Chapter 3

Results and Discussion

1. Primary screening of antioxidant activities in water extracts of mulberry green teas

The changes in absorbance of β-carotene in the absence and presence of different mulberry green tea water extracts are shown in Figure 13. A sharp decrease in OD_{470} was obtained in the control (without extract), indicating a rapid oxidation of β-carotene/linoleic acid. However, a decrease in OD_{470} was retarded when mulberry green tea water extracts were added. This suggested that some antioxidants were present in the extracts and played an essential role in prevention of oxidation. The results were in agreement with Duh and Yen (1997) who reported that water extracts of three herbs, including *Chrysanthemum morifolium* Ramat, *Hibiscus sabdariffa* L. and *Hordeum vulgare* L. showed significant antioxidative activity in linoleic acid system, suggesting that the extracts retarded the rate of peroxide formation.

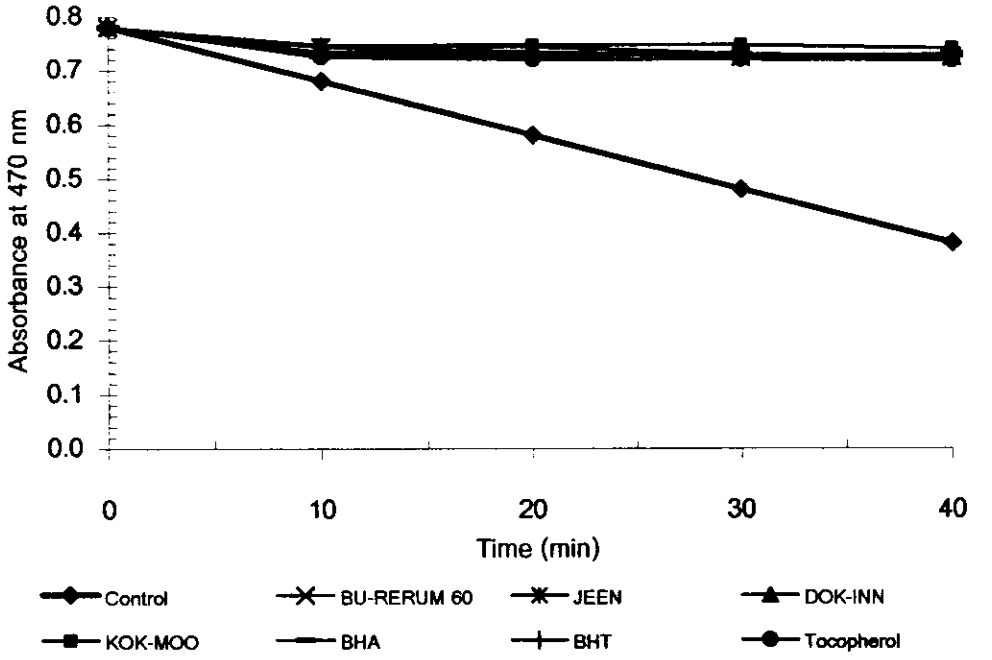

Figure 13 The decrease in absorbance of β-carotene in the presence of mulberry green tea water extract

The antioxidative activities of extracts from 4 different varieties of mulberry green tea are presented in Table 5. All water extracts from mulberry green tea showed high antioxidant activities ranging from 90.11 to 93.28 %. "KOK-MOO" extract had highest antioxidant activity, followed by "JEEN", "BU-REERUM 60" and "DOK-INN" extracts, respectively. "KOK-MOO" and "JEEN" extracts exhibited higher antioxidant activity than BHA, BHT and α-tocopherol. However, "BU-REERUM 60" and "DOK-INN" extracts exhibited lower antioxidant activities than BHA and BHT but higher than α-tocopherol. Duh and Yen (1997) found that the herbal water extracts (5 mg) showed stronger antioxidative activity than tocopherol or BHA (200 ppm). In general, BHA, BHT, and TBHQ have been used in food at levels of 100-200 ppm (Jadhav *et al.*, 1996). The quality of natural extracts and their antioxidative performances depends not only on the quality of the original plant, the geographic origin, climatic condition, harvesting date and storage, but also environmental and technological factors (Cuvelier *et al.*, 1996). In addition, Liao and Yin (2000) reported that flavonoid which interacted with biomembranes deeper displayed less interaction with free radicals present in the aqueous phase. From the result, differences in antioxidative activity were presumed to be due to the different antioxidative compounds in the extract. Saija *et al.* (1995) observed that quercetin showed a greater protective effect than rutin in inhibiting autoxidation of rat cerebral membranes, while quercetin showed less antioxidant activity than rutin in inhibiting Fe^{2+}-induced linoleate peroxidation. The higher partition coefficient of quercetin as compared to that of rutin supported that quercetin was able to interact with deeper biomembranes.

Differences in total phenolic content were observed among mulberry green tea varieties (Table 5). Total phenolic content of mulberry green teas in this study varied from 126.66 to 306.05 mg/100 g. "DOK-INN" extract contained the highest total phenolic content but it had a lower antioxidant activity than "KOK-MOO", "JEEN" and "BU-REERUM 60" respectively. This result indicated that phenolic compounds in mulberry green tea extract may partially contribute to inhibition of lipid peroxidation. In other words, not all phenolic compounds exhibited antioxidant activity. No correlation

between antioxidant activity and phenolic content was found in malts (Maillard and Berset, 1995). Differences in total phenolic content between the varieties of mulberry green tea may be due to differences in genetic, maturity and other factors (Hagerman *et al.*, 1998). Total polyphenol content and antioxidant activity was found to vary among different parts (leaf, phloem, bark, cork, needle) of trees (Kahkonen *et al.*, 1999). However, Gutfinger (1981) and Ramarathnam *et al.* (1986) discovered that among natural antioxidants, phenolics have been known as antioxidants widely distributed in the plant and play an important role in inhibiting oxidation of the oils. Yen and Chen (1995) noted that polyphenols are the most abundant group of compounds in the tea leaf and seem to be responsible for antioxidative activity. Pure catechins and phenolic acids found in tea are more powerful than vitamins C, E and β-carotene *in vitro* lipoprotein oxidation (Vinson and Dabbagh, 1998). Benzie and Szeto (1999) observed that different tea leaves were widely different *in vitro* antioxidant power and the antioxidant capacity was correlated with the total phenolic content. Furthermore, phenolic antioxidants of plant materials are reported to quench oxygen derived free radicals as well as the substrate derived free radicals by donating a hydrogen atom or an electron to the free radical in various system (Yen and Duh, 1994).

Different mulberry green tea extracts had different reducing power (Table 5). "DOK-INN" extract showed highest reducing power, while "BU-REERUM 60" extract exhibited weakest reducing power. From the result, reducing power did not correlate well with antioxidant activity. Therefore, many factors were presumed to determine antioxidant activity in mulberry green tea extact. Yen *et al.* (2000) reported that the anthraquinones, which also possessed antioxidant activity, showed almost no reducing power, indicating that reducing power of anthraquinones can not be considered as contributing to their antioxidative effect.

The results clearly indicated that mulberry green tea was rich in natural antioxidants. Different qualities or quantities of antioxidants in water extracts depended on the varieties of mulberry leaves. "KOK-MOO" and "JEEN" showed high level of antioxidant activity in β-carotene/linoleic acid emulsion system. However, "KOK-MOO"

extracts had higher total phenolic content and reducing power than "JEEN" extracts. Therefore, "KOK-MOO" variety was chosen for further study.

Table 5 Antioxidant activities, total phenolic content and reducing power of mulberry green tea water extracts*

Mulberry green tea/ Commercial antioxidants	Antioxidant activity (%)	Total phenolic content (mg/100 g)	Reducing power (ABS. at 700 nm)
KOK-MOO	93.28 ± 1.04[a] b[b]	203.86 ± 8.31 b	0.42 ± 0.02 c
JEEN	92.85 ± 0.47 b	126.66 ± 3.45 a	0.34 ± 0.02 b
BU-REERUM 60	90.70 ± 0.14 a	140.00 ± 12.08 a	0.30 ± 0.01 a
DOK-INN	90.11 ± 2.14 a	306.05 ± 4.57 c	0.67 ± 0.01 d
BHA (200 ppm)	91.06 ± 0.00 a	-	-
BHT (200 ppm)	91.06 ± 0.00 a	-	-
α–tocopherol (200 ppm)	89.36 ± 0.00 a	-	-

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

*Extraction at 100°C for 5 min;

The ratio of mulberry green tea powder:water was 1:20 (W/V)

2. Extraction of antioxidants from selected mulberry green tea

2.1 Effect of extraction temperature, time and repetition on antioxidant activity of mulberry green tea water extract

Antioxidant activity, reducing power and total phenolic content of mulberry green tea extracts prepared at different temperatures, times and repetitions are shown in Figure 14, 15 and 16, respectively.

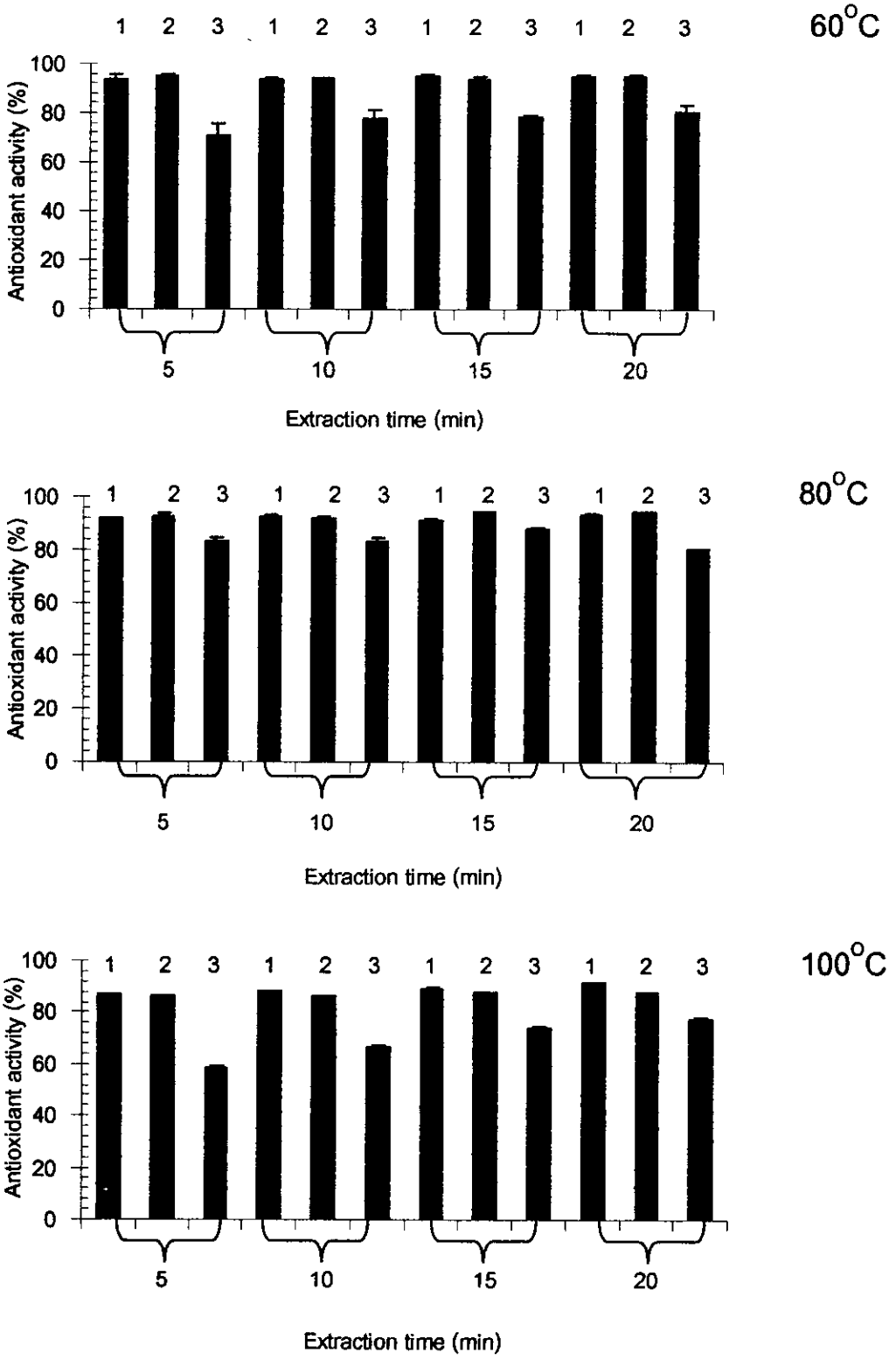

Figure 14 Antioxidant activity of mulberry green tea water extracts prepared at different extraction temperatures, times and repetitions

*Numbers on the bar represent repetition of extract

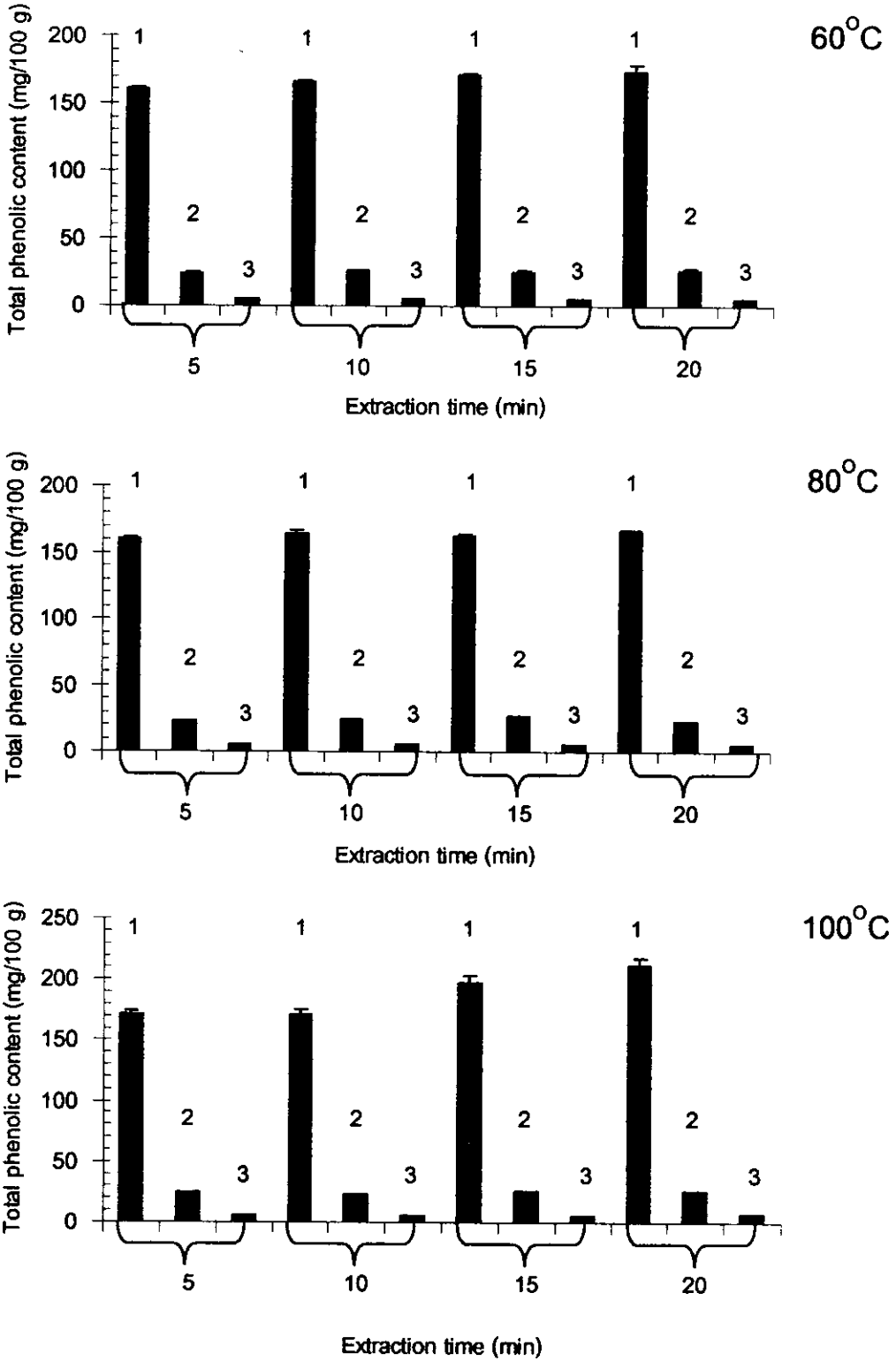

Figure 15 Total phenolic content of mulberry green tea water extracts prepared at different extraction temperatures, times and repetitions

