CHAPTER IV

RESULTS AND DISSCUSSION

A. Extraction and Isolation of Active Constituents from *Centella asiatica.*

1. Extraction of Chemical Components from Fresh Leaves of *Centella asiatica*

First, the ethanol was used for extraction of glycosides. The yields of ethanol extracts from 5.99 kg fresh leave of *Centella asiatica* was 520 g with black color. After that, chloroform was used for further extract non-polar substances such as chlorophyll, resinous substances from ethanol extract. While, the polar substances in water phase were taken and n-buthanol was added for extraction of saponins. The dried butanol extract was 40 g with brown color and passed through Sephadex ^{TM}-LH 20. Column with packing by Sephadex^{TM}-LH 20 was used for separation of substances by molecular weight. Collecting asiaticoside, madecassoside, madecassic acid and asiatic acid fractions were 26 g.

2. Extraction of Chemical Components from Dried Leaves of *Centella asiatica*

The weight of 2 kg of dried leaves of *Centella asiatica* was obtained from fresh leaves of 20 kg. The mixture of methanol and water at the ratio of 7:3 was used for extraction of glycosides. Next, the extract was partition with dichloromethane for separating non-polar group. Afterthat, the aqueous phase was partitioned with n-

butanol for separating more polar groups. Subsequently, 0.2 N sodium carbonate was used for washing base-soluble colors and then water was used to neutralize the system. The last step, ethylacetate was used for precipitation of the active compounds. That are mixture of asiaticoside and madecassoside. The weight of final yield was 21.31 g.

B. Determination of Active Constituents from *Centella asiatica.*

1. Thin Layer Chromatographic (TLC) Method

The isolated compounds from fresh leaves extract of *Centella asiatica* was identified by TLC. TLC is the simplest of all widely used chromatographic methods to perform the optimization of techniques and materials with highly efficient separation. The chromatograms of these compounds compared with the standard compounds are shown in Figure 10. In this chromatogram, the Rf values of asiaticoside, madecassoside, madecassic acid and asiatic acid are 0.4082, 0.3197, 0.7347 and 0.7755, respectively. From the TLC chromatrogram, madecassoside was found in fraction 5, followed by asiaticoside in fraction 6. Madecassic acid and asiatic acid were found in fractions afterthat. Based on these data, collected fractions from fractions 5-15, corresponding to asiaticoside, madecassoside, madecassic acid and asiatic acid were used to incorporate in microemulsions in method 4.1.

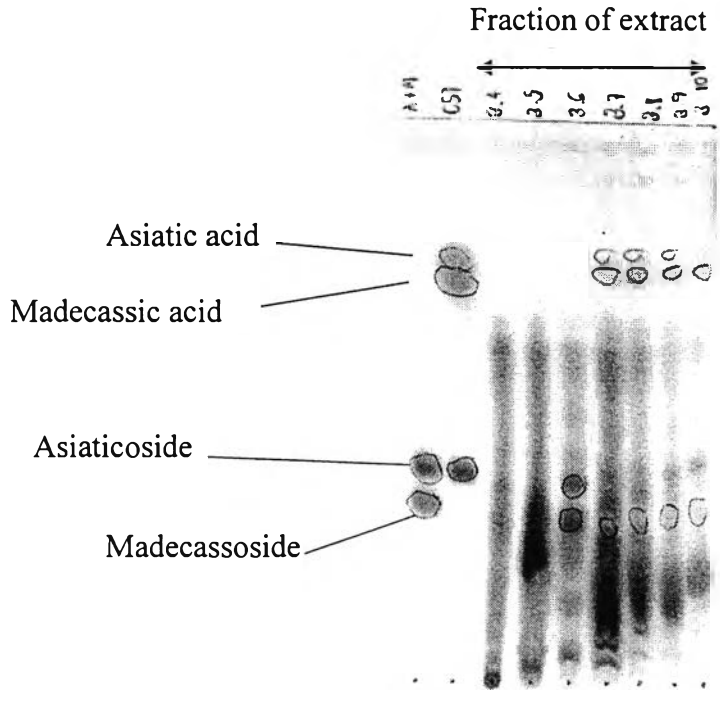


Figure 10 TLC chromatogram of asiaticoside, madecassoside, madecassic acid and asiatic acid

2. High Performance Liquid Chromatrographic (HPLC) Method

In the preliminary study, it was found that the isocratic system could not achieve a separation of the three compounds of the *Centella asiatica* extract such as asiaticoside, madecassic acid, asiatic acid and the internal standard, prednisolone using as internal standard. However, due to the large difference in polarity of the triterpene acids and their glycosides, a linear gradient system was chosen to apply in the study. After adjusting the type of mobile phase and the gradient system, four compounds were separated in a single run. The chromatograms of the mixture of their standard active compounds asiaticoside, madecassic acid, asiatic acid and prednisolone are shown in Figures 11-15, respectively.

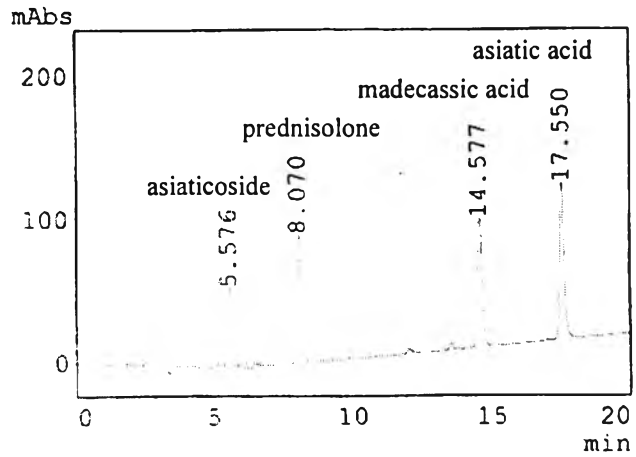


Figure 11 HPLC chromatogram of mixtures of 400 μg/ml asiaticoside, 50 μg/ml prednisolone, 300 μg/ml madecassic acid and 300 μg/ml asiatic acid

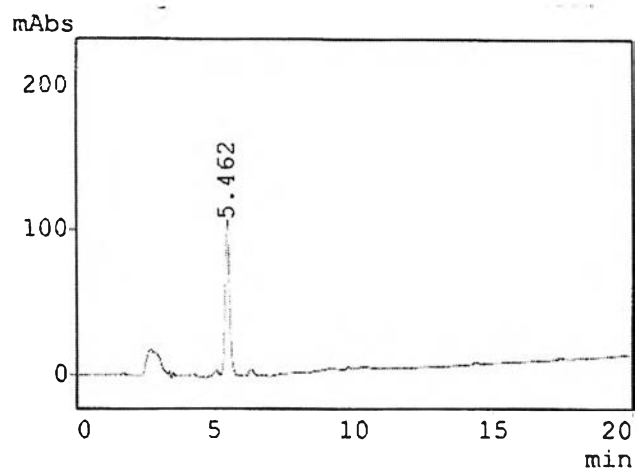


Figure 12 HPLC chromatogram of 400 μg/ml asiaticoside standard solution

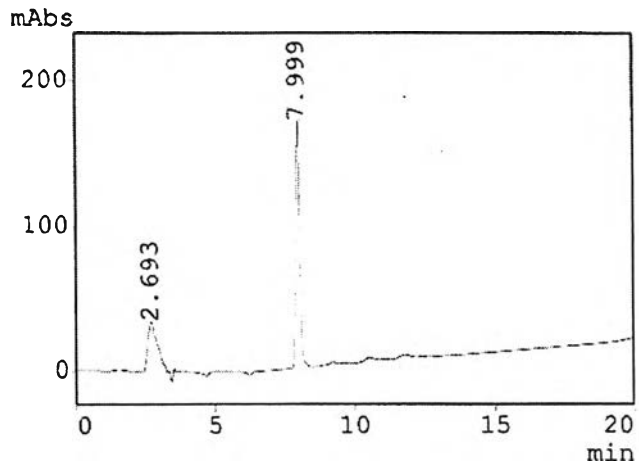


Figure 13 HPLC chromatogram of 100 μg/ml prednisolone standard solution

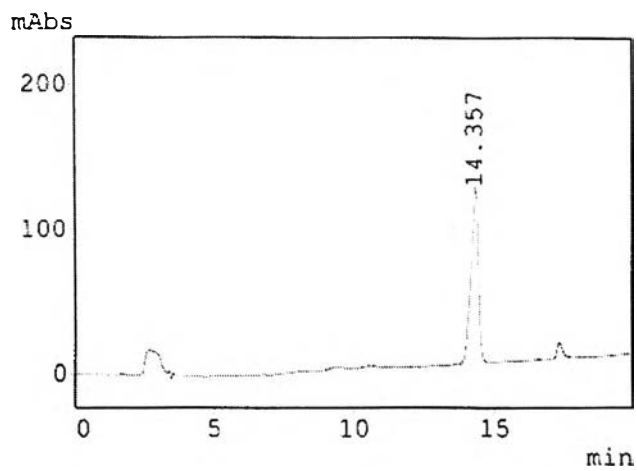


Figure 14 HPLC chromatogram of 300 μg/ml madecassic acid standard solution

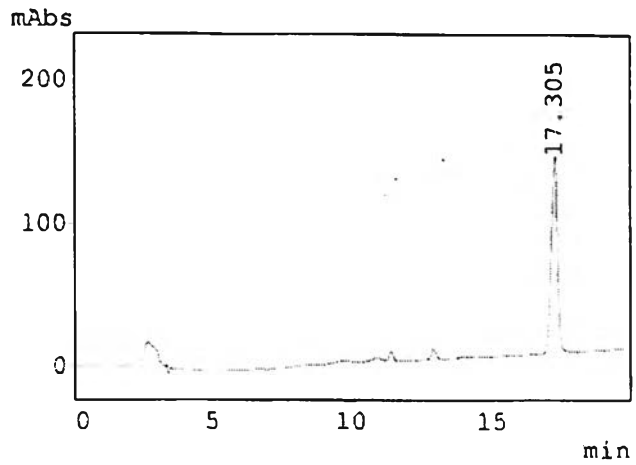


Figure 15 HPLC chromatogram of 300 μg/ml asiatic acid standard solution

The developed HPLC system was applied to the analysis of the extracts from fresh leaves and dried leaves of *Centella asiatica*. From Figures 16 and 17, one gram of the extract prepared from dried leaves from Topic A 2 gave the higher content of asiaticoside, 44.92 mg than the extract from Topic A1 with asiaticoside of 19.83 mg.

The extracts from fresh and dried leaves were identified with HPLC method. Figures 16 and 17 it was found that in crude extract 1 g, extract followed in topic A.2 gave content of asiaticoside higher than extract followed in topic A.1, 44.92 mg and 17.83 mg, respectively. However, the content of madecassic acid and asiatic acid in extract followed in topic A.2 (0.07 mg and 1.43 mg) lower than extract followed in topic A.1, (0.76 mg and 7.73 mg).

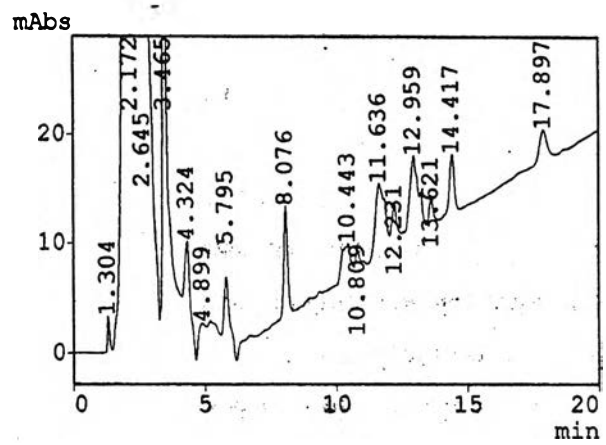


Figure 16 HPLC chromatogram of components from fresh leaves extraction following A.1

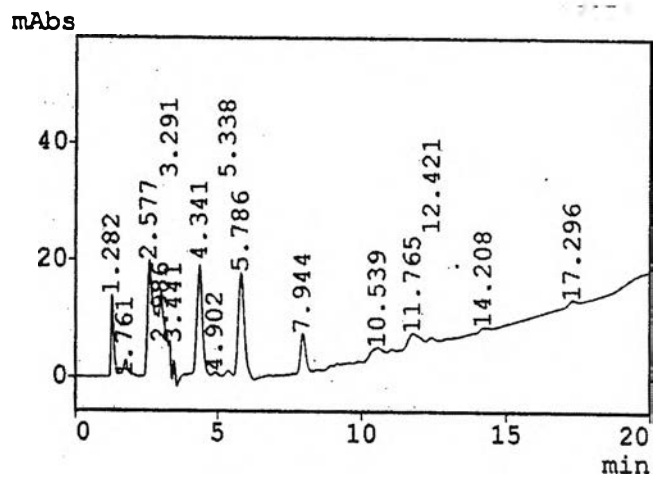


Figure 17 HPLC chromatogram of components from fresh leaves extraction following A.2

2.1 Validation of HPLC Method

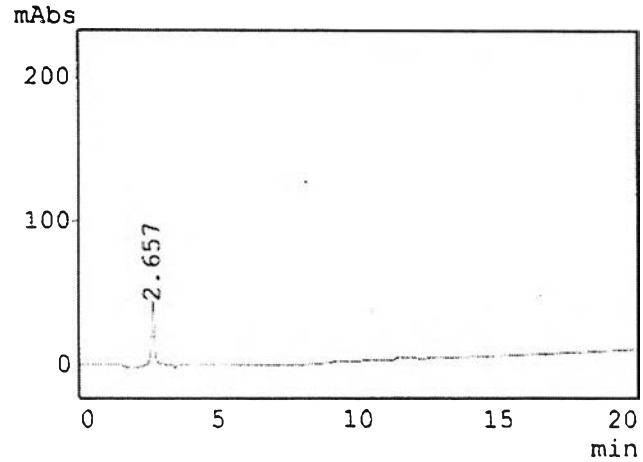
Validation of an analytical method is the process for evaluation that the method is suitable and reliable for the intended analytical applications. For HPLC assay, the analytical parameters to be considered were specificity, linearity, accuracy and precision.

