

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Materials

##### 2.1.1 Plant material

Fresh calyxes of *Hibiscus sabdariffa* Linn. (Roselle) were obtained from Amphur Jana, Songkhla province, Thailand in April 2004. The specimen (No. SKP 1090819) was deposited in the herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

##### 2.1.2 Chemicals and reagents

###### 2.1.2.1 Antioxidant and total phenolic assay

Absolute ethanol, AR grade (Merck<sup>®</sup>, Darmstadt, Germany)

Ascorbic acid, AR grade (Riedel-deHaen<sup>®</sup>, Seelze, Germany)

Bovin brain extracts (Fluka<sup>®</sup>, Buchs, Switzerland)

*n*-Butanol (Merck<sup>®</sup>, Darmstadt, Germany)

Butylated hydroxytoluene, AR grade (Sigma<sup>®</sup>, St.Louis, USA)

1,1-diphenyl-2-picrylhydrazyl (Sigma<sup>®</sup>, St.Louis, USA)

Folin & Ciocalteu's phenol reagent (Fluka<sup>®</sup>, Buchs, Switzerland)

Ferric chloride, AR grade (Fluka<sup>®</sup>, Buchs, Switzerland)

Gallic acid, AR grade (Fluka<sup>®</sup>, Buchs, Switzerland)

Hydrochloric acid (Merck<sup>®</sup>, Darmstadt, Germany)

Propyl Gallate, AR grade (Fluka<sup>®</sup>, Buchs, Switzerland)

Sodium carbonate (Riedel-deHaen<sup>®</sup>, Seelze, Germany)

Sodium hydroxide, AR grade (Merck<sup>®</sup>, Darmstadt, Germany)

$\alpha$ -Tocopherol, AR grade (Fluka<sup>®</sup>, Buchs, Switzerland)

Thiobarbituric acid, AR grade (Fluka<sup>®</sup>, Buchs, Switzerland)

#### 2.1.2.2 Total anthocyanin assay

Acetic acid, glacial (Merck<sup>®</sup>, Darmstadt, Germany)

Boric acid (Merck<sup>®</sup>, Darmstadt, Germany)

Potassium chloride (Vidhyasom, BKK, Thailand)

Phosphoric acid, AR grade (Merck<sup>®</sup>, Darmstadt, Germany)

Sodium acetate-3 hydrate, AR grade (AnalaR<sup>®</sup>, Poole, England)

Sodium metabisulfite, AR grade (UNIVAR, NSW, Australia)

Sodium phosphate, monobasic, AR grade (Merck<sup>®</sup>, Darmstadt, Germany)

Sodium phosphate, dibasic, AR grade (Merck<sup>®</sup>, Darmstadt, Germany)

Sodium acetate, anhydrous, AR grade (AnalaR<sup>®</sup>, Poole, England)

Sodium hydroxide (Merck<sup>®</sup>, Darmstadt, Germany)

Sodium borate-10 hydrate (Merck<sup>®</sup>, Darmstadt, Germany)

#### 2.1.2.3 Formulation development

Ceteryl octanoate, Lanol 1688<sup>®</sup> (ADINOP Co., Ltd., BKK, Thailand)

Cetomacrogol 1000 (BASF, Ludwigshafen, USA)

Cetostearyl alcohol (Srichand Co., LTD, BKK, Thailand)

Cetyl alcohol (Vidhyasom, BKK, Thailand)

Cremophor A 25 (BASF, Ludwigshafen, USA)

Cremophor A 6 (BASF, Ludwigshafen, USA)

Disodium EDTA (AnalaR<sup>®</sup>, Poole, England)

Glyceryl monostearate, SE (Vidhyasom, BKK, Thailand)

Glycerin (Vidhyasom, BKK, Thailand)

Hard paraffin (Srichand Co., LTD, BKK, Thailand)

Isopropyl myristate (Vidhyasom, BKK, Thailand)

Mineral oil (Vidhyasom, BKK, Thailand)

