ข้อกำหนดทางเภสัชเวทและเอกลักษณ์ทางโมเลกุลของใบอินทนิลน้ำ

นาย วรธัช ฐิติกรพงศ์

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

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PHARMACOGNOSTIC SPECIFICATION AND MOLECULAR AUTHENTICATION OF *LAGERSTROEMIA SPECIOSA* LEAVES

Mr. Woratouch Thitikornpong

ศูนย์วิทยทรัพยากร

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacognosy
Department of Pharmacognosy and Pharmaceutical Botany
Faculty of Pharmaceutical Sciences
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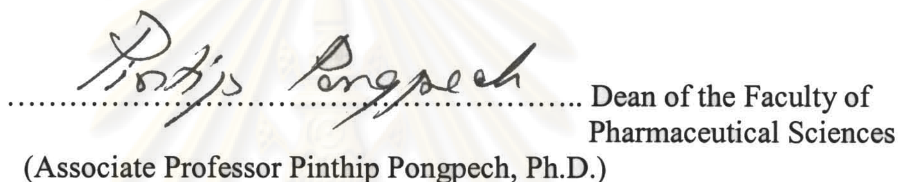
Thesis Title	Pharmacognostic specification and molecular authentication of *Lagerstroemia speciosa* leaves
By	Mr. Woratouch Thitikornpong
Field of Study	Pharmacognosy
Thesis Advisor	Assistant Professor Suchada Sukrong, Ph.D.
Thesis Co-Advisor	Associate Professor Thatree Padungcharoen, M.Sc. in Pharm.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree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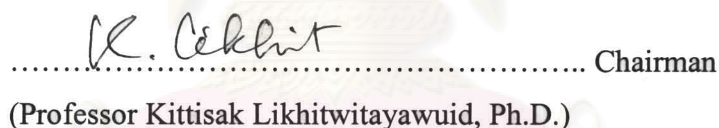
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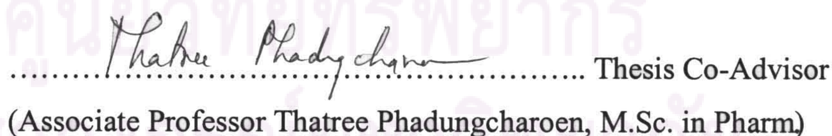
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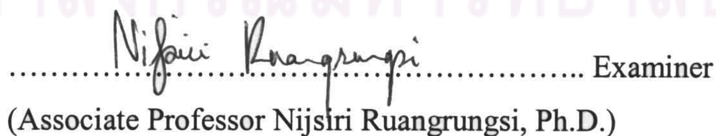
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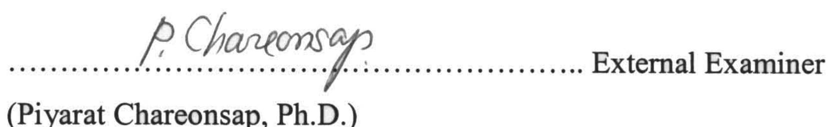
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จุฬาลงกรณ์มหาวิทยาลัย

วรธัช ฐิติกรพงศ์ : ข้อกำหนดทางเภสัชเวทและเอกลักษณ์ทางโมเลกุลของใบอินทนิลน้ำ. (PHARMACOGNOSTIC SPECIFICATION AND MOLECULAR AUTHENTICATION OF *LAGERSTROEMIA SPECIOSA* LEAVES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ร.ต.อ. หญิง ดร. สุชาดา สุขหร่อง, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ธาตรี ผดุงเจริญ, 131 หน้า.

อินทนิลน้ำ (*Lagerstroemia speciosa* L. Pers.) จัดอยู่ในวงศ์ Lythraceae ใช้ใบชงดื่มเป็นยาแก้เบาหวาน สารสำคัญในใบได้แก่โคโรโซลิค แอซิด ซึ่งเป็นสารในกลุ่มไทรเทอร์พีน การวิจัยนี้เป็นการศึกษาถึงคุณสมบัติทางเภสัชเวทของใบอินทนิลน้ำที่เก็บจากแหล่งปลูกและร้านขายยาแผนโบราณจำนวน 17 ตัวอย่าง และจัดทำมาตรฐานตามข้อกำหนดของตำรามาตรฐานยาสมุนไพรไทย จากการศึกษาลักษณะทางกายวิภาคและเนื้อเยื่อวิทยา พบปากใบชนิดอะนอโมไซติกและผลึกแคลเซียมออกซาเลตรูปดอกกุหลาบสะสมในชั้นพาเรงคิมา ค่าเฉลี่ยของปริมาณน้ำหนักที่หายไปเมื่อทำให้แห้ง ความชื้น เถ้ารวม เถ้าที่ไม่ละลายในกรด มีค่าร้อยละ 8.2141, 7.8593, 7.4725 และ 1.2176 โดยน้ำหนักแห้งตามลำดับ ขณะที่ค่าปริมาณสิ่งสกัดในเอทานอล สิ่งสกัดในน้ำ และสิ่งสกัดในไดคลอโรมีเทนมีค่าอยู่ที่ร้อยละ 9.0280, 2.9442 และ 13.1895 โดยน้ำหนักแห้งตามลำดับ นอกจากนี้ยังได้ศึกษาลักษณะทางโครมาโทกราฟีโดยแสดงกระสวนขององค์ประกอบทางเคมีบนรงคเลขผิวบางของสารสกัดเมทานอล และศึกษาเชิงปริมาณวิเคราะห์โดยการตรวจสอบปริมาณสารสำคัญโคโรโซลิค แอซิดโดยเทคนิคไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี

การวิจัยนี้ยังได้วิเคราะห์ลายพิมพ์ดีเอ็นเอของสมุนไพรอินทนิลน้ำและพืชสกุลใกล้เคียง ได้แก่ อินทนิลบก ตะแบกนา เสลาใบใหญ่ โดยใช้เทคนิค Amplified Fragment Length Polymorphism ผลการศึกษาพบแถบดีเอ็นเอที่เป็นเอกลักษณ์ของสมุนไพรอินทนิลน้ำ ข้อมูลทั้งหมดจากงานวิจัยนี้สามารถนำไปใช้พิสูจน์เอกลักษณ์สมุนไพรอินทนิลน้ำเพื่อให้เกิดประสิทธิผลและความปลอดภัยต่อผู้ใช้สมุนไพร

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต..
สาขาวิชา เภสัชเวท ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก........................
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KEYWORDS : *LAGERSTROEMIA SPECIOSA* / LYTHRACEAE / PHARMACOGNOSTIC SPECIFICATION / DNA FINGERPRINT / AFLP

WORATOUCH THITIKORNPONG : PHARMACOGNOSTIC SPECIFICATION AND MOLECULAR AUTHENTICATION OF *LAGERSTROEMIA SPECIOSA* LEAVES. THESIS ADVISOR: ASST. PROF. SUCHADA SUKRONG, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. THATREE PHADUNGCHAROEN, M.Sc. in Pharm., 131 pp.

Lagerstroemia speciosa L. Pers. (Lythraceae) leaf is used as infusion tea for antidiabetes. Corosolic acid, a triterpene compound, was found in this leaf. In this study, seventeen dried leaves of *L. speciosa* were collected from natural sources and traditional drugstores for pharmacognostic specifications and setting up the standardization according to Thai Herbal Pharmacopoeia. Anatomical and histological characteristics were the presence of anomocytic stomata and parenchyma containing rosette aggregate crystals of calcium oxalate. The mean contents of loss on drying, moisture content, ash content, acid-insoluble ash were 8.2141, 7.8593, 7.4725, 1.2176% of dry weight respectively. Whereas the ethanol extractive value, water extractive value and dichloromethane extractive value were 9.0280, 13.1895, and 2.9442% w/w, respectively. In addition, chromatographic pattern was investigated. Thin layer chromatographic patterns of methanolic extract were demonstrated. The quantitative analysis by high performance liquid chromatography method using corosolic acid as a marker was also reported.

Moreover, this study gave detailed analysis of the DNA fingerprinting of *Lagerstroemia*. *L. speciosa* and closely related species, *L. macrocarpa*, *L. floribunda* and *L. loudinii* using the AFLP technique. A species-specific band of *L. speciosa* was found. All of these results provide highly useful information for the authentication of *L. speciosa* leaves. It will contribute to effectiveness and safety prior to use.

Department : Pharmacognosy and Pharmaceutical botany
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Student's Signature Woratouch Thitikornpong
Advisor's Signature Suchada Sukrong
Co-Advisor's Signature Thatree Phadungcharoen

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ABBREVIATIONS

°C	=	degree Celsius
µg	=	microgram
µl	=	microliter
µM	=	micromolar
µm	=	micrometer
A, T, G, C	=	nucleotides containing the base adenine, thymine, guanine and cytosine, respectively.
AFLP	=	Amplified Fragment Length Polymorphism
AP-PCR	=	Arbitrarily Primed-PCR
ARMS	=	amplification refractory mutation system
AUC	=	area under curve
bp	=	base pair
BP	=	British Pharmacopoeia
CAPS	=	Cleaved Amplified Polymorphic Sequence
cm	=	centimeter
DALP	=	Direct Amplification of Length Polymorphism
DAMD	=	Directed Amplification of Minisatellite-region
ddNTPs	=	dideoxynucleotide triphosphates (ddATP, ddTTP, ddGTP, ddCTP)
DNA	=	deoxyribonucleic acid

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dNTPs	=	deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
EP	=	Europe Pharmacopoeia
h	=	hour
HPLC	=	High Performance Liquid Chromatography
kb	=	kilobase
L	=	liter
M	=	mole
mg	=	milligram
$MgCl_2$	=	Magnesium chloride
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimole
mm^2	=	square millimeter
ng	=	nanogram
nm	=	nanometer
PCR	=	Polymerase Chain Reaction
PCR-RFLP	=	Polymerase chain reaction- Restriction Fragment Length Polymorphism
ppm	=	part per million
r^2	=	correlation coefficient
RFLP	=	Restriction Fragment Length Polymorphism

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RP-PCR = Random Primed-Polymerase Chain Reaction

RT = retention time

SCAR = Sequence Characterized Amplified Regions

SD = standard deviation

sec = second

THP = Thai Herbal Pharmacopoeia

TLC = Thin-Layer Chromatography

USP = United State Pharmacopoeia

UV = ultraviolet light

V = volt

v = volume

w = weight

W = watt

WHO = World Health Organization

CHAPTER I

INTRODUCTION

Lagerstroemia speciosa (L.) Pers. (Lythraceae), locally known in Thai as "อินทนิลน้ำ", is a folkloric medicine. This medicinal plant, popularly known as banaba, is an ornamental plant that grows widely in the Philippines, India and South East Asian countries. The leaves of this tropical plant have been used as a folk medicine for treatment diabetes mellitus (Matsuyama, 2001; Quisumbing, 1978; Yamaguchi *et al.*, 2006). Banaba extracts are also known to have antiobesity (Suzuki *et al.*, 1999), anti-oxidant (Unno *et al.*, 1997) and anti-gout (Unno *et al.*, 2004) effects. Corosolic acid, an active ingredient of the extract, displays a potential anti-diabetic activity (Fukushima *et al.*, 2006; Judy *et al.*, 2003; Kakuda *et al.*, 1996; Lui *et al.*, 2001; Miura *et al.*, 2004; Miura *et al.*, 2006; Murakami *et al.*, 1993; Shi *et al.*, 2008), anti-oxidative stress, anti-inflammation, and antihypertention (Yamaguchi *et al.*, 2006).

L. speciosa has long been used in traditional medicines. However, scientific standards or pharmacognostical parameters are not yet available to ascertain the identification and to determine the quality of this herb. At present, *L. speciosa* tea from some traditional drugstores had decreased anti-diabetic activity. It may be occurred from incorrect herbal identification. Pharmacognostic specification and DNA fingerprinting could be used as tools for authentication and detection of adulterants.

Examinations of macroscopic and microscopic characteristics are the first step towards establishing the herbal standardization of materials, and should be carried out before any further tests are undertaken. In addition, the constant number of leaf is parameters that are unique to the plant (Radhika *et al.*, 2010). A chemical profile, the thin layer chromatographic pattern, was produced with the aim for testing the purity. R_f values indicated the position at which the substance was located on the chromatogram. The advantage of R_f value was widely recognized as a guide for identification of medicinal plants. Phytochemical screening portrays that most of the natural products tested for were present in the plant material. Physicochemical parameters of the crude drugs were important parameters to prevented adulteration or improper handling of drugs. The moisture content and loss on drying of crude drugs

should be minimized in order to prevent spoilage due to microbial contamination or decomposition of chemical. Ash contents are accountable for controlling the admixture of foreign inorganic matter. The extractive values also determine the quality control as well as purity of crude drugs. Furthermore, corosolic acid had been used for chemical marker for quantitative assessment of crude drugs, which were collected and purchased from traditional drugstores throughout Thailand. Hence, all of pharmacognostical parameters are major and reliable criteria for confirmation of the identity and determination of quality and purity of the crude drugs.

The accurate identification and quality control of the plant material is, therefore, an essential prerequisite for ensuring the quality, safety, and efficacy of *L. speciosa* leaves and other herbal medicines. General approaches to herbal identification depend on morphological, anatomical, and chemical analyses, but these characteristics are often affected by environmental and/or developmental factors during plant growth (Li *et al*., 1994). Nevertheless, the use of chromatographic techniques and marker compounds to standardize botanical preparations has limitations because of the varied sources and chemical complexity of such preparations. In particular, many extrinsic factors such as methods of cultivation, harvesting, drying and storing may affect the ultimate chemical profile of a given herb. So, DNA based polymorphism assay may offer an alternative method to identify herbal medicines. The analysis of DNA has the advantages of being applicable to all parts of plants and not being affected by conditions of culture (Shim *et al*., 2003). A number of recent studies have indicated that DNA markers are ideal tools for elucidating the molecular evolution and phylogeny of the species concerned, as well as for identifying crude herbal materials (Xue *et al*., 2006). Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique that approaches the ideal as a marker system for resolving genetic diversity among individuals, populations and species (Muller and Wolfenbarger, 1999). This technique is highly reproducible, and can be used to survey overall genetic difference in the genome without any prior sequence knowledge (Jones *et al.*, 1997)

The goal of this study is to develop various necessary pharmacognostic specification and DNA fingerprint of *L. speciosa* leaves for medicinal plant authentication. The present investigation of *L. speciosa* leaves is undertaken to establish pharmacognostic profiles of the leaves which will be useful in crude drug identification as well as in standardization of the quality and purity. The DNA

fingerprint analysis was designed to investigate the genetic relationship among species belonging to *Lagerstroemia* using the AFLP marker. The results of this study will provide useful information for its correct identification and may enable those who handle this plant to maintain its quality control. In addition, the results of this present study could be useful for preparation of a Pharmacopoeial Monograph of this plant.

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CHAPTER II

LITERATURE REVIEWS

2.1 Botanical aspects of *Lagerstroemia speciosa* (L.) Pers.

The genus *Lagerstroemia* belongs to the family Lythraceae. *Lagerstroemia* species are distributed in tropical and subtropical parts of Madagascar, Asia, New Guinea and Australia (Everett *et al.*, 1972). There are at least eighteen valid species of *Lagerstroemia* in Thailand according to Smitinand (2001), as listed in **Table 2.1**.

Leaves of the tropical plant, *Lagerstroemia speciosa* , have been used as a folk medicine for treatment of diabetes (Lui *et al.*, 2001). *L. speciosa* was distributed in China, Indo-china, Burma, Siam, Peninsular Malaysia, Sumatra, Java, Celebes, Perak, Pahang southwards, Langkawi, and the Philippines (Matsuyama, 2001; Quisumbing, 1978). There ecology was in lowland secondary forest on sandy soil. In Flora of Thailand, there is no information about *Lagerstroemia* characteristics. The Tree Flora of Sabah and Sarawak (Yii, 1996) described the characteristic of *L. speciosa* as follows;

"Tree to 10 m tall, 30 cm diameter. Leaves elliptic-oblong, 5 – 19 x 4 – 8 cm; base cuneate or almost rounded, apex acute or obtuse; glabrous, grayish green above and brown beneath; lateral veins 8-15 pairs, distinctly looped and joined to the next pair; petioles 4-9 mm long. Inflorescences 15 – 40 cm long, 10 – 20 cm across, covered with caduceus, ashy or rusty hairs; pedicels c. 1.5 mm long. Flower buds subglobose or pyriform, 10 – 15 x 6 – 10 mm, shortly apiculate at the apex, 12 – 14-ridged, ridges of the same length; calyx ashly or rusty hairy, lobes recurved; petals 6, suborbicular, 15 – 30 x 10 – 20 mm, tapering towards the slender claw; stamen numerous, subequal; ovary globose, glabrous or slightly scaly, style filiform, stigma capitates. Fruits woody, globose, glabrous, c. 22 mm across; persistent calyx glabrous or slightly hairy, lobes recurved."

Table 2.1 The list of *Lagerstroemia* species in Thailand.

No.	Scientific name	Thai name
1	*Lagerstroemia balansae* Koehne.	Ta baek kriap (ตะแบกเกรียบ)
2	*L. calyculata* Kurz.	Ta baek daeng (ตะแบกแดง)
3	*L. collinsae* Craib.	Ta baek bai lek (ตะแบกใบเล็ก)
4	*L. cuspidate* Wall.	Ta baek (ตะแบก)
5	*L. duperreana* Pierre ex Gagnep.	Ta baek plueak bang (ตะแบกเปลือกบาง)
6	*L. floribunda* Jack.	Ta baek na (ตะแบกนา)
7	*L. indica* L.	Yi Kheng (ยี่เข่ง)
8	*L. loudinii* Teijsm. & Binn.	In thara chit (อินทรชิต)
9	*L. macrocarpa* Wall.	Inthanin bok (อินทนิลบก)
10	*L. ovalifolia* Teijsm. & Binn.	Ta baek dong (ตะแบกดง)
11	*L. siamica* Gagnep.	Ta baek na (ตะแบกนา)
12	*L. speciosa* (L.) Pers.	Inthanin nam (อินทนิลน้ำ), (syn. *L. flos-reginae* Retz.)
13	*L. spireana* Gagnep.	Pueai nam (เปื๋อยน้ำ)
14	*L. tomentosa* C.Presl	Salao Khao (เสลาขาว)
15	*L. undulata* Koehne var. undulate	Salao dam (เสลาดำ)
16	*L. undulata* Koehne var. subangulata Craib.	Samo rong (สมอร่อง)
17	*L. venusta* Wall.	Salao plueal bang (เสลาเปลือกบาง)
18	*L. villosa* Wall. ex Kurz	Salao plueak na (เสลาเปลือกหนา)

Figure 2.1 *Lagerstroemia speciosa* (L.) Pers. (a) habitat (b) leaves (c) infructescence (d) fruit and seeds (e) inflorescences.

Figure 2.2 Twig of *Lagerstroemia speciosa*, inflorescence and fruit.

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2.2 Chemical constituents and biological activity of *L. speciosa*

The chemical constituents and biological activities of *L. speciosa* leaves are summarized in **Table 2.2**.

Table 2.2 The chemical constituents of *L.speciosa* leaves and its biological activity.

Chemical constituent	Category	Biological activity	Reference
Campesterol	Sterol	-	Sato *et al*., 1981
Sitosterol	Sterol	-	Sato *et al*., 1981
Stigmasterol	Sterol	-	Sato *et al*., 1981
31-norargerenol acetate	Triterpene	-	Ragasa *et al.*, 2005
24-methylenecycloartanol	Triterpene	-	Ragasa *et al.*, 2005
Corosolic acid	Triterpene	Hypoglycemic activity and glucose transport activator	Fukushima *et al*., 2006: Judy *et al.*, 2003; Kakuda *et al.*, 1996; Lui *et al.*, 2001; Miura *et al.*, 2004; Miura *et al.*, 2006; Murakami *et al.*, 1993; Shi *et al.*, 2008
		Anti-oxidant	Yamaguchi *et al.*, 2006
		Anti-inflammation	Yamaguchi *et al.*, 2006
		Anti-hypertension	Yamaguchi *et al.*, 2006
Cycloeucalenol acetate	Triterpene	-	Ragasa *et al.*, 2005

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Chemical constituent	Category	Biological activity	Reference
Maslinic acid	Triterpene	-	Murakami *et al.*, 1993
Largerenol acetate	Triterpene	-	Ragasa *et al.*, 2005
3,3',4-tri-O-methylellagic acid	Tannin	Antioxidation	Takahashi *et al.*, 1977; Malaisree *et al.*, 2006
3-O-methylellagic acid	Tannin	Antioxidation	Takahashi *et al.*, 1977; Malaisree *et al.*, 2006
Ellagic acid	Tannin	Treatment of hyperuicemia	Sato *et al.*, 1990; Unno *et al.*, 2004
Flosin B	Tannin	Activator of glucose transport in fat cells,	Hayashi *et al.*, 2001
Lagerstroemin	Tannin	Activator of glucose transport in fat cells, Activated insulin receptor	Hattori *et al.*, 2003; Hayashi *et al.*, 2001
Reginin A	Tannin	Activator of glucose transport in fat cells,	Hayashi *et al.*, 2001
Valoneic acid dilactone	Tannin	Treatment of hyperuicemia	Unno *et al.*, 2004

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Previous chemical investigations indicated that *L. speciosa* leaves contained terpenoids (Murakami *et al.*, 1993; Ragasa *et al.*, 2005), tannins (Tanaka *et al.*, 1992; Xu *et al.*, 1991[a]; Xu *et al.*, 1991[b]) and ellagic acids (Takahashi *et al.*, 1977). The corosolic acid was considered to be the active components responsible for the hypoglycemic activity of banaba (Hamamoto *et al.*, 1999; Lui *et al.*, 2005; Miura *et al.*, 2004; Murakami *et al.*, 1993).

