PHARMACOGNOSTIC SPECIFICATION AND BRAZILIN CONTENT IN *CAESALPINIA SAPPAN* HEARTWOODS, MACROSCOPIC, MICROSCOPIC AND MOLECULAR IDENTIFICATION OF SELECTED *CAESALPINIA* SPECIES IN THAILAND

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ข้อกำหนดทางเภสัชเวทและปริมาณบราซิลินของแก่นฝาง การประเมินลักษณะทางมหทรรศน์ จุลทรรศน์และอณูโมเลกุลของพืชบางชนิดในสกุลซีซาลพิเนียในประเทศไทย

น.ส.สุวิภา อินต๊ะเขียว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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สุวิภา อินต๊ะเขียว : ข้อกำหนดทางเภสัชเวทและปริมาณบราซิลินของแก่นฝาง การประเมินลักษณะทางมหทรรศน์ จุลทรรศน์และอณูโมเลกุลของพืชบางชนิดในสกุลซีซาลพิเนียในประเทศไทย. (PHARMACOGNOSTIC SPECIFICATION AND BRAZILIN CONTENT IN *CAESALPINIA SAPPAN* HEARTWOODS, MACROSCOPIC, MICROSCOPIC AND MOLECULAR IDENTIFICATION OF SELECTED *CAESALPINIA* SPECIES IN THAILAND) อ.ที่ปรึกษาหลัก : รศ. ดร.ชนิดา พลานุเวช, อ.ที่ปรึกษาร่วม : รศ. ดร.นิจศิริ เรืองรังษี,รศ. ดร.กาญจนา รังษีหิรัญรัตน์

พืชสกุลซีซาลพีเนีย (*Caesalpinia* L.) จัดอยู่ในวงศ์ Caesalpiniaceae กระจายทั่วทวีปเอเชียและเอเชียตะวันออกเฉียงใต้ ในประเทศไทยพบจำนวน 18 ชนิด กระจายทั่วภูมิภาค พืชบางชนิดจะมีลักษณะทางพฤกษศาสตร์โดยเฉพาะส่วนใบ รวมถึงชื่อพื้นเมืองที่มีความคล้ายคลึงกันอาจทำให้ยากต่อการจำแนก ฝาง คือ พืชชนิดหนึ่งในสกุลซีซาลพีเนีย เป็นที่รู้จักอย่างกว้างขวาง มีสรรพคุณทางยามากมายทั้งยังใช้ในอุตสาหกรรมอาหาร เครื่องดื่ม ยา และเครื่องสำอาง โดยเฉพาะส่วนแก่นที่มีสารสำคัญชื่อ บราซิลิน เป็นสารกลุ่มฟลาโวนอยด์ วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อจัดทำข้อกำหนดทางเภสัชเวทของแก่นฝาง วิเคราะห์ปริมาณสารบราซิลินในแก่นฝางโดยวิธีโครมาโทกราฟีชนิดแผ่นบาง-เด็นซิโทเมทรีเทียบกับการวิเคราะห์เชิงภาพด้วยโปรแกรม ImageJ ร่วมกับการศึกษาลักษณะทางจุลทรรศน์และลายพิมพ์ทางดีเอ็นเอ เพื่อจำแนกความแตกต่างของพืชในสกุล ซีซาลพีเนีย แก่นฝางมีลักษณะภายนอกที่แข็งและหยาบ มีสีส้มแดง การวิเคราะห์สารบราซิลินจากสองวิธี พบว่าในแก่นฝางมีปริมาณสารบราซิลินโดยเฉลี่ย 1.259 ± 0.455 และ 1.256 ± 0.405 กรัม ใน สมุนไพรแห้ง 100 กรัม ตามลำดับ ทั้งสองวิธีให้ค่าปริมาณสารบราซิลินที่ไม่ต่างกันอย่างมีนัยสำคัญทางสถิติ (P> 0.05) ทดสอบความเชื่อถือได้ของวิธีวิเคราะห์ทั้งสองวิธีในด้านความจำเพาะ ความแม่นยำ ความเที่ยงตรง ขีดจำกัดในการตรวจสอบ ขีดจำกัดในการวัดเชิงปริมาณและความคงทน ผลที่ได้มีความน่าเชื่อถือและยอมรับได้ การศึกษาเอกลักษณ์ทางเคมี-ฟิสิกส์ของแก่นฝางพบว่ามีปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด น้ำหนักที่หายไปเมื่อทำให้แห้งและปริมาณน้ำไม่เกินร้อยละ 0.87, 0.44, 8.50 และ 8.52 กรัม ใน สมุนไพรแห้ง 100 กรัม ตามลำดับ ปริมาณสารสกัดด้วยเอทานอลและปริมาณสารสกัดด้วยน้ำไม่น้อยกว่าร้อยละ 2.94 และ 3.77 กรัม ใน สมุนไพรแห้ง 100 กรัม ตามลำดับ ลักษณะทางพฤกษศาสตร์และภาคตัดขวางของเส้นกลางใบแสดงในรูปแบบภาพวาดลายเส้น ประเมินค่าคงที่ของใบได้แก่ จำนวนปากใบ ค่าดัชนีปากใบ ค่าอัตราส่วนเซลล์รั้ว ค่าพื้นที่เซลล์ผิว จำนวนขนและค่าดัชนีขนผลการทดลองพบว่าลักษณะทางจุลทรรศน์สามารถนำมาเป็นกุญแจในการแยกชนิดของพืชในสกุลซีซาลพีเนีย รวมทั้งผลจากการประเมินทางอณูโมเลกุลด้วยลายพิมพ์ชนิดไอเอสเอสอาร์ จากไพรเมอร์ที่นิยมใช้ทั่วไป 15 ไพรเมอร์ มี 7 ไพรเมอร์ที่นำมาใช้ในการศึกษา ทำให้เกิดแถบดีเอ็นเอที่มีความแตกต่างและคมชัดทั้งหมด 217 แถบ ให้ค่าดัชนีความคล้ายคลึงทางพันธุกรรมของพืชในสกุลซีซาลพีเนียอยู่ระหว่าง 0.192 ถึง 0.454 ข้อมูลที่ได้จากการศึกษาครั้งนี้สามารถนำไปประยุกต์ใช้ในการพิสูจน์เอกลักษณ์ของพืชสกุลซีซาลพีเนียที่พบในประเทศไทยและทำให้ทราบถึงข้อกำหนดทางเภสัชเวทรวมถึงปริมาณบราซิลินในแก่นฝางในประเทศไทย

สาขาวิชา	วิทยาศาสตร์สาธารณสุข	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
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Suwipa Intakhiao : PHARMACOGNOSTIC SPECIFICATION AND BRAZILIN CONTENT IN *CAESALPINIA SAPPAN* HEARTWOODS, MACROSCOPIC, MICROSCOPIC AND MOLECULAR IDENTIFICATION OF SELECTED *CAESALPINIA* SPECIES IN THAILAND. Advisor: Assoc. Prof. CHANIDA PALANUVEJ, Ph.D. Co-advisor: Assoc. Prof. Nijsiri Ruangrungsi, Ph.D.,Assoc. Prof. KANCHANA RUNGSIHIRUNRAT, Ph.D.

The genus *Caesalpinia* L. belongs to Caesalpiniaceae family. This genus was spread throughout Asia and South East Asia. In Thailand, 18 species of *Caesalpinia* L. genus were reported which were spread in every parts. Due to the similar morphology especially the leaf part and closely vernacular name of eight *Caesalpinia* species, the identification of these species is complicated. *Caesalpinia sappan* L. is the well-known species which has many important drug properties. It was used as food, beverage, and cosmetic industries especially the heartwood part that contained brazilin, one of the flavonoid group. This study aimed to establish the pharmacognostic specification, develop the quantitative analysis of brazilin in *C. sappan* heartwoods by TLC analysis using TLC-densitometry compared to TLC-image analysis. Microscopic leaf characteristics including leaf constant numbers, and phylogenetic relationship by ISSR markers were used to differentiate the selected eight *Caesalpinia* species in Thailand. The heartwood of *C. sappan* was hard and rough with orange-red color. The contents of brazilin using TLC-densitometry and TLC-image analysis were 1.259 ± 0.455 and 1.256 ± 0.405 g/100 g crude drug, respectively. Brazilin contents analyses from two methods were not statistically significantly different (P> 0.05). The analytical methods were validated to confirm that the processes were appropriate, reliable, and gain an accurate data in term of specificity, accuracy, precision, limit of detection, limit of quantitation and robustness. The physico-chemical parameters including total ash, acid insoluble ash, ethanol and water soluble extractive values, loss on drying, and water content should not be more than 0.87, 0.44, 8.50, and 8.52 g/100 g crude drug, respectively whereas the content of ethanol and water soluble extractives should not be less than 2.94 and 3.77 g/100 g crude drug. Botanical characteristics and transverse cross section of midrib were illustrated by drawing. Microscopic leaf characteristics including leaf constant numbers (stomatal number, stomatal index, palisade ratio, epidermal cell area, trichome number, and trichome index) were evaluated. The results from microscopic characteristics can be used as the key to classification plants in *Caesalpinia* species. For ISSR fingerprinting, 15 universal primers were screened and 7 primers were used in this study. This technique produced a total of distinct and reproducible 217 bands. The genetic relationship among *Caesalpinia* L. genus presented the similarity index between 0.192 and 0.454. The information from this study provided the beneficial data for identification of selected eight *Caesalpinia* species, provided the pharmacognostic specification and brazilin content in *C. sappan* heartwoods in Thailand.

Field of Study:	Public Health Sciences	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....
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Suwipa Intakhiao

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
bp	base pair
°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
ISSR	inter-simple sequence repeat
kg	kilogram
L	Liter
LOD	limit of detection
LOQ	limit of quantification
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
ml	milliliter

mm	millimeter
mM	millimolar
mm^2	square milliliter
µl	microliter
µm	micrometer
µM	micromolar
µg	microgram
ng	nanogram
nm	nanometer
PCR	polymerase chain reaction
rpm	round per minute
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
SD	standard deviation
SSR	simple sequence repeat
Taq	*Thermus aquaticus*
TBE buffer	Tris-boric and EDTA buffer
TE	Tris-EDTA buffer
TLC	Thin-layer chromatography
Tris	Tris (hydroxymethyl) aminomethane

Tris-HCl	Tris-hydrochloride buffer
UPGMA	unweighted pair group method with arithmetic mean
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Background and rationale

The Caesalpiniaceae family comprises of the spiny trees, shrubs, or incessant herbs. This family contains around 153 genera with 2,175 species (Mabberley 1997). In Thailand, only 20 genera with 113 species were found (Smitinand 1984). The genus *Caesalpinia* L. is a genus of trees, shrubs and climbers consisting of approximately 150 species distributed throughout the world (Verdcourt 1979). In 1793, Linnaeus reported on the genus *Caesalpinia* at the first time, and many species have been later on added to this genus (Lewis 1994). In 1984, Smitinand reported only 18 species of *Caesalpinia* L. genus presented in Thailand (Smitinand 1984). There are several researches about pharmacological activities, ethnomedicinal practices, and phytochemical studies. Chemical compounds such as flavonoids, steroids, chalcones, quercetin, etc., have been isolated from numerous species of the genus *Caesalpinia* (Zanin 2012).

Plants have been used before recorded history for treatment or medicinal intention. The study on plant secondary metabolites which are pharmacologically active chemical compositions in each part of plant is considerable as a basic knowledge for herbal medicine development. Nowadays, although medicinal plants and herbal products have been used in many ways in Thailand, few are subjected to

quality control. It might be because the standard compounds are quite expensive or the interested chemicals of crude samples are hard to be extracted. Crude drug should have good quality, contain active constituents for treatment, and none of any contaminated substances or foreign matters. Pharmacognostic specification is one of the most crucial tools to classify and indicate the quality of the plant material *via* many techniques for evaluation of the macroscopic and microscopic characteristics, genetic information, chemical composition, etc.

Morphological characterization is the first step which is simple and can be observed by naked eyes, magnifying glass and microscope. Macroscopic characteristics is based on the evaluation of crude drug by color, size, odor, taste and surface (texture and fracture). Microscopic characterization of medicinal plant is based on the observation of the cellular structure and storage products in the cells using microscope. The observation consists of stomatal type, trichome type, epidermal cell, transverse section of each part of plant, leaf constant number such as stomatal number, stomatal index, palisade ratio, epidermal cell area, trichome number, and trichome index.

The data obtained from only morphological characteristics might have limitations due to the variation of sample collecting and environmental effect. As a consequence, DNA fingerprint is a forceful tool to assess the identity of plant species and also the genetic diversity and a genetic relationship of plants with less effect

from environmental condition and plant collection. DNA fingerprint is a method used to identify an individual by consider the unique patterns in its DNA. This is a technique that simultaneously detects lots of minisatellites in the genome to generate a pattern unique to an individual. Nowadays, there are various molecular marker techniques such as ISSR, RAPD, AFLP, RFLP, and SSR. ISSR or inter-simple sequence repeat is an applied PCR technique to investigate DNA using only one primer in many positions and does not require any sequence information. Because of its rapidness and inexpensiveness, it is pervasively used for DNA studies (Ng 2015). There are not many studies on the molecular characteristics of *Caesalpinia* species. The previous studies were reported such as using RAPD (Rodrigues 2012) and AFLP (Mukherjee 2006) techniques for identification of plants species. In Thailand, there are no report on the plant identification by ISSR marker of these species.

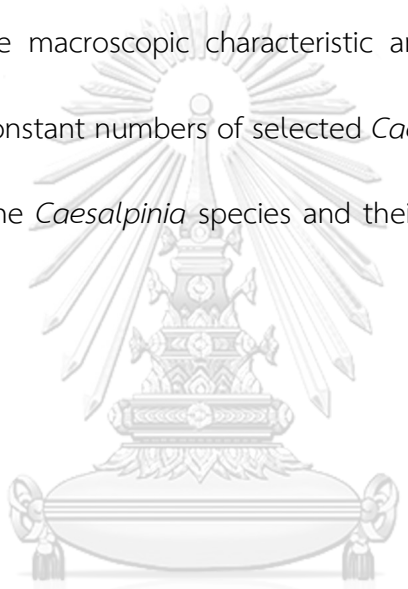
Caesalpinia sappan L., or Fang in Thai, is a Thai medicinal plant which is generally used for blood circulation enhancement, swelling reduction, anti-thirsty, and pain relief. This plant is widely distributed in Thailand, Indonesia, Vietnam, Burma, India and China (Flora of China 1988). *Caesalpinia sappan* is the most well-known species in the genus *Caesalpinia*. *Caesalpinia sappan* heartwood has been informed for its pharmacological activities such as antibacterial (Xu 2004), antimicrobial, antidiabetic (Mohan 2011), anti-anaemic (Badami 2004), and antioxidant activities (Sarumathy 2011). The wood is very hard, rough and has deep red color. In

addition, the red color from the heartwood is used various ways in cosmetics, fabrics, and foods. *Caesalpinia sappan* heartwood contains many phenolic constituents such as chachone, triterpinoids, and flavonoids (Cuong 2012). The substance named "brazilin" is the major compound in this plant (Gilbody 1902). Brazilin is a natural pigment commonly used as a dye, which is found to be particularly abundant in *C. sappan* (8-22% w/w) (Zanin 2012). The compound brazilin is homoisoflavonoids, collectively isolated from alcoholic extraction of the *C. sappan* heartwood. For the quantitative analysis, TLC or thin layer chromatography is a basic technique to detect the important compound from crude drugs or herbal plants, because it is simple, rapid and inexpensive (Waksmundzka-Hajnos 2008).

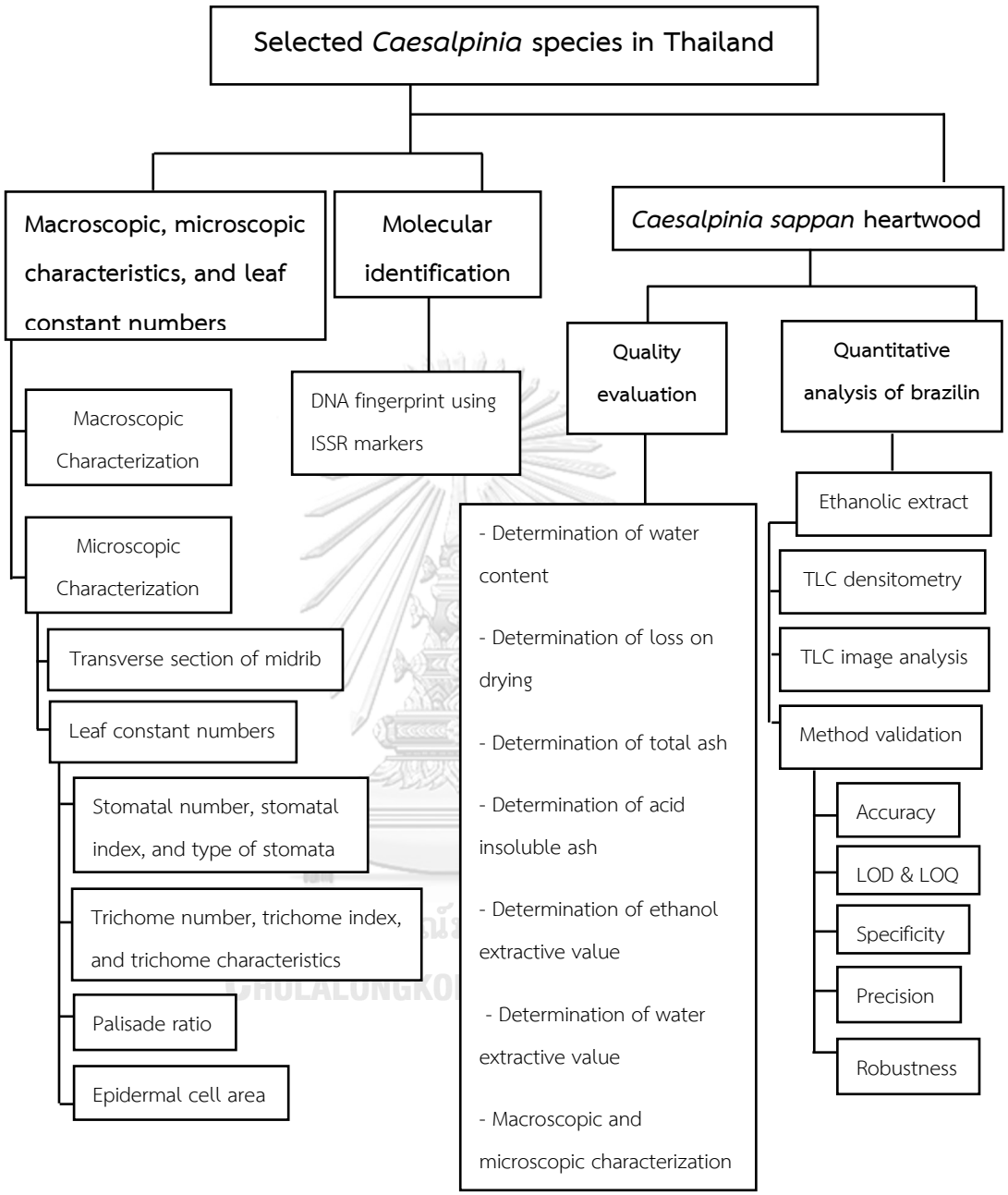
Consequently, this research aimed to evaluate the selected *Caesalpinia* species found in Thailand through the identifications of macroscopic characteristics and microscopic characteristics including leaf constant numbers, and ISSR fingerprinting. Other goals of the study were to examine the *Caesalpinia sappan* heartwood in terms of pharmacognostic specification, and to quantitate brazilin with chromatographic analysis using TLC densitometry by winCATS software and TLC image analysis by ImageJ free software. The methods were validated to provide the beneficial data for classification and identification of the genus *Caesalpinia* L. The assessment of an important compound brazilin was established.

Objectives of the study

1. To investigate the quantitative analysis of brazilin in *Caesalpinia sappan* heartwood by TLC analysis using TLC densitometry compared to TLC image analysis.
2. To establish the pharmacognostic specification of *Caesalpinia sappan* heartwood in Thailand.
3. To evaluate the macroscopic characteristic and microscopic characteristics including leaf constant numbers of selected *Caesalpinia* species in Thailand.
4. To distinguish the *Caesalpinia* species and their phylogenetic relationship by ISSR markers.



Conceptual framework



CHAPTER II

LITERATURE REVIEWS

The genus *Caesalpinia* L.

The description of the genus *Caesalpinia* L. was reported by Smitinand and Larsen from Flora of Thailand volume four part one as follows;

"This genus is a climber or shrub or small tree, usually prickly. Stipules present or absent, persistent or caduceus. Leaves alternate, bipinnate, the rhachis often prickly; pinnae opposite, usually even in number, the rhachis sometimes prickly; leaflets opposite or alternate, sessile or petiolulate. Flowers paniculate or racemose, axillary or terminal usually bisexual. Receptacle short, persistent or shed. Sepals 5, usually unequal, the lowest one hood-shaped, imbricate. Petals 5, unequal, the upper one (standard) different in shape and size, sometimes with a liguliform appendage. Stamens 10, free, equal or alternately unequal, mostly hairy at the base. Pistil sessile or shortly stalked; ovary pubescent or glabrous, 1-10-ovulate; style slender; stigma terminal, funnel-shaped or bilobed. Pods dehiscent or indehiscent, thin or thick, winged or wingless, sometimes spiny or twisted or furrowed. Seeds globose or flattened, orbicular or elliptic or reniform in outline; albumen usually absent."

Scientific classification (Cronquist 1981)

Kingdom: Plantae- Plants

Subkingdom: Viridiplantae

Division: Magnoliophyta

Subdivision: Magnoliophytina

Class: Rosopsida

Subclass: Rosidae

Superorder: Fabanae

Order: Fabales

Family: Caesalpiniaceae

Subfamily: Caesalpinioideae

Genus: *Caesalpinia* L.

A key to the species

Identification keys for the genus *Caesalpinia* L. based on flowering and fruiting specimens are shown in Table 1 and Table 2.

Table 1 A key to the species (flowering specimens) in Thailand (Smitinand 1984)

Statement	**Goes to**
1 Plants unarmed or nearly so	2, 2’
1’ Plants armed with recurved prickles	3, 3’
2 Leaves paripinnate; leaflets petiolulate (1- 2 mm), 6- 12 pairs, 10- 20 by 6- 10 mm. Pedicles 3- 7 cm. Petals 10- 25 mm	*C. pulcherrima* (L.) Sw.
2’ Leaves imparipinnate; leaflets sessile, 15- 28 pairs, 5- 9 by 1- 2 mm. Pedicles 2- 3 mm. Petals 3- 4 mm. (Cultivated, from South America)	*C. coriaria* (Jacq.) Willd.
3 Leaflets sessile or subsessile (petiolule less than 0.5 mm)	4, 4’
3’ Leaflets petioluate (petiolule more than 0.5 mm)	9
4 Pedicels not jointed near the top	5, 5’
4’ Pedicels jointed near the top	6, 6’
5 Pedicels spreading horizontally, glabrous, 15- 20 mm. Standard bilobed. Ovary glabrous, 2- 4-ovulate. Leaflet 8- 12 pairs	*C. digyna* Rottler
5’ Pedicels not spreading horizontally ± pubescent, 10- 20 mm. Standard emarginated at the top. Ovary pubescent or glabrous, 4- 7-ovulate. Leaflets 12- 40 pairs	*C. tortuosa* Roxb.

6 Pedicels long-hairy and armed with small prickles, 20- 30 mm. Ovary pubescent, 2-ovulate. Leaflets 10- 20 pairs	*C. mimosoides* Lam.
6’ Pedicels without long hairs and prickles	7, 7’
7 Pedicels 5- 6 mm. Leaflets 6- 18 pairs	*C. parviflora* Prain
7’ Pedicels 10- 40 mm	8, 8’
8 Ovary 3- 6-ovulate. Pedicel 15- 20 mm. Leaflets 10 -20 mm Tree or shrub	*C. sappan* L.
8’ Ovary 8- 10-ovulate. Pedicel 20- 40 mm. Leaflets 8 -12 pairs. Climber or shrub	*C. decapetala* (Roth) Alston
9 Standard rounded at the top	10, 10’
9’ Standard bilobed or emarginate at the top	21, 21’
10 Ovary glabrous	11, 11’
10’ Ovary ± pubescent or bristly	16, 16’
11 Ovary 1- 3-ovulate. Leaflets obtuse or slightly acute at the top, 2- 6 by 1- 3 cm	*C. crista* L.
11’ Ovary 4- 10-ovulate	12, 12’
12 Ovary 4- 6-ovulate	13, 13’
12’ Ovary 8- 10-ovulate. Buds pubescent. Standard without a ligule, hairy inside. Pedicels 20- 40 mm	*C. decapetala* (Roth) Alston
13 Buds glabrous	14, 14’
13’ Buds pubescent. Standard with a bilobed or erose,	*C. hymenocarpa*

glabrous ligule. Pedicels 8- 15 mm	(Wight & Arn. ex Prain) Hattink
14 Leaflets alternate, 15- 40 by 10- 20 mm. Standard with a hairy ligule. Pedicels 5- 15 mm	*C. andamanica* (Prain) Hattink
14’ Leaflets opposite	15, 15’
15 Standard with a bilobed, glabrous ligule. Pedicels 10- 20 mm	*C. enneaphylla* Roxb.
15’ Standard without a ligule, hairy inside. Pedicels 20- 25 mm	*C. furfuracea* (Prain) Hattink
16 Ovary bristly. Buds pubescent	17, 17’
16’ Ovary ± pubescent. Buds pubescent or glabrous	19, 19’
17 Stipules pinnate consisting of 3- 5 small leaflets. Leaflets unequal at the base. Bracts subulate, recurved, 6- 12 mm. Ovary 2-ovulate	*C. bonduc* (L.) Roxb.
17’ Stipules subulate or linear or absent	18, 18’
18 Stipules subulate, 1- 3 mm, or absent. Leaflets ± equal-sided at the base. Bracts subulate, 3- 5 mm. Ovary 3- 4-ovulate	*C. major* (Medik.) Dandy & Exell
18’ Stipules limear, 8 mm. Leaflets unequal at the base. Bracts ovate, 15- 20 by 5- 7 mm. Ovary 7- 8-ovulate	*C. minax* Hance
19 Ovary 1- 2-ovulate. Pinnae 2- 4 pairs. Leaflets 2- 4 pairs, 2- 6 by 1- 3 cm, obtuse or slightly acute at the top	*C. crista* L.

19’ Ovary 4- 10-ovulate	20, 20’
20 Ovary 4- 6-ovulate. Standard with a glabrous ligule. Bracts subulate, 10- 12 mm. Leaflets opposite or alternate, 10- 15 by 5- 8 mm	*C. pubescens* (Desf.) Hattink
20’ Ovary 8- 10-ovulate. Standard without ligule, hairy inside. Bracts ovate-lanceolate, 5- 8 mm. Leaflets opposite, 10- 25 by 4- 10 mm	*C. decapetala* (Roth) Alston
21 Standard bilobed, butterfly-shaped. Leaflets opposite, 5- 10 by 3- 6 cm, acute or acuminate at the top. Pedicels 5- 7 mm. Buds glabrous. 1- 2-ovulate	*C. cucullata* Roxb.
21’ Standard emarginated, not butterfly-shaped	22, 22’
22 Pedicels 6- 8 mm. Buds pubescent. Ovary pubescent, 2- 3-ovulate. Leaflets opposite or alternate, 15- 35 by 10- 15 mm, rounded at the top	*C. godefroyana* Kuntze
22’ Pedicels 20- 25 mm. Buds glabrous. Ovary glabrous, 4- 5-ovulate. Leaflets opposite, 18- 24 by 7- 13 mm, retuse	*C. furfuracea* (Prain) Hattink

Table 2 A key to the species (fruiting specimens) in Thailand (Smitinand 1984)

Statement	**Goes to**
1 Pods wingless or with a narrow wing (less than 5 mm)	2, 2’
1’ Pods with a wide wing (more than 5 mm) along the upper margin	14, 14’
2 Pods not fleshy, dehiscent	3, 3’
2’ Pods fleshly, indehiscent	12, 12’
3 Shrub or small tree unarmed or nearly so. Pedicels ± 5 cm ; pod 7- 12 by 1.5- 2 cm. Seeds 8- 10	*C. pulcherrima* (L.) Sw.
3’ Climbers or shrubs armed with recurved prickles	4, 4’
4 Pods not spiny	5, 5’
4’ Pods spiny	10, 10’
5 Pods ± truncate or enlarged at the top. Seeds mostly as long as wide or longer than wide. Leaflets usually many (6- 20 pairs), small (less than 35 mm in length), unequal at the base	6, 6’
5’ Pods ± rhombic or oval in outline, 4- 7 by 2.5- 3.5 cm. Seeds 1 (-2), wider than long (12 by 20 mm). Leaflets 2- 4 pairs, 2- 6 by 1- 3 cm, equal-sided or slightly unequal at the base	*C. crista* L.
6 Pedicels 10- 40 mm	7, 7’
6’ Pedicels 5- 8 mm	9, 9’
7 Pedicels pubescent or tomentose, unarmed	8, 8’
7’ Pedicels long-hairy and armed with small prickles. Seeds 2, ellipsoid, 10 by 7 mm. Pods bladder-like, enlarged at the top, 4- 5 by 2- 2.5 cm. Leaflets opposite, 10- 20 pairs, very unequal	*C. mimosoides* Lam.

at the base, 10 by 4 mm. Pedicels 20- 30 mm	
8 Seeds 2- 4, flattened, elliptic in outline, 18 by 10 mm. Pods enlarged at the top, 7- 9 by 3- 4 cm. Leaflets opposite, 10- 20 pairs, very unequal at the base, 10- 20 by 6- 10 mm. Pedicels 15- 20 mm	*C. sappan* L.
8’ Seeds 4- 8, ellipsoid, 10 by 6 mm. Pods oblong in outline, 6- 10 by 2.5- 3 cm, sometimes slightly longitudinally winged or ridged on the upper side. Leaflets opposite, 8- 10 pairs, unequal at the base, 10- 25 by 4- 10 mm. Pedicels 20- 40 mm	*C. decapetala* (Roth) Alston
9 Pinnae 4- 5 pairs. Leaflets 5- 6 pairs, subopposite or alternate, 15- 35 by 10- 15 mm. Pedicels 6- 8 mm. Pods enlarged at the top, 6- 7 by 2- 2.5 cm. Seeds 1- 3, flattened,	*C. godefroyana*
9’ Pinnae 8- 12 pairs. Leaflets 6- 20 pairs, opposite , 8- 12 by 4- 8 mm. Pedicels 5- 6 mm. Pods enlarged at the top, 4.5 by 2 cm. Seeds 1- 2, orbicular, ± 2 cm	*C. parviflora* Prain
10 Pods 5- 10 mm, stalked above the receptacle	11, 11’
10’ Pods not or very short-stalked above the receptacle. Leaflets unequal at the base, 25- 40 by 10- 18 mm. Stipules linear, 8 mm. Pedicels 20- 25 mm. Seeds 6- 7, ellipsoid, 18 by 10 mm, black	*C. minax* Hance
11 Leaflets unequal at the base, 2- 4 by 1- 2 cm. Stipules pinnate; consisting of 3- 5 small leaflets. Pedicels 4- 6 mm. Seeds 1- 2, globose, 15- 20 mm, grayish	*C. bonduc* (L.) Roxb.
11’ Leaflets equal-sided at the base, 2- 8 by 1- 5 cm. Stipules subulate, 1- 3 mm, or absent. Pedicels 4- 6 mm. Seeds 2- 4, subglobose, 20 by 15 mm, gray-green to brownish	*C. major* (Medik.) Dandy & Exell
12 Plants armed with recurved prickles. Pedicels 10- 25 mm	13, 13’

12’ Plants unarmed or nearly so. Pedicels 2- 3 mm. Leaves imparipinnate. Leaflets 15- 28 pairs, 5- 9 by 1- 2 mm. Pods twisted.	*C. coriaria* (Jacq.) Willd.
13 Pinnae 8- 12 pairs. Leaflets 8- 12 pairs, 8- 12 by 3- 4 mm, ± pubescent. Pedicels 15- 25 mm. Pods thickened along both sutures, 3.5- 5 by 1.5- 2 cm, constricted between the seeds. Seeds 1- 3, subglobose	*C. digyna* Rottler
13’ Pinnae 10- 20 pairs. Leaflets 12- 40 pairs, 6- 20 by 2- 6 mm, glabrous. Pedicels 10- 20 mm. Pods thickened along both sutures, 4- 8 by 2- 3 cm, transversely clefted	*C. tortuosa* Roxb.
14 Seeds close together in the middle of the pod, the space between them not conspicuous, or pods 1-seeded	15, 15’
14’ Seeds separate from each other, the space between them conspicuous	16, 16’
15 Pedicels 12- 15 mm, glabrous, jointed about 1/3 from the top. Wing ca 6 mm wide. Seeds 1 (-2). Leaflets 4- 5 pairs, 5- 10 by 3- 6 cm, acute or acuminate, equal-sided at the base. Petiolule 3- 5 mm	*C. cucullata* Roxb.
15’ Pedicels 20- 30 mm, pubesceent, jointed near the top. Wing 10- 15 mm wide. Seeds 4- 7. Leaflets 6- 8 pairs, 10- 15 by 5- 8 mm, retuse, unequal at the base. Petiolule 0.5- 1 mm	*C. pubescens* (Desf.) Hattink
16 Pedicels jointed near the top or about ¼ from the top	17, 17’
16’ Pedicels not conspicuously jointed, 20- 35 mm. Dorsal and ventral edges of the receptacle recurved, Wing 12- 20 mm wide. Leaflets opposite, 18- 25 by 7- 13 mm	*C. furfuracea* (Prain) Hattink
17 Pedicels glabrous or subglabrous. Receptacle glabrous or subglabrous, rarely shed. Pods not stalked	18, 18’

17’ Pedicels pubescent, 10- 25 mm. Receptacle pubescent, usually shed. Pods stalked, dull or shining. Wing 6- 12 mm wide. Leaflets 6- 8 pairs, opposite or subopposite, 12- 25 by 6- 15 mm	*C. hymenocarpa* (Wight & Arn. ex Prain) Hattink
18 Leaflets alternate, 6- 10 per pinna, 15- 40 by 10- 20 mm. Dorsal and ventral edges of the receptacle recurved. Pedicels 10- 25 mm	*C. andamanica* (Prain) Hattink
18’ Leaflets opposite	19, 19’
19 Pods shining. Dorsal and ventral edges of the receptacle not recurved. Wing 5- 7 mm wide. Pedicels 12- 20 mm, Leaflets 7- 12 pairs, 15- 25 by 5- 8 mm	*C. enneaphylla* Roxb.
19’ Pods dull. Dorsal and ventral edges of the receptacle recurved. Wing 12- 20 mm wide. Pedicels 20- 35 mm, obscurely jointed. Leaflets 7- 12 pairs, 18- 25 by 7- 13 mm	*C. furfuracea* (Prain) Hattink

There are other keys from many countries according to the genus *Caesalpinia* L. such as a key to the species in Bangladesh (Khatun 2006). In Bangladesh, the genus *Caesalpinia* L. has been represented 12 species. Key based on flowering and fruiting specimens in China has been studied by Dezhao and team, 2010 (Dezhao 2010). In China, from about 100 species: pantropical distribution; 20 species (six endemic, two introduced) of *Caesalpinia* were presented. In 1988, Orchard and team reported a key to the species in Australia. From a tropical genus of *Caesalpinia* around 100 species, most numerous in the New World, there are 12 species in Australia, of which three are endemic and two naturalized (Orchard 1998).

Name list of eighteen *Caesalpinia* species in Thailand

In Thailand, from various parts, there are many local names used and in other countries also presented different names of each *Caesalpinia* species as shown in Table 3.

Table 3 Name list of eighteen *Caesalpinia* species in Thailand (Smitinand 2012)

No.	Scientific Name	Common Name	Thai Name
1	*Caesalpinia andamanica* (Prain) Hattink		**ง้ายแดง ngai daeng** (Peninsular) ง้ายใหญ่ ngai yai (Peninsular) สวาด sawat (Peninsular)
2	*Caesalpinia bonduc* (L.) Roxb.	Grey nickers Nicker bean	ดามั้ด da-mat (Malay-Satun) บ่าขี้แฮด ba khi hat (Northern) มะกาเล็ง ma-ka-leng (Shan-Chiang Mai) **สวาด sawat** (Central) หวาด wat (Peninsular)
3	*Caesalpinia coriaria* (Jacq.) Willd.	Divi-divi	**ตันหยง tan yong** (Bangkok)

4	*Caesalpinia crista* L.	Nicker nut	ฆอระแอ ko-ra-ae (Malay-Narathiwat) **เทพี the phi** (Chumphon)
5	*Caesalpinia cucullata* Roxb.		กำจาย kam chai (Northern) ขี้แรด khi raet (Southwestern) วัลย์ทะลึงใหญ่ wan tha lueng yai (Nakhon Ratchasima) **หนามขี้แรด nam khi raet** (Ratchaburi) หนามค่า nam kha (Loei) หนามโค้ง nam khong (Central) หนามจั่น nam chan (Northern) หนามจาย nam chai (Central)
6	*Caesalpinia*	Cat's claw	กระจาย krachai (Central)

	decapetala (Roth) Alston	Mysore thorn Wait-a-bit	กำจาย kam chai (Central) หนามโค้ง nam khong (Central) **หนามจาย nam chai** (Central)
7	*Caesalpinia digyna* Rottler		กระจาย krachai (Phrae) **กำจาย kam chai** (Phrae) ขี้คาก khi khak (Phrae) ขี้แรด khi raet (Central) งาย ngai (Pattani, Nakhon Si Thammarat) จิงจ่าย ching chai (Nakhon Si Thammarat) ตาลู่แม ta-lu-mae (Karen-Mae Hong Son) บ่าเบน ba ben (Northern) มะเบ่น ma-ben (Shan-Northern) มะหนามจาย ma nam chai (Tak)

		สื่อกีพอ sue-ki-pho (Karen-Mae Hong Son) หนามจาย nam chai (Northern) หนามแดง nam daeng (Trat) หนามหัน nam han (Chanthaburi) ฮาย hai (Songkhla) ฮายปูน hai pun (Nakhon Si Thammarat)
8	*Caesalpinia enneaphylla* Roxb.	กำจาย kam chai (Ratchaburi) **ขี้แรดใหญ่ khi raet yai** (Kanchanaburi) หนามจาย nam chai (Ratchaburi)
9	*Caesalpinia furfuracea* (Prain) Hattink	งวม nguam (Uttaradit) นวม nuam (Lampang) พาย่วม pha yuam

		(Northern) **หนามโค้ง nam khong** (Northern)
10	*Caesalpinia godefroyana* Kuntze	เถาละนามหนาม thao la nam nam (Sing Buri) ฝางกา fang ka (Eastern) ฝางปีกไก่ fang pik kai (Nakhon Ratchasima) พังก๋า phangka (Buri Ram) หนามหัน nam han (Chanthaburi) **หนามหืนหนามหัน nam huen nam han** (Northern, Ratchaburi)
11	*Caesalpinia hymenocarpa* (Wight & Arn. ex Prain) Hattink	**หนามโก๊ง nam kong** (Northern) หนามงาย nam ngai (Southeastern) หนามจั่น nam chan (Northern)

		หนามยั้ง nam yang (Southeastern)
12	*Caesalpinia major* (Medik.) Dandy & Exell	**ว้าด wat** (Peninsular) เวียด wiat (Peninsular)
13	*Caesalpinia mimosoides* Lam.	**ช้าเรือด cha rueat** (General) ทะเน้าซอง thanao song (Northern) ผักกาดหญ้า phak kat ya (Prachin Buri) ผักขะยา phak khaya (Nakhon Phanom) ผักคายา phak khaya (Loei) ผักปู่ย่า phak puya (Northern) หนามปู่ย่า nam puya (Northern)
14	*Caesalpinia minax* Hance	ขี้แฮด khi haet (Chiang Mai, Phrae) **คำผีแปง kham phi paeng**

			(Chiang Mai) มะดำ ma dam (Northern)
15	*Caesalpinia parviflora* Prain		**วัลย์ทะลึง wan tha lueng** (Nakhon Ratchasima) วัวทะลึง wua tha lueng (Nakhon Ratchasima)
16	*Caesalpinia pubescens* (Desf.) Hattink		**กระแดะ kradae** (Surat Thani) กาแด๊ะ ka dae (Surat Thani)
17	*Caesalpinia pulcherrima* (L.) Sw.	Flower fence Peacock flower Peacock's crest Pride of Barbados	ขวางยอย khwang yoi (Nakhon Ratchasima) จำพอ cham pho (Northern) ซมพอ som pho (Northern) ซำพอ sam pho (Northern) นกยูงไทย nok yung thai (Central) พญาไม้ผุ phaya mai phu

			(Northern) ส้มผ่อ som pho (Northern) ส้มพอ som pho (Northern) หนวดแมว nuat maeo (Mae Hong Son) **หางนกยูงไทย hang nok yung thai** (Central)
18	*Caesalpinia sappan* L.	Sappan tree	ง้าย ngai (Karen-Kanchanaburi) **ฝาง fang** (General) ฝางส้ม fang som (Kanchanaburi)

Plant description of eight species of *Caesalpinia* (Smitinand 1984), (Hou 1996)

Eight species of *Caesalpinia* including *C. bonduc*, *C. coriaria*, *C. decapetala*, *C. digyna*, *C. mimosoides*, *C. minax*, *C. pulcherrima*, and *C. sappan* were selected in this study. Distribution and ecology were shown in Table 4. The chemical constituents were shown in Table 5 and the pharmacological activities were presented in Table 6, respectively.

Plant description

Caesalpinia bonduc (L.) Roxb.

"*Caesalpinia bonduc* is a climber armed with straight or recurved prickles. Stipules are pinnate consisting of 3-5 leaflets up to 2 cm. Leaves: rhachis 30-50 cm; pinnae 3-9 pairs; leaflets 8-12 pairs, opposite or subopposite, petiolulate (0.8 mm), ovate-lanceolate, 2-4 by 1-2 cm, acute or rounded and mucronalate at the tip, unequal at the base. Racemes are supra-axillary, sometimes branched, some with male flowers, others with female flowers (anthers without pollen). Bracts linear, 6-12 mm, recurved, lately caduceus. Pedicles 4-6 mm, pubescent, faintly jointed near the top. Sepals are pubescent, subequal. Petals are yellow, the standard constricted and hairy inside towards the middle. Filaments are hairy. Ovary ca 1 mm, stalked, hairy and bristly, 2-ovulate, in male flowers rudimentary. Pods stalked above the receptacle (0.5-1 cm), elliptic in outline, 5-8 by 4 cm, set with hairy 7-9 mm long bristles. Seeds 2, subglobular, 15-20 mm, greyish."

Caesalpinia coriaria (Jacq.) Willd.

"*Caesalpinia coriaria* is a small tree or shrub, unarmed. Leaves often impairipinnate, with 4-8 pairs of pinnae, often with additional terminal one; leaflets 15-28 pairs per pinna, subsessile, oblong, 4-9 by 1-3.5 mm, rounded to truncate at the apex, obliquely subcordate at the base, glabrous. Stipules minute, subulate. Hypanthium 1-2 mm deep. Sepals 3-4 mm long. Flowers fragrant, in terminal and axillary, short, amost sessile condensed racemes, 2-6 cm long; pedicels 2-4 mm. Petals are yellow or cream, 3-6 mm long. Pods are oblong to ovate, 6 by 1.5-3 cm, inflated, often becoming twisted or contorted. Seeds 1-10, valves black, thick, becoming fibrous-pulpy."

Caesalpinia decapetala (Roth) Alston

"This plant is a climber or shrub up to 10m, armed with recurved or straight prickles. Stipules are obliquely ovate, 4-20 by 2-8 mm, lately caduceus. Leaves: rhachis 12-40 cm; pinnae 4-10 pairs leaftets 8-12 pairs, opposite, subsessile or petiolalate up to 1 mm, elliptic-oblong, 10-15 by 4-10 mm, rounded at the tip, unequal at the base. Racemes terminal and axillary. Bracts ovate-lanceolate, 5-8 mm, caduceus. Pedicels 20 (-40) mm, pubescent, jointed near the top. Sepals are glabrous or pubescent, the lowest one hood-shaped. Petals are yellow, the standard smaller, rounded at the top, consitricted towards the middle into a claw, hairy inside and along the margins. Filaments hairy. Ovary is glabrous or pubescent, 8-10-ovulate.

Pods are subsessile on the receptacle, elliptics-oblong in outline, 6-10 by 2.5-3 cm, prolonged in a beak up to 2 cm, ± keeled or winged on the upper side (2-3 mm). Seeds present 4-8, ellipsoid, 10 by 6 mm."

Caesalpinia digyna Rottler

"*Caesalpinia digyna* is a climber or scandent shrub, armed with recurved prickles. Stipules are linear, awl-shaped, ca 2 mm, caduceus. Leaves: rhachis 15-20 cm; pinnae 8-12 pairs; leaflets 8-12 pairs, opposite, subsessile, oblong, 8-12 by 3-4 mm, rounded, slightly emarginate and mucronalate at the tip, unequal at the base. Racemes are axillary and combined into terminal panicles. Bracts awl-shaped, ca 5 mm, caduceus. Pedicels are slender, spreading, 15-25 mm, glabrous, not jointed above the base. Sepals are glabrous, dotted, the lowest one hood-shaped. Petals are yellow, the standard constricted towards the middle, hairy inside and along the margins of the claw. Filaments are densely hairy. Ovary is glabrous or somewhat hairy on the sutures, 2-4-ovulate. Pods are indehiscent, subsessile on the persistent receptacle, elliptic-oblong in outline, 35-50 by 15-20 mm, constricted between the seeds, thickened on both sutures. Seeds (-1) 2-3 (-4), subglobose, 12 by 9 mm, dark brown."

Caesalpinia mimosoides Lam.

"*Caesalpinia mimosoides* is an erect or climbing shrub, densely hispid and bristy on all parts. Stipules are awl-shaped, 7-15 mm, caduceus. Leaves: rhachis 25-40 cm; pinnae 10-30 pairs; leaflets 10-20 pairs, opposite, subsessile, oblong, ca 10 by 4 mm, rounded-mucronulate at the tip, unequal at the base. Racemes present at the terminal. Bracts awl-shaped, 4 mm, villous. Pedicels 2-3 cm, hispid and bristly, jointed near the top. Sepals villous, dotted outside, the lowest one more concave. Petals are yellow, the standard obovate, the others suborbicular. Filaments densely hairy. Ovary is pilose, 2-ovulate. Pods are subsessile on the receptable, bladder-like, narrowed at the base, rounded-truncate and prolonged into a beak at the top. Seeds 2, ellipsoid, ca 10 by 7 mm."

Caesalpinia minax Hance

"This plant is a climber armed with straight and recurved prickles. Stipules are linear with 8 mm. Leaves: rhachis 30-40 cm; pinnae 5-8 pairs; leaflets 6-12 pairs, opposite, shortly petiolulate (± 1 mm), elliptic-oblong, 25-40 by 10-18 mm, acute, rarely rounded, mucronate at the tip, unequal at the base. Racemes simply or branched, terminal. Bracts are ovate-lanceolate, acute, 15-20 by 5-7 mm, caduceus. Pedicels are 20-25 mm, pubescent, jointed near the top. Sepals pubescent, the lowest one hood-shaped. Petals are white-yellowish, the standard reddish, smaller, constricted towards the middle, glabrous inside. Filaments are hairy. Ovary is hairy

along the sutures, bristly, 7-8 ovulate. Pods are subsessile on the receptacle, elliptic-oblong in outline, 10-15 by 4-4.5 cm, set with 12 mm-long, pubescent or glabrous bristles. Seeds 6-7, ellipsoid, 18 by 10 mm, black."

Caesalpinia pulcherrima L.

"This plant is shrub or small tree, unarmed or nearly so, glabrous. Stipules has 2 mm, caduceus. Leaves present 10-40 cm of rhachis; pinnae 3-10 pairs; leaflets 6-12 pairs, opposite, petiolulate (1-2 mm), elliptic-oblong, 10-20 by 6-10 mm, rounded-emarginate at the tip, unequal at the base. Racemes axillary and terminal. Bracts linear, 3-7 mm, caduceus. Pedicels 3-7 mm. Sepals unequal, the lowest hooded, larger. Petals are red or yellow, up to 25 mm long, the standard smaller. Stamens exserted; filaments red, 5-6 cm, hairy in the basal part. Ovary 10-20-ovulate; style 5-6 cm. Pods short stalked above the receptacle (2-5 mm), flattened, 7-12 by 1.5-2 cm. Seeds 8-10, 9 by 7 mm."

Caesalpinia sappan L.

"This plant is a small tree or shrub up to 10 m, armed with recurved prickles. Stipules 3-4 mm, caduceus. Leaves: rhachis 15-40 cm; pinnae 8-16 pairs, leaflets 6-12 pairs, leaflets 6-12 pairs, opposite, subsessile, oblong, 10-20 by 6-10 mm, rounded-emarginate at the tip, very unequal at the base. Racemes supra-axillary and combined into terminal panicles. Bracts lanceolate, acuminate 6 mm, caduceus. Pedicles 15-20 mm, pubescent, jointed near the top. Sepals glabrous, the lowest one

more concave and larger. Petals are yellow, obovate, the standard smaller, constricted into a claw and hairy inside towards the middle. Filaments hairy. Ovary pubescent, 3-6-ovulate. Pods sessile on the receptacle, flattened, 7-12 by 1.5-2 cm, widest and truncate-acuminate towards the top, rounded at the base. Seeds had 2-4, elliptic in outline, 18 by 10 mm."

The distribution of eight *Caesalpinia* species in Thailand and other countries and also their ecology were shown in Table 4.

Table 4 Distribution and ecology of eight *Caesalpinia* species

Species	Thailand	Other country	Ecology
Caesalpinia bonduc (L.) Roxb.	Chiang Mai, Nakhon Phanom, Bangkok, Prachin Buri, Rayong, Chanthaburi, Surat, Thani, Trang, Pattani	Ceylon, India, Nepal, Burma, Laos, Cambodia, Vietnam, China, Hongkong, Taiwan, Malay peninsula and archipelago	Along sea beaches as well as in inland scrub jungle and hedges
Caesalpinia coriaria (Jacq.) Willd.	Chiang Mai, Bangkok, Pattani	South and Central America, South East Asia, Indonesia,	In dry or warm temperature climates to wet

		Philippine	tropical climates
Caesalpinia decapetala (Roth) Alston	Prachin Buri, Phuket, Chiang Mai, Phrae	Ceylon, India, Nepal, Bhutan, Burma, Laos, Vietnam, China, Japan, Malay peninsula and archipelago	Only two specimens recorded from Thailand without ecological information
Caesalpinia digyna Rottler	Chiang Mai, Mae Hong Son, Phrae, Loei, Khonkhaen, Nakhon Ratchasima, Bangkok, Prachinburi, Chonburi, Chanthaburi, Kanchanaburi, Ratchaburi, Phetchaburi,	Ceylon, India, Nepal, Burma, Laos, Cambodia, Vietnam, China, Malay peninsula and archipelago	In clearings, thickets, forest fringes, open and dry places up to 700 m

	Prachuap Khiri Khan, Surat Thani, Nakhon Si Thammarat, Pattani		
Caesalpinia mimosoides Lam.	Chiang Rai, Phrae, Lampang, Nan, Phitsanulok, Loei, Nakhon Phanom, Nakhon Ratchasima, Kanchanaburi, Chumphon, Prachinburi	India, Burma, Laos, Vietnam, China	In thickets, clearing and hedges.
Caesalpinia pulcherrima L.	Chiang Mai, Nakhon Ratchasima, Bangkok, Mae Hong Son, Ratchaburi, Loei	South America, South East Asia	Slopes, close to water, roadsides; in sunny locations

Caesalpinia sappan L.	Chiang Mai, Bangkok, Khanchanaburi, Ratchaburi, Phetchaburi, Chumphon	Ceylon, India, Burma, Indochina, Laos, Vietnam, China, Malay peninsula and archipelago	In scrub jungle, limestone hills and in cultivation around the villages

Chemical constituents

The chemical constituents from various plant parts of eight *Caesalpinia* species were listed in Table 5.

Table 5 List of chemical constituents from eight *Caesalpinia* species

Species	Plant part	Chemical constituent	Reference
Caesalpinia bonduc (L.) Roxb.	Whole plant	- Steroidal saponin - Fatty acids - Phytosterol - Isoflavone - Amino acid - Phenolic - Caesalpinolide-C-E	(Yadav 2009), (Nazeerullah 2012)

	- Cassane furanoditerpene	
Bark	- Homoisoflavonoids	(Ata 2009)
	- Caesalpinianone	
	- 6-*O*-methylcaesalpinianone	
	- Hematoxylol	
	- Stereochenol A	
	- 6'-*O*-acetyllogenic acid	
	- 4'-*O*-acetyllogenic acid	
	- 2-*O*-β-**D**-glucosyloxy-4-methoxybenzenepropanic acid	
Kernel	- 2-Acetoxycaesaldekarin E	(Pudhom 2007)
	- Bonducellpin B	
	- Bonducellpin C	
	- Bonducellpin E	
	- Bonducellpin F	
	- Bonducellpin G	
	- Caesalmin B	
	- Caesalmin D	
	- Caesalmin E	

		- α-Caesalpin	
		- ε-Caesalpin	
		- Caesalpin C	
		- 14(17)-Dehydrocaesalpin F	
		- Caesalpinin I	
		- Caesalpinin K	
	Seed	- Neocaesalpin W	(Wu 2007),
		- β-Amyrin	(Kinoshita 2000)
		- Neocaesalpin C	
		- Neocaesalpin D	
Caesalpinia coriaria (Jacq.) Willd.	Seed	- Linoleic acid	(Kumar 2014)
		- Malvalic acid	
		- Myristic acid	
		- Oleic acid	
		- Palmitic acid	
		- Sterculic acid	
		- Amino acid	
		- Aucubin	
		- Brasilein	
		- Brasilin	

		- Hematoxylin	
		- Tannin	
	Pod	- Carbohydrate	(Anandhi 2013)
		- Tannin	
		- Saponin	
		- Flavonoid	
		- Quinone	
		- Terpenoids	
		- Phenol	
		- Coumarin	
		- Steriods and phytosteroid	
		- Anthraquinone	
Caesalpinia	Bark	- Homoisoflavones	
decapetala		- Chalcones	
(Roth) Alston	Root	- Caesaljapin	(Ogawa 1992)
		- Andrographolide	(Wei 2013)
		- Quercetin	
		- β-sitosterol	
		- Bergenin	
		- Rutin	

		- Emodin - Betulin - Stigmaserol - Baicalein - Polydatin - Salicin - Apigenin - Epicatechin - Cinnamic acid	
	Stem	- Octacosyl 3, 5-dihydroxycinnamate - 2', 4, 4'-trihydroxychalocone - Bonducellin - 7, 3', 5'-trihydroxyflavanone - Daucosterin - β-sitosterol	(Zhang 2008)
Caesalpinia digyna Rottler	Root	- Alkaloids - Glycosides - Tannins - Terpenoids	(Mitra 2006)

		- Saponins - Fatty acids	
	Pod-case	- Tannin - Bonducin - Saponin	(Chopra 1992)
Caesalpinia mimosoides Lam.	Not specified	- Quercetin - Gallic acid	(Tangsaengvit 2013), (Chanwitheesuk 2007)
	Twig	- 10-methoxyprotosappanin B - 10-methoxyisoprotosappanin B - Eucomin - Intricatinol - 8-methoxybonducellin - Quercetin - Quercetin-3O-glucoside - Sulfuretin - Isoliquiritigenin - Butein	(He 2017)

		- Bergenin	
		- Bergenin 11-O-(E)ferulate	
		- Sitoindoside I	
		- Friedelin	
		- Coniferaldehyde	
		- Ethyl gallate	
		- Gallic acid	
Caesalpinia	Seed	- Diterpenes	(Ma 2013),
minax Hance		- Caesalpinolide F	(Zheng 2013),
		- Caesalpinolide G	(Wu 2014),
		- Cassane diterpenoids	(Liu 2013)
		- Caesalminaxins A–L	
		- Caesalpins A-H	
		- Spirocaesalmin B	
		- Caesalpinin M1	
		- Caesalpinin M2	
		- Caesalmin E1	
		- Caesalmin E2	
		- Caesalmin E3	
		- Caesalpinin F1	

		- Neocaesalpin A - Neocaesalpin L - Neocaesalpin L1 - 1α,7βdiacetoxy-5α-hydroxy-12α-methoxycass-13(15)-en-16,12olide-17β-carboxylate	
Caesalpinia pulcherrima L.	Root	- Pulcherrimins A - Pulcherrimins B - Cassane-type furanoditerpenes	
	Seed	- Lectins - Galactomannan - Protease inhibitor	
	Leaf	- Isovouacapenol E - Caesaldekarin A - Spathulenol - Caryophyllene oxide - Phytol - Sitosterol	(Ragasa 2003), (Perumalla 2012)

	- Phenolic acids - Flavonoids - Tannins	
Stem	- Pulcherrimin - 6methoxypulcherrimin - Homoisoflavonoids - Bonducellin	(McPherson 1983)
Aerial part	- (3*E*)-3-(1,3-Benzodioxol-5-ylmethylene)-2,3-dihydro-7-hydroxy-4*H*-1-benzopyran-4-one - (3*E*)-3-(1,3-Benzodioxol-5-ylmethylene)-2,3-dihydro-7-methoxy-4*H*-1-benzopyran-4-one - (3*E*)-2,3-Dihydro-3-[(3,4-dimethoxyphenyl)methylene]-7-methoxy-4*H*-1-benzopyran-4-one - (3*E*)-2,3-Dihydro-	(Das 2009)

		6,7dimethoxy-3[(3-hydroxy-4- methoxyphenyl)methylene]- 4*H*-1-benzopyran-4-one - (3*E*)-2,3-Dihydro-7-hydroxy-3- [(3-hydroxy-4- methoxyphenyl)methylene]- 4*H*-1-benzopyran-4-one - Bonducellin - Sappanone A - 7-*O*-Methyl bonducellin - 2-Methoxybonducellin	
Caesalpinia *sappan* L.	Heartwood	- Epicaesalpin J - 7,10,11- trihydroxydracaenone - 3'-Deoxy-4-O- methylepisappanol - Protosappanin A - Sappanchalcone - Sappanone B - Palmitic acid	(Zhao 2014), (Fu 2008), (Shu 2008), (Washiyama 2009)

		- Protosappanin A - Protosappanin B - Protosappanin C - Protosappanin D - Protosappanin E - Osoprotosappanin B, 3'-O-methylbrazilin - Brazilin - 7, 3',4'-Trihydroxy-3-benzyl-2*H*-chromene - 4-*O*-Methylsappanol - Caesappanin A - Caesappanin B - 3'-*O*-Methylbrazilin - Brazilein - 3-Deoxysappanchalcone - 3-Deoxysappanone B - Isoprotosappanin	
	Seed	- Phanginin A - Phanginin B	(Yodsaoue 2008), (Xu 2013)

- Phanginin C
- Phanginin D
- Phanginin E
- Phanginin F
- Phanginin G
- Phanginin H
- Phanginin I
- Phanginin J
- Phanginin K
- Phangininoxy A

Pharmacological activity

Caesalpinia L. genus has many reports related to their pharmacological activities and the pharmacological activities of eight *Caesalpinia* species were shown in Table 6.

