CHAPTER 3

RESULTS AND DISCUSSION

3.1 Preparation of Roselle dried extract

Roselle was extracted by boiling their fresh calyxes in water for 15 minutes and then dried using vacuum dry at 40 °C for 8-10 hours. The yield (based on the dried extract) was 4.1% by weight of fresh calyxes. Figure 3.1 illustrates the dried extract of Roselle. They were attractively red in color with a typical odor of Roselle. After leaving uncovered for 1 hr, the powders were hygroscopic leading to powder aggregating and loss of their flow ability. The dried extract was packed in tight containers and kept in desiccator at room temperature.

Figure 3.1 Dried Roselle extract

3.2 Antioxidant activities of Roselle extract

The antioxidant activity of Roselle extract was determined using two different methods, the DPPH radical scavenging assay and the lipid peroxidation (LPO) of liposome assay. The DDPH assay has been used for rapid chemical

screening and guidance for antioxidant activity since it is simple and reproducible (Katsube *et al.*, 2004). However, it is not able to evaluate the capability of physiological antioxidant. In other words, the LPO of liposome assay has been modeled for more physiological significance (Caillet *et al.*, 2005). In this assay, antioxidant activity was measured by inhibition of lipid peroxidation in liposome. The results are summarized in table 3.1.

Table 3.1 Comparative antioxidant activities of Roselle extract, BHT, ascorbic acid and α-tocopherol

Sample	**EC_{50} (μg/mL)**	
	DPPH assay	**LPO of liposome assay**
Roselle dried extract	8.45 ± 0.35	170.42 ± 39.94
BHT	9.14 ± 0.42	6.05 ± 0.22*
Ascorbic acid	3.19 ± 0.57*	-
α-Tocopherol	3.55 ± 0.27*	1.58 ± 0.58*

The values are Mean ± S.D. (n=3)

* $P<0.05$ vs. Roselle dried extract in the same assay (Student's *t*-test)

As shown in Table 3.1, the water extract of Roselle exhibited impressive antioxidant effect with DPPH assay. No significant difference was found between its antioxidant activity and that of BHT which is usually used as a standard synthetic antioxidant in the same system. However, it was less effective than commercial antioxidants such as ascorbic acid and α-tocopherol. The EC_{50} values have been reported in another study by Rathee *et al* .(2005) whose their EC_{50} values

found to be 5.32 ± 0.02 μg/mL in the DPPH assay and 3.73 ± 0.4 μg/mL in the LPO of rat liver mitochondria. The results of the DPPH assay indicated that Roselle achieves its antioxidant activity by acting as a free radical scavenger. Notice that the antioxidant activity of dried Roselle extract observed in our study was much better than that reported by Duh and Yen, 1997 where the water extract was previously decolorized by passing through the C_{18} cartridge before analysis. In their study, the decolorized extract at 5 mg/mL exhibited 49.9% inhibition of DPPH radicals. This clearly indicates that the red pigments of Roselle are the key factors for its antioxidant capacity.

Although Roselle extract showed good activity in the DPPH assay (EC_{50} 8.45 ± 0.35 μg/mL), it displayed remarkably low activity in inhibition of LPO of liposome (EC_{50} 170.42 ± 39.94 μg/mL). This activity was over 30 times less than that of BHT (EC_{50} 6.05 ± 0.22 μg/mL) and α-tocopherol (EC_{50} 1.58 ± 0.58 μg/mL). The significant higher potency of BHT and α-tocopherol might be due to their better lipophilicity. Most components in the water extract of Roselle are highly hydrophilic and, therefore, they are not likely to penetrate into the liposome resulting in less inhibition of LPO observed. However, contradictory results were found by Duh and Yen (1997) that 1 mg of the extract showed 62.8% inhibition of LPO of egg lecithin which was better than the action of 200 ppm of DL-α-tocopherol.

3.3 Total phenolic contents of Roselle extract

Since polyphenolics represent one of the major groups of compounds acting as primary antioxidant or free radical terminator in most plants, it was necessary to interpret their total amount in the Roselle extract. Color development using a Folin-Ciocalteu assay which is the generally preferred method for measuring phenolic contents was used in our study.

As shown in Figure 3.2, the standard calibration curve of gallic acid was linear over the range of concentrations used. The linear regression fit to the data was represented as $y = 0.0059x + 0.0031$ with the correlation coefficient (r^2) value of 0.9995. The content of phenolic compounds (mg/g) in Roselle dried extract, expressed as gallic acid equivalents (GAE) was found to be 50.1 ± 1.42 (n=6). This is higher than the previous value (23 mg/g dry weight) reported by Tsai *et al.* (2002). Moreover, it has been shown by Duh and Yen (1997) that the total phenolic contents of Roselle water extract was 14.4 mg/g of herb, but using catechin as a standard.

High linear correlation coefficient was observed between the total phenolic contents and the DPPH radical scavenging activity of Roselle ($r^2 = 0.9638$), as shown in Figure 3.3. This is similar to the result that Katsube *et al.* (2002) found by correlating the total phenolic contents and the DPPH radical scavenging activities of the aqueous-ethanol extracts of 52 kinds of edible plants ($r^2 = 0.969$). This implied that phenolic compounds are likely to contribute to the radical scavenging activity of Roselle extract and the DPPH radical scavenging assay can be credibly predicted on the basis of the Folin-Ciocalteu assay.

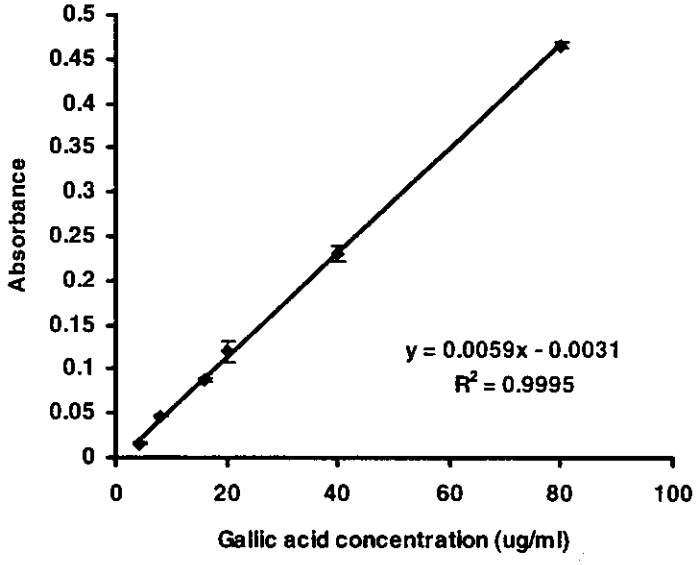


Figure 3.2 A standard calibration curve of gallic acid solutions. The plotted data are means ± SD (n=3). Solid line is a linear regression fit to the data.

