CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Butylated hydroxytoluene (BHT)	(Sigma)
2. Caffeine	(Sigma)
3. Chloroform	(Lab-Scan)
4. 1,1-diphenyl-2-picrylhydrazyl (DPPH)	(Sigma)
5. (-)-Epicatechin	(Sigma)
6. (-)-Epicatechin gallate	(Sigma)
7. (-)-Epigallocatechin	(Sigma)
8. (-)-Epigallocatechingallate	(Sigma)
9. Ethanol	(Merck)
10. Ethyl acetate	(Lab-Scan)
11. Ethylenediaminetetra acetic acid (EDTA)	(Merck)
12. Fetal bovine serum (FBS)	(Gibco)
13. Green tea (Japan)	(Shincha Ohashiri)
14. Green tea (Myanmar)	(Pinsali)
15. Green tea (Thailand)	(Choicest Tea)
16. Hydrochloric acid (HCl)	(Merck)
17. Melanoma cell line (A375)	(ATCC)
18. Methanol (HPLC grade)	(Lab-Scan)
19. Methyl thiazoletetrazolium (MTT)	(Sigma)
20. Penicillin G sodium	(Gibco)
21. Phosphate buffer saline (PBS)	(Gibco)
22. RPMI medium 1640	(Gibco)

23. Isopropanol (GR grade) (Merck)
24. Streptomycin (Gibco)
25. Sodium bicarbonate (Merck)
26. Total antioxidant status kit (Randox)
27. Triton X-100 (Sigma)
28. Trypsin (Gibco)
29. Water (HPLC grade) (Lab-Scan)

EQUIPMENTS

1. Analytical balance (BA2105) (Sartorius)
2. Autoclave oven (Hirayama)
3. Lamina airflow hood (Nuaire)
4. Brightline hemacytometer (0.100 MM. Deep) (Hausser)
5. Centrifuge (IEC)
6. CO_2 water-jacketed incubator (Nuaire)
7. Freeze dryer (Dura-dry)
8. High performance liquid chromatography (HPLC):
    - 8.1 Nova-Pak® C_{18}, 4 μm (3.9 X 150 mm) column (Waters)
    - 8.2 Sentry™ Guard column and universal holder (Waters)
    - 8.3 Pump (LC-3A) (Shimadzu)
    - 8.4 UV-visible detector (LDC 4100) (Shimadzu)
    - 8.5 Recorder-integrator (C-R1A) (Shimadzu)
9. Hot air oven (EYELA)
10. Inverted microscope (CK-30) (Olympus)
11. Microplate reader (Bio-Rad)
12. pH meter (Beckman)
13. UV spectrophotometer (UV-160A) (Shimadzu)

14. Sonicator	(Branson)
15. Water Bath	(Grant)
16. Vortex mixer	(Genie)
17. Rotary evaporator N-N series	(EYELA)

METHODS

1. Preparation of green tea extract

For the whole study, green tea polyphenols were extracted from dried green tea leaves of three different origins (Japan, Myanmar and Thailand). Extraction were performed by using aqueous-alcoholic extraction method (Agarwal *et al.*, 1992). Green tea extracts from three different sources were obtained as follow (see Figure 5). Briefly, 50 g of dried green tea leaves were grinded. Aqueous extract was obtained from the extraction of 50 g of powdered dried green leaves in 500 ml of distilled water for 30 min. at 80°C. Then ethanol extraction was carried out in 500 ml of 80% aqueous ethanol for 30 min. Both the extraction of aqueous and ethanol parts were repeated three times. Then two extracts were mixed together and the mixed solution was concentrated to half its volume by evaporation under vacuum using rotary evaporator. The concentrated solvent was extracted thrice with equal volume of chloroform to remove pigment and caffeine. Aqueous part was triple extracted with ethyl acetate. Then ethyl acetate part was concentrated under vacuum and reconstituted the residue with the small amount of distilled water and freeze-dried. The light-brown solid green tea extracts were obtained.

