CHAPTER IV

RESULTS AND DISCUSSION

1. Extraction of Asiaticoside from *Centella asiatica* L.(Urb.)

The 3 g of white powders of asiaticoside was obtained from *Centella asiatica* dried plant 3 kg.

1.1 Determination of Asiaticoside Extract Using HPLC

The asiaticoside demonstrated a major peak at 220 nm at the resolution time 7.795 min. The chromatogram of asiaticoside was shown in figure 8.

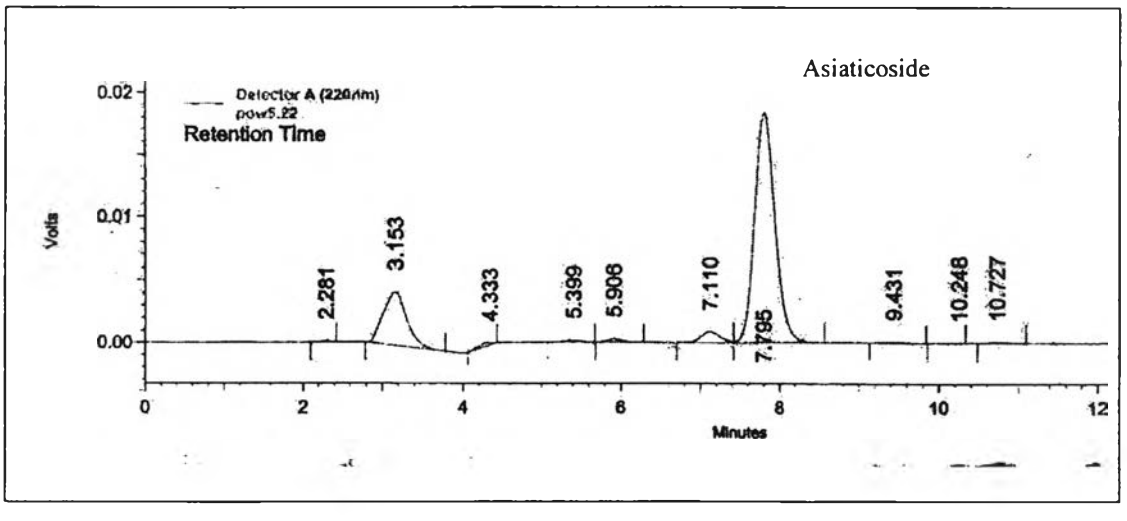


Figure 8 The HPLC Chromatogram of Asiaticoside

1.2 Determination of Suitable Solubilizing Agent of Asiaticoside

The 1g of asiaticoside was dissolved in 9.5 mL propylene glycol. The tween 20 was used as a solubilizing agent. The asiaticoside used in this study was 0.5 g the concentration of Tween 20 was 1.5g (1.5 times of asiaticoside)

2. Preparation of Polysaccharide Gel (PG) from Durian Fruit-Hulls

The PG mixture in deionize water solution was a clear, viscous and homogeneous mixture. The viscosity of the 4% w/w PG mixture was 1086±126 cps. The pH of the mixture was 2.74 ± 0.05.

3. Preparation of PG Facial-Patch Film Base

3.1 Determination of the Amount of Plasticizer

Table 4 and 5 showed the ingredients in each formula containing different concentrations of glycerin and sorbitol which varying from 5-20% w/w. In this study, due to the solubility of asiaticoside, the propylene glycol was used as solubilizing agent as well as plasticizer in the formulation in the concentration of 4.75% w/w in PG mixture solution. The mixture was dried in hot air oven to obtain the film.

The films were translucent pale brown in color, smooth and flexible film product. The thickness of the film was in the range 0.05±0.005 to 0.08±0.005. The mechanical properties of all films were shown in Table 6.

The several of the mechanical properties parameter (tensile strength, % elongation and Young's modulus) gave the different in film characteristics. The parameter was shown the strength and elasticity of the film. A soft and weak polymer was characterized by a low tensile strength, low % elongation and Young's modulus; a hard and brittle film was defined by a moderate tensile strength, low % elongation and high Young's modulus, a soft and tough film was determined by a moderate tensile strength, high % elongation and low Young's modulus finally, a hard and tough film was characterized by high tensile strength, % elongation and Young's modulus (Peh and Wong, 1999). In addition, the Facial-patch should have a moderate tensile strength, low Young's modulus and high % elongation.

