CHAPTER IV

RESULTS AND DISCUSSION

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

The developed HPLC system was applied to analyze the extract from *Artocarpus lakoocha* heartwood extract (Puag-Haad). It was found that the extract had similar chromatogram to the standard oxyresveratrol. However, the extract (Lot no.25/10/04) gave the percent oxyresveratrol of 85.11±0.25 % w/w of the standard oxyresveratrol.

1.1 Validation of HPLC method

Validation of the analytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended analytical applications. The performance characteristics are expressed in terms of analytical parameters. For HPLC assay validation, these include specificity, linearity, accuracy and precision.

1.1.1 Specificity

The specificity of an analytical method is its ability to measure the analyte accurately and with specificity in the presence of other components in the sample.

The internal standard technique was performed by determining the peak area ratio of oxyresveratrol to furazolidone (internal standard) to give the complete seperation, appropiate resolution and sharp peaks of all components. The methanol-water mixture (40:60%v/v) was used as the mobile phase. The typical chromatograms of blank solution, internal standard solution, oxyresveratrol standard

solution, Puag Haad extract solution and resveratrol standard solution are shown in Figures 11-17. All chromatogram are shown under the same attenuation (9) and scale.

The retention times of the internal standard solution, oxyresveratrol standard solution, Puag Haad extract solution and resveratrol standard solution were around 5.51, 11.49, 11.47 and 19.45 min, respectively. In addition, there was no interference from other components in the chromatogram. Also, it is interesting to note that the Puag-Haad extract showed extremely small amount of resveratrol, as seen from the chromatograms in which of peak of resveratrol was practically absent (Figure 14 and 16).

Moreover, to ensure that the degraded product of oxyresveratrol did not interfere with the intact substance, the 5 samples of Puag-Haad solution were prepared by adding 2-3 drops of water, hydrogen peroxide, 3N hydrochloric acid, 5N sodium hydroxide and dry powder. The samples were kept under storage temperature at 80°C for 30 minutes and diluted with mobile phase (adapted from จุไรรัตน์ รักวาทิน, 2004). The solutions were examined for the chromatogram as shown in Figures 18-22. It is demonstrated the decreased of peak intensity of oxyresveratrol and showed no other peak at this wavelength.

1.1.2 Linearity

The linearity of an analytical method is the ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in the samples within a given range. The linearlity is usually expressed in terms of variance around the slope of the regression line calculated according to an established mathematical relationship from the test results obtained by the analysis of samples with varying concentration of analyte. The calibration curve data of oxyresveratrol standard solutions are shown in Table 7. The plot of oxyresveratrol concentrations versus the peak area ratios of oxyresveratrol to its internal standard furazolidone illustrated the linear correlation in the concentration range of 50-300 μg/ml (Figure 23). The coefficient of determination (R^2) of this line was 0.9995. These results indicated that the HPLC method was acceptable for quantitative analysis of oxyresveratrol in the range studied.

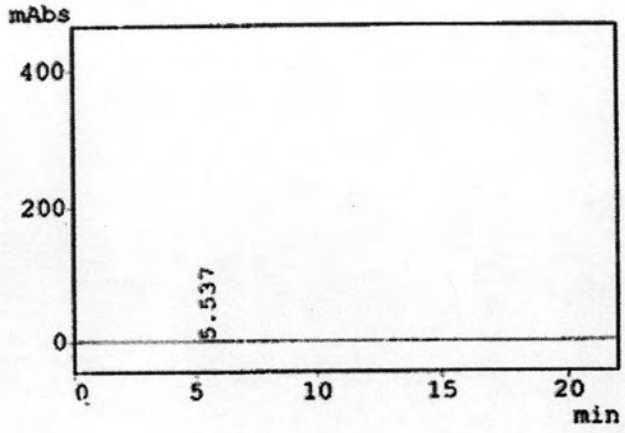

Figure 11. HPLC chromatogram of blank solution

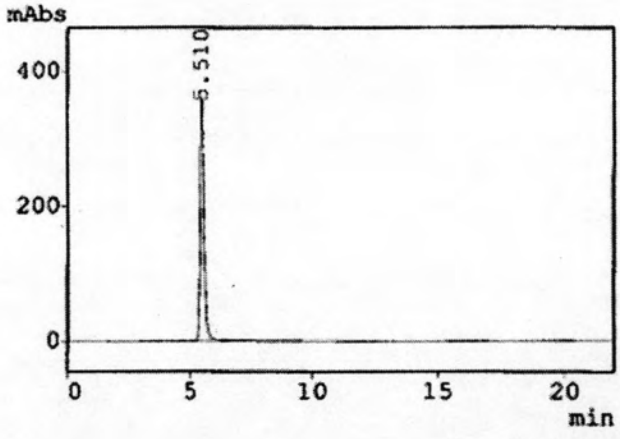

Figure 12. HPLC chromatogram of internal standard solution (furazolidone)

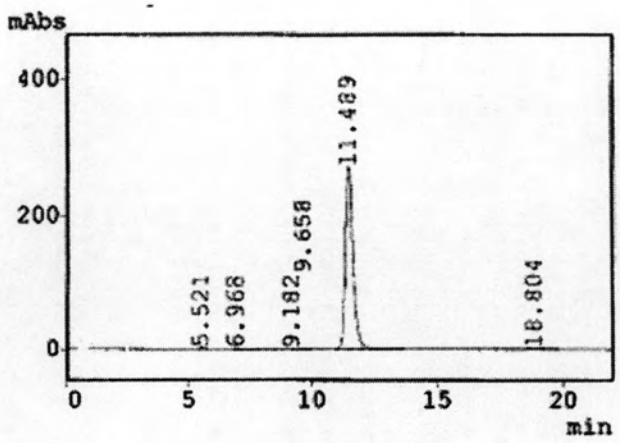

Figure 13. HPLC chromatogram of oxyresveratrol standard solution

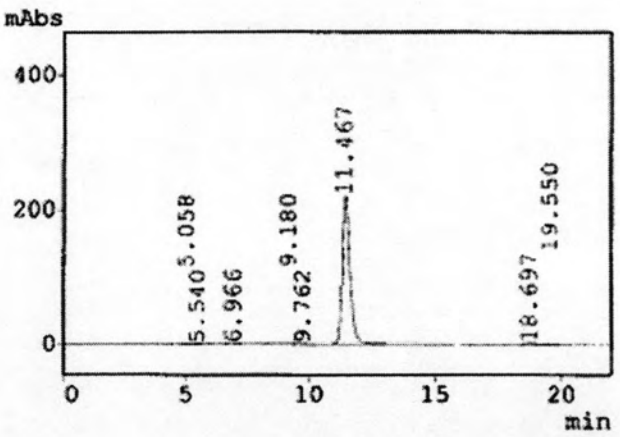

Figure 14. HPLC chromatogram of Puag Haad extract solution

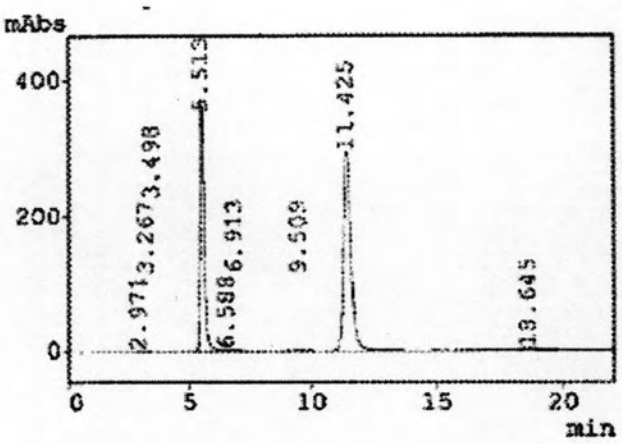

Figure 15. HPLC chromatogram of mixture of oxyresveratrol and internal standard

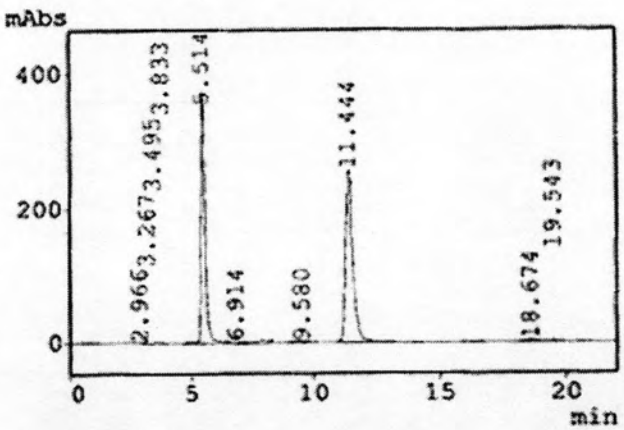

Figure 16. HPLC chromatogram of mixture of PuagHaad extract and internal standard

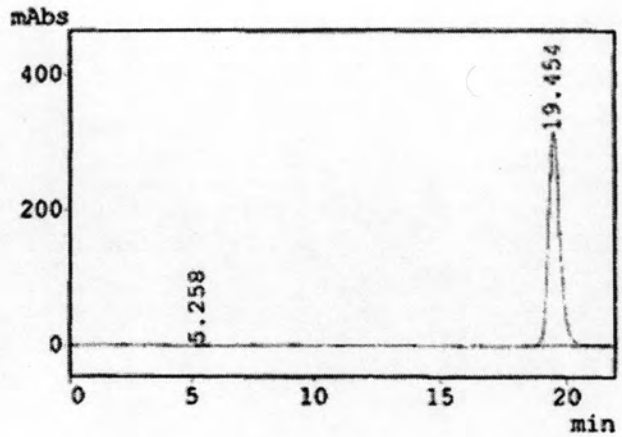

Figure 17. HPLC chromatogram of resveratrol standard solution

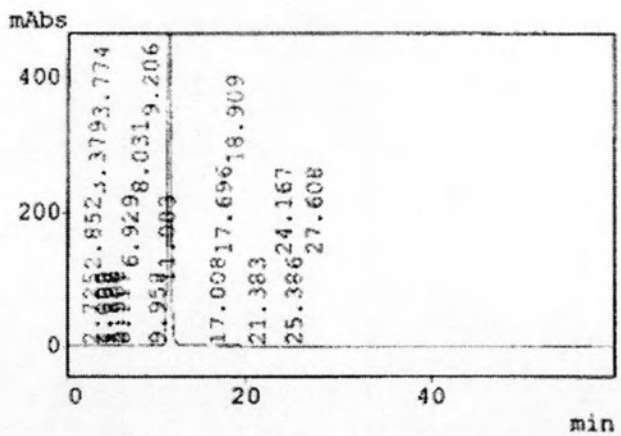

Figure 18. HPLC chromatogram of oxyresveratrol in water and storage at 80°C for 30 minutes

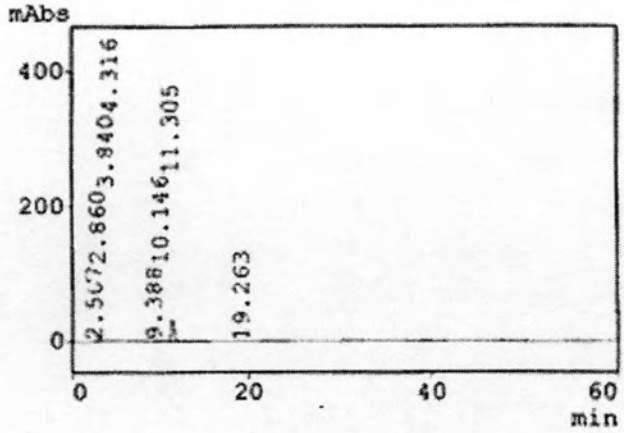

Figure 19. HPLC chromatogram of oxyresveratrol in hydrogen peroxide and storage at 80°C for 30 minutes

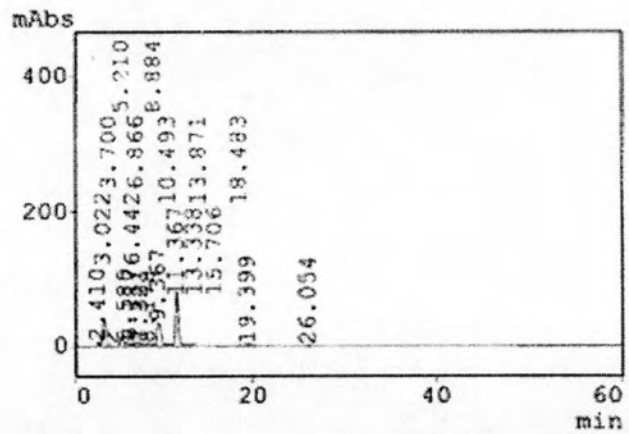

Figure 20. HPLC chromatogram of oxyresveratrol in 3N hydrochloric acid and storage at 80°C for 30 minutes

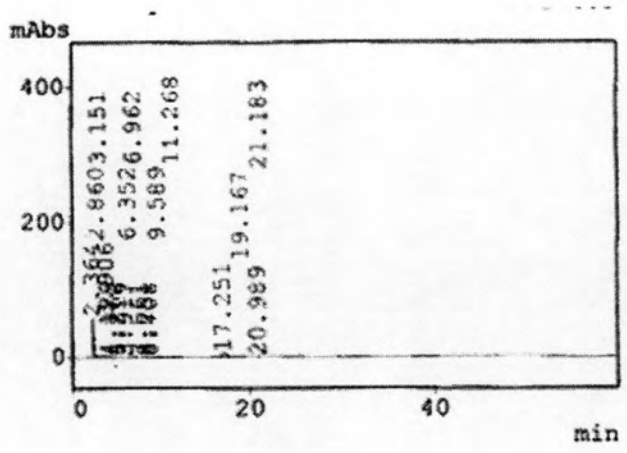

Figure 21. HPLC chromatogram of oxyresveratrol in 5N sodium hydroxide and storage at 80°C for 30 minutes

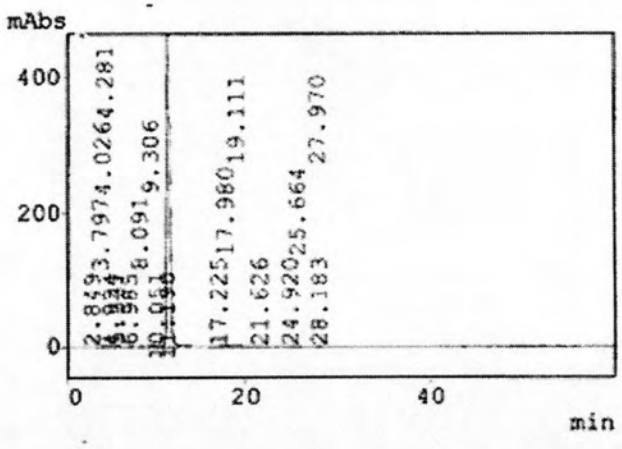

Figure 22. HPLC chromatogram of oxyresveratrol dry powder storage at 80°C for 30 minutes

Table 7. Data for calibration curve of oxyresveratrol by HPLC method

Concentration of oxyresveratrol (μg/ml)	Peak area ratio (oxyresveratrol to furazolidone)			Mean	SD
	Set 1	Set 2	Set3		
50	0.5609	0.5746	0.5460	0.5605	0.0143
100	1.2826	1.2642	1.2667	1.2712	0.0099
150	2.1370	2.0305	2.0708	2.0794	0.0538
200	2.8888	2.8498	2.9890	2.9092	0.0718
250	3.7963	3.6623	3.7181	3.7256	0.0674
300	4.4671	4.4365	4.5749	4.4928	0.0727

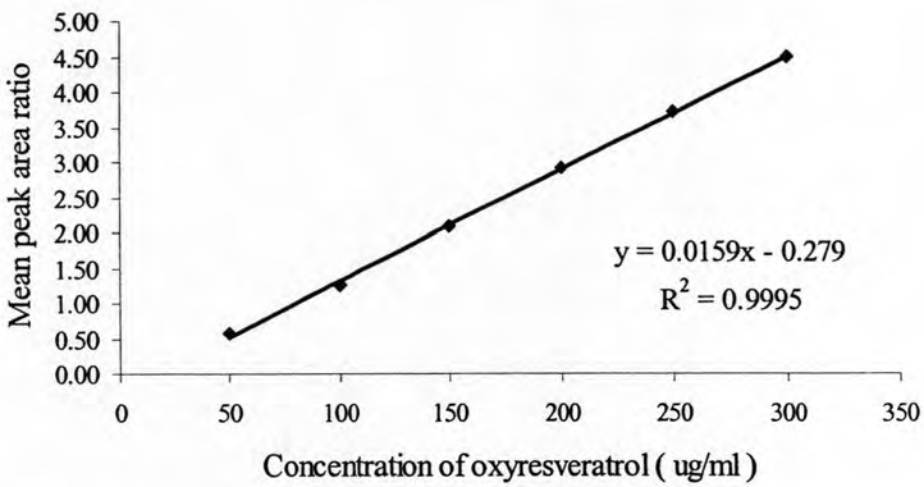

Figure 23. Calibration curve of oxyresveratrol by HPLC method

1.1.3 Accuracy

The determination of accuracy was performed by analyzing fives sets of three concentrations (60, 180, 240 μg/ml). The inversely estimated concentration and percentage of analytical recovery for each concentration are shown in Table 8 and Table 9, respectively. The percentages of analytical recovery were in the range of 100.71 – 101.01% , which indicated that this method could be used for analysis of oxyresveratrol at all concentrations studied with high accuracy.

Table 8. The inversely estimated concentrations of oxyresveratrol by HPLC method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean±SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
60	61.1314	60.9363	60.3908	59.2653	61.0596	60.5567±0.78
180	181.9336	179.2034	182.6173	182.0342	183.3076	181.8192±1.56
240	244.5473	242.3010	239.4484	241.6353	240.5377	241.6939±1.93

Table 9. The percentage of analytical recovery of oxyresveratrol by HPLC method

Concentration (μg/ml)	% Analytical recovery					Mean±SD
	Set 1	Set 2	Set 3	Set 4	Set 5	
60	101.89	101.56	100.65	98.78	101.77	100.93±1.30
180	101.07	99.56	101.45	101.13	101.84	101.01±0.87
240	101.89	100.96	99.77	100.68	100.22	100.71±0.80

1.1.4 Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation, %CV) of a series of measurements.

Tables 10 and 11 illustrate the data of with-run precision and between-run precision, respectively. All coefficient of variation values were small, 1.08-1.60% and 1.68-1.96%, respectively. The coefficient of variation of an analytical method should generally be less than 2% . Therefore, the HPLC method was precise for the quantitative analysis of oxyresveratrol in the range studied.

Table 10. Data of within-run precision by HPLC method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean	SD	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
60	59.5584	57.6760	58.4807	59.4951	59.3421	58.9105	0.81	1.38
180	175.8628	176.7381	176.3200	172.1742	176.5346	175.5259	1.90	1.08
240	247.9069	249.3759	244.3759	239.6761	242.3420	244.7039	3.92	1.60

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Table 11. Data of between-run precision by HPLC method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean	SD	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
60	59.5584	59.8603	60.3908	62.5068	59.9830	60.4599	1.18	1.96
180	175.8628	175.5227	182.6173	178.6975	174.1912	177.3783	3.36	1.89
240	247.9069	241.6933	239.4484	237.4126	243.8091	242.0541	4.06	1.68

In conclusion, the analysis of oxyresveratrol by HPLC method developed in this study showed good specificity, linearity, accuracy and precision. Thus this method was used for determination of the content of oxyresveratrol in the Puag-Haad extract to evaluate its stability.

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

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

The purpose of this part was to evaluate the physical and chemical stability of *Artocarpus lakoocha* heartwood extract. Because of the objective of this study was to formulate the lotion of *Artocarpus lakoocha*, the pH of the lotion must be compatible with human skin, so the pH range between 5.5-7 that is not harmful to human skin was selected to use in this study. Buffer system is chosen to maintain constant pH. Two types of buffers were chosen i.e, the citrate and phosphate buffers due to its popularity and compatibility with the skin. They were divided into three systems, namely, citrate buffer pH 5.5, phosphate buffer pH 5.5 and phosphate buffer pH 7 (citrate buffer pH 7 could not be prepared because the highest pKa (pKa3) of citric acid is 5.19). The buffer concentration chosen in this study was 50 mM because it was not too strong for the skin and provided adequate buffer capacity (วัชรี เนติสิงหะ, 1984). The freshly prepared Puag-Haad solution was also used as a control sample in every period. Solutions of Puag-Haad were prepared in mixture of either 20% propylene glycol and 80% water or 20% propylene glycol and 80% buffer . The test samples were coded as follows:

P = 0.10%w/v Puag-Haad in 20% propylene glycol and 80% water

P+ A1 = 0.10%w/v Puag-Haad in 20% propylene glycol and 80% 50 mM citrate buffer pH 5.5

P+ A2 = 0.10%w/v Puag-Haad in 20% propylene glycol and 80% 50 mM phosphate buffer pH 5.5

P+ A3 = 0.10%w/v Puag-Haad in 20% propylene glycol and 80% 50 mM phosphate buffer pH 7

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

2.1.1. Physical stability of *Artocarpus lakoocha* heartwood extract in three buffer systems

Table 12 shows changes in color of Puag-Haad sample upon storage at accelerated temperature (45°C) in dark condition (4 weeks). The initial color of 0.10% Puag-Haad solution was pale yellow (graded by number 0). Upon storage, the solution of pure Puag-Haad (solution P) gradually darkened to light brown (graded as +2) after 4 weeks. The color of all the stored Puag-Haad solutions was always compared with that of the freshly prepared 0.1% pure Puag-Haad. Only one sample, phosphate buffer pH 7 (P+A3), imparted a slightly darker yellow color in Puag-Haad solution at the initial time (week 0).

Addition of buffers, particularly citrate buffer pH 5.5 (P+A1) was able to protect 0.1% Puag-Haad from increased coloration after prolonged storage. As seen from Table 12, solution of P+A1 did not increase in color intensity after 4 weeks storage at accelerated temperature (45°C) while solution of pure Puag-Haad (P) became slightly brown (graded +2) after 4 weeks. On the other hand, solutions of Puag-Haad containing phosphate buffer (P+A2 and P+A3) did not protect Puag-Haad from discoloration. Solution of Puag-Haad in phosphate buffer pH 5.5 (P+A2) became brown (graded +3) and solution of Puag-Haad in phosphate buffer pH 7 (P+A3) became dark brown (graded +4) even at week 2. These results showed that the type of buffer had an effect on the change in color of Puag-Haad solutions. The citrate buffer especially at pH 5.5 can help stabilize the color along the study period

but the phosphate buffer failed to protect the discoloration at both pH value. Photographs of different Puag-Haad solutions taken after storage for 0, 2 and 4 weeks are also provided for visual comparison in Figure 24. Data from phosphate buffer pH 5.5 and 7 also suggested that pH also had on effect on the color stability, with pH 5.5 giving less change in color than pH 7.

