CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Solvents for extraction

A few different extraction solvents were tried to maximize the antioxidant activity in pomegranate fruit peel extract. The fruit peels were extracted under reflux conditions using $CHCl_3$, EtOAc, MeOH and 70% acetone as extraction solvents. The result showed that all extracts possessed antioxidant activity (Table 4.1). The MeOH extract, however, exhibited the strongest antioxidant activity with ED_{50} value of 68.99 μg/ml. This indicates that MeOH can be acceptable as the solvent for extraction of antioxidant constituents from pomegranate fruit peels.

Table 4.1 Antioxidant activity of the pomegranate fruit peel extracts and quercetin evaluated by DPPH radical scavenging assay

Extracts	Yield of the extract (%w/w)	ED_{50} (μg/ml)
$CHCl_3$ ext.	5.57	180.93 ± 1.36
EtOAc ext.	20.25	91.16 ± 1.18
MeOH ext.	26.17	68.99 ± 1.40*
70%Acetone ext.	35.91	76.16 ± 1.54
Quercetin[a]	-	3.07 ± 0.05

[a] Positive control

*Significant difference (P<0.05)

4.2 Determination of suitable solvent for fractionation

Although the MeOH extract of pomegranate fruit peels possessed strong antioxidant activity, the activity could be improved by simple fractionation method. Improvement of the antioxidant activity was achieved using liquid-liquid extraction (partition). Variation of the organic solvents, including EtOAc, n-BuOH and n-BuOH : EtOAc (1:1), for liquid-liquid

extraction found that all tested organic solvents were capable of producing more potent antioxidant fractions (Table 4.2). However, the EtOAc fraction showed the strongest antioxidant activity with the ED_{50} value of 6.33 μg/ml. These results indicate that liquid-liquid extraction between EtOAc and water is a simple method to improve the antioxidant activity of the MeOH extract of pomegranate fruit peels.

Table 4.2 Antioxidant activity of the fractions obtained from liquid-liquid extraction evaluated by DPPH radical scavenging assay

Extracts/Compound	Yield of the extract (%w/w)	ED_{50} (μg/ml; Mean ± S.D.)
Crude MeOH extract	26.17	68.99 ± 1.40
EtOAc fraction	6.75	6.33±0.13*
n-BuOH fraction	8.21	32.23±2.37
n-BuOH:EtOAc (1:1) fraction	7.89	24.53±1.39
Quercetin[a]	-	3.07 ± 0.05

[a] Positive control

*Significant difference (P<0.05)

4.3 Determination of extraction method

Extraction methods including maceration and extraction under reflux conditions were examined for the suitable method for antioxidant constituent extraction. It was found that both methods were capable of producing the pomegranate fruit peel extracts, which possessed equal antioxidant activity (Table 4.3). However, extraction under reflux conditions consumed markedly less extraction time (3.5 hours) than maceration method (10 days). These results suggest that extraction under reflux conditions is suitable for extraction of antioxidant constituents from pomegranate fruit peels.

Table 4.3 Comparison of extraction time and antioxidant activity of the extracts obtained from two extraction methods

Extract methods	Time used for extraction (hour)	Antioxidant activity ED_{50} (μg/ml Mean ± S.D.)	
		MeOH extract	EtOAc fraction
Maceration	240	65.92 ± 0.04	6.33 ± 0.13
Extract under reflux conditions	3.5	67.88 ± 0.70	7.36 ± 0.32

4.4 Preparation of antioxidant active extract of pomegranate fruit peels

After the extraction and fractionation methods for preparation of a high potency antioxidant extract of pomegranate fruit peels was optimized, the established method was applied to prepare the pomegranate fruit peel extract for anti-aging cream formulation. Dried powder of pomegranate fruit peels (1 kg) was reflux in methanol (5 L) for 1 hour (x2). The extract was then evaporated to dryness *in vaccuo*. The methanolic extract was further partitioned between ethyl acetate and distilled water to produce a high potency antioxidant extract (EtOAc fraction). The obtained extract was brown powder after evaporated to dryness (Figure 4.1) and the yield of the extract was 6.75% w/w. The extract was then subjected to evaluation of antioxidant activity again. The results showed that the extract still exhibited a satisfactory antioxidant activity with the ED_{50} value was 10.03 and 12.10 μg/ml when evaluated by DPPH radical scavenging assay and β-carotene bleaching test, respectively (Table 4.4, Figure 4.2, 4.3 and 4.4).

Figure 4.1 Antioxidant active extract of pomegranate fruit peels

Table 4.4 Antioxidant activity of the EtOAc fraction evaluated by DPPH radical scavenging assay and β-carotene bleaching assay

Extract/Compound	**DPPH radical scavenging assay** ED_{50} **(μg/ml; Mean ± S.D.)**	**β-carotene bleaching assay** ED_{50} **(μg/ml; Mean ± S.D.)**
EtOAc fraction	10.03 ± 1.14	12.10 ± 1.76
Ellagic acid	2.53 ± 0.94	1.32 ± 0.97
Quercetin[a]	3.05 ± 0.87	1.57 ± 0.26

[a] Positive control

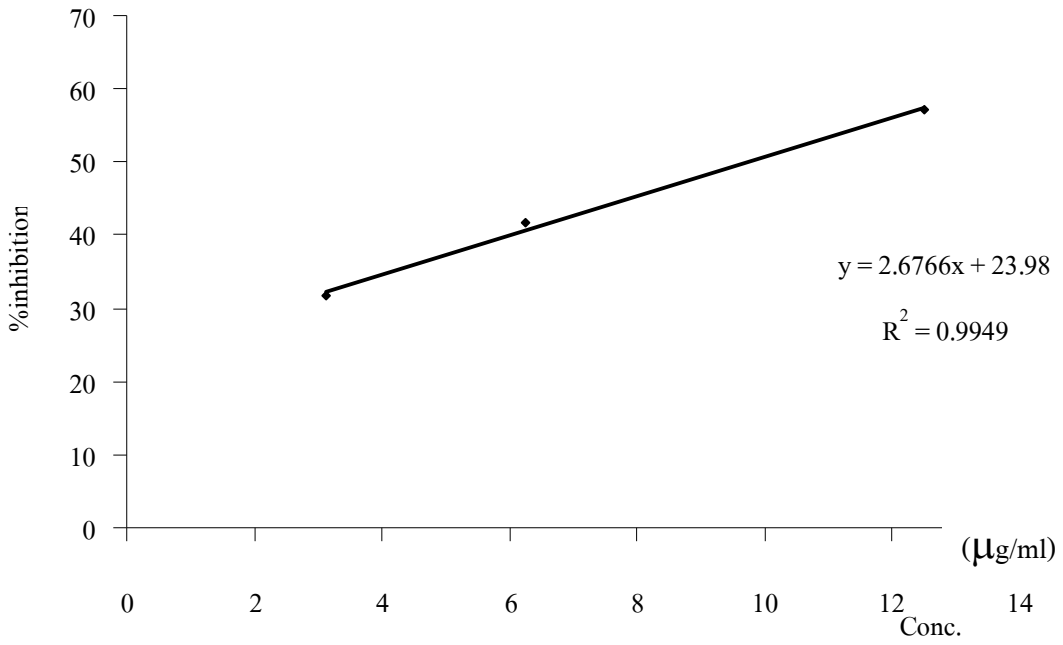


Figure 4.2 Correlation between concentration of EtOAc fraction (μg/ml) determined by DPPH radical scavenging assay and its inhibition (%)

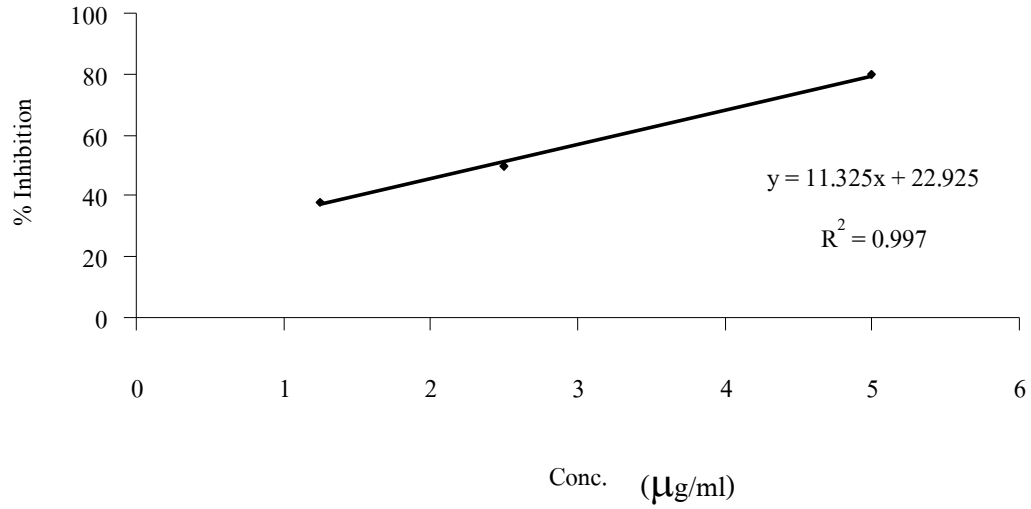


Figure 4.3 Correlation between concentration of ellagic acid (µg/ml) determined by DPPH radical scavenging assay and its inhibition (%)

