# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

### Chemicals

1. Denature ethyl alcohol 96% (DEB 96, Hong Huat Co., Ltd, Thailand)
2. Methanol (AR grade) (Lab-Scan, Thailand)
3. Hexane (AR. grade) (Mallinckrodt Chemical, U.S.A.)
4. Chloroform (AR. grade) (Lab-Scan, Thailand)
5. Ethyl acetate (AR. grade) (Lab-Scan, Thailand)
6. Ethanol (AR. grade) (Lab-Scan, Thailand)
7. Methanol (HPLC grade) (Lab-Scan, Thailand)
8. Petroleum ether (bp 40-60°C) (Mallinckrodt Chemical, U.S.A.)
9. Formic acid 98/100% (AR. grade) (Fisher Scientific, U.K.)
10. Vanillin (Fluka, Germany)
11. Phosphoric acid (Lab-Scan, Thailand)
12. Ethyl ether (Lab-Supplies, Thailand)
13. Aluminiunchloride krist. Reinst ($AlCl_3 \cdot 6H_2O$) (Lab-Scan, Thailand)
14. Distilled Ionized water
15. Silica gel 60 $GF_{254}$ (Merck, Germany)
16. TLC silica gel $GF_{245}$ aluminium sheet 20×20 cm (Merck, Germany)
17. Rutin (Fluka, Germany)

18. Quercetin Anhydrous (Fluka, Germany)
19. Geraniol 99% (Acros, U.S.A.)
20. Corn starch
21. Silicified microcrystalline cellulose 90 (Prosolv®, SMCC 90, JRS Pharma LP., Germany)
22. Microcrystalline cellulose (Avicel® pH 101, JRS Pharma LP., Germany)
23. Sodium Carboxymethyl Starch (Explotab®)
24. Crosslinked Sodium Carboxymethylcellulose (Ac-Di-Sol®)
25. Purified talcum
26. Magnesium stearate
27. Silicon dioxide (Aerosil 200®, Evonik Degussa Gambit, Germany)
28. Dibasic Calcium Phosphate (Emcompress®)
29. Hard gelatin capsule No. 0

**Apparatus**

1. Drying oven (HA-20, Kan Seng Lee Mechinery (1960) Ltd. Part., Thailand)
2. Cutting miller (Nippon Takin Kugro Co., Ltd, Japan)
3. Rotating vacuum Evaporator (Heidolph WB, type LABORTA 4001,)
4. Vacuum pump (MEDI-PUMP, THOMAS Industries, U.S.A.)
5. Cooling source (EYELA cool ACE type CA-111, Japan)
6. Whatman filter no.1, 150mm diameter (Whatman®, England)
7. Ultraviolet spectrophotometer (Milton Roy Spectronic 1001 plus, U.S.A.)
8. High Performance Liquid Chromatography (HPLC) instrument (HPLC-1100, Agilent Technology, U.S.A.)

9. Chromatographic column (Alltech, Apollo C18 column, U.S.A.)
10. Analytical balance (Scaltec type SBC 31 (0.01g-220g), Germany)
11. Electronic balance (Sartorius type LA 230 s, Germany)
12. Hot air oven (BINDER ED240/E2, Germany)
13. Sieve (U.S.A. standard testing sieve no. 80, U.S.A.)
14. Moisture balance (Sartorius type MA 50, Germany)
15. Jolting volumeter (J. Engelsmann AG, Germany)
16. Hydraulic press (Carver Laborotory Press type C, U.S.A.)
17. Single stroke tabletting machine (Charatchai Machinery Ltd. Part., Thailand)
18. Erweka tester (ERWEKA® type TBH 100, Germany)
19. Roche friabilator (Pharma Test ® type PTF 20 E, Germany)
20. USP Disintegration tester (Pharma test® type PTZ-AUTO 3, Germany)
21. Environmental testing chamber (TSE SANYO®, Japan)
22. Micropipette (Gilson, 0-20 µl, 0-100µl and 0-1000 µl, France)
23. Vibratory Capsulate filter machine (PANVIV. A01 M. No.653, Thailand)
24. Digital micrometer
25. Scanning electron microscope (SEM JEOL JSM-5910LV, France)
26. Stand and clamp
27. Thermometer
28. Dedicator
29. Mortar and pestle
30. Glass tubes and racks
31. Buchner funnel, Volumetric flasks, beakers and cylinders

**Plant Material**

1. Fresh HC (Sun-Phi-Sua, San Sai, Chiangmai)

## 2.2 Methods

### 2.2.1 Plant Material and Identification

Fresh HC was obtained from Sun-Phi-Sua, San Sai, Chiang Mai in August-October 2008. The identity of the plant was authenticated and a herbarium voucher specimen was prepared and deposited at the Faculty of Pharmacy, Chiang Mai University.

