QUALITY ASSESSMENT OF SELECTED LIRIODENINE
BEARING PLANTS ENDEMIC TO THAILAND

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การประเมินคุณภาพพืชสมุนไพรบางชนิดที่มีสารลิริโอเดนีนในประเทศไทย

นางสาวศรัญธินี มงคลรัตน์
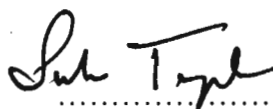
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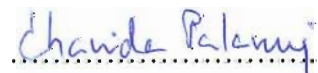Accepted by the College of Public Health Sciences, Chulalongkorn
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ศรัญธินี มงคลรัตน์ : การประเมินคุณภาพพืชสมุนไพรบางชนิดที่มีสารลิริโอเดนีนใน ประเทศไทย (QUALITY ASSESSMENT OF SELECTED LIRIODENINE BEARING PLANTS ENDEMIC TO THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร. ชนิดา พลานุเวช, อ. ที่ ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. นิจศิริ เรืองรังษี, 158 หน้า.

ลิริโอเดนีนจัดเป็นสารอัลคาลอยด์ไอโซควิโนลีน ที่มีฤทธิ์ทางชีวภาพที่หลากหลาย ได้แก่ การต้านการ เกาะกลุ่มของเกล็ดเลือด มีฤทธิ์ต้านเชื้อราและจุลชีพ นอกจากนี้ ยังมีการศึกษาพบศักยภาพในการออกฤทธิ์ใน การทำลายเซลล์มะเร็งหลายประเภท งานวิจัยนี้มีวัตถุประสงค์เพื่อจัดทำข้อกำหนดทางเภสัชเวท และวิเคราะห์ หาปริมาณสารลิริโอเดนีน ในเปลือกต้นจำปี จำปา และ ใบบัวหลวง โดยเทคนิคทางทินเลเยอร์โครมาโทกราฟี เปรียบเทียบกับวิธีโครมาโทกราฟีของเหลวสมรรถนะสูง โดยเก็บตัวอย่างพืชสมุนไพร 28 ชนิดที่อยู่ในวงศ์แมกโน เลีย วงศ์กระดังงา และวงศ์บัวปทุมชาติ จากแหล่งธรรมชาติในประเทศไทย เตรียมสารสกัดโดยวิธีสกัดต่อเนื่อง แบบซ็อกเล็ตด้วยเอทานอล 95 % วิเคราะห์หาปริมาณลิริโอเดนีน โดยเทคนิควิเคราะห์ทั้งสองซึ่งทดสอบความ ถูกต้องของการวิเคราะห์ โดยแสดงค่าความเป็นเส้นตรง ความถูกต้อง ความเที่ยงตรง และ ความไวของการ ทดสอบ ตามข้อกำหนดแนวทาง ICH จากการศึกษาพบ เปลือกต้นจำปี จำปา และใบบัวหลวง มีปริมาณสารลิริ โอเดนีนสูงสุดในสามอันดับแรก อีกทั้งเป็นพืชสมุนไพรที่ใช้เป็นเครื่องยาในการแพทย์แผนไทย โดยเก็บตัวอย่าง จาก 15 แหล่งทั่วประเทศไทย เพื่อกำหนดมาตรฐานทางเภสัชเวทและประเมินคุณภาพ การศึกษาเอกลักษณ์ทาง เภสัชเวทของเปลือกต้นจำปี พบว่ามีปริมาณเถ้าที่ไม่ละลายในกรด เถ้ารวม น้ำหนักที่หายไปเมื่อทำแห้ง และ ปริมาณน้ำ ไม่ควรเกินร้อยละ 3.49, 5.74, 7.17 และ 6.67 ของน้ำหนักแห้ง ตามลำดับ ปริมาณสารสกัดด้วยเอ ทานอลและน้ำ ไม่ควรน้อยกว่าร้อยละ 3.85 และ 8.25 ของน้ำหนักแห้ง ตามลำดับ สำหรับเปลือกต้นจำปา พบว่ามีปริมาณเถ้าที่ไม่ละลายในกรด เถ้ารวม น้ำหนักที่หายไปเมื่อทำแห้ง และ ปริมาณน้ำ ไม่ควรเกินร้อยละ 2.98, 6.25, 5.62 และ 7.37 ของน้ำหนักแห้ง ตามลำดับ ปริมาณสารสกัดด้วยเอทานอลและน้ำ ไม่ควรน้อยกว่า ร้อยละ 6.71 และ 12.38 ของน้ำหนักแห้ง ตามลำดับ การศึกษาใบบัวหลวง พบว่ามีปริมาณเถ้าที่ไม่ละลายใน กรด เถ้ารวม น้ำหนักที่หายไปเมื่อทำแห้ง และ ปริมาณน้ำ ไม่ควรเกินร้อยละ 2.61, 9.62, 7.69 และ 7.06 ของ น้ำหนักแห้ง ตามลำดับ ปริมาณสารสกัดด้วยเอทานอลและน้ำ ไม่ควรน้อยกว่าร้อยละ 6.24 และ 9.51 ของ น้ำหนักแห้ง ตามลำดับ โดยสรุป ผลการทดสอบค่าความเป็นเส้นตรง ความถูกต้อง ความเที่ยงตรง และ ความไว ของวิธีวิเคราะห์ ได้กราฟมาตรฐาน ในช่วงความเป็นเส้นตรงของความเข้มข้นระหว่าง 5 - 200 ไมโครกรัมต่อ มิลลิลิตร มีค่าสัมประสิทธิ์สหสัมพันธ์ที่ดีคือ มากกว่า 0.995 จากผลการยืนยันความถูกต้องของการวิเคราะห์สาร จากทั้งสองวิธีที่พัฒนาขึ้นมานี้ พบว่ามีความถูกต้อง แม่นยำ และความไวอยู่ในเกณฑ์ดี ภาพที่ได้จากแผ่นทินเล เยอร์โครมาโทกราฟี แสดงการเรืองแสงของสารลิริโอเดนีนอย่างชัดเจน ภายใต้การดูดกลืนแสงอัลตราไวโอเลตที่ ความยาวคลื่น 365 นาโนเมตร ได้ค่า R_f ที่ 0.75 ในขณะที่โครมาโทแกรมจากวิธีโครมาโทกราฟีของเหลว สมรรถนะสูง แสดงพีคของสารลิริโอเดนีน ที่เวลาประมาณ 11 นาที จากผลการศึกษา แสดงให้เห็นว่าทั้งสองวิธี นี้ สามารถนำมาใช้ในการวิเคราะห์ เชิงปริมาณของสารลิริโอเดนีนในพืชสมุนไพรได้เป็นอย่างดี โดยไม่พบความ แตกต่างอย่างมีนัยสำคัญ เมื่อเปรียบเทียบผลการทดลอง ระหว่างสองวิธี ($p>0.05$) ยิ่งไปกว่านั้น การวิเคราะห์ เชิงปริมาณด้วยภาพจากทินเลเยอร์โครมาโทกราฟี สามารถนำมาประยุกต์ใช้ได้ เนื่องจากเป็นเครื่องมือที่ ไม่ ยุ่งยาก สะดวก รวดเร็ว และ มีราคาไม่แพง อีกทั้งการเรืองแสงของสารลิริโอเดนีน ยังช่วยให้การวิเคราะห์ด้วยวิธี นี้ง่ายขึ้น สะดวก และเหมาะสมแก่การนำมาใช้ในการพัฒนาต่อไป

สาขาวิชา วิทยาศาสตร์สาธารณสุข   ลายมือชื่อนิสิต _____

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SARANTHINEE MONGKOLRAT: QUALITY ASSESSMENT OF SELECTED LIRIODENINE BEARING PLANTS ENDEMIC TO THAILAND. ADVISOR: CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 158 pp.

Liriodenine, an aporphine isoquinoline alkaloid, has many biological activities, including anti-platelet, anti-fungal and anti-microbial actions. Additionally previous studies revealed that it had potent cytotoxicity against a number of cancer cell lines. This study aimed to determine liriodenine content in selected Thai medicinal plants by TLC image analysis compared to HPLC. Twenty-eight plant materials in Magnoliaceae, Annonaceae and Nelumbonaceae were collected from natural sources in Thailand. Crude extracts were prepared by Soxhlet extraction with 95% ethanol. Validations of both methods were performed including linearity, accuracy, precision and sensitivity based on International Conference of Harmonization (ICH) guideline. Top three highest contents of liriodenine were found in *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaf) which are also used as crude drug in various Thai traditional recipes. Fifteen different locations throughout Thailand of each medicinal plant were then examined for pharmacognostic specification to establish their standardization for quality assessment. Pharmacognostic parameters from *Michelia longifolia* bark revealed that the acid-insoluble ash, total ash, loss on drying and water content should be not more than 3.49, 5.74, 7.17 and 6.67 % of dry weight respectively; while ethanol and water-soluble extractive should be not less than 3.85 and 8.25 % of dry weight respectively. *Michelia champaca* bark showed that the acid-insoluble ash, total ash, loss on drying and water content should be not more than 2.98, 6.25, 5.62 and 7.37 % of dry weight respectively; while ethanol and water-soluble extractive should be not less than 6.71 and 12.38 % of dry weight respectively. Pharmacognostic parameters of *Nelumbo nucifera* leaves revealed that the acid-insoluble ash, total ash, loss on drying and water content should be not more than 2.61, 9.62, 7.69 and 7.06 % of dry weight respectively; while ethanol and water-soluble extractive should be not less than 6.24 and 9.51 % of dry weight respectively. In summary, calibration showed good linear correlation coefficients (R^2 > 0.995) over the range of concentration 5-200 µg/mL. Method validation of both methods was successfully developed performing reliability and sensitivity. TLC image showed a well-defined fluorescent spot of liriodenine at the R_f value of 0.75 under UV 365 nm, while HPLC chromatogram indicating liriodenine peak at 11 min of retention time. Both proposed methods could be used as a tool for the quantification of liriodenine in medicinal plants. There was no significant difference between the results of both analytical methods (p>0.05). TLC image could be applied for analysis of the content of this compound because it is simple, rapid and inexpensive. Moreover, the fluorescent coloring spot which was a dominant characteristic of liriodenine on TLC plate allowed TLC image analysis to be more suitable and convenient for further development.

Field of Study: Public Health Sciences   Student's signature _____

AcademicYear: 2012 _____   Advisor's Signature _____

                                                     Co-advisor's Signature _____

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

A	=	Absorbance
AUC	=	Area under curve
B	=	Blume
c	=	Concentration
C-18	=	Octadecyl carbon chain
$CDCl_3$	=	Deuterated chloroform
CI	=	Confidence interval
cm	=	Centimeter
CPHS	=	College of Public Health Sciences
$^{\circ}$ c	=	Degree Celsius
^{13}C-NMR	=	Carbon – thirteen nuclear magnetic resonance
DAD	=	Diode array detector
DNA	=	Deoxyribonucleic acid
g	=	Gram
HCl	=	Hydrochloric acid
HPLC	=	High performance liquid chromatography
hr	=	Hour
^{1}H-NMR	=	Proton nuclear magnetic resonance
ICH	=	International conference on harmonization
kg	=	Kilogram
L	=	Liter
LC	=	Liquid chromatography
LC-MS	=	Liquid chromatography mass spectrometer

LOD	=	Limit of detection
LOQ	=	Limit of quantitation
Max	=	Maximun
MC	=	*Michelia champaca*
mg	=	Milligram
MHz	=	Megahertz
min	=	Minute
Min	=	Minimum
ML	=	*Michelia longifolia*
mL	=	Milliliter
mm	=	Millimeter
n.	=	Number
Nm	=	Nanometer
NMR	=	Nuclear magnetic resonance
NN	=	*Nelumbo nucifera*
ODS	=	Octa- decyl silane
R^2	=	Correlation coefficient
RSD	=	Relative standard deviation
s	=	Slope
SD	=	Standard deviation
spp	=	Species
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
v/v	=	Volume by volume
var	=	Variety

w/w	=	Weight by weight
WHO	=	World Health Organization
%	=	Percentage
μg	=	Microgram
μL	=	Microliter
μm	=	Micrometer
β	=	Beta
λ	=	Lambda
σ	=	Sigma
δ	=	Chemical shift
J	=	Coupling constant

CHAPTER I

INTRODUCTION

Background and rationale

Traditional medicine is the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses. Traditional medicine that has been adopted by other populations (outside its indigenous culture) is often termed alternative or complementary medicine [1].

Herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients.

After decades of serious obsession with the modern medicinal system, people have started looking at the ancient healing systems like Ayurveda, Siddha and Unnani. This is because of the adverse effects associated with synthetic drugs. Herbal drugs play an important role in health care programs especially in developing countries. Ancient Indian literature incorporates a remarkably broad definition of medicinal plants and considers all plant parts to be potential sources of medicinal substances [2].

However a key obstacle, which has hindered the acceptance of the alternative medicines in the developed countries, is the lack of documentation and stringent quality control. There is a need for documentation of research work carried out on traditional medicines [3]. Importantly, of about 300 types of frequently used Thai crude drugs, less than 10% have official pharmacognostic specifications in Thai Herbal Pharmacopeia.

With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as medicine. The process of standardization can be achieved by stepwise pharmacognostic studies [4]. These studies help in identification and authentication of the plant material. Correct identification and quality assurance of the starting materials is an essential

prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy. Simple pharmacognostic techniques used in standardization of plant material include its morphological, anatomical and biochemical characteristics [5].

Generally, the standardization of the plant material is not required when used by the rural communities for their primary health care. But, regardless of whether the medicinal plant is to be used by local communities or by industry, a systematic approach is required for a plant identified from traditional medicine, as is done in modern medicine. It is necessary to focus on all aspects of medicinal plant research: from cultivation, ethno-pharmacology, utilization, isolation and identification of active constituents to efficacy evaluation, pharmacology, safety, standardization, formulation and clinical evaluation. Animal toxicity studies are required to establish the potential adverse effects [6].

Nowadays quality assessment is important because there is a synergy among all the different chemical compounds in a medicinal plant. It's not only the one on the label that's important, but also it's really the whole plant that's important.

Phytochemical studies of the plant preparations are necessary for standardization, which helps in understanding the significance of phytoconstituents in terms of their observed activities. It also helps in standardizing the herbal preparations so as to get the optimal concentrations of known active constituents, and in preserving their activities.

Chemical fingerprints through chromatographic techniques are more commonly used for standardization and are obtained in terms of one or more marker compounds. It would be ideal to use the active constituent in the plant as the marker compound; however, in cases where active constituents are not known, the marker compound can be independent of the therapeutic activity. Furthermore, the plant extracts can also be standardized *via* class of compounds e.g. ginsenosides in ginseng, kava lactones in kava, or oxindole alkaloids in cat's claw [7].

According to European Medicines Agency guidelines, quantification of substances with known therapeutic activity or markers is obligatory [8]. As stated in the European Pharmacopoeia, marker compound should be characteristic or unique for the herbal material or herbal preparation, have an established chemical structure, be present in the starting material as well as the finished product in sufficient amounts and be accessible to quantification with common analytical methods.

The modern analytical and isolation methods that are used for screening and isolation of plant constituents are the chromatographic and spectroscopic techniques such as thin layer chromatography (TLC), thin layer electrophoresis, high performance liquid chromatography (HPLC), nuclear magnetic resonance, high performance thin layer chromatography (HPTLC) etc. These techniques have been proved for the usefulness in isolation and proper identification of the active constituents in the plant extracts [9]. TLC and HPLC are the most commonly used methods for obtaining chemical fingerprints and identification of the crude plant extracts.

Alkaloids are abundant secondary metabolites in plants and represent one of the most widespread class of compounds endowed with multiple, varied pharmacological properties. Among alkaloids, the aporphinoids constitute a broad subgroup of benzylisoquinoline compounds, with more than 500 alkaloids isolated up to now. They are widely distributed in a large number of plant families including Annonaceae, Lauraceae, Monimiaceae, Menispermaceae, Hernandiaceae, Ranunculaceae and so on [9]. Since 1975 a few reviews on chemical structures, spectral data, botanical sources and pharmacological activities have been published on aporphinoids, including proaporphines, aporphines, and related naturally occurring derivatives [10-15].

Liriodenine as a marker compound in this study is a class of isoquinoline alkaloid belonging to the aporphine subgroup. The effect of liriodenine has been tested for many years, it showed good activity *in vitro* and might be metabolized *in vivo* into inactive metabolites. Alternatively, its extract may only show *in vivo* activity due to the metabolism of inactive compounds into active forms. In addition, liriodenine showed potent cytotoxic against various cancer cell lines, it became an active

component of the anticancer traditional Chinese medicine [16] while its activities have been tested increasingly in past few years related to new findings in cancer treatment. According to drugs employed in chemotherapy and radiotherapy for treating cancer, they can have undesirable side effects as well as DNA damage in normal cells. Then it would be useful to investigate compounds that inhibit or reduce these effects. Therefore, it is necessary to find other drugs, and natural products are a good source. From 1983 to 1994, more than 60% of the approved anticancer drugs were found in natural products [17]. The new drugs are also necessary in the treatment of trypanosomiasis and leishmaniasis, since current chemotherapy is still inadequate and causes many undesirable side effects [18].

This study aimed to find good natural sources of liriodenine endemic to Thailand. HPLC and TLC image analysis techniques for qualitative and quantitative analysis of liriodenine in plant materials were applied and validated for developed system. Three plant families bearing liriodenine, Magnoliaceae, Annonaceae and Nelumbonaceae were selected to be examined, not only because they were benefit in traditional recipes, but also were accessible throughout Thailand. Moreover, traditional Thai crude drugs: *Michelia longifolia* Blume (bark), *Michelia champaca* Linn. (bark) and *Nelumbo nucifera* Gaernt. (leaves) were pharmacognostic studied to identify their quality assessment.

Research questions

1. How many percent of liriodenine contents in Thai medicinal plants belonging to Magnoliaceae, Annonaceae and Nelumbonaceae?

2. What are the pharmacognostic parameters leading to quality standardization of selected liriodenine bearing medicinal plants: *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaf)?

3. How to analyze qualitatively and quantitatively liriodenine in plant materials using HPLC and TLC image analysis?

Objectives of study

1. To develop HPLC and TLC image analysis techniques for qualitative and quantitative analysis of liriodenine in plant materials.

2. To determine liriodenine content in Thai medicinal plants belonging to Magnoliaceae, Annonaceae and Nelumbonaceae.

3. To develop pharmacognostic specifications of *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaf).

Expected Benefit & Application

To set the tools for the quality assurances of Thai medicinal crude drugs including chemical quantitative analysis by conventional TLC using image analysis from Scion software.

Selected plants in 3 families:

| Magnoliaceae | Annonaceae | Nelumbonaceae |

Plants authenticated by expert and national herbarium comparison and air dried.
All voucher specimens were deposited at CPHS, Chulalongkorn University.

⇩

Liriodenine screening by ethanolic extraction using soxhlet apparatus and
quantitative determination using HPLC and TLC image software

⇩

3 species with liriodenine in major:
- *Michelia longifolia* (bark)
- *Michelia champaca* (bark)
- *Nelumbo nucifera* (leaf)

⇩

Quality assessment

Plant specimens collected from 15 habitats throughout Thailand, authenticated as above mentioned.

**Quality parameters investigation
according to WHO Quality Control
Methods for Medicinal Plant Materials**

- Macroscopic examination

- Microscopic examination

- Determination of total ash

- Determination of acid-insoluble ash

- Determination of loss on drying

- Determination of ethanol extractive values

- Determination of water extractive values

- Determination of water content

- Determination of volatile oil content

- Thin layer chromatographic identification

Liriodenine content determination

Exhaustively ethanol extraction
by Soxhlet apparatus

HPLC   TLC image analysis

Method validation

Figure 1 Conceptual framework

CHAPTER II

LITERATURE REVIEWS

Liriodenine

Liriodenine was first isolated as a bright yellow constituent of the heartwood of the tulip tree *Liriodendron tulipifera* Linn. (Magnoliaceae) distributed throughout the eastern region of the United States [19-20]. Liriodenine (8H-benzo[g]-1,3-benzodioxolo [6,5,4-de]-quinolin-8-one), shown in Figure 2, was anoxoaporphine isoquinoline alkaloid which previously reported found from plant species in many genera as shown in Table1.
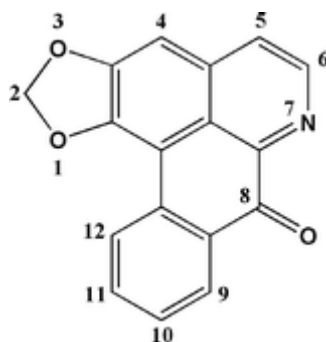
Figure 2 Chemical structure of liriodenine

Table 1 Plant species containing liriodenine

Family	Species	Plant part	Reference
Annonaceae	*Annona amazonica*	Stem	21
	Annona bullata	Bark	22
	Annona cherimola	Arial part	23
	Annona diversifolia	Root	24
	Annona foetida	Branch	25
	Annona glabra	Bark	26
	Annona reticulate	Leaf	27

Family	Species	Plant part	Reference
Annonaceae	*Annona senegalensis*	Root	28
	Annona squamosa	Unripe fruits	29
		Bark	30
	Artabotrys maingayi	Bark	31
	Artabotrys zeylanicus	Stem bark	32
	Cananga latifolia	Stem	33
	Cananga odorata	Bark	34
	Cleistopholis patens	Root bark	35
	Cyathostemma argenteum	Root	36
	Duguetia furfuracea	Leaf	37
	Enantia pilosa	Stem bark	38
		Root bark	
	Enicosanthellum pulchrum	Root	39
	Enicosanthum cupulare	Plant	40
	Fissistigma glaucescens	Leaf	41
	Fissistigma latifolium	Bark	42
	Goniothalamus amuyon	Stem	43
	Guatteria hispida	Bark	44
	Guatteria multivenia	Root	45
	Guatteriopsis friesiana	Stem	46
	Hornschuchia oblique	Stem	47
		Root	
	Lettowianthus stellatus	Root bark	48
	Melodorum punctulatum	Leaf	49
		Bark	
	Miliusa balansae	Stem	50
	Miliusa sinensis	Leaf	51
		Twig	
		Flower	
	Mitrephora glabra	Stem bark	52

Family	Species	Plant part	Reference
Annonaceae	*Mitrephora maingayi*	Leaf Stem	53
	Polyalthia cauliflora var. *cauliflora*	Stem bark	54
	Polyalthia longifolia var. *pendula*	Leaf	55
	Polyalthia nemoralis	Branch Leaf	56
	Popowia pisocarpa	Bark	57
	Pseudomalmea boyacana	Stem	58
	Pseuduvaria cf. grandifola	Leaf	59
	Pseuduvaria setosa	Arial part	60
	Rollinia leptopetala	Root	61
	Rollinia pittieri	Stem	58
	Schefferomitra subaequalis	Bark	59
	Unonopsis buchtienii	Stem bark	62
	Unonopsis lindmanii	Leaf Seed Bark	63
	Uvaria kurzii	Branch Leaf	64
	Uvaria versicolor	Stem	65
	Xylopia aethiopica	Leaf Twig Stem bark	66
Atherospermataceae	*Laurelia sempervirens*	Bark	67
	Doryphora sassafras	Bark	68
Eupomatiaceae	*Eupomatia laurina*	Root bark	69
Hernandiaceae	*Illigera luzonensis*	Stem	70

Family	Species	Plant part	Reference
Lauraceae	*Phoebe chinensis*	Stem bark	71
	Phoebe formosana	Bark	72
Magnoliaceae	*Kmeria duperreana*	Plant	73
	Liriodendron tulipifera	Heartwood	20
	Magnolia grandiflora	Trunk	74
	Magnolia obovata	Bark	75
		Leaf	76
	Magnolia officinalis	Bark	77
	Michelia champaca	Bark	78
	Michelia compressa	Leaf	79
	Michelia floribunda	Plant	80
	Michelia formosana	Heartwood	81
	Michelia longifolia	Bark	82
	Michelia rajaniana	Bark	83
	Paramichelia baillonii	Bark	84
	Talauma hodgsoni	Root bark	85
	Talauma ovata	Trunk bark	86
		Leaf	
	Tsoongiodendron odorum	Root bark	87
		Twig	
		Heartwood	
Menispermaceae	*Cissampelos sympodialis*	Root	88
	Limaciopsis loangensis	Root	89
		Stem	
		Leaf	
		Fruit	
	Pachygone ovate	Root	90
	Rhigiocarya racemifera	Root	91
	Sinomenium acutum	Root	92
		Stem	

Family	Species	Plant part	Reference
Menispermaceae	*Stephania dielsiana*	Root	93
	Stephania dinklagei	Stem	94
	Stephania rotunda	Tuber	95
Monimiaceae	*Glossocalyx brevipes*	Leaf	96
	Mollinedia brasiliensiis	Stem	97
	Mollinedia glaziovii	Stem	97
	Mollinedia gligiana	Stem	97
	Mollinedia marliae,	Stem	97
	Mollinedia salicifolia	Stem	97
	Mollinedia schottiana	Stem	97
	Siparuna apiosyce	Leaf Bark	98
	Siparuna guianensis	Fruit	99
Moraceae	*Broussonetia papyrifera*	Fruit	100
Nelumbonaceae	*Nelumno nucifera*	Leaf	101 102
Papaveraceae	*Papaver arachnoideum*	Aerial part	103
	Papaver heldreichii	Plant	104
Phyllanthaceae	*Actephila merrilliana*	Leaf	105
Rubiaceae	*Saprosma hainanense*	Stem	106
Rutaceae	*Zanthoxylum nitidum*	Stem bark	107
	Zanthoxylum cuspidatum	Bark	108

Literally, liriodenine widely distributes in many natural plants of a series of families and genera, but the content is very low [109].

Characterization of liriodenine demonstrated as yellow rosette crystals. It was slightly soluble in chloroform, soluble in ethyl alcohol and insoluble in petroleum ether. Color reaction showed orange color with Dragendroff's reagent. Melting point was 278-282°c, molecular weight 275 [82].

Liriodenine has a potent anticancer activity. Owing to its planar aromatic structure, liriodenine may intercalate between the neighboring base pairs of the DNA double helix, to which its significant antitumor activities can be primarily attributed. Moreover, it also catalytically inhibits topoisomerase II to block DNA synthesis and increases p53 and induces nitric oxide synthase expression as well as cell cycle G1 arrest [110–116]. Although there are some other biological targets in tumor cells, it is generally accepted that DNA is the primary target for many anticancer drugs [117-118]. Interactions between small molecules and DNA rank among the primary action mechanisms of antitumor activity.

Additionally liriodenine has a wide range of pharmacological activities such as anti-platelet, anti-fungal, and anti-microbial actions [114-115]. Previous studies revealed that liriodenine had cardio-protective efficacy with reducing the extent of cardiovascular injuries under ischemia-reperfusion conditions [119]. The compound also showed probably a promising drug for treatment of cardiac arrhythmia with heart failure [120].

Liriodenine bearing plants endemic to Thailand

There is still no data about liriodenine evaluation in Thai medicinal plants. Although it was found in many plant species, the content was still very low [109]. This study aimed to find good natural sources of this compound available in Thailand. Literally, there are some families containing liriodenine (Table 1) which generally distribute in Thailand including Magnoliaceae, Annonaceae and Nelumbonaceae. Thus these three families were selected for liriodenine content investigation.

The first part of the research was to explore liriodenine bearing plants endemic to Thailand. TLC image analysis was applied for liriodenine quantification, and the results were compared to those of HPLC. In addition, it was the first study applying TLC image analysis to this compound; hence, the procedure required to be confirmed with another referential method as HPLC as following described:

Chemical quantitative methods

Two chromatographic methods, TLC and HPLC (the referential method), were developed and used for separation and quantitative determination of liriodenine in crude extracts. All necessary validation tests for both methods were developed for their comparison. This part is to explain the concept of both methods and the validation.

High performance liquid chromatography (HPLC)

It is a chromatographic technique that can separate a mixture of compounds and is used in herbal medicines, medicinal plants and herbal sciences to identify, quantify and purify the individual components of the mixture [121]. Preparation and analytical HPLC are widely used in pharmaceutical industry for isolation and purification of herbal compound [122]. This is important nowadays for the industry because new product as natural synthetic have to be introduced to the market as quickly as possible. Having available such a powerful purification technique make it possible to spend less time on the synthesis conditions [123- 124].

The details of two differential detectors for HPLC system were described as followings:

- Ultraviolet (UV) detector

    The UV detector is by far the most popular and useful LC detector that is available to the analyst at this time. It is used for a wide range of application and it is the standard equipment of an HPLC system. Fixed wavelength UV detector employs a UV light source to measure components showing an absorption in the ultraviolet or visible region. Different components show a different spectrum. According to the optical system, light from lamp is shone onto the diffraction grating and dispersed in desirable wavelength. By monitoring the reference light divided from the light in front of the flow cell, and this is output as absorbance [125].
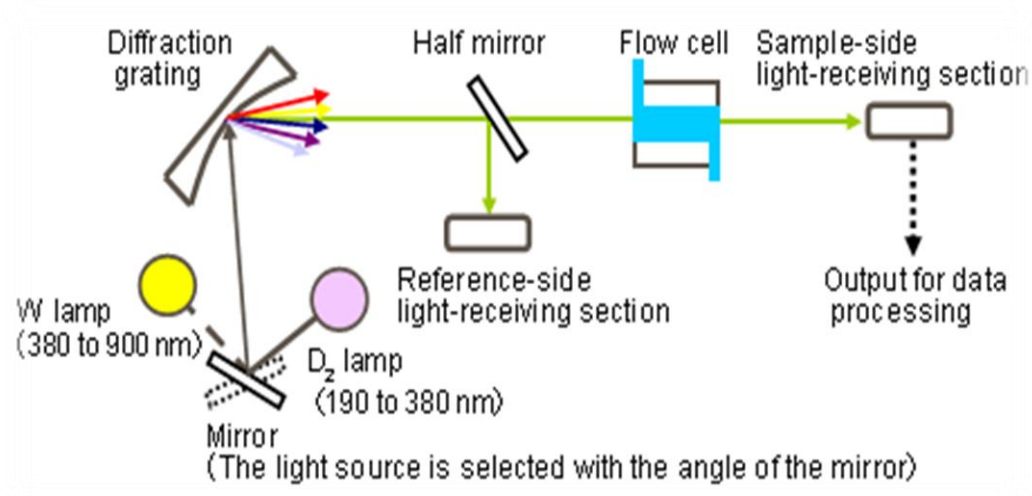
Figure 3 Diagrammatic illustration of a UV detector optical system

- Diode array detector (DAD)

  While the UV detector has only one sample-side light receiving section, a DAD has multiple to obtain information over a wide range of wavelengths at one time which is the advantage of DAD. DAD differs from UV detector in that light from the lamps is shone directly onto the flow cell; light passed through the flow cell is dispersed by the diffraction grating and the amount of the dispersed light is estimated for each wavelength [125].
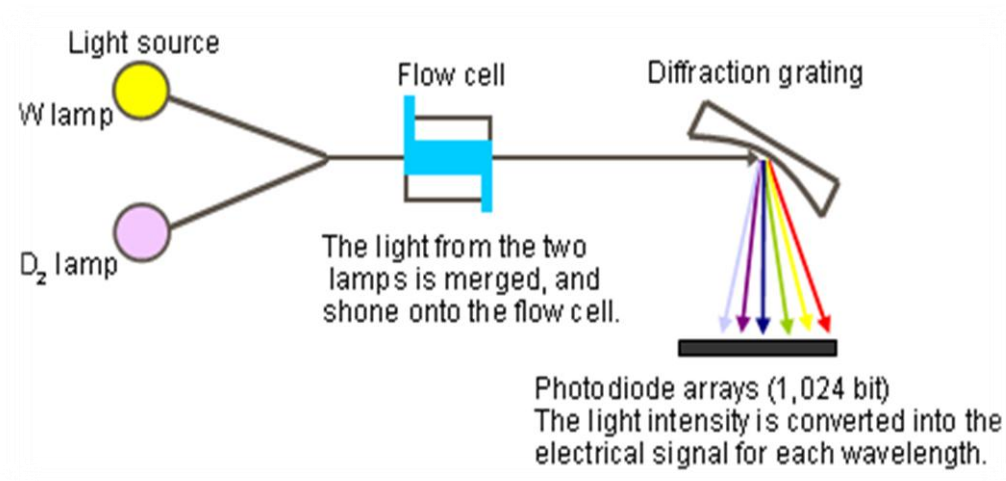
Figure 4 Diagrammatic illustration of a DAD detector optical system

If the measurement is performed at a fixed wavelength, components are identified from only their retention time; hence a minor deviation in retention time can make identification of components difficult. In such case the DAD can be used to identify components by a comparison of the spectrum. However, DAD still has some disadvantage when compared to UV detector; the noise is large as the amount of light is small while DAD is susceptible to various changes such as lamp fluctuations because the reference light cannot be received. DAD has been improved to reduce its different performance from UV detector. HPLC has general advantages and disadvantages listed in Table 2 [126]:

Table 2 Advantages and disadvantages of HPLC

Advantages	Disadvantages
- **High speed**	- Expensive instrumentation
- **High resolution**	- Experience required
- **High sensitivity**	- Lack of universal and sensitive detectors
- **High accuracy**	- Expensive supplies
- **Automated systems**	- Not good for qualitative analysis

Thin layer chromatography (TLC) and image analysis

It is a chromatographic technique used to separate mixtures. Separation of compounds is based on the composition of the solute and the mobile phase for either binding places on the stationary phase or dissolving in the mobile solvent. The retardation factor (R_f) value is used to quantify the movement of compound on plate. R_f is equal to the distance traveled by the substance divided by the distance traveled by the solvent [126]. In practice, different solvents or mixtures of solvent were tried until a good separation was observed. Typically effective solvent is one that gives R_f's in the range of 0.3-0.7. Referring to stationary phase (TLC plate), a thin layer of silica gel was used in this experiment as its simplicity and highly polar that is capable of hydrogen bonding. The main use of TLC is for qualitative analysis and screening, and it is performed as quick and simple assay. Manual TLC is also used for fast screening of mixtures and for surveying methods for possible use in HPLC. The advantages and disadvantages of TLC were shown in Table 3 [126]:

Table 3 Advantages and disadvantages of manual TLC

Advantages	Disadvantages
- **Fast**	- Flow is not constant.
- **Simple, inexpensive apparatus**	- Flow cannot be controlled easily.
- **High sample throughput using multiple samples per plate**	- Temperature and solvent gradients can exist.
- **Variable shapes of plates**	- Not always in predictable with theory.
- **Detection and quantitation are static.**	- Not automated; labor intensive
- **All analytes are on plate.**	- Less accurate quantitation

In TLC, if the solute of interest is colorless, a suitable colored derivative must be formed before the separation or commonly after the separation by treating the plate with an appropriate reagent. Providing the separation is clean and a suitable colored derivative is employed, the visual comparative procedure can give quantitative results with an accuracy of about +/-10 %. However, such accuracy is generally considered inadequate. As the retinic response of the human eye is not linear and varies widely with the wavelength of the reflected light. Visual estimation is further complicated by the variation of the iris with light intensity. The visual comparative technique has been largely replaced by instrumental methods of spot density measurement [124]. Calibration curve was analyzed using the least square regression equation derived from peak area.

TLC image software (SCION)

Scion image software was used to analyze TLC image due to its free download software and simple application; particularly it has been applied for any other chemical compounds prior to the present study [129]. The convenient TLC image analysis method using computer technology was applied and compared to the advance high technology technique using HPLC in this study. A digital image of a TLC chromatogram was obtained with a digital camera, and densities of the TLC spots were analyzed using Scion image program, which was public computer software modified from NIH image software of the National Institutes of Health, USA (Scion

Corporation, 2000-2001). Its main application is in the field of biological image analysis having been reported [127-129].

When combining digital photograpgy with regular TLC, it is to improve the compound's quantitative technique as well as provide the accuracy of the quantitative TLC analysis for also lower cost than any other high commercial equiptments [130] (Good quality digital camera costs is about 700$, and the software is free).

The result from TLC image analysis is related to the compound's concentration. The pixel density of spot image can be used as a detector signal. The intensity is proportional to the amount of substance and can be in accordance with the peak area when quantified with reference tool like HPLC.

Some alkaloids including quinolines and isoquinolines which have conjugated aromatic rings in the structure can produce the dark spot on green background fluoresced under excitation wavelength of 254 nm. Quenching spot is due to light absorption property of these compounds at that wavelength [131-132]. In addition, some isoquinoline alkaloids possess fluorescent property under excitation of longer wavelength. Fluorescent emission of the spot against dark background increases the sensitivity and accuracy of image analysis in quantitative thin layer chromatography from previous study [133-134].

Method validation
New chemical quantitative method should be validated according to International Conference of Harmonization (ICH) guideline. Typical validation characteristics and their definitions which should be considered are listed below [135]:

- *Accuracy* expresses the closeness of agreement between value which is accepted either a conventional true value or an accepted reference value and the value found.

- *Specificity* is to assess unequivocally the analyte in the presence of components which may be expected to be present. To demonstrate specificity in TLC image analysis, the sample, and standard liriodenine were chromatographic. Matrix

effects must be eliminated to obtain undisturbed spot-shape and Rf values. In case of HPLC, retention time, purity value and spectra were used to determine a probability of identifying a chemical peak.

- *Precision* expresses the closeness of agreement between a series of measurement. Repeatability and intermediate precision (different days) was conducted and presented as relative standard deviation (RSD).

- *Limit of detection* (LOD) is the lowest amount of analyte in a sample which can be detected, but not necessarily quantitated.

- *Limit of quantitation* (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

- *Linearity* is the ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte.

- *Range* is the interval between upper and lower concentration of analyte.

After chromatographic conditions were optimized and the purposed methods were validated, liriodenine, as a marker compound, was screened using TLC and HPLC methods from methanolic crude extracts. The study revealed that three highest content of liriodenine were found in *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaf). These medicinal plants are not only accessible in Thailand, but they are also used in Thai traditional medicine; as a consequence, their pharmacognostic parameters were investigated for the quality standardization. These selected medicinal plants have been reviewed in literature as followings:

Michelia longifolia Blume

Michelia longifolia (syn. *M. alba* DC.), shown in Figure 5, belongs to the family Magnoliaceae and has been used in the traditional medicine of Southeast Asia. It is known in Thai as "Champi" and English as "White Champaca" [136-137]. *Michelia longifolia* has been used in traditional medicine in Malay Peninsula; an infusion of the flowering buds is given to women for sapremia following a miscarriage [138]. They are also employed in the manufacture of perfume.

Previously reported constitutents of the essential oil were linalool, nerol, limonene, benzyl acetate, hydroxycitronellal, benzaldehyde, benzyl benzoate, methyl eugenol and eugenol [139] along with the alkaloids ushinsunine, oxoushinsunine (liriodenine), salicifoline, and michelalbine [140]. In traditional Thai medicine, *Michelia longifolia* stem bark has been used as antipyretics [82]. Its flower has been used for fainting and fever treatment and also for heart and blood circulation system benefit [141]. The preliminary study on biological activity of *Michelia longifolia* stem bark found that its crude ethanolic extract exhibited strong cytotoxicity to KB-cell culture assay. The aqueous extract of *Michelia longifolia* exhibited antimicrobial activity [142].