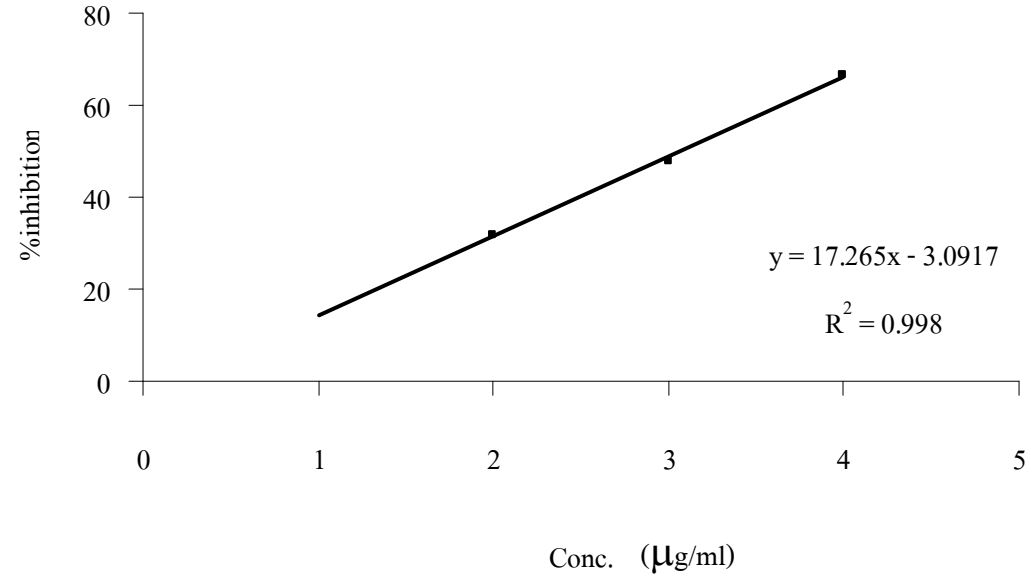
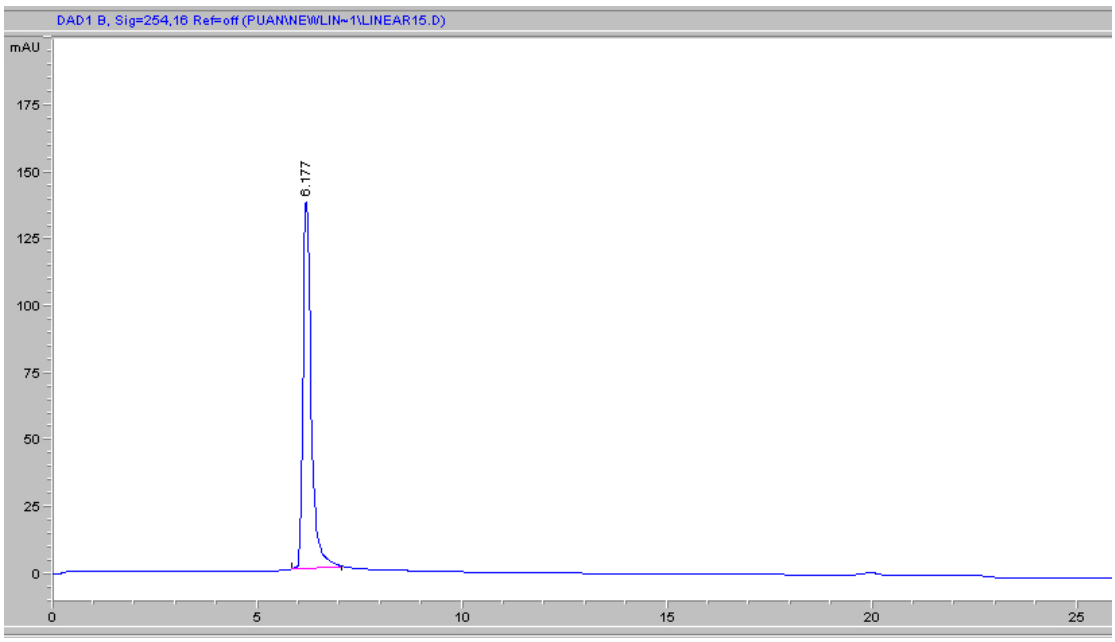


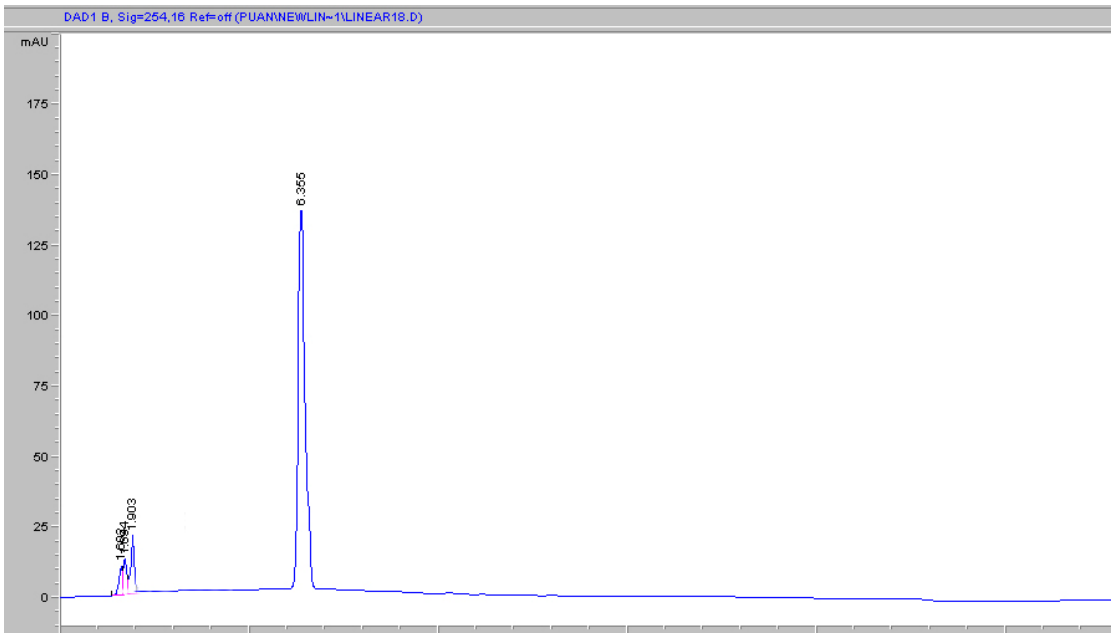
Figure 4.4 Correlation between concentration of quercetin (µg/ml) determined by DPPH radical scavenging assay and its inhibition (%)

4.5 Quantitative determination of ellagic acid in the antioxidant active extract of pomegranate fruit peels

It has been reported that ellagic acid, the antioxidant active compound was isolated from pomegranate fruit peels by antioxidant assay-guided purification (Panichayupakaranant *et al*., 2005). In this study, the antioxidant activities of ellagic acid were also determined using DPPH radical scavenging assay and β-carotene bleaching assay. The data are showed in Table 4.4. Standardization of the antioxidant active extract of pomegranate fruit peels was performed by quantitative determination of ellagic acid in the extract using HPLC method previously established and validated by Issuriya *et al.* (2007). Baseline separation of ellagic acid was achieved within 10 minutes. The retention time of ellagic acid was 6.1 minutes (Figure 4.5). The identity of ellagic acid peak was confirmed by comparison of its absorption spectrum produced by photo-diode array detector with the authentic compound (Figure 4.6). Linearity of the HPLC method was evaluated using standard samples over six calibration concentrations between 0.625 to 10 mg/ml (Figure 4.7). It exhibited good linearity over the evaluated ranges with correlation coefficient (r^2) of 0.9996. It was found that the content of ellagic acid in the antioxidant active extract of pomegranate fruit peels was 21.93 $\pm$ 0.85 %w/w. The extracts that used through this study were therefore standardized the ellagic acid content to not less than 21.0 %w/w.



