CHAPTER IV

RESULTS AND DISCUSSION

Part 1. Preparation of crude extracts from fresh roots of *Raphanus sativus* L. locally grown in Thailand

1.1 Percent yields of freeze-dried water extract

Percent yields of freeze-dried water extract (FDWE) were calculated from

$$\%\text{yield} = \frac{\text{weight of freeze-dried water extract}}{\text{weight of fresh radish root}} \times 100$$

They were 4.21% w/w. The extract was a pale-yellow powder. The freeze-dried water extract was stored in a well-closed container with silica gel at -20° C for further studies.

1.2 Percent yields of methanol extract

Percent yields of methanol extract (ME) were calculated from

$$\%\text{yield} = \frac{\text{weight of methanol extract}}{\text{weight of fresh radish root}} \times 100$$

They were 2.59% w/w. The extract was a brown powder. The methanol extract was stored in a well-closed container with silica gel at -20° C for further studies.

Part 2. Quantitative analysis of *Raphanus sativus* L. extracts: Freeze-dried water extract and methanol extract

2.1 Analysis of total phenolics content in *R. sativus* extracts

2.1.1. Validation of UV spectrophotometric method

The validation of analytical method is the process to evaluate that the method is suitable and reliable for the intended analytical application. For UV spectrophotometric assay validation, these include linearity, accuracy and precision.

2.1.1.1 Linearity

The calibration curve of sinapic acid in absolute ethanol is shown in Figure 12. Linear regression analysis of the absorbances versus the corresponding concentrations was performed and the coefficient of determination (R^2) was calculated as 0.9993. Calibration data were found to be linear with excellent coefficient of determination. These results indicated that UV spectrophotometric method was acceptable for quantitative analysis of sinapic acid in the range studied.

Table 5. Data for calibration curve of sinapic acid by UV spectrophotometric method

Concentration	Absorbance			Mean	SD.
(μg/ml)	Set1	Set2	Set3		
80	0.2025	0.2078	0.2106	0.2070	0.0041
60	0.1503	0.1509	0.1530	0.1514	0.0014
40	0.1019	0.1018	0.1017	0.1018	0.0001
20	0.0443	0.0437	0.0441	0.0440	0.0003
10	0.0182	0.0189	0.0183	0.0185	0.0004
5	0.0092	0.0095	0.0092	0.0093	0.0002
R^2	-	-	-	0.9993	

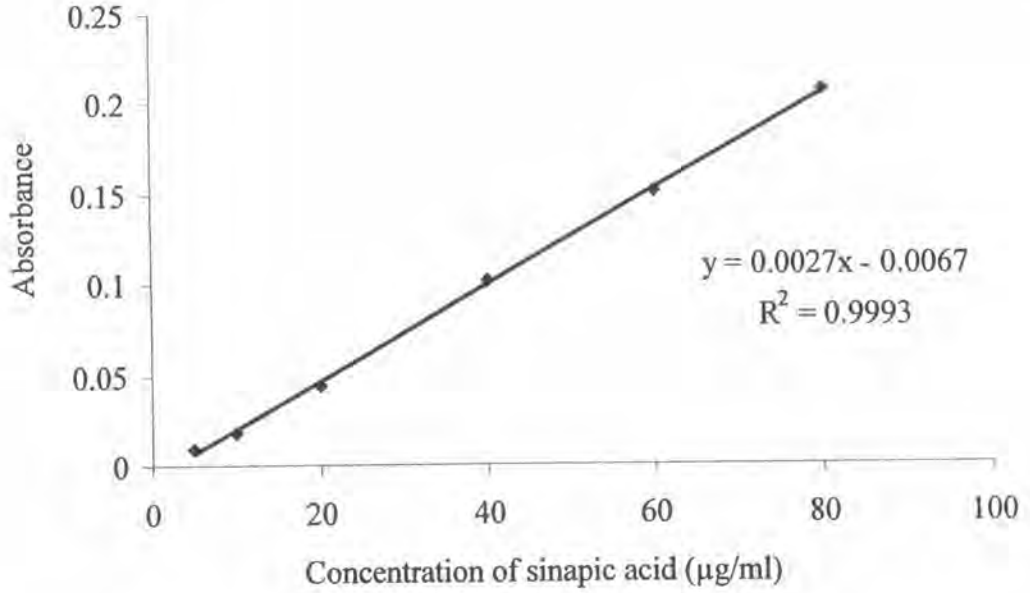


Figure 12. Data obtained from the average of 3 sets calibration curve of sinapic acid by UV spectrophotometric method

2.1.1.2 Accuracy

Sinapic acid solutions were prepared at the concentration of 16, 36 and 64 µg/ml in five sets. Each individual sample was analyzed by UV spectrophotometer. The inversely estimated concentrations and percentages of analytical recovery of each sinapic acid concentration are shown in Tables 6 and 7, respectively. All the percentages analytical recovery were in the range of 98.66 - 99.91%, which indicated the high accuracy of this method. Thus, it could be used for analysis of sinapic acid in all concentrations studied.

Table 6. The inversely estimated concentrations of sinapic acid by UV spectrophotometric method

Concentration	Inversely estimated concentration (µg/ml)					Mean ± SD
(µg/ml)	Set1	Set 2	Set 3	Set 4	Set 5	
16.00	15.786	15.750	16.000	16.071	16.321	15.99 ± 0.23
36.00	35.536	35.679	35.714	35.679	35.750	35.67 ± 0.08
64.00	63.893	63.179	62.786	62.893	62.965	63.14 ± 0.44

Table 7. The percentage of analytical recovery of sinapic acid by UV spectrophotometric method

Concentration	% Analytical recovery					Mean±SD
(µg/ml)	Set1	Set 2	Set 3	Set 4	Set 5	
16.00	98.66	98.44	100.00	100.44	102.00	99.91 ± 1.45
36.00	98.71	99.11	99.21	99.11	99.31	99.09 ± 0.23
64.00	99.83	98.72	98.10	98.27	98.38	98.66 ± 0.69

2.1.1.3 Precision

The precision of sinapic acid analyzed by UV spectrophotometric method was determined for within-run precision and between-run presicion as illustrated in Tables 8-11. For within-run precision, the coefficient of variation values of absorbance was in the range of 0.39-1.55% and the coefficient of variation values of inversely estimated concentration were in the range of 0.38-1.47%, respectively(Tables 8-9). For between-run precision, the coefficient of variation values of absorbance was in the range of 0.51-1.23% and the coefficient of variation values of inversely estimated concentration were in the range of 1.06-1.91%, respectively (Tables 10-11). The coefficient of variation of an analytical method should generally be less than 2%. Therefore, UV spectrophotometric method was of good precision for quantitative analysis of sinapic acid in the range studied.

Table 8. Data of absorbance of within-run precision of sinapic acid by UV spectrophotometric method

Concentration	Absorbance					Mean	SD	%CV
(µg/ml)	Set 1	Set 2	Set 3	Set 4	Set 5			
16.00	0.0393	0.0386	0.0396	0.0398	0.0399	0.0394	0.001	1.33
36.00	0.0969	0.0977	0.0978	0.0983	0.0945	0.0970	0.002	1.55
64.00	0.1731	0.1735	0.1738	0.1741	0.1749	0.1739	0.000	0.39

Table 9. The inversely estimated concentration of within-run precision of sinapic acid by UV spectrophotometric method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean	SD	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
16.00	15.96	15.71	16.07	16.14	16.18	16.01	0.19	1.18
36.00	36.54	36.82	36.86	37.04	35.68	36.59	0.54	1.47
64.00	63.75	63.89	64.00	64.11	64.39	64.03	0.24	0.38

Table 10. Data of absorbance of between-run precision of sinapic acid by UV spectrophotometric method

Concentration (μg/ml)	Absorbance					Mean	SD	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
16.00	0.0394	0.0387	0.0385	0.0388	0.0381	0.0387	0.000	1.23
36.00	0.0946	0.0920	0.0918	0.0931	0.0925	0.0928	0.001	1.21
64.00	0.1704	0.1685	0.1682	0.1687	0.1688	0.1689	0.000	0.51

Table 11. The inversely estimated concentration of between-run precision of sinapic acid by UV spectrophotometric method

Concentration (μg/ml)	Inversely estimated concentration (μg/ml)					Mean	SD	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
16.00	16.00	16.04	16.35	16.07	15.89	16.07	0.17	1.06
36.00	35.71	35.78	36.85	36.19	36.04	36.11	0.46	1.26
64.00	62.79	64.11	66.23	64.19	64.30	64.32	1.23	1.91

In conclusion, the analysis of sinapic acid by UV spectrophotometric method developed in this study showed good linearity, accuracy and precision. Thus, this method was used for the determination of the content of total phenolics in the extracts.

2.1.2 Determination of total phenolics in *R. sativus* extracts

The total phenolics in *R. sativus* extracts were determined as the amount equivalent to standard sinapic acid using the linear equation $y = 0.0027x - 0.0048$ ($R^2 = 1.000$), where y is the absorbance and x is the total phenolics content in μg/ml. The absorbances of each 2 mg/ml of freeze-dried water extract and methanol extract solutions are shown in Table 12 and the inversely estimated concentrations of total phenolics equivalent to sinapic acid are shown in Table 13.

Table 12. Data of absorbance of freeze-dried water extract and methanol extract at 2 mg/ml concentration by Folin-denis method

Sample	Absorbance					Mean±SD
	N1	N2	N3	N4	N5	
Freeze-dried water extract	0.0496	0.0495	0.0492	0.0502	0.0499	0.0497 ± 0.000
Methanol extract	0.0306	0.0304	0.0309	0.0311	0.0309	0.0308± 0.000

Table 13. The inversely estimated concentrations of total phenolics equivalent to sinapic acid in freeze-dried water extract and methanol extract at 2 mg/ml concentration

Sample	Inverse estimated concentration (μg/ml)					Mean±SD
	N1	N2	N3	N4	N5	
Freeze-dried water extract	20.15	20.11	20.00	20.37	20.26	20.18 ± 0.14
Methanol extract	13.11	13.04	13.22	13.30	13.22	13.18 ± 0.10

One mg of freeze–dried water extract and methanol extract were found to contain total phenolics equivalent to sinapic acid 10.09 ± 0.07 μg and 6.59 ± 0.05 μg, respectively.

2.2 Analysis of total flavonoids content in *R. sativus* extracts

2.2.1. Validation of UV spectrophotometric method

The validation of analytical method is the process to evaluate that the method is suitable and reliable for the intended analytical application. For UV spectrophotometric assay validation, these include linearity, accuracy and precision

2.2.1.1 Linearity

The calibration curve of quercetin in absolute ethanol is shown in Figure 13. Linear regression analysis of the absorbance versus the corresponding concentration was performed and the coefficient of determination (R^2) was calculated as 0.9994. The calibration data were found to be linear with excellent coefficient of determination. These results indicated that UV spectrophotometric method was acceptable for quantitative analysis of quercetin in the range studied.

Table 14. Data for quercetin calibration curve by UV spectrophotometric method

Concentration	Absorbance			Mean	SD.
(µg/ml)	Set1	Set2	Set3		
100	0.3946	0.4058	0.4048	0.4017	0.006
80	0.3258	0.3251	0.3258	0.3256	0.000
60	0.2374	0.2361	0.2359	0.2365	0.001
40	0.1580	0.1596	0.1578	0.1585	0.001
20	0.0833	0.0832	0.0836	0.0834	0.000
10	0.0356	0.0366	0.0373	0.0365	0.001
R^2	-	-	-	0.9994	

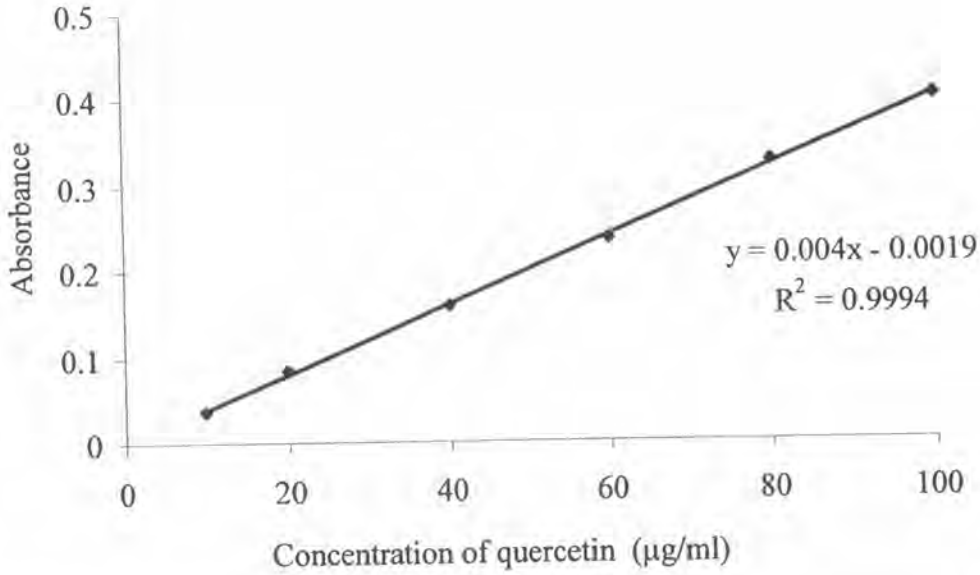


Figure 13. Data obtained from the average of 3 sets calibration curve of quercetin by UV spectrophotometric method

2.2.1.2 Accuracy

Quercetin solutions were prepared at the concentration of 25, 50 and 75 µg/ml in five sets. Each individual sample was analyzed by UV spectrophotometer. The inversely estimated concentrations and percentages of analytical recovery of each quercetin concentration are shown in Tables 15 and 16, respectively. The percentages analytical recovery were in the range of 99.24 – 100.66%, which indicated the high accuracy of this method.

Table 15. The inversely estimated concentrations of quercetin by UV spectrophotometric method

Concentration	Inversely estimated concentration (µg/ml)					Mean±SD
(µg/ml)	Set1	Set 2	Set 3	Set 4	Set 5	
25.00	24.610	25.244	24.878	24.537	24.781	24.81 ± 0.28
50.00	49.902	49.805	50.000	49.829	49.902	49.89 ± 0.08
75.00	75.585	75.073	75.659	75.512	75.634	75.49 ± 0.24

Table 16. The percentage of analytical recovery of quercetin by UV spectrophotometric method

Concentration	% Analytical recovery					Mean ± SD
(µg/ml)	Set1	Set 2	Set 3	Set 4	Set 5	
25.00	98.44	100.98	99.51	98.15	99.12	99.24 ± 1.11
50.00	99.81	99.61	100.00	99.66	99.81	99.78 ± 0.15
75.00	100.78	100.10	100.88	100.68	100.85	100.66 ± 0.32

2.2.1.3 Precision

The precision of quercetin analyzed by UV spectrophotometric method was determined for both within-run precision and between-run precision as illustrated in Tables 17-20. For within-run precision, the coefficient of variation values of absorbance was in the range of 0.15-1.12% and the coefficient of variation values of inversely estimated concentration were in the range of 0.16-1.11%, respectively (Tables 17-18). For between-run precision, the coefficient of variation values of absorbance was in the range of 1.12-1.53%, and the coefficient of variation values of inversely estimated concentration were in the range of 0.97-1.17%, respectively (Tables 19-20). The coefficient of variation of an analytical method should generally be less than 2%. Therefore, the UV spectrophotometric method demonstrated acceptable precision for the quantitative analysis of quercetin in the range studied.

Table 17. Data of absorbance of within-run precision of quercetin by UV spectrophotometric method

Concentration	Absorbance					Mean	SD	%CV
(µg/ml)	Set 1	Set 2	Set 3	Set 4	Set 5			
25.00	0.1010	0.1036	0.1021	0.1007	0.1017	0.1018	0.001	1.12
50.00	0.2047	0.2043	0.2051	0.2044	0.2047	0.2046	0.000	0.15
75.00	0.3100	0.3079	0.3103	0.3097	0.3102	0.3096	0.001	0.32

Table 18. The inversely estimated concentration of within-run precision of quercetin by UV spectrophotometric method

Concentration (µg/ml)	Inversely estimated concentration (µg/ml)					Mean	SD	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
25.00	24.61	25.24	24.88	24.54	24.78	24.81	0.28	1.11
50.00	49.90	49.80	50.00	49.83	49.90	49.89	0.08	0.16
75.00	75.59	75.07	75.66	75.51	75.63	75.49	0.24	0.32

Table 19. Data of absorbance of between-run precision of quercetin by UV spectrophotometric method

Concentration (µg/ml)	Absorbance					Mean	SD	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
25.00	0.0994	0.1021	0.1026	0.1036	0.1024	0.1020	0.002	1.53
50.00	0.2002	0.2051	0.2052	0.2059	0.2047	0.2042	0.002	1.12
75.00	0.3025	0.3079	0.3096	0.3121	0.3076	0.3079	0.004	1.15

Table 20. The inversely estimated concentration of between-run precision of quercetin by UV spectrophotometric method

Concentration (µg/ml)	Inversely estimated concentration (µg/ml)					Mean	SD	%CV
	Set 1	Set 2	Set 3	Set 4	Set 5			
25.00	24.22	24.88	24.95	24.68	24.59	24.66	0.29	1.17
50.00	48.80	50.00	49.98	49.63	49.54	49.59	0.49	0.98
75.00	73.76	75.07	75.44	75.54	74.63	74.89	0.72	0.97

In conclusion, the analysis of quercetin by UV spectrophotometric method developed in this study showed good linearity, accuracy and precision. Thus this method was used for the determination of the content of total flavonoids in the extracts.

2.2.2. Determination of total flavonoids in *R. sativus* extracts

The total flavonoids in *R. sativus* extracts were quantitated as amounts equivalent to standard quercetin from the equation y = 0.0041x + 0.0024, (R^2= 0.9995), where y is the absorbance and x is the total flavonoids content in µg/ml. Each 30 mg/ml of freeze-dried water extract and methanol extract were measured for their absorbances as shown in Table 21 and the inversely estimated concentrations of total flavonoids equivalent to quercetin are shown in Table 22.

Table 21. Absorbance data of freeze-dried water extract and methanol extract at concentration of 30 mg/ml by $AlCl_3$ method

Sample	Absorbance					Mean±SD
	N1	N2	N3	N4	N5	
Freeze-dried water extract	0.0660	0.0652	0.0653	0.0644	0.0668	0.0655 ±0.001
Methanol Extract	0.0430	0.0434	0.0425	0.0421	0.0428	0.0428 ±0.000

Table 22. The inversely estimated concentrations of total flavonoids equivalent to quercetin in freeze-dried water extract and methanol extract at 30 mg/ml concentration

Sample	Inverse estimated concentration (µg/ml)					Mean±SD
	N1	N2	N3	N4	N5	
Freeze-dried water extract	15.51	15.32	15.34	15.12	15.71	15.40 ± 0.22
Methanol extract	9.90	10.00	9.78	9.68	9.85	9.84 ± 0.12

One mg of freeze–dried water extract and methanol extract were equivalent to 0.51 ± 0.007 µg and 0.33 ± 0.004 µg of quercetin, respectively.

2.3 Analysis of vitamin C content in *R. sativus* extracts

The method based on the oxidation of ascorbic acid to dehydroascorbic acid by iodine was used for the determination of vitamin C content in test samples.

Standardization of 0.1 N iodine solution with arsenic trioxide (As_2O_3) is shown in Table 23.

Table 23. Data of standardization of 0.1 N iodine solution with arsenic trioxide (As_2O_3)

No.	Weight of As_2O_3 (mg)	Milliliter of 0.1 N iodine
1	185	37.00
2	185	37.00
3	185	37.00

$$\text{Normality} = \frac{(\text{wt } As_2O_3, \text{mg})}{(\text{ml of iodine})\ (49.46)}$$

Each ml of 0.1N iodine equivalent to 4.946 mg arsenic trioxide. The vitamin C content in freeze-dried water extract and methanol extract was first determined by direct titration with iodine. One gram each of the freeze-dried water extract and methanol extract in 25 ml distilled water was directly titrated with standardized 0.1N iodine. Each ml of 0.1N iodine is equivalent to 8.806 mg ascorbic acid (USP XX). The average (n=5) vitamin C content in 200 mg of freeze-dried water extract and methanol extract was found to be 4.02 and 2.07 mg, respectively.

