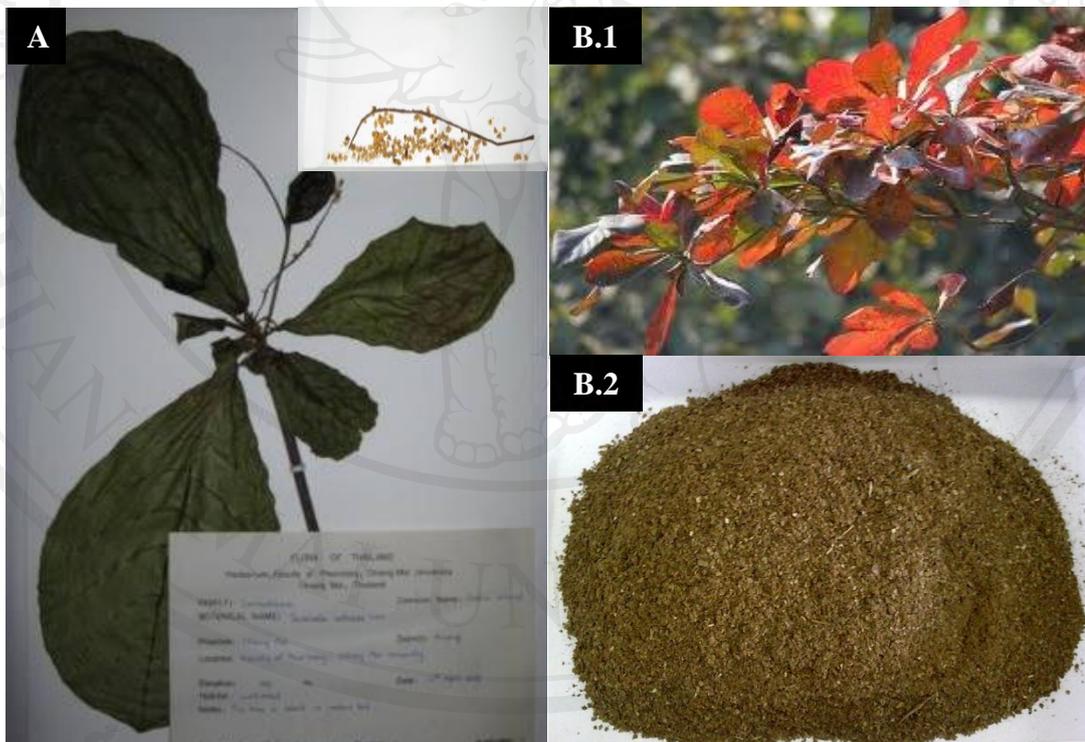


## CHAPTER 4

### RESULTS AND DISCUSSION

#### 4.1 Macroscopic characteristic

*T. catappa* Linn. (Hu-Kwang) tree is about 12 meters tall. The leaf is an obovate type and the flower is an inflorescence type. Fruit is hard and has a single seed. The collected *T. catappa* Linn. specimen is kept in the Herbarium, Faculty of Pharmacy Chiang Mai University (Figure 4.1). The collection number of the specimen is 023144.



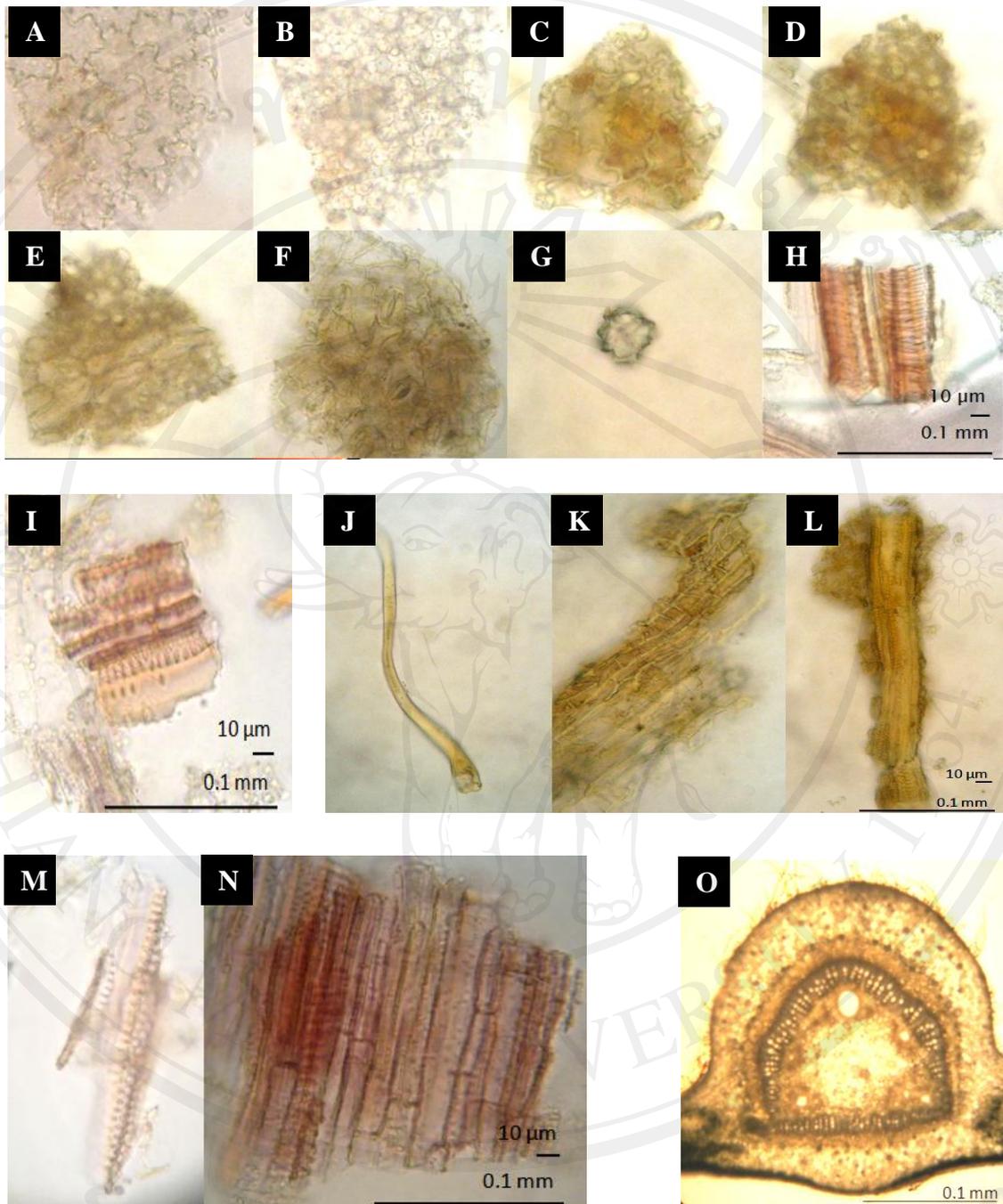
**Figure 4.1** A: Specimen of *T. catappa* Linn.; B: *T. catappa* Linn. red leaves

#### 4.2 Microscopic characteristic of red leaf powder

##### 4.2.1 Plant powder character

The red leaves powder was examined under a microscope and it was found to display trichomes located in the lower epidermis, calcium oxalate crystals in rosette form and anomocytic stoma.

The Figure 4.2 shows character of the leaf powder under microscope view.



**Figure 4.2** A: Upper epidermis mounted in chloral hydrate; B: Palisade cells found under the epidermis mounted in chloral hydrate; C: Upper epidermis; D: Palisade cells under the epidermis; E: Epidermis; F: Lower epidermis with anomocytic stoma; G: Calcium oxalate crystal in rosette forms; H; Spiral vessel mounted in phloroglucinol solution; I, M, N: Vessels; L: Vein; J: Trichome; K: Epidermis; O: Cross section of midrib

#### 4.2.2 Leaf constants

The leaf constants were repeated determination for 30 times in each value including stomatal number, stomatal index, palisade ratio, vein islet number and veinlet terminal number. The calculated leaf constants are shown in the Table 4.1. The Table 4.2 shows the raw data of leaf constants determination.

**Table 4.1** Leaf constants of *T. catappa* Linn.

Leaf constants	Mean±SD
Stomatal number	454.01±53.58
Stomatal index	0.21±0.02
Palisade ratio	11.78±1.75
Vein islet number	7.66±1.65
Veinlet terminal number	19.18±2.63

**Table 4.2** The leaf constants data of *T. catappa* Linn.

No	Stomatal (S)	Epidermis (E)	Stomatal number	S+E	Stomatal index	Palisade	Palisade ratio	Vein islet	Vein islet number	Veinlet terminal	Veinlet terminal number
1	49	189	453.70	238	0.21	39	9.75	28	7.00	71	17.75
2	45	185	416.67	230	0.20	53	13.25	35	8.75	62	15.50
3	52	213	481.48	265	0.20	53	13.25	26	6.50	85	21.25
4	47	191	435.19	238	0.20	45	11.25	24	6.00	80	20.00
5	50	194	462.96	244	0.20	45	11.25	43	10.75	72	18.00
6	44	176	407.41	220	0.20	49	12.25	28	7.00	52	13.00
7	50	215	462.96	265	0.19	62	15.50	28	7.00	74	18.50
8	50	176	462.96	226	0.22	53	13.25	31	7.75	52	13.00
9	58	200	537.04	258	0.22	45	11.25	34	8.50	71	17.75
10	59	192	546.30	251	0.24	47	11.75	27	6.75	78	19.50
11	51	176	472.22	227	0.22	39	9.75	26	6.50	82	20.50
12	47	197	435.19	244	0.19	37	9.25	29	7.25	100	25.00
13	48	163	444.44	211	0.23	41	10.25	30	7.50	66	16.50
14	51	197	472.22	248	0.21	58	14.50	29	7.25	77	19.25
15	33	160	305.56	193	0.17	55	13.75	28	7.00	81	20.25
16	55	179	509.26	234	0.24	45	11.25	30	7.50	75	18.75
17	47	177	435.19	224	0.21	49	12.25	26	6.50	85	21.25
18	37	198	342.59	235	0.16	41	10.25	22	5.50	88	22.00
19	52	185	481.48	237	0.22	42	10.50	33	8.25	81	20.25
20	49	196	453.70	245	0.20	58	14.50	28	7.00	74	18.50
21	55	178	509.26	233	0.24	52	13.00	28	7.00	77	19.25
22	51	210	472.22	261	0.20	44	11.00	46	11.50	80	20.00
23	57	193	527.78	250	0.23	41	10.25	35	8.75	66	16.50
24	52	180	481.48	232	0.22	39	9.75	34	8.50	76	19.00
25	43	163	398.15	206	0.21	57	14.25	22	5.50	70	17.50
26	51	192	472.22	243	0.21	37	9.25	29	7.25	77	19.25
27	42	170	388.89	212	0.20	46	11.50	18	4.50	82	20.50
28	51	191	472.22	242	0.21	42	10.50	39	9.75	88	22.00
29	53	181	490.74	234	0.23	57	14.25	38	9.50	88	22.00
30	42	148	388.89	190	0.22	43	10.75	45	11.25	91	22.75
<b>Mean±SD</b>			<b>454.01±53.58</b>		<b>0.21±0.02</b>		<b>11.78±1.75</b>		<b>7.66±1.65</b>		<b>19.18±2.63</b>

### 4.3 Phytochemical test

The extract showed a positive test for tannins (condensed and hydrolysable tannins), flavonoids and triterpenoids. The results related to phytochemicals groups which have been found in *T. catappa* Linn. leaves as following: tannins (punicalagin, corilagin), flavonoids (quercetin, kaempferol) and triterpenoids (ursolic acid) (65). These phytochemicals have also been reported to possess anti-inflammatory activity and expected to be active compounds for reducing the symptoms of inflammatory diseases.

