MICROSCOPIC AND MOLECULAR AUTHENTICATION OF SELECTED *CASSIA* SPECIES ENDEMIC TO THAILAND AND EVALUATION OF ALOE-EMODIN CONTENTS IN *CASSIA GARRETTIANA* AND *CASSIA GRANDIS* LEAVES

Miss Anusara Sihanat

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จุลทรรศนลักษณะและอณูโมเลกุลของพืชสกุลแคสเซียบางชนิดในประเทศไทย
และปริมาณอะโล-อีโมดินของใบแสมสารและใบกาลพฤกษ์

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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By	Miss Anusara Sihanat
Field of Study	Public Health Sciences
Thesis Advisor	Assistant Professor Kanchana Rungsihirunrat, Ph.D.
Thesis Co-Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.
	Piyarat Parinyapong Chareonsap, Ph.D.

Accepted by the College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

_____________________Dean of the College of Public Health Sciences

(Professor Sathirakorn Pongpanich, Ph.D.)

THESIS COMMITTEE

_____________________Chairman

(Assistant Professor Naowarat Kanchanakhan, Ph.D.)

_____________________Thesis Advisor

(Assistant Professor Kanchana Rungsihirunrat, Ph.D.)

_____________________Thesis Co-Advisor

(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

_____________________Thesis Co-Advisor

(Piyarat Parinyapong Chareonsap, Ph.D.)

_____________________Examiner

(Assistant Professor Chanida Palanuvej, Ph.D.)

_____________________Examiner

(Tepanata Pumpaibool, Ph.D.)

_____________________External Examiner

(Orawan Theanphong, Ph.D.)

อนุสรา สีหนาท : จุลทรรศนลักษณะและอณูโมเลกุลของพืชสกุลแคสเซียบางชนิดในประเทศไทยและปริมาณอะโล-อีโมดินของใบแสมสารและใบกาลพฤกษ์ (MICROSCOPIC AND MOLECULAR AUTHENTICATION OF SELECTED *CASSIA* SPECIES ENDEMIC TO THAILAND AND EVALUATION OF ALOE-EMODIN CONTENTS IN *CASSIA GARRETTIANA* AND *CASSIA GRANDIS* LEAVES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. กาญจนา รังษีหิรัญรัตน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นิจศิริ เรืองรังษี, ดร. ปิยรัษฎ์ ปริญญาพงษ์ เจริญทรัพย์, 345 หน้า.

พืชสกุล *Cassia* L. จัดอยู่ในวงศ์ Caesalpiniaceae ถูกนำมาใช้เป็นสมุนไพร อาหาร และไม้ประดับ มาเป็นเวลานาน เนื่องจากลักษณะทางพฤกษศาสตร์และชื่อพื้นเมืองมีความคล้ายคลึงกัน ทำให้ยากต่อการจำแนกชนิด วัตถุประสงค์ในการศึกษาครั้งนี้ เพื่อจำแนกความแตกต่างของพืชในสกุลแคสเซีย จำนวน 16 ชนิดที่พบในประเทศไทย โดยวิธีทางมหทรรศนลักษณะ จุลทรรศนลักษณะ และลายพิมพ์ดีเอ็นเอชนิดเอเอฟแอลพี ร่วมกับการวิเคราะห์ปริมาณสารอะโล-อีโมดินในใบแสมสารและใบกาลพฤกษ์ที่เก็บจาก 15 แหล่งทั่วประเทศไทย โดยวิธีโครมาโตกราฟฟี่ชนิดแผ่นบาง-เด็นซิโตเมทรีและวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ ลักษณะทางพฤกษศาสตร์และภาคตัดขวางของเส้นกลางใบแสดงในรูปแบบภาพวาดลายเส้น การศึกษาภาคตัดขวางของเส้นกลางใบภายใต้กล้องจุลทรรศน์ แสดงลักษณะเซลล์ผิว เซลล์แพลิเซด เซลล์สปันจี มัดท่อลำเลียงพาเรนไคมา และคลอเรนไคมา การศึกษาลักษณะและจำนวนขนบนแผ่นใบภายใต้กล้องจุลทรรศน์และกล้องจุลทรรศน์อิเล็คตรอนชนิดส่องกราด พบลักษณะของขนแบบเซลล์เดียวและหลายเซลล์ ไม่มีต่อมขนและขนแบบหลายเซลล์ มีต่อมขน อย่างไรก็ตาม พืชสกุลแคสเซียบางชนิดไม่พบว่ามีขน ชัยพฤกษ์ มีจำนวนขนทั้งด้านหลังใบและด้านท้องใบมากที่สุด (78.94±2.86, 127.39±2.46) ขณะที่ทรงบาดาล มีจำนวนขนด้านท้องใบน้อยที่สุด (3.46±0.80) การศึกษาลายพิมพ์ดีเอ็นเอชนิดเอเอฟแอลพีในพืชสกุลแคสเซียทั้ง 16 ชนิดที่พบในประเทศไทย พบว่าจำนวนไพรเมอร์ 11 คู่สามารถทำให้เกิดแถบดีเอ็นเอที่มีความแตกต่างและคมชัดทั้งหมด 849 แถบ เฉลี่ย 77.18 แถบต่อคู่ไพรเมอร์ ความสัมพันธ์ทางพันธุกรรมจากลายพิมพ์ดีเอ็นเอชนิดเอเอฟแอลพีมีค่าดัชนีความคล้ายคลึงทางพันธุกรรมของพืชในสกุลแคสเซียอยู่ระหว่าง 0.25 ถึง 0.78 การวิเคราะห์ปริมาณสารอะโล-อีโมดินในใบแสมสารและใบกาลพฤกษ์จาก 15 แหล่งทั่วประเทศไทยโดยวิธีโครมาโตกราฟฟี่ชนิดแผ่นบาง-เด็นซิโตเมทรีร่วมกับโปรแกรม winCATS และวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ พบว่าใบแสมสารมีปริมาณสารอะโล-อีโมดินเฉลี่ย 0.035±0.007 และ 0.035±0.006 กรัม/100 กรัม ในขณะที่ใบกาลพฤกษ์มีปริมาณสารอะโล-อีโมดินเฉลี่ย 0.412±0.067 และ 0.413±0.075 กรัม/100 กรัม จากการวิเคราะห์ทั้งสองวิธี ตามลำดับ โดยปริมาณวิเคราะห์ทั้งสองวิธีนั้นมีความเชื่อถือได้ในด้านความจำเพาะ ความสัมพันธ์เชิงเส้น ความแม่นยำ ความเที่ยง ขีดจำกัดในการตรวจสอบ ขีดจำกัดในการวัดเชิงปริมาณและความคงทน ข้อมูลที่ได้จากงานวิจัยครั้งนี้สามารถนำไปใช้ในการพิสูจน์เอกลักษณ์ของพืชสกุลแคสเซียที่พบในประเทศไทยและทำให้ทราบถึงปริมาณสารอะโล-อีโมดินในใบแสมสารและใบกาลพฤกษ์

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	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....
	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

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5579056153 : MAJOR PUBLIC HEALTH SCIENCES
KEYWORDS: CASSIA SPECIES / TRICHOME NUMBER / AFLP FINGERPRINT / TLC-DENSITOMETRY / TLC-IMAGE ANALYSIS

ANUSARA SIHANAT: MICROSCOPIC AND MOLECULAR AUTHENTICATION OF SELECTED *CASSIA* SPECIES ENDEMIC TO THAILAND AND EVALUATION OF ALOE-EMODIN CONTENTS IN *CASSIA GARRETTIANA* AND *CASSIA GRANDIS* LEAVES. ADVISOR: ASST. PROF. KANCHANA RUNGSIHIRUNRAT, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., PIYARAT PARINYAPONG CHAREONSAP, Ph.D., 345 pp.

The genus *Cassia* L, belongs to the Caesalpiniaceae family, has been widely used as herbs, foods and ornamental plants for a long time. Due to the similar morphology and vernacular name, the identification of these species is perplexed. This study aimed to distinguish 16 *Cassia* spp. existing in Thailand using macroscopic examination, microscopic examination and AFLP fingerprinting as well as the quantitative analysis of aloe-emodin contents in *C. garrettiana* and *C. grandis* leaves collected from 15 different locations in Thailand was also developed and validated using thin-layer chromatography densitometry and thin-layer chromatography image analysis with ImageJ software. The macroscopic characteristics and transverse section of leaf through midrib were illustrated by drawing. Transverse section of leaf through midrib observed under the light microscope showed the anatomical characteristics of epidermis, palisade cells, spongy cells, vascular bundle, parenchyma and collenchyma. Leaf trichome characteristic and number observed under the light microscope and scanning electron microscopy showed the uniseriate, uni- or multicellular non-glandular and multicellular glandular trichome. However, some *Cassia* spp. had absent of trichome. *Cassia javanica* L. had the highest trichome number in both dorsal (78.94±2.86) and ventral (127.39±2.46) surfaces of the leaf whereas *Cassia surattensis* Burm. f. had the lowest trichome number found only on ventral (3.46±0.80) surface. The AFLP fingerprint among 16 selected *Cassia* spp. indicated that eleven primer combinations produced a total of distinct and reproducible 849 bands with an average 77.18 bands per primer combinations. The genetic relationship based on amplified AFLP bands showed the similarity index (SI) ranged from 0.25 to 0.78. The quantitative analysis of aloe-emodin contents from 15 sources of *Cassia garrettiana* Craib and *Cassia grandis* L. f. using TLC-densitometry with winCATS software and TLC-image analysis with ImageJ software found that *C. garrettiana* dried crude drug had 0.035±0.007 and 0.035±0.006 g% of aloe-emodin contents whereas in *C. grandis* dried crude drug had 0.412±0.067 and 0.413±0.075 g% of aloe-emodin contents, respectively. Both methods were developed and validated in term of specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness. The data obtained from this study provided useful information for identification of selected Thai *Cassia* spp. and provided the aloe-emodin contents in *C. garrettiana* and *C. grandis* leaves.

Field of Study: Public Health Sciences — Student's Signature ....
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LIST OF ABBREVIATIONS

A, T, C, G	nucleotide containing the base adenine, thymine, cytosine, and guanine, respectively
AFLP	Amplified fragments length polymorphism
APS	Ammonium persulfate
ATP	Adenosine triphosphate
Avg	Average
bp	Base pair
%C	Crosslinker concentration
°C	Degree Celsius
CTAB	Cetyl trimethyl ammonium bromide
cm	Centimeter
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
EDTA	Ethylenediaminetetraacetic acid
g	Gram
gDNA	genomic DNA
ISSR	Inter- simple sequence repeat
ICH	International Conference on Harmonization
kg	Kilogram
L	Liter
LM	Light microscope
LOD	Limit of detection

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LIST OF ABBREVIATIONS

LOQ	Limit of quantification
M	Molar
mg	Milligram
$Mgcl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
mm^2	Square millimeter
µl	Microlitre
µm	Micrometer
µM	Micromolar
µg	Microgram
ng	Nanogram
nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
rpm	Round per minute
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SD	Standard deviation

LIST OF ABBREVIATIONS

SSCP	Single-strand conformation polymorphism
SCAR	Sequence characterized amplified region
SEM	Scanning electron microscope
SSR	Simple sequence repeat
SI	Similarity index
sp./spp.	Species
%T	Total acrylamide-bisacrylamide monomer concentration
Taq	*Thermus aquaticus*
TBE buffer	Tris-boric and EDTA buffer
TE	Tris-EDTA buffer
TLC	Thin-layer chromatography
TEMED	N, N, N', N', -tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris-hydrochloride buffer
UPGMG	Unweighted pair group method with arithmetic average
UV	Ultraviolet
v/v	Volume by volume
WHO	World Health Organizatio

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CHAPTER I

INTRODUCTION

Background and rationale

Medicinal plants become popular over the past decades. It has been used in the preparation of medicines, neutraceutical and cosmeceutical products. Herbal medicine must be administered with accuracy which is right plant, right part, right dose and right method. Thus, the identification of plant material is the first priority for ensuring the quality, safety and efficacy of herbal medicine.

The genus *Cassia*, the tropical flowering plant belongs to the family Caesalpiniaceae, is the largest genus that comprise of 500 - 600 species[1]. Thirty three species of *Cassia*, both native plants and imported from abroad, were distributed throughout Thailand[2]. *Cassia* species have been commonly used as ornamentals, food and traditional medicines in many countries such as India, China, East Africa, South Africa, America, Mexico, Brazil and Thailand. Some herbal plants in genus *Cassia* i.e. *Cassia fistula* L., *Cassia siamea* Lam. and *Cassia alata* L. have been enrolled as a Thai medicinal plant in primary health care and has been listed in Thai traditional household drug[3]. In addition, *Cassia alata* L. and *Cassia angustifolia* Vahl are included in the list of Herbal Medicinal Products A.D. 2006 of Thailand for their laxative, purgative properties and treatment of skin diseases[4]. General pharmacological studies from many parts of plant in genus *Cassia* revealed their antifungal, antibacterial, anti-inflammatory, anti-cancer, antipyretic, antihelmintic, antioxidant, relieved anxiety, promoting restful sleep and relaxes properties[5-8].

Several taxonomists classified *Cassia* genus in different systems based on various morphological characteristics. Irwin and Barneby[9] first classified the subtribe

Cassiinae into three genera; *Cassia, Senna* and *Chamaecrista*. However, in 1984, Larsen *et al*.[10] suggested in Flora of Thailand that all 21 species from the three genera should be grouped into one genus, *Cassia*. However, many botanists have still argued about this long time taxonomic problem. The Flora Malesianna was re-investigated an intergenetic relationship of the subtribe *Cassiinae* (*Cassia/Senna/Chamaecrista*) and then moved ten *Cassia* species into the genus *Senna*[11]. These confusing and problematic arguments rely heavily on a variability of their morphological characteristics. Moreover, some plants in genus *Cassia* have similar morphology and vernacular name but significantly vary in their medicinal properties. Misidentification might cause inconsistent results because of the different therapeutic effects of these species and may be poisonous if taken in excess[12].

There are many methods used for examination of medicinal plant such as macroscopic, microscopic, chemical compound and genetic information. Macroscopic and microscopic examinations are major methods used for herbal authentication because they are simple, rapid and inexpensive. Macroscopic evaluation is based on the morphological features such as shape, size, color, texture and other characteristics, which always used to distinguish various species or evaluate their quality. They are conducted by observing, touching, smelling, tasting and testing by other ways. Microscopic examination is a conventional method for identification of plant structural feature under microscope observation. In addition, the constant numbers of leaf are the parameters that are unique to the plant and can be used for identification. However, due to the similarity in their morphological characteristics and the processing of medicinal plants into powder for preparation in several dosage forms such as capsulation or pills resulting in morphological and anatomical changes. It is difficult to identify each species by morphological observation. In addition, a

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complementary with other analytical methods such as chemical component, molecular technology provides important supporting evidence[13]. Anthraquinone compounds which are the largest group of natural quinones have long been used as laxatives and antifungal drug for skin diseases[14]. Plants in genus *Cassia* are one of the main sources of anthraquinone compounds. Several anthraquinones and anthraquinone derivatives from *Cassia* species have been reported such as rhein, emodin, senosides and aloe-emodin[15]. Aloe-emodin is an anthraquinone found in plants (*Aloe* spp., *Rhamnus* spp., and *Cassia* spp.), fungi, lichens, and insects. Aloe-emodin has interested biological activity such as antiviral, antimicrobial, anticancer and hepatoprotective activities[16-21]. Moreover, from the previous studies main anthraquinone isolated from *Cassia garrettiana* Craib and *Cassia grandis* L. f. leaves growing in Thailand was identified as aloe-emodin[22, 23]. Quantitative analysis can be performed using scanning densitometry and image analysis method. This method is easy, rapid, and widely used method for investigation of the number of compounds in a mixture. Nevertheless, the use of chromatographic technique and marker compounds to standardize herbal preparation has some limitation due to many factors such as age, physiological conditions, environmental factors, harvest, storage and processing may affect to chemical profile[24].

Recently, DNA fingerprinting assay has been applied and also introduced for identification of medicinal plants. Individual plant DNA carries the same genetic information, which is not affected by environmental factors. Amplified Fragment Length Polymorphism (AFLP), an efficient DNA fingerprinting technique, is very useful for the assessment of genetic diversity and identification of herbal plant species because it is highly reproducible, and can be used for whole genome analysis without any prior sequence knowledge[25]. Besides the identification of medicinal plant can be

done via the implication of macroscopic and microscopic techniques, it also can be investigated using chemical constituents and genetic information. Hence, a combination of several methods is recommended because no single method can be making a conclusive result.

Therefore, this study aim to distinguish *Cassia* species using various methods including macroscopic examination, microscopic examination and AFLP fingerprinting molecular analysis for identification of *Cassia* species and investigation the phylogenetic relationship among *Cassia* species existing in Thailand. In addition, this study was to develop and validate thin-layer chromatography densitometry with winCATS software and thin-layer chromatography image analysis with ImageJ free software for quantitative analysis of aloe-emodin contents in *C. garrettiana* and *C. grandis* leaves collected from different locations in Thailand. The result of this study will provide useful information for its correct identification and provide the contents of aloe-emodin in *C. garrettiana* and *C.grandis* leaves.

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Research questions

1. Are macroscopic and microscopic examinations able to distinguish plants in *Cassia* genus?
2. Is the AFLP fingerprinting able to distinguish plants in *Cassia* genus?
3. Is quantitation of aloe-emodin by TLC-image analysis using ImageJ free software comparable to TLC-densitometric method?

Objectives

1. To distinguish the characteristics of *Cassia* species by the macroscopic and microscopic examinations.
2. To distinguish *Cassia* species and their phylogenetic relationship by AFLP fingerprinting.
3. To evaluate the contents of aloe-emodin in *C. garrettiana* and *C. grandis* leaves by TLC-image analysis using ImageJ free software compared to TLC-densitometric method.

Expected benefit and application

1. This research provides the macroscopic and microscopic characteristics of selected *Cassia* species in Thailand.
2. This research provides the phylogenetic relationship of selected *Cassia* species by AFLP fingerprinting.
3. This research provides the contents of aloe-emodin in *C. garrettiana* and *C. grandis* leaves.
4. This research provides the simple, less expensive and valid method of TLC-image analysis for aloe-emodin quantitation in *C. garrettiana* and *C. grandis* leaves.

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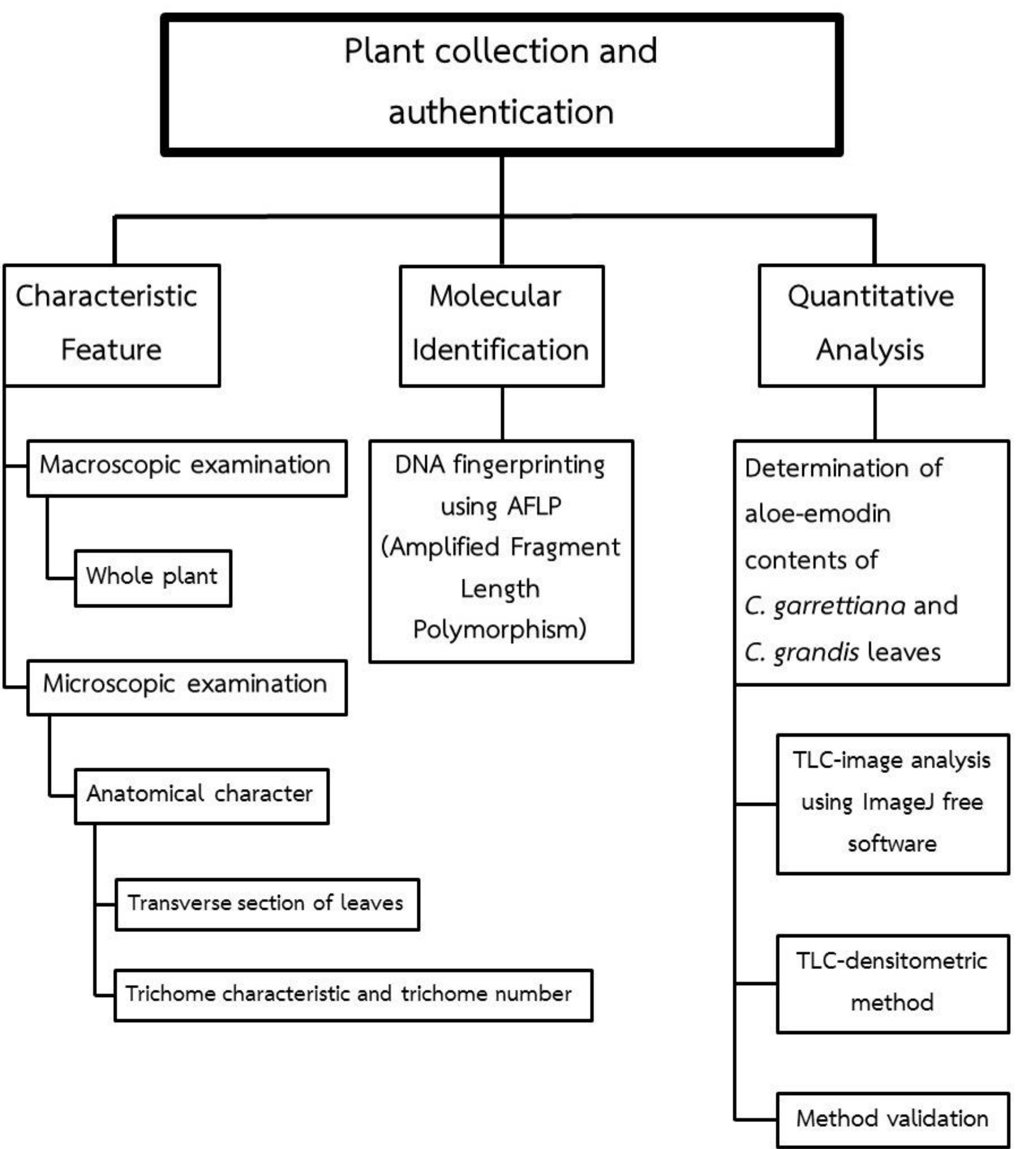

Figure 1 The conceptual framework

CHAPTER II

LITERATURE REVIEWS

2.1 The genus *Cassia*

2.1.1 Morphology of genus *Cassia*

The taxonomic description of genus *Cassia* was reported by Larsen *et al*.[10] as follows;

"It is a tropical plant consists of trees, shrubs, and herbs. Leaves are paripinnate. Foliar glands are often present. Flowers are in simple racemes or panicles, bisexual, yellow or pink to red. Receptacle is very short. Sepals are imbricate in bud. Petal 5. Stamen 10-5; anthers opening by pores or by a short slit. Ovule numerous. Fruit is varying in shape, indehiscent or dehiscent, albuminous."

According to Irwin and Barneby[9] classification, *Casiinae* subtribe was firstly classified into three genera *Cassia*, *Senna* and *Chamaecrista* using the characteristic of filaments and the presence or absence of bracteoles. Lock[26] investigated the African members of the *Casiinae* subtribe followed by Irwin and Barneby's classification and re-classified the subtribe into three genera. However, in 1984, Larsen *et al*.[10] advised in Flora of Thailand that all 21 species from the three genera should be only group into one genus, *Cassia*. There are many previous studies of the classification of the genus *Cassia* that are summarized in Table 1.

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Table 1 The classification of the genus *Cassia*

Classification system	No. of proposed genus	Generic name	Classification criteria
Bentham (1871)	1	*Cassia*	Morphological characteristics[27]
Irwin and Barneby (1981)	3	*Cassia, Senna* and *Chamaecrista*	Filaments and the presence or absence of bracteoles[9]
Lock (1988)	3	*Cassia, Senna* and *Chamaecrista*	Filaments and the presence or absence of bracteoles[26]
Tucker (1996)	3	*Cassia, Senna* and *Chamaecrista*	Floral ontogeny[28]
Doyle *et al.* (1997)	3	*Cassia, Senna* and *Chamaecrista*	*rbc*L sequence[29]
Ghareeb *et al.* (1999)	1	*Cassia*	Seed protein, Chromosome numbers, Morphological characteristics[30]
Mondal *et al.* (2000)	1	*Cassia*	Seed protein, RFLP[31]
Bruneau *et al.* (2001)	3	*Cassia, Senna* and *Chamaecrista*	*trn*L intron sequence[32]

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Table 1 The classification of the genus *Cassia* (Cont.)

Classification system	No. of proposed genus	Generic name	Classification criteria
Kidyue (2003)	3	*Cassia, Senna* and *Chamaecrista*	Anatomy of stems, leaves and flower[33]
Petchsri (2003)	3	*Cassia, Senna* and *Chamaecrista*	Numerical taxonomic[34]
Srisawat (2004)	3	*Cassia, Senna* and *Chamaecrista*	*trn*L intron, ITS region, DNA sequencing[35]
Boonkerd *et al.* (2005)	3	*Cassia, Senna* and *Chamaecrista*	Morphological characteristics using cluster analysis and canonical discriminant analysis[36]
Mohanty *et al.* (2006)	1	*Cassia*	Chromosome, 4C nuclear DNA[37]
Mohanty *et al.* (2010)	1	*Cassia*	RAPD, ISSR, SSR marker[38]
Tripathi and Goswami (2011)	3	*Cassia, Senna* and *Chamaecrista*	RAPD marker[39]

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Table 1 The classification of the genus *Cassia* (Cont.)

Classification system	No. of proposed genus	Generic name	Classification criteria
Monkheang *et al.* (2011)	1	*Senna*	*trn*H-*psb*A, Morphological characteristics[40]
Acharya *et al*. (2011)	3	*Cassia, Senna* and *Chamaecrista*	RAPD, ISSR, AFLP marker[41]
Abdel-Hameed *et al.* (2013)	2	*Cassia, Senna*	RAPD marker, Isozyme, Morphological characteristics[42]
Purushothaman *et al.* (2014)	1	*Cassia*	DNA barcoding[43]

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Among these taxonomic arguments about generic classification in the *Cassiinae*, the relationship between the genera *Cassia* and *Senna* is still confused. Recently, twelve species of *Cassia* and seventeen species of *Senna* in Thailand were reported[2]. However, some species are rarely found or completely absent. Among these, sixteen of *Cassia* plants including, *C. bakeriana*, *C. fistula*, *C. grandis*, *C. javanica*, *C. alata*, *C. angustifolia*, *C. garrettiana*, *C. hirsuta*, *C. occidentalis*, *C. spectabilis*, *C. siamea*, *C. sophera*, *C. sulfurea*, *C. surattensis*, *C. timoriensis* and *C. tora* have been commonly found and widely distributed in Thailand and most of them have been used as medicinal plants. The list of sixteen *Cassia* species commonly found in Thailand is shown in Table 2.

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Table 2 The list of sixteen *Cassia* species in Thailand

No.	Scientific Name (Larsen *et al.*, 1984)	Scientific Name (Irwin and Barneby, 1982; Thai plant names Tem Smitinand revised edition 2014)	Thai Name
1	*Cassia bakeriana* Craib	*Cassia bakeriana* Craib	Kalapaphruek (กัลปพฤกษ์) Chaiyaphruek (ชัยพฤกษ์) Daug-kapi (ดอกกะปิ)
2	*Cassia fistula* L.	*Cassia fistula* L.	Ratchaphruek (ราชพฤกษ์) Khuun (คูน) Lomlaeng (ลมแล้ง) Ku-phe-ya (กุเพยะ) Chaiyaphruek (ชัยพฤกษ์)
3	*Cassia grandis* L.f.	*Cassia grandis* L. f.	Kanlaphruek (กาลพฤกษ์) Kalapaphruek (กัลปพฤกษ์)
4	*Cassia javanica* L.	*Cassia javanica* L.	Chaiyaphruek (ชัยพฤกษ์) Ratchaphruek (ราชพฤกษ์) Kalapaphruek (กัลปพฤกษ์) Lak khoei lak klua (ลักเกยลักเกลือ)

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Table 2 The list of sixteen *Cassia* species in Thailand (Cont.)

No.	Scientific Name (Larsen *et al.*, 1984)	Scientific Name (Irwin and Barneby, 1982; Thai plant names Tem Smitinand revised edition 2014)	Thai Name
5	*Cassia alata* L.	*Senna alata* (L.) Roxb.	Chum het thet (ชุมเห็ดเทศ) Chum het (ชุมเห็ด) Chum het yai (ชุมเห็ดใหญ่) Khi khak (ขี้คาก) Lap muen luang (ลาบมึนหลวง) Mak kaling thet (หมากะลิงเทศ) Ta-see pho (ตะสีพอ)
6	*Cassia angustifolia* Vahl *Cassia acutifolia* Delile	*Senna alexandriana* Mill.	Makhaam khaek (มะขามแขก) Som khaek (ส้มแขก)

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Table 2 The list of sixteen *Cassia* species in Thailand (Cont.)

No.	Scientific Name (Larsen *et al.*, 1984)	Scientific Name (Irwin and Barneby, 1982; Thai plant names Tem Smitinand revised edition 2014)	Thai Name
7	*Cassia garrettiana* Craib	*Senna garrettiana* (Craib) H. S. Irwin & Barneby	Samae san (แสมสาร) Khi lek khok (ขี้เหล็กโคก) Khi lek phae (ขี้เหล็กแพะ) Khilek pa (ขี้เหล็กป่า) Khi lek san (ขี้เหล็กสาร) Ngai-san (ไงซาน) Kabat (กะบัด)
8	*Cassia hirsuta* L.	*Senna hirsuta* (L.) H. S. Irwin & Barneby	Dap phit (ดับพิษ) Phong pheng (โผงเผง)
9	*Cassia occidentalis* L.	*Senna occidentalis* (L.) Link	Chum het lek (ชุมเห็ดเล็ก) Chum het thet (ชุมเห็ดเทศ) Khi lek phuak (ขี้เหล็กเผือก) Lap muen noi (ลับมืนน้อย)

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Table 2 The list of sixteen *Cassia* species in Thailand (Cont.)

No.	Scientific Name (Larsen *et al*., 1984)	Scientific Name (Irwin and Barneby, 1982; Thai plant names Tem Smitinand revised edition 2014)	Thai Name
9	*Cassia occidentalis* L.	*Senna occidentalis* (L.) Link	Mak kaling thet (หมากกะลิงเทศ) Kheelek phee (ขี้เหล็กผี) Phrom dan (พรมดาน) Phak chit (ผักจิ๊ด)
10	*Cassia spectabilis* DC.	*Senna spectabilis* (DC.) H. S. Irwin & Barneby	Khee lek american (ขี้เหล็กอเมริกัน)
11	*Cassia siamea* Lam.	*Senna siamea* (Lam.) H. S. Irwin & Barneby	Kheelek (ขี้เหล็ก) Khee lek ban (ขี้เหล็กบ้าน) Khee lek luang (ขี้เหล็กหลวง) Khee lek yai (ขี้เหล็กใหญ่) Ya ha (ยะหา)
12	*Cassia sophera* L.	*Senna sophera* (L.) Roxb.	Phak khet (ผักเค็ด) Phak khlet (ผักเคล็ด)

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Table 2 The list of sixteen *Cassia* species in Thailand (Cont.)

No.	Scientific Name (Larsen *et al*., 1984)	Scientific Name (Irwin and Barneby, 1982; Thai plant names Tem Smitinand revised edition 2014)	Thai Name
12	*Cassia sophera* L.	*Senna sophera* (L.) Roxb.	Phak wan ban (ผักหวานบ้าน)
13	*Cassia surattensis* Burm. f. subsp. *glauca* (Lam.) K. Larsen & S. S. Larsen *Cassia glauca* Lam. *Cassia sulfurea* DC. ex Collad.	*Senna sulfurea* (DC. ex Collad.) H. S. Irwin & Barneby	Trueng badaan (ตรึงบาดาล) Sakeng (สะเก๋ง) Sakong (สะโก้ง)
14	*Cassia surattensis* Burm. f.	*Senna surattensis* (Burm. f.) H. S. Irwin & Barneby	Song badan (ทรงบาดาล) Khee lek wan (ขี้เหล็กหวาน)
15	*Cassia timoriensis* DC.	*Senna timoriensis* (DC.) H. S. Irwin & Barneby	Kheelek luead (ขี้เหล็กเลือด) Cha kheelek (ช้าขี้เหล็ก)

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Table 2 The list of sixteen *Cassia* species in Thailand (Cont.)

No.	Scientific Name (Larsen et al., 1984)	Scientific Name (Irwin and Barneby, 1982; Thai plant names Tem Smitinand revised edition 2014)	Thai Name
15	*Cassia timoriensis* DC.	*Senna timoriensis* (DC.) H. S. Irwin & Barneby	Makluea luead (มะเกลือเลือด) Kheelek daeng (ขี้เหล็กแดง) Kalaeng ngaen (กะแลงเงิน)
16	*Cassia tora* L.	*Senna tora* (L.) Roxb.	Chumhet thai (ชุมเห็ดไทย) Chumhet na (ชุมเห็ดนา) Chumhet lek (ชุมเห็ดเล็ก) Chumhet khwaai (ชุมเห็ดควาย) Phromdan (พรมแดน) Lap muen noi (ลับมืนน้อย)

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2.2 Ethnomedical uses of *Cassia* plants

Medicinal plants in genus *Cassia* have long been widely used as ethnomedicine in many countries such as India, China, East Africa, South Africa, America, Mexico, Brazil and Thailand. The history of herbal medicine or traditional Thai medicine has presented from the Sukhothai period basically in the utilization of Thai people in primary health care system. Plants used in traditional medicine usually constitute of an important source of new biologically active compounds. Numerous useful drugs have been discovered from higher plants by following up ethnomedical uses[44]. The Ethnomedical uses of *Cassia* plants are summarized in Table 3.

Table 3 Ethnomedical uses of *Cassia* plants

Species	Part uses	Ethnomedical uses
Cassia bakeriana	Leaf	Laxative[45]
	Pod	Laxative[46]
C. fistula	Leaf	Antitussive[47]
		Laxative[48]
		Wound healing[49]
		Relief headache[49]
		Treatment of scabies[50]
		Treatment of leprosy[51]
		Treatment of eczema[49]
		Treatment of jaundice[49]
		Treatment of dyspepsia[52]
		Treatment of paralysis[52, 53]
		Treatment of skin diseases[51, 54]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. fistula	Leaf	Treatment of pneumonia[52]
		Treatment of bronchitis[52]
		Treatment of liver disorder[52]
		Treatment of rheumatism[53]
	Pod	Antitussive[47]
		Wound healing[55]
		Laxative[48, 50]
		Antifungal[50]
		Antimalarial[56]
		Antifertility activity[56]
		Treatment of amoebiasis[50]
		Treatment of urinary disorder[50]
		Treatment of liver disorder[57]
		Treatment of cold[56]
		Treatment of leprosy[56]
		Treatment of asthma[58]
	Seed	Antidote[53]
		Laxative[56]
		Antidiabetic[56]
		Treatment of skin diseases[59]
		Treatment of jaundice[59]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. fistula	Leaf	Treatment of paralysis[53]
		Treatment of rheumatism[53]
		Treatment of pneumonia[52]
		Treatment of bronchitis[52]
		Treatment of liver disorder[52]
		Treatment of amoebiasis[60]
	Root	Treatment of skin diseases[61]
		Treatment of syphilis[61]
		Treatment of cold[50, 57]
	Flower	Laxative[62]
		Treatment of cold[56]
C. grandis	Leaf	Anti-inflammatory[47, 63]
		Antifungal[63]
		Analgesic[63]
		Antioxidant[64]
		Purgative[64]
		Treatment of skin diseases[64]
		Treatment of epistaxis[65]
		Treatment of anemia[66]
		Treatment of cold[65]
		Treatment of liver disorder[67]
		Treatment of urinary disorder[67]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. grandis	Pod	Laxative[68-70]
		Treatment of anemia[66]
		Treatment of epistaxis[65]
		Treatment of cold[65]
		Treatment of liver disorder[67]
		Treatment of urinary disorder[67]
	Seed	Laxative[64, 68-70]
	Bark	Wound healing[71]
		Treatment of rheumatism[67]
		Treatment of anemia[66]
		Treatment of epistaxis[65]
		Treatment of cold[65]
		Treatment of liver disorder[67]
		Treatment of urinary disorder[67]
	Root	Wound healing[67]
		Treatment of cold[67]
		Treatment of skin diseases[67]
C. javanica	Leaf	Treatment of *Herpes zoster*[72]
		Treatment of bad breath[73]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. javanica	Leaf	Treatment of chickenpox[74]
		Treatment of cold[75]
		Treatment of gastric pain[75]
		Treatment of measles[75]
		Antimalarial[75]
	Pod	Laxative[76]
		Treatment of cold[76]
	Bark	Antidiabetic[72]
C. alata	Leaf	Laxative[1, 40]
		Analgesic[77]
		Antimicrobial[78-82]
		Wound healing[40]
	Seed	Antidiabetic[83]
		Treatment of tinea[84]
		Treatment of scabies[57]
		Treatment of ringworm[40, 57]
		Treatment of allergy[85]
		Treatment of abscesses[85]
	Flower	Antibacterial[40]
		Antifungal[86]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. alata	Bark	Antimicrobial[40, 81]
		Laxative[40]
	Stem	Antibacterial[40, 81]
	Root	Antibacterial[40, 81]
C. angustifolia	Whole plant	Laxative[40, 53, 87]
		Antipyretic[87]
		Anthelmintics[87]
		Diuretic[87]
		Treatment of anemia[87]
		Treatment of gout[87]
		Treatment of rheumatism[87]
		Treatment of jaundice[87]
		Treatment of typhoid[87]
		Treatment of cholera[87]
C. garrettiana	Leaf	Antidiabetic[88]
		Anti-leukemia[88]
	Flower	Treatment of insomnia[88]
	Heartwood	Emmenagogue[89]
		Blood tonic for women[89]
		Laxative[90]
		Treatment of leukemia[90]
		Treatment of *Herpes zoster*[90]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. garrettiana	Heartwood	Anthelmintics[90]
C. hirsuta	Leaf	Antimicrobial[91]
		Anthelmintic[50, 91]
		Antidote to snake bite[50]
		Relief of stomachache[50, 91]
		Treatment of *Herpes zoster*[50]
		Treatment of bone fractures[50]
		Treatment of abscesses[91]
		Treatment of rheumatism[91]
		Treatment of haematuria[91]
		Treatment of liver disorder[92]
		Treatment of cold[91]
	Seed	Protect teeth and gums from plaque[50]
		Substituted for coffee[93]
	Bark	Treatment of chronic ulcer[50]
		Used for flavoring purposes in soaps, candy and perfumery[94]
C. occidentalis	Leaf	Laxative[95]
		Anti-inflammatory[96]
		Antifungal[96]
		Antiulcer[96]
		Antidote to snake bite[96]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. occidentalis	Leaf	Treatment of cold[96]
		Treatment of hepatitis[97]
		Treatment of asthma[51]
		Treatment of cough[51]
		Treatment of skin diseases[85]
		Treatment of gall bladder[95]
		Treatment of ringworm[57]
		Antidote to poisoning[95]
	Seed	Treatment of asthma[55]
		Treatment of skin diseases[85]
	Root	Treatment of asthma[51]
		Treatment of cough[51]
	Whole plant	Treatment of haematuria[98]
		Treatment of rheumatism[98]
		Treatment of asthma[98]
		Treatment of typhoid[98]
		Treatment of haemoglobin disorder[98]
C. spectabilis	Leaf	Antifungal[98]
		Antibacterial[98]
		Relief of edema[50]
		Laxative[50]
		Antidote[50]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. spectabilis	Leaf	Treatment of insomnia[99]
		Treatment of epilepsy[99]
	Flower	Antifungal[50]
	Pod	Antifungal[50]
	Stem	Antifungal[50]
C. siamea	Leaf	Treatment of liver disorder[50]
		Treatment of insomnia[50]
		Treatment of asthma[100]
		Antidiabetic[101]
		Antimalarial[102]
		Anxiolytic[103]
		Antidiabetic[100]
		Antihypertensive[100]
	Flower	Antihypertensive[50, 100]
		Anxiolytic[103]
		Antidiabetic[100]
		Treatment of asthma[100]
	Bark	Antimalarial[102]
C. sophera	Leaf	Treatment of ringworm[57]
		Antidote to insect bite[57]
C. surattensis	Leaf	Treatment of dysentery[104]
		Laxative[105]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. surattensis	Leaf	Treatment of sore throat and cough[105]
		Antidiabetic[106]
	Flower	Purgative[84]
	Bark	Antidiabetic[106]
	Root	Antidote to snake bite[104]
		Treatment of gonorrhea[104]
C. timoriensis	Leaf	Treatment of scabies[107]
		Treatment of itch[107]
		Anthelmintics[107]
		Treatment of menstrual disorders[40, 108]
		Treatment of blood stasis[108]
		Treatment of cough[108]
		Tonic[108]
		Antitumor[108]
	Bark	Treatment of itch[107]
	Heartwood	Treatment of menstrual disorders[40]
C. tora	Leaf	Treatment of asthma[53, 57]
		Treatment of skin diseases[85, 109]
		Treatment of throat infection[52]
		Treatment of ringworm[57]
		Treatment of leprosy[109]
		Treatment of cough[110]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. tora	Leaf	Treatment of cardiac disorder[110]
		Treatment of liver disorder[1]
		Laxative[1, 109]
		Antiperiodic[1]
		Antihelmintic[1]
		Antibacterial[1]
		Analgesic[1]
		Antifungal[1]
		Antidiabetic[1]
	Seed	Treatment of asthma[53]
		Treatment of skin diseases[61, 85]
		Treatment of throat infection[52]
		Treatment of stroke[55, 111]
		Treatment of leprosy[61, 85]
		Laxative[52]
		Anti-inflammatory[52]
	Stem	Antibacterial[1]
		Analgesic[1]
		Antifungal[1]
		Antidiabetic[1]
	Bark	Antibacterial[1]

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Table 3 Ethnomedical uses of *Cassia* plants (Cont.)

Species	Part uses	Ethnomedical uses
C. tora	Bark	Analgesic[1]
		Antifungal[1]
		Antidiabetic[1]
	Root	Antibacterial[1]
		Analgesic[1]
		Antifungal[1]
		Antidiabetic[1]

2.3 pharmacological activities of *Cassia* plants

Various medicinal plants have been used for years in daily life to treat diseases all over the world. *Cassia* plants possess valuable traditional and medicinal properties. Different parts of the plant are reported for their medicinal value. The pharmacological activities of *Cassia* species are included antifungal, antibacterial, antiviral, antimalarial, anti-inflammatory, antiemetic, antidiabetic, antipyretic, antioxidant, analgesic, hepatoprotective and laxative[1, 87, 112, 113]. The pharmacological activities of *Cassia* plants are summarized in Table 4.

Table 4 The pharmacological activities of *Cassia* plants

Species	Part uses	Pharmacological activities
Cassia bakeriana	Leaf	Antimicrobial[114, 115]
	Bark	Antimicrobial[114, 115]
	Wood	Antimicrobial[115]
C. fistula	Leaf	Wound healing[116]
		Hypocholesterolemic[56]

Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. fistula	Leaf	Hepatoprotective[117]
		Antitumor[56]
		Antioxidant[56, 118]
		Antidiabetic[56]
		Antifungal[119]
		Antitussive[120]
		Antipyretic[120]
		Anti-ulcer[121]
	Flower	Antioxidant[56, 122]
		Antifungal[56, 122]
		Antibacterial[56, 122]
	Pod	Laxative[123]
		Antiparasitic[124]
	Seed	Sedative[125]
		Antitumor[126]
		Antifertility[127]
		Anti-leishmaniatic[128]
	Bark	Antitumor[113]
		Anti-inflammatory[129]
		Antioxidant[129]
		Hepatoprotective[113]
	Stem bark	Antibacterial[130]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. grandis	Leaf	Antifungal[131]
		Anti-inflammatory[132]
		Antinociceptive[132]
		Antioxidant[133]
		Analgesic[132]
	Pod	Antiemetic[131]
	Stem	Antidiabetic[134]
	Bark	Antifungal[131]
C. javanica	Leaf	Hypoglycemic agent[135]
		Inhibits *Herpes simplex* Virus Type 2[136]
		Antimicrobial[137]
		Antibacterial[138]
		Antioxidant[139]
		Anti-inflammatory[140]
		Anti-ulcer[121]
	Flower	Anticancer[141]
		Antimycotic[141]
		Antimicrobial[142]
		Antioxidant[140]
		Anti-inflammatory[140]
	Bark	Antipyretic[138]
		Antioxidant[140]
		Anti-inflammatory[140]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. javanica	Seed	Antipyretic[138]
		Antioxidant[140]
		Anti-inflammatory[140]
C. alata	Leaf	Wound healing[143]
		Hepatoprotective[144, 145]
		Muscle relaxant[146]
		Laxative[147]
		Analgesic[148]
		Antibacterial[149-151]
		Antifungal[148, 151-153]
		Antioxidant[154]
		Anti-inflammatory[154]
		Antimalarial[155]
		Antidiabetic[156]
		Anti-cryptococcus[157]
		Antiallergic[158]
		Antigenotoxic[146]
		Treatment of skin diseases[159]
	Flower	Laxative[160]
		Antifungal[161]
		Anthelmintic[160]
	Root	Antibacterial[150]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. alata	Root	Antifungal[162]
		Antioxidant[163]
	Seed	Antifungal[149]
		Antibacterial[164]
C. angustifolia	Leaf	Antibacterial[165]
		Antidiabetic[166]
		Anti-emetic[112]
		Antiviral[167]
		Antitumor[167]
		Antioxidant[167]
		Anti-inflammatory[167]
		Treatment of osteoarthritis[167]
		Laxative[168]
		Stop bleeding[168]
		Hepatoprotective[169]
		Hypolipidemic agents[170]
	Seed	Anticancer[171]
		Antioxidant[171]
		Antimicrobial[171]
		Antidiabetic[172]
	Pod	Laxative[173]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. garrettiana	Heartwood	Anti-cancer[174]
		Antitumor[175]
		Antioxidant[176]
		Antifungal[177]
		Antimetastatic[175]
		Anti-HIV-1 integrase[178]
		Anti-HIV-1 protease[179]
C. hirsuta	Leaf	Antimicrobial[180, 181]
		Antimalarial[182]
		Antioxidant[183]
	Seed	Antioxidant[184]
C. occidentalis	Leaf	Antimicrobial[185]
		Antioxidant[186]
		Anti-inflammatory[187]
		Antianxiety[188]
		Antidepressant[188]
		Antipyretic[189]
		Analgesic[189]
		Antidiabetic[190, 191]
		Wound healing[192]
		hepatoprotective[186]
		Muscle relaxant[190]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. occidentalis	Flower	Antimicrobial[193]
	Pod	Antimicrobial[193]
	Seed	Antimicrobial[193]
		Antioxidant[194]
		Hepatoprotective[195]
	Bark	Antimicrobial[193]
	Root bark	Antimalarial[196, 197]
	Whole plant	Antidiabetic[198]
		Antioxidant[194]
		Antimicrobial[199]
		Anticancer[199]
		Anti-allergic[200]
		Anti-inflammatory[201]
		Immunosuppression[202]
C. spectabilis	Leaf	Antibiofilm[203]
		Antibacterial[204]
		Antioxidant[205]
		Anticonvulsant[206]
		Sedative[206]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
	Flower	Antifungal[204]
		Antibacterial[204]
		Antioxidant[205]
	Pod	Antifungal[204]
		Antibacterial[204]
		Antioxidant[205]
	Stem	Antifungal[204]
		Antibacterial[204]
		Antioxidant[205]
	Whole plant	Anti-nociceptive[207]
		Anti-inflammatory[207]
C. siamea	Leaf	Laxative[208]
		Anxiolytic[209-211]
		sedative[210, 211]
		Analgesic[212]
		Antipyretic[212]
		Anti-inflammatory[212]
		Antimalarial[213]
		Anti-lipemic[214]
		Antidiabetic[215]
		Antioxidant[216]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
	Leaf	Antitumor[217]
		Antihypertensive[218]
		Antidepressant[210, 211]
		Antibacterial[219]
		Antifungal[219]
	Flower	Anxiolytic[209-211]
		sedative[210, 211]
		Laxative[208]
		Antidepressant[210, 211]
		Antimalarial[220]
		Antioxidant[221]
	Stem bark	Analgesic[212]
		Antipyretic[212]
		Anti-inflammatory[212]
		Antimalarial[222]
		Antitumor[217]
		Antifugal[223]
	Root	Antidiabetic[215]
		Anti-lipemic[214]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. sophera	Leaf	Hepatoprotective[224]
		Analgesic[225]
		Antioxidant[226]
		Anti-inflammatory[227]
		Antiasthmatic[228]
		Antidiabetic[229]
		Antibacterial[230]
	Seed	Antibacterial[231]
C. surrattensis	Leaf	Antimicrobial[232]
		Antidiabetic[233]
		Antihemorrhagic[234]
		Antihyperlipidemic[235]
	Flower	Antimicrobial[105, 232]
		Antioxidant[236]
	Stem	Antimicrobial[232]
	Bark	Antihemorrhagic[234]
	Root	Antimicrobial[232]
C. sulfurea	Leaf	Antidiabetic[237]
		Antimicrobial[238]
		Antioxidant[239]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. sulfurea	Leaf	Cardio-protective[239]
		Nephro-protective[239]
	Seed	Antimicrobial[240]
		Antioxidant[240]
	Bark	Antidiabetic[241]
	Stem	Antimicrobial[238]
C. timoriensis	Heartwood	Antitumor[106]
		Antioxidant[106]
		Inhibition of Heinz body induction[106]
C. tora	Leaf	Antioxidant[242-244]
		Antifungal[245]
		Antibacterial[246]
		Anti-inflammatory[247]
		Antinociceptive[248]
		Antiproliferative[249]
		Antiulcer[250]
		Hepatoprotective[251]
		Laxative[252]

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Table 4 The pharmacological activities of *Cassia* plants (Cont.)

Species	Part uses	Pharmacological activities
C. tora	Seed	Antioxidant[242, 243]
		Antidiabetic[253]
		Antigenotoxic[254]
		Antiulcer[255]
		Antifungal[256]
		Anthelmintic[257]
		Antimutagenic[258]
		Antibacterial[259]
		Antiplasmodial[260]
		Hepatoprotective[261]
		Hypolipidemic[262]
		Immunostimulatory activity[263]
		Hypotensive[264]
	Root	Antishigellosis[265]
	Whole plant	Hepatoprotective[266]

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2.4 Chemical constituents of *Cassia* plants

The nature has provided abundant plant wealth, which possess medicinal virtues for all living creatures. Phytochemicals, naturally occurring in the medicinal plants, play a role in defense mechanisms and protection from various diseases. Primary phytochemical compounds are proteins, chlorophyll, and common sugars and secondary phytochemical compounds are terpenoids, alkaloids, phenolics and anthraquinones[176]. Previous phytochemical investigations on various *Cassia* species revealed the presence of anthraquinones, anthrones, flavonoids, triterpenes alkaloids, chromones hydroanthracenes and naphthalenic compounds[267-269]. Anthraquinone, also called 9,10-anthracenedione, 9,10-anthraquinone and 9,10-dioxoanthracene are group of functionally diverse aromatic chemicals[270]. It has the appearance of yellow or light gray to gray-green crystal powder[1]. Plant extracts containing anthraquinone compounds are being increasingly used for cosmetics, food, dyes and pharmaceuticals due to their therapeutic and pharmacological properties[271]. In addition, anthraquinones and its derivatives are frequently found in slimming agents and have been valued for their cathartic and presumed detoxifying action. However, anthraquinone may cause nausea, vomiting, abdominal cramps and diarrhea with both therapeutic dose and over dose[272]. The plant families which are rich of anthraquinone compounds are Rubiaceae, Rhamnaceae, Polygonaceae, Caesalpiniaceae (especially in *Cassia*), Verbenaceae and Liliaceae[273].

Many types of anthraquinone compound such as sennosides, chrysophanic acid, emodin, physion, rhein and aloe-emodin are present in several *Cassia* and their chemical compounds and structures are summarized in Table 5 and Figures 2, respectively.

Table 5 Anthraquinone compound from *Cassia* species

Species	Plant parts	Anthraquinone compounds
C. fistula	Leaf	Rhein, rhein glucoside, sennoside A&B, chrysophanol, physcion[60, 274-276]
	Fruit pulp	Rhein, rhein glucoside, sennoside A&B, fistulic acid[60, 277]
	Pod	Fistulic acid, 3-formyl-1-hydroxy-8-methoxyanthraquinone, rhein, sennidin, emodin, sennosides, aloe-emodin[277]
	Flower	Rhein, rhein glucoside, fistulin, fistulin rhamnoside[60]
	Stem bark	Rhein glucoside, 1,8-dihydroxy-6-methoxy-3-methyl anthraquinone[278]
	Heartwood	Rhein, chrysophanol[279]
	Seed	Chrysophanol, chrysophanein[280]
	Root and root bark	Rhamnetin-3-O-gentiobioside, emodin, chrysophanic acid fistuacacidin, barbaloin, rhein[281]

Table 5 Anthraquinone compound from *Cassia* species (Cont.)

Species	Plant parts	Anthraquinone compounds
C. grandis	Leaf	Aloe-emodin[68]
	Pod	1,3,4-trihydroxy-6,7,8-trimethoxy-2-methy anthraquinone-3-O-β-D-glucopyranoside[282]
	Stem	Emodin-9-anthrone[70]
	Seed	Chrysophanol, 1,2,4,8-tetrahydroxy-6-methoxy-3-methylanthraquinone-2-O-β-D-glucopyranoside, 3-hydroxy-6,8-dimethoxy-2-methylanthraquinone-2- O- β-D-glucopyranoside[68, 279]
C. javanica	Leaf	Emodin, rhein, chrysophanic acid, aloe-emodin, chrysophanol, physcion[283, 284]
	Root	Emodin-8-rhamnoside; 5-hydroxyemodin-8-rhamnoside, 1,3-dihydroxy-5,6,7-trimethoxy-2-methyl anthraquinone, 1,4-dihydroxy-8-methoxy-2-methylanthraquinone-3-O-β-D glucopyranoside, 1,8-dihydroxy-6,7-dihydroxy-2-methyl anthraquinone[285, 286]

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Table 5 Anthraquinone compound from *Cassia* species (Cont.)

Species	Plant parts	Anthraquinone compounds
C. javanica	Seed	Chrysophanol, physcion, 1,5-dihydroxy-4,7-dimethoxy-2- methylanthraquinone-rhamnopyranoside, 1,3,6,7,8-pentahydroxy-4-methoxy-2- methylanthraquinone[279, 287, 288]
	Stem bark	1,2-Dihydro-1,3-dihydroxyl, 6,8-dimethoxy-2-methylanthraquinone, 1,3,5,8-tetrahydroxy-6-methoxy-2- methylanthraquinone, 1,3,4,6-tetrahydroxy-5,8-dimethoxy-2-methylanthraquinone, 1,4- dimethoxy-6,7,8-trimethoxy-2-methylanthraquinone, 1-hydroxy-3,6,7,8-tetramethoxy-2-methylanthraquinone, 4,4'-bis(1,5-dihydroxy-7-hydroxymethyl-2-methyl-3-methoxy) anthraquinone[289-291]
C. angustifolia	Leaf	Aloe-emodin, aloe-emodin dianthraone, chrysophanol, emodin 8-O-sopharoside, Sennoside A, B, C, D, rhein, rheum-emodin glycoside[292-295]

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Table 5 Anthraquinone compound from *Cassia* species (Cont.)

Species	Plant parts	Anthraquinone compounds
C. angustifolia	Pod	Aloe-emodin, chrysophanol, rhein, Sennoside A, B[167]
	Root, seedling	Chrysophanol, physcion, emodin, aloe-emodin, rhein, Sennoside A, B, C, gluco- aloe-emodin, gluco-rhein[279]
C. alata	Leaf	Aloe-emodin, chrysophanol, chrysophanic acid, isochrysophanol, emodol, rhein Physcion glucoside, 4,5-dihydroxy-1-hydroxy-methylanthrone, 4,5-dihydroxy-2-hydroxy-methylanthraquinone[79, 82, 296]
	Pod	Aloe-emodin, rhein, emodin[279]
	Seed	Chysophanol, 2-hydroxy methylanthraquinone[279]
	Root	Aloe-emodin, chysophanol , emodin, physcion, 1,5-Dyhydroxy-8-methoxy-2-methyl-antraquinone-3-O-β-D-glucopyranoxide 1,3,8-Dyhydroxy-2 methylantraquinone[279, 297]

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Table 5 Anthraquinone compound from *Cassia* species (Cont.)

Species	Plant parts	Anthraquinone compounds
C. alata	Stem	1,5,7-trimethoxy-3- methylantraquinone, 2-formyl-1,3,8-trimethoxy-3-antraquinone[298-300]
C. garrettiana	Leaf	Aloe-emodin[21]
	Heartwood	Cassiaon, chrysophanol, chrysophanol dianthone, chrysophanol benzanthrone[89, 301]
C. hirsuta	Seed	4,4'-bis(1,3,8-trihydroxy-2-methyl anthraquinone)[302]
C. occidentalis	Leaf	Chrysophanol, emodin, physcion, bianthraquinones[279]
	Flower	Emodin, physcion[115, 140]
	Seed	Chrysophanol, emodin, physcion, rhein[303, 304]
	Root	Chrysophanol, emodin, physcion, emodol, rhein, aloe-emodin, Islandicin[305, 306]

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Table 5 Anthraquinone compound from *Cassia* species (Cont.)

Species	Plant parts	Anthraquinone compounds
C. spectabilis	Leaf	Chrysophanol, physcion, 1,3,8-trihydroxy-2-methylantraquinone[279]
	Flower	Chrysophanol, 1,8-dihydroxy-6-methoxy-3-methylantraquinone[307]
C. siamea	Leaf	Chrysophanol, physcion, rhein, sennoside, cassiamin A, barakol[103, 279]
	Heartwood	Cassiamin A, Chrysophanol, emodin[279, 308]
	Stem bark	Cassiamin A, B, C, Chrysophanol, physcion, siameanin, siameadin, rhein[309]
	Root bark	Chrysophanol, cassiamin A, B emodin[310-312]
	Root	1-hydroxy-6,8-dimethoxy-2-methylantraquinone-3-O-rutinoside, 1,5,8-trimethoxy-2-methylantraquinone-3-O-β-D glucopyranoside[300]
C. sophera	Leaf	Sennoside[279]
	Flower	Chrysophanol[279]

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Table 5 Anthraquinone compound from *Cassia* species (Cont.)

Species	Plant parts	Anthraquinone compounds
C. sophera	Heartwood	1,2,7-Trihydroxy-6,8-dimethoxy-3-methylantraquinone, 1,2,6-trihydroxy-7,8-dimethoxy-3- methylantraquinone, chrysophanol, physcion, emodin, sopheranin[313]
	Root bark	1,8-Dihydroxy-2- methylantraquinone, 3-neohesperidoside, chrysophanol, physcion, 1,8-dihydroxy-3,6-dimethoxy-2-methyl-7-vinylantraquinone, 1,3-dihydroxy-5,7,8-trimethoxy-2- methylantraquinone[314, 315]
C. timoriensis	Leaf	Aloe-emodin, barakol[68]
C. tora	Leaf	Aloe-emodin, emodin, 1,8-dihydroxy-3-hydroxymethylanthraquinone[279, 316]

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Table 5 Anthraquinone compound from *Cassia* species (Cont.)

Species	Plant parts	Anthraquinone compounds
C. tora	Seed	Chrysoobtusin, arurantio-obtustin, obtustin, chryso-obtustin-2-O-β-D-glucoside, physcion, emodin, chrysophanol, obtusifolin, obtusifolin-2-O-β-D-glucoside, rhein, 1-methylaurantio-obtusin, 1-methylchryso-obtusin, aloe-emodin, chrysophanic acid, alaternin[84, 110, 279, 317-319]
	Root	1,3,5-Trihydroxy-6,7-dimethoxy-2-methylanthraquinone[279]
	Stem	Rhein, 1-hydroxy-5-methoxy-2-methylanthraquinone, 5-methoxy-2-methylanthraquinone-1-O-α-L-rhamnoside, chrysophanol, emodin[320]

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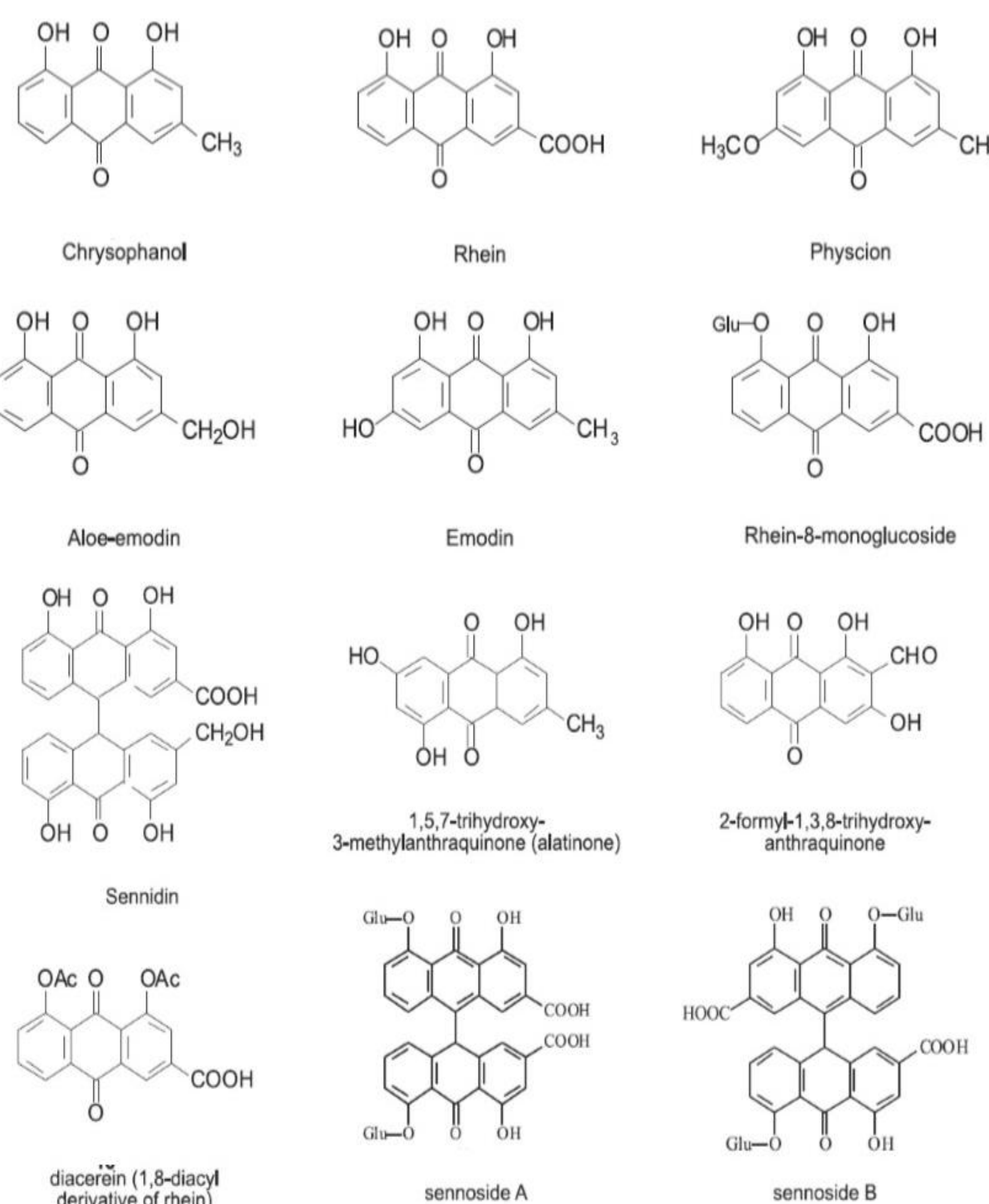

Figure 2 Chemical structures of some anthraquinones present in *Cassia* species[1]

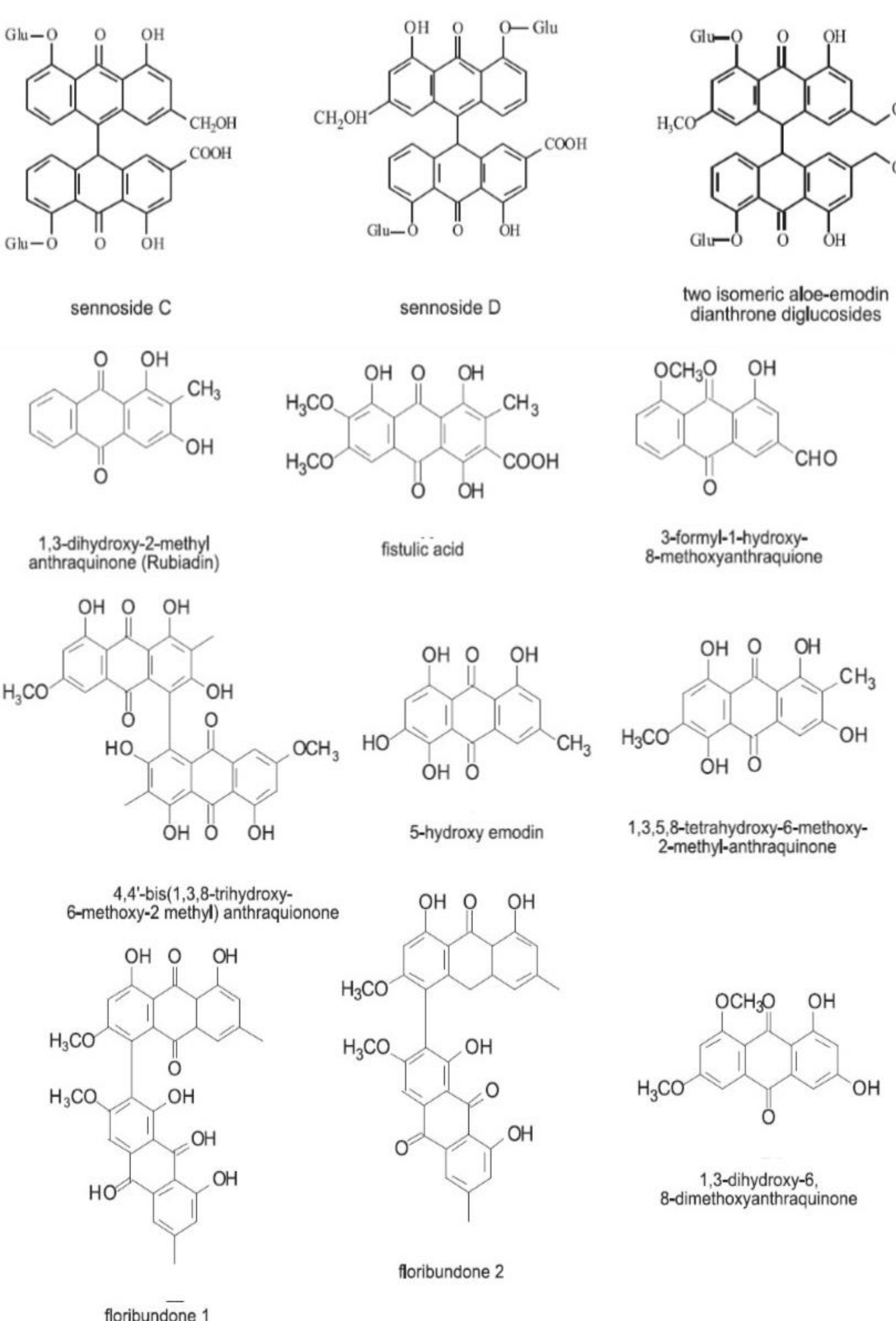

Figure 2 Chemical structures of some anthraquinones present in *Cassia* species[1] (Cont.)

