CHAPTER 4

RESULTS

4.1 TD extracts preparation

The yield of the extracts was shown in Table 9. The results found that the fractionation with ethanol showed the highest yield.

Table 9 Yield of the extracts

Extracts	Abbreviation	Yield (% w/w)
Crude ethanolic extract	TDE	5.0
Crude alkaloid extract	TDC	4.8*
Hexane fractionated extract	HF	0.7
Ethyl acetate fractionated extract	EAF	1.5
Ethanol fractionated extract	EF	6.5

* Yield of TDC was calculated base on TDE.

4.2 *In vitro* anti-inflammatory activity of TD extracts

The results of *in vitro* anti-inflammation study indicated that TDE showed the highest inhibition against COX-2 comparing with the extracts from electrocoagulation and fractionation method at the same concentration (4.00 mg/ml) as shown in Figure 5.

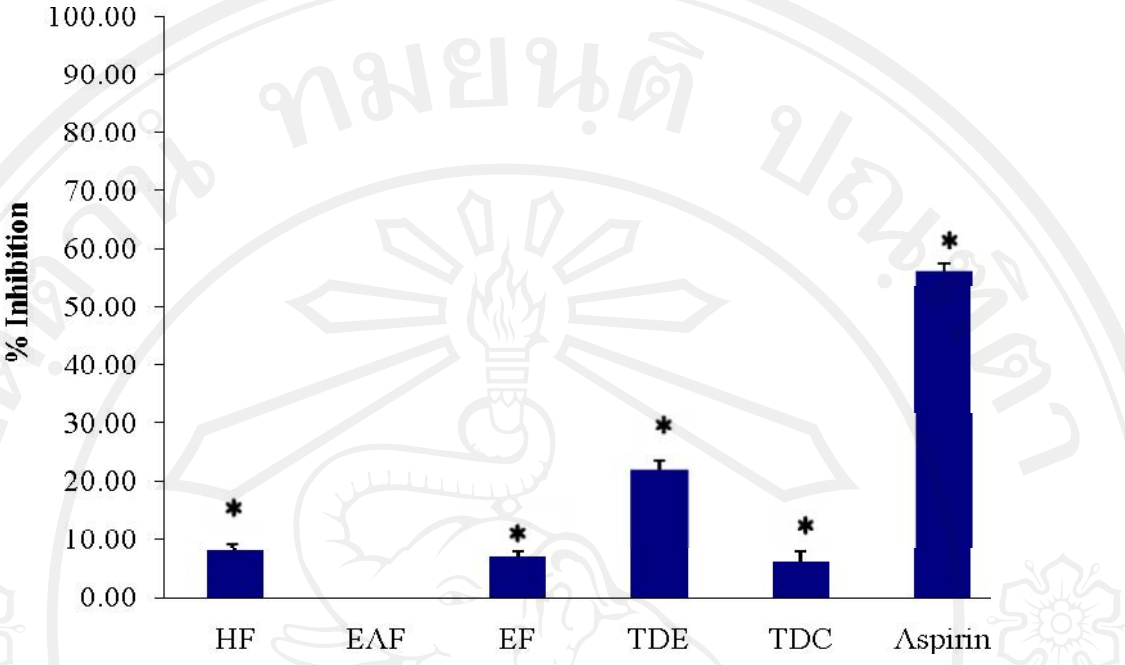


Figure 5 Percentage of inhibition of TD extracts against COX-2

4.3 Physicochemical properties of TDE

TDE revealed the highest inhibitory activity against COX-2 so it was selected for the further studies. The results are as shown in Figure 5.

4.3.1 Appearance of TDE

The obtained extract as shown in Figure 6 was an intense brown semi-solid with viscous mass and herbal odor.

Figure 6 Appearance of TDE

4.3.2 Solubility of TDE

The results demonstrated that TDE was freely soluble in alcohol, DMSO and glycerin, soluble in propylene glycol, PEG 400 and Tween 80, sparingly soluble in DI water, isopropyl myristate and liquid paraffin, very slightly soluble in n-butanol and practically insoluble in ethyl acetate, acetone and hexane (Table 10).

Table 10 Solubility of TDE at 27 °C

Solvent	**Solubility (solute: solvent, w/v)**	**Descriptions**
water	1:40	Clear yellow solution
Ethanol 95 %	1:10	Clear brown solution
Absolute alcohol	1:10	Clear brown solution
DMSO	1:10	Clear yellowish solution
Ethyl acetate	> 1:10000	Practically insoluble and suspended in solvent
Propylene glycol	1:20	Viscous yellow solution
n-butanol	1:10000	Clear yellow solution
Acetone	> 1:10000	Practically insoluble and precipitated in solvent
Polyethylene glycol 400	1:20	Viscous yellow solution
Tween 80	1:20	Viscous yellow solution
Glycerin	1:10	Viscous yellow solution
Liquid paraffin	1:100	Viscous yellow solution
Span 80	1:10	Viscous yellow solution

Solvent	Solubility (solute: solvent, w/v)	Descriptions
Hexane	> 1:10000	Practically insoluble and suspended in solvent
Polyethylene glycol 400	1:20	Viscous yellow solution
Tween 80	1:20	Viscous yellow solution
Glycerin	1:10	Viscous yellow solution
Isopropyl myristate	1:100	Viscous yellow solution

4.3.3 Thermal behavior of TDE

In this study, the result indicated that the TDE showed the endothermic behavior at about 125 °C. After that the thermogram was represented in the irregular pattern. This reaction could be concerned the degradation of TDE. So, it could be considered that the compounds in the extract were degraded when exposed to heat at 125 °C as shown in Figure 7.

4.3.4 Crystalline characteristic of TDE

The result of crystalline characteristic of TDE under taken by PXRD was shown in Figure 8. The X-ray diffractogram of TDE demonstrated the halo pattern. The result indicated that the structure of TDE was irregular arrangement as an amorphous form.

4.3.5 Study of the factors influencing the characteristic of TDE solution

The results indicated that the characteristic of TDE was changed upon pH, oxidizing-reducing agents and light exposure. It was found that color and pH of TDE solutions were changed and the precipitates occurred when the TDE solution was exposed to the acid-base solution or oxidizing-reducing agents. (Figure 9 and Tables 11-13).

4.3.6 Study of primary active compounds screening of TDE

The results indicated that the chemical constituents found in TDE consisting of alkaloid and glycosides such as cardiac and sterol/ triterpene as shown in Table 14 and 15.

4.3.7 Study of finger print of TDE using high performance liquid chromatography (HPLC)

4.3.7.1 HPLC finger print of TDE

The two alkaloids, vobasine and 19**,** 20 dehydroervatamine, were used as markers for quality control of the extract. The analysis by HPLC was developed under isocratic condition. The chromatogram of markers from TDE was shown in Figure 10. Retention time of vobasine and 19**,** 20 dehydroervatamine were 12.51 ± 0.05 and 13.95 ± 0.11 min, respectively.

4.3.7.2 Standard curve of TDE

The standard curves of vobasine and 19**,** 20 dehydroervatamine were produced for quantitative analysis. The stock TDE solution was prepared and diluted in the concentrations range of 0.0125-0.800 mg/ml. The standard curves were plotted between the peak area (y) and their amount (x). The regression analysis of standard curves of vobasine and 19**,** 20 dehydroervatamine were found to be linear between 1534.13x-18.80 and 2838.29x-2.24, respectively. The standard curves were shown in Figure 11.

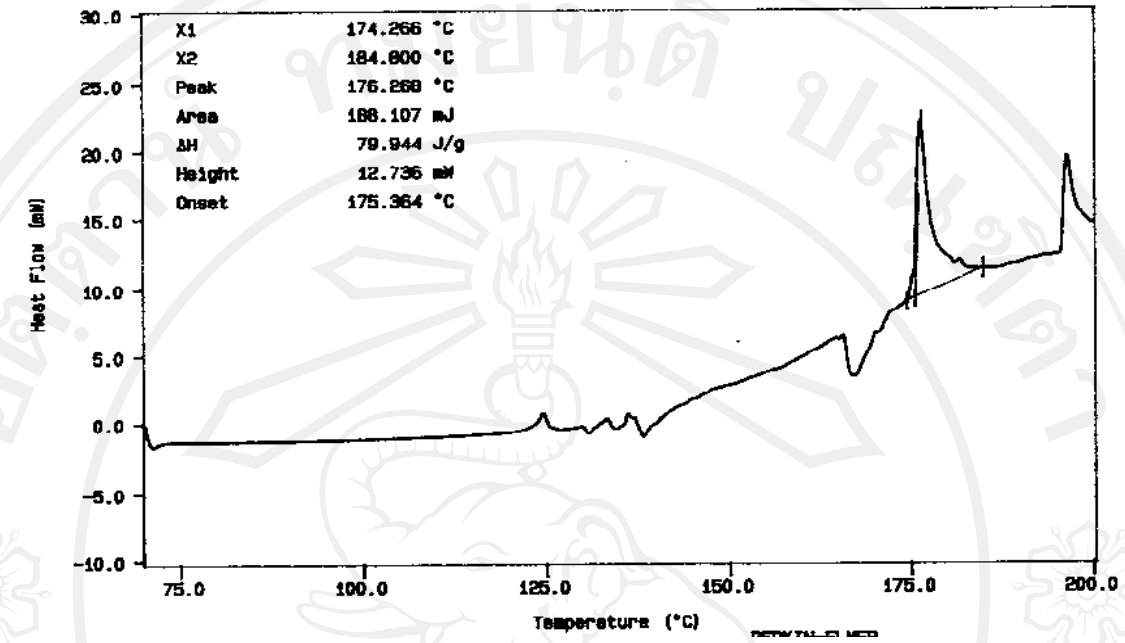


Figure 7 DSC thermogram of TDE

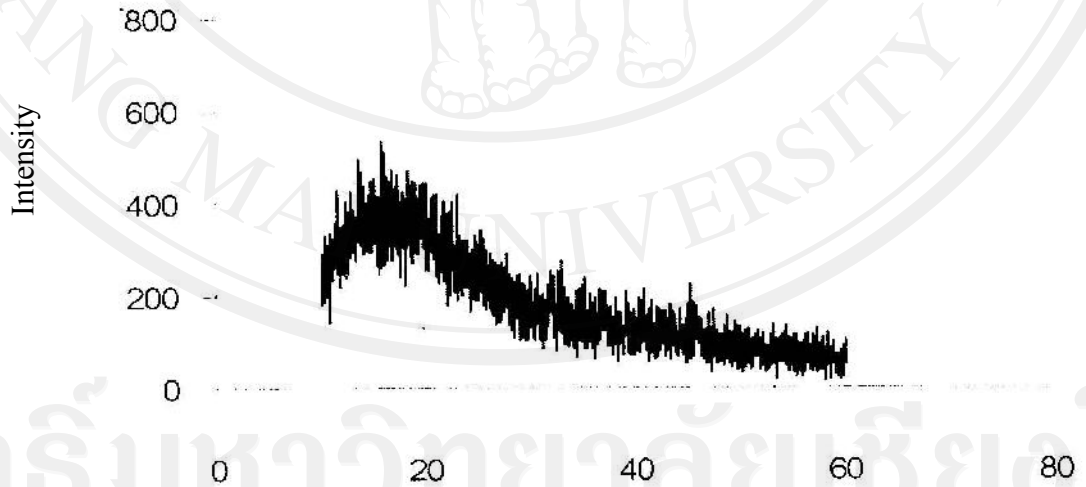
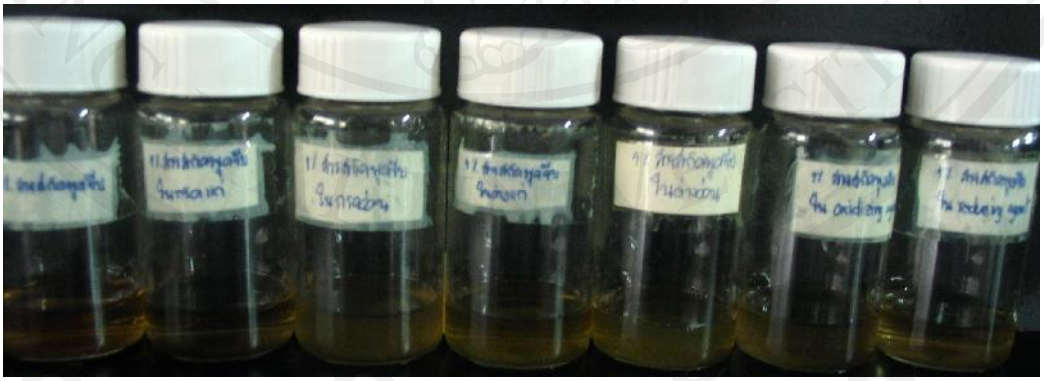


Figure 8 X-ray diffraction pattern of TDE

A

B



C

Figure 9 Appearances of TDE solution in various conditions at 27 (A), 45 (B) and 4 °C (C)

Table 11 pH value and appearances of TDE solution stored at 27 °C in various conditions

Condition	pH value		Appearances					
			Color of solution		Turbidity		Color of precipitates	
	Day 0	Day7	Day 0	Day 7	Day 0	Day7	Day 0	Day7
0.4 mM HCl	0.44	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
10% v/v acetic acid	2.1	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
1.0 N NaOH	12.74	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
30.0% v/v NH_4OH	12.48	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
10% v/v $Na_2S_2O_3$	7.43	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
3% v/v H_2O_2	3.72	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
1% w/v TDE	4.5	4.56	Yellowish solution	Yellowish solution	NF	NF	NF	NF
1% w/v TDE. in 0.4 mM HCl (1:1)	0.63	1.04	Light yellow solution	Yellowish solution	+++	++	White	White
1% w/v TDE. in 10 % v/v acetic acid (1:1)	2.49	2.79	Light yellow solution	Light yellow solution	+++	++	White	White

Table 11 (Continued)

Condition	pH value		Appearances					
			Color of solution		Turbidity		Color of precipitates	
	Day 0	Day7	Day 0	Day7	Day 0	Day7	Day 0	Day7
1% w/v TDE in 1.0 N NaOH (1:1)	10.66	12.07	Yellow solution	Yellow solution	++	++	White	White
1% w/v TDE in 30.0% v/v NH_4OH (1:1)	12.27	11.47	Yellow solution	Yellow solution	+	+	White	White
1% w/v TDE in 10% v/v $Na_2S_2O_3$ (1:1)	5.02	8.8	Light yellow solution	Light yellow solution	++	++	White	White
1% w/v TDE in 3% v/v H_2O_2 (1:1)	3.9	3.86	Light yellow solution	Light yellow solution	++	+++	White	White
Exposed with light	4.5	3.92	Yellowish solution	Yellowish solution	NF	NF	NF	NF

N/A; not available

NF; not found

Turbidity; Slight (+), Mild (++), Moderate (+++) and High (++++)

Table 12 pH value and appearances of TDE solution stored at 45 °C in various conditions

Condition	pH value		Appearances					
			Color of solution		Turbidity		Color of precipitates	
	Day 0	Day7	Day 0	Day 7	Day 0	Day7	Day 0	Day7
0.4 mM HCl	0.44	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
10% v/v acetic acid	2.1	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
1.0 N NaOH	12.74	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
30.0% v/v NH_4OH	12.48	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
10% v/v $Na_2S_2O_3$	7.43	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
3% v/v H_2O_2	3.72	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
1% w/v TDE	4.5	4.56	Yellowish solution	Yellowish solution	NF	NF	NF	NF
1% w/v TDE. in 0.4 mM HCl (1:1)	0.63	1.04	Light yellow solution	Yellowish solution	+++	+++	White	White
1% w/v TDE. in 10 % v/v acetic acid (1:1)	2.49	2.79	Light yellow solution	Light yellow solution	+++	+	White	White

Table 12 (Continued)

