CHAPTER IV

RESULTS AND DISCUSSION

Part 1. Preparation of crude extracts from fruit juice of *Phyllanthus emblica* locally grown in Thailand

Extraction of natural plants can be done by various methods depending on the polarity of active constituents to be extracted. Many solvents can be used for extraction such as hexane, ethyl acetate, acetone, butanol, ethanol, methanol and water. In this study, ethyl acetate, acetone and ethanol were used as extracting solvents for *P. emblica*. The ethyl acetate extracts of *P. emblica* had been reported to exhibit the highest DPPH scavenging activity compared with the hexane and methanol extracts (Mahattanapokai, 2004). Acetone and ethanol had also been used to isolate hydrolysable tannins of *P. emblica* by Ghosal (1996) and these extracts were found to exhibit strong protective action on erythrocytes against asbestos fibre-induced oxidative stress. Therefore, the spray-dried powder of *P. emblica* was investigated by extraction with these three solvents to compare the type of solvent and the extraction process on the extract's antioxidant, anti-collagenase and anti-tyrosinase activities. In this part of the study, the spray-dried powder of *P. emblica* (50 g) was extracted successively by solvents with different polarities starting from ethyl acetate, acetone and followed by ethanol using Soxhlet extractor. Moreover, the spray-dried powder (15 g) was also directly extracted by individual solvents (acetone and ethanol). The extracted solutions were concentrated by rotary evaporator to give five fractions of *P. emblica* extract including ethyl acetate, acetone (successive), ethanol (sucessive), acetone (direct) and ethanol (direct) extracts. The extraction condition and the yield of these five fractions are shown in Table 9. The pictures of Sohxlet extractor and evaporator are shown in Figure 16. The ethyl acetate extract had dark-brown color and gave 4.96 % w/w yield. Acetone (direct) and acetone (successive) extracts had yellow-brown color and gave 9.79 % and 10.38 % w/w yield, respectively. Ethanol (direct) and ethanol (sucessive) extracts had brown color and gave 14.00 % and 14.32 % w/w yield. The picture of spray-dried powder and five fractions of *P. emblica* are respectively shown in Figures 17 and 18. Although the

extraction procedure was different (successive or direct extraction), the yield results were similar if the same solvent was used. For example, both the successive and direct ethanol extracts gave the highest yield. This was followed by the acetone (successive and direct) and the ethyl acetate extracts. This indicated that the spray-dried powder of *P. emblica* the higher contents of the more polar components than the less polar ones.

Table 9. The extraction of spray-dried powder of *P. emblica*

Process	Ethyl acetate	Acetone (successive)	Ethanol (successive)	Acetone (direct)	Ethanol (direct)
1. Extraction					
Spray-dried *P.emblica* (g)	50	47.51*	40.62**	15	15
Volume of extraction (ml)	300	300	300	300	300
Time (min)	318	395	670	274	495
2.Concentration (rotary evaporator)					
Temperature (°C)	50	50	50	50	50
Rotate (cycle/sec)	2-4	2-4	2-4	2-4	2-4
Time (min)	30	45	60	30	30
3. Crude extract weight (g)	2.4804	4.9293	5.8150	1.468	2.1001
4. % yield	4.96	10.38	14.32	9.79	14.00

* weight of ethyl acetate insoluble solid

** weight of acetone insoluble solid

Figure 16. Soxhlet extractor and evaporator

Figure 17. Spray-dried *P. emblica* powder

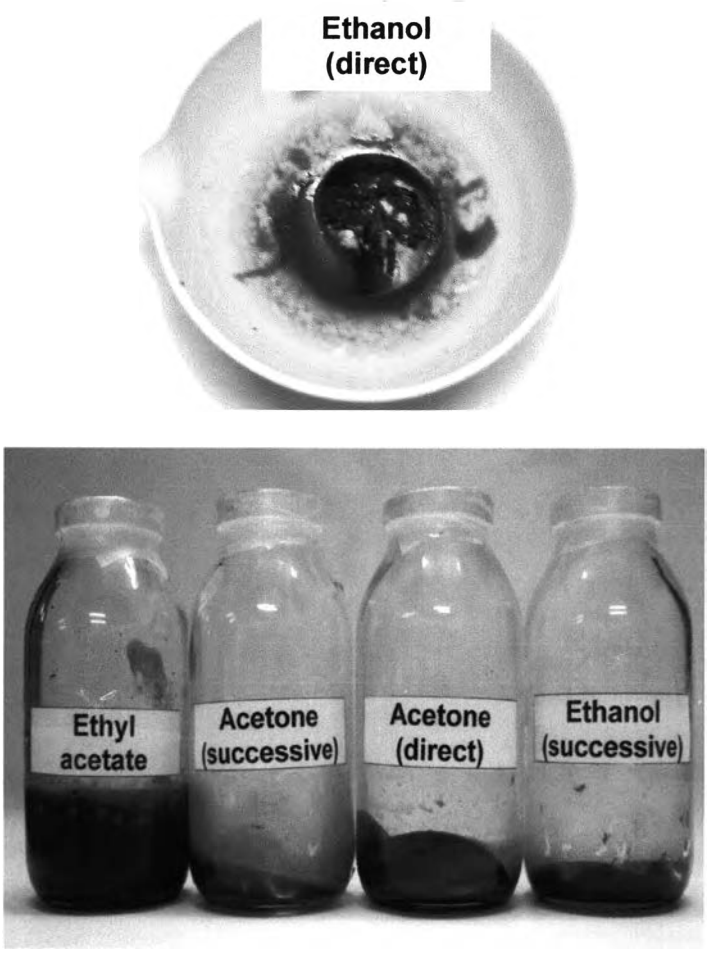

Figure 18. Five fractions of *P. emblica*: ethyl acetate, acetone (successive), acetone (direct), ethanol (successive) and ethanol (direct) extracts.

Part 2. Evaluation of different *Phyllanthus emblica* extracts for antioxidant activities

2.1. Hydrogen-donating activity (DPPH radical scavenging activity)

The role of an antioxidant is to remove free radicals. One mechanism through which this is achieved is by donating hydrogen to a free radical in its reduction to a non-reactive species. Addition of hydrogen would remove the odd electron feature which is responsible for radical reactivity. The hydrogen-donating activity of spray-dried *P. emblica*, five solvent extracts from the spray-dried *P. emblica* as well as a commercial *P. emblica* extract, l-ascorbic acid, Trolox® and ECGC was determined in terms of their ability to scavenge the DPPH stable free radical, as a hydrogen acceptor. The degree of DPPH discoloration is attributed to the hydrogen donating ability of the test compounds which is indicative of their scavenging potential (Negi et al., 2005). The change in color of the free radical DPPH absorbance, measured at 517 mn, was evaluated by comparison with a control sample without hydrogen-donating compounds. The result indicated that all the various solvent extracts of *P. emblica*, commercial *P. emblica* product, l-ascorbic acid, Trolox® and EGCG possessed a varying degree of free radical scavenging activity. The plots of percentages of DPPH scavenging activity (% inhibition of free radical) at various concentrations (0, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 20.0, 50.0 and 100.0 µg/mL) of the test samples are shown in Figure 19. It was found that EGCG exhibited the highest DPPH scavenging activity followed by l-ascorbic acid, Trolox® and the *P. emblica* extracts. The spray-dried *P. emblica* possessed the DPPH radical inhibition (H-donating activity) quite similar to the commercial *P. emblica* extract. However, both the spray-dried and the commercial *P. emblica* extracts exhibited lower inhibition when compared with the acetone and the ethanol extracts. The ethyl acetate extract showed the lowest scavenging activity on DPPH as seen in Figure 19 and Table 10.

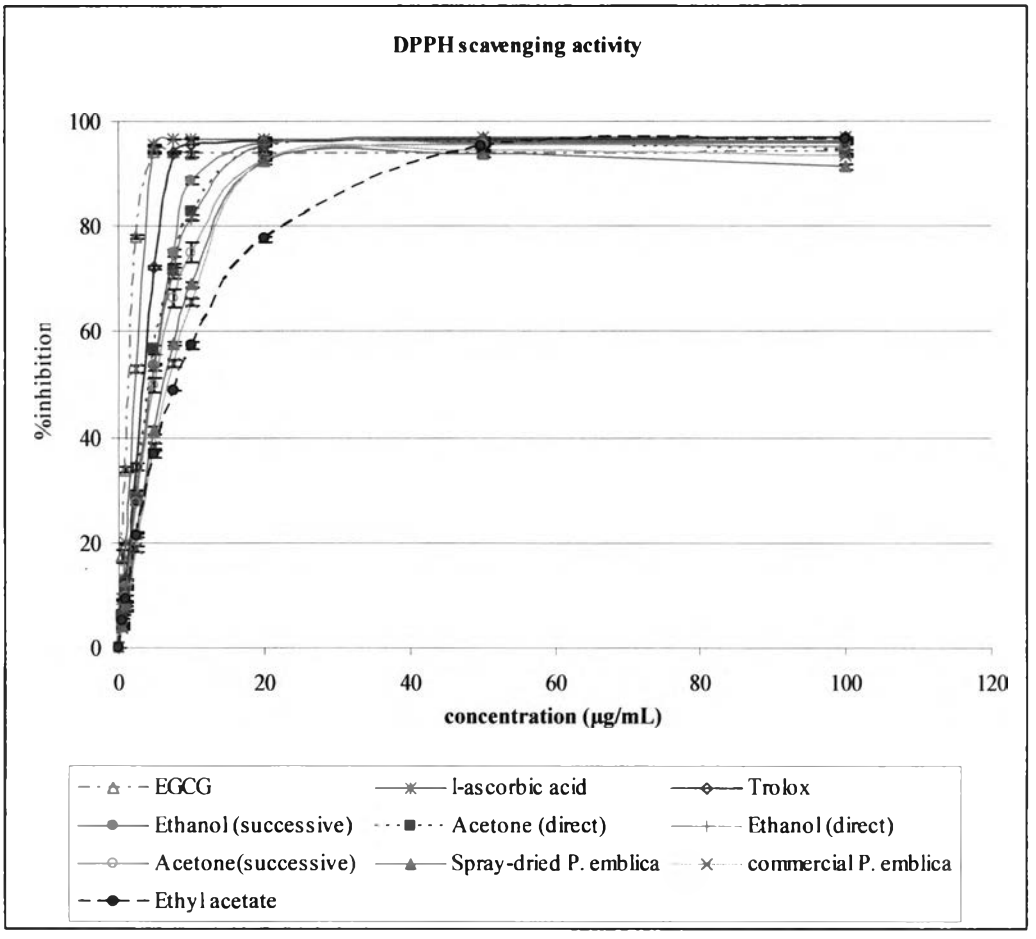

Figure 19. DPPH radical inhibition of various solvent extracts of *P. emblica* compared to commercial *P. emblica* extract and other antioxidants at different concentrations (Mean ± SD, n = 3).

It was found that as the concentration was increased, the extent of DPPH inhibition also increased for all antioxidants until certain concentrations where the plots reached a plateau and the inhibition became steady. The clearly relationship between DPPH radical inhibition and the concentration of the individual antioxidants is shown in Figure 20 and Table 10. The percent DPPH radical inhibition increased to 92.45 ± 0.59, 92.69 ± 0.77, 95.26 ± 0.14, 95.72 ± 0.00, 95.54 ± 0.13 % at 20.0 µg/mL for spray-dried *P. emblica* and acetone (successive), acetone (direct), ethanol (successive) and ethanol (direct) extracts of *P. emblica*, respectively, then reached a plateau between 50.0 and 100.0 µg/mL. Likewise, the commercial *P. emblica* extract also showed the increase in percent DPPH radical inhibition to 92.71 ± 0.14 at 20.0

μg/mL concentration and then reached a plateau between 50.0 and 100.0 μg/mL. On the other hand, the percent DPPH radical inhibition of the ethyl acetate extract at 20.0 μg/mL was only 77.54 ± 0.45% and increased to 95.06 ± 0.21 at 50.0 μg/mL and remained relatively unchanged up to 100.0 μg/mL, where the percent inhibition was 96.49 ± 0.12%.

The antioxidant activity against DPPH radical of Trolox® reached a plateau at lower concentration than that of *P. emblica* extracts. It exhibited the increase in percent DPPH radical scavenging activity to 93.91± 0.25% at 7.50 μg/mL and the inhibition remained unaffected by the increase in concentration to 100.0 μg/mL, with the values in the range of 95.48-96.77%.

On the other hand, l-ascorbic acid and EGCG reached a plateau of scavenging activity at lower concentration (5.0 μg/mL) than other antioxidants, with the inhibition values of 95.38 ± 0.25% and 94.00 ± 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 96.52-96.80% and 94.00-94.15%, respectively. The raw data for percent DPPH radical inhibition are provided in Appendix A.

Additionally, the DPPH radical inhibition profiles of the same solvent extract including acetone (direct) compared with acetone (successive) and ethanol (direct) compared with ethanol (successive) were nearly identical. This could be due to the presence of similar major components that might have been extracted by the same solvent, thereby making the extracts possessing similar DPPH scavenging activity. In addition, the other components that might have been co-extracted (the contaminating substances) may have a little scavenging effect on DPPH.

Table 10. DPPH radical inhibition of various solvent extracts of *P. emblica* compared to other antioxidants at different concentrations (Mean ± SD, n= 3).

No	Conc.	Spray-dried *P. emblica*		Ethyl acetate		Acetone (successive)		Acetone (direct)		Ethanol (successive)		Ethanol (direct)		Commercial *P. emblica*		Trolox®		l-ascorbic acid		EGCG	
	(µg/mL)	%inh	SD	%inh	SD	%inh	SD	%inh	SD	%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	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.50	4.28	0.13	5.01	0.33	4.48	0.17	5.72	1.16	6.28	0.25	5.99	0.33	3.69	0.14	6.95	0.25	9.17	0.21	17.49	1.18
3	1.00	8.33	0.36	9.44	0.43	9.75	1.18	10.78	0.65	11.63	0.25	11.84	0.22	7.05	0.14	12.97	0.12	19.83	0.21	33.83	0.37
4	2.50	21.73	0.23	21.17	0.33	27.39	0.17	28.27	1.13	28.34	0.45	29.61	0.22	18.75	0.42	34.34	0.66	52.88	0.64	77.87	0.33
5	5.00	41.20	0.75	36.77	0.69	49.71	1.34	56.54	0.99	53.68	0.12	53.29	0.58	38.22	0.48	72.19	0.33	95.38	0.25	94.00	0.21
6	7.50	57.71	0.23	48.78	0.12	66.37	1.78	71.49	1.50	75.02	0.65	71.49	0.76	53.85	0.64	93.91	0.25	96.52	0.12	94.15	0.12
7	10.00	68.93	0.47	57.51	0.64	74.95	1.88	82.84	0.65	88.65	0.74	81.51	0.46	65.54	0.69	95.48	0.00	96.66	0.12	93.65	0.49
8	20.00	92.45	0.59	77.54	0.45	92.69	0.77	95.26	0.14	95.72	0.00	95.54	0.13	92.71	0.14	96.34	0.00	96.73	0.12	93.93	0.25
9	50.00	93.69	0.47	95.06	0.21	95.42	0.17	95.92	0.14	96.22	0.12	95.98	0.13	94.47	0.00	96.70	0.12	96.80	0.00	93.93	0.25
10	100.00	91.28	0.59	96.49	0.12	95.03	0.51	94.61	0.00	96.29	0.12	95.91	0.13	93.43	0.28	96.77	0.00	96.80	0.00	94.00	0.37

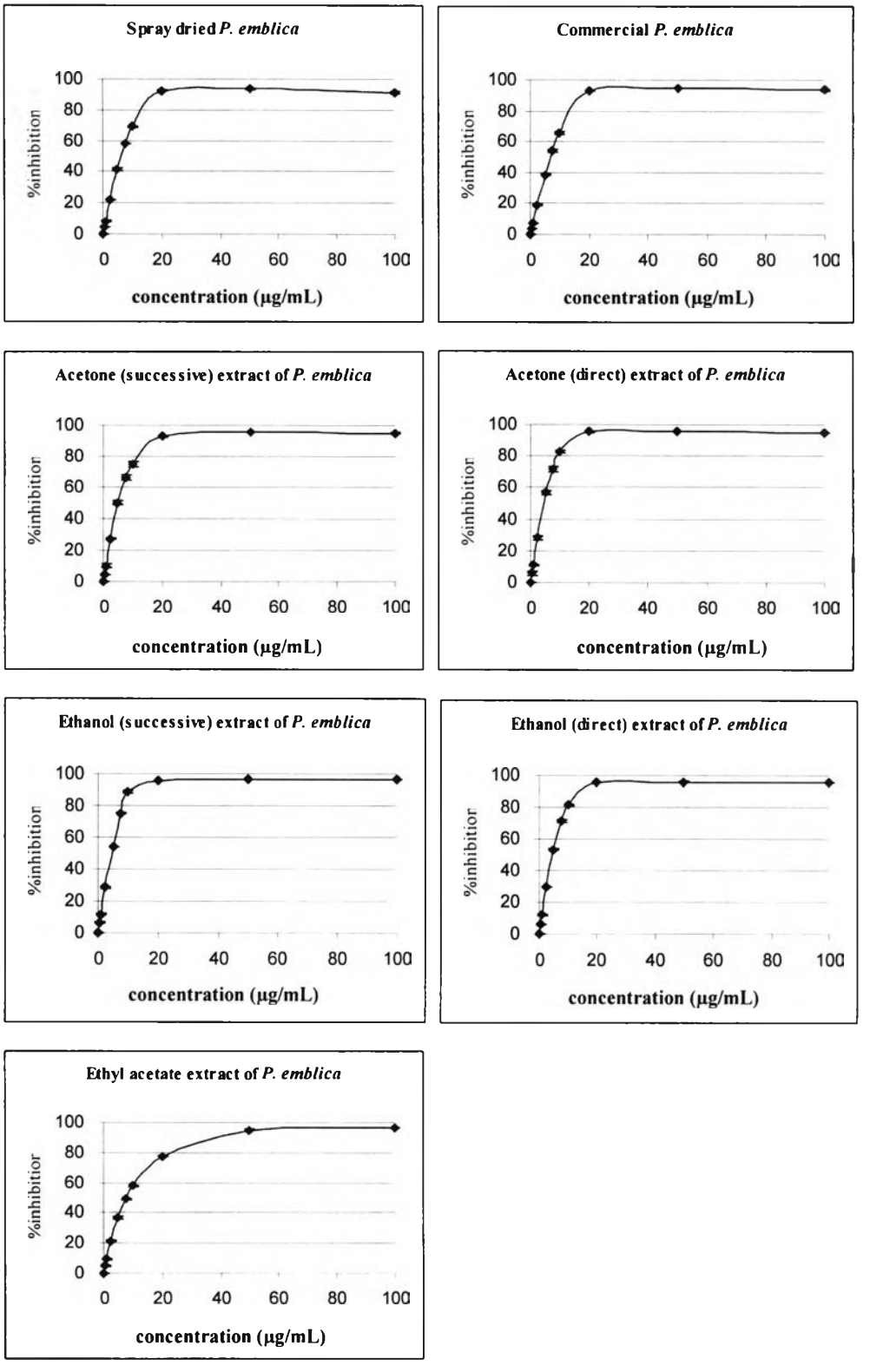

Figure 20. Relationship between percent DPPH radical inhibition and the concentration of the individual antioxidants (Mean ± SD, n = 3).

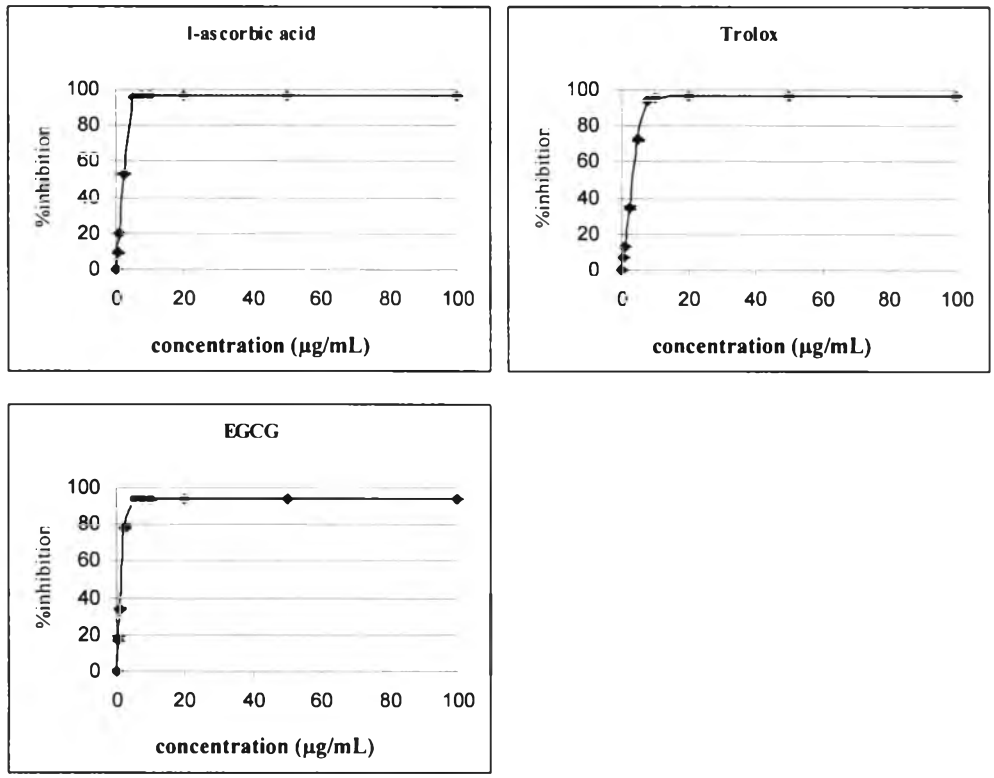

Figure 20 (cont.). Relationship between percent DPPH radical inhibition and the concentration of the individual antioxidants (Mean ± SD, n = 3).

The concentration at 50% inhibition (IC_{50}) of each sample was calculated from the equation of the partial polynomial plot and given in Figure 21 and Table 11. The estimated IC_{50} can be ranked from the lowest to highest, as follows: EGCG (1.52 µg/mL ± 0.02), l-ascorbic acid (2.37 µg/mL ± 0.03), Trolox® (3.57 µg/mL ± 0.03), acetone (direct) extract (4.43 µg/mL ± 0.07), ethanol (direct) extract (4.62 µg/mL ± 0.03), ethanol (successive) extract (4.63 µg/mL ± 0.02), acetone (successive) extract (5.00 µg/mL ± 0.15), spray-dried *P. emblica* (6.29 µg/mL ± 0.07), commercial *P. emblica* (6.87 µg/mL ± 0.06), ethyl acetate extract (7.74 µg/mL ± 0.11). Since the lower IC_{50} values give the greater scavenging potency of the antioxidant, therefore, EGCG presented the highest DPPH radical scavenging activity, followed by l-ascorbic acid and Trolox®. This result has also been observed by Gerhauser et al. (2003) who reported that the IC_{50} for DPPH scavenging activity of EGCG (3.4 µM) < l-ascorbic acid (8.5 µM) < Trolox® (9.7 µM), similar to the ranking of the data from this study (IC_{50} of EGCG = 3.3 µM, l-ascorbic acid = 13.5 µM, Trolox® = 14.3 µM).