**Table 4.3** Phytochemical test for 95% ethanol extract of *T. catappa* Linn.

<i>T. catappa</i> Linn. (Hu-Kwang)	
Part of use: <i>T. catappa</i> Linn. red leaves	
Test	95% Ethanol extract
1. Tannin	
1.1 0.5% Gelatin solution	White precipitate
1.2 1% Lead acetate solution	White precipitate
1.3 1% Quinine sulfate solution	White precipitate
1.4 Ferric Chloride T.S. (Hydrolysable tannin)	Blue precipitate
1.5 Lime water (Hydrolysable tannin)	Gray precipitate
1.6 Vanillin-HCl (Condensed tannin)	Crimpson
1.7 Formalin-HCl (Condensed tannin)	Red-orange
2. Glycoside	
2.1 Flavonol, Flavone (Shiba's reaction)	Red solution
2.2 Steroid (Lieberman Burchard's)	-
2.3 Terpene (Lieberman Burchard's)	Red-pink solution

#### 4.4 Specification of raw materials

The specification of *T. catappa* Linn. red leaf powder was determined following the Thai Pharmacopoeia Vol.1, 1987, the Thai Herbal Pharmacopoeia Vol. 1, 1995 and Specification of Thai Medicinal Plant V.1. There were moisture content ( $8.07 \pm 0.65\%$ ), total ash value ( $12.71 \pm 0.05\%$ ), acid-insoluble ash value ( $3.28 \pm 0.03\%$ ), 95% ethanol extractive value ( $16.04 \pm 0.23\%$ ) and water extractive value ( $18.42 \pm 0.30\%$ ), these results are shown in the Table 4.4-4.6.

Water may contribute more crude extract than 95% of ethanol, however there was research on anti-inflammatory activity in rats showed that the ethanol extract inhibited edema by 52.11% more than water extract by 45.66%, the results of Pauly *et al.*, 2001. Therefore, the extraction of *T. catappa* Linn. red leaves in this study was done by 95% ethanol as a solvent.

##### 4.4.1 Moisture content by gravimetric method

% Loss on drying of *T. catappa* Linn. red leaf powder was 8.07%. The acceptance value follow WHO is not more than 10% which was conformed.

**Table 4.4** % Loss on drying of *T. catappa* Linn. red leaf powder by gravimetric method

Data	Repeat		
	1	2	3
<u>Before drying process</u>			
Weight of plant powders (g)	2.0067	1.9986	2.0008
<u>After drying process</u>			
Weight of plant powders (g)	1.8591	1.8262	1.8365
Weight loss (g)	0.1476	0.1724	0.1643
<b>% Loss on drying</b>	7.36	8.63	8.21
<b>Mean <math>\pm</math> SD</b>	8.07 $\pm$ 0.65		

#### 4.4.2 Total ash and acid-insoluble ash values

Total ash and acid-insoluble ash of *T. catappa* Linn. red leaves were 12.71 and 3.28 %, respectively.

**Table 4.5** The data of total ash and acid-insoluble ash of *T. catappa* Linn. red leaves

Data	Repeat		
	1	2	3
<b>Total ash</b>			
Weight of plant powders (g)	2.0034	2.0032	2.0036
Weight of total ash (g)	0.2556	0.2548	0.2536
<b>% Total ash</b>	12.76	12.72	12.66
<b>Mean±SD</b>	<b>12.71 ± 0.05</b>		
<b>Acid-insoluble ash</b>			
Weight of acid-insoluble ash (g)	0.0663	0.0654	0.0655
<b>% Acid-insoluble ash</b>	3.31	3.26	3.27
<b>Mean±SD</b>	<b>3.28±0.03</b>		

#### 4.4.3 Solvent extractive values

Ethanol extractive value and water extractive value of *T. catappa* Linn. red leaves were 16.04 and 18.42%, respectively as shown in the Table 4.6. The results showed that the water extract did dissolve more compounds from the leaves.

**Table 4.6** Solvent extractive values of *T. catappa* Linn. red leaves

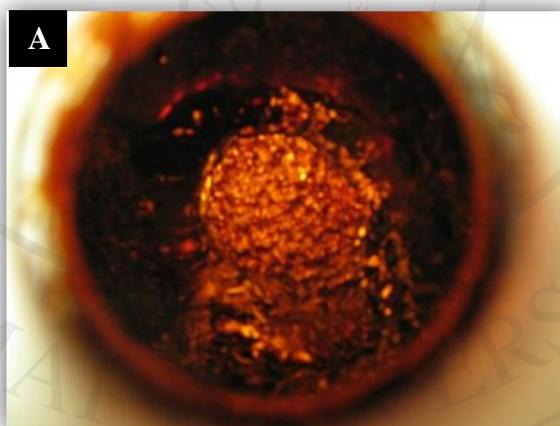
Data	Solvent					
	95% Ethanol			Distilled water		
	1	2	3	1	2	3
Weight of plant powders (g)	5.0022	5.0054	5.0088	5.0006	5.0015	5.0070
Weight of the extract (g)	0.7890	0.8110	0.8085	0.9305	0.9040	0.9305
<b>% Yield of the extract</b>	15.77	16.20	16.14	18.61	18.07	18.58
<b>Mean± SD</b>	16.04 ± 0.23			18.42 ± 0.30		

#### 4.5 Plant extraction

The yield of the 95% ethanol extract using soxhlet apparatus was 22.02% which was more than 95% ethanol extractive value due to thermal extraction and longer time can contribute more yield. The data is shown in the Table 4.7.

**Table 4.7** The character and percent yield of 95% ethanol extract from *T. catappa* Linn. red leaves

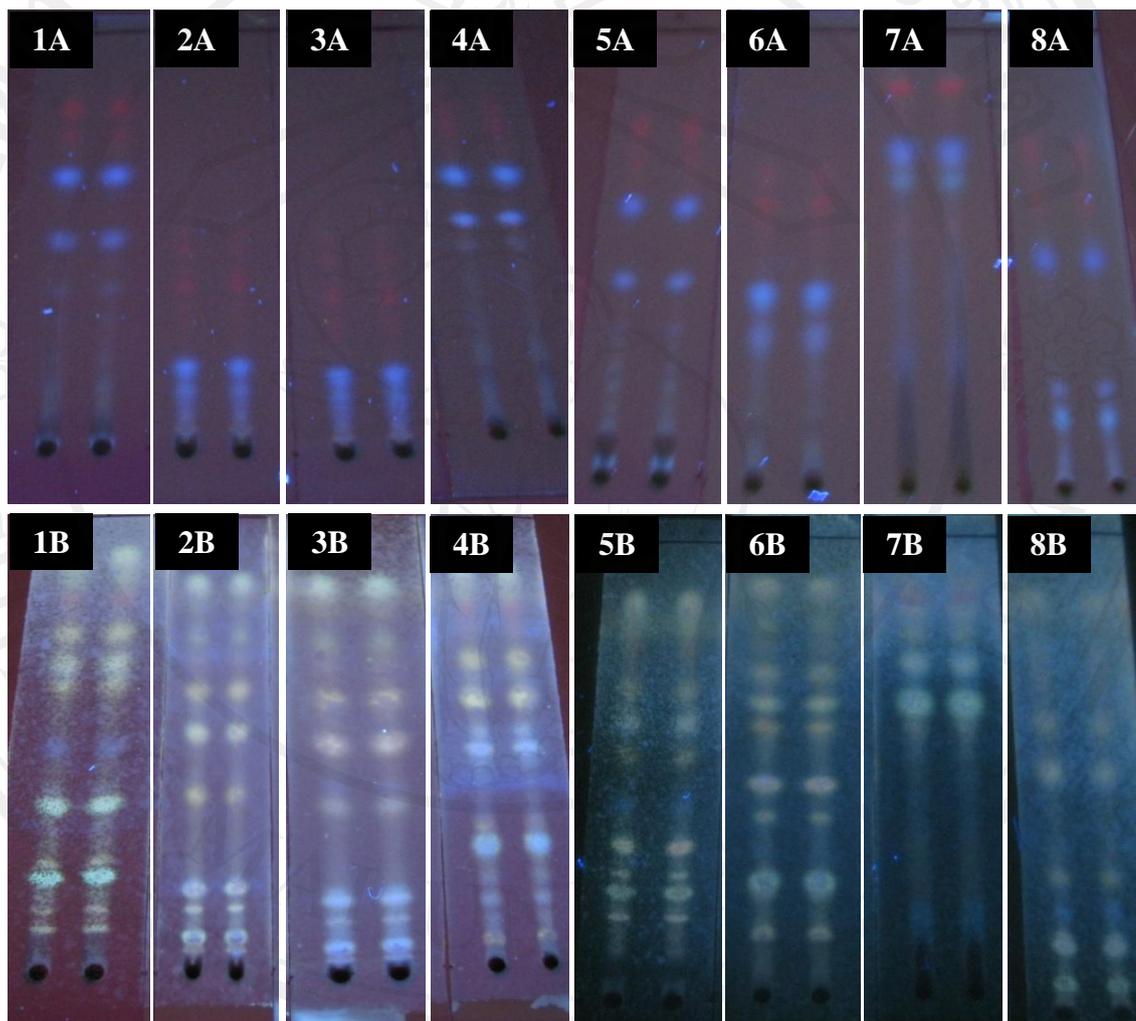
Data	Recorded data
Physical character	Hard powder with specific odor and amber to dark brown color as the Figure 4.3
Weight of plant powders (g)	500.0
Weight of the extract (g)	110.11
<b>% Yeild of the extract</b>	22.02



**Figure 4.3** A: The extract viewed in round flask; B: Physical character of the extract

#### 4.6 TLC chromatogram

The eight TLC conditions were tried. The condition 1 was chosen since it gave a clearer spot and better interval between each spot. The TLC chromatograms of condition 1-8 are shown as the Figure 4.4.



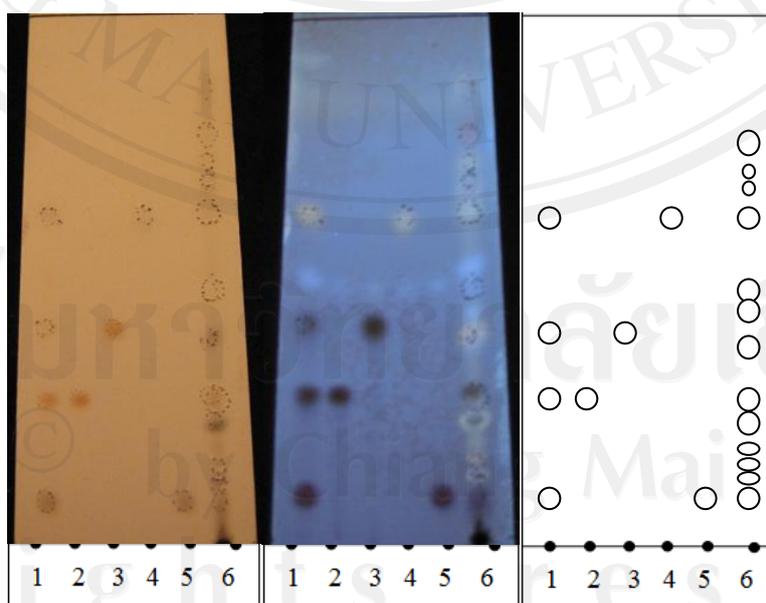
**Figure 4.4** TLC chromatograms for condition 1-8 under 365 nm light, A: Before sprayed by sulfuric acid; B: After sprayed by sulfuric acid

The TLC chromatogram was compared between the extract and 3 standards including quercetin (1), kaempferol (2), ursolic acid (3), gallic acid (4) and mixture of 3 standards (5) is shown in the Figure 4.5. The chromatogram of *T. catappa* Linn. red leaf extract was similar to the spot of quercetin, ursolic acid and gallic acid. The

other spots were unknown. However, it is supposed that there are at least 13 phytochemicals in the extract. The Rf value of each spot is also shown in the Table 4.8.