Table 6 Pharmacological activities of eight *Caesalpinia* species

Species	Pharmacological activity	Reference
Caesalpinia bonduc (L.) Roxb.	Antidiabetic	(Parameshwar 2002), (Kannur 2006), (Patil 2011)

	Antihelmintic	(Wadkar 2010), (Jabbar 2007)
	Anti-estrogenic	(Kanchan 2011)
	Anti-inflammatory	(Shukla 2010)
	Antimalarial	(Linn 2005), (Kalauni 2006) (Pudhom 2007)
	Antimicrobial	(Simin 2001), (Arif 2009), (Tusharkanti 2011), (Sagar 2010)
	Antibacterial	(Khan 2011), (Saeed 2001)
	Antioxidant	(Shukla 2009), (Mandal 2009), (Kumar 2005)
	Anticonvulsant	(Ali 2009)
	Analgesic	(Archana 2005), (Devi 2008)
	Antitumor	(Gupta 2004)
Caesalpinia coriaria (Jacq.) Willd.	Antioxidant	(Anandhi 2014)
	Antibacterial	(Mohana 2006, Mohana 2008), (Jeeva 2014) (Anandhi 2014)
Caesalpinia	Antioxidant	(Wei 2013), (Bhadoriya 2012)

decapetala (Roth) Alston		
Caesalpinia digyna Rottler	Antidiabetic	(Kumar 2012), (Kumar 2012)
	Spermatotoxic Effect	(Chandiran 2008)
	Antioxidant	(Srinivasan 2007)
Caesalpinia mimosoides Lam.	Anti-inflammatory	(Yodsaoue 2010), (Lakshmi 2015)
	Antimicrobial	(Chanwitheesuk 2007), (Manasa 2014), (Supriya 2013)
	Antioxidant	(Chanwitheesuk 2005), (Tangsaengvit 2013), (Ranjith 2014)
	Neuroprotective Activity	(Tangsaengvit 2013)
Caesalpinia minax Hance	Antiviral	(Jiang 2001)
	Anti-inflammatory	(Dong 2015)
Caesalpinia pulcherrima L.	Anti-inflammatory	(Patel 2010), (Bose 2011), (Rao 2005), (Kumbhare 2011)
	Analgesic	(Patel 2010)

	Antibacterial	(Prakash 2009), (Pulipati 2012)
	Antifertility	(Kumar 2013), (Deshmukh 2014)
	Antimicrobial	(Vivek 2013)
	Antioxidant	(Vivek 2013), (Pawar 2009)
	Anti-ulcer	(Ali 2013)
	Antiviral	(Chiang 2003)
	Immunomodulatory Effects	(Madagundi 2012)
	Neuroprotective Activity	(Bose 2011)
Caesalpinia sappan L.	Anti-acne	(Batubara 2010)
	Anti-allergic	(Yodsaoue 2009)
	Anti-arthritic	(Wang 2011)
	Antibacterial	(Xu 2004)
	Anticonvulsant	(Baek 2000)
	Anti-inflammatory	(Washiyama 2009), (Cuong 2012), (Wu 2011)
	Antimicrobial	(Mohan 2011), (Saravanakumar 2013), (Srinivasan 2012),

		(Kim 2004)
	Antioxidant	(Sarumathy 2011), (Badami 2003), (Wetwitayaklunga 2005), (Saenjum 2010)
	Anticancer	(Hung 2014)

***Caesalpinia sappan* heartwood**

The wood is orange red, hard, very heavy, straight grained with a fine and even texture (Figure 1).

Figure 1 *Caesalpinia sappan* heartwood

Traditional uses

Caesalpinia sappan L. heartwood is widely used in traditional Thai medicine and spread in every parts of Thailand. Sappanwood is used for promoting blood circulation, declining hypertension, antithirsty, etc. It can be mixed with other herbals for cure other diseases.

Disturbances of menstrual functions have been treated by *Caesalpinia sappan* L. in China and Malaysia. The heartwood is reputed to have blood vitalizing activity and used in the treatment of toxic side effects resulting from radiation and

chemotherapy in traditional Chinese medicine. In Philippines, it used as anti-anaemic agent and in Malaysia, it is used for antimalarial purpose (Subehan 2013).

In ancient traditional Chinese medicine, the powdered wood of *Caesalpinia sappan* L. has been used for anti- inflammatory agent and as an analgesics.

In South Korea, the traditional oriental medicine uses *Caesalpinia sappan* L. as a haemostatic, anti-inflammatory, and analgesic agents. Thrombosis and confusion are treated by this plant also (Subehan 2013).

In Indonesia, *Caesalpinia sappan* L. is used for dysentery treatment, hemoptysis, and ophthalmic disease and as a depurative; the Buginese tribe add it to daily drinking water to prevent and treat osteoporosis (Mohan 2011).

In traditional Ayurvedic medicine, the heartwood is used for treatment of burning sensation, wounds, ulcer, leprosy, skin disease, diarrhea, dysentery, diabetes, and etc. In Kerala, India, the heartwood's decoction is usually used for antithirsty, blood purifying, and antidiabetics (Xie 2000).

The heartwood of *Caesalpinia sappan* L. has been used in Oriental folk medicines for promoting blood circulation, as an emmenagogue, analgesics or anti-inflammatory agent. Many compounds have been purified and brazilin and hematoxylin are two of the most abundant (Nadkarni 1994).

Pharmacological activities

One of the major constituents of *Caesalpinia sappan* called brazilein had some potential immunosuppressive abilities that inhibited lymphocyte proliferation and had the property of inducing apoptosis of mice spleen lymphocytes. The ethanol extract of *C. sappan* could suppress mice humoral immune response by plaque forming cell test and immune organs (spleen and thymus). It also inhibited the proliferation of B lymphocyte stimulated by lipopolysaccharides and proliferation of T lymphocyte stimulated by Concanavalin A (Xu 2004).

The result of the phytochemical screening of *Caesalpinia sappan* L. (bark) presented alkaloids, flavonoids, tannins, steroids, terpenoids, carbohydrates, and proteins. Tannin was able to break down the bacterial colonies, it could inhibit the microbial growth (Xie 2000).

Xu and Lee, 2004 examined the antibacterial activity and toxicity with the 14 clinical bacterial species using a bioassay-directed purification scheme. This study reported that brazilin which was an important compound from *C. sappan* heartwood had no cytotoxicity to mammalian cells. It was highly active against a variety of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and multi-drug resistant *Burkholderia cepacia*, *Streptococcus mutans*, *Prevotella intermedia* whereas it had less an inhibitory effect

on the Gram-negative bacteria such as *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* (Xu 2004).

Wang *et al*., 2011 studied about the anti-arthritis effect and explored the potential mechanism of dried heartwood of *C. sappan* on collagen-induced arthritis in male Wistar rats. The rats in the onset of arthritis were treated daily with the ethanol extract from *C. sappan* heartwood at different doses. Paw swelling, arthritis index, radiographic and histopathogenic changes were examined. The results indicated that the ethanol extract from sappanwood significantly attenuated collagen-induced arthritis in rats by decreasing the levels of interleukin-1 beta, interleukin, tumor necrosis factor alpha and prostaglandin E2 in serum and the expression of cyclooxygenase-2 and transcription factor NF-**K**B in paw cartilage (Wang 2011).

Beak *et al*., 2000 examined the compound which isolated from the wood of *C. sappan* for sedative or antispasmodic effects and its inhibitory effect on GABA degrative enzymes isolated from bovine brain. The ethyl acetate fraction significantly inhibited the activities of two GABA degrative enzymes, SSADH and SSAR. The crude extracts of *C. sappan* presented the inhibitory effect on brain SSADH and SSAR by 90% and 70%. Sappanchalcone and brazilin from *C. sappan* showed higher inhibition effect than valproate and might be an effective anti-convulsant and anti-epileptic therapeutic drug (Baek 2000).

Seven compounds (brazilin, sappanchalcone, protosappanin A, protosappanin B, protosappanin C, protosappanin D, and protosappanin E) from the methanolic extract of Sappan Lignum was in *vitro* tested on the inhibition of the chemical mediators of inflammation using J774.1 cell line. The compounds were examined for the inhibitory effect on nitric oxide and prostaglandin E2 production. They were also evaluated for the suppressive effect on tumor necrosis factor-**α**, interleucin-6, cyclooxygenase-2, and inducible nitric oxide synthease mRNA expression. The results showed that brazilin inhibited nitric oxide production, and almost no inhibition in prostaglandin E2 whereas sappanchalcone, protosappanin D, and protosappanin E inhibited both nitric oxide and prostaglandin E2 production and suppressed tumor necrosis factor-**α**, interleukin-6, cyclooxygenase-2, and inducible nitric oxide synthase mRNA expression (Washiyama 2009).

Brazilin

Brazilin is also known as: Brasilin; Braziletto; Superbresiline; Hypernic extract; Limawood extract; Pernambuco extract; BRASILINE; CHEBI:3170. Molecular formula is $C_{16}H_{14}O_5$ and the molecular weight is 286.27936. Their chemotaxonomic group is flavonoids. The CAS number is 474-07-7.

Brazilin is a natural pigment commonly used as a dye and found in *Caesalpinia sappan* L., where is particularly abundant (8-22% w/w). The compound

brazilin is homoisoflavonoids, collectively isolated from alcoholic extraction of heartwood of *Caesalpinia sappan* L. (Gilbody 1902).

There is long history about the substance called “Brazilin” that is the major compound of *Caesalpinia sappan* wood. Since the year 1190, brazilin from dye wood was mentioned by Kimichi (Spanish writer). In 1500, this plant was discovered in South America. In the East Indies, central and South America, the Antilles and Africa, the brazil- wood tree (*Leguminosae*, species *Caesalpinia*) is widely classified in the tropics. The wood is very hard and deep red colour. It rapidly changes to red when exposed the air (Rondao 2013).

In 1808, Brazilin was isolated in the first time by Chevreul and in 1864, Bolly suggested the formula of Brazilin that is $C_{22}H_{20}O_7$. Ten years later, Lybermann and Burg reported the correct $C_{16}H_{14}O_5$ formula (Oliveira 2002).

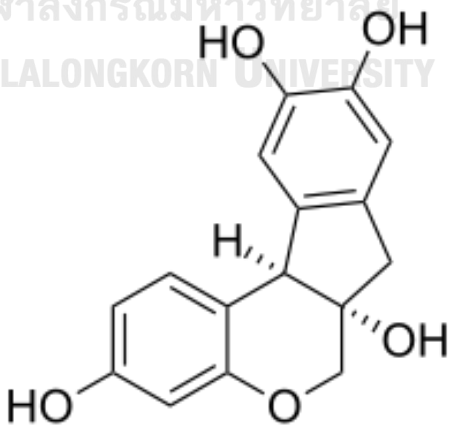


Figure 2 brazilin structure consist of tetracyclic with 2 aromatic rings, 1 pyrone, 1-5 membered ring.

Flavonoids are polyphenol that contain 15 carbon atoms. Flavonoids showed two benzene rings which are linked with a short three carbon chain. One of the carbons always connects to a carbon of the benzene rings, either directly or through an oxygen bridge, and forming a third middle ring, which can be five or six-membered. Flavonoids consist of 6 major subgroups are chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids (Weston 2013), (Laszlo 2015).

Isoflavonoids are subclass of flavonoids and have been isolated from a wide variety of leguminous and non-leguminous plants. Isoflavones are characterized by having the B-ring attached at C3 rather than C2 position (Figure 3). They are resulting from the phenylpropanoid pathway and are synthesized predominantly in leguminous plants. Isoflavonoids form a distinct class among flavonoids and have a characteristic structure with very limited distribution in nature.

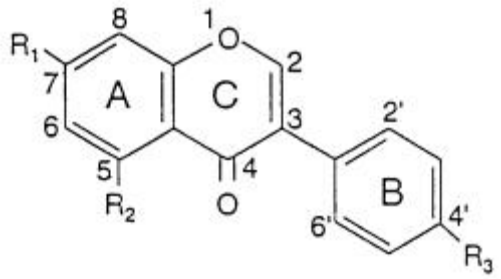


Figure 3 structure of isoflavones: isoflavones have three phenolic rings referred as A, B, C (or pyrane), with different hydroxyl group substitution

A number of homoisoflavonoids have been isolate from several general in Liliaceae and Caesalpinioideae (Legumiosae). The structural features of homoisoflavonoid can generally by classified in to three types, there are eucomin type (3-ben2ylidenechroman-4-one), dihydroeucomin type (3-benzylchroman-4-one) and eucomol type (3-hydroxy-3-benzylchroman-4-one). Homoisoflavonoids are a type of phenolic compounds arising in plants. They have the general structure of a 16-carbon skeleton, which consists of two phenyl rings and heterocyclic ring.

"Homoisoflavonoid is a special type of flavonoid with one more carbon atom than the common C15 flavonoids, and the perspectives of both chemical diversity and significant biological activities have been reported" (Heller 1981), (Abegaz 2007).

Brazilin isolated from *Caesalpinia sappan* had several biological properties such as anti- inflammation, antibacterial, and antiplatelet aggregation, etc. Furthermore, using human multiple myeloma U266 cells, the anticancer mechanism of brazilin were developed (Nazeerullah 2012).

Brazilin showed inhibitory activity against a difference of bacteria, ranging from bacteria responsible for dental caries (*Streptococcus mutans*) and periodontal disease (Prevotella intermedia) to bacteria causing strep throat (group A strep). It also inhibited antibiotic- resistant bacteria for example, MRSA, VRE and multidrug resistant *Burkholderia cepacia*. Nevertheless, brazilin showed less inhibitory effect on other

Gram- negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Escherichia coli,* and *Enterobacter aerogenes* (Sarumathy 2011).

Brazilin isolated from methanolic extracts of *Caesalpinia sappan* wood showed a potential anti-acne agent because of its antibacterial activity against *Propionibacterium acne* (MIC and MBC values of 0.50 mg/ml). It also had good lipase inhibitory activity and antioxidant activity (Baek 2000).

Macroscopic evaluation

Macroscopic evaluation is the description of plant morphology by naked eye or magnifying lens. Morphology is the study of the form of plants or crude drugs. This evaluation procedure provides the simplest and quickest means to establish the identity and purity and thereby ensure quality of a particular sample (Chanda 2014), (Kunle 2012). The example of macroscopic shapes of leaf, the margin, and leaf venation were shown in Figure 4- 6 (Nix 2017).

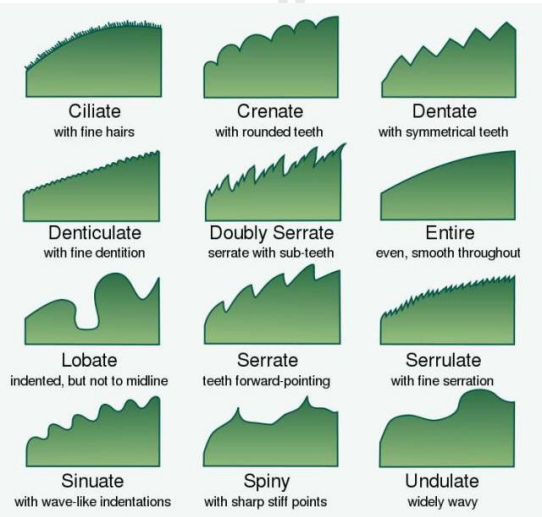


Figure 4 Leaf margin

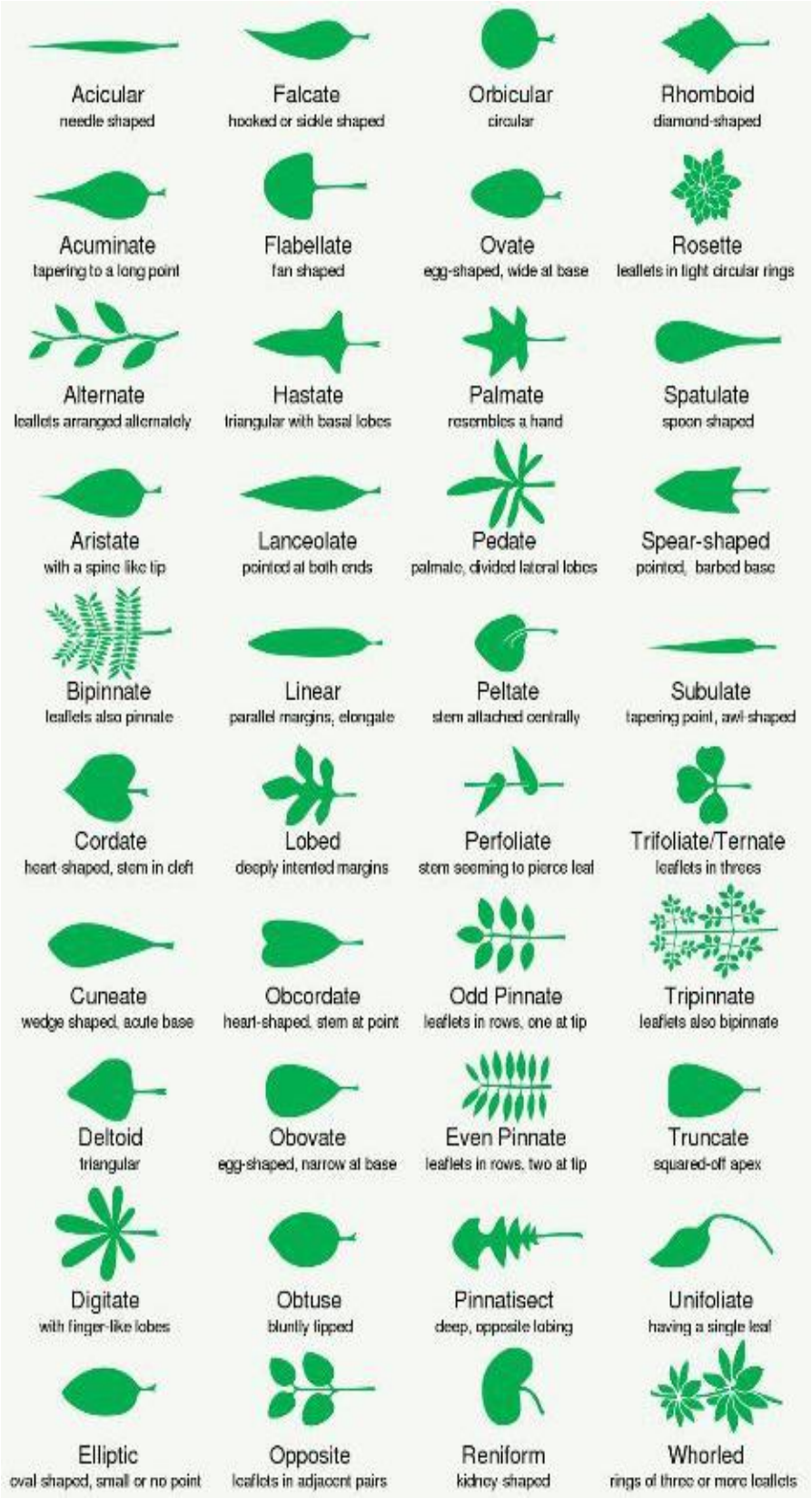


Figure 5 Leaf macroscopic shape

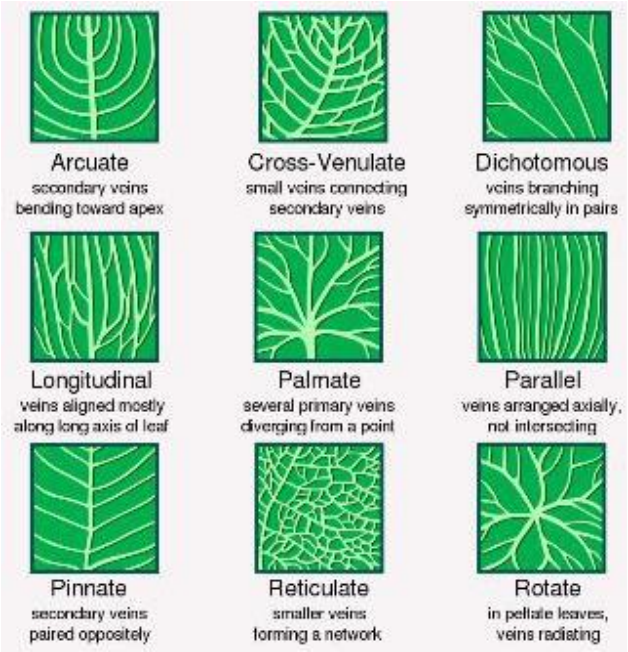


Figure 6 Leaf venation

Microscopic evaluation

Microscopic evaluation is the examination of cellular form and arrangement in a medicinal plant material using microscope.

The microscopic leaf constant numbers are also used to differentiate among species from the same genus (Evans 2002). The constant number might be assessed by several parameters such as stomatal number, stomatal index, palisade ratio, epidermal cell number, epidermal cell area, trichome number, and trichome index (Pitakpawasutthi 2018).

Stomatal number

Stoma is a cell functions as general opening in leaf and herbaceous stems for gas exchange (water vapor, carbon dioxide and oxygen). Stomatal number is the number of stomata per unit area of leaf. Timmermann (1927) and Rowson (1943) were amongst the first few to investigate leaf drugs for stomatal number and stomatal index (Timmerman 1927), (Rowson 1943). In 1927, Timmerman studied about the value of stomatal numbers and distinguished plant species from the *Datura* genus. He suggested that these data should be added for use as a diagnostic character (Timmerman 1927).

Types of stomata [Figure 7]

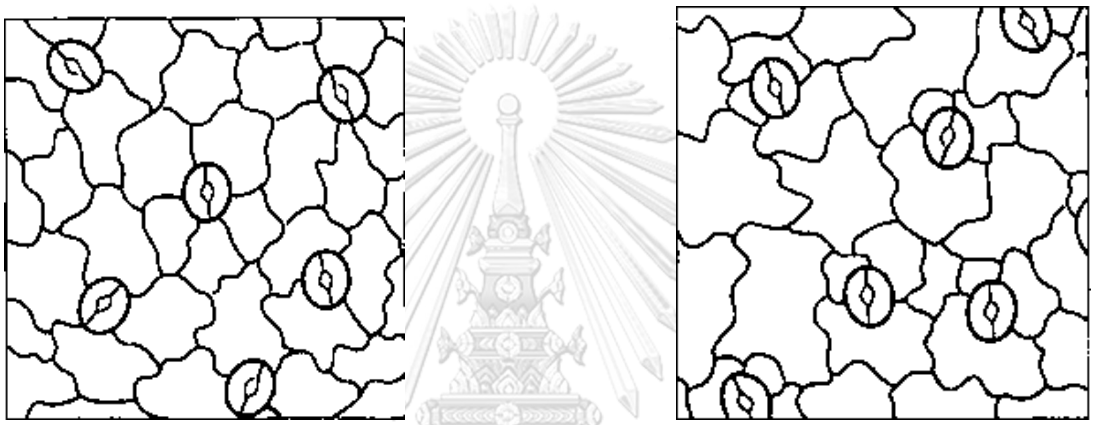
There are four significantly different types of stomata distinguished by their form and arrangement of surrounding cells (WHO 1998).

- **The anomocytic type** (irregular-called): the stomata is surrounded by a varying number of cells, generally not different from those of epidermis [Figure 7(a)].

- **The anisocytic type** (unequal-called): the stomata is usually surrounded by three or four subsidiary cells, one of which is markedly smaller than others [Figure 7(b)].

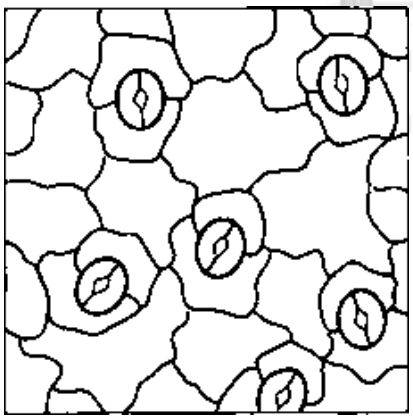
- **The diacytic type** (cross-called): the stomata is accompanied by two subsidiary cells, the common wall of which is at right angles to the stomata [Figure 7(c)].

- **The paracytic type** (parallel-called): the stomata has two subsidiary cells, of which the long axes are parallel to the axis of the stoma [Figure 7(d)].

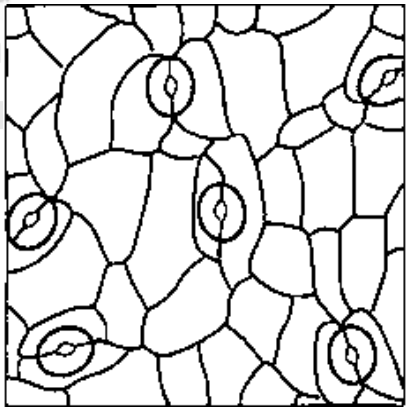


(a)

(b)



(c)



(d)

Figure 7 Types of stomata

Stomatal index

Stomatal index is a percentage of stomatal number to the total number of epidermal cells (each stoma is counted as one cell).

Palisade ratio

Palisade cells are type of photosynthetic cells of the mesophyll of leaf occurring mostly just beneath the upper epidermal surface layer (Wallis 1960). The cells 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 (Eames 1925). Palisade ratio is an average number of palisade cells beneath each epidermal cells of a leaf. This ratio will be assessed from the palisade cells beneath four continuous epidermal cells.

Trichome number

Trichomes are epidermal protuberances located on aerial parts of plant, that protect plant from excess transpiration, high temperature, radiation, ultraviolet (UV) light, and herbivore attack (Wagner 2004). Trichome anatomy is a huge significance in classification at all the levels, from the family relation down to the separation of species and even varieties (Stace 1980). Trichomes or plant hairs, known to present on the surface of leaf, stem, fruit and trichome number has been used for

identification of some plants that have trichomes covering their leaf. The example of trichome types were shown in Figure 8 (John 2018).

Trichome index

Trichome index is a percentage of the proportion between trichome number and epidermal cells in one square millimeter.

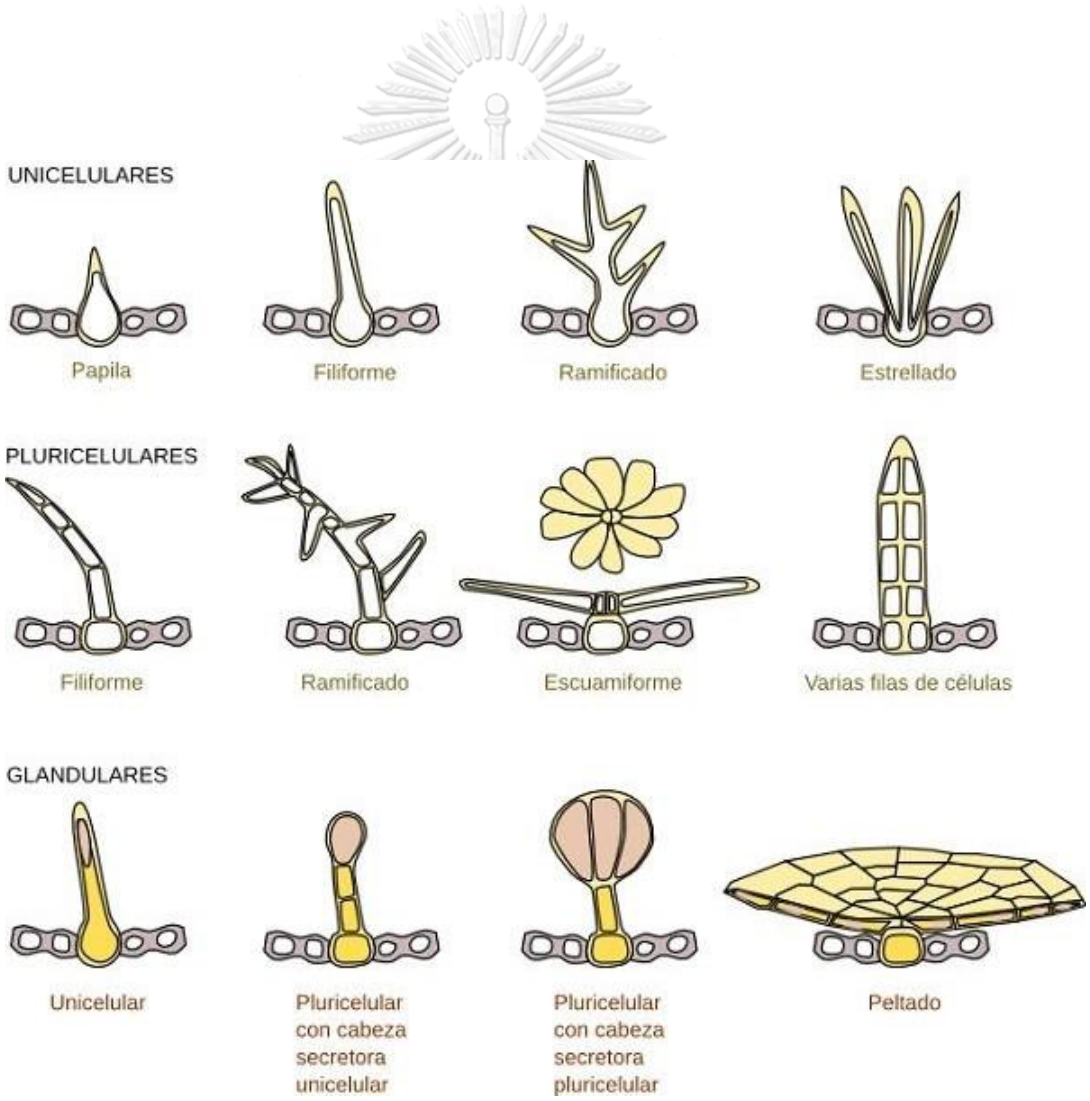


Figure 8 Trichome types

Anatomical characters of leaf

Leaf is the thin with a large surface area. Their cells contain chlorophyll in chloroplasts. It has short distances for gases to diffuse but presented large area for absorption of light. It is a plant's food factory and also is the main site of photosynthesis. Leaf has a great diversity of the internal structures that can possible present information for differentiation patterns (Judd 2008), (Metcalfe 1950). One of them is the midrib, which clearly divides between species in its shape and composition of basic tissues (Evert 2006). Leaf midrib is considered as a stable region regarding the conservation of its structures when submitted to the image acquirement process (Niinemets 2007).

With the invention of the microscope, there were many attempts to observe the structure of plants using anatomical cross-sections by microtome, or by hand (Smith 1915). Transverse section is obtained by cutting along the radial plane of a cylindrical portion of the stem, root, stolon, and perpendicular to the long axis (Khandelwal 2011). The mature leaves (midrib) are thinly cross sectioned with a razor blade and separately placed a complete piece on the glass slide and covered under microscope with magnification of 10X to 40X to evaluate the fine details. Figure 9 and 10 were presented the picture of leaf cross section (Paet 2010), (Raven 2005).

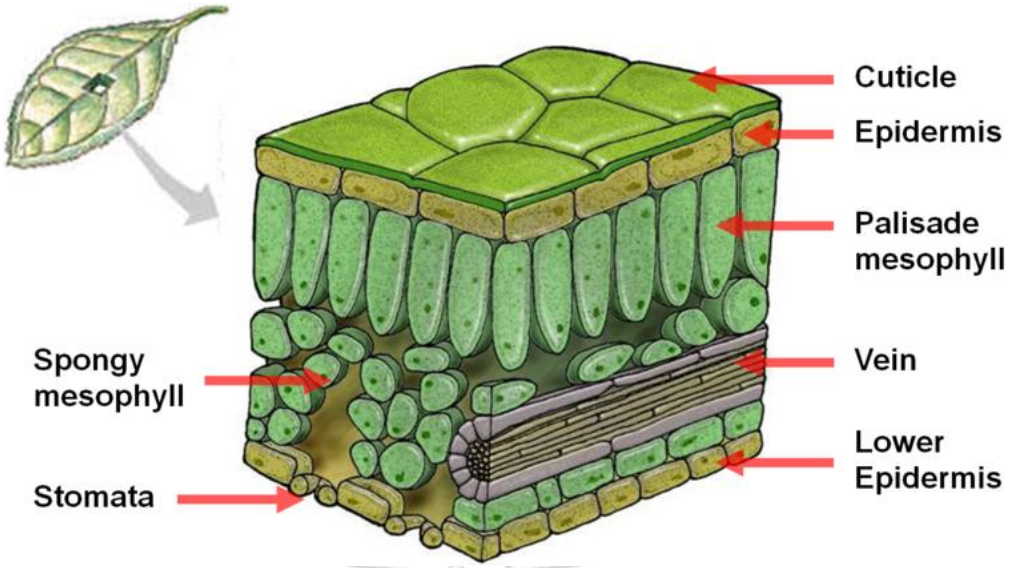


Figure 9 A cross section of leaf

A transverse section of typical leaf presents that it is covered by epidermis on both sides. Epidermis is interrupted by large number of opening cells called stomata. Each stoma is surrounded two kidney shaped cells called Guard cells. It regulates the exchange of gases and loss of water vapor from leaf. Mesophyll is the tissues that enclosed between the two epidermal layers and it consists of elongated cells arranged in rows called the palisade parenchyma. It also has irregularly arranged cells with large intercellular spaces known as spongy parenchyma. The mesophyll cell contain abundant chloroplasts. More palisade tissue occurs in leaves exposed to bright light that those exposed to shade. Vascular bundles in the leaf are located in the midrib and the veins. In each vascular bundle of the leaf, phloem is located towards the lower side and xylem towards upper side (Farabee 2000).

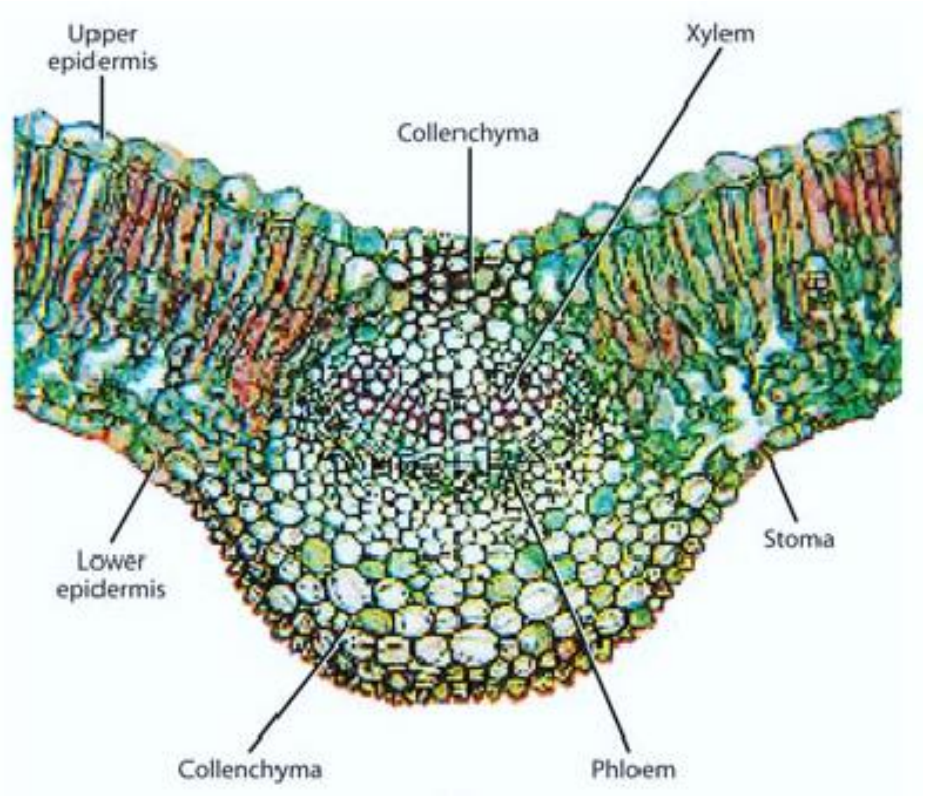


Figure 10 Midrib leaf cross section

Quality control for herbal material (WHO 1998)

Quality control method for medicinal plant materials published by WHO describes about analytical tests for assessment of the quality of herbal medicine and the modern analytical techniques should be applied for the evaluation.

Determination of extractive matters

Extractable matters stand for the value of active compounds in the medicinal plants. Phytochemicals can be extracted from plant material by maceration with particular solvent. Generally, water is a solvent used for polar compounds whereas ethanol is suitable for the slightly non-polar compound.

Determination of water content and loss on drying

The water or moisture content in medicinal plant material is directly measured by azeotropic distillation with water immiscible solvent such as toluene or xylene. To avoid water absorption in solvent, toluene or xylene must be saturated with water before use for the accuracy of result. Moisture content considerably affects medicinal plant quality. An extra amount of water leads to plant material spoilage due to the growth of microorganisms. Oven drying under specified condition is a method to determine total solids of plant material that remain after removal of water and volatile matters by dry heating in oven at 100- 105 °C.

Determination of total ash and acid insoluble ash

Total ash content represents the total amount of inorganic components in plant material remaining after removal of water and organic matters by incineration at 500 °C. Acid insoluble ash represents the remaining inorganic matters which are not be solubilized in hydrochloric acid, for example silica. The adulterants in crude drug such as foreign plant samples, adherent extraneous foreign matters, or chemical fertilizer can affect the contents of total ash and acid insoluble ash.

Thin layer chromatography

Chromatography is an ordinarily analytical technique used to separate the chemical compounds in the mixture (Cimpan 2010). It is a method of separation in which the components to be separated are distributed between two phases, the first one is stationary phase and another one is the mobile phase which is moves in definite direction (Waksmundzka-Hajnos 2008).

Thin layer chromatography or TLC is a planar chromatographic technique which broadly used for analysis of organic compounds. This technique is also used for an isolation of the unique compound from the mixture components. This is usually used to apply in phytochemistry, drug researches, and quality control of many products etc. TLC consists of a solid support coated with thin-layer stationary phase. Samples are spotted onto the TLC plate. The plate is placed in a closed chamber containing the solvents (mobile phase) which moves upward by capillary action (development of TLC plate). The developed TLC plate can be scanned with a beam of light of a specified wavelength for measurements of UV or visible light absorption or fluorescence, providing signal which can be used for the qualitative and quantitative analyses of separated compounds. The visualization step of TLC can be performed by UV viewing cabinet, TLC visualizer and TLC scanner (densitometer) (Figure 11). TLC is a fast technique to identify and separate any compounds (ICH 2005), (Waksmundzka-Hajnos 2008).

Retardation factors (R*f*)

The retardation factor is a parameter that calculated from the distance travelled of compound from an origin divided by the total distance travelled by the solvent (Equation 1). The R*f* value is used to identify each compound under the same solvent system or same condition. It is commonly presented as hR*f* (R*f* x 100).

$$Rf = \frac{distance\ of\ compound\ from\ an\ origin}{distance\ of\ solvent\ front\ from\ an\ origin} \quad \text{—— Equation 1}$$

TLC densitometry (TLC scanner)

This instrument is used for qualitative and quantitative measurement of the compounds separated on developed TLC according to the light absorption and/ or excitation/ emission fluorescence properties of each compound (Stroka 2002). The amounts of samples are evaluated by comparing of each sample with a standard curve from reference material chromatographed concurrently under the same condition. TLC densitometer can be used as fixed and scan wavelengths in both absorption and fluorescence detection modes. The range of absorbance starts from 190 to 900 nm. It contains three light sources (deuterium lamp for UV region, halogen-tungsten lamp for the visible region, and high pressure mercury lamp for fluorescence) (Sherma 2005), (CAMAG 2017), (Washiyama 2009). The densitometer consists of the software for computation and display the results as chromatographic

peak with peak area. The component of CAMAG TLC scanner 4 is shown in Table 7 (CAMAG 2017).

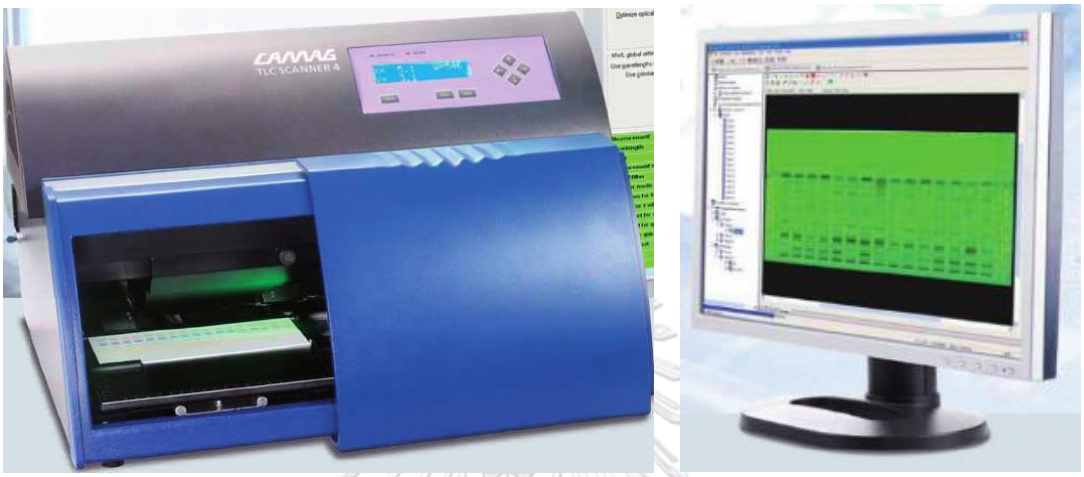


Figure 11 CAMAG TLC scanner 4

Table 7 Technical data of CAMAG TLC scanner 4 (CAMAG 2017)

Topic	Information
Light sources	- Deuterium lamp, usable continuum 190 – 450 nm - Halogen-tungsten lamp, usable continuum 350 – 900 nm - High-pressure mercury lamp, line spectrum 254 – 578 nm The lamp, which is positioned in the light path, is automatically ignited. All lamps are current stabilized.

Pilot lamp and compartment illumination	The slit is automatically illuminated with visible light when the compartment illumination is switched on. The scanning compartment is illuminated with a 4 watt fluorescent tube UV 254 nm which the user can replace by a UV 366 nm or a white light tube.
Optical system	Apochromatic suprasil-fluorite lens system, transmission range 190 – 900 nm, astigmatic entry lens for optimal slit illumination; automatic switching between micro and macro position for optimal light intensity.
Monochromator	Concave holographic grating, 1200 lines/mm, bandwidth selectable 5 or 20 nm, wavelength range 190 – 900 nm; monochromator driven by stepper motor, reproducibility of wavelength setting better than 0.2 nm, accuracy better than 1 nm; connector for flushing with nitrogen. Maximum speed of spectra recording 100 nm/s, positioning at 200 nm/s.
Secondary filter	Motor-driven filter wheel with three automatically selected filters for the elimination of second order wavelengths; 400 nm cut-off filter for fluorescence measurements; three positions for user selected filters.

Scanning slit	Revolving disk with 20 fixed apertures; length of slit images selectable between 0.2 and 12 mm, width between 0.1 and 1.2 mm in 42 combinations.
Detector	Two matched broad band photo multipliers, multi alkali type, spectral sensitivity 185 – 900 nm.
Stage drive	Independent in both directions by stepper motors, micro step driven for smooth movement; reproducibility of positioning better than 50 µm in Y-direction, better than 100 µm in X-direction; maximum scanning speed 100 mm/s, positioning at 150 mm/s.
Mains voltage	115 V and 230 V selectable; 50/60 Hz; maximum energy use 180 W (tungsten and mercury lamp ignited).
A/D converter	16 bit, 2-channel A/D converter, 100 ms per double conversion.
Connections/interfaces	Serial interface RS232 for communication to the computer, EquiLink for connection to winCATS software.
Dimensions	Width = 590 mm, depth = 650 mm, height = 367 mm; net weight 39 kg.

TLC image analysis

The alternative technique of TLC is using image analysis software to calculate the pixel number of spot image and create the chromatographic peak which can be quantitated. Image analysis is one of the densitometric chromatogram quantification that can analyze an image using a digital camera to gain images of the chromatograms on a plate. Images will be uploaded on computer before quantitative and qualitative analysis by several available software programs. There are many options for image analysis software and the popular one is ImageJ free software. This technique can compute the area and the pixel value statistics of determined selection by user (Ferreira 2012), (Schneider 2012).

Method validation (ICH 2005)

Method validation is necessary to confirm that the analytical procedure developed for quantification of active compound in the crude drug is suitable. According to the ICH guideline, method validation consists of specificity, accuracy, precision, detection limit, quantitation limit, linearity, range, and robustness.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

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. It might be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability expresses the precision under the same operating conditions over a short interval of time. It is also termed intra-assay precision.

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment etc.

Reproducibility expresses the precision between laboratories.

Specificity

Specificity is the ability to distinguish and quantify the response of the analyte (target compound) from the response of other compounds such as impurities, degradants, matrix etc. to ensure the identity of an analyte.

Linearity

The linearity of an analytical procedure is its ability to gain test results which are directly proportional to the concentration of analyte in the sample.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Limit of detection

The limit of detection is the lowest amount of analyte in sample which can be detected but not necessarily quantitated as an exact value.

Limit of quantitation

The limit of quantitation is the lowest amount of analyte in a sample which can be determined with suitable precision and accuracy.

Molecular evaluation

The development of biomolecular markers evaluation is one of the most crucial things in the field of herbal sciences. DNA-based markers have been prosperously used to study plant genetic relationship (Botstein 1980). Many techniques based on DNA have been modified for precise species identification (Karp 1997). Properties acceptable for ideal DNA markers include highly polymorphic nature, codominant inheritance, frequent occurrence in the genome, selective neutral behavior, easy access, high reproducibility, and easy exchange of data between laboratories (Joshi 1999).

Nowadays, there are various types of DNA markers such as Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), and Inter-Simple Sequence Repeats (ISSR). The various molecular markers might be classified into different groups based on mode of transmission, mode of gene action, and the method of analysis (Weising 2005), (Semagn 2006). The comparison of four widely used of genetic makers in plant were concluded in Table 8.

Table 8 Comparison of four widely used of genetic makers in plant (Semagn 2006)

Criterion	**RFLP**	**AFLP**	**RAPD**	**ISSR**
Genomic abundance	High	Very high	Very high	Medium
Part of genome surveyed	Low copy coding regions	Whole genome	Whole genome	Whole genome
Amount of DNA required	High	Medium	Low	Low
Type of polymorphism	Single base changes, insertion, deletion	Single base changes, insertion, deletion	Single base changes, insertion, deletion	Single base changes, insertion, deletion
Level of	Medium	Very high	High	High

polymorphism				
Inheritance	Codominant	Dominant	Dominant	Dominant
Detection of alleles	Yes	No	No	No
Ease of use	Labor intensive	Difficult initially	Easy	Easy
Automation	Low	Medium	Medium	Medium
Reproducibility	High	High	Intermediate	Medium to high
Type of probes/ primers	Low copy genetic DNA or cDNA clones	Specific sequence	Usually 10 bp random nucleotides	Specific repeat DNA sequence
Cloning and/ or sequencing	Yes	No	No	No
Radioactive detection	Usually yes	Yes/ No	No	No
Development/ startup costs	High	Medium	Low	Medium
Utility for genetic	Species specific	Cross specific	Cross specific	Cross specific

mapping				
Proprietary right status	No	Licensed	Licensed	No

Plants of *Caesalpinia* species were studied for their genetic evaluation using various molecular makers.

Cardoso *et al*. (1998) studied *C. echinata* and demonstrated that RAPD markers were effective in establishing a clear correlation between genetic and geographical distance and in identifying areas of maximum diversity, and might be used as an initial approach to assess the partitioning of genetic variation in this species (Cardaso 1998).

Jena *et al*. (2004) identified RAPD markers among five legume mangroves belonging to the sub-family Papilinoideae (*Dalbergia spinosa*, *Derris heterophylla* and *Derris indica*) and Caesalpinioideae (*Caesalpinia crita*, *Cynometra ramiflora*). The dendrogram showed clustering of *Caesalpinia crita* and *Cynometra ramiflora* into one groups. In second group, *Derris heterophylla* and *Derris indica* were more similar than *Dalbergia spinosa* (Jena 2004).

Mukherjee *et al.*, 2006 assessed the genetic diversity in 31 species of mangroves including *Caesalpinia bonduc* and found that AFLP markers could analyze the genetic variability and establish phylogenetic relationships among different genera and species (Mukherjee 2006).

In 2012, Rodrigues and team reported that RAPD technique was not an efficient method for detecting polymorphisms in *C. pulcherrima* that produced flowers of different colour (Rodrigues 2012).

Polymerase chain reaction (PCR)

In 1986, Mullis and their team described about the process of polymerase chain reaction (PCR) for the first time. PCR is quite popular, simple method, and spend a short period of time for the copy of selected DNA fragments. There are three steps of PCR which are repeated a number of times (cycles). The first step is DNA denaturation, second step is primer annealing, follow by primer extension step (Mullis 1994). The process of PCR in one cycle was shown in Figure 12 and PCR thermal cycler was shown in Figure 13.

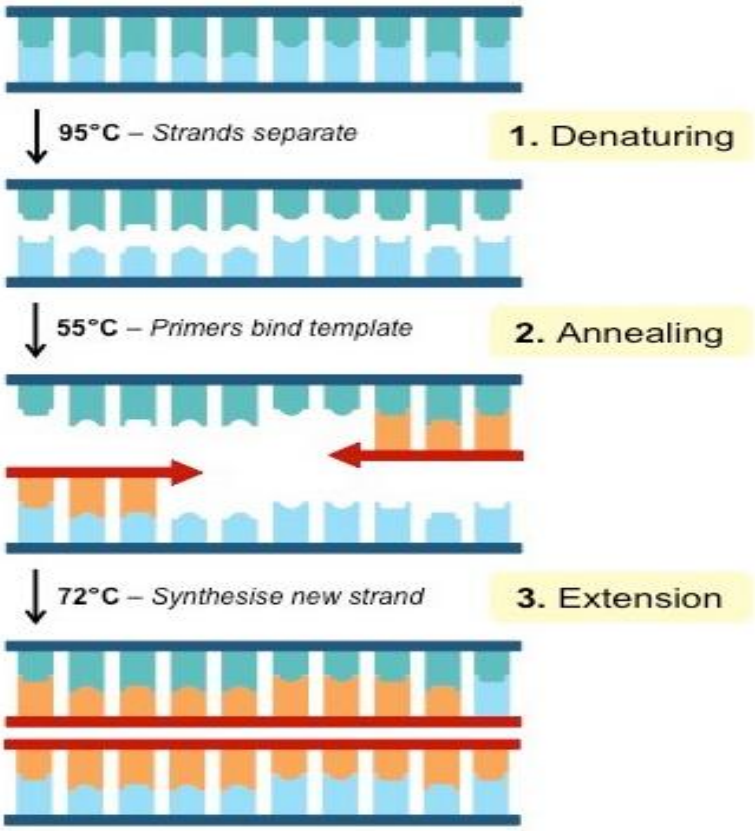


Figure 12 The process of Polymerase Chain Reaction in one cycle

Figure 13 PCR Thermal Cycler

Inter-Simple Sequence Repeats (ISSRs)

Inter-Simple Sequence Repeats or ISSRs are regions in the genome flanked by microsatellite sequences. This technique implicates amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions aimed in opposite direction. ISSR usually 16- 25 bp long as primers in a single primer PCR reaction targeting multiple genomic loci to amplify different sizes of inter-SSR sequences. ISSR PCR is a technique which conquers the problem such as high cost of AFLP, low reproducibility of RAPD, or the flanking sequences to develop species specific primers for SSR polymorphism (Idrees 2014). ISSR have been used since 1994 for a wide range of organisms (Zietkiewicz 1994). In plant genetics, there are many researches on the application of ISSR markers such as Godwin and team studied about the application of ISSR markers to plant genetics in 1997 (Godwin 1997), and in 2002, Reddy and the team studied the ISSR polymorphism and its application in plant bleeding (Reddy 2002). The procedure to conduct the ISSR genotyping method is quite simple. In 2015, Ng & Tan suggested the process flow chart for ISSR genotyping experiment (Ng 2015) (Figure 14).

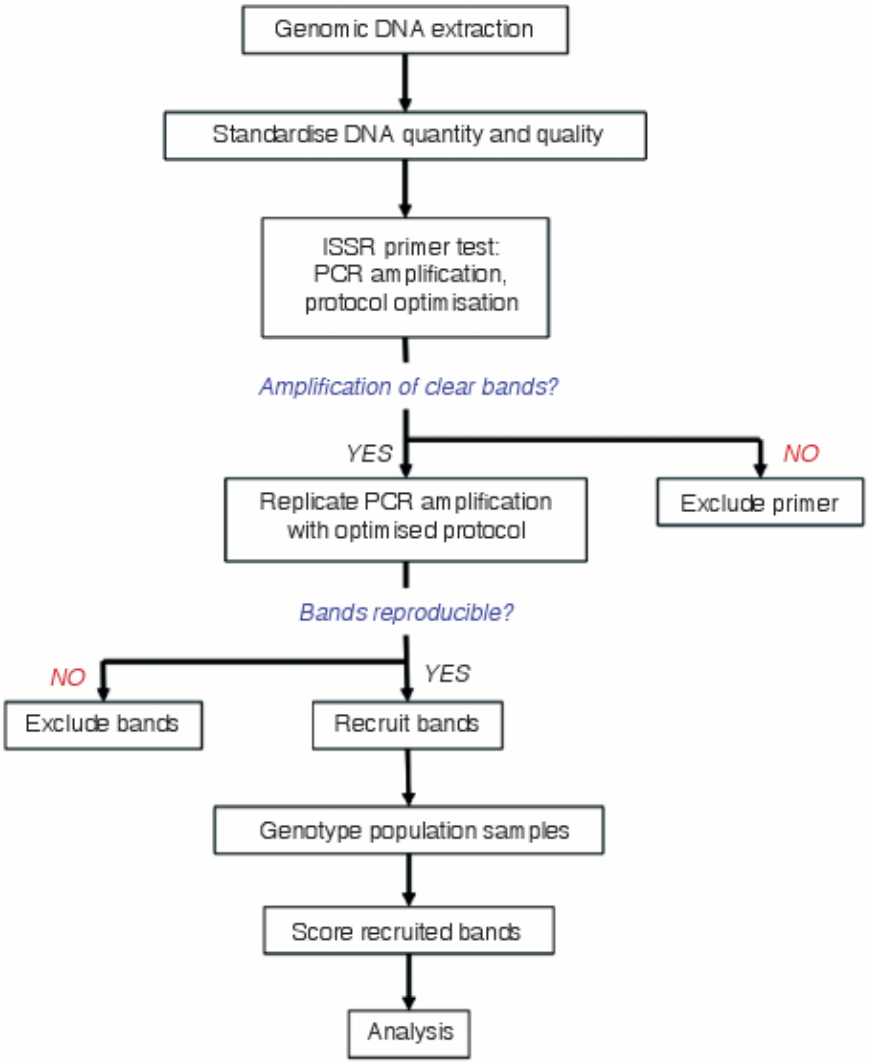


Figure 14 The process flow chart for ISSR genotyping

Genomic DNA is usually used as a template for ISSR-PCR. Sometimes, the extraction of DNA including extraction methods and different types of sample might contain a mark of cell debris and elements that inherent inhibit PCR reactions. In general, the conventional DNA extraction methods would be adequate to gain a good quality DNA. If not, an additional purification of DNA extract by the commercial

DNA extraction or kits might be help. Regularly, 10- 50 ng of DNA is enough for each reaction (Ng 2015).