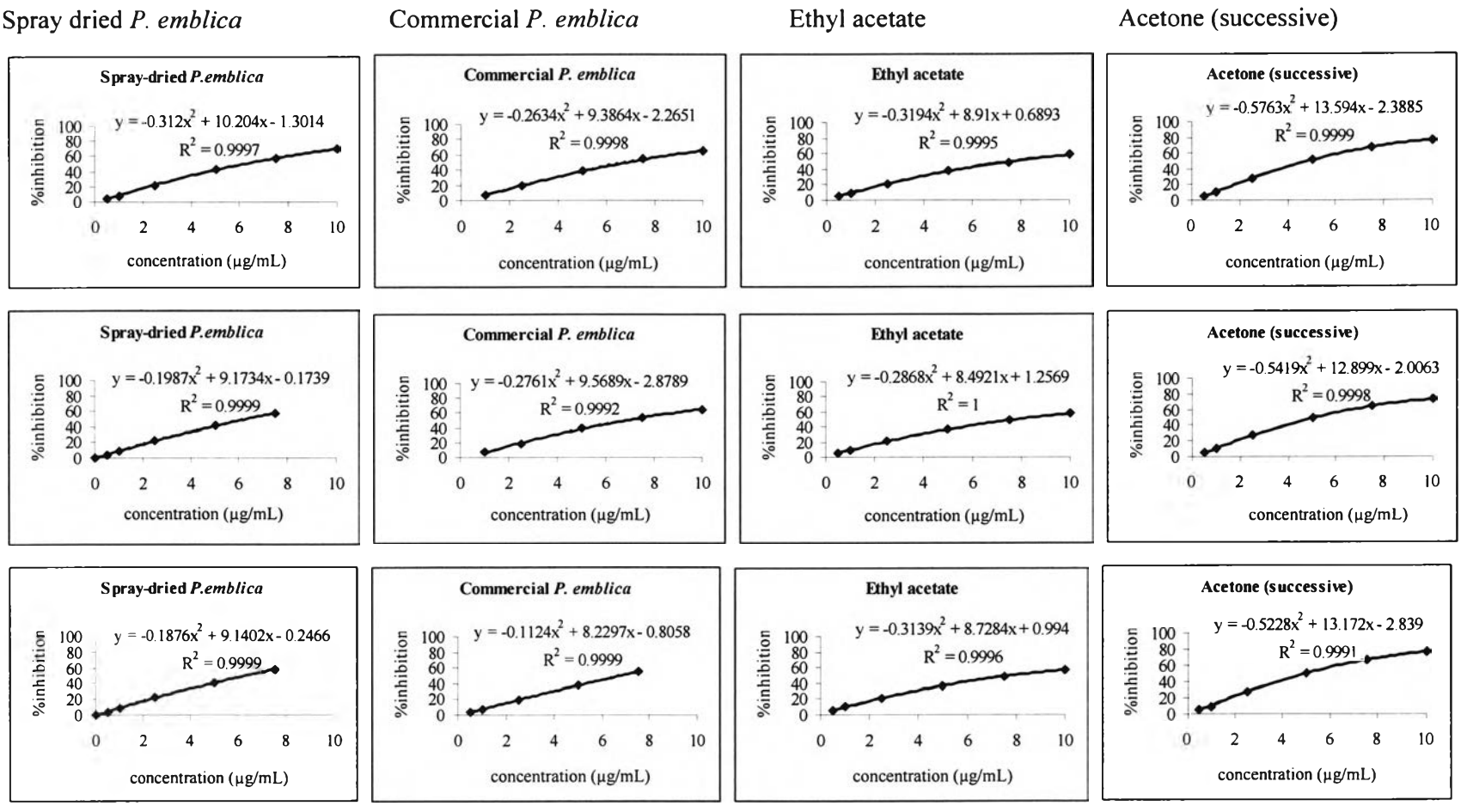

Figure 21. The relation of the %DPPH inhibition-concentration profile of the individual antioxidants. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants.

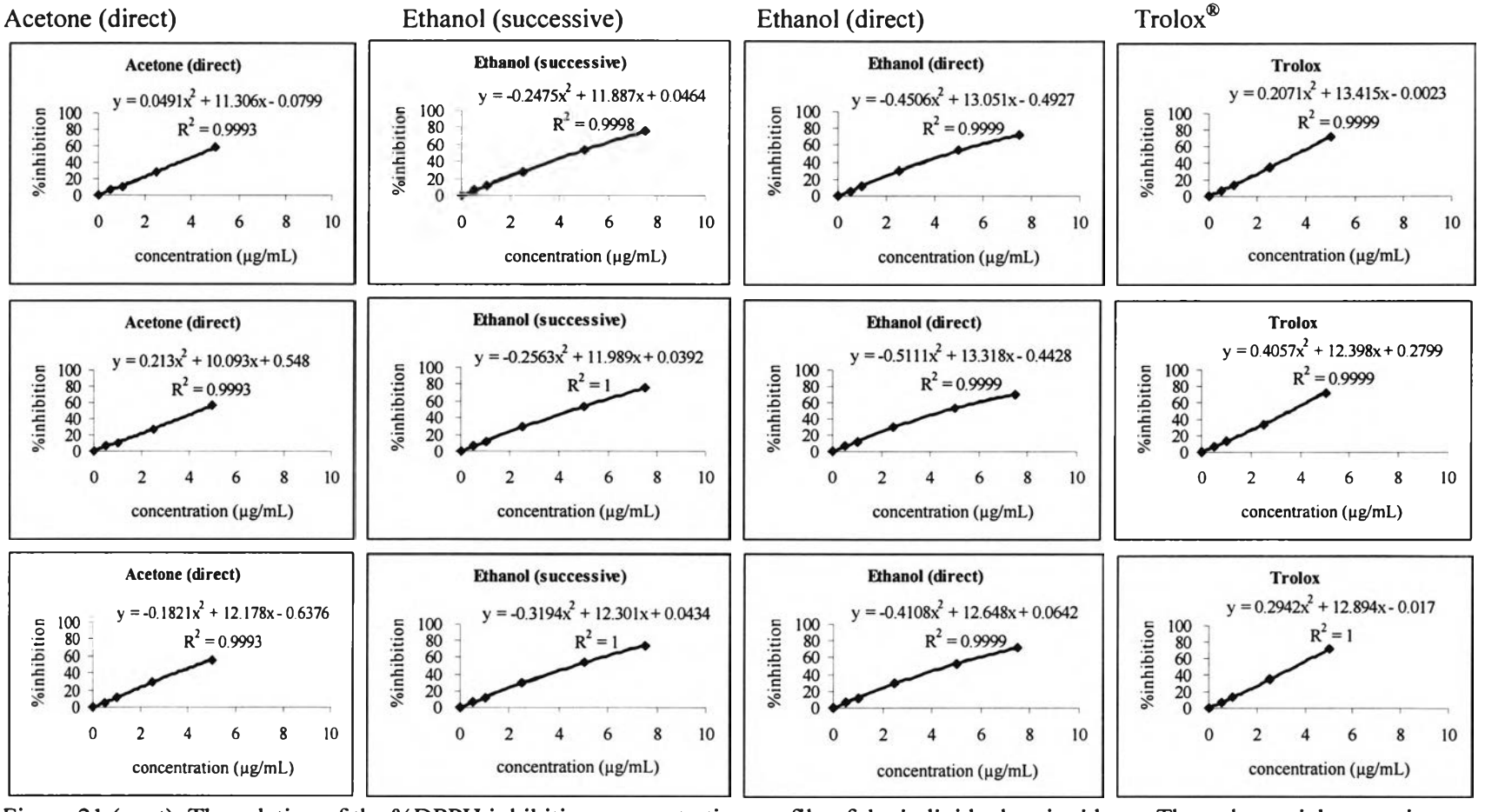

Figure 21 (cont). The relation of the %DPPH inhibition-concentration profile of the individual antioxidants. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants.

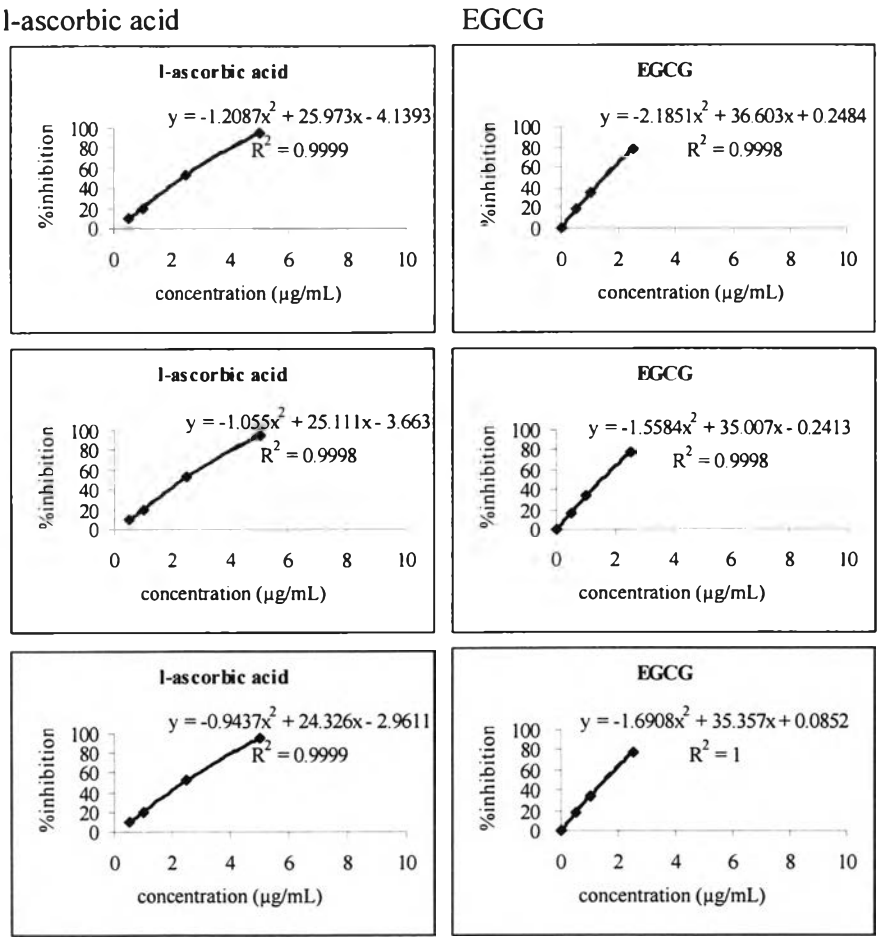

Figure 21 (cont). The relation of the %DPPH inhibition-concentration profile of the individual antioxidants. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants.

Table 11. The IC_{50} values for DPPH radical scavenging activity of each antioxidant. The R^2 is the regression coefficient obtained from polynomial regression of the rising portion of the plot between the inhibition percentage and the concentration of each antioxidant (Mean ± SD, n = 3).

Sample	Polynomial equation (partial)					
	IC_{50} (μg/mL)	Mean (μg/mL)	SD	R^2	Mean	SD
EGCG	1.49	1.52	0.02	0.9998	0.9999	0.00
	1.54			0.9998		
	1.52			1.0000		
l-ascorbic acid	2.34	2.37	0.03	0.9999	0.9999	0.00
	2.37			0.9998		
	2.40			0.9999		
Trolox®	3.53	3.57	0.03	0.9999	0.9999	0.00
	3.59			0.9999		
	3.59			1.0000		
Acetone (direct)	4.35	4.43	0.07	0.9993	0.9993	0.00
	4.48			0.9993		
	4.45			0.9993		
Ethanol (direct)	4.60	4.62	0.03	0.9999	0.9999	0.00
	4.60			0.9999		
	4.65			0.9999		
Ethanol (successive)	4.65	4.63	0.02	0.9998	0.9999	0.00
	4.62			1.0000		
	4.61			1.0000		
Acetone (successive)	4.85	5.00	0.15	0.9999	0.9996	0.00
	5.14			0.9998		
	5.01			0.9991		
Spray-dried *P. emblica*	6.20	6.29	0.07	0.9997	0.9998	0.00
	6.34			0.9999		
	6.32			0.9999		
Commercial *P. emblica*	6.91	6.87	0.06	0.9998	0.9996	0.00
	6.90			0.9992		
	6.81			0.9999		
Ethyl acetate	7.61	7.74	0.11	0.9995	0.9997	0.00
	7.79			1.0000		
	7.81			0.9996		

From Figure 22, it can be seen that the DPPH scavenging activity of the acetone (direct) and the ethanol (direct and successive) were quite comparable in terms of their IC_{50} and that they apparently had greater activity than the spray-dried and the commercial *P. emblica* extracts as well as the ethyl acetate extract, which showed the least activity (highest IC_{50}). However, their activity was still less than Trolox®, l-ascorbic acid and EGCG.

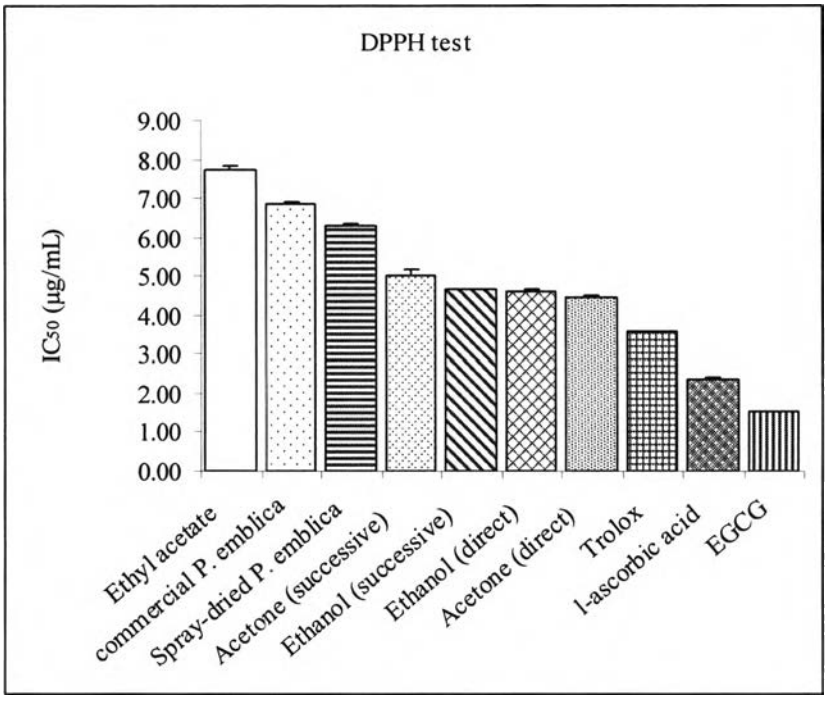

Figure 22. The IC_{50} (µg/mL) of each antioxidant for DPPH radical inhibition (Mean ± SD, n = 3).

Figure 22 also shows that EGCG give the lowest IC_{50} and thus possesses the strongest DPPH scavenging activity. L-ascorbic acid and Trolox® produce slightly higher IC_{50} values than EGCG, the IC_{50} being 1.56 and 2.35 folds higher than EGCG, respectively. Acetone (direct), ethanol (direct), ethanol (successive) and acetone (successive) extracts provided the nearly identical IC_{50} values, which are 2.91, 3.04, 3.05 and 3.29 times higher than EGCG. Their IC_{50} values are also higher than Trolox® and l-ascorbic acid. The IC_{50} of the spray-dried *P. emblica* is a little lower than that of the commercial *P. emblica* extract indicating that the locally grown fruits and their locally produced, spray-dried crude extract of the fruit juice is as effective as the

commercial extract in terms of DPPH scavenging activity, which were 4.14 and 4.52 times higher than EGCG, respectively. On the other hand, the IC_{50} value of ethyl acetate extract exhibited the highest value which was 5.09 folds higher than EGCG, indicating its weakest DPPH scavenging activity among the samples tested.

The IC_{50} data were subsequently analyzed by one-way analysis of variance (ANOVA) at 95% confidence followed by Tukey's test. It was found that there were significant differences among the antioxidants studies ($P < 0.05$). According to Tukey's test, the antioxidants could be divided into 9 different groups ($P < 0.05$) regarding their ability to inhibit or scavenge DPPH radicals. The IC_{50} ranking is as follows:

EGCG < l-ascorbic acid < Trolox® <

1.52 2.37 3.57 μg/mL

acetone (direct) extract ≈ ethanol (direct) extract ≈ ethanol (successive) extract <

4.43 4.62 4.63 μg/mL

acetone (successive)< spray-dried *P. emblica* < commercial *P. emblica* < ethyl acetate

5.00 6.29 6.87 7.74 μg/mL

The antioxidants joined by the same underline are within the same group and not statistically different in terms of their DPPH inhibition ($P > 0.05$). Thus, both direct and successive ethanol extracts of *P. emblica* provided the similar DPPH radical scavenging activity (H-donating property), whereas the acetone extracts of *P. emblica* exhibited significant difference between direct and successive processes. Although the statistical ranking showed that the IC_{50} of acetone (direct) was significant less than that of its successive counterpart, the difference was actually not much (4.43 versus 5.00 μg/mL) in terms of numerical values. Additionally, the DPPH radical scavenging activity of the ethanol (direct) extract exhibited no significant difference from the acetone (direct) extract. The overlapping activity of the ethanol (direct) extract indicated that both the ethanol extracts provided somewhat similar H-donating property on DPPH radical to the acetone (direct) solvent extract. The statistical results of the individual antioxidants against DPPH radical are shown in Appendix A. However, considering the graphical comparison among all test samples

in Figure 22, the four extracts (acetone (direct), acetone (successive), ethanol (direct) and ethanol (successive) extracts can be visually regarded as having relatively similar DPPH scavenging activity.

For the comparison with the commercial *P. emblica* extract, all of the various *P. emblica* extracts obtained here gave higher DPPH scavenging activity (lower IC_{50} values) than the commercial *P. emblica* product, except the ethyl acetate extract which provided the lowest potency. The locally obtained spray-dried powder of *P. emblica* was significantly more active than the commercial *P. emblica* extract as seen from the significantly lower IC_{50} value ($P < 0.05$). This may be due to the physical and chemical properties of the materials.

Mahattanapokai (2004) also evaluated the antioxidant of *P. emblica* ethyl acetate extract using the same DPPH test. She reported the IC_{50} of the ethyl acetate extract in scavenging the DPPH radical to be 2.07 μg/mL, which is smaller than the value obtained here (7.74 μg/mL). However, extraction procedure was also different. In Mahattanapokai's study the whole dried fruits were ground and first extracted in Soxhlet with hexane. After hexane extraction, the powder was further extracted with ethyl acetate to obtain the ethyl acetate extract. In our study, the aqueous fruit juice was spray-dried and directly extracted with ethyl acetate. It is possible that different sources and part of the starting raw materials contributed to the antioxidant activity due to differences in the type and amount of active constituents present in each extract.

2.2 Hydroxyl radical scavenging activity

Antioxidants that are able to protect lipids against free radical chain reactions of peroxidation can be evaluated by their ability to prevent damage of carbohydrates using 2-deoxy-D-ribose as a substrate (Li and Xie, 2000). Because deoxyribose is a DNA sugar, the scavenging effect of an antioxidant on hydroxyl radical-mediated degradation of DNA can thus be evaluated using this sugar also.

The effect of various solvent extracts of *P. emblica* on the inhibition of free radical-mediated DNA-sugar damage was assessed by deoxyribose method (Halliwell et al., 1987). Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damages to the adjacent bio-molecules. The scavenging effect against hydroxyl radical was investigated by using the Fenton reaction (Sakanaka et al., 2005). The Fenton reaction generates hydroxyl radicals ($OH^{\cdot}$) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. Attack at a sugar ultimately leads to sugar fragmentation, base loss and strand break with a terminal fragment sugar residue. Addition of low concentrations of transition metal ions such as iron to DNA causes degradation of the sugar into malondialdehyde and other related compounds which form a chromogen with thiobarbituric acid (TBA), into a thiobarbituric acid reactive substance (TBARS) (Desmarchelier et al., 1997).

Figure 23 depicts the hydroxyl radical scavenging percentage of various solvent extracts of *P. emblica* compared with the commercial *P. emblica* extract and other antioxidants by observing changes in the absorbance of pink chromogen at 532 nm. This result shows that the presence of higher concentrations of all test samples in the reaction mixture prevented the oxidation of deoxyribose (DNA-sugar). As presented in Table 12, it was found that as the concentration was increased, the extent of hydroxyl radical scavenging activity also increased for all antioxidants. The percent hydroxyl radical inhibition of the acetone (successive) extract indicated the highest potency, with the inhibition extent increasing to 92.44 ± 0.38 % at 2.0 mg/mL concentration, whereas the acetone (direct) extract and the spray-dried powder gave slightly lower extent (90.74 ± 0.56 % and 89.35 ± 0.79 % at 3.0 mg/mL, respectively). Other solvent extracts of *P. emblica* including the ethanol (successive), ethanol

(direct) and ethyl acetate extracts did not exhibit high hydroxyl radical scavenging activity. At 3.0 mg/mL concentration, their inhibition percentages were 66.06 ± 0.73 %, 50.29 ± 0.25 % and 52.94 ± 0.80 %, respectively.

Ethyl acetate extract gave the lowest inhibition-concentration profile compared with all other antioxidants, indicating the lowest preventive effect on the deoxyribose degradation (Figure 23).

Trolox®, or the water soluble form of α-tocopherol, showed the highest hydroxyl radical scavenging of 68.53 ± 0.67 % at 2.0 mg/mL. Maximal concentration of Trolox® was limited by its solubility in phosphate buffer (pH 7.4). Thus, the extent of hydroxyl radical inhibition for Trolox® could not be assessed at higher concentrations.

Likewise, the maximal concentration of EGCG was also limited by its solubility in phosphate buffer (pH 7.4). The percent hydroxyl radical inhibition of EGCG increased to 72.55 ± 0.54 % at 2.0 mg/mL the highest concentration studied.

For comparison at 2.0 mg/mL which is the highest measurable concentration for all test samples, the acetone (successive) extract showed the highest percent inhibition (92.44 ± 0.38%). This was followed by EGCG (72.55 ± 0.54%), spray-dried powder (70.89 ± 0.73%), Trolox® (68.53 ± 0.67%) and the acetone (direct) extract (62.15 ± 0.65%) which was slightly higher than the commercial *P. emblica* extract (61.77 ± 1.79%). Both of the acetone (direct) and commercial extracts showed higher antioxidant effect than the ethanol (successive), ethanol (direct) and the ethyl acetate extracts which gave 52.00 ± 0.32%, 41.72 ± 0.25% and 32.67 ± 0.34% hydroxyl radical inhibition, respectively, at the 2.0 mg/mL concentration.

In this system, addition of ascorbic acid greatly increased the rate of $OH^{\cdot}$ generation by reducing iron and maintaining a supply of Fe^{2+} (Li and Xie, 2000). Therefore, the hydroxyl radical scavenging activity of l-ascorbic acid could not be assessed under this experimental condition.

The relationship between the percentages of hydroxyl radical inhibition and the concentration of the individual antioxidants is shown in Figure 24. The plots indicated that all the antioxidants gave a hydroxyl radical scavenging activity in a dose-dependent manner. Similar dose-dependent relationships between plant phenolics and their hydroxyl radical scavenging activity have been reported in the

studies of Laughton et al., (1989), Desmarchelier et al., (1997) and Hagerman et al., (1998). However, the successive extract appeared to show higher potency on hydroxyl radical scavenging than the direct extract. It is possible that the successive extraction process might be able to give higher contents of the active compounds and less occurrence of other contaminating co-extracted materials than the direct extraction process. The raw data for hydroxyl radical inhibition percentages are provided in Appendix B.

