CHAPTER IV

RESULTS AND DISCUSSION

1. Extraction of three green teas leaves from the three different origins

Extraction was carried out with 50 g of dried green tea leaves from three geographical regions, resulting in the light-brown solid matters after freeze-dried. The total yield of the light-brown solid matter from the extraction was approximately above 10% (w/w) of the dried green tea leaves for all green tea extracts.

2. Quantitative analysis of green tea polyphenols from each extract

For the quantitative analysis of green tea polyphenol in green tea extracts, five standard substances, EGC, Caffeine, EGCG, EC and ECG were used.

2.1 UV spectrophotometer

The UV absorption spectrum of each of five standard substances dissolved in 10% aqueous methanol solution were observed as follows: EGC: 269 nm, caffeine: 272 nm, EGCG: 273 nm, EC: 278 nm and ECG: 277 nm respectively (see Appendix I). To get the optimal absorbance for all substances, 280 nm was set up as UV detector wavelength for quantitative analysis of green tea extracts by HPLC assay.

2.2 Mixed standard samples solution

In order to calculate the amount of five substances present in each of green tea extract, five standard substances were mixed together and prepared in 10% aqueous methanol and the stock solution was injected into the reverse-phase HPLC column, and peak area were recorded (see Figure 6). Then five standard calibration curves were obtained by plotting the peak areas against the amount of each of the standard substances (see figure 7 to 11).

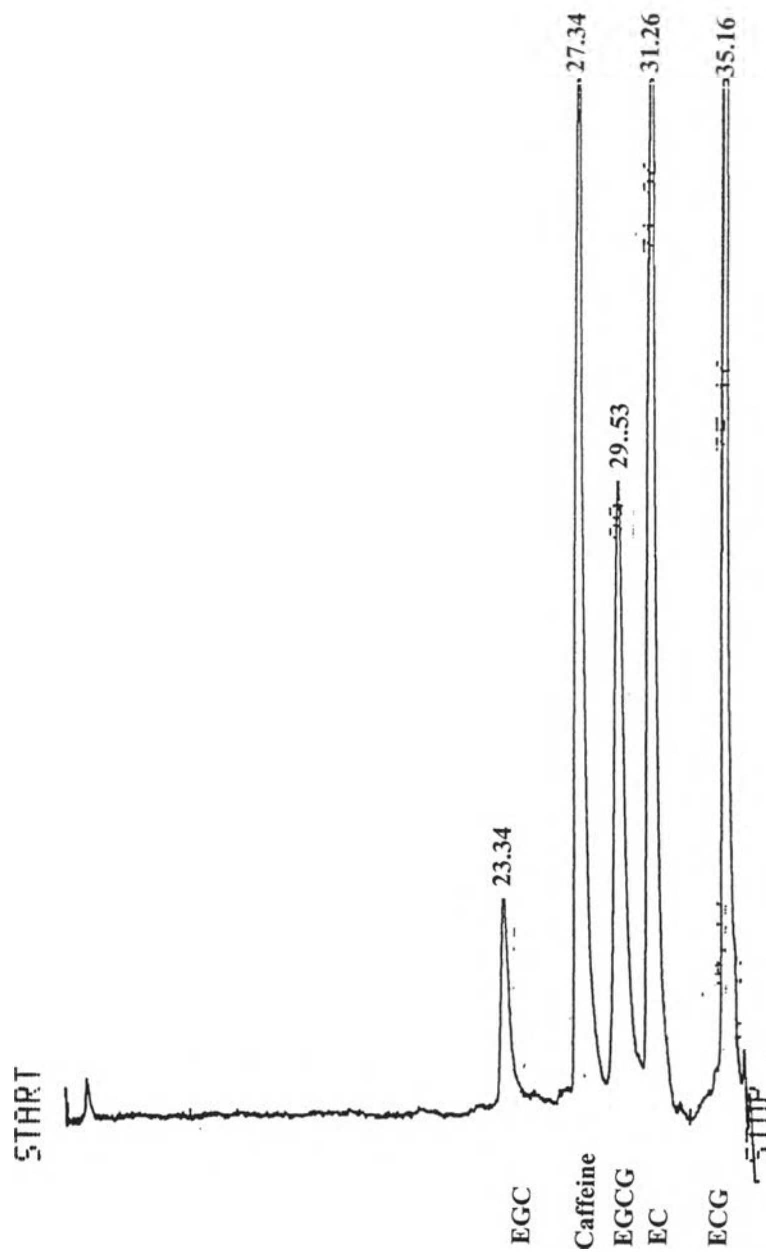


Figure 6. High-performance liquid chromatogram of standard catechins and caffeine mixture. EGC = (-)-epigallocatechin, EGCG = (-)-epigallocatechin 3-*O*-gallate, EC = (-)-epicatechin, ECG = (-)-epicatechin 3-*O*-gallate.