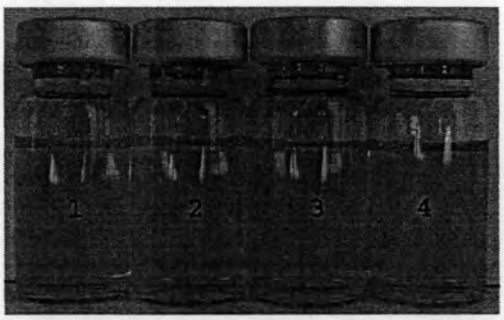
The pH of each solution was also measured in triplicate and the data are shown in Table 13. Pure Puag-Haad solutions showed a decreased in pH upon storage time, from 6.27 at week 0 to 5.67 at week 4, which was equivalent to a 0.59 unit drop in pH. Puag-Haad solutions with citrate and phosphate buffer (P+A1, P+A2 and P+A3) gave a slight decrease in pH from an initial value of 5.49 to 5.46 for P+A1, 5.48 to 5.43 for P+A2 and 6.96 to 6.86 for P+A3, which was equivalent to 0.02, 0.05 and 0.09 unit change in pH, respectively. A very little change in pH unit upon storage time was a result of its buffer capacity to maintain the pH of the solutions.

Table 12. Changes in color of Puag-Haad solutions upon storage at accelerated temperature (45°C)

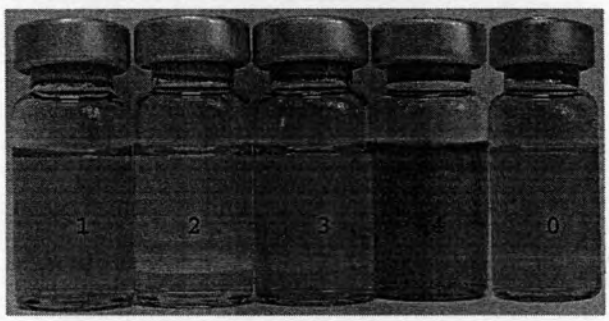
No.	Samples	Time (week)		
		0	2	4
0	Fresh P	0	0	0
1	P	0	+1	+2
2	P+A1	0	0	0
3	P+A2	0	+2	+3
4	P+A3	1	+4	+4

P = 0.1% Puag-Haad, A1 = 50 mM citrate buffer pH 5.5, A2 = 50 mM phosphate buffer pH 5.5 , A3 = 50 mM phosphate buffer pH 7

0 = normal (pale yellow); without change, +1 = slightly (light yellow) changed, +2 = noticeably (light brown) changed, +3 = markedly (brown) changed, +4 = seriously deteriorated (dark brown), +5 = almost or completely deteriorated (intense deep brown)



0 week



2 weeks

4 weeks

Figure 24. Physical appearance of Puag-Haad solutions in different buffers upon storage at accelerated temperature (45°C) for 4 weeks

0 = Freshly prepared 0.10% Puag-Haad , 1 = 0.10% Puag-Haad without buffer (P), 2 = P + 50 mM citrate buffer pH 5.5 (A1), 3 = P + 50 mM phosphate buffer pH 5.5 (A2), 4 = P + 50mM phosphate buffer pH 7 (A3)

Table 13. Changes in pH values of Puag-Haad solutions in different buffers upon storage at accelerated temperature (45°C) for 4 weeks

No.	Puag-Haad Samples	Time (week) 0	2	4	ΔpH
1	P	6.38 6.29 6.14	5.97 6.03 6.09	5.86 5.52 5.65	0.59
	Mean ± SD	6.27 ± 0.12	6.03 ± 0.06	5.67 ± 0.17	
2	P+A1	5.52 5.47 5.47	5.43 5.51 5.47	5.45 5.50 5.44	0.02
	Mean ± SD	5.49 ± 0.03	5.47 ± 0.04	5.46 ± 0.03	
3	P+A2	5.46 5.54 5.46	5.41 5.50 5.42	5.41 5.46 5.44	0.05
	Mean ± SD	5.49 ± 0.05	5.44 ± 0.05	5.44 ± 0.02	
4	P+A3	6.98 6.97 6.92	6.89 6.85 6.93	6.92 6.79 6.88	0.09
	Mean ± SD	6.96 ± 0.03	6.89 ± 0.04	6.86 ± 0.07	

P = 0.1% Puag-Haad, A1 = 50 mM citrate buffer pH 5.5, A2 = 50 mM phosphate buffer pH 5.5 , A3 = 50 mM phosphate buffer pH 7

2.1.2 Chemical stability of *Artocarpus lakoocha* heartwood extract in three buffer systems

The chemical stability of Puag-Haad solutions were determined by HPLC method at initial time and at week 2 and 4. Table 14 shows percent oxyresveratrol remaining (relative to initial value) in different Puag-Haad solutions during storage at 45 °C. After 4 weeks, the percent oxyresveratrol remaining in pure Puag-Haad (P) decreased to 73.22% whereas in P+A1, P+A2 and P+A3 were 91.77%, 75.59% and 36.39%, respectively. The value of percent oxyresveratrol are also graphically represented for each sample in Figure 25. These results showed that P+A1 could provide the highest content of oxyresveratrol in agreement with the result of color stability (Table 12 and Figure 24), in which no color change was observed after 4-week storage. Therefore, citrate buffer pH 5.5 appeared to provide the best protection in terms of both the physical and chemical stability. On the other hand, the degradation extent of oxyresveratrol in P+A2 seemed to be similar to pure Puag-Haad (P), suggesting that the phosphate buffer pH 5.5 failed to protect oxyresveratrol from chemical degradation as well as to prevent the solution from discoloration.

Furthermore, the degradation of oxyresveratrol in P+A3 solution, which contained the same phosphate buffer as P+A2 but at higher pH value (7.0), was even more rapid, resulting in more than 60% loss of oxyresveratrol after 4 weeks in line with the dark brown color (graded +4) of the solution. Thus, the phosphate buffer not only failed to provide protection against chemical degradation and discoloration, it even promoted them especially at higher pH. This could be due to the general base catalysis exerted by the phosphate species.

It could be assumed from the above observations that the condition that could stabilize both the physical and chemical stability of Puag-Haad solutions was a slightly acidic pH using citrate buffer pH 5.5. To see if a more acidic condition could provided further protection against degradation, stability study of Puag-Haad solution in citrate buffer pH 4.0 was carried out in the same manner. The percent of oxyresveratrol after 4 weeks was found to be 91.32% which was almost identical to the citrate buffer pH 5.5. Also the color change was similar to pH 5.5. Thus, the present data suggested that optimum pH for Puag-Haad physical and chemical stability was about 4-5.5 using citrate buffer. However, more extensive studies especially the pH-rate profile are required to elucidate the mechanisms of oxyresveratrol degradation, and how different buffer species and pH can protect against or promote its degradation. Nevertheless, the pH value of 5.5 was selected as the pH of chiose for the subsequence studies due to its compatibility with the human skin pH and ability to retain skin acid mantle (Gil and Howard, 1996).

Data on pH change in Table 13 also suggested that as the oxyresveratrol and other components in Puag-Haad deteriorated, the degradation product(s) could be acidic as there was a drop in pH about 0.59 from the initial pH of the pure Puag-Haad solution (P). These degradation product(s) also made the solution more discolored. However, when the buffer had been added to Puag-Haad solutions (P+A1, P+A2, P+A3), the drop in pH became minimal regardless of the type of buffer and the extent of oxyresveratrol degradation. The same concentration of citrate and phosphate buffer (50 mM) was always used thereby providing the Puag-Haad solutions with similar buffer capacity.

Table 14. Percent oxyresveratrol remaining (relative to initial value) in Puag-Haad solutions at accelerated temperature (45°C) (Mean ± SD, n=3)

Time	% oxyresveratrol remaining			
(week)	P	P+A1	P+A2	P+A3
0	100.00	100.00	100.00	100.00
(SD)	0.53	1.55	2.19	3.39
2	84.08	97.02	84.77	56.52
(SD)	2.69	3.01	5.46	2.12
4	73.22	91.77	75.59	36.39
(SD)	1.35	2.47	2.88	1.73

P = 0.1% Puag-Haad, A1 = 50 mM citrate buffer pH 5.5, A2 = 50 mM phosphate buffer pH 5.5 , A3 = 50 mM phosphate buffer pH 7

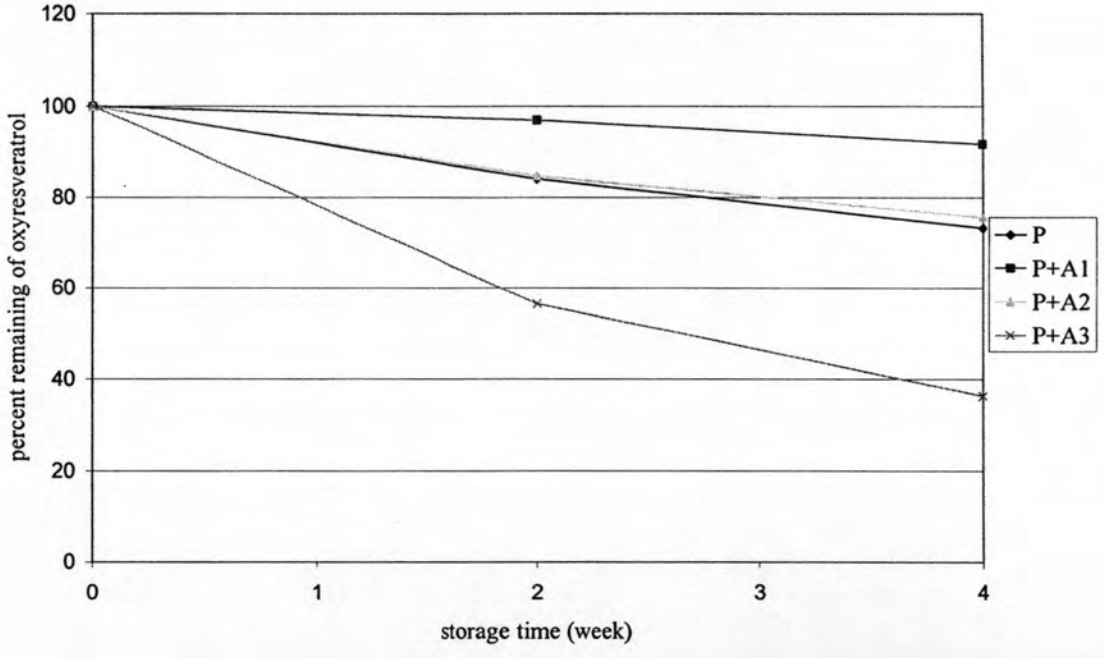


Figure 25. Percent oxyresveratrol remaining (relative to initial value) in Puag-Haad solutions at accelerated temperature (45°C)

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

Solutions of Puag-Haad at 0.10% w/v concentration dissolved in the mixture of 20% v/v propylene glycol and 80 % v/v 50 mM citrate buffer pH 5.5 (selected from 2.1) were chosen for stability test. The purpose of this part was to evaluate the physical and chemical stability of Puag-Haad solutions in the presence of various antioxidants upon storage at accelerated temperature (45°C).

Since the active component of Puag-Haad (2,4,3',5'-tetrahydroxystillbene) is a polyphenol derivative, it was thus expected to be degraded by oxidation (Tiptabiankarn, 1967). Solutions of 0.10% Puag-Haad with and without the four antioxidants (sodium metabisulfite, butylated hydroxyanisole (BHA), propyl gallate and ethylenediaminetetraacetic acid (EDTA)) as well as their combination, were prepared in the same solvent (20% propylene glycol and 80% v/v 50 mM citrate buffer pH 5.5). The freshly prepared Puag-Haad solution in 20% propylene glycol: 80% water (without buffer) was also used as control sample in every period. The test samples were as follows:

Sample No.	Puag-Haad samples	50 mM citrate buffer pH 5.5	0.10% sodium metabisulfite	0.02% BHA	0.10% PG	0.05% EDTA
1	P	-	-	-	-	-
2	PB	√	-	-	-	-
3	P+A1	-	√ (0.15%)*	-	-	-
4	P+A2	-	√	-	-	-
5	PB+A1	√	√ (0.15%)*	-	-	-
6	PB+A2	√	√	-	-	-
7	PB+A3	√	-	√	-	-
8	PB+A4	√	-	-	√	-
9	PB+A5	√	-	-	-	√
10	PB+A2+A3	√	√	√	-	-
11	PB+A2+A4	√	√	-	√	-
12	PB+A2+A5	√	√	-	-	√
13	PB+A3+A4	√	-	√	√	-
14	PB+A3+A5	√	-	√	-	√
15	PB+A4+A5	√	-	-	√	√
16	PB+A2+A3+A4	√	√	√	√	-
17	PB+A2+A3+A5	√	√	√	-	√
18	PB+A2+A4+A5	√	√	-	√	√
19	PB+A3+A4+A5	√	-	√	√	√
20	PB+A2+A3+A4+A5	√	√	√	√	√

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

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 20% propylene glycol and 80% v/v 50 mM citrate buffer pH 5.5, A1 = 0.15% sodium metabisulfite, A2 = 0.10% sodium metabisulfite, A3 = 0.02% BHA, A4 = 0.10% propyl gallate, A5 = 0.05% EDTA

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 0.10% (Wachiranuntasin, 2005) and 0.15% (Pengrungruangwong, 2001), butylated hydroxyanisole (BHA) at 0.02% and propyl gallate at 0.10% were used as 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 as a single agent as well as in double and triple combination to see if there was any enhancement in the protective effect against oxidation.

The selection of sodium metabisulfite and BHA as stabilizer of Puag-Haad solution was based on the previous result by Pengrungruangwong (2001), who reported that combination of the two antioxidants provided the best stabilization of 0.25% Puag-Haad solution in term of both the color and antityrosinase activity. In addition, Wachiranuntasin (2005) also reported the same result in term of the color stability. Propyl gallate was selected in this study because it provided a synergistic effect when used in combination with BHA (Kibbe, 2000), whereas EDTA was used as chelating agent in this study.

2.2.1. Physical stability of aqueous solutions of *Artocarpus lakoocha* heartwood extract in the presence of various antioxidants

Table 15 shows changes in color of Puag-Haad samples upon storage at 45 °C in dark condition for 4 weeks. The initial color of all samples was pale yellow (graded by number 0). Upon storage time, the solution of pure Puag-Haad (P) gradually darkened to dark brown (graded as +3) after 4 weeks, whereas solution of Puag-Haad in citrate buffer pH 5.5 (PB) became light brown (graded as +1) after 4 weeks due to color stabilization by citrate buffer from the result of 2.1. The color of

all stored Puag-Haad solutions was always compared with that of a freshly prepared 0.10% pure Puag-Haad (P).

Addition of antioxidants, particularly 0.15 % and 0.10% sodium metabisulfite was able to protect Puag-Haad from increased coloration after prolong storage. From Table 15, P+A1, P+A2, PB+A1 and PB+A2 had no changed in color (graded as 0) after 4 weeks storage. It could be observed that A1 (0.15% sodium metabisulfite) did not provide furthur protection in discoloration than A2 (0.10% sodium metabisulfite), so it is not necessary to use the higher concentration of sodium metabisulfite.

Samples no.10,11,12,16,17,18,and 20 also did not increased in color (graded as 0) after 4 weeks storage. All these solutions contain 0.10% sodium metabisulfite as a common antioxidant. These result agreed with Pengrungruangwong (2001) and Wachiranuntasin (2005), who observed that sodium metabisulfite was the best stabilizer especially against discoloration. Sodium metabisulfite has been reported to be an effective anti-browning agent and is used in many pharmaceutical and cosmetic preparations (Kibbe, 2000). On the other hand, 0.02% BHA, 0.10% propyl gallate and 0.05% EDTA alone (PB+A3, PB+A4 and PB+A5) hardly protected Puag-Haad from discoloration. After 4 weeks storage, these solutions became light brown in color but were better than pure Puag-Haad solution (P) and equally to PB which contained only citrate buffer as stabilizer. This suggested that BHA, propyl gallate and EDTA could not provide additional color stabilization over PB.

For double combination of antioxidants, the color of 0.02% BHA + 0.10% propyl gallate (PB+A3+A4) and 0.02% BHA + 0.05% EDTA (PB+A3+A5) became light brown after 4 weeks (graded as +2) and the color did not differ from the samples that contained single antioxidant, whereas the color of 0.10% propyl gallate + 0.05% EDTA (PB+A4+A5) becamed darkened than used alone, equally to pure Puag-Haad (graded as +3). Similar result was also observed in PB+A3+A4+A5, in which 0.02% BHA was added. Thus, BHA can not help further stabilize the darkened color of propyl gallate and EDTA combination. It might be that the darkened color of Puag-Haad solutions in the presence of these two antioxidant were caused by the color complex formation between propyl gallate and sodium in EDTA (EDTA that was used in this study was sodium form)(Kibbe, 2000). Citric acid can be used as sequestering agent to solve this problem. In this study, however, we also had citric acid as buffering agent. It was possible that the concentration of citric acid used may be too low to protect the color complex formation.

For triple combination (PB+A2+A3+A4, PB+A2+A3+A5 and PB+A2+A4+A5) did not change in color after 4 weeks because sodium metabisulfite was included in these samples, whereas the color of PB+A3+A4+A5 became brown due to the absence of sodium metabisulfite. PB+A2+A3+A4+A5 that contained all four antioxidants did not change in color along the storage time. Photographs of different Puag-Haad solutions taken after 2 and 4 weeks are also provided for visual comparison in Figures 26. 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 are given in Table 16. Pure Puag-Haad solution without buffer (P) showed minor decrease in pH upon storage at 45 °C, from 6.27 at 0 week to 5.68 at 4 weeks, which was equivalent to 0.59 unit drop in pH. This value agreed with that of Pengrungruangwong (2001), who also reported a pH drop of 0.74 after 4 week-storage at 45 °C for 0.25% pure Puag-Haad solution. She also reported that addition of sodium metabisulfite caused a further drop in pH of Puag-Haad solution (about 1.40 decrement), whereas BHA or EDTA had very slight effect on the pH. Since her study did not use the buffer in the medium. The aim of this part was to evaluate the effect of buffer (citrate pH 5.5) in the presence and absence of different antioxidants on the pH stability of Puag-Haad solutions. To compare the effect of sodium metabisulfite more clearly, Puag-Haad solutions containing sodium metabisulfite were also prepared in both the buffered and non-buffered medium.

As seen from Table 16, all Puag-Haad solutions containing citrate buffer pH 5.5 were able to maintain pH throughout the 4-week study period, either alone (PB) or in the presence of different antioxidants. The extent of pH drop was very small within the range of 0.02 – 0.05 indicating a very good buffer capacity of citrate buffer pH 5.5. On the other hand, Puag-Haad with either 0.15% or 0.10% sodium metabisulfite in non-buffered medium (P + A1, P + A2), resulted in greater drop in pH than the pure Puag-Haad (P), in agreement with Pengrungruangwong (2001). Adding the citrate buffer to these solutions resulted in near complete reversal of pH drop as seen in PB + A1 and PB + A2. To further clarify the contribution of the individual antioxidants on the pH of Puag-Haad, pure solutions of sodium metabisulfite (0.15 and 0.10%), 0.02% BHA, 0.10% propyl gallate and 0.05% EDTA in 20% propylene glycol: 80% water were prepared and similarly kept at 45°C. The values of pH are shown in Table 17. From this table, it can be seen that only sodium metabisulfite (A1

and A2) could contribute to further drop in pH of Puag-Haad solutions whereas the rest of the antioxidants did not seem to affect the pH, indicating that BHA, propyl gallate and EDTA were quite stable in the solution for at least up to 4 weeks.

The results from this part agreed with Pengrungruangwong (2001), who suggested that 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., 1986). This reaction has obviously led to a drop in pH of the solution regardless of whether Puag-Haad was present or not. Nevertheless, future studies are required to elucidate the mechanisms by which sodium metabisufite or its degraded products can interact with the components of Puag-Haad extract. Also, a longer study period may be necessary since Pengruangruangwong (2001) could detect difference in pH stability between BHA and EDTA after 12 week-storage at 45 °C, in which EDTA induced a much smaller drop in pH than BHA, probably as a result of its buffering capacity.