*Numbers on the bar represent repetition of extract

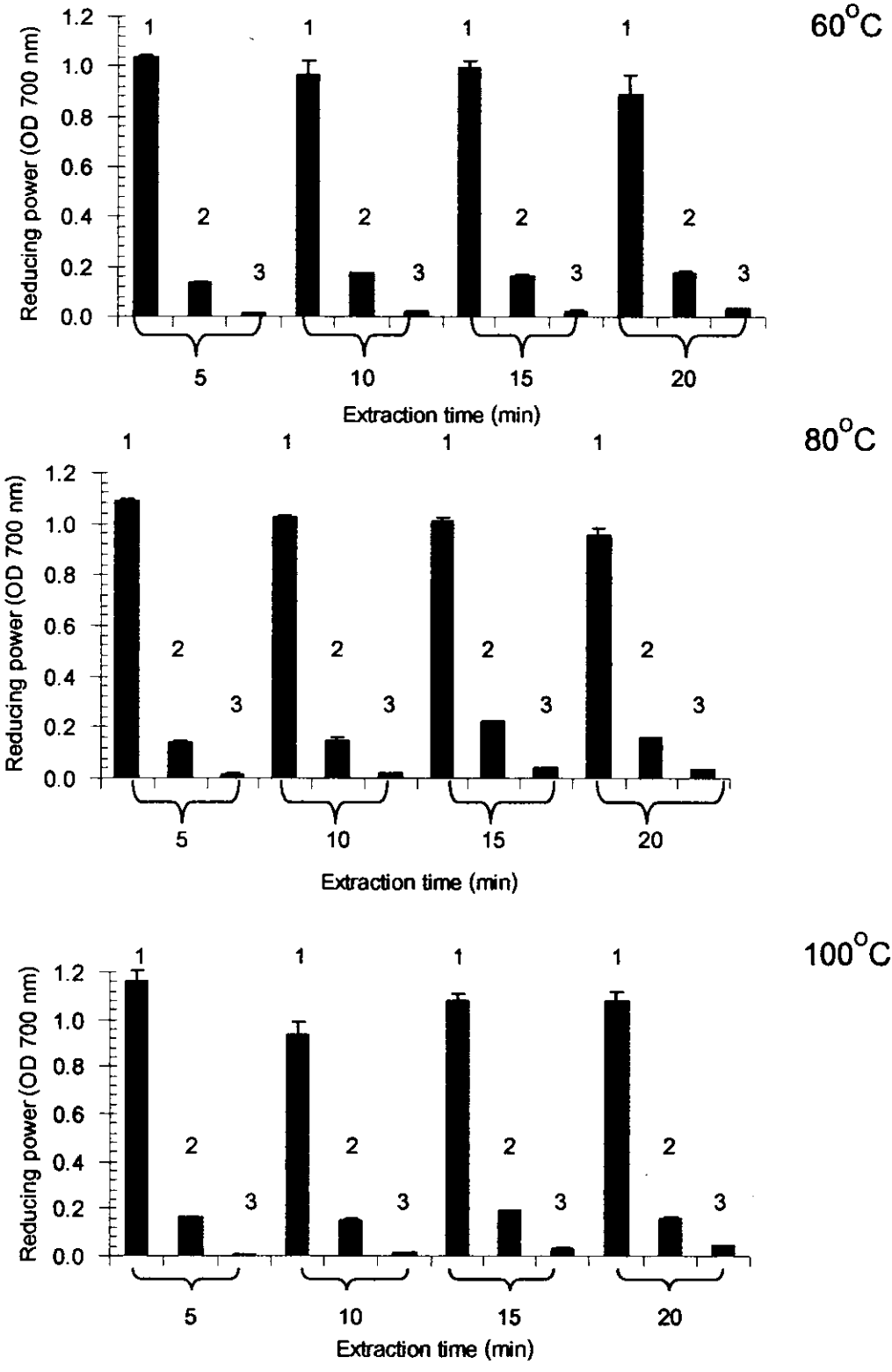

Figure 16 Reducing power of mulberry green tea water extracts prepared at different extaction temperatures, times and repetitions

*Numbers on the bar represent repetition of extract

Mulberry green tea powder was extracted with water at 60, 80 and 100 ^{o}C for 5, 10, 15 and 20 min with 1, 2 and 3 repetitions. The antioxidant activity of mulberry green tea water extract was slightly reduced when the extraction temperature increased ($p < 0.05$) (Figure 14). In general, extraction temperature at 60 and 80 ^{o}C rendered the similar extraction efficacy. However, extraction at temperature above 80 ^{o}C resulted in the slight decrease in activity. The temperature during extraction affects the extractable compounds differently. Boiling and resting increased the total phenol content in *Quercus suber* cork (Conde *et al.*, 1998). Maillard and Berset (1995) observed 20% reduction in antioxidant activity during kilning at 90 ^{o}C for bound and free polyphenols. The reduction in antioxidant activity was higher than that expected from the reduction in polyphenol content, probably due to the synergistic effect of natural phenols (Maillard and Berset, 1995). The evaporation and heat decomposition were the main mechanisms for the loss of activity of synthetic antioxidants (Hamama and Nawar, 1991). The amount of flavonoids in fresh mulberry leaves was higher for air-dried than for oven-dried, probably due to decomposition after storage or to lowered extractability due to modification of the matrix (Zhishen *et al.*, 1999). The highest antioxidant activity was observed for extract prepared at 60 ^{o}C for 15 min with 1 repetition. At the same extraction temperature, no significant differences in antioxidant activity were observed with increased extraction time from 5 to 20 min ($p > 0.05$). Nevertheless, lower activity was observed when the extraction was repeated. This was due to the fact that antioxidants were extracted to a higher extent with the first or second extraction. The lower antioxidant content was retained after repeated extraction.

Total phenolic content in the mulberry green tea water extract increased as the extracting temperature increased ($p < 0.05$), especially with increasing extraction time (Figure 15). Jukunen-Titto (1985) reported that 95% of the phenolic were extracted from the leaves of *Salix* sp. within 6 h and an additional 14 h gave only about 5% increase in total extractable phenolic compounds. However, prolonged exposure at moderate temperatures can also cause phenolic degradation during their enzyme-assisted extraction from grape pomace after 48 h of hydrolysis (40 ^{o}C and pH 5),

whereas no degradation was observed with hydrolysis time of 1-8 h (Cilliers and Singleton, 1991).

Significant decrease in total phenolic content was obtained with increased repetition ($p < 0.05$). Total phenolic content of extracts prepared with one repetition ranged from 160.137 to 211.699 mg/100 g. Extracts prepared with two and three repetitions contained phenolics of 22.631 - 26.619 and 4.794 - 6.700 mg/100 g, respectively. Highest total phenolic content was obtained by extraction at 100 ^{o}C for 20 min in the first repetition. Considerable decrease in phenolic content was generally observed with the subsequent extraction.

Reducing power of mulberry green tea water extract is shown in Figure 16. Reducing power of the extact was found to be increased as the temperature increased ($p < 0.05$). Reducing power of extracts prepared with one repetition ranged from 0.888 to 1.163 (absorbance at 700 nm). Extracts prepared with two and three repetitions contained reducing power of 0.136 - 0.220 and 0.005 - 0.041, respectively. The extract with extracting temperature at 100 ^{o}C for 5 min possessed highest reducing power ($p < 0.05$).

From the result, although total phenolic content and reducing power were increased as the temperature increased ($p < 0.05$), but the antioxidant activity of extract with extracting temperature of 100 ^{o}C showed lower activity than those of extracts prepared at 80 and 60 ^{o}C, respectively. An extracting temperature of 60 ^{o}C resulted in higher activity than 80 ^{o}C and no significant differences in antioxidant activity were observed with increased extraction time ($p > 0.05$). Therefore, the optimum condition involved extracting mulberry green tea at 60 ^{o}C for 5 min with one repetition.

2.2 Effects of extracting solvents on the antioxidant activities, total phenolic content and reducing power

The decrease in absorbance of β-carotene in the presence of extracts from "KOK-MOO" mulberry green tea prepared by using different organic solvents is shown in Figure 17.

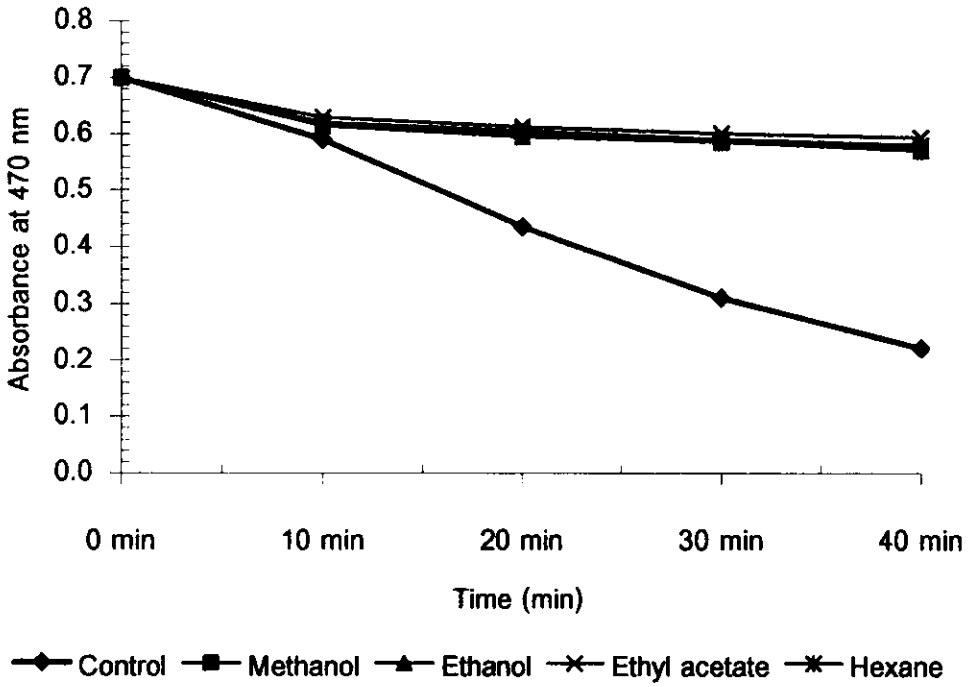

Figure 17 The decrease in absorbance of β-carotene in the presence of mulberry green tea extracts prepared with different organic solvents

A sharp decrease in OD_{470} was obtained in the control, however a decrease in OD_{470} was retarded when different organic solvents extracts of mulberry green teas were added. Similar pattern was observes among all solvent extracts. Therefore, it suggested that all extracts possessed antioxidant activity

Antioxidant activity, reducing power and total phenolic content of mulberry green tea extracts are shown in Table 6. Among four solvent extracts, ethyl acetate extracts had highest antioxidant activity ($p < 0.05$). The efficiency of solvents on the

antioxidant extraction from mulberry green tea was in the order of ethyl acetate > hexane > methanol > ethanol. Nevertheless, no significant differences in antioxidant activity among all solvent extracts and synthetic antioxidants were observed ($p > 0.05$). Meyer *et al.* (1998) reported that the antioxidant activity also depends on the type and polarity of the extracting solvent, the isolation procedures, purity of active compounds, as well as the test system and substrate used. Ethyl acetate and diethyl ether have been used for extraction of low molecular weight phenols from oak wood (Fernandez de Simón *et al.*, 1996) and the polyphenols extracted with ethyl acetate from natural materials were reported to have strong antioxidant activity (Marinova and Yanishlieva, 1997). Gamez *et al.* (1998) reported that an ethyl acetate-soluble extract of the leaves of *Daphniphyllum calycinum* was found to exhibit significant antioxidant effects, based on the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Moreover ethyl acetate extracts of tamarind seed showed the stronger antioxidant activity than methanol, ethanol and 1:1 ethyl acetate:ethanol extracts (Tsuda *et al.*, 1994b).

Methanol extract had highest total phenolic content ($p < 0.05$) (Table 6). The efficiency of solvents on the total phenolic content from mulberry green tea was in the order of methanol > ethyl acetate > hexanel > ethanol. Maximum total phenolics extraction yields were attained with methanol, whereas 50% acetone extracted more selectively leucoanthocyanins and no significant effects were observed in the extraction of glycosides (Julkunen-Titto, 1985). Methanol is widely used as effective solvent for extracting antioxidant components such as phenolics, flavonoids, and other polar materials from plant materials (Economou *et al.*, 1991; Kim *et al.*, 1994). Kallithraka *et al.* (1995) reported that methanol was the best solvent for the quantitative extraction of (+)-catechin, (-)-epicatechin and epigallocatechin from grape seeds. Many phenolic compounds are soluble in polar solvents. Thus, the choice of solvents depends on the number of hydroxyl groups and sugars in the molecules. For crude total phenolic extacts, aqueous alcohols and acetone have often been used as solvents (Julkunen-Titto, 1985). However, the reducing power of all mulberry green tea extracts was very low (Table 6).

