CHAPTER III

EXPERIMENTAL

1. Material

The following materials obtained from commercial sources were used.

1.1 Plant drug

Coarse dried powdered of *Butea superba* Roxb. It was collected from Kanchanaburi Province, Thailand, in November 2005. It was bought from traditional herb drug store (Thai Hua Chan), Bangkok, Thailand.

1.2 Additives of tablet

- Ac-Di-Sol® (Lot. No.T934, FMC, USA.)
- Aerosil® 200 (Lot. No. 635912F, Wacker Chemie GMBH, Germany)
- Avicel® PH 101 (Lot. No. 1292, FMC, USA)
- Avicel® PH 102 (Lot. No. 2352, FMC, USA)
- Corn starch (Lot. No. 05204005492, Friendship Corn Starch, Thailand)
- Polyvinylpyrolidone K-30 (BASF, Germany)
- Polyvinylpyrolidone K-90 (BASF, Germany)
- Lactose (WYNDALE™, Fonterra, New Zealand)
- Stearic acid (Lot. No. 6116 F34, Symex, Australia)
- Tapioca starch (Rose®, Thai-Wah Company Co.,Ltd., Thailand)

1.3 Chemicals

- Acetonitril, HPLC grade (Batch: 0565053, Fisher Scientific, England)
- Dichrolomethane, AR grade (Batch: 0444401, Fisher Scientific, England)
- Ethanol, Commercial grade (Samchai Chemical, Thailand)
- Ethylacetate, AR grade (Lot No. A 3511U, Lab-Scan Analytical Sciences, Bangkok)
- Hexane, AR grade (Mallinckrodt Chemicals, USA)

- Methanol, AR grade (Lot No. K31725209, Merk KGaA, Geramany)
- Methanol, HPLC grade (Batch: 0579328, Fisher Scientific, England)
- Vanilin powder (Lot. No. 1158944, Fluka Chemie GmbH, France)
- Sulfuric acid (Lot No. A8302, Lab-Scan Analytical Sciences, Bangkok)
- Silica gel 60 (0.063-0.200 mm, No. 9385, E. Merck, Germany)

1.4 Commercial *Butea superba* tablets

- Dokwhan® (Lot. No. 3604, Mfg. 26/02/05, Khal-La-Or Laboratories Co.,Ltd., Thailand.)

2. Equipment

- Analytical balance (Model A200s, Sartorius GmbH, Germany and Model PB3002, Mettler, Switzerland)
- Dissolution apparatus (Model VK-7000, Vankel®, Germany)
- Disintegration apparatus (Model ZT 31, Erweka®, Germany)
- Hardness Tester (Model TBH 30, Erweka®, Germany)
- Friabilator (Erweka TAR 20, Germany)
- High performance liquid chromatography (Model SPD-20 A and LC- 20 AB, Shimadzu, Japan)
- TLC aluminium sheet silica gel 60 F_{254} (Lot. No. OB541000, Merk KGaA, Germany)
- Glass column chromatography (40 cm × 5 cm)
- Hypersil® BDS (C18),column 250x4.6mm., 5um. (Thermohypersil, UK)
- Tray-dryer (Yheahang®, Thailand)
- Modified tap density tester (Chanchai Engineering, Thailand)
- Hot air oven (Model BM 600, Memmert, Germany)
- Micromass Platform II spectrometer (Altrincham®, Nation Science and Technology Development Agency).
- Nuclear Magnetic Resonance Spectrometer (Bruker®, Model AV-500 NMR, Nation Science and Technology Development Agency)
- Rotary evaporator (Model R-220, BUCHI®, Switzerland)

The experiment was performed in 5 parts as the following.

1. Purification of indicative substance in *Butea superba* Roxb.

1.1 Source of Plant material

The coarse powder of dried tuber of *Butea superba* Roxb. was collected from Kanchanaburi Province, Thailand, in November 2005. It was bought from the traditional herb drug store (Thai Hua Chan), Bangkok, Thailand.

1.2 Chromatographic Techniques

1.2.1 Thin Layer Chromatography (TLC)

The fingerprint of pure compound and crude extract were identified by thin layer chromatography (TLC) as the following condition.

