CHAPTER IV

RESULTS AND DISCUSSION

1. Extraction and Isolation of Chemical Components from *C.asiatica*

1.1 Extraction of Chemical Components from ***C.asiatica*** by using Ethanol, Hexane, Chloroform and Isobuthanol.

The crude extract from fresh leaves of ***C. asiatica*** was collected as a dry powder. Each batch was weight of 16.6 gm. Then it was kept for further examination for the composition of chemical component.

1.2 Extraction of Chemical Component from ***C. asiatica*** using Ethanol Chloroform and Buthanol and Purification.

The weight of 18.5 kilogram of clean fresh leaves of C. ***asiatica*** was macerate with ethanol. The dried extract of ethanol was received as 8 kilogram and chloroform extract was received as 3 kilogram. The final buthanol extract was 500 gram. The purified asiaticoside with white crystalline was 8 gram.

2. Characterization of **Asiaticoside** from ***C. asiatica***

2.1 Melting Point Determination

The purified **asiaticoside** received by recrystallization in methanol as a white crystalline needle gave melting point of 235-237° C

2.2 UV Visible Spectroscopy

The ethanol extract and hexane extract from method 1.1 were investigated using UV visible spectroscopy scanning the UV spectrums of ethanol and hexane extract were shown in figure 1A and 1B in Appendix I. The ethanol extract presented maximum peak at 323 nm and the shoulder around 220 nm while hexane extract could not find the peak. This indicated that in ethanol extract contains some **asiaticoside** while could not find in hexane extract. Figure 1C presented UV spectrum of chloroform extract in the third steps in method 1.1. The spectrum show more than 1 peak, it demonstrates the peak at 201, 205 and 322 nm. in isobuthanol extract it shows only one peak at 322 nm. as shown in Figure 1D. From crude extract asiaticoside cannot identify by using UV spectrophotometer. This finding results leading to change the solvent for extraction asiaticoside. Though the solvent used to extract asiaticoside was chloroform and buthanol.

The another method for extraction as in 1.2 was further study. The purified asiaticoside received from buthanol extract and recrystallized in methanol showed maximum absorption at 220 nm. as shown in Figure 1E.

2.3 High Resolution of LC-MS Analysis

The crude extract from fresh leaves of ***C. asiatica*** was examined for the chemical component by using LC-MS connected with electrospray ionization. The fingerprint of the extraction from fresh ***C. asiatica*** leaves and crude extract from drug store were shown in Figure 13 and 14 respectively. The sample prepared from fresh leaves of ***C. asiatica*** showed the **asiaticoside** of higher amount within the sample which has the molecular weight of 980.82 in which electrospray ionization contain $[M+Na^+]$then asiaticoside will show molecular weight of sodium salt whilst molecular weight of **asiaticoside** in Merck Index was 959.13. The sample of crude extract from drug store showed small amount of **asiaticoside**.

2.4 HPLC Analysis of **Asiaticoside**

Table 3 presents the peak area response of standard solution of **asiaticoside** and Figure 15 showed the calibration curve of **asiaticoside**. Linear regression analysis of the peak area response was performed with a coefficient of determination (R^2) of 0.9192

The HPLC method was also used to determined **asiaticoside** content in the extraction of each successive fraction. Figure 16 shows chromatogram of

asiaticoside in ethanol extract of fresh *C. asiatica* leaves, figure 17 shows chromatogram of **asiaticoside** in isobuthanol extract of fresh *C. asiatica* leaves. The chromatogram of authentic asiaticoside received from France was shown in figure 18 and the chromatogram of purified of **asiaticoside** from fresh *C. asiatica* leaves with recrystallized in methanol was shown in figure 19. **Asiaticoside** could not find in hexane extract.

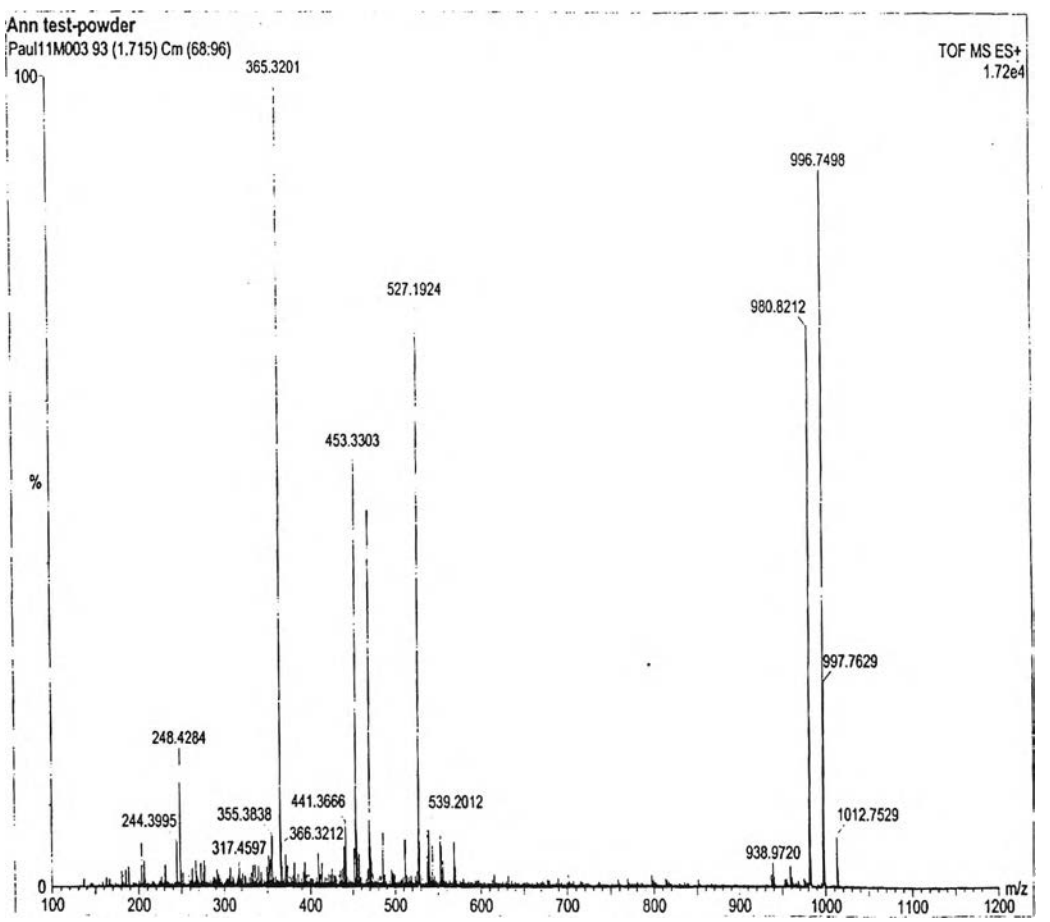

Figure 13 Chromatogram of high resolution LC-MS and electrospray ionization of extract from fresh leaves of *C. asiatica*