2.3.1 Method validation

Direct titration with iodine was employed for determining the vitamin C content in test samples (freeze-dried water extract and methanol extract). The method was validated for linearity, precision, accuracy, limit of detection and limit of quantitation.

2.3.1.1 Linearity

The linearity of the method was determined by adding standard ascorbic acid 10, 20, 30, 40 and 50 fold of the ascorbic acid amount originally found in each 200 mg of test samples (freeze-dried water extract and methanol extract). A linear

relationship of the amount of standard ascorbic acid found and the amount of standard ascorbic acid added over concentration of 10-50 fold in the test samples was obtained for both extracts (Figures 14 and 15).

Table 24. Data for calibration curve of vitamin C content in freeze-dried water extract between amount of ascorbic acid added and total amount of ascorbic acid recovered (amount found)

Strength (fold)	Set 1(mg)			Set 2 (mg)			Set 3 (mg)		
	Added	Total found	Intrinsic amount in sample	Added	Total found	Intrinsic amount in sample	Added	Total found	Intrinsic amount in sample
10	40.1	44.9	4.8	40.2	44.5	4.3	43.2	48.4	5.2
20	80.2	84.5	4.3	82.5	87.2	4.7	79.9	84.5	4.6
30	121.3	126.8	5.5	119.5	125.0	5.5	121.0	125.9	4.9
40	160.4	165.6	5.2	160.2	165.6	5.4	160.5	165.6	5.1
50	200.5	205.2	4.7	200.0	204.3	4.3	200.5	205.2	4.7

Note * Intrinsic amount of ascorbic acid was calculated from:

Total ascorbic acid found in 200 mg of test samples – original amount of ascorbic acid added to 200 mg test samples

Table 25. Data for calibration curve of vitamin C content in methanol extract between ascorbic acid amount added and ascorbic acid amount found recovered

Strength (fold)	Set 1(mg)			Set 2 (mg)			Set 3 (mg)		
	Added	Total found	Intrinsic amount in sample	Added	Total found	Intrinsic amount in sample	Added	Total found	Intrinsic amount in sample
10	26.0	27.7	1.7	30.2	31.7	1.5	30.2	32.1	1.9
20	50.5	52.0	1.5	52.0	53.7	1.7	55.0	56.8	1.8
30	76.8	78.4	1.6	77.0	79.3	2.3	78.0	80.1	2.1
40	104.5	106.6	2.1	100.8	103.0	2.2	100.0	101.7	1.7
50	129.5	131.2	1.7	129.9	131.6	1.7	130.0	132.1	2.1

Table 26. Calibration curve parameters of vitamin C by direct titration

Set	freeze-dried water extract			methanol extract		
	Slope	y-intercept	R^2	Slope	y-intercept	R^2
1	1.0018	4.6852	0.9999	1.0024	1.5350	1
2	1.0017	4.6315	0.9999	1.0033	1.6227	0.9999
3	0.9988	5.0473	1	1.0014	1.8120	1
Mean of 3 sets	1.0050	4.8300	1	1.0023	1.6623	1

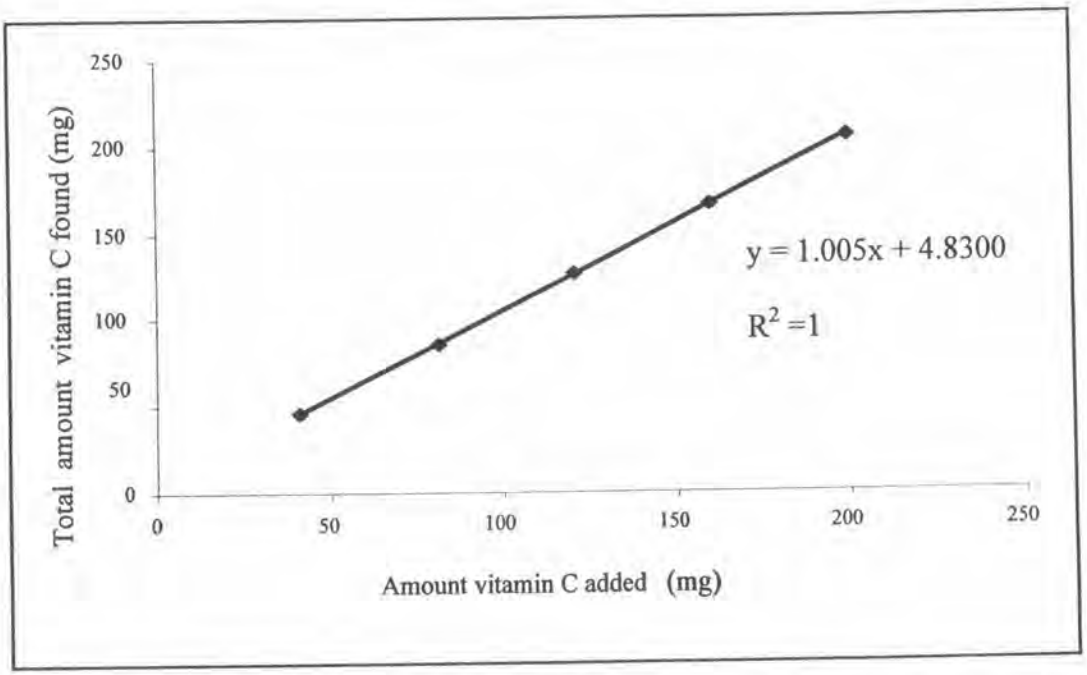


Figure 14. Data obtained from the average of 3 sets calibration curve of freeze-dried water extract by direct titration method

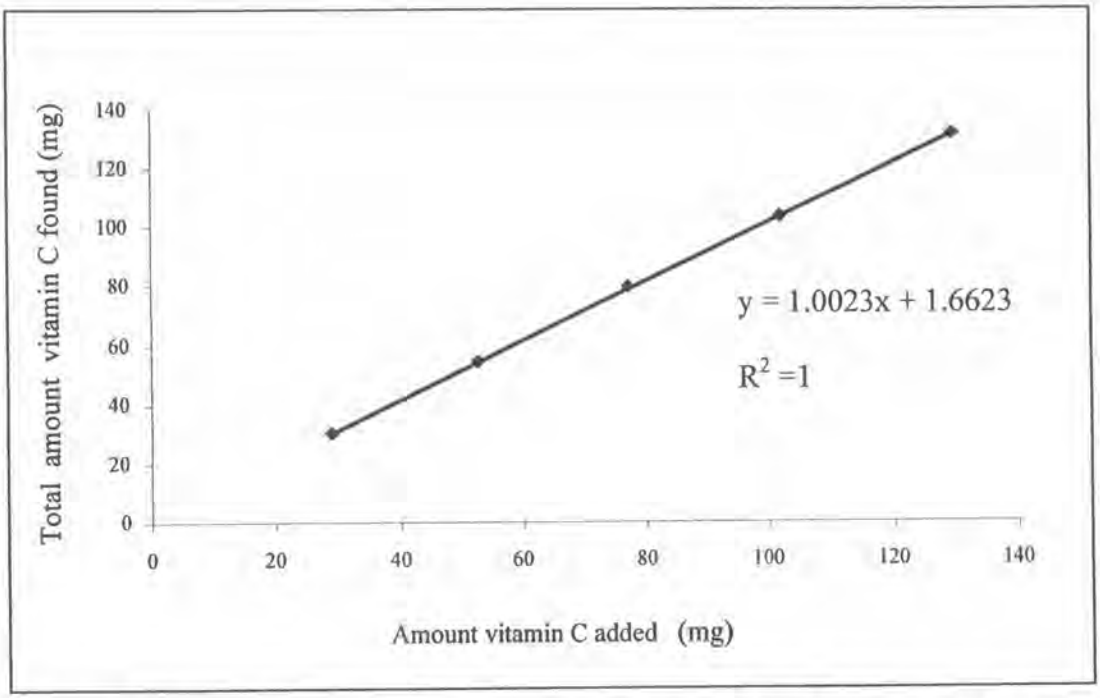


Figure 15. Data obtained from the average of 3 sets calibration curve of methanol extract by direct titration method

The validating parameters of each calibration curve are present in Table 26. The method showed good linearity. From the intercept in the calibration curve of freeze-dried water extract and methanol extract were found that each 200 mg of test samples, freeze-dried water extract and methanol extract were equivalent to vitamin C 4.83 and 1.66 mg, respectively.

2.3.1.2. Accuracy

The accuracy calculated from percentages of bias and recovery was determined using standard addition method. Standard ascorbic acid 10, 20, 30, 40 and 50 fold of each 200 mg test sample (freeze-dried water extract and methanol extract) was added into the test solution. Triplicate titrations were made for each standard solution added. The percentage bias and recoveries were calculated using the following equations:

$$\% \text{ Recovery} = \frac{\text{Amount of standard recovered}}{\text{Amount of standard added}} \times 100$$

Accuracy data are shown in Tables 27 and 28. The percent recoveries of standard ascorbic acid were within 98.51-101.90% and 99.27-101.99% for freeze-dried water extract and methanol extract, respectively, which indicated high accuracy of this method. Thus, it could be used for analysis of vitamin C in all concentrations studied.

Table 27. Recovery of standard ascorbic acid from freeze-dried water extract

Amount of standard added (mg)	Total amount found (mg)	Amount of standard recovered (mg)	Recovery (%)
40.10	44.93	40.50	101.00
40.20	45.03	39.60	98.51
43.20	48.03	44.00	101.85
80.20	85.03	81.00	101.00
82.50	87.33	83.70	101.45
79.90	84.73	79.30	99.25
121.30	126.13	123.30	101.65
119.50	124.33	118.90	99.50
121.00	125.88	123.30	101.90
160.40	165.23	160.30	99.94
160.20	165.03	158.50	98.94
160.50	165.33	158.50	98.75
200.50	205.33	202.50	101.00
200.00	204.83	198.10	99.05
200.50	205.33	202.50	101.00

Note* Amount of standard recovered = Total amount found – y-intercept

Table 28. Recovery of standard ascorbic acid from methanol extract

Amount of standard added (mg)	Total amount found (mg)	Amount of standard recovered (mg)	Recovery (%)
26.00	27.66	26.4	101.54
30.20	31.86	30.8	101.99
30.20	31.86	30.8	101.99
50.50	52.16	51.1	101.19
52.00	53.66	52.9	101.73
55.00	56.66	54.6	99.27
76.80	78.46	77.5	100.91
77.00	78.66	78.4	101.82
78.00	79.66	79.3	101.67
104.50	106.16	105.7	101.15
100.80	102.46	101.3	100.50
100.00	101.66	101.3	101.30
129.50	131.16	130.3	100.62
129.90	131.56	132.1	101.69
130.00	131.66	130.3	100.23

2.3.1.3. Precision

The within-run and between-run precision was studied by determining vitamin C content in freeze-dried water extract and methanol extract on the same and different days. Five titrations were made for 800 mg of each sample. Precision was expressed as % coefficient of variation (% CV). The method was precise and the %CV. for within-run precision was 1.02% for freeze-dried water extract and 1.20% for methanol extract, whereas % CV for between-run precision was 0.53-1.23 % for freeze-dried water extract and 0.92-1.72% for methanol extract as shown in Tables 29-30.

Table 29. Precision determination of vitamin C content in 800 mg of freeze-dried water extract

Number of sample	freeze dried water extract (mg)				
	1 day	5 days	10 days	20 days	30days
1	19.15	19.25	19.35	19.20	19.18
2	19.35	19.10	19.00	19.18	19.20
3	18.85	19.15	19.26	19.21	19.16
4	19.30	19.50	19.20	19.15	19.14
5	19.10	18.85	19.15	19.45	18.95
Mean (n=5)	19.15	19.17	19.19	19.30	19.13
S.D.	0.20	0.24	0.13	0.13	0.10
CV (%)	1.02	1.23	0.68	0.66	0.53

Table 30. Precision determination of vitamin C content in 800 mg of methanol extract

Number of sample	methanol water extract (mg)				
	1 day	5 days	10 days	20 days	30days
1	6.50	6.70	6.80	6.65	6.85
2	6.65	6.65	6.50	6.60	6.70
3	6.60	6.55	6.70	6.75	6.55
4	6.70	6.65	6.65	6.50	6.65
5	6.55	6.70	6.75	6.70	6.70
Mean (n=5)	6.60	6.65	6.68	6.64	6.69
S.D.	0.08	0.06	0.12	0.10	0.11
CV (%)	1.20	0.92	1.72	1.45	1.62

2.3.1.4 Limit of detection and quantitation

The limit of detection (LOD) was determined by decreasing the concentration of standard ascorbic acid 10 fold each time. The amount of standard ascorbic acid which could be detected by observation of end-point, was considered. The limit of quantitation (LOQ) was the lowest amount of standard ascorbic acid that could be quantified with reasonable precision and accuracy. The LOD and LOQ of vitamin C content were 2.0 and 6.2 mg/ml, respectively.

The high LOQ value is typical of volumetric titration method. This has necessitated the use of standard addition technique to increase the sensitivity of the assay. Since the intrinsic amount of L-ascorbic acid was previously estimated to be around 2 and 4 mg of methanol extract and freeze-dried water extracts, respectively.

Therefore the standard L-ascorbic acid had been added to the sample at least 10 times the intrinsic amount.

2.3.2. Determination of vitamin C content in test samples

The validated method was employed for the determination of vitamin C content in freeze-dried water extract and methanol extract. Eight hundred mg of freeze-dried water extract and methanol extract in 25 ml of distilled water were directly titrated with standardized 0.1N iodine. Each ml of 0.1N iodine is equivalent to 8.806 mg of ascorbic acid. The milliliters of 0.1 N iodine used in the assay for vitamin C in freeze-dried water extract and methanol extract are shown in Table 31 and the inversely estimated vitamin C content in freeze-dried water extract and methanol extract shown in Table 32.

Table 31. Quantities in milliliter of 0.1 N iodine used in the assay for vitamin C in 800 mg of freeze-dried water extract and methanol extract

Sample	Milliliter of 0.1 N iodine					Mean±SD
	N1	N2	N3	N4	N5	
Freeze-dried water extract	2.20	2.10	2.15	2.35	2.15	2.19 ± 0.10
Methanol extract	0.75	0.73	0.75	0.78	0.75	0.75 ± 0.02

Table 32. The inversely estimated vitamin C content in 800 mg of freeze-dried water extract and methanol extract

Sample	vitamin C content (mg)					Mean±SD
	N1	N2	N3	N4	N5	
Freeze-dried water extract	19.373	18.493	18.933	20.694	18.933	19.285 ± 0.85
Methanol extract	6.604	6.428	6.604	6.869	6.604	6.622 ± 0.16

One mg of freeze–dried water extract and methanol extract were equivalent to 24.11± 0.01 and 8.28 ± 0.20 µg of vitamin C, respectively.

Part 3. Determination of anti-tyrosinase enzyme activity of *Raphanus sativus* L. extracts

The anti-tyrosinase activity of *Raphanus sativus* L. extracts was determined by the DOPAchrome enzymatic method using L-DOPA as a substrate and mushroom tyrosinase as the enzyme. DOPAchrome is one of the intermediate substances in the melanin biosynthesis. The red color of DOPAchrome can be detected by visible light. In this experiment a microplate reader (VICTOR®) with 492 nm interference filter was used. A potential tyrosinase inhibitor would show a decreased DOPAchrome absorption. The percentage of tyrosinase inhibition of *Raphanus sativus* L. extracts (freeze-dried water extract and methanol extract) at various concentrations (1.0, 2.5, 5.0, 10.0, 20.0 and 40.0 mg/ml) compared to those of L-ascorbic acid at (5.0, 10.0, 25.0, 50.0, 100.0 and 200.0 μg/ml) and licorice extract at (0.25, 0.50, 1.00, 2.50, 5.00 and 10.00 μg/ml) are shown in Figure 16. It was found that as the concentration was increased, the percent inhibition was also increased for all samples. The percent tyrosinase inhibition of freeze-dried water extract increased to 97.87 ± 0.04 % at the concentration of 10.0 mg/ml, then reached a plateau at 20.0 and 40.0 mg/ml, where the inhibition percentages were 98.55 ± 0.38 and 98.84 ± 0.18 %, respectively. Methanol extract gave tyrosinase inhibition up to 86.51 ± 0.15 % at the concentration of 40.0 mg/ml. Freeze-dried water extract apparently showed higher extent of tyrosinase inhibition than methanol extract at all concentrations.

On the other hand, the percentage of tyrosinase inhibition of licorice extract and L-ascorbic acid showed much stronger inhibition than the freeze-dried water extract and methanol extract. Licorice extract gave tyrosinase inhibition up to 94.56 ± 0.27 % at the very low concentration of 10.0 μg/ml and l-ascorbic acid gave tyrosinase inhibition of 98.46 ± 0.31 % at the concentration of 200.0 μg/ml. The raw data for the absorbance and tyrosinase inhibition percentages are provided in Appendix A.

After the plots of % tyrosinase inhibition versus concentration were constructed for each anti-tyrosinase agent, its IC_{50} value or the concentration at which the tested anti-tyrosinase inhibits half the original tyrosinase activity was calculated from the regression analysis of the initial portion of each curve. It was found that a polynomial regression equation provided better result than a linear equation, with a higher R^2 (coefficient of determination), as shown in Table 33 and Figures 17-20.

The estimated IC_{50} values can be ranked into two groups between the reference anti-tyrosinase (licorice extract and L-ascorbic acid) and the extracts (freeze-dried water extract and methanol extract) in terms of microgram and milligram. The two reference anti-tyrosinases show the average IC_{50} values as follows: licorice extract 1.23 ± 0.02 μg/ml and L-ascorbic acid 73.57 ± 1.26 μg/ml, while the extracts gave the mean IC_{50} values as follows: freeze-dried water extract 3.09 ± 0.05 mg/ml and methanol extract 9.62 ± 0.08 mg/ml. Since the IC_{50} values indicate the inhibitory potency of the individual anti-tyrosinases, the licorice extract apparently demonstrated the strongest anti-tyrosinase activity.