Techniques	:	one way ascending
Stationary phase	:	TLC aluminium sheet silica gel 60F 254, layer thickness 0.2 mm
Distance	:	5 cm
Temperature	:	28-35°C (room temperature)
Detection	:	1) UV light at the wavelengths of 254 and 356 nm 2) 10% vanillin-sulfuric acid in ethanol and heating at 110 °C
Solvent	:	Various solvent systems depending on materials for example (CH_2Cl_2-MeOH, 97:3), (hexane:EtOAc, 3:2)

The general parameter used for describing the migration is the Rf value which defines as the following equation.

$$\text{Rf value} = \frac{\text{Distance moved by the solute}}{\text{Distance moved by mobile phase front}} \quad \ldots\ldots\ldots (1)$$

1.2.2 Column Chromatography (CC)

Isolation of pure compound was separated by column chromatography (CC) as the following condition.

Column	:	Flat bottom glass column (various diameters)

Stationary phase	:	Silica gel 60 (No. 9385, E. Merck®) particle size 0.040-0.063 mm (230-400 mesh ASTM)
Packing method	:	Dry and wet packing
Sample loading	:	1) Dry packing: The sample was dissolved in a small amount of suitable organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then loaded on top of the column. 2) Wet packing: The sample was dissolved in a small amount of the eluent, then loaded on top of the column
Solvent system	:	Various solvent systems depending on materials.
Detection	:	Fractions were examined by TLC observing under UV light at the wavelengths of 254 and 356 nm, then the TLC plate was sprayed with 10% vanillin-sulfuric acid in ethanol and heated at 110 °C. The fractions of similar TLC pattern were combined.

1.3 Spectroscopy

1.3.1 Mass Spectra (MS)

Electron impact mass spectra (EIMS) of pure compound were measured on a Micromass Platform II spectrometer at 70 ev. at the Nation Science and Technology Development Agency (NSTDA).

1.3.2 Nuclear Magnetic Resonance (NMR) spectra

The NMR spectra of pure compound were recorded at ambient temperature in $CDCl_3$, with a Bruker® AV-500 spectrometer at the Nation Science and Technology Development Agency (NSTDA). Instrument operating at 500 MHz. NMR solvents used in this study was deuterated chloroform ($CDCl_3$). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

1.3.3 High performance liquid chromatography (HPLC)

Quantitative and qualitative analysis of pure compound and crude extract were determined by high performance liquid chromatography (HPLC) as the following condition.

Column	:	Hypersil® BDS (C18) ,column 250x4.6mm., 5um.
Detector	:	UV-detector at 287 nm.
Injection volume	:	20 µl.
Mobile phase	:	Isocratic system as water -acetonitrile (50 : 50)

Mobile phase was filtered through a membrane filter with a pore size of 0.45 um and degassed for 30 minutes prior to use. Validation of HPLC method was typical analytical parameter to be considered for assay. Method validations were specificity, linearity, and precision. Since the amount of indicative substances was limited. Therefore only "linearity" was performed in this experiment.

1.3.3.1 Linearity

Triplicate injections of solution containing indicative substance in various concentrations ranging from 20 to 150 µg/ml in methanol HPLC grade were prepared and analysed. The linear equation of the curve obtained by plotting the peak area at each level prepared versus the concentrations of each standard was calculated using the least square method.

1.4 Solvents

Organic solvents used in extraction were commercial grade. For column chromatography and thin layer chromatography solvents were AR grade. Finally, solvent for high performance liquid chromatography was HPLC grade.