2.1.1 Specificity

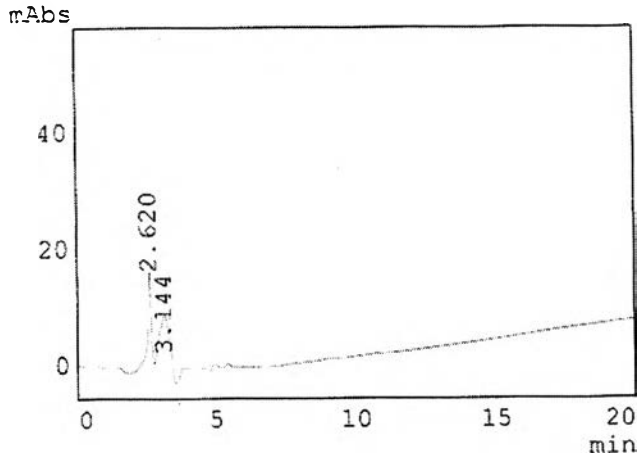
The specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Figure 18 shows typical chromatogram of blank solutions of methanol and 40% ethanol in isotonic PBS. It showed that both methanol and ethanol in isotonic PBS did not interfere with the analysis of all active compounds of Centella extract.

2.1.2 Linearity

The linearity of an analytical method is a directly mathematical transformation, proportional to the concentration of the analyte in samples within a given range. The linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. The standard curve data of asiaticoside, madecassic acid and asiatic acid in methanol and 40% ethanol in PBS are shown in Tables 8 and 9, respectively. The plots of concentration and mean peak area ratio of asiaticoside, madecassic acid and asiatic acid in methanol and 40% ethanol in PBS are shown in Figures 19 and 20. The standard curves were found to be linear with excellent



A



B

Figure 18 HPLC chromatogram of sample solutions of blank solutions

(A; methanol, B; 40% ethanol in isotonic PBS)

coefficients of determination (R^2). The coefficients of determination (R^2) were 0.9997, 0.9997, 0.9998, 0.9997, 0.9999 and 0.9998, respectively.

Table 8 Data for standard curve of asiaticoside, madecassic acid and asiatic acid in Methanol

Concentration of (mg/ml)	Peak area ratio Set1	Set2	Set3	Mean	SD	%CV
Asiaticoside						
9.1042	0.2656	0.2689	0.2707	0.2684	0.0026	0.9637
18.2084	0.5988	0.6012	0.5887	0.5962	0.0066	1.1126
27.3126	0.9125	0.9198	0.9164	0.9162	0.0037	0.3987
36.4168	1.2129	1.2145	1.2074	1.2116	0.0037	0.3074
45.5210	1.5614	1.5574	1.5533	1.5574	0.0041	0.2601
54.6252	1.8774	1.8773	1.8825	1.8791	0.0030	0.1583
Madecassic acid						
9.3760	0.7829	0.7831	0.7828	0.7829	0.0002	0.0195
18.7520	1.6060	1.5976	1.5951	1.5996	0.0057	0.3570
28.1280	2.3724	2.3744	2.3790	2.3753	0.0034	0.1425
37.5040	3.1049	3.1064	3.0843	3.0985	0.0123	0.3986
46.8800	3.9453	3.9475	3.9325	3.9418	0.0081	0.2055
56.256	4.6951	4.7033	4.6817	4.6934	0.0109	0.2323
asiatic acid						
9.6020	0.7357	0.7347	0.7383	0.7362	0.0019	0.2524
19.2040	1.5936	1.6017	1.5897	1.5950	0.0061	0.3838
28.8060	2.3753	2.3722	2.3760	2.3745	0.0020	0.0852
38.4080	3.1985	3.1960	3.1921	3.1955	0.0032	0.1009
48.0100	4.0757	4.0707	4.0817	4.0760	0.0055	0.1351
57.6120	4.8710	4.8609	4.8632	4.865	0.0053	0.1088

2.1.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. Accuracy is calculated as percent recovery by the assay of known added amount of analyte. The percentages of analytical recovery of asiaticoside, madecassic acid and asiatic acid in methanol and 40% ethanol in PBS are shown in Tables 10 and 11. The percentages analytical recovery of asiaticoside, madecassic acid and asiatic acid concentrations were in the range of 99.75-100.44%, 98.14-100.62% and 99.06-99.58%, respectively. which indicated that this method could be used for analysis in all concentrations studied with a high accuracy.

Table 9 Data for standard curve of asiaticoside, madecassic acid and asiatic acid in 40% ethanol in PBS

Concentration of (mg/ml)	Peak area ratio Set1	Set2	Set3	Mean	SD	%CV
Asiaticoside						
9.1042	0.3131	0.3225	0.3174	0.3177	0.0047	1.4813
18.2084	0.6249	0.6241	0.6162	0.6217	0.0048	0.7734
27.3126	0.9262	0.9311	0.9211	0.9261	0.0050	0.5399
36.4168	1.2130	1.2273	1.2245	1.2216	0.0076	0.6204
45.5210	1.5141	1.5221	1.5216	1.5193	0.0045	0.2950
54.6252	1.8580	1.8493	1.8510	1.8528	0.0046	0.2489
Madecassic acid						
9.3760	0.9655	0.9585	0.9641	0.9627	0.0037	0.3848
18.7520	1.7269	1.7204	1.7458	1.7310	0.0132	0.7623
28.1280	2.4753	2.4729	2.4722	2.4735	0.0016	0.0657
37.5040	3.2749	3.2525	3.2506	3.2593	0.0135	0.4146
46.8800	4.0123	4.0247	4.0287	4.0219	0.0086	0.2126
56.2560	4.7391	4.7493	4.7502	4.7462	0.0062	0.1299
asiatic acid						
9.6020	0.8915	0.8908	0.8946	0.8923	0.0020	0.2266
19.2040	1.7439	1.7384	1.7487	1.7437	0.0052	0.2956
28.8060	2.5464	2.5475	2.5440	2.5460	0.0018	0.0703
38.4080	3.4099	3.4108	3.3466	3.3891	0.0368	1.0861
48.0100	4.2579	4.2573	4.2562	4.2571	0.0009	0.0203
57.6120	5.0224	5.0350	5.0225	5.0266	0.0072	0.1442

2.1.4 Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurement. In this context, within run precision refers to the use of the analytical procedure with the same equipment in the same day. Tables 12 and 13 display the data of the analyses of asiaticoside, madecassic acid and asiatic acid in methanol and 40% ethanol in PBS in the same day, respectively. Between run precision expresses within laboratory variation as on different days. As given in

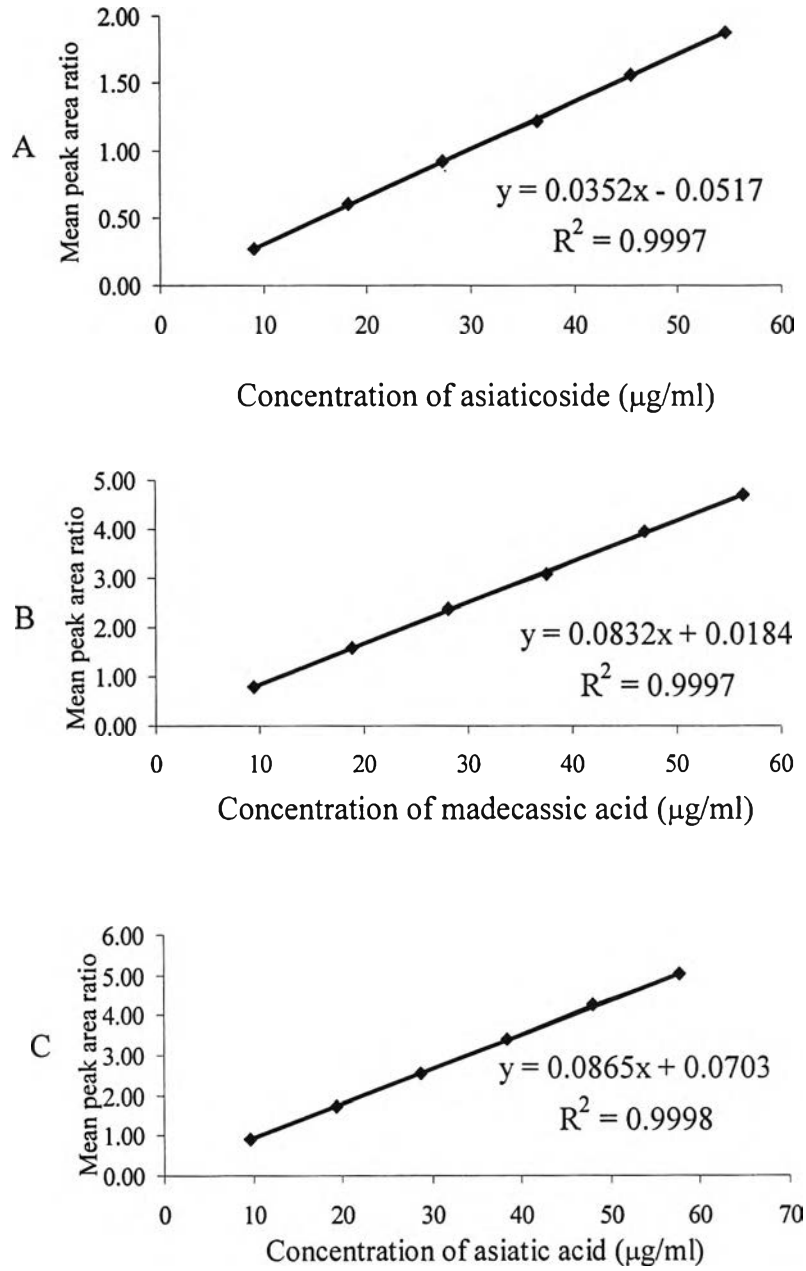


Figure 19 Standard curve of asiaticoside, madecassic acid and asiatic acid in methanol by HPLC method (A; asiaticoside, B; madecassic acid and C; asiatic acid)

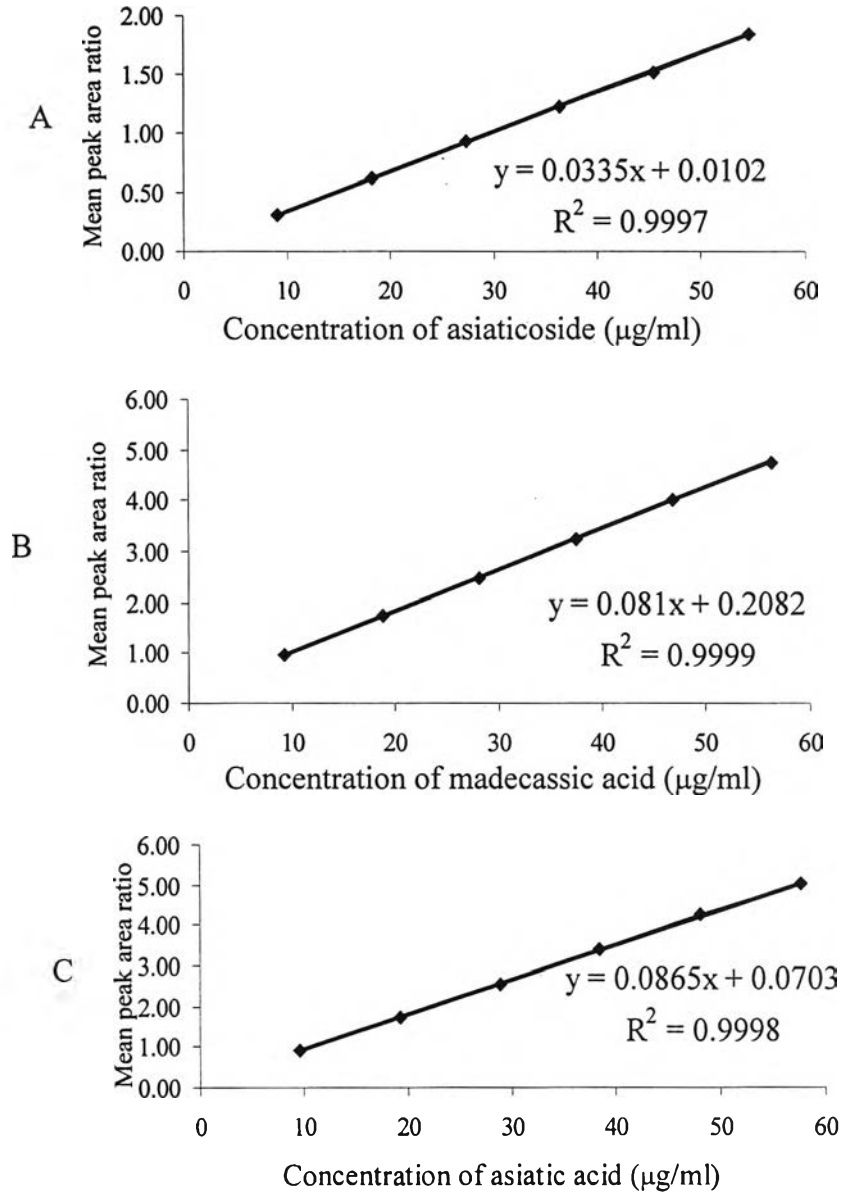


Figure 20 Standard curve of asiaticoside, madecassic acid and asiatic acid in 40% ethanol in PBS by HPLC method A; asiaticoside, B; madecassic acid and C; asiatic acid)

Table 10 The percentage of analytical recovery of asiaticoside, madecassic acid and asiatic acid in methanol

Actual concentration	%Analytical recovery					Mean ± SD
(μg/ml)	1	2	3	4	5	
asiaticoside						
9.1042	98.99	100.02	100.58	100.52	98.64	99.75 ± 0.89
27.3126	100.27	101.03	100.67	100.50	99.87	100.47 ± 0.43
54.6252	100.30	100.30	100.57	99.89	100.57	100.33 ± 0.28
madecassic acid						
9.3760	98.01	98.04	98.00	98.28	98.36	98.14 ± 0.17
28.1280	100.60	100.68	100.88	100.68	100.29	100.62 ± 0.21
56.2560	99.93	100.10	99.64	99.86	99.78	99.86 ± 0.17
asiatic acid						
9.6020	98.91	98.79	99.22	99.81	98.88	99.12 ± 0.42
28.8060	98.93	98.81	98.96	99.23	99.37	99.06 ± 0.23
57.6120	99.67	99.47	99.51	99.71	99.55	99.58 ± 0.10

Table 11 The percentage of analytical recovery of asiaticoside, madecassic acid and asiatic acid in 40% ethanol in PBS