Condition	pH value		Appearances					
			Color of solution		Turbidity		Color of precipitates	
	Day 0	Day7	Day 0	Day7	Day 0	Day7	Day 0	Day7
1% w/v TDE in 1.0 N NaOH (1:1)	10.66	12.07	Yellowish solution	Yellowish solution	++	+++	White	White
1% w/v TDE in 30.0% v/v NH_4OH (1:1)	12.27	11.47	Yellowish solution	Yellowish solution	+	++	White	White
1% w/v TDE in 10% v/v $Na_2S_2O_3$ (1:1)	5.02	8.8	Light yellow solution	Yellowish solution	++	+++	White	White
1% w/v TDE in 3% v/v H_2O_2 (1:1)	3.9	3.86	Light yellow solution	Light yellow solution	++	++++	White	White
Exposed with light	ND	ND	ND	ND	ND	ND	ND	ND

N/A; not available

NF; not found

ND; not determined

Turbidity; Slight (+), Mild (++), Moderate (+++) and High (++++)

Table 13 pH value and appearances of TDE solution stored at 4 °C in various conditions

Condition	pH value		Appearances					
			Color of solution		Turbidity		Color of precipitates	
	Day 0	Day7	Day 0	Day 7	Day 0	Day7	Day 0	Day7
0.4 mM HCl	0.44	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
10% v/v acetic acid	2.1	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
1.0 N NaOH	12.74	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
30.0% v/v NH_4OH	12.48	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
10% v/v $Na_2S_2O_3$	7.43	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
3% v/v H_2O_2	3.72	N/A	Clear solution	N/A	N/A	N/A	N/A	N/A
1% w/v TDE	4.5	4.56	Yellowish solution	Yellowish solution	NF	++	NF	Yellow
1% w/v TDE. in 0.4 mM HCl (1:1)	0.63	1.04	Light yellow solution	Yellowish solution	+++	+	White	White
1% w/v TDE. in 10 % v/v acetic acid (1:1)	2.49	2.79	Light yellow solution	Light yellow solution	+++	++++	White	White

Table 13 (Continued)

Condition	pH value		Appearances					
			Color of solution		Turbidity		Color of precipitates	
	Day 0	Day7	Day 0	Day7	Day 0	Day7	Day 0	Day7
1% w/v TDE in 1.0 N NaOH (1:1)	10.66	12.07	Yellowish solution	Yellowish solution	++	+++	White	White
1% w/v TDE in 30.0% v/v NH_4OH (1:1)	12.27	11.47	Yellowish solution	Yellowish solution	+	++	White	White
1% w/v TDE in 10% v/v $Na_2S_2O_3$ (1:1)	5.02	8.8	Light yellow solution	Light yellow solution	++	+	White	White
1% w/v TDE in 3% v/v H_2O_2 (1:1)	3.9	3.86	Light yellow solution	Light yellow solution	++	+++	White	White
Exposed with light	ND	ND	ND	ND	ND	ND	ND	ND

N/A; not available

NF; not found

ND; not determined

Turbidity; Slight (+), Mild (++), Moderate (+++) and High (++++)

Table 14 Alkaloids screening results

Substances	Before adding reagent	After adding reagents				
		Dragendroff	**Mayer**	**Wagner**	**Marme**	**Kraut**
Preliminary test						
Standard strychnine	Clear solution	Orange precipitates	White precipitates	Reddish brown precipitates	White precipitates	White precipitates
Standard atropine	Clear solution	Clear solution	Clear solution	Clear solution	Clear solution	White precipitates
Standard scopolamine HBr	Clear solution	Orange-red precipitates	White precipitates	Clear solution	Clear solution	White precipitates
DI water	Clear solution	Orange-red precipitates	Clear solution	Yellow solution	Clear solution	Clear solution
95% ethanol	Clear solution	Orange solution	Clear solution	Yellow solution	Clear solution	White precipitates
1% w/v TDE	Light yellow solution	Orange-red precipitates	White precipitates	Reddish brown precipitates	Light yellow solution	Light yellow solution
Confirm test						
1% w/v TDE	Clear solution	Orange solution	White precipitates	Reddish brown solution	White precipitates	White precipitates

Table 15 Glycosides screening results

Substances	Glycosides results								
	Anthraquinone	Coumarin	Sterol / Triterpene	Cardiac	Saponin	Flavonoid	Anthocyanine		
							Acidic	Neutral	Basic
DI water	Clear solution	NF	Clear solution	Clear solution	NF	Clear solution	Clear solution	Clear solution	Clear solution
95% EtOH	Clear solution	NF	Clear solution	Clear solution	NF	Clear solution	Clear solution	Clear solution	Clear solution
TDE	Clear solution	NF	Red-brown -> violet -> violet	Violet ring between solution layer	NF	Clear solution	Yellow solution	Yellow solution	Yellow solution

NF, not found

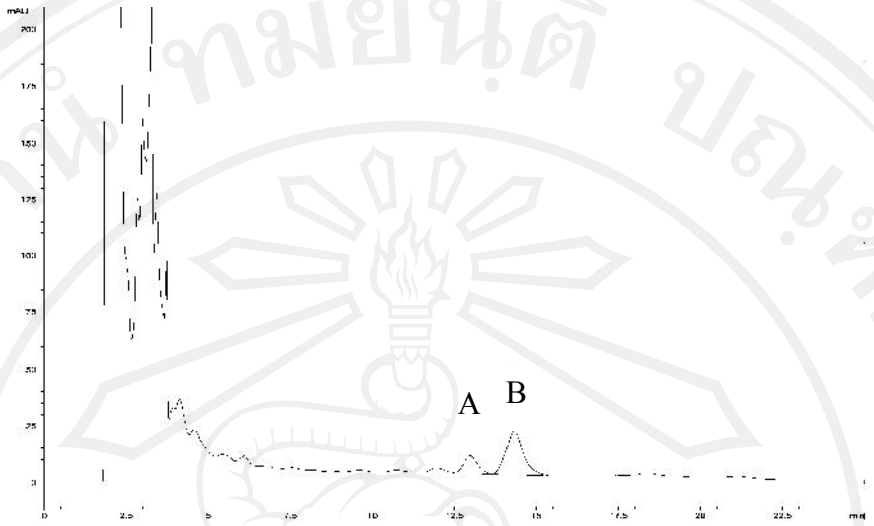


Figure 10 Chromatogram of vobasine (A) and 19, 20 dehydroervatamine (B)

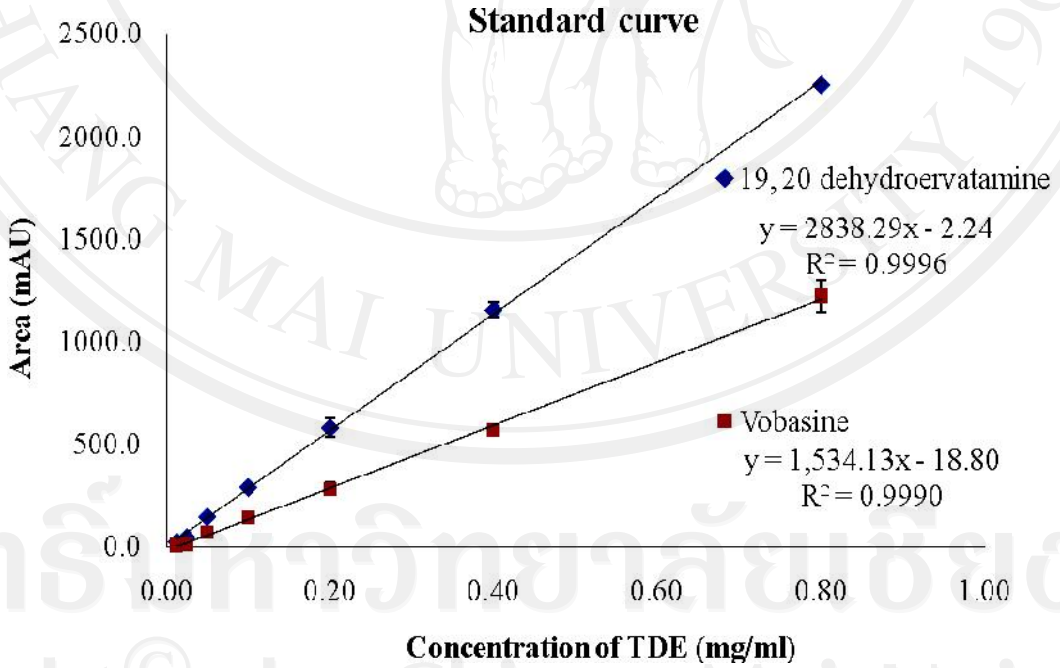


Figure 11 Standard curves of vobasine (■) and 19, 20 dehydroervatamine (♦)

4.4 Development of nanocream base

4.4.1 Effect of surfactant and homogenization cycle on the particle size, size distribution and zeta potential

The results showed that all the particle sizes were in nanometer range for the initial day. The sizes decreases were found when increasing the homogenization cycles. After 90 days, all the particle sizes increased. The polydispersity index of formulation 1A tended to increase and zeta potential was negative in range of 20-40 mV in all conditions. The polydispersity index of formulation 2A tended to increase at 4 and 45 °C while the polydispersity index tended to decrease at 27 °C. The zeta potential was negative in a range of 20-25 mV. The results were shown in Tables 16-21.

4.4.2 Effect of surfactant and homogenization cycle on the viscosity and rheological behavior

The results indicated that the viscosity of formulation 1A was increased when the number of homogenization cycles increased and their rheological behavior was pseudoplastic flow with thixotropy. After storing at 4 and 30 °C for 90 days, the viscosity of formulation 1A was decreased. On the other hand, the viscosity of formulation 1A was increased when stored at 45 °C. However, they also showed the pseudoplastic flow with thixotropy property. As shown in Tables 22-24 and Figures 12-14. The viscosity of formulation 2AC0 (before passing the high pressure homogenizer) was higher than that of 2AC3 and 2AC6 (after passing the high pressure homogenizer for 3 and 6 homogenization cycles, respectively). However, the viscosity of formulation 2A tended to increase and was in line with an increase of number of homogenization cycles. The formulation 2A showed the pseudoplastic flow with thixotropy property. After 90 days, the viscosity of formulation 2A tended to decrease as same as formulation 1A which was stored at 4 and 27 °C. Whereas, the viscosity of formulation 2A was increased when kept at 45 °C, however, they also represented the pseudoplastic flow with thixotropy property as shown in Tables 25-27 and Figures 15-17.

The particle size and size distribution results of formulation 1A comparing with 2A showed that formulation 2A was more stable than 1A when stored for 90 days. Both of formulations 1A and 2A were decreased in viscosity at 4 and 27 °C while their viscosity tended to increase at 45 °C but they also showed the pseudoplastic flow with thixotropy property. According to the above results, the formulation 2A was selected for being nanocream base. When consider the number of homogenization cycles, the characteristics of formulation 2AC3 showed a good stability as well as formulation 2AC6. Therefore, the formulation 2AC3 was selected for the further use.

Table 16 Particle size, polydispersity index and zeta potential of formulation 1A after 90 days of storage at 27 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	Cream 1AC0	124.3±1.7	0.216±0.013	-28.8±2.0
0	Cream 1AC3	95.9±3.5	0.236±0.040	-26.7±2.2
0	Cream 1AC6	127.4±7.6	0.409±0.047	-22.2±1.3
90	Cream 1AC0	184.4±7.6	0.287±0.049	-29.2±9.5
90	Cream 1AC3	284.2±96.1	0.356±0.073	-30.7±8.2
90	Cream 1AC6	219.1±35.3	0.367±0.088	-22.5±4.9

Annotation: The number shown in the sample name was referred to formulation 1A/the number of homogenization cycles, Mean ± SD, *n = 3*

Table 17 Particle size, polydispersity index and zeta potential of formulation 1A after 90 days of storage at 4 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	Cream 1AC0	124.3±1.7	0.216±0.013	-28.8±2.0
0	Cream 1AC3	95.9±3.5	0.236±0.040	-26.7±2.2
0	Cream 1AC6	127.4±7.6	0.409±0.047	-22.2±1.3
90	Cream 1AC0	293.8±50.8	0.426±0.135	-38.9±6.7
90	Cream 1AC3	228.1±56.8	0.311±0.060	-43.7±0.8
90	Cream 1AC6	141.6±17.0	0.251±0.083	-14.0±4.1

Annotation: The number shown in the sample name was referred to formulation 1A/the number of homogenization cycles, Mean ± SD, *n = 3*

Table 18 Particle size, polydispersity index and zeta potential of formulation 1A after 90 days of storage at 45 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	Cream 1AC0	124.3±1.7	0.216±0.013	-28.8±2.0
0	Cream 1AC3	95.9±3.5	0.236±0.040	-26.7±2.2
0	Cream 1AC6	127.4±7.6	0.409±0.047	-22.2±1.3
90	Cream 1AC0	625.3±33.9	0.503±0.035	-39.9±2.6
90	Cream 1AC3	1195.7±179.8	0.808±0.108	-35.8±1.0
90	Cream 1AC6	1742.4±1269.6	0.842±0.161	-39.4±2.8

Annotation: The number shown in the sample name was referred to formulation 1A/the number of homogenization cycles, Mean ± SD, *n = 3*

Table 19 Particle size, polydispersity index and zeta potential of formulation 2A after 90 days of storage at 27 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	Cream 2AC0	170.2±5.6	0.269±0.021	-26.3±0.5
0	Cream 2AC3	79.9±1.2	0.353±0.011	-31.0±0.7
0	Cream 2AC6	74.1±10.5	0.295±0.022	-33.1±0.4
90	Cream 2AC0	189.6±0.6	0.138±0.035	-28.8±0.6
90	Cream 2AC3	84.5±0.9	0.152±0.019	-24.4±0.7
90	Cream 2AC6	64.8±1.6	0.215±0.038	-12.2±4.0

Annotation: The number shown in the sample name was referred to formulation 2A/the number of homogenization cycles, Mean ± SD, *n = 3*

Table 20 Particle size, polydispersity index and zeta potential of formulation 2A after 90 days of storage at 4 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	Cream 2AC0	170.2±5.6	0.269±0.021	-26.3±0.5
0	Cream 2AC3	79.9±1.2	0.353±0.011	-31.0±0.7
0	Cream 2AC6	74.1±10.5	0.295±0.022	-33.1±0.4
90	Cream 2AC0	195.3±17.6	0.394±0.011	-27.0±1.4
90	Cream 2AC3	157.1±10.6	0.381±0.034	-33.1±1.0
90	Cream 2AC6	182.6±3.8	0.503±0.084	-27.5±2.3

Annotation: The number shown in the sample name was referred to formulation 2A/the number of homogenization cycles, Mean ± SD, *n = 3*

Table 21 Particle size, polydispersity index and zeta potential of formulation 2A after 90 days of storage at 45 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	Cream 2AC0	170.2±5.6	0.269±0.021	-26.3±0.5
0	Cream 2AC3	79.9±1.2	0.353±0.011	-31.0±0.7
0	Cream 2AC6	74.1±10.5	0.295±0.022	-33.1±0.4
90	Cream 2AC0	528.0±25.7	0.543±0.023	-32.9±3.6
90	Cream 2AC3	358.8±15.1	0.411±0.076	-23.8±0.5
90	Cream 2AC6	298.5±0.9	0.348±0.029	-25.5±0.9

Annotation: The number shown in the sample name was referred to formulation 2A/the number of homogenization cycles, Mean ± SD, *n = 3*

Table 22 Viscosity and rheological behavior of formulation 1AC0 after 90 days of storage at 4, 27 and 45 °C

