CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Punica granatum fruits were collected from Mengzhi pomegranate garden, Yunnan, China. The fruit peels were dried at 50-60 ^{o}C in hot air oven for 24 hours, and reduced to coarse powder by using a grinder. Dried plant powder was kept in well-closed container at 4 °C.

3.1.2 Chemicals

All solvents for general purposes were commercial grade and redistilled prior to use. The solvent for HPLC analysis was HPLC grade from Merck, Germany. The chemicals for formulation study were kindly provided from the Department of Pharmaceutical Technology and Department of Pharmacognosy and Pharmaceutical botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. Silica gel precoated aluminium sheets, silica gel were perchased from Merck, Germany. Ellagic acid was purchased from Fluka, Switzerland. Quercetin was purchased from Sigma, Switzerland.

3.1.3 General instrumental equipment

Quantitative determination of active substances was performed using HPLC Agilent series 1100, U.S.A.

pH of cream was measured with pH meter, Orion Model 410A, U.S.A.

Viscosity was measured using viscometer, Brookfield dial reading model RVT, U.S.A.

Stability test was performed in Stability Chamber HI 150, Q. nic CO. LTD, Thailand.

3.2 Methods

3.2.1 Solvents for extraction

Dried powder of *P. granatum* fruit peel (100 g) was extracted by maceration with various organic solvents, including chloroform ($CHCl_3$), ethyl acetate (EtOAc), methanol (MeOH) and 70% acetone in water for 3 days (500 ml x3). The pooled extracts of the same solvent were concentrated *in vaccuo*, and subjected to evaluation of antioxidant activity.

3.2.2 Antioxidant activity assay

3.2.2.1 DPPH radical scavenging assay

The antioxidant activity of the extracts was evaluated by DPPH radical scavenging assay which was originally described by Blois (1958).

DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered as a stable radical because of the paramagnetism conferred by its odd electron (delocalization of the spare electron over the molecule as a whole). The solution (in absolute ethanol) appears as a deep violet colour and shows a strong absorption band at 520 nm. DPPH radical can accept an electron or hydrogen radical to become a stable diamagnetic molecule and has pale violet. If substance for testing antioxidant activity is mixed with DPPH solution and gives rise to pale violet, it suggests that this substance has antioxidant effect by mechanism of free radical scavenging activity. The following assay procedure was modified from those described by Blois (1958) and Yamasaki, *et al*. (1994).

1. Dissolved MeOH, $CHCl_3$ and EtOAc extracts in absolute ethanol and water extract in distilled water.
2. Diluted each sample for at least 5 concentrations (two-fold dilutions).
3. Prepared $6x10^{-5}$ M of DPPH in absolute ethanol.
4. Transferred 500 μl of each sample solution into an eppendorf tube. Each concentration was tested in triplicate.
5. Transferred 500 μl of DPPH solution to mix with sample solution.
6. Shake and stand at the room temperature for 30 minutes.

7. Measured absorbance at 520 nm, using a mixture of 500 µl sample solution and 500 µl absolute ethanol as blank.

8. Prepared standard solution and control in each experiment as follows:

   - **Control ethanol:** mixture of absolute ethanol (500 µl) and $6x10^{-5}$ M DPPH in absolute ethanol (500 µl); **blank:** absolute ethanol.

   - **Control water:** mixture of distilled water (500 µl) and $6x10^{-5}$ M DPPH in absolute ethanol (500 µl); **blank:** mixture of distilled water (500 µl) and absolute ethanol (500 µl).

9. Calculation of % inhibition.

$$\text{\% inhibition} = \frac{\text{OD control-OD sample}}{\text{OD control}} \times 100$$

10. Plotted dose-response curve between % inhibition and concentrations.

11. Linear regression analysis is carried out for calculating the effective concentration of sample required to scavenge DPPH radical by 50 % (ED_{50} value).

12. In each experiment quercetin, a well known natural antioxidant is used as the positive control.

3.2.2.2 β-carotene bleaching assay

Antioxidant activity of the extract was also determined using β-carotene bleaching test (Sacchetti *et al*., 2005) as follows:

1. Approximately 10 mg of β-carotene was dissolved in 10 ml of chloroform.
2. The β-carotene solution (0.2 ml) was transferred into a boiling flask containing linoleic acid (20 mg) and Tween 40 (200 mg).
3. Chloroform was removed using a rotary evaporator at 40 ^{o}C for 5 min and, to the residue; 50 ml of distilled water was added, slowly with vigorous agitation, to form an emulsion.