Corosolic acid, a triterpenoid named 2α-hydroxyursolic acid (2α,3β-dihydroxyurs-12-en-28-oic acid), has been found in *L. speciosa* leaves. Recently, it has been reported to have antidiabetic activity in some animal experiments and clinical trials. Corosolic acid reduced the blood glucose levels and significantly lowered plasma insulin levels in KK-Ay mice 2 weeks after a single oral dose of 2 mg/kg. In addition, blood glucose in KK-Ay mice treated with corosolic acid significantly decreased in an insulin tolerance test (Miura *et al.*, 2006). The muscle GLUT4 translocation from low-density microsomal membrane to plasma membrane was significantly increased in the orally corosolic acid-treated mice when compared with that of the controls (Miura *et al.*, 2004). It has an effect on lowering postchallenge plasma glucose level *in vivo* in human (Fukushima *et al.*, 2006). Corosolic acid treatment subjects demonstrated lower glucose levels from 60 minutes until 120 minutes and reached statistical significance at 90 minutes (Judy *et al.*, 2003). So, corosolic acid was suggested to be a promising lead compound for diabetes treatment.

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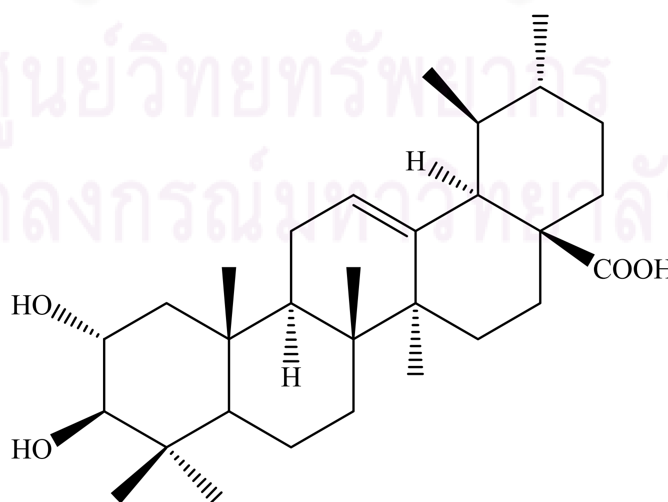

Figure 2.3 The chemical structure of corosolic acid.

2.3 Quality control methods for medicinal plant materials

Plant materials are used throughout developed and developing countries as home remedies, over-the-counter drug products and raw materials for the pharmaceutical industry, and represent a substantial proportion of the global drug market. It is essential to establish guidelines for assessing their quality. Thai Herbal Pharmacopoeia has emphasized the need to ensure the quality of medicinal plant products by using modern control techniques and applying suitable standards.

Pharmacognostic study is used to characterize and analyze the quality and quantity of crude drugs. In the macroscopic method, organoleptic sensation is used to determine the size, shape, color, odor, taste, *etc*. of the crude drugs, while the microscopic method revealed plant histological characteristics. 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 (THP) and WHO guidelines.

2.3.1 Macroscopic examination

Medicinal plant materials are categorized according to sensory, macroscopic and microscopic characteristics. An examination to determine these characteristics is the first step towards establishing the identity and the degree of purify of such materials, and should be carried out before any further tests are undertaken. Wherever possible, authentic specimens of the material in question and samples of pharmacopoeia quality should be available to serve as a reference.

Visual inspection provides the simplest and quickest means by which to establish identity, purity and quality. If a sample is found to be significantly different, in terms of color, consistency, odor or taste, from the specifications, it is considered as not fulfilling the requirements. However, judgment must be exercised when considering odor and taste, owing to variability in assessment from person to person or by the same person at different times.

2.3.2 Inspection by microscopy

Macroscopic identity of medicinal plant materials is based on shape, size, color, surface characteristics, texture, and fracture characteristics. Microscopic inspection of medicinal plant materials is indispensable for the identification of broken or powdered materials. An examination by microscopy alone cannot provide complete identification, though when used in association with other analytical methods it can frequently supply invaluable supporting evidence. Microscopic measurements can be carried out using a stage micrometer in conjugation with an eyepiece micrometer and drawing adjustment.

The stage micrometer was used to measure the size of small objects. Two scales are required, known respectively as a stage micrometer and an eyepiece ocular micrometer. The stage micrometer is a glass slide 7.6 x 2.5 cm (3 x 1 inch) with a scale engraved on it. The scale is usually 1 mm long and is divided into 0.1 and 0.01 parts of a millimeter. The eyepiece micrometer may be a linear scale and the scale 0-10 or it may be ruled in squares. The value of one eyepiece division is determined for every optical combination to used, a note being made in each case of the objective eyepiece and length of raw-tube.

To calibrate micrometer, unscrew the upper lens of the eyepiece, place the eyepiece micrometer on the right inside, and replace the lens. Put the stage micrometer on the stage and focus it in the ordinary way, the two micrometer scales now appear (**Figure 2.4**), when the objective (x4) is in used. It will be seen that when the 7 line of the stage micrometer coincides with the 0 of the eyepiece, the 10 of the stage coincides with 7.7 of the eyepiece. As the distance between 7 and 10 on the stage scale is 0.3 mm, 77 of the small eyepiece division equal 0.3 m or 300 µm; therefore, 1 eyepiece division of this eyepiece micrometer which is used with the objective (x4) of this microscope equals 300/77 or 3.9 µm (Trease and Evans, 2009).

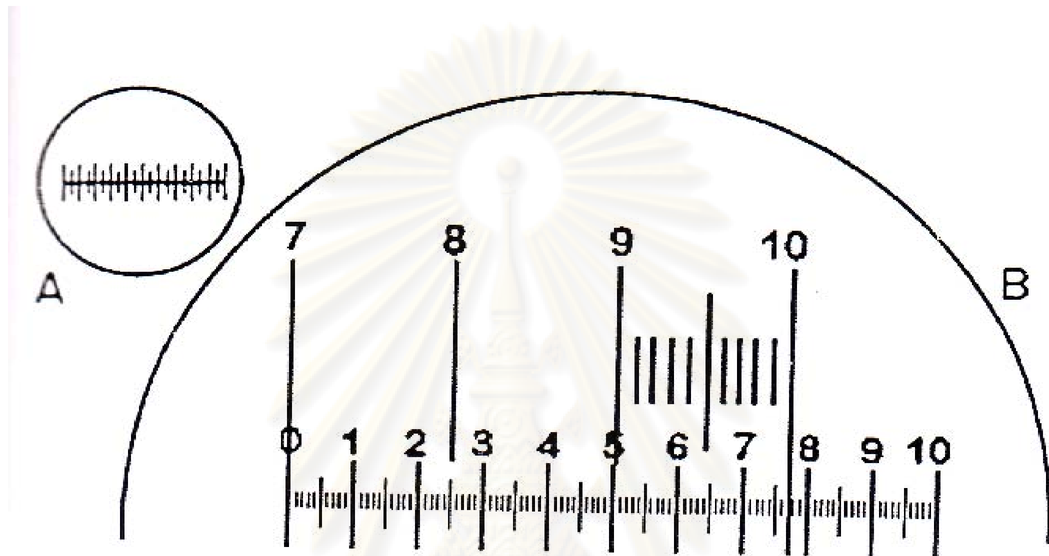

Figure 2.4 A. Eyepiece micrometer.

B. Eyepiece micrometer superimposed on portion of stage micrometer scale.

(Trease and Evans, 2009)

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2.3.3 Constant values of leaves

A number of leaf measurements are used to distinguish between some closely related species not easily characterized by general microscopy.

2.3.3.1 Determination of stomatal index and stomatal number

Stomata are openings (the stomatal pores or apertures) epidermis bounded by two specialized epidermal cells, the guard cells, which by changes in shape result in the opening and closure of the aperture. It is convenient to apply the term stoma to the entire unit, the pore and two guard cells. The stoma may be surrounded by cells that do not differ from other guard cells of the epidermis. On the other hand, in many plants the stomata are flanked or surrounded by cells that differ in shape and sometimes also in content from the ordinary epidermal cells. These distinct cells are called subsidiary cell of the stoma. The subsidiary cells may or may not be closely related onto genetically to the guard cells. (Esau, 1960; Eames and MacDaniels, 1974)

In the mature leaves, four significant types of stomata are distinguished by their form and the arrangement of the surrounding cells, especially the subsidiary cells (WHO, 1998):

- The anomocytic or ranunculaceous (irregular-celled) type; the stoma is surrounded by a varying number of cells, generally not different from those of the epidermis (figure 2.5A).
- The anisocytic or cruciferous (unequal-celled) type; the stoma is usually surrounded by three or four subsidiary cells, one of which is markedly smaller than the others (figure 2.5B)
- The diacytic or caryophyllaceous (cross-celled) type; the stoma is accompanied by two subsidiary cells, the common wall of which is at right angles to the stoma (figure 2.5C).
- The paracytic or rubiaceous (parallel-celled) type; the stoma has two subsidiary cells, of which the long axes are parallel to the axis of the stoma (figure 2.5D).

In describing an epidermis where certain stomata differ from the predominant type, the term applying to the majority of stomata is used.

Stomatal number is the number of stomata per unit area of leaves. It was investigated by Timmerman in 1927 (Youngken, 1948). The actual number of stomata per square millimeter is variable for the same plant, this brings especially noticeable if records are made for different year (Wallis, 1960). The average number of stomata per square millimeter of epidermis is termed the stomata number. In recording result the range as well as the average value should be recorded for each surface of the leaf and the ratio of values for the two surfaces. In certain cases this ratio may be of diagnostic importance (Trease and Evans, 2009).

Stomatal Index is the percentage proportion of the ultimate divisions of the epidermis of a leaves which have been converted into stomata (Youngken, 1948).

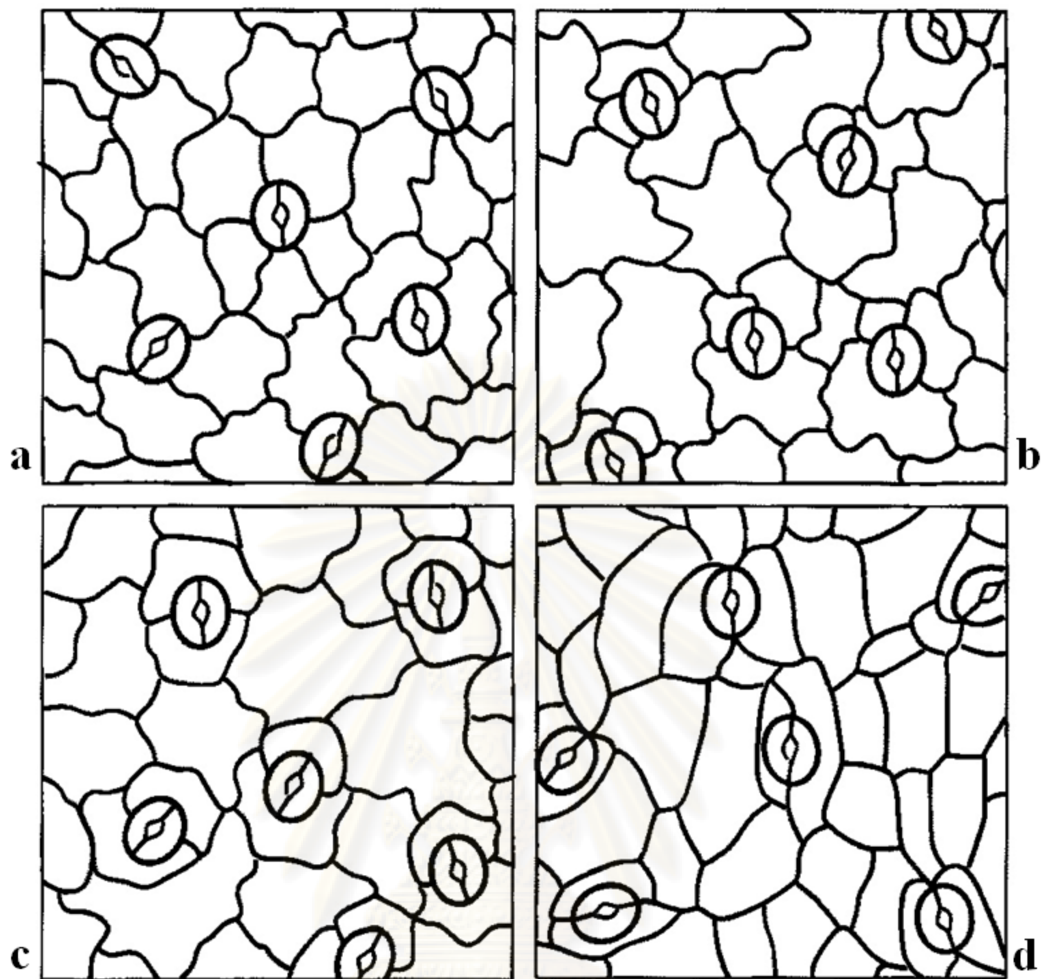

Figure 2.5 Epidermis in surface view illustrating patterns formed b guard cells and surrounding cells. a: anomocytic; b: anisocytic; c: diacytic; d: paracytic (WHO, 1998).

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2.3.3.2 Palisade ratio examination

Palisade cells are a type of photosynthetic cells of the mesophyll of a leaf occurring mostly just beneath the upper epidermis surface layer (Esau, 1972; Wallis, 1960). The cells are elongated and more or less cylindrical and arranged in one or more rather regular, relatively compact layers near the ventral, or upper side of the leaf with the long axis of the cells perpendicular to the leaf surface (Eames and MacDaniels, 1974).

The term "palisade ratio" was introduced by two British pharmacognosists, T.E. Wallis and T. Dewar, in 1933. It represents a figure obtained by counting the total number of palisade cells beneath four upper epidermal cells and dividing the number by four (Youngken, 1948). The average number of palisade cells beneath each upper epidermal cell is termed the palisade ratio. Quite fine powders can be used for the determination (Trease and Evans, 2009).

2.3.3.3 Inspection of Vein-islet number

Vein-islets are divisions of green leaf tissue formed by the ultimate divisions of the conducting strands of vascular bundles which either completely or partially surround areas of the chlorenchyma. The islets increase in size as the leaf matures, the full grown leaf showing constancy in vein-islet number (Youngken, 1948). The term "vein-islet" is used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein-islet/mm^2 calculated from four contiguous square millimeters in the central part of the lamina, midway between the midrib and the margin, is termed the vein-islet number. When determined on whole leaves, the area examined should be form the central part of the lamina, midway between the margin and midrib (Trease and Eans, 2009).

2.3.3.4 Investigation of Veinlet termination number

Hall and Melville (1951) determined veinlet termination number. It means to the number of veinlet terminations per mm^2 of leaves surface. A vein termination is the ultimate free termination of a veinlet or branch of a veinlet. (Trease and Evans, 1996).

2.3.4 Thin-Layer Chromatography (TLC)

TLC, which together with paper chromatography comprises “planar” or “flatted” chromatography, is the simplest of all of the widely used chromatographic methods to perform. A suitable closed vessel-containing solvent and a coated plate are all that are required to carry out separations and qualitative and semi-quantitative analysis. With optimization of techniques and materials, highly efficient separations and accurate and precise quantification can be achieved. TLC can be used also for preparative scale separations by employing specialized apparatus and techniques.

Basic TLC is carried out as follows. An initial zone of mixture is placed near one end of the stationary phase, a thin layer; the sample is dried; and the end of the stationary phase with the initial zone is placed into a mobile phase, usually a mixture of pure solvents, inside a closed chamber. The components of the mixture migrate at different rates during movement of the mobile phase through the stationary phase, which is termed the development of the chromatogram. When the mobile phase has moved an appropriate distance, the stationary phase is removed, the mobile phase is rapidly dried, and the zones are detected by application of a suitable visualization reagent.

Differential migration is the result of varying degrees of affinity of the mixture components of the stationary and mobile phases. Different separation mechanisms are involved, the predominant forces depending on the exact nature of the two phases and the solutes. The interactions involved in determining chromatographic retention and selectivity include hydrogen bonding, electron-pair donor/electron pair acceptor (charge transfer), ion-ion, ion-dipole, and van der Waals interactions. Among the latter are dipole-dipole, dipole-induced dipole, and instantaneous dipole-induced dipole interactions.

Sample collection, preservation, and purification are problems common to TLC and all other chromatographic methods. For complex samples, the TLC development will usually not completely resolve the analyses (the substance to be determined) from interference unless a prior purification is carried out. This is most often done by selective extraction and column chromatography. In some cases, substances are converted, prior to TLC, to a derivative that is more suitable for separation, detection, and/or quantification than the parent compound.

Detection is most simple when the compounds of interest are naturally colored or fluorescent or absorb ultraviolet (UV) light. However, application of a

location or visualization reagent by spraying or dipping is usually required to produce color or fluorescence for most compounds. Absorption of UV light is common for many compounds, e.g., aromatics and those with conjugated double bonds. This leads to a simple, rather universal detection method on layers impregnated with a fluorescence indicator (fluorescence quench detection).

Compound identification in TLC is based initially on R_f values compared to authentic standards. R_f values are generally not exactly reproducible from laboratory to laboratory or even in different runs in the same laboratory, so they should be considered mainly as guides to relative migration distances and sequences. Factors causing R_f values to vary include: dimensions and type of the chamber, nature and size of the layer, direction of mobile phase flow, the volume and composition of the mobile phase, equilibration conditions, humidity, and sample preparation methods preceding chromatography (Sherma, 1991).

2.3.5 Phytochemical screening

The importance of plant-derived medicinal in modern medicines is often underestimated. A knowledge of the biological activities and/or chemical constituents of plants is desirable, not only for the discovery of new therapeutic agents but because such information may be of value in disclosing new sources of such economic materials. A knowledge of the chemical constituents of plants would further be valuable to those interested in the expanding area of chemotaxonomy (biochemical systematic), to those interested in biosynthesis, and to those interested in deciphering the actual value of folkloric remedies.

Phytochemical screening is the process of separation and isolation of active principle from plant sources. These screening are helpful to get the lead for discovery of the therapeutic agents.

The method for use in phytochemical screening should be (a) simple, (b) rapid, (c) designed for a minimum of equipment, (d) reasonably selective for the class of compounds under study, (e) quantitative in so far as having a knowledge of the lower limit of detection is concerned, and if possible, (f) should give additional information as to the presence or absence of specific members of the group being evaluated (Farnsworth, 1966)

2.3.6 Determination of physicochemical values

One possible problem in devising standards for crude drugs concerns the requirement for an assay of the active constituents when the latter may not have been precisely ascertained. Moreover, one of the principles of the herbal medicine is that the maximum effectiveness of the drug derives from the whole drug or its crude extract rather than from isolated components. In cases where an assay is lacking it is therefore of paramount importance that the crude drug is properly authenticated, its general quality verified and all formulations of it prepared in accordance with good manufacturing practice. Although official standards are necessary to control the quality of drugs their use doses raise certain problems. Of necessity, to accommodate is necessary to set relatively low standards which allow the use of commercial materials available in any season. There are a number of standards, numerical in nature, which can be applied to evaluation of crude drug either in the whole or the powdered condition (Trease and Evans, 2009). For this investigation, these are following:

2.3.6.1 Loss on drying

This is employed in the WHO guideline, EP, BP, USP and THP. Although the loss in weight, in the samples so tested, principally is due to water, small amounts of other volatile materials will also lead to the weight loss. For materials, which certain little balance combines the drying, process and weight recording; it is suitable where great numbers of samples are handled and where a continuous record of loss in weight with time is required (Trease and Evans, 2009).

2.3.6.2 Moisture content

The "loss on drying" methods can be made more specific for the determination of water by separating and evaluating the water obtained from a sample. This can be achieved by passing a dry inert gas through the heated sample and using an absorption train (specific for water) to collect the water carried forward; such method can be extremely accurate, as shown in their use for the determination of hydrogen in organic compounds by combustion analysis. Method based on distillation has been widely used for moisture determination. This method is employed in the WHO guideline, USP, BP and in THP and EP for some volatile oil-containing drugs (Trease and Evans, 2009).

2.3.6.3 Ash content

The determination of ash is useful to detecting low-grade products, exhausted drugs and excess of sandy or earthy matter; it is more especially applicable to powder drugs. Different types of ash figures are used such as total ash, acid-insoluble ash and water soluble ash. A total ash usually consists mainly of carbonates, phosphates, silicates and silica. If the total ash were treated with dilute hydrochloric acid, the percent of acid-insoluble ash may be determined. This usually consists mainly of silica and a high acid-insoluble ash in drug (Trease and Evans, 1996).

2.3.6.4 Extractive value

The determination of water-soluble or ethanol-soluble extractives is used as a means of evaluating drugs the constituents of which are not readily estimated by other means. In certain cases extraction of the drug is by maceration, in others by a continuous extraction process. For the latter the soxhlet extractor is particularly useful and has been in use for many years, not only for the determination of extractives (e.g. fixed oil in seed) but also for small-scale isolations (Trease and Evans, 2009).

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2.4 DNA Fingerprinting

DNA technology provides a useful and independent tool to complement chemical analyses for the authentication and quality assurance of medicinal materials. DNA technology offers four advantages: (1) DNA-based markers are less affected by age and physiological conditions; (2) any part of the herb can be collected for analysis; (3) only a small sample is necessary for analysis; and (4) some DNA regions may be species-specific, whereas others may be family-specific. Benefiting of polymerase chain reaction (PCR) technique, DNA marker has now become a popular means for identification of herbal medicines.