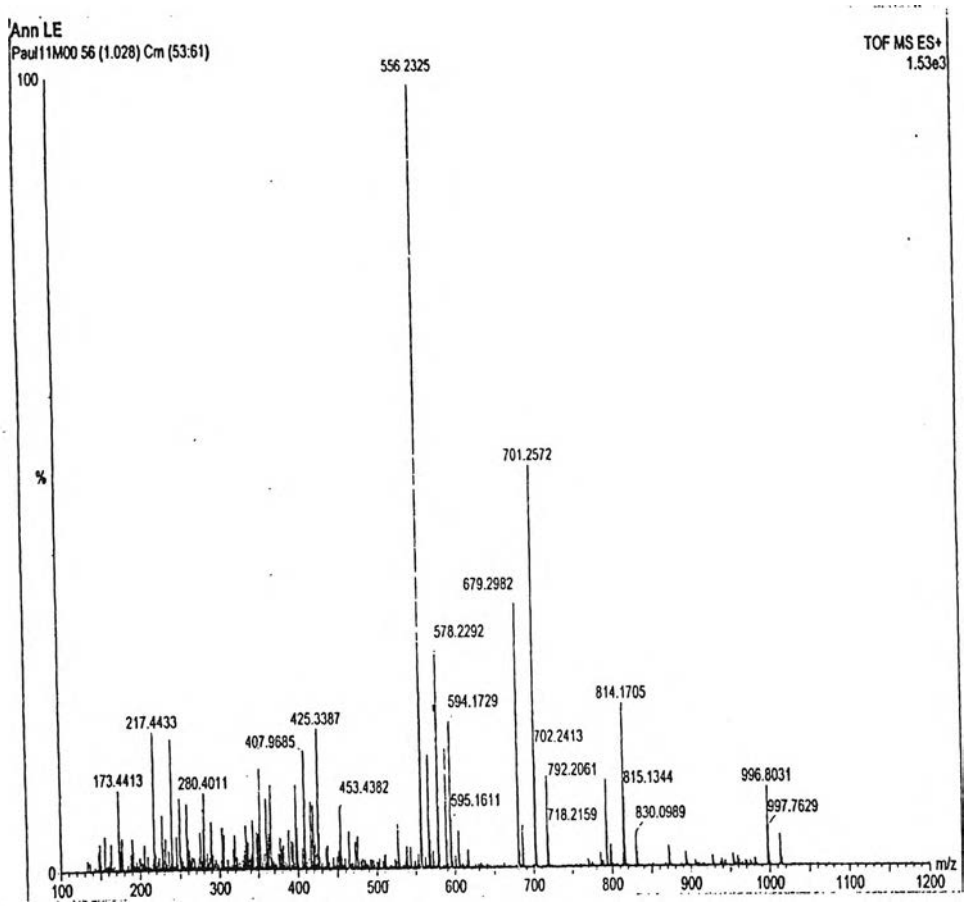

Figure 14 Chromatogram of high resolution LC-MS and electrospray ionization of ***C. asiatica*** extract received from drug store

Table 3 The peak area response of authentic **asiaticoside**

Concentration (mg/ml)	0.2	0.4	0.6	0.8
Area response under curve (1)	105670	1755465	10050015	11955421
Area response under curve (2)	104875	1720482	9874892	12038078
Area response under curve (3)	106465	1685499	10225138	12120735
Average	105670	1720482	10050015	12038078

