CHAPTER III

PHARMACOGNOSTIC SPECIFICATION

Pharmacognostic specifications are determination of medicinal plants for identification and qualitative control. Common pharmacognostical tests are simple, cost effective and easy to operate. The macroscopic method together with organoleptic sensation is used to determine the size, shape, color, odor, taste, etc. of the crude drugs, while the microscopic method reveals plant histology. The thin-layer chromatographic technique is used to differentiate extracts of different biological origins. Methods for quality control of crude drugs are described in Thai Herbal Pharmacopoeia. Moreover, the quantitative analysis by high performance liquid chromatography (HPLC) using corosolic acid as a marker is described.

The scope of this investigation was illustrated. The macroscopic and microscopic characterizations of *Lagerstroemia speciosa* leaves were determined. Microscopic determinations of leaf parameters were studied. Thin-layer chromatographic examination of methanol extracts of *Lagerstroemia speciosa* leaves from several sources was presented. Phytochemical screening test were done in various methods. The qualitative analysis of crude drugs according to the Thai Herbal Pharmacopoeia was examined. In addition, the quantitative determination of corosolic acid in *Lagerstroemia speciosa* leaves was also detected by high performance liquid chromatography.

3.1 Materials and methods

3.1.1 Macroscopic and microscopic characterizations of *L. speciosa* leaves.

The fresh *L. speciosa* leaves (semi-mature leaves) were collected from several sources and were authenticated by comparison with the herbarium specimens. Eight locations have been selected for plant collection as follows:

Table 3.1 The fresh samples of *L. speciosa* used in this study.

No.	Voucher no.	Locations
LS1	LS1WT200908	Rajamangala University of Technology Phra Nakhon. Bangkok
LS2	LS2WT220908	The Faculty of Pharmaceutical Sciences, Chulalongkorn university, Bangkok
LS3	LS3WT070109	The Faculty of Veterinary Sciences. Chulalongkorn university, Bangkok
LS4	LS4WT150109	The Royal Turf Club of Thailand, Bangkok
LS5	LS5WT130309	The Central Botanical Garden (Pukae), Saraburi Province
LS6	LS6WT270309	The Intanin field, Maejo University, Chiang Mai Province
LS7	LS7WT270309	The Maharaj Nakorn Chiang Mai Hospital, Chiang Mai Province
LS8	LS8WT140409	Wat Pa-ngun, Sawee District, Chumporn Province

Nine samples of crude drugs which are called "Intanin nam" were purchased randomly from traditional drugstores in four regions of Thailand, as follows:

Table 3.2 Intanin nam which were purchased from traditional drugstores.

No.	Voucher no.	Locations
LS9	LS9WT151008	Vechapong drugstore, Sampanthawong district, Bangkok
LS10	LS10WT151008	Jao-krom-per drugstore, Sampanthawong district, Bangkok
LS11	LS11SW170409	Silapa Osoth drugstore, Ubonrachathanee province
LS12	LS12TB200609	Ea-sae drugstore, Khonkaen Province
LS13	LS13PK120309	Wanchai Osoth drugstore, Pranakorn Sri Ayutthaya Province
LS14	LS14CK150209	Buan-un-tung, Ratchaburi Province
LS15	LS15WT131009	Lampang Rak Samunprai drugstore, Lampang Province
LS16	LS16WT131009	Kirisamunpai drugstore, Nakorn Sri Thammarat Province
LS17	LS17WT161009	Thai Osoth drugstore, Buriram Province

The leaves were dried in a hot air oven at 50°C and kept in a well-closed container for macroscopic and microscopic study. The macroscopic method were determined the shape, color, odor and taste of crude drugs. The microscopic methods were found out the characteristic cells and tissues were traced using a digital camera.

The leaves were dried, then ground and passed through a sieve with mesh number 60. The powdered sample was mounted in water to determine the characteristic cell and tissue. For unclear fragments, chloral hydrate solution was added onto the powdered sample and then powder sample was mounted in glycerin to prevent the formation of chloral hydrate crystals during the examination. The tissue and cell inclusion were photographed by photo-micrographic equipment which is attached to microscope.

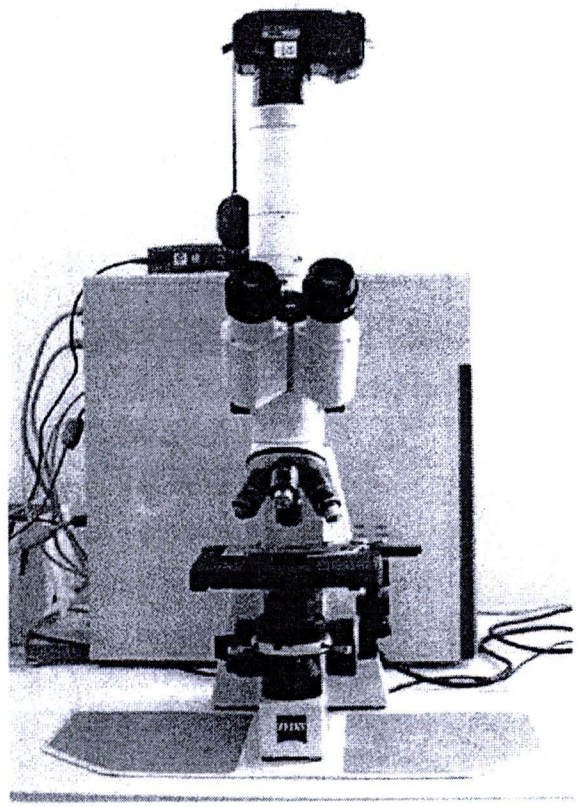

Figure 3.1 Compound microscope Zeiss model Axiostar attached with digital camera Sony Cyber-shot DSC-S85

3.1.2 Microscopic determination of the constant numbers of leaf

The constant values of are used as character for identification concerning their constant value in each species.

Preparation of leaves

The fresh mature leaves were collected from eight locations as described in **Table 3.1** and were used for leaf constant vales determination. The pieces of leaves from the middle of the lamina about 25 mm square were cleared by gently warming in chloral hydrate solution (4 g/ml in distilled water). This solution was frequently shaken and changed for rapid removing of chlorophyll from the leaf fragments. When the leaf fragments were cleared, they were rinsed off in distilled water at least 2 times and finally kept in glycerin to maintain the structure and moisture of cells.

Method for stomatal number

The stomatal number means to the average number of stomata per square millimeter of epidermis.

The number of stomata was counted in the circle field of view and incomplete part of the cells in one semicircle. The incomplete part of the cells in the other semicircle shouldn't count. There are 30 fields to be determined for each sample and from knowledge of the area of the circle filed was able to calculate the stomatal number.

$$\text{Stomatal number} = \frac{\text{Number of stomata}}{\text{Area of epidermal cell } (mm^2)}$$

Method for stomatal index

Counts are made of the numbers of epidermal cells and of stomata (the two guard cells and ostiole being considered as one unit) within the square grid.

The percentage proportion of the ultimate divisions of the epidermis of a leaf which have been converted into stomata is term the stomatal index:

$$I = \frac{S}{E + S} \times 100$$

Where S = number of stomata per unit area

E = number of ordinary epidermal cells in the same unit area

Method for palisade ratio

The average number of palisade cells beneath each upper epidermis cell is termed the palisade ratio.

First, a number of groups each of four epidermal cells are traced then the palisade cells in each group are focused and trace.

Then, the palisade cells in each group are counted, those being included in the count which are more than half-covered by the epidermal cells; the figure obtained divided by 4 gives the palisade ratio of that group.

Method for vein-islet number and veinlet termination number

A piece of leaf, bladed approximately 4 square mm, was cleared by chloral hydrate solution. The number of vein-islets in the square mm is counted. The islets which are intersected by the sides of square are included on two adjacent sides and excluded on other two sides. The average numbers of vein islets from four squares are found, and average numbers of vein islets are calculated.

The veinlet termination determination was same process as the determination of the Vein-islet number. The average number of vein terminations present with in the square was counted from four different squares to get the value for one square mm.

3.1.3 Thin-layer chromatographic patterns of leaves extract.

The dry leaves were chopped into small pieces, then ground and passed through a sieve with mesh number 20, kept in a well-closed container. The purchased sample also did as described above.

