CHAPTER IV

RESULTS AND DISCUSSION

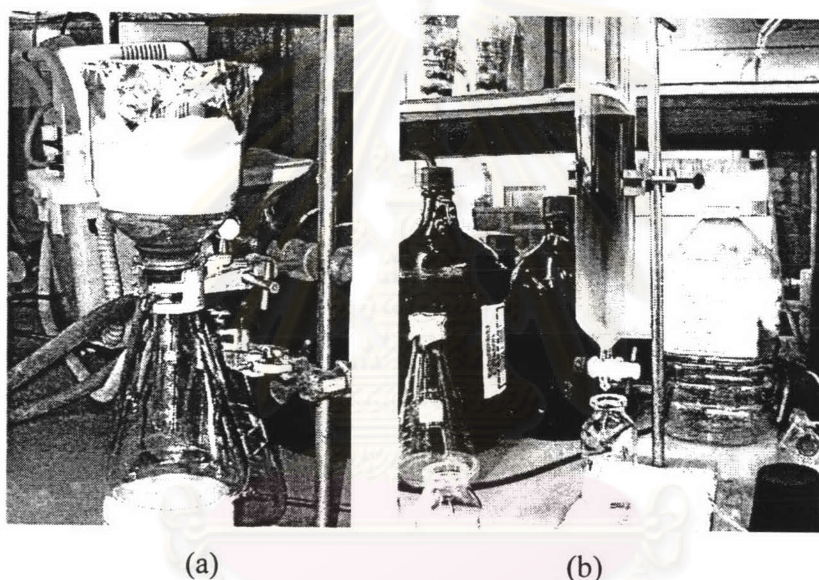
Part 1. Extraction and Isolation of Active Constituent (oxyresveratrol) from Aqueous Extract of *Artocarpus lakoocha* Heartwood (Puag-Haad)

Extraction of constituents from plants can be done by various methods depending on the polarity of the components to be extracted. Two chromatographic techniques used in this study were vacuum liquid column chromatography (VLC) or quick column chromatography and gel filtration chromatography (Figure 11(a) and 11(b). In the quick column chromatography, the dried aqueous extract of *Artocarpus lakoocha* heartwood or Puag-Haad powder (10.02 g) was separated in a polarity gradient manner with mixtures of chloroform and methanol as the solvents. Then the eluents were examined by TLC using 15% chloroform in methanol as developing solvent. Twenty-five fractions of the eluents were collected and sorted by TLC before being combined to yield six fractions with similar chromatographic patterns. The details and pictures of the TLC chromatograms are demonstrated in Table 5 and Figure 12, respectively. Fractions 10-13, were pooled and concentrated under reduced pressure using a rotary evaporator. This semi-pure extracts were weighed and calculated for the percentage yield of the extract . The results of the percentage yields were 69.13 % w/w .

Table 5. The extraction of Puag-Haad by quick column chromatography

Fraction	Weight (g)	% yield
1-2	-	-
3-8	0.0599	0.60
9	0.2052	2.05
10-13	6.9266	69.13*
14-18	1.1630	11.61
19-25	0.0602	1.21

Then fractions 10-13 were further purified or fractionated by gel filtration chromatography using Sephadex LH20 as adsorbent and methanol as eluent in an isocratic system. The fractions were collected and examined by aboved TLC condition. This method was repeated until the TLC chromatogram of the fractions showed a single spot under UV light at 254 nm. Finally, all fractions with the same single spots were pooled and evaporated under reduced pressure. The obtained compound was as a pale-yellow crystal and gave $R_f = 0.24$ in the TLC chromatogram (Figures 13 and 14). The UV spectrum (Figure 15) showed a maximum absorbance at λ_{max} 329 nm.



(a) (b)

Figure 11. Quick column chromatography (a) and gel column chromatography (b)

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Figure 12. TLC chromatogram of the fractions under UV 254 nm

Figure 13. TLC chromatogram of the pure compound under UV 254 nm

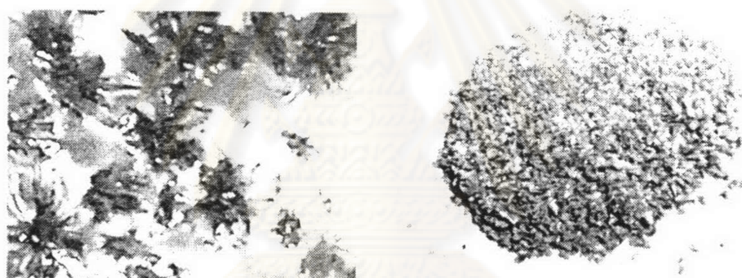

Figure 14. Photograph of oxyresveratrol or 2, 4, 3′, 5′-tetrahydroxystilbene

From the Nuclear Magnetic Resonance (NMR) data, ^{1}H NMR and ^{13}C NMR spectra (Figures 16 and 17) were obtained using deuterated dimethylsulfoxide (DMSO d_6) as a solvent. From the spectrum and the chemical shift of the compound, and according to the above data, the compound was identified as oxyresveratrol or 2, 4, 3′, 5′-tetrahydroxystilbene which agreed with the recently reported data (Sritularak, 1998). The weight of the final yield was 1.4863 g, which was calculated as 14.83 % yield. Then, oxyresveratrol was ground into fine powder for further studies. It was kept in a well-closed container at -20 °C. The purity of oxyresveratrol was estimated to be less than 95% w/w since the HPLC chromatogram indicated that the ratio of the area of oxyresveratrol peak to the total area (oxyresveratrol peak plus all minor peaks) was 98.64% w/w (Wanawatanakun, 2005).

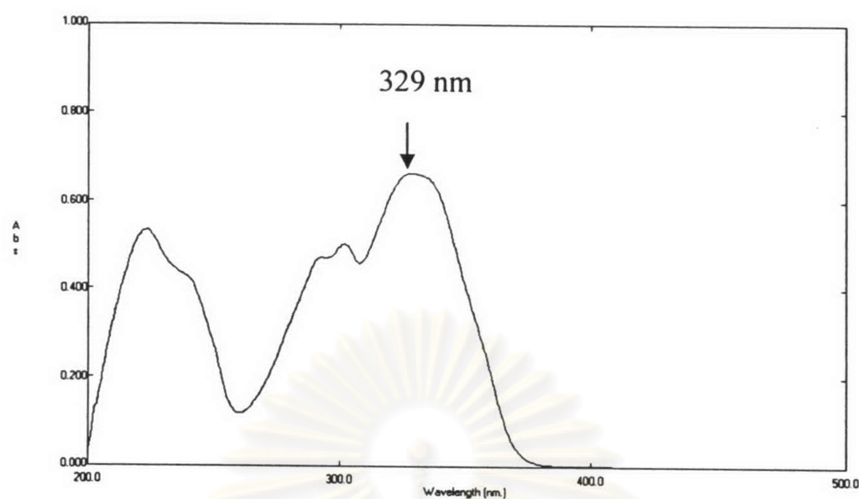

Figure 15. The UV spectrum of oxyresveratrol (0.001 mg/ml in MeOH)

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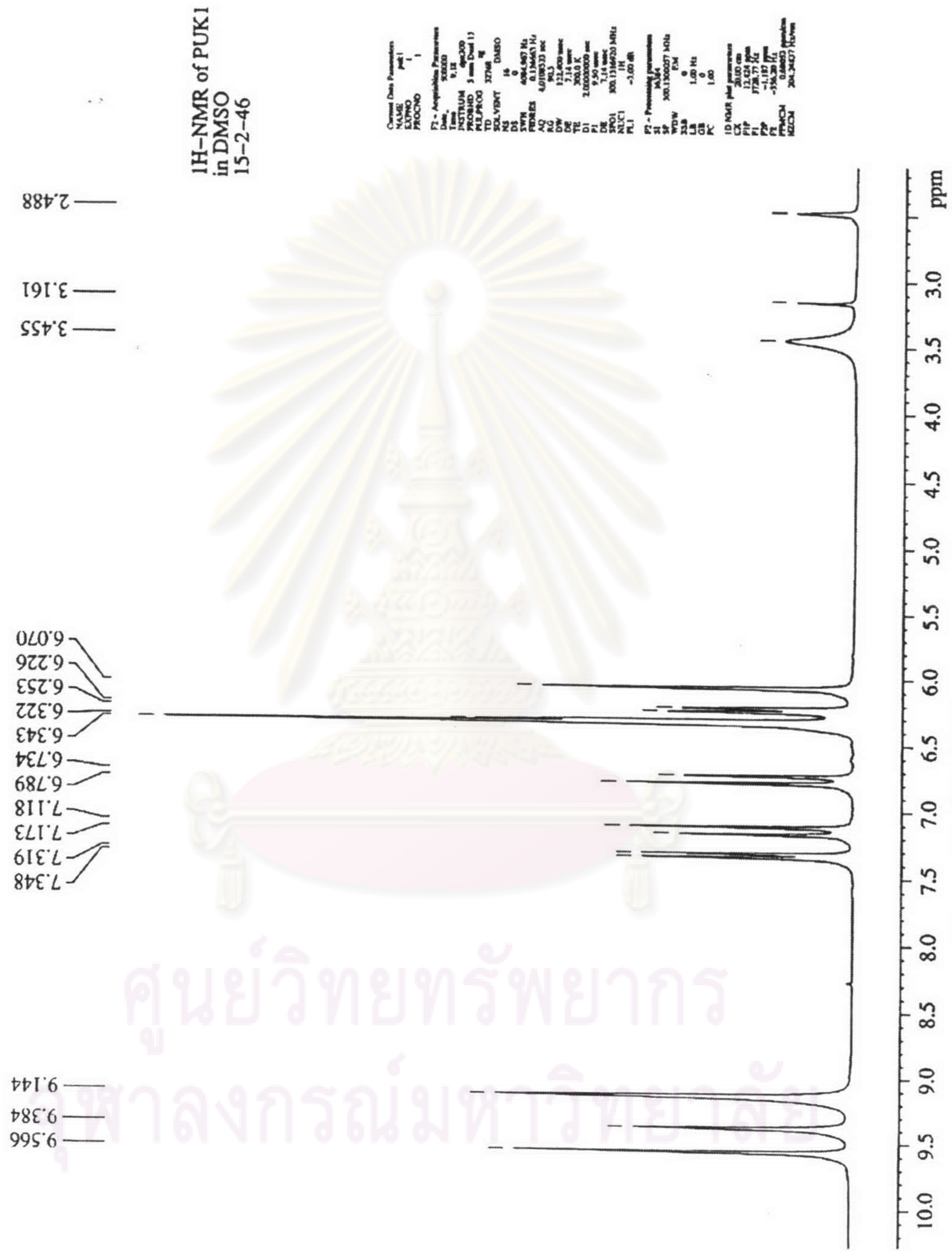

Figure 16. ^{1}H NMR spectrum of oxyresveratrol

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158.334
157.956
155.859
139.921
127.080
124.541
123.112
115.211
107.209
103.975
102.548
101.344
40.153
39.877
39.600
39.319
39.042

13C-PUK
in DMSO
15-2-46

170 160 150 140 130 120 110 100 90 80 70 60 50 40 ppm

Figure 17. ^{13}C NMR spectrum of oxyresveratrol

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Part 2. Stability Evaluation of Aqueous Extract of *Artocarpus lakoocha* Heartwood (Puag-Haad) Solutions

The purpose of this part was to evaluate the physical and biochemical stability of Puag-Haad solutions upon storage at ambient temperature (≈ 28 °C). Physical stability was evaluated with respect to clarity, color and pH value whereas biochemical stability was determined through measurements of its in vitro tyrosinase inhibitory activity.

Solutions of Puag-Haad with and without various antioxidants were prepared in propylene glycol in water (20% v/v) since it was shown to provide adequate solubility (Pengrungrangwong, 2001). Concentration of Puag-Haad was fixed at 0.25% w/v in this part because this concentration was found to give the most promising antityrosinase effect (Pengrungrangwong, 2001). In addition a solution of 0.25% w/v licorice extract was also prepared in the same solvent to serve as the reference antityrosinase agent.

Since the active component of Puag-Haad (2, 4, 3′, 5′-tetrahydroxystilbene) is a polyphenolic compound, it was thus expected to degrade by oxidation (Tibtabiankarn, 1967; Pengrungrangwong, 2001). Solutions of 0.25% Puag-Haad in various types of antioxidants were prepared in the same solvent and coded as follows:

P	=	0.25% w/v Puag-Haad
P + A1	=	0.25% w/v Puag-Haad + 0.1% w/v sodium metabisulfite
P + A2	=	0.25% w/v Puag-Haad + 0.01% w/v BHA
P + A3	=	0.25% w/v Puag-Haad + 0.4% w/v Rovisome®
P + A4	=	0.25% w/v Puag-Haad + 0.1% w/v sodium metabisulfite + 0.01% w/v BHA
P + A5	=	0.25% w/v Puag-Haad + 0.1% w/v sodium metabisulfite + 0.01% w/v BHA + 0.4% w/v Rovisome®

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 0.1%. BHA at 0.01% was used as a representative of true antioxidant whereas 0.4% w/v Rovisome® (5.0% magnesium ascorbyl phosphate in liposome suspension) was added for a possible synergistic effect.

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 terms of both the color and antityrosinase activity (80% of initial activity remaining after 24 weeks at room temperature). Pengrungruangwong (2001) also found that addition of EDTA as the third antioxidant did not further enhance the stability whereas a preliminary study using Rovisome® in combination with sodium metabisulfite and BHA was able to provide physical stability of 0.25% and 0.1% Puag-Haad lotions for at least 6 months. Therefore, Rovisome® was chosen in this study to replace EDTA in a triple antioxidant combination to see if there was any further enhanced stabilization of Puag-Haad solution in terms of both physical and antityrosinase activity. The stabilizing effect of Rovisome® alone was also investigated.

1. Physical stability

Table 6 shows changes in color of Puag-Haad samples upon storage at ambient temperature in dark condition (6 months). The initial color of 0.25% 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 8 weeks. The color of all the stored Puag-Haad solutions was always compared with that of the freshly prepared 0.25% pure Puag-Haad. Only one antioxidant, Rovisome®, which originally possessed a dark brown color, imparted a slightly darker yellow color in Puag-Haad solution at the initial time (0 week).

Addition of antioxidants, particularly 0.1% sodium metabisulfite was able to protect 0.25% Puag-Haad from increased coloration after prolonged storage. As seen from Table 6, solutions P+A1, P+A4, P+A5 did not increase in color after 24 weeks storage at ambient temperature. All these solutions contain 0.1% sodium metabisulfite as a common antioxidant. The results agreed with that of Pengrungruangwong (2001) 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, 0.01% BHA did not protect Puag-Haad from discoloration, this solution

became slightly brown (graded +2) similarly to pure Puag-Haad solution (P). Also, 0.4% Rovisome® (P+A3), equivalent to 2.0% Magnesium ascorbyl phosphate did not show any protecting effect. It became even more discolored than pure Puag-Haad solution after 24 weeks. However, when it was in combination with sodium metabisulfite and BHA (P+A5), the color was restored to the original color of pure Puag-Haad solution regardless of the storage time. Thus, Rovisome® alone failed to protect Puag-Haad solution against discoloration but when combined with other stabilizers, especially sodium metabisulfite, this undesirable effect disappeared in agreement with the preliminary data that the triple combination of sodium metabisulfite, BHA and Rovisome® could stabilize the physical appearance of Puag-Haad lotions, which are oil-in-water emulsions. Therefore, sodium metabisulfite appeared to give the best protection against Puag-Haad discoloration, especially in an aqueous system. It could even protect the discoloration due to Rovisome® (P+A3 versus P+A5). Photographs of different Puag-Haad solutions taken after storage for 0 and 24 weeks are also provided for visual comparison in Figure 18.

The pH of each solution was also measured in triplicate and the data are shown in Table 7. Pure Puag-Haad solution showed a decrease in pH upon storage time, from 5.75 at week 0 to 4.78 at week 24, which was equivalent to a 0.97 unit change in pH. Addition of 0.01% BHA (P+A2) also resulted in a slightly decrease in pH from an initial value of 5.46 to 4.63, equivalent to 0.83 unit drop in pH similar to pure Puag-Haad solution. The solutions containing sodium metabisulfite (P+A1) and Rovisome® (P+A3) showed a drop in pH values at week 24 of 1.30 and 1.61 unit, respectively. Similar results were also observed in double combination solutions containing sodium metabisulfite and BHA (P+A4) and triple combination containing sodium metabisulfite, BHA and Rovisome® (P+A5), with a drop in pH after 24 weeks of 1.10 and 1.21, respectively. The pH of the individual antioxidant solutions (without Puag-Haad) at the concentrations used in this study was also investigated for the same period, as seen in Table 8. The pH values of sodium metabisulfite and Rovisome® alone decreased from 5.05 at week 0 to 4.10 at week 24 (ΔpH = 0.95), and from 7.07 at week 0 to 5.48 at week 24 (ΔpH = 1.57), respectively. On the other hand, the pH of BHA alone gave a smaller

drop in pH (ΔpH = 0.89) similar to pure Puag-Haad solution (P) and Puag-Haad plus BHA (P+A2).

Thus, from the observed data, 0.01% BHA appeared to have a minor effect on the pH of Puag-Haad solution whereas addition of Rovisome® caused the greatest drop in the pH of Puag-Haad solution, which was probably due to the hydrolysis of phospholipids in the liposome bilayer to give free fatty acids. Sodium metabisulfite also could further cause the drop in pH but its effect was smaller than that of Rovisome®. Interestingly, when BHA was combined with sodium metabisulfite (P+A4) or with sodium metabisulfite and Rovisome® (P+A5), the drop in pH was improved as seen from data in Table 7. However, the reasons for this observation are not clearly understood.

In conclusion, after 24-week storage time it was found that the change in pH value of the Puag-Haad solution with BHA as a stabilizer was similar to the pure Puag-Haad solution. Addition of sodium metabisulfite or Rovisome® gave a further decrease in pH value. It might be that the decrease in pH value of Puag-Haad solution in the presence of these two substances were caused by the decrease in pH value of the individual stabilizer (metabisulfite and Rovisome®) in the solution at the same investigation time. The reason for this could be the properties of the antioxidants themselves such as sodium metabisulfite's reaction with oxygen and other oxidizing agents resulting in a fall in the pH of the solution. For Rovisome®, which consists of vitamin C derivative in liposome, oxidation of vitamin C may have occurred to give more acidic degradation products, or the phospholipid components of the liposome may have hydrolyzed to give free fatty acids as previously discussed. In any way, the concentration of the stabilizers must be optimized in order to provide a desired protective effect. If the concentration is too low, the capacity of the stabilizing antioxidant might not be enough to delay the degradation of Puag-Haad. But if the concentration is too high, the excess antioxidant may exert a pro-oxidant effect, leading to a greater physical change (such as pH and color) or even a further decrease in biological activity of the active compounds.