(A)



(B)

Figure 4.5 HPLC-chromatogram of the authentic ellagic acid **(A)** and the antioxidant active extract of pomegranate fruit peels **(B)**

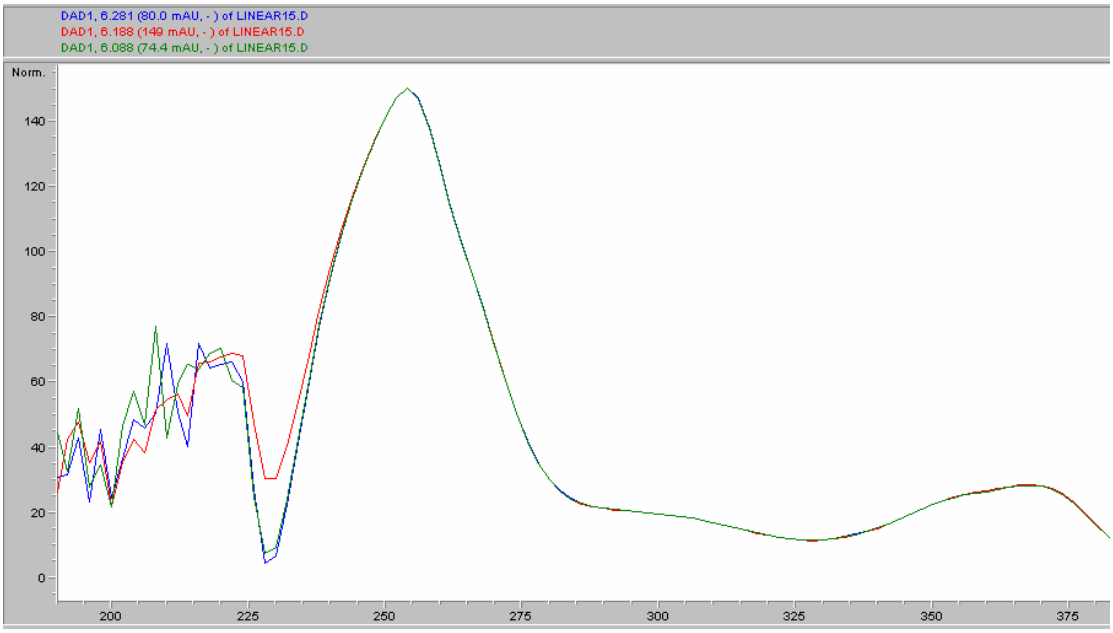


Figure 4.6 Absorption spectra of the peak at retention time 6.28 minutes (——) and the authentic ellagic acid (——)

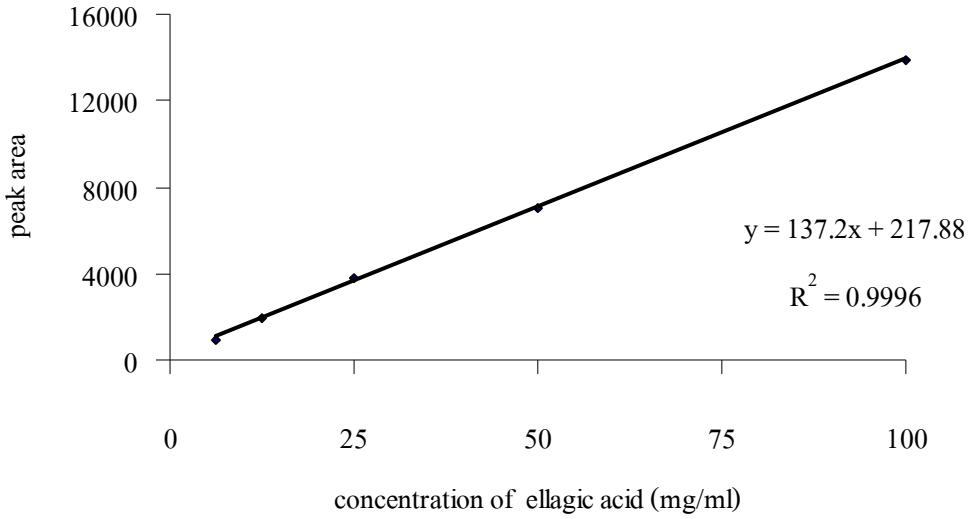


Figure 4.7 Calibration curve of ellagic acid

4.6 Solubility of the antioxidant active extract of pomegranate fruit peels

Since there are various solvents and co-solvents used in cream base, the solubility test was performed in order to determine the solubility of the antioxidant active extract of pomegranate fruit peels to ensure the homogeneity of the extract in cream base. A solubility study showed that the extract was very slightly soluble in ethanol and water, slightly soluble in propylene glycol, and practically insoluble in glycerin and mineral oil (Table 4.5). The results indicate that the antioxidant active extract of pomegranate fruit peels has limit solubility in both polar and nonpolar solvents.

Table 4.5 Solubility of pomegranate fruit peel extract with various cosmetic solvents

Solvent	Volume of solvent in ml/g of solute	Level of solubility
Ethanol	600	Very slightly soluble
Glycerine	>1000	Practically insoluble
Mineral oil	>1000	Practically insoluble
Propylene glycol	80	Slightly soluble
Water	700	Very slightly soluble

4.7 Stability of the antioxidant active extract of pomegranate fruit peels

Light, temperature and humidity have influence on affecting the stability of the antioxidant active extract of pomegranate fruit peels.

4.7.1 Effect of light

The effect of light on the stability of the extract was examined under fluorescent light as compared with light protection for a period of 60 days. Physical appearance of the extract, the content of ellagic acid and antioxidant activity of extract were examined weekly. The results showed that under light conditions, the color of the antioxidant active extract of pomegranate fruit peels gradually increased, but the color did not change, when stored under protected from light (Figure 4.8). However, a significant decrease of ellagic acid content was observed at weeks 5 and weeks 7, when the extracts were stored exposed to light and protected from light, respectively (Table 4.6, Figure 4.9). In both conditions, the ellagic acid content gradually decreased by 14 - 15

% after the extract was kept for the period of 60 days. In addition, the antioxidant activities of the extracts evaluated by DPPH assay and β-carotene bleaching assay also gradually decreased in both conditions. The antioxidant activity, evaluated by DPPH assay, began to decrease significantly after stored for 2 or 3 weeks, while that evaluated by β-carotene bleaching assay began to decrease significantly after stored for 6 week (Table 4.7, Figure 4.10 and 4.11). This finding suggests that the antioxidant active extracts of pomegranate fruit peels were not stable at 45 ± 2°C under either exposed to light or protected from light.

(A) **(B)**

Figure 4.8 Color of the antioxidant active extract of pomegranate fruit peels stored under protected from light **(A)** and stored under light condition **(B)** after storage at 45 ± 2°C

Table 4.6 Ellagic acid content in the antioxidant active extract of pomegranate fruit peels stored at 45 ± 2°C, under exposed to light and protected from light conditions

Week	Ellagic acid content (% w/w; Mean ± S.D.)	
	Light	**Protected from light**
0	21.93 ± 0.85	21.93 ± 0.85
1	21.97 ± 0.71	22.23 ± 0.72
2	21.70 ± 0.67	22.20 ± 0.78
3	20.59 ± 0.42	22.12 ± 0.35
4	20.79 ± 0.95	20.41 ± 0.59
5	19.28 ± 0.63*	21.07 ± 0.99
6	19.17 ± 0.84*	20.92 ± 0.89
7	18.70 ± 0.81*	19.75 ± 0.70*
8	18.20 ± 0.63*	19.15 ± 0.97*
9	18.93 ± 0.93*	18.77 ± 0.87*

* Significance at $P < 0.05$ when compared with the content at initial time

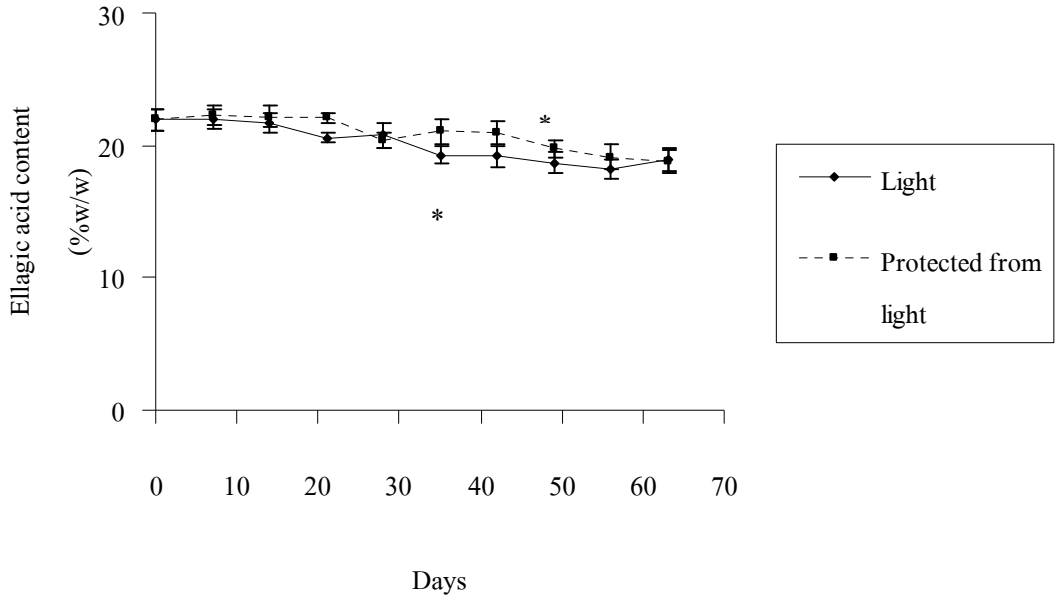


Figure 4.9 Ellagic acid content in the antioxidant active extract of pomegranate fruit peels stored at 45 ± 2°C, under exposed to light and protected from light conditions (*Significance at $P < 0.05$ when compared with the content at initial time)

Table 4.7 Antioxidant activity of the antioxidant active extract of pomegranate fruit peels stored at 45 ± 2 °C, under exposed to light and protected from light conditions