Table 15. Changes in color of Puag-Haad solutions in the presense of various antioxidants upon storage at accelerated temperature (45°C)

No.	Puag-Haad samples	Time (week)		
		0	2	4
0	Fresh P	0	0	0
1	P	0	+3	+3
2	PB	0	+1	+2
3	P+A1	0	0	0
4	P+A2	0	0	0
5	PB+A1	0	0	0
6	PB+A2	0	0	0
7	PB+A3	0	+1	+2
8	PB+A4	0	+1	+2
9	PB+A5	0	+1	+2
10	PB+A2+A3	0	0	0
11	PB+A2+A4	0	0	0
12	PB+A2+A5	0	0	0
13	PB+A3+A4	0	+1	+2
14	PB+A3+A5	0	+1	+2
15	PB+A4+A5	0	+2	+3
16	PB+A2+A3+A4	0	0	0
17	PB+A2+A3+A5	0	0	0
18	PB+A2+A4+A5	0	0	0
19	PB+A3+A4+A5	0	+2	+3
20	PB+A2+A3+A4+A5	0	0	0

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, A1 = 0.15% sodium metabisulfite, A2 = 0.10% sodium metabisulfite, A3 = 0.02% BHA, A4 = 0.10% propyl gallate, A5 = 0.05% EDTA

0 = normal (pale yellow); without change, +1 = slightly (light yellow) changed, +2 = noticeably (light brown) changed, +3 = markedly (brown) changed, +4 = seriously deteriorated (dark brown), +5 = almost or completely deteriorated (intense deep brown)

0 week

2 week

4 week

Figure 26. Physical appearance of Puag-Haad solutions in the presense of various antioxidants upon storage at accelerated temperature (45°C) for 4 weeks

0 = Fresh P, 1 = P, 2 = PB, 3 = P+A1, 4 = P+A2, 5 = PB+A1, 6 = PB+A2, 7 = PB+A3, 8 = PB+A4, 9 = PB+A5, 10 = PB+A2+A3, 11 = PB+A2+A4, 12 = PB+A2+A5, 13 = PB+A3+A4, 14 = PB+A3+A5, 15 = PB+A4+A5, 16 = PB+A2+A3+A4, 17 = PB+A2+A3+A5, 18 = PB+A2+A4+A5, 19 = PB+A3+A4+A5, 20 = PB+A2+A3+A4+A5

Table 16. Changes in pH values of Puag-Haad solutions in in the presense of various antioxidants upon storage at accelerated temperature (45°C) for 4 weeks

No.	Puag-Haad samples	Time (week)			ΔpH
		0	2	4	
1	P	6.38 6.29 6.14	5.97 6.03 6.06	5.86 5.52 5.65	0.59
	Mean ± SD	6.27±0.12	6.03±0.06	5.68±0.17	
2	PB	5.48 5.49 5.48	5.45 5.48 5.46	5.44 5.47 5.46	0.02
	Mean ± SD	5.48±0.01	5.46±0.02	5.46±0.02	
3	P+A1	6.20 6.24 6.18	5.25 5.20 5.24	5.02 5.08 5.05	1.16
	Mean ± SD	6.21±0.03	5.23±0.03	5.05±0.03	
4	P+A2	6.25 6.22 6.20	5.32 5.28 5.15	5.20 5.14 5.04	1.09
	Mean ± SD	6.22±0.03	5.25±0.09	5.13±0.08	
5	PB+A1	5.50 5.52 5.48	5.48 5.50 5.50	5.45 5.48 5.45	0.04
	Mean ± SD	5.50±0.02	5.49±0.01	5.46±0.02	
6	PB+A2	5.47 5.52 5.51	5.47 5.49 5.48	5.46 5.50 5.46	0.03
	Mean ± SD	5.50±0.03	5.48±0.01	5.47±0.02	
7	PB+A3	5.50 5.49 5.48	5.48 5.48 5.50	5.49 5.47 5.46	0.02
	Mean ± SD	5.49±0.01	5.49±0.01	5.47±0.02	
8	PB+A4	5.48 5.49 5.48	5.45 5.48 5.46	5.44 5.47 5.46	0.02
	Mean ± SD	5.48±0.01	5.49±0.02	5.46±0.02	
9	PB+A5	5.49 5.52 5.50	5.48 5.47 5.50	5.45 5.47 5.48	0.03
	Mean ± SD	5.50±0.02	5.48±0.02	5.47±0.02	
10	PB+A2+A3	5.49 5.50 5.49	5.47 5.46 5.48	5.44 5.48 5.47	0.03
	Mean ± SD	5.49±0.01	5.47±0.01	5.46±0.02	
11	PB+A2+A4	5.51 5.50 5.48	5.48 5.49 5.47	5.48 5.45 5.46	0.04
	Mean ± SD	5.50±0.02	5.48±0.01	5.46±0.02	
12	PB+A2+A5	5.48 5.50 5.50	5.47 5.52 5.51	5.49 5.47 5.46	0.02
	Mean ± SD	5.49±0.01	5.50±0.03	5.47±0.02	
13	PB+A3+A4	5.48 5.50 5.48	5.48 5.47 5.50	5.48 5.45 5.46	0.03
	Mean ± SD	5.49±0.01	5.48±0.02	5.46±0.02	
14	PB+A3+A5	5.52 5.50 5.48	5.48 5.48 5.47	5.48 5.47 5.45	0.03
	Mean ± SD	5.50±0.02	5.48±0.01	5.47±0.02	

No.	Puag-Haad Samples	Time (week)			ΔpH
		0	2	4	
15	PB+A4+A5	5.49 5.52 5.50	5.49 5.49 5.47	5.45 5.48 5.47	0.03
	Mean ± SD	5.50±0.02	5.48±0.01	5.47±0.02	
16	PB+A2+A3+A4	5.48 5.52 5.49	5.49 5.50 5.48	5.47 5.48 5.48	0.02
	Mean ± SD	5.50±0.02	5.49±0.01	5.48±0.01	
17	PB+A2+A3+A5	5.50 5.50 5.51	5.48 5.47 5.49	5.45 5.46 5.48	0.04
	Mean ± SD	5.50±0.01	5.48±0.01	5.46±0.02	
18	PB+A2+A4+A5	5.53 5.51 5.51	5.49 5.50 5.48	5.46 5.48 5.48	0.05
	Mean ± SD	5.52±0.01	5.49±0.01	5.47±0.01	
19	PB+A3+A4+A5	5.52 5.50 5.48	5.50 5.47 5.49	5.48 5.47 5.47	0.03
	Mean ± SD	5.50±0.02	5.49±0.02	5.47±0.01	
20	PB+A2+A3+A4+A5	5.48 5.50 5.52	5.49 5.48 5.49	5.47 5.47 5.48	0.03
	Mean ± SD	5.50±0.02	5.49±0.01	5.47±0.01	

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, A1 = 0.15% sodium metabisulfite, A2 = 0.10% sodium metabisulfite, A3 = 0.02% BHA, A4 = 0.10% propyl gallate, A5 = 0.05% EDTA

Table 17. Changes in pH values of antioxidants upon storage at accelerated temperature (45°C) for 4 weeks

No.	Antioxidants	Time (week) 0	2	4	ΔpH
1	A1	6.24 6.22 6.23	5.15 5.20 5.13	4.88 4.95 4.92	1.31
	Mean ± SD	6.23±0.01	5.16±0.04	4.92±0.04	
2	A2	6.22 6.20 6.20	5.24 5.32 5.29	5.04 5.09 5.02	1.16
	Mean ± SD	6.21±0.01	5.28±0.04	5.05±0.04	
3	A3	6.47 6.52 6.45	6.45 6.48 6.44	6.44 6.47 6.45	0.03
	Mean ± SD	6.48±0.04	6.46±0.02	6.45±0.02	
4	A4	6.45 6.50 6.48	6.46 6.48 6.48	6.45 6.47 6.45	0.02
	Mean ± SD	6.48±0.03	6.47±0.01	6.46±0.01	
5	A5	6.02 6.08 6.05	5.98 6.04 6.04	6.01 6.03 6.02	0.03
	Mean ± SD	6.05±0.03	6.02±0.03	6.02±0.01	

A1 = 0.15% sodium metabisulfite, A2 = 0.10% sodium metabisulfite, A3 = 0.02% BHA, A4 = 0.10% propyl gallate, A5 = 0.05% EDTA

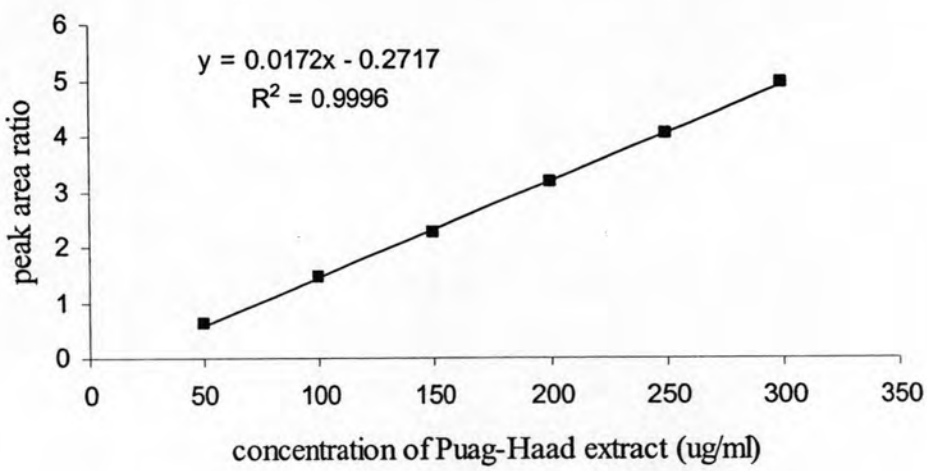
The medium = 20% propylene glycol and 80% water

2.2.2. Chemical stability of aqueous solutions of *Artocarpus lakoocha* heartwood extract in the presence of various antioxidants

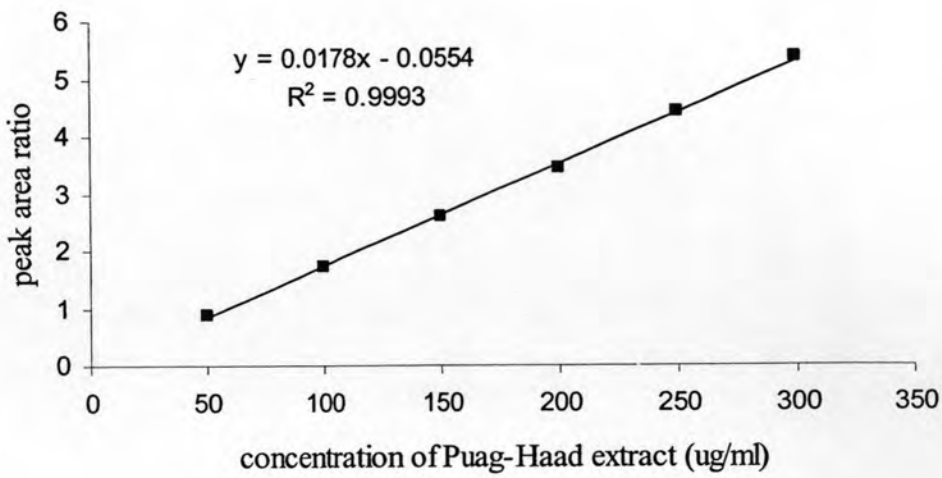
The chemical stability of Puag-Haad solutions with various antioxidants were determined by HPLC method at initial time and at week 2 and 4. The chromatograms of the individual antioxidants are shown in Appendix A. The results showed no interferences from other components in the chromatograms. Table 18 shows percent oxyresveratrol remaining in Puag-Haad solutions in the presence of various antioxidants at accelerated temperature (45°C) for 4 weeks. From this data, it is interesting to note that the initial value of percent oxyresveratrol remaining in PB+A4, PB+A2+A4, PB+A3+A4, PB+A4+A5, PB+A2+A3+A4, PB+A2+A4+A5, PB+A3+A4+A5 and PB+A2+A3+A4+A5 , all of which contained 0.10% propyl gallate, appeared to be higher than other solutions, although the peak of propyl gallate did not visibly interfere with the peak of oxyresveratrol (Figure A6, Appendix A). To see if propyl gallate could somehow interfere with the peak area of oxyresveratrol, standard curves of Puag-Haad extract with and without propyl gallate were carried out. As seen from Figure 27b which contained propyl gallate, the standard curve gave a higher slope (0.0178) than Figure 27a which had no propyl gallate (0.0172) and also gave a higher peak area ratio (PAR). From these results, it is possible that propyl gallate had an interaction with oxyresveratrol but the mechanism was not clearly known. However, it was assumed that the extent of interference was similar in all propyl gallate containing solutions.

The value of the absolute percent oxyresveratrol remaining were subsequently normalized to percent oxyresveratrol remaining relative to the initial value (Table 19). It was found that the percent oxyresveratrol remaining in pure Puag-Haad (P) decreased to 71.19% . Puag-Haad solutions that gave the high content of oxyresveratrol were PB, PB+A3, PB+A4, PB+A5, PB+A3+A4 and PB+A3+A5, the percent oxyresveratrol remaining was in the range of 78.15 to 89.45%. These solutions contained 0.02% BHA, 0.10% propyl gallate and 0.05% EDTA as antioxidants. In contrast, the oxyresveratrol content of PB+A4+A5 and PB+A3+A4+A5 that also contained BHA, propyl gallate and EDTA dropped to 38.74% and 45.45%, respectively. This result might be related with the result of color stability (Figure 26), in which change in color to markedly brown was observe after 4-week storage due to color complex formation between propyl gallate and EDTA. It is possible that the color complex formation might accelerate the degradation of Puag-

Haad solutions resulting in loss of oxyresveratrol and increased coloration. The values of percent oxyresveratrol are also graphically represented for each sample in Figure 28. Results from Table 19 and Figure 28 also suggested that the use of multiple antioxidants may not provide better chemical stability than the single antioxidant. The use of 0.02% BHA in citrate buffer (PB+A3) appeared to provide the best stabilization, followed by EDTA (PB+A5) and propyl gallate (PB+A4). In fact, only the citrate buffer (PB) was already to give substantial stabilization.



(a) without propyl gallate



(b) with propyl gallate

Figure 27. Standard curve of Puag-Haad extract with and without propyl gallate

Table 18. Percent oxyresveratrol remaining in Puag-Haad solutions in the presence of various antioxidants at accelerated temperature (45°C) (Mean ± SD, n=3)

Time	% oxyresveratrol remaining (% w/v)									
(weeks)	P	PB	P+A1	P+A2	PB+A1	PB+A2	PB+A3	PB+A4	PB+A5	PB+A2+A3
0	80.57	83.29	84.13	82.10	81.81	81.81	82.77	91.99	82.40	83.56
(SD)	0.69	1.58	1.93	0.65	2.08	0.42	2.17	3.00	1.16	1.18
2	67.69	74.15	39.53	50.15	18.73	26.56	78.38	84.49	76.57	44.98
(SD)	1.56	2.86	0.35	1.77	0.53	0.41	2.29	0.45	0.74	0.47
4	57.35	70.75	25.19	38.59	13.50	20.38	74.00	76.40	73.00	41.32
(SD)	0.31	0.47	0.18	0.35	0.03	0.03	0.15	0.47	0.34	0.90

Time	% oxyresveratrol remaining (%w/v)									
(weeks)	PB+A2+A4	PB+A2+A5	PB+A3+A4	PB+A3+A5	PB+A4+A5	PB+A2+A3+A4	PB+A2+A3+A5	PB+A2+A4+A5	PB+A3+A4+A5	PB+A2+A3+A4+A5
0	92.40	83.10	94.83	84.72	92.99	100.45	81.77	98.06	96.05	102.55
(SD)	1.43	0.95	2.09	1.02	0.87	0.94	1.42	2.64	0.83	3.19
2	42.41	49.06	82.50	75.66	62.99	52.99	58.62	33.82	64.88	40.47
(SD)	0.24	0.57	1.64	1.61	3.59	0.83	0.27	0.76	2.31	1.94
4	36.85	44.03	74.09	72.58	36.02	42.89	48.78	28.24	43.66	33.71
(SD)	0.93	0.34	0.37	1.16	0.45	0.45	0.60	0.12	0.23	0.35

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, A1 = 0.15% sodium metabisulfite, A2 = 0.10% sodium metabisulfite, A3 = 0.02% BHA, A4 = 0.10% propyl gallate, A5 = 0.05% EDTA

Table 19. Normalized percent oxyresveratrol remaining in Puag-Haad solutions in the presence of various antioxidants at accelerated temperature (45°C) (Mean ± SD, n=3). Extent of discoloration after 4 weeks are also provided for comparison

Time (weeks)	% oxyresveratrol remaining									
	P	PB	P+A1	P+A2	PB+A1	PB+A2	PB+A3	PB+A4	PB+A5	PB+A2+A3
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
(SD)	0.69	1.58	1.93	0.65	2.08	0.42	2.17	3.00	1.16	1.18
2	84.02	89.06	47.00	61.07	22.89	32.47	94.69	91.92	92.93	53.84
(SD)	2.66	4.29	0.67	1.70	0.24	0.62	0.91	3.31	1.17	0.86
4	71.19	84.97	29.96	47.01	16.51	24.91	89.45	83.13	88.61	49.45
(SD)	1.00	1.27	0.86	0.58	0.44	0.16	2.55	3.18	1.58	0.40
Degree of discoloration	+3	+2	0	0	0	0	+2	+2	+2	0

Time (weeks)	% oxyresveratrol remaining									
	PB+A2+A4	PB+A2+A5	PB+A3+A4	PB+A3+A5	PB+A4+A5	PB+A2+A3+A4	PB+A2+A3+A5	PB+A2+A4+A5	PB+A3+A4+A5	PB+A2+A3+A4+A5
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
(SD)	1.43	0.95	2.09	1.02	0.87	0.94	1.42	2.64	0.83	3.19
2	45.91	59.04	87.00	89.30	67.72	52.75	71.70	34.52	67.57	39.50
(SD)	0.91	1.20	1.65	1.53	3.26	0.87	1.20	1.70	2.99	2.50
4	39.90	52.98	78.15	85.66	38.74	42.70	59.67	28.81	45.45	32.89
(SD)	1.61	0.26	1.79	0.88	0.64	0.49	1.53	0.76	0.53	1.11
Degree of discoloration	0	0	+2	+2	+3	0	0	0	+3	0

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, A1 = 0.15% sodium metabisulfite, A2 = 0.10% sodium metabisulfite, A3 = 0.02% BHA, A4 = 0.10% propyl gallate, A5 = 0.05% EDTA

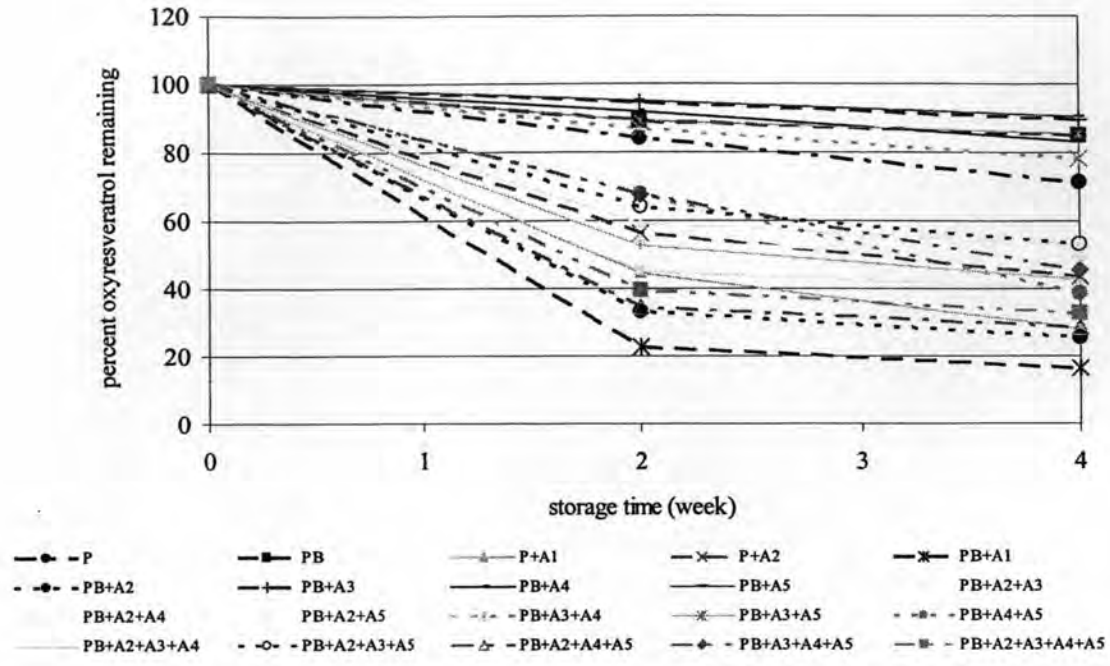


Figure 28. Percent oxyresveratrol remaining (relative to initial value) in Puag-Haad solutions in the presence of various antioxidants at accelerated temperature (45°C)
P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, A1 = 0.15% sodium metabisulfite, A2 = 0.10% sodium metabisulfite, A3 = 0.02% BHA, A4 = 0.10% propyl gallate, A5 = 0.05% EDTA

On the other hand, Puag-Haad solutions that had 0.10% sodium metabisulfite (A2) as an antioxidant gave a surprising results. All these solutions had a marked decrease in percent oxyresveratrol remaining since week 2. At week 4, the percent oxyresveratrol remaining was in the range of 24.91 to 59.67%. The results were in contrast to the results of color stability that sodium metabisulfite could provide the best stabilization in term of color. In addition, the results also did not agree with the biochemical stability. Pengrungruangwong (2001) also reported that the combination of sodium metabisulfite and BHA provided the best anti-tyrosinase activity (about 80% remaining after 24 weeks relative to the initial value). However, the anti-tyrosinase activity was not performed in this study and needed further investigation.

Since the solvent of Puag-Haad solutions in this study were propylene glycol in citrate buffer whereas the previous study was propylene glycol in water. P+A1 and P+A2, which were Puag-Haad solutions containing sodium metabisulfite without

buffer, were also prepared and evaluated. The result of percent oxyresveratrol remaining was 29.96 and 47.01%, respectively. Addition of citrate buffer into the medium did not increase the chemical stability of P+A1 and P+A2 as seen from the low % oxyresveratrol in PB+A1 and PB+A2 (Table 19). In addition, % oxyresveratrol further decreased as the concentration of sodium metabisulfite was increased from 0.10% to 0.15% regardless of the medium. These data thus indicated that sodium metabisulfite, despite its stability to stabilize the color of Puag-Haad solutions, was able to react with oxyresveratrol resulting in its rapid degradation. Comparasion of both the color and % oxyresveratrol among P, PB, P+A1, P+A2, PB+A1, PB+A2 revealed that degradation products of other unknown components of the extract apart from oxyresveratrol more likely contributed to the discoloration. For example, pure Puag-Haad solution (P) gave the highest extent of discoloration (+3) after 4 weeks but the content of remaining oxyresveratrol was still impressively high (71.19%), whereas solution P+A1 (Puag-Haad plus 0.15% sodium metabisulfite), which showed no change in color (0), had % oxyresveratrol remaining after 4 weeks storage of only about 30%. Therefore, concomitant degradation of other unidentified components of the extract significantly contributed to the color change and that sodium metabisulfite was somehow able to inhibit or stabilize this pathway, resulting in no change in color. However, more detailed studies are needed to further clarify how sodium metabisulfite promoted the degradation of oxyresvetrol with concomitant stabilization of color. Stability evaluation of pure oxyresveratrol solution should also be conducted in the future to determine its degradation products and their effects on discoloration.

Because sodium metabisulfite was necessary to protect the discoloration of Puag-Haad solution , an experiment was set up to find the optimum concentration of sodium metabisulfite that could provide the best physical and chemical stability. Concentration of sodium metabisulfite is varied in the range of 0.01 to 0.10%. The test samples were coded as follows :

P = 0.10%w/v Puag-Haad in 20% propylene glycol and 80% water

PB = 0.10%w/v Puag-Haad in 20% propylene glycol and 80% 50 mM citrate buffer pH 5.5

PB+0.10% SM = PB + 0.10% sodium metabisulfite

PB+0.07% SM = PB + 0.07% sodium metabisulfite

PB+0.05% SM = PB + 0.05% sodium metabisulfite
PB+0.03% SM = PB + 0.03% sodium metabisulfite
PB+0.02% SM = PB + 0.02% sodium metabisulfite
PB+0.01% SM = PB + 0.01% sodium metabisulfite

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

1. Physical stability

From Figure 29, all of Puag-Haad solutions containing sodium metabisulfite (vial 3-8) demonstrated a good color stability, with a very slight increase in yellow color from the initial time. The final color after 4-week storage appeared to be similar whereas that of pure Puag-Haad (P) and Puag-Haad in buffer (PB) showed a marked increased in color. Concentration of sodium metabisulfite in the range of 0.01 to 0.05% thus appeared to be the optimum concentration. The chemical stability was further investigated to confirm this result.

week 0

week 2

week 4

Figure 29. Physical appearance of Puag-Haad solutions with and without sodium metabisulfite upon storage at accelerated temperature (45°C) for 4 weeks
0 = Freshly prepared 0.10% Puag-Haad , 1 = 0.10% Puag-Haad without buffer (P), 2 = P + 50 mM citrate buffer pH 5.5 (PB), 3 = PB + 0.10% sodium metabisulfite, 4 = PB + 0.07% sodium metabisulfite, 5 = PB + 0.05% sodium metabisulfite, 6 = PB + 0.03% sodium metabisulfite, 7 = PB + 0.02% sodium metabisulfite, 8 = PB + 0.01% sodium metabisulfite

2. Chemical stability

The chemical stability of Puag-Haad solutions in the presence of sodium metabisulfite was determined by HPLC method at initial time and at week 2 and 4. Table 20 shows percent oxyresveratrol remaining in Puag-Haad solutions containing different concentrations of sodium metabisulfite. After 4 weeks, the percent oxyresveratrol remaining in PB + sodium metabisulfite were in the range of 48.15 to 91.43 %. The degradation of oxyresveratrol in PB+0.10% SM, which contained the highest percent of sodium metabisulfite, was the most rapid. Thus, the high concentration of sodium metabisulfite failed to protect Puag-Haad against chemical degradation but, on the contrary, promoted the reaction. The percent of sodium metabisulfite that provided the highest content of oxyresveratrol was 0.01%. However, the color became darkened pale yellow after extended storage period (longer than 4 weeks). So, 0.02% sodium metabisulfite was selected instead to provide the best protection in terms of both physical and chemical stability (87.69% after 4 weeks). The mechanism of oxyresveratrol degradation by sodium metabisulfite is not clearly known at present.