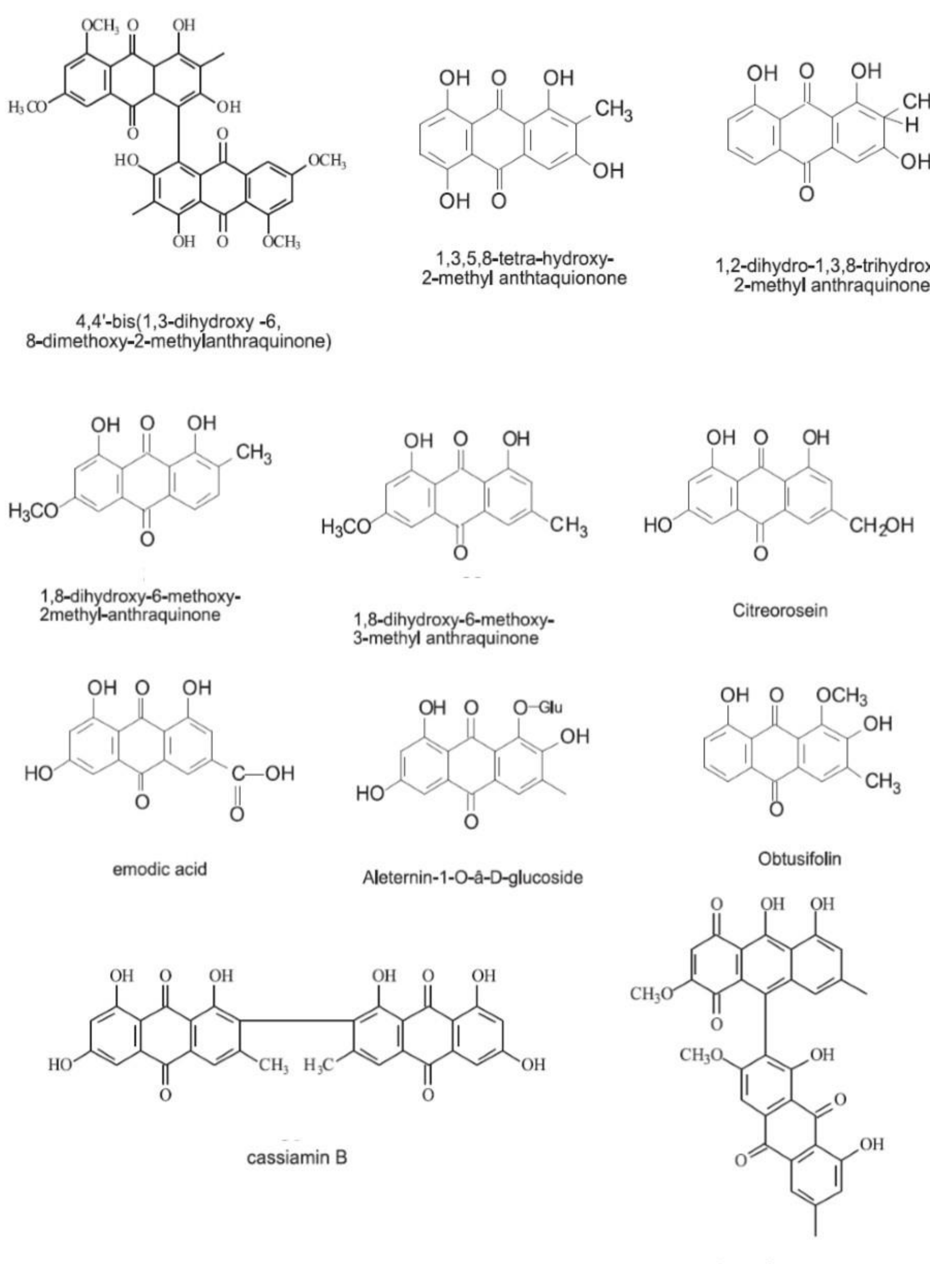

Figure 2 Chemical structures of some anthraquinones present in *Cassia* species[1] (Cont.)

2.4.1 Aloe-emodin

Aloe-emodin belongs to the anthraquinones, a group of more than 170 natural compounds that make up the largest group of natural quinones[321-323]. More than half of the natural anthraquinones are found in lower fungi, particularly in *Penicillium* and *Aspergillus* species, and lichens. Others anthraquinones are found in higher plants, and insects[321, 322, 324]. The family Rubiaceae, Rhamnaceae, Caesalpiniaceae, Polygonaceae, Bignoniaceae, Verbenaceae, Scrophulariaceae and Liliaceae are particularly rich in anthraquinones[325]. The most common naturally occurring anthraquinone aglycones in higher plants are emodin, rhein, chrysophanol, physcion and aloe-emodin[323, 324]. Aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone; 1,8-dihydroxy-3-(hydroxymethyl)-9,10 anthracenedione; 3-hydroxyme-thylchrysazin; rhabarberone)[270] (Figure 3) is mainly reported in three plant families: Caesalpiniaceae (*Cassia* spp.), Polygonaceae (*Rheum*, *Rumex* and *Polygonum* spp.) and Rhamnaceae (*Rhamnus* and *Ventilago* spp.)[326-328]. It is an orange-yellow crystalline cathartic compound of $C_{15}H_{10}O_5$[270]. Aloe-emodin has antiviral, antimicrobial, hepatoprotective activities[329], anticancer activity in neuroectodermal tumors[330], lung squamous cell carcinoma[331], hepatoma cells[332], a glia cell line[333] and a human glioma cell line[334]. It has been reported that aloe-emodin suppressed N-methyl-D-aspartate (NMDA)-induced apoptosis of retinal ganglion cells through regulation of extracellular signal-regulated kinase (ERK) phosphorylation and aloe-emodin-induced apoptosis in rat hepatic stellate cells transformed by simian virus 40 (t-HSC/Cl-6) involved a mitochondria-mediated pathway[335, 336]. Moreover, it has been reported that aloe-emodin-induced apoptotic cell death via oxidative stress and sustained Jun N-terminal kinase (JNK) activation and aloe-emodin-induced apoptosis in human gastric carcinoma

cells by a reduced phosphorylation of BH3 interacting domain death agonist (Bid), a downstream substrate of casein kinase II and a pro-apoptotic molecule[337, 338].

Figure 3 Structure of aloe-emodin

2.4.2 Phytochemical screening of anthraquinones

Phytochemical screening is the process of separation and isolation of active compounds from plant sources. These techniques are helpful for discovering the lead compounds of the therapeutic agents.

Borntrager's test is a chemical test for the identification of anthraquinone aglycones in the extract. Dilute hydrochloric acid (2M) was added to the sample and the mixture was heated on a hot water bath for 15 minutes, then cooled and filtered. The filtrate was then extracted with dichloromethane. The dichloromethane layer was separated and shaken with ammonium hydroxide. Pink to red color was developed in alkali layer[339]. In some case, the anthraquinones may negative for borntrager's test due to its reduced form thus, modified borntrager's test

were used[340]. Modified borntrager's test are employed using ferric chloride with dilute hydrochloric acid to bring about oxidation hydrolysis. Heated on a hot water bath for 5 minutes, then cooled and filtered. The filtrate was extracted with dichloromethane or any organic solvent. Shake and separate organic layer then ammonium hydroxide was added. Pink to red color was developed in alkali layer[341, 342].

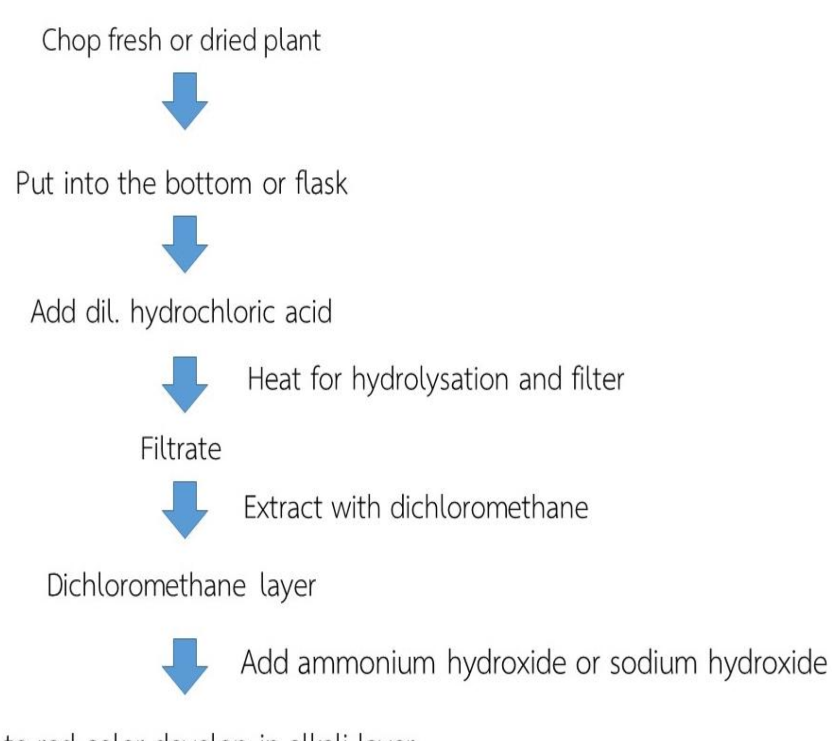

Figure 4 Borntrager's test for anthraquinone glycoside

2.5 Plant identification

The first step to categorize the herbal plant materials is the determination according to their macroscopic and microscopic characteristics for establishing the identity and the degree of purity of herbal plant materials. Visual by eye based on the appearance of morphological characteristic provides the simplest and quickest inspection. However, macroscopic examination is sometime inadequate. It is often necessary to combine with other methods such as microscopic, chemical constituents or molecular analysis.

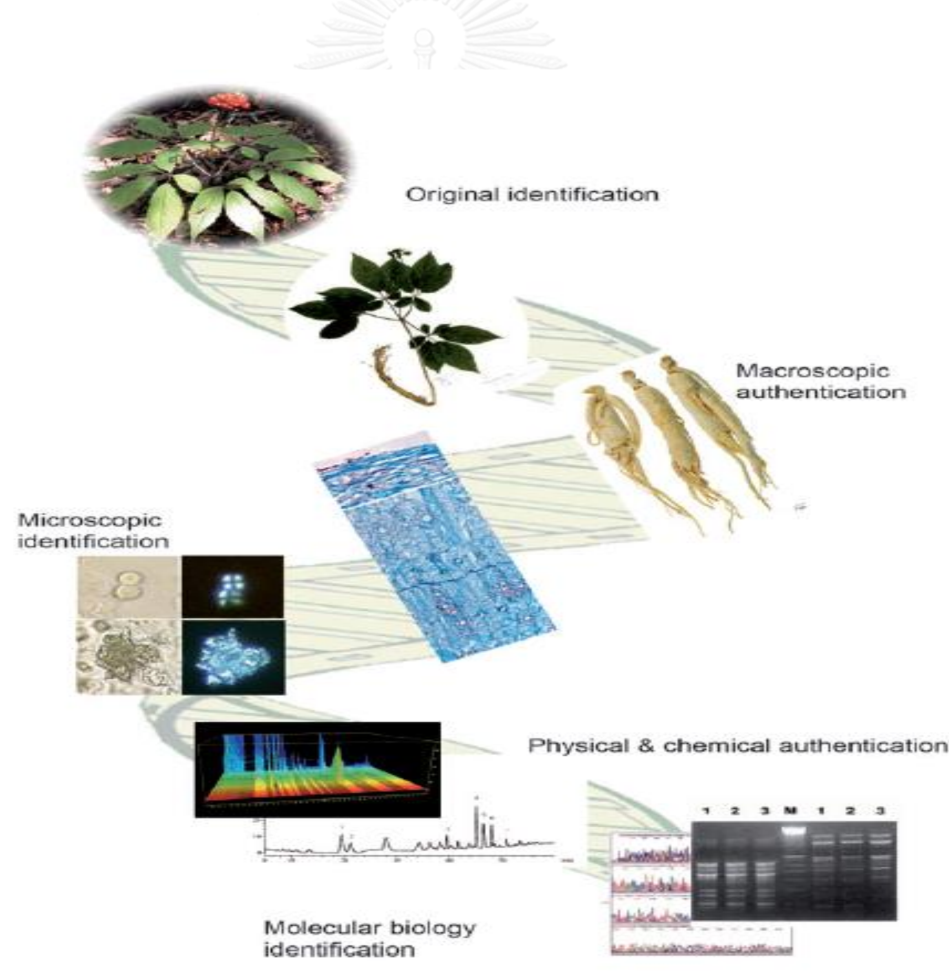

Figure 5 The herbal medicines authentication methods[343]

2.5.1 Morphological characteristics

Medicinal plant materials are categorized according to sensory, macroscopic and microscopic characteristics. The characteristics examination is the first step towards establishing the identity and the degree of purity of material and should be carried out before any further tests are undertaken.

2.5.1.1 Macroscopic examination

Macroscopic examination are based on their morphological features such as shape, size, color, texture and other characteristics, which always used to distinguish various species or evaluate their quality. There are conducted by observing, touching, smelling, tasting and testing by other ways.

Macroscopic examination of medicinal plant have been previously reported in *Malva parviflora* (family Malvaceae), *Combretum albidum* (family Combretaceae) and *Limonia acidissim* (family Rutaceae)[344-346].

2.5.1.2 Microscopic examination

Microscopic examination is a method using a microscope to identify the structural features, cells, and ergastic substances of herbal samples with application of the knowledge of plant morphology and anatomy so as to authenticate plant species. Microscopic characteristic such as the arrangement of tissue in transverse and longitudinal sections, types of cell, stoma, trichomes vessels etc. are important anatomical characteristics of medicinal plants[347]. The procedure of identification contains three main steps: firstly, selection of typical materials, secondly, preparation of sample on the slide and lastly, observation of features. The drawings will be made using microscope and drawing attachment. Microscopic examination is a conventional method for identification of plant structural feature under microscope due to their

simple, rapid and inexpensive procedures. Photomicroscope is usually used for anatomical and histological characters determination.

Microscopic examination of medicinal plant have been previously reported in *Gaultheria trichophylla* (family Ericaceae), *Adenanthera pavonina* (family Mimosaceae) and *Adhatoda vasica* (family Acantheceae) [348-350].

2.5.1.2.1 Photomicroscope

The microscope evaluation was commonly conducted using a digital camera attached with the microscope. The photograph is recorded with an attached digital camera and examined under the photomicroscope using appropriated objective lens (10X, 20X and 40X magnifications) and eyepiece lens of 10X magnification. The images were recorded using AxioVision Release 4.8.2 program. The photomicrography is uniquely qualified to be used for routine and advanced microscopic investigation of medicinal plant materials.

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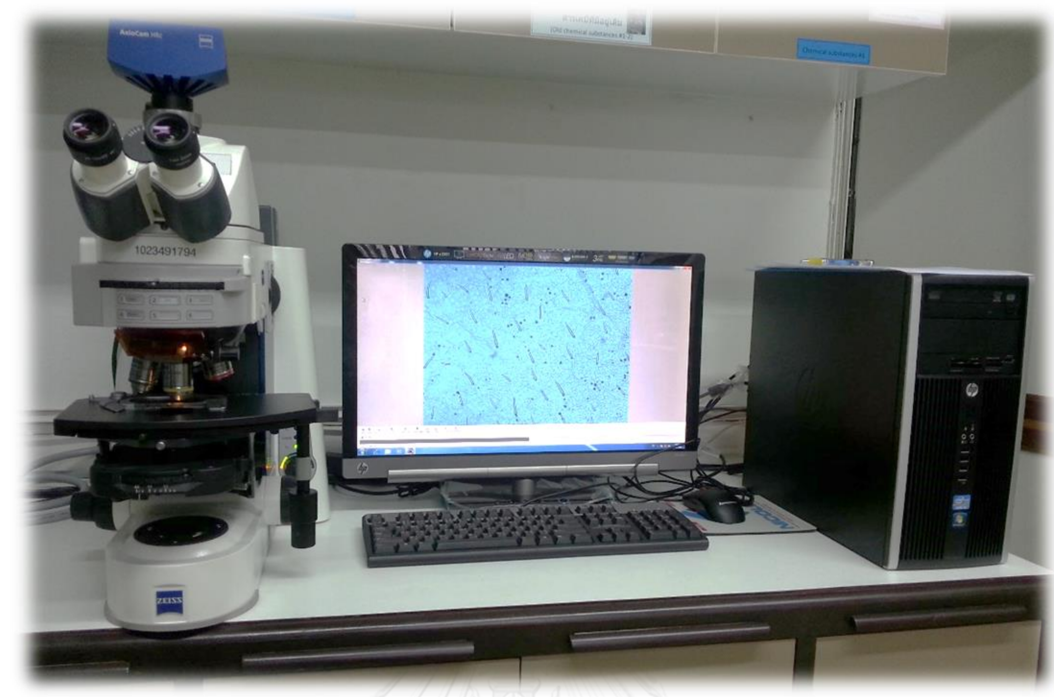

Figure 6 The photomicroscope (Zeiss Imager A.2 Axio, Germany) attached with digital camera (Cannon Power shot A640, Japan)

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2.5.1.2.2 Clearing reagents for microscopic examination

The presence of various content within the cell such as starch gain, plastid and oil etc., may result to non-translucent section and obscure certain characteristics. There are some reagents that can dissolve of these contents and have been used to make an infiltrating effect. Some of the most frequently used reagents are sodium hypochlorite and chloral hydrate as described below[351].

Sodium hypochlorite solution

Sodium hypochlorite is used for bleaching deeply colored sections for removing chlorophyll from the leaves[340]. The sections were immersed in sodium hypochlorite solution for a few minutes until sufficiently bleached, then washed with water and mounted with glycerol on the glass slide.

Chloral hydrate solution

Chloral hydrate is used as an aqueous solution, often added to glycerol to prevent crystallization of the reagent when used as a temporary mounting reagent for examination a variety of plant structures[352]. Chloral hydrate solution with gentle heating dissolves starch grains, plastids and volatile oils and expands collapsed and delicate tissue without causing any undue swelling of cell walls or distortion of the tissues.

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2.5.1.3 Leaf measurement

Leaf constant numbers are used to identify between some closely related species. They have great value for a quality of the medicinal plants based on their specific characters. Leaf constant numbers can be measured by the stomatal number, stomatal index, cicatrix number, cicatrix index, trichome number, trichome index, vein-islet number, vein termination number and palisade ratio[347].

2.5.1.3.1 Stomata classification

Stomata are frequently present in the lower epidermis of the leaf. The structure and shape of the epidermis and stomata are the first investigation in the microscopic examination of leaf[340]. In the mature leaves, four significantly different types of stomata are distinguished by their forms and the arrangement of the surrounding cells, especially the subsidiary cells. Four types of stomata are demonstrated in Table 6.

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Table 6 Stomata classification

Types of stomata	The arrangement of the surrounding cells	Surface view of epidermis
Anomocytic or ranunculaceous (irregular-celled) type	The stoma is surrounded by a varying number of cells, generally not different from those of the epidermis.	
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.	
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.	

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Table 6 Stomata classification (Cont.)

Types of stomata	The arrangement of the surrounding cells	Surface view of epidermis
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.	

2.5.1.3.2 Determination of stomatal number and stomatal index

Stomatal number is the average number of stomata cells per a square millimeter (mm^2) calculated from thirty determinations. The stomatal index is a percentage of the proportion between stomatal number and epidermal cells in one square millimeter. Stomatal index can be calculated as;

$$\text{Stomatal index} = \frac{S}{E+S} \times 100$$

Where; S = number of stomata per unit area

E = number of epidermal cells per unit area

Stomata index is not affected by various factors such as size of the leaf, environmental conditions etc. It is relatively constant and consequent parameter.

2.5.1.3.3 Determination of palisade ratio

Palisade cells are a type of photosynthetic cells in the mesophyll of leaf occurring mostly just beneath the upper epidermal surface layer. The cell are elongated and more cylindrical and arranged in one or more rather regular, relatively compact layer near the ventral, or upper side of the leaf with the long axis of the cells perpendicular to the leaf surface[353]. Palisade ratio is the average number of palisade cells beneath one epidermal cell of a leaf by counting the palisade cell beneath four continuous epidermal cells. Then divided by four gives the palisade ratio of that group.

2.5.1.3.4 Determination of vein-islet number

A vein-islet is the small area of green tissue surrounded by the veinlets. The vein-islet number is the average number of vein-islet per square millimeter of a leaf surface[339].

2.5.1.3.5 Determination of vein termination number

A vein termination is the ultimate free termination of a veinlet or branch of a veinlet. Vein termination number is defined as the number of veinlet termination per square millimeter of a leaf surface[339].

2.5.1.3.6 Determination of trichome number

Trichomes are also called plant hairs, known to present on the surfaces of leaves, stems, and fruits. Trichome number has been used for identification of some plants (Solanaceae, Lamiaceae) that have trichomes covering their leaves[354]. Previous reports have shown that there are glandular and non-glandular trichomes that function in plants to reduce heat load, increase tolerance to freezing, enhance water absorption, protect plant structures from the harmful effects of UV, serve as taxonomical criteria, serve as insect repellent, and offer a means of protection

against herbivores and pathogens[355-359]. The greatest significance of trichomes is applied in the identification of angiospermic plants[354]. They are constant in a species when present or show a constant range of form. Trichome number is the average number of trichome per mm^2 by counting the trichome number in the define area of the epidermis.

2.5.1.3.7 Determination of trichome index

The trichome index is a percentage of the proportion between trichome number and epidermal cells in one square millimeter. Trichome index can be calculated as;

$$\text{Trichome index} = \frac{T}{E+T+S}$$

Where; T = number of trichome per unit area

E = number of epidermal cells per unit area

S = number of stomata per unit area

Leaf constant numbers were previous used for identification of *Dodonaea* species, *Morinda* species and *Senna* species from other closely related species[360-362]. Microscopic examination alone cannot provide complete evaluation profile of a medicinal plant but it can provide supporting evidence[363].

2.5.2 Molecular identification

The molecular method or DNA-based techniques have been wildly used for herbal medicine technology and authentication of medicinal plant species. These methods are useful in case of medicinal plants which are frequently substituted or adulterated with other species or their morphological or phytochemical

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indistinguishable because of their variable sources and chemical complexity. These techniques have been found to be useful and accurate for determination of genetic variation in plants. DNA methods are suitable for identifying medicinal plant materials because genetic composition is unique for each individual irrespective of the physical forms of samples and are less affected by age, physiological conditions, environmental factors, harvest, storage and processing[13].

A number of recent studies have indicated that DNA markers are ideal tool in the characterization and evaluation of genetic diversity within and between species and population. Many previous studies have been identified the herbal medicines by DNA fingerprinting such as authentication of each six *Panax* species and differentiation from one another and from some of their adulterants by restriction fragment length polymorphism (RFLP) technique[364]. Molecular techniques have been used to discriminate and construct the genetic relationship between twelve *Phyllanthus* species existing in Thailand by random amplified polymorphic DNA (RAPD) marker and genetic relationship and species authentication of *Boesenbergia* (Zingiberaceae) in Thailand based on amplified fragments length polymorphisms (AFLP) and single-strand conformation polymorphism (SSCP) analyses[365, 366].

2.5.2.1 DNA extraction

There are many alternative protocols for DNA extraction and the choice of protocol depends on the quality and quantity of DNA.

2.5.2.1.1 CTAB method

DNA isolation by CTAB method is one of the most popular protocols. Many different methods and technologies are available for the isolation of genomic DNA[367]. In general, all methods involve disruption and lysis of the start material followed by the removal of proteins and other contaminants and finally

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recovery of DNA. Fresh young leaves are frozen rapidly in liquid nitrogen and grounded into powder then lysed with the ionic detergent CTAB (cetyltrimetylammonium bromide), which form an insoluble complex with nucleic acid in a low-salt environment. Under these conditions, polysaccharide, phenolic compound and other contaminants remain in the supernatant and can be washing away. Removal of proteins is typically achieved by organic solvent extraction. The DNA complex is solubilized by raising the salt concentration and precipitated with ethanol or isopropanol.

2.5.2.1.2 DNA extraction kit

Besides DNA isolation by CTAB method, the commercial instant DNA extraction kit is considered to be a widely isolation method. The technology makes use of spin columns, which contain a silica-gel-based membrane that binds the DNA. The DNA while bound to the membrane can be washed and cleaned from contaminants and then eluted from the column (membrane) using water. This method is relatively simple, save time, do not contain harmful chemicals such as phenol or chloroform, involves minimal handling, higher percent yields and the high quality of DNA but this method is expensive.

DNA quantity was also checked spectrophotometrically from the absorbance data of the sample DNA at 260 nm and 280 nm. The purity of DNA sample will be calculated from OD260/OD280 and it ratio ranged from 1.8-2.0.

The obtained genomic DNA is then used as a DNA template for amplification. There are several regions in the DNA from various origins that used for studying the divergence or identity of plants, such as nuclear genome, chloroplast genome and mitochondrial genome[368].

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Molecular authentication has more advantages over typical phenotype markers and reliable for informative polymorphisms as well as environmental factors. Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphism. These are hybridization-based method, polymerase chain reaction (PCR)-based methods and sequencing-based methods.

2.5.2.2 Polymerase chain reaction (PCR)

PCR-based methods are generated by Mullis and coworkers[369]. PCR technique is the amplification of the interested region in the genome *in vitro* by using thermostable DNA polymerase and either random or specific primers. It is become the most popular technique in many researches. The PCR principle is the amplification of a piece of DNA and generating thousands to millions copies of particular DNA sequences[370]. A basic PCR set up requires several components and reagents such as, DNA template that contains the DNA region to be amplified, two primer that are complementary to the 3' ends of the sense and anti-sense strand of the DNA target, deoxynucleoside triphophates (dNTPs; nucleotides containing triphophates groups) which acts like the building-blocks from which the DNA polymerase synthesizes a new DNA strand, buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase, divalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mg^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mg^{2+} concentration increases the error rate during DNA synthesis[371], monovalent cation potassium ions and *Taq* polymerase or another DNA polymerase with a temperature optimum at around 70 °C. *Taq* DNA polymerase is a highly thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus*. The enzyme catalyzes 5' to 3' synthesis of DNA, it has no proofreading activity which is no detectable 3' to 5' exonuclease and possesses

low 5’ to 3’ exonuclease activity. In addition, *Taq* DNA polymerase exhibits deoxynucleotidyl transferase activity, which frequently results in the addition of extra adenines at the 3’–end of PCR products. Recombinant *Taq* DNA polymerase is ideal for standard PCR of templates 5 Kilo base (kb) or shorter. The error rate of *Taq* DNA polymerase in PCR is 2.2×10^{-5} errors per nucleotide (nt) per cycle, as determined by a modified method that was described. Accordingly, the accuracy of PCR is 4.5×10^{4}. Accuracy is an inverse of the error rate and shows an average number of correct nucleotides incorporated before an error occurs.

The PCR is commonly carried out in a reaction volume of 10-100 μl in small reaction tubes in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. A typical set of reactions might have a pre denaturation then, followed by 30-40 cycles of each comprising denaturation, annealing, extension and final extension. Then, evaluate the PCR product in 1.5% agarose gel electrophoresis which can separate nucleic acid molecules by size. Agarose gel that contains buffer is formed by a meshwork of molecules and nucleic acids are driven through it by an electric field from negative charge to positive charge then visualize by staining the gel in ethidium bromide and observed under UV light[372]. There are some factors affect to the PCR exponential progression such as existing phenol or enzymes found in sample which are inhibitors of polymerase chain reaction, reagent limitation, accumulation of pyrophosphate molecules and self-annealing of the accumulating product.

The advantage of PCR is requiring tiny amount of DNA samples in experiment effort, for each analysis due to the high sensitivity of PCR and has ability to produce large within short periods. There have been many publications which success of this technique[368]. PCR-based methods include sequence characterized

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amplified regions (SCAR), random amplified polymorphic DNA (RAPD), PCR-restriction fragment length polymorphism (PCR-RFLP), simple sequence repeat (SSR), inter- simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP).

2.5.2.3 Amplified fragment length polymorphism (AFLP)

Amplified Length Fragments Polymorphisms (AFLPs) technique, a PCR-based molecular marker, was first developed in 1993 by Vos and Zabeau[373]. AFLPs analysis was a powerful technique in terms of its ability to identify a large number of polymorphic bands without any prior knowledge of organisms. The ability of this technique to generate many markers with minimum primer testing and the high resolution are features that make AFLP attractive as genetic markers.

AFLPs marker is extensively used for studying genetic diversity in different plant species[373, 374]. Comparative studies using restriction RFLPs, RAPD, AFLP and microsatellite techniques have shown that AFLP method is the most efficient method to estimate genetic diversity due to 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[375]. 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. Microsatellite 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. The comparison of four popular genetic markers is mentioned in Table 7.

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Table 7 The comparison of four popular genetic markers

Criterion	AFLP	RAPD	SSR	RFLP
Quantity of information	High	High	High	Low
Replicability	High	High	High	High
Resolution of genetic differences	High	High	High	Moderate
Ease of use and development	Moderate	Easy	Difficult	Difficult
Development time	Short	Short	Long	Long

AFLPs techniques are being widely used for genetic diversity studies because it shows significantly polymorphism and is robust and reliable for molecular genetic marker. AFLP fingerprinting analysis has been used to discriminate between accessions of a number of plants such as *Panax japonicas*, *Panax notoginseng*, *Fritillaria cirrhosa*, *Swertia* spp., *Zingiber officinale, Zingiber montanum*, *Zingiber zerumbet*, *Curcuma comosa*, *Zanthoxylum acanthopodium* and *Zanthoxylum oxyphyllum*[376-382].

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AFLP procedure

The technique involves five steps (Figure 7)

- The first step is a restriction digest in which genomic DNA is cut by two restriction enzymes to generate small DNA fragments.
- Second step is a ligation in which double-stranded DNA adapters are ligated to the ends of the restricted DNA fragments to generate templates for amplification.
- Third step is a pre-amplification in which two primers, complementary to the adapter-ligated ends with one pre-selected nucleotide, are employed to amplify containing the primer binding site and the restriction site.
- Fourth step is a selective amplification in which selective primers, with an additional one to three nucleotides.
- Finally, selective amplification products were separated by electrophoresis on denaturing polyacrylamide gel and the separated AFLP were visualized by silver staining.

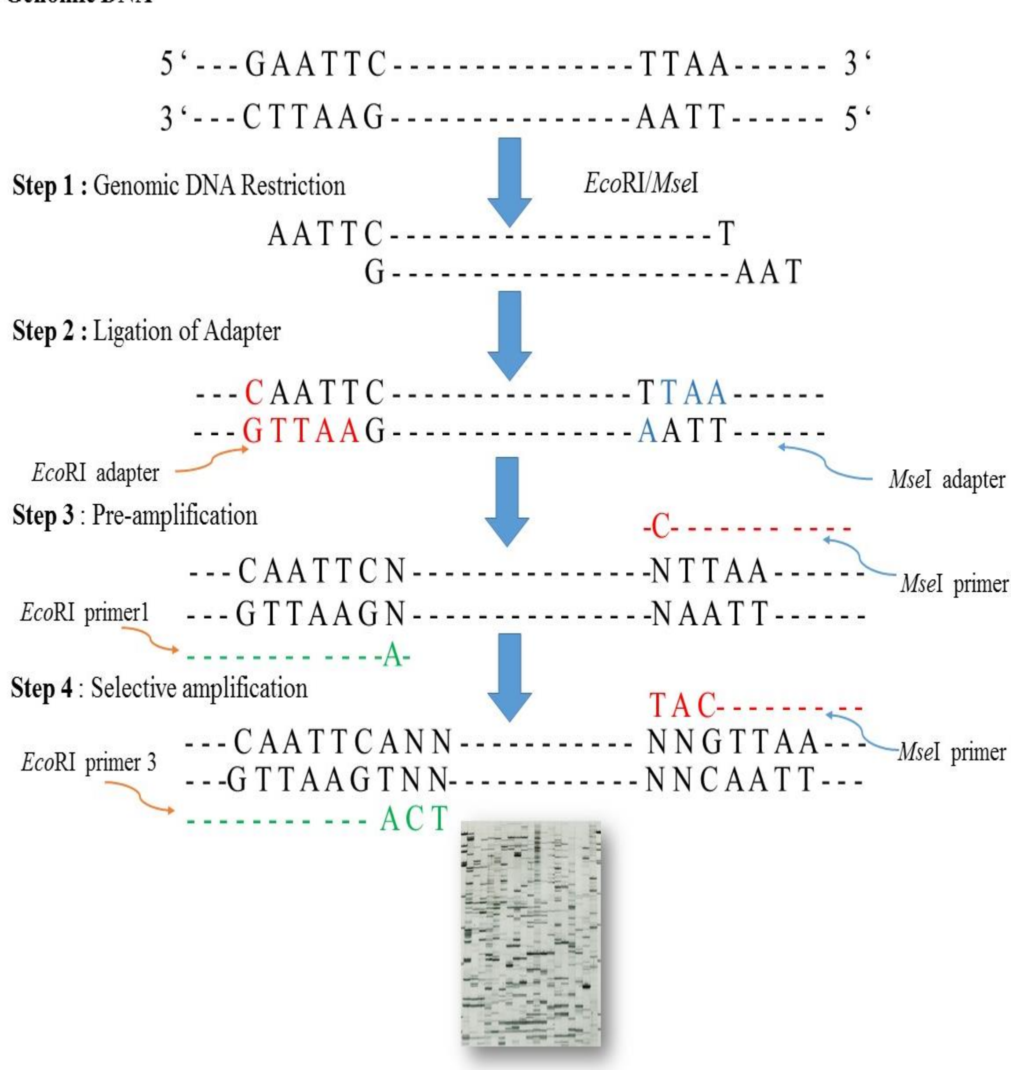

Figure 7 The Amplified Fragment Length Polymorphism (AFLP) process

2.5.2.4 Phylogenetic tree construction methods

2.5.2.4.1 Phylogenetic tree construction

Phylogenetic is one branch of systematic taxonomy and it is a field of biology concerning with identifying and understanding the evolutionary relationships among many different kinds of organism. Phylogenies are constructed using all kinds of data such as morphological characteristics data, molecular data and geographical data[383]. A phylogenetic tree is a tree diagram presenting evolutionary relationships among various biological species that are believed to have common ancestor. Construction of a phylogenetic tree is controlled by computer operations. Many phylogenetic computer programs such as PHYLIP, NTSYS and Free Tree software are common easily to conduct a phylogenetic[384-386].

Some commonly employed molecular marker methods such as RAPD, ISSR and AFLP generate a fingerprinting pattern obtained from a particular DNA material. Polymorphisms between the fingerprinting patterns of individuals are scored as presence (1) or absence (0) of particular sized fragments.

2.5.2.4.2 Similarity index

As the first step of similarity analysis, multilocus band patterns are applied to various procedures to quantify a pairwise similarity of genotypes represented in the different fingerprinting patterns. Commonly, a similarity index is calculated from band sharing data of each pair of the fingerprints[368].

There are many similarity coefficients used in molecular marker analysis. The examples of the similarity coefficients are described as follow;

- Jaccard's coefficient : $J = a/(a+b+c)$

Where; a = the number of 1-1 matches

b = the number of 1-0 matches

c = the number of 0-1 matches

(1= band present, 0 = band absent)

- Nei and Li's coefficient : N = 2a/(a+b)(a+c)

Where; a = the number of 1-1 matches

b = the number of 1-0 matches

c = the number of 0-1 matches

(1 = band present, 0 = band absent)

In the formulas of Jaccard's coefficient and Nei and Li's coefficient are derived from comparing the number of bands shared between individuals or population. One of the most commonly used similarity indices is Jaccard's coefficient which avoid including shared absents bands in the calculation of similarity index was chosen to use in this thesis.

2.5.2.4.3 Tree construction using distance matrix method

The distance matrix uses evaluation distances in a matrix from between all pairs of species in a data set to construct a phylogenetic tree. One widely used method is the Unweighted Pair Group Method of the Arithmetic Average method, used distance method for phylogenetic tree construction.

UPGMA (Unweighted Pair Group Method of the Arithmetic Average) was originally developed for constructing taxonomic phenograms which are trees that reflect the phenotypic similarities between operational taxonomic units (OTUs)[387]. This method involves clustering of closely species. At each stage of clustering, tree branches are being built and the branch lengths are calculated. UPGMA assumes a constant evolutionary rate thus, the two species in cluster are given the same branch length from the node. It is a simple and fast method of tree construction.

2.5.2.4.4 Bootstrap analysis

A bootstrap analysis is a simple and effective computer-based technique for assessing the accuracy of almost any statistical estimation[388]. It is one of tree evaluation method with provide measure of support for each branch in phylogenetic tree[389]. A bootstrap data matrix is created by randomly selecting a column from the original matrix with replacement. Pseudoreplicate datasets are generated by randomly sampling the original character matrix to create new matrices of the same size as the original. The whole process is repeated independently a large number of times (approximately 100-1000 replications). A bootstrap value is count (or percentage) of how often each branch presents in the resampled trees. These bootstrap confidence value can be considered as a reasonable assessment of errors for the estimated tree[390].

2.5.3 Quantitative analysis

2.5.3.1 Chromatography

Chromatography is general technique for separation of mixture compounds. Common chromatography methods used for chemical fingerprinting include thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC). All chromatographic techniques have a two-part operation in common. In each technique, a sample mixture is moved by a liquid or gas, called a mobile phase. The mobile phase carries the sample through a solid support, which contains an adsorbent or another liquid called the stationary phase. The different compounds in the sample mixture move through the stationary phase at different rates, due to different attractions for the mobile and stationary phases.

Thus, individual compounds in the mixture separate as they move through the stationary phase. The separated compounds can be collected or detected, depending on the particular chromatographic technique involved[391]. Chemical fingerprint is a method for the quality control of herbal samples that has been accepted by WHO[351]. It's suitable for detect adulterations and identify plant species[392]

2.5.3.1.1 Thin layer chromatography

Thin layer chromatography (TLC) is a fast screening method to separate and identify compounds in herbal extracts. TLC has some advantages more than other chromatographic techniques such as low cost of instrumentation, short time for analysis and easy to use[393-395]. The commonly adsorbents for TLC plates are silica, alumina, cellulose, polyamides etc. Silica gel is most commonly used[396]. An adsorbent is layered onto a glass, plastic or aluminum plate. The solvent for dissolve the sample must be proper to viscosity and volatility. Application of samples must be accurate, precise volumes and without damage the surface of TLC plate. The polarity of the solvent used for extraction should be similar with the compound mixture to be separated and analyzed[393, 395]. Mobile phase is a mixture of two to five different solvents selected experimental using trial and error guided by prior personal experiences and literature reports of similar separations. Samples can be detected on TLC plate analysis under the ultraviolet light with 254 and 365 or 366 nm wavelengths[393, 394]. Standard compounds of the known major of characteristic components in the herb are normally used as references for comparison when TLC is used to identify of herbal extracts. An important qualitative parameter, which characterizes the position of a spot on TLC plate, is the retardation factor (R_f) value. It is define as[394]:

$$R_f = \frac{\text{Distance of the compound from original spot travelled to the developed spot}}{\text{Distance of the solvent from original line travelled to the developed line}}$$

The TLC fingerprints with a visible character of bands provides fundamental data and is typically used to demonstrate the consistency and stability of herbal material. For detection of TLC plate after development, spot of compounds are detected on the plate. The first detection is direct observation of colored materials with eyes but most of compounds are colorless under daylight. Thus, they must be detected under other conditions[397]. Detection can be done by illuminating TLC plate UV light or by color reaction with suitable reagent. The adsorbent containing fluorescent additive can glow under specified UV light, usually at the wavelength of 254 nm. Many aromatic compounds having double bond can absorb this short wave UV (quenching) resulting in dark spot on a bright background detection. Some compounds with native fluorescence can be exited to fluorescence appear as bright spots, often differently colored, on dark background under UV 365 or 366 nm i.e. chlorophylls have red fluorescence, coumarins show blue fluorescence and anthraquinones appear as yellow fluorescence. The compounds that do not absorb UV light at 254 nm or 365 nm can be invisible and require detection with reagent. The staining reagents can be separated into two type, the universal staining reagents and the specific staining reagents. For known compounds, specific staining reagents for compounds of interest should be used such as dragendorff, vanillin, ninhydrin, natural product and 10% KOH. In case of crude extracts with unknown compound, universal spray reagents are recommended such as *p*-anisaldehyde/sulfuric acid and potassium permanganate[398]. The detection reagent solution is usually applied by spraying or dripping the layer. The mechanism of reagent detection is color reaction between the

compound of interest on TLC and selected reagent. In some case, heat is required to assist the color reaction on TLC plates and this can be supplied in the form of hair dryer or a drying oven[396, 398-400]. TLC is frequently used as a qualitative and quantitative method. Qualitative method can be determined by the number of compounds in a mixture and identified substances. Whereas quantitative method is used for content determination of require testing substances[394].

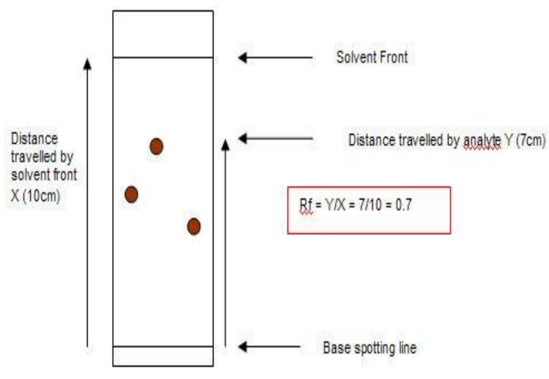

Figure 8 Thin-layer chromatography (TLC)

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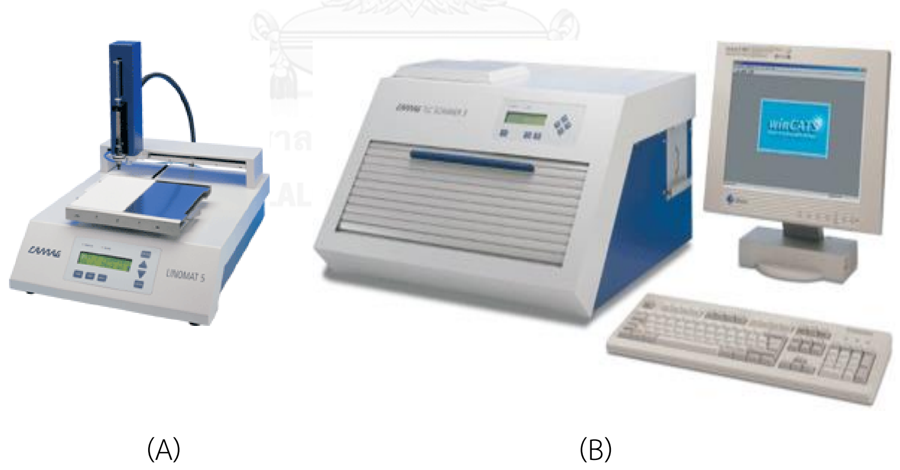
2.5.3.1.2 Quantitative detection of TLC

Quantitative analysis can be performed with data from TLC densitometry and image analysis method. Quantitative evaluation of a TLC plate is always performed densitometrically, either in absorption or fluorescence mode. The signal of each substance zone is compared to the substance free plate background. For calibration, the obtained peak data of the unknowns are compared against data obtained for standards on the same plate. Quantitative evaluation can be performed with data from classical densitometry or with those from electronic image acquisition. Classical densitometry uses monochromatic light and a slit of selectable length and width to scan the tracks of a chromatogram, measuring the diffusely reflected light.

The CAMAG TLC Scanner 3 uses the entire spectral range from 190 to 800 nm with high spectral selectivity for data acquisition (Figure 9). Different sources must be used to cover the entire UV-vis range. Halogen or tungsten lamps are light sources for visible wavelengths of 400-800 nm, deuterium lamps for UV wavelengths of 190-400 nm and high-intensity mercury or xenon sources for fluorescence excitation[398]. Absorption spectra for substance identification and for selection of the most suitable measurement wavelength can be recorded within this range[401].

Measurement of the amount of substance in the TLC plates are often used by densitometry. This technique has been successfully used for the analysis of active constituents in herbal plants such as *Strychnos* spp. and *Cassia fistula*[402, 403]. However, due to the high price it may not be suitable for some laboratories.



(A) (B)

Figure 9 (A) Linomat V, (B) TLC scanner III and wincats software

TLC image analysis, digitally enhanced TLC (DE-TLC) is introduced as an inexpensive, new technique for qualitative and quantitative analysis that can be used in any laboratory that cannot afford a commercial densitometer[404].

TLC-image analysis method has been developed and applied for quantitative assay with good accuracy and precision[405]. The DE-TLC equipment consisted of a UV lamp in a cabinet, CCD camera and image analysis software. This is based on the use of a charge-coupled device (CCD) sensor in camera as a detector that offer more than a million small detectors (pixels) and combination with image analysis software for converting pixel intensity to chromatographic peak. Commercial and free image analysis software for TLC image analysis are available in which performances are based on sensitivity of spot detection, background compensation algorithms, intensity resolution, precision and accuracy of image analysis[406-408]. The CCD camera is sufficiently sensitive to detect changes in spot fluorescence intensity from a UV light source. The CCD cameras are detectors containing an array of sensors that can image an area in fraction of seconds or real time[409]. The quantitative evaluation of the TLC plates using the proposed image analysis methods is based on the assumption that the color intensity and the area size of the each individual spot on the plate are function of the quantity of that particular compound in the corresponding spot. Due to the spot color intensity is evaluated by comparing it to the plate color background and the analysis should include the entire TLC plate[404]. The developed TLC plate is illuminated and recorded by a CCD camera under UV light. The TLC plate is positioned in the system, UV cabinet and CCD camera is aligned for optimal pixel resolution of the images. The images are processed using image analysis software or common photo-editing software which gives the peak area of each spot. After that, peak area value is substituted in the equation taken from the standard curve. The result shows the content of active constituent in each sample.

There are at least 20 different versions of software for quantitative evaluation of TLC with images analyzing systems written by different

companies such as Photoshop, Sorbfit TLC Video densitometer software, Scion Image software and ImageJ. ImageJ is one of several image analysis softwares that image from CCD camera are required for analysis[410] (Figure 10). ImageJ is open source software, which was developed in Java programs, that users can develop program and fix the program. It was used in many fields, for example medical researches and biological microscopy. It can be used in both Windows and Macintosh, available free download from website of the US National Institute of Mental Health. (http://rsbweb.nih.gov/ij/index.html)[411]. It can display, edit, analyze, process, save and print 8-bit, 16-bit and 32-bit image and can read many image formats including TIFF, GIF, JPEG, BNP, DICOM, FITS[412, 413]. ImageJ software can calculate area and pixel value statistics of user-defined selections, measure distances and angles, create density histograms and line profile plots. The software supports standard image processing functions such as contrast manipulation, sharpening, smoothing, edge detection and median filtering, geometric transformations such as scaling, rotation and flips. For analyzing the amount of compound on TLC plate by ImageJ software, firstly, the user must convert the spot on TLC plate to peak area. A rectangular tool is used to select interested spot and its area and pixel value statistics are calculated. Density histograms and line profile plots are created to obtain a peak from each spot with peak area calculation.

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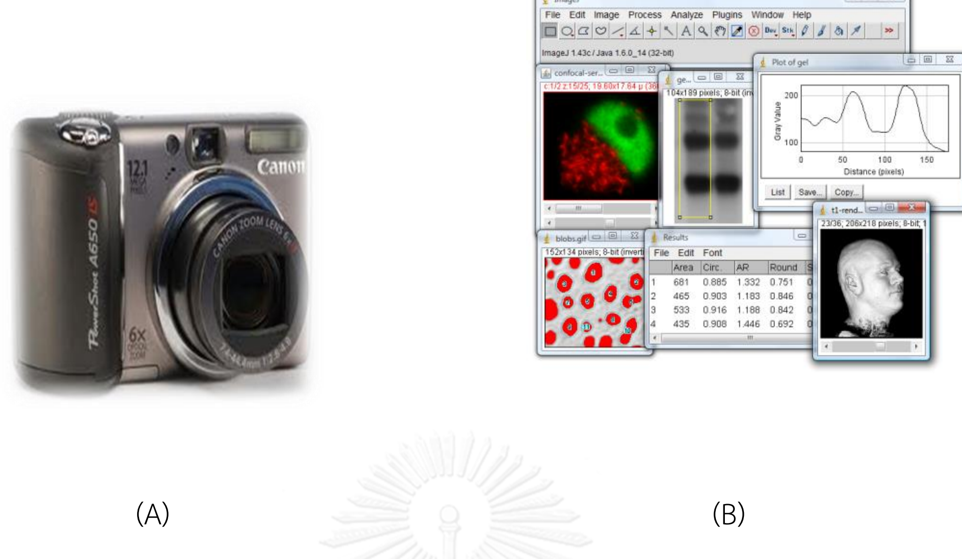

(A) (B)

Figure 10 (A) Charge-couple device (CCD) camera (B) ImageJ free software

2.5.3.2 Validation of analytical procedures

Process used to confirm and demonstrate the performance characteristics of an analytical methodology is method validation. The aim of method validation is to ensure that the methodology is accurate, specific, reproducible and robust. Accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), specificity, linearity and robustness are used for validate quantitative method according to the International Conference on Harmonization (ICH) guidelines[414].

2.5.3.2.1 Accuracy

The accuracy is expressed the closeness of the test results obtained by the analytical procedure to agreement between the value which is accepted either as a conventional true value or an accepted reference value and

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the value found. Accuracy is reported as percent recovery by the assay of spiked sample with known amount of analyte.

2.5.3.2.2 Specificity

Specificity is an ability to determine impurities in analyte. Purity test is commonly used to certify that all the analytical methods performed allow an accurate statement of the impurity content of an analytical method.

2.5.3.2.3 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

2.5.3.2.4 Linearity

Linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyze in the sample. A minimum of 5 concentration levels is recommended for founding of linearity.

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2.5.3.2.5 Range

Range of procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

2.5.3.2.5 Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) is the lowest amount of an analyst of interest which can be detected but not necessarily quantitated as an exact value. The limit of quantitation (LOQ) is the lowest amount of an analyst of interest which can be quantitatively determined with acceptable precision and accuracy. LOD and LOQ can be determined based on the SD of the blank, the residual SD of a regression line, or the SD of y-intercepts of a regression lines.

2.5.3.2.6 Robustness

Robustness of an analytical procedure is a measure of its capacity to remain an affected by small, but deliberate variations in method parameters and provide an indication of its reliability during normal usage.

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CHAPTER III

MATERIALS AND METHODS

Part I Morphological Characteristics

Chemicals and reagents

Glycerine	Imax glycerine, Honghat Co., Thailand

Materials

Cover slips	Menzel, Glazer
Drawing board	
Drawing paper 100 gram	Master art, Thailand
HB pencil and eraser	Pental, Thailand
Microscope slide	Sail Brand, China
Razor blade	
0.20 mm line width black micro-pigment pen	Sakura, Japan

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Instruments and equipments

Digital camera	Cannon Power shot A640, Japan
Photomicroscope	Zeiss Imager A.2 Axio, Germany

Part II Molecular identification

Chemicals and reagents (DNA extraction)

Absolute ethanol	Merck, Daemstadt, Germany
Agarose	Ultrapure TM, Life technologies, U.S.A.
Boric acid	Merck, Daemstadt, Germany
Bromophenol blue	Fermentas, U.S.A.
Chloroform	Merck, Daemstadt, Germany
Cyltrimethylammonium bromide	Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.
Ethidium bromine	Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.
Ethylene diamene tetaacetic acid	Merck, Daemstadt, Germany
Isoamyl alcohol	Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.
Polyvinylpyrrolidone	Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.
Sodium Chloride	BDH Laboratory supplies, Poole, England
Sodium acetate	BDH Laboratory supplies, Poole, England
Tris (hydroxymethly)-aminomethane	Fluka, Biochemika, Germany
1 kb DNA Ladder	Promega. U.S.A.

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Chemicals and reagents (DNA extraction Cont.)

100 kb DNA Ladder	Promega. U.S.A.,
	Fermentas, U.S.A.
2 Mercaptoethanol	Sigma-Aldrich Company Co.,
	St. Louis, MO, U.S.A.

Chemicals and reagents (AFLP procedure)

Acetic acid	BDH Laboratory supplies,
	Poole, England
Acrylamide	Sigma-Aldrich Company Co.,
	St. Louis, MO, U.S.A.
Bind-silane	Sigma-Aldrich Company Co.,
	St. Louis, MO, U.S.A.
Bisacrylamide	Sigma-Aldrich Company Co.,
	St. Louis, MO, U.S.A.
Bromophenol blue	Sigma-Aldrich Company Co.,
	St. Louis, MO, U.S.A.
dNTPs	Fermentas, Canada
*EcoR*I	Boehringer Mannheim
	GmbH, Germany
ER adapter	Eurofins MWG Operon,
	Germany
Ethanol	Merck Daemstadt,
	Germany

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Chemicals and reagents (AFLP procedure Cont.)

$MgCl_2$	Invitrogen, U.S.A.
MS adapter	Eurofins MWG Operon, Germany
*phi*X174 DNA Marker	Promega. U.S.A.
Repel- silane	Healthcare Bio-Sciences AB, UK.
Silver nitrate	BDH Laboratory supplies, Poole, England
Sodium carbonate	Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.
Sodiumthiosulphate	Merck, Daemstadt, Germany
Taq DNA polymerase	Fermentas, U.S.A.
Taq DNA polymerase	Invitrogen, U.S.A
TEMED	Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.
*Tru9*I	Roche Diagnostice GmbH Mannheim, Germany
T4 DNA ligase	NEB, United Kingdom
Urea	Merck Daemstadt, Germany
Xylene cyanole	Sigma-Aldrich Company Co., St. Louis, MO, U.S.A.
10% Ammonium persulphate	BDH Laboratory supplies, Poole, England

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Chemicals and reagents (AFLP procedure Cont.)

10x buffer A	Roche Diagnostice GmbH Mannheim, Germany
10x ligase buffer	NEB, United Kingdom
37% formaldehyde	Merck Daemstadt, Germany

Materials

Microcentrifuge tube	Axygen, U.S.A.
Micropipette	Eppendorf, Germany
Mortar and pestle	
Pipet tips	Axygen, U.S.A.

Instruments and equipments

Centrifuge	Labnet international, Inc. U.S.A.
DyNA Quant 200 Fluorometer	Hoefer Sc. Instr., Pharmacia, U.S.A.
Gel electrophoresis apparatus and power supply	Labnet international, Inc. U.S.A.
Microwave	Sharp, Thailand
NanoDrop Spectrophotometer ND-1000	NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.
Orbital incubator SI 50	GENEO BioTechProducts GmbH, Germany
PCR Thermal	Px2 Thermal Cycler; Thermo Electron Corporation, U.S.A.
Sequi-GEN GT Sequencing and power supply cycler	Bio-Rad, USA

UV transilluminator and analyzed with a gel documentation system

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Instruments and equipments (Cont.)

Waterbath GFL1083	GFL Gesellschaft für Labortechnik mbH, Germany
Vertex mixer, shaking incubator and etc.	

Part III Quantitative analysis

Chemicals and reagents

Ammonium hydroxide	RCI Labscan Limited, Bangkok, Thailand
Chloroform	J.T. Baker Chemical Co., Phillipsburg, U.S.A.
Dichloromethane	RCI Labscan Limited, Bangkok, Thailand
Ethanol	RCI Labscan Limited, Bangkok, Thailand
Ethyl acetate	RCI Labscan Limited, Bangkok, Thailand
Hexane	RCI Labscan Limited, Bangkok, Thailand
Hydrochloric acid	RCI Labscan Limited, Bangkok, Thailand
Methanol	RCI Labscan Limited, Bangkok, Thailand

The chemicals used were of analytical grade.

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Materials

Filter paper No.4	Whatman™ Paper, UK
Filter paper No.40 ashless	Whatman™ Paper, UK
TLC aluminium sheet 20 × 20 cm	Merck, Darmstadt, Germany
Silica gel 60 F_{254}, 200 µm thickness	

Instruments and equipments

Aqua-shaker	Adolf Kühner AG, Switzerland
Balance readability 0.0001 g	SI-234, Denver Instrument, Germany
Balance readability 0.01 g	Ohaus Corp. Pine Brook, NJ, USA (Pioneer™, PA2102)
UV viewing Cabinet, Model CC-80	Spectronics Corp., USA
CAMAG Linomat 5	CAMAG, Switzerland
CAMAG TLC Chamber	CAMAG, Switzerland
CAMAG TLC Scanner 3	CAMAG, Switzerland
CAMAG TLC Visualizer	CAMAG, Switzerland
Digital camera (Canon PowerShot A650 IS)	Canon Marketing (Thailand) Co., LTD, Bangkok
Free tree software version 0.9.1.50	

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Instruments and equipments (Cont.)

Hot air oven	WTC Binder tuttlingen, Germany
ImageJ software	National Institutes of Health, USA (Version: 1.46r)
Incinerator	Carbolite, UK
Rotary vacuum evaporator	Büchi, Switzerland
Scanning electron microscope (Model JSM-5410LV)	JEOL Ltd., Tokyo, Japan
Soxhlet apparatus	
TLC syringe	Hamilton Company, USA
Tree view software version 1.6.6	
Ultrasonic bath	Analytical Lab Science Co., LTD, Bangkok
Ultraviolet fluorescence analysis	Spectronics corp., USA
Water bath	Brinkmann, USA
winCATS software (Version: 1.4.6.2002)	CAMAG, Switzerland

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Scope of investigation

1. Macroscopic and microscopic examination of 16 *Cassia* species.

2. AFLP fingerprinting and phylogenetic relationship of 16 *Cassia* species.

3. Quantitative analysis of aloe-emodin content in *C. grandis* and *C. garrettiana* leaves by TLC-image analysis using ImageJ free software compared to TLC-densitometry.

Part I Characteristics feature

Sixteen *Cassia* species collected from different locations in Thailand were examined by macroscopic and microscopic examinations according to World Health Organization (WHO) guidelines standard methods.

Plant sample

The fresh mature leaves of sixteen *Cassia* species were collected from several locations in Thailand and authenticated by Associate Professor Nijsiri Ruangrangsi, Ph.D., College of Public Health Sciences, Chulalongkorn University and compared to the herbarium specimens at The Botanical Garden Organization, Ministry of Natural Resource and Environment, Bangkok, Thailand. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. All of the collected plant materials and their localities are listed in Table 8.

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Table 8 The detail of 16 *Cassia* species used in this study

No.	Scientific Name	Locality
1	*Cassia bakeriana*	Bangkok
		Phitsanulok
		Phetchaburi
2	*C. fistula*	Bangkok
		Phitsanulok
		Si Sa Ket
3	*C. grandis*	Bangkok
		Phitsanulok
		Si Sa Ket
4	*C. javanica*	Bangkok
		Phitsanulok
		Pathumthani
5	*C. alata*	Bangkok
		Phitsanulok
		Si Sa Ket

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Table 8 The detail of 16 *Cassia* species used in this study (Cont.)

No.	Scientific Name	Locality
6	*C. angustifolia*	Bangkok
		Phitsanulok
		Si Sa Ket
7	*C. garrettiana*	Bangkok
		Phitsanulok
		Si Sa Ket
8	*C. hirsuta*	Bangkok
		Phitsanulok
		Chachoengsao
9	*C. occidentalis*	Bangkok
		Phitsanulok
		Si Sa Ket
10	*C. spectabilis*	Bangkok
		Phitsanulok
		Si Sa Ket
11	*C. siamea*	Bangkok
		Phitsanulok
		Si Sa Ket

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Table 8 The detail of 16 *Cassia* species used in this study (Cont.)

No.	Scientific Name	Locality
12	*C. sophera*	Bangkok
		Phitsanulok
		Si Sa Ket
13	*C. surattensis*	Bangkok
		Phitsanulok
		Si Sa Ket
14	*C. sulfurea*	Bangkok
		Phitsanulok
		Si Sa Ket
15	*C. timoriensis*	Bangkok
		Phitsanulok
		Si Sa Ket
16	*C. tora*	Bangkok
		Phitsanulok
		Si Sa Ket

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1.1 Macroscopic examination

Procedure

Visual characters of sixteen *Cassia* species were observed and the whole plant were illustrated by hand drawing for its shape, size and botanical morphology.

1.2 Microscopic analysis

Procedure

Anatomical characters of transverse section and trichome number of sixteen *Cassia* species were investigated under photomicroscope observation under objective lens with a 10X, 20X and 40X magnifications and eyepiece lens of 10X magnification.

1.2.1 Anatomical character

1.2.1.1 Transverse section of leaves through midrib

Each leaf from sixteen *Cassia* species were thinly transverse sectioned with razor blade by hand, separately placed a complete piece on the glass slide and cover with a cover glass. Each leaf were investigated under photomicroscope observation with objective lens of 10X, 20X and 40X magnifications and eyepiece lens of 10X magnification to evaluate the fine details and the image were recorded by digital camera and illustrated by hand drawing in the proportion size related to the original scale in drawing paper.

1.2.1.2 Trichome number of leaves

The light microscope (LM) attached a digital camera and scanning electron microscope (SEM) were used in this study. The central lamina of cleaned fresh mature leaf were cut, soaked in bleaching agent as water : Haiter

bleaching solution (1:1) to remove the chlorophyll until it was clear, rinsed with water 2-3 times then the trichomes was investigated on both surfaces by wet mounting in glycerin and examining under the LM. The images were recorded using AxioVision Release 4.8.2 program. The studied area was avoided from the veinlet, margin, or unclear field. The trichome characteristics were investigated and the trichomes in 1 mm^2 area were counted. Thirty fields of each species from three different sources were examined. Mean, minimum, maximum, and standard deviations of trichome numbers on both surfaces were calculated and discussed**.** The trichome number within *Cassia* species were compared by Tukey HSD test ($p < 0.01$).

Part II Molecular identification

Plant materials

The fresh young leaves of 16 selected *Cassia* species and outgroup plants (*Andrographis paniculata*, Family Acanthaceae) were collected from different locations in Thailand. Plant specimens were authenticated by Associate Professor Nijsiri Ruangrangsi, Ph.D. College of Public Health Sciences, Chulalongkorn University and compared to the herbarium specimens at The Botanical Garden Organization, Ministry of Natural Resource and Environment, Bangkok, Thailand. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The list of plant samples information used in this study were shown in Table 8.

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Methods

Preparation of CTAB buffers

Genomic DNA were individually extracted from the fresh young leaves using Modified CTAB Method[415]. The preparation of 2x CTAB buffers was shown in Table 8. Four µl of 2-mercaptoethanol was immediately added to each 1 ml of 2XCTAB buffers before used.

Table 9 The preparation of 2x CTAB buffers

Stock reagent	Final concentration	Final amount
CTAB	2% (w/v)	2 g
1M Tris-HCL pH8	100 mM	10 ml
0.5 M EDTA	20 mM	4 ml
5M NaCl	1.4 M	28 ml
Adjust with distilled water to 100 ml		

Genomic DNA Extraction

For genomic DNA extraction, approximately 5 g of fresh young leaves were ground in liquid nitrogen with mortar and pestle. The leaves powder was transferred into microcentrifuge tubes and then 700 µl of CTAB buffer was added, incubated at 65 °C for 1 hour in water bath. After incubation, microcentrifuge tubes were centrifuged at 10,000 rpm for 10 minutes to spin down cell debris. After that, the supernatant was transfered to new microcentrifuge tubes and 600 µl of chloroform were added to each tube, mix the solution by vortexing. After mixing, microcentrifuge tubes were centrifuged at 10,000 rpm for 10 minutes and the upper aqueous phase was transfered to new microcentrifuge tubes. Six hundred µl of chloroform/isoamyl

alcohol (24:1) were added to each tube, and mix the solution by vortexing. After mixing and centrifugation at 10,000 rpm for 10 minutes, the upper aqueous phase was transfer to a new microcentrifuge tube. 3M sodium acetate pH 5 in the ratio of 1:10 volume was added to each new microcentrifuge tubes, followed by adding 2 volume of cold absolute ethanol. The microcentrifuge tubes were gently inverted several times to precipitate the DNA and incubated at -20 °C for 1 hour. After precipitation, microcentrifuge tubes were centrifuged at 10,000 rpm for 10 minutes and the supernatant was removed, the precipitated DNA was sticked at the bottom of the tube. The precipitated DNA was washed with 1 ml of 70% cold ethanol, gently inverted the tube and centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and the DNA pellet was allowed to dry at room temperature. Finally, the DNA was resuspend in 100 µl or optimal volume of TE buffer and stored at -20 °C. DNA quantification was performed using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). The DNA concentration and purity was recorded. The schematic of plant DNA extraction protocol was shown in Figure 11.