**Table 4.8** Rf values of substances of *T. catappa* Linn. red leaf extract and the standards

No.	Sample	Rf values of substances													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Mixture of standards	0.07					0.23		0.35			0.57			
2	Quercetin						0.23								
3	Kaempferol								0.35						
4	Ursolic acid											0.57			
5	Gallic acid	0.07													
6	Extract	0.07	0.10	0.13	0.15	0.19	0.23	0.34		0.38	0.43	0.57	0.63	0.73	0.83



**Figure 4.5** TLC chromatogram of *T. catappa* Linn. red leaf extract

#### **4.7 Anti-inflammatory study and determination of antioxidant activity**

The extract from *T. catappa* Linn. red leaf inhibited COX-1 and COX-2 enzyme lower than the standard aspirin and ibuprofen (Appendix A). Moreover, the results from EPP - induced ear edema in rat study showed that the dose of 1 and 3 mg/ear of the extract inhibited edema more than 50%. However anti-inflammatory activity of the extract via COX-1 and COX-2 was lower that may not be a major mechanism, the activity may occur via antioxidation activity on hydroxyl radical which is one factor of inflammation and inflammatory-related diseases.

##### **4.7.1 Anti-inflammatory screening test on cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) enzymes inhibition**

The inhibitory effects of *T. catappa* Linn. red leaf extract at the concentrations of 10-5 g/mL on COX-1 and COX-2 enzymes were 37.43 and 27.53%, respectively (Appendix A). However, the COX inhibitory activity of the plant extract was less than those of ibuprofen and aspirin, the well known COX-inhibitors.

##### **4.7.2 Ethyl phenylpropiolate (EPP) - induced ear edema in rat**

The inhibitory effects of *T. catappa* Linn. red leaf extract and phenylbutazone, on EPP-induced ear edema are shown in Table 4.9. In both control groups, rat ear thickness increased gradually with time up to 60 minutes then slightly decreased at 120 minutes after EPP application. Phenylbutazone as well as *T. catappa* Linn. red leaf extract significantly inhibited the EPP-induced edema formation of the rat ear at all assessment times. However, the inhibitory effect of *T. catappa* Linn. red leaf extract on ear edema formation was less than that of phenylbutazone. The results indicated that *T. catappa* Linn. red leaf extract possess inhibitory effect on acute phase of inflammation.

**Table 4.9** Effect of *T. catappa* Linn. red leaf extract and phenylbutazone on rat ear edema induced by EPP

Group	Dose (mg/ear)	Edema thickness ( $\mu\text{m}$ )				% edema inhibition			
		15 min	30 min	1 h	2 h	15 min	30 min	1 h	2 h
Control (Acetone)	-	93.33 $\pm$ 4.22	173.33 $\pm$ 8.43	173.33 $\pm$ 8.43	110.00 $\pm$ 8.56	-	-	-	-
Control (Ethanol)	-	86.67 $\pm$ 4.22	163.33 $\pm$ 3.33	166.67 $\pm$ 4.22	103.33 $\pm$ 6.15	-	-	-	-
Phenylbutazone	1	26.67 $\pm$ 5.17*	66.67 $\pm$ 6.67*	63.33 $\pm$ 8.03*	40.00 $\pm$ 7.30*	71.43	61.54	63.46	63.64
The extract	0.5	56.67 $\pm$ 8.03 <sup>#</sup>	116.67 $\pm$ 8.03 <sup>#</sup>	123.33 $\pm$ 9.55 <sup>#</sup>	83.33 $\pm$ 8.03 <sup>#</sup>	34.62	28.57	26.00	19.35
	1	40.00 $\pm$ 0.01 <sup>#</sup>	83.33 $\pm$ 8.03 <sup>#</sup>	96.67 $\pm$ 3.33 <sup>#</sup>	76.67 $\pm$ 3.33 <sup>#</sup>	53.85	48.98	42.00	25.81
	3	33.33 $\pm$ 4.22 <sup>#</sup>	80.00 $\pm$ 5.17 <sup>#</sup>	90.00 $\pm$ 4.47 <sup>#</sup>	53.33 $\pm$ 4.22 <sup>#</sup>	61.54	51.02	46.00	48.39

Note: Results are expressed as mean  $\pm$  S.E.M. (N of ears = 6)

Significantly different from the control group (Acetone): \*  $p < 0.05$

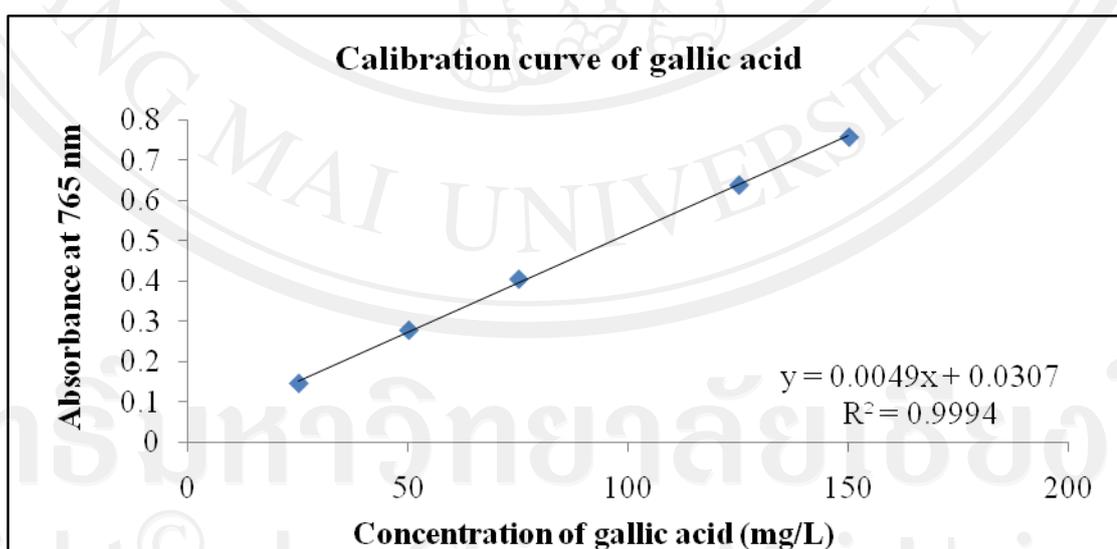
Significantly different from the control group (Ethanol): #  $p < 0.05$

### 4.7.3 Total phenolic contents

The total phenolic contents measurement of 95% ethanol extract of dried red leaves was established in term of gallic acid equivalent (GAE) at 256.64 mg/g dry sample. The water extract from a previous study of Sirisa-Ard *et al.*, 2009 showed more total phenolic contents measuring 498.53 mg/g dry sample. This difference might be due to the solvent used for the extraction and the collection of sample during a different season. These reasons will affect the quantity of active compounds.

**Table 4.10** Absorbance of varied concentration of gallic acid

Concentration of gallic acid (mg/L)	Absorbance at 765 nm				
	1	2	3	SD	Mean
25	0.145	0.143	0.147	0.002	0.145
50	0.270	0.277	0.285	0.008	0.277
75	0.396	0.407	0.408	0.007	0.404
125	0.638	0.633	0.637	0.003	0.636
150	0.758	0.756	0.757	0.001	0.757



**Figure 4.6** Calibration curve of gallic acid

**Table 4.11** The data of total phenolic contents of *T. catappa* Linn. red leaves

Sample	Concentration (g/L)	Absorbance at 765 nm				Total phenolic contents (mg/g)
		1	2	3	Mean±SD	
The extract	0.64	0.679	0.694	0.688	0.687±0.008	256.64

#### 4.7.4 Determination of antioxidant activity

*T. catappa* Linn. red leaf extract showed hydroxyl radical scavenging activity compared to standard quercetin, DPPH radical scavenging activity compared to standard trolox and ABTS scavenging activity expressed as trolox equivalent antioxidant capacity (TEAC). The results were shown in the Table 4.14, 4.17 and 4.19, respectively.

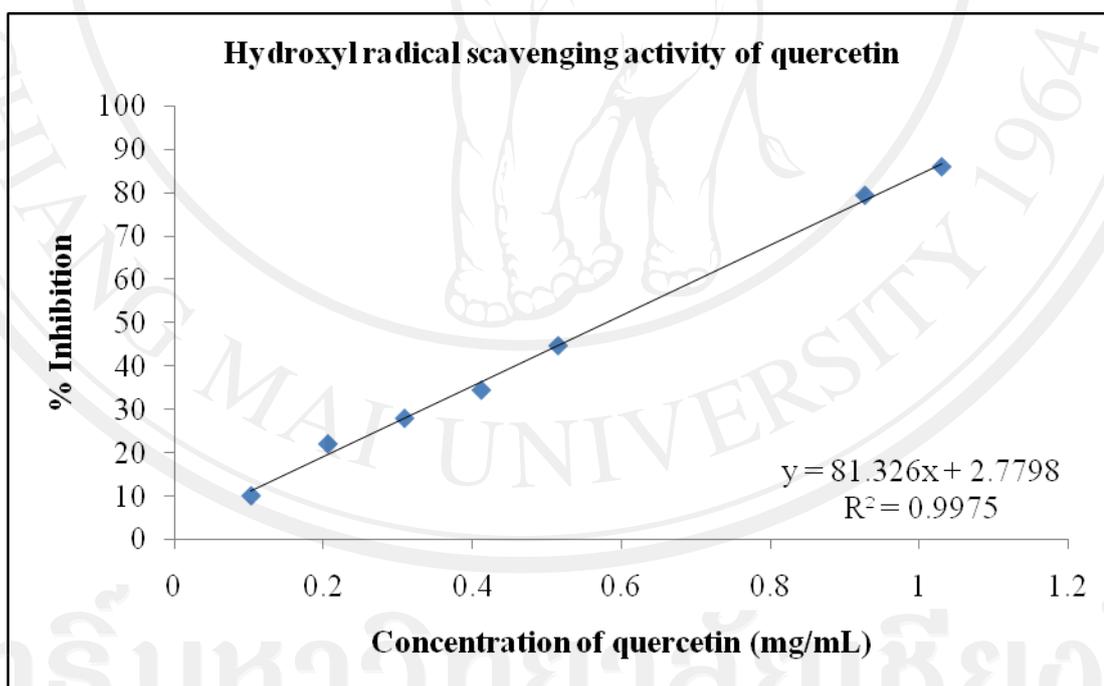
##### 4.7.4.1 Hydroxyl radical (OH<sup>•</sup>) scavenging activity

Hydroxyl radical (OH<sup>•</sup>) scavenging activity of quercetin and the extract were dose-dependent manner. In comparison of hydroxyl radical (OH<sup>•</sup>) scavenging activity, the extract was lower than quercetin as IC<sub>50</sub> of 0.6435 and 0.5806 mg/mL for the extract and quercetin, respectively.

The previous study of Chyau and colleagues (2006), hydroxyl radical scavenging of aqueous extract at 3 minutes boiled of the red leaves at 1 mg/mL was 70.0–75.5%. Whereas in this study, the 95% ethanol extract of the red leaves at 1 mg/mL was 82.70%. It can be indicated that using longer time for extraction and alcoholic solvent can contribute stronger inhibition extract on hydroxyl radical.

