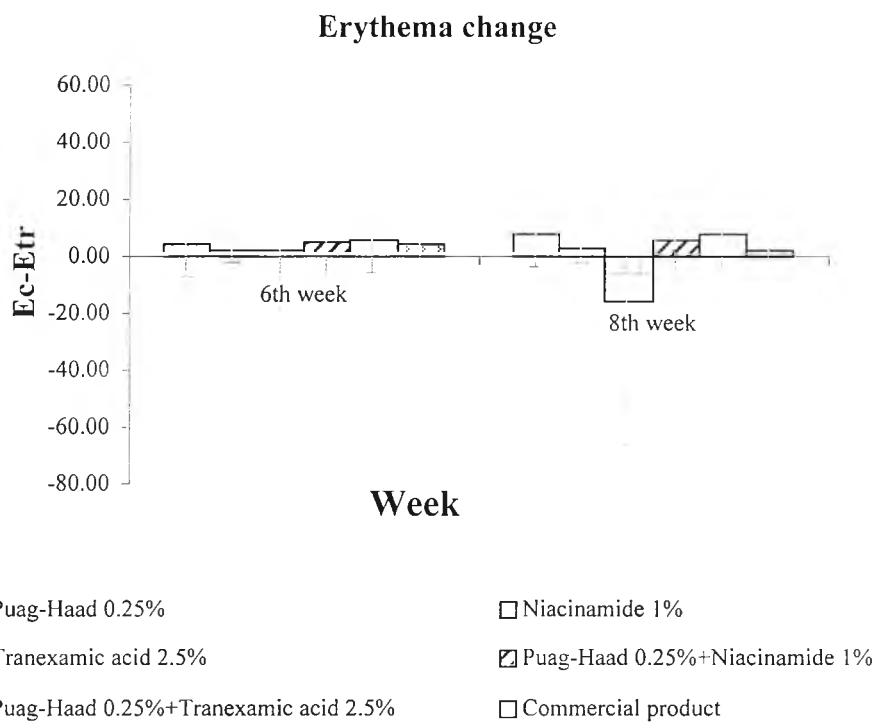


Figure 35. Comparison of erythema change (Ec-Etr) of each test substance in each week. Data = mean \pm SD (n = 6-7 /group)