Table 4 Chemical investigations of *Michelia longifolia* Blume

Plant part	Chemical substance	Category	Reference
Trunk bark, Root	Ushinsunine	Alkaloid (Aporpine)	143
	Liriodenine	Alkaloid (Oxoaporpine)	143
	Salicifoline	Alkaloid (Phenethylamine)	143
	Michelalbine	Alkaloid(Aporpine)	143
Flower	Acetaldehyde	Miscellaneous	144
	iso-Aristolene	Sesquiterpene	145
	Allocimene	Monoterpene	144
	Benzyl Acetate	Miscellaneous	146
	Benzyl Benzoate	Miscellaneous	146
	Benzaldehyde	Miscellaneous	146
	beta- Bisabolene	Sesquiterpene	145
	Butyric acid methyl ester	Miscellaneous	146 – 148
	Butyric acid,2-methyl-methyl ester	Miscellaneous	145
	Butyric acid,3-methyl-methyl ester	Miscellaneous	147
	Butyric acid,alpha-methyl methyl ester	Miscellaneous	144, 147
	iso-Butyric acid, methyl ester	Miscellaneous	147
	Buthyl pentanoate	Miscellaneous	144
	delta-Cadinene	Sesquiterpene	145
	Camphene	Monoteroene	145
	Car-3-ene	Monoterpene	145
	trans-Carveol	Monoterpene	145

Plant part	Chemical substance	Category	Reference
Flower	*cis*-Caryophyllene	Sesquiterpene	145
	1, 3-Cineol	Monoterpene	145
	alpha-Cubebene	Sesquiterpene	145
	beta-Cubebene	Sesquiterpene	145
	ortho-cymene	Monoterpene	144, 147
	Ethanol	Miscellaneous	144, 147
	Ethyl-α-methyl butyrate	Miscellaneous	144
	Eugenol methyl ether	Lignan	145
	iso-Eugenol methyl ether	Lignan	145
	Hydroxy Citronellol	Monoterpene	146
	Limonene	Monoterpene	144 – 146
	Linalool	Monoterpene	146, 148
	cis-Linalool oxide	Monoterpene	145
	trans-Linalool oxide	Monoterpene	145
	Methyl acetate	Miscellaneous	144
	Methyl benzoate	Miscellaneous	144
	Methyl-2-pentenoate	Miscellaneous	144
	Methyl hexanoate	Miscellaneous	144
	beta-Myrcene	Monoterpene	145
	Nerol	Monoterpene	146
	Ocimene	Monoterpene	144 – 145
	alpha-Phellandrene	Monoterpene	145
	beta-Phellandrene	Monoterpene	144
	beta-Pinene	Monoterpene	145
	Propionic acid methyl ester	Miscellaneous	144, 147
	beta-Selinene	Sesquiterpene	145
	Terpenolene	Monoterpene	144
	Undecane agarol	Monoterpene	144
	alpha-Ylangene	Sesquiterpene	145

Figure 5 Macroscopic characteristics of *Michelia longifolia* Blume

A) Whole plant, B) Twig with flowers and C) Fruits

Michelia champaca **Linn.**

Michelia champaca, shown in Figure 6, belongs to the family Magnoliaceae. This evergreen tree is native to South and Southeast Asia. It is best known as strongly fragrant yellow or white flowers. It is primarily cultivated for timber, and used in urban landscaping. Its aril-covered seeds are highly attractive to birds [149]. Common names include champaca, champak, sonchaaphaa or golden champa, champa, cempaka, sampenga, sampangi, sampige and shamba. All other names above were applied to plumeria varieties as well, except sonchaaphaa. Some half a dozen varieties of plumeria along with *Michelia champaca* (three varieties) and ylang ylang (two varieties) covered under the generic name chaaphaa in Marathi, and some given independent names ending in the generic chaaphaa.

Table 5 Chemical investigations of *Michelia champaca* Linn.

Plant part	Chemical substance	Category	Reference
Trunk bark	**Liriodenine**	Alkaloid (Oxoaporphine)	150 – 151
	Magnoflorine	Alkaloid (Aporphine)	150
	Ushinsunine	Alkaloid (Aporphine)	150
	β-sitosterol	Steroid	151
Root bark	Costunolide	Sesquiterpene Lactone	152
	Parthenolide	Sesquiterpene Lactone	152
	Dihydroparthenolide	Sesquiterpene Lactone	152
	Micheliolide	Sesquiterpene Lactone	152

The flowers are used in Southeast Asia for several purposes. They are primarily used for worship at temples whether at home or out and more generally worn in hair by girls and women as a means of beauty ornament as well as a natural perfume. Moreover, after globalization opening, flowers are used in many ways such as being floated in water bowl to scent the room, as a fragrant decoration for bridal beds, and for garlands [153].

The flower is the main scent which presented in the commercial "joy" perfume, and is sometimes commonly called the "joy perfume tree". The flowers are expectorant and useful in cough and rheumatism; the smell of the flowers is a good stimulant; the perfumed oil prepared from the flowers is considered cephalalgia, ophthalmia and gout [154]. The flowers and fruits are considered bitter and cool remedies, and are used in dyspepsia, nausea and fever. The flowers mixed with sesame oil form an external application, which is often prescribed in vertigo and also applied to foetid discharges from the nostrils. They are useful as a diuretic in renal diseases and in gonorrhea, stimulant, antispasmodic, tonic, stomachic and carminative [154].

The bark of *Michelia champaca* is bitter with a sharp acrid taste; causes warmth in the abdomen; destroys poisons; removes worms; facilitates maturation. Its properties compose of diuretic, diaphoretic, aphrodisiac, stimulant, expectorant, astringent and febrifuge [150, 154]. A decoction is administered as a part partum protective medicine. The leaves in combination with other drugs remove the odour of vaginal discharges and the juice of the leaves is given with honey in case of colic [150, 154]. The dried root and root-bark, mixed with curdled milk, is useful as an application to abscesses, clearing away or maturing the inflammation. Its infusion is used as emmenagogue and purgative. The seeds and fruits are useful for healing cracks in the feet [154].

In Thai traditional medicine, *Michelia champaca* has been used according to its part. In summary, for internal application, the leaf is used for neural disorders. The flower is used to treat fever, chronic fatigue and low immunity. It is also prescribed as a tonic for the heart, blood and nervous system. Both flower and fruit are diuretic, antiemetic, antipyretic, and considered to be general tonic for four elements. The stem bark is antipyretic. The wood is menstrual tonic. For topical application, flowers' decoction is applied in temple to relieve headache. Decoction of dried ground root in milk is applied for abscesses [155-156].

Figure 6 Macroscopic characteristics of *Michelia champaca* Linn.

A) Whole plant, B) Twig with flowers and C) Fruits

Nelumbo nucifera **Gaertn.** [157-159]

Nelumbo nucifera, shown in Figure 7, belongs to the family Nelumbonaceae, known by a common name in Thai as "Bua luang". Common name in English called as "Sacred lotus" or "Indian lotus". This plant is an aquatic perennial herb with stout, creeping rhizome. Under favorable circumstances its seeds may remain viable for many years, with the oldest recorded lotus germination being from that of seeds 1,300 years old recovered from a dry lakebed in northeastern China. Native to Tropical Asia and Queensland, Australia, it is commonly cultivated in water gardens. The white and pink lotuses are national flowers of India and Vietnam, respectively.
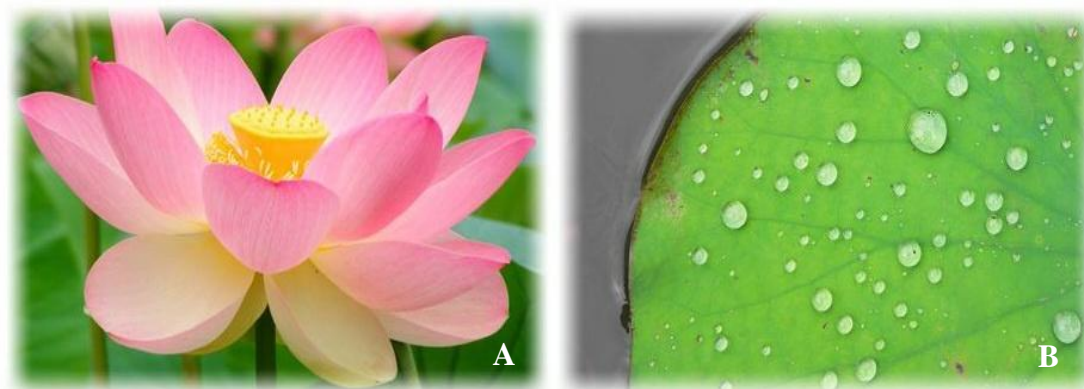
Figure 7 Macroscopic characteristics of *Nelumbo nucifera* Gaertn.

A) Flower and B) Leaf

It plays an important role in religious and cultural ceremonies. Many Thais believe its flowers are good for the heart, make people strong and alleviate body heat. Leaves can also be used as food, cigarette wrapper, herbal remedy and, when necessary, as an umbrella. Lotus is the foremost symbol of beauty, prosperity and fertility. Lotus flower has for thousands of year symbolized spiritual enlightenment. For centuries, sacred lotus is particularly valued for Thai medicinal properties and Thais treat lotus plants as part of their repertoire of medicinal herbs. Virtually all parts of the versatile plant including stamens, pollens, flowers, stems, tubers and leaves are used.

The leaf containing nuciferine like substance had antispasm action. The leaf extract and decoction showed direct vaso - dilatation action and moderate hypotensive effect

on experimental animals. Crystal liensinine isolated from the dried plumule of ripened seeds showed transient hypotensive effect and strong and prolonged action when changed into n-methyl-dendronbium while the non-crystal alkaloid Nn-9 had stronger hypotensive action with tachyphylaxis. The dried lotus stamen showed antibacterial effect. Their pollens are believed to help eliminate vertigo and dizziness. The tubers give one sweet taste and good flavor, and alleviate diarrhea in children. The green, bitter embryo of the seed is believed to reduce phlegm; thirst and body heat [160-169].

In brief, lotus leaf is a calming decoction that is said to benefit several organs and conditions that are characterized by what Chinese medicine calls "dampness accumulation". It also is a remedy that treats the stomach, spleen and liver, and to treat summer heat syndrome, which is characterized by overheating and sometimes accompanied by a heat rash. Lotus leaf is also effective against high blood pressure because of the tea's alkaloid content, according to the Institute of Traditional Medicine [170].

Nelumbo nucifera has already been investigated by many groups. Several alkaloids and some flavonoids have been isolated. Most alkaloids which included liensinine [164, 171-172], isoliensinine [180, 188], neferine [180, 189-192], lotusine [180], anonaine [182, 193], armepavine [182-183], asimilobine [178, 184], higenamine [185], lirinidine [184, 186] liriodenine [186-190], roemerine [177, 182, 187, 190], nuciferine [172, 182], dehydroroemerine, dehydronuciferine, dehydroanonaine, *N*-methylisococlaurine [182], pronuceferine [182, 191], *N*-nornuciferine, nornuciferine, *N*-methylcoclaurine [182] were isolated from embryo [192-194], leaf [172, 184], plumule [164] and seed [164, 192] while most flavonoids like quercetin [194-197], hyperoside, luteolin 7-glucoside, rutin, isoquercetrin, leucocyanidin, leucodelphinidin, luteolin, derivatives of quercetin were found in leaf, embryo, flower and seed [195]. Their interesting bioactivities such as antihypertensive [192], hypertropic scar fibroblast inhibiting [173], anti-depressive [178], serotonin antagonist [184], antiplatelet [186], CNS stimulant [198], anti-cancer [199], anti-HIV [172],

antispasmodic [200], anti-oxidative [180] and anti-microbial [179, 181, 201] activities were mentioned [202-207].

Quality control methods for medicinal plant materials [208-210]

WHO has developed a series of technical guidelines relating to quality control of herbal medicinal plants grown in commercial plantation. The following identification facilitates the methodology to monitor or to ensure raw material quality leading to product safety, efficiency and quality preparation.

- Macroscopic and microscopic examination

Medicinal plant materials are categorized according to sensory, macroscopic and microscopic characteristics. An examination to determine these characteristics is the first step towards establishing the identity and the degree of purity of such materials, and should be carried out before any further tests are undertaken. Visual inspection provides the simplest and quickest means by which to establish identity, purity and possibly, quality.

Macroscopic identity of medicinal plant materials is based in shape, size, colour, surface characteristics, texture, fracture characteristics and appearance of the cut surface. Microscopic inspection is indispensable for the identification of broken or powdered materials; the specimen may have to be treated with chemical reagents.

- Determination of foreign matter

Medicinal plant materials should be entirely free from visible signs of contamination by moulds or insects, and other animal contamination, including animal excreta. Macroscopic examination can conveniently be employed for determining the presence of foreign matter in whole or cut plant materials. However, microscopy is indispensable for powdered materials.

Any soil, stones, sand, dust and other foreign inorganic matter must be removed before medicinal plant materials are cut or ground for testing. Foreign matter is material consisting of any or all of the following:

- parts of the medicinal plant material or materials other than those named with the limits specified for the plant material concerned;
- any organism, part or product of an organism, other than that named in the specification and description of the plant material concerned;
- mineral admixtures not adhering to the medicinal plant materials, such as soil, stones, sand, and dust.

- Determination of total ash

This method is designed to measure the total amount of material remaining after ignition. In addition, ashing involves an oxidation of components of the product. A high ash value is indicative of contamination, substitution, adulteration or carelessness in preparing the crude drug for marketing.

- Determination of acid-insoluble ash

Acid-insoluble ash is the residue obtained after boiling the total ash with 2N hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

- Determination of solvent extractive values

This method determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material. It is employed for materials for which as yet no suitable chemical or biological assay exists.

- Determination of loss on drying

An excess of water in medicinal plant materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. The test determines both water and volatile matter. Drying can be carried out either by heating to 100-105°C or in a desiccator under atmospheric or reduced pressure at room temperature for a specified period of time.

- Determination of water content

The preparation of crude drug from the harvested drug plants involves cleaning or garbling to remove soil or other extraneous material followed by drying which play a very important role in the quality as well as purity of the material. Furthermore, the moisture requirements for the active growth of some of the common molds and bacteria that may be found in or on drugs are relatively low. Thus, the drying process should reduce the moisture content of the drug below this critical, or threshold level (mean value from the examination).

- Determination of volatile oil content

Volatile oils are characterized by their odour, oil-like appearance and ability to volatilize at room temperature. In order to determine the volume of oil, the plant material is distilled with water and the distillate is collected in a graduated tube. The aqueous portion separates automatically and is returned to the distillation flask. If the volatile oils possess a mass density higher than or near to that of water, or are difficult to separate from the aqueous phase owing to the formation of emulsions, a solvent with a low mass density and a suitable boiling-point may be added to the measuring tube. The dissolved volatile oils will then float on top of the aqueous phase.

- Thin layer chromatographic fingerprint

Thin-layer chromatography is particularly valuable for the qualitative determination of chemical characteristics of plant materials. The principles of thin-layer chromatography and application of the technique in medicinal plant researches are described below:

TLC is a basic tool for identification of natural compounds given in various pharmacopoeias. It is often used to provide the first characteristic fingerprint of herbs. TLC analysis is mostly performed on silica gel as stationary phase. Different compounds in the sample travel at different rates due to the differences in their attraction to the stationary phase, and because of differences in solubility in the solvent. The specific retardation factor (R_f) of each chemical can be used to aid in the

identification. By changing the solvent, or mixture, the separation of components (measured by the R$_f$ value) can be adjusted to get the better fingerprint [211].

The advantages of using TLC to construct the fingerprint of herbal medicine are its simplicity, versatility, high velocity, specific sensitivity, simple preparation and time-saving method. Hence, this method is convenient for determination the quality and possible adulteration of herbal products [212].

CHAPTER III

MATERIALS AND METHODS

Materials

Company, country of product

Beaker and other scientific glass materials	Pyrex, Germany and USA
Drawing paper (200 g)	Masterart, Thailand
Filter paper grade No.4	Whatman, Englang
Filter paper grade No.40, Ashless	Whatman, England
Filtration membrane 0.45µm	Millipore corporation, USA
HPLC syringe	Hamilton, Switzerland
Microscope cover slips	Menzel-Glaser, USA
Microscope slide	Sail brand, China
Parafilm	Brand, Germany
Pipettes	Transferpette, Germany
TLC silica gel 60 GF$_{254}$ (0.25 mm thickness, 20 x 20 cm)	Merck, Germany

Chemicals and reagents

Company, country of product

Acetonitrile (HPLC grade)	RCI Labscan, Thailand
Anisaldehyde	Sigma-Aldrich, Germany
Chloroform (HPLC grade)	J.T. Baker Chemical, USA
Diethylamine (HPLC grade)	RCI Labscan, Thailand
Ethanol	Thailand
Formic acid (Analytical grade)	Fisher Scientific, England
Glacial acetic acid (Analytical grade)	BDH Chemicals, England
Hydrochloric acid (Analytical grade)	RCI Labscan, Thailand
Methanol (HPLC grade)	J.T. Baker Chemical, USA
Sulfuric acid (Analytical grade)	BDH Chemicals, England
Toluene (Analytical grade)	RCI Labscan, Thailand
Trifluoroacetic acid	BDH Chemicals, England

Equipments and instruments

<div align="right">Company, country of product</div>

Ashing furnace	Carbolite, England
Azeotropic distillation	Buchi Glas Uster, Switzerland
Centrifuge	Thermo scientific, England
Digital camera (power shot A640)	Canon, Japan
Filtration instrument for HPLC	Millipore corporation, USA
Hot air oven	WTC Binder tuttlingen, Germany

HPLC-DAD instrument

HPLC column   Inertsil, Japan

- ODS-3, C-18, particle size of the packing 5µm, 4.6 x 250 mm

HPLC computerize system   Hewlett-Packard (HP), USA

HPLC-DAD instrument (Gradient system)   Shimadzu, Japan

- Communications Bus Module (CBM-20A)
- HPLC auto-sampler (SIL-20A HT)
- HPLC DAD detector (SPD-M20A)
- HPLC column oven (CTO-20AD)
- HPLC pumps (LC-20AD)
- Prominence degasser (DGU-20A$_3$)

HPLC-UV instrument (Isocratic system)   Shimadzu, Japan

- HPLC pump (LC-20AD)
- HPLC UV/VIS detector (SPD-20A)

Microscope	Zeiss, Germany
NMR Spectrometer	Varian, Inova, USA
pH meter	Denver instrument, USA
Rotary evaporation instrument	Buchi Glas Uster, Switzerland
Scanner	Hewlett-Packard (HP), USA
Scion image software	Scion Corporation, Maryland, USA

Equipments and instruments

Company, country of product

Shaker	Adolf Kuhner AG, Switzerland
Soxhlet apparatus	Buchi Glas Uster, Switzerland
TLC Chamber	Camag, Switzerland
Ultrasonic bath	Analytical Lab Science, Thailand
Ultraviolet fluorescence analysis cabinet	Spectronics Corporation, USA
Vortex	Scientific industries, USA
Water bath	Brinkmann, USA
Weighing analytical and precision balance	Ohaus, USA

Standard liriodenine

Standard liriodenine was purchased from Specs, Kluyverweg 6, 2629 HT Delft, The Netherlands (Lot no. AK-693/21087012), and then reconfirmed by NMR. The compound was prepared in deuterated chloroform. The procedure was conducted at Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University. The purity of this compound was 100% by LC-MS.

Preparation of standard solutions

The stock solution of liriodenine was prepared by dissolving accurately weighed 2 mg in 2 mL of methanol to obtain final concentration of 1 mg/mL. Standards in the range of 5-200 µg/mL of concentration were freshly prepared prior to analysis.

Instrumentation and chromatographic conditions

Liriodenine determination was analyzed using TLC image and HPLC. Both methods' conditions and system instrumentations were described as below:

- **Determination of liriodenine by TLC image analysis**

TLC separation was performed on a pre-coated silica gel 60 F_{254} plate. The extracted sample was dissolved in methanol. After applying a 2 µL of each extract solution onto TLC plates, they were firstly developed using 100% methanol of distance of 6 mm to expand the band length. Ensuring air-drying, the plates then were developed with

various mobile phase. The suitable one was using chloroform and methanol (9:1) to a distance of 80 mm; then dried and visualized the plate under UV 365 nm. An image of TLC chromatogram was taken using digital camera (Canon PowerShot A650) that was set at UV cabinet. The distance from camera lens to TLC plate, when capturing the picture, was controlled at 52 cm. The picture was taken with TV mode, ISO 200, shutter speed 3 s and saved as .tiff format. The image file was opened with Scion image for Windows (Version Alpha 4.0.3.2, Scion Corporation, Maryland, USA). After importing images, they were resized with scale to fit windows mode and modified grayscale selection with 10 times smoothing to reduce noises of image. Using the rectangular selection tool and load macros command to create the plot profile, then the areas and gray value of the selection were measured as square pixels [131, 213]. Calibration curve was constructed with least square regression analysis between peak area and standard concentration.

- **Determination of liriodenine by HPLC**

Choosing the optimum mobile-phase polarity is important by using a mixture of liquids, the polarity can be adjusted to the necessary level. Chromatography was performed with a Shimadzu (Japan) series. Compounds separated in C-18 packed column and run in the appropriate HPLC condition. All analyses were performed under same conditions at room temperature, demonstrating chromatogram with retention time. All measurement was done in triplicate. The peak area was used to calculate an amount of liriodenine. There were two detectors, UV detector and DAD, with different instrumentations used in this study for HPLC system.

❖ **HPLC quantitation using UV-detector**

HPLC was performed with an isocratic system (A separation in which the mobile phase composition remains constant throughout the procedure) [214] with UV detector set at λ_{max} 248 nm. Separation was carried out with a C-18 column as described above. The mobile phase consists of a mixture of methanol and water at 70:30 ratio and adjusted to pH 3 with trifluoroacetic acid. After that, filtered with filtration membrane 0.45 μm of pore size; then degassed with ultrasonic bath prior to use. All methanolic extracts were

filtered through 0.45 μm syringe filter. The injection was performed manually with 20 μL of each sample at flow rate of 1 mL/min [139]. There were no controlled oven temperature and degasser in this HPLC-UV system.

❖ **HPLC quantitation using diode array-detector**

A gradient HPLC system (A separation in which the mobile phase composition is changed during the separation process) [214] using a diode array detector (DAD) was set λ in the range of 190-800 nm. The chromatographic separation was performed on a column as described above with column temperature set at 25ºC. A linear gradient elution of A (formate buffer consisting of 1% formic acid, adjusted to pH 4.5 with diethylamine) and B (100% acetonitrile) was applied from 60:40 to 40:60 over 35 min [109]. Each solution was prepared as mentioned before and injected automatically at volume 10 μL with 1 mL/min of flow rate.

Method Validation

To validate quantitative methods before quantitative analysis, linearity, specificity, accuracy, precision (repeatability and intermediate precision), detection and quantification limits were evaluated according to ICH guidelines specification [135].

- **Linearity and calibration curve**

It was estimated by regression line; correlation coefficient (R^2), y-intercept and slope. Standard solutions of liriodenine were prepared in methanol. Aliquots of each solution were spotted (2 μL) onto a TLC plate to obtain the concentrations of standard curve which were analyzed using the least square regression equation derived from peak area [131]. In addition to HPLC analysis, standard solutions were also prepared in methanol to provide serial concentration. Linear regression calculated from peak area was employed in the construction of the calibration curve. Final concentrations of standard liriodenine from both methods were at 5, 25, 50, 100 and 200 μg/mL that was the interval between the upper and lower levels of analyte concentration.

- **Specificity**

Specificity was evaluated by HPLC-DAD (190-800 nm) of the detected peaks with samples and standard liriodenine by indicating at same retention time, peak purity index and peak spectra which is a compound characteristic. While TLC required to show a clear and good separated spot at same Rf.

- **Precision and accuracy**

Referring to developed TLC and HPLC method for liriodenine determination, precision was determined by repeatability (intra-day) and intermediate precision (inter-day) for 3 consecutive days. Three different concentrations of liriodenine, within the range of 5-200 µg/mL, were analyzed in six determinations each. Every sample was done in triplicate. Data were expressed as relative standard deviation (%RSD) as below:

$$\% \, RSD \; = \; \frac{Standard \; deviation \; (SD)}{Mean} \; x \; 100$$

Accuracy was evaluated as % Accuracy for liriodenine in addition to the equation:

$$\% \; Accuracy \; = \; \frac{Observed \; concentration}{Nominal \, concentration} \; x \; 100$$

Furthermore, accuracy were accessed on blank sample (sample without studied compound) spiked with known amount of liriodenine. Each spiked sample was done in triplicate and the amount of recovery was calculated as below:

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

A = Amount of compound found in spiked sample

B = Amount of compound in sample

C = Amount of compound added

Additionally, due to the later changed HPLC system for the better outcome, this study revealed the comparison of the results between HPLC-UV and HPLC-DAD analysis to confirm the accuracy when using different instrument and condition for liriodenine determination. Both methods were statistically compared by Student paired *t*-test.

- **Limit of detection (LOD) and limit of quantification (LOQ)**

According to TLC image analysis, LOD and LOQ were calculated based on the standard deviation (σ) of the y-intercept and the slope (*s*) as $3\sigma/s$ and $10\sigma/s$ respectively. The calculation obtained from the linear regression in low concentrations of liriodenine.

Otherwise, HPLC method, LOD and LOQ values were analyzed by signal to noise from the instrument. As a result of using dual wavelength to identity liriodenine peak, LOD and LOQ were accessed and compared between 248 nm and 407 nm.

Sample collection

Sample collection for liriodenine content in liriodenine bearing plants endemic to Thailand

Twenty-eight plant materials selected from the families of Magnoliaceae, Annonaceae and Nelumbonaceae, according to their accessibility throughout Thailand were collected to investigate the liriodenine content (Table 6).

Table 6 Sampling of liriodenine bearing plants endemic to Thailand

Family	Thai name (central) [137]	Scientific name [137]	Part	No.
Magnoliaceae	Champi	*Michelia longifolia* Blume	Leaf	1
	Champi	*Michelia longifolia* Blume	Bark	2
	Champa	*Michelia champaca* Linn.	Leaf	3
	Champa	*Michelia champaca* Linn.	Bark	4
	Champi khaek	*Magnolia figo* (Lour.)	Leaf	5
	Champi sirinthon	*Magnolia sirindhorniae* Noot. & Chalermglin	Leaf	6
	Champi sirinthon	*Magnolia sirindhorniae* Noot. & Chalermglin	Bark	7
	Montha	*Magnolia liliifera* (L.) Baill. Var. *liliifera*	Leaf	8
	Montha	*Magnolia liliifera* (L.) Baill. Var. *liliifera*	Bark	9
	Yihup nu	*Magnolia coco* (Lour.) DC.	Leaf	10
Annonaceae	Kradang nga thai	*Cananga odorata* (Lam.) Hook.f.&Thomson var. *odorata*	Leaf	11
	Kradang nga songkhla	*Cananga odorata* (Lam.) Hook.f.&Thomson var. *fruticosa* (Craib)	Leaf	12
	Nom meaw	*Rauwenhoffia siamensis* Scheff.	Leaf	13
	Champoon	*Anaxagorea javanica* Blume	Leaf	14
	Champoon	*Anaxagorea javanica* Blume	Bark	15
	Lamduan	*Melodorum fruticosum* Lour.	Leaf	16
	Lamduan	*Melodorum fruticosum* Lour.	Bark	17
	Karawek (Kradungnga cheen)	*Artabotrys hexapetalus* (L.f.) Bhandari	Leaf	18
	Saiyood	*Desmos chinensis* Lour.	Leaf	19
	Nom woa (north)	*Anomianthus dulcis* (Dunn) J. Sinclair	Leaf	20
	Kao lam	*Goniothalamus macanii* Craib	Leaf	21
	Noina	*Annona squamosa* L.	Leaf	22
	Noina	*Annona squamosa* L.	Bark	23
	Noi nong	*Annona reticulata* L.	Leaf	24
	Noi nong	*Annona reticulata* L.	Bark	25
	Thurain thet	*Annona muricata* L.	Leaf	26
	Nang daeng (Porkeehad)	*Mitrephora maingayi* Hook f. & Thomson	Leaf	27
Nelumbonaceae	Bua luang	*Nelumbo nucifera* Gaertn.	Leaf	28

Sample collection of selected medicinal plant materials for pharmacognostic specification

Three species containing high liriodenine content were collected from 15 habitats throughout Thailand during February to October in 2010 for quality assessment.

The bark of *Michelia longifolia* Blume

Collected place (n):

Yasothon (2), Phetchabun (1), Chiang Rai (1), Uthai Thani (1), Chanthaburi (1), Rayong (2), Buri Ram (2), Prachin Buri (1), Lampang (2), Nakhon Ratchasima (1), Nong Khai (1)

The bark of *Michelia champaca* Linn.

Collected place (n):

Nakhon Pathom (2), Phetchabun (2), Chanthaburi (1), Rayong (2), Lampang (1), Bangkok (1), Uthai Thani (1), Buri Ram (2), Nong Khai (2), Samut Prakan (1)

The leaves of *Nelumbo nucifera* Gaertn.

Collected place (n):

Lampang (2), Uthai Thani (1), Buri Ram (2), Chanthaburi (1), Nakhon Ratchasima (2), Phetchabun (1), Yasothon (1), Bangkok (1), Prachin Buri (1), Rayong (1), Nakhon Pathom (1), Nong Khai (1)

All samples were collected from natural sources and authenticated by the expert as well as national herbarium comparison. Samples were cleaned from soil, dust and any foreign materials and kept for further analyses. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Ethanolic extract preparation and liriodenine quantification

Each authentic plant material was air dried and ground to coarse powders. Approximately 10 g of plant materials were extracted successively with 95% ethanol in a Soxhlet apparatus and then evaporated the solvent on water bath until leftover

residue. The percentage yield of crude ethanolic extracts was calculated as this formula:

$$\% \text{ Yield } = \frac{\text{Weight of the extract}}{\text{Weight of plant material}} \times 100$$

The liriodenine content in ethanolic extract was determined by validated TLC image analysis and HPLC-DAD method as mentioned above. The liriodenine content was expressed as gram per 100 gram of dried plant material.

Crude drug quality analysis methods [209-210]

Macroscopic and microscopic examination

Size, colour and other visual inspection were identified for macroscopic examination. The morphology of each medicinal plant was botanically pictured by hand drawing and scaled comparing to the actual size.

Microscopic inspection was performed with labeled drawing including: anatomical characteristic of selected plant parts examined in transverse section by a razor blade, and histological characteristic examined in ground powder materials, sifted through a 250 micro sieve. The inspection was done under microscope with a magnification of 4x, 10x, 20x and 40x, compared the scale with the 0.01 mm micrometer.

Determination of total ash

Three grams of the ground sample were accurately weighted and placed in a tarred crucible. The sample was spread in an even layer and incinerated by gradually increasing the heat to 500-600°C until white, then cooled in a desiccator and weighted without delay.

Determination of acid-insoluble ash

To the crucible containing the total ash, added 25.0 mL of hydrochloric acid (70 g/L), then covered with a watch-glass and boiled gently for 5 minutes. Rinsed the watch glass with 5 mL of hot water and added this liquid to the crucible. The insoluble matter was collected on an ashless filter paper and washed with hot water until the

filtrate was neutral. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and incinerated to constant weight. After the residue was cooled in desiccators, and then weighted without delay.

Determination of solvent extractive values

Determination of ethanol-soluble extractive value

Five grams of the ground sample were macerated with 100.0 mL of 95% ethanol in a closed conical flask for 6 hours in shaking bath then for 18 hours in standing. They were filtered rapidly to avoid loss of ethanol. Twenty milliliter of the filtrate were evaporated to dryness in a tarred small beaker and dried with heat to constantly weight.

Determination of water-soluble extractive value

Five grams of the ground sample were macerated with 100.0 mL of distilled water in a closed conical flask for 6 hours in shaking bath then for 18 hours in standing. They were filtered rapidly to avoid loss of distilled water. Twenty milliliter of the filtrate were evaporated to dryness in a tarred small beaker and dried with heat to constantly weight.

Determination of loss on drying

Five grams of the ground sample were weighted in a tarred small crucible and dried with heat to constantly weight.

Determination of water content

Fifty grams of the ground sample were added with 200.0 mL of water-saturated toluene, then distilled by azeotropic distillation. As soon as water was completely distilled, rinsed the inside of the condenser tube with toluene and continued the distillation for 5 more minutes. The receiving tube was cooled to room temperature; showing the water and toluene layers separation, and then read off the volume of water.

Determination of volatile oil content

A hundred gram of the ground sample added with 600.0 mL of water was distilled by Clevenger apparatus. When volatile oil was completely distilled and the receiving tube was cooled to room temperature, read off the volume of volatile oil which separated from water layers.

Thin-layer chromatographic identification

The extracted sample was dissolved in methanol (20 mg/mL); then applied 5 μL to a pre-coated silica gel 60 F_{254} plate. The chromatogram was developed in the saturated TLC chamber with the solvent systems as chloroform and methanol (with the ratio of 9:1 for *Michelia longifolia*, *Michelia champaca* and *Nelumbo nucifera* extracts). Then removed the plate and allowed it to dry in air. The produced spots were observed in daylight, under short wave (254 nm) and long wave (365 nm) ultraviolet light and sprayed the spots with anisaldehyde reagent (prepared with anisaldehyde 0.5 mL, glacial acetic acid 10 mL, methanol 85 mL and sulfuric acid 5 mL) with heating the plate at 120ºc for 10 minutes after spraying.

Data Analysis

Quality parameters were conducted as grand mean ± pooled standard deviation (SD). Calibration curve was analyzed using regression equation. Liriodenine content between 2 methods: HPLC and TLC Image were statistically compared using Student's paired *t*-test.

CHAPTER IV

RESULTS

The present chapter divided into 3 sections: first part presents method validation of TLC image as well as HPLC analyses to demonstrate whether the developed methods are suitable for the intended purposes. Second part is the preliminary study for quantitative analysis of liriodenine bearing plants endemic to Thailand by TLC image analysis and HPLC. Later three medicinal plant parts were selected for further standardization according to their highest amount of liriodenine containing. Third part then reveals pharmacognostic study of *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaf) as well as their determinations of liriodenine content from both quantitative analyses.

Standard liriodenine was confirmed by NMR. The spectra were obtained in $CDCl_3$ with chemical shifts expressed in δ and coupling constant (J) in Hertz, showing very similar to literature [215] as ^{1}H-NMR (500 MHz) δ: 8.90 (1H, *d*, *J* = 5.5 Hz, H-5), 8.67 (1H, *d*, *J* = 8.0 Hz, H-11), 8.60 (1H, *d*, *J* = 8.0 Hz, H-8), 7.76 (1H, *d*, *J* = 7.5 Hz, H-10), 7.59 (1H, *t*, *J* = 15.5 Hz, H-9), 7.20 (1H, *s*, H-3) 6.38 (2H, *s*, $-OCH_2O-$), ^{13}C-NMR (125 MHz, $CHCl_3$): 180.0 (C-7), 152.5 (C-2), 147.3 (C-1), 146.4 (C-3a), 145.3 (C-5), 135.1 (C-6a), 134.0 (C-10), 132.8 (C-11a), 131.1 (C-7a), 129.1(C-9), 128.4 (C-8), 127.4 (C-11), 124.1 (C-4), 123.3 (C-1b), 103.3 (C-3), 102.0 ($O-CH_2-O$).

Method Validation

As new method was developed to find an easy and simple tool for liriodenine determination, thus it was necessary to access the quality in comparison with the standard method. To understand how the analytical performance changes between methods, it is common to compare the validation parameters; specificity, accuracy, precision, sensitivity and linearity. Therefore, this study compared a TLC image analysis by Scion image software to the HPLC-DAD method (reference method) for the simultaneous determination of liriodenine.

Linearity and calibration curve

Final concentrations of standard liriodenine from both methods were at 5, 25, 50, 100 and 200 µg/mL that was the interval between the upper and lower levels of analyte concentration. Linearity was constructed by at least square regression as illustrated in Figure 8 – 9. The correlation coefficient (R^2) for standard calibration curves of liriodenine from HPLC-DAD and TLC image analysis were 0.999 and 0.9995 respectively.
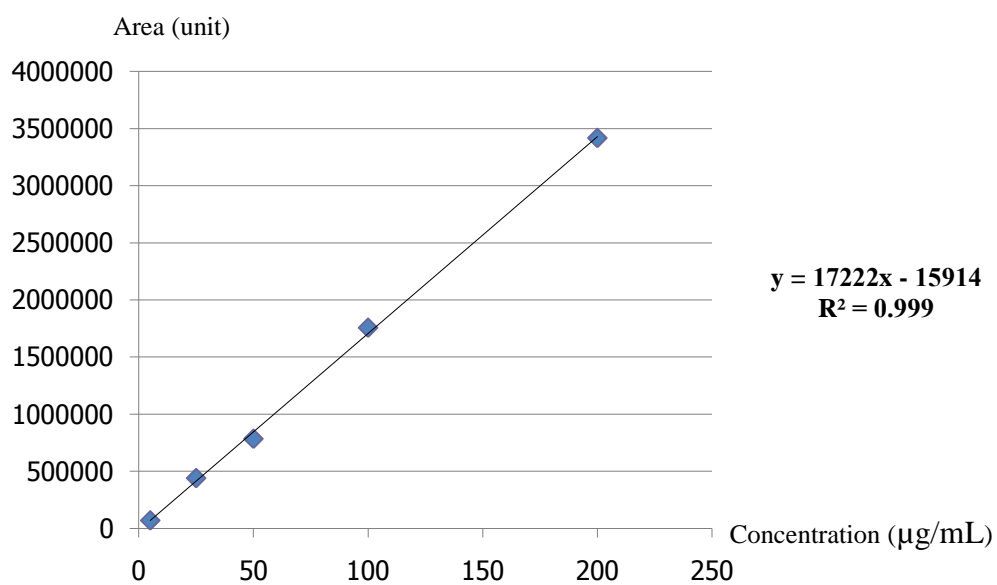
Figure 8 Calibration curve of liriodenine from HPLC-DAD method

$$y = -0.1573x^2 + 61.392x + 151.38$$
$$R^2 = 0.9995$$
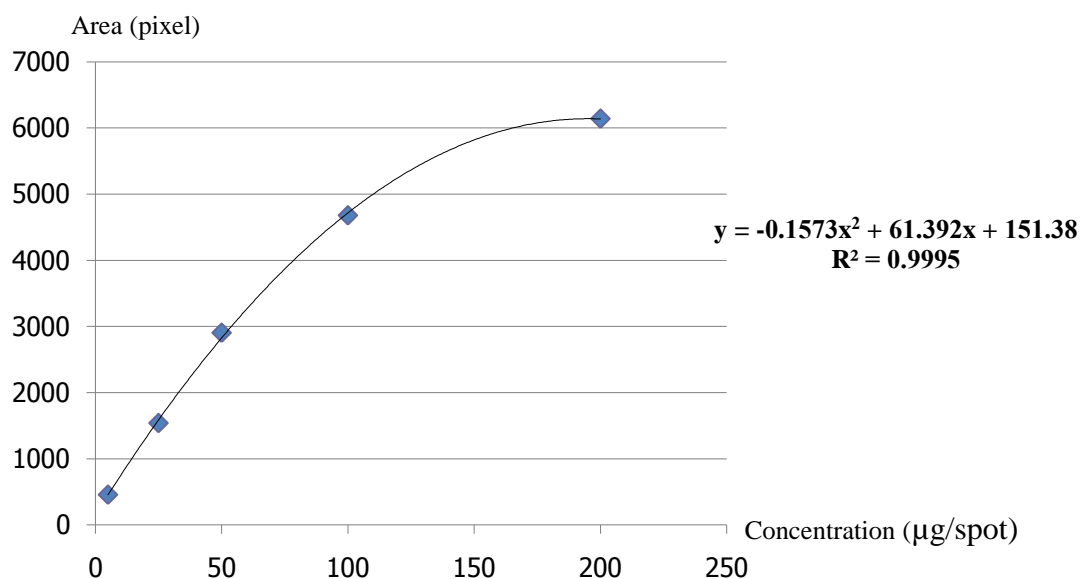
Figure 9 Calibration curve of liriodenine from TLC image analysis

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

According to HPLC-DAD, the LOD and LOQ was obtained from signal to noise over the range of 5-200 μg/mL. The results demonstrated in 248 nm and 407 nm due to using dual wavelength to identity the liriodenine peak. In addition to TLC image analysis, the low concentration range 5-50 μg/spot of liriodenine were calculated, giving $y = 55.577x + 186.28$ ($R^2=1$). The calculation of LOD and LOQ were 3 x SD and 10 x SD above y-intercept, respectively. Table 7 shows the LOD and LOQ from HPLC-DAD and TLC image analysis.

Table 7 Linearity and sensitivity for liriodenine determined using HPLC-DAD and TLC image analysis

Method	HPLC-DAD		TLC image analysis
Slope	17222.1		55.57
Y-intercept	-15914.4		186.28
R^2	> 0.995		> 0.995
	248 nm	**407 nm**	**365 nm**
LOD	0.816 μg/mL	0.994 μg/mL	3.767 μg/spot
LOQ	2.472 μg/mL	3.013 μg/mL	12.557 μg/spot

Precision and accuracy

Table 8 - 9 perform precision and accuracy for standard liriodenine during the intra- and inter-day. The data were expressed as % RSD for precision and % Accuracy for accuracy. Three concentrations were done in 6 determinations each in 3 consecutive days.