Week	ED_{50} μg/ml[a]		%Antioxidant activity[b]	
	Light	Protect from light	Light	Protect from light
0	10.03 ± 1.14	10.03 ± 1.14	64.15 ± 2.29	64.15 ± 2.29
1	11.46 ± 0.94	11.17 ± 0.23	62.76 ± 3.84	63.77 ± 3.03
2	12.11 ± 0.54*	11.73 ± 1.01	62.41 ± 1.67	63.01 ± 2.78
3	13.75 ± 0.94*	12.16 ± 0.94*	60.52 ± 1.10	61.58 ± 1.74
4	13.87 ± 1.38*	12.47 ± 1.06*	60.16 ± 1.79	60.87 ± 0.83
5	15.02 ± 1.24*	12.83 ± 1.10*	60.09 ± 1.77	60.41 ± 1.99
6	14.95 ± 1.17*	13.04 ± 1.01*	59.73 ± 1.20*	59.98 ± 0.95*
7	15.12 ± 1.08*	13.57 ± 1.18*	57.14 ± 1.05*	59.74 ± 1.23*
8	15.48 ± 0.84*	13.79 ± 1.04*	58.76 ± 1.87*	58.09 ± 1.91*
9	15.97 ± 0.71*	14.01 ± 0.98*	58.08 ± 1.25*	58.11 ± 1.03*

* Significance at $P < 0.05$ when compared with the content at initial time

[a] evaluated by DPPH radical scavenging assay

[b] evaluated by β-carotene bleaching assay

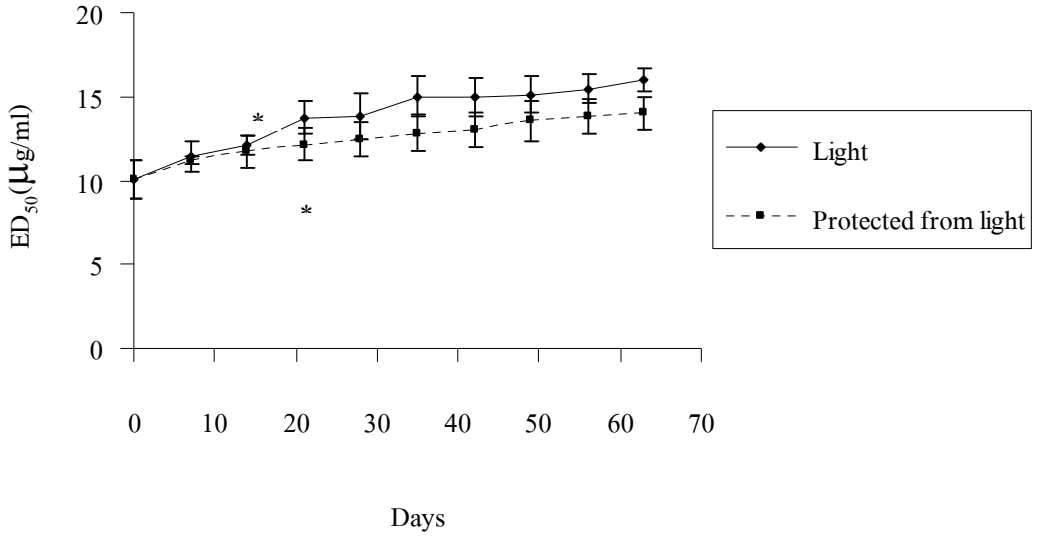


Figure 4.10 Antioxidant activity evaluated by DPPH assay of antioxidant active extract pomegranate fruit peel stored at 45 ± 2 °C, under exposed to light and protected from light conditions (*Significance at $P < 0.05$ when compared with the content at initial time)

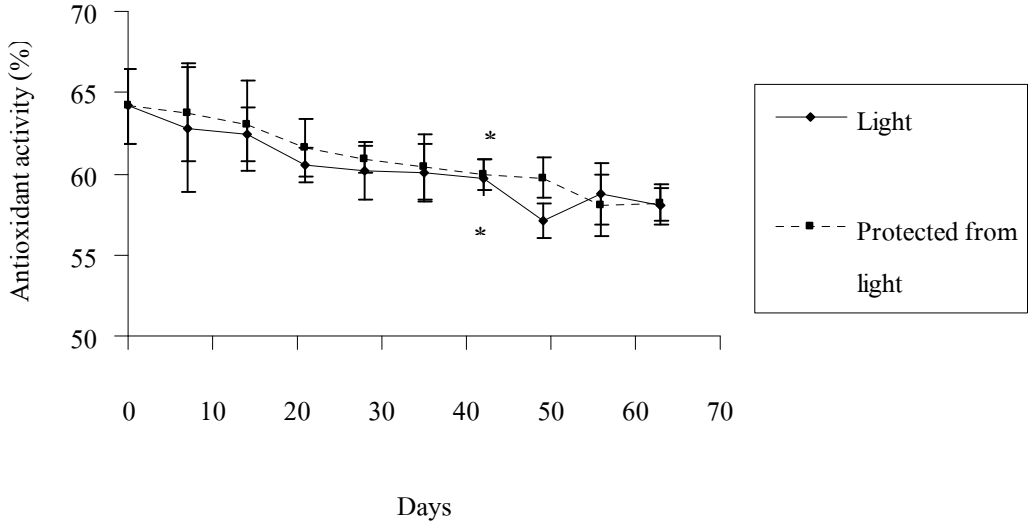


Figure 4.11 Antioxidant activity evaluated by β-carotene bleaching assay of antioxidant active extract pomegranate fruit peel stored at 45 ± 2 °C, under exposed to light and protected from light conditions (*Significance at $P < 0.05$ when compared with the content at initial time)

4.7.2 Effect of temperature

The effect of temperature on the stability of the antioxidant active extract of pomegranate fruit peels was examined at the temperatures of 25°C and 45°C, under light protecting condition for a period of 60 days. Physical appearance of the extract, the content of ellagic acid and antioxidant activity of extract were examined every week. The results showed that all tested temperatures did not affect the physical appearance of the extracts. Incontrast, the ellagic acid of the extracts was significantly decreased after kept for 7 weeks in both temperatures (Table 4.8, Figure 4.12). The ellagic acid content decreased by 12% and 14% when the extracts were kept under 25°C and 45°C, respectively, for the period of 60 days. However, the antioxidant activities evaluated by both assays of the extract kept under 25°C did not change through the period of 60 days, while those of the extract kept under 45°C were significantly decreased after kept for 3 or 6 weeks (Table 4.9, Figure 4.13 and 4.14). Although, the ellagic acid content of the extract kept under 25°C was significantly decreased, the antioxidant activity of the extract was not affected. This implies that the degraded product of ellagic acid may still exhibit antioxidant activity, or the antioxidant activity of the extract also strong contributed from the other compounds in the extract. This finding suggests that the antioxidant active extract of pomegranate fruit peels was not stable under high temperature. Therefore, the extract should be kept in cool place or low temperature.

Table 4.8 Ellagic acid content in the antioxidant active extract of pomegranate fruit peels stored at 25 ± 2 °C and 45 ± 2 °C, protected from light

Week	**Ellagic acid content (% w/w; Mean ± S.D.)**	
	25 ± 2 °C	**45 ± 2 °C**
0	21.93 ± 0.85	21.93 ± 0.85
1	21.82 ± 0.84	22.23 ± 0.72
2	21.48 ± 1.38	22.20 ± 0.78
3	20.96 ± 1.04	22.12 ± 0.35
4	21.87 ± 1.20	20.41 ± 0.59
5	20.02 ± 0.88	21.07 ± 0.99
6	20.58 ± 0.73	20.92 ± 0.89
7	19.82 ± 0.43*	19.75 ± 0.70*
8	19.01 ± 0.99*	19.15 ± 0.97*
9	19.26 ± 0.76*	18.77 ± 0.87*

* Significance at $P < 0.05$ when compared with the content at initial time

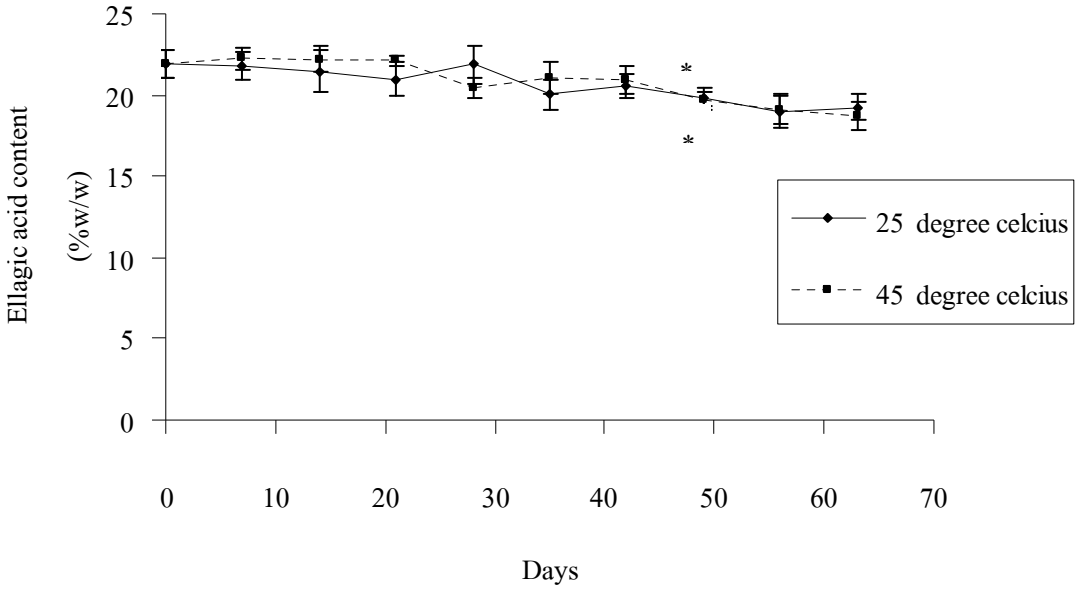


Figure 4.12 Ellagic acid content in the antioxidant active extract of pomegranate fruit peels stored at 25 ± 2 °C and 45 ± 2 °C, protected from light (*Significance at $P < 0.05$ when compared with the content at initial time)