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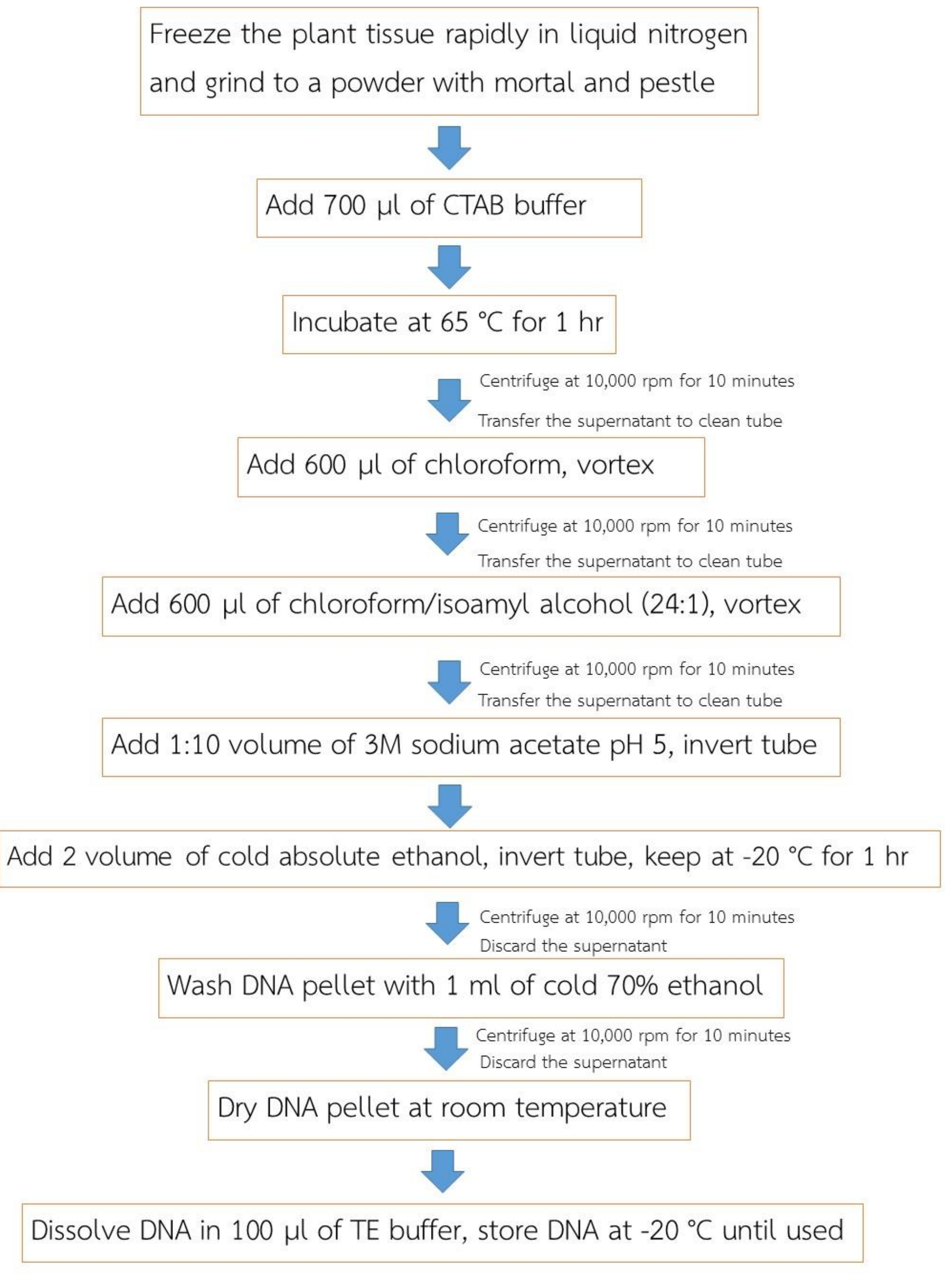

Figure 11 Schematic of plant DNA extraction protocol

AFLPs procedure

The AFLP procedure was carried out as previously reported by Vos *et al* [373] with some modification.

1. Digestion of genomic DNA

Approximately 100 ng/μl of genomic DNA were digested with two restriction enzymes, *EcoR*I and *Tru9*I in 10x buffer A (Roche). The digested reaction was incubated at 37 °C for 1 hour. The component of reaction mixture for genomic DNA digestion was mentioned in Table 10.

Table 10 Reaction mixture for digesting genomic DNA with restriction enzymes

Digestion components	**Stock concentration**	**Final concentration**	**Final amount**
*Tru9*I	10 U/μl	5 U	0.5 μl
*EcoR*I	10 U/μl	5 U	0.5 μl
10x buffer A	10x	1x	4 μl
ddH_2O			30 μl
gDNA (100 ng/μl)	100 ng/μl	500 ng	5 μl
	Total		40 μl

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2. Ligation of genomic DNA

The digested genomic DNA was ligated with *EcoR*I adapter and *Mse*I adapter by adding 10 µl of ligation master mix. The reaction was incubated at 37 ˚C for 3 hours (preferably overnight). The completeness of ligation process was detected by 1% agarose gel electrophoresis in 0.5x TBE buffer. Each ligation reaction was diluted as ten-folded with ddH_2O and the aliquots ligation reaction were stored at -20 ˚C. The component of reaction mixture for nucleotide adapter ligation and sequences of adapters and primers was mentioned in Table 11 and Table 12, respectively.

Table 11 Reaction mixture for nucleotide adapter ligation

Digestion components	Stock concentration	Final concentration	Final amount
ER adapter	5 pmol/µl	5 pmol	1 µl
MS adapter	50 pmol/µl	50 pmol	1 µl
T4 DNA ligase	5U/µl	1U	0.2 µl
dATP	10mM	1mM	1 µl
10x ligase buffer	10x	1x	1 µl
ddH_2O			5.8 µl
	Total		10 µl

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Table 12 Sequences of adapters and primers used for AFLPs analysis

Name/Abbreviation	Type	Sequence
*EcoR*I adapter		5'- CTC GTA GAC TGC GTA CC -3'
		3'- CTG ACG CAT GGT TAA -5'
*Mse*I adapter		5'- GAC GAT GAG TCC TGA G -3'
		3'- TAC TCA GGA CTC AT -5'
ER1A	Primer +1	5'- AGA CTG CGT ACC AAT TCA -3'
ER3AAC	Primer +3	5'- AGA CTG CGT ACC AAT TCA AC -3'
ER3ACG	Primer +3	5'- AGA CTG CGT ACC AAT TCA CG -3'
ER3AGC	Primer +3	5'- AGA CTG CGT ACC AAT TCA GC -3'
ER3ACC	Primer +3	5'- AGA CTG CGT ACC AAT TCA CC -3'
ER3AAG	Primer +3	5'- AGA CTG CGT ACC AAT TCA AG -3'
ER3ACT	Primer +3	5'- AGA CTG CGT ACC AAT TCA CT -3'
ER3ACA	Primer +3	5'- AGA CTG CGT ACC AAT TCA CA -3'
MS1C	Primer +1	5'- GAT GAG TCC TGA GTA AC -3'
MS3CCA	Primer +3	5'- GAT GAG TCC TGA GTA ACC A -3'

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Table 12 Sequences of adapters and primers used for AFLPs analysis (Cont.)

Name/Abbreviation	Type	Sequence
MS3CTA	Primer +3	5’- GAT GAG TCC TGA GTA ACT A -3’
MS3CAC	Primer +3	5’- GAT GAG TCC TGA GTA ACA C -3’
MS3CTC	Primer +3	5’- GAT GAG TCC TGA GTA ACT C -3’
MS3CAA	Primer +3	5’- GAT GAG TCC TGA GTA ACA A -3’
MS3CAG	Primer +3	5’- GAT GAG TCC TGA GTA ACA G -3’
MS3CAT	Primer +3	5’- GAT GAG TCC TGA GTA ACA T -3’
MS3CTG	Primer +3	5’- GAT GAG TCC TGA GTA ACT G -3’
MS3CGT	Primer +3	5’- GAT GAG TCC TGA GTA ACG T -3’
MS3CCC	Primer +3	5’- GAT GAG TCC TGA GTA ACC C -3’
MS3CCG	Primer +3	5’- GAT GAG TCC TGA GTA ACC G -3’
MS3CCT	Primer +3	5’- GAT GAG TCC TGA GTA ACC T -3’
MS3CGA	Primer +3	5’- GAT GAG TCC TGA GTA ACG A -3’
MS3CGC	Primer +3	5’- GAT GAG TCC TGA GTA ACC G -3’

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3. Pre-selective amplification

Five microliters of diluted ligation was used as template for PCR pre-amplification using ER1A and MS1C primer. Each PCR reaction was composed of 50 μl pre-amplification primer mixture (Table 13). PCR were performed in a PCR thermal cycler (Applied Biosystems, USA) with an initial pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 sec, annealing at 56 °C for 1 min, extension at 72 °C for 1 min with 20 cycles and final extension at 72 °C for 10 min. The pre-amplification PCR product were diluted to ten-folded with ddH_2O, mixed and stored at -20 °C.

Table 13 Reaction mixture for pre-amplification

Digestion components	Stock concentration	Final concentration	Final amount
PCR buffer	10x	1x	5 μl
$MgCl_2$	50 mM	1.5 mM	1.5 μl
dNTPs	1 mM	0.2 mM	10 μl
ER1A	70 ng/μl	1.4 ng/μl	1 μl
MS1C	70 ng/μl	1.4 ng/μl	1 μl
Taq DNA polymerase	5U/μl	1 U	0.2 μl
ddH_2O			26.3
gDNA (ligation product)			5
Total			50 μl

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4. **Selective amplification**

Three microliters of diluted pre-selective PCR product was used as selective amplification in a reaction tube containing 20 μl selective amplification mixtures (Table 14). The selective amplification mixtures were performed in a PCR thermal cycler (Applied Biosystems, USA) with an initial pre-denaturation at 95 ˚C for 2 min, 36 cycles of denaturation at 95 ˚C for 30 sec, annealing at 65 ˚C for 30 sec, extension at 72 ˚C for 1 min. Annealing were initiated at a temperature of 65 ˚C, which will be then reduced by 0.7 ˚C for the next 12 cycles and maintained at 56 ˚C for 30 sec subsequent 23 cycles. When the selective PCR amplification was finished, 10 μl of sequencing dye was added to the selective amplification product. The selective amplified PCR products were determined using 1% agarose gel electrophoresis in 0.5x TBE. The selective PCR products were run on 4.5% denaturing polyacrylaminde gel electrophoresis.

Table 14 Reaction mixture for selective-amplification

Digestion components	**Stock concentration**	**Final concentration**	**Final amount**
PCR buffer	10x	1x	2 μl
$MgCl_2$	50 mM	1.5 mM	0.6 μl
dNTPs	1 mM	0.2 mM	4 μl
ER3A_ _	30 ng/μl	1.5 ng/μl	1 μl
MS3C_ _	30 ng/μl	1.5 ng/μl	1 μl
Taq DNA polymerase	5U/μl	0.5 U	0.1 μl
ddH_2O			8.3 μl

Table 14 Reaction mixture for selective-amplification (Cont.)

Digestion components	Stock concentration	Final concentration	Final amount
gDNA (ligation product)			3 µl
	Total		20 µl

5. Detection of AFLP bands using denaturing polyacrylamide gel electrophoresis

Selective amplification products were separated by 4.5% denaturing polyacrylamide gel electrophoresis in 1x TBE buffer in a Sequi-GEN GT Sequencing (Biorad, USA). The AFLPs bands on polyacrylamide gel were detected by silver nitrate staining solution[416].

6. Data analysis

For the genetic similarity analysis, AFLP fragments were visually scored as present (1) or absent (0) to create a binary data set. The data were entered into a binary data matrix as discrete variables. Jaccard's coefficient of similarity were calculated for all pair-wise comparisons among the *Cassia* species as follows: Jaccard = $N_{AB}/ (N_{AB}+N_A+N_B)$, where N_{AB} is the number of fragments shared by two cultivars (A and B), N_A represents amplified fragments in cultivar A and N_B represents fragments in cultivar B[417]. A dendrogram was constructed using the Unweighted Pair Group Method of the Arithmetic Average (UPGMA), clustering by FreeTree software[384]. To evaluate the strength of the resulting branches, bootstrap probabilities were calculated using 1,000 bootstrap resampling data by FreeTree software.

Part III Quantitative analysis

Plant Materials

The fresh mature leaves of *C. grandis* and *C. garrettiana* were collected from 15 locations throughout Thailand. Plant specimens were authenticated by Associate Professor Nijsiri Ruangrangsi, Ph.D., College of Public Health Sciences, Chulalongkorn University Thailand and compared to the herbarium specimens at The Botanical Garden Organization, Ministry of Natural Resource and Environment, Bangkok, Thailand. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. Each authentic sample was dried in hot air oven at 45 °C and ground to powders. The collected locations of *C. grandis* and *C. garrettiana* were listed in Table 15 and Table 16, respectively.

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Table 15 The localities of *C. grandis* used in this study

No.	Localities
1	Ubon Ratchathani
2	Surin
3	Si Sa Ket
4	Chaiyaphum
5	Nakhon Ratchasima
6	Nakhon Sawan
7	Phichit
8	Phitsanulok
9	Sukhothai
10	Uttaradit
11	Pathum Thani
12	Nakhon Pathom
13	Prachin Buri
14	Phra Nakhon Si Ayutthaya
15	Bangkok

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Table 16 The localities of *C. garrettiana* used in this study

No.	Localities
1	Phetchabun
2	Lop Buri
3	Nonthaburi
4	Chon Buri
5	Sukhothai
6	Nakhon Pathom
7	Rayong
8	Kanchanaburi
9	Uttaradit
10	Nakhon Ratchasima
11	Khon Kaen
12	Maha Sarakham
13	Prachuap Khiri Khan
14	Prachin Buri
15	Bangkok

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Procedure

1. Quantitative analysis of aloe-emodin in *C. grandis* and *C. garrettiana* leaves

1.1 Preparation of standard solutions

The stock solution of standard aloe-emodin (0.5 mg/ml) were prepared in dichloromethane containing 10% methanol. The stock solutions were appropriately diluted to obtain the series of standard solutions of concentration 0.04, 0.08, 0.12, 0.16, 0.20 mg/ml, respectively and stored in refrigerator at 4 °C.

1.2 Preparation of sample extracts

Six gram of the dried leaf powders of *C. grandis* and *C. garrettiana* were extracted with dichloromethane by soxhlet apparatus. The extract was filtered and the solvent was evaporated by rotary evaporator. The yield of each plant sample was calculated and recorded. The extracts of *C. grandis* and *C. garrettiana* was dissolved in dichloromethane containing 10% methanol to obtain the final concentration of 5 mg/ml of *C. grandis* and 20 mg/ml of *C. garrettiana*.

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1.3 TLC-densitometry

Three microliters of 15 dichloromethane extracted samples of *C. grandis* and of *C. garrettiana* and aloe-emodin standard solutions were applied on the silica gel 60 GF254 20x10 cm TLC plate using a CAMAG Linomat 5 automatic sample spotter (Camag, Switzerland) under a flow of N_2 gas. Each sample band was set at 10 mm and distance between bands was 8.9 mm. The TLC plate were developed in a CAMAG glass twin-through chamber (20x10 cm) (Camag, Switzerland) which were presaturated in mobile phase of hexane-ethyl acetate (1:1 v/v) for 1 hours at room temperature. The development distance were 8 cm from the base. The plate was

scanned under wavelength at 434 nm using TLC scanner 3 (Camag, Switzerland) with winCATS software. Aloe-emodin contents in *C. grandis* and *C. garrettiana* leaf extract were quantitated by peak area. The test was done in triplicate.

1.4 TLC image analysis by ImageJ software

TLC plate was photographed under ultraviolet light at 254 nm by a digital camera and saved as tiff files. Quantitative analysis of the aloe-emodin contents in *C. grandis* and *C. garrettiana* leaf extract were determined as the color intensity of spot on TLC plate using ImageJ free software (Department of Health and Human Services, National Institutes of Health (NIH) in the United State). The test was done in triplicate.

2. Method validation

The method validation including accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), specificity, linearity and robustness were validated according to International Conference on Harmonization (ICH) guidelines.

2.1 Accuracy

The accuracy of the method was tested by carrying out recovery studied at different spike levels of known aloe-emodin standard solutions. Three known different levels of aloe-emodin standard solutions (low, medium, and high) were added into the sample. The accuracy was determined as recovery of aloe-emodin contents in percentage by using following formula;

$$\% \text{ Recovery} = [C_1/C_2+C_3] \times 100$$

where, C_1 = the amount of aloe-emodin found in spiked sample

C_2 = the amount of aloe-emodin found in un-spiked sample

C_3 = the amount of standard aloe-emodin added to the sample

2.2 Precision

The precision of low, medium and high levels of analytes were examined by the same day (repeatability) and different days (intermediate precision) and expressed in terms of % relative standard deviation (% RSD) by following formula;

%RSD = SD x 100/Mean

Where, SD = the standard deviation of each measurement

2.3 Linearity

The linearity of the method was calculated by plotting peak areas *versus* concentrations of aloe-emodin standard solutions.

2.4 LOD and LOQ

LOD and LOQ values were determined by standard deviation method. The LOD were calculated based on residual standard deviation of regression lines (SD) and the slope of the calibration curve (*S*) following the formula: LOD = 3.3(SD/*S*). The LOQ were calculated based on residual standard deviation of regression lines (SD) and the slope of the calibration curve (*S*) following the formula: = 10(SD/*S*).

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2.5 Robustness

The method was tested for the small changes in the mobile phase. The solvent compositions were changed in the ratio of hexane-ethyl acetate as 1:1, 0.9:1.1 and 1.1:0.9 v/v. Each test was performed in triplicate (n=3). The robustness was interpreted as %RSD of peak areas.

2.6 Specificity

Specificity were performed by the comparison of UV absorbance spectra at the peak apex among samples and standard (peak identity) and the comparison of UV spectra were recorded at up-slope, apex and down-slope of the peak (peak purity).

2.7 Data analysis

The aloe-emodin contents between TLC image analysis and TLC densitometry method were compared by paired *t-test* statistical analysis.

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CHAPTER IV

RESULTS

Part I Characteristics feature

1. The results of investigation of aerial part

The aerial parts including leaf, flowers and pod of 16 *Cassia* species were observed and illustrated by hand drawing in the proportion size related to the original scale.

1.1 *Cassia bakeriana* Craib

Thai name: Kalapaphruek (กัลปพฤกษ์), Chaiyaphruek (ชัยพฤกษ์), Daug-kapi (ดอกกะปิ)

Location found in Thailand: Chiang Mai (Doi Suthep), Phrae, Lampang, Phitsanulok, Sukhothai (Thung Salaeng Luang), Nakhorn Rachasima, and Saraburi

Distribution: Myanmar and Thailand

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Description: "*Tree up to 10 m high; all younger parts densely hairy. Leaves with 5-7 pairs of leaflets; rhachis 15-40 cm, light-brown velvety pubescent as the 2-4 cm long petiole. Stipules narrow-lanceolate, pointed towards both ends, attached in the middle. Leaflets oblong-oblanceolate, 6-8 by 1.5-3 cm, both ends ± rounded; apex with a small sharp point; on both sides ± densely velvety hairy; petiole 3 mm long. Racemes lateral, 5-12 cm long, 1-few together; main axis yellowish pubescent. Bracts lanceolate, apex long-pointed, hairy on both sides, 7-12 by 3 mm at base; bracteoles same shape but only ± of the length. Flowers large (the largest Cassia flower in Thailand) on a 6 cm, thinly pubescent pedicel, more densely hairy in a ring just below the calyx. Sepals ovate-lanceolate with acute apex, 9-12 by*

2-3 mm, hairy on both surfaces. Petals ovate-lanceolate, pinkish 3.5-4.5 by 1-2.5 cm with a 5 mm long, narrow claw. Stamens 10;3 long with filament 3.5-5 cm long, swollen in the middle, anther 5 mm long, ovoid, opening by apical and basal slit; 4 with filament only half the length but with anther nearly twice as long opening by slits; reduced stamen 3 small, with filament 1-1.5 cm long and very small anthers. Ovary 4 cm long, recurved, on a 1-1.5 cm long stipe, white pubescent with the subapical, punctiform stigma. Pods terete, softly grey to brownish velvety pubescent, 30-40 cm long, 1-1.5 cm diam. Seeds 30-40, separated by spongy septa."

Figure 12 Photography of *C. bakeriana*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pod

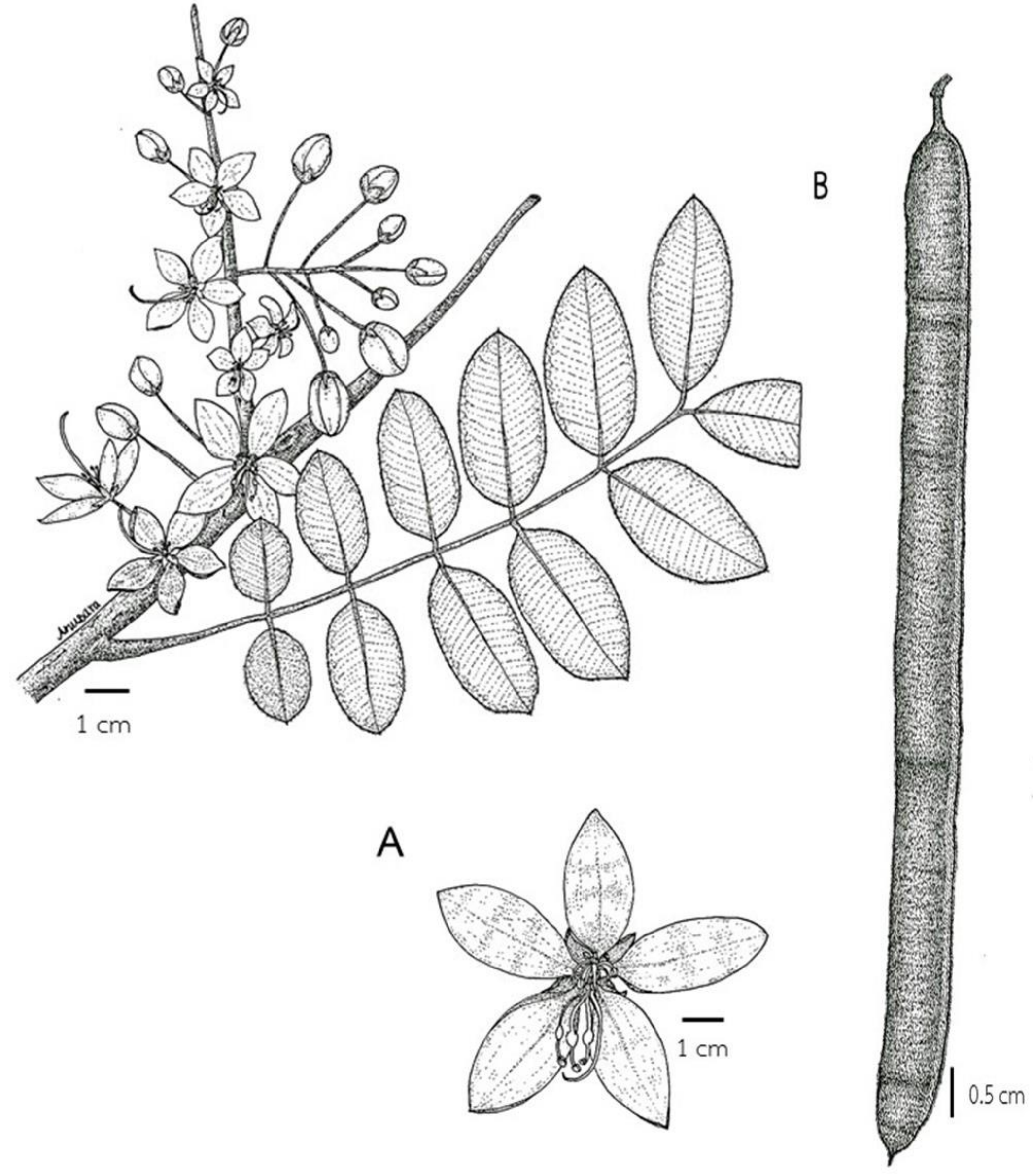

Figure 13 Twig of *C. bakeriana*; (A) Flower and (B) pod

1.2 *Cassia fistula* L.

Thai name: Ratchaphruek (ราชพฤกษ์), Khuun (คูน), Lomlaeng (ลมแล้ง), Ku-phe-ya (กุเพยะ), Chaiyaphruek (ชัยพฤกษ์)

Location found in Thailand: All over the country, often planted as an ornamental.

Distribution: India, Malesia, Thailand, China and Egypt.

Description: "***Tree****, rarely above 10-15 m high with glabrous branches.* ***Leaves*** *with 3-8 pairs of leaflets, large.* ***Petioles*** *7-10 cm; rhachis 15-25 cm. Leaflets ovate-oblong, 7-12 by 4-8 cm, glabrous when mature; petiolules 5-10 mm. Stipules small, caduceus.* ***Racemes*** *axillary, few together, pendent, lax, 20-40 cm long. Pedicels glabrous, 15-35 mm.* ***Bracts*** *8-10 mm long, caduceus.* ***Sepals*** *ovate-elliptic, velutinous outside, 7-10 mm long.* ***Petals*** *yellow, ovate, 30-35 by 10-15 mm, sub equal, short-clawed.* ***Stamens*** *10; 3 long ones with filaments 3 cm long, anthers opening by apical and basal slits; 4 shorter with filaments 8-10 mm long, anthers opening by a basal pore; reduce stamens 3 with filament 5 mm long and minute anther.* ***Ovary*** *and style velutinous; stigma small.* ***Pods*** *terete, glabrous, black, 20-60 cm long, 1.5-2 cm diam., with numerous seeds separated by spongy septs. Seeds elliptic, flattened, glossy brown, 8-9 by 5 mm.*"

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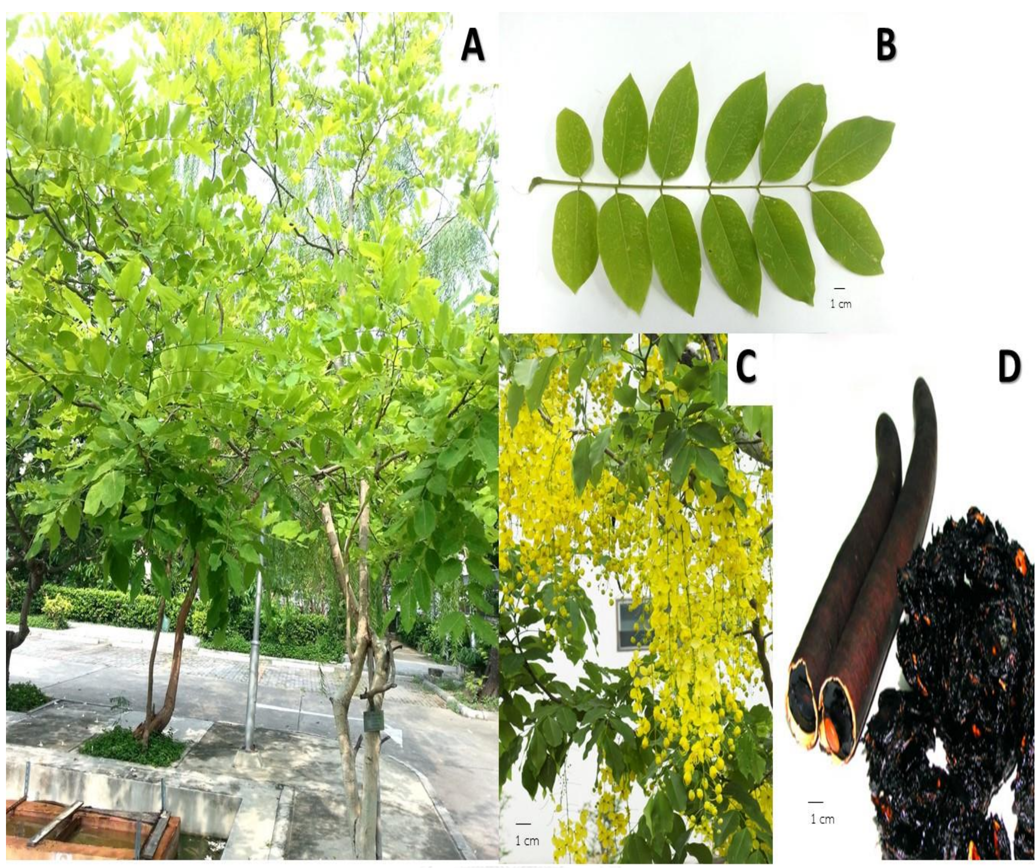

Figure 14 Photography of *C. fistula*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pod and ripe pod

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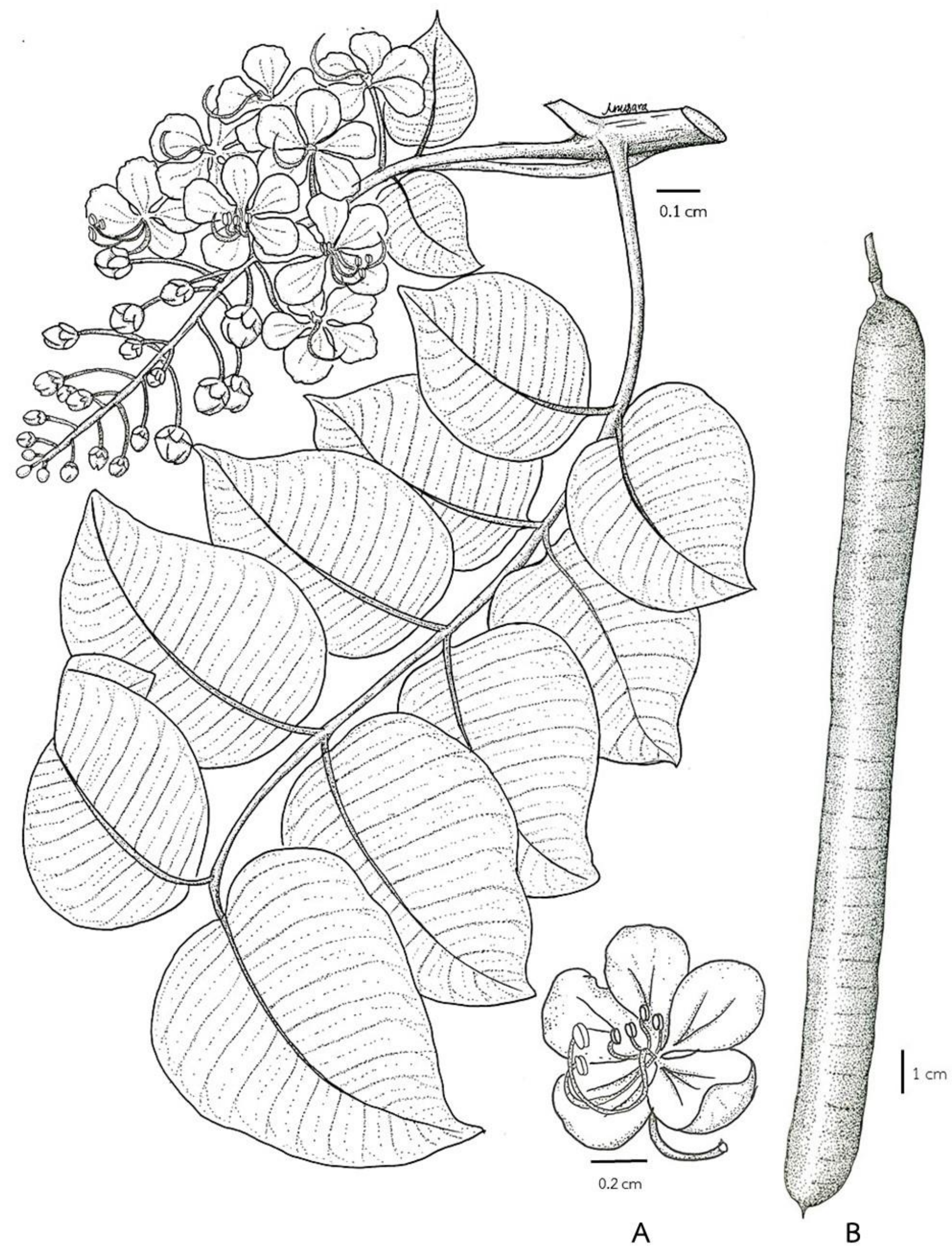

Figure 15 Twig of *C. fistula*; (A) Flower and (B) pod

1.3 ***Cassia grandis* L. f.**

Thai name: Kanlaphruek (กาลพฤกษ์), Kalapaphruek (กัลปพฤกษ์)

Location found in Thailand: Widely cultivated (Buri Ram, Sukhothai, Pathum Thani, and Phitsanulok), sometimes escape from garden.

Distribution: tropical America (cultivated throughout the tropics) Cambodia, Java, Malaysia, Vietnam and Thailand

Description: *"Deciduous tree up to 20 m high with buttressed trunk; young branches and inflorescence covered with short brownish to white wool.* ***Leaves*** *with 10-20 pairs of leaflets.* ***Petioles*** *2-3 cm, woolly; rhachis 10-25 cm.* ***Stipules*** *minute.* ***Leaflets*** *with short petiolules, sub coriaceous, elliptic-oblong, 3-5 by 1-2 cm, upper surface glossy. Lower woolly; apex and base rounded.* ***Racemes*** *lateral, 10-20 cm long with ca 20 flowers.* ***Pedicels*** *glabrous, 15-35 mm.* ***Bract****s ovate, acute, 5 mm long, caduceus; bracteoles smaller inserted at base of the 1-2 cm long pedicel.* ***Sepals*** *pubescent on both side, obovate, rounded, 5-8 mm long, finally reflexed.* ***Petals*** *obovate, 15 mm long, short-clawed, first red, lateral pink finally orange.* ***Stamens*** *10; 3 long with recurve filaments 3 cm long, anthers pubescent, 2.5 mm long, opening by apical large and smaller, basal slits; 5 shorter with straight and smaller anther; 2 reduce stamens2 mm long.* ***Ovary*** *silky tomentose; style short; stigma small.* ***Pods*** *woody, rugose, glabrous, compressed-cylindric, blackish, 20-40 cm long, 3-4 cm diam.* ***Seeds*** *are 20-40."*

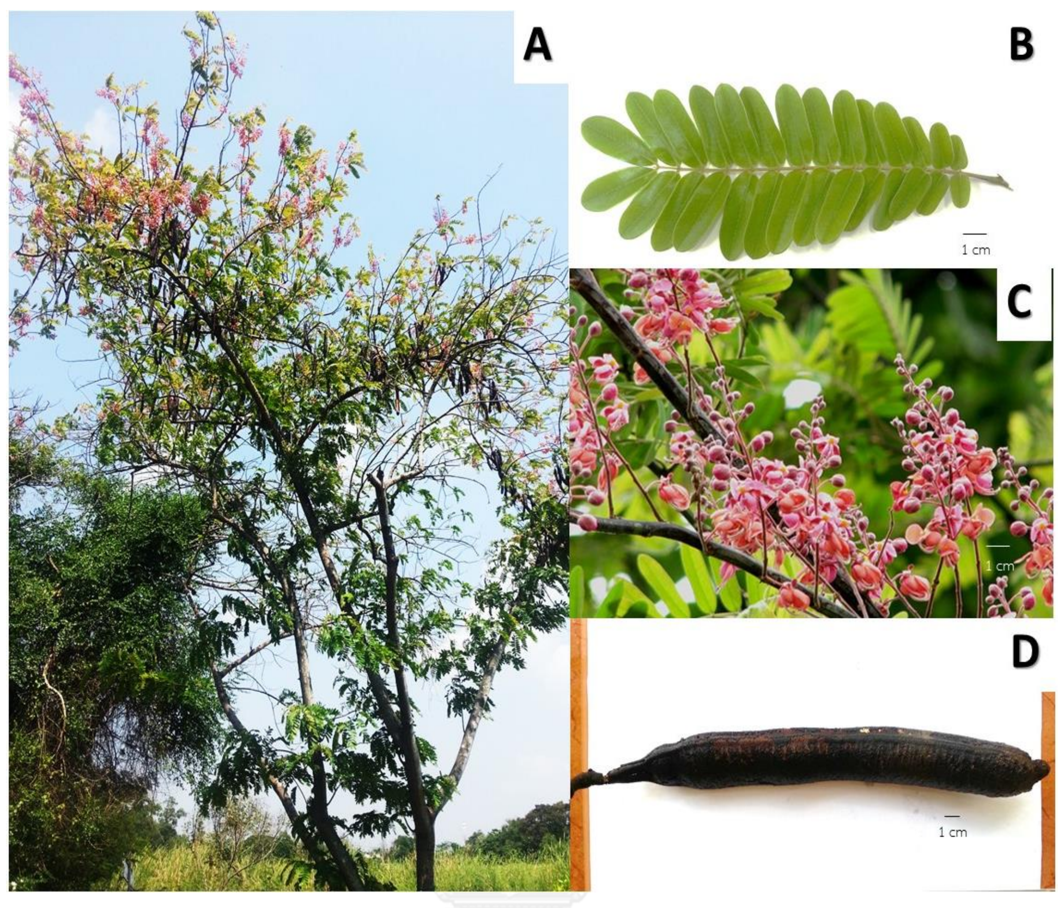

Figure 16 Photography of *C. grandis*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pod

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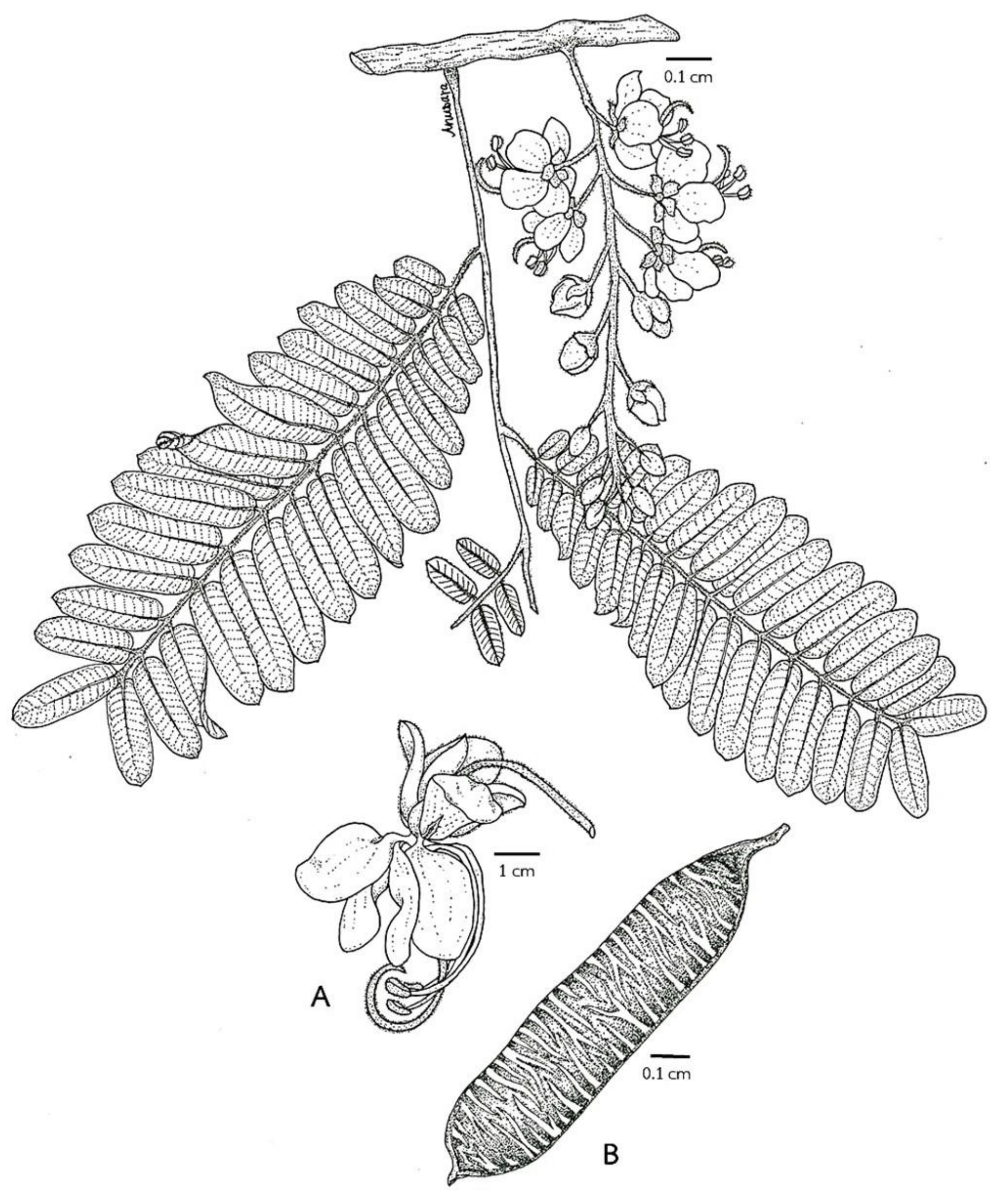

Figure 17 Twig of *C. grandis*; (A) Flower and (B) pod

1.4 *Cassia javanica* L.

Thai name: Chaiyaphruek (ชัยพฤกษ์), Ratchaphruek (ราชพฤกษ์) Kalapaphruek (กัลปพฤกษ์), Lak khoei lak klua (ลักเกยลักเกลือ)

Location found in Thailand: Cultivated (Nakhon Pathom, Phitsanulok, Pathum Thani, Ratchaburi, and Ubon Ratchathani)

Distribution: Indonesia, Philippines and Thailand

Description: "*Deciduous tree up to 15 m high; young specimens with the trunk armed with stump of branches; branches nearly glabrous.* ***Leaves*** *with 5-15 pairs of leaflets.* ***Stipules*** *falcate to point, elliptic, attached in the middle. Pedicels 1.5-4 cm nearly glabrous; rhachis 20-30 cm.* ***Leaflets*** *on a short petiolule, elliptic-ovate to oblong, 2.5-5 by 1.5-2.5 cm, apex rounded to blunt, base usually broadly round; upper surface feebly shining, lower dull, finely appressed pubescent.* ***Racemes*** *arising laterally from the branch, forming a corymb, patent or deflexed, 5-16 cm long.* ***Peduncle*** *2-3 cm.* ***Bracts*** *ovate-acute, 10-15 mm long; bracteoles axillary, linear oblong, 4-5 mm long.* ***Pedicel*** *3-5 cm.* ***Sepals*** *ovate-acute, dark red to reddish brown, 7-10 mm.* ***Petals*** *first pink later dark red, finally pale, obovate, 25-35 by 7-8 mm with a 3 mm long claw.* ***Stamens*** *10; 3 long recurved with a spherical enlargement near the middle of the 20 mm long filament, anther 4 mm long opening by apical and basal slits; 4 shorter ones ca 10 mm long with larger anther opening by basal pore; reduced stamens 3 ca 10 mm long with minute anthers.* ***Ovary*** *pubescent, slender, recurve on a thin stipe; stigma indistinct.* ***Pods*** *terete, glabrous, black, indehiscent, 20-60 cm long, 1-1.5 cm diam.* ***Seeds*** *are 50-75 glossy corky, brown, flat, ± orbicular, embedded in a flat disc.*"

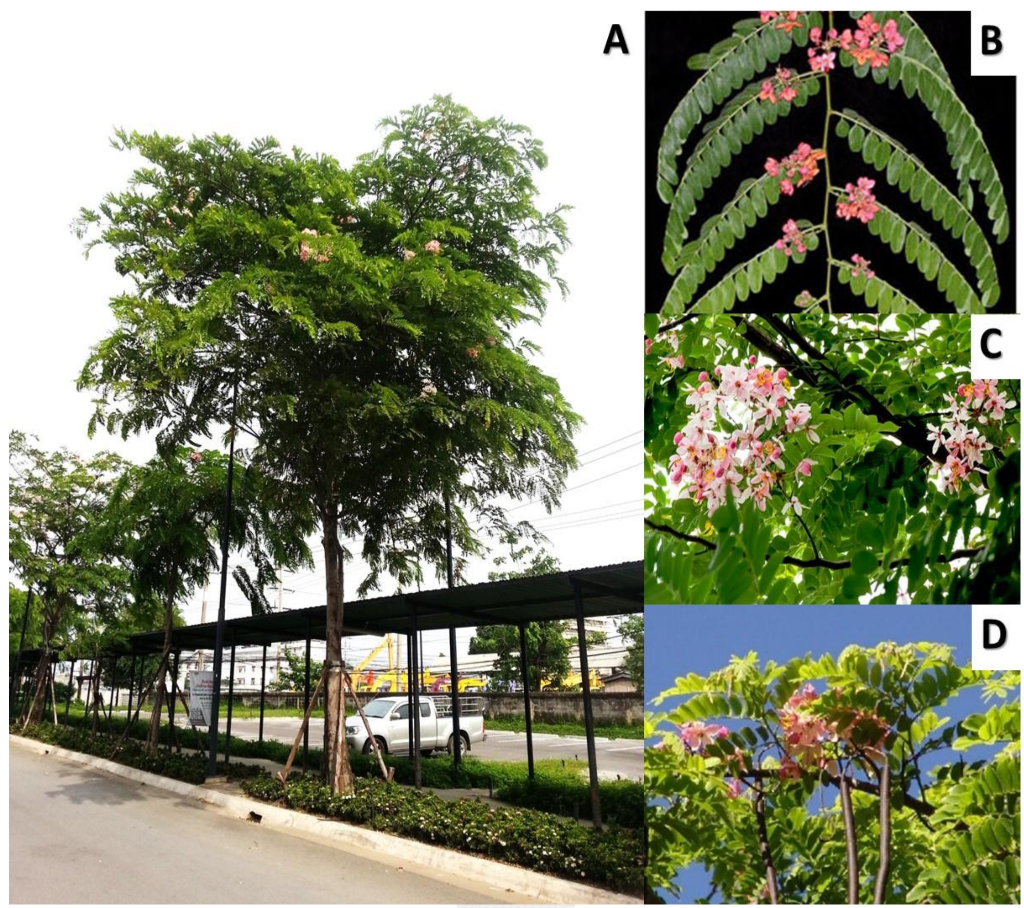

Figure 18 Photography of *C. javanica*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pod

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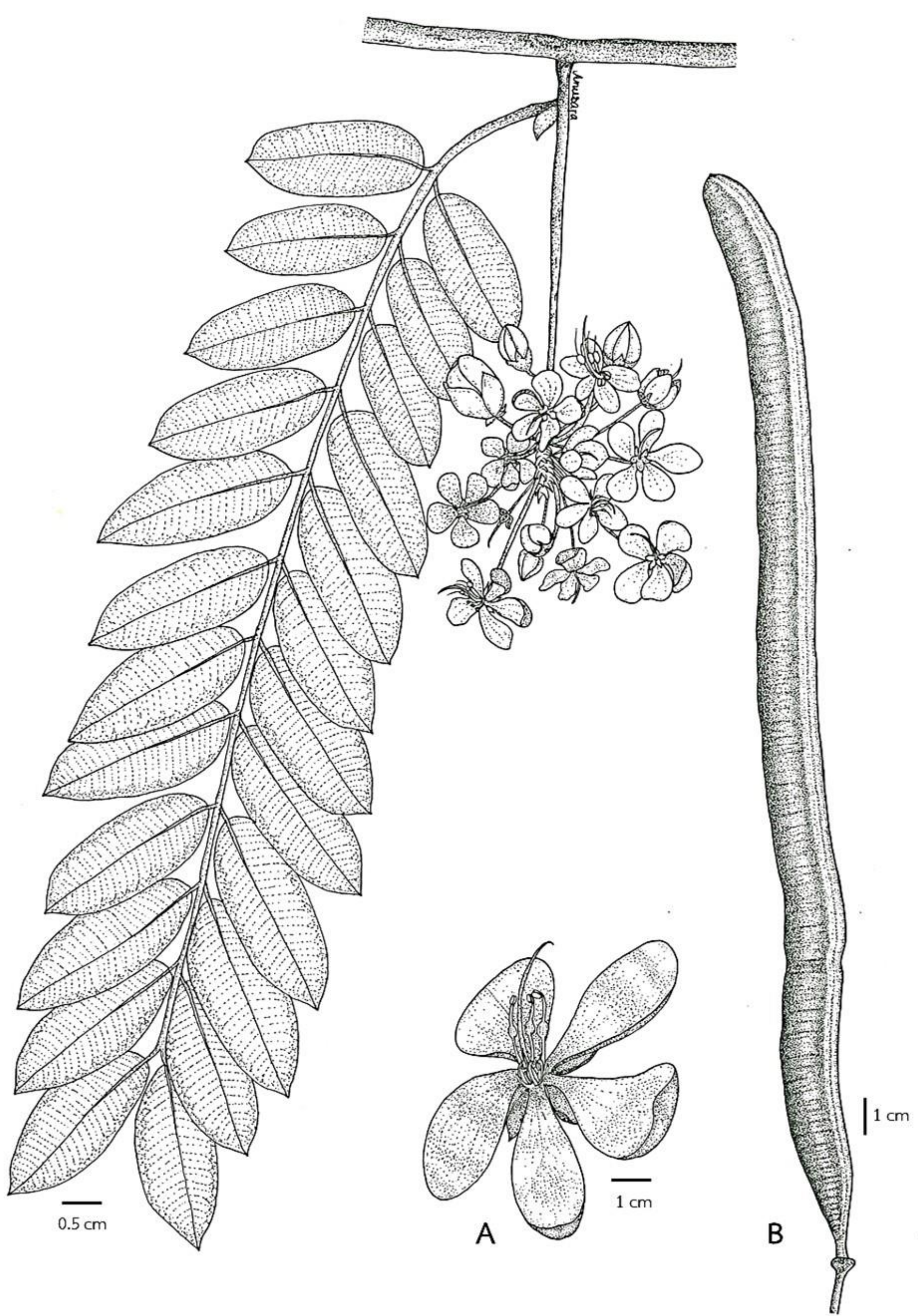

Figure 19 Twig of *C. javanica*; (A) Flower and (B) pod

1.5 *Cassia alata* L.

Thai name: Chum het thet (ชุมเห็ดเทศ), Chum het (ชุมเห็ด), Chum het yai (ชุมเห็ดใหญ่), Khi khak (ขี้คาก), Lap muen luang (ลาบมึนหลวง), Mak kaling-thet (หมากะลิงเทศ), Ta-see pho (ตะสีพอ)

Location found in Thailand: Found all over the country up to 1500 m; sometimes cultivated for medical purpose

Distribution: America, India, Indonesia, Malaysia, Thailand, Brazil and Africa

Description: *"**Shrub** 1-2 (-5) m high with pubescent, horizontally spread branches. **Leaves** with 8-20 pairs of leaflets. Petioles robust ca 2 cm; rhachis 30-60 cm. **Stipules** auriculate, persistent, deltoid, 6-8 mm long. **Leaflets** oblong-elliptic, rounded at both ends, 5-15 by 3-7 cm, glabrous; petiolules robust, 2-3 mm. Racemes axillary. Dense, robust, 20-50 cm long, 3-4 cm board. Bracts caducous, 2-3 by 1-2 cm. **Pedicels** very short ca 2-4 mm. **Sepals** unequal, oblong, 10-20 by 6-7 mm. **Petals** bright yellow, ovate-orbicular to spathulate, short-claw, 2 by 1.0-1.5 cm. **Stamens** 9-10; 2 largest with thick filaments, 4 mm long and anthers 4-5 mm opening by apical pore; 4 with filament 2 mm long and anther 12-13mm long opening by apical pores; 3-4 stamens reduced. **Ovary** and style glabrous; stigma small. Pods thick, flattened, winged, glabrous, septate, 10-15 by 1.5-2 cm; wing 5 mm. **Seeds** ca 50, flattened ± quadrangular 7-10 by 5-8 mm."*

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Figure 20 Photography of *C. alata*; (A) habitat, (B) leaves,
(C) inflorescences, and (D) pod

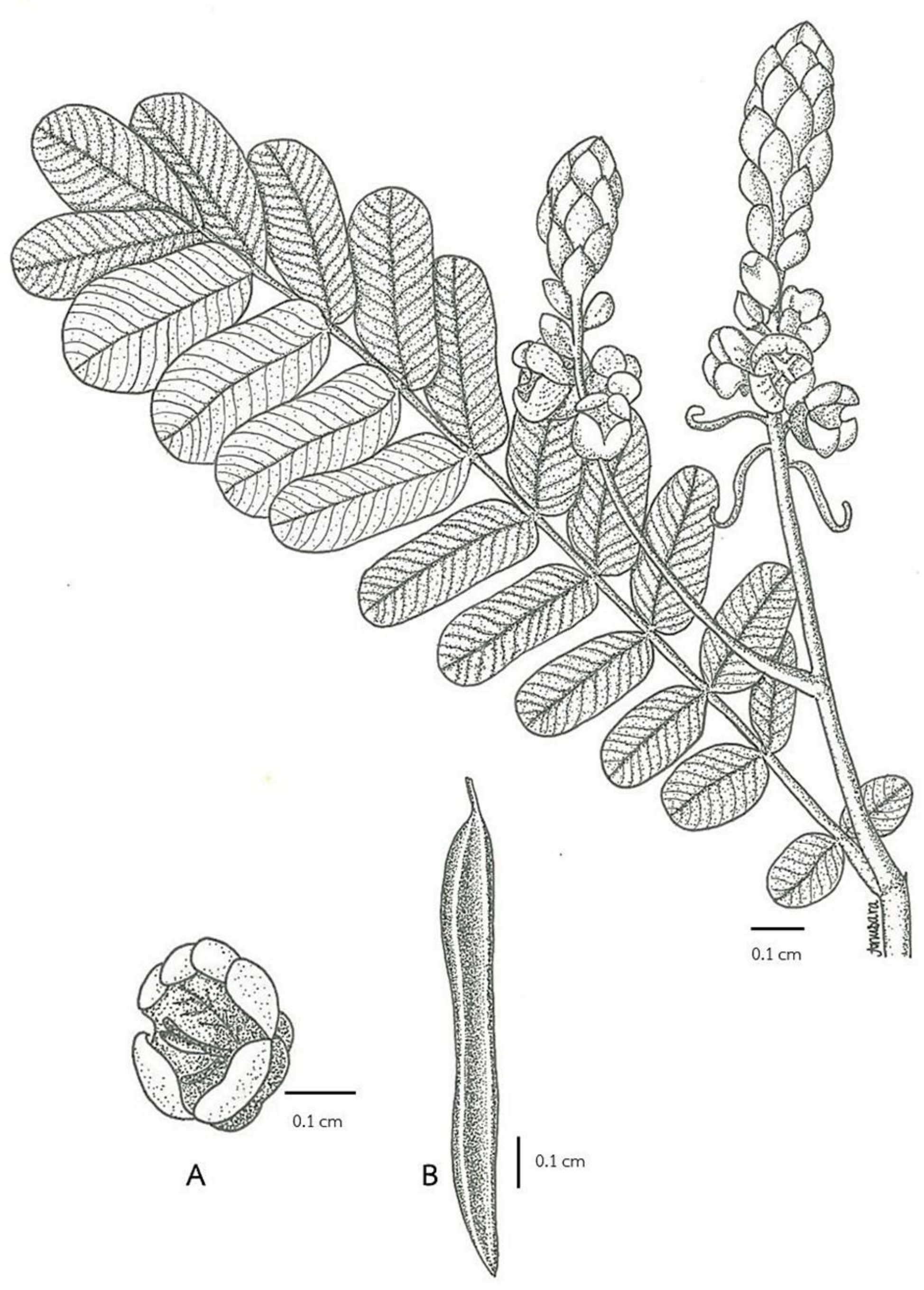

Figure 21 Twig of *C. alata;* (A) Flower and (B) pod

1.6 *Cassia angustifolia* Vahl

Thai name: Makhaam khaek (มะขามแขก), Som khaek (ส้มแขก)

Location found in Thailand: Si Sa Ket, Phitsanulok, and Sukhothai

Distribution: Saudi, Arabia, India and Thailand

Description: *"Perennial, up to 90 cm tall. **Branches** glabrous to sub glabrous. **Stipules** lateral, c. 1.5 mm long, acute. **Leaves** are paripinnate, c. 4.5-11.5 cm long. **Leaflets** 5-9 pairs, petiolule c. 1 mm long, lamina c. 1.2-4 cm long, c 3.5-10 mm wide, glabrous to sparingly hairy on both sides, lanceolate to ovate, tip acute. Inflorescence termical or axillary raceme, up to 15 cm long. **Young flowers** covered with c. 7-8 mm long cup-shaped bracts. **Pedicel** 3-4 cm long. **Sepals** 5, sub equal, 10-13 mm long, c. 6-8 mm broad, spoon shaped or cup shaped, light yellow in color. **Petals** 5, sub equal, 14-17 mm long, 7-10 mm wide, obovate, shortly clawed, deep yellow, veins becoming prominent after drying. **Stamens** 10, upper 3 reduced to staminodes, rest perfect, 2 lower largest. **Ovary** densely hairy, stipitate. Fruits c. 4-5 cm long, c. 16-22 mm broad, sparsely hairy, turning black at maturity, generally 4-10 seeded; stipe 2-3 mm."*

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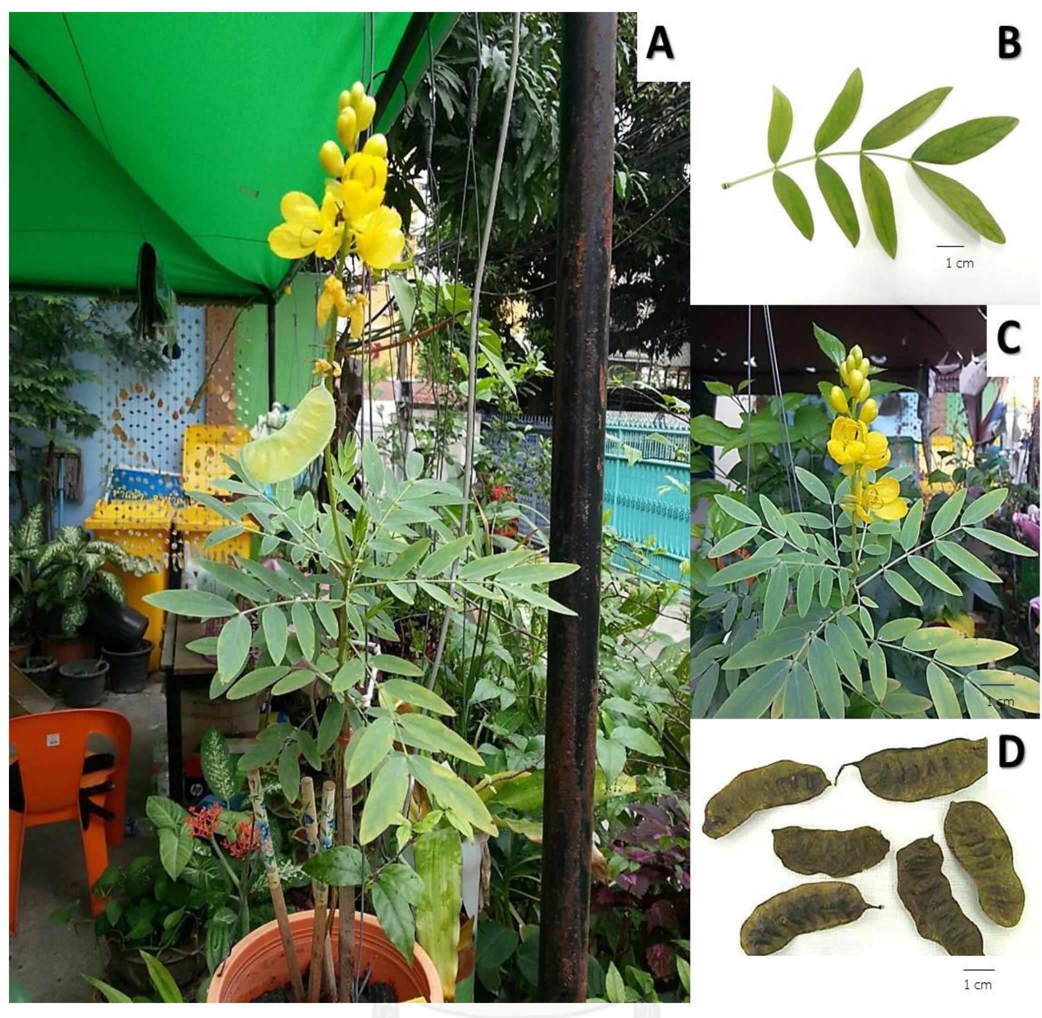

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Figure 22 Photography of *C. angustifolia*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pod

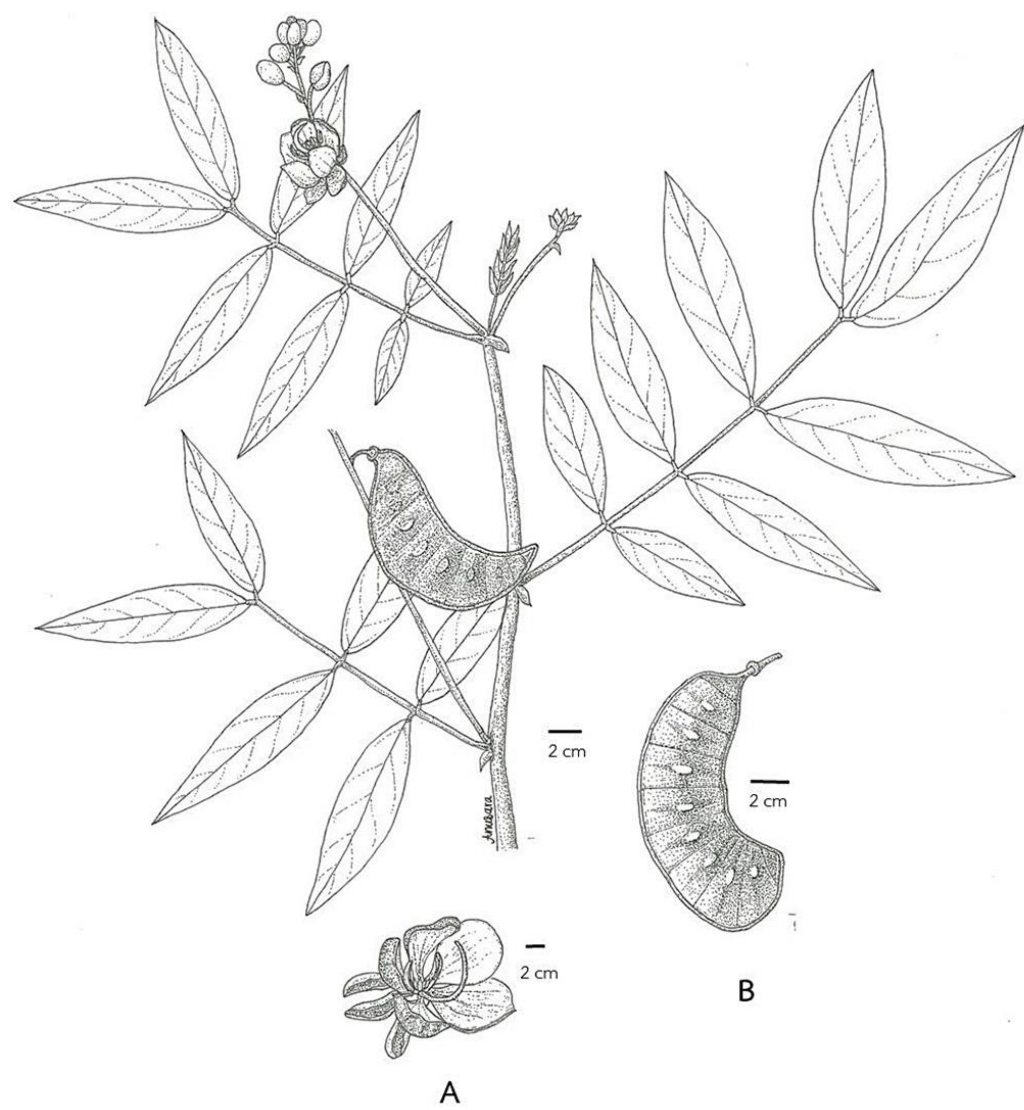

Figure 23 Twig of *C. angustifolia*; (A) Flower and (B) pod

1.7 *Cassia garrettiana* Craib

Thai name: Samae san (แสมสาร), Khi lek khok (ขี้เหล็กโคก), Khi lek phae (ขี้เหล็กแพะ), Khilek pa (ขี้เหล็กป่า), Khi lek san (ขี้เหล็กสาร), Ngai-san (ไงซาน), Kabat (กะบัด)

Location found in Thailand: Common in Northern Thailand down to the Plain of Bangkok. Often planted as a way side tree.

