

Chapter 2

Materials and Methods

Materials

1. **Mulberry green tea (*Morus spp.*) (4 varieties) was obtained from Lumpang Crop Research Station. Those are shown as follows:**
 - BU-RERUM 60
 - DOK-INN
 - JEEN
 - KOK-MOO
2. **Chemicals**
 - 2.1 **Solvents used for extraction of the antioxidants from mulberry green tea.**
 - Methanol
 - Ethanol
 - Ethyl acetate
 - Hexane
 - 2.2 **Chemicals for determination of antioxidant activity.**
 - β -carotene
 - Linoleic acid
 - Tween 40
 - 2.3 **Chemicals for determination of total phenolic compound content.**
 - Folin and Ciocalteu's Phenol Reagent
 - Sodium carbonate
 - 2.4 **Chemicals for determination of mode of action.**
 - 1,1-Diphenyl-2-picrylhydrazyl (DPPH)
 - 2-deoxyribose
 - Sodium phosphate

- Trichloroacetic acid (TCA)
- Sodium hydroxide
- Potassium ferricyanide
- Ferric chloride

2.5 Chemicals for oxidation analysis.

- Thiobarbituric acid
- Hydrochloric acid
- Sodium thiocyanate
- Starch
- Acetic acid : Chloroform 3 : 2 (vol/vol)
- Iso - octane

2.6 Chemicals for separation of compounds in mulberry green tea.

- Thin-layer chromatography (precoated silica gel plate, 20 x 20 cm, Kieselgel 60 F254, 0.20 mm, E.Merck, Dramstadt, Germany)
- Benzene : Ethyl formate : Formic acid (75:24:1 vol/vol)
- 1% solution of potassium ferricyanide + 1% solution of ferric chloride

2.7 Chemicals for study the inhibition of Low Density Lipoprotein (LDL) oxidation

- Disodium hydrogen phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)
- Ethylenediamine tetraacetic acid disodium salt dehydrate (EDTA)
- Potassium bromide (KBr)
- Potassium chloride (KCl)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Sodium azide (NaN_3)
- Sodium chloride (NaCl)

3. Instruments

Instruments	Model	Company	Country
Centrifuge	Hermle	Technical Science & Service	U.S.A.
Freeze-dryer	Dura-Dry™ $\mu\rho$	FIS System	U.S.A.
pH meter	Denver 15	Denver Instrument	U.S.A.
Shaker	GEL 1038	Gesellschaft fur	Germany
Shaker water bath	WB/OB 7-45	Memmert	Germany
Spectrophotometer	UV-1601	Shimadzu	Japan
Spectrophotometer	Spectronic 21	Spectronic	U.S.A.
Ultracentrifuge	L8-70M	Beckman	U.S.A.
Blender	MX-T2GN	National	Taiwan

Methods

1. Preparation of mulberry green tea extracts

Dried mulberry green tea was ground to a particle size of 20 mesh. The samples were kept in screw capped bottle in dark at 4 °C until used.

To extract antioxidant, mulberry green tea powder (5.00 g) was mixed with boiling distilled water (100 ml), in a shaking water bath at 100 °C for 5 min. The extract was filtered with Whatman No.1 filter paper and the filtrate was made up to a volume of 100 ml with distilled water (Figure 12). Water extracts were prepared in duplicates for each mulberry green tea variety and stored at 4 °C until analysis.

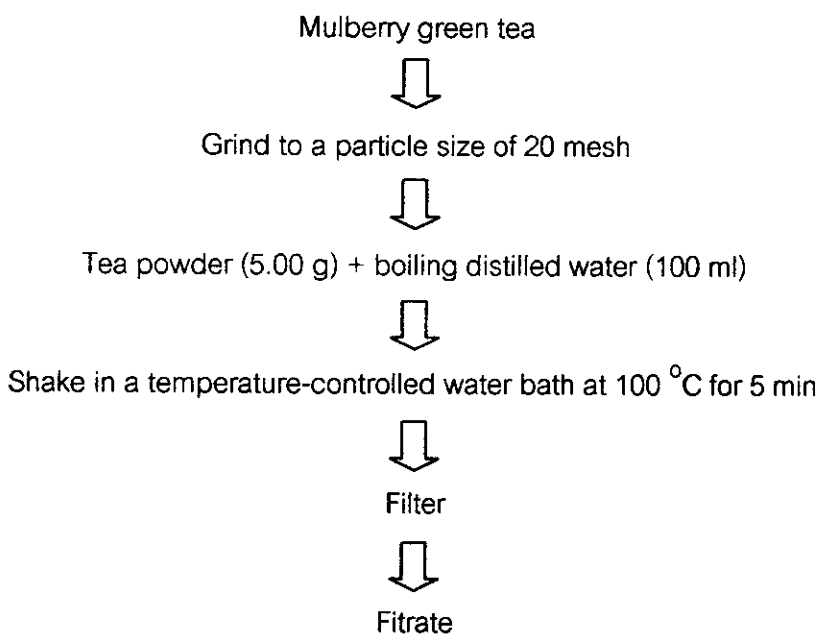


Figure 12 Extraction of antioxidants from mulberry green tea

Sources: Modified from Yen *et al.* (1997)