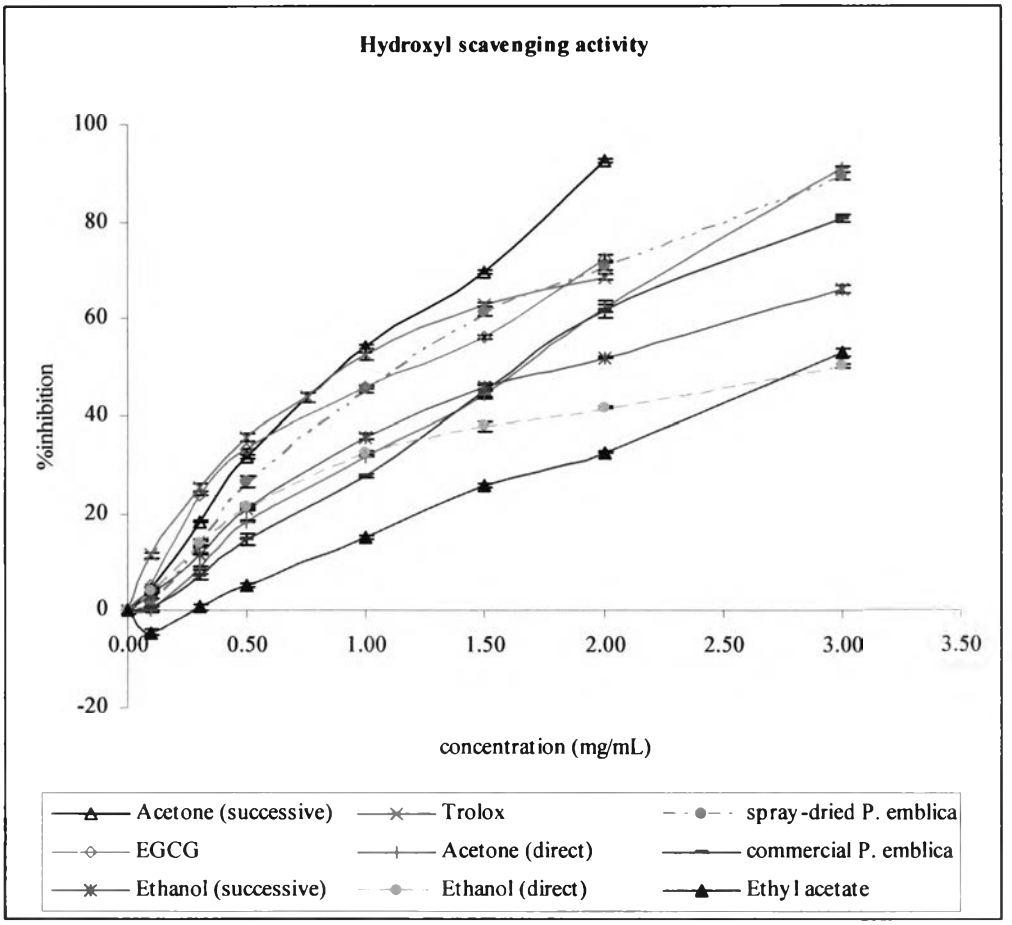

Figure 23. The extent of hydroxyl radical inhibition of various solvent extracts of *P. emblica* in comparison with other antioxidants at different concentrations (Mean ± SD, n = 3).

After the plots of % hydroxyl radical inhibition versus concentration were constructed for each antioxidant, their IC_{50} value was calculated from the equation of

the partial polynomial regression of each curve as shown in Figure 25 and Table 13. The estimated IC_{50} (mg/mL) can be ranked from the lowest to highest as follows: acetone (successive) extract (0.88 ± 0.01), Trolox® (0.92 ± 0.03), spray-dried *P. emblica* (1.12 ± 0.03), EGCG (1.19 ± 0.01), commercial *P. emblica* extract (1.62 ± 0.05), acetone (direct) extract (1.67 ± 0.02), ethanol (successive) extract (1.79 ± 0.03), ethyl acetate extract (2.88 ± 0.03) and ethanol (direct) extract (2.97 ± 0.03).

Table 12. Hydroxyl radical inhibition of various solvent extracts of *P. emblica* compared to other antioxidants at different concentrations (Mean ± SD, n= 3).

No	Concentration	Spray dried *P. emblica*		Ethyl acetate		Acetone (successive)		Acetone (direct)		Ethanol (successive)		Ethanol (direct)		Commercial *P. emblica*		Trolox®		EGCG	
	(mg/mL)	%inh	SD	%inh	SD	%inh	SD	%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	0.00	0.00	0.00	0.00	0.00	0.00
2	0.10	1.59	0.79	-4.49	0.64	4.35	0.74	0.31	0.59	3.23	0.56	4.12	0.44	0.46	0.48	11.51	0.64	5.08	0.11
3	0.30	13.83	0.63	0.73	0.57	18.41	0.25	8.83	0.56	11.67	0.21	14.13	0.53	7.08	0.74	25.38	0.76	23.73	0.11
4	0.50	26.69	1.25	5.28	0.23	31.64	0.28	18.40	0.21	21.01	0.24	21.36	0.39	14.77	1.07	35.62	0.89	33.46	0.00
5	0.75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	43.91	1.02	-	-
6	1.00	45.37	0.79	15.12	0.46	54.15	0.49	31.90	0.37	35.84	0.49	32.46	0.39	27.69	0.48	52.79	1.16	45.79	0.11
7	1.50	61.34	0.98	25.87	0.46	69.52	0.38	44.48	0.53	45.89	0.32	37.85	0.81	45.00	1.54	62.86	0.53	56.20	0.39
8	2.00	70.89	0.73	32.67	0.34	92.44	0.38	62.15	0.65	52.00	0.32	41.72	0.25	61.77	1.79	68.53	0.67	72.55	0.54
9	3.00	89.35	0.79	52.94	0.80	*	*	90.74	0.56	66.06	0.73	50.29	0.25	80.77	0.74	**	**	**	**

* could not be accurately detected due to possible interference from the direct interaction between Fe^{3+} and phenolic components in the extract at high concentration

** could not dissolve in phosphate buffer at 3mg/mL final concentration

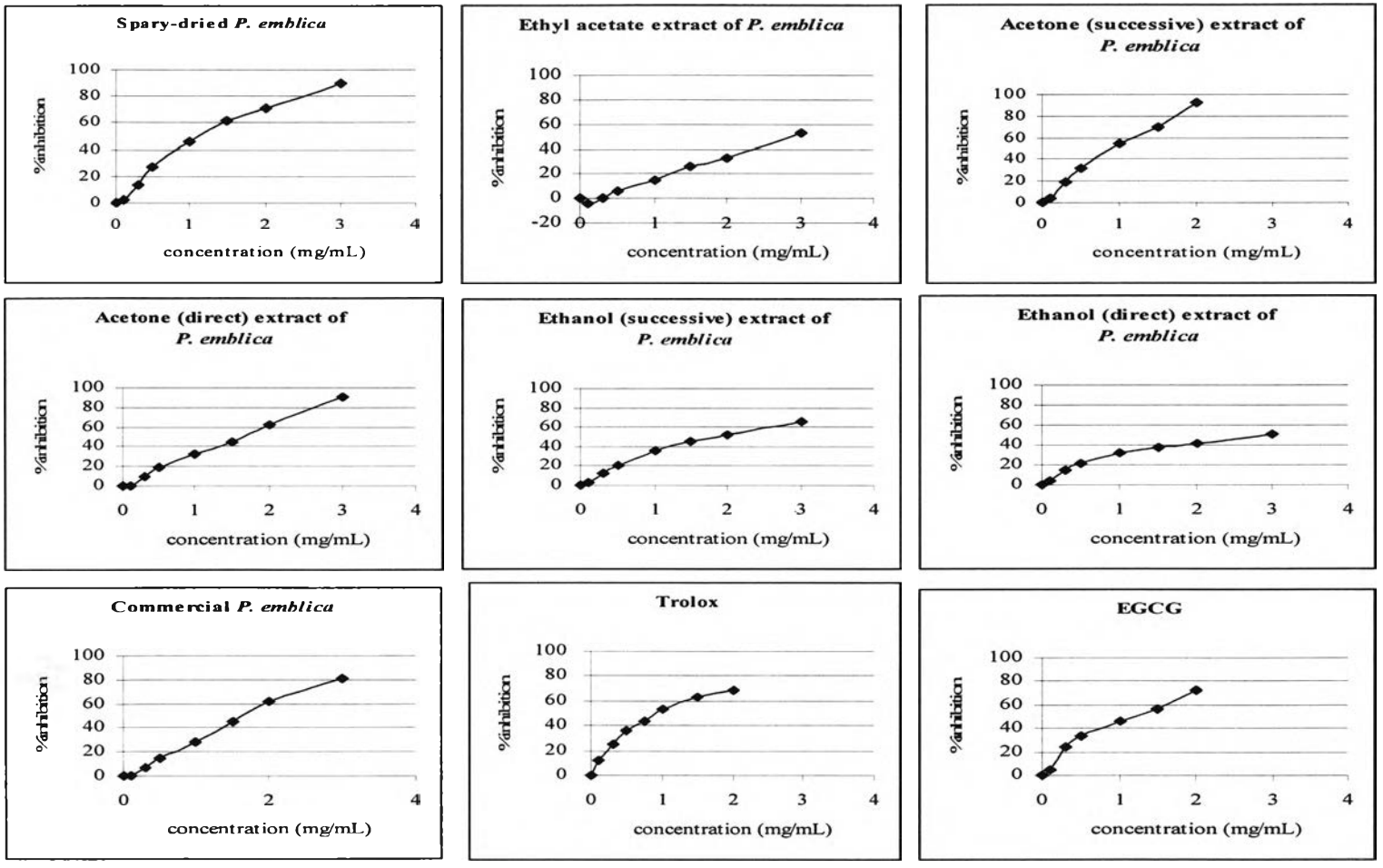

Figure 24. Relationship between hydroxyl radical inhibition extent and concentration of antioxidants (Mean ± SD, n = 3).

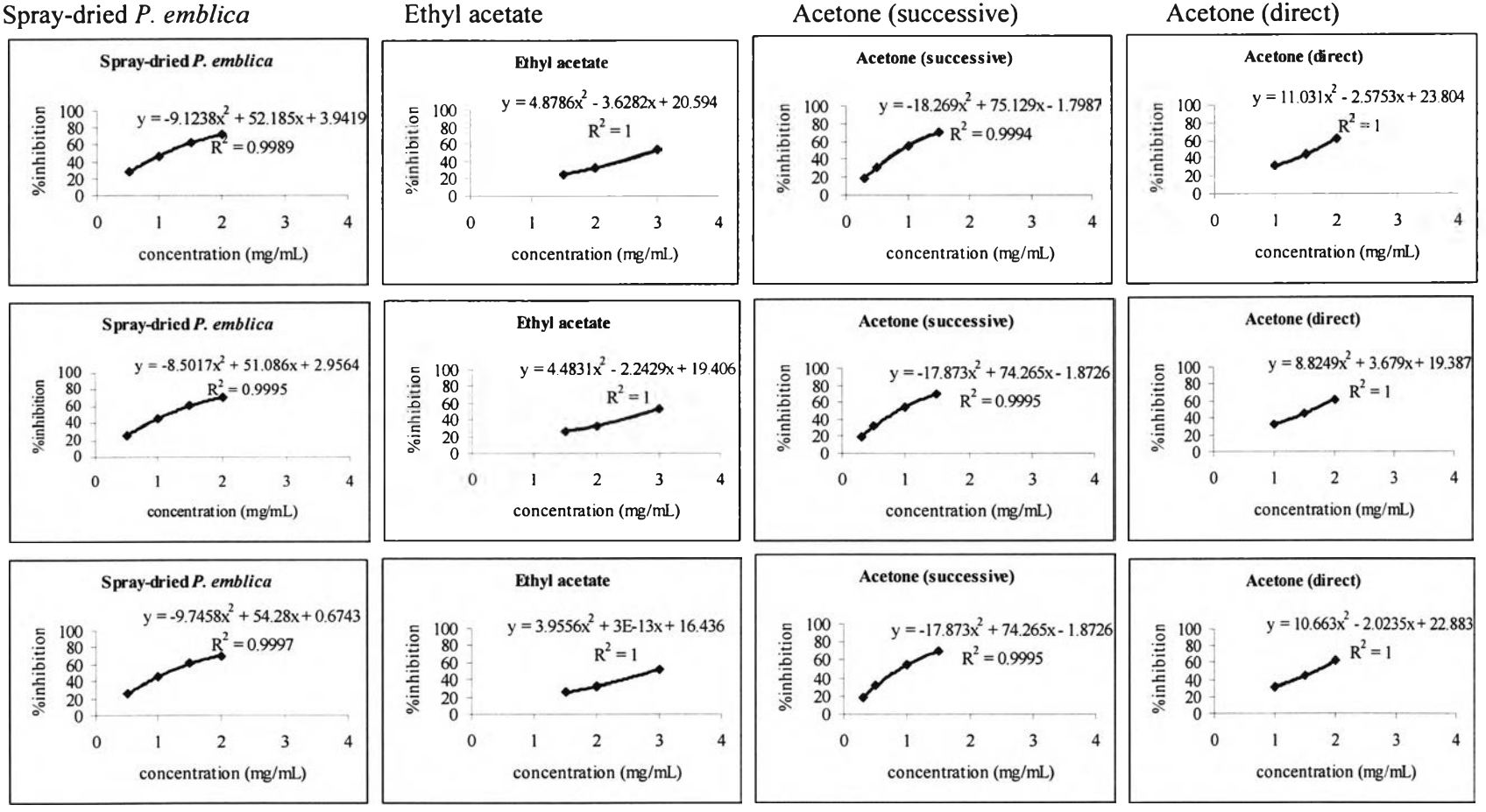

Figure 25. The relation of the %hydroxyl radical inhibition-concentration profile of the individual antioxidants. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants.

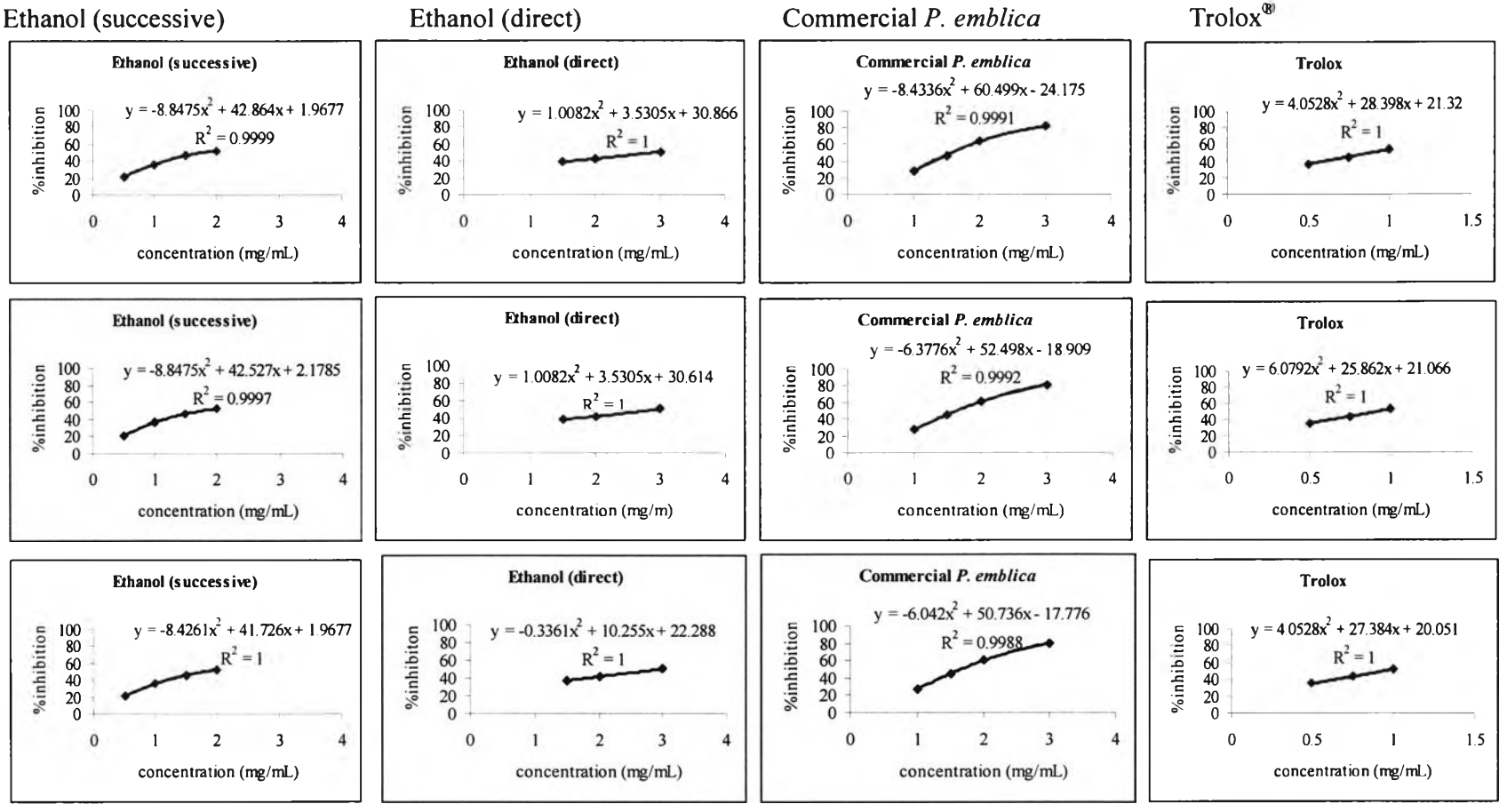

Figure 25 (cont). The relation of the %hydroxyl radical inhibition-concentration profile of the individual antioxidants. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants.

EGCG

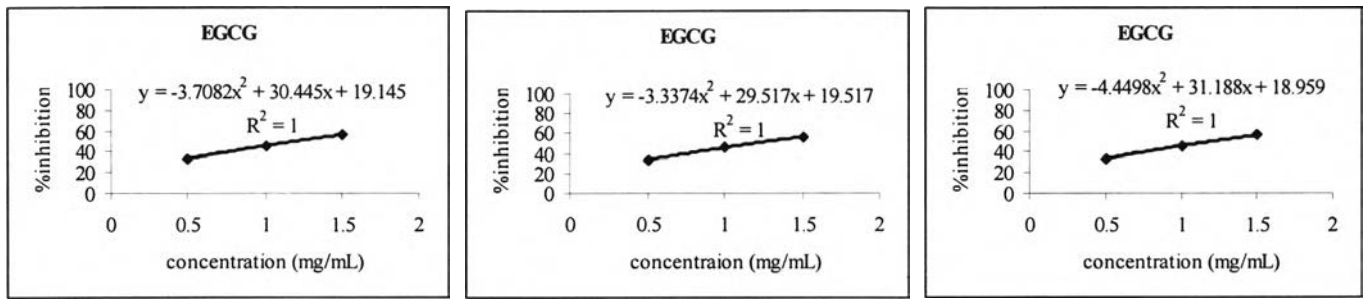

Figure 25 (cont). The relation of the %hydroxyl radical inhibition-concentration profile of the individual antioxidants. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual antioxidants.

Table 13. The IC_{50} value for hydroxyl radical scavenging activity of each antioxidant. The R^2 is the regression coefficient obtained from polynomial regression of the partial of the plot between the inhibition percentage and the concentration of each antioxidant (Mean ± SD, n = 3).

Sample	Polynomial equation (partial)					
	IC_{50} (mg/mL)	Mean	SD	R^2	Mean	SD
Acetone (successive)	0.88	0.88	0.01	0.9994	0.9995	0.00
	0.89			0.9995		
	0.89			0.9995		
Trolox®	0.90	0.92	0.03	1.0000	1.0000	0.00
	0.92			1.0000		
	0.96			1.0000		
Spray-dried *P. emblica*	1.09	1.12	0.03	0.9989	0.9994	0.00
	1.14			0.9995		
	1.14			0.9997		
EGCG	1.18	1.19	0.01	1.0000	1.0000	0.00
	1.19			1.0000		
	1.20			1.0000		
Commercial *P. emblica*	1.57	1.62	0.05	0.9991	0.9990	0.00
	1.64			0.9992		
	1.67			0.9988		
Acetone (direct)	1.66	1.67	0.02	1.0000	1.0000	0.00
	1.67			1.0000		
	1.69			1.0000		
Ethanol (successive)	1.76	1.79	0.03	0.9999	0.9999	0.00
	1.79			0.9997		
	1.82			1.0000		
Ethyl acetate	2.85	2.88	0.03	1.0000	1.0000	0.00
	2.87			1.0000		
	2.91			1.0000		
Ethanol (direct)	2.94	2.97	0.03	1.0000	1.0000	0.00
	2.97			1.0000		
	3.00			1.0000		

The IC_{50} result of Trolox® (3.69mM) in this study was quite similar to the result reported by Chaudhuri, 2004 (2.70mM), who also evaluated the hydroxyl radical scavenging activity of *P. emblica* water-based extract. However, he reported the IC_{50} of this extract in the unit of mM (0.92mM), which can not be compared to the value obtained here (mg/mL). Since the lower IC_{50} values indicate greater inhibitory potency of the antioxidant, therefore, the relative hydroxyl radical scavenging activity of all test samples decreased in the following order: acetone (successive) extract > Trolox® > spray-dried *P. emblica* > EGCG > commercial *P. emblica* > acetone (direct) extract > ethanol (successive) extract > ethyl acetate extract > ethanol (direct) extract. As these results, acetone (successive) extract had the highest hydroxyl radical ($OH^{\cdot}$) scavenging activity when compared to all other antioxidants under test. The commercial *P. emblica* exhibited lower hydroxyl radical scavenging activity than the acetone (successive) extract, Trolox®, spray-dried *P. emblica* and EGCG, respectively, but gave higher activity than the acetone (direct) extract, ethanol (successive) extract, ethyl acetate extract and ethanol (direct) extract. The extraction process appeared to have an influence on the hydroxyl radical scavenging activity. The successive extract showed higher inhibition effect on $OH^{\cdot}$ radical compared with the direct extract. The IC_{50} (mg/mL) profile of each antioxidant for hydroxyl radical ($OH^{\cdot}$) inhibition is shown in Figure 26.

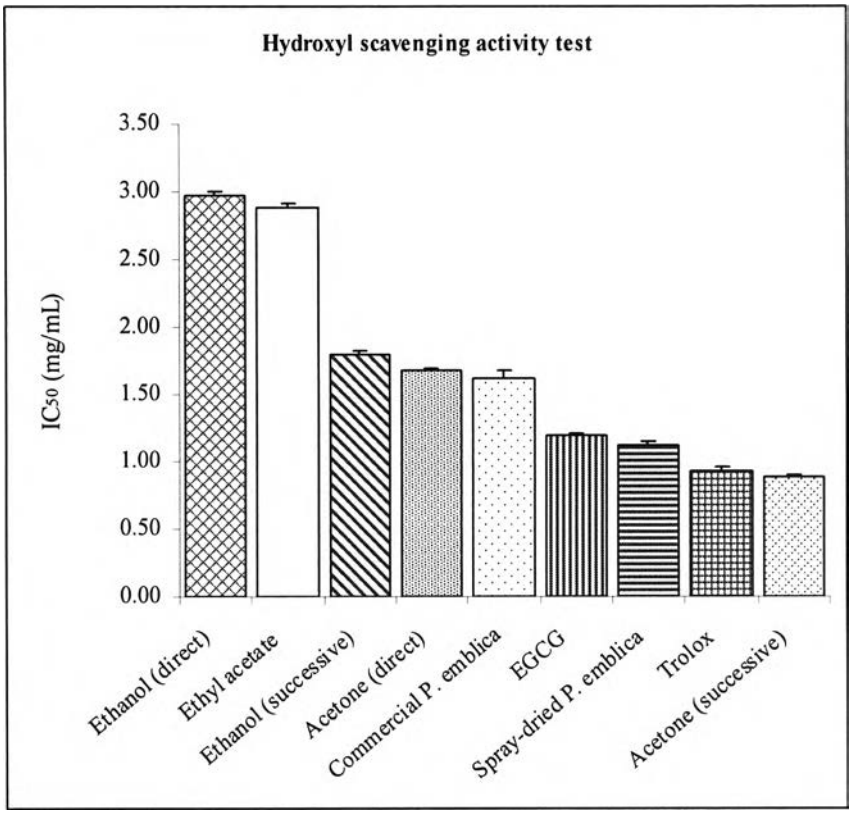

Figure 26. The IC_{50} (mg/mL) of each antioxidant for hydroxyl radical inhibition (Mean ± SD, n = 3).

As presented in Figure 26, the ethanol (direct) extract and ethyl acetate extract exhibited higher IC_{50} values than the ethanol (successive) extract, acetone (direct) extract and commercial *P. emblica*, which, in turn, were greater than EGCG, spray-dried *P. emblica*, Trolox®, and acetone (successive) extract, respectively. It can be seen that acetone (successive) extract gave the lowest IC_{50} and thus possessed the strongest hydroxyl radical ($OH^{\cdot}$) scavenging activity. Trolox® provided similar IC_{50} to the acetone (successive) extract with the value only 1.05 folds that of the acetone (successive) extract. The IC_{50} value of the spray-dried *P. emblica* was also close to that of EGCG, which were 1.27 and 1.35 times that of acetone (successive) extract, respectively. Commercial *P. emblica* extract, acetone (direct) and ethanol (successive) extracts gave the IC_{50} values which were 1.84, 1.90 and 2.03 times that of the acetone (successive) extract, respectively. The ethyl acetate extract gave a higher IC_{50} than the ethanol (successive) extract, its value being 3.27 times that of the acetone (successive) extract. The ethanol (direct) extract, on the other hand, provided the highest IC_{50} value

which was 3.38 times that of the acetone (successive) extract, indicating that it was the least potent hydroxyl radical scavenger.