The polymerase chain reaction (PCR) by Mullis *et al*. (1987) has opened a new approach for molecular genetic studies. PCR is a method for an *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. Million copies of the target DNA sequence can be synthesized from a low amount of starting DNA template within a few hours. The PCR reaction components consist of genomic DNA used as DNA template for copies, a pair of primers for amplified in target sequences, dNTPs, buffer and *Taq* DNA polymerase. The amplification reaction consists of three steps; denaturation of dsDNA at high temperature, annealing to allow primers to form hybrid molecules at the optimal temperature, and extension of the anneal primers by heat-stable *Taq* DNA polymerase. The cycle is repeated for 20–40 times. Finally, the amplification products are examined by electrophoresis.

Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphisms. In terms of the mechanisms involved, DNA methods can be classified into three types, namely hybridization-based method, PCR-based method and sequencing-based method (Joshi *et al*., 2004, Yip *et al*., 2007)

Hybridization-based method

Restriction Fragment Length Polymorphism (RFLP) is the most widely used hybridization-based molecular marker (Semagn et al., 2006). The technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms. On an agarose gel, RFLP can be visualized using radiolabeled complementary DNA sequences. Polymorphisms are analyzed after hybridization by observing presence or absence of bands (Joshi *et al*., 2004). The strength points of

RFLP markers are high reproducibility, codominant inheritance, no sequence information required, and relatively easy to score due to large size difference between fragments (Semagn *et al*., 2006). There are, however, some problems with the RFLP method of DNA fingerprinting. Firstly, the results do not specifically indicate the chance of a match between two organisms. Secondly, the process involves a lot of money and labor, which not many laboratories can afford (Vasudevan, 2007).

DNA sequencing-based method

Polymorphism at the DNA level can be studied by several methods but the direct strategy is determination of nucleotide sequences of a defined regions. There are two general methods for sequencing of DNA segments: the “chemical cleavage” procedure (Maxam and Gilbert, 1977) and the “chain termination” procedure (Sanger, 1977). Nevertheless, the latter method is more popular because chemical cleavage method requires the use of several hazardous substances. DNA fragments generated from PCR can be directly sequenced or alternatively, those fragments can be cloned and sequenced. This eliminates the need to establish a genome library and searching of a particular gene in the library.

Nowadays, the sequencing method developed by Fred Sanger forms the basis of automated "cycle" sequencing reactions. DNA sequencing reaction is like the PCR reaction for replicating DNA. The reaction mix contains template DNA, primers, DNA polymerase, four nucleotides (dGTP, dCTP, dATP and dTTP). In addition, a second type of nucleotide called dideoxynucleotide, are added in the reaction mix. These dideoxynucleotide are labeled with fluorescent dye and can be recognized by DNA sequencer. To start the reaction, the mixture is heated until the two stands of DNA separated. Then the primer sticks to its intended location and DNA polymerase starts elongating the primer. If allowed to go to completion, a new strand of DNA would be the result. The enzyme makes no difference between dNTPs or ddNTPs. When a ddNTP is included, the synthesis stops. Because billions of DNA molecules are present in the tube, the stand can be terminated at any position, so different lengths of DNA stands are emerged. Then, the reaction is transferred to polyacrylamide gel. The gel is placed into a DNA sequencer for electrophoresis and analysis. The fragments migrate according to size, and each is detected as it passes a laser beam at the bottom of gel. Each dideoxynucleotide emits colored light of a

characteristic wavelength and is recorded as a colored band on a simulated gel image (Weising *et al*., 2005)

DNA sequencing is the most optimal method for population genetic applications particular phylogenetic studies of organisms. This technique provides high resolution and facilitating interpretation. However, sequencing of a large number of individuals using conventional method is extremely tedious and prohibitively possible. The sequencing method has been facilitated by the direct and indirect use of DNA fragments generated through PCR. At present, automatic DNA sequencing has been introduced and commonly used. This greatly allows wider application of DNA sequencing analysis for population genetic and systematic studies.

PCR-based method

PCR-based methods use amplification of particular DNA sequences or loci, with the oligonucleotide primers and the thermostable DNA polymerase enzymes (Joshi *et al*., 2004). PCR-based methods include PCR-restriction fragment length polymorphism (PCR-RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single strand conformation polymorphism (SSCP), sequence characterized amplified regions (SCAR), amplification refractory mutation system (ARMS), simple sequence repeat (SSR) analysis, direct amplification of length polymorphism (DALP), inter-simple sequence repeat (ISSR), directed amplification of minisatellite-region DNA (DAMD) (Yip *et al*., 2007)

Amplified Fragment Length Polymorphisms (AFLPs) technique, a PCR-based molecular marker, was first developed by Vos and Zabeau, researchers of Keygene N.V. company in Netherlands, and registered a patent in 1993 (Vos *et al*., 1995). AFLPs analysis was clearly a powerful technique in terms of its ability to identify a large number of polymorphic bands without any prior knowledge of the organisms. The ability of this technique to generate many markers with minimum primer testing and the system's high resolution (i.e., band clarity and relative low lane background) are features that make AFLP attractive as genetic markers (Crouch *et al*., 1999). Moreover, AFLP analysis is used in variety of fields, including plants, animal breeding, medical diagnostics, forensic analysis, microbial typing, *etc*.

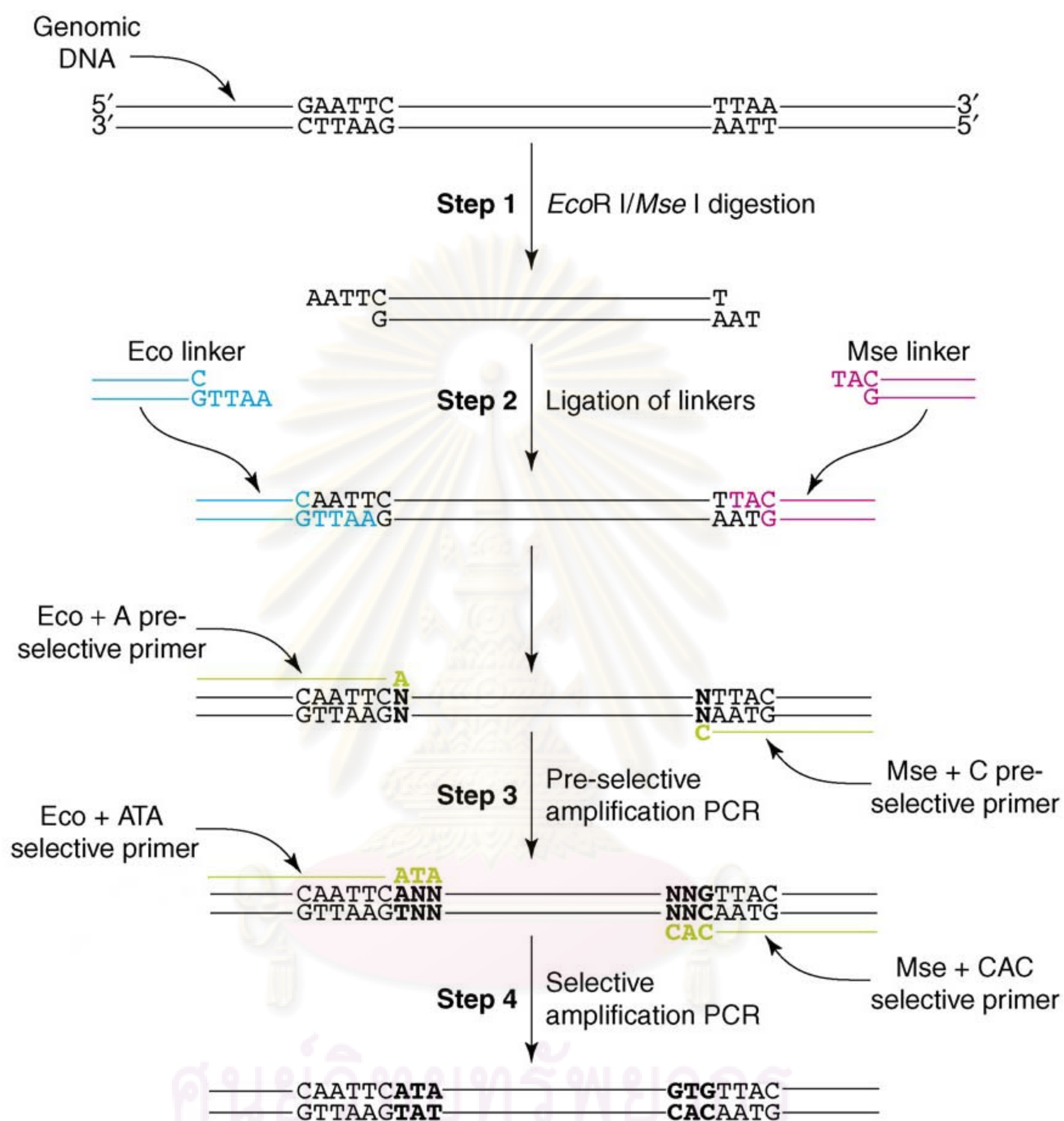

Figure 2.6 The process of Amplified Fragment Length Polymorphism (AFLP) technique. (Meudt *et al.*, 2007)

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AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos *et al*., 1995). The technique combined the reliability of the Restriction Fragment Length Polymorphism (RFLP) analysis with the power of the PCR technique. The technique involves three steps (**Figure 2.6**). The first step is restriction endonuclease digestion of genomic DNA with a restriction enzyme that cuts frequently (*Mse*I, 4 bps recognition sequence) and the other that cut less frequently (*EcoR*I, 6 bps recognition sequence). The following step is ligation of double stranded adapters to ends of the restriction fragments. The frequent cutter is used to generate small fragments. It is amplified in the optimal size range for separation on a polyacrylamide gel. The rare cutter is used to position the attachment of two primers in the next PCR amplification. Selective amplification of sets of restriction fragments using selective AFLP primers is performed. This step increases the number of fragments by selective primers which are the same base sequence with adapters. The PCR amplification uses primer to add nucleotides at 3′ ends, which adds two more nucleotides with two steps selective amplification. The first step is selective amplification using three nucleotides containing further additional primer bases. This step uses selective amplification of sets of restriction fragments and reduces the number of fragments because a single 3′-extension on both primers reduce the number of fragments amplified by a factor of 16, a two-base extension reduce the number by 256, and a three-base extension reduces it by 4,096. The last step, analysis of the amplified fragments on polyacrylamide gels and DNA band visualization is generated.

AFLPs marker is extensively used for studying genetic diversity in different plant species (Steiger *et al*., 2002; Vos *et al*., 1995). Comparative studies using restriction RFLPs, RAPD, AFLP, and microsatellite techniques have shown that AFLP method is the most efficient method to estimate genetic diversity because of its high reproducibility, high quantity of information throughout multiple loci on the genome, high resolution enough to determine some small genetic differences and generate multiplex ratio of data for numerical analysis. RAPD markers have the high variable of replicability, but easy to use. RFLP technique provides low quantity of information, but has higher replicability and resolution of genetic differences when compared to RAPD technique, but lower than AFLP and microsatellite techniques. Microsattellite method has good qualification as well as AFLP, but needs some

knowledge about genetic information which takes development time, difficult to use and develop the process. However, AFLP and microsatellite techniques, coupled with sequencing information for systematic analyses, could be synergized as main tools for the analysis of genetic variation (Mueller and Wolfenbarger, 1999).

AFLPs technique is being widely used for genetic diversity studies because it shows significantly polymorphism and is robust and reliable for molecular genetic marker assay. AFLP fingerprinting analysis has been used to discriminate between accessions of a number of plants *i.e*. olive (Angiolillo *et al.*, 1999), jackfruit (Schnell *et al.*, 2001), Neem; *Azadirachta indica* (Singh *et al.*,2002), *Erythroxylum* (Johnson *et al.*, 2003), *Prunus* (Aradhya *et al.*, 2004), *Gardenia jasminoides* (Han *et al.*, 2007), *Boesenbergia* (Techaprasan *et al.*, 2008), pomegranate (Jbir *et al.*, 2008), *Panax japonicus* (Choi *et al.*, 2008) Tunisian Fig; *Ficus carica* (Baraket *et al.*, 2009), *Fritillaria cirrhosa* (Zhang *et al.*, 2010).

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.

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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

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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.

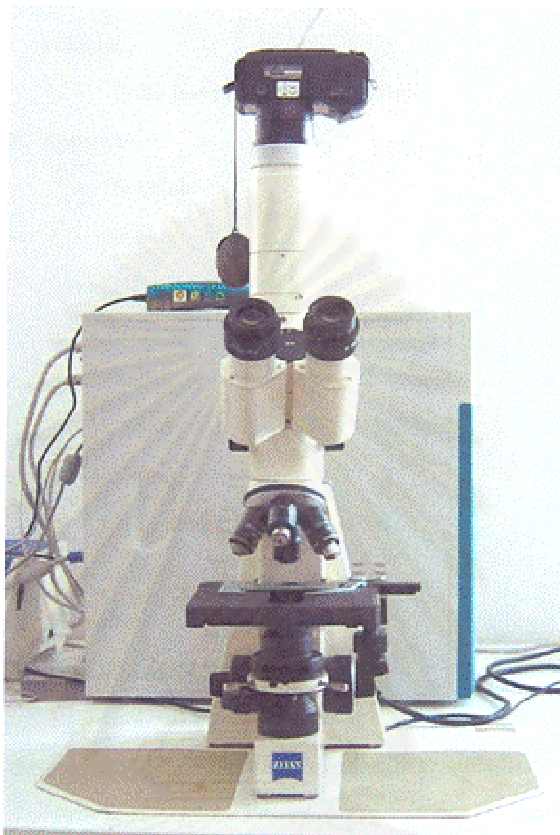

Figure 3.1 Compound microscope Zeiss model Axiostar attached with digital camera Sony Cyber-shot DSC-S85

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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.

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

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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°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.

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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.

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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.

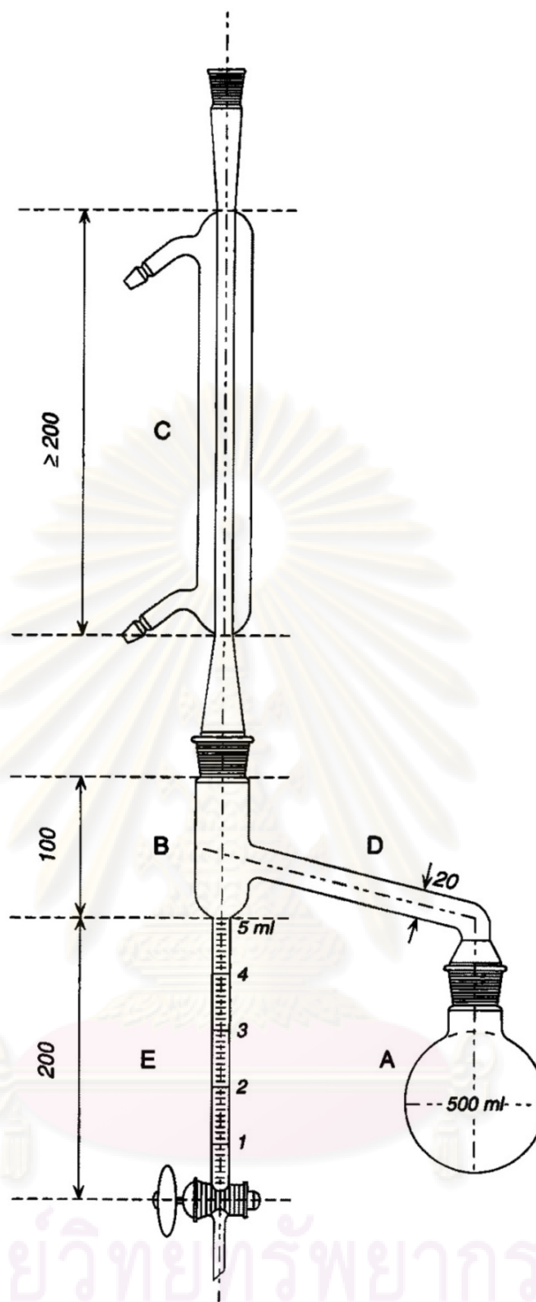

Figure 3.2 Azeotropic distillation method (THP, 1995).

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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**).

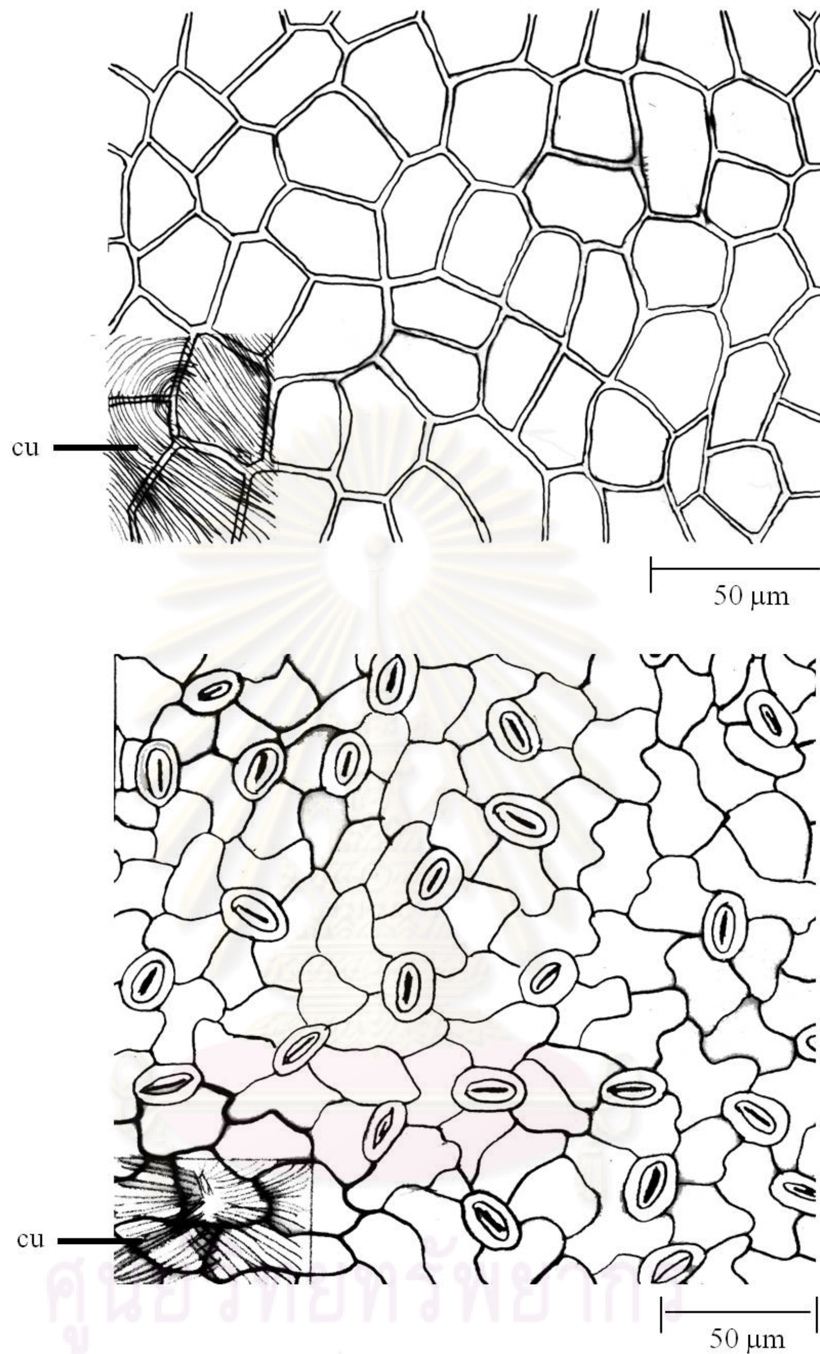

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Figure 3.3 Epidermal cells of *L. speciosa* leaf. A: upper epidermis of the lamina; B: lower epidermis of the lamina.

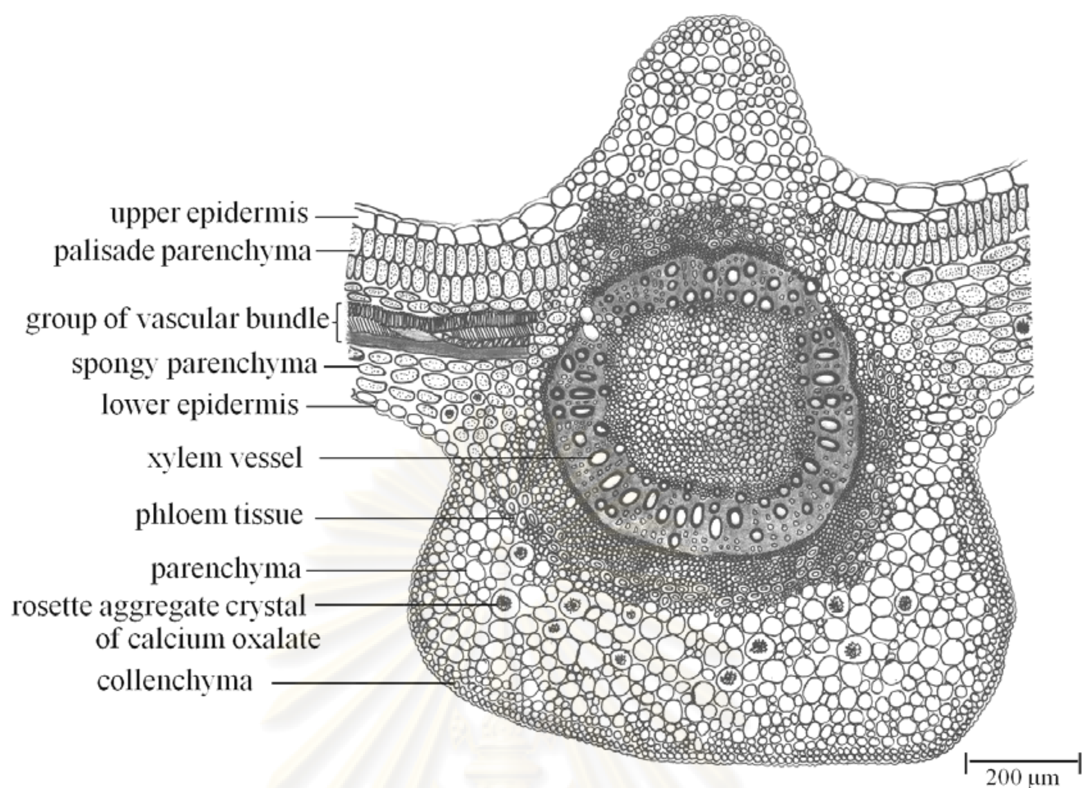

Figure 3.4 Transverse section of midrib of *L. speciosa* leaves.