Day	Sample name	Eta (Pas)	Rheological behavior
0	Cream 1AC0	0.732±0.054	Pseudoplastic flow with thixotropy
90	Cream 1AC0_27 °C	0.564±0.036	Pseudoplastic flow with thixotropy
90	Cream 1AC0_4 °C	0.675±0.011	Pseudoplastic flow with thixotropy
90	Cream 1AC0_45 °C	0.972±0.011	Pseudoplastic flow with thixotropy

Annotation: The number shown in the sample name was referred to formulation 1A/the number of homogenization cycles

Table 23 Viscosity and rheological behavior of formulation 1AC3 after 90 days of storage at 4, 27 and 45 °C

Day	Sample name	Eta (Pas)	Rheological behavior
0	Cream 1AC3	0.909±0.008	Pseudoplastic flow with thixotropy
90	Cream 1AC3_27 °C	0.733±0.018	Pseudoplastic flow with thixotropy
90	Cream 1AC3_4 °C	0.627±0.033	Pseudoplastic flow with thixotropy
90	Cream 1AC3_45 °C	1.124±0.021	Pseudoplastic flow with thixotropy

Annotation: The number shown in the sample name was referred to formulation 1A/the number of homogenization cycles

Table 24 Viscosity and rheological behavior of formulation 1AC6 after 90 days of storage at 4, 27 and 45 °C

Day	Sample name	Eta (Pas)	Rheological behavior
0	Cream 1AC6	0.933±0.012	Pseudoplastic flow with thixotropy
90	Cream 1AC6_27 °C	0.797±0.127	Pseudoplastic flow with thixotropy
90	Cream 1AC6_4 °C	0.869±0.018	Pseudoplastic flow with thixotropy
90	Cream 1AC6_45 °C	1.173±0.016	Pseudoplastic flow with thixotropy

Annotation: The number shown in the sample name was referred to formulation 1A/the number of homogenization cycles

Table 25 Viscosity and rheological behavior of formulation 2AC0 after 90 days of storage at 4, 27 and 45 °C

Day	Sample name	Eta (Pas)	Rheological behavior
0	Cream 2AC0	1.136±0.042	Pseudoplastic flow with thixotropy
90	Cream 2AC0_27 °C	0.663±0.023	Pseudoplastic flow with thixotropy
90	Cream 2AC0_4 °C	0.602±0.060	Pseudoplastic flow with thixotropy
90	Cream 2AC0_45 °C	1.041±0.068	Pseudoplastic flow with thixotropy

Annotation: The number shown in the sample name was referred to formulation 2A/the number of homogenization cycles

Table 26 Viscosity and rheological behavior of formulation 2AC3 after 90 days of storage at 4, 27 and 45 °C

Day	Sample name	Eta (Pas)	Rheological behavior
0	Cream 2AC3	0.920±0.035	Pseudoplastic flow with thixotropy
90	Cream 2AC3_27 °C	0.562±0.018	Pseudoplastic flow with thixotropy
90	Cream 2AC3_4 °C	0.628±0.036	Pseudoplastic flow with thixotropy
90	Cream 2AC3_45 °C	1.006±0.098	Pseudoplastic flow with thixotropy

Annotation: The number shown in the sample name was referred to formulation 2A/the number of homogenization cycles

Table 27 Viscosity and rheological behavior of formulation 2AC6 after 90 days of storage at 4, 27 and 45 °C

Day	Sample name	Eta (Pas)	Rheological behavior
0	Cream 2AC6	0.980±0.028	Pseudoplastic flow with thixotropy
90	Cream 2AC6_27 °C	0.583±0.026	Pseudoplastic flow with thixotropy
90	Cream 2AC6_4 °C	0.657±0.025	Pseudoplastic flow with thixotropy
90	Cream 2AC6_45 °C	0.987±0.054	Pseudoplastic flow with thixotropy

Annotation: The number shown in the sample name was referred to formulation 2A/the number of homogenization cycles

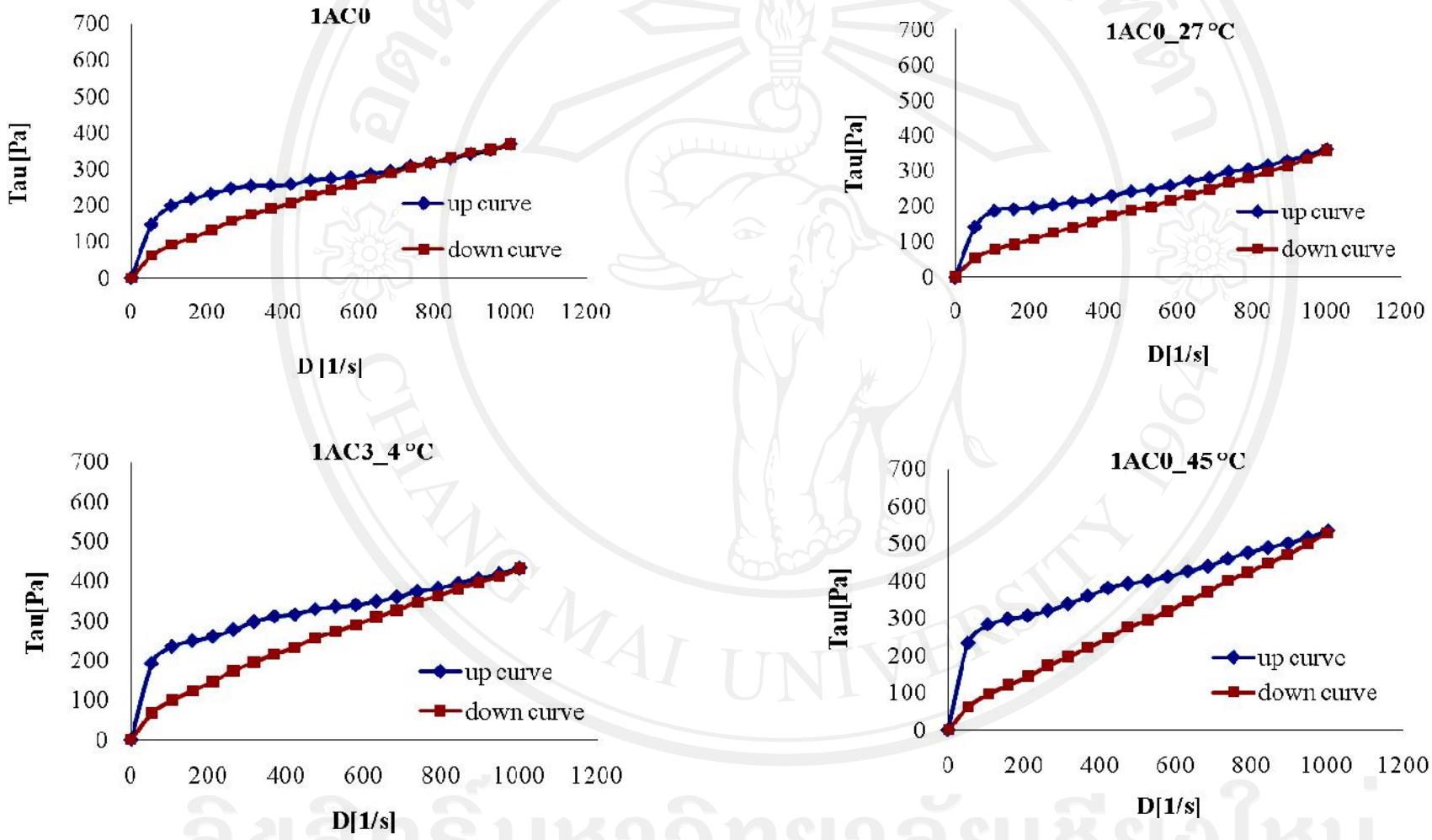


Figure 12 Rheogram of formulation 1AC0 after 90 days of storage at 4, 27 and 45 °C

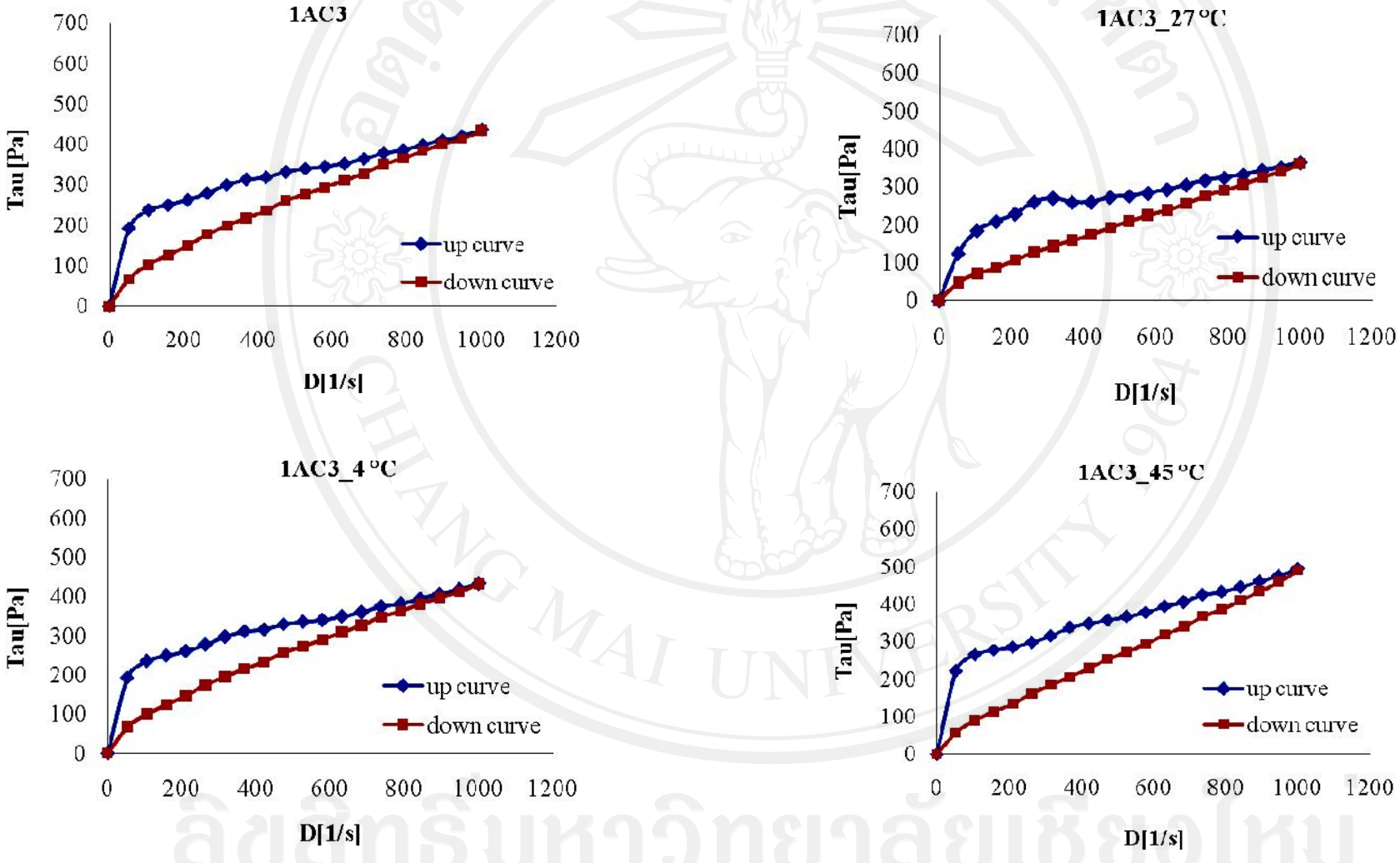


Figure 13 Rheogram of formulation 1AC3 after 90 days of storage at 4, 27 and 45 °C

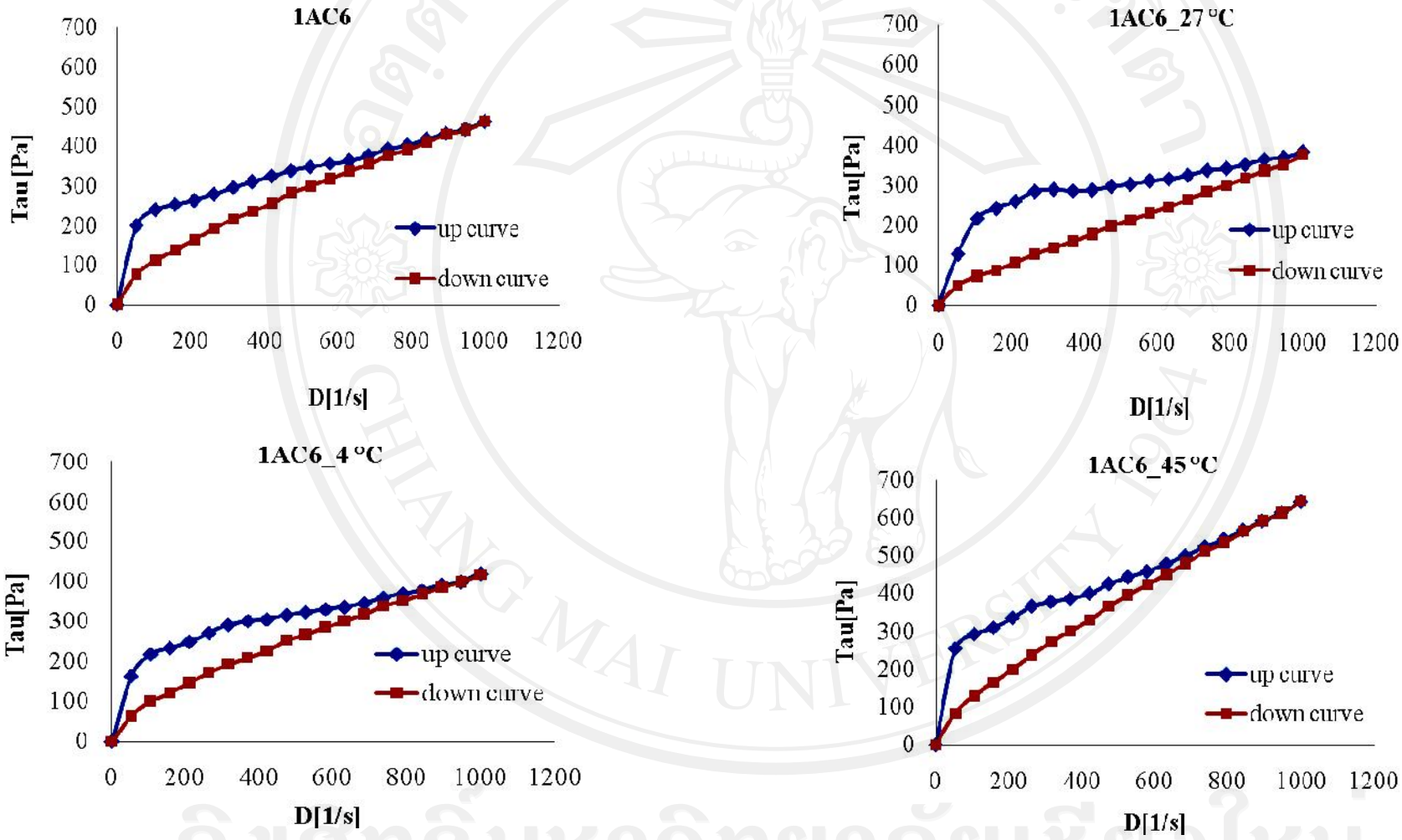


Figure 14 Rheogram of formulation 1AC6 after 90 days of storage at 4, 27 and 45 °C

