CHAPTER IV

EXPERIMENTAL

Scopes of investigation

1. Identification of authentic (+)-tetrandrine.

2. Study of the macroscopic and microscopic characters of *Cyclea barbata* roots.

3. Thin-layer chromatographic patterns (one-dimensional and two-dimensional TLC) of methanol extract of *Cyclea barbata* roots from several sources.

4. Quality controls of crude drugs, which were collected from several sources according to the Thai Herbal Pharmacopoeia: loss on drying, moisture content, total ash, acid-insoluble ash and extractive value.

5. Quantitative determination of tetrandrine in *Cyclea barbata* roots from several sources by TLC-densitometer and Capillary Electrophoresis.

Part I. Identification of standard (+)-tetrandrine

Materials

a) 98% (+)-Tetrandrine (Analytical grade, Aldrich Chem.)

b) Chloroform (Analytical grade, Lab-scan Asia)

c) Deuterated chloroform (Analytical grade, J.T. Baker chemical)

Proton and Carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C-NMR) spectra

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained on a JEOL JNM-ECP400 Spectrometer (Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan)

Solvent for NMR spectra was deuterated chloroform ($CDCl_3$). Chemical shifts were reported in ppm scale using the chemical shift of the solvent and internal standard, tetramethylsilane (TMS), as the reference signals.

Optical rotations

Optical rotations were measured on a Perkin Elmer 341 polarimeter (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University). Solvent for optical rotation was chloroform.

Part II. The macroscopic and microscopic characterization of *Cyclea barbata* roots

Materials.

a) Crude drugs, which were purchased from 7 traditional drugstores in Thailand (Table 4)

b) Chloral hydrate B.P.

c) Distilled water

d) Iodine solution

e) Phloroglucinol solution-hydrochloric acid

Table 4. Krung Kha Mao which were purchased from traditional drugstores.

Number of samples	Place	Code	Purchased date
1	Bangkok province, Jao-krom-per drugstore	J-1	August, 2004
2	Bangkok province, Vechapong drugstore	V-1	August, 2004
3	Ubonrachathanee province, Ae-sare-osod drugstore	U-1	August, 2004
4	Nakornrachasima province	N-1	August, 2004
5	Songkla province, Baan-chaiya-osod drugstore	S-1	August, 2004
6	Surachthanee province, Peng-un-tuang drugstroe	R-1	August, 2004
7	Chiang-Mai province	C-1	August, 2004

Procedure

Roots of *Cyclea barbata* were collected from the the Faculty of Pharmaceutical Sciences, Chulalongkorn University, in August, 2004. These plant materials were authenticated by comparison with herbarium specimens in Botanical Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperative

The authentic roots were chopped into small pieces and dried in a hot air oven at 50 °C, then ground and passed through a sieve with mesh number 60, kept in a well-closed container. The purchased samples also did as described above.

The macroscopic method:

Determine the size, shape, color, odor and taste of the seven crude drugs, purchased from traditional drugstore.

The microscopic method: plant histology

The drawings were made using microscope and drawing attachment. In preparing the drawings the objective has been to emphasize the most diagnostic characters by which each powder may be identified, particularly within the morphological group to which it belongs. The cells and cell inclusion were taken photographs by Photo-micrographic equipment which is attached to microscope. Most of particles were drawn from preparations of the powdered, which had been first mounted in water. Subsequently sections should be cleared by means of chloral hydrate and some stained as follows.

Chloral hydrate solution BP

Reagent: Dissolved chloral hydrate 80 g in 20 ml water, using gentle heat if necessary.

Chloral hydrate is a valuable and widely used clearing agent. This dissolves starch, proteins, chlorophyll, resins and volatile oils, and causes shrunken cells to expand. Chloral hydrate may be used, not only for sections but also for whole leaves, flower, pollen grain etc. It does not dissolve calcium oxalate and is therefore a good reagent for detection of the crystals.

Iodine solution

Reagent: Mixed 2 g of iodine and 3 g of potassium iodine and add about 5 ml of water, alginate until dissolve, slowly dilute with water to 100 ml.

This gives a blue color with starch and hemicelluloses.

Phloroglucinol solution and Hydrochloric acid

Phloroglucinol solution: Dissolve 1 g phloroglucin in 100 mL of 95% ethanol.

Hydrochloric acid: concentrate Hydrochloric acid.