2.2.1 The standard EGC solution

Table 1. The peak area of the standard EGC

Amount (μg)	Peak area
1	3,025
3	19,392
5	30,611
7	49,375

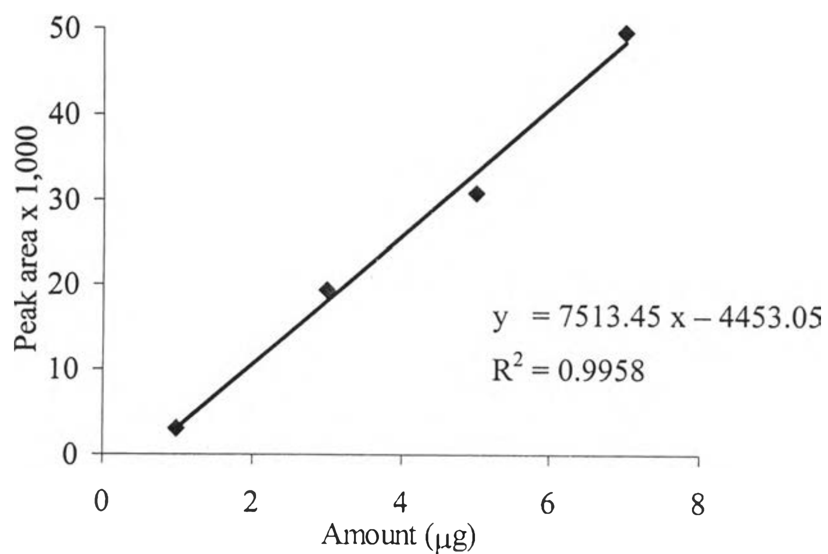


Figure 7. The calibration curve of the standard EGC

2.2.2 The standard caffeine solution

Table 2. The peak area of the standard caffeine

Amount (μg)	Peak area
0.59	51,329
1.18	117,439
1.77	165,032
2.36	216,126

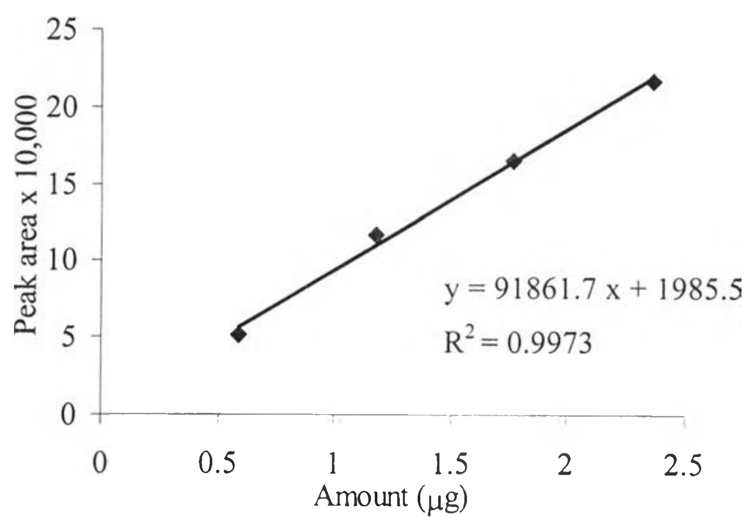


Figure 8. The calibration curve of the standard caffeine

2.2.3 The standard EGCG solution

Table 3. The peak area of the standard EGCG

Amount (μg)	Peak area
4.1	67,675
6.15	131,020
10.25	275,383
14.35	427,015
16.4	546,619