Polyacrylamide/c 13, 14 Isoparaffin/Laureth-7-Seppic, Sepigel 305<sup>®</sup>  
(ADINOP Co., Ltd., BKK, Thailand)

Polyoxyethylene (20) sorbitan monooleate, Tween 80<sup>®</sup> (Srichand Co.,  
Ltd., BKK, Thailand)

Potassium sorbate (UNIVAR, NSW, Australia)

Stearic acid (Srichand Co., Ltd., BKK, Thailand)

Sodium metabisulfite, AR grade (UNIVAR, NSW, Australia)

Sorbitan monooleate (Span 80<sup>®</sup>, Srichand Co., Ltd., BKK, Thailand)

## 2.2 Instruments

Instrument	Model	Company
Microplate reader	PowerWave X	Bio-TEK Instruments Inc.
UV-visible spectrophotometer	Genesis 5	Miltonroy, USA
Hot air oven	DIN 12880-KI	Memmert, Germany
pH meter	PHM 82	Radiometer, Denmark
Water bath	WB-14	Memmert, Germany
Vortex	G-560E	Scientific, USA

## 2.3 Methods

### 2.3.1 Preparation of Roselle dried extract

The extraction was carried out by boiling fresh calyces of Roselle (5 kg) in water (30 L) for 15 minutes. The water extracts were filtered through nylon cloth and then dried using vacuum dry at 40°C for 8-10 hours. The yield (calculated on the dried extract) was 4.1% of the fresh Roselle calyces. Dried Roselle extract was packed in tight containers and kept in a desiccator at room temperature.

### 2.3.2 Antioxidant assays

#### 2.3.2.1 DPPH radical scavenging assay

The antioxidant activity of Roselle extract was determined according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method described by Yamasaki *et al.*, 1994. DPPH can be 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). DPPH is reduced when it reacts with an antioxidant

compound which can donate hydrogen (Miliauskas *et al.*, 2004). A solution of DPPH in absolute ethanol gives a deep violet color and provides a strong absorption band at 520 nm. After accepting an electron or hydrogen radical to become a stable diamagnetic molecule, its absorption band (at 520 nm) vanishes and the resulting decolorization is stoichiometric with the number of electrons taken up (Blois, 1958).

### Testing procedure and data analysis

A sample for testing was dissolved in water to obtain a stock solution (1 mg/mL). The sample was further diluted for at least four dilutions (2, 20, 100 and 200  $\mu\text{g/mL}$ ). A portion of sample solution (100  $\mu\text{L}$ ) was mixed to an equal volume of  $6 \times 10^{-5}$  M DPPH solution (in absolute ethanol) in 96-well plate. After 30 minutes at room temperature, the mixture was measured absorbance (A) at 520 nm by UV spectrophotometer. Each sample was done in triplicate. Butylated hydroxytoluene (BHT), ascorbic acid and  $\alpha$ -tocopherol, a commercial antioxidant, was used as a positive standard in the same system.

The free radical scavenging activity of each sample was determined corresponding to the intensity of quenching DPPH. The result was expressed as the percentage inhibition calculated as the following equation:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

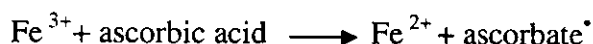
Where  $A_{\text{control}}$  : absorbance of DPPH solution without sample solution

$A_{\text{sample}}$  : absorbance of DPPH solution with sample solution

The value of EC<sub>50</sub> (effective concentration of sample requires scavenging DPPH radical by 50%) was obtained by linear regression analysis of dose response curve plotting between % inhibition and concentrations.

