CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Extraction and Preparation

3.1.1 HCE

The obtained HCE was greenish-black, soft, with fishy smell and high viscosity. The yield of extraction was 5.85%.

3.1.2 HCE and Fraction for analysis

The HCE in methanol was prepared to an equivalent of 0.03125 mg/ml for TLC analysis and 0.025 mg/ml for HPLC analysis. Partition of HCE by hexane, chloroform, ethyl acetate and water yielded F1, F2, F3 and F4, respectively. All fractions were dissolved in methanol.

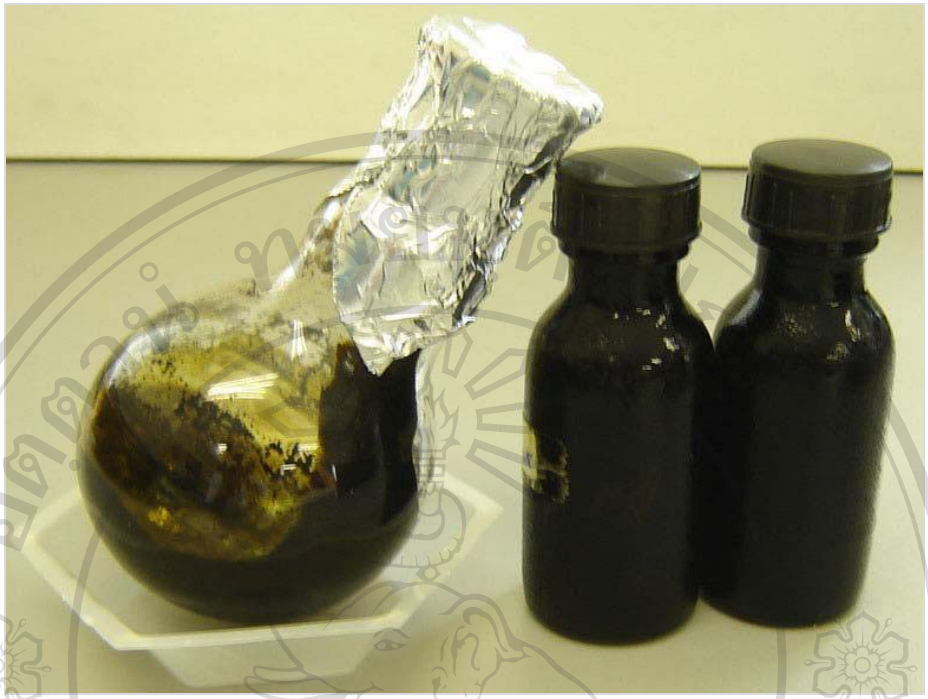

Figure 3.1 The extraction of HC

3.2 Identification of HCE

3.2.1 Identification of HCE with TLC

TLC for Terpenes Test

Terpene chromatograms (Figure 3.2) exhibited retardation factor (R_f) of standard geraniol at 0.10. There were violet-blackish and yellow fluorescence spots after spraying on visible light and UV-365 nm detections. Others were violet-blackish and gray spots with R_f 0.91 and 0.71 in HCE and F1 (Figure 3.2 (c)). However, a few unknown terpenes were detected in HCE and HCE fractions.

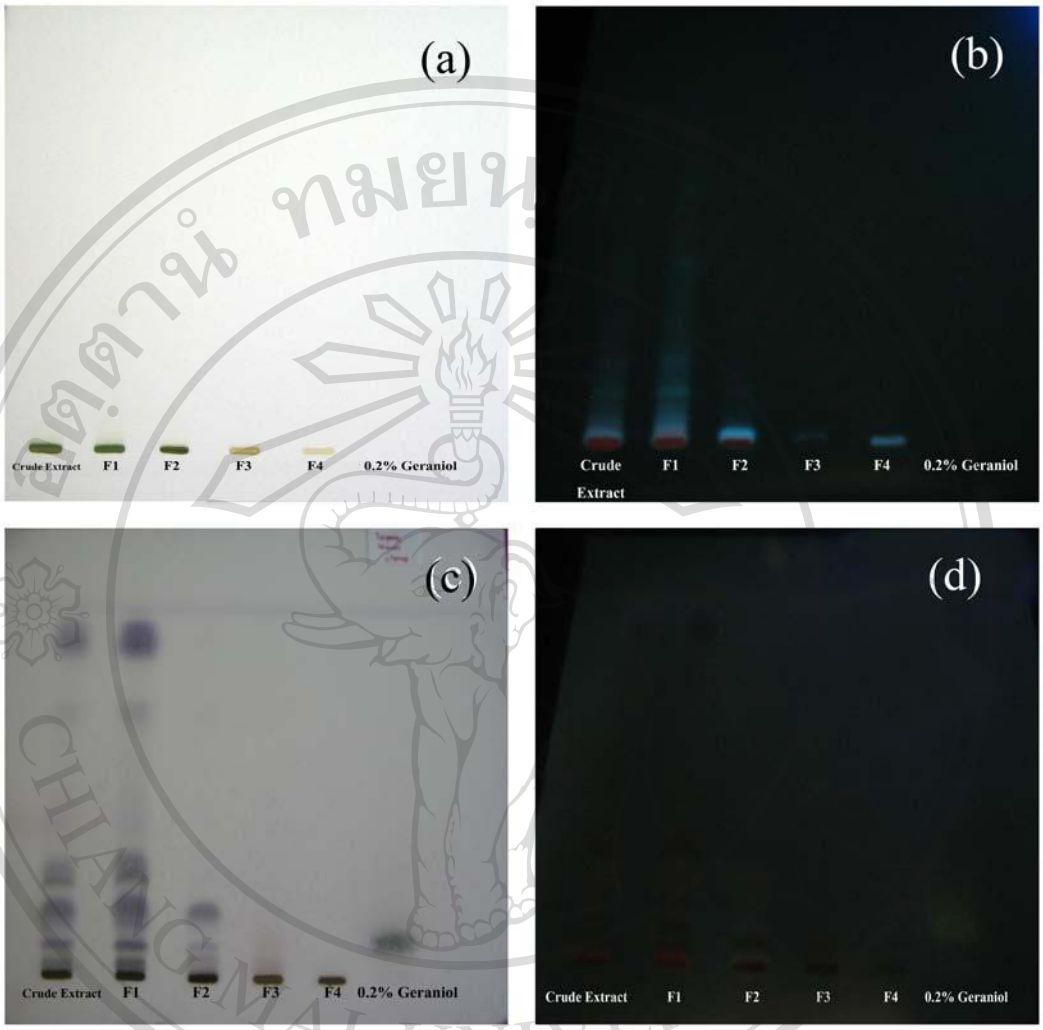

Figure 3.2 TLC chromatograms of HCE and fractions, in comparison with geraniol standard; (a) and (b) were not sprayed, (c) and (d) were sprayed, (a) and (c) were observed under visible light, while (b) and (d) were examined under UV light (365 nm).