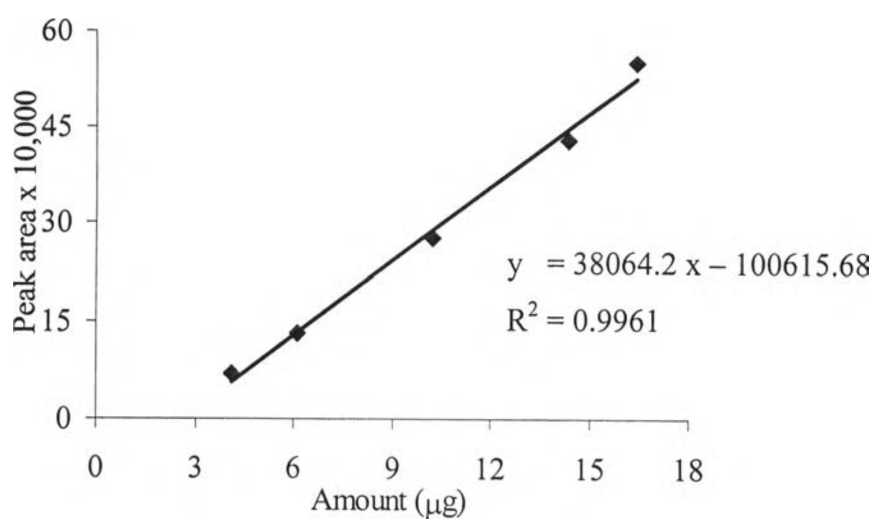


Figure 9. The calibration curve of the standard EGCG

2.2.4 The standard EC solution

Table 4. The peak area of the standard EC

Amount (μg)	Peak area
0.5	12,280
1.5	56,565
2.5	94,795
3.5	138,334

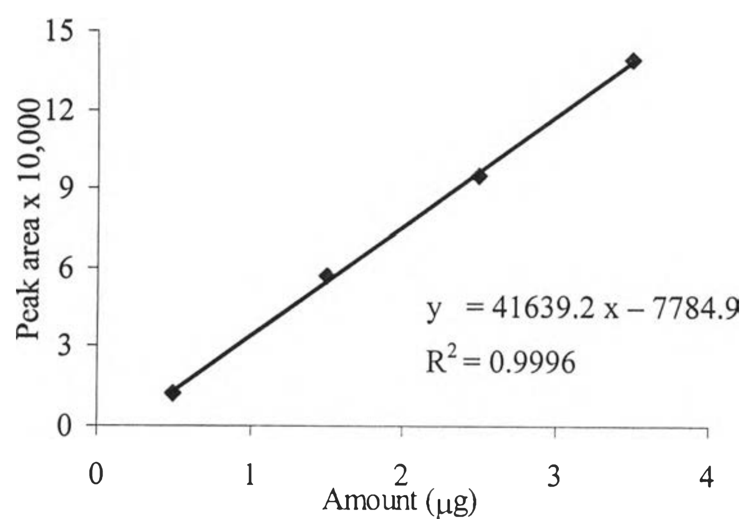


Figure 10. The calibration curve of the standard EC

2.2.5 The standard ECG solution

Table 5. The peak area of the standard ECG

Amount (μg)	Peak area
1.45	69,608
2.9	153,059
4.35	276,858
5.8	389,712

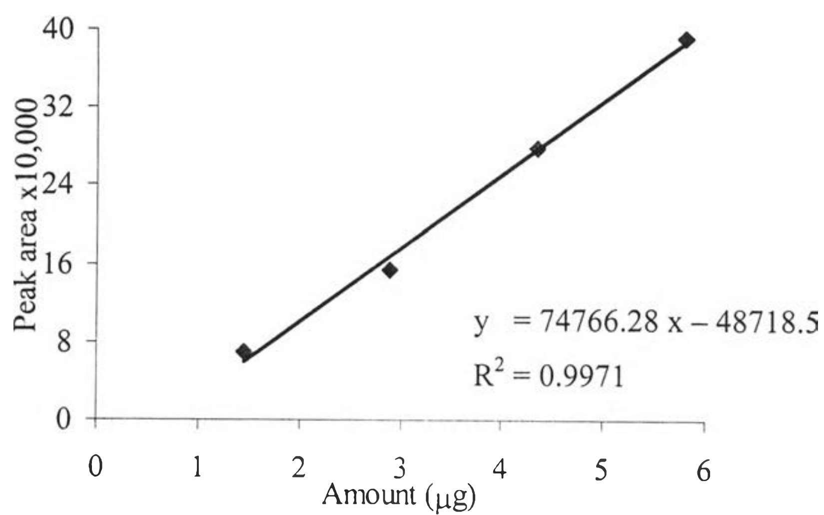


Figure 11. The calibration curve of the standard ECG

2.3 HPLC analysis of total polyphenol contents in green tea extracts

Each of three green tea extracts solution was injected into the reverse-phase HPLC column. Epicatechin derivatives present in green tea extracts were identified by comparing their retention times with the corresponding retention time from the standard mix sample (see figure 6). Five sharp peaks were eluted from the injection of each extract, whose retention time were in the range of 22.77 - 23.29 min for EGC, 27.34 – 27.61 min for caffeine, 29.77 – 29.96 min. for EGCG, 31.60 – 31.92 min. for EC and 35.17 – 35.29 min. for ECG. Reverse-phase HPLC chromatograms of green tea extracts were shown in Appendix II.

The amount of epicatechin derivatives present in green tea extracts were calculated from the peak area of each substance obtained from the HPLC chromatogram with the substitution in the equation obtained from each of standard calibration curve. Calculated data were reported in table 6, where total polyphenol content of three green tea extracts were in the order of JGT>MGT> TGT, however, no significant difference was observed in comparison of total polyphenol contents in green tea extracts. From this study, it was shown that epicatechin derivatives of JGT was in the order of EGCG>EGC>ECG>EC whereas MGT and TGT were identical in the sense that EGCG>ECG>EGC>EC. However, the main constituent EGCG stood the highest while EC ranked the lowest in all green tea extracts from three different origins.

According to Agarwal *et al.*, (1992), the quantitative analysis of green tea polyphenol by HPLC assay reported that total polyphenol contents in green tea extract accounted for 68.5% and one of the main epicatechin derivatives; EGCG constitutes the maximum. In the present finding, following the same extraction and analytical method, where total polyphenol contents for three green tea extracts were in the range of 54 – 58 %(w/w) of the dry solid weight and EGCG represented as the highest content and which was found to be in close agreement with the previous findings. However, little difference in this investigation could be the result of choice of green tea, method of sampling and varies in season, climate and horticultural practices.

Table 6. Qualitative and quantitative analysis of total polyphenol content present in each of green tea extract by reverse-phase HPLC

Substance	% yield of green tea polyphenol (w/w)[a]			% yield of total epicatechin derivatives (w/w)[b]		
	JGT	MGT	TGT	JGT	MGT	TGT
Total polyphenol content	58 *	55 *	54 *	100	100	100
EGC	12.78	8.89	9.83	22.23	16.21	18.37
EGCG	34.84	28.10	20.02	60.60	51.24	37.42
EC	4.11	5.85	6.80	7.15	10.67	12.71
ECG	5.76	12.00	16.85	10.02	21.88	31.50

JGT : Japanese green tea, MGT : Myanmar green tea and TGT : Thai green tea

[a] % yield of green tea polyphenol from injection of 20 μl of dark brown solid matter solution of three different green tea extracts

[b] Calculations are based on the total epicatechin derivatives as 100% of total green tea polyphenols

* Total polyphenol content among each of green tea extracts are not significant different, $p>0.05$

3 Determination of the radical scavenging activity of green tea extracts by using DPPH assay

Another strategy in this study was to evaluate the antioxidant activity of green tea extracts. Generally, because of the complex nature of phytochemicals, the antioxidant activities of plant extracts cannot be evaluated by only a single method. In the meantime, many strategies have been developed and well established to assess the antioxidant activities. In the present study, the antioxidant activity of green tea extracts was evaluated *in vitro* by using two methods; 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay and total antioxidant status assay.