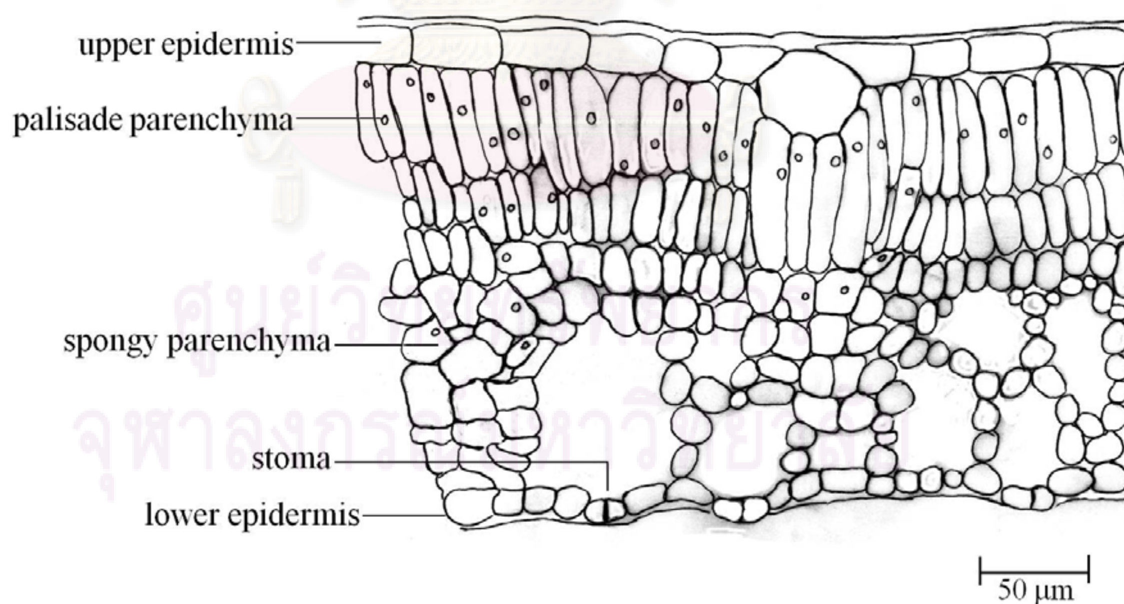

Figure 3.5 Transverse section of lamina of *L. speciosa* leaves.

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.

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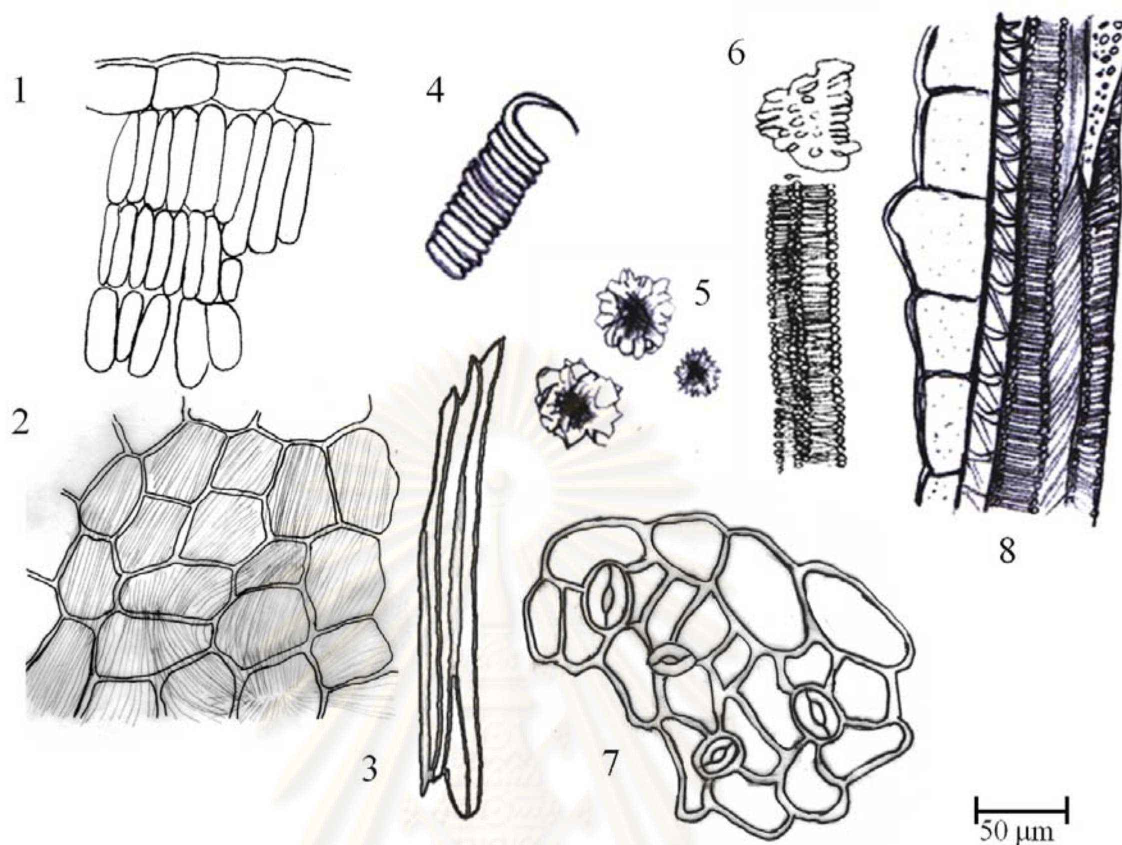

Figure 3.6 Powdered drug of the leaf of *L. speciosa*.

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.

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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.**

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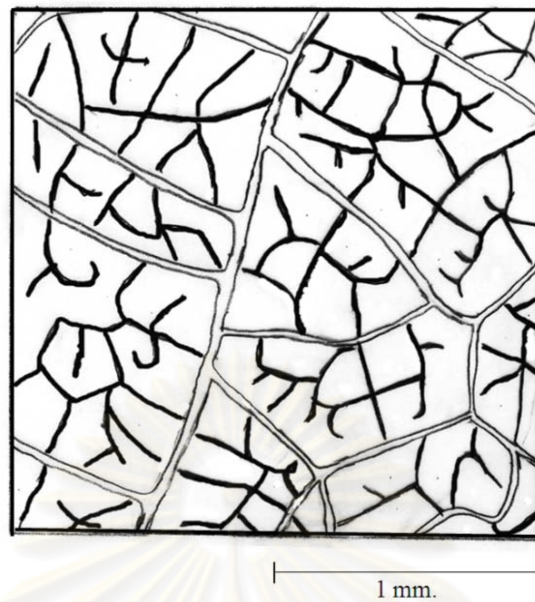

Figure 3.7 Vein-islet and veinlet termination of the *L. speciosa* leaves.

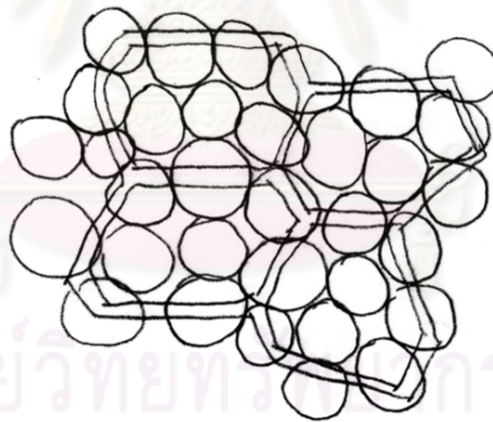

Figure 3.8 Four upper contiguous epidermal cells with underlying palisade cells of *L. speciosa* leaves.

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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

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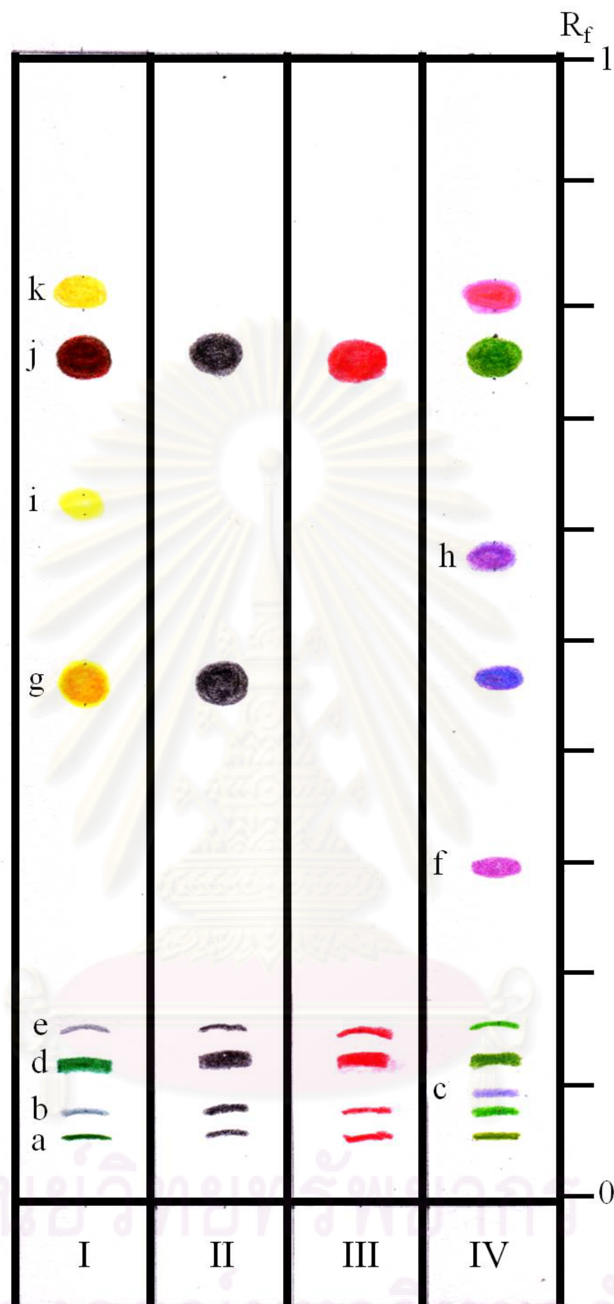

Figure 3.9 TLC patterns of methanolic extract of *L. speciosa* leaves which using chloroform and acetone (4:1) as solvent system.

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

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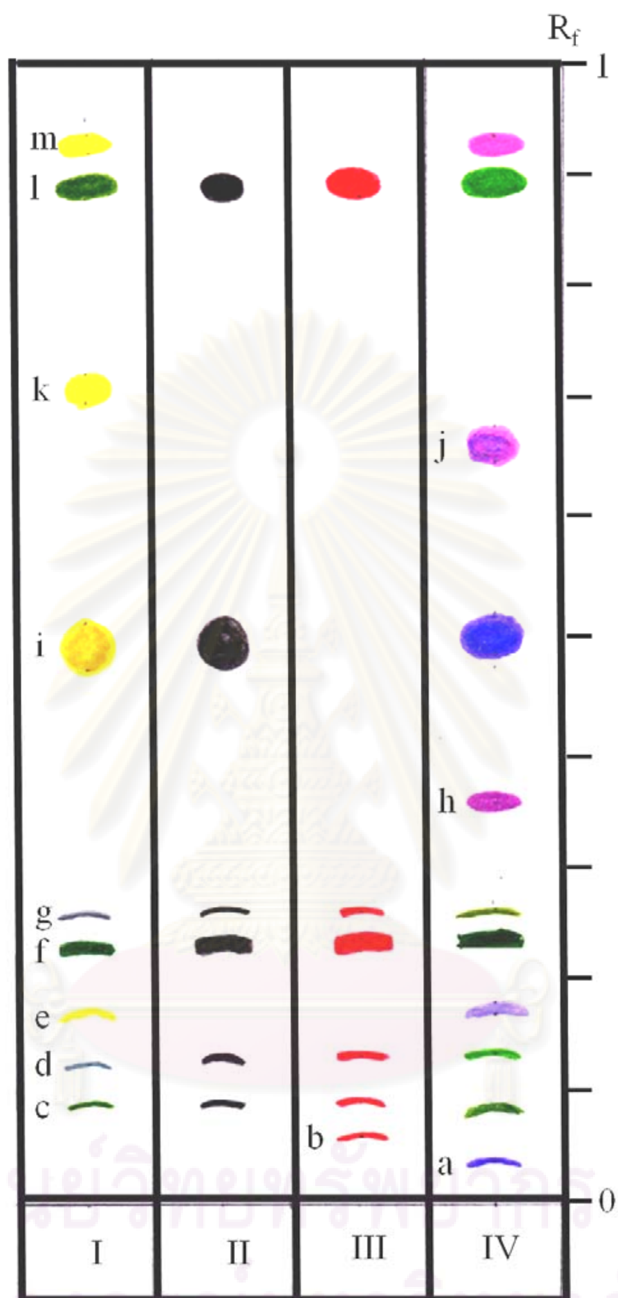

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Figure 3.10 TLC patterns of methanolic extract of *L. speciosa* leaves which using chloroform and methanol (95:5) as solvent system.

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

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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)

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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.

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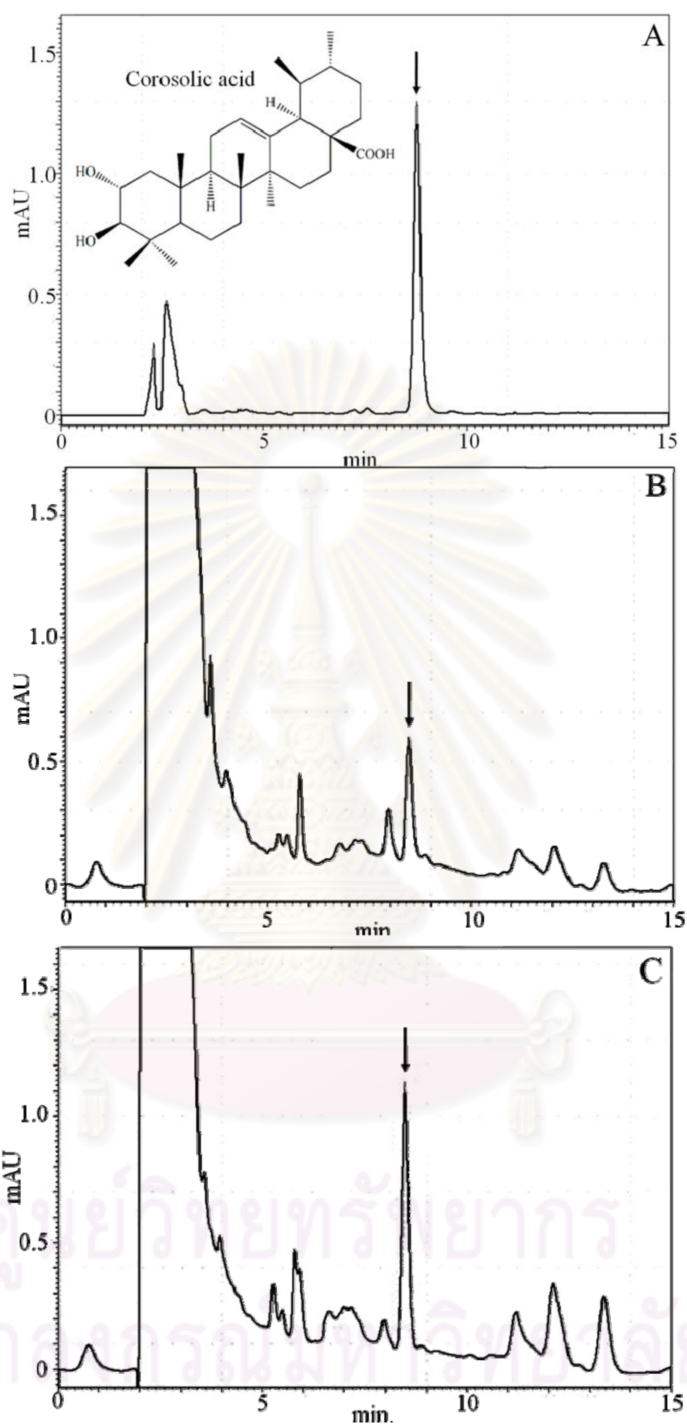

Figure 3.11 HPLC/DAD chromatograms detected at 204 nm of:

a) corosolic acid,

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

c) crude methanolic extract spiked with corosolic acid.

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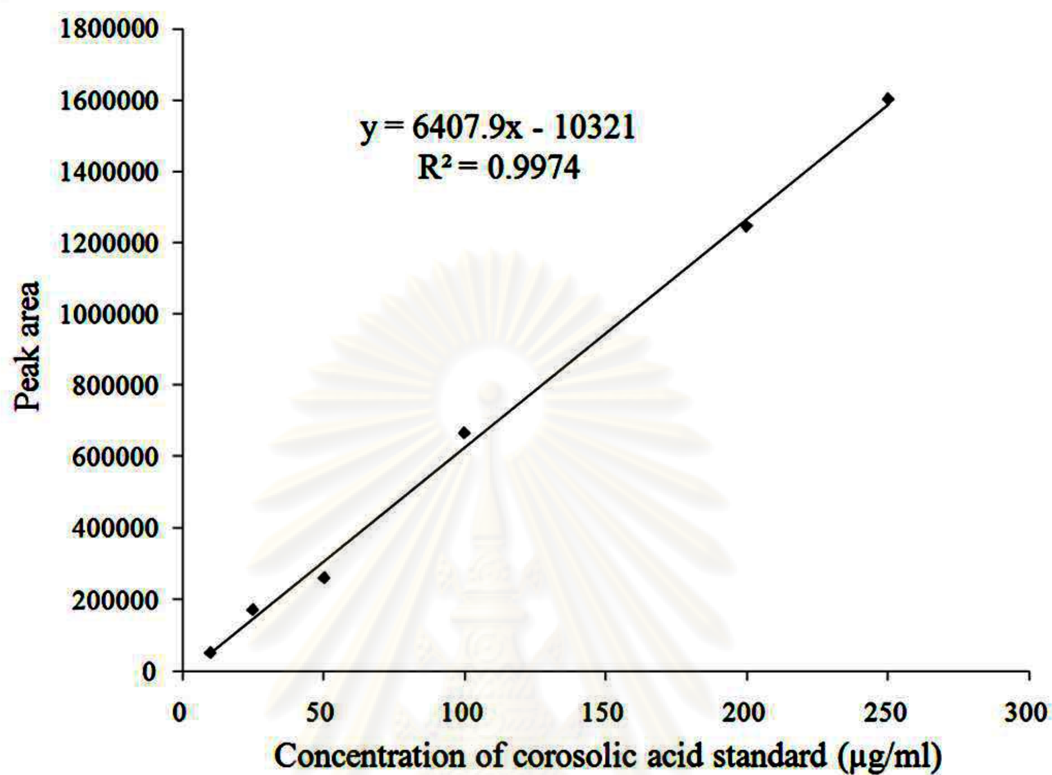

Figure 3.12 Standard curve of corosolic acid, peak area at 204 nm.

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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).

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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.

CHAPTER IV

Amplified Fragment Length Polymorphisms (AFLPs)

AFLP technique has been identified as a robust DNA fingerprinting technique (Vos *et al*., 1995) that detects significant levels of polymorphism between accessions. AFLP markers are highly repeatable (Breyne *et al.*, 1997), provide broad genomic coverage and a virtually limitless number of genetic markers. AFLP can be utilized not only to determine the relationship of closely related species, but as a tool to authenticate material in herbal remedies through the use of genetic fingerprinting. The present work utilized the primers combinations. The polymorphic bands were generated. Then, AFLP amplification bands showed a species-specific band of *L. speciosa* for authentication of plant materials. In addition, AFLP binary data was used for studying the genetic relationship in *Lagerstroemia.*

4.1 Plant materials

The leaves of four *Lagerstroemia species*, *L. speciosa*, *L. macrocarpa*, *L .loudonii* and *L. floribunda* were used in this study. A closely related species, *Lawsonia inermis*, was used as an outgroup. All of the collected plant materials and their localities are listed in **Table 4.1**. The genuine leaves specimens were authenticated by Associate Professor Thatree Phadungchareon. The specimens have been deposited in the department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

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Table 4.1 Plant materials for AFLPs evaluation.