### 2.2.2 Extraction and Preparations

**Extraction of HC**

All HC plant was dried in drying oven.

↓ At temperature 50°C for 48 hours.

Dried HC was milled with cutting miller

↓

Dried HCP was macerated in 95% ethanol (plant:solvent 1:10).

↓ For 20 hours a time (3 times).

Obtainable solvent was filtered with whatman® no.1 filter papers.

↓

Filtrate was concentrated with a vacuum evaporator.

↓ At temperature 50°C,

↓ rotating 90 rounds per minute (rpm).

Concentrated HCE for whole study

**Preparation of HCE**

32 ml methanol was added into 1 g of concentrated HCE as harmonious mixture of 0.03125 g/ml HCE for chromatography.

**Preparation of HCE by Liquid-Liquid Partition**

The concentrated HCE has many important active compounds which may be interfere one another. It was appropriated to separate their compound through their polarity property and like-dissolve-like property, so liquid-liquid partition technique was applied (Figure 2.1). Each of fractions (F1-F4) was removed solvent out and diluted with methanol for chromatographic analysis.

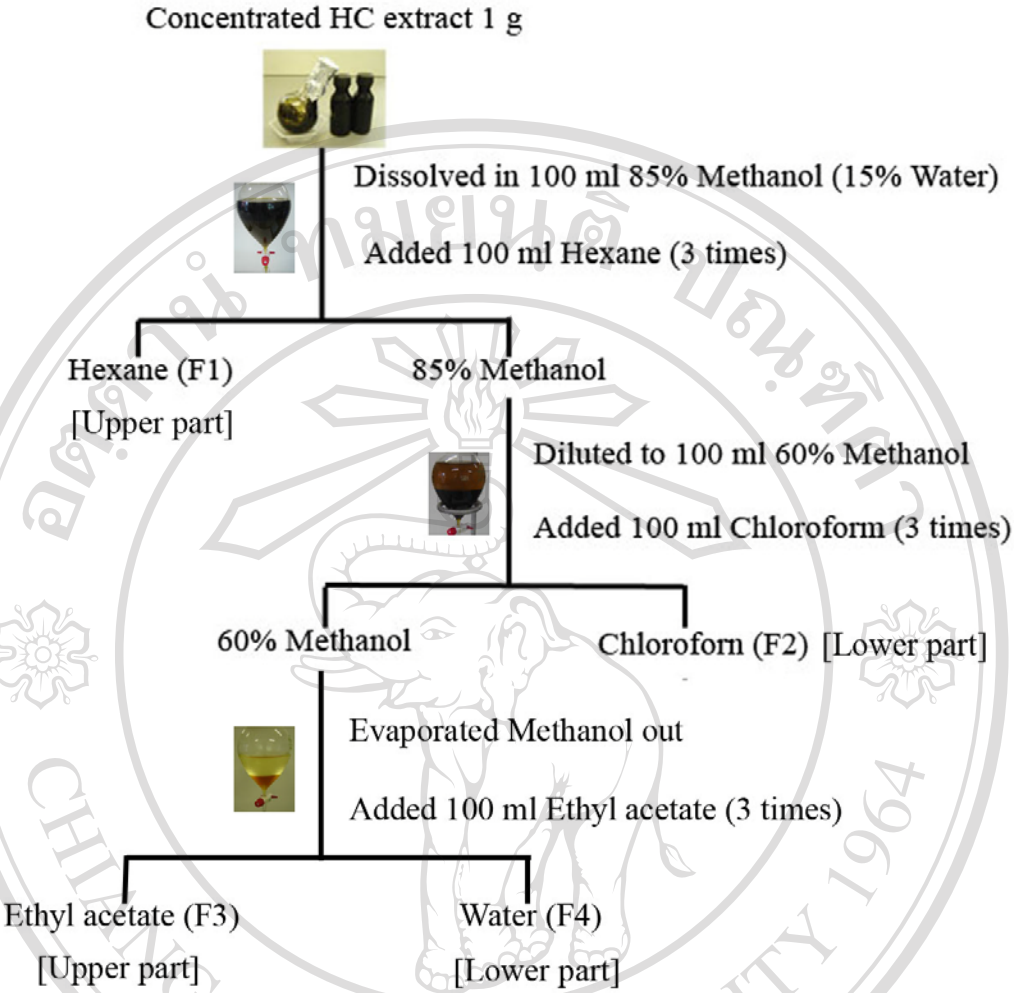