Technique for thin-layer chromatography (TLC)

The conditions used for the analytical TLC used in this work are as follows:

Technique	: One-dimentional TLC
Adsorbent	: Silica gel 60 F_{254} (Merck) precoated plate
Solvent system	: (A) Chloroform and Acetone (4:1)
	(B) Chloroform and Methanol (95:5)
Layer thickness	: 0.2 mm
Distance	: 8.5 cm
Temperature	: Laboratory temperature (25-28^{O}C)
Detection	: Visible daylight, UV 254 nm, UV 365 nm,
	Anisaldehyde-sulfuric acid TS

The detail of each step was described. Ten grams of dried leaves powdered drug were macerated in 100 ml of methanol for 24 hours, then filtered through filter paper (Whatman, No.1) and evaporated to dryness. The crude extract was then partitioned twice between dichloromethane and water. The CH_2Cl_2 layer were combined and concentrated under vacuum to yield CH_2Cl_2 soluble extracted part until dryness, then kept in well-closed container prior to spot on TLC plate. The residue was dissolved in 0.5 ml of methanol. Six microlitres of the extract was applied on TLC plate by micropipette and allowed to dry by air. Chloroform and acetone (4:1) and chloroform and methanol (95:5) were selected to solvent system that could provide the pattern for separation and identification. The chromatogram was developed after solvent system saturating in chamber. The plate was removed from the tank, allowed to dry by air. The developing distance was 8.5 cm. The chromatogram was detected.

Special methods were used to detect compounds, which could not be directly distinguishable, by their own colors. Many compounds became visible when the chromatogram was viewed under short (254 nm) and long (356 nm) waves ultraviolet

light. Some of them had to be visualized by spraying with special detection reagents *i.e.* anisaldehyde acid reagent.

The locations and colors of the spots were recorded after each treatment. R_f values were determined from the mean of a series of independent observations undertaken on three chromatograms of the same solvent system development.

$$R_f \text{ value} = \frac{\text{Distance of spot moving from starting point}}{\text{Distance of solvent front starting point}}$$

3.1.4 Phytochemical screening.

The dried coarse powdered drugs were tested with various method such as Froth test, Shinoda test, Alkaloid test, Lieberman-Burchard test, Ferric chloride TS.

Flavonoids test (Shinoda test, Cyanidin reduction test)

Five grams of dried coarse powdered drug was macerated in 50 ml of 95% ethanol for 24 hours, filtered and then evaporated to dryness on water bath. The residue was triturated with petroleum ether, decanted 2 times. The residue was dissolved in 50% ethanol, filtered and separated the filtrate into 2 portions:

Portion1: as control

Portion2: One millilitres of conc. HCl and 2-3 pieces of Magnesium ribbon was added into the filtrate. Pink red or red coloration of the solution indicates the presence of flavonoids in the drug.

Alkaloid test

Twenty grams of dried coarse powdered drug was macerated in 50 ml of 95% ethanol for 24 hours, filtered and then evaporated to dryness on water bath. The crude extract was dissolved in 10 ml of dilute sulfuric acid (2%), filtered and separated the filtrate into 2 portions:

Portion 1: Few drops of Mayer's reagent were added. Formation of a white cream precipitate indicates the presence of alkaloids.

Portion 2: Few drops of Dragendorff's reagent were added. An alkaloid-positive reaction gives orange colored precipitate.

The precipitate should not dissolve in alcohol.

Froth test

Two grams of dried coarse powdered drug was macerated in 10 ml of distilled water, filtered into the tubes. The filtrate was shaken rapidly and then allowed standing. The positive test with honeycomb froth was persisted for at least 30 minutes.

Liebermann-Burchard test for detection triterpene and steroidal group

The 0.5 grams of the dried coarse powder were macerated with chloroform. The chloroform extracts were treated with a few drop of acetic anhydride solution, followed by a few drops of sulphuric acid. A blue green color shows the presence of phytosterols.

Ferric chloride solution test for tannins

Two grams of dried coarse powdered drug was macerated in 10 ml of methanol, filtered in to the tube. Methanolic extract was treated with 10% ferric chloride test solution. A blue color indicates condensed tannins, a green color indicated hydrolysable tannins.

3.1.5 Physicochemical determination.

The dry leaves were ground and passed through a sieve with mesh number 20, kept in a well-closed container. The purchased samples were also treated as described above. The constant numbers due to quality of *L. speciosa* leaves were examined by standard methods of the World Health Organization (WHO) guideline (WHO, 1998) and Thai Herbal Pharmacopoeia (THP, 1995)

3.1.5.1 Loss on drying

The ground sample (2.0 g) was accurately weighted in a small crucible or weighing bottle and then dried at 105°C to constant weight. The percentage of loss on drying was calculated with reference to the air-dried substance.

3.1.5.2 Total ash

The ground sample (3.0 g, accurately weighted) was placed in a previously ignited and tared the crucible. The sample was spread in an even layer and ignited by gradually increasing the heat to 500-600°C until white ash was obtained. The ash was then cooled in a desiccator and weighed without delay. The percentage of loss on drying was calculated with reference to the air-dried substance.

3.1.5.3 Acid-insoluble ash

To the crucible containing the total ash was added 25.0 ml of 2N hydrochloric acid. The crucible was then covered with a watch-glass, and the mixture was boiled gently for 5 minutes. The watch-glass was rinsed with hot water, and this liquid was added into the crucible. The insoluble matter was collected on ashless filter-paper and washed with hot water until the filtrate was neutral. The filter-paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to constant weight. The residue was allowed to cool in desiccators and weighed without delay. The percentage of acid-insoluble ash was calculated with reference to the air-dried substance.

3.1.5.4 Extractive value

Ethanol-soluble extractive

The ground sample (5.0 g) was macerated with absolute ethanol (100.0 ml) in a closed conical flask in shaking bath for 6 hours and allowed to stand for 18 hours. The extract was filtered rapidly to avoid loss of ethanol. The filtrate (20.0 ml) was evaporated to dryness in an evaporating disc and then dried with the heat to constant weight.

Water-soluble extractive

The process of water-soluble extraction was preceded as directed for ethanol-soluble extractive, but using chloroform water in place of ethanol.

Dichloromethane-soluble extractive

The process of dichloromethane-soluble extraction was preceded as directed for ethanol-soluble extractive, but using dichloromethane in place of ethanol.

3.1.5.5 Determination of water (Azeotropic Distillation Method)

The apparatus (**Figure 3.2**) was consisted of a round bottom flask (A) connected by a tube (D) to a cylindrical tube (B) fitted with a graduated receiving tube (E) and a reflux condenser (C). The receiving tube (E) was graduated in 0.1 ml sub-divisions so that the error of reading was not greater than 0.05 ml. The source of heat was preferably an electric heater with rheostat control or an oil-bath. The upper portion of the flask and the connecting tube might be insulated with asbestos.

The receiving tube and the condenser of the apparatus were cleaned by a suitable method, thoroughly rinsed with water, and dry.

The ground sample (20.0 g) in water-saturated toluene (200.0 ml) was subjected to azeotropic distillation. As soon as the water was completely distilled, the inside of condenser tube was rinsed with toluene, and the distillation was continued for 5 more minutes. The heat was then removed, and the receiving tube was allowed to cool to room temperature. The water and toluene layers were allowed to separated, and then the volume of water was read off.

The percentage present in the substance using the formula:

$$\text{Moisture content} = \frac{100(n'-n)}{P}$$

where P = the weight in g of the substance to be examined,

n = the volume in ml of water obtained in the first distillation, and

n′ = the total volume in ml of water obtained in the two distillations.

3.1.6 Quantitative analysis of corosolic acid by HPLC method

This study aimed to develop a simple, rapid and sensitive reverse phase high performance liquid chromatography (HPLC) method to quantify corosolic acid in *L. speciosa* leaves.

Sample preparation

Dry *L. speciosa leaves* were ground into a fine powder. The tissue was sieved, and 200 mg of the ground leaves was weighed into a test tube and macerated in 2 ml of methanol for 24 hours. The extract was filtered through a 0.45 µm filter for high performance liquid chromatography (HPLC) analysis. The filtrate was transferred to clean glass vials and used directly for HPLC analysis.