Table 20. Percent oxyresveratrol remaining (relative to initial value) in Puag-Haad solutions in the presence of sodium metabisulfite at accelerated temperature (45°C) (Mean ± SD, n=3)

Time (weeks)	% oxyresveratrol remaining							
	P	PB	PB+0.10% SM	PB+0.07% SM	PB+0.05% SM	PB+0.03% SM	PB+0.02% SM	PB+0.01% SM
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
(SD)	1.98	2.02	0.87	0.75	2.02	0.52	1.44	1.98
2	76.07	91.36	54.74	67.01	75.57	83.83	90.81	91.06
(SD)	2.17	1.25	2.85	0.77	1.93	0.65	2.45	0.29
4	75.29	91.21	48.15	62.93	73.94	83.14	87.69	91.43
(SD)	2.19	1.10	2.39	0.62	2.41	1.68	1.80	0.35

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, SM = sodium metabisulfite

In conclusion, based on the 4 week-stability evaluation, the antioxidant that could provide the best chemical stabilization was 0.02% BHA alone or 0.05% EDTA alone in citrate buffer pH 5.5. Sodium metabisulfite, although detrimental to oxyresveratrol at high concentration, may be necessary in solution at 0.01%-0.02%, at which concentration the high content of oxyresveratrol was maintained in addition to a very good color stability.

Thus, in an attempt to achieve optimum stability of Puag-Haad from both the chemical and color intregity viewpoints, a combination of these antioxidants appeared to be highly attractive for further study. Solution of Puag-Haad containing triple combination of 0.02% BHA, 0.05% EDTA and 0.02% sodium metabisulfite was prepared and investigated for long term stability. Propyl gallate was not the choice of selected antioxidant because it could interfere with the peak of oxyresveratrol as discussed above as well as it could form color complex with EDTA. EDTA was necessary in the system due to its property of chelating agents. The selected combination of the above antioxidants were expected to provided a more thorough protection with BHA as a true antioxidant, sodium metabisulfite as a reducing agent and EDTA as a synergist or chelating agent.

2.3 Long term stability study of aqueous solutions of *Artocarpus lakoocha* heartwood extract in the presence of various antioxidants

From 2.2, the result showed that 0.02 % BHA alone or 0.05% EDTA alone provided the best chemical stabilization along 4-week storage and sodium metabisulfite at low concentration (0.02%) was necessary in the solutions to stabilize the color. To achieve optimum stability of Puag-Haad from both the chemical and color, solutions of Puag-Haad containing triple combination of 0.02% BHA, 0.05% EDTA and 0.02% sodium metabisulfite was prepared and investigated for long term stability. Nitrogen purge was also used in this study to see if it could help stabilize the solutions against oxidation. The study was performed to evaluate the physical and chemical stability upon storage at accelerated temperature (45°C) for 12 weeks and at room temperature (30 °C) for 24 weeks. The result of the stability at 45°C was used to select the antioxidant to develop the lotion in Part IV.

Solutions of 0.10% Puag-Haad with and without the three antioxidants as well as their combination, were prepared in the same solvent (20% propylene glycol and 80% v/v 50 mM citrate buffer pH 5.5). The freshly prepared Puag-Haad solution

(without buffer) was also used as control sample in every period. The test samples were as follows:

P = 0.10%w/v Puag-Haad in 20% propylene glycol and 80% water

PB = 0.10%w/v Puag-Haad in 20% propylene glycol and 80% 50 mM citrate buffer pH 5.5

PB+ N_2 = PB with Nitrogen purged

PB+C1 = PB + 0.02% sodium metabisulfite

PB+C2 = PB + 0.02% BHA

PB+C3 = PB + 0.05% EDTA

PB+C1+C2 = PB + 0.02% sodium metabisulfite + 0.02% BHA

PB+C1+C3 = PB + 0.02% sodium metabisulfite + 0.05% EDTA

PB+C1+C2+C3 = PB + 0.02% sodium metabisulfite + 0.02% BHA + 0.05% EDTA

The test samples were kept at accelerated temperature (45°C) for 12 weeks and at 30 °C for 24 weeks in tightly closed glass vials and protected from light. At the start and every 4 weeks, their physical properties (color/clarity and pH values) and chemical property (content of active constituent) were investigated. Each of the above solutions was prepared and assayed in triplicate (3 vials per solution).

2.3.1. Physical stability of aqueous solutions of *Artocarpus lakoocha* heartwood extract in the presence of various antioxidants (long term study)

Tables 21 and 22 show changes in coloring of Puag-Haad solutions upon storage at 45°C for 12 weeks and at 30 °C for 24 weeks. The initial color of 0.10% Puag-Haad solution was pale yellow (graded as 0). Upon storage, all Puag-Haad solutions darkened with time but to a different level to markedly brown (graded as +3). The color of all stored Puag-Haad solutions was always compared with the freshly prepared 0.10% pure Puag-Haad (fresh P).

At 45°C, P was gradually darkened to dark brown (graded as +4) and PB was marked brown (graded as +3) after 12 weeks storage, whereas PB+N_2 had slightly decreased to light brown (graded as +2). It indicated that the darkened color of Puag-Haad was due to the presence of atmospheric oxygen. The color of Puag-Haad solutions containing single antioxidants, C1, C2 and C3, was graded as +2, +3 and +4, respectively. The results agreed with the result of color stability in 2.2 in which sodium metabisulfite (PB+C1) was able to protect Puag-Haad from discoloration and BHA (PB+C2), EDTA (PB+C3) were not effective in protecting the discoloration in

long term stability. In addition, the color of solutions containing EDTA either single or in combination, was more intense than other samples. PB+C3 was gradually to dark brown (graded as +4) after 12 weeks. PB+C1+C3 and PB+C1+C2+C3 which also containing sodium metabisulfite were gradually darkened to marked brown (graded as +3), whereas PB+C1+C2 was gradually darkened to light brown (graded as +2). These results indicated that EDTA failed to protect Puag-Haad from discoloration even if in the presence of citrate buffer or sodium metabisulfite. Similar pattern was observed at 30 °C, except the color of PB+N_2 was equal to PB (graded as +2).

These results indicated that sodium metabisulfite either single or in combination with BHA appeared to provide the best color stabilization, followed by nitrogen purge, sodium metabisulfite in combination with EDTA which was equal with BHA and triple combination. EDTA alone provided the worst color stabilization. Photographs of Puag-Haad solutions taken after storage for 12 weeks (45°C) and 24 weeks (30°C) are also provided for visual comparison in Figures 30-31, respectively.

The pH of each solution was also measured in triplicate and the data at 45°C and 30°C are respectively given in Tables 23-24. Pure Puag-Haad without buffer (P) showed a decrease in pH upon storage at 45 °C, from 6.27 at week 0 to 5.28 at week 12, which was equivalent to 0.99 unit drop in pH. At 30 °C, the pH decreased from 6.27 at week 0 to 5.90 at week 24, which was equivalent to 0.37 unit drop in pH.

Table 21. Changes in color of Puag-Haad solutions in the presence of various antioxidants upon storage at accelerated temperature (45°C) for 12 weeks

No.	Puag-Haad samples	Time (week)			
		0	4	8	12
0	Fresh P	0	0	0	0
1	P	0	+2	+3	+4
2	PB	0	+2	+3	+3
3	PB+N_2	0	0	+1	+2
4	PB+C1	0	0	+1	+2
5	PB+C2	0	+1	+2	+3
6	PB+C3	0	+2	+3	+4
7	PB+C1+C2	0	0	+1	+2
8	PB+C1+C3	0	+1	+2	+3
9	PB+C1+C2+C3	0	0	+2	+3

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, N_2 = nitrogen purge, C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

0 = normal (pale yellow); without change; +1 = slightly (light yellow) changed; +2 = noticeably (light brown) changed; +3 = markedly (brown) changed; +4 = seriously deteriorated (dark brown); +5 = almost or completely deteriorated (intense deep brown)

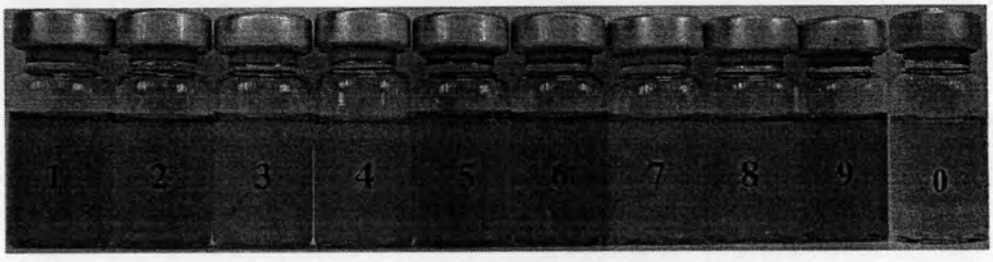
Table 22. Changes in color of Puag-Haad solutions in the presence of various antioxidants upon storage at room temperature (30°C) for 24 weeks

No.	Puag-Haad samples	Time (week)						
		0	4	8	12	16	20	24
0	Fresh P	0	0	0	0	0	0	0
1	P	0	+1	2	+2	+3	+3	+4
2	PB	0	0	+1	+1	+2	+2	+2
3	PB+N_2	0	0	+1	+1	+2	+2	+2
4	PB+C1	0	0	+1	+1	+1	+1	+1
5	PB+C2	0	0	+1	+1	+2	+2	+2
6	PB+C3	0	+1	+2	+2	+2	+2	+3
7	PB+C1+C2	0	0	+1	+1	+1	+1	+1
8	PB+C1+C3	0	0	+1	+1	+1	+1	+2
9	PB+C1+C2+C3	0	0	0	+1	+1	+1	+2

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, N_2 = nitrogen purge, C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

0 = normal (pale yellow); without change; +1 = slightly (light yellow) changed; +2 = noticeably (light brown) changed; +3 = markedly (brown) changed; +4 = seriously deteriorated (dark brown); +5 = almost or completely deteriorated (intense deep brown)

0 week



12 weeks

Figure 30. Physical appearance of Puag-Haad solutions in the presence of various antioxidants upon storage at accelerated temperature (45°C) for 12 weeks
0 = Freshly prepared 0.10% Puag-Haad , 1 = 0.10% Puag-Haad without buffer (P), 2 = P + 50 mM citrate buffer pH 5.5 (PB), 3 = PB + nitrogen purge, 4 = PB + 0.02% sodium metabisulfite, 5 = PB + 0.02% BHA, 6 = PB + 0.05% EDTA, 7 = PB + 0.02% sodium metabisulfite + 0.02% BHA, 8 = PB + 0.02% sodium metabisulfite + 0.05% EDTA, 9 = PB + 0.02% sodium metabisulfite + 0.02% BHA + 0.05% EDTA

0 week

24 weeks

Figure 31. Physical appearance of Puag-Haad solutions in the presence of various antioxidants upon storage at room temperature (30°C) for 24 weeks
0 = Freshly prepared 0.10% Puag-Haad , 1 = 0.10% Puag-Haad without buffer (P), 2 = P + 50 mM citrate buffer pH 5.5 (PB), 3 = PB + nitrogen purge, 4 = PB + 0.02% sodium metabisulfite, 5 = PB + 0.02% BHA, 6 = PB + 0.05% EDTA, 7 = PB + 0.02% sodium metabisulfite + 0.02% BHA, 8 = PB + 0.02% sodium metabisulfite + 0.05% EDTA, 9 = PB + 0.02% sodium metabisulfite + 0.02% BHA + 0.05% EDTA

All Puag-Haad solutions containing citrate buffer pH 5.5 were able to maintain pH throughout the 12 weeks at 45 °C and 24 weeks at 30 °C, either alone or in the presence of antioxidants. The extent of pH drop was very small within the range of 0.04 – 0.07 at 45 °C and 0.02 – 0.09 at 30 °C. The results were inagreement with part 2.2 indicating a very good buffer capacity of citrate buffer pH 5.5.

The pH of individual antioxidants was also investigated. The values of pH are shown in Table 23/1 and 24/1. The results from these tables showed the similar patterns as in part 2.2, in which sodium metabisulfite gave a greater drop in pH at both temperatures. BHA and EDTA gave a little drop in pH at 30 °C upon 24 week storage, in which EDTA induced a much smaller drop in pH than BHA. Pengrungruangwong (2001) also found the same result after 12 week- storage at 45 °C. She discussed that this was probably due to the buffer capacity of EDTA. However, the result at 45 °C in this study could not detect the difference in pH stability between BHA and EDTA after 12 week-storage. The reason for this discrepancy was not clearly known at present.

Table 23. Changes in pH values of Puag-Haad solutions in the presence of various antioxidants upon storage at accelerated temperature (45°C) for 12 weeks

No.	Puag-Haad samples	Time (week)				ΔpH
		0	4	8	12	
1	P	6.38 6.29 6.14	5.86 5.52 5.65	5.66 5.56 5.49	5.32 5.25 5.28	0.99
	Mean ± SD	6.27±0.12	5.67±0.17	5.57±0.08	5.28±0.03	
2	PB	5.52 5.45 5.47	5.45 5.55 5.44	5.43 5.51 5.43	5.42 5.48 5.4	0.05
	Mean ± SD	5.48±0.04	5.48±0.06	5.46±0.05	5.43±0.04	
3	PB+N_2	5.56 5.42 5.46	5.46 5.45 5.48	5.48 5.45 5.42	5.45 5.44 5.39	0.05
	Mean ± SD	5.48±0.07	5.46±0.01	5.45±0.03	5.43±0.03	
4	PB+C1	5.52 5.41 5.53	5.45 5.41 5.48	5.44 5.39 5.45	5.45 5.42 5.41	0.06
	Mean ± SD	5.49±0.07	5.45±0.03	5.43±0.03	5.43±0.02	
5	PB+C2	5.54 5.45 5.52	5.50 5.41 5.45	5.48 5.45 5.50	5.45 5.42 5.47	0.06
	Mean ± SD	5.50±0.05	5.45±0.04	5.48±0.02	5.45±0.02	
6	PB+C3	5.48 5.54 5.46	5.43 5.51 5.41	5.40 5.52 5.45	5.40 5.47 5.43	0.06
	Mean ± SD	5.49±0.04	5.45±0.05	5.46±0.06	5.43±0.03	
7	PB+C1+C2	5.41 5.48 5.40	5.45 5.42 5.50	5.45 5.41 5.46	5.42 5.44 5.40	0.04
	Mean ± SD	5.46±0.05	5.46±0.04	5.44±0.03	5.42±0.02	
8	PB+C1+C3	5.55 5.48 5.46	5.52 5.50 5.43	5.48 5.52 5.40	5.46 5.40 5.42	0.07
	Mean ± SD	5.50±0.05	5.48±0.05	5.47±0.06	5.43±0.04	
9	PB+C1+C2+C3	5.44 5.45 5.50	5.40 5.41 5.45	5.38 5.47 5.45	5.44 5.41 5.37	0.06
	Mean ± SD	5.46±0.03	5.42±0.03	5.43±0.05	5.41±0.03	

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, N_2 = nitrogen purged, C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

Table 23/1. Changes in pH values of antioxidants upon storage at accelerated temperature (45°C) for 12 weeks

No.	Antioxidants	Time (week)				ΔpH
		0	4	8	12	
1	C1	6.35 6.40 6.40	5.75 5.88 5.82	5.60 5.69 5.63	5.38 5.48 5.50	0.93
	Mean ± SD	6.38±0.03	5.82±0.07	5.64±0.05	5.45±0.06	
2	C2	6.45 6.50 6.43	6.44 6.47 6.45	6.22 6.20 6.23	6.01 5.95 5.98	0.48
	Mean ± SD	6.46±0.04	6.45±0.02	6.22±0.02	5.98±0.03	
3	C3	6.13 6.24 6.25	6.01 6.03 6.02	5.90 5.92 5.89	5.83 5.87 5.82	0.37
	Mean ± SD	6.21±0.07	6.02±0.01	5.90±0.02	5.84±0.03	

C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

Table 24. Changes in pH values of Puag-Haad solutions in the presence of various antioxidants upon storage at room temperature (30°C) for 24 weeks

No.	Puag-Haad samples	Time (week)							ΔpH
		0	4	8	12	16	20	24	
1	P	6.38 6.29 6.14	5.45 5.58 5.78	5.84 6.10 6.02	5.96 5.82 5.78	5.88 5.80 5.81	5.96 5.82 5.80	6.04 5.86 5.81	0.37
	Mean ± SD	6.27±0.12	5.52±0.09	5.99±0.13	5.85±0.04	5.83±0.04	5.86±0.09	5.90±0.12	
2	PB	5.52 5.45 5.47	5.48 5.46 5.44	5.41 5.50 5.40	5.40 5.42 5.45	5.42 5.39 5.47	5.40 5.42 5.45	5.38 5.45 5.39	0.07
	Mean ± SD	5.48±0.04	5.46±0.02	5.44±0.06	5.42±0.03	5.43±0.04	5.42±0.03	5.41±0.04	
3	PB+N_2	5.56 5.42 5.46	5.52 5.45 5.40	5.47 5.37 5.48	5.44 5.39 5.44	5.44 5.40 5.42	5.42 5.37 5.42	5.40 5.37 5.44	0.08
	Mean ± SD	5.48±0.07	5.46±0.06	5.44±0.06	5.42±0.03	5.42±0.02	5.40±0.03	5.40±0.04	
4	PB+C1	5.52 5.41 5.53	5.46 5.50 5.51	5.48 5.41 5.43	5.39 5.42 5.47	5.44 5.43 5.42	5.42 5.41 5.39	5.40 5.42 5.38	0.09
	Mean ± SD	5.49±0.07	5.49±0.03	5.44±0.04	5.43±0.04	5.43±0.01	5.41±0.02	5.40±0.02	
5	PB+C2	5.54 5.45 5.52	5.42 5.48 5.47	5.40 5.45 5.41	5.40 5.41 5.43	5.42 .42 5.45	5.43 5.41 5.40	5.43 5.43 5.39	0.09
	Mean ± SD	5.50±0.05	5.46±0.03	5.42±0.03	5.41±0.02	5.43±0.02	5.41±0.02	5.42±0.02	
6	PB+C3	5.48 5.44 5.46	5.39 5.42 5.41	5.42 5.38 5.46	5.40 5.44 5.41	5.42 5.37 5.42	5.42 5.41 5.39	5.45 5.40 5.39	0.07
	Mean ± SD	5.49±0.04	5.41±0.02	5.42±0.04	5.42±0.02	5.40±0.03	5.41±0.02	5.42±0.03	
7	PB+C1+C2	5.41 5.48 5.50	5.40 5.43 5.49	5.46 5.38 5.42	5.42 5.43 5.43	5.44 5.40 5.42	5.40 5.40 5.41	5.38 5.40 5.41	0.07
	Mean ± SD	5.46±0.05	5.44±0.05	5.42±0.04	5.43±0.01	5.42±0.02	5.40±0.01	5.40±0.02	
8	PB+C1+C3	5.55 5.48 5.46	5.44 5.49 5.52	5.42 5.46 5.47	5.40 5.44 5.47	5.42 5.43 5.45	5.43 5.41 5.44	5.42 5.41 5.43	0.08
	Mean ± SD	5.50±0.05	5.48±0.04	5.45±0.03	5.44±0.04	5.43±0.02	5.43±0.02	5.42±0.01	
9	PB+C1+C2+C3	5.44 5.45 5.50	5.46 5.42 5.42	5.44 5.40 5.43	5.43 5.37 5.42	5.42 5.39 5.40	5.44 5.40 5.43	5.40 5.46 5.46	0.02
	Mean ± SD	5.46±0.03	5.43±0.02	5.42±0.02	5.41±0.03	5.40±0.02	5.42±0.02	5.44±0.02	

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, N_2 = nitrogen purged, C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

Table 24/1. Changes in pH values of antioxidants upon storage at room temperature (30°C) for 24 weeks

No.	Puag-Haad samples	Time (week)							ΔpH
		0	4	8	12	16	20	24	
1	C1	6.35 6.40 6.40	6.15 6.22 6.13	6.09 6.18 6.12	6.07 6.12 6.15	6.01 6.02 6.08	5.98 5.96 6.02	5.98 5.93 5.97	0.42
	Mean ± SD	6.38±0.03	6.17±0.05	6.13±0.05	6.11±0.04	6.04±0.04	5.99±0.03	5.96±0.03	
2	C2	6.45 6.50 6.43	6.42 6.46 6.38	6.38 6.40 6.32	6.36 6.37 6.29	6.34 6.30 6.20	6.31 6.28 6.20	6.29 6.26 6.19	0.21
	Mean ± SD	6.46±0.04	6.42±0.04	6.37±0.04	6.34±0.04	6.28±0.07	6.26±0.06	6.25±0.05	
3	C3	6.13 6.24 6.25	6.11 6.23 6.25	6.10 6.21 6.23	6.09 6.19 6.20	6.07 6.19 6.18	6.08 6.20 6.15	6.07 6.18 6.16	0.07
	Mean ± SD	6.21±0.07	6.20±0.08	6.18±0.07	6.16±0.06	6.15±0.07	6.14±0.06	6.14±0.06	

C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

2.3.2. Chemical stability of aqueous solutions of *Artocarpus lakoocha* heartwood extract in the presence of various antioxidants (long term study)

The chemical stability of Puag-Haad solutions with three antioxidants were determined by HPLC method at initial time and every 4 weeks at 45 °C and 30 °C. Table 25 shows percent oxyresveratrol remaining in Puag-Haad solutions upon storage at accelerated temperature (45 °C) for 12 weeks. It was found that the percent oxyresveratrol remaining in pure Puag-Haad (P) decreased to 44.60% that gave the lowest content of oxyresveratrol, whereas PB + N_2 gave the highest content of oxyresveratrol (70.12%), followed by PB (63.85%). This result agreed with previous preliminary study (2.2) in that only citrate buffer was already able to give substantial stabilization. In addition, PB+ N_2 gave further chemical stabilization due to the absence of oxygen that could stimulate the oxidation reactions.