### 2.3.2.2 Lipid peroxidation (LPO) of liposome assay

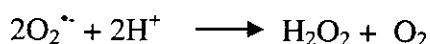
The antioxidant activity of Roselle extract was also evaluated by LPO of liposome assay described by Uchiyama and Mihara, 1978. Ascorbic acid with pro-oxidant property will play a key role in the initiation of the lipid peroxidation reaction in biological system. In the presence of a transition metal such as iron (Fe), it can reduce Fe<sup>3+</sup> to Fe<sup>2+</sup>:



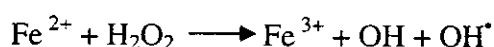
Fe<sup>2+</sup> will react with atmospheric oxygen giving rise to the formation of superoxide:



Then, superoxide can dismutate to form hydrogen peroxide:



Hydrogen peroxide can in turn react with Fe<sup>2+</sup> to form hydroxyl radical



These reactions caused the lipid peroxidation of liposome assay and are utilized to test for antioxidant behavior. The formation of hydroxyl radicals induces damage to liposome. However, if an antioxidant is added, it will scavenge free radicals and prevent this damage. The extent of liposome damage and hence the antioxidant efficacy of a test compound can be monitored and quantified with the aid of the thiobarbituric acid (TBA) test (Uchiyama and Mihara, 1978; Halliwell and Gutteridge, 1989).

The basis of TBA test is on the fact that peroxidation of most membrane systems leads to formation of small amounts of free malondialdehyde (MDA). MDA can further react with thiobarbituric acid and cause a pink colored product (Figure 3.1), which can be measured spectrophotometrically at 532 nm. The yield of colored product is proportional to the MDA formed, and thus to the extent of lipid peroxidation.

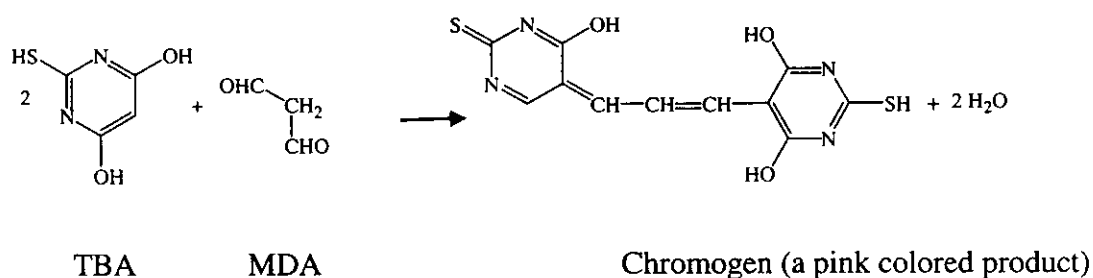


Figure 2.1 Reaction between TBA and MDA to produce a pink colored product.

Overall, lipid peroxidation can be quantified, as the protective effect of any compound that acts to prevent it from occurring (Halliwell and Gutteridge, 1989).

### Testing procedure

Roselle extract solution was prepared as a 10% w/v stock solution in water and then diluted to 1.0, 0.5, 0.1 and 0.01 mg/mL solutions. Liposomes were prepared from a bovine extract suspension in phosphate buffered saline (PBS; 5 mg/mL). The extract test reaction mixture consisted of 0.2 mL of liposomes, 0.1 mL FeCl<sub>3</sub>, 0.1 mL ascorbic acid (1 mM), 0.5 mL PBS and 0.1 mL of each test extract solution. Propyl gallate ( $1 \times 10^{-4}$  M) is used as positive control. All test tubes were incubated at 37 °C for 20 minutes. The LPO of liposomes should occur within the incubation period, unless the test substance exerted a protective antioxidant effect. For a control, the extract was omitted and solvent (water) was added instead to form the so-called full reaction mixture (FRM). The absorbance of liposome suspension alone was read after incubation in case it self-peroxidised during the incubation period. In addition, the extract alone was also assayed for its absorbance reading to be taken in consideration at a later stage. The assay was approved by a positive test performed by adding 0.1 mL propyl gallate ( $1 \times 10^{-4}$  M), as antioxidant that should prevent lipid peroxidation. Three replicates were carried out for each mixture. The TBA test was performed after 20 minutes incubation by adding 0.1 mL of 2% BHT in ethanol (another antioxidant to prevent lipid peroxidation during the TBA test itself) followed by 0.5 mL of 1% w/v TBA in 50 mM NaOH and 0.5 mL of 25% HCl. The full reaction mixture was completed after adding the TBA reagents, heated to 85 °C for 30 minutes and then allowed to cool to room temperature. At the end of this incubation period, TBA should be formed a colored product with MDA. Since the lipid suspension was turbid and unsuitable for spectroscopic analysis, the chromogen was extracted into 2.5 mL butanol. The mixture was vortexed to ensure complete



extraction of the chromogen and then centrifuged at 3500 rpm for 20 minutes at room temperature in order to separate the two layers. The upper layer of each sample which contained the chromogen, was pipetted into a cuvette to measure the absorbance at 532 nm.