Table 4.9 Antioxidant activity of the antioxidant active extract of pomegranate fruit peels stored at 25 ± 2 °C and 45 ± 2 °C, protected from light

Week	ED_{50} μg/ml[a]		%Antioxidant activity[b]	
	25±2 °C	45±2 °C	25±2 °C	45±2 °C
0	10.03 ± 1.14	10.03 ± 1.14	64.15 ± 2.29	64.15 ± 2.29
1	10.53 ± 0.75	11.17 ± 0.23	63.98 ± 1.79	63.77 ± 3.03
2	10.98 ± 1.04	11.73 ± 1.01	63.87 ± 2.56	63.01 ± 2.78
3	10.84 ± 1.23	12.16 ± 0.94*	62.88 ± 1.19	61.58 ± 1.74
4	10.91 ± 1.43	12.47 ± 1.06*	63.57 ± 0.98	60.87 ± 0.83
5	10.89 ± 1.02	12.83 ± 1.10*	61.43 ± 1.05	60.41 ± 1.99
6	10.99 ± 1.04	13.04 ± 1.01*	61.78 ± 0.79	59.98 ± 0.95*
7	10.87 ± 1.03	13.57 ± 1.18*	62.36 ± 1.41	59.74 ± 1.23*
8	10.97 ± 1.15	13.79 ± 1.04*	62.01 ± 1.23	58.09 ± 1.91*
9	10.95 ± 1.01	14.01 ± 0.98*	61.55 ± 1.39	58.11 ± 1.03*

* Significance at $P < 0.05$ when compared with the content at initial time

[a] evaluated by DPPH radical scavenging assay

[b] evaluated by β-carotene bleaching assay

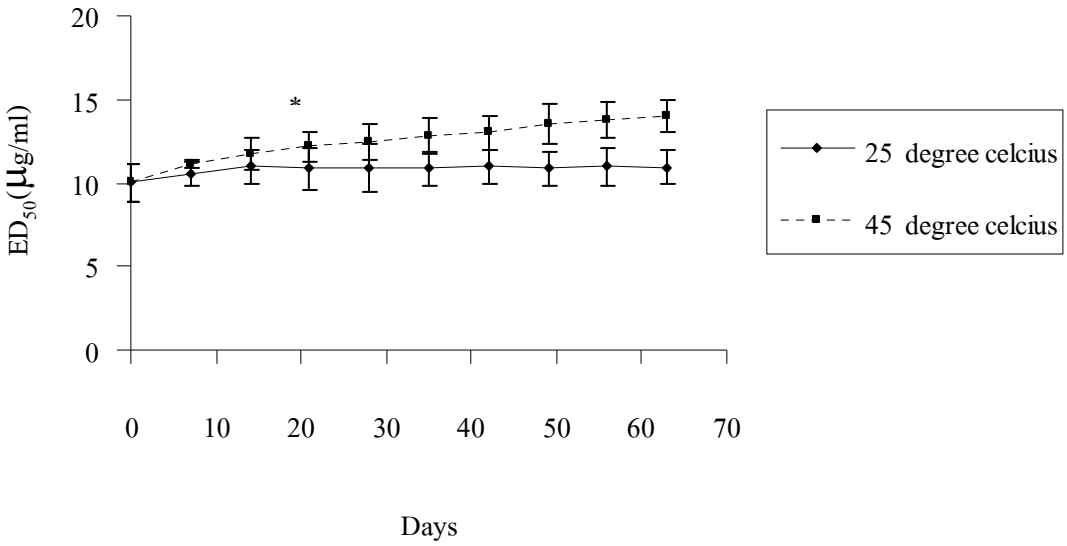


Figure 4.13 Antioxidant activity evaluated by DPPH assay of the antioxidant active extract of pomegranate fruit peels stored at 25 ± 2 °C and 45 ± 2 °C, protected from light (*Significance at $P < 0.05$ when compared with the content at initial time)

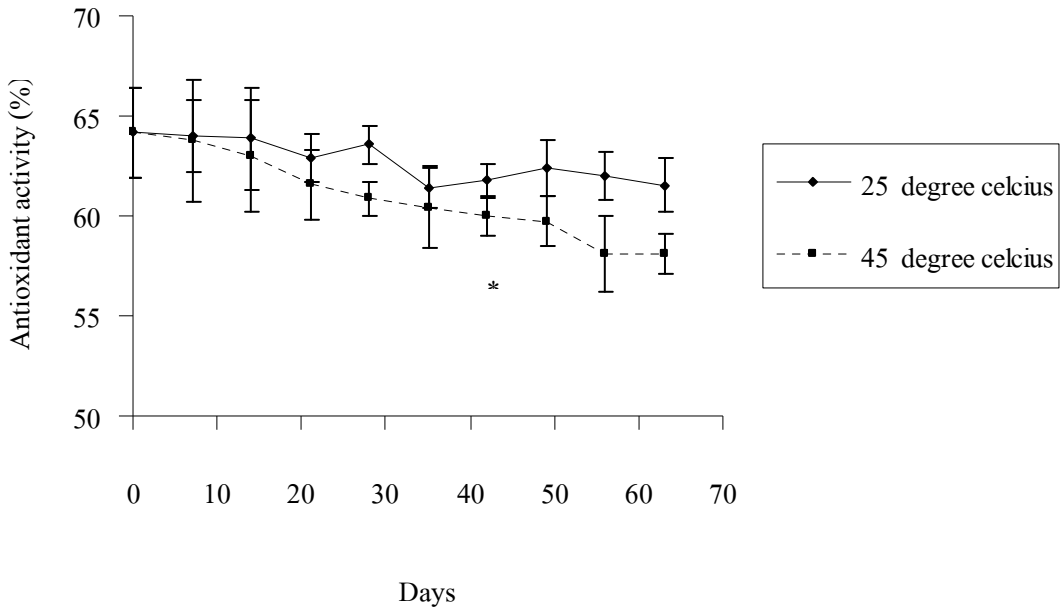


Figure 4.14 Antioxidant activity evaluated by β-carotene bleaching assay of the antioxidant active extract of pomegranate fruit peels stored at 25 ± 2 °C and 45 ± 2 °C, protected from light (*Significance at $P < 0.05$ when compared with the content at initial time)

4.7.3 The effect of humidity

The effect of humidity on the stability of the antioxidant active extract of pomegranate fruit peels was examined under 75% relative humidity and protected from humidity conditions at temperatures of 25°C for a period of 60 days. Physical appearance of the extract, the content of ellagic acid and antioxidant activity of the extract were examined weekly. The result showed that the physical appearance of the extract was changed from brown powder to dark brown sticky powder when kept under 75% relative humidity conditions (Figure 4.15). Moreover, the ellagic acid content was significantly decreased after 3-week storage when the extract was kept under 75% relative humidity conditions (Table 4.10, Figure 4.16). And the ellagic acid content decreased by 33% of the period of 60 days. As shown in Figure 4.19, the major peak of ellagic acid (7 min) was decreased comparing between the initial time and after the stability that period. The major peak of degradation products of ellagic acid was found in the HPLC chromatograms at the retention time of 5.8 minutes. It has been reported that ellagic acid was not stable in aqueous solution and undergoes hydrolysis reaction. These reactions produced hexahydroxydiphenic acid as hydrolysis products (Figure 4.20) (Quideau and Feldman, 1996). In addition, the antioxidant activity of the extract evaluated by DPPH assay was also significantly decreased after 3 weeks. However, the antioxidant activity of the extract evaluated by β-carotene bleaching assay was not affected by the high humidity conditions (Table 4.11, Figure 4.17 and 4.18). This implies that the degraded product of ellagic acid may still exhibit inhibitory effect of lipid peroxidation.