There are three forms ISSR primers depending on the usage

(1) Unanchored: the primer consist only of a repeated motif

(2) 5'-anchored: the primer consist of a repeated motif with one or several non-motif nucleotides at the 5'-end

(3) 3'-anchored: the primer consist of a repeated motif with one or several non-motif nucleotides at the 3'-end

In 2002, Reddy and team reported that ISSR-PCR is usually conducted with an annealing temperature of 45- 60 °C, depends on the melting temperature of ISSR primer (Reddy 2002). Ng and Szmidt, 2014 suggested that the touch-down PCR method is also prosperously used to gain a good amplification with ISSR primers which were difficult to optimize using standard PCR method (Ng 2015).

For the gel electrophoresis method, ISSR-PCR amplification products are usually electrophoresed range 1.5- 2.0 % w/v agarose gel. From that range, the result will be completely sufficient separation of DNA bands for easy counting. The band also determined by polyacrylamide gel electrophoresis (Godwin 1997), (Reddy 2002). For scoring of bands, there are many points to observe. Firstly, score only the clear particular bands because the smeared band could be the result of unspecific binding of ISSR primers causing unexpected amplification. It might be overlapping of other

bands with a closely DNA fragment sizes and it would make scoring difficult and incongruent. Second, score the band with strong intensities because a weak intensity bands lean to present a low reproducibility. Lastly, arrange standard band-scoring size range before counting. The range that generally used was 100- 2000 base pairs. Eventually, the bands will be recorded into the binary symbols, 1, for the present band whereas 0, for the absence following analysis.

The strong point of ISSRs is that no needed a sequence data for primer formation. ISSR marker is a useful tool for the analysis of genetic diversity in diverse species (Gupta 2000). It has been confirmed as a reliable, rapid, simple, cost effective, easy to generate, and useful set of marker that is not clear previous knowledge of the genome sequence to generate DNA markers (Zietkiewicz 1994), (Gupta 1994), (Bornet 2001), (Bornet 2002). Sheppard and Smith, 2000 suggested that the marker is easy to generate by using minimal equipment and are hypervariable, so it is a sensible cost to the researcher (Sheppard 2000). This technique involves polymerase chain reaction (PCR) to amplify DNA fragments between two simple sequence repeats (SSRs) with contrary position using primers with a single SSR motifs anchored at the 30- or 50-end by a few nucleotides (Zietkiewicz 1994). Peng and team (2006) said that ISSR markers are usually highly polymorphic in plant populations, providing a genotyping system with features of consistency, reliability and codominancy. Moreover, ISSR markers are occur in both nuclear and organellar

genomes. On the other hand, ISSRs also have a weakness because it is a multilocus technique which might be present a non-homology of similar sized fragment causing reproducibility problems (Peng 2006).

CHAPTER III

MATERIALS AND METHODS

Chemicals and reagents

Absolute ethanol	Merck, Daemstadt, Germany
Agarose	SeaGar®, Cambridge, UK
Brazilin	TAUTO BIOTECH, China
Chloral hydrate	Ajax Finechem Pty. Ltd., New Zealand
Chloroform	RCI Labscan Limited, Thailand
DNA marker	Thermo Fisher Scientific Inc., USA
DNeasy plant mini kit	QIAGEN, USA
dNTPs	Thermo Fisher Scientific Inc., USA
EDTA	Ajax Finechem Pty. Ltd., New Zealand
Ethanol	RCI Labscan Limited, Thailand
Ethidium bromide	Bio Basic Canada, Canada
Ethyl acetate	RCI Labscan Limited, Thailand

Formic acid	Merck, Germany
GeneRuler 1 kb DNA ladder	Thermo Fisher Scientific Inc., USA
Haiter solution (6% sodium hypochlorite)	Kao Corp., Japan
Hydrochloric acid	RCI Labscan Limited, Thailand
Isoamyl alcohol	Sigma-Aldrich Company Co., St. Louis, MO, USA
ISSR primers	Eurofins MWG Operon Inc., USA
Liquid nitrogen	
Loading dye	Thermo Fisher Scientific Inc., USA
Magnesium chloride	Thermo Fisher Scientific Inc., USA
Mercaptoethanol	
Methanol	RCI Labscan Limited, Thailand
Sodium acetate	BDH Laboratory supplies, Poole, England
Sodium chloride	BDH Laboratory supplies, Poole, England
Taq DNA polymerase	Thermo Fisher Scientific Inc., USA

Toluene	RCI Labscan Limited, Thailand
Tris (hydroxymethyl)-aminomethane	Fluka, Biochemika, Germany
Ultrapure water	NW20VF, Heal Force, China
1 kb DNA ladder	Promega, USA
100 kb DNA ladder	Promega, USA

Materials, instruments and equipment

CAMAG TLC Chamber	CAMAG, Switzerland
CAMAG TLC Scanner 4	CAMAG, Switzerland
CAMAG TLC Visualizer	CAMAG, Switzerland
Centrifuge machine	Labnet International, Inc., USA
Combi-Spin	BioSan, USA
Digital camera (Canon PowerShot A650 IS)	Canon Inc., Japan
Filter paper No.4	Whatman™paper, UK
Filter paper No.40 ashless	Whatman™paper, UK
Gel electrophoresis apparatus and power supply	Biometra
Hot air oven	WTB binder, Tuttlingen, Germany

ImageJ sofeware	National Institutes of Health, USA
Incinerator	Carbolite, UK
InGenius 3 with GeneSis software	Syngene, UK
Microscope (Axio imager A2)	Zeiss Inc., Germany
NanoDrop Spectrophotometer ND- 1000	NanoDrop Technologies, Inc., Wilmington, DE, USA
PCR Thermal Cycler	GenePlus, Thailand
Photomicroscope	Zeiss Imager A.2 Axio, Germany
Proflex PCR system thermocycler	Thermo Fisher Scientific Inc., USA
Rotary vacuum evaporator	Buchi, Switzerland
TLC aluminium sheet Silica gel 60 F254	Merk, Germany
Ultraviolet fluorescence analysis	Spectronic corp., USA
Ultralviolet viewing cabinet	Spectronics Corporation, USA
UV visualize gel documentation machine	Auto Chemi System, USA
winCATS software (Version: 1.4.6.2002)	CAMAG, Switzerland

Methodology

Part I: Quality evaluation of *Caesalpinia sappan* heartwoods

The quality parameters including macroscopic-microscopic characteristics, total ash and acid insoluble ash, ethanol and water soluble extractive values, loss on drying, water content were examined in accordance with WHO guideline (WHO 1998).

Plant materials

The heartwoods of *Caesalpinia sappan* were collected from 15 different places in Thailand. All samples were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi and voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Macroscopic examination

Macroscopic examination of medicinal plant refers to the evaluation of a crude drug by color, odor and taste, size, surface characteristics and other visual observation. All characteristics were examined by naked eyes.

Microscopic examination

The sections and powders of crude drug were mounted with water on slide. The anatomical and histological characters of *C. sappan* heartwood were investigated by microscope under the objective lens including 10X, 20X, and 40X magnifications and the eyepiece lens magnification of 10X.

Determination of loss on drying, total ash, and acid insoluble ash

Three grams of *C. sappan* heartwood powders in a pre-weighed crucible were dried at 105°C until constant weight was obtained then keep in the desiccator at the room temperature. The value of loss on drying were calculated in a percentage of dried crude drug.

The aforementioned crucible were incinerated at 500 °C until the carbon matters are absent. The percentage of total ash were calculated.

The crucible which contains the total ash were boiled with 25 ml of hydrochloric acid (70g/ L) for 5 minutes. The insoluble matters were collected on an ashless filter paper No.40, transferred into the original crucible and incinerated to ash again. Acid insoluble ash were weighed and calculated in percentage.

Determination of water content

Fifty grams of crude drug were mixed with 200 ml of water-saturated toluene and heated for azeotropic distillation. The volume of water was measured and water content was calculated in percentage.

Determination of solvent extractive value

Five grams of crude drug were macerated with 70 ml of the solvent (95% ethanol or water) in a closed flask. It was shaken frequently for 6 hours and allowed to stand for 18 hours. The marc was rinsed with the solvent and the filtrate was adjusted to 100 ml. The aliquot (25 ml) of the filtrate was transferred into a pre-weighed beaker and evaporated till dryness. The extract was dried at 105°C for 6

hours in a hot air oven and cooled for 30 minutes in a desiccator. The solvent soluble extractive value was weighed and calculated in percentage of the crude drug.

Thin layer chromatographic fingerprint

One gram of powdered sample was macerated with 20 ml of methanol for 30 minutes at room temperature (shaking). The sample was filtered and evaporated to dryness. Then, the residue was dissolved in 0.5 ml of methanol and applied with the volume of 3 µl onto the TLC silica gel 60 GF_{254} plate and developed in an appropriate solvent system chloroform: methanol (9: 1). The plate was examined by under ultraviolet light 254, 365 nm and dipped with suitable reagent (p-anisaldehyde/ sulfuric acid reagent).

Data analysis

The physico- chemical parameters of each sample were performed in triplicate and reported as grand mean ± pooled standard deviation as following formulas.

Formulas:

$$Grand\ mean = \frac{\bar{x}_1 n_1 + \bar{x}_2 n_2 + \ldots + \bar{x}_k n_k}{n_1 + n_2 + \ldots + n_k}$$

$$Pooled\ SD = \sqrt{\frac{\left((n_1 - 1)\ x\ SD_1^2\right) + ((n_2 - 1) x\ SD_2^2 + \ldots + ((n_k - 1) x\ SD_k^2)}{(n_1 + n_2 + \ldots + n_k) - k}}$$

Part II: Quantitative analysis of brazilin in *Caesalpinia sappan* heartwood

Ethanolic extraction

Caesalpinia sappan heartwood powders (5.0 g) were extracted with 95% ethanol by Soxhlet apparatus. After the exhaustive extraction, the extracts were filtered through the filter paper No.4 and evaporated till dryness in *vacuo.* The yield was recorded before storing the extract at -20°C. The extract was dissolved with 95% ethanol to obtain the concentration of 1 mg/ml and kept at 4°C for TLC densitometry and TLC image analysis.

Preparation of standard solution

The standard brazilin was weighed at 1.0 g and dissolved in 95% ethanol. The stock solution was diluted to gain the series of standard solution and stored at 4°C in the dark.

Thin-layer chromatography densitometry

Three microliters of the extract (1 mg/ml) and brazilin standard solutions were spotted on TLC plate (Silica gel 60 GF_{254}). The plate was developed in TLC chamber (10x20 cm) with the mobile phase of chloroform: ethyl acetate: formic acid (10: 8: 2). TLC plate was scanned under the wavelength of 525 nm by TLC Scanner 4 using winCATS software. Brazilin was quantitated by peak area, the calibration curve of brazilin was prepared by plotting peak area versus concentrations of brazilin in µg/ spot. The peak area of each band was examined and calculated the content of brazilin. The test was done in triplicate.

Thin-layer chromatography image analysis

The TLC plate was photographed under short wave ultraviolet light at 254 nm by digital camera and saved as tiff files. Color of the band was changed to chromatographic peak areas by ImageJ software and brazilin content was calculated. Brazilin content was examined by comparing peak area with the calibration curve gain from the same TLC plate. This test was done in triplicate.

Method validation

The method validation consisting of accuracy, repeatability, intermediate precision, limit of detection (LOD), limit of quantitation (LOQ), calibration range, specificity and robustness were performed according to ICH guideline (ICH 2005).

Calibration range

The calibration range was examined by regression line of peak area *versus* brazilin concentration and coefficient of determination was examined by Excel program.

Specificity

The specificity of brazilin quantitative analysis in *C. sappan* heartwood was examined by comparing the absorption spectra of 15 sample spots to the brazilin standard bands under the range from 200 - 700 nm using TLC scanner 4 (CAMAG) as well as comparison of the absorption spectra at up- slope, apex and down- slope of the chromatographic peak.

Accuracy

The accuracy was tested by fulfilling recovery studies with spike method. Brazilin solution was spiked into the extract to gain three different levels (low, medium, and high) in calibration range. An un-spiked and spiked samples were examined in the same condition. This test was done in triplicate and estimated percent recovery as following formula:

$$\%\ Recovery = \frac{A}{B + C} \times 100$$

Where, A = the amount of brazilin found in spiked sample

B = the amount of brazilin found in un-spiked sample

C = the amount of standard brazilin spiked into sample

Precision

The precision of brazilin quantitative analysis was evaluated by repeatability (Intra-day) and intermediate (Inter-day) precision. The method was done by analyzing the sample solution of three concentrations with three replicates at same day and three different days, respectively. The precision was calculated in term of percent relative standard deviation (%RSD) of brazilin content following formula:

$$\%\ RSD = \frac{Standard\ deviation}{Mean} \times 100$$

Robustness

The robustness was examined by slightly changing of a mixture ratio of mobile phase. The variation was calculated by %RSD of peak area of brazilin in the extract.

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

Limit of detection is the lowest concentration that can be detected but not quantified whereas limit of quantitation is the lowest concentration that can be quantified. They were evaluated from the residual standard deviation of regression line (**σ**) and the slope of regression line (S).

$$LOD = \frac{3.3\ \sigma}{S}$$

$$LOQ = \frac{10\ \sigma}{S}$$

Data analysis

The quantity of brazilin in *C. sappan* heartwood between TLC densitometry and TLC image analysis were compared by paired *t*-test statistics.

Part III: Plant morphology and microscopic characteristics including leaf constant numbers of selected *Caesalpinia* species

Plant collection

The mature leaves of selected *Caesalpinia* species were collected from three different sources throughout Thailand. All samples were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi and compared with herbarium specimens at Forest Herbarium Thailand (BKF). Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The samples were evaluated by macroscopic and microscopic evaluation as follows:

Macroscopic evaluation

Macroscopic evaluation of selected *Caesalpinia* species was done by observing their visual characters such as shape, size, and other botanical morphology. The botanical characters were illustrated by hand drawing.

Microscopic leaf anatomical evaluation

The transverse section of the midrib of each *Caesalpinia* species was examined. The leaf samples were cleaned before used. The cross section of the midrib was done by hand with a razor as thin as possible, transferred onto a slide, added two drops of water and observed the anatomical characters under a photomicroscope. All pictures were recorded by digital camera and also illustrated by hand drawing with dimensions of a specific ratio relative to the actual size.

Microscopic leaf constant numbers

The stomatal number, stomatal index, trichome number, trichome index, epidermal cell number, epidermal cell area and palisade ratio were evaluated to identify each *Caesalipinia* species. The fresh mature leaf was soaked in a bottle that contain Haiter solution which contains sodium hypochloride (6%): water (1:1 v/v) for 24 hours or until it clear to remove the chlorophyll. Leaf was transferred into a beaker containing chloral hydrate: water (4:1 w/v) and heated on water bath about 1-2 hours. Leaf was transferred on a slide and placed 2-3 drops of water and examined the cells under microscope. The labeled image was recorded using an AxioVision software. For the magnification, the 40X was examined palisade cell while the 20X power was used with stomatal and epidermal cells and 10X was evaluated trichome cell, respectively. Every examination was investigated on both sides of the leaf. Thirty fields of each selected *Calsalpinia* species from three locations were assessed. The average of 90 fields from three places were performed. The maximum, minimum, mean, and standard deviation of results were calculated. The data was fulfilled in average value.

Stomatal number and stomatal index

Stomatal number was an average number of stomata per square millimeter (mm^2) of epidermis of the leaf was calculated from ninety determination (30 determinations per each location). The value was reported both upper and lower parts of leaf.

Stomatal index was defined as the ratio of stomata number to all the epidermal cell number (including stomata cell and trichome) in unit area of leaf was calculated as following formula:

$$Stomatal\ index = \frac{Sx100}{E + S}$$

Where, S= the number of stomata in a given area of leaf

E= the number of epidermal cells in the same area of leaf including trichomes or cicatrix

Palisade ratio

Palisade ratio, the average number of palisade cells beneath one epidermal cell of a leaf was determined by counting the palisade cells beneath four contiguous epidermal cells then divided by four to obtain the palisade ratio.

Epidermal cell area

The epidermal cells per one square millimeter of upper epidermis were counted and the epidermal cell area was calculated from the reciprocal of the epidermal cell number.

Trichome number and trichome index

Trichome cells or cicatrices per one square millimeter of epidermis were counted. Trichome index, a percentage proportion of trichome number to all of the epidermal cell number in one square millimeter was calculated as following formula:

$$Trichome\ index = \frac{Tx100}{E + S}$$

Where, T= the number of trichome or cicatrix per unit area

E= the number of epidermal cells in the same unit area including trichome or cicatrix

S= the number of stomatal cells in the same unit area of leaf

Part IV: Molecular identification

Plant collection

The young leaves of selected *Caesalpinia* species were collected from three different sources throughout Thailand. *Annona squamosa* was used as outgroup in this study. All samples were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi and compared with herbarium specimens at Forest Herbarium Thailand (BKF).

Preparation of CTAB buffer

2x CTAB (hexadecyltrimethylammonium bromide) was prepared before DNA extraction step (Table 9).

Table 9 Preparation of 2x CTAB buffer

Stock	Amount	Final Concentration
CTAB	2 g	2% (w/v)
1M Tris-HCl pH8	10 ml	100 mM
0.5 M EDTA	4 ml	20 mM
5 M NaCl	28 ml	1.4 M
Adjust to 100 ml with H_2O		

After preparation of CTAB buffer, 4 µl of 2-mercaptoethanol was added to each 1 ml of 2x CTAB buffer before used.

DNA extraction

Plant genomic DNA extraction using CTAB

The genomic DNA was extracted from the fresh young leaf tissue using CTAB method. Five grams of plant tissue were cleaned and frozen rapidly in liquid nitrogen, then ground to powder with mortar and pestle. After that, the sample was transferred into a 1.5 ml microcentrifuge tube and added 700 µl of CTAB buffer. The mixture of CTAB buffer stock solution consisting 2% (w/v) CTAB, 100 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl, and 2% (w/v) β-mercaptoethanol. Next, the mixture of sample and CTAB buffer was incubated at 65°C for 1 hour. The mixture was centrifuged at 10,000 rpm for 10 minutes, and transferred the supernatant phase into

a new clean 1.5 ml microcentrifuge tube. Five hundred microliters of chloroform was added to the supernatant, then mixed using a vortex mixer and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube, added with 500 µl of chloroform/ isoamyl alcohol (24: 1) and mixed with a vortex mixer. The mixture tube was centrifuged for 10 minutes at 10,000 rpm. The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube, added with 1: 10 volume of 3M sodium acetate pH 5 then, genetic mix by inverting tube. Two volumes of ice cold absolute ethanol (-20°C) was added into the tube, gentle mix by inverting tube, and kept at -20°C for 1 hour. After this method, the tube was centrifuged at 10,000 rpm for 10 minutes and discarded the supernatant. DNA pellet was washed with 1 ml of 70% cold ethanol for twice then, centrifuged the tube at 10,000 rpm for 10 minutes and discarded the supernatant. The DNA pellet was dried at room temperature around 30 minutes or until it is dry. DNA was dissolved in 100 µl of TE buffer and stored at 4°C for 2 days to make sure that DNA is completely dissolved before kept it at -20°C for further use.

DNA concentration and purity was checked using NanoDrop One spectrophotometer to determine the absorbance ratio at λ260/ 280 nm prior to amplification.

Preparation of PCR reaction mixture

The PCR components were mixed in the 1.5 microcentrifuge tube with the total volume at 20 µl in the final concentration (Table 10).

Table 10 Preparation of PCR reaction mixture

PCR components (Stock)	Amount	Final concentration
H_2O	14.2 µl	
10X PCR buffer	2 µl	1X
25 mM $MgCl_2$	2.4 µl	3 mM
10 µM dNTPs	0.4 µl	0.2 µM
10 µM primer	0.8 µl	0.4 µM
5 Unit *Tag* DNA polymerase	0.2 µl	1 Unit
Total volume	20 µl	

PCR amplification

PCR amplification using ISSR markers

Inter simple sequence repeat or ISSR markers was applied for this study to identify the relationship among *Caesalpinia* species in Thailand through PCR amplification. Primers were screened from 15 ISSR primers (Table 11). The genomic DNA (1 µl) of each species was mixed with 19 µl of final mixture. PCR amplification was performed in a Proflex PCR system thermocycler. PCR was accomplished with 5 steps consisting an initial denaturation, denaturation, annealing, extension, and final extension steps. Firstly, the initial denaturation step was done for 5 minutes at 95°C. Next, denaturation step was performed at 95°C for 30 seconds, follow by annealing

step with 45 seconds at different annealing temperature of each primer. The extension step was performed at 72°C for 30 seconds and the last step is final extension was performed at 72°C for 5 minutes. The second step to the fourth step was done with 45 cycles. The negative control was added in every testing to assess contaminations.

Table 11 ISSR primers, primer sequences and annealing temperatures

Primer	Primer sequence*	Annealing Tm (C°)
ISSR02	AGAGAGAGAGAGAGAGC	52
ISSR06	ACACACACACACACACC	52
ISSR08	CTCTCTCTCTCTCTCTC	52
ISSR09	ACACACACACACACACT	50
ISSR10	ACACACACACACACACA	50
ISSR13	AGAGAGAGAGAGAGAGYA	48
ISSR14	GAGAGAGAGAGAGAGAYT	48
ISSR17	CACACACACACACACARg	48
ISSR19	ACACACACACACACACYt	48
ISSR21	ACACACACACACACACYg	52
ISSR22	TGTGTGTGTGTGTGTGRc	52
ISSR23	AGCAGCAGCAGCAGCAGC	60
ISSR27	GGATGGATGGATGGAT	48
ISSR31	AGAGAGAGAGAGAGT	44
ISSR44	GACAGACAGACAGACA	48

* Single letter abbreviation for mixed-base position: Y = (C or T), R = (A or G)

Gel electrophoresis

PCR product was examined by gel electrophoresis. PCR product was mixed with loading dye before visualized on 1% agarose gel. The fragment size of DNA was evaluated by GeneRuler 1 Kb and 100 bp DNA ladders. The gel with DNA fragment was developed in the gel electrophoresis chamber at 100 Volt. The gel was stained with ethidium bromide (2mg/ml) then, move the gel into a de-stained chamber. The result was determined under UV visualize gel documentation machine using program GeneSys and the picture was saved as JPEG image file.

Data analysis

DNA fragment sizes were evaluated after photographed an agarose gel. The reproducible amplified bands were used to analyze the result. Amplification profiles were scored as present (1) or absent (0) in a binary code. Similarity index was assessed and pairwise distance matrix was originated a dendrogram by cluster analysis using UPGMA ground on the character deviations.

CHAPTER IV

RESULTS

Part I: Quality evaluation of *Caesalpinia sappan* heartwoods

Macroscopic characteristics

Caesalpinia sappan L., a tree 6-9 m high; stem prickly, 15-25 cm diam.; branches rufous-pubescent, armed with a few small prickles; leaves 20-38 cm long, pinnae 8-12 pairs, 10-15 cm long, subsessile, with small prickles at the base; leaflets 10-18 pairs, 1.3-2 by 1 cm, subsessile, close, oblong, rounded at the apex, attached at the lowest corner, very inequilateral, glabrous above, more or less puberulous beneath; flowers in panicles, which are terminal and in the axils of the upper leaves, 30-40 cm long; pedicles 1.3-1.5 cm long; bracts lanceolate, 8 mm long, caducous; calyx 11 mm long, leathery, glabrous; corolla 2 cm across; petals orbicular, subequal, yellow, the upper with a red spot at the base; ovary grey-velvety; pods 7.5-10 by 3.8-5 cm, woody, obliquely oblong, subcompressed, polished, indehiscent, with a hard recurved short beak at the upper angle of the obtuse apex; seeds 3-4 (Figure 16) (Kirtikar 1975). The heartwood of *C. sappan* was hard and rough with orange-red color (Figure 15).

Microscopic characteristics

The histological characteristics in powdered form showed the fragment of wood, fragment of xylem, fragment of bordered pitted vessel, and calcium oxalate (Figure 17). The anatomical characteristics of *C. sappan* heartwood including transverse section, tangential longitudinal section, and radial longitudinal section presented the wood of parenchyma, vessel, and wood fiber structures shown in Figure 18.

Figure 15 *Caesalpinia sappan* heartwoods

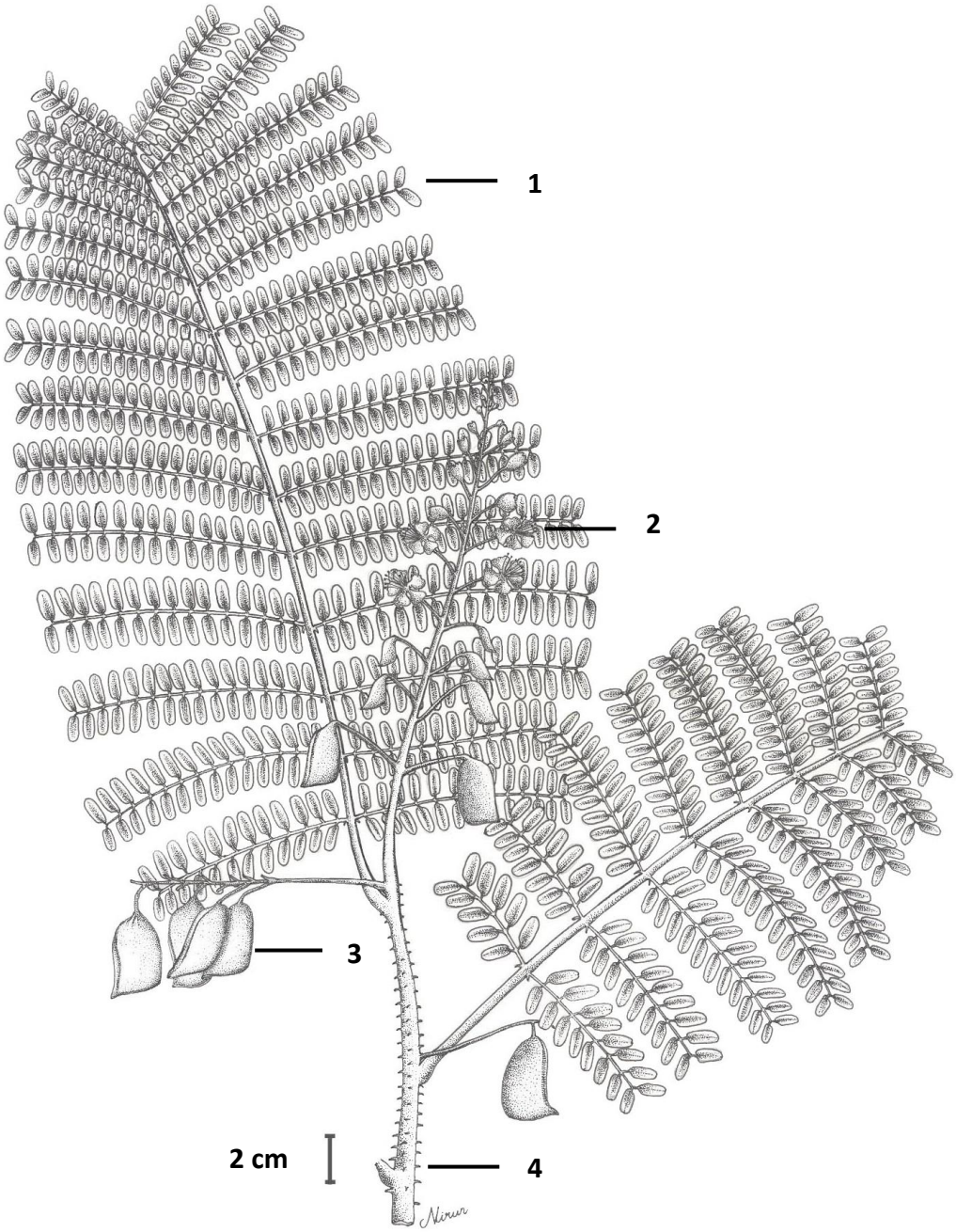


Figure 16 *Caesalpinia sappan* L. 1. Pinnate leaf, 2. Flower, 3. Pod, 4. Branch

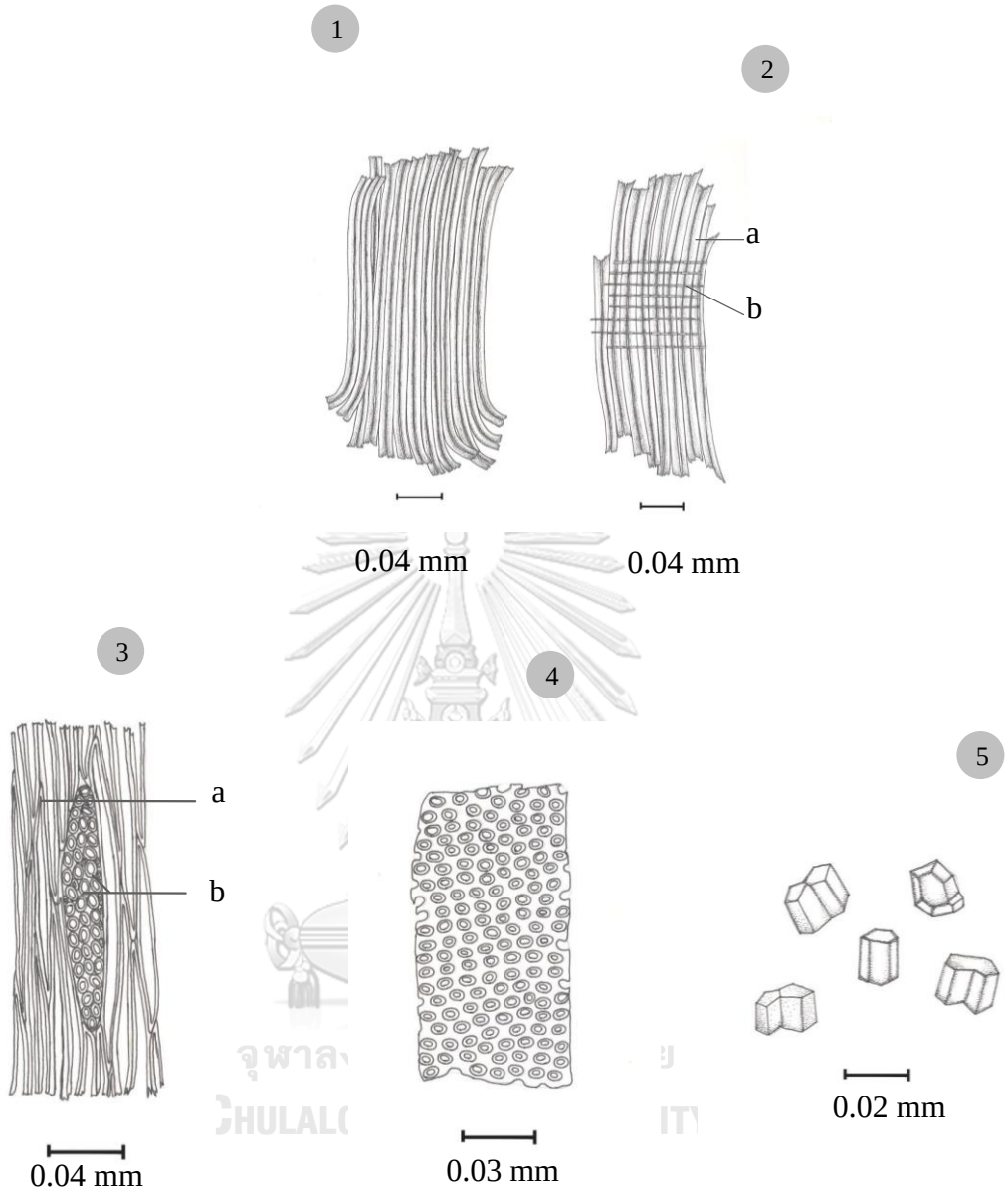


Figure 17 Powdered *Caesalpinia sappan* L. 1. fragment of wood fibers 2. fragment of xylem ray in radial longitudinal view (2a. wood fiber, 2b. wood parenchyma) 3. fragment of xylem ray in tangential longitudinal view (3a. wood fiber, 3b. wood parenchyma)

4. fragment of bordered pitted vessel 5. prism crystals of calcium oxalate

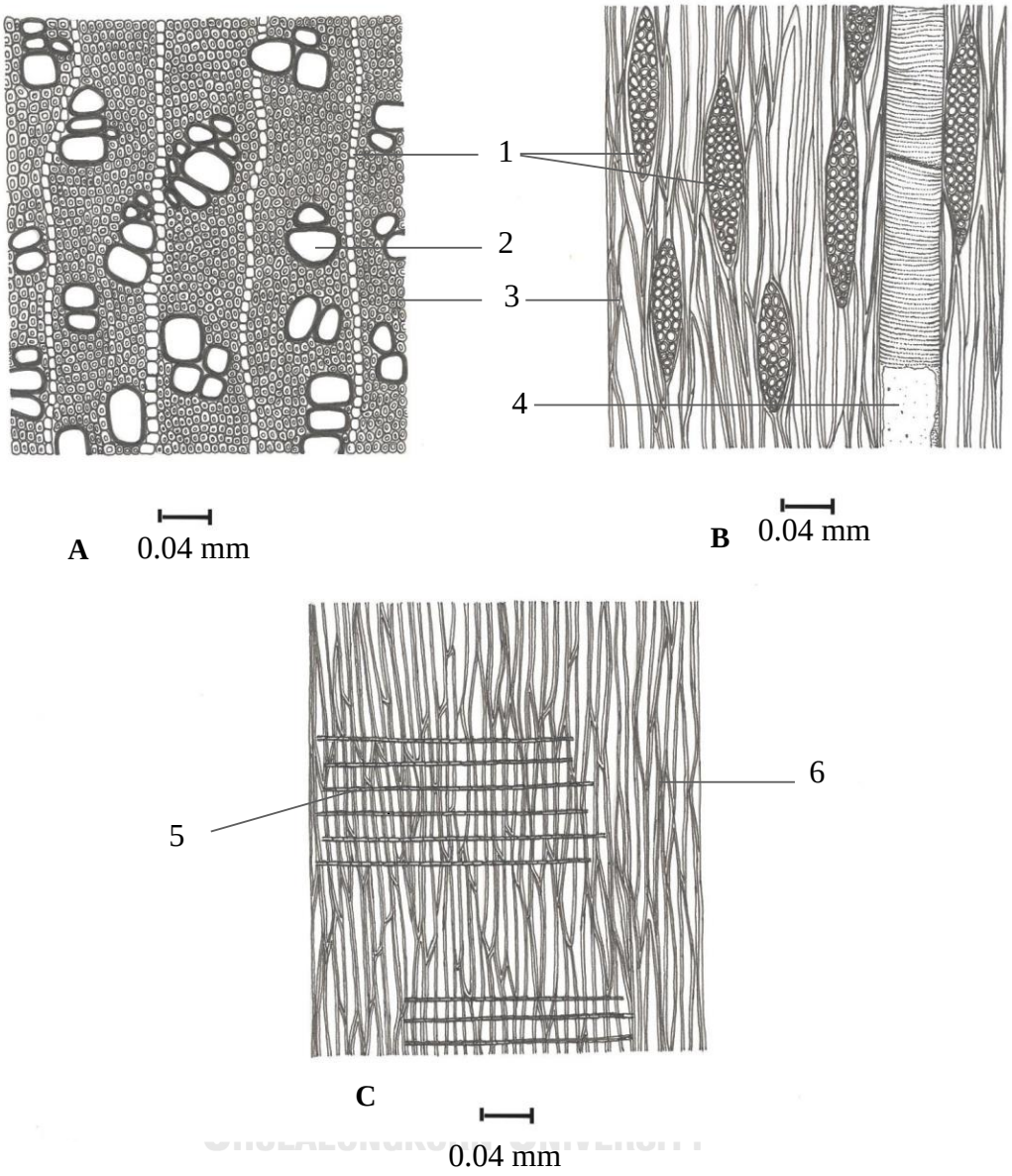


Figure 18 *Caesalpinia sappan* L. A. transverse section, B. tangential longitudinal section, C. radial longitudinal section, 1. wood parenchyma, 2. vessel, 3. wood fiber, 4. vessel, 5. wood parenchyma, 6. wood fiber

Physico-chemical parameters

The physico-chemical parameters of *C. sappan* heartwood showed the values of acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, and water content in Table 12. Thin layer chromatographic fingerprint was shown in Figure 19.

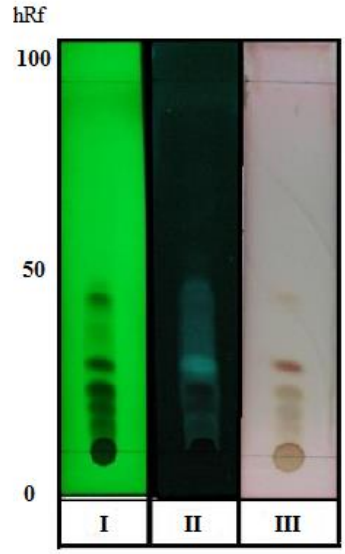


Figure 19 Thin-layer fingerprint of methanolic extract of the wood of *Caesalpinia sappan* L.

Stationary phase: Silica gel 60 GF_{254}

Mobile phase: Chloroform: Methanol 9: 1

Detection I = Detection under UV light 254 nm

II = Detection under UV light 365 nm

III = Detection with p-anisaldehyde/ sulfuric acid reagent

Table 12 The quality parameters of *Caesalpinia sappan* heartwood

Content (% by weight)	***Mean ± SD***	***Min - Max***
Acid-insoluble ash	0.44 ± 0.14	0.22 – 0.71
Total ash	0.88 ± 0.14	0.66 – 1.12
Ethanol-soluble extractive	2.94 ± 0.57	2.00 – 4.00
Water-soluble extractive	3.78 ± 0.63	2.70 – 4.99
Loss on drying	8.51 ± 0.37	7.98 – 9.58
Water content	8.50 ± 0.40	7.80 – 9.20

Part II: Quantitative analysis of brazilin in *Caesalpinia sappan* heartwood

Caesalpinia sappan heartwood ethanolic extracts from 15 different sources in Thailand were performed by soxhlet extraction. The average yield of the ethanolic extract of *C. sappan* heartwood was 15.52 ± 2.14 g/100g dried crude drug (Table 13).

Yield of ethanolic extract

Table 13 Yield of ethanolic extract of *C. sappan* heartwoods from 15 different locations throughout Thailand

Source	**Dried crude drug**	**Ethanolic extract (g)**	**%yield (g/100g)**
1. Bangkok 1	5.00	0.76	15.20
2.Kamphaeng Phet	5.01	0.90	18.03
3. NakhonSi thammarat	5.00	0.84	16.82
4. Nakhon Sawan	5.00	0.72	14.32
5. Bangkok 2	5.00	0.70	13.91

6. Nakhon Ratchasima	5.00	0.78	15.66
7. Phichit	5.00	0.70	14.09
8. Chiang Rai	5.01	0.84	16.78
9. Nakhon Pathom	5.00	0.56	11.10
10. Phuket	5.01	0.79	15.75
11. Chanthaburi	5.00	0.78	15.56
12. Phetchabun	5.00	0.99	19.76
13. Chiang Mai	5.00	0.64	12.75
14. Ubon Ratchathani	5.00	0.86	17.15
15. Nong Khai	5.01	0.80	15.91
Average			**15.52± 2.14**

To determine brazilin content in the ethanolic extracts by TLC technique, the silica gel 60 GF_{254} was used as a stationary phase and the solvents including chloroform: ethyl acetate: formic acid (10: 8: 2) were used as mobile phase. The band of each sample which contained brazilin was confirmed by comparing the Rf value with standard brazilin. TLC chromatograms of 15 samples and standard brazilin under UV 254 nm, under UV 365 nm, and under daylight were demonstrated in Figure 20. TLC densitogram scanned at λmax of 525 nm was shown in Figure 21.

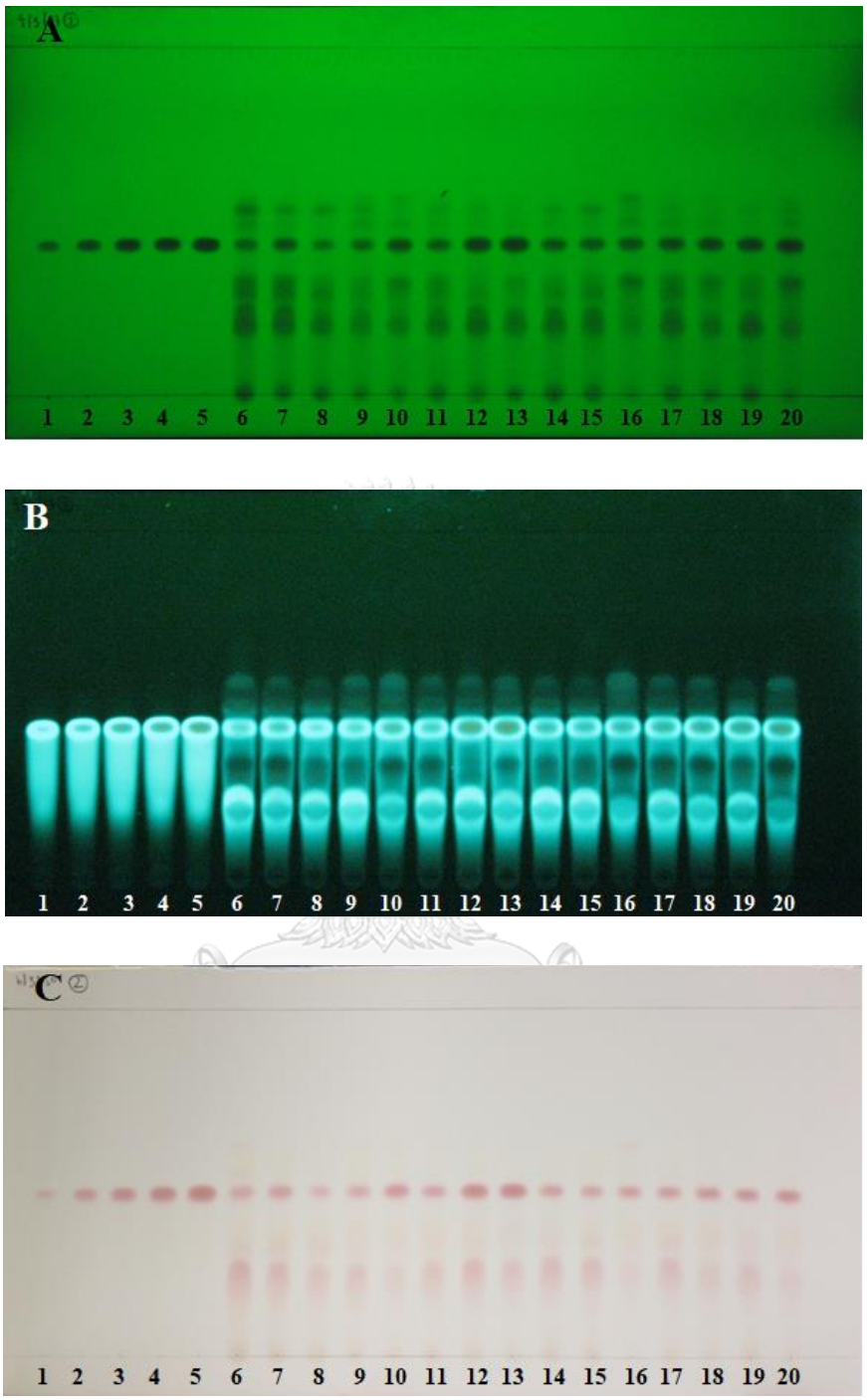


Figure 20 TLC plate under UV 254 nm (A), under UV 365 nm (B), under daylight (C) [track 1-5 = brazilin, track 6-20 = *C. sappan* heartwood ethanolic extracts from 15 different sources in Thailand]

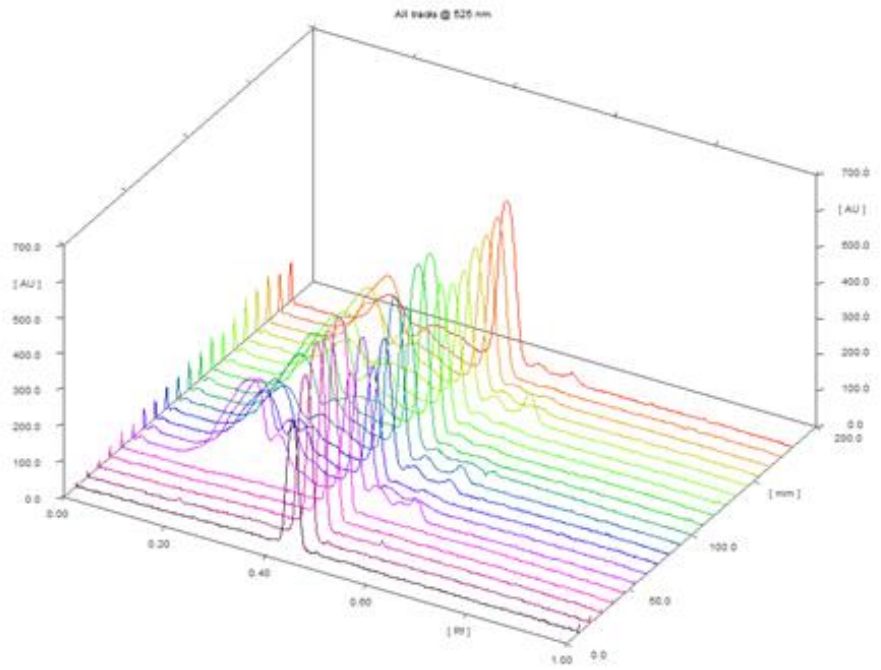


Figure 21 TLC densitogram of brazilin standard and brazilin in *Caesalpinia sappan* heartwood samples

The amount of brazilin in *C. sappan* heartwoods by TLC-densitometry

Brazilin contents in *C. sappan* heartwoods were determined in triplicate by TLC-densitometry and the average of brazilin in crude drug was 1.259 ± 0.455 % (Table 14).

Table 14 Amount of brazilin in *C. sappan* heartwoods by TLC-densitometry (% by dried weight)

Sample	Brazilin in ethanolic extract (g/g)	Yield of ethanolic extract (g/100g of crude drug)	Brazilin in crude drug (g/100 g crude drug)
Bangkok 1	0.072	13.911	0.996
Nakhon Ratchasima	0.087	15.662	1.368
Nakhon Sithammarat	0.055	16.820	0.917
Nakhon Sawan	0.072	14.320	1.031
Chiang Mai	0.099	12.746	1.267
Phichit	0.101	14.095	1.431
Ubon Ratchathani	0.118	17.145	2.024
Phuket	0.090	15.754	1.424
Kamphaeng Phet	0.072	18.025	1.290
Bangkok 2	0.076	15.200	1.157
Nakhon Pathom	0.076	11.103	0.847
Chanthaburi	0.074	15.558	1.155
Chiang Rai	0.075	16.782	1.262
Phetchabun	0.075	19.762	1.482
Nong Khai	0.078	15.911	1.238
Average			**1.259 ± 0.455**

Method validation of TLC-densitometry

Calibration curve

The calibration curve of brazilin was performed at of 0.150, 0.225, 0.300, 0.375, and 0.450 µg/spot. Regression equation of brazilin was $y = -71405x^2 + 82212x - 1388$ and the coefficient of determination (R^2) was 0.9953. Figure 22 presented the calibration curve of brazilin by TLC-densitometry.

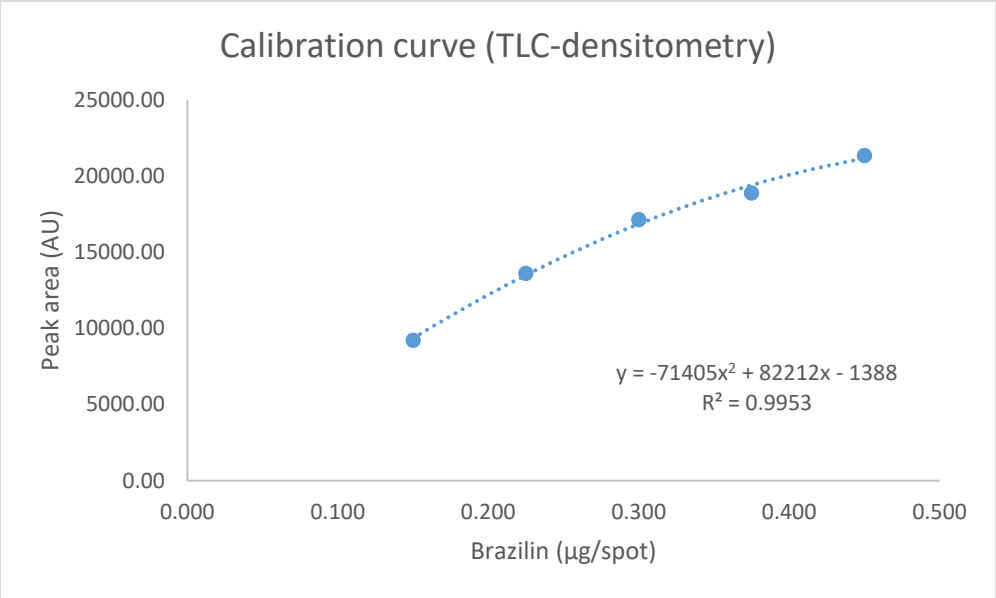


Figure 22 Calibration curve of brazilin by TLC-densitometry

Accuracy

Accuracy was evaluated in percentage of recovery. This method was done by spiking the standard of brazilin at three different concentrations (0.03, 0.09, 0.18 µg/sport) in the sample. The recovery was presented between 94.11 to 103.68 % (Table 15).

Table 15 Percent recovery of brazilin by TLC-densitometry

Brazilin added (µg/spot)	Brazilin found (µg/spot)	% Recovery
0.00	0.196	-
0.03	0.234	103.675
0.09	0.276	96.506
0.18	0.354	94.106

Precision

Precision consisted of repeatability and intermediate precision. Repeatability was performed on samples in the same day with three different concentrations while an intermediate precision was determined in different days. The results were shown as the percentage of relative standard deviation (% RSD). Repeatability and intermediate precision were between 2.230 to 4.336 %RSD and 2.918 to 7.686 %RSD, respectively. The results were shown in Table 16.

Table 16 Precision of brazilin quantitation by TLC-densitometry

Repeatability		Intermediate precision	
Brazilin (µg/spot)	%RSD	Brazilin (µg/spot)	%RSD
0.168 ± 0.006	3.481	0.178 ± 0.012	7.017
0.220 ± 0.010	4.336	0.224 ± 0.017	7.686
0.294 ± 0.007	2.230	0.285 ± 0.012	4.137
0.376 ± 0.010	2.622	0.370 ± 0.011	2.918

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

Limit of detection and limit of quantitation were calculated based on the residual standard deviation of a regression line and slope of calibration curve. LOD and LOQ of brazilin were 0.0211 and 0.0640 µg/spot, respectively.

Robustness

This method was assessed by slightly changing in mobile phase ratio and each variation was evaluated in triplicate. The result showed 2.12 %RSD of robustness. Peak area of brazilin was between 18831.61 and 19140.27 (Table 17).

Table 17 Robustness of brazilin by TLC-densitometry

Mobile phase ratio (v/v)			**Peak area of brazilin**
Chloroform	**Ethyl acetate**	**Formic acid**	
10.2	8.2	1.8	18831.61
10.0	8.0	2.0	18350.70
9.8	7.8	2.2	19140.27
Average			**18774.20 ± 397.90**
%RSD			**2.12**

Specificity

The absorbance spectra of brazilin scanned in the range of 200- 700 nm demonstrated the λmax of 525 nm. The identity in absorbance spectra assessed at the peak apex among brazilin standards and the spot of each sample at the same *Rf* value (Figure 23). The purity evaluated at up-slope, apex, and down-slope of the sample peak (Figure 24).

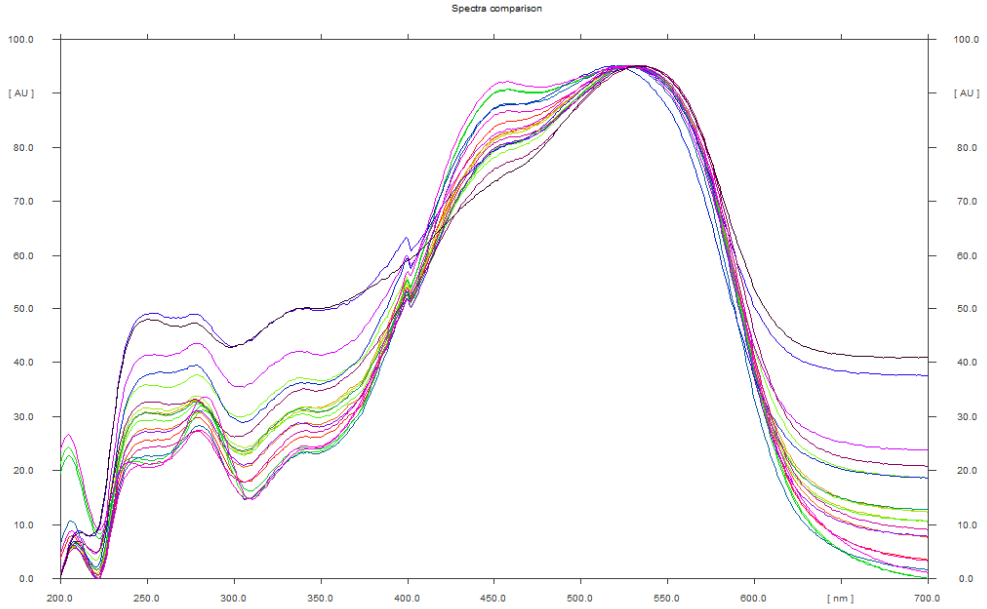


Figure 23 The absorbance spectra of brazilin in standard and sample bands

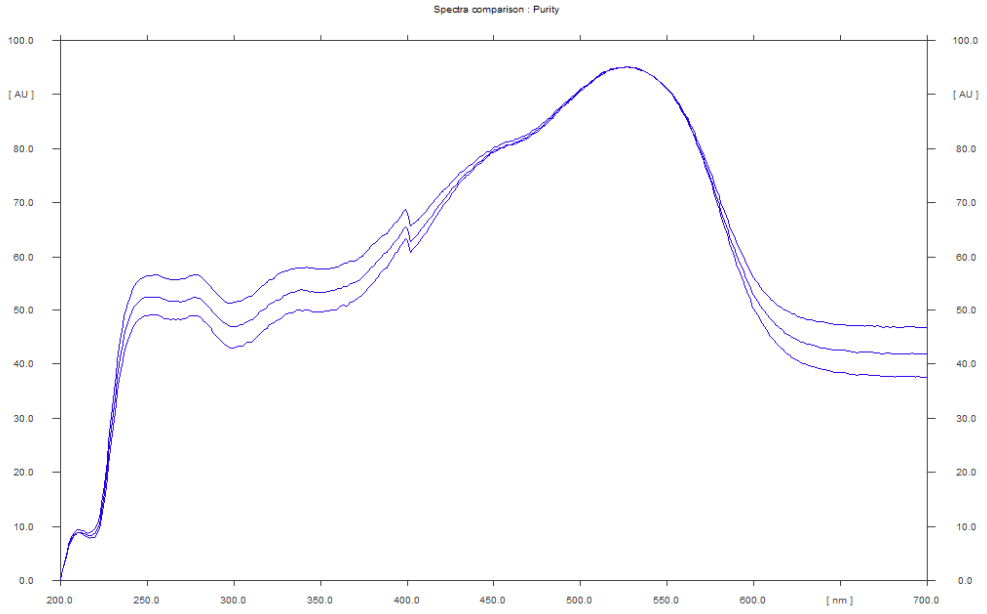


Figure 24 The absorbance spectra of brazilin in the extract detected at apex, up-slope, and down-slope of the peak

The amount of brazilin in *C. sappan* heartwoods by TLC-image analysis

Brazilin contents in *C. sappan* heartwoods were determined in triplicate by TLC-image analysis and the average of brazilin in crude drug was 1.256 ± 0.405 % (Table 18).

Table 18 Amount of brazilin in *C. sappan* heartwoods by TLC-image analysis (% by dried weight)

Sample	Brazilin in ethanolic extract (g/g)	Yield of ethanolic extract (g/100g of crude drug)	Brazilin in crude drug (g/100 g crude drug)
Bangkok 1	0.072	13.911	0.996
Nakhon Ratchasima	0.088	15.662	1.386
Nakhon Sithammarat	0.056	16.820	0.934
Nakhon Sawan	0.074	14.320	1.057
Chiang Mai	0.098	12.746	1.248
Phichit	0.101	14.095	1.424
Ubon Ratchathani	0.114	17.145	1.953
Phuket	0.090	15.754	1.418
Kamphaeng Phet	0.070	18.025	1.267
Bangkok 2	0.078	15.200	1.191
Nakhon Pathom	0.078	11.103	0.865
Chanthaburi	0.074	15.558	1.156
Chiang Rai	0.073	16.782	1.233
Phetchabun	0.075	19.762	1.480
Nong Khai	0.077	15.911	1.228
Average			**1.256 ± 0.405**

Method validation of TLC-image analysis

Calibration curve

The calibration curve of brazilin was in the range of 0.150 - 0.450 µg/spot. Regression equation of brazilin was $y = -75987x^2 + 87386x - 2840.8$ and the coefficient of determination (R^2) was 0.9977. Figure 25 presented the calibration curve of brazilin by TLC-image analysis.