Plant species	No.	Voucher no.	Locations
L. speciosa	LS1	LS1WT200908	Rajamangala University of Technology Phra Nakhon. Bangkok
	LS2	LS2WT220908	The Faculty of Pharmaceutical Sciences, Chulalongkorn university
	LS3	LS3WT070109	The Faculty of Veterinary Sciences. Chulalongkorn university
	LS4	LS4WT150109	The Royal Turf Club of Thailand Under Royal Patronage, 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
L. macrocarpa	LM1	LM1WT051008	The Faculty of Political Sciences, Chulalongkorn university
	LM2	LM2TP261208	Blood Bank Section, Faculty of Medicines, Chulalongkorn university
	LM3	LM3WT270329	The Intanin field, Maejo University, Chiang Mai Province
	LM4	LM4WT270309	The Maharaj Nakorn Chiang Mai Hospital, Chiang Mai Province
L. loudonii	LL1	LL1WT130309	The Central Botanical Garden (Pukae), Saraburi Province
	LL2	LL2WT190409	Rajavinit Mathayom school, Bangkok
L. floribunda	LF1	LF1WT130309	The Central Botanical Garden (Pukae), Saraburi Province
	LF2	LF2WT190409	The Royal Turf Club of Thailand Under Royal Patronage, Bangkok
Lawsonia inermis	LAW	LAWSW180809	Jatujak market, Bangkok

4.2 Methods

4.2.1 Genomic DNA extraction

Approximately 3 g of leaves was ground in liquid nitrogen with mortar and pestle. Isolation of total genomic DNA followed the protocol described by Doyle and Doyle (1990), which is a CTAB-based extraction protocol. The concentration of genomic DNA was estimated by using spectrophotometric measurement of UV absorbance at 260 nm.

4.2.2 AFLPs procedure

The AFLPs procedure was carried out as reported by Vos *et al.* (1995) with a few modifications.

4.2.2.1 Digestion of genomic DNA

Approximately 100 ng/µl of genomic DNA was digested with two restriction enzymes, *EcoR*I HF and *Mse*I in 10x buffer 4. The reaction was incubated at 37°C for 2 hours. The complete digestion products were investigated by 1% agarose gel electrophoresis in 0.5x TBE using 10 µl of digestion product.

Table 4.2 Reaction mixture for digesting genomic DNA with restriction enzymes.

Digestion components	Volumes (µl)
100 ng/µl Genomic DNA	5.00
10 unit/µl *EcoR*I (NEB, United Kingdom)	0.50
10 unit/µl *Mse*I (NEB, United Kingdom)	0.50
10X buffer 4 (NEB, United Kingdom)	4.00
ddH_2O	30.00
Total	**40.00**

4.2.2.2 Ligation of genomic DNA

The digestion genomic DNA fragment was ligated to *EcoR*I-adapter and *Mse*I-adapter (**Table 4.3**) by adding 10 µl of ligation master mix. The reaction was incubated at 37°C for at least 3 hours (preferably overnight). The completeness of ligation process was detected by 1% agarose gel electrophoresis in 0.5x TBE using 5 µl of ligation reaction and 1 µl of 6x loading dye. Each ligation reaction was diluted as ten-folded with ddH_2O. The aliquots were stored at -20°C

Table 4.3 Sequences of adapters and primers used for AFLPs analysis.

Name/Abbreviation	Type	Sequence (5′--- 3′)
*EcoR*I-adapter		CTC GTA GAC TGC GTA CC AAT TGG TAC GCA GTC TAC
MseI-adapter		GAC GAT GAG TCC TGA G TAC TCA GGA CTC AT
ER1A	Primer +1	AGA CTG CGT ACC AAT TCA
ER3ACC	Primer +3	AGA CTG CGT ACC AAT TCA CC
ER3AAC	Primer +3	AGA CTG CGT ACC AAT TCA AC
ER3AAG	Primer +3	AGA CTG CGT ACC AAT TCA AG
MS3C	Primer +1	GAT GAG TCC TGA GTA AC
MS3CTA	Primer +3	GAT GAG TCC TGA GTA ACT A
MS3CAG	Primer +3	GAT GAG TCC TGA GTA ACA G
MS3CAT	Primer +3	GAT GAG TCC TGA GTA ACA T
MS3CTT	Primer +3	GAT GAG TCC TGA GTA ACT T
MS3CTG	Primer +3	GAT GAG TCC TGA GTA ACT G
MS3CAC	Primer +3	GAT GAG TCC TGA GTA ACA C

Table 4.4 Reaction mixture for nucleotide adapter ligation.

Ligation components	Volume (µl)
5 µM EcoRI-adapter (Eurofins MWG Operon, Germany)	1.00
50 µM MseI-adapter (Eurofins MWG Operon, Germany)	1.00
T4 DNA Ligase (NEB, United Kingdom)	0.40
10x ligation buffer (NEB, United Kingdom)	1.00
ddH_2O	6.60
Total	10.00

4.2.2.3 Pre-selective amplification

Five microliters of diluted ligation product were amplified using ER1A and MS1C primers (**Table 4.3**). Each reaction was composed of 50 µl pre-amplification primer mixture (**Table 4.5**). PCR was performed in a C1000 Themal Cycler (Biorad, USA) and used the following pre-selective PCR program. The pre-amplification PCR product was diluted to ten-folded with ddH_2O, mixed and placed in -20°C or proceeds to next step.

Table 4.5 Reaction mixture for pre-amplification reaction.

Ligation components	Volume (µl)
Digestion – ligation product	5.00
10 µM ER1A (Eurofins MWG Operon, Germany)	1.00
10 µM MS1C (Eurofins MWG Operon, Germany)	1.00
10x PCR buffer (Invitrogen, USA)	5.00
50 mM $MgCl_2$ (Invitrogen, USA)	1.50
10 mM dNTPs (Fermentas, Canada)	1.00
5 U/µl Taq polymerase (Invitrogen, USA)	0.50
ddH_2O	35.00
Total	50.00

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Pre-amplification PCR program (C1000 Thermal Cycler, Biorad)

Step 1:	Predenaturation step	$95^{O}C$	3 min.
Step 2:	Denaturation step	$95^{O}C$	30 sec.
Step 3:	Annealing step	$56^{O}C$	1 min
Step 4:	Extension step	$72^{O}C$	1 min
Step 5:	20 cycles; step 2 to step 4		
Step 6:	Final extension step	$72^{O}C$	10 min
Step 7:	Hold	$4^{O}C$	∞
Step 8:	End		

4.2.2.4 Selective amplification

Three microliters of the diluted pre-selective PCR product were used as selective amplification in a reaction tube containing 20 µl selective amplification mixtures (**Table 4.6**). Eight primer pairs were used for the selective amplification (**Table 4.3**). Selective amplification mixtures were performed the following selective PCR program. The selective amplification product was added with 10 µl of sequencing dye. The selective amplified PCR products were determined by using 1% agarose gel electrophoresis in 0.5x TBE. The selective PCR products were run on 4.5% denaturing polyacrylamide gel electrophoresis.

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Table 4.6 Reaction mixture for selective amplification reaction.

Ligation components	**Volume (µl)**
Dilute pre-amplification product	3.00
10 µM ER3A_ _ (Eurofins MWG Operon, Germany)	1.00
10 µM MS3C_ _ (Eurofins MWG Operon, Germany)	1.00
10x PCR buffer (Invitrogen, USA)	2.00
50 mM $MgCl_2$ (Invitrogen, USA)	1.00
10 mM dNTPs (Fermentas, Canada)	0.40
5 U/µl Taq polymerase (Invitrogen, USA)	0.10
ddH_2O	11.00
Total	20.00

Selective amplification PCR program (C1000 Thermal Cycler, Biorad)

Step 1:	Predenaturation step	$95^{O}C$	3 min.
Step 2:	Denaturation step	$95^{O}C$	30 sec.
Step 3:	Annealing step	$65^{O}C$	30 sec. (starting at 65 ^{O}C and reducing each step 0.6 ^{O}C until 56 ^{O}C)
Step 4:	Extension step	$72^{O}C$	1 min
Step 5:	12 cycles; step 2 to step 4		
Step 6:	Denaturation step	$95^{O}C$	30 sec
Step 7:	Annealing step	$56^{O}C$	30 sec
Step 8:	Extension step	$72^{O}C$	1 min
Step 9:	23 cycles; step 6 to 8		
Step 10:	Final extension step	$72^{O}C$	10 min
Step 11:	Hold	$4^{O}C$	∞
Step 12:	End		

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4.2.3 Detection of AFLPs bands using denaturing polyacrylamide gel electrophoresis

Selective amplification products (AFLPs products) were separated by 4.5% denaturing polyacrylamide gel electrophoresis in 0.5X TBE in a Sequi-Gen GT Sequencing cell (Biorad, USA). The AFLPs bands on polyacrylamide gel were detected by silver nitrate staining. The silver nitrate staining was followed the protocol described by Bassam *et al.* (1991).

4.2.4 Data analysis

For genetic diversity analysis, a band was considered polymorphic if it was present in at least one genotype and absent in others. AFLP amplification products were scored for their presence (1) or absence (0) across 17 accessions for all the primer combinations employed to generate binary matrix. Genetic similarity (GS) was calculated by making a pairwise comparison among the accession using Jaccard's coefficient (Jaccard, 1908).The formula is given as follows: Gs_{ij} = a/a + b + c, where Gs_{ij} is the measure of GS between individuals i and j, a is the number of polymorphic fragments are shared by i and j, b is the number of fragments present in i and absent in j, and c is the number of fragments present in j and absent in i. A dendrogram was constructed from the matrix of similarity coefficients using the Unweighted Pair-Group Method of the Arithmetic average (UPGMA) technique (Sneath and Sokal, 1973) of the NTSYS-pc software package version 2.11T (Rohlf, 2000). The cophenetic correlation coefficient was generated by means of COPH routine in order to check the goodness of fit between the cluster in the dendrogram and the similarity coefficient matrix.

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4.3 Results

4.3.1 AFLP analysis

Genomic DNA was extracted from leaves of each sample using a CTAB extraction method. The quality of extracted genomic DNA was electrophoretically determined using 0.8% agarose gel (w/v). High molecular weight DNA at 23.1 Kbs along with sheared DNA was obtained from that leaves. Some DNA samples contained RNA contamination as visualized by the smear at the bottom of gel.

A total of 28 primer combinations were screened, and the amplification products of eight primer combinations (**Table 4.7**) were further genotyped across all investigated taxa. In total, 461 amplified fragments ranging from 40 to 700 bps in size were generated from respective primer combinations. The average number of polymorphic bands per primer was 58, while the amounts for the eight primers range from 24 to 107 bands (**Table 4.7**). The AFLP profiles can be used to distinguish between the *Lagerstroemia* accessions by their unique band patterns (**Figure 4.1 – 4.8**).

Table 4.7 Primer combination, the number of AFLP bands, size range and the percentage of polymorphic bands resulted from AFLP analyses of this study.

Primer combination	**Number of AFLP bands**	**Size range (bps)**	**Percentage of polymorphic bands**
ER3AAC / MS3CAG	88	40 – 700	100
ER3AAC / MS3CAT	67	40 – 700	98
ER3AAC / MS3CTT	24	60 – 600	100
ER3AAG / MS3CTG	54	40 – 700	96.30
ER3ACC / MS3CAC	43	40 – 600	100
ER3ACC / MS3CAT	51	40 – 700	96.08
ER3ACC / MS3CTA	27	60 – 600	100
ER3ACC / MS3CTT	107	40 – 700	100
Total	461	40 – 700	98.9795

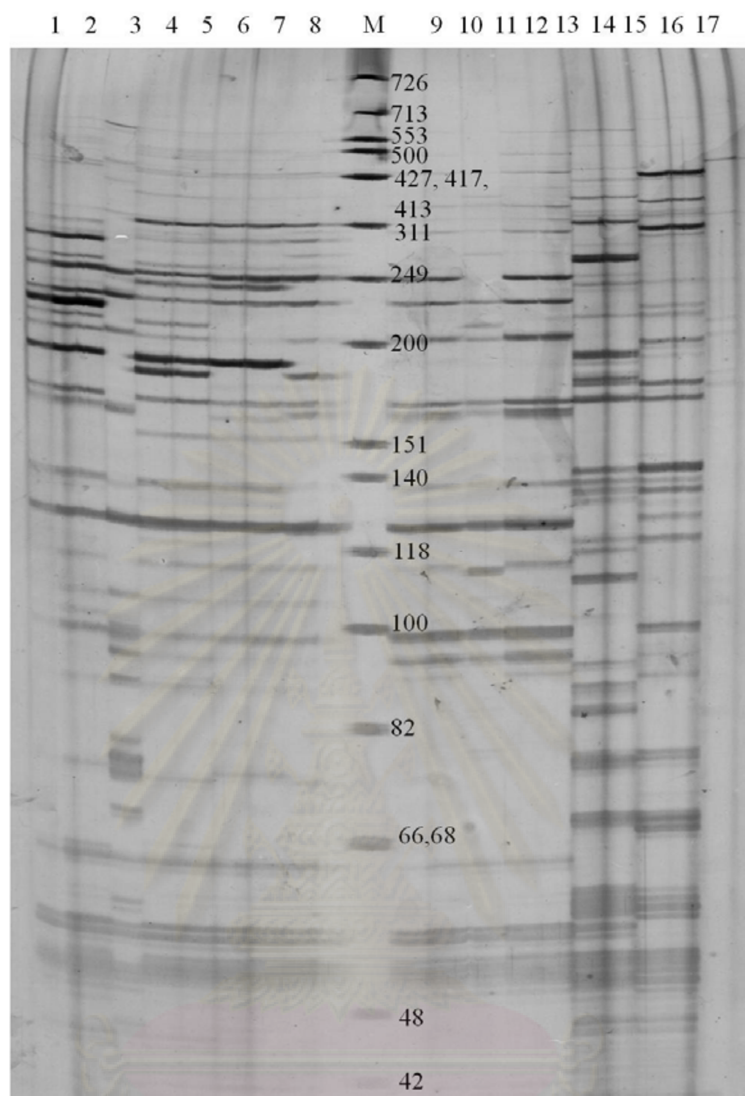

Figure 4.1 AFLP profile generated by primer combination of ER3AAC, MS3CAG. Lane designations with accession number in parenthesis are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1

Lane 2: *L. speciosa* sample LS2

Lane 3: *L. speciosa* sample LS3

Lane 4: *L. speciosa* sample LS4

Lane 5: *L. speciosa* sample LS5

Lane 6: *L. speciosa* sample LS6

Lane 7: *L. speciosa* sample LS7

Lane 8: *L. speciosa* sample LS8

Lane 9: *L. macrocarpa* sample LM1

Lane 10: *L. macrocarpa* sample LM2

Lane 11: *L. macrocarpa* sample LM3

Lane 12: *L. macrocarpa* sample LM4

Lane 13: *L. loudonii* sample LL1

Lane 14: *L. loudonii* sample LL2

Lane 15: *L. floribunda* sample LF1

Lane 16: *L. floribunda* sample LF2

Lane 17: *Lawsonia inermis* sample LAW

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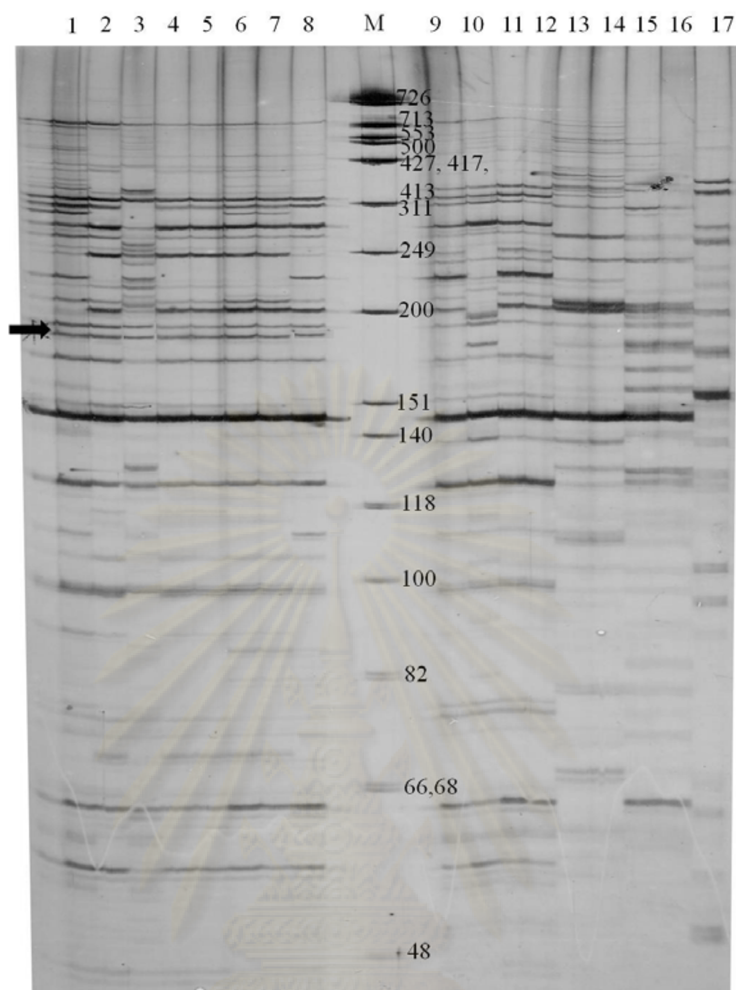

Figure 4.2 AFLP profile generated by primer combination of ER3AAC, MS3CAT. Arrow indicates unique bands of *Lagerstroemia speciosa*. Lane designations with accession number in parenthesis are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

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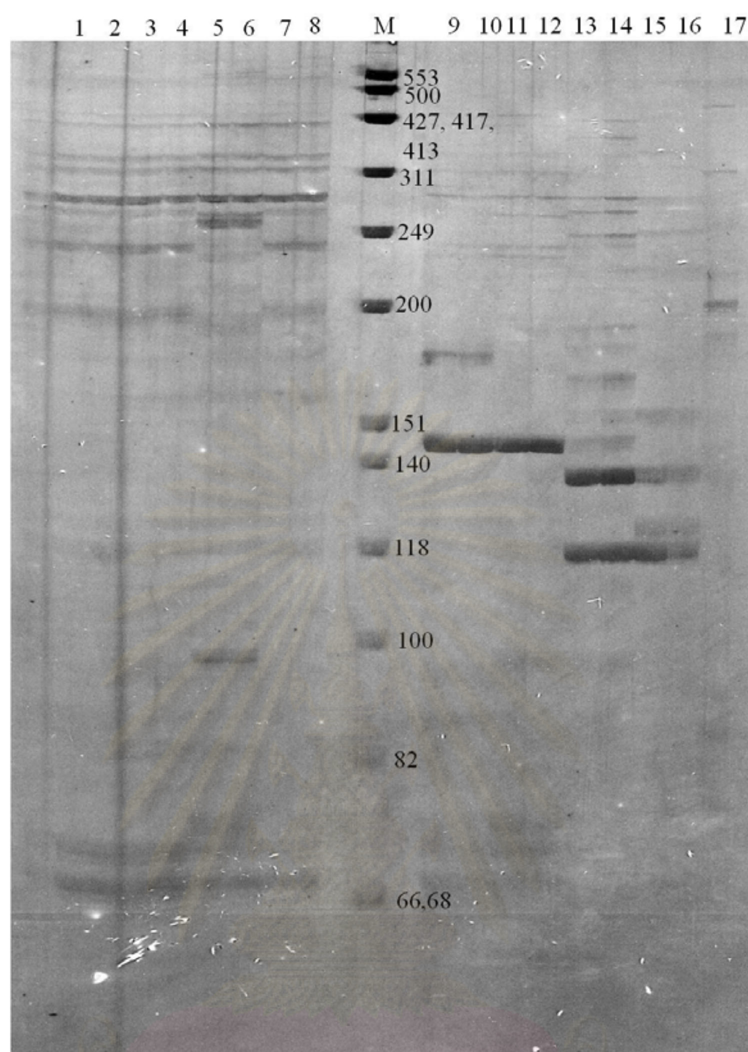

Figure 4.3 AFLP profile generated by primer combination of ER3AAC, MS3CTT. Lane designations with accession number in parenthesis are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

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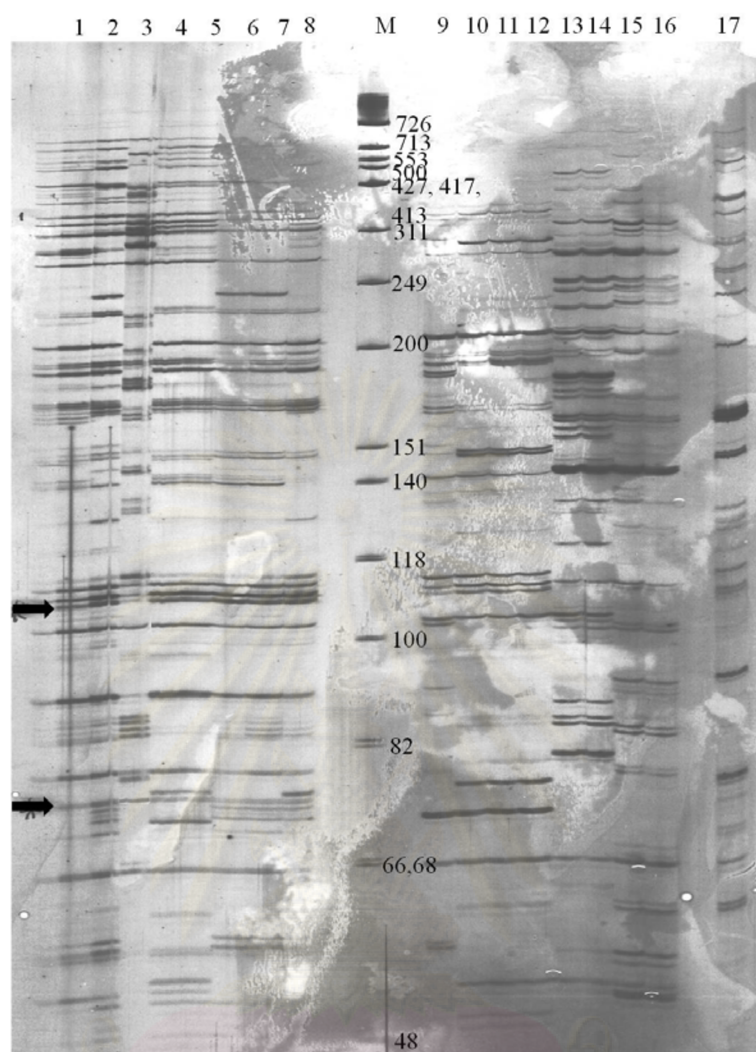