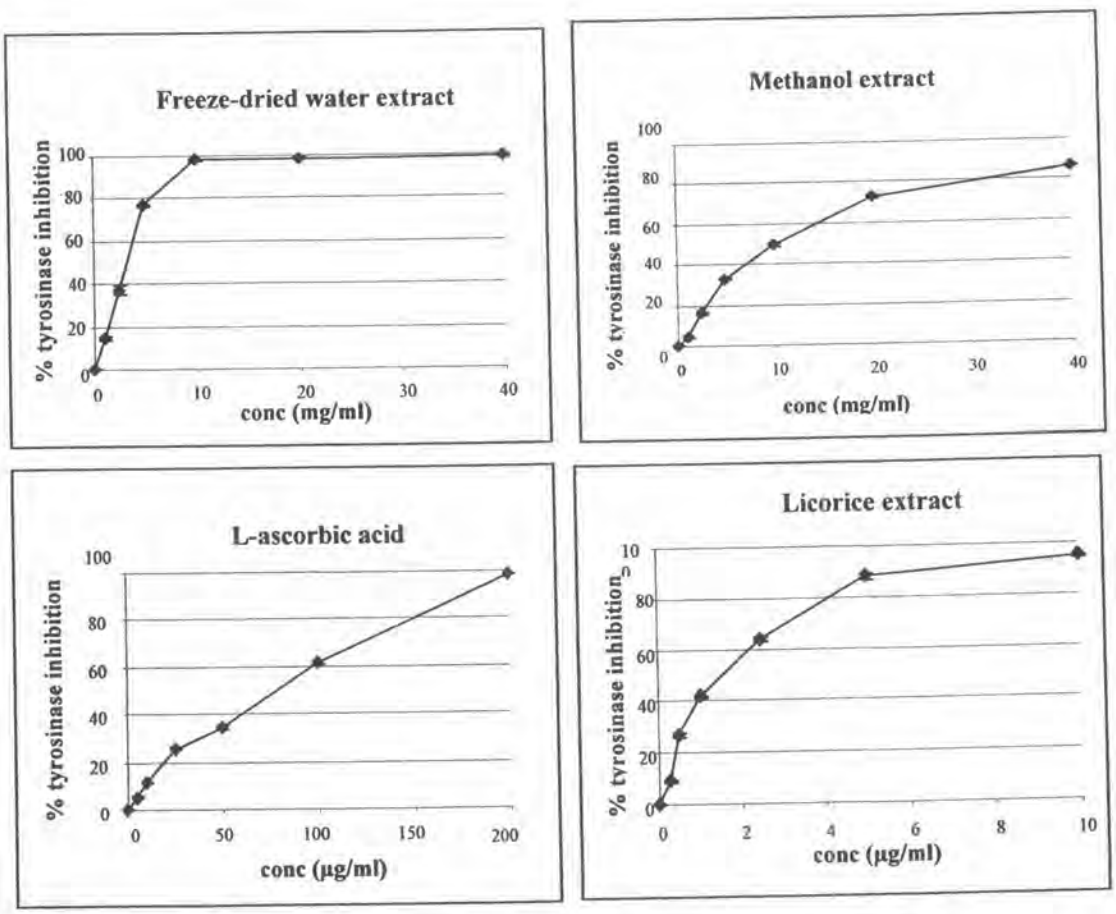


Figure 16. The relationship between % tyrosinase inhibition and the concentration of freeze-dried water extract, methanol extract and the reference anti-tyrosinase L-ascorbic acid and licorice extract (mean ± SD, n=3)

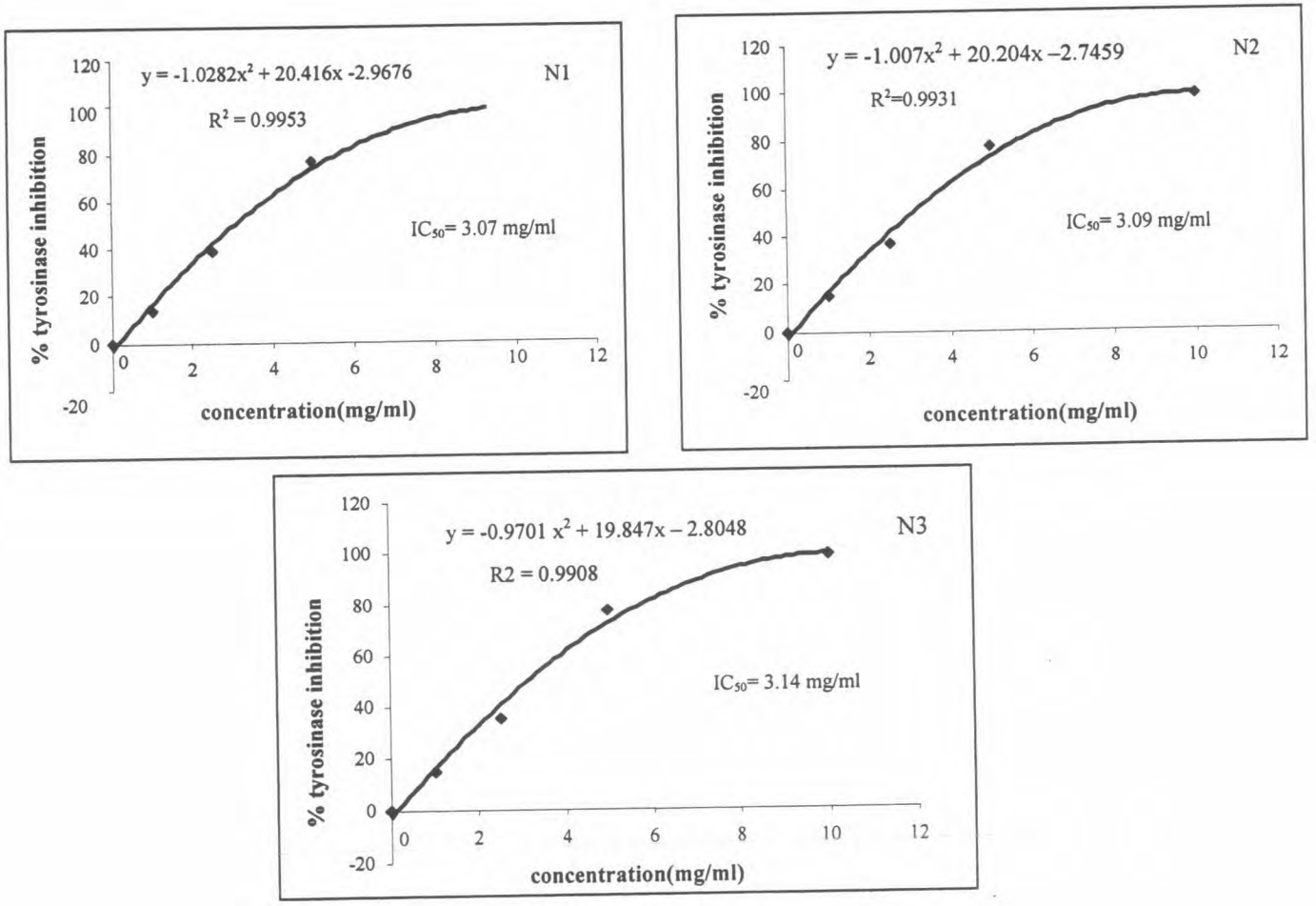


Figure 17. The regression curve of the initial portion of the % tyrosinase inhibition-concentration profile of freeze-dried water extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

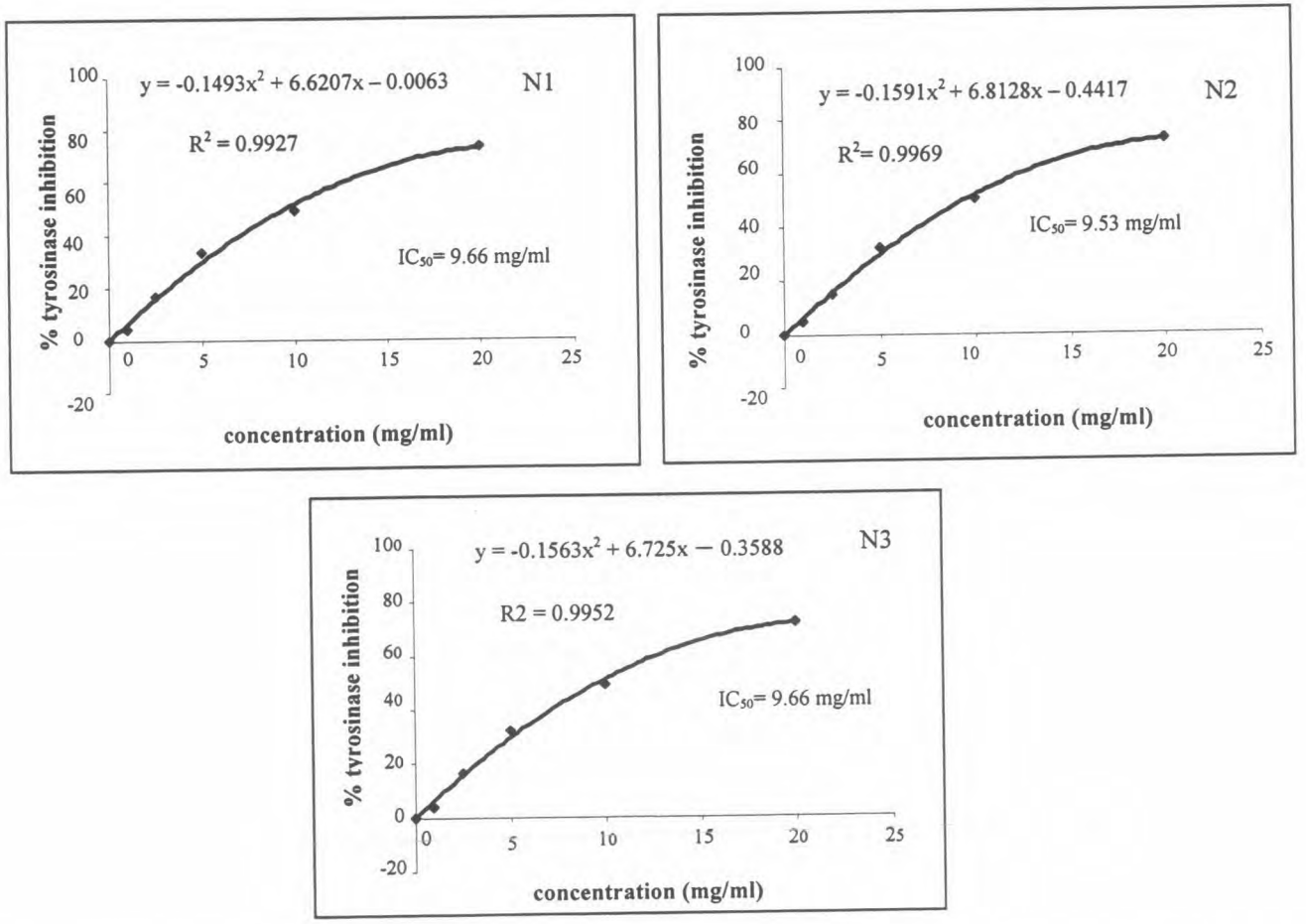


Figure 18. The regression curve of the initial portion of the % tyrosinase inhibition-concentration profile of methanol extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

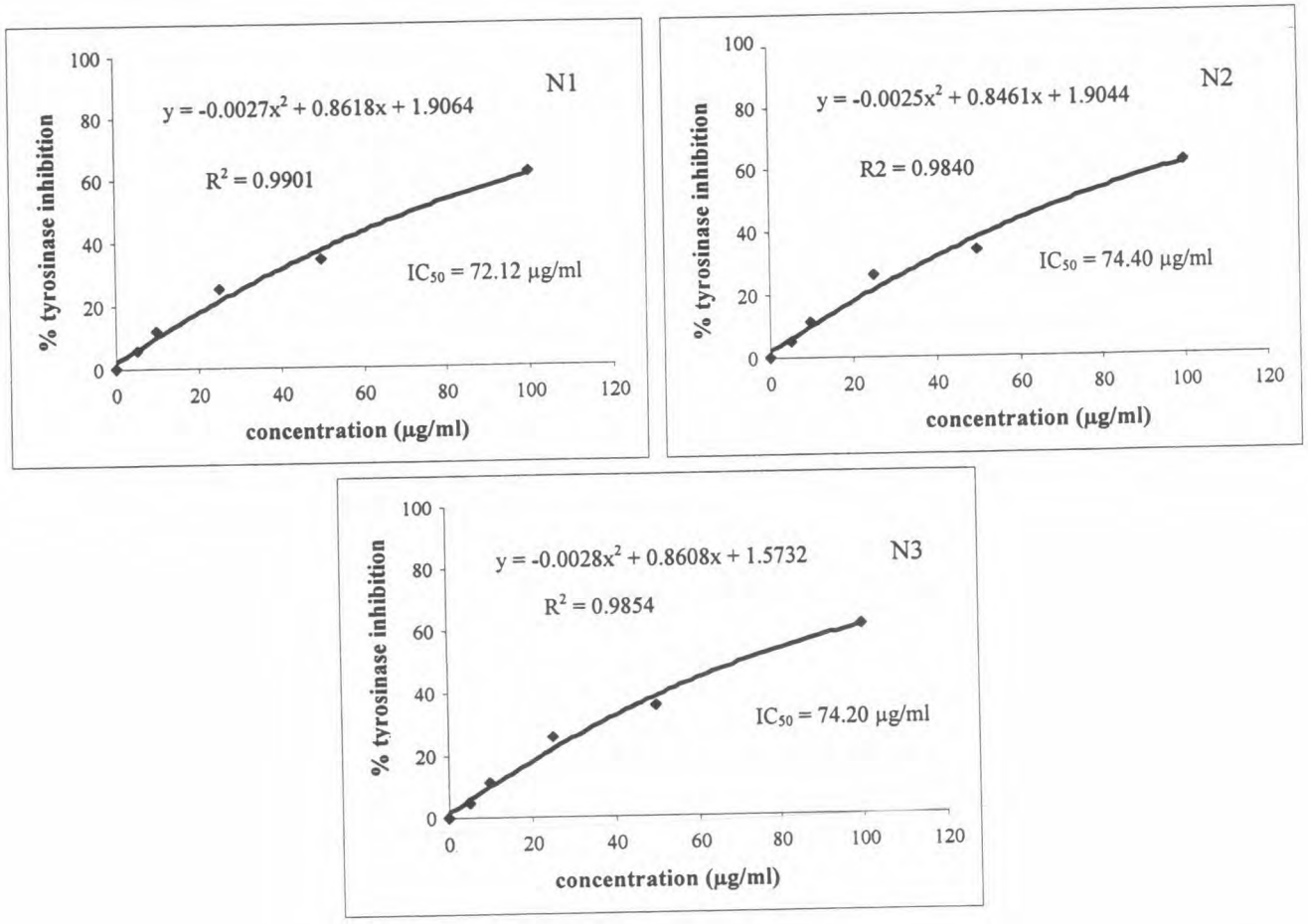


Figure 19. The regression curve of the initial portion of the % tyrosinase inhibition-concentration profile of L-ascorbic acid. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

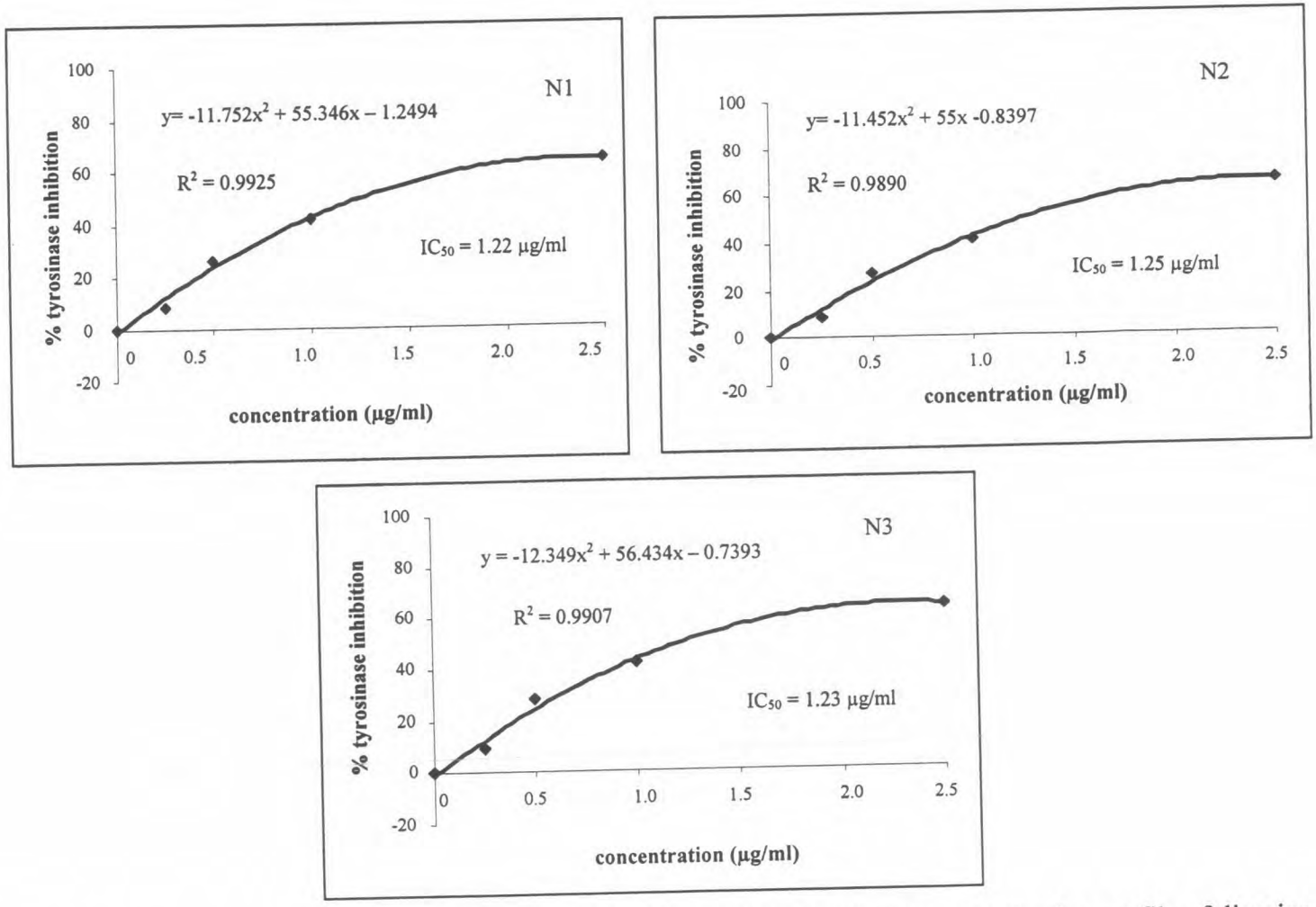


Figure 20. The regression curve of the initial portion of the % tyrosinase inhibition-concentration profile of licorice extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

Table 33. The IC_{50} values of tyrosinase inhibition of each anti-tyrosinase, and coefficient of determination (R^2) obtained from polynomial regression of the initial portion of the plot concentration (n=3)

Sample	Polynomial equation (partial)					
	IC_{50} (mg/ml)	Mean	SD	R^2	Mean	SD
Freeze-dried water extract	3.07	3.10	0.036	0.9953	0.9931	0.002
	3.09			0.9931		
	3.14			0.9908		
Methanol extract	9.66	9.62	0.075	0.9927	0.9949	0.002
	9.53			0.9969		
	9.66			0.9952		

Sample	Polynomial equation (partial)					
	IC_{50} (µg/ml)	Mean	SD	R^2	Mean	SD
Licorice extract	1.22	1.23	0.015	0.9925	0.9907	0.002
	1.25			0.9890		
	1.23			0.9907		
L-ascorbic acid	72.12	73.57	1.263	0.9901	0.9865	0.003
	74.40			0.9840		
	74.20			0.9854		

Compared to the reference anti-tyrosinasees, the freeze-dried water extract and methanol extract appeared to give lower potency, as judged from their IC_{50} values which were expressed in mg/ml. However, the freeze-dried water extract was a more effective anti-tyrosinase than the methanol extract. The IC_{50} data of the freeze-dried water extract and methanol extract were analyzed by independent t-test at 95% confidence interval. It was found that there were significant differences among the two extracts studied ($p < 0.05$), supporting the greater anti-tyrosinase potency of the freeze-dried water extract.

Since freeze-dried water extract and methanol extract were crude extracts and not pure compounds, it is possible that their anti-tyrosinase activity was a result of many constituents of these extracts. Determination of polyphenols and vitamin C contents in the extracts showed that one mg of each freeze-dried water extract and methanol extract was equivalent to 10.09 and 6.59 μg of sinapic acid and 24.11 and 8.28 μg of vitamin C, respectively. Since the main constituent in both extracts is vitamin C, it is possible that the vitamin may play an important role in exerting the anti-tyrosinase activity in both extracts. This was also supported by the fact that L-ascorbic acid has been shown to be a strong tyrosinase inhibitor (Zhai and Maibach, 2001).

Jang et al. (1997) also reported the IC_{50} of L-ascorbic acid in inhibiting tyrosinase enzyme which was nearly identical to the data from this study (70 μg/ml). In this study it was found that the average IC_{50} of freeze-dried water extract and methanol extract of *R. sativus* were 3.10 and 9.62 mg/ml, respectively. Since the content of vitamin C in the extracts were previously found to be 24.11 and 8.28 μg/mg for freeze-dried water extract and methanol extract, respectively, the IC_{50} of the two extracts could be expressed in terms of vitamin C equivalent. This was obtained by multiplying the IC_{50} of each extract with its respective vitamin C content. Thus, the IC_{50} of the two extracts expressed as vitamin C content were determined to be 74.74 and 79.65 μg/ml, respectively. Thus values were pure vitamin C (73.57 μg/ml), thereby suggesting that the anti-tyrosinase activity of the two extracts was mainly due to the presence of vitamin C. This result also explained why the freeze-dried water extract was more active than methanol extract. However, other polyphenols in the extract might also show some anti-tyrosinase activity but their effect could be very small compared to that due to vitamin C.

Part 4. Determination of antioxidant and free radical scavenging activities of *Raphanus sativus* L. extracts.

4.1 Determination of DPPH free radical scavenging activity

The antioxidant and free radical scavenging activities of the freeze-dried water extract, methanol extract and reference substances were determined in terms of their ability to scavenge different reactive oxygen species. The first test involved determination of their ability to scavenge DPPH free radical in ethanolic solution, which resulted in a decrease in absorbance at 517 nm. The loss of DPPH radical was evaluated by comparison with a control sample containing no test substance that could act as a hydrogen-donating compound.