**Figure 2.1** Liquid-liquid partition chart of concentrated HCE as 4 fractions (F1-F4)

### 2.2.3 Identifications of HCE with TLC

**TLC Terpene Test**

***Preparation of Solvents and Standard for Terpene Test***

- Standard terpenes:0.2% geraniol standard in methanol (v/v)
- Sample: HCE, F1, F2, F3 and F4
- Developing solvent (DVS): petroleum ether (bp 40-60°C):ethyl Acetate:Formic acid ratio [47:2:1]
- Spray reagent: Vanillin-Phosphoric acid solution which was provided from 1 g vanillin, 25 ml ethanol, 25 ml water and 35 ml phosphoric acid.

***TLC for Terpene Test***

DVS was filled into chromatographic tank

↓

Space over DVS was full of solvent vapor and equilibrated for 1 hour.

↓

Samples: HCE, F1, F2, F3, F4 and standard terpenes were spotted on Silica gel 60 $GF_{254}$ TLC aluminium sheets with 1 cm spaces between each sample.

(2 TLC sheets as control and tester)

↓

Sheets were rested to dry and placed into chromatographic tank.

↓

DVS run to 15 cm solvent front.

↓

TLC aluminium sheets were taken out and dried.

↓

A TLC sheet was sprayed with Vanillin-Phosphoric acid

↓

It was placed into Hot air oven at 120°C for 10 minutes.

A TLC sheet was not spray

↓

Both were observed to identify terpenes compounds from HCE.