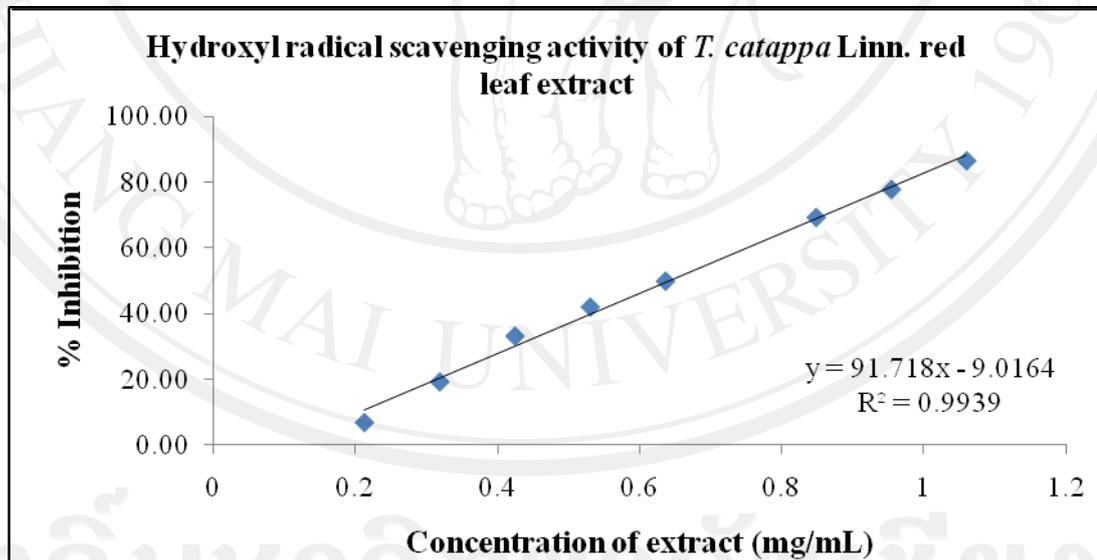
**Table 4.12** The percentage inhibition on hydroxyl radical (OH<sup>•</sup>) of quercetin

Concentration of quercetin (mg/mL)	Absorbance at 536 nm			
	Blank (A <sub>0</sub> )	Control (A <sub>1</sub> )	Standard (A <sub>s</sub> )	% Inhibition
0.103	1.066	0.421	0.486	10.08
0.206	1.066	0.421	0.563	22.02
0.309	1.066	0.421	0.601	27.91
0.412	1.066	0.421	0.643	34.42
0.515	1.066	0.421	0.709	44.65
0.927	1.066	0.421	0.933	79.30
1.03	1.066	0.421	0.975	85.89

**Figure 4.7** Hydroxyl radical scavenging activity of quercetin

**Table 4.13** The percentage inhibition on hydroxyl radical (OH<sup>•</sup>) of *T. catappa* Linn. red leaf extract

Concentration of the extract (mg/mL)	Absorbance at 536 nm			
	Blank (A <sub>0</sub> )	Control (A <sub>1</sub> )	Sample (A <sub>s</sub> )	% Inhibition
0.212	1.110	0.443	0.489	6.90
0.318	1.110	0.443	0.571	19.24
0.424	1.110	0.443	0.664	33.18
0.53	1.110	0.443	0.723	42.03
0.636	1.110	0.443	0.776	49.88
0.848	1.110	0.443	0.905	69.27
0.954	1.110	0.443	0.962	77.81
1.06	1.110	0.443	1.020	86.51



**Figure 4.8** Hydroxyl radical scavenging activity of *T. catappa* Linn. red leaf extract

**Table 4.14** Hydroxyl radical scavenging activity as IC<sub>50</sub> of quercetin and the extract

Sample	Hydroxyl radical scavenging activity (IC <sub>50</sub> , mg/mL)
Quercetin	0.5806
The extract	0.6435

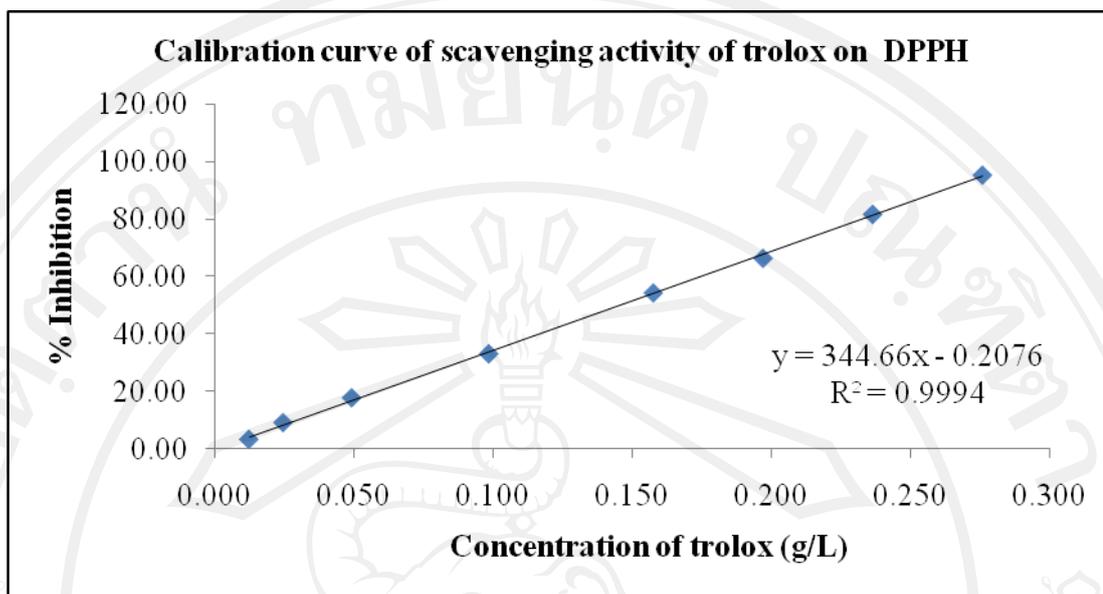
#### 4.7.4.2 DPPH radical scavenging assay

The DPPH radical scavenging activity of the extract and trolox expressed as IC<sub>50</sub> were 0.1154 and 0.1457 mg/mL, respectively. It indicates that DPPH radical scavenging activity of the extract was higher than trolox.

Moreover the percentage inhibition on DPPH radical of the extract is dose-dependent manner and reaches to steady state at a concentration of 0.2 g/L for 80% inhibition (Figure 4.10). While Umale and colleagues (2012) found that at 0.1 mg/mL of 90% ethanol extract scavenged DPPH radical about 92.5-95.7%. The results in this study showed lower scavenging activity on DPPH than Umale found. Chyau (2002, 2006) showed that scavenging activity on DPPH radical were 66.5% and 74.8% for 5mg/mL aqueous and 0.2 mg/mL methanolic extract of the red leaves. It showed that alcoholic extract exhibited stronger inhibition on DPPH than aqueous extract.

**Table 4.15** The percentage inhibition on DPPH radical of trolox

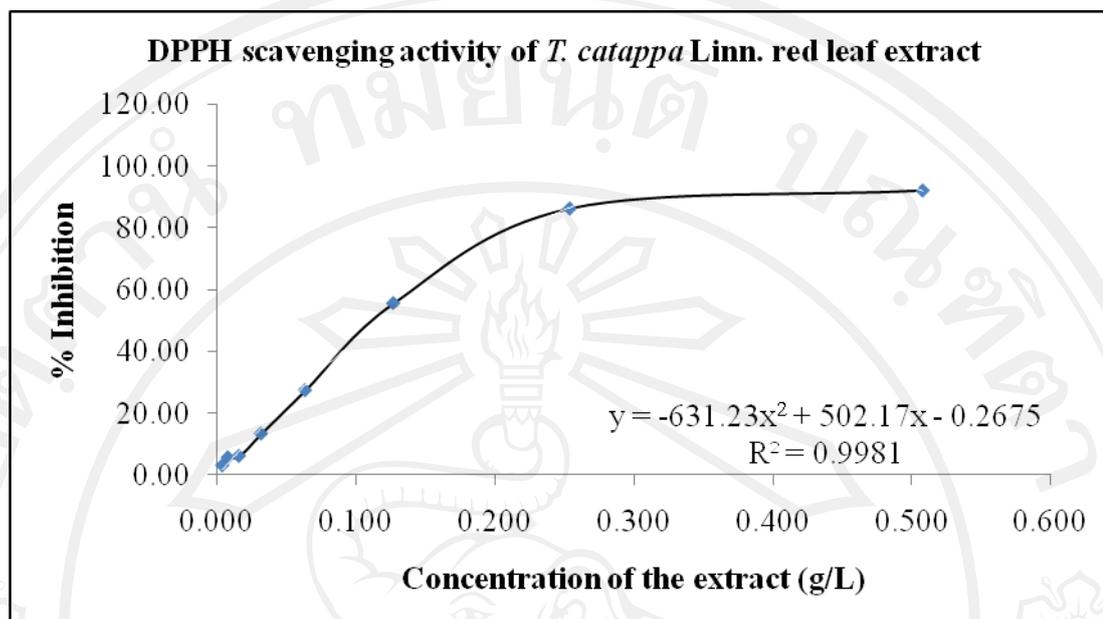
Concentration of trolox (g/L)	Absorbance at 520 nm				% Inhibition
	1	2	3	Mean	
0.012	1.2471	1.2625	1.2601	1.2566	3.23
0.025	1.1684	1.2064	1.169	1.1813	9.07
0.049	1.0683	1.0613	1.0871	1.0722	17.71
0.099	0.879	0.9006	0.8684	0.8827	33.07
0.158	0.6442	0.6045	0.6247	0.6245	54.33
0.197	0.4577	0.4685	1.3391*	0.4631	66.41
0.237	0.2984	0.2516	0.258	0.2693	81.77
0.276	0.1232*	0.0995	0.0873	0.0934	95.46



**Figure 4.9** DPPH radical scavenging activity of trolox

**Table 4.16** The percentage inhibition on DPPH radical of *T. catappa* Linn. red leaf extract

Concentration of the extract (g/L)	Absorbance at 520 nm				% Inhibition
	1	2	3	Mean	
0.508	0.1478	0.1535	0.151	0.151	91.94
0.254	0.2164	0.2022	0.2219	0.214	86.13
0.127	0.5802	0.628	0.569	0.592	55.48
0.064	0.9461	0.9404	0.9292	0.939	27.35
0.032	1.1277	1.1077	1.1052	1.114	13.30
0.016	1.1986	1.2179	1.1827	1.200	6.07
0.008	1.2064	1.2374	1.1947	1.213	5.58
0.004	1.2456	1.2407	1.2353	1.241	3.02



**Figure 4.10** DPPH radical scavenging activity of the extract

**Table 4.17** DPPH radical scavenging activity of the extract and trolox

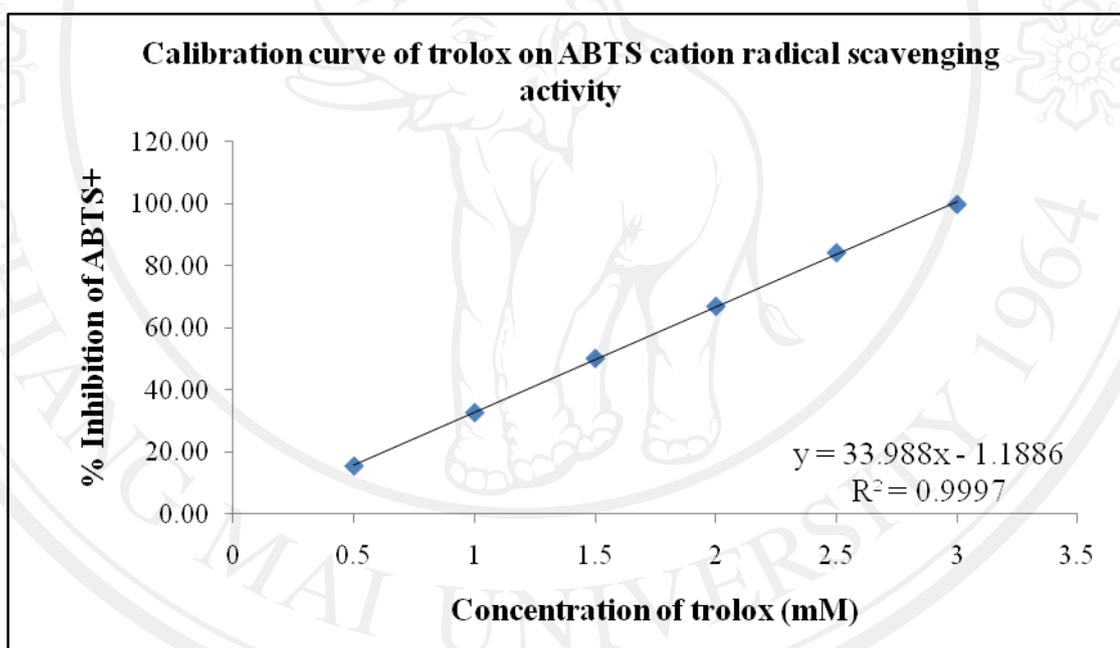
Sample	IC <sub>50</sub> (g/L)
Trolox	0.1457
<i>T. catappa</i> Linn. red leaf extract	0.1154

#### 4.7.4.3 ABTS cation radical scavenging assay

ABTS cation radical scavenging activity in term of trolox equivalent antioxidant capacity (TEAC) of the extract was 1,255.88 mg/g of sample. The inhibition was dose dependent same as Annegowda (2010) found. Annegowda and colleagues (2010) showed that 99.5% ethanol extract of fresh leaf using sonication, ABTS cation radical scavenging activity in term of vitamin C equivalent antioxidant capacity (VCEAC) was 396.23-388.01 mg/g extract. By using soxhlet for 48 hours, at 10 µg/mL of the extract exhibited about 80-90% inhibition. In comparison, the similar extraction method of Annegowda (2010) and this study can be indicated that fresh leaf possesses more scavenging effect on ABTS cation radical than the red leaf.