Table 6. Changes in color of Puag-Haad solutions upon storage at ambient temperature

No.	Samples	Time (week)						
		0	4	8	12	16	20	24
0	Fresh P	-	0	0	0	0	0	0
1	P	0	+1	+2	+2	+2	+2	+2
2	P+A1	0	0	0	0	0	0	0
3	P+A2	0	+1	+2	+2	+2	+2	+2
4	P+A3	+1	+2	+3	+3	+4	+4	+4
5	P+A4	0	0	0	0	0	0	0
6	P+A5	0	0	0	0	0	0	0

P = 0.25% Puag-Haad, A1 = 0.1% w/v sodium metabisulfite, A2 = 0.01% w/v BHA, A3 = 0.4% w/v Rovisome®, A4 = A1+A2, A5 = A1+A2+A3

0 = normal (pale yellow); no change, +1 = slightly (light yellow) changed, +2 = noticeably (light brown) charged, +3 = markedly (brown) charged, +4 = seriously deteriorated (dark brown), +5 = almost or completely deteriorated (intense deep brown)

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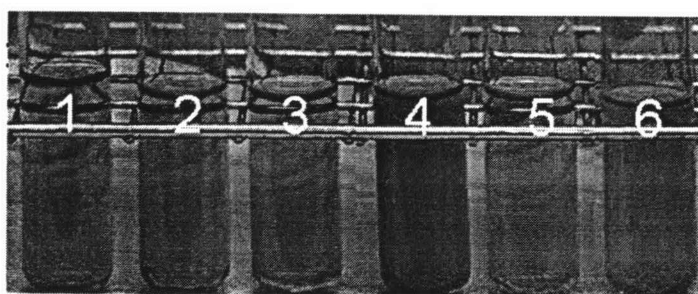
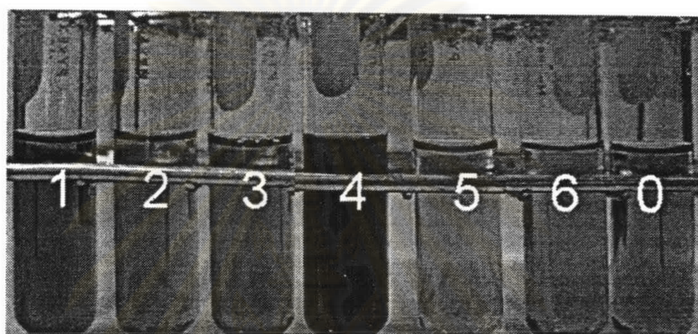


0 week



24 weeks

Figure 18. Physical appearance of Puag-Haad solutions upon storage at ambient temperature for 24 weeks

0 = Fresh 0.25% Puag-Haad (P), 1 = Pure P, 2 = P+0.1% w/v sodium metabisulfite (A1), 3 = P+0.01% w/v BHA (A2), 4 = P+0.4% w/v Rovisome® (A3), 5 = P+A1+A2 (A4), 6 = P+A1+A2+A3 (A5)

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Table 7. Changes in pH values of Puag-Haad solutions upon storage at ambient temperature for 24 weeks

No.	Puag-Haad samples	Time (week) 0	4	8	12	16	20	24	ΔpH (0-24 week)
1	P	5.89 5.68 5.68	5.38 5.43 5.45	5.49 5.55 5.50	4.92 4.94 4.74	4.65 4.72 4.73	4.80 4.75 4.72	4.73 4.82 4.78	0.97
	Mean ± SD	5.75 ± 0.12	5.42 ± 0.04	5.51 ± 0.03	4.87 ± 0.11	4.70 ± 0.04	4.76 ± 0.04	4.78 ± 0.05	
2	P+A1	5.07 5.26 5.30	4.71 4.83 4.78	4.71 4.74 4.75	4.41 4.44 4.39	4.10 4.16 4.13	3.99 3.98 3.98	3.88 2.90 2.95	1.30
	Mean ± SD	5.21 ± 0.12	4.77 ± 0.06	4.73 ± 0.02	4.41 ± 0.03	4.13 ± 0.03	3.98 ± 0.01	3.91 ± 0.04	
3	P+A2	5.51 5.46 5.40	5.36 5.98 5.56	5.14 5.22 5.22	5.24 5.23 5.19	4.79 4.79 4.81	4.72 4.69 4.68	4.69 4.60 4.61	0.83
	Mean ± SD	5.46 ± 0.06	5.63 ± 0.32	5.19 ± 0.05	5.22 ± 0.03	4.80 ± 0.01	4.70 ± 0.02	4.63 ± 0.05	
4	P+A3	6.93 6.91 6.95	6.61 6.61 6.66	6.29 6.34 6.31	5.89 6.03 6.01	5.70 5.71 5.71	5.57 5.57 5.56	5.30 5.31 5.34	1.61
	Mean ± SD	6.93 ± 0.02	6.63 ± 0.03	6.31± 0.03	5.98 ± 0.08	5.71 ± 0.01	5.57 ± 0.01	5.32 ± 0.02	
5	P+A4	5.17 5.13 5.16	4.98 4.77 4.89	4.51 4.54 4.55	4.32 4.31 4.34	4.17 4.80 4.78	4.11 4.12 4.13	4.03 4.08 4.05	1.10
	Mean ± SD	5.15 ± 0.02	4.88 ± 0.11	4.53 ± 0.02	4.32± 0.02	4.58 ± 0.36	4.12 ± 0.01	4.05 ± 0.03	
6	P+A5	5.85 6.19 6.06	5.81 6.18 5.98	5.29 5.30 5.31	5.09 5.08 5.12	5.04 5.04 5.03	4.99 4.98 4.97	4.86 4.81 4.80	1.21
	Mean ± SD	6.03 ± 0.17	5.99 ± 0.19	5.30 ± 0.01	5.10 ± 0.02	5.04 ± 0.01	4.98 ± 0.01	4.82 ± 0.03	

P = 0.25% Puag-Haad, A1 = 0.1% w/v sodium metabisulfite, A2 = 0.01% w/v BHA, A3 = 0.4% w/v Rovisome®, A4 = A1+A2, A5 = A1+A2+A3

Table 8. Changes in pH values of antioxidant solutions without Puag-Haad upon storage at ambient temperature for 24 weeks

No.	Antioxidant	Time (week)							ΔpH
		0	4	8	12	16	20	24	(0-24 week)
1	A1	5.05 5.03 5.06	5.05 5.02 5.03	4.86 4.88 4.89	4.62 4.70 4.61	4.28 4.36 4.26	4.11 4.23 4.18	4.05 4.11 4.15	0.95
	Mean ± SD	5.05 ± 0.02	5.03 ± 0.02	4.88 ± 0.02	4.64 ± 0.05	4.30 ± 0.05	4.17 ± 0.06	4.10 ± 0.05	
2	A2	6.03 6.09 6.05	6.08 6.05 6.05	5.88 5.76 5.92	5.35 5.35 5.34	5.44 5.35 5.31	5.20 5.11 5.23	5.18 5.18 5.16	0.89
	Mean ± SD	6.06 ± 0.03	6.06 ± 0.02	5.85 ± 0.08	5.35 ± 0.01	5.37 ± 0.07	5.18 ± 0.06	5.17 ± 0.01	
3	A3	7.04 7.13 7.05	6.859 6.89 6.99	6.44 6.53 6.55	6.33 6.31 6.22	5.73 5.76 5.76	5.43 5.45 5.56	5.43 5.45 5.56	1.57
	Mean ± SD	7.07 ± 0.05	6.91 ± 0.07	6.51 ± 0.06	5.29 ± 0.06	5.75 ± 0.02	5.48 ± 0.07	5.48 ± 0.07	

A1 = 0.1% w/v sodium metabisulfite, A2 = 0.01% w/v BHA, A3 = 0.4% w/v Rovisome®

2. Biochemical stability

The biochemical stability of Puag-Haad solutions were determined by quantitating their in vitro anti-tyrosinase activities upon storage. Tyrosinase inhibitory activity was determined by the DOPAchrome enzymatic method using L-DOPA as a substrate and mushroom tyrosinase as the enzyme. Dopachrome is one of the intermediate substances in the melanin biosynthesis. The red color of DOPAchrome can be detected by visible light. In this experiment a microplate reader (Model, Bio-Rad) with 492 nm interference filter was used. A potential tyrosinase inhibitor would show decreased DOPAchrome absorption. The first step was conducted by screening the appropriate range of the incubation time from 0 to 30 min. The absorbance of the positive control (only enzyme and substrate) reached a plateau at 10 min and its calculated % tyrosinase inhibition started to decrease after 10 min standing. So the incubation time for the assay of the sample's tyrosinase inhibitory activity was set at 10 min (Figure 19a and 19b). A freshly prepared solution of 0.25% licorice extract was used as a reference solution to validate for accuracy and precision of the enzymatic assay. It was assayed at the same intervals as the Puag-Haad solutions.

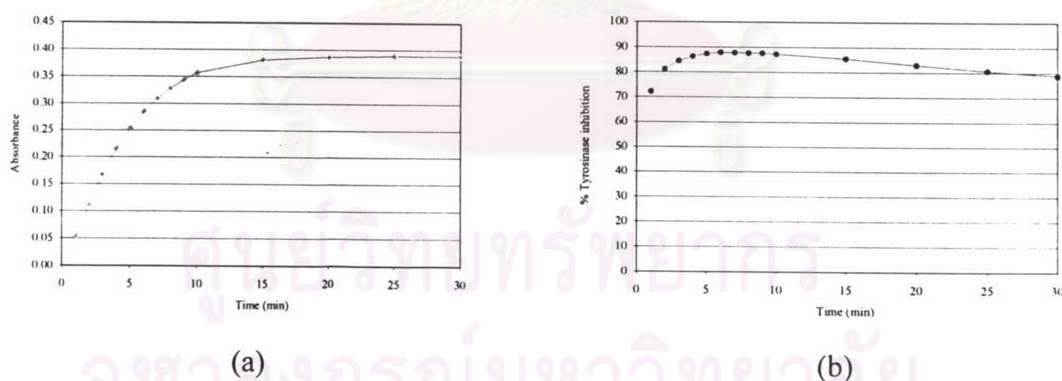

Figure 19. (a) Absorbance of positive control (enzyme and substrate) (b) Tyrosinase inhibition percentage at difference time (enzyme plus substrate and licolice extract)

Table 9 shows the individual and the average values (mean, SD and %CV) of % tyrosinase inhibition by the freshly prepared 0.25% licorice extract. The values of the tyrosinase inhibitory activity were consistent both within each run and between different runs, giving the overall mean of 91.73 % inhibition. The within-run coefficient of variation (CV) was in the range of 0.06 – 3.25% and the between-run CV was 1.67%. These values were much smaller than the 15% limit generally allowed for biological assay, indicating that the method had good within-run and between-run precision.

Table 9. Precision of the enzymatic method used in determining tyrosinase inhibitory activity. 0.25% freshly prepared licorice extract solution was used as a reference standard

Week of Assay	% inhibition					
	n1	n2	n3	Mean	SD	Within-run % CV
0	93.67	94.00	91.77	93.15	0.98	1.05
4	92.34	89.62	93.99	91.98	1.80	1.96
8	87.85	87.39	89.73	88.32	1.01	1.14
12	94.37	94.52	94.46	94.45	0.06	0.06
16	88.64	84.90	91.09	88.21	2.55	2.89
20	90.40	92.23	97.56	93.40	3.04	3.25
24	93.44	90.83	93.49	92.59	1.24	1.34

Table 10 shows the average % inhibition (Means ± SD of triplicate determinations) of various Puag-Haad solutions kept at ambient temperature. From this table, it can be seen that addition of sodium metabisulfite (A1) and its combination with BHA (P+A4) and with BHA plus Rovisome® (P+A5) caused a slight increase in tyrosinase inhibitory activity from 91.51% of pure Puag-Haad (P) to 96.75, 97.38, and 97.16%, respectively, whereas BHA (P+A2) and Rovisome® (P+A3) did not cause any noticeable increase in tyrosinase inhibition at the initial time (0 week). The mechanism by which sodium metabisulfite could slightly enhance the initial activity of Puag-Haad solution was not clearly known. Nevertheless, it could be assumed from the above observations that the contribution from the individual stabilizers and their combinations to Puag-Haad's anti-tyrosinase activity was negligible in this study.

After 24 week-storage, the tyrosinase inhibitory activity of pure Puag-Haad solution was slightly decreased from 91.51% to 88.23%. Addition of different antioxidants was shown to give a varied degree of % tyrosinase inhibition. Since the initial values of the individual samples were not the same, the values of the absolute percent inhibition were subsequently normalized to percent inhibition relative to the initial value (Table 11). From this table, the relative tyrosinase inhibition data show that there was not any difference in the extent of tyrosinase inhibition in any samples. All the test samples, including the pure Puag-Haad solution, similarly gave the remaining % relative inhibition values in the range of 91.13 to 96.33%. The values of both the absolute and relative % tyrosinase inhibition at different times are shown graphically for each sample in Figures 20 and 21, respectively.

Thus, all the antioxidants tested apparently were unable to provide extra stabilization of the extract in terms of its anti-tyrosinase activity. The reasons as to this observation were not understood as the current data were in contrast to those of Pengrungruangwong (2001), who 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). She also reported that the solution of pure Puag-Haad without any addition of stabilizer showed a significant drop in its activity to about 50% at 24 weeks. This is also in contrast with the current study in which the pure Puag-Haad solution was found to be quite stable, with its anti-tyrosinase activity dropped to only 96.33% of the initial concentration (0.25%) after 24 weeks.

Since the storage conditions and Puag-Haad concentration (including the final concentration in the reaction mixture after dilution) were the same as the previous study, the discrepancy might have come from the enzymatic assay employed in this study. Although the same type of enzyme was used (mushroom tyrosinase), there could be a lot-to-lot variation in its bioactivity. In the present study, the labeled bioactivity was 3960 units/mg solid, which was diluted by buffer to 480 units/ml similar to that used by Pengrungruangwong (2001). However, there seemed to be a saturation of the enzyme activity occurring this time, i.e., the concentration of enzyme might not be in excess to accommodate its interactions with various concentrations of the test substance (inhibitor). It was thus postulated that due to the lower-than-expected amount of enzyme present in

the reaction mixture, the inhibitor (active component of Puag-Haad) might be able to fully saturate it regardless of the inhibitor concentration. Even in the case where the inhibitor might have degraded to some extent, its remaining concentration could be high enough to still saturate the enzyme, resulting in the similar value of % inhibition, which was already in the maximum range (always greater than 80% inhibition).

To support this hypothesis, an experiment was set up to establish the IC_{50} of Puag-Haad extract. As seen from Figure 22a, which is a plot between % inhibition of tyrosinase activity versus concentration of Puag-Haad solution. The curve reaches plateau level in the concentration range of 0.2-50 μg/ml and the IC_{50} was interpolated from the curve to be about 0.76 μg/ml, which is equivalent to oxyresveratrol content of 2.36 μM. Since after dilution, the final concentration of Puag-Haad in the reaction mixture was 50 μg/ml in this study. This value was in the plateau region of the IC_{50} curve. Therefore, it is possible that to counterbalance the lower-than-expected activity of the enzyme, more dilution of the Puag-Haad samples should have been made so that the final concentration would not lie in the plateau region to be able to observe the 'real' extent of tyrosinase inhibition and thus the 'real' remaining antityrosinase activity of the extract.

In addition, the IC_{50} of oxyresveratrol (244 g/mole) was also investigated from a plot between % inhibition of tyrosinase activity versus concentration of oxyresveratrol (Figure 22b). The IC_{50} of oxyresveratrol was interpolated from the curve to be about 0.83 μg/ml, which is equivalent to 3.23 μM. The value was slightly higher than 1.5 μM previously reported by Sritularak (1998). It is also interesting to note that the IC_{50} value of Puag-Haad was slightly lower than that of oxyresveratrol regardless of the unit. Since Puag-Haad was a crude extract and thus not a pure compound, it is possible that other undetected substance(s) may have exitsted in the extract which contributed to its anti-tyrosinase activity.

Therefore, it can be concluded that the tyrosinase inhition activity may not be due solely to oxyresveratrol. Transforming the value from μg/ml to μM was obtained using the following equation.

$$\mu M \text{ for oxyresveratrol} = \frac{(\mu g/ml) \times \text{purity } (\%w/w) \times 1000}{\text{molecular weight} \times 100}$$

$$\mu M \text{ for extract} = \frac{(\mu g/ml) \times \%\text{oxyresveratrol} \times \text{purity } (\%w/w) \times 1000}{\text{molecular weight} \times 100 \times 100}$$

where %purity was taken as 95% w/w and %oxyresveratrol in extract was taken as 80% w/w

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Table 10. Stability of Puag-Haad with and without antioxidants as determined from % tyrosinase inhibitory activity (Mean ± SD, n = 3)

Time	% Tyrosinase inhibitory activity					
	P	P+A1	P+A2	P+A3	P+A4	P+A5
0	91.51	96.75	90.00	91.01	97.38	97.16
(SD)	1.07	1.01	0.69	1.85	0.90	1.92
4	90.95	96.65	89.88	89.76	95.63	93.02
(SD)	1.76	1.16	0.83	0.49	0.65	2.16
8	89.42	97.02	89.67	89.73	97.30	93.93
(SD)	1.64	0.81	0.18	0.39	0.62	1.29
12	87.43	96.33	87.56	86.83	96.95	92.93
(SD)	1.40	0.28	2.56	1.24	2.11	0.66
16	90.93	96.83	90.09	88.30	94.57	90.76
(SD)	0.30	0.58	1.56	2.03	0.70	1.32
20	85.56	90.99	81.34	80.45	86.49	78.17
(SD)	2.73	0.59	4.56	3.20	3.61	4.01
24	88.23	91.01	86.33	84.97	90.18	88.54
(SD)	0.68	0.69	2.57	0.41	2.14	1.11

P = 0.25% Puag-Haad, A1 = 0.1% w/v sodium metabisulfite, A2 = 0.01% w/v BHA, A3 = 0.4% w/v Rovisome®, A4 = A1+A2, A5 = A1+A2+A3

Table 11. Stability of Puag-Haad with and without antioxidants as determined from % tyrosinase inhibitory activity relative to their initial value (Mean ± SD, n = 3)

Time	% Tyrosinase inhibitory activity					
	P	P+A1	P+A2	P+A3	P+A4	P+A5
0	100.00	100.00	100.00	100.00	100.00	100.00
(SD)	1.07	1.01	0.69	1.85	0.90	1.92
4	99.30	99.89	99.86	98.63	98.20	95.74
(SD)	1.76	1.16	0.83	0.49	0.65	2.16
8	97.63	100.28	99.63	98.59	99.91	96.68
(SD)	1.64	0.81	0.18	0.39	0.62	1.29
12	95.46	99.56	97.29	95.41	99.56	95.65
(SD)	1.40	0.28	2.56	1.24	2.11	0.66
16	99.28	100.08	100.10	97.03	97.11	93.42
(SD)	0.30	0.58	1.56	2.03	0.70	1.32
20	93.41	94.04	90.38	88.40	88.82	80.46
(SD)	2.73	0.59	4.56	3.20	3.61	4.01
24	96.33	94.07	95.92	93.37	92.60	91.13
(SD)	0.68	0.69	2.57	0.41	2.14	1.11

P = 0.25% Puag-Haad, A1 = 0.1% w/v sodium metabisulfite, A2 = 0.01% w/v BHA, A3 = 0.4% w/v Rovisome®, A4 = A1+A2, A5 = A1+A2+A3

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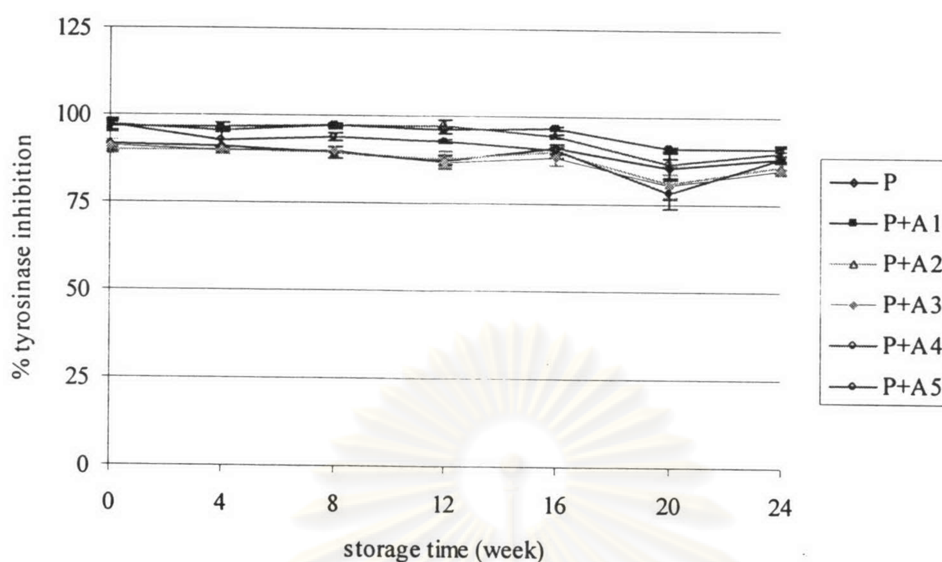