Hence, the result suggested that the antioxidant activity and total phenolic content of mulberry green tea was greatly dependent on the type of solvents used for the extraction. Reducing power in solvent extract was very low, especially when compared with that of water extract. This was presumed to be due to the differences in solubility of antioxidative components of mulberry green tea in different solvents.

From the result, ethyl acetate extracts showed the highest antioxidant activity. Therefore, ethyl acetate was chosen as extracting solvent for further study.

Table 6 Antioxidant activity, phenolic content and reducing power of mulberry green tea extracts prepared with different organic solvents

Solvent	Antioxidant activity (%)	Phenolic content (mg/100 g)	Reducing power (ABS. at 700 nm)
Methanol	88.74 ± 2.55[a] a[b]	75.00 ± 0.06 d	0.13 ± 0.00 c
Ethanol	87.91 ± 1.26 a	56.31 ± 1.16 a	0.04 ± 0.00 b
Ethyl acetate	90.04 ± 1.09 a	63.85 ± 0.52 c	0.02 ± 0.01 a
Hexane	89.02 ± 0.81 a	57.89 ± 0.18 b	0.02 ± 0.00 a
BHA (200 ppm)	91.06 ± 0.00 a	-	-
BHT (200 ppm)	91.06 ± 0.00 a	-	-
α–tocopherol (200 ppm)	89.36 ± 0.00 a	-	-

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

2.3 Effect of extraction time on the extraction of mulberry green tea ethyl acetate extract

The effect of extraction time on the antioxidant activity of mulberry green tea ethyl acetate extract is shown in Table 7. Extraction time had no significant effect on the antioxidant activity ($p > 0.05$). The antioxidant activity of extracts was ranged from 88.25 to 90.27 %.

Longer extraction times increased the possibility of oxidation of phenolic unless reducing agents were added to the solvent system (Khanna *et al.*, 1968). Therefore, the optimum condition for extracting of antioxidant from mulberry green tea involved extracting tea powder with ethyl acetate for 0.5 h.

Table 7 Antioxidant activity of mulberry green tea extracted with ethyl acetate under different extracting times

Extracted time (hour)	Antioxidant activity (%)
0.5	89.13 ± 0.61[a] a[b]
1	88.25 ± 1.72 a
2	89.97 ± 0.89 a
3	90.27 ± 0.69 a
5	88.55 ± 0.90 a
8	90.24 ± 1.58 a
10	89.48 ± 0.16 a

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

3. Some properties of mulberry green tea extracts

Natural antioxidants extracted from plants such as tea catechin can be used as alternatives to the synthetic antioxidants because of their equivalent or greater effect on inhibition of lipid oxidation (Namiki, 1990). Mulberry leaves extract also have a potential used as a medicinal herb (Yen *et al.*, 1996). If the mulberry leaves extract are used in food system, their effectiveness will depend on various factors such as the concentration of the antioxidants, pH of food, pH stability of antioxidant, or the synergistic substance in the food. Therefore, these factors must be evaluated to more thoroughly understand the feasibility of using the mulberry green tea extract in the food system.

3.1 Effect of mulberry green tea water and ethyl acetate extracts at different concentrations on antioxidant activities

Figure 18 and 19 depict the antioxidant activity of the mulberry green tea extract at different concentrations. The antioxidant activities of extracts were compared with three commercial antioxidants including BHA, BHT and α-tocopherol at a level of 20 ppm.

Antioxidant activity of mulberry green tea water extract increased with increasing concentration (Figure 18). Mulberry green tea water extract at all concentrations had lower antioxidant activity than BHA, BHT and α-tocopherol at a level of 20 ppm ($p < 0.05$). However, no significant differences in antioxidant activity between the mulberry green tea water extract with concentration from 900 to 1500 ppm and all commercial antioxidants tested were observed ($p > 0.05$). For mulberry green tea ethyl acetate extract, the similar result was observed with water extract. The antioxidant activity of ethyl acetate extract increased with increasing concentration up to 300 ppm ($p < 0.05$) (Figure 19). Ethyl acetate extract at a concentration of 300 to 1,500 ppm had significantly higher antioxidant activity than BHA, BHT and α-tocopherol at a level of 20 ppm ($p < 0.05$).

At the same concentration, the mulberry green tea ethyl acetate extract exhibited stronger antioxidant activity than water extract. This was postulated to be due to different antioxidative compounds in both extracts. Koketsu (1997) reported that tea polyphenols clearly showed a suppressive effect on oxidation of oil, and the effect was concentration-dependent, while tocopherol did not show concentration-dependent, and the addition of more than 400 ppm of tocopherol did not produce any additional antioxidative effect under these conditions. The inhibitory effect of tea polyphenols on rancid formation of soybean oil was also determined under the same condition as lard. Tocopherol showed no effect for depressing rancid formation of soybean oil while tea polyphenols were distinctly effective and suppressed the oxidation of the oil. Antioxidative effect of tea polyphenols was also concentration-dependent. Also, tea polyphenols were effective in suppressing the discoloration of magarine and fish meat (Koketsu, 1997).

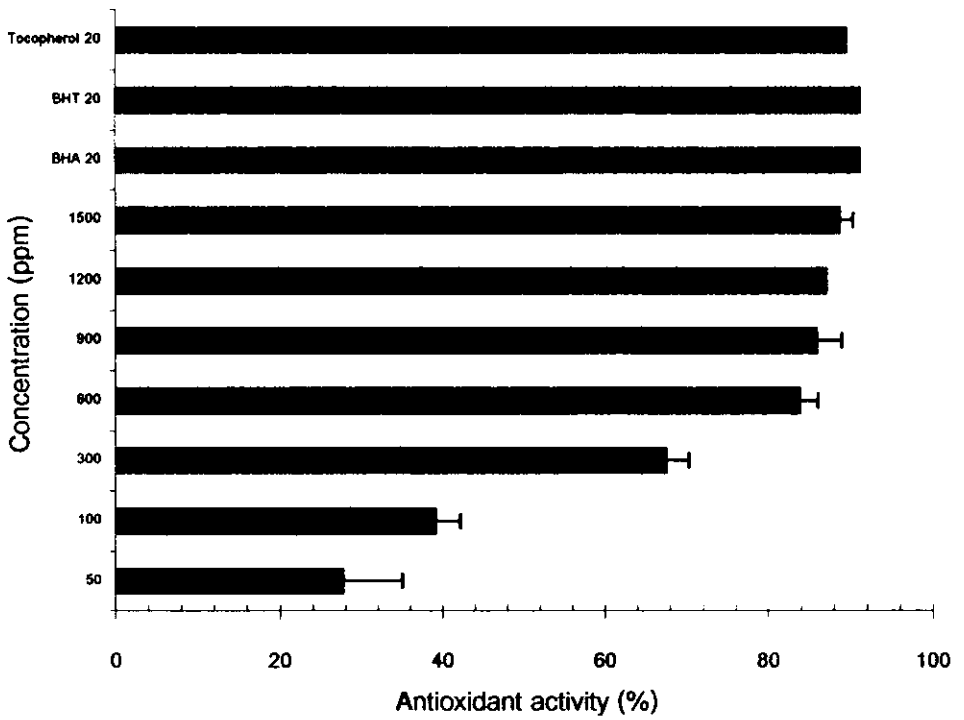

Figure 18 Antioxidant activity of mulberry green tea water extract at different concentrations

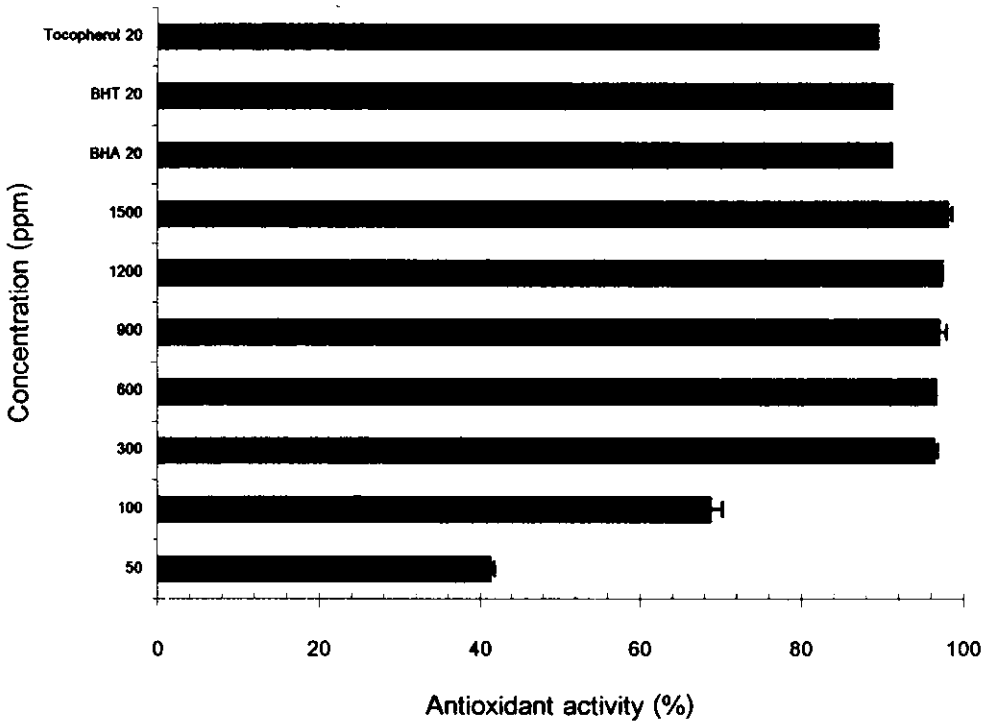

Figure 19 Antioxidant activity of mulberry green tea ethyl acetate extract at different concentrations

From this result, antioxidant activities of mulberry green tea water and ethyl acetate extracts were dependent on concentrations used. This result was in agreement with Yen and Lee (1997) who found that antioxidant activity of the extract from *Aspergillus candidas* broth filtrate increased with an increasing concentration, but it reached maximum at 200 ppm. Similarly, Duh *et al.* (1997) observed that antioxidant activity of mung bean hull extract increased with increasing concentration up to 100 ppm ($p < 0.05$) and no significant differences in activity were observed with concentration ranging from 100 to 500 ppm ($p > 0.05$). In general, the antioxidant activity increases with the antioxidant concentration, but only up to certain level, which depend on both the antioxidant and the test. In liposomes, the optimal concentration of grape seed and rose hip extracts was 0.1 mM, whereas for BHT it was 0.02 mM and for catechin a steady state was observed in the range of 0.05-0.2 mM (Gabrielska *et al.*,

1997). For most natural extracts, maximum antioxidant activity was achieved using a 0.05 % concentration.

3.2 Effect of pH on antioxidant activities of mulberry green tea water and ethyl acetate extracts

The effect of pH on antioxidant activity of mulberry green tea water and ethyl acetate extracts is shown in Figure 20 and 21. Both mulberry green tea extracts exhibited strong antioxidant activity at neutral and alkaline pHs and the activity was decreased at acidic pH ($p < 0.05$). At pH 3.0, the mulberry green tea water and ethyl acetate extracts showed the lowest antioxidant activity while the highest antioxidant activity was found at pH 9.0. However, the antioxidant activity was slightly decreased at pH 11.0 ($p < 0.05$). No significant differences in antioxidant activity of mulberry green tea ethyl acetate extract tested at pH 7 and 11 were found ($p > 0.05$). At neutral or alkaline pH, tea catechins increase their proton-donating potential and become easier to form the corresponding semiquinone free radicals (Yoshioka *et al.*, 1991). This may explain why the free-radical scavenging capacity of green tea catechins increases with increasing pH. EGCG has been shown to be more susceptible to formation of a semiquinone free radical than ECG in 1 mol of NaOH solution (Guo *et al.*, 1996; Yoshioka *et al.*, 1991). Lee *et al.* (1986) found that protection efficiency value of ginger extract depended on pH and concentration of the extract. At low concentration, the protection factor value was decreased at alkaline pH, whereas at high concentration, the protection factor value was increased with increasing pH values. The different activity of mulberry green tea extract at various pH was presumed to be due to the conformational changes, which altered partitioning of the extracts into the emulsified carotene/linoleic acid globule (Marco, 1968).

From the result, the activity of mulberry green tea water and ethyl acetate extract at alkaline and neutral pH were stronger than that at acidic pH, possibly due to the different conformation and charges of antioxidant compounds under different pHs.

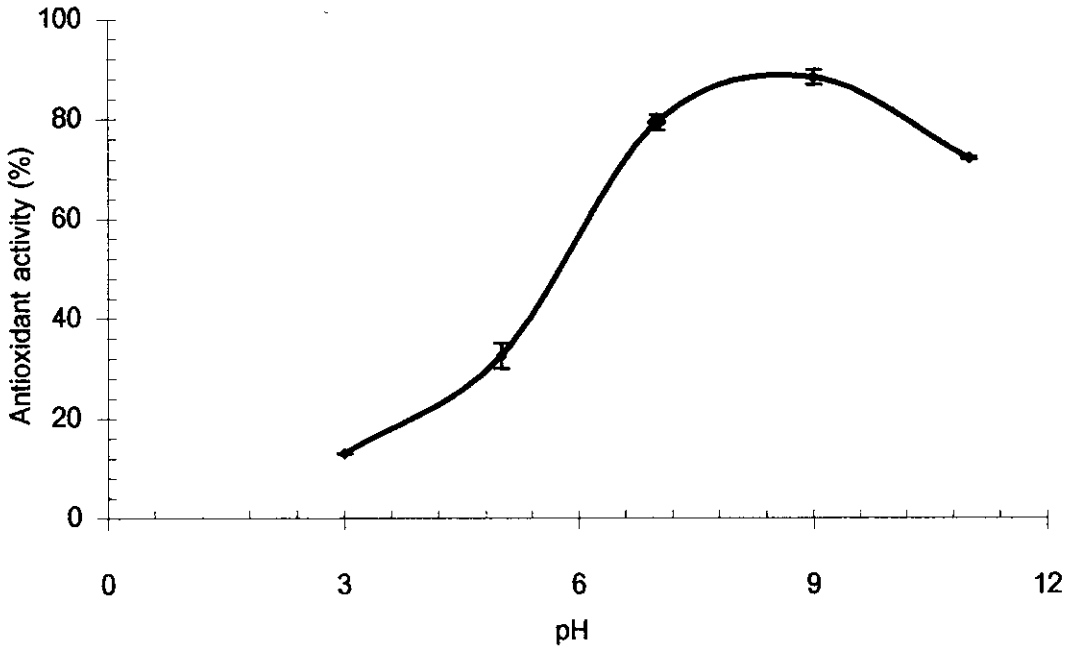

Figure 20 Effect of pH on antioxidant activity of mulberry green tea water extract

