Pharmacognostic Specification and Lupinifolin Content of

Derris reticulata Stem Wood

Miss Siriprapa Chansuwan

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ข้อกำหนดทางเภสัชเวทและปริมาณวิเคราะห์สารลูพินิโฟลินในลำต้นชะเอมเหนือ

นางสาวสิริประภา ชั้นสุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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สิริประภา ชั้นสุวรรณ : ข้อกำหนดทางเภสัชเวทและปริมาณวิเคราะห์สารลูพินิโฟลินในลำต้นชะเอมเหนือ (Pharmacognostic Specification and Lupinifolin Content of *Derris reticulata* Stem Wood) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: นิจศิริ เรืองรังษี, 103 หน้า.

ชะเอมเหนือ มีชื่อทางวิทยาศาสตร์ว่า *Derris reticulata* Craib ชะเอมเหนือถูกนำมาใช้เป็นเครื่องยาสมุนไพรมาหลายศตวรรษ การศึกษาครั้งนี้เพื่อจัดทำข้อกำหนดทางเภสัชเวทของเครื่องยาลำต้นชะเอมเหนือทั้งทางด้านคุณภาพและปริมาณวิเคราะห์ทดสอบความเป็นพิษต่อเซลล์มะเร็งและฤทธิ์ต้านออกซิเดชัน วิเคราะห์ปริมาณสารสำคัญลูพินิโฟลินในลำต้นแห้งของชะเอมเหนือที่เก็บจาก 15 แหล่งทั่วประเทศไทย ผลการศึกษาแสดงลักษณะทางมหทรรศน์และจุลทรรศน์ของลำต้นชะเอมเหนือ การศึกษาค่าคงที่ของใบพบค่าอัตราส่วนเซลล์รั้วด้านท้องใบเท่ากับ 8.95±1.96 พบปากใบชนิดแอนไอโซไซติกเฉพาะด้านท้องใบ จำนวนของปากใบและดัชนีปากใบต่อพื้นที่ 1 ตารางมิลลิเมตรมีค่าเท่ากับ 316.53±23.03 และ 20.21±1.53 ตามลำดับ พื้นที่ของเซลล์ผิวใบ มีขนาด 1173.13±56.25 ตารางไมโครเมตร การศึกษาเอกลักษณ์ทางกายภาพและเคมีของลำต้นชะเอมเหนือ พบว่า มีน้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด ปริมาณสิ่งสกัดด้วยน้ำ ปริมาณสิ่งสกัดด้วยเอทานอล และปริมาณความชื้น ร้อยละ 5.7 ± 0.1, 4.6 ± 0.1, 1.0 ± 0.1, 15.5 ± 0.7, 6.8 ± 0.3 และ8.5 ± 0.3 โดยน้ำหนักตามลำดับ จัดทำลายพิมพ์องค์ประกอบทางเคมีโดยวิธีทินเลเยอร์โครมาโทกราฟี สกัดลำต้นชะเอมเหนือด้วยเอททานอลเข้มข้นร้อยละ 95 โดยใช้เครื่องสกัดต่อเนื่อง วิเคราะห์ลูพินิโฟลินในสิ่งสกัดจากเอทานอลด้วยเทคนิคทินเลเยอร์โครมาโทกราฟี โดยใช้ตัวทำละลายเฮกเซนต่อเอทิลอะซิเตท (6:4) เป็นวัฏภาคเคลื่อนที่ วิเคราะห์ปริมาณสารลูพินิโฟลินโดยวิธีเด็นซิโตมีทรีภายใต้แสงอัลตราไวโอเลตที่ความยาวคลื่น 275 นาโนเมตรและวิธีภาพถ่ายวิเคราะห์ภายใต้แสงอัลตราไวโอเลตที่ความยาวคลื่น 254 นาโนเมตรโดยใช้โปรแกรมอิมเมจเจพบสารลูพินิโฟลินปริมาณร้อยละ8.07±2.41 และ 7.76±2.21 โดยน้ำหนัก โดยทั้งสองวิธีตามลำดับ เปรียบเทียบปริมาณลูพินิโฟลินระหว่าง 2 วิธี โดยใช้สถิติ paired *t*-test พบว่าปริมาณลูพินิโฟลินที่วิเคราะห์โดยวิธีทั้งสองไม่แตกต่างกัน (p=0.11) การทดสอบความเที่ยงตรงของวิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโตมีทรีและวิธีทินเลเยอร์โครมาโทกราฟีโดยวิเคราะห์ภาพถ่ายโดยใช้โปรแกรมอิมเมจเจพบว่ามีช่วงวิเคราะห์แบบโพลีโนเมียล 0.6-3 ไมโครกรัมต่อจุดโดยมีค่าสัมประสิทธิ์การตัดสินใจเท่ากับ 0.9985 และ 0.9989 ค่าเฉลี่ยการคืนกลับร้อยละ 97.68 – 110.59 และ 94.65 – 111.79 ค่าความสามารถในการวัดซ้ำ มีค่าระหว่างร้อยละ 0.9-2.38และ 0.74-4.18 ค่าความแม่นยำ มีค่าระหว่างร้อยละ 0.8-4.77 และ 0.6-3.59 ขีดจำกัดของการตรวจพบมีค่า 0.14 และ 0.10 ไมโครกรัมต่อจุด และขีดจำกัดของการหาปริมาณ มีค่า 0.41 และ 0.30 ไมโครกรัมต่อจุด ค่าความคงที่มีค่าสัมประสิทธิ์ของการกระจายร้อยละ 2.98 และ 3.04 ตามลำดับ การทดสอบความเป็นพิษต่อเซลล์มะเร็งของสิ่งสกัดเอทานอลของลำต้นชะเอมเหนือโดยใช้เซลล์มะเร็งทั้งหมด 5 ชนิด คือ เซลล์มะเร็งเต้านม (BT-474) เซลล์มะเร็งปอด (CHAGO-K1) เซลล์มะเร็งลำไส้ (SW-620) เซลล์มะเร็งกะเพาะอาหาร (KATO-3) เซลล์มะเร็งตับ (HEP-G2) และเซลล์ปกติ 1 ชนิด คือ เซลล์ปอด (Wi-38) พบว่าสิ่งสกัดเอทานอลของลำต้นชะเอมเหนือไม่แสดงความเป็นพิษต่อเซลล์มะเร็งและเซลล์ปกติ โดยมีค่า IC_{50} มากกว่า 20 ไมโครกรัมต่อมิลลิลิตร การศึกษาฤทธิ์ต้านออกซิเดชั่นด้วยวิธีการต้านอนุมูลอิสระดีพีพีเอชพบว่าสิ่งสกัดเอทานอลจากลำต้นชะเอมเหนือ มีค่า IC_{50} เท่ากับ 907.39 ไมโครกรัมต่อมิลลิลิตร ผลการวิเคราะห์ฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นด้วยวิธี Ferric reducing antioxidant power (FRAP) assay พบว่ามีค่า FRAP เทียบเท่า 0.132 มิลลิโมลาร์เฟอรัสไอออนต่อมิลลิกรัมสิ่งสกัด และผลการวิเคราะห์ปริมาณฟีนอลลิครวม พบว่าค่าเทียบเท่า 44.37 ± 0.99 มิลลิกรัมกรดแกลลิคต่อกรัมสิ่งสกัด จากการศึกษานี้สามารถจัดทำเป็นข้อกำหนดทางเภสัชเวทของลำต้นชะเอมเหนือในประเทศไทยได้ นอกจากนี้วิธีวิเคราะห์ด้วยเทคนิคทางทินเลเยอร์โครมาโทกราฟี-เด็นซิโตมีทรีและวิธีทินเลเยอร์โครมาโทกราฟีโดยวิเคราะห์ภาพถ่ายโดยใช้โปรแกรมอิมเมจเจสามารถนำมาประยุกต์หาปริมาณของลูพินิโฟลินในพืชสมุนไพรชนิดนี้ได้ ซึ่งจะเป็นประโยชน์ต่อการควบคุมคุณภาพวัตถุดิบสมุนไพรและการศึกษาวิจัยพัฒนาเครื่องยานี้ต่อไป

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: LUPINIFOLIN / DERRIS RETICULATA / PHARMACOGNOSTIC SPECIFICATION / TLC DENSITOMETRY / TLC IMAGE ANALYSIS

SIRIPRAPA CHANSUWAN: Pharmacognostic Specification and Lupinifolin Content of *Derris reticulata* Stem Wood. ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 103 pp.

Derris reticulata Craib is used in traditional Thai medicine for centuries. This study was carried out to investigate the pharmacognostic parameters by qualitative, quantitative analyses, cytotoxic and antioxidant activities as well as lupinifolin content of *D. reticulata* dried stem wood collected from 15 habitats located at various regions throughout Thailand. Macroscopic and microscopic evaluation of *D. reticulata* stem wood were demonstrated. For leaf measurement of *D. reticulata*, palisade ratio was 8.95±1.96. Upper epidermal cell area was 1173.13±56.25 μm^2. Anisocytic typed stomata were found only at lower epidermis. The stomata number and stomata index in 1 mm^2 were 316.53±23.03 and 20.21±1.53, respectively. Physico-chemical parameters including loss on drying, total ash, acid-insoluble ash, water soluble extractive, ethanol soluble extractive and water contents were found to be 5.7 ± 0.1, 4.6 ± 0.1, 1.0 ± 0.1, 15.5 ± 0.7, 6.8 ± 0.3 and 8.5 ± 0.3% by dry weight, respectively. Thin layer chromatographic fingerprint was also established. *D. reticulata* stem woods were extracted in 95% ethanol using Soxhlet apparatus. Lupinifolin in the ethanolic extracts were analyzed by thin layer chromatography (TLC) using silica gel 60 GF_{254} as stationary phase and hexane : ethyl acetate (6 : 4) as mobile phase. For quantitative analysis of lupinifolin, the contents were evaluated by TLC-densitometry under UV 275 nm and TLC image analysis under UV 254 nm using image J software which were respectively found to be 8.07±2.41 and 7.76±2.21 % by dry weight. The method validity of TLC-densitometry and TLC image analysis were shown that the calibration range were polynomial with 0.6 – 3 µg/spot (R^2=0.9985 and R^2=0.9989). The accuracy was 97.68 – 110.59 %recovery and 94.65 – 111.79 % recovery. The repeatability was 0.9-2.38%RSD and 0.74-4.18%RSD. The intermediate precision was 0.8-4.77%RSD and 0.6-3.59%RSD. LOD were 0.14 and 0.10 and LOQ were 0.41 and 0.30 µg/spot. The robustness was 2.98%RSD and 3.04%RSD, respectively. The comparison of the total lupinifolin in both methods was not significant different (p=0.11). Additionally, the ethanolic extract of *D. reticulata* stem wood was tested for *in vitro* cytotoxic activity against 5 human cancer cell lines including breast ductal carcinoma (BT-474), undifferentiated lung carcinoma (CHAGO-K1), colon adenocarcinoma (SW-620), gastric carcinoma (KATO-3), hepatocarcinoma (HEP-G2) and one human normal cell line, lung fibroblast (Wi-38). The ethanolic extract of *D. reticulata* stem wood exhibited no significant activity against the five cancer cell lines as well as one normal cell line with an IC_{50} more than 20 µg/ml. Furthermore, the free radical scavenging potentials of the ethanolic extract of *D. reticulata* stem wood was demonstrated with the IC_{50} of 907.39 µg/ml for DPPH. FRAP assay indicated that the ethanolic extract of *D. reticulata* stem wood had a reducing power value equivalent to 0.132 mM Fe(II)/mg extract. The total phenolic content of the ethanolic extract of *D. reticulata* stem wood was 44.37 ± 0.99 mg GAE/g extract. This study provided pharmacognostic specification toward fundamental standardization of *D. reticulata* stem wood in Thailand. Moreover, the simple TLC-densitometry and TLC with image analysis can be applied to quantitatively determine lupinifolin in this plant material.

Field of Study: Public Health Sciences
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	=	Percent
µg/ml	=	Microgram per milliliter
µl	=	Microliter
ATCC	=	American type culture collection
BHT	=	Buthylated hydroxytolen
BT-474	=	Ductal carcinoma breast
Chago-K1	=	Undifferentiated lung carcinoma
cm	=	Centimeter
DMSO	=	Dimethyl sulfoxide
DPPH	=	2,2-Diphenyl-1 picryl hydrazyl
Fe^{2+}	=	Iron (II)
Fe^{3+}	=	Iron (III)
$FeSO_4$	=	Iron (II) sulfate
FRAP	=	Ferric reducing antioxidant power
g	=	Gram
g/mol	=	Gram per mole
HCl	=	Hydrochloric acid
Hep-G2	=	Liver hepatoblastoma
hr	=	Hour
IC_{50}	=	Fifty percent inhibitory concentration
ICH	=	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

LIST OF ABBREVIATIONS

KATO-III	=	Gastric carcinoma
kg	=	Kilogram
l	=	Litre
LOD	=	Limit of detection
LOQ	=	Limit of quantification
m	=	Meter
mg	=	Milligram
mg/ml	=	Milligram per milliliter
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
mm^2	=	Square Millimeter
nm	=	Nanometer
NSS	=	Normal saline solution
°C	=	Degree Celsius
pH	=	Potential of hydrogen ion
R^2	=	Correlation coefficients
Rf	=	Retention factor
rpm	=	Revolutions per minute
RSD	=	Relative standard deviation

LIST OF ABBREVIATIONS

SD	=	Standard deviation
ß	=	Beta
SW-620	=	Colon adenocarcinoma
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
v/v	=	Volume in a volume
WHO	=	World Health Organization
Wi-38	=	Lung fibroblast

CHAPTER I

INTRODUCTION

Background and significance of the study

Herbal medicines have been popular in many countries around the world since the past decades. Both folklore healing practice and herbal medicine have been promoted as the basic health projects by World Health Organization [1]. Herbs have been also used as herbal remedies or raw materials for pharmaceutical industry. However, the quality of herb was not considered to be respectable in character or appearance, these may affect confidence of the consumer. Therefore, there is a need to ensure the quality control of the herb using modern technique for standardization of the herbal remedies.

The genus *Derris* belongs to the family Leguminosae, sub-family Papilionoideae and tribe Dalbergieae. It contains over 80 species which are chiefly distributed in the tropical region of South-East Asia, but a few species occur also in the warmer areas of Australia, Africa and America. Representatives of the genus are mostly shrubs or woody climbers, but some are trees [2].

Derris reticulata is a medicinal plant locally known in Thai as Cha-Aem-Nuea. It is a climbing plant which can be found in semi-shaded area of dry evergreen forest, edge of evergreen mixed (dipterocarp) forest, in bamboo forest, or along streams, flowering is from August to September, and fruiting is from September to January [3, 4]. Stem wood of *D. reticulata* has been used as cough suppressant and expectorant [5, 6].

A flavanone named lupinifolin has been isolated from the ethanolic extract of *D. reticulata* stem wood, and it possibly is a main active constituent. The determination of free sugar in the aqueous portion of the *D. reticulata* stem's extract revealed that the presence of sucrose might be the major sweetener of this plant [3]. The previous study investigated the gel preparation of 5% alcoholic extract of *D. reticulata.* The results demonstrated that *D. reticulata* ethanolic extract showed moderately anti HSV-

1 activity [7]. Furthermore, the study of Chivapat *et al.*, 2010 revealed that *D. reticulata* have an active potency on antimicrobial activities [8]. Whereas, Vongnam *et al.*, 2013 studied an anti-inflammatory activity by interfering with nitric oxide production of macrophage, they found that the ethanolic extract from stem of *D. reticulata* demonstrated the active potency on anti-inflammatory activity [9].

Although, *D. reticulata* stem wood has widely used in traditional Thai medicine, but its pharmacognostic specification have never been established. This research aimed to develop the standardization parameters of *D. reticulata* crude drug, to study the lupinifolin which is an active component in *D. reticulata* by TLC image analysis using ImageJ free software compared to TLC densitometry, and to evaluate its *in vitro* biological activities.

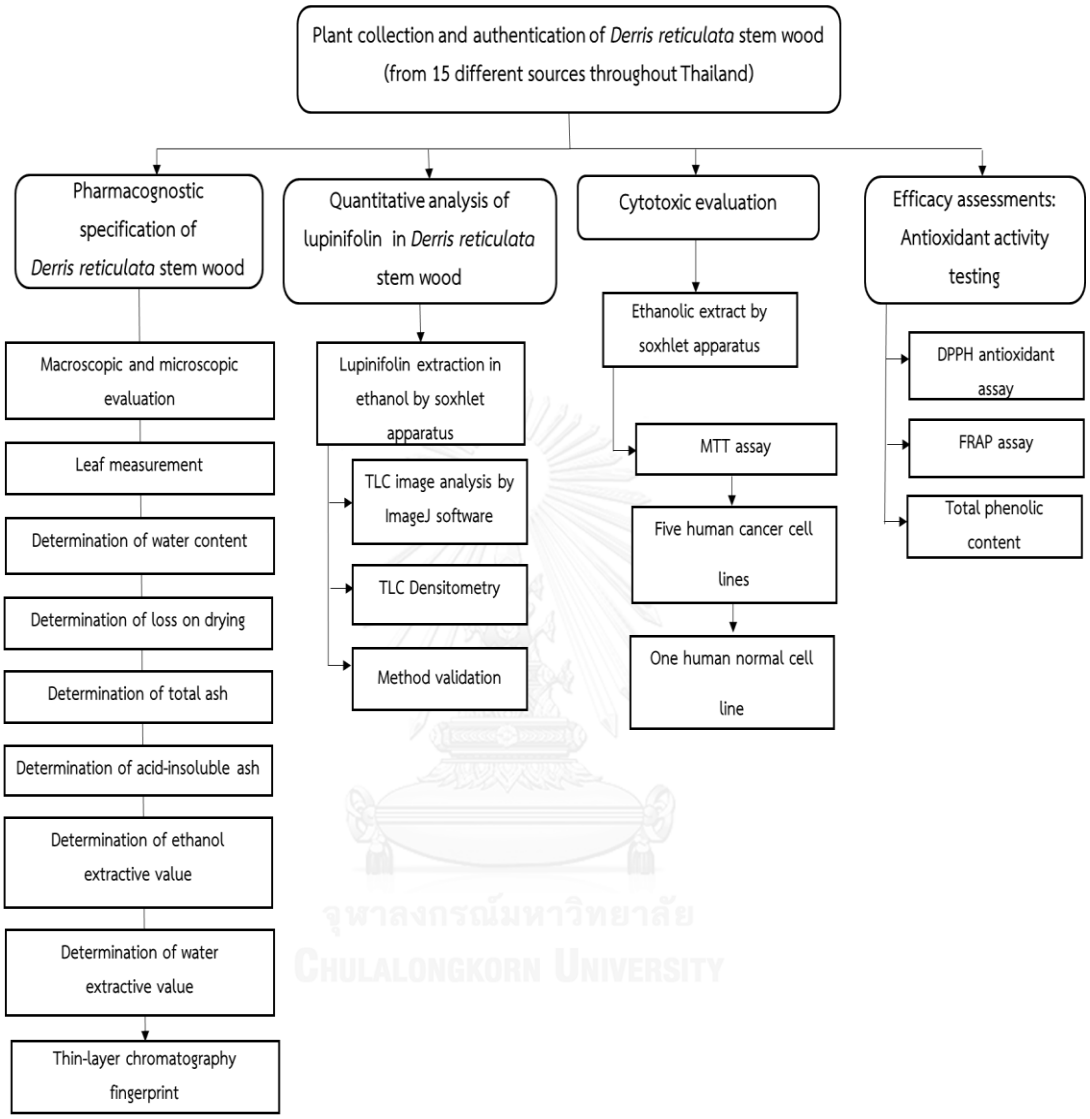
Research gap

The pharmacognostic specification parameters and lupinifolin content of *D. reticulata* crude drug in Thailand have never been determined.

Objectives

1. To establish the pharmacognostic specification the *D. reticulata* stem wood in Thailand.
2. To examine leaf constant value including stomatal index, stomatal number and palisade ratio of *D. reticulata* in Thailand.
3. To evaluate lupinifolin contents in *D. reticulata* stem wood by TLC image analysis using ImageJ free software compared to TLC densitometry.
4. To evaluate antioxidant activity of *D. reticulata* stem wood.
5. To evaluate cytotoxicity against various cancer cell lines of *D. reticulata* stem wood by MTT assay.

Conceptual framework



CHAPTER II

REVIEW OF LITERATURE

Taxonomy of the genus *Derris*

The taxonomic hierarchy of the genus *Derris* can be classified as follows :

Kingdom Plantae
Subkingdom Tracheobionta
Infrakingdom Spermatophyta
Division Magnoliophyta
Class Magnoliopsida
Subclass Rosidae
Order Fabales
Family Fabaceae
Genus *Derris*
Species *Derris reticulata* Craib

There are 13 species which can be found in Thailand including *Derris alborubra* Hemsl. (Thao taa plaa), *Derris amoena* Benth. (Yaan saao kham), *Derris dalbergioides* Bak. (Khaang ten), *Derris elliptica* Benth. (Haang lai daeng), *Derris indica* Bennet. (Yee nam), *Derris kerrii* Craib (Kaang khee mot), *Derris malaccensis* Prain (Haang lai khao), *Derris robusta* Benth. (Khee mot), *Derris scandens* Benth. (Thao wan priang), *Derris thorelii* Craib (Khruea taa plaa), *Derris thyrsiflora* Benth. (Tang me), *Derris trifoliata* Lour. (Thop thaep nam), *Derris reticulata* Craib (Cha aem nuea) [10].