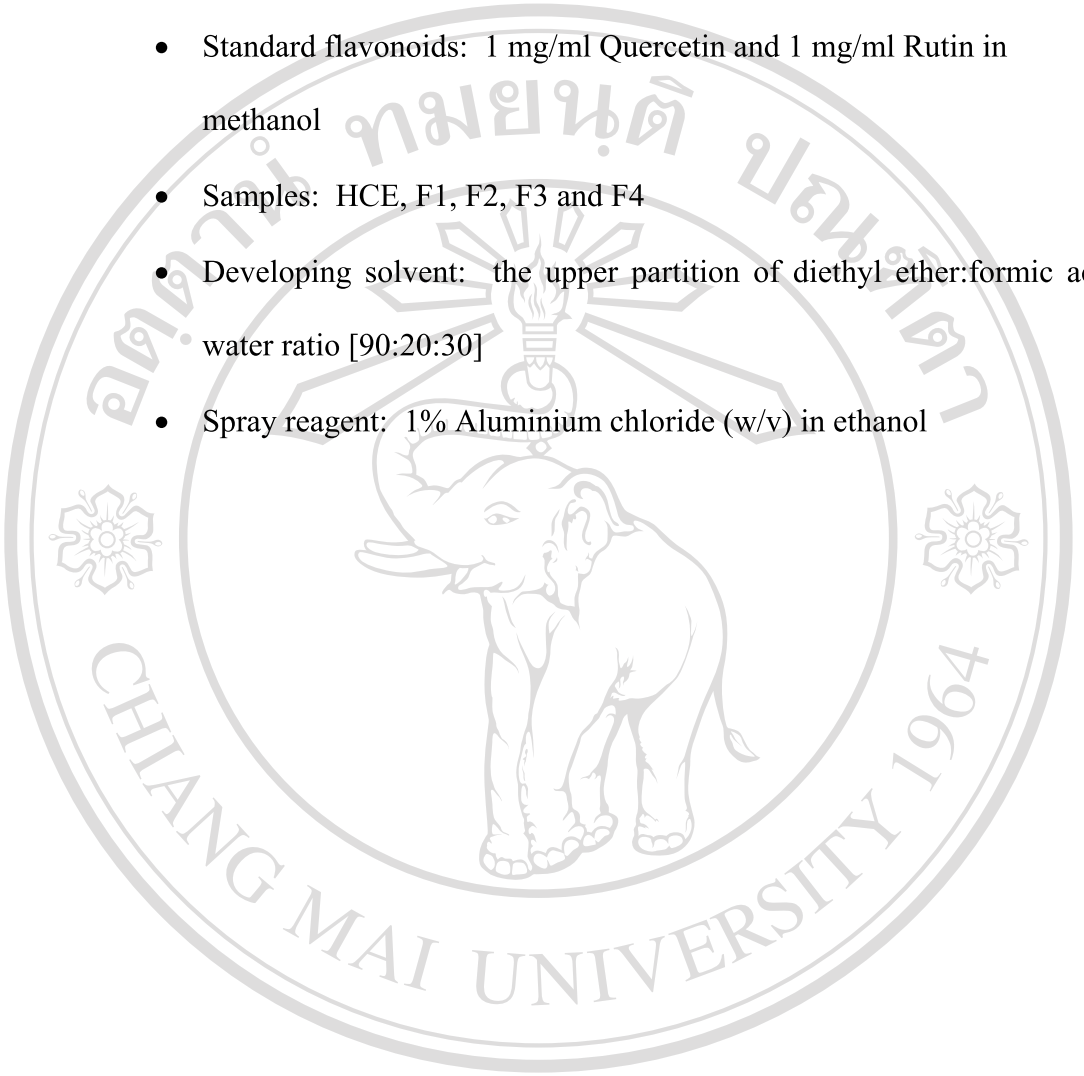
Observation condition: Visible light

Ultraviolet (365 nm)

**TLC Flavonoid Test**

***Preparation of Solvents and Standard for Flavonoid Test***

- Standard flavonoids: 1 mg/ml Quercetin and 1 mg/ml Rutin in methanol
- Samples: HCE, F1, F2, F3 and F4
- Developing solvent: the upper partition of diethyl ether:formic acid: water ratio [90:20:30]
- Spray reagent: 1% Aluminium chloride (w/v) in ethanol



***TLC for Flavonoid Test***

DVS was filled into chromatographic tank

↓

Space over DVS was full of solvent vapor and equilibrated for 1 hour.

↓

Samples: HCE, F1, F2, F3, F4 and standard terpenes were spotted on Silica gel 60 $GF_{254}$ TLC aluminium sheets with 1 cm spaces between each sample.

(2 TLC sheets as control and tester)

↓

Sheets were rested to dry and placed into chromatographic tank.

↓

DVS run to 15 cm solvent front.

↓

TLC aluminium sheets were taken out and dried.

↓

A TLC sheet was sprayed with 1% Aluminium chloride

A TLC sheet was not spray

↓

Both were observed to identify flavonoids compounds from HCE.