Distribution: Cambodia, Laos, Vietnam and Thailand

Description: *"**Tree** up to 10 m high; branches puberulous, later glabrous. **Leaves** with 6-9 pairs of leaflets. **Petioles** 4-5 cm long; rhachis 10-20 cm long. Stipules early caducous (not seen). **Leaflet** lanceolate to broadly ovate, acuminate with rounded base, 5-9 by 2-5 cm, glabrous or nearly so; petiolules 4-6 mm. **Inflorescences** terminal, leafy, compound raceme, 9-20 cm long, many-flowered; axis densely yellowish velutinous. **Pedicels** 3 cm, pubescent. Bracts ovate, acute, caducous 4 mm long; bracteoles minute. **Sepals** unequal, 2 outer smaller, ca 5 mm long, 3 inner twice as long, broadly elliptic, puberulous outside. **Petals** yellow, obovate, 15-18 mm long, with a 4 mm long clawed. **Stamens** 10, 2 largest with flattened filaments 7 mm long and anther 7-9 mm long curve, opening by apical pores; 5 shorter with smaller anther; reduced stamens 3, ca 2 mm long. **Ovary** and style glabrous or puberulous along the margin; stigma indistinct. **Pods** flat, glabrous, thin-valve, 15-22 by 2-4 cm, often twisted. Seeds ca 20 by 55 mm, brownish."*

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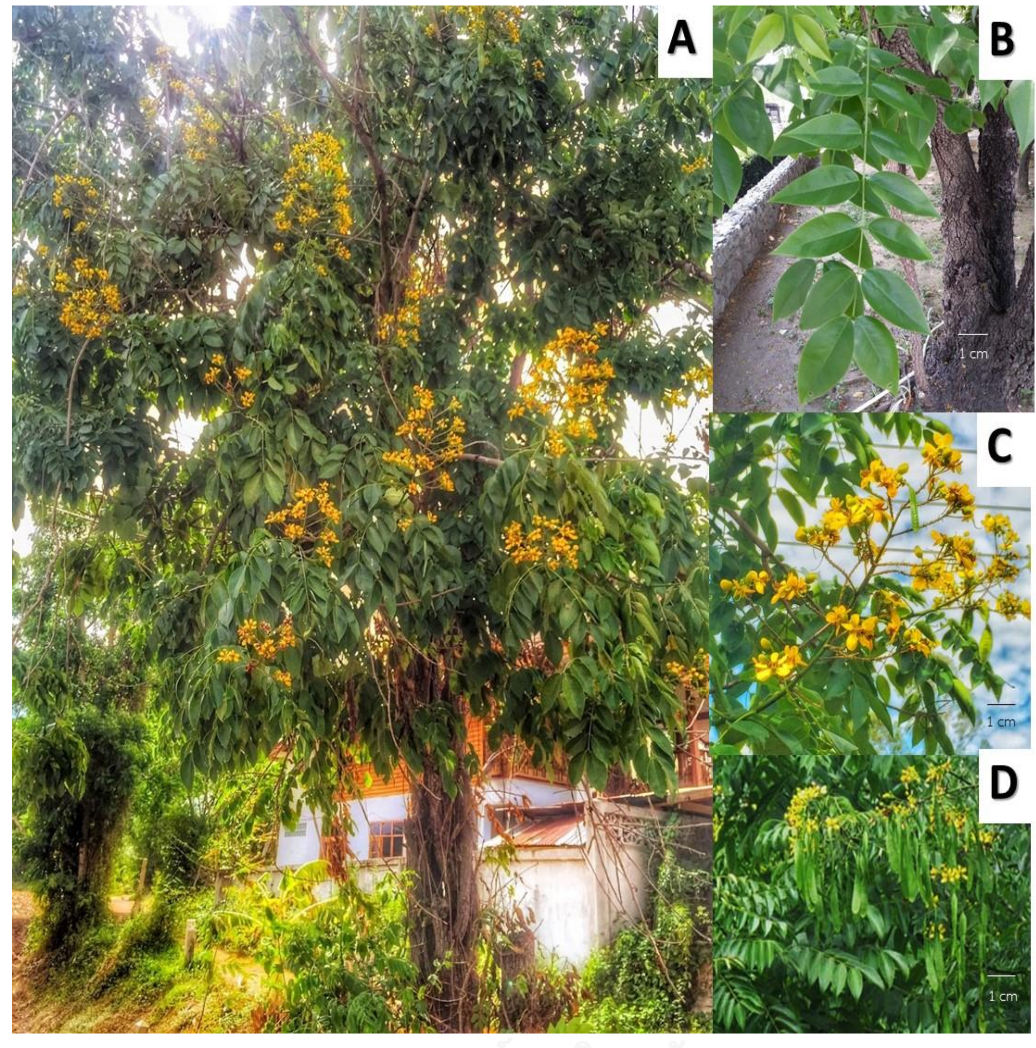

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Figure 24 Photography of *C. garrettiana;* (A) habitat, (B) leaves, (C) inflorescences, and (D) pod

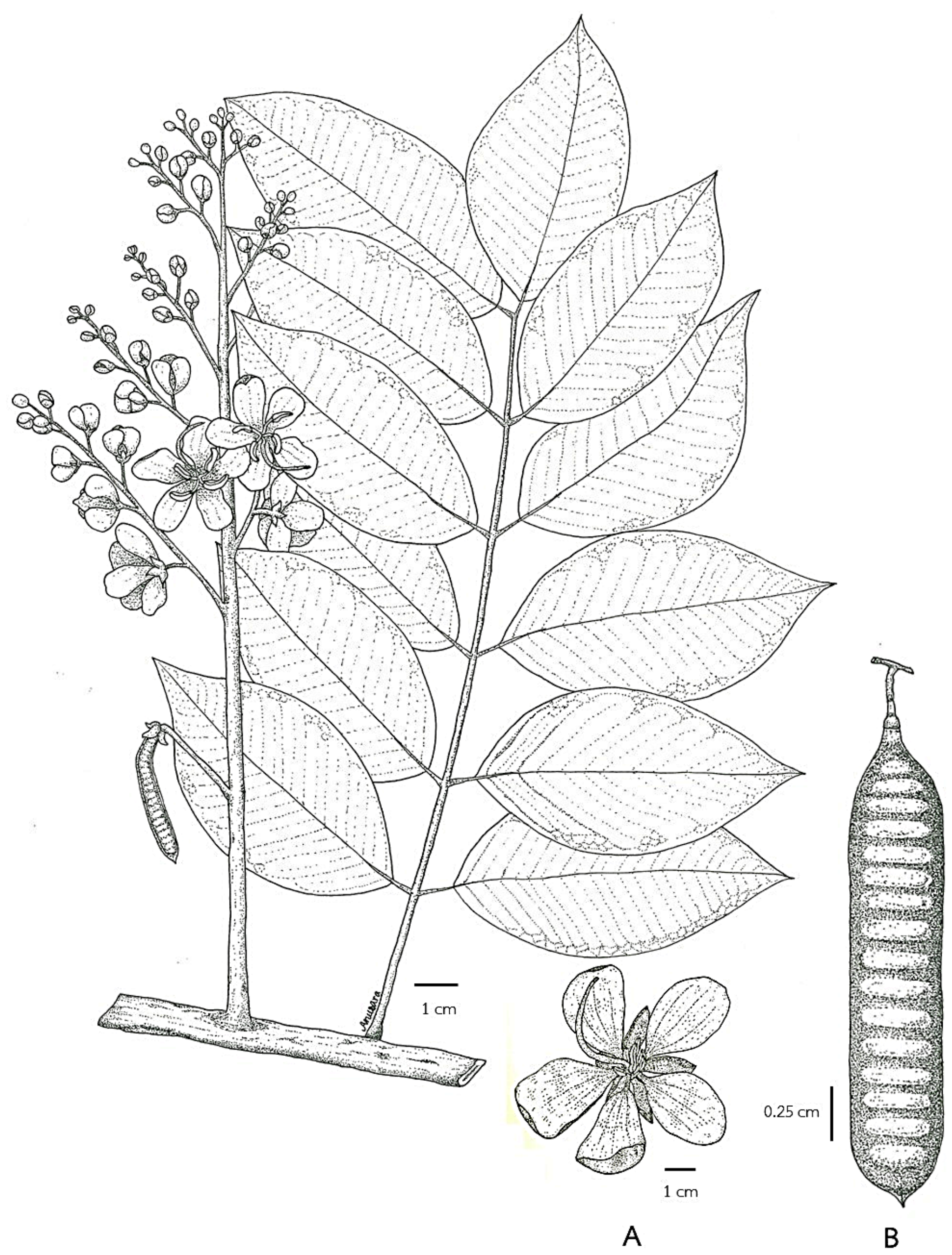

Figure 25 Twig of *C. garrettiana;* (A) Flower and (B) pod

1.8 *Cassia hirsuta* L.

Thai name: Dap phit (ดับพิษ), Phong pheng (โผงเผง)

Location found in Thailand: Found all over the country as a weed, but not common.

Distribution: tropical America and Thailand

Description: *"**Herb** or undershrub up to 2.5 m high, hirsute, with a foetid smell. **Leaves** with 4-5 pairs (rarely more or less) of sub opposite or opposite leaflets. **Petioles** 5-6 cm, villous with a sessile, oblong gland above the joint; rhachis 7-10 cm. **Stipules** linear, acute hairy, ± caduceus, 5-15 mm long. **Leaflets** lanceolate-acuminate, 5-9 by 2-3 cm, hirsute in both surfaces; apex acute, base rounded; the upper pair larges; petiolules short. **Racemes** few-flowers, short, from the upper leaf-axil. **Bracts** 4-5 mm long, hirsute. **Flowers** on a 1-2 cm pubescent, filiform pedicel. **Sepals** unequal; 2 outer smaller, orbicular 5-6 mm; 3 inner larger, 7-9 mm. **Petals** yellow, unequal, obovate, 15-28 mm long glabrous, short- clawed. **Stamens** 10, 2 large with flat filaments 5-7 mm long and anthers 7-8 mm long, opening by apical pores; 4 smaller opening the same way; reduced stamens 4, 3 mm. **Ovary** grayish woolly, recurve; style glabrous; stigma slightly enlarge, ciliate. **Pods** falcate to nearly straight, 6-13 by 0.5 cm, hirsute, angulate. **Seeds** numerous dark olive, orbicular, ca 3 mm diam."*

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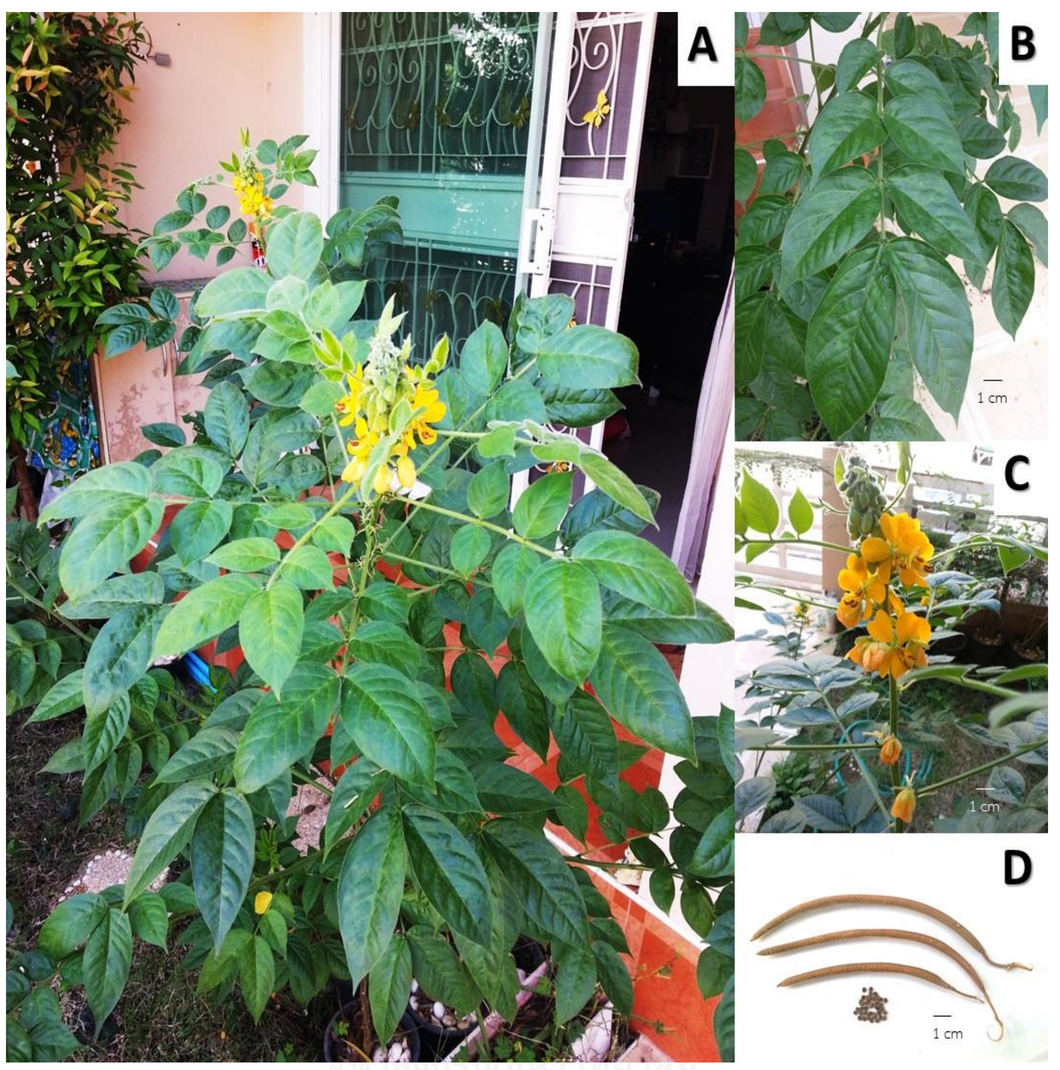

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Figure 26 Photography of *C. hirsuta*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pods and seeds

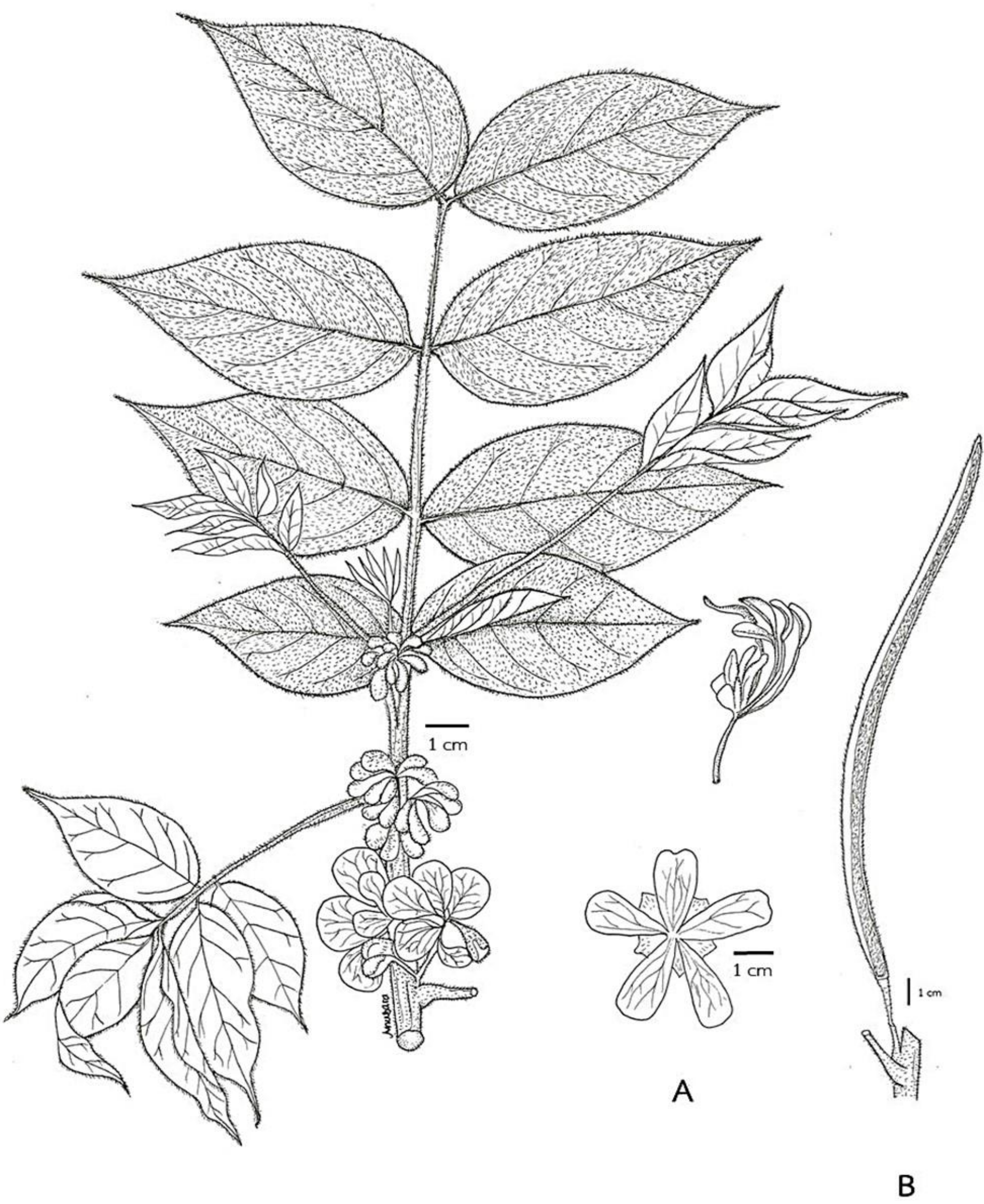

Figure 27 Twig of *C. hirsuta*; (A) Flower and (B) pod

1.9 *Cassia occidentalis* L.

Thai name: Chum het lek (ชุมเห็ดเล็ก), Chum het thet (ชุมเห็ด-เทศ), Khi lek phuak (ขี้เหล็กเผือก), Lap muen noi (ลับมืนน้อย), Chum het lek (ชุมเห็ดเล็ก) Chum het thet (ชุมเห็ดเทศ), Khi lek phuak (ขี้เหล็กเผือก), Lap muen noi (ลับมืนน้อย)

Location found in Thailand: Common weed all over the country from sea level up to 1000 m

Distribution: tropical America and Thailand

Description: *"**Herb** or undershrub up to 150 (-250) cm ± glabrous. **Leaves** with 3-5 pairs of leaflets. **Petioles** 3-4 cm long with a relatively large, ovoid gland just above the petiole joint; rhachis 8-12 cm. **Stipules** linear-acute, ± falcate, 1-2 cm long. **Leaflets** membranous, ovate-oblong, ± unequal-side, 4-10 by 2-3 cm, apex acuminate, base round; petiolues 2 mm. **Racemes** short- short-peduncled, 2-4 flowered, mainly terminal. Bracts linear-acute, caducous. **Pedicels** 5-10 mm. **Sepals** unequal, outer one orbicular, 6 mm diam., inner ovate larger. **Petals** yellow with violet veins 2 outer slightly larger, 1-2 by 1.5 cm, short-clawed. **Stamens** 9-10, 2 long with filaments 5-6 mm long, anther 5-7 mm long opening the same way; reduced stamens 3-4 with filaments 1-2 mm long and minus anthers. **Ovary** tomentose; style glabrous; stigma lateral small. **Pods** flattened, glabrous, brown with pale margins, 10-12 by 1 cm, hirsute, angulate. Seeds 30-40, flat, orbicular, 3-4 mm diam."*

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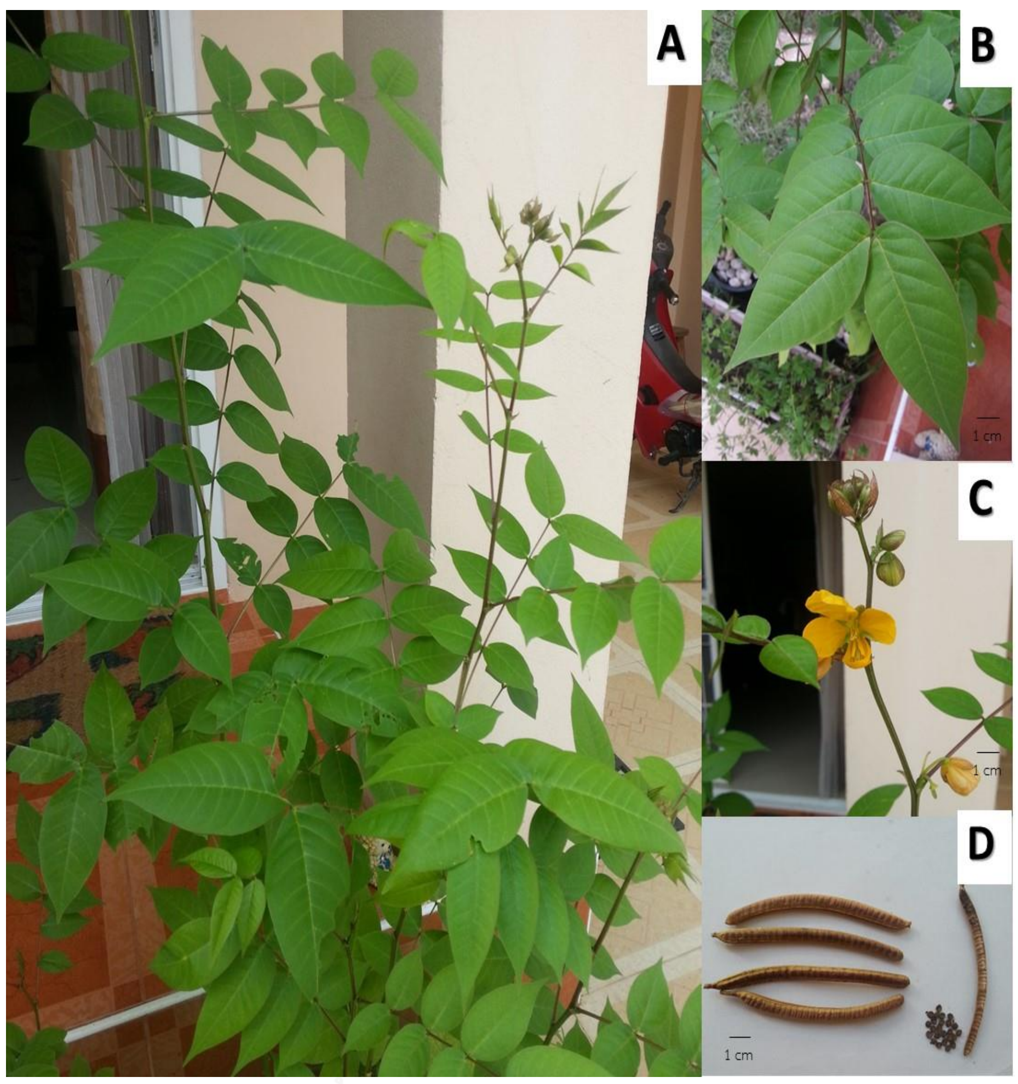

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Figure 28 Photography of *C. occidentalis*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pods and seeds

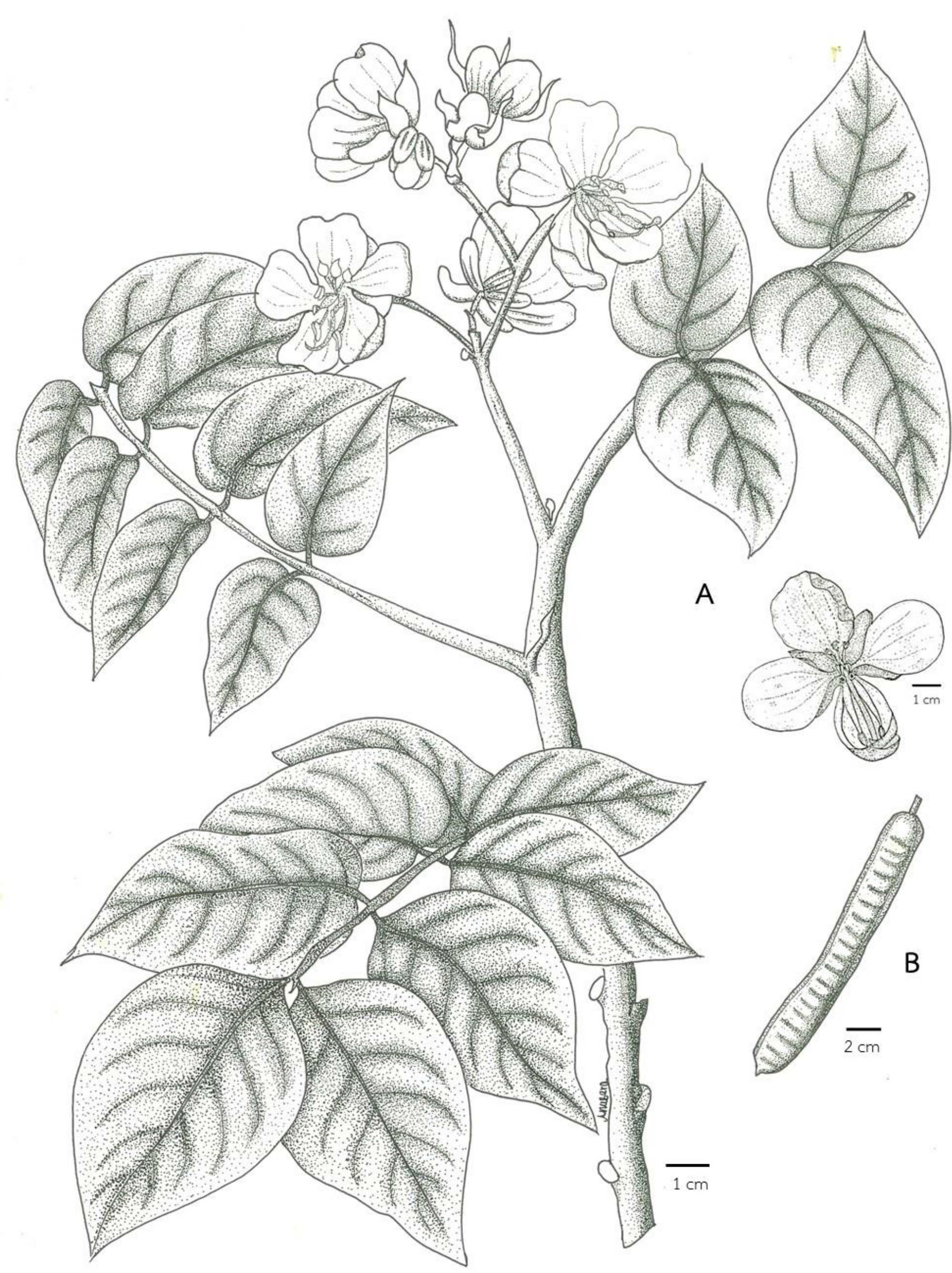

Figure 29 Twig of *C. occidentalis*; (A) Flower and (B) pod

1.10 *Cassia spectabilis* DC.

Thai name: Khee lek American (ขี้เหล็กอเมริกัน)

Location found in Thailand: Commonly cultivated (Phetchabun, Chiang Mai, Lampang, Nakhon Sawan, Phitsanulok and Nakhon Ratchasima)

Distribution: Central America, Brazil and Thailand

Description: *"**Small tree** up to 7 m high with long, spreading leafy branches; young parts softly pubescent. **Leaves** with 10-15 pairs of leaflets. **Petioles** 3-4 cm; rhachis 20-30 cm. **Stipules** linear, falcate, early caducous, 1 cm long. **Leaflets** with short petiolute, narrow elliptic, 3-7 by 1-2 cm, base rounded, apex acute, mucronate; upper surface glabrous, lower finely appressed pubescent. **Inflorescences** large, terminal, leafy panicles, 20-30 cm. **Bracts** ovate, 4-5 mm long, canducous. Pedicels 2-3 cm, velutinous. **Sepals** unequal, 2 outer pubescent, 3 inner glabrous, larger, board falcate, 2-2.5 mm long. **Petals** yellow, spathulate, short-clewed, the lower one larger, broad falcate, 2-2.5 mm long. **Stamens** 10; 7 large with filaments 2-3 cm long anthers opening by apical pore and a slit; reduce stamens 3, with reniform anthers much smaller. **Ovary** glabrous, recurved; style and stigma inconspicuous. **Pods** ± terete glabrous, glossy, black, annulate septate, 18-25 by 1 cm. **Seeds** 50-70, suborbicular, pointed at one end, 5 mm diam.; septa papery."*

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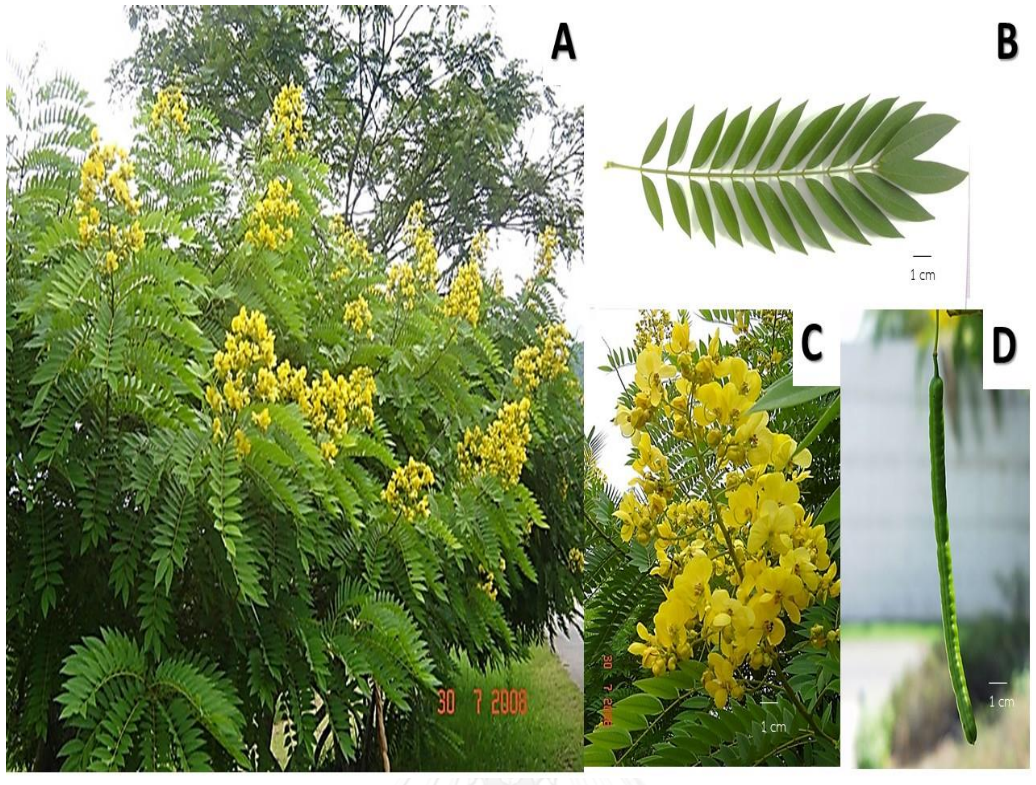

Figure 30 Photography of *C. spectabilis*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pods and seeds

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Figure 31 Twig of *C. spectabilis;* (A) pod and (B) Flower

1.11 *Cassia siamea* Lam.

Thai name: Kheelek (ขี้เหล็ก), Khee lek ban (ขี้เหล็กบ้าน), Khee-lek luang (ขี้เหล็กหลวง), Khee lek yai (ขี้เหล็กใหญ่), Ya ha (ยะหา)

Location found in Thailand: All over the country. **Distribution:** Southeast Asia, India and Thailand

Description: *"**Medium-size tree**; young branches striate, finely pubescent. **Leaves** with 7-10 (-15) pairs of leaflets. **Petiole** 2-3 cm; rhachis 10-25 cm long. **Stipules** minute, subulate, caduceus. **Leaflets** on a short petiolule, ovate-oblong, base round, apex rounded or emarginated with a short, mucronate tip, glabrous on upper surface, ± finely pubescent on lower; 3-7 by 1-2 cm. **Flowers** in large terminal panicles on a robust 5-7 cm long peduncle. **Bracts** obovate with long acte apex, 5 mm long; bracteoles absent. **Pedicels** 2-3 cm, velutinous. **Sepals** 5, orbicular, thick, unequal, 2 outer small, 5 mm long, 3 inner up to 9 mm long, hairy on the outer side. **Petals** yellow broadly obovate, 1.5-2 cm long, short clawed. **Stamens** 10, 2 with filaments 2-4 mm long and anther 5-6 mm opening the same way; reduced stamens 3, ca 2-4 mm. **Ovary** finely pubescent; style glabrous; stigma indistinct. **Pods** flat, glabrescent, longitudinally wave with raised sutures, 20-30 by 1-1.5 cm. **Seeds** 20-30 flat, oval, light brown, 10-15 by 5-6 mm."*

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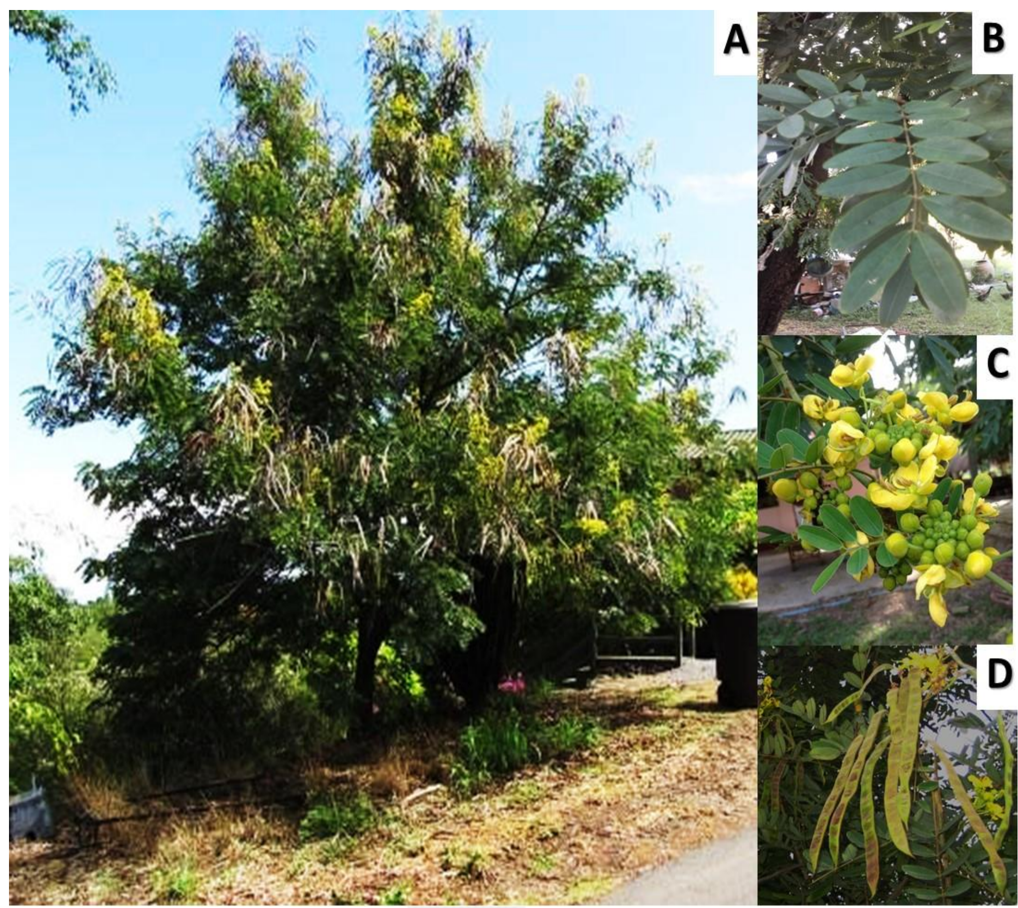

Figure 32 Photography of *C. siamea*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pods and seeds

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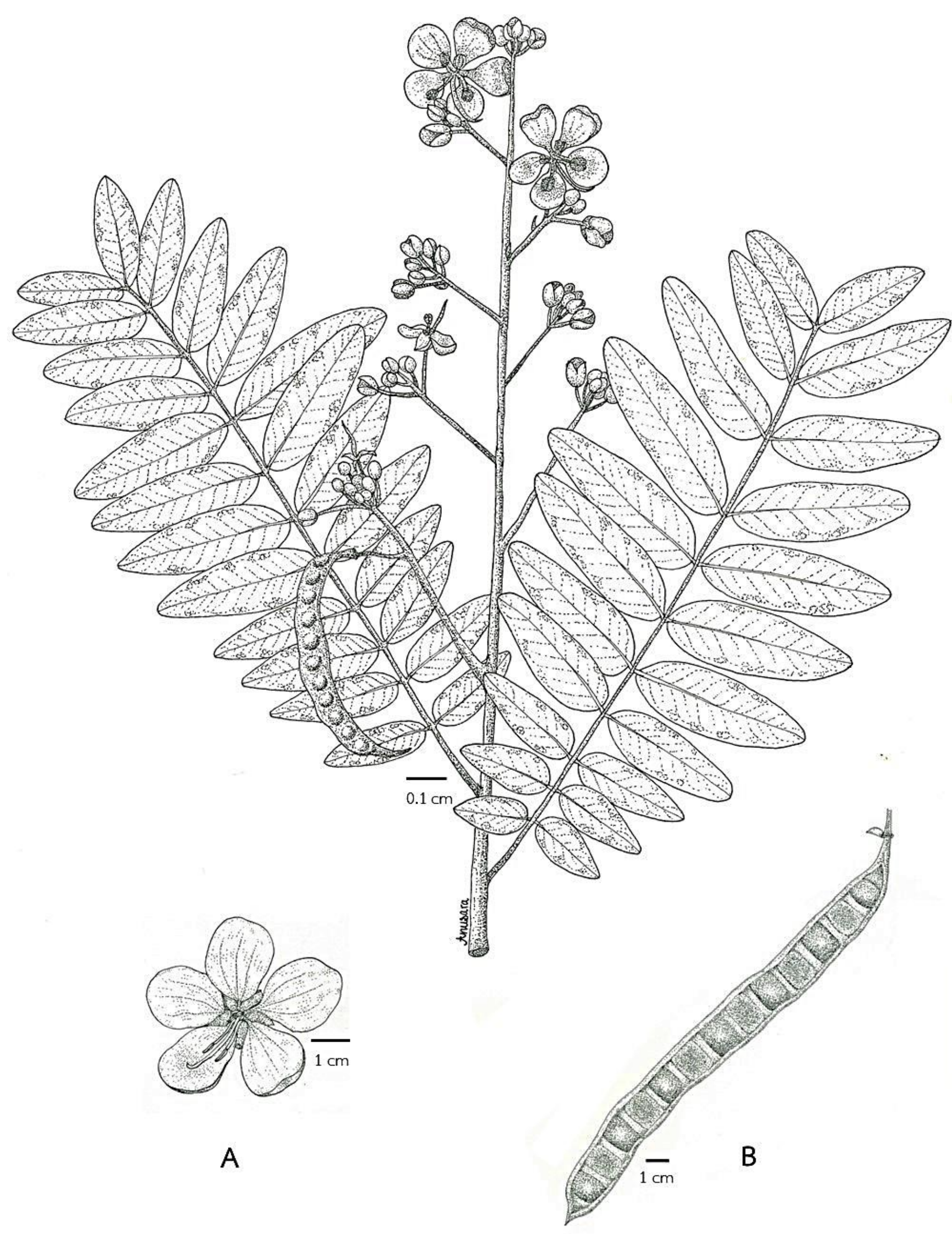

Figure 33 Twig of *C. siamea*; (A) Flower and (B) pod

1.12 *Cassia sophera* L.

Thai name: Phak khet (ผักเค็ด), Phak khlet (ผักเคล็ด), Phak wan ban (ผักหวานบ้าน)

Location found in Thailand: All over the country a common weed.

Distribution: India and Thailand

Description: *"**Shrub** up to 1-3 m high, nearly glabrous. **Leaves** with 4-9 pairs of relatively narrow leaflets. **Petioles** 3-5 cm long with thin, subulate gland, 0.5-1 cm above the petiole joint; rhachis 9-15 cm. **Stipules** ovate, caducous, ca 5 mm long. **Leaflets** membranous, lanceolate; apex acute, base rounded, 2-5 (-8) by 1-2 cm, the upper leaflet largest. **Flowers** in axillary, few flowered corymbs. Bracts ovate, ca 5 mm long. **Peduncles** 1-2 cm; pedicels 1-1.5 cm; bracteoles absent. Sepals ovate-rounded, 5 mm long. **Petals** yellow, obovate, 10-14 by 7 mm, short-clawed. **Stamens** 9-10, 2 long with filaments 5-7 mm long, anther 5-6 mm long, curve opening by apical pores; 4 shorter with filaments 2 mm long and anthers 5 mm long, opening the same way; reduced stamens 3-4 ca 2 mm. **Ovary** finely pubescent; style thin, glabrous; stigma slightly swollen. **Pods** ± swollen, straight or nearly so, 10 by 0.5-1 cm, glabrous. **Seeds** 30-40, ovoid, compressed, ca 4 mm long."*

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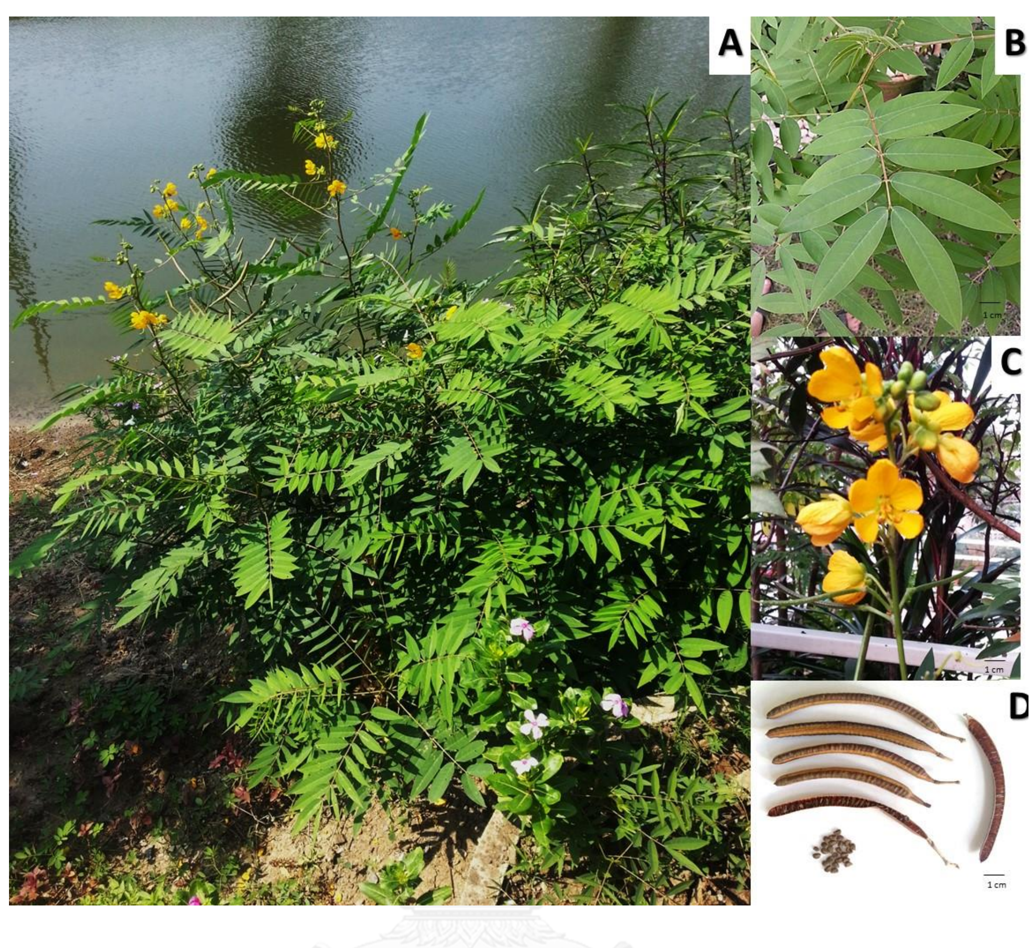

Figure 34 Photography of *C. sophera*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pods and seeds

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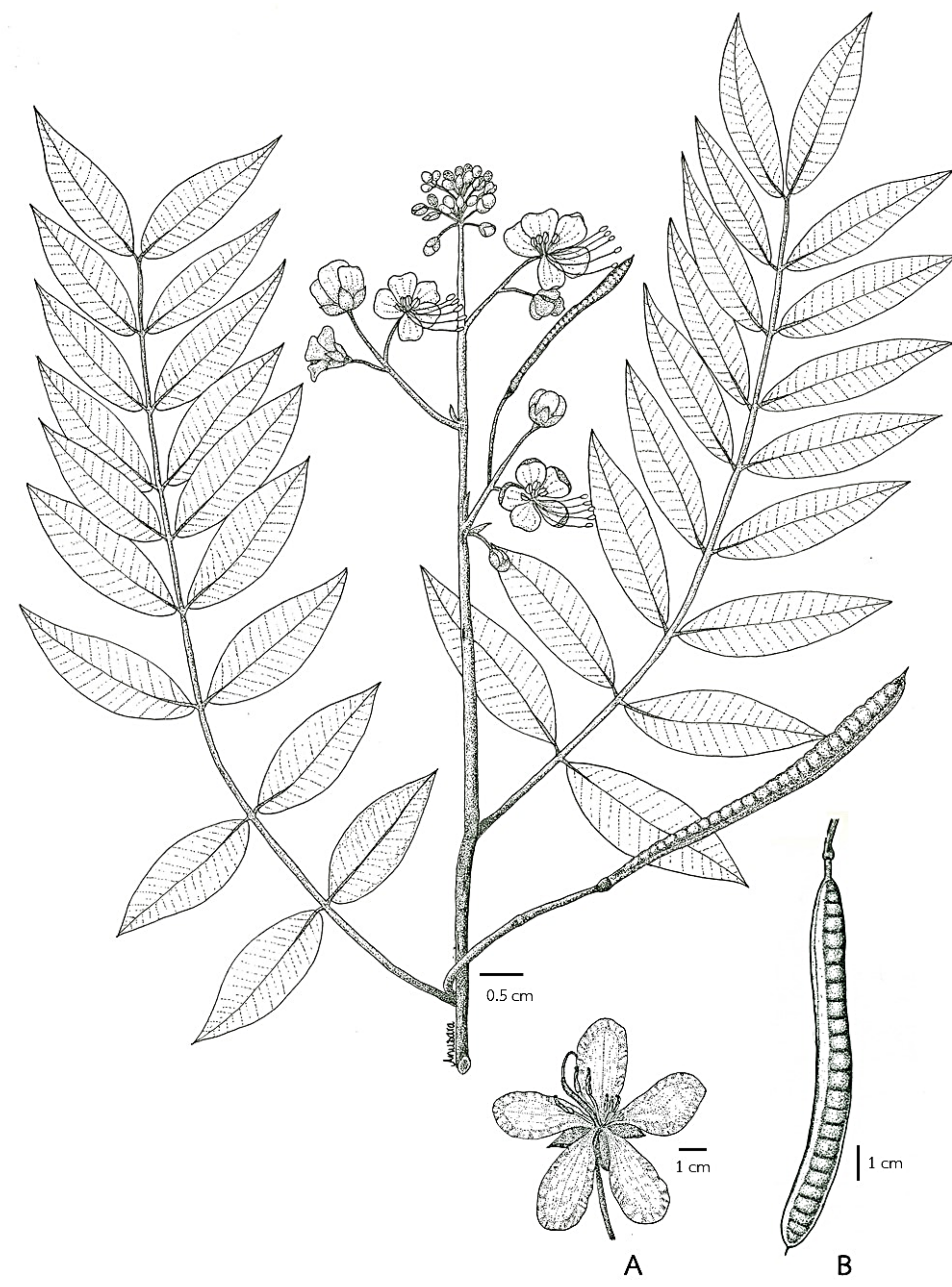

Figure 35 Twig of *C. sophera;* (A) Flower and (B) pod

1.13 *Cassia sulfurea* DC. ex Collad.

Thai name: Trueng badaan (ตรึงบาดาล), Sakeng (สะเก๋ง), Sakong (สะโก้ง)

Location found in Thailand: Often planted as a way side tree all over the country. Cultivated perhaps less frequently than *C. surattensis.*

Distribution: Southeast Asia, India and Thailand

Description: *"**Shrub** or small tree up to 7 m high; young branches puberulous. **Leaves** with 4-6 pairs of larger, lanceolate leaflets with acute apex, 5-10 by 2.3-5 cm. **Petioles** 1.5-3cm; rhachis up to 15 cm with a elevate, 1-2 mm long gland between the 2-3 lower pairs of leaflets. **Stipules** linear-falcate, puberulous, 5-10 mm long, subpersistent. **Leaflets** with a short petiolue, ovate to ovate-oblong, 2.5-4 by 1-1.7 cm; upper surface glabrous, lower sparsely pubescent; apex rounded ± slightly emarginated, base rounded, rarely cuneate. **Raceme** from the upper leaf axil, 3-6 cm long, 10-15 flowered. **Peduncles** 2.5-5 cm. Bracts ovate-acute, 4-5 mm long, finally reflex. **Pedicels** 1-2 cm. **Sepals**; 2, outer ± orbicular, 3 mm long; 3 inner increasing to 6-7 mm in length. **Petals** yellow sub equal, ovate-obovate, 1.5-2 cm long with a 1-1.5 mm long; anthers ± equal, 5-7 mm long, opening by longituditional slit. **Ovary** appressed uberulous, filiform, recurve; style with anindistinct stigma. **Pods** flat glabrous, thin valve, dehiscent, 7-10 by 1-1.5 cm. **Seeds** 15-25, glossy, flattened, 8 by 4 mm."*

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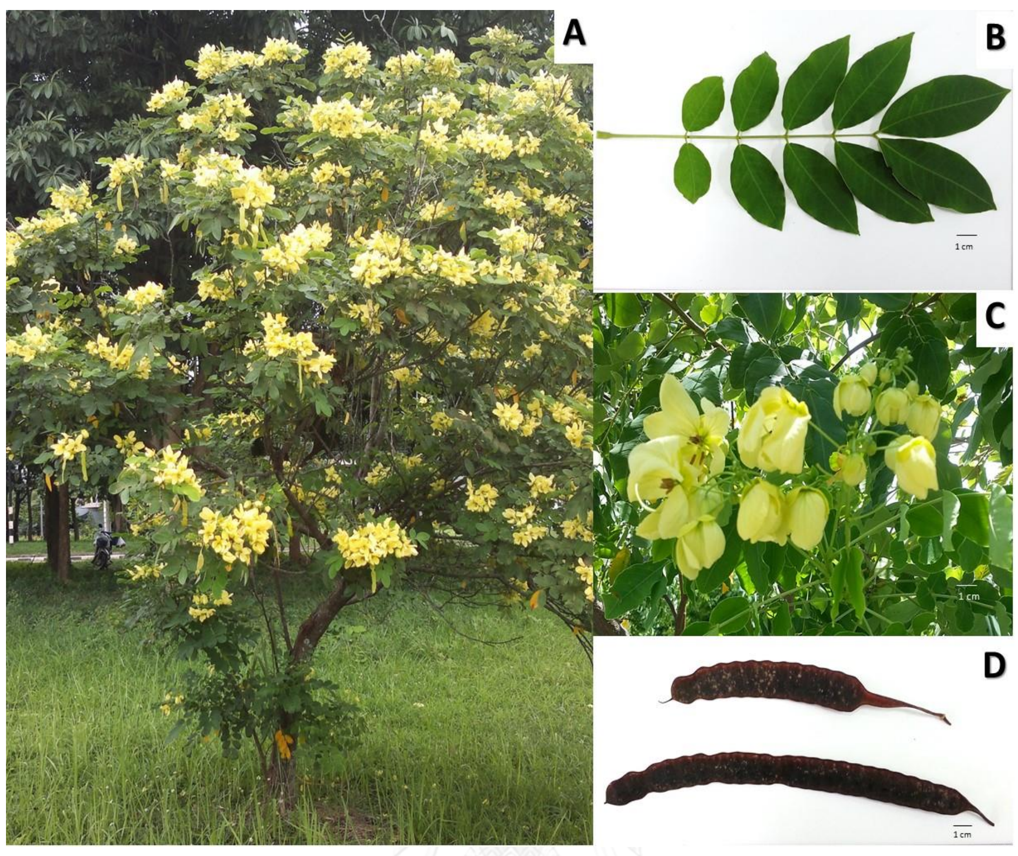

Figure 36 Photography of *C. sulfurea;* (A) habitat, (B) leaves, (C) inflorescences, and (D) pods

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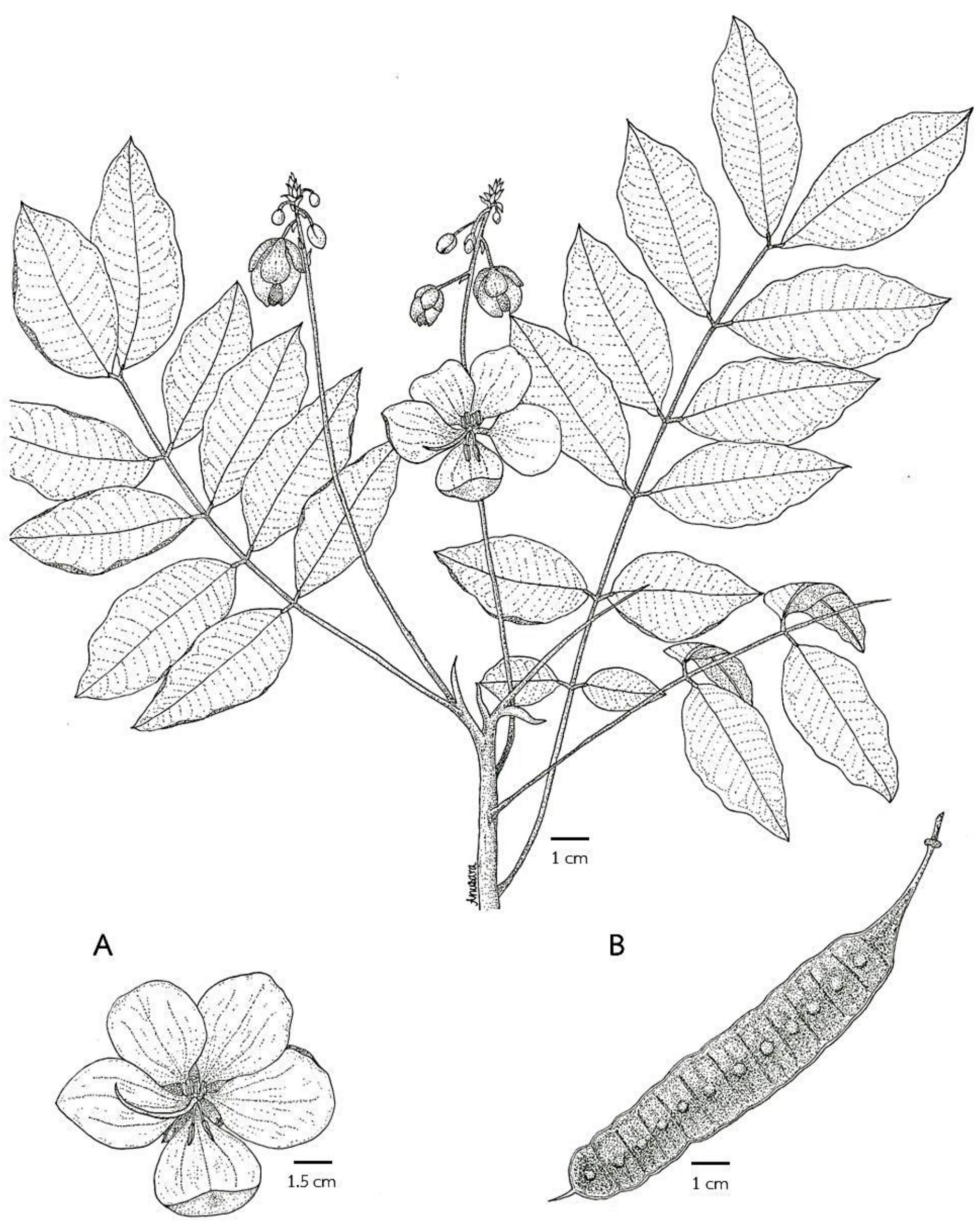

Figure 37 Twig of *C. sulfurea*; (A) Flower and (B) pod

1.14 *Cassia surattensis* Burm. f.

Thai name: Song badan (ทรงบาดาล), Khee lek wan (ขี้เหล็กหวาน)

Location found in Thailand: Often planted as a way side tree all over the country.

Distribution: Southeast Asia, India and Thailand

Description: *"**Shrub** or small tree up to 7 m high; young branches puberulous. **Leaves** with 6-9 pairs of leaflets. **Petioles** 1.5-3 cm; rhachis up to 15 cm with a clavate, 1-2 mm long gland between the 2-3 lower pairs of leaflets. **Stipules** linear-falcate, puberulous, 5-10 mm long, subpersistent. **Leaflets** with a short petiolue, ovate to ovate-oblong, 2.5-4 by 1-1.7 cm; upper surface glabrous, lower sparsely pubescent; apex rounded ± slightly emarginated, base rounded, rarely cuneate. **Raceme** from the upper leaf axil, 3-6 cm long, 10-15 flowered. **Peduncles** 2.5-5 cm. **Bracts** ovate-acute, 4-5 mm long, finally reflex. **Pedicels** 1-2 cm. Sepal; 2, outer ± orbicular, 3 mm long; 3 inner increasing to 6-7 mm in length. **Petals** yellow sub equal, ovate-obovate, 1.5-2 cm long with a 1-1.5 mm long; anthers ± equal, 5-7 mm long, opening by longituditional slit. **Ovary** appressed puberulous, filiform, recurve; style with anindistinct stigma. **Pods** flat glabrous, thin valve, dehiscent, 7-10 by -1.5 cm. **Seeds** 15-25, glossy, flattened, 8 by 4 mm."*

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Figure 38 Photography of *C. surattensis*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pods

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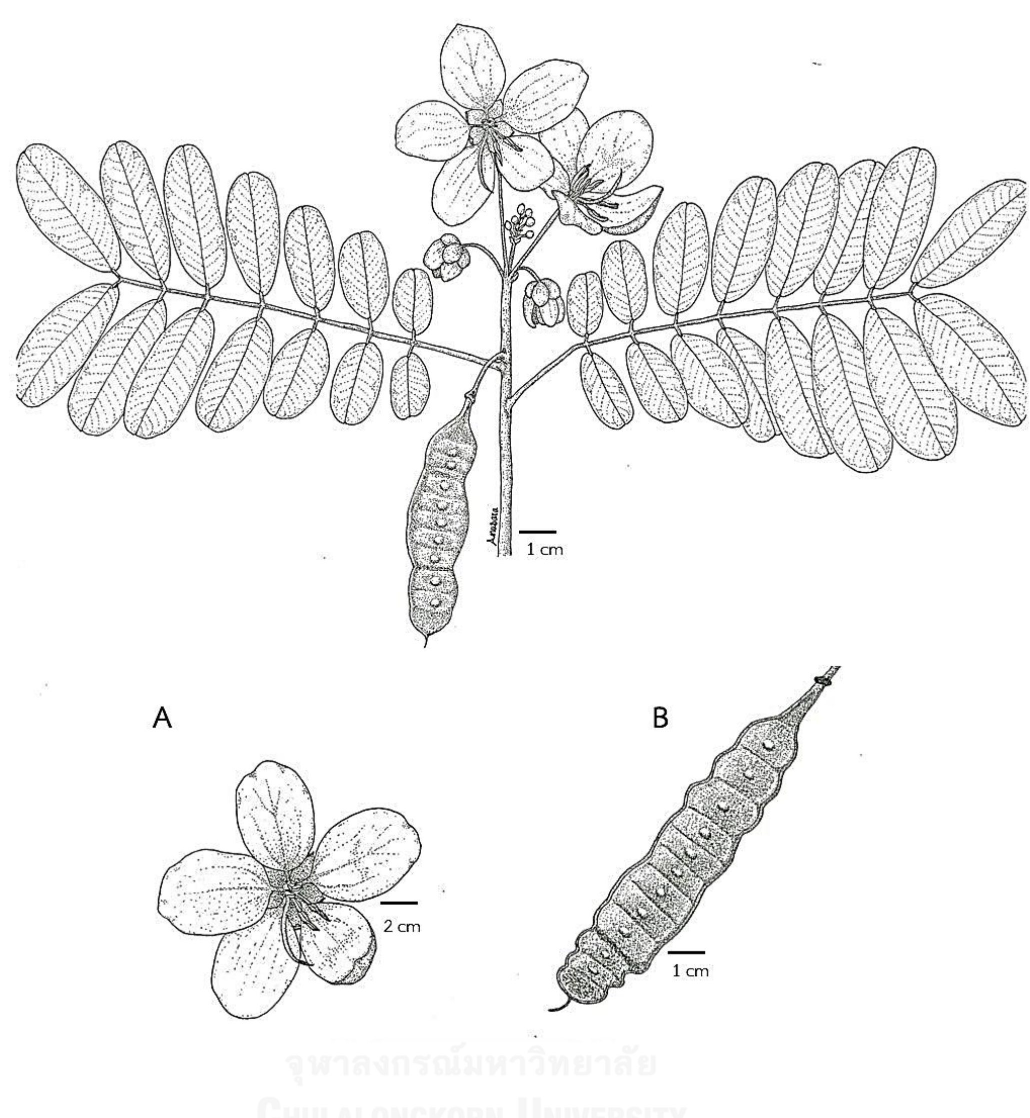

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Figure 39 Twig of *C. surattensis;* (A) Flower and (B) pod

1.15 *Cassia timoriensis* DC.

Thai name: Kheelek luead (ขี้เหล็กเลือด), Cha kheelek (ช้าขี้เหล็ก), Makluea luead (มะเกลือเลือด), Kheelek daeng (ขี้เหล็กแดง), Kalaeng ngaen (กะแลงเงิน)

Location found in Thailand: All over the country

Distribution: Ceylon, Southeast Asia, Thailand and Australia

Description: *"Small tree up to 7 m high with long; young branches and leaves varying in Indumentums from nearly glabrous to yellowish and golden hairy.* ***Leaves*** *with 10-20 pairs of leaflets.* ***Petioles*** *1-2 cm; rhachis 20-30 cm, pubescent.* ***Stipules*** *large, auticulate, 1.5-2.0 cm long.* ***Leaflets*** *with short petiolule oblong, 2-6 by 1-1.5 cm with rounded base and subacute to mucronate apex, from nearly glabrous to yellowish pubescent on both side.* ***Inflorescences*** *axillary, dense raceme 10-30 cm long; axis ± glabrous to yellowish pubescent.* ***Bracts*** *caducous, ovate, acute up to 20 by 15 mm.* ***Pedicels*** *yellowish pubescent outside.* ***Petals*** *yellow, obovate, short clawed 15-20 by 10-15 mm.* ***Stamens*** *10, 2 largest with filaments 2-4 mm long and anther 8-10 mm long opening by apical pores; 5 somewhat smaller opening the same way; reduced stamens 3, ca 2 mm.* ***Ovary*** *± glabrous; style glabrous; stigma inconspicuous.* ***Pods*** *flat, glabrous, dehiscent, 8-16 by 1-1.5 cm.* ***Seeds*** *10-30, elliptic, glossy flattened, 7 by 5 mm."*

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Figure 40 Photography of *C. timoriensis*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pods

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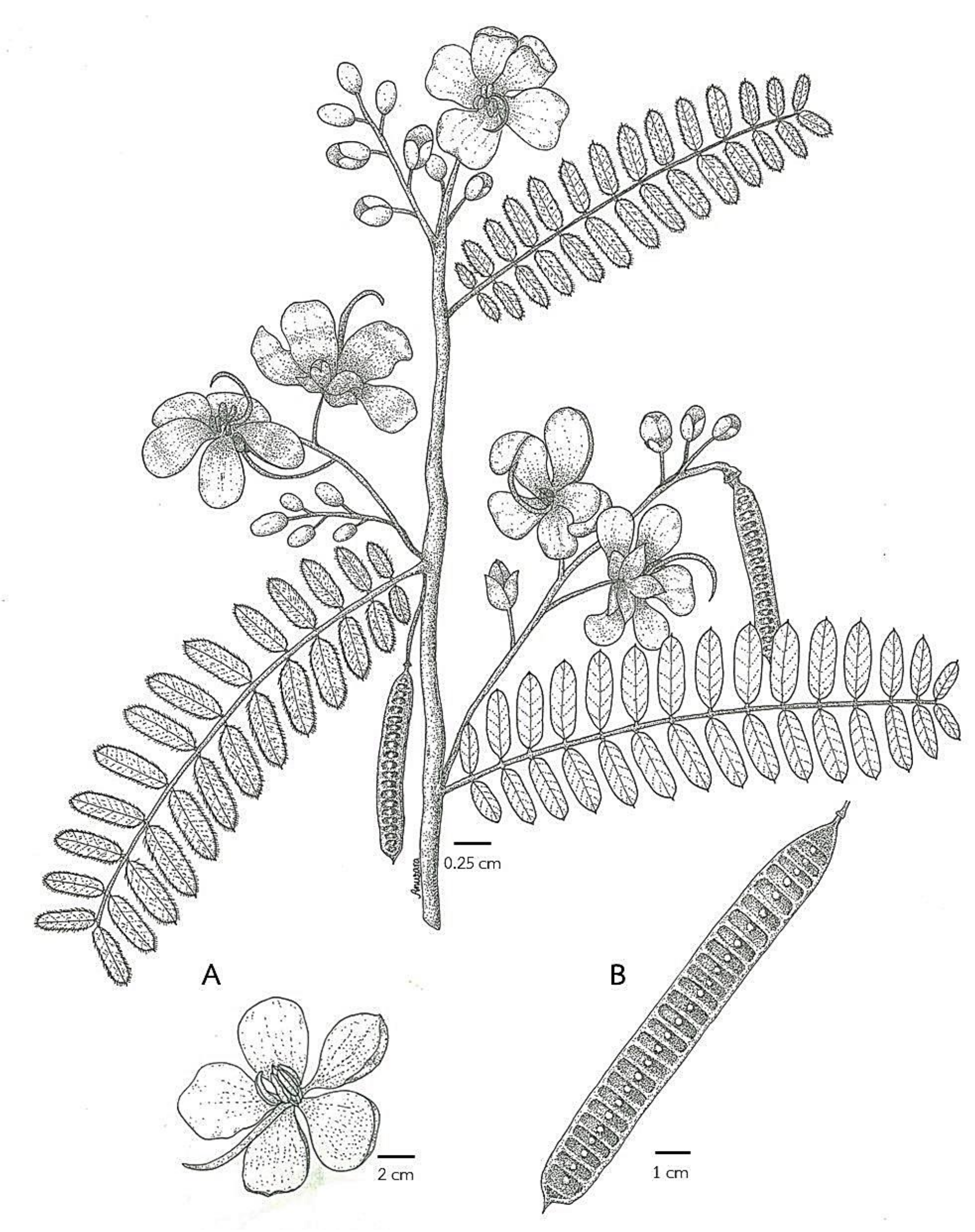

Figure 41 Twig of *C. timoriensis*; (A) Flower and (B) pod

1.16 *Cassia tora* L.

Thai name: Chumhet thai (ชุมเห็ดไทย), Chumhet na (ชุมเห็ดนา) Chumhet lek (ชุมเห็ดเล็ก), Chumhet khwaai (ชุมเห็ดควาย), Phromdan (พรมแดน) Lap muen noi (ลับมืนน้อย)

Location found in Thailand: Common weed throughout the country.