The radical scavenging activity of each green tea extract was determined by using DPPH assay where the stable DPPH radical (an odd electron) was used. It becomes paired off in the presence of a free radical scavenger and the absorption fades out. Reduction in DPPH radical from the tested samples were measured by monitoring the decrease in the absorbance at 520 nm with UV-spectrophotometer. In the present study, antioxidant BHT was selected to compare the radical-scavenging activities of green tea extracts toward the stable free radical DPPH. Dose-response curves were plotted with various concentrations against calculated percent inhibition of tested samples on DPPH radical and reported in table 7 to 10. The linear correlation relationship between the concentrations of each sample and the scavenging activity against DPPH radical was obtained where x-axis represented the concentration of tested compounds and y-axis represented the average percent inhibition of scavenging capacity on DPPH radical from three replicates measurements (see figure 12 to 15). The findings from these curves proved that all green tea extracts inhibit the radical DPPH activity in dose-dependent manner.

The effective concentration of 50% inhibition on DPPH radical was expressed in terms of EC_{50}. Calculated the EC_{50} of each green tea extracts and BHT on DPPH radical from their linear regression of plots.

3.1 The radical scavenging activity of BHT

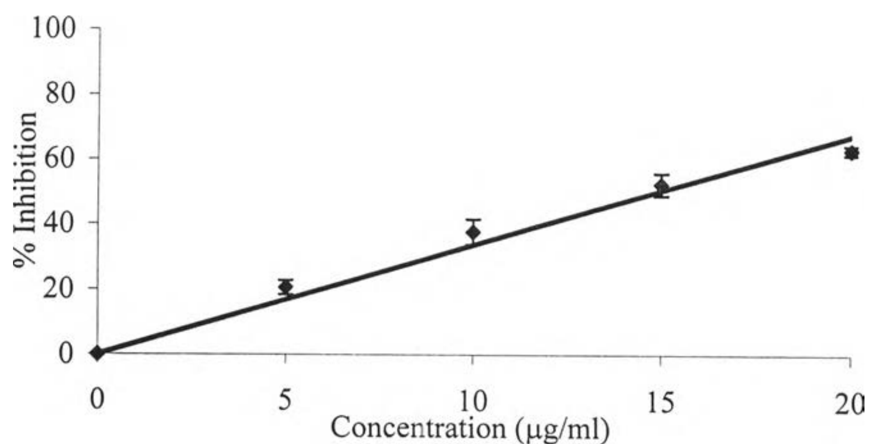
The radical scavenging activity of antioxidant BHT on DPPH radical was studied with different concentrations (0, 5, 10, 15 and 20 μg/ml) and % inhibition values were obtained as follows:

Table 7. The radical scavenging activity of the antioxidant BHT on the DPPH radical.

Concentration (μg/ml)	% Inhibition			Mean
	N_1	N_2	N_3	
0	0.00	0.00	0.00	0.00 ± 0.00
5	21.25	22.24	18.09	20.53 ± 2.17
10	33.76	37.19	41.47	37.47 ± 3.86
15	48.11	53.92	54.27	52.10 ± 3.46
20	62.78	61.08	63.91	62.59 ± 1.42

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = % inhibition values from three times measurements



EC_{50} of BHT = 14.95 ± 0.53 μg/ml

Figure 12. The dose-response representative curve of the radical scavenging activity of antioxidant BHT on DPPH radical

3.2 The radical scavenging activity of Japanese green tea extract

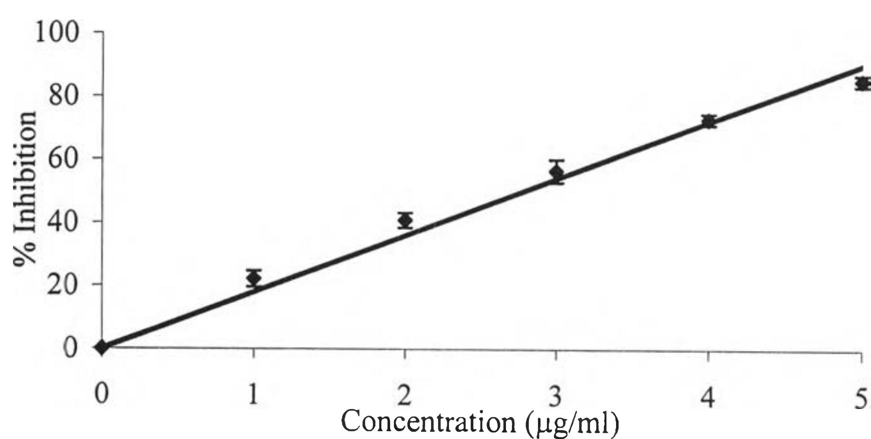
The radical scavenging activity of Japanese green tea extract on DPPH radical was studied with different concentrations (0, 1, 2, 3, 4 and 5 μg/ml) and % inhibition values were obtained as follows:

Table 8. The radical scavenging activity of Japanese green tea extract on the DPPH radical

Concentration (μg/ml)	% Inhibition			Mean
	N_1	N_2	N_3	
0	0.00	0.00	0.00	0.00 ± 0.00
1	24.83	21.41	19.87	22.04 ± 2.54
2	42.71	40.92	38.07	40.57 ± 2.34
3	59.23	56.66	52.05	55.98 ± 3.64
4	74.19	71.55	70.80	72.18 ± 1.78
5	85.24	82.32	85.61	84.39 ± 1.80

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = % inhibition values from three times measurements



EC_{50} of JGT = 2.80 ± 0.07 μg/ml

Figure 13. The dose-response representative curve of the radical scavenging activity of Japanese green tea extract on DPPH radical

3.3 The radical scavenging activity of Myanmar green tea extract

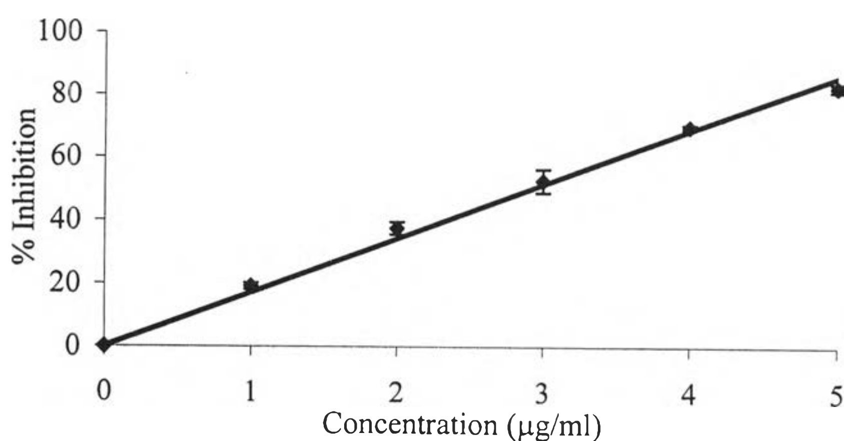
The radical scavenging activity of Myanmar green tea extract on DPPH radical was studied with different concentrations (0, 1, 2, 3, 4 and 5 μg/ml) and % inhibition values were obtained as follows:

Table 9. The radical scavenging activity of Myanmar green tea extract on the DPPH radical

Concentration (μg/ml)	% Inhibition			Mean
	N_1	N_2	N_3	
0	0.00	0.00	0.00	0.00 ± 0.00
1	19.97	17.93	19.15	19.02 ± 1.03
2	39.52	36.34	35.97	37.28 ± 1.95
3	54.81	53.59	47.88	52.09 ± 3.70
4	70.01	68.75	68.61	69.12 ± 0.77
5	82.25	82.64	80.18	81.69 ± 1.32

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = % inhibition values from three times measurements



EC_{50} of MGT = 2.94 ± 0.07 μg/ml

Figure 14. The dose-response representative curve of the radical scavenging activity of Myanmar green tea extract on DPPH radical

3.4 The radical scavenging activity of Thai green tea extract

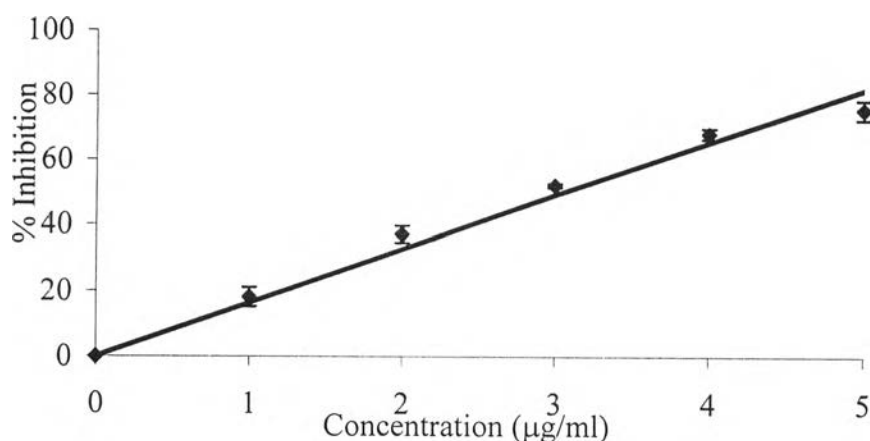
The radical scavenging activity of Thai green tea extract on DPPH radical was studied with different concentrations (0, 1, 2, 3, 4 and 5 μg/ml) and % inhibition values were obtained as follows:

Table 10. The radical scavenging activity of the Thai green tea on the DPPH radical

Concentration (μg/ml)	% Inhibition			Mean
	N_1	N_2	N_3	
0	0.00	0.00	0.00	0.00 ± 0.00
1	18.53	14.92	20.79	18.08 ± 2.96
2	35.67	40	35.58	37.08 ± 2.53
3	51.09	52.24	52.15	51.83 ± 0.64
4	67.67	69.7	66.39	67.92 ± 1.67
5	77.97	72.08	75.84	75.30 ± 2.98