A solution of Phloroglucinol with hydrochloric acid as a test for lignin. Mounted the section in the solution of Phloroglucinol and allowed to stand for about 2 minutes; removed any alcohol which has not evaporated with a piece of filter paper; added concentrated hydrochloric acid covered and examined. All lignified walls stain pink or red.

Hydrochloric acid is a powerful clearing agent and it must be remembered that it will dissolve many cell contents, including calcium oxalate. To prevent damage to the microscope either by liquid contact or by vapors, preparations mounted in concentrated hydrochloric acid should be free of excess acid and must be removed from the microscope stage as soon as possible.

Part III. Thin-layer Chromatographic patterns of root extract

Materials:

a) Authentic sample and crude drugs samples as part II

b) (+)-Tetrandrine 98% (Analytical grade, Aldrich Chem.)

c) Dichloromethane (Commercial grade, INEOS Chlor.)

d) Methanol (Commercial grade, INEOS Chlor.)

e) Ethylacetate (Commercial grade, INEOS Chlor.)

f) Hexane (Commercial grade, INEOS Chlor.)

g) Toluene (Commercial grade, Modern chemical)

h) Chloroform (Commercial grade, INEOS Chlor.)

i) 28% Ammonia solution (Analytical grade, Lab-scan Asia)

j) aluminium sheet silica gel 60 F254 (precoated, Merck)

k) Anisaldehyde acid reagent (AS)

l) Dragendorff TS 2, Modified

Procedure

a) The dry roots 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.

b) Prepared 0.02% tetrandrine in methanol as control.

One-dimensional thin-layer chromatography

a) Five grams of dried stem powdered drug were macerated in 10 ml of methanol for 24 hours, then filtered through filter paper (Whatman, No.1) and kept in well-closed container prior to spot on TLC plate.

b) Selected suitable solvent system that could provide the pattern for separation and identification.

System 1: Dichloromethane: Hexane: Methanol: Ammonia (5:4:1:0.3)

System 2: Ethylacetate: Hexane: Methanol: Ammonia (4:4:1:0.3)

System 3: Toluene: Ethylacetate: Methanol: Ammonia (10:10:5:0.3)

System 4: Chloroform: Methanol: Ammonia (5: 1: 0.3)

c) Spotted the extract amount 4 µl of each sample by micropipette on TLC plate and allowed to dry by air.

d) Developed the chromatogram after solvent system saturating in chamber. In system 1, developed the chromatogram after first solvent system saturating in chamber and removed the plate from the tank, allowed to dry by air. Re-developed the same plate in the same direction in the second solvent system then removed the plate and allowed to dry by air. The developing distance was 15 cm at room temperature (30-35°C).

e) Detection of the chromatogram.

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.

Treatments and detection reagents applied were as follows:

Colour in Daylight.

In order to provide a uniform light intensity over the whole chromatograms, a 20-Watts (Daylight) Fluorescence lamp was used.

Fluorescence

The chromatograms were examined under 254 nm and 365 nm ultraviolet light in UV chamber.

Anisaldehyde acid reagent (AS)

Reagent: 0.5 ml Anisaldehyde was mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulfuric acid, in that order.

The reagent has only limited stability, and was no longer usable when the color has turned to red-violet.

Procedure: The TLC plate was sprayed with Anisaldehyde acid reagent about 10 ml, heated at 100°C for 5-10 mm, and then evaluated under UV-365 nm.

Color: various color

Detection: Essential oil, pungent principles, bitter principles, *etc*.

Dragendorff TS 2, Modified

Solution A: Dissolved 0.85 g of bismuth subnitrate in a mixture of 40 ml of water and 10 ml of acetic acid.

Solution B: Dissolved 8 g of potassium iodide in 20 ml of water.

Mixed 5 ml each of solution A and solution B with 20 ml of glacial acetic acid and added sufficient water to make 100 ml.

Color: orange

Detection: Alkaloid

f) Recording of R_f Value and Colour

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.

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

Two-dimensional thin-layer chromatography

a) Crude extract of authentic sample was prepared as one-dimensional chromatography.

b) Solvent systems in this present work, two thin-layer chromatogram patterns of chemical constituents were illustrated for the plants. Two difference pairs of solvent systems, as listed in Table 5, were used for developing the chromatograms.

c) Spotted the extract amount 30 µl of each sample by micropipette on TLC plate at the left side angle of plate and allowed to dry by air.