Preparation of Standard Solutions for Calibration Curve

Five milligrams of corosolic acid was dissolved in 5 ml of methanol to give a 1 mg/ml stock solution. The stock solution was diluted to various concentrations ranging from 10 to 250 µg/ml. The solutions were then used to construct a calibration curve of corosolic acid using HPLC.

HPLC analysis condition

The HPLC system was composed of a Shimadzu SIL-20 AHT pump equipped with a ZORBAX Eclipse XDB-C18 column (250 × 0.46 mm, i.d. 5 µm) and a guard column (Agilent Technologies, USA). The column contents were eluted with acetonitrile and 0.1% phosphoric acid in water (75:25) at a flow rate of 1 ml/min. The eluent was monitored at 204 nm using diode array detector (DAD). The amount of corosolic acid in the crude extracts was estimated using the standard curves. All of the measurements were done in triplicate.

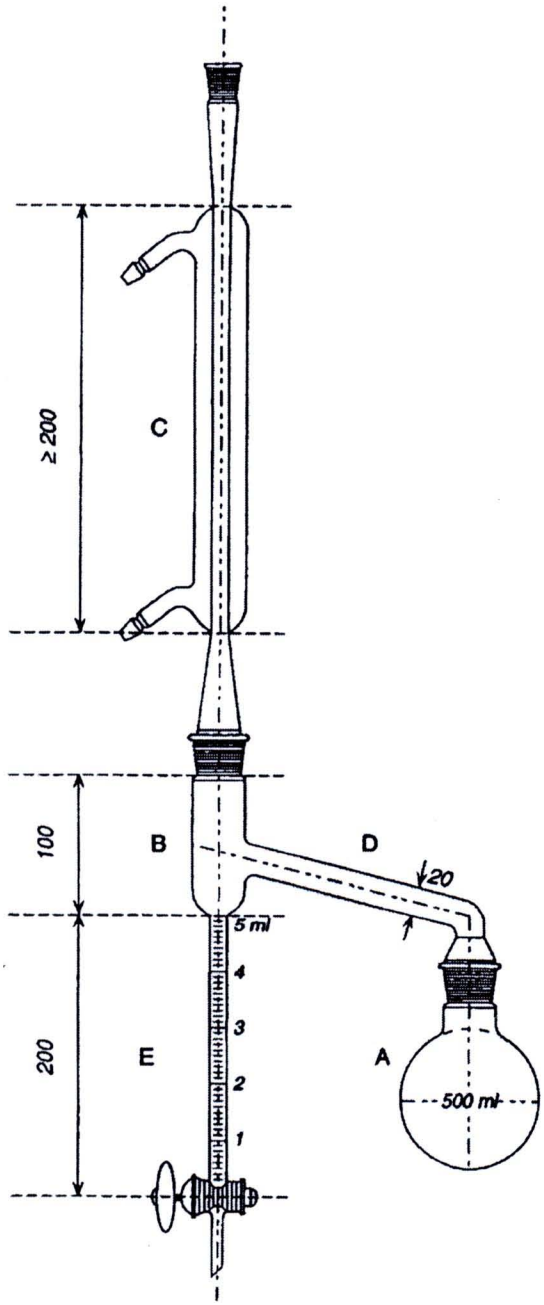

Figure 3.2 Azeotropic distillation method (THP, 1995).

3.2 Results

3.2.1 Organoleptic and microscopic investigation of *L. speciosa* leaves

Organoleptic investigation

Organoleptic investigation of *L. speciosa* leaves from traditional drugstore was described. The color of their leaves was shown in olive green to yellowish brown. The nearly perfect leaves were 7-12 cm wide and 11 – 26 cm long. The petiole was 1 cm long. The shape of leaves was broadly, ovate or oblong. There were some fragments of leaves. Some of crude drugs from traditional drugstore were chopped in small pieces. The odor was slightly characteristic and the taste was slightly bitter. (see **Appendix A)**

Microscopic investigation

Microscopic characters of *L. speciosa* leaves were studied in the upper epidermis, the lower epidermis, transverse section of midrib and leaf, and the powdered drug.

The upper epidermal cells were slightly thick-walled polygonal cells. The cell length was about as long as it width (**Figure 3.3A**). The lower epidermal cells were slightly thick-walled polygonal cell in various size and anomocytic stomata (**Figure 3.3B**).

The transverse section of leaves showed the upper epidermis, a single layer of cuticularized rectangular cells. Mesophyll was consisted of 1-4 layers of palisade parenchyma, several layers of spongy parenchyma, some containing a small rosette aggregate crystal of calcium oxalate, and small vascular bundle. Lower epidermis was a single layer of rectangular cell. Transverse section through midrib of lamina showed group of collenchymas scattered in parenchyma layers underneath the epidermis, collateral vascular bundles (**Figure 3.4, 3.5**).

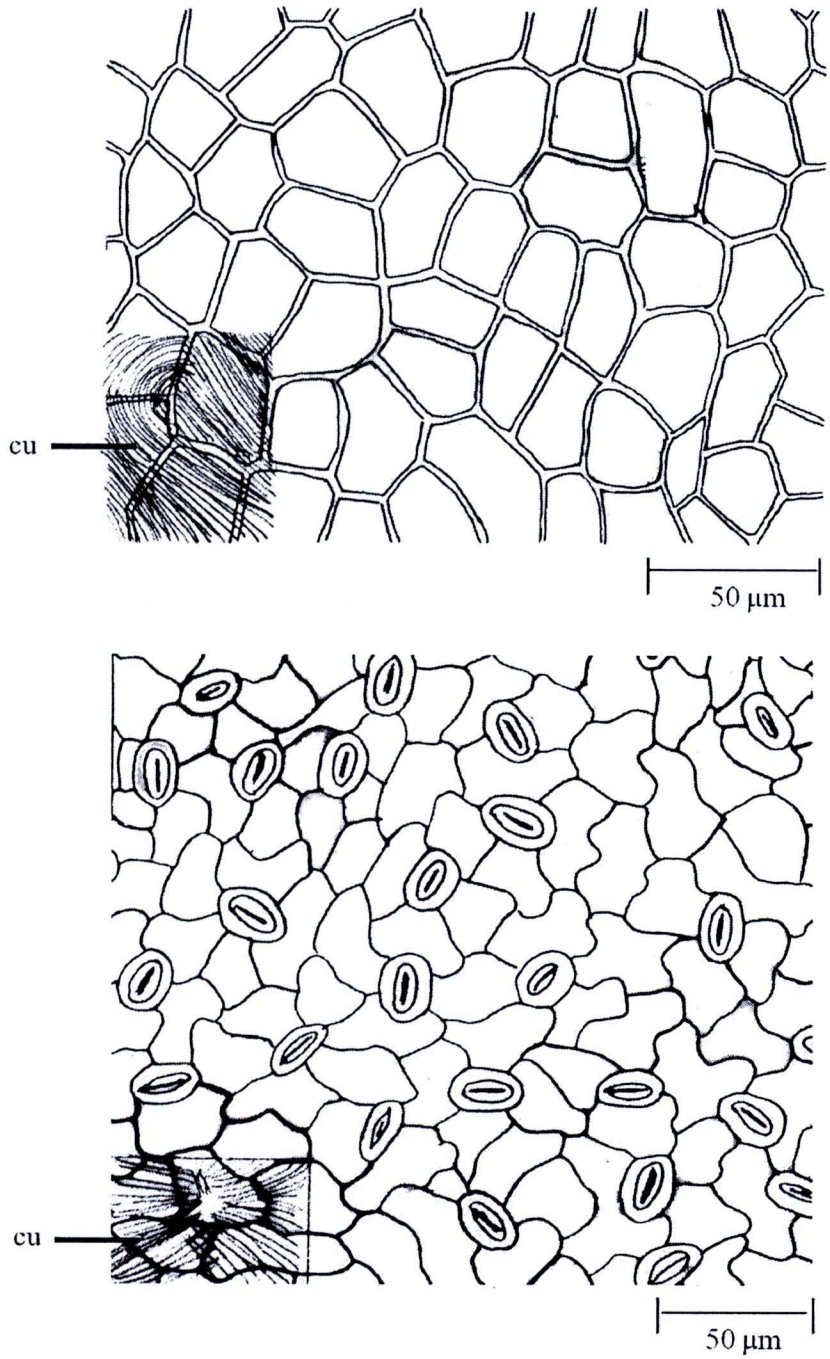

Figure 3.3 Epidermal cells of *L. speciosa* leaf. A: upper epidermis of the lamina; B: lower epidermis of the lamina.