The recoveries in percentage of liriodenine spiked in specimen without this compound were analyzed in 3 concentrations which done in triplicate for both quantitative methods: TLC image analysis by Scion and HPLC-DAD as shown in Table 10.

Furthermore, two systems of HPLC showed consistency, there was no significant difference between liriodenine content of three selected medicinal plants determined by 2 HPLC-detector methods (Table 11).

Table 8 Intra-day precision and accuracy of liriodenine determined by HPLC-DAD and TLC image analysis

Nominal concentration	Repeatability (n=6)			
HPLC-DAD (µg/mL)	Day	Mean ± SD	% RSD	% Accuracy
25	1	25.75 ± 0.17	0.68	103.02 ± 0.70
	2	25.37 ± 0.15	0.59	101.46 ± 0.60
	3	25.60 ± 0.08	0.29	102.38 ± 0.30
50	1	49.28 ± 0.13	0.26	98.57 ± 0.26
	2	50.17 ± 0.07	0.14	100.34 ± 0.14
	3	50.20 ± 0.06	0.13	100.40 ± 0.13
100	1	101.33 ± 0.07	0.07	101.33 ± 0.07
	2	101.87 ± 0.05	0.05	101.87 ± 0.05
	3	101.06 ± 0.17	0.17	101.06 ± 0.17
TLC image (µg/spot)	Day	Mean ± SD	% RSD	% Accuracy
25	1	22.95 ± 0.66	2.89	91.79 ± 2.66
	2	23.56 ± 0.53	2.27	94.25 ± 2.14
	3	24.58 ± 0.96	3.89	98.31 ± 3.82
50	1	53.06 ± 0.33	0.62	106.11 ±0.65
	2	49.69 ± 0.91	1.83	99.37 ± 1.82
	3	49.00 ± 1.68	3.43	97.99 ± 3.36
100	1	99.89 ± 2.86	2.86	99.89 ± 2.86
	2	98.43 ± 1.02	1.04	98.43 ± 1.02
	3	102.19 ± 1.20	1.17	102.19 ± 1.20

Table 9 Inter-day precision and accuracy of liriodenine determined by HPLC-DAD and TLC image analysis

Nominal concentration	Inter-day (3 days, n=18)		
HPLC-DAD (µg/mL)	Mean ± SD	%RSD	% Accuracy
25	25.57 ± 0.20	0.82	102.29 ± 0.84
50	49.89 ± 0.45	0.90	99.77 ± 0.89
100	101.42 ± 0.36	0.36	101.42 ± 0.36
TLC image (µg/spot)	Mean ± SD	%RSD	% Accuracy
25	23.70 ± 0.98	4.14	94.78 ± 3.92
50	50.58 ± 2.11	4.16	101.16 ± 4.21
100	100.17 ± 2.38	2.38	100.17 ± 2.38

Table 10 Recovery of liriodenine determined by HPLC-DAD and TLC image analysis

Method	Initial amount (µg/mL)	Added (µg/mL)	Found (µg/mL)	Recovery (%)	Mean ± SD (%)	RSD (%)
HPLC-DAD	0.012	12.50	11.80	94.32	94.81 ± 0.65	0.62
			11.83	94.56		
			11.96	95.55		
		25.00	24.47	97.83	98.14 ± 0.30	0.29
			24.56	98.17		
			24.62	98.42		
		50.00	52.48	104.94	105.28 ± 0.30	0.32
			52.76	105.49		
			52.72	105.42		
Method	**Initial amount (µg/spot)**	**Added (µg/spot)**	**Found (µg/spot)**	**Recovery (%)**	**Mean ± SD (%)**	**RSD (%)**
TLC image	0.000	12.50	11.76	94.08	101.12 ± 6.71	6.78
			13.43	107.44		
			12.73	101.84		
		25.00	25.43	101.72	101.97 ± 3.39	3.45
			26.37	105.48		
			24.68	98.72		
		50.00	50.63	101.26	103.09 ± 2.03	2.10
			51.37	102.74		
			52.64	105.28		

Sample without liriodenine was spiked with standard at 3 concentrations. Each spiked sample was done in triplicate.

Table 11 Accuracy of liriodenine content (%w/w) in *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaves) determined by HPLC-DAD method compared to HPLC-UV method

Sources	*Michelia longifolia* (bark)		*Michelia champaca* (bark)		*Nelumbo nucifera* (leaves)	
no.	HPLC-DAD	HPLC-UV	HPLC-DAD	HPLC-UV	HPLC-DAD	HPLC-UV
1	0.0056	0.0046	0.0226	0.0240	0.0033	0.0040
2	0.0026	0.0020	0.0934	0.0724	0.0073	0.0121
3	0.0027	0.0022	0.0119	0.0128	0.0034	0.0023
4	0.0490	0.0322	0.0069	0.0062	0.0069	0.0099
5	0.0244	0.0146	0.0368	0.0369	0.0009	0.0009
6	0.0158	0.0118	0.0089	0.0086	0.0016	0.0031
7	0.0287	0.0180	0.0180	0.0177	0.0025	0.0033
8	0.0434	0.0262	0.0933	0.0719	0.0084	0.0093
9	0.0104	0.0098	0.0068	0.0096	0.0055	0.0051
10	0.0065	0.0059	0.0368	0.0283	0.0022	0.0024
11	0.0039	0.0080	0.0041	0.0034	0.0066	0.0077
12	0.0043	0.0060	0.0473	0.0242	0.0085	0.0096
13	0.0051	0.0076	0.0112	0.0106	0.0110	0.0148
14	0.0219	0.0253	0.0171	0.0146	0.0024	0.0028
15	0.0044	0.0065	0.0203	0.0346	0.0021	0.0077
Mean	0.0153	0.0120	0.0290	0.0250	0.0049	0.0063
Paired *t*-test	*p>*0.05		*p>*0.05		*p>*0.05	

Each batch was done in triplicate. Each medicinal plant was collected from 15 sources. There was no significant difference between the results of both quantitative methods ($p>0.05$) as analyzed by Student's paired *t*-test.

Specificity

In addition to HPLC-DAD, dual wavelength at 248 nm and 407 nm was optimum for liriodenine detection. Specificity was evaluated by 3 criteria: the peak should be shown in both wavelengths. Then the retention time (T_R) of that peak should be as same as T_R of standard liriodenine peak as shown in chromatograms in Figure 11. Finally, peak purity index (Table 12) and peak spectra (Figure 10) were observed to indicate the compound. While TLC required to show a clear and good separated spot at same Rf of liriodenine. Figure 12 demonstrated the process of TLC image analyzed by Scion image software for liriodenine determination.

Table 12 Specificity of HPLC-DAD method

Wavelength (nm)	Peak purity index	Single point threshold
248	0.99850	0.99936
407	0.99833	0.99935

Standard liriodenine at concentration of 50 µg/mL. High purity was indicated as the value close to 1.
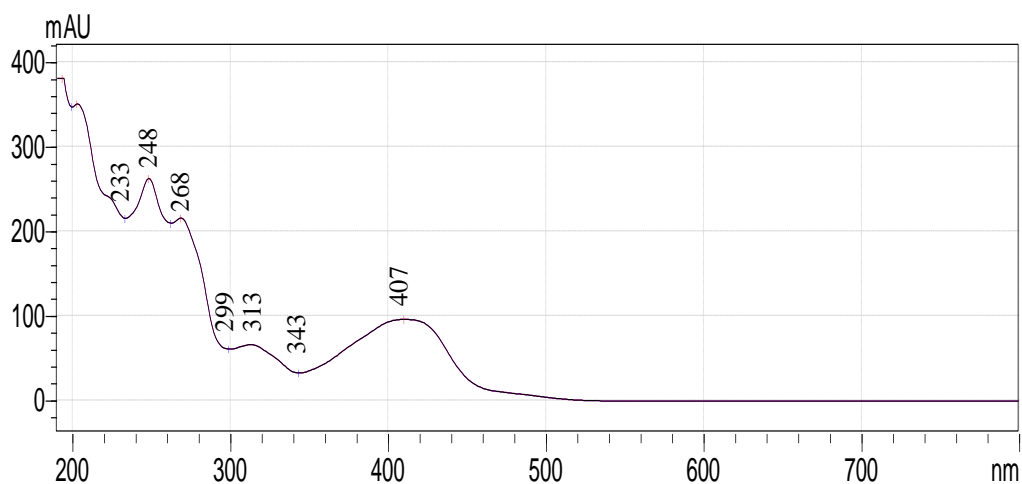
Figure 10 The peak spectra of standard liriodenine detected by HPLC-DAD
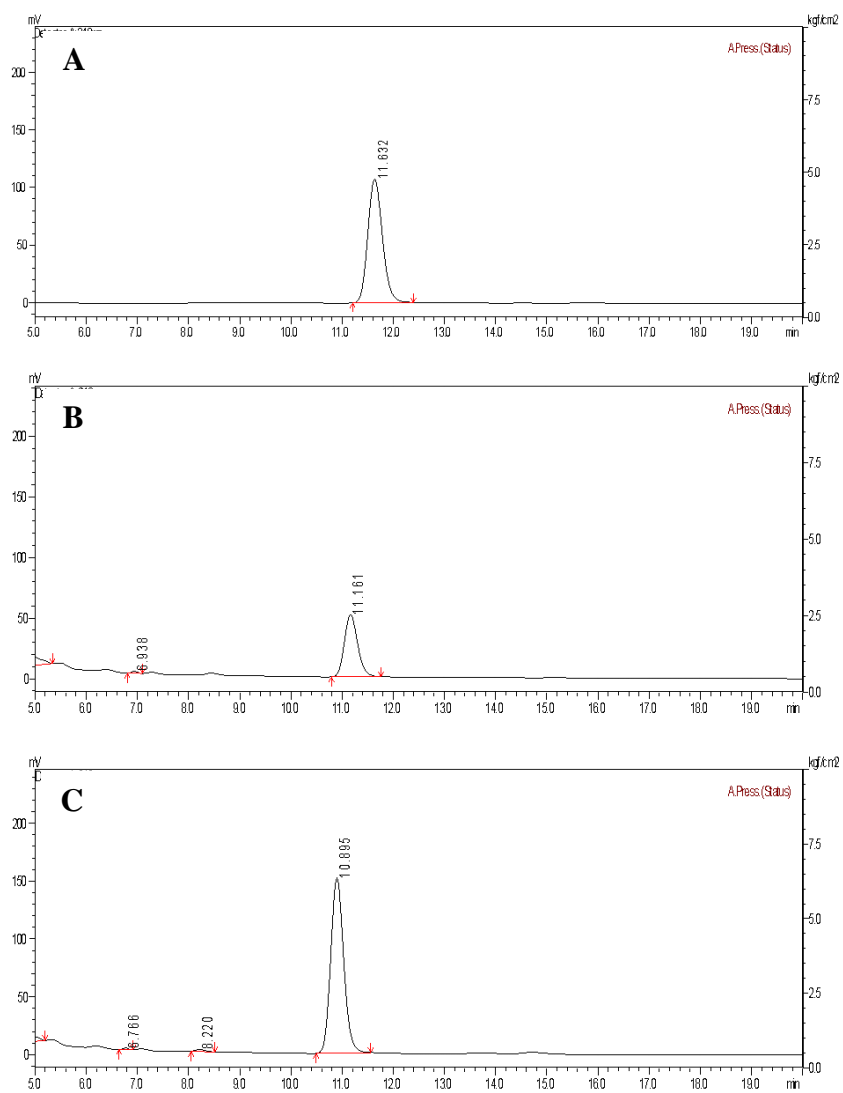
Figure 11 The HPLC chromatograms at 407 nm by HPLC-DAD method

      A) Standard liriodenine
      B) Crude methanolic extract of *Michelia longifolia* bark
      C) Crude methanolic extract spiked with standard liriodenine
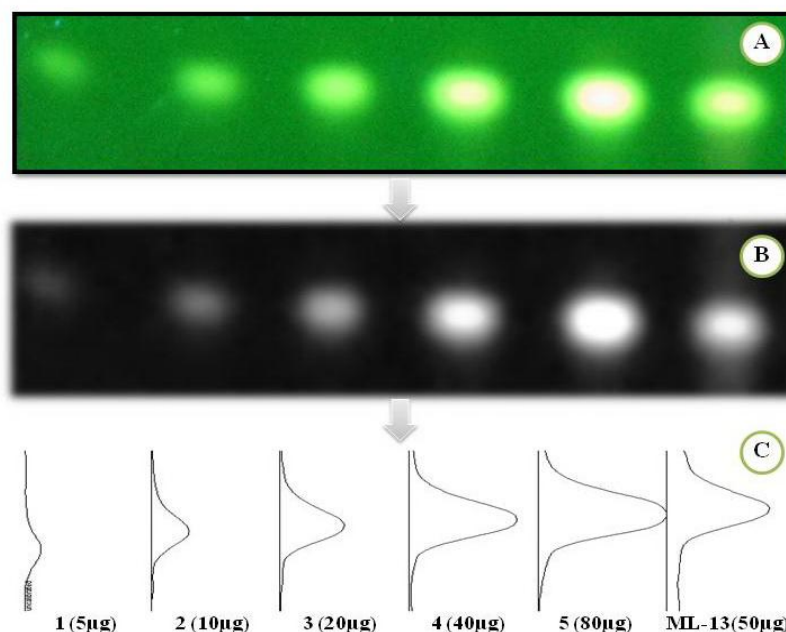
Figure 12 Process of TLC image analyzed by Scion image software

A) Importing TLC image saved as TIFF file

B) Converting to grayscale and smoothing image

C) Chromatograms obtained from plotting profile. (From left to right lane: standard liriodenine 5, 10, 20, 40, 80 µg/spot and *Michelia longifolia* sample no. 13, 50µg/spot)

Quantitative analysis of liriodenine bearing plants endemic to Thailand

Twenty-eight plant samples from the families of Magnoliaceae, Annonaceae and Nelumbonaceae were collected according to their accessibility throughout Thailand. All crude extracts were authenticated as mentioned before and performed with 95 % ethanol in a Soxhlet apparatus. The screening of liriodenine content was analyzed by TLC image analysis as well as HPLC. All measurements were done in triplicate. All specimens' yields were shown in Table 13.

Table 13 Quantitative analysis of liriodenine bearing plants endemic to Thailand

Family	Scientific name (Thai name)	no.	Part	% Yield	Liriodenine content (% w/w)	
					HPLC	TLC image analysis
Magnoliaceae	*Michelia longifolia* **Blume** (Champi)	1	leaf	23.42	0.0011	0.0009
		2	bark	10.23	0.0158 *	0.0153 *
	Michelia champaca **Linn.** (Champa)	3	leaf	30.42	0.0019	0.0019
		4	bark	17.71	0.0119 *	0.0116 *
	Magnolia figo **Lour.** (Champi khaek)	5	leaf	36.61	0.0014	0.0014
	Magnolia sirindhorniae **Noot. & Chalermglin** (Champi sirinthon)	6	leaf	30.96	0.0013	0.0015
		7	bark	12.80	0.0003	0.0003
	Magnolia liliifera (**L.**) **Baill. var.** *liliifera* (Montha)	8	leaf	25.91	0.0008	0.0007
		9	bark	6.48	0.0058	0.0060
	Magnolia coco (**Lour.**) **DC.** (Yihup nu)	10	leaf	25.14	0.0003	0.0003
Annonaceae	*Cananga odorata* (**Lam.**) **Hook.f.&Thomson var.** *odoratam* (Kradang nga thai)	11	leaf	43.85	0.0006	0.0009
	Cananga odorata (**Lam.**) **Hook.f.&Thomson var.** *fruticosa* (Kradang nga songkhla)	12	leaf	30.85	0.0004	0.0006
	Rauwenhoffia siamensis **Scheff.** (Nom meaw)	13	leaf	32.94	-	-
	Anaxagorea javanica **Blume** (Champoon)	14	leaf	25.07	-	-
		15	bark	14.41	-	-
	Melodorum fruticosum **Lour.** (Lamduan)	16	leaf	25.34	-	-
		17	bark	24.79	-	-
	Artabotrys hexapetalus (**L.f.**) **Bhandari** (Karawek, Kradang nga cheen)	18	leaf	20.85	-	-
	Desmos chinensis **Lour.** (Saiyood)	19	leaf	26.78	-	-
	Anomianthus dulcis **J. Sinclair** (Nom woa)	20	leaf	13.18	-	-
	Goniothalamus macanii **Craib** (Kao lam)	21	leaf	6.28	-	-
	Annona squamosa **L.** (Noina)	22	leaf	14.42	-	-
		23	bark	12.08	0.0005	0.0005
	Annona reticulata **L.** (Noi nong)	24	leaf	14.50	-	-
		25	bark	11.59	-	-
	Annona muricata **L.** (Thurain thet)	26	leaf	13.97	-	-
	Mitrephora maingayi **Hook f. & Thomson** (Nang daeng, Porkeehad)	27	leaf	20.53	-	-
Nelumbonaceae	*Nelumbo nucifera* **Gaertn.** (Bua luang)	28	leaf	21.09	0.0069 *	0.0074 *

(**-**) Not found or undetectable for liriodenine
(*) Top 3 medicinal plant species found highest content of liriodenine

Quality assessment

As a result of liriodenine determination, it found that the content were high in *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaf). Therefore, these three medicinal was selected to further pharmacognostic study as followings:

Michelia longifolia Blume

Common name	Champi [136]
Other name	White champaka
Scientific Name	*Michelia longifolia* Blume
Synonym	*Michelia alba* DC.
Family	Magnoliaceae
Distribution	Native to tropical and subtropical south and south-east Asia (Indomalaya), including southern China
Used Part	Stem bark
Ethomedical Uses	Flower: perfumery, cough relieving

Description of Plant

This plant is evergreen or semi-deciduous, having small to medium sized tree up to 20 m. It is tall, bole straight, cylindrical, without buttresses. The bark surface is smooth showing grey to greyish-white color while inner bark fibrous has yellow to brown color with conical to cylindrical crown. Leaves are simple, thick and entire arranged spirally; stipules agnate free from the petiole. Leaves are 5-8 inches long and about 2 to 3.5 inches wide. Flowers are short, axillary brachyblast presenting solitary or rarely in pairs with 6-21 tepals. Its color is white to yellow. It has many stamens, anthers with a short to prominently elongated connective; gynoecium stipitate with spirally arrangement. The carpels are free or connate containing with many ovules. Fruiting carpels dehisce along the dorsal suture forming a fleshy or woody syncarp. Seed is hanged from its funicle [216].

Macroscopic evaluation

2 cm
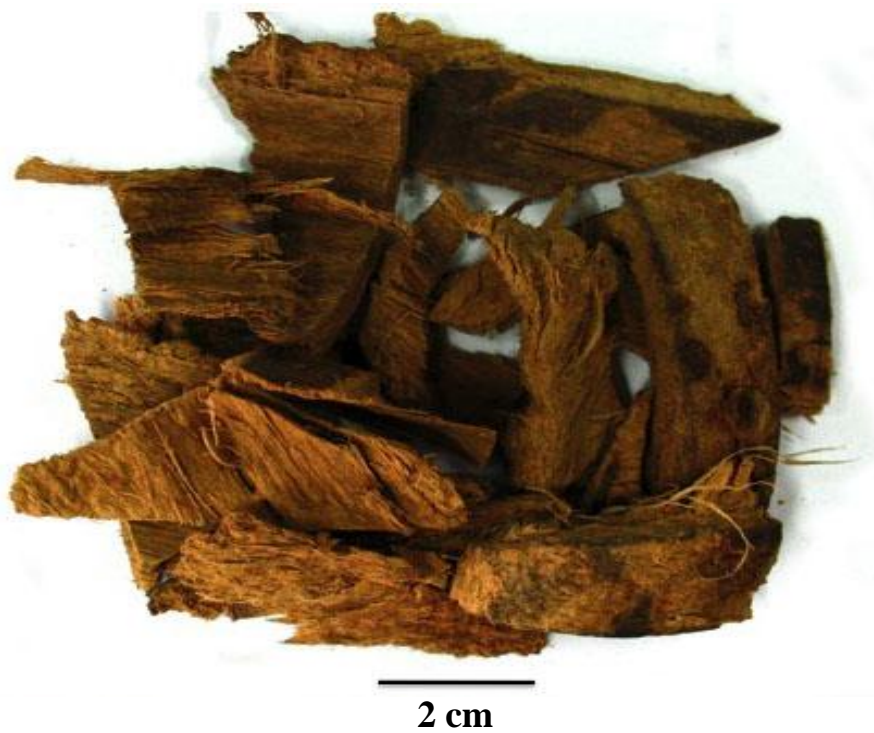
Figure 13 Dried stem bark of *Michelia longifolia* Blume

Description: bark surface was smooth, grey to greyish-white in color, inner bark fibrous showed yellow to brown color.
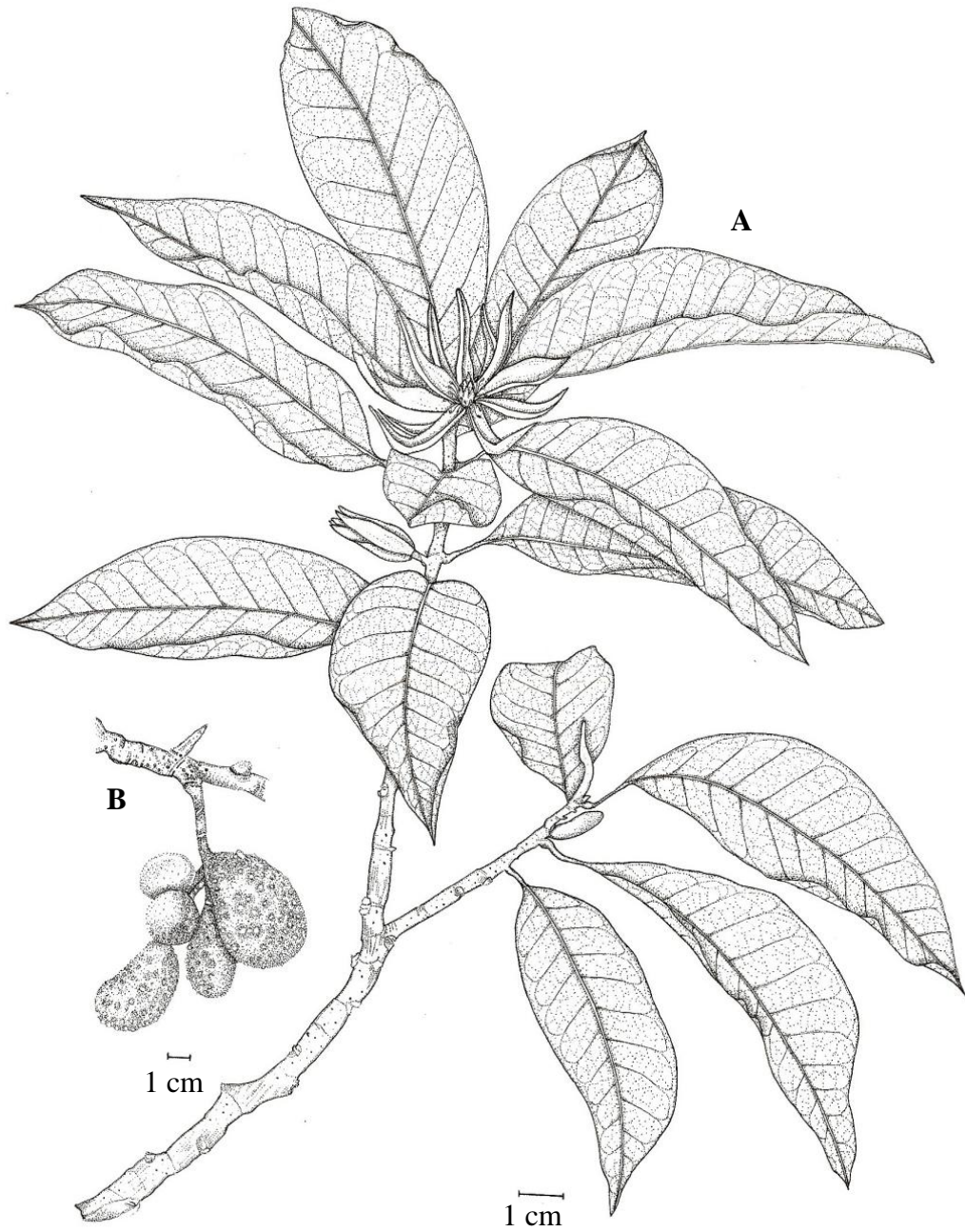
Figure 14 Whole plant of *Michelia longifolia* Blume

    A. Twig with flowers
    B. Fruits

Microscopic evaluation

Anatomical character was studied in both stem bark and leaf because its leaf was used to distinguish *Michelia longifolia* Blume from *Michelia champaca* Linn.
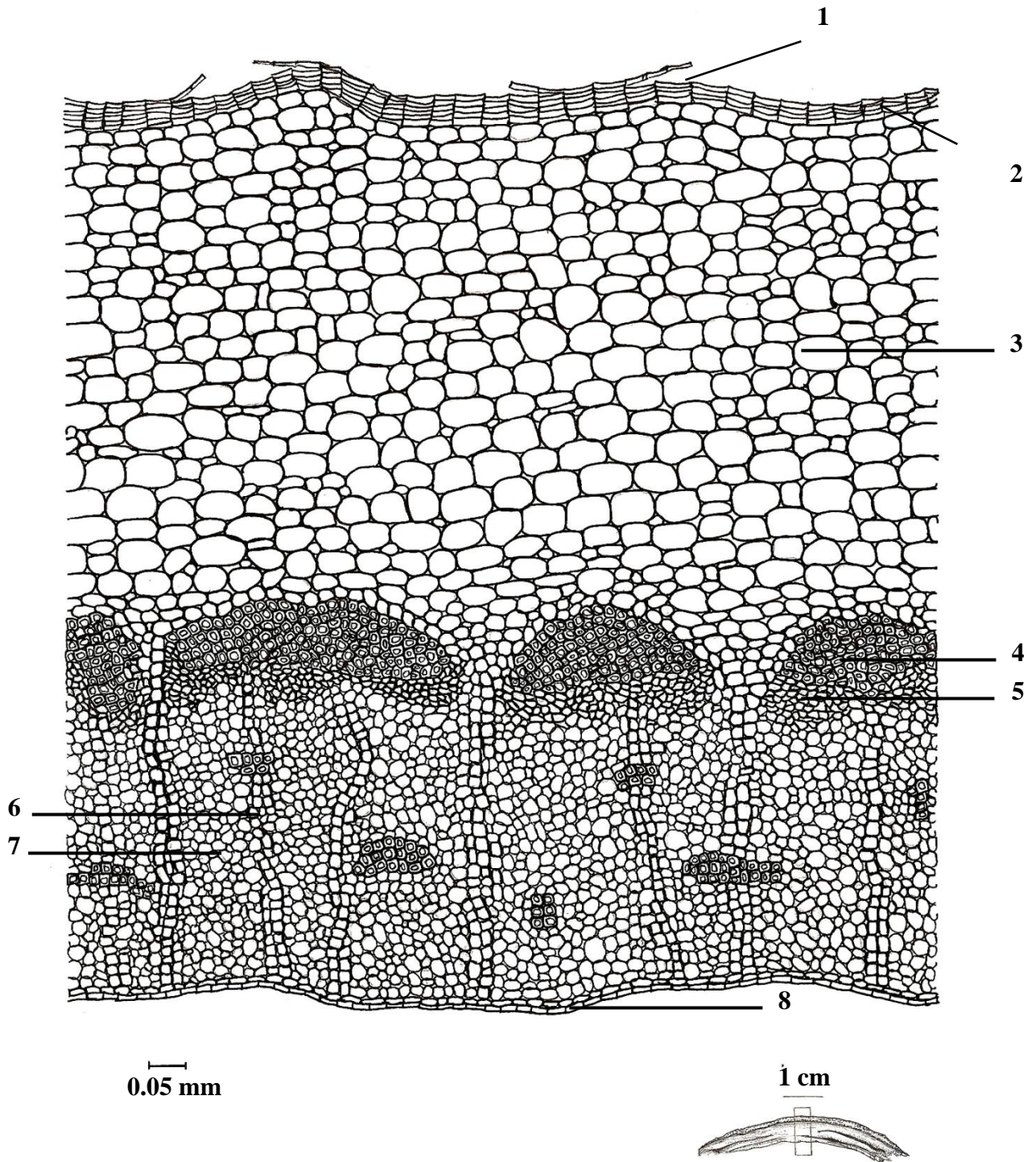
Figure 15 Transverse section of stem bark of *Michelia longifolia* Blume:
1. epidermis 2. periderm 3. parenchyma 4. cortical fiber 5. phloem tissue 6. xylem ray
7. xylem vessel 8. endodermis
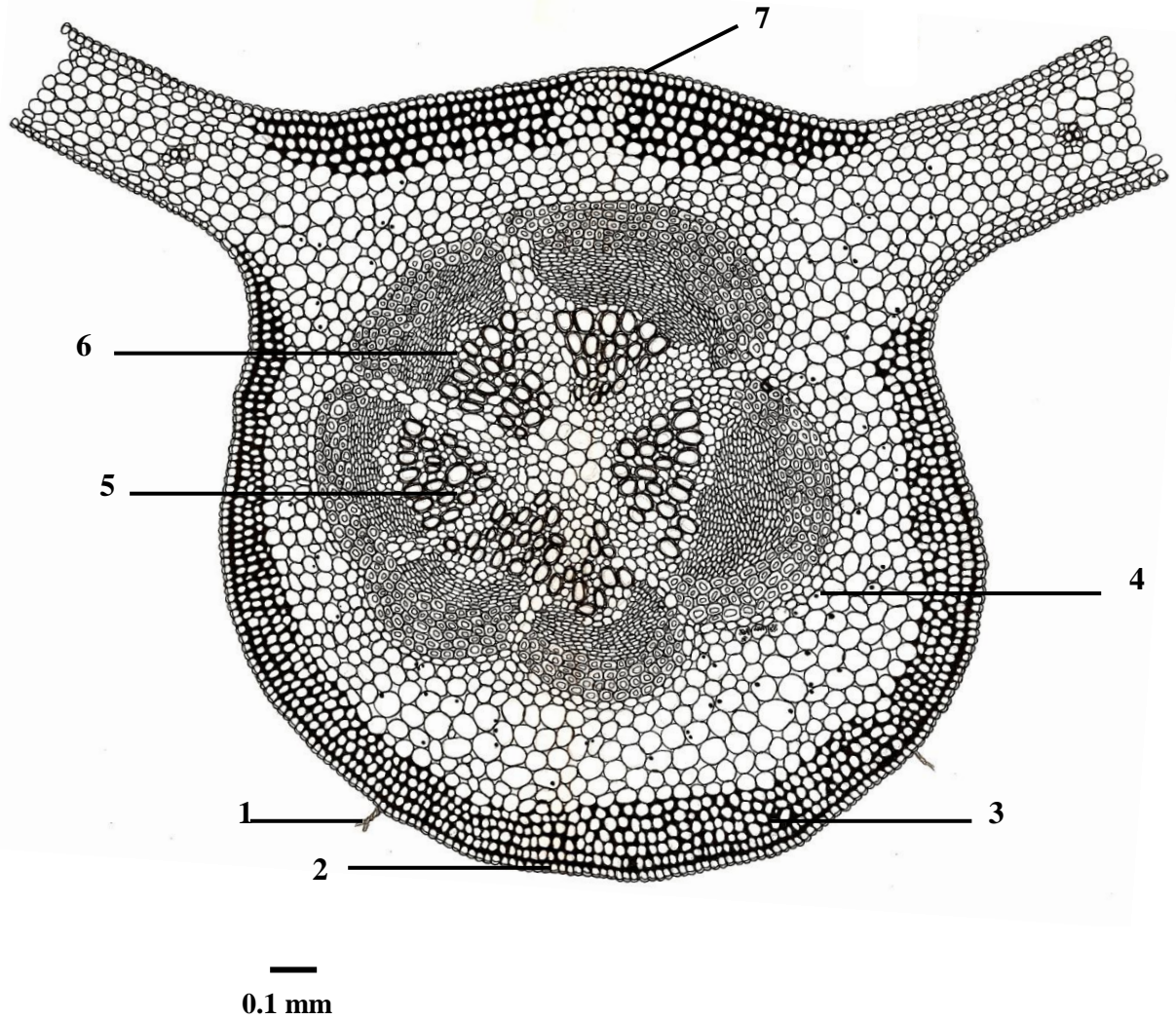
Figure 16 Transverse section of leaf of *Michelia longifolia* Blume:
1. trichome 2. upper epidermis 3. collenchyma 4. fiber 5. xylem tissue 6.phloem tissue
7. lower epidermis

Microscopic evaluation
     Histological character
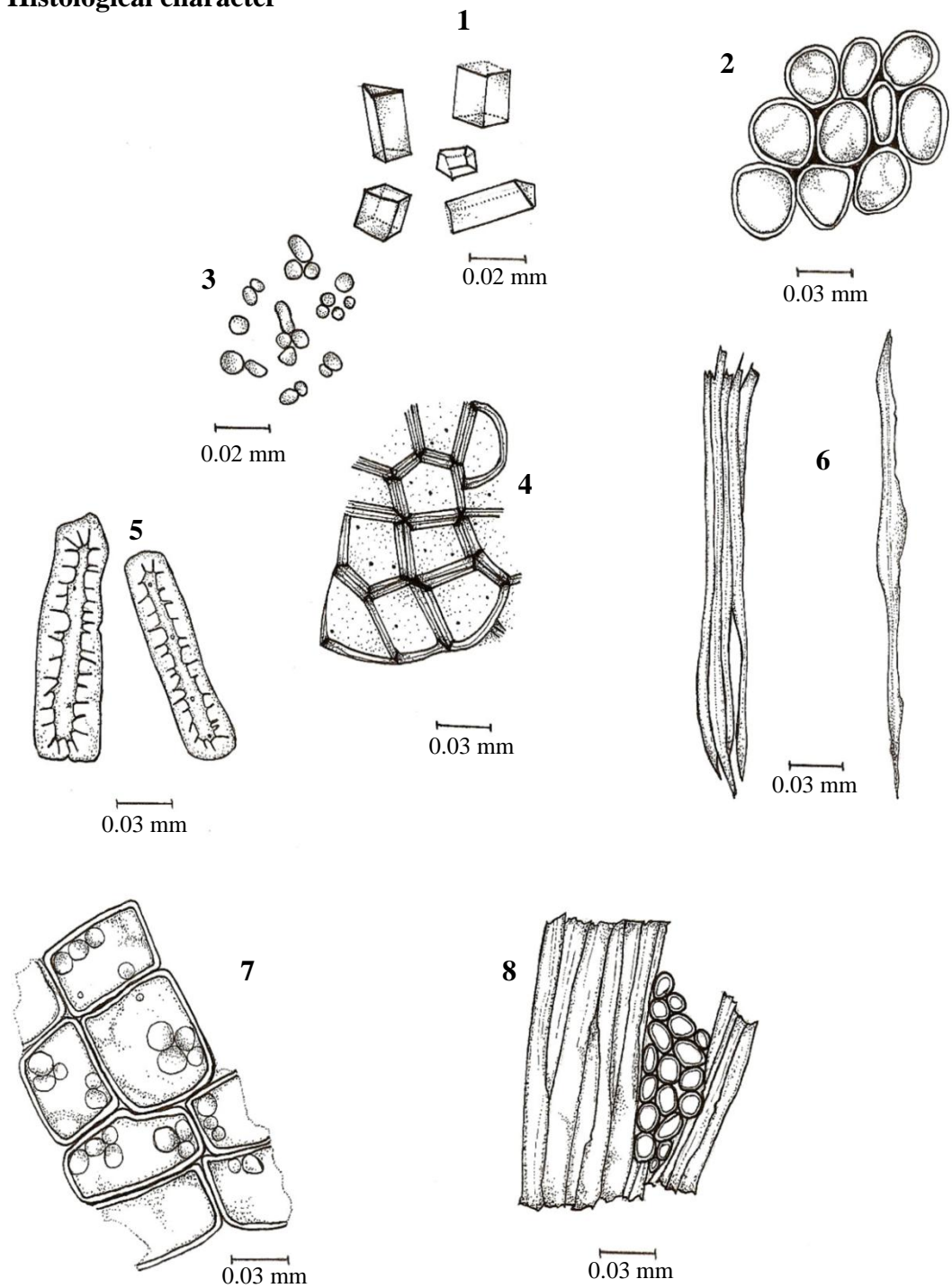
Figure 17 Powdered dried stem bark of *Michelia longifolia* Blume:
1. prism crystals 2. parenchyma in surface view 3. starch granules 4. cork in surface view 5. sclerenchymatous sclereids 6. fragment of fibers 7. parenchyma containing starch granules 8. part of the xylem in tangential longitudinal section showing wood fiber and radullary ray cells
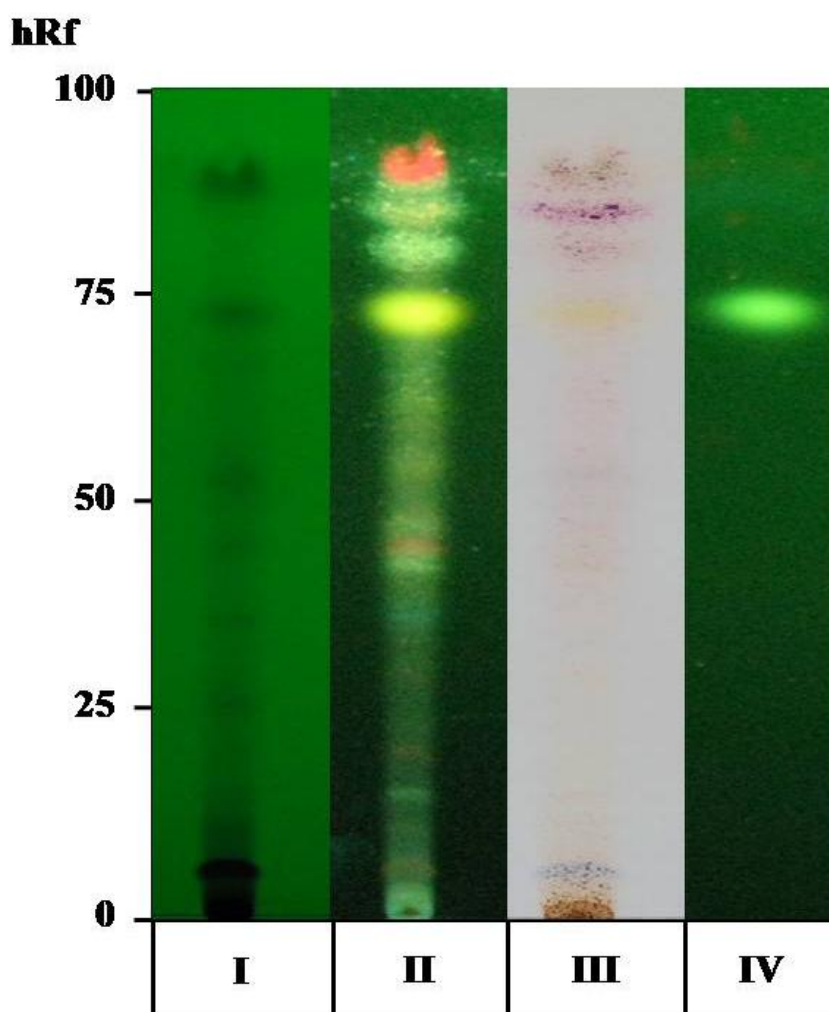
Figure 18 TLC fingerprint of methanolic extract of stem bark of *Michelia longifolia* Blume

Solvent system - **chloroform : methanol   9 : 1**
Detection   I -   detection under UV light 254 nm
II -   detection under UV light 365 nm
III -   detection with Anisaldehyde *,**
IV -   standard liriodenine

*Anisaldehyde reagent preparation:
anisaldehyde (0.5 mL), glacial acetic acid (10 mL), methanol (85 mL), sulfuric acid (5 mL)
** Spot color development:
Heat the plate 120 °C for 10 minutes after spraying.
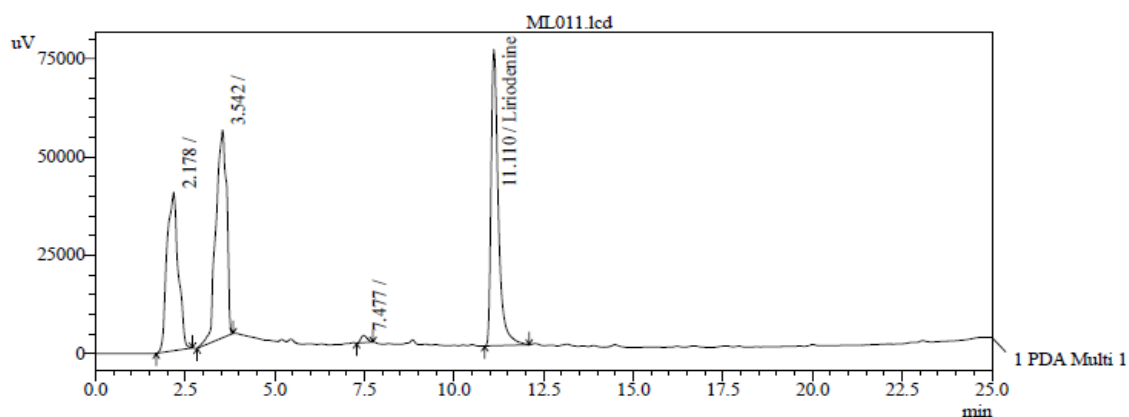
Figure 19 The HPLC chromatogram of methanolic stem bark of *Michelia longifolia* Blume (source no.11); sample concentration was 0.1 g/mL of crude material. Liriodenine peak was at 11.1 min of retention time, analyzed using SHIMADZU gradient system with Inersil ODS-3, C-18 column (4.6 x 250 mm), DAD detector at 407 nm. Mobile phase consisted of A (formate buffer consisting 1% formic acid, adjusted to pH 4.5 diethylamine) and B (100% acetonitrile) from 60:40 to 40:60 over 35 min. at 25°c, flow rate 1 mL/min.