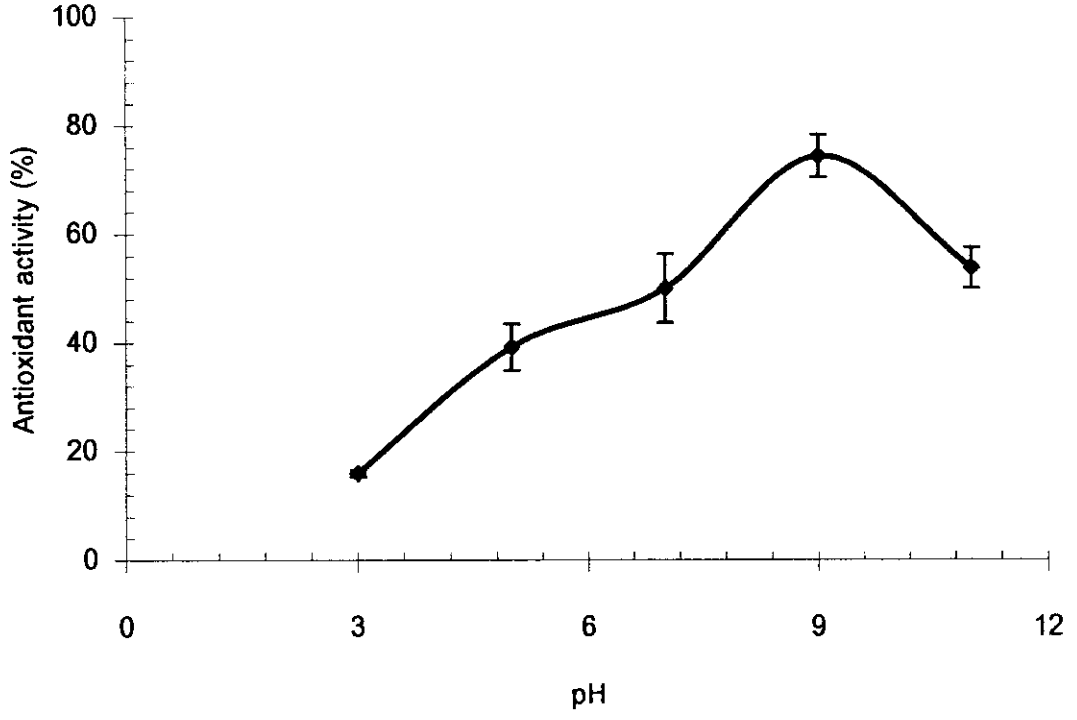

Figure 21 Effect of pH on antioxidant activity of mulberry green tea ethyl acetate extract

3.3 pH stability of mulberry green tea water and ethyl acetate extracts

pH stability of mulberry green tea water and ethyl acetate extracts is shown in Figure 22 and 23. Mulberry green tea water extract had highest stability at pH 9.0 but the stability was slightly decreased in acidic pH ranges and at pH 11.0 (Figure 22). While the mulberry green tea ethyl acetate extract had high stability at pH 7 but the stability was slightly decreased in acidic pH ranges and at pH 11.0 (Figure 23). From the result, it indicated that mulberry green tea extracts were stable in a neutral pH range. Strongly acidic and alkaline conditions were able to reduce the antioxidant property of the extracts. Zhu *et al.* (1997) reported that green tea catechins extracts were unstable in Krebs-Ringer bicarbonate buffer (pH 7.4). When green tea catechins were dissolved in Krebs-Ringer bicarbonate buffer, more than 75% of total green tea catechins were degraded within a first half hour. In contrast, the same amount of green tea catechins dissolved in HPLC grade H_2O remained unchanged for a period of 3 h under the same incubation conditions. In addition, Zhu *et al.* (1997) found that in alkaline solution (pH > 8) green tea catechins was extremely instable and degraded almost completely in a few minutes, whereas in the acidic solution (pH < 4) green tea catechins were stable at least 18 h. Therefore, stability of green tea catechins were pH-dependent (Zhu *et al.*, 1997).

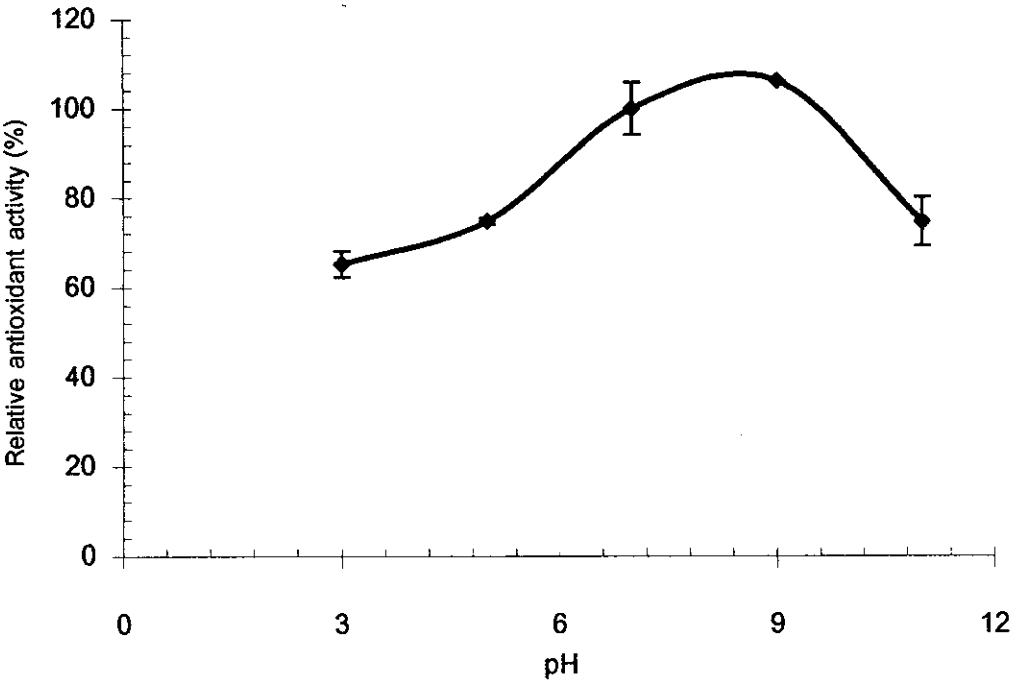

Figure 22 pH stability of antioxidant activity of mulberry green tea water extract

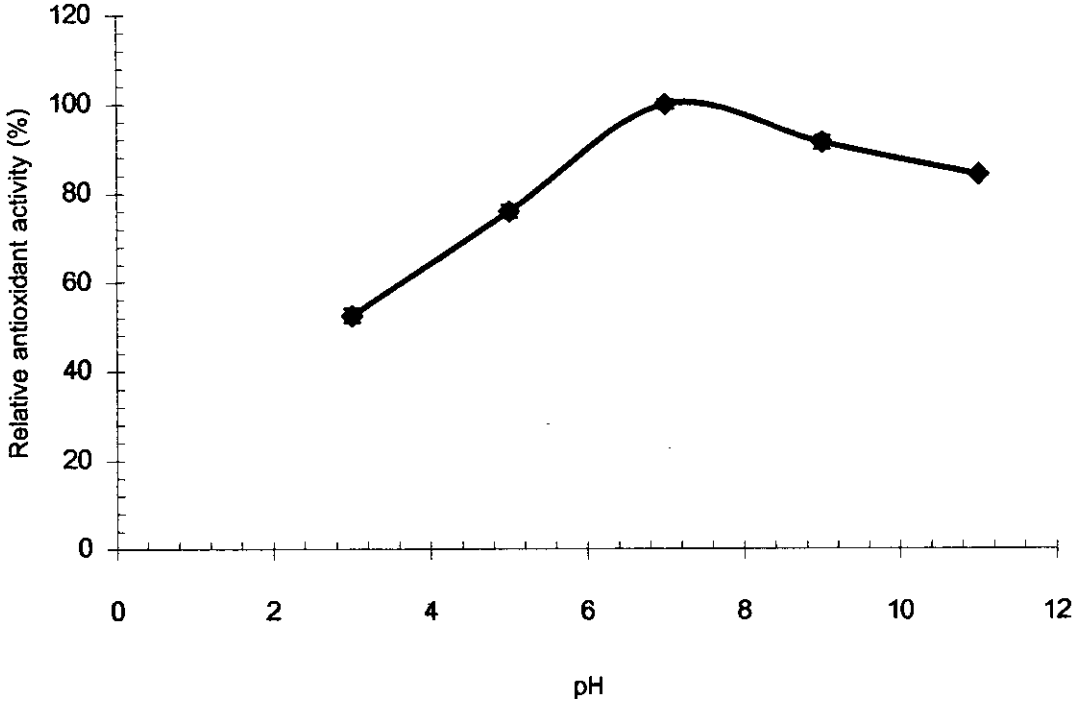

Figure 23 pH stability of antioxidant activity of mulberry green tea ethyl acetate extract

3.4 Synergistic effect of mulberry green tea extract with some compounds

Synergistic antioxidant effects between the compounds found in natural extracts are probably responsible for the higher antioxidant activities observed for the crude extracts than that measured in simulated extracts. Rodríguez de Sotillo *et al.* (1994) reported that higher antioxidant activity of freeze-dried potato peel extract in sunflower oil was observed, compared to the synthetic mixture of individual compounds. In general, the action of antioxidants is influenced by the synergistic components in the food system, such as α–tocopherol, citric acid and ascorbic acid. Therefore, the synergistic effects of α–tocopherol, citric acid and ascorbic acid on the antioxidant activity of mulberry green tea extract was evaluated in emulsion system using β–carotene bleaching method.

3.4.1 Synergistic effect of mulberry green tea water and ethyl acetate extracts with α-tocopherol

The synergistic effects of α-tocopherol, citric acid and ascorbic acid on the antioxidant activity of mulberry green tea extract evaluated in emulsion system using β-carotene bleaching method are depicted in Figure 24, 25, 26, 27, 28 and 29. Synergistic effect of mulberry green tea water extract with α-tocopherol is shown in Figure 24. α-Tocopherol generally showed no synergistic effect on mulberry green tea extract ($p > 0.05$).

No synergistic effect was also found between mulberry green tea ethyl acetate extract and α-tocopherol (Figure 25). From this result, no synergistic effect was postulated to be due to the use of exceed initial concentration of both extracts and α-tocopherol. Mulberry green tea ethyl acetate extract (200 ppm and 300 ppm) and α-tocopherol (20 and 30 ppm) exhibited slightly higher antioxidant activity than only α-tocopherol or mulberry green tea extract ($p < 0.05$). The differences in synergist effect of α-tocopherol between water extract and ethyl acetate extract was postulated to be due to the differences in antioxidants in both extracts. Synergistic antioxidant effects were observed for mixtures of carotenoids and tocopherol (Stahl *et al.*, 1998).

The reaction of α-tocopherol with a peroxyl radical leads to a relatively stable α-tocopheroxyl radical. The α-tocopheroxyl radical can be reduced to α-tocopherol by vitamin C, bile pigments or thiols (Stahl *et al.*, 1998). α-Tocopherol behaves as a chain breaking antioxidant by competing with the substrate for the chain carrying peroxy radicals, normally present in the highest concentration in the meat system (Frankel, 1996). α-Tocopherol can act as antioxidant or pro-oxidant depending on the test system, the concentration, the oxidation time and the method used to follow oxidation (Frankel, 1996). On the basis of hydroperoxide formation (measured by conjugated diene formation) in bulk corn oil, α-tocopherol exhibited optimum antioxidant activity at lower concentration (100 μg/g) than in the corresponding oil-in-water emulsion (250-500 μg/g). However, on the basis of hydroperoxide decomposition (measured by hexanal formation), the antioxidant activity of α-tocopherol increased with concentration in both bulk and emulsified oil (Huang *et al.*, 1994).

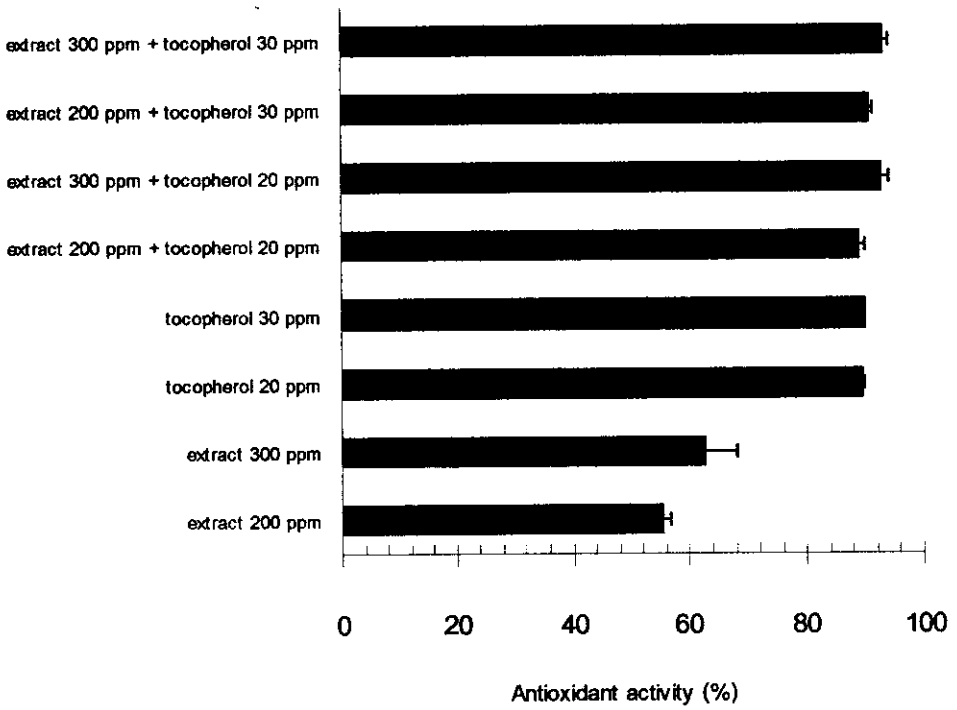

Figure 24 Synergistic antioxidant activity of mulberry green tea water extract with α-tocopherol in β-carotene/linoleic acid emulsion