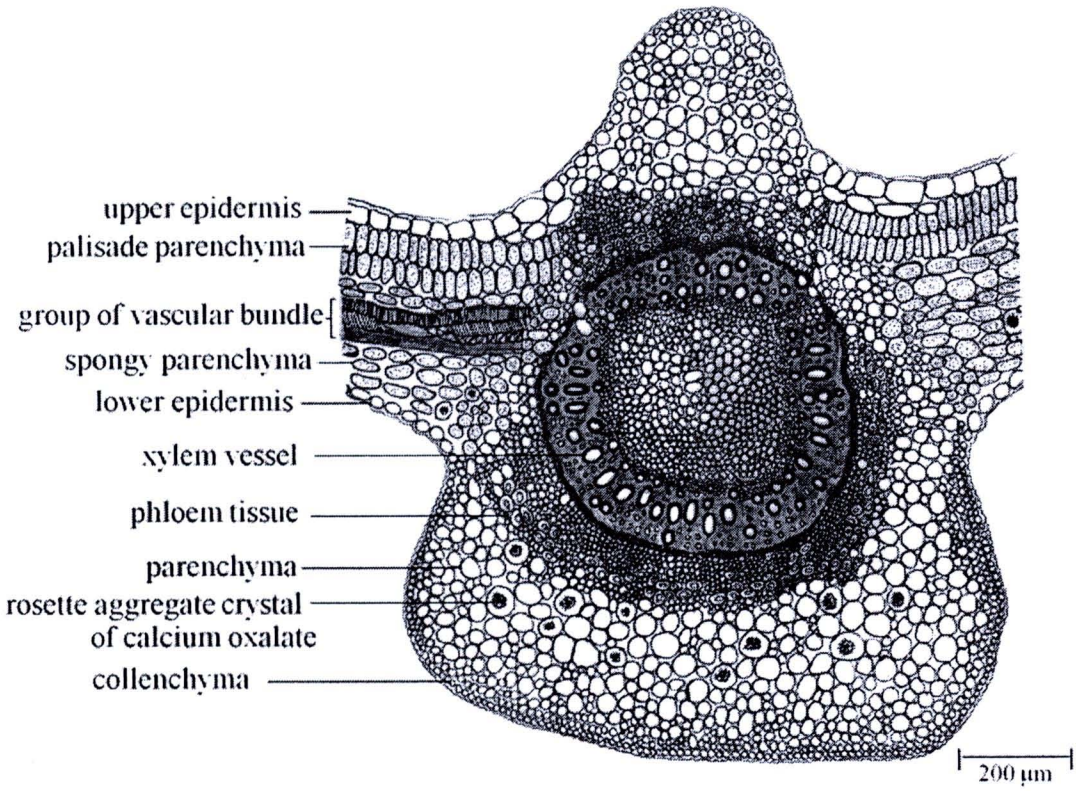

Figure 3.4 Transverse section of midrib of *L. speciosa* leaves.

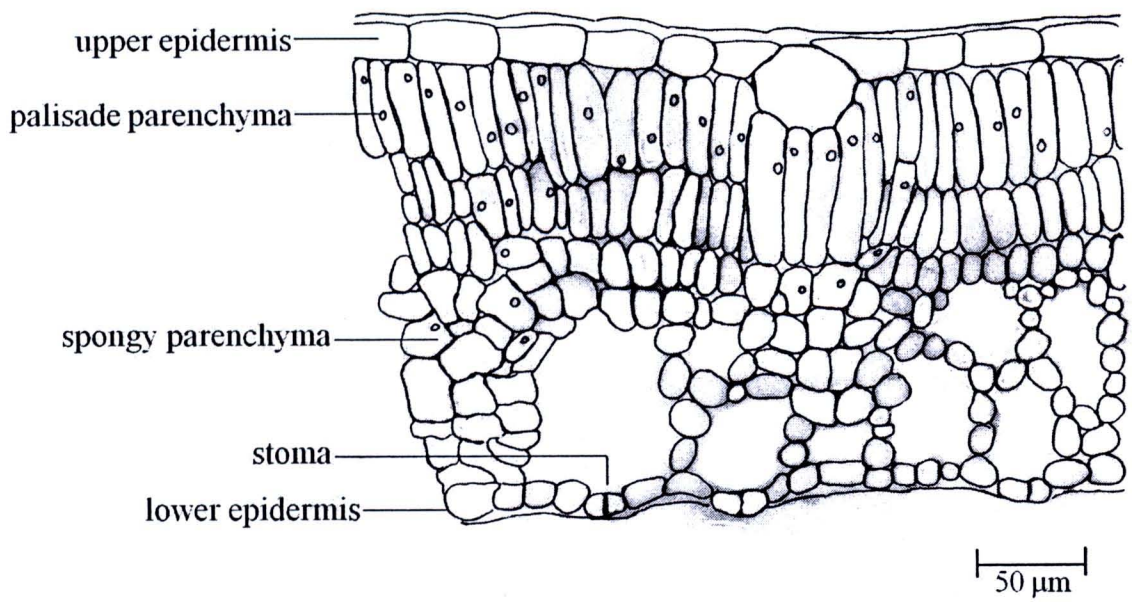

Figure 3.5 Transverse section of lamina of *L. speciosa* leaves.

The powdered drugs of *L. speciosa* are dark green color. The odor was slightly characteristic. The taste is slightly bitter. The microscopic characteristics of powdered drugs of *L. speciosa* (**Figure 3.6**) were as follows:

a) the fragment of upper epidermal cells, which were polygonal in surface views, occasionally found to be swollen cells which containing mucilage. They showed the pink or red color with ruthenium red reagent.
b) the rosette aggregate crystals of calcium oxalate found scattered and occasionally found in spongy parenchyma. The calcium oxalate prisms were rarely found in the powder.
c) the fragment of lignified fibrovascular tissues, group of fiber and vessel, reticulate vessel, spiral vessel, rarely found scaraliform vessel and bordered pits.
d) the fragment of lower epidermis in surface view, showing anomocytic stomata.
e) the fragment of the lamina in sectional view, showing the thick, striated cuticle (particularly over the upper epidermis) and two to four rows of palisade cell.