### **Data analysis**

The % inhibition of lipid peroxidation was assessed by comparing the absorbance of the full reaction mixtures with that of the extract test reaction mixtures.

The calculation can be carried out as follows:

$$\% \text{ inhibition} = ((\text{FRM}-\text{B})-(\text{ET}-\text{b}-\text{EA})) / (\text{FRM}-\text{B}) \times 100$$

Where; FRM :       absorbance of the full reaction mixture (liposome and iron source plus solvent without the test substance)

B :                 absorbance of the blank mixture (liposome only)

b :                 absorbance of butanol

ET :                absorbance of the extract test mixture (full reaction mixture plus test substance)

EA :                absorbance of the extract alone

The EC<sub>50</sub> value was again obtained by linear regression analysis of the dose response curve plotting % inhibition against concentration using prism program.

Mean and standard deviation (SD) values (n=3) of the experiments were calculated.

### 2.3.3 Total phenolic assay

The content of total phenolic compounds in Roselle extract was determined by the Folin & Ciocalteu's assay as described by Miliauskas *et al.* (2004) using gallic acid as a standard.

#### Testing procedure and data analysis

A solution (500  $\mu\text{g/mL}$ ) of Roselle extract was prepared in water. 100  $\mu\text{L}$  of this solution were then mixed with 500  $\mu\text{L}$  of Folin-Ciocalteu's phenol reagent (diluted ten-fold) and 400  $\mu\text{L}$  of sodium carbonate (75 g/L). After a 30 minute incubation period at room temperature, the absorption at 756 nm was measured. Each sample was assayed in triplicate. Mean and SD values of the experiments were calculated. The concentration of the total phenolic compounds in the test sample was interpreted from calibration curve of gallic acid.

Calibration curves were determined using five standard solutions of gallic acid, which covered a concentration range of 4, 8, 16, 20, 40, 80  $\mu\text{g/mL}$ . Absolute ethanol was used as a blank. Each concentration was tested in triplicate. The standard curve was constructed by plotting absorbance versus gallic acid concentration.

The contents of total phenolic in Roselle extract were expressed as milligrams of gallic acid equivalent (GAE) per gram of the dried extract and calculated by the following formula:

$$C = c \times (V/W)$$

Where; C : total phenolic contents (mg/g) of Roselle extract in GAE

c : concentration of gallic acid established from the calibration curve  
(mg/mL)

V : volume of extract (mL)

W : weight of dried Roselle extract (mg)

### 2.3.4 Total anthocyanin assay

Anthocyanin contents in the Roselle extract were determined using pH-differential method described by Gusti and Wrolstad, 2001. This method of analysis based on structural transformations of anthocyanin chromophore as a function of pH. As shown in Figure 2.2, the flavylium cation (colored oxonium form) is in the majority at pH 1.0 while the carbinol-pseudobase (colorless hemiketal form) predominates at pH 4.5. To determine total anthocyanin contents, the absorbance at pH 1.0 and 4.5 are measured at the maximal wavelength (520 nm) and at 700 nm for some colloidal material suspended in the samples.