**Table 4.18** The percentage inhibition on ABTS cation radical of trolox

Concentration of trolox (mM)	Absorbance at 734 nm					A <sub>0</sub> -A <sub>1</sub>	% Inhibition
	A <sub>0</sub>	1	2	3	Mean		
0.5	0.774	0.684	0.639	0.641	0.655	0.119	15.42
1	0.774	0.536	0.518	0.509	0.521	0.253	32.69
1.5	0.774	0.429	0.363	0.364	0.385	0.389	50.22
2	0.774	0.253	0.257	0.257	0.255	0.519	67.05
2.5	0.774	0.121	0.118	0.124	0.121	0.653	84.37
3	0.774	0.000	0.000	0.000	0.000	0.774	100.00

**Figure 4.11** ABTS cation radical scavenging activity of the trolox**Table 4.19** ABTS cation radical scavenging activity of *T. catappa* Linn. red leaf extract in term of trolox equivalent antioxidant capacity (TEAC)

Sample	Absorbance at 734 nm					% Inhibition	TEAC (mg/g of Sample)
	A <sub>0</sub>	1	2	3	Mean		
The extract	0.792	0.103	0.102	0.141	0.115	85.44	1,255.88

#### 4.8 Preformulation study

##### 4.8.1 Physicochemical properties

###### (1) Characteristic of the crude extract

The character of the extract is shown in the Table 4.7 and as the Figure 4.3.

###### (2) pH

pH value of the extract solution in water was  $3.90 \pm 0.02$  as shown in the Table 4.20.

**Table 4.20** pH value of *T. catappa* Linn. red leaf extract in DI water

Sample	pH value			
	1	2	3	Mean $\pm$ SD
DI water	6.87	6.81	6.72	6.80 $\pm$ 0.08
The extract	3.90	3.91	3.88	3.90 $\pm$ 0.02

###### (3) Solubility

The extract was soluble in propylene glycol, ethanol 95% and PEG 400. It was slightly soluble in phosphate buffer, pH 7.4 and 1% Tween 20. It was very slightly soluble in distilled water. It was insoluble in mineral oil, isopropyl myristate, castor oil and glycerin. The data is shown in the Table 4.21.

The solvent in the formula were isopropyl myristate, 95% ethanol and propylene glycol that also play a role as skin enhancers by modified lipid domains in stratum corneum (66). The extract was able to dissolve in the mixture of ethanol and iso propylmyristate.

**Table 4.21** Solubility of *T. catappa* Linn. red leaf extract in various solvents

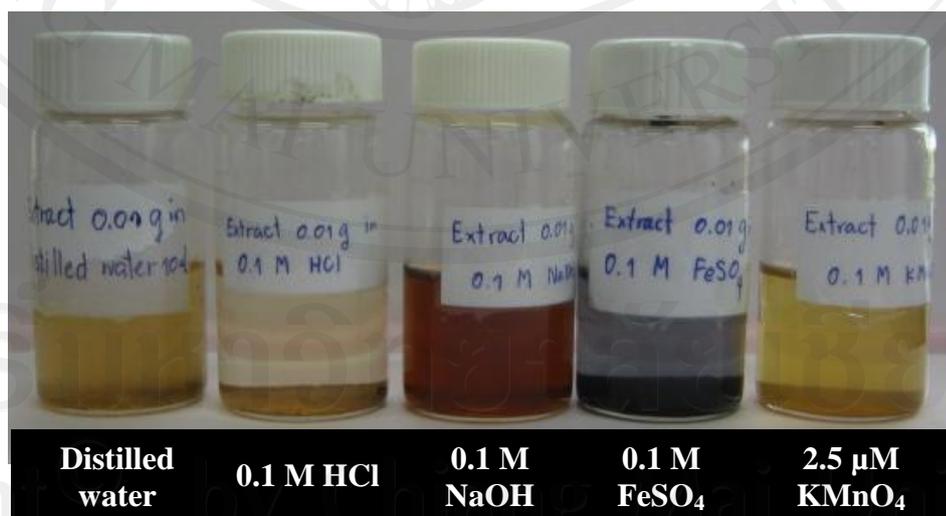
Solvents	Solubility
Distilled water	Very slightly soluble
95% Ethanol	Soluble
Glycerin	Insoluble
Propylene glycol	Soluble

**Table 4.21** Solubility of *T. catappa* Linn. red leaf extract in various solvents (Cont.)

Solvents	Solubility
PEG 400	Soluble
Phosphate Buffer pH 7.4	Slightly soluble
1% Tween 20	Slightly soluble
Mineral oil	Insoluble
Isopropyl myristate	Insoluble
Caster oil	Insoluble

**(4) Chemical reaction**

The extract could dissolve in the basic solution more than in acid solution due to the acidity of the extract (Table 4.22). The reaction did occur between the extract and ferrous sulfate solution, lead to precipitation and colored changed in solution. It was expected that tannin in the extract was precipitated with ferrous salt. Meanwhile the reaction with strong oxidizing agent, potassium permanganate ( $\text{KMnO}_4$ ), the mixture solution was changed from purple to colorless. It was expected that the permanganate ion ( $\text{MnO}_4^-$ ) with purple color was reduced to  $\text{Mn}^{2+}$  which was colorless. The data is shown in the Table 4.22.

**Figure 4.12** Chemical change of the extract in various solutions

**Table 4.22** Chemical reaction of *T. catappa* Linn. red leaf extract and 0.1 M HCl, 0.1 M NaOH, 0.1 M FeSO<sub>4</sub> and 2.5 μM KMnO<sub>4</sub>

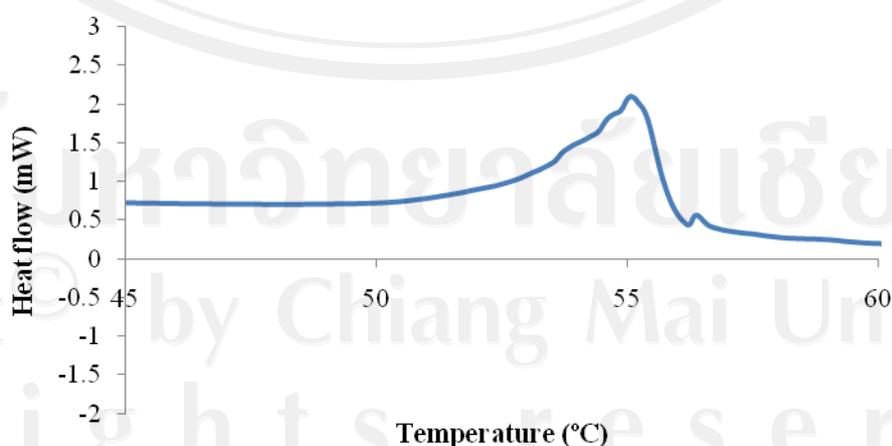
Extract solution in	Results	
	Change in color	Solubility
Distilled water	++	+
0.1 M HCl	-	-
0.1 M NaOH	+++	+++
0.1 M FeSO <sub>4</sub>	+++	+
2.5 μM KMnO <sub>4</sub>	+++	+

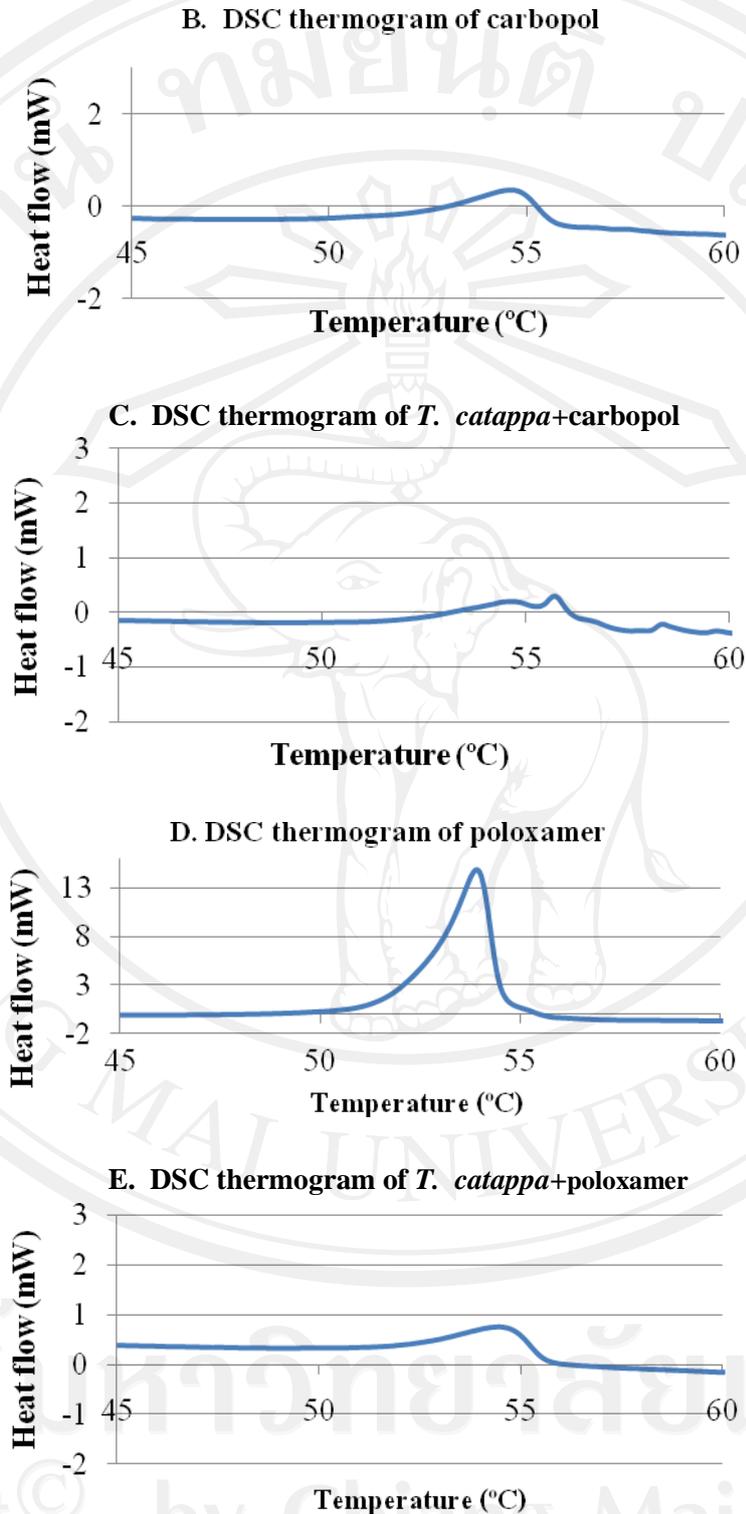
**Note:** -: No change; +: small change; ++: partially change; +++: clearly change

#### 4.8.2 Compatibility study

The DSC thermograms show that the extract and poloxamer were incompatible by decrease endothermic change and physical change after mixing by decrease in melting point. The incompatibility between the extract and poloxamer may due to the phenol structure compounds in the extract react to poloxamer with unknown mechanism. The extract and carbopol ultrez 21 were compatible as shown in the Figure 4.13.