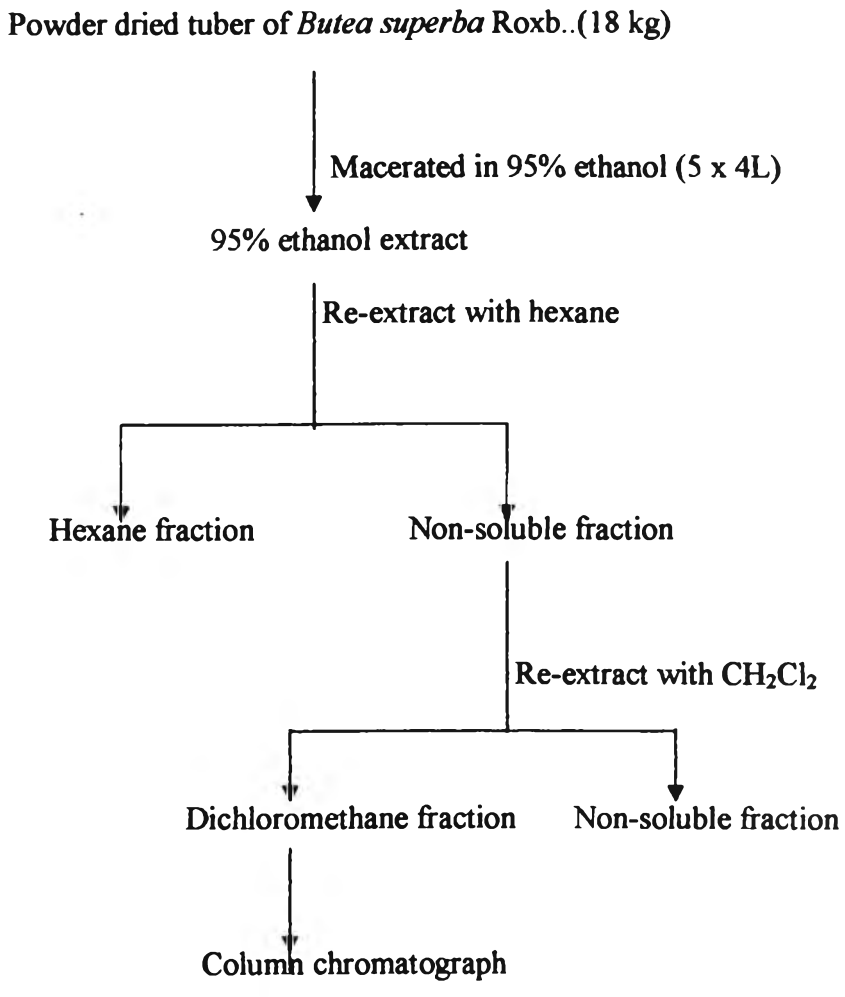
1.5 Extraction and Isolation

1.5.1 Extraction of the dried tuber of *Butea superba* Roxb.

Dried tuber of *Butea superba* Roxb.(18 kg) was powdered and macerated by 95% ethanol (5 x 20L) at room temperature. The 95% ethanol extract solution was filtered and evaporated under reduced pressure to obtain a dark-red gummy residue (500g) of 95% ethanol crude extract. This extract was repeatedly re-extracted by hexane and soluble fraction was discarded. The non-soluble hexane fraction was then

repeatedly re-extracted by dichloromethane (CH_2Cl_2) and the final insoluble fraction was discarded. The dichloromethane fraction was evaporated under reduced pressure to give dichloromethane extract. These extracts were subjected to column chromatography for future purification.

Extraction of the dried tuber of *Butea superba* Roxb. is summerized in Scheme 1.



Scheme 1. Extraction of the dried tuber of *Butea superba* Roxb.

1.5.2 Isolation

1.5.2.1 Fractionation of the dichloromethane extract

The dichloromethane crude extract (22.60 g) was applied to a silica gel column chromatograph (100 g, 5 x 40 cm) using gradient elution with dichloromethane (CH_2Cl_2) and methanol (MeOH) in a stepwise fashion, with 20 ml

fractions being collected as follows: Fractions 1-4 (CH_2Cl_2, 100%), 5-29 (CH_2Cl_2-MeOH, 93.5:6.5). Thirty fractions (20 ml each) were collected and combined according to their TLC pattern into ten major fractions (C2) as shown in Table 9. Finally, the column was washed down with methanol to give fraction C4.

Table 9. Combined fractions from the dichloromethane extract.

Fractions	Number of eluates	Weight (g)
C1	1-4	0.34
C2	5-15	0.42
C3	16-29	0.55
C4	methanol eluate	

1.5.2.2 Isolation of compound BS1

Fraction C2 (0.42 g) was applied to a silica gel column chromatography (20 g, 1 x 20 cm) using gradient elution with dichloromethane (CH_2Cl_2) and methanol (MeOH) in a stepwise fashion, with 5 ml subfractions being collected as follows: subfractions 1-13 (CH_2Cl_2, 100%), 14-16 (CH_2Cl_2-MeOH, 98:2), 17-26 (CH_2Cl_2-MeOH, 95:5), 27-31 (CH_2Cl_2-MeOH, 90:10). Thirty-two subfractions (5 ml each) were collected and combined according to their TLC pattern into ten major fractions (C2.1) as shown in Table 10. Finally, the column was washed down with methanol to give fraction C2.6.

Table 10. Combined fractions from fraction C2.