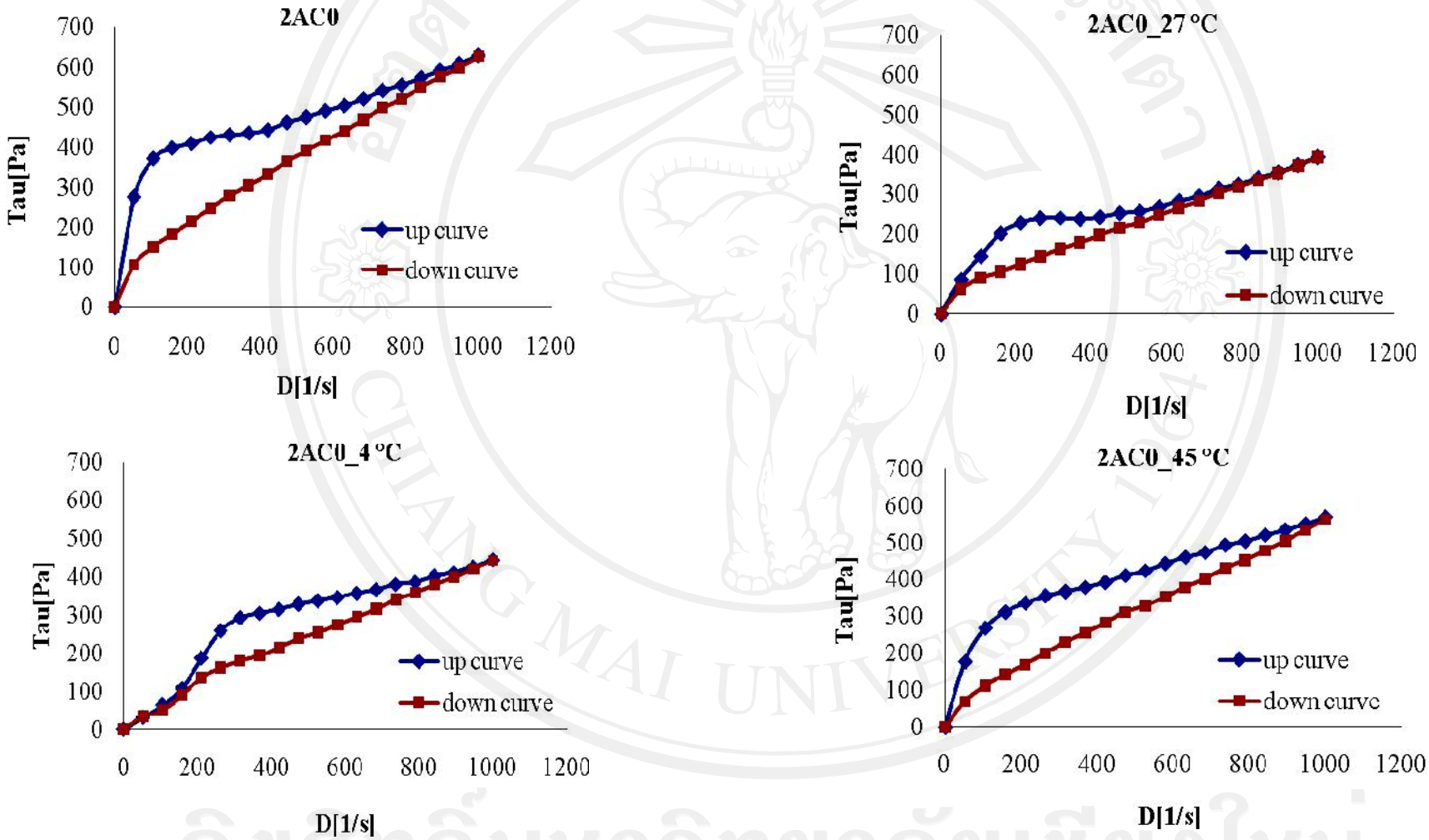


Figure 15 Rheogram of formulation 2AC0 after 90 days of storage at 4, 27 and 45 °C

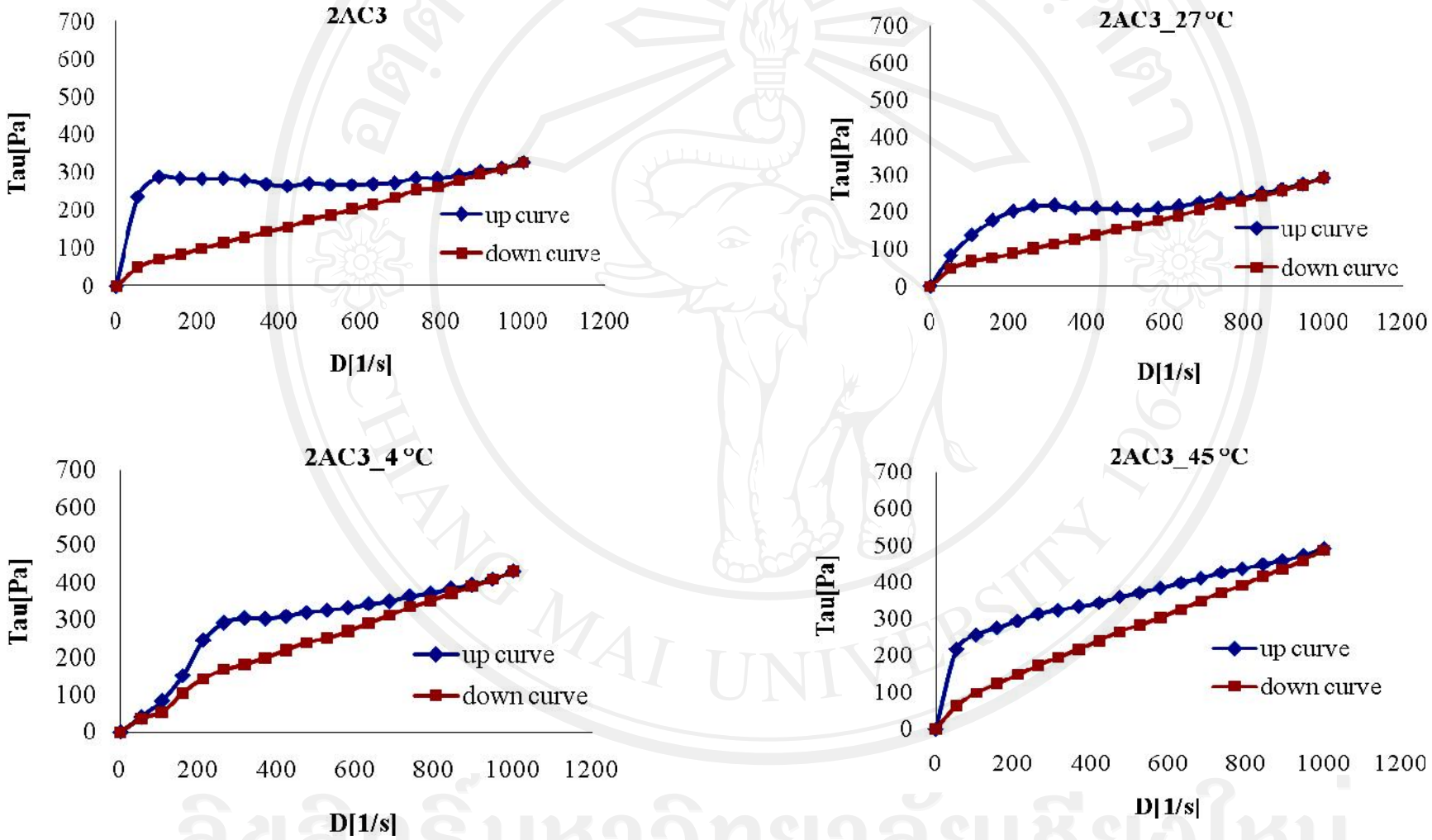


Figure 16 Rheogram of formulation 2AC3 after 90 days of storage at 4, 27 and 45 °C

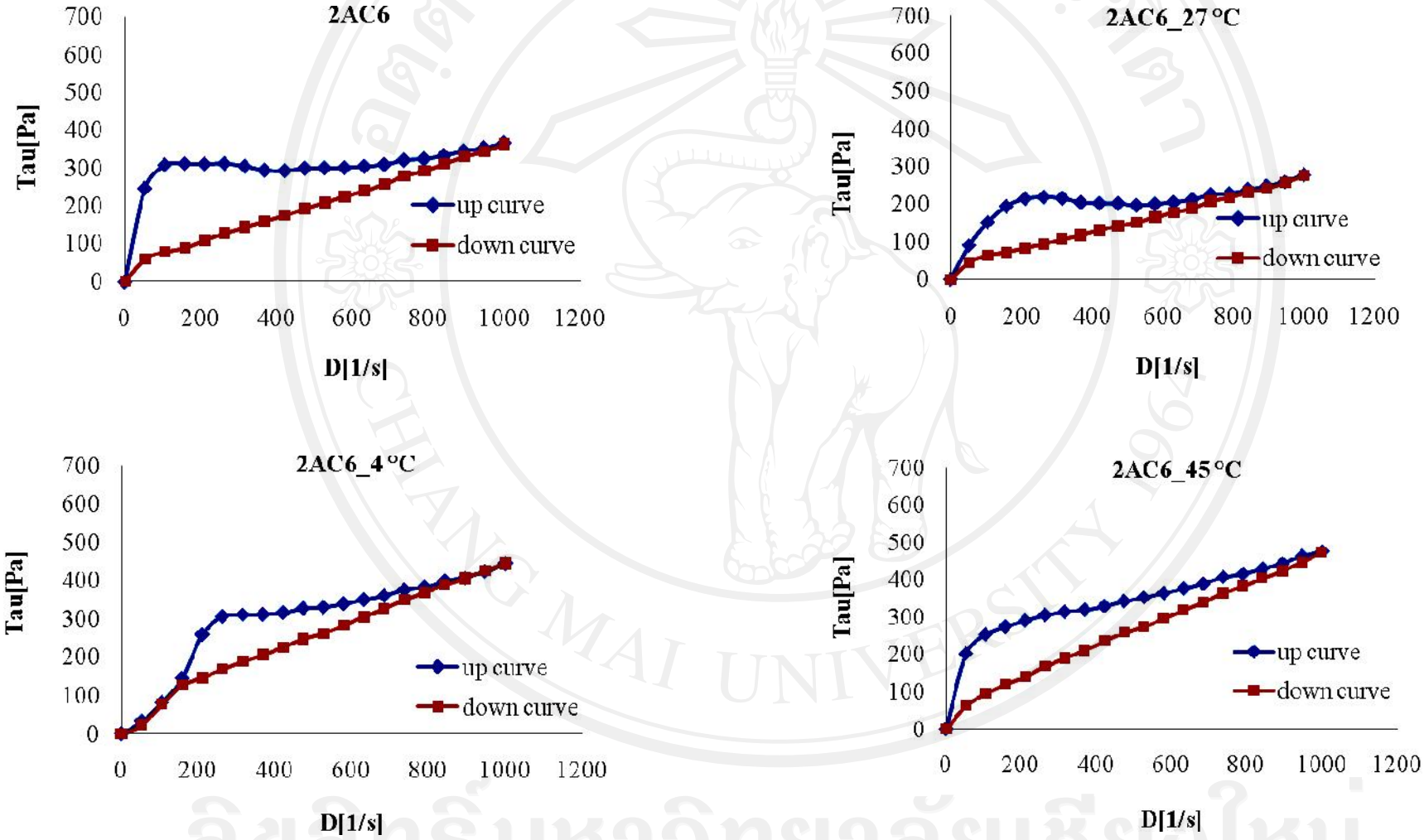


Figure 17 Rheogram of formulation 2AC6 after 90 days of storage at 4, 27 and 45 °C

4.5 Development of TDE loaded solid lipid nanoparticles (SLN)

4.5.1 Effect of surfactant and the numbers of homogenization cycles

This study interested in the mixture of surfactant, Tween80 and Span80, at concentrations of 10, 15 and 20% w/w and the numbers of homogenization at 3-or 6-homogenization cycles. The result indicated that the amounts of surfactant and the numbers of homogenization cycles affected the physiochemical properties including particle size, size distributions and zeta potential of nanoparticles. It was found that the particle sizes at the surfactant mixture concentration of 10% w/w were in the range of 180-200 nm in both 3 and 6 homogenization cycles (Figure 18 and Table 28). Table 28 showed that the 3 homogenization cycles gave more narrow size distribution than 6 homogenization cycles. All zeta potential were in negative charge and the value closed to 30 mV. According to obtained results, at surfactant mixture concentration of 10% w/w was decided to be used for the further SLN formulation.

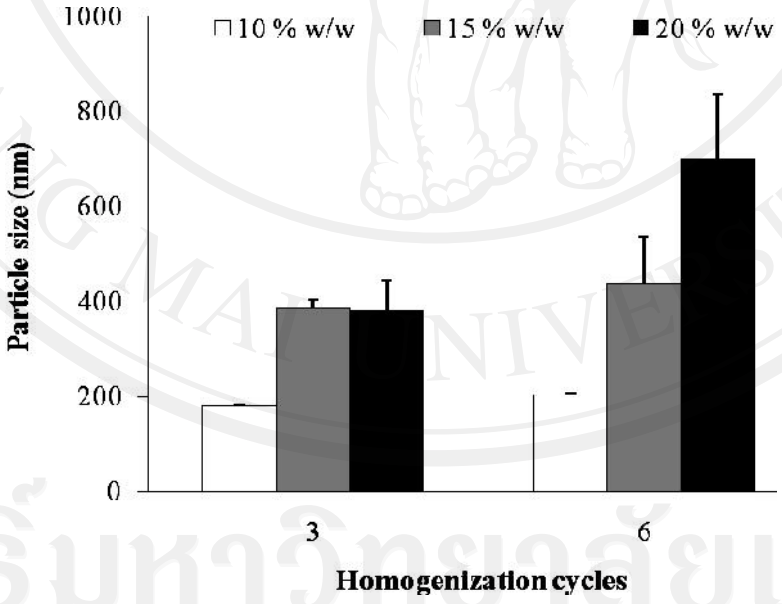


Figure 18 Effect of surfactant and numbers of homogenization cycles on the particle size

Table 28 Effect of surfactant on particle size, polydispersity index and zeta potential of SLN prepared at 3 and 6 cycles

Concentration of surfactants (% w/w)	Particle size (nm)		Polydispersity index		Zeta potential (mV)	
	3 cycles	6 cycles	3 cycles	6 cycles	3 cycles	6 cycles
10	180.2 ± 3.7	202.0 ± 5.4	0.199 ± 0.025	0.261 ± 0.020	-31.0 ± 2.6	-22.2 ± 1.2
15	385.7 ± 19.7	437.0 ± 97.5	0.531 ± 0.010	0.574 ± 0.108	-31.7 ± 2.4	-33.7 ± 1.5
20	379.9 ± 64.2	697.1 ± 138.6	0.513 ± 0.027	0.656 ± 0.076	-37.1 ± 1.6	-38.7 ± 1.9

Mean ± SD, *n=3*

4.5.2 Effect of solid lipid

The SLN formulation comprised of 2.5 to 7.5% w/w of cetyl palmitate was investigated while the amount of surfactant mixture was fixed at a concentration of 10% w/w. The results demonstrated that the particle size of all formulation were in the range of 140-200 nm with narrow size distribution (Figure 19 and Table 29). All the zeta potential was in negative charge in the range of 26-30 mV as shown in Table 29. From these results, the amounts of solid lipid of 2.5 to 7.5% w/w cetyl palmitate were used and prepared using high pressure homogenizer under the most suitable condition at 1000 bars and passing through for 3 homogenization cycles for further entrapment efficiency study.