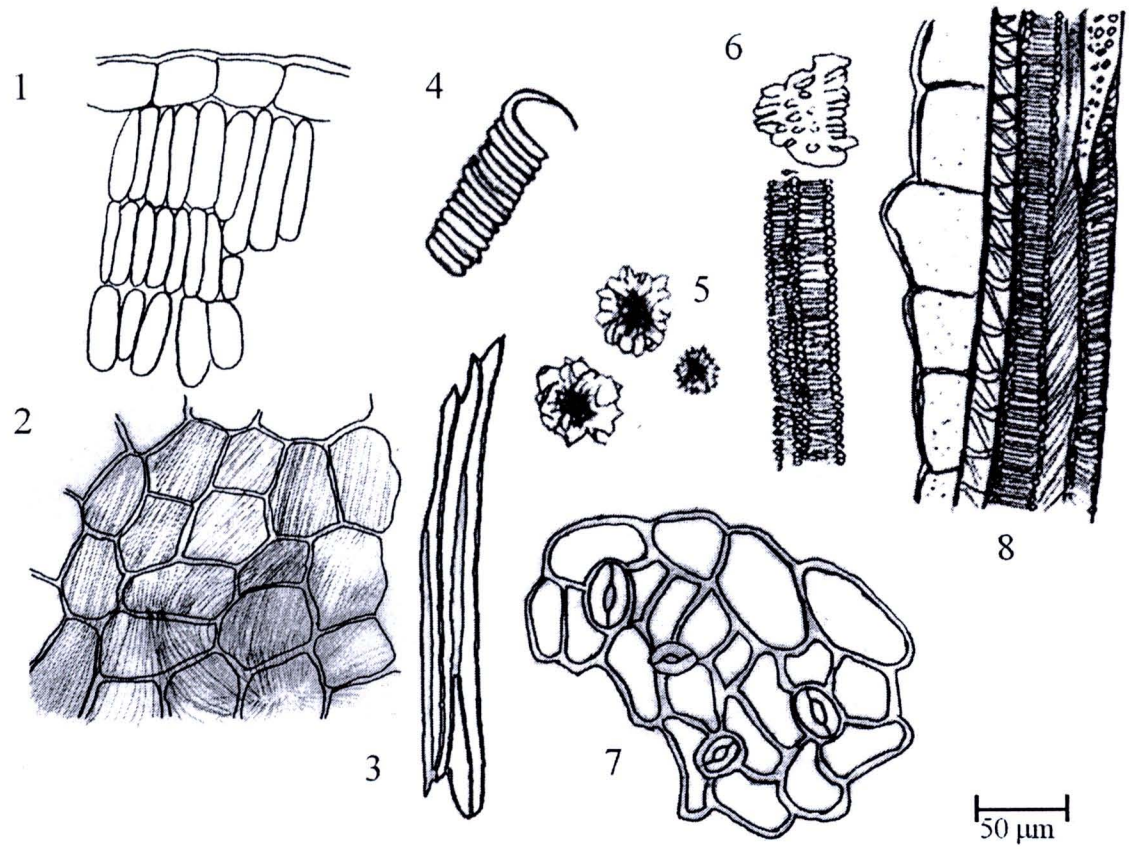

Figure 3.6 Powdered drug of the leaf of *L. speciosa*.

1. sectional view of lamina.
2. upper epidermis in surface view, showing the striated cuticle.
3. group of lignified fiber.
4. spiral vessel.
5. rosette aggregate crystals of calcium oxalate.
6. reticulate vessel.
7. lower epidermis in surface view showing anomocytic stomata.
8. fibrovascular tissue and parenchyma cell.

3.2.2 The constant values of leaves

The constant values of leaves data are shown in **Appendix B**. The leaves constant values were summarized (**Table 3.3**). The outline drawing of constant value of leaves including vein structure and four epidermal cells with underlying palisade cells (**Figure 3.7 – 3.8**).

3.2.3 TLC analysis

The result of one-dimensional TLC patterns of methanol extract with dichloromethane-water partitions of *L. speciosa* leaves were shown as follows:

The chromatogram, R_f value and color detection of methanolic extract which used chloroform:acetone (4:1) (**Figure 3.9, Table 3.4**) and chloroform:methanol (95:5) (**Figure 3.10, Table 3.5**) as solvent system were displayed.

3.2.4 Preliminary phytochemical test

The results of various tests of powdered *L.speciosa* leaves were shown in **Table 3.6**. It revealed that *L. speciosa* leaves might contain triterpene and steroid, phenolic compound.

3.2.5 Physico-chemical parameter

The physicochemical constant of *L. speciosa* leaves which were collected and purchased from traditional drugstores are shown as in **Table 3.7**. Raw data was tabulated in **Appendix C.**

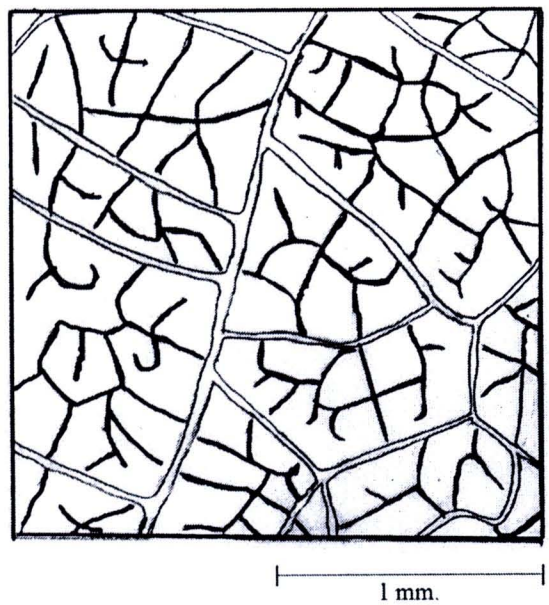

Figure 3.7 Vein-islet and veinlet termination of the *L. speciosa* leaves.

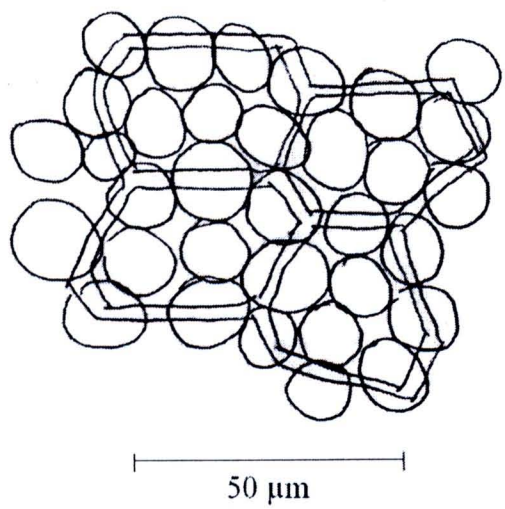

Figure 3.8 Four upper contiguous epidermal cells with underlying palisade cells of *L. speciosa* leaves.

Table 3.3 The constant values of *L. speciosa* leaves.

Sample no.	Stomatal index	Stomatal number	Palisade ratio	Vein-islet number	Veinlet termination number
LS1	14.22±1.26	261.67±17.32	5.31±0.25	14.88±0.62	6.40±0.86
LS2	13.99±0.97	257.43±17.18	5.35±0.31	14.59±0.74	6.94±0.61
LS3	14.11±1.22	261.67±18.48	5.31±0.27	14.93±0.66	6.63±0.84
LS4	14.24±1.59	264.97±22.88	5.27±0.23	14.95±0.74	6.37±0.82
LS5	13.75±1.35	258.84±20.73	5.28±0.28	15.06±0.68	6.62±0.89
LS6	13.71±1.57	257.43±25.41	5.34±0.23	14.08±0.94	6.31±0.88
LS7	13.67±1.48	255.54±22.88	5.31±0.28	13.98±0.95	5.98±0.78
LS8	13.76±1.61	259.31±29.56	5.32±0.25	14.83±0.68	6.40±0.88

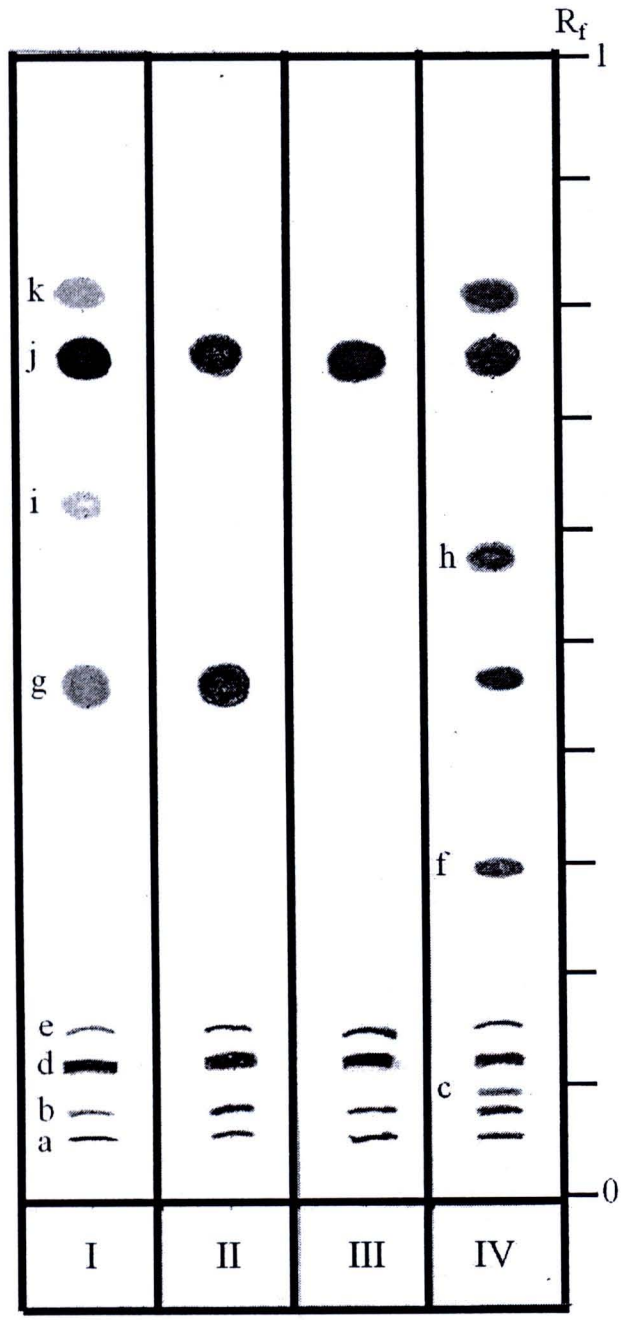

Figure 3.9 TLC patterns of methanolic extract of *L. speciosa* leaves which using chloroform and acetone (4:1) as solvent system.