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = % inhibition values from three times measurements



EC_{50} of TGT = 3.07 ± 0.03 μg/ml

Figure 15. The dose-response representative curve of the radical scavenging activity of Thai green tea extract on DPPH radical

3.5 Comparison of antioxidant BHT and green tea extracts

Table 11. Effective concentration at 50% inhibition (EC_{50}) of BHT and green tea extracts from the DPPH assay

Sample	EC_{50} (μg/ml)			Mean
	N_1	N_2	N_3	
BHT	15.49	14.92	14.44	14.95 ± 0.53
JGT	2.72	2.83	2.85	2.80 ± 0.07
MGT	2.88	2.92	3.02	2.94 ± 0.07
TGT	3.04	3.09	3.08	3.07 ± 0.03

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = EC_{50} of each extracts and BHT from three times measurements

As shown in table 11, concentration at 50% inhibition (EC_{50}) on DPPH radical of all green tea extracts were round about 3 μg/ml however statistically significant was observed in comparison among green tea extracts. In comparison with selected antioxidant BHT, all green tea extracts exhibited the lowest EC_{50} on DPPH radical. This findings indicated that all three green teas extracts have potent radical scavenging activity on DPPH radical which is approximately 5 times higher in activity than synthetic antioxidant BHT. Significant difference was observed in comparison with BHT and green tea extracts at EC_{50} level ($p < 0.05$).

Toit *et al.* (2001) reported the radical scavenging activity of water soluble vitamin C at EC_{50} on DPPH radical was 55 μg/ml. In related to this fact green tea extracts from the present study also proved the better radical scavenging activity than vitamin C at EC_{50} level on DPPH radical.

4. Total antioxidant status assay

Another strategy to evaluate the antioxidant activity, total antioxidant activity of each green tea extracts were measured by using the commercialized Randox kit. In which total antioxidant activities of green tea extracts were assessed with the ABTS $^{\cdot+}$ radical cation activated with H_2O_2 and Trolox; water-soluble vitamin E analog, was used as a standard and the units were expressed in terms of mmol/l at the concentration 50 μg/ml of extract solution. The results were reported in table 12. In comparison in total antioxidant activity of green tea extracts was shown in figure 16, the difference was not significant.

Table 12.The total antioxidant activity of green tea extracts by Randox kit

Extract (50 μg/ml)	Total antioxidant activity (mmol/l)			Mean
	N_1	N_2	N_3	
JGT	1.02	0.87	0.95	0.94 ± 0.07
MGT	0.99	0.76	0.84	0.86 ± 0.12
TGT	0.72	0.87	0.91	0.83 ± 0.10

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = total antioxidant activity of triplicate measurements

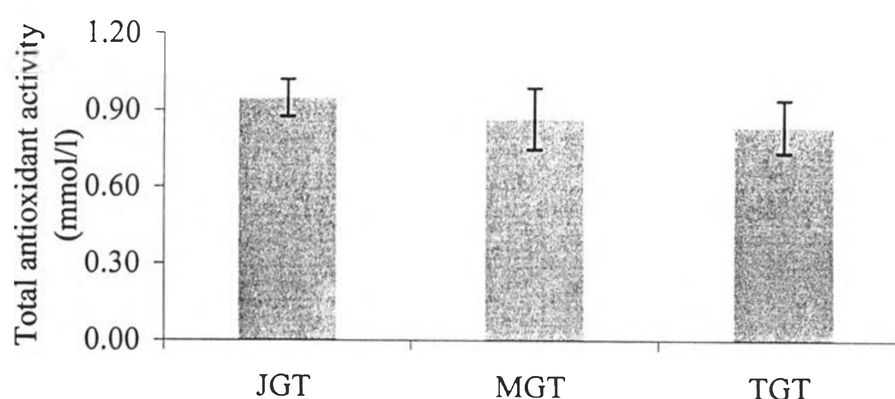


Figure 16. Comparison of the total antioxidant activity among green tea extracts by Randox kit. No significant difference was observed in comparison among green tea extracts, p>0.05.

5. MTT assay

Compare the cytotoxicity of green tea extracts on melanoma cell line (A375) cultured in a 24-well plate and the cell viability was determined by using MTT assay. Cells were subjected to exposure with different concentrations of green tea extracts up to a maximum concentration of 1000 μg/ml. Then the viability of the cells was measured with MTT at 550 nm.

The different concentration of green tea extracts against their % inhibition on melanoma cell line was monitored and the experimental results were summarized in tables 13 to 15. The dose-response curve of each extract with the calculated % inhibition on the growth of melanoma cell against different concentrations was plotted.