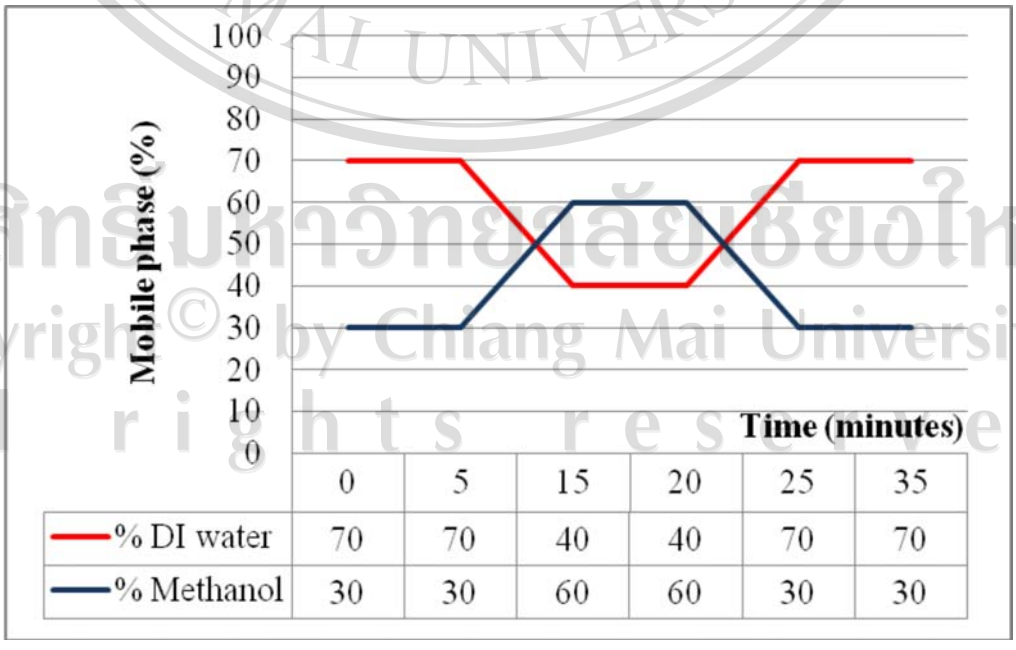
Observation condition: Visible light

Ultraviolet (365 nm)

### 2.2.4 Identification of HCE with HPLC

Samples and standard flavonoids were measured through Agilent technology HPLC series 1100. HCE was identified flavonoids compounds via peak characteristics and retention times of flavonoids makers as standard rutin and standard quercetin were compared with these in HCE.

- Standard flavonoids: Quercetin 1 mg/ml and Rutin 1 mg/ml
- Sample: HCE 0.025 g/ml, F1, F2, F3 and F4
- Injection volume: 10 µl
- Mobile phase: methanol: DI water [Gradient HPLC]
- Flow rate: 1.00 min/ml
- Pressure: 0-250 Bar
- Column: Alltech, Apollo C18 5u, 4.6 mm x 150 mm
- Wave length: 350 nm (UV-Visible HPLC)




| | 0 | 5 | 15 | 20 | 25 | 35 |
|---|---|---|---|---|---|---|
| % DI water | 70 | 70 | 40 | 40 | 70 | 70 |
| % Methanol | 30 | 30 | 60 | 60 | 30 | 30 |

**Figure 2.2** Mobile phase ratios for gradient HPLC

### 2.2.5 Method Validations

**Calibration curves**

Two flavonoids; rutin and quercetin at five concentrations were measured using HPLC analysis and the linear calibration curves were created though the known concentrations of standard flavonoids as Y axis (vertical axis) and peak areas of each concentration of absorbance spectrum as X axis (horizontal axis). Concentrations' standards of measurement depended on theirs in HCE so the concentrations for testing were multiple as 0.125-2.000 mg/ml and 0.009375-0.15 mg/ml for rutin and quercetin consecutively.

**Precision and Accuracy**

The method reproducibility was investigated in virtue of 50%, 100% and 150% concentrations of each marker in HCE were spiked into 0.025 g/ml HCE. The inter-assay precision were analyzed triple over 3 days and data of the first day were investigated the intra-assay precision. In additional the same sets of samples were examined on rate of recovery as accuracy.

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

The limit of detection and the limit of quantitation were tested on a signal-to-noise ratio of 3:1 and 10:1 respectively for the least quantitative detection.



### 2.2.6 Development of HCE Tablet Formulations

**Calculation of HCE Tablet Formulations**

Calculation of HCE tablets was compared with GPO NaturePlex® capsules and HCP capsules which based on percentage of extract and reported as mg per a tablet.

1. GPO Natureplex® in capsule form has been copyright of Government Pharmaceutical and was composed *Borassus flabellifer* Linn., *Houttuynia cordata* Thunb., *Randia Saimnsis* Craib., *Combretum quadragulare* Kurz. and *Minmusops elegy* Linn. A capsule was filled 350 mg which consisted in 65.3% total extract. HCE was 7.69% of total extract.

2. HCP was loaded into 20 capsules (No.0) and determined mean of loaded weight to estimate HCE that base on percentage of yield of extraction as 5.85%.

**Improvement of HCEP**

As a result of HCE was consistency or high viscosity, direct compression was so difficultly applied to table form. HCE was appropriately adapted as powder and performed with direct compression, respectively. This section focused on absorbents: corn starch and Prosolv® (SMCC 90) which completely absorbed at least ratio and optimized for HCE formulation.