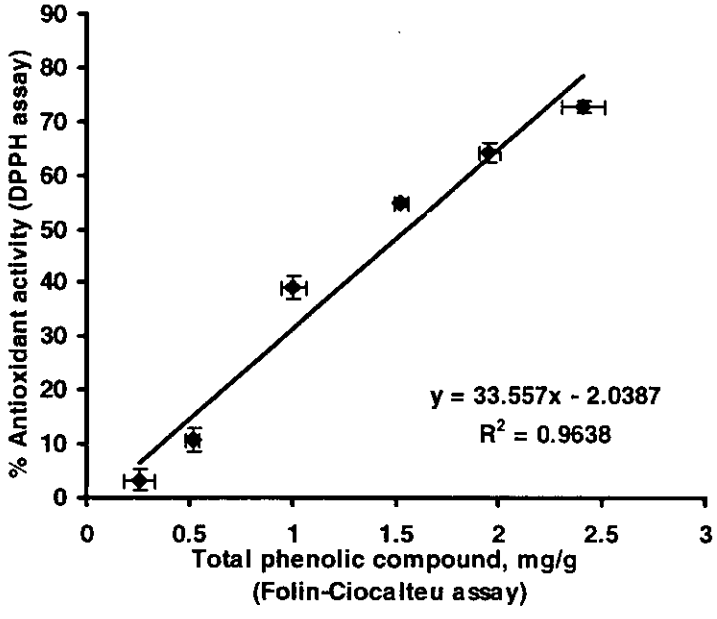


Figure 3.3 Correlation between total phenolic compounds (mg/g) of Roselle extract determined by Folin-Ciocalteu assay and its antioxidant activity determined by DPPH radical scavenging assay. The plotted data are means ± SD (n=3). Solid line is a linear regression fit to the data.

3.4 Quantitative determination of monomeric anthocyanins

The differential method was utilized for determining the amount of monomeric anthocyanins in Roselle extract. The absorbance of anthocyanin chromophores which rely on structure transformation at two different pH values was measured. Those are the colored oxonium form at pH 1.0 and the colorless hemiketal form at pH 4.5. This method has been described as fast and easy for quantitative determination of monomeric anthocyanins (Giusti and Wrolstad, 2001).

The content of total monomeric anthocyanin pigments, calculated as cyanidin-3-glucoside in mg/g of dried Roselle extract was found to be 3.46 ± 0.12 (n=6). A linear relationship between the monomeric anthocyanin content in Roselle extract and its DPPH activity was observed with the correlation coefficient (r^2) of 0.9525 as shown in Figure 3.4. This is better than the result that Tsai *et al.* (2002) found by correlating absorbance at 520 nm of Roselle extract with the ferric reducing of plasma (FRAP) assay (r^2 = 0.8375). This result again suggested that anthocyanins seem to be one of the major sources of antioxidant capacity in Roselle extract. Roselle with its higher anthocyanin content gives higher antioxidant activity.

The red pigment of Roselle was identified as delphinidine-3-sambubioside (85% of the anthocyanin) and the pink pigment was cyanidine-3-sambubioside (Tsai *et al.*, 2002; Tsai and Ou, 1996). In addition, it was found that these two pigments contributed to about 51% of the total FRAP capacity while the remainder of the activity (about 24%) was due to the phenolic brown pigments (Tsai *et al.*, 2002).

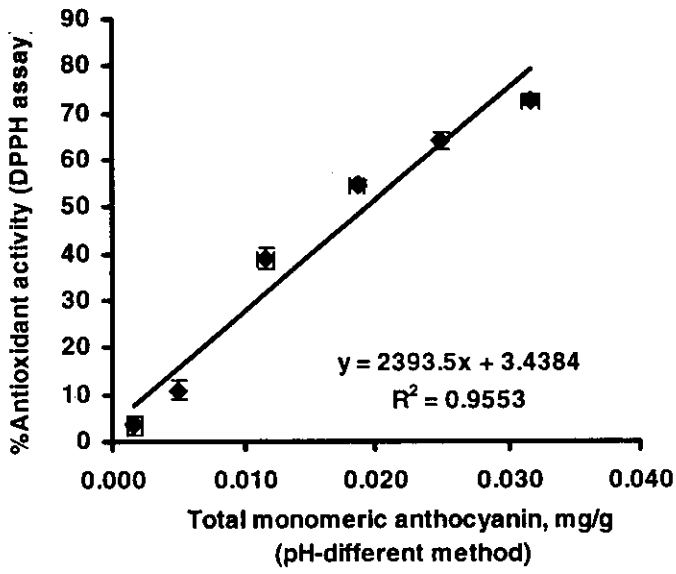


Figure 3.4 Correlation between total monomeric anthocyanins (mg/g) determined by pH-differential method and its antioxidant activity determined by DPPH radical scavenging assay. The plotted data are means ± SD (n=3). Solid line is a linear regression fit to the data.

3.5 Stability of Roselle extract in aqueous solutions

3.5.1 Effect of pH on color stability

The color of Roselle extract solutions with pH ranging from 1-9 were demonstrated in Figure 3.5. It was attractively pink-red up to pH 4 and then the red hue decreased from pH 4 to pH 6. However, this red shade can be returned upon acidification. Above pH 7, Roselle colorant gradually became toward more bluish as pH increased and finally turned to brown and yellow tone in alkaline pH. In an aqueous solution, common anthocyanins exist in equilibrium as a mixture of several structures including flavylium cation (red), quinonoidal anhydrobase (blue), carbinol