The IC_{50} data were then analyzed by one-way analysis of variance (ANOVA) at 95% confidence followed by Tukey's test. Significant differences were found among the antioxidants studied ($P < 0.05$). According to the subsequent Tukey's test, these antioxidants could be divided into 6 different groups ($P < 0.05$) regarding their ability to inhibit or scavenge the hydroxyl radical. The IC_{50} ranking is as follows:

acetone (successive) extract ≈ Trolox® <		spray-dried *P. emblica* ≈ EGCG <	
0.88	0.92	1.12	1.19 mg/mL
commercial *P. emblica* ≈ acetone (direct) extract <		ethanol (successive) extract <	
1.62	1.67	1.79 mg/mL	
ethyl acetate extract < ethanol (direct) extract			
2.88	2.97 mg/mL		

The antioxidants joined by the same underline are within the same group and not statistically different in terms of their hydroxyl radical inhibition ($P > 0.05$). Thus, both the acetone (successive) extract of *P. emblica* and Trolox® demonstrated the highest potency of hydroxyl radical inhibition with the smallest IC_{50} values. Their potency was significantly greater than the group of spray-dried *P. emblica* and EGCG which, in turn, was more potent than the group of commercial *P. emblica* extract and acetone (direct) extract. The ethanol (successive and direct) and the ethyl acetate extracts constituted the next three groups having even lower activity. Although the ethanol (direct) extract was shown to give the highest IC_{50} values indicating its lowest activity in scavenging $OH^{\cdot}$ radical in term of concentration of 50% inhibition, in considering the hydroxyl radical inhibition profiles of test samples, the ethyl acetate extract still provided the lowest inhibition concentration profile. The results obtained here can be concluded that ethyl acetate extract exhibited the lowest antioxidant activities against DPPH and hydroxyl radicals compared to all other antioxidants. The statistical results of the individual antioxidants against the hydroxyl radical are shown in Appendix B.

2.3 Pro-oxidant activity

The pro-oxidant activity is a result of the ability to reduce metals, such as Fe^{3+}, to forms ion that react with O_2 or H_2O_2 generated the initiators of oxidation (Yen et al., 1997). Modification of deoxyribose method was made to evaluate the pro-oxidant activity by omitting ascorbic acid. Ascorbic acid acts as a pro-oxidant in this method via reducing properties, it generates an oxidative potential in the presence of transitional metal ions such as Fe^{3+} and Cu^{2+}. The reduction of Fe^{3+} to more reactive Fe^{2+} salts gave greater rate of $OH^{\cdot}$ generation (Laughton et al., 1989) which has a very high oxidative potential (Majd, Goldberg, and Stanislawski, 2003), leading to increase deoxyribose degradation. Therefore, compounds that can reduce transition metals to promote $OH^{\cdot}$ generation, show the pro-oxidant activity in this simple model system which consists of deoxyribose, Fe^{3+}-EDTA and H_2O_2. This model can be used to assess whether an antioxidant, or any compound, also possesses a concomitant pro-oxidant property.

The effects of spray-dried and various solvent extracts of *P. emblica* on the oxidative damage of deoxyribose was evaluated in comparison with the commercial *P. emblica* extract, Trolox® and EGCG by observing an increase in the formation of pink chromogen over the ascorbic acid-free controls at 532 nm. The ascorbic acid (0.1 mM) was also used as a reference pro-oxidant. The relationship between the absorbance and the concentration of the individual test samples are shown in Figures 27 (above), 28 and Table 14.

It was found that an increase in the absorbance (production of the deoxyribose fragments) was observed for all of the test samples at lower concentrations. However, at higher concentration the deoxyribose degradation decreased. This may be due to the predominance of the hydroxyl radical scavenging activity associated with the higher contents of the test samples in a dose-dependent manner.

At 1.00 mg/mL, acetone (successive) extract exhibited the average absorbance of 0.282 ± 0.002, which was slightly higher than the ascorbic acid-free control (0.274 ± 0.001). However, at the higher concentration range of 1.50-3.00 mg/mL, acetone (successive) extract exhibited lower absorbance than the ascorbic acid-free control, indicating lower deoxyribose degradation.

Likewise, the data showed that the absorbances of the spray-dried *P. emblica*, acetone (direct) extract and EGCG at 1.00 mg/mL concentration were higher than ascorbic acid-free control. However, they gradually decreased to 2.81 ± 0.005, 0.203 ± 0.001 and 0.153 ± 0.003, respectively at 3.00 mg/mL. This result suggested the pro-oxidant effect on deoxyribose degradation at the concentration below 2.00 mg/mL. As presented in Figure 27 and Table 14, although EGCG exhibited the lowest absorbance at concentration of 3.00 mg/mL compared with the spray-dried *P. emblica* and acetone (direct) extracts, but at lower concentrations (1-2 mg/mL) EGCG was able to provide the powerful pro-oxidant activity as seen in the higher absorbance values when compared with its ascorbic acid-free control.

The ethyl acetate, ethanol (successive), ethanol (direct) and commercial *P. emblica* extracts also showed a marked increase in absorbance at lower concentration range (1-2 mg/mL) but at the highest concentration studied (3 mg/mL), a decrease in absorbance was observed, with the values of 0.429 ± 0.001, 0.316 ± 0.004, 0.334 ± 0.001 and 0.379 ± 0.005, respectively. However, their absorbance values at this concentration were still higher than their corresponding ascorbic acid-free control. Therefore, these three extracts of *P. emblica* and the commercial *P. emblica* extract promoted the deoxyribose degradation in all of the experimental concentrations. Figure 27 also suggested that the ethyl acetate extract exhibited the highest pro-oxidant activity followed by the commercial *P. emblica*, ethanol (direct) and ethanol (successive) extracts, respectively.

As displayed in Figure 28 and Table 14, Trolox® presented lower absorbance in the range of 0.75-2.00 mg/mL compared with its ascorbic acid-free control. However, Trolox® clearly exhibited pro-oxidant activity with higher absorbance at the lower concentration range of 0.05-0.50 mg/mL (200 μM-2 mM). This range of concentration is in agreement with a previous study by Mayo et al. (2003), which reported the pro-oxidant effect of Trolox® in the range of 250μM-4mM. The result indicated that Trolox® exhibited pro-oxidant activity at lower concentration range than the *P. emblica* extracts and other antioxidants. The raw data for absorbance of each test sample in the pro-oxidant test using l-ascorbic acid as a reference standard are provided in Appendix C.

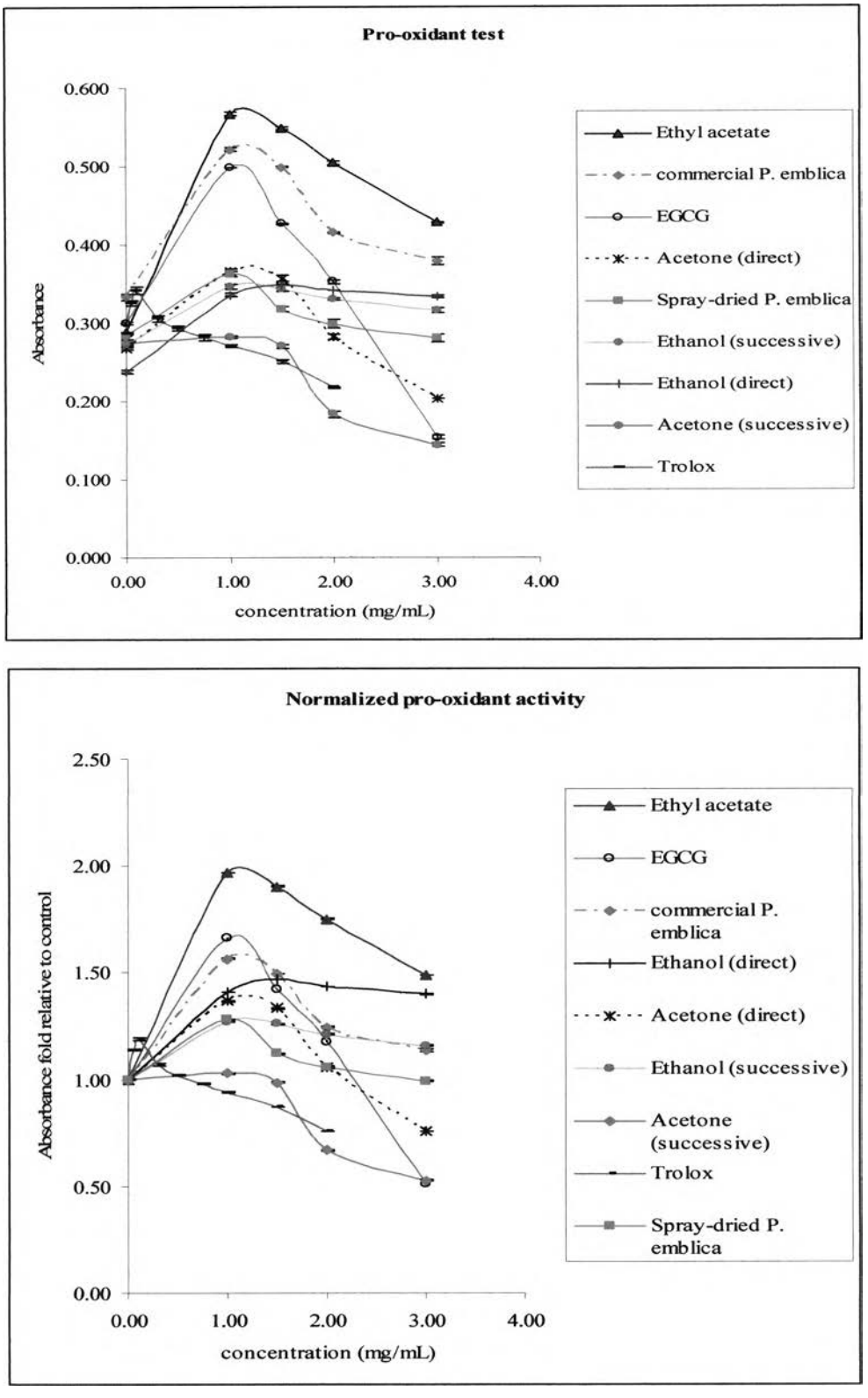

Figure 27. The extent of pro-oxidant activity of various solvent extracts of *P. emblica* in comparison with other antioxidants at different concentrations (Mean ± SD, n = 3).

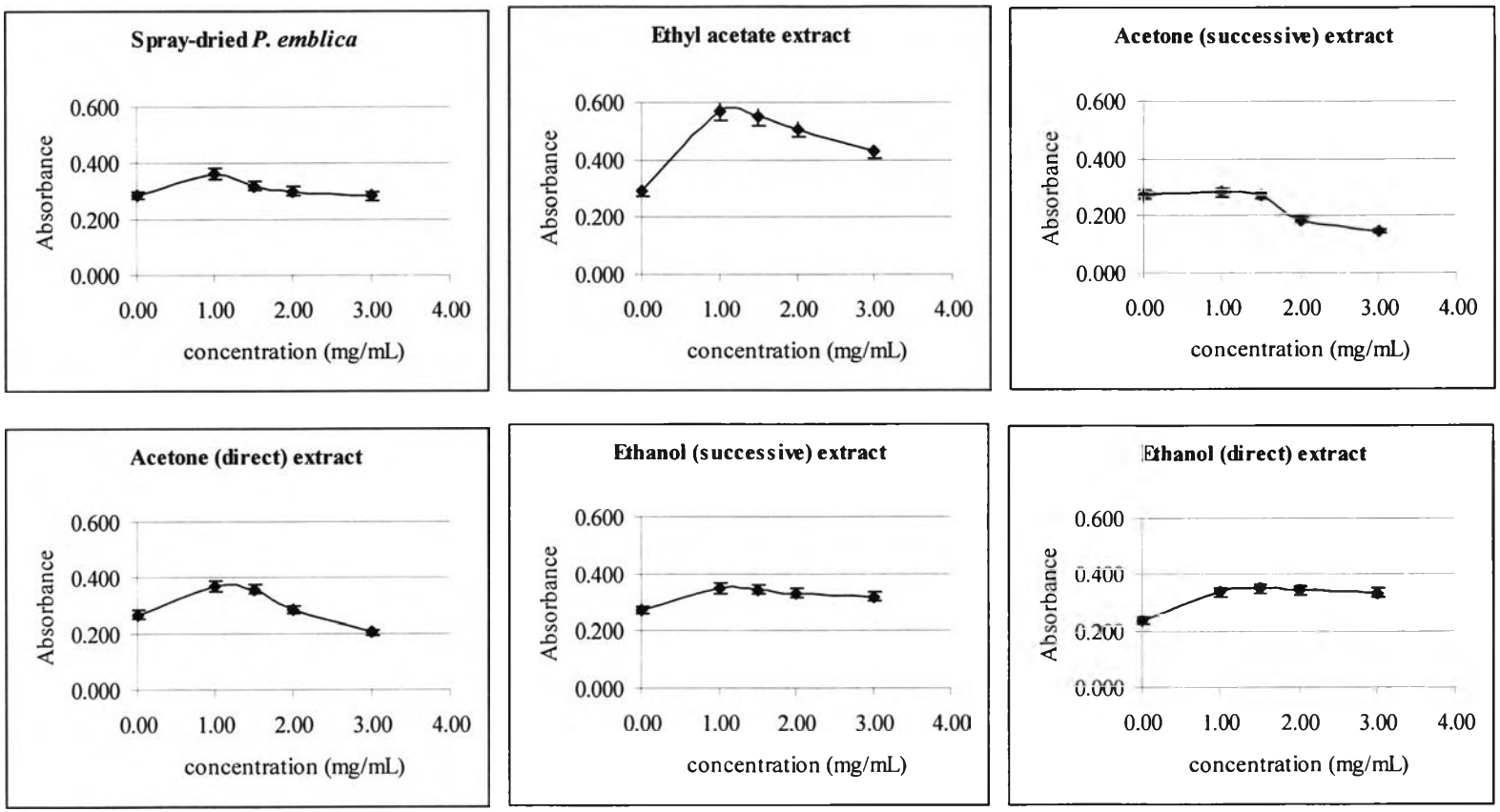

Figure 28. Relationship between the absorbance at 532 nm (pro-oxidant effect) and the concentration of the individual antioxidants (Mean ± SD, n = 3).

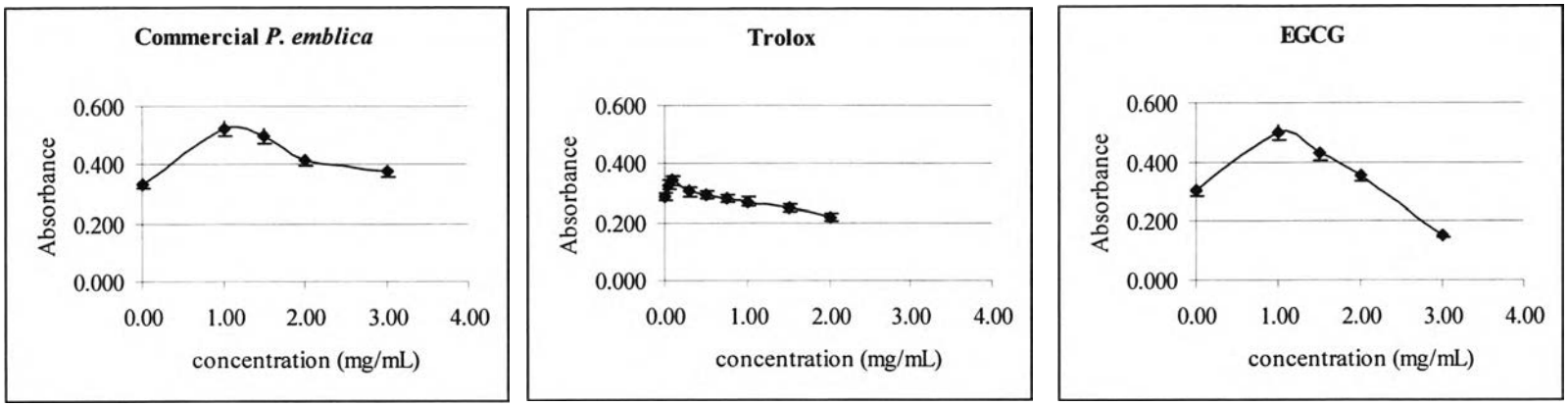

Figure 28 (cont.). Relationship between the absorbance at 532 nm (pro-oxidant effect) and the concentration of the individual antioxidants (Mean ± SD, n = 3).

Table 14. The absorbance and normalized pro-oxidant activity relative to the ascorbic acid-free control (0 mg/mL sample) of various solvent extracts of *P. emblica* and other antioxidants at different concentrations, in comparison with ascorbic acid reference standard (Mean ± SD, n = 3).

Test sample	Spray-dried *P. emblica*		Ethyl acetate		Acetone (successive)		Acetone (direct)		Ethanol (successive)		Ethanol (direct)		Commercial *P. emblica*		EGCG		Trolox®	
mg/mL	Abs ± SD	fold over (C)	Abs ± SD	fold over (C)	Abs ± SD	fold over (C)	Abs ± SD	fold over (C)	Abs ± SD	fold over (C)	Abs ± SD	fold over (C)	Abs ± SD	fold over (C)	Abs ± SD	fold over (C)	Abs ± SD	fold over (C)
Control (C) 0.00	0.283 ±0.005	1.00	0.288 ± 0.001	1.00	0.274 ± 0.001	1.00	0.268 ± 0.002	1.00	0.273 ± 0.002	1.00	0.238 ± 0.003	1.00	0.333 ± 0.004	1.00	0.300 ± 0.001	1.00	0.287 ± 0.001	1.00
0.05	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.326 ±0.003	1.14
0.10	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.342 ±0.005	1.19
0.30	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.306 ±0.004	1.07
0.50	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.294 ±0.003	1.02
0.75	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.282 ±0.004	0.98
1.00	0.363 ±0.004	1.28	0.567 ±0.002	1.97	0.282 ± 0.002	1.03	0.366 ± 0.003	1.37	0.347 ±0.003	1.27	0.336 ±0.003	1.41	0.522 ±0.002	1.57	0.498 ± 0.001	1.66	0.271 ±0.002	0.94
1.50	0.318 ±0.003	1.12	0.548 ± 0.002	1.90	0.270 ± 0.002	0.99	0.357 ± 0.004	1.33	0.344 ±0.004	1.26	0.349 ±0.001	1.47	0.499 ± 0.001	1.50	0.427 ± 0.001	1.42	0.251 ±0.002	0.87
2.00	0.299 ±0.006	1.06	0.504 ± 0.002	1.75	0.184 ± 0.004	0.67	0.283 ± 0.003	1.06	0.331 ±0.002	1.21	0.342 ±0.000	1.44	0.415 ± 0.002	1.25	0.353 ± 0.003	1.18	0.217 ±0.001	0.76
3.00	0.281 ±0.005	0.99	0.429 ± 0.001	1.49	0.144 ± 0.003	0.53	0.203 ± 0.001	0.76	0.316 ±0.004	1.16	0.334 ±0.001	1.40	0.379 ± 0.005	1.14	0.153 ± 0.003	0.51	NA	-
ascorbic acid 0.1mM	0.699 ±0.003	2.47	0.679 ± 0.002	2.36	0.676 ± 0.005	2.47	0.713 ± 0.002	2.66	0.657 ±0.001	2.41	0.549 ±0.001	2.31	0.716 ± 0.003	2.15	0.714 ± 0.002	2.38	0.684 ±0.003	2.38

Where; NA = not available

Since the absorbances of ascorbic acid-free control of each sample were not the same, the values of the absolute absorbance were subsequently normalized to absorbance relative to their corresponding control as shown in Figure 27 (below) and Table 14. Comparative pro-oxidant potency was made among different *P. emblica* extracts, and other test samples in terms of their increases in absorbance fold over their respective ascorbic acid-free controls.

At concentration of 1.00 mg/mL, except Trolox®, all of test samples exhibited absorbance fold over their corresponding controls that demonstrated pro-oxidant activity. At this concentration, acetone (successive) extract showed the lowest pro-oxidant activity with the absorbance 1.03 times that of its ascorbic acid-free control, whereas the absorbances of the spray-dried *P. emblica*, acetone (direct) extract and EGCG at 1.00 mg/mL were 1.28, 1.37 and 1.66 times their corresponding controls. However, the ethyl acetate, ethanol (successive), ethanol (direct) and commercial *P. emblica* extracts exhibited the pro-oxidant activity at all concentrations studied, showing absorbance at 1.00 mg/mL 1.97, 1.27, 1.41 and 1.57 times that of their ascorbic acid-free controls, as presented in Table 14. At 1.00 mg/mL concentration, the pro-oxidant activity of the test samples, relative to their corresponding controls, increased in the following order:

acetone (successive) extract < ethanol (successive) extract <
1.03 1.27 fold
spray-dried *P. emblica* < acetone (direct) extract < ethanol (direct) extract <
1.28 1.37 1.41 fold
commercial *P. emblica* < EGCG < ethyl acetate extract
1.57 1.66 1.97 fold

The absorbance data were analyzed by one-way analysis of variance (ANOVA) at 95% confidence followed by Dunnett's test to compare the test samples with the control. The hypothesis was that any substance possessed no pro-oxidant activity should exhibit equal or lower absorbance when compared with the absorbance of its corresponding ascorbic acid-free control. Therefore, the higher the absorbance

of the test substance over the control, the greater was its pro-oxidant effect which may become significant at 5% significance level.

H_0 : the test substance was not a pro-oxidant
absorbance $\leq$ absorbance control

H_1 : the test substance was a pro-oxidant
absorbance > absorbance control

Table 15. The absorbance and significant level of various solvent extracts of *P. emblica* and other antioxidants at different concentrations in comparison with ascorbic acid-free control (0 mg/mL sample) and ascorbic acid reference standard (Mean ± SD, n = 3).

Test sample	Spray-dried *P. emblica*		Ethyl acetate		Acetone (successive)		Acetone (direct)		Ethanol (successive)		Ethanol (direct)		Commercial *P. emblica*		EGCG		Trolox®	
mg/mL	Abs ± SD	Sig. level	Abs ± SD	Sig. level	Abs ± SD	Sig. level	Abs ± SD	Sig. level	Abs ± SD	Sig. level	Abs ± SD	Sig. level	Abs ± SD	Sig. level	Abs ± SD	Sig. level	Abs ± SD	Sig. level
Control 0.00	0.283 ±0.005	-	0.288 ± 0.001	-	0.274 ± 0.001	-	0.268 ± 0.002	-	0.273 ± 0.002	-	0.238 ± 0.003	-	0.333 ± 0.004	-	0.300 ± 0.001	-	0.287 ± 0.001	-
0.05	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.326 ±0.003	S
0.10	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.342 ±0.005	S
0.30	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.306 ±0.004	S
0.50	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.294 ±0.003	S
0.75	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-	0.282 ±0.004	NS
1.00	0.363 ±0.004	S	0.567 ±0.002	S	0.282 ± 0.002	S	0.366 ± 0.003	S	0.347 ±0.003	S	0.336 ±0.003	S	0.522 ±0.002	S	0.498 ± 0.001	S	0.271 ±0.002	NS
1.50	0.318 ±0.003	S	0.548 ± 0.002	S	0.270 ± 0.002	NS	0.357 ± 0.004	S	0.344 ±0.004	S	0.349 ±0.001	S	0.499 ± 0.001	S	0.427 ± 0.001	S	0.251 ±0.002	NS
2.00	0.299 ±0.006	S	0.504 ± 0.002	S	0.184 ± 0.004	NS	0.283 ± 0.003	S	0.331 ±0.002	S	0.342 ±0.000	S	0.415 ± 0.002	S	0.353 ± 0.003	S	0.217 ±0.001	NS
3.00	0.281 ±0.005	NS	0.429 ± 0.001	S	0.144 ± 0.003	NS	0.203 ± 0.001	NS	0.316 ±0.004	S	0.334 ±0.001	S	0.379 ± 0.005	S	0.153 ± 0.003	NS	NA	-
ascorbic acid 0.1mM	0.699 ±0.003	S	0.679 ± 0.002	S	0.676 ± 0.005	S	0.713 ± 0.002	S	0.657 ±0.001	S	0.549 ±0.001	S	0.716 ± 0.003	S	0.714 ± 0.002	S	0.684 ±0.003	S

Where; NA = not available, S = significantly higher than ascorbic acid-free control (having pro-oxidant activity) ($p < 0.05$), NS = not significantly higher than ascorbic acid-free control (not a pro-oxidant) ($p > 0.05$).