(A) **(B)**

Figure 4.15 Color of the antioxidant active extract of pomegranate fruit peels stored under protected from humidity conditions **(A)** and stored under 75% relative humidity **(B)**

Table 4.10 Ellagic acid content in the antioxidant active extract of pomegranate fruit peels stored under 75% relative humidity and protected from humidity conditions, at 25 ± 2 °C

Weeks	**Ellagic acid content (% w/w; Mean ± S.D.)**	
	Protected from humidity	**75% Relative humidity**
0	21.93 ± 0.85	21.93 ± 0.85
1	21.82 ± 0.84	21.07 ± 0.76
2	21.48 ± 1.38	20.35 ± 0.98
3	20.96 ± 1.04	17.20 ± 1.01*
4	21.87 ± 1.20	17.38 ± 0.73*
5	20.02 ± 0.88	16.77 ± 0.67*
6	20.58 ± 0.73	16.49 ± 0.92*
7	19.82 ± 0.43*	16.92 ± 0.34*
8	19.01 ± 0.99*	14.72 ± 0.91*
9	19.26 ± 0.76*	14.65 ± 0.58*

* Significance at $P < 0.05$ when compared with the content at initial time

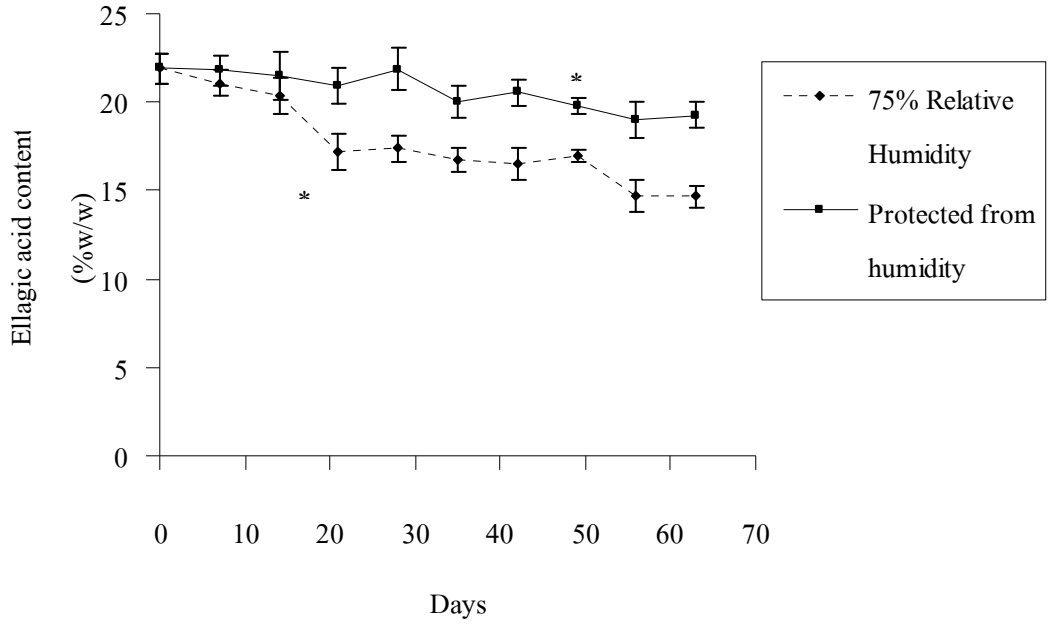


Figure 4.16 Ellagic acid content in the antioxidant active extract of pomegranate fruit peels stored under 75% relative humidity and protected from humidity conditions, at 25 ± 2 °C (*Significance at $P < 0.05$ when compared with the content at initial time)

Table 4.11 Antioxidant activity of the antioxidant active extract of pomegranate fruit peels stored under 75% relative humidity and protected from humidity conditions, at 25 ± 2 °C

Week	ED_{50} μg/ml[a]		%Antioxidant activity[b]	
	Protect from humidity	**75% Relative humidity**	**Protect from humidity**	**75% Relative humidity**
0	10.03 ± 1.14	10.03 ± 1.14	64.15 ± 2.29	64.15 ± 2.29
1	10.53 ± 0.75	10.76 ± 0.89	63.98 ± 1.79	63.89 ± 1.94
2	10.98 ± 1.04	11.72 ± 1.19	63.87 ± 2.56	63.35 ± 2.43
3	10.84 ± 1.23	12.37 ± 1.45*	62.88 ± 1.19	62.53 ± 1.75
4	10.91 ± 1.43	13.12 ± 1.35*	63.57 ± 0.98	61.66 ± 0.98
5	10.89 ± 1.02	13.39 ± 0.64*	61.43 ± 1.05	61.69 ± 1.48
6	10.99 ± 1.04	13.27 ± 0.75*	61.78 ± 0.79	61.75 ± 1.06
7	10.87 ± 1.03	13.48 ± 0.99*	62.36 ± 1.41	60.25 ± 1.19
8	10.97 ± 1.15	13.75 ± 1.16*	62.01 ± 1.23	61.08 ± 1.21
9	10.95 ± 1.01	13.94 ± 1.02*	61.55 ± 1.39	60.39 ± 1.18

***** Significance at $P < 0.05$ when compared with the content at initial time

[a] evaluated by DPPH radical scavenging assay

[b] evaluated by β-carotene bleaching assay

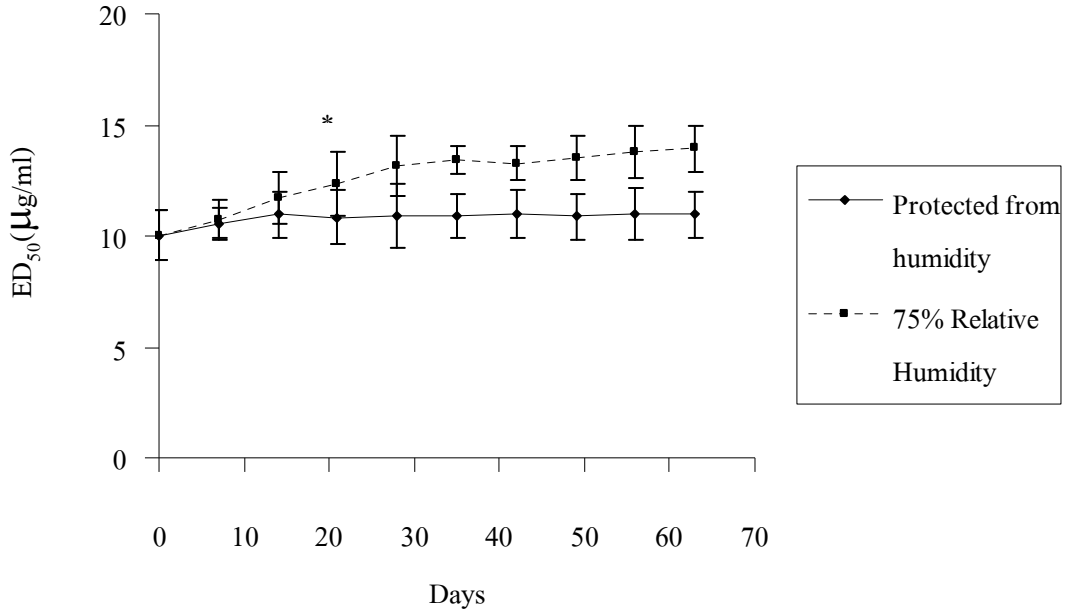


Figure 4.17 Antioxidant activity evaluated by DPPH assay of the antioxidant active extract of pomegranate fruit peels stored 75%RH and protected from humidity conditions, at 25 ± 2 °C (*Significance at $P < 0.05$ when compared with the content at initial time)

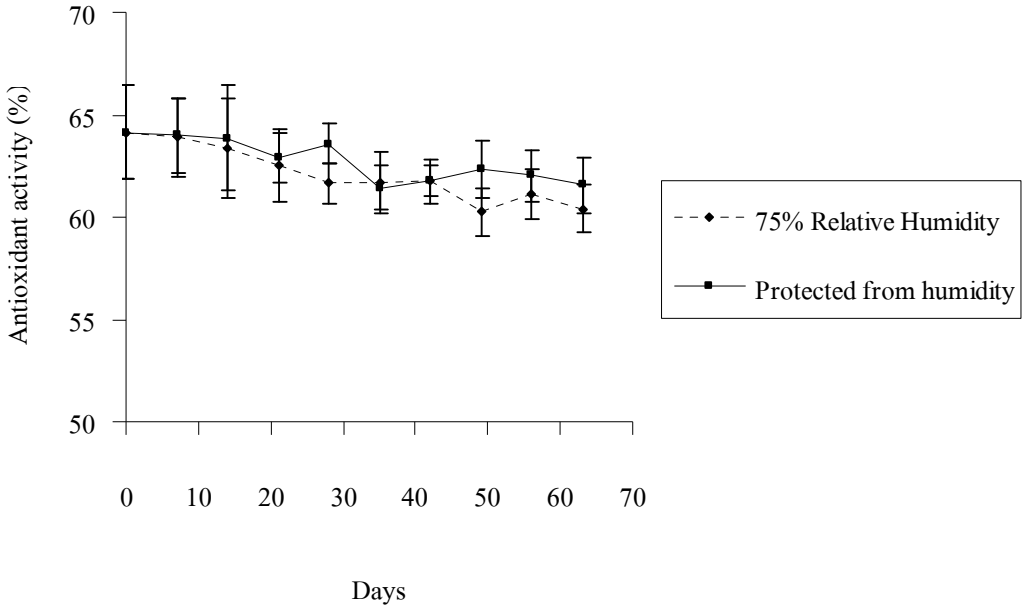


Figure 4.18 Antioxidant activity evaluated by β-carotene bleaching assay of the antioxidant active extract of pomegranate fruit peels stored 75%RH and protected from humidity conditions, at 25 ± 2 °C