Actual concentration	%Analytical recovery					Mean ± SD
(μg/ml)	1	2	3	4	5	
asiaticoside						
9.1042	98.29	104.34	99.69	99.59	100.53	99.89 ± 1.14
27.3126	99.08	99.61	98.53	98.53	98.69	98.89 ± 0.46
54.6252	99.94	99.47	99.56	99.07	99.89	99.58 ± 0.35
madecassic acid						
9.3760	99.56	98.64	99.37	99.7	99.78	99.41 ± 0.46
28.1280	99.35	99.24	99.21	99.00	99.79	99.12 ± 0.22
56.2560	99.27	99.50	99.52	99.29	99.28	99.36 ± 0.13
asiatic acid						
9.6020	98.71	98.63	99.09	99.71	99.23	99.07 ± 0.44
28.8060	99.21	99.26	99.12	99.33	99.08	99.20 ± 0.10
57.6120	99.21	99.46	99.21	99.21	99.13	99.25 ± 0.13

Table 12 Data of within run precision of asiaticoside, madecassic acid and asiatic acid in methanol

Actual concentration (mg/ml)	Peak area ratio					Mean	SD	%CV
	Set1	Set2	Set3	Set 4	Set5			
asiaticoside								
9.1042	0.2656	0.2689	0.2707	0.2705	0.2645	0.2680	0.0028	1.06
27.3126	0.9125	0.9198	0.9164	0.9147	0.9087	0.9144	0.0042	0.46
54.6252	1.8774	1.8773	1.8825	1.8695	1.8825	1.8778	0.0053	0.28
madecassic acid								
9.3760	0.7829	0.7831	0.7828	0.7850	0.7856	0.7839	0.0013	0.17
28.1280	2.3724	2.3744	2.3790	2.3744	2.3652	2.3731	0.0050	0.21
56.2560	4.6951	4.7033	4.6817	4.6918	4.6880	4.6920	0.0081	0.17
asiatic acid								
9.6020	0.7357	0.7347	0.7383	0.7432	0.7355	0.7375	0.0035	0.47
28.8060	2.3753	2.3722	2.3760	2.3826	2.3861	2.3784	0.0057	0.24
57.6120	4.8710	4.8609	4.8632	4.8731	4.8649	4.8666	0.0052	0.11

Table 13 Data of within run precision of asiaticoside, madecassic acid and asiatic acid in 40% ethanol in PBS

Actual concentration (mg/ml)	Peak area ratio					Mean	SD	%CV
	Set1	Set2	Set3	Set 4	Set5			
asiaticoside								
9.1042	0.3131	0.3225	0.3174	0.3171	0.3200	0.3180	0.0035	1.11
27.3126	0.9262	0.9311	0.9211	0.9211	0.9226	0.9244	0.0043	0.46
54.6252	1.8580	1.8493	1.8510	1.8419	1.8571	1.8515	0.0065	0.35
madecassic acid								
9.3760	0.9655	0.9585	0.9641	0.9666	0.9672	0.9644	0.0035	0.36
28.1280	2.4753	2.4729	2.4722	2.4674	2.4625	2.4701	0.0051	0.21
56.2560	4.7391	4.7493	4.7502	4.7379	4.7393	4.7432	0.0060	0.13
asiatic acid								
9.6020	0.8908	0.7347	0.8946	0.8998	0.8958	0.8945	0.0036	0.40
28.8060	2.5464	2.5475	2.5440	2.5494	2.5431	2.5461	0.0026	0.10
57.6120	5.0224	5.0350	5.0225	5.0223	5.0184	5.0241	0.0063	0.13

Tables 14 and 15, there were the data of between run precision in methanol and 40% ethanol in PBS, respectively. All coefficient of variation values were small as shown to be in the range of 0.05-1.77%. The coefficient of variation of an analytical method should generally be less than 2 %. Therefore, the HPLC method could be used precisely for quantitative analysis in the range studied.

In conclusion, the analysis of asiaticoside, madecassic acid and asiatic acid either in methanol or 40% ethanol in PBS by HPLC method with the gradient system developed in this study showed good specificity, linearity, accuracy and precision. Thus this method was used for the determination of the content of asiaticoside, madecassic acid and asiatic acid in the study.

Table 14 Data of between run precision of asiaticoside, madecassic acid and asiatic acid in methanol

Actual concentration	Peak area ratio					Mean	SD	%CV
(mg/ml)	Set1	Set2	Set3	Set 4	Set5			
asiaticoside								
9.1042	0.2555	0.2519	0.2586	0.2591	0.2567	0.2564	0.0029	1.13
27.3126	0.9170	0.9197	0.9746	0.9162	0.9187	0.9172	0.0020	0.22
54.6252	1.9421	1.9439	1.9372	1.9455	1.9363	1.9410	0.0041	0.21
madecassic acid								
9.3760	0.7755	0.7710	0.7717	0.7830	0.7817	0.7766	0.0056	0.72
28.1280	2.4207	2.4226	2.4194	2.4213	2.4248	2.4218	0.0021	0.08
56.2560	4.8663	4.7390	4.7509	4.8580	4.8557	4.8140	0.0633	1.31
asiatic acid								
9.6020	0.7384	0.7354	0.7350	0.7366	0.7387	0.7368	0.0017	0.23
28.8060	2.4218	2.4237	2.4291	2.4194	2.4180	2.4224	0.0043	0.18
57.6120	5.0224	5.0405	4.9546	5.0257	5.0270	5.0140	0.0339	0.68

Table 15 Data of between run precision of asiaticoside, madecassic acid and asiatic acid in 40% ethanol in PBS

Actual concentration (mg/ml)	Peak area ratio					Mean	SD	%CV
	Set1	Set2	Set3	Set 4	Set5			
asiaticoside								
9.1042	0.3213	0.3290	0.3265	0.3238	0.3140	0.3252	0.0058	1.77
27.3126	0.9220	0.9281	0.9348	0.9147	0.9218	0.9249	0.0076	0.82
54.6252	1.8545	1.8552	1.8546	1.8489	1.8531	1.8533	0.0026	0.14
madecassic acid								
9.3760	0.9546	0.9650	0.9636	0.9603	0.9594	0.9609	0.0041	0.42
28.1280	2.4661	2.4618	2.4608	2.4626	2.4628	2.4218	0.0027	0.11
56.2560	4.7695	4.7518	4.7550	4.7416	4.7355	4.7545	0.0131	1.28
asiatic acid								
9.6020	0.9056	0.9025	0.8983	0.8976	0.8983	0.9010	0.0035	0.38
28.8060	2.5445	2.5378	2.5316	2.5437	2.5442	2.5394	0.0056	0.22
57.6120	5.0222	5.0270	5.0260	5.0243	5.0217	5.0249	0.0023	0.05

C. Microemulsion Formulation

1. Physical Characterization

The microemulsion structure is greatly influenced by physicochemical properties of the component used, the nature of surfactants, the system composition temperature, and presence of cosurfactants (Malmsten, 2002). As the size of microemulsion aggregates typically less than 250 nm, which is smaller than the wavelength of visible light, direct examination of microemulsion structures is limited. Therefore, indirect measurement techniques are often employed to obtain information about the internal structure. The order of mixing of various components is not expected to influence the formation of microemulsion if the system is indeed thermodynamically stable (Constantinides and Scalart, 1997). It is the low interfacial

tension of microemulsions that favors the formation of a thermodynamically stable dispersion.

1.1 Visual inspection

Visual inspection could be instantly used to identify the physical characteristics of the formulation prepared. Visually, macroemulsions are milky white appearances and propensity to separate into their two original liquid phases upon standing. In the other hand, microemulsions or liquid crystals are not white, they are translucent or transparent to the eye and they do not separate upon standing (Malinsten, 2002). Recording data of ternary and pseudo-ternary phase diagram were shown in Appendixs B.

1.2 Polarized light microscopy

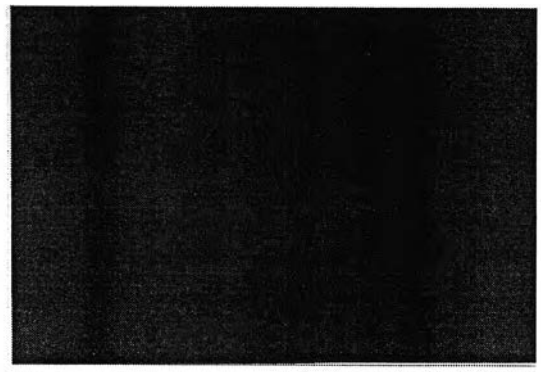
Polarized light microscopy is useful in the study of clear liquid system. Figure 21 illustrates birefringent characteristics of liquid crystals and nonbirefringence of microemulsion under polarized light microscopy. The systems which exhibited birefringence showing oily streaks, maltese crosses or fan shaped which were designed as systems containing a lamellar phases (Alany, et al., 2000)

2. Study of Ternary and Pseudo-ternary Phase Diagrams

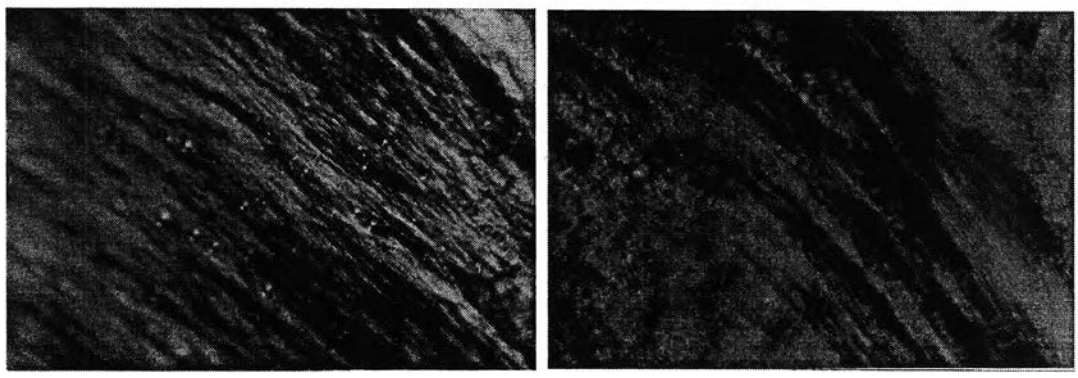
2.1 Determination of ternary and pseudo-ternary phase diagrams

2.1.1 Effects of oil and surfactant on ternary phase diagrams

Figure 22 shows the ternary phase or pseudo-ternary diagrams of ten



A



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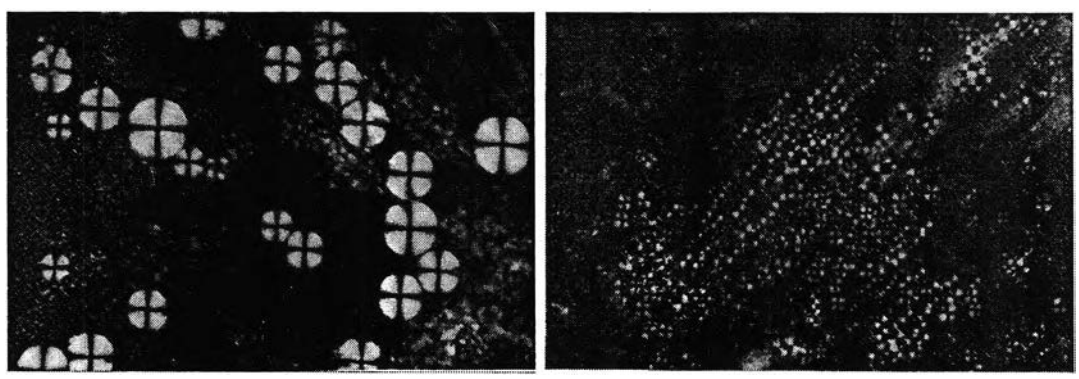


3 4

B

Figure 21 Characterization of liquid crystals and microemulsion under polarized light (A; nonbirefringence of microemulsion, B; birefringence of liquid crystals, B1, B2; oily streaks, B3; fan shape, B4; maltese cross)

systems using either caprylic/capric triglyceride or IPM as oils and four types surfactants. Overall, although differences in phase behavior were seen on using different oil components, these differences were generally small. Interestingly, the phase diagrams obtained using IPM as oil show virtually higher isotropic areas than when using the caprylic/capric triglyceride. It appears that the molecular size of oil is the major factor influencing phase behavior, which microemulsions formation tending to be seen when the smaller oils were used. Due to penetration of smaller oil molecules between the hydrocarbon tails of surfactant would be expected to increase the effective surfactant volume resulting in reduced effective HLB, whereas larger molecule volume oils would not be expected to exert much effect (Swarbrick and Boylan, 1994; Aboolazeli et al., 1995).

Comparison of isotropic regions between the system containing different surfactants, surfactants alone was able to reduce the oil/water interfacial tension to form microemulsion, Brij 30 and Arlatone T gave the high isotropic zone in the systems using a both of caprylic/capric triglyceride and IPM. This results may be involved HLB character, HLB of either Brij 30 and Arlatone T in range 9-9.7.