Because the aim of this part was to select the antioxidants to formulate lotion in Part IV. Although PB+ N_2 gave the highest content of oxyresveratrol, it is necessary to use antioxidant(s) to stabilize the emulsion system, so Puag-Haad solutions with antioxidant(s) were still considered. Percent oxyresveratrol remaining in Puag-Haad solutions containing sodium metabisulfite (PB+C1) and in combination with BHA (PB+C1+C2) dropped to 57.31 and 59.35%, respectively. The result showed that even at the low concentration of sodium metabisulfite (0.02%), the dropped of oxyresveratrol was observed. PB+C3 (EDTA) gave a 60.77% oxyresveratrol remaining , whereas in combination with other antioxidant, espectially with sodium metabisulfite (PB+C1+C3 and PB+C1+C2+C3) gave a further dropped in % oxyresveratrol, decreasing to 57.30% and 54.62%. The result agreed with chemical stability in 2.2 that addition of EDTA (PB+C3) did not improve chemical stability and seemed to darkened the color of Puag-Haad.

When considering both the chemical and color stability, the antioxidants that could provide the compromised results appeared to be combination of sodium metabisulfite and BHA (PB+C1+C2) which gave a moderate % of oxyresveratrol but the best color stabilization. However, since the presence of sodium metabisulfite caused the deterioration of oxyresveratrol despite the clear improvement in color, the double combination (C1+C2) system was not selected for further study. Thus, base on the data at 45 °C, the optimum antioxidant system which could provide sufficient chemical and color stabilization appeared to be BHA alone (PB+C2). As a result, the

single antioxidant system containing BHA (PB+C2) was selected for further formulation into lotions in the next part.

Similar pattern was observed at 30 °C . Table 26 shows percent oxyresveratrol remaining in Puag-Haad solutions at room temperature (30 °C) for 24 weeks. PB+N_2 gave the highest percent oxyresveratrol remaining (88.14%), whereas the second highest was observed with PB (86.91%), followed by PB+C2 (84.30%) , PB+C3 (83.76%) and PB+C1+C2 (81.92%). From the data at 30 °C, the optimum antioxidant systems may include not only PB+C2, which gave the high percent of oxyresveratrol remaining but PB+C1+C2 as well, in which a negligible drop in percent oxyresveratrol remaining was observed and yet providing better color stability (+1). Addition of EDTA (PB+C3) again caused too much discoloration (+3) and could not be selected for further formulation studies. The values of percent oxyresveratrol remaining at 45 °C and 30 °C are also graphically represented for each sample in Figures 32 and 33, respectively. In addition, future formulation approach should include nitrogen flushing of the product before sealing since the data from both 45 °C and 30 °C showed that this procedure greatly enhance the chemical stability of oxyresveratrol.

Table 25. Normalized percent oxyresveratrol remaining in Puag-Haad solutions in the presence of various antioxidants at accelerated temperature (45°C) for 12 weeks. (Mean ± SD, n=3). Extent of discoloration after 12 weeks are also provided for comparison

Time (weeks)	% oxyresveratrol remaining								
	P	PB	PB+ N_2	PB+C1	PB+C2	PB+C3	PB+C1+C2	PB+C1+C3	PB+C1+C2+C3
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
(SD)	0.27	0.78	0.64	1.24	1.04	1.08	2.32	1.14	0.42
4	73.76	91.70	94.24	80.81	89.19	87.99	82.27	79.76	78.63
(SD)	0.64	2.55	1.46	1.10	1.27	0.61	0.59	2.02	1.12
8	55.31	73.54	84.04	70.96	76.14	73.36	71.92	68.34	65.85
(SD)	1.22	2.70	1.08	1.75	1.61	1.26	0.90	1.75	1.43
12	44.60	63.85	70.12	57.31	63.77	60.77	59.36	57.30	54.62
(SD)	0.91	0.60	2.07	0.65	2.81	0.97	1.36	0.81	1.86
Degree of discoloration	4	3	2	2	3	4	2	3	3

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, N_2 = nitrogen purged, C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

Table 26. Normalized percent oxyresveratrol remaining in Puag-Haad solutions in the presence of various antioxidants at room temperature (30°C) for 24 weeks. (Mean ± SD, n=3). Extent of discoloration after 24 weeks are also provided for comparison

Time	% oxyresveratrol remaining								
(weeks)	P	PB	PB+ N_2	PB+C1	PB+C2	PB+C3	PB+C1+C2	PB+C1+C3	PB+C1+C2+C3
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
(SD)	0.27	0.78	0.64	1.24	1.04	1.08	2.32	1.14	0.42
4	86.27	93.80	95.19	83.40	94.56	92.69	88.61	88.49	89.03
(SD)	1.01	1.72	1.07	1.84	2.97	2.47	1.18	1.61	2.57
8	81.48	90.35	95.50	81.29	91.60	89.56	85.88	85.52	85.25
(SD)	0.72	0.67	1.95	0.65	1.93	3.25	1.10	2.48	1.06
12	76.86	89.06	92.17	77.18	90.43	88.73	84.45	79.18	81.53
(SD)	1.19	1.20	2.46	1.25	2.49	1.24	3.03	2.07	1.17
16	75.94	89.08	92.45	77.26	90.03	89.55	84.09	76.83	80.39
(SD)	1.54	1.56	1.42	1.22	1.81	0.91	0.63	1.71	0.86
20	70.02	87.00	89.31	74.58	85.74	84.25	82.18	76.16	72.02
(SD)	0.66	2.97	0.01	3.21	1.80	0.66	2.65	1.54	0.47
24	69.89	86.91	88.14	73.76	84.30	83.76	81.92	76.41	68.97
(SD)	1.93	2.15	1.85	1.62	1.87	1.45	2.14	0.76	2.47
Degree of discoloration	3	2	2	1	2	3	1	2	2

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, N_2 = nitrogen purged, C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

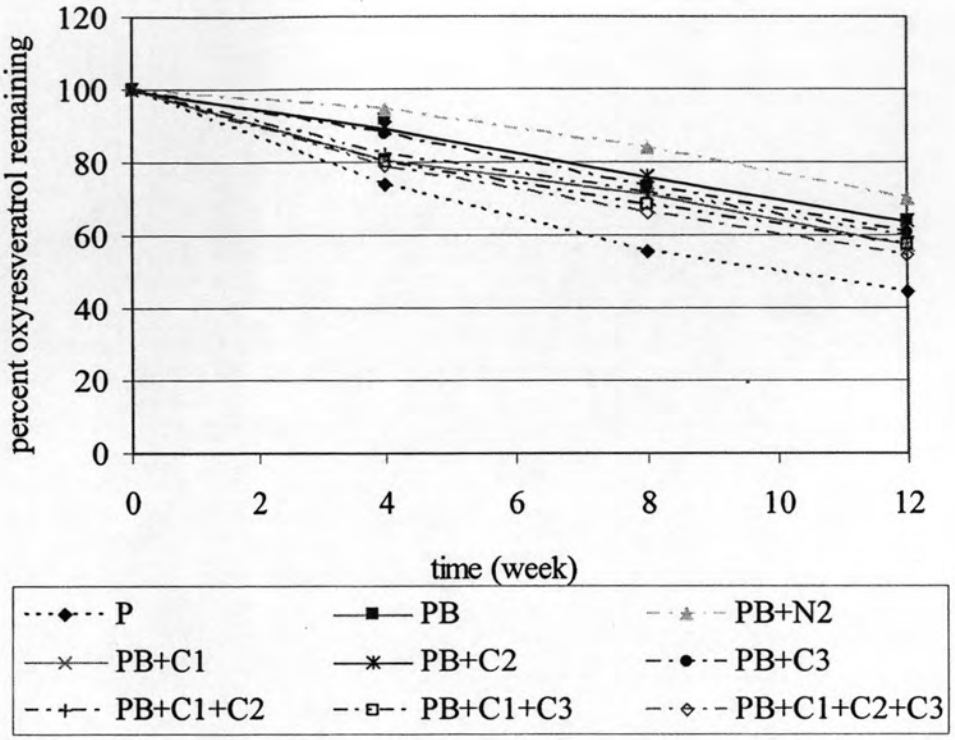


Figure 32. Percent oxyresveratrol remaining (relative to initial value) in Puag-Haad solutions in the presence of various antioxidants upon storage at accelerated temperature (45°C) for 12 weeks

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, N_2 = nitrogen purge, C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

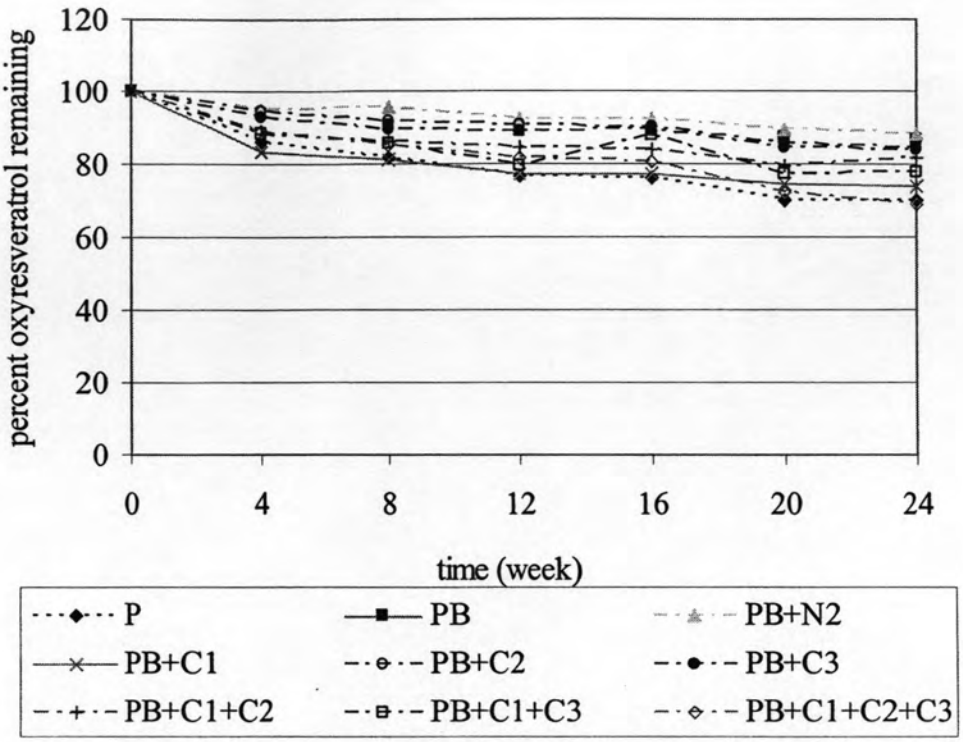


Figure 33. Percent oxyresveratrol remaining (relative to initial value) in Puag-Haad solutions in the presence of various antioxidants upon storage at room temperature (30°C) for 24 weeks

P = 0.10% pure Puag-Haad solution (without buffer), PB = 0.10% Puag-Haad solution in 50 mM citrate buffer pH 5.5, N_2 = nitrogen purge, C1 = 0.02% sodium metabisulfite, C2 = 0.02% BHA, C3 = 0.05% EDTA

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

The purpose of this part was to demonstrate the in *vivo* anti-wrinkle efficacy of *Artocarpus lakoocha* extract or Puag-Haad solutions at two different concentrations in healthy volunteers. Two concentrations of Puag-Haad were chosen in this study, i.e., 0.25% and 0.10% w/v solutions in 20% v/v propylene glycol in water. The decision for selecting these two concentrations were based on the data obtained from the whitening efficacy study, in which Pengrungruangwong (2001) reported that 0.25% w/v solution provided the best whitening efficacy compared to 0.5% and 1.0 % w/v Puag-Haad and also better than kojic acid and licorice extract. The lower concentration of 0.10% w/v was also found to give better whitening activity when compared to 0.10% w/v licorice in the lotion dosage form (Tengamnuay et al., 2006). In addition, the lower concentration of Puag-Haad solution would be less yellow in color, thereby giving a better appearance.

As the reference anti-wrinkle agents, epigallocathechin gallate (EGCG) and vitamin C were concomitantly evaluated in this part of study. Both EGCG and vitamin C are widely used as anti-wrinkle agents in many cosmetic products. The concentration of EGCG and vitamin C used in this part was 0.10% w/v in order to compare their anti-wrinkle efficacy at the same concentration as Puag-Haad.

Ninety female volunteers participated in this parallel study with self-control. They were allocated into four groups of 23, 23, 22 and 22 subjects. Each group was randomly assigned to the treatments, either A, B, C or D. The group assigned to treatment A received 0.25% w/v Puag-Haad (or simply called group A) on their right cheeks. Group B, also on the right cheeks, applied 0.10% w/v Puag-Haad, whereas group C and D respectively received 0.10% w/v EGCG and 0.10% w/v vitamin C. All treatment solutions were prepared using 20% v/v propylene glycol in water as solvent. Thus, all the subjects in each group also applied the solvent as the self-control on their remaining left cheeks.

Before starting the experiment, patch tests were performed in all subjects. Each subject was given 0.05 ml of the test solutions and the control solvent to the inner side of their arms. The right side was the test solution and the left side was the control solvent. After 24 hr, it was found that two subjects had adverse reactions.

The first one who was in group B showed a mild erythema (+1) and the second one who was in group D showed erythema and papules (+2) on the right sides which applying the test solutions. These two subjects were thus withdrawn from the study. The other two subjects (one in group C and one in group D) withdrew themselves from the study due to their personal reasons not related to adverse reactions after two weeks of application. Hence, the number of subjects who eventually completed the study was 23, 22, 21 and 20 for groups A, B, C and D, respectively.

Before application of the treatments, each subject was monitored for their baseline roughness parameters for 4 weeks to observe for any fluctuation. The average baseline values of circular roughness and mean roughness parameters measured from intact cheeks are shown in Tables 27 and 28.

As seen from Tables 27 and 28, the average baseline values of both the circular and mean roughness were similar among the four groups during the pre-treatment period (week -4 to week 0) prior to the start of experiment. One-way ANOVA on either the left or right cheeks reveals that there was no significant difference among the four groups with respect to both roughness parameters at any week within this period ($P > 0.05$), indicating that the subjects were equally distributed along the four groups such that their baseline group means were similar. Furthermore, when randomized block ANOVA was applied to test for the effect of time on the baseline value within each group, no significant difference in circular roughness values was found among the values at week -4, -2 and 0 ($P > 0.05$) in either side of the cheeks for all groups (Table 27). Similar result was also observed for the mean roughness values in Table 28. The results indicated that the baseline roughness values had not changed with time.

Paired t-test was also applied to test for any difference in roughness parameters (circular and mean roughness) between the left and the right cheeks at week -4, -2 and 0. No significant difference ($P > 0.05$) was found between the two sides of the cheek in all groups indicating the stability of the baseline values with respect to the application site in addition to the baseline integrity with respect to group and time previously observed.

Table 29 shows the average values of circular roughness parameter for each treatment group. As seen from this table, the values of the propylene glycol-treated cheek (PG-self control) appeared to increase slightly from the initial time (week 0) in all groups, indicating that the solvent alone might increase the skin roughness. For

example, the average circular roughness in the self-control cheek of subjects in group A was 57.10 at the start of the study. Upon application of the 20% propylene glycol, the values were 56.54, 57.00, 57.58, and 58.34 at weeks 2, 4, 6 and 8, respectively. Similar results were also observed for the self-control cheeks in other groups in which the average circular roughness tended to increase over time from week 0 through week 8. The values at week 0 versus week 8 were 58.08 vs 59.19, 58.24 vs 59.73, and 56.95 vs 59.68 for groups B, C and D, respectively (Table 29). However, the magnitude of the increase in circular roughness with time was very small and not significantly different from the initial values at week 0 ($P > 0.05$, randomized block ANOVA on the period effect).

It is known that high amount of propylene glycol could induce skin dryness and decrease skin permeability (Roberts and Walker, 1993). Although the percentage of propylene glycol used here (20%) did not exceed the maximum limit recommended for use in topical and cosmetic preparations (Dr. Jellinek, 1970), its daily prolonged application may have resulted in reduced skin moisture content, which in turn could affect the skin texture and increase its surface roughness.

When the circular roughness of the antioxidant-treated cheeks was examined, the values did not appear to increase with time as opposed to the self-control cheeks in all groups. Randomized block ANOVA also revealed that there was no significant difference in the average circular roughness values among different weeks of application for each antioxidant ($P > 0.05$). However, closer examination of the data revealed that the average circular roughness of all the antioxidant-treated cheeks did not increase with application time and in some groups (such as B and C) even appeared to decrease slightly. For example, in the cheeks treated with 0.25% Puag-Haad (group A), the average circular roughness value was 55.96, 54.65, 54.58, 55.52, and 55.75 at weeks 0, 2, 4, 6 and 8, respectively. In the cheeks treated with 0.10% Puag-Haad (group B), the value slightly decreased from 56.90 at week 0 to 55.33 at week 8, whereas the cheeks treated with 0.10% EGCG (group C) showed a decrease from 57.35 at week 0 to 56.36 at week 8. For group D, the cheeks treated with 0.10% vitamin C gave the average circular roughness of 56.33, 55.97, 56.42, 58.43, and 56.63 at weeks 0, 2, 4, 6, and 8, respectively. The observation that the circular roughness of the antioxidant-treated cheeks did not tend to increase over application time as opposed to the solvent self-control may suggest the ability of the test

antioxidants to delay or reduce the possible detrimental effects of the solvent on the skin.

The values of circular roughness were then compared between the antioxidant-treated-cheek and its self-control cheek within each group using paired student's t-test at each time point. As previously discussed, there was no significant difference in the initial circular roughness values (week 0) between the left and the right cheeks in all subjects ($P > 0.05$). However, when the antioxidant solution was applied to the subjects, the circular roughness value became significantly lower than the PG-treated side for all antioxidants but after different weeks of application (Table 29). For example, the value of average circular roughness of the cheek treated with 0.25% Puag-Haad (A) and 0.10% EGCG (C) became significantly lower than their respective self-control at the last week (week 8) of application whereas 0.10% vitamin C gave a rather erratic result, with significant decrease in roughness observed only at week 4 and became non-significant again at weeks 6 and 8. Application of 0.10% Puag-Haad (B), on the other hand, appeared to be the most effective, with the roughness value becoming significantly lower than its self-control after only 2 weeks of application and remained significant until the last week of the study.

The values of the mean roughness parameter were also obtained using Visioscan and the data are shown in Table 30. Similar to the circular roughness values, there were no significant changes in the mean roughness values with time for the self-control cheeks in all groups ($P > 0.05$, randomized block ANOVA on the period effect). However, the values tended to increase very slightly as previously observed with the circular roughness values, especially in the self-control of groups A and C. For example, the mean roughness for the self-control of group A slightly dropped from 55.56 at week 0 to 54.59 at week 2, but slowly increased to 54.94, 55.19, and 56.53 after 4, 6, and 8 weeks of solvent application, respectively. The self-control of group C showed a slight increase from 55.90 at week 0 to 56.05, 56.75, 56.48 and 57.51 at weeks 2, 4, 6 and 8. Thus, the data from the mean roughness parameter agreed with circular roughness in that 20% propylene glycol in water used as solvent might have some dehydrating effect on the skin and this could result in a slight increase in the skin roughness. The self-controls of group B and D also showed the same trend although the increase in mean roughness was observed only in some weeks.

On the other hand, the mean roughness of the cheeks of subjects treated with antioxidant solutions did not appear to increase with time but remained relatively constant throughout the study period. For example, the mean roughness value in subjects treated with 0.25% Puag-Haad (A) was 53.91, 53.09, 53.12, 53.79 and 53.92 at weeks 0, 2, 4, 6, and 8, respectively, whereas it was 54.83, 52.54, 54.80, 54.98 and 53.83 for 0.10% Puag-Haad (B) and was from 55.73 at week 0 to 55.17 at week 8 for 0.10% EGCG (C). This was substantiated by the randomized block ANOVA which revealed no significant difference in the mean circular roughness values among different application times within each of the antioxidant-treated groups ($P > 0.05$). Thus, in agreement with the previous result on the circular roughness parameter, it seems that the mean roughness of the antioxidant-treated cheeks did not increase over application time whereas application of PG solvent to the skin tended to produce increased roughness at some points after application.

Similarly, the values of mean roughness were then compared between the antioxidant-treated-cheek and its self-control cheek within each group using paired student's t-test at each time point. As previously discussed, there was no significant difference in the initial mean roughness values (week 0) between the left and the right cheeks in all subjects ($P > 0.05$). However, when the antioxidant solution was applied to the subjects, the mean roughness value became significantly lower than the PG-treated side for all antioxidants but after different weeks of application (Table 30). For example, the value of average mean roughness of the cheek treated with 0.25% Puag-Haad (A) and 0.10% EGCG (C) became significantly lower than their respective self-control at week 8. This was similar to the circular roughness results. 0.10% Puag-Haad (B) also yielded the mean roughness values significantly lower than its respective PG control at all weeks after the start of the study (from week 2 through week 8), giving it the most effective anti-wrinkle treatment. On the contrary, treatment with 0.10% vitamin C (D) failed to decrease the skin roughness as there was no significant difference between the self-control and the treated cheeks at all time points of application ($P > 0.05$, paired student's t-test).

Thus, the results from this part of study, suggested that all the test antioxidants may be able to counteract the possible negative effects of the solvent and reduce the skin roughness. The most effective antioxidant observed in this study was 0.10% Puag-Haad, followed by 0.25% Puag-Haad and 0.10% EGCG which demonstrated similar anti-wrinkling efficacy. 0.10% vitamin C, on the other hand, appeared to be

the least effective. Histograms summarizing the average values of circular roughness and mean roughness parameters for the individual antioxidants and their respective controls at various times are shown in Figures 34 – 41.

Figure 42 is a representative of the picture taken from the Visioscan showing the skin surface after treatment with 0.10% Puag-Haad for 8 weeks in comparison with its PG self-control taken at the same period (More pictures are shown in Appendix D).