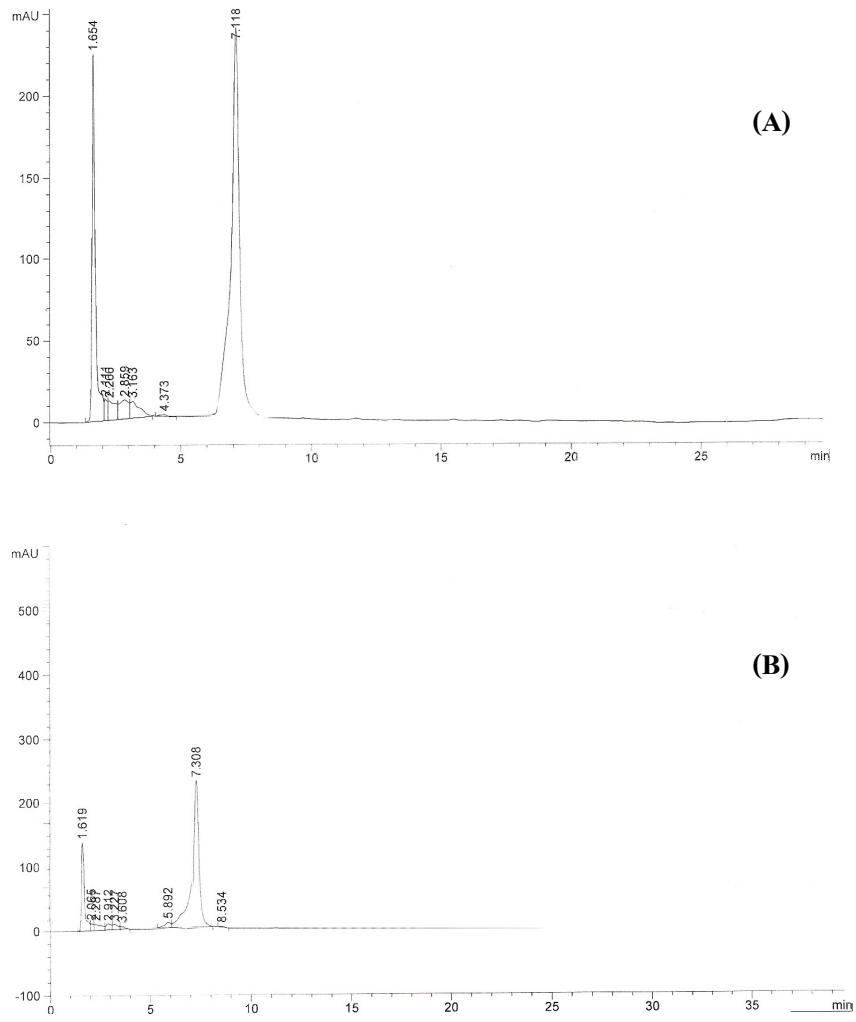
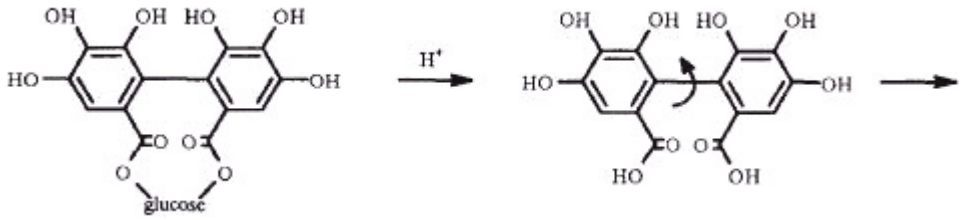
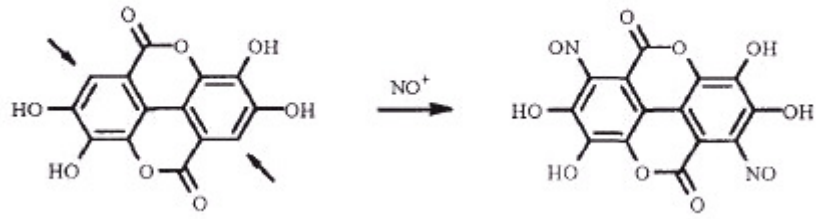


Figure 4.19 HPLC-chromatogram of the extracts at initial time **(A)** and after stability test **(B)**



Ellagitannin Hexahydroxydiphenic acid



Ellagic acid Hexahydroxydiphenic acid

Figure 4.20 Hydrolysis of ellagic acid

4.8 Preliminary formulation study of pomegranate extract cream

Five cream bases were prepared and tested for their stability by heating-cooling test method as described in section 3.2.11. An observation of the physical appearance, including color, smoothness, phase separation, viscosity and pH, before and after heating-cooling test led to the selection of a suitable cream base (Table 4.12, Figure 4.21). The cream base Rx 1, Rx 2, Rx 3 and Rx 4 were not suitable due to their poor dispersion on skin and high basic property. The cream base Rx 5 showed good physical appearance, good dispersion and the pH of the cream bases (5.75) was closed to the skin pH (5.5). In addition, their pH and viscosity did not significantly change after the test. Therefore, the cream bases Rx 5 were selected for further formulation studies.

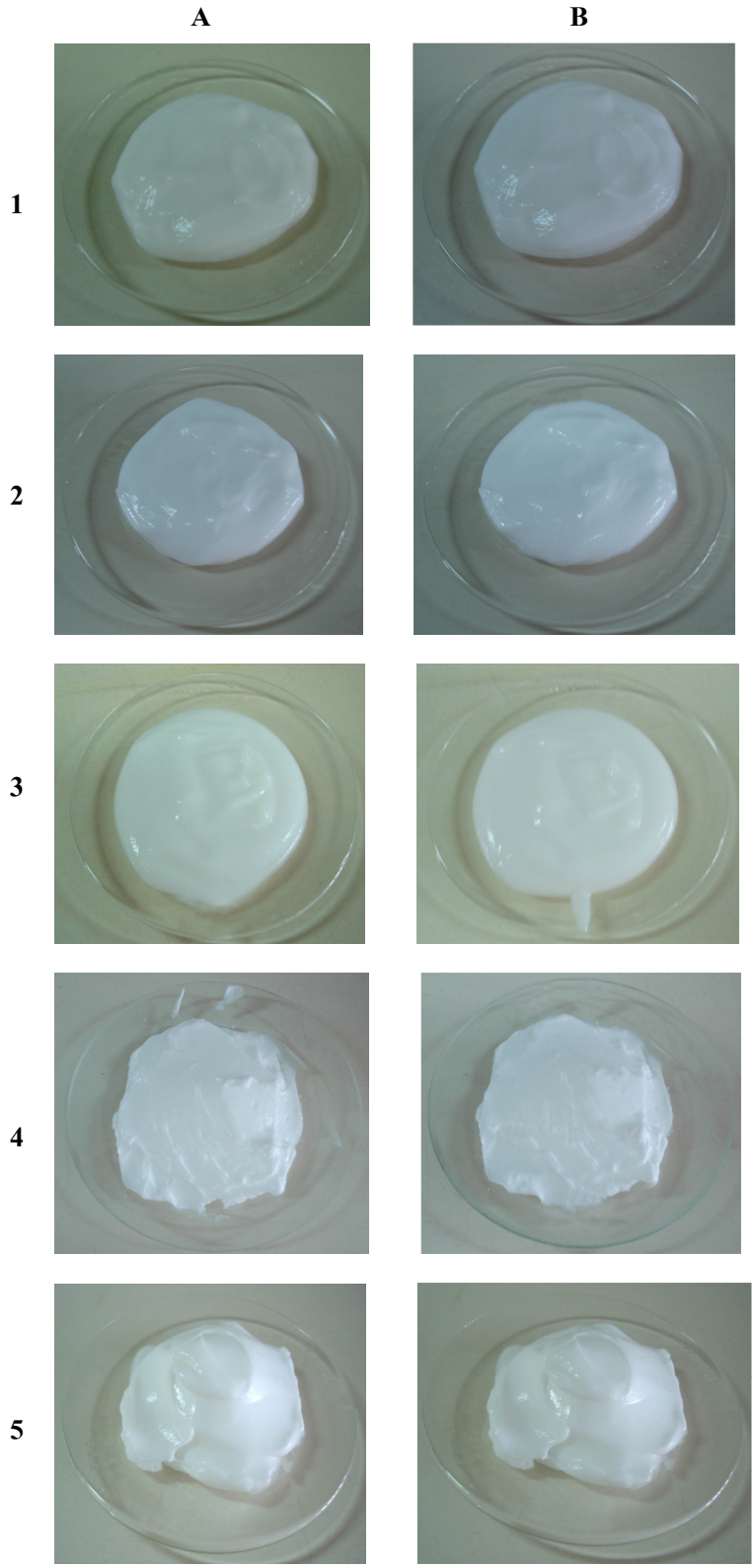


Figure 4.21 Physical appearances of cream bases before **(A)** and after **(B)** heating and cooling test (8 cycles): Rx1 (1), Rx 2 (2), Rx 3 (3) Rx 4 (4), Rx 5 (5)

In order to formulate an antioxidant cream, pomegranate extract creams were prepared from ethyl acetate fraction of pomegranate at the concentration of 0.5% and 1% w/w, equivalent to ellagic acid concentration. The ethyl acetate fraction of the extract was added into selected cream base Rx 5 to produce antioxidant cream preparations (Rx 5-1 and Rx 5-2). The physical appearances (color, smoothness, and phase separation) before and after 8 cycles of heating cooling test, as well as its pH and viscosity were shown in Table 4.13. Both preparations showed good appearance with a little odor at initial time. The colors of preparations are yellow brown. The tone of color depended on the concentration of the extract in the preparation. However, the colors of all preparations are acceptable as skin creams. After a heating and cooling test, it was found that the colors of all preparations were little changed. Phase separation was not observed in all preparations. However, little changes of pH and viscosity were observed in all preparations. The pictures of 0.5% and 1% pomegranate extract creams before and after heating and cooling test were shown in Figure 4.22.