2.1.2 Effects of cosurfactant on pseudo-ternary phase diagrams

For many nonionic surfactant systems, a sufficiently low surface tension are obtained for the surfactant/oil/water systems. An addition of a cosurfactant is not so necessary as in the ionic surfactant systems. A cosurfactant is

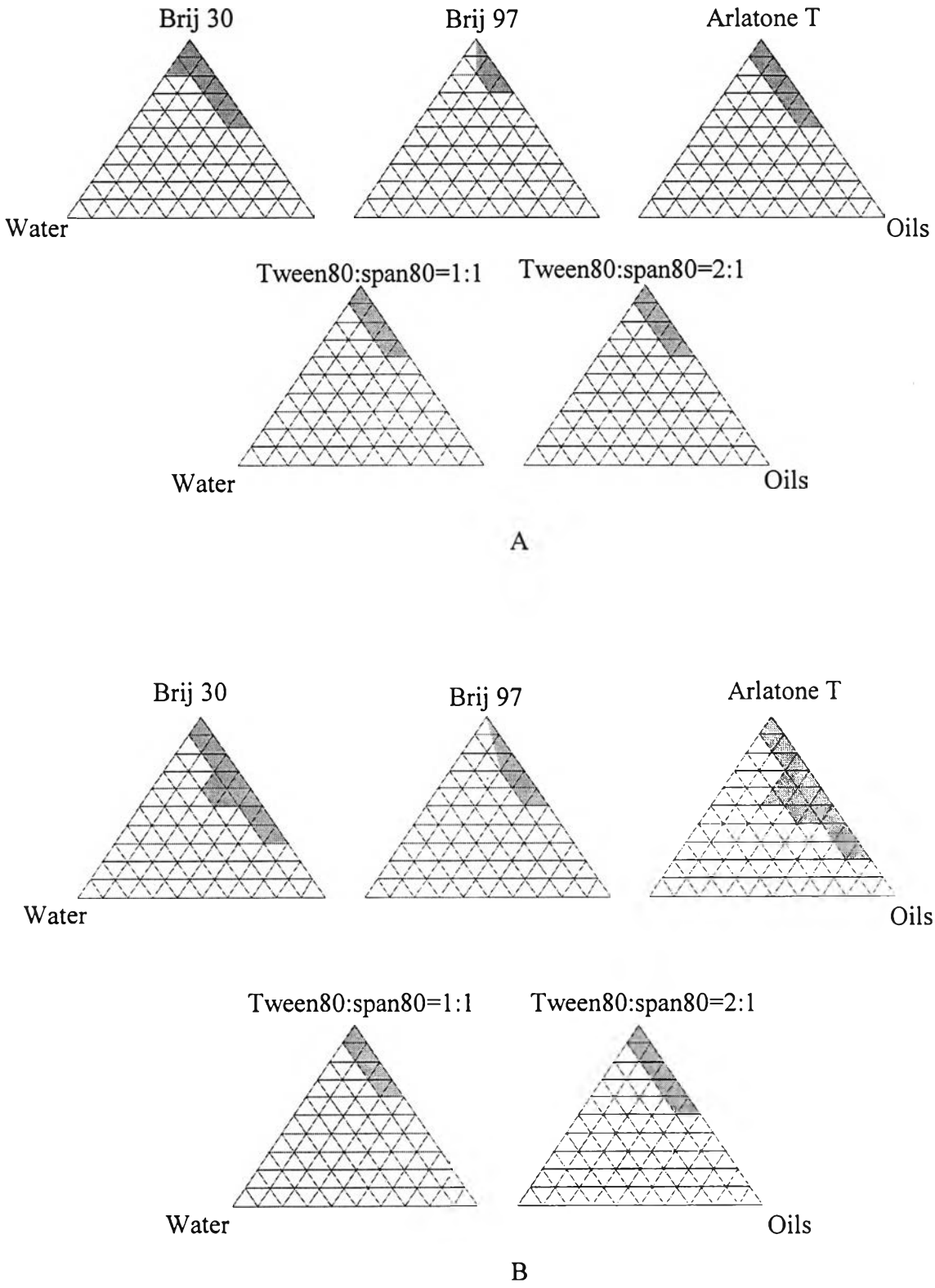


Figure 22 Ternary and pseudo-ternary phase diagrams with different surfactants

(A; caprylic/capric triglyceride as oil, B; IPM as oil)

added in order to achieve a dense packing at the interface and thereby reach a sufficiently low interfacial tension for microemulsion formation to occur (Malmsten, 2002). As shown in Figure 23 (A, B), the addition of propan-2-ol showed the obviously increasing capacity to microemulsion formation as compared to those without propan-2-ol (Figure 21 A, B). Commonly, the cosurfactant is also amphiphilic with an affinity for both the oil and aqueous phases and partitions to an appreciable extent into the surfactant interfacial monolayer present at the oil-water interfacial. In this study, the molecules which function as cosurfactants including short chain alcohols and alkanediols. Figure 23 shows the isotropic regions obtained by using propan-2-ol as a cosurfactant at various weight ratios while Figure 24 shows the isotropic regions obtained by using propylene glycol as cosurfactant.

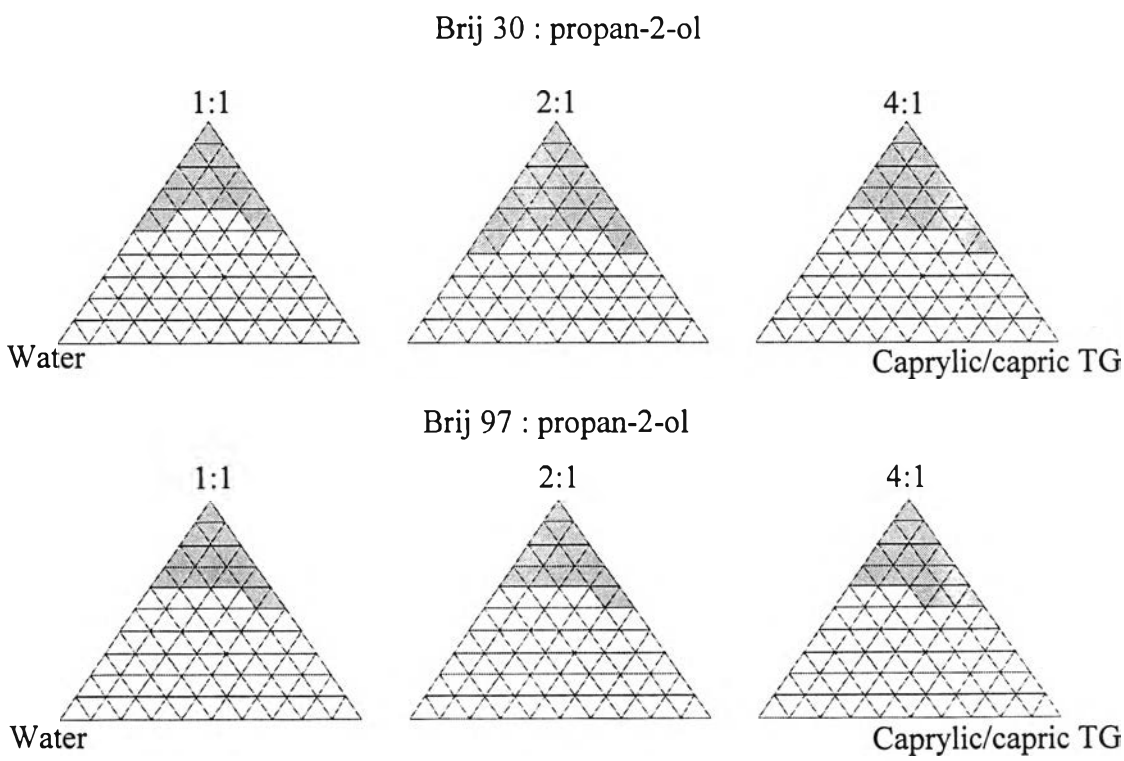


Figure 23 Pseudoternary phase diagram with propan-2-ol as cosurfactant

(A; caprylic/capric triglyceride as oil, B; IPM as oil)

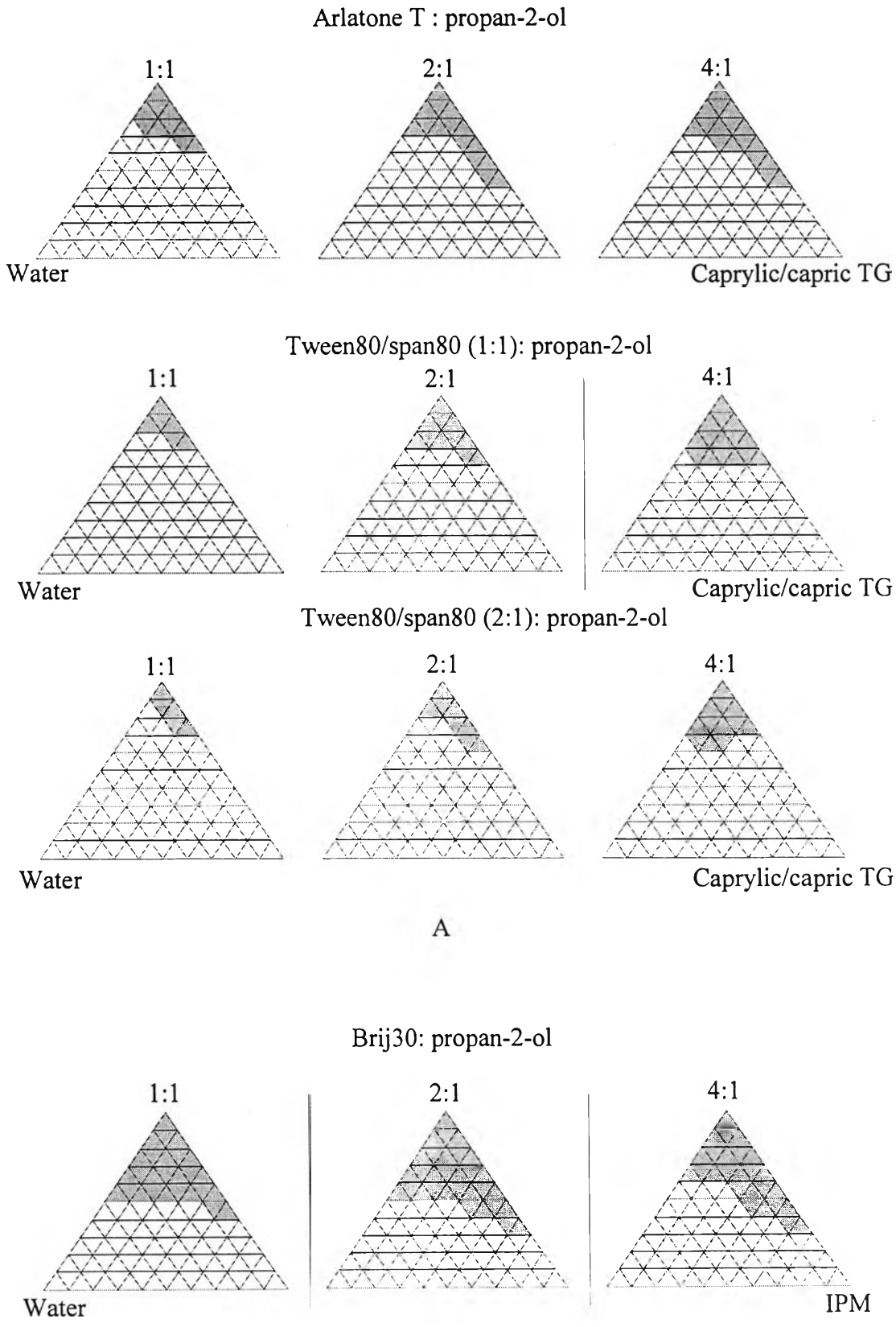


Figure 23 Pseudoternary phase diagram with propan-2-ol as cosurfactant (cont.)

(A; caprylic/capric triglyceride as oil, B; IPM as oil)

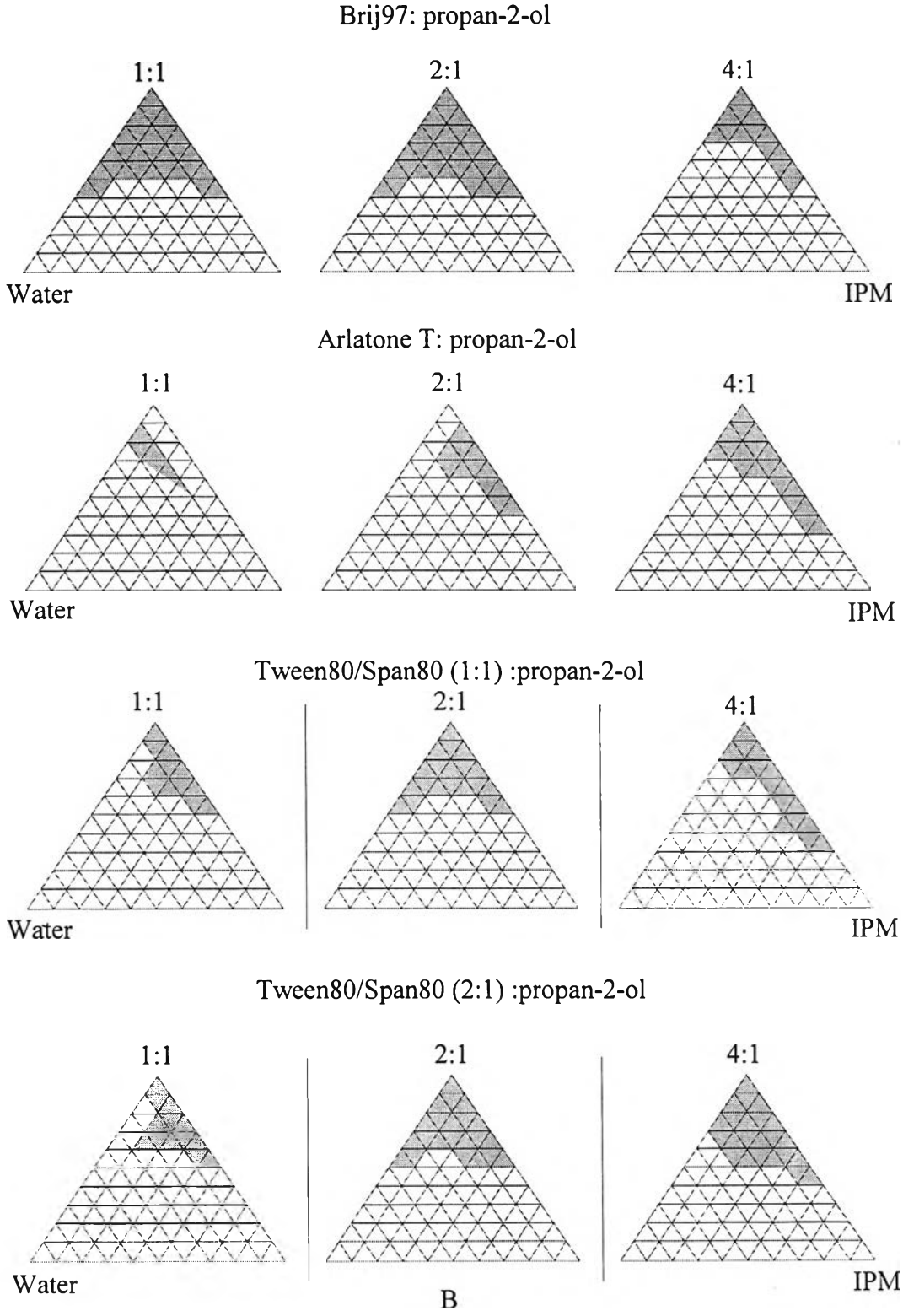


Figure 23 Pseudoternary phase diagram with propan-2-ol as cosurfactant (cont.)