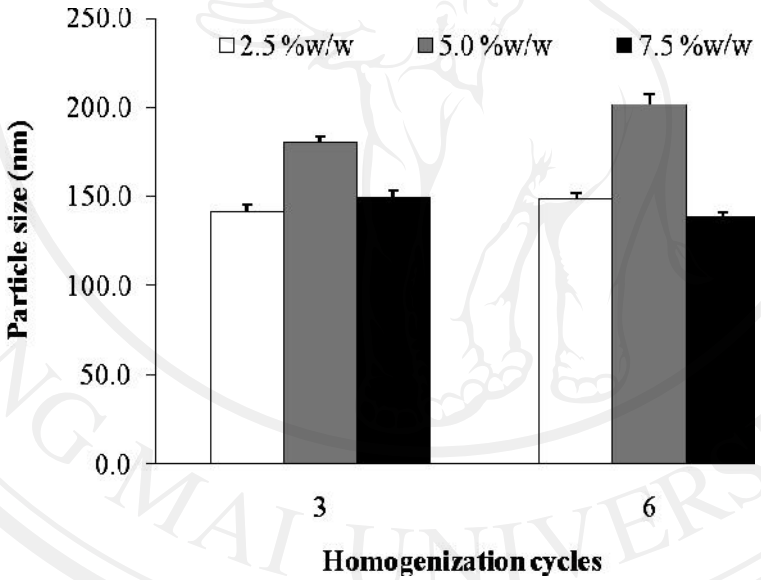


Figure 19 Effect of solid lipid on the particle size of SLN prepared at 3 and 6 homogenization cycles

Table 29 Effect of solid lipid on particle size, polydispersity index and zeta potential of SLN prepared at 3 and 6 cycles

Concentration of cetyl palmitate (% w/w)	Particle size (nm)		Polydispersity index		Zeta potential (mV)	
	3 cycles	6 cycles	3 cycles	6 cycles	3 cycles	6 cycles
2.5	143.1 ± 3.7	149.2 ± 2.8	0.154 ± 0.020	0.197 ± 0.018	-28.2 ± 1.8	-27.8 ± 1.9
5.0	180.2 ± 3.7	202.0 ± 5.4	0.199 ± 0.025	0.261 ± 0.020	-31.0 ± 2.6	-22.2 ± 1.2
7.5	149.9 ± 3.6	138.8 ± 2.8	0.247 ± 0.021	0.149 ± 0.002	-26.5 ± 0.5	-27.0 ± 0.5

Mean ± SD, *n=3*

4.5.3 Effect of TDE and solid lipid

Physicochemical properties of SLN prepared using various amounts of cetyl palmitate, i.e. 2.5, 5.0, and 7.5% w/w, and TDE i.e. 0.25 and 0.50% w/w were investigated. In this study, surfactant was fixed at 10% w/w and SLN were produced at 1000 bars with 3 homogenization cycles were investigated. The results indicated that the particle size increased when the amount of TDE increased at condition of 3 homogenization cycles. However, the particle size decreased when the amount of solid lipid increased. Both the increase of solid lipid and TDE amounts in formulation influenced their physicochemical properties such as the size distribution and the zeta potential of nanoparticles. It was found that the size distribution was broad and the zeta potential became more negative (Table 30).

4.5.4 Entrapment efficiency of TDE in SLN formulation

The entrapment efficiency (EE) of TDE in various SLN formulations with various amounts of cetyl palmitate, i.e. 2.5, 5.0, and 7.5% w/w, various amounts of TDE (0.25 and 0.50% w/w), and 10% w/w of surfactant were determined. An entrapment efficiency analysis showed that it tended to increase with the increase of the amount of solid lipid. At 7.5% w/w of cetyl palmitate, the highest EE value was shown (Table 30).

Thus, the optimized SLN formulation for the preparation of TDE loaded SLN were 7.5% w/w of solid lipid and 10% w/w of surfactant mixture under the suitable condition at 1000 bars for 3 homogenization cycles.

Table 30 Effect of the amounts of TDE and solid lipid on percentage of entrapment efficiency of 3 homogenization cycles

Concentration of TDE (% w/w)	Concentration of cetyl palmitate (% w/w)	Particle size (nm)	Polydispersity index	Zeta potential (mV)	EE (%)
0.25	2.5	143.1 ± 3.7	0.154 ± 0.020	-28.2 ± 1.8	47.46 ± 0.23
	5.0	180.2 ± 3.7	0.199 ± 0.025	-31.0 ± 2.6	47.64 ± 2.48
	7.5	149.9 ± 3.6	0.247 ± 0.021	-26.5 ± 0.5	66.89 ± 1.43
0.50	2.5	262.8 ± 11.6	0.528 ± 0.063	-31.4 ± 0.6	34.42 ± 1.33
	5.0	286.8 ± 7.1	0.482 ± 0.061	-32.9 ± 1.5	26.94 ± 11.33
	7.5	193.8 ± 5.9	0.251 ± 0.009	-32.9 ±0.3	81.95 ± 0.57

Mean ± SD, *n=3*

4.6 Development of TDE loaded SLN in nanocream

Appearance of TDE loaded SLN in nanocream

TDE loaded SLN were incorporated to nanocream at concentrations of 0.5, 1.0 and 2.5% w/w to obtain concentrations of TDE after incorporating with 0.25, 0.50 and 1.25% w/w respectively. Their physical appearances were shown in Figure 20 and Table 31. The shape of nanoparicles which was viewed with SEM at 10 kV (20,000×) found that their shapes were sphere particles as shown in Figure 21.

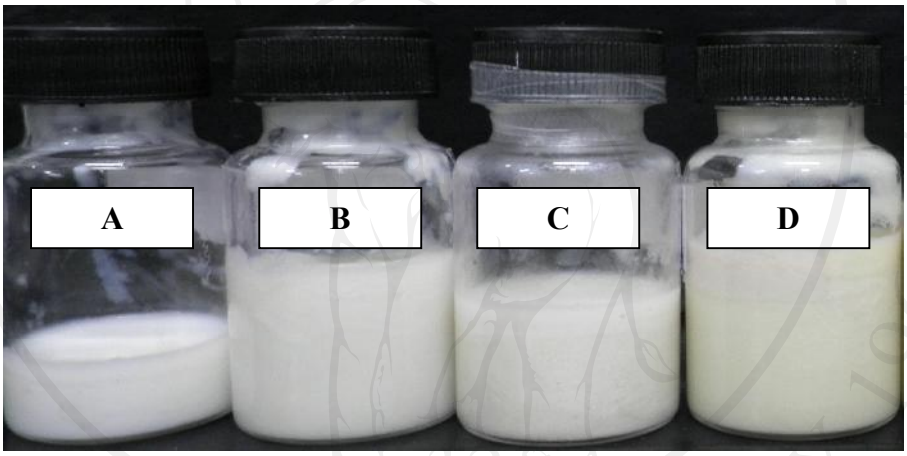
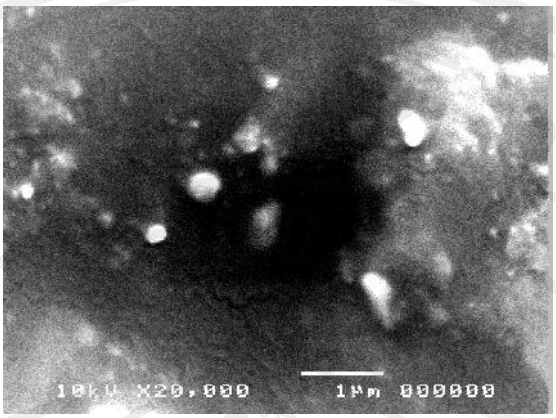
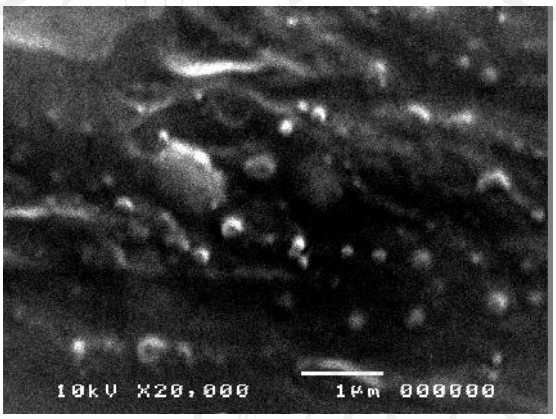


Figure 20 Appearance of SLN nanocream base (A), 0.25% TDE loaded SLN nanocream (B), 0.50% TDE loaded SLN nanocream (C) and 1.25% TDE loaded SLN nanocream (D)

A



B



C

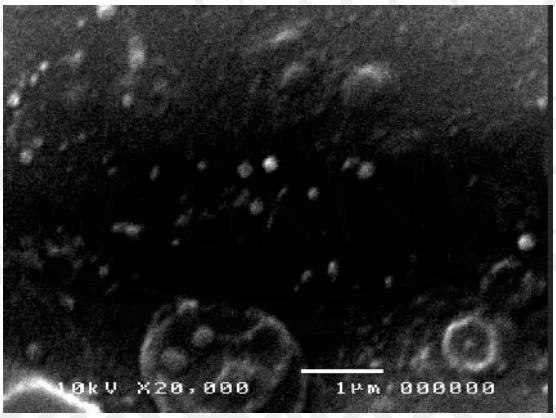


Figure 21 SEM micrographs of TDE loaded SLN incorporated in nanocream at 0.25% (A), 0.50% (B) and 1.25% w/w (C)

Table 31 Appearance of SLN nanocream formulations

Formulation	Appearance				
	Color	**Odor**	**Texture**	**Spreading**	**Phase separation**
SLN nanocream base	White	Wax	Good	Good with white opaque	NF
0.25% TDE loaded SLN nanocream	Yellow-white	Herbal	Good	Good with white opaque	NF
0.50% TDE loaded SLN nanocream	Light yellow	Herbal	Good	Good with white opaque	NF
1.25% TDE loaded SLN nanocream	Light yellow	Herbal	Good	Good with white opaque	NF

NF, not found

4.7 Study of TDE and TDE loaded SLN nanocream on EPP-induced mouse ear edema model

The results after topical application of all test samples were shown in Table 34. At 15 min after application of EPP, TDE solution and TDE loaded SLN nanocream were applied to reduce the ear edema. It was found that the dose increased from 0.25% to 0.50% exhibited the greater ear edema inhibition but the dose of 1.25% w/w TDE loaded SLN nanocream showed low inhibition. TDE loaded SLN nanocream showed higher inhibitory effect than that of TDE solution at the same dose (Table 32). This indicated that the SLN could enhance solubility and penetration of the extract. The concentrations of 0.25% and 0.50% w/w of TDE loaded SLNs were selected for the further studies.

Table 32 Effects of TDE solution and TDE loaded SLN nanocream on EPP-induced mouse ear edema

Group	Dose (mg/ear)	Time after topical application of EPP							
		15 min		30 min		60 min		120 min	
		ED (µm)	EDI (%)	ED (µm)	EDI (%)	ED (µm)	EDI (%)	ED (µm)	EDI (%)
Control	-	155.33±4.56	-	165.33±4.29	-	160.00±8.84	-	130.67±12.86	-
Indomethacin	2.000	77.67±5.44[a]	50	43.00±9.55[a]	74	49.33±5.98[a]	69	47.00±5.67[a]	64
TDE	0.125	79.33±5.84[a]	49	82.67±6.77[a]	50	67.33±11.4[a]	58	60.33±6.59[a]	54
	0.250	76.33±5.44[a]	51	79.33±5.59[a]	52	67.33±8.83[a]	58	64.00±9.00[a]	51
	0.625	74.67±10.40[a]	52	74.33±5.44[a]	55	64.00±6.27[a]	60	61.33±7.00[a]	53
TDE loaded SLN nanocream	0.125	63.67±5.64[a]	59	72.67±4.63[a]	56	56.00±8.52[a]	65	64.00±6.32[a]	51
	0.250	55.67±4.14[a]	64	69.33±4.64[a]	58	54.33±9.41[a]	66	52.33±8.37[a]	60
	0.625	57.33±9.74[a]	63	67.67±8.70[a]	59	62.33±6.25[a]	61	60.00±9.08[a]	54

Value are expressed as MEAN ± S.E.M., *n=6*

[a] significantly different from control group, *p<0.05*

ED, edema thickness (µm) at time

EDI, edema inhibition of test substances at time

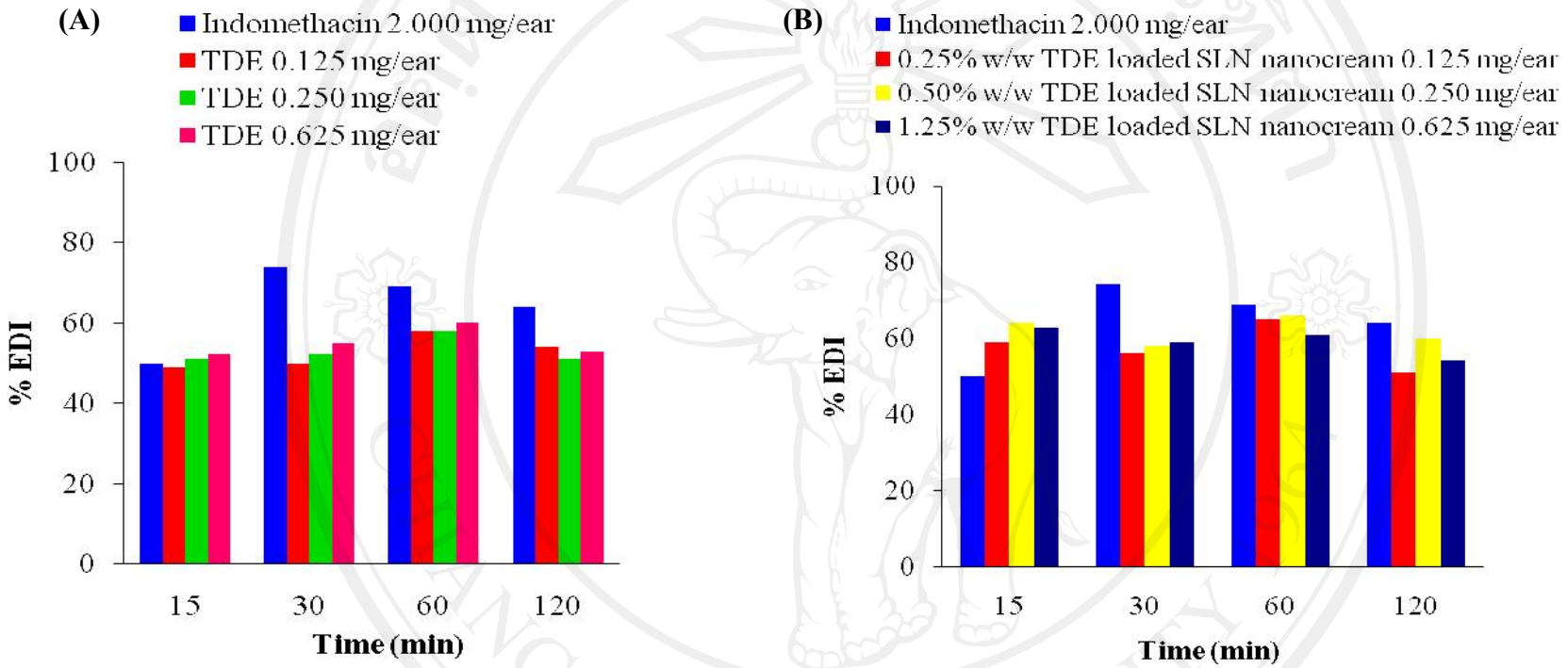


Figure 22 Percentage of ear edema inhibition (EDI) of TDE solution (A) and TDE loaded SLN nanocream (B)

4.8 Characteristics of SLN nanocream formulation

At concentration of 0.25% and 0.50% w/w of TDE loaded SLN nanocream and SLN nanocream base were characterized.

4.8.1 Particle size, size distribution and zeta potential

The results found that all the mean particle size was in nanometer (less than 200 nm). The particle size was increased with the increase of the TDE amount. The SLN nanocream base showed more narrow size distribution than that of the nanocream containing TDE loaded SLN. The zeta potential of all formulations was negative charge with the value nearly 30 mV. After at heating and cooling condition, the particle sizes were increased but their sizes were still in the nanometer size. The size distribution of all formulations exhibited broader distribution. The zeta potential was negative and tended to decrease as shown in Table 33. After stored for 90 days (at 4 and 27 °C conditions), the particle sizes were in the nanometer range (200 nm) but their sizes were larger than the initial one. The size distribution of all formulations was broader. The zeta potential was negative and tended to increase as shown in Table 34 and 35. At 45 °C, the particle sizes were in the range of 200-300 nm and broader. Moreover, the zeta potential was decreased as shown in Table 36.