Plant description of *Derris reticulata*

The botanical characteristics of *D. reticulata* (Figure 1) are described as follows [3, 11]: *Leaves are odd pinnately compound with 5 leaflets. Leaflets are oblong-ovate, ovate lanceolate or oblong-lanceolate. The uppermost tips are round, base round or cuneate. The petiole is 1.5-4.0 cm long and rachis with a longitudinal groove is on the upper side. Leaves have 6-8 lateral nerves anastomosing into an intramarginal nerve on both sides. Lateral nerves slightly rise above and below. The thin network veinlet presents on both surfaces. The branchlets cover with ash grey or straw-yellow-ash grey bark and pale brown lenticel. Legume is green or straw yellow and short oblong pod. The seed is thick. The inflorescence flowers are papilonaceous corolla.*

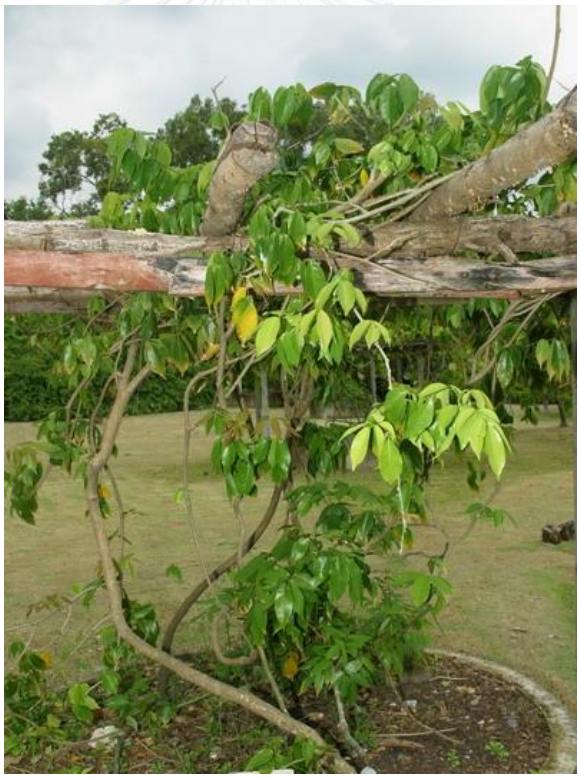


Figure 1 *Derris reticulata* Craib [11]

Medicinal uses of *Derris reticulata*

Pharmacological properties of *D. reticulata* are well known for a long time. It has been traditionally used as an expectorant to relieve cough [12]. Its antidiabetic application has been performed by some local medicinal plant practitioners in Prachinburi province as alternative medicine [13]. The activities include antioxidant, anti-inflammatory and antimicrobial activities. The previous studies found that lupinifolin was a flavonone compound expressing high efficacy against Herpes simplex virus 1 (HSV-1) [8].

Biological activity of *D. reticulata*

Antioxidant and anti-inflammatory activities

The ethanolic extract of *D. reticulata* stem has a potential on anti-inflammatory activity. *D. reticulata* ethanolic extract inhibited production of several known inflammatory mediators in LPS-activated macrophages. The extract at the concentrations of 50 and 100 µg/ml significantly inhibited zymosan phagocytosis of LPS-activated cells in a concentration dependent manner. The results demonstrated that flavonoids from *D. reticulata* might have potential to be an inflammatory agent [9]. Furthermore, the aqueous extract from *D. reticulata* stem was also investigated using ABTS-DPPH scavenging assays, and FRAP method for in vitro antioxidant activities. The results revealed that the extract possessed moderate radical scavenging activities [14].

Anti-diabetic activity

D. reticulata extract exerts an anti-diabetic activity *via* cytoprotective effect on pancreatic ß-cells and inhibitory action on glucose absorption with a relatively wide margin of safety [13]. In addition, the effects of the aqueous extract of *D. reticulata* has been investigated using alpha-glucosidase activity and insulin releasing activity. The results found that the aqueous extract did not stimulate insulin secretion, but it could inhibit alpha-glucosidase compared to acarbose which was served as positive control [14].

D. reticulata is one of medicinal plants which used in traditional Thai medicine. It is distributed in the tropical regions of Asia and East Africa. It is used for the relief of thirst and as an expectorant. The Leguminosae is known to be a rich source of flavonoids and most of the prenyl derivatives have been found in this family It has flavonoids as major active compounds similar to other plants in genus *Derris*. The isolation and structural characterization of four prenylated flavanones were lupinifolin, epoxylupinifolin, dereticulatin and hydroxyl epoxylupinifolin from the stems of *Derris reticulata.* Further investigation of this herb has led to the isolation of three new cytotoxic flavonoids, dihydroxy-hydroxymethyl-dimethylpyrano flavanone, dihydroxylupinifolin and lupiwighteone. [3, 15]

Lupinifolin (Figure 2) was major compound which isolated from the stem wood of this plant. The structures of these compounds were deduced from various spectroscopic analyses, especially 1D and 2D NMR as well as chemical transformations [4, 15].

Lupinifolin

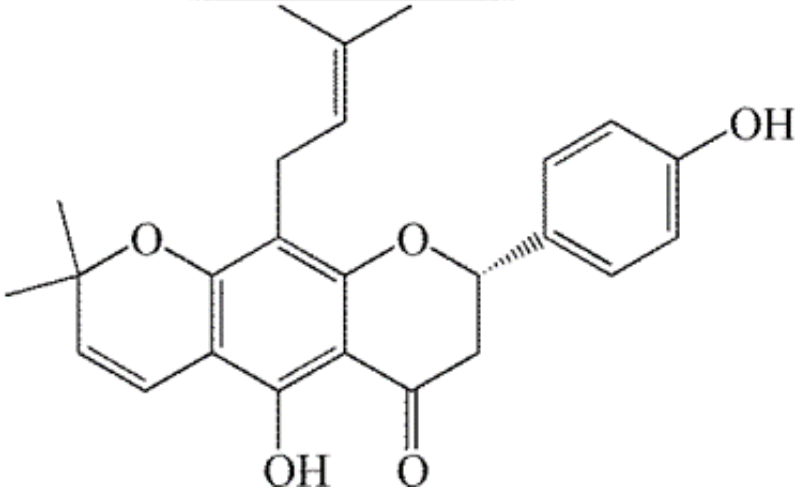


Figure 2 Structure of lupinifolin

IUPAC Name: 5-hydroxy-8-(4-hydroxyphenyl)-2,2-dimethyl-10-(3-methylbut-2-enyl)-7,8-dihydropyrano[3,2-g] chromen-6-one

Synonyms: (8S)-7,8-Dihydro-5-hydroxy-8-(4-hydroxyphenyl)-2,2-dimethyl-10-(3-methyl-2-buten-1-yl)-2H,6H-benzo[1,2-b:5,4-b']dipyran-6-one; 2S-Lupinifolin; Flemichin B

Molecular formular: $C_{25}H_{26}O_5$

Molecular weight: 406.478 g/mol

UV/VIS: λ_{max} : 254, 274, 311, 367 nm.

Description: Yellow needle

Boiling Point: 627.5±55.0 °C at 760 mmHg

Lupiwighteone

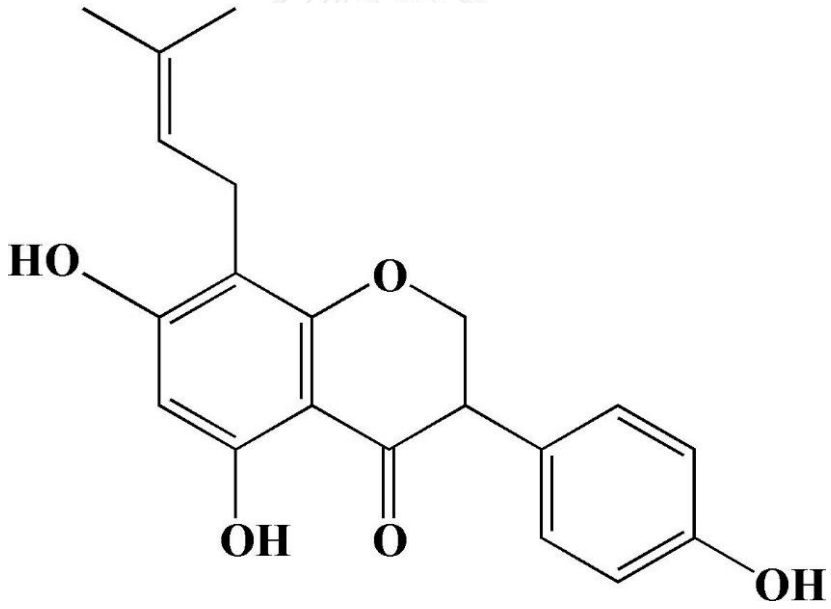


Figure 3 Structure of lupiwighteone

IUPAC Name: 5,7-dihydroxy-3-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)chromen-4-one

Synonyms: 8-prenyl-5,7,4'-trihydroxyisoflavone

Molecular formular: $C_{20}H_{17}O_5$

Molecular weight: 338.359 g/mol

Description: Yellow powder

In the investigation on phytochemical study, two compounds were isolated from the stem wood of *D. reticulata.* The compounds DR-1 was found to be lupinifolin and DR-2 was an isoflavone namely lupiwighteone. The identification of these isolated compounds was based on data from various spectroscopic techniques. In addition to the isolation of the compounds, the determination of free sugars in the aqueous portion had been made. Sucrose may be the major principle which provide a sweet taste to *D. reticulata.* [3]

Biological activities of lupinifolin

Lupinifolin is a prenylated flavanone which was found to be a major compound in stem and stem wood of *D. reticulata* [15]. However, it can be isolated from other medicinal plants, such as *Myriopteron extensum* [16], *Eriosema chinense* [17] and *Albizia myriophylla* [18]. Lupinifolin is a compound of whose characteristics is less soluble in water, but easily soluble in methanol, ethanol, trichoromethane, and other organic solvents. It has been shown to exhibit antimicrobial activities against several pathogens such as herpes simplex virus type 1 [16], *Mycobacterium tuberculosis* [19], *Bacillus cereus, Corynebacterium diphtheria* and *S. epidermidis* [16, 19].

Cytotoxic and Anti-mycobacterial

Cytotoxicity against small-cell lung (NCI-H187) and oral epidermal carcinoma (KB) human cell lines and antimycobacterial activity against Mycobacterium tuberculosis H37Ra of the isolates were evaluated, with the result were found that lupinifolin were the most active antimycobacterial substances, and all exhibited a MIC value of 12.5 μg/mL. [19]

Antibacterial activities

Antibacterial activities of *A. myriophylla* components against *S. mutans* ATCC 25175 were presented as MIC and MBC values. All tested compounds were effective against *S. mutans* ATCC 25175 with MIC and MBC values ranging from 1-256 and 2-256 lg/ml, respectively. However, MIC and MBC values of penicillin G and chlorhexidine were 0.0156 and 0.0156 lg/ml and 0.5 and 1 lg/ml, respectively. Among the compounds isolated from *A. myriophylla*, lupinifolin displayed the highest activity with MIC and MBC of 1 and 2 lg/ml, respectively. [18]

Anti-inflammatory activity

Myriopteron extensum and lupinifolin were tested for anti-COX-2 and anti-COX-1 activities at 105- g/ml using radioimmunoassay. Lupinifolin and ethanolic extract showed inactive in the anti-inflammatory assay at concentration 200, 10 and 50 µg/ml, respectively. [16]

Plant material quality control [20]

Macroscopic and microscopic examination

Macroscopic identity of medicinal plant materials is based on shape, size, color, surface characteristics, texture, fracture characteristics and appearance of the cut surface. This method can be observed using visual observation or hand lens.

Microscopic inspection of medicinal plant materials is necessary for the identification of the anatomical and histological characters of the plant. It is suitable for examination of the fraction or powdered materials [21].

Determination of stomata type, stomatal number and stomatal index

The leaf crude drug can be specified, identified and characterized by the stomatal number and stomatal index. The stoma is a pore that surrounded by two guard cells. They help to regulate the rate of transpiration by opening and closing the stomata. The stomatal number and the stomatal index are very specific criteria for identification and characterization of leaf crude drugs. Stomatal number is the average number of stomata per mm^2 of epidermis on each surface of leaf. Four different type of stomata (Figure 4) are often available for matured leaves that are distinguished by there from and arrangement in the surrounding cells.

Anomocytic type is the stomata type that the stomata remains surrounded by a limited number of subsidiary cells which are quite alike the remaining epidermal cells.

Anisocytic type is the stomata type that the stoma remains surrounded by three accessory or subsidiary cells of which one is distinctly smaller than the other two.

Diacytic type is the stomata type that the stoma remains surrounded by a pair of subsidiary or accessory cells and whose common wall is at right angles to the guard cells.

Paracytic type is the stomata type that the stoma remains surrounded by two subsidiary or accessory cells which are parallel to the long axis of the pore and guard cells [20].

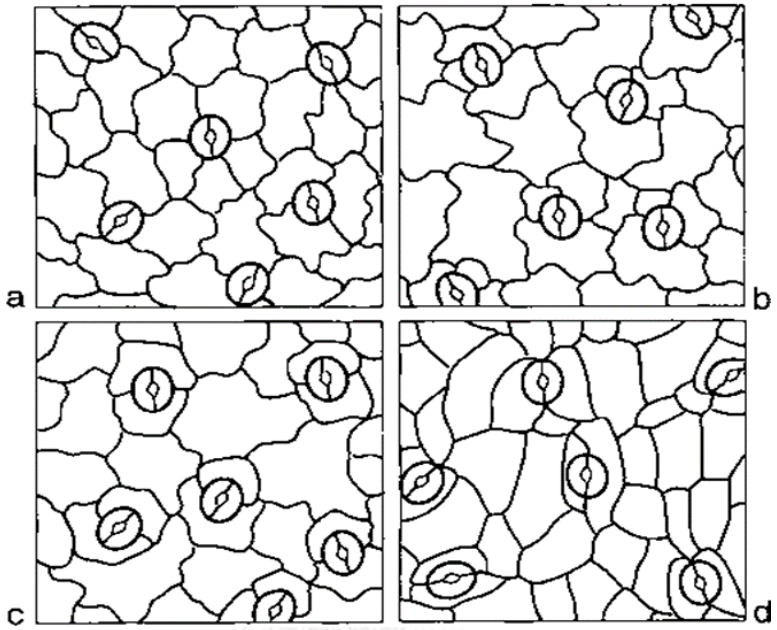


Figure 4 Types of stomata; a. Anomocytic type, b. Anisocytic type, c. Diacytic type, d. Paracytic type [20]

Stomatal number

Stomatal number is the number of stomata per 1 mm^2 of epidermal cell area. Two guarding cells are counted into single unit of a stoma. It was designed by Timmerman in 1927 [22].

Stomatal index

Stomatal index is the percentage of stomata number per total number of epidermal cell [21]. The calculation of the stomatal index can be explained following formula.

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

Where; S = the number of stoma per unit area

E = the number of ordinary epidermal cells in the same unit area

Palisade ratio

Palisade ratio can be calculated by average number of palisade cell beneath each epidermal cell. It can be determined with powdered drugs [22]. The determination was obtained by counting the total number of palisade cells beneath four upper epidermal cells and dividing the number by four. The value of the palisade ratio in same species gives the same result. This value is useful diagnostic feature for identification and characterization of the different plant species [21].

Foreign matter

Foreign matter referred to any matter which does not from a needed part of the plant sample. Foreign matter consists of any of the matter coming from the source plant but not defined as plant sample. It may be not coming from the source plant such as any organism, and it may include matter of mineral origin such as sand, stone, soil, dust, etc. Medicinal plant materials should be free from any contamination [20].

Determination of water content

Azeotropic method is specifically used for measurement of the water content in crude drug. The crude drug is distilled with a water-immiscible solvent such as toluene or xylene. However, the organic solvents can absorb a small amount of water. Therefore, the solvent should be saturated with water before use for accurate result [20].

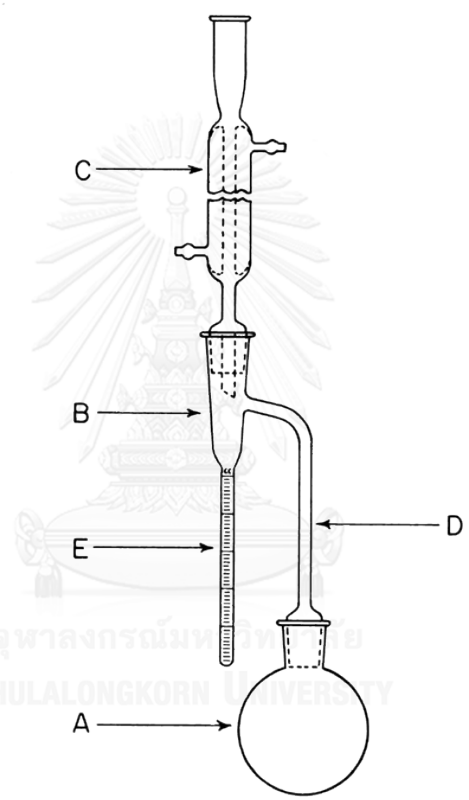


Figure 5 Apparatus used to determine water content by the azeotropic method [23]

Determination of extractable matter

Determination of extractable matter was used for determination of constituent from plant materials. The plant material was extracted with specified solvent such as water and ethanol. Water was used for the polar substances while ethanol was used for the slightly non-polar substances [20].

Determination of loss on drying

Loss on drying is one of quality parameters of the plant material. Drying method can be performed in several ways such as heating or keeping in desiccator with phosphorus pentoxide under atmospheric or reducing of pressure at room temperature for a specified period of time. The easiest way to determined water and volatile matters in plant materials is heating method. The heating method can be done by heating in oven at the temperature around 100-105 °C [20].

Determination of ash

Ash is the inorganic substances in plant material. The ash remaining following incineration of plant materials is determined by two different methods which measure total ash and acid-insoluble ash.

The total ash method determined the total non-volatile inorganic matters remaining after combustion.

Acid-insoluble ash is the residue obtained after boiling the total ash with hydrochloric acid (70 g/L) and incineration of the remaining insoluble matters. This measures the amount of some inorganic matters which are not solubilized in hydrochloric solution [20].

Soxhlet extractor

Extraction using Soxhlet apparatus is a continuous maceration which uses a Soxhlet apparatus to macerate plant sample with fresh distilled solvent to prepare crude plant extracts. The advantages of this method are automatic, continuous method that does not required further manipulation other than concentration of the extractive, saves solvent and not time consuming [24].

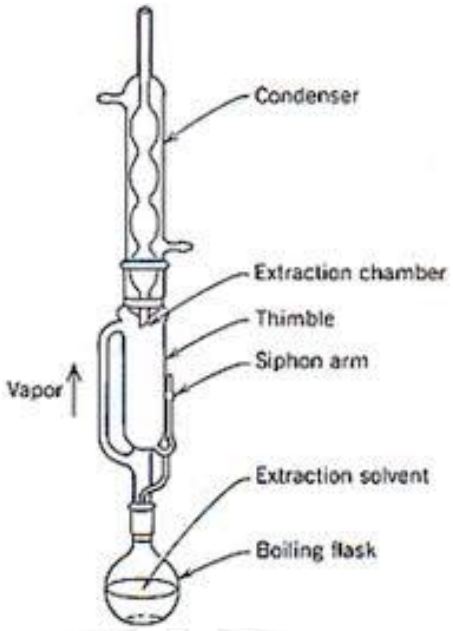


Figure 6 Soxhlet apparatus [25]

Thin Layer Chromatography

Thin-layer chromatography (TLC) is a planar chromatography and based on a distribution process. It can be used to check purity of compounds, to identify the compounds in the extract and to achieve a quantitative analysis of the compounds. TLC is a popular chromatographic technique because it is simple and inexpensive. It is used for qualitative and quantitative results. TLC system is composed of stationary phase and mobile phase. The stationary phase in TLC is an adsorbent and spongy particles coated with silica gel, aluminum or magnesium silicate. Mobile phase is the mixture of solvents for separation components in extract. The suitability of stationary and mobile phase will be effectively separated the extract. The result of TLC can be detected when separated compound absorb UV light or illuminate fluorescence. Moreover, the developed TLC plates will be sprayed or dipped into the chemical reagent for ensuring a homogenous and reproducible [26].

Retention factor

The retention factor (Rf) is a calculated value for the distance of the spots from compound appear from origin in TLC plates and the distance moved of the solvent

from origin. The Rf value can be used for identify the compounds under the same conditions. The Rf values can be calculated using the formula below [26].

$$Rf = \frac{distance\ of\ compound\ from\ origin}{distance\ of\ solvent\ front\ from\ origin}$$

TLC quantitative analysis of lupinifolin in *D. reticulata*

TLC quantitative analysis can be executed by TLC scanning using densitometer or TLC Image analysis. Densitometry is the qualitative and quantitative measurement of the substance on a TLC plate according to its fluorescence or UV-visible light absorption properties [27]. ImageJ is a public domain Java image processing and analysis program. It is a software to calculate pixel intensity in digital image of TLC spot and transform to chromatographic peak [28].

MTT assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was originally described by Mosmann (1983) [29]. It has been used successfully to quantitate cell survival and proliferation in macrophage-mediated cytotoxicity. Colorimetric assay is used based on the capacity of succinate dehydrogenase in mitochondria of viable cells to transform the MTT tetrazolium salt into MTT formazan (5- (4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan). The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The advantages of this method is savings in costs, reagents and equipment, reducing labor through the elimination of sample processing steps for liquid scintillation and gamma counting as well as avoiding use of radioisotopes. The result will be expressed as a concentration required for inhibiting cell growth by 50% (IC_{50} value). According to the American NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with $IC_{50} \leq 20$ µg/ml, while this value was deemed at ≤ 4 µg/ml for a pure compound [30].

DPPH free radical-scavenging assay

DPPH is stable free radical. Unlike *in vitro* generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. The freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably *via* a free-radical attack on the DPPH molecule) and convert them into a colorless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or substituted analogous hydrazine), resulting in decrease in absorbance at 517 nm [31].

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) method is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$, to the intensely blue colored Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium. Results are obtained as absorbance increases at 593 nm and can be expressed as μmolar of Fe^{2+} equivalents or relative to an antioxidant standard [32].