Table 5. Developing solvent system for two-dimensional thin-layer chromatography

Pattern	Dimension	Solvent System
A	1	Dichloromethane: Hexane: Methanol: Ammonia (5: 4: 1: 0.3)
	2	Ethylacetate: Hexane: Methanol: Ammonia (4: 4: 1: 0.3)
B	1	Toluene: Ethylacetate: Methanol: Ammonia (10: 10: 5: 0.3)
	2	Chloroform: Methanol: Ammonia (5: 1: 0.3)

d) Developed the chromatogram after first solvent system saturating in chamber. The developing distance is 15 cm, removed the plate from the tank and allowed to dry by air. Re-developed the same plate in the second direction commenced in the perpendicular direction with the second solvent system until ascended 15 cm, removed the plate and allowed to dry by air.

e) Detection of the chromatogram

- Visible under daylight
- Fluorescence under UV 254 and 365 nm
- Detection with Dragendorff's reagent
- Detection with Anisaldehyde-sulfuric acid reagent

f) Recording R_f value for two-dimensional chromatography

R_f grid was used as an aid in recording the values as shown in Figure 8. The R_f values were coded as follows (Thanapat, 1995, and Supattra, 2001).

00 (Zero, Zero)	Corresponds to R_f	0.000 to .075
08	"	0.075 to.I25
10	"	0.I25 to.175
18	"	0.I75 to.225
20	"	0.225 to .275
28	"	0.275 to .325
30	"	0.325 to .375
38	"	0.375 to .425
40	"	0.425 to .475
48	"	0.475 to .525
50	"	0.525 to .575
58	"	0.575 to .625
60	"	0.625 to .675
68	"	0.675 to .725
70	"	0.725 to .775
78	"	0.775 to 825
80	"	0.825 to .875
88	"	0.875 to .925
90	"	0.925 to 1.000

The colors were arranged in a continuous disc system according to the solar spectrum colors. It was often difficult to indicate color with precision (e.g. shades of dark and pale violet), but moderate errors would not invalidate the present system. Number 1 to 7 were used, the first digit for the basic color itself and the second digit "0" (zero) to "5" for light or pale shade to darker such as (Vichiara Jirawongse, 1964)

10	pink	13	purplish-red	15	red
20	pale orange	21	pinkish-orange		
		24	yellowish-orange	25	orange
30	brown			35	dark brown
				38	yellowish-brown
40	light yellow			45	yellow
50	green	53	brownish-green	55	dark green
60	blue			65	dark blue
70	heliotrope	73	bluish-violet	75	violet
80	gray			85	black
90	quenching			95	strong quenching (under UV)
00	nil				