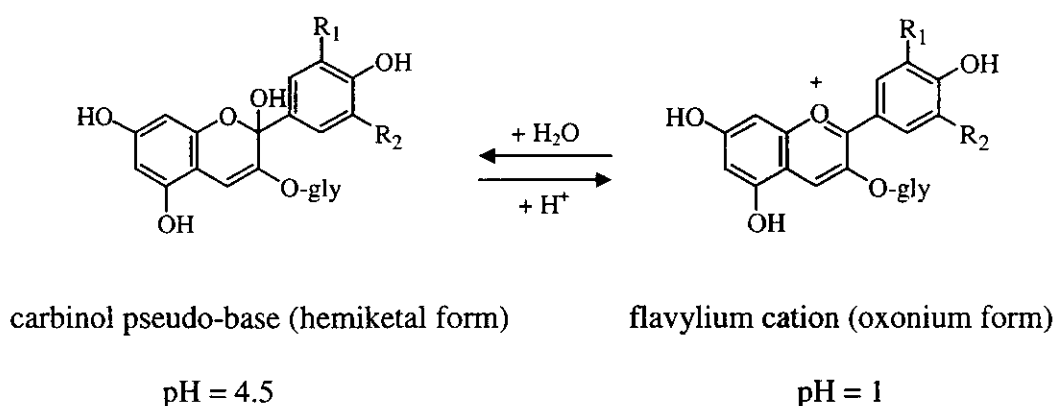


Figure 2.2 Structural transformation of anthocyanin chromophores as a function of pH.

### Testing procedure and data analysis

A stock solution (5 mg/mL) of Roselle extract was prepared in water. Two diluted solutions (1 mg/mL) were prepared from the stock solution. The first one was diluted with 0.025 M potassium chloride buffer pH 1.0, and the other was diluted with 0.4 M sodium acetate buffer pH 4.5. After equilibration for 15 minutes at room temperature, 200  $\mu$ l of each diluted sample were transferred to a 96-well plate and measured the UV absorbance at 520 and 700 nm, using distilled water as a blank. The experiment was performed in triplicate. The absorbance of the sample was calculated as follows:

$$A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$$

Where;  $A$  : absorbance of diluted sample

$A_{520}$  : absorbance of diluted sample at 520 nm

$A_{700}$  : absorbance of diluted sample at 700 nm

Total monomeric anthocyanins in dried Roselle extract were calculated as cyanidin-3-glucoside

$$\text{Monomeric anthocyanin pigment (mg/g)} = \frac{[(A \times MW \times DF \times 1000) / (\epsilon)]}{W}$$

Where;  $A$  : absorbance of diluted sample

$MW$  : molecular weight of cyanidin-3-glucoside (449.2 g/mol)

$DF$  : dilution factor

$\epsilon$  : the molar absorptivity of cyanidin-3-glucoside (26,900 mol/L)

$W$  : weight of dried Roselle extract (g)

## **2.3.5 Preformulation study**

### **2.3.5.1 Stability of Roselle extract in aqueous solution**

#### **a. Influence of pH on color and UV spectra of Roselle extract**

##### **Preparation**

Buffer solutions ranging in pH from 1 to 10 were prepared using acetate buffers (pH 4-5), phosphate buffers (pH 1-3 and pH 6-8) and borate buffers (pH 9-10). Buffer concentrations were 0.03 M. A constant ionic strength of 0.1 was maintained by adding an appropriate amount of sodium chloride (Martin *et al.*, 1993).

##### **Testing procedure**

A stock solution of Roselle extract (5 mg/mL) was prepared in water. Test solutions were prepared by diluting the stock solution with each buffer solution to obtain a final concentration of 1 mg/mL. The UV spectra of the resulting solutions were determined using water as a blank. The UV scan was performed at the wavelength from 400 to 700 nm.

#### **b. Effect of pH on colorant stability and antioxidant activity**

Roselle extract solutions of 1 mg/mL were prepared in three different buffer systems (acetate buffer pH 5 and phosphate buffer pH 2 and pH 7). The accurate pH of each test solution was measured using a pH meter. All test solutions were sealed in clear glass ampoules and stored in a refrigerator (8-10 °C) for about 1 month. At suitable time intervals (0, 7, 13, 21, 28, 35 days), the samples were withdrawn. Then, their absorbances at the maximal wavelength of 520 nm were assessed along with the determination of antioxidant activities and total phenolics

using the methods described in section 2.3.2.1 and 2.3.3, respectively. All determinations were performed in triplicate at room temperature.