Table 4 Formulations of PG Facial-Patch Containing Different Concentration of Glycerin

Ingredients	Concentration of ingredients in facial-patch formula				
	1	2	3	4	5
PG	4	4	4	4	4
Propylene glycol	4.75	4.75	4.75	4.75	4.75
Glycerin (% based on PG)	-	5 (0.2)	10 (0.4)	15 (0.6)	20 (0.8)
DI water qs.to	100	100	100	100	100

Table 5 Formulation of PG Facial-Patch Containing Different Concentration of Sorbitol

Ingredients	Concentration of ingredients in facial-patch formula			
	6	7	8	9
PG	4	4	4	4
Propylene glycol	4.75	4.75	4.75	4.75
Sorbitol (% based on PG)	5 (0.2)	10 (0.4)	15 (0.6)	20 (0.8)
DI water qs.to	100	100	100	100

Table 6 Mechanical Property Data of Film No.1-9 (mean ± SD, n = 5)

Formula	Thickness (mm.)	Tensile strength (Mpa)	%Elongation	Young's Modulus (Mpa)	Work of failure (MJ)	Appearance of the patch
1	0.06±0.002	33.75±10.60	15.32±2.852	315.2±57.9	1.368±0.514	Hard, brittle
2	0.05±0.002	37.24±7.65	13.05±2.294	405.1±21.11	1.016±0.4215	Hard, brittle
3	0.06±0.001	29.08±6.52	12.29±2.427	350.5±41.08	0.825±0.4072	Hard, brittle
4	0.09±0.002	16.23±1.520	49.82±9.94	95.80±17.02	4.749±1.132	Soft, tough
5	0.05±0.001	29.97±2.506	13.58±1.671	376.3±19.55	0.688±0.1195	Hard, brittle
6	0.05±0.002	42.34±4.113	21.50±3.283	356.1±31.23	2.430±0.509	Hard, brittle
7	0.05±0.003	32.19±3.897	32.28±5.94	245.6±42.50	3.147±0.961	Soft, tough
8	**0.05±0.003**	**29.14±2.15**	**45.81±7.04**	**177.3±25.54**	**4.096±0.700**	**Soft, tough**
9	0.08±0.002	9.70±0.899	86.90±14.46	18.23±5.12	4.008±0.771	Soft, tough

In Table 6, the formula 8 which contained 4% PG, 4.75% propylene glycol, 15 % sorbitol solution demonstrated the moderate tensile strength, high % elongation but low Young's modulus, the film was soft and tough.

The plasticizer effect the film property to make them softer and more flexible depend on the decrease in the glass transition temperature of the polymer. The combined plasticizer produced more satisfactory film property than one plasticizer used.

3.2 Determination of a Suitable Concentration of PG

According to the film no.8 was selected the formula composed of 15% w/w sorbitol and 4.75% w/w of propylene glycol. The concentrations of 4, 5, 6 % w/w of PG were prepared in film no. 8, 10, 11. The thickness of film No.8, 10, 11 were 0.06±0.003, 0.06±0.003 and 0.12±0.003 mm. respectively. The mechanical properties of all the three films were shown in Table 7. The formula no.8 and no.10 gave the similar mechanical

properties of film in the tensile strength value and % elongation but have a little different in Young's modulus value. The film No.8 was more brittle than film No.10. In addition, film No.11 was most satisfactory property film for facial-patch.

Table 7 Mechanical Properties Data of Film No.8, 10 and 11 (mean ± SD, n = 5)

Formula	% PG	Thickness (mm.)	Tensile strength (Mpa)	%Elongation	Young's modulus (Mpa)	Work of failure (MJ)	Appearance of the patch
8	4	0.06±0.003	53.10±4.03	20.69±2.032	361.10±6.39	3.031±0.81	Hard, brittle
10	5	0.06±0.003	55.20±5.01	31.16±2.447	332.2±15.63	6.08±0.725	Hard, strong
11	6	0.12±0.003	19.24±0.78	36.96±4.201	124.4±5.38	5.17±0.698	Soft,tough

3.3 Determination of the Amount of PG Aqueous Solution for Casting

The facial-patch thickness is important to give the good satisfactory application. The facial-patch should be an appropriate thickness. The PG mixture solution in the formula 11 was chosen to cast in the amount of 8, 10 and 12 g and were cast into the 9 mm diameter petridish as film No. 12, 13 and 14. The thickness of the films were 0.04±0.005, 0.07±0.005 and 0.08±0.005 mm. respectively. The mechanical properties of the film were shown in Table 8.