I. visible in daylight

II. detection under UV 254 nm

III. detection under UV 365 nm

IV. detection with anisaldehyde-sulfuric acid and heat

Table 3.4 R_f values of components in methanol extract of the leaves of *L. speciosa* leaves. Chloroform and acetone (4:1) was used as solvent system.

spot	R_f	Detection with			
		Visible day light	UV 254	UV 365	Anisaldehyde – sulfuric acid TS
a	0.06	Green	Quenching	Red	Olive green
b	0.08	Pale grey	Quenching	Red	green
c	0.09	-	-	-	light purple
d	0.13	Green	Quenching	Red	green
e	0.15	Grey	Quenching	Red	green
f*	0.29	-	-	-	Purple
g	0.44 – 0.47	Yellow	Quenching	-	Blue purple
h	0.55 – 0.58	-	-	-	Pink purple
i	0.60 – 0.61	Yelow	-	-	-
j	0.72 – 0.75	Brownish green	Quenching	Red	emerald green
k	0.78 – 0.8	Yellow	-	-	Reddish pink

* f = corosolic acid

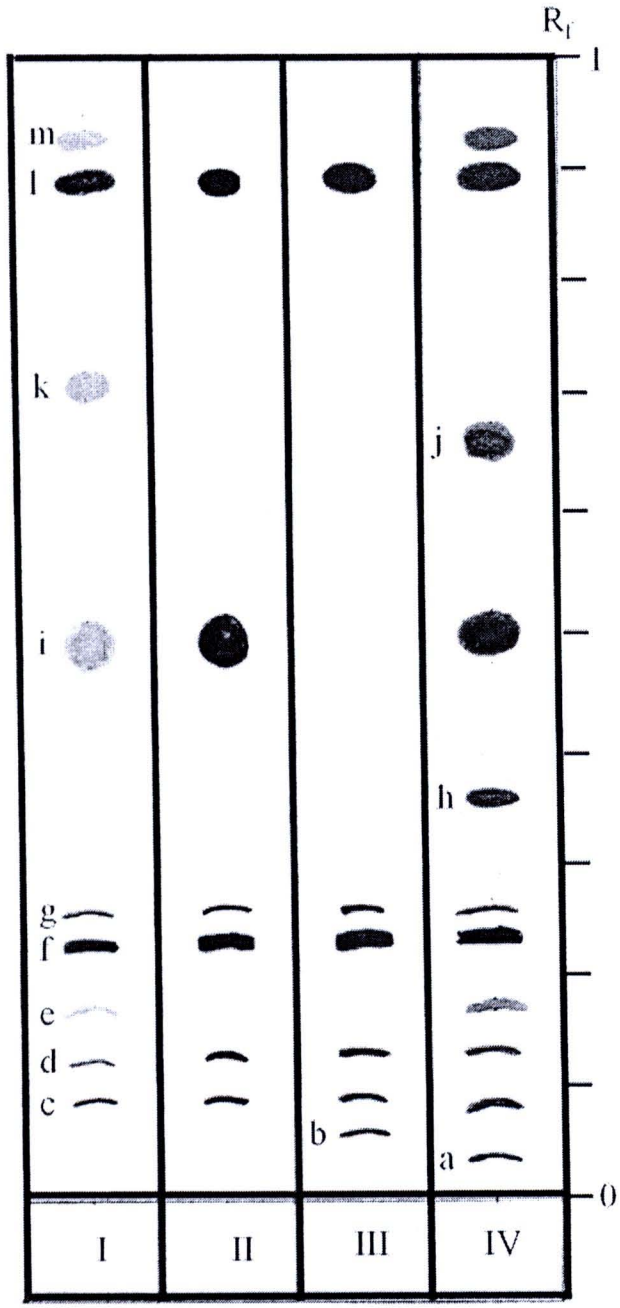

Figure 3.10 TLC patterns of methanolic extract of *L. speciosa* leaves which using chloroform and methanol (95:5) as solvent system.

I. visible in daylight

II. detection under UV 254 nm

III. detection under UV 365 nm

IV. detection with anisaldehyde-sulfuric acid and heat.

Table 3.5 R_f values of components in methanol extract of the leaves of *L. speciosa* leaves. Chloroform and methanol (95:5:1) was used as solvent system.

spot	R_f	Detection with			
		Visible day light	UV 254	UV 365	Anisaldehyde – sulfuric acid TS
a	0.04	-	-	-	Purple
b	0.06	-	-	Red	-
c	0.09	Green	Quenching	Red	Olive green
d	0.13	Pale grey	Quenching	Red	green
e	0.16	Yellow	-	-	light purple
f	0.22	Green	Quenching	Red	green
g	0.26	Grey	Quenching	Red	green
h*	0.35	-	-	-	Purple
i	0.48 – 0.53	Yellow	Quenching	-	Blue purple
j	0.65 – 0.67	-	-	-	Pink purple
K	0.71 – 0.73	Yelow	-	-	-
l	0.86 – 0.88	Green	Quenching	Red	Emerald green
m	0.93	Yellow	-	-	Reddish pink

*h = corosolic acid

Table 3.6 Chemical test of powdered *L. speciosa* leaves.

Detection method	Positive test	Results
Froth test	Honey comb froth which persists for at least 30 minutes	Negative (less than 30 min.)
Ferric chloride TS	Green or blue precipitation	Positive (violet precipitation)
Gelation solution	Gelatin precipitation	Positive
Shinoda's test	Pink to red color	Negative (green color)
Alkaloid test	Orange precipitate (Dragendorff's reagent) Cream white precipitate (Mayer's reagent)	Negative Negative
Liebermann-Burchard test	Red, pink, purple or violet and green	Positive (violet and green)

Table 3.7 Physicochemical values (% w/w) in 17 samples of *L. speciosa* leaves.