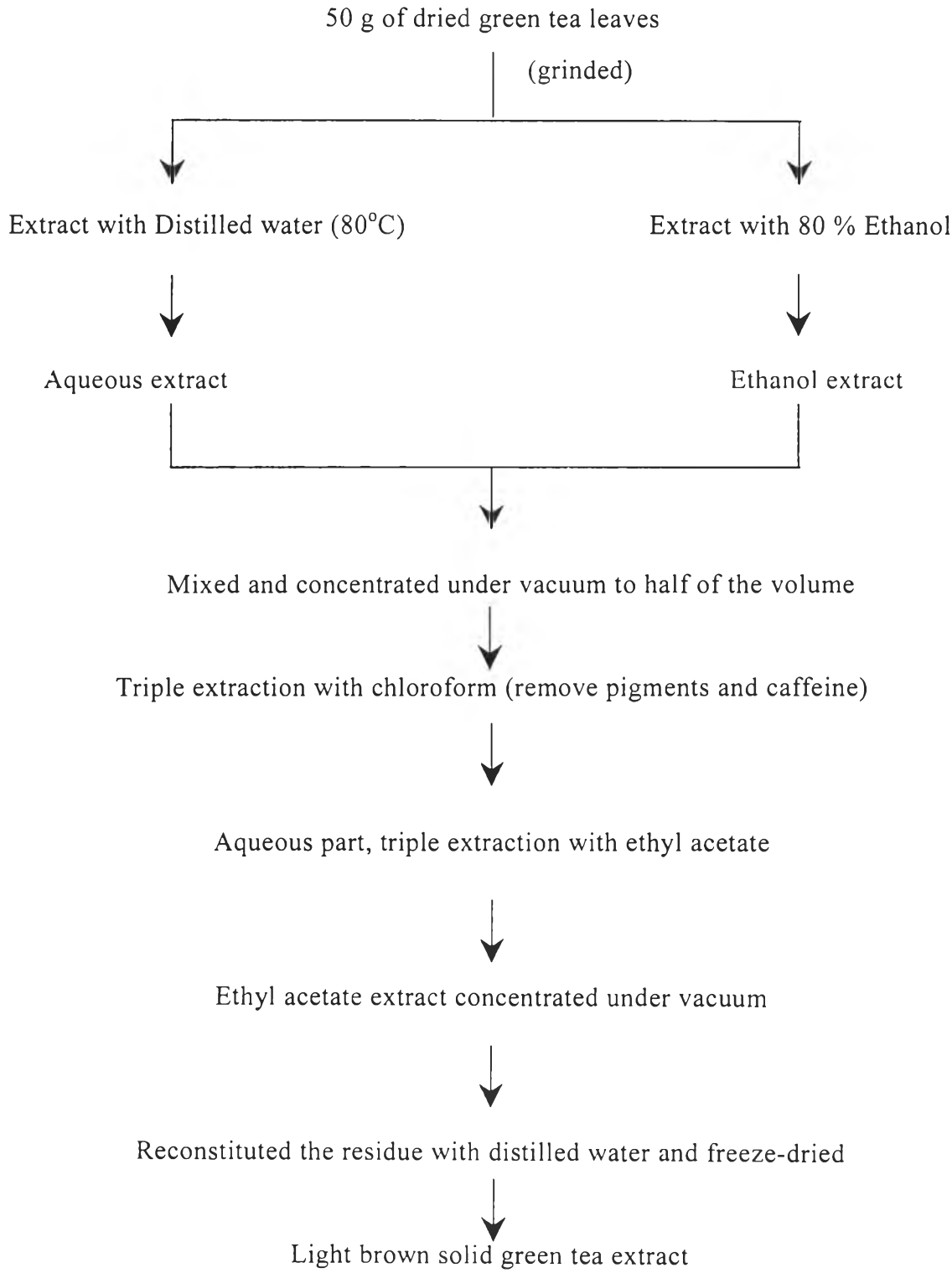

Figure 5. Schematic presentation of the extraction of the green tea polyphenols from dried green tea leaves.

2. Quantitative analysis of green tea polyphenols by using HPLC assay

Reverse phase high performance liquid chromatography (HPLC) assay was used for the analysis of the total polyphenols content in each of the green tea extracts, following the method used by Agarwal *et al.,* (1992) with some modification.

2.1 HPLC condition

HPLC column	:	Nova-Pack® C_{18} 3.9 x 150 mm Steel column
HPLC guard column	:	Waters Sentry™ guard column and guard holder
Mobile phase	:	water and methanol (HPLC grade)
Detector wave-length	:	280 nm
Flow rate	:	1.2 ml/min
Gradient set up	:	0 min. ; 100 % water,
		0 - 36 min. ; linear increase to 18% methanol,
		36 - 40 min.; 50 % methanol

2.2 Preparation of the mixed standard solution and green tea extracts

Stock solution was prepared by mixing 1mg each of the five standard samples (EGC, caffeine, EC, EGCG and ECG) in 10% aqueous methanol. Then the solution was adjusted to 5 ml in a volumetric flask with 10% aqueous methanol.

Green tea extract solutions were prepared at 1 mg/ml concentration with 10% aqueous methanol solution.

2.3 Analysis of total polyphenol content in greeen tea extracts

Total polyphenol contents (without caffeine) present in green tea extracts were expressed in terms of % yield (w/w) of the total dry solid powder.