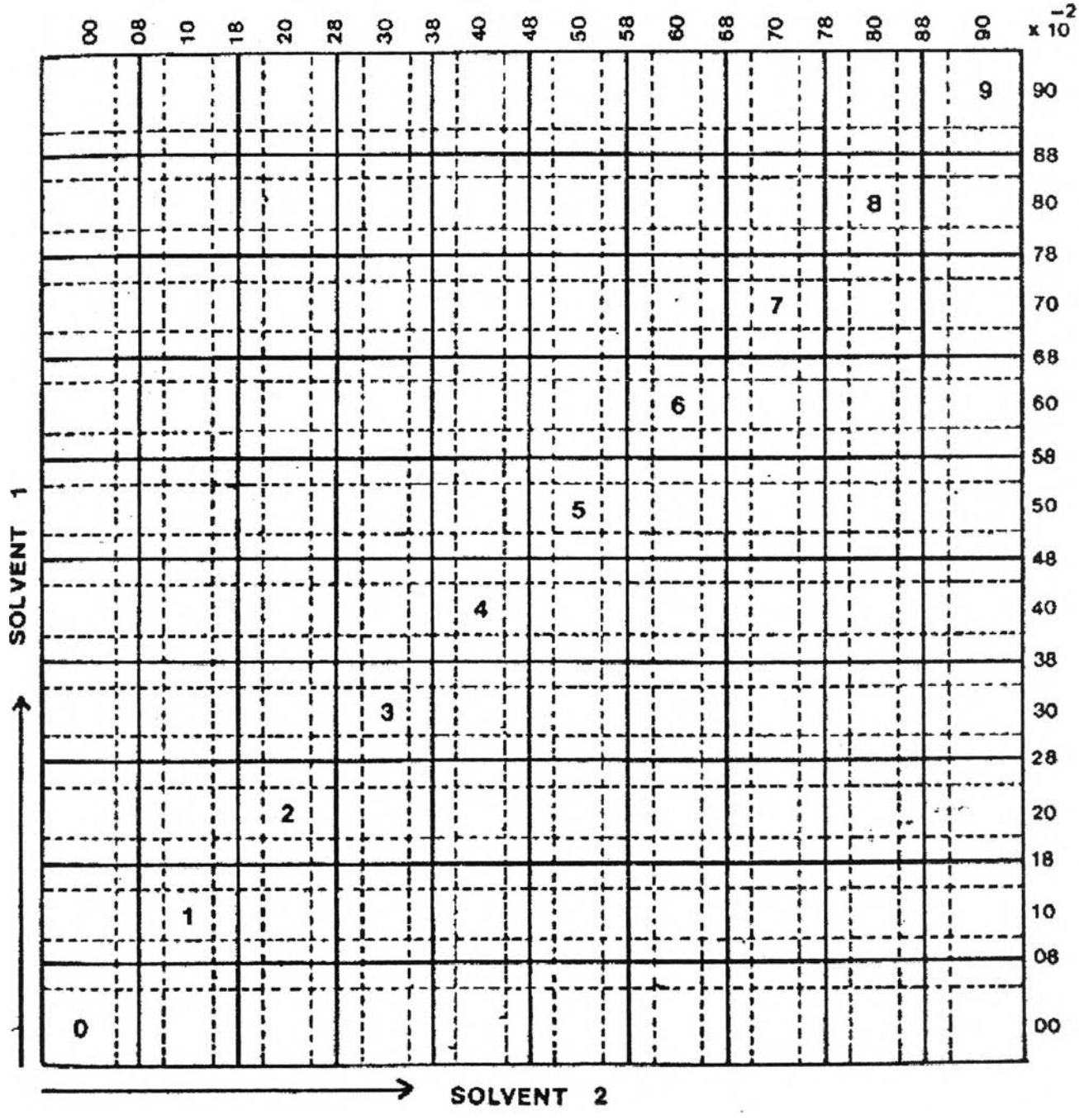

Figure 8. R_f grid (1:1)

Part IV. General quality controls

Materials:

a) Crude drug samples as part II

b) Hydrochloric acid (Lab-scan Asia)

c) Ethanol 95% (INEOS Chlor.)

d) Chloroform (INEOS Chlor.)

e) Toluene (Modern chemical)

Procedure

The dry roots 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.

Loss on drying (Thai Herbal Pharmacopoeia, 1995)

Conducted the determination on 2 to 5 grams of crude drugs, previously mixed and accurately weighed. Tare a glass-stopper, shallow weighing bottle that has been dried until constant weight or 30 minutes under the same condition to be employed in the determination. Put the test specimen in the bottle, replaced the cover, and accurately weighed the bottle and the contents. Dried the test specimen at the temperature 105°C to constant weight. Upon opening the chamber closed the bottle promptly and allowed it to come to room temperature in the desiccators before weighing. Calculated the percentage of loss on drying with reference to the air-dried substance.

Total ash and acid-insoluble ash (Thai Herbal Pharmacopoeia, 1995)

Total ash

Placed 2 to 4 g of the ground sample accurately weighed in a suitable tare crucible (usually of platinum or silica), previously ignited, cooled and weighed. Incinerated the sample by gradually increasing the temperature, not exceeding 450°C, until free from carbon; cooled and weighed. If a carbon-free ash cannot be obtained in this way, cooled the crucible and moisten the residue with about 2 ml of water or a saturated solution of ammonium nitrated. Dried on a water-bath and then on a hot plate and incinerate to constant weight. Calculated the percentage of total ash with reference to the air-dried substance.

Acid-insoluble ash

Boiled the total ash for 5 minutes with 25 ml of diluted hydrochloric acid, collected the insoluble matter on an ashless filter paper, washed with hot water until the filtrate was neutral, and ignited at about 500°C. Calculated the percentage of acid-insoluble ash with reference to the air-dried substance.