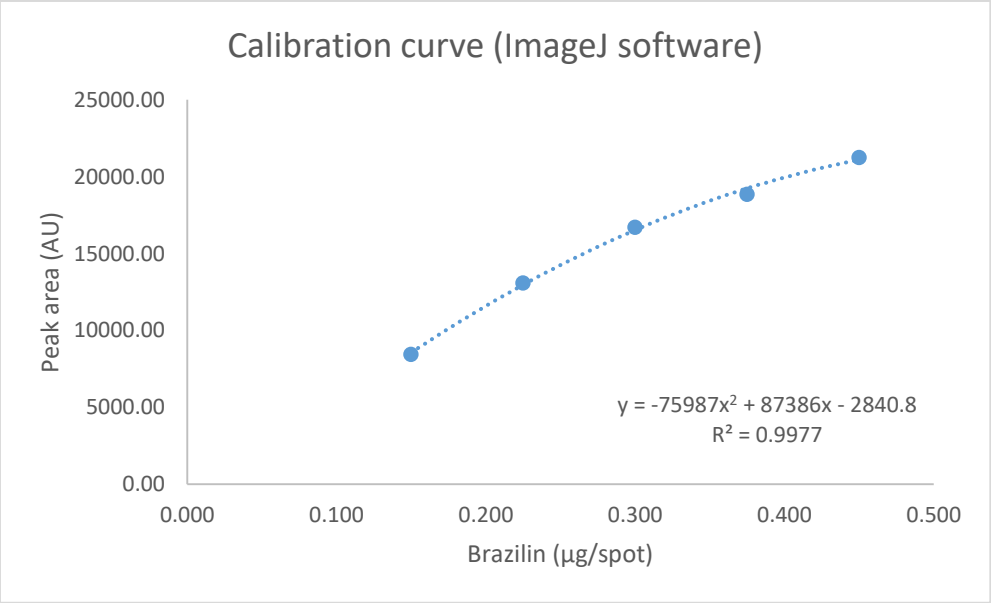


Figure 25 Calibration curve of brazilin by TLC-image analysis

Accuracy

Accuracy was evaluated in percentage of recovery. The recovery was presented between 93.69 to 104.61 % (Table 19).

Table 19 Recovery of brazilin by TLC-image analysis

Brazilin added (µg/spot)	Brazilin found (µg/spot)	% Recovery
0.00	0.193	-
0.03	0.233	104.611
0.09	0.270	95.279
0.18	0.350	93.694

Precision

The results were shown as the percentage of relative standard deviation (% RSD). Repeatability and intermediate precision were between 3.012 to 4.949 %RSD and 6.454 to 13.514 %RSD, respectively. The results were shown in Table 20.

Table 20 Precision of brazilin quantitation by TLC-image analysis

Repeatability		Intermediate precision	
Brazilin (µg/spot)	%RSD	Brazilin (µg/spot)	%RSD
0.187 ± 0.015	8.183	0.180 ± 0.010	5.556
0.233 ± 0.012	4.949	0.237 ± 0.015	6.454
0.300 ± 0.010	3.333	0.300 ± 0.036	12.019
0.383 ± 0.012	3.012	0.370 ± 0.050	13.514

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

Limit of detection and limit of quantitation were calculated based on the residual standard deviation of a regression line and slope of calibration curve. LOD and LOQ of brazilin were 0.0148 and 0.0448 µg/spot, respectively.

Robustness

By slightly changing in mobile phase ratio the result showed 3.96 %RSD of robustness. Peak area of brazilin was between 11490.74 and 12340.74 (Table 21).

Table 21 Robustness of brazilin by TLC-image analysis

Mobile phase ratio (v/v)			**Peak area of brazilin**
Chloroform	**Ethyl acetate**	**Formic acid**	
10.2	8.2	1.8	12340.74
10.0	8.0	2.0	11490.74
9.8	7.8	2.2	11577.99
Average			**11803.16 ± 467.60**
%RSD			**3.96**

The comparison of brazilin content between TLC-densitometry and TLC-image analysis

Brazilin from TLC-densitometry and TLC-image analysis was 1.259 ± 0.455 and 1.256 ± 0.405 g/100g dried heartwood, respectively (Table 22). The result showed that both techniques were not statistically significantly different (P> 0.05) using paired *t*-test.

Table 22 Comparison of brazilin content between TLC-densitometry and TLC-image analysis

Sample	Brazilin in C. sappan heartwoods (g/100 g crude drug)	
	TLC-densitometry	TLC-image analysis
Bangkok 1	0.996	0.996
Nakhon Ratchasima	1.368	1.386
Nakhon Sithammarat	0.917	0.934
Nakhon Sawan	1.031	1.057
Chiang Mai	1.267	1.248
Phichit	1.431	1.424
Ubon Ratchathani	2.024	1.953
Phuket	1.424	1.418
Kamphaeng Phet	1.290	1.267
Bangkok 2	1.157	1.191
Nakhon Pathom	0.847	0.865
Chanthaburi	1.155	1.156
Chiang Rai	1.262	1.233
Phetchabun	1.482	1.480
Nong Khai	1.238	1.228
Average (Mean ± SD)	**1.259 ± 0.455**	**1.256 ± 0.405**

Part III: Plant morphology and microscopic characteristics including leaf constant numbers of selected *Caesalpinia* species

The results of macroscopic evaluation

Eight *Caesalpinia* species including *C. bonduc* (L.) Roxb., *C. coriaria* (Jacq.) Willd., *C. decapetala* (Roth) Alston, *C. digyna* Rottler, *C. mimosoides* Lam., *C. minax* Hance, *C. pulcherrima* (L.) Sw., and *C. sappan* L. were observed, photographed, and illustrated by hand drawing in the proportion size related to an original scale.

***Caesalpinia bonduc* (L.) Roxb.**

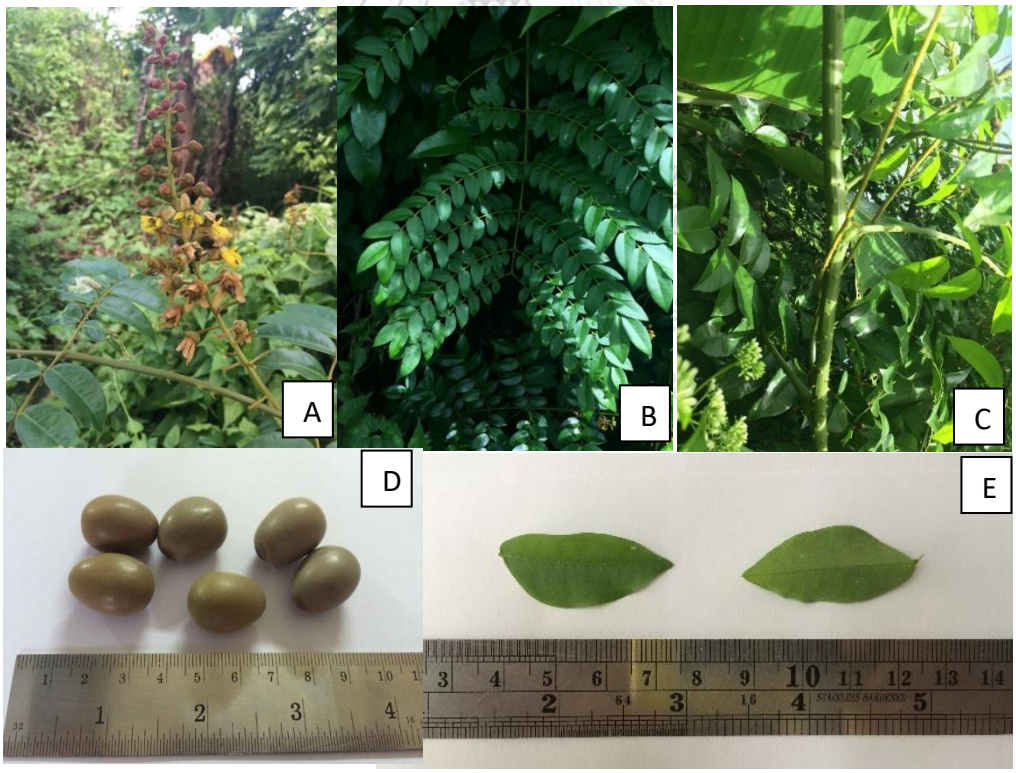


Figure 26 *Caesalpinia bonduc* (L.) Roxb.; (A) Flowers, (B) & (E) Leaves, (C) Branches, (D) Seeds

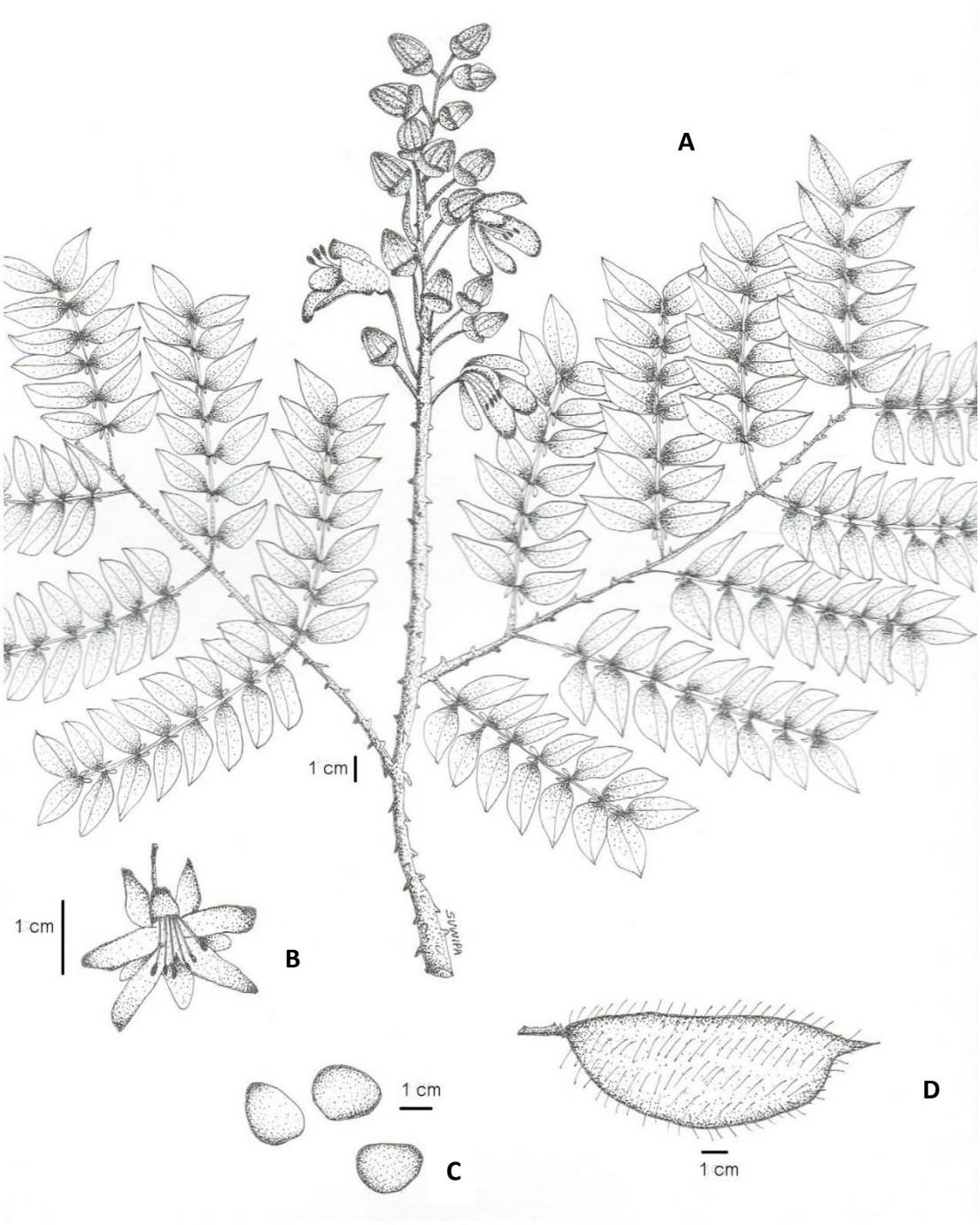


Figure 27 *Caesalpinia bonduc* (L.) Roxb.; A. whole plant, B. flower, C. seeds, D. pod

***Caesalpinia coriaria* (Jacq.) Willd.**

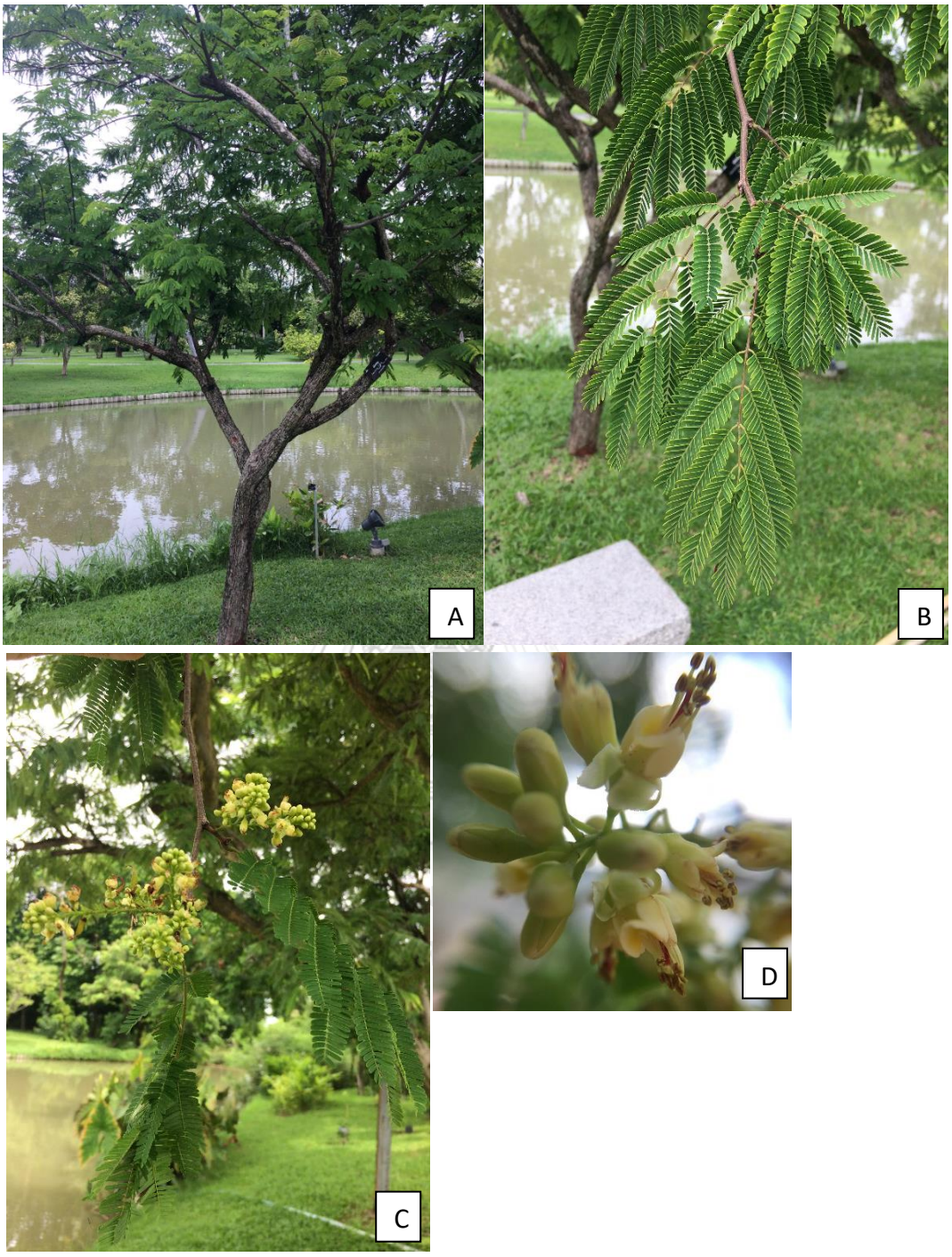


Figure 28 *Caesalpinia coriaria* (Jacq.) Willd.; (A) whole plant, (B) Leaves, (C) & (D) Flowers

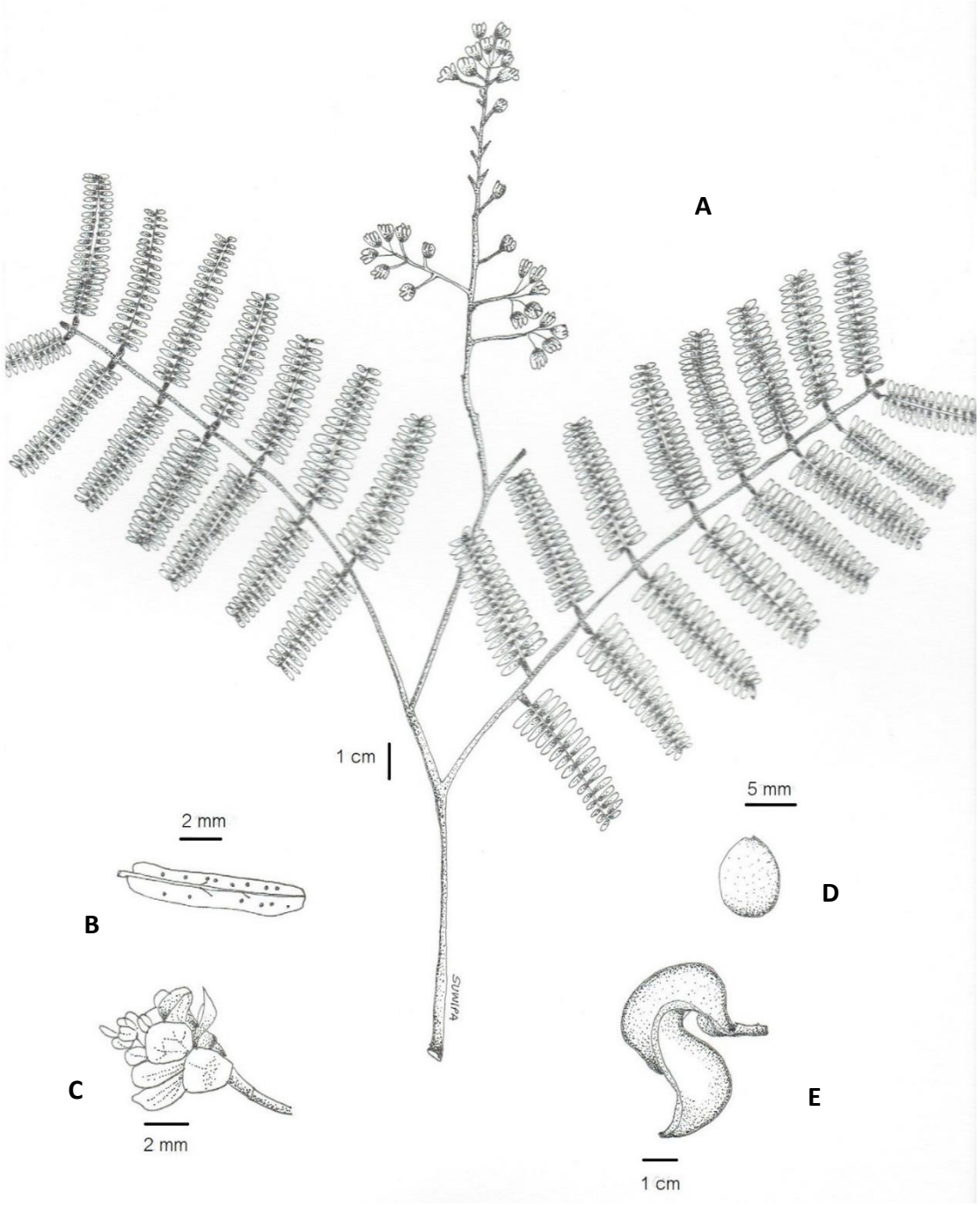


Figure 29 *Caesalpinia coriaria* (Jacq.) Willd.; A. whole plant, B. leaf, C. flower, D. seed, E. pod

Caesalpinia decapetala (Roth) Alston

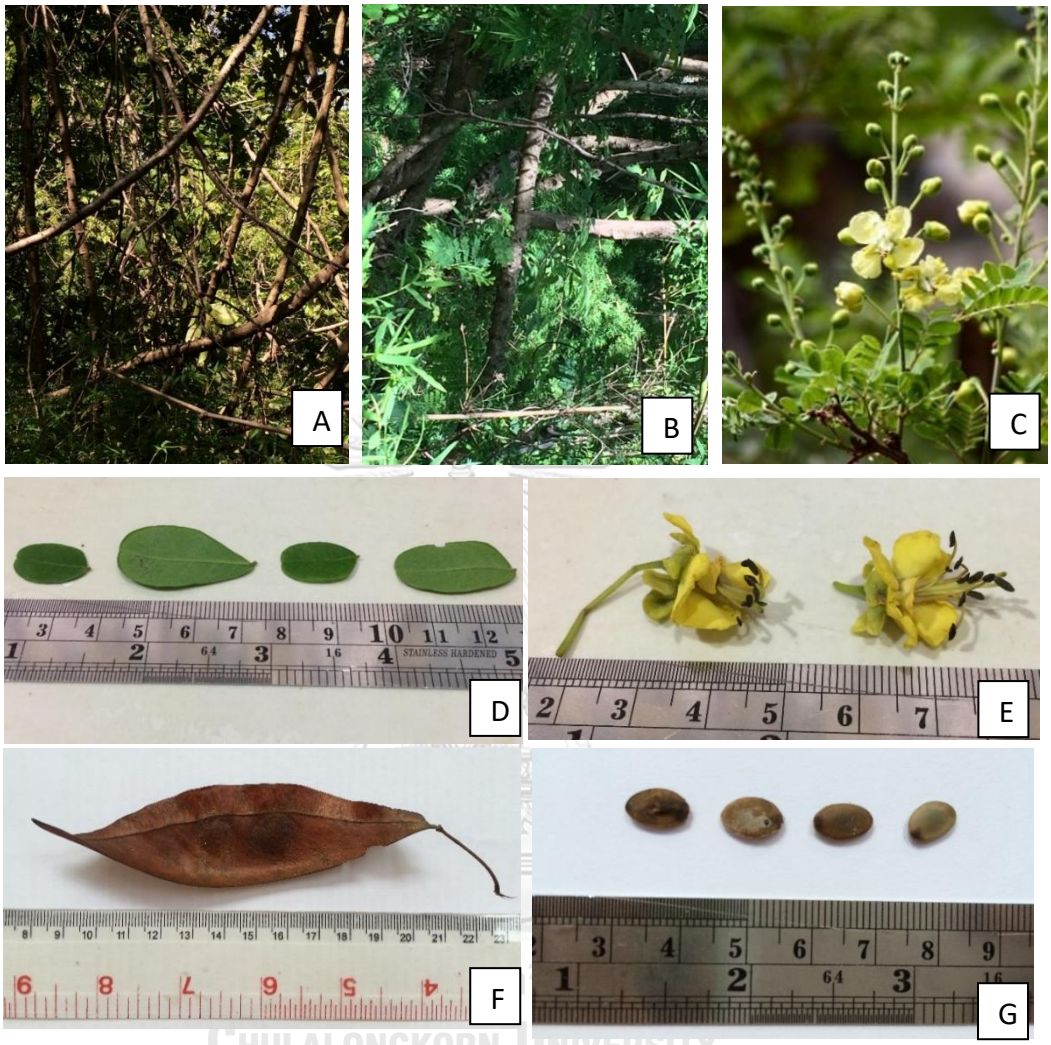


Figure 30 *Caesalpinia decapetala* (Roth) Alston; (A) & (B) stems, (C) & (E) Flowers, (D) Leaves, (F) Pod, (G) Seeds

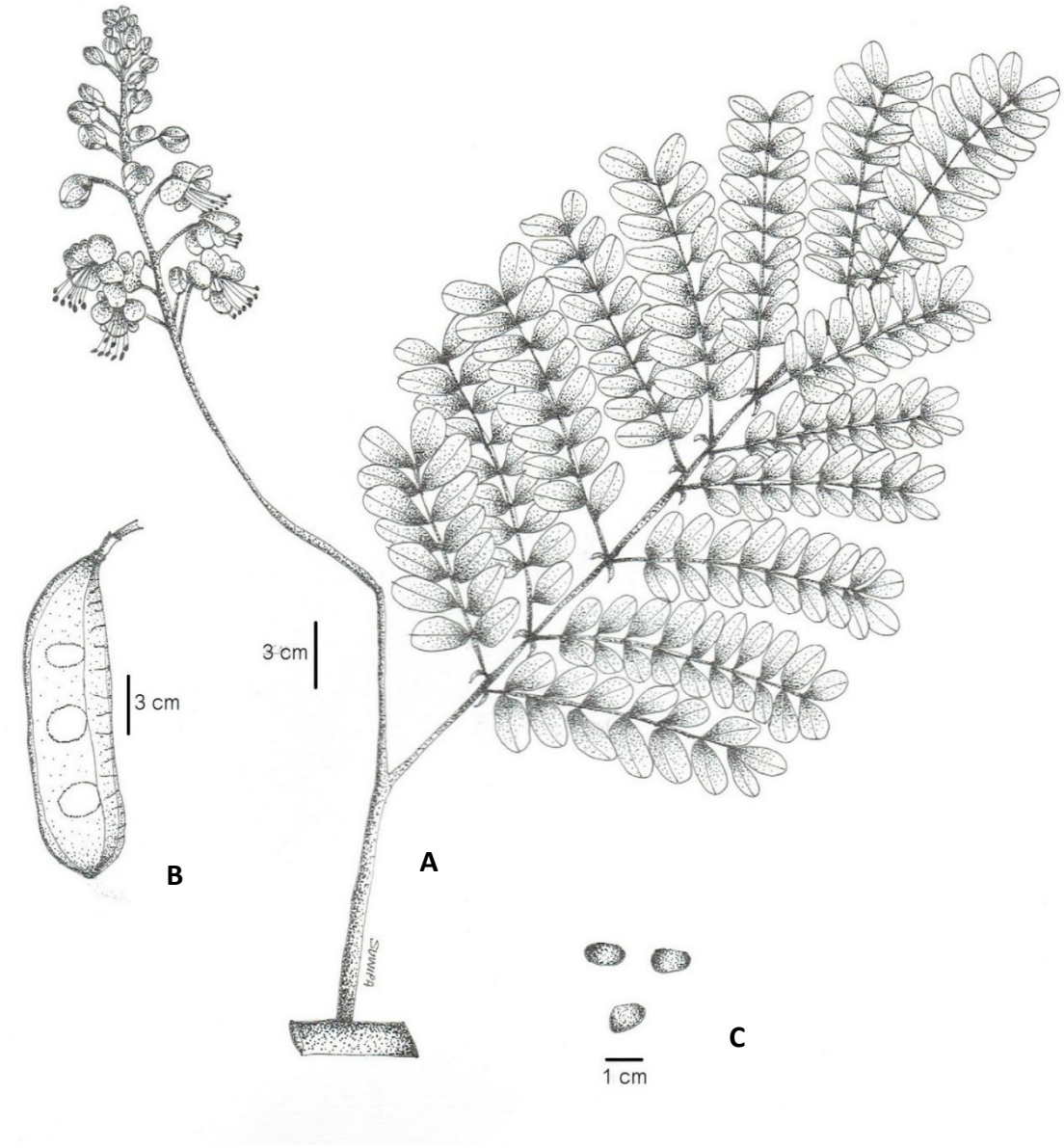


Figure 31 *Caesalpinia decapetala* (Roth) Alston; A. whole plant, B. pod, C. seeds

***Caesalpinia digyna* Rottler**

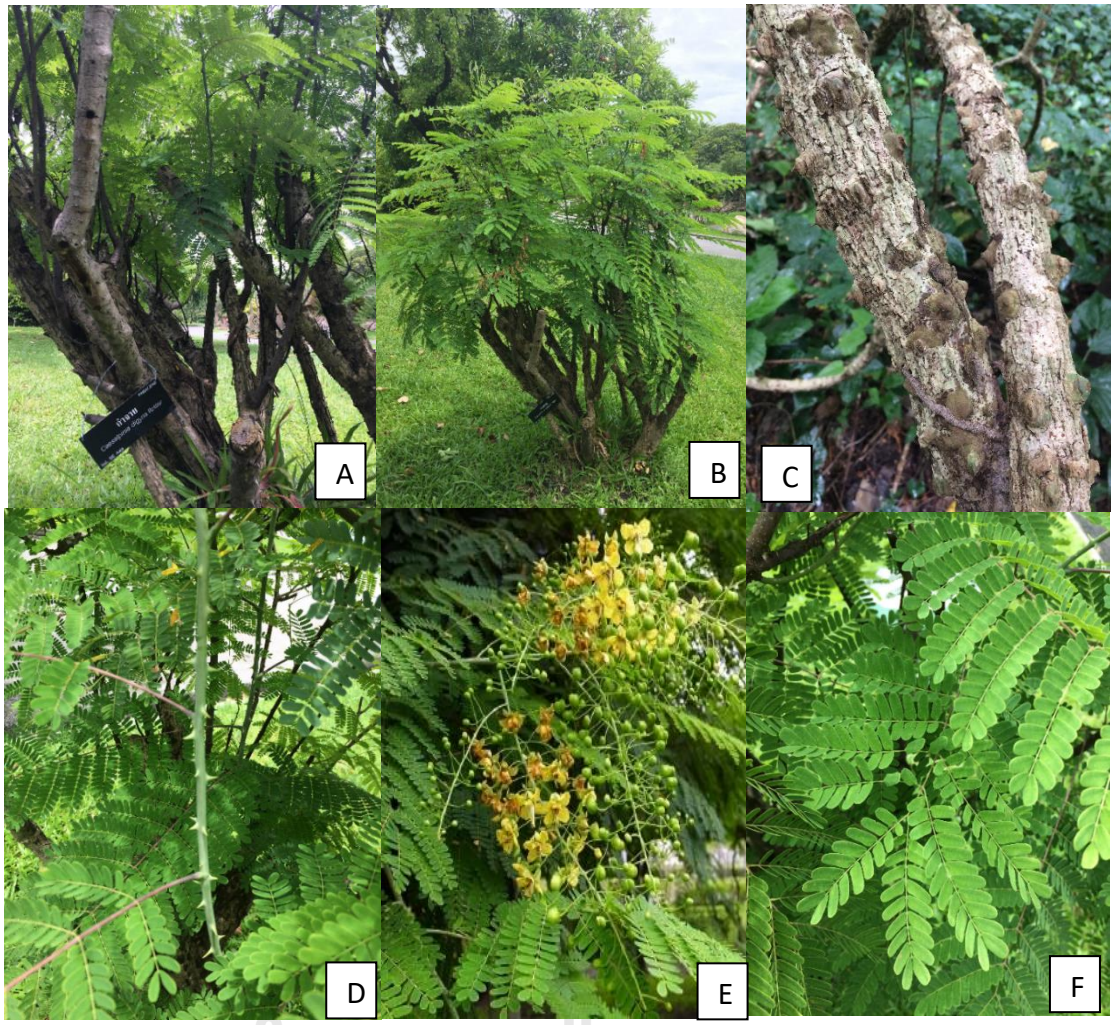


Figure 32 *Caesalpinia digyna* Rottler; (A) Stem, (B) Whole plant, (C) Trunk, (D) Branch, (E) Flowers, (F) Leaves

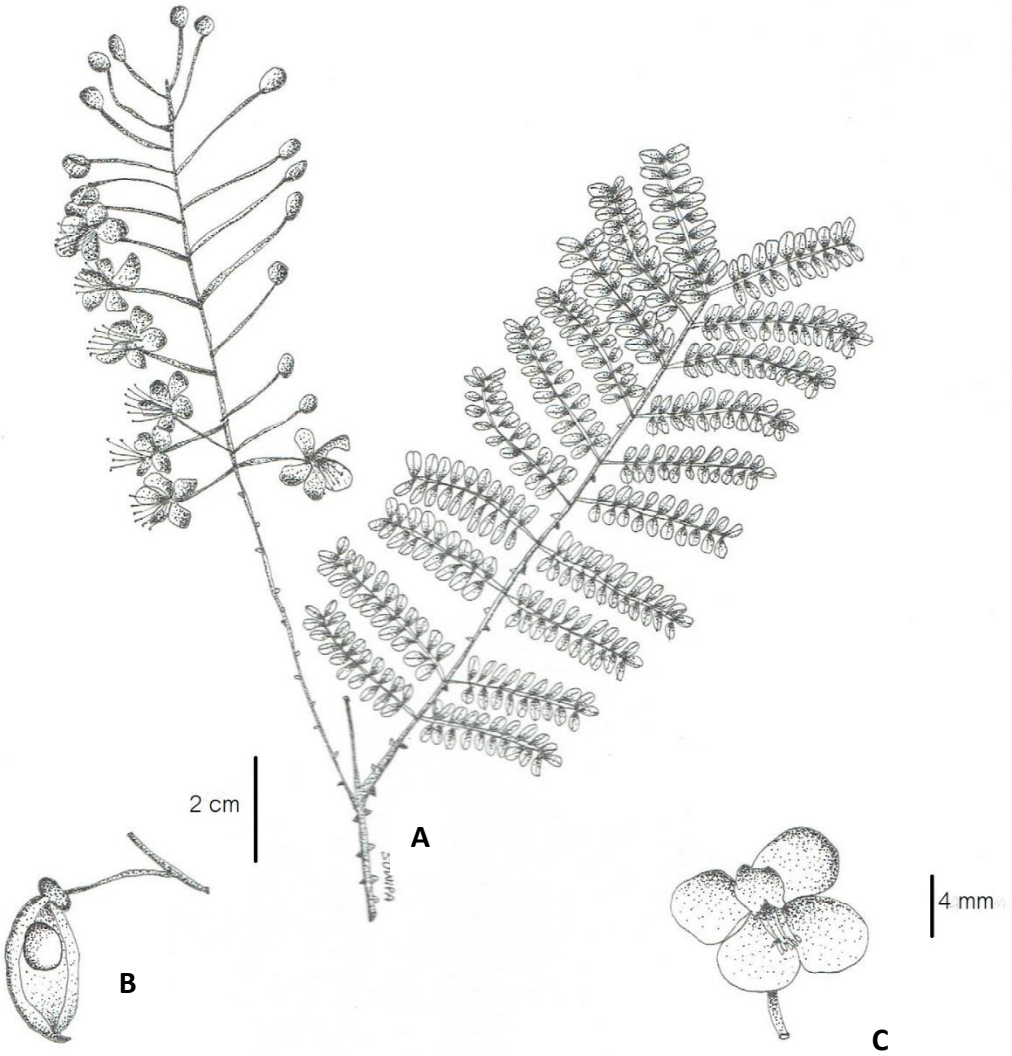


Figure 33 *Caesalpinia digyna* Rottler; A. whole plant, B. pod, C. flower

***Caesalpinia mimosoides* Lam.**

Figure 34 *Caesalpinia mimosoides* Lam.; (A) & (B) Flowers, (C) Branches, (D) Leaves, (E) Pods

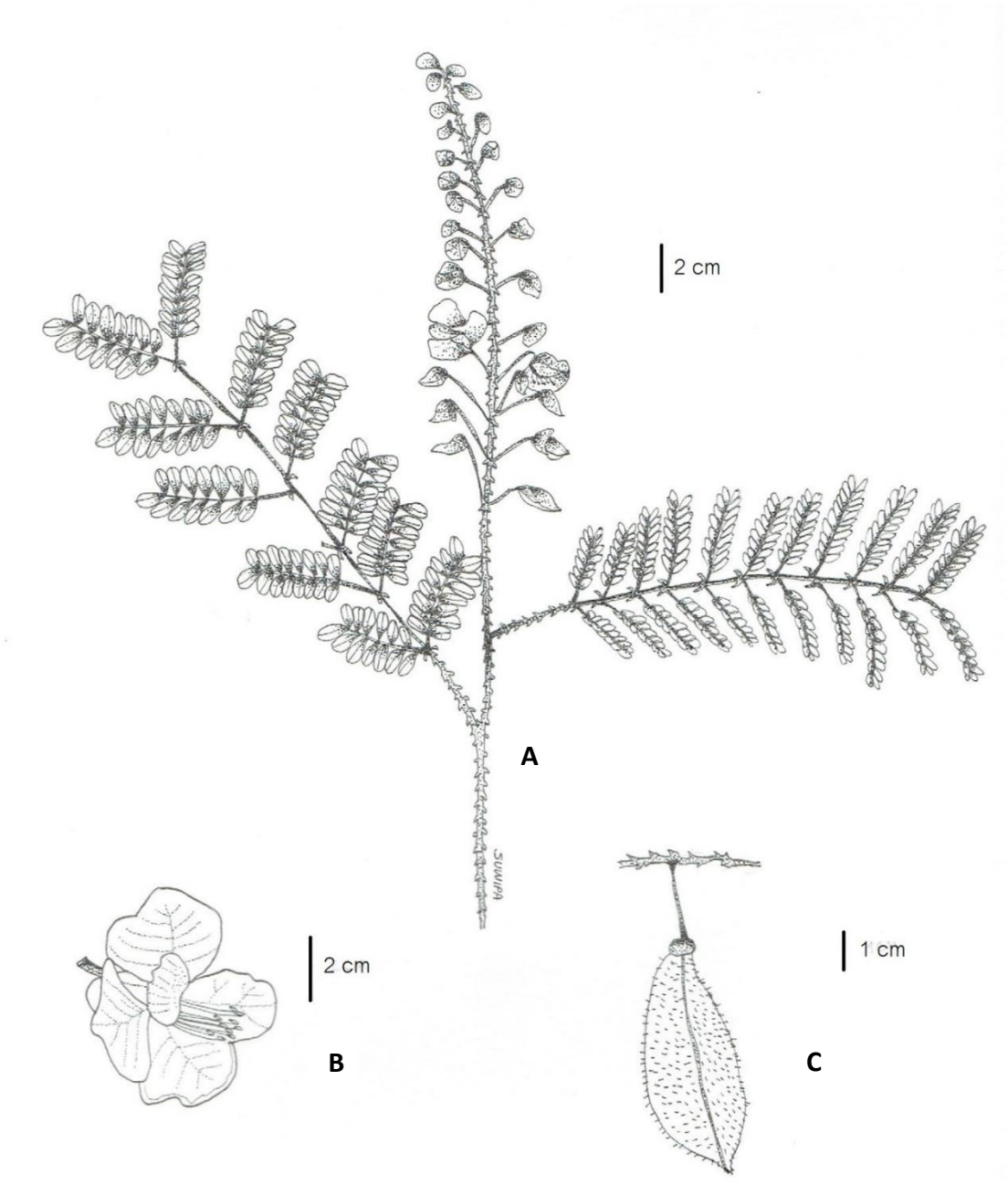


Figure 35 *Caesalpinia mimosoides* Lam.; A. whole plant, B. flower, C. pod

Caesalpinia minax Hance

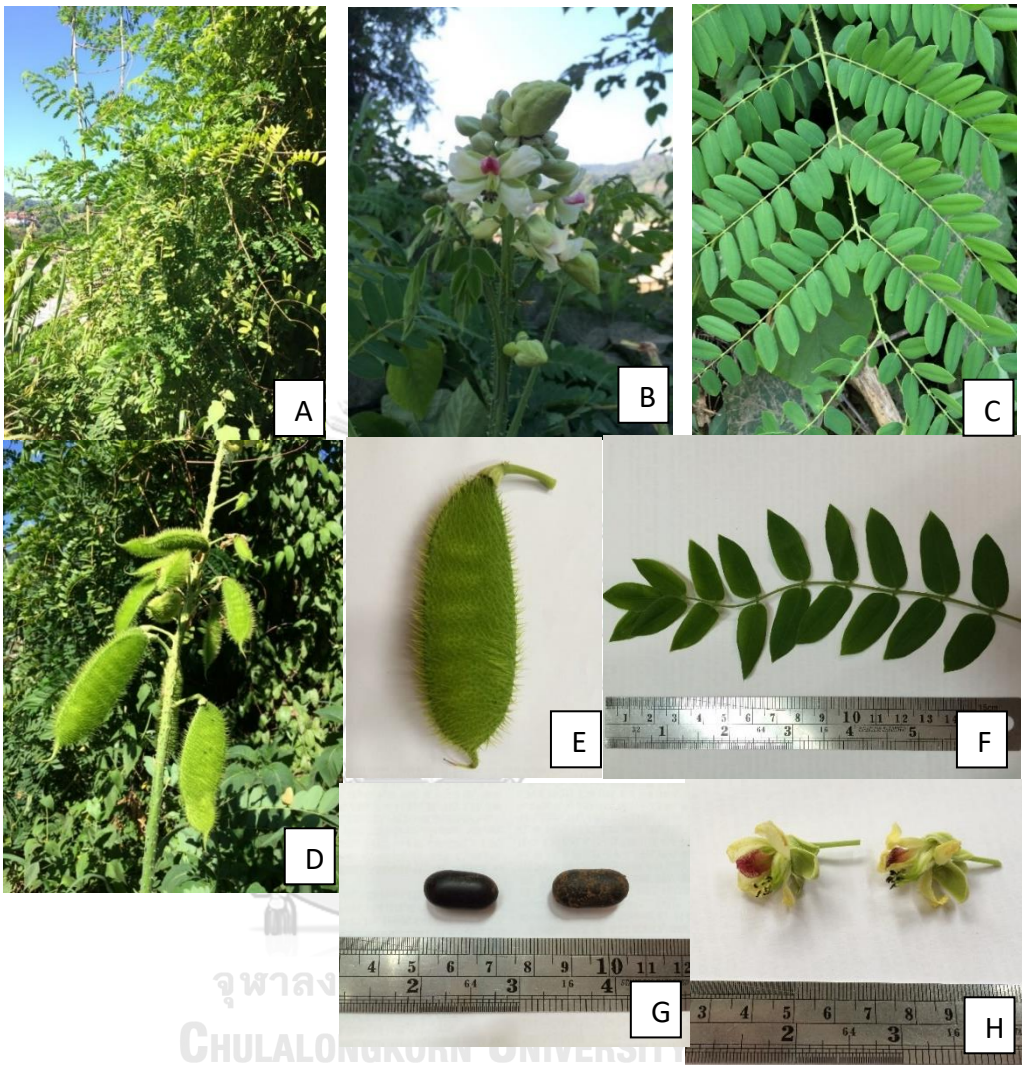


Figure 36 *Caesalpinia minax* Hance; (A) Whole plant, (B) & (H) Flowers, (C) & (F) Leaves, (D) & (E) Pods, (G) Seeds

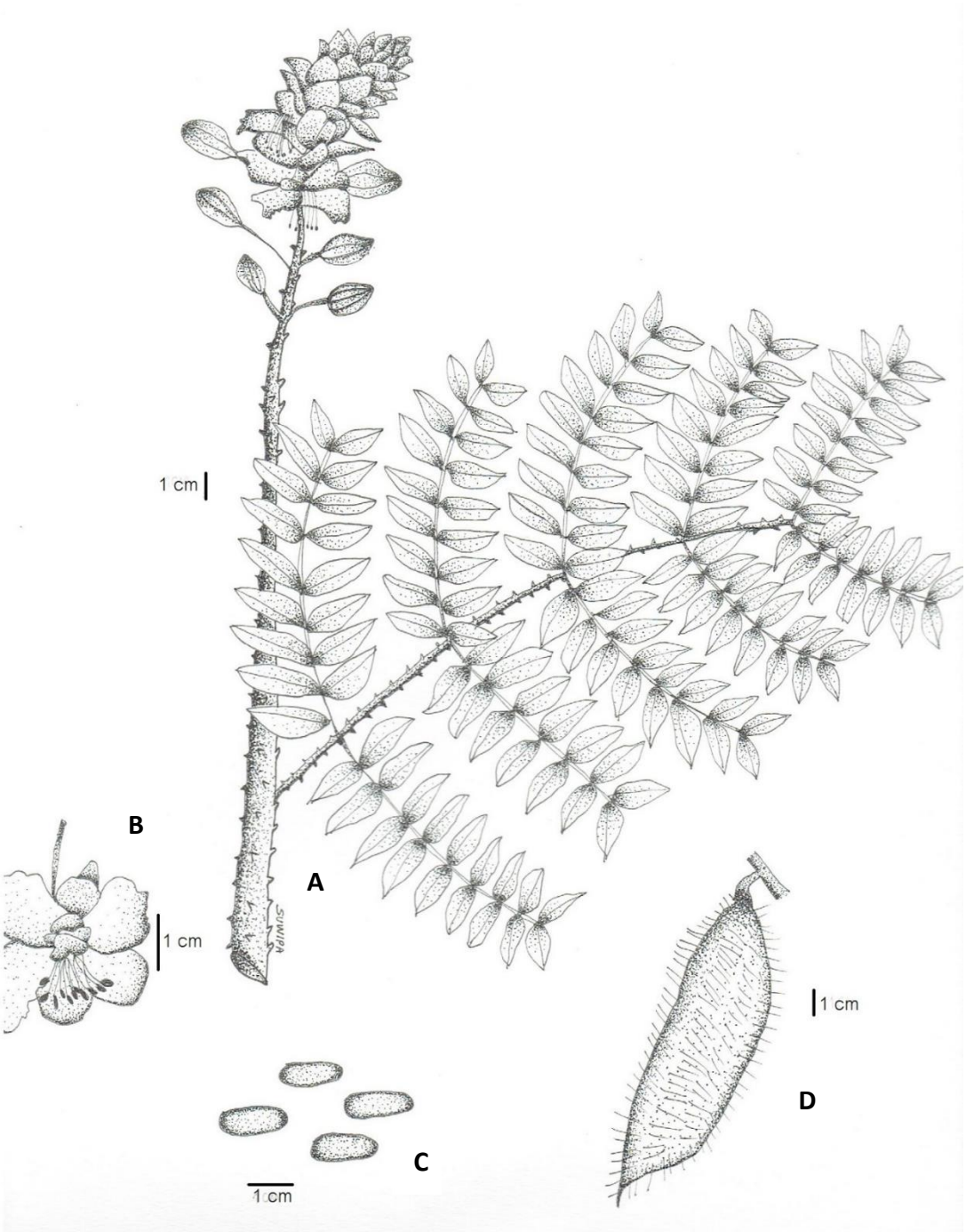


Figure 37 *Caesalpinia minax* Hance; A. whole plant, B. flower, C. seeds, D. pod

Caesalpinia pulcherrima (L.) Sw.

Figure 38 *Caesalpinia pulcherrima* (L.) Sw.; (A) & (B) Whole plant, (C) & (G) Leaves, (D) Trunks, (E) Flowers, (F) Pod

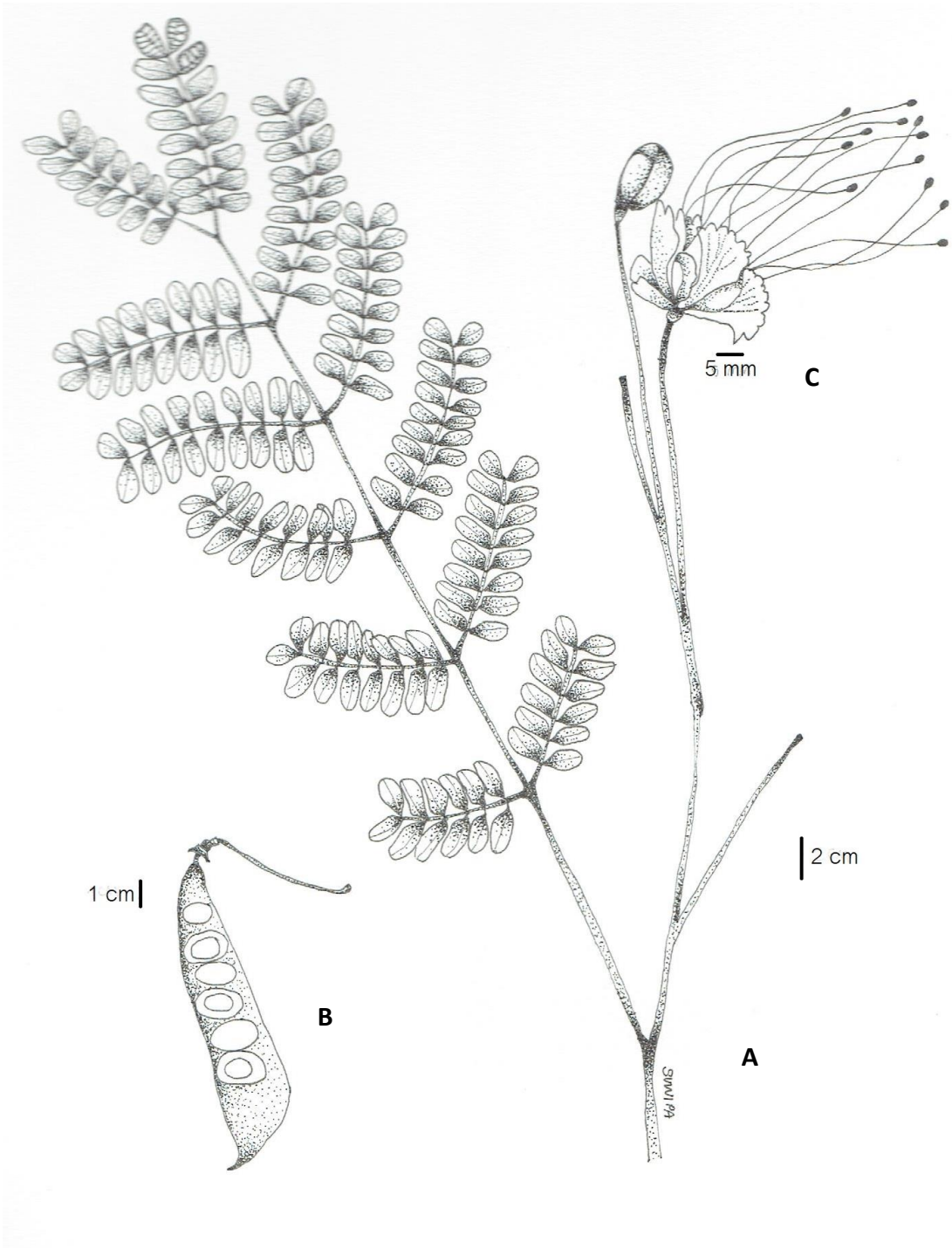


Figure 39 *Caesalpinia pulcherrima* (L.) Sw.; A. whole plant, B. pod, C. flower

Caesalpinia sappan L.

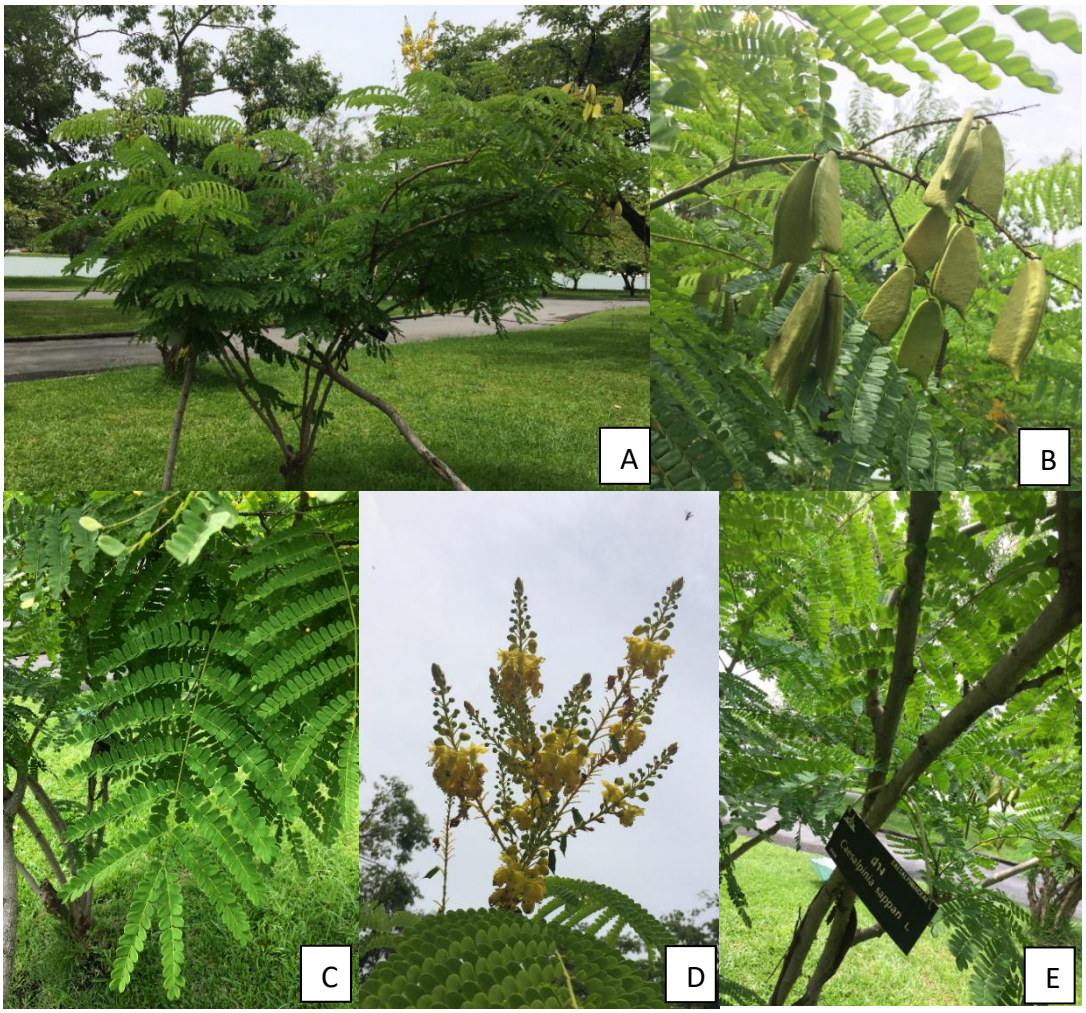


Figure 40 *Caesalpinia sappan* L.; (A) Whole plant, (B) Pods (C) Leaves, (D) Flowers, (E) Trunks

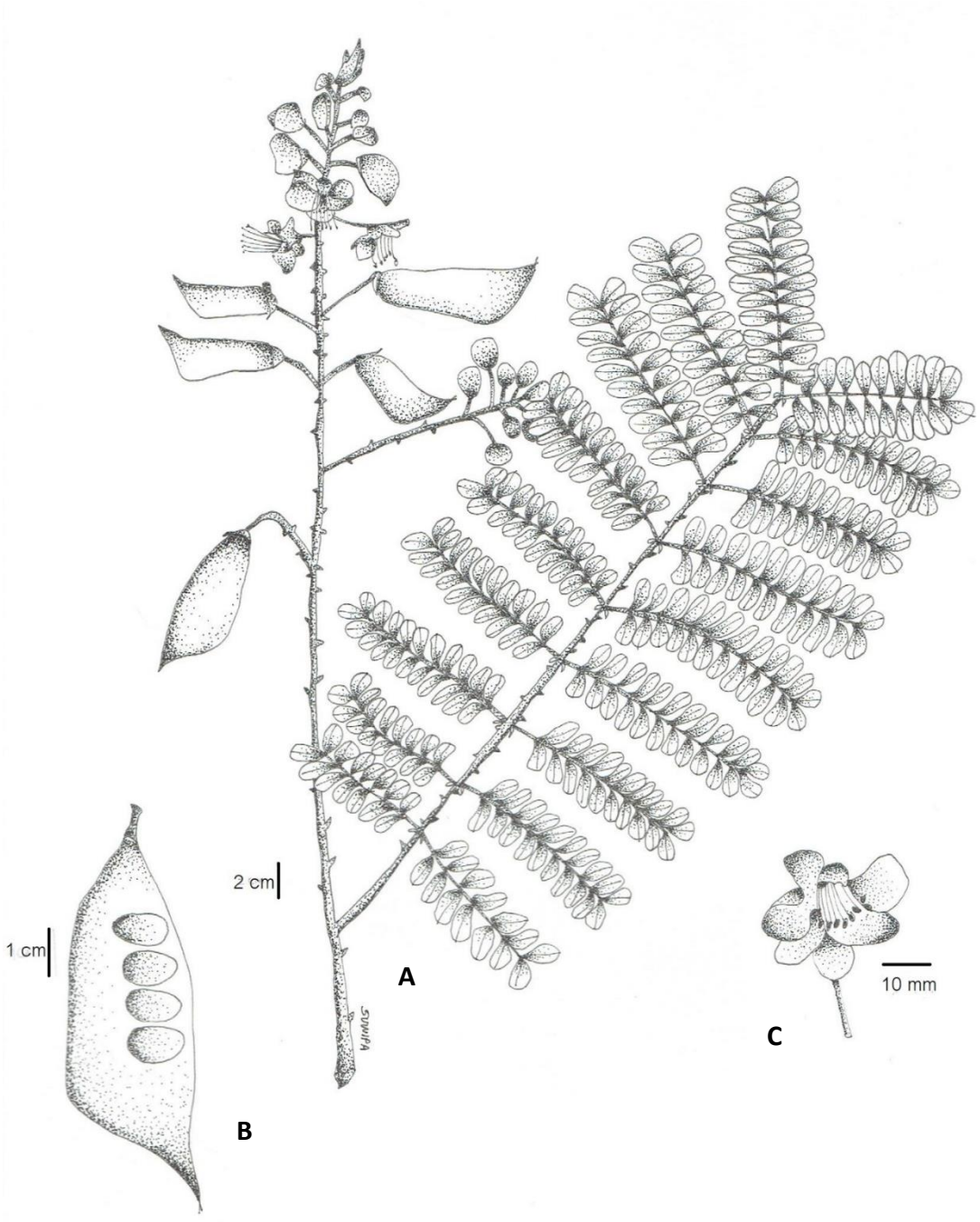


Figure 41 *Caesalpinia sappan* L.; A. whole plant, B. pod, C. flower

The results of microscopic evaluation

The results of microscopic leaf anatomical evaluation

The transverse sections of the midrib of eight *Caesalpinia* species were examined. The midrib region of all eight species including upper epidermis, palisade cells, spongy cells, sclerenchyma, xylem tissue, phloem tissue, parenchyma, collenchyma, lower epidermis, and trichomes were established. Among eight species, there were four species presenting unicellar non-glandular trichomes (*C. bonduc, C. digyna, C. minax, and C. decapetala*). The palisade cells of *C. coriaria* were found at both sides of epidermis (isolateral or isobilateral leaf) whereas the rest of species were found only at upper epidermis (dorsiventral or bifacial leaf).