Table 27. The baseline values of circular roughness parameter at 4 and 2 weeks before application of treatment (week -4 and week -2). The values at the start of the experiment (week 0) are also shown (Mean ± SD)

Subject group		Left cheek				Right cheek			
		week -4	week -2	week 0	P-value	week -4	week -2	week 0	P-value[a]
A	Mean	57.38	59.84	57.10	0.10	58.43	58.86	55.96	0.10
(n = 23)	SD	11.26	9.53	10.08	NS*	10.71	8.59	10.73	NS*
B	Mean	58.62	58.74	58.08	0.87	59.02	56.89	56.91	0.40
(n = 22)	SD	10.07	8.86	9.08	NS*	11.29	9.09	11.00	NS*
C	Mean	60.90	59.40	58.24	0.21	60.03	58.25	57.35	0.28
(n = 21)	SD	12.41	10.22	11.44	NS*	11.55	9.11	10.53	NS*
D	Mean	62.10	59.43	58.95	0.29	61.92	59.08	56.33	0.06
(n = 20)	SD	8.44	10.23	12.17	NS*	8.73	10.24	10.26	NS*
P-value[b]		0.465	0.986	0.954		0.731	0.865	0.974	
		NS**	NS**	NS**		NS**	NS**	NS**	

a = P-value for randomized block ANOVA, b = P-value for one-way ANOVA

* No significant time effect on the baseline circular roughness values (P > 0.05) after randomized block ANOVA.

** No significant difference in the baseline circular roughness values between the four groups (P > 0.05) after one-way ANOVA

Table 28. The baseline values of mean roughness parameter at 4 and 2 weeks before application of treatment (week -4 and week -2). The values at the start of the experiment (week 0) are also shown (Mean ± SD)

Subject group		Left cheek				Right cheek			
		week -4	week -2	Week 0	P-value	week -4	week -2	week 0	P-value[a]
A	Mean	55.88	57.94	55.57	0.17	56.61	56.77	53.91	0.07
(n =23)	SD	10.86	9.29	9.76	NS*	10.75	8.73	10.54	NS*
B	Mean	56.82	56.74	56.36	0.94	57.38	54.98	54.83	0.21
(n = 22)	SD	9.79	8.48	9.60	NS*	10.78	8.69	10.60	NS*
C	Mean	59.02	57.70	55.90	0.08	57.81	56.68	55.70	0.42
(n = 21)	SD	11.83	9.77	10.13	NS*	10.93	8.73	10.13	NS*
D	Mean	60.05	57.17	56.48	0.17	59.93	57.27	54.60	0.06
(n = 20)	SD	7.73	9.71	10.98	NS*	8.55	9.34	9.76	NS*
P-value[b]		0.519	0.974	0.990		0.756	0.846	0.950	
		NS**	NS**	NS**		NS**	NS**	NS**	

a = P-value for randomized block ANOVA, b = P-value for one-way ANOVA

* No significant time effect on the baseline mean roughness values ($P > 0.05$) after randomized block ANOVA.

** No significant difference in the baseline mean roughness values between the four groups ($P > 0.05$) after one-way ANOVA

It is also interesting to note that 0.10% Puag-Haad gave the best anti-wrinkle efficacy, with significant effect detected after only 2 weeks of application whereas the higher concentration of Puag-Haad (0.25%) showed much slower onset of efficacy, about 6 weeks later which was similar to 0.10% EGCG. This result agreed with Pengrungruangwong (2001), who studied the whitening efficacy of Puag-Haad solution in the same propylene glycol-water system. She reported that the whitening extent of 0.50% Puag-Haad solution was obviously less than 0.25% Puag-Haad. The data from this study and that of Pengrungruangwong (2001) seem to suggest the importance of Puag-Haad concentration on its anti-wrinkle and whitening efficacy and that there might exist an optimal concentration range for this plant extract. Future experiments are thus needed to establish the most effective concentration of Puag-Haad in cosmetic preparations. Also, the reason as to the weak anti-wrinkle efficacy observed with vitamin C was not clearly known. It could be due to the possible degradation of vitamin C in this solvent system or otherwise its concentration used

may be too low, due to vitamin C has been used in commercial anti-wrinkle cream at 5% concentration (Farris, 2005).

From the above data, it could be concluded that 0.10% Puag-Haad gave a higher anti-wrinkle activity than 0.25% Puag-Haad and 0.10% EGCG. Vitamin C appeared to give the weakest anti-wrinkle effect during the 8-week study period. 0.10% Puag-Haad and 0.10% ECGC were therefore selected to be formulated into lotions and evaluated in human volunteers to further confirm their anti-wrinkle efficacy.

Table 29. Circular roughness values (mean ± SD) measured from female volunteers' cheeks treated with different substances for 8 weeks (n = 20 - 23 subjects per treatment group)

Treatment group		Circular roughness					P-value[a]
		Week 0	Week 2	Week 4	Week 6	Week 8	
A	PG-control	57.10 ±10.08	56.54 ± 11.61	57.00 ± 9.19	57.58 ± 9.81	58.34 ± 9.48	0.824
	A	55.96 ± 10.73	54.65 ± 10.87	54.58 ± 8.56	55.52 ± 9.63	55.75 ± 7.08	0.834
(n = 23)	P-value[b]	0.355	0.131	0.056	0.076	0.028*	
B	PG-control	58.08 ± 9.08	59.62 ± 10.08	60.42 ± 10.71	60.51 ± 9.95	59.19 ± 10.14	0.526
	B	56.90 ± 11.00	54.10 ± 9.83	56.45 ± 8.65	57.26 ± 10.19	55.33 ± 9.66	0.270
(n = 22)	P-value[b]	0.36	0.000*	0.010*	0.013*	0.011*	
C	PG-control	58.24 ± 11.44	58.05 ± 9.44	59.06 ± 10.33	58.51 ± 9.04	59.73 ± 9.05	0.155
	C	57.35 ± 10.53	57.14 ± 10.67	56.68 ± 8.75	56.35 ± 9.19	56.36 ± 10.11	0.975
(n = 21)	P-value[b]	0.407	0.482	0.190	0.058	0.015*	
D	PG-control	56.95 ± 12.17	58.55 ± 10.18	58.92 ± 8.10	57.45 ± 8.56	59.68 ± 8.63	0.765
	D	56.33 ± 10.26	55.97 ± 9.14	56.42 ± 7.73	58.43 ± 9.05	56.63 ± 9.94	0.503
(n = 20)	P-value[b]	0.077	0.069	0.012*	0.481	0.057	

a = P-value for randomized block ANOVA, b = P-value for paired student's t-test

PG-control = 20% propylene glycol self-control. A = 0.25% Puag-Haad, B = 0.10% Puag-Haad, C = 0.10% EGCG, D = 0.10% Vitamin C

* Significantly less than PG-control at the same week ($P < 0.05$, paired t-test)

Table 30. Mean roughness values (mean ± SD) measured from female volunteers' cheeks treated with different substances for 8 weeks (n = 20 - 23 subjects per treatment group)

Treatment group		Mean roughness					P-value[a]
		Week 0	Week 2	Week 4	Week 6	Week 8	
A	PG-control	55.56 ± 9.76	54.59 ± 11.23	54.94 ± 9.01	55.19 ± 9.48	56.53 ± 8.89	0.741
	A	53.91 ± 10.54	53.09 ± 10.67	53.12 ± 8.57	53.79 ± 9.30	53.92 ± 6.69	0.950
	P-value[b]	0.162	0.177	0.131	0.192	0.019*	
B	PG-control	56.36 ± 9.60	57.70 ± 9.58	58.47 ± 10.27	57.56 ± 8.96	56.56 ± 9.71	0.676
	B	54.83 ± 10.60	52.54 ± 9.39	54.80 ± 8.31	54.98 ± 8.74	53.83 ± 9.24	0.333
	P-value[b]	0.212	0.000*	0.011*	0.046*	0.045*	
C	PG-control	55.90 ± 10.80	56.05 ± 9.38	56.75 ± 9.70	56.48 ± 9.19	57.51 ± 8.50	0.113
	C	55.73 ± 10.13	55.33 ± 10.19	55.19 ± 862	54.86 ± 9.36	55.17 ± 10.57	0.987
	P-value[b]	0.864	0.550	0.347	0.119	0.036*	
D	PG-control	56.48 ± 10.98	56.42 ± 9.56	56.60 ± 7.69	55.32 ± 8.32	58.02 ± 10.55	0.657
	D	54.60 ± 9.76	54.52 ± 8.80	54.77 ± 7.53	56.33 ± 8.35	55.80 ± 11.03	0.697
	P-value[b]	0.169	0.139	0.057	0.432	0.126	

a = P-value for randomized block ANOVA, b = P-value for paired student's t-test

PG-control = 20% propylene glycol self-control. A = 0.25% Puag-Haad, B = 0.10% Puag-Haad, C = 0.10% EGCG, D = 0.10% Vitamin C

* Significantly less than PG-control at the same week ($P < 0.05$, paired t-test)

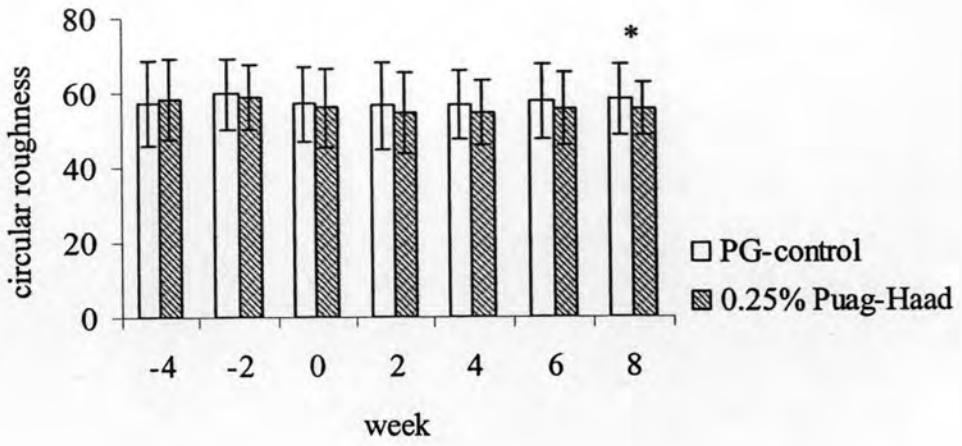


Figure 34. Circular roughness values before and after applying 0.25% Puag-Haad and 20% propylene glycol (PG-control) to the cheek area for different times. Data = mean ± SD (n = 23)

* Values are significantly different between the antioxidant-treated and the control cheeks at the same week ($P < 0.05$).

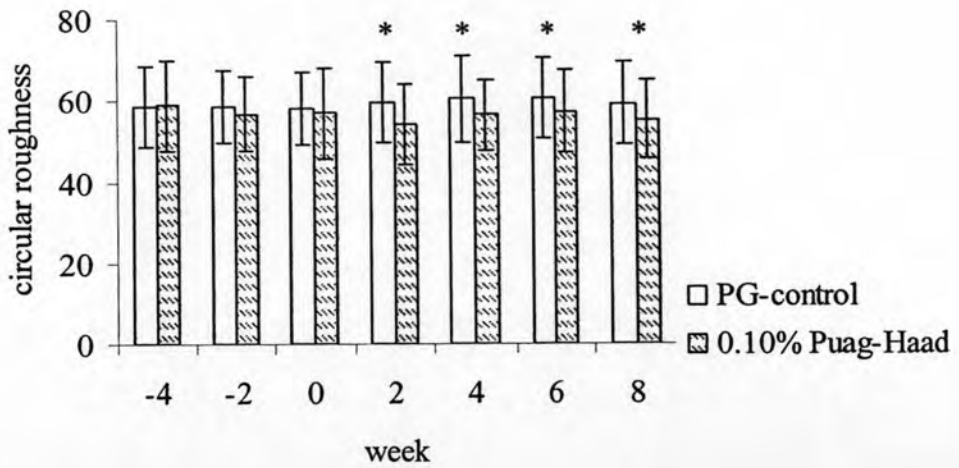


Figure 35. Circular roughness values before and after applying 0.10% Puag-Haad and 20% propylene glycol (PG-control) to the cheek area for different times. Data = mean ± SD (n = 22)

* Values are significantly different between the antioxidant-treated and the control cheeks at the same week ($P < 0.05$).

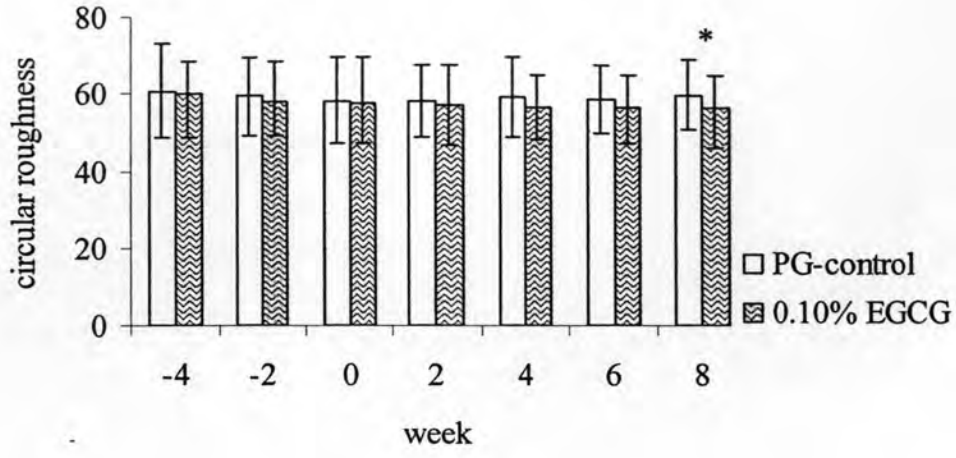


Figure 36. Circular roughness values before and after applying 0.10% EGCG and 20% propylene glycol (PG-control) to the cheek area for different times. Data = mean ± SD (n = 21)

* Values are significantly different between the antioxidant-treated and the control cheeks at the same week ($P < 0.05$).

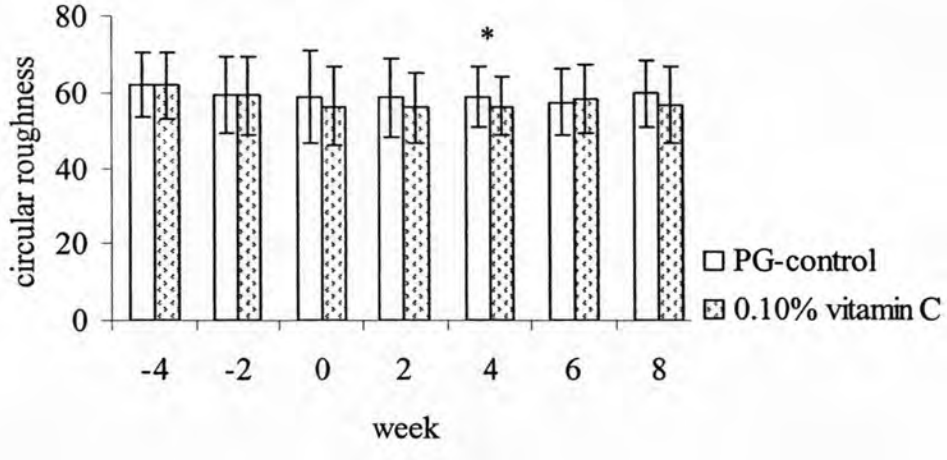


Figure 37. Circular roughness values before and after applying 0.10% Vitamin C and 20% propylene glycol (PG-control) to the cheek area for different times. Data = mean ± SD (n = 20)

* Values are significantly different between the antioxidant-treated and the control cheeks at the same week ($P < 0.05$).

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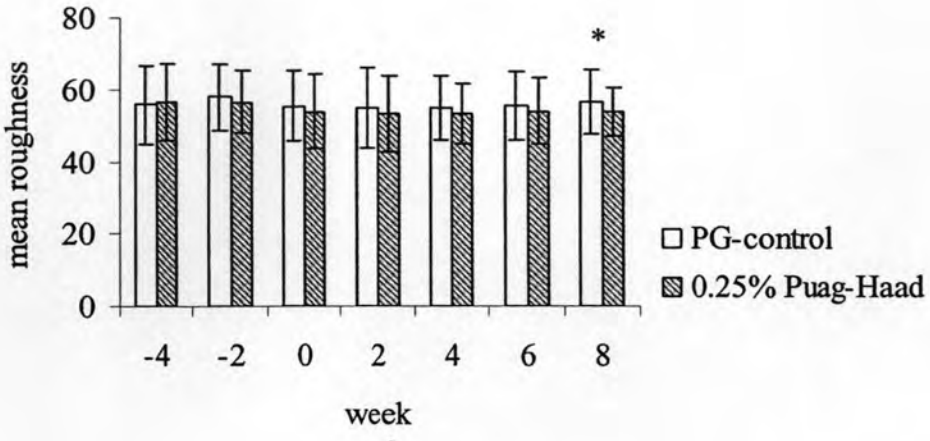


Figure 38. Mean roughness values before and after applying 0.25% Puag-Haad and 20% propylene glycol (PG-control) to the cheek area for different times. Data = mean ± SD (n = 23)

* Values are significantly different between the antioxidant-treated and the control cheeks at the same week ($P < 0.05$).

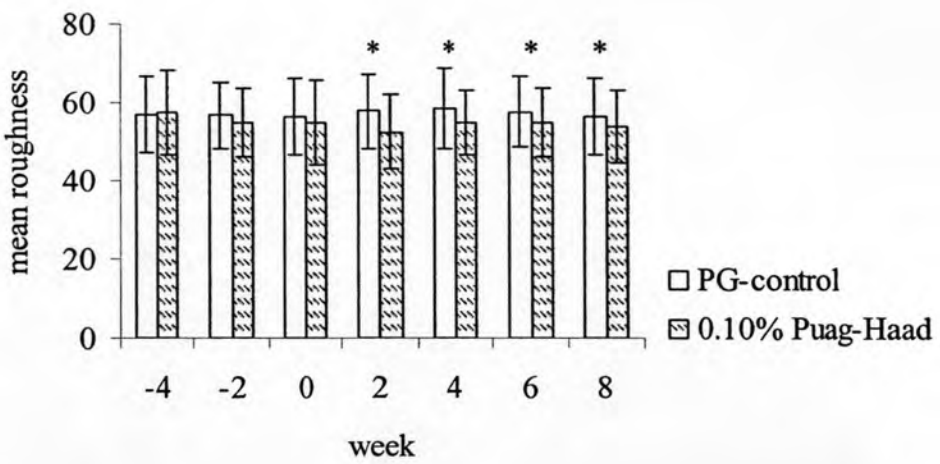


Figure 39. Mean roughness values before and after applying 0.10% Puag-Haad and 20% propylene glycol (PG-control) to the cheek area for different times. Data = mean ± SD (n = 22)

* Values are significantly different between the antioxidant-treated and the control cheeks at the same week ($P < 0.05$).

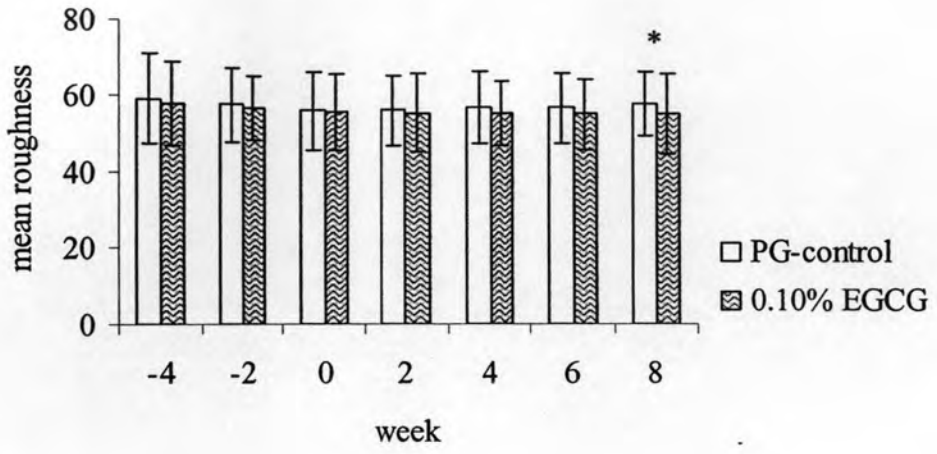


Figure 40. Mean roughness values before and after applying 0.10% EGCG and 20% propylene glycol (PG-control) to the cheek area for different times. Data = mean ± SD (n = 21)

* Values are significantly different between the antioxidant-treated and the control cheeks at the same week ($P < 0.05$).

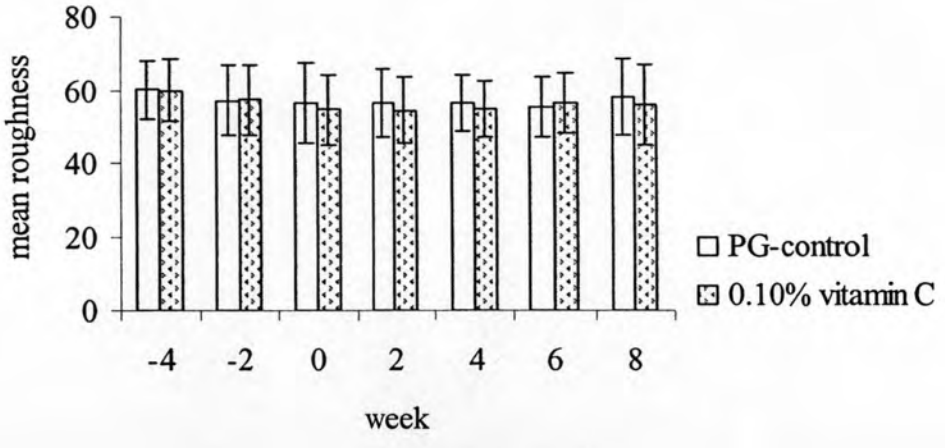
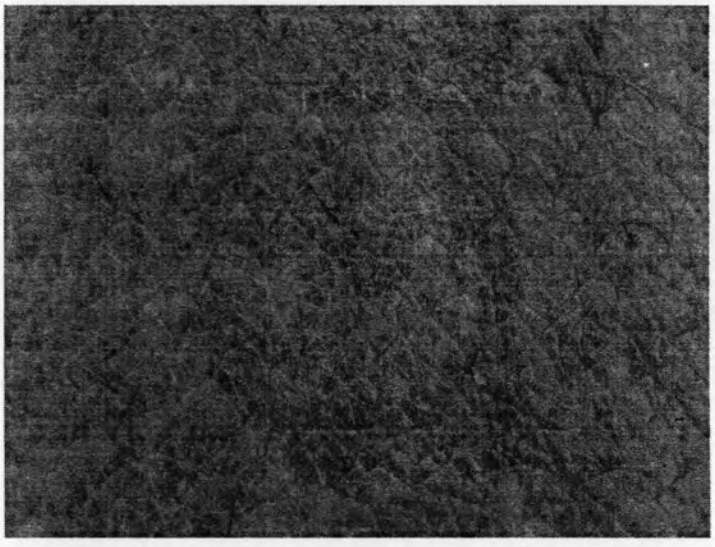


Figure 41. Mean roughness values before and after applying 0.10% Vitamin C and 20% propylene glycol (PG-control) to the cheek area for different times. Data = mean ± SD (n = 20)

Left cheek (control)

circular roughness = 66.00, mean roughness = 63.33



Right cheek (treatment)

circular roughness = 55.33, mean roughness = 53.67

Figure 42. Representative skin surface of one subject after treatment with 0.10% Puag-Haad for 8 weeks in comparison with its PG self-control taken at the same period

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

Lotions containing 0.10% w/w Puag-Haad were formulated by development from a guide formulation originally developed by Pheansri (2001). Summary of the modified Puag-Haad formulations used in this study has already been given in Table 6 (Chapter III). Table 31 shows the physical appearances (phase seperation and color) before and after 6 heat-cool cycles of the prepared Puag-Haad lotions (One cycle consisted of storage at 45 °C for 24 hr and at 4 °C for another 24 hr).