TLC for Flavonoids Test

For flavonoid TLC chromatograms, rutin and quercetin standard demonstrated R_f as 0.43 and 0.98 with yellow and green fluorescence respectively (Figure 3.3 (d)). The same characteristic spots found in HCE, F2, F3 and F4. HCE and F3 showed green and yellow spots with R_f 0.55 and 0.68 (Figure 3.3 (d)) but others did not. The unknown components also presented as blue fluorescence and blue spot with R_f 0.50 and 0.70 in F2, F4 and HCE. It can be deduced that HCE composed of rutin, quercetin and several unknown compounds.

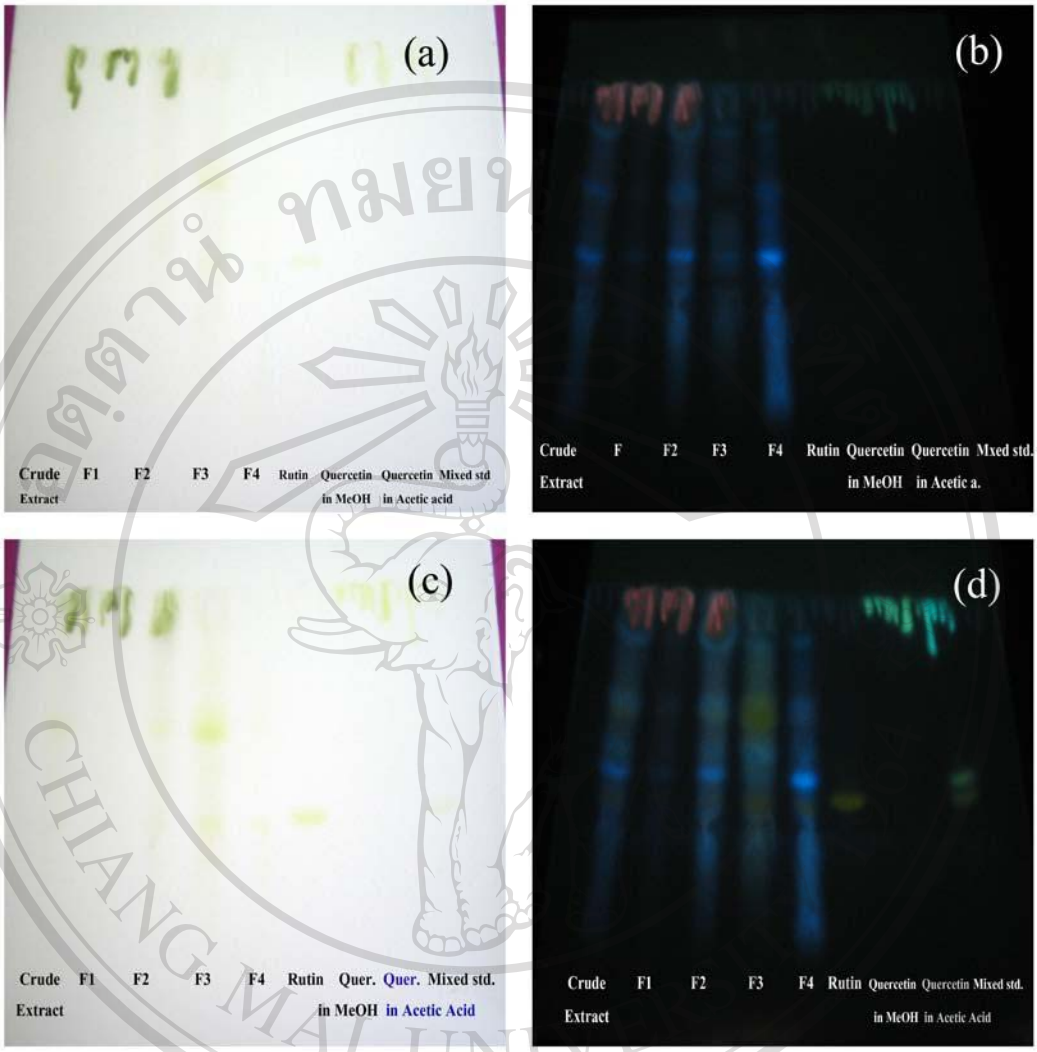

Figure 3.3 TLC chromatograms of HCE and fractions, in comparison with rutin and quercetin standard; (a) and (b) were not sprayed, (c) and (d) were sprayed, (a) and (c) were observed under visible light while (b) and (d) were examined under UV-365 nm.

3.2.2 Identification of HCE with HPLC

HCE was analyzed to identify flavonoids markers with HPLC-UV (350 nm). Results showed that rutin and quercetin amounts in HCE were 0.8547 (1.709%) and 0.0375 mg/ml (0.0751%), respectively. Retention time showed at 6.2 and 12.4 minutes, respectively (Figure 3.4).

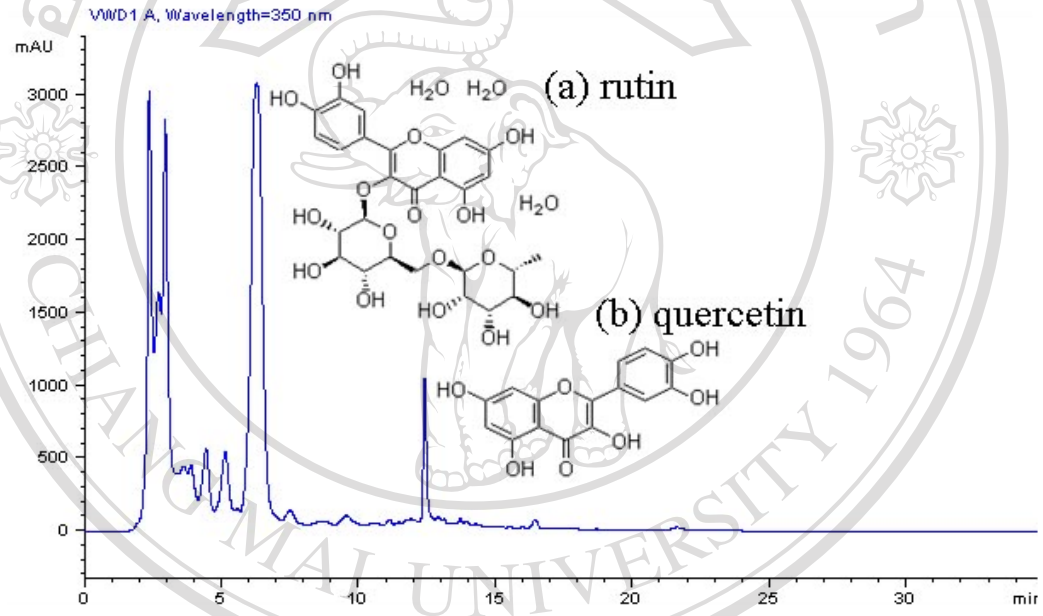

Figure 3.4 HCE chromatogram from HPLC-UV (350 nm) analysis for rutin (a) and quercetin (b)