(A; caprylic/capric triglyceride as oil, B; IPM as oil)

a) Using propan-2-ol as a cosurfactant

Mostly, in case of addition of propan-2-ol in the system used with caprylic/capric triglyceride as oil resulted increasing in the isotropic area compared with those in the absence of cosurfactant. However, different results were obtained in the system with Tween 80: Span 80 (1:1 and 2:1) and the ratio of surfactant: cosurfactant of 1:1, the isotropic area decreased. These were consistent with the results obtained from the systems with IPM as oil, however every ratio of propan-2-ol in Arlatone T showed decreasing isotropic areas.

To investigate the effect of the surfactant: cosurfactant weight ratios of 1:0, 1:1, 2:1 and 4:1, it was demonstrated that there were only certain appropriate ratios that gave a good capacity of microemulsion formation. In most surfactants used, the increasing amount of propan-2-ol (from 4:1 to 2:1 and 1:1), the isotropic regions decreased. The effects of the surfactant: cosurfactant weight ratio may be explained by the opposing effects of the surfactant and the surfactant causes the interfacial film to condense while the addition of the cosurfactant would cause the film to expand (Swarbrick and Boylan, 1994). Thus, this might results to the limited the concentration of cosurfactant added.

The amphiphilic nature, short hydrophobic chain and hydroxy group of propan-2-ol may enable to interact with the surfactant at the interface thereby affecting their packing, which leading to more positive curvature of the surfactant monolayer. The low molecular weight propan-2-ol also enables them to distribute between the aqueous and oil phase thereby altering the chemical composition.

Moreover, a decrease in the polarity of the polar medium and the incorporation of alcohol molecules in the lipid, would produce a change in the HLB of surfactant that can be shifted as appropiate to form microemulsions (Swarbrick and Boylan, 1994; Lawrence and Rees, 2000).

Furthermore, propan-2-ol has the effects to reducing the interfacial tension, whilst increasing the fluidity of the interface thereby increasing the entropy of the system. The mobility of the hydrocarbon tail also increases and penetration of the oil allows greater into this region. Propan-2-ol present may also influence the solubility properties of the aqueous and oily phases due to its partitioning between these phases (Kreuter, 1994).

b) Using propylene glycol as cosurfactant

It was seen that increasing isotropic areas of pseudoternary phase diagrams of both oils when added propylene glycol was obtained only in the system using Brij 30 and Brij 97 as surfactants. Surprisingly, in the systems with Alatone T and Tween 80: Span 80 systems, the addition of propylene glycol as cosurfactant could not give a beneficial effect in increasing isotropic region (Figure 24 A, B).

The result of the present study offered the support for the belief that propylene glycol lead to long range ordered packing of the surfactant combination films to a zero curvature (Alany et al., 2000), so liquid crystal was observed

when using propylene glycol in systems (Appendix B). Tables 16-18 summarize the percentage of miroemulsion area for cosurfactant free and cosurfactant system.

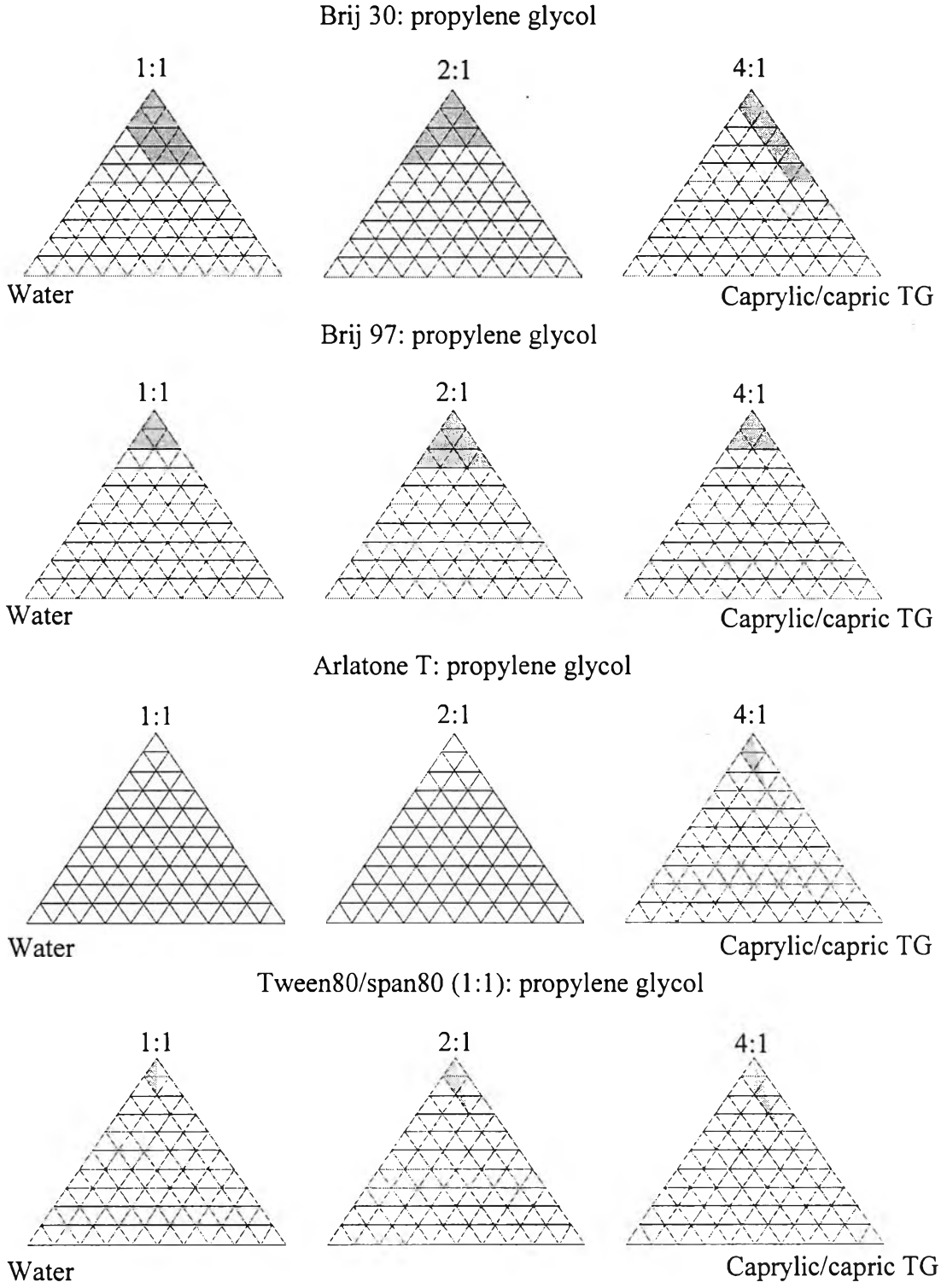


Figure 24 Pseudoternary phase diagram with propylene glycol as cosurfactant

(A; caprylic/capric triglyceride as oil, B; IPM as oil)

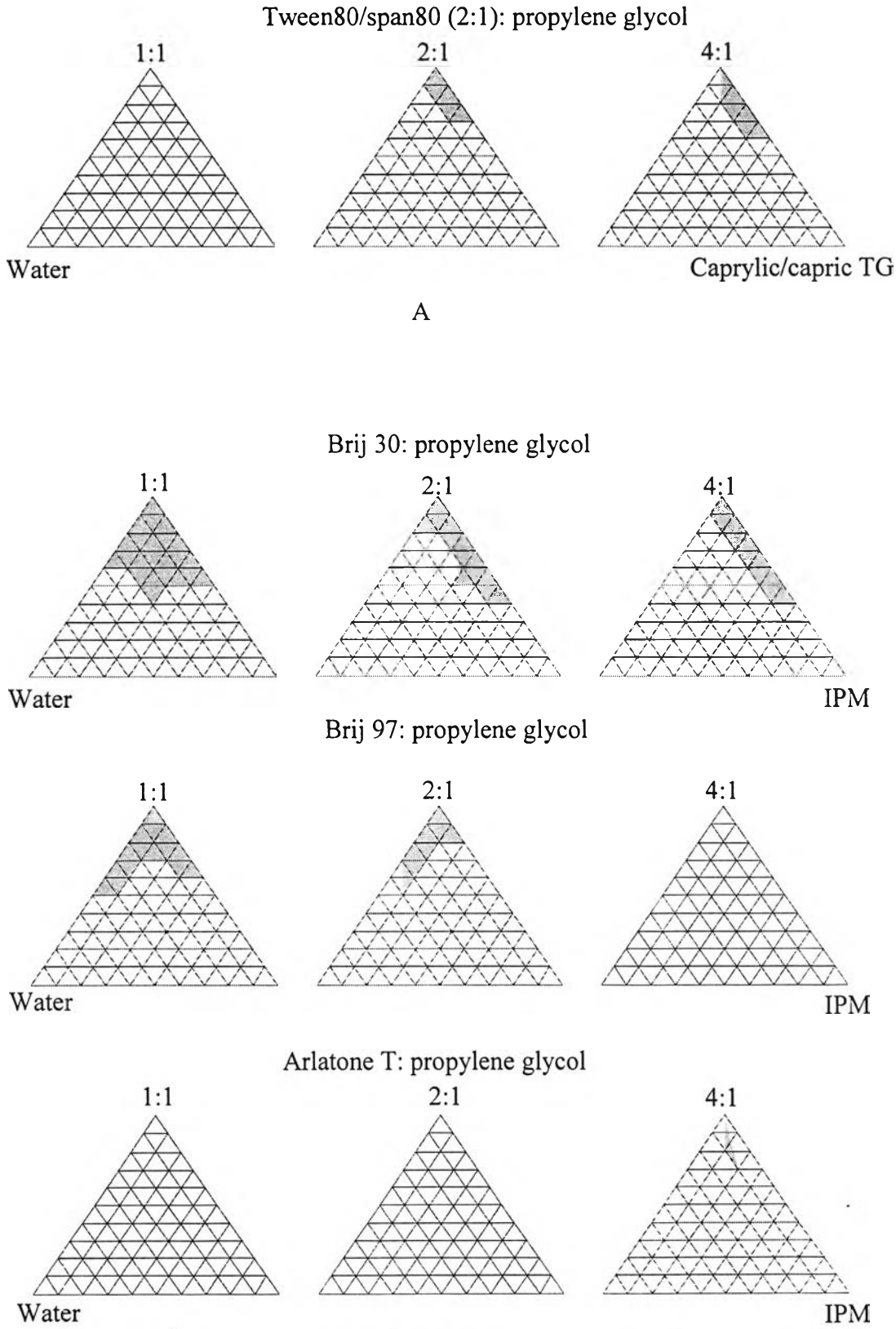


Figure 24 Pseudoternary phase diagram with propylene glycol as cosurfactant (cont.)

(A; caprylic/capric triglyceride as oil, B; IPM as oil)

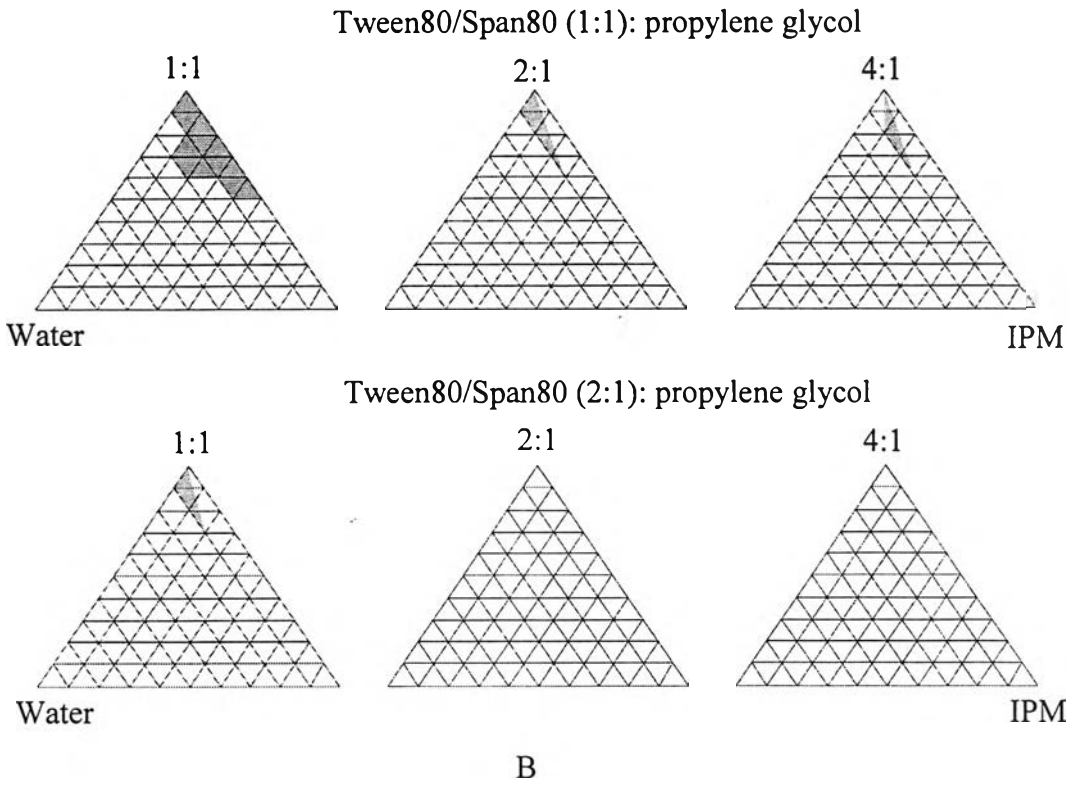


Figure 24 Pseudoternary phase diagram with propylene glycol as cosurfactant (cont.)

(A; caprylic/capric triglyceride as oil, B; IPM as oil)

From Table 16, pseudonary phase diagrams showed highest percentage isotropic area at 30.56% using IPM as oil and Arlatone T as cosurfactant. However in the system with caprylic/capric triglyceride as oil, Brij 30 and Arlatone T exhibibited the same percentage microemulsion area at 11.11%, which was much lower than that obtained in the IPM systems.

Table 16 The percentage of isotropic area in pseudoternary phase diagram for cosurfactant free system.