Total phenolic content by Folin-Ciocalteu method

Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins. These compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators, contributors to plant pigmentation, antioxidants, and protective agents against UV light, among others.

Colorimetric reactions are widely used in the UV/VIS spectrophotometric method, which is easy to perform, rapid and applicable in routine laboratory use, and low-cost. However, it is important that colorimetric assay need to use a reference substance, then this method measures the total concentration of phenolic hydroxyl

groups in the plant extract. Polyphenols in plant extracts react with specific redox reagents (Folin-Ciocalteu reagent) to form a blue complex that can be quantified by visible-light spectrophotometry. The Folin-Ciocalteu reaction forms a blue chromophore constituted by a phosphotungsticphosphomolybdenum complex, where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds. [33]

CHAPTER III

MATERIALS AND METHODOLOGY

Chemicals and reagent

2, 2-Dihenyl-1-picrylhydrazyl	Sigma-Aldrich, St Louis, USA
3, 5-Di-*tert*-4-butylhydroxytoluene (BHT)	Sigma-Aldrich, St Louis, USA
Acetic acid	BDH Chemicals Ltd., England
Chloral hydrate	Ajax Finechem Pty. Ltd., New Zealand
Dimethylsulfoxide (DMSO)	RCI Labscan Limited, Bangkok, Thailand
Doxorubicin	Sigma-Aldrich., Germany
Ethanol	RCI Labscan Limited, Bangkok, Thailand
Ethyl acetate	RCI Labscan Limited, Bangkok, Thailand
Ferric chloride	Ajex Finechem Pty Ltd, New Zealand
Fetal calf serum	Biochrom Gmbh, Germany
Folin-Ciocalteu reagent	Sigma Chemical, Co., USA
Haiter Bleach	Kao industrial, Thailand
Hexane	RCI Labscan Limited, Bangkok, Thailand
Hydrochloric acid	RCI Labscan Limited, Bangkok, Thailand
Lupinifolin	Isolation by Assoc. Prof. Nijsiri Ruangrungsi, Ph.D., Chulalongkorn University
Methanol	RCI Labscan Limited, Bangkok, Thailand
MTT	Invitrogen, USA
Normal saline	General Hospital Product Public Co., Ltd., Thailand

Quercetin	Sigma-Aldrich, St Louis, USA
RPMI 1640	Biochrom Gmbh, Germany
Toluene	RCI Labscan Limited, Bangkok, Thailand
Trypsin	Sigma-Aldrich., Germany

All of chemicals and reagents were analytical grade.

Materials

Cover glasses	Menzel-Glaser, Germany
Filter paper No.4	WhatmanTM Paper, UK
Filter paper No.40 ashless	WhatmanTM Paper, UK
Microplate with 96 wells	Costar, USA
Microscope Slide	Sail Brand, China
Tissue culture flask 25 cm^2	Constar, USA
Tissue culture plate 96 well	Constar, USA
TLC aluminum sheet 20 x 20 cm silica gel 60 GF_{254}, 200 µm thickness	Merck, Darmstadt, Germany

Equipment and instruments

Aqua-shaker	Adolf Kühner AG, Switzerland
Ashing furnance	Carbolite, England
Balance readability 0.0001 g	SI-234, Denver Instrument, Germany
Balance readability 0.01 g (PioneerTM, PA2102)	Ohaus Corp. Pine Brook, NJ, USA
CAMAG Linomat 5	CAMAG, Switzerland

CAMAG TLC Chamber	CAMAG, Switzerland
CAMAG TLC Scanner 3	CAMAG, Switzerland
CAMAG TLC Visualizer	CAMAG, Switzerland
CO_2 incubator forma series II	Thermo Electron Corporation, USA
Digital camera (Canon PowerShot A650 IS)	Canon Marketing (Thailand) Co., Ltd, Bangkok
Hemocytometer deep 1/10 nm	BOECO, Germany
Hot air oven	WTC Binder tuttlingen, Germany
ImageJ software (Version: 1.48)	National Institutes of Health, USA
Incinerator	Carbolite, UK
Incubator	Memmert, Germany
Invert microscope	Nikon, Japan
Microplate reader Multiskan FC 540 nm	Thermo Scientific, USA
Microscope	Zeiss Axioskop, Germany
Plate mixer SH30	Bio-Active Co., Ltd.
Rotary vacuum evaporator	Büchi Glas Uster, Switzerland
TLC syringe	Hamilton Company, USA
Ultrasonic sonicator	Analytical Lab Science Co., Ltd, Bangkok
Ultraviolet viewing cabinet (Model CC-80)	Spectronics corp., USA
Vortex mixer	Scientific Industries, USA
winCATS Software (Version: 1.4.6.2002)	CAMAG, Switzerland

Cell line

Five human cancer cell lines

BT-474 (Ductal carcinoma breast)	ATCC, USA
CHAGO-K1 (Undifferentiated lung carcinoma)	ATCC, USA
Hep-G2 (Liver hepatoblastoma)	ATCC, USA
KATO-3 (Gastric carcinoma)	ATCC, USA
SW-620 (Colon adenocarcinoma)	ATCC, USA

One human normal cell line

Wi-38 (Lung fibroblast)	ATCC, USA

Plant materials

The stem woods of *D. reticulata* were collected from 15 different locations throughout Thailand. They were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. The stem wood materials were spread on a cleaned paper. Foreign matters were sorted either visual inspection. The remainder sample was made to free from dust by passing through suitable mesh sieve. The stem wood was air dried and pulverized for further analysis [34].

Standardization parameters

The determination of standardization parameter including macroscopic and microscopic examination, leaf measurement, water content, loss on drying, total ash values, acid insoluble ash, ethanol extractive values, water extractive values were studied for specifying the characteristic of crude plant samples.

Macroscopic and microscopic examination of *D. reticulata* stem wood

Macroscopic identity of *D. reticulata* materials was based on shape, size, color, surface characteristics, texture and fracture characteristics.

Microscopic characteristics were examined in cross section and in powdered form. The stem wood of *D. reticulata* was transverse sectioned using razor blade and mounted onto a slide in water. The anatomical characters were observed under microscope with 10X, 20X, and 40X objective lens and 10x eyepiece lens magnifications. The stem wood was pulverized then observed for histological structures under microscope as mentioned above.

Leaf constant number measurement

D. reticulata leaf section was prepared by cutting from the middle lamina of the fresh mature leaf and soaked in the mixture of water and Haiter bleach solution (50% in water) about 1-2 days until chlorophyll removal. It was further boiled in the 10% hydrochloric acid for 15 minutes and respectively boiled in chloral hydrate solution (4 g/ml in water) for 30 minutes until it was transparent. The transparent section was rinsed with water before observation of the palisade number, stomatal number and epidermal cell number under the microscope. The palisade ratio, stomatal index and epidermal cell area were calculated.

Determination of water content (Azeotropic method)

D. reticulata dried stem wood powders (30 g) were added with 200 ml of water-saturated toluene in flask. The flask was heated for water distillation. The toluene and water in receiving tube were completely separated [12, 20].The content of water was calculated in percentage.

Determination of loss on drying

D. reticulata dried stem wood powders (3 g) were weighed in the pre-weighed crucible. Loss on drying was done in an oven at 105 ºC for 6 hours until constant weight. After cooling in a desiccator for 30 minutes, the loss on dying was weighed and calculated in percentage [12, 20].

Determination of total ash

D. reticulata dried stem wood powders (3 g) were weighed in the pre-weighed crucible. The sample was incinerated at 500 ºC until it is become white which indicating the absence of carbon. After cooling in a desiccator for 30 minutes, the total ash was weighed and calculated in percentage [12, 20].

Determination of acid insoluble ash

Twenty-five milliliters of hydrochloric acid solution (70 g/l) were added to the crucible containing the total ash and boiled gently for 5 minutes. The insoluble matter was collected on an ashless filter-paper No.40. The filter-paper containing the insoluble matter was transferred to the original crucible, dried on a hot-plate and incinerated at 500 ºC until constant weight. After cooling in a desiccator for 30 minutes, the acid insoluble ash was weighed and calculated in percentage [12, 20].

Determination of ethanol soluble extractive matter

D. reticulata dried stem wood powders (5 g) were macerated with 70 ml of 95% ethanol in a closed conical flask for 6 hours under shaking, then standing for 18 hours. The marc was filtered and washed. The filtrate was adjusted to 100 ml with ethanol. Twenty milliliters of the filtrate were transferred to a pre-weighed beaker. The sample was evaporated to dryness on a water-bath and then dried in oven at 105 ºC for 6 hours. After cooling in a desiccator for 30 minutes, the ethanol soluble extractive matter was weighed and calculated in percentage [12, 20].

Determination of water soluble extractive matter

D. reticulata dried stem wood powders (5 g) were macerated with 70 ml of water in a closed conical flask for 6 hours under shaking, then standing for 18 hours. The marc was filtered and washed. The filtrate was adjusted to 100 ml with water. Twenty milliliters of the filtrate were transferred to a pre-weighed beaker. The sample was evaporated to dryness on a water-bath and then dried in oven at 105 ºC for 6 hours. After cooling in a desiccator for 30 minutes, the water soluble extractive matter was weighed and calculated in percentage [12, 20].

Thin layer chromatography fingerprint

Twenty milliliters of the aforementioned ethanolic filtrate were evaporated and re-dissolved in 1 ml of ethanol. Three microliters were applied to the thin layer aluminum plate coat with silica gel 60 GF_{254}. The chromatogram was developed in the chamber with the solvent system of hexane : ethyl acetate (6:4 v/v). The plate was removed and allowed to dry in air and observed the produced spots in daylight, under short wave (254 nm) and long wave (365 nm) ultraviolet light. The spots were detected by spraying with 10 % sulfuric acid in ethanol and heated.

Quantitative analysis of lupinifolin in *D. reticulata* stem wood

Standard lupinifolin was isolated by Assoc. Prof. Dr. Nijsiri Ruangrungsi then compared with the previous studies of pharmacological properties and absolute configuration of *D. reticulata*

One milligram of lupinifolin standard was dissolved in 1 ml of 95% of ethanol and diluted concentration to 0.2, 0.4, 0.6, 0.8 and 1 mg/ml to prepare the series of stock solution. These standard solutions were kept in refrigerator at 4 ºC.

Preparation of ethanol extracts of *D. reticulata* stem wood

The accurate 5 g of *D. reticulata* dried stem wood powders were exhaustively extracted with 250 ml of 95% ethanol in a Soxhlet apparatus. The ethanolic extract

was filtered and evaporated under reduced pressure in a rotary evaporator till dryness. The extracted sample was stored at 4 ºC for TLC-densitometry and TLC image analyses.

TLC image analysis by ImageJ software

Three microliters of *D. reticulata* extract and standard lupinifolin solution in ethanol were applied on the 20 x 10 cm silica gel 60 GF_{254} TLC plate, developed in hexane : ethyl acetate (6:4). After development, the plate was observed under short wave ultraviolet light (254 nm) and photographed using digital camera.

Quantitative analysis of the lupinifolin spots on TLC plate was performed using ImageJ software. The calibration curve of lupinifolin was examined by plotting peak areas and concentrations of lupinifolin in µg/spot.

TLC-densitometry

The lupinifolin spots on the developed TLC plates were quantitatively analyzed by scanning with CAMAG TLC densitometer at optimal wavelength. The calibration curve of lupinifolin was examined by plotting peak areas and concentrations of lupinifolin in µg/spot.

Method validation

According to the ICH guidelines [35], the method validation including calibration range, accuracy, precision, specificity, LOD, LOQ and robustness were performed.

Calibration range

The calibration range was calculated by plotting peak areas and concentrations of standard lupinifolin applied.

Accuracy

The accuracy was tested by recovery method. Standard lupinifolin solution was spiked into the extract to have three different levels of lupinifolin (low, medium and high). The spiked and un-spiked sample were analyzed under the same conditions in triplicate. The accuracy was determined by using following formula.

$$\%Recovery = \left[\frac{A}{B+C}\right] \times 100$$

A = actual calculated amount in recovery sample

B = amount un-spiked into the sample

C = amount standard lupinifolin added to the sample

Precision

The precision was examined by repeatability (intra-day) and intermediate (inter-day) precision. The method was performed by analyzing sample solution of three concentrations in three replicates on the same day and three different days respectively. The content calculated by measurement of peak area was determined for % relative standard deviation (% RSD) by following formula.

$$RSD = \frac{SD \times 100}{Mean}$$

SD = the standard deviation of each measurement

Specificity

The TLC plates after development were scanned for absorption spectra under the range of 200-700 nm by TLC scanner. The specificity was evaluated by comparison of the absorption the standard lupinifolin and each sample as well as comparison of the absorption spectra at up-slope, apex and down-slope of the peak.

Limit of detection

The limit of detection (LOD) was determined from the calibration range using this formula.

$$LOD = \frac{3.3(SD)}{S}$$

SD = the residual standard deviation of regression line

S = the slope of regression line

Limit of quantitation

The limit of quantitation (LOQ) was determined from the calibration range using this formula.

$$LOQ = \frac{10(SD)}{S}$$

SD = the residual standard deviation of regression line.

S = the slope of regression line

Robustness

The robustness was examined by changing the mobile phase ratio. The selected mobile phase ratio of hexane : ethyl acetate at the ratio of 5.9 : 4.1 , 6.1 : 3.9 , 6.0 : 4.0 were examined and calculated for % RSD of peak area.

Cytotoxicity determination

Five human cancer lines and 1 human normal cell line including BT-474 (breast ductal carcinoma), CHAGO-K1 (undifferentiated lung carcinoma), SW-620 (colon adenocarcinoma), KATO-3 (gastric carcinoma), Hep-G2 (Liver hepatoblastoma) and Wi-38 (Lung fibroblast) respectively were used in cytotoxic assay. Cell survival was measured using the MTT method.

The crude ethanolic extract was dissolved in DMSO to obtain the concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 µg/ml. Five milligrams of doxorubicin positive control drug were dissolved in normal saline to obtain the concentrations of 0.001, 0.01, 0.1, 1 and 10 µg/ml. MTT was dissolved in normal saline at 5 mg/ml, stored in the dark at 4 ºC and used within 1 month.

The cell line was cultured in tissue culture flask in RpMI-1640 supplemented with 5% (v/v) fetal calf serum and incubated at 37 ºC in 5 % CO_2 for 3 days. Two hundred microliters of cell were seeded in a 96-well culture plates at a density of $1x10^4$ cells/well and cultured in 5 % CO_2 incubator at 37 ºC and 100% relative humidity for 24 hours.

The sample solution (2 µl) was dispensed into the appropriate wells. This analysis was performed in 4 replicates (control cells group, n=4; each sample treatment group, n=4; Doxorubicin/DMSO n=4). Culture plates were incubated for 72 hours prior to the addition of MTT solution (10 µl). Plates were incubated for 4 hours at 37 ºC, 5% CO_2 incubator. After incubation, the formazan produced in the cell was captured as dark crystal in the bottom of the wells. The supernatant from culture medium was removed from wells. DMSO (150 µl) was added to dissolve the resulting formazan and mixed for 2-3 minutes on an orbital shaker. Following formazan solubilization, the absorbance was measured using a microplate reader at the wavelength of 540 nm. The cell survival was calculated in percentage using the following formula:

$$Percentage\ of\ cell\ survival = \frac{Mean\ absorbance\ in\ test\ wells}{Mean\ absorbance\ in\ control\ wells} \times 100$$

The cytotoxic activity was expressed as a concentration required for inhibiting cell growth by 50% (IC_{50} value).

Determination of antioxidant activity

DPPH free radical-scavenging assay

The antioxidant activity of plant extracts were assessed by ability to scavenge DPPH free radical as described by Brand-William *et al*. [36]. Various concentrations of samples were dissolved in methanol, and were added to DPPH radical methanolic solution (120 μM). After 30 minutes of incubation at room temperature in the dark. The absorbance was measured at the wavelength of 517 nm using a 96 well microplate reader. BHT and Quercetin were used as positive controls. Three replicates were made for each test sample. The scavenging activity was evaluated from the decrease in absorbance value at 517 nm and calculated using the following formula.

$$DPPH\ radical\ inhibition\ (\%) = \frac{(absorbance\ of\ control - \ absorbance\ of\ sample)}{absorbance\ of\ control} \times 100$$

The activity was expressed as IC_{50} values which indicate the concentration of sample required to scavenge 50% of DPPH free radical.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was examined following Benzie and Stain (1996) with minor modification. The FRAP working solution was freshly prepared by mixing: 300 mM of acetate buffer at pH 3.6, 10 mM of TPTZ in 40 mM HCL and 20 mM $FeCl_3$ solution at (10:1:1) parts per volume, respectively. Twenty-five microliters of the extract, quercetin or BHT (0.5 mg/ml) and 175 μl of FRAP reagent were added in 96 well plate and were incubated in room temperature for 30 minutes. The absorbance was recorded at 593 nm using microplate reader. The calibration curve of $FeSO_4$ was conducted as mentioned protocol. Antioxidant power of the extracts and positive controls were calculated in term of mM of ferrous iron (Fe (II)) per mg extract [37].

Total phenolic content

The total phenolic content was determined using spectrophotometric method. In brief, 25 µl of extracts (0.5 mg/ml) was mixed with 125 µl of 10% Folin- Ciocalteu's phenol reagent in with a 96 well microplate reader. After 5 min, 100 µl of a 7.5% Na_2CO_3 solution was added to the mixture then incubation at room temperature at 60 min, after which the absorbance was read at 756 nm [38].

Data analysis

The parameters due to standardization were expressed as grand mean ± pooled standard deviation. The lupinifolin contents between TLC image analysis and TLC-densitometry were compared by paired *t*-test statistical analysis. The results of antioxidant activities were expressed as IC_{50} in DPPH radical scavenging, the total phenolic content of extract was determined from a standard curve of gallic acid and results were expressed as mg gallic acid equivalents (GAE) per gram extract and as mM of ferrous iron (Fe (II)) per milligram extract in FRAP assay.

Scope of the study

Quality assessments are performed according to Thai herbal pharmacognostic specification. Moreover, the biological activities in this study were performed *in vitro*. This study was not applied for ethical consideration because of no methodologies related to animal or human.

CHAPTER IV

RESULTS

Pharmacognostic specifications

Common Name	CHA-AEM-NUEA
Scientific Name	*Derris reticulata* Craib
Family	PAPILIONACEAE
Distribution	The tropical region of South-East Asia
Used Part	Stem wood
Ethnomedical Use	Expectorant, antitussive, treatment of throat diseases, tonic

Description of plant

Woody climbers less commonly trees or shrubs. Leaf 5-foliate, petiole 1.5-4 cm, imparipinnate, leaflets opposite, stipels present or absent; stipules small and caducous. Inflorescence a raceme or panicle. Bract and bracteoles small. Calyx cupulate and subtruncate or with very short teeth. Corolla white, pink or purple. Vexillum ovate to obovate or suborbicular, usually with auricles or calluses at the base. Wing adhering to keel petal. Stamens monadelphous, vexillary stamen free at the base only. Ovary often subsessile, few-ovulate, style glabrous above, stigma terminal. Fruit flat, indehiscent, 4 x (1.7-2) cm, upper or both the sutures winged. Seed reniform or orbicular.

Macroscopic evaluation

The dried stem wood of *D. reticulata* was yellow and brown color, 2 cm in width (Figure 7). The taste was sweet. The drawing of twig of *D. reticulata* was shown in Figure 8.

Crude drugs

Figure 7 Dried stem wood of *Derris reticulata* Craib

Figure 8 Twig of *Derris reticulata* Craib

Microscopic evaluation

The anatomical characterization of *D. reticulata* stem wood showed epidermis, periderm, parenchyma containing prism crystal, sclereid, phloem fiber, vascular cambium, xylem vessel, xylem fiber and xylem ray (Figure 9). The histological evaluation of *D. reticulata* stem wood powder demonstrated fragment of xylem ray in radial longitudinal view, sclereid, cork in surface view, cork in sectional view, fragment of fiber, fragment of bordered pitted vessel, fragment of parenchyma, ray parenchyma, prism crystal of calcium oxalate and calcium oxalate prism sheath (Figure 10).