The chemical stability of the freeze-dried water extract and methanol extract during 3-month storage was also determined by quantitating their *in vitro* DPPH free radical scavenging activities. The percentages of DPPH scavenging (% inhibition of free radical) of the freeze-dried water extract and methanol extract at concentrations of 0, 0.05, 0.10, 0.50, 1.00, 2.00, 3.00 and 4.00 mg/ml are shown in Tables 34 and 35. The average values of % DPPH scavenging activity of the freeze-dried water extract (freshly prepared, 0 month-storage) were 88.78 ± 0.58 at 4 mg/ml concentration, 87.70 ± 0.24 at 3 mg/ml concentration, 84.44 ± 0.88 at 2 mg/ml concentration, 69.66 ± 0.30 at 1 mg/ml concentration, 38.88 ± 0.90 at 0.5 mg/ml concentration, 11.93 ± 0.65 at 0.1 mg/ml concentration and 5.69 ± 0.40 % at 0.05 mg/ml concentration, respectively. The average values of % DPPH scavenging activity of methanol extract (freshly prepared, 0 month-storage) were 89.15 ± 0.25 at 4 mg/ml concentration, 86.39 ± 0.52 at 3 mg/ml concentration, 66.71 ± 0.56 at 2 mg/ml concentration, 41.31 ± 0.42 at 1 mg/ml concentration, 25.32 ± 0.39 at 0.5 mg/ml concentration, 8.12 ± 0.57 at 0.1 mg/ml concentration and 4.32 ± 0.29% at 0.05 mg/ml concentration, respectively. The values of % DPPH scavenging activity were consistent for both the freeze-dried water extract and methanol extract in every concentrations. The freeze-dried water extract appeared to be more active than the methanol extract as seen from the higher percent inhibition. The IC_{50} values of DPPH inhibition were also determined for each extract after polynomial regression of the initial portion of the plots between % inhibition and extract concentration. The IC_{50} values were also re-determined after storage at 1 and 3 months. The average IC_{50} of the freeze-dried water

extract were found to be 0.643 mg/ml, 0.648 mg/ml and 0.643 mg/ml after 0, 1 and 3 month-storage, respectively. For the methanol extract the average IC_{50} values were 1.248 mg/ml, 1.238 mg/ml and 1.236 mg/ml after 0, 1 and 3 month-storage, respectively (Table 36). From the short term stability evaluation, the result showed that the storage conditions used in this study, i.e., keeping the extracts in a well-closed container with silica gel at -20° C, can protect the product during the time of study.

Table 34. The average percentage of DPPH inhibition by freeze-dried water extract after 0, 1 and 3 month-storage (Mean ± SD, n=3)

No	conc (mg/ml)	% inhibition		
		month 0	month 1	month 3
1	4.00	88.78 ± 0.58	88.89 ± 0.46	89.06 ± 0.30
2	3.00	87.70 ± 0.24	87.07 ± 0.12	87.29 ± 0.49
3	2.00	84.44 ± 0.88	84.12 ± 0.30	84.24 ± 0.61
4	1.00	69.66 ± 0.30	69.17 ± 0.34	69.57 ± 0.36
5	0.50	38.88 ± 0.90	38.72 ± 0.51	38.68 ± 1.02
6	0.10	11.93 ± 0.65	12.65 ± 1.13	12.43 ± 0.34
7	0.05	5.69 ± 0.40	4.66 ± 0.04	5.24 ± 0.82
8	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 35. The average percentage of DPPH inhibition by methanol extract after 0, 1 and 3 month-storage (Mean ± SD, n=3)

No	conc (mg/ml)	% inhibition		
		month 0	month 1	month 3
1	4.00	89.15 ± 0.25	89.29 ± 0.21	89.11 ± 0.15
2	3.00	86.39 ± 0.52	86.52 ± 0.58	86.36 ± 0.62
3	2.00	66.71 ± 0.56	66.74 ± 0.60	67.02 ± 0.20
4	1.00	41.31 ± 0.42	41.77 ± 0.60	41.69 ± 0.63
5	0.50	25.32 ± 0.39	25.53 ± 0.34	25.65 ± 0.38
6	0.10	8.12 ± 0.57	8.62 ± 0.97	8.87 ± 0.71
7	0.05	4.32 ± 0.29	4.61 ± 0.44	4.74 ± 0.33
8	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 36. The IC_{50} values of DPPH radical inhibition by freeze-dried water extract and methanol extract after 0, 1 and 3 month-storage. Coefficient of determination (R^2) from polynomial regression of the initial portion of the plots between inhibition percentage and concentrations are also provided (n=3)

Month	Samples	IC_{50}(mg/ml)	Mean	SD	R^2	Mean	SD
0	Freeze-dried water extract	0.635 0.650 0.643	0.643	0.007	0.9983 0.9974 0.9987	0.9981	0.001
	Methanol Extract	1.244 1.247 1.254	1.248	0.005	0.9984 0.9971 0.9969	0.9974	0.001
1	Freeze-dried water extract	0.643 0.655 0.645	0.648	0.006	0.9966 0.9972 0.9987	0.9975	0.001
	Methanol extract	1.249 1.244 1.220	1.238	0.02	0.9973 0.9980 0.9960	0.9971	0.001
3	Freeze-dried water extract	0.636 0.648 0.644	0.643	0.006	0.9979 0.9973 0.9974	0.9975	0.000
	Methanol extract	1.245 1.238 1.225	1.236	0.01	0.9969 0.9968 0.9960	0.9966	0.000

The percentage of DPPH scavenging (% inhibition of free radical) by the freeze-dried water extract and methanol extract at various concentrations were compared to Trolox® (water-soluble form of vitamin E) and L-ascorbic acid. The relationships between DPPH radical inhibition and the concentration of the individual

antioxidants are shown in Figure 21. It was found that as the concentration was increased, the extent of DPPH inhibition also increased for all antioxidants.

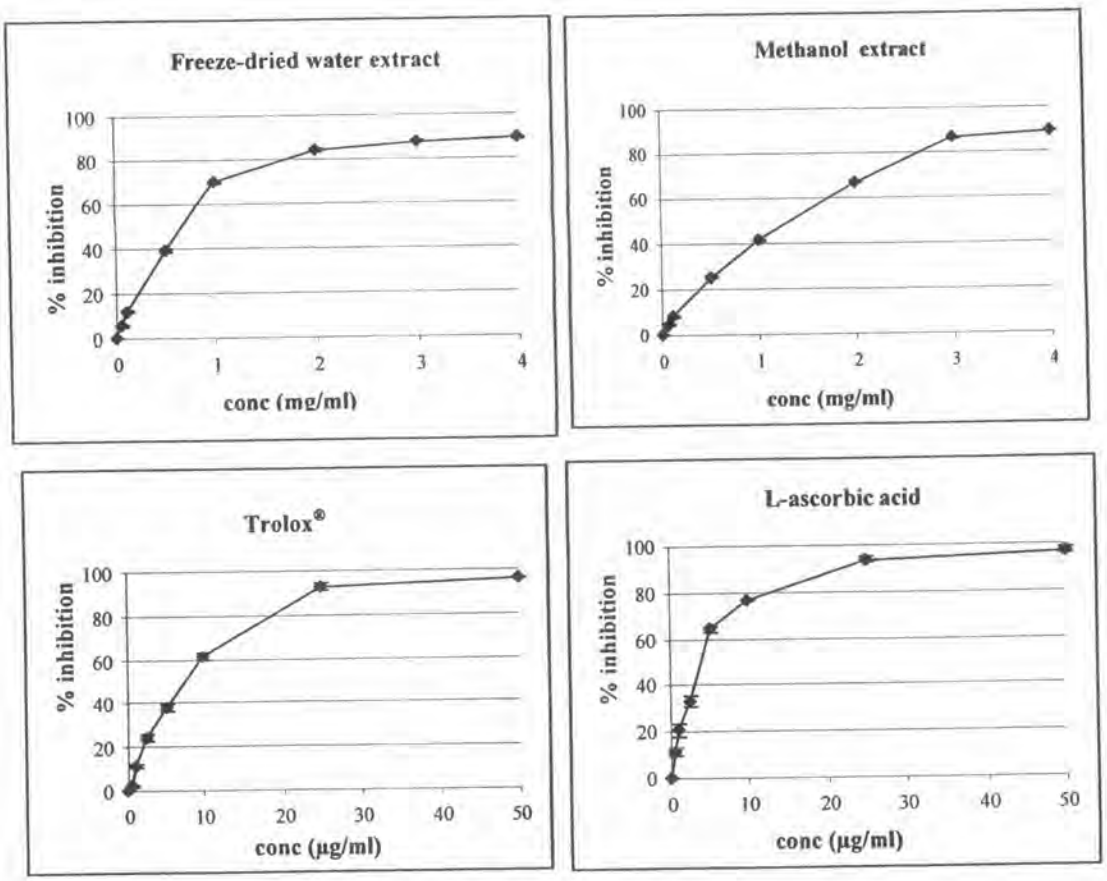


Figure 21. The relationship between percentage DPPH inhibition and the concentration of freeze-dried water extract, methanol extract and reference antioxidants Trolox ® and L-ascorbic acid (mean ± SD, n=3)

The percent DPPH radical inhibition of freeze-dried water extract increased to 84.44 ± 0.88 % at the concentration of 2.0 mg/ml, then reached a plateau at 3.0 and 4.0 mg/ml, where the inhibition percentages were 87.70 ± 0.24 and 88.78 ± 0.58 %, respectively. Methanol extract gave scavenging activity of 66.71 ± 0.56 % at the concentration of 2.0 mg/ml. When the concentration was increased to 3.0 and 4.0 mg/ml, the % inhibition increased sharply to 86.39 ± 0.52 and to 89.15 ± 0.25 %, respectively. The freeze-dried water extract thus showed higher extent of inhibition than the methanol extract.

Trolox ® and L-ascorbic acid showed much higher extent of DPPH radical inhibition than the two extracts. The percent inhibition at the concentration of 10.0

μg/ml was 61.32 ± 1.34 % for Trolox ® and 76.54 ± 0.19 % for L-ascorbic acid. When the concentration was increased to 25.0 μg/ml, the values of % inhibition rapidly increased (92.55 ± 1.42 for Trolox ® and 93.62 ± 1.18 % for L-ascorbic acid). When the concentration was further increased to 50.0 μg/ml, the values of % inhibition barely increased (96.06 ± 0.15 for Trolox ® and 97.03 ± 1.02 % for L-ascorbic acid). However, the inhibition extent of both vitamins was still much higher than that of the freeze-dried water extract and methanol extract. The raw data for the absorbance and DPPH radical inhibition percentages are provided in Appendix B.

After the plots of % free radical inhibition versus concentration were constructed for each antioxidant, its IC_{50} value or the concentration that the tested antioxidant could cause 50% inhibition was calculated from the regression analysis of the initial portion of each curve. It was found that a polynomial regression equation provided a better result than a linear equation, with a higher R^2 (coefficient of determination), as shown in Table 37 and Figures 22-25.

The estimated IC_{50} can be divided into two groups of different activities, i.e., the reference antioxidants (Trolox ® and L-ascorbic acid) and the extracts (freeze-dried water extract and methanol extract). The reference antioxidants exhibited the average IC_{50} values as follows: L-ascorbic acid 3.60 ± 0.19 μg/ml and Trolox ® 6.92 ± 0.29 μg/ml and the extracts showed the IC_{50} values as follows: freeze-dried water extract 0.643 ± 0.01 mg/ml and methanol extract 1.248 ± 0.01 mg/ml. Since the IC_{50} values indicate the inhibitory potency of the individual antioxidants, the methanol extract apparently demonstrated the weakest DPPH radical scavenging activity. The IC_{50} of Trolox ® and L-ascorbic acid were similar to that reported by Jang et al. (1997), which gave the IC_{50} for Trolox ® of 9.5μg/ml and the IC_{50} for L-ascorbic acid of 3.3 μg/ml, respectively.

The IC_{50} data, then, were analyzed by independent t-test at the 95% confidence level between Trolox ® and L-ascorbic acid. It was found that, there were significant differences in the antioxidants studied ($p<0.05$) with L-ascorbic acid being more active. According to the IC_{50} data of the freeze-dried water extract and the methanol extract, analysis by the same independent t-test at the 95% confidence level, there were also significant differences between the two ($p<0.05$), with the aqueous freeze-dried extract being more active.

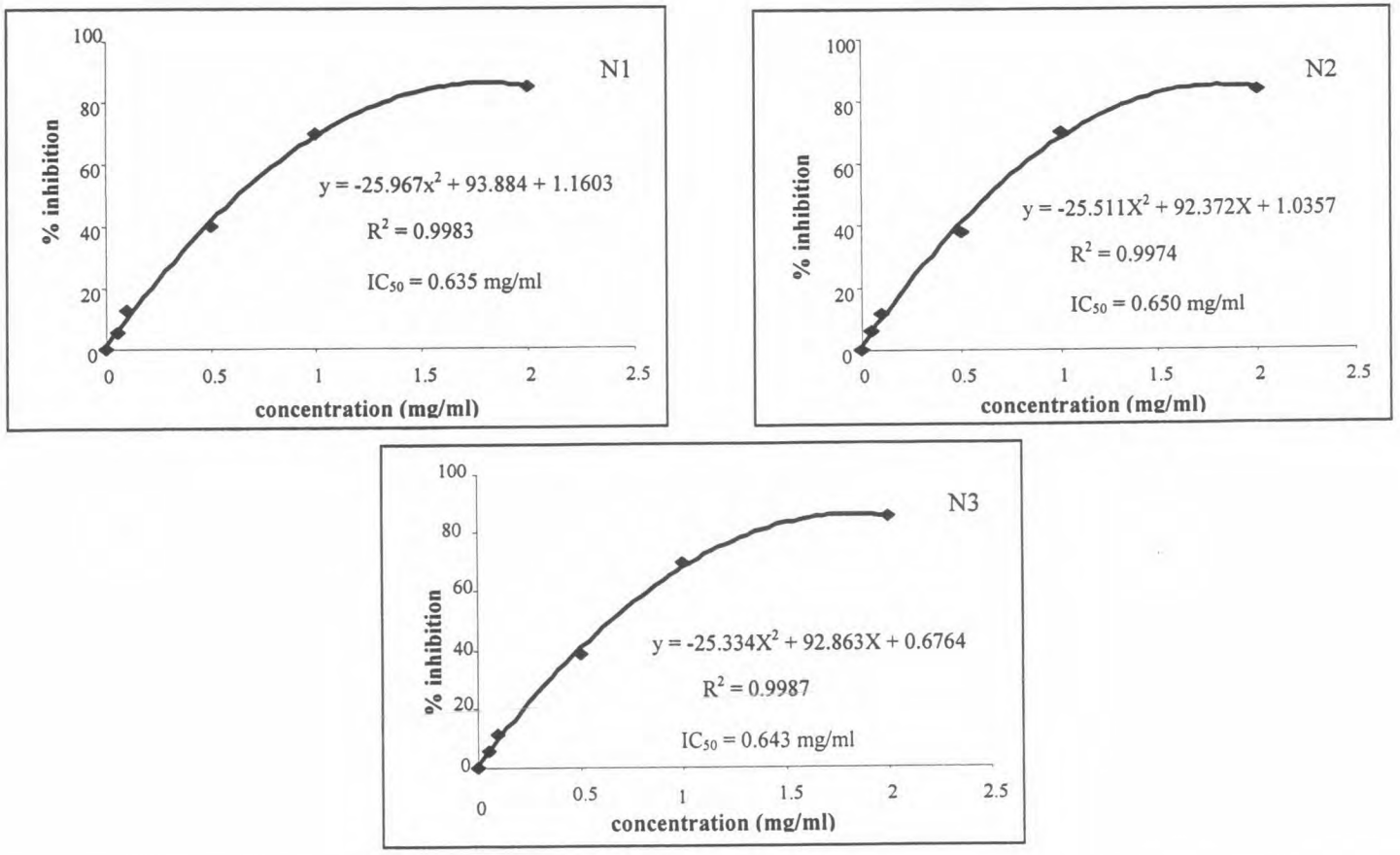


Figure 22. The regression curve of the initial portion of the % DPPH inhibition-concentration profile of freeze-dried water extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

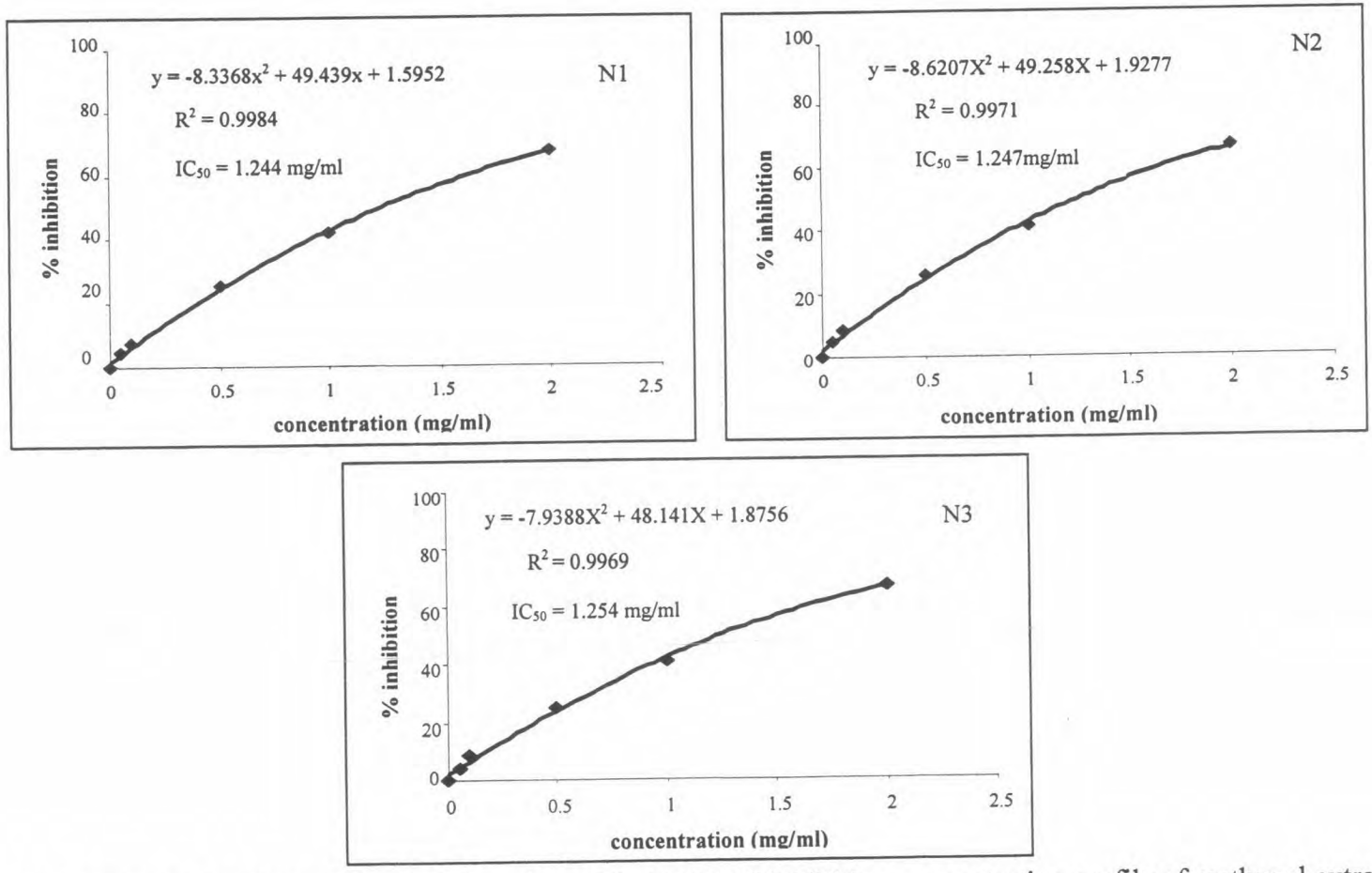


Figure 23. The regression curve of the initial portion of the % DPPH inhibition-concentration profile of methanol extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provide