2. Primary screening of antioxidant activities in water extracts of mulberry green tea

Evaluation of antioxidant activity based on coupled oxidation of β -carotene and linoleic acid was conducted as described by Taga *et al.* (1984) with some modification. β -carotene (1 mg) was dissolved in 10 ml of chloroform. A 1.5 ml aliquot of the solution was added to a 50 ml-beaker with 20 mg linoleic acid and 200 mg

Tween 40. Chloroform was removed by purging with nitrogen. Oxygenated distilled water (50 ml) was added into the β -carotene emulsion and mixed well used magnetic stirrer. Aliquot (3 ml) of the oxygenated β -carotene emulsion and 0.2 ml of the water extract were transferred into spectrophotometer tubes and mixed thoroughly. The tubes were immediately placed in a water bath and incubated at 50 °C. Oxidation of β -carotene emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. Absorbance was measured at 0, 10, 20, 30 and 40 min. A control was prepared by using 0.2 ml distilled water instead of mulberry green tea extracts. Degradation rate of mulberry green tea extract was calculated according to first order kinetics with the following equation (Al-Saikhan *et al.*, 1995):

$$\ln (a/b) \times 1/t = \text{sample degradation rate}$$

where \ln = natural log

a = initial absorbance (470 nm) at time zero

b = absorbance (470 nm) at time 40 min

t = time (min)

Antioxidant activity (AA) was expressed as % inhibition relative to the control using the following equation:

$$AA = \frac{\text{Degradation rate of control} - \text{Degradation rate of sample} \times 100}{\text{Degradation rate of control}}$$

The antioxidant activity of the extracts was compared with BHA, BHT and α -tocopherol at the level of 200 ppm. Total phenolic content in mulberry green tea extracts was determined spectrophotometrically using Folin-Ciocalteu reagent as described by Julkunen-Titto (1985) and Weurman and Swain (1955). Reducing power was measured according to the method of Oyaizu (1986). Tea samples which exhibited the highest antioxidant activity was chosen for further study.

3. Extraction of antioxidants from selected mulberry green tea

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

Ground mulberry green tea was mixed with distilled water at different temperatures (60, 80 and 100 °C) using a sample/water ratio of 1:20 (w/v). Different extraction times (5, 10, 15 and 20 min) were used for antioxidant extraction. The sample was extracted repeatedly up to 3 times. The extracts (3 x 4 x 3 treatments) prepared as above were evaluated for antioxidant activity by using β -carotene bleaching method (Taga *et al.*, 1984). Reducing power was measured according to the method of Oyaizu (1986) and phenolic content was determined spectrophotometrically using Folin-Ciocalteu reagent as described by Julkunen-Titto (1985) and Weurman and Swain (1955). Optimum extraction time that rendered the highest antioxidant activity was chosen.

Mulberry green tea water extract was prepared under optimum extracting condition. The extracts were freeze-dried to obtain the antioxidant powder.

3.2 Effect of extracting solvents and extracting times

3.2.1 Screening for optimum extracting solvents

The various solvents including methanol, ethanol, ethyl acetate and hexane were used for extracting antioxidants from selected mulberry green tea. The mulberry green tea powder was mixed with various solvents by using a sample/solvent ratio of 1:20 (w/v) and shaken at 300 rpm for 2 h at room temperature (26-28 °C). The extracts were evaluated for antioxidant activity, total phenolic content and reducing power according to the method of Taga *et al.* (1984), Weurman and Swain (1955) and Oyaizu (1986), respectively. The solvent rendering the highest antioxidant activity was chosen for further studies.

3.2.2 Effect of extracting times on selected solvent

Various extraction times (0.5, 1, 2, 3, 5, 8 and 10 h) was used to extract the antioxidants. The extracts obtained were evaluated for antioxidant activity by using β -carotene bleaching method (Taga *et al.*, 1984).

4. Some properties of the extracts from selected mulberry green tea

4.1 Effect of concentration on antioxidant activity

The antioxidant activity of both water and solvent mulberry green tea extract at different concentrations ranging from 50 to 1,500 ppm were tested in comparison with BHT, BHA and α -tocopherol at a level of 20 ppm. The antioxidant activity was determined using β -carotene bleaching method (Taga *et al.*, 1984).