Fractions	Number of eluates	Weight (mg)
C2.1	1-10	210
C2.2	11-13	185
C2.3	14-17	174
C2.4	18-27	146
C2.5	28-31	102
C2.6	methanol eluate	

Subfractions C2.1 (210 mg) was applied to a silica gel column chromatography (20 g, 1 x 20 cm) using gradient elution with hexane and ethylacetate (EtOAc) in a stepwise fashion , with 5 ml subfractions being collected as follows: subfractions 1-2 (hexane,100%), 3-5(hexane:EtOAc, 98:2), 5-10 (hexane:EtOAc, 95:5), 11-27 (hexane:EtOAc, 90:10), 28-39 (hexane:EtOAc, 85:15), 40-42 (hexane:EtOAc, 80:20). Forty-three subfractions (5 ml each) were collected and combined according to their TLC pattern into one major subfractions (C2.1.4) as shown in Table 11. Finally, the column was washed down with methanol to give fraction C2.1.12.

Table 11. Combined subfractions from fraction C2.1

Fractions	Number of eluates	Weight (mg)
C2.1.1	1-10	16.80
C2.1.2	11-12	5.70
C2.1.3	13-14	17.88
C2.1.4	15	20.10
C2.1.5	16	16.40
C2.1.6	17-18	22.14
C2.1.7	19-21	19.63
C2.1.8	22	10.39
C2.1.9	23-26	17.24
C2.1.10	27-39	12.58
C2.1.11	40-42	14.35
C2.1.12	methanol eluate	

Subfractions C2.1.4 (20.10 mg) displayed one major spot (R_f = 0.73) on TLC plate when detected under UV light and after sprayed with 10% vanillin-sulfuric acid in ethanol and heating at 110 °C. **Compound BS 1** as whitish powder.

2. Formulation of dry powder *Butea superba* extract

2.1 Extraction of *Butea superba* Roxb in ethanol (EtOH)

2.1.1 Preparation of *Butea superba* fluidextract

Dried tuber of *Butea superba* Roxb.(18 kg) was powdered and macerated by 95% ethanol and 50% ethanol (5 x 20L) at room temperature for about 7 days The 50% and 95% ethanol extract solution were filtered and evaporated under reduced pressure to obtain a dark-red gummy and dark-brown residue (500g) respectively. Both extracts were identified by thin layer chromatography (TLC) method. These extracts were subjected to transform to dry powder extracts with various inert pharmaceutical excipients.

2.1.2 Determination of water content in *Butea superba* fluidextract

The method was modified from gravimetric method (Method III) from USP 29/NF 24. Fluidextract of about 10g were placed and accurately weighed in a tared evaporating dish (wc_1). After 5 hours on the water bath at 100°C, evaporating dish was cool down and weighed. Evaporating dish was moved to desiccator and weighed by the balance everyday until weigh constant was obtained (wc_2). The percentage of water content was calculated based on the following equation.

$$\text{Water content (WC)} = \frac{(wc_1 - wc_2)}{wc_1} \times 100 \quad \ldots\ldots\ldots\ldots (2)$$

2.2 Preparation of dry powder *Butea superba* extract

The inert pharmaceutical excipients were chosen to incoporate in the *Butea superba* extract made from 50% and 95% ethanol. Prior to mixing, the excipients such as tapioca starch, corn starch, Avicel® PH101, Avicel® PH102, PVP k-30 were individually sieved through an 80-mesh screen. The transformation was prepared by wet granulation method. From the preliminary the optimize ratio liquid extract and excipient was 1:2. Because the ratio 1:1 resulted in hard granule and more than 1:3 provided a lot of fine powder. The formulation of dry powder extract made from 50% and 95% ethanol were listed in Table 12 and 13.

Table 12. Formulation composition of *Butea superba* 95% ethanol dry extract.

Ingradient (g)	Formulation (% w/w)			
	F1-BS-95	F2-BS-95	F3-BS-95	F4-BS-95
B.S. 95% ethanol	33.33	33.33	33.33	33.33
Tapioca starch	66.66	-	33.33	31.66
Corn starch	-	66.66	33.33	31.66
PVP k-30	-	-	-	3.33

Table 13. Formulation composition of *Butea superba* 50% ethanol dry extract.