Anatomical character

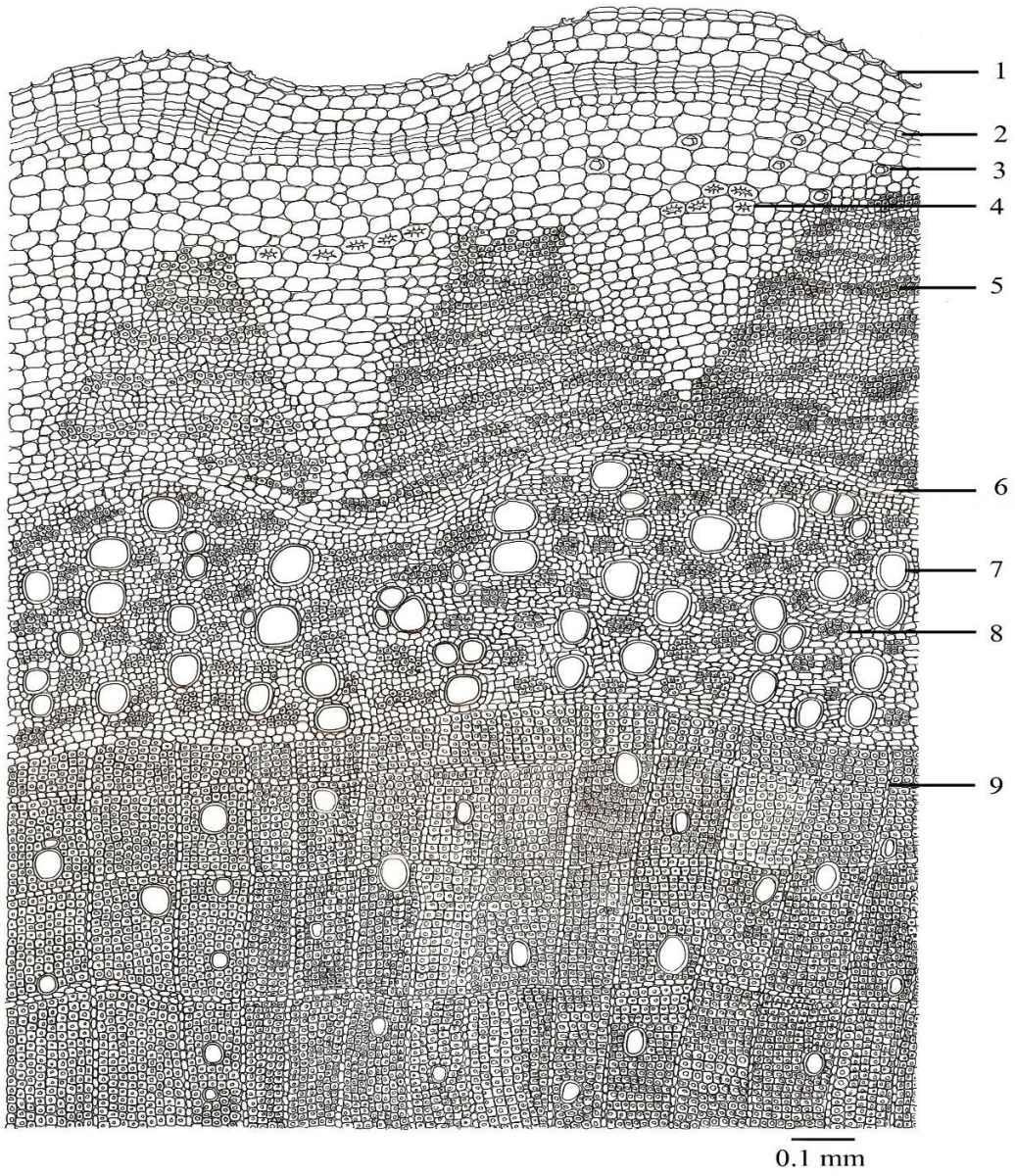


Figure 9 Anatomical character of *D. reticulata* stem wood transverse section

1. Epidermis
2. Periderm
3. Parenchyma containing prism crystal
4. Sclereid
5. Phloem fiber
6. Vascular cambium
7. Xylem vessel
8. Xylem fiber
9. Xylem ray

Histological character

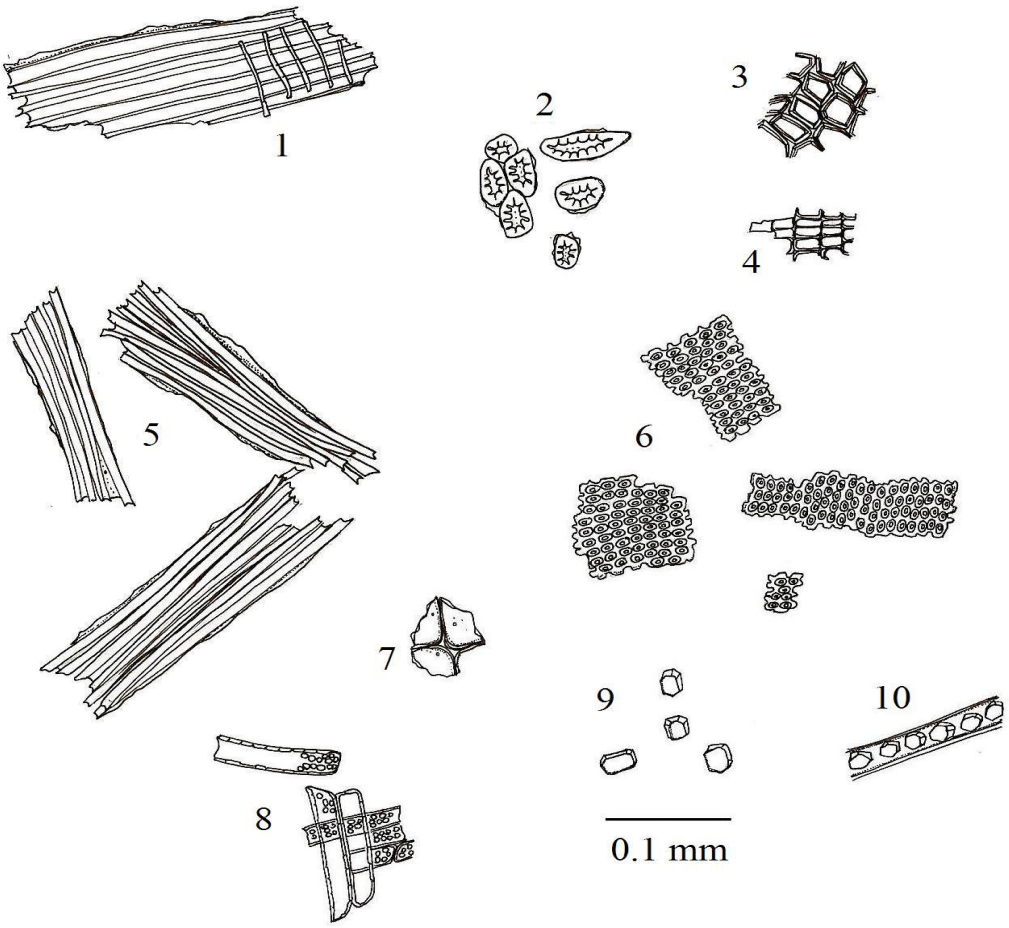


Figure 10 Histological character of *D. reticulata* stem wood powder

1. Fragment of xylem ray in radial longitudinal view
2. Sclereid
3. Cork in surface view
4. Cork in sectional view
5. Fragment of fiber
6. Fragment of bordered pitted vessel
7. Fragment of parenchyma
8. Ray parenchyma
9. Prism crystal of calcium oxalate
10. Calcium oxalate prism sheath

Thin layer chromatographic fingerprint

The ethanolic extract of *D. reticulata* was spotted on TLC silica gel 60 GF_{254} plate developed in hexane : ethyl acetate (6 : 4) and observed under ultraviolet light (254, 365 nm) and dipped with 10% sulfuric acid in ethanol then heated at 100 ºC for 15 minutes.

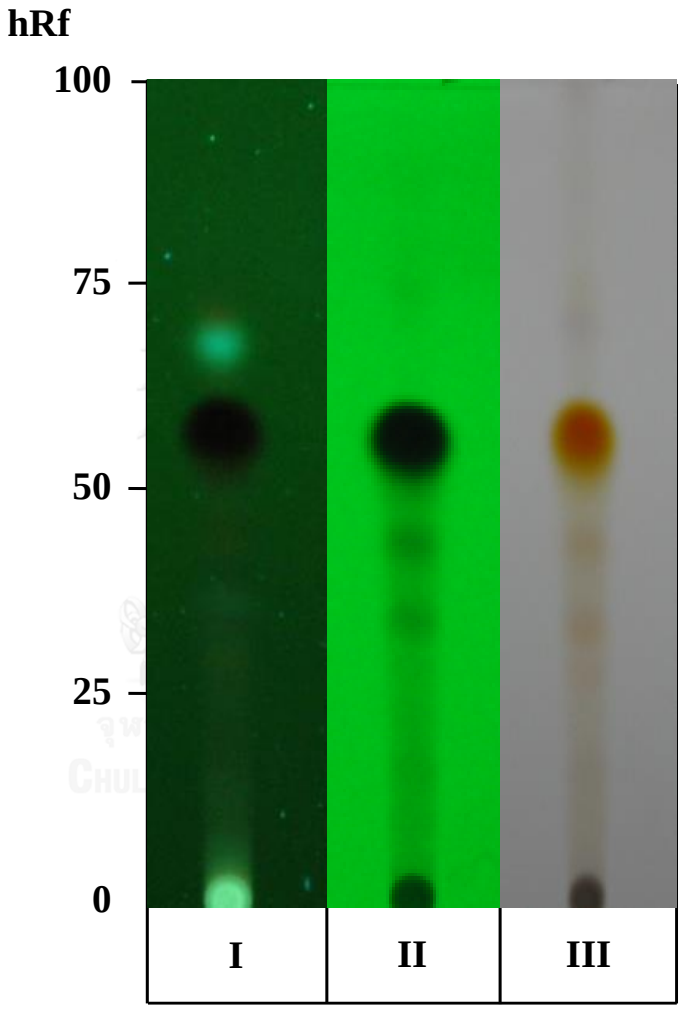


Figure 11 Thin layer chromatographic fingerprint of ethanolic extract of *D. reticulata*

Stationary phase : Silica gel 60 GF_{254}

Solvent system hexane : ethyl acetate (6:4)

Detection I = detection under UV 365 nm

II = detection under UV 254 nm

III = detection with 10 % sulfuric acid in ethanol and heated

Physico-chemical parameters of dried *D. reticulata* stem wood

The results of physico-chemical parameters of *D. reticulata* stem wood were shown in Table 1. The loss on drying, total ash, acid-insoluble ash and water content should be not more than 5.7, 4.6, 1.0 and 8.5 % of dry weight respectively. The water soluble extractive value and ethanol soluble extractive value should be not less than 15.5 and 6.8 % by dry weight respectively.

Table 1 Physico-chemical content of *D. reticulata* stem wood (% by weight)

Specification	**Content (% dry weight) ***
Water Content	8.5 ± 0.3
Loss on Drying	5.7 ± 0.1
Total Ash	4.6 ± 0.1
Acid Insoluble Ash	1.0 ± 0.1
Water Soluble Extractive Value	15.5 ± 0.7
Ethanol Soluble Extractive Value	6.8 ± 0.3
Volatile oil	0

*The parameters were shown as grand mean ± pooled SD. Samples were collected from 15 different sources in Thailand. Each sample was tested in triplicate.

Leaf measurement

The fresh mature leaf was observed for palisade, stomata, epidermal cells in both sides. Palisade was determined in the upper side. Anisocytic stomata was found in the lower side. The quantitative analyses of palisade ratio, stomatal number, stomatal index, epidermal number and epidermal cell were done in thirty fields and averaged. The results were shown in Figure 12-14 and Table 2.

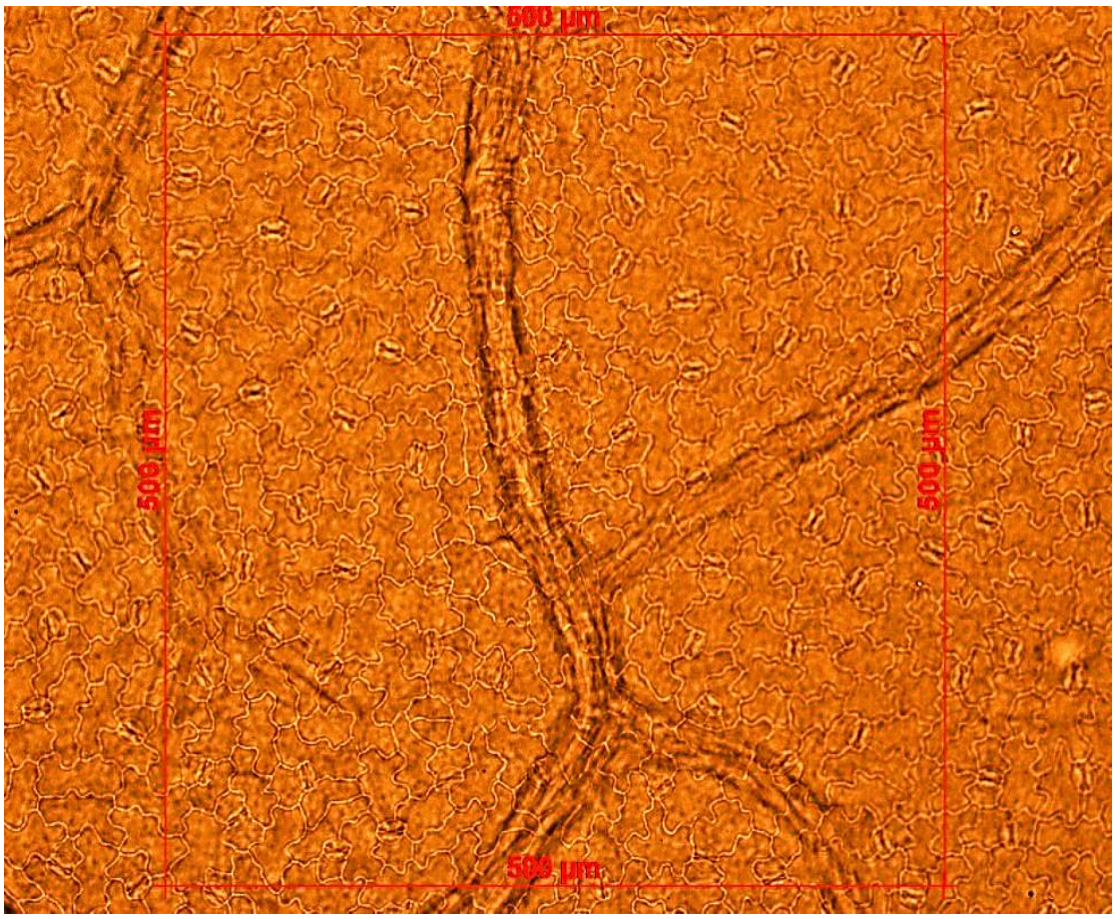


Figure 12 Stomata of *Derris reticulata* Craib leaf (Anisocytic type)

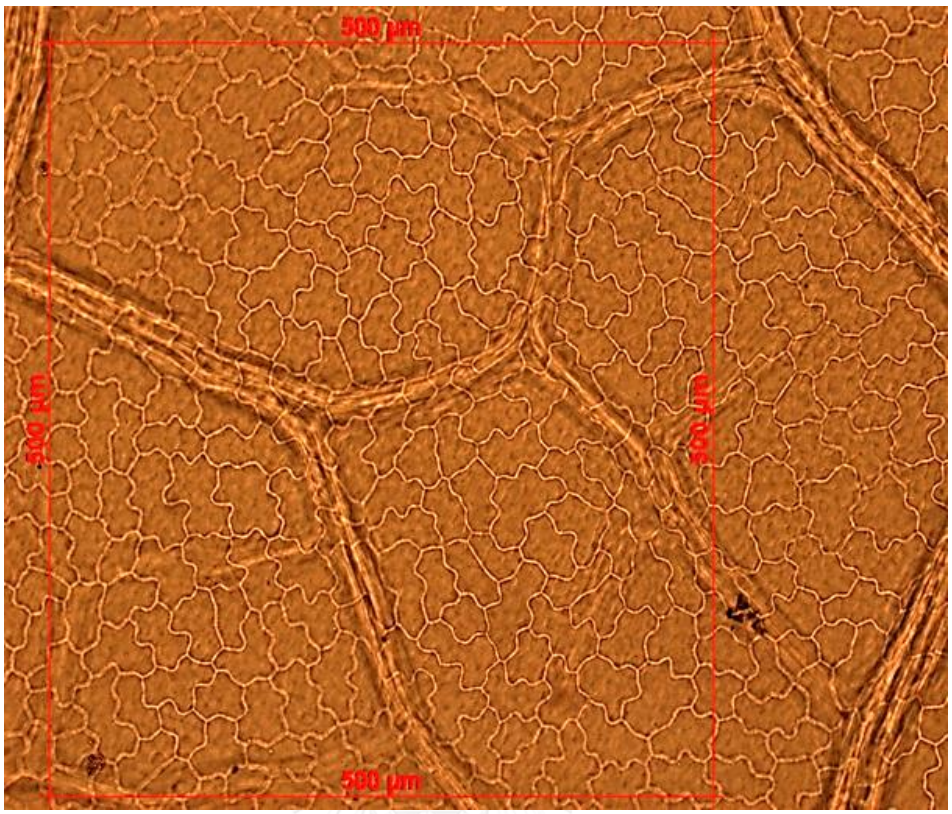


Figure 13 Epidermal cell in the upper side of *Derris reticulata* Craib leaf

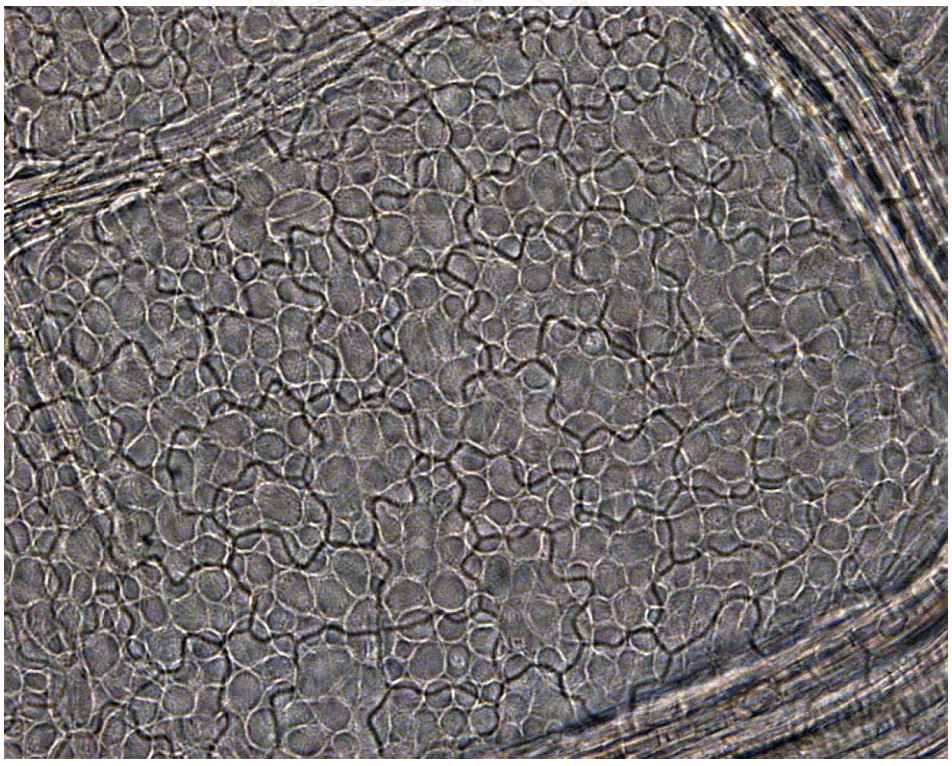


Figure 14 Palisade in the upper epidermis of *Derris reticulata* Craib leaf

Table 2 Stomatal number, stomatal index, palisade ratio, epidermal cell number and stomatal cell area of *D. reticulata* leaves

No.	Epidermal cell number in 1 mm^2	Epidermal cell area (μm^2)	Stomatal number in 1 mm^2	Stomatal Index (mm^2)	Palisade ratio
1	822	1216.55	268	19.03	6.5
2	842	1187.65	316	20.52	6.625
3	856	1168.22	264	18.70	6.5
4	864	1157.41	280	17.07	6.25
5	852	1173.71	300	18.12	7
6	848	1179.25	288	17.43	12.25
7	838	1193.32	292	20.74	12
8	812	1231.53	284	17.49	9
9	904	1106.19	336	19.18	10
10	836	1196.17	352	19.56	9.25
11	882	1133.79	320	18.35	6.75
12	830	1204.82	356	19.82	9
13	862	1160.09	324	21.15	10.75
14	968	1033.06	348	19.55	9.25
15	890	1123.60	332	22.02	9.5
16	864	1157.41	320	21.51	8
17	872	1146.79	312	20.53	10.75
18	890	1123.60	328	21.30	7.5
19	764	1308.90	312	21.20	8
20	796	1256.28	316	21.35	8.25
21	802	1246.88	320	21.00	9.75
22	852	1173.71	316	20.63	9
23	814	1228.50	320	21.16	8.75
24	834	1199.04	328	21.47	9.25
25	902	1108.65	324	22.07	9.5
26	866	1154.73	328	21.35	8
27	924	1082.25	320	21.16	7.75
28	866	1154.73	312	18.57	12.75
29	808	1237.62	336	22.11	13.75
30	870	1149.43	344	22.28	6.75
MIN	764	1033.06	264	17.07	6.25
MAX	968	1308.90	356	22.28	13.75
MEAN	**854.33**	**1173.13**	**316.53**	**20.21**	**8.95**
SD	**41.37**	**56.25**	**23.03**	**1.53**	**1.96**

Ethanolic extraction of *D. reticulata* stem wood

The percent yield of 95 % ethanolic extract of *D. reticulata* stem wood by Soxhlet extraction was 17.08 ± 2.69 % by weight in average (Table 3).

Table 3 The percent yield of 95 % ethanolic extract of *D. reticulata* stem wood from 15 different sources throughout Thailand

Source	Weight of sample (g)	Weight of extractive matter (g)	% yield
1	5.00	0.94	18.79
2	5.00	0.93	18.52
3	5.00	0.81	16.10
4	5.00	0.96	19.23
5	5.00	1.01	20.24
6	5.00	1.05	20.92
7	5.00	0.87	17.29
8	5.00	0.70	13.95
9	5.00	0.94	18.73
10	5.00	0.96	19.16
11	5.00	0.60	12.08
12	5.00	0.65	12.94
13	5.00	0.88	17.62
14	5.00	0.75	14.99
15	5.00	0.78	15.66
		Average	**17.08 ± 2.69**

The content of lupinifolin in *D. reticulata* stem wood by TLC densitometry

Standard lupinifolin and the ethanolic extracts were developed in hexane : ethyl acetate (6:4). TLC plate was scanned by CAMAG TLC scanner under 275 nm. The peak areas were computed by WinCATS software. The ethanolic extrats of *D. reticulata* stem wood were determined for the lupinifolin content in triplicate by TLC densitometry and calculated as grams per 100 grams of the crude drug (Table 4).