4.8.2 Viscosity and rheological behavior

The results found that the rheological behavior of all formulations was pseudoplastic flow with thixotropy property. Moreover, the SLN nanocream base exhibited higher viscosity than that of the TDE loaded SLN after incorporated in nanocream. It was found that the viscosity increased when the amounts of TDE in formulation increased. After at heating and cooling condition, the viscosity of all formulations was increased but they also showed the pseudoplastic flow with thixotropy property. As shown in Table 37 and Figures 23-25, after kept for 90 days, their rheological behaviors were pseudoplastic flow with thixotropy property in all conditions (Figures 26-28). At 4 and 27 °C, the viscosity of all formulations tended to be slightly increased as shown in Tables 38 and 39. At 45 °C condition, the viscosity of all formulations was more increased as shown in Table 40.

Table 33 Particle size, polydispersity index and zeta potential of SLN nanocream at heating-cooling condition for 6 cycles

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	SLN nanocream base	128.7±9.8	0.136±0.028	-28.8±1.2
0	0.25% TDE loaded SLN nanocream	170.9±3.0	0.346±0.014	-30.4±0.8
0	0.50% TDE loaded SLN nanocream	186.5±4.1	0.220±0.015	-33.2±0.4
12	SLN nanocream base	227.8±4.3	0.355±0.012	-29.1±1.4
12	0.25% TDE loaded SLN nanocream	278.1±19.4	0.331±0.028	-21.9±2.4
12	0.50% TDE loaded SLN nanocream	299.8±12.2	0.317±0.031	-27.6±2.8

Mean ± SD, *n=3*

Table 34 Particle size, polydispersity index and zeta potential of SLN nanocream at 27 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	SLN nanocream base	128.7±9.8	0.136±0.028	-28.8±1.2
0	0.25% TDE loaded SLN nanocream	170.9±3.0	0.346±0.014	-30.4±0.8
0	0.50% TDE loaded SLN nanocream	186.5±4.1	0.220±0.015	-33.2±0.4
90	SLN nanocream base	159.6±15.4	0.303±0.014	-29.9±0.5
90	0.25% TDE loaded SLN nanocream	195.1±7.5	0.341±0.013	-33.6±1.6
90	0.50% TDE loaded SLN nanocream	202.4±8.1	0.379±0.013	-22.7±1.1

Mean ± SD, *n=3*

Table 35 Particle size, polydispersity index and zeta potential of SLN nanocream at 4 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	SLN nanocream base	128.7±9.8	0.136±0.028	-28.8±1.2
0	0.25% TDE loaded SLN nanocream	170.9±3.0	0.346±0.014	-30.4±0.8
0	0.50% TDE loaded SLN nanocream	186.5±4.1	0.220±0.015	-33.2±0.4
90	SLN nanocream base	143.6±3.1	0.422±0.005	-32.0±0.9
90	0.25% TDE loaded SLN nanocream	187.8±0.7	0.326±0.023	-30.5±1.2
90	0.50% TDE loaded SLN nanocream	206.3±6.1	0.302±0.004	-43.1±1.8

Mean ± SD, *n=3*

Table 36 Particle size, polydispersity index and zeta potential of SLN nanocream at 45 °C

Day	Sample name	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	SLN nanocream base	128.7±9.8	0.136±0.028	-28.8±1.2
0	0.25% TDE loaded SLN nanocream	170.9±3.0	0.346±0.014	-30.4±0.8
0	0.50% TDE loaded SLN nanocream	186.5±4.1	0.220±0.015	-33.2±0.4
90	SLN nanocream base	360.2±5.1	0.374±0.035	-28.9±3.0
90	0.25% TDE loaded SLN nanocream	231.4±18.0	0.362±0.025	-28.7±1.0
90	0.50% TDE loaded SLN nanocream	255.1±11.1	0.430±0.059	-24.8±2.9

Mean ± SD, *n=3*

Table 37 Viscosity and rheological property of SLN nanocream at heating-cooling condition for 6 cycles

Day	Sample name	Eta (Pas)	Rheogram
0	SLN nanocream base	0.793±0.035	Pseudoplastic flow with thixotropy
0	0.25% TDE loaded SLN nanocream	0.355±0.006	Pseudoplastic flow with thixotropy
0	0.50% TDE loaded SLN nanocream	0.380±0.011	Pseudoplastic flow with thixotropy
12	SLN nanocream base	0.810±0.015	Pseudoplastic flow with thixotropy
12	0.25% TDE loaded SLN nanocream	0.548±0.055	Pseudoplastic flow with thixotropy
12	0.50% TDE loaded SLN nanocream	0.517±0.018	Pseudoplastic flow with thixotropy

Mean ± SD, *n=3*

Table 38 Viscosity and rheological property of SLN nanocream at 27 °C

Day	Sample name	Eta (Pas)	Rheogram
0	SLN nanocream base	0.793±0.035	Pseudoplastic flow with thixotropy
0	0.25% TDE loaded SLN nanocream	0.355±0.006	Pseudoplastic flow with thixotropy
0	0.50% TDE loaded SLN nanocream	0.380±0.011	Pseudoplastic flow with thixotropy
90	SLN nanocream base	0.813±0.022	Pseudoplastic flow with thixotropy
90	0.25% TDE loaded SLN nanocream	0.381±0.021	Pseudoplastic flow with thixotropy
90	0.50% TDE loaded SLN nanocream	0.399±0.032	Pseudoplastic flow with thixotropy

Mean ± SD, *n=3*

Table 39 Viscosity and rheological property of SLN nanocream at 4 °C

Day	Sample name	Eta (Pas)	Rheogram
0	SLN nanocream base	0.793±0.035	Pseudoplastic flow with thixotropy
0	0.25% TDE loaded SLN nanocream	0.355±0.006	Pseudoplastic flow with thixotropy
0	0.50% TDE loaded SLN nanocream	0.380±0.011	Pseudoplastic flow with thixotropy
90	SLN nanocream base	0.621±0.056	Pseudoplastic flow with thixotropy
90	0.25% TDE loaded SLN nanocream	0.348±0.002	Pseudoplastic flow with thixotropy
90	0.50% TDE loaded SLN nanocream	0.394±0.008	Pseudoplastic flow with thixotropy

Mean ± SD, *n=3*

Table 40 Viscosity and rheological property of SLN nanocream at 45 °C

Day	Sample name	Eta (Pas)	Rheogram
0	SLN nanocream base	0.793±0.035	Pseudoplastic flow with thixotropy
0	0.25% TDE loaded SLN nanocream	0.355±0.006	Pseudoplastic flow with thixotropy
0	0.50% TDE loaded SLN nanocream	0.380±0.011	Pseudoplastic flow with thixotropy
90	SLN nanocream base	0.893±0.078	Pseudoplastic flow with thixotropy
90	0.25% TDE loaded SLN nanocream	0.465±0.037	Pseudoplastic flow with thixotropy
90	0.50% TDE loaded SLN nanocream	0.471±0.029	Pseudoplastic flow with thixotropy

Mean ± SD, *n=3*

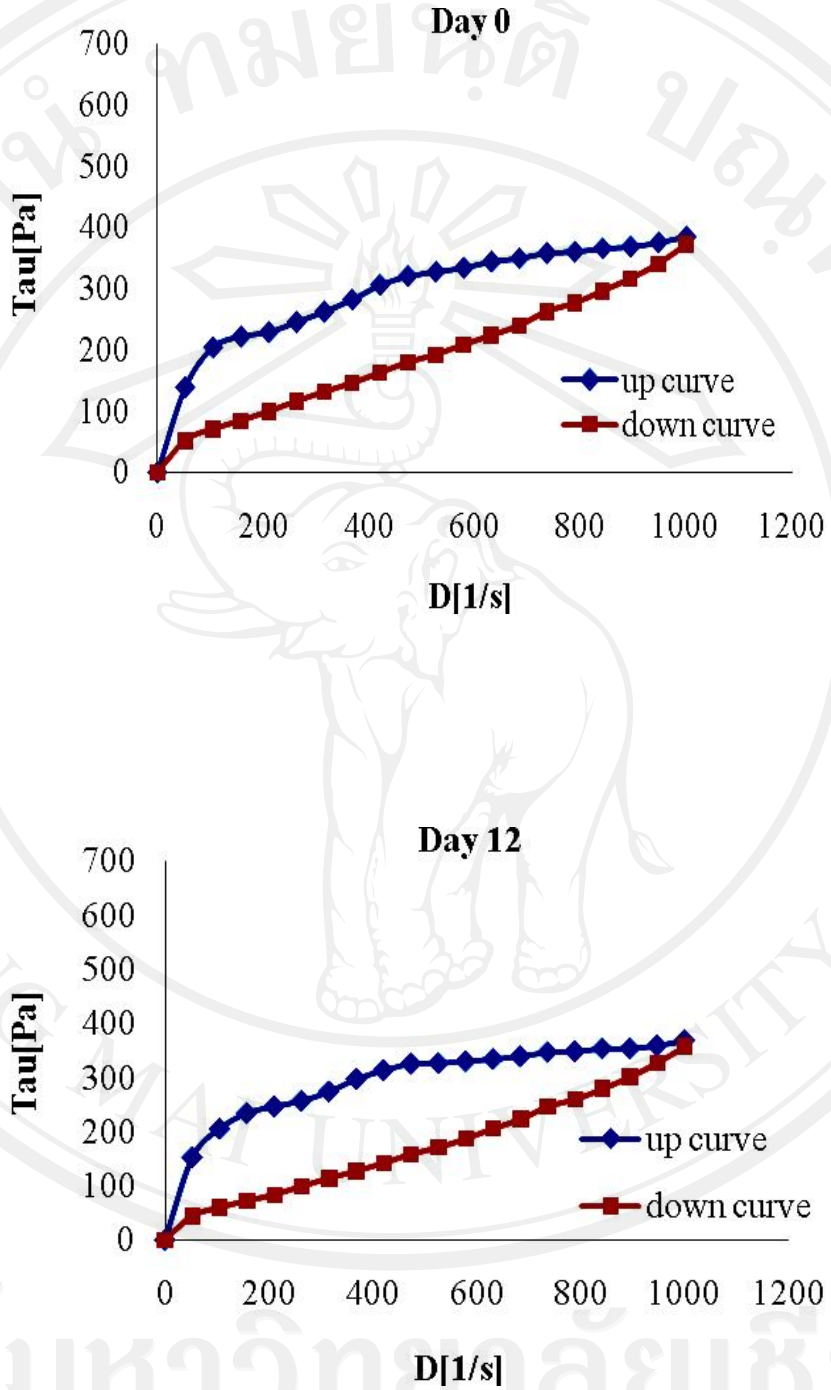


Figure 23 Rheogram of SLN nanocream base after 6 cycles of storage at heating-cooling condition

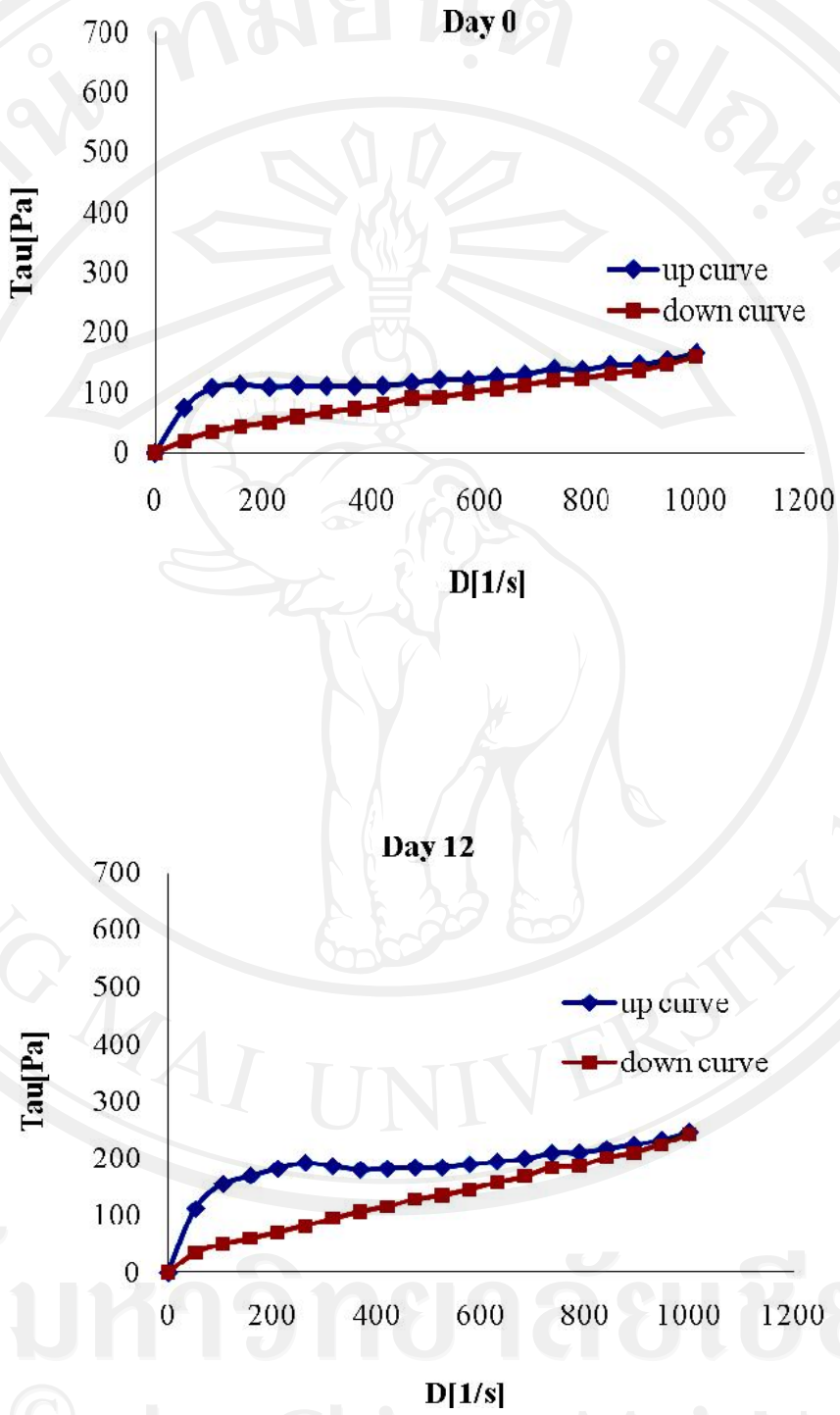


Figure 24 Rheogram of 0.25% TDE loaded SLN nanocream after 6 cycles of storage at heating-cooling condition