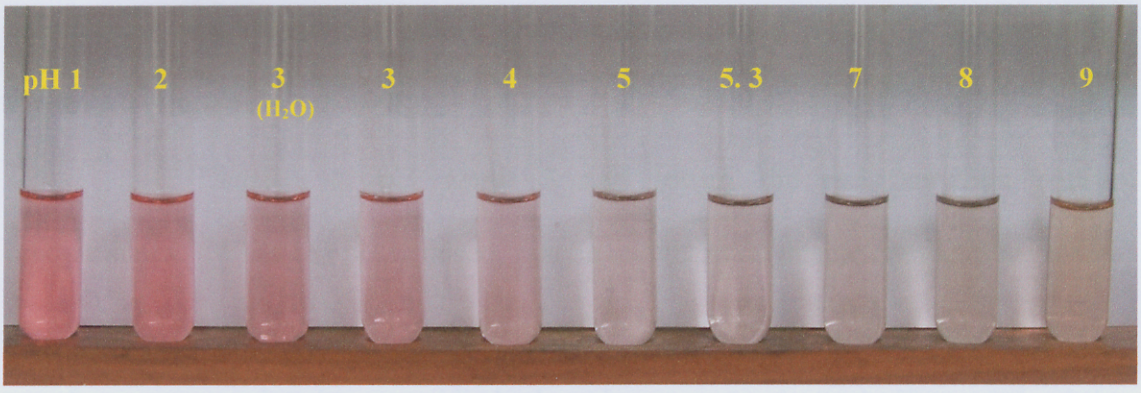
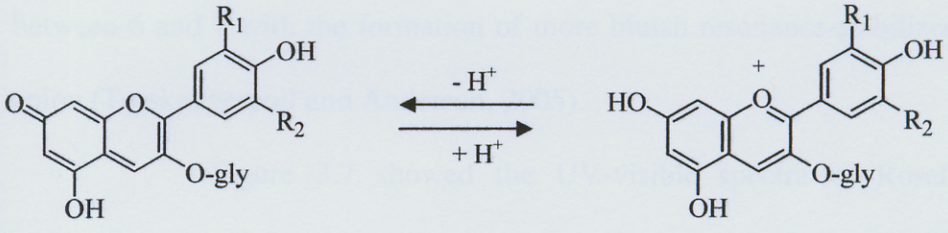


Figure 3.5 Color of Roselle extract at concentration of 1 mg/mL in aqueous solutions with different pH values

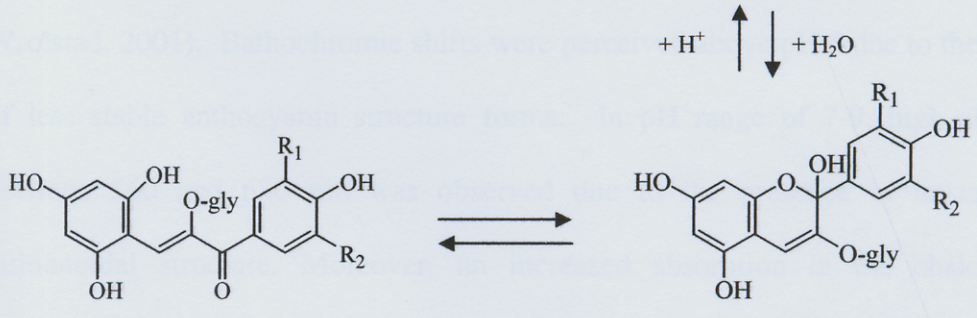


quinonoidal base: blue

pH = 7

flavylium cation (oxonium form): orange to purple

pH = 1



chalcone: colorless to yellow

pH = 4.5

carbinol pseudo-base (hemiketal form): colorless

pH = 4.5

Figure 3.6 Predominant structural forms of anthocyanins presented at different pHs

pseudo-base (colourless) and chalcone (colourless or light yellow) as illustrated in Figure 3.6 (Heredia *et al,* 1998).

Usually, at pH about 3 and lower, the red flavylium cation predominates. When the pH is raised, kinetic and thermodynamic competition occurs between the hydration reaction on position 2 of the flavylium cation and the proton transfer reactions related to its acidic hydroxyl group. As shown in Figure 3.6, while the first reaction gives a colourless carbinol pseudobase, which can undergo ring opening to yellow retro-chalcones, the latter reactions give rise to more violet quinonoidal bases. Further deprotonation of quinonoidal bases can take place at pH between 6 and 7 with the formation of more bluish resonance-stabilized quinonoidal anion (Torskangerpoll and Anderson, 2005).

Figure 3.7 showed the UV-visible spectra of Roselle extract at different pH. As can be seen, the absorbance readings decreased as pH increased up to pH 5. This indicated the decrease of red anthocyanin pigments which have a typical absorption band in 490 to 550 nm region of visible spectra (Giusti and Wrolstad, 2001). Bathochromic shifts were perceived above pH 5 due to the merging of less stable anthocyanin structure forms. In pH range of 7-9, high absorption between 560 and 630 nm was observed due to the presence of unstable blue quinonoidal structure. Moreover, an increased absorption in the chalcone and browning regions at 380 to 420 nm was detected (Cevallos-Casals and Cisneros-Zevallos, 2004).