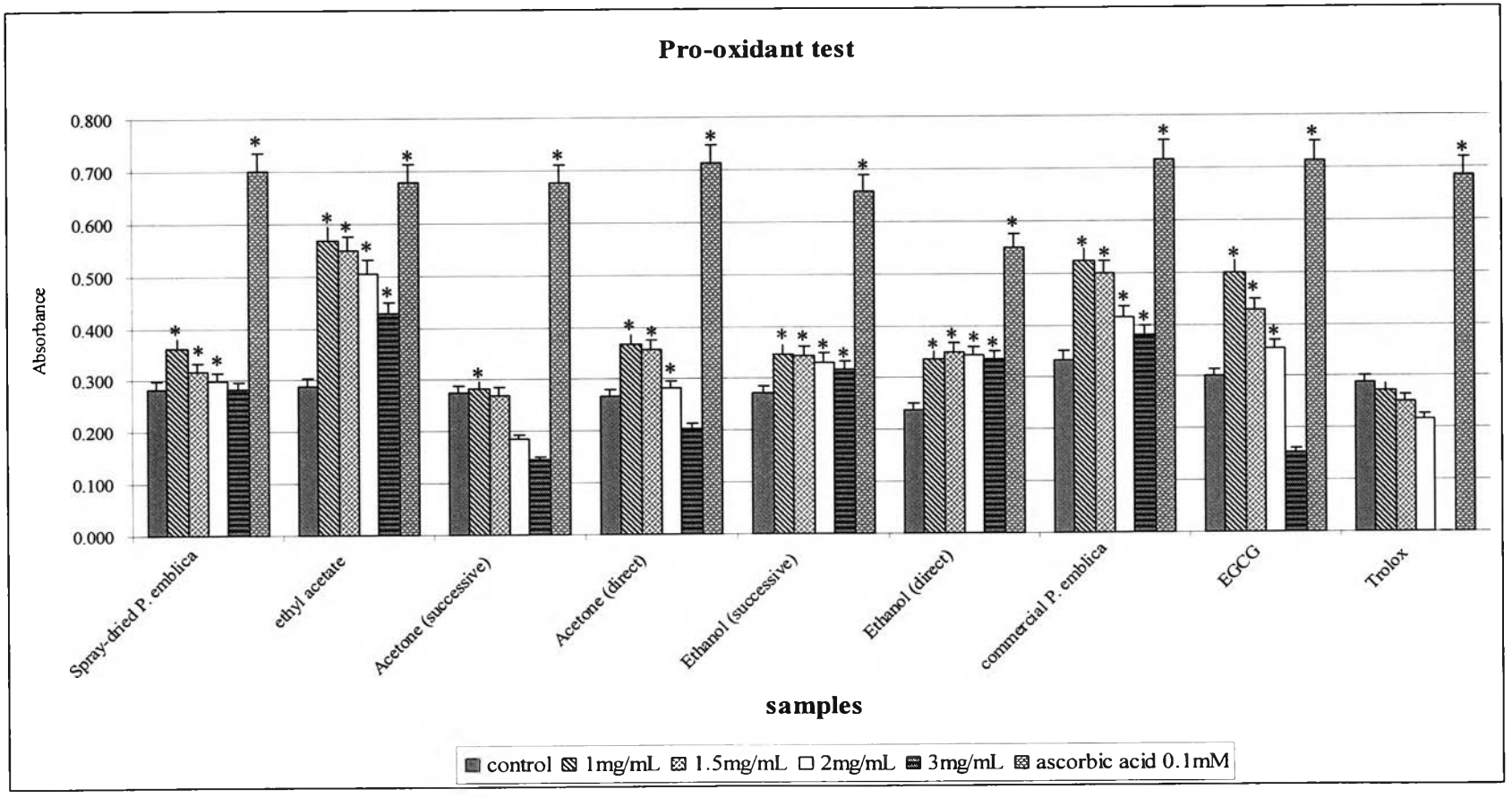

Figure 29. The absorbance of various solvent extracts of *P. emblica* and other antioxidants in the range of 1.00-3.00 mg/mL compared to their respective ascorbic acid-free controls and ascorbic acid reference standard (Mean ± SD, n = 3).

* = significantly higher than the ascorbic acid-free control ($p < 0.05$) by Dunnett's test

In Table 15 and Figure 29, it was found that the acetone (successive) extract did not show upper significant difference ($P > 0.05$) in absorbance from its control in the range of 1.50-3.00 mg/mL (absence of pro-oxidant effect) but exhibited greater significant difference ($P < 0.05$) at the lowest concentration studied of 1.00 mg/mL compared with its ascorbic acid-free control (presence of pro-oxidant effect). This result indicated that the acetone (successive) extract presented the pro-oxidant activity at 1.00 mg/mL but at higher concentrations it was free from pro-oxidant effect. It may be due to the scavenging effect of its active components at high concentrations. This result agrees with the study of Hagerman et al., (1998) who found that plant polyphenolics (tannin) were highly active in reducing oxidation of DNA sugar at higher concentrations, but the deoxyribose degradation increased over the control at lower doses suggesting a pro-oxidant property.

For the spray-dried *P. emblica*, acetone (direct) extract and EGCG, only at the concentration of 3.00 mg/mL that these three materials exhibited no significantly higher absorbance than the ascorbic acid-free control ($P > 0.05$), showing absence of pro-oxidant activity, whereas in the range of 1.00-2.00 mg/mL they showed upper significant difference ($P < 0.05$), indication of the presence of pro-oxidant activity compared with their respective controls. This result indicated that the spray-dried *P. emblica*, the acetone (direct) extract and EGCG provided the pro-oxidant effect on the deoxyribose degradation in the range of 1.00-2.00 mg/mL but at the highest concentration studied of 3.00 mg/mL they became free from pro-oxidant activity. The result of EGCC was also in agreement with Furukawa et al. (2003), who postulated that EGCG can act as a pro-oxidant at a low concentration (1-5 μM) in the presence of H_2O_2 and metal ions.

Ethyl acetate, ethanol (successive), ethanol (direct) and commercial *P. emblica* extracts demonstrated significantly higher absorbance values than their respective ascorbic acid-free controls ($P < 0.05$) at all the experimental concentrations (1.00-3.00 mg/mL). Therefore, these three extracts and the commercial *P. emblica* extract exhibited relatively high pro-oxidant activity in all of the experimental concentrations studied. The higher absorbance compared with the ascorbic acid-free control implied the higher deoxyribose degradation.

As presented in Figure 29, the pro-oxidant effect of Trolox® could not be observed in the range of 0.75-2.00 mg/mL. The result showed no upper significant difference ($P > 0.05$) from the ascorbic acid-free control in this concentration range. However, as the concentration was lowered below 0.75 mg/mL, Trolox® showed pro-oxidant effect on the deoxyribose degradation with the absorbance becoming significantly higher than the control as seen in Figure 30 ($P < 0.05$). Trolox® exhibited the pro-oxidant activity at lower concentrations in the range of 0.05-0.5 mg/mL (200μM-2mM) which displayed an upper significant difference ($P < 0.05$) compared with ascorbic acid-free control. The statistical results of the individual test samples for the pro-oxidant test are shown in Appendix C.

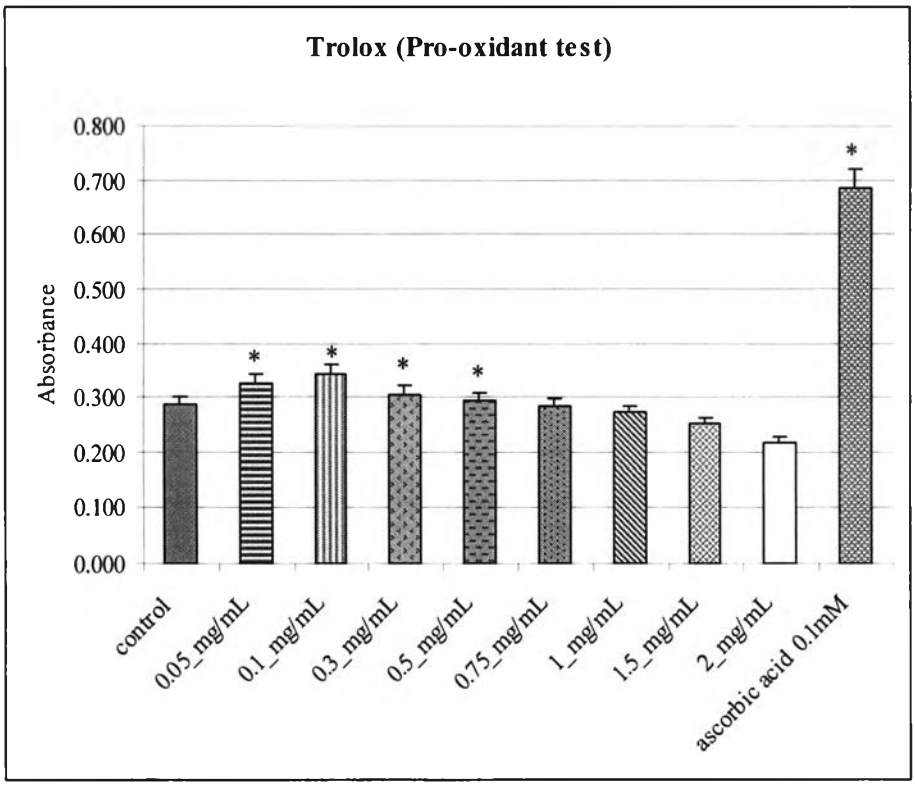

Figure 30. The absorbance of Trolox® at various concentrations (Mean ± SD, n = 3)

* = significantly greater than the ascorbic acid-free control ($p < 0.05$)

As depicted in Figures 29 and 30, Tables 14 and 15, ascorbic acid at the concentration of 0.1 mM exhibited a substantial pro-oxidant effect on the deoxyribose degradation via reducing property to generate hydroxyl radical by Fenton reaction. It

had been reported that pro-oxidant activity of ascorbic acid increased in the range of 0.004-0.24 mM and reached a maximum when the concentration of ascorbic acid was 1.65 mM (Yen et al., 2002).

When comparing among different *P. emblica* extracts and commercial *P. emblica* extract, the acetone (successive) extract seemed to be safer than other extracts or the commercial *P. emblica* extract because it showed lower absorbance compared with the control (lack of pro-oxidant effect) in the wider concentration range (1.50-3.00 mg/mL) and exhibited a slightly higher absorbance than the control (minimum pro-oxidant activity) only at the lowest concentration studied of 1.00 mg/mL (1.03 times). However, the spray-dried *P. emblica* and the acetone (direct) extract showed the pro-oxidant property at concentrations below 2.00 mg/mL and their pro-oxidant activity disappeared at the higher concentration of 3.00 mg/mL in the presence of the transition metal. Additionally, the ethyl acetate, ethanol (successive), ethanol (direct) and the commercial *P. emblica* extract exhibited pro-oxidant effects on deoxyribose degradation at all concentrations studied but the effects also appeared to gradually decrease as the concentration was increased.

Part 3. Evaluation of different *Phyllanthus emblica* extracts for anti-collagenase activity

Collagenase is a metalloproteinase of which the active site zinc ion is important in facilitating the interaction with an inhibitor (Bigg, Clark, and Cawston, 1994). Quantification of anti-collagenase activity of spray-dried *P. emblica* and various solvent extracts was determined by using an EnzChek® gelatinase/collagenase assay kit and the result was compared with the commercial *P. emblica* extract. The strong metal chelator e.g. 1,10-phenanthroline (Bigg et al., 1994) was used as a control inhibitor to optimize incubation time for screening the potential collagenase inhibitors. The fluorescence microplate reader, used to observe the decrease in fluorescence due to the inhibitor compared with Type IV collagenase enzyme activity without inhibitor, was set for excitation at 485 nm and emission detection at 535 nm.

As shown in Table 16 and Figure 31, it was found that during the first hour, slight fluctuation in the percentage of collagenase inhibition due to 1,10-phenanthroline was observed. Then, the extent of inhibition remained relatively stable throughout 180 min at all concentrations except at 0.05 mM where slight decrease was observed. Therefore, the optimized incubation time of 90 min was selected. The result was in agreement with the product information of EnzChek® gelatinase/collagenase assay kit which suggested appropriate incubation time for the reaction of about 1-2 hours.

The percentage of anti-collagenase activity (% collagenase inhibition) of spray-dried *P. emblica* and its various solvent extracts compared to commercial *P. emblica* extract at 90 min incubation time are shown in Figure 32 and Table 17. It was found that the increase in concentration of all test samples led to the increase in anti-collagenase activity. The plot indicated that both ethanol extracts gave similar profiles to the two acetone extracts, whose inhibition efficacy was higher than the spray-dried *P. emblica*, the ethyl acetate and the commercial *P. emblica* extracts, respectively.

Table 16. Percent of collagenase inhibition by various concentrations of 1,10-phenanthroline at different time periods (Mean ± SD, n = 3).

Time	Concentration (mM)									
	0.4		0.3		0.2		0.1		0.05	
(min)	%inh	SD	%inh	SD	%inh	SD	%inh	SD	%inh	SD
10	99.76	0.65	98.42	2.21	96.03	0.74	77.18	2.11	39.92	2.51
15	99.81	1.53	98.57	3.15	95.36	0.23	80.87	0.82	40.30	1.66
20	99.85	1.45	98.80	1.95	95.04	1.03	80.39	1.22	41.01	1.58
30	99.68	0.66	98.24	1.47	94.90	0.15	79.60	1.03	39.84	1.67
45	99.82	0.70	97.97	0.87	94.92	0.62	79.60	1.21	39.62	1.48
60	99.50	0.50	98.41	0.22	95.05	0.72	78.99	0.95	39.90	1.33
75	99.63	0.83	98.33	0.31	94.91	0.91	79.06	0.46	39.62	1.46
90	99.64	0.73	98.30	0.28	94.92	0.91	78.77	0.57	39.46	1.48
105	99.60	0.88	98.28	0.09	94.72	0.74	78.38	0.52	38.94	1.65
120	99.60	0.68	98.30	0.21	94.53	0.87	78.07	0.61	38.40	0.83
135	99.58	0.73	98.28	0.19	94.46	0.88	77.51	0.69	37.96	0.77
150	99.54	0.73	98.22	0.22	94.37	1.04	77.35	0.72	37.24	0.76
165	99.48	0.61	98.23	0.19	94.43	0.80	77.34	1.01	37.03	0.79
180	99.55	0.69	98.21	0.12	94.24	0.90	78.20	2.70	36.44	0.98

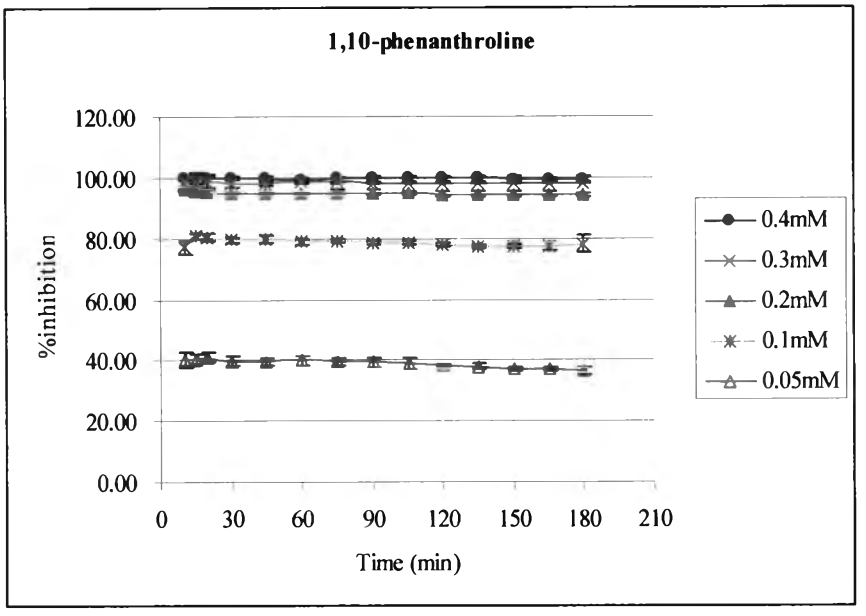

Figure 31. Percent of collagenase inhibition at different times of various concentrations of 1,10-phenanthroline (Mean ± SD, n = 3).

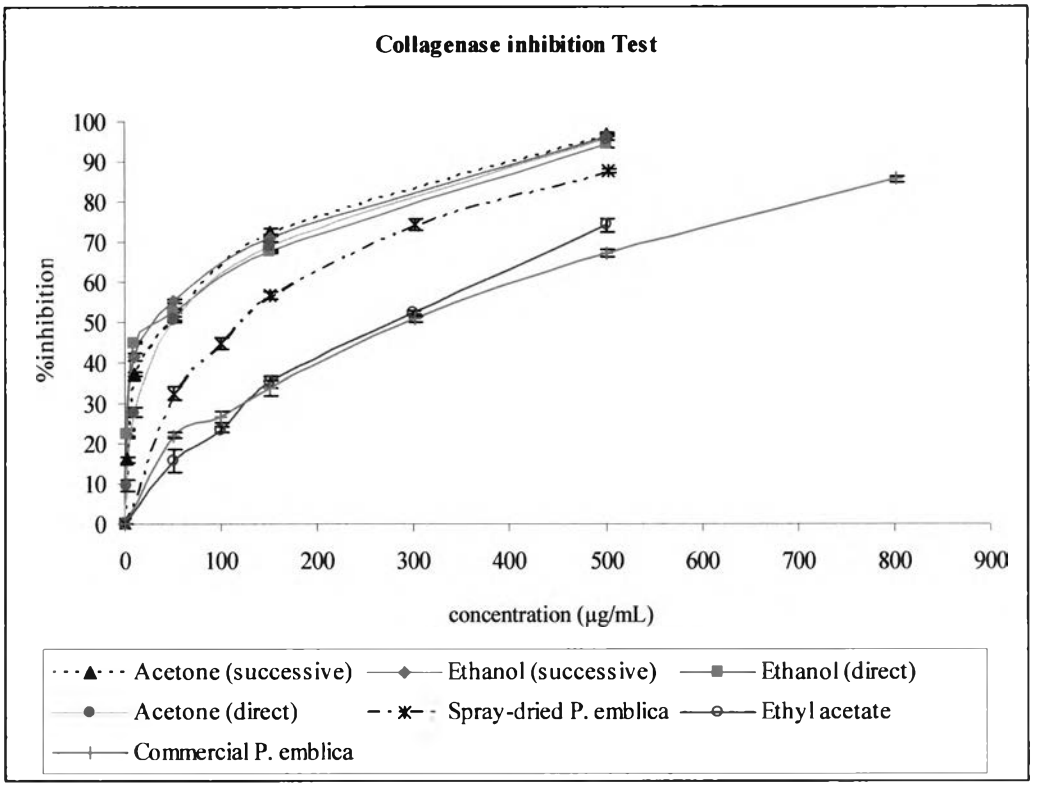

Figure 32. The extent of % collagenase inhibition of spray-dried *P. emblica* and its various solvent extracts compared to commercial extract at various concentrations (Mean ± SD, n = 3).

The ethanol (successive) extract exhibited the percent maximum inhibition of 96.08 ± 0.82% at the concentration of 500μg/mL. This inhibition was slightly lower than that of acetone (successive) extract (96.61 ± 0.33%) and slightly higher than those of acetone (direct) extract (95.68 ± 0.62%) and ethanol (direct) extract (94.19 ± 0.86%) at the same concentration. At this concentration, spray-dried *P. emblica* and its ethyl acetate extract showed the percent collagenase inhibition of 87.49 ± 0.51% and 74.18 ± 1.70%, respectively.

The commercial *P. emblica* extract, on the other hand, had the percent inhibition of only 67.13 ± 0.74% at 500 μg/mL and the inhibition activity could be increased to 85.51 ± 0.74% when the concentration was extended to 800 μg/mL. The raw data for collagenase inhibition percentages are provided in Appendix D.

Table 17. Collagenase inhibition of spray-dried *P. emblica*, its various solvent extracts and the commercial extract at various concentrations (Mean ± SD, n = 3).

No	Conc. (µg/mL)	Spray-dried *P. emblica*		Ethyl acetate		Commercial *P. emblica*		Conc (µg/mL)	Acetone (successive)		Acetone (direct)		Ethanol (successive)		Ethanol (direct)	
		% inh	SD	% inh	SD	% inh	SD		% inh	SD	% inh	SD	% inh	SD	% inh	SD
1	0	0.00	0.00	0.00	0.00	0.00	0.00	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	50	32.52	1.63	15.77	2.74	22.09	0.60	2	16.01	0.73	9.65	1.47	22.53	0.81	22.47	1.12
3	100	44.95	1.44	23.49	0.69	26.70	1.45	10	37.13	0.45	27.82	1.20	41.45	1.04	44.93	0.33
4	150	56.50	0.93	35.32	1.29	33.84	1.75	50	51.40	1.11	50.62	0.58	55.43	0.49	52.57	1.00
5	300	74.17	1.45	52.36	0.35	50.72	0.56	150	72.37	0.75	68.96	1.23	70.86	0.81	67.51	0.42
6	500	87.49	0.51	74.18	1.70	67.13	0.74	500	96.61	0.33	95.68	0.62	96.08	0.82	94.19	0.86
7	800	-	-	-	-	85.51	0.74	-	-	-	-	-	-	-	-	-

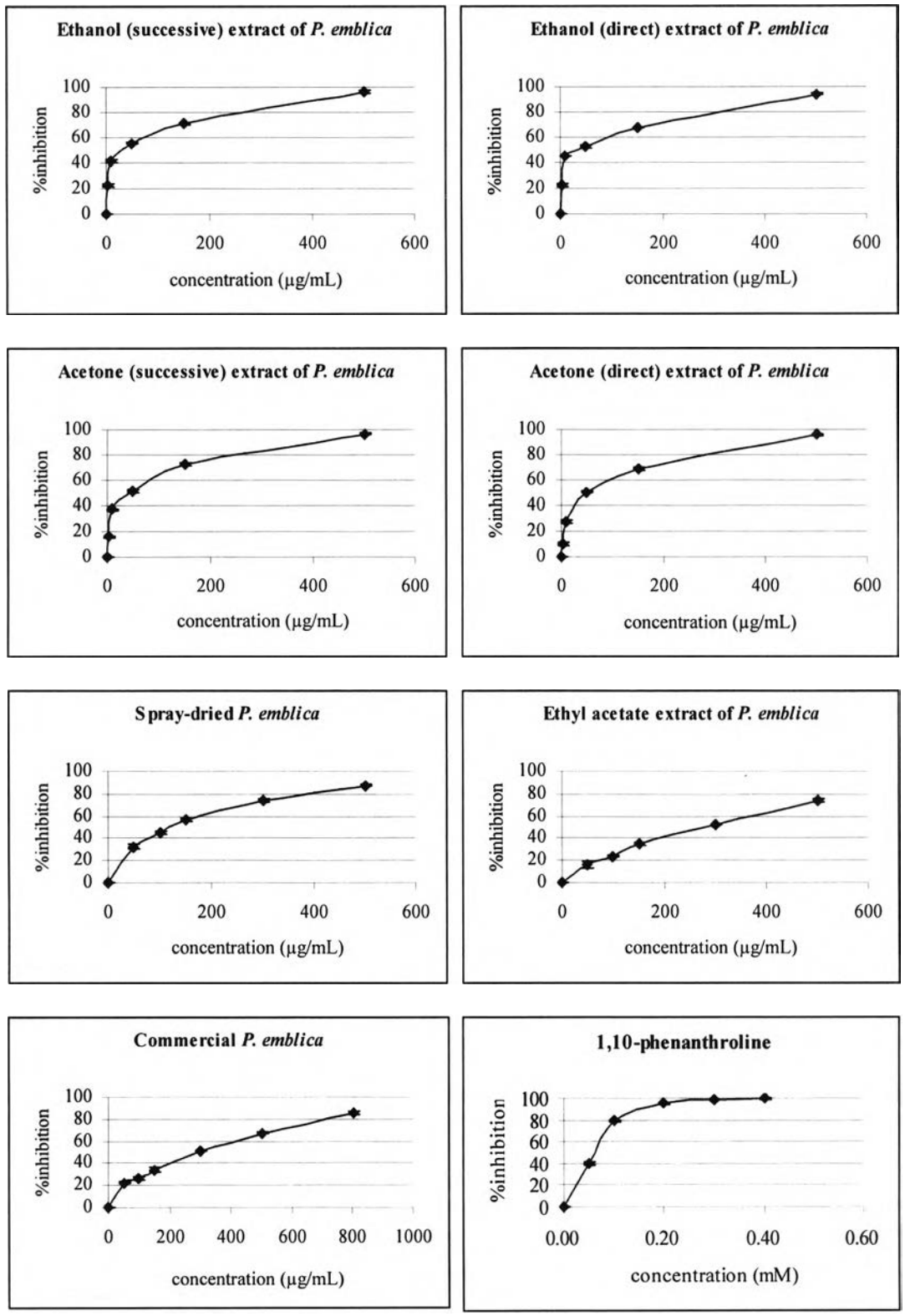

Figure 33. The relationship between percent collagenase inhibition and the concentration of the collagenase inhibitors (Mean ± SD, n = 3).