Ingradient(g)	Formulation (% w/w)							
	F1-BS-50	**F2-BS-50**	**F3- BS-50**	**F4- BS-50**	**F5- BS-50**	**F6- BS-50**	**F7- BS-50**	**F8- BS-50**
***B.S.* 50% ethanol**	33.33	33.33	33.33	33.33	33.33	33.33	33.33	33.33
Tapioca starch	66.66	-	30.55	27.77	30.55	31.66	29.57	32.07
Corn starch	-	66.66	30.55	27.77	30.55	31.66	29.57	32.07
Avicel® PH 101	-	-	5.55	11.11	-	-	-	-
Avicel® PH 102	-	-	-	-	5.55	-	-	-
PVP K-30	-	-	-	-	-	3.33	7.50	-
PVP K-90	-	-	-	-	-	-	-	2.5

A batch size about 80g was produced by manually mixing by mortar and pestle. First, *Butea superba* ethanolic extract, tapioca starch, corn starch, Avicel® PH101, Avicel® PH102, PVP k-30 and PVP k-90 were individually and accurately weighed. The powder ingredients were blended by geometric dilution for 5 minutes to obtain a homogeneous powder mixture. Afterward, *Butea superba* ethanolic extract was added and blended until a homogeneous wet mass mixture was obtain. Beaker contain ethanolic extract was rinsed with 10 ml water. The dispersion was then poured into the wet mass and blended until homogeneous. The wet mass mixture was then screened through a 12-mesh sieve. The wet granules were dried for 3 hours at 50° C in a hot air oven. After that, the dried granules were passed through a 16-mesh screen and stored in a dessicator.

2.3 Evaluation of dry powder *Butea superba* extract.

2.3.1 Organoleptic properties

The dried granules of about 20g were poured on the white paper and spreaded by spatula. The color of dried granules was examined and compared under diffuse daylight. The odor of dried granules was placed in a small beaker with continuous inhalation of the air over it to determine distinct odor or smell. The taste of dried granules was masticated by oral cavity for at lest 10-30 sec.

2.3.2 Moisture content

The moisture content (MC) of the granules were determined by using a hydrogen moisture analyzer. About 5 g of samples were spreaded uniformly in the thin layer of aluminium plate. The accurate weight was recorded (wc_1) prior to exposure to an infrared lamp until constant weight was obtained (wc_2). The percentage of moisture content was calculated based on the previous equation (2).

2.3.3 Density and compressibility

To determine the bulk density of the sample, a known quantity of the granule (10 g) was gently poured through a 25-ml graduate cylinder. The volume was then read directly from the cylinder. The bulk density (ρb) was calculated as the ratio between weight (g) and volume (ml).

To determine the ultimate tapped density (ρt), the cylinder contain granules was tapped on a tap density tester from a height of 1.3 cm until no measurable change in volume was noticed. The constant volume was read and used to calculate the Carr's index. The samples were determined in triplicate. The percent compressibility of granule was evaluated using the Carr's index in the following equation.

$$\text{Carr's index} = \frac{(\rho t - \rho b)}{\rho t} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

2.3.4 Determination of flow rate

Ten grams of granule were filled in a 1.5-cm internal orifice diameter paper funnel that fixed on a clamp. The time was recorded when granule started to

flow until all granules were passed through the orifice. The averaged flow rate was reported in g/sec.

2.3.5 Determination of the amount of medicarpin compared with fluid extract

The dried granules of about 400 mg were transfered to 50-volunmetric flask and 25 ml of methanol was added. After 15 minutes of sonication, the volumetric flask was filled up to volume with methanol and mixed. Solution was filtered through 0.45 μm filter paper. Each sample was determined in duplicate by HPLC method.

3. Formulation development of *Butea superba* extract tablets

Good formulations of dry extract granule were selected for tablets formulation process. Dry extract granule had agglomerate granule, good flowing, gave compressibility and not more than moisture content that caused hygroscopic. Prior to mixing, the excipients (expect dry granule *Butea superba* extract) Ac-Di-Sol®, Aerosil®, stearic acid were screened through an 80-mesh sieve. Tablet each contained 400 mg of powder *Butea superba* ethanolic extract were prepared by direct compression.

A batch size of about 60 gram was produced by manually mixing in a plastic bag. First, granule of *Butea superba* extract were blended in a plastic bag with Ac-Di-Sol® for 5 minutes. Next, Aerosil® and stearic acid were thoroughly mixed with the previous mixture for additional 5 minutes. Finally, the lubricanted mixture was compressed into 434 mg tablets using a round concave punch of 10.5 mm in diameter on a manually single punch tableting machine. The compress force as well as tablet weight were controlled in order to obtain the tablet hardness within the acceptable range of 4-7 kp. The tablets were evaluated following the specification in topic 4. The formulations are listed in Table 14.