**Table 2.1** Ratios of HCE and absorbent

| Formulation | HCE:absorbent | | | | | | |
|---|---|---|---|---|---|---|---|
| Absorbents | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Corn starch | 1:21 | 1:12 | 1:8 | 1:3 | 1:1 | - | - |
| Prosolv® | - | - | - | - | - | 1:2 | 1:3 |

Ratios of HCE and absorbents were calculated.

↓

Absorbents were sieved and weight also HCE were weighted following table2.1

↓

Adsorbent was mixed HCE to HCEP

by Tritulation method and Geometric dilution technique

↓

High moisture and mold ability mixtures

↓

Mixtures were sieved (12 mesh) to granules

Low moisture and unable mold mixtures

↓

Mixtures were dehydrated at 50 °C for 20 hours in hot air oven.

↓

Mixtures were weighted and observed.

↓

The appropriable formulation was selected.

↓

250 mg of HCEP for a tablet were compressed with 1 ton and 2 tons pressure via hydraulic press.

↓

Tablets were measured hardness by Erweka tester (ERWEKA® type TBH 100)

↓

Their data were evaluated for tablet formulation

**HCE Tablet Formulations**

The optimistic mixed compound from (2.2.6) which was the best property powder was an initial HCEP for tablet formulations.

**Table 2.2** Development of HCE tablet formulations

| Formulations | HCEP | Avicel pH101® | Emcompress® | Purified Talcum | Magnesium Stearate | Ac-Di-Sol® | Explotab® | Aerosil 200® |
|---|---|---|---|---|---|---|---|---|
| | Substance ratios (%) | | | | | | | |
| 1 | 80.0 | 16.0 | 1.6 | 2.0 | 0.4 | - | - | - |
| 2 | 80.0 | 16.0 | - | 2.0 | 0.4 | 1.6 | - | - |
| 3 | 80.0 | 16.0 | - | 2.0 | 0.4 | - | 1.6 | - |
| 4 | 80.0 | 12.0 | - | 4.0 | 0.4 | - | 1.6 | 2.0 |
| 5 | 80.0 | 16.0 | - | 2.0 | 0.4 | - | - | 1.6 |
| 6 | 80.0 | 13.6 | - | 2.0 | 0.4 | 1.6 | - | 2.4 |
| 7 | 80.0 | 16.0 | - | 2.0 | 0.4 | - | 1.6 | - |
| 8 | 80.0 | 12.0 | - | 4.0 | 0.4 | - | 1.6 | 2.0 |
| 9 | 80.0 | 12.0 | - | 4.0 | 0.4 | - | 1.6 | 2.0 |

*Remark*: Ratio of HCE:Prosolv® were 3:1, 2:1 and 4:1 for formulation 1-4, 5-8 and 9 respectively.

All substances except HCEP and Aerosol 200® were sieved and weighted according to Table 2.2.

↓

Avicel® pH 101, Purified Talcum, Emcompress® or Ac-Di-sol® or Explotab®, Magnesium stearate and Aerosil 200® was respectively tumbled with HCEP to consistency.

↓

250 mg of bulk powder were compacted at 1 ton pressure via hydraulic press.

↓

Tablets were evaluated weight variations, friability test by Roche friabilator (Pharma Test® type PTF 20 E), hardness by Erweka tester (ERWEKA® type TBH 100) and disintegrating time by USP disintegrating tester (Pharma test® type PTZ-AUTO 3).

The optimistic formulation was evaluated and selected to reproduce for this study. All processes there were initial herbal plant, HCE, HCP, bulk powder and HCE tablets were qualitatively controlled.

**HCE Tablets Compression**

A suitable formulation was collected and pressed by hydraulic press and single stroke tabletting machine.

**Table 2.3** HCE tablet formulations

| Substances | Ratio (%) |
|---|---|
| HCEP | 80.0 |
| Avicel pH101® | 12.0 |
| Purified talcum | 4.0 |
| Magnesium Stearate | 0.4 |
| Explotab® | 1.6 |
| Aerosil 200® | 2.0 |

***HCE Tablets by Hydraulic Press***

All substances except HCEP and Aerosol 200® were sieved and weighted following Table 2.3

↓

Avicel® pH 101, Purified Talcum, Explotab®, Magnesium stearate and Aerosil 200® was respectively tumbled with HCEP to consistency.

↓

250 mg of bulk powder were compacted at 1 ton pressure via hydraulic press.

↓

Tablets were controlled the quality follow USP 25/NF 18.