Table 4.13 Physical properties of pomegranate-extract cream containing 0.5% (Rx 5-1) and 1% (Rx 5-2) the antioxidant active extract of pomegranate fruit peels before and after heating and cooling test (8 cycles)

Physical properties	**Pomegranate-extract cream**			
	Rx 5-1		**Rx 5-2**	
	Before	**After**	**Before**	**After**
Color	YB	YB	YB	YB
Phase separation	No	No	No	No
pH	5.14	5.15	5.09	5.11
Viscosity (cps)	90,000	91,000	76,000	78,000

YB= Yellow brown

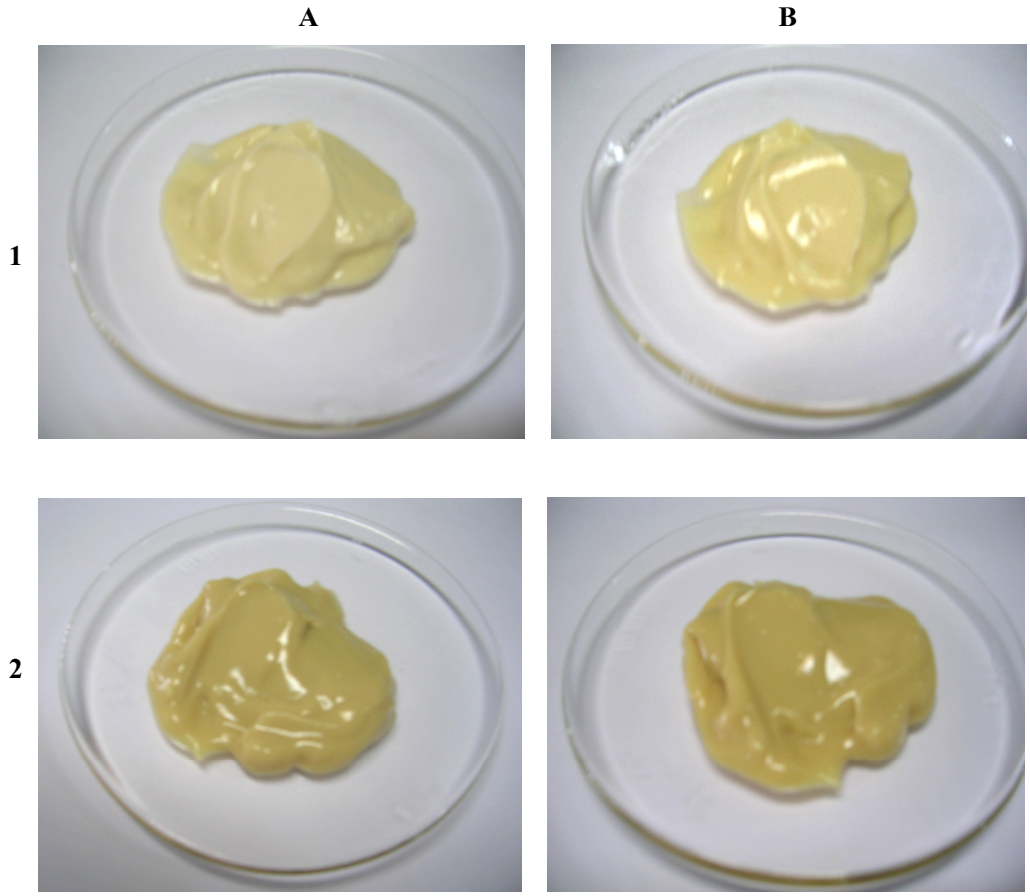


Figure 4.22 Pomegranate extract creams, Rx 5-1 (1), Rx 5-2 (2) before **(A)** and after **(B)** heating and cooling test

Regarding of chemical stability of the preparations, quantitative determination of ellagic acid content and antioxidant activity of the preparations before and after heating-cooling test were determined. It was found that at the initial time all preparations contained of ellagic acid in range of 17.96 – 18.75 %w/w. Unfortunately, after heating-cooling test the content of ellagic acid in all preparations was significantly decreased by 2 – 3 %w/w (Table 4.14).

Table 4.14 Ellagic acid content in pomegranate extract creams, before and after heating-cooling test (8 cycles)

preparations	**Concentration of the extract (%w/w)**	**Content (%w/w)**	
		Before heating-cooling test	**After heating-cooling test**
Rx 5-1	0.5	17.96 ± 0.25	15.18 ± 0.14*
Rx 5-2	1.0	18.75 ± 0.19	16.82 ± 0.23*

***** Significance at $P < 0.05$ when compared with the content at initial time

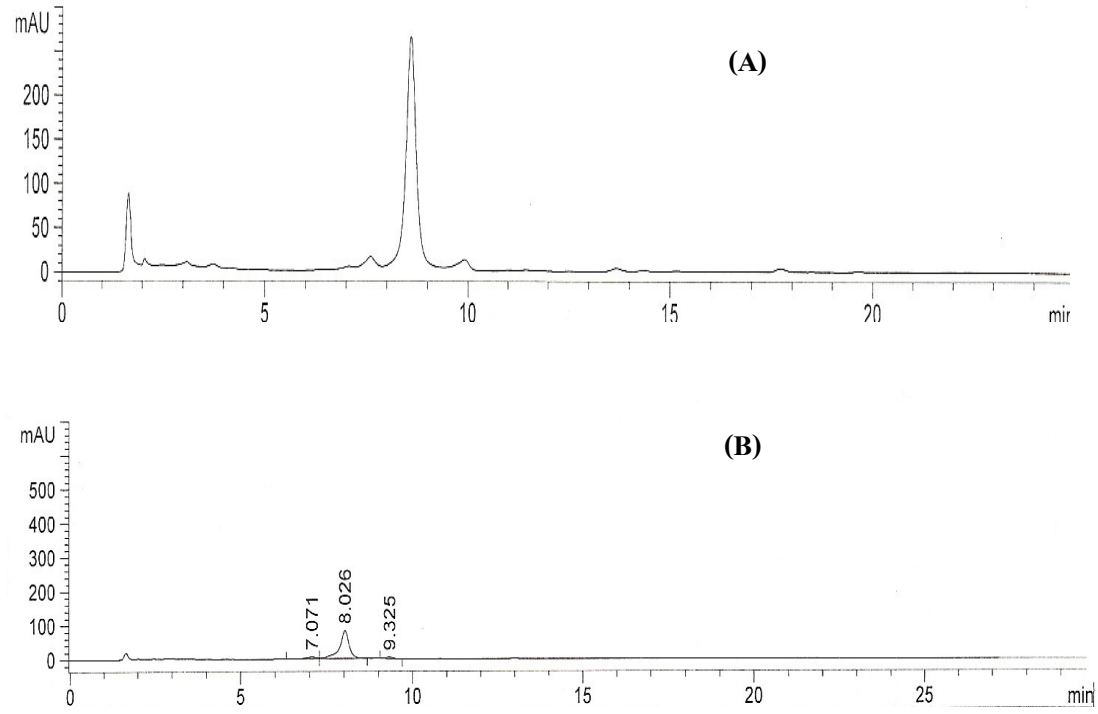


Figure 4.23 HPLC-chromatogram of the preparation extracts at initial time **(A)** and after heating-cooling test **(B)**

In contrast, the investigation of antioxidant activity of the pomegranate extract creams showed that the ED_{50} and antioxidant activity of the preparations Rx 5-1 and Rx 5-2 were not significantly different when compared between before and after heating and cooling test (Table 4.15 and 4.16).

Table 4.15 Antioxidant activity of pomegranate extract creams evaluated by DPPH assay

Formulation	**Concentration of the extract (% w/w)**	**ED_{50} (μg/ml)**	
		Before heating-cooling test	**After heating-cooling test**
Rx 5-1	0.5	13.85 ± 1.12	12.91 ± 1.07
Rx 5-2	1.0	12.49 ± 1.35	12.01 ± 0.93
Quercetin[a]		3.76 ± 1.04	

[a] Positive control

Table 4.16 Antioxidant activity of pomegranate extract creams evaluated by β-carotene bleaching assay

Formulation	**Concentration of the extract (% w/w)**	**%AA**	
		Before heating-cooling test	**After heating-cooling test**
Rx 5-1	0.5	52.93 ± 1.16	49.59 ± 1.38
Rx 5-2	1.0	65.25 ± 1.02	63.96 ± 0.97
Quercetin[a]		90.34 ± 0.89	

[a] Positive control

Preliminary formulation study of antioxidant cream using the antioxidant active extract of pomegranate fruit peels found that although, the pomegranate extract cream exhibited inhibitory effect after heating and cooling test, ellagic acid may not be stable in the oil in water cream bases. Stability of the pomegranate extract cream in this study was not success. This may due to the lack of stability agent such as antioxidant of chelating agent in preparation. Therefore,