Distribution: America, India and Thailand

Description: *"Herb or undershrub up to 1 m high nearly glabrous.* ***Leaves*** *with 3 pairs of leaflets.* ***Petioles*** *1-4 cm, rhachis 2-3 cm with a subulate, 2 mm long gland between the 2 lower 3 pairs of leaflets.* ***Stipules*** *setaceous, 10-15 mm long ± caducous.* ***Leaflets*** *increasing in size distally with a short petiolule, membranous, obovate; apex broadly round, base cuneate-rounded, 2-5 by 1.5-2 cm long.* ***Raceme*** *axillary, short, 1-3 flowered.* ***Bracts*** *linear-acute, 2-3 mm long.* ***Pedicels*** *4-10 mm (enlarging in fruit).* ***Sepals*** *sub equal, ovate 5 by 2-4 mm.* ***Petals*** *yellow, unequal, obovate, short claw with rounded apex, up to 10 by 6 mm.* ***Stamens*** *7 nearly equal; filaments 1.5-2 mm; anther 1.5-2.5 mm long, opening by apical pores; reduced stamens absent.* ***Ovary*** *densely pubescent; style glabrous with truncate apex (stigma).* ***Pods*** *terete, linear, ± falcate, 10-15 by 0.5 cm.* ***Seeds*** *20-30, glossy, rhomboidal, 5 mm diam."*

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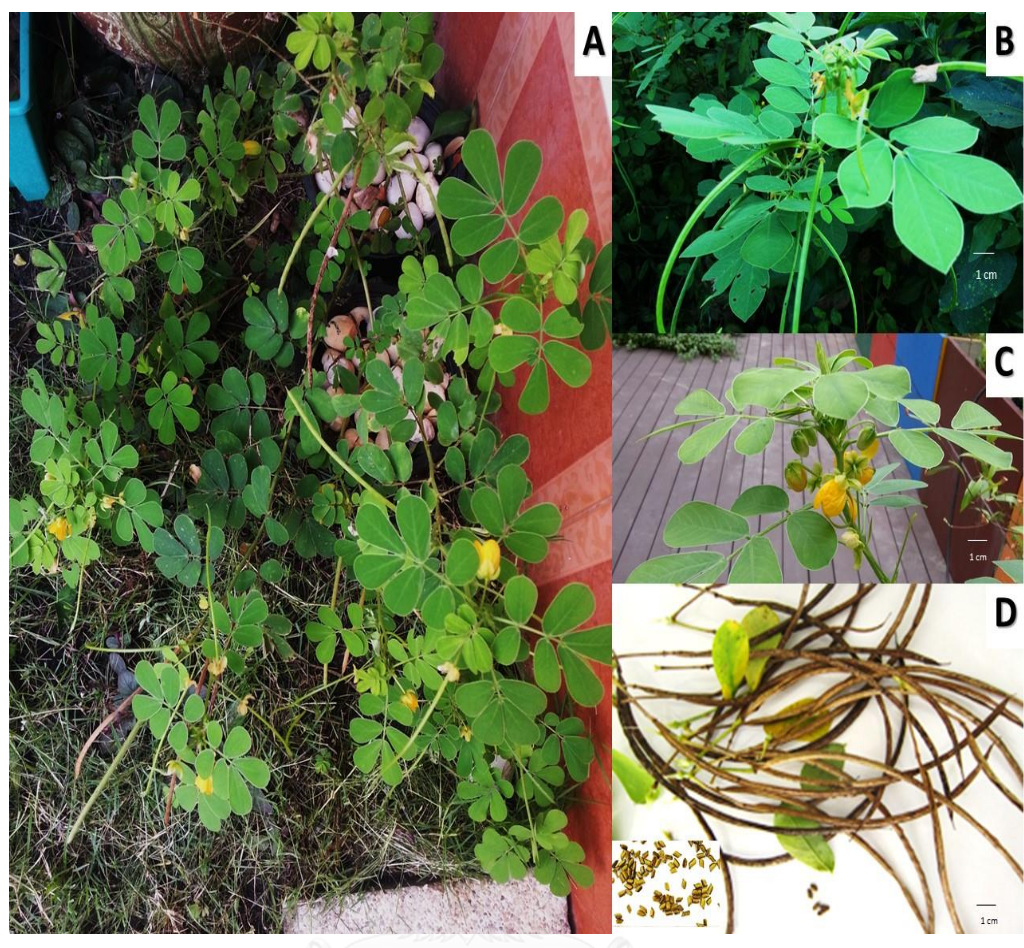

Figure 42 Photography of *C. tora*; (A) habitat, (B) leaves, (C) inflorescences, and (D) pods

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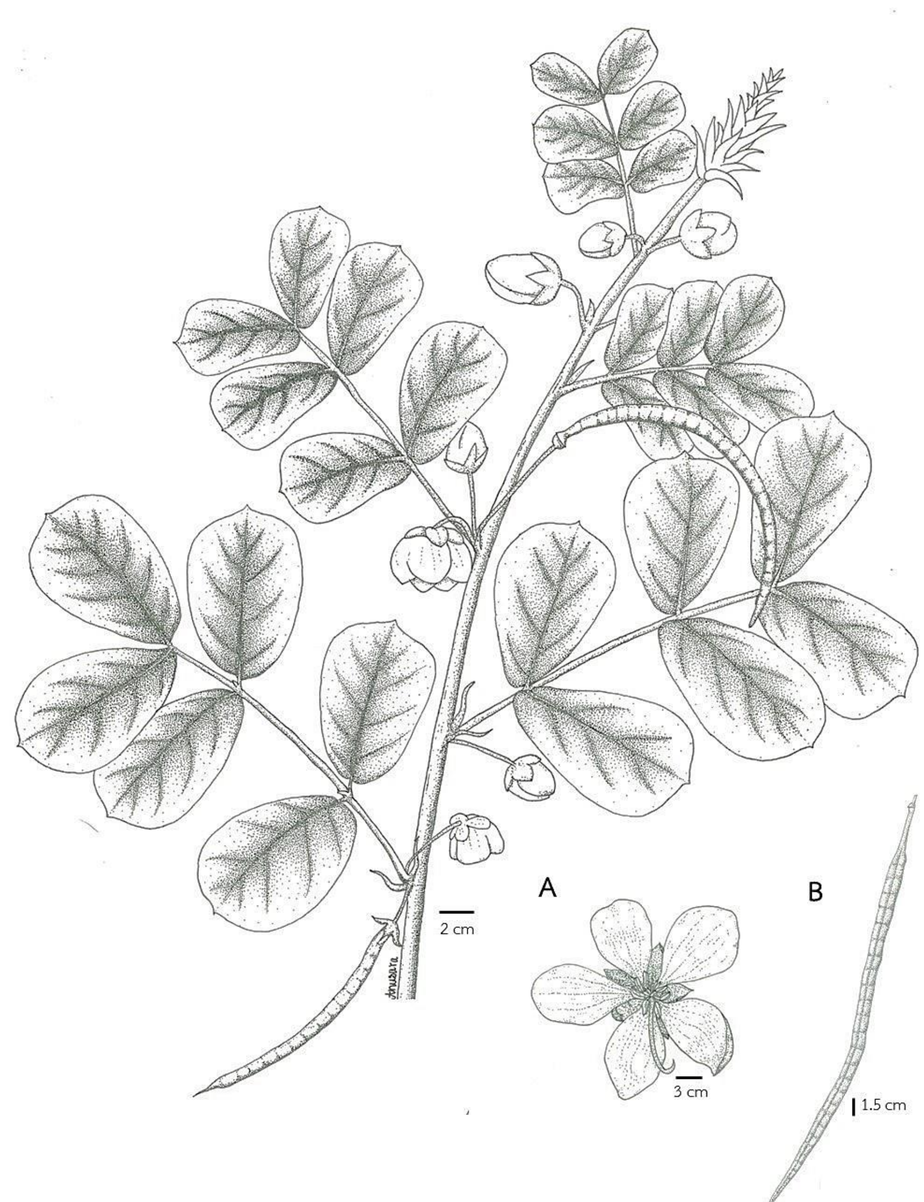

Figure 43 Twig of *C. tora*; (A) Flower and (B) pod

According to the important morphological characteristics, sixteen selected *Cassia* species in Thailand have paripinnate leaves. Foliar grand are absent in ten *Cassia* species (*C. bakeriana, C fistula, C. grandis, C. javanica, C. alata, C. angustifolia, C. garrettiana, C. spectabilis, C. siamea* and *C. timoreinsis*). Flowers are three types; raceme type found in 13 species (*C. bakeriana, C. fistula, C. grandis, C. javanica, C. alata, C. angustifolia, C. garrettiana, C. hirsuta, C. occidentalis, C. sulfurea, C. surattensis, C. timoriensis* and *C. tora*, panicle type found in 2 species (*C. spectabilis* and *C. siamea*) and corymb type found in *C. sophera*. Fruit is varying in shape, indehiscent or dehiscent. The important morphological characteristics of 16 *Cassia* species were shown in Table 17.

Table 17 The important morphological characteristics of 16 *Cassia* species

No.	Plant sample	Plant habit	Foliar gland	Pair of leaflet	Leaf			Flower		Pod	
					Leaf Shape	Leaf Base	Leaf Apex	Type	Colour	Pod shape	Colour
1	*C. bakeriana*	Tree	Absent	5-7	Oblong-lanceolate	Rounded	Rounded	Raceme	Pink	Terete, softly	grey-brown
2	*C. fistula*	Tree	Absent	3-8	Ovate-oblong	Rounded	Acute	Raceme	Yellow	Terete, glabrous	Black
3	*C. grandis*	Tree	Absent	10-20	Elliptic-oblong	Rounded	Rounded	Raceme	First red, later pink and finally orange	Woody, rugose glabrous	Black
4	*C. javanica*	Tree	Absent	5-15	Elliptic-ovate to oblong	Rounded	Rounded	Raceme	First pink, later dark red and finally pale	Terete, glabrous	Black
5	*C. alata*	Shrub	Absent	8-20	Oblong-obovate	Rounded	Rounded-emarginate	Raceme	Yellow	Thick, flattened, winge glabrous, septate	Black
6	*C. angustifolia*	Shrub	Absent	10-15	Oblong-lanceolate	Rounded	Acute/ mucronate	Raceme	Yellow	Broad, smooth or slightly puberulous	Brown-black
7	*C. garrettiana*	Tree	Absent	6-9	Ovate-broadly lanceolate	Rounded	Acuminate	Raceme	Yellow	Flat, glabrous, twisted	Brown
8	*C. hirsuta*	Herb/ Undershrub	Present	4-5	Ovate-broadly lanceolate	Rounded	Acuminate	Raceme	Yellow	Falcate to nearly straight, hirsute	Brown

Table 17 The important morphological characteristics of 16 *Cassia* species (Cont.)

No.	Plant sample	Plant habit	Foliar gland	Pair of leaflet	Leaf			Flower		Pod	
					Leaf Shape	Leaf Base	Leaf Apex	Type	Colour	Pod shape	Colour
9	*C. occidentalis*	Herb/ Undershrub	Present	3-5	Ovate-broadly lanceolate	Rounded	Acuminate	Raceme	Yellow	Flat, glabrous	Brown
10	*C. spectabilis*	Tree	Absent	10-15	Narrow elliptic	Rounded	Acute/ mucronate	Panicle	Yellow	Terete, glabrous, glossy	Black
11	*C. siamea*	Tree	Absent	7-10	Ovate-oblong	Rounded	Rounded/ emarginate	Panicle	Yellow	grabrescent,longitudinally waved with raised sutures	Brown-black
12	*C. sophera*	Shrub	Present	4-9	Narrowly lanceolate to oblong	Rounded	Acute	Corymb	Yellow	Swollen, straight glabrous	Brown-black
13	*C. surattensis*	Shrub/ Small tree	Present	6-9	Ovate to ovate-oblong	Rounded	Rounded	Raceme	Yellow	Flat, glabrous, dehiscent	Brown-black
14	*C. sulfurea*	Shrub/ Small tree	Present	4-6	Ovate-elliptic	Rounded	Acute	Raceme	Yellow	Flat, glabrous, dehiscent	Brown-black
15	*C. timoriensis*	Tree	Absent	10-20	Oblong	Rounded	Subacute-mucronate	Raceme	Yellow	Flat, glabrous, dehiscent	Brown-red
16	*C. tora*	Herb	Present	3	Obovate	Rounded	Rounded	Raceme	Yellow	Terete, falcate	Brown-black

2. The results of investigation of anatomical character

2.1 Transverse sections of leaves through midrib

The transverse sections of the lamina of the leaflet of sixteen *Cassia* species showed the bifacial structure. The outline drawings of transverse section of leaves through midrib of each species were found and described as below;

2.1.1 *C. bakeriana*; the dorsal and ventral epidermis composed of single layer, slightly thick walled epidermal cells with cuticle and numerous multicellular non-glandular trichomes. The dorsal epidermis was absent of stomata cell whereas, the ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 3-4 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed of two to four collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many multicellular non-glandular trichomes were found on both sides of epidermis. The illustration of *C. bakeriana* by hand drawing in the proportion size related to the original scale was showed in Figure 44.

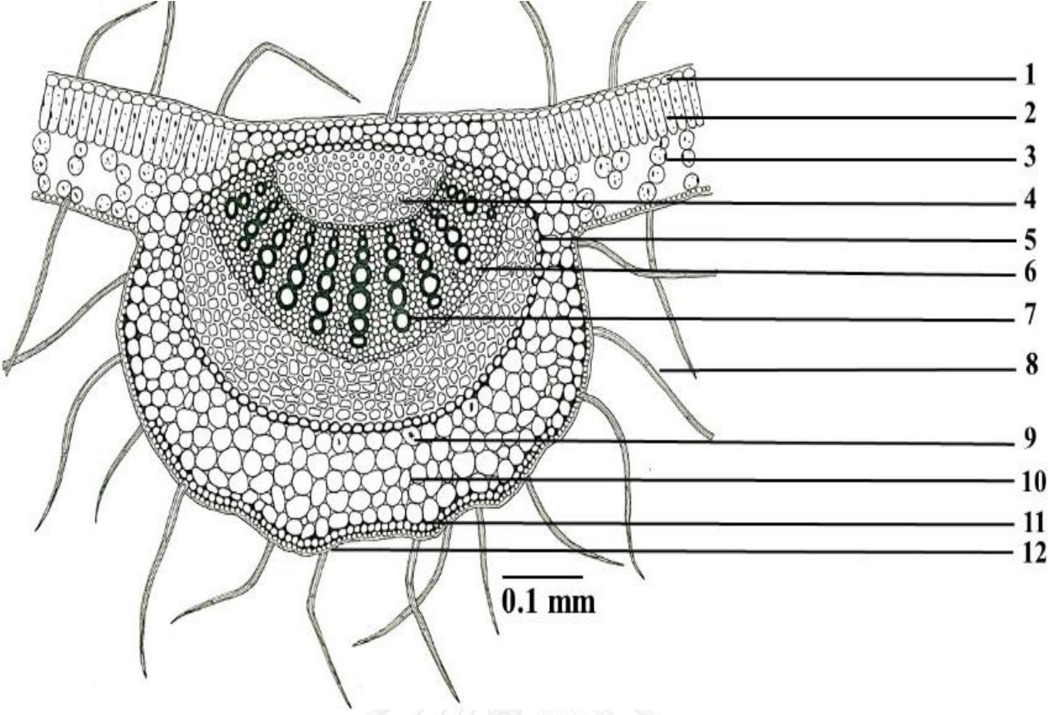

Figure 44 Transverse section of leaf through midrib of *C. bakeriana*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Prism crystal, 5. Sclerenchyma, 6. Phloem tissue, 7. Xylem tissue, 8. Multicellular non-glandular trichome, 9. Druse crystal, 10. Parenchyma, 11. Collenchyma, 12. Lower epidermis

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2.1.2 *C. fistula*; epidermis of both surfaces consisting of single layer, ellipse to circle cells shape with thin cuticle and numerous unicellular non-glandular trichomes. The dorsal epidermis was absent of stomata cell whereas, the ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 3-4 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed of two to five collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many unicellular non-glandular trichomes were found on both sides of epidermis. The illustration of *C. fistula* by hand drawing in the proportion size related to the original scale was showed in Figure 45.

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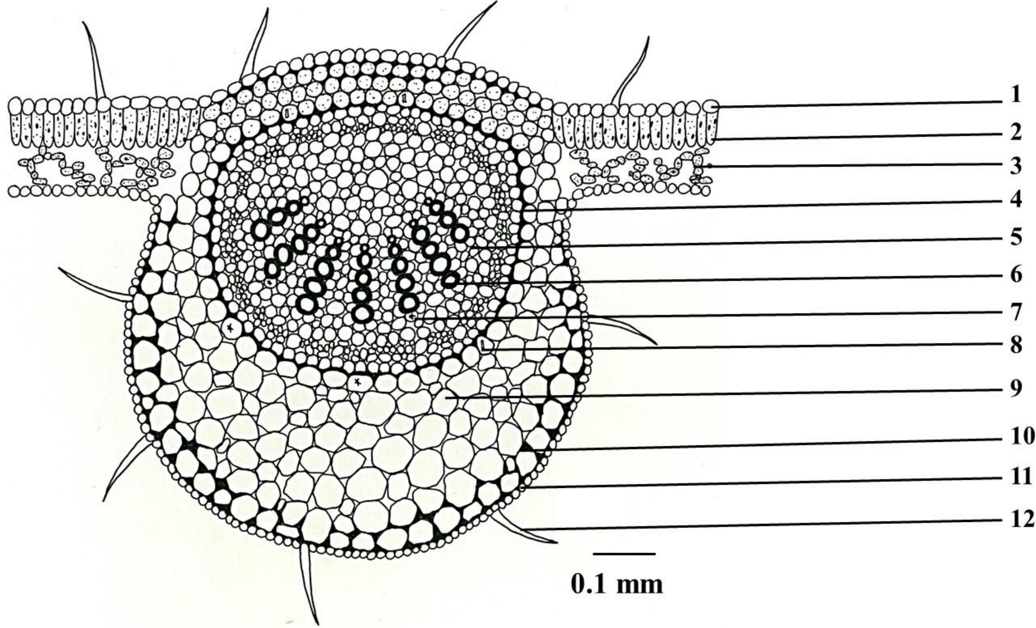

Figure 45 Transverse section of leaf through midrib of *C. fistula;* 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Phloem tissue, 6. Xylem tissue, 7. Druse crystal, 8. Prism crystal, 9. Parenchyma, 10. Collenchyma, 11. Lower epidermis, 12. Unicellular non-glandular trichome

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2.1.3 *C. grandis*; the epidermis of both surfaces consisting of single layer, rectangular cells with cuticle and numerous multicellular non-glandular trichomes. The dorsal epidermis was absent of stomata cell whereas, the ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll had regularity size. The midrib was composed of two to five collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many multicellular non-glandular trichomes were found on both sides of epidermis. The illustration of *C. grandis* by hand drawing in the proportion size related to the original scale was showed in Figure 46.

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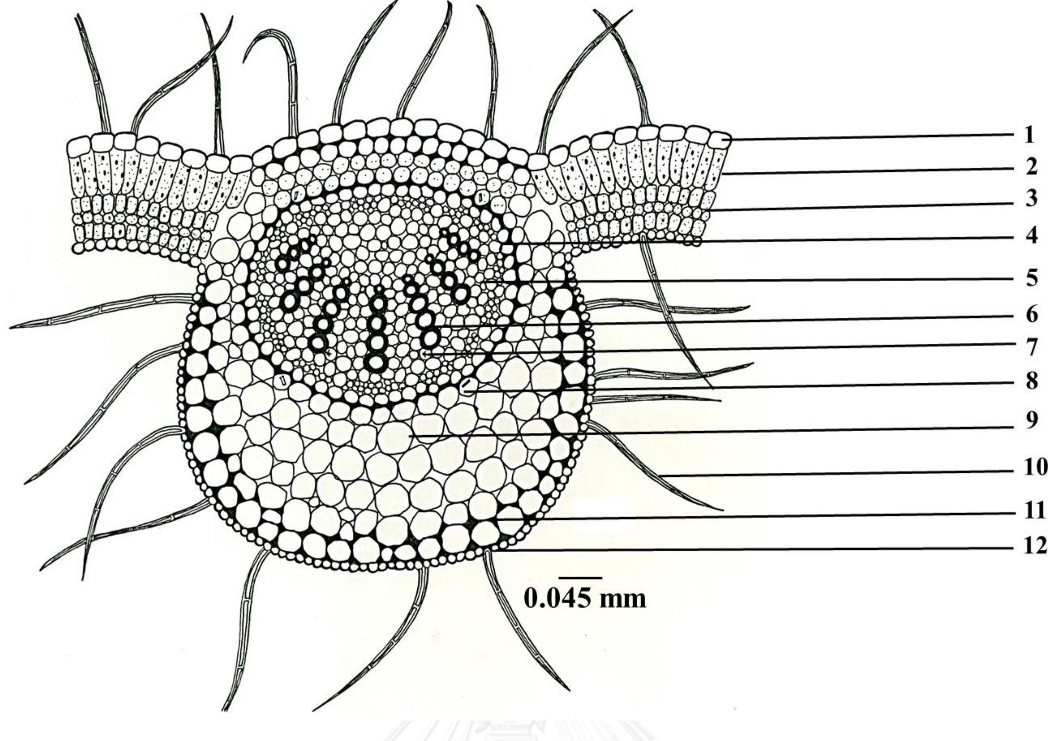

Figure 46 Transverse section of leaf through midrib of *C. grandis;* 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Phloem tissue, 6. Xylem tissue, 7. Druse crystal, 8. Prism crystal, 9. Parenchyma, 10. Multicellular non-glandular trichome, 11. Collenchyma, 12. Lower epidermis

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2.1.4 ***C. javanica*****;** the epidermis of both surfaces consisting of single later, rectangular cells with cuticle and numerous multicellular non-glandular trichomes. The dorsal epidermis was absent of stomata cell whereas, the ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll is three layered arranged regularity. The midrib was composed of one to three collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many multicellular non-glandular trichomes were found on both sides of epidermis. The illustration of *C. javanica* by hand drawing in the proportion size related to the original scale was showed in Figure 47.

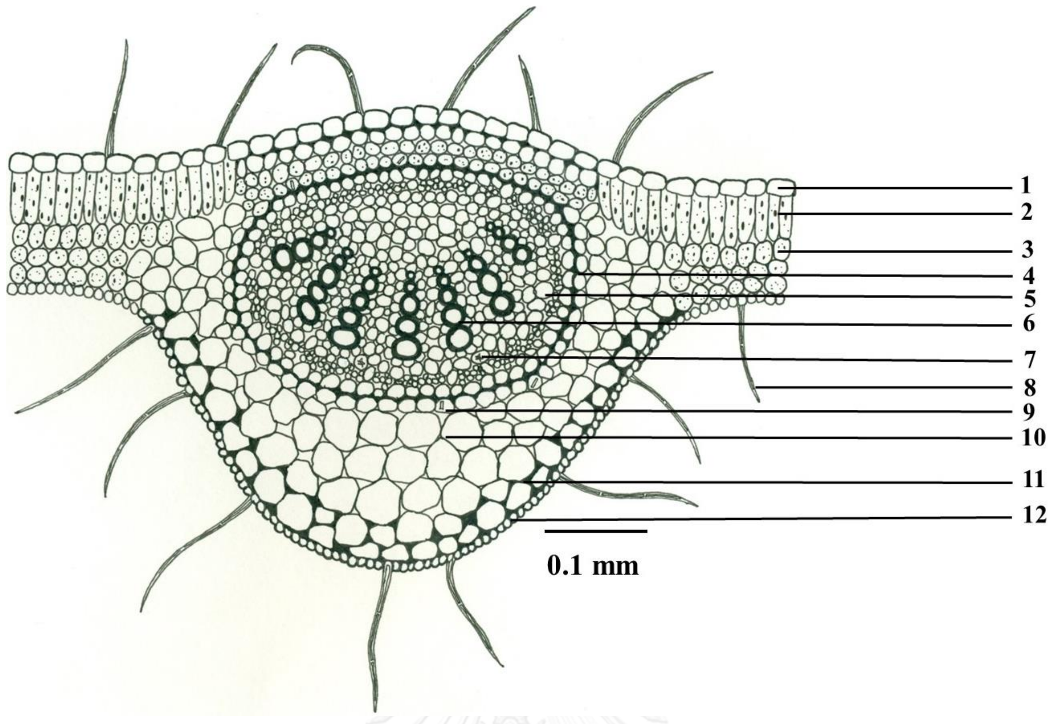

Figure 47 Transverse section of leaf through midrib of *C. javanica;* 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Phloem tissue, 6. Xylem, 7. Druse crystal tissue, 8. Multicellular non-glandular trichome, 9. Prism crystal, 10. Parenchyma, 11. Collenchyma, 12. Lower epidermis

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2.1.5 *C. alata*; the epidermis of both surfaces is composed of single layer, rectangular, tangentially elongated cells and numerous of unicellular non-glandular trichomes. The palisade mesophyll is single layered and made up of columnar closely arranged cells. The spongy mesophyll composed of 4-7 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The dorsal epidermis and ventral epidermis was composed of numerous paracytic stomata cells. The midrib was composed of one to three collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many unicellular non-glandular, conical trichome were found on both sides of epidermis. The illustration of *C. alata* by hand drawing in the proportion size related to the original scale was showed in Figure 48.

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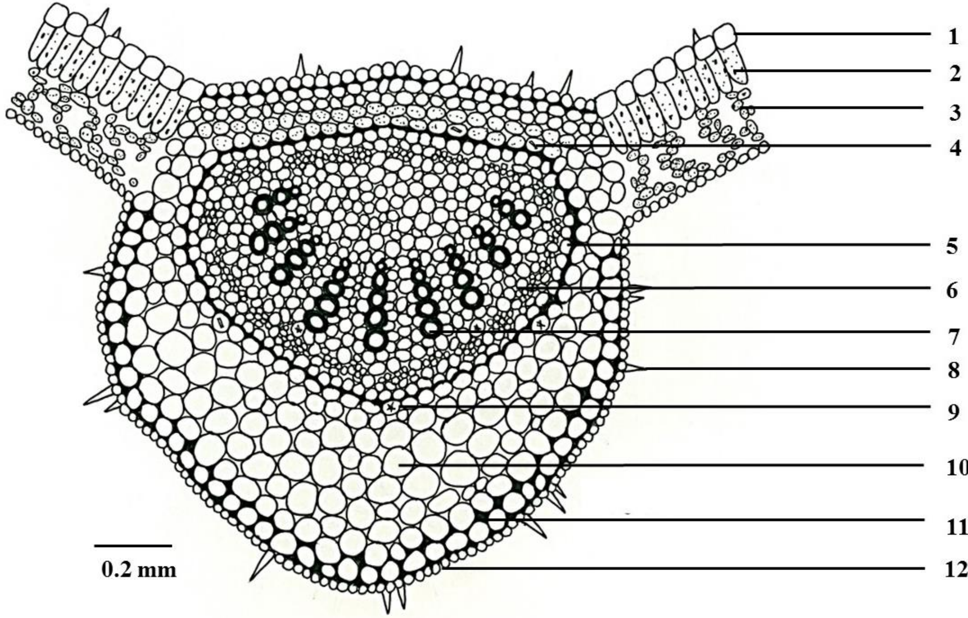

Figure 48 Transverse section of leaf through midrib of *C. alata;* 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Prism crystal, 5. Sclerenchyma, 6. Phloem tissue, 7. Xylem tissue, 8. Unicellular non-glandular trichome, 9. Druse crystal, 10. Parenchyma, 11. Collenchyma, 12. Lower epidermis

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2.1.6 *C. angustifolia*; the epidermis of both surfaces, consisting of rectangular cells with prominent cuticle and numerous of unicellular non-glandular, curved and warty trichome. The palisade mesophyll is single layered of columnar cells containing chloroplast. The spongy mesophyll consisting of rather loosely arranged rounded cells. The dorsal epidermis and ventral epidermis was composed of numerous paracytic stomata cells. The midrib was composed of several layers of collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal structure. Many unicellular non-glandular were found on both sides of epidermis. The illustration of *C. angustifolia* by hand drawing in the proportion size related to the original scale was showed in Figure 49.

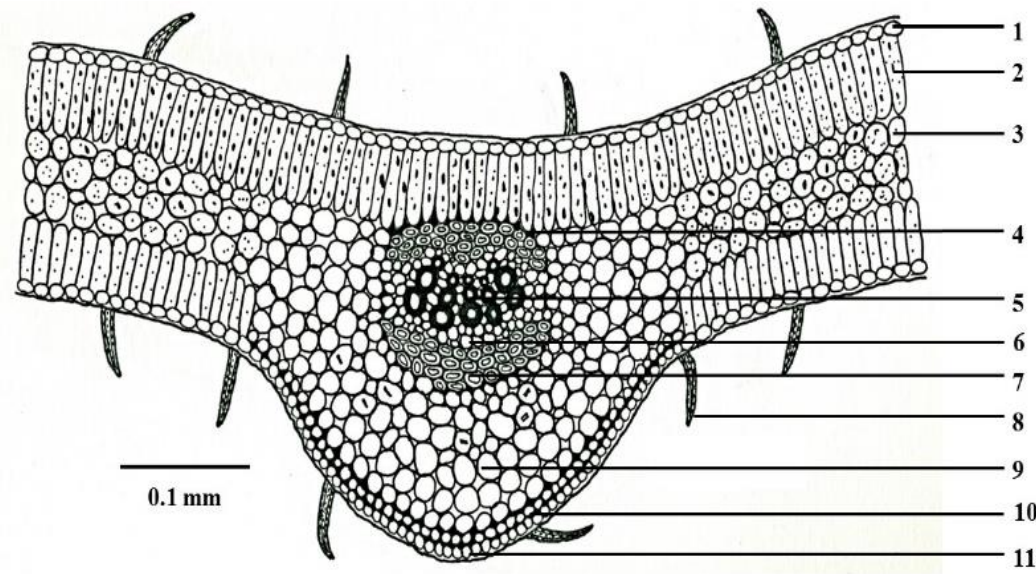

Figure 49 Transverse section of leaf through midrib of *C. angustifolia;* 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Prism crystal, 8. Unicellular non-glandular trichome, 9. Parenchyma, 10. Collenchyma, 11. Lower epidermis

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2.1.7 *C. garrettiana*; the epidermis of both surfaces consisting of single layer rectangular cells with prominent cuticle without any trichome. The palisade mesophyll is single layered of columnar cells containing chloroplast. The spongy mesophyll composed of 3-4 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The dorsal epidermis was absent of stomata cell whereas, the ventral epidermis was composed of numerous paracytic stomata cells. The midrib was composed of several layers of collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. The illustration of *C. garrettiana* by hand drawing in the proportion size related to the original scale was showed in Figure 50.

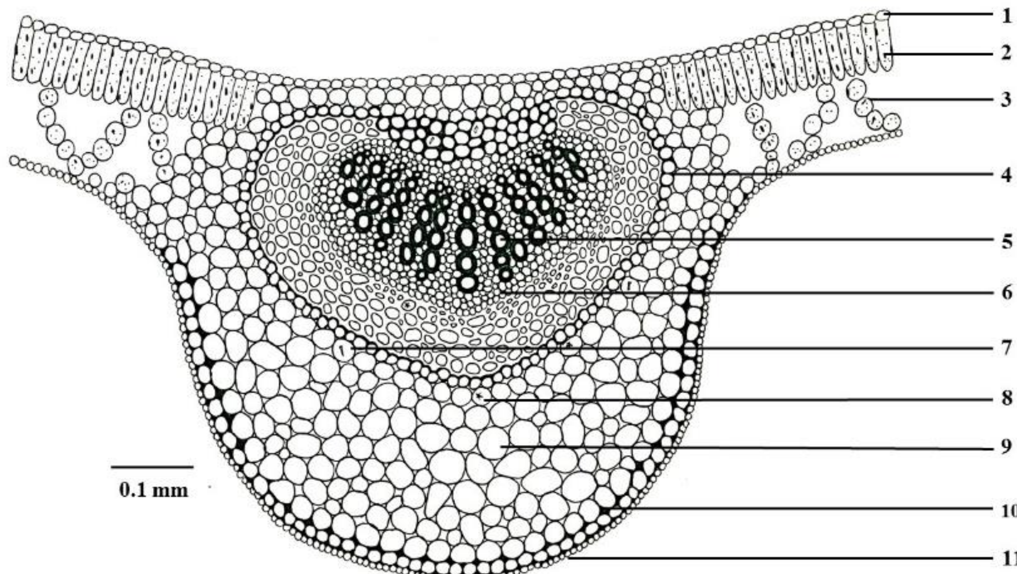

Figure 50 Transverse section of leaf through midrib of *C. garrettiana;* 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Prism crystal, 8. Druse crystal, 9. Parenchyma, 10. Collenchyma, 11. Lower epidermis

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2.1.8 *C. hirsuta*; the epidermis of both surfaces consisting of single layer, circle to ellipse cells shape with thick cuticle and numerous multicellular non-glandular trichomes and multicellular glandular trichomes. The dorsal and ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 3-4 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed of one to three collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal, druse crystal and raphide crystal structure. Many multicellular non-glandular trichomes were found on both sides of epidermis. The illustration of *C. hirsuta* by hand drawing in the proportion size related to the original scale was showed in Figure 51

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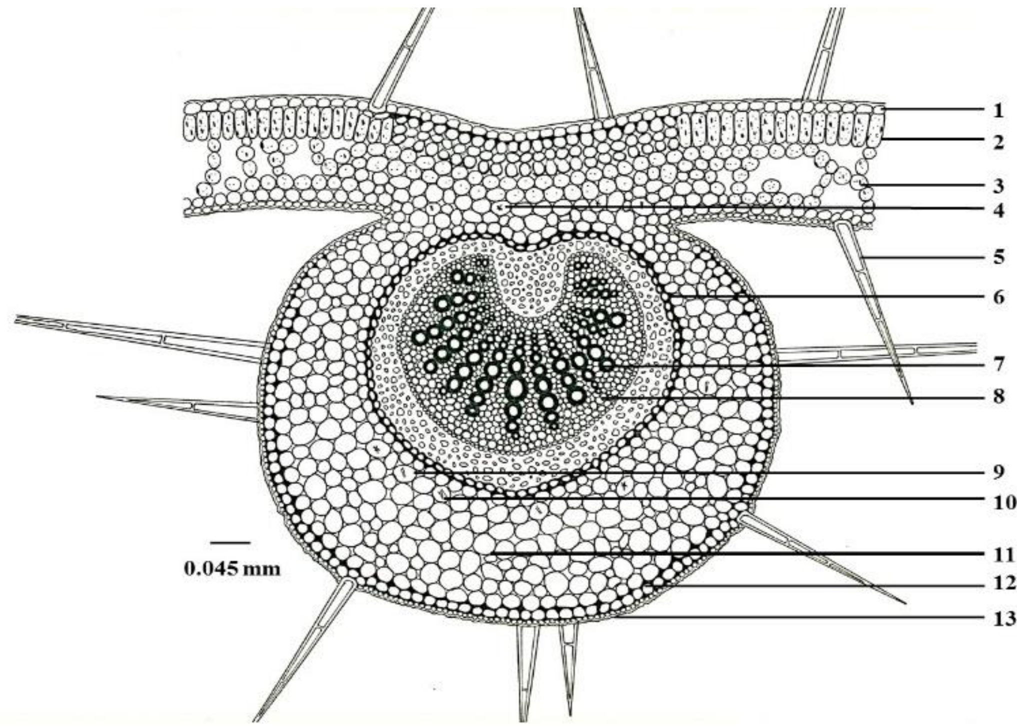

Figure 51 Transverse section of leaf through midrib of *C. hirsuta;* 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Druse crystal, 5. Multicellular non-glandular trichome, 6.Sclerenchyma, 7. Xylem tissue, 8. Phloem tissue, 9. Prism crystal, 10. Raphide crystal, 11. Parenchyma, 12. Collenchyma, 13. Lower epidermis

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2.1.9 *C. occidentalis*; the epidermis of both surfaces, consisting of single layer, circle to ellipse cells shape with prominent cuticle without any trichome. The palisade mesophyll is single layered of columnar cells containing chloroplast. The spongy mesophyll composed of 3-4 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The dorsal and ventral epidermis was composed of numerous paracytic stomata cells. The midrib was composed of one to two layers of collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. The illustration of *C. occidentalis* by hand drawing in the proportion size related to the original scale was showed in Figure 52.

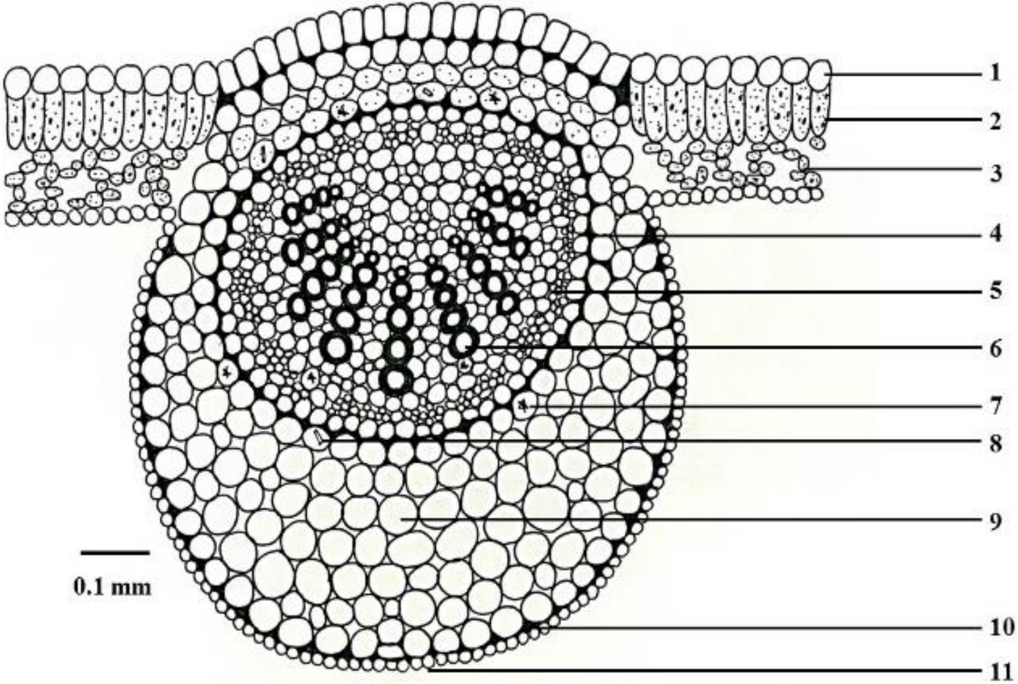

Figure 52 Transverse section of leaf through midrib of *C. occidentalis*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Phloem tissue, 6. Xylem tissue, 7. Druse crystal, 8. Prism crystal, 9. Parenchyma, 10. Collenchyma, 11. Lower epidermis

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2.2.10 *C. spectabilis*; the dorsal and ventral epidermis composed of single layer, circle to ellipse cells shape with cuticle and numerous multicellular non-glandular trichomes. The dorsal epidermis was absent of stomata cell whereas, the ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 4-7 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed of two to four collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many multicellular non-glandular trichomes were found on both sides of epidermis. The illustration of *C. spectabilis* by hand drawing in the proportion size related to the original scale was showed in Figure 53.

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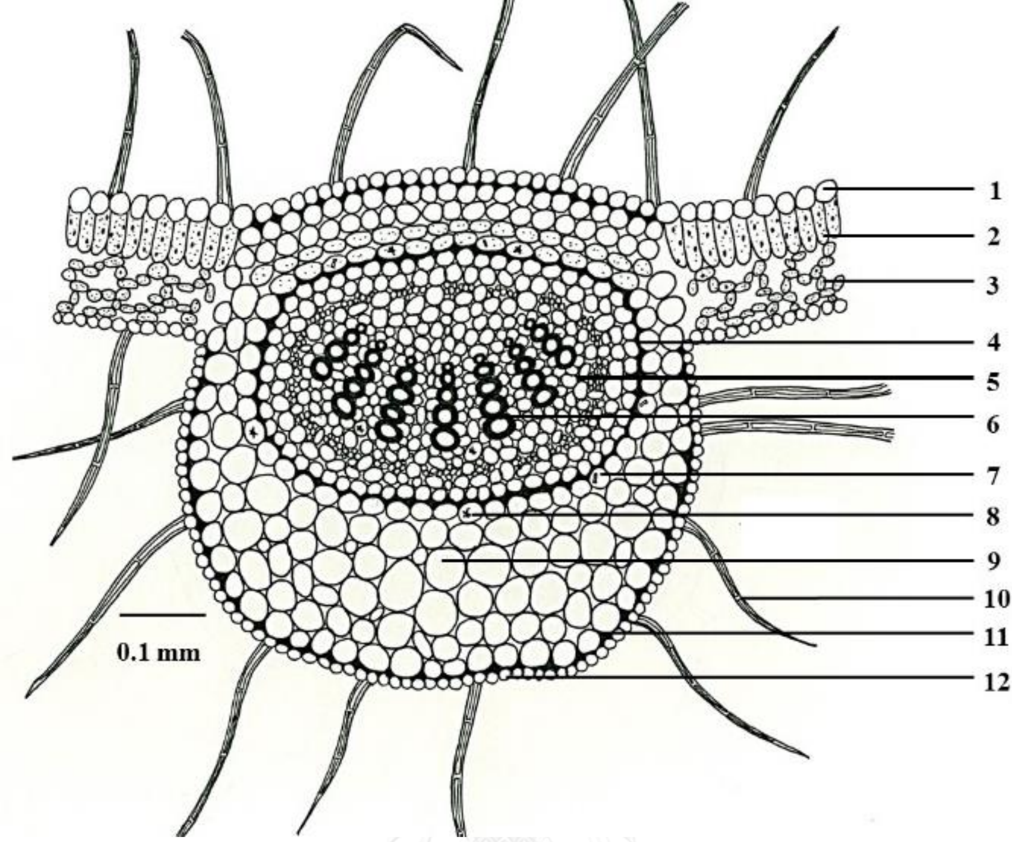

Figure 53 Transverse section of leaf through midrib of *C. spectabilis*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Phloem tissue, 6. Xylem tissue, 7. Prism crystal, 8. Druse crystal, 9. Parenchyma, 10. Multicellular non-glandular trichome, 11. Collenchyma, 12. Lower epidermis

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2.1.11 *C. siamea*; epidermis of both surfaces consisting of single layer, ellipse to circle cells shape with cuticle and numerous unicellular non-glandular trichomes. The dorsal epidermis was absent of stomata cell whereas, the ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 4-7 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed of two to three collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many unicellular non-glandular trichomes were found on both sides of epidermis. The illustration of *C. siamea* by hand drawing in the proportion size related to the original scale was showed in Figure 54.

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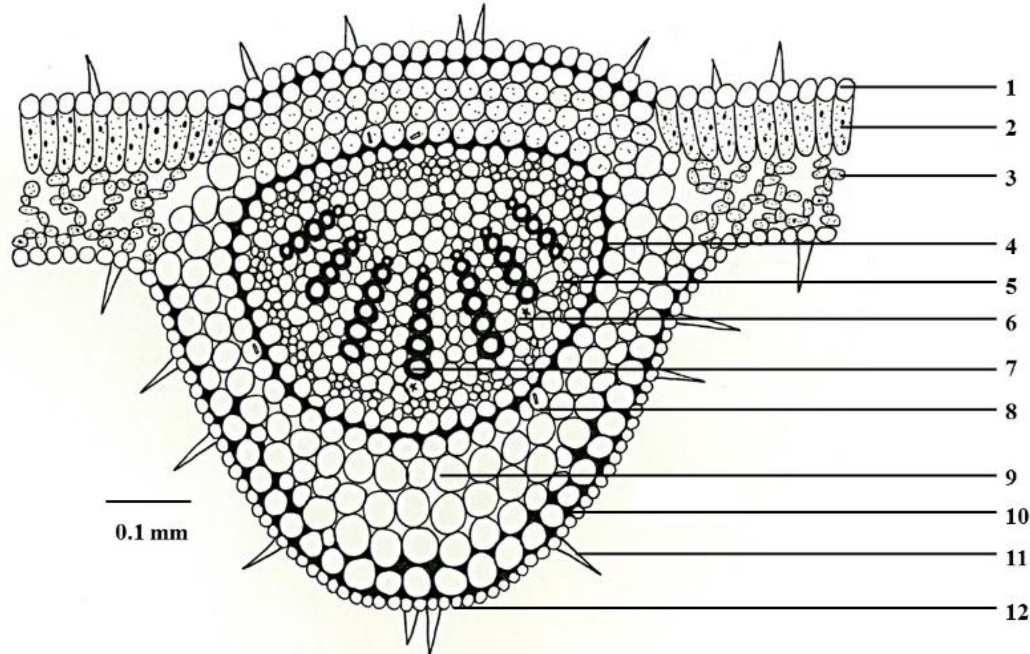

Figure 54 Transverse section of leaf through midrib of *C. siamea*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Phloem tissue, 6. Druse crystal, 7. Xylem tissue, 8. Prism crystal, 9. Parenchyma, 10. Unticellular non-glandular trichome, 11. Collenchyma, 12. Lower epidermis

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2.1.12 ***C. sophera***; the epidermis of both surfaces consisting of single layer, circle to ellipse cells shape with prominent cuticle without any trichome. The palisade mesophyll is single layered of columnar cells containing chloroplast. The spongy mesophyll composed of 3-4 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The dorsal and ventral epidermis was composed of numerous paracytic stomata cells. The midrib was composed of one to two layers of collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. The illustration of *C. sophera* by hand drawing in the proportion size related to the original scale was showed in Figure 55.

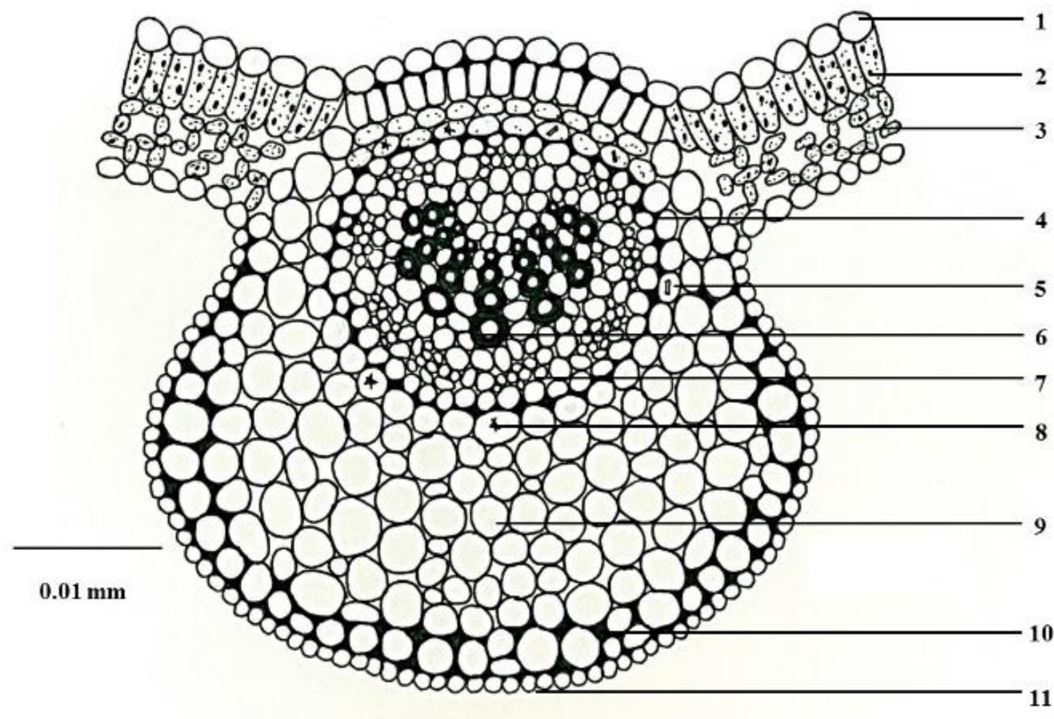

Figure 55 Transverse section of leaf through midrib of *C. sophera*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Prism crystal, 6. Xylem tissue, 7. Phloem tissue, 8. Druse crystal, 9. Parenchyma, 10. Collenchyma, 11. Lower epidermis

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2.2.13 ***C. surattensis***; the dorsal and ventral epidermis composed of single layer, circle to ellipse cells shape with cuticle. Multicellular non-glandular trichomes was found only on ventral epidermis. The dorsal and ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 3-4 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed of two to four collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many multicellular non-glandular trichomes were found on ventral sides of epidermis. The illustration of *C. surattensis* by hand drawing in the proportion size related to the original scale was showed in Figure 56.

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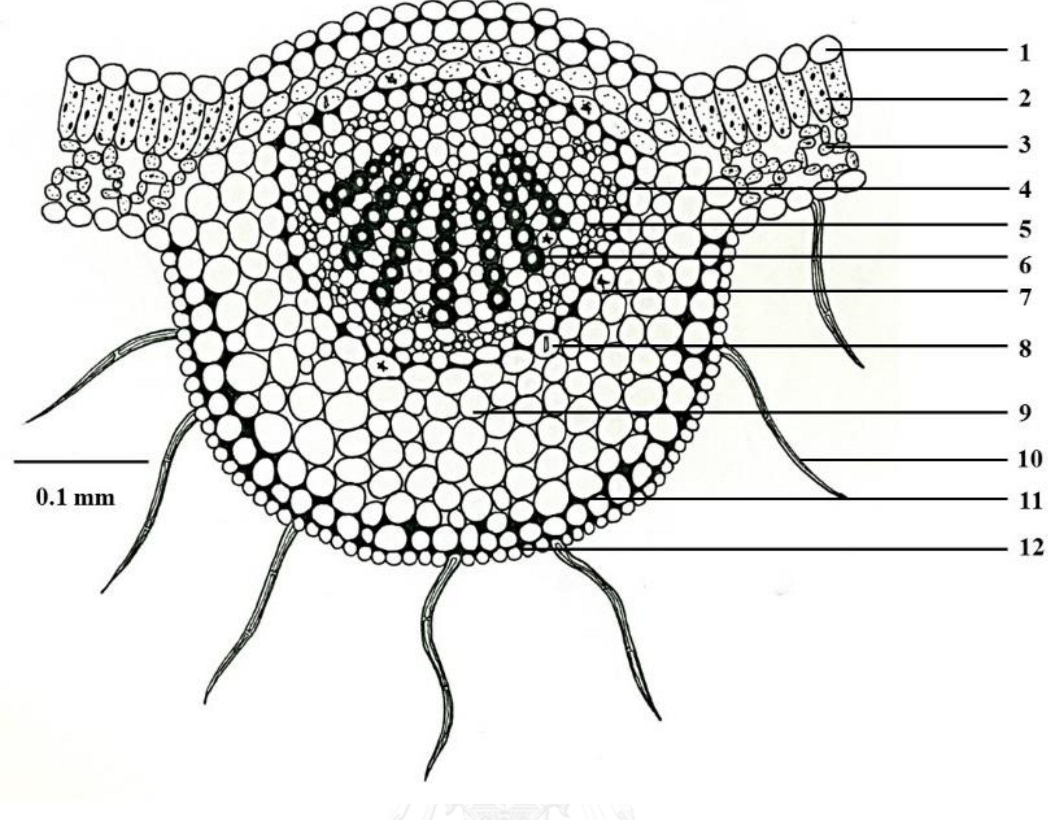

Figure 56 Transverse section of leaf through midrib of *C. surattensis*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Phloem tissue, 6. Xylem tissue, 7. Druse crystal, 8. Prism crystal, 9. Parenchyma, 10. Multticellular non-glandular trichome, 11. Collenchyma, 12. Lower epidermis

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2.2.14 *C. sulfurea*; the dorsal and ventral epidermis composed of single layer, circle to ellipse cells shape with cuticle. Multicellular non-glandular trichomes was found only on ventral epidermis. The dorsal and ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 3-4 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed of two to four collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many multicellular non-glandular trichomes were found on ventral sides of epidermis. The illustration of *C. sulfurea* by hand drawing in the proportion size related to the original scale was showed in Figure 57.

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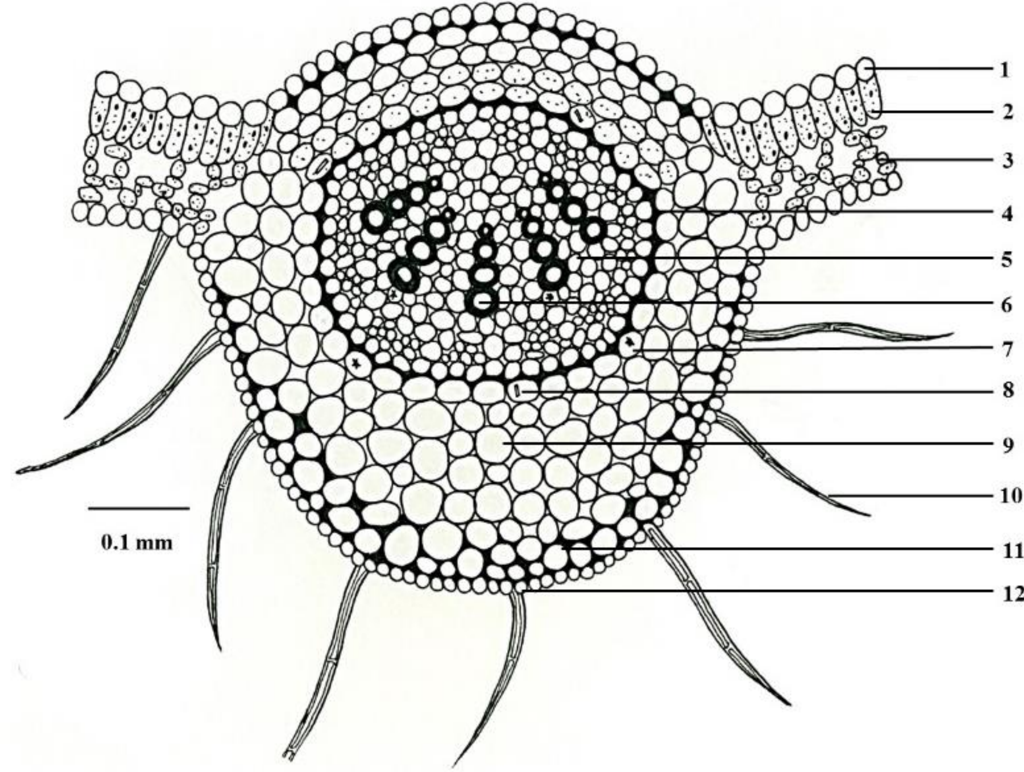

Figure 57 Transverse section of leaf through midrib of *C. sulfurea*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Phloem tissue, 6. Xylem tissue, 7. Druse crystal, 8. Prism crystal, 9. Parenchyma, 10. Multticellular non-glandular trichome, 11. Collenchyma, 12. Lower epidermis

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2.2.15 ***C. timoriensis***; the dorsal and ventral epidermis composed of slightly thick walled epidermal cells with cuticle and numerous multicellular non-glandular trichomes. The dorsal epidermis was absent of stomata cell whereas, the ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of bilayer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 3-6 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed several layered of collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many multicellular non-glandular trichomes were found on both sides of epidermis. The illustration of *C. timoriensis* by hand drawing in the proportion size related to the original scale was showed in Figure 58.

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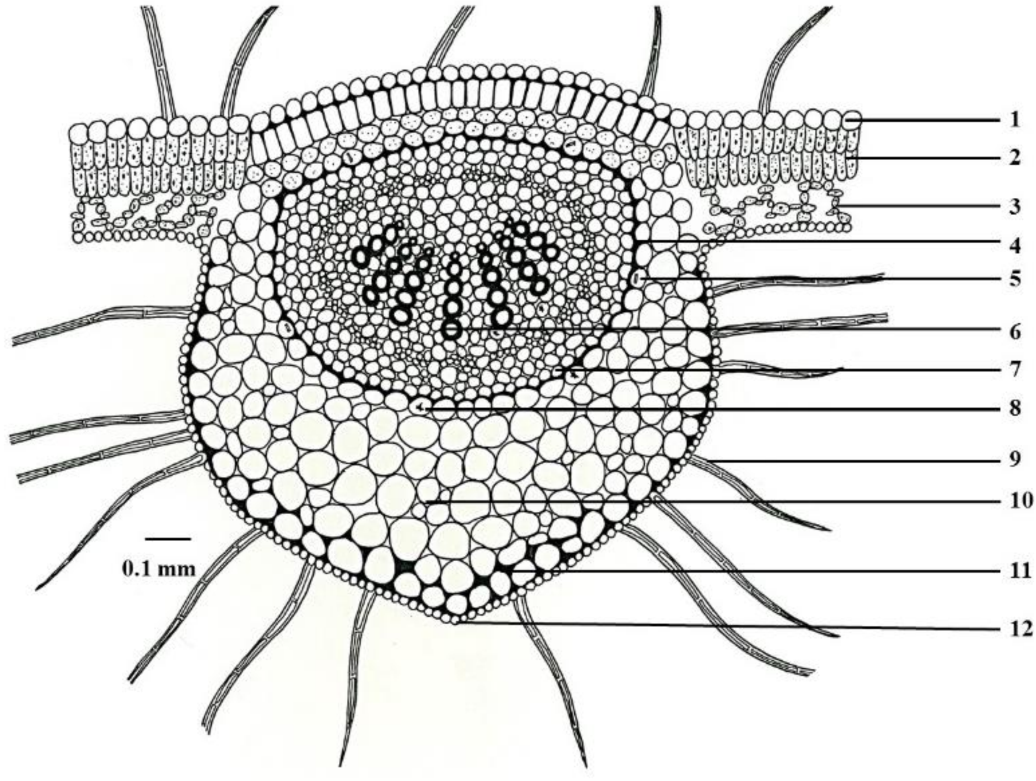

Figure 58 Transverse section of leaf through midrib of *C. timoriensis*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Prism crystal, 6. Xylem tissue, 7. Phloem tissue, 8. Druse crystal, 9. Multticellular non-glandular trichome, 10. Parenchyma, 11. Collenchyma, 12. Lower epidermis

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2.2.16 *C. tora*; the dorsal and ventral epidermis composed of circle to ellipse cells shape with cuticle. Multicellular non-glandular trichomes was found only on ventral epidermis. The dorsal and ventral epidermis was composed of numerous paracytic stomata cells. The palisade mesophyll composed of a single layer of columnar cell rich in chloroplasts. The spongy mesophyll composed of 4-6 layers of loosely arranged rounded to oval shaped with intercellular air-spaces. The midrib was composed of two to four collenchyma cells underneath the epidermis of both dorsal and ventral epidermal. Collateral vascular bundles, surrounded with bundle of fiber sheath, were situated in the center of the ground parenchyma. The parenchyma cell comprised prism crystal and druse crystal structure. Many multicellular non-glandular trichomes were found on ventral sides of epidermis. The illustration of *C. tora* by hand drawing in the proportion size related to the original scale was showed in Figure 59.

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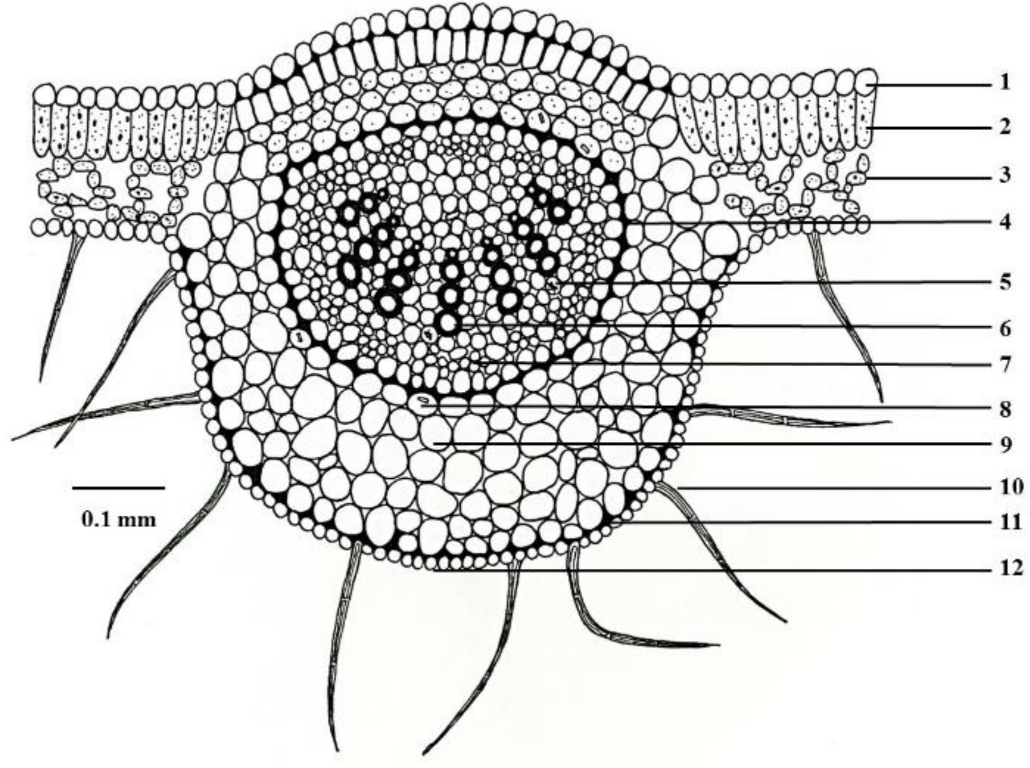

Figure 59 Transverse section of leaf through midrib of *C. tora*; 1. Upper epidermis, 2. Palisade cells, 3. Spongy cells, 4. Sclerenchyma, 5. Druse crystal, 6. Xylem tissue, 7. Phloem tissue, 8. Prism crystal, 9. Parenchyma, 10. Multticellular non-glandular trichome, 11. Collenchyma, 12. Lower epidermis

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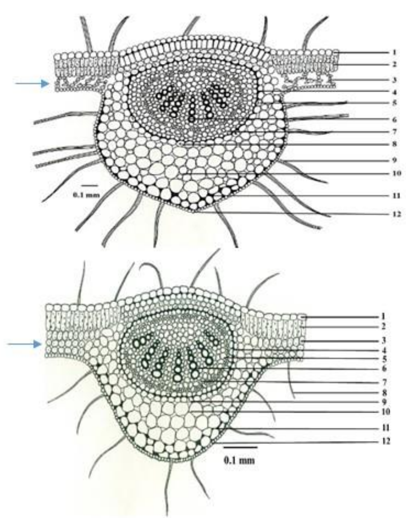
The arrangement of palisade

Fifteen *Cassia* species (*C. bakeriana*, *C. fistula*, *C. grandis*, *C. javanica*, *C. alata*, *C. angustifolia*, *C. garrettiana*, *C. hirsuta*, *C. occidentalis*, *C. spectabilis*, *C. siamea*, *C. sophera*, *C. sulfurea*, *C. surattensis* and *C. tora*) had single layer of palisade cells.

C. timoreinsis had bilayer of palisade cells.

The arrangement of spongy cells in mesophyll

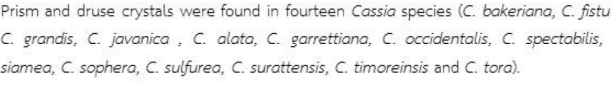


Thirteen *Cassia* species (*C. bakeriana*, *C. fistula*, *C. alata*, *C. garrettiana*, *C. hirsuta*, *C. occidentalis*, *C. spectabilis*, *C. siamea*, *C. sophera*, *C. sulfurea*, *C. surattensis*, *C. timoreinsis* and *C. tora*) shown loosely arranged rounded to oval shaped with intercellular air-space.

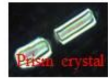
Three *Cassia* species (*C. grandis*, *C. javanica*, *C. angustifolia* shown regularity arrangement.

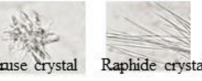
Calcium oxalate crystal structure in transverse section of leaves through midrib

Prism crystals were found only in *C. angustifolia*.



Prism and druse crystals were found in fourteen *Cassia* species (*C. bakeriana*, *C. fistula*, *C. grandis*, *C. javanica* , *C. alata*, *C. garrettiana*, *C. occidentalis*, *C. spectabilis*, *C. siamea*, *C. sophera*, *C. sulfurea*, *C. surattensis*, *C. timoreinsis* and *C. tora*).





Prism, druse and raphide crystals were found only in *C. hirsuta*.

Figure 60 Summarization of microscopic characteristics of 16 *Cassia* species

2.2 The results of stomatal classification

The type of stomata in 16 *Cassia* species were classified as paracytic type (stomata surrounded by two subsidiary cells by parallel to the long axis of guard cells). In *C.alata, C. occidentalis, C. sophera, C. hirsuta, C. angustifolia, C. tora, C.surattensis* and *C. sulfurea*, stomata were found on both dorsal and ventral epidermis. In *C. bakeriana, C. fistula, C. grandis, C. javanica, C. siamea. C. spectabilis, C. timoriensis* and *C. garrettiana*, stomata were found on only at ventral epidermis. The paracytic type of stomata were showed in Figure 61.

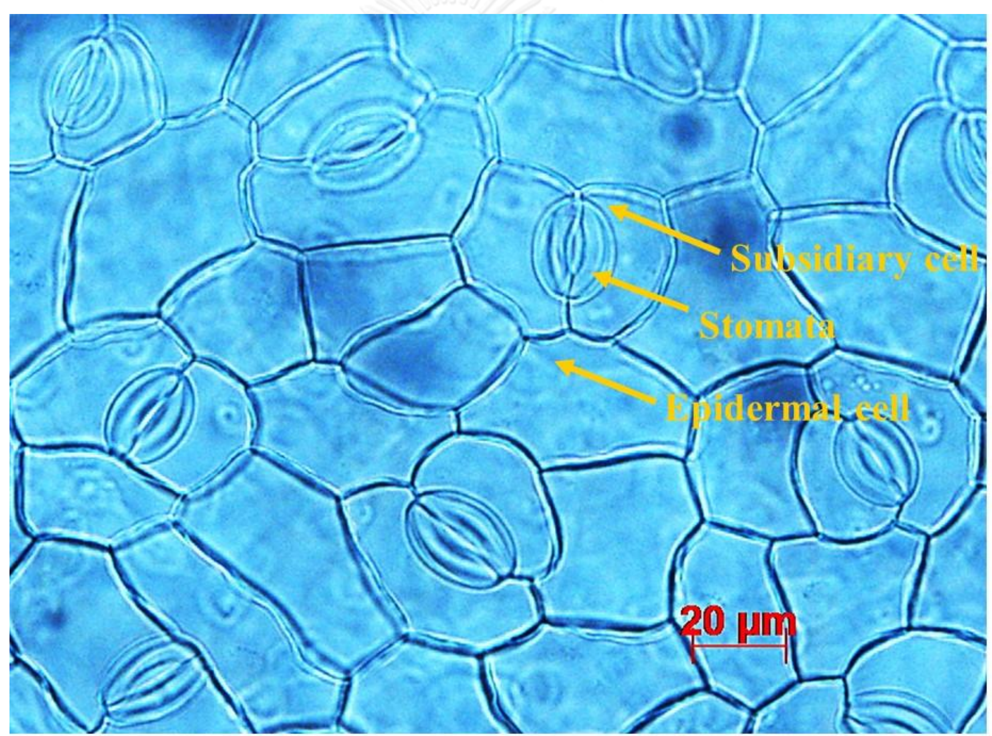


Figure 61 LM micrographs of paracytic type in *C. angustifolia*

2.3 Trichome numbers and trichome characteristics

Forty eight samples of *Cassia* species were examined. Trichome numbers and trichome characteristics were examined as shown in Table 18. Micrographs of *Cassia* species taken from SEM were demonstrated in Figure 62-64.

Table 18 The trichome number and trichome characteristics of *Cassia* species*

No.	*Cassia* species	Trichome number Mean ± SD (Min-Max)		Trichome characteristics	
		Dorsal surface	Ventral surface	Dorsal surface	Ventral surface
1	*C. bakeriana*	42.21 ± 1.31 (39-44)	71.48 ± 2.64 (66-77)	multicellular non-glandular	multicellular non-glandular
2	*C. fistula*	32.00 ± 1.56 (29-34)	94.47 ± 2.21 (90-98)	unicellular non-glandular	unicellular non-glandular
3	*C. grandis*	22.04 ± 2.13 (19-25)	46.36 ± 2.95 (42-52)	multicellular non-glandular	multicellular non-glandular
4	*C. javanica*	78.94 ± 2.86 (72-88)	127.39 ± 2.46 (124-135)	multicellular non-glandular	multicellular non-glandular
5	*C. alata*	8.59±1.47 (6-12)	7.29±1.08 (5-10)	unicellular non-glandular	unicellular non-glandular
6	*C. angustifolia*	12.42±2.28 (9-18)	49.43±3.64 (42-58)	unicellular non-glandular	unicellular non-glandular
7	*C. siamea*	31.39±2.45 (26-35)	57.44±2.60 (52-65)	unicellular non-glandular	unicellular non-glandular
8	*C. spectabilis*	20.37±2.30 (16-26)	55.67±2.48 (52-62)	multicellular non-glandular	multicellular non-glandular
9	*C. timoriensis*	11.33±1.73 (8-14)	22.31±1.65 (19-25)	multicellular non-glandular	multicellular non-glandular
10	*C. hirsuta*	5.00±0.50 (4-6)	8.21±0.95 (6-10)	multicellular non-glandular	multicellular non-glandular
		n.c.	n.c.	multicellular glandular	multicellular glandular

Table 18 The trichome number and trichome characteristics of *Cassia* species* (Cont.)