3.3 Method Validation

3.3.1 Calibration curves

Two flavonoid standards, rutin and quercetin presented in HPLC-UV (350 nm) chromatogram at retention time 6.1 and 11.7 minutes, respectively (Figure 3.5). Because rutin was more polar than quercetin, it was detected first with methanol:water as mobile phase and C-18 column as stationary phase. This was in accordance with like-dissolve-like principle for chromatography and their retention time was compared with compounds in HCE within the same testing condition.

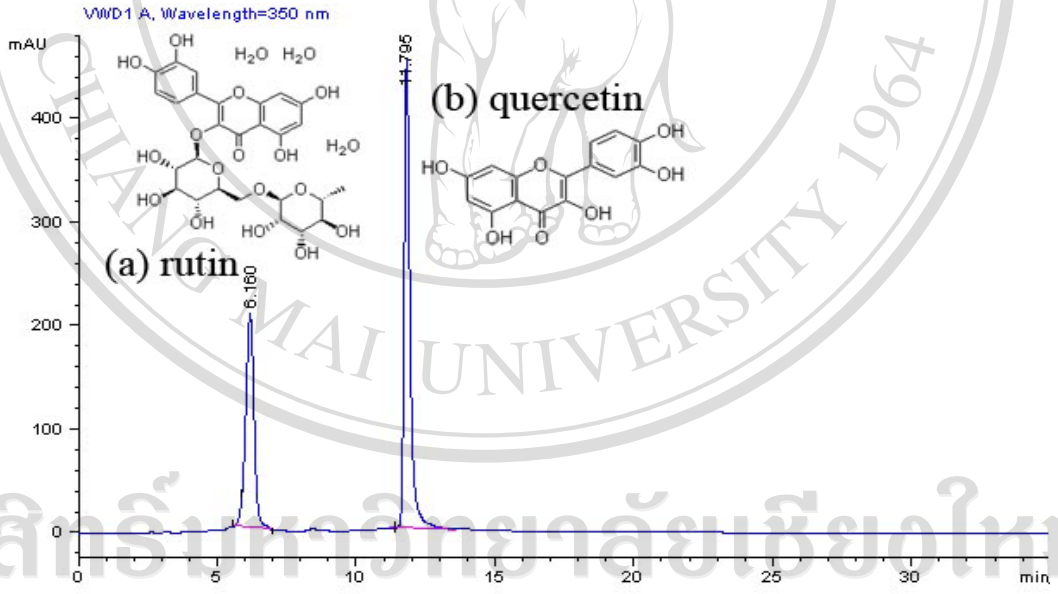

Figure 3.5 HPLC chromatogram of (a) rutin and (b) quercetin standards on HPLC-UV (350 nm)

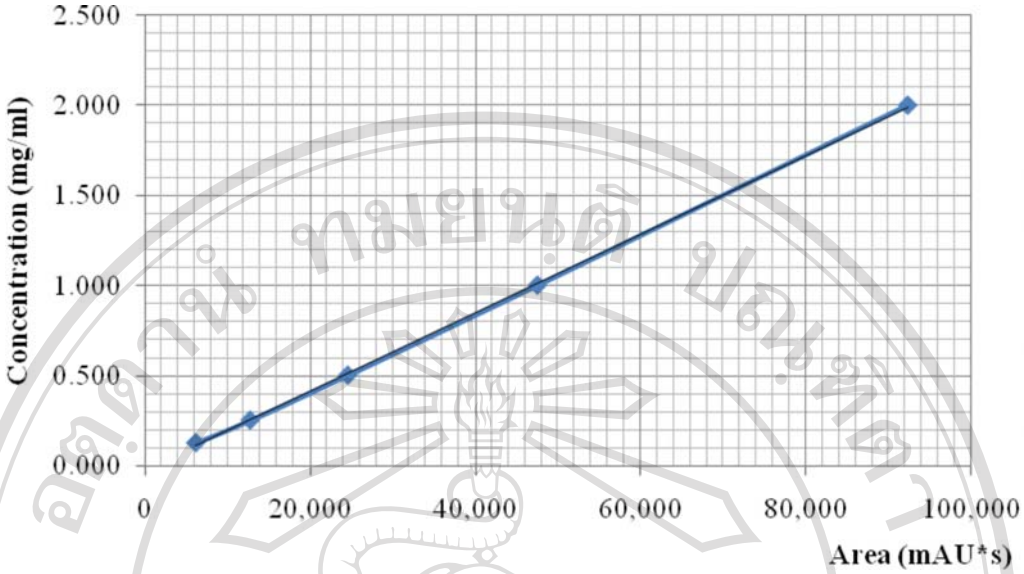

Figure 3.6 Standard curve of rutin

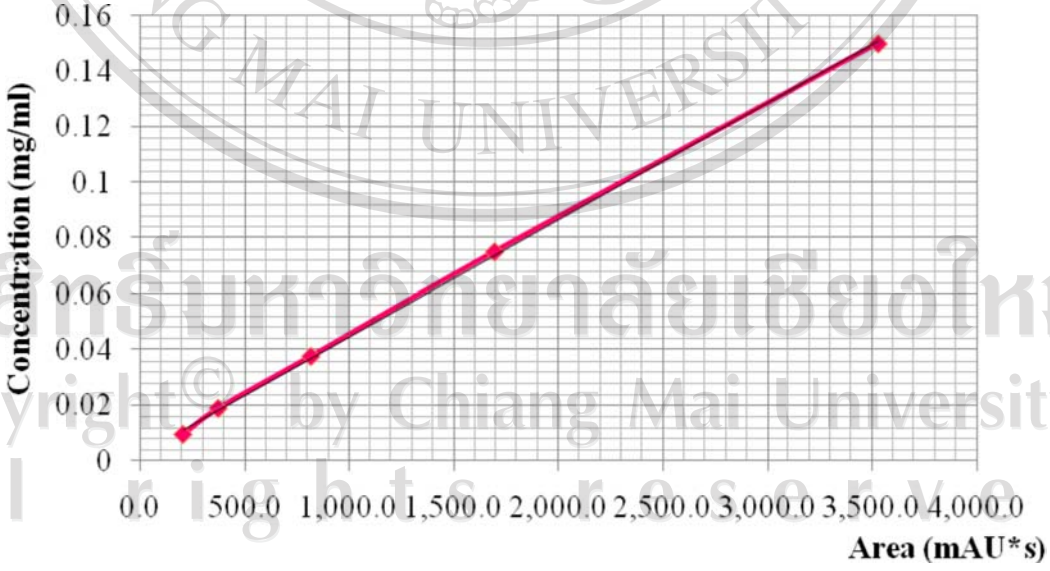

Figure 3.7 Standard curve of quercetin

Table 3.1 Linear regression analysis of flavonoid standards

Standard flavonoids	Linear regression models	R^2	Linear range (mg/ml)
Rutin	$y=2.18\times10^{-5}(x)-0.025$	0.999 7	0.125-2.00
Quercetin	$y=4.20\times10^{-5}(x)+0.002$	0.999 5	0.009375-0.15