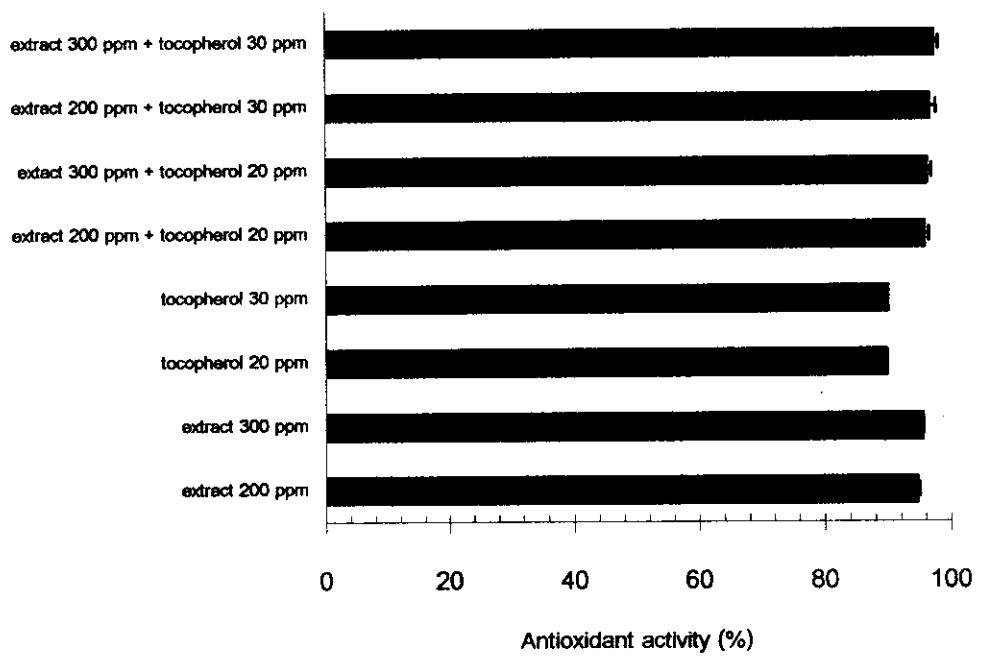

Figure 25 Synergistic antioxidant activity of mulberry green tea ethyl acetate extract with α-tocopherol in β-carotene/linoleic acid emulsion

3.4.2 Synergistic effect of mulberry green tea water and ethyl acetate extracts with citric acid

Figure 26 shows the antioxidant activity of mulberry green tea water extract (200 and 300 ppm), citric acid (20 and 30 ppm) and the combination of the extract and citric acid. Citric acid exhibited low antioxidant effect in β-carotene/linoleic acid emulsion system, compared to tocopherol at the same level used (Figure 24 and 25). However, citric acid showed synergistic effect on antioxidant of mulberry green tea water extract. Citric acid has been used in oils as a synergist antioxidants and chelator but this component is not hydrogen donor (Labuza, 1971). The antioxidant activity from mulberry green tea ethyl acetate extract (200 and 300 ppm), citric acid (20 and 30 ppm) and the combination of the extract and citric acid is shown in Figure 26. No synergistic action was found between mulberry green tea ethyl acetate extract and citric acid. Conversely, a lower antioxidant activity of mulberry green tea ethyl acetate extract – citric acid combination was found, compared to that of mulberry green tea ethyl acetate extract alone. Citric acid possibly caused an acidic pH. As the result, mulberry green tea ethyl acetate extract exhibited weak antioxidant activity in such an acidic pH cause by citric acid added. Furthermore, the simultaneous presence of some compounds may result in lower antioxidant activity. For example, antagonist effects were observed between ellagic acid and catechin (Meyer *et al.*, 1998).

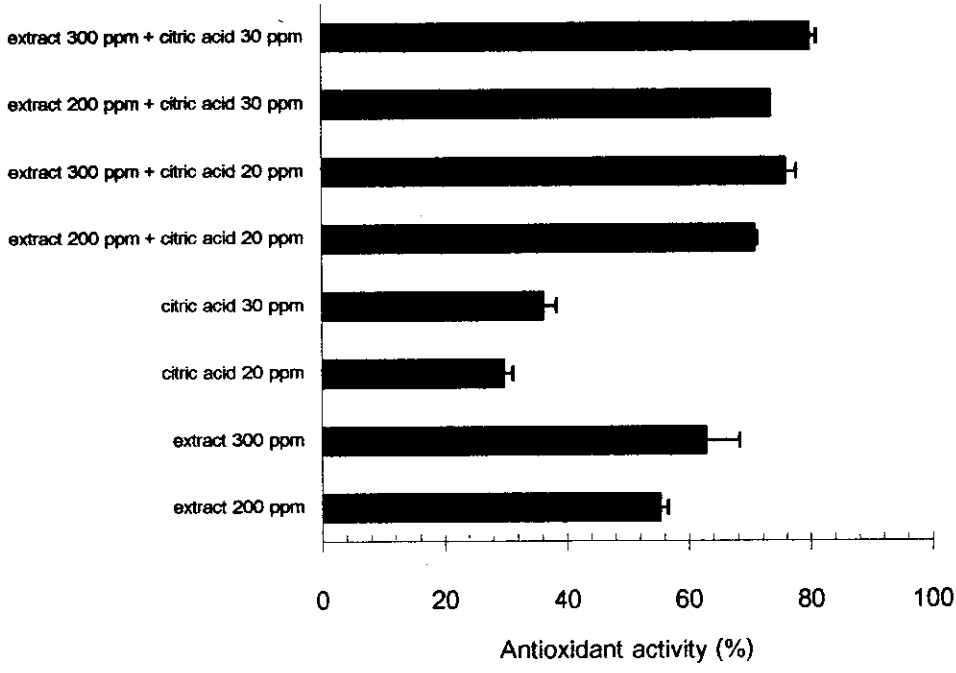

Figure 26 Synergistic antioxidant activity of mulberry green tea water extract with citric acid in β-carotene/linoleic acid emulsion

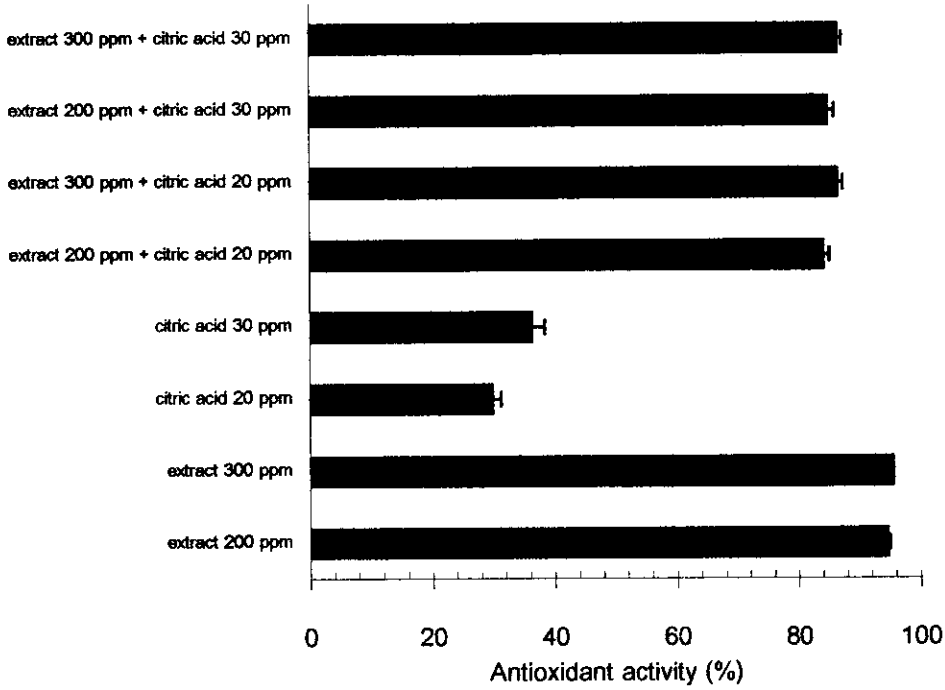

Figure 27 Synergistic antioxidant activity of mulberry green tea ethyl acetate extract with citric acid in β-carotene/linoleic acid emulsion

3.4.3 Synergistic effect of mulberry green tea water and ethyl acetate extracts with ascorbic acid

Synergistic effect of ascorbic acid (20 and 30 ppm) on the antioxidant activity of mulberry green tea water and ethyl acetate extract (200 and 300 ppm) is shown in Figure 28 and 29, respectively. Ascorbic acid showed no synergistic effect on antioxidant from both mulberry green tea extracts but exhibited prooxidative effect. Uri (1961) reported that ascorbic acid can act as an antioxidant, a pro-oxidant, a metal chelator, a reducing agent or as an oxygen scavenger, depending on conditions. In aqueous systems containing metals, ascorbic acid can act as a pro-oxidant by reducing the metals, which become more active catalysts of oxidation in their lower valence state. In the absence of added metals, ascorbic acid is an effective antioxidant at high concentrations (Cort, 1982). In non-aqueous media, ascorbic acid and esters are not good antioxidants (Porter, 1980).

Mitusumoto *et al.* (1991) reported that 500 ppm ascorbic acid decreased lipid peroxidation in ground beef. However, 50 ppm ascorbic acid increased lipid peroxidation. Ascorbic acid functioned as an oxygen scavenger, which is particularly useful in canned or bottled products with headspace of air (Dziezak, 1986). In this study, oxygenated water was added in β-carotene emulsion system. Weak antioxidant activity of ascorbic acid was caused by excess oxygen in β-carotene emulsion system. The extract from roots of *R. japonicus* Houtt has a synergistic effect with tocopherol, but no synergism was observed for the combination with L-ascorbic acid (Nishina *et al.*, 1991). No synergistic effect of ascorbic acid, citric acid, cystein or α-tocopherol was observed on the inhibitory effect of the extract from peanut hull (Yen and Duh, 1993).

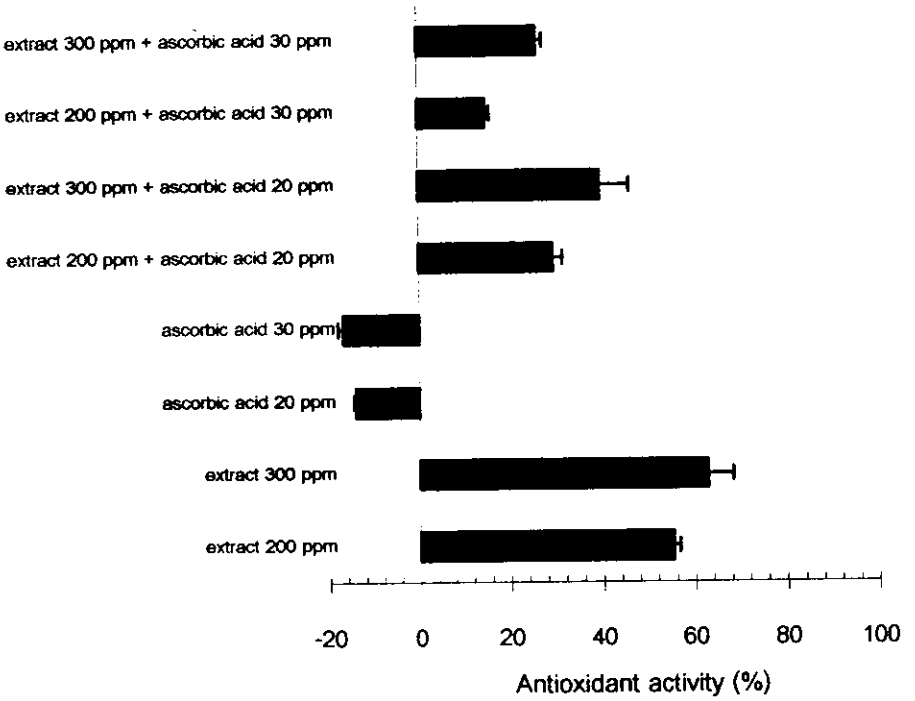

Figure 28 Synergistic antioxidant activity of mulberry green tea water extract with ascorbic acid in β-carotene/linoleic acid emulsion

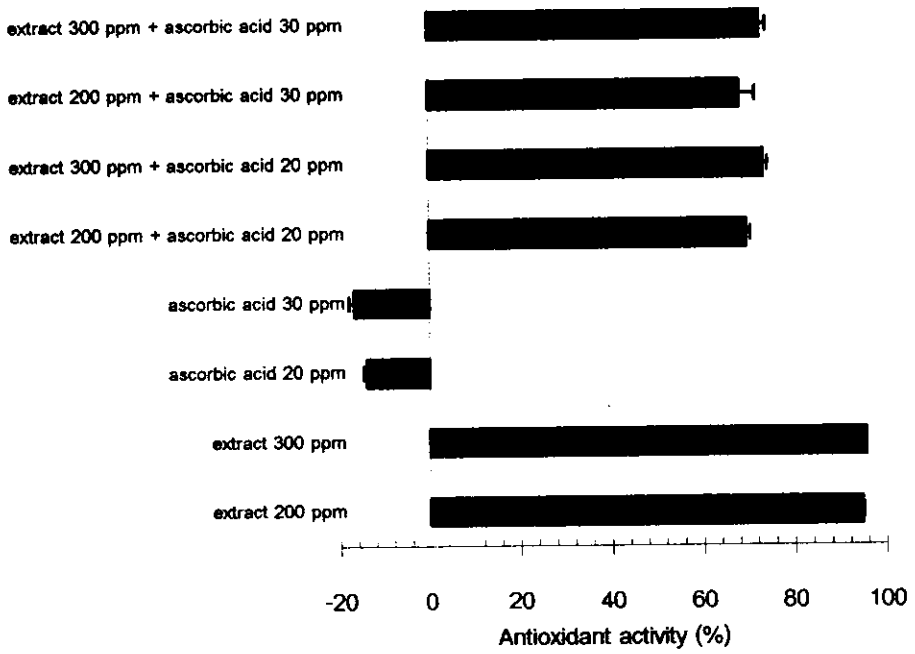

Figure 29 Synergistic antioxidant activity of mulberry green tea ethyl acetate extract with ascorbic acid in β-carotene/linoleic acid emulsion

4. Mode of action

4.1 Radical-scavenging activity of mulberry green tea water and ethyl acetate extracts

It is well known that free radicals cause autoxidation of unsaturated lipids in food (Kaur and Perkin, 1991). On the other hand, antioxidants are believed to interrupt the free-radical chain of oxidation and to donate hydrogen from phenolic hydroxyl group, thereby, forming stable free radicals, which do not initiate or propagate further oxidation of lipids (Sherwin, 1978). Elimination of 1,1 – diphenyl – 2 – picrylhydrazyl radicals (DPPH) is used to indicate the presence of hydrogen donors in a reaction system. Ionized DPPH produces a color which is changed, when radicals are removed from the system and is measured by diminishing absorption at 515 nm (Brand-Wiliams *et al.*, 1995)

Scavenging effects of mulberry green tea water extract at different concentrations on the DPPH radical is presented in Table 8. The absorbance at 517 nm decreased with increasing concentration of the extracts. However, scavenging activity of water extract at all concentrations tested was significantly lower than that of BHA, BHT and α-tocopherol at a level of 20 ppm ($p < 0.05$).