Table 14 Pharmacognostic specification of stem bark of *Michelia longifolia* Blume

Parameter	Mean ± SD (%w/w)	Min – Max (%w/w)
Acid – insoluble ashes	3.49 ± 0.20	0.92 - 8.68
Total ash	5.74 ± 0.08	3.01 - 10.53
Loss on drying	7.17 ± 0.06	4.17 - 13.24
Ethanol-soluble extractive	3.85 ± 0.25	1.67 - 6.64
Water-soluble extractive	8.25 ± 0.14	4.80 - 12.70
Moisture content	6.67 ± 0.13	2.80 - 14.66
Volatile oil	-	-

Crude drug specimens were from 15 different sources. Each sample was done in triplicate according to WHO guildeline.

Table 15 The percentage of liriodenine content in stem bark of *Michelia longifolia* Blume obtained from TLC image analysis and HPLC-DAD.

Batch code	Source	TLC image analysis (%w/w)	HPLC-DAD (%w/w)
ML1	Yasothon	0.0047	0.0056
ML2	Phetchabun	0.0027	0.0026
ML3	Chiang rai	0.0023	0.0027
ML4	Uthai thani	0.0438	0.0490
ML5	Chanthaburi	0.0224	0.0244
ML6	Rayong	0.0153	0.0158
ML7	Buriram	0.0302	0.0287
ML8	Prachin buri	0.0425	0.0437
ML9	Rayong (2)	0.0109	0.0104
ML10	Lampang	0.0066	0.0065
ML11	Nakorn ratchasima	0.0040	0.0039
ML12	Yasothon (2)	0.0041	0.0043
ML13	Lampang (2)	0.0054	0.0051
ML14	Buriram (2)	0.0206	0.0219
ML15	Nong khai	0.0044	0.0044
Grand mean ± pooled S.D		0.0147 ± 0.0143	0.0153 ± 0.0152
Min - Max		0.0023 – 0.0438	0.0026 – 0.0490

Each batch was done in triplicate. There was no significant difference between the results of both quantitative methods ($p > 0.05$) as analyzed by Student's paired *t*-test.

Liriodenine content (%w/w)
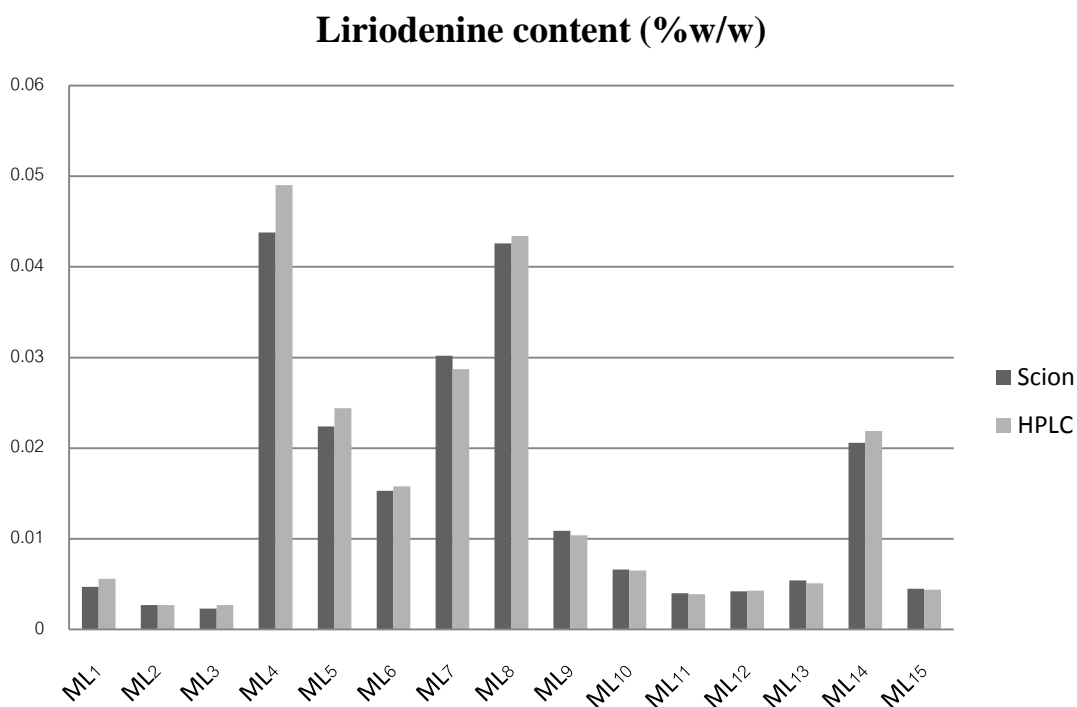
Figure 20 Comparison of liriodenine content of stem bark of *Michelia longifolia* Blume in between TLC image analysis and HPLC-DAD

Each batch was done in triplicate. There was no significant difference between the results of both quantitative methods ($p>0.05$) as analyzed by Student's paired *t*-test. Higher content of liriodenine were found in Uthai thani, Prachin buri and Buriram provinces.

Michelia champaca **Linn.**

Common name	Champa [136]
Other names	Champa khao (Trang), champa thong (Nakhon Si Thammarat), champa pa (Surat Thani), champak, orange champaka, sonchampa
English name	Golden champa, yellow champa, fragrant champaka, orange champaka
Scientific Name	*Michelia champaca* Linn.
Family	Magnoliaceae
Distribution	India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Sri Lanka, Thailand, Vietnam
Used Part	Stem bark
Ethomedical Uses	Dried flower: cardiotonic, nerve and blood tonic, anti-emetic, antipyretic, diuretic
	Leaf: treatment of neural disorder
	Stem bark: antipyretic

Description of Plant

It is an evergreen tree and tall with sized tree up to 50 m. Stems are bole straight and cylindrical, up to 200 cm in diameter, without buttresses; bark is smooth in surface, grey to greyish-white in color. The inner bark is fibrous, yellow to brown in color. Leaves are simple, hairy, entire and arranged spirally. Leaves are 5-8 inches long and about 2.5 inches wide. The stipules are agnate or free from the petiole. Flowers are short, axillary brachyblast presenting solitary or rarely in pairs. Flowers show yellow to dark yellow in color; has many stamens, anthers with a short to prominently elongated connective; gynoecium stipitate, with spirally arranged. Carpels are free or connate containing many ovules. Fruiting carpels dehisce along the dorsal suture and form a fleshy or woody syncarp. Seed is hanged from its funicle [216].

Macroscopic evaluation

2 cm

Figure 21 Dried stem bark of *Michelia champaca* Linn.

Description: bark surface was smooth, grey to greyish-white color; inner bark fibrous showed yellow to brown color.

Figure 22 Whole plant of *Michelia champaca* Linn.

    A. Twig with flowers
    B. Fruits

Microscopic evaluation

Anatomical character was studied in both stem bark and leaf because its leaf was used to distinguish *Michelia champaca* Linn. from *Michelia longifolia* Blume
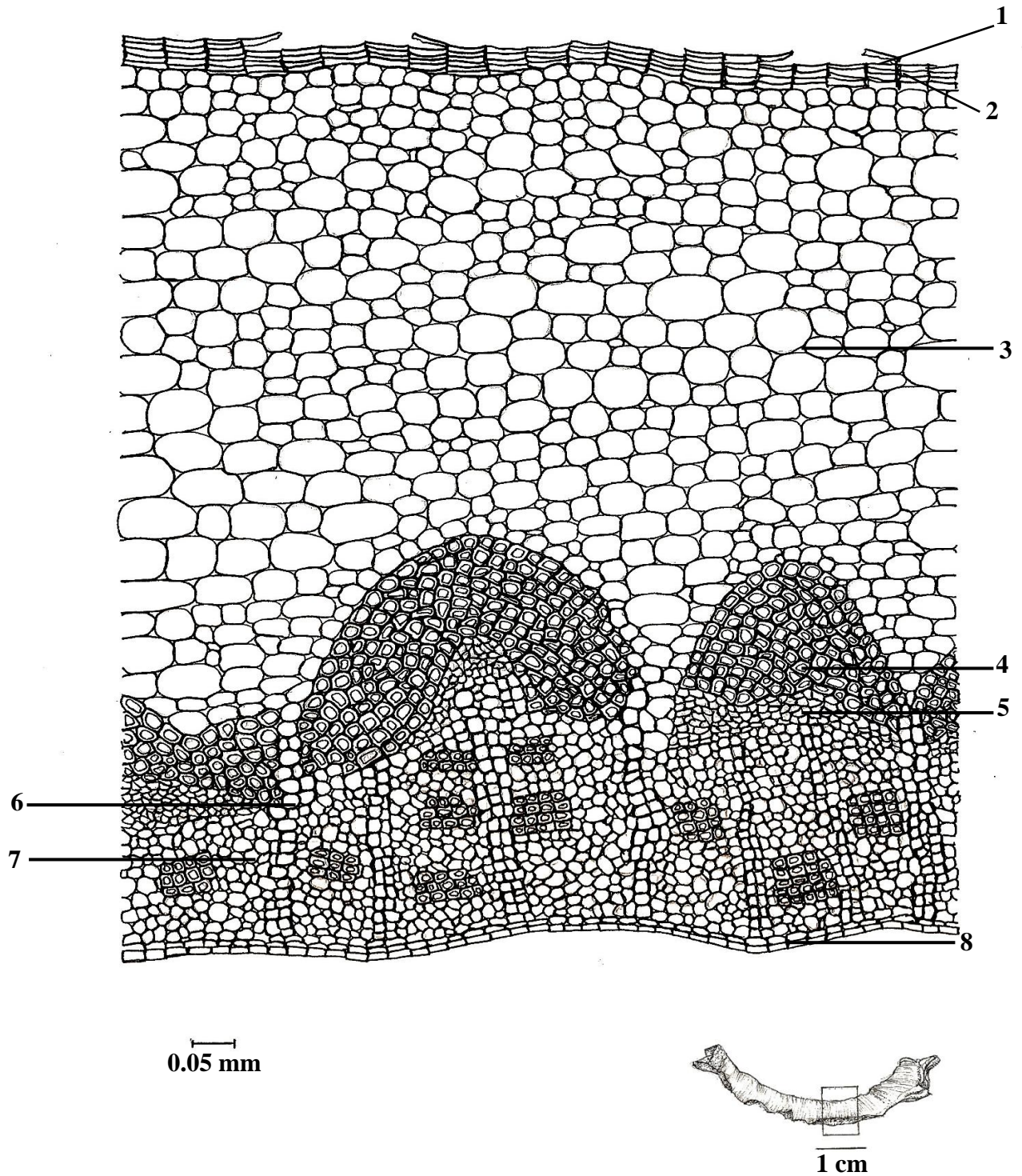
Figure 23 Transverse section of stem bark of *Michelia champaca* Linn.:
1.epidermis 2. periderm 3. parenchyma 4. cortical fiber 5. phloem tissue 6. xylem ray
7. xylem vessel 8. endodermis
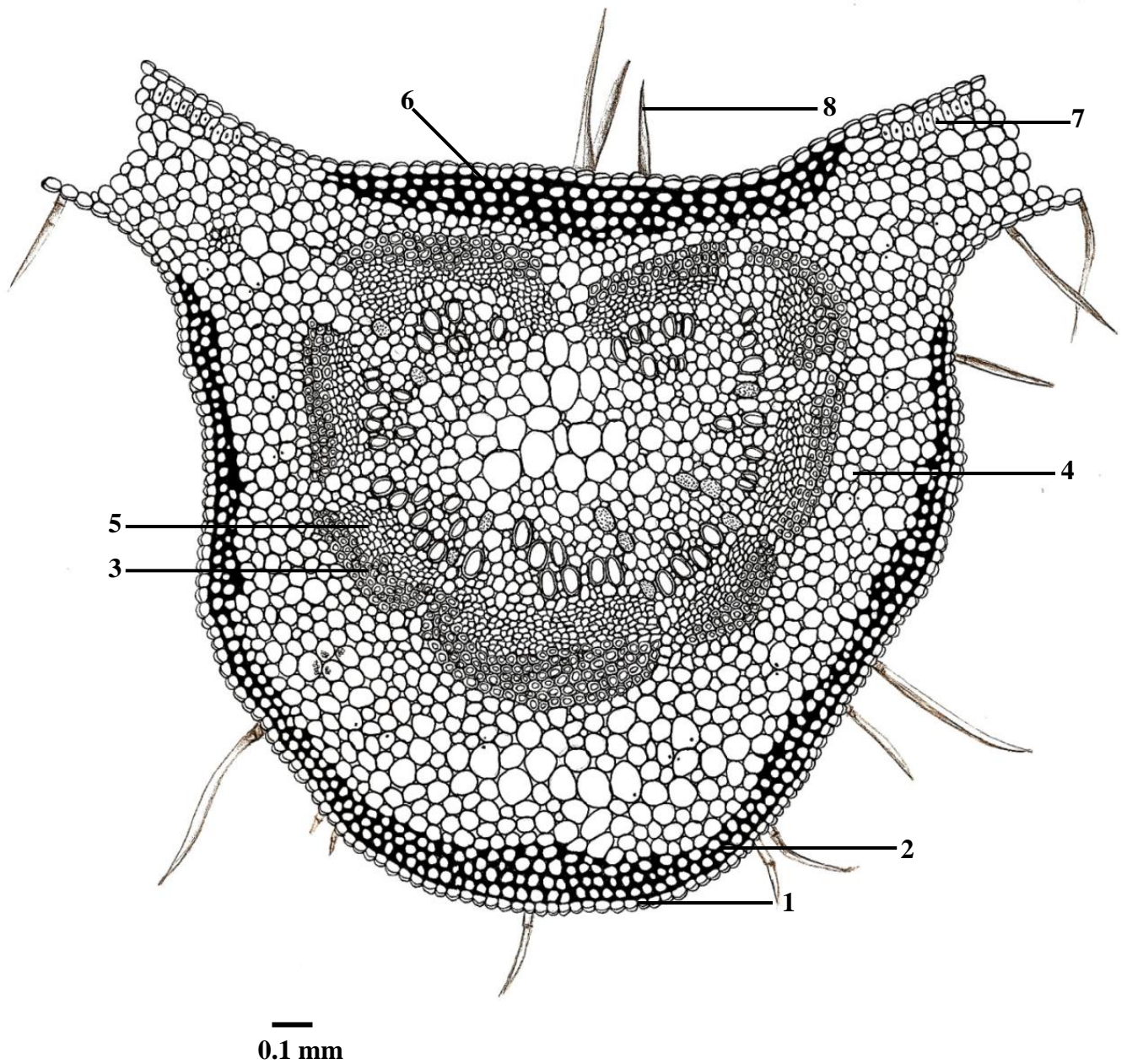
Figure 24 Transverse section of leaf of *Michelia champaca* Linn:
1. upper epidermis 2. collenchyma 3. fiber 4. xylem tissue 5. phloem tissue
6. lower epidermis 7. palisade cell 8. trichome

Microscopic evaluation
  Histological character
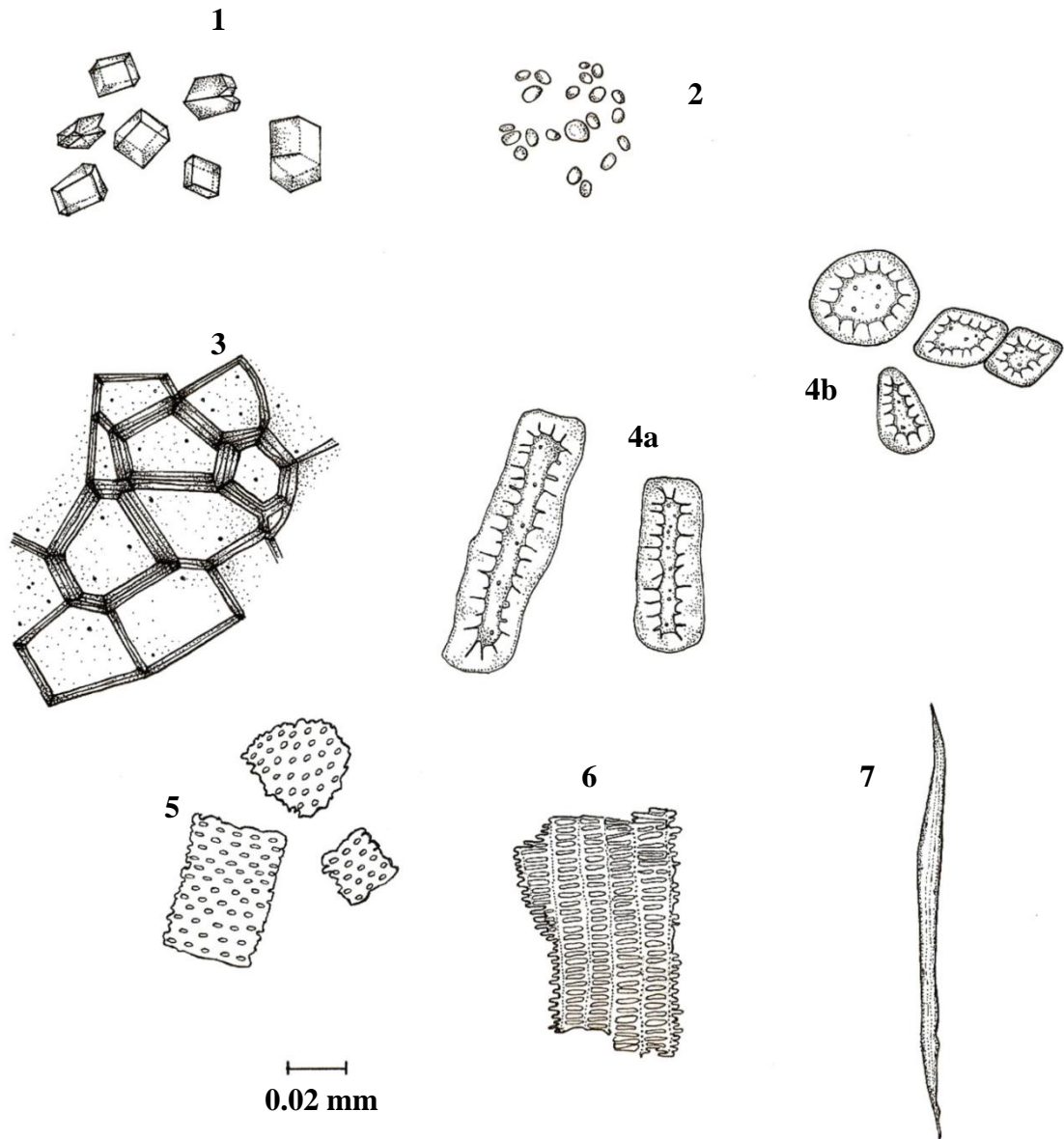
Figure 25 Powdered dried stem bark of *Michelia champaca* Linn.:
1. prism crystals of calcium oxalate 2. starch granules 3. cork in surface view
4. sclerenchymatous sclereids a.) top view b.) side view 5. pitted cells 6. reticulate
vessels 7. fragment of fibers
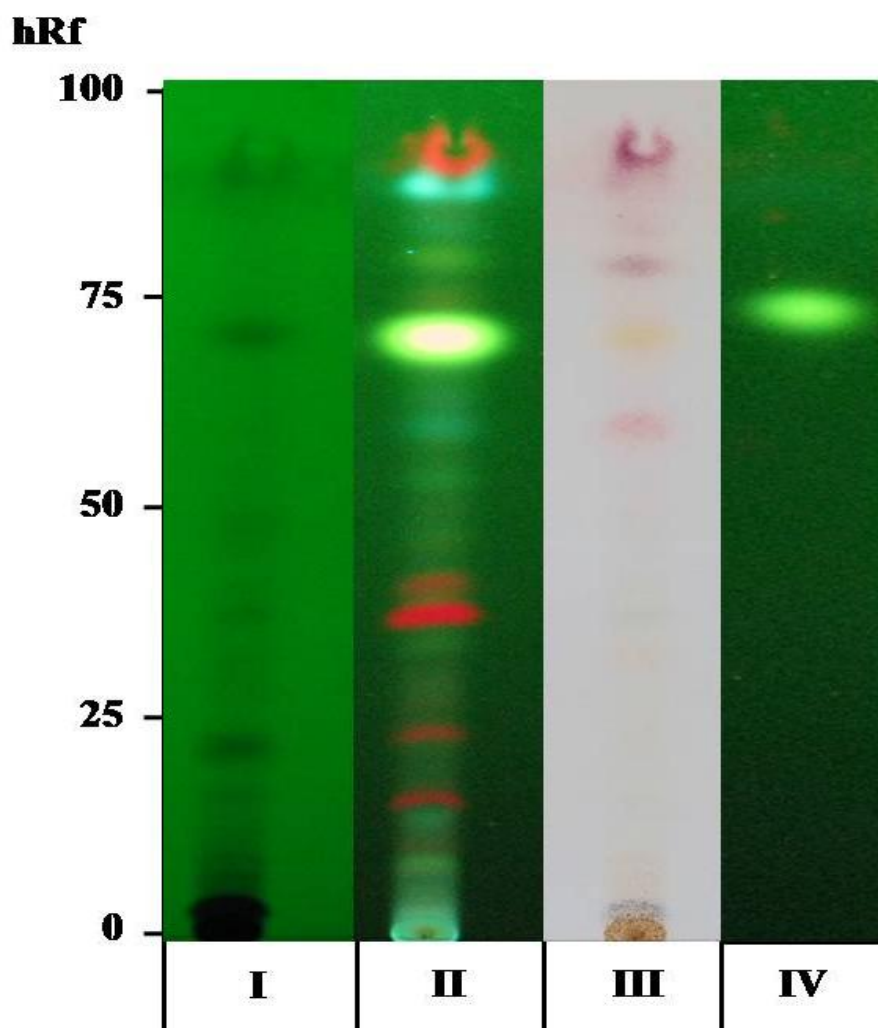
Figure 26 TLC fingerprint of methanolic extract of stem bark of *Michelia champaca* Linn.

Solvent system - **chloroform : methanol   9 : 1**
Detection **I** - detection under UV light 254 nm
II - detection under UV light 365 nm
III - detection with Anisaldehyde *,**
IV - standard liriodenine

*Anisaldehyde reagent preparation:
anisaldehyde (0.5 mL), glacial acetic acid (10 mL), methanol (85 mL), sulfuric acid (5 mL)
** Spot color development:
Heat the plate 120 ºC for 10 minutes after spraying.
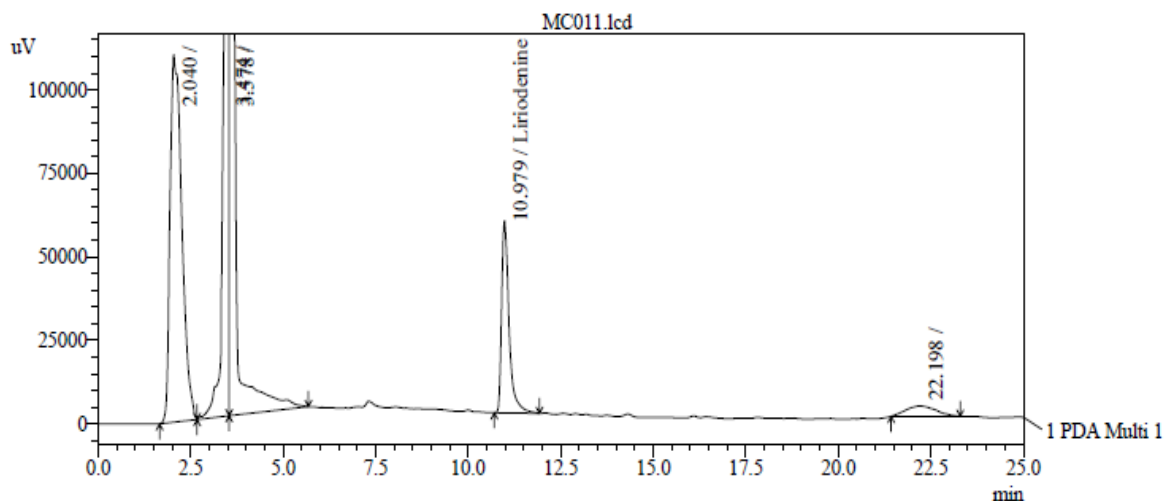
Figure 27 The HPLC chromatogram of methanolic stem bark of *Michelia champaca* Linn. (source no.11); sample concentration was 0.1 g/mL of crude material. Liriodenine peak was at 10.9 min of retention time, analyzed using SHIMADZU gradient system with Inersil ODS-3, C-18 column (4.6 x 250 mm), DAD detector at 407 nm. Mobile phase consisted of A (formate buffer consisting 1% formic acid, adjusted to pH 4.5 diethylamine) and B (100% acetonitrile) from 60:40 to 40:60 over 35 min. at 25ºc, flow rate 1 mL/min.

Table 16 Pharmacognostic specification of stem bark of *Michelia champaca* Linn.

Parameter	Mean ± SD (%w/w)	Min – Max (%w/w)
Acid – insoluble ashes	2.98 ± 0.17	1.53 - 5.29
Total ash	6.25 ± 0.07	3.61 - 7.92
Loss on drying	5.62 ± 0.08	0.55 - 9.86
Ethanol-soluble extractive	6.71 ± 0.32	1.48 - 19.24
Water-soluble extractive	12.38 ± 0.19	4.28 - 26.45
Moisture content	7.37 ± 0.08	2.99 - 9.90
Volatile oil	-	-

Crude drug specimens were from 15 different sources, each sample was done in triplicate according to WHO guideline.

Table 17 The percentage of liriodenine content in stem bark of *Michelia champaca* Linn. obtained from TLC image analysis and HPLC-DAD

Batch code	Source	TLC image analysis (%w/w)	HPLC-DAD (%w/w)
MC1	Nakhon Pathom	0.0213	0.0226
MC2	Phetchabun	0.0790	0.0934
MC3	Chanthaburi	0.0116	0.0119
MC4	Rayong	0.0075	0.0069
MC5	Rayong (2)	0.0352	0.0368
MC6	Lampang	0.0091	0.0089
MC7	Bangkok	0.0172	0.0180
MC8	Phetchabun (2)	0.0901	0.0933
MC9	Nakhon Pathom (2)	0.0073	0.0068
MC10	Uthai thani	0.0366	0.0368
MC11	Buriram	0.0037	0.0040
MC12	Nong khai	0.0473	0.0473
MC13	Buriram (2)	0.0104	0.0112
MC14	Samutprakarn	0.0159	0.0171
MC15	Nong khai (2)	0.0182	0.0203
Grand mean ± pooled S.D		0.0274 ± 0.0264	0.0290 ± 0.0290
Min - Max		0.0037 – 0.0901	0.0040 – 0.0934

Each batch was done in triplicate. There was no significant difference between the results of both quantitative methods ($p > 0.05$) as analyzed by Student's paired *t*-test.
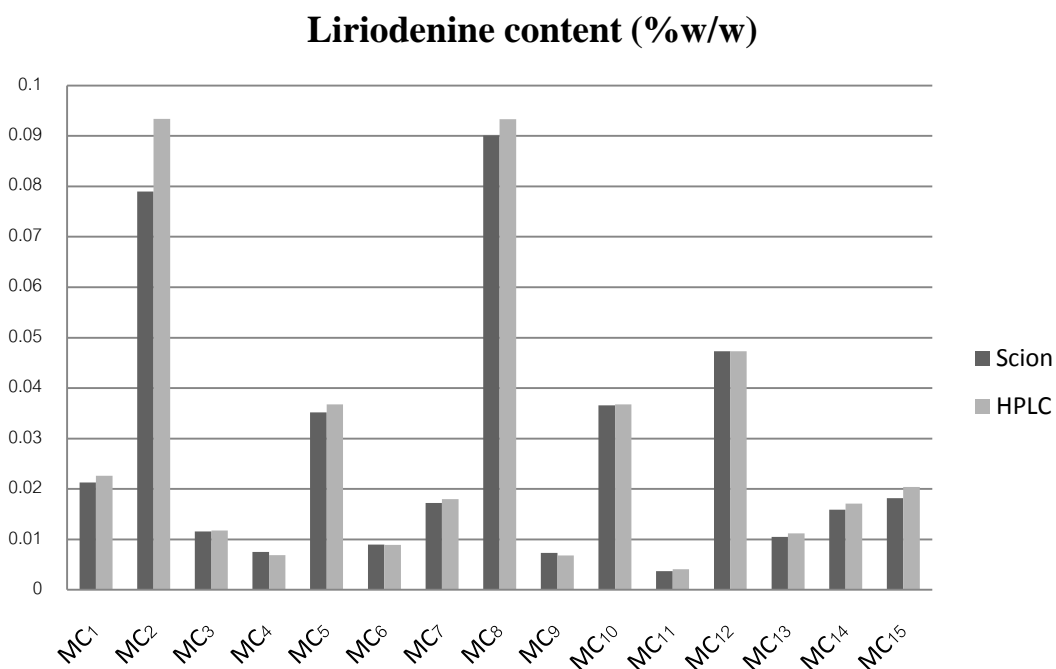
Figure 28 Comparison of liriodenine content of stem bark of *Michelia champaca* Linn. between TLC image analysis and HPLC-DAD

Each batch was done in triplicate. There was no significant difference between the results of both quantitative methods ($p>0.05$) as analyzed by Student's paired t-test. Higher content of liriodenine were found in Phetchabun and Nong khai provinces.

Nelumbo nucifera **Gaertn.**

Common name	Bua luang [136]
Other names	Bua, satta bongkoch, satta but, ubon, chok (Khmer Buriram)
English name	Sacred lotus**,** Indian lotus, sacred lotus, bean of India
Scientific Name	*Nelumbo nucifera* Gaertn.
Family	Nelumbonaceae
Distribution	Throughout the world
Used Part	Leaf
Ethomedical Uses	Embryo: coronary dilator action, otherwise used as anti-inflammation, antidiarrhea and hemotonic.

Description of Plant

This plant is an aquatic perennial. The roots of *Nelumbo nucifera* are planted in the soil of the pond or river bottom, while the leaves float on top of the water surface. The flowers are usually found on thick stems rising several centimeters above the water, fragrant; 2 sepals, petals pink, white inserted at base of receptacle. The plant normally grows up to a height of about 150 cm and a horizontal spread of up to 3 meters, but some unverified reports place the height as high as over 5 meters. The leaves may be as large as 60 cm in diameter, while the showy flowers can be up to 20 cm in diameter. Leaves borne on subterranean, creeping, long-jointed rhizome; orbicular, glaucous above, purinose, erect, exactly peltate, entire, radiately nerved glabrous, partly flat, floating, with weak petioles, partly shallowly cup-shaped on rigid petioles raised above the water, petiole very long, rough with small distant prickles, milky latex [217-218].

Macroscopic evaluation

Figure 29 Dried leaves of *Nelumbo nucifera* Gaertn.

Description: leaves surface were smooth, greenish brown to brown color in dried leaves.

Vertical section of receptacle and carpels

Anther

Embryo, one lobe removed to show the plumule

Embryo with spreading lobes

Root

2 cm

1 cm

5 cm

2 cm
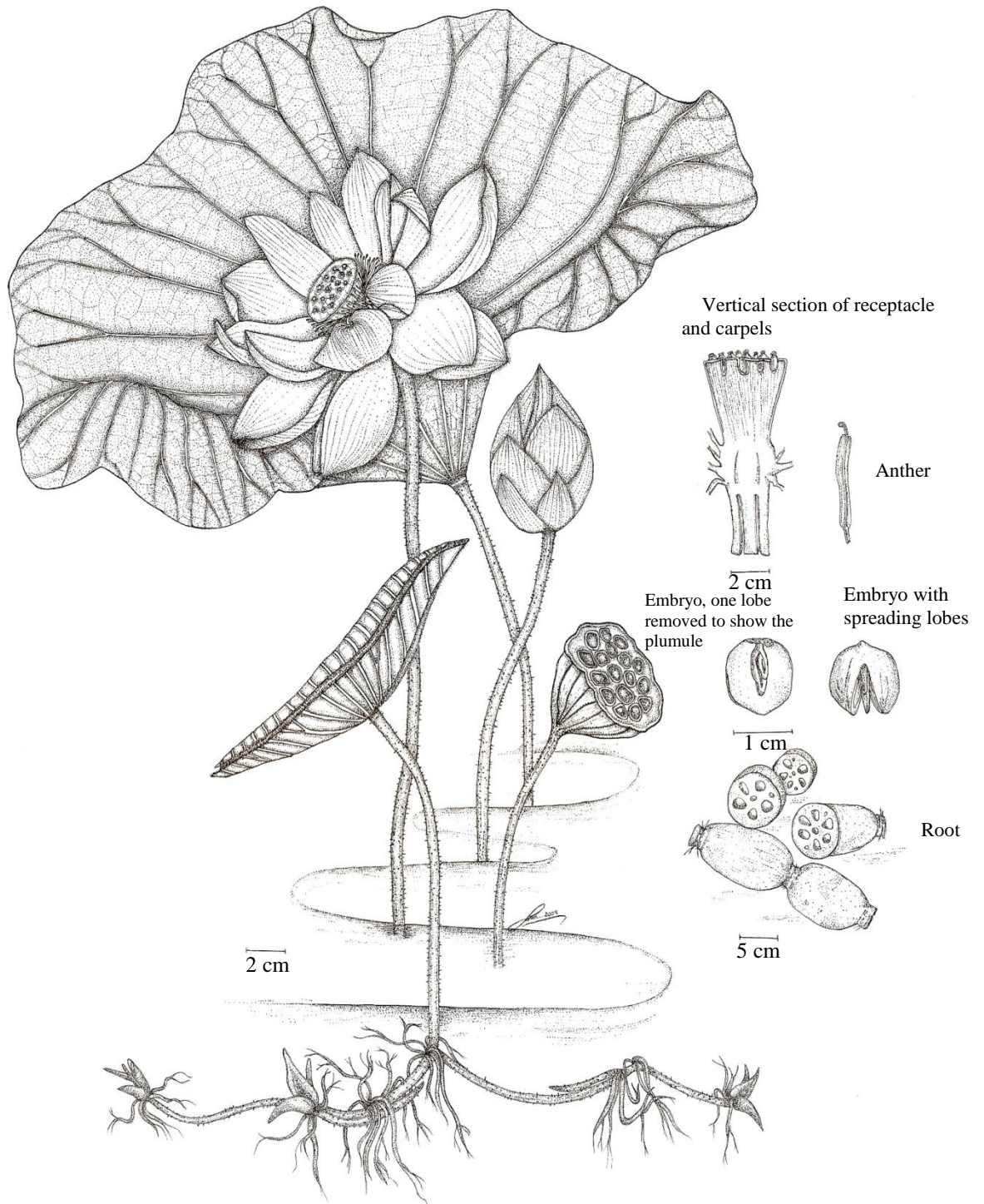
Figure 30 Whole plant of *Nelumbo nucifera* Gaertn.