X = peak area; y= concentration

Both rutin and quercetin at a series dilution of concentrations were analyzed and created as standard curves (Figure 3.6-3.7). Linear regression models and correlation coefficients (R^2) of calibration curves are presented in Table 3.1. There were applied for quantitative measurement of each marker in HCE. R^2 of linear regression model was in the range of 0.999-1.00.

3.3.2 Precision and Accuracy

The intra-assay precisions were evaluated in triplicate over 3 days. The results presented as percentage of RSD (%RSD) which were 0.53-1.83% and 0.41-1.63% for rutin and quercetin, respectively (Table 3.2). The inter-assay precisions were investigated on the same set of data on the first day which revealed 0.53-1.04%RSD and 0.45-1.06%RSD for rutin and quercetin, resdpectively. The RSD values were less than 2.00% for both of rutin and quercetin.

The external standard measurements of HCE were calculated as rates of recovery which were 98.93-103.52% and 99.62-103.07% for rutin and quercetin, respectively.

Table 3.2 Recoveries of flavonoids from HCE over 3 days

% Add	day	Rutin			Quercetin		
		mean±s.d.	%RSD	% Recovery	mean±s.d.	%RSD	% Recovery
50	1	50.57±0.52	1.04	101.14±1.04	51.54±0.60	1.16	103.07±1.20
	2	51.76±0.95	1.83	103.52±1.90	51.01±0.83	1.63	102.02±1.66
	3	51.72±0.82	1.58	103.44±1.64	50.84±0.70	1.37	101.67±1.39
100	1	101.36±0.54	0.53	101.36±0.54	100.84±0.46	0.46	100.84±0.46
	2	101.11±1.71	1.69	101.11±1.71	100.54±0.41	0.41	100.54±0.41
	3	100.96±1.29	1.27	100.96±1.29	101.04±0.67	0.66	101.04±0.67
150	1	150.70±0.82	0.54	100.47±0.55	150.28±0.64	0.45	100.18±0.45
	2	148.39±1.82	1.23	98.93±1.21	149.43±0.80	0.55	99.62±0.53
	3	152.06±1.59	1.04	101.37±1.06	149.64±0.38	0.41	99.76±0.25

3.3.3 Limits of Detection (LOD) and Limits of Quantitation (LOQ)

Rutin and quercetin standard were prepared as dilutions to investigate the LOD and LOQ via signal-to-noise ratios for 3:1 and 10:1, respectively. The limits of detection were 0.00029 and 0.000646 mg/ml for rutin and quercetin, respectively. The limits of quantitation were 0.00109 mg/ml for rutin and 0.001777 mg/ml for quercetin.

3.4 Development of HCE Tablet Formulation

3.4.1 Calculation of HCE Tablet Formulation

HCE tablet formulations were calculated to compare with capsule forms, as shown below;

1. GPO Natureplex® in capsule form, a product of the Government Pharmaceutical Organization, composed *Borassus flabellifer, Houttuynia cordata, Randia siamensis, Combretum quadragulare* and *Mimusops elang*i.

- Total herbal extracts 65.3%
- HCE in Total herbal extract 7.69%
- A capsule 350 mg
- Dietary intake recommendation 2 capsule/day as 700 mg/day
- HCE intake per a day 35.16 mg/day

When a HCE tablet contained 40 mg or 80 mg HCE, this tablet was equivalent to 2 or 4 Natureplex® capsules, respectively.

2. HCP was loaded into 20 capsules (No.0) and determined the weight of loading. HCE content per a capsule were calculated base on 5.85% yield of extraction.

- Mean of loaded weight 278.455±1.99 mg/a capsule
- HCE (base on 5.85% of yield) 16.38 mg/a capsule

When a HCE tablet contained 40 mg or 80 mg HCE, this tablet was equivalent to approximately 2 or 4 HCP capsules, respectively.

3.4.2 Improvement of HCEP

Despite high ratio of extract-to-adsorbent, HCEP obtained from formulations 1 and 2 exhibited poor appearance and flow property. Formulation 2-5, HCE was not completely absorbed by corn starch as there was some remained HCE in the mortar, especially formulations 4 and 5. At low ratio in formulations 3-5, wetness was observed and production of powder was difficult. So this mixture substance was pre-granulation. Corn starch is known to be a principle excipient for oral solid dosage form and a generally absorbent in pharmaceutical products (Rowe *et al*., 2003), with adsorption ability resulting from the interaction between free hydroxyl groups (OH) in glucose unit and water molecules via hydrogen bond (Beery and Ladisck, 2001). However, this interaction occurred only on the surface (van den Berg *et al*., 1975; Beery and Ladisck, 2001) and thus, a high amount of corn starch was required to obtain HCEP with proper characteristics. In contrast, formulations 6 and 7 which utilized Prosolv® as adsorbent showed better results and showed a good appearance as

powder (Figure 3.8). Both of formulations 6 and 7, HCE did not remain in sorption process. Formulation 6 was the least ratio of HCE on adsorbent to completely adsorb. This is in agreement with a report by Rowe *et al*. (2003) which suggested the use of Prosolv® as filler for both capsule and tablet forms to improve the compressibility in wet-granulation and direct compression. Prosolv® was obtained from silicification of 2% colloidal silicon dioxide (CSD) and 98% microcrystalline cellulose (MCC). Although its polymorphism, porosity and particle size were not different from those of MCC (Tobyn *et al*., 1998; Luukkonen *et al*., 1999), the surface area of Prosolv® was five times higher than that of MCC. Prosolv® was a more effective adsorbent than corn starch because CSD on Prosolv® possessed high affinity sorption sites (Kachrimanis *et al*., 2000). The surface area of CSD in Prosolv® was at 50-380 m^2/g (BET method) while that of corn starch was 0.41-0.43 m^2/g. In addition, corn starch was insoluble in both cold water and cold 95% ethanol while Prosolv® was soluble in water, organic solvent and acid (Rowe *et al*., 2003). As a result, less amount of Prosolv® was required to obtain similar HCEP compared to the use of corn starch. This allowed the formulation of smaller size tablet with the same or higher amount of HCE. Formulation 6 and 7 were collected to tabulating formula process, although the flowability remained poor and required further improvement in the formulation process.