Table 8 Mechanical Properties Data of Film No.12, 13 and 14 (mean ± SD, n = 5)

Formula	Amount of PG mixture (g)	Thickness (mm.)	Tensile strength (Mpa)	%Elongation	Young's Modulus (Mpa)	Work of failure (MJ)	Appearance of the patch
12	8	0.04±0.005	65.90±13.28	17.68±1.441	530.00±44.98	2.322±0.55	Hard, brittle
13	10	0.07±0.005	39.21±6.640	35.12±7.25	250.9±16.46	5.61±2.61	Soft,tough
14	12	0.08±0.005	55.4±5.60	31.76±1.571	283.2±11.79	7.79±1.07	Soft,tough

In Table 8, the film No. 13 that contained 4% w/w PG, 15% v/v sorbitol and 4.75% w/w propylene glycol was selected based on the amount of mixture solution which performed the greatest mechanical properties value and appearance had a suitable thickness of the film.

4. Preparation of PG Facial-Patch Backing Layer

4.1 Determination of a Suitable Film Forming for Backing Layer

The polymeric film forming agents that were used to form the backing layer were shown in Table 9. The backing layer should be compatible to the drug and other excipients in the formulation. The most useful backing materials are conform with the skin and provide sufficient resistance to transepidermal water loss to allow some hydration of the stratum corneum(Aulton,2002). The suitable film forming agent was selected from the formula containing each of 2 %w/w of Polyvinyl alcohol (PVA) 72,000 cps, Polyvinylpyrrolidone K-90 (PVP-K90), Polyvinylpyrrolidone K-30 (PVP-K30), Methylcellulose (MC) 100 cps and 20% w/w glycerin. In the formula containing 2% w/w of Ethylcellulose (EC) 45 the 20% dibutyl phthalate was used as plasticizer

Table 9 The Ingredient of Backing Layer Film (DBP=Dibutyl phthalate)

Ingredients	PVA	PVP K-90	PVP K-30	MC	EC
% of polymer	20	20	20	20	20
DBP	-	-	-	-	20
Glycerin (% based on polymer weight)	20	20	20	20	20
DI water qs.to	100	100	100	100	-
Ethanol qs.to	-	-	-	-	100
Appearance of film	Tough, Greasy	Non-film form	Non-film form	Transparent film	Transparent film

The MC and EC film introduced the good appearance that suitable for using in backing layer. In the study of Guo (1998), the ethylcellulose gave an effective in retarding drug release from the matrix systems. The EC is a hydrophobic polymer, has very low water permeability and moderate flexibility. Therefore, the EC was chosen to use as backing layer.

4.2 Determination the Concentration of Film Forming for Backing Layer

The concentration of 2, 5, 10 % w/w ethylcellulose (45cps) was dispersed in absolute ethanol, 10% w/w dibutyl phathalate (DBP) as plasticizer was added. The 5 % w/w of the EC gave the best characteristic of the film.

4.3 Determination of the Amount of Plasticizer for Backing Layer

The 10, 20 and 30% w/w of DBP were added to selected the suitable concentration to form the best backing layer. The 30% w/w of the DBP gave a satisfactory film with soft and tough.

4.4 Determination of the Amount of EC Solution for Backing Layer

The amount of EC in ethanol solution for casting the film that gave the appropriate thickness of the PG Facial-patch was 5 g.

5. Preparation of PG Facial-Patch Containing Asiaticoside

The film base No.13 was selected as PG facial-patch film base and the 5% EC was selected as a backing layer. The concentration of asiaticoside in facial-patch was analyzed by HPLC method. The concentration of asiaticoside was 20 mcg/cm^2.

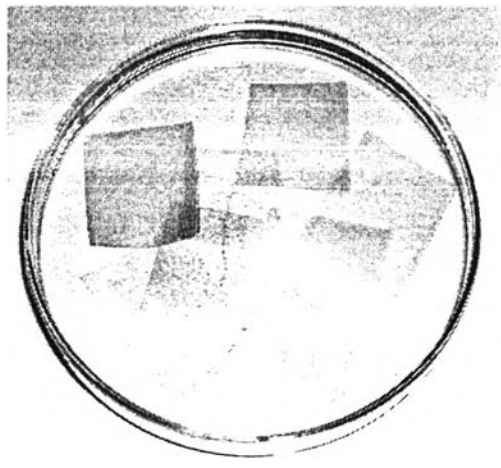


Figure 9 Polysaccharide Gel Facial-Patch Formula No.15

6. Physical Evaluation of PG Facial-Patch

The PG Facial-patch containing asiaticoside gave a translucent pale brown color film. The mechanical properties of PG facial-patch containing asiaticoside were shown in Table 10.