4.2 Effect of pH on antioxidant activity

The influence of pH on antioxidant activity of mulberry green tea water extract and solvent extract was studied using 0.2 M phosphate buffer, pH 3, 5, 7, 9 and 11. Mulberry green tea water extract was dissolved in 0.2 M phosphate buffer using a extract/buffer ratio of 1:10 (w/v) while mulberry green tea ethyl acetate extract (evaporated form) was dissolved in methanol using a extract/methanol of 1:1000 (w/v) and a dissolved extract/buffer ratio of 1:10 (v/v) was used. The antioxidant activity was determined by β -carotene bleaching method (Taga *et al.*, 1984). To reduce interference on β -carotene caused by pH, the control was performed using the same buffer without addition of extract samples. The net absorbance was used to calculate antioxidant activity as shown in section 2.

4.3 pH stability

Mulberry green tea water extract was dissolved in 100 mM phosphate buffer at various pHs (3, 5, 7, 9 and 11) using a extract/buffer ratio of 1:10 (w/v). For mulberry green tea ethyl acetate extract (evaporated form), it was dissolved in methanol using a extract/methanol of 1:1000 (w/v). This diluted extract was mixed with 100 mM phosphate buffer with various pHs (3, 5, 7, 9 and 11) at a ratio of 1:10 (v/v). This mixtures were kept

at room temperature for 1 h. The samples (200 μ l) were then added with 0.2 M phosphate buffer at pH 7 (200 μ l) prior to analysis. The antioxidant activity was determined by β -carotene bleaching method (Taga *et al.*, 1984).

4.4 Synergistic effect

Chemical compounds, including ascorbic acid, citric acid and α -tocopherol at different concentrations (20 and 30 ppm) were used to study the synergism with the mulberry green tea extracts. Synergism of these compounds on antioxidant of the extracts was determined in an aqueous system by using β -carotene bleaching method (Taga *et al.*, 1984). Some factors affecting antioxidant activity including extract concentration (200 and 300 ppm) and type of chemical compounds (ascorbic acid, citric acid and α -tocopherol) were evaluated.

5. Mode of action

5.1 Scavenging effect on DPPH radicals

The radical-scavenging activity was measured from the reaction mixture containing 1,1-diphenyl-2-picrylhydrazyl (DPPH), used as a radical source according to the method of Blois (1958). Different concentrations of mulberry green tea extracts were tested, and the activity was compared with BHA, BHT and α -tocopherol at a level of 20 ppm.

5.2 Scavenging effect on hydroxyl radicals

The deoxyribose degradation caused by hydroxyl radicals was determined by the formation of TBARS (Aruoma, 1994). Different concentrations of mulberry green tea extracts were tested, and the activity was compared with BHT and α -tocopherol at a level of 20 ppm.

5.3 Reducing power

The reducing power of mulberry green tea extracts at different concentrations were measured according to the method of Oyaizu (1986). The reducing power of the extracts was compared with that of ascorbic acid at a level of 50 ppm.

6. Separation of antioxidative compounds in mulberry green tea ethyl acetate extract

Thin - Layer Chromatography (TLC) with silica gel plate (20x20 cm, Kieselgel 60 F, 0.20 mm thick, E.Merck) was used. An aliquot of ethyl acetate extract (20 μ L) was spotted on precoated which had been activated for 30 min at 100 °C. The plate was developed in the ascending direction for 10 cm with the solvent system benzene/ethyl formate/formic acid (75:24:1 v/v/v).

After separation, the chromatograms were dried and different sprays were used to identify chemical compounds as follows.

Spray 1: ferric chloride - potassium ferricyanide (equal volumes of 1% (w/v) aqueous solution of each salt: an orange-brown colored solution). Phenols react immediately to give blue color with this reagent (Barton *et al.*, 1952).

Spray 2: 2% solution of ferric chloride in ethanol indicated the presence of trihydroxy-phenolics by turning blue, dihydroxy-phenolics by turning green or other phenolics by turning red or brown (Reio, 1958).

Bands of interest were scraped from the plate. The silica gel residues which contained the separated compounds were soaked in 10 ml methanol for 30 min, filtered using Whatman No. 42 filter paper. Antioxidant was determined by using the coupled oxidation of β -carotene and linoleic acid assay (Taga *et al.*, 1984).

7. Application of mulberry green tea ethyl acetate extracts in lard and partially purified fish oil

The lard was purchased from local market in Hat Yai, Songkhla and the fish oil was obtained from CMC factory. After the lard was simmered and the fish oil was partially purified, ethyl acetate mulberry green tea extract was added into the lard and fish oil at concentrations of 100 and 200 ppm.