Table 3.3 Properties of HC granules/powder through adsorption

Formulations	Properties
1	powder, poor flowability, light-green
2	powder, very poor flowability, green
3	dried granules, Soft, very poor flowability, dark green
4	dried granules, soft, very poor flowability, dark green
5	dried granules, hard, passable flowability, dark green
6	powder, poor flowability, dark green
7	powder, poor flowability, green

Remark: Absorbent of formulation 1-5 was corn starch and 6-7 was prosolv®

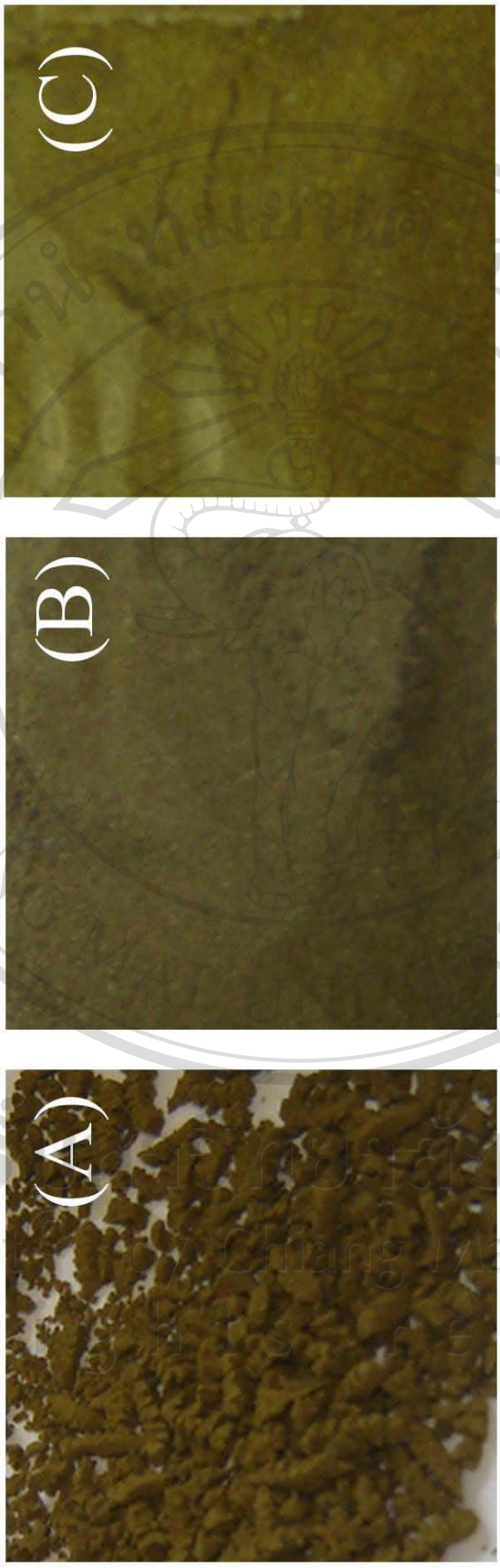

Figure 3.8 HCE with absorbent as granules of formulation 4 (A) and powder of formulation 6 (B) and formulation 7 (C)

Table 3.4 Hardness of HCEP/granules as tablets for 3 formulations

Formulation	Pressure (T)	Hardness (N)
4	1	Not applicable (due to tablet softness)
	2	Not applicable (due to tablet softness)
6	1	53.95±8.84
	2	48.60±10.64
7	1	32.80±3.11
	2	30.10±0.00

mean±s.d.

HCEP/granule from formulation 4, 6 and 7 were compacted and their hardness was shown in Table 3.4. Formulation 4, tablets were immediately broken when plunger of hardness tester touched, after that the plunger stopped instantly. Thus, the hardness was reported as “not applicable” because it was too weak. This weakness was due to the use of corn starch as adsorbent. The hardness of pure Prosolv® was too high; the tablets did not break during testing (data not shown). Tablets of formulation 6 were harder than those of formulation 7 because of higher ratio of Prosolv®. While the hardness was not different between compression forces of 1 and 2 tons. The tablets of formulation 6 and 7 were selected to HCE tablet developments.

3.4.3 HCE Tablet Formulations

Hardness of HCE tablets from formulation 1-4 that HCEP composed of HCE:Prosolv® ratio 3:1 were harder than there from formulation 5-8 that HCEP composed of HCE:Prosolv® 2:1 so result assumed that Prosolv® can be increased the hardness. According to Table 3.5, HCE tablets form formulation 5-8 were not acceptable for hardness but bulk powder from formulation 8 was good flowability with visible observation which optimized for direct compression. Formulation 9 was created though based on formulation 8 with higher ratio of Prosolv® in HCEP as 4:1. Although formulation 9 had more Prosolv®, the HCE in an obtained tablet was still higher to approximately 2 capsules of GPO Natureplex product or HCP capsules.

HCE tablets from almost formulations disintegrated within 15 minute except these from formulation 4 cleared only 16 from 18 tablets, perhaps tablets from formulation 4 was Aerosil® and high Prosolv® which could be decreased disintegration although Explotab® quickly absorbed water.

Friability of tablets from formulation 6-8 was not over 0.1% that acceptable while tablet from formulation 9 reported in negative value that mean there were absorbed moisture during testing. The properties of tablets from formulation 9 were satisfactory although those were moistening in testing condition. Thus this problem could prevent and solve in the future study or commercial production.

Because of poor flow and compression properties of HCEP, the pharmaceutical excipients were required to enhance powder properties to suitable for direct compression. These applied excipients were chemically inert, causing neither interaction with nor decomposition to the active compounds in the extract (Jivraj *et al*., 2000). In the formulation, MCC (Avicel PH101®) was used as

binder/disintegrant. A study by Palma *et al*. (2002) on a formulation of tablet from plant extract showed that Avicel PH101® facilitated the disintegration of tablets. Dibasic calcium phosphate (Emcompress®), with a good flowability and compact property was utilized as glidant (Rowe *et al*., 2003). Magnesium stearate served as lubricant and antiadherent (Eilalifa *et al*., 2009). Sodium carboxymethyl starch (Explotab®) or crosslinked sodium carboxymethylcellulose (Ac-Di-Sol®) was employed as superdisintegrant. Purified talcum possessed lubricating and antiadherent properties. CSD (Aerosil 200®) was used as glidant and antiadherent to improve flowability and content uniformity of pre-compressed powder (Gierer, 2002; Rowe *et al*., 2003; Teng *et al*., 2009). A decrease of Avicel PH101® and increases of purified talcum and addition of Aerosil 200® in formulation 4, resulted in tablets with higher hardness and longer disintegration time (>15 min) than other formulations. Tablets of formulations 8 and 9 appeared to have acceptable hardness, disintegration time (<15 min) and friability (<1%) (USP25/NF18). The properties of formulated tablets are compiled in Table 3.5.