Sample No.	Loss on drying	Moisture content	Total ash	Acid-insoluble ash	Ethanol extractive value	Water extractive value	CH_2Cl_2 extractive value
LS1	7.4506±0.0328	7.1357±0.0890	7.1198±0.0669	1.4089±0.0023	8.5289±0.0268	12.1505±0.0922	3.3529±0.0350
LS2	6.7272±0.0277	6.3677±0.2119	7.3809±0.0793	1.4822±0.0012	12.0931±0.0061	13.6080±0.1362	5.2492±0.0364
LS3	6.5125±0.0327	6.4883±0.0612	7.2116±0.0724	1.4388±0.0026	11.2704±0.1807	13.6328±0.0138	4.7024±0.0303
LS4	7.7544±0.1899	7.5303±0.1325	7.7502±0.0412	1.6430±0.0050	7.5656±0.2915	11.7109±0.0109	2.4181±0.0079
LS5	8.1594±0.2746	7.4736±0.1621	8.4600±0.0330	0.4265±0.0003	7.3989±0.0487	12.0077±0.0490	2.3313±0.0077
LS6	8.4266±0.2519	8.0791±0.0937	8.8701±0.0420	1.0559±0.0005	7.1475±0.0863	12.4645±0.0981	2.2393±0.0100
LS7	8.8310±0.2827	8.5233±0.2093	8.2532±0.0574	2.4863±0.0131	8.6499±0.1522	10.0191±0.0248	2.2478±0.0238
LS8	8.4989±0.0705	8.2181±0.1029	6.8888±0.0076	0.1844±0.0038	7.2665±0.2898	13.6106±0.0167	2.2746±0.0151
LS9	9.9292±0.0228	9.2675±0.1384	6.3839±0.0123	0.9033±0.0054	11.2065±0.2295	14.8795±0.0232	2.6987±0.0100
LS10	9.4312±0.0501	9.0519±0.1130	6.5786±0.0110	0.8043±0.0052	12.3388±0.3776	15.6160±0.1205	2.4240±0.0053
LS11	8.2098±0.1590	8.0925±0.0510	6.7901±0.0392	1.3885±0.0086	10.7395±0.3114	17.8087±0.0340	2.4377±0.0121
LS12	9.5513±0.1092	8.3635±0.0625	7.4391±0.0660	2.3530±0.0303	12.8143±0.3510	16.1044±0.0568	3.5090±0.0101
LS13	8.7763±0.1914	8.3481±0.2232	7.1828±0.0383	1.1671±0.0013	7.4315±0.2803	12.5076±0.0838	2.6322±0.0154
LS14	8.3579±0.2419	8.1696±0.0973	8.4065±0.0160	0.9215±0.0016	9.4392±0.3424	12.4212±0.0165	2.4776±0.0204
LS15	7.6689±0.2470	7.0101±0.1395	6.9669±0.0717	1.5188±0.0092	5.2661±0.0383	11.9048±0.0841	2.6660±0.0207
LS16	7.6987±0.0798	7.6283±0.0981	7.1410±0.0523	0.3522±0.0019	5.7746±0.1221	10.6390±0.0268	2.7547±0.0233
LS17	7.6525±0.2718	7.5874±0.1740	8.2096±0.0408	1.1842±0.0020	8.4941±0.2344	13.1366±0.0396	3.6354±0.0110

Each values (in % w/w) represented the mean ± SD of analysis (The experiments were done in triplicate)

3.2.6 Corosolic acid content in *L. speciosa* leaves

A characteristic of HPLC-DAD chromatogram of corosolic acid standard and methanol crude extracts of *L. speciosa* leaves monitored at 204 nm (**Figure 3.11A, 3.11B** respectively). Retention time of peak obtained with this chemical marker was used to identify the corresponding peaks in methanol crude extracts of *L. speciosa* plant. The retention time of its standard was 8.681 min.

Beside relative retention time, corosolic acid identification was confirmed by carried out through standard addition. It was identified by co-elution after spiking of crude extract with standard corosolic acid (**Figure 3.11C**)

The calibration curve showed good linearity, with a regression coefficient of 0.9974. The regression equation for corosolic acid was $y = 6407.9x - 10321$ (**Figure 3.12**). The contents in methanol extracts were calculated with this regression equation obtained from calibration curve. The results were tabulated (**Table 3.8**). The quantity of the corosolic acid varied. It was ranged from 0.0010 to 0.7496% dry weight.

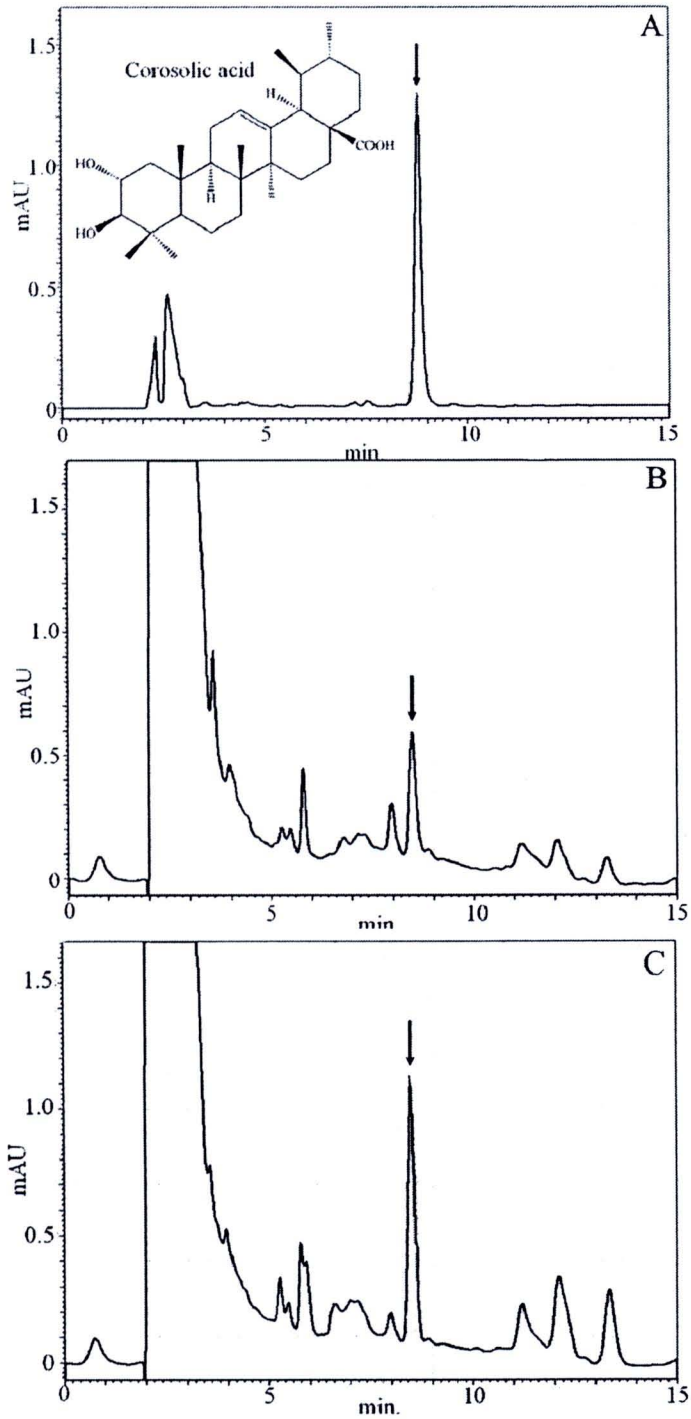

Figure 3.11 HPLC/DAD chromatograms detected at 204 nm of:

a) corosolic acid,

b) crude methanolic extract of *L. speciosa* leaves, and

c) crude methanolic extract spiked with corosolic acid.

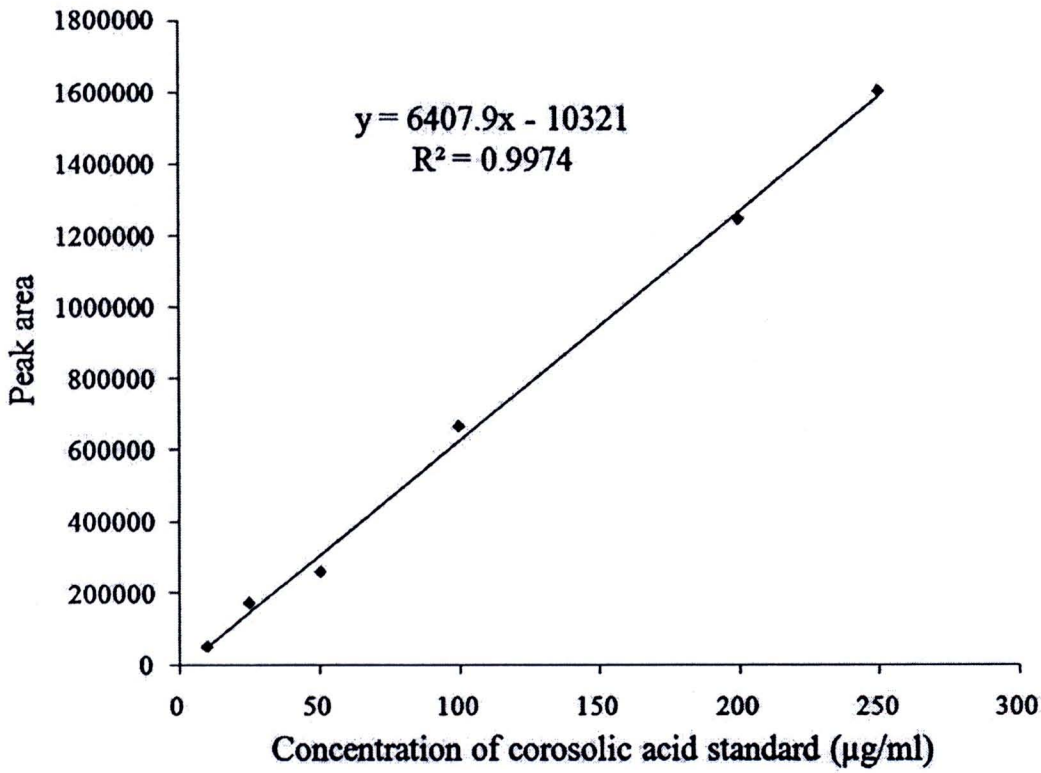

Figure 3.12 Standard curve of corosolic acid, peak area at 204 nm.