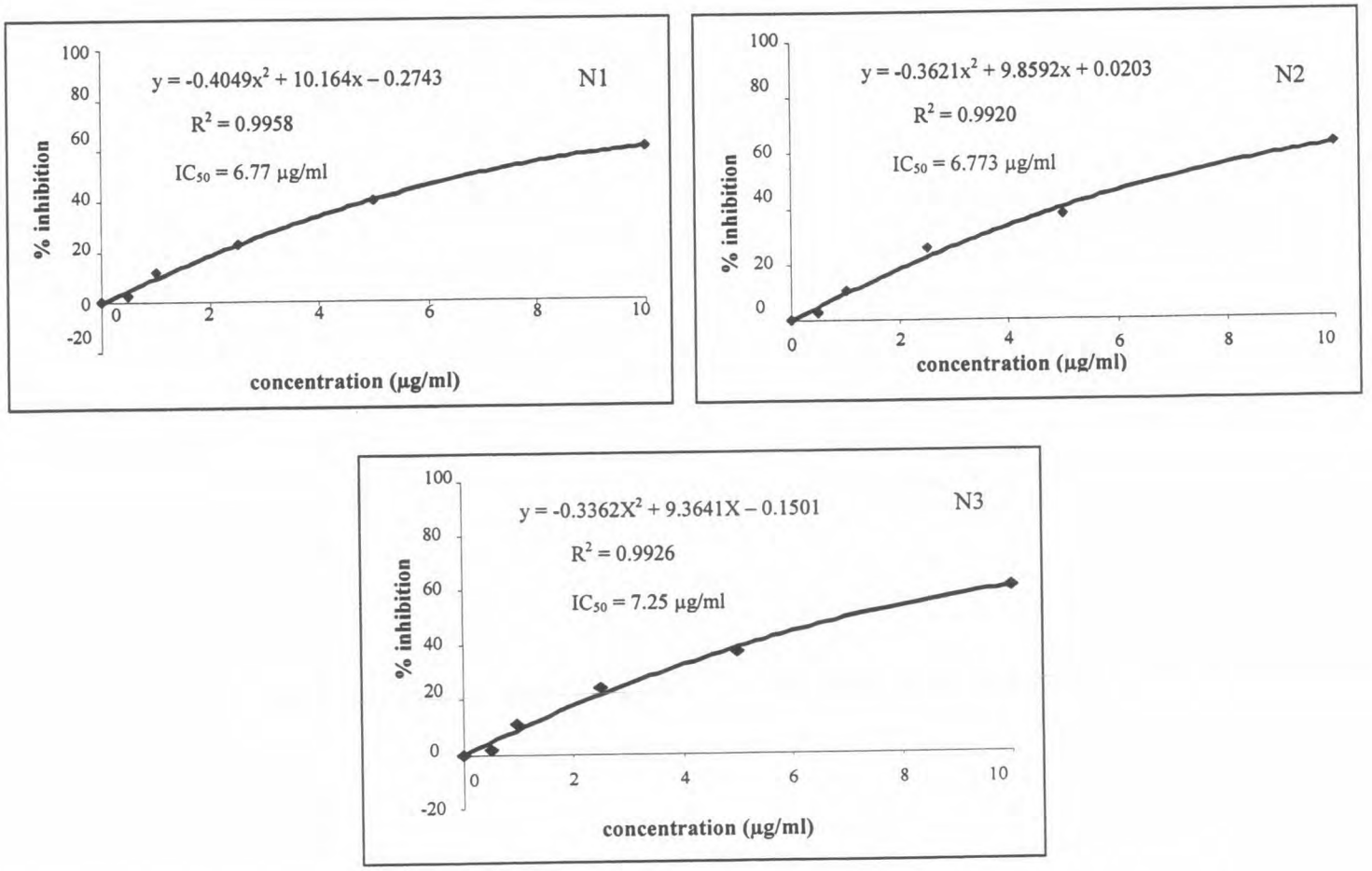


Figure 24. The regression curve of the initial portion of the % DPPH inhibition-concentration profile of Trolox®.

The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provide

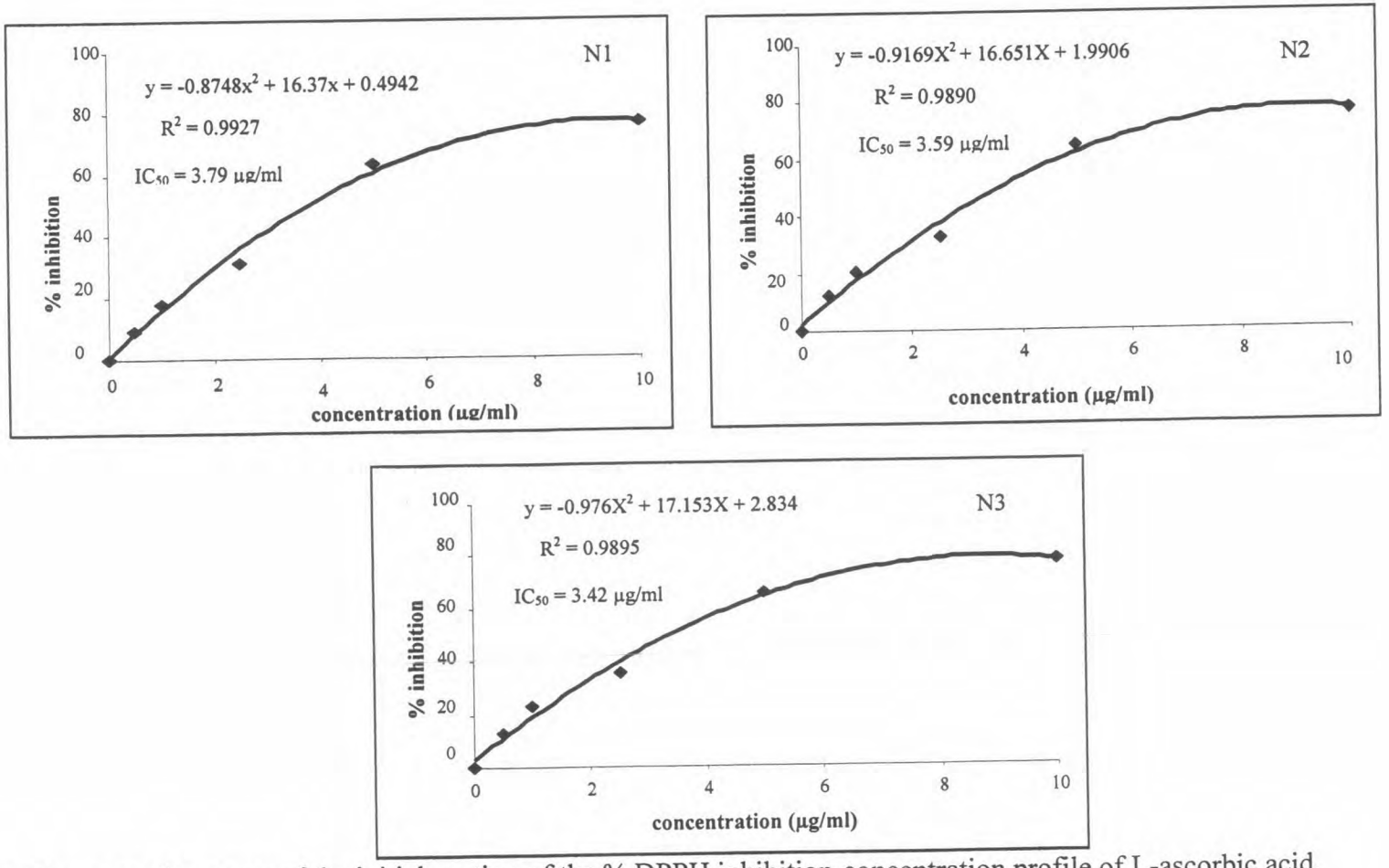


Figure 25. The regression curve of the initial portion of the % DPPH inhibition-concentration profile of L-ascorbic acid. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

Table 37. The IC_{50} of DPPH radical inhibition of each antioxidant, and coefficient of determination (R^2) obtained from polynomial regression of the initial portion of the plots between inhibition percentage and the concentrations of each antioxidant (n=3)

Sample	Polynomial equation (partial)					
	IC_{50} (mg/ml)	Mean	SD	R^2	Mean	SD
Freeze-dried water extract	0.635	0.643	0.007	0.9983	0.9981	0.001
	0.650			0.9974		
	0.643			0.9987		
Methanol extract	1.244	1.248	0.005	0.9984	0.9974	0.001
	1.247			0.9971		
	1.254			0.9969		

Sample	Polynomial equation (partial)					
	IC_{50} (µg/ml)	Mean	SD	R^2	Mean	SD
Trolox®	6.77	6.92	0.29	0.9958	0.9935	0.002
	6.73			0.9920		
	7.25			0.9926		
L-ascorbic acid	3.79	3.60	0.19	0.9927	0.9924	0.001
	3.59			0.9890		
	3.42			0.9895		

By comparing with Trolox ® and L-ascorbic acid, the freeze-dried water extract and methanol extract appeared to have much lower potency as judged from their IC_{50} values, which are in mg/ml range. There was correlation between IC_{50} values of freeze-dried water extract and methanol extract. The data show that the average IC_{50} values of freeze-dried water extract and methanol extract were 0.643 and 1.248 mg/ml, respectively. Similar to anti-tyrosinase data, the IC_{50} of the two extracts were also transformed into L-ascorbic acid equivalent, which were calculated to be 15.50 µg/ml and 10.33 µg/ml for freeze-dried water extract and methanol extract, respectively. However, the IC_{50} of pure L-ascorbic acid in scavenging DPPH was found to be 3.60 µg/ml, which was much different from the two extracts. The data seem to suggest that, unlike the anti-tyrosinase activity, the DPPH scavenging effects of the two extracts were not due solely to L-ascorbic acid content, but could as well result from other components especially the phenolic contents available in the extracts.However, it is not possible at this stage to know if the other components were more or less active than L-ascorbic acid and if they could enhance or counteract its effect. Despite multi-components responsible for the antioxidant activity, the DPPH scavenging potency of the two extracts was still quite low compound to well-known antioxidants like L-ascorbic acid and Trolox®. This may suggest that the contents of active antioxidant components present in the two extracts might be rather small. It was found that one mg of each freeze-dried water extract and methanol extract was equivalent to 10.09 and 6.59 µg of sinapic acid, 0.51 and 0.33 µg of quercetin and 24.11 and 8.28 µg of vitamin C, respectively. Transformation of the two extracts in terms of % w/w that was equivalent to sinapic acid, quercetin and vitamin C were 1.01, 0.05 and 2.41 % w/w for freeze-dried water extract and 0.66, 0.03 and 0.83% w/w for methanol extract, respectively. The freeze-dried water extract contained more total phenolics, total flavonoids and vitamin C than the methanol extract. The freeze-dried water extract accordingly showed stronger antioxidant activity by DPPH radical inhibition than the methanol extract. Several studies have been made concerning the relationship between the total phenolic contents and the antioxidant activity (Velioglu et al., 1998; Deighton et al., 2000). Their studies clearly indicated that it is important to measure the antioxidant activity by taking into account the phenolic contents when evaluating the antioxidant potential of plant extracts.

4.2 Inhibition of Superoxide anion from riboflavin-light-NBT system or riboflavin-photo-oxidation method

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

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

The data on percentage of superoxide anion inhibition by freeze-dried water extract and methanol extract compared to other antioxidants /radical scavengers such as Trolox® and L-ascorbic acid are graphically shown in Figure 26. Freeze-dried water extract and methanol extract were prepared at various concentrations of 0.00, 1.00, 2.00, 4.00, 6.00, 8.00 mg/ml. Trolox® and L-ascorbic acid were prepared at the concentrations of 0.00, 10.00, 20.00, 50.00, 100.00, 200.00 µg/ml.

Figure 26 demonstrates that when the concentration of the antioxidants was increased, the mean inhibition or the scavenging extent of superoxide anion was also increased. The freeze-dried water extract and the methanol extract exhibited the respective percent maximum inhibition of 84.33 ± 1.42 and 72.03 ± 0.59 %, both at the concentration of 8.0 mg/ml (Table 38). This maximum inhibition was lower than Trolox ®, which gave maximum inhibition of 92.63 ± 0.14 % at the concentration of only 200.0 µg/ml. L-ascorbic acid gave lower extent of maximum inhibition than Trolox ®, with the mean inhibition extent of 57.27 ± 0.49 % at 100.0 µg/ml. When the concentration was increased to 200.0 µg/ml, the inhibition percentage did not increase. Rather, it decreased slightly to 50.92 ± 1.03 % (Table 39). However, the inhibition extent exerted by L-ascorbic acid was still much higher than the two extracts.

Figures 27, 28, 29 and 30 show the individual plots between % superoxide anion inhibition and concentration, constructed separately for each antioxidant. The plots clearly indicate that the freeze-dried water extract and the methanol extract gave inhibition profiles similar to Trolox ®, whose inhibition efficacy increased

consistently with the concentration within the range studied. On the other hand, the inhibition of L-ascorbic acid was concentration-dependent only for a short range of concentration and became saturated at 100.0 µg/ml. At the higher concentration of 100.0 µg/ml, L-ascorbic interacted direct by NBT resulting in the solution becoming visibly blue in color without the added riboflavin or stimulation by fluorescent lamp. The raw data of the absorbance values and % superoxide anion inhibition of the individual antioxidants are presented in Appendix C.

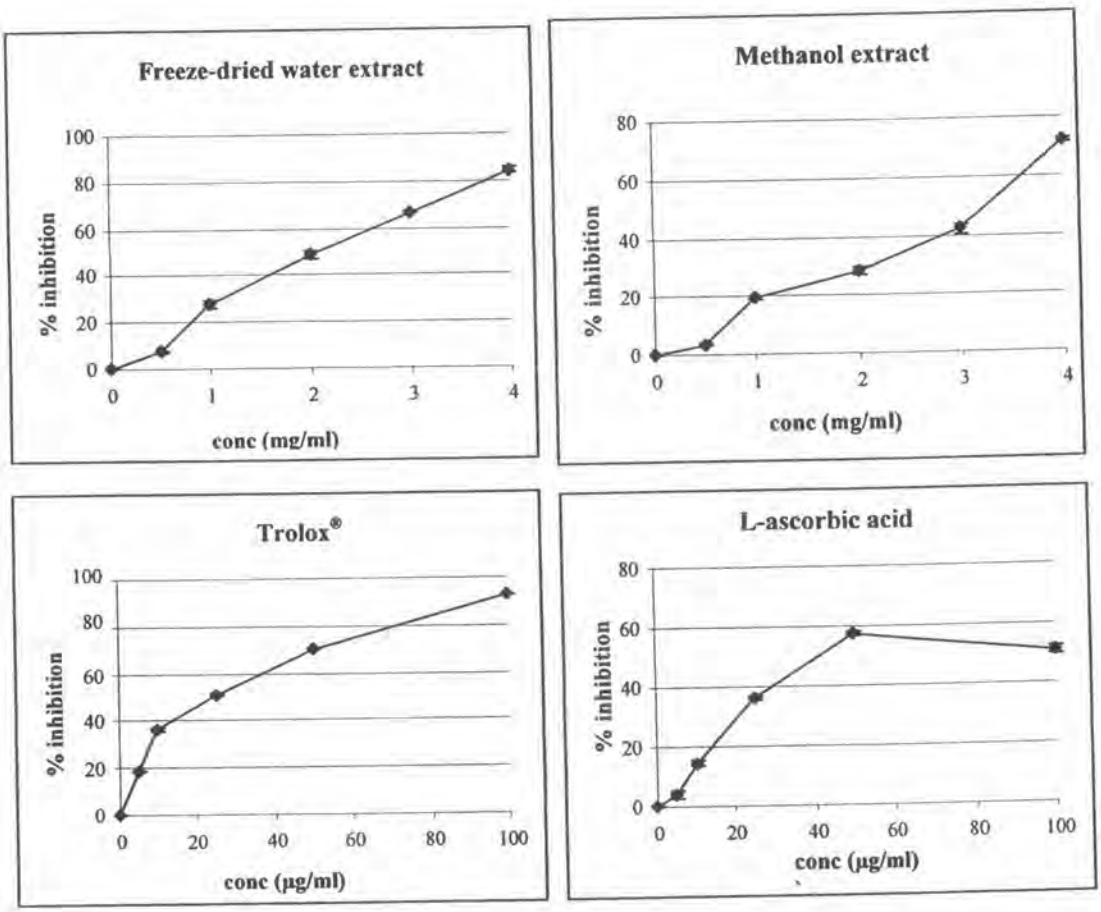


Figure 26. The relationship between superoxide anion inhibition and the concentration of freeze-dried water extract, methanol extract and two reference antioxidants Trolox ® and L-ascorbic acid (mean ± SD, n=3)

Table 38. Inhibition of superoxide anion from riboflavin photo-oxidation by freeze-dried water and methanol extracts at various concentrations (Mean ± SD)

No	conc (mg/ml)	Freeze-dried water extract		Methanol extract	
		% inhibition	SD	%inhibition	SD
1	0.00	0.00	0.00	0.00	0.00
2	1.00	7.45	0.32	3.50	0.47
3	2.00	27.86	1.64	19.60	0.90
4	4.00	48.67	1.97	28.44	1.22
5	6.00	66.53	0.54	42.22	1.72
6	8.00	84.33	1.42	72.03	0.59

Table 39. Inhibition of superoxide anion from riboflavin photo-oxidation by Trolox® compared to L-ascorbic acid at various concentration (Mean ± SD)

No	conc (µg/ml)	Trolox®		L-ascorbic acid	
		% inhibition	SD	%inhibition	SD
1	0.00	0.00	0.00	0.00	0.00
2	10.00	18.48	0.33	3.96	1.34
3	20.00	36.22	0.68	14.48	0.91
4	50.00	51.10	0.20	36.15	0.36
5	100.00	70.12	0.19	57.27	0.49
6	200.00	92.63	0.14	50.92	1.03

The IC_{50} or a concentration at which an antioxidant can cause 50% inhibition was calculated for each antioxidant by regression analysis of the % inhibition versus concentration plots. It was found that a polynomial equation gave a better R^2 (coefficient of determination) than a linear model (Table 40).