3. To determine the radical scavenging activity of each green tea extract by DPPH assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay based on the scavenging of the stable DPPH radical was used to determine the free radical scavenging activity of the extract. The radical scavenging activity of each of green tea extracts was determined by reacting with the stable DPPH radical and their effectiveness were compared with the well known antioxidant; butylated hydroxytoluene (BHT). Quenching activity of each tested sample against DPPH radical was measured by using UV spectrophotometer, with the absorbance at 520 nm. The decrease in its characteristic wavelength during the reaction was monitored and found to be related to the reduction activity of DPPH.

3.1 Preparation of the test samples

DPPH solution was freshly prepared with the absolute ethanol ($6x10^{-5}$ M). Standard BHT solutions were prepared with absolute ethanol in different concentrations (5, 10, 15, and 20 μg/ml). Green tea extract solutions were prepared in different concentrations (1, 2, 3, 4 and 5 μg/ml) with distilled water.

3.2 Assay procedure

Firstly, DPPH solution (500 μl) was mixed in equal volume with standard BHT solution test and equal volume of DPPH solution and extract solution were mixed for green tea extracts measurement. As a control, DPPH solution (500 μl) and equal volume of absolute ethanol were used. Determination of DPPH concentration was carried out spectrometric by using the absorbance at 520 nm after 20 min. incubation at room temperature. The mixture of absolute ethanol and distilled water was used to correct background absorbance of each extract sample (Hatano *et al.*, 1989; Hu *et al.*, 2001; Robards *et al.*, 1999)

3.3 Calculation of inhibition percentage of BHT and green tea extracts

Green tea extracts were examined for their radical scavenging capacity with respect to the stable free radical DPPH. The radical scavenging activity was expressed in terms of % inhibition. The relative inhibition of the antioxidant against DPPH was calculated according to the following equation.

$$\% \text{ inhibition of the sample} = \frac{[A_{control} - A_{sample}]}{A_{control}} \times 100$$

Where, $A_{control}$ = the absorbance of the control

A_{sample} = the absorbance of the standard or test sample

Calculated % inhibition values from each test were plotted against its concentrations (μg/ml).

3.4 Comparison of the 50% inhibition concentration by BHT and three green tea extracts

The effective radical scavenging activity concentration on the DPPH radical at 50% inhibition was expressed in terms of EC_{50}. The EC_{50} (μg/ml) of BHT and three green tea extracts were calculated.

4. Total antioxidant activity assay

Total antioxidant activity for each extract was investigated by using commercialized Randox® total antioxidant status kit (Randox Laboratories Ltd, UK). This Randox kit provides an indication of overall antioxidant protection. The Randox kit includes (1) phosphate buffer saline, (2) chromogen; contain metmyoglobin and ABTS®, (3) substrate; H_2O_2 as in stabilized form and the

hydrogen-donating antioxidant (6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid) as a standard.

4.1 Assay principle

This assay is based on the interaction of 2,2'-azinobis[3 ethylbenzothiazo line-6-sulphate (ABTS) with the ferrylmyoglobin radical species, resulting in $ABTS^{®}$. Then $ABTS^{®}$ is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce a radical cation $ABTS^{®\cdot+}$. This has a relatively stable blue-green color at 600 nm and the absorbance is inversely proportional to the antioxidant capacity of the substance tested. Antioxidants in the added sample cause suppression of $ABTS^{®,+}$ formation (color production) to a degree which is proportional to their concentration. Both positive and negative controls were used (see Appendix IV) (Bright *et al.*, 1999; Hu *et al.*, 200; Zhang *et al.*, 1999). Trolox (water-soluble vitamin E) was used as standard.

4.2 Preparation of the solutions from the Randox® kit and green tea extracts

Preparation of the solutions for the assay was carried out according to Randox® kit instructions.

Reconstitute one vial of chromogen with 10 ml of buffer solution.

Reconstitute 1 ml of substrate with 1.5 ml of buffer.

Reconstitute one vial of standard with 1 ml of double deionized water.

Green tea extracts were freshly prepared with deionized water in the concentration of 50 µg/ml.

4.3 Assay procedure

All the measurements were carried out at 600 nm against air and automatic analyzer system was used.

For the blank reagent; firstly the absorbance (A_1) of the mixture of 20 µl of double deionized water and 1 ml of the chromogen solution was measured, and then substrate 200 µl was added. After 3 min., the absorbance (A_2) of the blank reagent was then measured.

For the standard sample; firstly the absorbance (A_1) of the mixture of 20 µl of standard solution and 1 ml of the chromogen solution was measured, and then substrate 200 µl was added. After 3 min., the absorbance (A_2) of the standard sample was measured.

For the extract sample; firstly the absorbance (A_1) of the mixture of 20 µl of test sample and 1 ml of the chromogen solution was measured, and then substrate 200 µl was added. After 3 min. and then the absorbance (A_2) of the test sample was measured.