Lipid oxidation was monitored by determining TBARS (Esterbauer and Cheeseman, 1990) and peroxide value (IUPAC, 1979). Antioxidant activity of ethyl acetate mulberry green tea extract in the lard and the fish oil was compared with those of BHT and α -tocopherol at concentration of 200 ppm.

8. Application of mulberry green tea extract on inhibition of Low Density Lipoprotein (LDL) oxidation

8.1 Plasma preparation

Blood samples were obtained by venipuncture from overnight fasting healthy volunteers and collected into tubes containing EDTA at a level of 1 g/l as an anticoagulant and antioxidant. Plasma was prepared by centrifuging blood in an Eppendorf 5804 R by using swing-bucket rotor at 2,200 rpm for 7 min at room temperature.

8.2 Short-run ultracentrifugation for LDL isolation (Kleinvelde *et al.*, 1992)

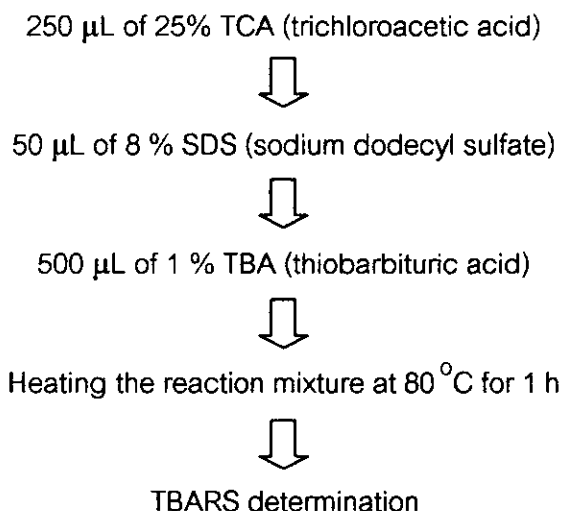
Lipoprotein fractions were separated by sequential ultracentrifugation technique. LDL was isolated by a short-run-ultracentrifugation (SRU) method based on nonequilibrium density-gradient ultracentrifugation. Plasma (0.9 mL) was adjusted to a density of 1.255 kg/L by adding 0.3465 g of KBr. Isotonic saline (5 mL; $d = 1.006$ kg/L) containing 0.1 kg/L EDTA, was layered on top plasma (1.8 mL) in a polycarbonate centrifuge tube. The tubes were centrifuges in a Beckman L8 – 70 M ultracentrifuge by using fixed-angle rotor at 30,000 rpm for 3 h. at 15 °C. After ultracentrifugation, the LDL-containing fraction, located in the center of the tube, was collected by aspiration. Further purification was achieved by a second ultracentrifugation step. EDTA (2 mL) containing saline (0.1 g/L) with density 1.10 kg/L (KBr 133.5 g/L) was layered on top of the LDL-containing fraction (4 mL) and was centrifuged at 30,000 rpm for 18 h at 15 °C. The LDL-containing top layer was aspirated and dialyzed in the dark for 48 h at 4 °C against 3 L of 0.01 mol/L phosphate buffer (pH 7.4). LDL obtained was used for further analysis. Protein in the lipoprotein fractions was determined by a modified method of

Lowry method (Markwell *et al.*, 1978). Protein content of LDL was adjusted to be 200 $\mu\text{g}/\text{ml}$ using 10 mM phosphate buffer saline (PBS) (pH 7.4).

8.3 The oxidation of Low Density Lipoprotein

In vitro oxidation of LDL was performed by using the procedure described by Williams *et al.* (1995) with a slight modification. To initiate the oxidation of LDL (200 $\mu\text{g}/\text{ml}$), a freshly prepared ascorbic acid (100 μM) and FeSO_4 (60 μM) were added. Then mulberry green tea extract (0.125 and 0.5 $\mu\text{g}/\text{ml}$) was added into reaction mixture and mixed thoroughly. The reaction mixture was immediately incubated in a temperature-controlled water bath at 37 $^{\circ}\text{C}$ for 6 h and the sample (200 μL) was taken every 30 min. The oxidation was then stopped by addition 50 μL of 2.1% EDTA and cooled at 4 $^{\circ}\text{C}$.

The degree of LDL-oxidation was monitored by measuring the production of TBARS as described by Asakawa and Matsushita (1980). Following reagents were added into the aliquots taken from the incubation mixture in the following order:



The TBARS was measured at excitation wavelength 532 nm. The calibration was done with a malondialdehyde (MDA) standard solution prepared in 50 mM H_2SO_4 . The value of TBARS was expressed as nmol MDA/mg LDL protein.