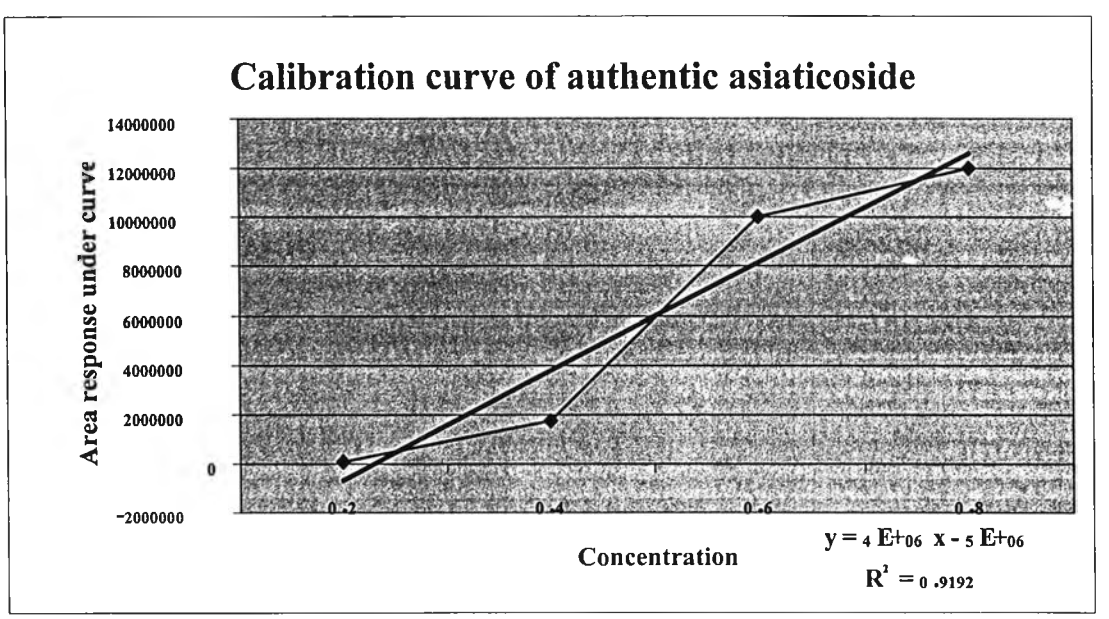

Figure 15; The calibration curve of **asiaticoside**

External Standard Report

Asiaticoside in Ethanal extract of fresh *Centella asiatica*

Method: C:\CLASS-VP\anne\methodAnne.met

Data File: C:\CLASS-VP\anne\Suvipha\11method1
User: **Suvipha**
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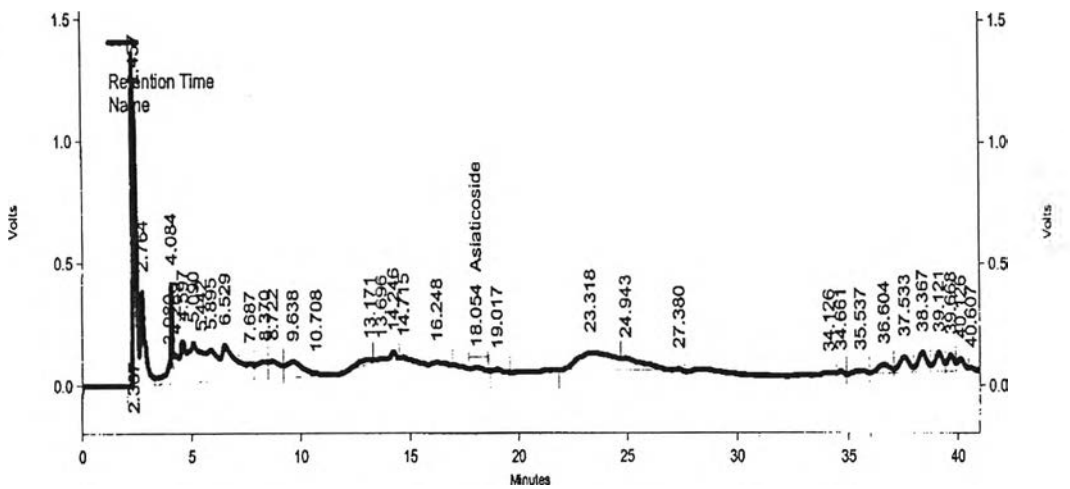

Detector A - 1 (220nm)

Pk #	Retention Time	Name	Area
22	18.054	Asiaticoside	207184

Totals			207184

External Standard Report

Asiaticoside in Isobuthanal extract of fresh *Centella asiatica*

Method: C:\CLASS-VP\anne\methodAnne.met

Data File: C:\CLASS-VP\anne\Suvipha\10method1
User: **Suvipha**
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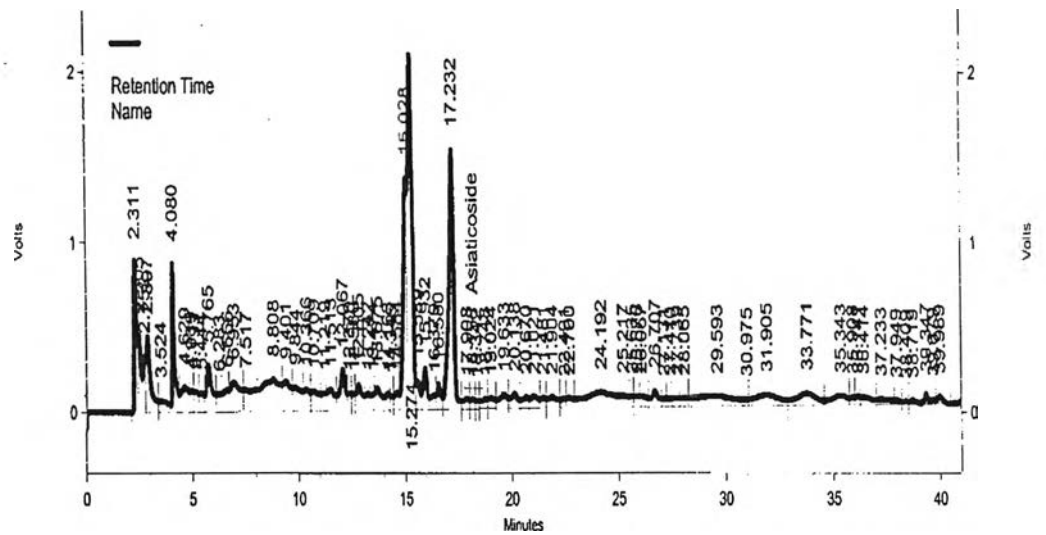

Detector A - 1 (220nm)

Pk #	Retention Time	Name	Area
41	18.158	Asiaticoside	792692

Totals			792692

Figure 17 Chromatogram of **asiaticoside** in isobuthanol extract of fresh ***C. asiatica*** leaves

External Standard Report

Asiaticoside1

Method: C:\CLASS-VP\anne\methodAnne.met
Data File: C:\CLASS-VP\anne\A1\Asiaticoside.stand.0.02-1
User: **Suvipha**
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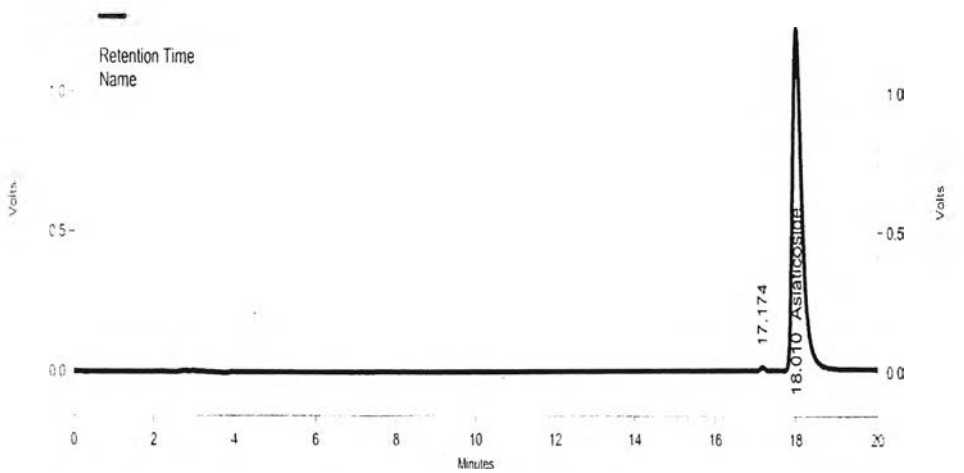

Detector A - 1
(220nm)

Pk #	Retention Time	Name	Area	ESTD concentration
2	18.010	Asiaticoside	19562187	0.020 CAL
Totals			19562187	0.020 CAL

Figure 18; Chromatogram of purified standard of **asiaticoside** from authentic drug at the retention time = 18.010 minutes.