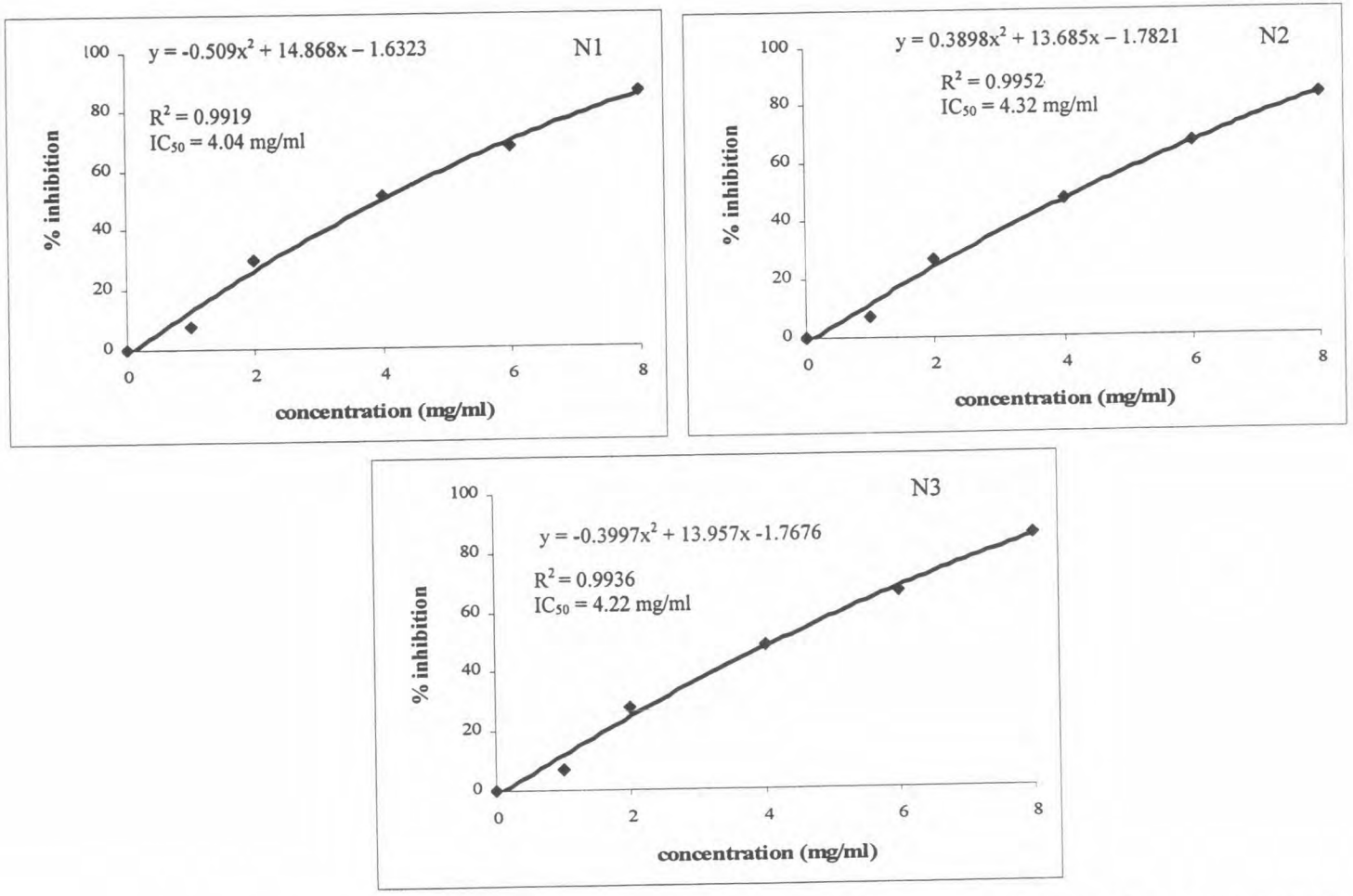


Figure 27. The regression curve of the initial portion of the % superoxide anion inhibition-concentration profile of freeze-dried water extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

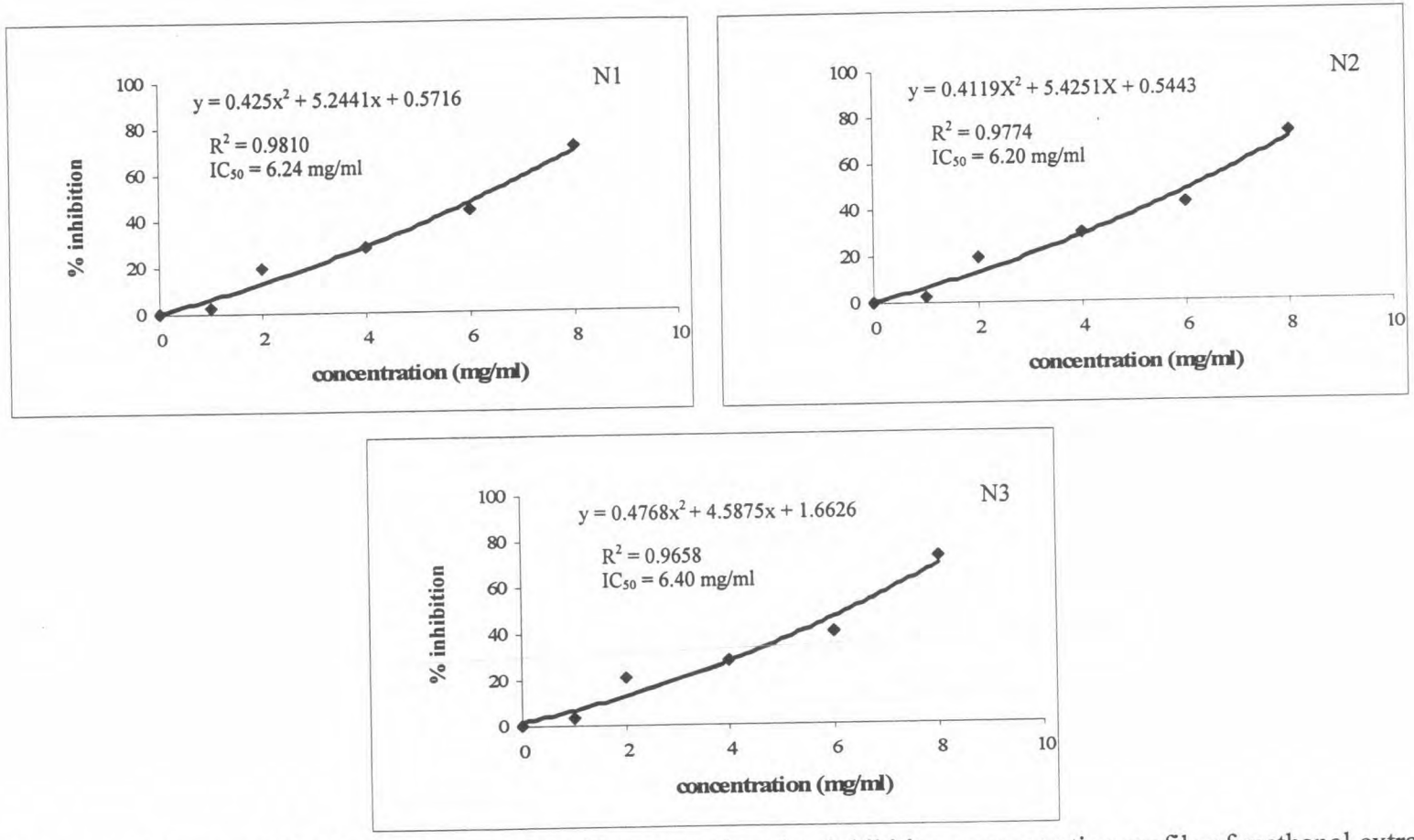


Figure 28. The regression curve of the initial portion of the % superoxide anion inhibition-concentration profile of methanol extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provide

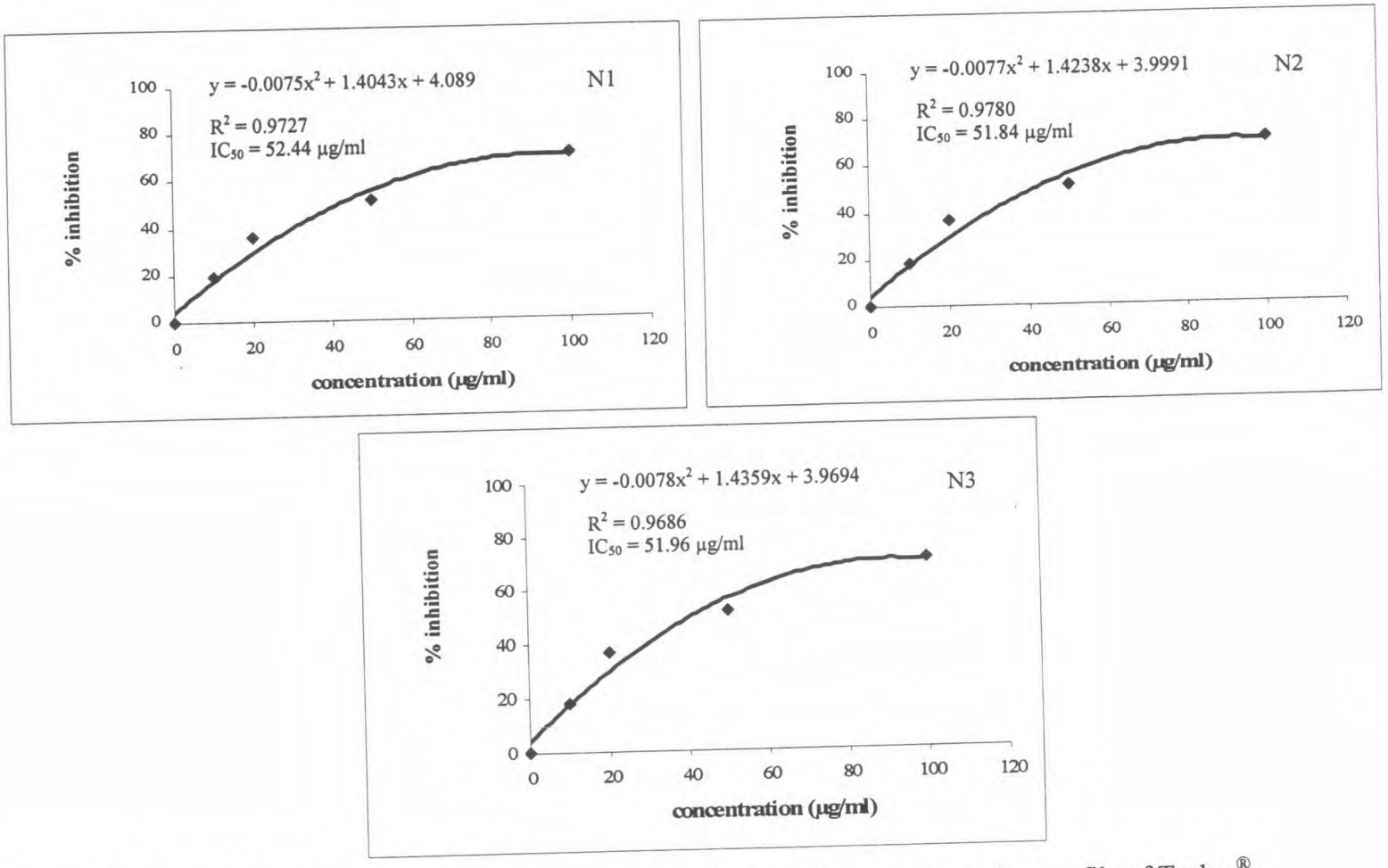


Figure 29. The regression curve of the initial portion of the % superoxide anion inhibition-concentration profile of Trolox®. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

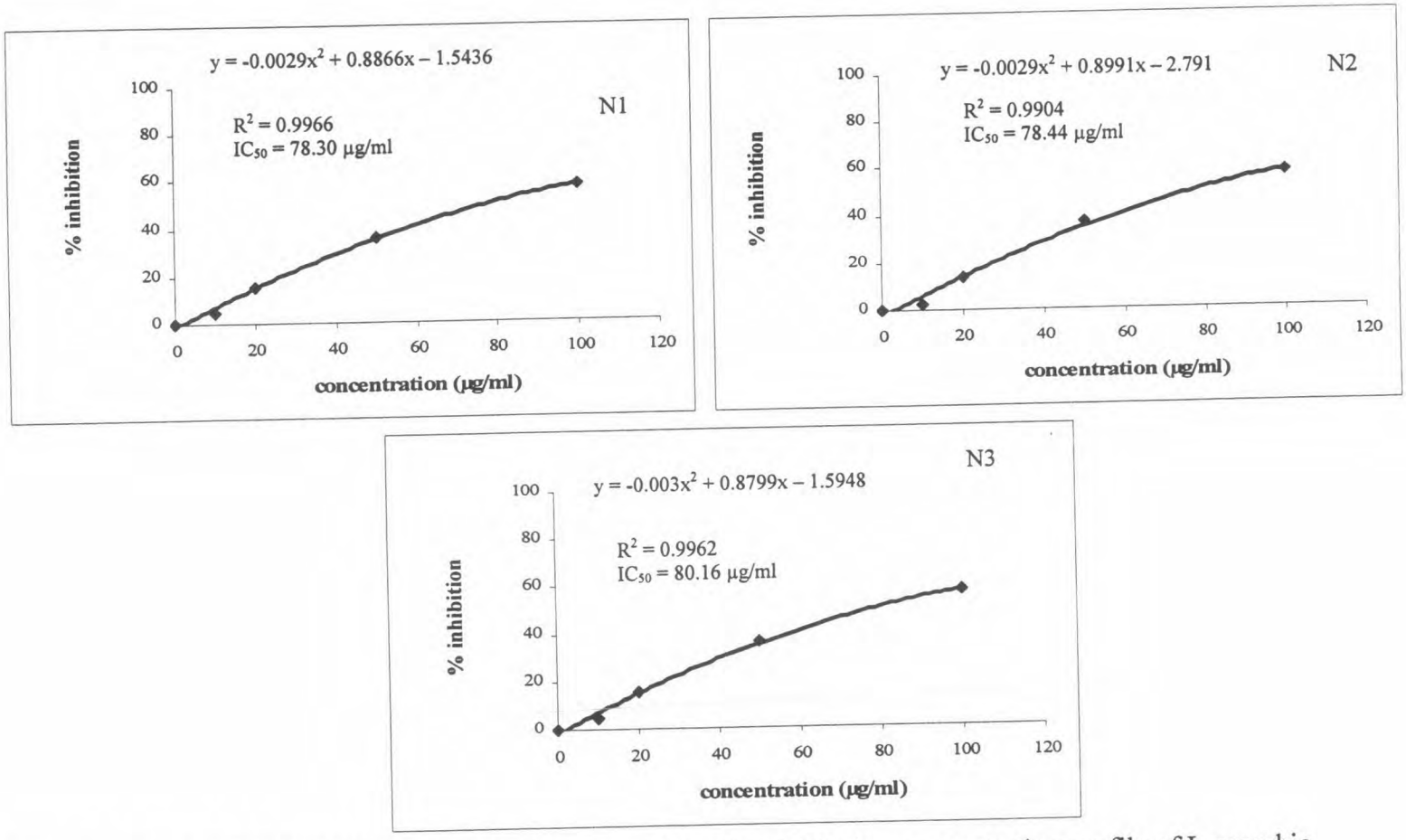


Figure 30. The regression curve of the initial portion of the % superoxide anion inhibition-concentration profile of L-ascorbic acid. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

Table 40. The IC_{50} and R^2 values of superoxide radical inhibition of each antioxidant obtained from polynomial regression of the initial portion of the plot between inhibition percentage and concentration of each antioxidant (Mean ± SD, n=3)

Sample	Polynomial equation					
	IC_{50} (mg/ml)	Mean	SD	R^2	Mean	SD
Freeze-dried water extract	4.04	4.20	0.142	0.9919	0.9930	0.002
	4.32			0.9952		
	4.22			0.9936		
Methanol extract	6.24	6.28	0.106	0.9810	0.9716	0.008
	6.20			0.9774		
	6.40			0.9658		

Sample	Polynomial equation					
	IC_{50} (µg/ml)	Mean	SD	R^2	Mean	SD
Trolox®	52.44	52.08	0.317	0.9727	0.9731	0.005
	51.84			0.9780		
	51.96			0.9686		
L-ascorbic acid	78.30	78.96	1.036	0.9966	0.9944	0.003
	78.44			0.9904		
	80.16			0.9962		

From Table 36, the means of estimated IC_{50} from the lowest to the highest value are: Trolox® 52.08 ± 0.16 µg/ml, L-ascorbic acid 78.96 ± 0.52 µg/ml, freeze-dried water extract 4.20 ± 0.07 mg/ml and methanol extract 6.28 ± 0.05 mg/ml. Since IC_{50} values is an indicator of the potency of an antioxidant, the freeze-dried water extract and methanol extract apparently showed weaker superoxide anion free radical scavenging activity than the reference antioxidants Trolox® and L-ascorbic acid. Therefore, higher concentrations were required for the freeze-dried water extract and the methanol extract to give similar inhibition percentage (50% inhibition) to other antioxidant.

The IC_{50} values of the freeze-dried water extract and the methanol extract were analyzed by independent t-test at 5% significant level. They were found to be significantly different ($p<0.05$). This suggested that the freeze-dried water extract was a more potent superoxide anion free radical scavenger than the methanol extract. Similarly, after applying the same t-test on their IC_{50} values, Trolox® was shown to be significantly more potent than L-ascorbic acid ($p<0.05$).

L-ascorbic acid is known to be a good radical scavenger and, thus it was expected to provide superoxide anion inhibition in concentration-dependent manner. However, the results obtained from this study appeared to be in conflict. The reasons for this discrepancy could be explained in terms of the ability of L-ascorbic acid to directly interact with NBT, even in the absence of riboflavin and light (Wachiranuntasin, 2005). Moreover, another explanation for this could be due to the formation of ascorbyl semiquinone radicals and oxidation of ascorbic acid by riboflavin in the presence of light. Photo-excited riboflavin can perform one electron oxidation of L-ascorbic acid, thereby generating a riboflavin radical (Geetha et al., 2004), or L-ascorbic acid itself could act as a pro-oxidant at high concentration (Konig and Ring, 2005). Therefore, the many unique properties of L-ascorbic acid might result in the saturation of its superoxide anion scavenging activity observed in this study.

Similar to DPPH test, the freeze-dried water extract gave a higher superoxide anion free radical scavenging activity than the methanol extract. The data seem to suggest that the superoxide anion free radical scavenging activities of the two extracts were probably a combined effect from L-ascorbic acid and other components like polyphenols. However, more studies are needed to characterize the type and amount of polyphenols contributing to the superoxide anion scavenging activity of the two extracts.

4.3 Singlet oxygen inhibition by $NaOCl/H_2O_2$ method

In this method, singlet oxygen inhibition was measured in the sodium hypochloride/hydrogen peroxide ($NaOCl/H_2O_2$) system. Sodium hypochloride and hydrogen peroxide can react to generate the singlet oxygen ($'O_2$). In this reaction, *N,N*-dimethyl-*p*-nitrosoaniline was used as a selective scavenger of $'O_2$ and histidine as a selective acceptor of $'O_2$ (stabilizer of singlet oxygen). The extent of $'O_2$ production was determined by measuring the decrease in absorbance of *N,N*-dimethyl-*p*-nitrosoaniline at 440 nm. The antioxidant activity of the extract, expressed as percent inhibition of singlet oxygen (% reduction of singlet oxygen), was calculated.

Figure 31 compares the extent of singlet oxygen inhibition exerted by freeze-dried water extract, methanol extract, Trolox® and L-ascorbic acid at various concentrations (0.1 to 4.0 mg/ml for freeze-dried water extract and methanol extract, 10.0 to 100.0 µg/ml for Trolox® and L-ascorbic acid). As seen from the data in Tables 41 and 42, as the concentration was increased, the singlet oxygen inhibition also increased for all antioxidants. The singlet oxygen inhibition by the freeze-dried extract increased to 68.32 ± 2.39 % at concentration of 4.0 mg/ml, whereas the methanol extract showed a similar inhibition extent of 67.30 ± 2.41 % at the same concentration.

Trolox® provided the highest singlet oxygen scavenging of 76.81 ± 1.05 % at concentration of 100.0 µg/ml, whereas L-ascorbic acid showed slightly lower inhibition extent of 63.73 ± 1.31 % at the same concentration.

After the plots of % singlet oxygen inhibition versus concentration were constructed for each antioxidant, the IC_{50} or the concentration at which an antioxidant can cause 50% inhibition was calculated by regression analysis using the data from initial part of the inhibition percentage plot of each antioxidant. Similar to the DPPH and superoxide anion tests, a polynomial equation was used and the results are given in Table 43 and Figures 32-35. The raw data for the absorbance and singlet oxygen inhibition percentages are provided in Appendix D.