Table 3.8. Corosolic acid content (% w/w) in seventeen *L. speciosa* leaves.

Sample no.	Corosolic aid content (%w/w)*
LS1	0.1290 ± 0.0028
LS2	0.1599 ± 0.0011
LS3	0.1175 ± 0.0030
LS4	0.7496 ± 0.0077
LS5	0.0790 ± 0.0019
LS6	0.1080 ± 0.0029
LS7	0.0638 ± 0.0024
LS8	0.1371 ± 0.0033
LS9	0.0109 ± 0.0004
LS10	0.0101 ± 0.0003
LS11	0.0100 ± 0.0003
LS12	0.0281 ± 0.0003
LS13	0.1073 ± 0.0009
LS14	0.0830 ± 0.0013
LS15	0.1928 ± 0.0027
LS16	0.1415 ± 0.0011
LS17	0.0959 ± 0.0024

* Each value represented the mean ± SD of analysis (The experiments were done in triplicate).

3.3 Discussion

Currently, there is an emphasis on the standardization of medicinal plant materials for their therapeutic potentials. The modern techniques available make the identification and evaluation of crude drugs by pharmacognostic studies reliable, accurate and inexpensive. According to the WHO, determinations of macroscopic and microscopic characteristics are the first steps towards establishing the identity and the purity of such materials, and these steps should be carried out before any further tests are undertaken.

This study dealt with the investigation of pharmacognostic specification of *Lagerstroemia speciosa* leaves. Macroscopic characters of *L. speciosa* from several sources in this study are slightly differences in length, due to the preparation of these crude drugs in each source, while the microscopic characters are similar including the TLC pattern.

Microscopically, the majority tissues were found as stomata, vessel, lamina view of leave and rosette aggregate calcium oxalate crystals. Leaf measurements were determined from 8 samples and statistic process was used for discussion of those particular data. The 95% confidence interval of those leaf measurements were calculated using total data of 8 samples (240 data) and representative information of them are shown in **Table 3.9**.

Table 3.9 The constant number of *L. speciosa* leaves.

	Data interval	Mean ± SD.	95% confidence interval
Stomatal index	10.71 – 18.25	13.75 ± 1.39	13.75 – 14.10
Stomatal number	212.16 – 325.32	259.61 ± 22.09	256.80 – 262.42
Palisade ratio	4.5 – 6.0	5.31 ± 0.26	5.27 – 5.34
Vein-islet number	11.50 – 17.00	14.67 ± 0.84	14.57 – 14.78
Veinlet termination number	4.25 – 8.25	6.45 ± 0.85	6.35 – 6.56

The constant values of leaf are used as character for identification concerning their constant value in each species. It is interesting to note that the represent data

should be used to authentication the banaba leaves. These data was the reliable information.

The thin layer chromatographic fingerprinting was performed to identify the individual substances in the mixture and to determine the purity of these substances. The TLC chromatogram showed characteristic fingerprint profiles that could be used as markers for quality evaluation and standardization of crude drug. The R_f values indicate the position at which the substance was located on chromatogram. The Rf value is widely recognised as a guide for the identification of medicinal plants. However, it is difficult to obtain exactly reproducible R_f values as a result of the variety of influences operation during chromatography.

Phytochemical screening was used to detect therapeutic compounds in the plants. Qualitative chemical examination of *L. speciosa* leaves revealed the presence of tannins, triterpenes and steroids, as previously reported (Murakami *et al.*, 1993; Ragasa *et al.*, 2005; Suzuki *et al.*, 1999; Unno *et al.*, 2004; Yamaguchi *et al.*, 2006). The phytochemicals detected in this investigation have a great deal of medicinal importance. The presence of tannins suggests the ability of this plant to play a major role in the treatment infectious diseases (Asquith and Butler, 1986), as tannins have shown anti-oxidant and protein-precipitating properties (Ruch *et al.*, 1989). Triterpenoids and sterols possess anti-inflammatory and anti-tumour activities (Lui *et al.*, 1995).

The physical constant evaluation of the crude drugs is an important parameter in detecting adulteration or improper handling of drugs. *L. speciosa* leaves from several locations were determined and concluded the data as an estimated percentage values. The physicochemical parameters could be used to form the standardization of this drug as shown in **Table 3.10**. The moisture content was employed to control the water in crude drug. On the other hand, loss on drying controlled the loss in weight (due to water and other volatile materials) of crude drug. The moisture content of the herbal raw materials should be determined and be controlled to make the solution of definite strength. The moisture content of crude drugs should be minimized in order to prevent spoilage due to microbial contamination or decomposition of chemical. The objective of drying of fresh materials was to fix their constituents i.e. to check enzymatic or hydrolysis reaction that might alter the chemical composition and to reduce the weight and bulk. The excessive content of water in crude drugs and

temperature were the promoter factors of fungal and bacterial growth which caused the spoilage. Therefore, drying should be accomplished as rapidly as is possible with good practices.

The ash of any organic material was composed of non-volatile inorganic components. The controlled incineration resulted in ash residue consisting of an inorganic material (metallic salt and silica). Ash content were accountable for controlling the admixture of foreign inorganic matter due to their storage, container or intentional add to disguise the appearance of crude drug. We could detect the extant of adulterations as well as set up the quality and purity of crude drug by using this method. The acid-insoluble ash gave an idea about the amount of silica present, especially as sand and siliceous earth.

The determination of ethanol-soluble, dichloromethane-soluble and water-soluble extractive values were used to control the constituents of crude drugs which caused inferiority from many factors such as moisture content, temperature, harvesting, drying process, kept duration and storage.

Table 3.10 General specification of *L. speciosa* leaves.

	Data interval (%)	**Mean ± SD (%)**
Loss on drying	6.4844 - 9.9510	8.2141 ± 0.9300
Moisture content	6.1440 - 9.3782	7.8593 ± 0.8141
Total ash	6.3714 - 8.9072	7.4725 ± 0.7277
Acid-insoluble ash	0.1700 - 2.4995	1.2176 ± 0.6223
Ethanol-soluble extractive value	5.2745 - 13.1487	9.0280 ± 2.2937
Dichloromethane extractive value	2.2992 - 5.2795	2.9442 ± 0.8827
Water-soluble extractive value	9.9998 - 17.8247	13.1895 ± 1.9934

In the present study, corosolic acid was quantified from the leaves of *L. speciosa* using HPLC. Corosolic acid could be used as a chemical marker for the standardisation of *L. speciosa*. Under the present chromatographic conditions, the run time for each sample was 15 min. The retention time of corosolic acid was 8.681 min. HPLC analyses of all samples were similar in pattern, but the quantity of corosolic acid ranged from 0.0100 to 0.7496% w/w. The fresh sample collected in Bangkok showed a higher corosolic acid content than those from Saraburi and Chiang Mai. The

crude drug sample from Lampang was greenish in colour, representing a high concentration of corosolic acid, while the lowest content was found in dry leaves with a brownish colour. The difference in corosolic acid content in the crude drugs may be due to the age of plants, the geographic conditions where the leaves were cultivated, the duration of storage, differences in the drying process, or genetic variations. Moreover, the season of collection and the storage conditions may also lead to fluctuations in the corosolic acid content (He *et al.*, 2009).

The results obtained from this study will play a significant role in setting standards for this medicinal plant. This study provides useful information for the identification of *L. speciosa* leaves and will help those who handle this plant to maintain its quality. Thus, the standards presented in this study will help minimize the adulteration of *L. speciosa* samples and will be of great use for future researchers in selecting correct herbal specimens. In addition, the results of this investigation may be useful in the preparation of a monograph for this plant.