External Standard Report

The purified standard of asiaticoside in concentration 0.02 g./ml.

Method: C:\CLASS-VP\anne\methodAnne.met

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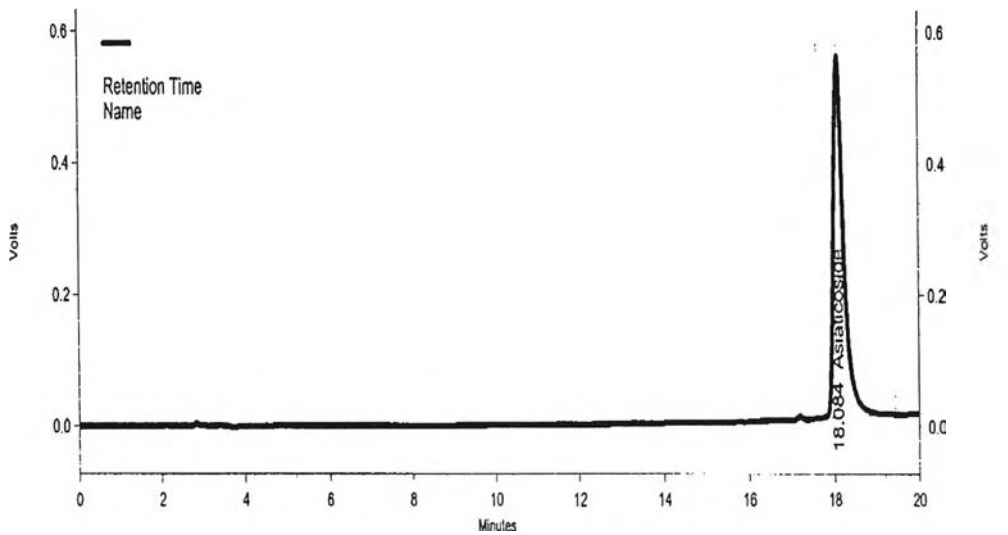


Detector A - 1 (220nm)

Pk #	Retention Time	Name	Area
1	18.084	Asiaticoside	9304810
Totals			9304810

Figure 19 Chromatogram of purified **asiaticoside** received from successive of fresh ***C. asiatica*** with recrystallization **asiaticoside**.

3. Formulation of Microemulsion Gel Containing 1% Asiaticoside

Six formulae of microemulsion gels were prepared according to the method of Ubonthip N., et al 2001. Formula 1 contained capric/caprylic triglyceride, which was the medium chain triglyceride. It is the component that found in epidermis. Formula 2 contained soy bean oil which is long chain triglyceride as same as formula 3. These formulae contained the same surfactant, which was polyoxyethylene 10-oleye ether, but different in the amount used. The physical appearance of three formulae was transparent with low viscosity. When **asiaticoside** was added the viscosity was increased and form gel. Formula 4, 5 and 6 contained the same oil that was isopropyl myristate but difference in surfactants. Formulation 4 contained polyoxyethylene 40 stearate while formulation 5 and 6 contained polyoxyethylene 20 sorbitan monooleate in different concentration. The physical appearance of formula 4 showed a transparent gel, after **asiaticoside** was added it still showed the same appearance as same as in formula 6. In formula 5 the physical appearance show low viscosity **asiaticoside** could not dissolved in formula 5. There are the precipitations of **asiaticoside** in the formula.

3.1 Stability Studied

All of six formulae of asiaticoside microemulsion gels were tested. Only formula 1,3 and 6 were passed 3 freeze-thaw cycles without separation of oil.

3.2 Viscosity Measurement

The viscosity of formula 1, 3 and 6 were measured at room temperature. The viscosity was shown in Table 4

Table 4 Viscosity of asiaticoside microemulsion gel of formula 1, 3 and 6

Formula	Viscosity (mPas)
1	1056.64
3	1536.77
6	15460.48

3.3 The pH Measurement

The pH of asiaticoside microemulsion gels were measured the pH was shown in Table 5

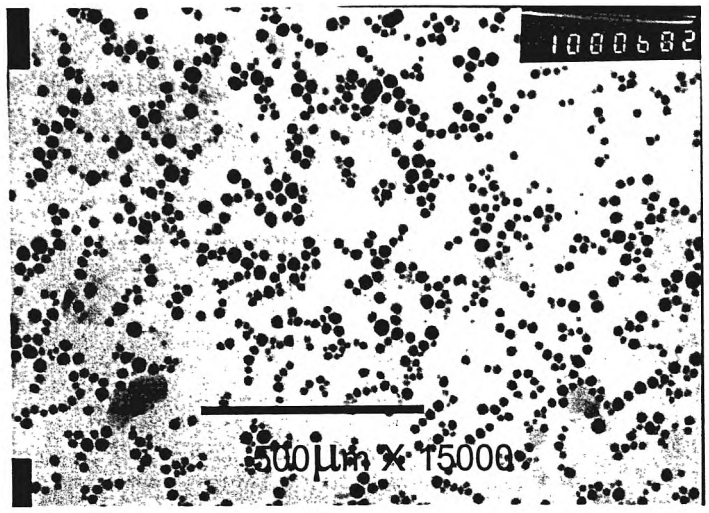
Table 5; pH of **asiaticoside** in microemulsion gel