Scavenging activity of mulberry green tea ethyl acetate extract (50-600 ppm) on DPPH radicals was evaluated (Table 9). From the result, ethyl acetate extract exhibited much higher DPPH scavenging activity than water extract (Table 8). Ethyl acetate extract of mulberry green tea was capable of scavenging of DPPH radicals in a concentration-dependent manner. The scavenging activity of ethyl acetate extract at 600 ppm was significantly higher than that α-tocopherol but was comparable to those of BHA and BHT at a level of 20 ppm.

Table 8 Scavenging effects of mulberry green tea water extract at different concentrations on the 1,1 diphenyl – 2 –picrylhydrazyl (DPPH) radical

Concentration (ppm)	Absorbance at 517 nm	Radical – scavenging activity (%)
0	0.293 ± 0.009[a] f[b]	0.000 a
50	0.205 ± 0.006 e	30.219 ± 1.898 b
100	0.197 ± 0.003 d	32.947 ± 0.902 c
300	0.195 ± 0.003 cd	33.515 ± 1.042 cd
600	0.194 ± 0.003 cd	33.970 ± 1.042 cde
900	0.189 ± 0.005 bcd	35.447 ± 1.682 def
1200	0.188 ± 0.005 bc	35.845 ± 1.644 ef
1500	0.184 ± 0.002 b	37.379 ± 0.591 f
BHA (20)	0.016 ± 0.002 a	94.658 ± 0.710 g
BHT (20)	0.017 ± 0.001 a	94.090 ± 0.197 g
α-tocopherol (20)	0.017 ± 0.002 a	94.204 ± 0.591 g

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

Table 9 Scavenging effects of mulberry green tea ethyl acetate extract at different concentrations on the 1,1 diphenyl – 2 – picrylhydrazyl (DPPH) radical

Concentration (ppm)	Absorbance at 517 nm	Radical – scavenging activity (%)
0	0.295 ± 0.005[a] e[b]	0.000 a
50	0.246 ± 0.003 d	16.723 ± 1.174 b
100	0.192 ± 0.003 c	34.915 ± 0.853 c
300	0.057 ± 0.003 b	80.791 ± 1.174 d
600	0.013 ± 0.001 a	95.706 ± 0.339 e
BHA (20)	0.016 ± 0.002 a	94.689 ± 0.706 e
BHT (20)	0.017 ± 0.001 a	94.124 ± 0.196 ef
α-tocopherol (20)	0.017 ± 0.002 a	94.237 ± 0.587 f

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

This result was in agreement with Thabrew *et al.* (1998) who reported that the *Osbeckia aspera* was shown to scavenge DPPH free radical in a concentration – dependent manner. Furthermore, phenolic extracts of plant could neutralize free radicals in various model systems (Wettasingha and Shahidi, 1999; Lissi *et al.*, 1999). The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability (Bran-Wiliams *et al.*, 1995). Yen and Duh (1994) reported that the DPPH radical scavenging effect of phenolic compounds was due to their hydrogen-donating ability, however, tea polyphenolics also have electron-donating antioxidant properties. The relative activity of different polyphenolic compounds was related to the number and location of the hydroxyl groups (Lin *et al.*, 1996). Chen and Ho (1995) reported that catechins, theaflavins, carnosol, carnosic acid, and BHT at a concentration of 10 μM exhibited scavenging activity. The catechins and theaflavins showed higher

DPPH radical scavenging activity than carnosic acid, carnosol, and BHT. The DPPH radical scavenging activity was proportional to the number of -OH groups in the catechins or theaflavins (Chen and Ho, 1994; Zhu *et al.*, 2001a). Kim *et al.* (2000) found that two flavonoids from the leaves of *Morus alba* (quercetin-3-O-D-glucopyranoside and quercetin-3,7-di-O-D-glucopyranoside) exhibited scavenging effects on DPPH radical.

The results demonstrated that the mulberry green tea water and ethyl acetate extracts functioned as hydrogen donors and free-radicals inhibitors, possibly as primary antioxidants that reacted with free radicals. This action was possibly the main factor to inhibit the peroxidation in β-carotene and linoleic acid system. Ethyl acetate extract showed much higher scavenging activity than water extract, suggesting the differences in antioxidative compounds in both extracts. Those compounds in the former possessed hydrogen donating ability to a higher extent, compared to the latter.

4.2 Hydroxyl radical ($^{\bullet}OH$) scavenging activity of mulberry green tea water and ethyl acetate extracts

Mulberry green tea water extract exhibited a concentration–dependent increase in hydroxyl radical scavenging activity up to 1500 ppm (Table 10). The hydroxyl radical scavenging activity of water extract with the concentration between 50 and 1200 ppm was significantly lower than that of BHT and α-tocopherol at a concentration of 20 ppm ($p < 0.05$). However, the activity of water extract at a concentration of 1500 ppm was not different from that of α-tocopherol. Mulberry green tea ethyl acetate extract also exhibited hydroxyl radical scavenging activity in a concentration-dependent manner up to a concentration of 150 ppm (Table 11). The hydroxyl radical scavenging activity of ethyl acetate extract at all concentrations tested was significantly higher than that of BHT and α-tocopherol at a level of 20 ppm ($p < 0.05$). The hydroxyl radical is an extreamely reactive free radical formed in biological systems. It can act on and damage almost every molecule found in living cells, such as sugars, amino acids, phospholipids, DNA bases and organic acid

(Namiki, 1990; Halliwell *et al.*, 1992). Lipid peroxidation is rapidly stimulated by hydroxyl radicals that are sufficiently reactive to abstract hydrogen atoms from unsaturated fatty acid (Halliwell *et al.*, 1992). Yang *et al.* (2000) reported that the scavenging effect of fermented soybean broth on hydroxyl radicals increased from 30 to 96 % with its concentrations ranging from 2 to 100 %. For anthocyanins and procyanidins, they were effective against hydroxyl radical at the level of 42 and 29 %, respectively (Ghiselli *et al.*, 1998). The relative extents of free radical-mediated inhibition of deoxyribose degradation will give an indication of hydroxyl radical scavenging potential (Miller *et al.*, 1996). Phenolic compounds in extract might play a role in hydroxyl radical scavenger because phenolic groups are excellent nucleophiles and also able to quench lipid peoxidation, acting as chain break antioxidants (Shi *et al.*, 1991).

The results demonstrated that the mulberry green tea water extract and ethyl acetate extracts inhibited deoxyribose degradation. Hydroxyl radicals are known to be responsible for the breakdown of deoxyribose (Ueda *et al.*, 1996). Therefore, it indicated that mulberry green tea extracts had hydroxyl radical scavenging activity, suggesting its efficiency in prevention of propagation in peroxidation process.

Table 10 Scavenging effect of mulberry green tea water extract at different concentrations on the hydroxyl radical in the deoxyribose assay

Concentration (ppm)	Absorbance at 532 nm	Hydroxyl radical – Scavenging activity (%)
0	0.367 ± 0.003[a] g[b]	0.000 a
50	0.342 ± 0.003 f	6.721 ± 0.832 b
100	0.315 ± 0.008 e	14.078 ± 2.202 c
300	0.292 ± 0.012 d	20.436 ± 3.315 d
600	0.287 ± 0.004 d	21.798 ± 1.090 d
900	0.281 ± 0.006 cd	23.433 ± 1.657 de
1200	0.273 ± 0.010 c	25.522 ± 2.836 e
1500	0.272 ± 0.003 c	25.976 ± 0.817 ef
BHT (20)	0.241 ± 0.002 a	34.423 ± 0.567 g
α-tocopherol (20)	0.261 ± 0.001 b	28.883 ± 0.272 f

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

Table 11 Scavenging effect of mulberry green tea ethyl acetate extract at different concentrations on the hydroxyl radical in the deoxyribose assay

Concentration (ppm)	Absorbance at 517 nm	Hydroxyl radical – scavenging activity (%)
0	0.367 ± 0.003[a] f[b]	0.000 a
20	0.185 ± 0.001 e	49.682 ± 0.157 b
40	0.162 ± 0.001 d	55.949 ± 0.157 c
60	0.159 ± 0.002 d	56.585 ± 0.416 c
80	0.147 ± 0.001 c	60.036 ± 0.157 d
100	0.147 ± 0.001 c	60.036 ± 0.315 d
150	0.146 ± 0.001 c	60.127 ± 0.315 d
BHT (20)	0.241 ± 0.002 a	34.423 ± 0.567 f
α-tocopherol (20)	0.261 ± 0.001 b	28.883 ± 0.272 e

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

4.3 Reducing power of mulberry green tea water and ethyl acetate extracts

The reducing power and antioxidant activity of mulberry green tea water and ethyl acetate extracts increased with an increasing concentration (Table 12 and 13). Water and ethyl acetate extracts at every concentration tested had significantly lower reducing power than ascorbic acid at a level of 50 ppm ($p < 0.05$). Antioxidant activity of both extracts correlated well with the reducing power of the extract. Correlation coefficient between antioxidant activity and reducing power of water extract and ethyl acetate extact was 0.89 (Figure 30) and 0.83 (Figure 31), respectively. The data indicated that the marked antioxidant action of mulberry green tea extract might be a result of their reducing power. Antioxidant activity in ethyl acetate extract was generally higher than that of water extract, though the reducing power was lower.

Therefore, other antioxidative component in ethyl acetate extract exhibiting other functions in prevention of oxidation apart from electron or hydrogen donating was possibly present.

Table 12 Reducing power and antioxidant activity of mulberry green tea water extract at different concentrations

Concentration (ppm)	Reducing power (Absorbance at 700 nm)	Antioxidant activity (%)
50	0.017 ± 0.001[a] a[b]	27.618 ± 7.404 a
100	0.072 ± 0.002 b	39.110 ± 3.044 b
300	0.121 ± 0.003 c	37.468 ± 2.829 c
600	0.182 ± 0.005 d	83.796 ± 2.240 d
900	0.228 ± 0.006 e	85.840 ± 3.092 d
1200	0.257 ± 0.005 f	87.034 ± 0.000 d
1500	0.291 ± 0.011 g	88.684 ± 1.628 d
Ascorbic acid (50)	0.334 ± 0.006 h	

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

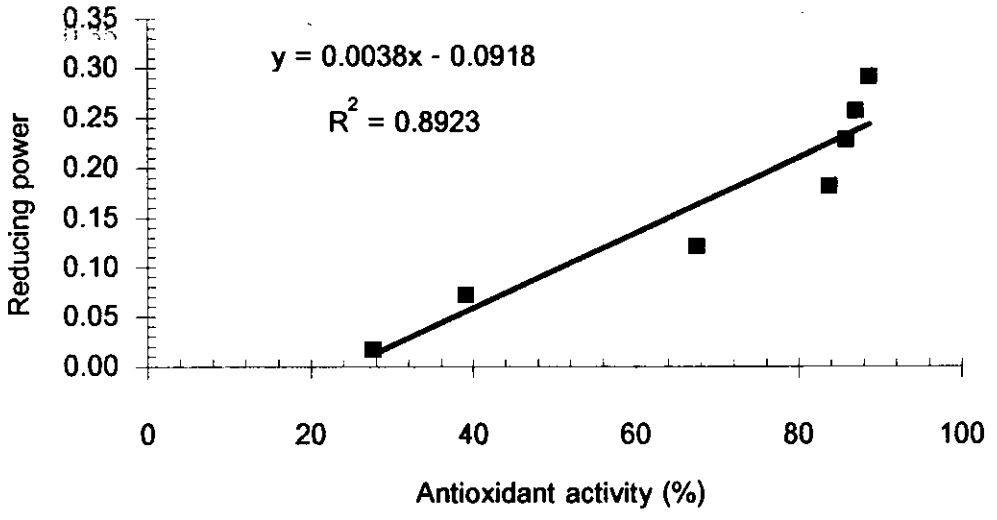

Figure 30 Relationship between antioxidant activity and reducing power of mulberry green tea water extract

Table 13 Reducing power and antioxidant activity of mulberry green tea ethyl acetate extract at different concentrations

Concentration (ppm)	Reducing power (Absorbance at 700 nm)	Antioxidant activity (%)
50	0.010 ± 0.001[a]a[b]	41.196 ± 0.595 a
100	0.043 ± 0.005 b	68.752 ± 1.526 b
300	0.082 ± 0.003 c	96.325 ± 0.505 c
600	0.122 ± 0.004 d	96.571 ± 0.000 cd
900	0.135 ± 0.001 e	96.937 ± 0.939 cd
1200	0.139 ± 0.003 e	97.288 ± 0.022 cd
1500	0.149 ± 0.005 f	97.952 ± 0.516 d
Ascorbic acid (50)	0.334 ± 0.006 g	

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column indicate significant differences ($p < 0.05$)

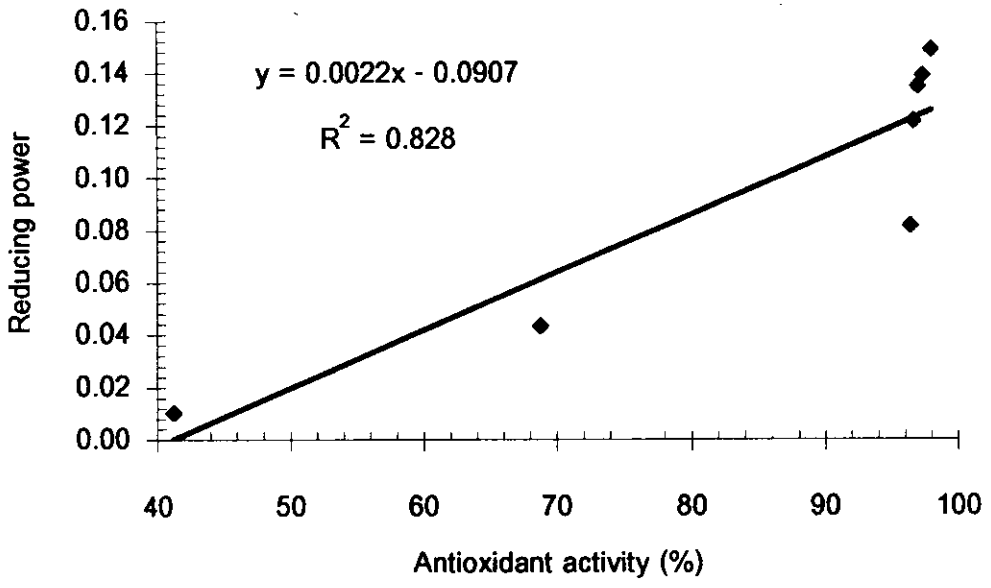

Figure 31 Relationship between antioxidant activity and reducing power of mulberry green tea ethyl acetate extract

This result was in accordance with Duh (1998) who found that the reducing power of burdock extract was dependent on concentration and correlated well with the antioxidant activity. Yen and Duh (1993) also reported that the reducing power of methanolic extracts of peanut hulls containing high levels of polyphenols was significantly correlated to the extent of antioxidative activity. In addition, Rajalakshmi and Narasimhan (1996) and Fejes *et al.* (2000) noted that the antioxidative effect exponentially increased as a function of the development of the reducing power, indicating that the antioxidative properties are concomitant with the development of the reducing power. The hsian-tsao leaf gum is an electron donor and could react with free radicals, convert them to more stable products, and terminate radical chain reaction (Yen and Chen, 1995). Antioxidative activity of reductones is believed to break radical chains by donating of hydrogen atom (Gordon, 1990). In addition, Eichner (1981) reported that the intermediate reductone compound of Maillard reaction products (MRP) were able to break the radical chain by donating of a hydrogen atom and they are also effective in reducing hydroperoxide to non-radical products.