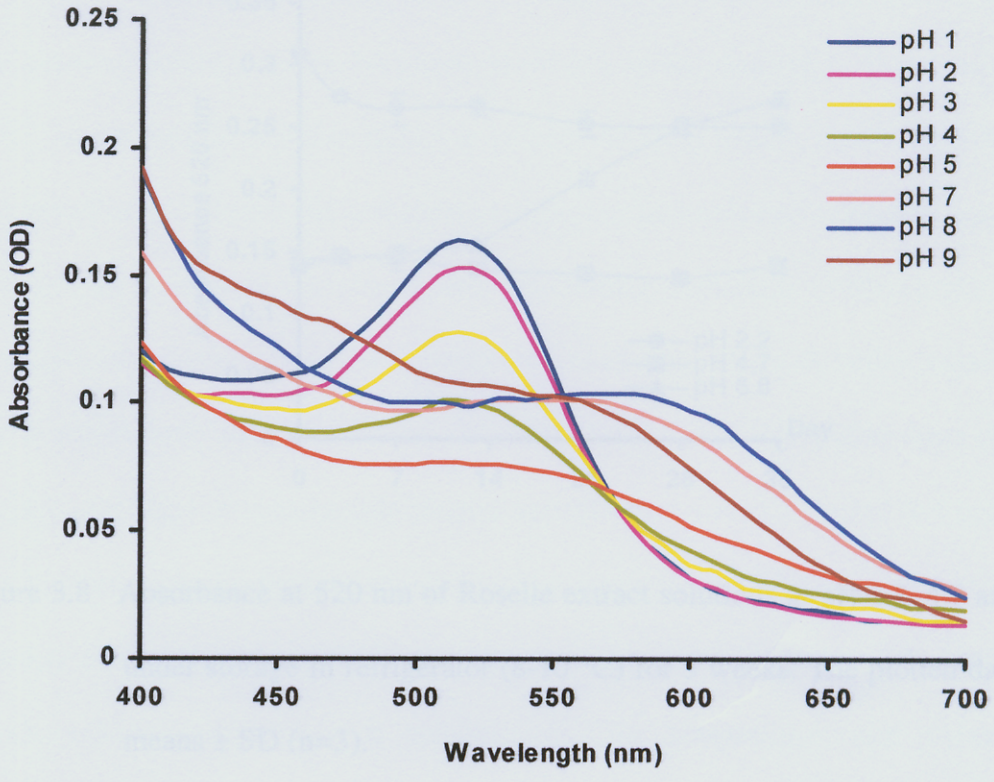


Figure 3.7 UV-visible spectra of Roselle extract at concentration of 1 mg/mL in aqueous solutions with different pH values.

The influence of pH on the color stability of Roselle extract was studied at pH 2.2, 4.7 and 6.8. The extract at pH 2.2 was selected due to its high color stability as described above. The extracts at pH 4.7 and 6.8 were studied to consider the usual pHs for developing dermatological preparations. All samples were kept in a refrigerator (8-10 °C) for 5 weeks. Figure 3.8 illustrated the changes in absorbance at λ_{max} (520 nm) as functions of pH and storage time. Since Roselle extract concentration in each solution was constant, these profiles, therefore, reflected the stability of red anthocyanin pigments. Absorbance of the extract at pH 2.2 decreased sharply during the first three days, followed by slower decreasing up to 35 days.

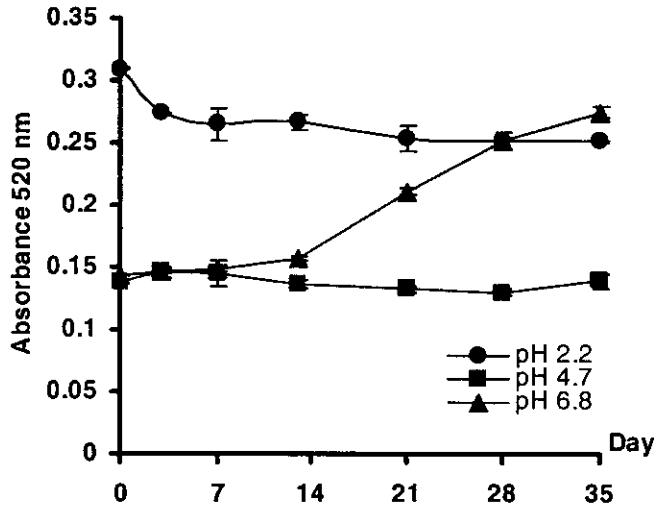


Figure 3.8 Absorbance at 520 nm of Roselle extract solutions at pH 2.2, 4.7 and 6.8 under storage in refrigerator (8-10 °C) for 5 weeks. The plotted data are means ± SD (n=3).

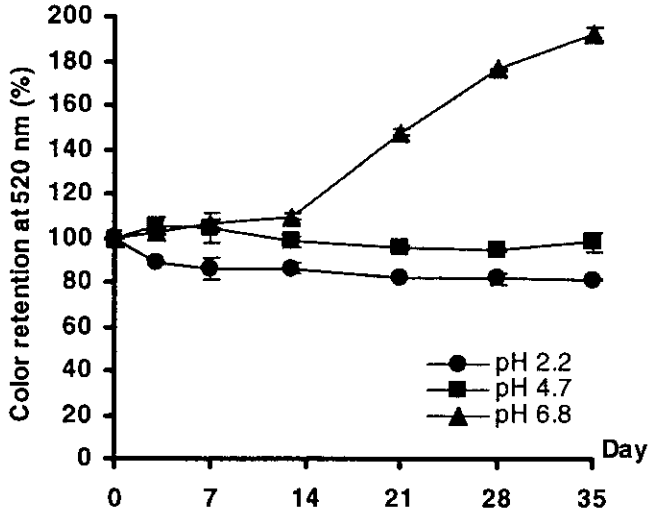


Figure 3.9 % Color retention of Roselle extract solutions at pH 2.2, 4.7 and 6.8 under storage in refrigerator (8-10 °C) for 5 weeks. The plotted data are means ± SD (n=3).

At pH 4.7, absorbance was slightly increased in the first week, and then retained within 5 weeks. However, at pH 6.8, an increased absorbance took place after 2 weeks and the solution turned to yellow. As these results, color degradation of anthocyanins at different pH and through time can be explained with three pathways including an increase in absorbance due to browning, a decrease in absorbance due to formation of a colourless carbinol pseudo-base, and the effect of bathochromic shift due to anthocyanin structure evolving into less stable forms. The percentages of color retention (at λ_{max}) of each sample plotted against times were illustrated in Figure 3.9. The highest color retention of the extract was observed at pH 4.7. In fact, the color stability of anthocyanin depends on a combination of various factors, such as the structure and concentration of anthocyanins, pH, temperature, and presence of complexing agents or copigments (Bakowska *et al.*, 2003). It has been reported that stability of anthocyanins can increase with intermolecular copigmentation (Bakowska *et al.*, 2003; Malien-Aubert *et al.*, 2001; Mazza and Brouillard, 1990). A copigment may be one of flavonoids, alkaloids, amino acids, organic acids, nucleotides, metals and anthocyanins themselves. These compounds have rich pi electrons, which are able to associate with the comparatively electron–poor flvylium ion. This association provides protection from the nucleophilic addition of water on the flavylium ion and, therefore, prevents the change of the flavylium ion into a colorless pseudobase, which consequently results in loss of color (Mazza and Brouillard, 1987; Bakowska *et al.*, 2003). Crude Roselle extract contains a mixture of different compounds that may serve as copigments for intermolecular association with anthocyanins. Therefore, copigmentation may play a significant role for color retention of roselle extract especially at pH 2.2 and 4.7. However, at higher pH (6.8), the browning reactions due

to the degradation of unstable quinonoidal bases predominated resulting in a change of solution color towards brown and yellow with time.