Figure 4.4 AFLP profile generated by primer combination of ER3AAG, MS3CTG. Arrow indicates unique bands of *Lagerstroemia speciosa*. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1

Lane 2: *L. speciosa* sample LS2

Lane 3: *L. speciosa* sample LS3

Lane 4: *L. speciosa* sample LS4

Lane 5: *L. speciosa* sample LS5

Lane 6: *L. speciosa* sample LS6

Lane 7: *L. speciosa* sample LS7

Lane 8: *L. speciosa* sample LS8

Lane 9: *L. macrocarpa* sample LM1

Lane 10: *L. macrocarpa* sample LM2

Lane 11: *L. macrocarpa* sample LM3

Lane 12: *L. macrocarpa* sample LM4

Lane 13: *L. loudonii* sample LL1

Lane 14: *L. loudonii* sample LL2

Lane 15: *L. floribunda* sample LF1

Lane 16: *L. floribunda* sample LF2

Lane 17: *Lawsonia inermis* sample LAW

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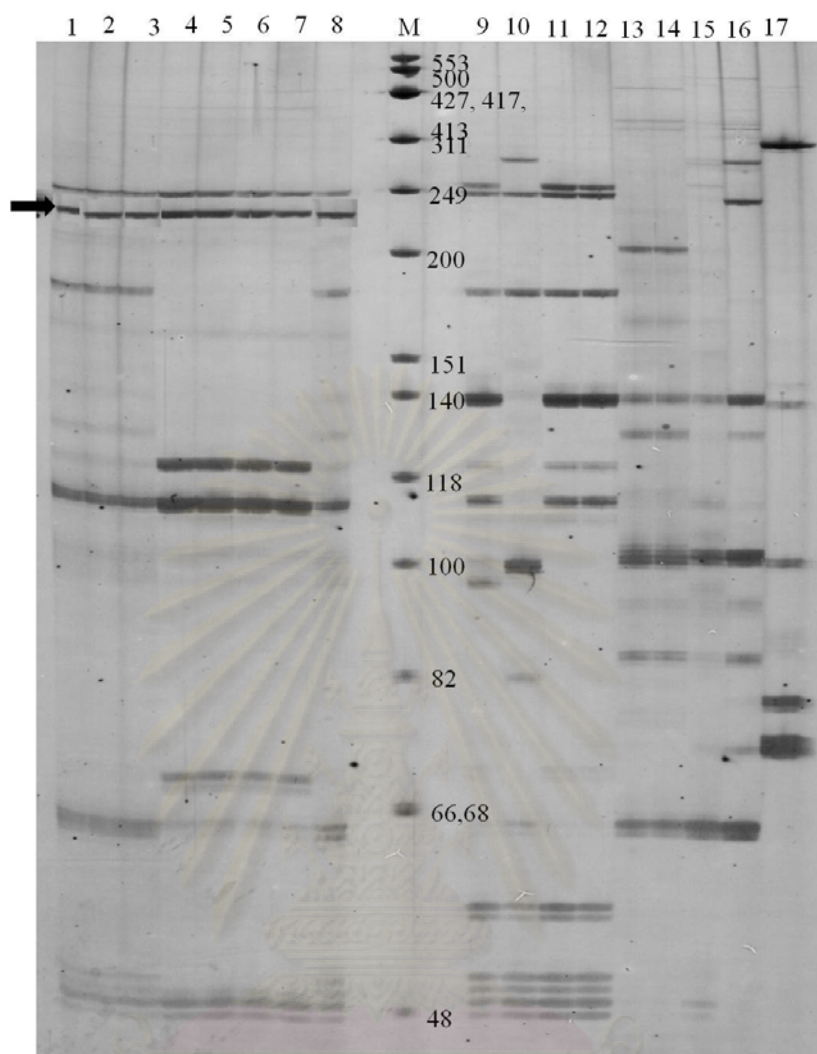

Figure 4.5 AFLP profile generated by primer combination of ER3ACC, MS3CAC. Arrow indicates unique bands of *Lagerstroemia speciosa*. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

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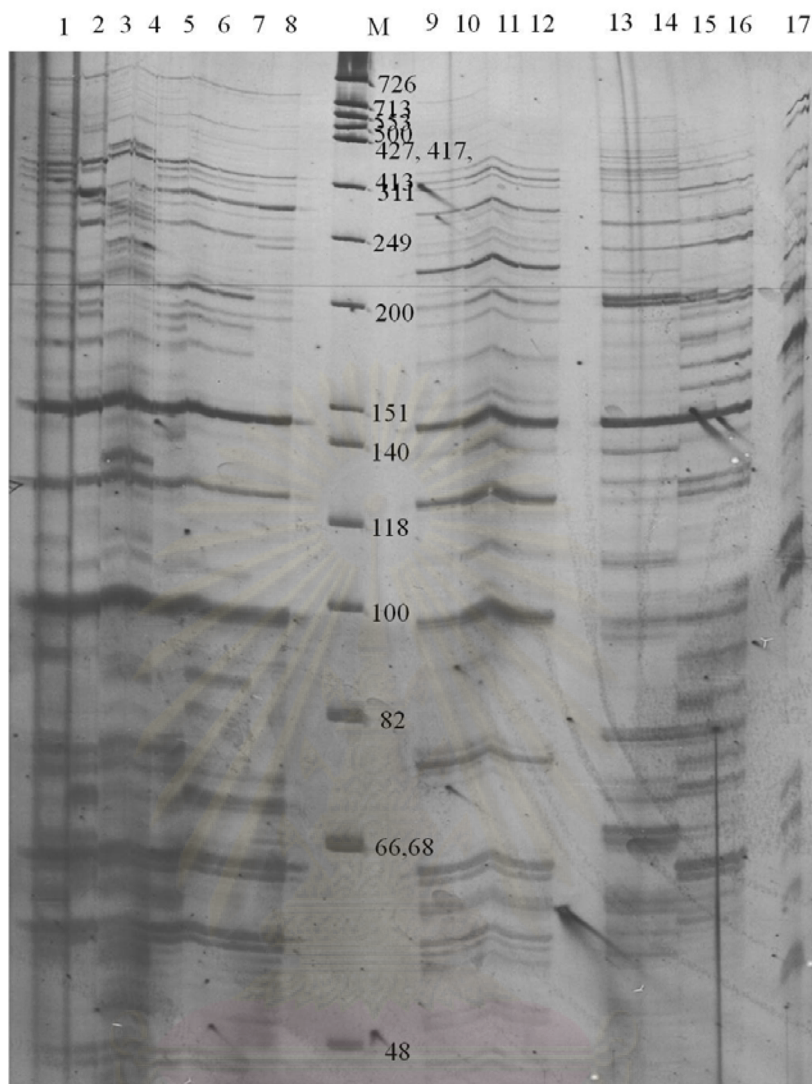

Figure 4.6 AFLP profile generated by primer combination of ER3ACC, MS3CAT.
Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

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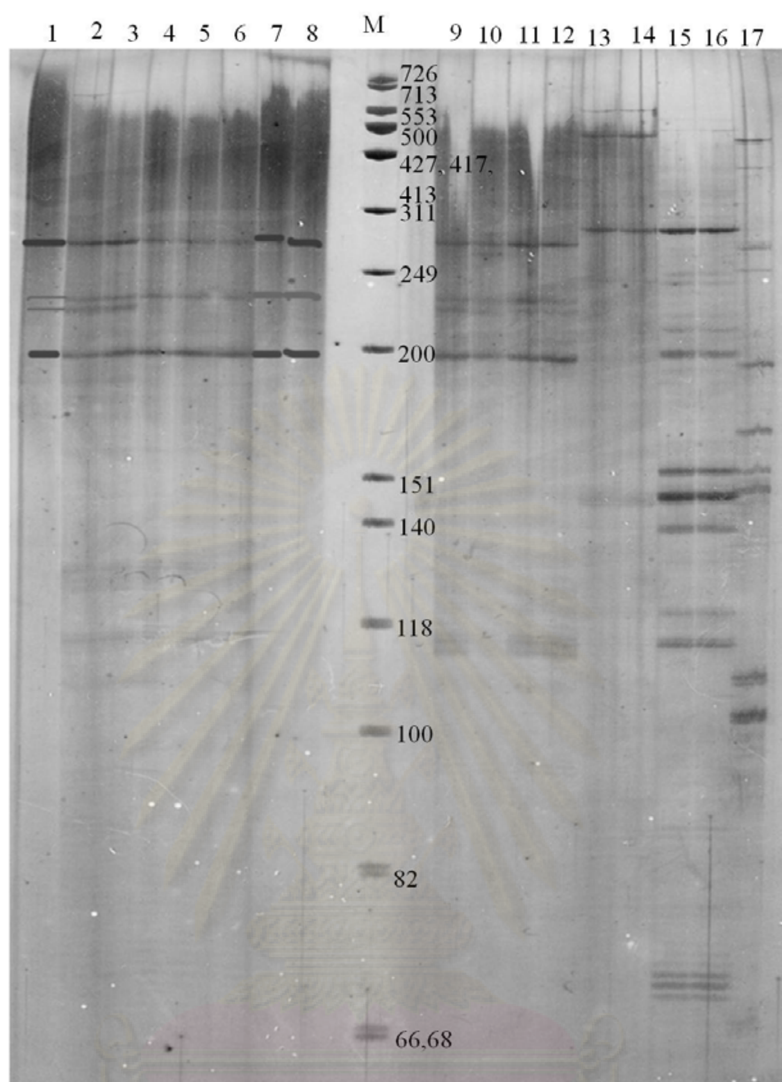

Figure 4.7 AFLP profile generated by primer combination of ER3ACC, MS3CTA. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

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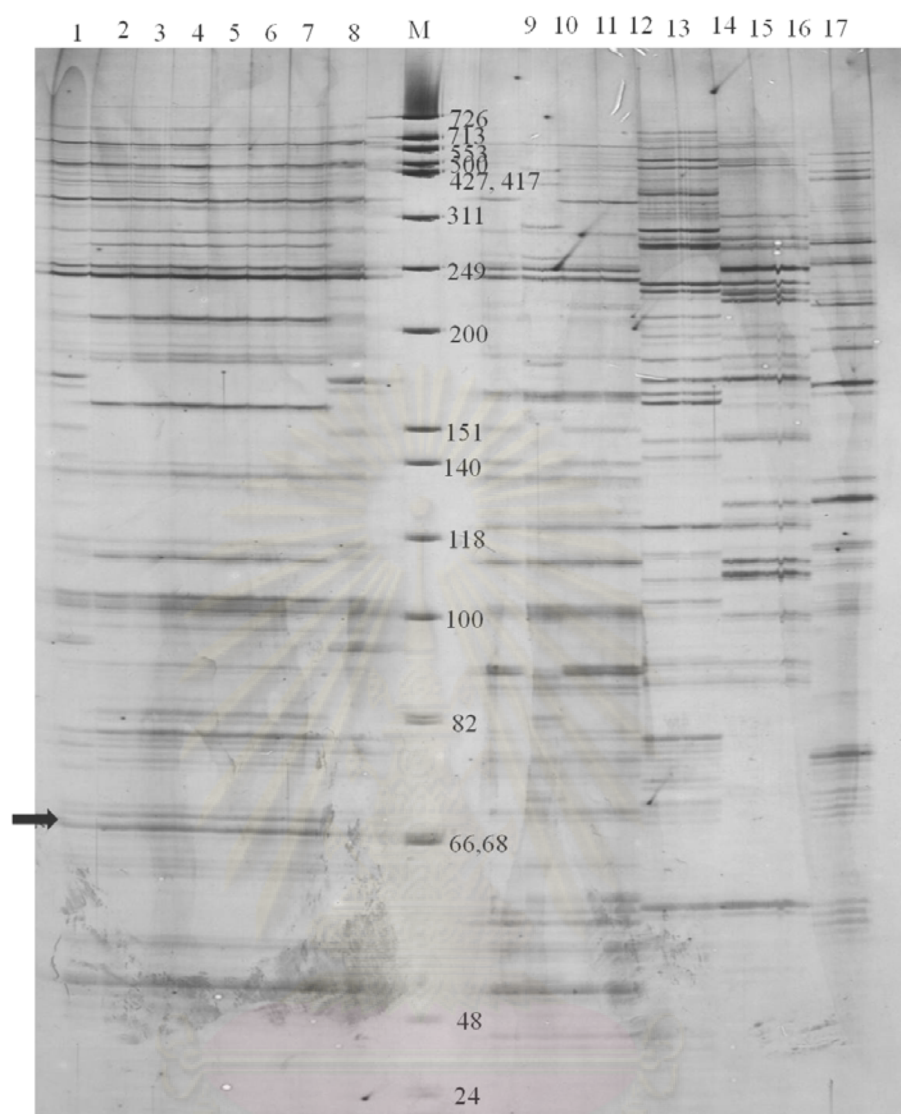

Figure 4.8 AFLP profile generated by primer combination of ER3ACC, MS3CTT. Arrow indicates unique bands of *Lagerstroemia speciosa*. Lane designations with accession number in parenthesis are as follows:
Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *L. speciosa* sample LS1
Lane 2: *L. speciosa* sample LS2
Lane 3: *L. speciosa* sample LS3
Lane 4: *L. speciosa* sample LS4
Lane 5: *L. speciosa* sample LS5
Lane 6: *L. speciosa* sample LS6
Lane 7: *L. speciosa* sample LS7
Lane 8: *L. speciosa* sample LS8
Lane 9: *L. macrocarpa* sample LM1
Lane 10: *L. macrocarpa* sample LM2
Lane 11: *L. macrocarpa* sample LM3
Lane 12: *L. macrocarpa* sample LM4
Lane 13: *L. loudonii* sample LL1
Lane 14: *L. loudonii* sample LL2
Lane 15: *L. floribunda* sample LF1
Lane 16: *L. floribunda* sample LF2
Lane 17: *Lawsonia inermis* sample LAW

4.3.2 Genetic relationship

The dendrogram was generated by the Jaccard's similarity matrix and UPGMA method revealed genetic relationship among four *Lagerstroemia* species and outgroup, *Lawsonia* (**Figure 4.9**). A high cophenetic correlation coefficient of 0.99837 between the Jaccard's similarity data matrix and the cophenetic matrix was obtained, indicating a good fit between the dendrogram clusters and the similarity matrices. According to the dendrogram, four main groups were produced. Cluster I was composed of *L. speciosa* and *L. macrocarpa*. This cluster was divided into two subgroups. *L. loudonii* were in cluster II, whereas, *L. floribunda* were in cluster III. The outlier, *Lawsonia inermis*, was classified in cluster IV.

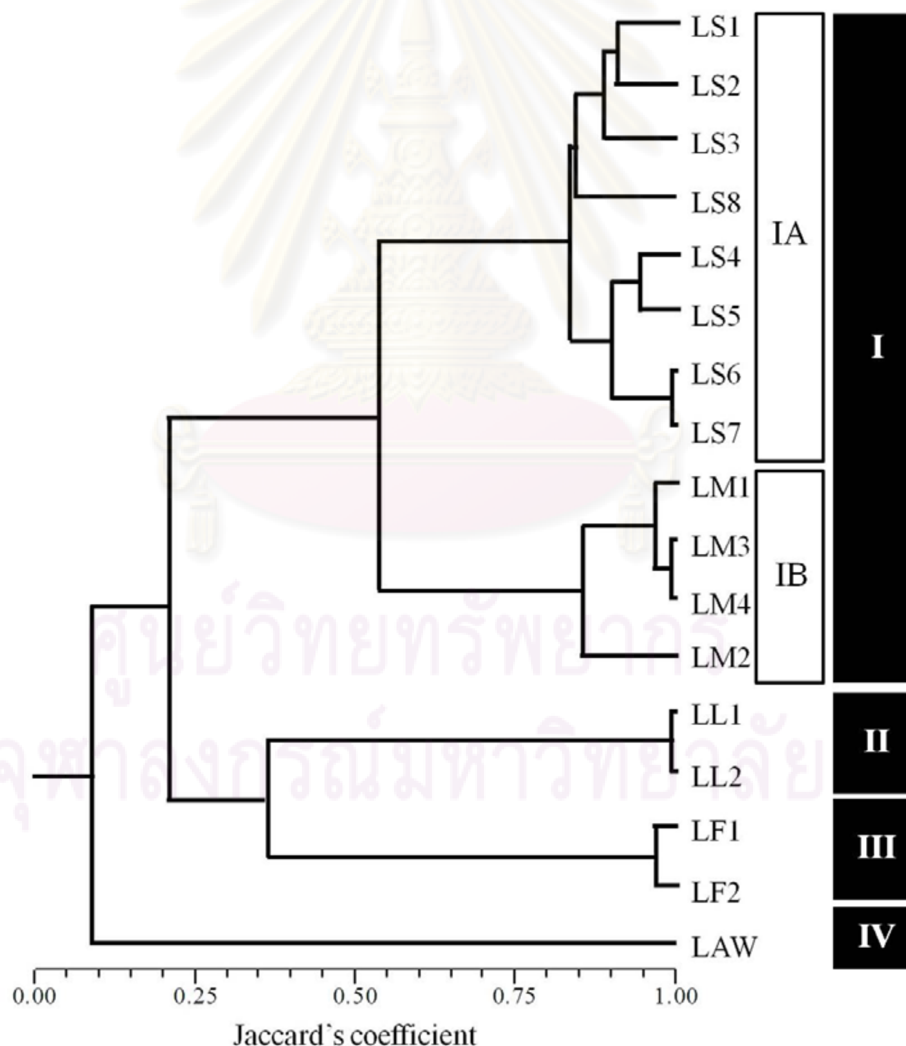

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Figure 4.9 UPGMA dendrogram based on Jaccard's similarity coefficient among *Lagerstroemia* and *Lawsonia inermis* accessions.

4.4 Discussion

Assessment of genetic diversity provides an efficient and effective way to estimate genetic variation and delineate phenetic relationships among accessions. DNA-based markers, such as RAPD (Williams *et al.*, 1990), AFLP (Vos *et al*., 1995) and SSR markers (Tautz *et al.*, 1984) have been routinely employed for analysis of variation in gene pool of herb and for medicinal plant identification. These molecular markers uncover variation at different genomic location (Powell *et al.*, 1996; Russel *et al.*, 1997). AFLP is based on selective PCR amplification of restriction fragments from a digest of total genomic DNA using PCR. The procedure of the AFLP analysis is more time-consuming than RAPD. However, a major advantage of AFLP markers is the capacity to reveal many polymorphic bands in one lane compared to RAPD markers (Barker *et al.*, 1999; Ha *et al.*, 2002). Because of the higher sensitivity of AFLP, the weak divergence can be efficiently detected by AFLP. Also, AFLP technique is a reliable, stable, and rapid assay for use in molecular marker screening (Jia *et al.*, 2001). Thus the AFLP technique has been increasingly used to assess genetic diversity and authentication in a variety of organisms (Cresswell *et al.*, 2001; Das *et al.*, 1999; Winfield *et al.*, 1998).

Three candidates species-specific of *L. speciosa*, a 230 bp fragment from ER3ACC/MS3CAC, a 110 bp fragment from ER3AAG/MS3CTG, and 190 bp fragment from ER3AAC/MS3CAT, should be developed further to SCAR marker. Conversion of AFLP marker to SCAR marker by sequencing of species-specific bands can provide effective methods for the identification and classification in a various species (He *et al.*, 2008; Kwon *et al.*, 2009; Radisek *et al.*, 2004; Sun *et al.*, 2005). Compared with AFLP markers, SCAR markers are stable, repeatable and convenient in practical application.

To the best of our knowledge, this is the first report on the application of the AFLP technique in the evaluation of the genetic relationship in the genus *Lagerstroemia* species. The dendrogram constructed on the basis of genetic similarity showed that all the *Lagerstroemia* accessions clustered in a way that is generally in agreement with the classification based on morphological characteristics.

The genetic similarity coefficient between *L. speciosa* and *L. macrocarpa* was 0.54, and they were clustered in a clade. The result suggested that these two species are closely related with other and they are very similar in morphological

characteristics. They all had monomorphic stamens with monomorphic pollen (Kim *et al.*, 1994). Compared with *L. speciosa*, *L. macrocarpa* had bigger fruit length (2.5–4 cm vs 1.5–2.5 cm) and flower size (5–7.5 cm vs. 6–10 cm) (Gardner *et al.*, 2007).

In this study, the dendrogram placed *L. loudonii* in clase II whereas *L. floribunda* was formed alone in clade III. The morphological characteristics displayed the softly grayish hairs below the leaves especially when it was young (Gardner *et al.*, 2007). *L floribunda* bark showed pale cream in color with smooth and flaking in thin plates (Gardner *et al.*, 2007).

The present contribution shows that AFLP is a promising approach to help in plant identification with three candidate species-specific primer combinations, ER3ACC/MS3CAC, ER3AAG/MS3CTG, and ER3AAC/MS3CAT, for *L. speciosa*. For further research, SCAR marker should be developed for identification of *L. speciosa*.