Table 4 The amount of lupinifolin in *D. reticulata* stem wood by TLC densitometry (% by weight)

Source	Lupinifolin in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100 g of dried crude drug)	Lupinifolin in *D. reticulata* stem wood (g/100 g of dried crude drug)
1	0.45	18.79	8.52
2	0.33	18.52	6.09
3	0.33	16.10	5.31
4	0.44	19.23	8.53
5	0.41	20.24	8.20
6	0.44	20.92	9.20
7	0.36	17.29	6.28
8	0.34	13.95	4.79
9	0.48	18.73	9.02
10	0.51	19.16	9.83
11	0.67	12.08	8.15
12	0.38	12.94	4.86
13	0.74	17.62	12.96
14	0.48	14.99	7.26
15	0.77	15.66	12.02
	Average		**8.07 ± 2.41**

Method validation (TLC densitometry)

The calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were examined for the validation of an analytical method followed by ICH guideline.

Calibration range

The calibration curve of lupinifolin was obtained by the peak area of standard (0.6 – 3 µg/spot). The polynomial with the regression equation was $y = -600.37x^2 + 9653.7x + 3643.1$. The coefficient of determination (R^2) of lupinifolin was 0.9985.

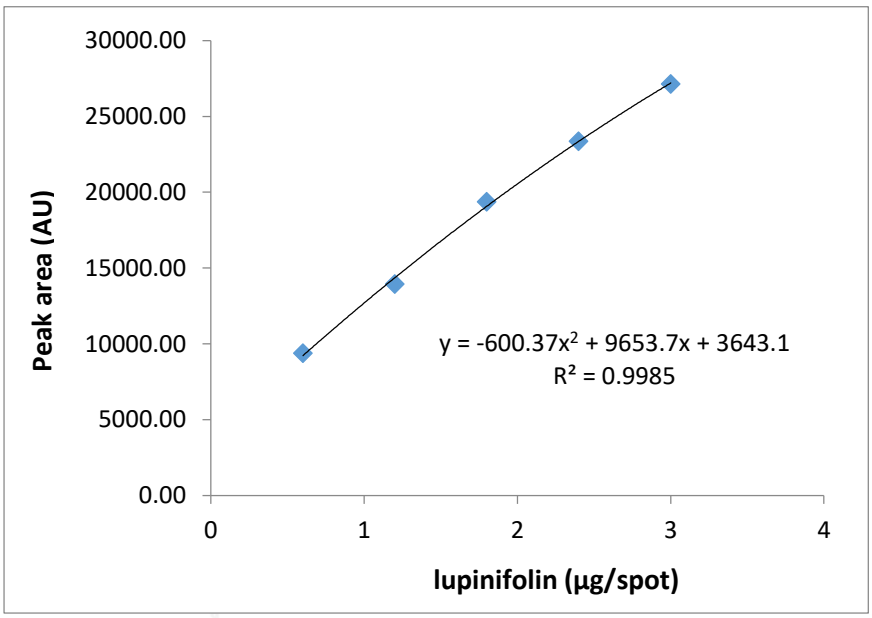


Figure 15 The calibration curve of lupinifolin in *D. reticulata* stem wood by TLC densitometry

Detection limit and quantitation limit

The detection limit and quantitation limit determination were based on the standard deviation of regression line and the slope of the calibration curve. The LOD value, the lowest concentration of analyze in a sample that could be detected was found to be 0.14 µg/spot. The LOQ value, the lowest concentration of analyze in a sample that could be quantitatively defined was 0.41 µg/spot.

Accuracy

The accuracy was tested by recovery method. Standard lupinifolin was spiked into the extract to have three different levels of lupinifolin (low, medium, high). The recovery values were 97.68 – 110.59 % as demonstrated in Table 5.

Table 5 Accuracy of quantitation of lupinifolin in *D. reticulata* stem wood by TLC densitometry (n=3)

Lupinifolin added (µg/spot)	**Lupinifolin found (µg/spot)**	**% Recovery**
0.00	0.76 ± 0.01	-
0.3	1.03 ± 0.01	97.68 ± 1.65
0.9	1.83 ± 0.02	110.59 ± 1.29
1.5	2.45 ± 0.06	108.60 ± 2.52
	Average	**105.62 ± 6.95**

Precision

The precision was examined by repeatability (intra-day) and intermediate precision (inter-day). The method was performed by analyzing sample solution of three concentrations in three replicates on the same day and three different days respectively. The repeatability and intermediate precision were showed in Table 6.

Table 6 Repeatability and intermediate precision of quantitation of lupinifolin in *D. reticulata* stem wood by TLC densitometry (n=3)

Repeatability		**Intermediate precision**	
Amount (µg/spot)	**%RSD**	**Amount (µg/spot)**	**%RSD**
1.03 ± 0.01	1.21	1.04 ± 0.04	3.93
1.83 ± 0.02	0.90	1.85 ± 0.01	0.80
2.45 ± 0.06	2.38	2.46 ± 0.12	4.77
Average	**1.30 ± 0.75**		**3.83 ± 2.16**

Specificity

Peak identity

The specificity was executed by peak identity and peak purity checking. The identity in absorbance spectra was determined at the peak apex among lupinifolin standards and samples. The maximum absorbance of lupinifolin was at the wavelength 275 nm. The result was illustrated in Figure 16.

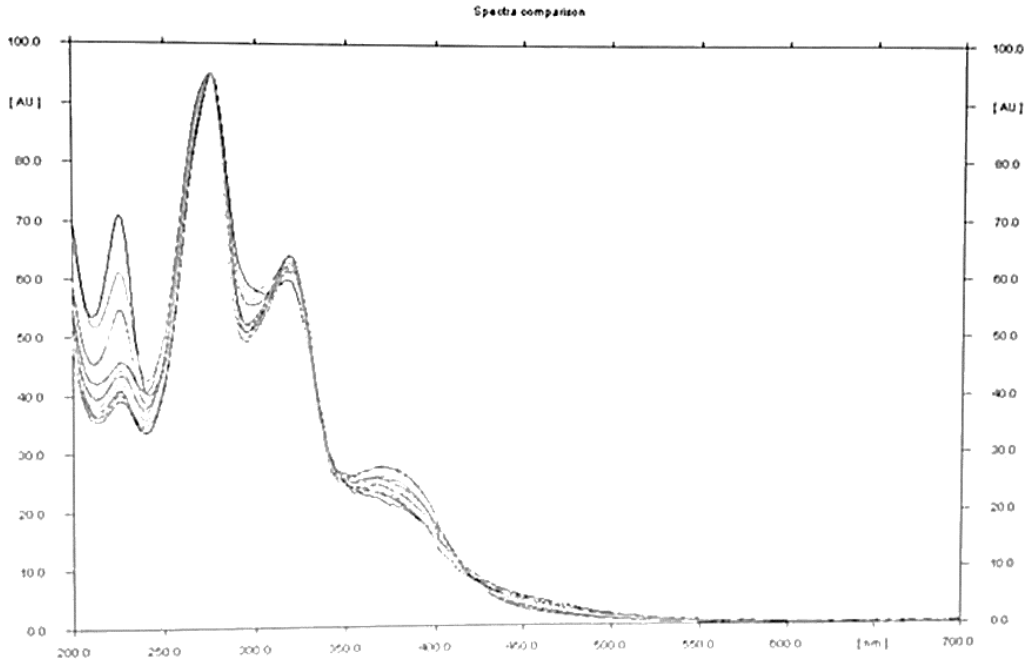


Figure 16 The absorbance spectra of lupinifolin in *D. reticulata* stem wood extracts from 15 different sources and standard lupinifolin representing peak identity

Peak purity

Peak purity of lupinfolin was represented in Figure 17. The absorbance spectra from up-slope, apex and down-slope of the peak were identical.

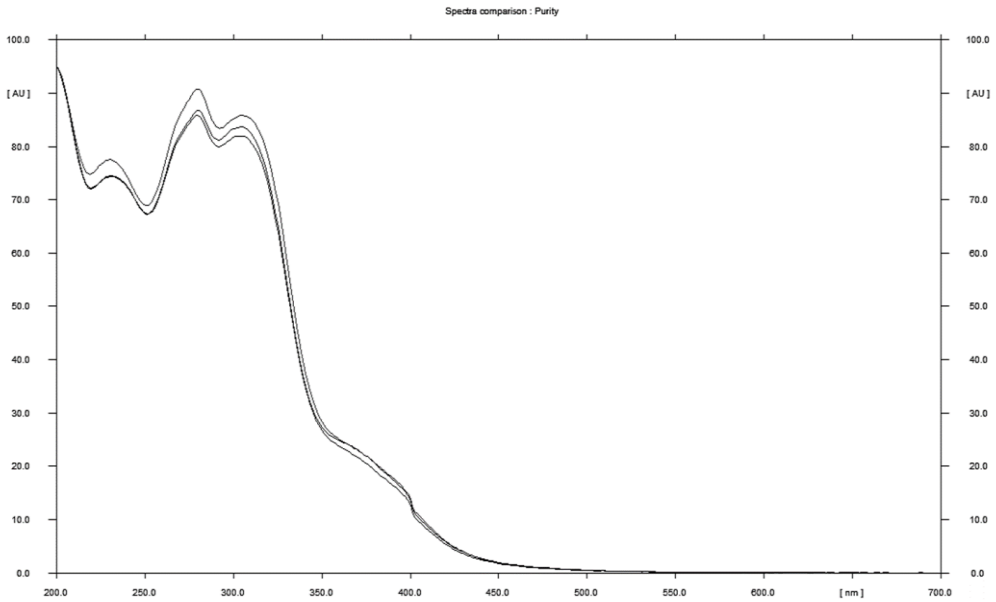


Figure 17 Peak purity measurement using up-slope, apex and down-slope of the peak.

Robustness

The robustness of lupinifolin quantitation in *D. reticulata* stem wood by TLC densitometric analysis was examined by changing the mobile phase ratio. The selected mobile phase ratio was shown in Table 7. The result of robustness was 2.98 % RSD of peak area.

Table 7 Robustness of lupinifolin in *D. reticulata* stem wood by TLC densitometry

Mobile phase composition **Hexane : Ethyl Acetate**	**Peak area**
5.9 : 4.1	33633.18
6.1 : 3.9	32730.20
6.0 : 4.0	34735.11
Mean ± SD	**33699.50 ± 1004.10**
% RSD	**2.98**

The content of lupinifolin in *D. reticulata* stem wood by TLC image analysis

Standard lupinifolin and the ethanolic extracts were developed in hexane : ethyl acetate (6:4). The image of TLC plates photographed under UV 254 nm were analyzed for lupinifolin of peak areas by ImageJ software. The amounts of lupinifolin were found to be 7.76 ± 2.21 grams in 100 grams of *D. reticulata* stem wood crude drug (Table 8).

Table 8 The amount of lupinifolin in *D. reticulata* stem wood by TLC image analysis (% by weight)

Source	Lupinifolin in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100 g of dried crude drug)	Lupinifolin in *D. reticulata* stem wood (g/100 g of dried crude drug)
1	0.45	18.79	8.52
2	0.31	18.52	5.75
3	0.36	16.10	5.75
4	0.44	19.23	8.49
5	0.36	20.24	7.32
6	0.48	20.92	9.94
7	0.44	17.29	7.58
8	0.40	13.95	5.65
9	0.41	18.73	7.64
10	0.45	19.16	8.57
11	0.50	12.08	6.02
12	0.32	12.94	4.16
13	0.70	17.62	12.35
14	0.49	14.99	7.37
15	0.72	15.66	11.24
	Average		**7.76 ± 2.21**

Method validation (TLC image analysis)

Calibration range

The calibration curve of lupinifolin was obtained by the peak area of standard (0.6 – 3 µg/spot). The polynomial with the regression equation was $y = -681.91x^2 + 12803x - 984.23$. The coefficient of determination (R^2) of lupinifolin was 0.9989.

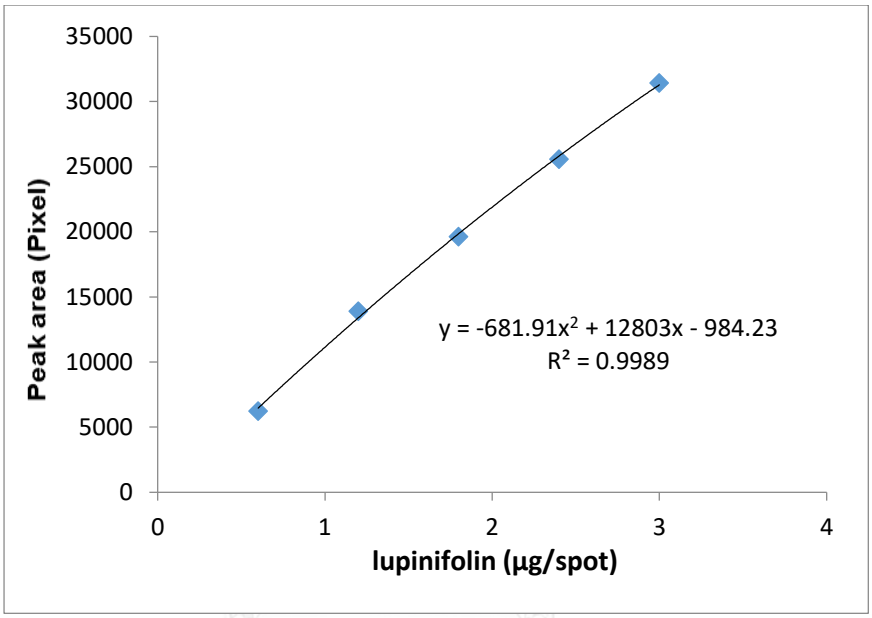


Figure 18 The calibration curve of lupinifolin in *D. reticulata* stem wood by TLC image analysis

Detection limit and quantitation limit

The detection limit and quantitation limit determination were based on the standard deviation of regression line and the slope of the calibration curve. The LOD value, the lowest concentration of analyze in a sample that could be detected was found to be 0.10 µg/spot. The LOQ value, the lowest concentration of analyze in a sample that could be quantitatively defined was 0.30 µg/spot.

Accuracy

The accuracy was tested by recovery method. Standard lupinifolin was spiked into the extract to have three different levels of lupinifolin (low, medium, high). The recovery values were 94.65 – 111.79 % as demonstrated in Table 9.

Table 9 Accuracy of quantitation of lupinifolin in *D. reticulata* stem wood by TLC image analysis (n=3)

Lupinifolin added (µg/spot)	**Lupinifolin found (µg/spot)**	**% Recovery**
0.00	0.68 ± 0.03	-
0.3	1.06 ± 0.02	108.16 ± 2.05
0.9	1.77 ± 0.01	111.79 ± 2.79
1.5	2.07 ± 0.09	94.65 ± 4.16
	Average	**104.86 ± 9.03**

Precision

The precision was examined by repeatability (intra-day) and intermediate precision (inter-day). The method was performed by analyzing sample solution of three concentrations in three replicates on the same day and three different days respectively. The repeatability and intermediate precision were showed in Table 10.

Table 10 Repeatability and intermediate precision of quantitation of lupinifolin in *D. reticulata* stem wood by TLC image analysis (n=3)

Repeatability		**Intermediate precision**	
Amount (µg/spot)	**%RSD**	**Amount (µg/spot)**	**%RSD**
1.06 ± 0.02	2.29	1.06 ± 0.01	0.69
1.77 ± 0.01	0.74	1.74 ± 0.06	3.41
2.07 ± 0.09	4.18	2.16 ± 0.08	3.59
Average	**2.86 ± 1.68**		**3.50 ± 2.29**

Robustness

The robustness of lupinifolin quantitation in *D. reticulata* stem wood by TLC image analysis was examined by changing the mobile phase ratio. The selected mobile phase ratio was shown in Table 11. The result of robustness was 3.04 % RSD of peak area.

Table 11 Robustness of lupinifolin in *D. reticulata* stem wood by TLC image analysis

Mobile phase composition **Hexane : Ethyl Acetate**	**Peak area**
5.9 : 4.1	10692.93
6.1 : 3.9	11053.10
6.0 : 4.0	10402.68
Mean ± SD	**10716.24 ± 325.84**
% RSD	**3.04**

The comparison of lupinifolin contents between TLC image analysis and TLC densitometry

The comparison of lupinifolin contents between TLC image analysis and TLC densitometry were analyzed using paired *t*-test. The lupinifolin contents in *D. reticulata* stem wood ethanolic extracts performed by TLC image analysis were in accordance with the one determine by TLC densitometry (Table 12). The result presented that the lupinifolin contents from two methods were not significantly different (p=0.11).

Table 12 The comparison of lupinifolin contents of *D. reticulata* stem wood between TLC image analysis and TLC densitometry

Source	Lupinifolin in *D. reticulata* stem wood (%w/w)	
	TLC image analysis	TLC densitometry
1	8.52	8.52
2	5.75	6.09
3	5.75	5.31
4	8.49	8.53
5	7.32	8.20
6	9.94	9.20
7	7.58	6.28
8	5.65	4.79
9	7.64	9.02
10	8.57	9.83
11	6.02	8.15
12	4.16	4.86
13	12.35	12.96
14	7.37	7.26
15	11.24	12.02
Average	**7.76 ± 2.21**	**8.07 ± 2.41**

MTT assay

The ethanolic extract of *D. reticulata* stem wood was tested for *in vitro* cytotoxic activity against 5 human cancer cell lines and 1 human. The cytotoxic effects of seven concentrations of sample were evaluated by micro-culture tetrazolium assay (MTT) and IC_{50} values were calculated. The results were shown in Table 13. The ethanolic extract of *D. reticulata* stem wood exhibited no significant activity against five cancer cell lines with IC_{50} more than 20 µg/ml. The criteria of cytotoxicity for the crude extract, as established by the U.S. National Cancer Institute (NCI), is an $IC_{50} < 20$ µg/ml in the preliminary assay. The U.S. National Cancer Institute (NCI) defined plants which give the extract with the IC_{50} values of < 20 µg/ml and < 4 µg/ml for pure compounds as the plants with cytotoxic activity [30], [39]. However, the ethanolic extract showed more potential to against hepatocarcinoma (HEP-G2) (IC_{50} = 39.21 µg/ml).

Table 13 IC_{50} of the ethanolic extract of *D. reticulata* stem wood on 5 human cancer cell lines and 1 human normal cell line

Sample/Cell	**IC_{50} (µg/ml)**					
	BT474	**CHAGO-K1**	**HEP-G2**	**KATO-3**	**SW620**	**Wi-38**
The ethanolic extract	50	>100	39.21	>100	60.64	78.65
Doxorubicin	0.40	0.50	0.07	8.81	0.23	0.17

IC_{50}: The concentration at which cell viability was reduced by 50%

Antioxidant activities

There were three assay for investigation of the potential of antioxidant activities including DPPH free radical scavenging activity, ferric ion reducing antioxidant power assay and total phenolic content.

DPPH radical scavenging activity

The results of DPPH radical scavenging activity of the ethanolic extract of *D. reticulata* stem wood was presented in Table 14. Quercetin and butylated hydroxyl toluene (BHT) which were used as positive control in this study showed high radical scavenging activity with IC_{50} of 6.92 and 106.54 µg/ml, respectively (Figure 19,20). The ethanolic extract of *D. reticulata* stem wood showed the DPPH radical scavenging activity with IC_{50} of 907.39 µg/ml (Figure 21).