3.5.2 Effect of pH on antioxidant activity and total phenolic contents

The antioxidant capacity of Roselle extract is pH dependent. As illustrated in Figure 3.10, the free radical scavenging activity of the extract decreased initially from about 81% at pH 4.7 to 69% and 48% at pH 2.2 and 6.8, respectively. This is due to the best activities of the flavylium cation and carbinol pseudo-base as free radical scavengers compared to the resonance-stabilized quinonoidal base. However, through the storage time, the antioxidant activity of the extract at each pH did not significantly decrease at $p < 0.05$. These results indicated that antioxidant activity of Roselle extract seemed to relate to its amount of red anthocyanin pigments. However, its remaining activity at each pH value may due to the activity of other anthocyanin structure forms as well as their degradation products.

The effect of pH on total phenolic contents of Roselle extract was illustrated in Figure 3.11. Initially, the amounts of total phenolic in the extract at each pH were about the same (40.52, 39.31 and 40.07 mg/g at pH 2.2, 4.7 and 6.8, respectively). At pH 2.2 and 4.7, there were no significant changes at $p < 0.05$ in phenolic contents during storage whereas the contents substantially decreased at pH 6.8.

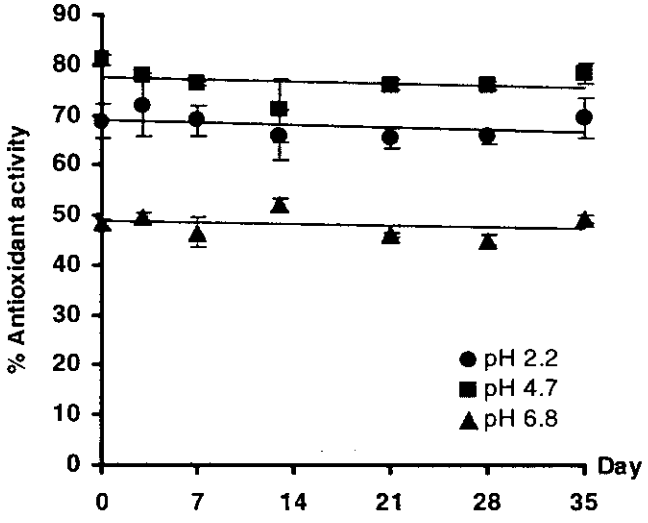


Figure 3.10 Effect of pH on antioxidant activity of Roselle extract solutions at pH 2.2, 4.7 and 6.8 under storage in refrigerator (8-10 °C) for 5 weeks. The plotted data are means ± SD (n=3).

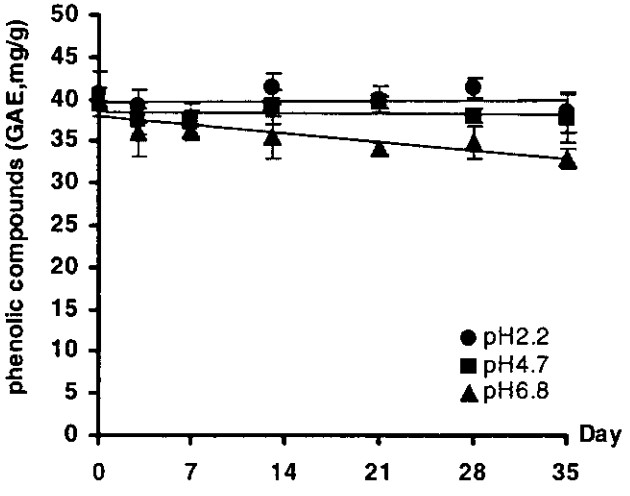


Figure 3.11 Effect of pH on total phenolic contents of Roselle extract solutions at pH 2.2, 4.7 and 6.8 under storage in refrigerator (8-10 °C) for 5 weeks. The plotted data are means ± SD (n=3).

3.6 Stability of Roselle extract dried powder

3.6.1 Moisture sorption

Dried Roselle extract absorbed moisture from the environment very rapidly. As shown in Figure 3.12, a sharp increase of moisture contents was observed in the first 50 hours, after that its increasing rate slowed down and then reached a plateau within 300 hours. The maximum moisture sorption of the extract determined by gravimetric method (AOAC, 1999) was found to be about 27 ± 0.4 % (w/w). In addition, it has been observed that, under high humidity, Roselle extract was hygroscopic, lost flow ability and finally turned to a hard cake with a dark brown color. These results indicate that moisture seems to affect the stability of Roselle extract in a dry state, especially for anthocyanin pigments. These are in agreement with the observation of Gradinaru *et al.* (2003) who showed freeze-dried anthocyanins from Roselle to be relatively stable in low moisture environments. Markakis *et al.* (1957) have postulated two hydrolytic mechanisms of degradation of anthocyanins at limit water concentration, one being hydrolysis of the glycosidic linkage to yield unstable aglycone and the other involving opening of the pyrilium ring to form a substituted chalcone and finally degradation products.

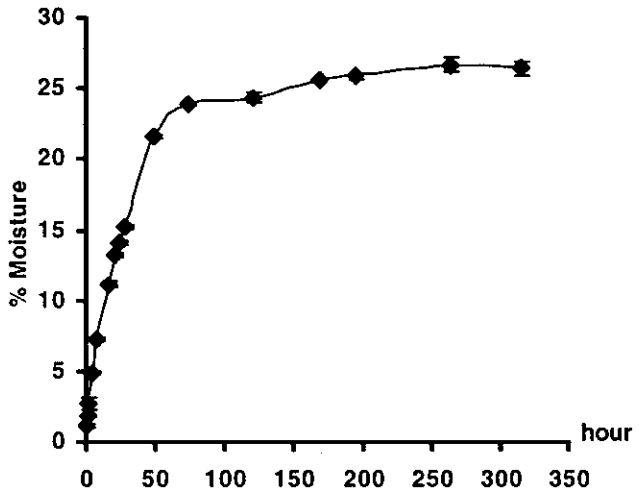


Figure 3.12 Moisture sorption profile of dried Roselle extract at room temperature and 75% RH. The plotted data are means ± SD (n=3).