***Caesalpinia bonduc* (L.) Roxb.**

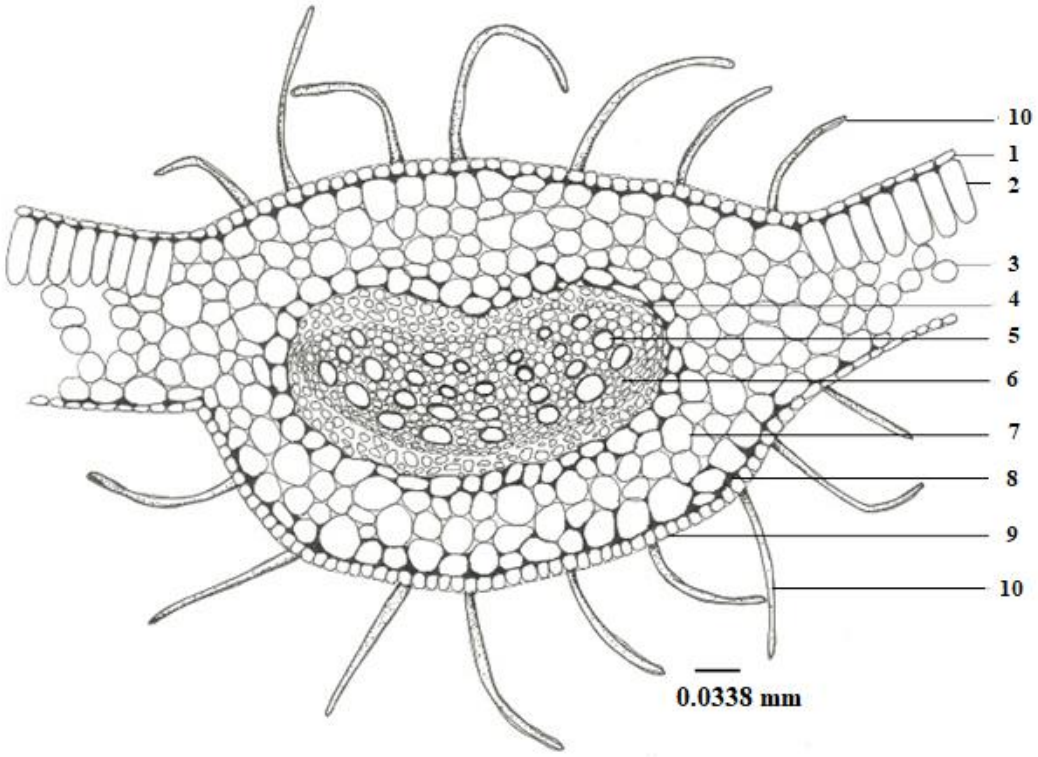


Figure 42 Midrib cross section of *C. bonduc*; 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis, 10. Trichome

***Caesalpinia coriaria* (Jacq.) Willd.**

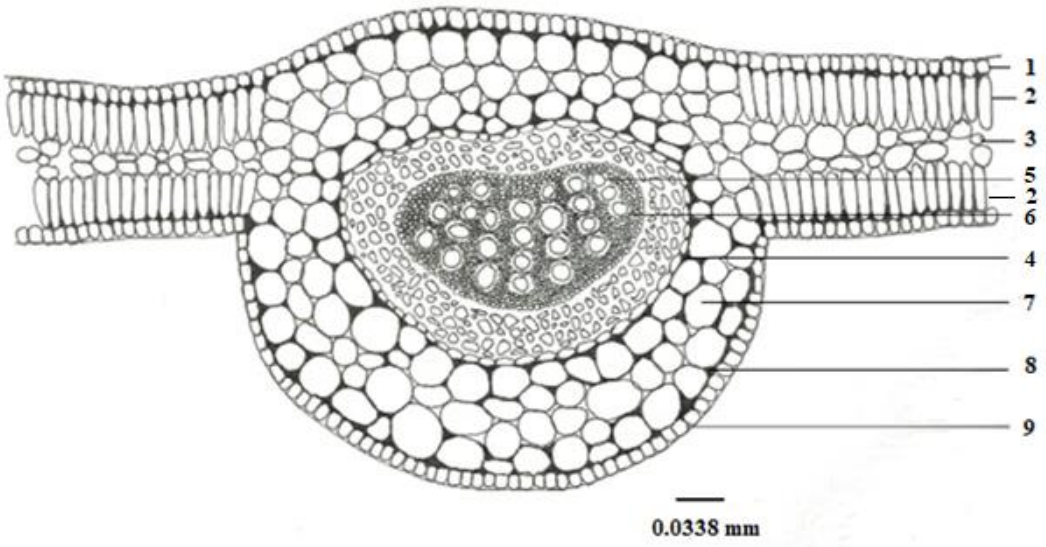


Figure 43 Midrib cross section of *C. coriaria*; 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis

Caesalpinia decapetala **(Roth) Alston**

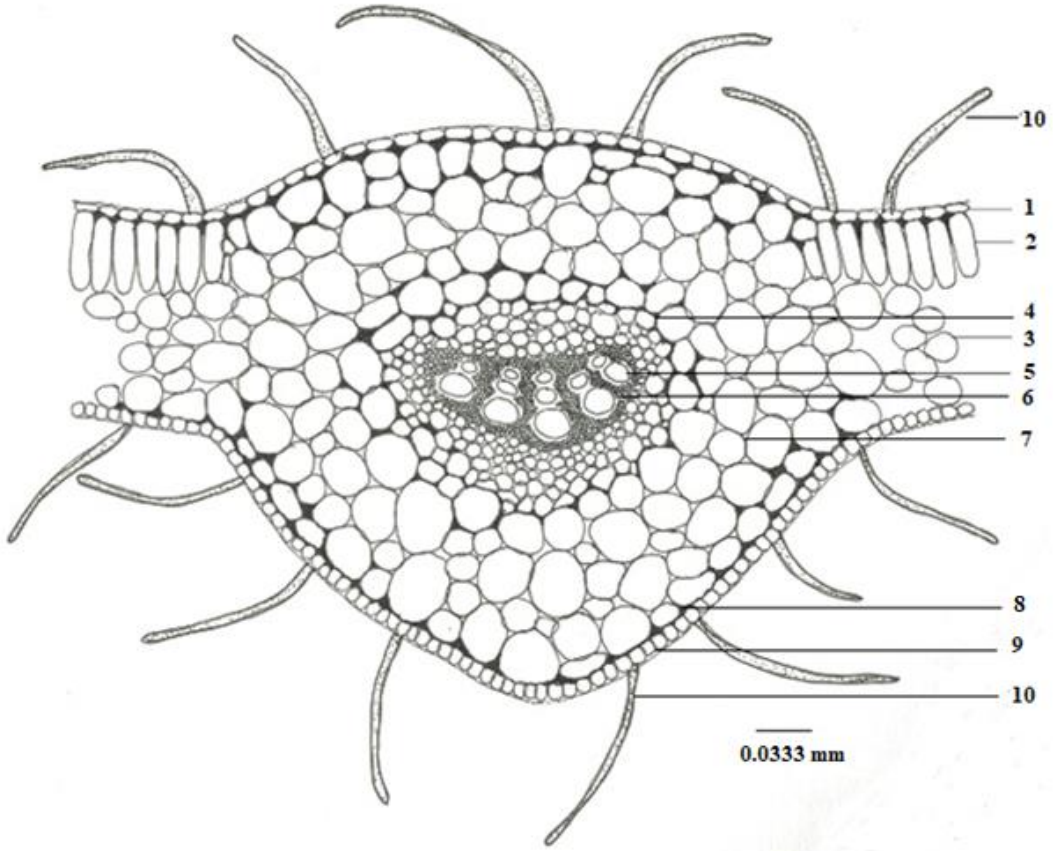


Figure 44 Midrib cross section of *C. decapetala*; 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis, 10. Trichome

***Caesalpinia digyna* Rottler**

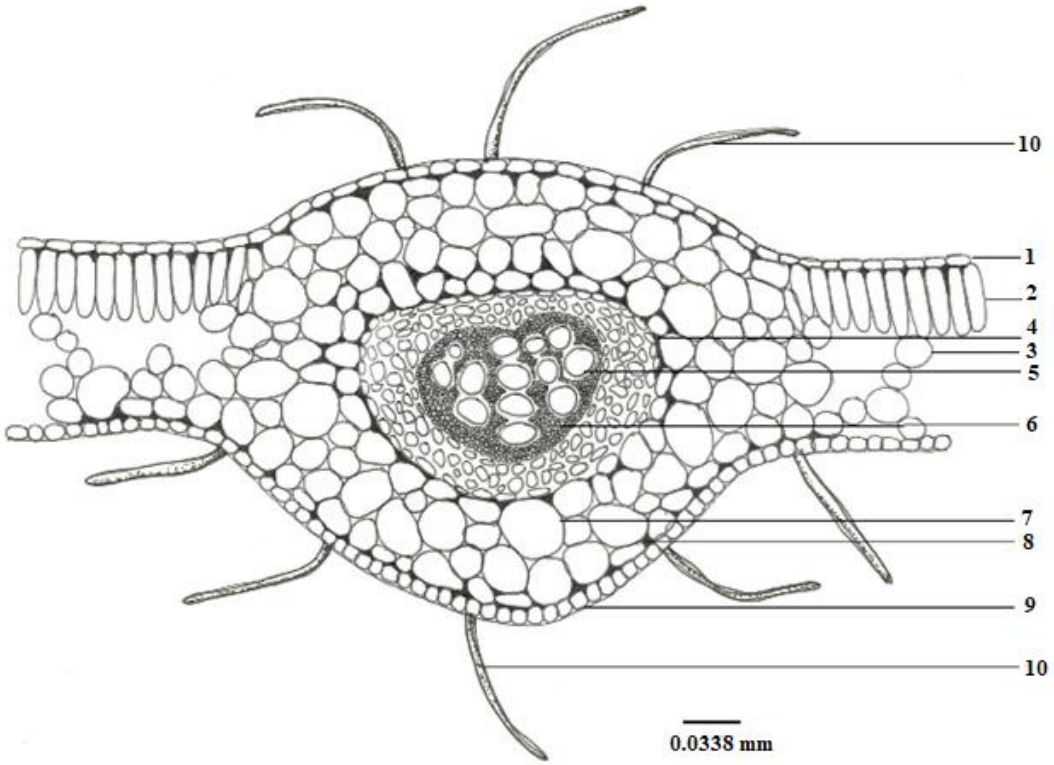


Figure 45 Midrib cross section of *C. digyna*; 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis, 10. Trichome

***Caesalpinia mimosoides* Lam.**

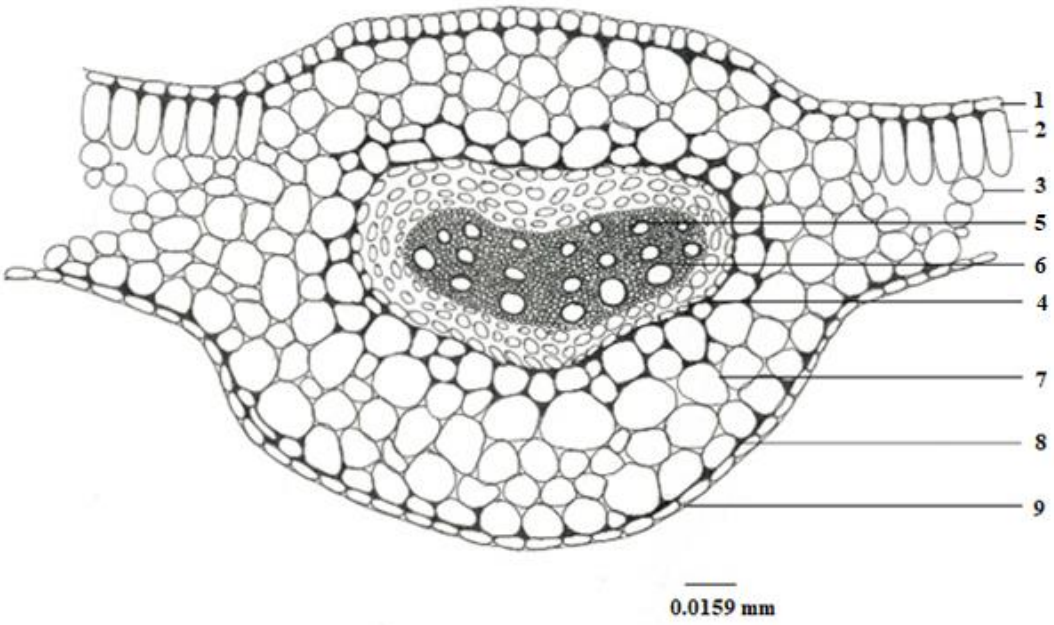


Figure 46 Midrib cross section of *C. mimosoides*; 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis

Caesalpinia minax Hance

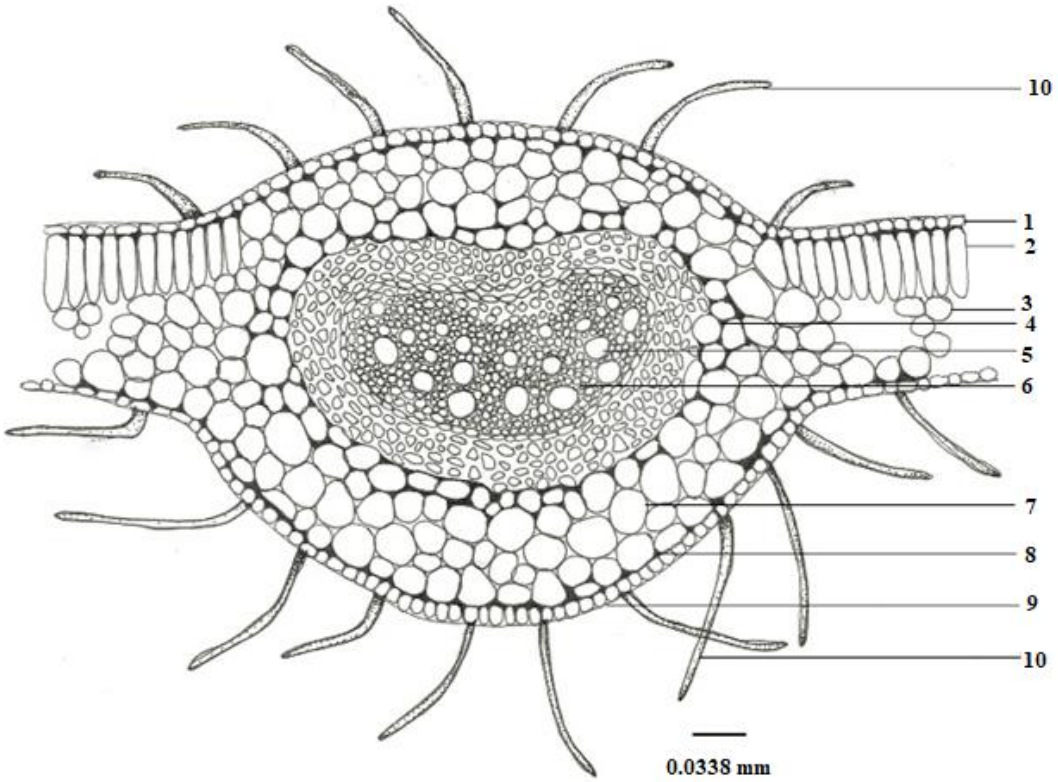


Figure 47 Midrib cross section of *C. minax*; 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis, 10. Trichome

Caesalpinia pulcherrima (L.) Sw.

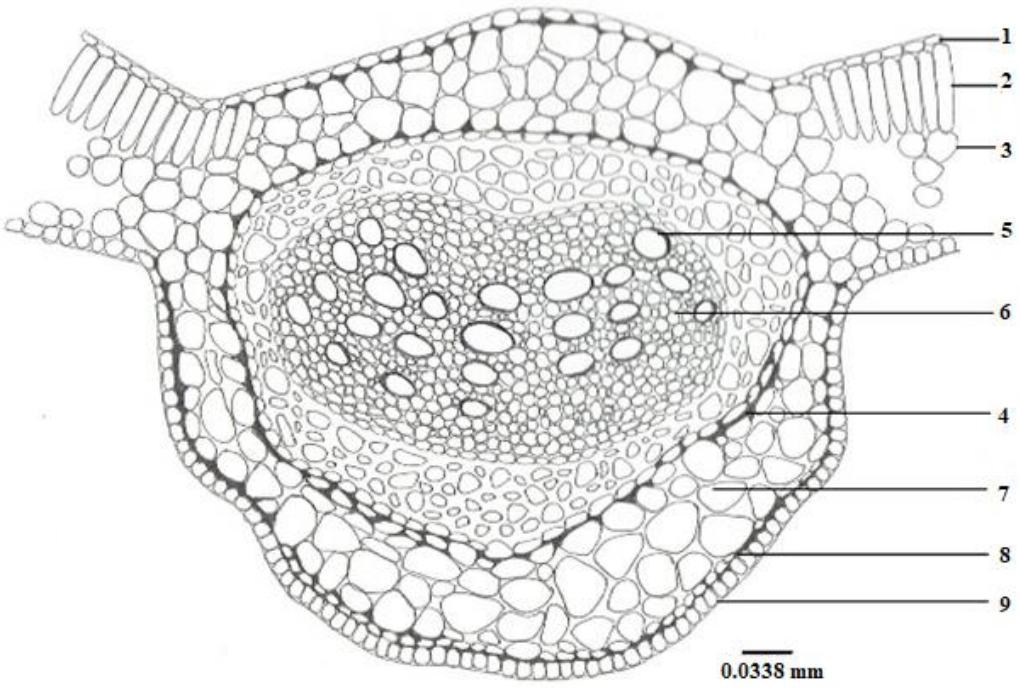


Figure 48 Midrib cross section of *C. pulcherrima*; 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis

***Caesalpinia sappan* L.**

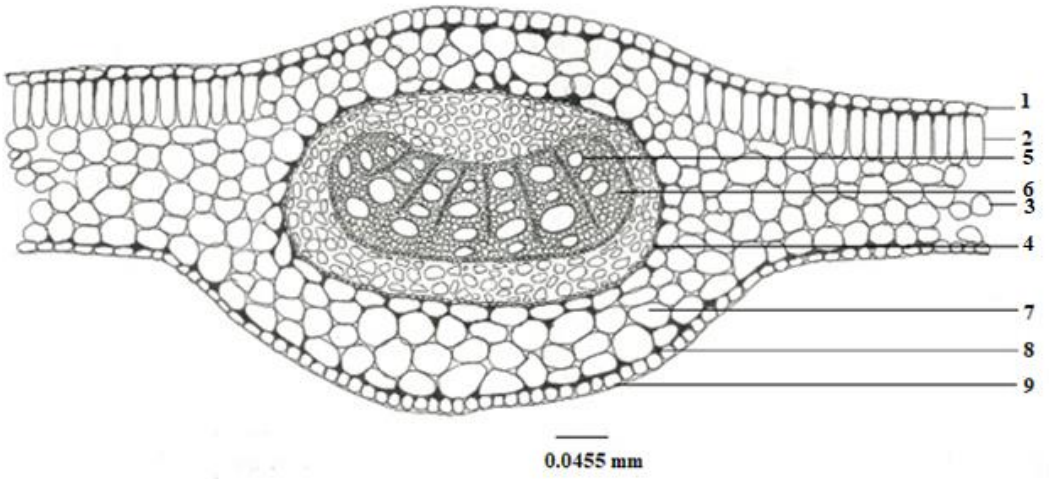
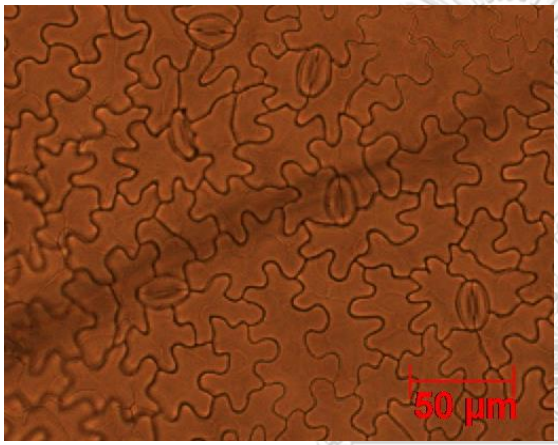


Figure 49 Midrib cross section of *C. sappan*; 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis

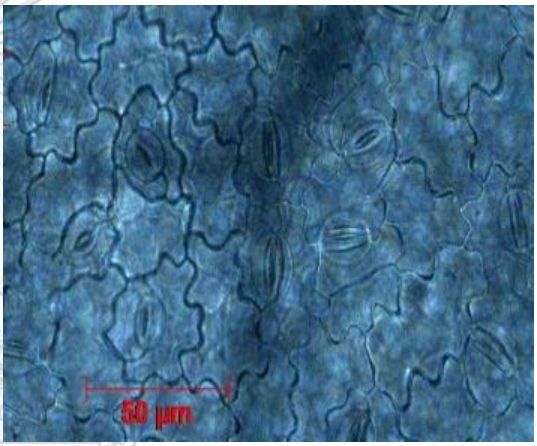
The results of microscopic leaf constant numbers

Stomatal cells

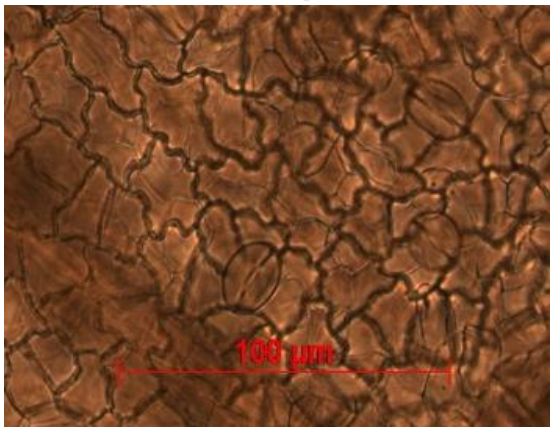
The stomatal cells of all eight *Caesalpinia* species were distributed only on the lower surface of the leaf. The photograph of stomatal cells of eight *Caesalpinia* species were presented in Figure 50 (A- H). Stomatal cell number and stomatal index values were shown in Table 23.



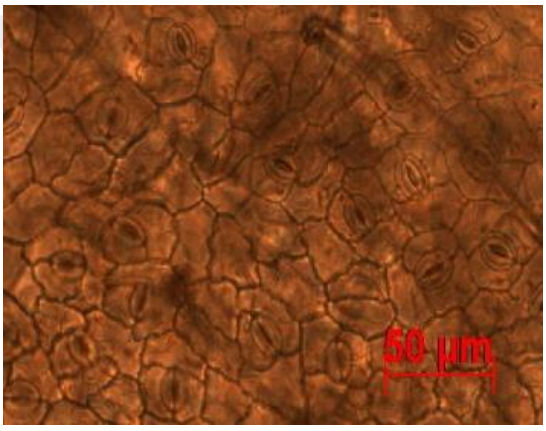
(A) Caesalpinia bonduc



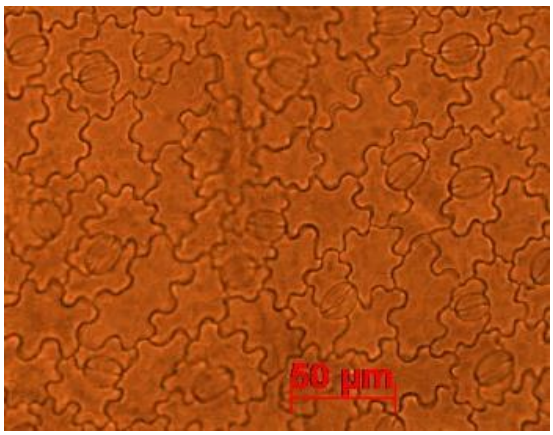
(B) Caesalpinia coriaria



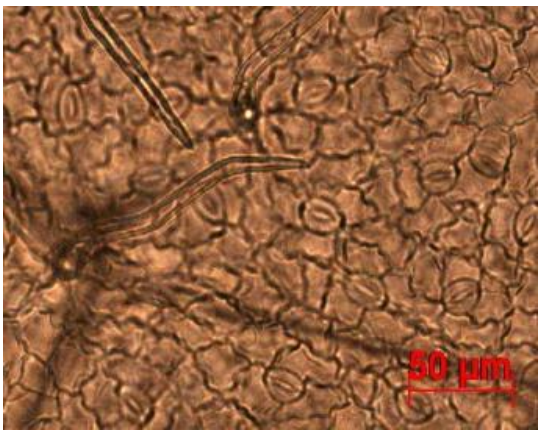
(C) Caesalpinia decapetala



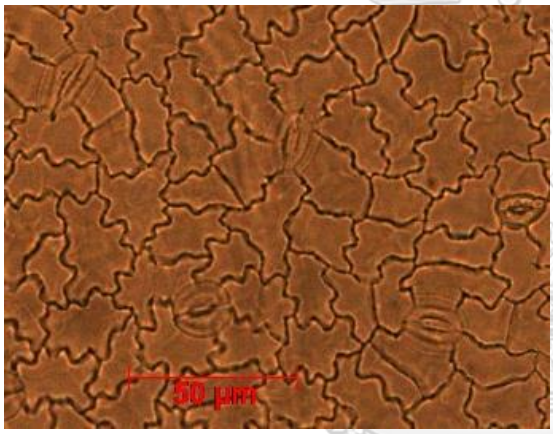
(D) Caesalpinia digyna



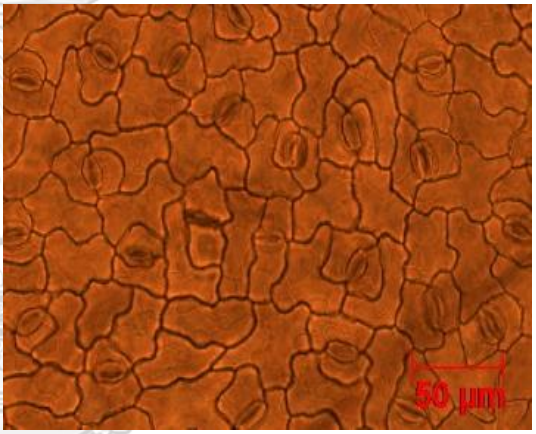
(E) Caesalpinia mimosoides



(F) Caesalpinia minax



(G) Caesalpinia pulcherrima



(H) Caesalpinia sappan

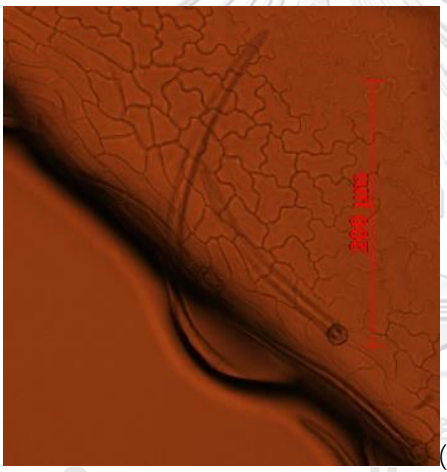
Figure 50 Photographs of stomatal cells; (A) *C. bonduc*, (B) *C. coriaria*, (C) *C. decapetala*, (D) *C. digyna*, (E) *C. mimosoides*, (F) *C. minax*, (G) *C. pulcherrima*, (H) *C. sappan*

Table 23 Microscopic leaf constant numbers of eight *Caesalpinia* species based on stomatal number and stomatal index

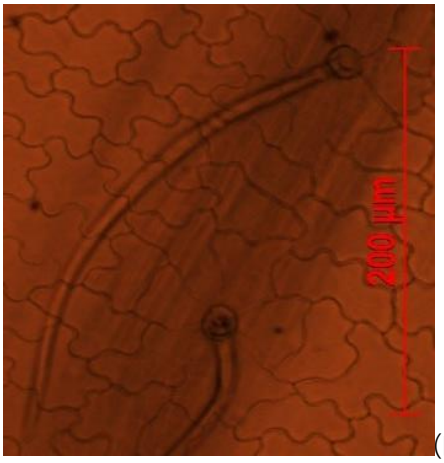
Caesalpinia **species**	**Stomatal number Mean ± S.D. (Min-Max)**	**Lower epidermal cell number Mean ± S.D. (Min-Max)**	**Stomatal index Mean ± S.D. (Min-Max)**
C. sappan	291.91 ± 23.63	1362.93 ± 63.73	17.62 ± 1.07
	(230 – 348)	(1214 – 1504)	(15.32 – 19.82)
C. pulcherrima	372.96 ± 57.17	3732.00 ± 454.41	9.07 ± 0.73
	(278 – 484)	(2938 – 4590)	(7.82 – 10.98)
C. bonduc	115.62 ± 17.34	1498.69 ± 390.42	7.53 ± 1.72
	(90 - 154)	(900 – 2002)	(4.81 – 10.87)
C. mimosoides	265.29 ± 73.49	1289.36 ± 251.65	16.80 ± 1.75
	(130 – 386)	(862 – 1600)	(11.93 – 19.84)
C. coriaria	342.67 ± 96.53	1500.31 ± 163.30	18.27 ± 2.58
	(226 – 562)	(1216 – 1832)	(14.89 – 24.96)
C. digyna	320.54 ± 48.54	1746.42 ± 244.21	15.52 ± 1.14
	(248 – 412)	(1372 – 2156)	(13.34 – 18.77)
C. minax	156.11 ± 15.15	1138.49 ± 56.53	12.06 ± 0.99
	(128 - 192)	(960 – 1276)	(10.19 – 14.89)
C. decapetala	254.82 ± 23.14	3472.13 ± 230.94	6.60 ± 0.59
	(208 – 312)	(2754 – 3958)	(5.45 – 8.65)

Trichomes

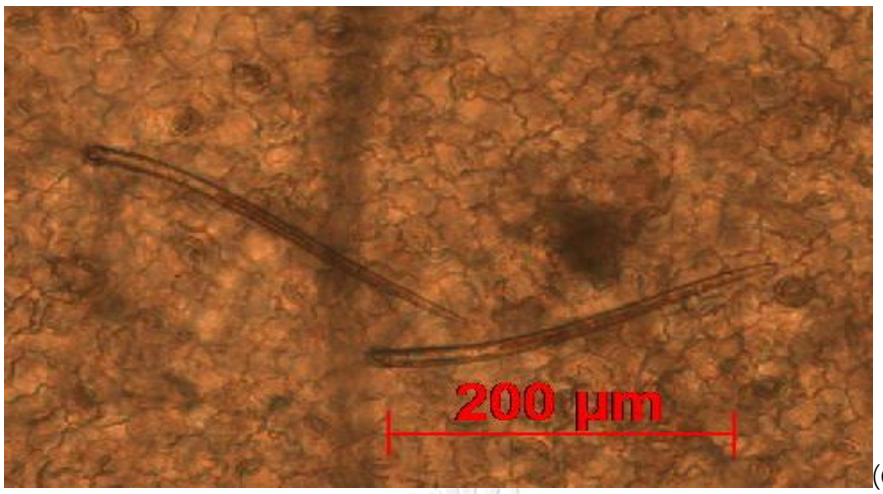
The trichome type that found in four *Caesalpinia* species was unicellar non-glandular trichome. Trichomes were found on both sides of the leaf of *C. bonduc, C. digyna, C. minax, and C. decapetala*. The photograph of trichomes found in four *Caesalpinia* species were presented in Figure 51 (A-E). Trichome number and trichome index were shown in Table 24 and Table 25. The trichomes of *C. bonduc* were found only at margin and midrib of the leaf, not at the lamina. So *C. bonduc* trichome number could not be determined.



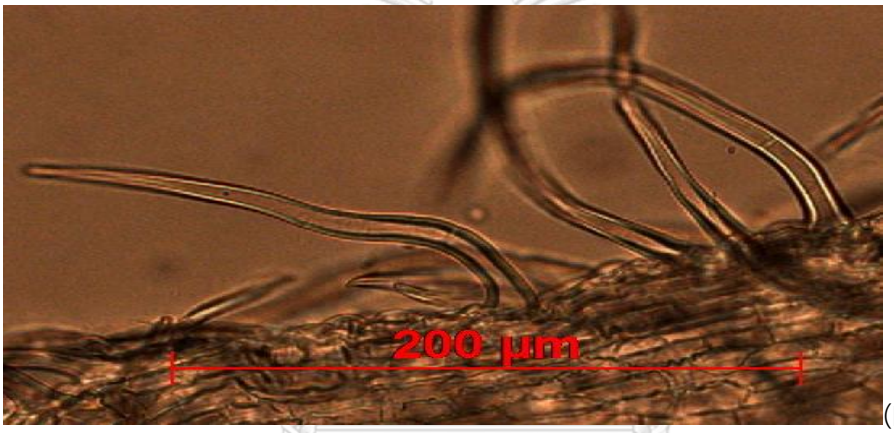
(A) *C. bonduc* (Margin)



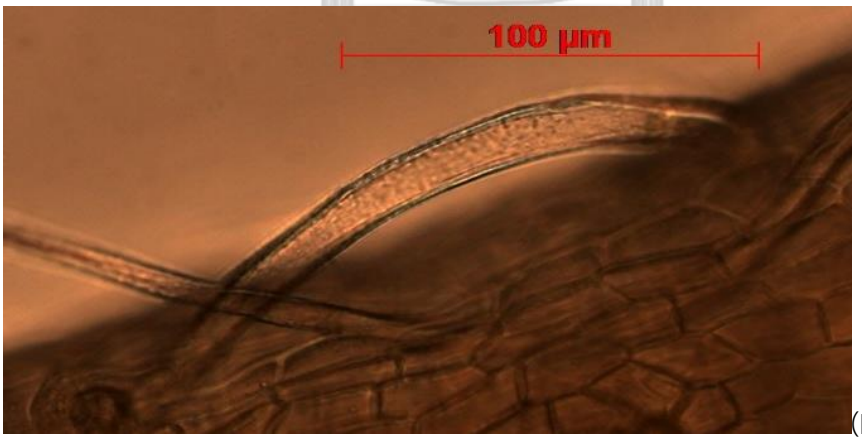
(B) *C. bonduc* (Midrib)



(C) *C. digyna*



(D) *C. minax*



(E) *C. decapetala*

Figure 51 Photographs of trichomes; (A) *C. bonduc* [at margin], (B) *C. bonduc* [at midrib], (C) *C. digyna*, (D) *C. minax*, (E) *C. decapetala*

Table 24 Microscopic leaf constant numbers of eight *Caesalpinia* species based on trichome number and trichome index evaluated from the lower part of leaf

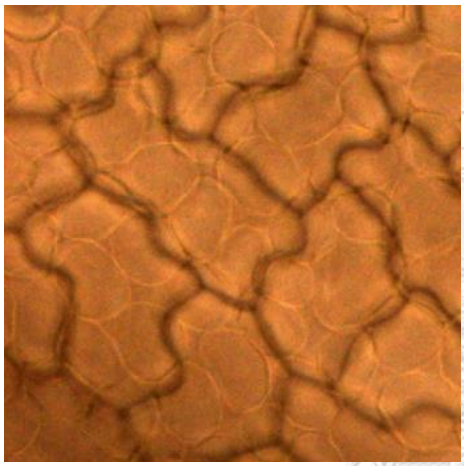
Caesalpinia **species**	**Trichome number Mean ± S.D. (Min-Max)**	**Lower epidermal cell number Mean ± S.D. (Min-Max)**	**Trichome index Mean ± S.D. (Min-Max)**
C. sappan	-	-	-
C. pulcherrima	-	-	-
C. bonduc	*Could not count but trichomes found at margin and midrib of the leaf*		
C. mimosoides	-	-	-
C. coriaria	-	-	-
C. digyna	15.15 ± 2.16	1694.16 ± 206.93	0.92 ± 0.17
	(11 – 21)	(1312 – 2122)	(0.66 – 1.41)
C. minax	10.61 ± 1.57	1624.73 ± 321.59	0.67 ± 0.16
	(8 – 15)	(1196 – 2374)	(0.35 – 1.06)
C. decapetala	18.69 ± 2.30	3098.08 ± 218.51	0.60 ± 0.07
	(15 – 27)	(2636 – 3510)	(0.47 – 0.81)

Table 25 Microscopic leaf constant numbers of eight *Caesalpinia* species based on trichome number and trichome index evaluated from the upper part of leaf

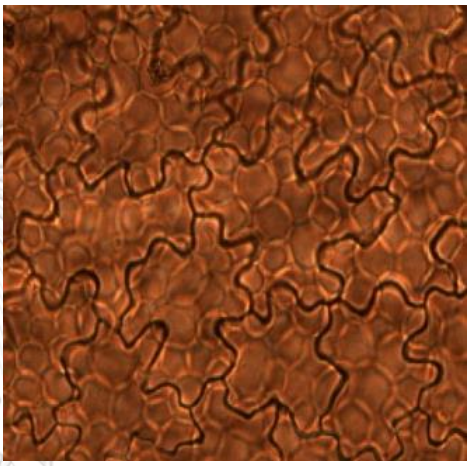
Caesalpinia **species**	**Trichome number Mean ± S.D. (Min-Max)**	**Upper epidermal cell number Mean ± S.D. (Min-Max)**	**Trichome index Mean ± S.D. (Min-Max)**
C. sappan	-	-	-
C. pulcherrima	-	-	-
C. bonduc	* Could not count but trichomes found at margin and midrib of the leaf*		
C. mimosoides	-	-	-
C. coriaria	-	-	-
C. digyna	30.93 ± 3.28	2245.33 ± 258.89	1.28 ± 0.23
	(23 – 39)	(1810 – 2800)	(0.89 – 2.00)
C. minax	18.02 ± 2.43	2090.12 ± 373.33	0.88 ± 0.18
	(14 – 24)	(1616 – 2816)	(0.56 – 1.31)
C. decapetala	30.72 ± 6.09	3338.83 ± 209.94	0.92 ± 0.18
	(20 – 42)	(2876 – 3812)	(0.57 – 1.36)

Palisade cells

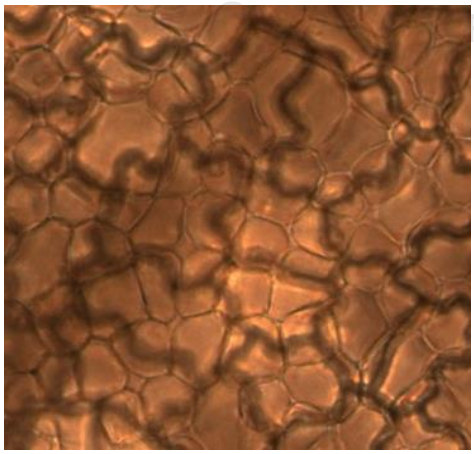
Caesalpinia palisade cells occur between upper and lower epidermis cells. Palisade ratio was slightly different among *Caesalpinia* species and the ratio was shown in Table 26. The photograph of palisade cells of selected *Caesalpinia* species were presented in Figure 52 (A-H).



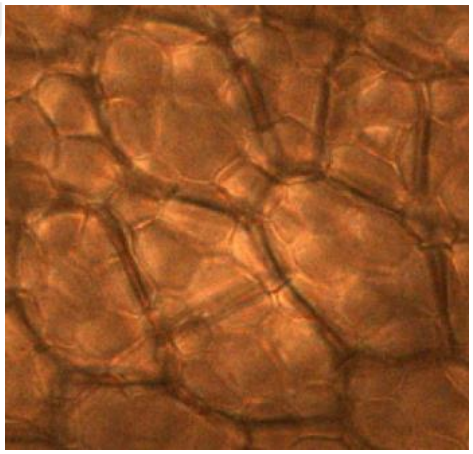
(A) Caesalpinia bonduc



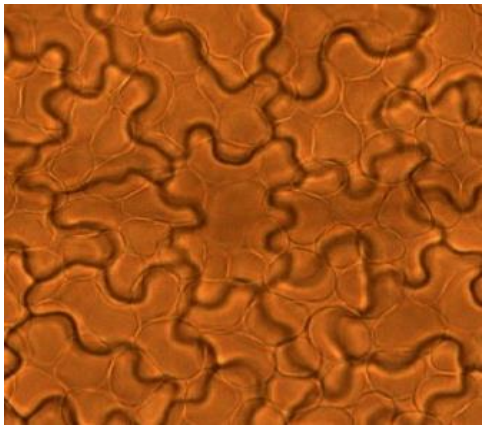
(B) Caesalpinia coriaria



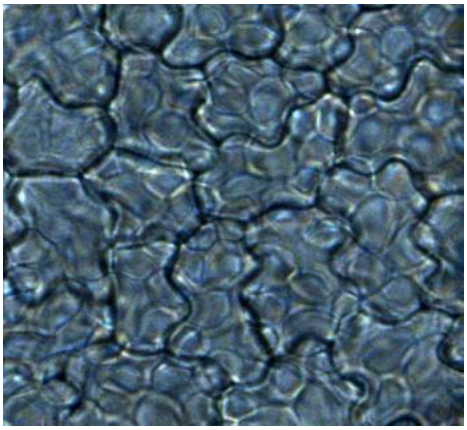
(C) Caesalpinia decapetala



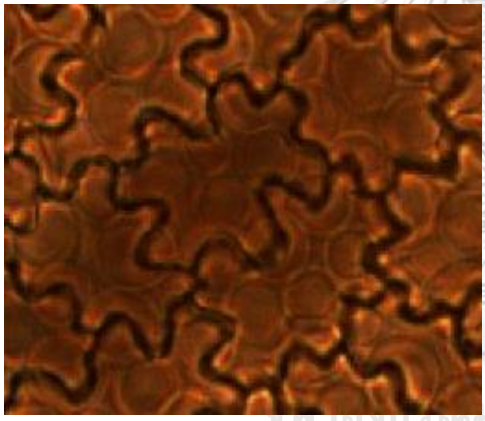
(D) Caesalpinia digyna



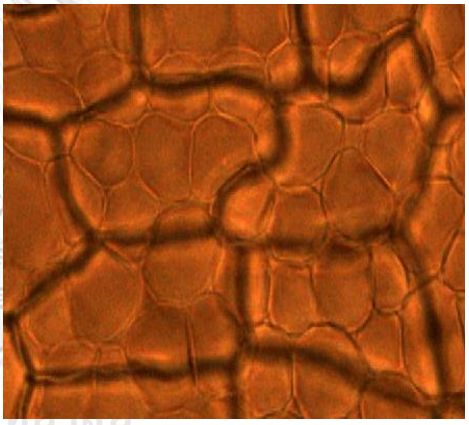
(E) Caesalpinia mimosoides



(F) Caesalpinia minax



(G) Caesalpinia pulcherrima

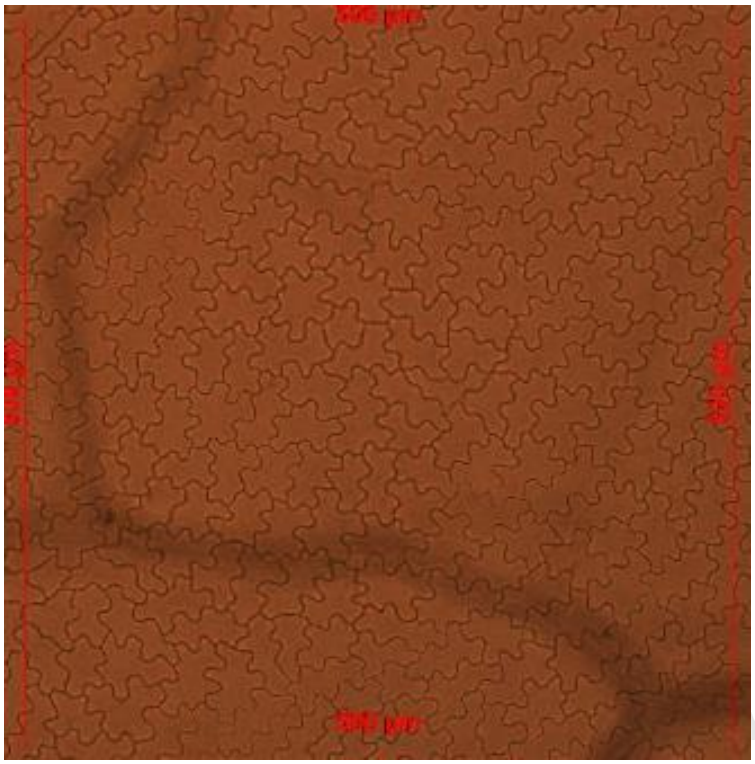


(H) Caesalpinia sappan

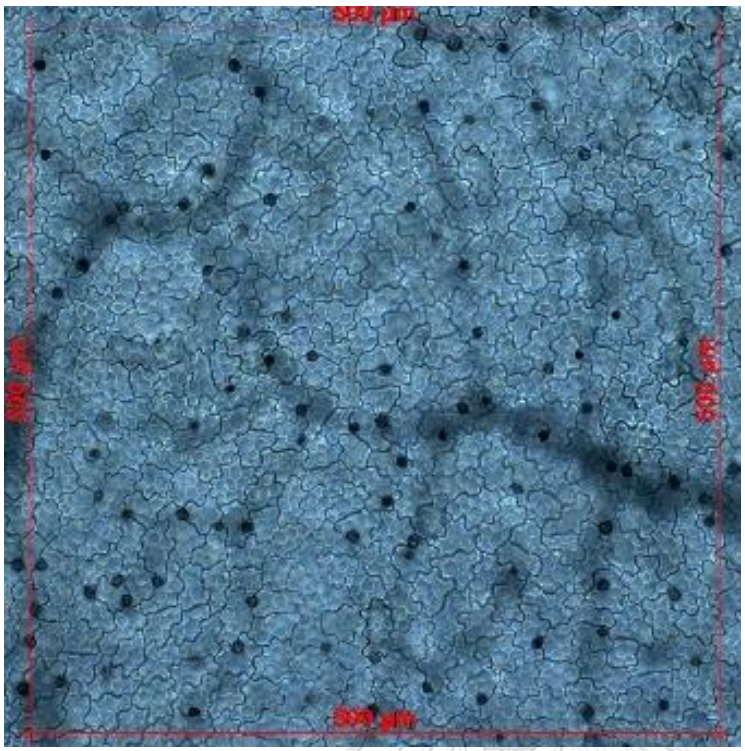
Figure 52 Photographs of palisade cells; (A) *C. bonduc*, (B) *C. coriaria*, (C) *C. decapetala*, (D) *C. digyna*, (E) *C. mimosoides*, (F) *C. minax*, (G) *C. pulcherrima*, (H) *C. sappan*

Upper epidermal cells

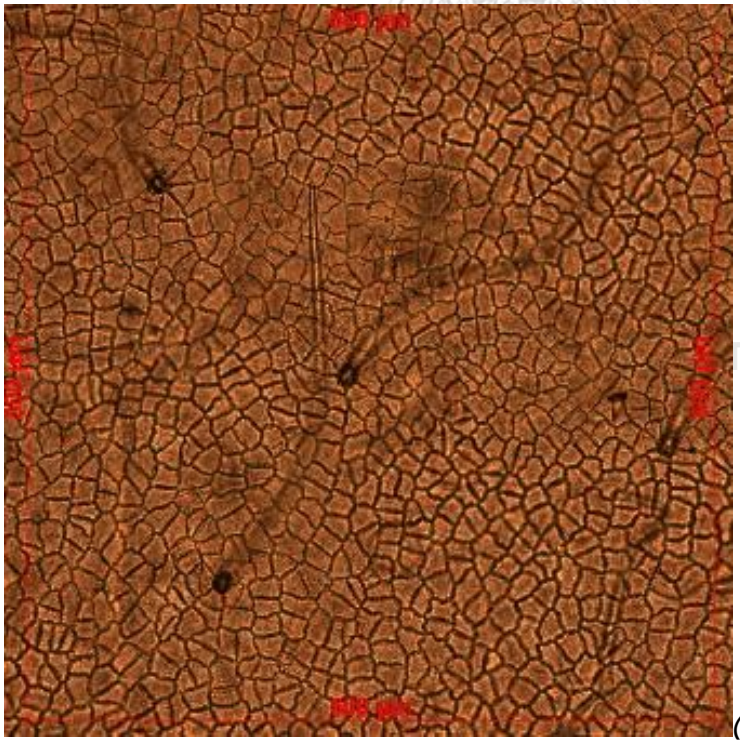
The photograph of upper epidermal cells of selected *Caesalpinia* species were presented in Figure 53 (A-H). The upper epidermis cell area were shown in Table 26.



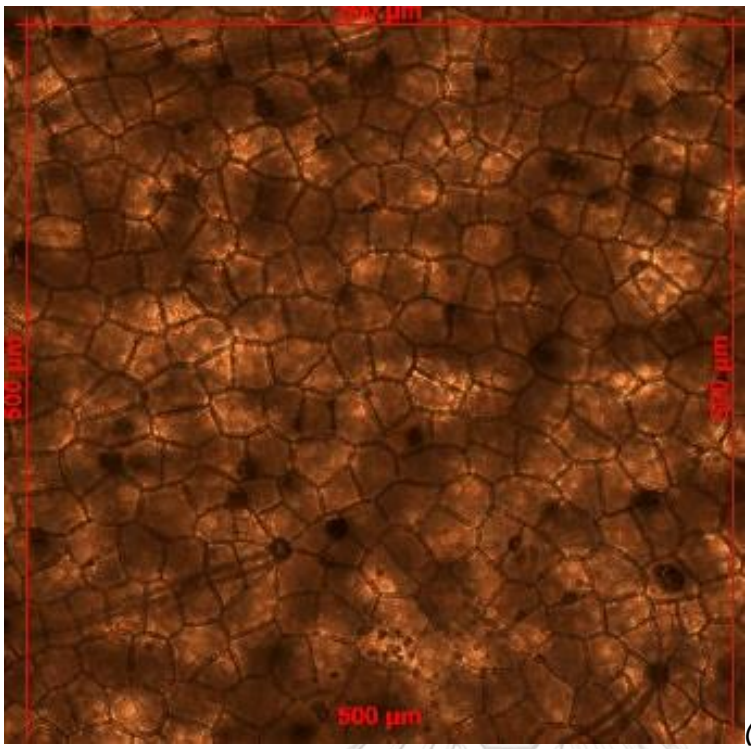
(A) Caesalpinia bonduc



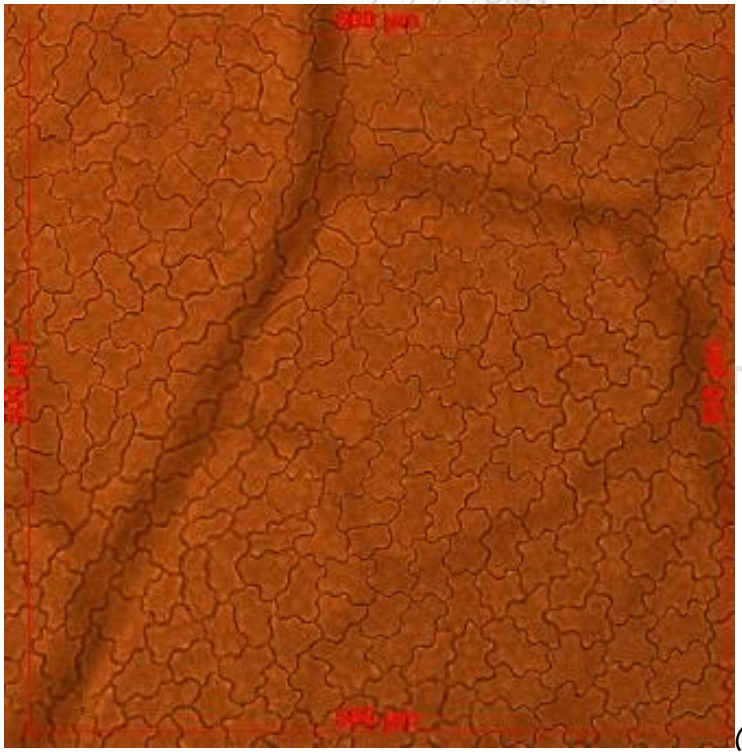
(B) Caesalpinia coriaria



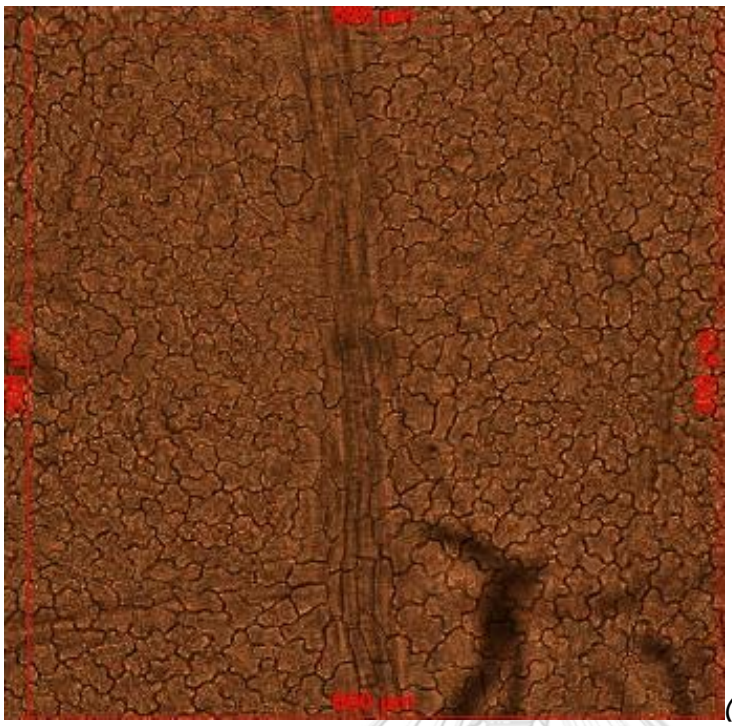
(C)Caesalpinia decapetala



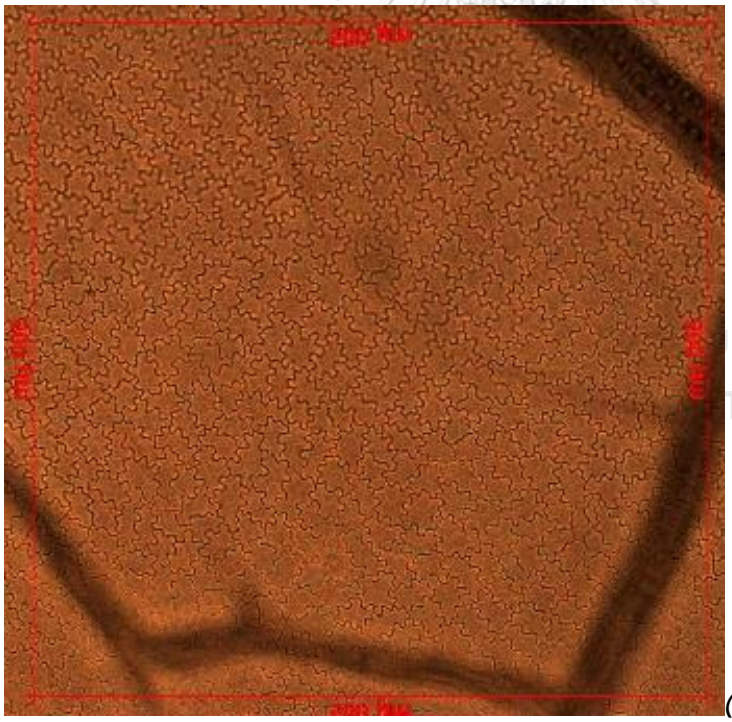
(D) Caesalpinia digyna



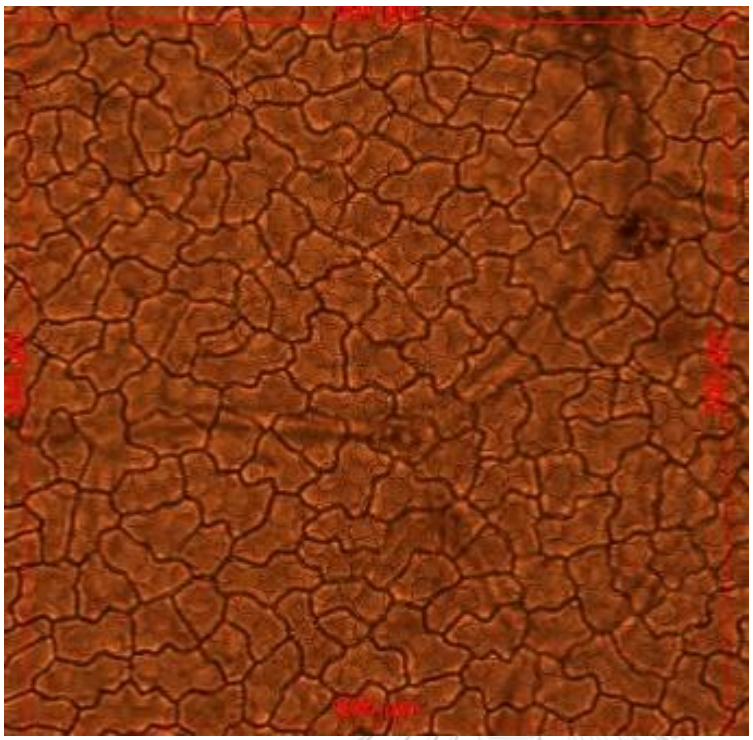
(E)Caesalpinia mimosoides



(F) Caesalpinia minax



(G)Caesalpinia pulcherrima



(H) Caesalpinia sappan

Figure 53 Photographs of upper epidermal cells; (A) *C. bonduc*, (B) *C. coriaria*, (C) *C. decapetala*, (D) *C. digyna*, (E) *C. mimosoides*, (F) *C. minax*, (G) *C. pulcherrima*, (H) *C. sappan*

Table 26 Microscopic leaf constant numbers of eight *Caesalpinia* species based on upper epidermal cell area and palisade ratio

Caesalpinia **species**	**Upper epidermis cell area (μm²) Mean ± S.D. (Min-Max)**	**Palisade ratio Mean ± S.D. (Min-Max)**
C. sappan	1452.50 ± 148.21	6.34 ± 0.63
	(1141.55 – 1724.14)	(5.00 – 7.50)
C. pulcherrima	457.24 ± 36.19	5.90 ± 0.69
	(386.10 – 524.66)	(4.75 – 7.50)
C. bonduc	947.79 ± 159.96	3.98 ± 0.84
	(687.76 – 1225.49)	(3.00 – 5.75)
C. mimosoides	886.80 ± 117.48	5.83 ± 0.80
	(657.03 – 1103.75)	(4.63 – 7.50)
C. coriaria	697.47 ± 53.43	9.78 ± 2.22
	(565.61 – 771.60)	(6.13 – 13.63)
C. digyna	808.68 ± 78.44	6.04 ± 0.58
	(666.67 – 957.85)	(5.13 – 7.88)
C. minax	905.86 ± 56.36	5.61 ± 0.51
	(796.17 – 1091.70)	(4.63 – 6.75)
C. decapetala	318.50 ± 36.55	4.20 ± 0.40
	(279.96 – 397.46)	(3.25 – 5.25)

The microscopic leaf constant numbers of eight *Caesalpinia* species were concluded and listed in Table 27 and 28. Trichome characteristics of *C. bonduc* could be used as an identification key for this species. Trichome number and trichome index determined from *C. minax*, *C. digyna*, and *C. decapetala* were found

to be overlapping. However, upper epidermal cell area was capable to identify *C. decapetala*. Furthermore, the stomatal number could differentiate *C. digyna* from *C. minax*. For non-trichome containing species, *C. sappan* and *C. pulcherrima* could be identified by stomatal index and upper epidermal cell area. Microscopic leaf constant numbers between *C. mimosoides* and *C. coriaria* were overlapping, however *C. coriaria* demonstrated its identity due to isolateral character (Figure 43).