Microscopic evaluation

Anatomical character was studied in the leaf of *Nelumbo nucifera* Gaertn.
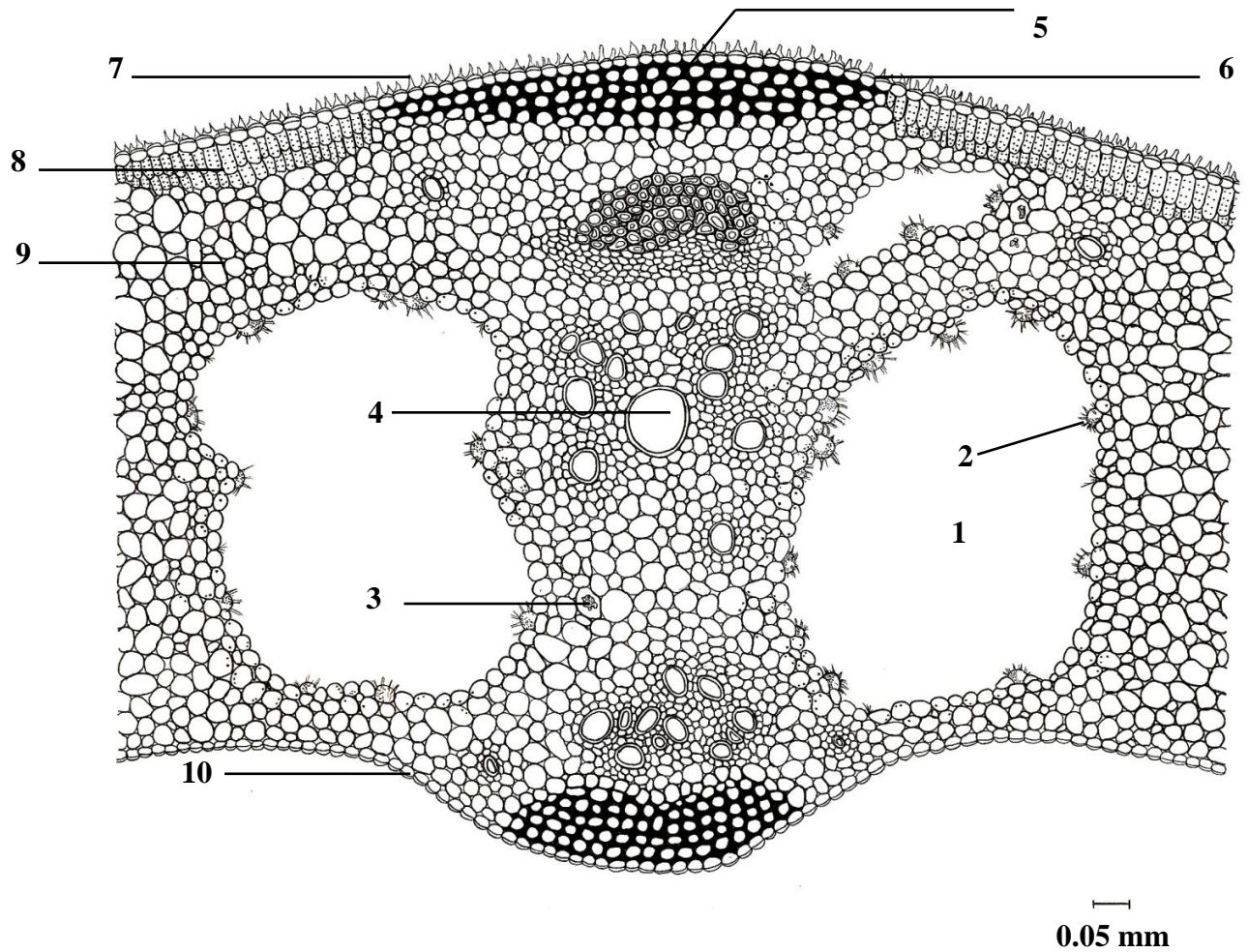
Figure 31 Transverse section of leaf of *Nelumbo nucifera* Gaertn.:
1. air chamber 2. trichosclereid 3. calcium oxalate 4. xylem vessle 5. collenchyma
6. upper epidermis 7. trichome 8. palisade cell 9. parenchyma 10. lower epidermis

Microscopic evaluation
    Histological character
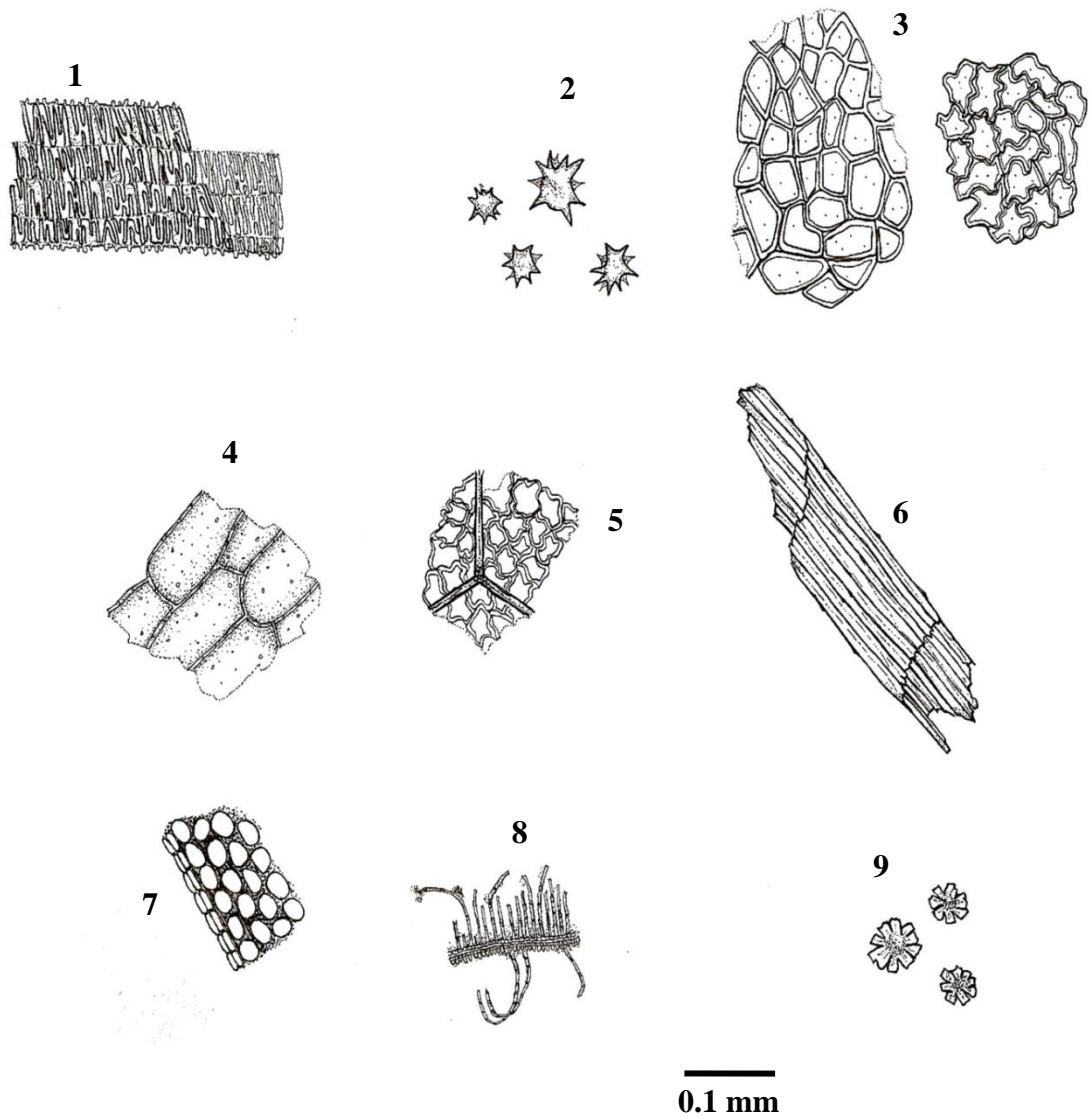
Figure 32 Powdered dried leaves of *Nelumbo nucifera* Gaertn.:
1. reticulate vessels 2. tricho-sclereids 3. epidermal cells 4. fragments of parenchyma longitudinal view 5. vein 6. fibers 7. collenchyma 8. vessels 9. calcium oxalate crystals
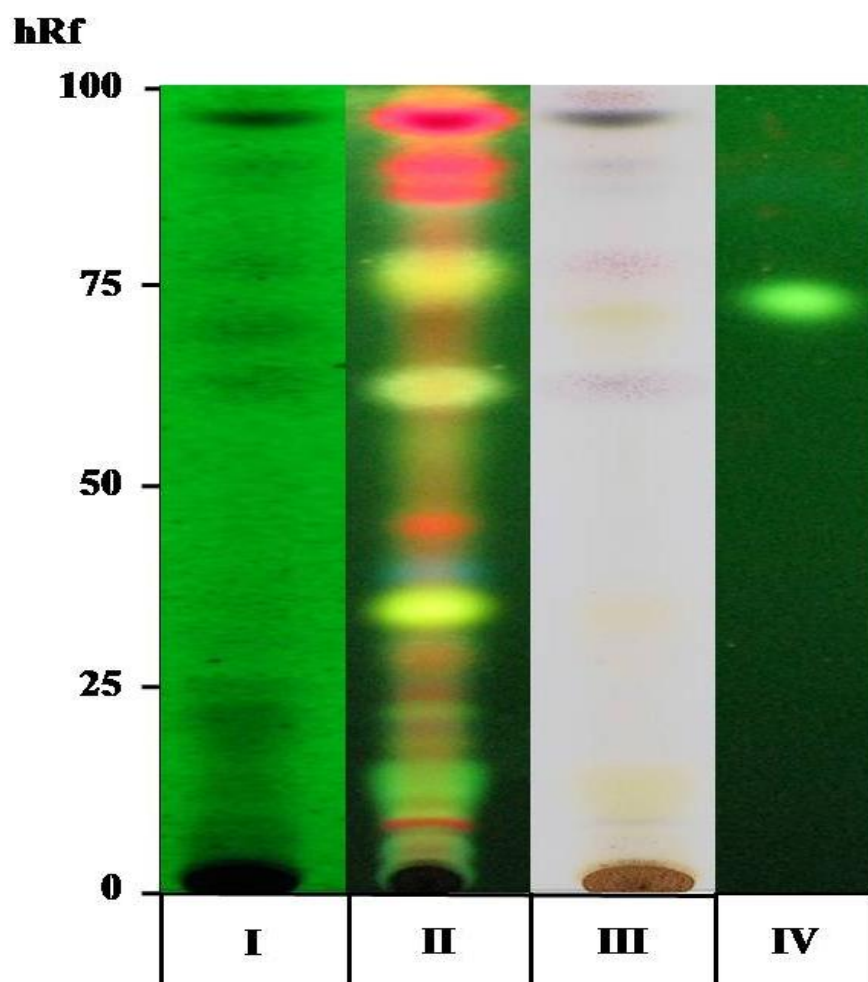
Figure 33 TLC fingerprint of methanolic extract of leaf of *Nelumbo nucifera* Gaertn.

Solvent system **-** **chloroform : methanol 9 : 1**
Detection **I** **-** detection under UV light 254 nm
II **-** detection under UV light 365 nm
III **-** detection with Anisaldehyde *,**
IV **-** standard liriodenine

*Anisaldehyde reagent preparation:
anisaldehyde (0.5 mL), glacial acetic acid (10 mL), methanol (85 mL), sulfuric acid (5 mL)
** Spot color development:
Heat the plate 120 °C for 10 minutes after spraying.
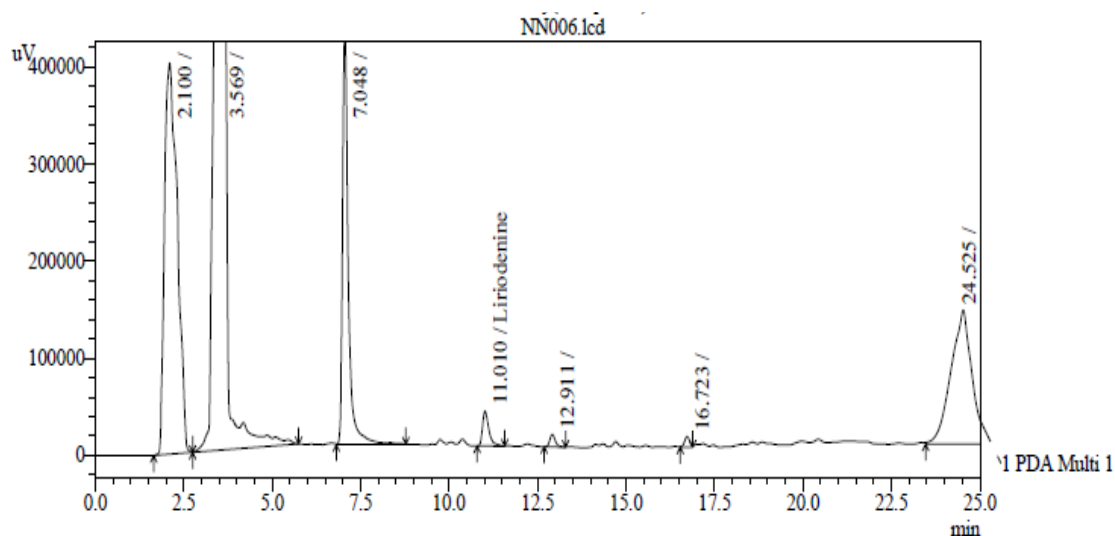
Figure 34 The HPLC chromatogram of methanolic leaf of *Nelumbo nucifera* Gaertn. (source no.6); sample concentration was 0.1 g/mL of crude material. Liriodenine peak was at 11 min of retention time, analyzed using SHIMADZU gradient system with Inersil ODS-3, C-18 column (4.6 x 250 mm), DAD detector at 407 nm. Mobile phase consisted of A (formate buffer consisting 1% formic acid, adjusted to pH 4.5 diethylamine) and B (100% acetonitrile) from 60:40 to 40:60 over 35 min. at 25°c, flow rate 1 mL/min.

Table 18 Pharmacognostic specification of leaf of *Nelumbo nucifera* Gaertn.

Parameter	Mean ± SD (%w/w)	Min - Max (%w/w)
Acid – insoluble ashes	2.61 ± 0.24	1.62 - 3.77
Total ash	9.62 ± 0.07	7.94 - 11.64
Loss on drying	7.69 ± 0.07	4.22 - 10.02
Ethanol-soluble extractive	6.24 ± 0.22	3.37 - 13.33
Water-soluble extractive	9.51 ± 0.24	5.24 - 12.92
Moisture content	7.06 ± 0.11	4.60 - 9.02
Volatile oil	-	-

Crude drug specimens were from 15 different sources. Each sample was done in triplicate according to WHO guideline.

Table 19 The percentage of liriodenine content in leaf of *Nelumbo nucifera* Gaertn. obtained from TLC image analysis and HPLC-DAD.

Batch code	Source	TLC image analysis (%w/w)	HPLC-DAD (%w/w)
NN1	Lampang	0.0037	0.0033
NN2	Uthai thani	0.0077	0.0073
NN3	Buriram	0.0033	0.0034
NN4	Chanthaburi	0.0074	0.0069
NN5	Nakorn Ratchasima	0.0009	0.0009
NN6	Petchaboon	0.0018	0.0016
NN7	Yasothon	0.0023	0.0025
NN8	Bangkok	0.0086	0.0084
NN9	Nakorn Ratchasima (2)	0.0058	0.0055
NN10	Prachinburi	0.0026	0.0022
NN11	Rayong	0.0063	0.0066
NN12	Lampang (2)	0.0080	0.0085
NN13	Nakhon-pathom	0.0104	0.0110
NN14	Nongkhai	0.0021	0.0024
NN15	Buriram (2)	0.0025	0.0021
Grand mean ± pooled S.D		0.0049 ± 0.0030	0.0049 ± 0.0031
Min – Max		0.0009 – 0.0104	0.0009 – 0.0110

Each batch was done in triplicate. There was no significant difference between the results of both quantitative methods ($p > 0.05$) as analyzed by Student's paired *t*-test.

Liriodenine content (% w/w)
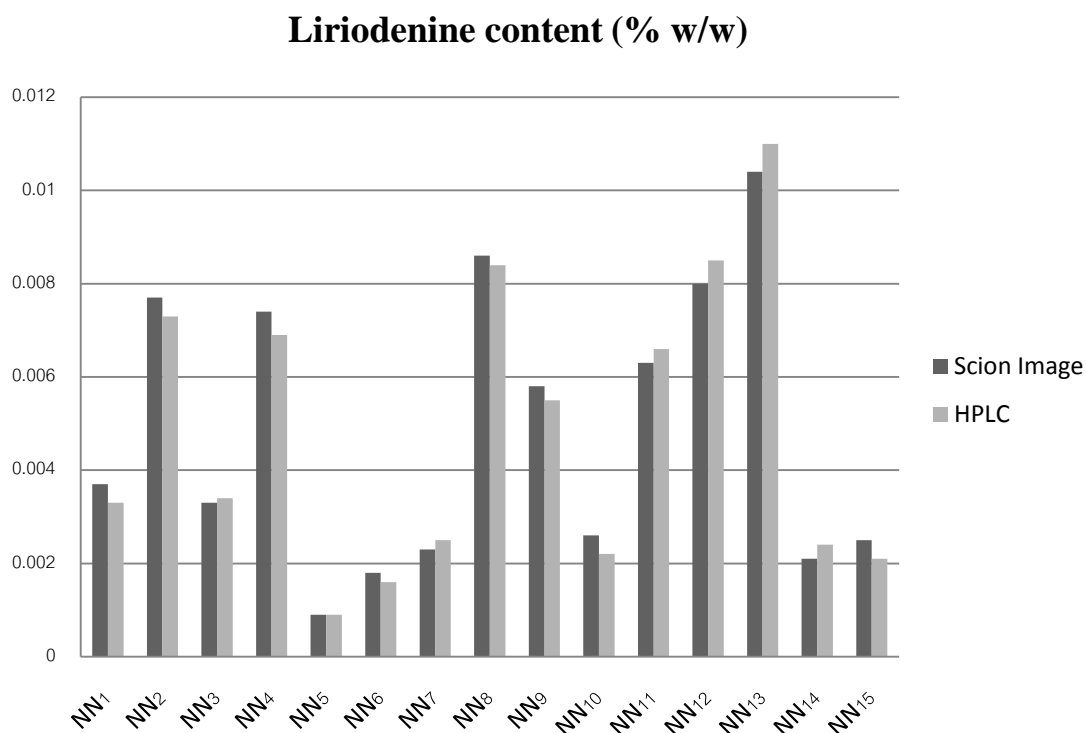
Figure 35 Comparison of liriodenine content of leaf of *Nelumbo nucifera* Gaertn. between TLC image analysis and HPLC-DAD

Each batch was done in triplicate. There was no significant difference between the results of both quantitative methods ($p>0.05$) as analyzed by Student's paired *t*-test. Higher content of liriodenine were found in Nakhon pathom, Bangkok and Lampang provinces.

CHAPTER V
DISCUSSION AND CONCLUSION

The interest and use of medicinal plants have witnessed a tremendous resurgence in past decade. Also phytochemical constituents have been studied for a role use in specific health problems as well as long term health promoting. Liriodenine, an oxoaporphine alkaloid, has a wide range of pharmacological activities, such as antibacterial, antifungal, antiviral activities and to which its significant antitumor activities can be primarily attributed. Thus, three approaches in this study firstly were to develop the quantification of liriodenine using TLC image analysis by Scion image software as well as HPLC method. Second was to screen the compound content in Thai medicinal plants to be a reference database as the source of supply. Three highest liriodenine contents were found in *Michelia longifolia* bark, *Michelia champaca* bark and *Nelumbo nucifera* leaf. Third approach provided their pharmacognostic parameters available for standardization according to WHO guideline.

The NMR spectra of standard liriodenine were in very similar to literature data [215]. Referring to HPLC system as a reference method in this study, HPLC-UV was first applied to liriodenine quantitation detected with the maximum absorbance wavelength at 248 nm when checking with spectrophotometry and literature reviewed [219]. The researcher found that there were some samples showing unclear liriodenine peak. Then the problem was solved by spiking sample with standard and compared to its retention time to identify the suspected peak. Nevertheless, the results still could not be confirmed due to HPLC-UV components; that were an isocratic system without controlled temperature oven and required manual mixture of mobile phase. Therefore, the drift retention problem could be from the poor temperature control and mobile phase changing [220]. Moreover, the output could not show the peak spectra and purity peak index to help identification. In addition, the identity of analyte might require confirmation. Whenever the retention time of the peak was not constant, the possibility existed for the error in interpreting data, and analysts would have less

confident in data [126]. Therefore, to solve the problem, HPLC-DAD was used for liriodenine quantitation instead. This HPLC component consisted of gradient system, which provided a very precise control of solvent composition to maintain a reproducible gradient profile, and controlled temperature oven to reduce the drift of retention time [220]. Additionally, the output could process the peak spectra and indicate the purity to confirm the data interpreting.

HPLC-DAD was employed at the wavelength range of 180-800 nm. The result supported that wavelength at 248 nm was the maximum absorption wavelenght for liriodenine [219]. However, it was found that liriodenine showed good absorbance at 407 nm as well. Thus, these dual wavelengths gave more reliable to detect liriodenine in selected plant extracts. Specifically, liriodenine containing plants should demonstrate the compound peak at both 248 nm and 407 nm.

In summary, as a reference method in this study, HPLC-DAD later was confirmed quantitative results with HPLC-UV; there were no statistically difference between both data indicating the accuracy of developed HPLC. Therefore, both developed system including isocratic and gradient system can be applied for future study depending on the availability of equipments, solvents and so on.

With regard to TLC image analysis, Scion image software was chosen for liriodenine determination due to its free download and user-friendly. This study is the first report of liriodenine quantitation by TLC image analysis. The TLC condition was developed using chloroform and methanol as previously described. The fluorescent illuminating spot of liriodenine was its dominant character on TLC plate detected at 365 nm; that suitably for liriodenine in quantitation. From Hess, Amber 2004, the study indicated that round spots were extreamly to get good quantitative results. All pictures should be taken at the same lens aperture, distance between UV lamp and TLC plate and the exposure setting. However, it is the good idea to test with different exposures to find the best one with a particular setup. It is also important to keep the room or setting area as dark as possible to avoid light altering the camera exposure [130].

Hence, the process and techniques during TLC preparation was all important to get the good image. Consequently the software is meaningless if the quality of image is poor. This study introduced the optimum condition for liriodenine analyzed by TLC image; all determinations were controlled in same techniques such as ensuring drying spot before developing the plate, the tank equillibium, the developing process, the spotting techniques; for instance, not only must sample areas be kept small but the volumes of sample solutions delivered the plate must be accurately known and controlled. Furthermore, the photographic process: lens aperture, resolution or shutter speed should be controlled. Moreover, when analyzing the TLC image using Scion image software, every pictures were applied with the smooting tool fixed at 10 times and similar adjusting baseline. Thus, all determinations should be done in the same settings to reduce the techniques error and bias.

TLC image analysis and HPLC-DAD were validated to confirm whether the analytical procedures employed were suitable for the purpose. This research focused on system validation to estimate whether the developed techniques was proper for liriodenine determination. Thus, the matrix variations of each sample were not presented that would describe in limitation of this study.

 Performance characteristics included specificity, accuracy, precision, linearity, limit of detection and quantitation. Calibration curve of standard compound showed good linearity relationship for both methods (r^2>0.995) over the range 5 – 200 µg/mL. The regression equation from TLC image and HPLC-DAD were $y = -0.1573x^2 + 61.392x + 151.38$ and $y = 17222.1x - 15914.4$ respectively where y is AUC and x is concentration. Average percentage of accuracy from TLC image analysis and HPLC-DAD revealed 98.57 – 103.02 % and 91.79 – 106.11 % respectively; high values indicated a satisfactory accuracy. RSDs of all parameters were less than 5 % for repeatability and reproducibility indicating that the proposed methods were precise. LOD and LOQ which calculated based on the standard deviation (σ) of the y-intercept and the slope (s) as $3\sigma/s$ and $10\sigma/s$ respectively were 3.767 and 12.557µg/spot from TLC image analysis; while LOD and LOQ values analyzed by signal to noise from HPLC showed 0.816 and 2.472 µg/mL at 248 nm and 0.994 and 3.013 µg/mL at

407nm respectively. Therefore, the results revealed that at both wavelengths providing good sensitivity and resolution for liriodenine detection. Specification was accessed using retention time, spectra and purity index of specific peak to confirm the data interpreting from HPLC. The purity peak values from both wavelengths were close to 1 indicating high purity and confirming the specificity of the method. However the chromatograms of all samples were also no interfering peak when using 407 nm; but found little interfering peaks in few samples when using 248 nm as shown in Appendix B. Beside, R_f and good separation of spot was evaluated in TLC image analysis. In conclusion,TLC image analysis as well as HPLC developed for liriodenine determination were sensitive and specific. Moreover, the accuracy and precision of methods were within the acceptable range.

Twenty-eight plant materials were determined for liriodenine content by developed TLC image analysis compared to HPLC method as shown in Table 6.There were 14 of 28 specimens found liriodenine. Among these, 6 plant's parts containing liriodenine were found related to previous studies [27, 30, 53, 78, 82, 102]; otherwise they were firstly found liriodenine in this study. However the highest content was shown in *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaf).

WHO guideline was performed as quality control methods in the three medicinal plants which containing high liriodenine content. Morphological study, TLC fingerprint and HPLC chromatogram were demonstrated for plants' identification.

According to morphological evaluation, Magnolia plant including *Michelia longifolia* and *Michelia champaca* are similar in the feature. Thus, microscopic examination for their anatomical characters was done in the stem bark as well as the leaf. As a result of trichome, it was found many in *Michelia champaca* leaf which in accordance with hairy in its leaf. Thus, to distinguish *Michelia champaca* to *Michelia longifolia*, the leaf was also collected together with the bark to identify the plant. Otherwise macroscopic study of these Magnolia plants showed that their leaf arrangement is in a spiral pattern; relatively the bark is thin and smooth [216].

Anatomical and histological observation of the leaf of *Nelumbo nucifera* showed air chamber, trichosclereid and abundant calcium oxalate. The upper leaf surface tends to have a very prominent cuticle, thereby permitting water to roll off, and not interfering with photosynthesis or promoting growth of epiphytic algae. Epidermis may be rich in chloroplasts, and a bifacial mesophyll (palisade and spongy layers) is formed. Floating leaves often have well-developed air chambers (lacunae**),** which provide buoyancy, and they may also have hard cells, sclereids, within the mesophyll that provide some toughness for the leaf and prevent the layers from becoming collapsed [221].

The yields of ethanolic extracts in *Michelia longifolia* bark ranged from 7.95 to 18.43 % (average of 12.63 %), while those of *Michelia champaca* bark ranged from 6.58 to 40.01 % (average of 18.65 %). The yields of ethanolic extracts in *Nelumbo nucifera* leaves ranged from 15.79 to 34.67 % (average of 24.47 %).

Pharmacognostic parameters from *Michelia longifolia* bark revealed that the acid-insoluble ash, total ash, loss on drying and water content should be not more than 3.49, 5.74, 7.17 and 6.67 % of dry weight respectively; while ethanol and water-soluble extractive should be not less than 3.85 and 8.25 % of dry weight respectively. *Michelia champaca* bark showed that the acid-insoluble ash, total ash, loss on drying and water content should be not more than 2.98, 6.25, 5.62 and 7.37 % of dry weight respectively; while ethanol and water-soluble extractive should be not less than 6.71 and 12.38 % of dry weight respectively. Pharmacognostic parameters of *Nelumbo nucifera* leaves revealed that the acid-insoluble ash, total ash, loss on drying and water content should be not more than 2.61, 9.62, 7.69 and 7.06 % of dry weight respectively; while ethanol and water-soluble extractive should be not less than 6.24 and 9.51 % of dry weight respectively. Whereas there were no volatile oil found in all collected medicinal plants. These parameters were useful for detecting low-grade products as well as for determining the extractive values [222].

This study supported that liriodenine was the major compound in the bark of *Michelia longifolia* and *Michelia champaca* [78, 82]; moreover, it constitutes the yellow

coloring matter in the bark. TLC image showed a well-defined yellow fluorescent spot of liriodenine at the R$_f$ value of 0.75 under UV 365 nm.

According to HPLC system, the results detected from UV and DAD system were compared and tested by statistical analysis. There were no significant differences between both detectors from three plant specimens. In case of TLC image, the results were confirmed with those of the reference method (HPLC-DAD).

Liriodenine content from TLC imageas well as HPLC-DAD analysis was averagely 14.7 and 15.3 %mg respectively and found comparatively high in Uthai Thani, Prachin Buri and Buri Ram province for *Michelia longifolia* bark. While *Michelia champaca* bark showed average content at 27.4 and 29.0 %mg respectively and found high in Phetchabun and Nong Khai province. Comparatively the average compound content was found higher in *Michelia champaca* bark than in *Michelia longifolia* bark. In addition to the leaves of *Nelumbo nucifera*, the content was 4.8 %mg from both methods and found comparatively high in Nakhon Pathom, Lampang and Bangkok province. Previous study revealed liriodenine content in *Mollinedia* genus (family Monimiaceae) indicating that the amount of compound increased according to the plant's phenophase, being less in sterile plants, increasing in plants with flowers and reaching its maximum in the fruiting plants [97]. However, variation of liriodenine content may be due to various factors such as effect of growth period and environment, maturity of plant, genetic variations [222]. Additionally, the season of collection, difference of drying process, storage time and condition could be lead to fluctuations in the liriodenine content [223].

TLC image demonstrated clearly fluorescent illuminating spot of liriodenine at R$_f$ 0.75 under UV 365 nm. The fluorescent coloring spot which was a dominant characteristic of liriodenine on TLC plate allowed TLC image analysis to be more suitable and convenient for the determination of liriodenine. The results supported that the image analysis software packages were proper to investigate the fluorescent compounds in quantitative thin layer chromatography [134].

In summary, these pharmacognostic parameters could be set as standardization of these medicinal plants. The quantitative methods of liriodenine content in the crude drug using TLC image and HPLC were developed; there was no significant difference between the results of both quantitative methods ($p>0.05$) as analyzed by Student's paired t-test. As compared to HPLC analysis, TLC-image analysis using Scion image software could be applied for screening the content of this compound as its low-cost, rapid and simple application.

LIMITATION

Method validation was conducted as the system precision using standard liriodenine according to the intended purpose of this study as to develop general methods for liriodenine determination. However, this study did not determine the matrix effect on an instrument response. Bonfiglio et al. [224] reported that the chemical nature of a compound had a significant effect on the degree of matrix effects.

Thus, the author strongly suggests that to remove or minimize matrix effects, modification to the sample extraction methodology and improved chromatographic separation must be performed for further study.

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APPENDICES

APPENDIX A

Pharmacognostic parameters (%w/w) of *Michelia longifolia* (bark), *Michelia champaca* (bark) and *Nelumbo nucifera* (leaves)

Table 20 Pharmacognostic parameters (%w/w) of *Michelia longifolia* bark

Sources	No.	Water content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
						EtOH	Water
1. Yasothon	1	6.19	7.35	5.30	2.87	4.72	9.49
	2	6.39	7.37	5.42	2.73	4.23	9.23
	3	6.50	7.31	5.40	2.87	4.08	9.52
2. Petchabun	1	5.29	6.51	5.43	2.81	5.30	10.26
	2	5.38	6.47	5.50	2.80	4.95	10.39
	3	5.28	6.59	5.58	2.91	4.53	10.25
3. Chiang Rai	1	7.68	7.16	6.42	4.10	3.71	11.88
	2	7.39	7.10	6.40	4.22	3.88	11.86
	3	7.49	7.05	6.49	4.19	3.85	11.91
4. Uthai Thani	1	7.30	8.63	7.30	5.64	2.43	5.62
	2	7.80	8.59	7.43	5.41	2.38	5.44
	3	7.39	8.63	7.30	5.70	2.40	5.48
5. Chanthaburi	1	6.49	8.22	3.89	1.24	3.08	6.94
	2	6.59	8.23	3.99	1.20	2.79	6.81
	3	6.49	8.22	3.93	1.23	2.88	6.86
6. Rayong	1	6.29	8.28	3.08	1.00	3.00	7.73
	2	6.59	8.21	3.01	0.92	3.36	7.15
	3	6.70	8.30	3.07	1.21	3.60	7.41
7. Buri Ram	1	6.49	7.27	10.38	8.57	4.12	4.80
	2	6.50	7.36	10.13	8.16	3.41	5.26
	3	6.49	7.35	10.53	8.68	3.56	5.05
8. Prachin Buri	1	14.66	13.24	3.48	1.42	3.94	7.17
	2	14.40	13.10	3.45	1.05	4.27	6.99
	3	14.39	13.11	3.36	1.79	3.95	7.13

Table 20 Pharmacognostic parameters (%w/w) of *Michelia longifolia* bark (cont.)

Sources	No.	Water content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
						EtOH	Water
9. Rayong2	1	3.00	4.21	4.83	2.56	4.39	8.19
	2	3.00	4.25	4.82	2.56	3.97	7.99
	3	2.80	4.17	4.81	2.68	4.50	8.12
10. Lampang	1	4.39	5.38	6.20	4.59	2.33	7.57
	2	4.39	5.27	6.24	4.53	2.57	7.85
	3	4.39	5.23	6.36	4.43	2.48	7.82
11. Nakhon Ratchasima	1	7.00	7.16	6.21	2.76	4.65	9.16
	2	7.09	7.18	6.18	2.77	5.12	9.28
	3	6.99	7.14	6.24	2.65	5.00	9.07
12. Yasothon2	1	5.00	6.11	4.82	2.39	6.64	12.55
	2	4.99	6.16	4.81	2.30	6.44	12.70
	3	4.90	6.14	4.77	2.45	6.08	12.64
13. Lampang2	1	5.29	5.89	6.15	4.15	2.36	7.03
	2	5.09	5.94	6.18	4.12	1.67	6.92
	3	5.40	5.94	6.08	4.89	2.13	6.63
14. Buri Ram2	1	7.39	5.83	7.97	5.52	2.88	6.14
	2	7.30	5.65	8.19	6.15	2.68	6.11
	3	7.40	5.79	8.05	6.00	2.83	6.08
15. Nong Khai	1	7.15	6.44	4.41	2.20	5.39	9.58
	2	7.40	6.40	4.48	2.21	5.19	9.61
	3	7.39	6.58	4.40	2.25	5.62	9.61
Min-Max		2.80-14.66	4.17-13.24	3.01-10.53	0.92-8.68	1.67-6.64	4.80-12.70
Grand mean ±pooled SD		6.67±0.13	7.17±0.06	5.74±0.08	3.49±0.21	3.85±0.25	8.25±0.14

Table 21 Pharmacognostic parameters (%w/w) of *Michelia champaca* bark

Sources	No.	Water content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
						EtOH	Water
1. Nakhon Pathom	1	8.79	9.70	7.50	3.76	3.52	11.52
	2	8.80	9.72	7.63	3.55	3.42	11.31
	3	8.80	9.86	7.55	3.44	2.74	11.03
2. Phetchabun	1	9.80	9.60	6.38	3.09	3.60	8.84
	2	9.79	9.62	6.50	3.04	3.56	8.99
	3	9.88	9.73	6.49	2.92	3.28	9.13
3. Chanthaburi	1	9.90	7.73	6.06	1.96	4.92	12.78
	2	9.80	7.98	6.08	1.94	5.98	13.03
	3	9.79	7.81	5.96	1.91	6.34	13.14
4. Rayong	1	7.69	5.60	3.70	2.04	4.16	8.05
	2	7.60	5.77	3.80	2.08	3.46	8.06
	3	7.70	5.74	3.61	2.21	3.60	8.01
5. Rayong2	1	4.10	1.60	4.17	2.14	3.38	6.93
	2	4.30	1.68	4.19	2.08	3.28	6.97
	3	4.20	1.57	4.07	2.13	3.35	7.17
6. Lampang	1	3.00	0.55	5.86	3.68	3.76	9.56
	2	2.99	0.69	5.81	3.61	3.83	9.65
	3	3.00	0.60	5.68	3.66	3.78	9.54
7. Bangkok	1	6.80	5.31	6.15	1.56	13.45	20.76
	2	7.00	5.32	6.02	1.64	13.04	20.83
	3	6.80	5.29	6.20	1.70	12.95	20.74
8. Phetchabun2	1	9.79	7.00	6.20	3.95	3.14	8.74
	2	9.79	7.14	6.26	3.90	2.81	8.55
	3	9.79	7.04	6.18	3.91	2.89	8.92

Table 21 Pharmacognostic parameters (%w/w) of *Michelia champaca* bark (cont.)

Sources	No.	Water content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
						EtOH	Water
9.Nakhon Pathom2	1	5.80	5.48	7.65	3.61	4.36	11.91
	2	5.70	5.58	7.61	3.61	4.69	11.88
	3	5.60	5.61	7.67	3.64	3.94	11.77
10.Uthai thani	1	6.00	5.09	5.85	1.67	12.98	21.50
	2	6.20	4.99	5.99	1.78	13.55	21.86
	3	6.00	4.91	5.95	1.67	13.22	21.92
11. Buri Ram	1	7.00	4.53	7.33	3.95	3.82	8.39
	2	6.80	4.69	7.28	4.08	3.52	8.04
	3	6.79	4.62	7.45	3.91	3.40	7.79
12. Nong Khai	1	7.66	5.10	6.16	2.05	19.24	14.15
	2	7.50	4.91	6.27	2.74	19.24	14.01
	3	7.70	4.99	6.25	1.97	18.68	14.61
13. Buri Ram2	1	6.80	4.52	7.92	5.26	3.77	11.23
	2	6.80	4.65	7.88	4.56	3.68	11.32
	3	6.80	4.70	7.72	5.29	3.49	11.59
14. Samut Prakan	1	9.50	6.61	6.83	4.58	1.55	4.89
	2	9.69	6.55	6.94	4.45	1.48	4.28
	3	9.58	6.59	6.86	4.46	1.57	4.73
15. Nong Khai2	1	7.99	5.30	5.89	1.78	15.91	26.30
	2	8.00	5.44	5.82	1.78	15.47	26.37
	3	8.10	5.48	5.90	1.53	16.14	26.45
Min-Max		2.99-9.90	0.55-9.86	3.61-7.92	1.53-5.29	1.48-19.24	4.28-26.45
Grand mean ±pooled SD		7.37±0.08	5.62±0.08	6.25±0.07	2.98±0.18	6.71±0.32	12.38±0.19

Table 22 Pharmacognostic parameters (%w/w) of *Nelumbo nucifera* leaves

Sources	No.	Water content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
						EtOH	Water
1. Lampang	1	5.99	7.07	9.29	2.42	6.50	10.80
	2	6.20	7.19	9.31	2.55	6.41	11.20
	3	6.00	7.12	9.41	2.63	6.16	10.38
2. Uthai Thani	1	6.29	7.97	10.29	2.03	4.54	10.70
	2	6.20	7.67	10.27	2.09	4.45	10.36
	3	6.20	7.63	10.38	2.19	4.60	10.58
3. Buri Ram	1	7.70	8.06	8.27	2.49	4.79	11.71
	2	7.40	8.17	8.37	2.53	4.91	11.13
	3	7.49	8.13	8.31	2.42	4.56	11.05
4. Chanthaburi	1	6.70	7.96	8.49	1.62	3.68	6.49
	2	6.80	7.93	8.68	2.64	4.27	6.79
	3	6.69	8.00	8.39	1.93	4.07	6.69
5. Nakhon Ratchasima	1	6.59	7.89	9.63	3.63	6.41	9.40
	2	6.61	7.86	9.69	3.64	6.35	9.51
	3	6.57	7.94	9.67	3.77	6.20	9.42
6. Phetchabun	1	7.08	8.35	9.87	1.98	5.70	9.79
	2	7.05	8.51	9.92	2.10	5.52	9.72
	3	7.38	8.39	9.99	2.06	5.94	9.60
7. Yasothon	1	8.83	10.02	8.30	2.89	3.97	5.54
	2	8.96	9.97	8.22	2.98	4.06	5.24
	3	9.02	9.94	8.31	2.86	3.37	5.24
8. Bangkok	1	7.69	7.10	10.90	3.61	5.05	12.13
	2	7.68	7.13	10.81	2.75	4.89	12.10
	3	7.73	7.13	10.78	3.32	4.84	12.12

Table 22 Pharmacognostic parameters (%w/w) of *Nelumbo nucifera* leaves (cont.)