3.6.2 Accelerated stability study

Solid state stabilities of dried Roselle extract were studied in terms of its antioxidant activity, total phenolics, total monomeric anthocyanins, and polymeric color. After storage under accelerated conditions (45 °C, 75% RH) for 4 months, the color of dried Roselle extract changed progressively from pink-red to dark brown and the overall loss of monomeric anthocyanins in the sample was observed. Figure 3.13 showed the linear plot of ln % monomeric anthocyanins in dried extract versus time which implies an apparent first order kinetic behavior. This observation is in good agreement with the findings of Gradinaru *et al.* (2003) where degradation of Roselle anthocyanins in both monomeric and co-pigmented forms followed first order kinetic at 40 °C and 75% RH. However, in our study, instead of a purified anthocyanin extract, the whole crude extract was used. At the same time, an increase of % polymeric color was detected in the sample as illustrated in Figure 3.14.

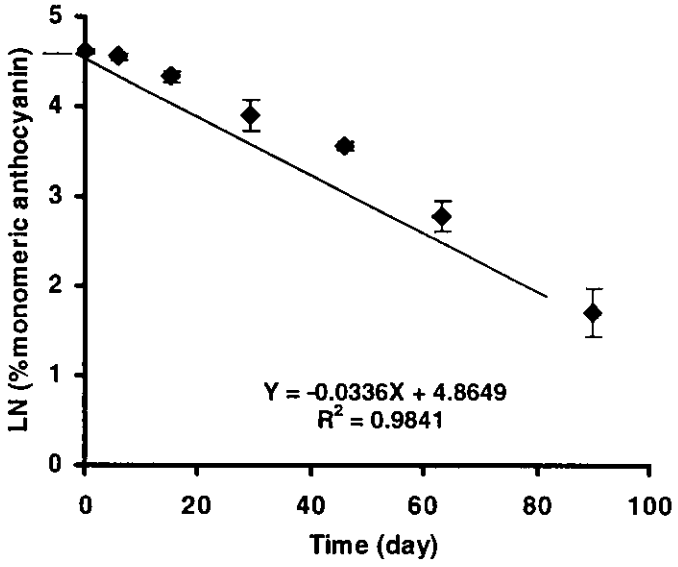


Figure 3.13 Apparent first order plot for the degradation of monomeric anthocyanins of dried Roselle extract (45 °C, 75% RH) indicating the initial lag time. The plotted data are means ± SD (n=3).

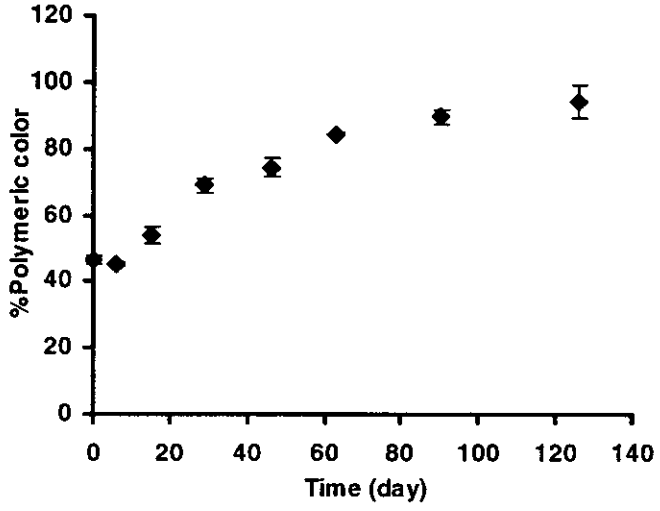


Figure 3.14 % Polymeric color occurred in dried Roselle extract (45 °C, 75% RH). The plotted data are means ± SD (n=3).

Generally, polymeric is an indicator of polymerized pigments including tannins and brown compounds (Cevallos-Casals and Cisneros-Zevallos, 2004). These results indicate that the degradation of monomeric anthocyanins may lead to the formation of brown polymeric compounds. Notice that, the initial lag times were observed in both the degradation profile of monomeric anthocyanins (Figure 3.13) and the incremental profile of polymeric color (Figure 3.14). These lag times indicate the times required for phase transformation of anthocyanins from solid to liquid before degradation occurred under the limited water activity environments. Moreover, it should be pointed out that there was an inversely proportional correlation between the content of monomeric anthocyanins and the polymeric color. High linear correlation coefficient was found ($r^2 = 0.9981$), as shown in Figure 3.15. In addition, the degradation index was examined as shown in Figure 3.16. The degradation index (DI) was obtained from the ratio between the absorbance reading at 420 nm (browning regions) and 520 nm (λ_{max}) of the reconstituted sample (Cevallos-Casals and Cisneros-Zevallos, 2004). Again, the more degradation the more polymeric color was observed in the sample. Although degradation of the dried extract was evidenced, there were no significant changes in its antioxidant capacity and total phenolic contents as illustrated in Figure 3.17 and 3.18, respectively. The DPPH scavenging activity of Roselle was still very high (over 90%) even in the totally brown samples. These results clearly indicated that the degradation products were active in the DPPH assay. In fact, it is reasonable to assume that these degradation products may be the primary cause of the increase in the radical scavenging ability of the degraded samples.

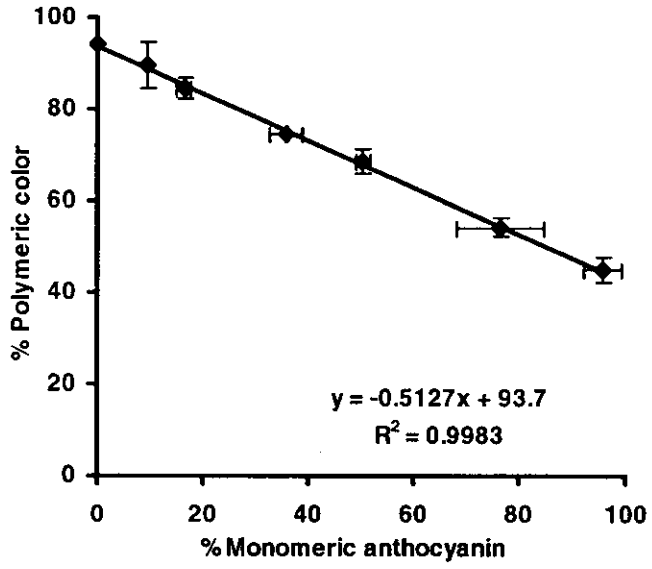


Figure 3.15 Inversely proportional correlation between % polymeric color and % monomeric anthocyanin contents in dried Roselle extract (45 °C, 75% RH). The plotted data are means ± SD (n=3). Solid line is a linear regression fit to the data.