Table 3.5 Hardness, disintegration times and friability of each formulation tablets

Formulation	Hardness (N)	Disintegration times (min)	Friability (%)
1	47.28±5.39	2.55	Unmeasured
2	31.72±2.56	0.35	Unmeasured
3	54.37±2.75	1.52	Unmeasured
4	97.06±8.53	>15	Unmeasured
5	21.61±2.62	1.02	Unmeasured
6	23.76±3.36	0.45	0.06
7	14.42±1.50	0.18	0.22
8	22.15±1.70	6.32	0.08
9	57.98±7.60	2.18	-0.04

3.5 Quality Controls

3.5.1 Quality controls of HCEP and bulk powder

Investigation of flow characteristic from Bulk density and tapped density of bulk powder showed in Table 3.6. The moisture content of bulk powder was 37.6% lower that of HCEP because bulk powder composed of HCEP and the low moisture excipients. High moisture content of herbal tablets reported to associate with the growth of microorganisms and possibility of degradation of active compounds (Sitthichai, 2004). Lower moisture content also contributed to the improvement of the flow property as evidenced by a significant decrease (p<0.05) in the angle of repose. The compressibility ratio of bulk powder was within a range (5-12%) that suggested excellent flowability. The properties of this bulk powder appeared to be suitable for direct compression (Jivraj *et al*., 2000). SEMs of Prosolv® and HCEP are shown in Figure 3.9-3.11. The appearances of HCEP after adsorption process were similar with that of Prosolv®.

Table 3.6. Quality controls of HCEP and bulk powder

Test	HCEP	Bulk powder
LOD (%)	5.29±0.01[a]	3.30±0.01[b]
Bulk density	-	0.398
Tapped density	-	0.431
Compressibility ratio	-	7.609
Repose angle (°)	46.20±4.80[a]	28.10±2.60[b]

[a, b] mean±s.d. in a same row with different letter are significantly different (p < 0.05)