Table 41. Inhibition of singlet oxygen by freeze-dried water extract compared to methanol extract at various concentrations (Mean ± SD)

No	conc (mg/ml)	Freeze-dried water extract		Methanol extract	
		% inhibition	SD	%inhibition	SD
1	0.00	0.00	0.00	0.00	0.00
2	0.10	2.68	0.28	1.57	0.17
3	0.50	15.73	2.33	10.44	0.47
4	1.00	45.65	0.42	27.15	0.49
5	2.00	59.09	1.13	42.29	0.81
6	4.00	68.32	2.39	67.30	2.41

Table 42. Inhibition of singlet oxygen by Trolox® compared to L-ascorbic acid at various concentrations (Mean ± SD)

No	conc (µg/ml)	Trolox®		L-ascorbic acid	
		% inhibition	SD	%inhibition	SD
1	0.00	0.00	0.00	0.00	0.00
2	10.00	10.31	0.70	3.80	0.75
3	25.00	16.41	0.63	10.07	0.39
4	50.00	23.37	0.86	24.31	1.25
5	75.00	40.69	1.36	39.89	1.25
6	100.00	76.81	1.05	63.73	1.31

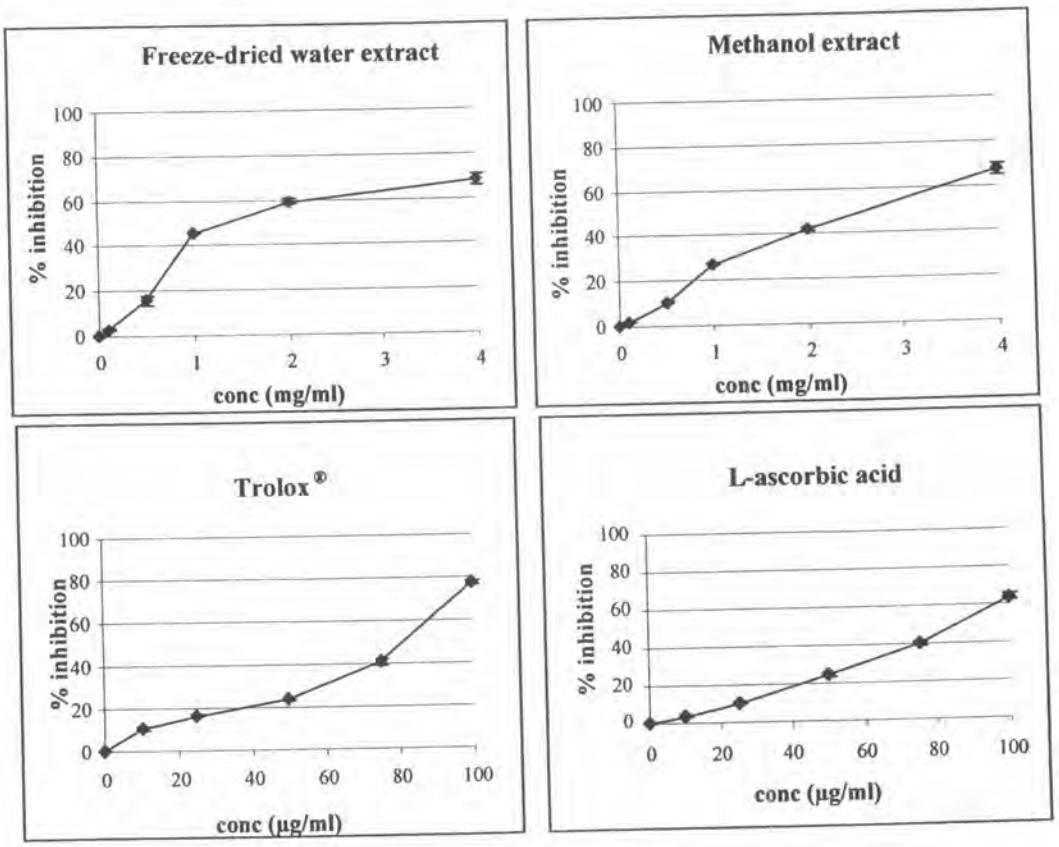


Figure 31. The relationship between singlet oxygen inhibition and the concentration of freeze-dried water extract, methanol extract and reference antioxidant Trolox ® and L-ascorbic acid (Mean ± SD, n=3)

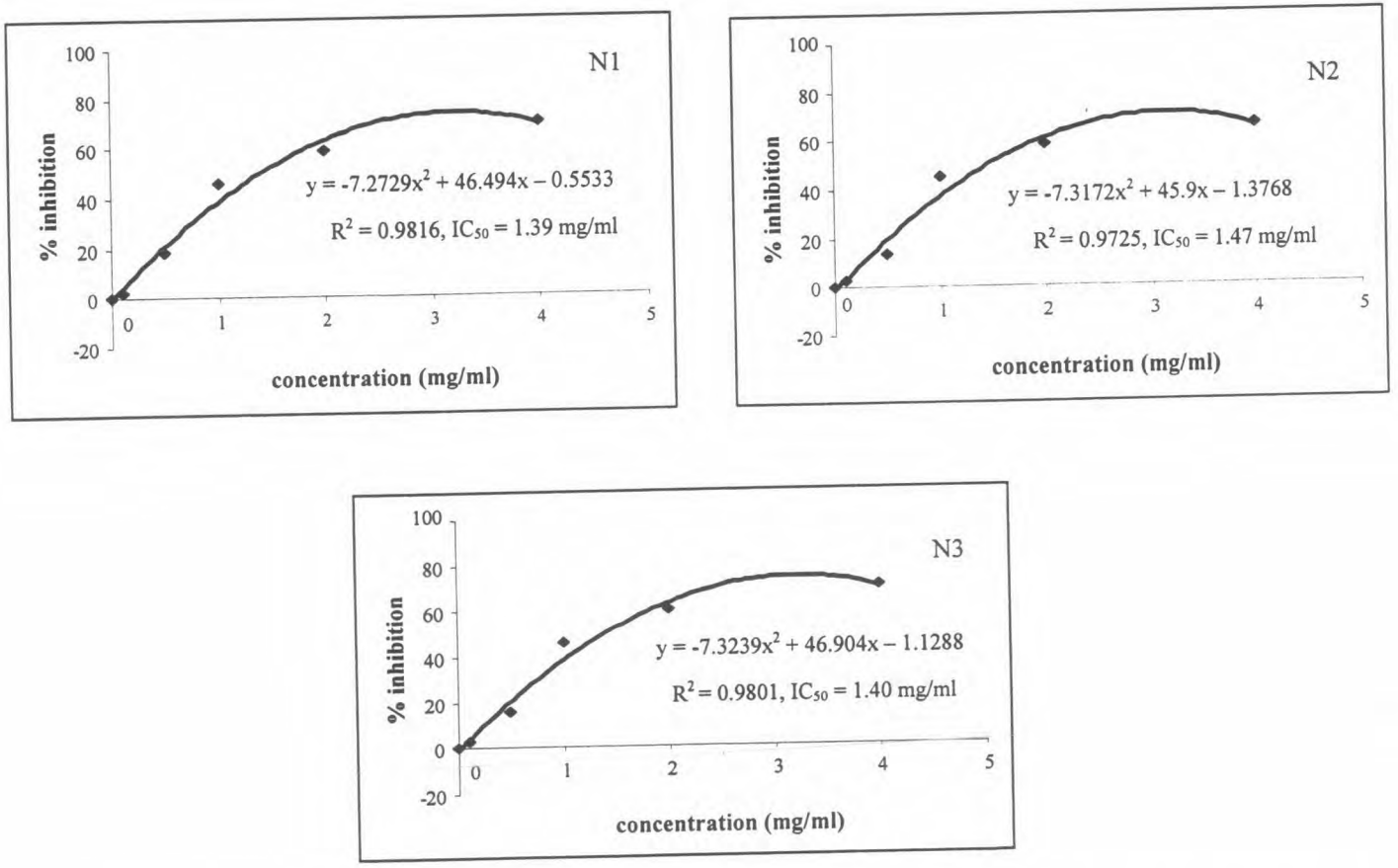


Figure 32. The regression curve of the initial portion of the % Singlet oxygen inhibition-concentration profile of freeze-dried water extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

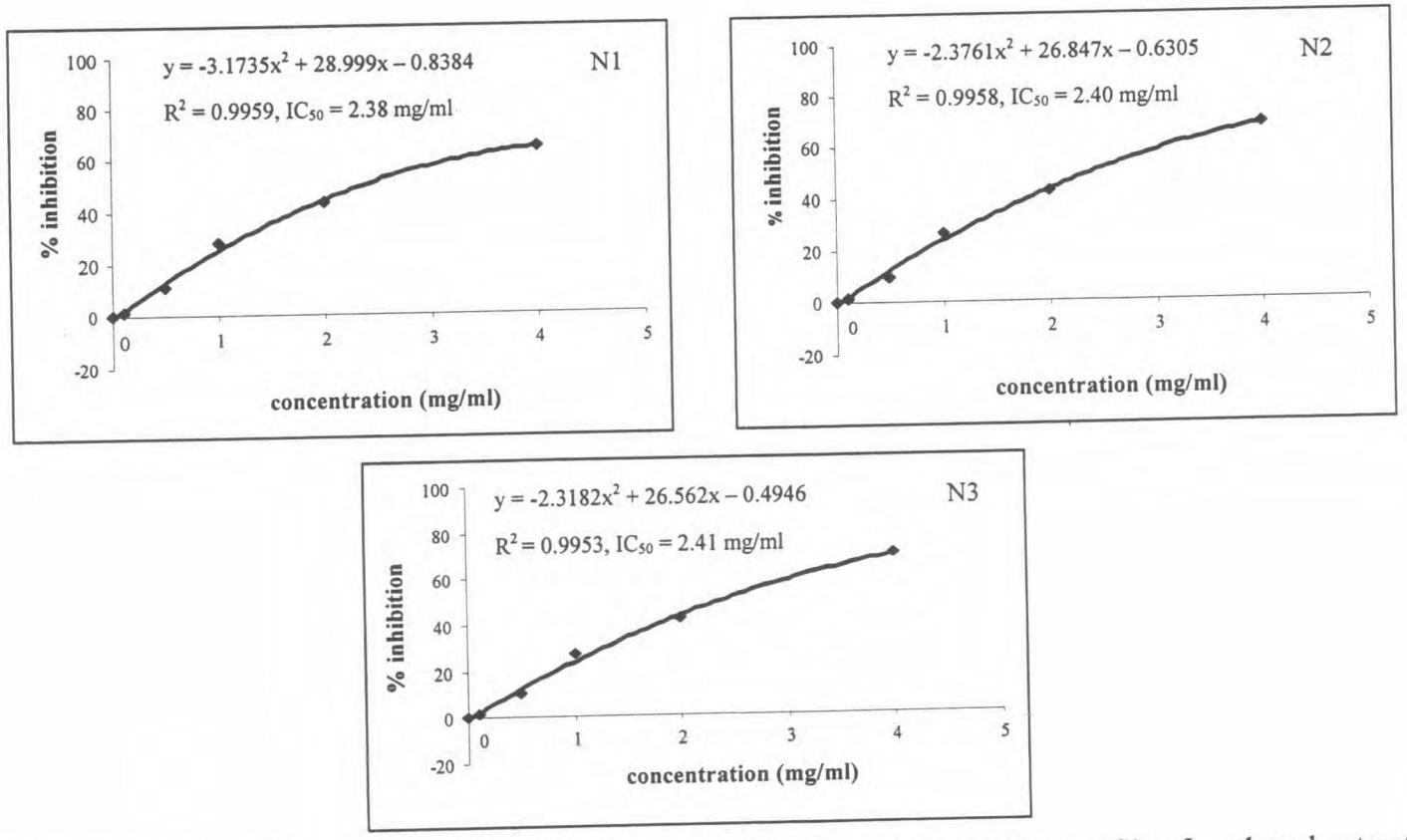


Figure 33. The regression curve of the initial portion of the % Singlet oxygen inhibition-concentration profile of methanol extract. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

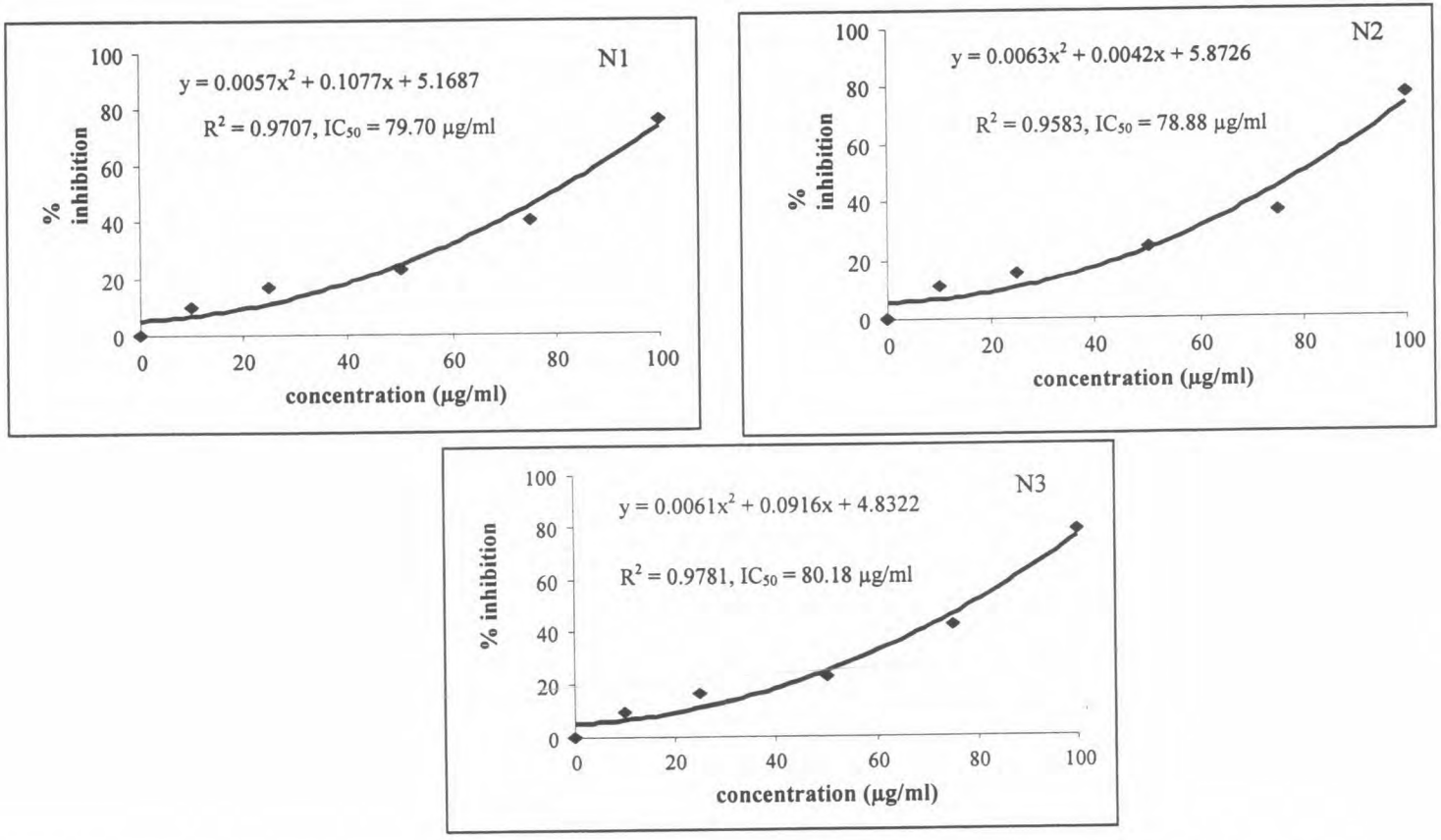


Figure 34. The regression curve of the initial portion of the % Singlet oxygen inhibition-concentration profile of Trolox®. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

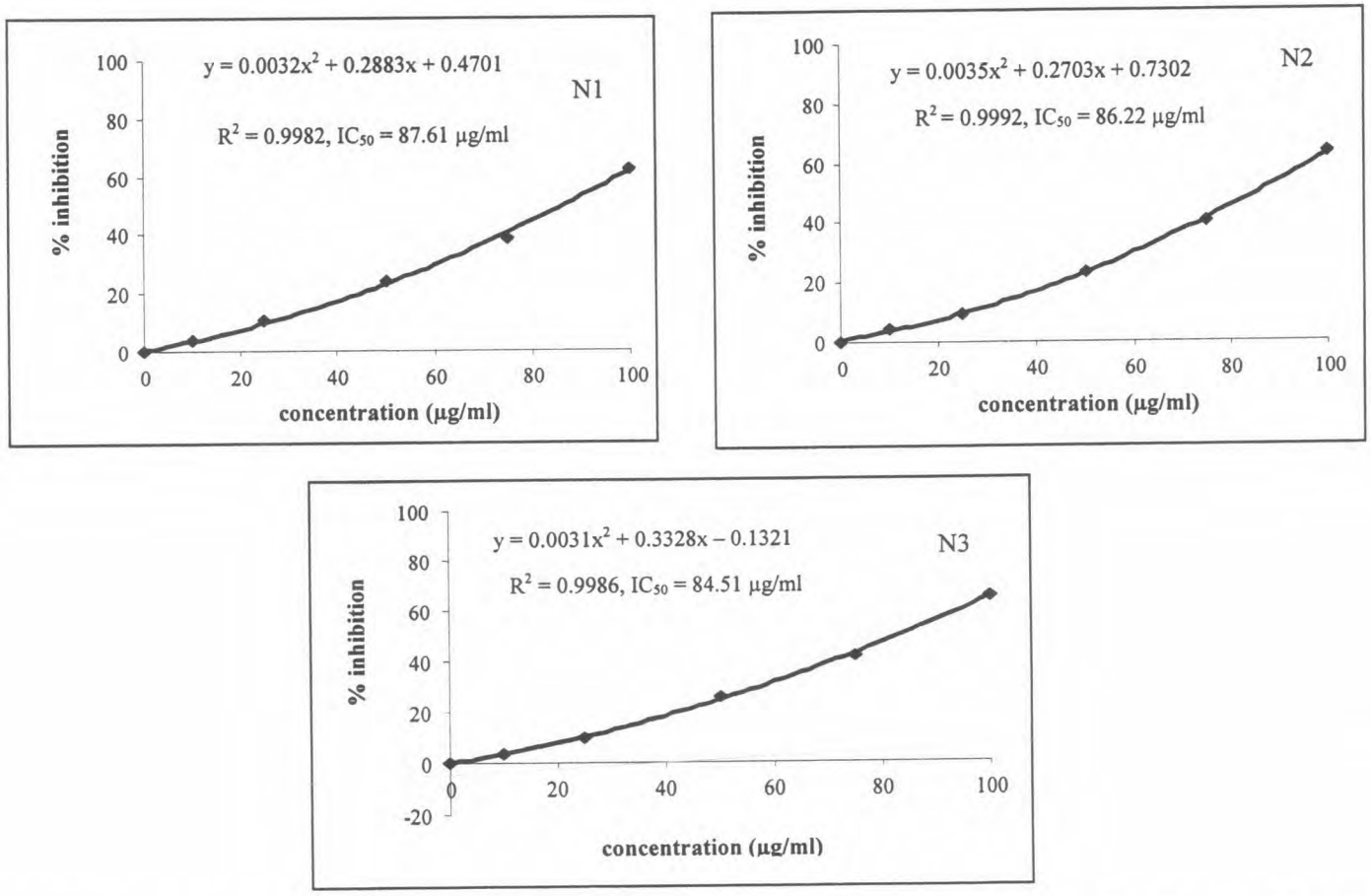


Figure 35. The regression curve of the initial portion of the % Singlet oxygen inhibition-concentration profile of L-ascorbic acid. The polynomial regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided

Table 43. The IC_{50} values of singlet oxygen inhibition by each antioxidant. The coefficient of determination (R^2) was obtained from polynomial regression of the initial portion of the plot between inhibition percentage and the initial concentrations of each antioxidant (Mean ± SD, n=3)

Sample	Polynomial equation (partial)					
	IC_{50} (mg/ml)	Mean	SD	R^2	Mean	SD
Freeze-dried water extract	1.39	1.42	0.04	0.9816	0.9781	0.01
	1.47			0.9725		
	1.40			0.9801		
Methanol extract	2.38	2.40	0.02	0.9959	0.9957	0.00
	2.40			0.9958		
	2.41			0.9953		

Sample	Polynomial equation (partial)					
	IC_{50} (µg/ml)	Mean	SD	R^2	Mean	SD
Trolox®	79.70	79.59	0.66	0.9707	0.9690	0.010
	78.88			0.9583		
	80.18			0.9781		
L-ascorbic acid	87.61	86.11	1.55	0.9982	0.9987	0.001
	86.22			0.9992		
	84.51			0.9986		

According to Table 43, the estimated IC_{50} values can be ranked from the lowest to the highest as follows: Trolox® 79.59 ± 0.66 µg/ml, L-ascorbic acid 86.11 ± 1.55 µg/ml, freeze-dried water extract 1.42 ± 0.04 mg/ml and methanol extract 2.40 ± 0.02 mg/ml. Since the IC_{50} value is an indicator of the potency of an antioxidant, the methanol extract which demonstrated the highest IC_{50} value thus appeared to be the least potent singlet oxygen scavenger. Trolox® gave the lowest IC_{50} and thus possessed the strongest singlet oxygen scavenging potency. The freeze-dried

water extract and the methanol extract gave distincly higher IC_{50} values than Trolox® and L-ascorbic acid indicating their markedly lower potency than the two vitamins.