No.	*Cassia* species	Trichome number Mean ± SD (Min-Max)		Trichome characteristics	
		Dorsal surface	Ventral surface	Dorsal surface	Ventral surface
11	*C. sulfurea*	-	10.20 ± 1.79 (6-15)	-	multicellular non-glandular
12	*C. surattensis*	-	3.46 ± 0.80 (2-5)	-	multicellular non-glandular
13	*C. tora*	-	63.49 ± 2.34 (58-69)	-	multicellular non-glandular
14	*C. garrettiana*	-	-	-	-
15	*C. occidentalis*	-	-	-	-
16	*C. sophera*	-	-	-	-

* n = Thirty fields of each specimen from 3 different sources were examined

\- = absence trichome

n.c. = not counted

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The results showed that there were differences in trichome numbers between 13 species. According to the presence and absence of trichome, three main groups were classified. The first group, the trichome of 10 *Cassia* spp. (*C. bakeriana*, *C. fistula*, *C. grandis*, *C. javanica*, *C. alata*, *C. angustifolia*, *C. siamea*, *C. spectabilis*, *C. timoriensis* and *C. hirsuta*) was shown trichome on both dorsal and ventral surfaces (Figure 62). The second group, three *Cassia* spp. (*C. sulfurea*, *C. surattensis* and *C. tora*) had shown trichome on ventral surface (Figure 63). The last group, three *Cassia* spp. (*C. garrettiana*, *C. occidentalis* and *C. sophera*) had no trichome (Figure 64). The highest value was found on both dorsal and ventral surface of *C. javanica* (78.94±2.86 and 127.39±2.46, respectively). The lowest trichome number was found on only ventral surface of *C. surattensis* (3.46±0.80).

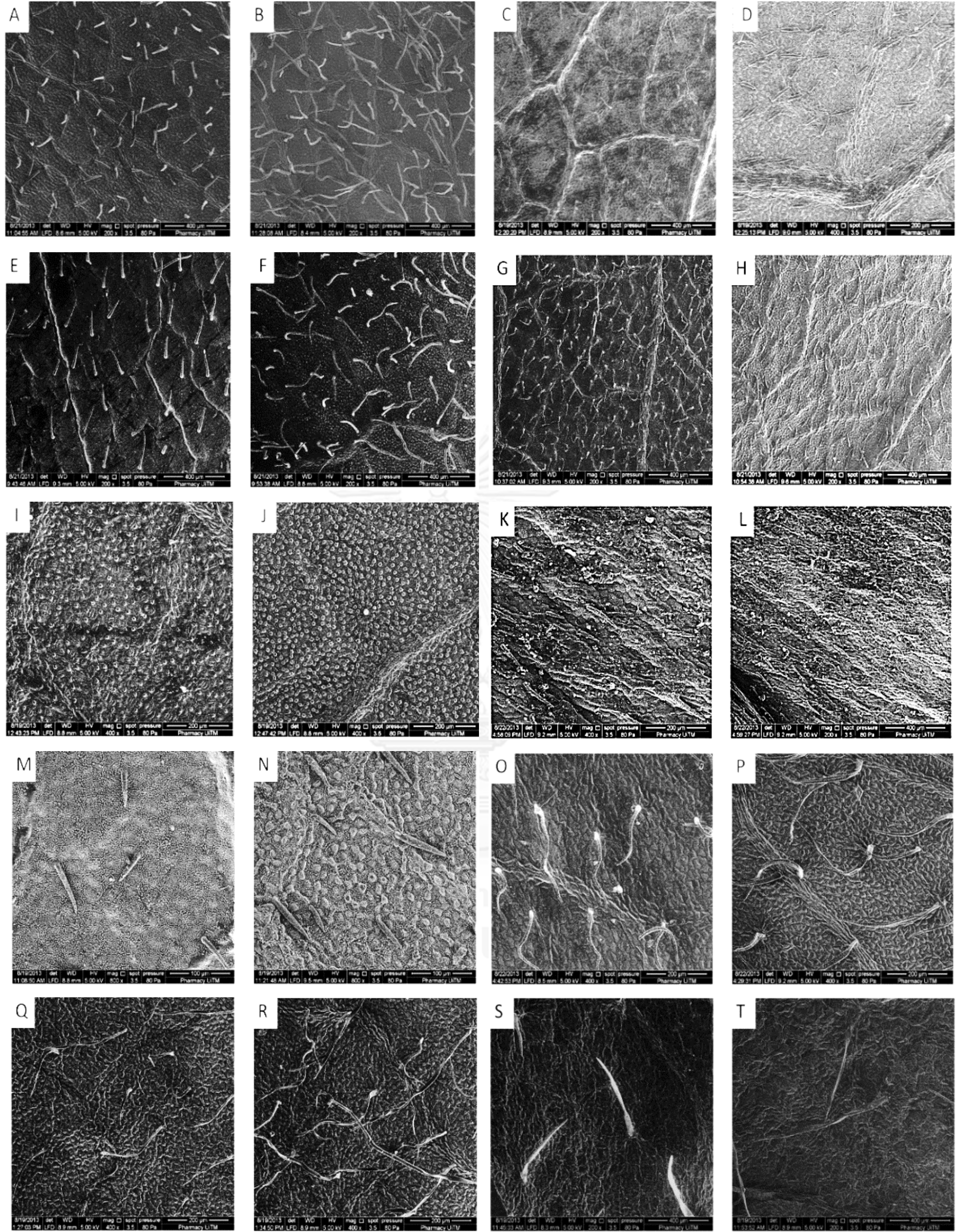

Figure 62 SEM micrographs of 10 *Cassia* species. Trichome was present on both dorsal and ventral surfaces: (A),(B) *C. bakeriana*, (C),(D) *C. fistula*, (E),(F) *C. grandis*, (G),(H) *C. javanica*, (I),(J) *C. alata*, (K),(L) *C. angustifolia*, (M),(N) *C. siamea*, (O),(P) *C. spectabilis,* (Q),(R) *C. timoriensis*, (S),(T) *C. hirsuta*

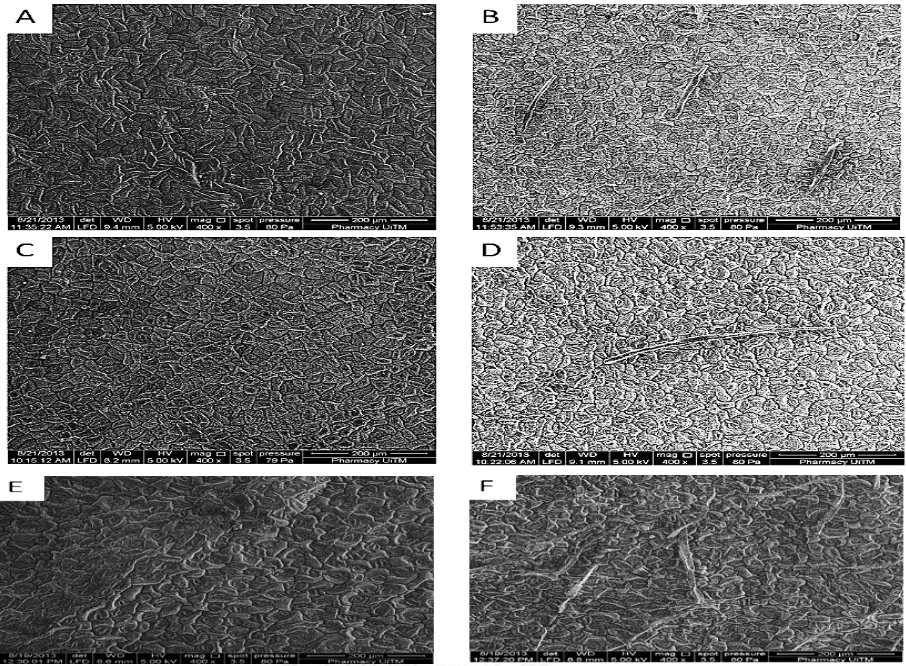

Figure 63 SEM micrographs of three Cassia species. Trichome was present only on ventral surfaces: (A),(B) *C. sulfurea* (C),(D) *C. surattensis*, (E),(F) *C. tora*

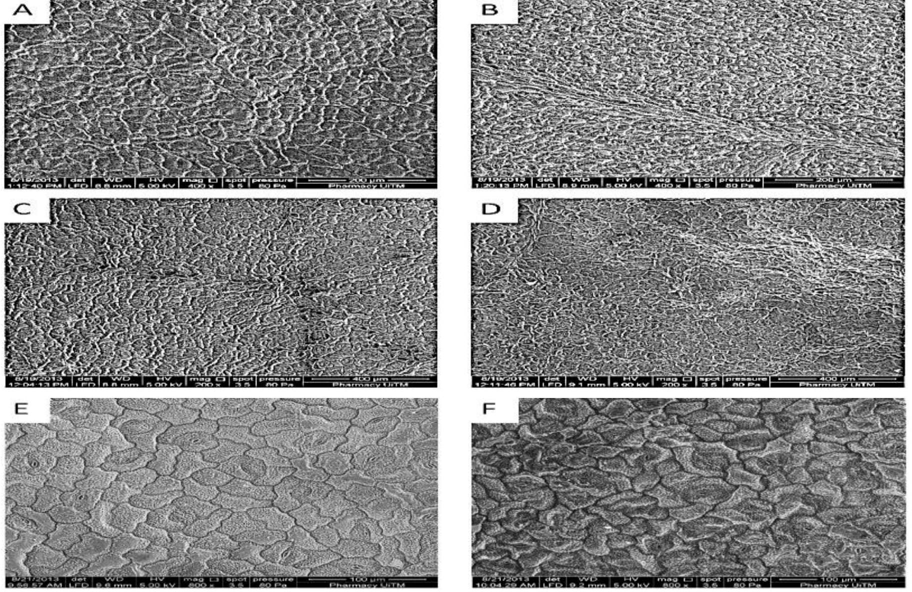

Figure 64 SEM micrographs of three Cassia species. Trichome was absent on both surfaces: (A),(B) *C. garrettiana*, (C),(D) *C. occidentalis*, (E),(F) *C. sophera*

In dorsal surface, the results showed that there were significant differences ($P < 0.01$) in trichrome numbers among eight species except in *C. fistula* and *C. siamea*. In thirteen species that trichome were found in ventral surface, trichrome numbers were significant differences ($P < 0.01$) except *C. alata* and *C. hirsuta* (Figure 65).

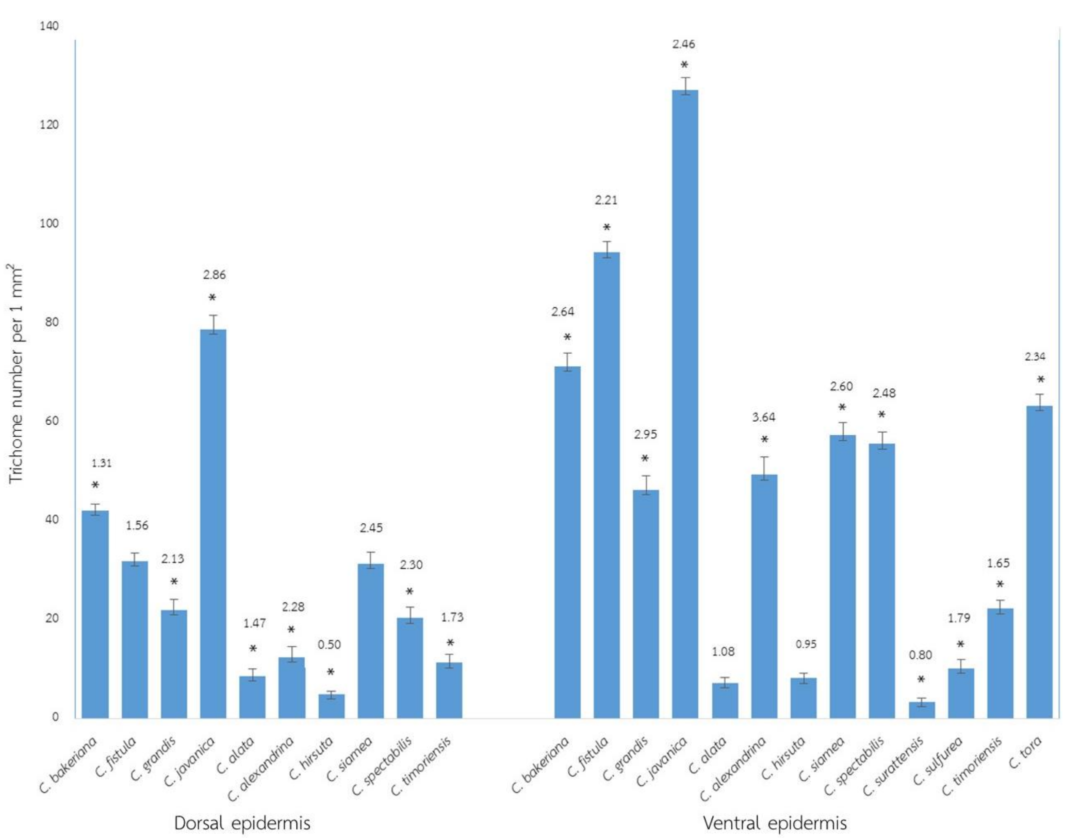

Figure 65 Trichome number in dorsal surface and ventral surface of *Cassia* species and expressed as mean ± SD. (* $P < 0.01$, Tukey HSD test)

Based on the number of cell present in structure and the presence or absence of glandular cell of trichome, the trichome characteristics of investigated *Cassia* species were uniseriate, uni- or multicellular non-glandular and multicellular glandular types (Figure 66).

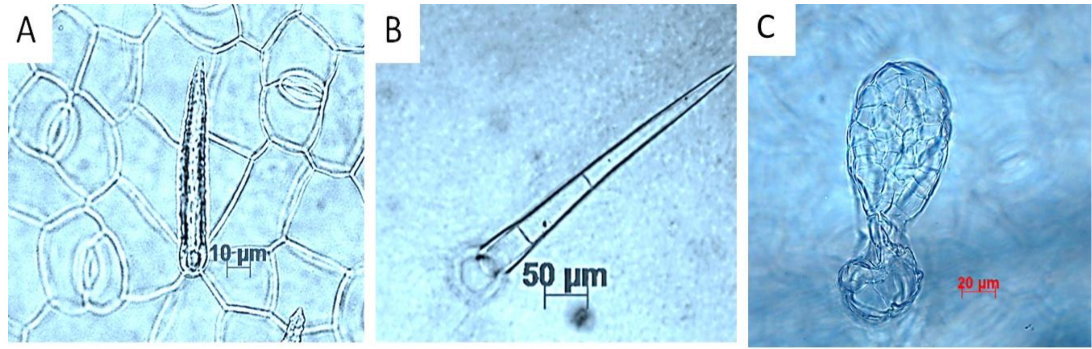

Figure 66 LM micrographs of the trichome characteristic: (A) unicellular non-glandular types, (B) multicellular non-glandular types, (C) globose glandular types

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Part II Molecular identification

1. DNA extraction

The genomic DNA was isolated from young leaves of each *Cassia* species using modified CTAB method as described in chapter 3. The genomic DNA was run on 1.5 % agarose gel electrophoresis and stained with ethidium bromide as showed in Figure 67.

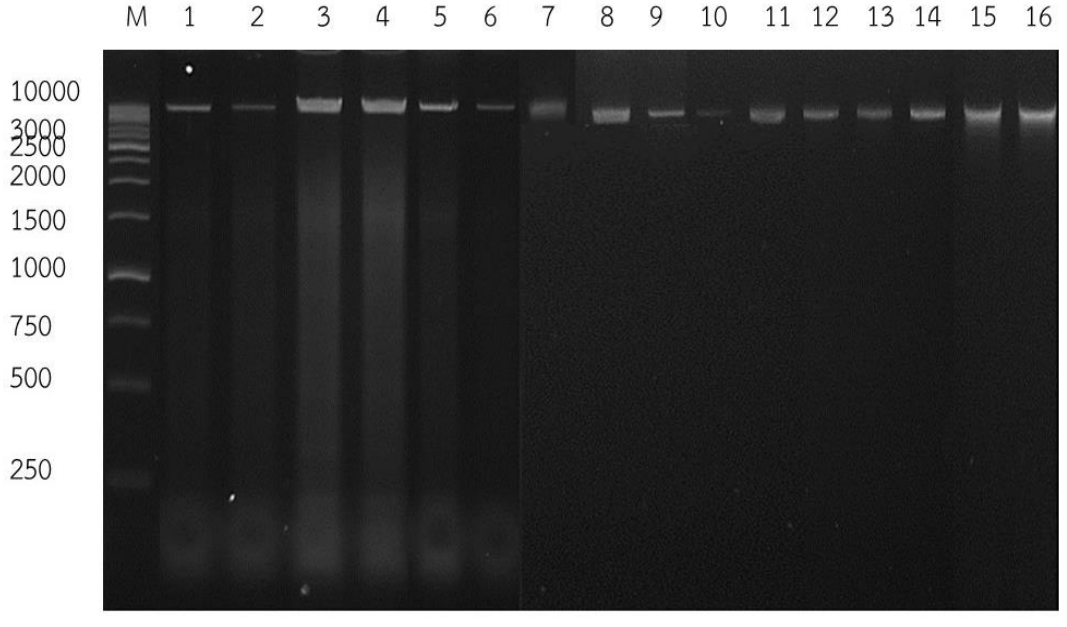

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Figure 67 Genomic DNA of 16 *Cassia* species on 1.5 % agarose gel electrophoresis

Lane M : 1 kb DNA ladder
Lane 1: *C. bakeriana*
Lane 2: *C. grandis*

Lane 3: *C. fistula*
Lane 4: *C. alata*
Lane5: *C. javanica*

Lane 6: *C. spectabilis*
Lane 7: *C. siamea*
Lane 8: *C. garrettiana*

Lane 9: *C. hirsuta*
Lane 10: *C. angustifolia*
Lane 11: *C. occidentalis*

Lane 12: *C. sophera*
Lane 13: *C. tora*
Lane 14: *C. timoriensis*

Lane 15: *C. surattensis*
Lane 16: *C. sulfurea*

2. AFLP analysis

A total of 70 primer combinations were initially screened among these 11 primer combinations which produced visible and clear bands in all plant samples (Table 19). Each species was collected from 3 different localities, but showed the same patterns of AFLP profiles, so an individual representative sample of each species was selected. The results demonstrated that different primers generate different fragment numbers and lengths. A total of 849 amplified fragments, ranging from 80 to 700 base pairs in size, were generated from 11 primer combinations (Table 19). The bands that were produced from the 11 primer combinations ranged from 60 to 100 bands with an average of 77.18 polymorphic bands per primer combinations and generated a high percentage (99.07%) of polymorphic bands. The highest number of the amplified fragments was obtained from the primer pair E+AAC/M+CAA (100 bands) (Figure 68), while the lowest number was obtained from the primer pair E+AAC/M+CCC (60 bands) (Figure 69). The primer pair E+AAC/M+CAA shown the monomorphic bands of *Cassia* species (sample number 1-4) and *Senna* species (sample number 5-16) (Figure 68). Moreover, this primer pair also shown the unique bands of *Cassia* species.

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Table 19 The list of 11 primer combinations and the number of AFLP bands, size ranges and percentages of polymorphic bands

Primer combination	Number of AFLP band	Size range (bps)	Percentage of polymorphic band
E+AAC/M+CCA	61	80-700	100
E+AAC/M+CAA	100	80-700	100
E+AAC/M+CGT	64	80-700	100
E+AAC/M+CCC	60	80-700	100
E+ACC/M+CAA	78	80-700	96.15
E+ACC/M+CCA	67	80-700	100
E+AAG/M+CCA	99	80-700	98.99
E+AAG/M+CAT	90	80-700	100
E+AAG/M+CAA	79	80-700	96.20
E+AGC/M+CCA	89	80-700	100
E+AGC/M+CAA	62	80-700	98.39
Total	**849**	**80-700**	**99.07**

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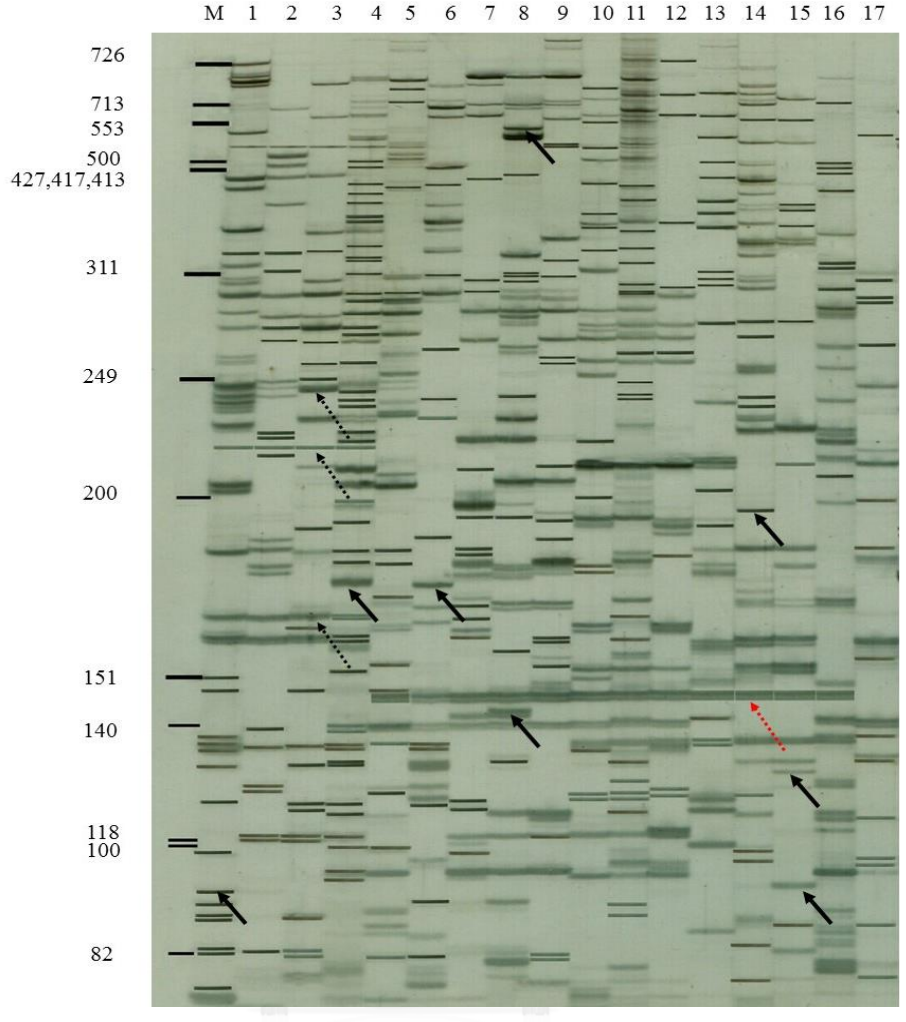

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Figure 68 AFLP fingerprint of 16 *Cassia* species and *Andrographis paniculata* (outgroup plants) obtained from E+AAC/M+CAA primer combinations

——▶ indicates unique bands of *Cassia* species

······▶ indicates monomorphic bands of *Cassia* species

▪▪▪▪▶ indicates monomorphic bands of *Senna* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker

Lane 1: *C. fistula*

Lane 2: *C. grandis*

Lane 3: *C. bakeriana*

Lane 4: *C. javanica*

Lane5: *C. alata*

Lane 6: *C. spectabilis*

Lane 7: *C. siamea*

Lane 8: *C. timoriensis*

Lane 9: *C. garrettiana*

Lane 10: *C. hirsuta*

Lane 11: *C. occidentalis*

Lane 12: *C. sophera*

Lane 13: *C. tora*

Lane 14: *C. surattensis*

Lane 15: *C. sulfurea*

Lane 16: *C. angustifolia*

Lane17: *A. paniculata*

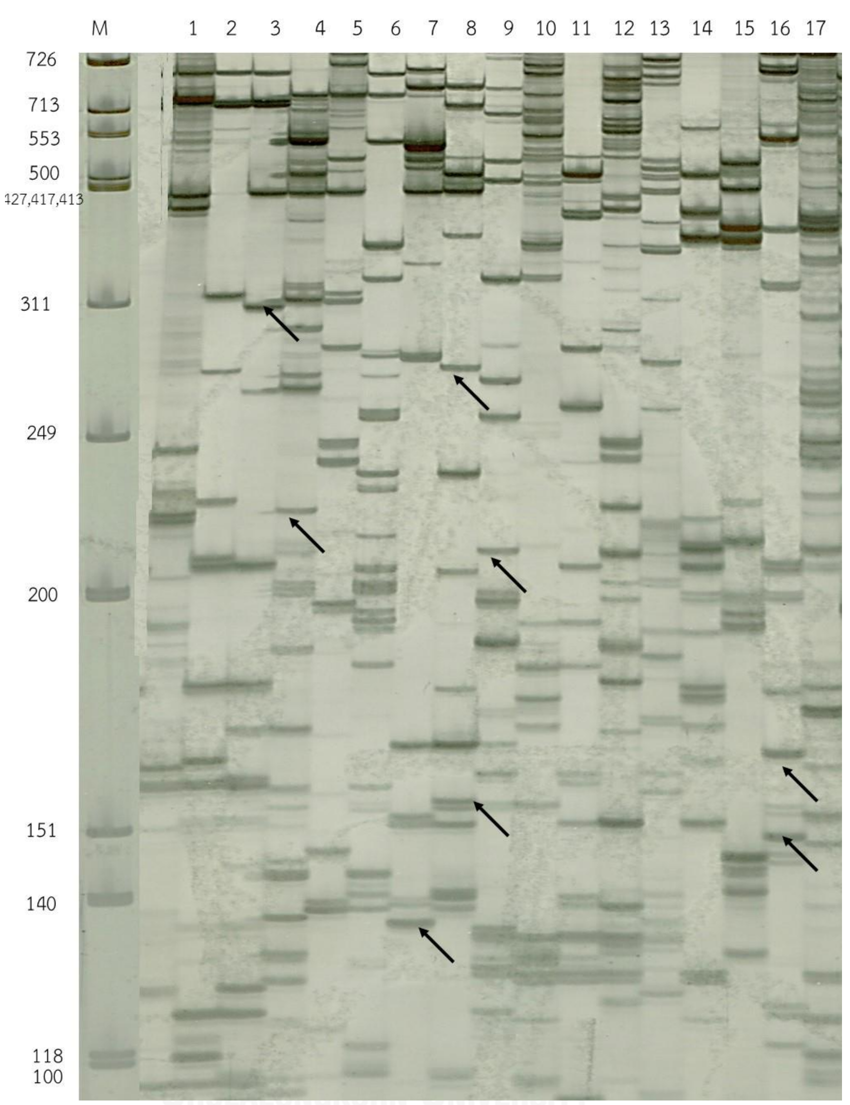

Figure 69 AFLP fingerprint of 16 *Cassia* species and *Andrographis paniculata* (outgroup plants) obtained from E+AAC/M+CCC primer combinations

⟶ indicates unique bands of *Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker
Lane 1: *C. fistula*
Lane 2: *C. grandis*
Lane 3: *C. bakeriana*
Lane 4: *C. javanica*
Lane5: *C. alata*
Lane 6: *C. spectabilis*
Lane 7: *C. siamea*
Lane 8: *C. timoriensis*
Lane 9: *C. garrettiana*
Lane 10: *C. hirsuta*
Lane 11: *C. occidentalis*
Lane 12: *C. sophera*
Lane 13: *C. tora*
Lane 14: *C. surattensis*
Lane 15: *C. sulfurea*
Lane 16: *C. angustifolia*
Lane17: *A. paniculata*

3. Genetic relationships

The dendrogram was generated by Jaccard's similarity matrix and the UPGMA method. According to the dendrogram, two major groups were classified as having bootstrap values higher than 80%. The bootstrap values of the different clusters and subclusters are displayed in the bootstrap tree (Figure 70). The first group is composed of *C. bakeriana, C. fistula, C. grandis* and *C. javanica* with the similarity index 0.54-0.72 and 100% bootstrap support. The second group can be divided into 3 subgroups (98% bootstrap) with the first subgroup being composed of *C. garrettiana, C. siamea, C. timoriensis, C. alata and C. spectabilis* with a 0.45-0.64 similarity index. *C. tora, C. surattensis* and *C. sulfurea* were clustered into a second subgroup with a 0.47-0.78 similarity index. The last subgroup belongs to *C. hirsuta, C. occidentalis, C. sophera* and *C. angustifolia* with the similarity index 0.39-0.63. According to the dendrogram, the outgroup plants (*A. paniculata*) were clearly separated from sixteen *Cassia* species with 100% bootstrap support.

The pair-wise comparisons of the AFLP profiles were based on both of the shared and unique amplification bands, and were used to generate a similarity index. Among the 48 accessions of 16 species, the genetic similarity ranges from 0.25 to 0.78 (Table 20). *C. surattensis* and *C. sulfurea* shown the highest genetic similarity value (0.78), whereas *C. fistula* and *C. hirsuta* shown the lowest genetic similarity value (0.25).

Table 20 Pair-wise genetic similarity index (SI) of 16 *Cassia* species and outgroup plants according to the index of Jaccard

	C. fistula	*C. grandis*	*C. bakeriana*	*C. javanica*	*C. alata*	*C. spectabilis*	*C. siamea*	*C. timoriensis*	*C. garrettiana*	*C. hirsuta*	*C. occidentalis*	*C. sophera*	*C. angustifolia*	*C. tora*	*C. surattensis*	*C. sulfurea*	*A. paniculata*
C. fistula	1.0000																
C. grandis	0.6069	1.0000															
C. bakeriana	0.6083	0.5866	1.0000														
C. javanica	0.6136	0.5354	0.7165	1.0000													
C. alata	0.3587	0.3586	0.3765	0.4116	1.0000												
C. spectabilis	0.3138	0.3225	0.3405	0.3707	0.5568	1.0000											
C. siamea	0.3402	0.3511	0.3288	0.3313	0.4900	0.4497	1.0000										
C. timoriensis	0.3070	0.3275	0.3191	0.3314	0.4783	0.4921	0.6421	1.0000									
C. garrettiana	0.3215	0.3511	0.3211	0.3338	0.4744	0.4656	0.6126	0.6121	1.0000								
C. hirsuta	0.2537	0.2766	0.2831	0.2838	0.3667	0.3899	0.4092	0.4149	0.4326	1.0000							
C. occidentalis	0.2928	0.3008	0.2884	0.3097	0.4004	0.3809	0.4072	0.4206	0.4236	0.6267	1.0000						
C. sophera	0.2596	0.2715	0.2786	0.2794	0.3467	0.3548	0.3732	0.3832	0.4095	0.5810	0.5961	1.0000					
C. angustifolia	0.2741	0.3050	0.2921	0.2620	0.3925	0.3321	0.3525	0.3518	0.3556	0.3946	0.4383	0.4512	1.0000				
C. tora	0.2784	0.2773	0.2766	0.3000	0.3495	0.3389	0.3259	0.3424	0.3354	0.4433	0.4838	0.4220	0.3712	1.0000			
C. surattensis	0.2955	0.3053	0.3023	0.3251	0.3570	0.3233	0.3624	0.3780	0.3552	0.3871	0.4647	0.3939	0.3650	0.5342	1.0000		
C. sulfurea	0.2909	0.3066	0.3008	0.3184	0.3331	0.3342	0.3408	0.3656	0.3588	0.3668	0.4061	0.3636	0.3580	0.4726	0.7750	1.0000	
A. paniculata	0.0267	0.0325	0.0492	0.0475	0.0202	0.0192	0.0301	0.0342	0.0395	0.0306	0.0332	0.0211	0.0165	0.0417	0.0359	0.0357	1.0000

SI value range from 0 – 1.0000 according to the increasing similarity index

According to the revised classification[2] the first groups (I) in the dendrogram are the genus *Cassia* whereas the second groups (II) are moved to the genus *Senna* (Figure 70).

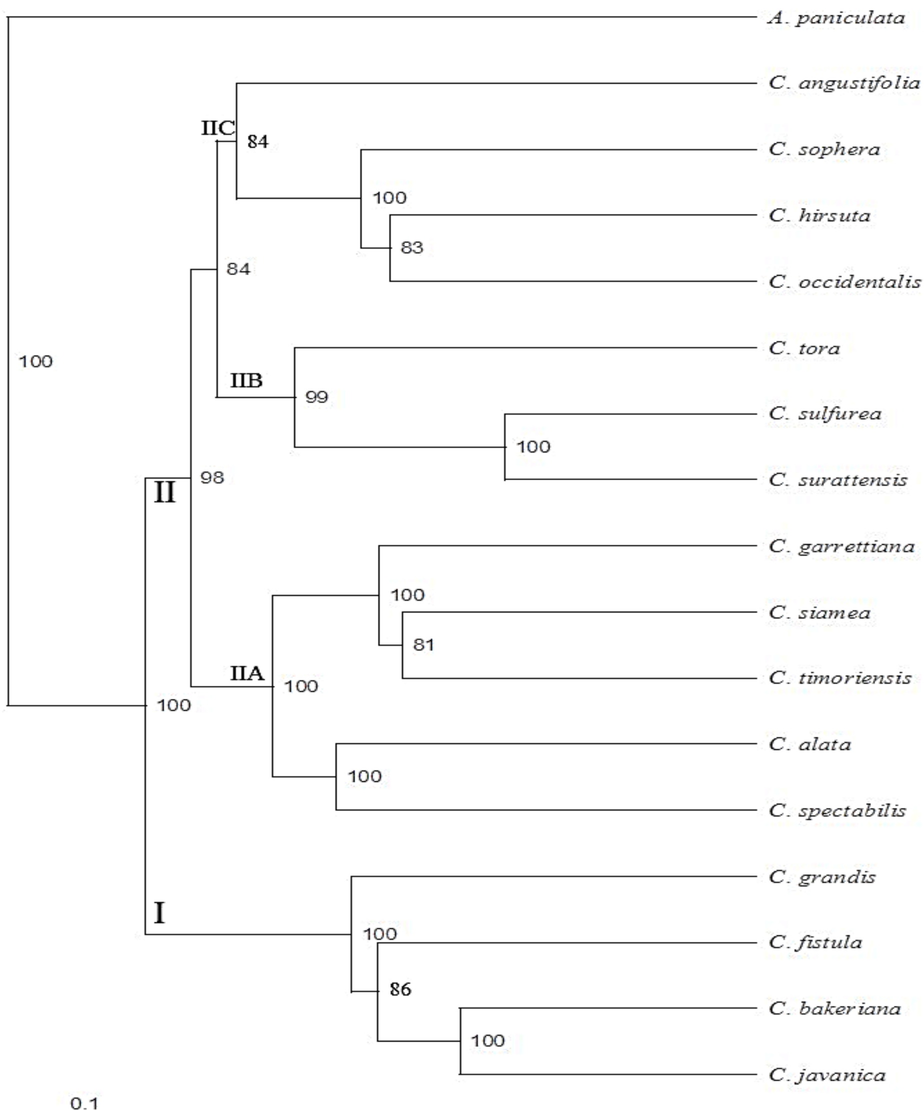

Figure 70 UPGMA dendrogram based on Jaccard's similarity coefficient among *Cassia* species and outgroup plants

Part III Quantitative analysis

1. Quantitation of aloe-emodin content in *C. grandis* leaves

1.1 Dichloromethane extracts of dried *C. grandis* leaves

The average percent yield of dichloromethane extracts of *C. grandis* leaves by soxhlet extraction was 13.9602±1.1229 % (Table 21).

Table 21 The percent yield of dichloromethane extract of dried *C. grandis* leaves from 15 different locations throughout Thailand

Source	Weight of sample (g)	Weight of extractive matter (g)	% Yield
1	6.002	0.812	13.525
2	6.001	0.825	13.751
3	6.001	0.839	13.981
4	6.002	0.893	14.876
5	6.001	0.725	12.076
6	6.002	0.821	13.675
7	6.002	0.821	13.676
8	6.001	0.879	14.641
9	6.001	0.861	14.349
10	6.001	0.853	14.216
11	6.002	0.737	12.281
12	6.001	0.906	15.100
13	6.002	0.947	15.778
14	6.001	0.916	15.266
15	6.001	0.733	12.212
	Average		13.960±1.123

1.2 The chromatographic condition for quantitating aloe-emodin in *C. grandis* leaves

The chromatographic condition for quantitating aloe-emodin was examined using silica gel 60 GF_{254}. The selected mobile phase, hexane-ethyl acetate (1:1, v/v) demonstrated the best separation of aloe-emodin in *C. grandis* leaf extracts with R_f value 0.50±0.007. The aloe-emodin band of the plant samples was confirmed by comparing an R_f value with standard aloe-emodin. The TLC chromatograms of 15 samples and standard aloe-emodin under UV 254 nm, under UV 365 nm and in visible light were demonstrated in Figure 71.

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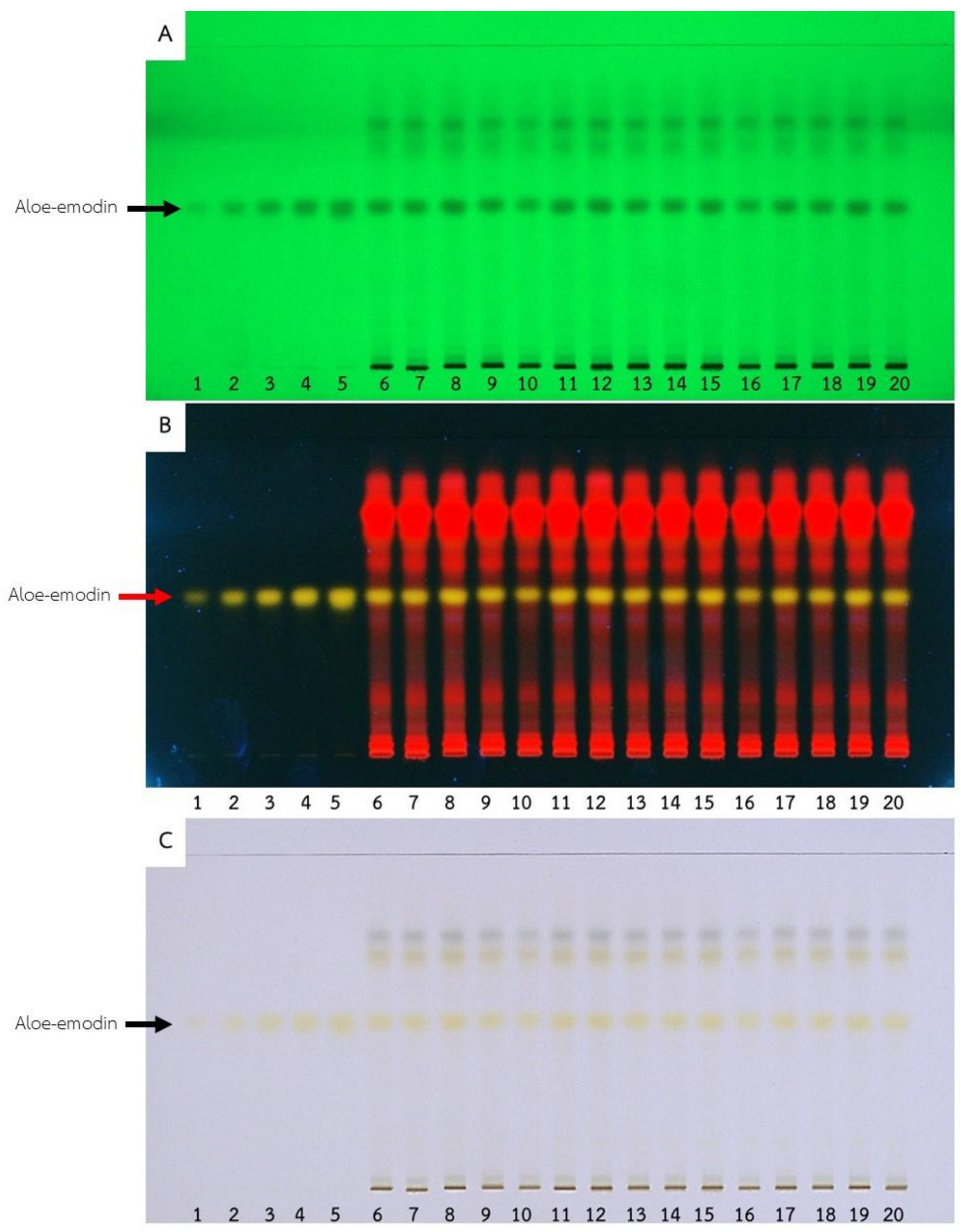

Figure 71 The TLC plate (A) under UV 254 nm, (B) under UV 365 nm, (C) in visible light; standard aloe-emodin (track 1 to 5) and *C. grandis* leaf extracts from 15 various locations in Thailand

1.3 The amount of aloe-emodin in *C. grandis* leaves by TLC-densitometry

The aloe-emodin contents in *C. grandis* leaf extracts from 15 different locations were determined in triplicate by TLC-densitometry. The average aloe-emodin contents in crude drugs were 0.412±0.067% (Table 22).

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Table 22 The amount of aloe-emodin in *C. grandis* leaves by TLC-densitometry (% by dried weight)

Source	Aloe-emodin in dichloromethane extract (g/g of crude extract)				Yield of the dichloromethane extract (g/100 g of dried crude drug)	Aloe-emodin in *C. grandis* leaves (g/100 g of dried crude drug)
	1	2	3	Mean		
1	0.028	0.027	0.028	0.028	13.525	0.374
2	0.028	0.029	0.028	0.028	13.751	0.386
3	0.028	0.028	0.029	0.028	13.981	0.393
4	0.028	0.027	0.028	0.028	14.877	0.412
5	0.022	0.022	0.022	0.022	12.076	0.266
6	0.031	0.032	0.033	0.032	13.675	0.432
7	0.034	0.033	0.035	0.034	13.676	0.467
8	0.029	0.030	0.031	0.030	14.641	0.440
9	0.030	0.030	0.029	0.030	14.349	0.427
10	0.031	0.032	0.033	0.032	14.216	0.455
11	0.026	0.027	0.027	0.027	12.281	0.329
12	0.029	0.031	0.032	0.031	15.100	0.467
13	0.033	0.032	0.034	0.033	15.778	0.523
14	0.031	0.030	0.032	0.031	15.266	0.478
15	0.027	0.027	0.028	0.027	12.212	0.335
					Mean	0.412
					SD	0.067

1.4 Method validation of TLC-densitometry

1.4.1 Calibration curve

The calibration curve of aloe-emodin standard solutions was linear in the range of 0.12, 0.24, 0.36, 0.48 and 0.60 µg/spot. The regression equation of aloe-emodin was y = 33534x + 1700. The coefficient of determination (R^2) of aloe-emodin was 0.9995.

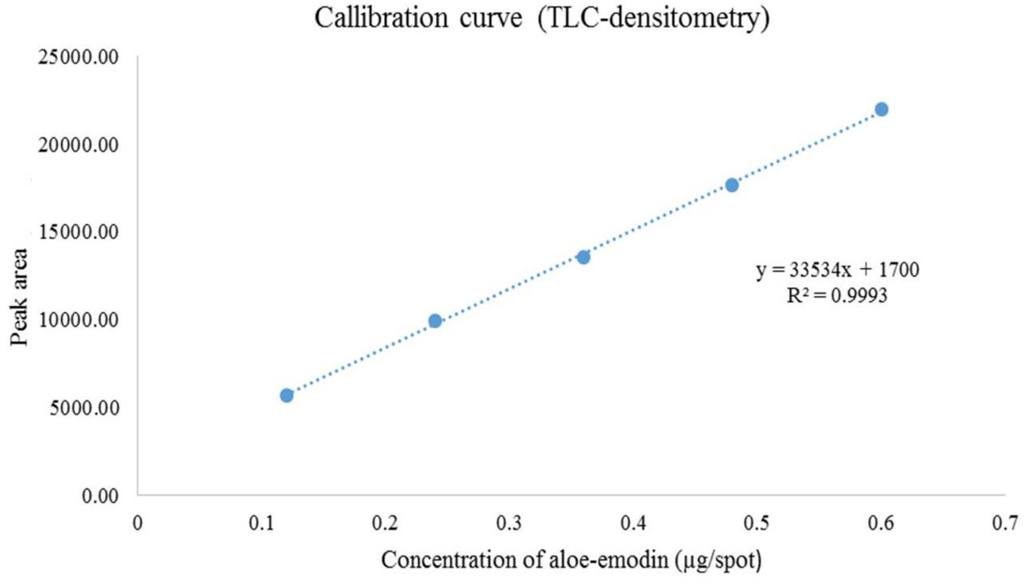

Figure 72 The calibration curve of aloe-emodin in *C. grandis* leaf extracts by TLC-densitometry

1.4.2 Accuracy

The accuracy of aloe-emodin quantitation by TLC-densitometry was evaluated in percentage of recovery. The recovery of aloe-emodin was performed on sample spiked with three different concentrations of aloe-emodin (0.06, 0.18 and 0.30 µg/spot). The recovery method was done in triplicate. The results were between 98.161-103.377% (Table 23).

Table 23 Recovery of aloe-emodin by TLC-densitometry (n=3)

Aloe-emodin added (µg/spot)	Aloe-emodin found (µg/spot)	%Recovery
0.00	0.207	-
0.06	0.276	103.377
0.18	0.387	100.003
0.30	0.498	98.161

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1.4.3 Precision

The precision of aloe-emodin quantitation by TLC-densitometry was evaluated in triplicate of each concentration group. The result was presented as the percentage of relative standard deviation which represented the error of the method. The repeatability was performed by three different concentrations on the same day. The intermediate precision was determined by three different concentrations on different days. The repeatability and intermediate precision were between 0.418-1.087% and 0.837-2.203%, respectively (Table 24).

Table 24 Repeatability and intermediate precision of aloe-emodin by TLC-densitometry (n=3)

Aloe-emodin (µg/spot)	Repeatability (%RSD)	Intermediate precision (%RSD)
0.276	1.087	2.203
0.387	0.517	2.194
0.498	0.418	0.837

1.4.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

In this study, LOD and LOQ in TLC-densitometry were measured based on the residual standard deviation of a regression line and the slope of calibration curve. The LOD and LOQ of aloe-emodin were assessed value as 0.0198 and 0.0601 µg/spot, respectively.

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1.4.5 Robustness

Robustness of the TLC-densitometry was performed by introducing small changes in the mobile phase complements (hexane: ethyl acetate). Each variation was determined in triplicate. The robustness of value was 0.28%RSD. The peak area of aloe-emodin in sample matrix was between 21667.88 and 21784.94 (Table 25).

Table 25 Robustness of aloe-emodin in *C. grandis* by the TLC-densitometry

Mobile phase (v/v)	Peak area of aloe-emodin
1.0 : 1.0	21749.25
0.9 : 1.1	21784.94
1.1 : 0.9	21667.88
Mean±SD	21734.02±60
%RSD	0.28

1.4.6 Specificity

The specificity was proved by peak identity and peak purity checking. The identity in absorbance spectra determined at the peak apex among aloe-emodin standards and a spot in the samples at the same R_f value was illustrated in Figure 73. The purity in absorbance spectra determined at up-slop, apex and down-slope of the sample peak was shown in Figure 74. The absorption spectra of aloe-emodin in all samples and standard were identical with the maximum absorption spectra at 434 nm which represented the method specificity.

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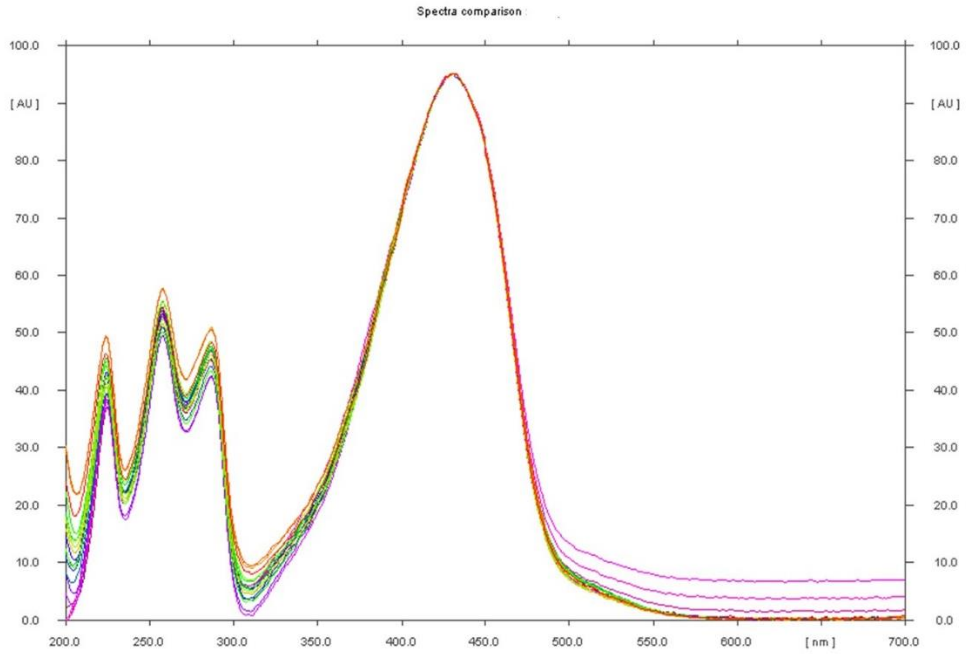

Figure 73 The absorption spectra of aloe-emodin in standard and sample bands *of C. grandis* leaves

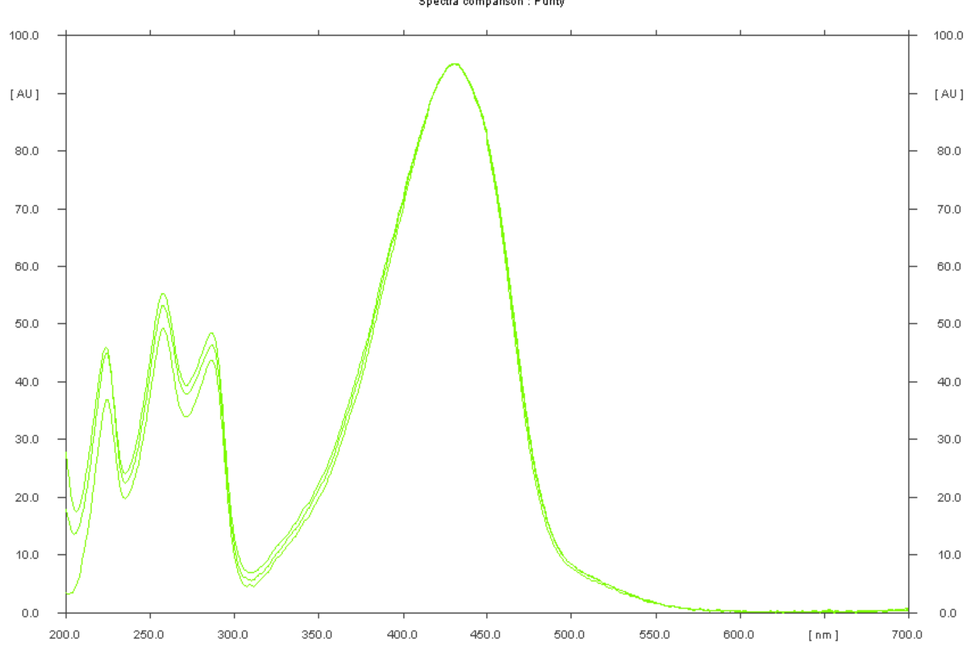

Figure 74 UV absorbance spectra of aloe-emodin in dichloromethane extract of *C. grandis* leaves using up-slope, apex and down-slope of the peak

1.5 The amount of aloe-emodin in *C. grandis* leaves by TLC-image analysis

The aloe-emodin contents in *C. grandis* leaf extracts from 15 different locations were determined in triplicate by TLC-image analysis. The average aloe-emodin contents in crude drugs were 0.413±0.075% (Table 26).

Table 26 The amount of aloe-emodin in *C. grandis* leaves by TLC-image analysis (% by dried weight)

Source	Aloe-emodin in dichloromethane extract (g/g of crude extract)				Yield of the dichloromethane extract (g/100 g of dried crude drug)	Aloe-emodin in *C. grandis* leaves (g/100 g of dried crude drug)
	1	2	3	Mean		
1	0.029	0.028	0.029	0.029	13.525	0.389
2	0.030	0.030	0.032	0.031	13.751	0.420
3	0.029	0.029	0.029	0.029	13.981	0.406
4	0.030	0.029	0.031	0.030	14.877	0.442
5	0.023	0.021	0.020	0.021	12.076	0.259
6	0.029	0.028	0.029	0.029	13.675	0.391
7	0.032	0.031	0.034	0.032	13.676	0.443
8	0.030	0.031	0.031	0.030	14.641	0.446
9	0.028	0.032	0.030	0.030	14.349	0.432
10	0.030	0.031	0.032	0.031	14.216	0.443
11	0.026	0.025	0.024	0.025	12.281	0.307
12	0.033	0.031	0.031	0.032	15.100	0.478
13	0.033	0.033	0.033	0.033	15.778	0.519
14	0.032	0.034	0.036	0.034	15.266	0.518
15	0.024	0.024	0.026	0.025	12.212	0.302
					Mean	0.413
					SD	0.075

1.6 Method validation of TLC-image analysis

1.6.1 Calibration curve

The calibration curves of aloe-emodin standard solutions was linear in the range of 0.12, 0.24, 0.36, 0.48 and 0.60 µg/spot. The regression equation of aloe-emodin was y = 43454x + 1023.8. The coefficient of determination (R^2) of aloe-emodin was 0.9995.

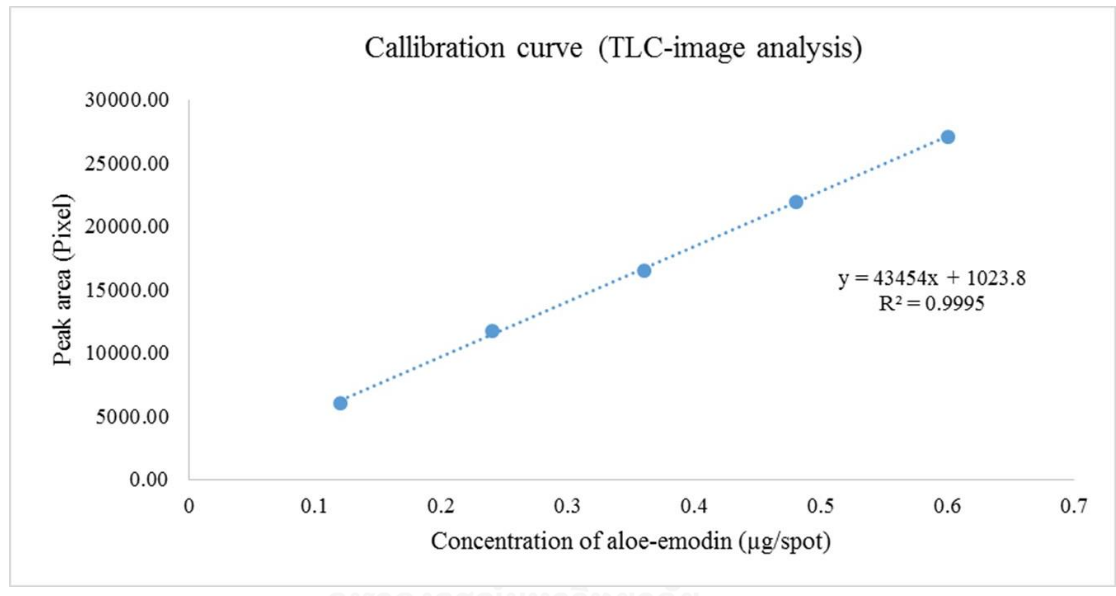

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Figure 75 The calibration curve of aloe-emodin in *C. grandis* leaf extracts by TLC-image analysis

1.6.2 Accuracy

The accuracy of aloe-emodin quantitation by TLC-image analysis was evaluated in percentage of recovery. The recovery of aloe-emodin was performed on sample spiked with three different concentrations of aloe-emodin (0.06, 0.18 and 0.30 µg/spot). The recovery method was done in triplicate. The results were between 97.578-107.863% (Table 27).

Table 27 Recovery of aloe-emodin by TLC-image analysis (n=3)

Aloe-emodin added (µg/spot)	Aloe-emodin found (µg/spot)	%Recovery
0.00	0.201	-
0.06	0.282	107.863
0.18	0.372	97.578
0.30	0.503	100.425

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1.6.3 Precision

The precision of aloe-emodin quantitation by TLC-image analysis was evaluated in triplicate of each concentration group. The result was presented as the percentage of relative standard deviation which represented the error of the method. The repeatability was performed by three different concentrations on the same day. The intermediate precision was determined by three different concentrations on different days. The repeatability and intermediate precision were between 0.420-0.967% and 0.913-2.395%, respectively (Table 28).

Table 28 Repeatability and intermediate precision of aloe-emodin by TLC-image analysis (n=3)

Aloe-emodin (µg/spot)	Repeatability (%RSD)	Intermediate precision (%RSD)
0.282	0.954	2.395
0.372	0.420	0.913
0.503	0.967	1.807

1.6.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

In this study, LOD and LOQ in TLC-image analysis were measured based on the residual standard deviation of a regression line and the slope of calibration curve. The LOD and LOQ of aloe-emodin were assessed value as 0.0171 and 0.0517 µg/spot, respectively.

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1.6.5 Robustness

Robustness of the TLC-image analysis was performed by introducing small changes in the mobile phase complements (hexane: ethyl acetate). Each variation was determined in triplicate. The robustness of value was 0.50%RSD. The peak area of aloe-emodin in sample matrix was between 17422.03 and 17594.32 (Table 29).

Table 29 Robustness of aloe-emodin in *C. grandis* by the TLC-image analysis

Mobile phase (v/v)	Peak area of aloe-emodin
1.0 : 1.0	17422.03
0.9 : 1.1	17594.32
1.1 : 0.9	17540.74
Mean±SD	17519.03±88.17
%RSD	0.50

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1.7 The comparison of aloe-emodin contents between TLC-densitometry and TLC-image analysis

The aloe-emodin contents between TLC-densitometry and TLC-image analysis using ImageJ software were compared by paired *t*-test statistical analysis. The comparison was found that the aloe-emodin by two methods were not statistically significant ($P > 0.05$).

Table 30 Comparison of aloe-emodin contents between TLC-densitometry and TLC-image analysis

Source	Aloe-emodin in *C. grandis* leaves (g/100 g of dried crude drug)	
	TLC-densitometry	TLC-image analysis
1	0.37	0.39
2	0.39	0.42
3	0.39	0.41
4	0.41	0.44
5	0.27	0.26
6	0.43	0.39
7	0.47	0.44
8	0.44	0.45
9	0.43	0.43
10	0.45	0.44
11	0.33	0.31
12	0.47	0.48
13	0.52	0.52
14	0.48	0.52
15	0.33	0.30
Average (Mean±SD)	0.412±0.067	0.413±0.075

2. Quantitation of aloe-emodin content in *C. garrettiana* leaves

2.1 Dichloromethane extracts of dried *C. garrettiana* leaves

The average percent yield of dichloromethane extracts of *C. garrettiana* leaves by soxhlet extraction was 8.571±0.885 % (Table 31).

Table 31 The percent yield of dichloromethane extract of dried *C. garrettiana* leaves from 15 different locations throughout Thailand

Source	Weight of sample (g)	Weight of extractive matter (g)	% Yield
1	6.001	0.566	9.439
2	6.001	0.551	9.187
3	6.001	0.536	8.938
4	6.001	0.478	7.967
5	6.001	0.424	7.066
6	6.001	0.539	8.986
7	6.001	0.575	9.588
8	6.003	0.513	8.537
9	6.004	0.591	9.846
10	6.001	0.478	7.964
11	6.002	0.489	8.140
12	6.002	0.434	7.233
13	6.001	0.477	7.946
14	6.002	0.483	8.046
15	6.003	0.581	9.673
	Average		8.571±0.885

2.2 The chromatographic condition for quantitating aloe-emodin in *C. garrettiana* leaves

The chromatographic condition for quantitating aloe-emodin was examined using silica gel 60 GF_{254}. The selected mobile phase, hexane-ethyl acetate (1:1, v/v) demonstrated the best separation of aloe-emodin in *C. garrettiana* leaf extracts with R_f value 0.50±0.009. The aloe-emodin band of the plant samples was confirmed by comparing an R_f value with standard aloe-emodin. The TLC chromatograms of 15 samples and standard aloe-emodin under UV 254 nm, under UV 365 nm and in visible light were demonstrated in Figure 76.

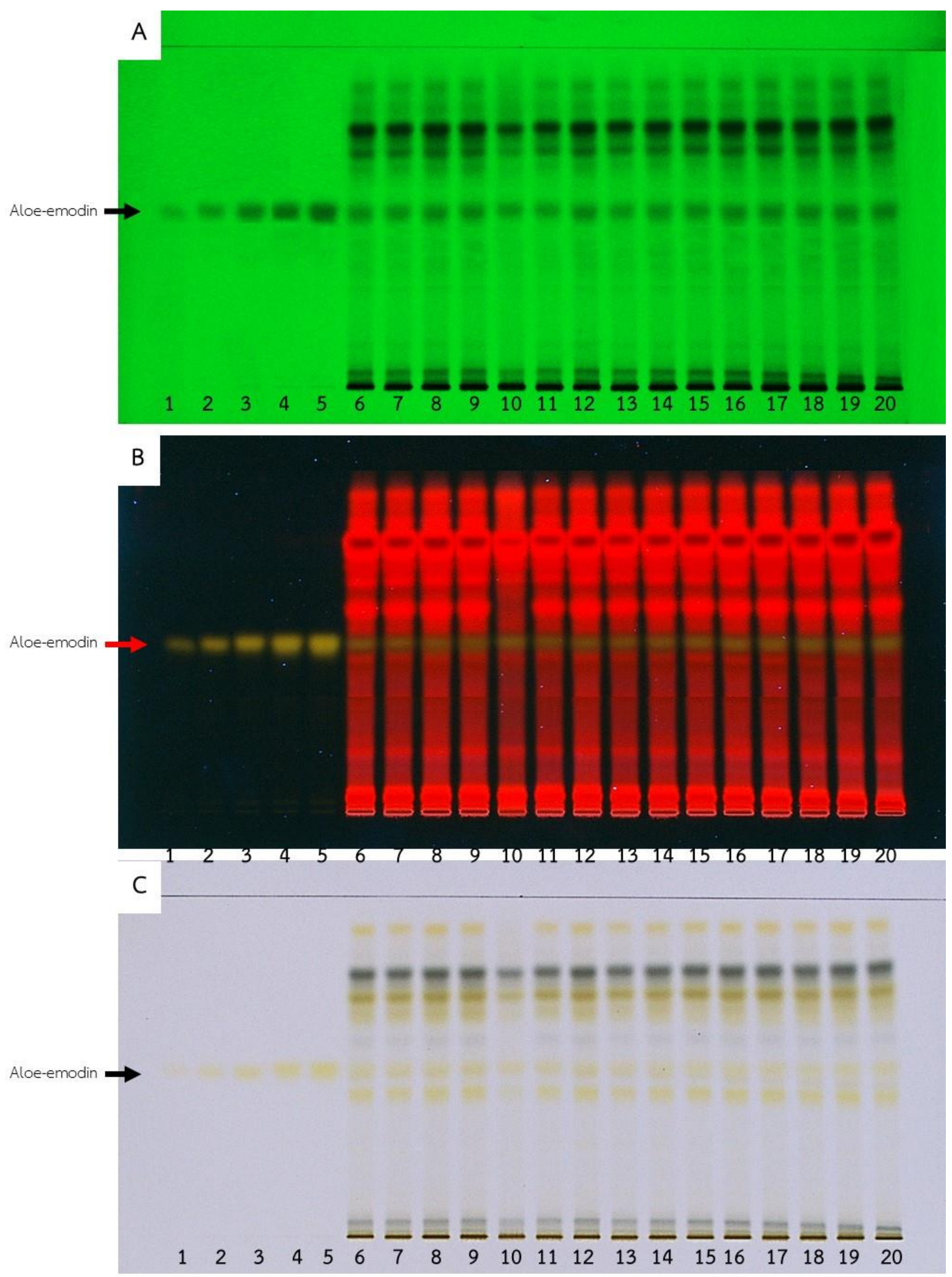

Figure 76 The TLC plate (A) under UV 254 nm (B) under UV 365 nm (C) in visible light; standard aloe-emodin (track 1 to 5) and *C. garrettiana* leaf extracts from 15 various locations in Thailand

2.3 The amount of aloe-emodin in *C. garrettiana* leaves by TLC-densitometry

The aloe-emodin contents in *C. garrettiana* leaf extracts from 15 different locations were determined in triplicate by TLC-densitometry. The average aloe-emodin contents in crude drugs were 0.035±0.007% (Table 32).

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Table 32 The amount of aloe-emodin in *C. garrettiana* leaves by TLC-densitometry (% by dried weight)

Source	Aloe-emodin in dichloromethane extract (g/g of crude extract)				Yield of the dichloromethane extract (g/100 g of dried crude drug)	Aloe-emodin in *C. garretiana* leaves (g/100 g of dried crude drug)
	1	**2**	**3**	Mean		
1	0.005	0.005	0.005	0.005	9.439	0.044
2	0.004	0.004	0.005	0.004	9.187	0.041
3	0.004	0.004	0.005	0.004	8.938	0.039
4	0.004	0.004	0.004	0.004	7.970	0.031
5	0.004	0.004	0.004	0.004	7.066	0.027
6	0.004	0.005	0.005	0.004	8.985	0.040
7	0.004	0.004	0.004	0.004	9.588	0.038
8	0.004	0.004	0.004	0.004	8.537	0.036
9	0.004	0.004	0.004	0.004	9.846	0.042
10	0.005	0.004	0.005	0.005	7.964	0.036
11	0.004	0.004	0.004	0.004	8.140	0.034
12	0.004	0.004	0.004	0.004	7.233	0.028
13	0.003	0.004	0.004	0.004	7.946	0.029
14	0.003	0.004	0.004	0.004	8.046	0.029
15	0.004	0.003	0.004	0.004	9.673	0.034
					Mean	0.035
					SD	0.006

2.4 Method validation of TLC-densitometry

2.4.1 Calibration curve

The calibration curve of aloe-emodin standard solutions was linear in the range of 0.12, 0.24, 0.36, 0.48 and 0.60 µg/spot. The regression equation of aloe-emodin was y = 33697x + 1822.5. The coefficient of determination (R^2) of aloe-emodin was 0.9992.