Sources	No.	Water content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
						EtOH	Water
9.Nakhon Ratchasima2	1	7.67	7.71	9.38	2.11	4.71	8.58
	2	7.59	7.65	9.41	2.06	4.00	8.27
	3	7.49	7.62	9.41	2.14	4.05	8.40
10.Prachin Buri	1	6.98	9.59	11.64	3.20	10.88	12.92
	2	6.99	9.50	11.48	3.16	10.66	12.87
	3	6.89	9.43	11.55	3.01	11.25	12.61
11. Rayong	1	7.16	7.87	10.68	2.01	8.65	9.34
	2	7.39	8.05	10.57	2.01	8.45	9.61
	3	7.39	7.93	10.57	2.15	8.25	9.48
12. Lampang2	1	4.87	4.22	10.55	2.93	6.99	9.37
	2	4.70	4.24	10.64	2.67	6.98	9.22
	3	4.60	4.34	10.57	3.03	7.17	8.98
13. Nakhon Pathom	1	7.99	8.51	9.65	1.75	3.68	6.47
	2	7.98	8.59	9.72	2.75	3.72	6.66
	3	8.09	8.51	9.80	2.56	3.77	6.77
14. Nong Khai	1	7.70	8.02	7.96	2.76	5.67	11.04
	2	7.40	8.05	7.94	2.71	5.52	12.10
	3	7.49	8.08	7.99	2.86	5.20	12.00
15. Buri Ram2	1	6.70	4.99	9.33	2.81	13.26	8.80
	2	6.80	5.07	9.36	2.75	13.33	8.82
	3	6.89	5.14	9.26	2.88	13.18	8.31
Min-Max		4.60-9.02	4.22-10.02	7.94-11.64	1.62-3.77	3.37-13.33	5.24-12.92
Grand mean ±pooled SD		7.06±0.11	7.69±0.08	9.62±0.07	2.61±0.25	6.24±0.22	9.51±0.24

APPENDIX B

HPLC chromatograms at 248 and 407nm

for liriodenine determination from 28 plant samples comprised of:

A) 14 plant extracts <u>without</u> liriodenine containing

B) 14 plant extracts <u>with</u> liriodenine containing

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing

Figure 36 The HPLC chromatogram of *Rauwenhoffia siamensis* Scheff. leaf at 248 nm
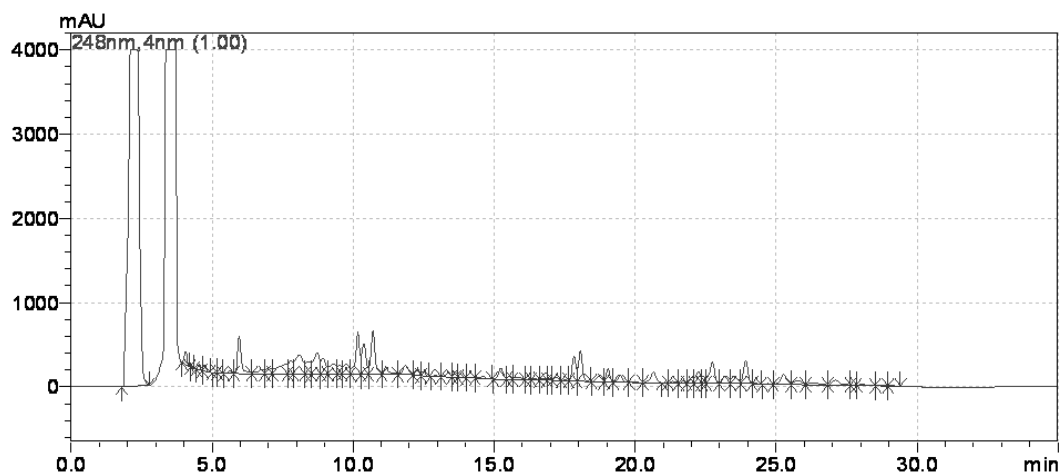
Figure 37 The HPLC chromatogram of *Rauwenhoffia siamensis* Scheff. leaf at 407 nm
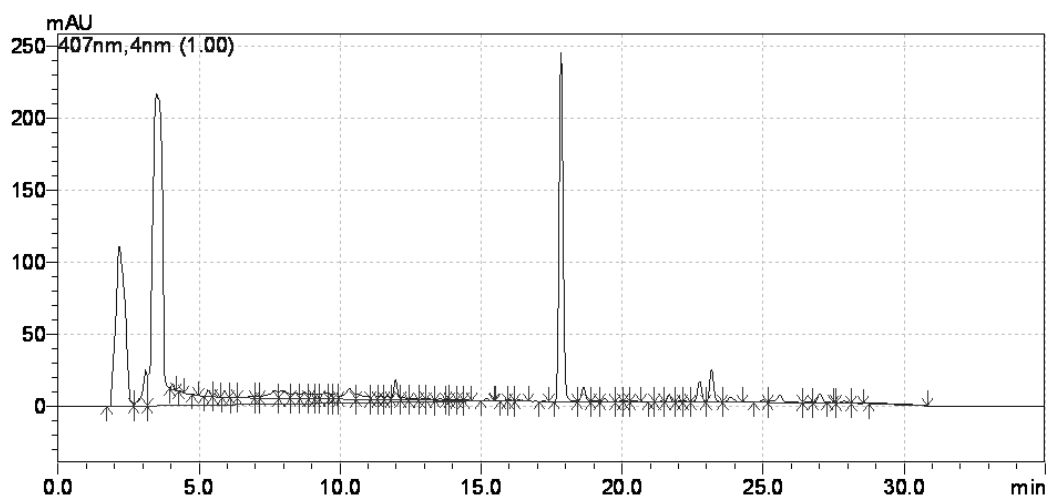
Description: Plant family: Annonaceae; Thai name: Nom meaw

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 38 The HPLC chromatogram *Anaxagorea javanica* Blume leaf at 248 nm
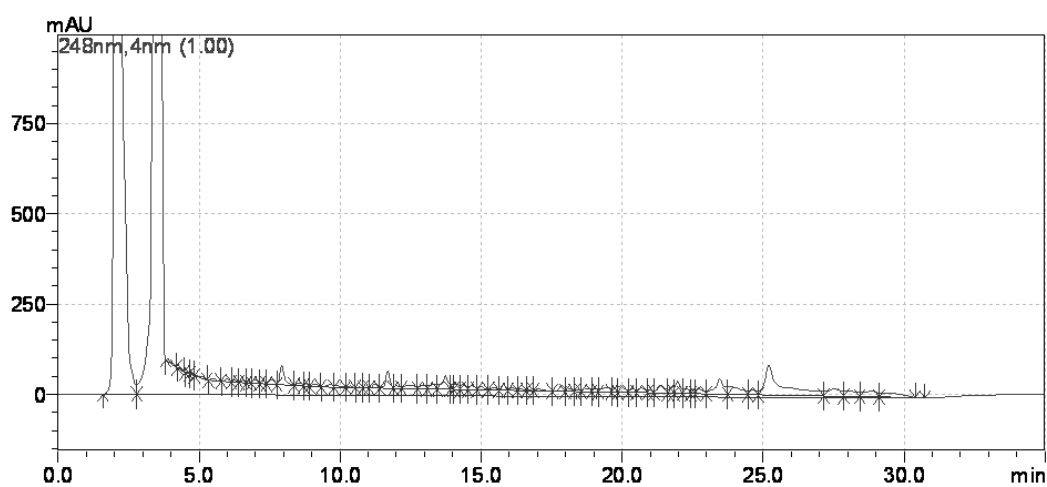
Figure 39 The HPLC chromatogram *Anaxagorea javanica* Blume leaf at 407 nm
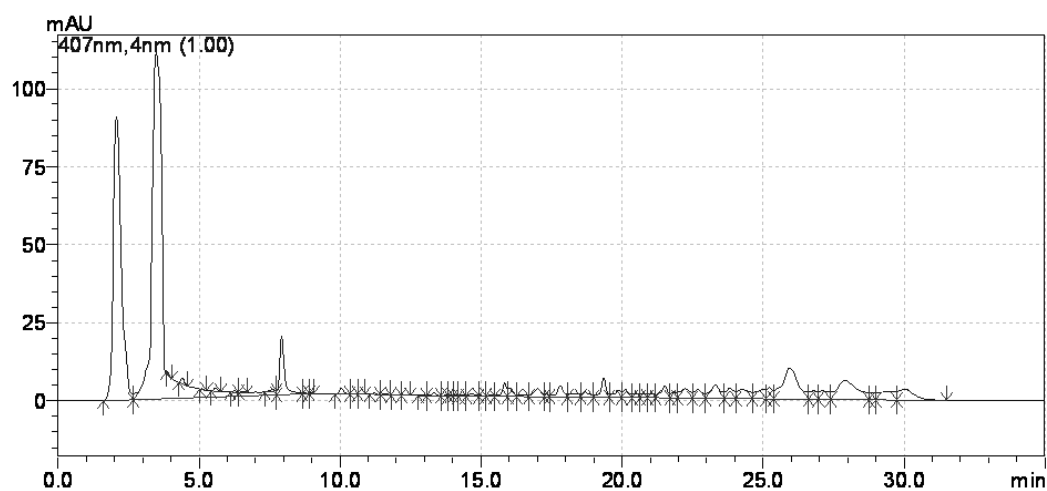
Description: Plant family: Annonaceae; Thai name: Champoon

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 40 The HPLC chromatogram *Anaxagorea javanica* Blume bark at 248 nm
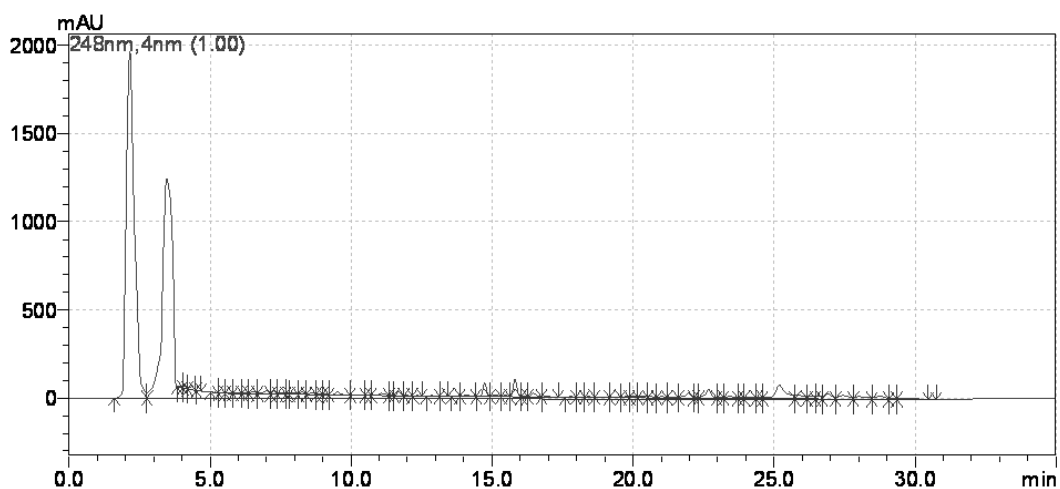
Figure 41 The HPLC chromatogram *Anaxagorea javanica* Blume bark at 407 nm
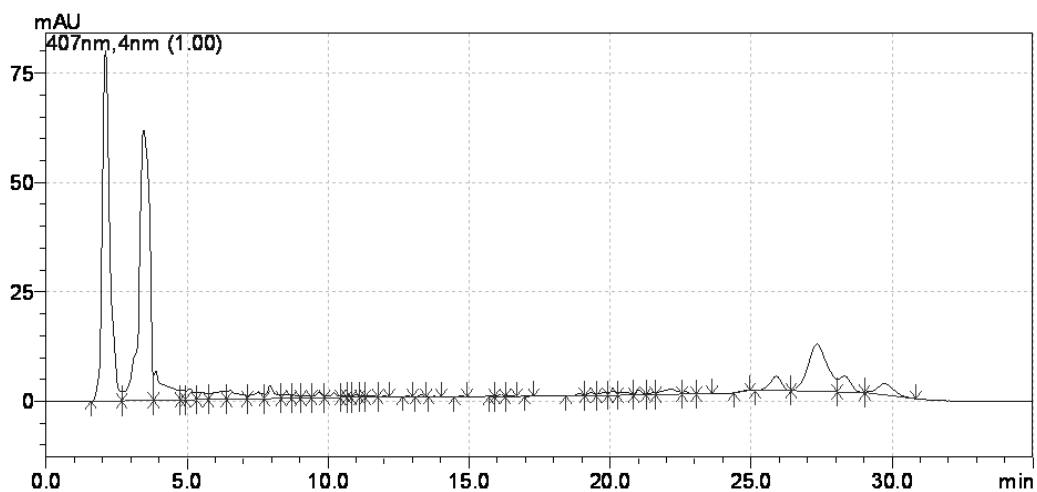
Description: Plant family: Annonaceae; Thai name: Champoon

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 42 The HPLC chromatogram *Melodorum fruticosum* Lour. leaf at 248 nm
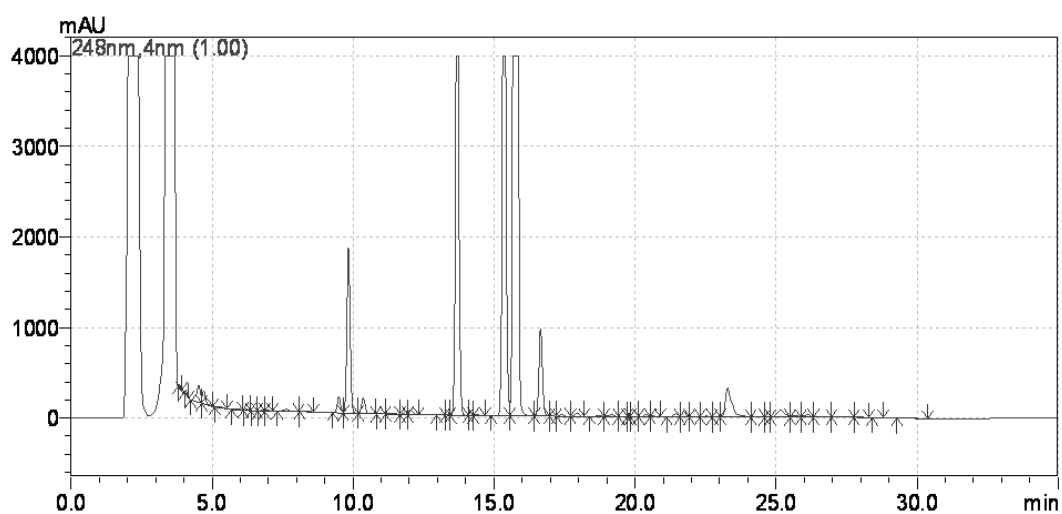
Figure 43 The HPLC chromatogram *Melodorum fruticosum* Lour. leaf at 407 nm
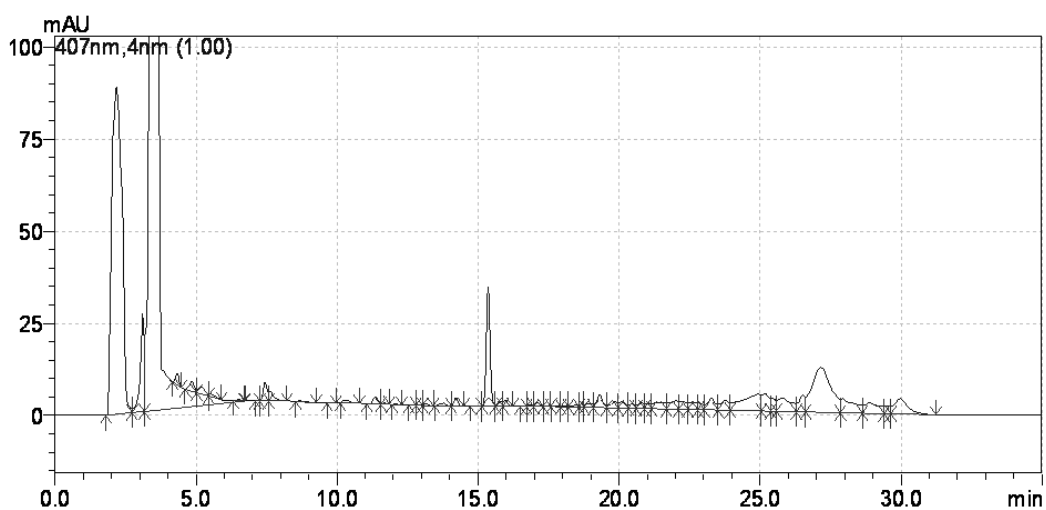
Description: Plant family: Annonaceae; Thai name: Lamduan

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 44 The HPLC chromatogram *Melodorum fruticosum* Lour. bark at 248 nm
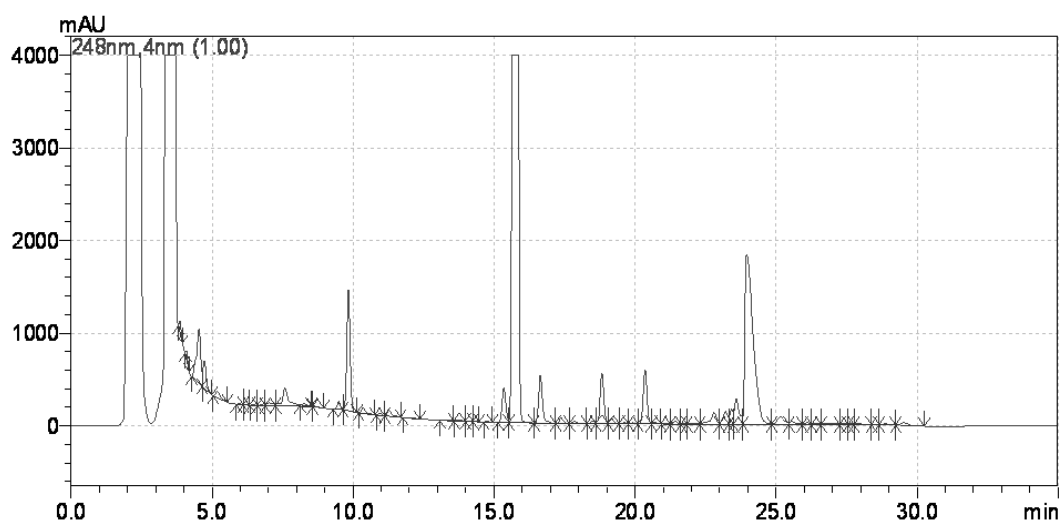
Figure 45 The HPLC chromatogram *Melodorum fruticosum* Lour. bark at 407 nm
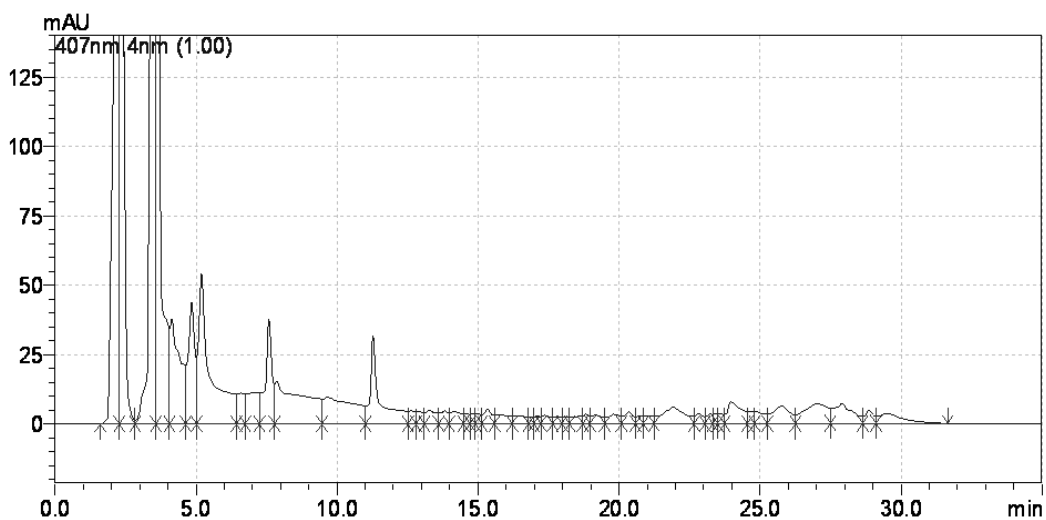
Description: Plant family; Annonaceae; Thai name: Lamduan

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 46 The HPLC chromatogram *Artabotrys hexapetalus* (L.f.) Bhandari leaf at 248 nm
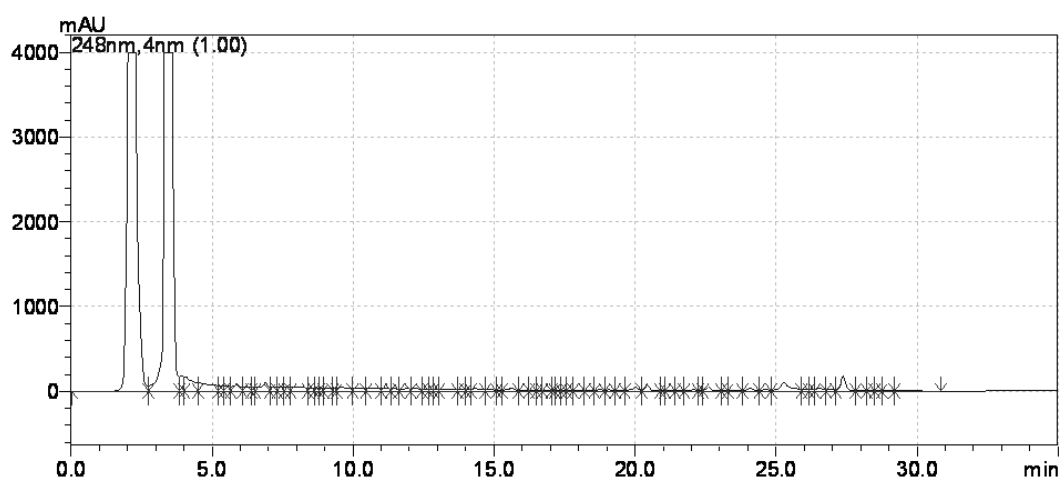
Figure 47 The HPLC chromatogram *Artabotrys hexapetalus* (L.f.) Bhandari leaf at 407 nm
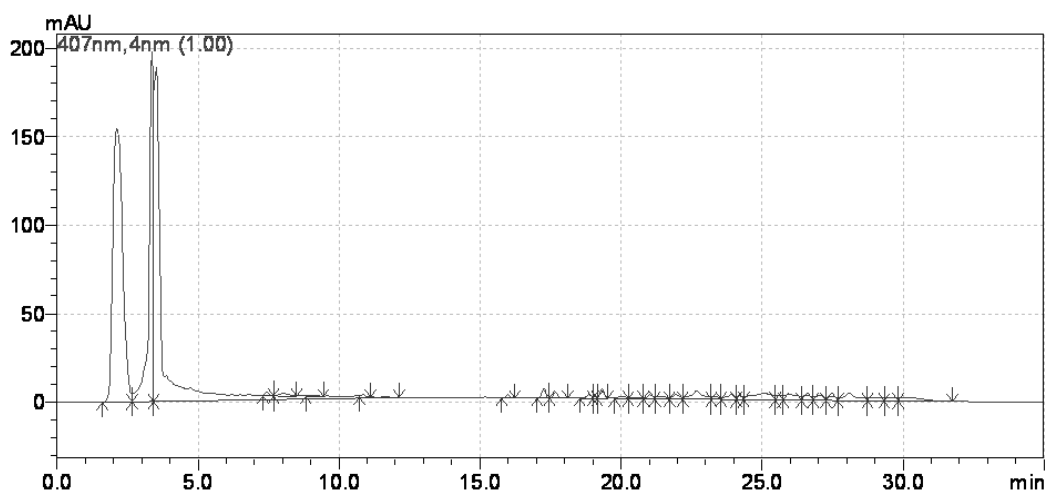
Description: Plant family: Annonaceae; Thai name: Karawek, Kradang nga cheen

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 48 The HPLC chromatogram *Desmos chinensis* Lour. leaf at 248 nm
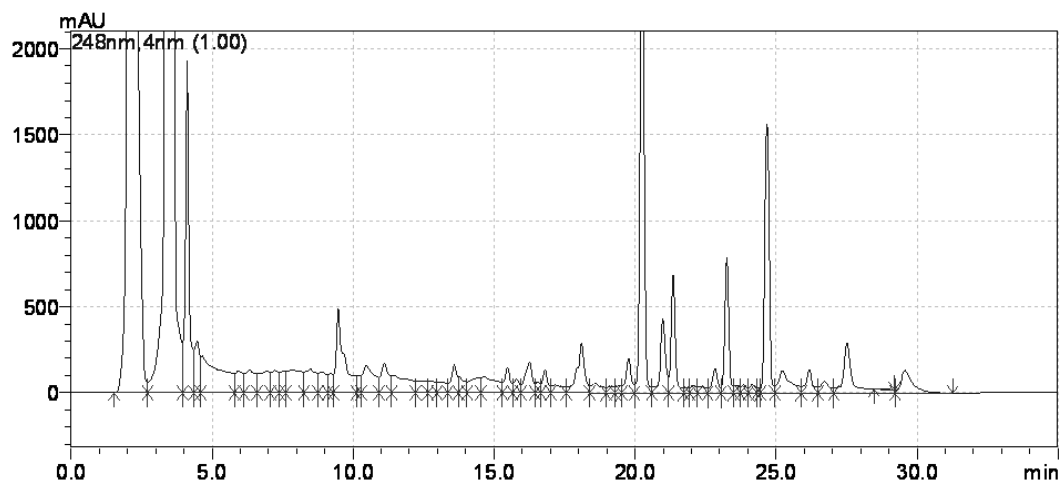
Figure 49 The HPLC chromatogram *Desmos chinensis* Lour. leaf at 407 nm
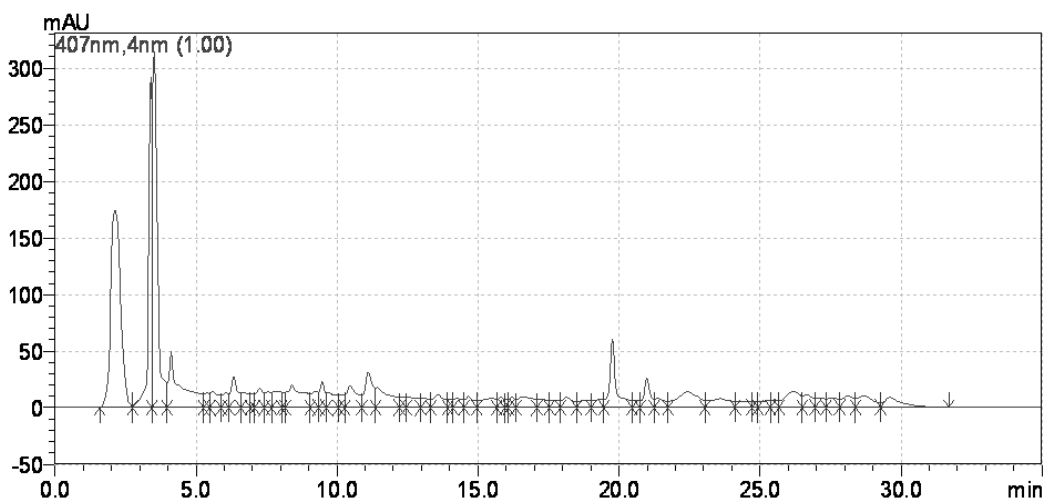
Description: Plant family: Annonaceae; Thai name: Saiyood

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 50 The HPLC chromatogram *Anomianthus dulci* (Dunn) J. Sinclair leaf at 248 nm
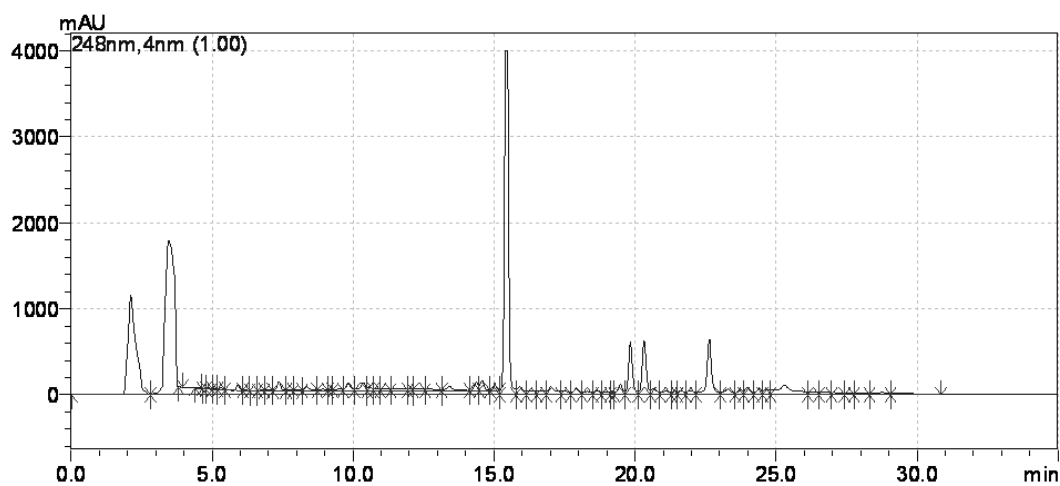
Figure 51 The HPLC chromatogram *Anomianthus dulcis* (Dunn) J. Sinclair leaf at 407 nm
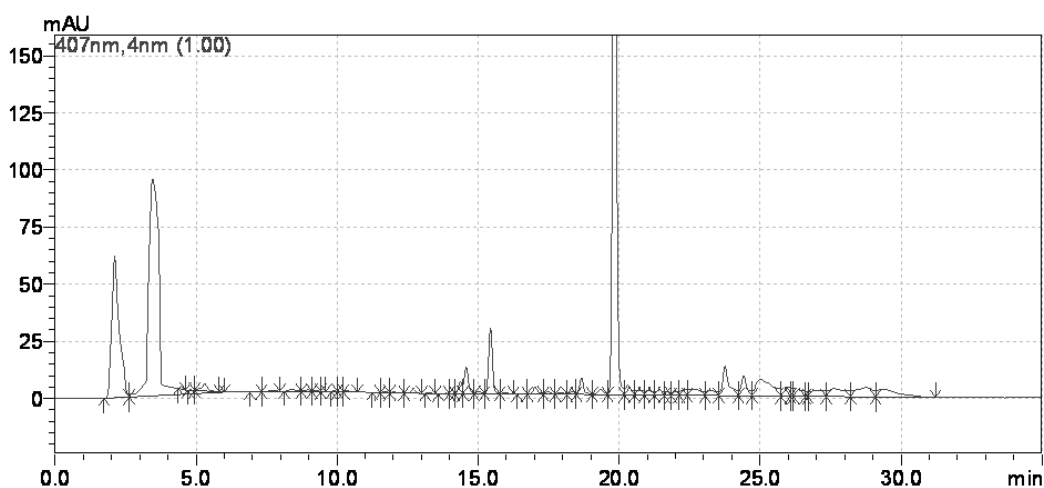
Description: Plant family: Annonaceae; Thai name: Nom woa

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 52 The HPLC chromatogram *Goniothalamus macanii* Craib leaf at 248 nm
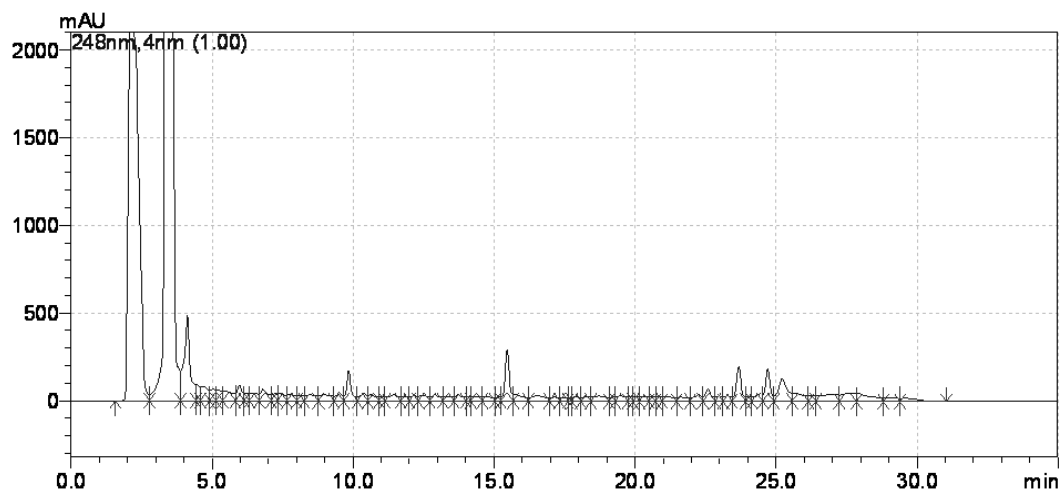
Figure 53 The HPLC chromatogram *Goniothalamus macanii* Craib leaf at 407 nm
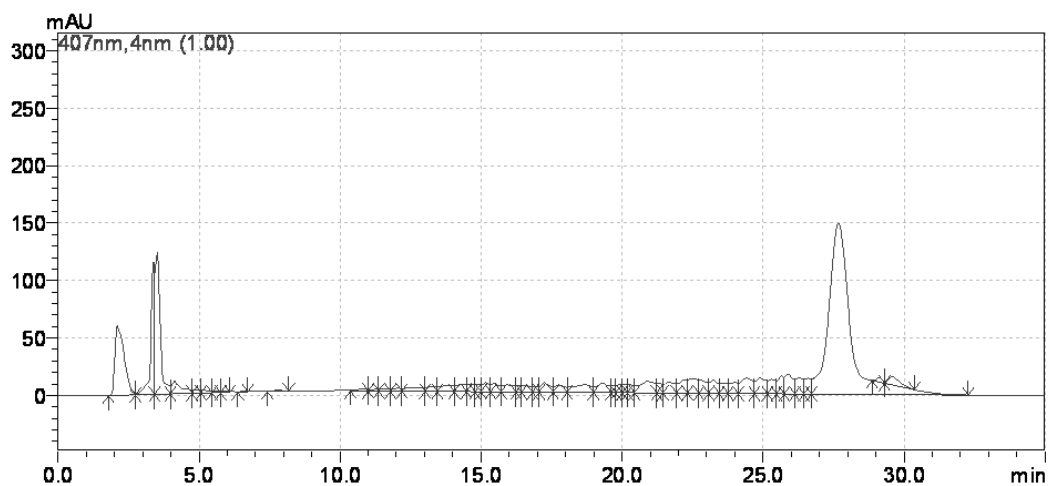
Description: Plant family: Annonaceae; Thai name: Kao lam

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 54 The HPLC chromatogram *Annona squamosa* L. leaf at 248 nm
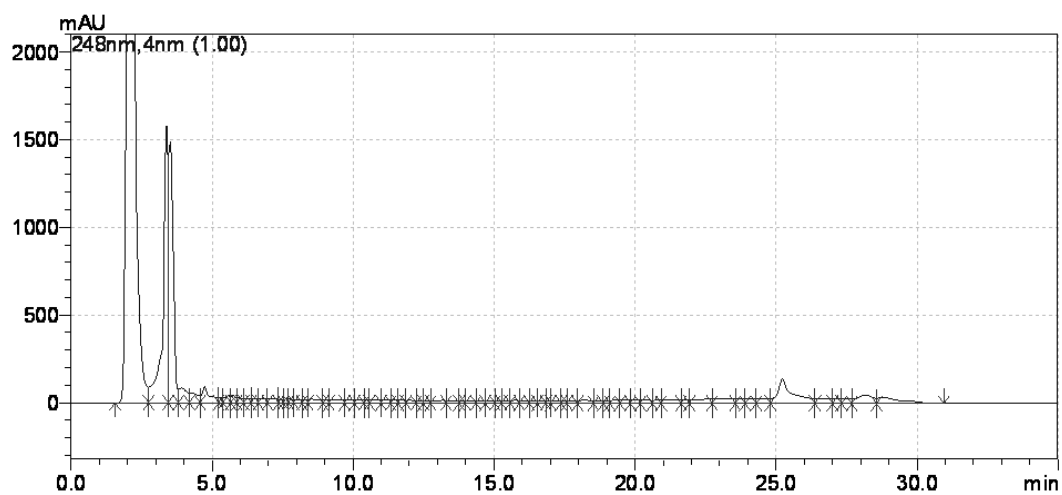
Figure 55 The HPLC chromatogram *Annona squamosa* L. leaf at 407 nm
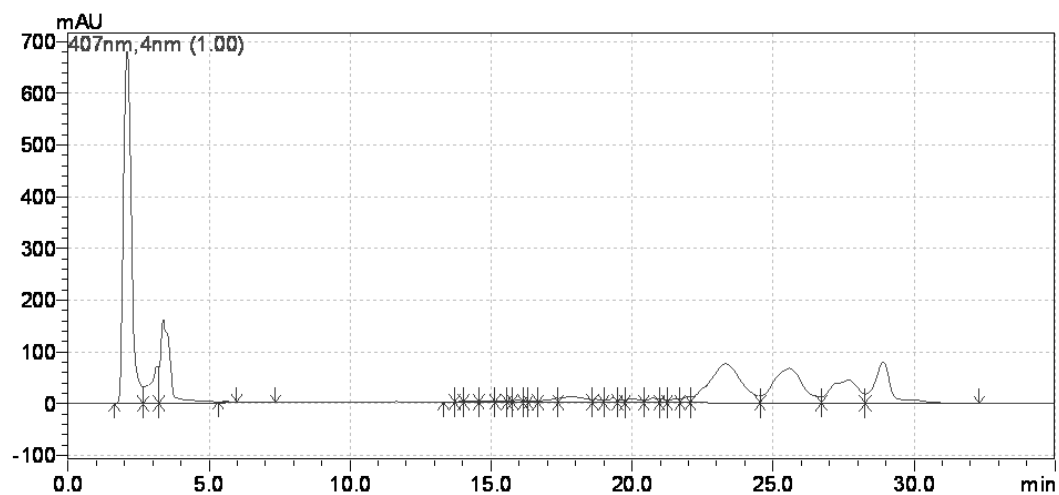
Description: Plant family: Annonaceae; Thai name: Noina

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 56 The HPLC chromatogram *Annona reticulata* L. leaf at 248 nm
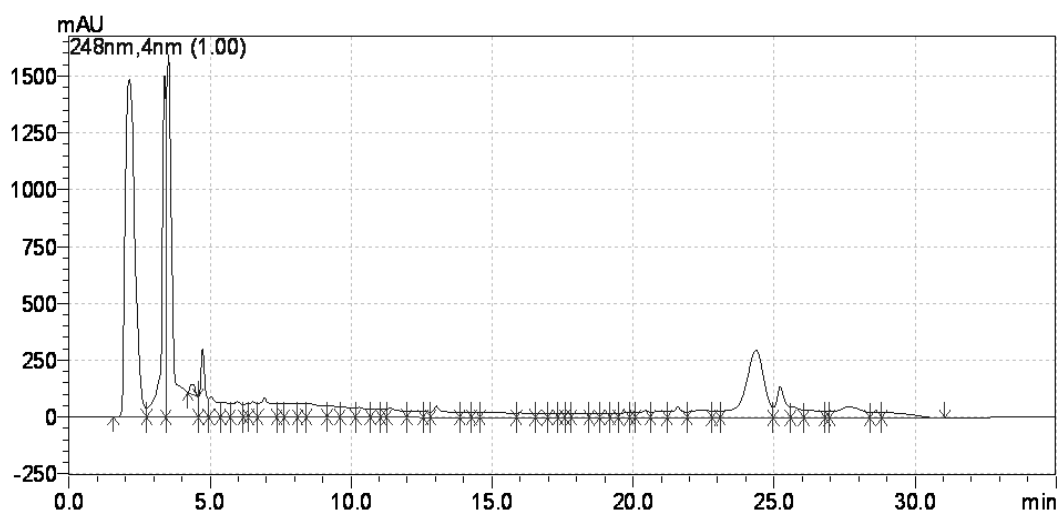
Figure 57 The HPLC chromatogram *Annona reticulata* L. leaf at 407 nm
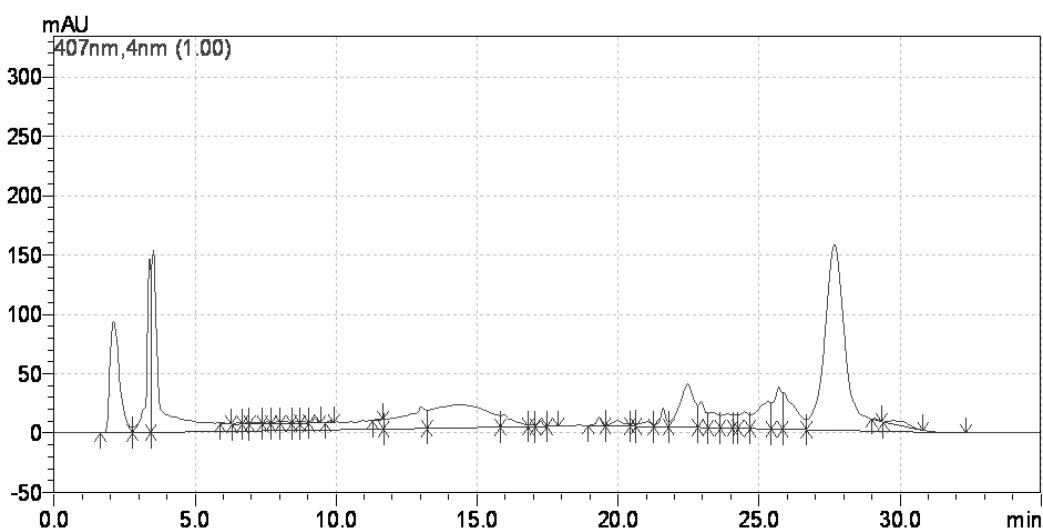
Description: Plant family: Annonaceae; Thai name: Noi nong

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 58 The HPLC chromatogram *Annona reticulata* L. bark at 248 nm
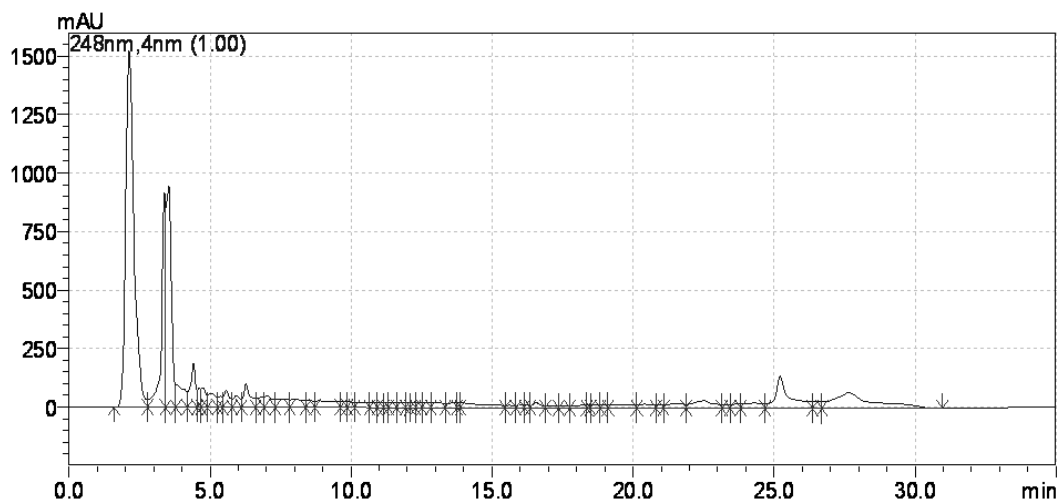
Figure 59 The HPLC chromatogram *Annona reticulata* L. bark at 407 nm
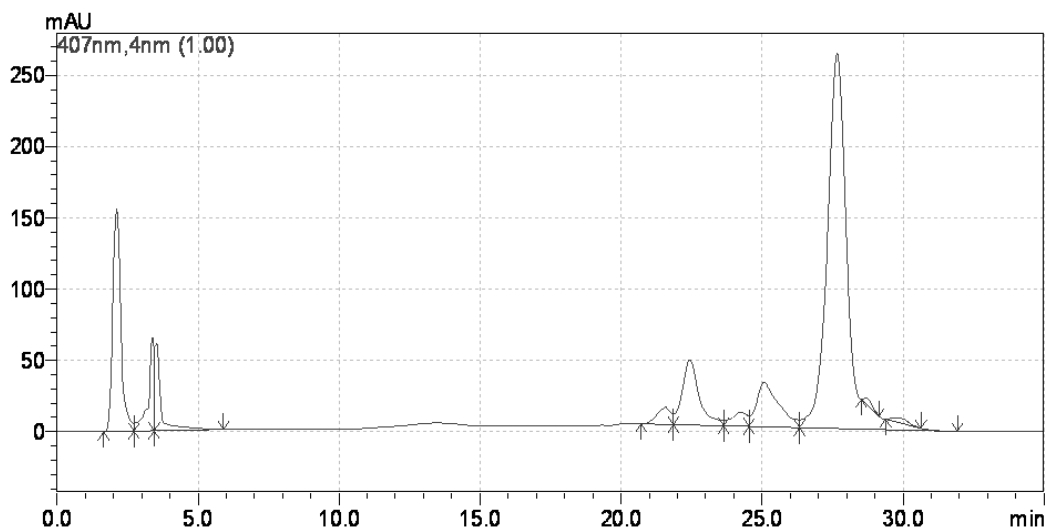
Description: Plant family: Annonaceae; Thai name: Noi nong

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 60 The HPLC chromatogram *Annona muricata* L. leaf at 248 nm
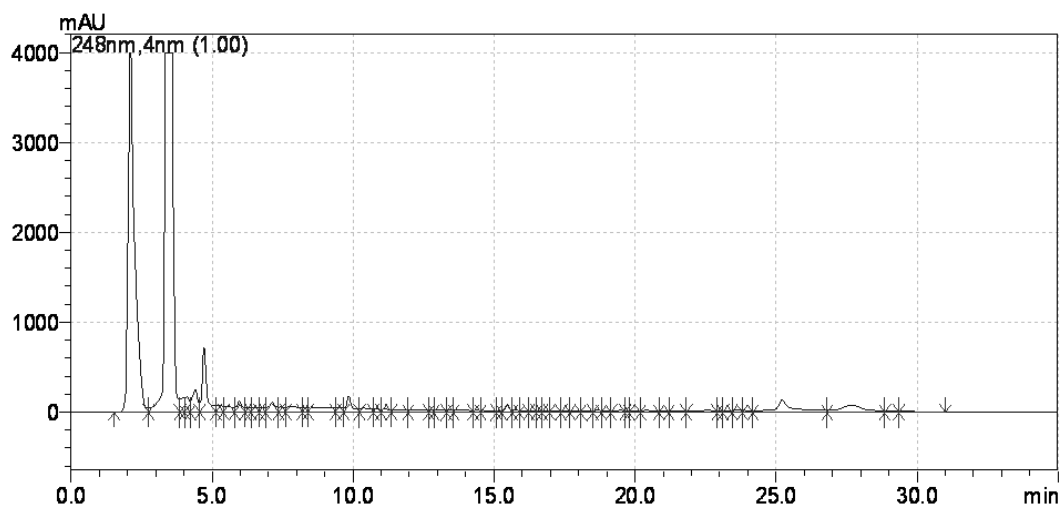
Figure 61 The HPLC chromatogram *Annona muricata* L. leaf at 407 nm
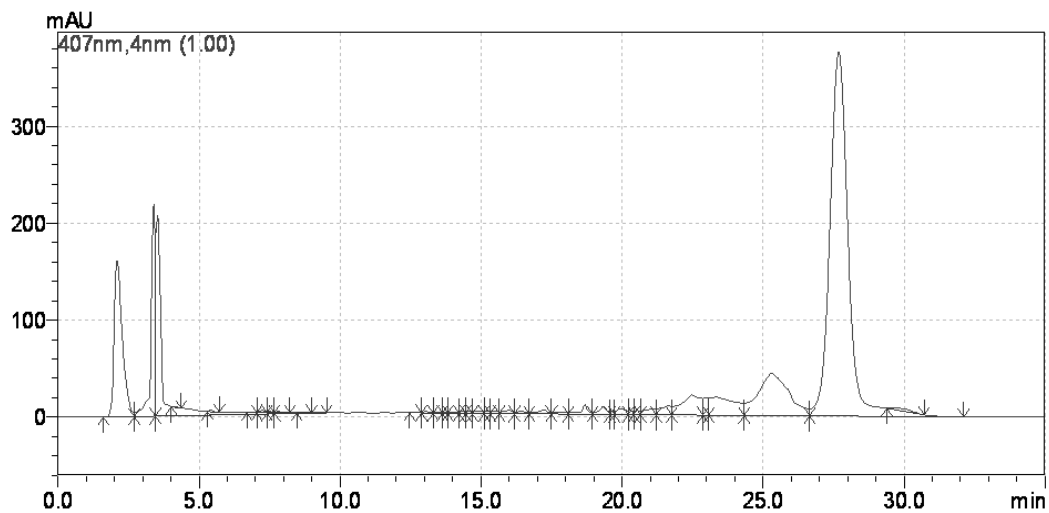
Description: Plant family: Annonaceae; Thai name: Thurain thet

Chromatograms of plant extracts for liriodenine screening

A.) Plant extracts without liriodenine containing (cont.)