Figure 20. Percent tyrosinase inhibitory activity remaining after storage up to 24 weeks. Each point represents Mean ± SD (n = 3). P = 0.25% Puag-Haad, A1 = 0.10% Sodium metabisulfite, A2 = 0.01% BHA, A3 = Rovisome®, A4 = A1+A2, A5 = A1+A2+A3

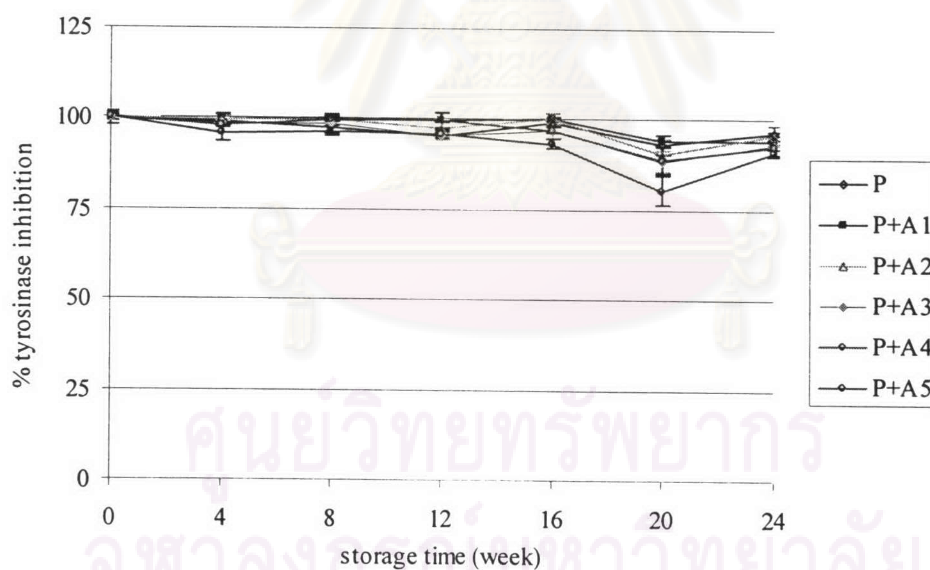

Figure 21. Percent tyrosinase inhibitory activity (relative to initial value) remaining after storage up to 24 weeks. Each point represents Mean ± SD (n = 3). P = 0.25% Puag-Haad, A1 = 0.10% Sodium metabisulfite, A2 = 0.01% BHA, A3 = Rovisome®, A4 = A1+A2, A5 = A1+A2+A3

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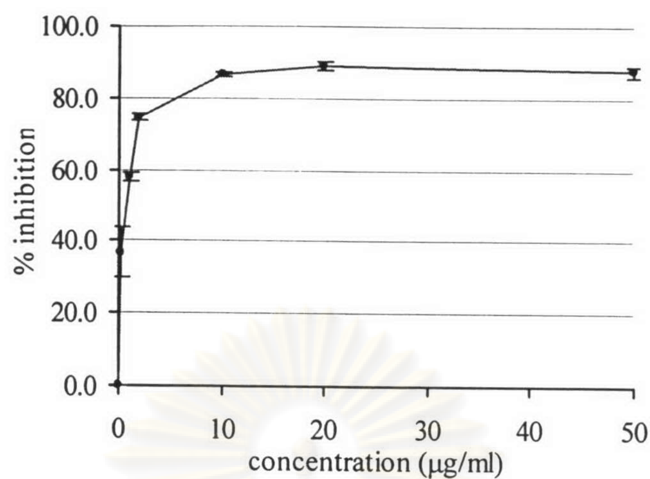
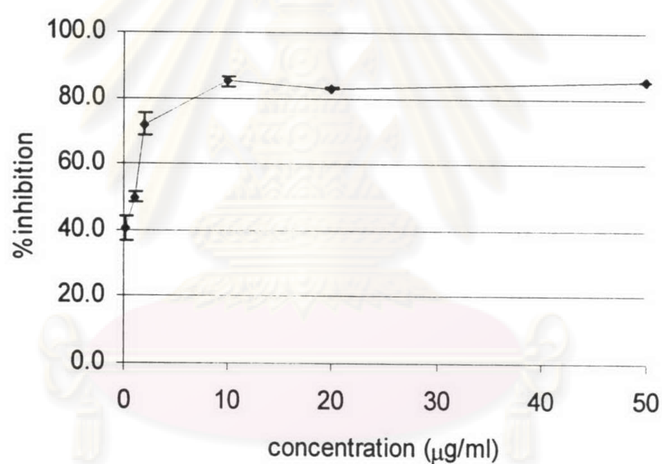


(a) IC_{50} = 0.76 μg/ml (equivalent to oxyresveratrol 2.36 μM)



(b) IC_{50} = 0.83 μg/ml (equivalent to oxyresveratrol 3.23 μM)

Figure 22. Percent tyrosinase inhibition of Puag-Haad solution (a) and oxyresveratrol (b)

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Part 3. Determination of Antioxidants and Free Radical Scavenging Activities of *Artocarpus lakoocha* Heartwood Extract (Puag-Haad)

3.1 Determination of DPPH free radical scavenging activity

The antioxidant and free radical scavenging activities of Puag-Haad and reference substances were determined in terms of their ability to scavenge different reactive oxygen species as well as their protective effect on the red blood cell model against oxidative stress inducers like UV. The first test involved determination of their ability to scavenge DPPH free radical in ethanolic solution, which resulted in a decrease in absorbance at 517 nm. The loss of DPPH radical was evaluated by comparison with a control sample which contained no test substance that could act as a hydrogen-donating compound.

The percentage of DPPH scavenging (% inhibition of free radical) of Puag-Haad compared to those of Trolox® (water-soluble form of vitamin E), l-ascorbic acid, EGCG, pine bark extract and oxyresveratrol (the active constituent of Puag-Haad) at various concentrations (0, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0 and 100.0 µg/ml) are shown in Figure 23.

It was found that as the concentration was increased, the extent of DPPH inhibition also increased for all antioxidants but until certain concentrations where the plots reached a plateau and the inhibition was steady (Figure 24 and Table 12). The percent DPPH radical inhibition of Puag-Haad increased to 73.33 ± 0.50 % at the concentration of 20.0 µg/ml, then reached a plateau at 50.0 and 100.0 µg/ml, where the inhibition percentages were 74.72 ± 0.45 and 74.17 ± 1.47 %, respectively.

Oxyresveratrol, the active component of Puag-Haad, also gave scavenging activity profile similar to Puag-Haad. Its activity appeared to remain stable at a concentration 20.0 µg/ml and beyond. However, oxyresveratrol showed higher extent of inhibition at this concentration, about 82.88 ± 2.83 %. When the concentration was increased to 50.0 and 100.0 µg/ml, the % inhibition changed only slightly to 84.47 ± 4.05 and 84.55 ± 3.25 %, respectively. Likewise, the plots of Trolox® and l-ascorbic acid reached a plateau at 20.0 µg/ml. However, the inhibition extent of both vitamins was higher than that of Puag-Haad and oxyresveratrol, i.e., 94.79 ± 1.13 % for Trolox® and 94.06 ± 2.15 % for l-ascorbic acid. When the concentration was increased to 100.0

μg/ml, the values of % inhibition barely increased (96.28 ± 0.36 for Trolox® and 95.53 ± 2.28 % for l-ascorbic acid.

On the other hand, EGCG and pine bark extract reached a plateau of scavenging activity at a lower concentration (5.0 μg/ml) than other antioxidants, with the inhibition values of 85.39 ± 1.17 and 86.16 ± 0.21 %, respectively. When the concentration was increased to 100.0 μg/ml, the inhibition did not notably increase with the values in the range of 87.25 – 88.17 % and 87.52 – 88.81 %, respectively.

The relationship between DPPH radical inhibition and the concentration of the individual antioxidants is shown in Figure 24. It was found that Puag-Haad gave a slightly lower DPPH radical inhibition than oxyresveratrol, especially at the saturation concentration from 20.0 to 100.0 μg/ml, but the overall plots were similar. This may be due to the nature of oxyresveratrol, which is a purified compound, whereas the extract contains a lesser amount of oxyresveratrol.

Additionally, the DPPH radical inhibitions of Trolox® and l-ascorbic acid are also alike (Table 12 and Figure 23). At the concentration of 10.0 μg/ml, their inhibition percentages were comparable, and their plots were nearly identical. Also, the plots indicate that both Trolox® and l-ascorbic acid gave their maximum inhibition at the concentration range of 20.0 - 100.0 μg/ml.

Likewise, the DPPH radical inhibition of EGCG and pine bark extract was very similar. Their similarity in the inhibition profiles started at the concentration of 5.0 μg/ml (85.39 vs 86.16 %), and continued until the highest concentration studied (88.17 vs 87.52 % at 100.0 μg/ml). It is noticeable that EGCG and pine bark extract showed stronger inhibitions than Trolox® and l-ascorbic acid at lower concentrations, especially at 2.5 and 5.0 μg/ml (Table 12). After that, the inhibitory activity of EGCG and pine bark extract became saturated, while that of other antioxidants still continued to increase until reaching plateau at 20.0 μg/ml (Figure 24). It was found the inhibition extents of EGCG and pine bark extract were lower than those of Trolox® and l-ascorbic acid, especially in the plateau region (Table 12 and Figure 23). The reasons for this observation are unclear. It is hypothesized that difference in the molecular structure of the antioxidants may result in their different ability to scavenge DPPH radical. The difference in their hydrogen-donating properties and the bonding potential of the

individual molecules may be the reason of this finding (Catherine et al., 1996). The raw data for the absorbance and DPPH radical inhibition percentages are provided in Appendix A.

After the plots of % free radical inhibition versus concentration were constructed for each antioxidant, its IC_{50} value or the concentration that the test antioxidant can cause 50% inhibition was calculated from the regression analysis of the initial portion of each curve. It was found that a polynomial regression equation provided a better result than a linear equation, with a higher R^2 (regression coefficient), as shown in Table 13 and Figure 25.

The estimated IC_{50} can be ranked from the lowest to the highest, as follows: EGCG (1.75 ± 0.18 μg/ml), pine bark extract (2.43 ± 0.17 μg/ml), l-ascorbic acid (3.83 ± 0.21 μg/ml), Trolox® (5.48 ± 0.55 μg/ml), Puag-Haad (8.25 ± 0.48 μg/ml) and oxyresveratrol (8.78 ± 0.66 μg/ml) (Table 13). Since the IC_{50} values indicate the inhibitory potency of the individual antioxidants, Puag-Haad and its active compound oxyresveratrol apparently demonstrated the weakest DPPH radical scavenging activity. This was in agreement with the report of Fremont et al. (1999), which showed that a stilbene derivative, resveratrol was also a less powerful DPPH scavenger than epicatechin compounds (Figure 26). Regarding to Trolox® and l-ascorbic acid, Jang et al. (1997) also reported the IC_{50} which were nearly of the data from this study (9.5 and 6.7 μg/ml, repectively).

Table 12. DPPH radical inhibition of Puag-Haad compared to other antioxidants at various concentrations (Mean ± SD, n = 3)

No	Concentration	Puag-Haad		oxyresveratrol		Trolox®		l-ascorbic acid		EGCG		pine bark extract	
	(μg/ml)	% inh	SD	% inh	SD	% inh	SD	% inh	SD	% inh	SD	% inh	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.50	7.17	3.81	5.85	1.39	3.32	1.17	13.04	2.21	16.76	0.81	9.96	1.98
3	1.00	10.17	2.82	8.97	2.09	8.16	1.52	22.65	3.51	30.02	3.71	20.83	0.85
4	2.50	20.06	1.53	17.42	2.58	20.34	1.33	30.19	2.88	65.83	4.73	52.32	3.65
5	5.00	34.80	2.50	30.22	2.31	45.25	3.88	63.53	1.13	85.39	1.17	86.16	0.21
6	10.00	55.79	1.46	56.16	4.01	87.14	1.82	85.72	4.10	87.25	0.92	88.17	0.02
7	20.00	73.33	0.50	82.88	2.83	94.79	1.13	94.06	2.15	87.32	0.98	88.26	0.48
8	50.00	74.72	0.45	84.47	4.05	96.08	0.58	95.44	1.93	87.67	0.33	88.81	0.60
9	100.00	74.17	1.47	84.55	3.25	96.28	0.36	95.53	2.28	88.17	0.38	87.52	1.01

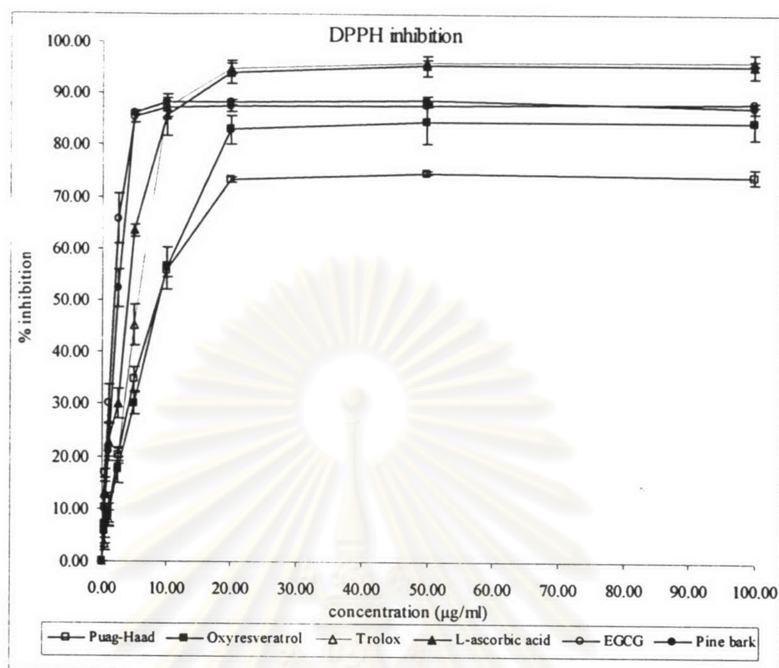

Figure 23. DPPH radical inhibition percentage of Puag-Haad compared to other antioxidants at various concentrations (mean ± SD, n = 3)

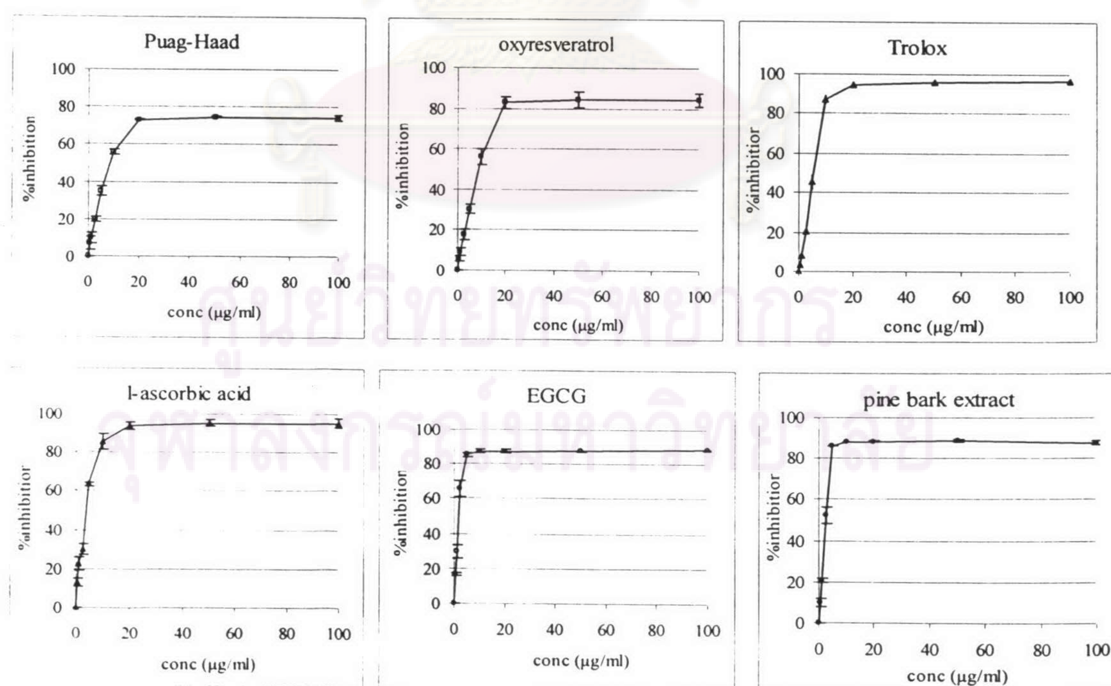

Figure 24. The relationship between DPPH radical inhibition percentage and the concentration of the antioxidants (mean ± SD, n = 3)

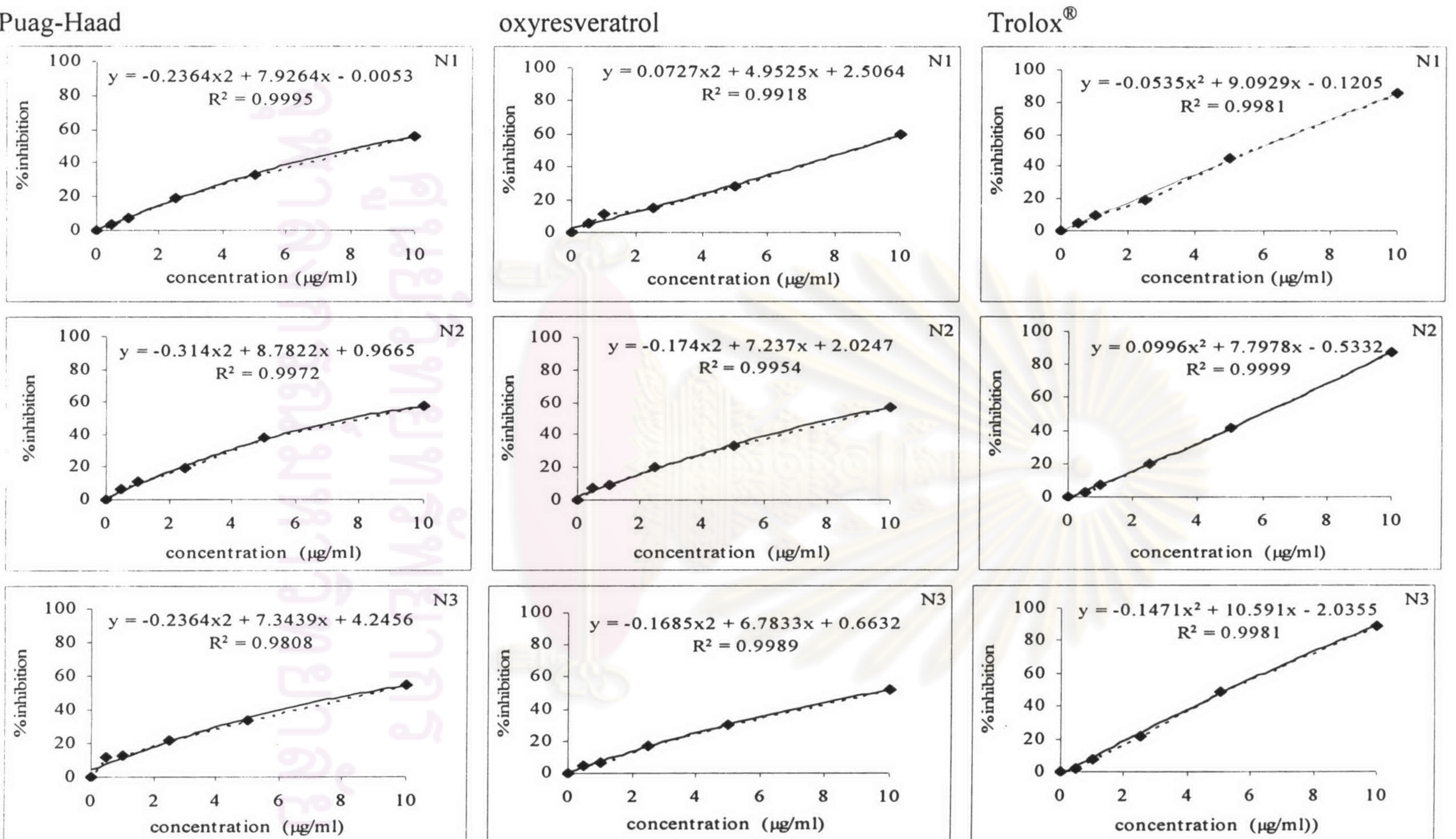

Figure 25. Comparison between the actual curve (dotted line) and the regression curve (solid line) of the initial portion of the % DPPH inhibition-concentration profile of each antioxidant. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants

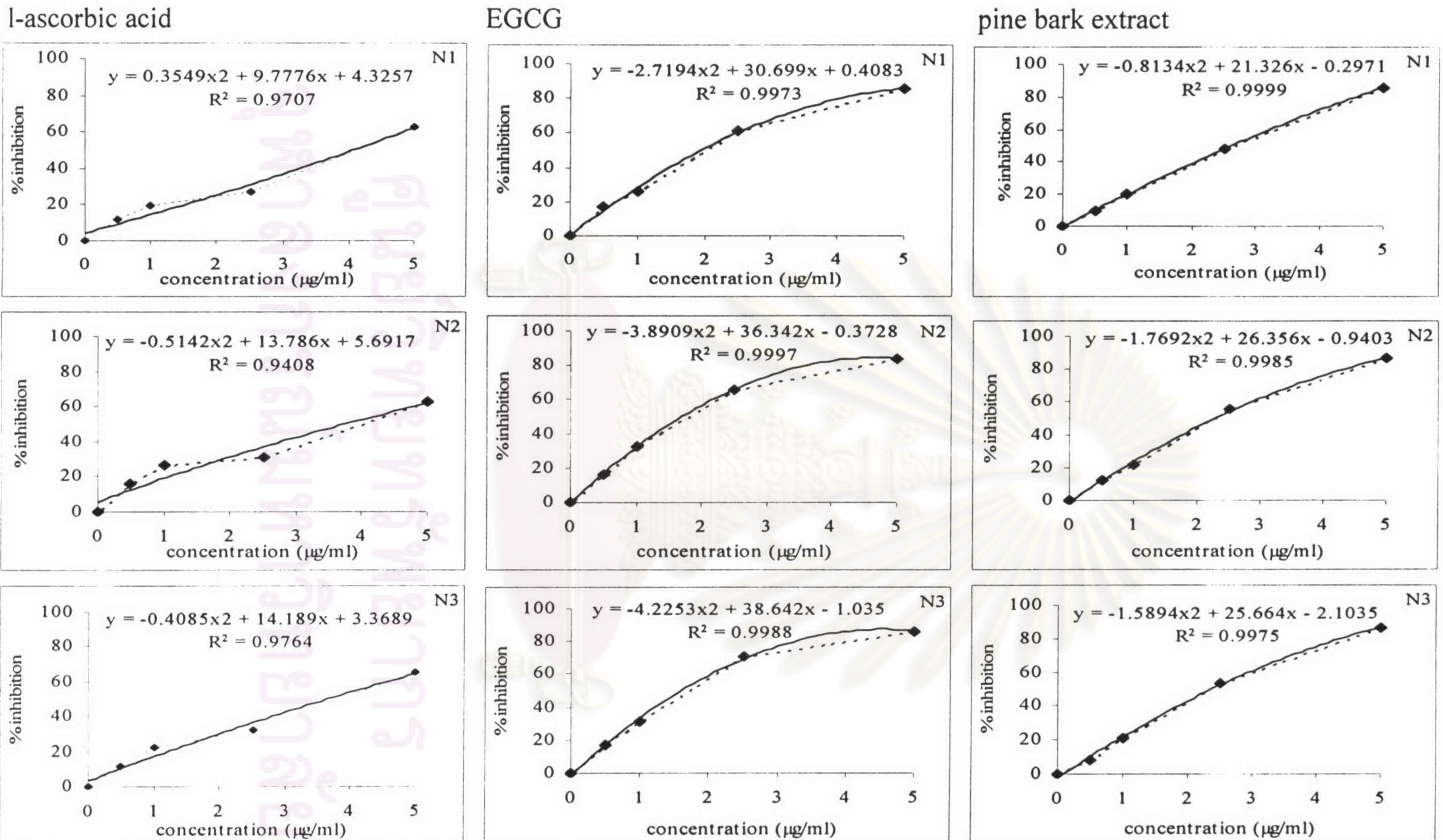

Figure 25. Comparison between the actual curve (dotted line) and the regression curve (solid line) of the initial portion of the % DPPH inhibition-concentration profile of each antioxidant. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants (Cont.)

The IC_{50} data, then, were analyzed by One-way analysis of variance (ANOVA) at the 95% confidence interval. It was found that, there were significant differences among the antioxidants studied ($P < 0.05$). Consequently, post-ANOVA analysis was done using Tukey's test. According to Tukey's test, the antioxidants could be divided into 4 different groups ($P < 0.05$) regarding their ability to inhibit or scavenge DPPH radicals, as follows:

<u>EGCG</u>	<u>≈ pine bark extract</u>	< l-ascorbic acid	< Trolox®	< <u>Puag-Haad</u>	<u>≈ oxyresveratrol</u>
1.75	2.43	3.83	5.48	8.25	8.78 µg/ml

The antioxidants joined by the same underline are within the same group and not statistically different in terms of their radical inhibition ($P > 0.05$). Thus, both the EGCG and pine bark extract demonstrated the highest potency of DPPH radical inhibition with the smallest IC_{50} values. Their potency was significantly greater than l-ascorbic acid which, in turn, was more potent than Trolox® ($P < 0.05$). Puag-Haad and oxyresveratrol, on the other hand, appeared to have the lowest potency since both exhibited the highest and similar values of IC_{50}. The statistical results of the individual antioxidants are shown in Appendix A.

Although EGCG and pine bark extract showed the highest potency in DPPH radical inhibition according to their IC_{50} values. However, when the values of % inhibition were compared among the 6 antioxidants, Trolox® and l-ascorbic acid appeared to give the highest inhibition percentage, especially at the concentration of 20.0 µg/ml onward (Figure 23). Figure 27 shows the histograms comparing % DPPH inhibition by the six antioxidants at various concentrations. It was found that at the concentrations of 20.0, 50.0 and 100.0 µg/ml, where all antioxidants reached saturation state, Trolox® and l-ascorbic acid demonstrated the highest DPPH radical inhibition.

The data on % DPPH inhibition at different concentrations were then analyzed by ANOVA and Tukey's test in order to differentiate the antioxidants and group them with respect to the extent of DPPH inhibition at the same concentration. It was found that at the concentrations of 1.0 and 2.5 µg/ml, EGCG showed the highest inhibition percentage, while pine bark extract and l-ascorbic acid had intermediate effect, and the rest

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demonstrated the similarly lower inhibition values. When the concentration was increased to 5.0 μg/ml, % DPPH inhibition of pine bark extract drastically increased until comparable to that of EGCG, which was followed by l-ascorbic acid and Trolox®. Puag-Haad and oxyresveratrol, on the other hand, showed the lowest inhibition extent at this concentration. When the concentration was increased to 10.0 μg/ml, % DPPH inhibition of Trolox® and l-ascorbic acid increased to the same level as pine bark extract and EGCG, while that of Puag-Haad and oxyresveratrol increased only slightly and were still lower than other substances (Figure 27). When the concentration was increased to 20.0 μg/ml, the extent of DPPH inhibition reached the plateau levels for all antioxidants. At this concentration, Trolox® and l-ascorbic acid were found to provide the highest inhibition extent, followed by pine bark extract, EGCG, oxyresveratrol and finally Puag-Haad, which demonstrated the lowest inhibition percentage. The statistical results at the concentrations of 50.0 and 100.0 μg/ml were not different from those at 20.0 μg/ml, i.e., Trolox® and l-ascorbic acid ranked highest, followed by the three antioxidants with similar inhibition, namely, EGCG, pine bark extract, and oxyresveratrol, while Puag-Haad always demonstrated the lowest extent of inhibition. The statistical data for the % DPPH inhibition at each concentration are shown in Appendix A.

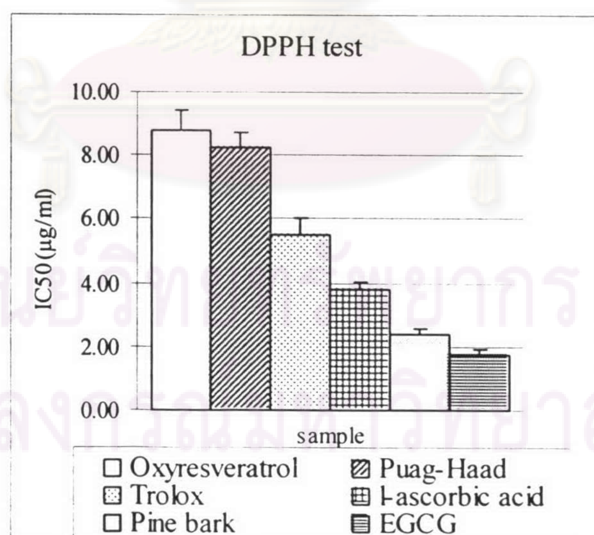

Figure 26. The IC_{50} (μg/ml) of each antioxidant in DPPH radical inhibition (mean ± SD, n = 3)

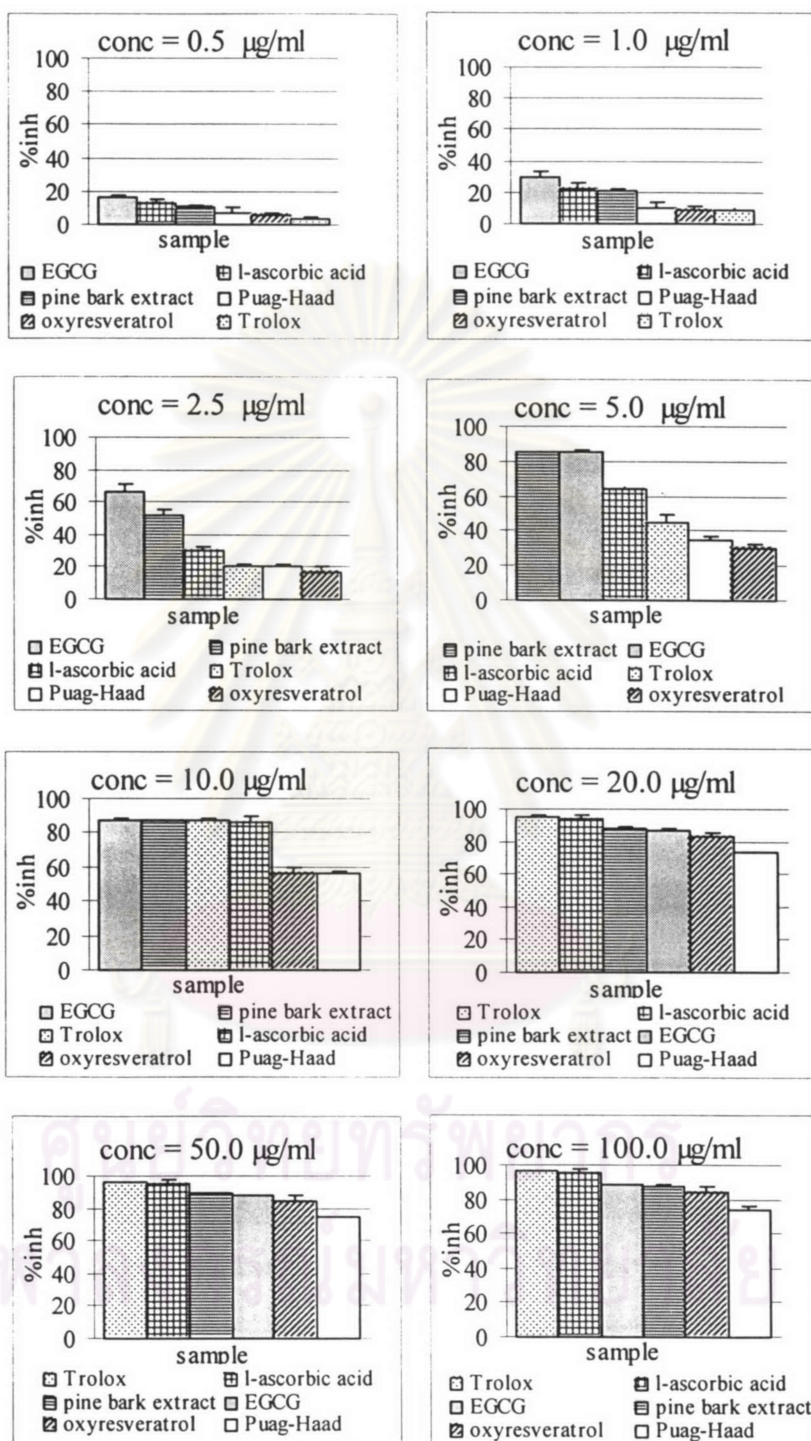

Figure 27. DPPH radical inhibition percentage of each antioxidant at various concentrations (mean ± SD, n = 3)

Although Puag-Haad and oxyresveratrol appeared to give the lowest potency as judged from their IC_{50} values, the concentration was expressed in μg/ml. To better characterize the relative potency of the individual antioxidants, the IC_{50} values should be expressed in terms of μM, in which the molecular weight of the substance was also taken into account. However, such transformation of the data from μg/ml to μM could not be made for all the test substances because Puag-Haad and pine bark extract are not pure compound. Nevertheless, if we assume that oxyresveratrol was the sole active and major constituent of Puag-Haad, the IC_{50} of the extract could be expressed in terms of μM. In this case, where the content of oxyresveratrol in Puag-Haad was taken as about 80 % w/w (Wanawatanakun, 2005), the IC_{50} of the extract equivalent to oxyresveratrol was calculated to be 25.71 μM. On the other hand, the IC_{50} in μM could not be determined for pine bark extract due to its multi-active components. Transforming the value from μg/ml in to μM was obtained using the same equation as in the tyrosinase inhibition test.

$$\mu M \text{ of extract} = \frac{(\mu g/ml) \times \text{purity } (\%w/w) \times \%\text{oxyresveratrol} \times 1000}{\text{molecular weight} \times 100 \times 100}$$

The following table shows the calculated values of IC_{50} in μM versus μg/ml for each of the five antioxidants under test:

Sample	IC_{50} (μg/ml)	IC_{50} (μM)
Puag-Haad	8.25 ± 0.48	25.71 ± 1.50
oxyresveratrol	8.74 ± 0.66	34.17 ± 2.58
Trolox®	5.48 ± 0.55	21.24 ± 2.12
l-ascorbic acid	3.83 ± 0.21	21.67 ± 1.20
EGCG	1.75 ± 0.18	3.77 ± 0.39

From the above data, the IC_{50} of oxyresveratrol was comparable with the value reported by Lorenz et al. (2003) with the IC_{50} of 28.9 μM. Oxyresveratrol was shown to be more effective scavenger than its derivative, resveratrol and tran-4-hydroxylstilbene. They also reported the slightly lower DPPH scavenging of oxyresveratrol than l-ascorbic acid (IC_{50} 18.0 μM) and Trolox® (IC_{50} 17.8 μM) (Lorenz et al., 2003). The IC_{50} value for

EGCG appeared to be smallest regardless of the expressed unit, indicating that EGCG was the most potent DPPH scavenger among the five antioxidants. Although l-ascorbic acid was more potent than Trolox® when judging from the IC_{50} in μg/ml, both seemed to be equivalent in potency in terms of μM which agreed with results from Lorenz et al. (2003). This observation was not to be considered as a discrepancy in data since both types of IC_{50} similarly indicated that l-ascorbic acid and Trolox® still demonstrated intermediate potency whereas Puag-Haad and oxyresveratrol exhibited the lowest potency in DPPH scavenging. It is also interesting to note that the IC_{50} value in μg/ml of Puag-Haad was very close to that of oxyresveratrol. Since Puag-Haad was a crude extract and thus not a pure compound, it is possible that other undetected substance(s) may have existed in the extract which contributed to its DPPH scavenging effect. Otherwise, its IC_{50} value in μg/ml would have been markedly higher than the pure compound oxyresveratrol. Transforming the value from μg/ml into μM further confirmed this suggestion since the IC_{50} for Puag-Haad became even much lower than oxyresveratrol (25.71 vs 34.17 μM). Therefore, it can be concluded that the DPPH scavenging property of Puag-Haad may not be due solely to oxyresveratrol and thus the use of the less expensive aqueous extract may have a greater benefit over the purified compound.

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3.2 Superoxide anion inhibition from riboflavin-light-NBT system or riboflavin-photo-oxidation method

Superoxide anion scavenging activity was assayed by nitroblue tetrazolium (NBT) method that was an indirect assay. The riboflavin photo-oxidation method was used to generate superoxide anion. Then, superoxide anion was allowed to reduce NBT to the blue formazan that was a spectrophotometrically detectable product at 560 nm.

The assay was based on the capacity of the samples to inhibit the reduction of NBT into formazan by superoxide anion generated from the riboflavin-light system. The production of the blue formazan was followed by measuring the absorbance by UV-visible spectrophotometer (Dasgupta and De, 2004; Geetha, et al., 2004; Banerjee, Dasgupta and Bratati, 2005).

The data on percentage of superoxide anion inhibition by Puag-Haad compared to other antioxidants or radical scavengers such as Trolox®, l-ascorbic acid, EGCG, pine bark extract and oxyresveratrol are graphically shown in Figure 28. Each test substance was prepared at various concentrations of 0, 5.0, 10.0, 25.0, 50.0 and 100.0 μg/ml.

The figure demonstrates that when the concentrations of the antioxidants were increased, the inhibition or the scavenging extent of superoxide anion also increased. Puag-Haad had the percentage of maximum inhibition of 88.42 ± 2.49 % at the concentration of 100.0 μg/ml. This inhibition is slightly lower than those of oxyresveratrol (94.51 ± 0.88 %) and Trolox® (92.78 ± 0.13 %) at the same concentration. L-ascorbic acid, on the other hand, had the percentage of maximum inhibition of only 57.31 ± 4.53 % at the concentration of 50.0 μg/ml. When the concentration was increased to100.0 μg/ml, the inhibition percentage did not increase; rather, it decreased slightly to 48.17 ± 4.15 % (Table 14).

Both EGCG and pine bark extract demonstrated good superoxide anion inhibition with respective maximum inhibition of 92.78 ± 0.27 % and 93.44 ± 0.83 % observed at 50.0 μg/ml. Both compounds also had similar inhibition extent of 89.47 – 92.78 % (EGCG) and 89.10 – 93.44 % (pine bark extract) at the concentration ranging from 25.0 – 100.0 μg/ml (Table 14).