Formula 1	pH
1	4.5
2	5.5
3	4.5
4	6.4
5	6.4
6	4.5

3.4 Transmission Electron Microscopy

The size and morphology of **asiaticoside** in microemulsion droplets were investigated by using transmission electron microscope. The sizes were uniform with the size range, which between 10-80 nm as present in Figure 20.

a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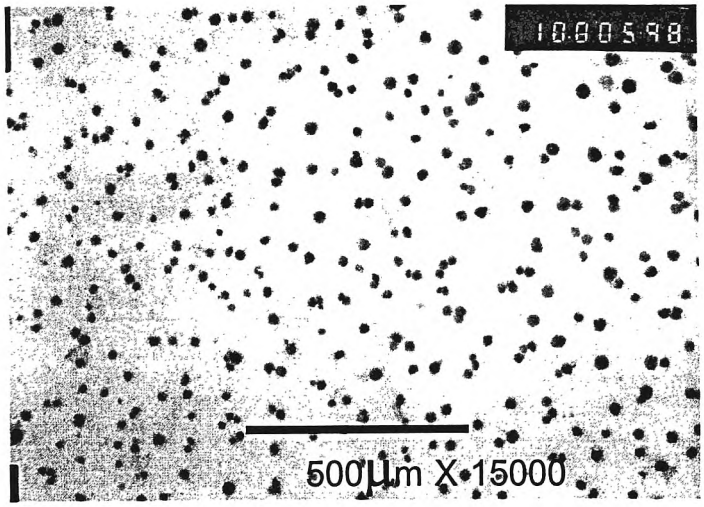
b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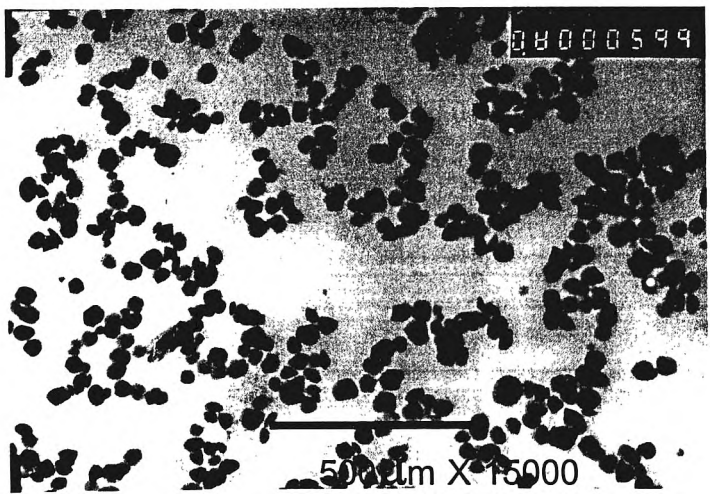
500µm X 15000

c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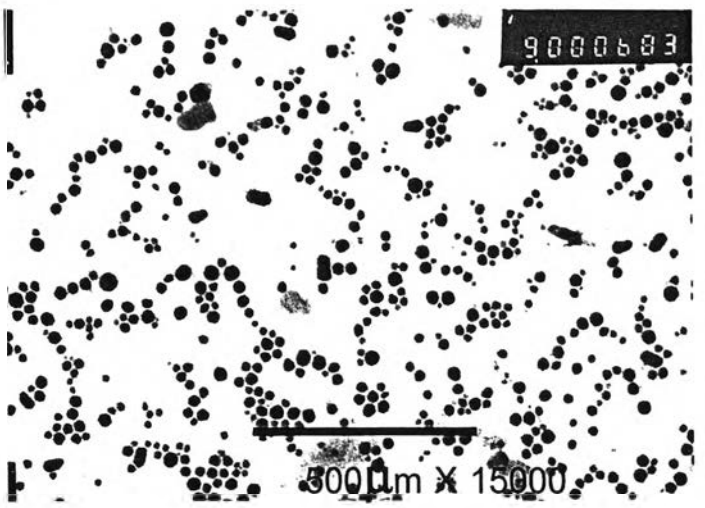
500µm X 15000

d



e

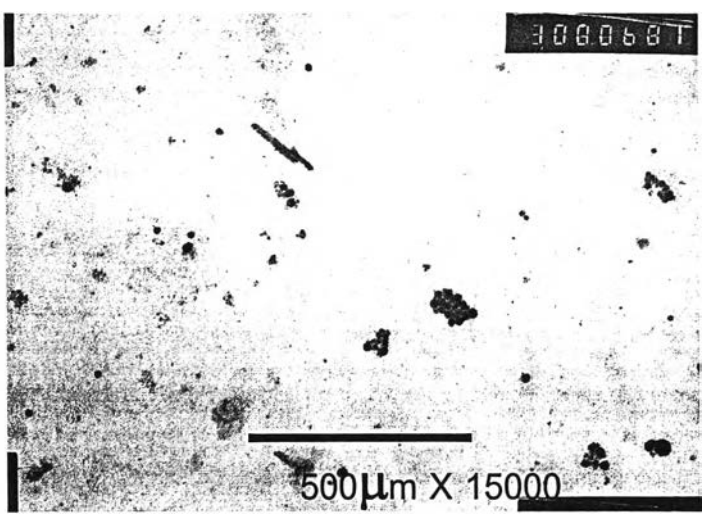


Figure 20; Transmission electron micrograph of **asiaticoside** microemulsion gel