4. Five ml of the emulsion were added to a tube containing 0.2 ml of sample solution (conc. 10 μg/ml) and the absorbance was immediately measured at 470 nm against a blank, consisting of the emulsion without β-carotene.
5. The tubes were placed in a water bath at 50 ^{o}C and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60 minute period.
6. Control sample contained 10 μl of water instead of sample solution.
7. Quercetin was used as a positive reference.
8. The antioxidant activity was expressed as percentage inhibition with reference to the control after a 60 min incubation using the following equation:

$$AA\ (\%) = \frac{DR_C - DR_S}{DR_C} \times 100$$

where AA = antioxidant activity

DR_C = degradation rate of the control = [ln(*a*/*b*)/60]

DR_S = degradation rate in percentage of the sample = [ln(*a*/*b*)/60];

a = absorbance at time 0; *b* = absorbance at 60 min.

3.2.3 Determination of suitable solvent for fractionation

The methanolic extract was further fractionated by liquid-liquid extraction. The extracts (3 g) were suspended in distilled water (50 ml), and were separately extracted with EtOAc (50 ml x3), n-butanol (n-BuOH) (50 ml x3) and a mixture of n-BuOH and EtOAc (1:1) (50 ml x3). The pooled organic fractions were evaporated to dryness *in vacco*, and subjected to evaluation of antioxidant activity.

3.2.4 Determination of extraction method

Maceration and reflux methods were examined by consideration of the antioxidant activity (ED_{50}) and the extraction time.

3.2.4.1 Extraction by maceration method

Dried powder of the fruit peel (100 g) was macerated in methanol for 3 days (500 ml x3). The pooled extracts were then evaporated to dryness *in vaccuo*, and subjected to partition between ethyl acetate and distilled water. The pooled ethyl acetate fractions were evaporated to dryness *in vaccuo* and subjected to evaluation of antioxidant activity.

3.2.4.2 Extraction under reflux conditions

Dried powder of the fruit peel (100 g) was extracted with methanol (500 ml x2) under reflux conditions for 1 hour. The pooled extract was evaporated to dryness *in vaccuo*, and subjected to partition between ethyl acetate and distilled water. The pooled ethyl acetate fractions were evaporated to dryness *in vaccuo* and subjected to evaluation of antioxidant activity.

3.2.5 Preparation of the antioxidant fraction of pomegranate fruit peels

Dried powder of pomegranate fruit peels (1 kg) were extracted with methanol (5 L) under reflux conditions for 1 hour (x2). The pooled extract was evaporated to dryness *in vaccuo*, and subjected to partition between ethyl acetate and distilled water. The pooled ethyl acetate fractions were evaporated to dryness *in vaccuo* and subjected to evaluation of antioxidant activity.

3.2.6 Quantitative determination of ellagic acid

Quantitative analysis of ellagic acid in pomegranate fruit peel extract was performed using HPLC method that was already validated (Issuriya *et al*., 2007). The calibration curve of ellagic acid was established from the authentic compound at the concentration range between 0.0625 to 10 mg/ml. The linear equation of ellagic acid was $Y = 137.2x + 217.88$ ($r^2 = 0.9996$). The conditions of HPLC were described below. All samples were analyzed in triplicate.

HPLC conditions:

HPLC analysis was carried out using Agilent 1100 series equipped with Agilent 1100 series Phodiode-array detector (PDA) and autosampler. Separation was achieved at 25 ^{o}C on a TSK-gel ODS-80Tm (4.6 mm X 15 cm) column. The mobile phase consisted of methanol and 2% aqueous acetic acid, gradient elution from 40% methanol to 60% methanol in 15 min at a flow rate of 1 ml/min. The injection volume was 20 µl. The quantitation wavelength was set at 254 nm.

3.2.7 Determination of solubility

The extract was accurately weighed to 10 mg and place in a vessel of at least 100 ml capacity. The vessel was placed in a constant temperature device, maintained at room temperature. Various solvents (propylene glycol, glycerine, mineral oil, ethanol and water) were examined by adding of the strength prescribed in the monograph by increments of 10 µl, shaking frequently and vigorously for 10 minutes. The volume of solvent added were recorded when a clear solution was obtained. If the solution was becomes cloudy or undissolved, the sample was continuously added up to 10 ml. After addition of 10 ml of solvents and the sample remained undissolved, the experiment had to be repeated in a 100 ml volumetric flask. In case of solubility, the longer of time required to dissolve a substance (at least 24 hours) should be allowed (British Pharmacopoeia Commission, 2001).

Descriptive terms of solubility and approximate volume of solvents required to completely dissolve a solute (in milliliters per gram of solute) are drawn as follow.

Solubility term	**Volume of solvent required To dissolve 1 g of solute (ml)**
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1,000
Very slightly soluble	From 1,000 to 10,000
Practically insoluble	More than 10,000

The term partly soluble is used to describe a mixture of which only some of the components dissolve.

3.2.8 Stability test

3.2.8.1 Effect of light

The antioxidant fractions of pomegranate fruit peels were kept in well-closed containers and stored in stability chamber under light protection and light explose for a period of 60 days. The temperature of chamber was set constant at 45 ºC (±2 ºC) (จุไรรัตน์ รักวาทิน, 2538). Physical appearances of the fraction were observed weekly and the aliquot of each sample was determined for ellagic acid using HPLC assay. The experiments were performed in triplicate.