The IC_{50} values of the freeze-dried water extract and the methanol extract were analyzed using independent t-test at 5% significant level. Significant differences were found among the IC_{50} values of the two extracts ($p<0.05$), suggesting that the freeze-dried water extract was a more potent singlet oxygen scavenger than the methanol extract. Similarly, Trolox® was significantly more potent than L-ascorbic acid in inhibiting the singlet oxygen using the same test ($p<0.05$).

According to report by Wang and Jiao (2000) L-ascorbic acid at 17.6 μg/ml was found to produce 6.18% inhibition of singlet oxygen. Using the % singlet oxygen inhibition versus concentration relationship obtained from this study, the concentration of L-ascorbic acid to generate the same 6.18% inhibition was estimated to be 16.55 μg/ml. This value agreed quite well with Wang and Jiao (2000).

From this study, singlet oxygen was the least quenched by methanol extract, with IC_{50} of 2.40 ± 0.02 mg/ml. The freeze-dried water extract, on the other hand, was a more powerful singlet oxygen quencher than the methanol extract, with IC_{50} of 1.42 ± 0.04 mg/ml. When compared to the pure reference antioxidants Trolox® and L-ascorbic acid, both extracts showed a remarkably lower scavenging activity of chemically generated active oxygen species. The data seem to suggest that the singlet oxygen quenching effects of the two extracts were also probably a combined effect of both L-ascorbic acid and other components present in the two extracts. The different contents of polyphenols and vitamin C in the extract is important for its ability to inhibit the reactive oxygen species. The relatively lower singlet oxygen scavenging activity of the methanol extract could be due primarily to its relatively lower content of polyphenols and L-ascorbic acid.

Part 5. Cytotoxicity test of *Raphanus sativus* L. extracts on normal human fibroblast cell line by LDH assay

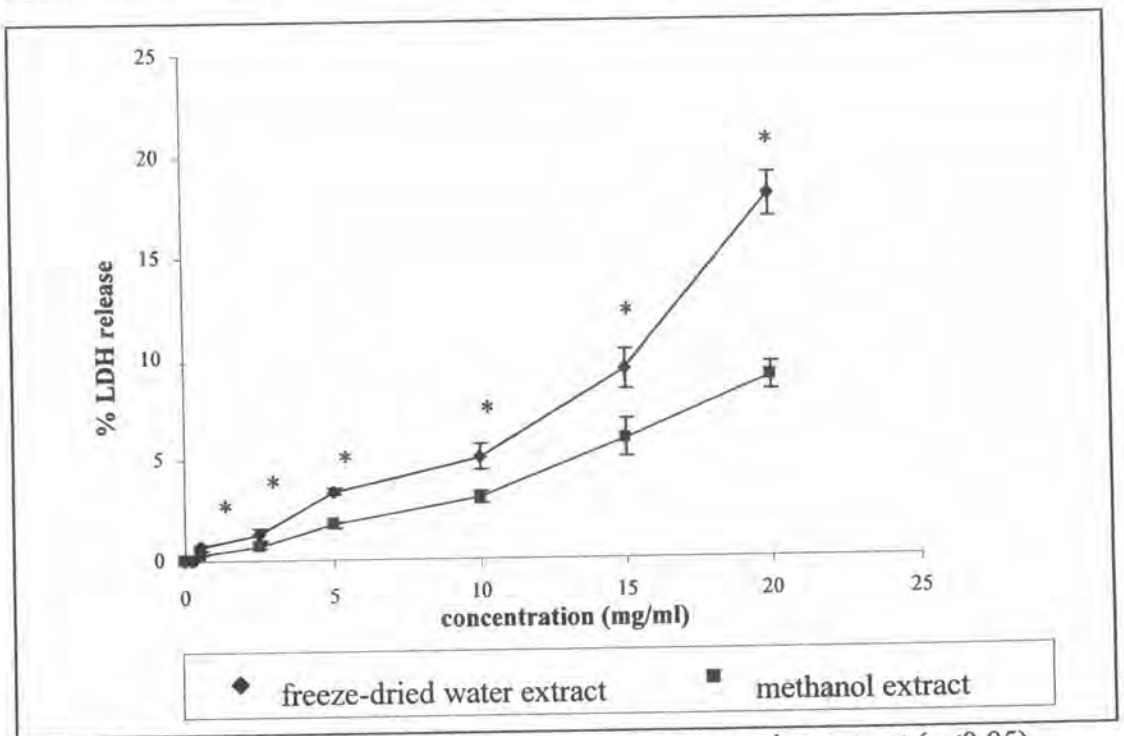
In vitro cytotoxicity assays detect the degree to which a treatment is toxic to cells. Lactate dehydrogenase (LDH) assay was another method used in this study to evaluate cell viability in terms of the degree of cell membrane integrity after exposure to the *R. sativus* extracts. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme which is not secreted outside the cells. But upon damage of the cell membrane LDH leaks out. Measurement of the release of LDH from cells is based on a colorimetric quantitation after an enzymatic reaction (Vihola et al., 2005). The assay is based on the reduction of nicotinamide adenine dinucleotide (NAD) by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of tetrazolium dye. LDH leakage was measured from normal human dermal fibroblast cells by using TOX-7 cytotoxicity assay-kit. The % LDH release of the freeze-dried water extract and the methanol extract were compared at various concentrations (0.25, 0.50, 2.50, 5.00, 10.00, 15.00 and 20.00 mg/ml). The average data are shown in Table 44 and Figure 36. As previously described in the method, the extent of % release was calculated by comparison of the individual absorbance with the average absorbance of the total LDH released from the lysed cells (mean value = 0.366 $\pm$ 0.020, n = 30, Table 45). The individual data on absorbance and % release are provided in Tables 46-47 for the freeze-dried extract and the methanol extract, respectively.

Table 44 and Figure 36 show the average values of percent LDH release from normal human dermal fibroblasts following 24 hr incubation with the freeze-dried water extract and the methanol extract. It was found that the freeze-dried water extract gave greater % LDH release than the methanol extract at all concentrations except at the lowest concentration studied of 0.25 mg/ml, where no LDH was detected in the incubation medium. At 0.50 mg/ml concentration the extent of LDH release was 0.73 $\pm$ 0.16 % for the freeze-dried water extract whereas it was 0.36 $\pm$ 0.16 % for the methanol extract. As the exposure concentration was further increased from 2.50 to 20.00 mg/ml, the extent of LDH release also increased for both extracts, with the values increasing from 1.37 $\pm$ 0.28 % to 17.94 $\pm$ 1.11 % for the freeze-dried extract and from 0.64 $\pm$ 0.16 % to 8.93 $\pm$ 0.68 % for the methanol extract. The higher extent

of LDH release suggested that the freeze-dried water extract might be able to interact with the plasma membrane to a greater degree than the methanol extract.

Table 44. The % LDH release from normal human fibroblast after exposure to various concentrations of freeze-dried water extract and methanol extract (Mean ± SD, n = 3)

conc (mg/ml)	Freeze-dried water extract		Methanol extract	
	% LDH released	SD	% LDH released	SD
0.00	0.00	0.00	0.00	0.00
0.25	0.00	0.00	0.00	0.00
0.50	0.73	0.16	0.36	0.16
2.50	1.37	0.28	0.64	0.16
5.00	3.37	0.16	1.73	0.16
10.00	5.01	0.64	3.01	0.28
15.00	9.29	0.98	5.92	0.96
20.00	17.94	1.11	8.93	0.68



* Significant between the two substance at same concentration extract ($p<0.05$)

Figure 36. The extent of LDH release from cultured fibroblasts after exposure to various concentrations of freeze-dried water extract and methanol extract (Mean ± SD, n=3)

The data on % LDH release at the same concentrations of freeze-dried water extract and methanol extract were analyzed by independent t-test at 5% significant level. Significant differences ($P < 0.05$) between the two substance were found at 0.5 mg/ml concentration regarding to the higher concentrations were also significant differences. The statistical results are shown in Appendix E.

According to sinapic acid and L-ascorbic acid, after applying the same independent t-test on % LDH release, there were significant differences ($P < 0.05$) at concentrations 25.0 µg/ml and the higher concentrations were also significant differences.

Since both extracts contained phenolic compounds and L-ascorbic acid as major active components, it would be interesting to know if these antioxidants were also responsible for the membrane interacting activities of the extract. To support this hypothesis, the extent of LDH release was also evaluated for L-ascorbic acid and sinapic acid (representative of total phenolic compounds) using the same fibroblast model. As seen in Tables 49 and Figure 37 show sinapic acid and L-ascorbic acid were also able to interact with the fibroblast membrane and resulted in a concentration-dependent release of LDH. At 250 µg/ml concentration, sinapic acid and L-ascorbic acid gave % LDH release of 1.82 ± 0.16 and 2.82 ± 0.42 %, respectively. Thus, the membrane-interacting activity of the two extracts was partly due to the presence of these antioxidants, with the freeze-dried extract being more active due to its higher contents of L-ascorbic acid and phenolic compounds.

However, other components apart from the phenolic compounds or vitamin C may also be responsible for the membrane-interacting activity of the two extracts. At 20.0 mg/ml, the freeze-dried extract had total phenolic contents (equivalent to sinapic acid) of 201.8 µg/ml and L-ascorbic acid content of 282.2 µg/ml, making a total of 484 µg/ml. For the methanol extract at the same concentration the contents of total phenolics and L-ascorbic acid were 131.8 and 165.6 µg/ml, making a total of 297 µg/ml. The combined concentrations of total phenolics and L-ascorbic acid found in the two extracts were less than 500 µg/ml. Yet at 20 mg/ml concentration, the extent of LDH release produced by the two extracts were far greater than that of the pure L-ascorbic acid or sinapic acid or the two substances in combination (Table 44 versus Table 49). This evidence thus suggested that other unidentified components in the two extracts also contributed to the total membrane toxicity.

It is interesting to note that the absolute absorbance values of the freeze-dried water extract, especially at higher concentrations of 15.00 and 20.00 mg/ml (0.834 – 1.038, Table 46), were greater than that of the total LDH release when no extract was added but the cells were intentionally lyzed to cause 100% LDH release (0.605 – 0.725, Table 45). This could be due to the ability of some components in the extract to directly interact with the reagents in LDH assay kit.

It was noticed that after addition of the extract, a red color was developed before the final addition of HCl. This was observed in both the cell-added wells and the wells in which no cells were cultured. Thus, some unknown components in the extract might directly interact with the reagents causing an increase in the absolute absorbance values. Since the interference was observed in both the cell-added and the blank wells, the effect appeared to be equal and balanced out. Therefore, when the absolute values were corrected by subtraction with their corresponding blank absorbance, the corrected absorbance of the freeze-dried extract became much lower than that of the total LDH, making the results acceptable under current comparison. On the other hand, the interference observed with the methanol extract was much less than the freeze-dried extract. Even at the highest concentration of 20 mg/ml, its absolute absorbance values (0.439 – 0.468, Table 47) were still lower than that of 100% LDH release, suggesting lesser contents of interfering components in the extract.

Table 45. The raw data for the absorbance of total LDH (100% release) obtained from lysed cells (n= 30 wells)

N	Abs	Blank	Diff	mean	SD
1	0.722	0.325	0.397		
2	0.661	0.303	0.358		
3	0.655	0.272	0.383		
4	0.691	0.293	0.398		
5	0.625	0.282	0.343		
6	0.650	0.306	0.344		
7	0.639	0.300	0.339		
8	0.690	0.304	0.386		
9	0.648	0.290	0.358		
10	0.605	0.265	0.340		
11	0.712	0.320	0.392		
12	0.664	0.302	0.362		
13	0.698	0.315	0.383		
14	0.637	0.274	0.363		
15	0.704	0.335	0.379	0.366	0.02
16	0.725	0.316	0.409		
17	0.662	0.282	0.380		
18	0.620	0.275	0.345		
19	0.655	0.286	0.369		
20	0.620	0.270	0.350		
21	0.695	0.312	0.383		
22	0.645	0.284	0.361		
23	0.680	0.310	0.370		
24	0.625	0.272	0.353		
25	0.710	0.305	0.396		
26	0.665	0.305	0.360		
27	0.620	0.284	0.336		
28	0.705	0.337	0.368		
29	0.675	0.320	0.355		
30	0.624	0.300	0.324		

Table 46. The raw data for the absorbance and LDH release percentages of freeze-dried water extract (Mean ± SD, n=3)

No.	Final conc (mg/ml)	n	Abs	Blank	Diff	% individual release	mean	SD
1	0.00	1	0.265	0.265	0.000	0.00	0.00	0.00
		2	0.269	0.269	0.000	0.00		
		3	0.272	0.272	0.000	0.00		
2	0.25	1	0.274	0.274	0.000	0.00	0.00	0.00
		2	0.270	0.270	0.000	0.00		
		3	0.269	0.269	0.000	0.00		
3	0.50	1	0.303	0.300	0.003	0.82	0.73	0.16
		2	0.322	0.320	0.002	0.55		
		3	0.287	0.284	0.003	0.82		
4	2.50	1	0.338	0.332	0.006	1.64	1.37	0.28
		2	0.335	0.330	0.005	1.37		
		3	0.349	0.345	0.004	1.09		
5	5.00	1	0.505	0.492	0.013	3.55	3.37	0.16
		2	0.460	0.448	0.012	3.28		
		3	0.427	0.415	0.012	3.28		
6	10.00	1	0.632	0.615	0.017	4.64	5.01	0.64
		2	0.618	0.597	0.021	5.74		
		3	0.573	0.556	0.017	4.64		
7	15.00	1	0.898	0.863	0.035	9.56	9.29	0.98
		2	0.834	0.804	0.030	8.20		
		3	0.885	0.848	0.037	10.11		
8	20.00	1	1.038	0.976	0.062	16.94	17.94	1.11
		2	1.033	0.963	0.070	19.13		
		3	0.935	0.870	0.065	17.76		

Table 47. The raw data for the absorbance and LDH release percentages of methanol extract (Mean ± SD, n=3)

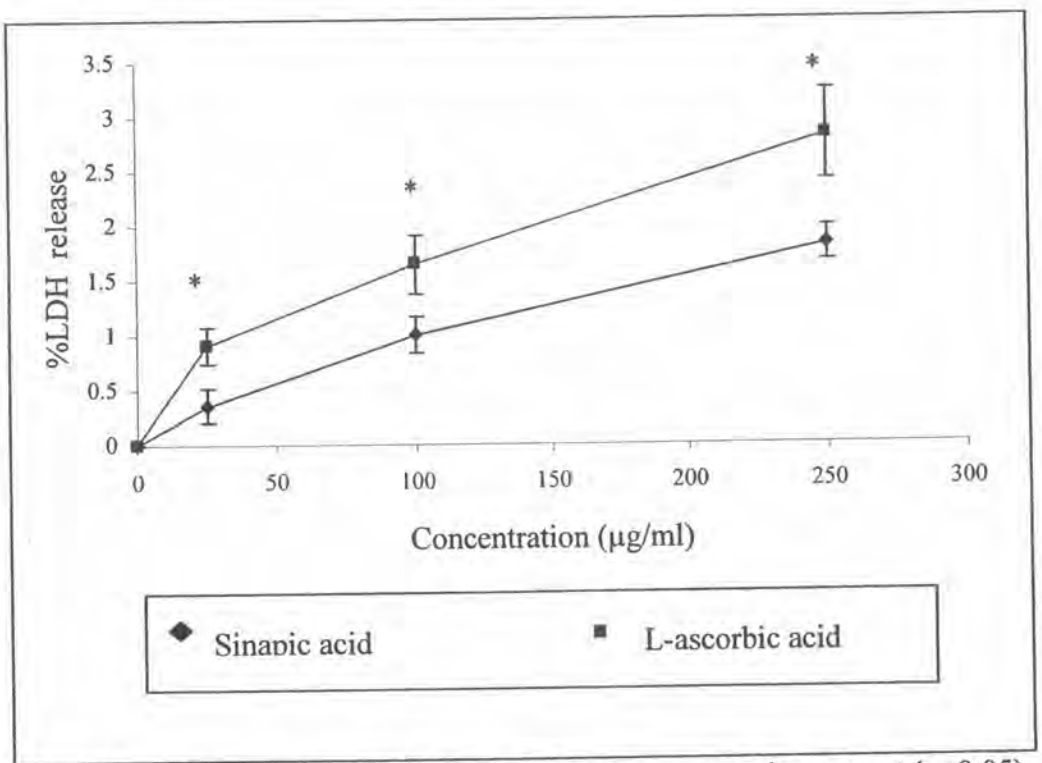
No.	Final conc (mg/ml)	n	Abs	Blank	Diff	% individual release	mean	SD
1	0.00	1	0.265	0.265	0.000	0.00	0.00	0.00
		2	0.262	0.262	0.000	0.00		
		3	0.260	0.260	0.000	0.00		
2	0.25	1	0.260	0.260	0.000	0.00	0.00	0.00
		2	0.265	0.265	0.000	0.00		
		3	0.261	0.261	0.000	0.00		
3	0.50	1	0.272	0.271	0.001	0.27	0.36	0.16
		2	0.265	0.263	0.002	0.55		
		3	0.280	0.279	0.001	0.27		
4	2.50	1	0.290	0.287	0.003	0.82	0.64	0.16
		2	0.284	0.282	0.002	0.55		
		3	0.295	0.293	0.002	0.55		
5	5.00	1	0.337	0.330	0.007	1.91	1.73	0.16
		2	0.317	0.311	0.006	1.64		
		3	0.324	0.318	0.006	1.64		
6	10.00	1	0.357	0.345	0.012	3.28	3.01	0.28
		2	0.348	0.338	0.010	2.73		
		3	0.375	0.364	0.011	3.01		
7	15.00	1	0.441	0.419	0.022	4.92	5.92	0.96
		2	0.454	0.434	0.020	6.01		
		3	0.421	0.396	0.025	6.83		
8	20.00	1	0.455	0.420	0.035	9.56	8.93	0.68
		2	0.439	0.406	0.033	9.02		
		3	0.468	0.438	0.030	8.20		

Table 48. The raw data for the absorbance and LDH release percentages of L-ascorbic acid and sinapic acid (Mean ± SD, n=3)

No.	Final conc (µg/ml)	n	Abs	Blank	Diff	% individual release	mean	SD
L-ascorbic acid	0.00	1	0.262	0.262	0.000	0.00	0.00	0.00
		2	0.260	0.260	0.000	0.00		
		3	0.266	0.266	0.000	0.00		
	25.0	1	0.267	0.264	0.003	0.82	0.91	0.16
		2	0.279	0.275	0.004	1.09		
		3	0.286	0.283	0.003	0.82		
	100.0	1	0.289	0.282	0.007	1.91	1.64	0.27
		2	0.278	0.273	0.005	1.37		
		3	0.275	0.269	0.006	1.64		
	250.0	1	0.279	0.270	0.009	2.46	2.82	0.42
		2	0.273	0.263	0.010	2.73		
		3	0.277	0.265	0.012	3.28		
Sinapic acid	0.00	1	0.268	0.268	0.000	0.00	0.00	0.00
		2	0.270	0.270	0.000	0.00		
		3	0.265	0.265	0.000	0.00		
	25.0	1	0.272	0.270	0.002	0.55	0.36	0.16
		2	0.276	0.275	0.001	0.27		
		3	0.284	0.283	0.001	0.27		
	100.0	1	0.277	0.274	0.003	0.82	1.00	0.16
		2	0.280	0.276	0.004	1.09		
		3	0.276	0.272	0.004	1.09		
	250.0	1	0.286	0.280	0.006	1.64	1.82	0.16
		2	0.288	0.281	0.007	1.91		
		3	0.287	0.280	0.007	1.91		

Table 49. The % LDH release by sinapic acid compared to L-ascorbic acid at various concentration (Mean ± SD)

No	conc (µg/ml)	sinapic acid		L-ascorbic acid	
		% LDH released	SD	% LDH released	SD
1	0.00	0.00	0.00	0.00	0.00
2	25.00	0.36	0.16	0.91	0.16
3	100.00	1.00	0.16	1.64	0.27
4	250.00	1.82	0.16	2.82	0.42



* significant between the two substance at same concentration extract ($p<0.05$)

Figure 37. The extent of LDH release from cultured fibroblasts after exposure to various concentrations of sinapic acid and L-ascorbic acid (Mean ± SD, n=3)