Table 14 DPPH scavenging activity (IC_{50}) of the ethanolic extract of *D. reticulata* stem wood

Substance	IC_{50} (µg/ml)
Quercetin	6.92
Butylated hydroxyl toluene (BHT)	106.54
The ethanolic extract of *D. reticulata* stem wood	907.39

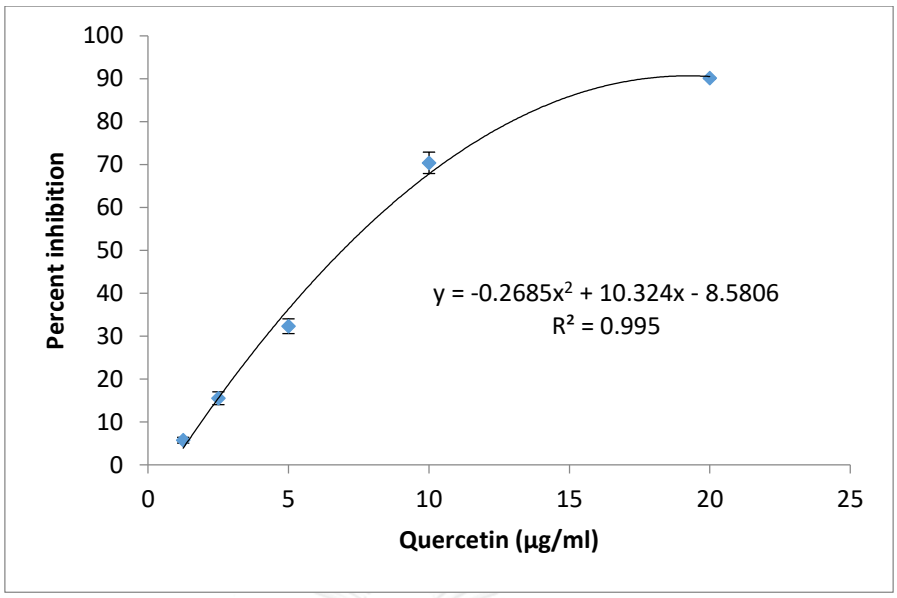


Figure 19 Percent DPPH inhibition of positive control (Quercetin) (IC_{50} 6.92 µg/ml)

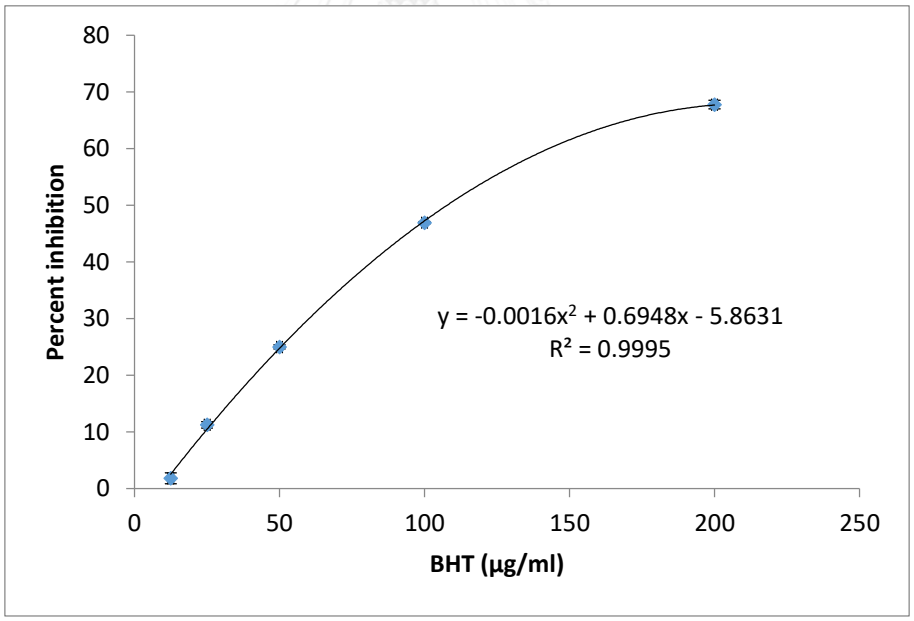


Figure 20 Percent DPPH inhibition of positive control (BHT) (IC_{50} 106.54 µg/ml)

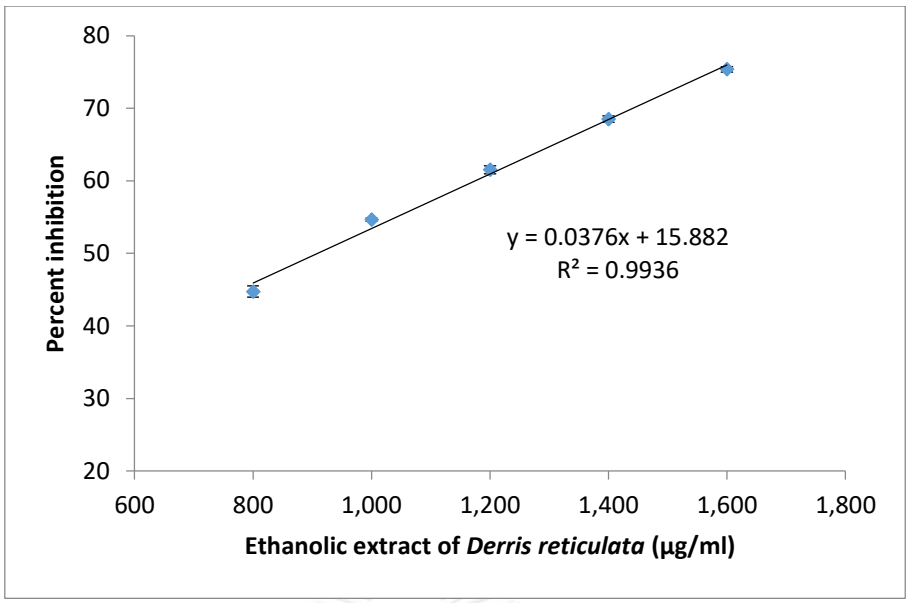


Figure 21 Percent DPPH inhibition of The ethanolic extract of *D. reticulata* stem wood (IC_{50} 907.39 µg/ml)

Ferric ion reducing antioxidant power assay

The ferrous sulfate ion concentration was calculated according to the equation of standard curve of ferrous sulfate as shown in Figure 22. The result of ferric ion reducing antioxidant power assay of the ethanolic extract of *D. reticulata* stem wood was presented in Table 15. Quercetin and butylated hydroxyl toluene (BHT) were used as positive control. The ethanolic extract of *D. reticulata* stem wood demonstrated reducing power ability with FRAP value of 0.132 mM Fe(II)/mg extract.

Table 15 The calculated FRAP value of *D. reticulata* ethanolic extract, quercetin and BHT, which calculated by using the equation from standard curve of ferrous sulphate

Substance	FRAP value (mM Fe(II)/mg extract)
Quercetin	0.914
Butylated hydroxyl toluene (BHT)	0.978
The ethanolic extract of *D. reticulata* stem wood	0.132

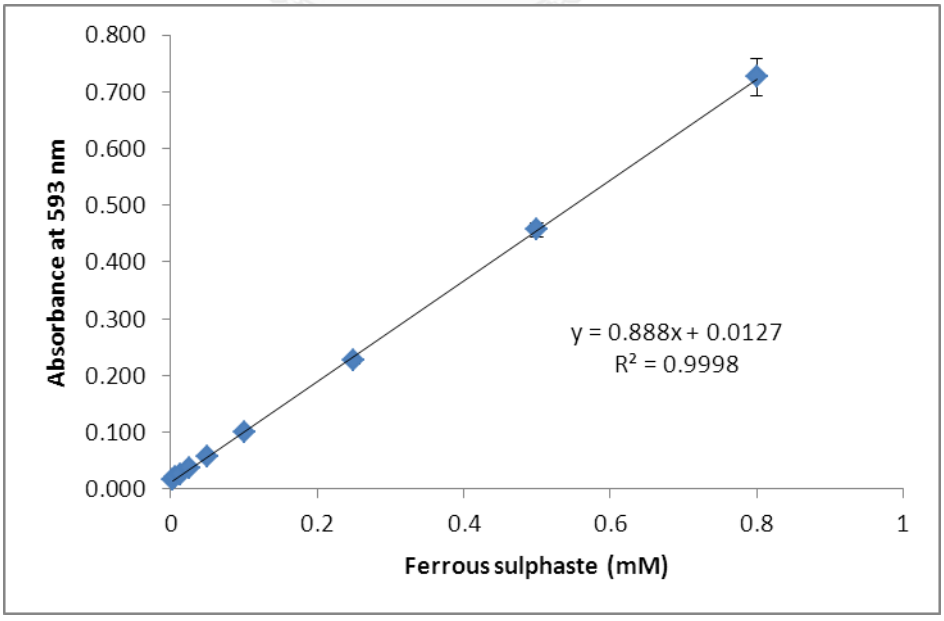


Figure 22 Standard curve for determination of antioxidant capacity by Ferric ion reducing antioxidant power

Total phenolic content

The ethanolic extract of *D. reticulata* stem wood was evaluated for the quantification of total phenolic content by Folin-Ciocalteu reagent. The total phenolic content of the ethanolic extract of *D. reticulata* stem wood was 44.37 ± 0.99 mg GAE/g extract (Table 16).

Table 16 Total phenolic content of the ethanolic extract of *D. reticulata* stem wood

Sample (0.5 mg/ml)	Gallic acid equivalents (mg GAE/g extract)
The ethanolic extract of *D. reticulata* stem wood (1)	43.51
The ethanolic extract of *D. reticulata* stem wood (2)	45.46
The ethanolic extract of *D. reticulata* stem wood (3)	44.16
mean ± SD	**44.37 ± 0.99**

GAE = Gallic acid equivalent

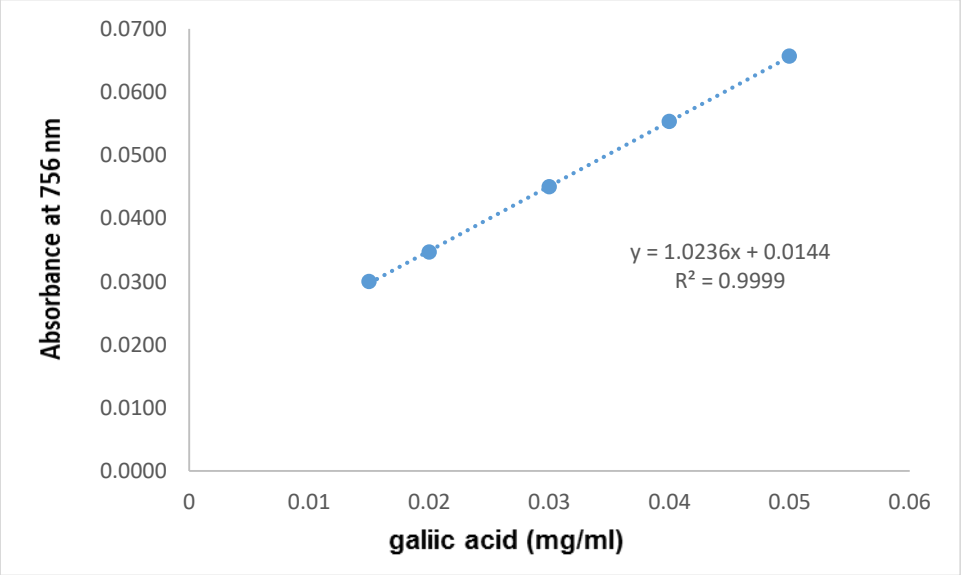


Figure 23 Gallic acid equivalents of *D. reticulata* ethanolic extract

CHAPTER V
DISCUSSION AND CONCLUSION

The pharmacognostic specification is fundamental standardization of herbal crude drug which is important for the quality control of herbal medicine. The pharmacognostic specification is set by macroscopic, microscopic characteristics, physico-chemical parameters, leaf constant numbers, chemical fingerprint profile and active chemical compound.

In Thailand, stem wood of *D. reticulata* has been used as cough suppressant and expectorant [5, 6]. It is a medicinal plant locally known in Thai as Cha-Aem-Nuea [3, 4]. This study provided the information of pharmacognostic specification, lupinifolin content of *D. reticulata* stem wood crude drug, cytotoxic potential and its antioxidant potential.

The characteristic specifications are the primary stage that can be benefit to authenticate and qualify plant materials. Macroscopic and microscopic methods are the simplest and cheapest method to establish the correct identification of plant materials[20]. These characteristics are subjectively substituted or adulterated, they may closely resemble the genuine material. It is often necessary to substantiate the findings by microscopy and/or physico-chemical analysis. This study presented macroscopic and microscopic characteristics of *D. reticulata* stem wood. Transverse sectioning of *D. reticulata* stem wood demonstrated the anatomical structures of epidermis, periderm, parenchyma containing prism crystal, sclereid, phloem fiber, vascular cambium, xylem vessel, xylem fiber and xylem ray (Figure 9). The histological evaluation of *D. reticulata* stem wood powder demonstrated fragment of xylem ray in radial longitudinal view, sclereid, cork in surface view, cork in sectional view, fragment of fiber, fragment of bordered pitted vessel, fragment of parenchyma, ray parenchyma, prism crystal of calcium oxalate and calcium oxalate prism sheath (Figure 10).

In addition, microscopic characteristics of leaf can also be used as primary screening test for identification and authentication of plant species. *D. reticulata* had

anisocytic stomata type that the stoma bordered by three accessory or subsidiary cells of which one is distinctly smaller than the other two. They were small size and presented only in the lower surface of the leaf. The palisade cells appeared below the upper epidermis. The stomatal number of *D. reticulata* leaf was found to be 316.53 ± 23.03 per 1 mm^2 and the stomatal index was 20.21 ± 1.53 per 1 mm^2. The previous study reported that the stomatal number was 372.81 per 1 mm^2 and the stomatal index was 11.69 per 1 mm^2 [3]. The variation in stomatal characters might be caused by genetic factors and environmental factors especially atmospheric CO_2 [40]. In this study, the palisade ratio was similar to the result of 8.95 ± 1.96 from the previous study which showed the result of 9.64 ± 0.768 [3]. Besides, this study revealed other leaf constant numbers including upper epidermal cell number and epidermal cell area of *D. reticulata* leaf. The results showed that the upper epidermal cell number was 854.33 ± 41.37 per 1 mm^2 and the upper epidermal cell area was 1173.13 ± 56.25 μm^2.

Physico-chemical parameters could be useful for the quality control and quality assurance of plant materials. Standardization parameters of *D. reticulata* stem wood including loss on drying, total ash, acid-insoluble ash, water soluble extractive value, ethanol soluble extractive value and water content were found to be 5.7 ± 0.1, 4.6 ± 0.1, 1.0 ± 0.1, 15.5 ± 0.7, 6.8 ± 0.3 and 8.5 ± 0.3% by dry weight, respectively (Table 1). The data from this study could be used as quality criteria of this crude drug. Ash contents were referred to inorganic components of plant materials. Acid insoluble ash value of this plant material was not exceeded 2 % similar to India [24]. Total ash and acid insoluble ash typically represent the non-volatile inorganic matters or minerals remaining after incineration of plant materials and are the important quality parameters. The ash investigation is helpful to determine the quality and purity of powdered crude drug. A larger total value indicates that the crude drug contains more of inorganic compounds such as calcium oxalate crystals [41]. These specifications are beneficial to control crude drug adulteration or contamination in crude drug preparation. The total ash represents mineral compounds such as phosphates, carbonates, oxides, or silicates in plant tissues whereas acid insoluble ash represents some mineral compounds that do not react with hydrochloric acid to form metal salts

such as silicates. Total ash and acid insoluble ash contents higher than the specifications indicate the adulteration from sand and siliceous earth [20]. Extractive matters in specified solvents indicate chemical components in crude drug. As water soluble extractive yield was higher than ethanol soluble extractive yield, it represented more polar compounds in *D. reticulata* stem wood. According to previous study, the aqueous extract of *D. reticulata* was shown the highest yield (10.56%) compared to the chloroform and hexane extracts with 2.68% and 2.02% yields, respectively [42]. Therefore, the result of previous study and in this study can conclude that chemical compounds of *D. reticulata* stem wood were mainly hydrophilic. Loss on drying value is used to determine the content of both volatile matters and water content in the crude drug [20]. Water content plays an important role in the stability of plant products. The water content should be minimized in order to prevent chemical degradation as well as microbial contamination [43]. The water content of *D. reticulata* stem wood (8.5 ± 0.3 % dry weight) was higher than loss on drying value (5.7 ± 0.1 % dry weight). This plant did not contain volatile substances. Hence, it might be due to the water within plant cells [44].

Thin layer chromatography is a simple technique for separation, illustrated pattern of many components and identification of crude drug. Each compound can be separated and presented at different R_f value which can be used as marker for quality control. TLC fingerprint of ethanolic extracts of *D. reticulata* stem wood was established for crude drug identification according to chemical components. The advantages of TLC are its simplicity, versatility, rapidity, specific, sensitivity and simple sample preparation [45]. Thus, TLC is a convenient method to determine the quality and possible adulteration of plant materials [46]. In this study, TLC fingerprint of ethanolic extracts of *D. reticulata* stem wood was performed using hexane : ethyl acetate (6:4) as mobile phase. The band was clearly detected under ultraviolet lights (254 nm and 365 nm) and 10 % sulfuric acid in ethanol reagent and heated. TLC is appropriate fast screening method in order to identify plant according to their chemical constituents [47].

In this study, Soxhlet apparatus with 95% ethanol was used to extract constituents from *D. reticulata* stem wood powder for the determination of lupinifolin content. The percentage of extract yields was 17.08 ± 2.69 % by weight in average. The advantages of this extraction include continuous process, basic, simple and cheap [48]. The disadvantages include time consuming, large amounts of solvent, inability to provide agitation to accelerate the process, no suitability for thermolabile compounds as long time heating may lead to degradation of compounds and exposure to hazardous and flammable liquid organic solvents with potential toxic emissions during extraction [49].

For the purpose of lupinifolin content in *D. reticulata* stem wood crude drug, TLC-densitometry and TLC image analysis by ImageJ software used for quantitative analysis. The quantitative parameters is useful for setting standards of crude drug [50]. TLC-densitometry is a quantitative technique with high reliability to measure in both UV and visible ranges. TLC image analysis could be used as an alternative method to TLC-densitometry to quantitate lupinifolin content in *D. reticulata* stem wood owing to its convenience and cost-effectiveness. In this study, TLC-densitometry and TLC image analysis using imageJ software were performed and validated to confirm that these analytical techniques provided reliable and accurate results. By TLC-densitometry and TLC image analysis in this study, lupinifolin content in *D. reticulata* stem wood were 8.07 ± 2.41 and 7.76 ± 2.21 % by dry weight, respectively (Table 12). According to previous study, the amount of active compound, lupinifolin (quantified by TLC-densitometry after ethanolic maceration) in crude drug was 3.47 % by dry weight [7]. Geographic variation is one of the factors influenced on chemical characteristics and chemical content of herbal medicine [51]. This study found that the lupinifolin contents among 15 different locations were between 4.79 – 12.96 % by dry weight.

In this study, TLC-densitometry and TLC image analysis were validated following ICH guideline which including accuracy, precision, specificity, detection limit (LOD), quantitation limit (LOQ) and robustness. The accuracy was performed by recovery of spiking known three concentrations of standard lupinifolin in sample

matrix. The recovery values were within acceptable limits (104.86-105.62%). Therefore, both methods were accurate with % recovery between 80-120% [35]. The repeatability or intra-day precision and the intermediate precision or inter-day precision were less than 4% [52]. The precision of lupinifolin quantitative analysis by TLC-densitometry and TLC image analysis method were conducted by determination of 4 concentrations × 3 replicates at the same and different days of tests. LOD and LOQ were calculated based on the residual standard deviation of a regression line. The LOD value of TLC-densitometry and TLC image analysis, regard as the lowest concentration of analyze in a sample which could be qualitative detected were found to be 0.14, 0.10 mg/spot, respectively. The LOQ value of TLC-densitometry and TLC image analysis, regard as the lowest concentration of analyze in a sample which could be quantitatively determined were 0.41, 0.30 mg/spot, respectively. The robustness estimated by analysis of results obtain after deliberate variation of mobile phase ratio showed %RSD of peak area < 5. The calibration curves were polynomial in both TLC densitometry and TLC image analysis with the range of 0.6 - 3 mg/spot (Figure 15, 18). Specificity of the methods was validated through UV absorbance spectra under the range of 200-450 nm among standard lupinifolin and lupinifolin in the ethanolic extract. In addition, the result also revealed the identical spectra obtained at up-slope, apex and down-slope of the peak which represented chromatographic peak purity of lupinifolin. In this study, maximum absorption of lupinifolin was 275 nm in agreement with the previous study of 274 nm [53]. The results from method validation indicated that TLC-densitometry and TLC image analysis could be efficient, reliable and suitable technique for quantitative analysis of lupinifolin in *D. reticulata* stem wood.

Cell viability assay using MTT is an uncomplicated and accurate screening method, to study eukaryotic cell proliferation or cytotoxic potential of natural product extracts. In order to evaluate of the cytotoxic effect of *D. reticulata* stem wood ethanolic extract on human cancer cells by using MTT assay, six cell lines were selected to be investigated in this study. Five human cancer cell lines included breast ductal carcinoma (BT-474), undifferentiated lung carcinoma (CHAGO-K1), colon adenocarcinoma (SW-620), gastric carcinoma (KATO-3), hepatocarcinoma (HEP-G2) and

one human normal cell line was lung fibroblast (Wi-38). The results showed that the extract exhibited no significant cytotoxic activity against five cancer cell lines with an IC_{50} more than 20 µg/ml (39.21-125.37 µg/ml) and the normal lung fibroblast (Wi-38) cells with IC_{50} of 78.65 µg/ml. *D. reticulata* stem wood ethanolic extract was non-toxic to human normal cell. US NCI guidelines set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation of less than 20 µg/ml after the exposure time of 72 hours [30, 39]. In previous study, the result from cytotoxicity demonstrated that the aqueous extract *D. reticulata* stem wood produced the least toxic effect to HEP-G2 cells since the highest dose up to 5,000 µg/ml did not cause significant cell damage compared to control. In contrast, at concentrations higher than 60 µg/ml, the hexane and chloroform *D. reticulata* extracts significantly reduced cell viability ($P<0.05$) [42].

Antioxidant compounds are important in medical part. It is a health protective factor along with the scientific evidences that antioxidant can be the diseases reducing factor particularly cancer [54]. Several antioxidant activity evaluating methods have been used to monitor and compare the antioxidant activities of natural products. Various types of reactive oxygen species have been used to estimate the capacity of herbs to scavenge or reduce free radical *in vitro*. The antioxidant capacities are influenced by many factors which cannot be fully described by only one method. It is necessary to investigate the various mechanisms of antioxidant action. Thus, the antioxidant activities of *D. reticulata* stem wood ethanolic extract was assessed based on characteristic radical reduction including DPPH radical scavenging activity, ferric ion reducing antioxidant power assay and total phenolic content.

DPPH radical scavenging activity, the stable and well-characterized free radical which used for estimation of the electron donation ability is popularly used for evaluation of radical scavenging potential of the natural substances [55]. The reduction of DPPH radical scavenging activity is used as an indicator of radical scavenger property. DPPH radical has a dark violet color in methanolic solution with the absorbance range between 515-520 nm and its becomes yellow when neutralized by hydrogen atom donated from the antioxidant [56]. The discoloration degree indicates the scavenging potential of plant extract. A large decrease in the absorbance of the reaction mixture

indicates significant free radical scavenging activity of the compound under test [57]. The color changing is monitored by spectrophotometry. In this study, the ethanolic extract of *D. reticulata* stem wood was able to decolorized DPPH free radical in methanolic solution with IC_{50} of 907.39 µg/ml compared to quercetin and butylated hydroxyl toluene (BHT) which were used as positive control with the IC_{50} of 6.92 µg/ml and 106.54 µg/ml, respectively. Principally, a lower DPPH radical scavenging activity is associated with a higher IC_{50} value. According to previous study, it was found that different types of solvent extracts of *D. reticulata* stem wood had DPPH radical scavenging activity with different degrees of potency depending on the solution. The results revealed that the aqueous extract had the lowest IC_{50} value (236.99 ± 10.98 µg/ml), followed by chloroform extract (570.56 ± 22.02 µg/ml) and hexane extract (717.37 ± 36.31 µg/ml) [42]. DPPH radical scavenging activity of the aqueous extract of *D. reticulata* stem wood in another previous study was reported the IC_{50} of 239.85 ± 0.13 µg/ml [14]. It might be related to the different location of collecting the sample, solution of extract, processing of extraction and other environment factors [58].