Therefore, the mulberry green tea extract was suggested to act as electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reactions. The result indicated that the marked antioxidant action of mulberry green tea extracts might be a result of their reducing power.

5. Separation of antioxidants from mulberry green tea ethyl acetate extract

Thin-layer chromatography (TLC) was used for separation of components in mulberry green tea ethyl acetate extract. Separated compounds in the extract was developed with benzene/ethyl formate/formic acid (75:24:1 v/v/v). Four bands were found, after drying and spraying with different reagents (Table 14) at R_f of 0.91 (band A), 0.83 (band B), 0.77 (band C) and 0.26 (band D) (Figure 32). Spray 1 revealed the presence of phenolic compound in mulberry green tea ethyl acetate extract. Spray 2 indicated that phenolic compound with free *ortho-* and *para-* hydroxyl groups were present. The green color produced also indicated that a phenolic compound consisted of two hydroxyl groups.

Table 14 Identification of antioxidant compounds in mulberry green tea ethyl acetate extract

Sprays	Band[a]	Color	Compound identified
1.) $FeCl_3$-$K_3Fe(CN)_6$	A, B, C, D	blue	Phenolics
2.) $FeCl_3$	A, C, D	green	Dihydroxy-phenolics

[a] The bands corresponded to positive identification of compounds at Rf values of 0.91 (band A), 0.83 (band B), 0.77 (band C) and 0.26 (band D)

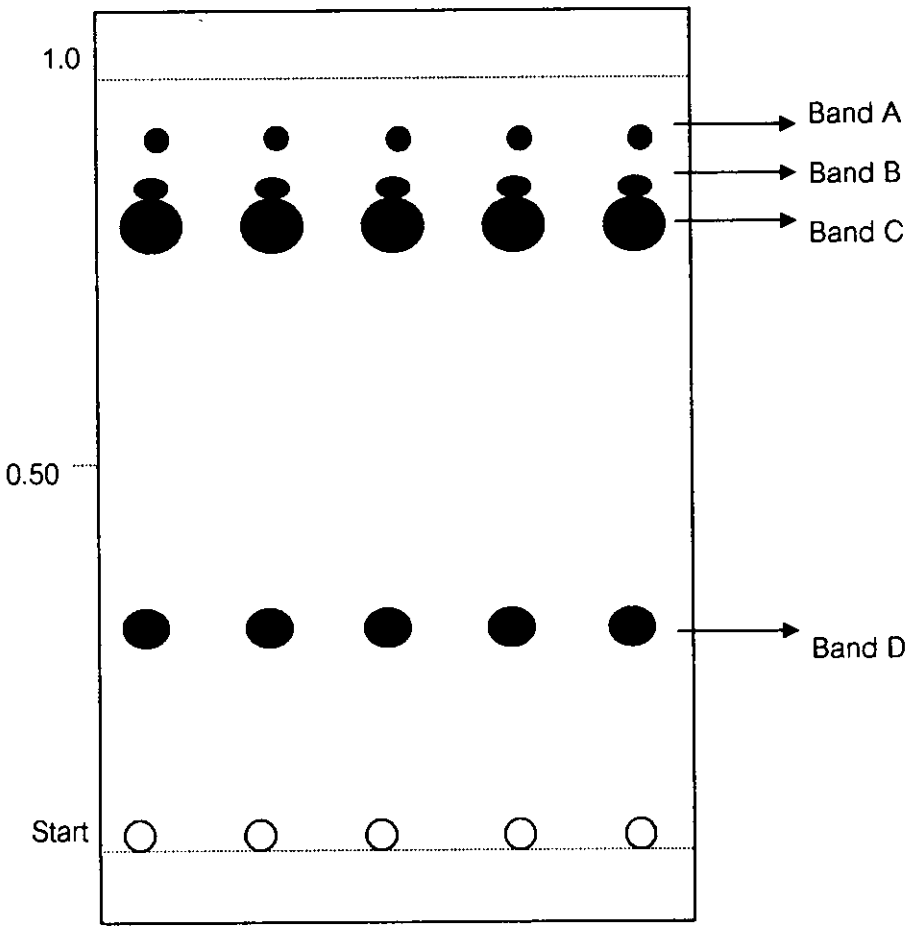

Figure 32 Thin-layer chromatography (TLC) pattern of mulberry green tea ethyl acetate extract

After the bands position were located by different reagents, the silica gel scrapings, which contained the interested identified compounds, were soaked in methanol (10 ml) for 30 min. The extract was filtered with Whatman No. 41 filter paper, then adjusted with methanol to volume 10 ml and antioxidant activity, total phenolic content and reducing power were determined. The decreases in absorbance of β-carotene in the presence of band A, B, C and D extract are shown in Figure 33.

The decrease in absorbance of β-carotene in the presence of different components in mulberry green tea ethyl acetate extract (Band A, B, C, D) is shown in Figure 33. A sharp decrease in OD_{470} was obtained in the control, indicating a rapid

oxidation of β-carotene/linoleic acid. However, a decrease in OD_{470} was retarded when scraping extracts were added. Extract from Band B (63.565 ± 1.428) had highest antioxidant activity when compared to the extracts from other bands ($p < 0.05$). An order of antioxidant activity was Band B > D > C >A. This result reconfirmed that different compounds with different antioxidant activity were present in the extracts and played an essential role in prevention of oxidation.

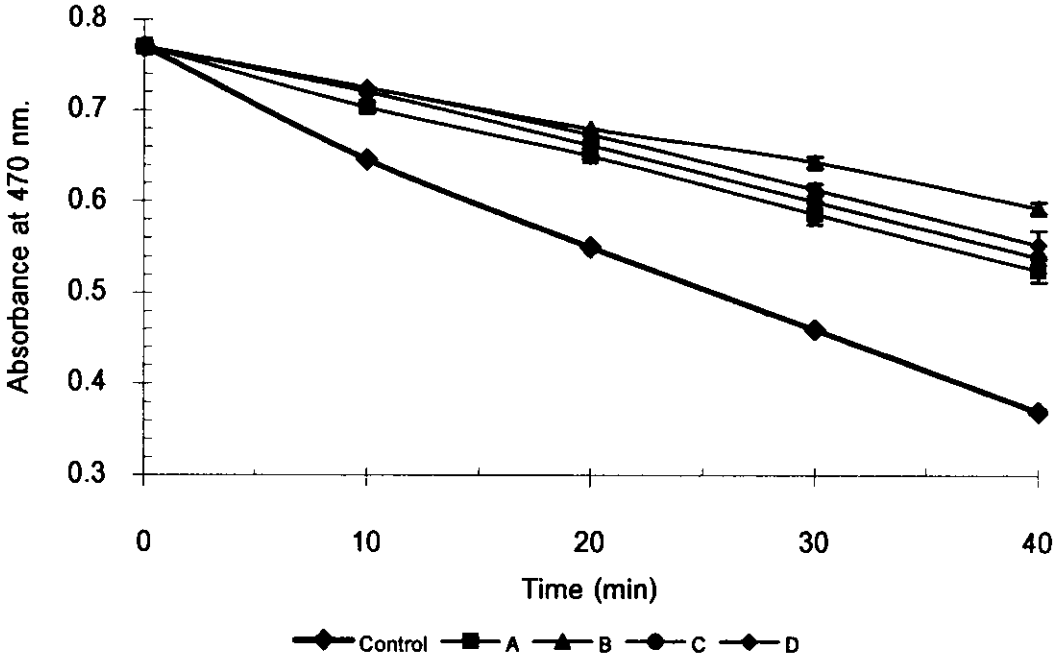

Figure 33 Antioxidant activity of components extracted from bands appeared on thin-layer chromatography

Differences in total phenolic content were observed among different scraping extracts (Table 15). Total phenolic content varied from 2.412 to 2.507 mg/100 g. Band C extract contained the highest total phenolic content but it had a lower antioxidant activity than Band B and D extracts, respectively. Among all scraping extracts, Band D extract showed the lowest total phenolic content. This result indicated that phenolic compounds in extract of mulberry green tea may partially contribute to inhibition of lipid peroxidation.

After 18 days of storage, significant differences in TBARS between samples added with the extracts, BHT and α-tocopherol were observed ($p < 0.05$). The sample added with BHT had lower TBARS than those added with other antioxidants. No differences in TBARS in sample added with extract at level of 100 and 200 ppm were observed. Although tocopherols are considered as safe natural antioxidants, they do not always provide effective protection against *in vitro* oxidation (Frankel, 1980).

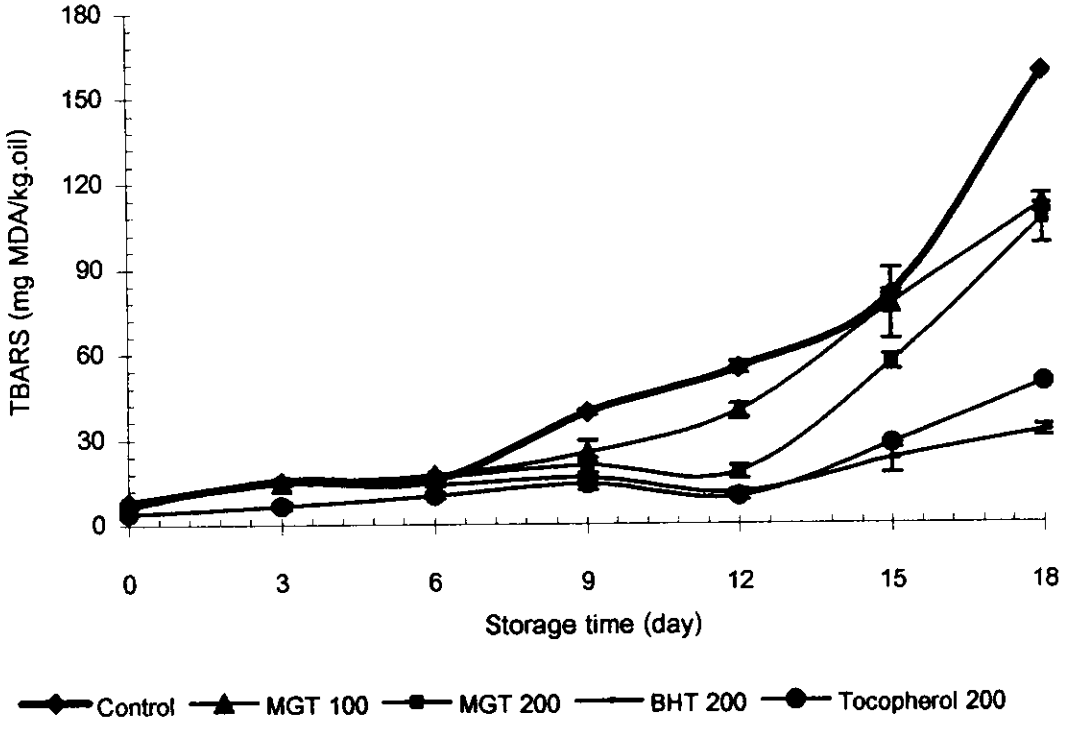

Figure 34 TBARS of lard added with mulberry green tea ethyl acetate extract and other commercial antioxidants during storage at 37 °C for 18 days

Peroxide values of lard added with mulberry green tea ethyl acetate extract (100 and 200 ppm), BHT (200 ppm) and α-tocopherol (200 ppm) are illustrated in Figure 35. No differences in peroxide value were found among all samples during the first 9 days of storage. All samples added with mulberry green tea ethyl acetate extract and other antioxidants had significantly lower peroxide value than the control ($p < 0.05$) after 12 days of storage. Mulberry green tea ethyl acetate extract inhibited the peroxide

formation in lard in a concentration-dependent manner. However, the efficiency in oxidation prevention was lower than α-tocopherol and BHT. From the result, BHT at a concentration of 200 ppm exhibited the highest antioxidative activity in lard, especially as storage time increased. Hara (1994) found that crude tea catechins reduced the formation of peroxides more effectively than α-tocopherol or BHA determined by active oxygen method. Green tea catechins showed an alternative in protecting fats and oils in foods from oxidative rancidity (Chen and Chan, 1996). The ethanol extracts from green tea and white teas exhibited a stronger inhibition on lipid oxidation in canola oil than BHT. In contrast, the ethanol extracts from black tea showed no or little antioxidative activity (Chen and Chan, 1996).

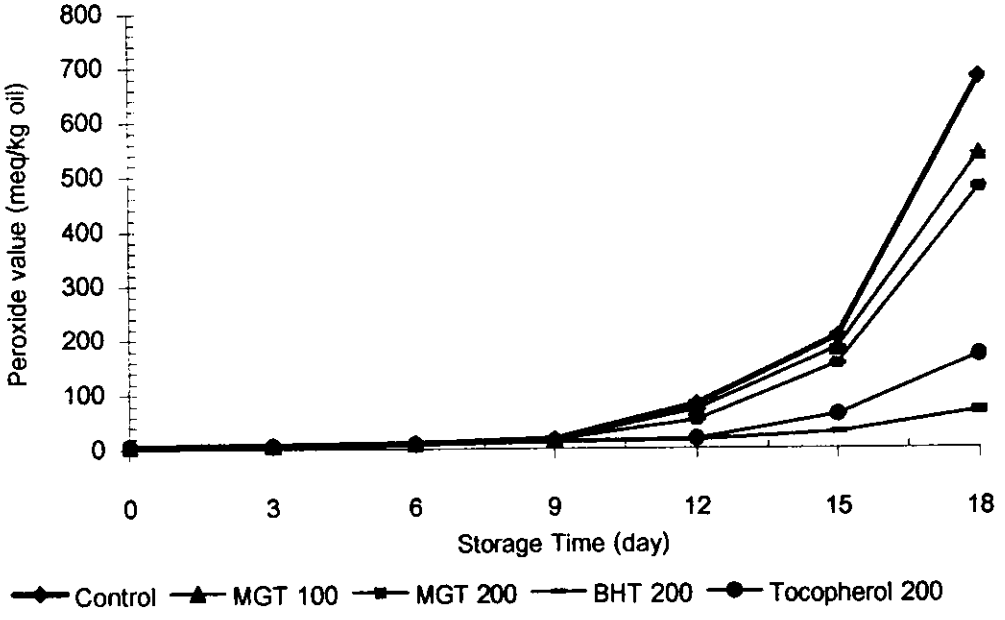

Figure 35 Peroxide value of lard added with mulberry green tea ethyl acetate extract and other commercial antioxidants during storage at 37 °C for 18 days

TBARS of partially purified fish oil added with mulberry green tea ethyl acetate extract (100 and 200 ppm), BHT (200 ppm) and α-tocopherol (200 ppm) are shown in Figure 36. All samples treated with mulberry green tea ethyl acetate extracts and other antioxidants had significantly lower TBARS than the control ($p < 0.05$) after 6 day of storage. However, at day 18, TBARS of the control slightly decreased and was

not different from other samples ($p > 0.05$). This was presumed to be due to the loss of volatile secondary oxidation products formed, resulting in the lowered amount of TBARS. Mulberry green tea ethyl acetate extract inhibited the oxidation in partially purified fish oil less effectively than BHT and α-tocopherol throughout the storage ($p < 0.05$). From the result, BHT inhibited the oxidation of partially purified fish oil to the higher extent, compared to other antioxidants ($p < 0.05$).