##### A. DSC thermogram of *T. catappa* Linn. red leaf extract





**Figure 4.13** DSC thermogram of the extract (A), carbopol ultrez 21 (B), the mixture of the extract and carbopol ultrez 21 (C), poloxamer 407 (D) and the mixture of the extract and poloxamer 407 (E).

#### 4.9 Formulation of gel containing *T. catappa* Linn. red leaf extract

The 7 formulas were prepared by varying enhancers ratios (95% ethanol, propylene glycol and isopropyl myristate). The ratio used was missible weight between 95% ethanol or propylene glycol and isopropyl myristate as followed in the Table 4.23. The role of each ingredient is shown in the Table 4.24. The 7 gel bases and 7 gel formulations containing the 3% w/w of the extract were evaluated as following parameter: stability, physical properties, antioxidant activity and releasing test.

**Table 4.23** The ratio weight between 95% ethanol, propylene glycol and isopropyl myristate

Enhancer	Formula number						
	1	2	3	4	5	6	7
EtOH : PG : IPM	0:0:0	20:0:0	20:5:0	20:5:5	0:5:0	20:5:10	20:10:5

**Table 4.24** The ingredients in the formulation and their role

Ingredients	Role in the formula (% w/w)
The extract	Active ingredient (3.0%)
Carbopol Ultrez 21	Gelling agent (0.5-2%)
95% Ethanol	Cosolvent, Enhancer (%)
Propylene glycol	Cosolvent, Humectant, Enhancer (5-15%)
Isopropyl myristate	Enhancer (1-10%)
Menthol	Cooling agent (0.05-10%)
BHT	Antioxidant (0.0075-0.1%)
EDTA	Chelating agent (0.005-0.1)
Conc. Paraben	Preservative ( $\leq 1\%$ )
Triethanolamine	Gel stabilizer (q.s.)
Water	Gelling medium (q.s.)

#### 4.9.1 Evaluation of gel containing *T. catappa* Linn. red leaf extract

##### 4.9.1.1 Stability test

From the results, all formulations of gel bases were not changed in physical character but gel base number 3 had a few changes in viscosity and gel base number 4-7 had a few changes in pH. All formulations containing the extract were changed in pH, viscosity and total phenolic contents. It was supposed that hydrolysis of tannins such as punicalagin and corilagin which were found in the extract lead to pH and viscosity decrease (67). Moreover using isopropyl myristate and increasing ethyl alcohol used, the formulation was more unstable. This maybe due to the nonpolar isopropyl myristate which dissolves in ethanol may reduce solubility of carbopol. Then it led to viscosity change. It was found that the formula number 1, 2 and 5 were not syneresis. Therefore these formulas were chosen to determine on *in vitro* releasing test, antioxidant activity and long term stability test at 4°C.

The data of stability test is shown in the Table 4.26 and 4.27. The statistical analysis using paired samples test of change in pH, viscosity and total phenolic contents are shown in the Appendix B.

#### 4.9.1.2 Antioxidant activity of gel containing *T. catappa* Linn. red leaf extract

Hydroxyl radical scavenging activity of gel containing *T. catappa* Linn. red leaf extract was determined. The results showed that *T. catappa* Linn. red leaf extract when incorporated in gel base of formula number 1, 2 and 5 were as shown in the Table 4.25.

**Table 4.25** Hydroxyl radical scavenging activity of gel containing *T. catappa* Linn. red leaf extract

Formular number	Concentration of gel (mg/mL)	% Inhibition
1	50 mg/mL	46.48
2	50 mg/mL	38.24
5	50 mg/mL	48.47

**Table 4.26** Physical properties and total phenolic contents of the formulations

Formulation No.	Physical character	Odor	Spreadability	Removeable	Weight (g)	Viscosity (Pas)	pH	Total phenolic contents (GAE, mg/g dry extract)
<b>Gel Base</b>								
1	+++	Cooling	+++++	++++	6.81±0.61	18.323±0.248	7.93±0.06	-
2	++++	Cooling	+++++	++++	7.41±0.44	17.557±0.485	7.90±0.20	-
3	+++++	Cooling	+++++	++++	7.24±0.39	18.141±0.057	7.66±0.09	-
4	++	Cooling	+++++	+++	7.00±0.32	17.941±0.378	7.53±0.12	-
5	+++	Cooling	+++++	++++	7.35±0.11	18.536±1.099	7.40±0.02	-
6	+	Cooling	+++++	+++	5.30±0.90	15.777±0.420	7.64±0.09	-
7	+	Cooling	+++++	+++	7.44±0.29	18.768±0.728	7.46±0.23	-
<b>Gel containing the extract</b>								
1	++++	Cooling	+++++	+++++	4.86±0.62	8.665±0.360	7.38±0.12	221.94±9.28
2	++++	Cooling	+++++	+++++	4.68±0.26	8.040±0.264	7.49±0.08	217.07±8.34
3	+++++	Cooling	+++++	+++++	5.29±0.29	3.906±0.077	6.14±0.15	221.92±8.11
4	++++	Cooling	+++++	+++++	4.51±0.90	6.011±0.370	6.31±0.05	242.63±6.03
5	++++	Cooling	+++++	+++++	4.83±0.16	4.684±0.194	5.98±0.04	212.02±7.22
6	++	Cooling	+++++	+++++	5.22±0.35	2.918±0.416	4.72±0.05	227.43±13.38
7	++	Cooling	+++++	+++++	4.71±0.12	2.021±0.093	5.73±0.06	261.63±8.74

**Table 4.27** Physical properties and total phenolic contents of the formulations after stability test

Formula No.	Physical character	Odor	Spreadability	Removeable	Weight (g)	Viscosity (Pas)	pH	Total phenolic contents (GAE, mg/g dry extract)
<b>Gel Base</b>								
1	+++	Cooling	+++++	++++	6.83±0.63	16.319±1.013	7.69±0.32	-
2	++++	Cooling	+++++	++++	7.47±0.29	14.804±0.342	7.64±0.10	-
3	+++++	Cooling	+++++	++++	7.24±0.40	15.103±0.708	6.87±0.28	-
4	++	Cooling	+++++	+++	6.98±0.33	16.004±0.894	6.86±0.02	-
5	+++	Cooling	+++++	++++	7.34±0.11	16.538±1.030	6.25±0.30	-
6	+	Cooling	+++++	+++	5.31±0.90	13.576±1.649	6.57±0.13	-
7	+	Cooling	+++++	+++	7.44±0.29	17.209±1.592	6.23±0.14	-
<b>Gel containing the extract</b>								
1	++++	Cooling	+++++	+++++	4.85±0.62	7.031±0.312	6.61±0.07	265.82±10.01
2	++++	Cooling	+++++	+++++	4.63±0.20	7.585±1.242	6.88±0.10	256.55±9.72
3	+++	Cooling	+++++	+++++	5.29±0.29	2.809±0.170	5.48±0.06	273.01±2.72
4	+++	Cooling	+++++	+++++	4.48±0.92	3.944±0.371	5.46±0.25	323.32±11.44
5	+++++	Cooling	+++++	+++++	4.89±0.26	2.332±0.347	4.94±0.11	296.51±9.76
6	+	Cooling	+++++	+++++	4.91±0.25	7.031±0.312	4.28±0.06	462.02±4.17
7	++++	Cooling	+++++	+++++	4.85±0.62	7.585±1.242	6.61±0.07	265.82±10.01

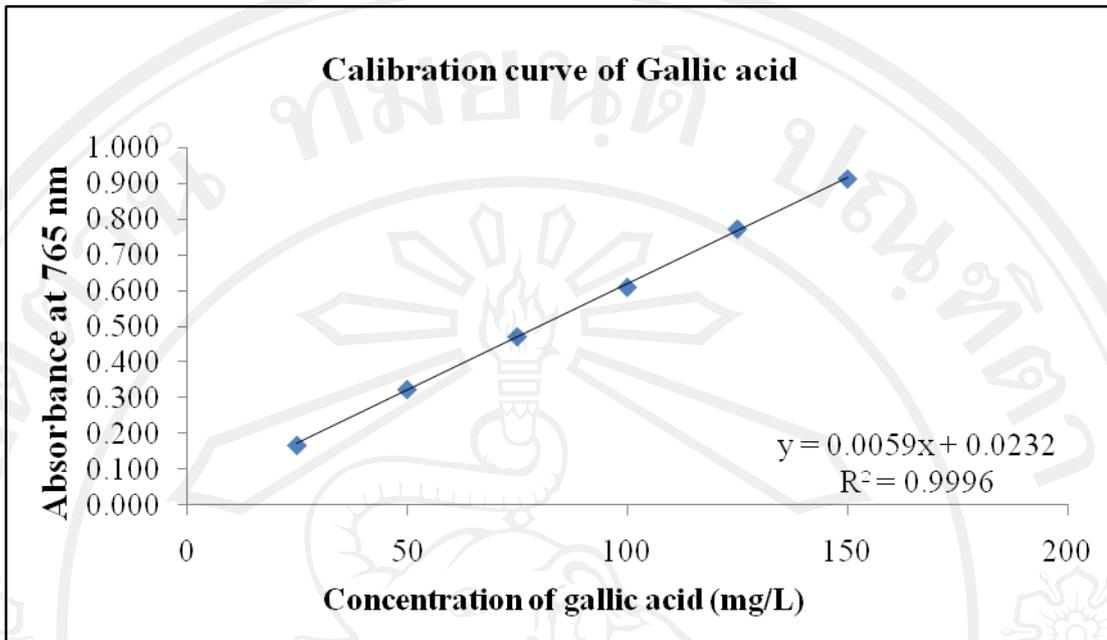
#### 4.9.2 *In vitro* releasing test using Franz static diffusion cells

From releasing profile, it was found that bioactive agent was released from formula number 1 equal to formula number 5 and more than formula number 2 (See in the Figure 4.15). The difference of releasing profile between 3 formulas was due to difference enhancer. The formula number 1 had only ethyl alcohol from the extract solution. The formula number 2 had ethyl alcohol more than formula number 1. The formula number 5 had only propylene glycol. From the releasing profile, it is supposed that the formula with lower ethyl alcohol can release more total phenolic contents which may be due to the less affinity of bioactive compounds to vehicle than in others.

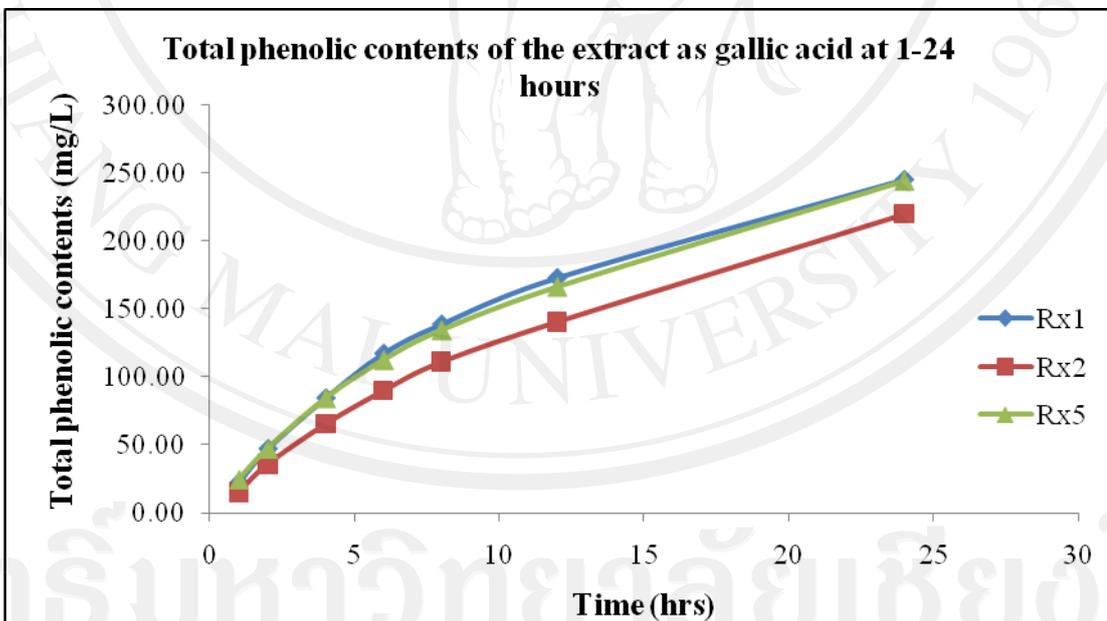
The calibration curve of gallic acid and the data of released total phenolic contents are shown in the Table 4.28 and 4.29, respectively. The calibration curve of gallic acid is shown as the Figure 4.14.