Figure 62 The HPLC chromatogram *Mitrephora maingayi* Hook f.&Thomson leaf at 248 nm
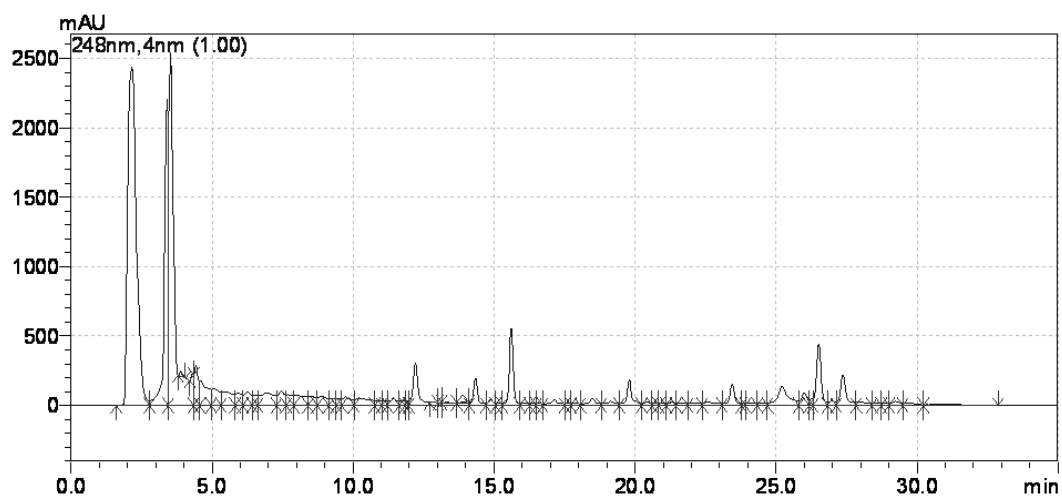
Figure 63 The HPLC chromatogram *Mitrephora maingayi* Hook f.&Thomson leaf at 407 nm
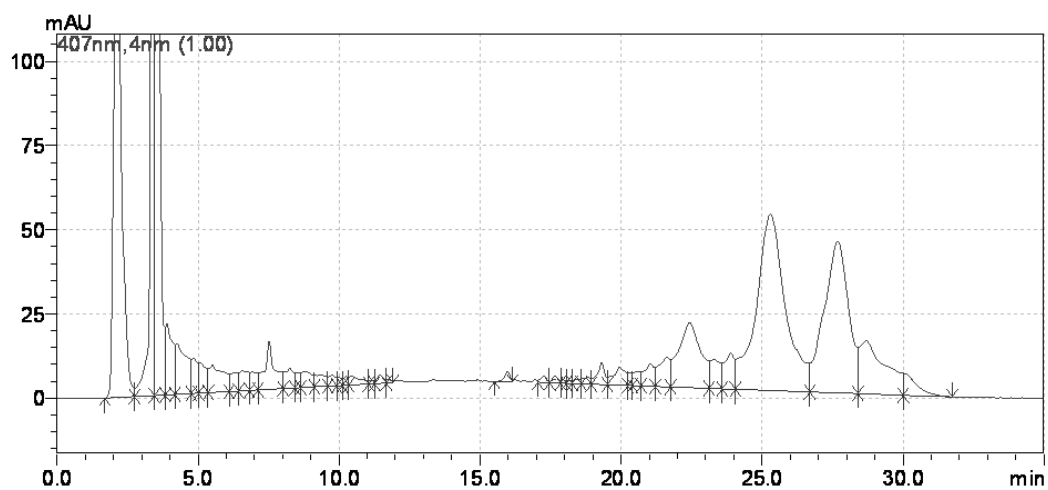
Description: Plant family: Annonaceae; Thai name: Nang daeng, Porkeehad

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing

Figure 64 The HPLC chromatogram *Michelia longifolia* Blume leaf at 248 nm
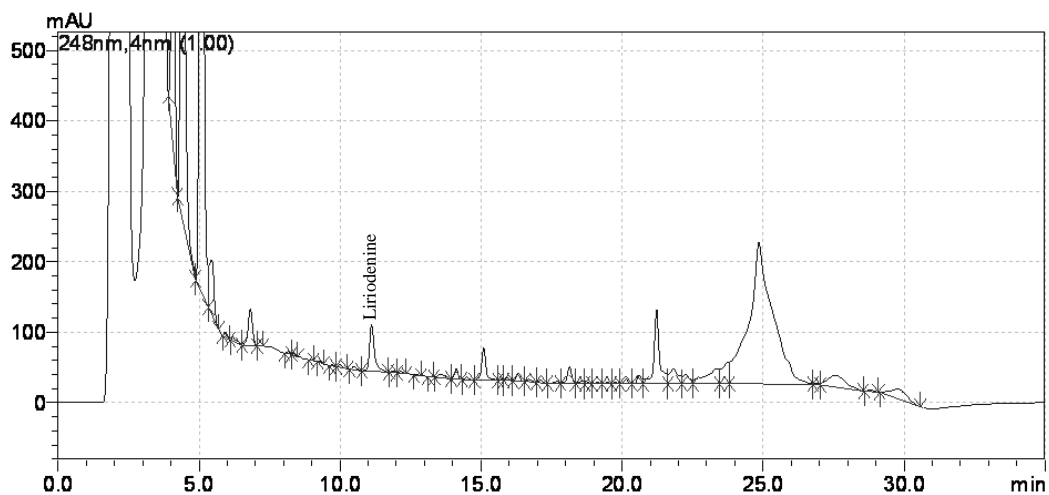
Figure 65 The HPLC chromatogram *Michelia longifolia* Blume leaf at 407 nm
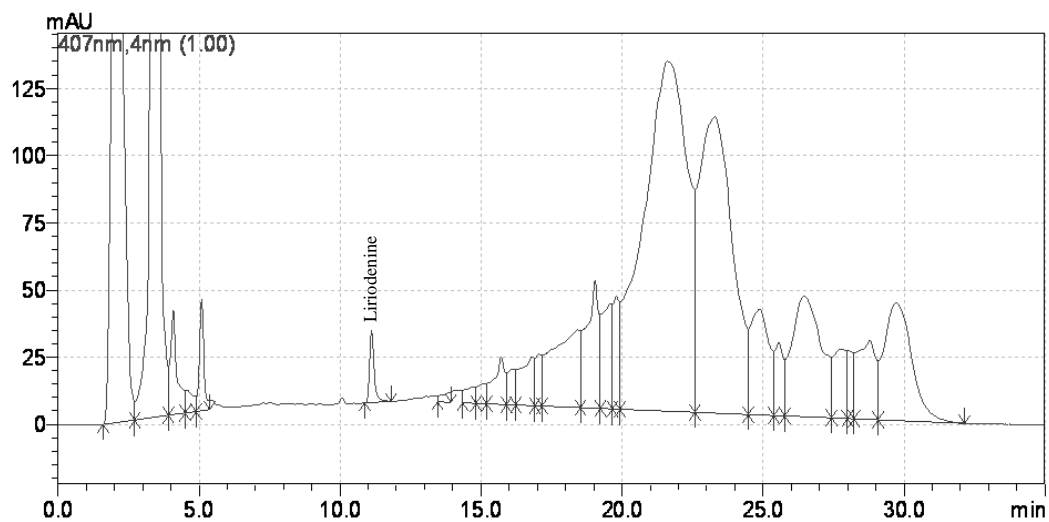
Description: Plant family: Magnoliaceae; Thai name: Champi

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 66 The HPLC chromatogram *Michelia longifolia* Blume bark at 248 nm
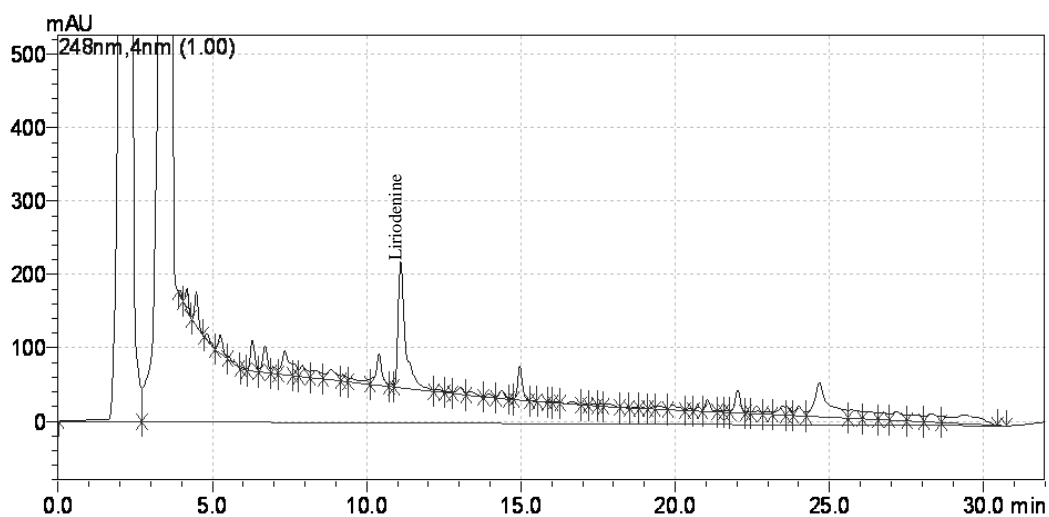
Figure 67 The HPLC chromatogram *Michelia longifolia* Blume bark at 407 nm
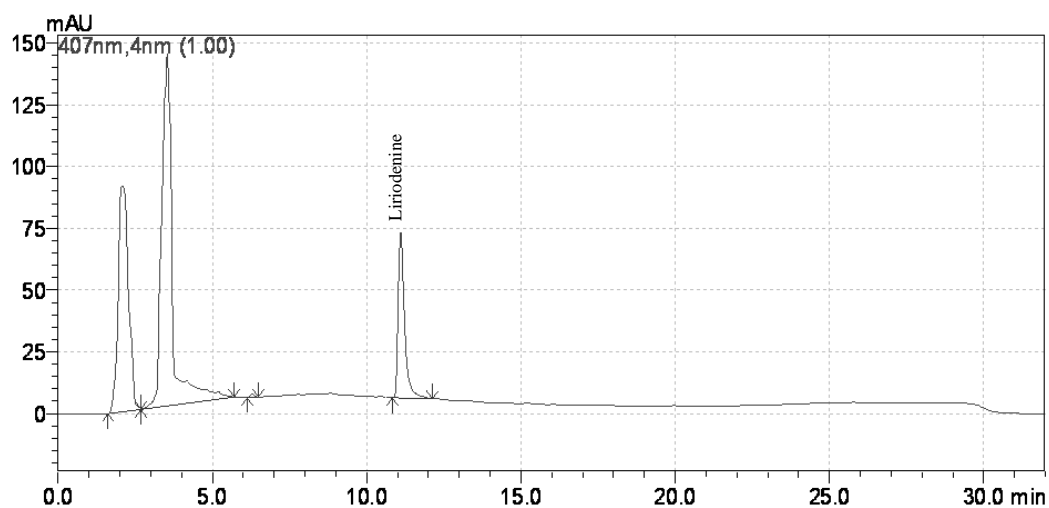
Description: Plant family: Magnoliaceae; Thai name: Champi

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 68 The HPLC chromatogram *Michelia champaca* Linn. leaf at 248 nm
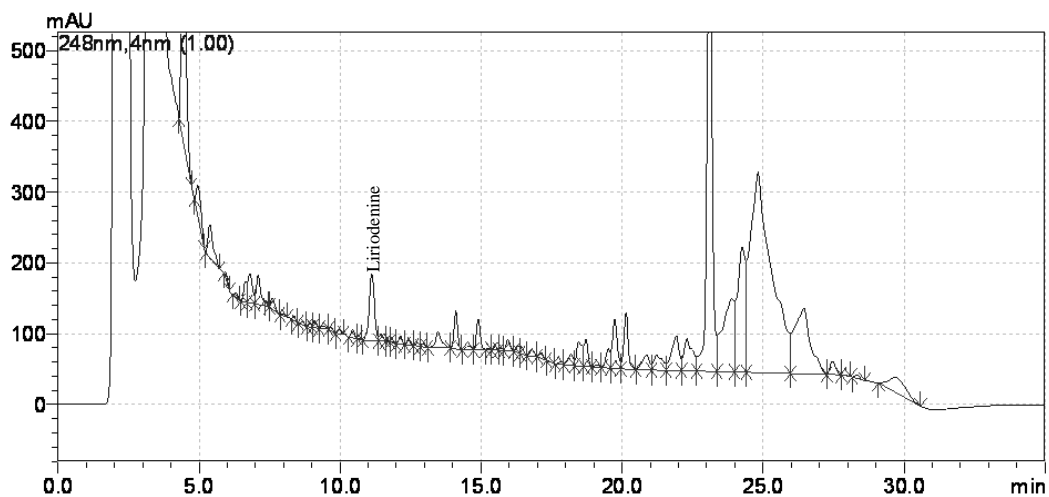
Figure 69 The HPLC chromatogram *Michelia champaca* Linn. leaf at 407 nm
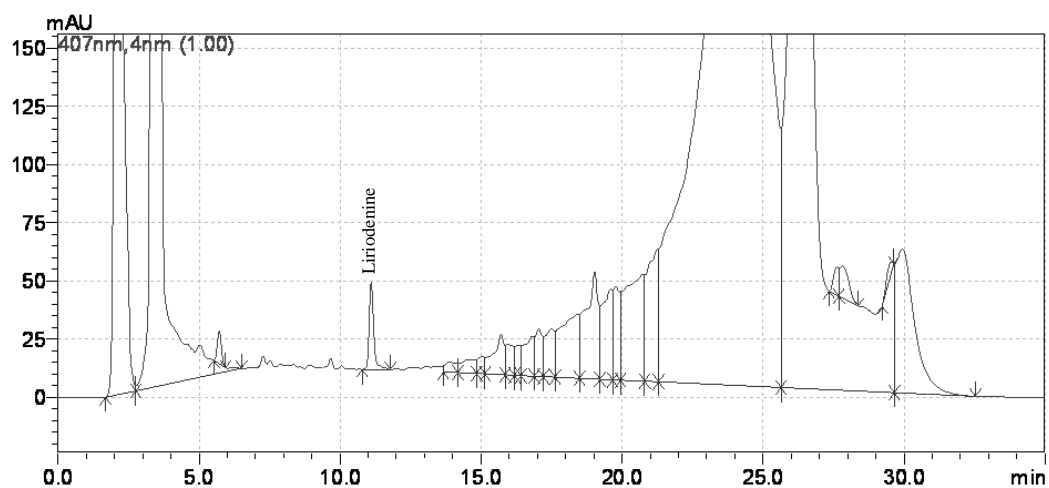
Description: Plant family: Magnoliaceae; Thai name: Champa

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 70 The HPLC chromatogram *Michelia champaca* Linn. bark at 248 nm
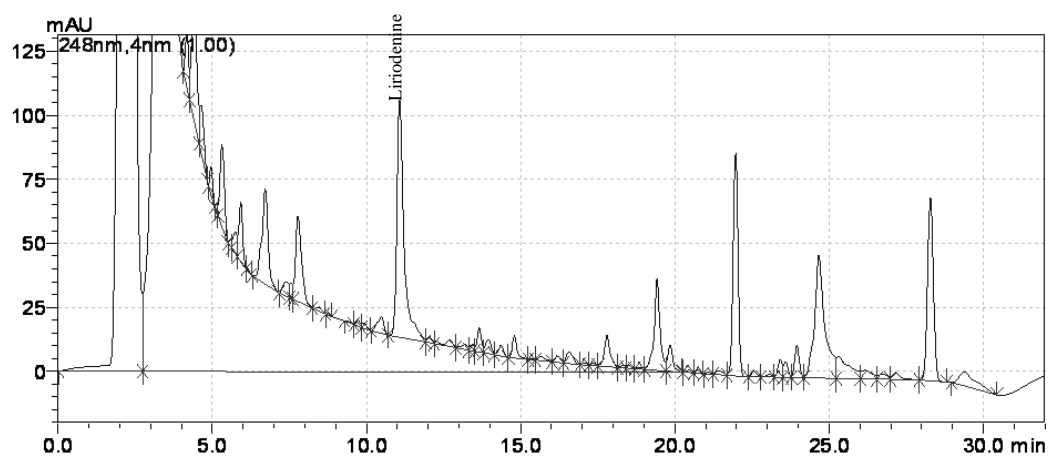
Figure 71 The HPLC chromatogram *Michelia champaca* Linn. bark at 407 nm
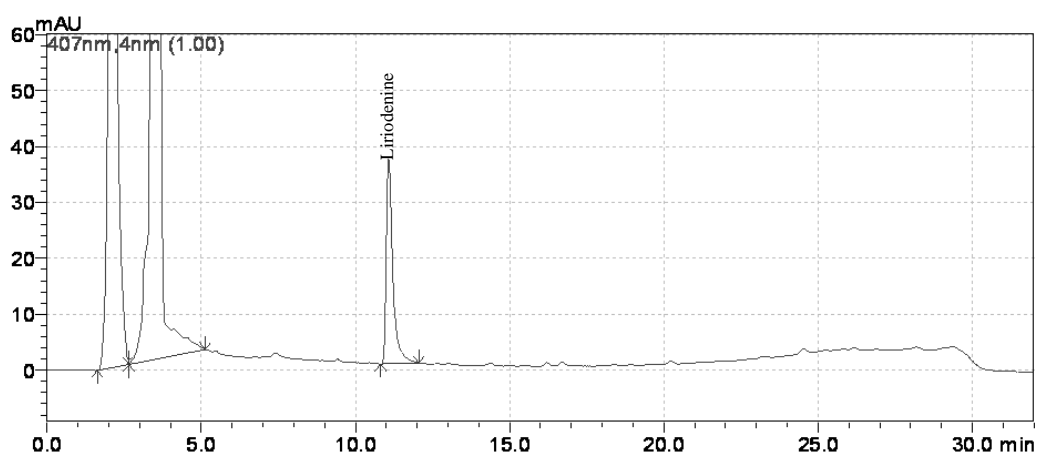
Description: Plant family: Magnoliaceae; Thai name: Champa

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

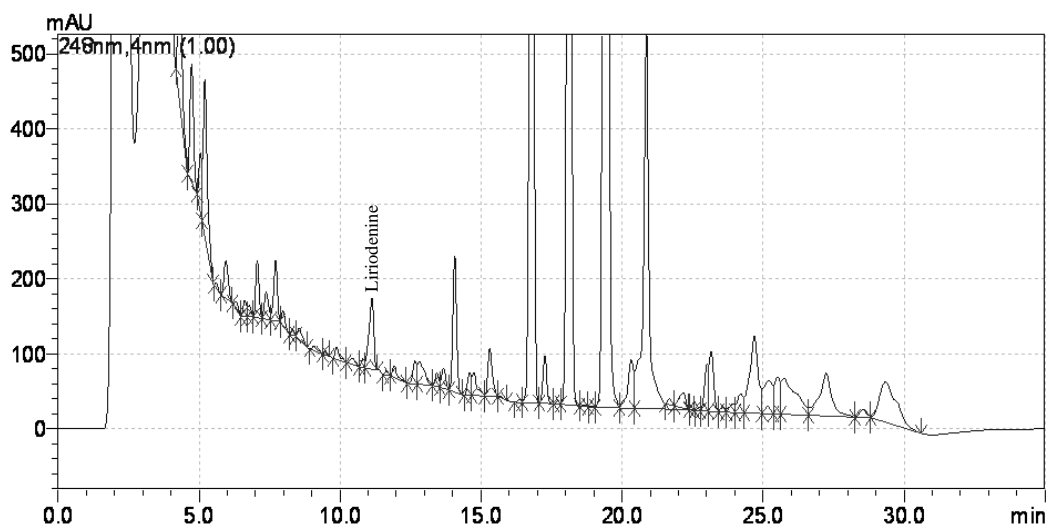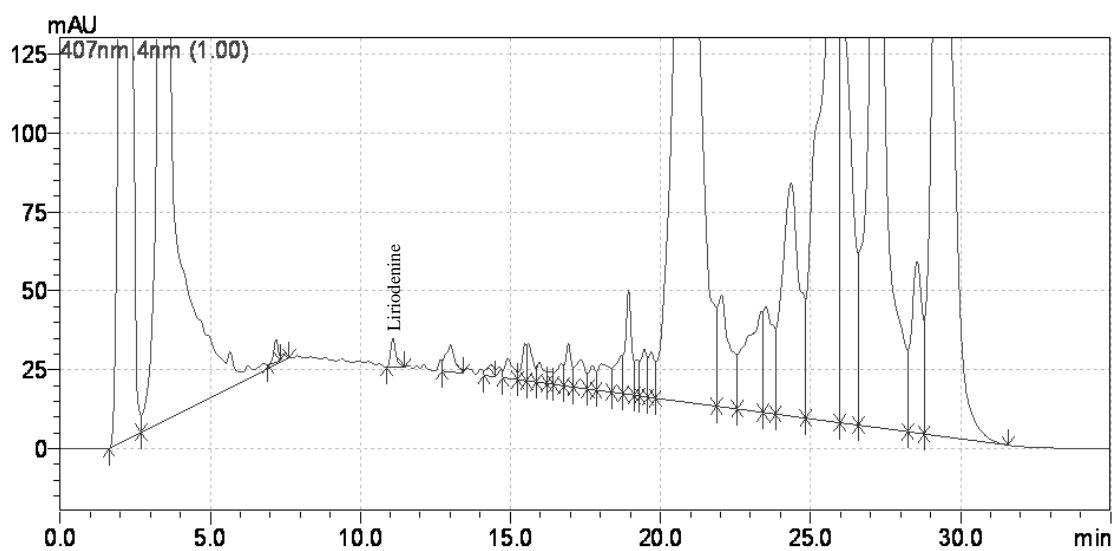**Figure 72** The HPLC chromatogram *Magnolia figo* Lour. leaf at 248 nm
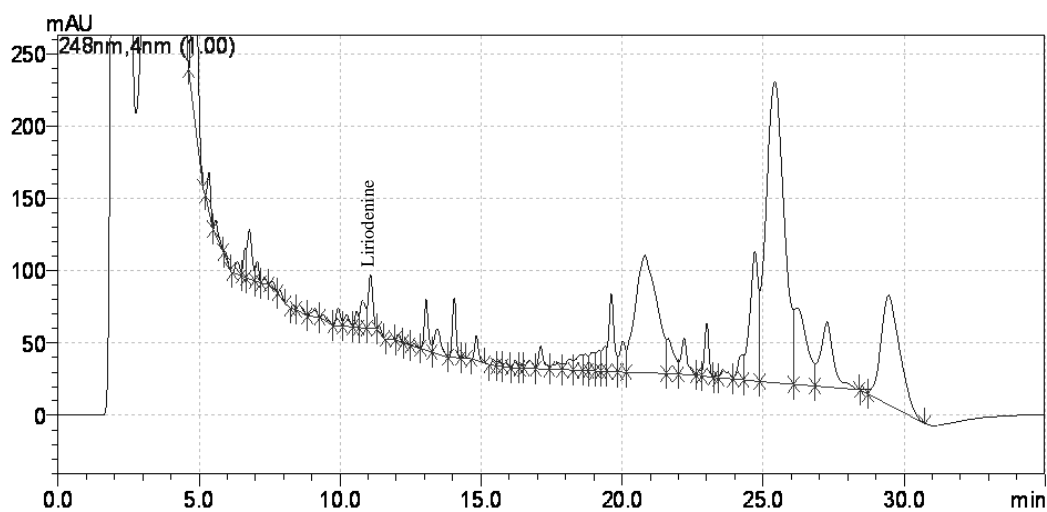
Figure 73 The HPLC chromatogram *Magnolia figo* Lour. leaf at 407 nm
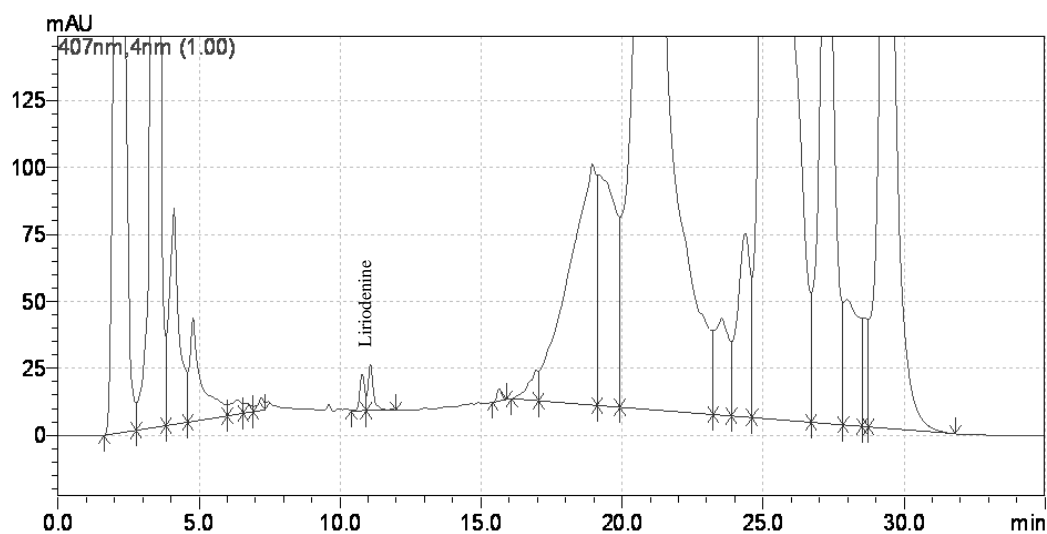
Description: Plant family: Magnoliaceae; Thai name: Champi khaek

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 74 The HPLC chromatogram *Magnolia sirindhorniae* Noot. & Chalermglin leaf at 248 nm
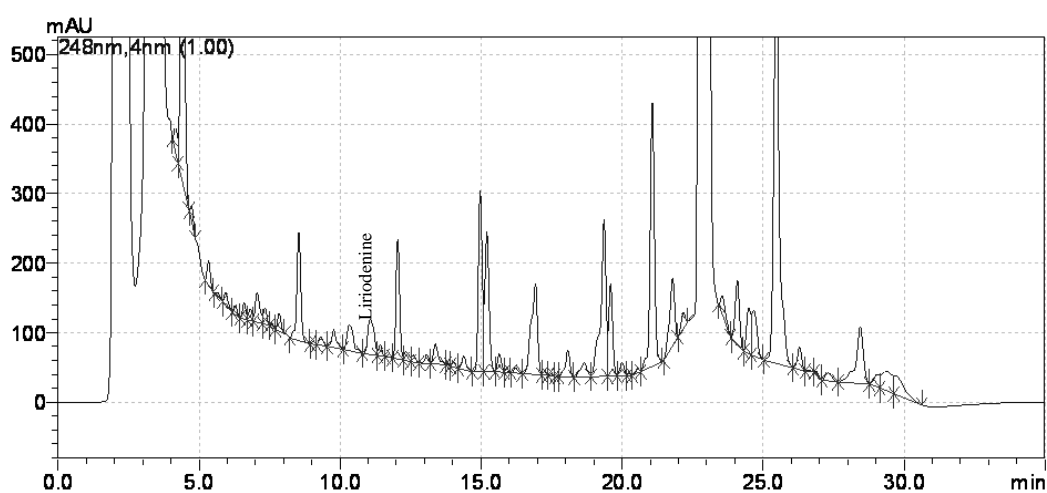
Figure 75 The HPLC chromatogram *Magnolia sirindhorniae* Noot. & Chalermglin leaf at 407 nm
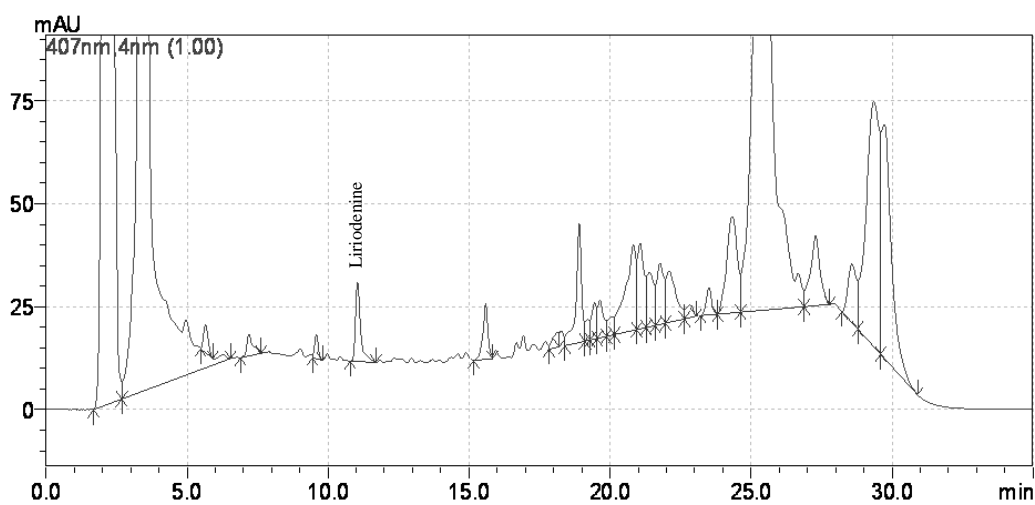
Description: Plant family: Magnoliaceae; Thai name: Champi sirinthon

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 76 The HPLC chromatogram *Magnolia sirindhorniae* Noot. & Chalermglin bark at 248 nm
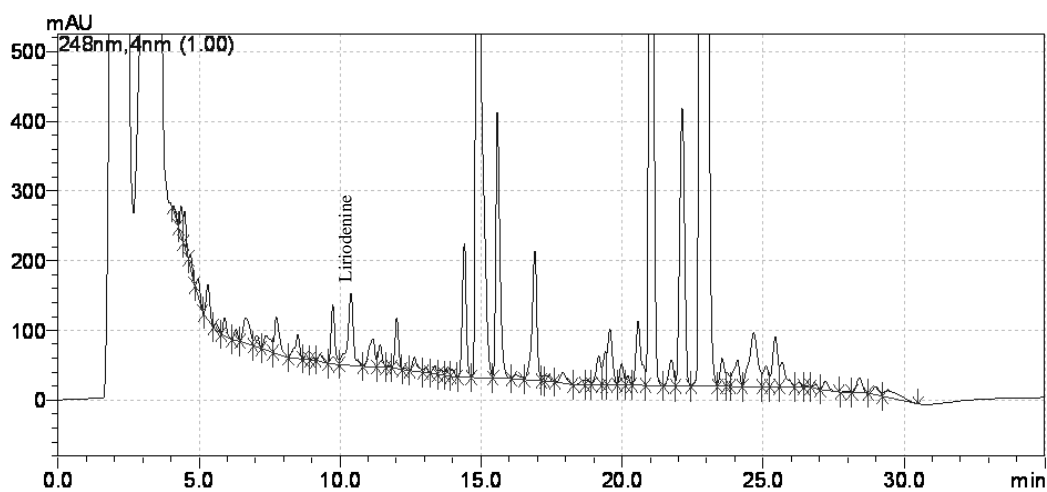
Figure 77 The HPLC chromatogram *Magnolia sirindhorniae* Noot. & Chalermglin bark at 407 nm
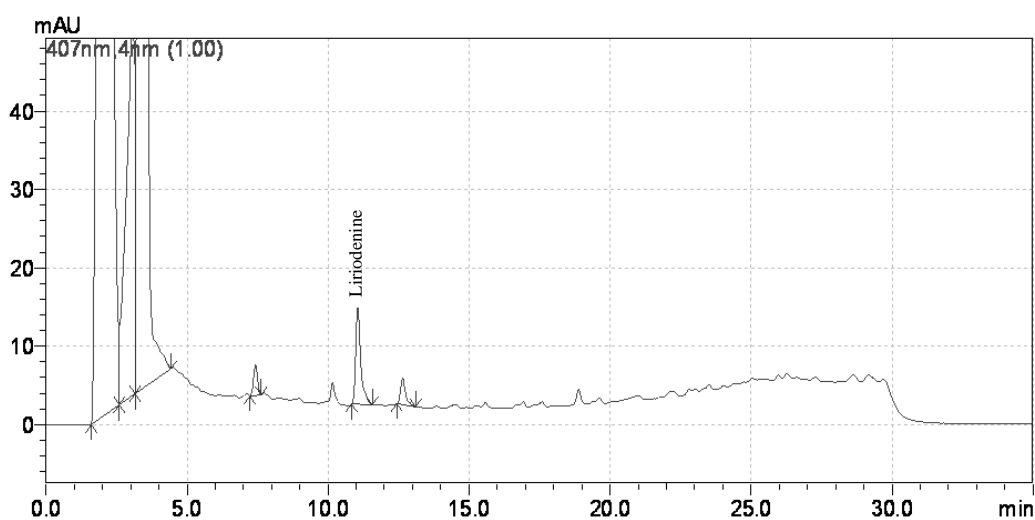
Description: Plant family: Magnoliaceae; Thai name: Champi sirinthon

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 78 The HPLC chromatogram *Magnolia liliifera* (L.) Baill. var. *liliifera* leaf at 248 nm
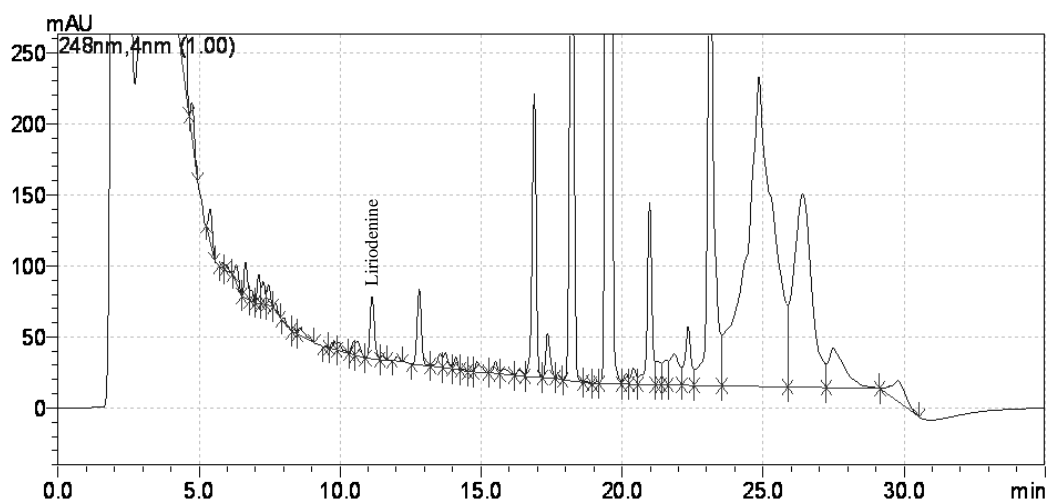
Figure 79 The HPLC chromatogram *Magnolia liliifera* (L.) Baill. var. *liliifera* leaf at 407 nm
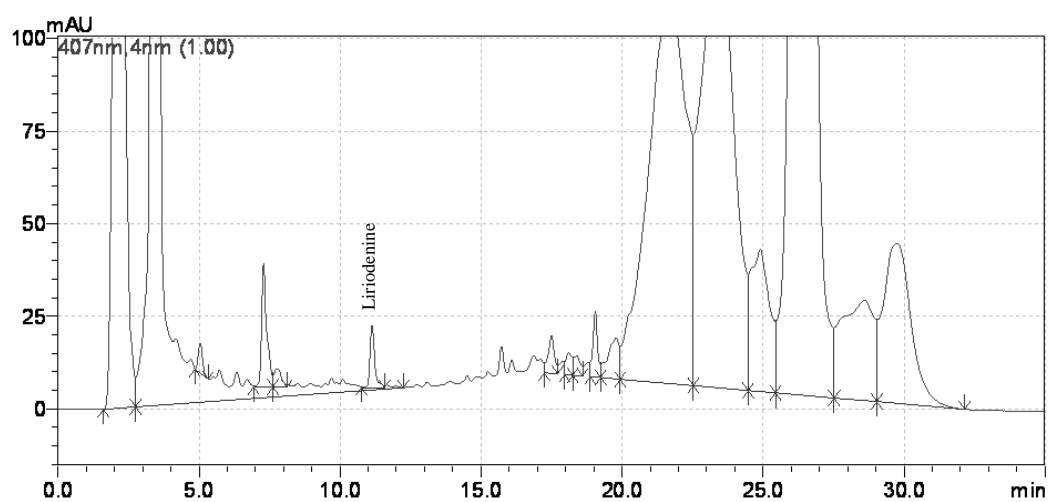
Description: Plant family: Magnoliaceae; Thai name: Montha