***HCE Tablets by Single Stroke Tabletting Machine***

Weight and hardness of HCE tablets were set on single stroke tabletting machine.

↓

All substances except HCEP and Aerosol 200® were sieved and weighted following Table 2.3

↓

Avicel® pH 101, Purified Talcum, Explotab®, Magnesium stearate and Aerosil 200® was respectively tumbled with HCEP to consistency.

↓

Tablets were controlled the quality follow USP 25/NF 18.

**2.2.7 Quality Controls**

**Quality Controls of HCEP**

***Moisture Content***

5 g of HCEP were measured moisture content at 120°C temperature by moisture balance (Sartorius LA 230 s, Germany) for 3 times and presented as % loss on drying (%LOD).

***Flowability Test***

50 g of HCEP were loaded into powder funnel, measured flow rate and investigated repose angle (°) in accordance with USP 25/NF 18 ( 3 times). The obtained data were compared with flowability.

***Scanning electron microscopy (SEM)***

A JSM-5910LV scanning electron microscope was applied in the studying of power surface, shape and size of Prosolv® and HCEP (HCE:Prosolv® as 1:2 and 1:8),

as well as drug formulation powders to determine their characteristics. Samples were prepared on carbon fixing paper and coated with gold by SPI-Module sputter coater for 15 min. The accelerating voltage was 30 kv under high vacuum mode and the samples were photographed at a 200x magnification.

**Quality Controls of Bulk Powder**

***Moisture Content***

5 g of bulk powder were measured moisture content at 120 °C temperature by moisture balance (Sartorius LA 230 s, Germany) and presented as %LOD (3 times).

***Flowability Test***

50 g of bulk powder were loaded into powder funnel, measured flow rate and investigated repose angle (°) in accordance with USP 25/NF 18 (3 times). The results were compared with flowability.

***Bulk Density, Tapped Density and Compressibility Ratio Test***

40 g of bulk powder was filled into a 100 ml-graduated cylinder to flat surface then hold up 1 inch from hard wood surface and dropped down depending on gravity force for 3 times at 2 seconds interval. The bulk density was evaluated from difference between initial volume and final volume of bulk powder.

Bulk powder within a 100 ml-graduated cylinder was set into jolting volumeter (JEL Stampfvolumeter STAV2003) before it was tapped to 500 times. The tapped density was measured initial volume and final volume. The compressibility ratio was calculated from bulk density and tapped density

**Quality Controls of Tablet**

***Weight Variation Test***

20 tablets of HCE tablets were weighted to calculate weight variation and mean for minimum and maximum percentage following USP 25/ NF 18.

***Size Test***

10 tablets of HCE tablets were measured dimension and thickness with digital micrometer and results were present as millimeter unit (mm).

***Hardness Test***

10 tablets of HCE tablets were measured by Erweka tester (ERWEKA® type TBH 100) and results were reported as Newton unit (N).

***Friability Test***

20 tablets of HCE tablets were appraised by Roche friabilitor (Pharma Test ® type PTF 20 E) with speed 25 rounds per a minute for 4 minutes therefore difference between pre-weight and post-weight were investigated. Friability property of HCE tablets was calculated and present as percentage unit (%).

***Disintegration Test***

6 tablets of HCE tablet were measured in tested for uncoated tablet regulation following USP 25/NF 18.

***TLC Tests***

5 tablets were dissolved in 100 ml methanol.

↓ For 5 minutes a time (3 times).

Obtainable solvent was filtered with whatman® no.1 filter papers.

↓

Filtrate was concentrated with a vacuum evaporator.

↓ At temperature 50°C, rotating 90 rpm.

Concentrated HCE was added 5 ml methanol

↓

There was measured by TLC flavonoid testing method.

**2.2.8 Stability Tests**

**Method of stability test**

The stability testing methods were performed similar to the quality control methods. Each of samples was measured below.

***HCEP and Bulk powder***

- Moisture content

***HCE Tablets***

- Weight Variation Test
- Size Test
- Hardness Test
- Friability Test
- Disintegration Test
- TLC Tests

**Condition of Stability Tests**

HCE tablets were packed into double layer polystyrene bags. The conditional storages see below.

- *Condition 1:* Room temperature
  Storage time: 3 months
- *Condition 2*: 45 °C temperature
  Storage time: Every month for 3 months