Table 10 Mechanical Properties Data of Film Formula No.15

Formula	Thickness (mm.)	Tensile strength (Mpa)	%Elongation	Y-mod (Mpa)	Work of failure (MJ)
15	0.07±0.002	50.3±4.32	42.4±6.31	246.1±14.21	3.12±4.3

7. HPLC Analysis

The calibration curve of standard asiaticoside was shown in Figure 9. The data of validation of HPLC method of asiaticoside was shown in Appendix I.

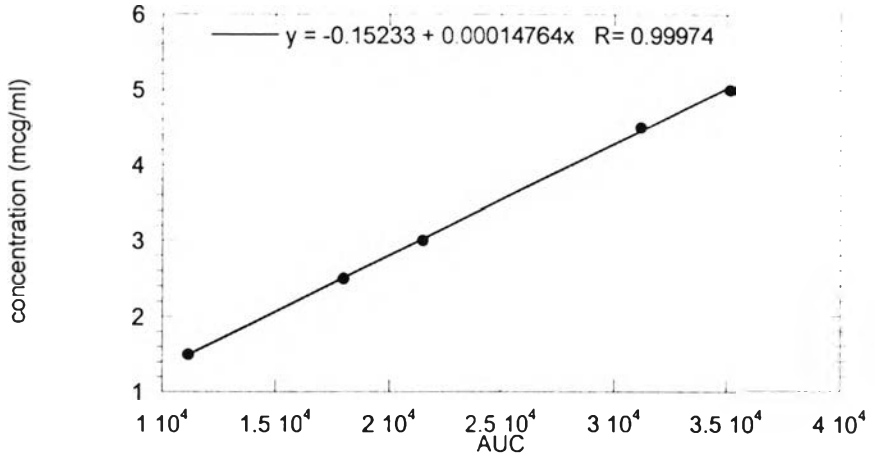


Figure 10 Calibration Curve of the Standard Asiaticoside

8. *In Vitro* Diffusion Study of Asiaticoside from the PG Facial-Patch

The *in vitro* release of asiaticoside from PG facial-patch was studied using modified Franz's diffusion cell. The release profile of the PG Facial-patches containing asiaticoside (n=3) were shown in Table 11. The concentration of asiaticoside in PG Facial-patch was 20 mcg/cm^2. It can be released 89.5% within 1 h.

Table 11 The Cumulative Amount of Asiaticoside Release from PG Facial-Patch

Time (min)	Concentration (mcg/ml)	Concentration at different Time(mcg/ml)	Concentration per area (mcg/ml/cm ^{2})	Cumulative Amount (mcg/cm^2)
0	0	0	0	0
30	0.76	0.76	0.378	4.537
45	2.71	2.77	1.379	16.557
60	2.71	2.999	1.492	17.905

9. *In Vivo* Skin Irritation of PG Facial-Patch

9.1 Draize Skin Irritation Test Method

The PG facial-patch containing asiaticoside were intact on the 6 rabbits that one site was abraded skin and other sites were normal skins. The result was shown in Table 12. The distilled water used as control. The score following the criteria in Table 2.

Table 12 Primary Skin Irritation Results in Normal Site of Rabbit

Rabbit No.	1			2			3			4			5			6		
Introduction Weight (kg)	2.8			2.9			3.0			3.1			3.2			3.2		
Hours	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
	Skin Irritation Score																	
I. Erythema and eschar formation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
II. Edema formation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The irritation were shown in 6 rabbits with abraded skin in the degree of +1 (very slightly edema)

Table 13 Primary Skin Irritation Results in Abraded Site of Rabbit

Rabbit No.	1			2			3			4			5			6		
Introduction Weight (kg)	2.8			2.9			3.0			3.1			3.2			3.2		
Hours	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
	Skin Irritation Score																	
I. Erythema and eschar formation	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1
II. Edema formation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

9.2 Prophetic Patch Testing

The 2 volunteers in 200 Thai healthy volunteers were irritated when used PG patch, the rest of them was non-irritation when used student's T-test to compare data. There was not significant difference in data. The statistical data was shown in appendix 2.

10. *In Vivo* Skin Efficacy of PG Facial-Patch

Moisturizing Effect of PG Facial-Patch Containing Asiaticoside

Skin hydration was evaluated by DermaLab® Moisture probe. Transepidermal water loss (TEWL) was measured. The forehead was used as tested area. The difference of TEWL was shown in Table 13.