From Table 31, it was found that before the heat-cool cycle, all lotions were physically stable and their color was pale yellow (graded as 0). After 6 heat-cool cycles the formulations 1, 2, 5, 6, 7, 8, 9, 10 and 11 had phase seperation so that a homogeneous color could not be obtained and accurately evaluated. The remaining formulations (3, 4, 12, 13, 14 and 15) had quite a stable consistency and the color had changed only a little (graded as +1) except formulations 14 and 15 of which their color was more intense (+2). This could be due to the color of xanthan gum that was included in these formulations as a thickening agent. Table 33 shows the viscosity and pH of Puag-Haad lotions before and after heat-cool cycles. Due to phase separation, the pH and viscosity of formulations 1, 2, 5, 6, 7, 8, 9, 10 and 11 could not be accurately determined. Formulations 3, 4, and 12 gave a slight drop in viscosity whereas that of formulations 14 and 15 remained relatively unchanged. On the other hand, the viscosity of formulation 13 decreased almost by 50% from the pre-heat-cool value. The pH of all lotions which did not give phase separation was quite stable after the six heat-cool cycles. This was probably due to the presence of citrate buffer components (pH 5.5) in the formulations. From the results of phase separation, color, viscosity and pH, formulations 4 and 12 appeared to be the formulation of choice since they could provide adequate physicochemical stability. Formulations 14 and 15, although their viscosity was quite stable, were not selected because they gave more intense color after the heat-cool cycles whereas the viscosity of formulation 3 was too low.

Table 31. Physical appearances (phase seperation and color) of Puag-Haad lotions before and after 6 heat-cool cycles

Formulation	Before heat-cool cycle		After 6 heat-cool cycles	
	phase seperation	color	phase seperation	Color
1	-	0	PS	N/A
2	-	0	PS	N/A
3	-	0	-	+1
4	-	0	-	+1
5	-	0	PS	N/A
6	-	0	PS	N/A
7	-	0	PS	N/A
8	-	0	PS	N/A
9	-	0	PS	N/A
10	-	0	PS	N/A
11	-	0	PS	N/A
12	-	0	-	+1
13	-	0	-	+1
14	-	0	-	+2
15	-	0	-	+2

- = no phase separation; PS = phase separation; N/A = not available due to PS

0 = normal (pale yellow); without change, +1 = slightly changed (light yellow), +2 = noticeably changed (light brown), +3 = markedly changed (brown), +4 = seriously deteriorated (dark brown), +5 = completely deteriorated (intense deep brown)

Table 32. Viscosity and pH of Puag-Haad lotions before and after 6 heat-cool cycles

Formulation	Before heat-cool cycle		After heat-cool cycle	
	Viscosity (cps)	pH	Viscosity (cps)	pH
1	1471.06	5.55	-	-
2	1813.40	5.48	-	-
3	1178.50	5.48	941	5.50
4	1343.73	5.47	1132.2	5.48
5	1008.00	5.47	-	-
6	1716.73	5.50	-	-
7	1350.27	5.50	-	-
8	1756.40	5.51	-	-
9	994.16	5.49	-	-
10	1550.95	5.50	-	-
11	758.76	5.44	-	-
12	1833.25	5.48	1637.50	5.48
13	2318.33	5.51	1181.50	5.50
14	1685.25	5.50	1663	5.48
15	1846.00	5.52	1809	5.49

- = was not measured due to phase seperation

The self-evaluation results of the formulation effects on the researcher's skin feel are given in Table 33, which reveal that after heat-cool cycles, formulation 12 gave greater satisfaction of the skin feel in terms of spreadability, absorption and stickiness than formulation 4. Formulation 12 was therefore selected for further evaluation in human volunteers in the next part. 0.10% EGCG lotion was also prepared in the same lotion base of formulation 12 and served as a positive control. The formula of formulation 12 is summarized again as shown in Table 34

Table 33. Skin feels of Puag-Haad lotions before and after 6 heat-cool cycles

Formulation	Before heat-cool cycle			After heat-cool cycle		
	spreadability	absorption	Stickiness	spreadability	absorption	Stickiness
1	+3	+2	+3	-	-	-
2	+3	+3	+3	-	-	-
3	+3	+3	+3	+2	+3	+3
4	+3	+3	+3	+3	+2	+2
5	+3	+3	+3	-	-	-
6	+3	+3	+3	-	-	-
7	+3	+3	+3	-	-	-
8	+3	+3	+3	-	-	-
9	+3	+3	+3	-	-	-
10	+3	+3	+3	-	-	-
11	+3	+3	+3	-	-	-
12	+3	+3	+3	+3	+3	+3
13	+3	+2	+1	+2	+2	+1
14	+3	+3	+3	+3	+3	+3
15	+3	+3	+3	+3	+3	+3

+3 = highly satisfied , +2 = moderately satisfied, +1 = least satisfied

- = phase seperation

Table 34. Composition of the test anti-wrinkle lotions (%w/w)

Ingredient	A	B
Phase A		
Puresyn No.2 *	3	3
Cetearyl alcohol	0.8	0.8
Glyceryl monostearate	1	1
Ceteareth-20	0.8	0.8
Emulgrade NI 1000 **	2.3	2.3
Myristol 318 ***	3	3
Dimethicone	1	1
Phase B		
Glycerine	2	2
Propylene glycol	3	3
DI water	81.2	81.2
Phase C		
Puag-Haad	0.1	-
EGCG	-	0.1
BHA	0.02	0.02
Buffer	1.28	1.28
Sepicide HB ****	0.5	0.5

* = Hydrogenated polydecene

** = Cetearyl alcohol/Ceteareth-20

*** = Caprylic/capric triglyderide,

**** = Phenoxyethanol + combined paraben

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

The purpose of this part was to confirm the *in vivo* anti-wrinkle efficacy in healthy volunteers of *Artocarpus lakoocha* heartwood extract or Puag-Haad lotion at 0.10% w/v concentration. The anti-wrinkle activity was also compared with 0.10% w/v EGCG in the same lotion base (formulation 12).

Fourty-four healthy female volunteers (selected from the same group as in Part 3), aged ranging from 30 – 55 years, were recruited by the same criteria as those in 3.1. They were randomly divided into two groups of 22 subjects each. Each group thus separately received different treatment sample lotion. All of them stopped using any cosmetics on the application areas for at least one week before the start of the experiments. The application sites were the whole area of both the left and the right face.

Puag-Haad lotion was assigned as treatment A whereas EGCG lotion was assigned as treatment B. The first group (group A) received Puag-Haad lotion on one side of their face whereas the remaining side received the lotion base as a self-control. Similarly, the second group (B) received EGCG lotion on one side of their face and the lotion base on the other side. The side of application was randomized such that half of the subject in each group (11 subjects) received the treatment lotion on their right side of the face whereas the remaining half received the treatment lotion on their left side. The application amount was always 0.2 g for both the treatment and the self-control samples.

Each volunteer would self-apply the self-control and the treatment lotions twice daily, in the morning and at night time for 8 consecutive weeks. They were monitored for any changes in skin roughness parameters and skin elasticity at week 0 and every two weeks interval using Visioscan® VC 98 and Cutometer® MPA 580, respectively. The melanin value and skin hydration extent were also monitored for any changes at week 0 and every four weeks using Mexameter® MX18 and Corneometer® CM 825, respectively.

Table 35 shows average circular roughness values for each group. As seen from this table, the value of the face treated with the test lotion in group A and B slightly decreased with time compared to their respective self-control. In group A, the average circular roughness value of the Puag-Haad treated side decreased from 63.50

at week 0 to 58.59 at week 8. In group B, the value also decreased from 64.05 at week 0 to 59.52 at week 8. However, the average circular roughness of the control for group A remained relatively unchanged, i.e. from 64.52 at week 0 to 65.05, 63.50, 64.61 and 63.11 at weeks 2, 4, 6 and 8 respectively. The values for the control side in group B also were very stable, starting from 63.21 at week 0 to 63.38 at week 8. When randomized block ANOVA was applied to test for the effect of time on the average roughness of control in groups A and B, no significant difference was found among the values along the study period ($P > 0.05$, randomized block ANOVA). The result thus indicated that the average circular roughness of the control side did not change with time. Similar behavior was also observed for the mean roughness parameter as shown in Table 36. Application of the control lotion base did not produce any significant changes in the mean roughness values with time in both groups ($P > 0.05$, randomized block ANOVA).

Paired student's t-test was applied every two weeks to compare the values of circular roughness and mean roughness between control and treatment of the same subject within each treatment group at 5% significant level. The results are also shown in Table 35 and 36 where significant difference ($P < 0.05$) is denoted by a single asterisk. It can be seen from these tables that subject treated with 0.10% Puag-Haad lotion gave the significant difference over their control after application for 6 weeks in both circular roughness and mean roughness. Thereafter, the roughness level continued to be significantly lower than the self-control until week 8, the last week of the study. Randomized block ANOVA was applied to test the effect of time on the treatment side. The significant difference was found in both the circular roughness and mean roughness values with respect to the application time, with the P-value of 0.017 and 0.015, respectively. Post-ANOVA Dunnett's test was subsequently applied to test the difference in values between the starting time (week 0) and the subsequent application time. The results showed significant differences in the roughness values between week 8 and week 0 for both roughness parameters ($P = 0.020$ for circular roughness and 0.008 for mean roughness). Thus, the results indicated that there was a significant anti-wrinkle effect after applying 0.10% Puag-Haad lotion for 6 weeks, with the values of both roughness parameters significantly lower than the control lotion base at week 6 and 8. Significant time effect and Dunnett's test result also implied that the roughness values also decreased with time following application of Puag-Haad lotion, especially after 8 weeks of application.

On the other hand, subjects treated with 0.10% EGCG lotion gave a significant anti-wrinkle effect compared to their self-control only at the last week (week 8) of application ($P < 0.05$, paired student's t-test) for both roughness parameters (Tables 35 and 36). However, when randomized block ANOVA was employed to test for the effect of time of EGCG application, significant difference was not found in both the circular and mean roughness values during the 8-week period ($P > 0.05$). This implied that the EGCG lotion might need a longer application time to exert a significant reduction in the roughness values from the initial state. Thus, 0.10% Puag-Haad lotion appeared to induce an anti-wrinkle effect faster than 0.10% EGCG lotion.

With respect to the extent of anti-wrinkle efficacy, percent improvement in skin roughness over self-control was used for comparison between the two lotions. Since EGCG gave significant anti-wrinkle effect only at week 8, the extent of anti-wrinkle effect was compared at this time point, using the formula:

$$\text{Percent improvement in roughness} = \frac{Rc - Rt}{Rc} \times 100\%$$

Rc = Roughness value at week 8 of the control-treated side

Rt = Roughness value at week 8 of the antioxidant-treated side

The average values of percent improvement in circular and mean roughness at week 8 are provided in Table 37. Unpaired t-test was further applied to test for difference in percent improvement. From the P-values in this table, the result shows that percent improvement in both the circular and the mean roughness values did not differ significantly between Puag-Haad and EGCG lotions, indicating the similar extent of anti-wrinkle efficacy.

Table 35. Circular roughness values (mean ± SD) in human volunteers treated with Puag-Haad or EGCG lotions for 8 weeks (n = 22 subjects per treatment group)

Treatment group		Circular roughness					P-value[a]
		Week 0	Week 2	Week 4	Week 6	Week 8	
A (n = 22)	Control	64.52 ± 11.73	65.05 ± 14.17	63.50 ± 11.83	64.61 ± 10.49	63.11 ± 11.98	0.715
	Puag-Haad	63.50 ± 10.30	62.98 ± 10.12	63.91 ± 8.65	61.23 ± 9.63	58.59 ± 10.91	0.017**
	P-value[b]	0.419	0.286	0.797	0.035*	0.03*	
B (n = 22)	Control	63.21 ± 9.09	62.93 ± 10.56	63.50 ± 10.32	63.53 ± 10.95	63.38 ± 8.31	0.996
	EGCG	64.05 ± 9.74	62.80 ± 10.53	63.09 ± 10.88	63.74 ± 8.50	59.52 ± 9.22	0.069
	P-value[b]	0.518	0.937	0.803	0.877	0.047*	

a = P-value for randomized block ANOVA, b = P-value for paired student's t-test

* Significant difference in the circular roughness values between the control and treatment at the same week ($P < 0.05$, pair student's t-test)

** Significant time effect on the circular roughness value ($P < 0.05$, randomized block ANOVA)

Table 36. Mean roughness values (mean ± SD) in human volunteers treated with Puag-Haad or EGCG lotions for 8 weeks (n = 22 subjects per treatment group)

Treatment group		Mean roughness					P-value[a]
		Week 0	Week 2	Week 4	Week 6	Week 8	
A	Control	62.27 ± 11.07	62.98 ± 12.92	60.77 ± 10.15	62.94 ± 10.35	60.70 ± 11.31	0.25
	Puag-Haad	61.41 ± 10.01	59.98 ± 10.47	61.32 ± 8.38	59.29 ± 8.99	56.58 ± 10.33	0.015**
	P-value[b]	0.436	0.098	0.695	0.007*	0.024*	
B	Control	61.24 ± 8.32	60.84 ± 9.99	61.25 ± 10.10	61.38 ± 10.24	61.39 ± 9.35	0.575
	EGCG	61.91 ± 8.95	60.68 ± 9.63	60.89 ± 10.56	60.92 ± 8.25	57.45 ± 8.57	0.059
	P-value[b]	0.528	0.917	0.803	0.700	0.045*	

a = P-value for randomized block ANOVA, b = P-value for paired student's t-test

* Significant difference in the mean roughness values between the control and treatment at the same week ($P < 0.05$, pair student's t-test)

** Significant time effect on the mean roughness value ($P < 0.05$, randomized block ANOVA)

Table 37. Percent improvement in circular and mean roughness values (mean ± SD, n = 22 subjects per group) after 8 weeks of application of lotions containing Puag-Haad and EGCG

Percent improvement	0.10% Puag-Haad	0.10% EGCG	P-value
Circular roughness	5.93 ± 14.77	5.64 ± 12.86	>> 0.05
Mean roughness	5.78 ± 13.65	2.11 ± 13.48	>> 0.05

Therefore, based on the circular and the mean roughness results, it could be concluded that 0.10% Puag-Haad lotion gave faster anti-wrinkle activity than 0.10% EGCG lotion. The extent of their anti-wrinkle effect, however, appeared to be similar. The data are also depicted graphically for each antioxidant in Figures 43 - 46.

Also, it is interesting to note that the values of both circular and mean roughness obtained from this part of study (Tables 35 - 36) were more predictable than the previous part, which was studied using solutions of antioxidants in 20% propylene glycol (Tables 29 - 30). In the previous part, the roughness values of the solvent (propylene glycol-control) tended to increase with time from the initial value, especially at week 8, whereas the values for the antioxidant solutions remained unchanged or decreased very slightly. This was explained in part by the skin-dehydrating effect of propylene glycol, which might counteract the anti-wrinkle effect of the antioxidants. In this part, the lotion base was used in place of 20% propylene glycol. The presence of the moisturizing oil phase in the lotion, which is an oil-in-water emulsion dosage form, may help alleviate the negative dehydrating effect of propylene glycol solvent, resulting in the roughness values in the control lotion-treated side being more stable as seen in Tables 35 - 36. The roughness values of the lotions containing Puag-Haad and EGCG also tended to decrease gradually with time, providing straightforward evidence that the added antioxidant had a beneficial effect of reducing the skin roughness. In addition, allocation of the treatment was more balanced in the lotion study, in which half of the subjects in each group received the antioxidant lotion on the right face and the control lotion on the left face (and vice versa for the other half). In the previous solution study, the right cheek was always applied with the antioxidant solutions and the left cheek with 20% propylene glycol control. Thus, the application position was better distributed in the lotion study, which might lead to less variable data.

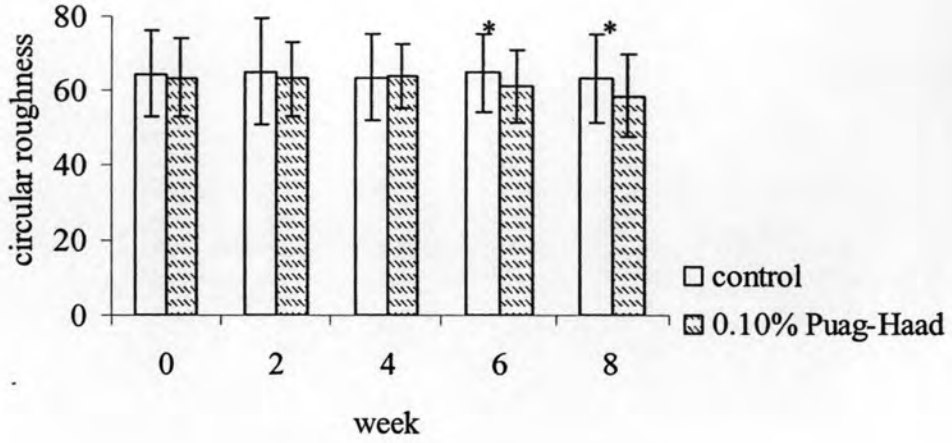


Figure 43. Circular roughness values after applying 0.10% Puag-Haad lotion and lotion base for different times. Data = mean ± SD (n = 22)

* Values are significantly different ($P < 0.05$, paired student's t-test)

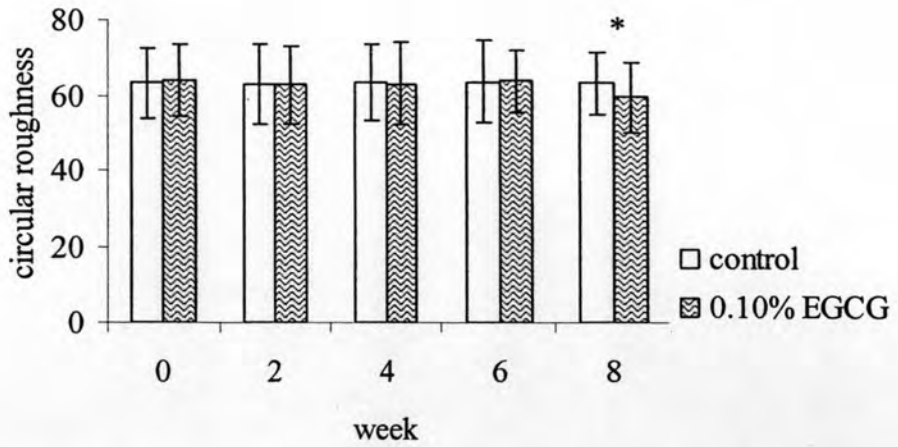


Figure 44. Circular roughness values after applying 0.10% EGCG lotion and lotion base for different times. Data = mean ± SD (n = 22)

* Values are significantly different ($P < 0.05$, paired student's t-test)

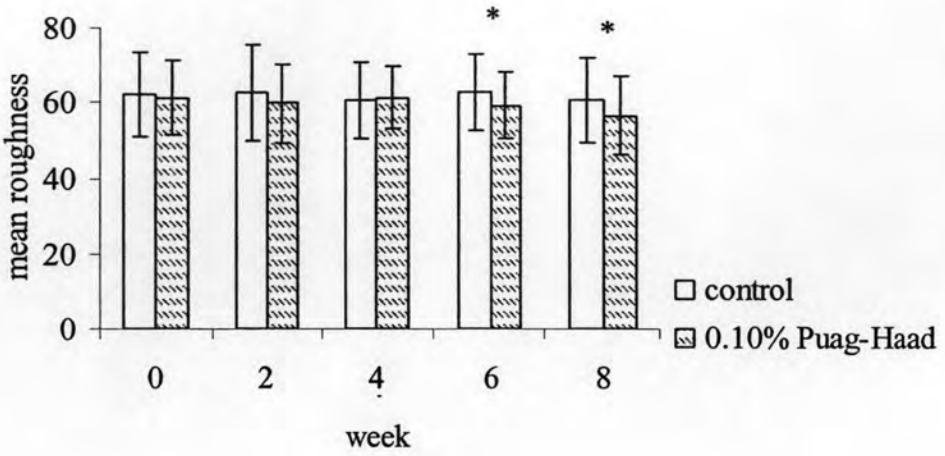


Figure 45. Mean roughness values after applying 0.10% Puag-Haad lotion and lotion base for different times. Data = mean ± SD (n = 22)

* Values are significantly different ($P < 0.05$, paired student's t-test)

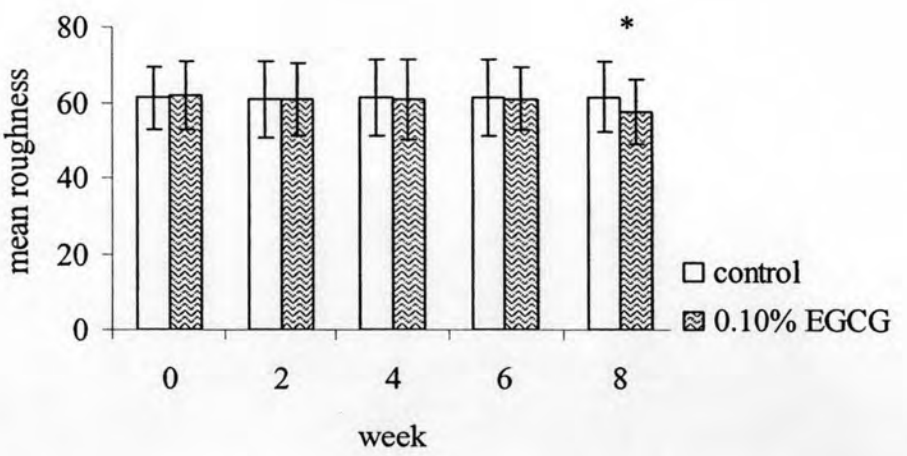


Figure 46. Mean roughness values after applying 0.10% EGCG lotion and lotion base for different times. Data = mean ± SD (n = 22)

* Values are significantly different ($P < 0.05$, paired student's t-test)

Measurement of skin elasticity

Skin elasticity was also monitored by Cutometer® MPA 580 every two weeks. The R2 parameter was determined using the built-in software. The higher value of R2 indicated greater elasticity of the skin. Table 38 shows the average values of skin elasticity for each treatment group. From the table, the R2 values did not seem to change from the initial values regardless of the type of the applied lotion. Randomized block ANOVA was applied to test the effect of application time on the R2 values of either the control or antioxidant-treated side. No significant differences were found in all cases ($P > 0.05$), suggesting that none of the control, Puag-Haad and EGCG lotions had any effect on this parameter. Paired student's t-test was also applied to the biweekly R2 data to compare the values of skin elasticity between control and treatment of the same subject within each treatment group. In agreement with the ANOVA results on the time effect, no significant differences were found between control and treatment in both Puag-Haad and EGCG groups at all weeks measured ($P > 0.05$). The R2 values for the control and the antioxidant-treated sides were similar at all weeks. Thus, the results from the Cutometer® indicated that both the Puag-Haad and EGCG lotions did not improve the skin elasticity over its self-control throughout the 8-week study period. The R2 data are graphically represented for each antioxidant in Figures 47 – 48.