(The magnification x 15,000)

a) Authentic **asiaticoside** in formula 1

b) Purified **asiaticoside** in formula 1

c) Purified **asiaticoside** in formula 3

d) Purified **asiaticoside** in formula 6

e) Crude extract from drug store in formula 1

4. Permeation Study

The permeation of **asiaticoside** in Franz cell was determined by analysis the amount of **asiaticoside** penetrated through the shed snake skin as shown in Table 7 the cumulative amount of **asiaticoside** at 72 hours from Formula 1 containing authentic **asiaticoside** form France and purified asiaticoside from fresh ***C. asiatica*** leaves extract illustrated higher amount of 67.7 mg and 62.3 mg respectively which is higher than formula 6 and formula 3. The cumulative amounts of purified **asiaticoside** in formula 3 and 6 were 42.9 mg and 54.5 mg respectively. The asiaticoside could not find in formula 1 containing crude extract of ***C. asiatica*** from drug store. The flux or the amount of **asiaticoside** penetrate per unit area per hour were calculated. The permeation profile of **asiaticoside** in 5 different formulas was shown in Figure 21, 22, 23, 24 and 25 respectively. Formula 1 containing authentic asiaticoside form France illustrated higher flux than formula 1 containing purified **asiaticoside** from fresh leaves extract with significant difference. It also showed the different of flux between 5 formula which degree of permeation was following, formula 1 containing **asiaticoside** from fresh ***C. asiatica*** leaves extract > formula 1 containing authentic **asiaticoside** from France > formula 6 containing purified **asiaticoside** from fresh leaves extract > formula 3 containing purified **asiaticoside** from fresh ***C. asiatica*** leaves extract > formula 1 containing crude extract of ***C. asiatica*** from drug store. From these result formula 1 was the best formula for preparing **asiaticoside** microemulsion gel.

From figure 20 the difference particle size of asiaticoside microemulsion gel was corresponded to the permeability of asiaticoside through shed snake skin. The particle of microemulsion gel formula 1 contained authentic **asiaticoside** received from France and purified **asiaticoside** from fresh ***C. asiatica*** leaves were smaller than the particle size of **asiaticoside** microemulsion gel formula 3. The particle size of purified **asiaticoside** in microemulsion gel formula 6 was the biggest resulted in lower diffusion of **asiaticoside** through shed snake skin. Then the rate of penetration was lower. From figure 20e, there was small amount of **asiaticoside** in microemulsion gel resulted in no penetration of **asiaticoside** through shed snake skin.

The rate of penetration or flux of **asiaticoside** in authentic **asiaticoside** microemulsion formula 1 and purified **asiaticoside** from fresh ***C. asiatica*** leaves in microemulsion formula 1 were higher than formula 6 and formula 3 respectively. No **asiaticoside** from crude extract of ***C. asiatica*** passed through shed snake skin.

The flux of 5 formulae were significant difference when analysis with method quadrati as shown in appendix V.

The composition of microemulsion gel formula 1 contained higher amount of oil phase than formula 3 and formula 6 which were 15 %, 10 % and 5 % respectively resulted in formula 1 have the more lipophilicity to the skin than formula

3 and formula 6 while the nature of skin comprise of skin lipid which was triglyceride.

Table 6; Cumulative amount of **asiaticoside** penetrate through Franz cell

Formula	Cumulative amount (mg)				Slope	Flux
	1 hr	24 hr	48 hr	72 hr	mg/hr	$mg/cm^2/hr$
Formula 1 with authentic **asiaticoside** from France	40.30 48.70 31.80	37.90 32.80 43.00	65.00 64.20 73.00	67.70 62.40 73.00		
Mean	40.30	37.90	65.00	67.70	0.62	0.31
Formula 1 containing purified **asiaticoside** from fresh ***C.asiatica*** leaves extract	28.90 21.70 36.10	35.10 39.40 30.80	53.50 61.10 45.90	62.30 52.40 72.20		
Mean	28.90	35.10	53.50	62.30	0.50	0.25
Formula 3 containing purified **asiaticoside** from fresh ***C.asiatica*** leaves extract	25.70 24.80 26.60	28.80 30.10 27.50	37.70 31.40 44.00	42.90 50.20 35.60		
Mean	25.70	28.80	37.70	42.90	0.26	0.13
Formula 6 containing purified **asiaticoside** from fresh ***C.asiatica*** leaves extract	27.90 31.40 24.40	34.30 31.10 37.50	53.50 60.90 46.10	54.50 50.60 58.40		
Mean	27.90	34.30	53.50	54.50	0.42	0.21
Formula 1 containing crude extract of ***C.asiatica*** from drug store	UN	UN	UN	UN		
Mean	UN	UN	UN	UN	UN	UN