4. Evaluation of *Butea superba* extract tablets

The following properties of *Butea superba* extract tablets were investigated and compared to commercial tablets (Dokwhan® tablets as each tablet 600 mg contained : *Butea superba* 180 mg, Thailand).

4.1 Physical appearance

Freshly prepared tablets, commercial *Butea superba* tablets and tablets stored in desiccator at ambient temperature for 1 year and 10 months were observed visually. Their diameter and thickness were measured by ERWEKA® tablet hardness tester (Model TBH 30).

4.2 Friability

The friability of tablets was determined by a tablet friabilator. Twenty tablets were weighed by an analytical balance and averaged (Wo). They were filled into the friability tester and rotated at 25 rpm for 4 minutes. The tablets were weighed again after the dust was eliminated (W). The percent friability was calculated based on the following equation.

$$\%\text{Friability} = \frac{(Wo-W)}{Wo} \times 100 \quad \ldots\ldots\ldots\ldots\ldots (4)$$

4.3 Hardness

Ten tablets were sampling and individually subjected to a hardness tester. The tablet hardness was expressed in kilopound (kp) unit. Data of mean and standard deviation of tablets hardness were calculated.

4.4 Weight variation

Twenty tablets were accurately weighed by an analytical balance. The average weight and standard deviation of tablets were calculated.

4.5 Disintegration time

The disintegration time was determined upon six tablets, using de-ionized water and maintained at temperature 37±1° C. Experiment was performed following the protocol with standard USP 29 . Results were reported as the time requirement for disintegration of tablets.

Table 14. Formulation composition of *Butea superba* 95% and 50% ethanolic extract tablets.

Formula-tion	Component (%w/w)	Granule (mg)	5% Ac-Di-Sol® (mg)	0.5% Aerosil® (mg)	3% Stearic acid (mg)	Weigh per tablet
F1-BS-95	Butea superba extract (33.33), Tapioca starch (66.66)	400	20	2	12	434
F2- BS-95	Butea superba extract (33.33), Corn starch (66.66)	400	20	2	12	434
F3- BS-95	Butea superba extract (33.33), Tapioca starch (33.33), corn starch (33.33)	400	20	2	12	434
F4- BS-95	Butea superba extract (33.33),Tapioca starch (31.66),corn starch (31.66),PVP K-30 (3.33)	400	20	2	12	434
F1- BS-50	Butea superba extract (33.33), Tapioca starch (66.66)	400	20	2	12	434
F2- BS-50	Butea superba extract (33.33), Corn starch (66.66)	400	20	2	12	434
F3- BS-50	Butea superba extract (33.33),Tapioca starch (30.55),Corn starch (30.55),AvicelPH101 (5.55)	400	20	2	12	434
F4- BS-50	Butea superba extract(33.33),Tapioca starch (27.77),Corn starch (27.77),AvicelPH101 (11.11)	400	20	2	12	434
F5- BS-50	Butea superba extract (33.33),Tapioca starch (30.55),Corn starch (30.55),AvicelPH102 (5.55)	400	20	2	12	434
F6- BS-50	Butea superba extract (33.33),Tapioca starch (31.66),Corn starch (31.66), PVP K-30 (3.33)	400	20	2	12	434
F7- BS-50	Butea superba extract (33.33),Tapioca starch (29.57),Corn starch(29.57),PVP K-30 (7.50)	400	20	2	12	434
F8- BS-50	Butea superba extract (33.33),Tapioca starch (32.07),Corn starch (32.07), PVP K-90 (2.5)	400	20	2	12	434

4.6 The uniformity of dosage form

Ten tablets were taken by sampling. Each tablets was weighed and placed into a 50-volunmetric flask and 25 ml of methanol HPLC grade was added. After 15 minutes of sonication, the volumetric flask was filled up to volume with methanol and mixed. Solution was filtered through 0.45 μm filter paper. Each sample was determined for indicative substance in duplicate by HPLC method.

4.7 Dissolution study

From the preliminary study 400 ml of 20% ethanol in water was used as the dissolution medium and maintained at temperature 37±0.5° C in the closed system. The dissolution apparatus as basket method were rotated by a speed of 100 rpm. A sampling of dissoluted tablets was at the end of 30 minutes and assayed for indicative by HPLC method.

5. Stability study

The *Butea superba* extract tablets were packed under plastic bags and stored in a glass desiccator and under ambient temperature for 1 year and 10 months. Tablets randomized for analyzing the percentage remaining of indicative substance by HPLC method. The sample preparation for analysis were followed as in topic 4.6