Figure 33 depicts the individual plots between % collagenase inhibition and concentration for each test sample. The plots clearly confirm that the ethanol (successive) and the ethanol (direct) extracts gave similar profiles to both the acetone (successive) and the acetone (direct) extracts, whose inhibition activity increased highly during the concentration range of 2-50 µg/mL. On the other hand, the spray-dried *P. emblica*, its ethyl acetate extract and the commercial *P. emblica* extract clearly exhibited lower anti-collagenase activity, although the increase was still in a concentration dependent manner.

As presented in Table 16 and Figure 33, at 90 min incubation time, 1,10-phenanthroline showed the increase in % collagenase inhibition, until the concentration of 0.20 mM, to 94.92 ± 0.91%. After that, the inhibitory activity of 1,10-phenanthroline became saturated, reaching a plateau between 0.30 and 0.40 mM, where the inhibition percentage were 98.30 ± 0.28 % and 99.64 ± 0.73 %, respectively.

The concentration at 50% collagenase inhibition (IC_{50}) of each sample was calculated from the equation of the partial polynomial regression and was presented in Figure 34 and Table 18. The means of the estimated IC_{50}, ranked from the lowest to the highest, are as follows: ethanol (successive) extract (32.77 µg/mL ± 1.84), ethanol (direct) extract (37.24 µg/mL ± 3.32), acetone (successive) extract (45.51 µg/mL ± 3.12), acetone (direct) extract (48.56 µg/mL ± 1.08), spray-dried *P. emblica* (121.13 µg/mL ± 4.46), ethyl acetate extract (277.27 µg/mL ± 4.88) and commercial *P. emblica* extract (290.74 µg/mL ± 4.92). Since the lower IC_{50} values reflect the greater inhibitory potency of the collagenase inhibitor, therefore, among *P. emblica* extracts, ethanol (successive) extract gave the highest anti-collagenase activity, whereas the commercial *P. emblica* extract presented the lowest inhibitory potency on the collagenase enzyme.

1,10-Phenanthroline, the control inhibitor, exhibited average IC_{50} value at 90 min of 0.061 ± 0.001 mM equivalent to 12.09 µg/mL. This indicated that 1,10-phenanthroline showed the highest anti-collagenase activity in terms of w/v in comparison with *P. emblica* extracts. However, this compound is used for research purposes only and is not intended for food, drug, household, agricultural or cosmetic use.

Among the various solvent extracts of *P. emblica*, the lowest collagenase inhibitory activity was observed for the ethyl acetate extract followed by the spray-dried *P. emblica*, which was the original substance in the extraction process. Successive extraction by both ethanol and acetone gave extracts with slightly higher inhibition on the collagenase enzyme compared with the direct extracts using these solvents. This result may be due to the successive extraction process which provided greater contents of the active components and less co-occurrence of other contaminating substances than those of the direct extraction process.

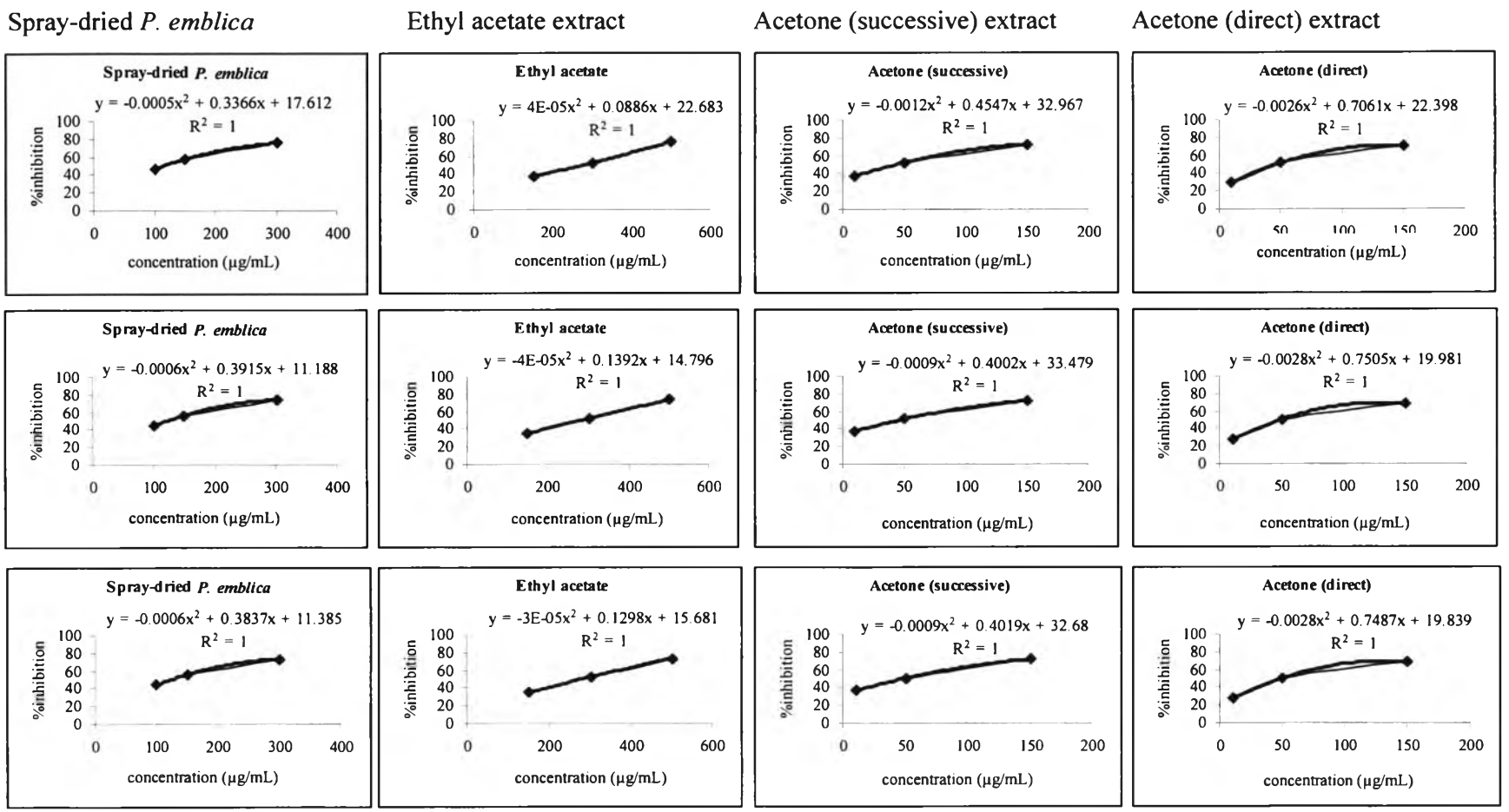

Figure 34. The relationship between percent collagenase inhibition and concentration of the individual inhibitors. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual collagenase inhibitors.

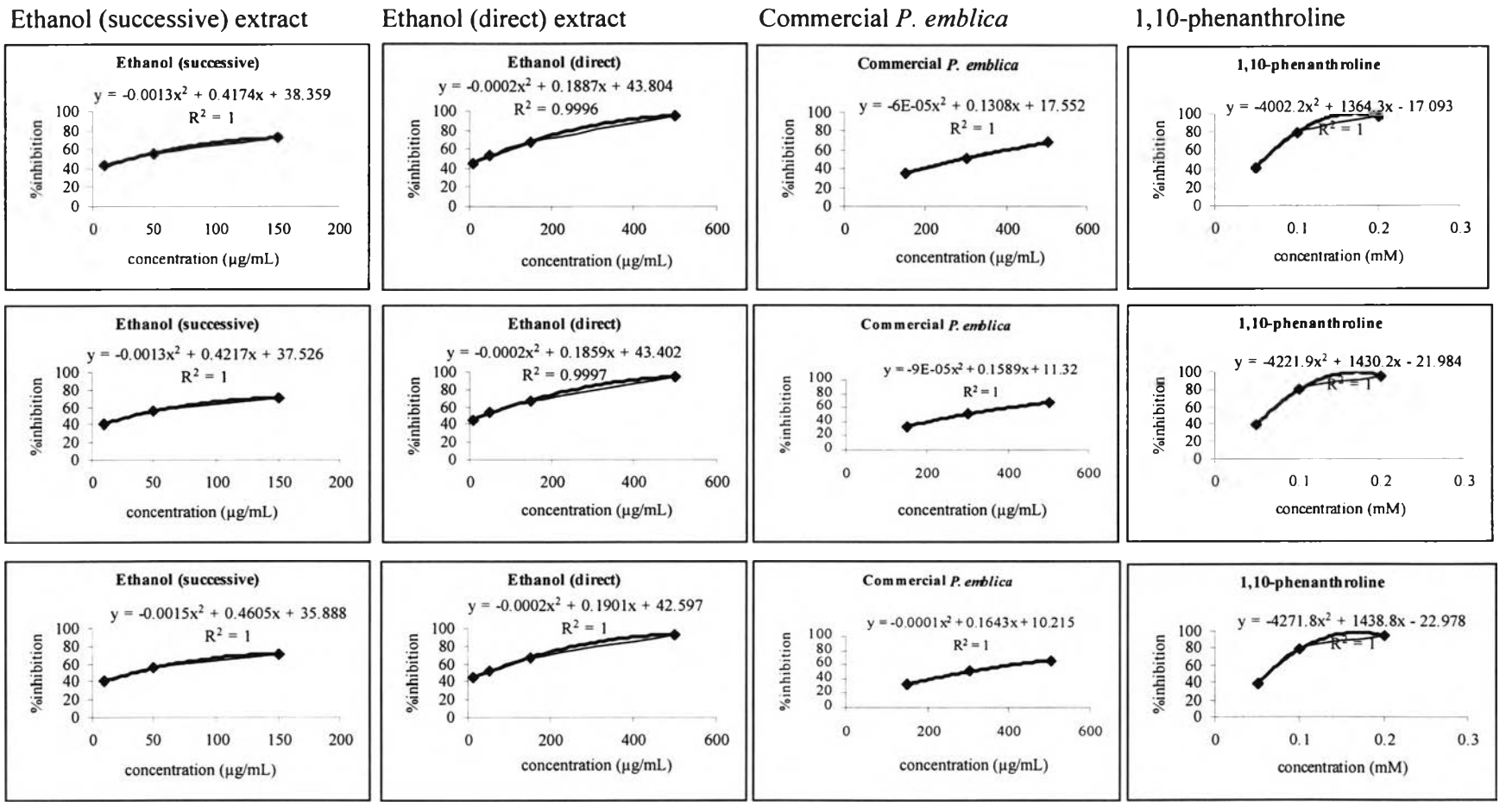

Figure 34 (cont). The relationship between percent collagenase inhibition and concentration of the individual inhibitors. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual collagenase inhibitors.

Table 18. The IC_{50} values for anti-collagenase activity of individual inhibitors. The R^2 is the regression coefficient obtained from polynomial regression of the partial plot between the inhibition percentage and the concentration of each inhibitor (Mean ± SD, n = 3).

Sample	Polynomial equation (partial)					
	IC_{50}(µg/mL)	Mean	SD	R^2	Mean	SD
Ethanol (successive)	30.85	32.77	1.84	1.0000	1.0000	0.00
	32.92			1.0000		
	34.53			1.0000		
Ethanol (direct)	34.07	37.24	3.32	0.9996	0.9998	0.00
	36.96			0.9997		
	40.68			1.0000		
Acetone (successive)	42.15	45.51	3.12	1.0000	1.0000	0.00
	46.05			1.0000		
	48.32			1.0000		
Acetone (direct)	47.34	48.56	1.08	1.0000	1.0000	0.00
	48.93			1.0000		
	49.42			1.0000		
Spray-dried *P. emblica*	116.32	121.13	4.46	1.0000	1.0000	0.00
	121.95			1.0000		
	125.12			1.0000		
Ethyl acetate	274.34	277.27	4.88	1.0000	1.0000	0.00
	274.56			1.0000		
	282.90			1.0000		
Commercial *P. emblica*	285.45	290.74	4.92	1.0000	1.0000	0.00
	291.58			1.0000		
	295.18			1.0000		
1,10-phenanthroline (mM)	0.060	0.061*	0.001	1.0000	1.0000	0.00
	0.061			1.0000		
	0.062			1.0000		

* average IC_{50} value of 1,10-phenanthrolin = 0.061 mM (12.09µg/mL)

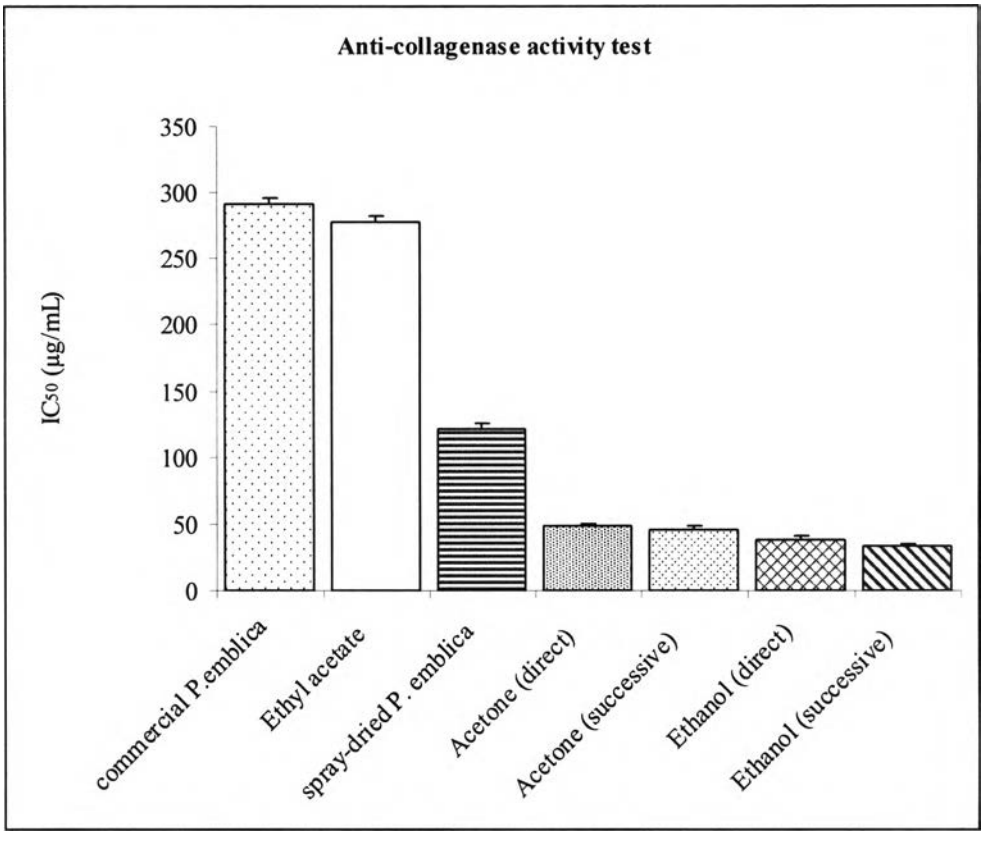

Figure 35. The IC_{50} (µg/mL) of each collagenase inhibitor (Mean ± SD, n = 3).

In Figure 35, it can be seen that the ethanol (successive) and the ethanol (direct) extracts gave the lowest IC_{50} and thus possessed the strongest collagenase inhibitory activity. Acetone (successive) and acetone (direct) extract gave slightly higher IC_{50} values than both the ethanol extracts. The spray-dried *P. emblica* provided higher IC_{50} value than both the ethanol and the acetone extracts, the mean IC_{50} being 3.70 times higher than the ethanol (successive) extract. On the other hand, the IC_{50} values of the ethyl acetate extract and the commercial *P. emblica* extract were 8.46 and 8.87 times, respectively, higher than the ethanol (successive) extract, indicating that they were the least potent collagenase inhibitors.

The IC_{50} data were subsequently analyzed by one-way analysis of variance (ANOVA) at 95% confidence level and Tukey's test. Significant differences were found among the inhibitors studied ($P < 0.05$). According to Tukey's test, the collagenase inhibitors could be divided into 6 different groups ($P < 0.05$) regarding

their ability to inhibit the enzyme, the IC_{50} ranking from the most potent inhibitor group to the least potent was as follows:

Group 1: ethanol (successive) extract ≈ ethanol (direct) extract
Group 2: ethanol (direct) extract ≈ acetone (successive) extract
Group 3: acetone (successive) extract ≈ acetone (direct) extract
Group 4: spray-dried *P. emblica*
Group 5: ethyl acetate extract
Group 6: commercial *P. emblica*

The collagenase inhibitors in the same group were not statistically different in terms of their collagenase inhibition ($P > 0.05$). Both ethanol extracts, thus, demonstrated the highest potency of collagenase inhibition with smallest IC_{50} values. However, their potency was overlapping with the group of acetone extracts. The ethanol (direct) extract was not significantly different from the acetone (successive) extract. A clear distinction among these groups (group 1, 2 and 3) was thus difficult.

On the other hand, the acetone extracts of *P. emblica* were significantly more potent in term of collagenase inhibition than the spray-dried *P. emblica*, which, in turn, was more potent than the ethyl acetate extract. The commercial *P. emblica* extract exhibited the lowest activity giving the highest average IC_{50} value. The statistical results of the individual test samples for collagenase inhibition are shown in Appendix D. Despite the statistical classification of the *P. emblica* extracts into six different groups by the Tukey's multiple comparison test, visual observation of the IC_{50} values in Figure 35 roughly indicated that the extracts could be divided into only three main groups, each with a marked difference in collagenase inhibitory potency, namely the most active inhibitors consiting of the four acetone and ethanol extracts, the intermediate group (spray-dried *P. emblica*), and the least active inhibitors (ethyl acetate and commercial extracts).

Since collagenases, especially type IV collagenase or MMP-2, are responsible for the degradation of several types of collagens involved in cell damage and aging (Creemers et al., 1998; Thibodeau, 2000), the higher the extent of collagenase inhibition, the lower the connective tissue degradation. Therefore, all the ethanol and

acetone extracts of *P. emblica* apparently have potential for use in preventing premature aging. Because the active site of type IV collagenase is the zinc ion (Morgunova et al., 2002), the mechanism of action of these extracts, may be due to their chelating property.

Part 4. Evaluation of different *Phyllanthus emblica* extracts for anti-tyrosinase activity

Tyrosinase is a copper-containing enzyme involved in producing high molecular weight brown pigments (melanins), which is the key physiological defense against sun-induced damages (No et al., 1999; Nerya et al., 2003). Tyrosinase inhibitors have become increasingly important in medication and in cosmetics to prevent hyperpigmentation by inhibiting tyrosinase enzyme (Nerya et al., 2004). The inhibitory effect on tyrosinase activity of the spray-dried *P. emblica* and its various solvent extracts in comparison with the commercial *P. emblica* extract was examined by the DOPAchrome enzymatic method using L-DOPA as the substrate (Sritularak, 2002; Nerya et al., 2004). Dopachrome is one of the intermediate substances in the melanin biosynthesis in which the red color can be detected by visible light. In the present study, a microplate reader (VICTOR®) was used to measure the changes in absorbance at 492 nm due to the formation of DOPAchrome. Licorice extract was used as a reference tyrosinase inhibitor in this study. The incubation time for anti-tyrosinase activity of the test samples was set at 10 min according to the study of Wachiranuntasin (2005). A potent tyrosinase inhibitor would cause a decrease in DOPAchrome absorption.

The percentages of anti-tyrosinase activity (%tyrosinase inhibition) of all test samples at various concentrations are shown in Figure 36 and Table 19. It was found that licorice extract exhibited the highest anti-tyrosinase activity.

Even at low concentration of 0.005 mg/mL licorice extract already exhibited the tyrosinase inhibitory activity of 80.56 ± 3.58 % as shown in Table 19. After that, the inhibitory activity of licorice extract became saturated, reaching a plateau in the range of 0.01-0.05 mg/mL, where the inhibition percentages were 84.97-88.59 %.

Among the *P. emblica* extracts, the ethyl acetate extract exhibited the highest tyrosinase inhibition, giving the maximum inhibition of 88.45 ± 2.60% at the highest concentration of 2.40 mg/mL. On the other hand, commercial *P. emblica* extract provided the % tyrosinase inhibition of 37.75 ± 1.39% at 1.20 mg/mL concentration, after which the inhibition extent increased gradually to 69.14 ± 2.37 % at 2 mg/mL, where the plateau was apparently reached as seen in Table 19 and Figure 36. Its anti-tyrosinase activity slightly increased to 71.54 ± 1.10% at 2.40 mg/mL.

At 2.40 mg/mL, the acetone (successive) extract provided the anti-tyrosinase activity similar to the ethanol (direct) extract, where the inhibition percentages were 77.37 ± 2.08% and 75.48 ± 2.07%, respectively. However, the plot of the acetone (successive) extract exhibited a slightly higher potency than that of the ethanol (direct) extract in the lower concentration range of 0.00-1.60 mg/mL, but at 2.00 mg/mL the ethanol (direct) extract converted to show a higher potency than the acetone (successive) extract.

The tyrosinase inhibitory activity of the acetone (direct) extract showed a lower profile than the ethyl acetate extract at all concentrations except at the concentration of 2.40 mg/mL where the acetone (direct) extract gave 93.38 ± 1.89 % inhibition which was slightly higher than that of the ethyl acetate extract (88.45 ± 2.60 %).

Spray-dried *P. emblica* showed a moderate increase in anti-tyrosinase activity. The percent tyrosinase inhibition of spray-dried *P. emblica* just increased to 57.67 ± 1.00% at 2.40 mg/mL. However, it was still higher than that of the ethanol (successive) extract which gave 51.40 ± 1.69% inhibition at the same concentration. The ethanol (successive) extract also exhibited the lowest potency compared to other *P. emblica* extracts since further increasing of its concentration to 2.80 mg/mL, the inhibition was only marginally increased its inhibitory potency to 60.47 ± 1.13%.

The relationships between the tyrosinase inhibition percentage and the concentration of the individual test samples are shown in Figure 37. The plots clearly indicated that both acetone (direct) and acetone (successive) extracts gave very similar profiles but at different levels, the direct extract being higher than the successive extract. The anti-tyrosinase activity increased in a concentration-dependent manner for both extracts and there appeared to be a sharp rise in activity from 2.0 to 2.40 mg/mL in both profiles without reaching saturation.