### **2.3.5.2 Stability of Roselle extract in solid state**

#### **a. Moisture sorption**

Moisture sorption of dried Roselle extract powders was determined using procedure as described in Association of Official Analytical Chemistry International (A.O.A.C., 1999). A moisture dish and its tight-fit cover were dried at 70°C for 3 hours in hot air oven or until the constant weight was obtained. One gram of the dried powders was accurately weighed in a dried moisture dish and then kept in a desiccator at room temperature with 75% relative humidity (RH). At suitable time intervals (0.5, 1, 2, 4, 7, 16, 21, 24, 28, 48, 73, 120, 168, 195 hour), the sample was withdrawn and weighted. The experiment was done in triplicate. The percentage of moisture sorption of test sample was calculated using the following formula:

$$\% \text{ Moisture} = [(\text{final weight} - \text{initial weight}) / \text{initial weight}] \times 100$$

#### **b. Accelerated stability Testing**

Twenty-five milligrams of dried Roselle extract powders were weighed and placed in well capped glass bottles. All samples were kept in a desiccator protected from light at 45 °C and 75% RH for 4 months. At regular time intervals (0, 6, 15, 29, 46, 63, 90 and 126 day), three samples were withdrawn and cooled to room temperature. They were then reconstituted in water to obtain stock solutions of 5 mg/mL before analysis. The experiment was performed in triplicate. Antioxidant

capacities, total phenolics, as well as total monomeric anthocyanins and their pigment degradation or polymeric color were determined as following:

**(1) Antioxidant capacity**

Each stock solution was further diluted to obtain a test solution of 0.1 mg/mL and then its antioxidant activity were determined using the DPPH radical scavenging assay as previously described in section 2.3.2.1.

**(2) Total phenolic content**

Test solutions of 0.5 mg/mL were prepared by diluting the stock solutions with water. Total phenolic contents were then determined in the same manner as previously described in section 2.3.3.

**(3) Total monomeric anthocyanins**

The contents of total monomeric anthocyanins were determined from the above stock solution using the pH-differential method as described in section 2.3.4.

**(4) Pigment degradation (Polymeric color)**

Pigment degradation of anthocyanins was determined in term of polymeric color using the subtractive method. This method is based on the use of bleaching agents that will discolor anthocyanins but not affect other interfering materials Monomeric anthocyanins are readily bleached by bleaching agents such as bisulfite, polymeric anthocyanin are resistant and will remain colored(Giusti and

Wrolstad, 2001). The absorbance of bisulfite-treated samples can be measured at 420 nm. The absorbance of anthocyanins and some colloidal materials suspended in the samples were obtained at 520 and 700, respectively. Color density is the sum of the absorbance at 520 nm and at 420 nm of the control sample, while polymeric color is the result from a bisulfite-treated sample.

### Testing procedure and data analysis

Each stock solution was further diluted with 0.025 M potassium chloride buffer solution pH 1.0 to obtain a test solution of 1 mg/mL. The bisulfite bleached sample was performed by mixing 1.4 mL of the test solution with 0.1 mL of 20% sodium metabisulfite solution. For control sample, a 1.4 mL of test solution was mixed with 0.1 mL of distilled water. The absorbances of both samples were measured at 420, 520 and 700 nm after equilibration for 15 minutes at room temperature, using distilled water as a blank. The experiments were performed in triplicate.