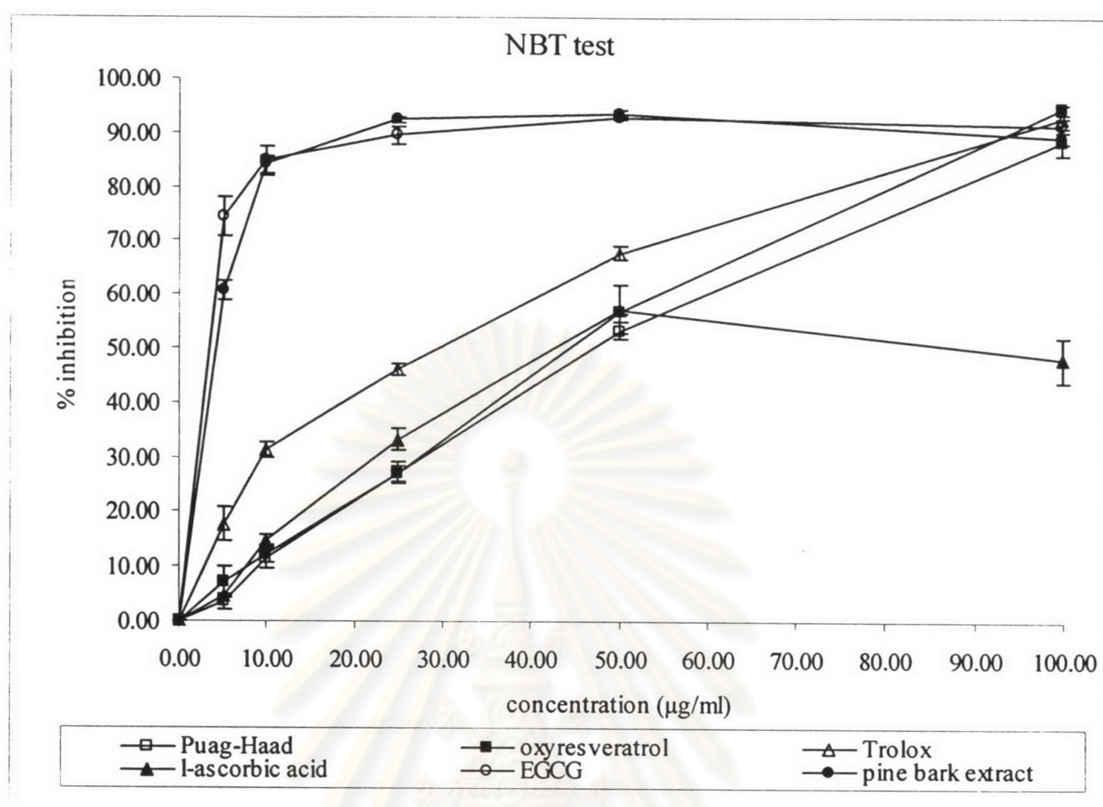

Figure 28. The extent of superoxide anion inhibition by Puag-Haad as a function of concentration in comparison with other antioxidants (mean ± SD, n = 3)

Table 14. Inhibition of superoxide anion from riboflavin photo-oxidation by Puag-Haad compared to other antioxidants at various concentrations (mean ± SD, n = 3)

No	Concentration	Puag-Haad		oxyresveratrol		Trolox*		l-ascorbic acid		EGCG		pine bark extract	
	(µg/ml)	% inh	SD	% inh	SD	% inh	SD	% inh	SD	% inh	SD	% inh	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	5.00	3.63	1.32	7.19	2.81	17.77	3.14	4.83	0.09	74.37	3.53	60.71	1.85
3	10.00	11.64	1.97	12.32	1.57	31.39	1.45	14.68	1.31	84.81	2.67	84.06	1.52
4	25.00	27.11	2.09	26.98	1.52	45.95	1.14	33.25	2.00	89.47	1.68	92.32	0.64
5	50.00	53.38	1.54	56.81	0.31	67.79	1.39	57.31	4.53	92.78	0.27	93.44	0.83
6	100.00	88.42	2.49	94.51	0.88	92.78	0.13	48.17	4.15	91.51	1.18	89.10	1.06

Figure 29 shows the individual plots between % superoxide anion inhibition and concentration, constructed separately for each antioxidant. The plots clearly indicate that Puag-Haad gave a similar profile to oxyresveratrol and Trolox®, whose inhibition efficacy increased consistently with the increased concentration and did not become saturated at all the concentrations studied. On the other hand, the inhibition of l-ascorbic acid, EGCG, and pine bark extract was concentration dependent only for a short range of concentration. For l-ascorbic acid, superoxide anion inhibition was saturated at the concentration of 50.0 μg/ml, while those of EGCG and pine bark extract reached the plateau region at a lower concentration of 10.0 μg/ml, with % inhibition of 84.81 ± 2.67 and 84.06 ± 1.52 %, respectively. The raw data of the absorbance values and % superoxide anion inhibition of the individual antioxidants are presented in Appendix B.

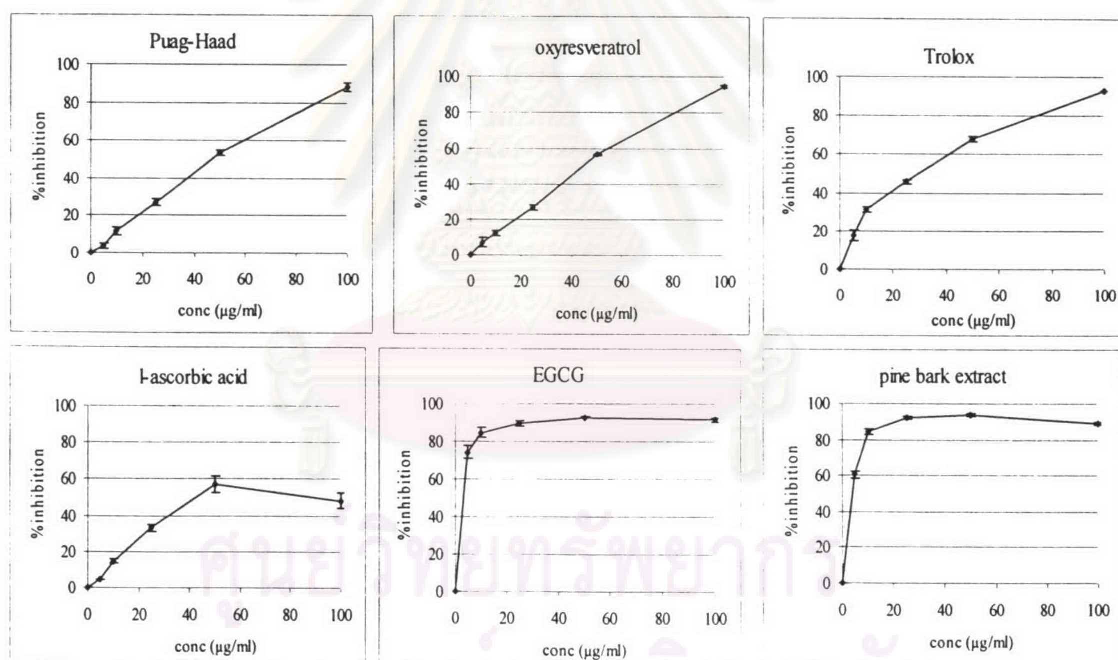

Figure 29. The extent of superoxide anion inhibition and concentration for each antioxidant (mean ± SD, n = 3)

The IC_{50} or a concentration at which an antioxidant can cause 50% inhibition was calculated for each antioxidant by regression analysis of the plots. It was found that a polynomial equation gave a better R^2 (regression coefficient) than a linear model. Moreover, the range of the plot used for calculation was also important. It was found that the initial range of concentration before saturation gave the highest R^2 of 0.9718 – 1.0000,

as shown in Table 15 and Figure 30. Also, the curves generated by partial polynomial regression were similar to the actual, visually observed plots. Thus, the polynomial regression equation was used to determine the IC_{50} values for all the tested antioxidants.

Table 15. The IC_{50} values for superoxide anion inhibition of each antioxidant. The R^2 is the regression coefficient obtained from polynomial regression of the initial portion of the plot between the inhibition percentage and the initial concentration of each antioxidant (mean ± SD, n = 3)

no.	Sample	polynomial equation (partial)					
		IC50(μg/ml)	Mean	SD	R^2	Mean	SD
		44.88			0.9953		
1	Puag-Haad	47.35	46.11	1.24	0.9948	0.9962	0.00
		46.11			0.9984		
		44.63			0.9942		
2	oxyresveratrol	43.59	44.31	0.63	0.9964	0.9967	0.00
		44.72			0.9994		
		24.56			0.9844		
3	Trolox®	24.80	24.90	0.39	0.9696	0.9718	0.01
		25.33			0.9613		
		44.07			0.9986		
4	l-ascorbic acid	38.54	40.24	3.32	0.9967	0.9974	0.00
		38.12			0.9970		
		2.63			1.0000		
5	EGCG	3.04	2.84	0.21	1.0000	1.0000	0.00
		2.86			1.0000		
		3.81			1.0000		
6	pine bark extract	3.70	3.85	0.17	1.0000	1.0000	0.00
		4.03			1.0000		

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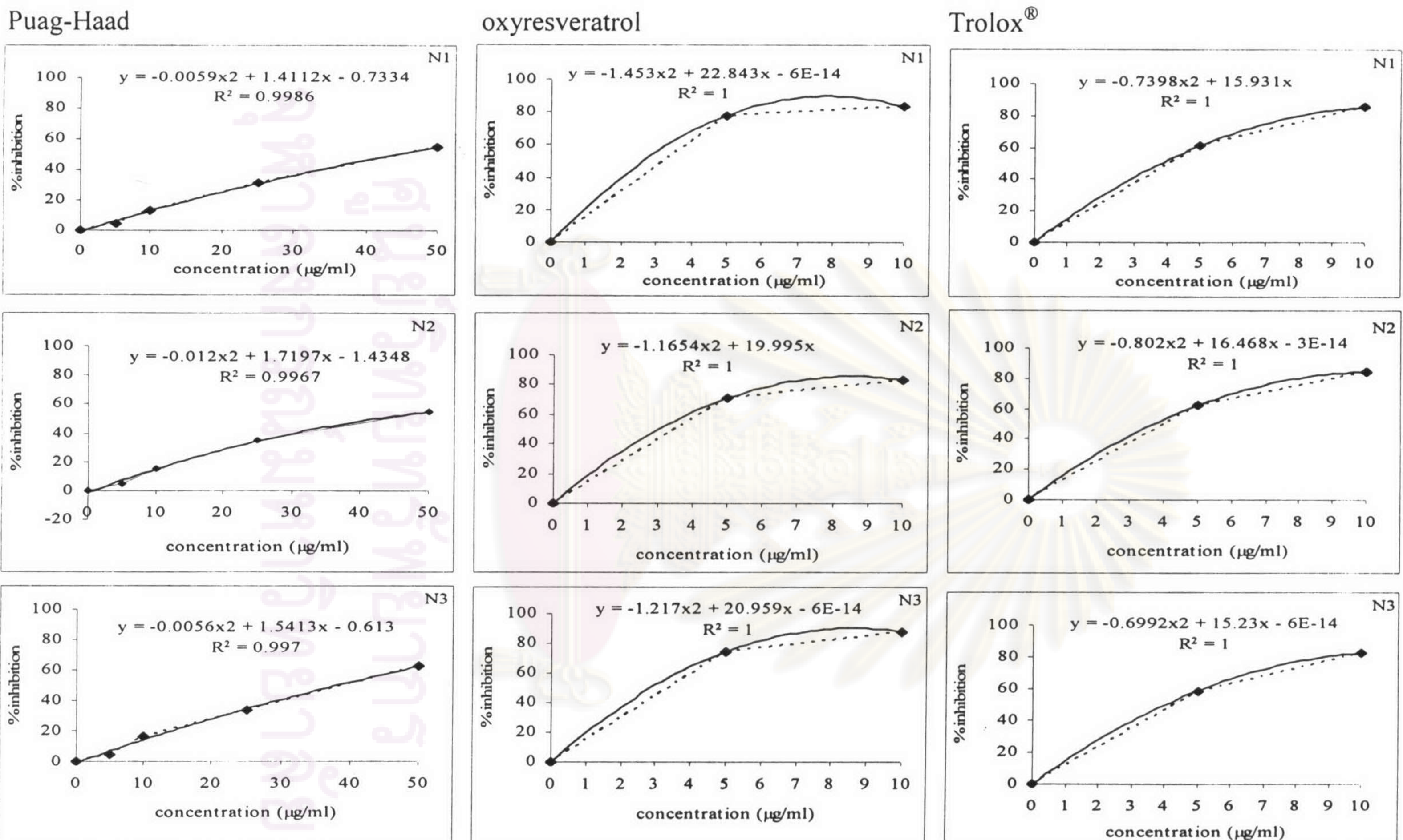

Figure 30. Comparison between the actual curve (dotted line) and the regression curve (solid line) of the initial portion of the % superoxide anion inhibition-concentration profile of each antioxidant. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants

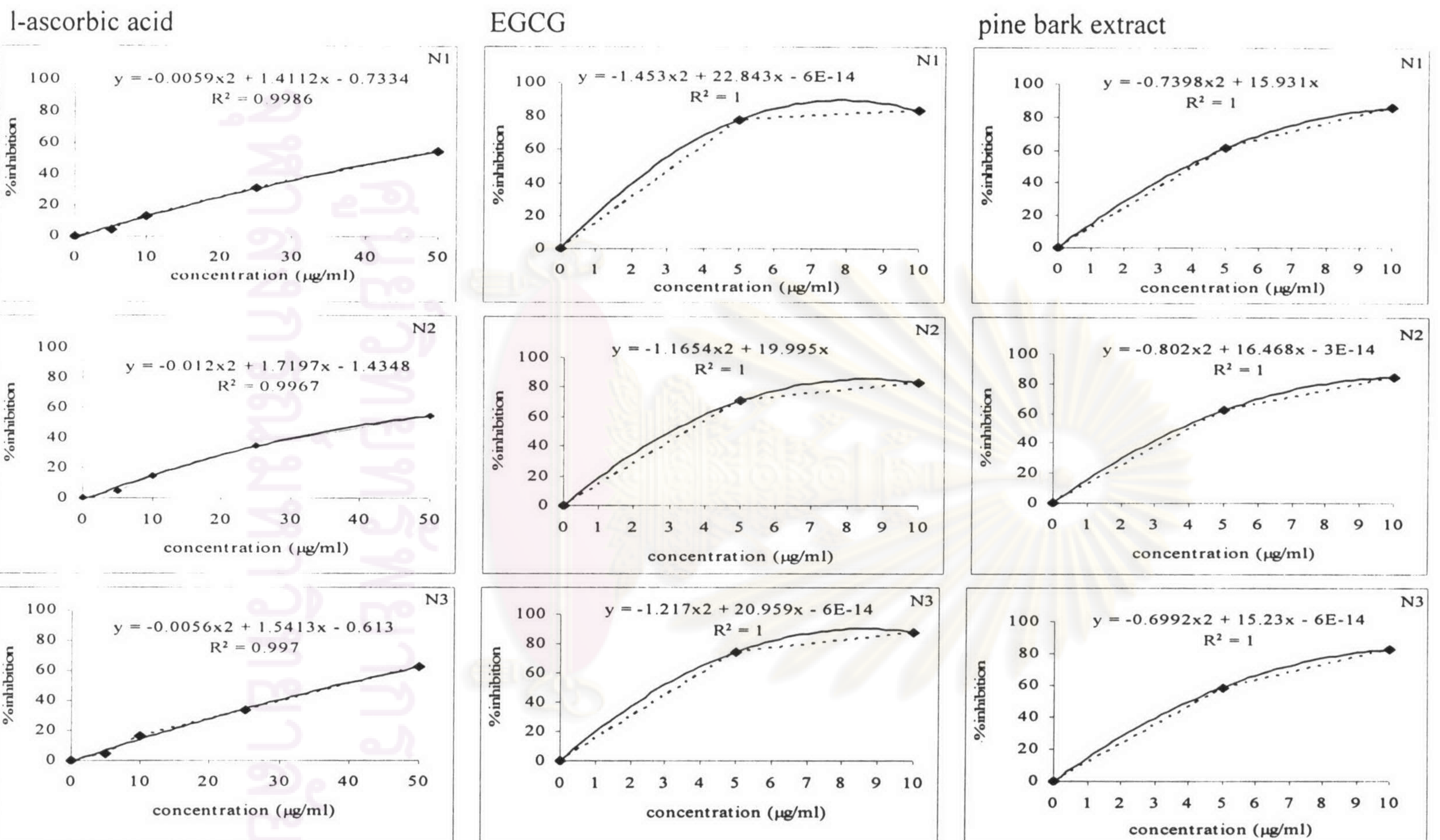

Figure 30. Comparison between the actual curve (dotted line) and the regression curve (solid line) of the initial portion of the % superoxide anion inhibition-concentration profile of each antioxidant. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants (Cont.)

From Table 15, the means of estimated IC_{50} ranking from the lowest to the highest value are: EGCG (2.84 ± 0.21 µg/ml), pine bark extract (3.85 ± 0.17 µg/ml), Trolox® (24.90 ± 0.39 µg/ml), l-ascorbic acid (40.24 ± 3.32 µg/ml), oxyresveratrol (44.31 ± 0.63 µg/ml) and Puag-Haad (46.11 ± 1.24 µg/ml). Since IC_{50} value is an indicator for antioxidation potency, Puag-Haad and its active compound, oxyresveratrol, showed weaker superoxide anion free radical inhibition than the other studied antioxidants. This is because higher concentrations were required for Puag-Haad and oxyresveratrol to give similar inhibition percentage (50% inhibition) to other antioxidants (Figure 31).

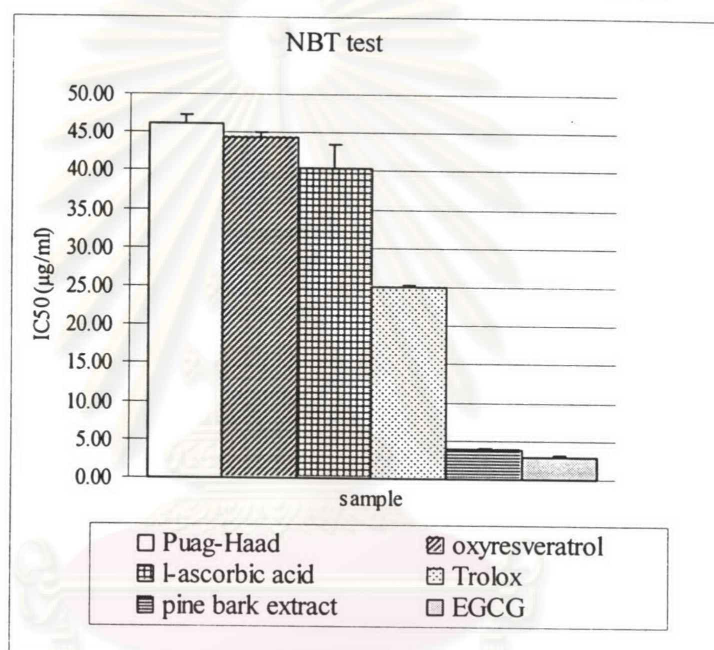

Figure 31. Comparison of IC_{50} (µg/ml) of the six antioxidants (mean ± SD, n = 3)

The IC_{50} values were analyzed by one-way ANOVA at 5 % significant level. They were found to be significantly different ($P << 0.05$). The data were then analyzed by Tukey's test to group the antioxidants according to their superoxide anion radical scavenging efficacy. The six antioxidants were classified into 4 groups with significant difference in superoxide scavenging activities ($P < 0.05$) as follows:

<u>EGCG ≈ pine bark extract</u>		< Trolox® <	l-ascorbic acid	< <u>oxyresveratrol ≈ Puag-Haad</u>	
2.84	3.85	24.9	40.24	44.31	46.11 µg/ml

The antioxidants that are joined by the same line are in the same group and not statistically different in terms of their radical inhibition. Thus, EGCG and pine bark extract were similar and possessed the highest superoxide anion radical inhibition efficacy, which was significantly different from Trolox® and l-ascorbic acid, respectively. Oxyresveratrol and Puag-Haad were also similarly active ($P > 0.05$) but their activity appeared to be the lowest against superoxide anion radical due to their higher IC_{50} values ($P < 0.05$). The detailed statistical results are provided in Appendix B.

Although Puag-Haad and oxyresveratrol gave the highest IC_{50} values, indicating a relatively low potency when compared with EGCG, pine bark and Trolox®. They still exerted a substantial extent of inhibition which was close to 100%, i.e., 88.42 and 94.51 % at 100μg/ml, respectively. According to the plot in Figure 28, their extent of inhibition increased consistently with concentration up to the last concentration studied (100 μg/ml). From Figure 32, which shows the bar graphs comparing the extent of inhibition among different antioxidants at each concentration, it can be seen that the extents of superoxide anion inhibition by Puag-Haad and oxyresveratrol are comparable to those of other antioxidants, especially at the highest concentration studied of 100 μg/ml. There is a possibility that the inhibition extent could further increase for Puag-Haad and oxyresveratrol if the concentration were increased above 100 μg/ml because the graphs had not yet reached a plateau at this concentration, while EGCG and pine bark extract already reached a plateau at a much lower concentration of 25 μg/ml (Figure 28). However, more studies should be done in the future using higher concentrations to determine the saturation concentrations of Puag-Haad and oxyresveratrol in scavenging superoxide anion radical.