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 80 The HPLC chromatogram *Magnolia liliifera* (L.) Baill. var. *liliifera* bark at 248 nm
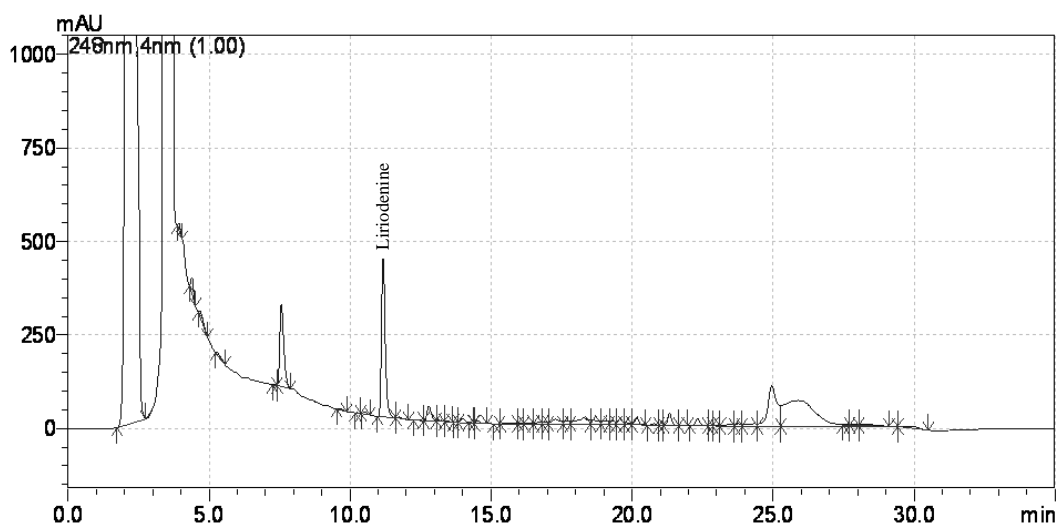
Figure 81 The HPLC chromatogram *Magnolia liliifera* (L.) Baill. var. *liliifera* bark at 407 nm
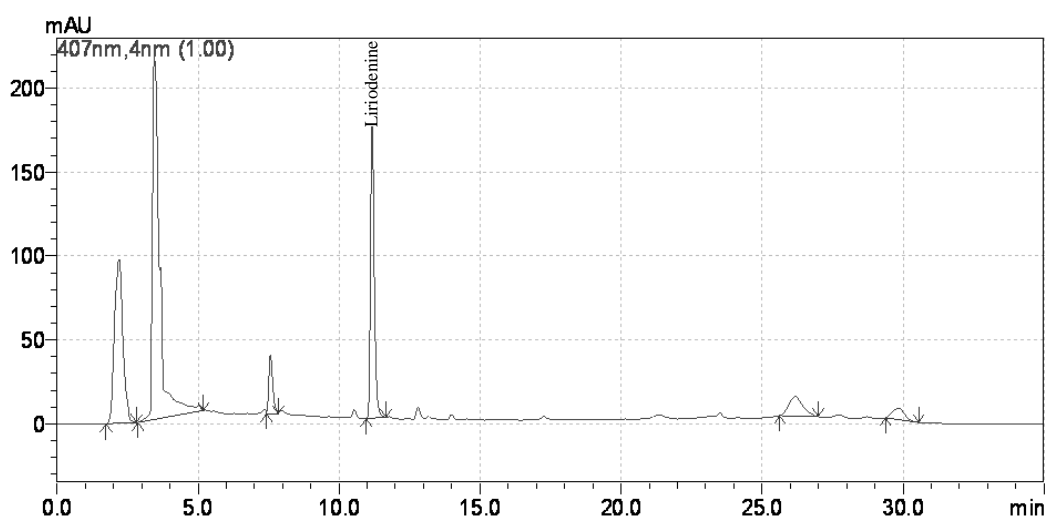
Description: Plant family: Magnoliaceae; Thai name: Montha

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 82 The HPLC chromatogram *Magnolia coco (*Lour.) DC. leaf at 248 nm

Figure 83 The HPLC chromatogram *Magnolia coco (*Lour.) DC. leaf at 407 nm

Description: Plant family: Magnoliaceae; Thai name: Yihup nu

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 84 The HPLC chromatogram *Cananga odorata* (Lam.) Hook.f. & Thomson var. *odorata* leaf at 248 nm
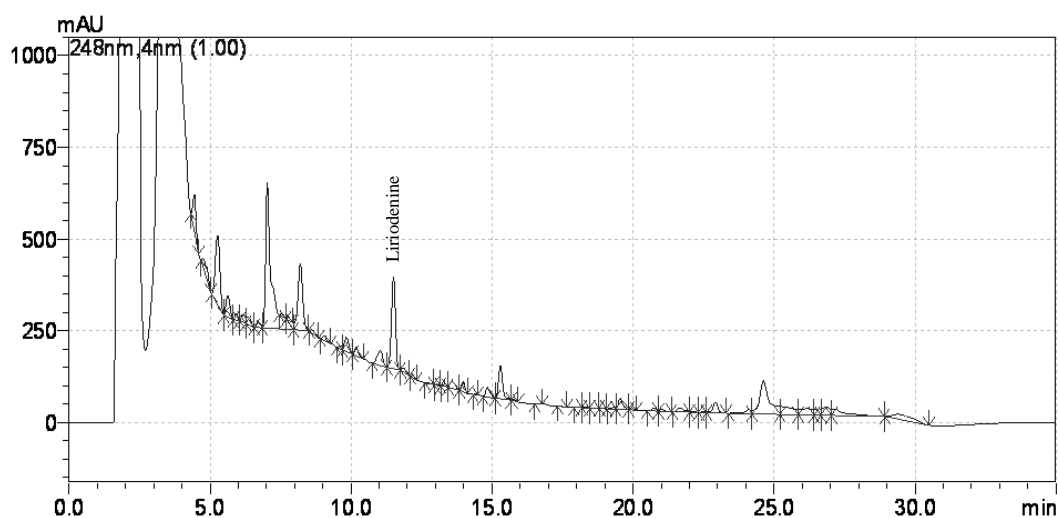
Figure 85 The HPLC chromatogram *Cananga odorata* (Lam.) Hook.f. & Thomson var. *odorata* leaf at 407 nm
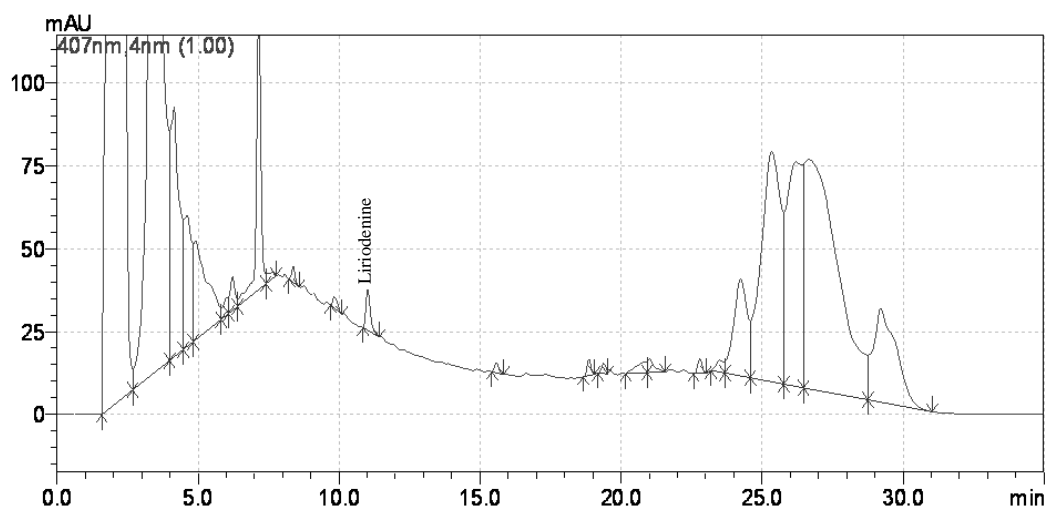
Description: Plant family: Annonaceae; Thai name: Kradang nga thai

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 86 The HPLC chromatogram *Cananga odorata* (Lam.) Hook.f. & Thomson var. *fruticosa* leaf at 248 nm
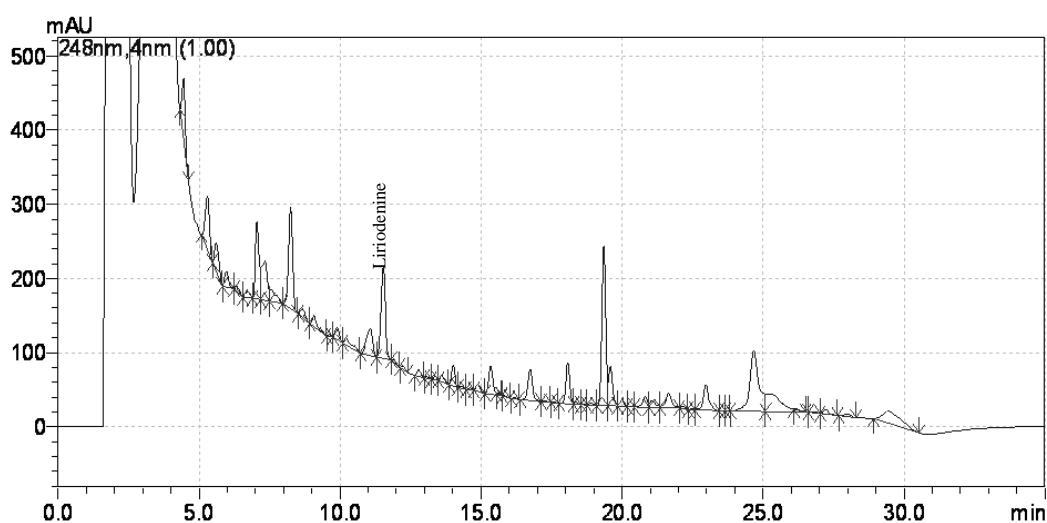
Figure 87 The HPLC chromatogram *Cananga odorata* (Lam.) Hook.f. & Thomson var. *fruticosa* leaf at 407 nm
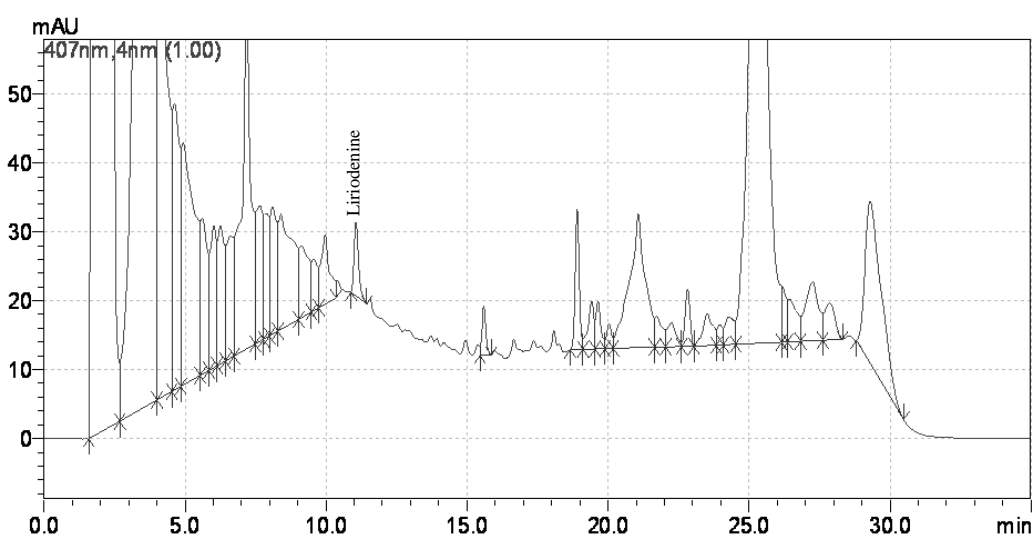
Description: Plant family: Annonaceae; Thai name: Kradang nga songkhla

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 88 The HPLC chromatogram *Annona squamosa* L. bark at 248 nm
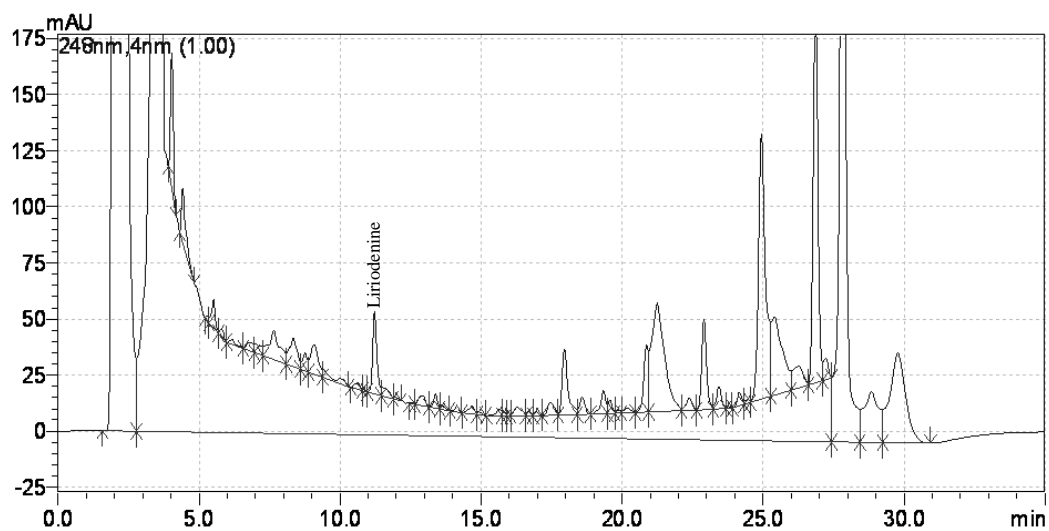
Figure 89 The HPLC chromatogram *Annona squamosa* L. bark at 407 nm
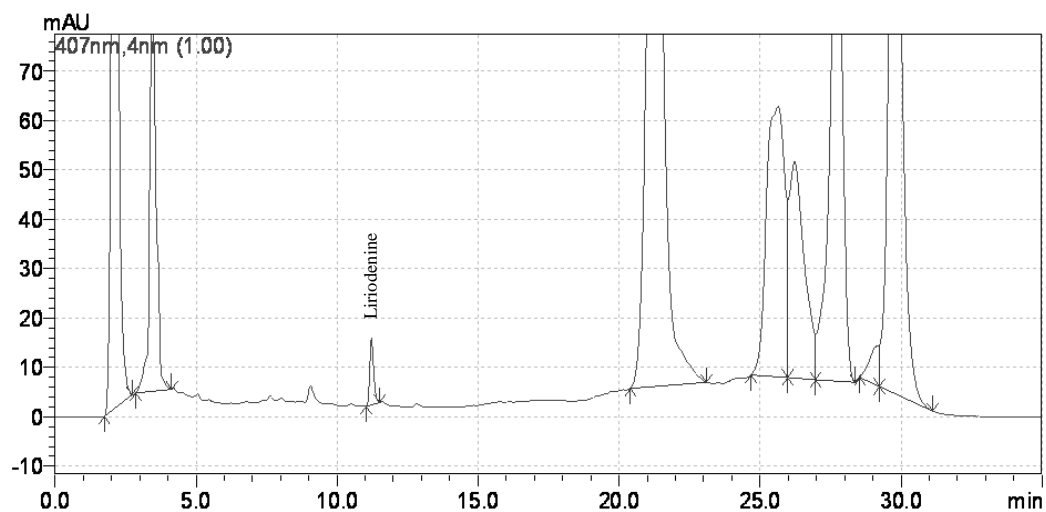
Description: Plant family: Annonaceae; Thai name: Noina

Chromatograms of plant extracts for liriodenine screening

B.) Plant extracts with liriodenine containing (cont.)

Figure 90 The HPLC chromatogram *Nelumbo nucifera* Gaertn. leaf at 248 nm
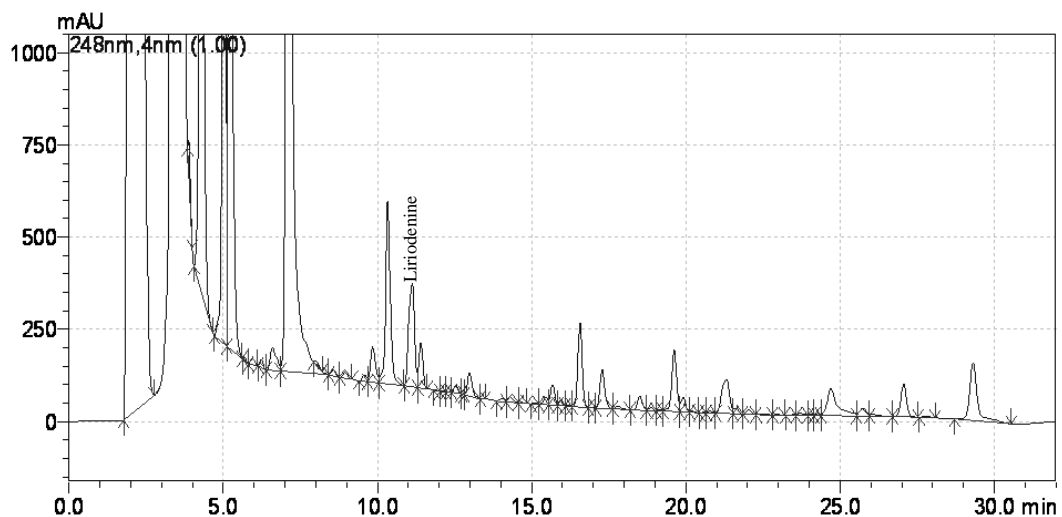
Figure 91 The HPLC chromatogram *Nelumbo nucifera* Gaertn. leaf at 407 nm
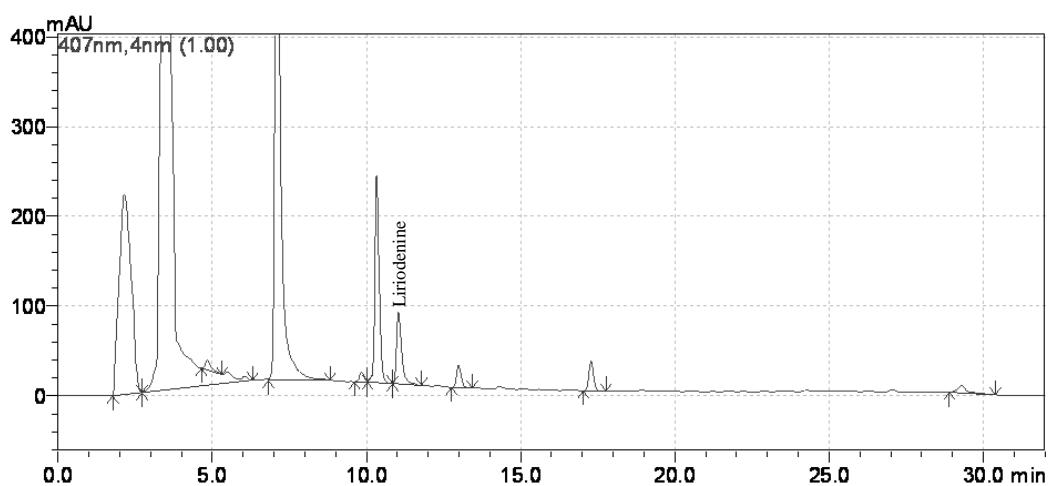
Description: Plant family: Nelumbonaceae; Thai name: Bua luang

APPENDIX C

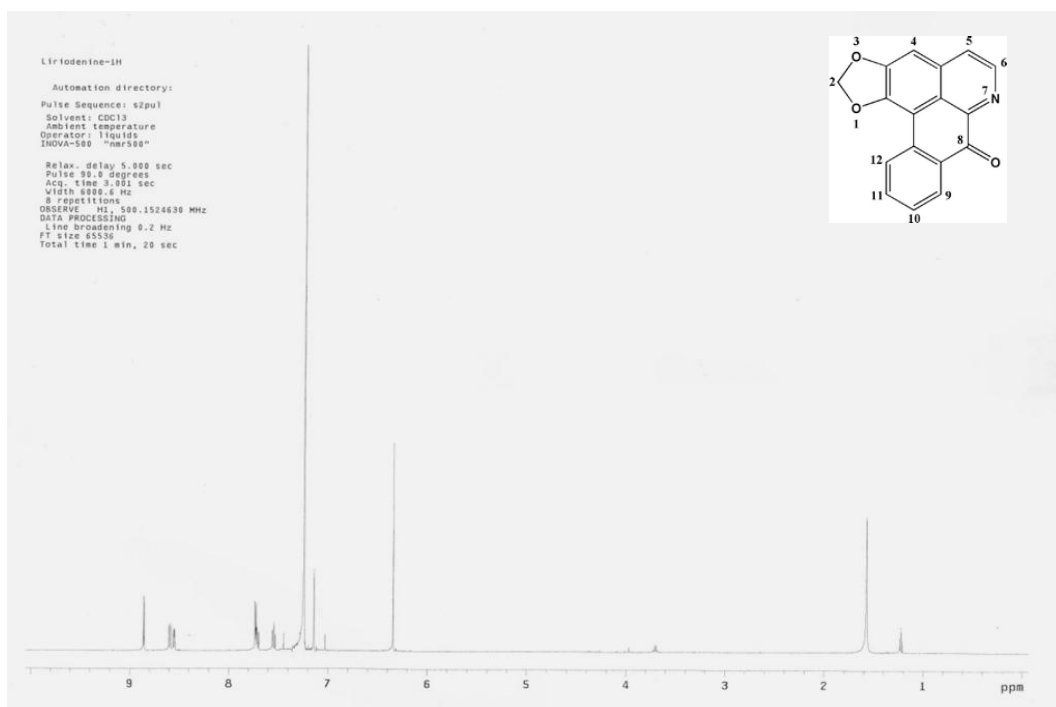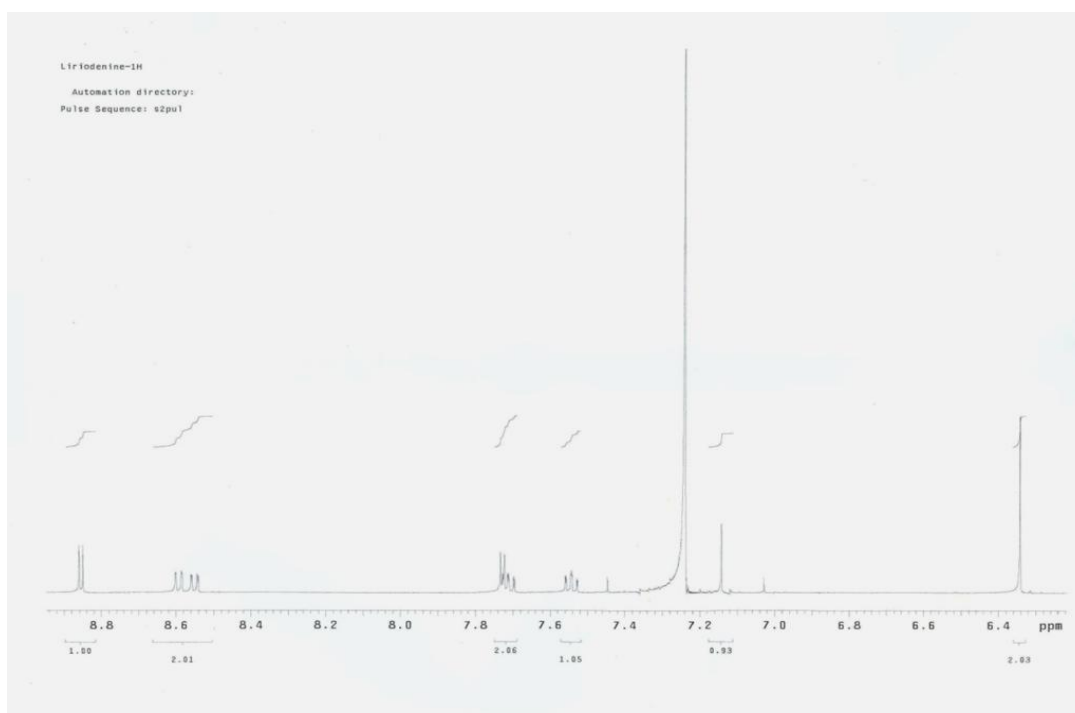**¹H NMR and ¹³C NMR spectra of standard liriodenine in CDCL₃**

Figure 92 The 500 MHz ^{1}H NMR spectrum of liriodenine in CDCl$_3$

Figure 93 The expanded 500 MHz ^{1}H NMR spectrum of liriodenine in CDCl$_3$
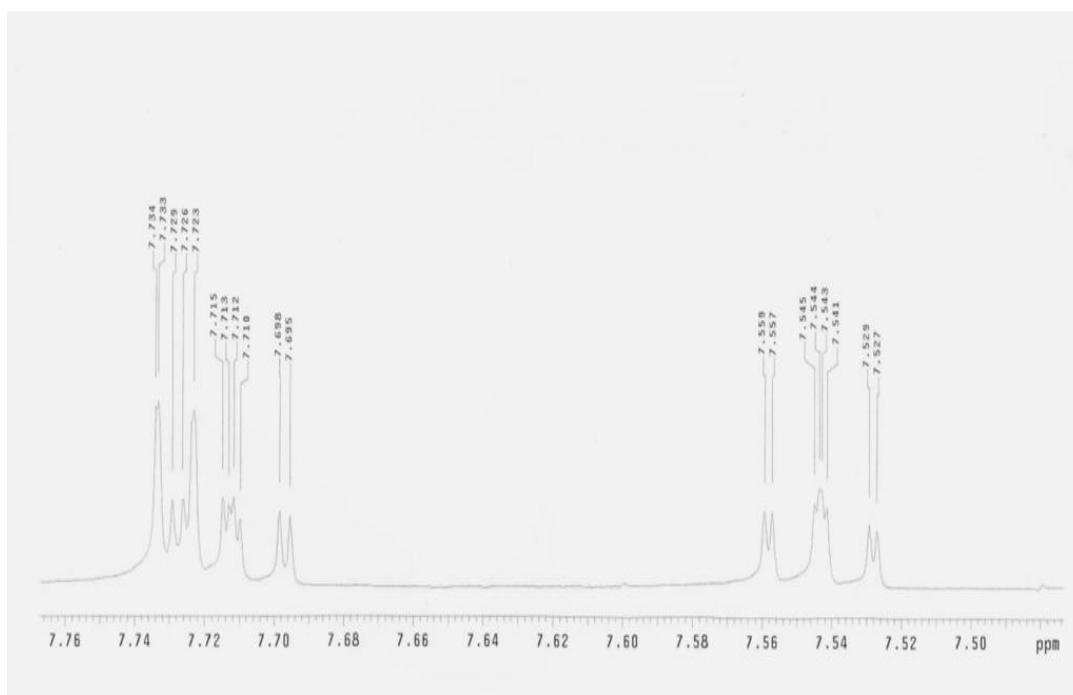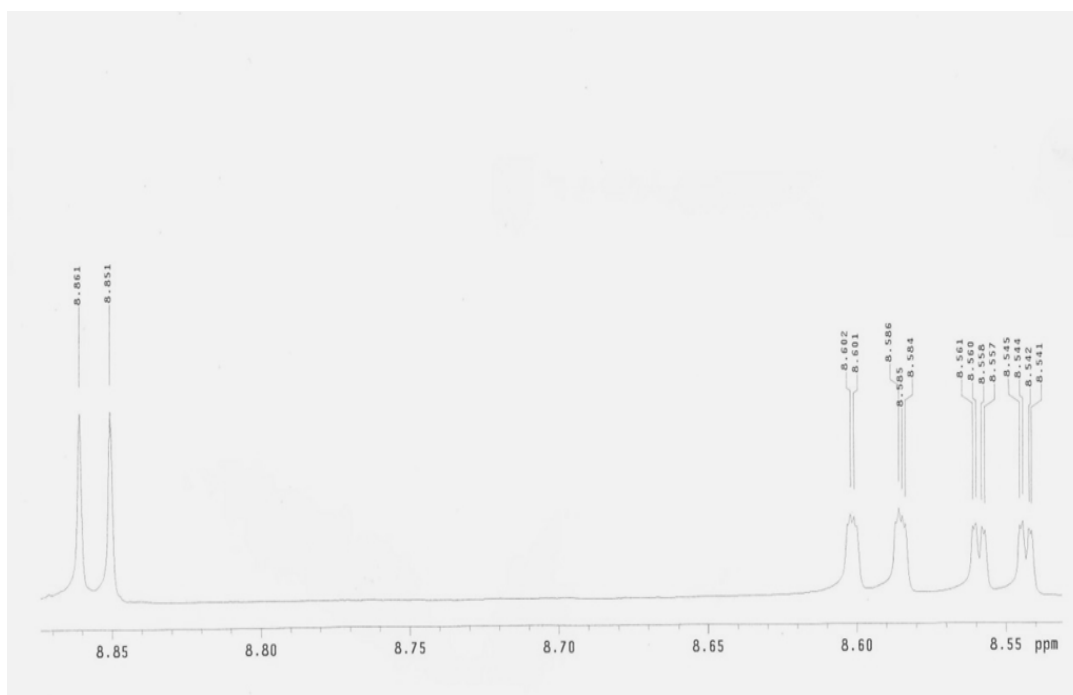
Figure 94 The expanded 500 MHz ^{1}H NMR spectrum of liriodenine in CDCl$_3$

Figure 95 The expanded 500 MHz ^{1}H NMR spectrum of liriodenine in CDCl$_3$

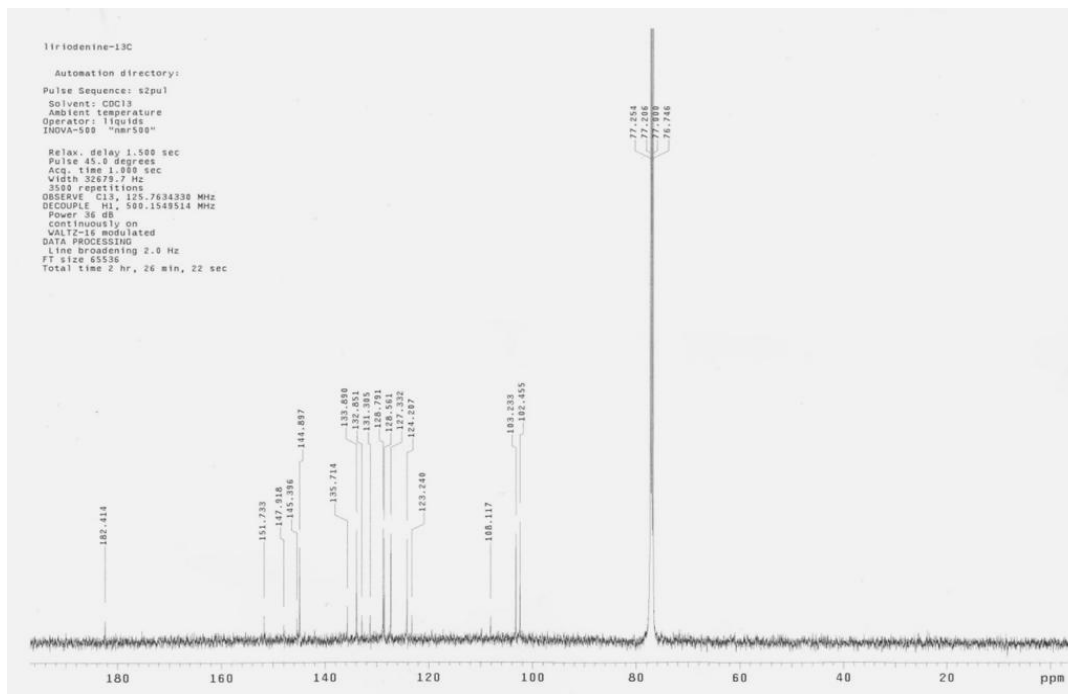**Figure 96** The 125 MHz ^{13}C NMR spectrum of liriodenine in CDCl$_3$

Figure 97 The expanded 125 MHz ^{13}C NMR spectrum of liriodenine in CDCl$_3$
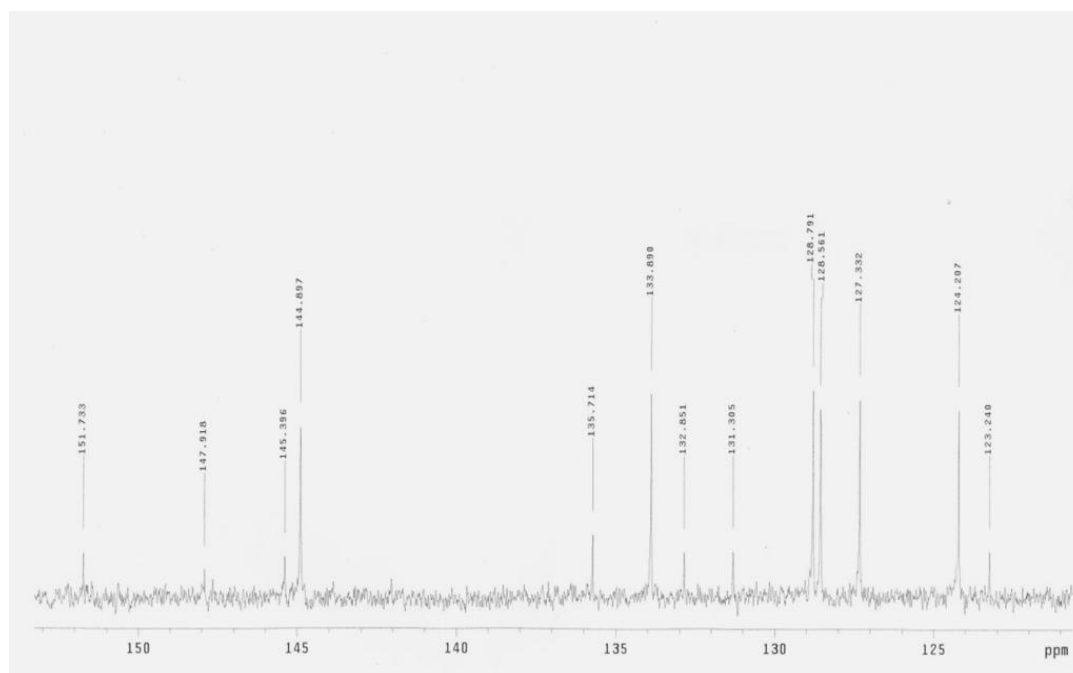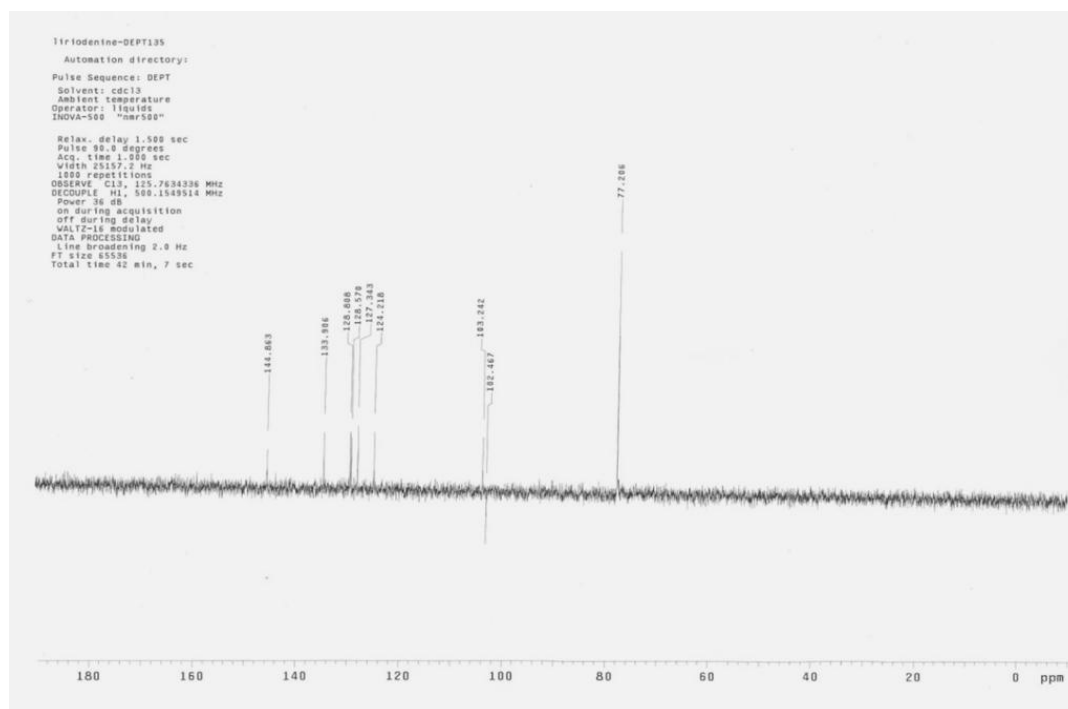
Figure 98 The 125 DEPT 90° ^{13}C NMR spectrum of liriodenine in CDCl$_3$

CURRICULUM VITAE

SARANTHINEE MONGKOLRAT

Personal data - Date of Birth: 21 April 1984, Bangkok, Thailand

Education

Chulalongkorn University, Bangkok, Thailand
- 2005   Bachelor of Science (M.T.), Faculty of Allied Health Sciences
- 2008   Master of Public Health in Health Systems Development,
                  College of Public Health Sciences
- 2009-2012   Ph.D. candidate, Major in Thai Traditional and Alternative
                  Medicine, College of Public Health Sciences

Experience and Accomplishment

2003, 2005   - Medical Technician Trainee at Theptarin Hospital
2006-08   - Coordinator at Heart Centre, BNH Hospital.
2009-10   - Assistant to Managing Director at Health Resort and Spa
2010-present   - Co-founder and Partner Bangkok Drugstore

Research

2005: Association of receptor advanced glycation end product polymorphism (G82S)
and diabetic complications in Thai population (senior project)
2008: Foreign patient customer satisfaction with private hospital service

Poster presentation and publication

- Mongkolrat, S., and Pongpanich S. Foreign patient customer satisfaction with
  private hospital service. 2009. Journal of Health Research 23: 49-52.

- Mongkolrat, S., Palanuvej, C., and Ruangrungsi, N. Quality Assessment
  of *Michelia longifolia* Bark. Proceeding 7th Indochina Conference on
  Pharmaceutical Sciences, pp. 350-355, Bangkok Thailand, 2011.

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  Michelia champaca Bark. The 28th Annual Research Conference in
  Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Chulalongkorn
  University, Bangkok, Thailand, 2011.

- Mongkolrat, S., Palanuvej, C., and Ruangrungsi, N. Quality Assessment of
  Nelumbo nucifera Leaf. The 12th Asian Conference on Clinical Pharmacy, 7-9
  July 2012, Hong Kong SAR.

Scholarship

1. Study scholarships for tuition fee 60/40 from Chulalongkorn University
2. Scholarships in Support of Academic Paper Presentations in a Foreign Country for
Doctorate candidate, Chulalongkorn University, 2012
3. Research fund for the 90th Anniversary of Chulalongkorn University Fund
(Ratchadaphiseksomphot Endowment Fund)