Table 27 The summary of microscopic leaf constant numbers of eight *Caesalpinia* species

Caesalpinia species	Lower epidermis			Upper epidermis		
	Stomatal number	Epidermal cell number	Stomatal index	Epidermis cell area (μm²)	Palisade ratio	Trichome
C. sappan	291.91 ± 23.63 (230 – 348)	1362.93 ± 63.73 (1214 – 1504)	17.62 ± 1.07 (15.32 – 19.82)	1452.50 ± 148.21 (1141.55 – 1724.14)	6.34 ± 0.63 (5.00 – 7.50)	-
C. pulcherrima	372.96 ± 57.17 (278 – 484)	3732.00 ± 454.41 (2938 – 4590)	9.07 ± 0.73 (7.82 – 10.98)	457.24 ± 36.19 (386.10 – 524.66)	5.90 ± 0.69 (4.75 – 7.50)	-
C. bonduc	115.62 ± 17.34 (90 - 154)	1498.69 ± 390.42 (900 – 2002)	7.53 ± 1.72 (4.81 – 10.87)	947.79 ± 159.96 (687.76 – 1225.49)	3.98 ± 0.84 (3.00 – 5.75)	+
C. mimosoides	265.29 ± 73.49 (130 – 386)	1289.36 ± 251.65 (862 – 1600)	16.80 ± 1.75 (11.93 – 19.84)	886.80 ± 117.48 (657.03 – 1103.75)	5.83 ± 0.80 (4.63 – 7.50)	-
C. coriaria	342.67 ± 96.53 (226 – 562)	1500.31 ± 163.30 (1216 – 1832)	18.27 ± 2.58 (14.89 – 24.96)	697.47 ± 53.43 (565.61 – 771.60)	9.78 ± 2.22 (6.13 – 13.63)	-
C. digyna	320.54 ± 48.54 (248 – 412)	1746.42 ± 244.21 (1372 – 2156)	15.52 ± 1.14 (13.34 – 18.77)	808.68 ± 78.44 (666.67 – 957.85)	6.04 ± 0.58 (5.13 – 7.88)	+
C. minax	156.11 ± 15.15 (128 - 192)	1138.49 ± 56.53 (960 – 1276)	12.06 ± 0.99 (10.19 – 14.89)	905.86 ± 56.36 (796.17 – 1091.70)	5.61 ± 0.51 (4.63 – 6.75)	+
C. decapetala	254.82 ± 23.14 (208 – 312)	3472.13 ± 230.94 (2754 – 3958)	6.60 ± 0.59 (5.45 – 8.65)	318.50 ± 36.55 (279.96 – 397.46)	4.20 ± 0.40 (3.25 – 5.25)	+

Table 28 The summary of microscopic leaf constant numbers of four *Caesalpinia* species containing trichome

Caesalpinia species	Lower epidermis			Upper epidermis		
	Trichome number	Epidermal cell number	Trichome index	Trichome number	Epidermal cell number	Trichome index
C. bonduc	-*	-	-	-	-	-
C. digyna	15.15 ± 2.16 (11 – 21)	1694.16 ± 206.93 (1312 – 2122)	0.92 ± 0.17 (0.66 – 1.41)	30.93 ± 3.28 (23 – 39)	2245.33 ± 258.89 (1810 – 2800)	1.28 ± 0.23 (0.89 – 2.00)
C. minax	10.61 ± 1.57 (8 – 15)	1624.73 ± 321.59 (1196 – 2374)	0.67 ± 0.16 (0.35 – 1.06)	18.02 ± 2.43 (14 – 24)	2090.12 ± 373.33 (1616 – 2816)	0.88 ± 0.18 (0.56 – 1.31)
C. decapetala	18.69 ± 2.30 (15 – 27)	3098.08 ± 218.51 (2636 – 3510)	0.60 ± 0.07 (0.47 – 0.81)	30.72 ± 6.09 (20 – 42)	3338.83 ± 209.94 (2876 – 3812)	0.92 ± 0.18 (0.57 – 1.36)

*Could not determine but trichomes found at midrib and margin of the leaf

Part IV: Molecular identification

DNA isolation

The genomic DNA was isolated from young fresh leaves of each *Caesalpinia* species using modified CTAB method (Doyle & Doyle, 1990). DNA concentration and purity was checked using NanoDrop One spectrophotometer and the results were shown in Table 29.

Table 29 DNA concentration and purity of DNA obtained from NanoDrop One spectrophotometer

Sample	DNA concentration (ng/ µL)	A260/ A280
C. sappan	70.00	1.85
C. pulcherrima	90.00	1.79
C. bonduc	444.10	1.89
C. mimosoides	187.00	1.98
C. coriaria	43.70	1.63
C. digyna	61.90	1.92
C. minax	474.50	1.88
C. decapetala	138.90	1.89
A. squamosa	16.85	1.94

ISSR analysis

In this study, fifteen ISSR primers consisting of di- and tri- nucleotide repeat primers were screened to amplify DNA fragments. Among these, seven primers that gave the clear band and reproducible were selected. Two hundred and seventeen reproducible polymorphic bands ranging from 23 to 38 bands were obtained. An average was 31 bands per primer. The fragment size range from 254 to 2263 base pairs (bps). Primer ISSR 09 revealed the highest polymorphic bands (38 bands) whereas primer ISSR 10 had the lowest number of the band (23 bands). No band was found in negative control amplification. The result was 100% polymorphism. The summary of ISSR primer sequences, the annealing temperatures of each ISSR primers and the number of ISSR products were presented in Table 30. The example of ISSR fingerprint of selected *Caesalpinia* species and outgroup obtained from primer ISSR 02 was shown in Figure 54.

Table 30 ISSR primer sequences, annealing temperatures, and the number of ISSR products of selected eight *Caesalpinia* plant samples

Primer	Primer sequence*	Annealing Tm (°C)	Fragment size range (bps)	Total band	Polymorphic band	Polymorphism (%)
ISSR02	AGAGAGAGAGAGAGAGC	50	354-1846	32	32	100
ISSR06	ACACACACACACACACC	50	411-1918	34	34	100
ISSR09	ACACACACACACACACT	50	254-1996	38	38	100
ISSR10	ACACACACACACACACA	50	498-2263	23	23	100
ISSR13	AGAGAGAGAGAGAGAGYA	50	264-1397	30	30	100
ISSR14	GAGAGAGAGAGAGAGAYT	50	310-1709	31	31	100
ISSR23	AGCAGCAGCAGCAGCAGC	60	306-1977	29	29	100
Total			**254-2263**	**217**	**217**	**100**

*Single letter abbreviation for mixed-base position: Y = (C, T)

The Jaccard's similarity matrix was used to analyze the genetic similarity coefficients among *Caesalpinia* species in Thailand. The highest similarity value was presented between *C. coriaria* and *C. mimosoides* at 0.454 while the lowest similarity index at 0.192 was found between *C. bonduc* and *C. sappan*. Outgroup plant (*Annona squamosa*) was separated from the *Caesalpinia* species. The similarity index of selected eight *Caesalpinia* species and outgroup was shown in Table 31.

ISSR fingerprint

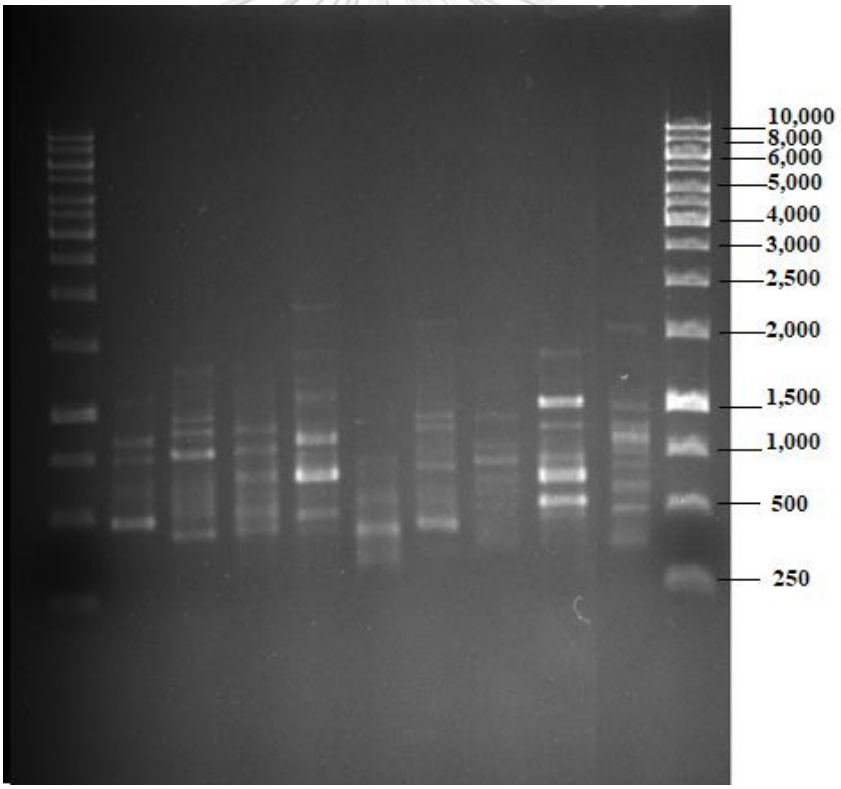


Figure 54 ISSR fingerprint of selected eight *Caesalpinia* species and one outgroup obtained from primer ISSR 02 [M = 1 kb molecular weight marker, lane1 = *C. sappan*, lane2 = *C. coriaria*, lane3 = *C. mimosoides*, lane4 = *C. pulcherrima*, lane5 = *C. bonduc*, lane6= *C. digyna*, lane7 = *C. minax*, lane8 = *C. decapetala*, and lane9 = *A. squamosa*]

Similarity index

Table 31 Similarity index of selected *Caesalpinia* species and outgroup

Caesalpinia		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Species										
C. mimosoides	**1**	1.000								
C. coriaria	**2**	0.454	1.000							
C. digyna	**3**	0.423	0.443	1.000						
C. decapetala	**4**	0.341	0.372	0.359	1.000					
C. pulcherrima	**5**	0.397	0.332	0.340	0.424	1.000				
C. sappan	**6**	0.365	0.315	0.347	0.272	0.265	1.000			
C. minax	**7**	0.282	0.290	0.319	0.216	0.373	0.317	1.000		
C. bonduc	**8**	0.239	0.311	0.355	0.283	0.255	0.192	0.372	1.000	
A. squamosa	**9**	0.300	0.182	0.238	0.231	0.301	0.247	0.189	0.139	1.000

To examine the genetic relationship of *Caesalpinia* species, ISSR-PCR bands gained from seven primers were scored and a phylogenetic dendrogram was clustered among eight *Caesalpinia* species. According to the dendrogram, all eight species were categorized into three major groups. Cluster I could be separated into two subgroups (Ia and Ib). Cluster Ia consisted of *C. mimosoides*, *C. coriaria*, and *C. digyna*. Among these *C. mimosoides* and *C. coriaria* presented the highest similarity index (0.454). Cluster Ib contained *C. decapetala* and *C. pulcherrima*. The Cluster II consisted only one species named *C. sappan*. The rest, *C. minax* and *C. bonduc* were listed in cluster III. From Cluster II and III, the lowest similarity index (0.192) was presented between *C. sappan* and *C. bonduc*. At last, one outgroup plant (*A. squamosa*) was clearly separated from all *Caesalpinia* species. The genetic relationship among selected eight *Caesalpinia* species and outgroup using UPGMA

cluster analysis based on the genetic similarities from seven ISSR primers was shown in Figure 55.

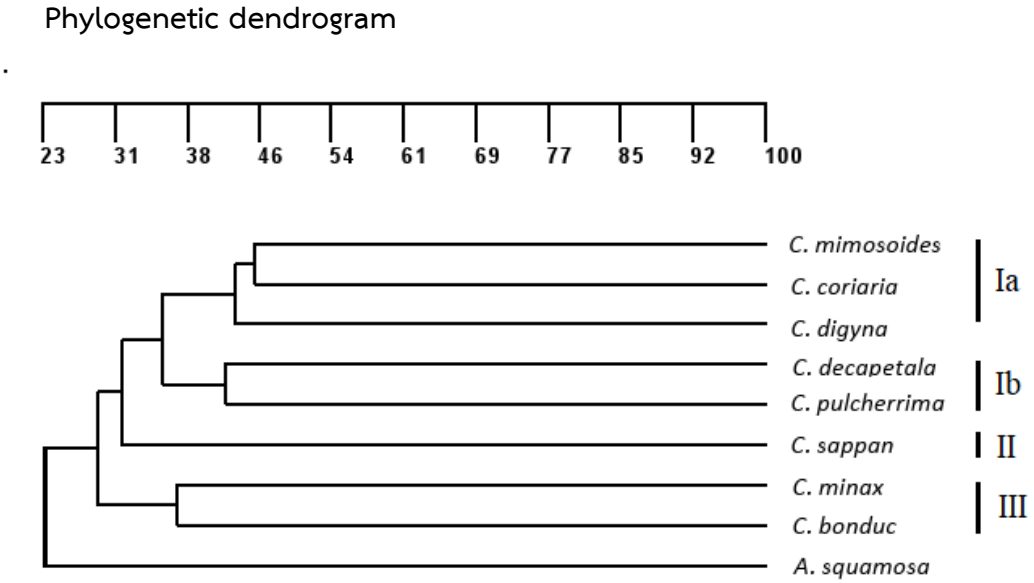


Figure 55 Phylogenetic dendrogram of eight *Caesalpinia* species and outgroup clustered by UPGMA

CHAPTER V

DISCUSSION AND CONCLUSION

Nowadays, plants become more popular and important in the field of drug development, integrative medicine, and also nutraceuticals and cosmetic industry. They have been used over the past decades for medicine, foods, and beverages. Accordingly, the identification and standardization of medicinal plants are required to control the quality, efficacy, and safety. There are various methods to identify plant. Macroscopic and microscopic characteristics are the first steps for plant material identification, for example the main structures that can be found in powder of heartwood are xylem and pitted vessel (Alax 2003). This research demonstrated microscopic characteristics of *Caesalpinia sappan* heartwood in both sections (tangential longitudinal and radial longitudinal) and powders which presented fragment of wood, fragment of xylem (wood fiber, wood parenchyma), fragment of bordered pitted vessel, and prism crystals of calcium oxalate.

The physico-chemical parameters including total ash, acid insoluble ash, ethanol and water soluble extractive values, loss on drying, and water content were specified to evaluate the quality of *C. sappan* heartwood in Thailand. The content of total ash and acid-insoluble ash, loss on drying, and water content should not be more than 0.87, 0.44, 8.50, and 8.52 g/100 g crude drug, respectively whereas the content of ethanol and water soluble extractives values should not be less than 2.94

and 3.77 g/100 g crude drug. In 2003, Badami and team reported the total ash, acid insoluble ash, water soluble ash, and sulphated ash values of *C. sappan* heartwood in India as 1.22, 0.13, 0.38, and 1.14 %. The ethanol and water soluble extractive values were 4.80 and 2.69 % (Badami 2003). In 2010, Chen and team established the quality standard of Sappan Lignum in China and the ethanol soluble extractives of 18 samples ranged from 6.4 to 11.3% (Chen 2010). The values might be different from other countries depending on intrinsic and extrinsic factors, for example, an atmospheric condition and the physical features of the land.

Brazilin is the major constituent of *C. sappan* heartwood. Therefore, brazilin quantification is essential for quality control of this crude drug. TLC-densitometry as well as TLC-image analysis were developed for brazilin quantification. Brazilin in *C. sappan* heartwood by TLC-densitometry compared to TLC-image analysis presented at 1.259 ± 0.455 and 1.256 ± 0.405 g/100 g crude drug, respectively. The quantity of brazilin in *C. sappan* heartwood from two methods were not statistically significantly different (P> 0.05). As a result, TLC-image analysis, which is inexpensive, can be used as another optional technique to examine brazilin compound in *C. sappan* heartwood.

According to International Conference on Harmonization guideline (Q2R1), analytical method was validated to confirm that the analytical process was appropriate, reliable, and obtain an accurate data (ICH 2005). For accuracy, the

sample was spiked with three different concentrations of standard brazilin to calculate the percent recovery. The results obtained from TLC-densitometry and TLC-image analysis were found to be 98.10% and 97.86%, respectively. In terms of precision, the repeatability and intermediate precision were performed within a day and at different days, consecutively. Each test was conducted in triplicate using four concentrations. The results for both intra-day and inter-day precision were displayed as % relative standard deviation (%RSD), and were found to be acceptable (less than 10%). The values of limit of detection obtained from TLC-densitometry and TLC-image analysis were 0.02 and 0.01 µg/spot, respectively; whereas, those of the limit of quantitation were 0.06 and 0.04 µg/spot. Robustness was done by varying the ratio of mobile phase (chloroform: ethyl acetate: formic acid). The three various ratios included 10.2: 8.2: 1.8, 10: 8: 2 and 9.8: 7.8: 2.2. Presented as %RSD, the results were not more than 5%. Consequently, the validated method proved that both TLC-densitometry and TLC-image analysis were effective and dependable to quantify the brazilin content in *C. sappan* heartwood.

Morphological characteristics is fundamental for plant authentication (Santhan 2014). Mostly, the keys to differentiate each plant include the flower, pod, seed, shape and size of leaf etc. Flowers, fruits and seeds depend upon the specific time and condition. *Caesalpinia* L. are evergreen plants, their leaves can be sampling throughout the year. However, the size and shape of each leaflet are similar and may

be affected by the environment condition. Leaf anatomy especially at the midrib part is considered as a stable region regarding the conservation of its structures when submitted to the image acquirement process (Niinemets 2007). Midrib cross section of the leaf of eight *Caesalpinia* species were investigated. The structures of upper epidermis, palisade cell, spongy cell, sclerenchyma, xylem tissue, phloem tissue, parenchyma, collenchyma, and lower epidermis were illustrated. Unicellular non-glandular trichomes were found in four species including *C. bonduc*, *C. digyna*, *C. minax*, and *C. decapetala* which were the same type found in other trichome containing *Caesalpinia* (Lersten 1994). In 2015, Mehra *et al*. reported that the unicellular trichomes were presented on the upper and lower epidermis from transverse section through midrib of the *C. bonduc* in India (Mehra 2015). *Caesalpinia bonduc* in this study also demonstrated the trichomes only at both epidermis of midrib cross section. So the trichome numbers and trichome index determined from the lamina could not be evaluated. This is an identity of *C. bonduc* beneficial for the species authentication.

Microscopic leaf constant numbers are useful parameter for plant identification in species level (Martin 2007). The palisade ratio has been used as a demonstrative value to differentiate plant species. This value can be affected by geographical variation but different from species to species (Gokhale 2009), (Komlaga 2014). However, among eight *Caesalpinia* species, the palisade ratio seemed to be

overlapping. Kundu *et al.*, 2011 reported the palisade ratio of *C. bonduc* leaves in India in the range of 14 to 18 which was higher than in Thailand (3-6) (Kundu 2011). Epidermal cell area is relatively constant within a diminish range of each species that permit a correct identification although some variation of overlapping with closely related species (Foroughbakhch 2008). In this study, upper epidermal cell area was demonstrated its benefit to differentiate *C. sappan* from *C. pulcherrima*.

Furthermore, molecular identification of selected eight *Caesalpinia* species was also achieved for evaluation of the genetic relationship in this genus. DNA markers provide an accurate and efficient identification of medicinal plants without an effect of environment factors (Joshi 2007). ISSR markers is one of popular technique that used to identify plant species, genetic diversity, molecular ecology etc. This technique provides a reliable and high informative system for DNA fingerprinting. ISSR primer is universal primer, easy to assess, and not require the previous sequence information. It is not complicated and suitable for beginner to study the relationship in plants (Wang 2002).

In this study, seven ISSR primers including ISSR 02, ISSR 06, ISSR 09, ISSR 10, ISSR 13, ISSR 14, and ISSR 23 were selected from fifteen primers. Gagnon and team reported that annealing temperatures were varied between 50 and 53 °C for *Caesalpinia* L. genus (Gagnon 2016). This research used the annealing temperature at 50 °C for all primers except ISSR 23 which used at 60°C. For the annealing

temperature, each primer should be optimized to obtain a good product. Thus, each primer might use the different annealing temperature for the best quality of analysis and suitable for individual plant species. In this study, the results from all seven ISSR primers demonstrated one hundred percent of polymorphism representing non-monophyletic group.

According to ISSR technique, eight *Caesalpinia* species in the study were classified into four groups. Cluster I was divided into two subgroups including cluster Ia (*C. mimosoides*, *C. coriaria*, and *C. digyna*) and cluster Ib (*C. decapetala* and *C. pulcherrima*). *Caesalpinia mimosoides* and *C. coriaria* were grouped in cluster Ia which display the highest similarity index of 0.454. Cluster II consisted of *C. sappan* and cluster III consisted of *C. minax* and *C. bonduc* with the similarity index 0.372. The lowest similarity index (0.192) was presented between *C. sappan* and *C. bonduc*. *Annona squamosa*, the outgroup plant was clearly separated from other *Caesalpinia* species. Gagnon *et al*., 2016 proposed the phylogeny of the *Caesalpinia* group using five plastid [rps16, the trnD-trnT intergenic spacer, ycf6-psbM, the matK gene and flanking 3'-trnK intron, and the trnL-trnF intron-spacer region] and one nuclear ribosomal marker [ITS1 and ITS2]. From 172 species, they were classified into 26 groups (Gagnon 2016). Eight *Caesalpinia* species were classified as I. *C. pulcherrima*; II. *C. mimosoides*; III. *C. minax* and *C. bonduc*; IV. *C. digyna*; V. *C. decapetala* and

C. sappan and VI. *C. coriaria*. From Gagnon study and this ISSR study, it was found similarly that *C. minax* and *C. bonduc* were closely relationship.

Conclusion

This research provides beneficial information for identification and confirmation of *Caesalpinia* L. genus based on macroscopic and microscopic evaluation including leaf constant numbers, and molecular identification by ISSR marker. The pharmacognostic specification with reference to brazilin content of *C. sappan* heartwood in Thailand were demonstrated. TLC-densitometry and TLC-image analysis were developed for brazilin quantitation. ImageJ software was used for processing of the chromatogram image and found that it could be an alternated method for quantification of brazilin in *C. sappan* heartwood. The qualitative and quantitative microscopic characteristics of selected eight *Caesalpinia* species in Thailand were established. These parameters could be used as a tool for these plants authentication. ISSR could be used as genetic diversity analysis for *Caesalpinia*.

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APPENDIX

Appendix A Leaf constant numbers

Table 32 Stomatal cell number, lower epidermal cell number, and stomatal index of *C. sappan* (Source1 = Bangkok, Source2 = Phetchabun, Source3 = Ratchaburi)

Field	Stomatal number			Lower epidermal cell number			Stomatal index		
	1	2	3	1	2	3	1	2	3
1	294.00	292.00	310.00	1334.00	1282.00	1480.00	18.06	18.55	17.33
2	284.00	286.00	316.00	1306.00	1272.00	1484.00	17.86	18.36	17.56
3	304.00	302.00	294.00	1348.00	1334.00	1502.00	18.40	18.46	16.37
4	298.00	300.00	260.00	1342.00	1340.00	1268.00	18.17	18.29	17.02
5	318.00	322.00	258.00	1384.00	1334.00	1262.00	18.68	19.44	16.97
6	310.00	294.00	252.00	1344.00	1296.00	1346.00	18.74	18.49	15.77
7	314.00	306.00	242.00	1368.00	1392.00	1304.00	18.66	18.02	15.65
8	314.00	322.00	230.00	1458.00	1472.00	1222.00	17.72	17.95	15.84
9	322.00	322.00	248.00	1462.00	1458.00	1232.00	18.05	18.09	16.76
10	306.00	288.00	238.00	1362.00	1392.00	1316.00	18.35	17.14	15.32
11	306.00	316.00	258.00	1370.00	1386.00	1314.00	18.26	18.57	16.41
12	284.00	282.00	248.00	1330.00	1322.00	1350.00	17.60	17.58	15.52
13	348.00	292.00	252.00	1464.00	1440.00	1346.00	19.21	16.86	15.77
14	332.00	294.00	274.00	1446.00	1404.00	1296.00	18.67	17.31	17.45
15	294.00	300.00	280.00	1438.00	1316.00	1404.00	16.97	18.56	15.63
16	312.00	316.00	268.00	1436.00	1420.00	1382.00	17.85	18.20	16.24
17	298.00	306.00	260.00	1316.00	1394.00	1368.00	18.46	18.00	15.97
18	306.00	292.00	272.00	1456.00	1330.00	1392.00	17.37	18.00	16.35
19	314.00	314.00	258.00	1380.00	1370.00	1376.00	18.54	18.65	15.79
20	300.00	298.00	272.00	1374.00	1334.00	1394.00	17.92	18.26	16.33
21	326.00	302.00	280.00	1350.00	1394.00	1482.00	19.45	17.81	15.89
22	298.00	280.00	310.00	1312.00	1312.00	1504.00	18.51	17.59	17.09
23	300.00	294.00	276.00	1380.00	1328.00	1382.00	17.86	18.13	16.65
24	280.00	304.00	256.00	1322.00	1310.00	1320.00	17.37	18.84	16.24
25	288.00	292.00	248.00	1320.00	1358.00	1314.00	17.91	17.70	15.88
26	302.00	318.00	274.00	1330.00	1306.00	1372.00	18.50	19.58	16.65
27	308.00	288.00	284.00	1296.00	1392.00	1432.00	19.20	17.14	16.75
28	294.00	298.00	314.00	1350.00	1362.00	1476.00	17.88	17.95	16.55
29	322.00	300.00	284.00	1328.00	1214.00	1352.00	19.52	19.82	17.54
30	292.00	312.00	256.00	1392.00	1358.00	1272.00	17.34	18.68	17.36
Min	230.00			1214.00			15.32		
Max	348.00			1504.00			19.82		
Mean	291.91			1362.93			17.62		
S.D.	23.63			63.73			1.07		

Table 33 Upper epidermal cell area, palisade cell number, and palisade ratio of *C. sappan*

Field	Number of palisade cell			Palisade ratio			Upper epidermal cell area (μm²)		
	1	2	3	1	2	3	1	2	3
1	22.00	20.50	25.50	5.50	5.13	6.38	1488.10	1552.80	1340.48
2	23.00	25.00	23.00	5.75	6.25	5.75	1529.05	1547.99	1388.89
3	25.50	24.00	24.50	6.38	6.00	6.13	1602.56	1650.17	1298.70
4	24.00	27.00	26.50	6.00	6.75	6.63	1618.12	1689.19	1204.82
5	23.00	28.00	23.50	5.75	7.00	5.88	1639.34	1694.92	1265.82
6	28.50	20.00	27.00	7.13	5.00	6.75	1639.34	1683.50	1272.26
7	25.50	24.00	27.00	6.38	6.00	6.75	1552.80	1724.14	1404.49
8	22.00	22.50	26.00	5.50	5.63	6.50	1483.68	1602.56	1336.90
9	24.50	24.00	26.00	6.13	6.00	6.50	1506.02	1543.21	1250.00
10	25.00	24.00	23.50	6.25	6.00	5.88	1582.28	1515.15	1305.48
11	24.50	23.50	29.50	6.13	5.88	7.38	1483.68	1587.30	1256.28
12	23.50	21.00	24.50	5.88	5.25	6.13	1474.93	1506.02	1295.34
13	27.00	27.00	27.00	6.75	6.75	6.75	1515.15	1552.80	1222.49
14	23.50	25.00	28.50	5.88	6.25	7.13	1623.38	1533.74	1272.26
15	30.00	29.00	26.50	7.50	7.25	6.63	1607.72	1582.28	1196.17
16	29.50	28.00	27.50	7.38	7.00	6.88	1524.39	1562.50	1149.43
17	26.50	20.50	27.00	6.63	5.13	6.75	1497.01	1506.02	1228.50
18	28.50	23.00	26.50	7.13	5.75	6.63	1470.59	1501.50	1288.66
19	30.00	25.00	25.50	7.50	6.25	6.38	1510.57	1567.40	1285.35
20	28.00	20.50	29.00	7.00	5.13	7.25	1533.74	1582.28	1420.45
21	22.50	27.00	25.50	5.63	6.75	6.38	1466.28	1420.45	1291.99
22	26.00	24.00	23.50	6.50	6.00	5.88	1412.43	1533.74	1305.48
23	22.00	24.00	30.00	5.50	6.00	7.50	1515.15	1519.76	1377.41
24	30.00	23.00	25.00	7.50	5.75	6.25	1524.39	1650.17	1170.96
25	27.00	22.00	28.50	6.75	5.50	7.13	1449.28	1557.63	1187.65
26	23.50	26.00	24.00	5.88	6.50	6.00	1567.40	1445.09	1210.65
27	24.50	25.00	28.50	6.13	6.25	7.13	1416.43	1597.44	1207.73
28	29.50	27.00	24.50	7.38	6.75	6.13	1501.50	1453.49	1373.63
29	21.50	26.00	23.50	5.38	6.50	5.88	1453.49	1533.74	1176.48
30	23.50	29.50	25.50	5.88	7.38	6.38	1533.74	1479.29	1141.55
Min	20.00			5.00			1141.55		
Max	30.00			7.50			1724.14		
Mean	25.35			6.34			1452.50		
S.D.	2.52			0.63			148.21		

Source1 = Bangkok, Source2 = Phetchabun, Source3 = Ratchaburi

Table 34 Stomatal cell number, lower epidermal cell number, and stomatal index of *C. pulcherrima*

Field	Stomatal number			Lower epidermal cell number			Stomatal index		
	1	2	3	1	2	3	1	2	3
1	412.00	302.00	446.00	3754.00	3280.00	4562.00	9.89	8.43	8.91
2	422.00	292.00	408.00	3748.00	3148.00	4212.00	10.12	8.49	8.83
3	422.00	312.00	466.00	3700.00	3114.00	4484.00	10.24	9.11	9.41
4	478.00	300.00	454.00	4092.00	3128.00	4468.00	10.46	8.75	9.22
5	380.00	308.00	468.00	3672.00	3212.00	4534.00	9.38	8.75	9.36
6	384.00	346.00	484.00	3560.00	3394.00	4590.00	9.74	9.25	9.54
7	374.00	314.00	428.00	3788.00	3406.00	4460.00	8.99	8.44	8.76
8	370.00	280.00	422.00	3876.00	2938.00	4184.00	8.71	8.70	9.16
9	362.00	278.00	438.00	3750.00	3132.00	4226.00	8.80	8.15	9.39
10	394.00	294.00	376.00	3520.00	3444.00	4170.00	10.07	7.87	8.27
11	352.00	318.00	448.00	3596.00	3228.00	4402.00	8.92	8.97	9.24
12	366.00	288.00	446.00	3664.00	3286.00	4328.00	9.08	8.06	9.34
13	362.00	318.00	402.00	3442.00	3268.00	4150.00	9.52	8.87	8.83
14	422.00	292.00	390.00	3560.00	3012.00	4168.00	10.60	8.84	8.56
15	350.00	318.00	408.00	3976.00	3376.00	4140.00	8.09	8.61	8.97
16	348.00	294.00	396.00	3504.00	3310.00	4168.00	9.03	8.16	8.68
17	392.00	310.00	414.00	3460.00	3052.00	4188.00	10.18	9.22	9.00
18	428.00	296.00	408.00	3918.00	3122.00	4246.00	9.85	8.66	8.77
19	430.00	282.00	400.00	3816.00	3032.00	4152.00	10.13	8.51	8.79
20	434.00	310.00	376.00	3616.00	3272.00	4158.00	10.72	8.65	8.29
21	436.00	334.00	408.00	4000.00	3352.00	4160.00	9.83	9.06	8.93
22	480.00	326.00	368.00	3890.00	3400.00	4164.00	10.98	8.75	8.12
23	458.00	336.00	404.00	3844.00	3294.00	4326.00	10.65	9.26	8.54
24	432.00	278.00	372.00	3864.00	3142.00	4384.00	10.06	8.13	7.82
25	384.00	282.00	424.00	3452.00	3250.00	4156.00	10.01	7.98	9.26
26	402.00	338.00	382.00	3546.00	3328.00	4156.00	10.18	9.22	8.42
27	358.00	344.00	410.00	3492.00	3320.00	4146.00	9.30	9.39	9.00
28	464.00	300.00	362.00	3900.00	3082.00	4212.00	10.63	8.87	7.91
29	372.00	286.00	360.00	3632.00	3136.00	4130.00	9.29	8.36	8.02
30	370.00	308.00	376.00	3548.00	3276.00	4142.00	9.44	8.59	8.32
Min	278.00			2938.00			7.82		
Max	484.00			4590.00			10.98		
Mean	372.96			3732.00			9.07		
S.D.	57.17			454.41			0.73		

Source1 = Bangkok, Source2 = Nonthaburi, Source3 = Samut Sakhon

Table 35 Upper epidermal cell area, palisade cell number, and palisade ratio of *C. pulcherrima*

Field	Number of palisade cell			Palisade ratio			Upper epidermal cell area (μm²)		
	1	2	3	1	2	3	1	2	3
1	28.50	25.50	22.50	7.13	6.38	5.63	429.55	490.20	457.46
2	21.00	28.00	19.50	5.25	7.00	4.88	456.62	497.02	432.90
3	23.00	26.00	19.50	5.75	6.50	4.88	406.17	494.07	453.72
4	19.50	30.00	21.50	4.88	7.50	5.38	424.81	487.33	486.38
5	25.00	26.50	25.50	6.25	6.63	6.38	439.75	520.83	477.55
6	22.00	24.00	23.00	5.50	6.00	5.75	425.89	513.35	476.19
7	23.50	28.50	21.00	5.88	7.13	5.25	442.09	505.56	462.96
8	21.50	25.00	23.50	5.38	6.25	5.88	442.48	477.55	450.45
9	20.00	23.50	23.00	5.00	5.88	5.75	437.45	441.31	435.16
10	21.50	25.00	23.00	5.38	6.25	5.75	441.31	502.01	446.03
11	23.00	27.50	21.00	5.75	6.88	5.25	425.89	495.05	440.14
12	20.50	29.50	21.00	5.13	7.38	5.25	412.88	511.25	457.04
13	21.50	24.00	24.50	5.38	6.00	6.13	423.73	524.66	494.07
14	21.50	27.50	21.50	5.38	6.88	5.38	401.93	515.46	453.72
15	24.50	26.00	24.00	6.13	6.50	6.00	442.09	523.01	448.03
16	19.50	24.50	20.50	4.88	6.13	5.13	404.86	513.35	432.53
17	20.50	28.00	19.50	5.13	7.00	4.88	410.51	515.46	434.40
18	25.00	27.50	20.00	6.25	6.88	5.00	412.88	507.61	435.16
19	20.50	28.00	22.00	5.13	7.00	5.50	410.17	520.83	435.16
20	21.50	27.50	21.00	5.38	6.88	5.25	418.76	479.85	495.54
21	20.50	24.00	25.00	5.13	6.00	6.25	408.50	493.10	472.14
22	20.00	29.00	21.00	5.00	7.25	5.25	410.51	469.48	478.01
23	24.00	27.50	25.00	6.00	6.88	6.25	386.10	480.77	451.67
24	24.00	26.50	22.50	6.00	6.63	5.63	399.04	461.25	450.86
25	22.00	24.50	21.50	5.50	6.13	5.38	406.17	492.61	445.24
26	24.50	25.00	21.50	6.13	6.25	5.38	411.52	461.68	429.92
27	19.00	28.00	22.50	4.75	7.00	5.63	418.76	445.63	457.88
28	23.50	24.00	24.00	5.88	6.00	6.00	429.92	468.16	488.76
29	25.50	25.00	21.00	6.38	6.25	5.25	392.77	496.52	489.24
30	26.00	25.00	20.50	6.50	6.25	5.13	432.15	509.17	464.25
Min	19.00			4.75			386.10		
Max	30.00			7.50			524.66		
Mean	23.61			5.90			457.24		
S.D.	2.76			0.69			36.19		

Source1 = Bangkok, Source2 = Nonthaburi, Source3 = Samut Sakhon

Table 36 Stomatal cell number, lower epidermal cell number, and stomatal index of *C. coriaria*

Field	Stomatal number			Lower epidermal cell number			Stomatal index		
	1	2	3	1	2	3	1	2	3
1	288.00	306.00	470.00	1446.00	1540.00	1682.00	16.61	16.58	21.84
2	272.00	304.00	516.00	1258.00	1482.00	1702.00	17.78	17.02	23.26
3	260.00	304.00	554.00	1374.00	1528.00	1700.00	15.91	16.59	24.69
4	234.00	300.00	454.00	1256.00	1546.00	1698.00	15.70	16.25	21.10
5	280.00	282.00	482.00	1290.00	1474.00	1696.00	17.83	16.06	22.13
6	272.00	282.00	522.00	1296.00	1472.00	1792.00	17.35	16.08	22.56
7	236.00	286.00	480.00	1292.00	1424.00	1718.00	15.45	16.73	21.84
8	274.00	290.00	482.00	1380.00	1514.00	1620.00	16.57	16.08	22.93
9	296.00	272.00	462.00	1410.00	1450.00	1660.00	17.35	15.80	21.77
10	268.00	298.00	442.00	1358.00	1496.00	1680.00	16.48	16.61	21.03
11	276.00	302.00	446.00	1340.00	1558.00	1730.00	17.08	16.24	20.50
12	244.00	284.00	466.00	1216.00	1406.00	1710.00	16.71	16.80	21.42
13	266.00	290.00	436.00	1296.00	1514.00	1782.00	17.03	16.08	19.66
14	244.00	292.00	474.00	1352.00	1512.00	1680.00	15.29	16.19	22.01
15	236.00	304.00	468.00	1288.00	1470.00	1680.00	15.49	17.14	21.79
16	244.00	292.00	462.00	1298.00	1466.00	1732.00	15.82	16.19	21.06
17	266.00	302.00	466.00	1396.00	1508.00	1670.00	16.00	16.69	21.82
18	278.00	286.00	470.00	1266.00	1450.00	1686.00	18.01	16.47	21.80
19	270.00	306.00	412.00	1350.00	1532.00	1728.00	16.67	16.65	19.25
20	252.00	308.00	432.00	1302.00	1444.00	1740.00	16.22	17.58	19.89
21	254.00	282.00	466.00	1290.00	1412.00	1776.00	16.45	16.65	20.79
22	232.00	284.00	464.00	1288.00	1464.00	1690.00	15.26	16.25	21.54
23	226.00	312.00	562.00	1292.00	1456.00	1832.00	14.89	17.65	23.48
24	236.00	310.00	476.00	1298.00	1532.00	1664.00	15.38	16.83	22.24
25	264.00	310.00	500.00	1312.00	1488.00	1712.00	16.75	17.24	22.60
26	250.00	296.00	412.00	1294.00	1456.00	1656.00	16.19	16.90	19.92
27	270.00	292.00	464.00	1328.00	1436.00	1726.00	16.90	16.90	21.19
28	270.00	292.00	482.00	1302.00	1428.00	1722.00	17.18	16.98	21.87
29	280.00	284.00	488.00	1266.00	1428.00	1690.00	18.11	16.59	22.41
30	268.00	290.00	482.00	1336.00	1424.00	1694.00	16.71	16.92	22.15
Min	226.00			1216.00			14.89		
Max	562.00			1832.00			24.69		
Mean	342.67			1500.31			18.27		
S.D.	96.53			168.30			2.58		

Source1 = Bangkok 1, Source2 = Prachin Buri, Source3 = Bangkok 2

Table 37 Upper epidermal cell area, palisade cell number, and palisade ratio of *C. coriaria*

Field	Number of palisade cell			Palisade ratio			Upper epidermal cell area (μm²)		
	1	2	3	1	2	3	1	2	3
1	26.50	53.50	36.00	6.63	13.38	9.00	749.63	651.89	608.27
2	26.50	54.00	37.00	6.63	13.50	9.25	734.21	755.29	702.25
3	24.50	54.50	37.00	6.13	13.63	9.25	738.55	705.22	642.67
4	27.00	51.50	36.50	6.75	12.88	9.13	744.05	709.22	641.85
5	28.00	51.00	35.00	7.00	12.75	8.75	750.75	652.74	763.36
6	27.50	46.00	39.50	6.88	11.50	9.88	704.23	647.67	713.27
7	30.00	52.00	35.50	7.50	13.00	8.88	664.89	693.48	727.80
8	26.50	52.50	39.00	6.13	13.13	9.75	626.57	727.80	748.50
9	28.50	47.00	41.00	7.13	11.75	10.25	670.24	738.55	736.38
10	31.00	45.00	42.00	7.75	11.25	10.50	718.39	739.65	711.24
11	30.50	49.50	40.50	7.63	12.38	10.13	720.46	742.94	608.27
12	27.00	49.00	42.50	6.75	12.25	10.63	710.23	753.01	603.86
13	29.50	49.50	36.50	7.38	12.38	9.13	715.31	771.60	603.14
14	31.00	52.00	39.00	7.75	13.00	9.75	771.60	725.69	609.76
15	30.00	51.50	38.50	7.50	12.88	9.63	748.50	769.23	614.25
16	29.50	50.00	40.00	7.38	12.50	10.00	763.36	708.22	683.06
17	31.50	46.00	38.50	7.88	11.50	9.63	747.38	765.70	758.73
18	30.50	48.00	35.00	7.63	12.00	8.75	762.20	744.05	719.42
19	31.00	51.00	37.00	7.75	12.75	9.25	739.65	703.23	640.20
20	31.50	54.50	35.50	7.88	13.63	8.88	741.84	764.53	698.32
21	31.50	47.50	35.00	7.88	11.88	8.75	733.14	711.24	603.86
22	30.50	45.50	42.50	7.63	11.38	10.63	728.86	706.21	620.35
23	28.50	49.50	42.00	7.13	12.38	10.50	710.23	700.28	565.61
24	29.00	49.00	35.50	7.25	12.25	8.88	723.59	714.29	593.12
25	31.50	53.50	37.50	7.88	13.38	9.38	667.56	735.29	638.06
26	31.00	49.00	40.00	7.75	12.25	10.00	643.50	736.38	606.80
27	27.00	49.50	39.50	6.75	12.38	9.88	641.03	740.74	616.52
28	31.50	48.00	38.00	7.88	12.00	9.50	663.13	721.50	706.21
29	29.50	53.00	36.00	7.38	13.25	9.00	651.04	729.93	644.33
30	30.50	48.00	38.00	7.63	12.00	9.50	670.24	730.99	591.72
Min	24.50			6.13			565.61		
Max	54.50			13.63			771.60		
Mean	39.16			9.78			697.47		
S.D.	8.86			2.22			53.43		

Source1 = Bangkok 1, Source2 = Prachin Buri, Source3 = Bangkok 2

Table 38 Stomatal cell number, lower epidermal cell number, and stomatal index of *C. mimosoides*

Field	Stomatal number			Lower epidermal cell number			Stomatal index		
	1	2	3	1	2	3	1	2	3
1	286.00	150.00	338.00	1512.00	928.00	1388.00	15.91	13.91	19.58
2	302.00	168.00	336.00	1574.00	946.00	1408.00	16.10	15.08	19.27
3	288.00	170.00	318.00	1530.00	948.00	1426.00	15.84	15.21	18.23
4	296.00	172.00	346.00	1528.00	918.00	1524.00	16.23	15.78	18.50
5	302.00	158.00	326.00	1516.00	926.00	1474.00	16.61	14.58	18.11
6	286.00	162.00	344.00	1442.00	930.00	1426.00	16.55	14.84	19.44
7	332.00	190.00	316.00	1466.00	948.00	1402.00	18.47	16.70	18.39
8	318.00	138.00	310.00	1448.00	862.00	1458.00	18.01	13.80	17.53
9	274.00	186.00	340.00	1402.00	936.00	1436.00	16.35	16.58	18.14
10	304.00	186.00	338.00	1562.00	960.00	1428.00	16.29	16.23	19.14
11	266.00	186.00	342.00	1494.00	974.00	1444.00	15.11	16.03	19.15
12	272.00	204.00	362.00	1494.00	954.00	1524.00	15.40	17.62	19.19
13	342.00	194.00	340.00	1494.00	960.00	1498.00	18.63	16.81	18.50
14	304.00	172.00	360.00	1442.00	926.00	1516.00	17.41	14.93	19.19
15	284.00	174.00	326.00	1324.00	920.00	1454.00	17.66	15.90	18.31
16	322.00	182.00	324.00	1534.00	944.00	1520.00	17.35	16.16	17.57
17	272.00	146.00	328.00	1460.00	980.00	1490.00	15.70	12.97	18.04
18	314.00	130.00	324.00	1524.00	960.00	1428.00	17.08	11.93	18.49
19	266.00	142.00	318.00	1410.00	966.00	1422.00	15.87	12.82	18.28
20	304.00	158.00	298.00	1486.00	988.00	1370.00	16.98	13.79	17.87
21	324.00	170.00	284.00	1478.00	982.00	1366.00	17.98	14.76	17.21
22	292.00	180.00	342.00	1438.00	1034.00	1446.00	16.88	14.83	19.13
23	304.00	184.00	340.00	1410.00	966.00	1422.00	17.74	16.00	19.30
24	292.00	166.00	330.00	1450.00	960.00	1382.00	16.76	14.74	19.28
25	254.00	190.00	308.00	1410.00	958.00	1358.00	15.26	16.55	18.49
26	266.00	144.00	320.00	1454.00	890.00	1362.00	15.47	13.93	19.03
27	290.00	162.00	386.00	1504.00	910.00	1560.00	16.17	15.11	19.84
28	302.00	178.00	368.00	1428.00	932.00	1600.00	17.46	16.04	18.70
29	276.00	164.00	366.00	1482.00	914.00	1588.00	16.65	15.21	18.73
30	286.00	150.00	322.00	1356.00	896.00	1554.00	17.42	14.34	17.16
Min	130.00			862.00			11.93		
Max	386.00			1600.00			19.84		
Mean	265.29			1289.36			16.80		
S.D.	73.49			251.65			1.75		

Source1 = Chiangrai, Source2 = Nakhon Ratchasima, Source3 = Chiangmai

Table 39 Upper epidermal cell area, palisade cell number, and palisade ratio of *C. mimosoides*

Field	Number of palisade cell			Palisade ratio			Upper epidermal cell area (μm²)		
	1	2	3	1	2	3	1	2	3
1	21.50	22.00	23.50	5.38	5.50	5.88	772.80	1020.41	789.89
2	22.50	25.00	25.50	5.63	6.25	6.38	847.46	1052.63	836.12
3	18.50	21.50	26.00	4.63	5.38	6.50	931.10	1041.67	900.90
4	21.00	20.00	26.50	5.25	5.00	6.63	860.59	1063.83	1008.06
5	20.00	21.50	24.00	5.00	5.38	6.00	770.42	1054.85	1020.41
6	20.00	24.00	23.50	5.00	6.00	5.88	775.19	1061.57	946.97
7	20.00	22.00	26.00	5.00	5.50	6.50	786.16	984.25	982.32
8	20.00	24.00	29.00	5.00	6.00	7.25	871.08	1075.27	929.37
9	19.50	27.00	27.00	4.88	6.75	6.75	851.79	1103.75	850.34
10	22.00	22.50	28.50	5.50	5.63	7.13	823.72	897.67	871.08
11	21.00	22.00	23.00	5.25	5.50	5.75	677.51	831.95	1016.26
12	20.50	21.50	28.00	5.13	5.38	7.00	671.14	924.21	957.85
13	19.00	24.00	25.50	4.75	6.00	6.38	657.03	826.45	862.07
14	19.00	20.50	26.50	4.75	5.13	6.63	695.41	904.16	823.72
15	18.50	22.00	29.00	4.63	5.50	7.25	737.46	945.18	821.02
16	19.00	20.00	27.50	4.75	5.00	6.88	714.29	946.97	854.70
17	20.00	23.00	25.00	5.00	5.75	6.25	728.86	996.02	871.08
18	23.50	23.00	26.00	5.88	5.75	6.50	715.31	957.85	841.75
19	19.00	22.00	28.00	4.75	5.50	7.00	705.22	986.19	875.66
20	22.50	24.00	27.50	5.63	6.00	6.88	700.28	968.99	833.33
21	21.00	23.00	25.50	5.25	5.75	6.38	751.88	1016.26	848.90
22	21.00	20.00	26.50	5.25	5.00	6.63	762.20	1000.00	934.58
23	20.50	25.50	28.00	5.13	6.38	7.00	766.87	1028.81	886.52
24	20.00	25.00	29.00	5.00	6.25	7.25	772.80	1008.06	875.66
25	20.00	26.50	27.50	5.00	6.63	6.88	782.47	972.76	836.12
26	19.00	27.00	26.50	4.75	6.75	6.63	777.61	990.10	936.33
27	20.00	20.50	28.00	5.00	5.13	7.00	780.03	978.47	956.02
28	19.50	21.00	30.00	4.88	5.25	7.50	754.15	1010.10	950.57
29	19.00	25.00	30.00	4.75	6.25	7.50	735.29	1072.96	1024.59
30	22.50	21.00	26.00	5.63	5.25	6.50	672.04	1096.49	1004.02
Min	18.50			4.63			657.03		
Max	30.00			7.50			1103.75		
Mean	23.31			5.83			886.80		
S.D.	3.19			0.80			117.48		

Source1 = Chiangrai, Source2 = Nakhon Ratchasima, Source3 = Chiangmai

Table 40 Stomatal cell number, lower epidermal cell number, and stomatal index of *C. bonduc*

Field	Stomatal number			Lower epidermal cell number			Stomatal index		
	1	2	3	1	2	3	1	2	3
1	100.00	118.00	110.00	960.00	1620.00	1754.00	9.43	6.79	5.90
2	108.00	128.00	106.00	956.00	1486.00	1790.00	10.15	7.93	5.59
3	96.00	136.00	118.00	970.00	1706.00	1844.00	9.01	7.38	6.01
4	100.00	148.00	128.00	900.00	1718.00	1832.00	10.00	7.93	6.53
5	104.00	128.00	96.00	942.00	2000.00	1766.00	9.94	6.02	5.16
6	98.00	116.00	98.00	932.00	1860.00	1878.00	9.51	5.87	4.96
7	100.00	140.00	112.00	932.00	1594.00	1798.00	9.69	8.07	5.86
8	100.00	116.00	90.00	938.00	1730.00	1782.00	9.63	6.28	4.81
9	106.00	132.00	106.00	966.00	1544.00	1790.00	9.89	7.88	5.59
10	118.00	146.00	92.00	996.00	1734.00	1758.00	10.59	7.77	4.97
11	114.00	138.00	92.00	960.00	1658.00	1744.00	10.61	7.68	5.02
12	108.00	138.00	116.00	912.00	1970.00	1798.00	10.59	6.55	6.06
13	98.00	144.00	130.00	996.00	2002.00	1680.00	8.96	6.71	7.18
14	108.00	126.00	96.00	1004.00	1754.00	1618.00	9.71	6.70	5.60
15	100.00	118.00	102.00	992.00	1630.00	1632.00	9.16	6.75	5.88
16	106.00	134.00	92.00	984.00	1796.00	1638.00	9.72	6.94	5.32
17	94.00	124.00	112.00	992.00	1850.00	1652.00	8.66	6.28	6.35
18	104.00	146.00	106.00	992.00	1964.00	1778.00	9.49	6.92	5.63
19	96.00	132.00	130.00	944.00	1840.00	1776.00	9.23	6.69	6.82
20	92.00	110.00	94.00	964.00	1930.00	1688.00	8.71	5.39	5.28
21	120.00	140.00	140.00	984.00	1740.00	1744.00	10.87	7.45	7.43
22	108.00	114.00	142.00	998.00	1862.00	1702.00	9.76	5.77	7.70
23	94.00	110.00	128.00	982.00	1746.00	1784.00	8.74	5.93	6.69
24	98.00	132.00	122.00	976.00	2000.00	1754.00	9.12	6.19	6.50
25	94.00	126.00	114.00	986.00	1908.00	1616.00	8.70	6.19	6.59
26	98.00	122.00	104.00	986.00	1798.00	1738.00	9.04	6.35	5.65
27	96.00	138.00	100.00	900.00	1698.00	1734.00	9.64	7.52	5.45
28	108.00	152.00	116.00	916.00	1778.00	1718.00	10.55	7.88	6.33
29	122.00	126.00	152.00	1004.00	1746.00	1882.00	10.83	6.73	7.47
30	104.00	154.00	138.00	978.00	1788.00	1822.00	9.61	7.93	7.04
Min	90.00			900.00			4.81		
Max	154.00			2002.00			10.87		
Mean	115.62			1498.69			7.53		
S.D.	17.34			390.42			1.72		

Source1 = Nakhon Ratchasima, Source2 = Ratchaburi, Source3 = Songkhla

Table 41 Upper epidermal cell area, palisade cell number, and palisade ratio of *C. bonduc*