The data were tested statistically in order to compare the inhibition extent of the six antioxidants using one-way ANOVA and Tukey's test at each concentration. It was found that at the concentrations of 5, 10, 25 and 50 μg/ml, EGCG and pine bark extract showed the highest superoxide anion inhibition. However, at 100 μg/ml, both Puag-Haad and oxyresveratrol exhibited an inhibition extent similar to pine bark extract, EGCG and Trolox® ($P > 0.05$), while l-ascorbic acid demonstrated the least efficacy ($P < 0.05$). Moreover, at 100 μg/ml, oxyresveratrol gave the highest inhibition percentage (94.51%),

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although it is not significantly different from those of pine bark extract (89.10%), EGCG (91.51%) and Trolox® (92.78%). The data are given in detail in Appendix B.

According to the data in Table 14 and Figure 28, the superoxide anion radical inhibition of l-ascorbic acid was lower than other antioxidants, especially at the highest concentration studied of 100 μg/ml, at which the inhibition percentage decreased to 48.17 % from a level of 57.31 % at 50 μg/ml, whereas the extent of inhibition for other antioxidants remained the same or further increased at this highest concentration. L-Ascorbic acid is known to be a good radical scavenger and thus, it was expected to provide superoxide anion inhibition in a concentration-dependent manner. Therefore, the results obtained from this study appeared to be in conflict. The reasons for this discrepancy could be explained in terms of the ability of l-ascorbic acid to directly interact with NBT, even in the absence of riboflavin and light.

The confirmation of this finding was done by adding NBT into l-ascorbic acid solutions at various concentrations. It was found that at concentrations of 100 μg/ml and higher, l-ascorbic acid caused a direct interaction with NBT resulting in the solution becoming visibly blue in color without the need to add riboflavin or be stimulated by fluoresecent lamp. Thus, at the concentration of 100 μg/ml or more, l-ascorbic acid was able to cause a substantial colour change by reacting with NBT, and hence, contribute to a rise in the reaction mixture's absorbance and a drop in % radical inhibition.

Moreover, another reason for this is proposed to be the formation of ascorbyl semiquinone radicals and oxidation of ascorbic acid by riboflavin in the presence of light. Photo-excited riboflavin can perform one electron oxidation of l-ascorbic acid generating a riboflavin radical (Geetha, 2004), or l-ascorbic itself could act as a pro-oxidant at high concentration (Konig and Ring, 2005). Due to the unique properties of l-ascorbic acid, less superoxide anion scavenging activity was thus observed at high concentrations.

Although l-ascorbic acid can have reaction with NBT at higher concentrations, this would not affect the calculation of IC_{50} for superoxide anion inhibition because the IC_{50} value of l-ascorbic acid was calculated using the data from the concentrations of 0-50 μg/ml, which were in the initial, rising part of the % inhibition-concentration curve.

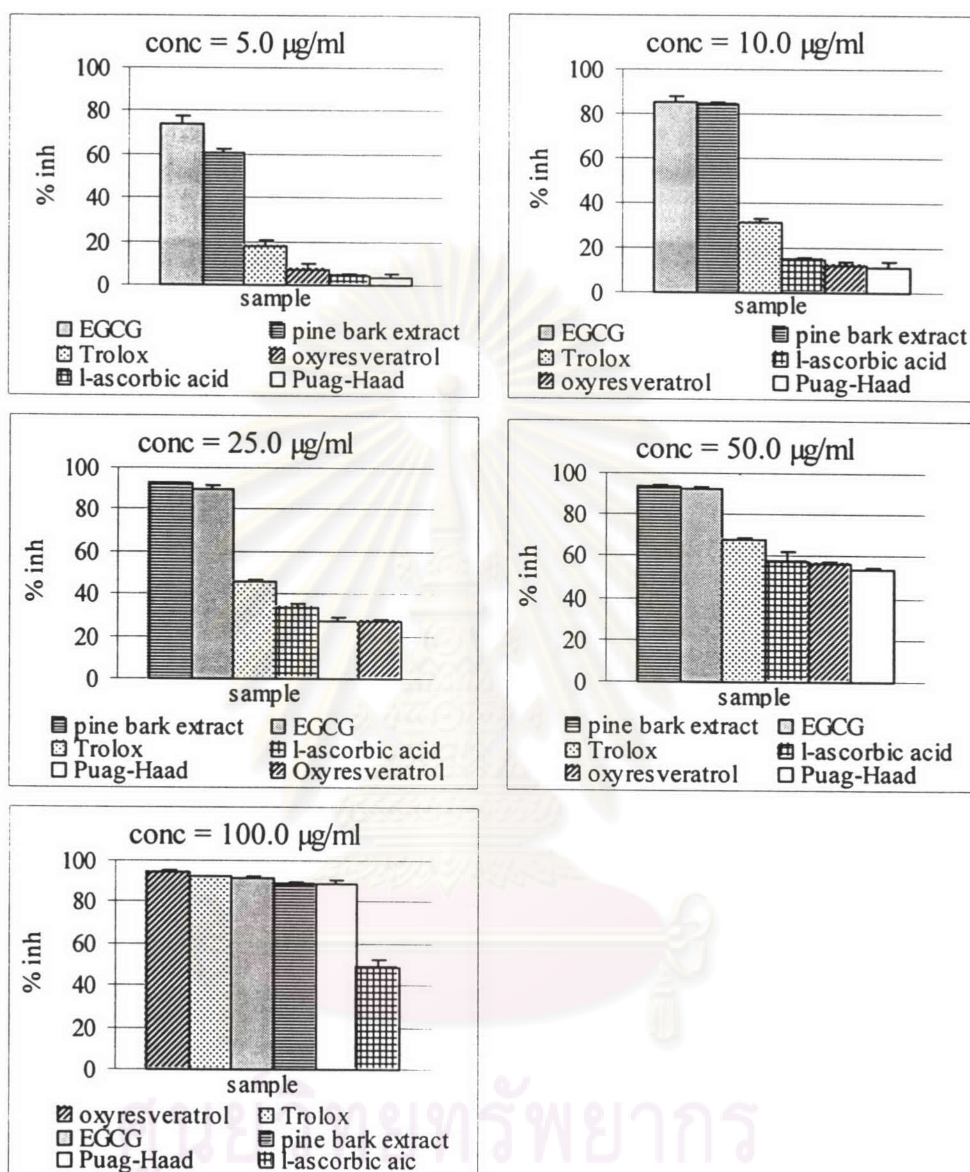

Figure 32. Comparison of superoxide anion inhibition extent of the six antioxidants at various concentrations (mean ± SD, n = 3)

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Similar to the DPPH test, the IC_{50} values of the five antioxidants were also transformed into μM unit to better compare their efficacy in terms of molar concentration in scavenging the superoxide anion free radical by the following equation.

$$\mu M \text{ of extract} = \frac{(\mu g/ml) \times \text{purity } (\%w/w) \times \%\text{oxyresveratrol} \times 1000}{\text{molecular weight} \times 100 \times 100}$$

The calculated values of the IC_{50} in μM versus μg/ml are as follows:

Sample	IC_{50} (μg/ml)	IC_{50} (μM)
Puag-Haad	46.11 ± 1.24	143.63 ± 3.85
oxyresveratrol	44.31 ± 0.63	172.53 ± 2.45
Trolox®	24.90 ± 0.39	96.48 ± 1.53
l-ascorbic acid	40.24 ± 3.32	227.92 ± 18.81
EGCG	2.84 ± 0.21	6.14 ± 0.44

EGCG gave the highest potency regardless of the IC_{50} unit similar to the DPPH test. However, obvious difference between the two types of IC_{50} can be seen with l-ascorbic acid. In μg/ml unit, l-ascorbic was slightly more potent than Puag-Haad and oxyresveratrol. But in μM unit, it appeared to be less potent than Puag-Haad and oxyresveratrol due to the much higher IC_{50} value (227.92 μM) although statistical test was not performed. Also, the similarity in IC_{50} values in μg/ml between Puag-Haad and oxyresveratrol suggested that some unidentified substance(s) in the extract apart from oxyresveratrol could have contributed to its superoxide anion scavenging activity. Transformation of the IC_{50} values from μg/ml into μM further confirmed this hypothesis since the μM unit for Puag-Haad (143.63) was also lower than oxyresveratrol (172.53).

The activity of all test compounds can be explained by its polyphenolic structures with can give hydrogen atom to the reactive species; superoxide anion. Some reports clarified this activity by the definition of reduction potentials of this compound that are lower than superoxide anion which means the phenolic compound may inactivate superoxide and prevent the deleterious consequences of their reactions. However,

superoxide anion scavenging activities can be determined in various tests such as xanthine/xanthine oxidase system. Thus, the results seem to be slightly different (Yuting et al., 1990; Cotelle et al., 1992; Hanasaki, Ogawa, and Fukui, 1994; Catherine et al., 1996).

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3.3 Hydroxyl radical inhibition by $ABTS/H_2O_2/FeCl_3$ method

In this method, hydroxyl radical scavenging activity was measured in the $ABTS/H_2O_2/FeCl_3$ system. Hydrogen peroxide can readily react with transition-metal catalysts to generate the hydroxyl radical ($HO^{\cdot}$). In this reaction, ABTS is oxidized in the presence of hydroxyl radical to generate an intermediate, a metastable radical form of oxidized ABTS yielding a green color solution that can be measured at 416 nm. The antioxidant activity of the extract, expressed as percent inhibition of hydroxyl radical (% reduction in intermediate ABTS radical), was calculated.

Figure 33 compares the extent of hydroxyl radical inhibition exerted by Puag-Haad, oxyresveratrol, Trolox®, l-ascorbic acid, EGCG and pine bark extract at various concentrations (0.5 to 200.0 μg/ml depending on each antioxidant). As seen from the data in Table 16 and Figure 33, as the concentration was increased the hydroxyl free radical scavenging activity also increased for all antioxidants (except for pine bark extract) until reaching a plateau at certain concentration. The hydroxyl radical scavenging activity of Puag-Haad increased to 84.89 ± 0.84% at concentration of 50.0 μg/ml, and then only slightly increased to 90.06 ± 1.72% when the concentration was raised further to 100.0 μg/ml. Its active compound, oxyresveratrol, also showed a similar profile, i.e., the inhibition extent started to reach a plateau at the concentration of 50.0 μg/ml (88.48 ± 1.02%), and when the concentration was increased to 100.0 μg/ml, the value of percent inhibition did not greatly increase (90.67 ± 0.96%). Thus, according to Figures 23, 28, and 33, the profiles of Puag-Haad were very much alike those of oxyresveratrol. These similarities verify the hypothesis that oxyresveratrol is the major, if not sole, active antioxidant component in Puag-Haad. It can scavenge all superoxide anion, DPPH radical and hydroxyl radical.

L-ascorbic acid and Trolox® provided the highest hydroxyl free radical scavenging activity because both vitamins reached the inhibition saturation at very low concentration and still showed high inhibition percentages. L-ascorbic acid reached a plateau at 5.0 μg/ml with 96.24 ± 1.70% inhibition, whereas Trolox® reached a plateau at 10.0 μg/ml with 96.12 ± 1.01% inhibition. EGCG demonstrated a similar scavenging activity to Puag-Haad and oxyresveratrol, which was in contrast to the previous two tests in which EGCG was more effective. The hydroxyl radical scavenging data are shown in Figure 33

and Table 16. EGCG showed inhibition saturation at the concentration of 50.0 μg/ml (81.43 ± 1.81%) similar to Puag-Haad and oxyresveratrol but its extent was slightly lower.

In this test the pine bark extract was found to possess the lowest hydroxyl radical scavenging potency. At the maximum concentration of 200.0 μg/ml, it gave percent inhibition of only 70.43 ± 1.36 %, which was lower than other antioxidants. Figure 34 shows the plots between the extent of hydroxyl radical inhibition and concentration of the individual antioxidants. The data on the absorbance values and hydroxyl free radical scavenging extent of each antioxidant are shown in Appendix C.

Table 16. Hydroxyl free radical inhibition of Puag-Haad compared to other antioxidants at various concentrations (mean ± SD, n = 3)

No	conc	Puag-Haad		oxyresveratrol		EGCG		pine bark extract		No	conc	Trolox®		ascorbic acid	
	(μg/ml)	% inh	SD	% inh	SD	% inh	SD	% inh	SD		(μg/ml)	% inh	SD	% inh	SD
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1	0.00	0.00	0.00	0.00	0.00
2	5.00	29.80	2.29	37.11	2.56	45.66	3.80	7.34	2.25	2	0.50	15.16	1.11	21.15	6.27
3	10.00	52.24	1.89	61.22	2.85	65.77	2.05	19.72	1.96	3	1.00	24.76	2.56	36.12	2.80
4	20.00	70.76	2.22	77.29	1.40	73.96	2.06	30.94	1.34	4	2.00	41.90	1.58	58.37	1.05
5	50.00	84.89	0.84	88.48	1.02	81.43	1.81	47.31	1.67	5	5.00	84.96	5.06	96.24	1.70
6	100.00	90.06	1.72	90.67	0.96	84.12	1.32	60.52	1.29	6	10.00	96.12	1.01	97.44	1.42
7	200.00	-	-	-	-	-	-	70.43	1.36	7	20.00	97.36	0.90	-	-

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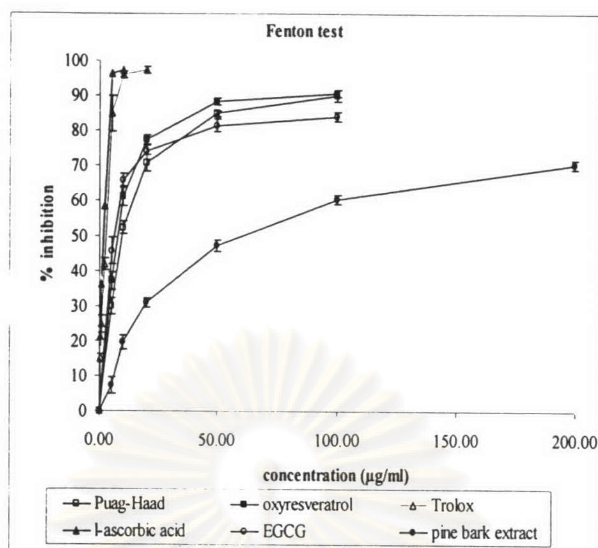

Figure 33. The extent of hydroxyl radical inhibition by Puag-Haad as a function of concentration in comparison with other antioxidants (mean ± SD, n = 3)

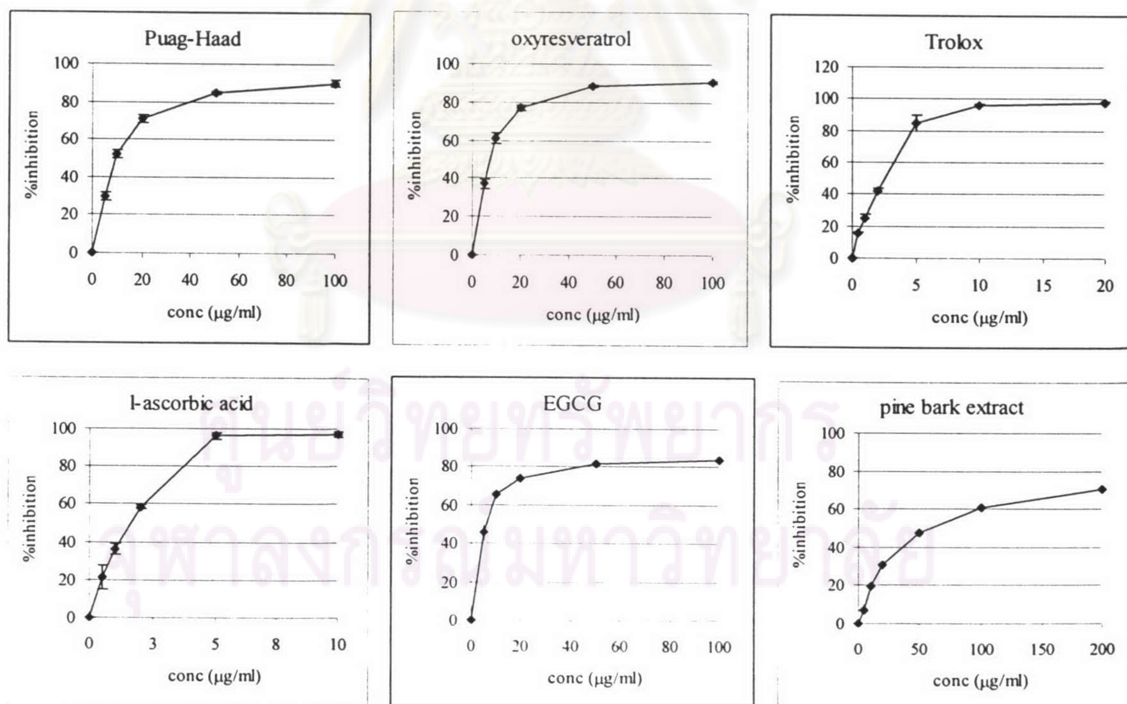

Figure 34. The extent of hydroxyl radical inhibition and concentration for each antioxidant (mean ± SD, n = 3)

Table 17. The IC_{50} values for hydroxyl radical inhibition of each antioxidant. The R^2 is the regression coefficient obtained from polynomial regression of the initial portion of the plot between the inhibition percentage and the initial concentration of each antioxidant (mean ± SD, n = 3)

no.	sample	polynomial equation (partial)					
		IC50 (µg/ml)	Mean	SD	R^2	Mean	SD
		9.54			1.0000		
1	Puag-Haad	10.05	9.47	0.61	0.9991	0.9997	0.00
		8.83			1.0000		
		6.86			0.9993		
2	oxyresveratrol	7.45	7.41	0.54	0.9999	0.9997	0.00
		7.93			0.9998		
		2.42			0.9937		
3	Trolox®	2.46	2.39	0.09	0.9989	0.9968	0.00
		2.29			0.9977		
		1.54			0.9929		
4	l-ascorbic acid	1.58	1.57	0.03	0.9882	0.9926	0.00
		1.59			0.9967		
		6.41			0.9999		
5	EGCG	6.35	6.20	0.31	0.9890	0.9925	0.01
		5.85			0.9885		
		45.23			0.9721		
6	pine bark extract	50.62	48.56	2.91	0.9707	0.9757	0.01
		49.82			0.9843		

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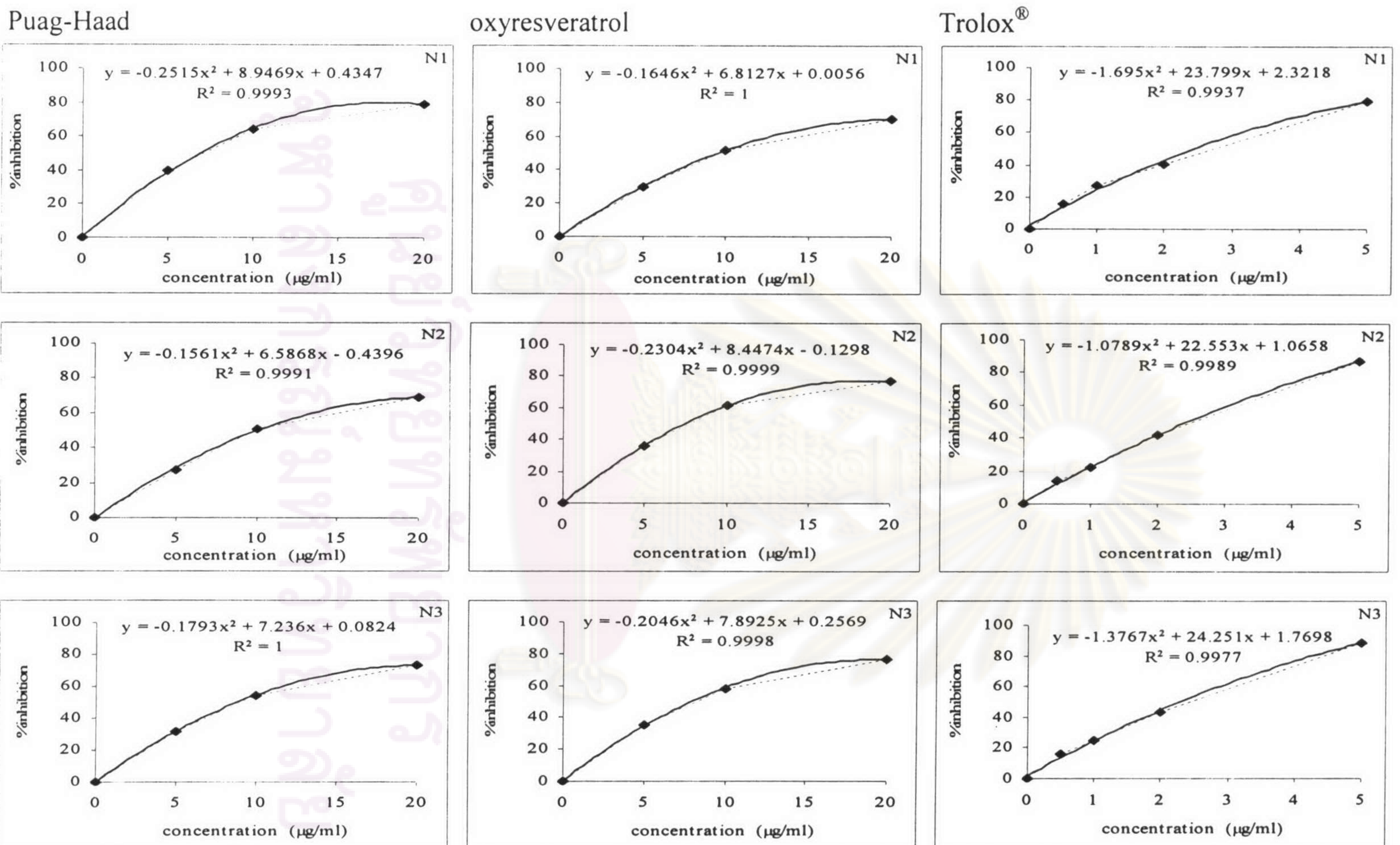