3.2.8.2 Effect of temperature

The antioxidant fractions of pomegranate fruit peels were kept in well-closed containers, protected from light. The extracts were then stored at 25±2 ºC and 45±2 ºC for a period of 60 days (จุไรรัตน์ รักวาทิน, 2538). Physical appearances of the fraction were observed weekly and the aliquot of each sample was determined for ellagic acid using HPLC assay. The experiments were performed in triplicate.

3.2.8.3 Effect of humidity

The antioxidant fractions of pomegranate fruit peels were kept in well-closed containers, protected from light. The extracts were then stored at 25±2°C under 75% relative humidity for a period of 60 days (จุไรรัตน์ รักวาทิน, 2538). Physical appearances of the fraction were observed weekly and the aliquot of each sample was determined for ellagic acid using HPLC assay. The experiments were performed in triplicate.

3.2.9 Preliminary formulation study and stability test

3.2.9.1 Formulation of cream bases

Five different cream bases were prepared. The compositions of the cream bases are shown in Table 3.1. All cream bases were prepared by beaker method (พิมพร ลีลาพรพิสิฐ, 2534). The ingredients in oil and water phases were accurately weighed and placed into two separated beakers. The oil phase ingredients were melted together in a water bath to 75 °C. Meanwhile, the water phase ingredients were warmed at 75 °C. Then the aqueous solution was slowly added, with constant stirring, to the oil phase mixture. The mixture was then slowly cooled with continuous stirring until the mixture was congealed at room temperature. The obtained cream base was packed in tight container and kept at room temperature.

3.2.9.2 Formulation of cream containing pomegranate extract

The antioxidant active fraction of *P. granatum* fruit peel was levigated using the selected cream base to make a smooth paste and added to cream base by geometric dilution method until mixing was complete. The pomegranate extract cream was formulated with two different concentrations, 0.5% and 1% w/w.

Table 3.1 The ingredients of the cream bases

Ingredients / **Formulation**	**Content (g)**				
	Rx1	**Rx2**	**Rx3**	**Rx4**	**Rx5**
Cetyl alcohol	-	-	2.0	-	1.0
Cetostearyl alcohol	2.0	-	5.0	-	-
Glyceryl monostearate SE	-	-	-	8.0	-
Glyceryl monostearate	4.0	8.0	1.0	-	1.0
Paraffin liquid USP XIX	-	-	-	11.0	-
Mineral oil	3.0	11.0	-	-	-
Propylene glycol	5.0	3.0	5.0	3.0	-
Stearic acid	-	5.0	-	5.5	1.5
Sodium citrate	-	-	0.2	-	-
Isopropyl palmitate	-	-	-	-	3.0
Triethanolamine	-	1.5	-	1.0	0.5
Petrolatum	-	-	-	-	2.0
Carbomer 934, 2% aqueous	-	-	-	-	35.0
Polysorbate 20	-	-	-	-	1.5
Dimethicone	-	-	-	-	2.0
Silicone oil	3.0	-	1.0	-	-
Polawax®	3.0	4.0	2.0	4.0	-
Veegum	1.0	-	-	-	-
Xanthan gum	0.5	-	-	-	-
Uniphen p-23	0.3	0.3	0.3	0.3	-
Paraben concentrate	-	-	-	-	1.0
Purified water qs to	100	100	100	100	100

3.2.9.3 Stability test

The heating and cooling test method (Rieger, 1976) was used for the stability determination of all cream bases. The cream bases were kept alternately at 4°C (48 h) and 45°C (48 h) for 8 cycles. The physical appearances of the cream bases such as color, smoothness and phase separation were observed before and after testing. The data were used to select the suitable cream base for preparing the pomegranate extract cream. For the formulations, the physical properties including viscosity and pH were measured before and after heating and cooling test (8 cycles). In addition, the content of ellagic acid and antioxidant activity of the pomegranate extract cream were determined.

3.2.9.4 Quantitative analysis of ellagic acid in pomegranate extract cream

The pomegranate extract cream (1.0 g) was dissolved in methanol (10 ml). The mixture was then centrifuged at 4°C, 4500 rpm for 10 minutes. The supernatant was filtered through membrane filter (0.45 micron) and subjected to quantitative determination of ellagic acid using HPLC as described in section 3.2.6.

3.2.10 Antioxidant activity evaluation of pomegranate-extract cream

Pomegranate extract cream (1.0 g) was dissolved in methanol (10 ml). Antioxidant activity of the cream was evaluated by DPPH as described in section 3.2.2.1. The assay was performed in triplicate.

3.2.11 Statistical analysis

The Statistic Package for Social Science (SPSS for windows) was used for data analysis. The data were analyzed and compared by t-test. The level of statistical significance was taken at *P*-value of less than 0.05.