Extractive value (Thai Herbal Pharmacopoeia, 1995)

Ethanol-soluble extractive

Macerated 5 g of the air-dried drug, coarsely powdered, with 100 ml of ethanol of the specified strength in a closed flask or 24 hours, shaking frequently during the first 6 hours and then allowing standing for 18 hours. Filtered rapidly, taking precautions against loss of ethanol, evaporated 20 ml of the filtrate to dryness in a tare, flat-bottomed, shallow dish and dried at 105°C to constant weight. Calculated the percentage of ethanol-soluble extractive with reference to the air-dried drug.

Water-soluble extractive

Proceeded as directed for ethanol-soluble extractive, but using chloroform water in place of ethanol.

Determination of water (Thai Herbal Pharmacopoeia, 1995)

Azeotropic Distillation Method

Apparatus The apparatus (Figure 9) consists of a glass 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) is graduated in 0.1 ml sub-divisions so that the error of reading is not greater than 0.05 ml. The source of heat is preferably an electric heater with rheostat control or an oil-bath. The upper portion of the flask and the connecting tube may be insulated with asbestos.

Cleaned the receiving tube and the condenser of the apparatus by a suitable method, thoroughly rinse with water, and dry.

Introduce 200 ml of toluene and about 2 ml of water into the dry flask. Distil for about 2 hours, allow cooling to room temperature and reading the water volume to an accuracy of 0.05 ml. Place in the flask a quantity of the substance, weighed to the nearest centigram, expected to give about 2 to 3 ml of water. If the substance is a pasty character, weigh it in a boat of metal foil. Add a few pieces of porous material and heat the flask gently for 15 minutes. When the toluene begins to boil, distil at the rate of 2 drops per second until most of the water has distilled over, and then increase the rate of distillation to about 4 drops per second.

When the water has all distilled over, rinse the inside of the condenser tube with toluene. Continue the distillation for 5 minutes, remove the heat, and allow the receiving tube to cool to room temperature and dissolved any droplets of water, which adhere to the walls of the receiving tube. When the water and toluene have completely

separated, read the volume of water and calculate 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.

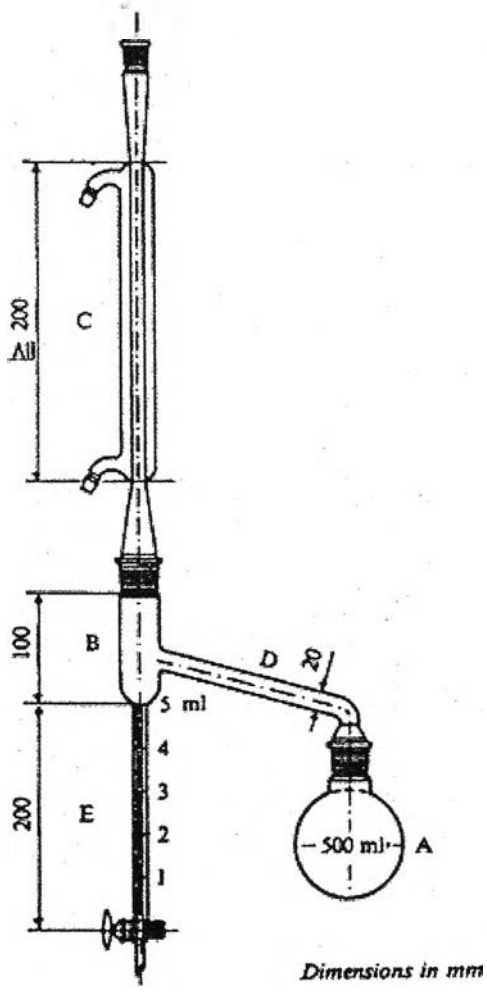

Figure 9. Azeotropic distillation method (Thai Herbal Pharmacopoeia, 1995).