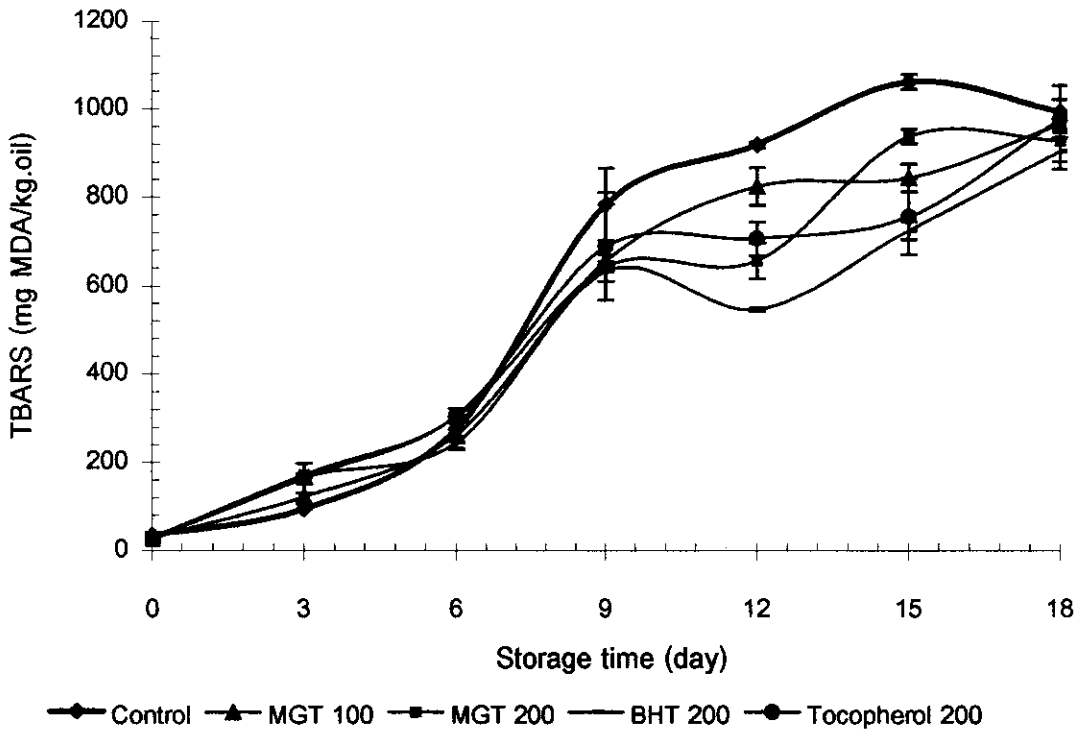

Figure 36 TBARS of fish oil added with mulberry green tea ethyl acetate extact and other commercial antioxidants during storage at 37 °C for 18 days

Peroxide values of partially purified fish oil added with mulberry green tea ethyl acetate extract (100 and 200 ppm), BHT (200 ppm) and α-tocopherol (200 ppm) are shown in Figure 37. No significant differences in peroxide value were found between the treatments throughout the storage. Since fatty acids in fish oil are mostly unsaturated fatty acids, which are prone to oxidation, antioxidant added might not enough to prevent the reaction. From the result, sample added with the extract tended to contain higher

peroxide value than other samples, suggesting the pro-oxidative effect of the extract. Wanasundara and Shahidi (1998) also reported the pro-oxidative effect of green tea extract in seal bubble oil and menhaden oil due to the presence of chlorophyll. Endo *et al.* (1985) have reported that chlorophylls and their derivatives promote oxidation of lipids during storage.

In this experiment, the ethyl acetate in mulberry green tea extract was evaporated with nitrogen gas and the residue was redissolved in methanol. Therefore, the low efficiency in prevention of lipid peroxidation of partially purified fish oil was possibly due to the use of methanol as redissolved solvent. Polarity of methanol is higher than ethyl acetate and oils. As a result, the extract was not totally dissolved. Due to the differences in polarity, methanol extract could not be mixed well with oil, leading to the marked decrease in antioxidative activity of non-polar antioxidant in the oil.

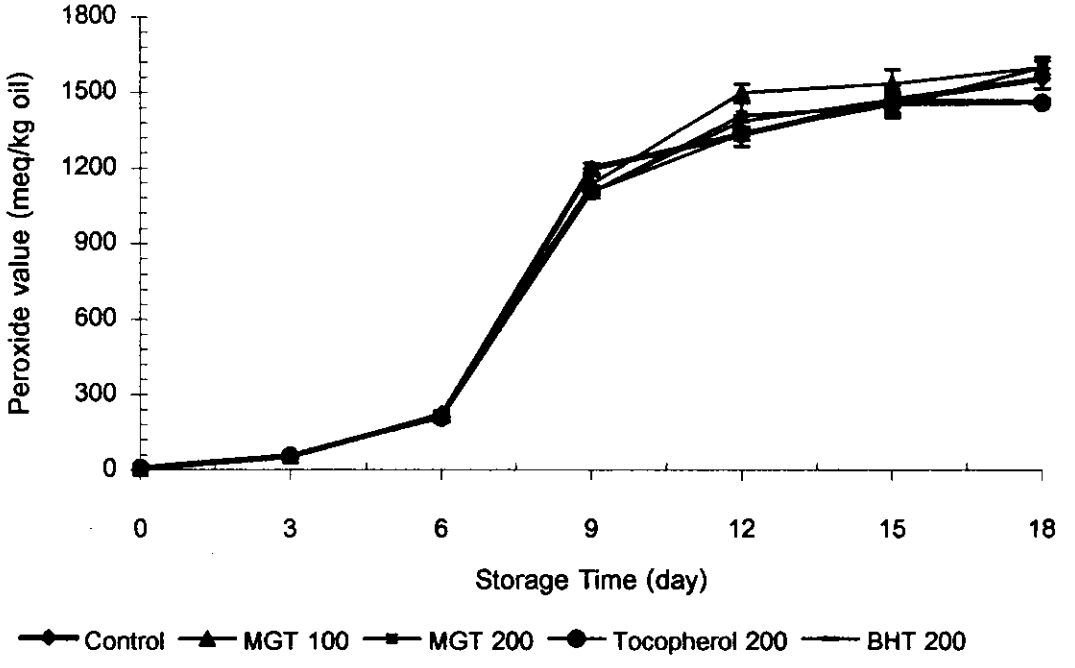

Figure 37 Peroxide value of fish oil added with mulberry green tea ethyl acetate extract and other commercial antioxidants during storage at 37 ^{o}C for 18 days

However, He and Shahidi (1997) have recently demonstrated that green tea extract, despite the presence of chlorophyll, had an antioxidant effect when applied to white muscle of mackerel. Therefore, it is evident that the antioxidant/pro-oxidant activity of green tea extract is system-dependent. Wanasundara and Shahidi (1998) reported that addition of dechlorophyllized green tea extracts (DGTE) to both seal bubble oils and menhaden oil significantly ($p < 0.05$) decreased their peroxide value and TBARS under accelerated oxidation conditions. Effectiveness of DGTE at 500 and 1000 ppm levels was superior to that of α-tocopherol at 500 ppm and BHA and BHT at 200 ppm.

7. Inhibition of Low Density Lipoprotein (LDL) oxidation by mulberry green tea extracts

Mulberry green tea water extract (0.125 and 0.5 μg/ml) and ethyl acetate extract (0.125 and 0.5 μg/ml) inhibited TBARS formation dose-dependently and α-tocopherol at a concentration of 0.5 μg/ml also inhibited TBARS formation, suggesting their inhibition of Fe^{2+}-induced LDL oxidation (Figure 38). All samples treated with mulberry green tea water extract, ethyl acetate extract and α-tocopherol had significantly lower TBARS than control ($p < 0.05$). Mulberry green tea water extract inhibited LDL oxidation less effectively than ethyl acetate extract and α-tocopherol, respectively throughout incubation times ($p < 0.05$). Doi *et al.* (2000) reported that quercetin and isoquercitrin, which extracted from *Morus alba* leaf, inhibited the formation of TBARS by copper-induced oxidative modification of rabbit and human LDL. Yang and Koo (2000) reported that Chinese tea extract, at 5 and 10 μg/ml, dose-dependently inhibited endothelial cell induced LDL oxidation and significantly lowered the TBARS values. Anti-oxidative effect of 10 μg/ml ethyl acetate extract fom Chinese tea was significantly stronger than the same concentration of chloroform extract. Ethyl acetate extract has the highest amount of catechins while the least amount was found in the remaining aqueous extract (Yang and Koo, 2000). In an epidermiological study of Japanese men, an inverse relationship was reported between green tea consumption and both serum cholesterol levels and systolic blood pressure (Kono *et al.*, 1992). Cholesterol-fed rats supplemented with crude green tea catechins had decreased

plasma total cholesterol levels compared to nonsupplemented rats (Muramatsu *et al.*, 1986). Doi *et al.* (2000) reported that rabbits fed with mulberry extract were found to have lower levels of blood fats, and the effect appears to prevent the development of a fat-rich liver. Quercetin reduced the oxidation of low density lipoprotein, suggesting that mulberry leaves may prevent arteriosclerosis (Doi *et al.*, 2000). Inhibition of LDL peroxidation by supplementation of antioxidants becomes an attractive therapeutic strategy to prevent and possibly to treat atherosclerosis and related diseases in human (Liu *et al.*, 2000).

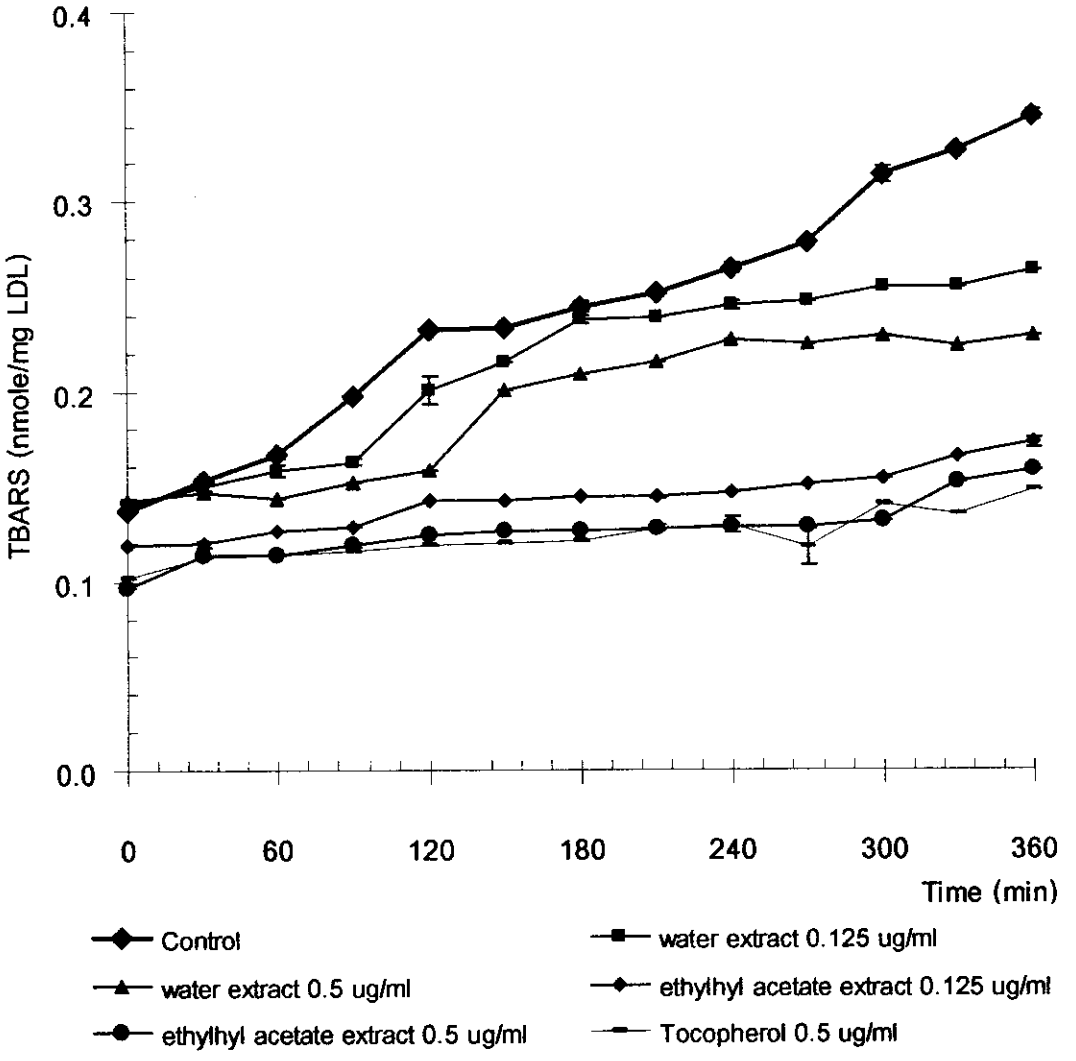

Figure 38 TBARS of LDL in presence of mulberry green tea water extact, ethyl acetate extract and α-tocopherol

From the results, it is possible that mulberry green tea extracts may delay atherogenesis and lower the risk of coronary heart disease by inhibiting LDL oxidation and foam cell formation. Mulberry green tea therefore may be serve as an neutraceutical beverage.