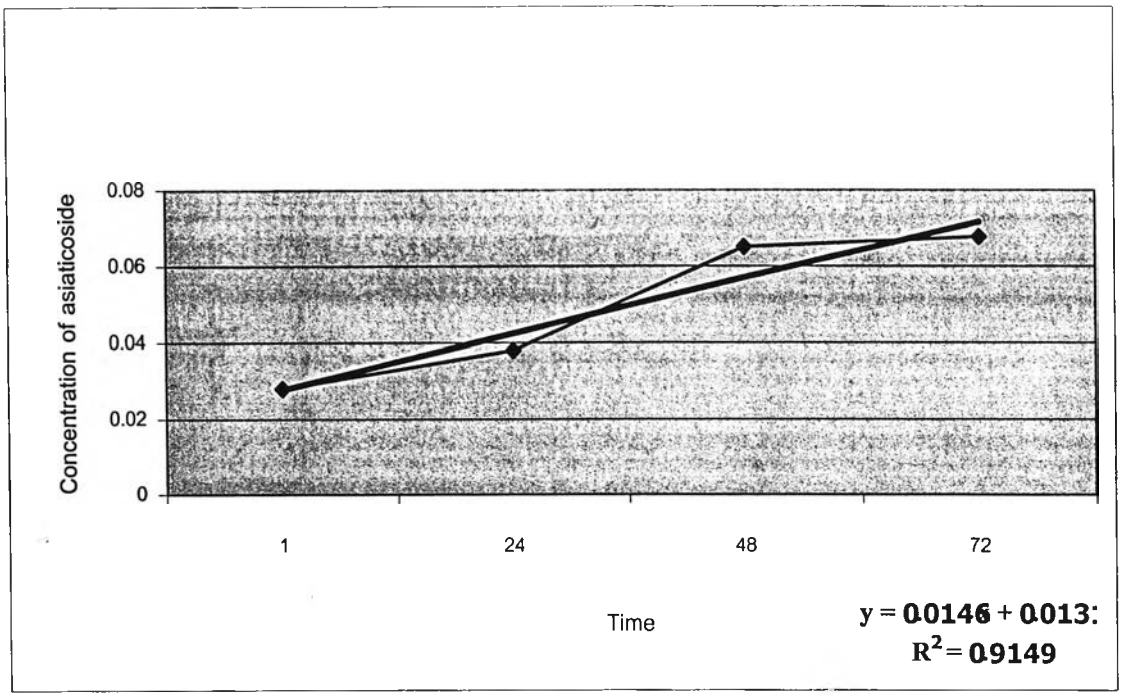


Figure 21 Permeation profile of authentic **asiaticoside** from France

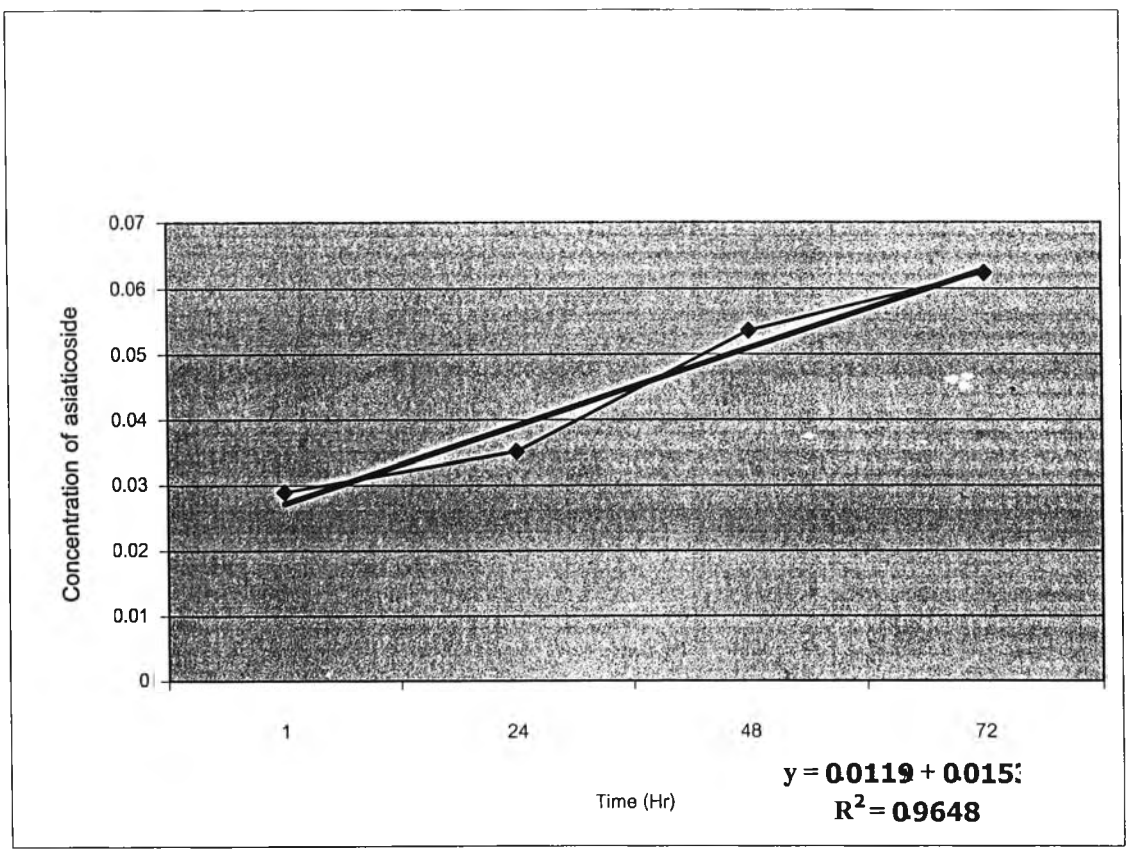


Figure 22 Permeation profile of purified **asiaticoside** from fresh leaves extract in formula 1

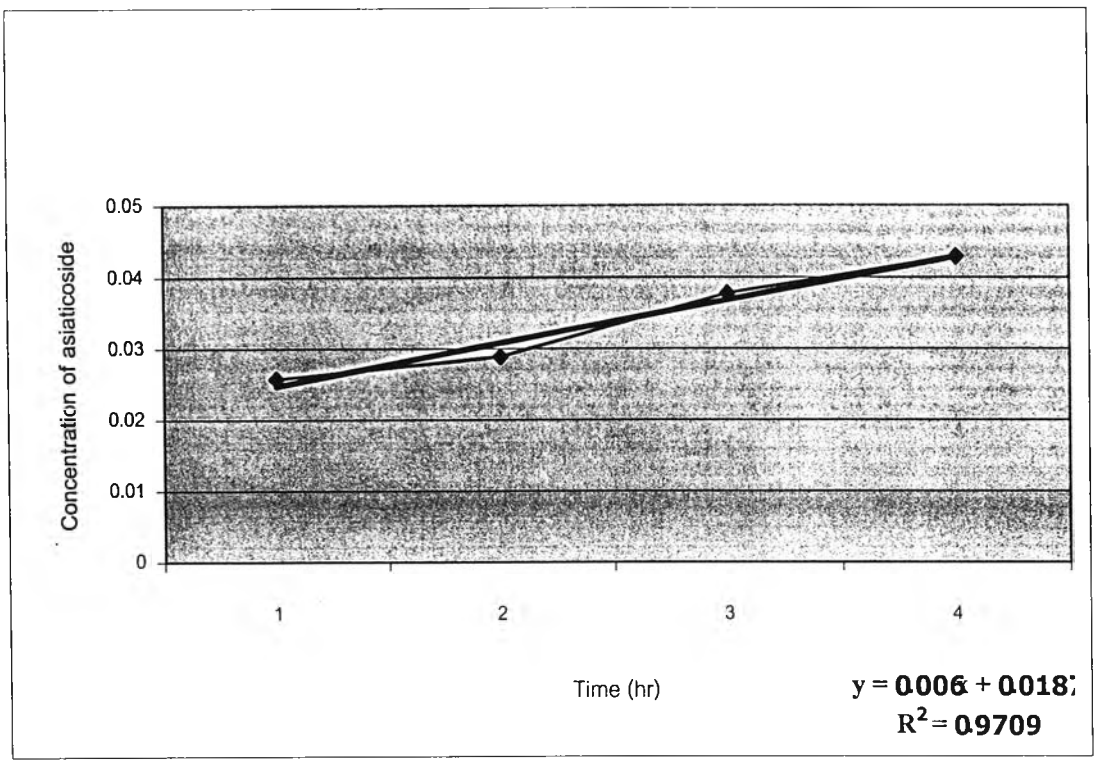


Figure 23 Permeation profile of purified **asiaticoside** from fresh leaves extract in formula 3