Likewise, the tyrosinase inhibition profiles of the ethanol (direct) extract and the ethanol (successive) extract were similar, although at different levels. The ethanol (direct) extract more efficiently inhibited tyrosinase enzyme than the ethanol (successive) extract. Their profiles showed a flat line in the beginning and tended to increase until reaching saturation at concentration of 2.00 mg/mL or higher.

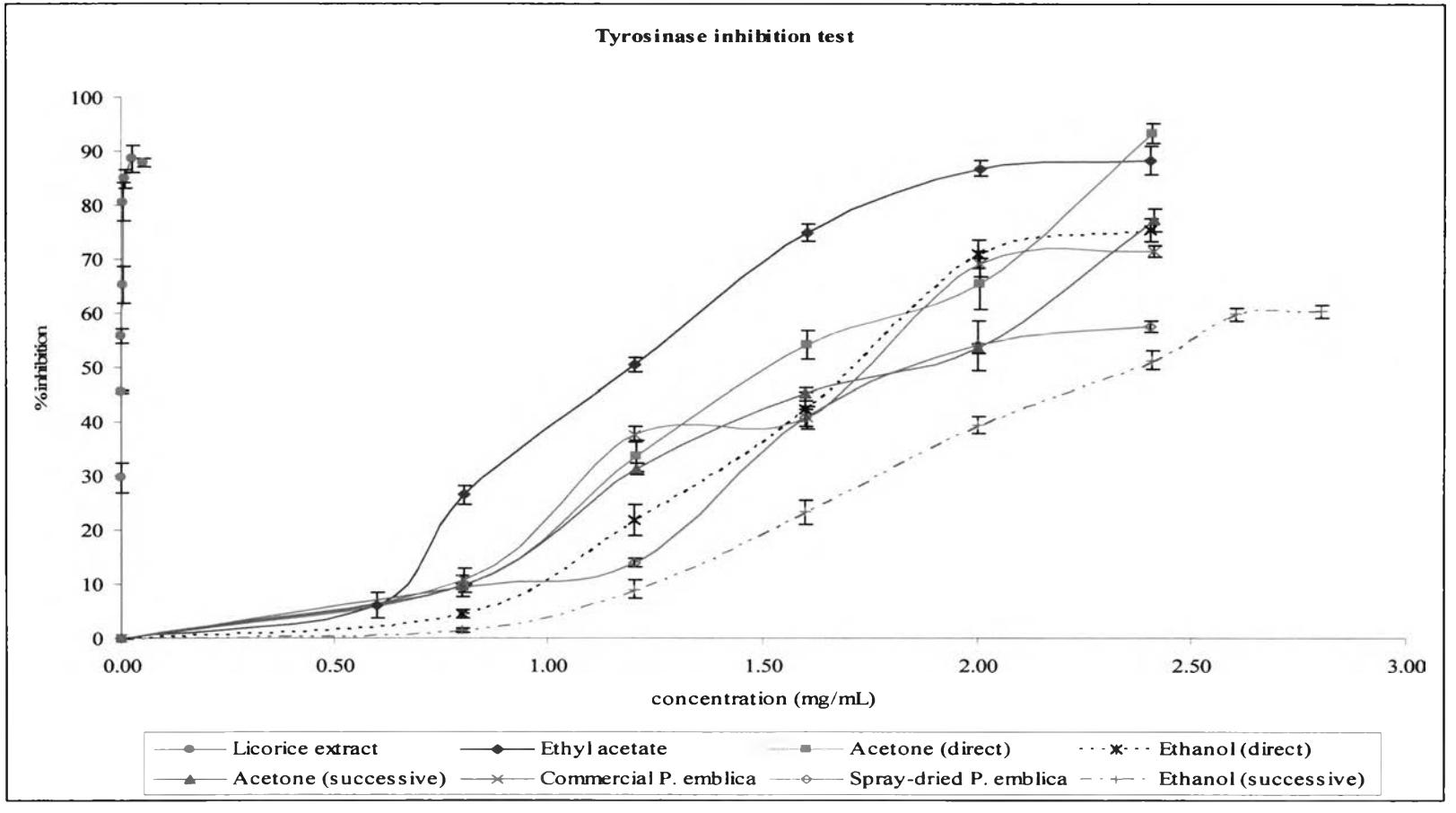

Figure 36. The extent of % tyrosinase inhibition of spray-dried *P. emblica* and its various solvent extracts compared to commercial extract and licorice extract at various concentrations (Mean ±SD, n = 3).

Table19. Tyrosinase inhibition of spray-dried *P. emblica* and its various solvent extracts compared to commercial extract and licorice extract at various concentrations (Mean ± SD, n = 3).

No	Conc	Ethyl acetate		Spray-dried *P. emblica*		Acetone (successive)		Acetone (direct)		Ethanol (successive)		Ethanol (direct)		Commercial *P. emblica*		Conc	Licorice extract	
	(mg/mL)	% inh	SD	% inh	SD	% inh	SD	% inh	SD	% inh	SD	% inh	SD	% inh	SD	(mg/mL)	% 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	0.00	0.00	0.0000	0.00	0.00
2	0.60	6.14	2.34	-	-	-	-	-	-	-	-	-	-	-	-	0.0003	29.65	2.72
3	0.80	26.46	1.82	9.60	1.93	9.73	0.29	9.80	0.13	1.51	0.37	4.48	0.79	10.69	2.27	0.0006	45.61	0.26
4	1.20	50.51	1.32	13.98	0.80	31.23	1.09	33.71	2.91	9.01	1.73	21.75	2.89	37.75	1.39	0.0013	55.83	1.40
5	1.60	74.91	1.61	40.77	1.50	45.21	1.21	54.24	2.59	23.35	2.18	42.36	3.16	40.73	2.17	0.0025	65.20	3.35
6	2.00	86.90	1.48	54.12	4.62	53.59	0.90	65.49	4.70	39.50	1.54	71.05	2.74	69.14	2.37	0.0050	80.56	3.58
7	2.40	88.45	2.60	57.67	1.00	77.37	2.08	93.38	1.89	51.40	1.69	75.48	2.07	71.54	1.10	0.0100	84.97	1.73
8	2.60	-	-	-	-	-	-	-	-	59.93	1.13	-	-	-	-	0.0250	88.59	2.48
9	2.80	-	-	-	-	-	-	-	-	60.47	1.13	-	-	-	-	0.0500	87.98	0.76

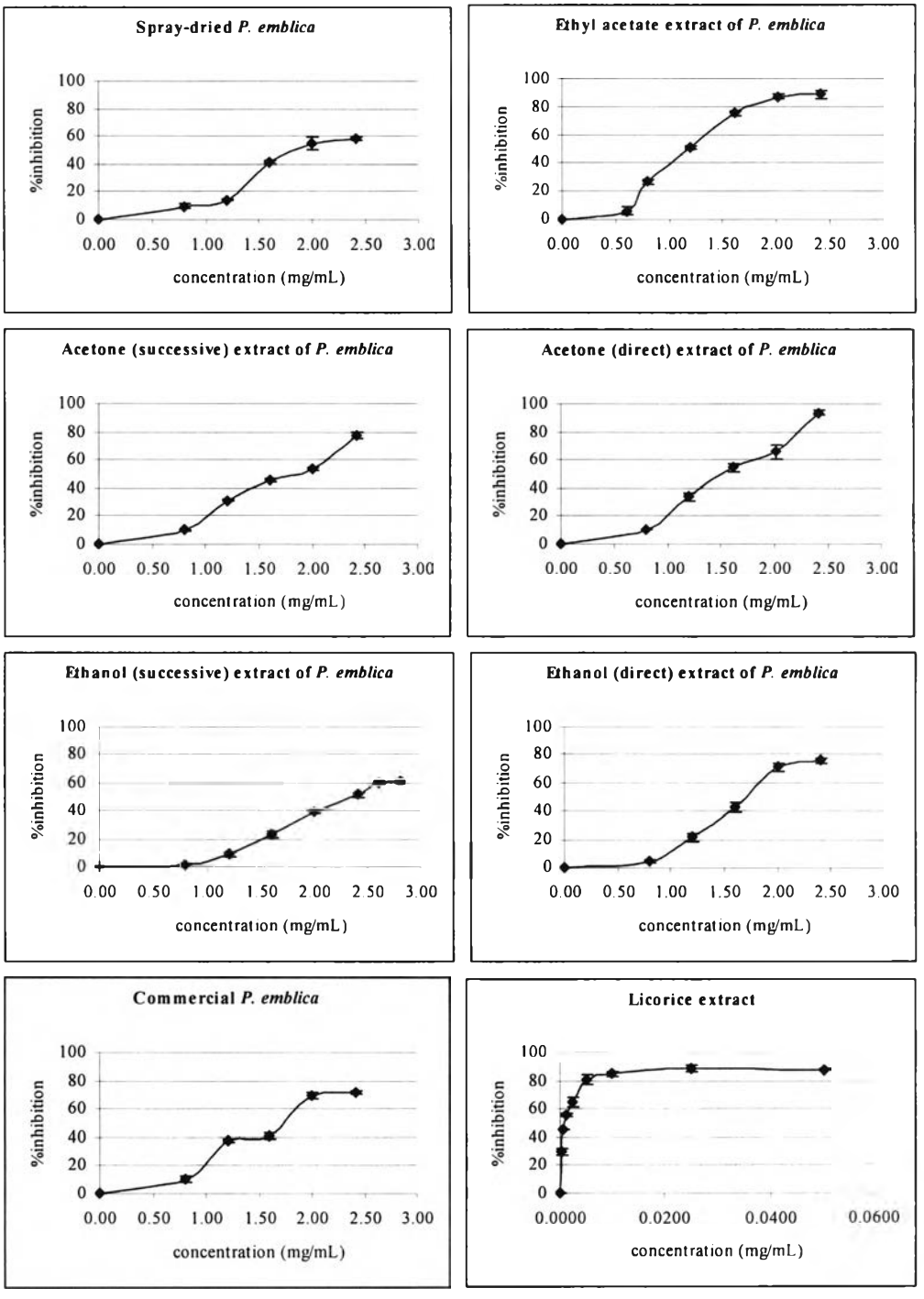

Figure 37. The relationship between percent tyrosinase inhibition and concentration of the individual tyrosinase inhibitors (Mean ± SD, n = 3).

Ethyl acetate extract also exhibited minimal inhibitory potency at the beginning of the concentration range tested. But at 0.80 mg/mL, its inhibition extent rose markedly and was higher than those of the acetone and the ethanol extracts. Spray-dried *P. emblica*, on the other hand, was much less effective, showing a small rise of activity in the concentration range of 0.00-1.20 mg/mL, after which a slight increase in the activity was observed until 2.40 mg/mL, although it was always lower than the ethyl acetate extract.

Commercial *P. emblica* extract showed a uniquely different profile, displaying the step increment of the curve. In the range of 1.20-1.60 mg/mL, the increase of its inhibitory activity was not much. However, the commercial *P. emblica* extract still provided greater anti-tyrosinase activity than the spray-dried *P. emblica* and the ethanol (successive) extract. The raw data for tyrosinase inhibition percentages are provided in Appendix E.

The concentration at 50% tyrosinase inhibition (IC_{50}) of each sample was calculated from the equation of the partial polynomial plot and was depicted in Figure 38 and Table 20. The means of the estimated IC_{50} can be ranked from the lowest to highest value as follows: licorice extract (0.88 μg/mL ± 0.00), ethyl acetate extract (1.19 mg/mL ± 0.02), acetone (direct) extract (1.51 mg/mL ± 0.05), ethanol (direct) extract (1.73 mg/mL ± 0.05), commercial *P. emblica* extract (1.78 mg/mL ± 0.04), acetone (successive) extract (1.79 mg/mL ± 0.05), spray-dried *p. emblica* (1.85 mg/mL ± 0.08) and ethanol (successive) extract (2.35 mg/mL ± 0.07). Since the lower IC_{50} values indicate greater inhibitory potency of a tyrosinase inhibitor. Therefore, the licorice extract exhibited the highest anti-tyrosinase activity. Among different *P. emblica* extracts, the ethyl acetate extract appeared to give the highest tyrosinase inhibitory activity, whereas the ethanol (successive) extract showed the lowest potency.

It is interesting to note that polarity of the solvent played an important role in the anti-tyrosinase activity of the extract. The less polar solvent used, the higher the anti-tyrosinase activity. Thus, ethyl acetate extract, the least polar solvent used in this study, provided an effect with the highest potency. Likewise, the less polar acetone extract was more potent than the ethanol extract, given the same extraction procedure. Similarly, the direct extraction process provided greater contents of the less polar

components than the successive extraction process, by which much of the active, less polar components had been removed from the extract.

As a result, the acetone (direct) extract exhibited higher inhibitory potency on the tyrosinase enzyme than the acetone (successive) extract. Also, the ethanol (direct) extract provided greater anti-tyrosinase activity than that of the ethanol (successive) extract.

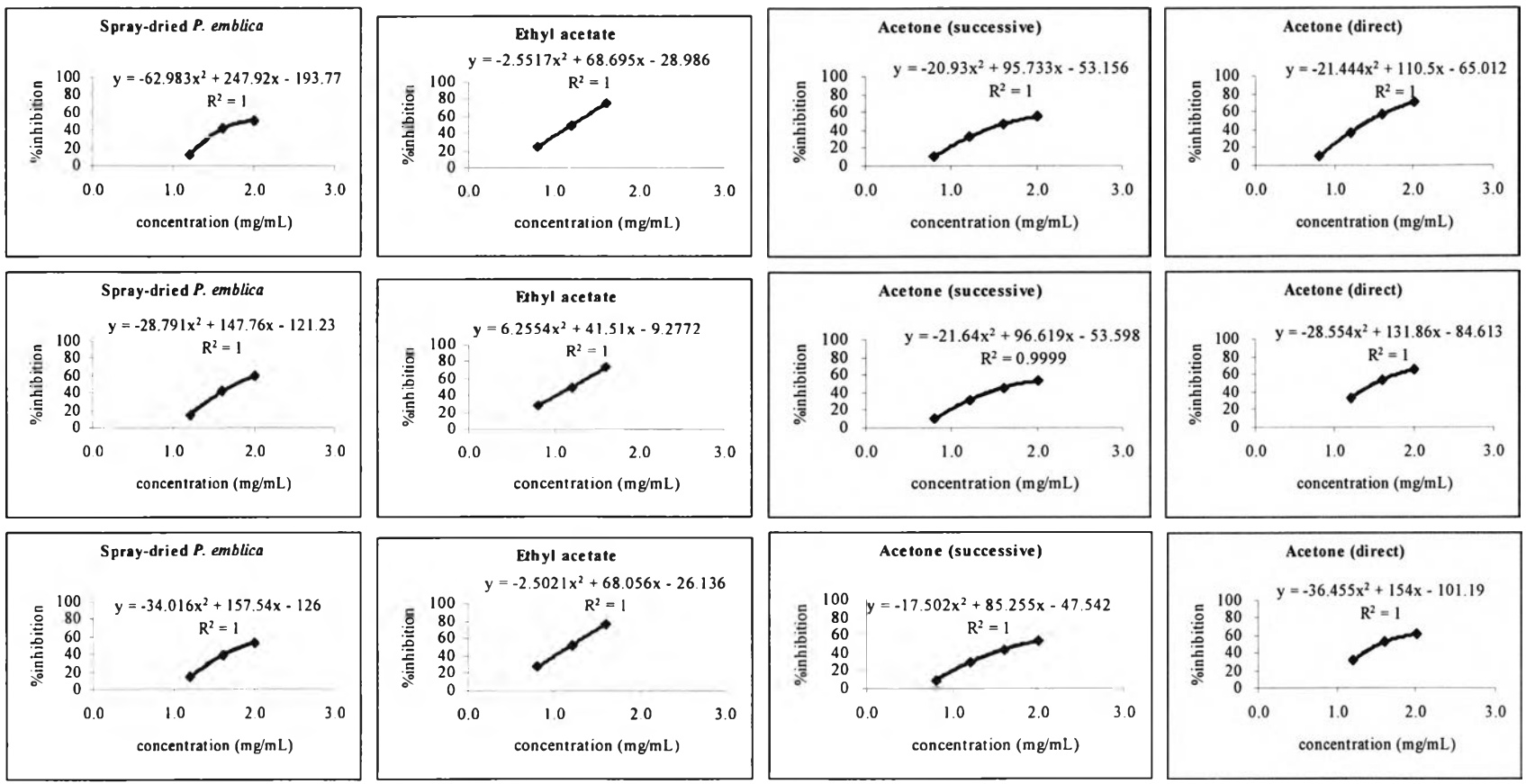

Figure 38. The relationship between percent tyrosinase inhibition and concentration of the individual inhibitors. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual tyrosinase inhibitors.

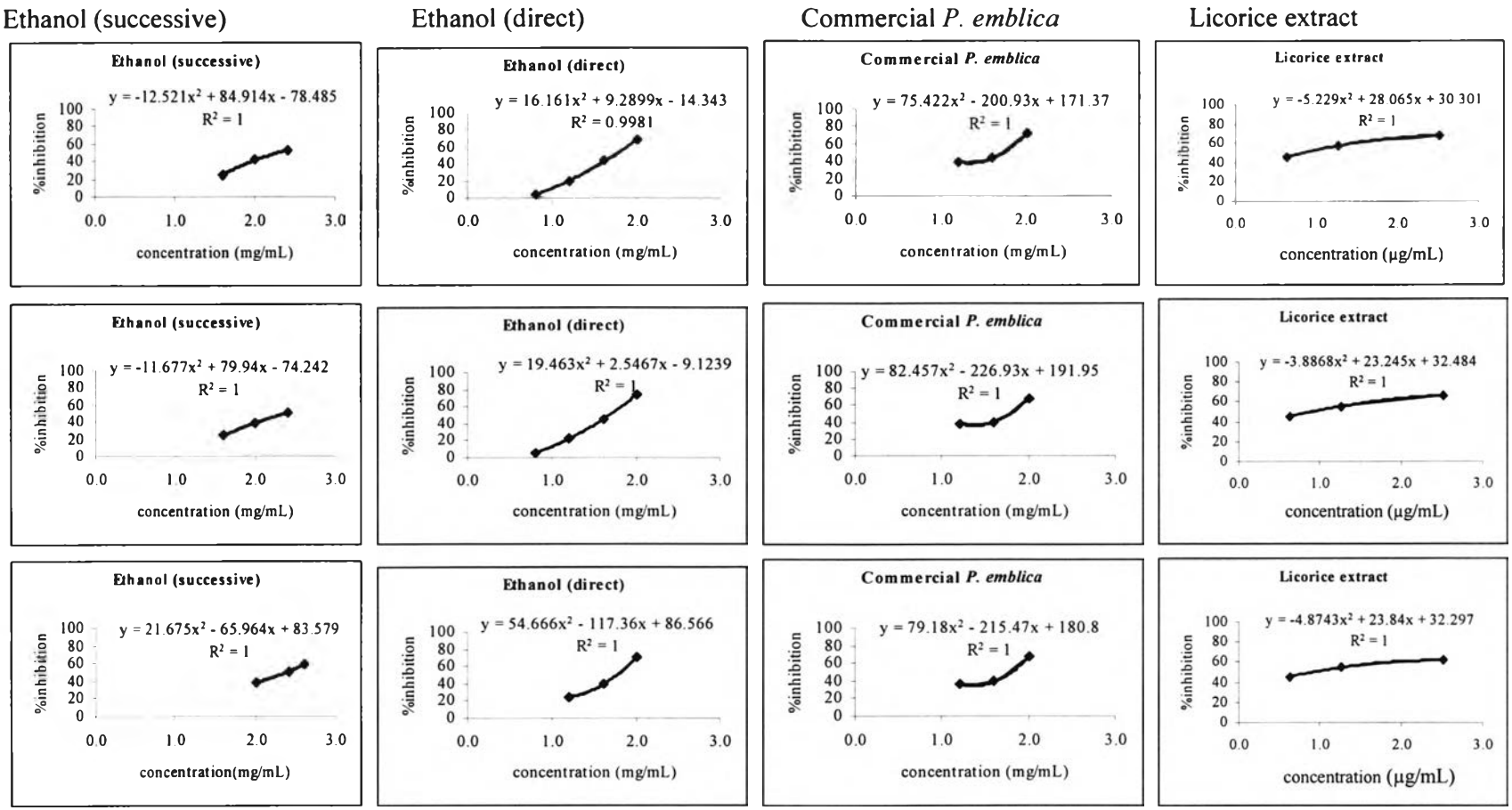

Figure 38 (cont). The relationship between percent tyrosinase inhibition and concentration of the individual inhibitors. The polynomial regression equation for determining the IC_{50} and the regression coefficient (R^2) are also provided for the individual tyrosinase inhibitors.

Table 20. The IC_{50} values for anti-tyrosinase activity of the individual inhibitors. The R^2 is the regression coefficient obtained from polynomial regression of the partial plot between the inhibition percentage and the concentration of each inhibitor (Mean ± SD, n = 3).

Sample	Polynomial equation (partial)					
	IC_{50} (mg/mL)	Mean	SD	R^2	Mean	SD
Licorice extract (μg/mL)	0.83 0.88 0.91	0.88	0.00	1.0000 1.0000 1.0000	1.0000	0.00
Ethyl acetate	1.20 1.21 1.17	1.19	0.02	1.0000 1.0000 1.0000	1.0000	0.00
Acetone (direct)	1.45 1.52 1.55	1.51	0.05	1.0000 1.0000 1.0000	1.0000	0.00
Ethanol (direct)	1.73 1.68 1.77	1.73	0.05	0.9996 0.9997 1.0000	0.9998	0.00
Commercial *P. emblica*	1.74 1.79 1.81	1.78	0.04	1.0000 1.0000 1.0000	1.0000	0.00
Acetone (successive)	1.74 1.79 1.84	1.79	0.05	1.0000 0.9999 1.0000	1.0000	0.00
Spray-dried *P. emblica*	1.91 1.77 1.88	1.85	0.08	1.0000 1.0000 1.0000	1.0000	0.00
Ethanol (successive)	2.28 2.39 2.40	2.35	0.07	1.0000 1.0000 1.0000	1.0000	0.00

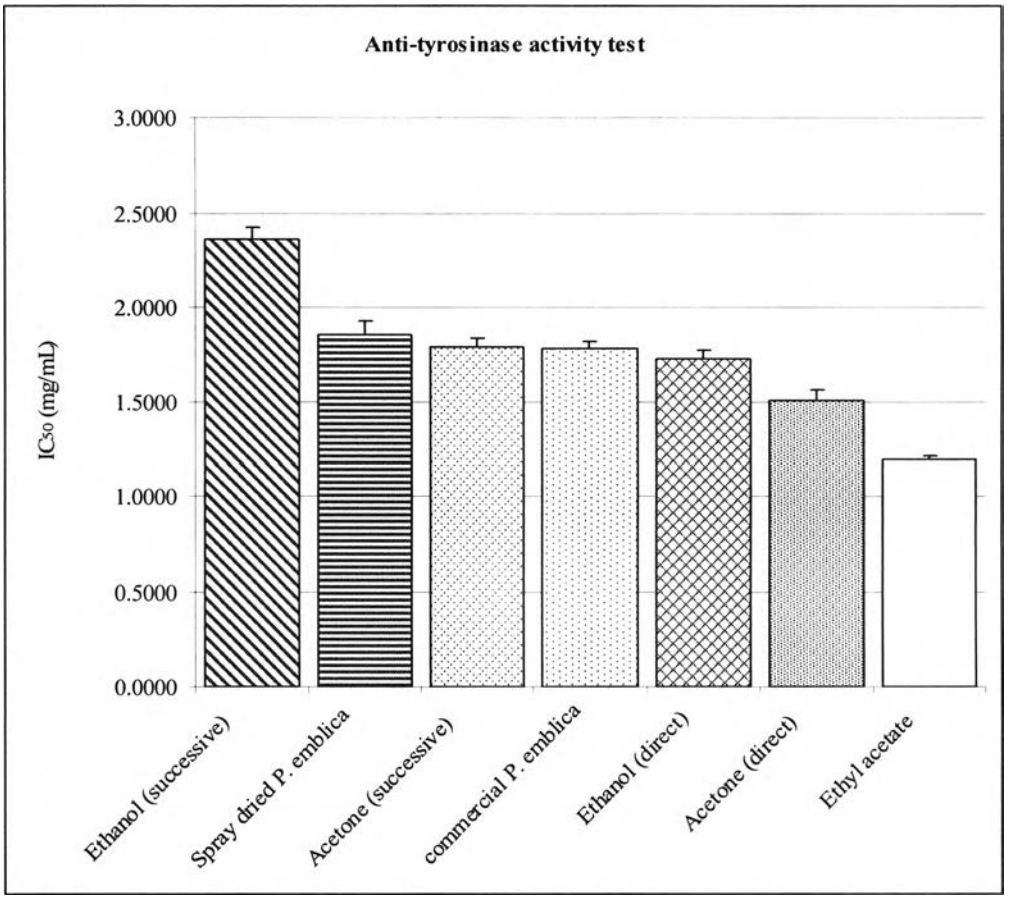

Figure 39. The IC_{50} (mg/mL) of each tyrosinase inhibitor (Mean ± SD, n = 3).