Table 14 The TEWL Value of Skin on Forehead in 20 Volunteers

Subject No.	TEWL Value		
	Week 0	Week 2	Week 4 *
1	15.49	11.27	8.97
2	12.13	11.47	8.10
3	12.53	11.47	15.53
4	5.53	5.97	7.90
5	11.30	6.73	10.87
6	13.30	11.97	6.07
7	26.77	9.53	12.00
8	8.30	7.33	5.57
9	7.23	5.77	6.60
10	13.53	11.57	6.33
11	25.60	20.40	12.07
12	12.63	14.57	9.30
13	9.23	8.10	9.53
14	7.33	7.93	6.83
15	6.33	3.83	3.37
16	6.27	4.70	3.43
17	8.10	3.93	3.63
18	15.97	4.40	3.83
19	15.30	8.47	4.83
20	8.33	4.40	4.00
Mean±SD	12.06±5.84	8.69±4.2	7.44±3.38

* = Significant difference ($p < 0.005$)

From the Table 14, the p-value < 0.005, therefore the TEWL value was significantly decreased. This result can explain that the moisture was more up taken into the skin, thus the PG facial-patch can keep the moisture content onto the skin.

The Elasticity Measurement using DermaLab® moisture probe

The elasticity (Young's modulus value) were measured by DermaLab® moisture probe, the differences of Young's modulus between week 0 and 4 were calculated. The wrinkle was observed using macroscopic photography. Three measurements were performed in each testing and were shown in Table 15. The Young's

modulus was reduced as a result to PG facial-patch which can increase the elasticity of the skin significantly (p- value < 0.05).

Table 15 The Young's Modules of Skin on Forehead in 20 Volunteers

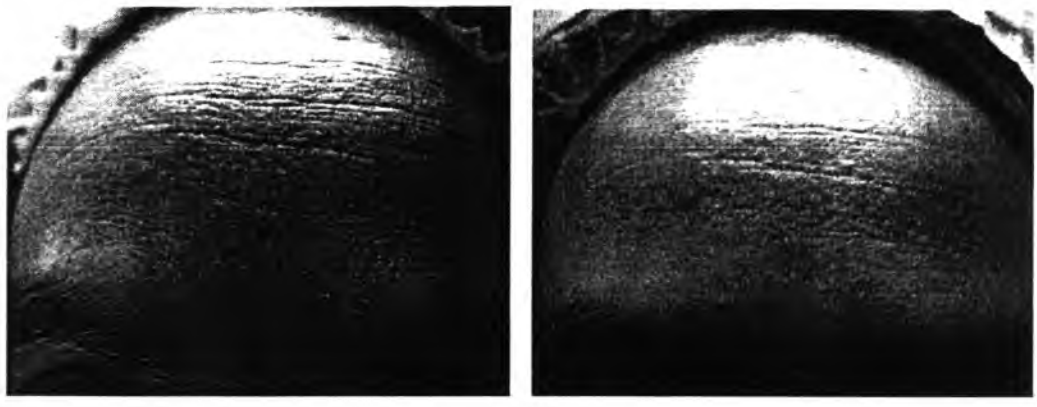
SUBJECT	Young's Modules		
	Week 0	**Week 2**	**Week 4 ***
1	12.97	8.85	9.41
2	14.99	15.31	13.43
3	13.9	13.16	10.2
4	9.37	12.6	10.78
5	4.2	4.49	3.99
6	12.56	11.83	9.37
7	11.62	11.16	11.28
8	12.49	13.14	10.45
9	12.51	12.99	12.47
10	13.64	14.12	13.22
11	10.39	10.97	12.06
12	11.03	7.83	8.12
13	14.28	14.35	14.51
14	13.56	13.26	11.78
15	13.53	12.95	11.25
16	14.1	13.35	12.17
17	11.18	10.33	10.2
18	15.31	8.12	7.87
19	12.99	13.28	12.8
20	9.56	9.2	7.2
Mean±SD	12.21±2.52	11.56±2.70	10.63±2.49

*=Significant Difference ($p < 0.005$)

The Young's modulus value of the skin was reduced. Thus, the elasticity of the skin is increasing resulted in reducing the wrinkle on the forehead that confirm by the photograph of the skin as shown in figure 11.

The Wrinkle Line Defined by Macroscopic photography

From the photograph of the wrinkle at the forehead area in 20 volunteers compared between before and after 4 weeks application. The PG patch can reduce the wrinkle line significantly from the observation. The photograph was shown in figure 11.



(a) **(b)**

Figure 11 The Macroscopic Photograph of the Wrinkle Line : (a) before application (b) and after application