Part V. Quantitative controls

Materials:

a) Crude drug samples as part II

b) 98% (+)-Tetrandrine (Analytical grade, Aldrich Chem.)

c) Methanol (Analytical grade, Lab-scan Asia)

d) 28% Ammonia hydroxide (Analytical grade, Lab-scan Asia)

e) Toluene (Analytical grade, Lab-scan Asia)

f) Ethylacetate (Analytical grade, Lab-scan Asia)

g) Aluminium sheet silica gel 60 F_{254} (1.0554.0001 precoated, Merck)

h) Phosphoric acid (Analytical grade, Farmitalia)

i) Triethanolamine (Analytical grade, Unilab)

j) Sodium hydroxide (Analytical grade, J.T. Baker chemical)

k) Ammonium acetate (Analytical grade, Merck)

l) Acetic acid (Analytical grade, B.H. chemical)

m) Acetonitrile (Analytical grade, J.T. Baker chemical)

Sample preparation

The dried roots of *Cyclea barbata* were ground to fine powder. After passing a sieve no. 40, two grams of the ground roots were weighed into the thimble of a Soxhlet extractor, wetted with 50 ml of a mixture of methanol-28% ammonia hydroxide (95:5) and left for 15 min. Then adding 250 ml of the same solvent started extraction and heating to 80°C. After 5 h the extract was cooled to room temperature, concentrated

with evaporator, transferred quantitatively to a volumetric flask and adjusted to 100 ml with methanol.

Procedure

Thin-layer chromatographic densitometric analysis

Five-microliter aliquot of each methanolic extract obtained from above was spotted on TLC plate. The stationary phase was silica gel 60 F_{254} plate (Merck, Damstadt, Germany) and the mobile phase was toluene: ethyl acetate: methanol: 28% ammonia hydroxide using the solvent ratio of 10:10:5:0.3. (+)-Tetrandrine in each sample in TLC plate was quantitated by the developed TLC-densitometric method and calculated based on its standard curve. TLC conditions for (+)-tetrandrine separation and densitometric analysis are described as follows.

Thin-layer chromatographic conditions for tetrandrine Separation

Technique	: one way, ascending, single development
Stationary phase	: aluminium sheet silica gel 60 F_{254} (precoated, Merck)
Plate size	: 10 x 20 cm^2
Solvent system	: toluene: ethyl acetate: methanol: 28% ammonia hydroxide (10:10:5:0.3)
Sample size	: 7 μl
Distance	: 8 cm
Temperature	: 25-30 ^{o}C
Detection	: Ultraviolet light 254 nm

Densitometric analysis

(+)-Tetrandrine spot obtained after thin layer chromatography was quantitative by the densitometric method as described below.

Instrumental model	: CAMAG TLC-Scanner II
Lamp	: Deuterium (D2)
Determination mode	: absorption
Scan width	: X = 6.00 mm
	Y = 0.40 mm
Sensitivity	: high
Wavelength detector	: 210 nm

Capillary electrophoresis (CE)

The accuracy and precision of TLC densitometric method was evaluated using capillary electrophoresis method, which could also be used for determination the (+)-tetrandrine content in *C. barbata* roots.

Capillary electrophoresis conditions

Tetrandrine in *C. barbata* roots was analysed by capillary electrophoresis. The conditions of CE are described below.

Instrumental model	: P/ACE System 5000 Beckman
Detector	: Ultraviolet light 214 nm
Capillary	: uncoated fuse silica capillary, 57 cm in length (50 cm to detector) x 50 μm I.D.
Temperature	: 25 ^{o}C

Voltage	: 30 kV
Injection pressure	: 0.5 psi for 2 sec
Running buffer solution	: prepared by 25 ml 200 mM Ammonia Acetate (methanol medium), 10 ml Acetic acid, and 20 ml Acetonitrile (ANC), then diluted to 100 ml with methanol solution in 100 ml volumetric flask, and filtered though a 0.45 μm filter.
Condition new capillary	: 1M NaOH for 15 min, 0.1M NaOH for 15 min, water for 15 min, 0.1M H_3PO_4 for 15 min , and running buffer for 15 min
Rinse capillary	: 0.1M NaOH for 20 min and running buffer for 20 min
Flush capillary	: 0.1M NaOH for 5 min, water for 5 min, and running buffer for 5 min.

Preparation of Standard Solutions for Calibration Curve

Five milligrams of (+)-tetrandrine was dissolved in 100 ml methanol to give 0.05 mg per ml stock solution. The stock solution was diluted to the concentration range of 0.0100-0.0225 mg/ml (50-112.5 ng/5 μl) for constructing their calibration curve of (+)-tetrandrine by TLC-densitiometric analysis. For capillary electrophoresis, the stock solution was diluted to concentration range of 0.01-0.05 mg/ml (10-50 mg/L) was used for constructing the calibration curve of (+)-tetrandrine.