Field	Number of palisade cell			Palisade ratio			Upper epidermal cell area (μm²)		
	1	2	3	1	2	3	1	2	3
1	15.00	14.00	19.50	3.75	3.50	4.88	1219.51	910.75	862.07
2	13.00	14.50	19.00	3.25	3.63	4.75	1190.48	851.79	865.05
3	12.50	12.50	19.00	3.13	3.13	4.75	1225.49	907.44	837.52
4	12.00	14.50	20.50	3.00	3.63	5.13	1136.36	875.66	788.64
5	13.00	14.00	19.50	3.25	3.50	4.88	1165.50	797.45	822.37
6	12.50	15.50	20.00	3.13	3.88	5.00	1152.07	806.45	782.47
7	12.00	12.00	20.00	3.00	3.00	5.00	1170.96	830.56	805.15
8	14.00	12.50	22.00	3.50	3.13	5.50	1157.41	846.02	830.56
9	13.00	14.00	19.50	3.25	3.50	4.88	1043.84	830.56	840.34
10	12.00	15.50	22.50	3.00	3.88	5.63	1141.55	868.06	796.18
11	16.00	13.50	18.00	4.00	3.38	4.50	1108.65	859.11	762.20
12	13.50	12.00	22.00	3.38	3.00	5.50	1154.73	766.87	746.27
13	14.00	15.00	20.50	3.50	3.75	5.13	1190.48	815.66	687.76
14	12.00	12.00	20.50	3.00	3.00	5.13	1173.71	811.69	798.72
15	14.50	13.50	19.50	3.63	3.38	4.88	1190.48	800.00	826.45
16	17.50	14.50	20.50	4.38	3.63	5.13	1219.51	762.20	848.90
17	12.50	14.00	18.50	3.13	3.50	4.63	1225.49	860.59	833.33
18	13.50	14.00	19.50	3.38	3.50	4.88	1168.22	853.24	889.68
19	13.50	14.50	19.50	3.38	3.63	4.88	1086.96	809.06	848.90
20	14.00	13.00	18.50	3.50	3.25	4.63	1094.09	871.08	811.69
21	16.00	13.00	21.00	4.00	3.25	5.25	1160.09	896.06	837.52
22	13.50	14.00	21.50	3.38	3.50	5.38	1179.25	880.28	863.56
23	12.00	13.00	19.00	3.00	3.25	4.75	1138.95	925.93	863.56
24	14.00	15.50	19.50	3.50	3.88	4.88	1204.82	880.28	837.52
25	13.50	15.50	20.50	3.38	3.88	5.13	1168.22	891.27	863.56
26	13.50	14.00	21.50	3.38	3.50	5.38	1184.83	884.96	859.11
27	15.50	13.00	23.00	3.88	3.25	5.75	1219.51	834.72	853.24
28	14.50	15.00	20.00	3.63	3.75	5.00	1210.65	840.34	830.56
29	17.00	13.00	22.50	4.25	3.25	5.63	1101.32	902.53	831.95
30	13.00	12.50	21.50	3.25	3.13	5.38	1133.79	899.28	889.68
Min	12.00			3.00			687.76		
Max	23.00			5.75			1225.49		
Mean	15.94			3.98			947.79		
S.D.	3.35			0.84			159.96		

Source1 = Nakhon Ratchasima, Source2 = Ratchaburi, Source3 = Songkhla

Table 42 Stomatal cell number, lower epidermal cell number, and stomatal index of *C. digyna*

Field	Stomatal number			Lower epidermal cell number			Stomatal index		
	1	2	3	1	2	3	1	2	3
1	360.00	322.00	266.00	2052.00	1644.00	1400.00	14.93	16.80	15.97
2	340.00	380.00	260.00	2126.00	1868.00	1452.00	13.79	16.90	15.19
3	348.00	332.00	286.00	2002.00	1634.00	1514.00	14.81	16.89	15.89
4	360.00	321.00	274.00	2122.00	1530.00	1480.00	14.50	16.94	15.62
5	354.00	298.00	272.00	2118.00	1548.00	1476.00	14.32	16.14	15.56
6	358.00	302.00	264.00	2010.00	1532.00	1438.00	15.12	16.47	15.51
7	336.00	288.00	248.00	2046.00	1600.00	1480.00	14.11	15.25	14.35
8	332.00	330.00	262.00	1960.00	1624.00	1482.00	14.49	16.89	15.02
9	324.00	302.00	276.00	1968.00	1558.00	1584.00	14.14	16.24	14.84
10	332.00	302.00	276.00	1970.00	1712.00	1590.00	14.42	15.00	14.79
11	364.00	312.00	294.00	1956.00	1702.00	1508.00	15.69	15.49	16.32
12	352.00	302.00	272.00	2020.00	1612.00	1500.00	14.84	15.78	15.35
13	366.00	288.00	288.00	2100.00	1720.00	1548.00	14.84	14.34	15.69
14	384.00	288.00	276.00	2062.00	1610.00	1498.00	15.70	15.17	15.56
15	374.00	296.00	260.00	2036.00	1700.00	1504.00	15.52	14.83	14.74
16	384.00	290.00	252.00	2008.00	1714.00	1480.00	16.05	14.47	14.55
17	368.00	320.00	258.00	1954.00	1710.00	1504.00	15.85	15.76	14.64
18	366.00	312.00	252.00	2150.00	1600.00	1550.00	14.55	16.32	13.98
19	360.00	290.00	258.00	2058.00	1636.00	1500.00	14.89	15.06	14.68
20	340.00	402.00	248.00	2010.00	1740.00	1436.00	14.47	18.77	14.73
21	332.00	370.00	248.00	2156.00	1766.00	1390.00	13.34	17.32	15.14
22	358.00	412.00	280.00	2108.00	1800.00	1462.00	14.52	18.63	16.07
23	378.00	384.00	250.00	2096.00	1804.00	1482.00	15.28	17.56	14.43
24	340.00	372.00	248.00	2034.00	1802.00	1508.00	14.32	17.11	14.12
25	360.00	392.00	250.00	2018.00	1836.00	1502.00	15.14	17.59	14.27
26	398.00	328.00	250.00	2020.00	1630.00	1372.00	16.46	16.75	15.41
27	366.00	400.00	298.00	2080.00	1810.00	1578.00	14.96	18.10	15.88
28	370.00	374.00	284.00	2066.00	1796.00	1504.00	15.19	17.24	16.35
29	352.00	410.00	298.00	2144.00	1868.00	1532.00	14.10	18.00	16.28
30	380.00	396.00	250.00	2040.00	1882.00	1446.00	15.70	17.38	14.74
Min	248.00			1372.00			13.34		
Max	412.00			2156.00			18.77		
Mean	320.54			1746.42			15.52		
S.D.	48.54			244.21			1.14		

Source1 = Bangkok, Source2 = Songkhla 1, Source3 = Songkhla 2

Table 43 Upper epidermal cell area, palisade cell number, and palisade ratio of *C. digyna*

Field	Number of palisade cell			Palisade ratio			Upper epidermal cell area (μm²)		
	1	2	3	1	2	3	1	2	3
1	22.00	20.50	24.00	5.50	5.13	6.00	915.75	793.65	714.29
2	26.00	25.00	24.50	6.50	6.25	6.13	912.41	780.03	770.42
3	24.50	22.50	21.50	6.13	5.63	5.38	957.85	827.81	765.70
4	29.00	21.50	25.50	7.25	5.38	6.38	934.58	838.93	758.73
5	23.00	21.50	26.50	5.75	5.38	6.63	822.37	801.28	759.88
6	23.00	25.50	24.50	5.75	6.38	6.13	871.08	837.52	766.87
7	22.50	25.00	24.00	5.63	6.25	6.00	859.11	830.56	759.88
8	27.50	22.50	24.50	6.88	5.63	6.13	878.73	806.45	741.84
9	25.00	24.00	22.50	6.25	6.00	5.63	889.68	797.45	742.95
10	27.00	23.00	21.00	6.75	5.75	5.25	899.28	815.08	742.94
11	27.00	20.50	25.00	6.75	5.13	6.25	919.12	734.21	738.55
12	26.00	24.50	25.00	6.50	6.13	6.25	891.27	725.69	773.99
13	26.50	26.00	22.00	6.63	6.50	5.50	948.77	727.80	751.88
14	22.50	23.00	22.50	5.63	5.75	5.63	929.37	733.14	751.88
15	30.50	23.00	25.00	7.63	5.75	6.25	938.09	739.65	742.94
16	26.00	24.00	25.50	6.50	6.00	6.38	927.64	750.75	741.84
17	27.50	22.00	25.00	6.88	5.50	6.25	920.81	733.14	775.19
18	30.50	23.50	24.00	7.63	5.88	6.00	929.37	747.38	776.40
19	31.50	20.50	25.50	7.88	5.13	6.38	919.12	744.05	803.86
20	26.50	23.00	22.00	6.63	5.75	5.50	840.34	740.74	801.28
21	28.50	21.50	22.50	7.13	5.38	5.63	874.13	722.54	773.99
22	27.50	26.00	25.00	6.88	6.50	6.25	909.09	720.46	745.16
23	25.00	25.50	23.00	6.25	6.38	5.75	925.93	702.25	781.25
24	23.00	21.00	22.50	5.75	5.25	5.63	924.21	699.30	770.42
25	26.50	21.50	25.50	6.63	5.38	6.38	950.57	666.67	769.23
26	24.00	20.50	23.00	6.00	5.13	5.75	945.18	683.06	780.03
27	23.00	21.50	21.00	5.75	5.38	5.25	915.75	694.44	794.91
28	24.00	22.00	23.00	6.00	5.50	5.75	897.67	745.16	777.61
29	25.50	21.00	23.00	6.38	5.25	5.75	915.75	751.88	787.40
30	23.50	24.00	24.50	5.88	6.00	6.13	843.17	780.03	742.94
Min	20.50			5.13			666.67		
Max	31.50			7.88			957.85		
Mean	24.14			6.04			808.68		
S.D.	2.33			0.58			78.44		

Source1 = Bangkok, Source2 = Songkhla 1, Source3 = Songkhla 2

Table 44 Upper trichome number, upper epidermal cell number and upper trichome index of *C. digyna*

Field	Trichome number			Epidermal cell number			Trichome index		
	1	2	3	1	2	3	1	2	3
1	17.00	18.00	14.00	1828.00	1856.00	1488.00	0.92	0.96	0.93
2	18.00	15.00	16.00	2036.00	1916.00	1548.00	0.88	0.78	1.02
3	20.00	18.00	20.00	1970.00	1726.00	1402.00	1.01	1.03	1.41
4	21.00	17.00	16.00	2048.00	1696.00	1466.00	1.02	0.99	1.08
5	17.00	16.00	14.00	1932.00	1798.50	1476.00	0.87	0.88	0.94
6	19.00	17.00	15.00	2009.00	1886.00	1518.00	0.94	0.89	0.98
7	19.00	13.00	13.00	1899.00	1791.00	1475.00	0.99	0.72	0.97
8	21.00	13.00	16.00	2042.00	1775.00	1507.00	1.02	0.73	1.05
9	15.00	14.00	17.00	1938.00	1806.00	1434.00	0.77	0.77	1.17
10	15.00	14.00	15.00	1970.50	1711.00	1477.00	0.76	0.81	1.01
11	13.00	15.00	16.00	1828.00	1774.00	1408.00	0.71	0.84	1.12
12	15.00	14.00	18.00	1938.00	1760.00	1406.00	0.77	0.79	1.26
13	17.00	12.00	11.00	1883.00	1646.00	1428.00	0.89	0.72	0.76
14	16.00	16.00	14.00	1852.00	1640.00	1360.00	0.86	0.97	1.02
15	14.00	13.00	14.00	1935.00	1767.00	1400.50	0.72	0.73	0.99
16	14.00	14.00	16.00	2122.00	1710.00	1407.00	0.66	0.81	1.12
17	15.00	13.00	15.00	2094.00	1707.00	1417.00	0.71	0.76	1.05
18	14.00	15.00	18.00	1895.00	1705.00	1383.00	0.73	0.87	1.28
19	15.00	16.00	16.00	1922.00	1638.00	1394.00	0.75	0.97	1.13
20	16.00	15.00	12.00	1976.00	1703.00	1418.00	0.80	0.87	0.84
21	14.00	16.00	12.00	1638.00	1780.00	1502.00	0.85	0.89	0.79
22	15.00	16.00	19.00	1664.00	1882.00	1548.00	0.89	0.84	1.21
23	16.00	18.00	18.00	1717.00	1728.00	1312.00	0.92	1.03	1.35
24	16.00	15.00	18.00	1615.00	1772.00	1400.00	0.98	0.84	1.27
25	15.00	16.00	12.00	1720.00	1790.50	1440.50	0.86	0.89	0.83
26	13.00	18.00	12.00	1740.50	1805.00	1525.00	0.74	0.99	0.78
27	14.00	17.00	15.00	1690.50	1827.00	1407.00	0.82	0.92	1.05
28	15.00	14.00	13.00	1694.50	1750.00	1451.00	0.88	0.79	0.89
29	15.00	14.00	19.00	1781.00	1831.00	1436.00	0.84	0.76	1.31
30	13.00	14.00	19.00	1851.00	1754.00	1480.00	0.70	0.79	1.27
Min	23.00			1810.00			0.89		
Max	39.00			2800.00			2.00		
Mean	30.93			2245.33			1.38		
S.D.	3.28			258.89			0.23		

Source1 = Bangkok, Source2 = Songkhla 1, Source3 = Songkhla 2

Table 45 Lower trichome number, lower epidermal cell number and lower trichome index of *C. digyna*

Field	Trichome number			Epidermal cell number			Trichome index		
	1	2	3	1	2	3	1	2	3
1	31.00	37.00	30.00	2746.00	2282.00	2190.00	1.12	1.60	1.35
2	26.00	39.00	30.00	2734.00	1982.00	2294.00	0.94	1.93	1.29
3	26.00	34.00	33.00	2800.00	1928.00	2106.00	0.92	1.73	1.54
4	27.00	27.00	32.00	2490.00	1810.00	2130.00	1.07	1.47	1.48
5	27.00	33.00	27.00	2692.50	2000.50	2182.00	0.99	1.62	1.22
6	31.00	32.00	31.00	2740.00	2132.00	2246.00	1.12	1.48	1.36
7	32.00	35.00	27.00	2773.00	2105.00	2152.00	1.14	1.64	1.24
8	35.00	31.00	36.00	2618.00	2046.00	2164.00	1.14	1.49	1.64
9	29.00	30.00	37.00	2612.00	1955.00	2118.00	1.10	1.51	1.72
10	28.00	31.00	33.00	2645.00	1896.00	2212.00	1.05	1.61	1.47
11	34.00	36.00	34.00	2594.00	2220.00	2158.00	1.29	1.60	1.55
12	33.00	28.00	27.00	2424.00	1966.00	2132.00	1.34	1.40	1.25
13	31.00	32.00	31.00	2366.00	2264.00	2002.00	1.29	1.39	1.52
14	28.00	27.00	27.00	2516.00	2034.00	2040.00	1.10	1.31	1.31
15	28.00	27.00	34.00	2509.00	2121.00	2069.00	1.10	1.26	1.62
16	30.00	32.00	35.00	2395.00	2242.00	2145.00	1.24	1.41	1.61
17	27.00	32.00	33.00	2441.00	2093.00	2066.00	1.09	1.51	1.57
18	27.00	31.00	30.00	2477.00	2127.00	2067.00	1.08	1.44	1.43
19	23.00	32.00	31.00	2555.00	2001.00	1993.00	0.89	1.57	1.53
20	24.00	33.00	36.00	2480.00	2149.00	2080.00	0.96	1.51	1.70
21	33.00	28.00	27.00	2672.00	1824.00	2054.00	1.22	1.51	1.30
22	34.00	34.00	33.00	2572.00	2246.00	2076.00	1.30	1.49	1.56
23	33.00	37.00	32.00	2512.00	1816.00	2166.00	1.30	2.00	1.46
24	30.00	29.00	34.00	2468.00	2150.00	1968.00	1.20	1.33	1.70
25	30.00	29.00	28.00	2556.00	2009.00	2066.00	1.16	1.42	1.34
26	27.00	36.00	36.00	2622.00	2035.00	2063.00	1.02	1.74	1.72
27	31.00	28.00	33.00	2542.00	2031.00	2110.00	1.20	1.36	1.54
28	30.00	36.00	30.00	2592.00	2198.00	2011.00	1.14	1.61	1.47
29	30.00	29.00	29.00	2570.00	1994.00	2121.00	1.15	1.43	1.35
30	29.00	28.00	31.00	2520.00	1987.00	2022.00	1.14	1.38	1.51
Min	11.00			1312.00			0.66		
Max	21.00			2122.00			1.41		
Mean	15.51			1694.16			0.92		
S.D.	2.16			206.93			0.17		

Source1 = Bangkok, Source2 = Songkhla 1, Source3 = Songkhla 2

Table 46 Stomatal cell number, lower epidermal cell number, and stomatal index of *C. minax*

Field	Stomatal number			Lower epidermal cell number			Stomatal index		
	1	2	3	1	2	3	1	2	3
1	140.00	162.00	158.00	1190.00	1166.00	1104.00	10.53	12.20	12.52
2	148.00	148.00	162.00	1142.00	1192.00	1108.00	11.47	11.04	12.76
3	160.00	148.00	152.00	1160.00	1054.00	1144.00	12.12	12.31	11.73
4	158.00	156.00	156.00	1094.00	1082.00	1162.00	12.62	12.60	11.84
5	136.00	164.00	144.00	1148.00	1184.00	1166.00	10.59	12.17	10.99
6	134.00	158.00	142.00	1142.00	1150.00	1156.00	10.50	12.08	10.94
7	140.00	174.00	148.00	1162.00	1188.00	1042.00	10.75	12.78	12.44
8	148.00	160.00	150.00	1176.00	1120.00	1062.00	11.18	12.50	12.38
9	134.00	138.00	148.00	1174.00	960.00	1068.00	10.24	12.57	12.17
10	158.00	182.00	152.00	1136.00	1206.00	1078.00	12.21	13.11	12.36
11	140.00	148.00	190.00	1104.00	1258.00	1086.00	11.25	10.53	14.89
12	168.00	186.00	156.00	1142.00	1132.00	1104.00	12.82	14.11	12.38
13	146.00	164.00	162.00	1136.00	1276.00	1094.00	11.39	11.39	12.90
14	136.00	166.00	160.00	1076.00	1120.00	1108.00	11.22	12.91	12.62
15	138.00	180.00	168.00	1196.00	1214.00	1120.00	10.34	12.91	13.04
16	152.00	160.00	168.00	1176.00	1168.00	1140.00	11.45	12.05	12.84
17	136.00	162.00	166.00	1136.00	1268.00	1100.00	10.69	11.33	13.11
18	150.00	160.00	152.00	1180.00	1218.00	1052.00	11.28	11.61	12.62
19	130.00	160.00	150.00	1146.00	1170.00	1056.00	10.19	12.03	12.44
20	146.00	166.00	148.00	1104.00	1210.00	1068.00	11.68	12.06	12.17
21	130.00	158.00	148.00	1072.00	1126.00	1102.00	10.82	12.31	11.84
22	156.00	182.00	146.00	1180.00	1186.00	1050.00	11.68	13.30	12.21
23	144.00	176.00	176.00	1088.00	1208.00	1150.00	11.69	12.72	13.27
24	166.00	186.00	142.00	1120.00	1222.00	1052.00	12.91	13.21	11.89
25	132.00	184.00	144.00	1126.00	1198.00	1136.00	10.49	13.31	11.25
26	170.00	162.00	146.00	1142.00	1186.00	1112.00	12.96	12.02	11.61
27	146.00	164.00	186.00	1180.00	1210.00	1082.00	11.01	11.94	14.67
28	150.00	192.00	148.00	1060.00	1222.00	1102.00	10.77	13.58	11.84
29	142.00	164.00	160.00	1202.00	1146.00	1058.00	10.57	12.52	13.14
30	128.00	160.00	190.00	1152.00	1168.00	1152.00	11.52	12.05	14.16
Min	128.00			960.00			10.19		
Max	192.00			1276.00			14.89		
Mean	156.11			1138.49			12.06		
S.D.	15.15			56.53			0.99		

Source1 = Chiangmai 1, Source2 = Chiangmai 2, Source3 = Chiangmai 3

Table 47 Upper epidermal cell area, palisade cell number, and palisade ratio of *C. minax*

Field	Number of palisade cell			Palisade ratio			Upper epidermal cell area (μm²)		
	1	2	3	1	2	3	1	2	3
1	21.00	21.50	20.00	5.25	5.38	5.00	970.87	821.02	857.63
2	20.50	25.00	20.50	5.13	6.25	5.13	889.68	875.66	833.33
3	20.50	25.50	21.50	5.13	6.38	5.38	982.32	877.19	814.33
4	23.00	22.50	20.00	5.75	5.63	5.00	1004.02	836.12	840.34
5	21.00	23.50	24.00	5.25	5.88	6.00	998.00	927.64	796.17
6	24.00	20.50	19.50	6.00	5.13	4.88	1006.04	972.76	853.24
7	20.50	24.50	20.00	5.13	6.13	5.00	1043.84	972.76	889.68
8	22.00	23.50	25.00	5.50	5.88	6.25	956.02	965.25	896.06
9	22.00	26.50	21.50	5.50	6.63	5.38	1091.70	948.77	896.06
10	20.50	21.50	20.00	5.13	5.38	5.00	1016.26	936.33	910.75
11	20.00	25.50	19.50	5.00	6.38	4.88	914.08	872.60	853.24
12	22.00	19.50	26.50	5.50	4.88	6.63	956.02	904.16	838.93
13	21.00	24.00	23.50	5.25	6.00	5.88	990.10	924.21	815.66
14	23.00	21.50	20.50	5.75	5.38	5.13	889.68	936.33	848.90
15	22.00	27.00	21.00	5.50	6.75	5.25	874.13	897.67	850.34
16	23.00	23.50	23.00	5.75	5.88	5.75	888.10	892.86	850.34
17	25.00	22.00	23.50	6.25	5.50	5.88	925.93	809.06	860.59
18	22.00	25.50	20.00	5.50	6.38	5.00	900.90	894.45	889.68
19	22.50	21.50	23.50	5.63	5.38	5.88	868.06	919.12	899.28
20	23.00	23.50	21.00	5.75	5.88	5.25	872.60	939.85	881.83
21	20.00	21.50	20.50	5.00	5.38	5.13	886.52	919.12	905.80
22	21.00	25.50	23.50	5.25	6.38	5.88	881.83	941.62	912.41
23	23.50	19.50	19.00	5.88	4.88	4.75	936.33	883.39	854.70
24	21.50	22.50	24.00	5.38	5.63	6.00	929.37	982.32	886.52
25	24.00	24.00	18.50	6.00	6.00	4.63	891.27	894.45	833.33
26	23.50	24.00	24.00	5.88	6.00	6.00	905.80	952.38	809.06
27	25.00	24.50	20.50	6.25	6.13	5.13	932.84	972.76	807.75
28	21.50	26.50	24.50	5.38	6.63	6.13	980.39	904.16	874.13
29	22.00	26.50	21.00	5.50	6.63	5.25	902.53	915.75	904.16
30	21.00	20.50	24.00	5.25	5.13	6.00	965.25	910.75	912.41
Min	18.50			4.63			796.17		
Max	27.00			6.75			1091.70		
Mean	22.42			5.61			905.86		
S.D.	2.02			0.51			56.36		

Source1 = Chiangmai 1, Source2 = Chiangmai 2, Source3 = Chiangmai 3

Table 48 Upper trichome number, upper epidermal cell number and upper trichome index of *C. minax*

Field	Trichome number			Epidermal cell number			Trichome index		
	1	2	3	1	2	3	1	2	3
1	13.00	10.00	10.00	2002.00	1428.00	1400.00	0.65	0.70	0.71
2	12.00	9.00	10.00	1926.00	1508.00	1444.00	0.62	0.59	0.69
3	9.00	9.00	11.00	1982.00	1438.00	1520.00	0.45	0.62	0.72
4	10.00	13.00	12.00	1892.00	1366.00	1598.00	0.53	0.94	0.75
5	11.00	10.00	13.00	1950.50	1435.00	1490.50	0.56	0.69	0.86
6	10.00	11.00	11.00	1964.00	1468.00	1422.00	0.51	0.74	0.77
7	9.00	9.00	13.00	1947.00	1473.00	1282.00	0.46	0.61	0.87
8	9.00	11.00	11.00	1992.00	1437.00	1521.00	0.45	0.76	0.72
9	10.00	10.00	12.00	1937.00	1402.00	1559.00	0.51	0.71	0.76
10	11.00	10.00	12.00	1909.00	1433.00	1499.00	0.57	0.69	0.79
11	11.00	12.00	11.00	1892.00	1362.00	1310.00	0.58	0.87	0.83
12	12.00	12.00	12.00	2224.00	1440.00	1388.00	0.54	0.83	0.86
13	10.00	12.00	12.00	2074.00	1434.00	1196.00	0.48	0.83	0.99
14	9.00	9.00	12.00	2010.00	1436.00	1230.00	0.45	0.62	0.97
15	9.00	10.00	11.00	2050.00	1418.00	1281.00	0.44	0.70	0.85
16	9.00	9.00	9.00	2058.00	1401.00	1349.00	0.44	0.64	0.66
17	11.00	9.00	10.00	2144.00	1437.00	1292.00	0.51	0.62	0.77
18	12.00	9.00	12.00	2117.00	1438.00	1309.00	0.56	0.62	0.91
19	14.00	15.00	11.00	2039.00	1398.00	1213.00	0.68	1.06	0.90
20	9.00	11.00	9.00	1984.00	1399.00	1270.00	0.45	0.78	0.70
21	12.00	14.00	10.00	2200.00	1592.00	1286.00	0.54	0.87	0.77
22	11.00	10.00	8.00	2374.00	1578.00	1400.00	0.46	0.63	0.57
23	10.00	11.00	12.00	1908.00	1582.00	1264.00	0.52	0.69	0.94
24	9.00	10.00	9.00	1994.00	1472.00	1260.00	0.45	0.67	0.71
25	10.00	14.00	13.00	2119.00	1556.00	1302.50	0.47	0.89	0.99
26	8.00	11.00	10.00	2287.00	1585.00	1343.00	0.35	0.69	0.74
27	8.00	9.00	10.00	2054.00	1580.00	1332.00	0.39	0.57	0.75
28	9.00	12.00	10.00	2098.00	1530.00	1330.00	0.43	0.78	0.75
29	11.00	14.00	9.00	2141.00	1527.00	1262.00	0.51	0.91	0.71
30	9.00	10.00	8.00	2184.00	1594.00	1273.00	0.41	0.62	0.62
Min	14.00			1616.00			0.56		
Max	24.00			2816.00			1.31		
Mean	18.02			2090.12			0.88		
S.D.	2.43			373.33			0.18		

Source1 = Chiangmai 1, Source2 = Chiangmai 2, Source3 = Chiangmai 3

Table 49 Lower trichome number, lower epidermal cell number and lower trichome index of *C. minax*

Field	Trichome number			Epidermal cell number			Trichome index		
	1	2	3	1	2	3	1	2	3
1	18.00	15.00	17.00	2624.00	1846.00	1770.00	0.68	0.81	0.95
2	18.00	15.00	24.00	2732.00	1878.00	1996.00	0.65	0.79	1.19
3	15.00	18.00	22.00	2554.00	1648.00	1696.00	0.58	1.08	1.28
4	17.00	20.00	21.00	2520.00	1864.00	1620.00	0.67	1.07	1.28
5	17.00	19.00	15.00	2607.50	1804.50	1770.50	0.65	1.04	0.84
6	19.00	16.00	17.00	2678.00	1862.00	1883.00	0.70	0.85	0.89
7	18.00	18.00	14.00	2643.00	1763.00	1846.00	0.71	1.01	0.75
8	18.00	21.00	18.00	2629.00	1862.00	1808.00	0.68	1.12	0.99
9	18.00	19.00	22.00	2537.00	1747.00	1658.00	0.70	1.08	1.31
10	16.00	21.00	21.00	2572.00	1846.00	1734.00	0.62	1.07	1.20
11	15.00	19.00	19.00	2676.00	1904.00	2021.00	0.56	0.99	0.93
12	18.00	15.00	14.00	2816.00	1776.00	1902.00	0.64	0.84	0.73
13	22.00	16.00	15.00	2452.00	1928.00	1770.00	0.89	0.82	0.84
14	20.00	15.00	19.00	2444.00	1616.00	1636.00	0.81	0.92	1.15
15	15.00	19.00	15.00	2597.00	1806.00	1832.50	0.57	1.04	0.81
16	24.00	15.00	21.00	2746.00	1840.00	1961.50	0.87	0.81	1.06
17	23.00	14.00	20.00	2634.00	1852.00	1836.00	0.87	0.75	1.08
18	21.00	19.00	19.00	2630.00	1696.00	1769.00	0.79	1.11	1.06
19	19.00	18.00	15.00	2564.00	1772.00	1703.00	0.74	1.01	0.87
20	21.00	18.00	16.00	2560.00	1916.00	1895.50	0.81	0.93	0.84
21	17.00	17.00	19.00	2682.00	1862.00	1782.00	0.63	0.90	1.06
22	17.00	18.00	17.00	2502.00	1950.00	1876.00	0.67	0.91	0.90
23	17.00	16.00	17.00	2432.00	1998.00	1964.00	0.69	0.79	0.86
24	24.00	16.00	21.00	2724.00	1808.00	1842.00	0.87	0.88	1.13
25	20.00	16.00	19.00	2585.00	1904.50	1866.00	0.77	0.83	1.01
26	20.00	18.00	17.00	2592.00	1906.00	1829.00	0.77	0.94	0.92
27	15.00	18.00	17.00	2467.00	1974.00	1920.00	0.60	0.90	0.88
28	16.00	21.00	18.00	2613.00	1879.00	1859.00	0.61	1.11	0.96
29	16.00	19.00	14.00	2578.00	1903.00	1903.00	0.62	0.99	0.73
30	18.00	19.00	19.00	2557.00	1930.00	1873.00	0.70	0.97	1.00
Min	8.00			1196.00			0.35		
Max	15.00			2374.00			1.06		
Mean	10.61			1624.73			0.67		
S.D.	1.57			321.59			0.16		

Source1 = Chiangmai 1, Source2 = Chiangmai 2, Source3 = Chiangmai 3

Table 50 Stomatal cell number, lower epidermal cell number, and stomatal index of *C. decapetala*

Field	Stomatal number			Lower epidermal cell number			Stomatal index		
	1	2	3	1	2	3	1	2	3
1	254.00	266.00	280.00	3516.00	3550.00	3810.00	6.74	6.97	6.85
2	240.00	214.00	256.00	3596.00	2928.00	3894.00	6.60	6.81	6.17
3	220.00	214.00	222.00	3624.00	2964.00	3600.00	5.72	6.73	5.81
4	228.00	220.00	252.00	3388.00	3110.00	3516.00	6.31	6.61	6.69
5	236.00	238.00	246.00	3586.00	3570.00	3488.00	6.17	6.25	6.59
6	232.00	272.00	254.00	3288.00	3430.00	3882.00	6.59	7.35	6.14
7	224.00	226.00	292.00	3436.00	3124.00	2754.00	6.12	6.75	7.22
8	270.00	210.00	266.00	3466.00	3058.00	3662.00	7.23	6.43	6.77
9	246.00	222.00	276.00	3584.00	3080.00	3804.00	6.42	6.72	6.76
10	264.00	216.00	236.00	3554.00	3062.00	3958.00	6.91	6.59	5.63
11	278.00	264.00	224.00	3308.00	3456.00	3888.00	7.75	7.10	5.45
12	252.00	258.00	266.00	3428.00	3486.00	3400.00	6.85	6.89	7.26
13	262.00	262.00	228.00	3628.00	3512.00	3528.00	6.74	6.94	6.07
14	234.00	270.00	232.00	3382.00	3564.00	3520.00	6.47	7.04	6.18
15	224.00	280.00	214.00	3240.00	3496.00	3584.00	6.47	7.42	5.63
16	234.00	244.00	232.00	3560.00	3428.00	3478.00	6.17	6.64	6.25
17	234.00	234.00	246.00	3578.00	3286.00	3954.00	6.14	6.65	5.85
18	230.00	278.00	258.00	3486.00	3484.00	3874.00	6.19	7.39	6.24
19	226.00	208.00	274.00	3556.00	3076.00	3534.00	5.98	6.33	7.20
20	224.00	210.00	256.00	3312.00	3146.00	3662.00	6.33	6.26	6.53
21	262.00	256.00	254.00	3396.00	3464.00	3602.00	7.16	6.88	6.58
22	280.00	234.00	262.00	3352.00	3434.00	3426.00	7.71	6.38	7.10
23	256.00	236.00	240.00	3510.00	3312.00	3768.00	6.80	6.65	5.99
24	262.00	232.00	244.00	3580.00	3234.00	3500.00	6.82	6.69	6.52
25	254.00	234.00	242.00	3540.00	3156.00	3570.00	6.69	6.90	6.35
26	216.00	214.00	220.00	3448.00	3332.00	3570.00	5.90	6.04	5.80
27	246.00	288.00	218.00	3422.00	3494.00	3564.00	6.71	7.62	5.76
28	208.00	304.00	230.00	3552.00	3212.00	3640.00	5.53	8.65	5.94
29	244.00	312.00	280.00	3576.00	3296.00	3800.00	6.39	8.65	6.86
30	250.00	262.00	256.00	3556.00	3232.00	3838.00	6.57	7.50	6.25
Min	208.00			2754.00			5.45		
Max	312.00			3958.00			8.65		
Mean	245.82			3472.13			6.60		
S.D.	23.14			230.94			0.59		

Source1 = Chiangmai 1, Source2 = Chiangmai 2, Source3 = Chiangmai 3

Table 51 Upper epidermal cell area, palisade cell number, and palisade ratio of *C. decapetala*

Field	Number of palisade cell			Palisade ratio			Upper epidermal cell area (μm²)		
	1	2	3	1	2	3	1	2	3
1	19.00	15.00	18.00	4.75	3.75	4.50	291.89	299.22	386.40
2	19.50	16.00	17.00	4.88	4.00	4.25	291.72	300.48	364.17
3	17.00	16.00	17.50	4.25	4.00	4.38	301.39	309.79	352.11
4	18.50	18.00	18.00	4.63	4.50	4.50	298.69	294.99	350.39
5	16.00	18.00	19.50	4.00	4.50	4.88	306.75	297.62	377.36
6	16.50	18.50	18.50	4.13	4.63	4.63	282.81	309.79	391.85
7	15.00	14.00	18.00	3.75	3.50	4.50	292.23	291.04	386.70
8	18.50	16.50	17.00	4.63	4.13	4.25	285.39	290.02	397.46
9	16.00	15.00	16.00	4.00	3.75	4.00	282.97	289.35	368.73
10	16.00	16.50	17.00	4.00	4.13	4.25	288.02	297.62	382.26
11	17.50	16.50	17.00	4.38	4.13	4.25	287.19	297.44	371.47
12	19.00	14.00	16.00	4.75	3.50	4.00	295.51	302.85	374.25
13	15.50	14.00	16.50	3.88	3.50	4.15	292.91	295.86	359.71
14	17.00	15.50	16.50	4.25	3.88	4.15	287.52	289.69	366.30
15	17.00	14.50	16.00	4.25	3.63	4.00	289.69	300.48	361.79
16	18.50	16.00	17.50	4.63	4.00	4.38	281.21	291.04	367.38
17	15.50	18.00	21.00	3.88	4.50	5.25	301.39	306.18	372.30
18	16.50	16.00	19.50	4.13	4.00	4.88	297.97	307.13	375.09
19	18.00	18.50	19.00	4.50	4.63	4.75	287.19	292.91	377.93
20	15.00	15.00	16.00	3.75	3.75	4.00	287.52	294.29	381.39
21	17.00	15.00	18.00	4.25	3.75	4.50	296.03	297.97	351.37
22	17.50	15.50	16.50	4.38	3.88	4.13	281.37	295.51	358.17
23	15.00	13.00	17.00	3.75	3.25	4.25	287.36	300.84	354.86
24	16.50	15.00	16.50	4.13	3.75	4.13	297.09	300.30	361.53
25	19.00	15.00	16.50	4.75	3.75	4.13	281.21	291.55	381.10
26	16.50	15.50	18.50	4.13	3.88	4.63	295.33	303.40	357.65
27	15.00	15.50	20.00	3.75	3.88	5.00	282.17	297.27	363.64
28	21.00	14.50	16.00	5.25	3.63	4.00	279.96	293.08	342.94
29	17.50	18.00	19.50	4.38	4.50	4.88	279.96	290.87	346.74
30	17.00	15.50	16.00	4.25	3.88	4.00	288.02	300.48	354.36
Min	13.00			3.25			279.96		
Max	21.00			5.25			397.46		
Mean	16.82			4.20			318.50		
S.D.	1.62			0.40			36.55		

Source1 = Chiangmai 1, Source2 = Chiangmai 2, Source3 = Chiangmai 3

Table 52 Upper trichome number, upper epidermal cell number and upper trichome index of *C. decapetala*

Field	Trichome number			Epidermal cell number			Trichome index		
	1	2	3	1	2	3	1	2	3
1	29.00	27.00	26.00	3568.00	3768.00	3418.00	0.81	0.71	0.75
2	31.00	31.00	22.00	3430.00	3502.00	3246.00	0.90	0.88	0.67
3	31.00	31.00	24.00	3499.00	3635.00	2876.00	0.88	0.85	0.83
4	33.00	27.00	25.00	3272.00	3274.00	3398.00	1.00	0.82	0.73
5	29.00	27.00	20.00	3112.00	3176.00	3023.00	0.92	0.84	0.66
6	32.00	26.00	36.00	3192.00	3430.00	3408.50	0.99	0.75	1.05
7	30.00	26.00	33.00	3345.50	3225.00	3137.50	0.89	0.80	1.04
8	29.00	33.00	37.00	3088.00	3521.00	3147.50	0.93	0.93	1.16
9	28.00	34.00	40.00	3252.00	3339.00	2902.00	0.85	1.01	1.36
10	27.00	33.00	33.00	3670.00	3536.00	3166.00	0.87	0.92	1.03
11	25.00	31.00	34.00	3194.00	3156.00	3458.00	0.78	0.97	0.97
12	25.00	31.00	35.00	3316.00	3346.00	3224.00	0.75	0.92	1.07
13	34.00	30.00	24.00	3255.00	3390.00	3336.00	1.03	0.88	0.71
14	40.00	37.00	20.00	3462.50	3086.00	2905.00	1.14	1.18	0.68
15	40.00	37.00	21.00	3376.00	3285.00	3578.50	1.17	1.11	0.58
16	37.00	30.00	22.00	3222.00	3247.00	3082.00	1.14	0.92	0.71
17	39.00	33.00	24.00	3299.00	3281.00	3166.50	1.17	1.00	0.75
18	40.00	35.00	23.00	3206.00	3247.00	3270.00	1.23	1.07	0.70
19	41.00	36.00	25.00	3138.00	3620.00	2991.50	1.29	0.98	0.83
20	41.00	36.00	27.00	3172.00	3724.00	3179.50	1.28	0.96	0.84
21	38.00	39.00	24.00	3135.50	3722.00	3490.00	1.20	1.04	0.68
22	32.00	34.00	25.00	3812.00	3272.00	2968.00	0.83	1.03	0.84
23	35.00	34.00	24.00	3402.00	3497.00	3376.00	1.02	0.96	0.71
24	42.00	40.00	21.00	3605.00	3037.00	3327.00	1.15	1.30	0.63
25	39.00	36.00	20.00	3364.00	3546.00	3489.00	1.15	1.01	0.57
26	37.00	39.00	23.00	3750.00	3670.50	3433.00	0.98	1.05	0.67
27	39.00	38.00	24.00	3557.00	3291.50	3425.00	1.08	1.14	0.70
28	26.00	26.00	25.00	3581.00	3381.00	3181.00	0.72	0.76	0.78
29	22.00	28.00	25.00	3600.00	3634.00	3488.00	0.61	0.76	0.71
30	34.00	27.00	26.00	3359.00	3151.50	3179.00	1.00	0.85	0.81
Min	20.00			2876.00			0.57		
Max	42.00			3812.00			1.36		
Mean	30.72			3338.83			0.92		
S.D.	6.09			209.94			0.18		

Source1 = Chiangmai 1, Source2 = Chiangmai 2, Source3 = Chiangmai 3

Table 53 Lower trichome number, lower epidermal cell number and lower trichome index of *C. decapetala*

Field	Trichome number			Epidermal cell number			Trichome index		
	1	2	3	1	2	3	1	2	3
1	18.00	19.00	15.00	2824.50	3228.00	3108.00	0.63	0.59	0.48
2	18.00	23.00	16.00	3118.00	3234.00	3012.00	0.57	0.71	0.53
3	19.00	20.00	17.00	3119.00	3231.00	3060.00	0.61	0.62	0.55
4	17.00	19.00	15.00	3232.00	2842.00	3082.00	0.52	0.66	0.48
5	18.00	18.00	18.00	3176.50	3099.50	3058.00	0.56	0.58	0.59
6	17.00	18.00	16.00	3257.00	3098.00	3070.00	0.52	0.58	0.52
7	16.00	16.00	16.00	3245.00	2966.50	3065.00	0.49	0.54	0.52
8	16.00	16.00	17.00	3088.00	3390.00	2740.00	0.52	0.47	0.62
9	20.00	21.00	15.00	3350.00	3494.00	2938.00	0.59	0.60	0.51
10	25.00	27.00	18.00	3068.00	3452.00	2839.00	0.81	0.78	0.63
11	21.00	23.00	17.00	3242.00	3160.00	2762.00	0.64	0.72	0.61
12	20.00	21.00	18.00	3340.00	3262.00	2878.00	0.60	0.64	0.62
13	18.00	17.00	17.00	3151.00	3328.00	2820.00	0.57	0.51	0.60
14	19.00	19.00	18.00	3243.50	3211.00	2829.50	0.58	0.59	0.63
15	21.00	22.00	21.00	3245.50	3510.00	3080.00	0.64	0.62	0.68
16	18.00	19.00	18.00	3434.00	3124.00	2780.00	0.52	0.60	0.64
17	18.00	18.00	20.00	3052.00	3317.00	2780.00	0.59	0.54	0.71
18	18.00	18.00	19.00	3243.00	3324.00	2700.00	0.55	0.54	0.70
19	18.00	19.00	18.00	3243.00	3266.00	2936.00	0.55	0.58	0.61
20	20.00	20.00	19.00	2936.00	3305.50	2818.00	0.68	0.60	0.67
21	24.00	24.00	17.00	3167.50	3295.00	2799.00	0.75	0.72	0.60
22	20.00	19.00	16.00	3089.50	3220.00	2762.00	0.64	0.59	0.58
23	24.00	24.00	17.00	3156.00	3446.00	2784.00	0.75	0.69	0.61
24	19.00	19.00	18.00	3250.00	3333.00	2773.00	0.58	0.57	0.64
25	20.00	19.00	17.00	3203.00	3012.00	2636.00	0.62	0.63	0.64
26	18.00	17.00	19.00	3054.00	3254.00	2640.00	0.59	0.52	0.71
27	18.00	18.00	16.00	3203.50	3137.50	2638.00	0.56	0.57	0.60
28	17.00	17.00	20.00	3350.00	3237.00	2705.50	0.50	0.52	0.73
29	18.00	19.00	17.00	3202.00	3228.50	2918.00	0.56	0.59	0.58
30	19.00	18.00	18.00	3295.00	3319.50	2912.50	0.57	0.54	0.61
Min	15.00			2636.00			0.47		
Max	27.00			3510.00			0.81		
Mean	18.69			3098.08			0.60		
S.D.	2.30			218.51			0.07		

Source1 = Chiangmai 1, Source2 = Chiangmai 2, Source3 = Chiangmai 3

Appendix B ISSR fingerprint of selected eight *Caesalpinia* species

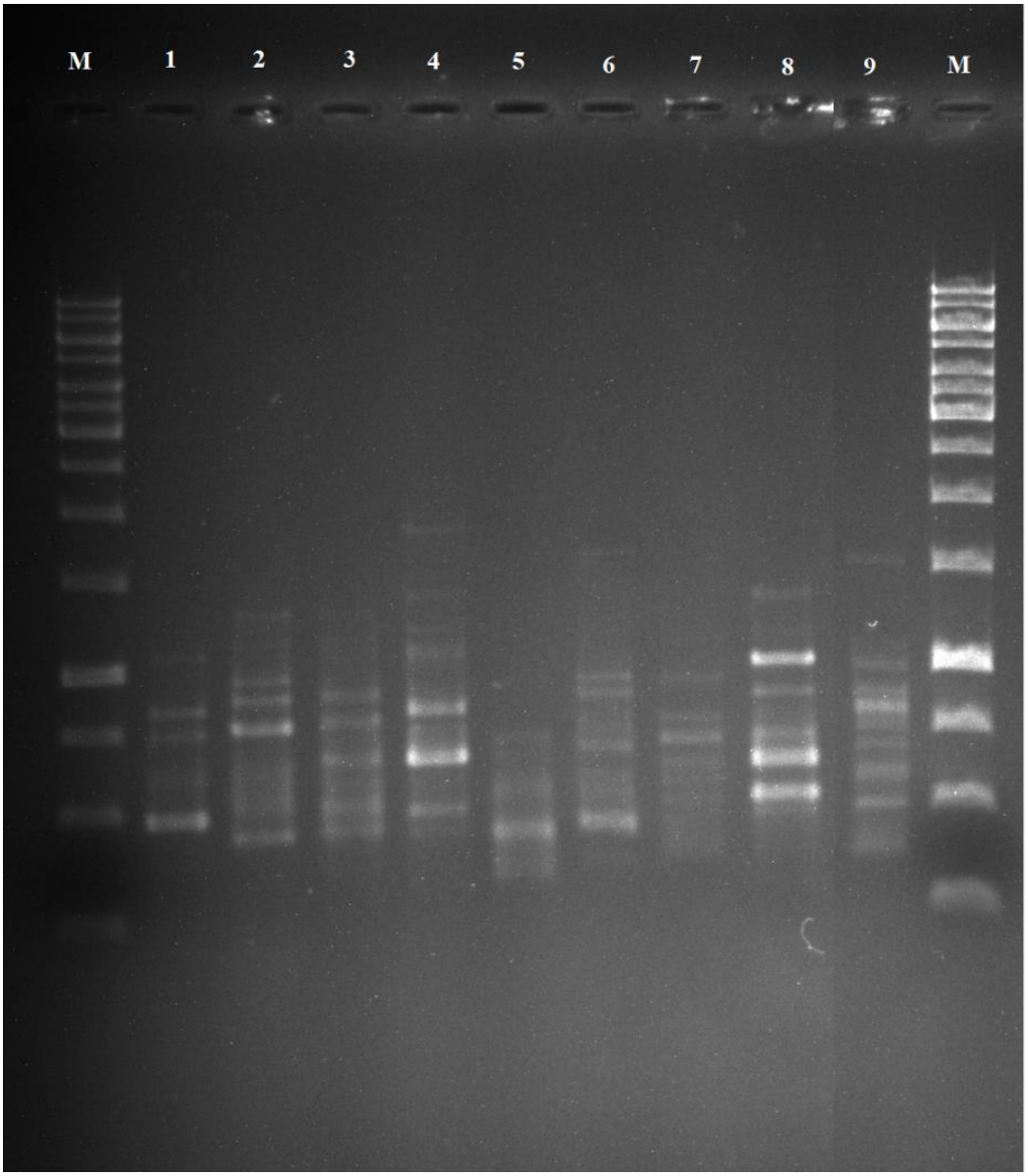


Figure 56 ISSR fingerprint of selected eight *Caesalpinia* species and one outgroup obtained from primer ISSR 02 [M = 1 kb molecular weight marker, lane1 = *C. sappan*, lane2 = *C. coriaria*, lane3 = *C. mimosoides*, lane4 = *C. pulcherrima*, lane5 = *C. bonduc*, lane6= *C. digyna*, lane7 = *C. minax*, lane8 = *C. decapetala*, and lane9 = *A. squamosa*]

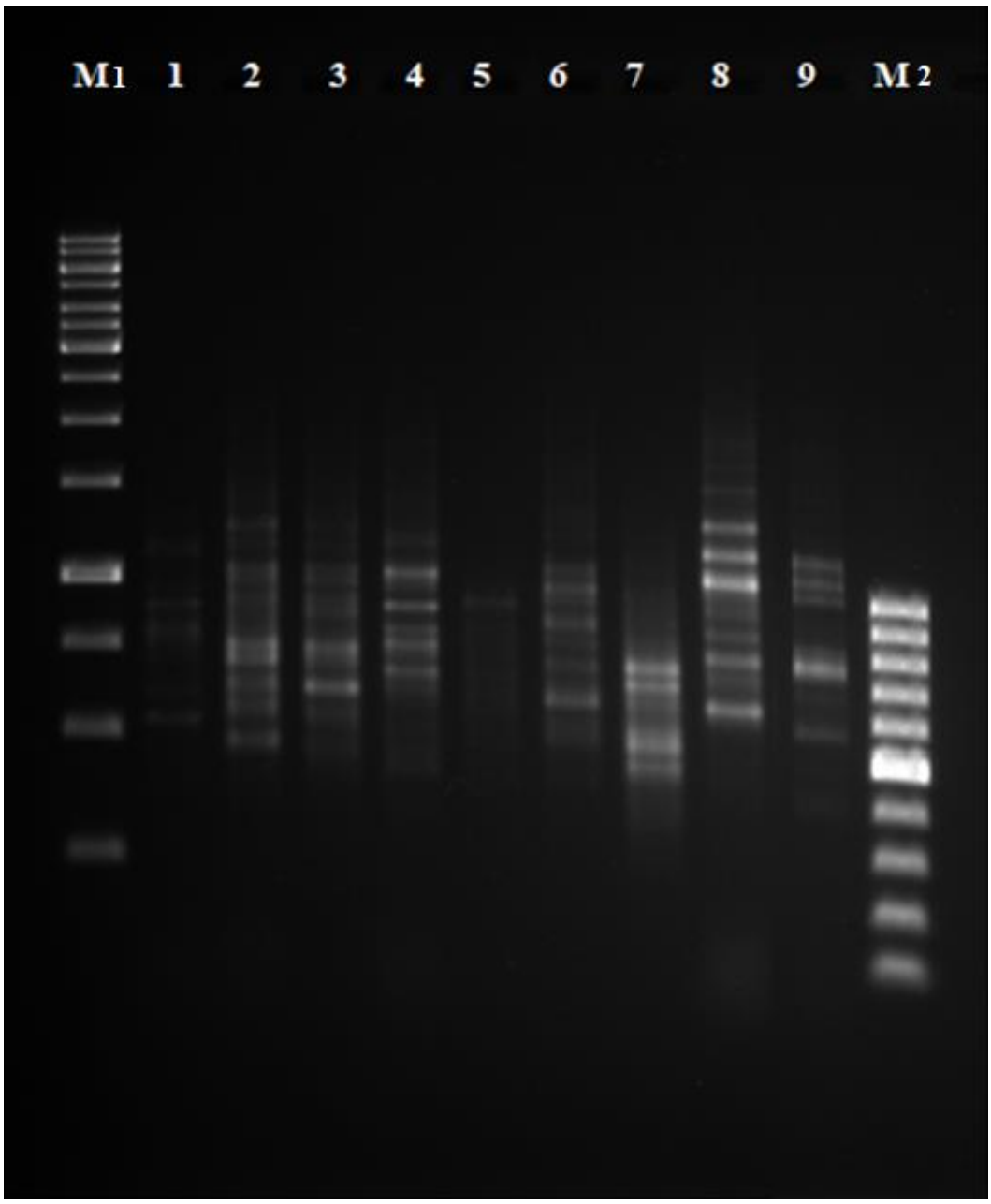


Figure 57 ISSR fingerprint of selected eight *Caesalpinia* species and one outgroup obtained from primer ISSR 06 [M1 = 1 kb molecular weight marker, lane1 = *C. sappan*, lane2 = *C. coriaria*, lane3 = *C. mimosoides*, lane4 = *C. pulcherrima*, lane5 = *C. bonduc*, lane6= *C. digyna*, lane7 = *C. minax*, lane8 = *C. decapetala*, and lane9 = *A. squamosa*, M2 = 100 bp molecular weight marker]

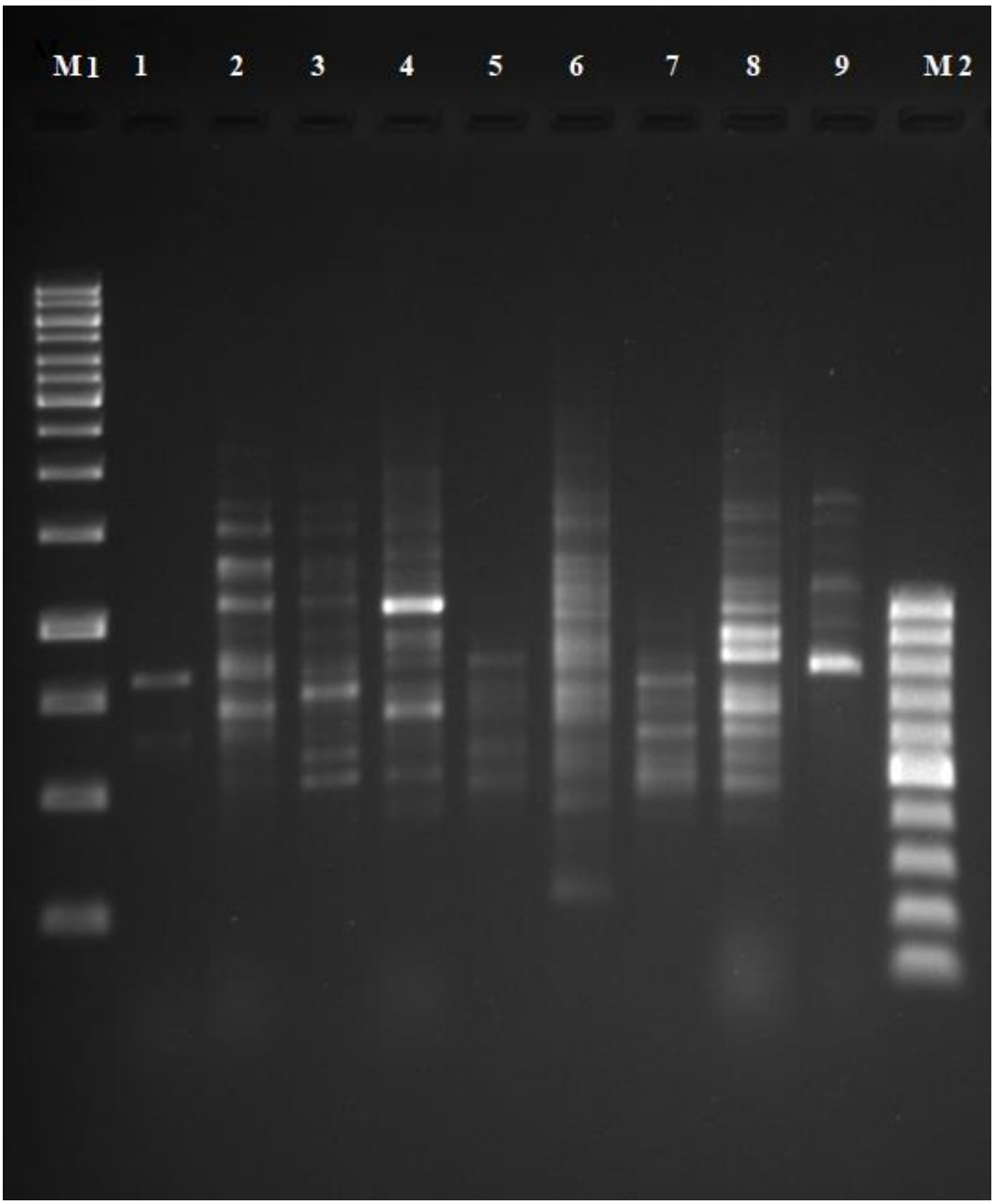


Figure 58 ISSR fingerprint of selected eight *Caesalpinia* species and one outgroup obtained from primer ISSR 09 [M1 = 1 kb molecular weight marker, lane1 = *C. sappan*, lane2 = *C. coriaria*, lane3 = *C. mimosoides*, lane4 = *C. pulcherrima*, lane5 = *C. bonduc*, lane6= *C. digyna*, lane7 = *C. minax*, lane8 = *C. decapetala*, and lane9 = *A. squamosa*, M2 = 100 bp molecular weight marker]