The data from the Cutometer® was therefore quite different from those of Visioscan© which indicated that both the Puag-Haad and EGCG lotions were quite effective in reducing the skin roughness. This could be primarily due to the difficulties in using the Cutometer®, which required a very experienced hand to precisely and reproducibly place the probe onto the subject's skin for each measurement. The pressure so generated as the probe was pushed against the skin surface could also vary greatly leading to fluctuation in elasticity responses. The sensitivity of the instrument could be another factor contributing to the current results. It is possible that changes in the skin elasticity due to the use of antioxidant lotions might be too small for the instrument to be able to distinguish. Green tea extract (containing 38% EGCG) has previously been reported to improve skin elasticity in human volunteers by skin biopsies method (Chui, 2005). However, the subjects received both topical and oral preparations of green tea extract at very high dose (10% green tea cream and 300 mg daily oral dose) in order to achieve detectable

improvement. The same paper also reported skin irritation from high percentage of green tea extract in topical preparation.

Measurement of melanin content (whitening) and skin capacitance

Other skin conditioning properties were also investigated such as skin whitening and skin hydration by monitoring the subjects for any changes in these parameters every four weeks. Skin whitening was monitored using Mexameter® MX18 and the data are shown in Table 39. From this table, it can be seen that the melanin values of the self-control lotion did not change from the initial value in both groups. However, the melanin values in the subjects treated with Puag-Haad lotion gradually decreased with time from 257.64 at week 0 to 252.95 at week 8. Significant time effect was also detected with Puag-Haad lotion but not for the control lotion. When paired student's t-test was applied to compare the melanin values between control and treatment at each week, the value of the Puag-Haad-treated side became significantly lower than control after 4 weeks of application ($P < 0.05$), indicating a whitening effect of Puag-Haad lotion on the subject's skin.

This result agreed with Tengamnuay et al. (2006), who reported that 0.10% Puag-Haad lotion gave a significant whitening effect over the self-control since the third week of application. It is well known that oxyresveratrol, the active constituent of Puag-Haad, is a potent tyrosinase inhibitor (Likhitwitayawuid, Sritularak and De-Eknamkul, 2000) and the extract had been previously shown to be a more effective skin whitening agent than kojic acid and licorice extract in both the guinea pig and human models (Pengrungruangwong, 2001). On the other hand, the melanin values in subjects treated with 0.10% EGCG remained relatively unchanged throughout the 8-week study period similar to its self-control ($P > 0.05$, randomized block ANOVA on time effect). Paired student's t-test also gave no significant difference between control and treatment at all weeks ($P > 0.05$). Thus, only Puag-Haad lotion was effective in reducing the melanin values whereas EGCG lotion did not have the whitening activity. The data are also graphically represented for each antioxidant in Figures 49 and 50.

Table 40 shows the skin capacitance for each lotion which was measured using Corneometer® CM 825. From this table, it can be seen that the skin capacitance clearly increased with time in all groups regardless of the type of lotion. For example, in subjects group A, the values for the control lotion increased from

48.08 at week 0 to 54.67 at week 8 whereas those for the Puag-Haad lotion increased from 43.60 to 54.97 during the same period. In group B, the control lotion gave an increase in value from 48.19 at week 0 to 55.46 at week 8 whereas the EGCG lotion increased from 50.96 to 56.72 during the same period. Randomized block ANOVA was further applied to evaluate the effect of application time on the capacitance values. A highly significant time effect was found in all lotions (control, Puag-Haad and EGCG) indicating that they were capable of increasing the skin capacitance with increasing application time ($P < 0.05$). Paired student's t-test was also applied to compare the values of skin capacitance between control and treatment at each week. No significant differences were found at all weeks for both groups ($P > 0.05$). The data thus indicated that the lotion base (control) and the antioxidant lotions (Puag-Haad and EGCG) were equally effective in increasing the skin capacitance. The improvement in skin capacitance was probably due to the moisturizing effect of the lotion base as this was the common vehicle in all lotions studied. The data on skin capacitance are also depicted graphically for each antioxidant in Figures 51 and 52.

Table 38. Skin elasticity values (R2) (mean ± SD) in human volunteers treated with Puag-Haad or EGCG lotions for 8 weeks (n = 22 subjects per treatment group)

Treatment group		Skin elasticity (R2)					P-value[a]
		Week 0	Week 2	Week 4	Week 6	Week 8	
A	Control	0.8749 ± 0.0563	0.8824 ± 0.0448	0.8754 ± 0.0525	0.8731 ± 0.0467	0.8891 ± 0.0527	0.375
	Puag-Haad	0.8745 ± 0.0486	0.8810 ± 0.0486	0.8722 ± 0.0548	0.8742 ± 0.0534	0.8821 ± 0.0517	0.646
	P-value[b]	0.968	0.781	0.544	0.852	0.516	
B	Control	0.8562 ± 0.0685	0.8552 ± 0.0759	0.8427 ± 0.0703	0.8597 ± 0.0527	0.8559 ± 0.0693	0.428
	EGCG	0.8490 ± 0.0838	0.8575 ± 0.0736	0.8443 ± 0.0756	0.8482 ± 0.0755	0.8581 ± 0.0686	0.492
	P-value[b]	0.498	0.818	0.815	0.182	0.838	

a = P-value for randomized block ANOVA, b = P-value for paired student's t-test

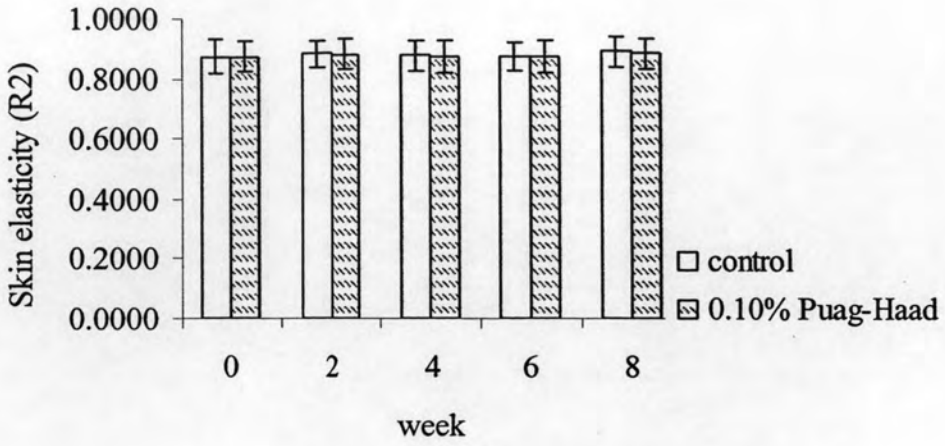


Figure 47. Skin elasticity (R2) values after applying 0.10% Puag-Haad lotion and lotion base for different times. Data = mean ± SD (n=22)

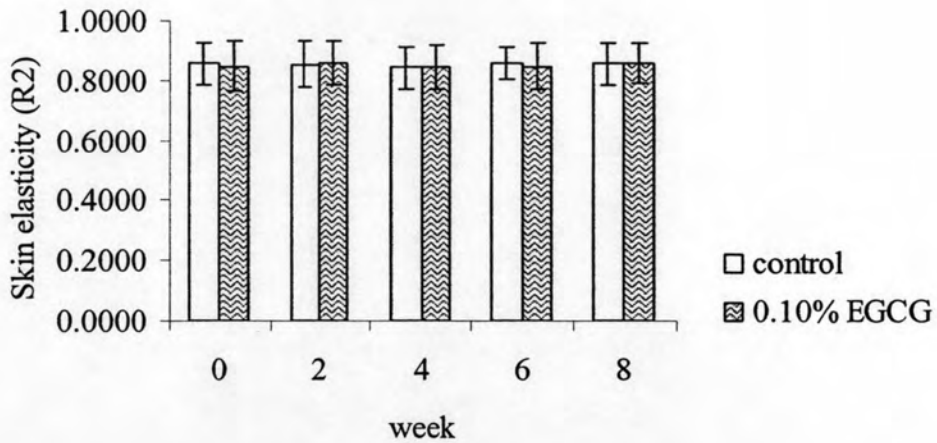


Figure 48. Skin elasticity (R2) values after applying 0.10% EGCG lotion and lotion base for different times. Data = mean ± SD (n=22)

Table 39. Skin melanin values (mean ± SD) of human volunteers treated with Puag-Haad or EGCG lotions for 8 weeks (n = 22 subjects per treatment group)

Treatment group		Melanin value			P-value[a]
		Week 0	Week 4	Week 8	
A	Control	258.16 ± 62.81	260.02 ± 66.36	259.47 ± 65.36	0.669
	Puag-Haad	257.64 ± 61.54	255.32 ± 67.40	252.95 ± 62.95	0.027**
	P-value[b]	0.633	0.005*	0.004*	
B	Control	255.51 ± 60.32	258.74 ± 60.25	255.87 ± 58.74	0.156
	EGCG	256.48 ± 71.54	257.06 ± 63.79	255.74 ± 69.01	0.449
	P-value[b]	0.867	0.612	0.984	

a = P-value for randomized block ANOVA, b = P-value for paired student's t-test

* Significant difference of melanin values between control and treatment in the same week ($P < 0.05$) after pair student's t-test.

** Significant time effect on melanin value ($P < 0.05$) after randomized block ANOVA.

Table 40. Skin capacitance values (mean ± SD) in human volunteers treated with Puag-Haad or EGCG lotions for 8 weeks (n = 22 subjects per treatment group)

Treatment group		Skin capacitance			P-value[a]
		Week 0	Week 4	Week 8	
A	Control	48.08 ± 13.63	52.73 ± 10.47	54.67 ±10.25	0.007**
	Puag-Haad	43.60 ± 12.89	50.92 ± 11.74	54.97 ± 7.96	0.000**
	P-value[b]	0.144	0.423	0.863	
B	Control	48.19 ± 12.24	54.23 ± 15.94	55.46 ±12.31	0.035**
	EGCG	50.96 ± 9.52	55.56 ± 10.45	56.72 ±11.05	0.034**
	P-value[b]	0.246	0.551	0.585	

a = P-value for randomized block ANOVA, b = P-value for paired student's t-test

** Significant time effect on skin capacitance value ($P < 0.05$) after randomized block ANOVA.

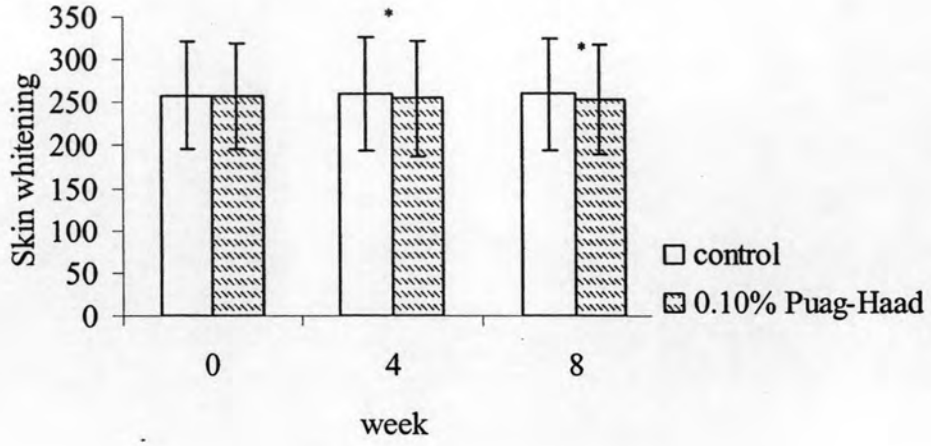


Figure 49. Skin whitening (melanin values) after applying 0.10% Puag-Haad lotion and lotion base for different times. Data = mean ± SD (n=22)

* Values are significantly different ($P < 0.05$, paired student's t-test)

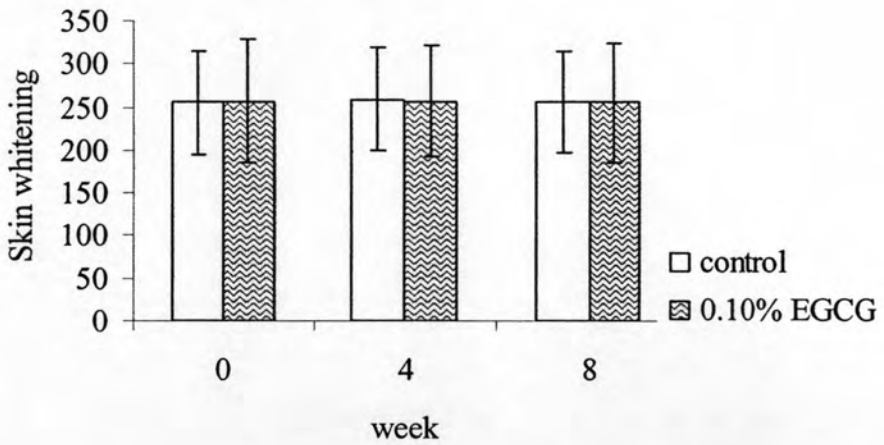


Figure 50. Skin whitening (melanin values) after applying 0.10% EGCG lotion and lotion base for different times. Data = mean ± SD (n=22)

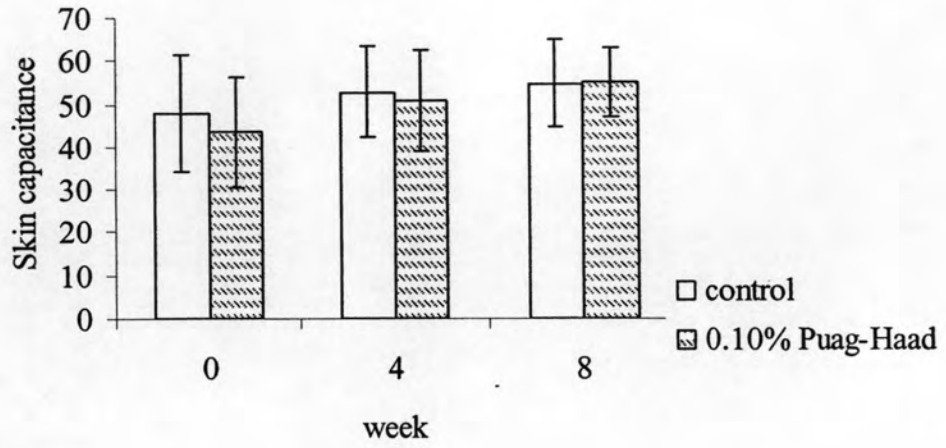


Figure 51. Skin capacitance values after applying 0.10% Puag-Haad lotion and lotion base for different times. Data = mean ± SD (n=22)

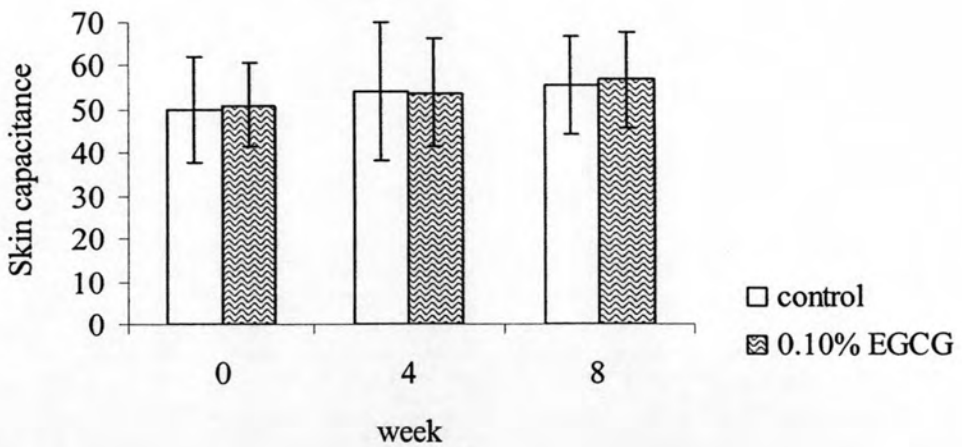


Figure 52. Skin capacitance values after applying 0.10% EGCG lotion and lotion base for different times. Data = mean ± SD (n=22)

Sensory evaluation of lotions

The lotions appeared to be well tolerated by the subjects. No skin irritation or hypersensitivity reactions were observed in all cases and none of subjects withdrew from the study. At the end of the study, the subjects were also asked to fill the questionnaire to evaluate their level of satisfaction on the product and its effect on their skin. Table 41 shows the level of product satisfaction assessed by the subjects with respect to ease of application, speed of absorption, stickiness and overall perception (expressed as percentage of total subjects). All the subjects were blinded and did not know which type of lotion they were using. Since all types of lotions (control, Puag-Haad and EGCG) had common lotion base, the results in this table were thus regarded as obtained from a single product. From this table, the majority of subjects (more than 60%) were highly satisfied with the product's ease of application, stickiness, and overall perception. About 43% of the subjects were highly satisfied with the speed of absorption and 57% with moderate satisfaction. Only about 2% graded stickiness as least satisfied.

Table 41. Level of product satisfaction evaluated by the subjects (% of total subject, n = 44)

Attributes	Level of satisfaction		
	most	mederate	least
Ease of application	68.18	31.82	0
Speed of penetration	43.18	56.82	0
Stickiness	72.73	25	2.27
Overall perception	70.45	29.55	0

* The subjects were blinded and did not know what type of lotion being used.

Table 42 shows results of the subjects' self-evaluation on their skin feel after using the lotion. The skin feel attributes were skin roughness, skin elasticity, skin whitening and skin softening. Regarding the skin roughness, 18.19% of subjects felt that the results were better on the Puag-Haad-treated side than on the control side whereas 27.27 % felt the opposite. 27.27% felt equal improvement in both sides whereas another 27.27% felt that there was no change in both sides. 22.73% of subjects treated with EGCG felt that the skin roughness improved better on the treated

side than on the control side whereas only 4.54% felt otherwise. 50% of subjects in this group felt that both the EGCG and control lotions equally improve the skin roughness whereas 22.73 % felt no change in both sides.

Table 42. Subjects' self evaluation results on the skin feel (% of total subjects, n = 22 per group)

Group	Skin feel	Percentage of satisfaction			
		Better on the control side	Better on the treatment side	Better equally on both sides	No changed
A	Skin roughness	27.27	18.19	27.27	27.27
	Skin elasticity	18.19	36.36	40.91	4.54
	Skin whitening	4.54	18.19	40.91	36.36
	Skin softening	4.54	9.10	86.36	0
B	Skin roughness	4.54	22.73	50.00	22.73
	Skin elasticity	18.19	22.73	45.45	13.63
	Skin whitening	13.63	13.63	54.55	18.19
	Skin softening	9.10	4.54	86.36	0

* The subjects were blinded and did not know what type of lotion being used.

Regarding the skin elasticity, 36.36% of subjects felt that the results were better on the Puag-Haad-treated side than on the control side whereas 18.19% felt the opposite. 40.91% felt equal improvement in both sides whereas only 4.54% felt that there was no change in both sides. 22.73% of subjects treated with EGCG felt that the skin elasticity improved better on the treated side than on the control side whereas 18.19% felt otherwise. 45.45% of subjects in this group felt that both the EGCG and control lotions equally improve the skin roughness whereas 13.63 % felt no change in both sides.

It can be noticed that the results from the self-evaluation of subjects on skin roughness and skin elasticity appeared to be not compatible with those using instruments. This is not surprising, however, because subject's self-evaluation is a highly subjective method. It is naturally difficult to discern a small change in skin condition, which requires a highly sensitive instrument to detect. In addition, all the subjects had not been trained beforehand to make a self-evaluation and no definitions about the skin roughness nor skin elasticity was given to them prior to evaluation. Thus, interpretation of these parameters may be different among the individual subjects.

Results on the skin whitening and skin softening were also very subjective. However, 18.19% of the subjects treated with Puag-Haad lotion felt that the Puag-Haad-treated side was better (whiter) than control whereas only 4.54% felt the opposite. The whitening effect of Puag-Haad felt by the subjects thus appeared to be compatible with the instrumental results. Although 13.63% of subjects treated with EGCG felt that the antioxidant provided better whitening effect than control, the same proportion of subjects in this group (13.63%) felt the opposite.

Regarding the skin softening effect, most of the subjects in the two groups (86.36%) felt that both sides of their face equally improved and none of them (0%) felt no change in both sides. This could be due to the moisturizing effect of the lotion base rather than due to the activity of the added antioxidants. Thus, the skin softening effect felt by the subjects appeared to be compatible with the skin capacitance data.

In conclusion, the results obtained from this part indicated that 0.10% Puag-Haad lotion gave better anti-wrinkle efficacy than 0.10% EGCG lotion based on the roughness values (both circular and mean roughness) using Visioscan©. Significant anti-wrinkle effect was observed after 6 weeks of Puag-Haad application whereas EGCG lotion required 8 weeks to observe significant effect. Puag-Haad lotion also had added benefit as a whitening agent. Both Puag-Haad and EGCG lotions did not produce improvement in skin elasticity as measured by Cutometer©. However, both lotions could improve skin hydration, as evaluated from the skin capacitance using Corneometer© due to the moisturizing effect of the lotion vehicle.

Therefore, judging from its anti-wrinkle and skin whitening activities, Puag-Haad seems to be a very attractive choice as a source of natural cosmetic ingredient, which is highly available and economical. Its market price is only about 400 - 600 baht/kg compared to 8,500-12,000 baht/kg for EGCG. It thus has a very promising potential for further development as an effective skin whitening, antioxidant and anti-wrinkle agent for cosmetic products.