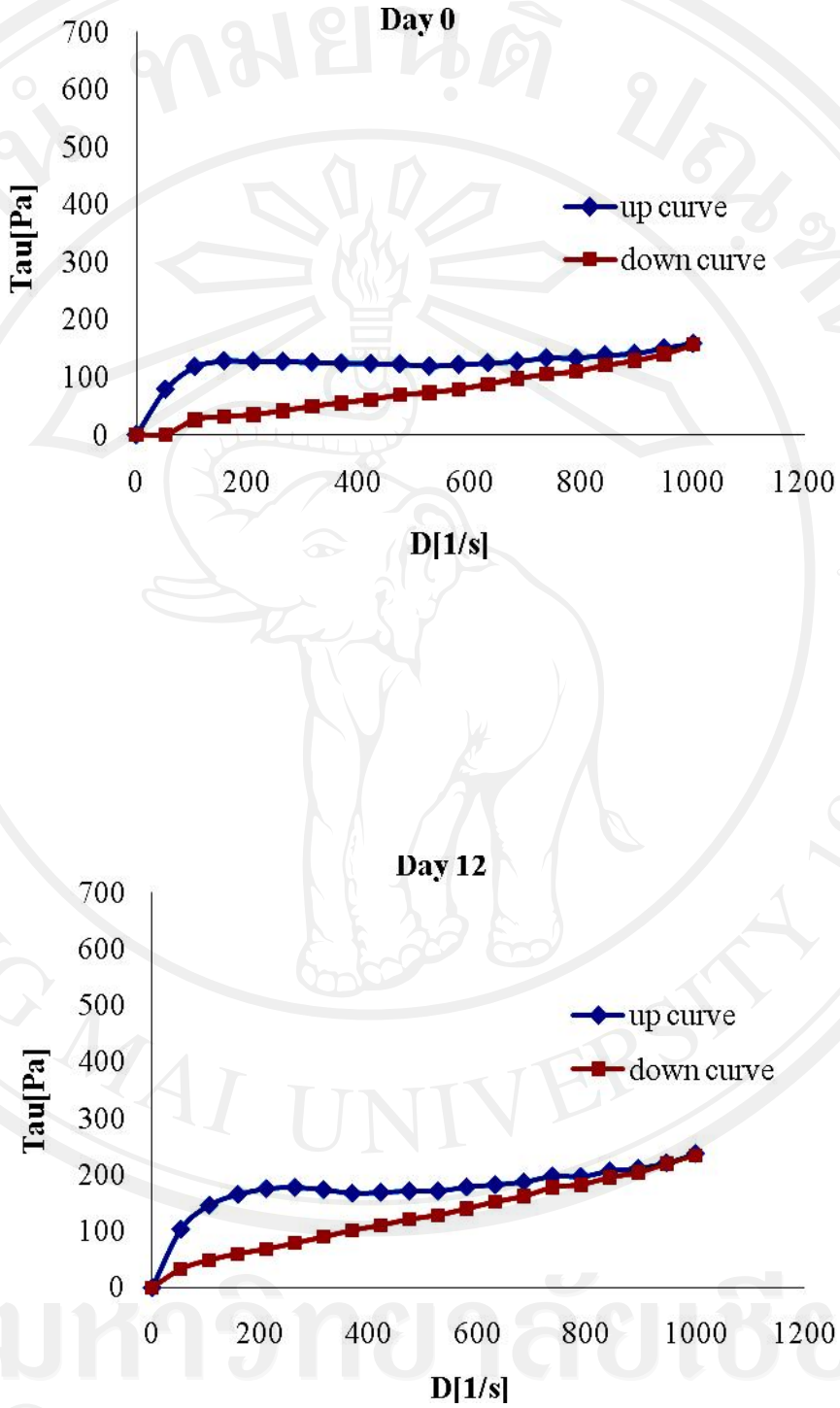


Figure 25 Rheogram of 0.50% TDE loaded SLN nanocream after 6 cycles of storage at heating-cooling condition

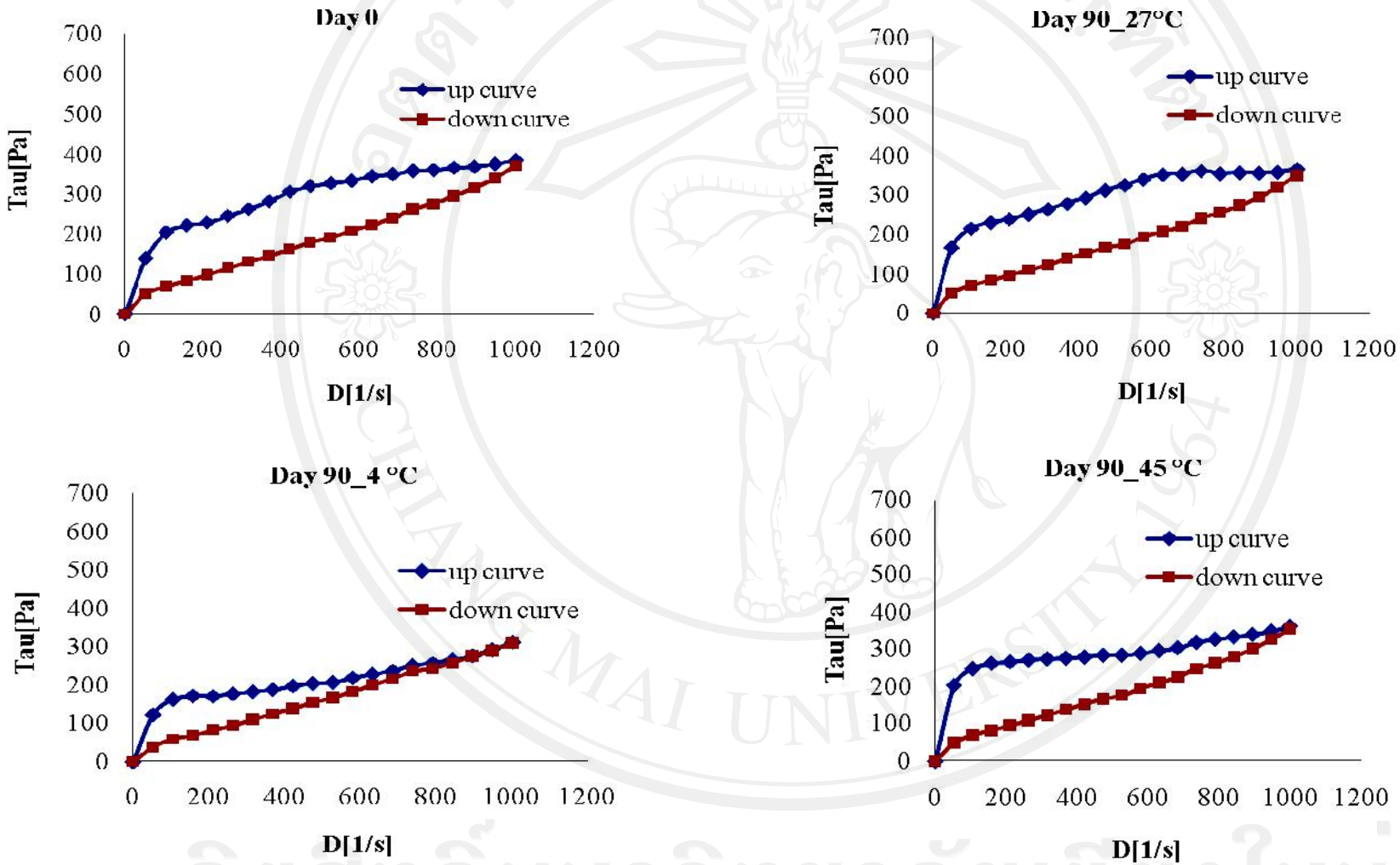


Figure 26 Rheogram of SLN nanocream base after 90 days of storage at 4, 27 and 45 °C

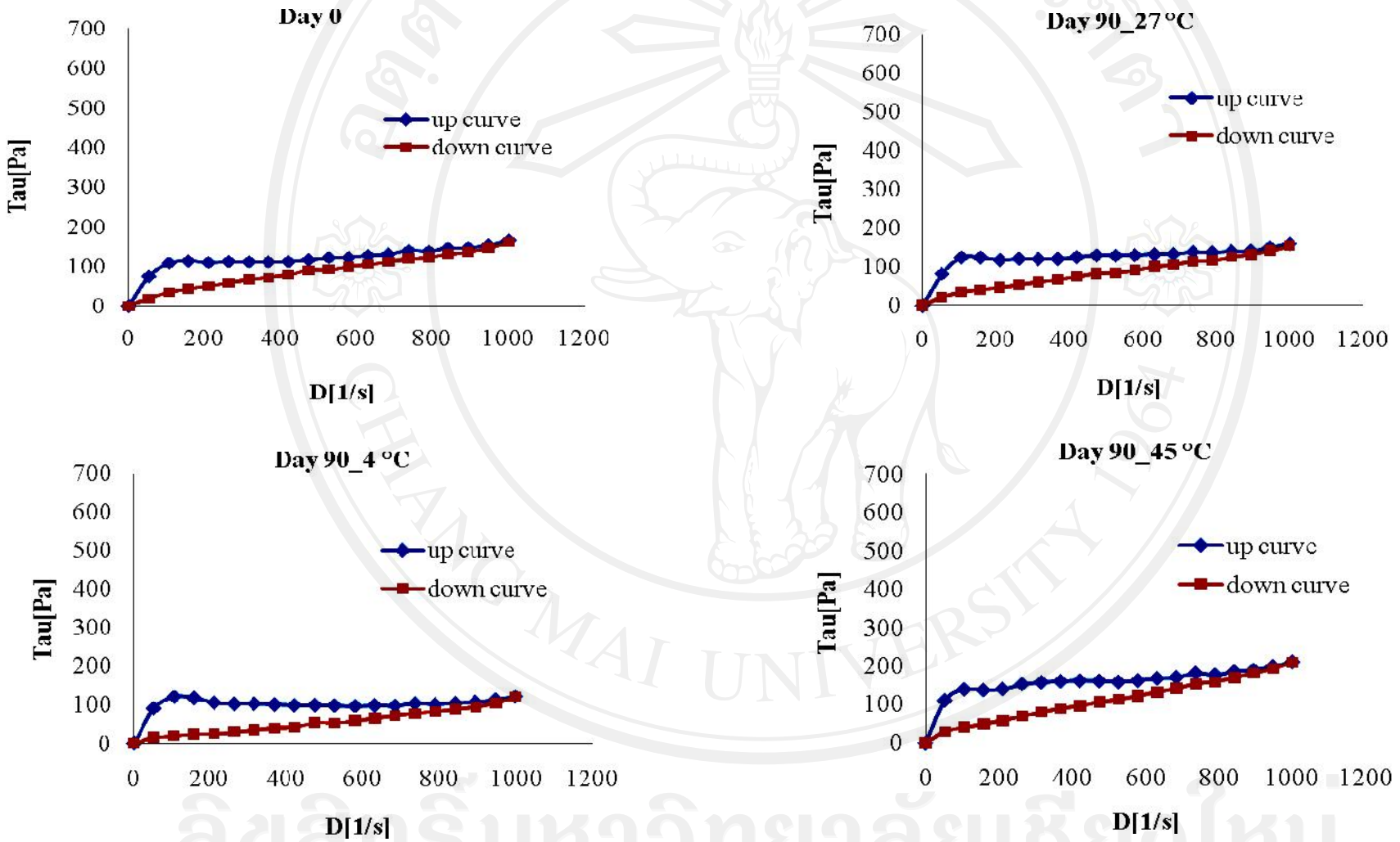


Figure 27 Rheogram of 0.25% TDE loaded SLN nanocream after 90 days of storage at 4, 27 and 45 °C

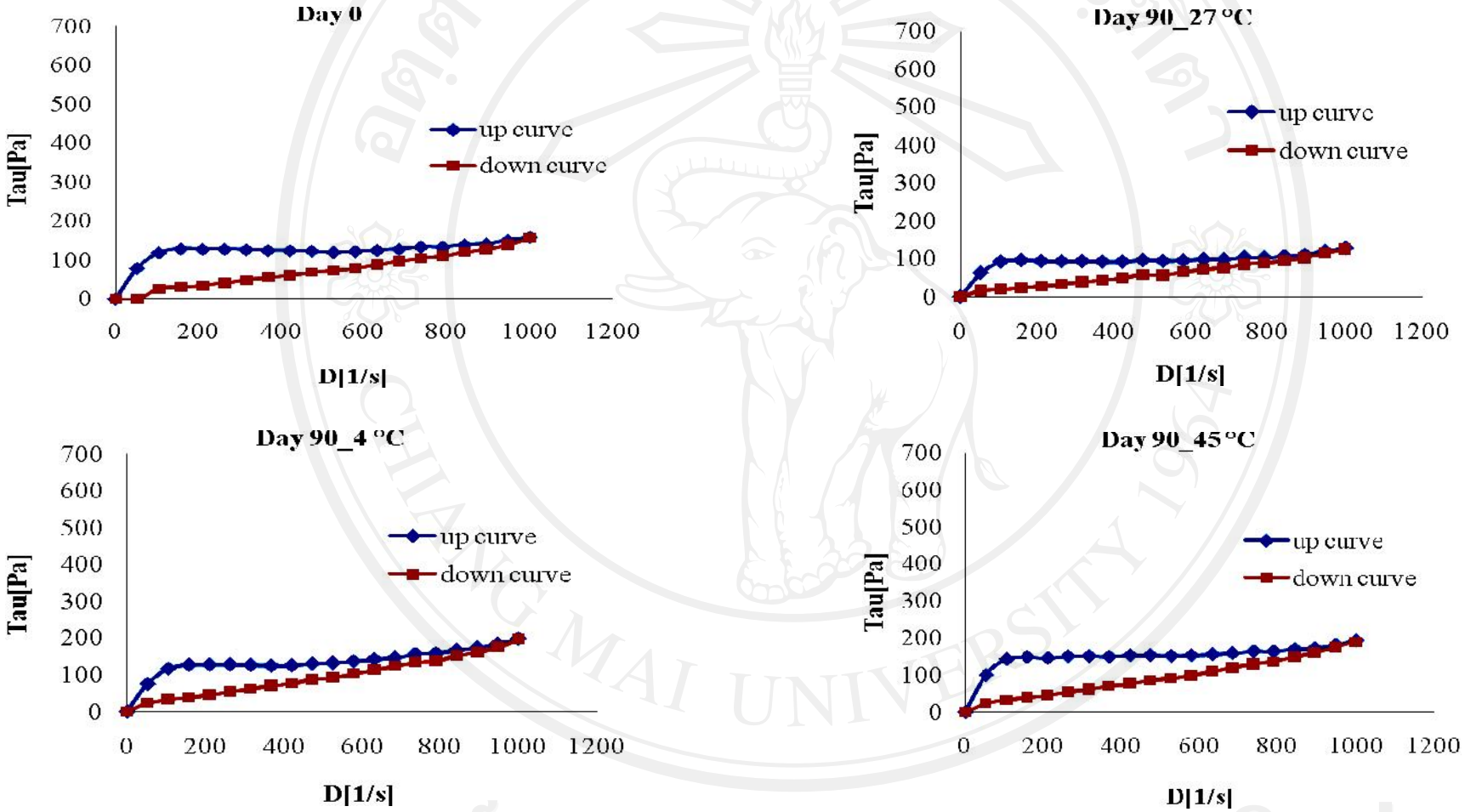


Figure 28 Rheogram of 0.50% TDE loaded SLN nanocream after 90 days of storage at 4, 27 and 45 °C

4.8.3 Determination of the degradation of TDE in SLN formulation and entrapment efficiency (EE) using a HPLC after stored for 90 days

Figures 29 and 30 showed the degradation of TDE in the intact and TDE loaded SLN nanocream, respectively after kept for 90 days. Figure 31 showed the percentage of entrapment efficiency of TDE which was entrapped in nanoparticles and incorporated in nanocream after stored at stability conditions.