4.4 Calculation of total antioxidant activity from the assay

ΔA of the sample, standard and blank was calculated as follows:

$A_2 - A_1$ = ΔA of sample / standard / blank

Where, A_1 = the absorbance before adding substrate

A_2 = the absorbance after adding substrate

The total antioxidant status was calculated as follows:

$$\text{Factor} = \frac{\text{Conc. of standard}}{(\Delta A \text{ blank} - \Delta A \text{ standard})}$$

Total antioxidant activity (mmol/l) = Factor X (ΔA blank - ΔA sample)

The total antioxidant activity of each sample was expressed in terms of mmol/l (TROLOX® UNITS – Trolox® is a water-soluble vitamin E analogue).

5. To compare the cytotoxicity on melanoma cell line by MTT assay

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to monitor the cytotoxicity of the extract samples on the melanoma cell line. This assay evidences the global viability of the cultured cell by measuring the metabolic activity of living cells via mitochondrial dehydrogenases. An increase or decrease in number of survival cell indicates the degree of cytotoxicity caused by the tested samples.

5.1 Assay principle

The main component MTT is a water-soluble pale yellow substrate. Active mitochondrial dehydrogenase of living cells cleaves the tetrazolium ring and converts the yellowish MTT to an insoluble purple formazan crystal. This conversion does not take place in dead cells. This water-insoluble formazan crystal can be solubilized by using MTT solubilization medium, and gave a homogenous blue solution, which is suitable for quantification by spectrophotometric means at 550 nm on microplate reader.

5.2 Cell culture preparation

Monolayer melanoma cell lines (A375) were obtained from ATTC and kept in liquid N_2 tank. Cells were thawed at room temperature and were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% antibiotic in cultured dish. Cells were incubated at 37°C in water-jacket incubator equilibrated with 5% CO_2 humidified incubator for 24 hours.

After removal of the supernatant medium from cultured dish, the cells were washed with PBS twice. Cultured attached cells were passaged by using trypsin EDTA solution to detach cells from the previous dish. Cells were transferred to sterile tube and centrifuged at 12,000 r.p.m for 5 min. Then supernatant medium was removed and new medium was added into the tube and mixed well. The cells were

counted on a hemacytometer and cell concentration of $10x10^4$ cells/well was seeded to obtain desirable 80-90% confluent cells in 24-well plate. The plate with cell suspension of 500 µl/well were cultured overnight at 37°C in CO_2 water-jacket incubator.

5.3 Preparation of the test sample

Green tea extract solutions were prepared by direct dissolution of it in the culture medium to make its concentrations to be 20, 40, 60, 80, 100, 200, 400, 600, 800 and 1000 µg/ml.

5.4 Assay procedure

80-90% confluent cells were obtained from cultured in 24-well plate. The cultured medium was washed out twice with phosphate buffer saline (PBS). Then cells were subjected to exposed with 500 µl/well of freshly prepared green tea extract solution in various concentrations and then followed by incubation for 24 hr., at 37°C with 5% CO_2 humidified incubator. Cells in the blank control well were cultured in culture medium without extracts.

After the removal of the supernatant fluid the cells were washed with PBS twice. Then 250 µl/well MTT solution (1.0 mg MTT per 10 ml cultured medium) was added. The cells were cultured for another 2 hrs. until purple crystals appeared. When the incubation was ended, MTT solubilization medium which includes triton, HCl and isopropanol (see Appendix V, 3), was added at 250 µl/well and mixed thorcughly by repeated pipetting up and down. After leaving the plate for another 2 hr., it was then transferred to 96-well plate and read the absorbance at 550 nm monitored (Lorea *et al.*, 1997, Zhu *et al.*, 2001).

The mixture of the MTT solution and solubilizing medium (yellow color) at the same condition was used as the blank sample.

5.5 Calculation of inhibition percentage on melanoma cell line by green tea extracts

The cytotoxicity was expressed in term of % inhibition on melanoma cell line from each tested sample by using the formula as follows:

$$\% \text{ inhibition of the sample} = \frac{[A_{control} - A_{sample}]}{A_{control}} \times 100$$

Where, $A_{control}$ = the absorbance of the control

A_{sample} = the absorbance of the standard or test sample

Calculated % inhibition values from each sample were plotted against its concentrations (μg/ml). The extract concentration, which gives 50% of growth inhibition, is represented as EC_{50}.

6. Statistical analysis among green tea extracts

Statistical comparison of the difference in total polyphenol content, the radical scavenging activity, the total antioxidant activity and the cytotoxicity among green tea extracts was made. One-way ANOVA was used to determine significance among different green tea extracts. Statistic data were expressed as mean ± SD. The differences were considered statistically at $p<0.05$.