**Table 4.28** Absorbance of varied concentration of gallic acid

Concentration of gallic acid (mg/L)	Absorbance at 765 nm			
	1	2	3	Mean±SD
25	0.166	0.170	0.170	0.169±0.002
50	0.321	0.326	0.327	0.325±0.003
75	0.459	0.472	0.486	0.472±0.014
100	0.564	0.634	0.633	0.610±0.040
125	0.752	0.783	0.784	0.773±0.018
150	0.908	0.912	0.919	0.913±0.006



**Figure 4.14** Calibration curve of gallic acid



**Figure 4.15** Total phenolic contents of the extract as gallic acid released from gel at 1-24 hours

**Table 4.29** The data of released total phenolic contents from *T. catappa* Linn. red leaf extract gel formula 1, 2, 5

Formula	Time	1		2		3		Mean	TPC (mg/L)	TPC in 1 mL	Cumulative TPC in each 1 mL	Cumulative TPC in 13 mL	Collected TPC (mg/L)
		Weight	A	Weight	A	Weight	A						
1	1	1.2735	0.166	1.0031	0.147	1.1141	0.147	0.153	22.06	0.02206	0.00000	0.2867	22.06
	2	1.2735	0.304	1.0031	0.305	1.1141	0.260	0.290	45.15	0.04515	0.02206	0.6089	46.84
	4	1.2735	0.506	1.0031	0.497	1.1141	0.467	0.490	79.06	0.07906	0.06721	1.0950	84.23
	6	1.2735	0.652	1.0031	0.638	1.1141	0.652	0.647	105.73	0.10573	0.14627	1.5207	116.98
	8	1.2735	0.746	1.0031	0.737	1.1141	0.697	0.726	119.18	0.11918	0.25200	1.8013	138.56
	12	1.2735	0.909	1.0031	0.827	1.1141	0.885	0.874	144.18	0.14418	0.37117	2.2456	172.74
	24	1.2735	1.242	1.0031	1.228	1.1141	1.235	1.235	205.39	0.20539	0.51536	3.1854	245.03
2	1	1.3588	0.122	1.1941	0.119	1.1166	0.101	0.114	15.35	0.01535	0.00000	0.1996	15.35
	2	1.3588	0.218	1.1941	0.237	1.1166	0.220	0.225	34.18	0.03418	0.02206	0.4665	35.88
	4	1.3588	0.376	1.1941	0.398	1.1166	0.376	0.384	61.10	0.06110	0.05624	0.8505	65.42
	6	1.3588	0.491	1.1941	0.514	1.1166	0.499	0.501	80.98	0.08098	0.11734	1.1701	90.01
	8	1.3588	0.576	1.1941	0.602	1.1166	0.589	0.589	95.92	0.09592	0.19832	1.4452	111.17
	12	1.3588	0.747	1.1941	0.708	1.1166	0.703	0.719	118.01	0.11801	0.29424	1.8283	140.64
	24	1.3588	1.110	1.1941	1.158	1.1166	1.133	1.134	188.25	0.18825	0.41225	2.8595	219.96
5	1	1.3414	0.168	1.1574	0.172	1.1453	0.161	0.167	24.41	0.02441	0.00000	0.3173	24.41
	2	1.3414	0.261	1.1574	0.313	1.1453	0.299	0.291	45.39	0.04539	0.02206	0.6121	47.09
	4	1.3414	0.460	1.1574	0.506	1.1453	0.501	0.489	78.99	0.07899	0.06745	1.0943	84.18
	6	1.3414	0.566	1.1574	0.645	1.1453	0.647	0.619	101.04	0.10104	0.14644	1.4600	112.30
	8	1.3414	0.631	1.1574	0.741	1.1453	0.736	0.703	115.16	0.11516	0.24748	1.7446	134.20
	12	1.3414	0.793	1.1574	0.846	1.1453	0.876	0.838	138.18	0.13818	0.36264	2.1589	166.07
	24	1.3414	1.143	1.1574	1.265	1.1453	1.298	1.235	205.45	0.20545	0.50082	3.1716	243.97

**Note:** A, Absorbance at 765 nm; TPC, Total phenolic contents

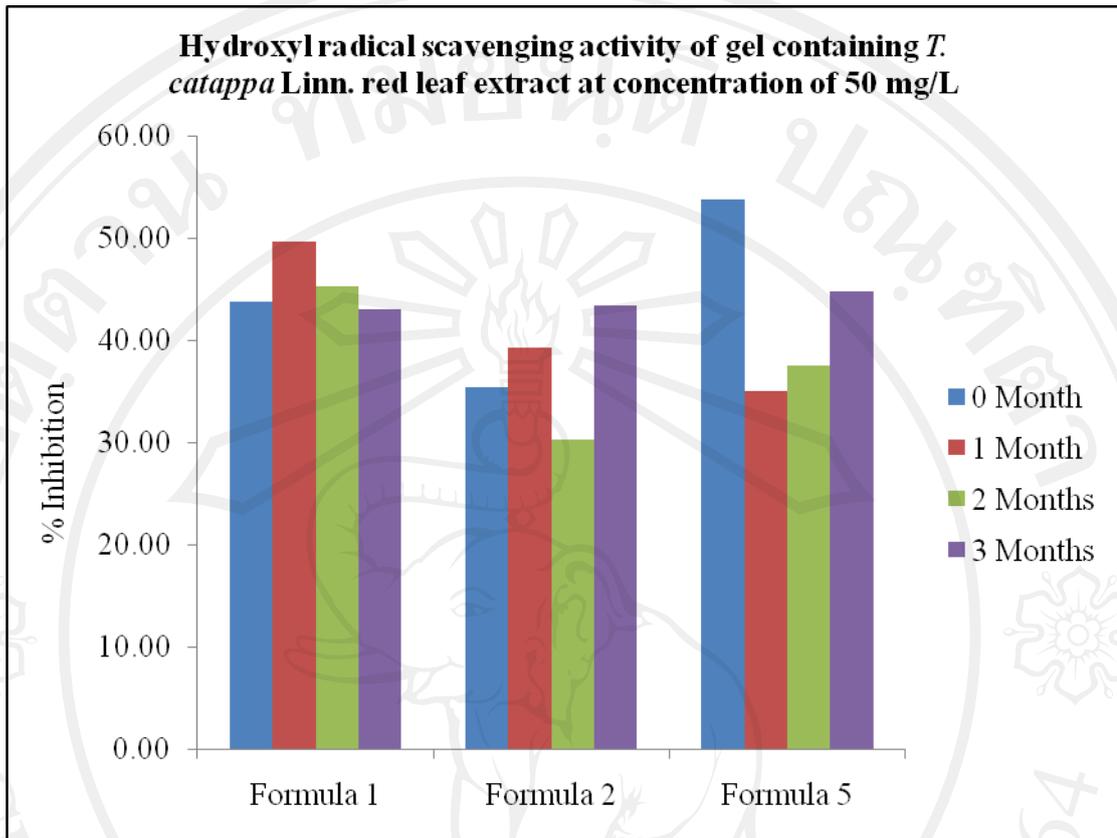
#### 4.9.3 Long term stability test of gel containing *T. catappa* Linn. red leaf extract at 4°C

Stability of gel containing *T. catappa* Linn. red leaf extract at 4°C showed that the formula number 1, 2 (95% ethanol added as enhancer) and 5 (propylene glycol added as enhancer) were not syneresis. After 3 months, the formula number 1 had no change in viscosity, pH, total phenolic contents and hydroxyl radical scavenging activity. Formula number 2 had a viscosity decreased and total phenolic contents was varied while hydroxyl radical scavenging activity was not changed. However total phenolic contents of formula number 2 was not significantly different to at 0 month. Formula number 5 had hydroxyl radical scavenging activity decreased and total phenolic contents increased. It can be supposed that formula number 5 had hydrolysis of bioactive agent which was to reduce in antioxidant activity similar to the founding of Annegowda *et al.*, 2010. Moreover, pH of formula number 5 was lower than others, this may influence to the antioxidant activity. The data was shown as the Figure 4.16- 4.19 and the Table 4.30- 4.33.

From this study, it showed that the formula number 1 was more stable than formula number 2 and 5 after keeping for 3 months at 4°C. It was suggested that the gel containing *T. catappa* Linn. red leaf extract should be kept in refrigerator.