CHAPTER V

CONCLUSION

Medicinal plant authentication is a quality assurance process. The proper authentication of herbal raw materials is critically important to the safety and efficacy of herbal medicines. Hence, the purpose of this study was to perform pharmacognostic evaluation and DNA fingerprinting of *L. speciosa*.

To reach the objective, an investigation was conducted in two steps. The first step, pharmacognostic specifications were performed. Macroscopical and microscopical characteristics, physicochemical properties, preliminary phytochemical screening, and TLC fingerprinting profiles were examined. The results indicated that the macroscopic and microscopic characteristics of leaves, and thin layer chromatographic patterns can be effectively used together as important tools in authentication of crude drugs sold in the crude drug market. The physicochemical evaluation of crude drug gives important parameters in detecting adulteration or improper handling of drugs. From this investigation, the requirements of the specification should be established (in integer). The loss on drying, moisture content, total ash and acid insoluble ash should not be more than 10%, 9%, 9% and 2% w/w respectively. These physicochemical parameters were useful for detecting low-grade products as well as the extractive values. The ethanol-, water- and dichloromethane-extractive values were determined to be not less than 8%, 10% and 2%, respectively. These specifications can also be used as quality assurance of the crude drug. *L. speciosa* leaves obtained from various sources of Thailand which contained corosolic acid ranging from 0.01-0.75 % base on dry weight. The difference in contents in the crude drugs may be due to the environment of the plant cultivation. Moreover, collected season and storage condition may also lead to fluctuation in corosolic acid content.

The second step, the DNA fingerprint of *L. speciosa* and closely related species, *L. macrocarpa*; *L. loudonii* and *L. floribunda*, was investigated using AFLP method. DNA based polymorphism assay may offer an alternative method to identify herbal medicines. This investigation found three candidates species-specific of *L.*

speciosa, a 230 bp fragment from ER3ACC/MS3CAC, a 110 bp fragment from ER3AAG/MS3CTG, and 190 bp fragment from ER3AAC/MS3CAT. It should be further developed to SCAR marker for identification of banaba. SCAR method proved to be a rapid and easy-to-perform analytical tool to achieve species authentication of crude drugs. In addition, this study was successful in constructing the dendrogram based on AFLP bands. Genetic relationship analyzed by Jaccard's similarity matrix and UPGMA method showed that the two species, *L. speciosa* and *L. macrocarpa*, formed in the same cluster. *L. loudonii* and *L. floribunda* were placed in other groups as an individual branch.

Based on the result from this study, it is reasonable to say that the pharmacognostic specifications and its DNA fingerprinting could serve as a basis for proper identification, collection and investigation of *L. speciosa* leaves. It will provide useful information for its correct identity and may enable those who handle this plant to maintain its quality control. Thus, these techniques developed here have been successfully proved as a powerful tool for the identification of *L. speciosa* and authentication of *L. speciosa* crude drugs.

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APPENDICES

ศูนย์วิทยทรัพยากร
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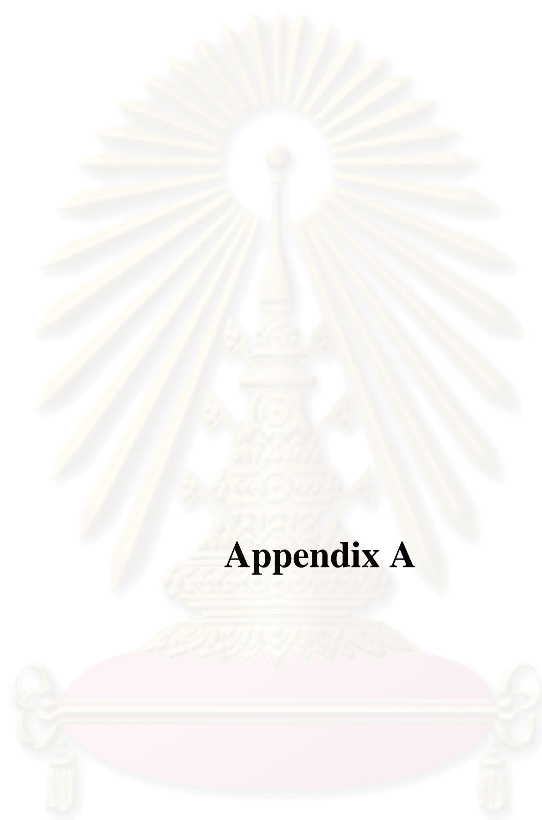

Appendix A

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Figure A.1 Morphology of *L. speciosa* crude drug (LS1).

Figure A.2 Morphology of *L. speciosa* crude drug (LS2).

Figure A.3 Morphology of *L. speciosa* crude drug (LS3).

Figure A.4 Morphology of *L. speciosa* crude drug (LS4).

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จุฬาลงกรณ์มหาวิทยาลัย

Figure A.5 Morphology of *L. speciosa* crude drug (LS5).

Figure A.6 Morphology of *L. speciosa* crude drug (LS6).

Figure A.7 Morphology of *L. speciosa* crude drug (LS7).

Figure A.8 Morphology of *L. speciosa* crude drug (LS8).

Figure A.9 Morphology of *L. speciosa* crude drug (LS9).

Figure A.10 Morphology of *L. speciosa* crude drug (LS10).

Figure A.11 Morphology of *L. speciosa* crude (LS11).

Figure A.12 Morphology of *L. speciosa* crude drug (LS12).

Figure A.13 Morphology of *L. speciosa* crude drug (LS13).

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จุฬาลงกรณ์มหาวิทยาลัย

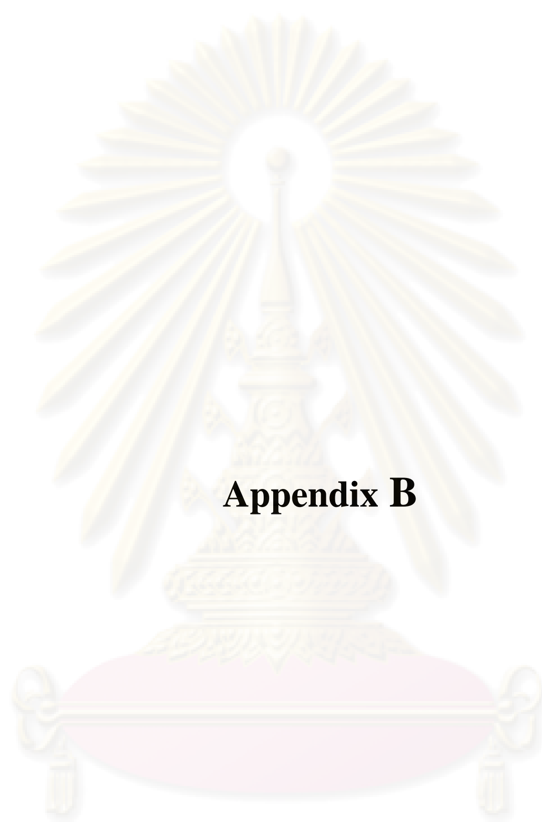
Figure A.14 Morphology of *L. speciosa* crude drug (LS14).

Figure A.15 Morphology of *L. speciosa* crude drug (LS15).

Figure A.16 Morphology of *L. speciosa* crude drug (LS16).

Figure A.17 Morphology of *L. speciosa* crude drug (LS17).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย



Appendix B

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table B.1 Stamatal number and stomatal index of *L. speciosa* (LS1). Area of determination = 0.0707 mm^2.

Number of stomata	Number of epidermal cells	Stomatal index	Stomatal number
17	115	12.88	240.45
18	112	13.85	254.60
21	110	16.03	297.03
20	111	15.27	282.89
16	118	11.94	226.31
18	115	13.53	254.60
18	113	13.74	254.60
17	114	12.98	240.45
17	112	13.18	240.45
18	108	14.29	254.60
18	112	13.85	254.60
17	116	12.78	240.45
19	107	15.08	268.74
18	109	14.17	254.60
18	113	13.74	254.60
19	108	14.96	268.74
17	114	12.98	240.45
17	112	13.18	240.45
18	107	14.40	254.60
17	112	13.18	240.45
18	110	14.06	254.60
17	116	12.78	240.45
19	108	14.96	268.74
19	111	14.62	268.74
19	110	14.73	268.74
18	115	13.53	254.60
18	113	13.74	254.60
19	112	14.50	268.74
20	109	15.50	282.89
21	116	15.33	297.03
	Mean	**13.99**	**257.43**
	SD	**0.97**	**17.18**

Table B.2 Stamatal number and stomatal index of *L. speciosa* (LS2). Area of determination = 0.0707 mm^2

Number of stomata	Number of epidermal cells	Stomatal index	Stomatal number
21	105	16.67	297.03
17	116	12.78	240.45
18	112	13.85	254.60
18	115	13.53	254.60
18	113	13.74	254.60
19	110	14.73	268.74
18	115	13.53	254.60
19	107	15.08	268.74
17	117	12.69	240.45
20	109	15.50	282.89
21	104	16.80	297.03
20	110	15.38	282.89
20	106	15.87	282.89
19	111	14.62	268.74
18	115	13.53	254.60
18	112	13.85	254.60
19	109	14.84	268.74
17	118	12.59	240.45
21	102	17.07	297.03
18	119	13.14	254.60
17	116	12.78	240.45
19	111	14.62	268.74
18	109	14.17	254.60
17	116	12.78	240.45
17	114	12.98	240.45
18	112	13.85	254.60
18	111	13.95	254.60
18	116	13.43	254.60
19	110	14.73	268.74
18	116	13.43	254.60
	Mean	**14.22**	**261.67**
	SD	**1.26**	**17.32**

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Table B.3 Stamatal number and stomatal index of *L. speciosa* (LS3). Area of determination = 0.0707 mm^2.

Number of stomata	Number of epidermal cells	Stomatal index	Stomatal number
22	106	17.19	311.17
21	110	16.03	297.03
18	113	13.74	254.60
17	116	12.78	240.45
19	101	15.83	268.74
19	106	15.20	268.74
18	116	13.43	254.60
19	113	14.39	268.74
18	114	13.64	254.60
18	117	13.33	254.60
17	119	12.50	240.45
19	111	14.62	268.74
17	121	12.32	240.45
18	115	13.53	254.60
19	113	14.39	268.74
20	109	15.50	282.89
18	112	13.85	254.60
18	114	13.64	254.60
19	110	14.73	268.74
17	114	12.98	240.45
18	112	13.85	254.60
19	114	14.29	268.74
21	108	16.28	297.03
20	110	15.38	282.89
17	116	12.78	240.45
18	114	13.64	254.60
18	111	13.95	254.60
19	116	14.07	268.74
17	118	12.59	240.45
17	115	12.88	240.45
	Mean	**14.11**	**261.67**
	SD	**1.22**	**18.48**

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Table B.4 Stamatal number and stomatal index of *L. speciosa* (LS4). Area of determination = 0.0707 mm^2.

Number of stomata	Number of epidermal cells	Stomatal index	Stomatal number
17	118	12.59	240.45
18	116	13.43	254.60
17	121	12.32	240.45
22	109	16.79	311.17
18	101	15.13	254.60
19	113	14.39	268.74
17	118	12.59	240.45
18	115	13.53	254.60
21	107	16.41	297.03
19	111	14.62	268.74
18	114	13.64	254.60
19	108	14.96	268.74
18	110	14.06	254.60
21	108	16.28	297.03
18	119	13.14	254.60
17	121	12.32	240.45
16	125	11.35	226.31
19	115	14.18	268.74
18	117	13.33	254.60
19	115	14.18	268.74
20	112	15.15	282.89
21	106	16.54	297.03
20	108	15.63	282.89
22	103	17.60	311.17
21	106	16.54	297.03
18	117	13.33	254.60
19	114	14.29	268.74
17	120	12.41	240.45
18	111	13.95	254.60
17	118	12.59	240.45
	Mean	**14.24**	**264.97**
	SD	**1.59**	**22.88**

Table B.5 Stamatal number and stomatal index of *L. speciosa* (LS5). Area of determination = 0.0707 mm^2.

Number of stomata	Number of epidermal cells	Stomatal index	Stomatal number
18	115	13.53	254.60
17	118	12.59	240.45
17	121	12.32	240.45
18	116	13.43	254.60
18	114	13.64	254.60
19	112	14.50	268.74
18	117	13.33	254.60
19	114	14.29	268.74
22	110	16.67	311.17
18	118	13.24	254.60
19	117	13.97	268.74
21	108	16.28	297.03
17	117	12.69	240.45
19	114	14.29	268.74
17	119	12.50	240.45
16	125	11.35	226.31
19	114	14.29	268.74
18	111	13.95	254.60
21	106	16.54	297.03
17	114	12.98	240.45
18	113	13.74	254.60
18	108	14.29	254.60
19	115	14.18	268.74
21	110	16.03	297.03
18	117	13.33	254.60
16	122	11.59	226.31
17	116	12.78	240.45
19	113	14.39	268.74
18	111	13.95	254.60
17	127	11.81	240.45
	Mean	**13.75**	**258.84**
	SD	**1.35**	**20.73**

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Table B.6 Stamatal number and stomatal index of *L. speciosa* (LS6). Area of determination = 0.0707 mm^2.

Number of stomata	Number of epidermal cells	Stomatal index	Stomatal number
17	116	12.78	240.45
17	114	12.98	240.45
18	112	13.85	254.60
19	108	14.96	268.74
16	120	11.76	226.31
22	106	17.19	311.17
23	103	18.25	325.32
18	117	13.33	254.60
18	115	13.53	254.60
21	112	15.79	297.03
19	115	14.18	268.74
17	116	12.78	240.45
17	119	12.50	240.45
16	121	11.68	226.31
19	117	13.97	268.74
18	116	13.43	254.60
17	118	12.59	240.45
15	125	10.71	212.16
18	116	13.43	254.60
17	118	12.59	240.45
18	114	13.64	254.60
17	119	12.50	240.45
18	115	13.53	254.60
19	112	14.50	268.74
17	113	13.08	240.45
17	111	13.28	240.45
18	109	14.17	254.60
19	113	14.39	268.74
22	114	16.18	311.17
19	119	13.77	268.74
	Mean	**13.71**	**257.43**
	SD	**1.57**	**25.41**

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Table B.7 Stamatal number and stomatal index of *L. speciosa* (LS7). Area of determination = 0.0707 mm^2.

Number of stomata	Number of epidermal cells	Stomatal index	Stomatal number
18	116	13.43	254.60
21	107	16.41	297.03
16	123	11.51	226.31
17	117	12.69	240.45
18	115	13.53	254.60
17	118	12.59	240.45
17	112	13.18	240.45
18	116	13.43	254.60
19	110	14.73	268.74
18	115	13.53	254.60
18	117	13.33	254.60
17	119	12.50	240.45
17	114	12.98	240.45
18	112	13.85	254.60
17	116	12.78	240.45
15	121	11.03	212.16
19	116	14.07	268.74
21	109	16.15	297.03
23	104	18.11	325.32
18	115	13.53	254.60
18	113	13.74	254.60
17	119	12.50	240.45
18	116	13.43	254.60
16	120	11.76	226.31
18	116	13.43	254.60
18	113	13.74	254.60
19	110	14.73	268.74
20	107	15.75	282.89
19	111	14.62	268.74
17	114	12.98	240.45
	Mean	**13.67**	**255.54**
	SD	**1.48**	**22.88**

Table B.8 Stamatal number and stomatal index of *L. speciosa* (LS8). Area of determination = 0.0707 mm^2.

Number of stomata	Number of epidermal cells	Stomatal index	Stomatal number
19	111	14.62	268.74
16	119	11.85	226.31
18	116	13.43	254.60
22	109	16.79	311.17
18	116	13.43	254.60
17	118	12.59	240.45
21	111	15.91	297.03
20	121	14.18	282.89
16	123	11.51	226.31
18	115	13.53	254.60
15	117	11.36	212.16
23	108	17.56	325.32
22	111	16.54	311.17
20	114	14.93	282.89
18	118	13.24	254.60
17	120	12.41	240.45
17	117	12.69	240.45
19	113	14.39	268.74
16	116	12.12	226.31
18	116	13.43	254.60
15	118	11.28	212.16
19	114	14.29	268.74
17	116	12.78	240.45
17	114	12.98	240.45
18	111	13.95	254.60
19	110	14.73	268.74
17	116	12.78	240.45
18	111	13.95	254.60
18	114	13.64	254.60
22	117	15.83	311.17
	Mean	**13.76**	**259.31**
	SD	**1.61**	**29.56**

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Table B.9 Palisade ratio, Vein-islet number and veinlet termination of *L. speciosa* (LS1).

Palisade cell		Vein-islet		Veinlet termination	
Number beneath 4 epidermal cell	**Palisade ratio**	**Count in 4 mm^2**	**Vein-islet number**	**Count in 4 mm^2**	**Veinlet termination number**
21	5.25	58	14.50	28	7.00
22	5.50	62	15.50	26	6.50
20	5.00	54	13.50	24	6.00
23	5.75	57	14.25	32	8.00
23	5.75	61	15.25	30	7.50
21	5.25	55	13.75	29	7.25
21	5.25	58	14.50	30	7.50
21	5.25	60	15.00	28	7.00
22	5.50	62	15.50	26	6.50
20	5.00	55	13.75	25	6.25
21	5.25	58	14.50	27	6.75
19	4.75	60	15.00	31	7.75
24	6.00	57	14.25	28	7.00
23	5.75	56	14.00	27	6.75
22	5.50	51	12.75	31	7.75
22	5.50	55	13.75	26	6.50
21	5.25	63	15.75	28	7.00
18	4.50	57	14.25	25	6.25
22	5.50	59	14.75	22	5.50
21	5.25	58	14.50	31	7.75
22	5.50	61	15.25	28	7.00
22	5.50	63	15.75	26	6.50
22	5.50	59	14.75	24	6.00
21	5.25	55	13.75	31	7.75
22	5.50	58	14.50	30	7.50
22	5.50	62	15.50	28	7.00
21	5.25	56	14.00	27	6.75
22	5.50	59	14.75	30	7.50
21	5.25	62	15.50	28	7.00
20	5.00	60	15.00	27	6.75
Mean	**5.35**		**14.59**		**6.94**
SD	**0.31**		**0.74**		**0.61**

Table B.10 Palisade ratio, Vein-islet number and veinlet termination of *L. speciosa* (LS2).

Palisade cell		Vein-islet		Veinlet termination	
Number beneath 4 epidermal cell	**Palisade ratio**	**Count in 4 mm^2**	**Vein-islet number**	**Count in 4 mm^2**	**Veinlet termination number**
22	5.50	60	15.00	30	7.50
22	5.50	62	15.50	26	6.50
21	5.25	58	14.50	28	7.00
21	5.25	56	14.00	20	5.00
21	5.25	63	15.75	22	5.50
21	5.25	61	15.25	31	7.75
21	5.25	60	15.00	26	6.50
20	5.00	58	14.50	28	7.00
19	4.75	59	14.75	27	6.75
20	5.00	54	13.50	29	7.25
20	5.00	63	15.75	31	7.75
21	5.25	58	14.50	25	6.25
20	5.00	55	13.75	21	5.25
22	5.50	59	14.75	28	7.00
22	5.50	61	15.25	21	5.25
21	5.25	58	14.50	26	6.50
21	5.25	63	15.75	25	6.25
21	5.25	59	14.75	21	5.25
22	5.50	57	14.25	18	4.50
22	5.50	62	15.50	26	6.50
23	5.75	63	15.75	25	6.25
22	5.50	60	15.00	27	6.75
19	4.75	57	14.25	22	5.50
21	5.25	63	15.75	31	7.75
22	5.50	57	14.25	28	7.00
22	5.50	59	14.75	25	6.25
21	5.25	60	15.00	22	5.50
22	5.50	62	15.50	26	6.50
23	5.75	60	15.00	28	7.00
22	5.50	58	14.50	25	6.25
Mean	**5.31**		**14.88**		**6.40**
SD	**0.25**		**0.62**		**0.86**

Table B.11 Palisade ratio, Vein-islet number and veinlet termination of *L. speciosa* (LS3).

Palisade cell		Vein-islet		Veinlet termination	
Number beneath 4 epidermal cell	**Palisade ratio**	**Count in 4 mm^2**	**Vein-islet number**	**Count in 4 mm^2**	**Veinlet termination number**
22	5.50	58	14.50	23	5.75
22	5.50	55	13.75	26	6.50
21	5.25	62	15.50	21	5.25
22	5.50	65	16.25	33	8.25
23	5.75	58	14.50	28	7.00
19	4.75	59	14.75	32	8.00
22	5.50	58	14.50	30	7.50
20	5.00	62	15.50	27	6.75
22	5.50	59	14.75	28	7.00
21	5.25	60	15.00	26	6.50
21	5.25	55	13.75	25	6.25
21	5.25	58	14.50	28	7.00
21	5.25	63	15.75	26	6.50
22	5.50	57	14.25	24	6.00
21	5.25	60	15.00	22	5.50
20	5.00	62	15.50	18	4.50
22	5.50	57	14.25	26	6.50
22	5.50	61	15.25	28	7.00
21	5.25	60	15.00	25	6.25
21	5.25	57	14.25	29	7.25
20	5.00	60	15.00	31	7.75
22	5.50	58	14.50	26	6.50
18	4.50	63	15.75	28	7.00
22	5.50	62	15.50	25	6.25
22	5.50	59	14.75	21	5.25
21	5.25	60	15.00	30	7.50
20	5.00	58	14.50	29	7.25
22	5.50	63	15.75	25	6.25
22	5.50	65	16.25	27	6.75
22	5.50	57	14.25	28	7.00
Mean	**5.31**		**14.93**		**6.63**
SD	**0.27**		**0.66**		**0.84**

Table B.12 Palisade ratio, Vein-islet number and veinlet termination of *L. speciosa* (LS4).