Metal ion transition such as Cu^{3+} /Cu^{2+} and Fe^{3+} /Fe^{2+} plays a role in oxidative stress pathway of living organism [59]. Metal reducing power is one of parameters representing antioxidant capacity. Ferric reducing antioxidant power assay is an inexpensive reagent, simple prepared, highly reproducible and linearly related to molar concentration of the antioxidants [60]. Ferric reducing antioxidant power assay is an antioxidant capacity assay that measures the power of ferric reduction. This method uses TPTZ as the coupling agent to form colorless ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex, which turns to a blue color complex of ferrous tripyridyltriazine (Fe^{2+}-TPTZ) by reducing power of antioxidant [37]. FRAP value is based on reducing ferric ion by antioxidants that are reducing agent. The reducing properties are associated with the presence of compounds which exert their actions by breaking the free radical chain by donating a hydrogen atom [61]. High FRAP value indicated that the compound in sample can break free radical chain as a reductant in a redox-linked colorimetric reaction [62]. In this study, the ethanolic extract of *D. reticulata* stem wood was able to reduce ferric ion then colorize FRAP working solution. The result indicated that the

reducing power of *D. reticulata* stem wood at the concentration of 0.5 mg/ml had a value equivalent to ferrous sulphate 0.132 mM Fe(II)/mg extract. The reducing power of quercetin and butylated hydroxyl toluene (BHT) which were used as the positive control, at the concentration of 0.5 mg/ml had a value equivalent to ferrous sulphate 0.914 and 0.978 mM Fe(II)/mg extract, respectively. From the previous study, the hexane extract, the chloroform extract and the aqueous of *D. reticulata* stem wood at the concentration of 5 mg/ml had a value equivalent to ferrous sulphate 0.000047, 0.000121 and 0.000091 mM Fe(II)/mg extract, respectively [42]. In another previous study, the aqueous of *D. reticulata* stem wood at the concentration of 5 mg/ml had a value equivalent to ferrous sulphate 0.00023 mM Fe(II)/mg extract [14]. The result was different from previous study. It might be affected by the quality of extracted compounds which depended on storage time, geographic origin, harvesting date, environment, technological factors and solution of extract. Besides, the temperature and light also contribute to antioxidant activity change during storage [63].

Phenolic compounds are considered secondary metabolites that be synthesized by plant. They are derived from phenylalanine and tyrosine [64]. Phenolic compounds of plants are also very important because their hydroxyl groups as able to scavenge free radicals. In this study, the ethanolic extract of *D. reticulata* stem wood at the concentration of 0.5 mg/ml had the total phenolic of 44.37 ± 0.99 mg GAE/g extract. From the previous study, it was found that the aqueous extract of *D. reticulata* stem wood at the concentration of 1.25 mg/ml contained fewer total phenolic compounds (33.21 ± 0.31 mg GAE/g extract) than the hexane extract (52.29 ± 1.33 mg GAE/g extract) and chloroform extract (53.89 ± 0.68 mg GAE/g extract) [42]. In another previous studies, total phenolic content of the aqueous extract of *D. reticulata* stem wood at the concentration of 5 mg/ml was 78.84 ± 0.01 mg GAE/g extract [14] whereas, the DMSO extract of *D. reticulata* stem wood at the concentration of 5 mg/ml had the total phenolic of 78.5 ± 5.6 mg GAE/g extract [65]. The result was different from previous study. It might be affected by the solution of extract and the concentration of *D. reticulata* stem wood. Furthermore, several previous studies indicated the

relationships between antioxidant activities of medicinal plant and their phenolic contents [14, 42, 65].

Regarding to the results of the present study, the physico-chemical parameters are important for quality control. The pharmacognostic specifications and lupinifolin content of *D. reticulata* stem wood in Thailand were established. Conventional TLC combined with image analysis software could be used for quantification of lupinifolin in this crude drug. In addition, these study provide important clues to help understand the cytotoxic and antioxidant activities of *D. reticulata* stem wood and their components which support in further clinical researches. The finding generated from this study would be useful in quality assurance and also for preparation of monograph on the *D. reticulata* stem wood.

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APPENDIX

APPENDIX A

Data of pharmacognostic specification and quantitative analysis

of lupinifolin in *Derris reticulata* stem wood

Table 17 The percent yield of *Derris reticulata* from 15 different sources throughout Thailand

Sources	Weight of sample (g)	Weight of evaporating dish (g)	Weight of evaporating dish and extractable matter(g)	Weight of extractive value (g)	% yield
1. Bangkok 1	5.0024	158.7234	159.6633	0.9399	18.7890
2. Bangkok 2	5.0015	163.7879	164.7143	0.9264	18.5224
3. Bangkok 3	5.0035	152.7340	153.5396	0.8056	16.1007
4.Uthai Thani	5.0014	146.8891	147.8507	0.9616	19.2266
5. Nakhon Sawan	5.0028	75.79850	76.8110	1.0125	20.2387
6. Nakhon Pathom	5.0039	152.6196	153.6662	1.0466	20.9157
7.Chiang Mai	5.0025	146.8791	147.7442	0.8651	17.2934
8.Chiang Rai	5.0064	163.1369	163.8355	0.6986	13.9541
9.Kalasin	5.0017	147.2682	148.2050	0.9368	18.7296
10.Rayong	5.0005	155.2713	156.2294	0.9581	19.1601
11.Cha choeng sao	5.0012	155.4762	156.0803	0.6041	12.0791
12.Ratchaburi	5.0018	156.2467	156.8938	0.6471	12.9373
13.Surat Thani	5.0021	148.5324	149.4140	0.8816	17.6246
14.Nakhon Si Thammarat	5.0054	163.6000	164.3504	0.7504	14.9918
15.Prachuap Khiri Khan	5.0083	163.6002	164.3843	0.7841	15.6560
				max	20.9157
				min	12.0791
				mean	17.0813
				SD	2.6857

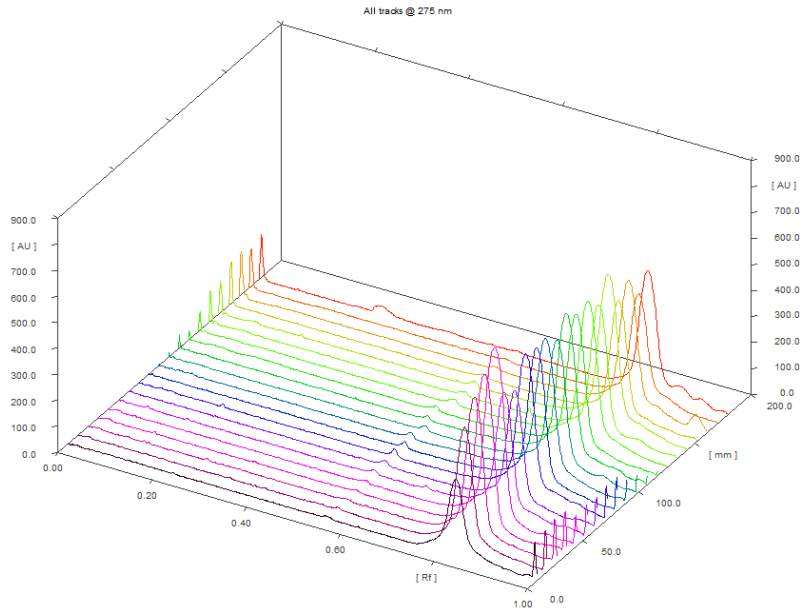


Figure 24 3D TLC-densitometry chromatogram of lupinifolin in *D. reticulata* stem wood extracts from 15 different sources and standard lupinifolin for calibration range (Plate A – standard lupinifolin NO.1-5, sample NO.6-20)

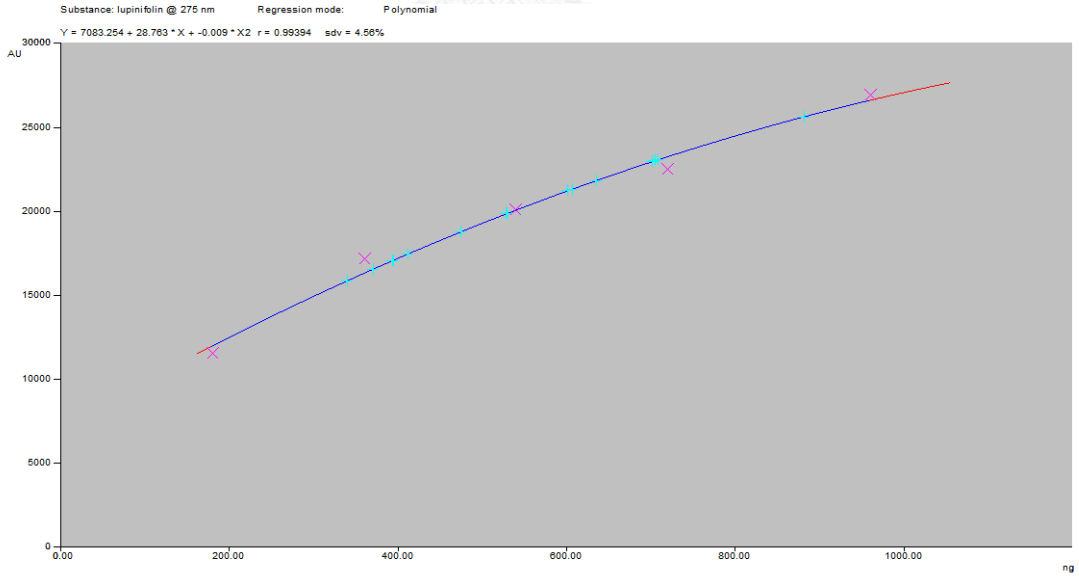


Figure 25 The calibration curve of lupinifolin in *D. reticulata* stem wood by TLC densitometry (Plate A)

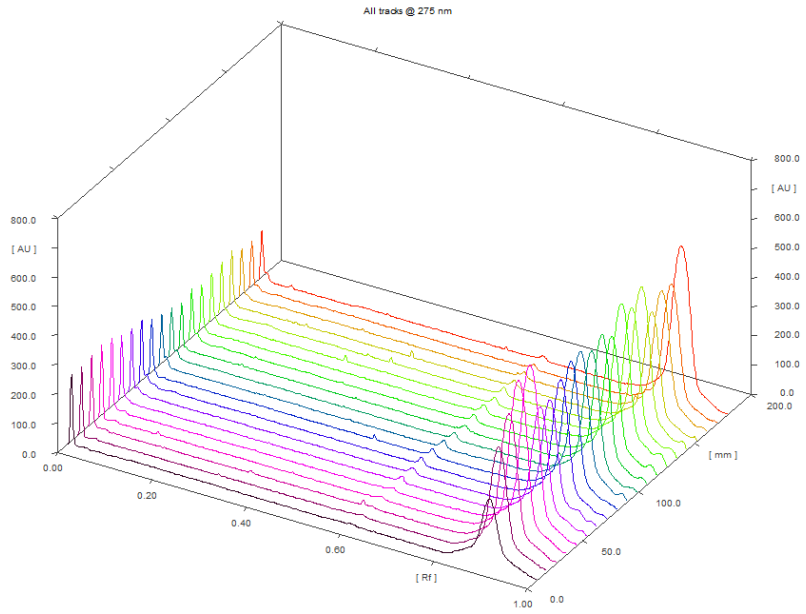


Figure 26 3D TLC-densitometry chromatogram of lupinifolin in *D. reticulata* stem wood extracts from 15 different sources and standard lupinifolin for calibration range (Plate B – standard lupinifolin NO.1-5, sample NO.6-20)

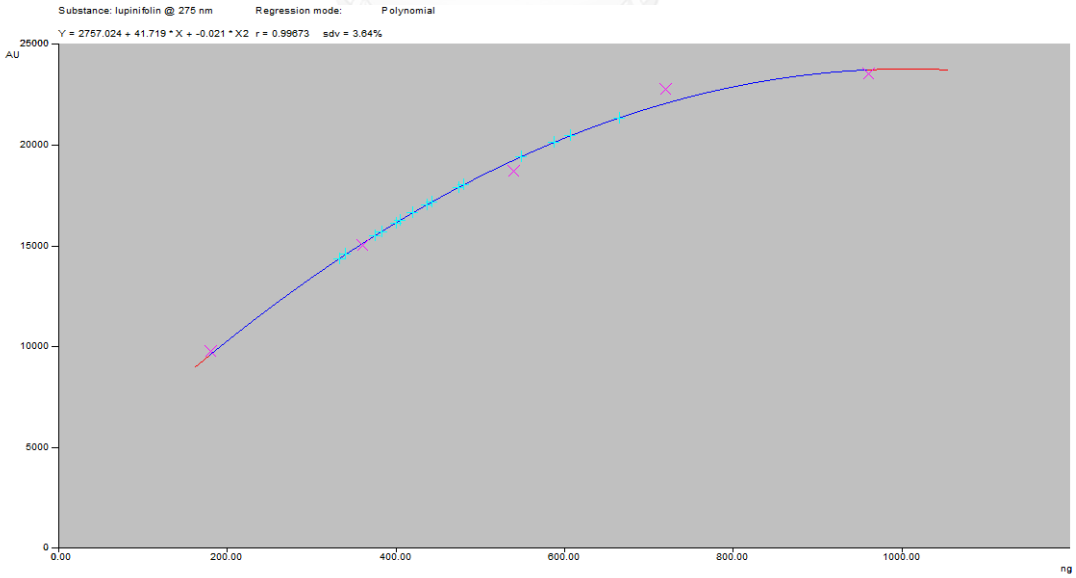


Figure 27 The calibration curve of lupinifolin in *D. reticulata* stem wood by TLC densitometry (Plate B)

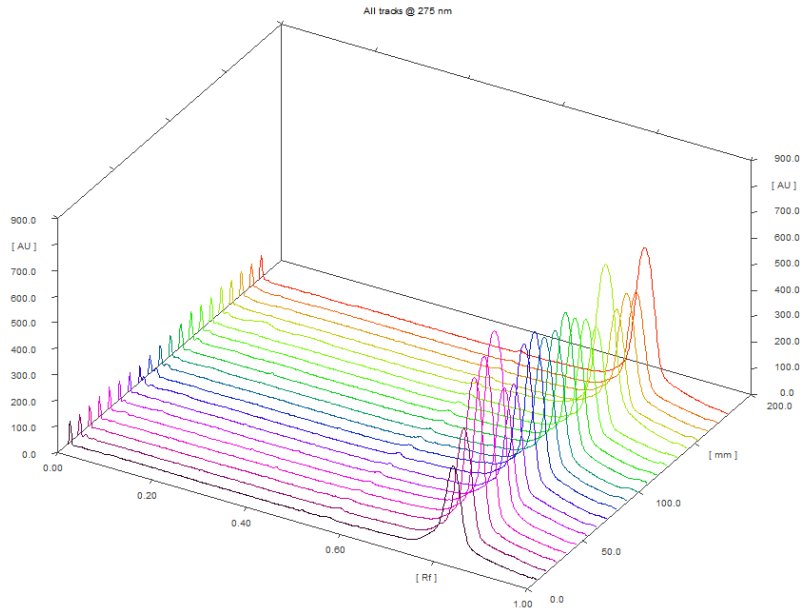


Figure 28 3D TLC-densitometry chromatogram of lupinifolin in *D. reticulata* stem wood extracts from 15 different sources and standard lupinifolin for calibration range (Plate C – standard lupinifolin NO.1-5, sample NO.6-20)

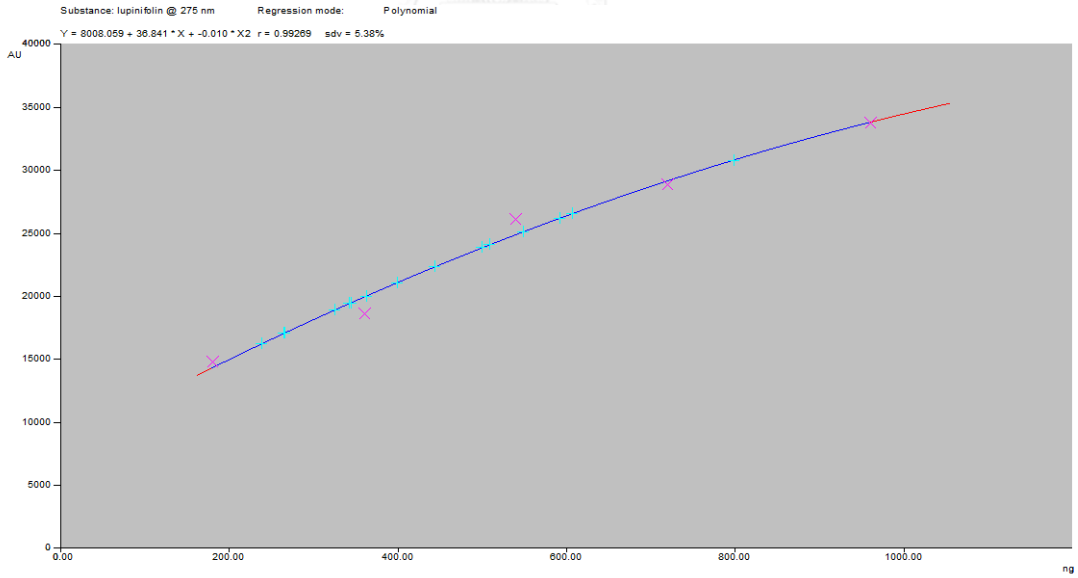


Figure 29 The calibration curve of lupinifolin in *D. reticulata* stem wood by TLC densitometry (Plate C)

Table 18 Accuracy by TLC densitometry

Lupinifolin added (µg/spot)	1	2	3	Average	%Recovery			
0.0	0.75	0.75	0.76	0.76	**1**	**2**	**3**	**Average**
0.3	1.04	1.04	1.02	1.03	98.493	98.763	95.772	97.68
0.9	1.83	1.85	1.82	1.83	110.856	111.730	109.184	110.59
1.5	2.39	2.50	2.46	2.45	105.977	111.008	108.824	108.60
							Average	105.62
							SD	6.95

Table 19 Repeatability by TLC densitometry

Lupinifolin added (µg/spot)	Repeatability					
	1	**2**	**3**	**Mean**	**SD**	**%RSD**
0.000	0.753	0.754	0.762	0.76	0.01	0.70
0.3	1.037	1.041	1.018	1.03	0.01	1.21
0.9	1.832	1.848	1.815	1.83	0.02	0.90
1.5	2.387	2.502	2.462	2.45	0.06	2.38
					Average	**1.30**
					SD	**0.75**

Table 20 Intermediate precision by TLC densitometry

Lupinifolin added (µg/spot)	Intermediate precision					
	1	2	3	Mean	SD	%RSD
0.000	0.679	0.693	0.756	0.71	0.04	5.82
0.3	1.003	1.084	1.032	1.04	0.04	3.93
0.9	1.858	1.856	1.832	1.85	0.01	0.80
1.5	2.586	2.353	2.451	2.46	0.12	4.77
					Average	3.83
					SD	2.16

Table 21 Robustness by TLC densitometry

Mobile phase (v/v)	Robustness					Peak area of lupinifolin (Pixel)
	0.6	1.2	1.8	2.4	3	
5.9 : 4.1	15602.62	22795.99	27438.19	29740.17	33633.18	17617.44
6.1 : 3.9	16730.97	22851.62	26578.07	29840.17	32730.20	15746.68
6.0 : 4.0	17624.33	24627.10	28652.88	32625.81	34735.11	17171.31
Mean	16652.64	23424.90	27556.38	30735.38	33699.50	16845.14
SD	1013.13	1041.50	1042.44	1637.92	1004.10	977.10
%RSD	6.08	4.45	3.78	5.33	2.98	5.80

Table 22 Accuracy by TLC image analysis

Lupinifolin added (µg/spot)	1	2	3	Average	%Recovery			
0.0	0.662	0.673	0.717	0.68	1	2	3	Average
0.3	1.036	1.075	1.081	1.06	107.742	110.392	106.354	108.16
0.9	1.774	1.780	1.755	1.77	113.621	113.156	108.579	111.79
1.5	2.140	1.971	2.089	2.07	98.992	90.702	94.243	94.65
							Average	104.86
							SD	9.03

Table 23 Repeatability by TLC image analysis

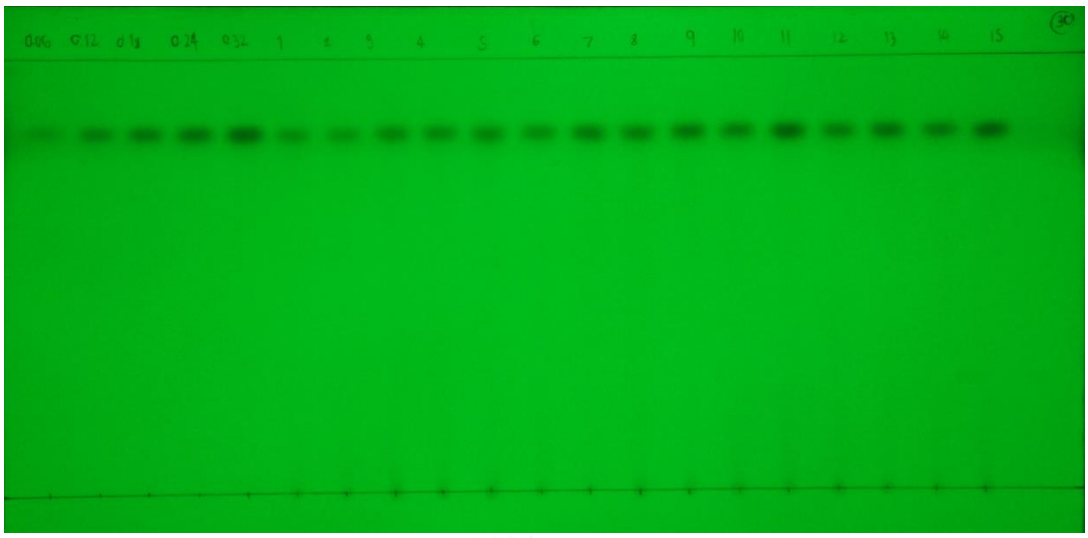
Lupinifolin added (µg/spot)	Repeatability					
	1	2	3	Mean	SD	%RSD
0.000	0.662	0.673	0.717	0.68	0.03	4.23
0.3	1.036	1.075	1.081	1.06	0.02	2.29
0.9	1.774	1.780	1.755	1.77	0.01	0.74
1.5	2.140	1.971	2.089	2.07	0.09	4.18
					Average	2.86
					SD	1.68