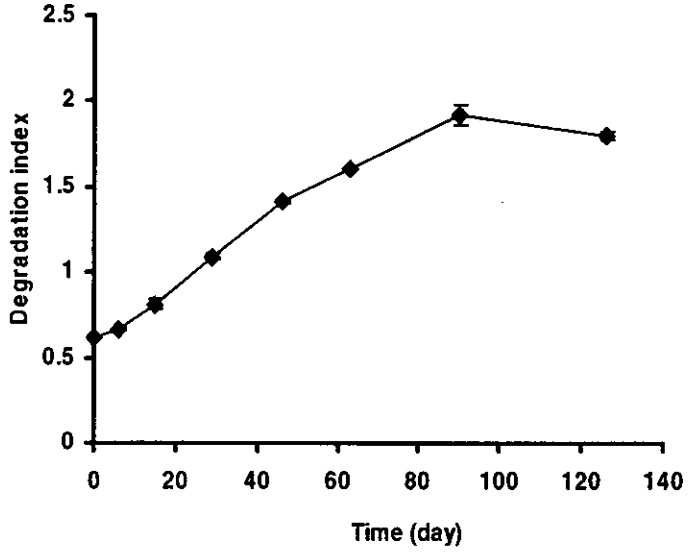


Figure 3.16 Degradation index (DI) of dried powder of Roselle extract (45 °C, 75% RH). The plotted data are means ± SD (n=3).

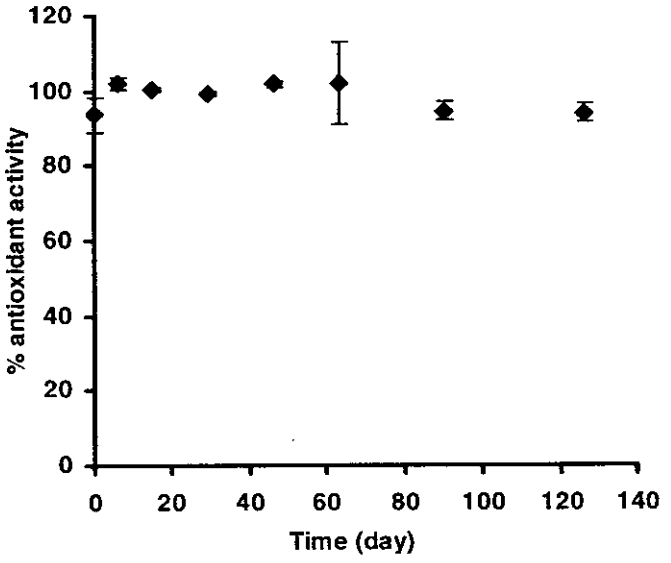


Figure 3.17 Antioxidant activity of dried powder of Roselle extract (45 °C, 75% RH). The plotted data are means ± SD (n=3).

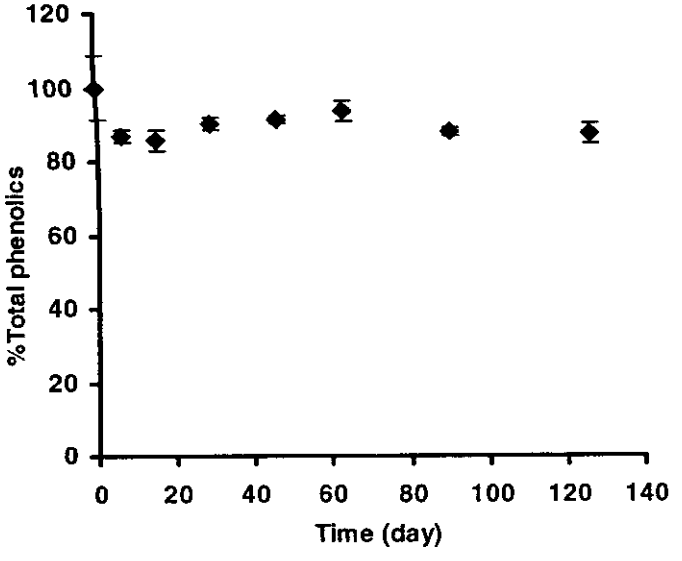


Figure 3.18 Total phenolic contents of dried powder of Roselle extract (45 °C, 75% RH). The plotted data are means ± SD (n=3).

3.7 Preliminary study for development of cream containing Roselle extract

Cream bases Rx 1 and Rx 2 were selected for developing creams containing Roselle extract because they showed better physical appearance in terms of color (white) and smoothness than Rx 3 and Rx4. In addition, they were physically stable after heating and cooling cycle test (6 cycles). In a preliminary study, Roselle extract was incorporated into a cream base at the concentrations of 5, 10, 15 and 20% w/w. The highest concentration of 20% w/w was chosen based on the regular concentration (5-20%) of vitamin C, a commercial antioxidant, in antioxidant creams. However, at this concentration, the color of cream was maroon and it was very rough and sticky, therefore not suitable for further formulation development. Color suitability was taken into account for selecting a suitable concentration of the extract in formulation.

As shown in Figure 3.19, the colors of creams containing 10 and 15 % w/w of the extract were still dark red and not quite suitable for skin preparations. However, at the concentration of 5% w/w of the extract, the cream formulation was pink-red in color and therefore may be acceptable as skin creams. In our study, the creams containing 5%w/w of the extract were prepared using cream bases Rx 1 and Rx 2 and used as formulation models for stability study. The physical appearances (color, smoothness, and phase separation) and pH of the cream formulations were examined before and after a heating and cooling cycle test. It has been found that both formulations seemed to be physically stable with no phase separation. However, increases of color intensity and pH changes were observed in both formulations as shown in Table 3.2. The pictures of 5% Roselle extract creams before and after the heating and cooling cycle tests are shown in Figure 3.20.

The antioxidant capacities and monomeric anthocyanin contents of the cream formulations were determined before and after stability testing. As given in Table 3.3, in both cream formulations, while the antioxidant activities were significantly increased, the monomeric anthocyanin contents were considerably decreased after the heating and cooling cycle tests. These results indicate the limit of the stability of anthocyanins in both cream bases. However, the increase of antioxidant capacities in the formulation was mainly due to the activity of degradation products occurring after the heating and cooling cycle test.