Palisade cell		Vein-islet		Veinlet termination	
Number beneath 4 epidermal cell	**Palisade ratio**	**Count in 4 mm^2**	**Vein-islet number**	**Count in 4 mm^2**	**Veinlet termination number**
23	5.75	62	15.50	25	6.25
22	5.50	60	15.00	28	7.00
22	5.50	57	14.25	18	4.50
21	5.25	55	13.75	24	6.00
22	5.50	62	15.50	32	8.00
20	5.00	60	15.00	29	7.25
22	5.50	59	14.75	27	6.75
20	5.00	58	14.50	22	5.50
21	5.25	59	14.75	26	6.50
20	5.00	61	15.25	19	4.75
20	5.00	64	16.00	31	7.75
20	5.00	57	14.25	26	6.50
22	5.50	59	14.75	28	7.00
21	5.25	52	13.00	22	5.50
22	5.50	58	14.50	25	6.25
21	5.25	63	15.75	21	5.25
21	5.25	60	15.00	22	5.50
21	5.25	58	14.50	27	6.75
21	5.25	61	15.25	26	6.50
20	5.00	59	14.75	28	7.00
20	5.00	62	15.50	23	5.75
20	5.00	58	14.50	27	6.75
21	5.25	61	15.25	25	6.25
22	5.50	58	14.50	26	6.50
20	5.00	60	15.00	28	7.00
21	5.25	68	17.00	26	6.50
22	5.50	62	15.50	24	6.00
20	5.00	60	15.00	22	5.50
22	5.50	63	15.75	28	7.00
22	5.50	58	14.50	29	7.25
Mean	**5.27**		**14.95**		**6.37**
SD	**0.23**		**0.74**		**0.82**

Table B.13 Palisade ratio, Vein-islet number and veinlet termination of *L. speciosa* (LS5).

Palisade cell		Vein-islet		Veinlet termination	
Number beneath 4 epidermal cell	**Palisade ratio**	**Count in 4 mm^2**	**Vein-islet number**	**Count in 4 mm^2**	**Veinlet termination number**
21	5.25	59	14.75	24	6.00
22	5.50	64	16.00	29	7.25
21	5.25	57	14.25	31	7.75
21	5.25	55	13.75	22	5.50
20	5.00	63	15.75	25	6.25
21	5.25	58	14.50	28	7.00
21	5.25	60	15.00	26	6.50
22	5.50	62	15.50	29	7.25
21	5.25	57	14.25	31	7.75
22	5.50	65	16.25	18	4.50
24	6.00	63	15.75	27	6.75
22	5.50	59	14.75	29	7.25
22	5.50	61	15.25	30	7.50
21	5.25	56	14.00	22	5.50
21	5.25	62	15.50	25	6.25
18	4.50	60	15.00	27	6.75
20	5.00	58	14.50	24	6.00
21	5.25	63	15.75	28	7.00
22	5.50	59	14.75	21	5.25
22	5.50	56	14.00	25	6.25
22	5.50	58	14.50	28	7.00
21	5.25	59	14.75	31	7.75
21	5.25	63	15.75	28	7.00
19	4.75	65	16.25	29	7.25
21	5.25	61	15.25	25	6.25
21	5.25	60	15.00	28	7.00
21	5.25	62	15.50	32	8.00
22	5.50	59	14.75	19	4.75
19	4.75	60	15.00	25	6.25
22	5.50	63	15.75	28	7.00
Mean	**5.28**		**15.06**		**6.62**
SD	**0.28**		**0.68**		**0.89**

Table B.14 Palisade ratio, Vein-islet number and veinlet termination of *L. speciosa* (LS6).

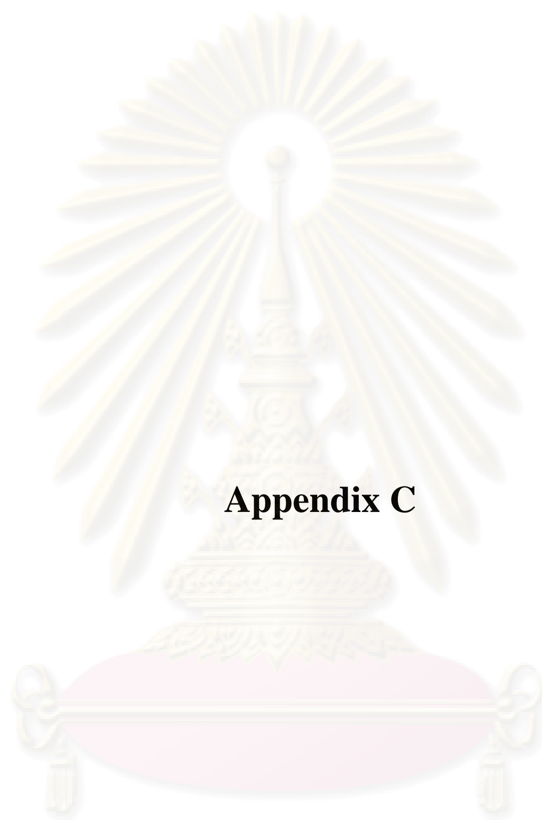
Palisade cell		**Vein-islet**		**Veinlet termination**	
Number beneath 4 epidermal cell	**Palisade ratio**	**Count in 4 mm^2**	**Vein-islet number**	**Count in 4 mm^2**	**Veinlet termination number**
22	5.50	55	13.75	28	7.00
22	5.50	57	14.25	25	6.25
22	5.50	59	14.75	31	7.75
21	5.25	58	14.50	26	6.50
22	5.50	51	12.75	24	6.00
20	5.00	50	12.50	29	7.25
22	5.50	56	14.00	26	6.50
21	5.25	58	14.50	26	6.50
22	5.50	59	14.75	21	5.25
21	5.25	59	14.75	29	7.25
21	5.25	56	14.00	30	7.50
21	5.25	58	14.50	24	6.00
21	5.25	63	15.75	18	4.50
21	5.25	59	14.75	21	5.25
21	5.25	57	14.25	27	6.75
19	4.75	59	14.75	29	7.25
20	5.00	58	14.50	19	4.75
22	5.50	60	15.00	22	5.50
21	5.25	58	14.50	18	4.50
22	5.50	58	14.50	29	7.25
22	5.50	54	13.50	26	6.50
22	5.50	48	12.00	28	7.00
21	5.25	59	14.75	25	6.25
22	5.50	57	14.25	22	5.50
22	5.50	49	12.25	25	6.25
22	5.50	51	12.75	26	6.50
23	5.75	60	15.00	28	7.00
19	4.75	51	12.75	27	6.75
22	5.50	59	14.75	26	6.50
22	5.50	53	13.25	22	5.50
Mean	**5.34**		**14.08**		**6.31**
SD	**0.23**		**0.94**		**0.88**

Table B.15 Palisade ratio, Vein-islet number and veinlet termination of *L. speciosa* (LS7).

Palisade cell		**Vein-islet**		**Veinlet termination**	
Number beneath 4 epidermal cell	**Palisade ratio**	**Count in 4 mm^2**	**Vein-islet number**	**Count in 4 mm^2**	**Veinlet termination number**
21	5.25	52	13.00	22	5.50
21	5.25	60	15.00	21	5.25
20	5.00	57	14.25	18	4.50
22	5.50	61	15.25	22	5.50
21	5.25	56	14.00	26	6.50
21	5.25	58	14.50	25	6.25
22	5.50	46	11.50	27	6.75
21	5.25	60	15.00	24	6.00
22	5.50	52	13.00	22	5.50
21	5.25	57	14.25	26	6.50
22	5.50	58	14.50	23	5.75
20	5.00	60	15.00	21	5.25
21	5.25	53	13.25	17	4.25
22	5.50	54	13.50	22	5.50
19	4.75	57	14.25	25	6.25
22	5.50	55	13.75	28	7.00
22	5.50	59	14.75	30	7.50
22	5.50	51	12.75	25	6.25
21	5.25	57	14.25	26	6.50
22	5.50	60	15.00	23	5.75
21	5.25	58	14.50	21	5.25
22	5.50	52	13.00	24	6.00
21	5.25	56	14.00	26	6.50
21	5.25	57	14.25	22	5.50
20	5.00	61	15.25	28	7.00
18	4.50	59	14.75	19	4.75
22	5.50	54	13.50	26	6.50
22	5.50	48	12.00	24	6.00
21	5.25	57	14.25	27	6.75
24	6.00	52	13.00	28	7.00
Mean	**5.31**		**13.98**		**5.98**
SD	**0.28**		**0.95**		**0.78**

Table B.16 Palisade ratio, Vein-islet number and veinlet termination of *L. speciosa* (LS8).

Palisade cell		Vein-islet		Veinlet termination	
Number beneath 4 epidermal cell	**Palisade ratio**	**Count in 4 mm^2**	**Vein-islet number**	**Count in 4 mm^2**	**Veinlet termination number**
22	5.50	56	14.00	28	7.00
21	5.25	59	14.75	25	6.25
22	5.50	62	15.50	22	5.50
21	5.25	61	15.25	19	4.75
21	5.25	58	14.50	28	7.00
21	5.25	60	15.00	31	7.75
21	5.25	57	14.25	22	5.50
20	5.00	58	14.50	25	6.25
19	4.75	63	15.75	27	6.75
19	4.75	60	15.00	28	7.00
22	5.50	62	15.50	31	7.75
22	5.50	59	14.75	22	5.50
23	5.75	60	15.00	24	6.00
22	5.50	63	15.75	28	7.00
22	5.50	57	14.25	29	7.25
21	5.25	58	14.50	27	6.75
21	5.25	60	15.00	31	7.75
20	5.00	59	14.75	28	7.00
21	5.25	53	13.25	26	6.50
21	5.25	66	16.50	27	6.75
22	5.50	57	14.25	22	5.50
20	5.00	55	13.75	19	4.75
22	5.50	63	15.75	24	6.00
21	5.25	58	14.50	22	5.50
21	5.25	60	15.00	19	4.75
22	5.50	62	15.50	27	6.75
23	5.75	58	14.50	23	5.75
21	5.25	60	15.00	27	6.75
22	5.50	57	14.25	29	7.25
22	5.50	59	14.75	28	7.00
Mean	**5.32**		**14.83**		**6.40**
SD	**0.25**		**0.68**		**0.88**



Appendix C

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Table C.1 Physiocochemical values of *L. speciosa* leaves (LS1).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	6.7233	6.3936	7.4445	1.4809	12.0976	13.7353	5.2594
2	6.7016	6.1440	7.2920	1.4824	12.0956	13.6167	5.2089
3	6.7566	6.5655	7.4062	1.4832	12.0861	13.4792	5.2795
mean	**6.7272**	**6.3677**	**7.3809**	**1.4822**	**12.0931**	**13.6080**	**5.2492**
SD	**0.0277**	**0.2119**	**0.0793**	**0.0012**	**0.0061**	**0.1362**	**0.0364**

Table C.2 Physiocochemical values of *L. speciosa* leaves (LS2).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	7.4789	7.0366	7.1483	1.4063	8.5095	12.1773	3.3491
2	7.4583	7.1619	7.1678	1.4109	8.5176	12.1490	3.3199
3	7.4146	7.2087	7.0434	1.4093	8.5595	12.1251	3.3896
mean	**7.4506**	**7.1357**	**7.1198**	**1.4089**	**8.5289**	**12.1505**	**3.3529**
SD	**0.0328**	**0.0890**	**0.0669**	**0.0023**	**0.0268**	**0.0922**	**0.0350**

Table C.3 Physiocochemical values of *L. speciosa* leaves (LS3).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	6.4844	6.5303	7.2941	1.4415	11.1889	13.5767	4.7290
2	6.5048	6.5165	7.1586	1.4363	11.4775	13.7348	4.7090
3	6.5484	6.4181	7.1821	1.4384	11.1449	13.5967	4.6693
mean	**6.5125**	**6.4883**	**7.2116**	**1.4388**	**11.2704**	**13.6328**	**4.7024**
SD	**0.0327**	**0.0612**	**0.0724**	**0.0026**	**0.1807**	**0.0138**	**0.0303**

Table C.4 Physiocochemical values of *L. speciosa* leaves (LS4).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	7.5570	7.5664	7.7338	1.6394	7.6242	11.6770	2.4250
2	7.7707	7.6410	7.7971	1.6294	7.8234	11.7569	2.4096
3	7.9356	7.3835	7.7197	1.6333	7.2493	11.6988	2.4199
mean	**7.7544**	**7.5303**	**7.7502**	**1.6430**	**7.5656**	**11.7109**	**2.4181**
SD	**0.1899**	**0.1325**	**0.0412**	**0.0050**	**0.2915**	**0.0109**	**0.0079**

Table C.5 Physiocochemical values of *L. speciosa* leaves (LS5).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	7.8849	7.3461	8.4328	0.4266	7.4000	11.9786	2.3243
2	8.1593	7.6561	8.4967	0.4267	7.4470	11.9878	2.3300
3	8.4341	7.4188	8.4504	0.4262	7.3496	12.0566	2.3395
mean	**8.1594**	**7.4736**	**8.4600**	**0.4265**	**7.3989**	**12.0077**	**2.3313**
SD	**0.2746**	**0.1621**	**0.0330**	**0.0003**	**0.0487**	**0.0490**	**0.0077**

Table C.6 Physiocochemical values of *L. speciosa* leaves (LS6).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	8.1651	7.9709	8.9072	1.0555	7.0968	12.4078	2.2492
2	8.4470	8.1328	8.8245	1.0556	7.0986	12.5777	2.2396
3	8.6676	8.1336	8.8785	1.0565	7.2471	12.4080	2.2292
mean	**8.4266**	**8.0791**	**8.8701**	**1.0559**	**7.1475**	**12.4645**	**2.2393**
SD	**0.2519**	**0.0937**	**0.0420**	**0.0005**	**0.0863**	**0.0981**	**0.0100**

Table C.7 Physiocochemical values of *L. speciosa* leaves (LS7).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	8.5415	8.7367	8.2097	2.4733	8.5750	10.0494	2.2747
2	8.8451	8.3184	8.3183	2.4995	8.5496	10.0082	2.2395
3	9.1063	8.5148	8.2317	2.4862	8.8250	9.9998	2.2294
mean	**8.8310**	**8.5233**	**8.2532**	**2.4863**	**8.6499**	**10.0191**	**2.2478**
SD	**0.2827**	**0.2093**	**0.0574**	**0.0131**	**0.1522**	**0.0248**	**0.0238**

Table C.8 Physiocochemical values of *L. speciosa* leaves (LS8).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	8.4916	8.1961	6.8808	0.1766	7.2246	13.5959	2.2745
2	8.5728	8.3302	6.8960	0.1766	7.5750	13.6373	2.2896
3	8.4324	8.1280	6.8895	0.1700	7.0000	13.5986	2.2595
mean	**8.4989**	**8.2181**	**6.8888**	**0.1844**	**7.2665**	**13.6106**	**2.2746**
SD	**0.0705**	**0.1029**	**0.0076**	**0.0038**	**0.2898**	**0.0167**	**0.0151**

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Table C.9 Physiocochemical values of *L. speciosa* leaves (LS9).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	9.9055	9.3121	6.3959	0.9089	10.9451	14.9567	2.6989
2	9.9510	9.1123	6.3845	0.8983	11.3750	14.7241	2.6885
3	9.9310	9.3782	6.3714	0.9026	11.2994	14.9576	2.7085
mean	**9.9292**	**9.2675**	**6.3839**	**0.9033**	**11.2065**	**14.8795**	**2.6987**
SD	**0.0228**	**0.1384**	**0.0123**	**0.0054**	**0.2295**	**0.0232**	**0.0100**

Table C.10 Physiocochemical values of *L. speciosa* leaves (LS10).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	9.4891	8.9786	6.5693	0.8099	12.7449	15.4997	2.4242
2	9.4041	9.1820	6.5907	0.7997	11.9982	15.6400	2.4292
3	9.4006	8.9949	6.5758	0.8032	12.2732	15.7084	2.4186
mean	**9.4312**	**9.0519**	**6.5786**	**0.8043**	**12.3388**	**15.6160**	**2.4240**
SD	**0.0501**	**0.1130**	**0.0110**	**0.0052**	**0.3776**	**0.1205**	**0.0053**

Table C.11 Physiocochemical values of *L. speciosa* leaves (LS11).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	8.1239	8.0292	6.8208	1.3795	10.5708	17.8150	2.4248
2	8.3933	8.1506	6.7460	1.3965	10.5489	17.7864	2.4487
3	8.1123	8..0487	6.8035	1.3894	11.0989	17.8247	2.4395
mean	**8.2098**	**8.0925**	**6.7901**	**1.3885**	**10.7395**	**17.8087**	**2.4377**
SD	**0.1590**	**0.0510**	**0.0392**	**0.0086**	**0.3114**	**0.0340**	**0.0121**

Table C.12 Physiocochemical values of *L. speciosa* leaves (LS12).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	9.5417	8.2913	7.4928	2.3797	13.1487	16.0913	3.4990
2	9.4472	8.4007	7.3645	2.3565	12.8455	16.1732	3.5088
3	9.6650	8.3984	7.4590	2.3197	12.4488	16.0587	3.5192
mean	**9.5513**	**8.3635**	**7.4391**	**2.3530**	**12.8143**	**16.1044**	**3.5090**
SD	**0.1092**	**0.0625**	**0.0660**	**0.0303**	**0.3510**	**0.0568**	**0.0101**

Table C.13 Physiocochemical values of *L. speciosa* leaves (LS13).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	8.5877	8.1038	7.1671	1.1662	7.4978	12.4568	2.6489
2	8.7796	8.3990	7.1547	1.1686	7.1239	12.4985	2.6292
3	8.9706	8.5414	6.2264	1.1666	7.6727	12.5675	2.6186
mean	**8.7763**	**8.3481**	**7.1828**	**1.1671**	**7.4315**	**12.5076**	**2.6322**
SD	**0.1914**	**0.2232**	**0.0383**	**0.0013**	**0.2803**	**0.0838**	**0.0154**

Table C.14 Physiocochemical values of *L. speciosa* leaves (LS14).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	8.1272	8.1633	8.3883	0.9224	9.3213	12.4298	2.4745
2	8.3371	8.0756	8.4130	0.9196	9.8250	12.4085	2.4590
3	8.6098	8.2699	8.4182	0.9224	9.1713	12.4253	2.4994
mean	**8.3579**	**8.1696**	**8.4065**	**0.9215**	**9.4392**	**12.4212**	**2.4776**
SD	**0.2419**	**0.0973**	**0.0160**	**0.0016**	**0.3424**	**0.0165**	**0.0204**

Table C.15 Physiocochemical values of *L. speciosa* leaves (LS15).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	7.4074	6.9084	7.0486	1.5190	5.2242	11.8783	2.6497
2	7.7042	7.1692	6.9146	1.5095	5.2995	11.8674	2.6892
3	7.8979	6.9528	6.0374	1.5280	5.2745	11.9686	2.6590
mean	**7.6689**	**7.0101**	**6.9669**	**1.5188**	**5.2661**	**11.9048**	**2.6660**
SD	**0.2470**	**0.1395**	**0.0717**	**0.0092**	**0.0383**	**0.0841**	**0.0207**

Table C.16 Physiocochemical values of *L. speciosa* leaves (LS16).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	7.6204	7.5180	7.1952	0.3499	5.5760	10.6298	2.7296
2	7.6958	7.6609	7.1367	0.3533	5.8680	10.6072	2.7592
3	7.7799	7.7060	7.0910	0.3532	5.8798	10.6800	2.7754
mean	**7.6987**	**7.6283**	**7.1410**	**0.3522**	**5.7746**	**10.6390**	**2.7547**
SD	**0.0798**	**0.0981**	**0.0523**	**0.0019**	**0.1221**	**0.0268**	**0.0233**

Table C.17 Physiocochemical values of *L. speciosa* leaves (LS17).

No.	Loss on drying (%)	Moisture content (%)	Ash content (%)		Extractive value (%)		
			Total ash	Acid insoluble ash	Ethanol	Water	Dichloro methane
1	7.3608	7.7617	8.1725	1.1865	8.2487	13.2083	3.6480
2	7.6981	7.5870	8.2533	1.1833	8.5180	13.0682	3.6285
3	7.8987	7.4136	8.2028	1.1828	8.7156	13.1332	3.6296
mean	**7.6525**	**7.5874**	**8.2096**	**1.1842**	**8.4941**	**13.1366**	**3.6354**
SD	**0.2718**	**0.1740**	**0.0408**	**0.0020**	**0.2344**	**0.0396**	**0.0110**

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VITA

Mr. Woraouch Thitikornpong was born on July 21, 1985 in Bangkok, Thailand. He received his Bachelor's degree of Science (Second Honors) in Pharmacy in 2008 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Poster Presentation

Thitikornpong, T., Padungcharoen, T., Vetchapongsa, A., Sukrong, S. 2010. Pharmacognostic study of intanin-nam (Lagerstroemia speciosa) for herbal raw materials identification. The 16th National Graduate Research Conference. Maejo University. March, 11, 2010. Chiang Mai, Thailand.

Oral Presentation

Thitikornpong, T., Padungcharoen, T., Vetchapongsa, A., Sukrong, S. 2010. Identification of Crude Drugs by Macroscopic and Microscopic Characteristics, Thin Layer Chromatography, and DNA Fingerprinting: *Lagerstroemia speciosa*. TRF-Master Research Congress IV, March, 31, 2010. Pattaya, Chonburi, Thailand.

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