Color density was calculated from the absorbance of the control sample as the following formula:

$$\text{Color density} = \left[ (A_{420nm} - A_{700nm}) + (A_{520nm} - A_{700nm}) \right] \times DF$$

Polymeric color was calculated from the absorbance of the bisulfite bleached sample as the following formula:

$$\text{Polymeric color} = \left[ (A_{420nm} - A_{700nm}) + (A_{520nm} - A_{700nm}) \right] \times DF$$



Where;  $A_{420\text{ nm}}$  : absorbance of sample at 420 nm

$A_{520\text{ nm}}$  : absorbance of sample at 520 nm

$A_{700\text{ nm}}$  : absorbance of sample at 700 nm

$DF$  : dilution factor

Percentage of polymeric color was then calculated using the following formula:

$$\% \text{ Polymeric color} = (\text{polymeric color} / \text{color density}) \times 100$$

### **2.3.6 Preliminary study for development of cream containing Roselle extract**

#### **2.3.6.1 Formulation of cream base**

Oil in water emulsion cream bases were formulated with different materials and emulsifying agents as shown in Table 2.1. All cream bases were prepared by fusion method. Each material was accurately weighed and placed into two separate beakers, one for the oil phase and the other one for the water phase. Then, they were heated to about 70-75 °C using a water bath. Once the desired temperature was reached, the contents from the water phase beaker were slowly added to the oil phase beaker with constant stirring. The mixture was stirred until it congealed at room temperature. All cream bases were evaluated under a heating and cooling cycle tests (45 °C, 24 hr – 4 °C, 24 hr; 6 cycles). Their physical appearances including color, smoothness and phase separation were observed before and after the stability testing. Cream bases with good appearances and promising stability were then selected for developing Roselle extract creams.

Table 2.1 Ingredients of the cream bases.

Ingredients	Content (g)			
	Rx1	Rx2	Rx3	Rx4
Cetyl alcohol	-	2	-	4
Stearic acid	-	2	-	-
Cetostearyl alcohol	-	-	5	-
Hard paraffin	-	-	9	-
Glyceryl monostearate	-	3.8	-	-
Isopropyl myristate	-	2	4	-
Cremophor A6	-	1.8	-	-
Cremophor A25	-	1.8	-	-
Cetomacrogol 1000	-	-	4	-
Sepigel	10	-	-	-
Tween 80	-	-	-	1.39
Span 80	-	-	-	5.61
Lanol 1688	12	-	-	-
Mineral oil	5	0.8	-	8
Glycerin	10	10	6	-
Disodium EDTA	0.1	0.1	0.1	0.1
Sodium metabisulfite	0.1	0.1	0.1	0.1
Potassium sorbate	0.2	0.2	0.2	0.2
Purified water to	100	100	100	100

### **2.3.6.2 Formulation of creams containing Roselle extract**

Creams of Roselle extract were prepared using suitable cream bases from section 2.3.6.1. Concentrations of the extract in cream formulations were varied from 5% to 20% in order to evaluate for color suitability. For preparation, the dried extract was added into the water phase and creams were prepared in the same manner as described above.

### **2.3.6.3 Stability evaluation**

Each of the cream formulations was evaluated for its physical stability under heating and cooling cycle tests (45 °C, 24 hr - 4° C, 24 hr; 6 cycles). The physical appearances of the cream formulations such as color, pH, smoothness and phase separation were observed before and after the stability testing. In addition, the antioxidant activity and monomeric anthocyanin contents of the cream formulation were determined. The experiments were performed in triplicate.

#### **Testing procedure**

One gram of each cream formulation was weighed and mixed with 10 mL of absolute ethanol. After 30 minute sonicating, it was centrifuged for 10 minutes. Then, the supernatant was collected and adjusted to 25 mL with absolute ethanol. This solution was subsequently diluted to a concentration of 0.1 mg/mL and determined for its antioxidant activity using the DPPH radical scavenging assay in the same method as described in section 2.3.2.1. Similarly, for monomeric anthocyanin assay, the solution was diluted to a concentration of 0.4 mg/mL and determined in the same manner described in section 2.3.4.

### 2.3.7 Statistical analysis

All experiment data were presented as mean  $\pm$  SD. The relationship between antioxidant activity from the DPPH assay and total monomeric anthocyanin contents were tested using linear regression analysis and presented in term of its correlation coefficient ( $r^2$ ). For stability testing, stability analysis was performed using Student's t-test, and the significant difference was set at  $p \leq 0.05$ .