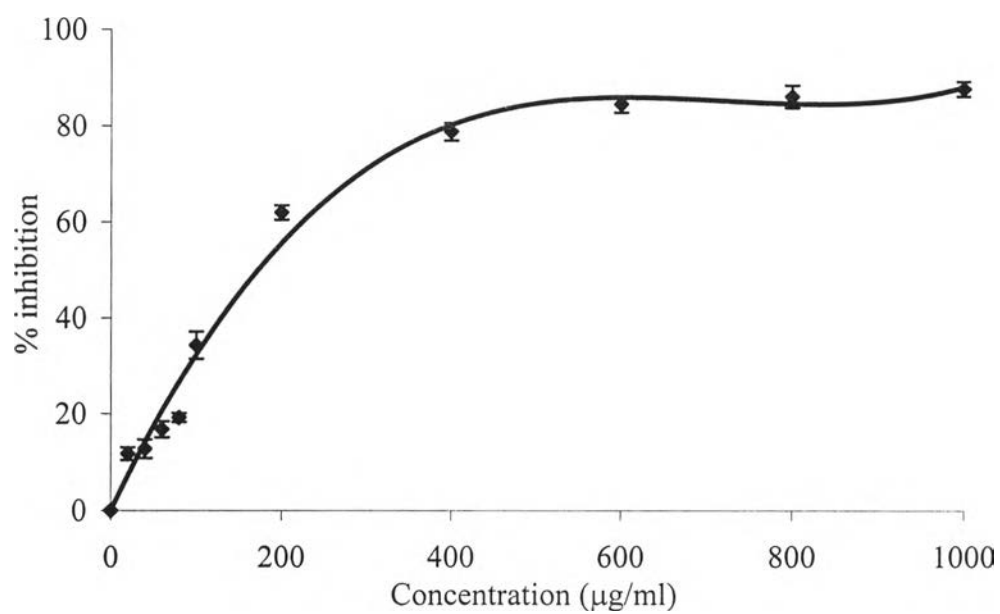
From all graphs as shown in figure 17 to 19, it was seen that percent inhibition on melanoma cell line by green tea extracts were found to gradually increase with increase in concentration and tendency towards a linear dose-response curve obtained up to the concentration of 400 μg/ml of green tea extracts. However, above 400 μg/ml concentration of green tea extracts, the graph tends to flatten and no remarkable change was observed up to 1000 mg/ml concentration.

Table 13. Percent inhibition data of Japanese green tea extract on Melanoma cell line by MTT assay

Concentration (μg/ml)	% Inhibition			Mean
	N_1	N_2	N_3	
0	0.00	0.00	0.00	0.00 ± 0.00
20	13.04	11.78	10.43	11.75 ± 1.31
40	14.84	12.27	11.11	12.74 ± 1.91
60	16.98	14.98	18.28	16.75 ± 1.66
80	20.13	18.26	19.20	19.20 ± 0.94
100	33.31	37.58	32.23	34.37 ± 2.83
200	63.46	60.48	61.86	61.93 ± 1.49
400	79.87	76.62	79.89	78.79 ± 1.88
600	82.80	86.25	84.69	84.58 ± 1.73
800	86.39	83.70	88.39	86.16 ± 2.35
1000	89.11	86.09	88.11	87.77 ± 1.54

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = %inhibition values from three times measurements



EC_{50} of JGT = 173 μg/ml

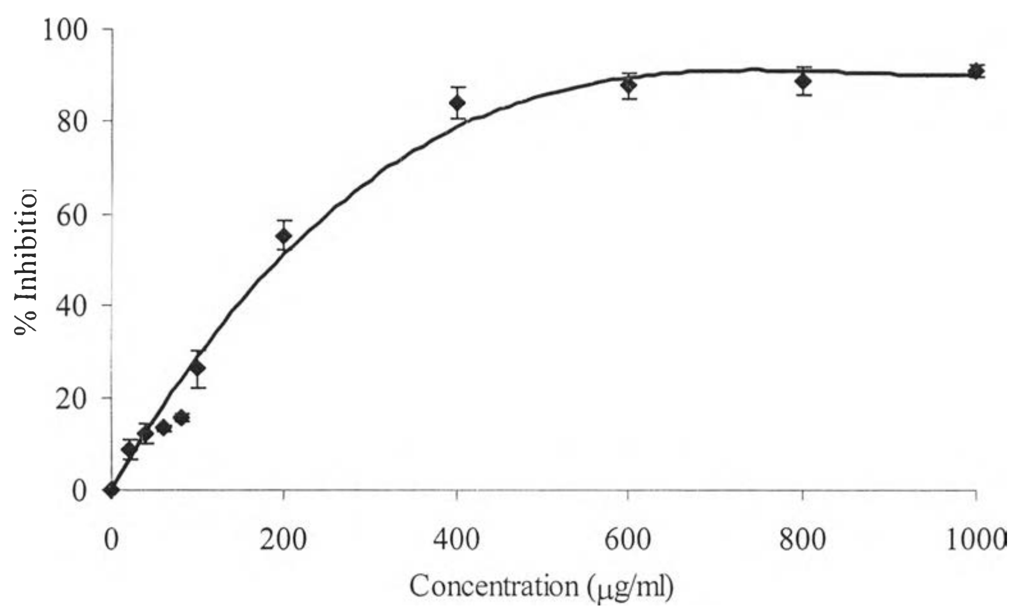
Figure 17. The dose-response representative curve from the cytotoxicity study of JGT extract on melanoma cell line by MTT assay

Table 14. Percent inhibition data of Myanmar green tea extract on Melanoma cell lines by MTT assay