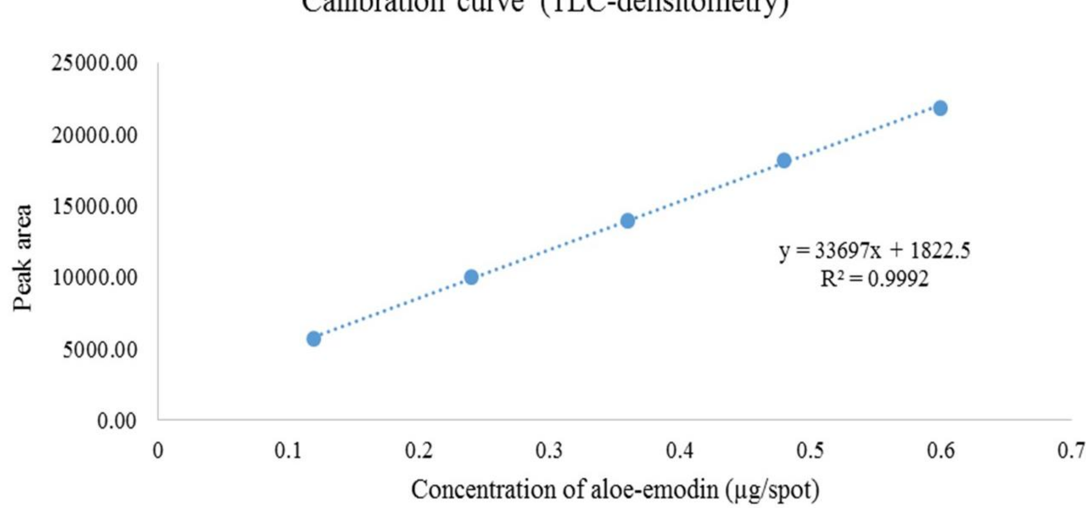

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Figure 77 The calibration curve of aloe-emodin in *C. garrettiana* leaf extracts by thin-layer chromatography densitometry

2.4.2 Accuracy

The accuracy of aloe-emodin quantitation by TLC-densitometry was evaluated in percentage of recovery. The recovery of aloe-emodin was performed on sample spiked with three different concentrations of aloe-emodin (0.06, 0.18 and 0.30 µg/spot). The recovery method was done in triplicate. The results were between 98.167-105.528% (Table 33).

Table 33 Recovery of aloe-emodin by TLC-densitometry (n=3)

Aloe-emodin added (µg/spot)	Aloe-emodin found (µg/spot)	%Recovery
0.00	0.199	-
0.06	0.273	105.528
0.18	0.372	98.167
0.30	0.495	99.168

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2.4.3 Precision

The precision of aloe-emodin quantitation by TLC-densitometry was evaluated in triplicate of each concentration group. The result was presented as the percentage of relative standard deviation which represented the error of the method. The repeatability was performed by three different concentrations on the same day. The intermediate precision was determined by three different concentrations on different days. The repeatability and intermediate precision were between 0.549-1.084% and 0.870-1.279%, respectively (Table 34).

Table 34 Repeatability and intermediate precision of aloe-emodin by TLC-densitometry (n=3)

Aloe-emodin (µg/spot)	Repeatability (%RSD)	Intermediate precision (%RSD)
0.273	0.751	1.279
0.372	1.084	0.870
0.495	0.549	1.111

2.4.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

In this study, LOD and LOQ in TLC-densitometry were measured based on the residual standard deviation of a regression line and the slope of calibration curve. The LOD and LOQ of aloe-emodin were assessed value as 0.0214 and 0.0188 µg/spot, respectively.

2.4.5 Robustness

Robustness of the TLC-densitometry was performed by introducing small changes in the mobile phase complements (hexane: ethyl acetate). Each variation was determined in triplicate. The robustness of value was 0.56%RSD. The peak area of aloe-emodin in sample matrix was between 21772.56 and 21992.22 (Table 35).

Table 35 Robustness of aloe-emodin in *C. gerrettiana* by the TLC-densitometry

Mobile phase (v/v)	Peak area of aloe-emodin
1.0 : 1.0	21772.56
0.9 : 1.1	21992.22
1.1 : 0.9	21979.37
Mean±SD	21914.72±123.28
%RSD	0.56

2.4.6 Specificity

The specificity was proved by peak identity and peak purity checking. The identity in absorbance spectra determined at the peak apex among aloe-emodin standards and a spot in the samples at the same R_f value was illustrated in Figure 78. The purity in absorbance spectra determined at up-slop, apex and down-slope of the sample peak was shown in Figure 79. The absorption spectra of aloe-emodin in all samples and standard were identical with the maximum absorption spectra at 434 nm which represented the method specificity.

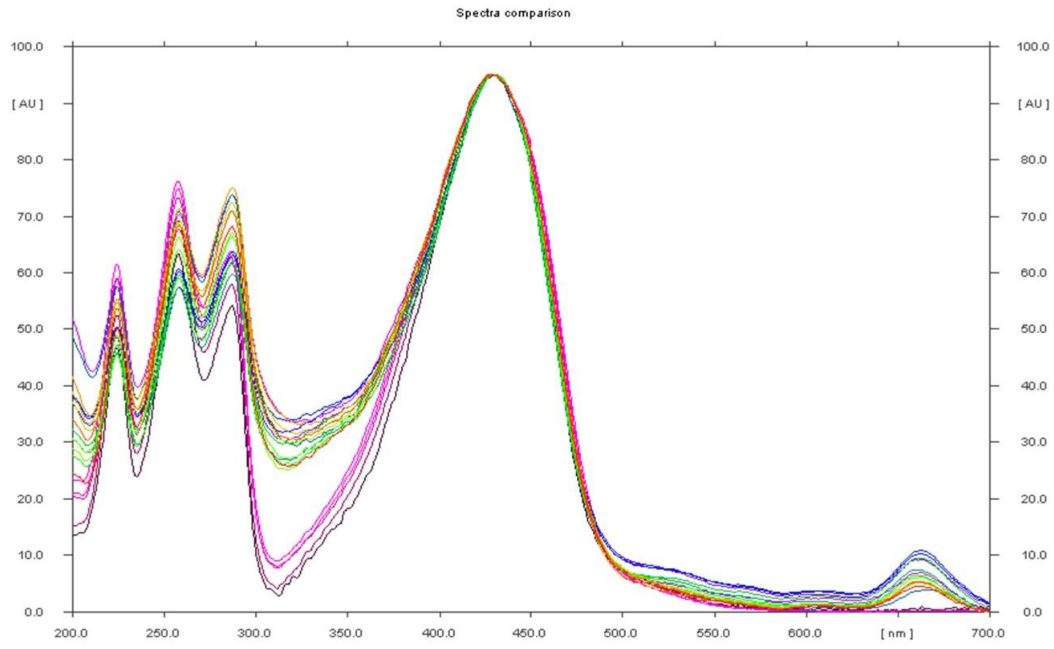

Figure 78 The absorption spectra of aloe-emodin in standard and sample bands of *C. garrettiana* leaves

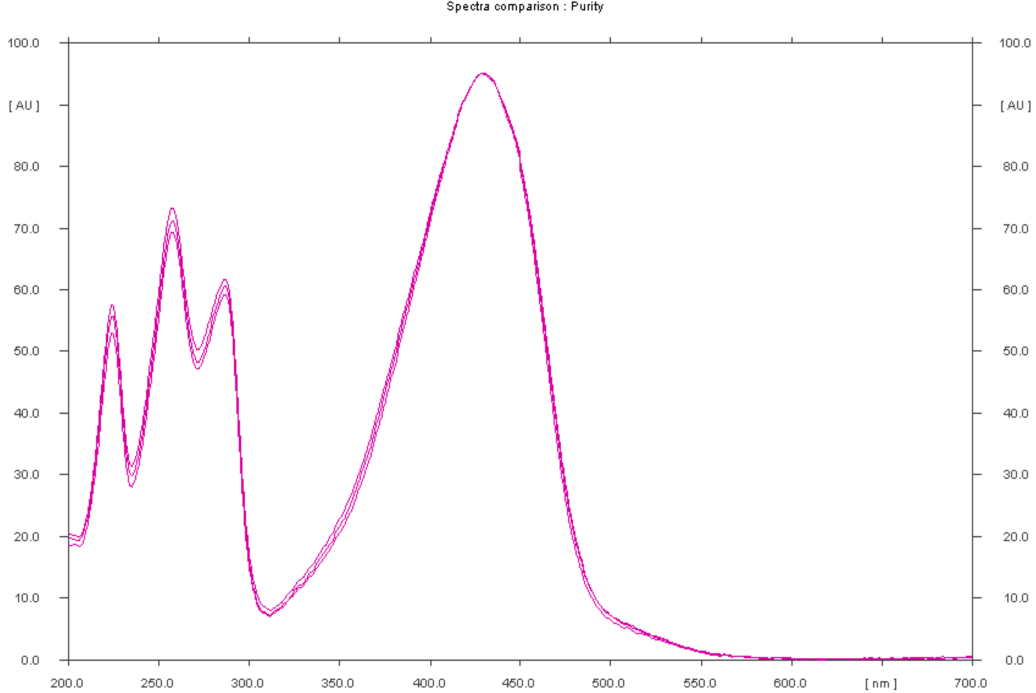

Figure 79 UV absorbance spectra of aloe-emodin in dichloromethane extract of *C. garrettiana* leaves using up-slope, apex and down-slope of the peak

2.5 The amount of aloe-emodin in *C. garrettiana* leaves by TLC-image analysis

The aloe-emodin contents in *C. garrettiana* leaf extracts from 15 different locations were determined in triplicate by TLC-image analysis. The average aloe-emodin contents in crude drugs were 0.035±0.006% (Table 36).

Table 36 The amount of aloe-emodin in *C. garrettiana* leaves by TLC-image analysis (% by dried weight)

Source	Aloe-emodin in dichloromethane extract (g/g of crude extract)				Yield of the dichloromethane extract (g/100 g of dried crude drug)	Aloe-emodin in *C. garretiana* leaves (g/100 g of dried crude drug)
	1	2	3	Mean		
1	0.005	0.004	0.005	0.005	9.439	0.043
2	0.005	0.004	0.004	0.004	9.187	0.041
3	0.004	0.004	0.005	0.004	8.938	0.039
4	0.004	0.004	0.005	0.004	7.970	0.035
5	0.003	0.003	0.004	0.003	7.066	0.024
6	0.004	0.004	0.004	0.004	8.985	0.035
7	0.004	0.004	0.005	0.004	9.588	0.042
8	0.004	0.004	0.004	0.004	8.537	0.036
9	0.005	0.005	0.005	0.005	9.846	0.046
10	0.004	0.004	0.004	0.004	7.964	0.033
11	0.004	0.004	0.004	0.004	8.140	0.033
12	0.003	0.003	0.004	0.003	7.233	0.024
13	0.003	0.003	0.004	0.003	7.946	0.027
14	0.003	0.003	0.004	0.003	8.046	0.028
15	0.004	0.004	0.004	0.004	9.673	0.036
					Mean	0.035
					SD	0.007

2.6 Method validation of TLC-image analysis

2.6.1 Calibration curve

The calibration curves of aloe-emodin standard solutions was linear in the range of 0.12, 0.24, 0.36, 0.48 and 0.60 µg/spot. The regression equation of aloe-emodin was y = 44776x + 918.79. The coefficient of determination (R^2) of aloe-emodin was 0.9994.

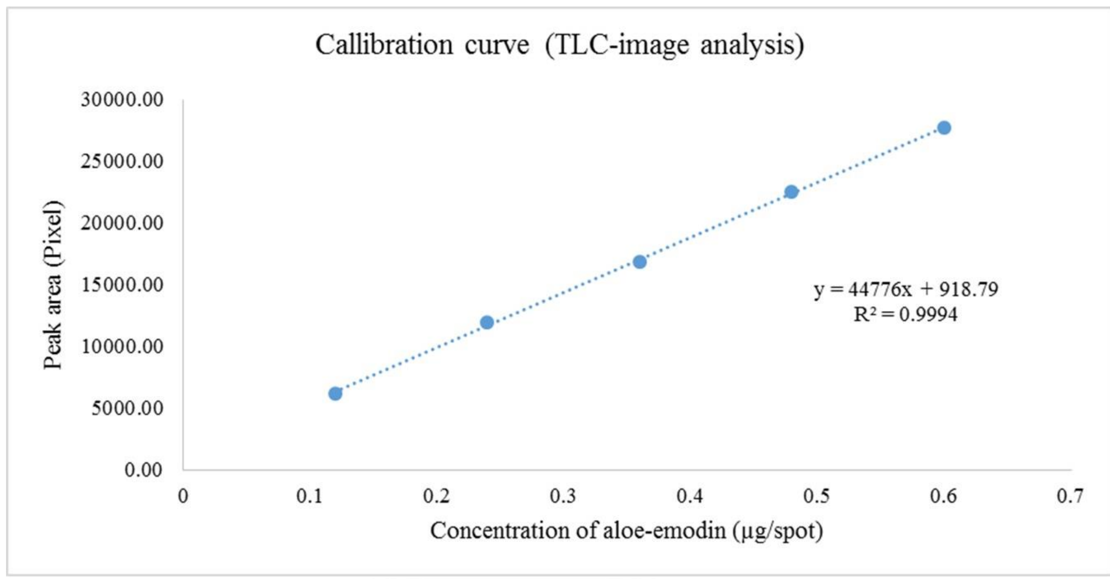

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Figure 80 The calibration curve of aloe-emodin in *C. garrettiana* leaf extracts by TLC-image analysis

2.6.2 Accuracy

The accuracy of aloe-emodin quantitation by TLC-image analysis was evaluated in percentage of recovery. The recovery of aloe-emodin was performed on sample spiked with three different concentrations of aloe-emodin (0.06, 0.18 and 0.30 µg/spot). The recovery method was done in triplicate. The results were between 97.351-105.935% (Table 37).

Table 37 Recovery of aloe-emodin by TLC-image analysis (n=3)

Aloe-emodin added (µg/spot)	Aloe-emodin found (µg/spot)	%Recovery
0.00	0.202	-
0.06	0.277	105.935
0.18	0.372	97.351
0.30	0.496	98.753

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2.6.3 Precision

The precision of aloe-emodin quantitation by TLC-image analysis was evaluated in triplicate of each concentration group. The result was presented as the percentage of relative standard deviation which represented the error of the method. The repeatability was performed by three different concentrations on the same day. The intermediate precision was determined by three different concentrations on different days. The repeatability and intermediate precision were between 0.277-0.498% and 1.032-1.300%, respectively (Table 38).

Table 38 Repeatability and intermediate precision of aloe-emodin by TLC-image analysis (n=3)

Aloe-emodin (µg/spot)	Repeatability (%RSD)	Intermediate precision (%RSD)
0.277	0.277	1.118
0.372	0.372	1.300
0.498	0.498	1.032

2.6.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

In this study, LOD and LOQ in TLC-image analysis were measured based on the residual standard deviation of a regression line and the slope of calibration curve. The LOD and LOQ of aloe-emodin were assessed value as 0.0188 and 0.0568 µg/spot, respectively.

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2.6.5 Robustness

Robustness of the TLC-image analysis was performed by introducing small changes in the mobile phase complements (hexane: ethyl acetate). Each variation was determined in triplicate. The robustness of value was 0.58%RSD. The peak area of aloe-emodin in sample matrix was between 28498.95 and 28827.95 (Table 39).

Table 39 Robustness of aloe-emodin in *C. garrettiana* by the TLC-image analysis

Mobile phase (v/v)	Peak area of aloe-emodin
1.0 : 1.0	28827.95
0.9 : 1.1	28498.95
1.1 : 0.9	28644.48
Mean±SD	28657.13±164.86
%RSD	0.58

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2.7 The comparison of aloe-emodin contents between TLC-densitometry and TLC-image analysis

The aloe-emodin contents between TLC-densitometry and TLC-image analysis using ImageJ software were compared by paired *t*-test statistical analysis. The comparison was found that the aloe-emodin by two methods were not statistically significant ($P > 0.05$).

Table 40 Comparison of aloe-emodin contents between TLC-densitometry and TLC-image analysis

Source	Aloe-emodin in *C. garrettiana* leaves (g/100 g of dried crude drug)	
	TLC-densitometry	TLC-image analysis
1	0.043	0.044
2	0.041	0.041
3	0.039	0.039
4	0.035	0.031
5	0.024	0.027
6	0.035	0.040
7	0.042	0.038
8	0.036	0.036
9	0.046	0.042
10	0.033	0.037
11	0.033	0.034
12	0.024	0.028
13	0.027	0.029
14	0.028	0.029
15	0.036	0.034
Average (Mean±SD)	0.035±0.007	0.035±0.006

CHAPTER V
DISCUSSION AND CONCLUSION

Cassia L. is a genus belonging to family Caesalpiniaceae. There are thirty-three species found in Thailand and there is a great diversity of habit within the genus ranging from trees to prostrate annual herbs. Medicinal plants have been used worldwide for health maintenance and diseases treatment. However, adulteration or substitution has become a major concern for safety and efficacy. Therefore, authentication of medicinal plants is important. The misidentification usage of plants can cause a risk practical application in both agriculture and medicine due to their side-effects and toxicity. Many methods have been employed for medicinal plant authentication such as morphological characteristics, anatomical characteristics, chemical profiling and DNA markers. Based on morphological characteristic, macroscopic examination is the observation of the entire plant characteristics or crude drug such as shape, size, colour, texture and odour which require highly skills and experience.

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In this study, the transverse sections of the lamina of the leaflet showed the bifacial structure. Transverse section of leaf through midrib of each investigated *Cassia* species showed the arrangement of upper epidermis, palisade cells, spongy cells, sclerenchyma, xylem tissue, phloem tissue, calcium oxalate crystal structure, parenchyma, collenchyma and lower epidermis. According to the arrangement of palisade, fifteen *Cassia* species had single layer of palisade cells except *C. timoreinsis* had bilayer of palisade cells. When considering the arrangement of spongy cells in mesophyll, thirteen *Cassia* species shown loosely arrangement of spongy cells with rounded to oval shaped and intercellular air-space whereas three *Cassia* species (*C. grandis, C. javanica* and *C. angustifolia*) shown regularity arrangement of spongy cells.

The presence of prism like crystals calcium oxalate crystal structure was found only in *C. angustifolia*, prism and druse like crystals were found in fourteen *Cassia* species and prism, druse and raphide like crystals were found only in *C. hirsuta*. The presence of calcium oxalate crystal structure and the arrangement of palisade and spongy cells in mesophyll showed individual characteristics in *Cassia* species that can be used as important characteristics for plant authentication (Figure 60). Moreover, the presence or absence of trichomes on the midrib and trichome number is also one of the important characteristics that can be used for differentiation of *Cassia* species. Both *C. sulfurea* and *C. surattensis* shown the similar morphology such as 5-10 m. tall, pinnately compound leaves, even pinnate, inflorescent racemes with 5 petals bright yellow but *C. sulfurea* had the trichome number in ventral surface (10.20±1.79) higher than *C. surattensis* (3.46±0.80). As well as the similar morphology of *C. hirsuta* and *C. occidentalis*, *C. hirsuta* and *C. occidentalis* as they were shrubs, 1.5-2 m. tall, pinnately compound leaves, leaflets arranged oppositely in 4-5 pairs, broadly lanceolate to ovate in shape, inflorescent racemes, brilliant yellow petal but *C. hirsuta* had covering trichome on both dorsal and ventral surfaces (5.00±0.50 and 8.21±0.95, respectively) whereas *C. occidentalis* had no covering trichome. Moreover, *C. hirsuta* can be easily distinguished from the other *Cassia* species by the presence of multicellular glandular trichomes with a globular head as similar as previous reported[362, 418]. However, the exact density of multicellular glandular trichome of *C. hirsuta* was not calculated because of their uneven distribution on a leaves. Trichome number has been previously used for identification of *Morinda* spp. and *Solanum* spp. from their closely related species[361, 419].

The type of trichome is usually consistent in many species. The presence or absence and types of trichomes on the epidermis have been used for classification based on the number of cell and the presence or absence of glandular cell of trichome. Among the 16 selected *Cassia* species, the trichome characteristics were uniseriate, uni- or multicellular non-glandular and multicellular glandular types and most of them are appressed to the epidermis. *C. fistula* had the uniseriate, unicellular non-glandular trichome types which is corroborated from the previously reported by Saheed and Illoh (2010), Pandya *et al*. (2012) and Rani and Satish (2014). *C. alata* had short unicellular non-glandular trichome, conical trichomes with bulbose base in the leaflet which is the important diagnostic features in this species. *C. angustifolia* had the unicellular non-glandular trichome, thick walled and conical in shape with cuticular warts and frequent distributed on both surfaces which is an important characteristic of *C. angustifolia* and can be used to distinguish *C. angustifolia* from the other *Cassia* species. The trichome characteristics in this recent study was in agreement with Kidyue et al. (2003) which reported that *C. bakeriana, C. grandis, C. javanica, C. spectabilis, C. timoriensis C. sulfurea, C. surattensis* and *C. tora* had uniseriate, multicellular non-glandular types whereas *C. hirsuta* had multicellular non-glandular and multicellular glandular types. Saheed and Illoh (2010) reported that the presence or absence of trichomes as well as their types could be used in characterizing some species in *Cassiinae.* Besides other morphological characteristics, the greatest significance of trichomes is an importance characteristic for the identification of angiospermic plants. The taxonomic value of the trichome and their significance in systematic and phylogenetic relationship is well known in Lamiaceae, Verbenaceae and Scrophulariaceae[354, 420]. The individual species of family Restionaceae and Centrolepidaceae can be distinguished by their unique characteristics of trichomes.

Moreover, the T-shaped trichomes of Malpighiaceae and Ericaceae family have been classified on the basis of leaf hair, as an aid for species identification[421].

The type of stomata in 16 *Cassia* species were classified as paracytic type (stomata surrounded by two subsidiary cells by parallel to the long axis of guard cells). *C.alata, C. occidentalis, C. sophera, C. hirsuta, C. angustifolia, C. tora, C.surattensis* and *C. sulfurea* were found stomata on both dorsal and ventral epidermis. In *C. bakeriana, C. fistula, C. grandis, C. javanica, C. siamea. C. spectabilis, C. timoriensis* and *C. garrettiana* were found stomata on only at ventral epidermis. The result of this study is in agreement with the previous reports that the paracytic type of stomata is commonly found in all sub genera of *Cassia*[33, 422].

DNA marker provides an efficient, accurate and simultaneously automation of many samples for quality control and safety monitoring of herbal pharmaceuticals products due to they are not affected by age, environmental factors, physiological conditions and harvesting a higher discrimination power compared to the phenotypic and chemical markers. A vast numbers of molecular markers are available for medicinal plant identification. The AFLP technique is commonly applied for plant classification, genetic relationships and genetic diversity in many plant species, such as *Curcuma comosa, Punica granatum* and *Panax notoginseng*[378, 380, 423]. Comparative studies using PCR- RFLP, RAPD and AFLP techniques have revealed that AFLP techniques are the most efficient and effective due to their high reproducibility, high quantity of information throughout multiple loci in the genome, and high resolution. This technique is the combination of both RFLP and RAPD based on the detection of restriction fragments by PCR amplification[373]. In the current study, eleven primer combinations produced clear and reproducible amplified bands. A total of 849

amplified fragments were detected. The high percentage (99.07%) of polymorphism indicates that there is a high level of genetic diversity among the 16 *Cassia* species. The dendrogram was created based on the genetic similarity index and showed that all of the *Cassia* species can be clustered into two main groups that have bootstrap values higher than 80%. Bootstrap analysis revealed that the branching in the tree was stable and robust. The first group consists of *C. bakeriana, C. javanica, C. grandis* and *C. fistula*. This result is similar to those previously reporting that *C. javanica*, and *C. fistula* had been clustered into the same group on the basis of RAPD fingerprints[39]. Based on SSR and ISSR fingerprints, *C. fistula, C. grandis* and *C. javanica* were also clustered together[38]. The second group can be divided into 3 subgroups with the first subgroup being composed of *C. garrettiana, C. siamea, C. timoriensis, C. alata* and *C. spectabilis*. The second subgroup is composed of *C. tora, C. surattensis* and *C. sulfurea*. The last subgroup belongs to *C. hirsuta, C. occidentalis, C. sophera* and *C. angustifolia*. The result is consistent with previous report regarding RAPD fingerprints that clustered *C. tora, C. surattensis* and *C. sulfurea* together[39]. Moreover, *C. hirsuta, C. occidentalis* were clustered together based on the SSR and RAPD fingerprints, whereas *C. siamea* and *C. spectabilis* were clustered into the same group based on the SSR, ISSR and RAPD fingerprints[38, 39]. The outgroup plant, *A. paniculata*, was clearly separated from the other *Cassia* species. The taxonomic relationships between *Cassia* and other genera in the *Cassiinae* subtribe have been discussed for a long time. Several taxonomists have classified *Cassia* genus into different systems based on various morphological characteristics. According to the classification of Irwin and Barneby (1981), the *Casiinae* subtribe was first classified into three genera, *Cassia, Senna* and *Chamaecrista*, using the characteristics of filaments and the presence or absence of bracteoles. The revised classification is widely accepted in many countries, including

Thailand. Thai plant names Tem Smitinand revised edition 2014, reveals that seventeen species out of the thirty-three species of *Cassia* distributed throughout Thailand had been moved into the genus *Senna*, which is supported by the results of the AFLP in this study (Figure 68). The monomorphic banding patterns derived by AFLP fingerprinting were clearly separated between the genus *Cassia* and *Senna*. In addition, the AFLP data was used as molecular characters for phylogenetic analyses to reveal the evolutionary relationships among the *Cassia* and *Senna* species. Moreover, the genetic relationships through the AFLP markers were also correlated with the morphological characteristics. The members of the first group have similar morphological characteristics when considering their curved filaments. Two bracteoles under the peduncles and pods are terete, whereas all members of the second group have similar short and straight filaments with no bracteole under the peduncles and pods being flat to terete. This was corroborated by the findings of Irwin and Barneby (1982), and Kidyue (2003). The results of the AFLP phylogenetic analysis could be an important basis for further taxonomic, evolutionary, breeding and pharmacological studies of the genus *Cassia*.

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Aloe-emodin is one of major components in the leaf extracts of *C. grandis* and *C. garrettiana* and this compound was employed as marker for TLC-densitometric method and TLC-image analysis in the present study. The amounts of aloe-emodin showed a variation quantities in plant materials collected from various locations in Thailand since the chemical constituent contents in herbal plant can vary with the plant origin, harvest season, environmental factor and herbal preparation method. These data will be also useful as guidance for finding good sources of both plants in Thailand. The aloe-emodin contents of *C. grandis* and *C. garrettiana* leaves from 15

various locations in Thailand that obtained from TLC-densitometric method and TLC-image analysis were compared using paired *t*-test statistical analysis. It was indicated that the aloe-emodin contents in *C. grandis* and *C. garrettiana* leaves from both methods were not significantly different with $P > 0.05$. From the results, it could be used TLC-image analysis as an alternative method for routine quantitative analysis of this compounds in the *C. grandis* and *C. garrettiana* leaf extracts. According to International Conference on Hamonization (ICH) guideline (Q2R1), the analytical method was validated to confirm that the analytical procedure employed reliable and accurate data. The calibration curves of aloe-emodin in *C. grandis* and *C. garrettiana* leaves by both methods shown good linearity relationships with coefficient of determination (r^2) more than 0.9990 in ranged of 0.12-0.60 μg/spot**.** The absorption spectrum of standard aloe-emodin in this study showed the maximum absorbance at 434 nm which in accordance to the previous study that maximum UV absorption spectrum of aloe-emodin could be detect at 434 nm[424]. The recovery assay in both methods of *C. grandis* and *C. garrettiana* showed that these methods were accurate. Determination of aloe-emodin contents in *C. grandis* and *C. garrettiana* repeatedly within and between set of experiments by both methods revealed acceptable precisions. LOD and LOQ value from both methods of *C. grandis* and *C. garrettiana* confirmed that the lowest concentration of standard aloe-emodin (0.12 μg/spot) used in this study were suitable. The robustness studied by changing composition of mobile phase indicated that changing composition of mobile phase was not affected in both methods.

This is the first report of the validated TLC-densitometric method and TLC-image analysis using ImageJ free software for quantitation of aloe-emodin in

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dichloromethane extracts of *C. grandis* and *C. garrettiana* leaves collected from 15 different locations in Thailand. These results may be valuable for indicating alternative sources of aloe-emodin. Due to the distinguished of *C. grandis* and *C. garrettiana* distributed throughout Thailand, the supply of the leaf materials will be easily available. Statistical analysis indicated that the aloe-emodin content determined using TLC-densitometric method and TLC-image analysis shown no significance; hence TLC-image analysis might be an alternative method for quantitative analysis of *C. grandis* and *C. garrettiana* leaves due to its rapidly, simplicity, precisely, accuracy and cost effectiveness.

This research provides useful information for its correct identification in term of macroscopic examination, microscopic examination and AFLP fingerprinting. AFLP fingerprinting is a useful technique for plant identification and confirmation of the phylogenetic relationships of selected *Cassia* species. The simple method of TLC-image analysis can be used to determine the active constituents of these medicinal plants.

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APPENDICES

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX A

The Stomata type of 16 *Cassia* species

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

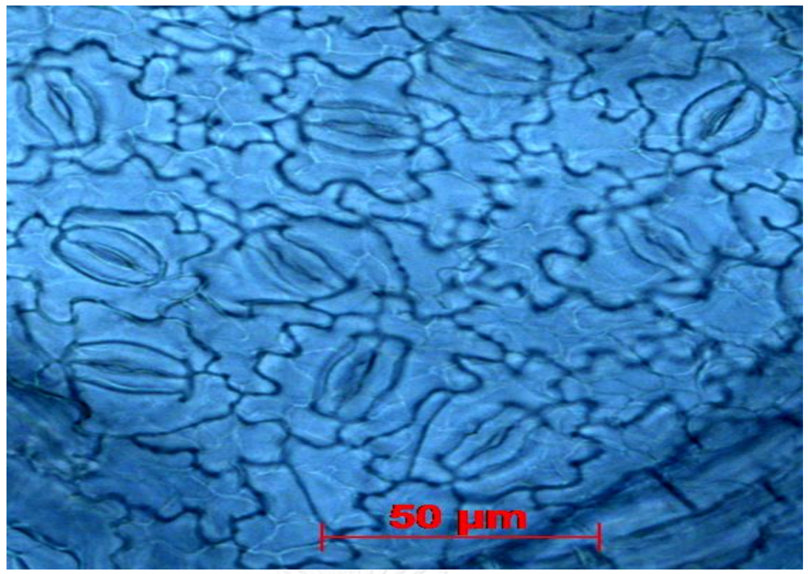

Figure 81 paracytic type of *C. bakeriana*

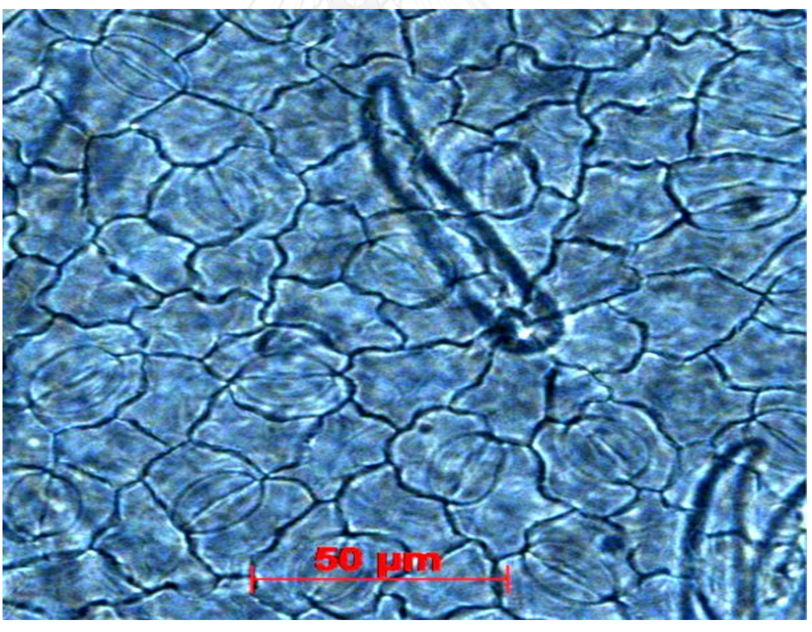

Figure 82 paracytic type of *C. fistula*

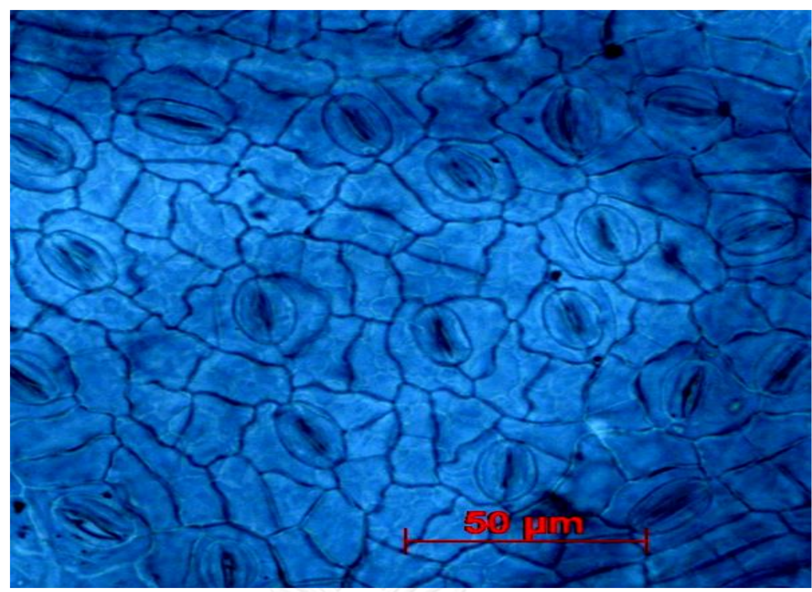

Figure 83 paracytic type of *C. grandis*

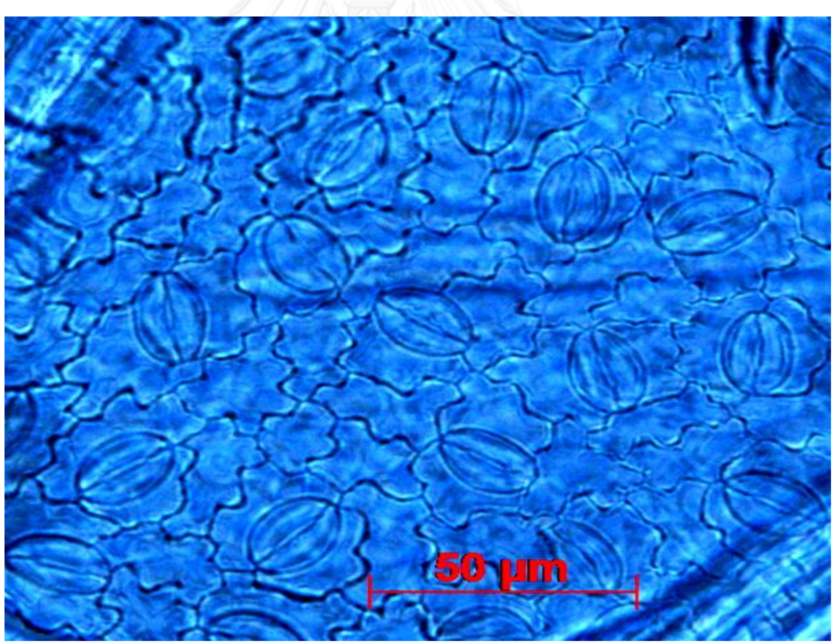

Figure 84 paracytic type of *C. javanica*

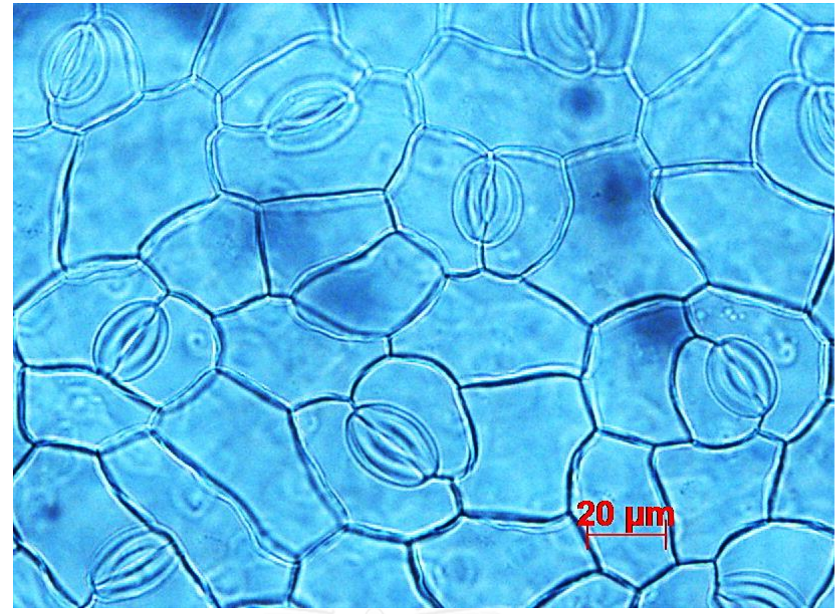

Figure 85 paracytic type of *C. angustifolia*

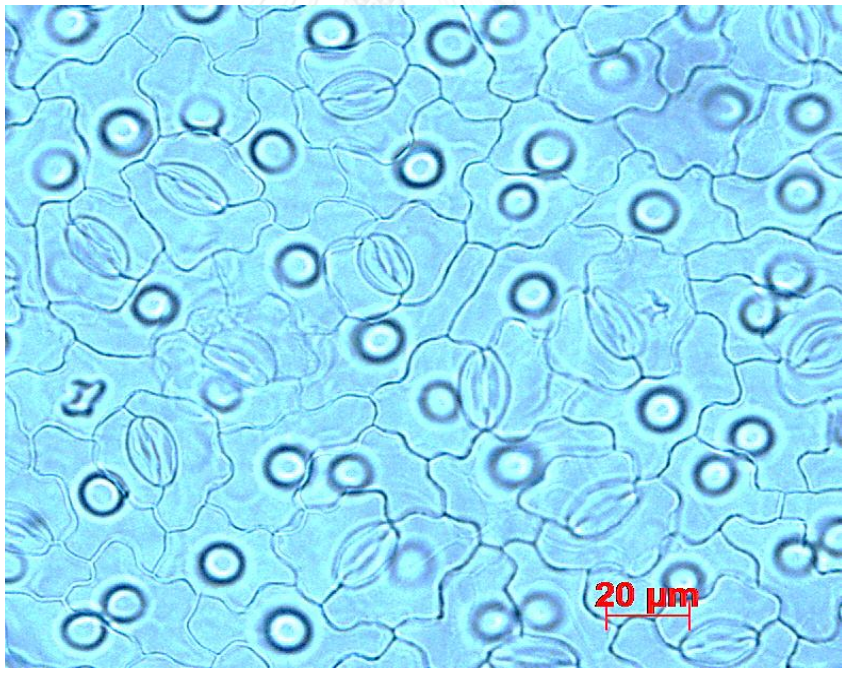

Figure 86 paracytic type of *C. alata*

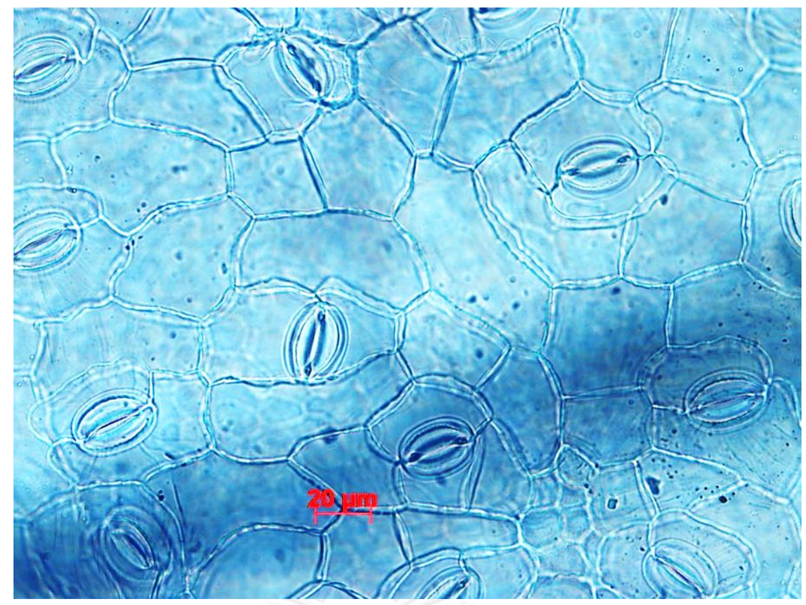

Figure 87 paracytic type of *C. hirsuta*

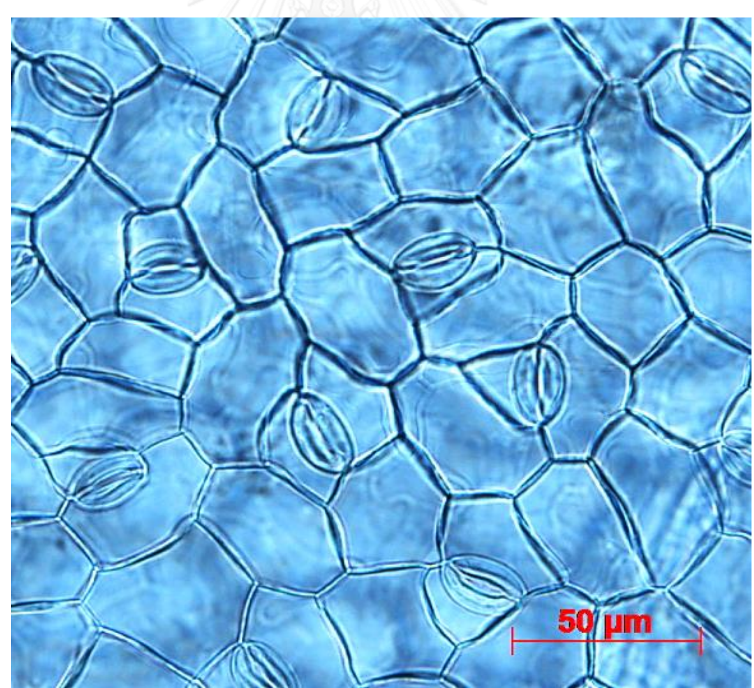

Figure 88 paracytic type of *C. garrettiana*

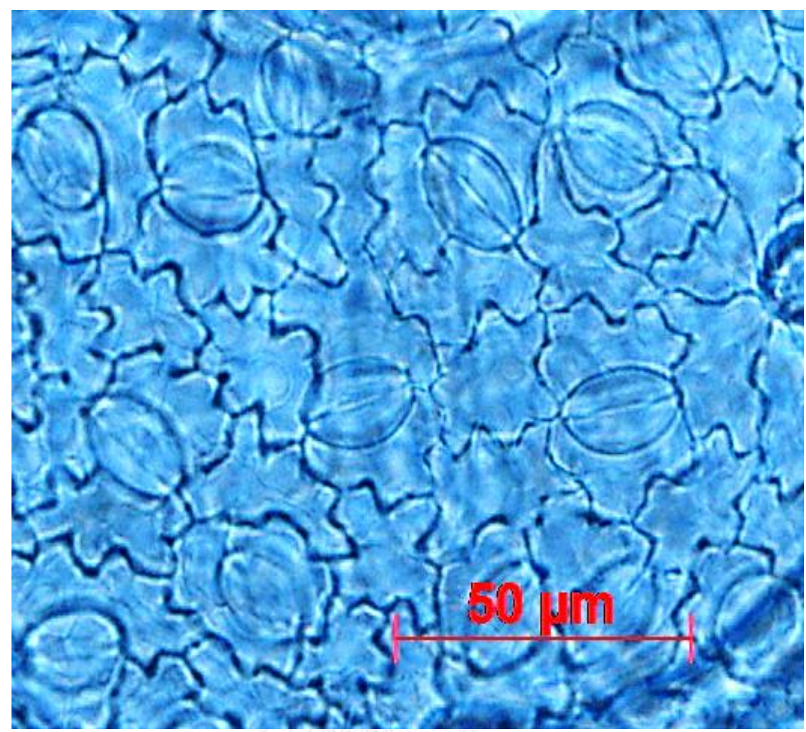

Figure 89 paracytic type of *C. siamea*

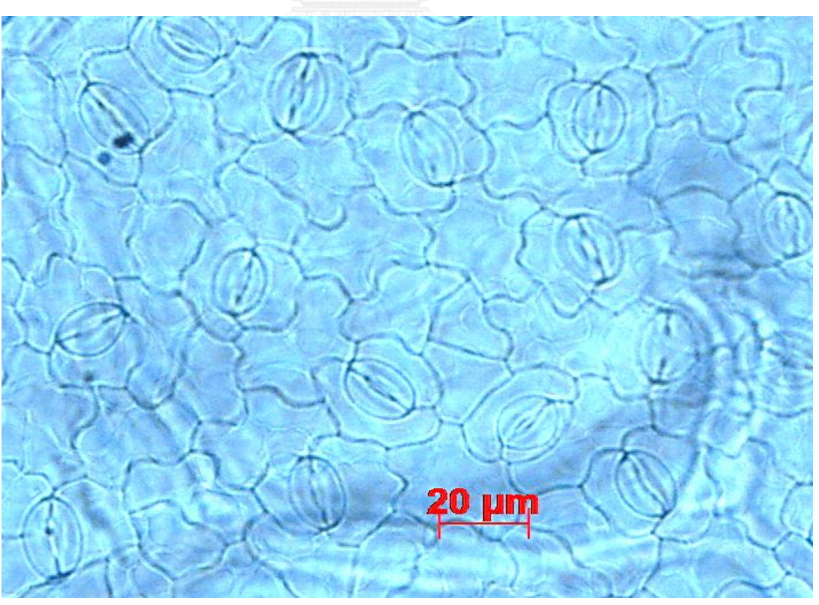

Figure 90 paracytic type of *C. spectabilis*

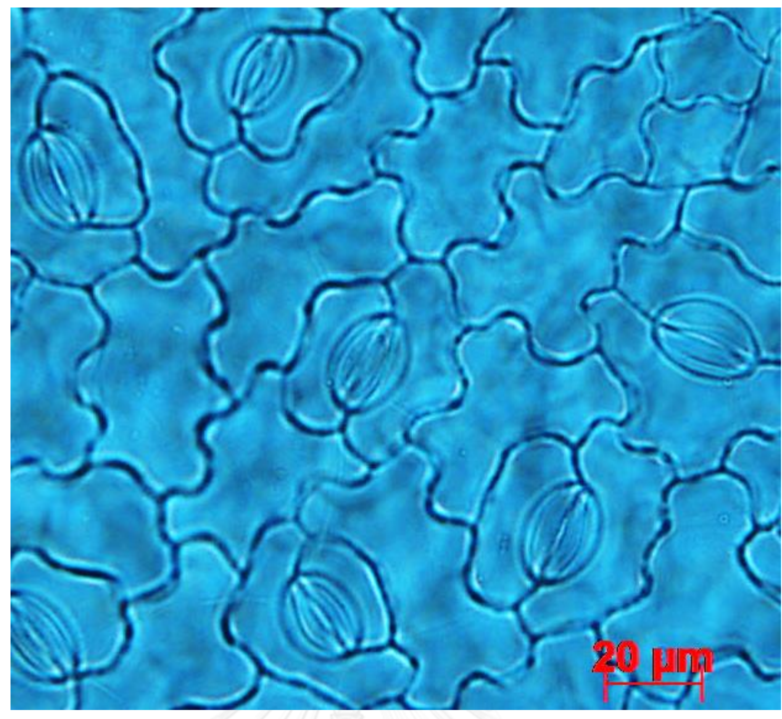

Figure 91 paracytic type of *C. surattensis*

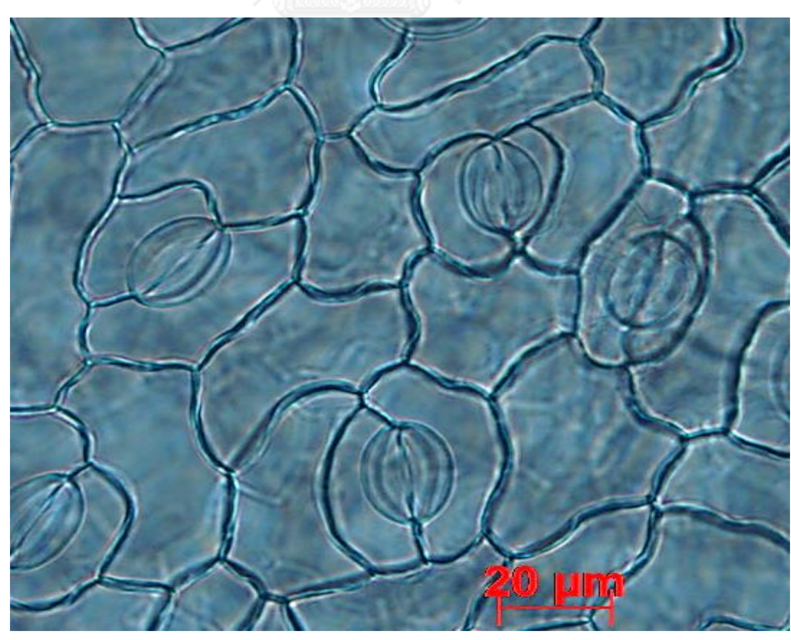

Figure 92 paracytic type of *C. sulfurea*

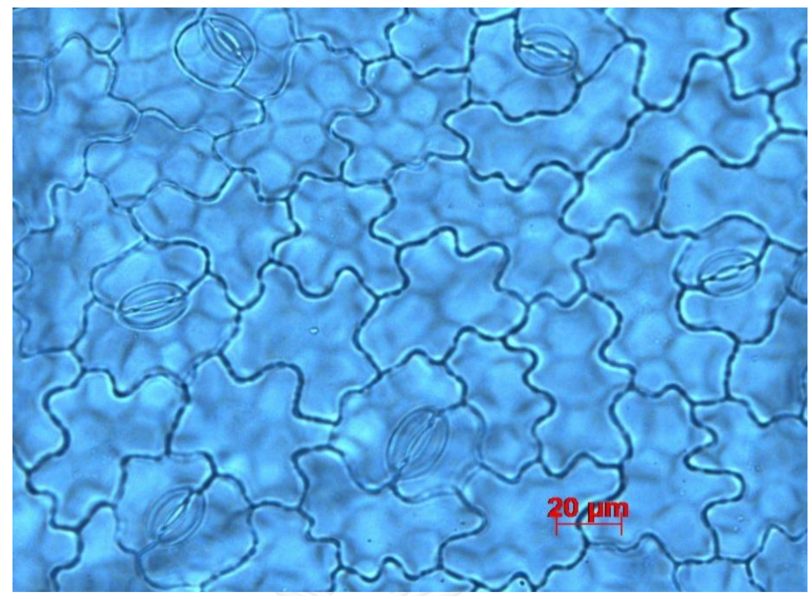

Figure 93 paracytic type of *C. occidentalis*

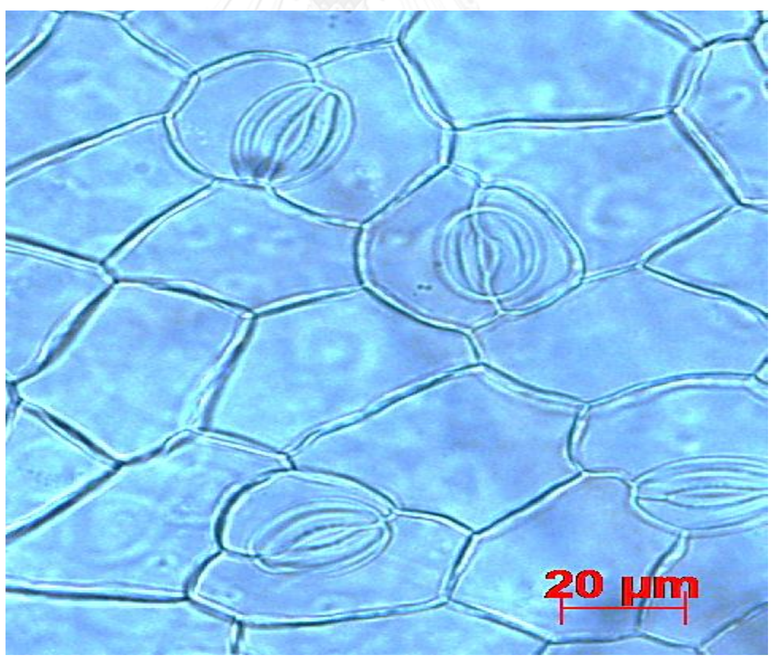

Figure 94 paracytic type of *C. sophera*

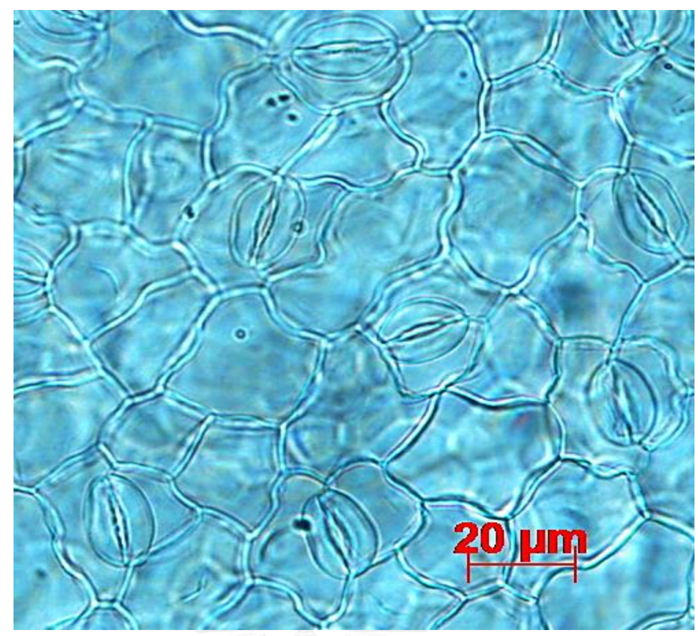

Figure 95 paracytic type of *C. timoriensis*

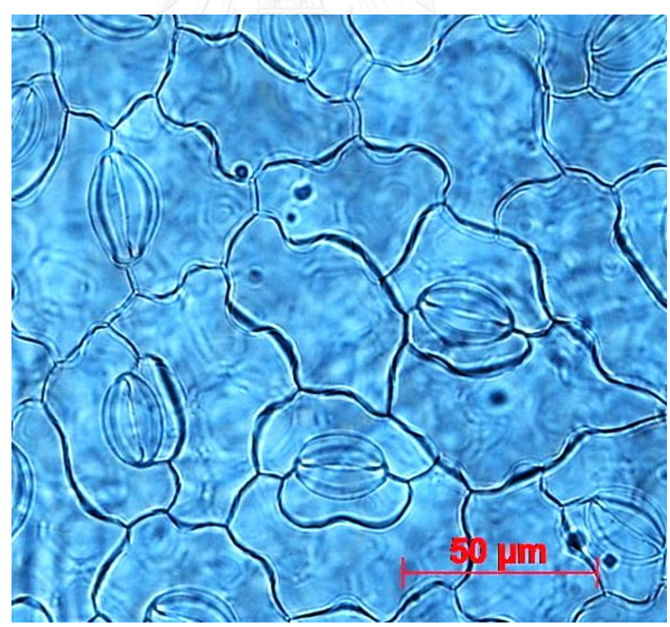

Figure 96 paracytic type of *C. tora*

APPENDIX B

The data of trichome number

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 41 Trichome number of *C. bakeriana* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Si Sa Ket	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	27	68	27	70	30	68
2	33	66	28	74	32	70
3	33	72	29	68	35	69
4	31	70	28	70	34	68
5	28	74	28	68	32	74
6	28	72	27	68	30	73
7	33	68	27	74	32	74
8	28	68	28	72	30	76
9	32	70	28	70	32	72
10	30	70	32	72	35	72
11	31	72	30	74	30	76
12	30	74	32	74	35	72
13	28	68	30	72	33	71
14	30	70	28	70	30	74
15	29	69	29	72	34	69
16	28	70	30	70	32	70
17	30	68	32	74	30	75
18	28	70	34	75	32	76
19	30	72	32	74	30	73
20	28	70	34	68	34	74
21	29	74	30	68	35	75
22	27	68	32	70	30	73
23	27	76	30	72	32	72
24	30	74	30	70	34	76
25	32	68	32	68	30	72
26	34	72	28	72	32	76
27	34	70	30	68	34	75
28	27	72	32	70	30	74
29	29	68	30	72	30	76
30	31	70	30	70	30	76
Min	27	66	27	68	30	68
Max	34	77	34	75	35	76
Mean	29.88	70.50	29.94	71.00	32.00	73.03
SD	1.78	1.95	1.59	1.96	1.57	2.10

Table 42 Trichome number of *C. fistula* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Si Sa Ket	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	54	94	50	98	54	92
2	56	96	52	96	50	94
3	52	90	54	94	52	92
4	54	92	52	92	54	94
5	50	96	54	96	54	94
6	54	95	60	92	50	92
7	50	96	58	94	52	94
8	56	94	50	98	52	94
9	54	96	57	94	50	96
10	52	90	58	96	52	92
11	54	93	54	98	54	96
12	56	94	52	97	52	94
13	53	96	52	94	48	96
14	56	96	52	94	48	92
15	54	95	54	96	50	94
16	52	94	50	97	52	96
17	50	90	54	96	50	98
18	54	92	52	92	52	94
19	56	95	54	94	54	92
20	54	94	52	97	48	92
21	56	96	50	90	52	94
22	55	94	56	98	50	92
23	54	92	54	94	54	96
24	56	96	52	96	52	98
25	52	96	58	98	52	94
26	56	90	54	96	48	96
27	54	94	52	90	50	94
28	50	90	54	94	48	96
29	52	92	56	97	48	98
30	50	96	60	98	52	96
Min	50	90	50	90	48	92
Max	56	96	60	98	54	98
Mean	53.53	93.80	53.90	95.20	51.13	94.40
SD	1.72	1.8	2.18	1.97	1.78	1.57

Table 43 Trichome number of *C. grandis* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Si Sa Ket	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	32	46	40	48	36	48
2	32	49	40	48	36	50
3	30	48	38	42	35	52
4	36	46	40	44	37	50
5	32	47	36	46	38	46
6	36	48	36	48	36	48
7	32	46	39	44	36	49
8	33	48	39	42	34	49
9	30	48	40	42	35	48
10	32	49	42	42	37	46
11	33	50	42	42	39	50
12	34	46	39	44	36	40
13	36	46	40	46	36	42
14	36	42	42	42	38	41
15	34	46	44	48	34	43
16	38	48	40	47	36	42
17	36	43	40	42	32	42
18	30	49	39	42	34	40
19	32	50	40	48	36	42
20	33	46	44	44	37	44
21	36	48	42	42	38	42
22	34	47	43	43	36	42
23	36	46	44	44	36	46
24	34	45	42	42	35	48
25	36	44	42	42	34	46
26	34	49	40	43	34	46
27	38	50	42	42	32	45
28	36	48	40	46	36	44
29	32	46	42	48	37	40
30	30	47	40	46	39	40
Min	38	42	36	42	32	40
Max	30	50	44	48	39	52
Mean	33.77	46.97	40.53	44.34	35.81	45.09
SD	2.36	1.97	2.03	2.41	1.74	3.59

Table 44 Trichome number of *C. javanica* collected from Bangkok, Phitsanulok and Phathumthani provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Phathumthani	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	78	129	78	125	76	126
2	82	128	72	126	80	128
3	84	126	74	124	76	126
4	82	124	72	128	82	128
5	80	128	76	126	78	126
6	78	130	80	128	78	134
7	79	124	78	126	76	128
8	80	126	74	124	82	132
9	78	130	78	128	84	126
10	80	128	78	126	80	126
11	80	126	76	125	78	130
12	78	126	80	127	76	126
13	83	129	76	124	78	134
14	78	130	80	126	80	128
15	78	125	78	128	76	132
16	82	126	76	127	82	130
17	78	128	78	125	80	126
18	88	130	80	124	78	126
19	80	130	78	128	80	128
20	78	124	76	127	78	130
21	80	128	80	125	76	126
22	83	124	76	125	77	128
23	82	126	80	126	80	134
24	88	130	78	127	82	133
25	80	128	76	125	76	130
26	86	124	78	128	82	128
27	78	125	76	127	80	130
28	82	128	78	124	76	126
29	78	126	79	128	78	130
30	80	130	80	125	80	132
Min	78	124	72	124	76	126
Max	88	130	80	128	84	134
Mean	80.84	127.19	77.22	126.06	78.91	128.97
SD	2.29	1.92	1.84	1.21	2.02	2.35

Table 45 Trichome number of *C. alata* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Si Sa Ket	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	8	7	9	6	8	7
2	6	7	8	7	12	7
3	8	6	7	6	10	8
4	6	6	8	6	8	8
5	6	6	11	7	9	10
6	6	7	9	8	10	9
7	8	8	10	8	10	7
8	9	9	12	8	9	9
9	9	7	8	8	8	8
10	9	8	8	7	10	8
11	9	7	11	9	9	9
12	10	7	7	9	10	9
13	8	6	8	6	9	7
14	6	6	9	6	8	7
15	6	6	8	7	10	7
16	8	6	8	6	9	8
17	6	8	10	7	12	7
18	6	5	9	7	8	8
19	8	5	9	6	10	7
20	8	5	7	6	9	8
21	10	7	8	7	10	8
22	9	8	8	7	12	7
23	8	7	9	8	10	7
24	8	8	8	8	9	8
25	7	9	8	6	9	8
26	6	8	7	6	10	9
27	8	7	9	7	8	7
28	7	7	8	7	12	8
29	7	8	8	6	10	10
30	8	7	9	7	9	9
Min	6	5	7	6	8	7
Max	10	9	12	9	12	10
Mean	7.60	6.93	8.60	6.97	9.57	7.97
SD	1.08	0.82	0.95	0.71	0.97	0.71

Table 46 Trichome number of *C. angustifolia* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Si Sa Ket	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	9	43	11	45	12	49
2	10	45	15	50	14	53
3	11	49	13	52	15	52
4	10	48	15	49	13	52
5	10	50	16	46	10	49
6	11	51	18	45	11	50
7	14	43	11	45	12	45
8	15	48	12	49	13	49
9	9	49	11	48	16	49
10	9	49	14	48	15	49
11	10	43	15	49	17	49
12	12	42	15	53	11	55
13	13	49	12	50	12	49
14	10	50	13	52	12	50
15	9	51	11	52	14	56
16	14	49	11	49	15	49
17	11	48	14	56	15	56
18	10	46	15	55	15	49
19	10	47	16	49	16	56
20	9	49	12	49	12	51
21	9	48	11	48	11	51
22	12	48	15	50	15	56
23	13	49	15	50	16	50
24	14	42	14	49	12	58
25	11	42	11	49	13	56
26	10	43	12	48	10	58
27	9	49	11	47	10	56
28	10	46	13	46	15	55
29	10	45	15	49	10	51
30	9	49	15	49	11	55
Min	9	42	11	45	10	45
Max	15	51	18	56	17	58
Mean	10.77	47.00	13.40	49.20	13.10	52.10
SD	1.79	2.88	1.98	2.66	2.12	3.43

Table 47 Trichome number of C. hirsuta collected from Bangkok, Phitsanulok and Chachoengsao provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Chachoengsao	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	6	7	4	7	5	9
2	5	8	5	8	5	9
3	5	8	5	8	4	8
4	5	6	5	9	5	8
5	5	8	6	8	5	9
6	5	8	5	8	5	9
7	5	6	5	8	4	9
8	6	8	5	9	5	9
9	5	8	5	7	5	10
10	5	6	5	7	5	10
11	5	7	5	8	4	9
12	5	9	6	8	5	9
13	5	6	6	9	5	9
14	5	8	5	9	5	9
15	6	8	5	8	4	10
16	5	7	5	8	4	9
17	6	7	4	9	5	9
18	5	8	6	8	5	9
19	5	9	5	7	5	7
20	5	7	5	7	5	9
21	5	7	5	9	5	8
22	5	8	5	9	4	9
23	5	8	5	8	5	9
24	5	8	6	9	4	9
25	6	6	5	9	5	9
26	4	8	5	8	5	8
27	6	7	5	9	5	10
28	5	7	5	9	5	9
29	5	8	5	9	5	9
30	5	8	4	8	5	9
Min	4	6	4	7	4	7
Max	6	9	6	9	5	10
Mean	5.17	7.47	5.07	8.23	4.77	8.93
SD	0.33	0.74	0.31	0.61	0.36	0.38

Table 48 Trichome number of *C. siamea* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Si Sa Ket	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	26	58	30	56	32	56
2	30	55	32	58	30	59
3	32	58	33	57	32	57
4	34	55	32	56	33	56
5	28	56	32	58	32	60
6	30	52	31	54	33	57
7	28	54	32	58	32	60
8	34	60	26	54	28	59
9	32	58	32	58	33	60
10	34	60	30	60	32	62
11	28	56	32	54	32	60
12	29	54	33	58	30	57
13	34	58	32	57	33	57
14	34	54	33	58	33	59
15	30	55	26	55	28	60
16	34	56	32	58	33	62
17	32	54	26	60	29	56
18	34	55	32	58	30	60
19	28	54	33	56	33	58
20	34	55	30	58	32	57
21	32	58	32	54	32	56
22	34	55	29	58	30	59
23	34	56	32	56	33	56
24	28	54	33	60	32	62
25	29	55	32	56	33	64
26	34	56	33	65	33	56
27	28	59	33	58	34	62
28	32	58	32	54	32	59
29	28	54	26	58	35	60
30	30	60	32	65	34	57
Min	26	52	26	54	28	56
Max	34	60	33	65	35	64
Mean	31.13	56.07	31.10	57.50	31.93	58.77
SD	2.39	1.76	1.73	1.90	1.24	1.86

Table 49 Trichome number of *C. spectabilis* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Si Sa Ket	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	19	56	18	52	18	56
2	23	57	22	54	20	55
3	18	56	18	52	22	57
4	20	56	20	54	22	56
5	22	55	22	52	24	58
6	19	54	19	54	20	60
7	22	55	22	52	22	57
8	21	56	20	54	19	56
9	21	54	19	56	18	54
10	20	55	21	52	20	54
11	17	56	17	58	22	60
12	17	54	17	54	20	62
13	18	55	18	54	23	56
14	20	56	18	60	20	58
15	18	52	18	54	18	57
16	24	56	22	52	20	54
17	21	57	21	56	24	56
18	23	52	23	60	22	56
19	21	54	20	54	18	58
20	20	56	19	56	22	54
21	17	55	17	52	20	56
22	20	54	19	54	24	54
23	18	60	18	56	19	60
24	16	52	17	54	18	58
25	20	54	20	60	20	56
26	25	52	25	58	25	54
27	22	54	23	56	24	55
28	21	58	22	54	18	56
29	26	54	25	56	20	60
30	21	62	22	58	19	62
Min	16	52	17	52	18	54
Max	26	62	25	60	25	62
Mean	20.38	55.34	20.07	54.93	20.70	56.83
SD	1.89	1.60	1.95	2.05	1.84	1.88