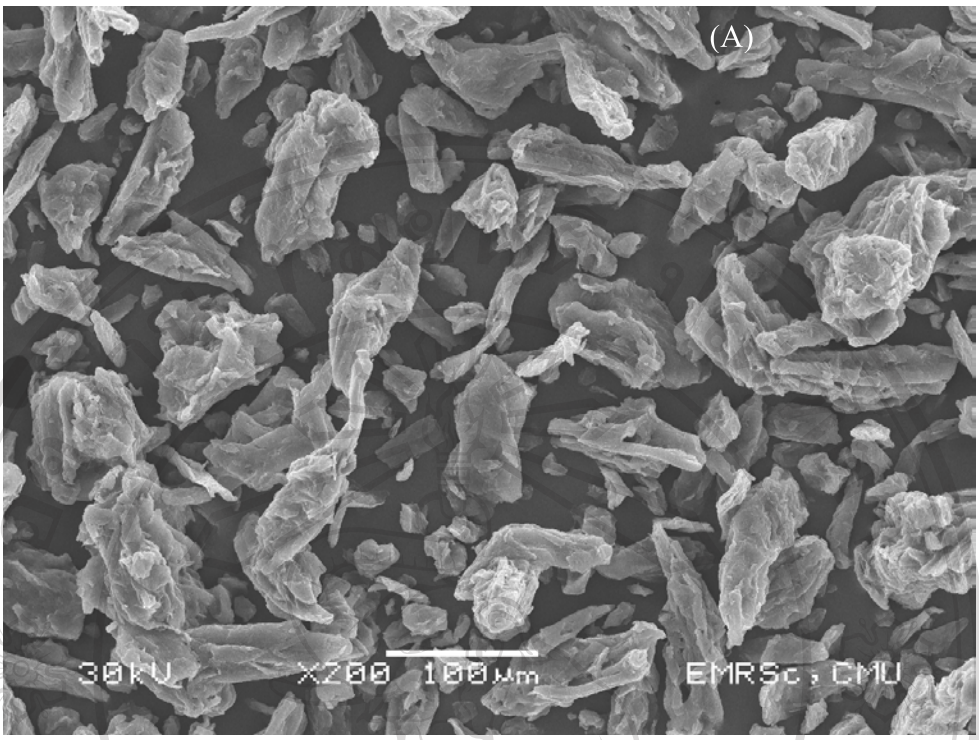

Figure 3.9 SEM of Prosolv®

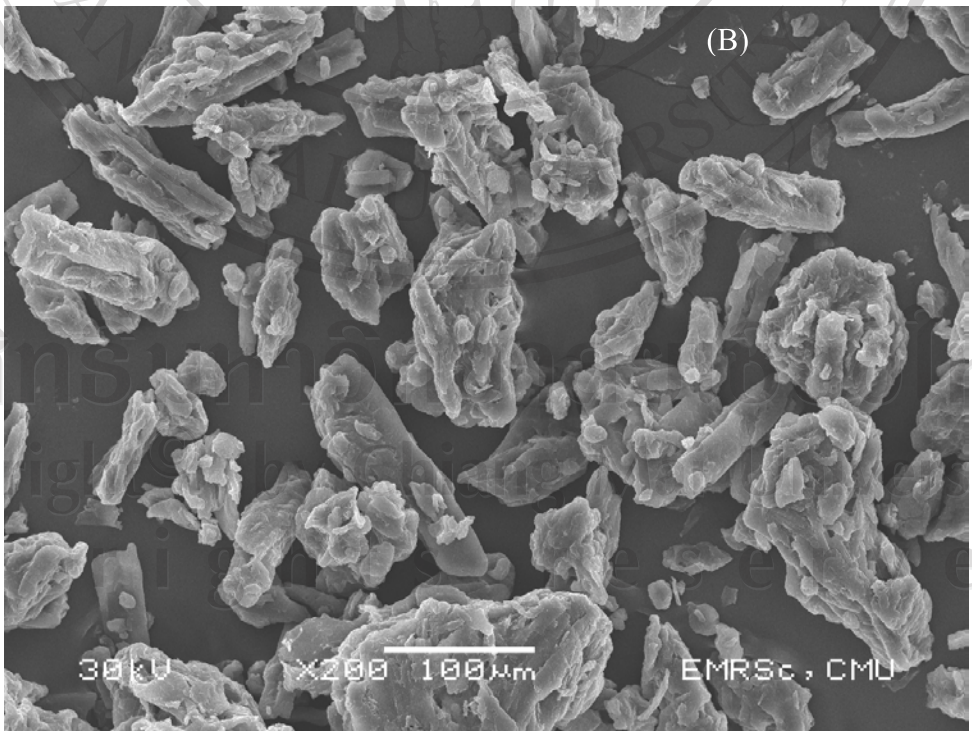

Figure 3.10 SEM of Prosolv®:HCE ratio as 1:2

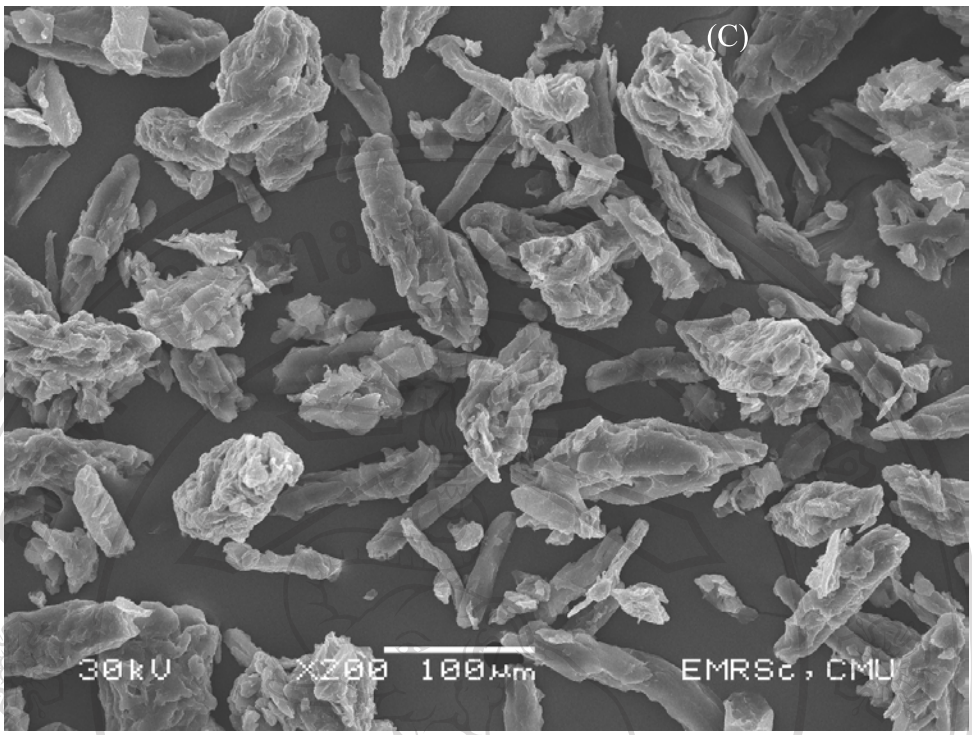

Figure 3.11 SEM of Prosolv®:HCE ratio as 1:8

3.5.2 Quality controls of HCE tablets

HCE tablets from hydraulic press

HCE tablets from hydraulic press showed good appearance (Figure 3.11). Weight variation of HCE tablets was within the range of 231.25-268.75 mg (USP 25/NF 18), thus ensured the consistency of the amount of active compounds of HCE tablets. Tablet dimension was not deviated while thickness was between 4.56-5.04 mm (USP 25/NF 18). The hardness of tablets was acceptable when the friability was less than 0.5-1.0% and the disintegration time was less than 15 minutes (USP25/NF18). The negative value of friability caused by moisture adsorption during friability test can be solved by several methods such as tablet coating, blister packaging etc. All parameters are important in commercial process and influence the quality and shelf-life of the products (Sitthichai, 2004). These results expressed that HCE tablets were good properties according tablet requirement for food supplement.

Figure 3.12 HCE tablets by hydraulic press

Table 3.7 Quality controls of HCE tablets by hydraulic press

Weight (mg)	Dimension (mm)	Thickness (mm)	Hardness (N)	Friability (%)	Disintegration time (min)
250.26±0.73	8.51±0.01	3.40±0.01	41.50±3.20	-0.04	1.23

HCE tablets from single stroke tabletting machine

Tablets showed a good appearance although there were some blotches on tablet surface (Figure 3.13) which caused by moisture attraction on surface. The results of quality controls are presented in Table 3.8. Weigh variation was fluctuated, resulting in a high standard variation. This probably caused by the electrical charging and/or moisture adsorbing of excipients in the compression process. The hardness was acceptable also the friability was less than 0.5%. Also the disintegration time was less than 15 minutes according to tablet drug requirement (USP 25/NF 18).

Figure 3.13 HCE tablets by single stroke tabletting machine

Table 3.8 Quality controls of HCE tablets by single stroke tabletting machine

Weight (mg)	Hardness (N)	Disintegration time (min)	Friability (%)
253.34±5.36	26.19±3.01	0.10	-0.22

3.6 Stability of HCE

3.6.1 Stability of HCEP and bulk powder

Under both 45°C and RT storage conditions, storage time affected the moisture content of HCEP and bulk powder. The longer the storage time, the lower the moisture content (Table 3.9). Compared to the value on 0 month, % LOD of HCEP kept for 3 months at 45°C and RT decreased 58.6 and 59.7%, respectively. The trend also applied to the bulk powders, in which the decreases were 33.6 and 37.6% for the 3-month storage at 45°C and RT, respectively. The appearances of the HCEP and bulk powder remained unchanged. In the 1st month period, the rate of moisture evaporation was the highest especially at 45°C. This was because HCEP and bulk powder exposed to a relatively high temperature, thus a rapid transfer of moisture within the system occurred to balance the temperature. As the temperature of the samples were in equilibrium with the storage system, the rate of moisture evaporation decreased (2nd and 3rd month) (Sirithunyalug *et al*., 2008).