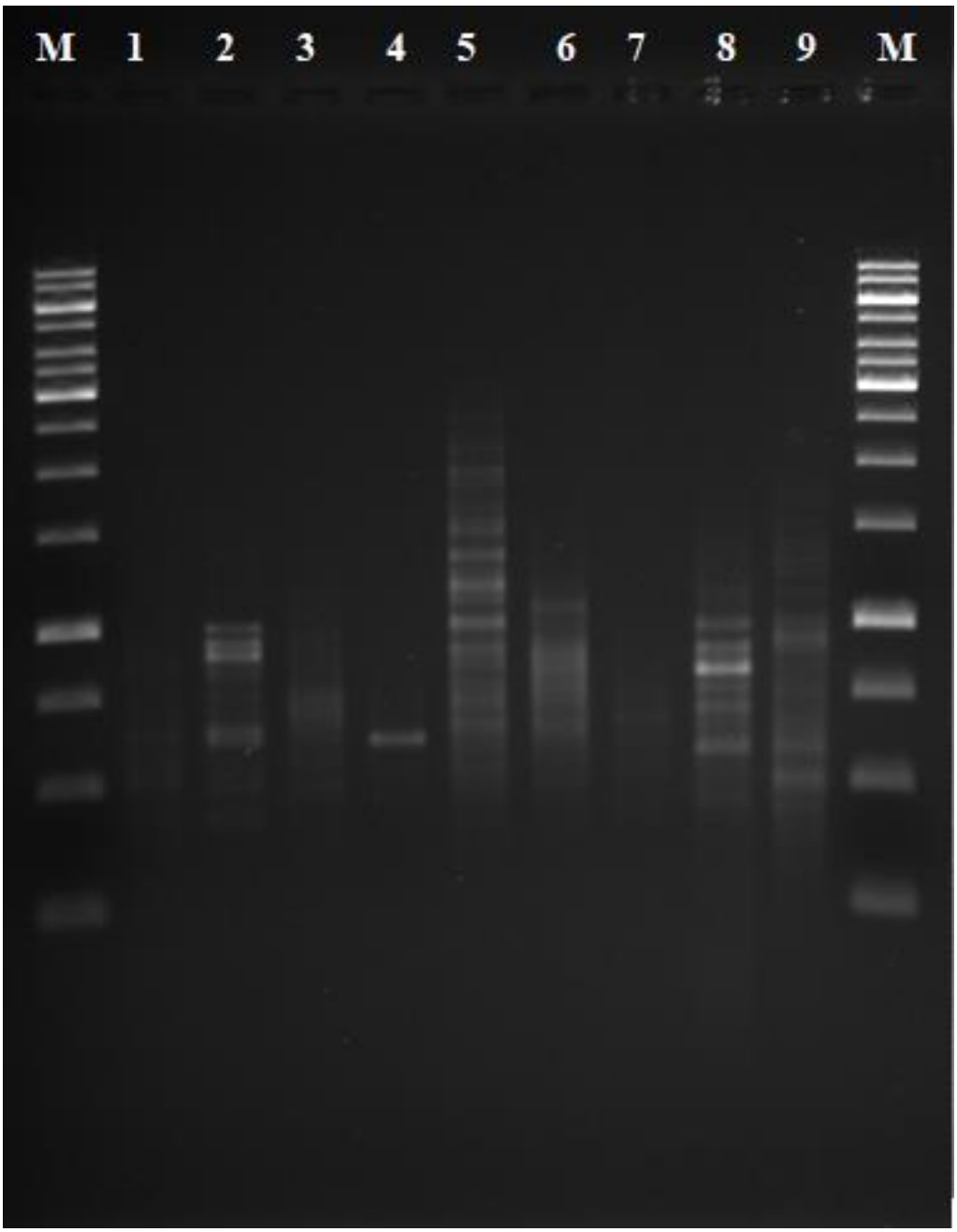


Figure 59 ISSR fingerprint of selected eight *Caesalpinia* species and one outgroup obtained from primer ISSR 10 [M = 1 kb molecular weight marker, lane1 = *C. sappan*, lane2 = *C. coriaria*, lane3 = *C. mimosoides*, lane4 = *C. pulcherrima*, lane5 = *C. bonduc*, lane6= *C. digyna*, lane7 = *C. minax*, lane8 = *C. decapetala*, and lane9 = *A. squamosa*]

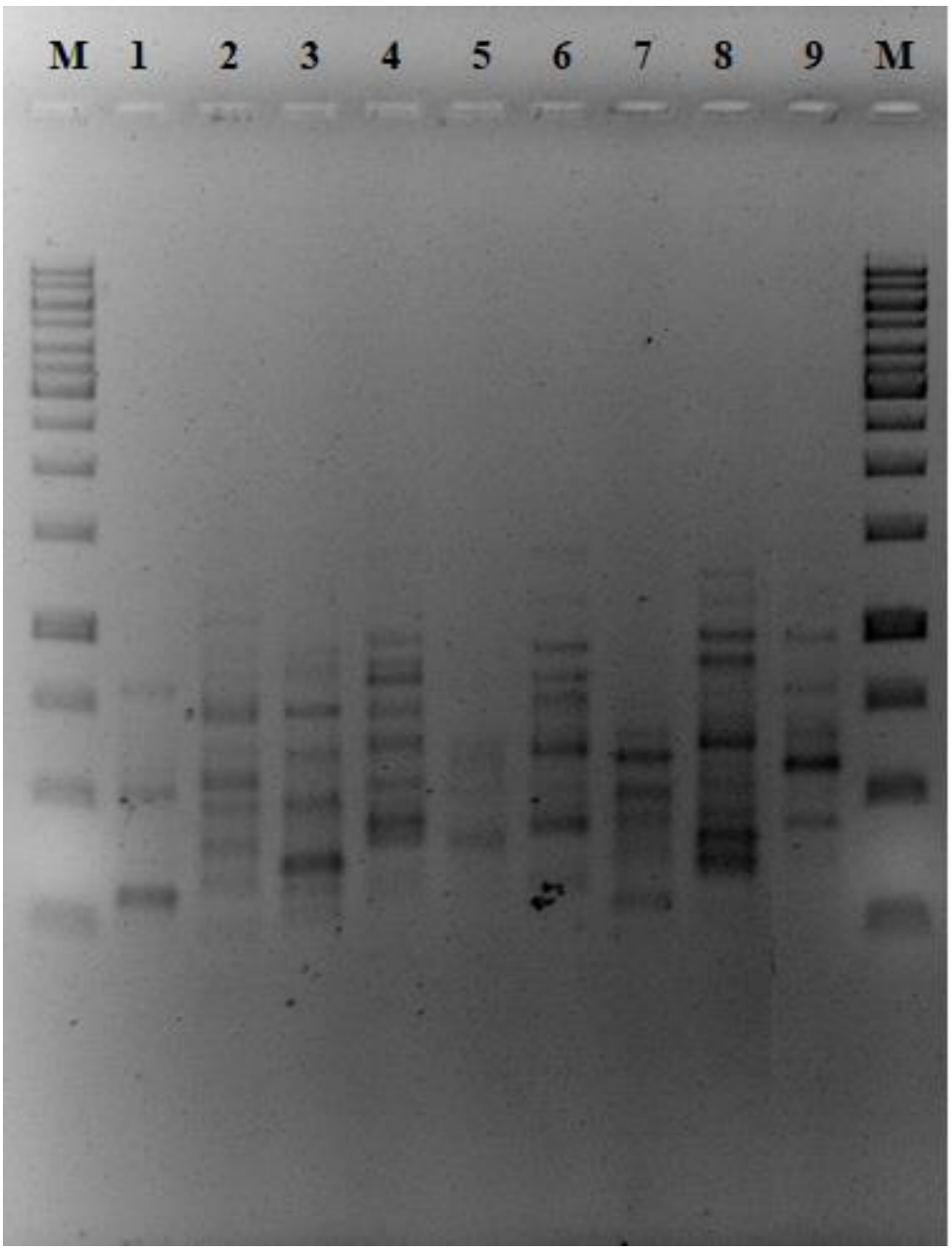


Figure 60 ISSR fingerprint of selected eight *Caesalpinia* species and one outgroup obtained from primer ISSR 13 [M = 1 kb molecular weight marker, lane1 = *C. sappan*, lane2 = *C. coriaria*, lane3 = *C. mimosoides*, lane4 = *C. pulcherrima*, lane5 = *C. bonduc*, lane6= *C. digyna*, lane7 = *C. minax*, lane8 = *C. decapetala*, and lane9 = *A. squamosa*]

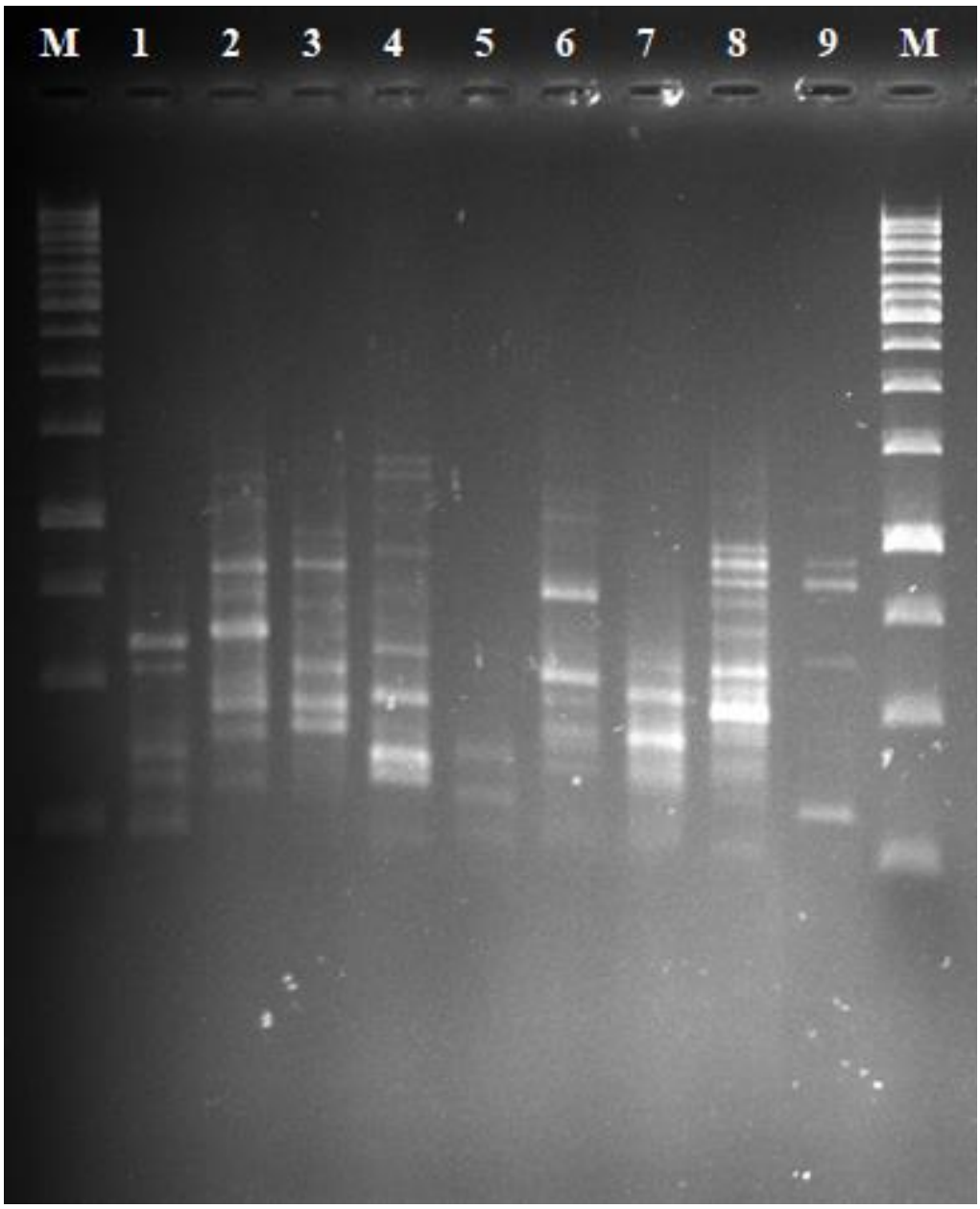


Figure 61 ISSR fingerprint of selected eight *Caesalpinia* species and one outgroup obtained from primer ISSR 14 [M = 1 kb molecular weight marker, lane1 = *C. sappan*, lane2 = *C. coriaria*, lane3 = *C. mimosoides*, lane4 = *C. pulcherrima*, lane5 = *C. bonduc*, lane6= *C. digyna*, lane7 = *C. minax*, lane8 = *C. decapetala*, and lane9 = *A. squamosa*]

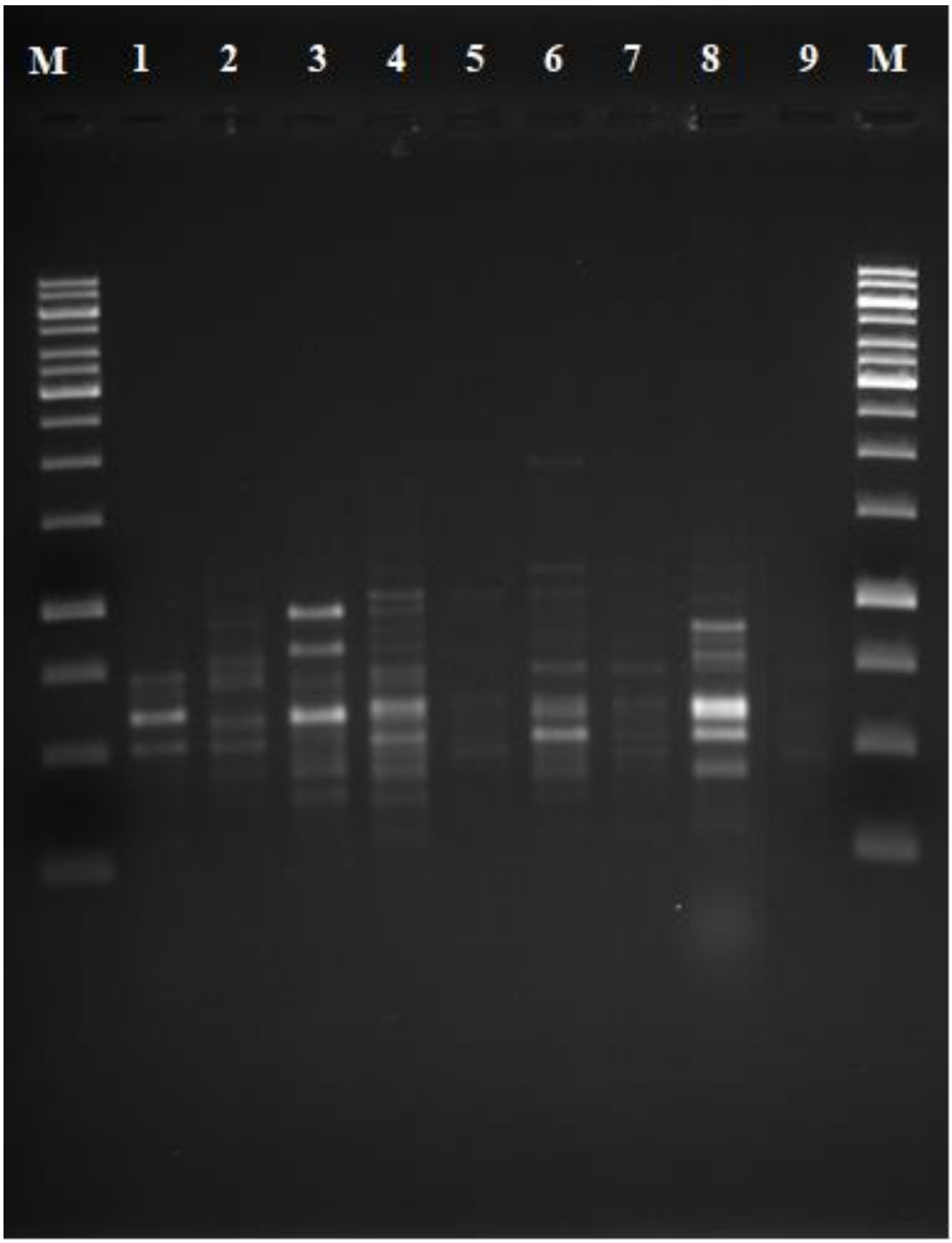


Figure 62 ISSR fingerprint of selected eight *Caesalpinia* species and one outgroup obtained from primer ISSR 23 [M = 1 kb molecular weight marker, lane1 = *C. sappan*, lane2 = *C. coriaria*, lane3 = *C. mimosoides*, lane4 = *C. pulcherrima*, lane5 = *C. bonduc*, lane6= *C. digyna*, lane7 = *C. minax*, lane8 = *C. decapetala*, and lane9 = *A. squamosa*]

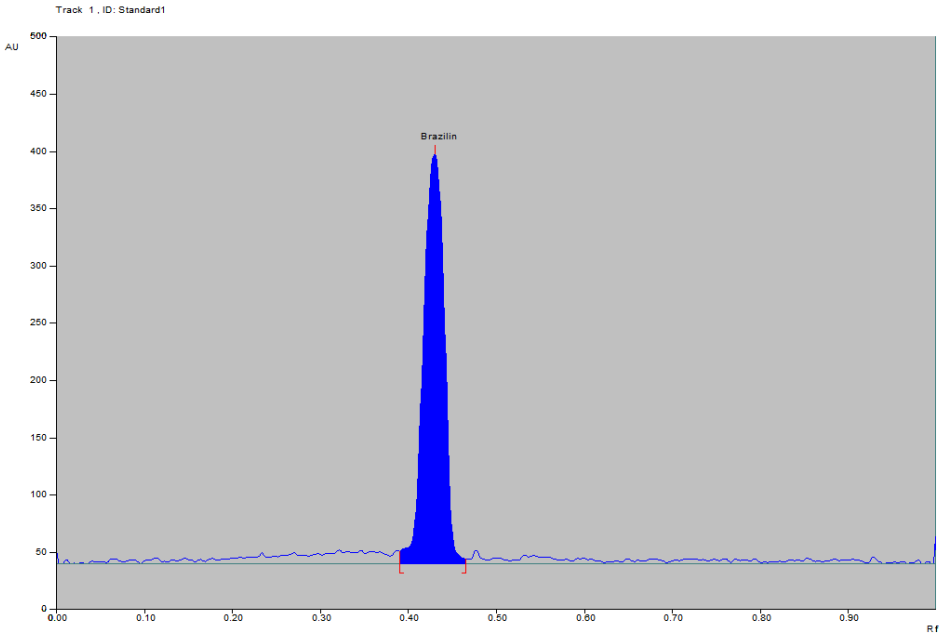
Appendix C Quality parameters of *Caesalpinia sappan* heartwoods

Table 54 Physicochemical parameters of *C. sappan* heartwoods

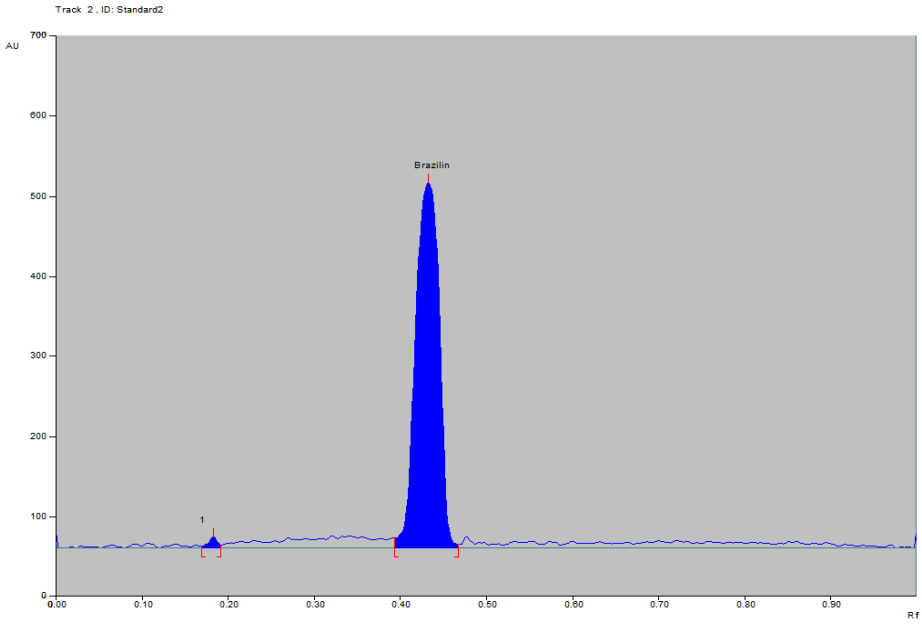
source	order	Loss on drying	Total ash	Acid-isoluble ash	Water extractable value	Ethanol extractable value	Moisture content
Chiangmai	1	9.100	0.975	0.556	3.700	2.100	9.20
	2	8.140	1.013	0.567	3.500	2.400	8.00
	3	8.540	1.001	0.573	3.700	2.400	9.00
Phichit	4	8.480	0.966	0.531	3.200	3.500	8.00
	5	8.480	1.022	0.601	3.300	3.000	9.16
	6	8.410	0.997	0.574	3.000	3.100	8.60
Kamphaeng Phet	7	9.140	0.989	0.485	3.300	3.200	9.20
	8	8.950	0.799	0.319	3.300	3.200	8.80
	9	8.980	0.850	0.378	3.600	3.100	8.80
Nakhon Sawan	10	8.280	0.992	0.541	3.900	2.800	8.00
	11	8.340	1.018	0.576	4.100	3.500	8.00
	12	8.230	1.000	0.524	4.800	2.500	8.00
Bangkok 1	13	8.560	1.058	0.654	2.900	2.990	8.48
	14	8.510	0.944	0.604	2.700	2.000	8.92
	15	8.430	0.988	0.555	3.100	2.200	8.80
Bangkok 2	16	8.180	0.775	0.371	3.590	2.500	8.40
	17	8.270	0.820	0.395	3.590	3.300	8.80
	18	8.270	0.819	0.373	3.600	3.300	8.40
Bangkok 3	19	8.440	0.698	0.285	3.800	3.000	8.80
	20	8.330	0.691	0.245	4.200	3.100	8.80
	21	8.370	0.727	0.283	4.150	3.400	8.20
Nakhon Pathom	22	9.090	0.699	0.270	3.300	2.000	9.20
	23	9.150	0.712	0.298	3.100	2.100	8.80
	24	9.010	0.710	0.279	2.900	2.200	8.60
Chon Buri	25	8.240	0.866	0.388	3.300	2.700	7.80
	26	8.240	0.914	0.429	3.000	2.900	8.00
	27	8.200	0.990	0.513	3.300	2.300	8.00

Nakhon Ratchasima	28	8.590	0.926	0.494	4.700	3.600	8.80
	29	9.580	0.960	0.554	4.890	3.800	8.60
	30	8.480	1.007	0.581	4.900	3.700	8.60
Roi Et	31	8.150	0.887	0.435	4.300	3.790	8.40
	32	8.010	0.833	0.467	3.600	3.800	8.00
	33	7.980	0.882	0.456	3.690	3.190	8.80
Ubon Ratchathani	34	8.450	0.763	0.359	4.900	3.190	8.60
	35	8.570	0.843	0.370	4.490	3.100	8.80
	36	8.870	0.845	0.396	4.600	3.700	8.60
Nakhon Si Thammarat	37	8.300	0.659	0.220	4.400	3.100	8.60
	38	8.060	0.685	0.248	3.100	3.290	8.40
	39	8.550	0.728	0.299	3.600	4.000	8.40
Songkhla	40	8.840	0.658	0.263	4.990	2.500	8.40
	41	8.840	0.684	0.250	3.990	2.200	8.00
	42	8.850	0.693	0.232	4.700	2.100	8.00
Trang	43	8.220	1.121	0.700	3.900	2.900	8.00
	44	8.150	1.098	0.672	3.900	3.300	8.20
	45	8.080	1.074	0.707	3.500	2.200	8.60
Min		**8.047**	**0.678**	**0.248**	**2.864**	**1.895**	**8.030**
Max		**9.083**	**1.098**	**0.693**	**4.696**	**3.983**	**8.973**
Grand mean		**8.510**	**0.875**	**0.442**	**3.780**	**2.939**	**8.501**
Pooled S.D.		**0.224**	**0.042**	**0.038**	**0.305**	**0.348**	**0.157**

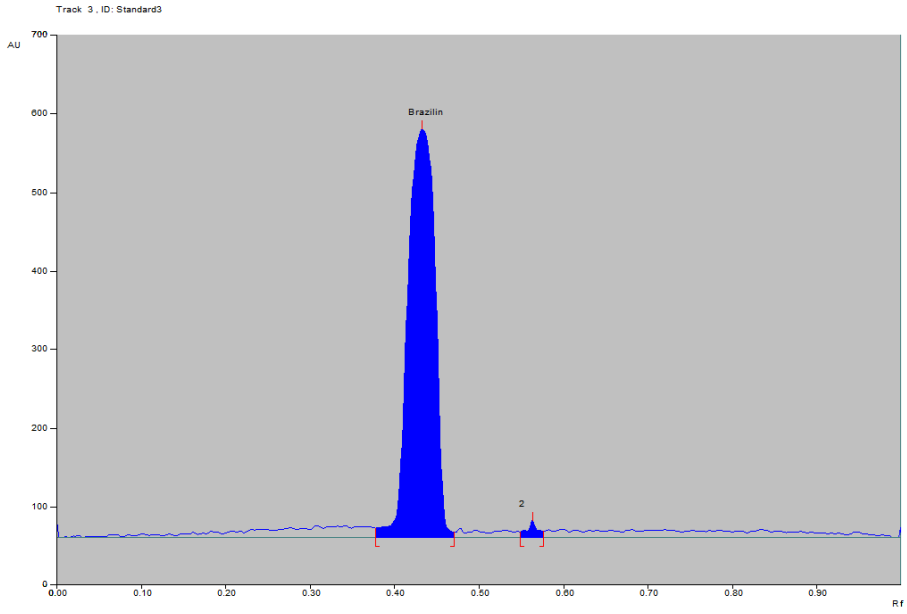
Appendix D Quantitative analysis of brazilin: TLC-densitometric chromatogram



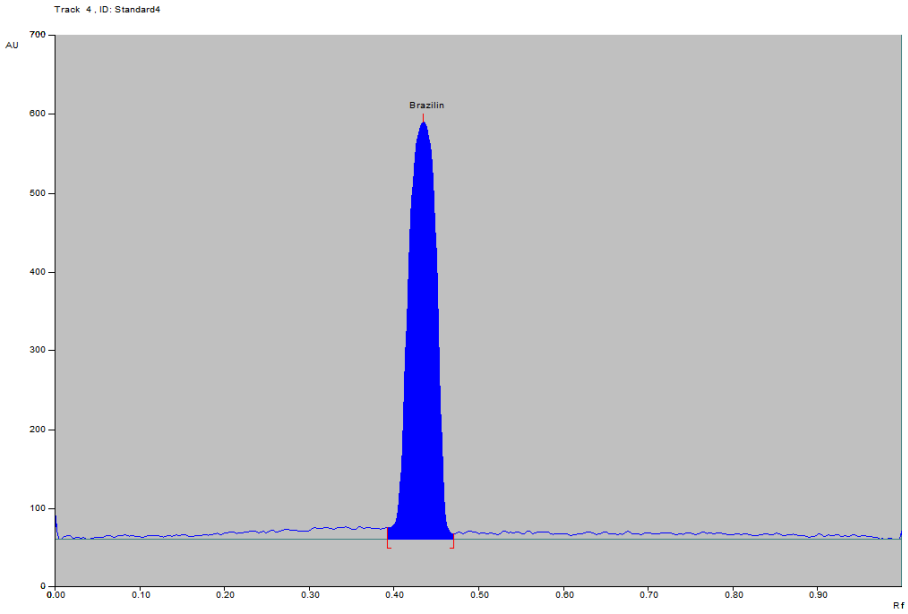
Track1: Standard brazilin 0.05 mg/ml



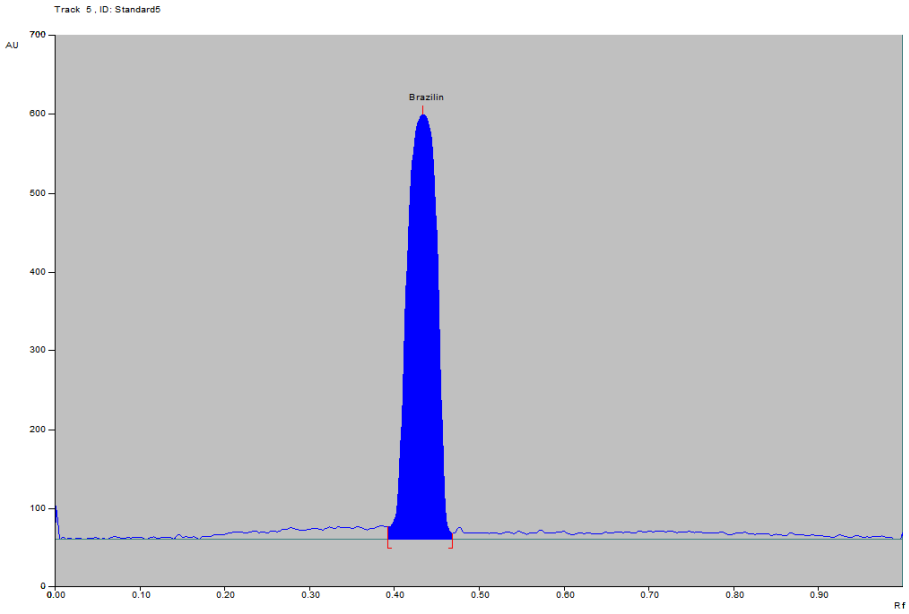
Track2: Standard brazilin 0.075 mg/ml



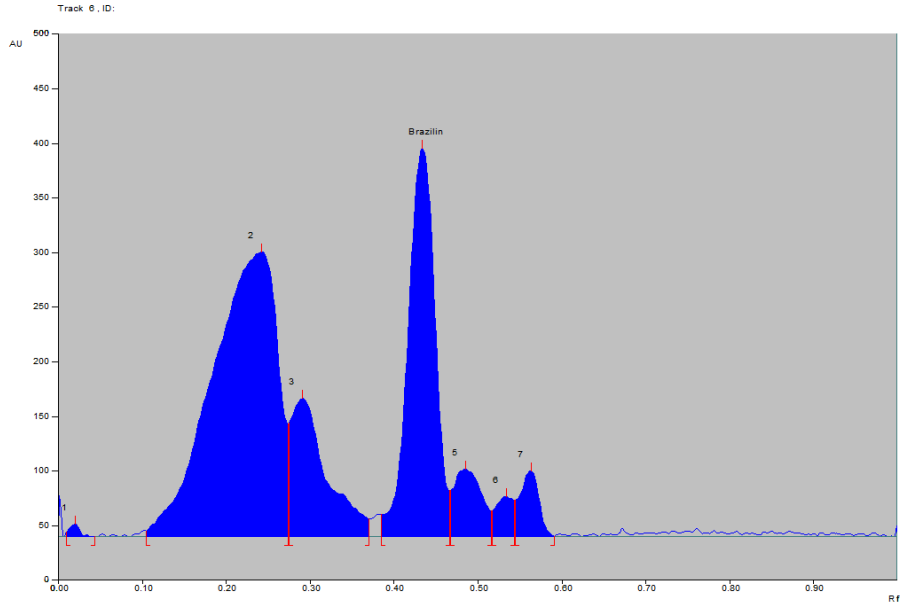
Track3: Standard brazilin 0.1 mg/ml



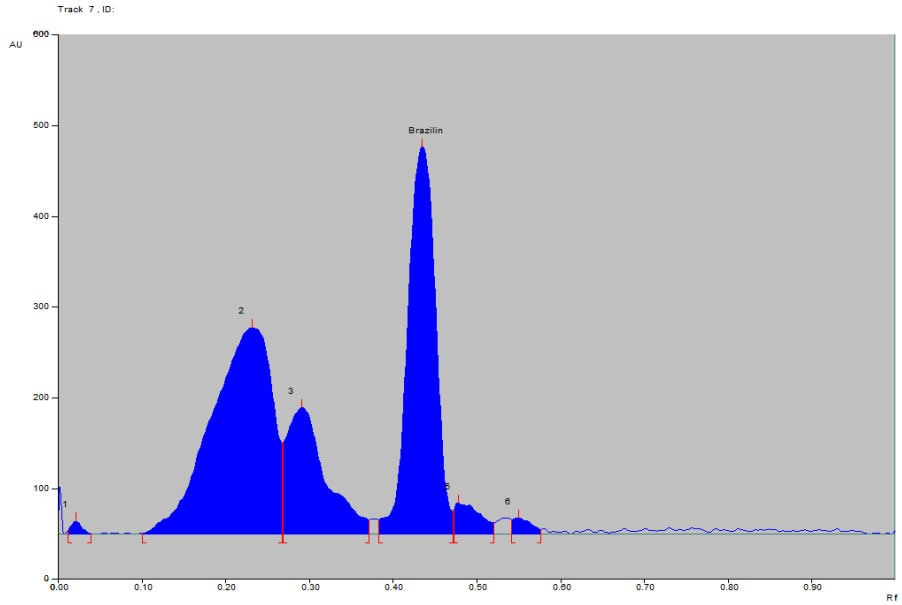
Track4: Standard brazilin 0.125 mg/ml



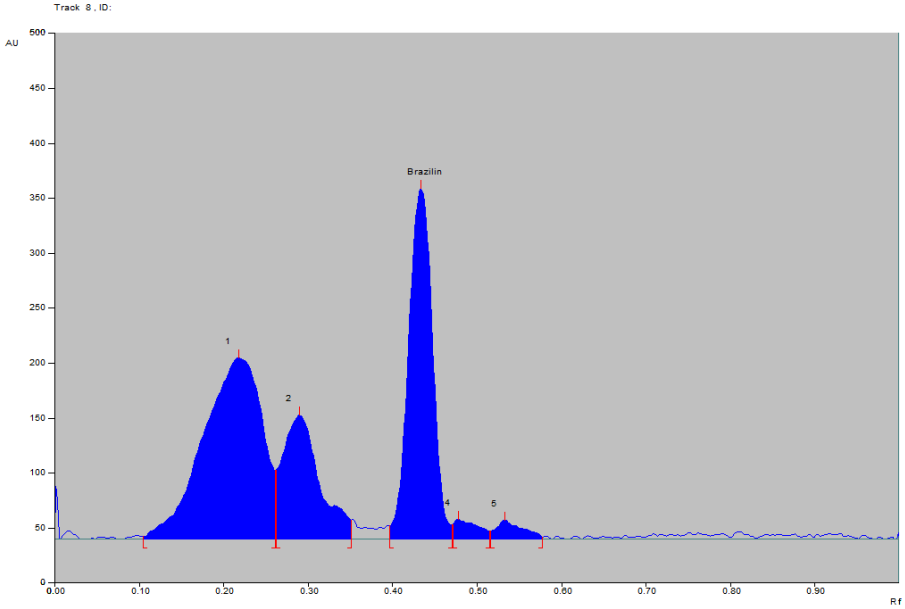
Track5: Standard brazilin 0.15 mg/ml



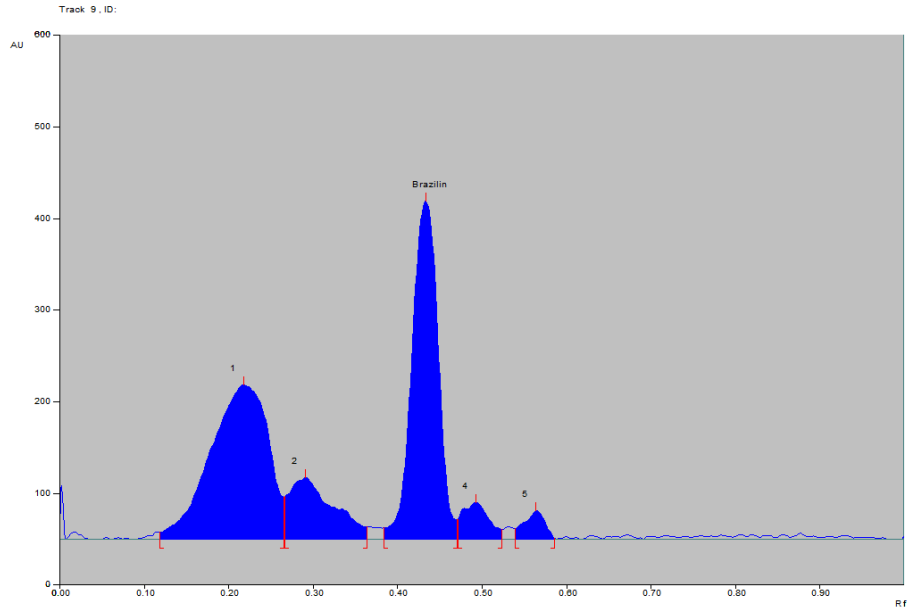
Track6: Sample 1 (1 mg/ml)



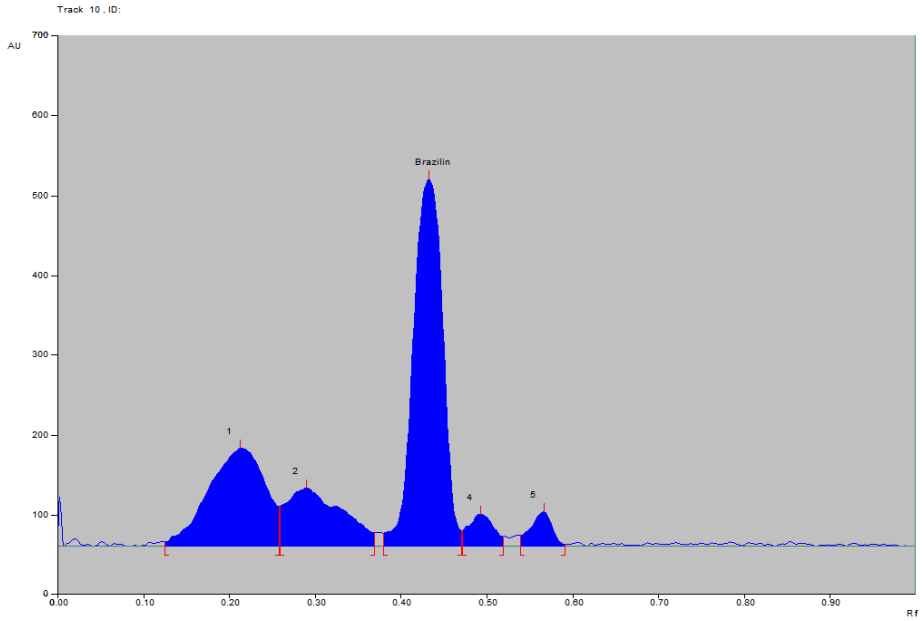
Track7: Sample 2 (1 mg/ml)



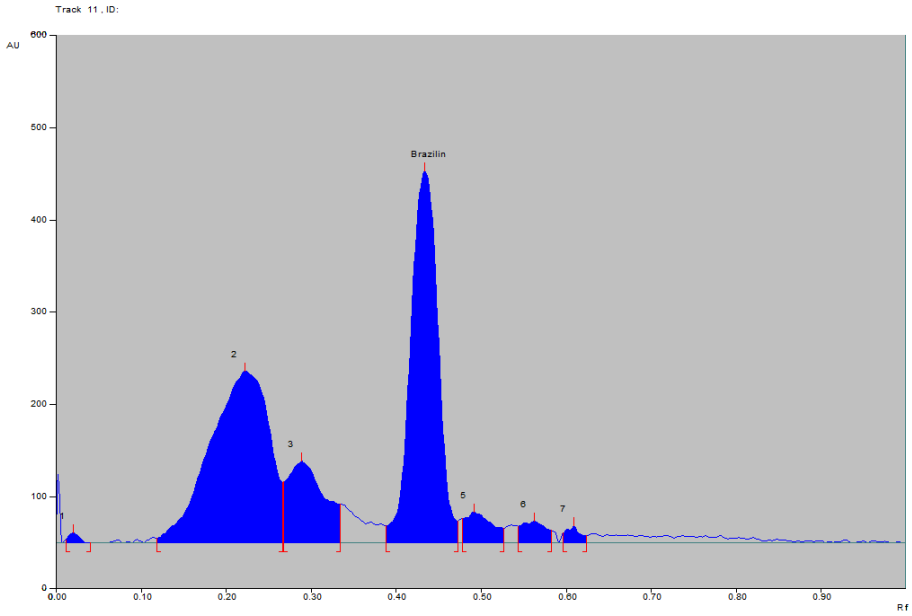
Track8: Sample 3 (1 mg/ml)



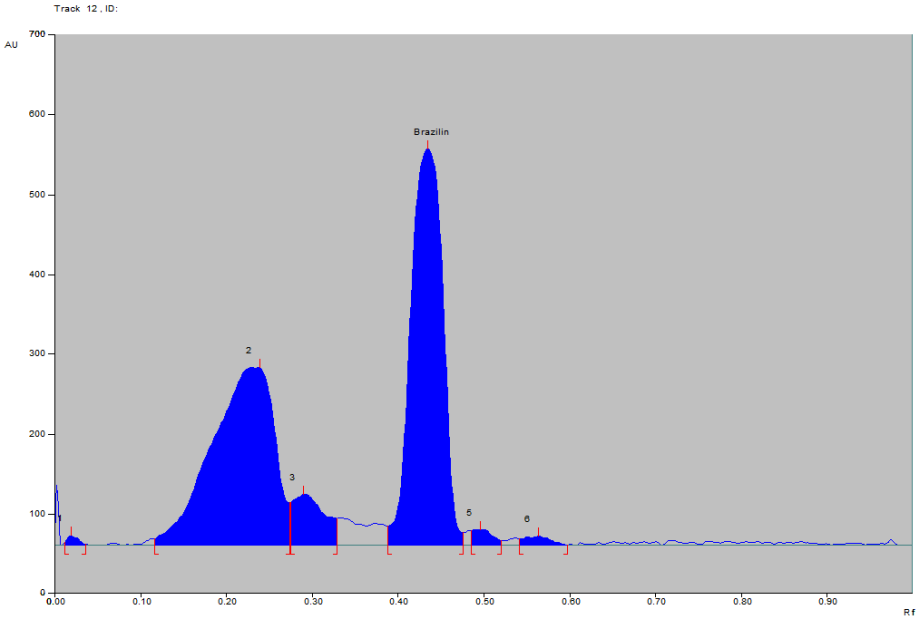
Track9: Sample 4 (1 mg/ml)



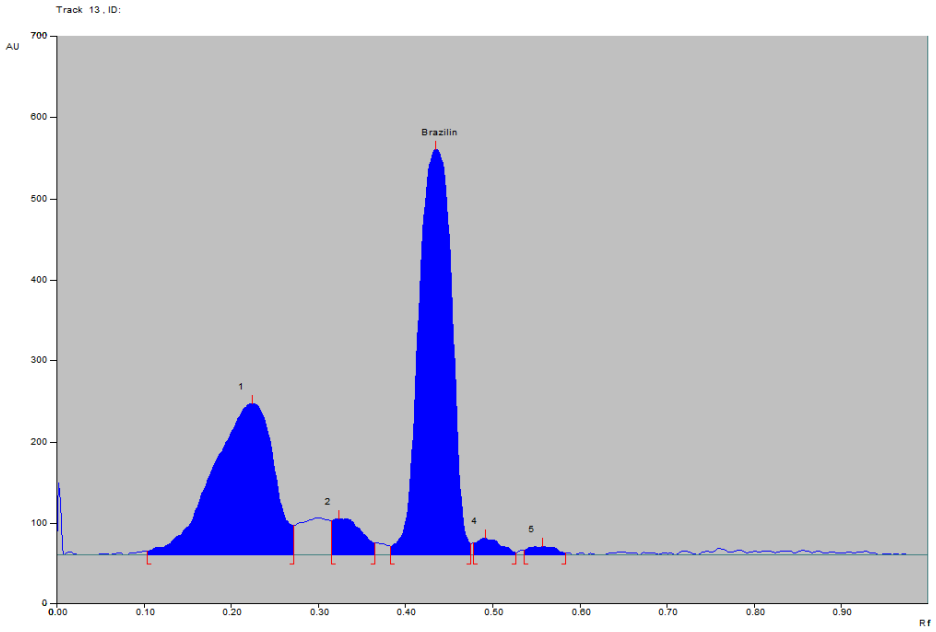
Track10: Sample 5 (1 mg/ml)



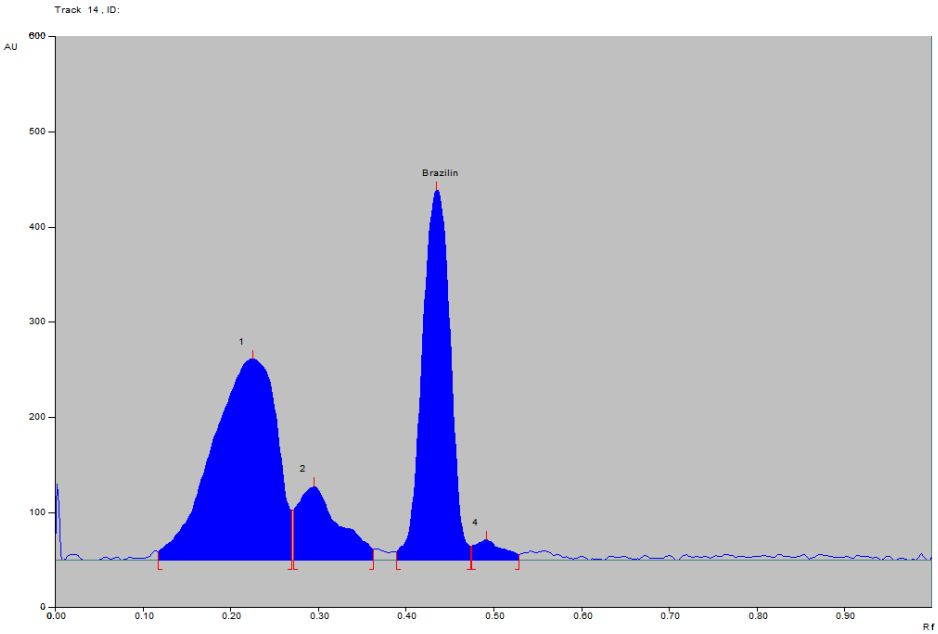
Track11: Sample 6 (1 mg/ml)



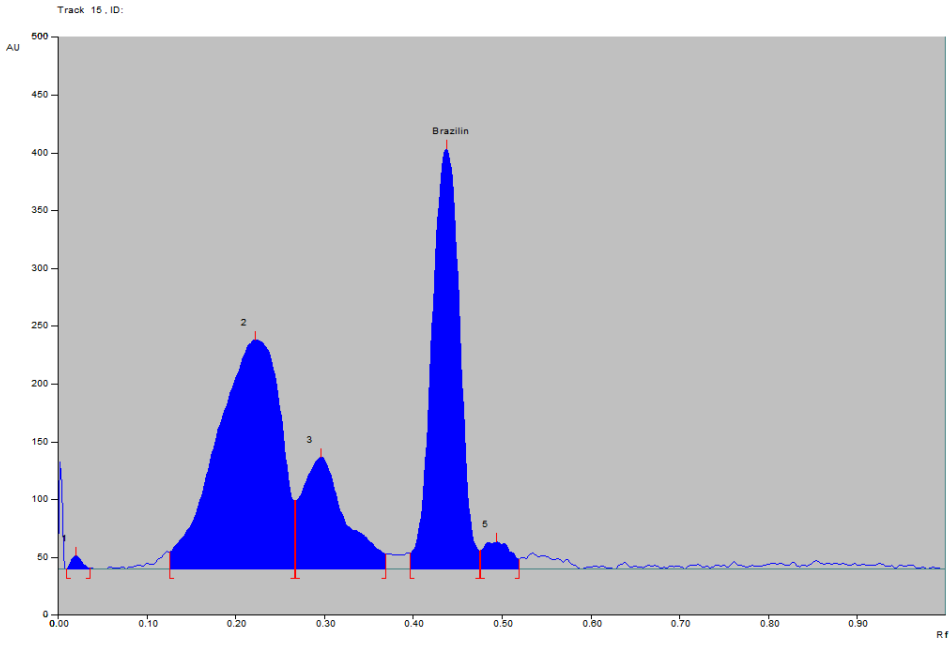
Track12: Sample 7 (1 mg/ml)



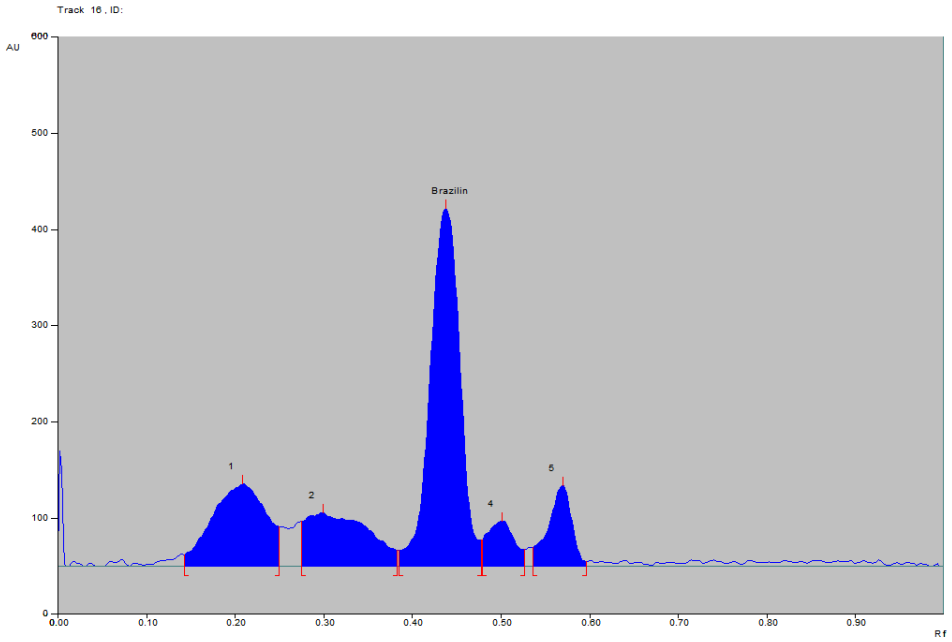
Track13: Sample 8 (1 mg/ml)



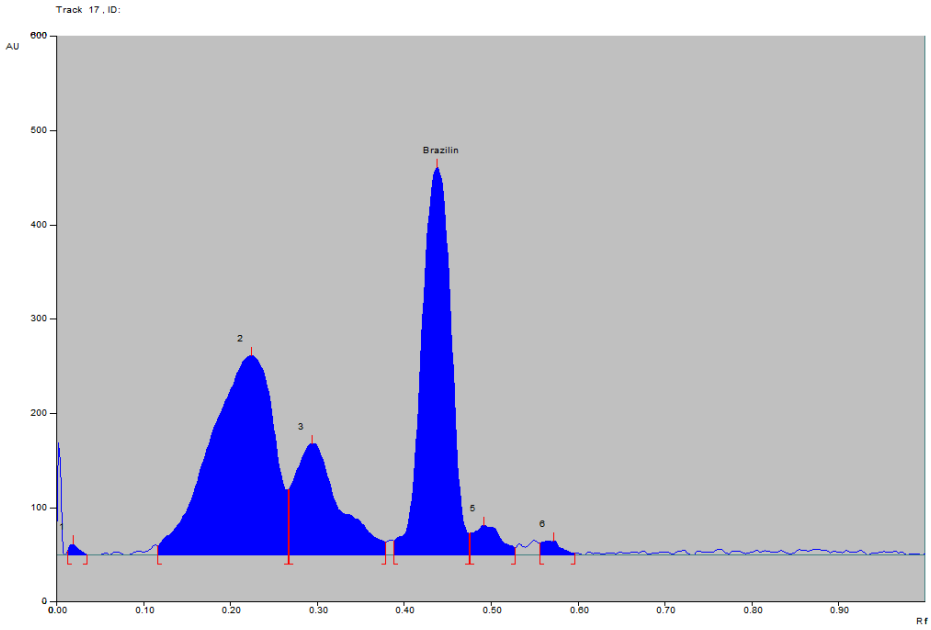
Track14: Sample 9 (1 mg/ml)



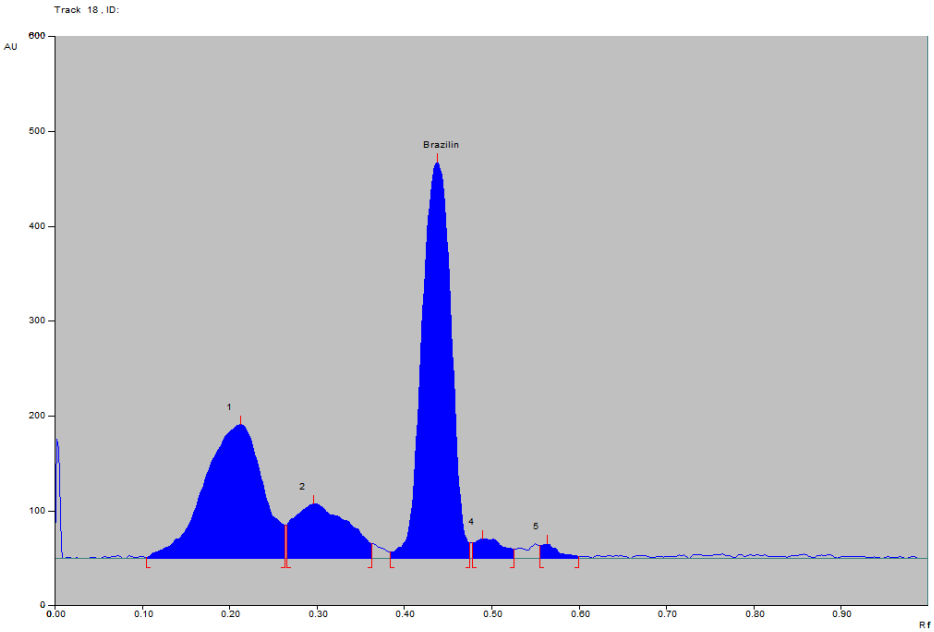
Track15: Sample 10 (1 mg/ml)



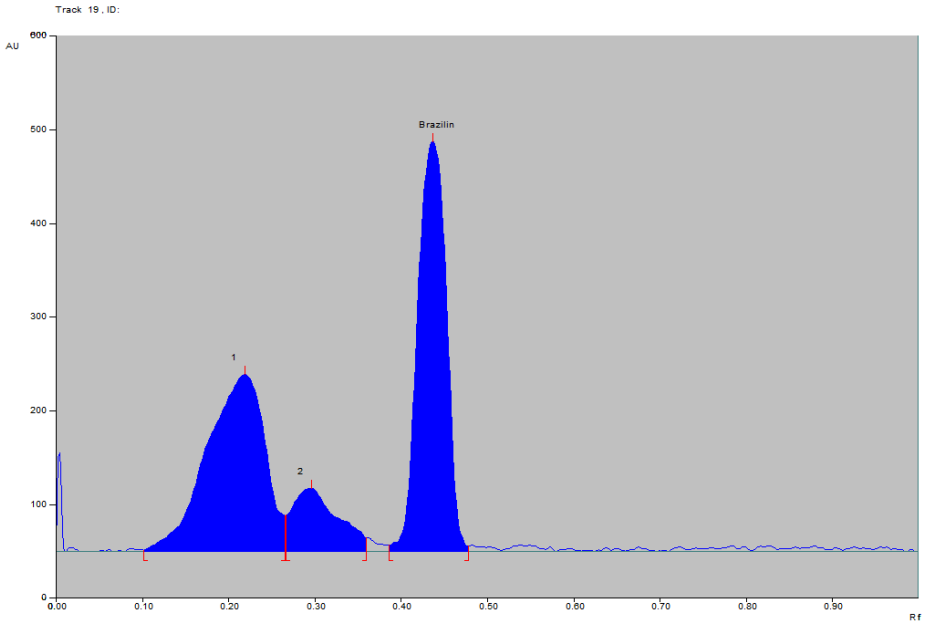
Track16: Sample 11 (1 mg/ml)



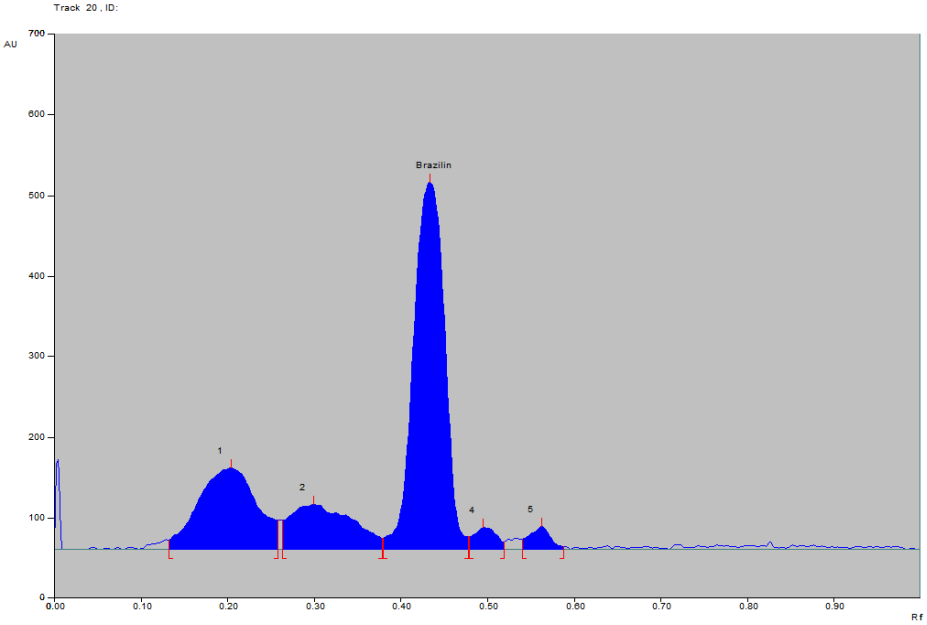
Track17: Sample 12 (1 mg/ml)



Track18: Sample 13 (1 mg/ml)



Track19: Sample 14 (1 mg/ml)



Track20: Sample 15 (1 mg/ml)

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