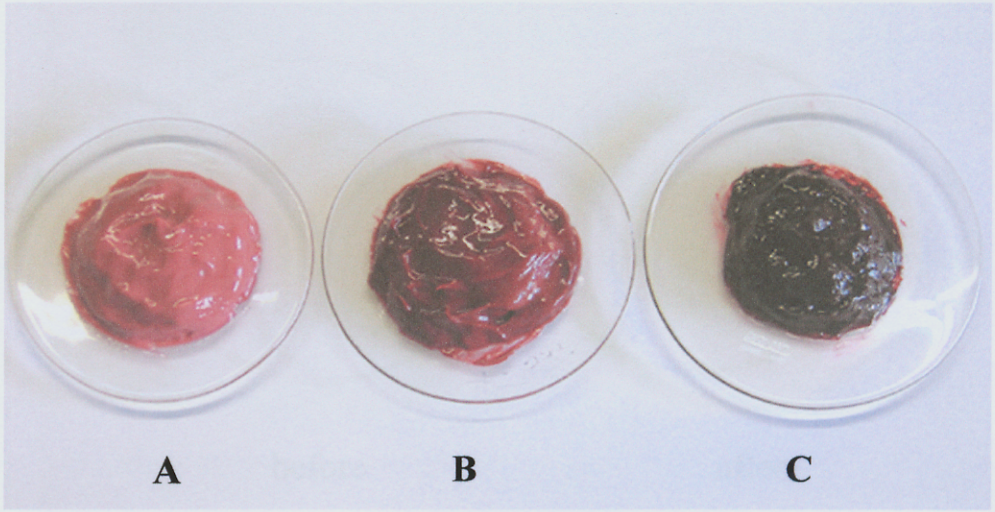


Figure 3.19 Formulation of cream containing Roselle extract (Rx1) at various concentrations: 5% (A), 10% (B) and 15% (C).

Table 3.2 Physical appearance of cream containing 5% of Roselle extract before and after heating and cooling cycle tests (6 cycles)

Physical properties	Rx1		Rx2	
	before	after	before	after
Color	red	dark Red	red	dark red
Phase separation	no	no	no	no
pH	2.69	2.99	2.72	2.68

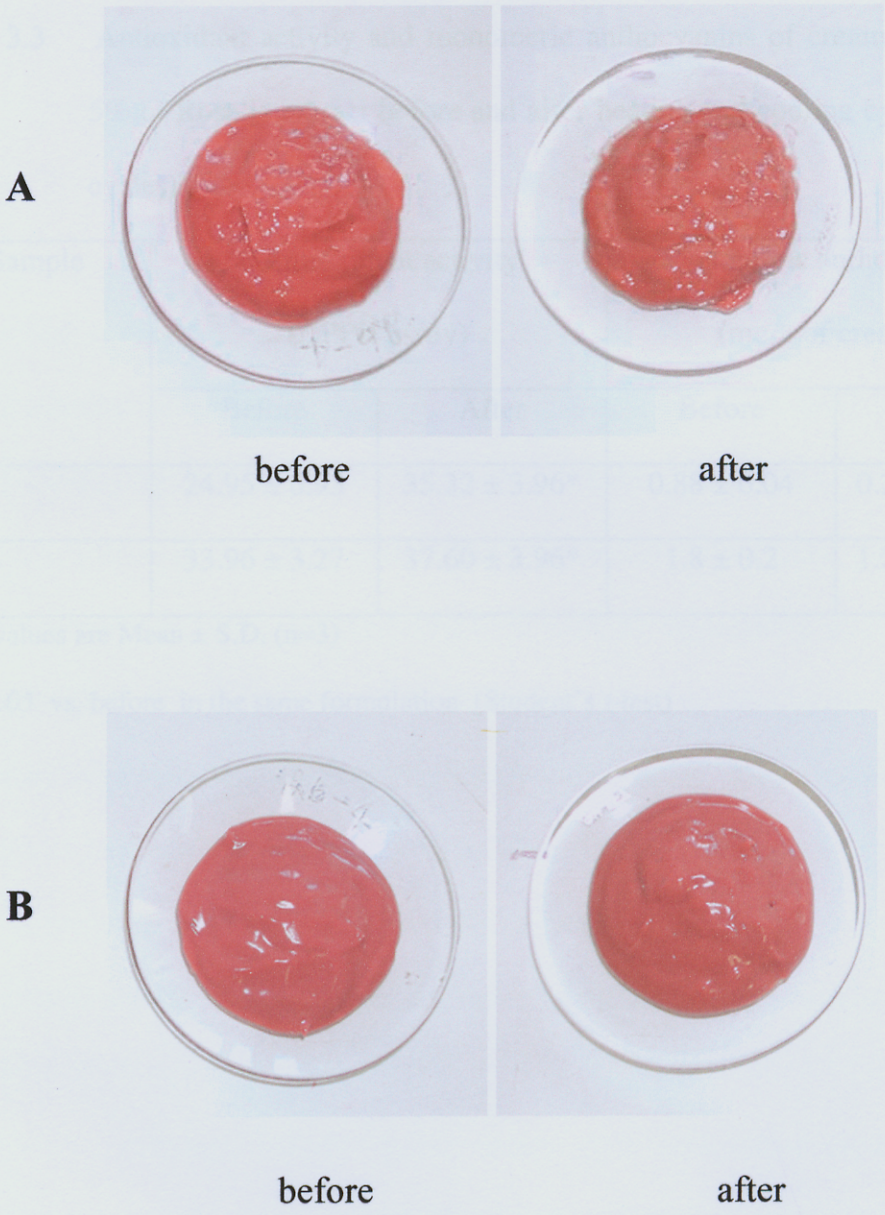


Figure 3.20 Formulation of creams containing 5% of Roselle extract in cream base Rx1 (A) and Rx2 (B) before and after heating and cooling cycle tests (6 cycles).

Table 3.3 Antioxidant activity and monomeric anthocyanins of cream containing 5% of Roselle extract before and after heating and cooling cycle tests (6 cycles)

Sample	% Antioxidant activity[a] (DPPH assay)		Monomeric anthocyanin[a] (mg/g of cream)	
	Before	After	Before	After
Rx 1	24.95 ± 0.95	35.32 ± 3.96*	0.88 ± 0.04	0.21 ± 0.04*
Rx 2	33.96 ± 3.27	37.60 ± 3.96*	1.8 ± 0.2	1.24 ± 0.15*

[a] The values are Mean ± S.D. (n=3)

* $P<0.05$ vs. before in the same formulation (Student's *t*-test)