Surfactant	% Isotropic	
	caprylic/capric triglyceride	IPM
Brij 30	11.11	19.44
Brij 97	5.56	8.33
Arlatone T	11.11	30.56
Tween 80: Span 80 (1:1)	8.33	8.33
Tween 80: Span 80 (2:1)	8.33	11.11

From Tables 17 and 18 either capryric/capric triglyceride or IPM system containing Brij 30: propan-2-ol gave high percentage microemulsion area at 33.33 %, 36.11% Propan-2-ol exhibited excellent cosurfactant increasing microemulsion area better than propylene glycol. It might be the influence of cosurfactant on molecular packing and film curvature. From the ternary and pseudo-ternary phase diagram study revealed that microemulsion system containing IPM as oil, Brij 30: propan-2-ol at ratio of 2:1 and Brij 97: propan-2-ol at ratio of 2:1 showed the highest percentage microemulsion area at 36.11.

Table 17 The percentage of isotropic area in pseudoternary phase diagram with capryric/capric triglyceride as oil

Surfactant	Surfactant : cosurfactant ratio	% Isotropic	
		caprylic/capric triglyceride	IPM
Brij 30	1:1	22.22	13.89
	2:1	33.33	11.11
	4:1	27.78	11.11
Brij 97	1:1	19.44	2.78
	2:1	19.44	8.33
	4:1	22.22	2.78
Arlatone T	1:1	11.11	0
	2:1	16.67	0
	4:1	19.44	11.11
Tween 80: Span 80 (1:1)	1:1	11.11	0
	2:1	16.67	0
	4:1	19.44	11.11
Tween 80: Span 80 (2:1)	1:1	5.56	0
	2:1	8.33	5.56
	4:1	13.89	8.33

Table 18 The percentage of isotropic area in pseudoternary phase diagram with IPM as oil

Surfactant	Surfactant : cosurfactant ratio	% Isotropic	
		caprylic/capric triglyceride	IPM
Brij 30	1:1	30.56	27.78
	2:1	36.11	16.67
	4:1	30.56	13.89
Brij 97	1:1	33.33	16.67
	2:1	36.11	11.11
	4:1	16.67	0
Arlatone T	1:1	13.89	0
	2:1	19.44	0
	4:1	22.22	5.56
Tween 80: Span 80 (1:1)	1:1	16.67	5.56
	2:1	22.22	8.33
	4:1	22.22	8.33
Tween 80: Span 80 (2:1)	1:1	19.44	5.56
	2:1	25.00	0
	4:1	27.78	0

3. Physical Stability

Under the stressed condition of six heating-cooling cycles, all the microemulsions did not show any change in physical appearances, including color and clarity.

D. Centella Extract Microemulsion

1. Determination of Physical Properties of Centella Triterpene Extract Microemulsion

Ten formulas of microemulsions were selected to incorporate with Centella

triterpene extract. The oil: surfactant: water ratio was selected and fixed in the regard of the lowest surfactant concentration that formed microemulsions.

As reported by Shukla (1999), 0.2% asiaticoside solution reported in an improvement of wound healing in guinea pigs. In this study 2% w/w Centella extract was used for the purpose to increase the accuracy of the analysis of active compounds in the permeation study.

1.1 pH

Table 19 showed pH values of microemulsion formulas with and without Centella triterpene extract. The pH values were in the range of 5-7. This revealed that the Centella triterpene extract did not affect the pH values of the microemulsion. Additionally, the pH values of all microemulsions were in the appropriate range for dermal preparation.

1.2 Viscosity

As summarized in Table 19, the viscosity values of microemulsions with Centella triterpene extract were slightly higher than those without the extract. These microemulsions were quite low viscous at ambient temperature and had a wide range of viscosity from 3.75-384.99 cp. From Table 19, it was obvious that the addition of cosurfactants (in formula 11-15) decreased the viscosity of microemulsion. The cosurfactants might lower the interfacial tension between the oil

and water phase so that increasing the fluidity of the interface (Lawrence and Rees, 1994).

Table 19 pH and viscosity results of microemulsion preparation with and without Centella triterpene extract at 23 ± 0.5 °C

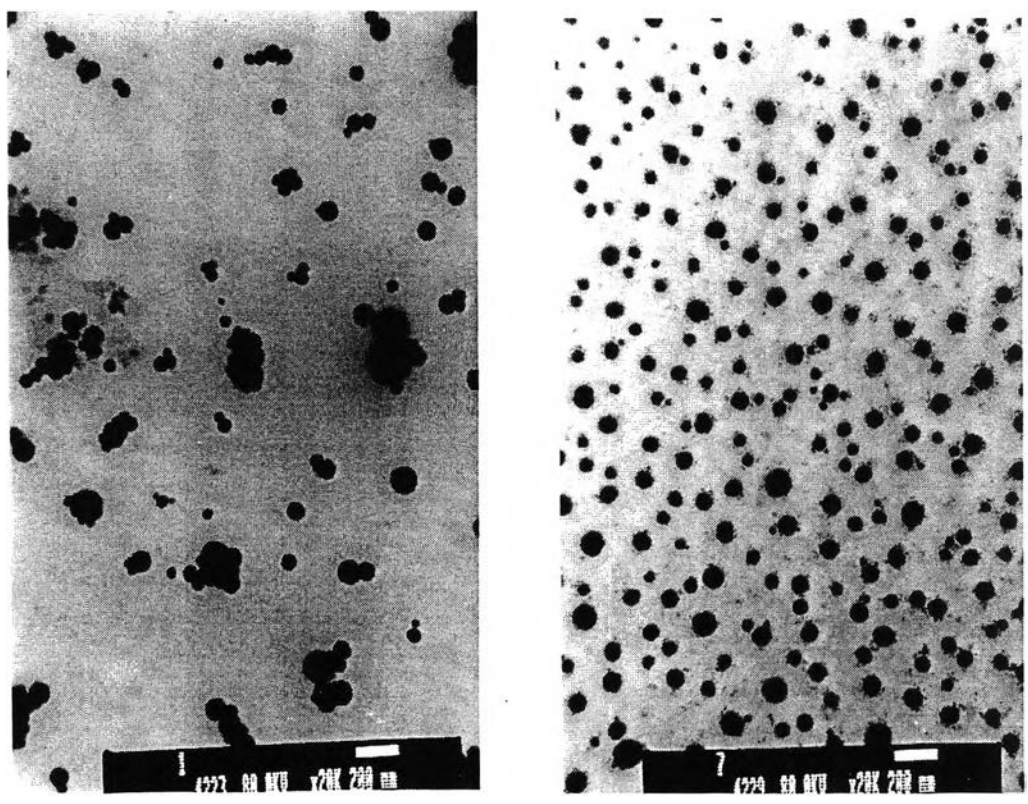
Formula	pH		Mean of viscosity (cp)	
	A*	B*	A*	B*
1	5.67	5.52	125.24	210.48
2	5.77	5.12	243.07	352.94
3	6.17	5.49	217.35	286.23
4	6.76	5.04	302.48	384.99
5	5.96	5.50	30.33	31.59
6	5.83	6.16	36.10	43.40
7	6.71	6.68	132.65	146.44
8	6.38	6.35	105.29	160.56
9	6.60	6.38	89.43	142.90
10	5.31	6.56	118.21	149.98
11	6.45	5.63	3.75	4.88
12	5.56	5.53	26.40	36.06
13	6.54	5.49	6.36	14.12
14	6.83	5.61	13.98	19.05
15	7.12	6.31	20.83	31.33

(A*; microemulsion formulation without Centella triterpene extract, B*;microemulsion formulation with Centella triterpene extract)

1.3 Droplet Size

Visualization of microemulsion droplets was performed by negative staining transmission electron microscopy. It demonstrated microemulsion droplets as spherical shape, having a diameter in the range of 30-200 nm (Figure 23 and Table 20).

From the determined droplet size range, this confirmed that the systems used in the further study were microemulsion. Different patterns between o/w and w/o microemulsion were observed (Appendix C). From the TEM images of the o/w microemulsion (Figure 25 B), smaller attack surroundly to larger droplets were observed. This was not observed in w/o microemulsion system. The type of microemulsion was confirmed by electrical conductivity value in the study Topic 1.4.



A B

Figure 25 TEM images of microemulsions (x 20 K)

(A; w/o microemulsion (IPM: Tween 80 : Span 80 (1:1) : Water = 30: 60:10), b; o/w microemulsion (IPM: Brij 97: Water = 30: 60 :10))

Obviously, the droplet sizes of o/w microemulsion in cosurfactant free system (formulas 7 and 8) were smaller than system with cosurfactant (formulas 11-14), the mechanism was not clear. However, it might involve in film spread properties of cosurfactant.

Table 20 Droplet size results of microemulsion preparation without centella selected triterpene extract

Formula	Droplet Size (nm)
1	50-200
2	50-120
3	50-110
4	90-180
5	40-180
6	40-120
7	40-120
8	30-60
9	60-100
10	50-100
11	110-240
12	80-200
13	60-240
14	40-140
15	60-120

1.4 Electrical Conductivity

Conductimetry is a useful tool to assess microemulsion structure. Usually, an extremely low conductance less than 1μs was employed to verify that the microemulsions formed were of the w/o type. Conductivity values of the compounds used and microemulsion preparations are shown in Tables 21 and 22.

In the study, the specific conductivity of the microemulsions varied

from 0.1-0.9 μs for the w/o system, and 4.4-21.1 μs for the o/w system. Conductivity of o/w microemulsion with Centella triterpene extract was lower than without Centella triterpene extract. The rather lower conductivity of microemulsion may be explained by the structural change associated with formulations close to the polar head of surfactant turned to polar molecules of Centella triterpene extract. However, conductivity of w/o microemulsion with Centella triterpene extract tended to be higher than those without the extract. This result may explained that Centella triterpene extract was solubilised by surfactant at oil-water interface.

Table 21 Conductivity results of control at 23 ± 0.5 °C

Control	Conductivity (μs)
Arlatone T	1.6
Brij 30	2.9
Brij 97	2
caprylic/capric triglyceride	1
IPM	1
Span 80	1.5
Tween 80	1.7
water	2.2
propan-2-ol	21.8
propylene glycol	22.1

In o/w microemulsion, the conductivity values were higher when added cosurfactant in the system (formulas 11-14) as comparing with those of absence cosurfactant (formulas 7,8). While in w/o microemulsion, the conductivity values did not change after the addition of cosurfactant. This might be the result of short chain cosurfactant that might flavor the head region of surfactant.

Table 22 Conductivity results of microemulsion preparations with and without Centella triterpene extract at 23 ± 0.5 °C

Formula	Conductivity (μs)	
	A*	B*
1	0.2	0.8
2	0.2	0.8
3	0.3	1.3
4	0.9	0.9
5	4.4	4.2
6	5.3	4.3
7	11.5	1.6
8	11.3	0.9
9	1.6	0.8
10	1.7	1.4
11	21.1	14.1
12	16.4	15.1
13	12.4	8.6
14	8.9	5.7
15	0.1	3.5

(A*; microemulsion formulation without Centella triterpene extract, B*; microemulsion formulation with Centella triterpene extract)

2. Permeation Study

Microemulsions have generated considerable interest as potential drug delivery systems. The existence of domains of different polarity within the same single-phase solution enables both water-soluble and oil-soluble materials to be solubilised. Water-soluble materials would be most likely to be incorporated into the dispersed aqueous phase of a water-in-oil droplet. The attraction of o/w microemulsion systems lies in their ability to incorporate hydrophobic drugs into the non polar oil phase thereby enhancing their solubility. The vast majority of drug

delivery investigations with topical microemulsions have been done in vitro, using the Franz type diffusion cells (Walters, 2002).

2.1 Solubility Studies of Receptor Fluids

Regarding solubility studies, it was found that the solubility of asiaticoside, madecassic acid and asiatic acid were increased as a function of ethanol concentrations (Table 23). The results showed the highest solubility when PBS with 40% ethanol was used and this solvent was selected as the receptor fluid for permeation study.

Table 23 Solubility of asiaticoside, madecassic acid and asiatic acid and various concentrations of ethanol in PBS

Solvent	Solubility [a](mg/ml)		
	Asiaticoside	Madecassic acid	Asiatic acid
PBS	0.1430 ± 0.0114	0.0133 ± 0.0038	0.0030 ± 0.0011
PBS+10%ethanol	0.2687 ± 0.0356	0.0158 ± 0.0020	0.0044 ± 0.0001
PBS+20%ethanol	0.8396 ± 0.0687	0.0562 ± 0.0130	0.0173 ± 0.0045
PBS+30%ethanol	1.4805 ± 0.0194	0.1474 ± 0.0200	0.0763 ± 0.0107
PBS+40%ethanol	1.5768 ± 0.0258	0.2736 ± 0.0657	0.1602 ± 0.0364

a-mean ± SD, n = 3

2.2 Effects of Oils

Microemulsions have been known to affect to the permeability of drug in the skin. The mechanisms of the effect have not clearly been explained. Several compounds used in microemulsions have been reported to improve the transdermal permeation by altering the structure of the stratum corneum. In addition, microemulsions could act as drug reservoirs where drug is released from the inner

phase to the outer phase and then further on the skin. Another possibility is that the microemulsion droplet breaks down on the surface of the stratum corneum and, subsequently, release its contents onto the skin (Rhee et al., 2001).

The permeation profiles of asiaticoside, madecassic acid and asiatic acid of microemulsions are presented in Figure 26. From permeation profiles calculated flux and permeability, statistical analysis by independent t-test comparison of permeability throughout 72 hours (Appendix D and E) showed that microemulsion using IPM as oil provided permeability of asiaticoside significantly higher ($p<0.05$) than caprylic/capric triglyceride in system using Tween 80: Span 80 (2:1), Brij 30, Brij 97 and Arlatone T. Similarity in permeability of madecassic acid, IPM showed significantly higher ($p<0.05$) than caprylic/capric triglyceride in system using Tween80: Span 80 (2:1), Brij 97 and Arlatone T. While, permeability of asiatic acid IPM gave significantly higher ($p<0.05$) than caprylic/capric triglyceride in system using Tween80: Span 80 (2:1)

In contrast to system using Brij 30, permeability of madecassic acid and asiatic acid, caprylic/capric triglyceride showed significantly higher ($p<0.05$) than IPM in system using Tween80: Span 80 (2:1), Brij 97 and Arlatont T. However, the beneficial effects of IPM on permeability seemed to be higher than caprylic/capric triglyceride. Thus, IPM was selected for further study.