Concentration (μg/ml)	% Inhibition			Mean
	N_1	N_2	N_3	
0	0.00	0.00	0.00	0.00 ± 0.00
20	6.94	11.02	8.16	8.71 ± 2.09
40	9.70	14.10	12.47	12.09 ± 2.22
60	12.45	13.58	13.49	13.17 ± 0.63
80	15.97	15.70	14.51	15.39 ± 0.78
100	27.56	29.21	21.66	26.14 ± 3.97
200	51.52	56.61	57.48	55.20 ± 3.22
400	81.56	82.47	87.86	83.96 ± 3.41
600	88.78	84.59	89.80	87.72 ± 2.76
800	90.87	85.32	90.36	88.85 ± 3.07
1000	92.40	89.77	91.04	91.07 ± 1.32

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = %inhibition values from three times measurements



EC_{50} of MGT = 198 μg/ml

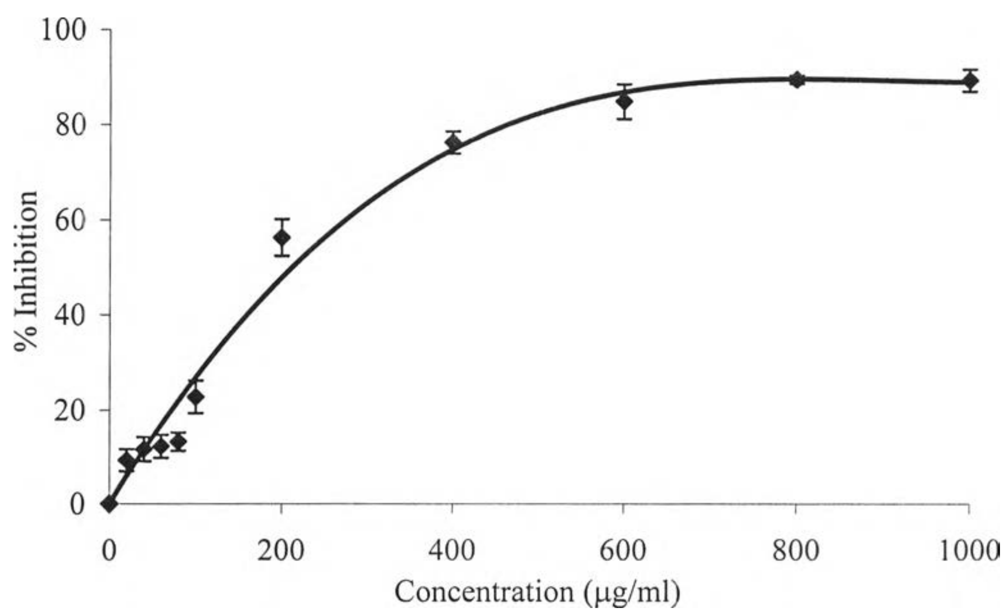
Figure 18. The dose-response representative curve from the cytotoxicity study of MGT extract on melanoma cell line by MTT assay

Table 15. Percent inhibition data of Thai green tea extract on Melanoma cell line by MTT assay

Concentration (μg/ml)	% Inhibition			Mean
	N_1	N_2	N_3	
0	0.00	0.00	0.00	0.00 ± 0.00
20	9.52	11.52	6.70	9.25 ± 2.42
40	10.19	14.66	10.05	11.63 ± 2.62
60	10.79	15.13	10.86	12.26 ± 2.49
80	12.35	15.48	11.89	13.24 ± 1.95
100	26.70	20.49	21.13	22.77 ± 3.42
200	54.10	54.01	60.74	56.28 ± 3.86
400	78.79	76.01	74.13	76.31 ± 2.34
600	80.73	86.50	87.53	84.92 ± 3.67
800	88.91	90.57	89.26	89.58 ± 0.88
1000	86.83	91.39	90.18	89.47 ± 2.36

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = %inhibition values from three times measurements



EC_{50} of TGT = 206 μg/ml

Figure 19. The dose-response representative curve from the cytotoxicity study of TGT extract on melanoma cell line by MTT assay

Table 16. Comparison of cytotoxicity of green tea extracts at 50% inhibition level (EC_{50}) on melanoma cell line by MTT assay

Sample	EC_{50} (μg/ml)			Mean
	N_1	N_2	N_3	
JGT	171	173	175	173 ± 2.00
MGT	199	195	200	198 ± 2.65
TGT	203	206	209	206 ± 3.00

The values are the means ± SD of triplicate measurements

N_1, N_2 and N_3 = %inhibition values from three times measurements

Significant difference was observed in cytotoxicity study among green tea extracts at EC_{50} level, $p < 0.05$.

The effective concentration at 50% inhibition (EC_{50}) of each extract was calculated (see table 16). EC_{50} on melanoma cell line of JGT, MGT and TGT are then compared and the significant difference was observed. From these investigations, we can summarize that the JGT achieved 50% inhibition with the lowest concentration: 173 μg/ml. MGT 198 μg/ml comes second followed by TGT 206 μg/ml. In other word, JGT ranked the best among three green tea extracts in terms of cytotoxicity on melanoma cell line.

Zhu *et al.*, (2001) reported that green tea extract with 96% green tea polyphenol showed IC_{50} at 115.2 μg/ml on breast carcinoma cell line MCF-7 by MTT assay. However, in the present study, green tea extract showed effectiveness at approximately in the range of 173 - 206 μg/ml on melanoma cell line at EC_{50} level. In fact that EC_{50} results is more compared to that of the above studies may be attributed to lower yield of polyphenol in our extract and different effect on different cell line was experienced.