**Table 4.30** Hydroxyl radical scavenging activity of the formula number 1, 2 and 5 at day of 0, 1, 2 and 3 months

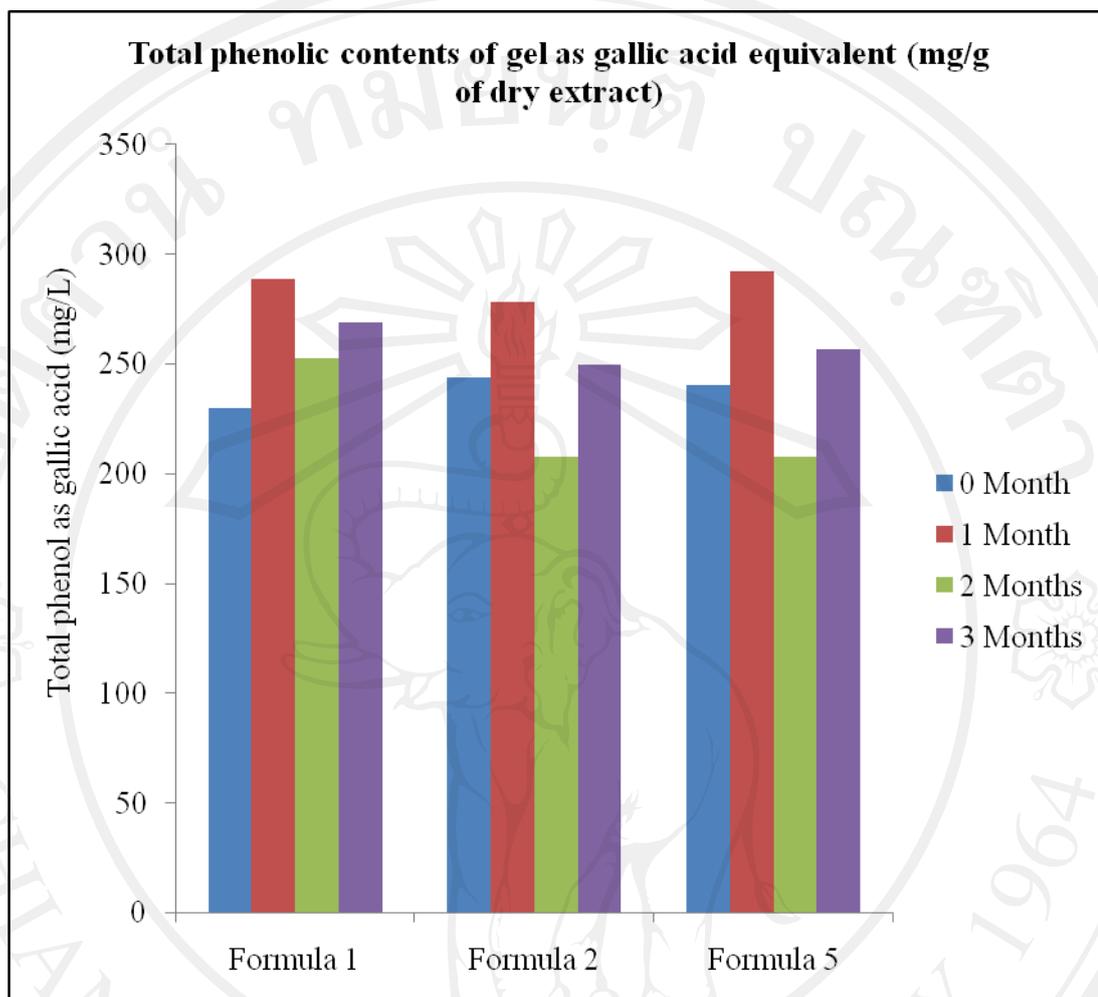
Month	Formula	Weight	Concentration (mg/mL)	Absorbance at 536 nm							% CV	%Inhibition	50 mg/mL
				A0	A1	1	2	3	x	SD			
0	1	0.3117	31.17000	1.135	0.348	0.567	0.556	0.566	0.563	0.006	1.080	27.3189	43.82
	2	0.3160	31.60000	1.135	0.348	0.53	0.531	0.511	0.524	0.011	2.151	22.3634	35.39
	5	0.3029	30.29000	1.135	0.348	0.602	0.628	0.582	0.604	0.023	3.819	32.5286	53.70
1	1	0.2832	28.32000	1.092	0.437	0.58	0.632	0.652	0.621	0.037	5.982	28.1425	49.69
	2	0.2846	28.46000	1.092	0.437	0.582	0.585	0.809	0.584	0.002	0.364	22.3664	39.29
	5	0.2856	28.56000	1.092	0.437	0.561	0.566	0.577	0.568	0.008	1.441	20.0000	35.01
2	1	0.3152	31.52000	1.142	0.386	0.599	0.608	0.599	0.602	0.005	0.863	28.5714	45.32
	2	0.3003	30.03000	1.142	0.386	0.521	0.518	0.532	0.524	0.007	1.408	18.2099	30.32
	5	0.3177	31.77000	1.142	0.386	0.561	0.561	0.576	0.566	0.009	1.530	23.8095	37.47
3	1	0.2975	29.75000	1.133	0.371	0.561	0.561	0.576	0.566	0.009	1.530	25.5906	43.01
	2	0.2947	29.47000	1.133	0.371	0.561	0.561	0.576	0.566	0.009	1.530	25.5906	43.42
	5	0.2862	28.62000	1.133	0.371	0.561	0.561	0.576	0.566	0.009	1.530	25.5906	44.71



**Figure 4.16** Hydroxyl radical scavenging activity of the formula number 1, 2 and 5 at day of 0, 1, 2 and 3 months

**Table 4.31** Total phenolic contents of the formula number 1, 2 and 5 at day of 0, 1, 2 and 3 months

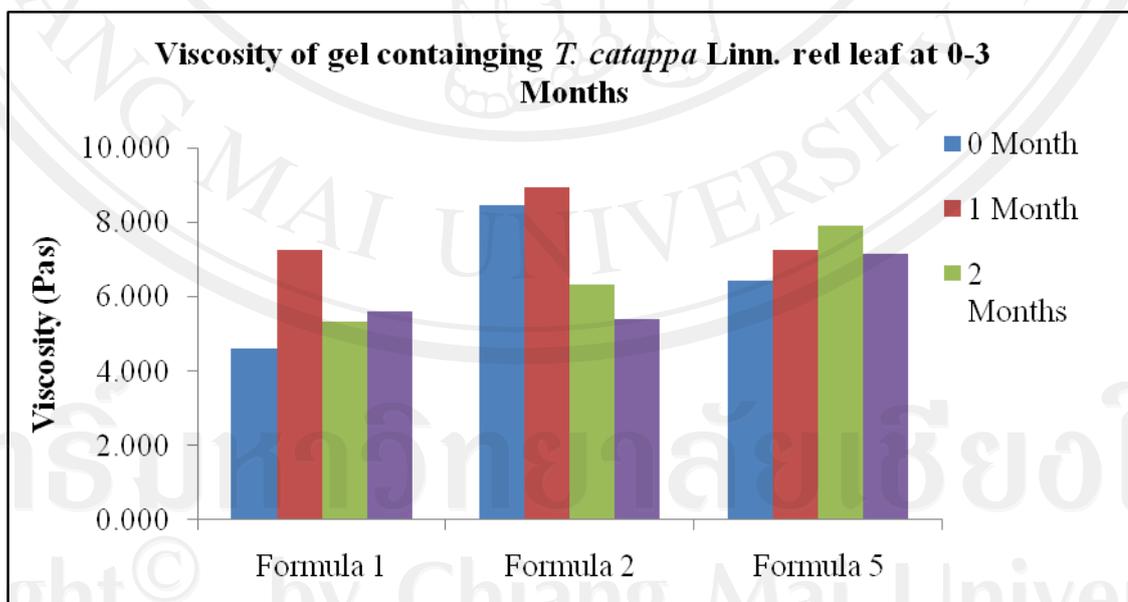
Month	Formula	Weight	Absorbance at 765 nm						% CV	TCP (GAE, mg/g dry extract)	
			a	b	1	2	3	x			SD
0	1	0.1726	0.0059	0.0232	0.7280	0.7780	0.6720	0.726	0.053	7.304	230.048
	2	0.1553	0.0059	0.0232	0.6720	0.7060	0.7040	0.694	0.019	2.749	244.033
	5	0.1823	0.0059	0.0232	0.7660	0.8140	0.8180	0.799	0.029	3.620	240.534
1	1	0.1406	0.0059	0.0232	0.6860	0.7570	0.7810	0.741	0.049	6.664	288.567
	2	0.1719	0.0059	0.0232	0.8190	0.8920	0.8980	0.870	0.044	5.057	278.202
	5	0.1497	0.0059	0.0232	0.7500	0.8210	0.8230	0.798	0.042	5.211	292.412
2	1	0.1576	0.0059	0.0232	0.6920	0.7420	0.7520	0.729	0.032	4.412	252.899
	2	0.1502	0.0059	0.0232	0.5640	0.5660	0.5960	0.575	0.018	3.116	207.683
	5	0.1588	0.0059	0.0232	0.6060	0.6080	0.6060	0.607	0.001	0.190	207.583
3	1	0.1488	0.0059	0.0232	0.7070	0.7400	0.7480	0.732	0.022	2.970	268.994
	2	0.1474	0.0059	0.0232	0.6610	0.6800	0.6850	0.675	0.013	1.875	249.957
	5	0.1431	0.0059	0.0232	0.6530	0.6880	0.6790	0.673	0.018	2.699	256.679



**Figure 4.17** Total phenolic contents of the formula number 1, 2 and 5 at day of 0, 1, 2 and 3 months

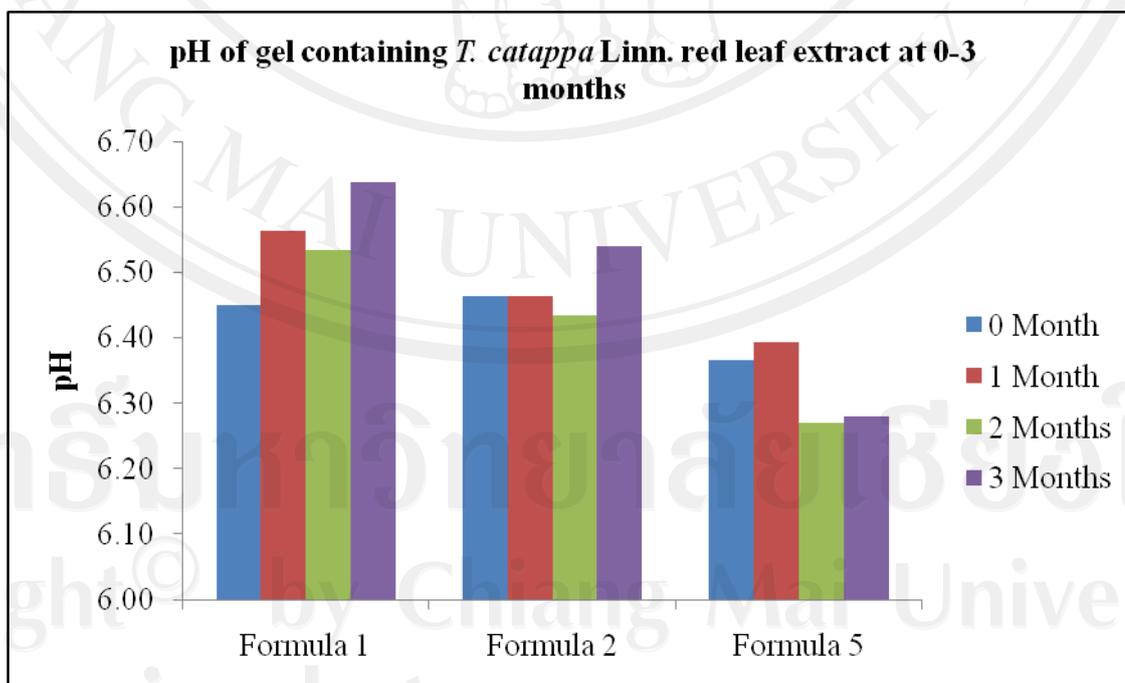
**Table 4.32** Viscosity of the formula number 1, 2 and 5 at day of 0, 1, 2 and 3 months

Month	Formula	Viscosity (Pas)					% CV
		1	2	3	x	SD	
0	1	4.829	3.715	4.368	4.599	0.326	7.089
	2	8.425	8.558	8.384	8.456	0.091	1.076
	5	6.733	6.748	5.854	6.445	0.512	7.942
1	1	5.603	7.145	7.391	7.268	0.174	2.393
	2	9.348	8.568	6.521	8.958	0.552	6.157
	5	6.721	7.634	7.467	7.274	0.486	6.683
2	1	5.764	4.829	5.444	5.346	0.475	8.889
	2	6.511	6.342	6.090	6.314	0.212	3.355
	5	7.977	6.055	7.848	7.913	0.091	1.153
3	1	5.898	7.098	5.305	5.602	0.41931	7.48575
	2	5.069	4.66	5.749	5.409	0.48083	8.88949
	5	7.074	7.062	7.277	7.138	0.12082	1.69264

**Figure 4.18** Viscosity of the formula number 1, 2 and 5 at day of 0, 1, 2 and 3 months

**Table 4.33** pH value of the formula number 1, 2 and 5 at day of 0, 1, 2 and 3 months

Month	Formula	pH					
		1	2	3	x	SD	% CV
0	Formula 1	6.40	6.47	6.48	6.45	0.04	0.68
	Formula 2	6.50	6.47	6.42	6.46	0.04	0.63
	Formula 5	6.38	6.37	6.35	6.37	0.02	0.24
1	Formula 1	6.57	6.56	6.56	6.56	0.01	0.09
	Formula 2	6.50	6.38	6.51	6.46	0.07	1.12
	Formula 5	6.39	6.39	6.40	6.39	0.01	0.09
2	Formula 1	6.63	6.59	6.38	6.53	0.13	2.06
	Formula 2	6.23	6.69	6.38	6.43	0.23	3.65
	Formula 5	6.42	6.39	6.00	6.27	0.23	3.74
3	Formula 1	6.66	6.62	6.63	6.64	0.02	0.31
	Formula 2	6.61	6.51	6.50	6.54	0.06	0.93
	Formula 5	6.35	6.12	6.37	6.28	0.14	2.21

**Figure 4.19** pH value of the formula number 1, 2 and 5 at day of 0, 1, 2 and 3 mont