Figure 35. Comparison between the actual curve (dotted line) and the regression curve (solid line) of the initial portion of the % hydroxyl radical inhibition-concentration profile of each antioxidant. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants

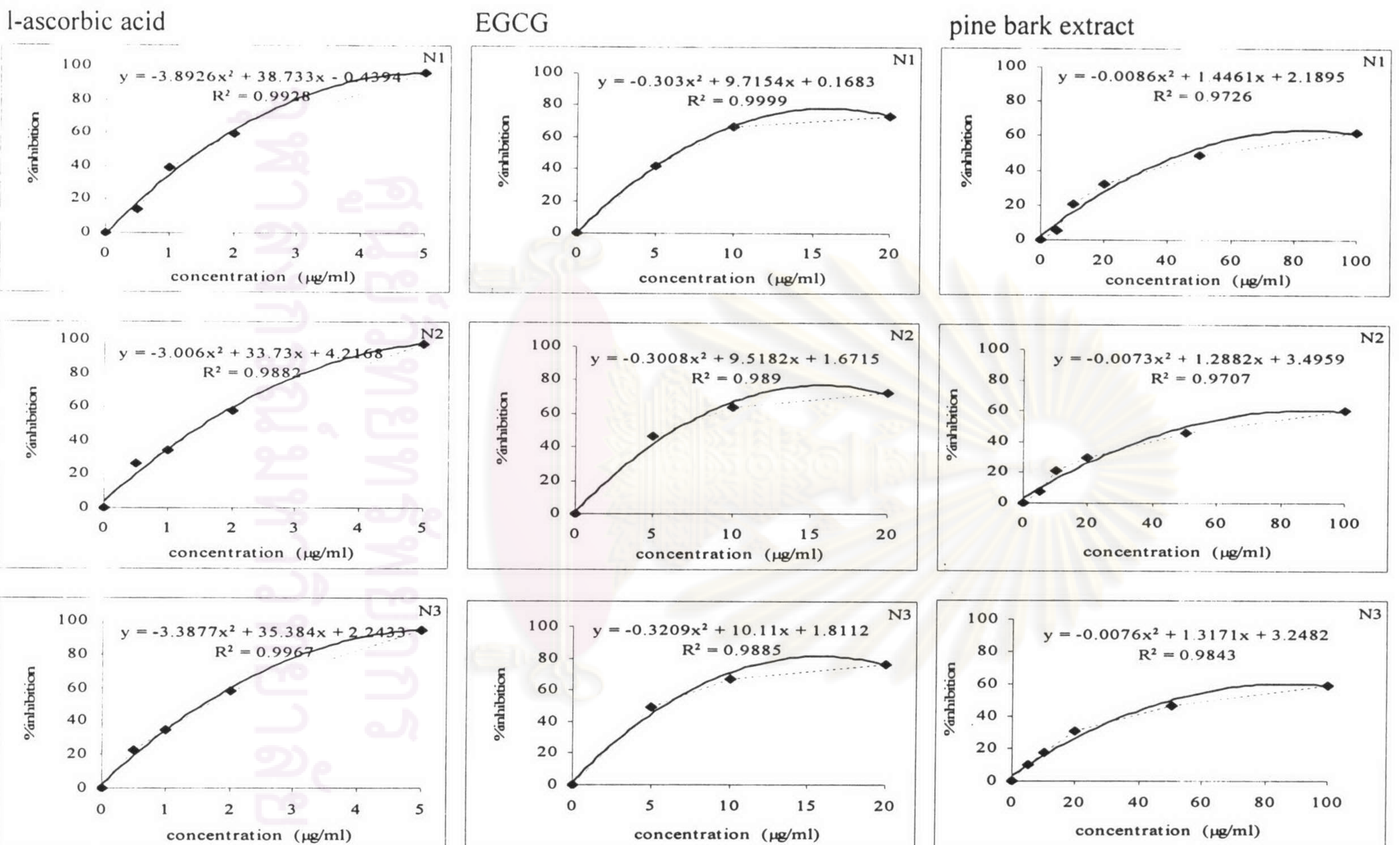

Figure 35. Comparison between the actual curve (dotted line) and the regression curve (solid line) of the initial portion of the % hydroxyl radical inhibition-concentration profile of each antioxidant. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants (Cont.)

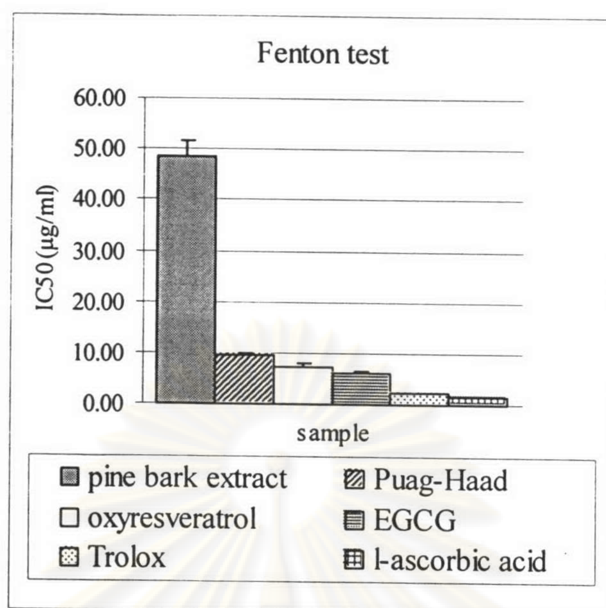

Figure 36. The IC_{50} values for hydroxyl free radical inhibition of each antioxidant (mean ± SD, n = 3)

Then, the IC_{50} or the concentration at which an antioxidant can cause 50% inhibition of hydroxyl radical was calculated by regression analysis using the data from the initial part of each inhibition percentage plot. Like the superoxide anion and DPPH tests, a polynomial equation was used and the results are given in Table 17 and Figure 35.

According to Table 17, the estimated IC_{50} values can be ranked from the lowest to the highest as follows: l-ascorbic acid (1.57 ± 0.03 μg/ml), Trolox® (2.39 ± 0.09 μg/ml), EGCG (6.20 ± 0.31 μg/ml), oxyresveratrol (7.41 ± 0.54 μg/ml), Puag-Haad (9.47 ± 0.61 μg/ml) and pine bark extract (48.56 ± 2.91 μg/ml). Since the IC_{50} value is an indicator of the potency of an antioxidant, the pine bark extract which demonstrated the highest IC_{50} value thus appeared to be the least potent hydroxyl radical scavenger. The data are also plotted as a bar graph in Figure 36.

From this figure it can be seen that l-ascorbic acid and Trolox® gave the lowest IC_{50} and thus possessed the strongest hydroxyl radical scavenging potency. EGCG, oxyresveratrol and Puag-Haad gave slightly higher IC_{50} values than l-ascorbic acid and Trolox® (3.9 – 6 times higher than l-ascorbic acid) indicating a slightly lower potency than the two vitamins. On the other hand, the IC_{50} value for the pine bark extract was 31

times higher than l-ascorbic acid, indicating that it was the least potent hydroxyl radical scavenger.

The IC_{50} values were analysed using one-way ANOVA at 5% significant level. Significant differences were found among the IC_{50} values of the studied antioxidants ($P \ll 0.05$). The data were subsequently further analyzed by Tukey's test in order to differentiate the antioxidants with respect to their hydroxyl radical scavenging potency. The results showed that the six antioxidants could be divided into three statistically different groups ($P < 0.05$) as follows:

l-ascorbic acid ≈	Trolox® <	EGCG ≈	oxyresveratrol ≈	Puag-Haad <	pine bark extract
1.57	2.39	6.20	7.41	9.47	48.56 μg/ml

The antioxidants that are joined by the same line are in the same group and not statistically different in terms of their radical inhibition. Thus, l-ascorbic acid and Trolox® was similar and possessed the highest hydroxyl radical inhibition potency, which was significantly different from the group of EGCG, oxyresveratrol and Puag-Haad. The activity of pine bark extract appeared to be the lowest due to its highest IC_{50} value, which was 30.93- and 5.13-folds less potent than l-ascorbic acid and Puag-Haad, respectively ($P < 0.05$). The detailed statistical results are provided in Appendix C.

L-ascorbic acid and Trolox® appeared to be the most potent inhibitors or scavengers of hydroxyl free radical, with the lowest IC_{50} values. Their high potency could be noticed from the plots in Figure 33 and data in Table 16, in which the studied concentration was ended at 10.0 μg/ml for l-ascorbic acid and at 20.0 μg/ml for Trolox® since the inhibition already reached a plateau level at 5.0 and 10.0 μg/ml for l-ascorbic acid and Trolox®, respectively. Since EGCG, oxyresveratrol and Puag-Haad demonstrated less potency than the two vitamins, the maximum studied concentration was extended to 100.0 μg/ml for these compounds.

On the other hand, the hydroxyl free radical inhibition of the pine bark extract increased only slightly when the concentration was increased. Therefore, the maximum studied concentration was much higher, ending at 200.0 μg/ml. Even at this concentration, it still provided the lowest inhibition (70.43 %) and the plateau level still

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was not reached. This was the reason why it was not feasible to compare the extent of hydroxyl radical inhibition of all six antioxidants at every concentration. The comparison was made only at 5.0 and 10.0 μg/ml, and also at the last concentration of each antioxidant, where the maximum inhibitory effect was observed (Figure 37).

After analysis with one-way ANOVA and Tukey's test at 5% significance level, the rankings of the hydroxyl free radical scavenging activity of the six antioxidants at these concentrations were the same, i.e., the most potent group consisting of l-ascorbic acid and Trolox®, the intermediate group consisting of EGCG, oxyresveratrol and Puag-Haad, and the least potent group was the pine bark extract. Statistical data comparing % inhibition at these concentrations are provided in Appendix C.

Results from this study agreed with Pumthong (1999) that l-ascorbic acid and Trolox® possess a strong quenching activity for hydroxyl radical. Both of these antioxidants possed the highest hydroxyl radical scavenging activity by delaying the reaction and giving a particular lag time in the first period of the reaction (90 s) when compared to the tamarind seed extracts. Oxyresveratrol, Puag-Haad and EGCG which are polyphenolic compounds possess this activity probably via their multi-hydroxy structures. On the other hand, the pine bark extract demonstrated the lowest hydroxyl radical scavenging activity despite it also being a polyphenol. This could be due to the subtle difference in the chemical nature of the extract, which consists mainly of proanthocyanidins. Nevertheless, the weak hydroxyl radical scavenging activity of pine bark extract reported here is only relative due to comparison with other antioxidants. Its activity was still significant over the control in which no pine bark was added to the system. Other reports also claimed good hydroxyl scavenging activity of pine bark extract (Virgili, Kobuchi, and Packer, 1998; Guo, Zhao, and Packer, 1999; Packer, Rimbach, and Virgili, 1999). They reported about the strong hydroxyl radical scavenging activity of this compound using in vitro test which also generated the free radicals by Fenton reaction, as assessed by EPR spectroscopy. However, the IC50 for the pine bark extract could not be calculated due to complex components of the mixture. Futher study could be carried out for the structure activities relationship of these compounds. Differences in the testing conditions probably also contributed to the variation in the results.

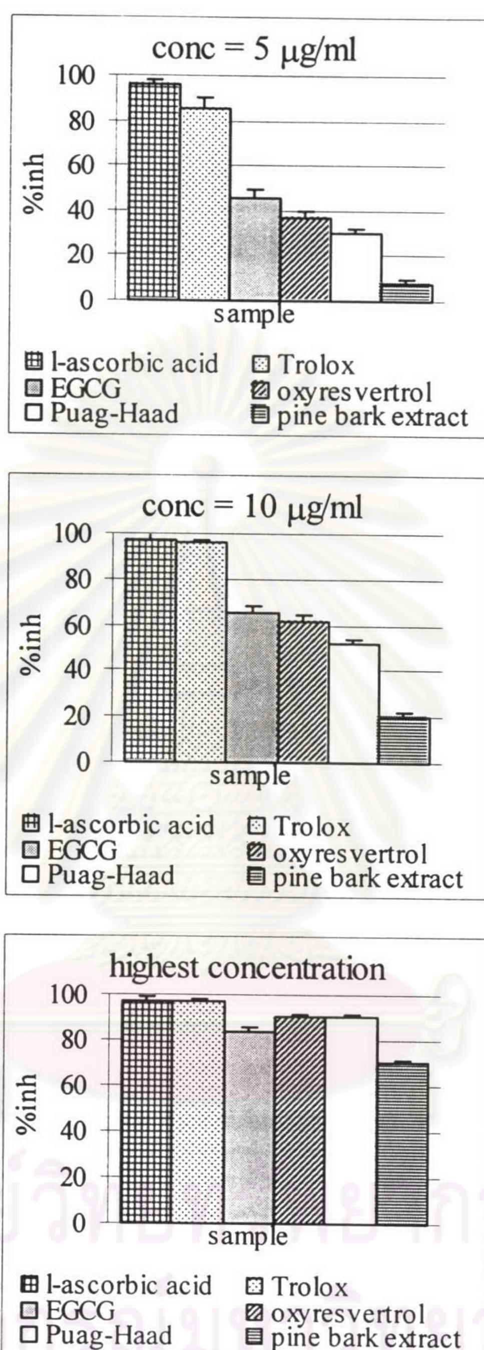

Figure 37. Comparison of hydroxyl radical inhibition extent of the six antioxidants at the concentration of 5.0 μg/ml, 10.0 μg/ml and the highest test concentraton; l-ascorbic acid (10 μg/ml), Trolox® (20 μg/ml), EGCG (100 μg/ml), oxyresveratrol (100 μg/ml), Puag-Haad (100 μg/ml) and pine bark extract (200 μg/ml) (mean ± SD, n = 3)

The IC_{50} values of the five antioxidants (except pine bark extract) were also transformed into μM unit to better compare their efficacy in terms of molar concentration in scavenging the hydroxyl free radical by the following equation.

$$\mu M \text{ of extract} = \frac{(\mu g/ml) \times \text{purity } (\%w/w) \times \%\text{oxyresveratrol} \times 1000}{\text{molecular weight} \times 100 \times 100}$$

The calculated values of the IC_{50} (μM versus μg/ml) are as follows:

Sample	IC_{50} (μg/ml)	IC_{50} (μM)
Puag-Haad	9.47± 0.61	29.51 ± 1.91
oxyresveratrol	7.41 ± 0.54	28.86 ± 2.09
Trolox®	2.39 ± 0.09	9.26 ± 0.34
l-ascorbic acid	1.57 ± 0.03	8.89 ± 0.15
EGCG	6.20 ± 0.31	13.40 ± 0.66

It is interesting to note that the IC_{50} of Puag-Haad in μg/ml was now higher than oxyresveratrol (9.47 versus 7.41), indicating that the hydroxyl radical scavenging activity of the extract was due mainly to oxyresveratrol and not to other minor component(s) that might be present in the extract. This was further confirmed by the IC_{50} values in μM which showed that Puag-Haad, when transformed into equivalent molar concentration of oxyresveratrol, almost gave the same value of IC_{50} as that of the pure compound (29.51 versus 28.86 μM). The smallest IC_{50} in μM also showed that l-ascorbic acid (8.89) and Trolox (9.26) were still the most potent hydroxyl radical scavengers. However, the IC_{50} value for EGCG appeared to be about two-times smaller than oxyresveratrol and Puag-Haad after transformation into μM unit. This was due to the fact that EGCG has a much higher molecular weight (MW = 458.4) than oxyresveratrol (MW = 244) and thus, based on a mole-by-mole basis, less molecules of EGCG are required to produce the same extent of inhibition as oxyresveratrol.

3.4 UV-induced lipid peroxidation (Hemolysis test)

Erythrocyte has a large amount of lipid membrane compared to other tissues, and hemolyis can be induced easily by singlet oxygen as a result of membrane damage via lipid peroxidation (Kawashima et al., 2003). In this method, singlet oxygen is produced in the system by exposing hematoporphyrin, a photosensitiser, to UV radiation. Singlet oxygen, then, can oxidize erythrocyte membrane lipid, causing lipid peroxidation, which leads to the degeneration of erythrocyte membrane and eventually hemolysis. Based on this assumption, any substance which is able to scavenge such singlet oxygen, could possibly reduce hemolysis.

During the preliminary study, it was found that the optimum period to irradiate the control system (red blood cells plus hematoporphyrin without antioxidant) until substantial hemolysis occurred was about 90 min. When UV irradiation was conducted for 60 min, the extent of hemolysis was less than 50%, which was not sufficient to evaluate the anti-hemolytic effect of the test substance. The 90 min-exposure time was found to consistently cause more than 90% hemolysis in all the control groups. The UV irradiation period of 120 min was also evaluated but the extent of hemolysis did not change significantly or only slightly increased. Thus, the irradiation time was fixed at 90 min in all subsequent studies. Table 18 shows average percentage of hemolysis before and after induction with UV radiation for 90 min, with and without antioxidative agents.

It is interesting to note that at time zero, when all the samples including controls had not yet been irradiated, a small amount of hemolysis was also observed. The extent of initial hemolysis ranged between 0.88% and 7.94% (Table 18). This pre-UV exposure hemolysis may have occurred from the damage of erythrocytes during sample preparation which involved pipetting, mixing and centrifugation. As such, deduction of this amount from the value obtained at 90 min was necessary for each sample to correct for any interference from the non-UV induced hemolysis at time zero. The acquired results concerning absorbance values and the percentage of hemolysis before and after UV-irradiation are provided in Appendix D for the individual antioxidants.

Table 19 shows the average value of percent hemolysis for each antioxidant after correction for the non-UV induced hemolysis. (The individual raw data are shown in

Appendix D). It can be seen that the extent of hemolysis was still above 80% in all the control groups (no antioxidants) even after correction.