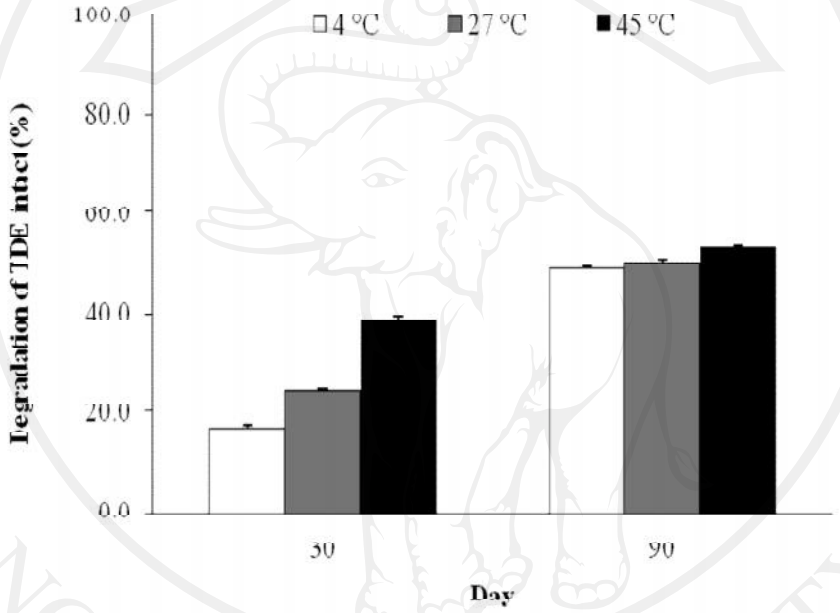


Figure 29 Degradation of TDE intact after 90 days of storage at 4, 27 and 45 °C

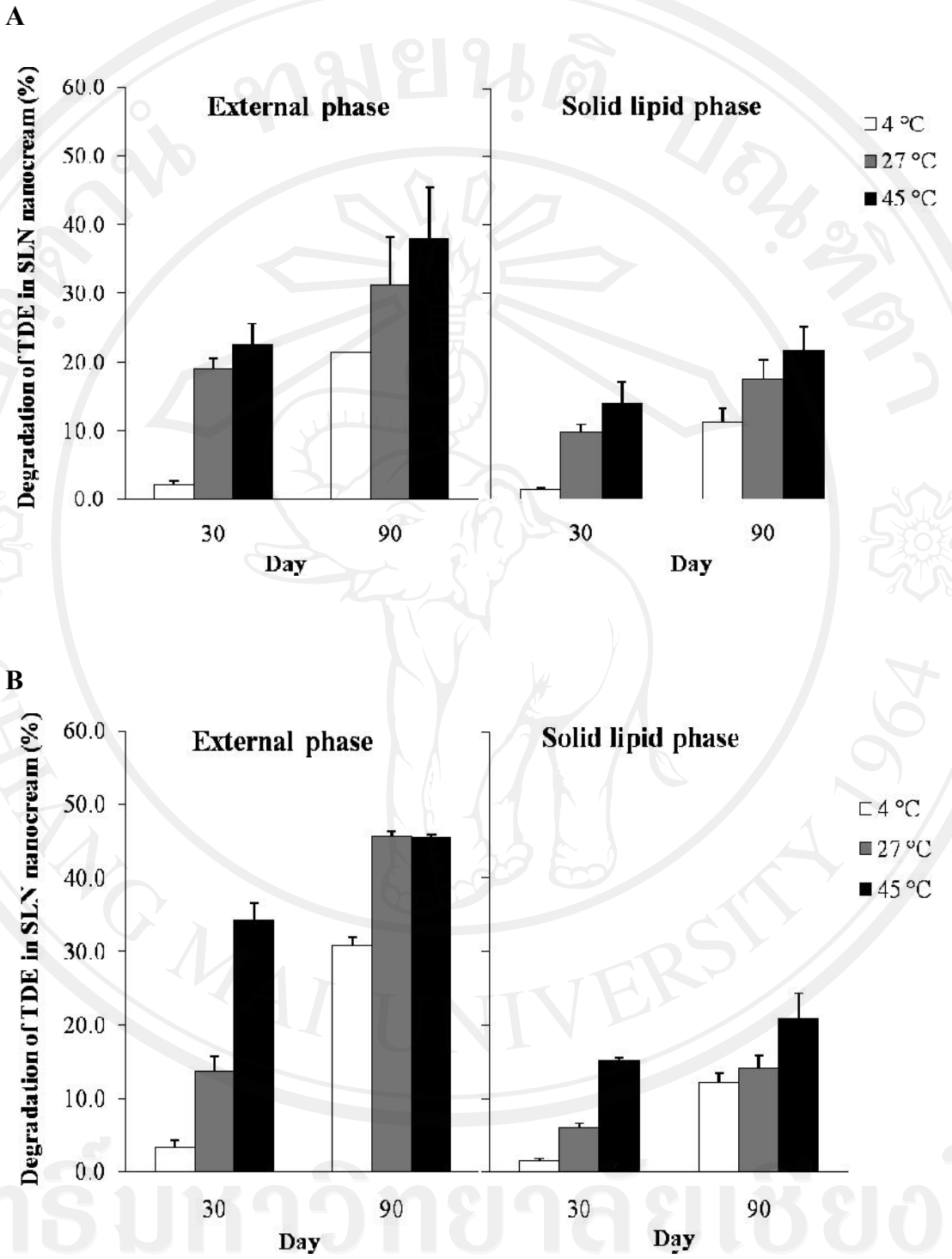


Figure 30 Degradation of TDE in 0.25% (A) and 0.50% (B) TDE loaded SLN nanocream after storage at 4, 27 and 45 °C for either 30, 90 days

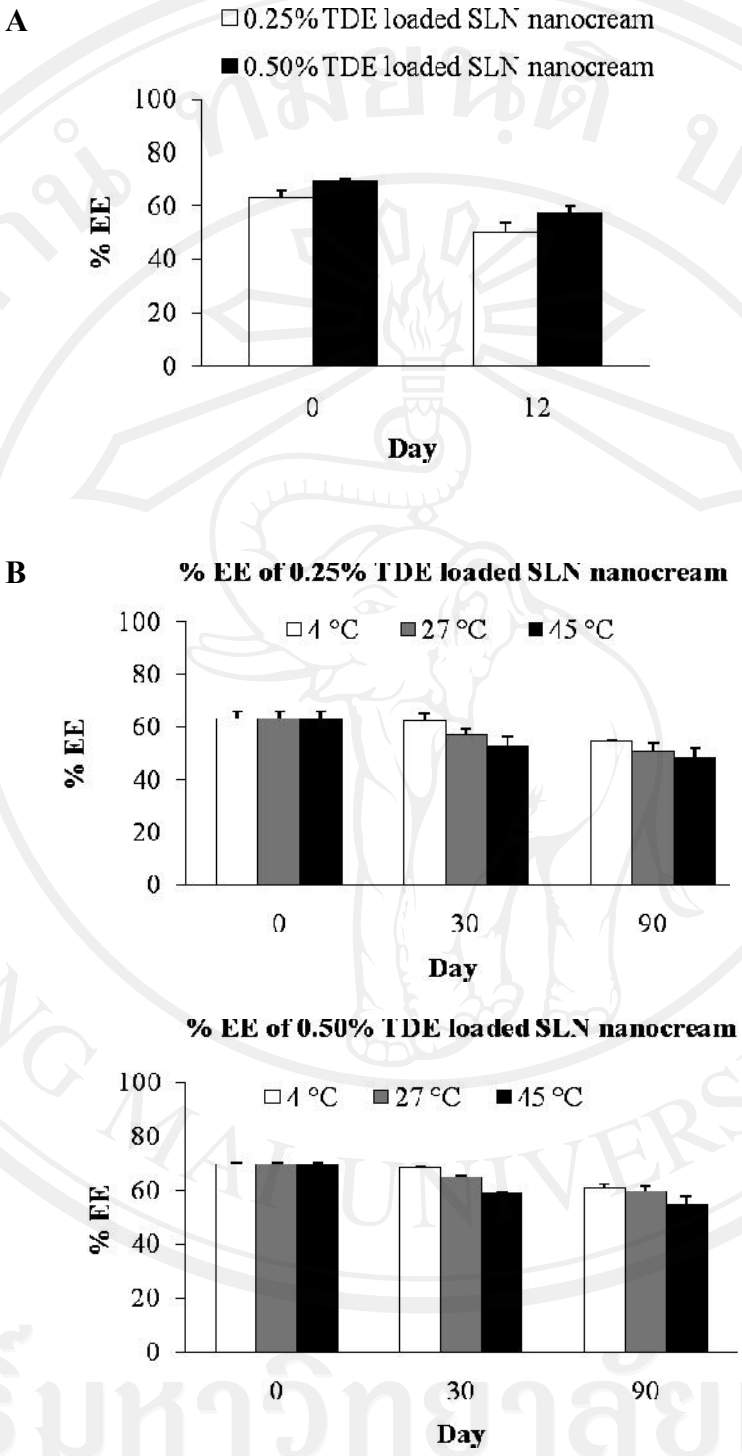


Figure 31 Percentage of EE of TDE loaded SLN nanocream after storage at heating-cooling for 6 cycles (A) and 4, 27 and 45 °C (B)

4.9 Study of releasing of TDE loaded SLN nanocream

It was found that the release rate of TDE was depended on concentration of TDE in the nanocream as shown in Figure 32. TDE loaded SLN in the nanocream was gradually released and increased when the concentration of TDE in the formulation increased. The % cumulative release maintained constant after 6 h.

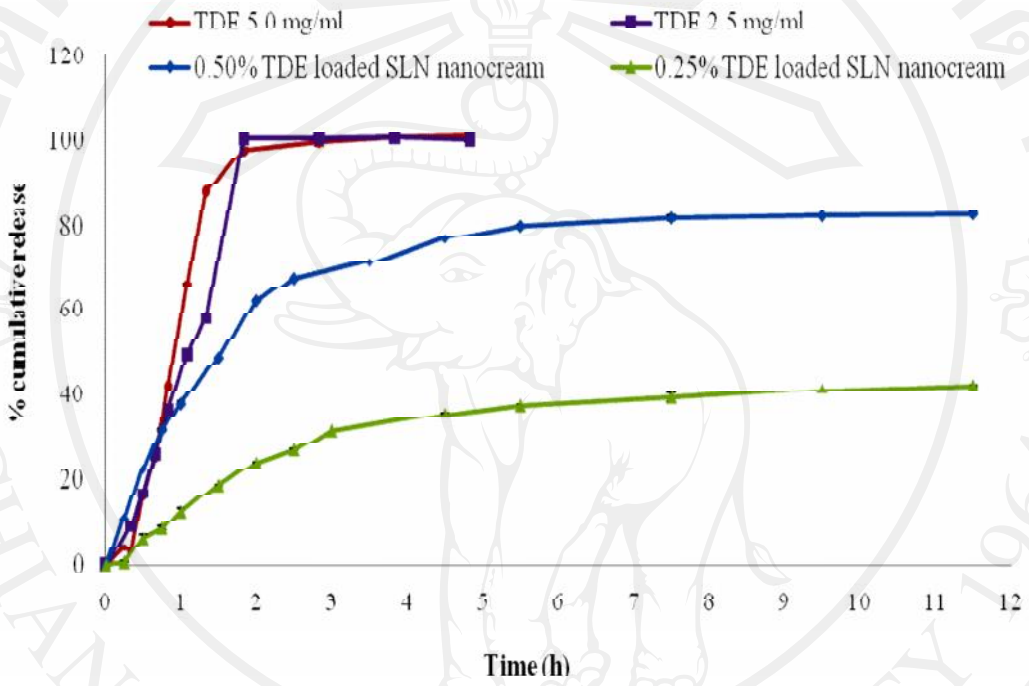


Figure 32 The percentage of cumulative release of TDE from TDE solution and TDE loaded SLN nanocream tested in 50% v/v ethanolic aqueous solution at 37 °C

4.10 Study of skin irritation

The irritation study by Draize's method indicated that the developed TDE nanocream showed no irritation to the skin of the tested animals as shown in Figure 33 and Table 41.

Table 41 *In vivo* skin irritation results

Sample	Observation time (h)	Combined score	Combined index
Control	1	0.00	0.00
	24	0.00	0.00
	48	0.00	0.00
	72	0.00	0.00
Absolute alcohol	1	0.00	0.00
	24	0.00	0.00
	48	0.00	0.00
	72	0.00	0.00
SLN nanocream base	1	0.00	0.00
	24	0.00	0.00
	48	0.00	0.00
	72	0.00	0.00
98% Lactic acid	1	0.00	0.00
	24	4.00	0.67
	48	8.00	1.33
	72	11.00	1.83
TDE 2.50 mg	1	0.00	0.00
	24	0.00	0.00
	48	0.00	0.00
	72	0.00	0.00
0.50% TDE loaded SLN nanocream	1	0.00	0.00
	24	0.00	0.00
	48	0.00	0.00
	72	0.00	0.00

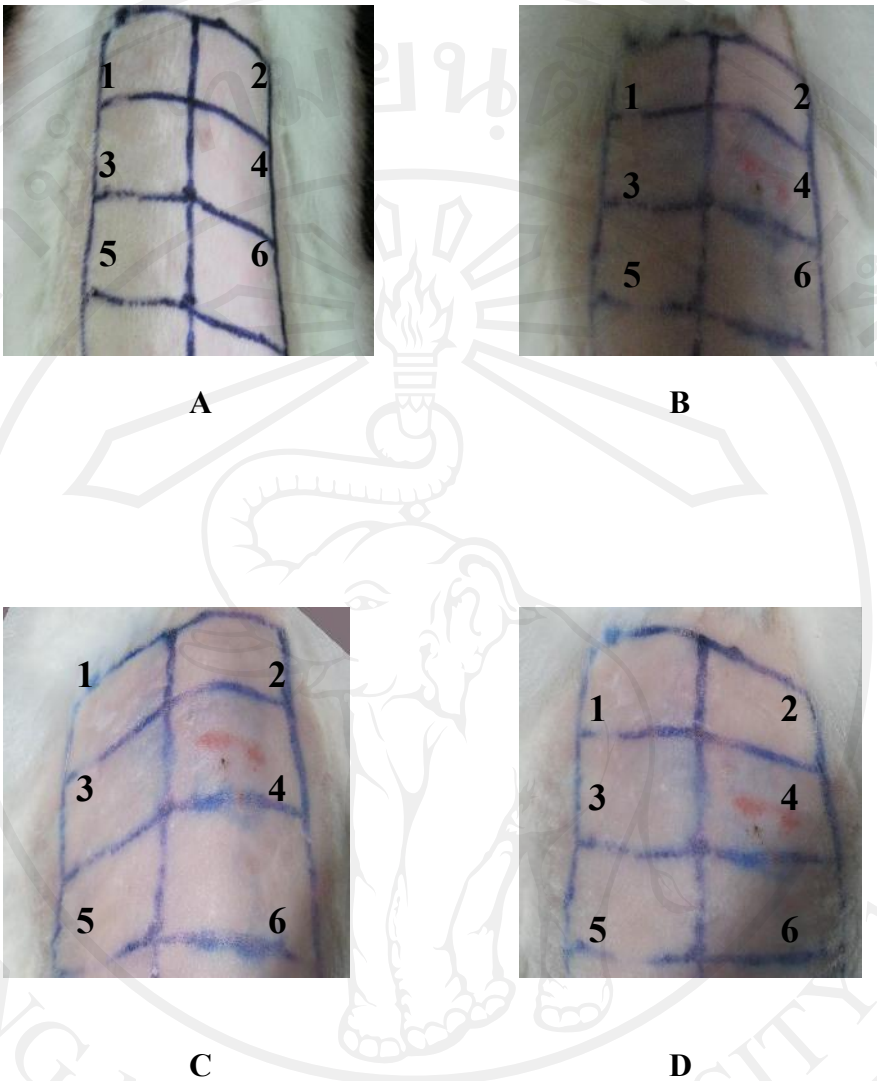


Figure 33 Skin irritation of TDE solution and TDE loaded SLN nanocream at 1 h (A), 24 h (B), 48 h (C) and 72 h (D)

Annotation: 1, control; 2, SLN nanocream base; 3, absolute alcohol; 4, 98% lactic acid; 5, 0.25% w/v TDE solution; and 8, 0.50% w/w TDE loaded SLN nanocream