Figure 39 is a histogram comparing the mean IC_{50} values among different *P. emblica* extracts. The IC_{50} of licorice extract is not included here because its value is too small. All the *P. emblica* extracts exhibited the IC_{50} values above 1.000 mg/mL. However, the ethyl acetate extract gave the lowest IC_{50}, thus possessing the strongest tyrosinase inhibitory activity. On the other hand, the ethanol (successive) extract showed the highest IC_{50} thereby suggesting that it was a weak tyrosinase inhibitor.

The IC_{50} data were subsequently analyzed by one-way analysis of variance (ANOVA) at 95% confidence followed by Tukey's test. Significant differences were found among the inhibitors studied ($P < 0.05$). According to Tukey's test, the tyrosinase inhibitors could be divided into 5 different groups ($P < 0.05$) with respect to their ability to inhibit the tyrosinase enzyme. The IC_{50} grouping, in an increasing order, is as follows:

Group 1: licorice extract

Group 2: ethyl acetate extract

Group 3: acetone (direct) extract

Group 4: ethanol (direct) extract ≈ commercial *P. emblica* ≈ acetone (successive) extract ≈ spray-dried *P. emblica*

Group 5: ethanol (successive) extract

The tyrosinase inhibitors in the same group are not statistically different in term of their tyrosinase inhibition ($P > 0.05$). Licorice extract used as a reference tyrosinase inhibitor was the most potent inhibitor with the smallest IC_{50} values. It potency was significantly greater than the ethyl acetate extract, which, in turn, was more potent than the acetone (direct) extract ($P < 0.05$). Spray-dried *P. emblica*, acetone (successive) extract, commercial *P. emblica* extract and ethanol (direct) extract were not significantly difference from each other in terms of their inhibitory potency ($P > 0.05$). However, this group was significantly more effective than the ethanol (successive) extract. The statistical analysis thus further confirmed our previous postulation that the anti-tyrosinase activity of *P. emblica* depends on the availability of the less polar components in the extract. The statistical results of the individual test samples for tyrosinase inhibition are shown in Appendix E.

Although the anti-tyrosinase activity of *P. emblica* extract was not high compared with the licorice extract, it has been suggested that it could provide the skin lightening activity in the clinical study by Chaudhuri (2004), in which 2% *P. emblica* extract was reported to be as effective as 2% hydroquinone after 6 and 9 weeks of application. Therefore, *P. emblica* may have additional pathway to augment its whitening activity. The possible mechanism could be due to its ability to scavenge reactive nitrogen species like nitric oxide ($NO^{\cdot}$). Nitric oxide is involved in the increase of cGMP and the activation of cGMP-dependent protein kinase, leading to the stimulation of melanogenesis. The nitric oxide ($NO^{\cdot}$) scavenging activity of triphala which is a mixture of *Terminalia chebula*, *Terminalia bellerica* and *Phyllanthus emblica* (1:1:1) was demonstrated in the study of Jagetia et al. (2004), thereby suggesting its potential for skin whitening activity through this mechanism.

Part 5. Preliminary stability evaluation of *Phyllanthus emblica* extracts

The 1,1-diphenyl-2-picrylhydrazine (DPPH) method was used to determine the solid-state stability in terms of antioxidant activity of spray-dried *P. emblica* and its various solvent extracts in comparison with commercial *P. emblica* extract. The decrease in absorbance at 517nm of the DPPH radical (deep purple) after the addition of an antioxidant compound in an ethanolic solution was measured at 10 µg/mL and 20 µg/mL of each test sample for the preliminary stability evaluation. The ethyl acetate extract was further investigated at the concentration of 50 µg/mL (concentration in the saturation region) to see if the experimental condition had any effect on the stability profiles.

The remaining antioxidant activity was investigated at various times (0, 6, 9 months). Table 21 depicts the average % inhibition (Mean ± SD of triplicate determinations) of all *P. emblica* extracts kept at ambient temperature. It was found that after 9-month storage, the DPPH scavenging activity of spray-dried *P. emblica* slightly decreased from 68.93% to 62.86 % at concentration of 10 µg/mL and 92.45 % to 89.16 % at 20 µg/mL. Likewise, commercial *P. emblica* extract presented a decrease in the DPPH inhibitory activity from 65.54 % to 59.75 at 10 µg/mL and 92.71 % to 90.77 % at 20 µg/mL after 9-month storage. On the other hand, five solvent extracts of *P. emblica* including ethyl acetate, acetone (successive), acetone (direct), ethanol (successive) and ethanol (direct) extracts provided the DPPH scavenging activity after 9-month storage comparable to initial value at all concentration. The profiles of the absolute % DPPH inhibition at different storage times are presented in Figures 40 and 41 for individual test samples at 10 and 20 µg/mL, respectively.

The percent DPPH inhibitory activity of each antioxidant during the 9-month storage period are shown in Figure 42. The plots clearly indicated that all solvent extracts of *P. emblica* were acceptably stable at both 10 and 20 µg/mL over the storage time studied. On the other hand, the spray-dried and the commercial *P. emblica* extract exhibited a slight decrease in % DPPH inhibition, especially at 10 µg/mL after 9 months because concentration at 20 µg/mL was near saturation point where DPPH was almost used up and caused about 90% absolute inhibition.

Addition of different antioxidants at different concentrations was shown to give varying degree of %DPPH inhibition. Since the initial values of each sample were not the same, the values of the absolute percent inhibition were subsequently normalized to percent inhibition relative to the initial value (Table 22). From this Table, spray-dried *P. emblica* and commercial *P. emblica* extract exhibited a slight decrease and comparable values in the remaining % relative DPPH scavenging activity at both 10 and 20 μg/mL concentrations. The spray-dried *P. emblica* exhibited the remaining % relative DPPH inhibition of 91.19 % and 96.44 % at 10 μg/mL and 20 μg/mL, respectively. Likewise, commercial *P. emblica* extract presented the remaining % relative DPPH inhibition as 91.17 % and 97.91 % at 10 and 20 μg/mL, respectively, after 9-month storage. On the other hand, the five solvent extracts of *P. emblica* did not show any decrease in the extent of DPPH inhibition, all of which gave the remaining % relative inhibition values in the range of 99.00-107.87 %. The profiles of the relative % DPPH inhibition at different storage times are presented in Figures 43 and 44 for individual test samples at 10 and at 20 μg/mL, respectively.

Slight difference in the stability between the five solvent extracts and the spray-dried extract might be due to the nature of the solvent and components of each extract.

The spray-dried extract was an aqueous extract and, thus, was likely to have high contents of moisture and water-soluble components that could be susceptible to hydrolysis or other water-induced degradation.

The solvent extracts, on the other hand, may contain less moisture and fewer components that were susceptible to aqueous degradation, resulting in greater stability. However, this should be further confirmed by comparing the moisture contents among different extracts. It is interesting to note that the commercial *P. emblica* extract also demonstrated a profile very similar to the spray-dried extract, suggesting that it might also be a water-based extract.

Table 21. Stability of spray-dried *P. emblica*, various solvent extracts of *P. emblica* and commercial *P. emblica* extract as determined from % DPPH inhibitory activity (Mean ± SD, n = 3).

Conc. (µg/mL)	Spray-dried *P. emblica*		Ethyl acetate			Acetone (successive)		Acetone (direct)		Ethanol (successive)		Ethanol (direct)		Commercial *P. emblica*	
	10	20	10	20	50	10	20	10	20	10	20	10	20	10	20
Time (month)	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD
0	68.93	92.45	57.51	77.54	95.06	74.95	92.69	82.84	95.26	88.65	95.72	81.51	95.54	65.54	92.71
	±0.47	±0.59	±0.64	±0.45	±0.21	±1.88	±0.77	±0.65	±0.14	±0.74	±0.00	±0.46	±0.13	±0.69	±0.14
6	63.46	88.54	57.48	80.98	94.77	79.73	94.19	81.07	94.82	90.28	95.27	89.53	95.27	65.45	89.87
	±0.76	±0.75	±0.38	±1.04	±0.25	±1.18	±0.29	±1.35	±0.56	±0.50	±0.00	±0.25	±0.00	±0.38	±0.29
9	62.86	89.16	58.25	81.08	94.12	80.85	94.35	82.12	94.23	89.97	94.12	84.54	94.58	59.75	90.77
	±0.20	±0.20	±0.40	±0.80	±0.00	±0.20	±0.20	±0.20	±0.20	±0.35	±0.00	±0.80	±0.20	±0.87	±0.20

Table 22. Stability of spray-dried *P. emblica*, various solvent extracts of *P. emblica* and commercial *P. emblica* extract as determined from % DPPH inhibitory activity relative to their initial value (Mean ± SD, n = 3).

Conc. (µg/mL)	Spray-dried *P. emblica*		Ethyl acetate			Acetone (successive)		Acetone (direct)		Ethanol (successive)		Ethanol (direct)		Commercial *P. emblica*	
	10	20	10	20	50	10	20	10	20	10	20	10	20	10	20
Time (month)	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD	%inh ±SD
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	±0.47	±0.59	±0.64	±0.45	±0.21	±1.88	±0.77	±0.65	±0.14	±0.74	±0.00	±0.46	±0.13	±0.69	±0.14
6	92.06	95.77	99.95	104.44	99.96	106.38	101.62	97.86	99.54	101.84	99.53	109.84	99.72	99.86	96.94
	±0.76	±0.75	±0.38	±1.04	±0.25	±1.18	±0.29	±1.35	±0.56	±0.50	±0.00	±0.25	±0.00	±0.38	±0.29
9	91.19	96.44	101.29	104.57	99.01	107.87	101.79	99.13	98.92	101.49	98.33	103.72	99.00	91.17	97.91
	±0.20	±0.20	±0.40	±0.80	±0.00	±0.20	±0.20	±0.20	±0.20	±0.35	±0.00	±0.80	±0.20	±0.87	±0.20

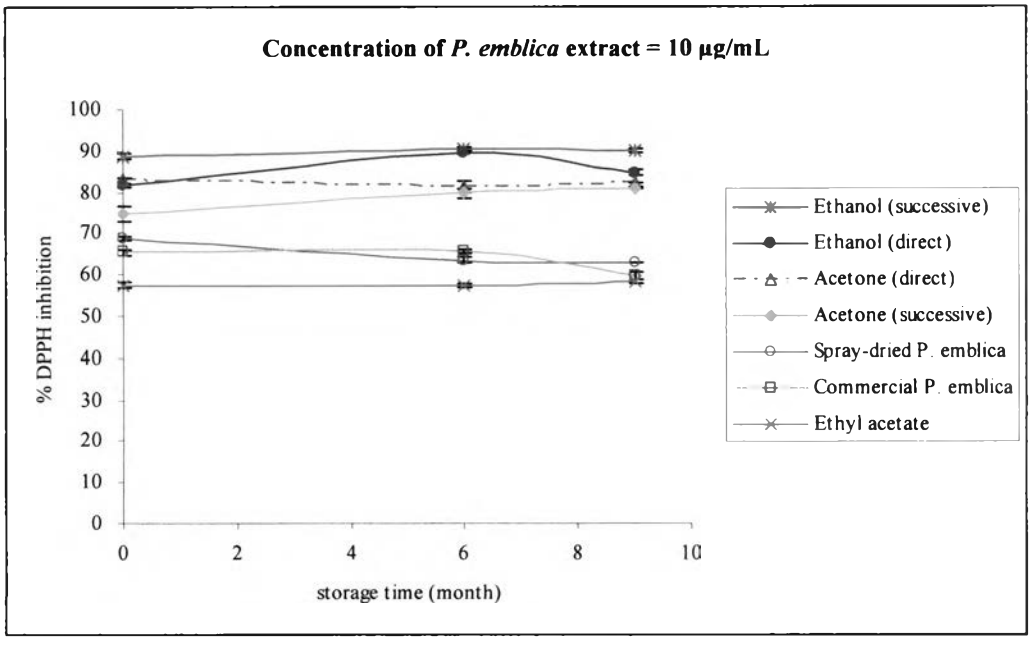

Figure 40. Percent DPPH inhibitory activity (prepared at 10 µg/mL) after storage up to 9 months (Mean ± SD, n = 3).

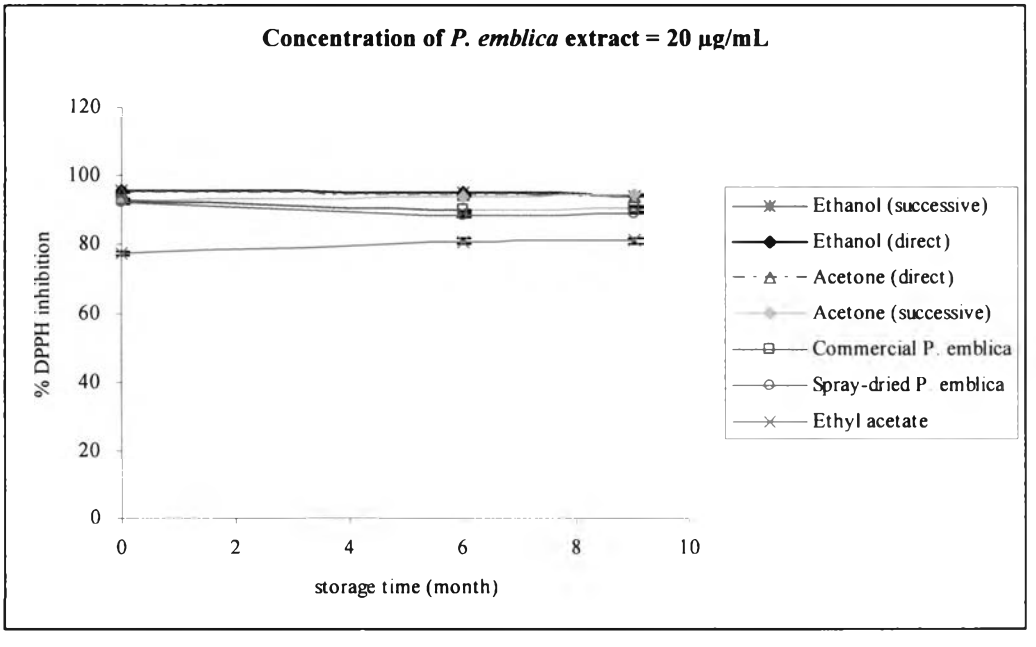

Figure 41. Percent DPPH inhibitory activity (prepared at 20 µg/mL) after storage up to 9 months (Mean ± SD, n = 3).

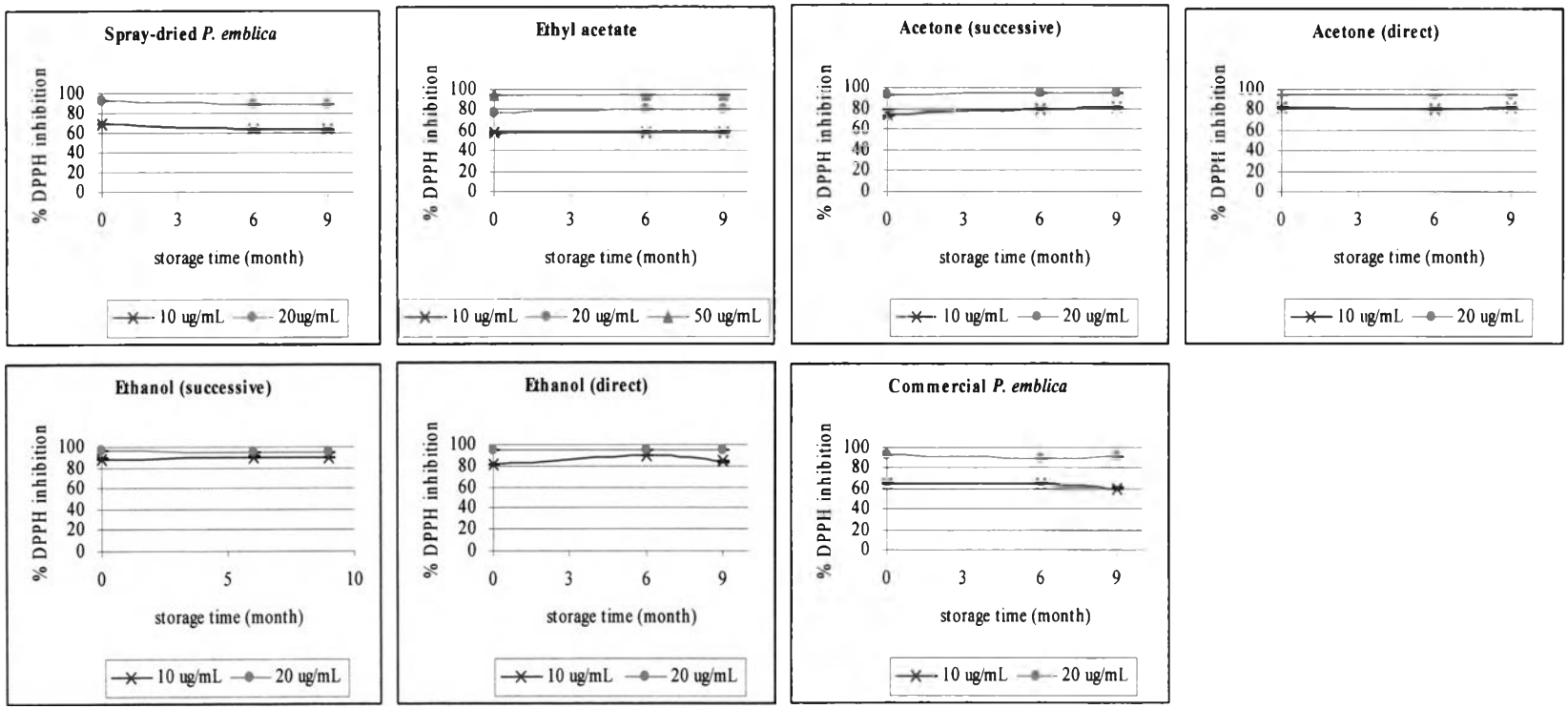

Figure 42. Percent DPPH inhibitory activity of each antioxidant after storage up to 9 months (Mean ± SD, n = 3).

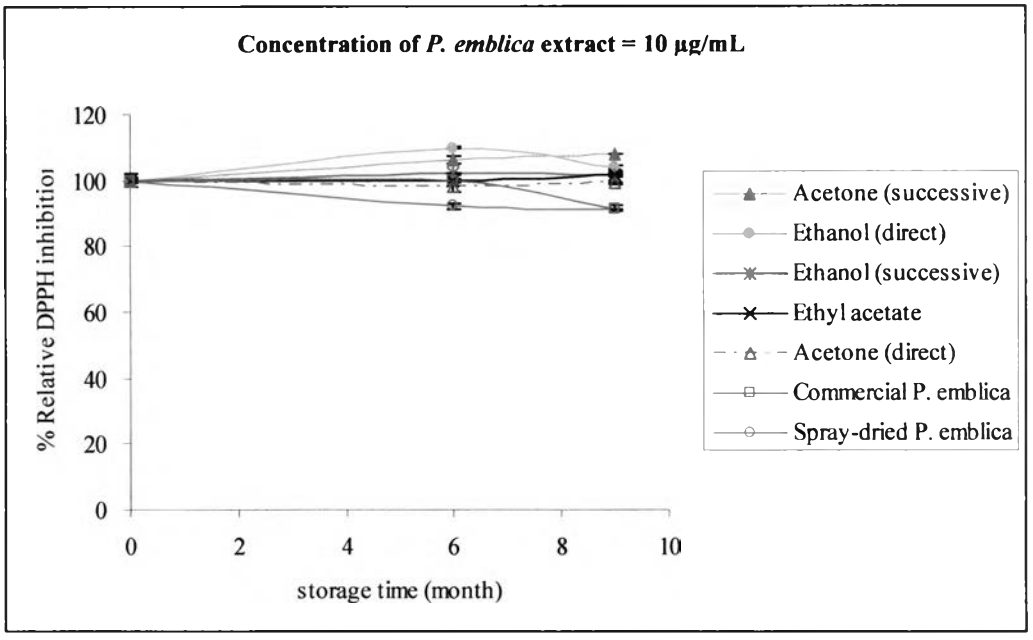

Figure 43. Percent DPPH inhibitory activity relative to initial value (prepared at 10 µg/mL) after storage up to 9 months (Mean ± SD, n = 3).

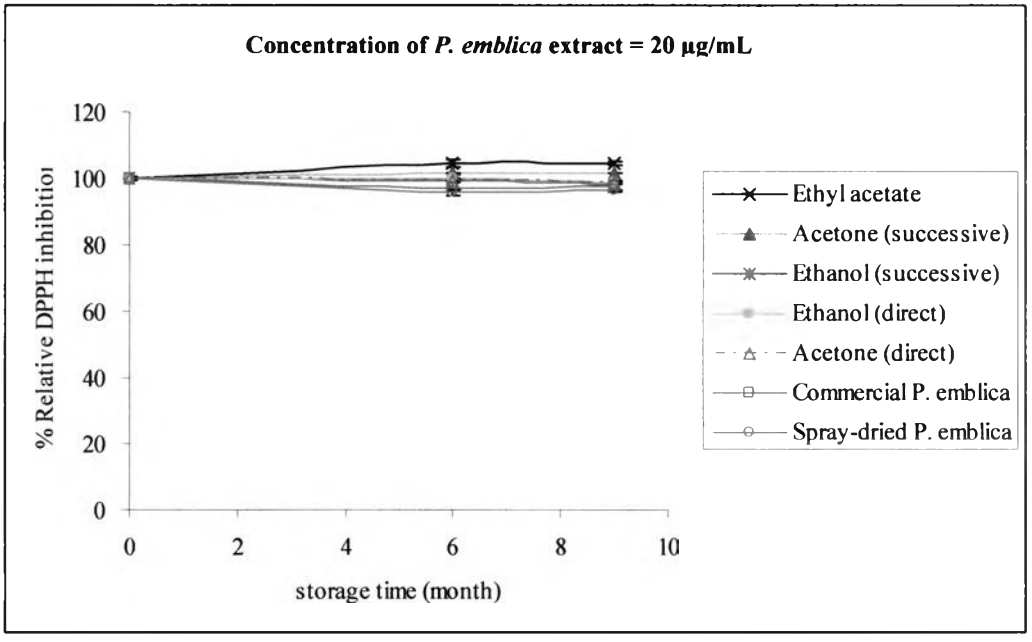

Figure 44. Percent DPPH inhibitory activity relative to initial value (prepared at 20 µg/mL) after storage up to 9 months (Mean ± SD, n = 3).

The results showing good stability of the extracts obtained in this study were consistent with that of Chaudhuri (2002), who reported the antioxidant stability of *P. emblica* aqueous-fruit extract against 0.1 mM DPPH during 12-month storage at 45°C (using 31 µg/mL of the extract). Therefore, it can be concluded that the solvent extracts of *P. emblica* appeared to have slightly better stability than the spray-dried powder, as seen from their % relative DPPH inhibition values in the range of 99.00-107.87 % after 9-month storage, whereas the spray-dried *P. emblica* gave % inhibition of 91.19% and 96.44% at 10 and 20 µg/mL, respectively, at the same storage time.