Table 18. Comparison of percent hemolysis before and after UV irradiation obtained from samples with and without antioxidants (mean ± SD, n = 3)

Sample	Conc (μg/ml)	% Hemolysis (Mean ± SD)	
		Before UV (0 min)	After UV (90 min)
1) Puag-Haad	0	5.83 ± 1.03	90.06 ± 4.60
	200	1.40 ± 0.66	61.15 ± 0.67
	400	7.94 ± 0.19	30.88 ± 0.93
	600	0.88 ± 1.05	13.48 ± 6.05
2) oxyresveratrol	0	3.50 ± 0.49	91.54 ± 0.82
	200	2.97 ± 0.47	51.35 ± 2.27
	400	3.23 ± 0.96	35.05 ± 2.46
	600	5.37 ± 0.30	13.34 ± 1.91
3) Trolox®	0	6.39 ± 1.63	96.74 ± 1.10
	200	4.80 ± 0.63	97.12 ± 1.55
	400	6.32 ± 0.54	97.02 ± 1.29
	600	6.13 ± 1.51	94.58 ± 1.67
4) l-ascorbic acid	0	4.40 ± 0.75	96.60 ± 3.06
	200	5.76 ± 2.86	92.73 ± 1.88
	400	6.57 ± 1.41	92.37 ± 2.11
	600	4.34 ± 0.57	92.46 ± 2.85
5) EGCG	0	5.02 ± 0.30	95.26 ± 1.05
	200	3.41 ± 0.75	90.98 ± 5.28
	400	3.37 ± 0.53	87.72 ± 1.25
	600	3.76 ± 1.79	71.46 ± 6.89
6) pine bark extract	0	6.41 ± 0.47	97.88 ± 2.67
	200	3.69 ± 0.78	42.97 ±1.13
	400	6.51 ± 1.45	32.02 ± 0.33
	600	1.33 ± 0.31	7.00 ± 1.12

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Table 19. Percent hemolysis at 90 min after correction for the non-UV induced hemolysis (mean ± SD, n = 3)

Conc	% Hemolysis (Mean ± SD)					
(μg/ml)	Puag-Haad	oxyresveratrol	Trolox®	l-ascorbic acid	EGCG	pine bark extract
0 (control)	84.23 ± 4.60	88.04 ± 0.82	90.35 ± 1.10	92.20 ± 3.06	90.24 ± 1.05	91.46 ± 2.67
200	59.75 ± 0.67	48.38 ± 2.27	92.32 ± 1.55	86.97 ± 1.88	87.57 ± 5.28	39.28 ± 1.13
400	22.94 ± 0.93	31.82 ± 2.46	90.70 ± 1.29	85.81 ± 2.11	84.34 ± 1.25	25.51 ± 0.33
600	12.60 ± 6.05	7.96 ± 1.91	88.45 ± 1.67	88.12 ± 2.85	67.70 ± 6.89	5.67 ± 1.12

Table 20. Comparison of percent relative hemolysis at 90 min among the six antioxidants. The data are normalized in relation to the individual antioxidant's control group (mean ± SD, n = 3)

Conc	% Relative hemolysis (Mean ± SD)					
(μg/ml)	Puag-Haad	Oxyresveratrol	Trolox®	l-ascorbic acid	EGCG	pine bark extract
0 (control)	100.00 ± 5.46	100.00 ± 0.93	100.00 ± 1.22	100.00 ± 3.32	100.00 ± 1.16	100.00 ± 2.92
200	70.93 ± 0.79	54.95 ± 2.58	102.18 ± 1.72	94.32 ± 2.04	97.04 ± 5.85	42.95 ± 1.24
400	27.24 ± 1.11	36.14 ± 2.80	100.39 ± 1.43	93.07 ± 2.29	93.46 ± 1.38	27.89 ± 0.36
600	14.96 ± 7.18	9.05 ± 2.17	97.89 ± 1.85	95.57 ± 3.10	75.02 ± 7.64	6.20 ± 1.23

Figure 38 illustrates the percentage hemolysis taken from the data in Table 19, which were plotted as bar graphs to compare the concentration effect on the individual antioxidants. It can be clearly seen from this figure and Table 19 that addition of Puag-Haad, oxyresveratrol, EGCG and pine bark extract to the red blood cells could decrease UV-induced hemolysis. This effect was also concentration-dependent as the hemolysis

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extent was found to decrease with increasing concentration in the range studied, which was 200-600 μg/ml. On the other hand, Trolox® and l-ascorbic acid could not decrease hemolysis in the same concentration range as the extent of hemolysis of the two vitamins barely differed from their respective control groups (0 μg/ml).

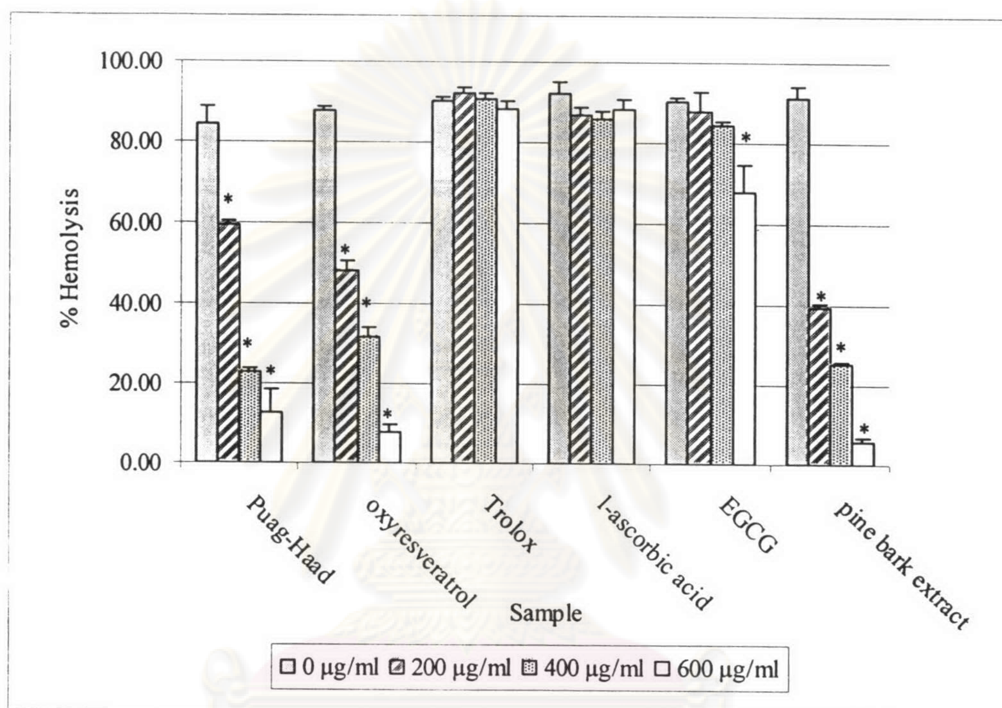

Figure 38. Percent hemolysis of the six antioxidants at various concentrations (mean ± SD, n=3)

* significantly different from the corresponding antioxidant's control group ($P < 0.05$)

Since the hemolysis extents of the individual antioxidants' control groups were not exactly the same due to biological variation (Table 19), it was necessary to normalize the values obtained for each antioxidant in relation to its own control in order to make a valid comparison among different antioxidants. The percent relative hemolysis was thus calculated for each concentration of the individual antioxidant using the following equation:

$$\% \text{ Relative hemolysis} = \frac{A}{B} \times 100\ \%$$

Where A = Percent hemolysis in the presence of antioxidant

B = Percent hemolysis of control (no antioxidant)

The average values of percent relative hemolysis and the individual data are tabulated in Table 20 and Appendix D, respectively.

The values of percent relative hemolysis were calculated for the convenience of statistical comparison in terms of concentrations and types of antioxidant effects. The data were also plotted as bar graphs as shown in Figure 39. The graph patterns were quite similar to those of Figure 36 with the exception that the values were now normalized in relation to the individual antioxidant's own control, which was considered to have 100% hemolysis.

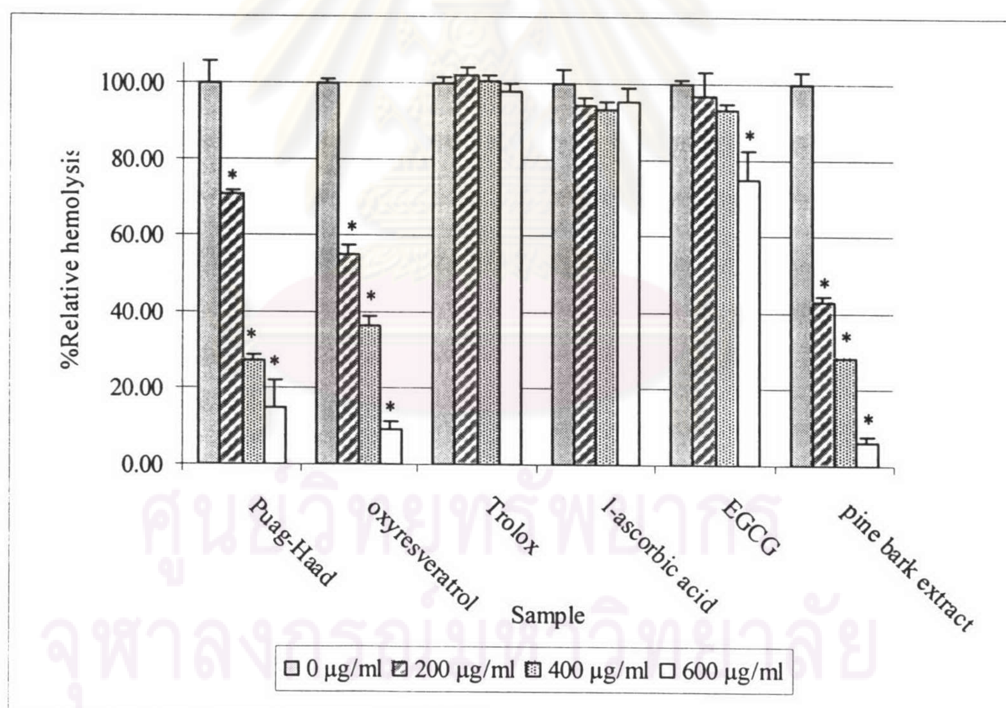

Figure 39. Percent relative hemolysis of the six antioxidants at various concentrations (mean ± SD, n=3)

* significantly different from control group ($P < 0.05$)

The percent hemolysis values both before (Table 19) and after normalization (% relative, Table 20) were then evaluated for each antioxidant using one-way ANOVA at 5% significant level in order to assess the effect of varying antioxidant concentration on hemolysis. The results show that Trolox® and l-ascorbic acid could not protect erythrocytes from hemolysis at all three concentrations studied since the hemolysis values obtained from the test samples and the control were not significantly different ($P > 0.05$). On the contrary, the other test antioxidants demonstrated significant concentration effect on hemolysis ($P < 0.05$). Tukey's multiple comparison was further applied to rank the effect of each antioxidant's concentration. The test results showed that Puag-Haad, oxyresveratrol and pine bark extract had a protective effect against UV-induced hemolysis in a concentration-dependent manner, in which the anti-hemolytic effect increased significantly from 0 to 200, 400 and 600 μg/ml, respectively ($P < 0.05$). Their protective effect was also maximum at 600 μg/ml, the highest concentration studied ($P < 0.05$), with the percent relative hemolysis decreasing from 100% to 14.96%, 9.05% and 6.20% for Puag-Haad, oxyresveratrol and pine bark extract, respectively. With respect to EGCG, the active ingredient in green tea leaves, Tukey's test result showed that only the highest concentration (600 μg/ml) was effective ($P < 0.05$), with percent relative hemolysis decreased from 100% to 75.02% (Table 20). ANOVA and Tukey's test results of the concentration effect on the percent hemolysis are shown in Appendix D. The statistical results were the same regardless of whether the non-normalized or the relative hemolysis data were used.

Subsequently, the four antioxidants (Puag-Haad, oxyresveratrol, EGCG and pine bark extract) which demonstrated significant protective effect against hemolysis were compared together using one-way ANOVA at the same effective concentration in order to distinguish the most effective antioxidant in terms of singlet oxygen scavenging/anti-hemolytic activity. The comparison was made at 600 μg/ml, which was the concentration that all four antioxidants gave their highest anti-hemolytic effect. Significant difference among groups was found ($P < 0.05$) and Tukey's test was applied to rank the anti-hemolytic effects (% relative hemolysis) as follows:

pine bark extract ~ oxyresveratrol ~ Puag-Haad < EGCG
6.20 9.05 14.96 75.02 %

Thus, pine bark extract, oxyresveratrol and Puag-Haad apparently demonstrated comparable protective effect against UV-induced hemolysis ($P > 0.05$), whereas EGCG demonstrated significantly lower anti-hemolytic activity ($P < 0.05$). For l-ascorbic acid and Trolox® the protective effect against hemolysis could not be observed, where the high and indifferent hemolysis values were obtained in both the samples and control groups. Appendix D also provides detailed statistical results of the anti-hemolytic effects of the four antioxidants at 600 μg/ml.

Additionally, the IC_{50} values to cause 50% reduction in hemolysis relative to the control were also calculated for the individual antioxidants in terms of both μg/ml and mM units as shown in Table 21. Transforming the value from mg/ml in to mM was conducted by the following equation.

$$mM = \frac{(\mu g/ml) \times \text{purity } (\%w/w) \times 1000 \times 1000}{\text{molecular weight} \times 100}$$

Table 21. The IC_{50} values for hemolysis inhibition of each antioxidant (mean±SD, n = 3)

Sample	IC_{50} (μg/ml)	IC_{50} (mM)
Puag-Haad	284.28	0.88
oxyresveratrol	257.53	1.00
pine bark extract	197.07	-
EGCG	802.32	1.73

The IC_{50} values were estimated from polynomial regression of the curve of the percentage relative inhibition and the antioxidant concentration. The calculated values were found to be in agreement with the visually observed values from the curve. It can be seen that the IC_{50} values in μg/ml obtained from Puag-Haad, oxyresveratrol and pine bark extract were similar, where the difference between Puag-Haad and pine bark extract was not more than 1.44 folds. On the other hand, the very high IC_{50} value of EGCG

(802.32 μg/ml) indicated that it was least effective among the four antioxidants tested, i.e., it was 4.07-, 2.82- and 3.12-fold less effective than pine bark extract, Puag-Haad and oxyresveratrol, respectively.

A closer examination of the data in Table 21 revealed that Puag-Haad had a slightly higher IC_{50} value (284.28 μg/ml) than oxyresveratrol (257.53 μg/ml) indicating that the pure compound could be the major component responsible for the singlet oxygen scavenging activity. When the data were transformed into mM unit, the IC_{50} values of the extract and the pure compound became almost identical (0.88 versus 1.00 mM), which further strengthened the previous suggestion that oxyresveratrol was the major, if not sole, active singlet oxygen scavenger of the extract. EGCG still gave a relatively high IC_{50} value in mM (1.73) when compared to Puag-Haad and oxyresveratrol indicating that a high MW compound like EGCG was still less effective than Puag-Haad and its active component. On the other hand, the IC_{50} in mM could not be determined for the pine bark extract since it contained too many active constituents and thus comparison on a molar basis could not be made.

Trolox® and l-ascorbic acid showed no protective effect against UV-induced hemolysis at all within the concentration range evaluated. The protective or anti-hemolytic effect evaluated in this study indicated the ability of an antioxidant to scavenge singlet oxygen, which is responsible for damage of erythrocyte membrane via lipid peroxidation. Thus, it is possible that the protective effects of the test antioxidants on lipid peroxidation observed in this study may as well be observed in other cell types because mammalian and human cell membranes also contain phospholipids as a major component. Erythrocytes were used in this study as an experimental model because their analysis was relatively simple, i.e., when the cells are destroyed (known as hemolysis), the red color of hemoglobin which is released into the supernatant can be easily detected by a visible spectrophotometer.

As demonstrated by this method, UV irradiation of a photosensitizer such as hematoporphyrin can generate singlet oxygen, which in turn, causes lipid peroxidation of the red blood cell membrane leading to hemolysis (Bibincova et al. 2001). Pine bark extract, which contains proanthocyanidins, was found to be a potent singlet oxygen scavenger or inhibitor in this study. It inhibited the hemolysis process to a much greater

extent than EGCG. Similar effect was also reported which showed that an extract of pine bark consisting of a mixture of flavonoids, mainly procyanidins and phenolic acid, displayed greater biological effects as a mixture than its individual, purified components, thereby indicating that the multi-components could interact synergistically (Facino, 1998; Carini, et al., 2000; Bergman, et al., 2003). Carini et al. (2000), using a gamma-irradiated liposome model, suggested this inhibitory effect of procyanidins was due to inhibition of lipid peroxidation and site-specific phospholipid interaction. It is a complexation mechanism, through electrostatic interactions between the nucleoplilic phenol groups of oligomeric catechins and the cationic polar heads of phospholipids in the membrane, that stabilized it against damage caused by free radicals, in this case the lipid peroxyl radicals generated from gamma-irradiation of the liposomes.

Similar to the pine bark extract, this study found that Puag-Haad and oxyresveratrol can provide a strong protective effect against hemolysis. The activity could be a result of the polyphenolic nature of oxyresveratrol (2, 4, 3′, 5′-tetrahydroxystilbene), which possesses several hydroxyl groups that somehow can readily formed by donation of phenolic hydrogens and subsequently react with singlet oxygen involved in lipid peroxidation (Haraguchi et al., 1998).

On the contrary, Trolox® and l-ascorbic acid failed to provide any protective effect or singlet oxygen scavenging activity, especially in the concentration range used in this study. Trolox® has been reported to increase fragility of the erythrocyte membrane in vitro under mechanical stress from ultrasound (Miller et al., 2003). They found that applying ultrasound in the presence of high concentration of Trolox® resulted in more damage to the erythrocyte membrane and subsequent hemolysis. They explained that ultrasound application could induce cavitation in the membrane and cause membranous antioxidant like Trolox® which was in excess to perturb the membrane integrity leading to increased fragility. Moreover, at high concentrations or under special condition, prooxidant effects of Trolox® and l-ascorbic acid have been widely discussed (Azzi and Stocker, 2000). Especially for Trolox®, some reports demonstrated that alpha-tocopherol accelerated lipid auto-oxidation in vitro and may act as an endogenous photosensitizer enhancieng light-induced oxidative damage (Jacob and Burri, 1996; Konig and Ring; 2005). Inaddition, the photo-oxidation of Trolox® and l-ascorbic acid is another possible

cause of failing the anti-hemolysis test. UV irradiation can induce the decay of antioxidants, especially for vitamin E and l-ascorbic acid (Allwood and Martin, 2000; Tanino et al., 2005). The weak anti-hemolysis activity of both antioxidants may be due to the degradation via UV irradiation resulting in the lowest protection activity. However, further studies should be conducted to confirm this hypothesis.

Hence, several possible mechanisms may be involved in the current observation of l-ascorbic acid and Trolox® effect on hemolysis. For example, Trolox® and l-ascorbic acid may have acted as a prooxidant in the current study in which high concentrations (200 – 600 μg/ml) were used. High concentration was necessary in order to facilitate direct comparison of their effects with other antioxidants which showed significant anti-hemolytic effects at these concentrations.

Moreover, lipid peroxidation of erythrocyte membrane is a chain-reaction process which can be directly induced by other sources such as enzymatic reactions, ionizing irradiation, and UV light. In this method, apart from singlet oxygen, other radicals may also be produced and could have a deleterious effect on the membrane as well. Various antioxidants can inhibit lipid peroxidation at different levels. Vitamin C and vitamin E have been reported to show the lowest lipid peroxyl radical scavenging activity based on the gamma-irradiated liposome model, while a stilbene derivative like resveratrol, which is a trihydroxystilbene, was able to inhibit lipid peroxidation to the same extent as epicatechin and flavonoid in agreement with the results of this study (Stojanovic, Sprinz, and Brede, 2001). However, some reports were different from Stojanovic et al. (2001) depending on the models being used (Frankel, Waterhouse, and Kinsella, 1993; Belguendouz, Fremont, and Linard, 1997; Sanchez-Moreno, Larrauri, and Calixto, 1999; Fremont, 2000). Frankel et al.(1993) reported that epicatechin can inhibit copper-catalyzed peroxidation of human low-density LDL twice better than resveratrol. On ther other hand, reports of Fremont et al. (1999) and Sanchez-Moreno et al. (1999) showed that resveratrol inhibited copper-catalyzed peroxidation of LDL much better than epicatechin. So, controversy among results still exist in the literature and more studies are inevitably requied to clarity the real mechanisms of these antioxidants.