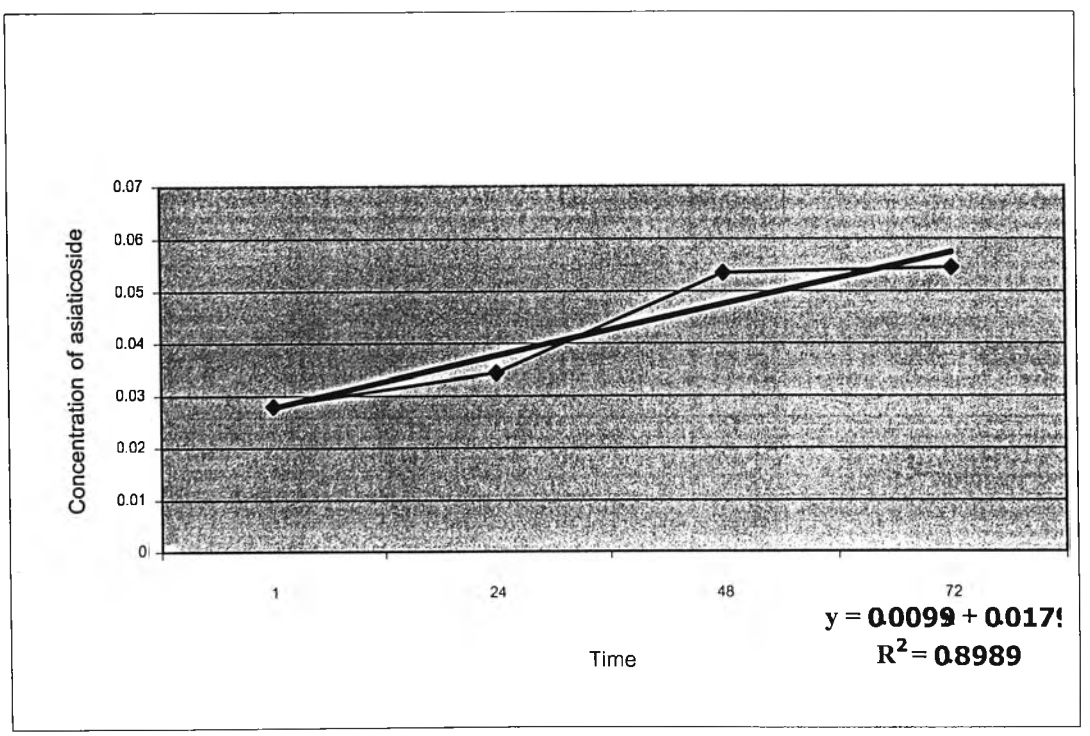


Figure 24; Permeation profile of purified **asiaticoside** from fresh leaves extract

in formula 6

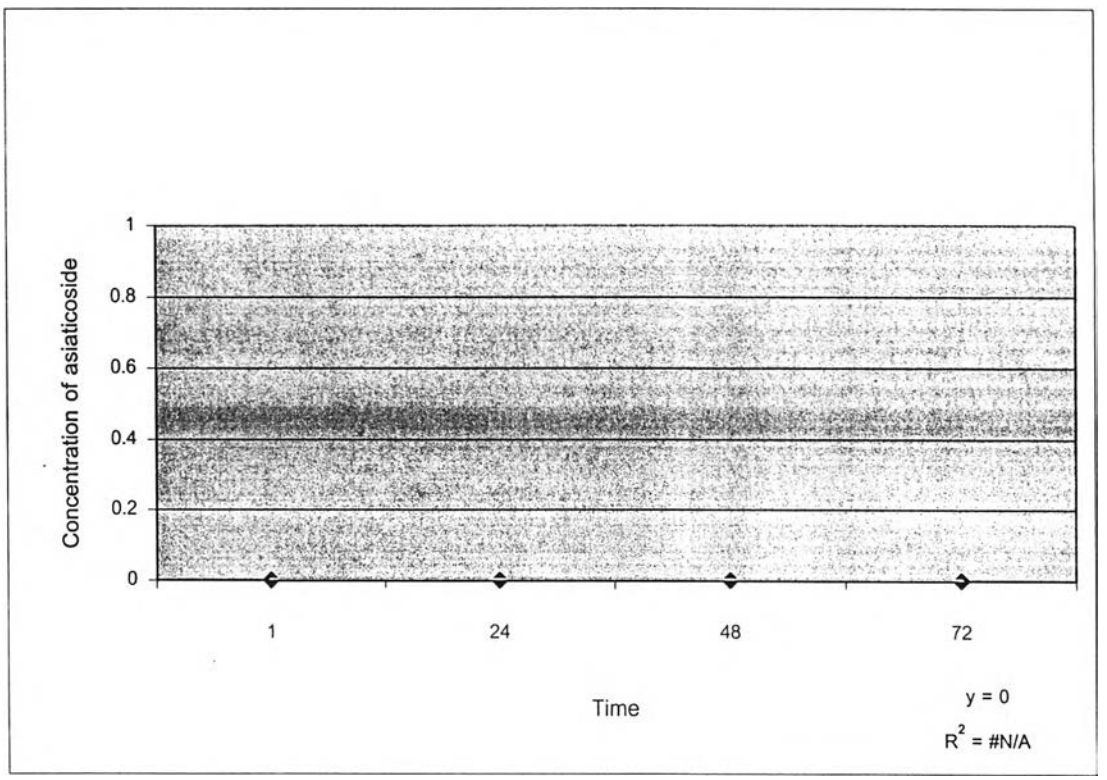


Figure 25; Permeation profile of crude extract from drug store