Table 50 Trichome number of *C. timoriensis* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces					
	Bangkok		Phitsanulok		Si Sa Ket	
	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis	Dorsal epidermis	Ventral epidermis
1	12	20	12	24	8	24
2	13	25	10	22	9	22
3	13	24	9	24	12	20
4	14	22	9	21	11	22
5	12	22	11	21	10	24
6	11	24	12	19	12	21
7	13	22	11	19	10	20
8	14	24	10	21	9	24
9	12	24	12	19	9	22
10	12	24	12	20	9	24
11	12	25	9	22	9	22
12	13	22	14	24	10	22
13	14	24	9	24	9	21
14	13	24	12	21	12	20
15	11	25	14	23	9	24
16	12	24	10	20	9	22
17	12	23	12	23	9	24
18	14	25	11	21	12	23
19	14	20	12	22	9	24
20	13	21	12	22	10	20
21	12	23	10	23	12	21
22	14	23	9	20	10	22
23	14	24	9	21	9	24
24	14	22	14	24	9	24
25	13	21	10	22	12	22
26	12	20	12	23	9	24
27	13	20	12	22	10	20
28	11	22	10	20	12	22
29	12	24	12	22	10	24
30	14	24	14	20	12	24
Min	11	20	9	19	8	20
Max	14	25	14	24	12	24
Mean	12.75	22.88	11.19	21.63	10.06	22.38
SD	0.86	1.39	1.37	1.32	1.09	1.32

Table 51 Trichome number of *C. sulfurea* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces		
	Bangkok	Phitsanulok	Si Sa Ket
	Ventral epidermis	Ventral epidermis	Ventral epidermis
1	14	12	6
2	11	10	6
3	9	11	7
4	9	12	9
5	10	10	9
6	11	12	9
7	12	13	8
8	12	10	10
9	12	10	11
10	10	13	10
11	10	11	10
12	9	9	12
13	11	11	12
14	11	12	11
15	10	11	9
16	9	9	9
17	9	9	9
18	12	9	7
19	14	8	7
20	11	7	10
21	12	8	9
22	13	10	12
23	8	12	9
24	8	11	8
25	9	9	9
26	15	9	9
27	12	11	9
28	10	11	10
29	10	12	11
30	11	13	12
Min	8	7	6
Max	15	13	12
Mean	10.80	10.50	9.30
SD	1.41	1.33	1.29

Table 52 Trichome number of *C. surattensis* collected from Bangkok, Phitsanulok and Si Sa Ket provinces, Thailand

Field	Souces		
	Bangkok	Phitsanulok	Si Sa Ket
	Ventral epidermis	Ventral epidermis	Ventral epidermis
1	2	4	4
2	3	4	4
3	3	3	5
4	2	4	4
5	2	2	4
6	3	4	4
7	3	4	4
8	3	4	5
9	3	4	4
10	2	3	4
11	3	3	4
12	2	4	3
13	3	4	4
14	3	4	4
15	3	4	4
16	3	4	4
17	2	3	2
18	3	4	4
19	2	4	4
20	3	4	4
21	2	4	3
22	3	4	4
23	3	5	4
24	3	4	4
25	2	4	5
26	3	4	4
27	3	4	4
28	2	4	4
29	3	4	4
30	3	2	3
Min	2	2	2
Max	3	5	5
Mean	2.67	3.77	3.93
SD	0.44	0.44	0.32

Table 53 Trichome number of *C. tora* collected from Bangkok, Phitsanulok and Si Sa-Ket provinces, Thailand

Field	Souces		
	Bangkok	Phitsanulok	Si Sa Ket
	Ventral epidermis	Ventral epidermis	Ventral epidermis
1	64	60	67
2	60	64	68
3	64	62	66
4	60	64	65
5	64	63	65
6	62	60	66
7	65	59	60
8	64	60	66
9	60	64	65
10	60	65	69
11	64	62	68
12	62	64	65
13	65	66	66
14	64	62	63
15	62	64	65
16	64	63	64
17	64	66	66
18	65	61	65
19	64	60	65
20	62	62	66
21	64	65	63
22	62	63	66
23	60	62	64
24	65	64	66
25	62	65	65
26	58	66	63
27	62	67	60
28	60	64	58
29	60	65	64
30	62	63	66
Min	58	59	58
Max	65	67	69
Mean	62.47	63.17	64.83
SD	1.70	1.70	1.63

```
ONEWAY VAR00002 BY VAR00001
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.01).
```

Oneway

[DataSet0]

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	388809.023	9	43201.003	1.149E4	.000
Within Groups	3345.567	890	3.759		
Total	392154.590	899			

Post Hoc

Multiple Comparisons

VAR00002
Tukey HSD

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
1	2	10.21111*	.28902	.000	9.1534	11.2688
	3	20.16667*	.28902	.000	19.1089	21.2244
	4	-36.73333*	.28902	.000	-37.7911	-35.6756
	5	33.62222*	.28902	.000	32.5645	34.6800
	6	29.78889*	.28902	.000	28.7312	30.8466
	7	37.21111*	.28902	.000	36.1534	38.2688
	8	10.82222*	.28902	.000	9.7645	11.8800
	9	21.84444*	.28902	.000	20.7867	22.9022
	10	30.87778*	.28902	.000	29.8200	31.9355
2	1	-10.21111*	.28902	.000	-11.2688	-9.1534
	3	9.95556*	.28902	.000	8.8978	11.0133
	4	-46.94444*	.28902	.000	-48.0022	-45.8867
	5	23.41111*	.28902	.000	22.3534	24.4688
	6	19.57778*	.28902	.000	18.5200	20.6355
	7	27.00000*	.28902	.000	25.9423	28.0577
	8	.61111	.28902	.518	-.4466	1.6688
	9	11.63333*	.28902	.000	10.5756	12.6911
	10	20.66667*	.28902	.000	19.6089	21.7244

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval Lower Bound	Upper Bound
3	1	-20.16667*	.28902	.000	-21.2244	-19.1089
	2	-9.95556*	.28902	.000	-11.0133	-8.8978
	4	-56.90000*	.28902	.000	-57.9577	-55.8423
	5	13.45556*	.28902	.000	12.3978	14.5133
	6	9.62222*	.28902	.000	8.5645	10.6800
	7	17.04444*	.28902	.000	15.9867	18.1022
	8	-9.34444*	.28902	.000	-10.4022	-8.2867
	9	1.67778*	.28902	.000	.6200	2.7355
	10	10.71111*	.28902	.000	9.6534	11.7688
4	1	36.73333*	.28902	.000	35.6756	37.7911
	2	46.94444*	.28902	.000	45.8867	48.0022
	3	56.90000*	.28902	.000	55.8423	57.9577
	5	70.35556*	.28902	.000	69.2978	71.4133
	6	66.52222*	.28902	.000	65.4645	67.5800
	7	73.94444*	.28902	.000	72.8867	75.0022
	8	47.55556*	.28902	.000	46.4978	48.6133
	9	58.57778*	.28902	.000	57.5200	59.6355
	10	67.61111*	.28902	.000	66.5534	68.6688
5	1	-33.62222*	.28902	.000	-34.6800	-32.5645
	2	-23.41111*	.28902	.000	-24.4688	-22.3534
	3	-13.45556*	.28902	.000	-14.5133	-12.3978
	4	-70.35556*	.28902	.000	-71.4133	-69.2978
	6	-3.83333*	.28902	.000	-4.8911	-2.7756
	7	3.58889*	.28902	.000	2.5312	4.6466
	8	-22.80000*	.28902	.000	-23.8577	-21.7423
	9	-11.77778*	.28902	.000	-12.8355	-10.7200
	10	-2.74444*	.28902	.000	-3.8022	-1.6867
6	1	-29.78889*	.28902	.000	-30.8466	-28.7312
	2	-19.57778*	.28902	.000	-20.6355	-18.5200
	3	-9.62222*	.28902	.000	-10.6800	-8.5645
	4	-66.52222*	.28902	.000	-67.5800	-65.4645
	5	3.83333*	.28902	.000	2.7756	4.8911
	7	7.42222*	.28902	.000	6.3645	8.4800
	8	-18.96667*	.28902	.000	-20.0244	-17.9089
	9	-7.94444*	.28902	.000	-9.0022	-6.8867
	10	1.08889*	.28902	.007	.0312	2.1466
7	1	-37.21111*	.28902	.000	-38.2688	-36.1534
	2	-27.00000*	.28902	.000	-28.0577	-25.9423
	3	-17.04444*	.28902	.000	-18.1022	-15.9867
	4	-73.94444*	.28902	.000	-75.0022	-72.8867
	5	-3.58889*	.28902	.000	-4.6466	-2.5312
	6	-7.42222*	.28902	.000	-8.4800	-6.3645
	8	-26.38889*	.28902	.000	-27.4466	-25.3312
	9	-15.36667*	.28902	.000	-16.4244	-14.3089
	10	-6.33333*	.28902	.000	-7.3911	-5.2756

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
7	1	-37.21111*	.28902	.000	-38.2688	-36.1534
	2	-27.00000*	.28902	.000	-28.0577	-25.9423
	3	-17.04444*	.28902	.000	-18.1022	-15.9867
	4	-73.94444*	.28902	.000	-75.0022	-72.8867
	5	-3.58889*	.28902	.000	-4.6466	-2.5312
	6	-7.42222*	.28902	.000	-8.4800	-6.3645
	8	-26.38889*	.28902	.000	-27.4466	-25.3312
	9	-15.36667*	.28902	.000	-16.4244	-14.3089
	10	-6.33333*	.28902	.000	-7.3911	-5.2756
8	1	-10.82222*	.28902	.000	-11.8800	-9.7645
	2	-.61111	.28902	.518	-1.6688	.4466
	3	9.34444*	.28902	.000	8.2867	10.4022
	4	-47.55556*	.28902	.000	-48.6133	-46.4978
	5	22.80000*	.28902	.000	21.7423	23.8577
	6	18.96667*	.28902	.000	17.9089	20.0244
	7	26.38889*	.28902	.000	25.3312	27.4466
	9	11.02222*	.28902	.000	9.9645	12.0800
	10	20.05556*	.28902	.000	18.9978	21.1133
9	1	-21.84444*	.28902	.000	-22.9022	-20.7867
	2	-11.63333*	.28902	.000	-12.6911	-10.5756
	3	-1.67778*	.28902	.000	-2.7355	-.6200
	4	-58.57778*	.28902	.000	-59.6355	-57.5200
	5	11.77778*	.28902	.000	10.7200	12.8355
	6	7.94444*	.28902	.000	6.8867	9.0022
	7	15.36667*	.28902	.000	14.3089	16.4244
	8	-11.02222*	.28902	.000	-12.0800	-9.9645
	10	9.03333*	.28902	.000	7.9756	10.0911
10	1	-30.87778*	.28902	.000	-31.9355	-29.8200
	2	-20.66667*	.28902	.000	-21.7244	-19.6089
	3	-10.71111*	.28902	.000	-11.7688	-9.6534
	4	-67.61111*	.28902	.000	-68.6688	-66.5534
	5	2.74444*	.28902	.000	1.6867	3.8022
	6	-1.08889*	.28902	.007	-2.1466	-.0312
	7	6.33333*	.28902	.000	5.2756	7.3911
	8	-20.05556*	.28902	.000	-21.1133	-18.9978
	9	-9.03333*	.28902	.000	-10.0911	-7.9756

*. The mean difference is significant at the 0.01 level.

Homogeneous

VAR00002

Tukey HSD

VAR00001	N	Subset for alpha = 0.01								
		1	2	3	4	5	6	7	8	9
7	90	5.0000								
5	90		8.5889							
10	90			11.3333						
6	90				12.4222					
9	90					20.3667				
3	90						22.0444			
8	90							31.3889		
2	90							32.0000		
1	90								42.2111	
4	90									78.9444
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	.518	1.000	1.000

Means for groups in homogeneous subsets are displayed.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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ONEWAY VAR00002 BY VAR00001
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.01).
```

Oneway

[DataSet0]

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1504398.663	12	125366.555	2.448E4	.000
Within Groups	5925.167	1157	5.121		
Total	1510323.830	1169			

Post Hoc

Multiple Comparisons

VAR00002
Tukey HSD

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-22.98889*	.33735	.000	-24.2683	-21.7094
	3	25.12222*	.33735	.000	23.8428	26.4017
	4	-55.91111*	.33735	.000	-57.1906	-54.6317
	5	64.18889*	.33735	.000	62.9094	65.4683
	6	22.04444*	.33735	.000	20.7650	23.3239
	7	63.26667*	.33735	.000	61.9872	64.5461
	8	14.03333*	.33735	.000	12.7539	15.3128
	9	15.81111*	.33735	.000	14.5317	17.0906
	10	61.27778*	.33735	.000	59.9983	62.5572
	11	68.02222*	.33735	.000	66.7428	69.3017
	12	49.16667*	.33735	.000	47.8872	50.4461
	13	7.98889*	.33735	.000	6.7094	9.2683
2	1	22.98889*	.33735	.000	21.7094	24.2683
	3	48.11111*	.33735	.000	46.8317	49.3906
	4	-32.92222*	.33735	.000	-34.2017	-31.6428
	5	87.17778*	.33735	.000	85.8983	88.4572
	6	45.03333*	.33735	.000	43.7539	46.3128
	7	86.25556*	.33735	.000	84.9761	87.5350
	8	37.02222*	.33735	.000	35.7428	38.3017
	9	38.80000*	.33735	.000	37.5206	40.0794
	10	84.26667*	.33735	.000	82.9872	85.5461
	11	91.01111*	.33735	.000	89.7317	92.2906
	12	72.15556*	.33735	.000	70.8761	73.4350
	13	30.97778*	.33735	.000	29.6983	32.2572

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
3	1	-25.12222*	.33735	.000	-26.4017	-23.8428
	2	-48.11111*	.33735	.000	-49.3906	-46.8317
	4	-81.03333*	.33735	.000	-82.3128	-79.7539
	5	39.06667*	.33735	.000	37.7872	40.3461
	6	-3.07778*	.33735	.000	-4.3572	-1.7983
	7	38.14444*	.33735	.000	36.8650	39.4239
	8	-11.08889*	.33735	.000	-12.3683	-9.8094
	9	-9.31111*	.33735	.000	-10.5906	-8.0317
	10	36.15556*	.33735	.000	34.8761	37.4350
	11	42.90000*	.33735	.000	41.6206	44.1794
	12	24.04444*	.33735	.000	22.7650	25.3239
	13	-17.13333*	.33735	.000	-18.4128	-15.8539
4	1	55.91111*	.33735	.000	54.6317	57.1906
	2	32.92222*	.33735	.000	31.6428	34.2017
	3	81.03333*	.33735	.000	79.7539	82.3128
	5	120.10000*	.33735	.000	118.8206	121.3794
	6	77.95556*	.33735	.000	76.6761	79.2350
	7	119.17778*	.33735	.000	117.8983	120.4572
	8	69.94444*	.33735	.000	68.6650	71.2239
	9	71.72222*	.33735	.000	70.4428	73.0017
	10	117.18889*	.33735	.000	115.9094	118.4683
	11	123.93333*	.33735	.000	122.6539	125.2128
	12	105.07778*	.33735	.000	103.7983	106.3572
	13	63.90000*	.33735	.000	62.6206	65.1794
5	1	-64.18889*	.33735	.000	-65.4683	-62.9094
	2	-87.17778*	.33735	.000	-88.4572	-85.8983
	3	-39.06667*	.33735	.000	-40.3461	-37.7872
	4	-120.10000*	.33735	.000	-121.3794	-118.8206
	6	-42.14444*	.33735	.000	-43.4239	-40.8650
	7	-.92222	.33735	.236	-2.2017	.3572
	8	-50.15556*	.33735	.000	-51.4350	-48.8761
	9	-48.37778*	.33735	.000	-49.6572	-47.0983
	10	-2.91111*	.33735	.000	-4.1906	-1.6317
	11	3.83333*	.33735	.000	2.5539	5.1128
	12	-15.02222*	.33735	.000	-16.3017	-13.7428
	13	-56.20000*	.33735	.000	-57.4794	-54.9206
6	1	-22.04444*	.33735	.000	-23.3239	-20.7650
	2	-45.03333*	.33735	.000	-46.3128	-43.7539
	3	3.07778*	.33735	.000	1.7983	4.3572
	4	-77.95556*	.33735	.000	-79.2350	-76.6761
	5	42.14444*	.33735	.000	40.8650	43.4239
	7	41.22222*	.33735	.000	39.9428	42.5017
	8	-8.01111*	.33735	.000	-9.2906	-6.7317
	9	-6.23333*	.33735	.000	-7.5128	-4.9539
	10	39.23333*	.33735	.000	37.9539	40.5128
	11	45.97778*	.33735	.000	44.6983	47.2572
	12	27.12222*	.33735	.000	25.8428	28.4017
	13	-14.05556*	.33735	.000	-15.3350	-12.7761

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
7	1	-63.26667*	.33735	.000	-64.5461	-61.9872
	2	-86.25556*	.33735	.000	-87.5350	-84.9761
	3	-38.14444*	.33735	.000	-39.4239	-36.8650
	4	-119.17778*	.33735	.000	-120.4572	-117.8983
	5	.92222	.33735	.236	-.3572	2.2017
	6	-41.22222*	.33735	.000	-42.5017	-39.9428
	8	-49.23333*	.33735	.000	-50.5128	-47.9539
	9	-47.45556*	.33735	.000	-48.7350	-46.1761
	10	-1.98889*	.33735	.000	-3.2683	-.7094
	11	4.75556*	.33735	.000	3.4761	6.0350
	12	-14.10000*	.33735	.000	-15.3794	-12.8206
	13	-55.27778*	.33735	.000	-56.5572	-53.9983
8	1	-14.03333*	.33735	.000	-15.3128	-12.7539
	2	-37.02222*	.33735	.000	-38.3017	-35.7428
	3	11.08889*	.33735	.000	9.8094	12.3683
	4	-69.94444*	.33735	.000	-71.2239	-68.6650
	5	50.15556*	.33735	.000	48.8761	51.4350
	6	8.01111*	.33735	.000	6.7317	9.2906
	7	49.23333*	.33735	.000	47.9539	50.5128
	9	1.77778*	.33735	.000	.4983	3.0572
	10	47.24444*	.33735	.000	45.9650	48.5239
	11	53.98889*	.33735	.000	52.7094	55.2683
	12	35.13333*	.33735	.000	33.8539	36.4128
	13	-6.04444*	.33735	.000	-7.3239	-4.7650
9	1	-15.81111*	.33735	.000	-17.0906	-14.5317
	2	-38.80000*	.33735	.000	-40.0794	-37.5206
	3	9.31111*	.33735	.000	8.0317	10.5906
	4	-71.72222*	.33735	.000	-73.0017	-70.4428
	5	48.37778*	.33735	.000	47.0983	49.6572
	6	6.23333*	.33735	.000	4.9539	7.5128
	7	47.45556*	.33735	.000	46.1761	48.7350
	8	-1.77778*	.33735	.000	-3.0572	-.4983
	10	45.46667*	.33735	.000	44.1872	46.7461
	11	52.21111*	.33735	.000	50.9317	53.4906
	12	33.35556*	.33735	.000	32.0761	34.6350
	13	-7.82222*	.33735	.000	-9.1017	-6.5428
10	1	-61.27778*	.33735	.000	-62.5572	-59.9983
	2	-84.26667*	.33735	.000	-85.5461	-82.9872
	3	-36.15556*	.33735	.000	-37.4350	-34.8761
	4	-117.18889*	.33735	.000	-118.4683	-115.9094
	5	2.91111*	.33735	.000	1.6317	4.1906
	6	-39.23333*	.33735	.000	-40.5128	-37.9539
	7	1.98889*	.33735	.000	.7094	3.2683
	8	-47.24444*	.33735	.000	-48.5239	-45.9650
	9	-45.46667*	.33735	.000	-46.7461	-44.1872
	11	6.74444*	.33735	.000	5.4650	8.0239
	12	-12.11111*	.33735	.000	-13.3906	-10.8317
	13	-53.28889*	.33735	.000	-54.5683	-52.0094

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	99% Confidence Interval	
					Lower Bound	Upper Bound
11	1	-68.02222*	.33735	.000	-69.3017	-66.7428
	2	-91.01111*	.33735	.000	-92.2906	-89.7317
	3	-42.90000*	.33735	.000	-44.1794	-41.6206
	4	-123.93333*	.33735	.000	-125.2128	-122.6539
	5	-3.83333*	.33735	.000	-5.1128	-2.5539
	6	-45.97778*	.33735	.000	-47.2572	-44.6983
	7	-4.75556*	.33735	.000	-6.0350	-3.4761
	8	-53.98889*	.33735	.000	-55.2683	-52.7094
	9	-52.21111*	.33735	.000	-53.4906	-50.9317
	10	-6.74444*	.33735	.000	-8.0239	-5.4650
	12	-18.85556*	.33735	.000	-20.1350	-17.5761
	13	-60.03333*	.33735	.000	-61.3128	-58.7539
12	1	-49.16667*	.33735	.000	-50.4461	-47.8872
	2	-72.15556*	.33735	.000	-73.4350	-70.8761
	3	-24.04444*	.33735	.000	-25.3239	-22.7650
	4	-105.07778*	.33735	.000	-106.3572	-103.7983
	5	15.02222*	.33735	.000	13.7428	16.3017
	6	-27.12222*	.33735	.000	-28.4017	-25.8428
	7	14.10000*	.33735	.000	12.8206	15.3794
	8	-35.13333*	.33735	.000	-36.4128	-33.8539
	9	-33.35556*	.33735	.000	-34.6350	-32.0761
	10	12.11111*	.33735	.000	10.8317	13.3906
	11	18.85556*	.33735	.000	17.5761	20.1350
	13	-41.17778*	.33735	.000	-42.4572	-39.8983
13	1	-7.98889*	.33735	.000	-9.2683	-6.7094
	2	-30.97778*	.33735	.000	-32.2572	-29.6983
	3	17.13333*	.33735	.000	15.8539	18.4128
	4	-63.90000*	.33735	.000	-65.1794	-62.6206
	5	56.20000*	.33735	.000	54.9206	57.4794
	6	14.05556*	.33735	.000	12.7761	15.3350
	7	55.27778*	.33735	.000	53.9983	56.5572
	8	6.04444*	.33735	.000	4.7650	7.3239
	9	7.82222*	.33735	.000	6.5428	9.1017
	10	53.28889*	.33735	.000	52.0094	54.5683
	11	60.03333*	.33735	.000	58.7539	61.3128
	12	41.17778*	.33735	.000	39.8983	42.4572

*. The mean difference is significant at the 0.01 level.

Homogeneous

VAR00002

Tukey HSD

VAR00001	N	Subset for alpha = 0.01											
		1	2	3	4	5	6	7	8	9	10	11	12
11	90	3.4556											
5	90		7.2889										
7	90		8.2111										
10	90			10.2000									
12	90				22.3111								
3	90					46.3556							
6	90						49.4333						
9	90							55.6667					
8	90								57.4444				
13	90									63.4889			
1	90										71.4778		
2	90											94.4667	
4	90												1.2739E2
Sig.		1.000	.236	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

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APPENDIX C

AFLP profile generated by primer combination

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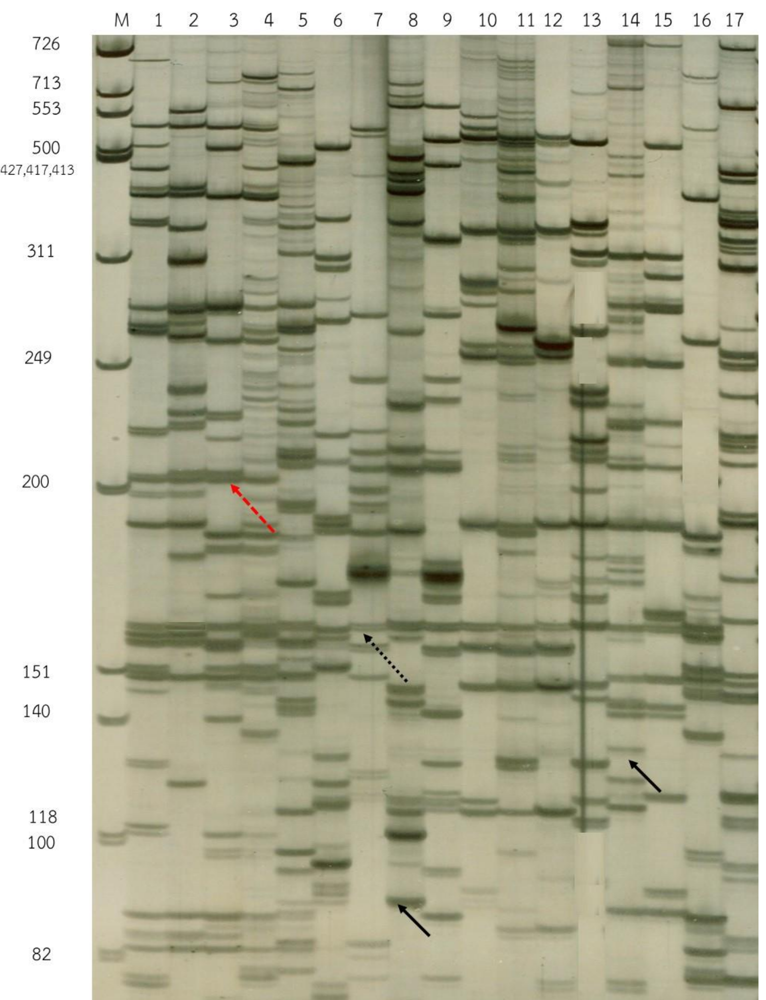

Figure 97 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+AAC/M+CCA primer combinations

→ indicates unique bands of *Cassia* species

▪▪▪▶ indicates monomorphic bands of four *Cassia* species

······▶ indicates monomorphic bands of *all Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker
Lane 1: *C. fistula*
Lane 2: *C. grandis*
Lane 3: *C. bakeriana*
Lane 4: *C. javanica*
Lane5: *C. alata*
Lane 6: *C. spectabilis*
Lane 7: *C. siamea*
Lane 8: *C. timoriensis*
Lane 9: *C. garrettiana*
Lane 10: *C. hirsuta*
Lane 11: *C. occidentalis*
Lane 12: *C. sophera*
Lane 13: *C. tora*
Lane 14: *C. surattensis*
Lane 15: *C. sulfurea*
Lane 16: *C. angustifolia*
Lane17: *A. paniculata*

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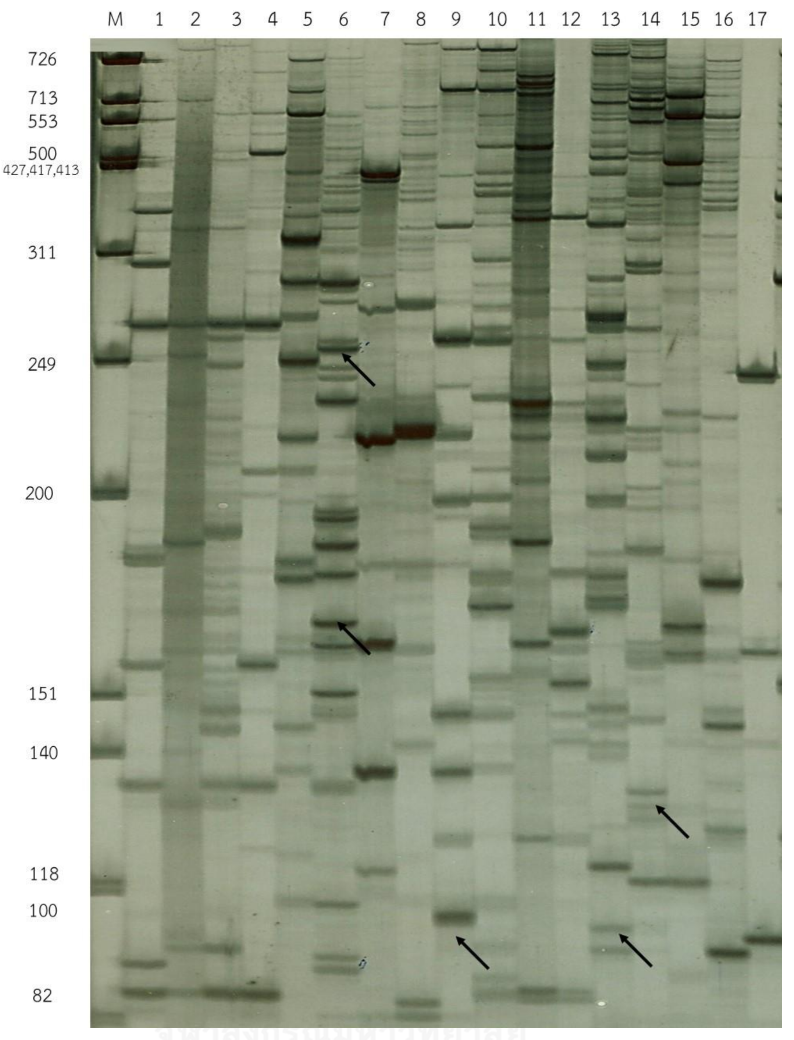

Figure 98 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+AAC/M+CGT primer combinations

⟶ indicates unique bands of *Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker	Lane 1: *C. fistula*	Lane 2: *C. grandis*
Lane 3: *C. bakeriana*	Lane 4: *C. javanica*	Lane5: *C. alata*
Lane 6: *C. spectabilis*	Lane 7: *C. siamea*	Lane 8: *C. timoriensis*
Lane 9: *C. garrettiana*	Lane 10: *C. hirsuta*	Lane 11: *C. occidentalis*
Lane 12: *C. sophera*	Lane 13: *C. tora*	Lane 14: *C. surattensis*
Lane 15: *C. sulfurea*	Lane 16: *C. angustifolia*	Lane17: *A. paniculata*

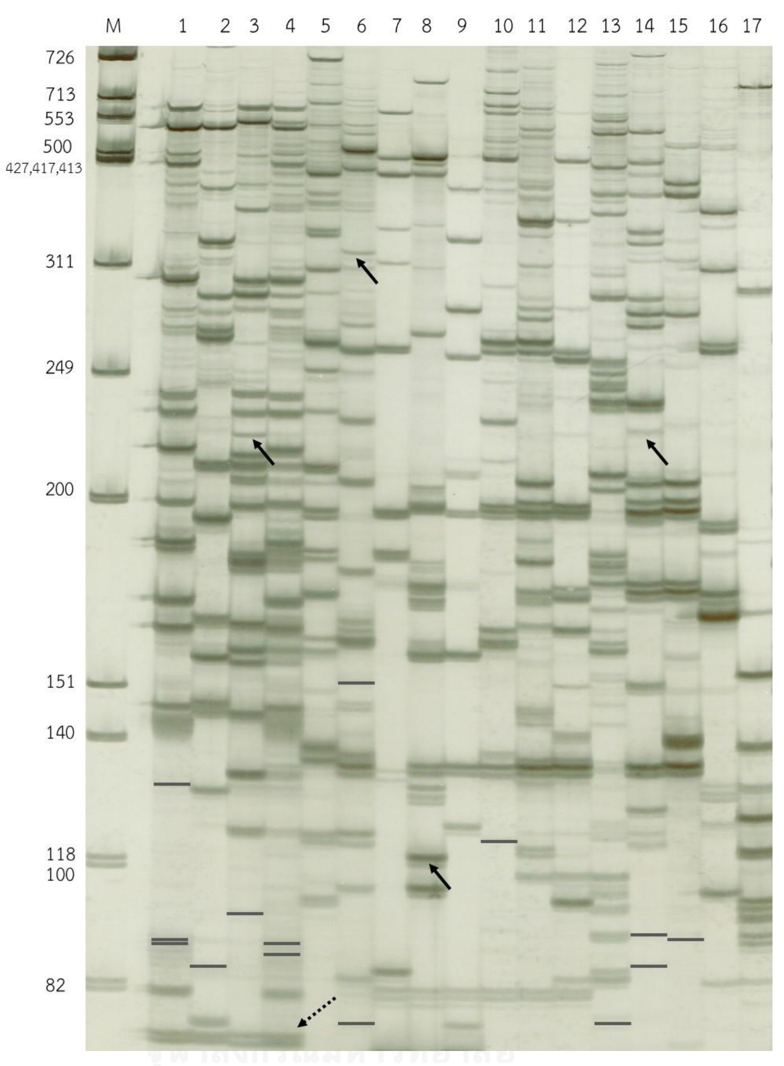

Figure 99 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+AGC/M+CCA primer combinations

⟶ indicates unique bands of *Cassia* species

⋯⋯▶ indicates monomorphic bands of *Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker
Lane 1: *C. fistula*
Lane 2: *C. grandis*
Lane 3: *C. bakeriana*
Lane 4: *C. javanica*
Lane5: *C. alata*
Lane 6: *C. spectabilis*
Lane 7: *C. siamea*
Lane 8: *C. timoriensis*
Lane 9: *C. garrettiana*
Lane 10: *C. hirsuta*
Lane 11: *C. occidentalis*
Lane 12: *C. sophera*
Lane 13: *C. tora*
Lane 14: *C. surattensis*
Lane 15: *C. sulfurea*
Lane 16: *C. angustifolia*
Lane17: *A. paniculata*

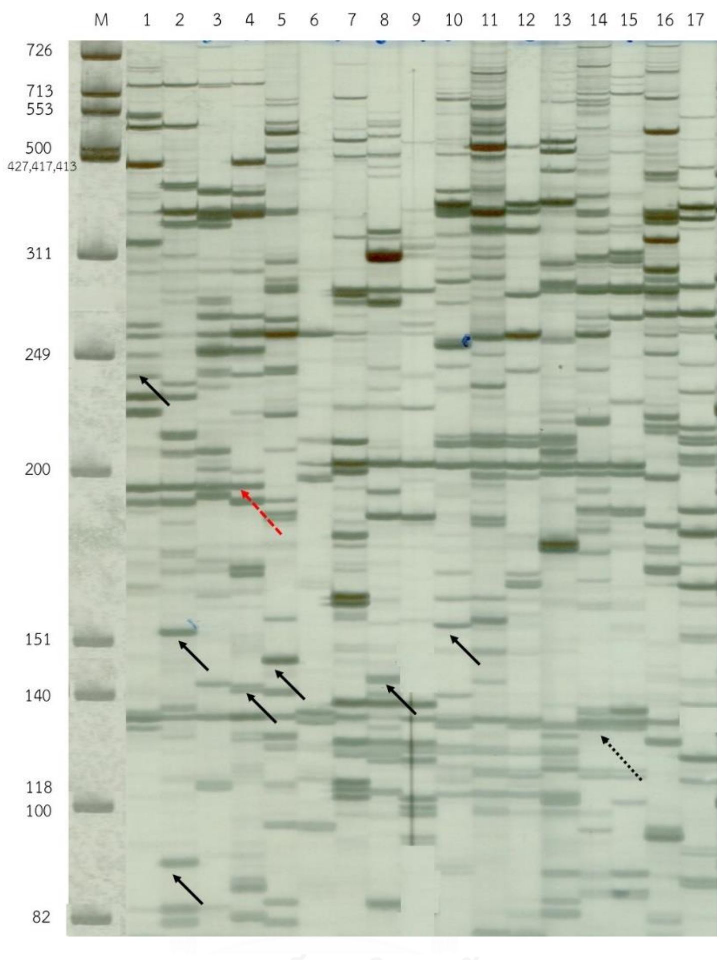

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Figure 100 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+AGC/M+CAA primer combinations

→ indicates unique bands of *Cassia* species

→ (red dashed) indicates monomorphic bands of four *Cassia* species

→ (black dotted) indicates monomorphic bands of *all Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker — Lane 1: *C. fistula* — Lane 2: *C. grandis*

Lane 3: *C. bakeriana* — Lane 4: *C. javanica* — Lane5: *C. alata*

Lane 6: *C. spectabilis* — Lane 7: *C. siamea* — Lane 8: *C. timoriensis*

Lane 9: *C. garrettiana* — Lane 10: *C. hirsuta* — Lane 11: *C. occidentalis*

Lane 12: *C. sophera* — Lane 13: *C. tora* — Lane 14: *C. surattensis*

Lane 15: *C. sulfurea* — Lane 16: *C. angustifolia* — Lane17: *A. paniculata*

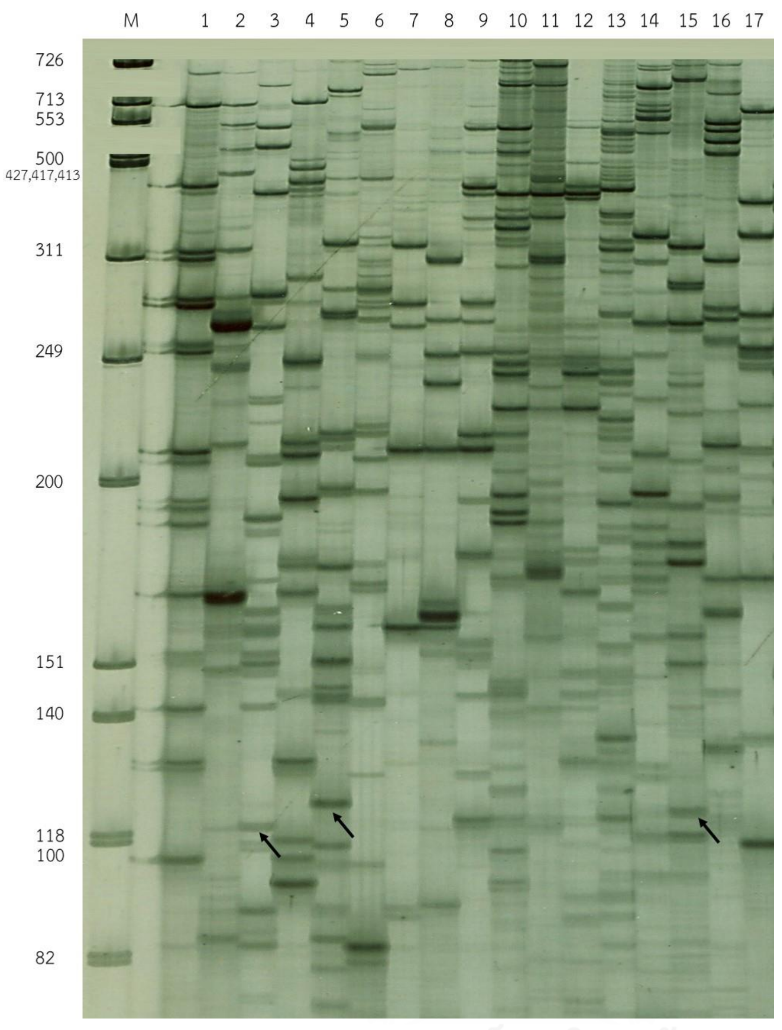

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Figure 101 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+ACC/M+CAA primer combinations

⟶ indicates unique bands of *Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker | Lane 1: *C. fistula* | Lane 2: *C. grandis*

Lane 3: *C. bakeriana* | Lane 4: *C. javanica* | Lane5: *C. alata*

Lane 6: *C. spectabilis* | Lane 7: *C. siamea* | Lane 8: *C. timoriensis*

Lane 9: *C. garrettiana* | Lane 10: *C. hirsuta* | Lane 11: *C. occidentalis*

Lane 12: *C. sophera* | Lane 13: *C. tora* | Lane 14: *C. surattensis*

Lane 15: *C. sulfurea* | Lane 16: *C. angustifolia* | Lane17: *A. paniculata*

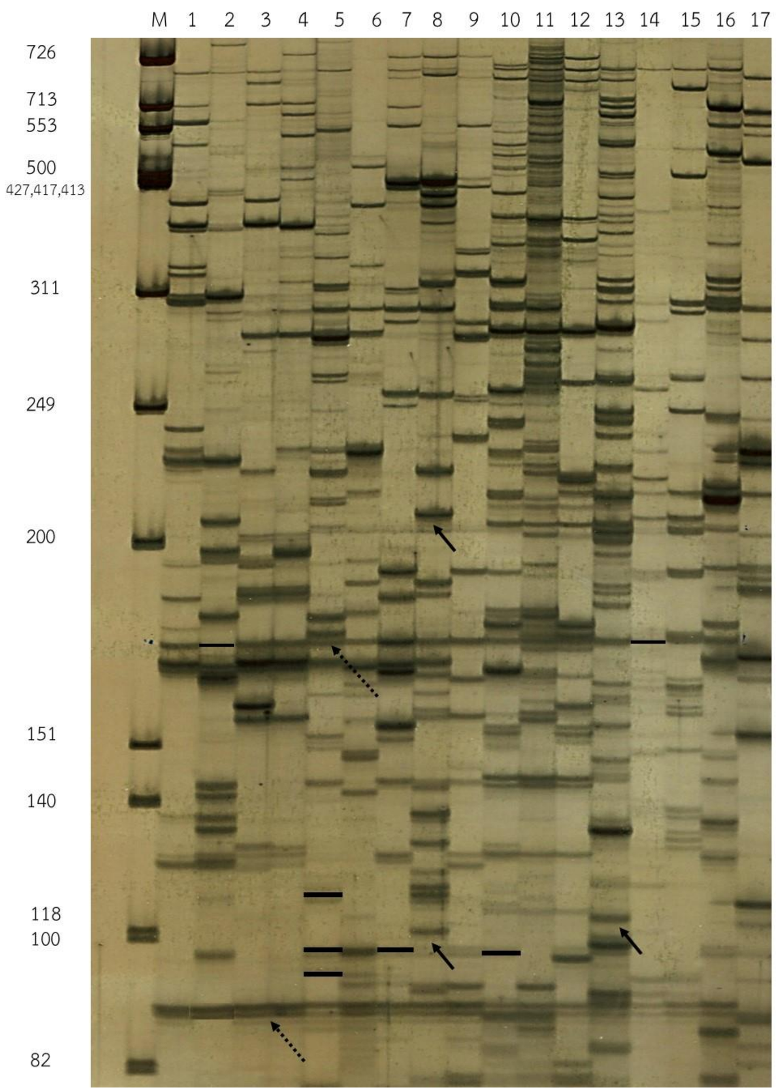

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Figure 102 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+ACC/M+CCA primer combinations

——▶ indicates unique bands of *Cassia* species

······▶ indicates monomorphic bands of *Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker
Lane 1: *C. fistula*
Lane 2: *C. grandis*
Lane 3: *C. bakeriana*
Lane 4: *C. javanica*
Lane5: *C. alata*
Lane 6: *C. spectabilis*
Lane 7: *C. siamea*
Lane 8: *C. timoriensis*
Lane 9: *C. garrettiana*
Lane 10: *C. hirsuta*
Lane 11: *C. occidentalis*
Lane 12: *C. sophera*
Lane 13: *C. tora*
Lane 14: *C. surattensis*
Lane 15: *C. sulfurea*
Lane 16: *C. angustifolia*
Lane17: *A. paniculata*

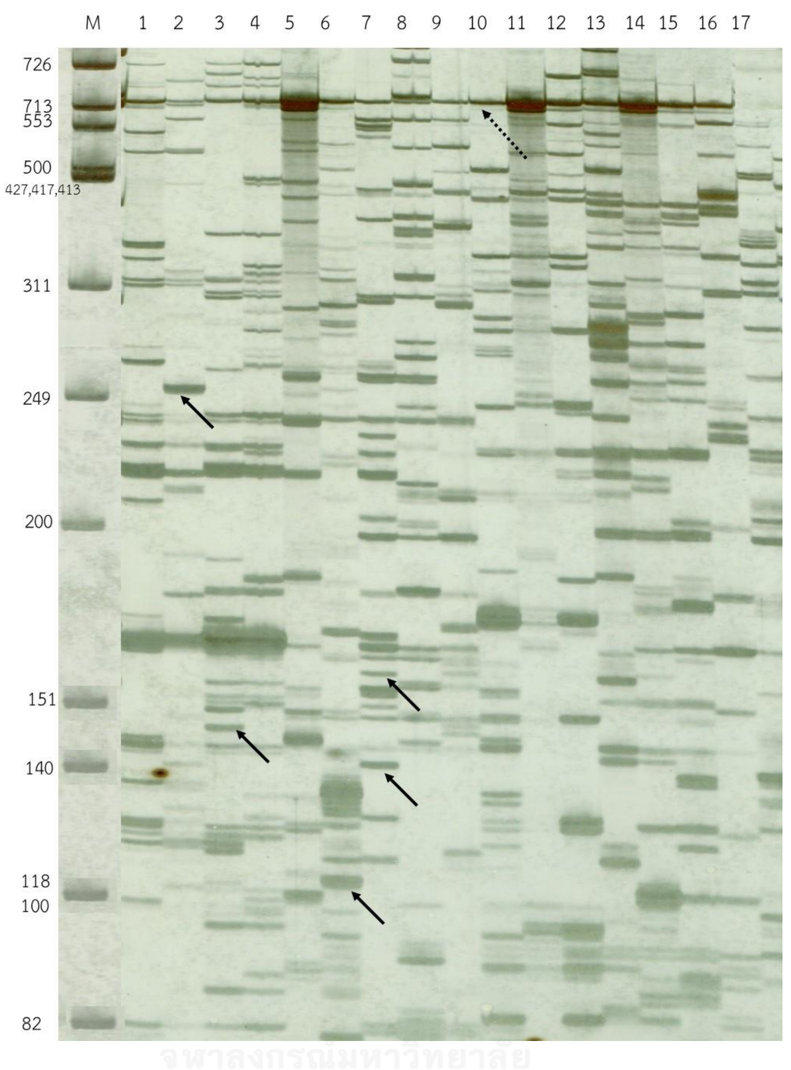

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Figure 103 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+AAG/M+CCA primer combinations

⟶ indicates unique bands of *Cassia* species

⋯⋯► indicates monomorphic bands of *Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker	Lane 1: *C. fistula*	Lane 2: *C. grandis*
Lane 3: *C. bakeriana*	Lane 4: *C. javanica*	Lane5: *C. alata*
Lane 6: *C. spectabilis*	Lane 7: *C. siamea*	Lane 8: *C. timoriensis*
Lane 9: *C. garrettiana*	Lane 10: *C. hirsuta*	Lane 11: *C. occidentalis*
Lane 12: *C. sophera*	Lane 13: *C. tora*	Lane 14: *C. surattensis*
Lane 15: *C. sulfurea*	Lane 16: *C. angustifolia*	Lane17: *A. paniculata*

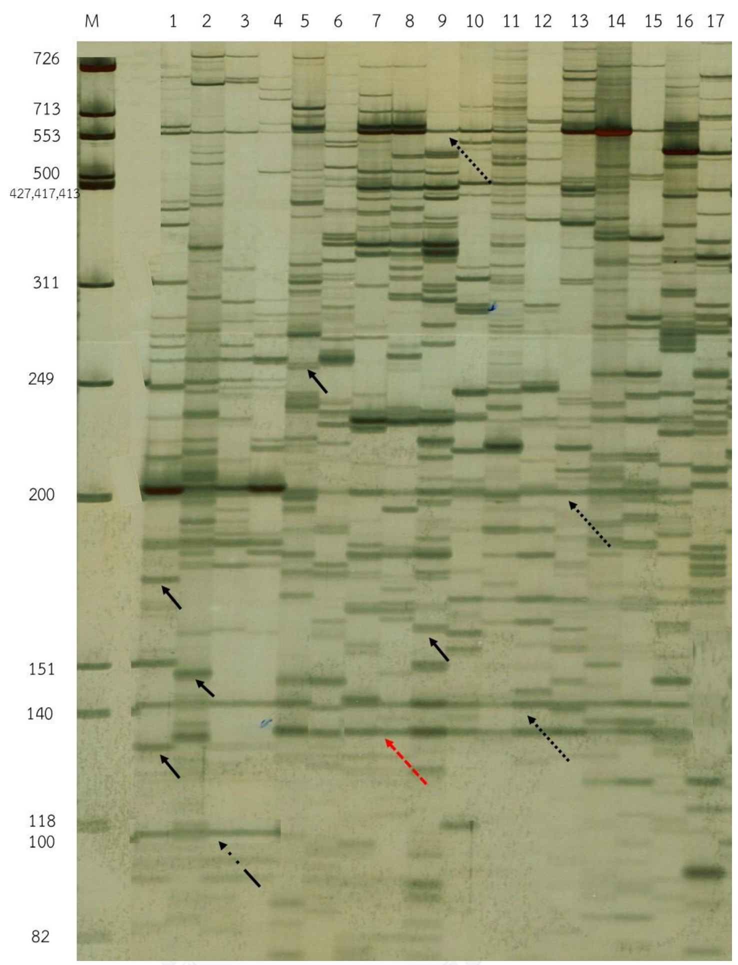

Figure 104 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+AAG/M+CAA primer combinations

→ indicates unique bands of *Cassia* species

⇢ indicates monomorphic bands of *Senna* species

⇢ indicates monomorphic bands of *Cassia* species

⇢ indicates monomorphic bands of *all Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker | Lane 1: *C. fistula* | Lane 2: *C. grandis*

Lane 3: *C. bakeriana* | Lane 4: *C. javanica* | Lane5: *C. alata*

Lane 6: *C. spectabilis* | Lane 7: *C. siamea* | Lane 8: *C. timoriensis*

Lane 9: *C. garrettiana* | Lane 10: *C. hirsuta* | Lane 11: *C. occidentalis*

Lane 12: *C. sophera* | Lane 13: *C. tora* | Lane 14: *C. surattensis*

Lane 15: *C. sulfurea* | Lane 16: *C. angustifolia* | Lane17: *A. paniculata*

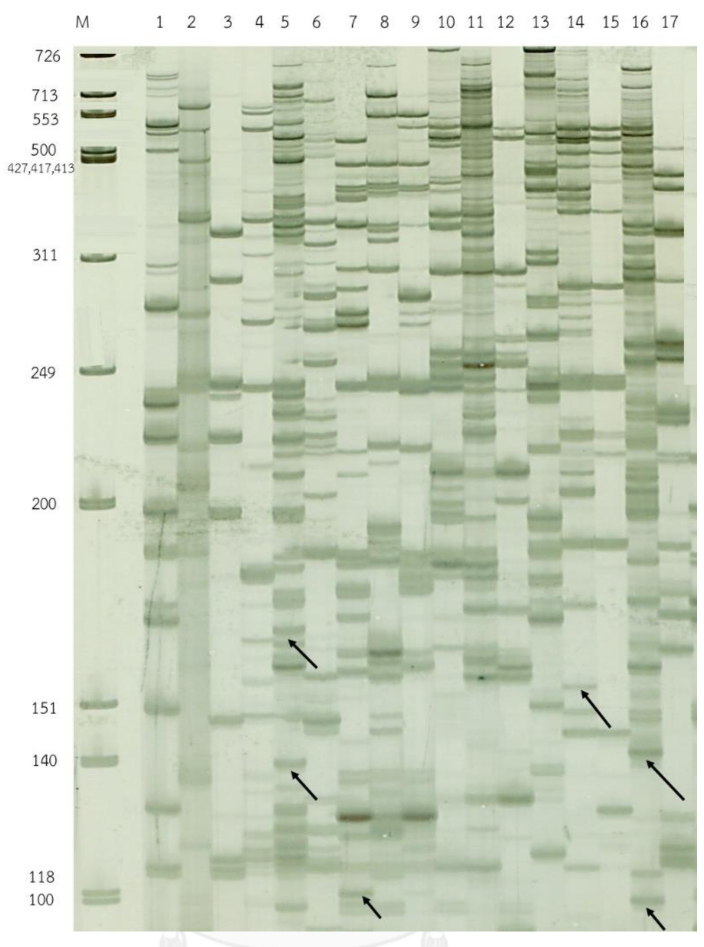

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Figure 105 AFLP fingerprint of 16 *Cassia* species and *A. paniculata* (outgroup plants) obtained from E+AAG/M+CAT primer combinations

⟶ indicates unique bands of *Cassia* species

Lane designations with accession number are as follows:

Lane M: phiX174 DNA/*Hinf*I Marker | Lane 1: *C. fistula* | Lane 2: *C. grandis*

Lane 3: *C. bakeriana* | Lane 4: *C. javanica* | Lane5: *C. alata*

Lane 6: *C. spectabilis* | Lane 7: *C. siamea* | Lane 8: *C. timoriensis*

Lane 9: *C. garrettiana* | Lane 10: *C. hirsuta* | Lane 11: *C. occidentalis*

Lane 12: *C. sophera* | Lane 13: *C. tora* | Lane 14: *C. surattensis*

Lane 15: *C. sulfurea* | Lane 16: *C. angustifolia* | Lane17: *A. paniculata*

APPENDIX D

Quantitative analysis

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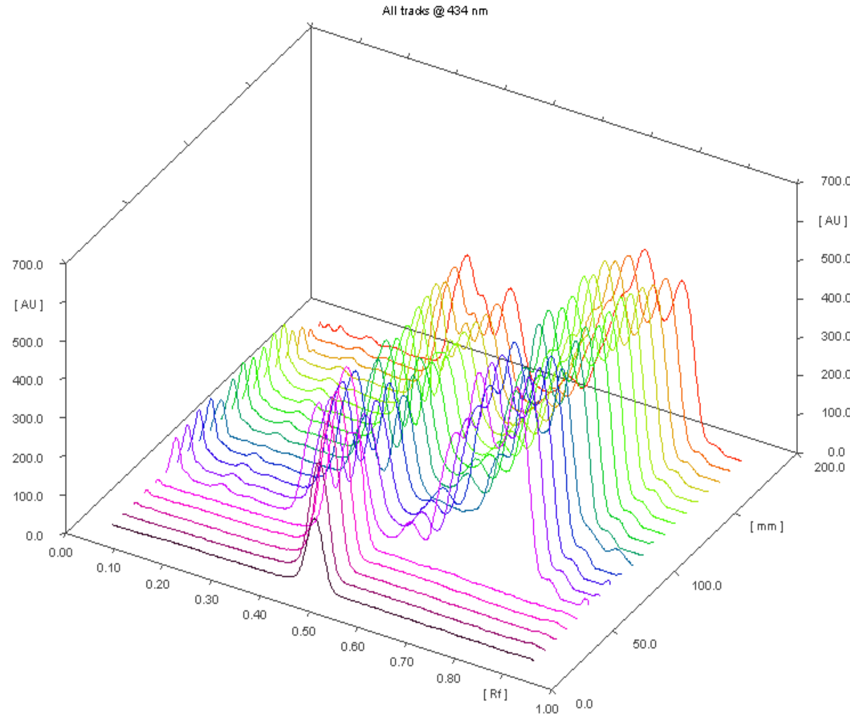

Figure 106 TLC densitogram of *C. garrettiana* (standard aloe-emodin No. 1-5, sample No. 6-20)

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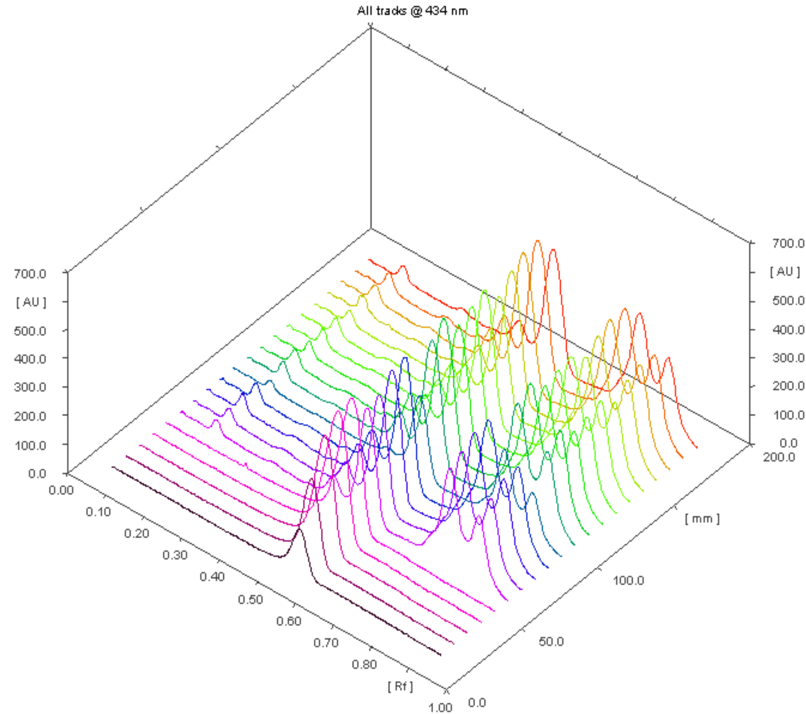

Figure 107 TLC densitogram of *C. grandis* (standard aloe-emodin No. 1-5, sample No. 6-20)

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Figure 108 *C. grandis* dried leaves crude drug

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Figure 109 *C. garrettiana* dried leaves crude drug

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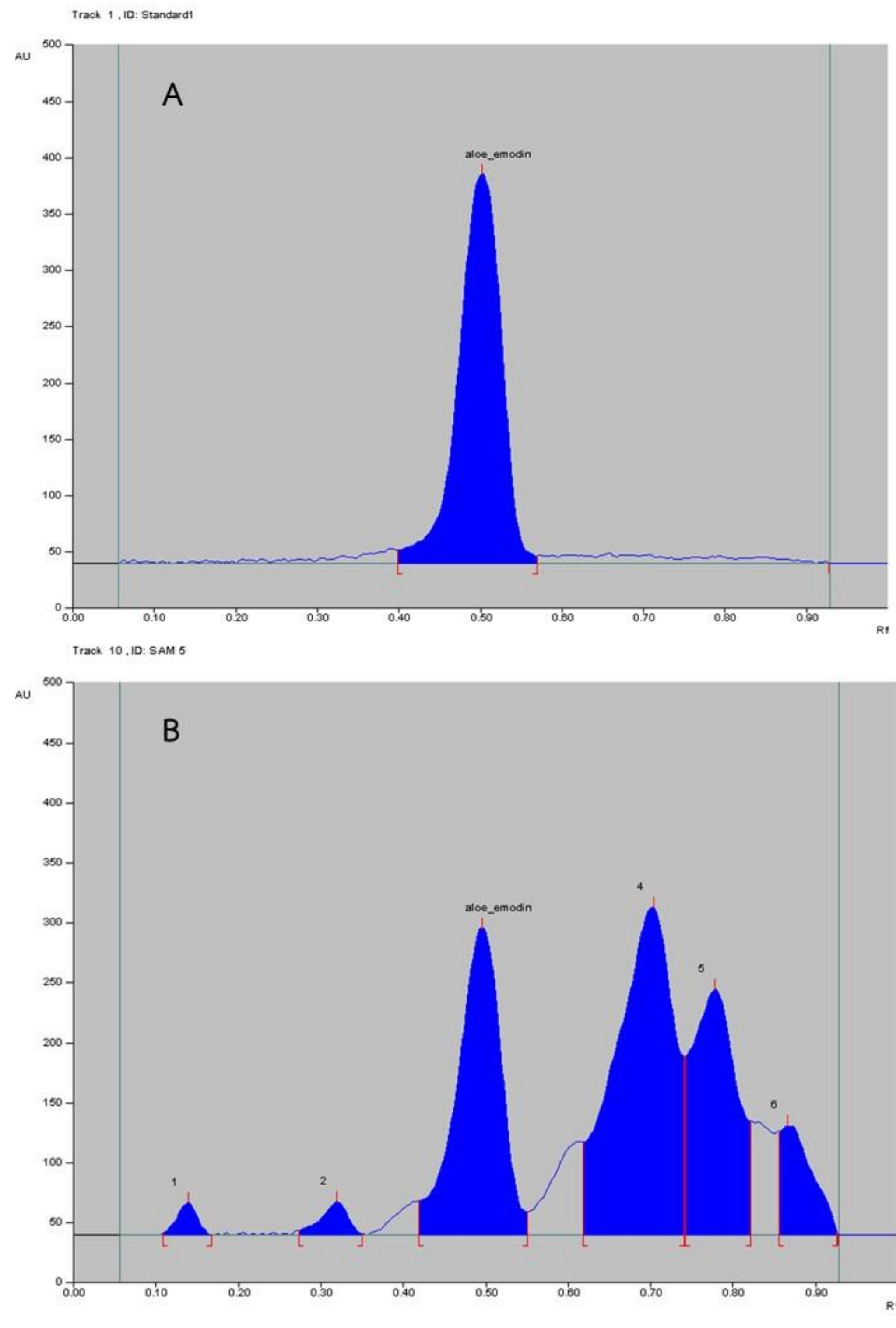
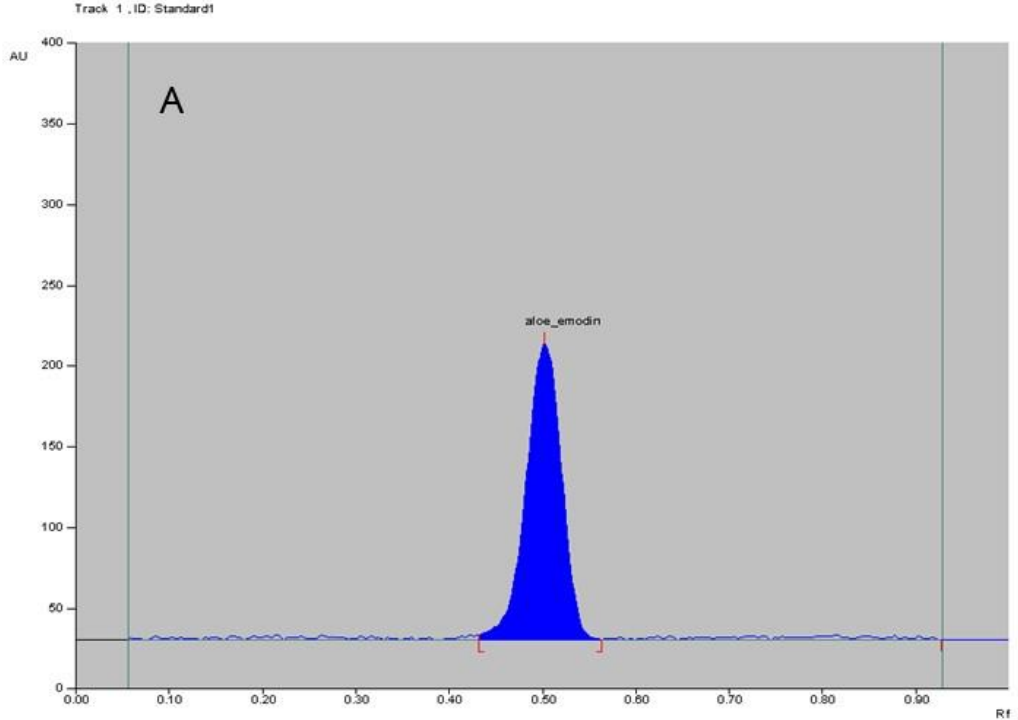


Figure 110 TLC chomatogram of **A:** standard aloe-emodin **B**: aloe-emodin in *C. grandis* leaf extract



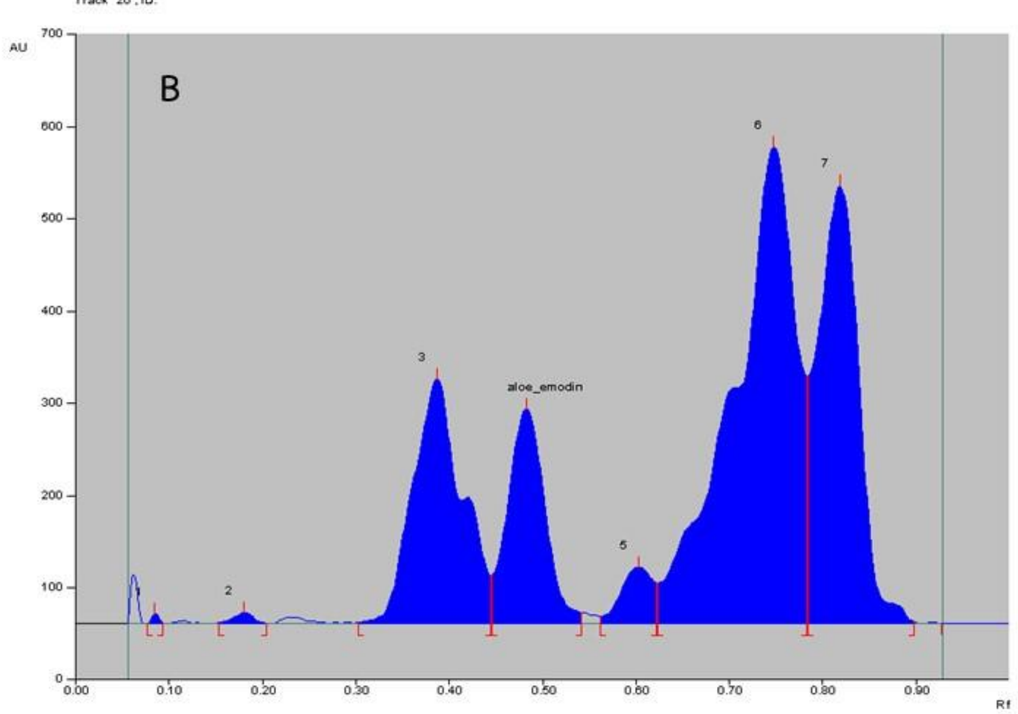


Figure 111 TLC chomatogram of **A:** standard aloe-emodin **B**: aloe-emodin in *C. garrettiana* leaf extract

VITA

Miss Anusara Sihanat was born on September 25, 1986 in Si Sa Ket, Thailand. She got a Bachelor's degree of Science (Applied Thai Traditional Medicine) first class honor from Faculty of Public Health, Naresuan University, Thailand in 2008.

Publications

1. Sihanat A, Ruangrungsi N, Palanuvej C, Chareonsap PP, Rungsihirunrat K. Leaf constant numbers of selected Cassia species in Thailand. Bulletin of Health Science and Technology. 2015;13(2):8-16.

2. Sihanat A, Rungsihirunrat K, Palanuvej C, Ruangrungsi N. Characteristics and number of trichome of leaves from selected Cassia spp. in Thailand. Bulletin of Health Science and Technology. 2016;14(1):10-20.

3. Sihanat A, Chareonsap PP, Ruangrungsi N, Rungsihirunrat K. Using AFLP to identify genetic relationships in Cassia species from Thailand. Pakistan Journal of Botany. 2016. (In press)

Oral presentations

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2. Sihanat A, Rungsihirunrat K, Palanuvej C, Ruangrungsi N. Characteristics and number of trichome of leaves from selected Cassia spp. in Thailand. Proceedings of The 3rd International Conference on Advanced Pharmaceutical Research (ICAPH); 2016 March 12; Rangsit University, Thailand. (2nd Award Poster Presentation).