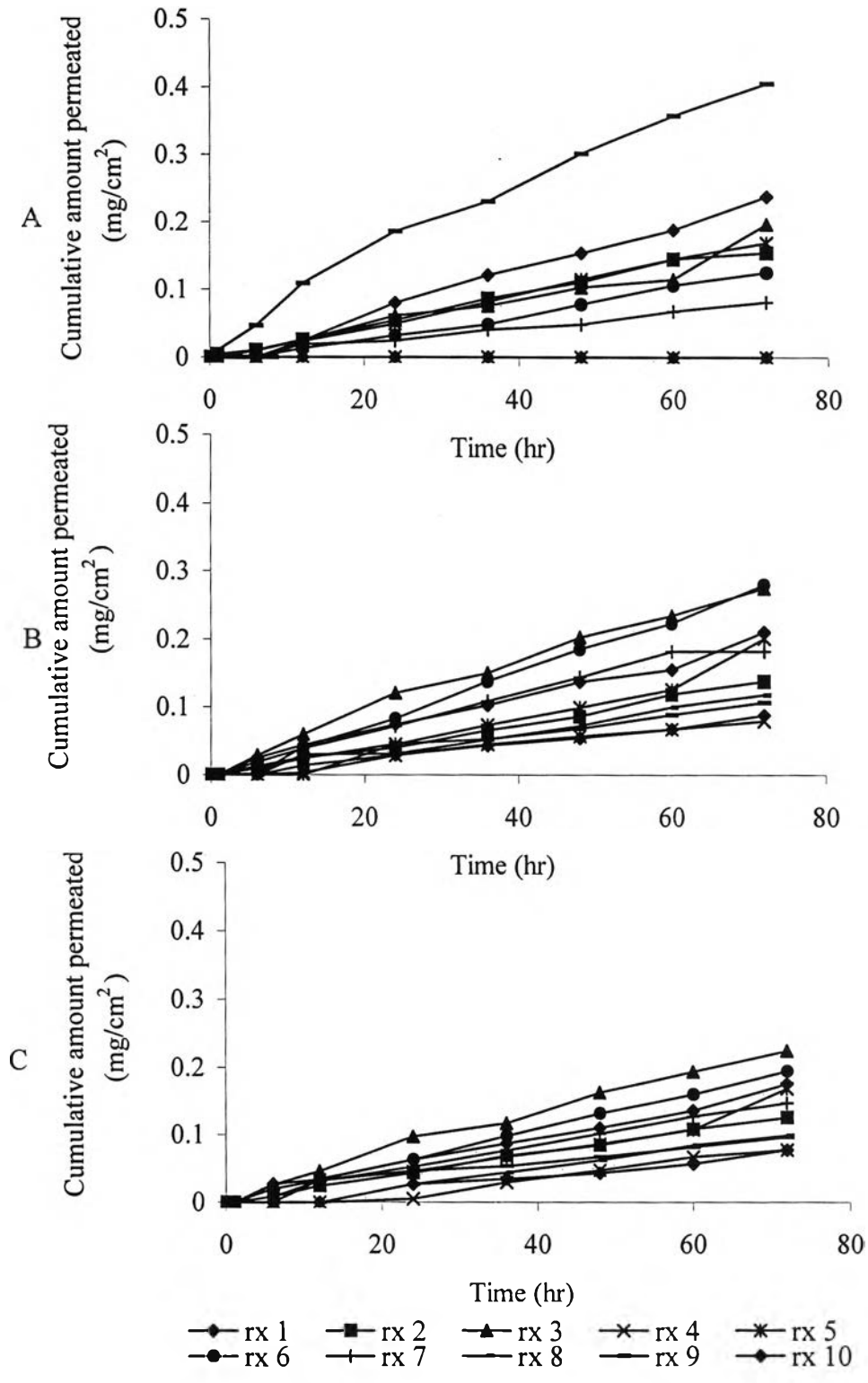


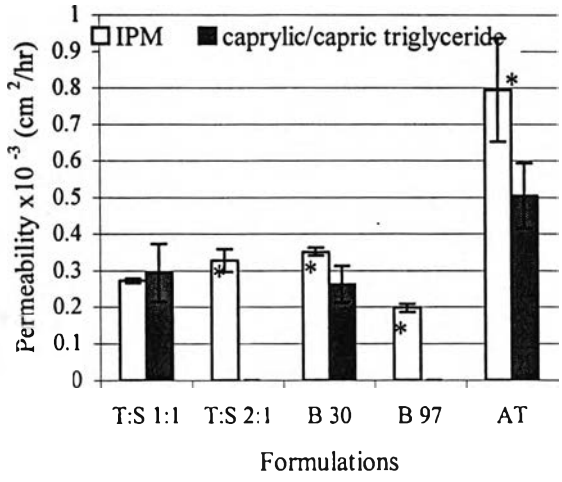
Figure 26 Permeation profiles of Centella triterpene extract from microemulsion without cosurfactant (A; asiaticoside, B; madecassic acid, C; asiatic acid)

2.3 Effects of Surfactants

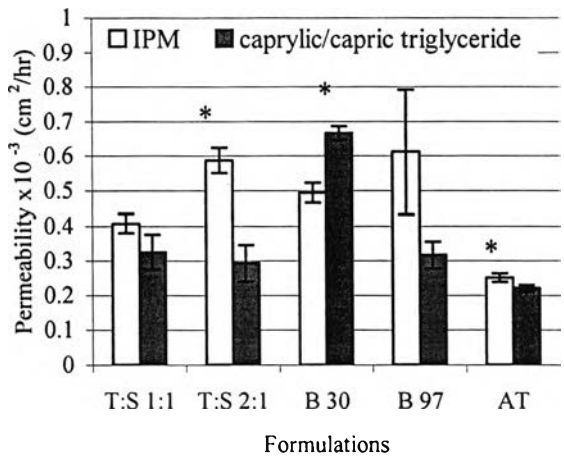
Surfactants, which are able to function as enhancers, are believed to penetrate the skin mainly in their monomer form. However, microemulsions are highly dynamic structures, it is possible that monomer surfactants or oil molecules can diffuse to the skin surface and act by disrupting the lipid structure of the stratum corneum, facilitating diffusion through the barrier phase, or by increasing the solubility of the drug in the skin. It is likely that microemulsions, which enhance cutaneous drug delivery by disruption of the stratum corneum lipids, will result in an increase in both dermal and transdermal drug delivery, while the vehicles that act as enhancers by means of increasing the partition of the drug into the skin, will mainly yield an increase in dermal drug delivery (Walters, 2002).

The permeabilities of asiaticoside, madecassic acid and asiatic acid from microemulsions using IPM with different surfactants are compared and depicted in Figure 27. Statistical analysis by one way ANOVA followed by Scheffe's multiple comparison test at a level of significance of $p<0.05$ was used. The microemulsion containing Arlatone T showed the obviously highest asiaticoside permeability (Figure 28 A, Appendix E). In contrast with the permeability of madecassic acid, Arlatone T gave the significantly lowest madecassic acid permeability ($p<0.05$). The different of permeability may be involves physicochemical properties of compound such as polarity, similar permeability in madecassic and asiatic acid that nearly polarity (Figure 28 B and C) were observed.

A



B



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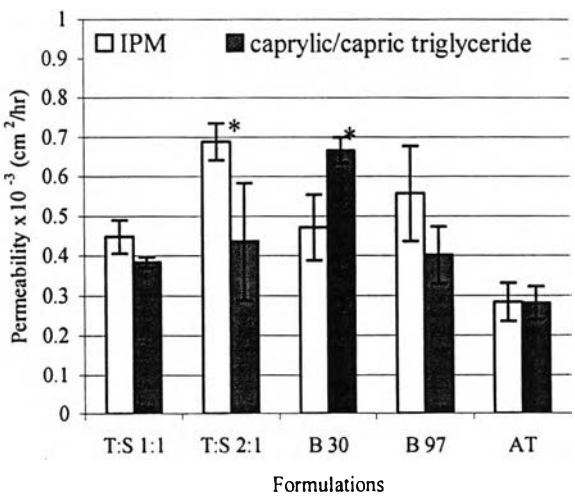

Figure 27 Comparison of permeability from microemulsion with IPM and caprylic/capric triglyceride as oils. (A; asiaticoside, B; madecassic acid and C; asiatic acid),* $p<0.05$

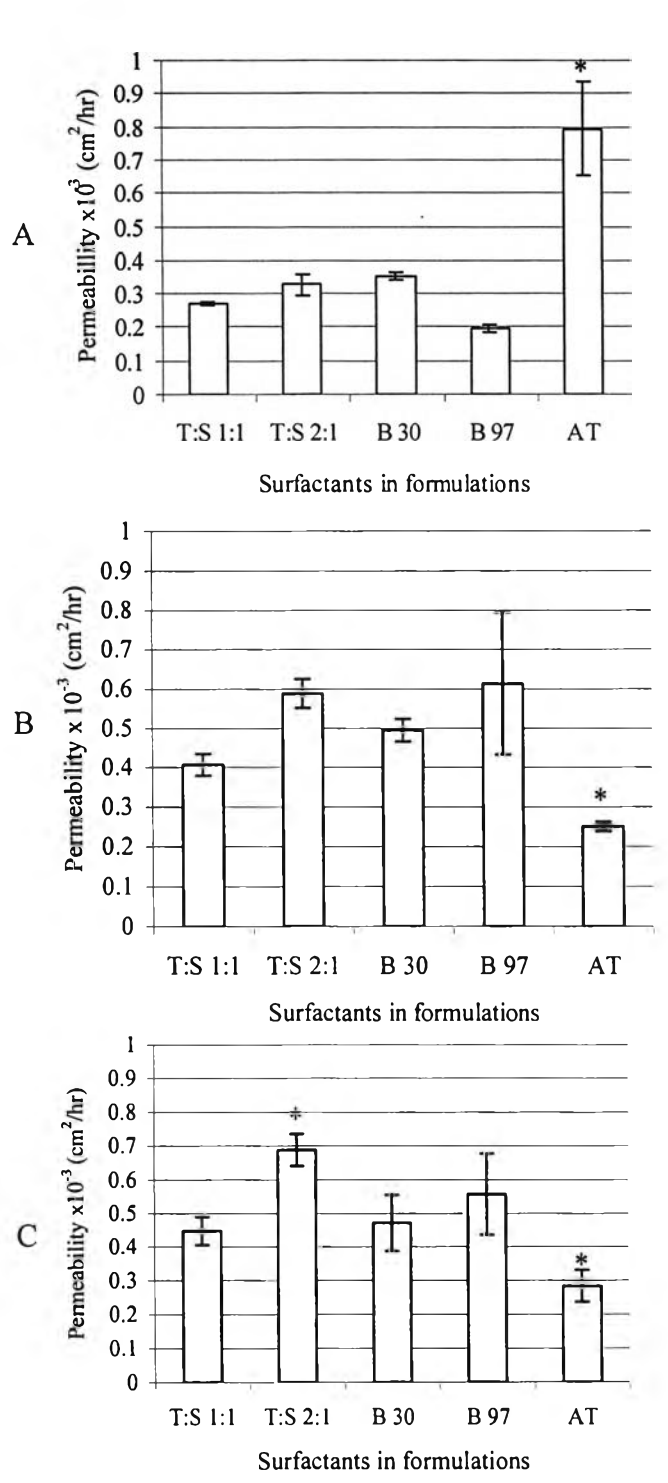

Figure 28 Comparison of permeability between different surfactant from microemulsion using IPM as oils (A; asiaticoside, B; madecassic acid and C; asiatic acid), * $p<0.05$

It was interesting that Tween 80: Span 80 (2:1) resulted to the significantly higher permeability of asiatic acid and madecassic acid ($p<0.05$) than Arlatone T. It is important because the therapeutic effects of asiaticoside may be mediated through conversion to asiatic acid (Bonte et al., 1993; Shim et al, 1996). However, as for the further study of the effect of cosurfactants, Arlatone T and Tween 80: Span 80 (2:1) could not form microemulsions in the range of concentration of studied, Brij 97 was chosen as a suitable model for next study.

2.4 Effects of Cosurfactants

The effect of cosurfactant seems to be based on improved solubility. Only the dissolved fraction of a drug in vehicle can enter the skin (Kreilgaard, 2002). In previous studies with Peltola et al. (2003) ethanol and isopropanol had substantial effects on estradiol flux across the skin. Al-suwayeh 2003, reported the ability of propylene glycol to enhance transdermal delivery. Propylene glycol could solvate the keratin structure of the cells and involved in the intercellular diffusion enhancement and disordered the lamellar lipid structure.