Table 3.9 Moisture contents of HCEP and bulk powder

Conditions		HCEP	Bulk powder
	0 Mo	5.29±0.00	3.30±0.00
45°C	1 Mo	2.71±0.00	2.64±0.01
	2 Mo	2.43±0.01	2.50±0.01
	3 Mo	2.19±0.00	2.19±0.00
RT	3 Mo	2.13±0.00	2.06±0.00

3.6.2 Stability of HCE tablets

Quality controls of HCE tablets after stability test are presented in Table 3.10. Compared to the data of 0-month tablets, the weights of tablets were significantly different as the storage time extended. Weight of RT kept tablets decreased less than the 45°C kept storage after 3 month-storage. The decrease of HCE tablet weight was caused of losing moisture content which was supported by the decline of moisture contents in HCEP and bulk powder stability tests (Table 3.9). Dimensions of tablets were not differences until 3 month-storage there were significantly decreasing both 45°C and RT conditions. The thickness of HCE tablets significantly decreased at 1st-month keeping and then there was constant at 2nd- and 3rd-month keeping. The results exploded that thickness inversely correlated with the hardness. The hardness of HCE tablets significantly decreased after one month of storage then continued to slowly and significantly decrease as the storage time extended on three months of storage. The hardness of tablets stored at 45°C affected to the storage time while the RT condition at three months of storage did not affect to hardness of HCE tablets. These were reflected to the friability properties. Friability values of HCE tablets decreased on 45°C condition while RT condition there was not different at 3rd- month storage. Disintegration time of HCE tablet slightly decreased at 45°C condition and distinctly increased at RT condition for tree month storage that reasoned hardness.

Table 3.10 Quality controls of HCE tablets after stability test

Conditions		Weight (mg)	Dimension (mm)	Thickness (mm)	Hardness (N)	Friability (%)	Disintegration time (min)
	0 Mo	250.26±0.73[a]	8.51±0.01[a]	3.40±0.01[a]	41.50±3.20[a]	-0.04	1.23
45°C	1 Mo	246.96±1.17[b]	8.52±0.01[a]	3.43±0.02[b]	37.50±2.10[b]	0.01	1.20
	2 Mo	246.13±1.48[c]	8.51±0.01[a]	3.43±0.01[b]	36.50±1.00[b]	-0.01	1.25
	3 Mo	245.76±1.10[c]	8.50±0.00[b]	3.43±0.02[b]	34.70±1.00[c]	-0.20	1.02
RT	3 Mo	246.84±1.22[b]	8.49±0.02[b]	3.37±0.01[c]	44.90±4.20[a]	-0.07	2.18

[a, b, c] mean±s.d. in a same column with different letter are significantly different ($p < 0.05$)

Stability of chemical components in HCE tablets was tested by TLC analysis using rutin and quercetin as standard markers. Tablet extracts showed TLC profiles that were practically identical to that of the HCE, at both RT and 45°C during the 3-month storage, suggesting that no degradation or decomposition of the extract components occurred (Figure 3.11). This is in part due to the elimination of solvent from the extract to prevent chemical reaction and the minimization of moisture content in the adsorption step which prevented the growth of microorganisms that could lead to fermentation and chemical decomposition (Sitthichai, 2004). The results also verified the compatibility between the chemical components in the extract and the excipients used in the formulation of tablets.

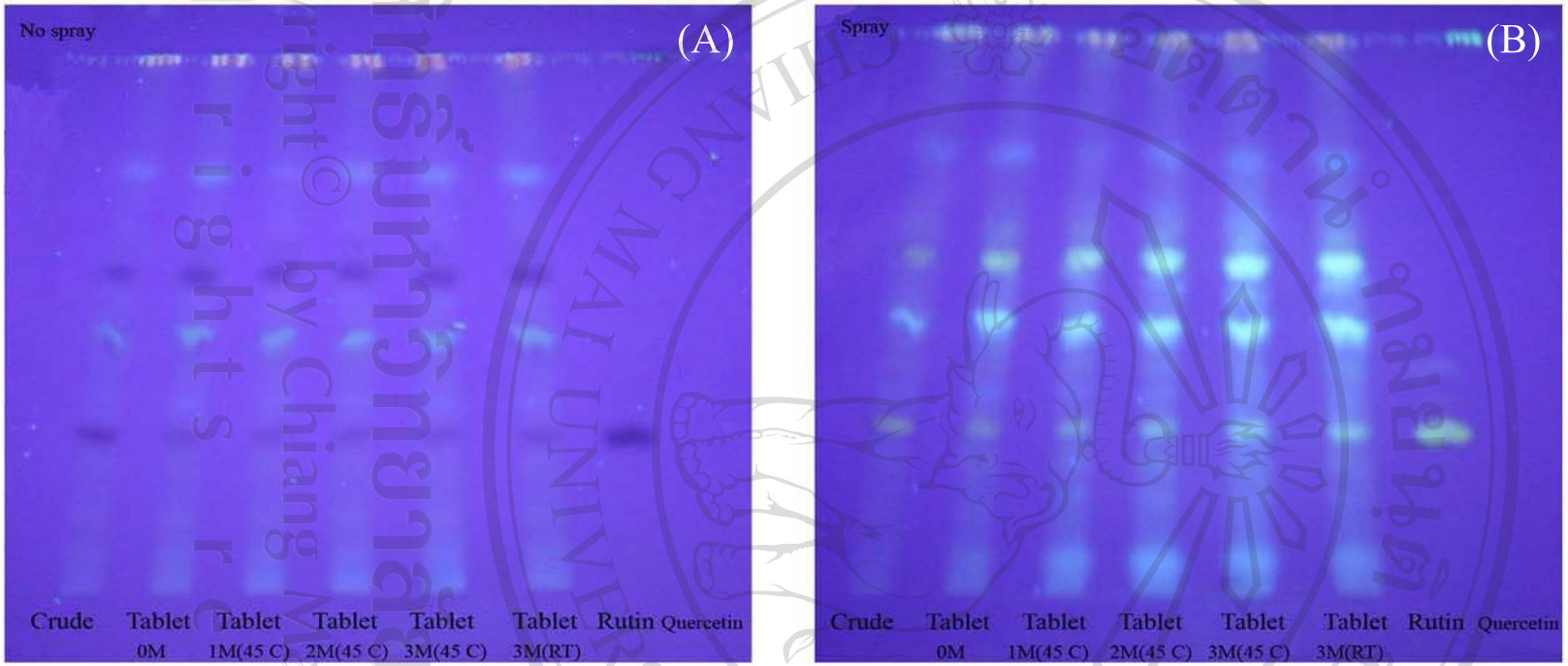

Figure 3.14 TLC chromatograms of HCE tablet extracts, compared with crude extract and standards. Chromatograms are visualized under UV-254 nm (A) and UV-365 nm (B). Lane 1: crude HCE, Lane 2: HCE tablet extract 0th Mo at 45°C, Lane 3: HCE tablet extract 1st Mo at 45°C, Lane 4: HCE tablet extract 2nd Mo at 45°C, Lane 5: HCE tablet extract 3rd Mo at 45°C, Lane 6: HCE tablet extract 3rd Mo at RT, Lane 7: rutin standard, Lane 8: quercetin standard.