In Figure 29 illustrates permeation profiles of microemulsions using propan-2-ol and propylene glycol as cosurfactant. The permeability of madecassic acid and asiatic acid from propan-2-ol were significantly greater than propylene glycol ($p<0.05$) (Appendix E). Oppositely for asiaticoside permeability, propan-2-ol and propylene glycol gave non different effect ($p>0.05$). The lower enhancing effect of propylene glycol might involve droplet size of microemulsions. It was noticed that as increasing the amount of cosurfactant from 4:1 (40-140 nm) to 2:1 and 1:1, the

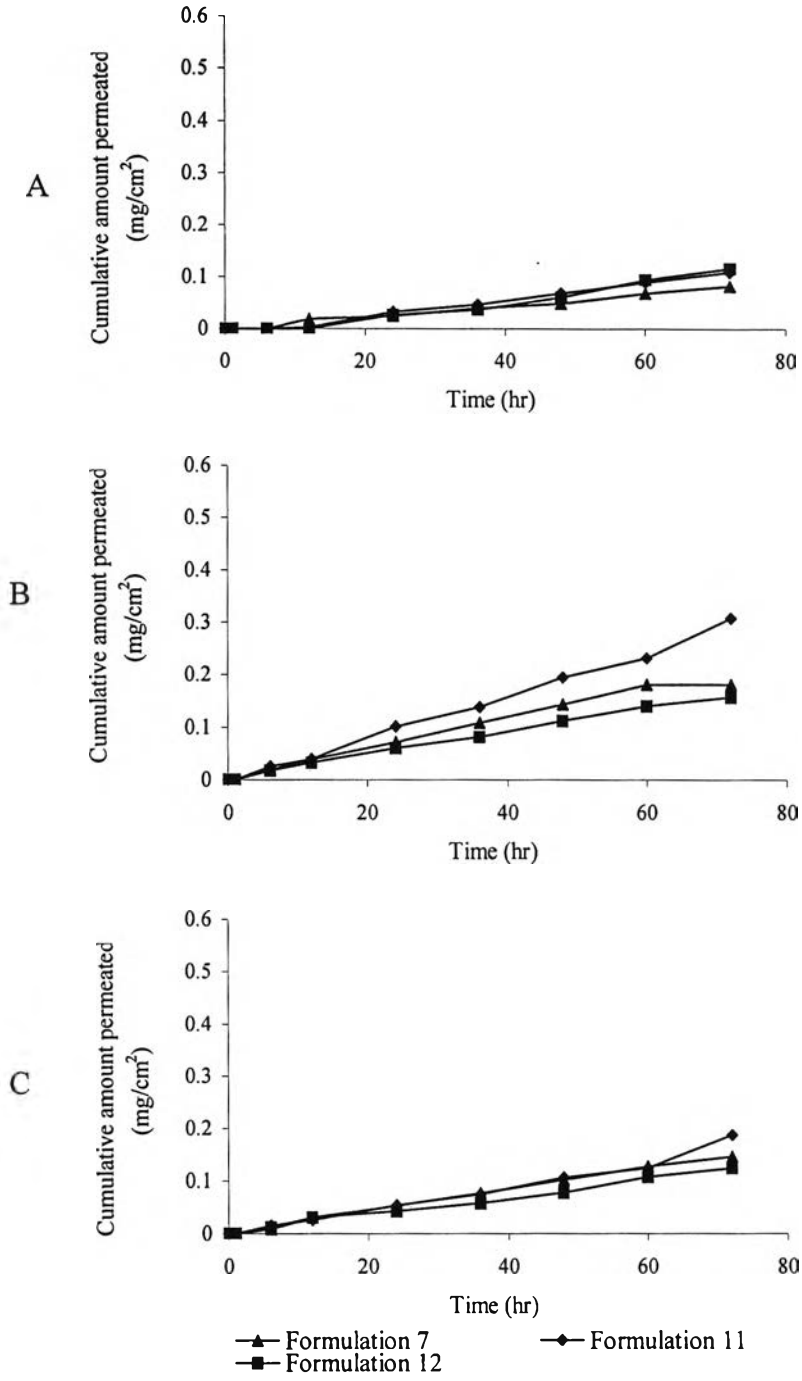

Figure 29 Permeation profiles of Centella extract from microemulsion using propan-2-ol (Formulation 11) and propylene glycol (Formulation 12) as cosurfactant comparing without non-cosurfactant (Formulation 7) (A; asiaticoside, B; madecassic acid and C; asiatic acid)

droplet sizes increased (60-240,110-240 nm), respectively. In this regard, propylene glycol gave rather large droplet size (80-200 nm).

Comparisons of the microemulsions using Brij 97: propan-2-ol at the ratios of 1:1, 2:1 and 4:1 was demonstrated in Figure 30. The result showed significant highest asiaticoside, madecasic acid and asiatic acid permeabilities at ratio 1:1 ($p<0.05$) (Appendix E). Whereas the permeabilities of madecasic acid and asiatic acid were not significantly different. The ratio of surfactant and cosurfactant of 1:1 was used in formulation of *Centella asiatica* microemulsion.

Drug transport from microemulsions is influenced by interactions within the vehicle as well as by interactions between vehicle and skin. In terms of the vehicle, physicochemical parameters of the drug influence the penetration process (Bronaugh and Maibach, 1999). Focusing in microemulsions using Brij 97, either added cosurfactants or none, it was found a slightly effect on the permeability of asiaticoside, whereas a great effect on madecassic acid and asiatic acid. This finding could explain that the polar group of asiaticoside flavor to solubilise in water phase more than oil phase, besides large molecule of asiaticoside and high polarity vehicle may be hard to partition through stratum corneum.

3. Formulation of *Centella asiatica* Extract Microemulsion

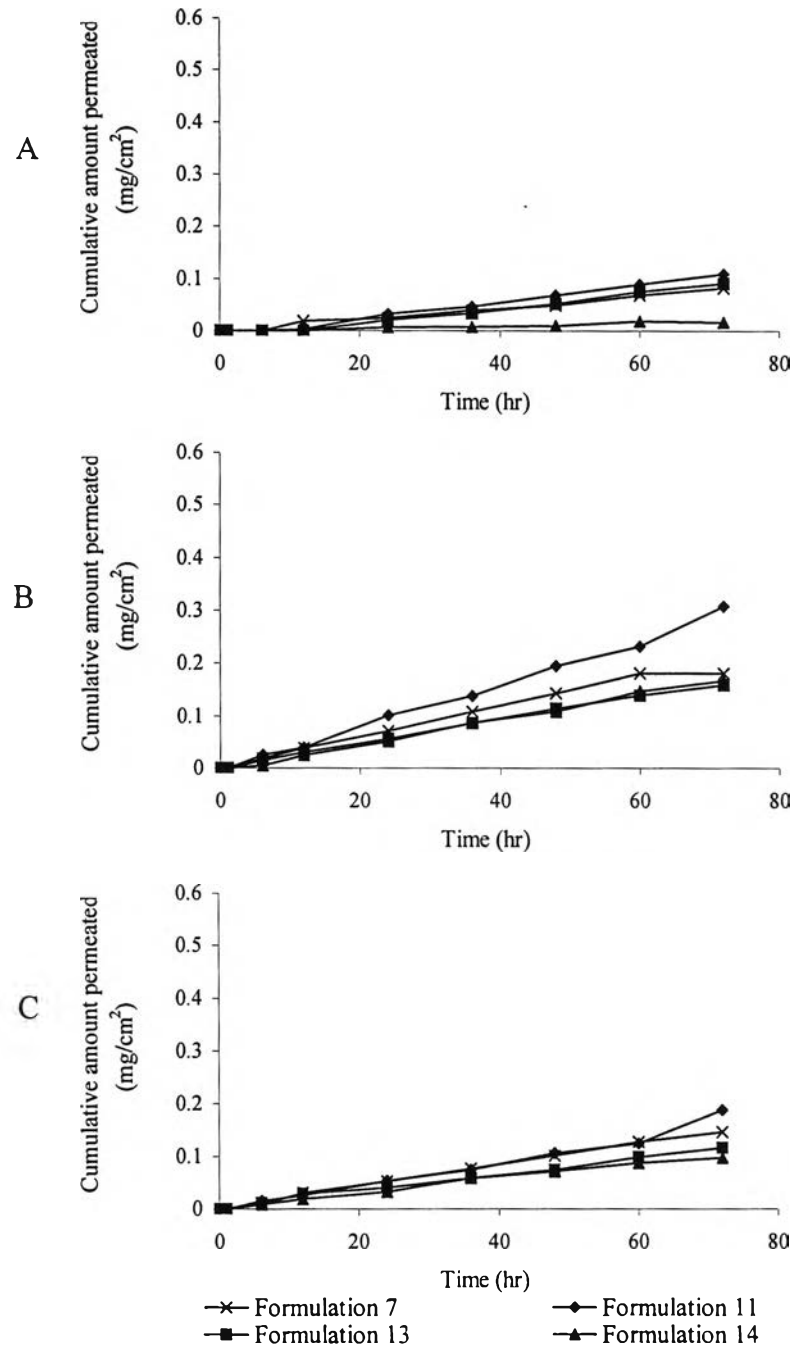

Figure 30 Permeation profiles of Centella triterpene extract from microemulsion using propan-2-ol as cosurfactant at different ratios. (A; asiaticoside, B; madecassic acid and C; asiatic acid), (1:1 in Formulation 11, 2:1 in Formulation 13, 4:1 in Formulation 14)

From the permeation study, selected type of oil, surfactant, cosurfactant and surfactant-cosurfactant raito were used to prepare a microemulsion formulation with *Centella asiatica* extract. IPM was used as oil and propan-2-ol as cosurfactant at the surfactant and cosurfactant ratio of 1:1. Although, Brij 97 was used for permeation study model for chosen cosurfactant, but actually, Arlatone T and Tween 80: Span 80 (2:1) gave higher beneficial in permeabilities. In this study, Tween 80: Span 80 (2:1) was selected in two reasons. Firstly, Tween 80: Span 80 (2:1) gave higher isotropic area than Arlatone T, thus they might result to higher resistance when composition of microemulsion change. Secondly, Tween 80: Span 80 (2:1) gave higher summation permeability value of asiaticoside, madecassoside and asiatic acid than Arlatone T.

3.1 Permeation Study of *Centella asiatica* Extract Microemulsion

Microemulsions with extract from *Centella asiatica* following method A1 and A2 were investigated for permeation profiles (Figure 30). The flux and permeability of asiaticoside, madecassic acid and asiatic acid are shown in Appendix D. *Centella asiatica* extract with method A2 gave asiaticoside permeability higher than from method A1. The reason was that the extract from method A2 gave higher content of asiaticoside than A1 thus, concentration gradient affected on the permeability.

3.2 Stability study

The stability of microemulsions containing IPM: Tween 80: Span 80: Propan-2-ol: water in the ratios of 30: 20: 10: 30 : 10 was determined. For the physical stability, phase separation and precipitation were not observed after heating-

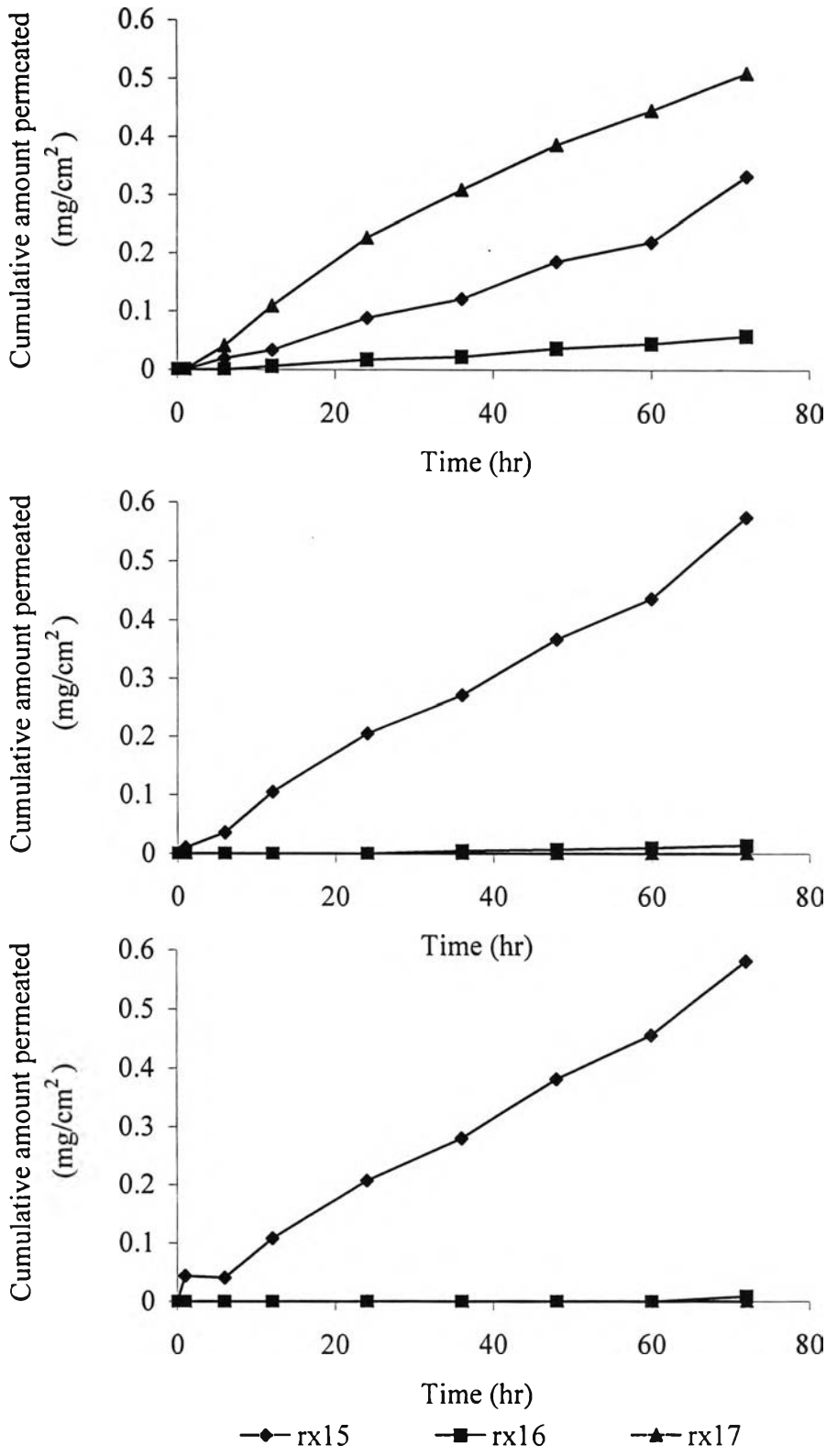

Figure 31 Permeation profiles of Centella triterpene extract from selected microemulsion (A; asiaticoside, B; madecassic acid and C; asiatic acid)

cooling cycle by visual observation. The percent remaining of asiaticoside, madecassic acid and asiatic acid in formula 15 were determined as 99.03 ± 0.60, 98.81 ± 0.66 and 99.19 ± 1.1 (n=3), respectively.

The stability of microemulsions containing Brij 97 as surfactants and propan-2-ol as cosurfactant at the ratios of 1:0, 4:1, 2:1, 1:1 (Formula 7, 11, 13 and 14) were also determined and compared in Table 24

Table 24 The percent remaining of asiaticoside, madecassic acid and asiatic acid

Formulation	Cycle	Percent remaining		
		Asiaticoside	Madecassic acid	Asiatic acid
15	0	100.00 ± 1.24	100.00 ± 1.86	100.00 ± 1.24
	6	99.03 ± 0.60	98.81 ± 0.66	99.19 ± 1.10
7	0	100.00 ± 0.80	100.00 ± 1.26	100.00 ± 1.13
	6	99.78 ± 0.74	99.83 ± 0.55	99.63 ± 1.12
11	0	100.00 ± 0.98	100.00 ± 1.44	100.00 ± 0.88
	6	99.57 ± 1.35	99.76 ± 1.20	99.46 ± 0.99
13	0	100.00 ± 0.88	100.00 ± 1.52	100.00 ± 1.31
	6	99.89 ± 1.47	99.56 ± 1.72	99.32 ± 1.58
14	0	100.00 ± 1.16	100.00 ± 0 .68	100.00 ± 1.35
	6	99.03 ± 1.39	99.19 ± 1.11	99.26 ± 0.86