Table 24 Intermediate precision by TLC image analysis

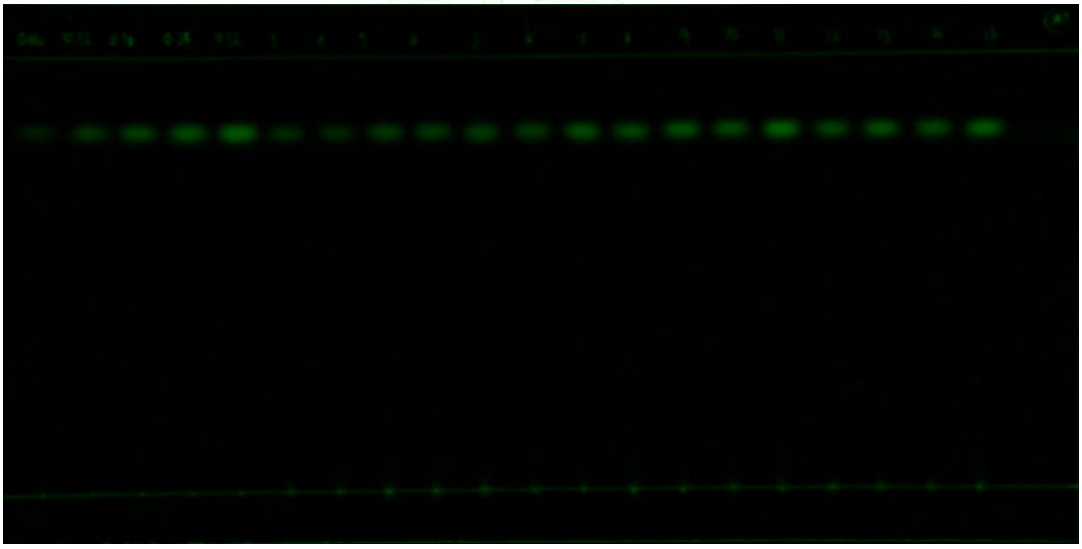
Lupinifolin added (µg/spot)	Intermediate precision					
	1	2	3	Mean	SD	%RSD
0.000	0.633	0.718	0.684	0.68	0.04	6.30
0.3	1.057	1.072	1.064	1.06	0.01	0.69
0.9	1.786	1.676	1.770	1.74	0.06	3.41
1.5	2.204	2.197	2.067	2.16	0.08	3.59
					Average	3.50
					SD	2.29

Table 25 Robustness by TLC image analysis

Mobile phase (v/v)	Robustness					Peak area of lupinifolin (Pixel)
	0.6	1.2	1.8	2.4	3	
5.9 : 4.1	5820.51	10692.93367	15731.79	20701.847	22187.91133	5997.586333
6.1 : 3.9	4218.47	11053.10	14449.02	18560.59067	21131.06	5154.14
6.0 : 4.0	4424.89	10402.68	13757.24	18662.04533	20816.857	4992.741
Mean	4821.29	10716.24	14646.02	19308.16	21378.61	5381.49
SD	871.48	325.84	1001.91	1208.03	718.27	539.62
%RSD	18.08	3.04	6.84	6.26	3.36	10.03



(A)

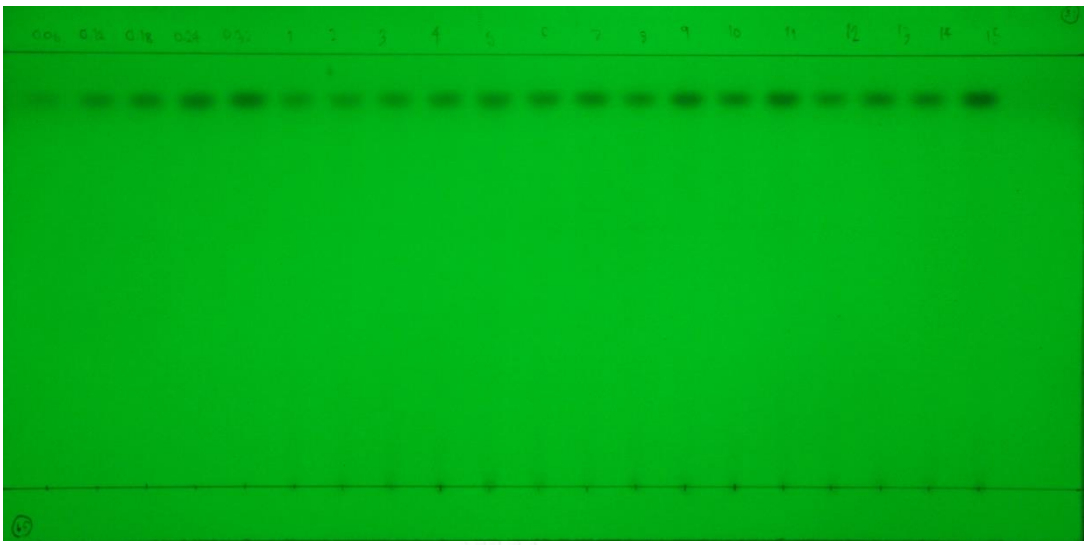


(B)

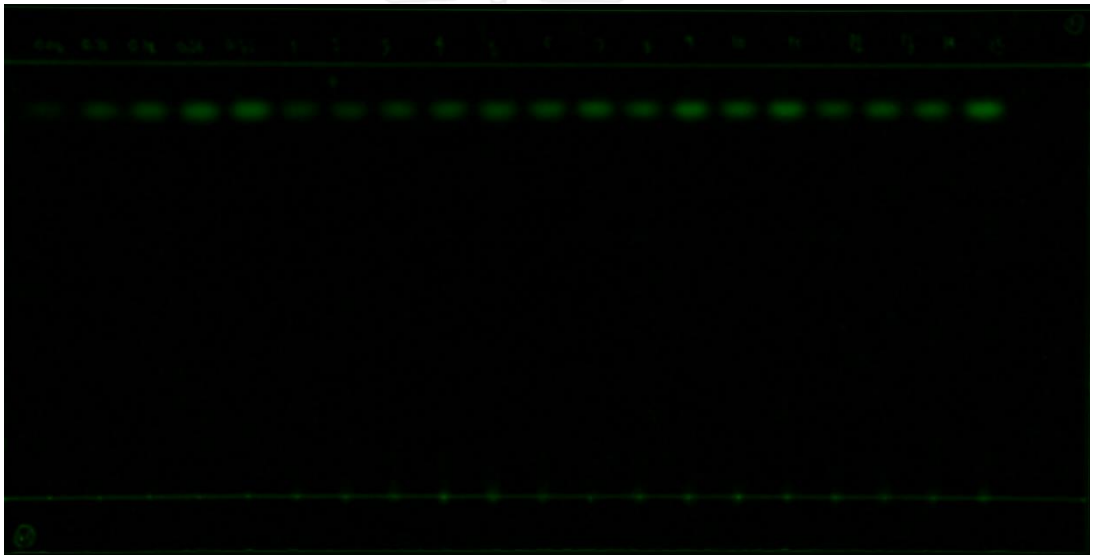
Figure 30 The TLC Plate A

(standard lupinifolin NO.1-5, sample NO.6-20)

visual under UV 254 nm (A), with subtract background by ImageJ software (B)



(A)

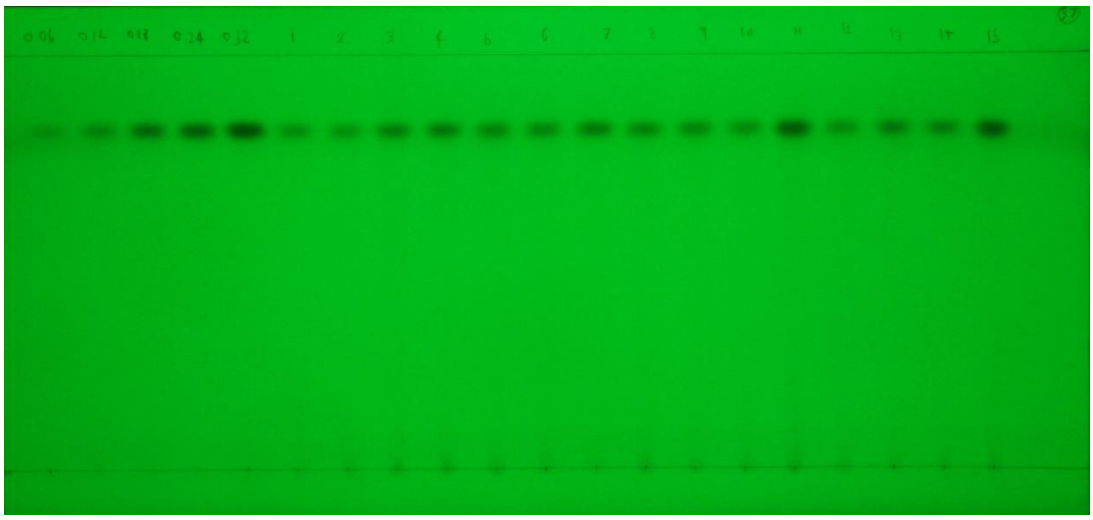


(B)

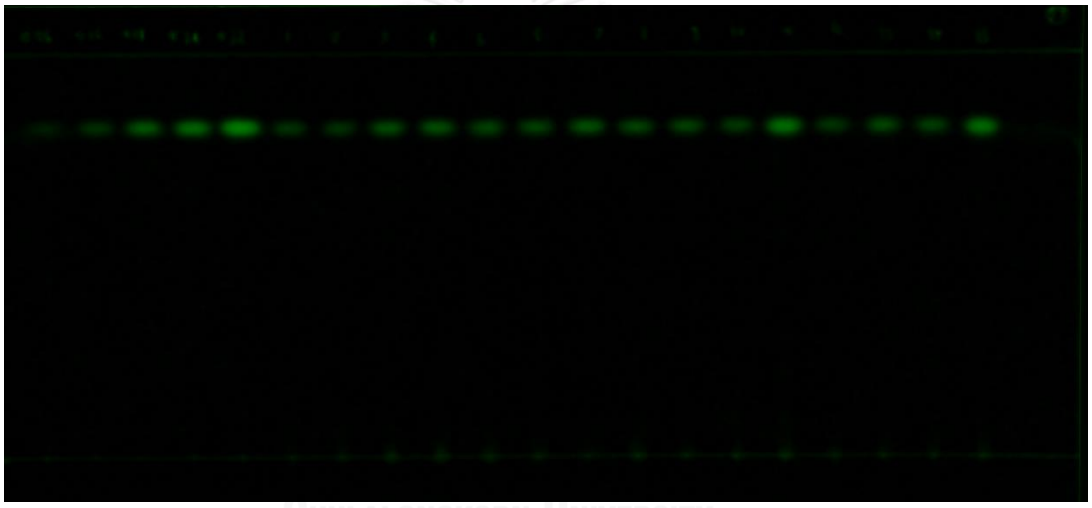
Figure 31 The TLC Plate B

(standard lupinifolin NO.1-5, sample NO.6-20)

visual under UV 254 nm (A), with subtract background by ImageJ software (B)



(A)

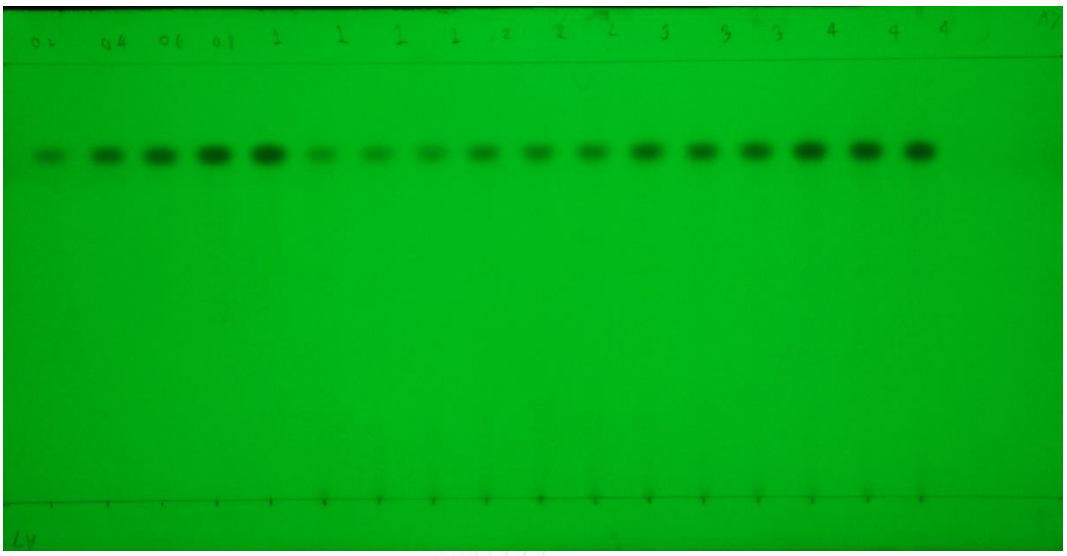


(B)

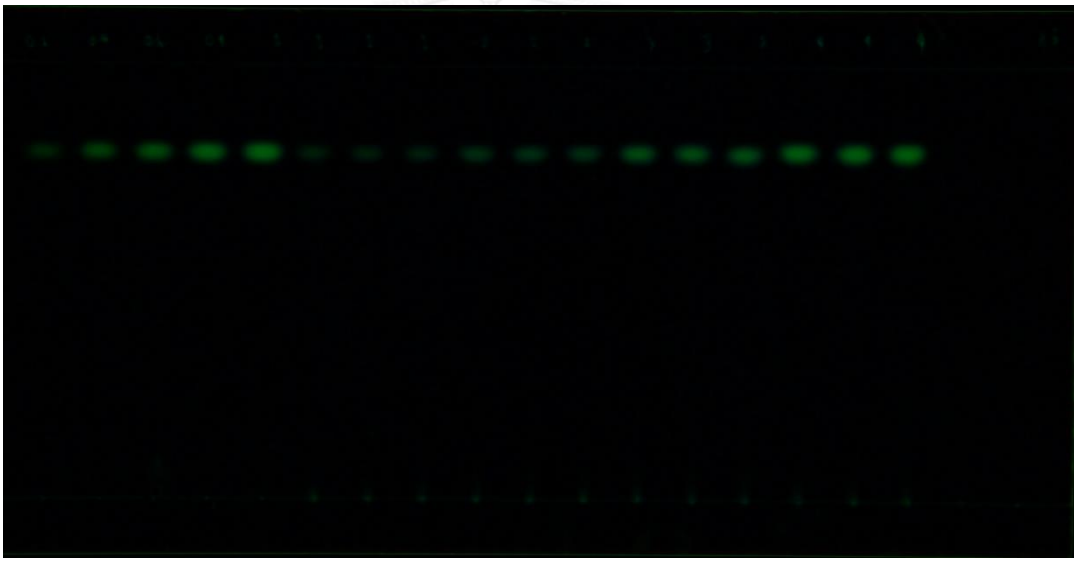
Figure 32 The TLC Plate C

(standard lupinifolin NO.1-5, sample NO.6-20)

visual under UV 254 nm (A), with subtract background by ImageJ software (B)



(A)



(B)

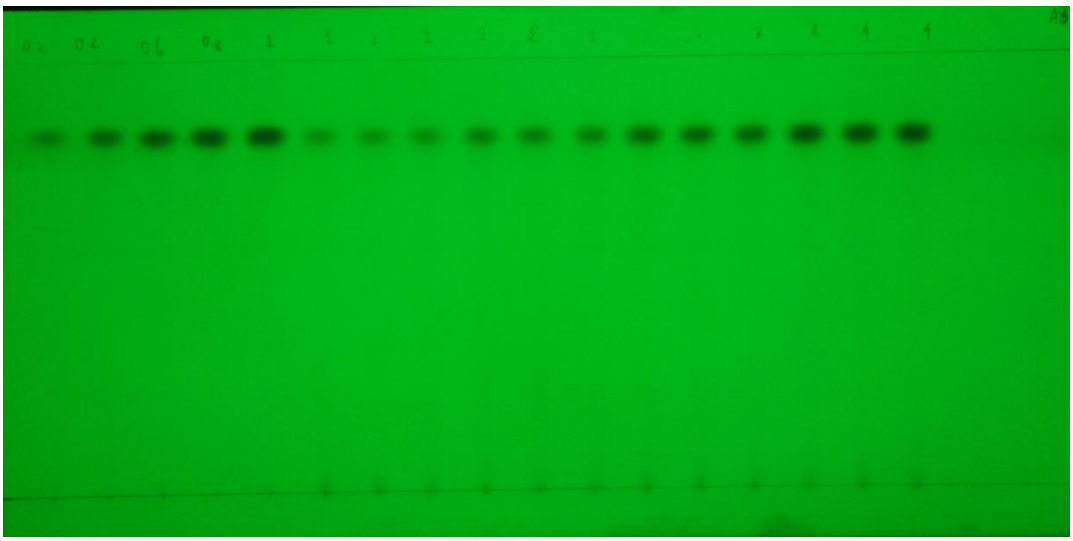
Figure 33 The TLC Plate D Accuracy

(standard lupinifolin NO.1-5, sample NO.6-8, spiked standard 0.3 µg/spot NO. 9-11,

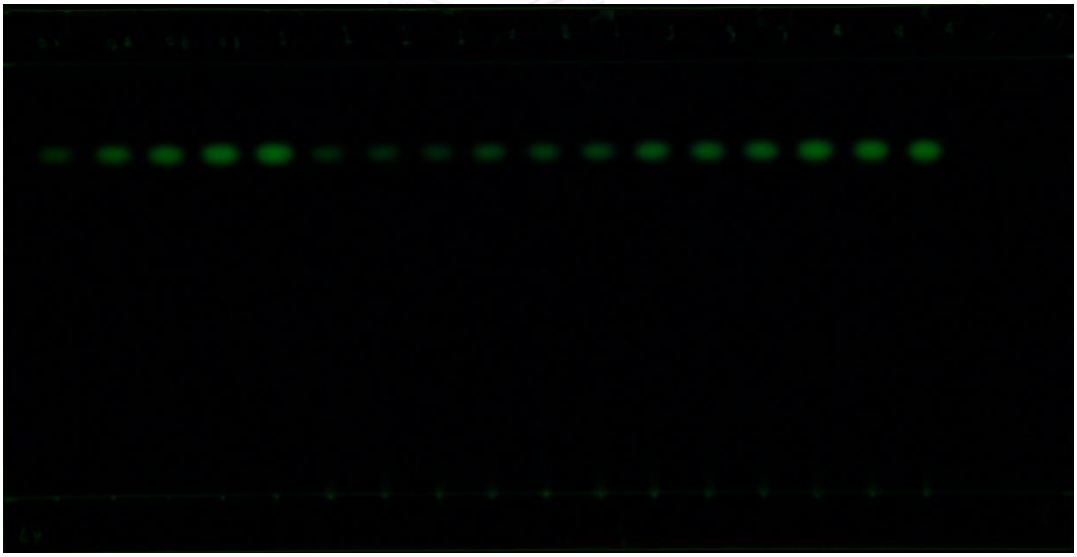
spiked standard 0.9 µg/spot NO. 12-14,

spiked standard 1.5 µg/spot NO. 15-17)

visual under UV 254 nm (A), with subtract background by ImageJ software (B)



(A)



(B)

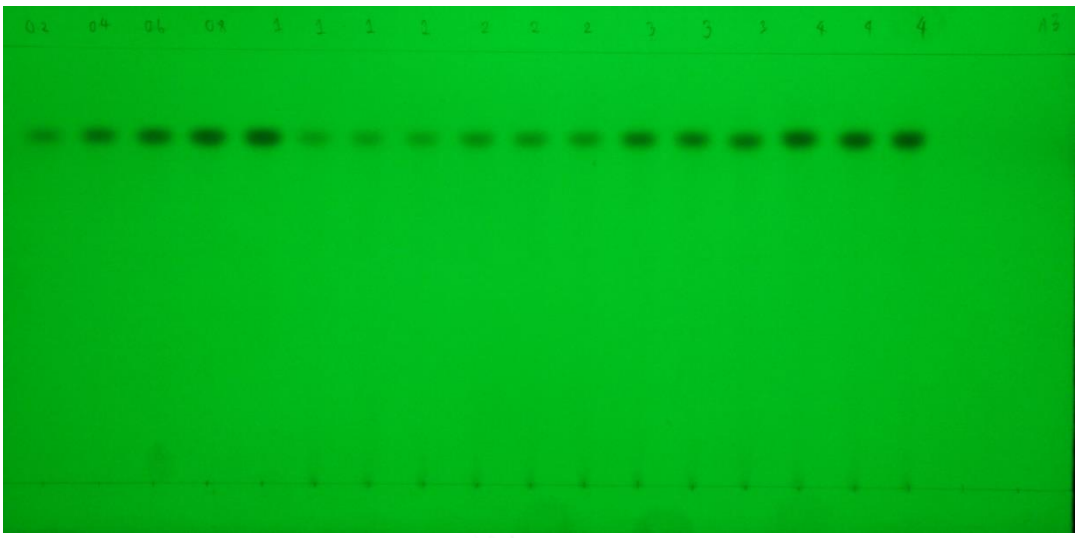
Figure 34 The TLC Plate E Precision

(standard lupinifolin NO.1-5, sample NO.6-8, spiked standard 0.3 µg/spot NO. 9-11,

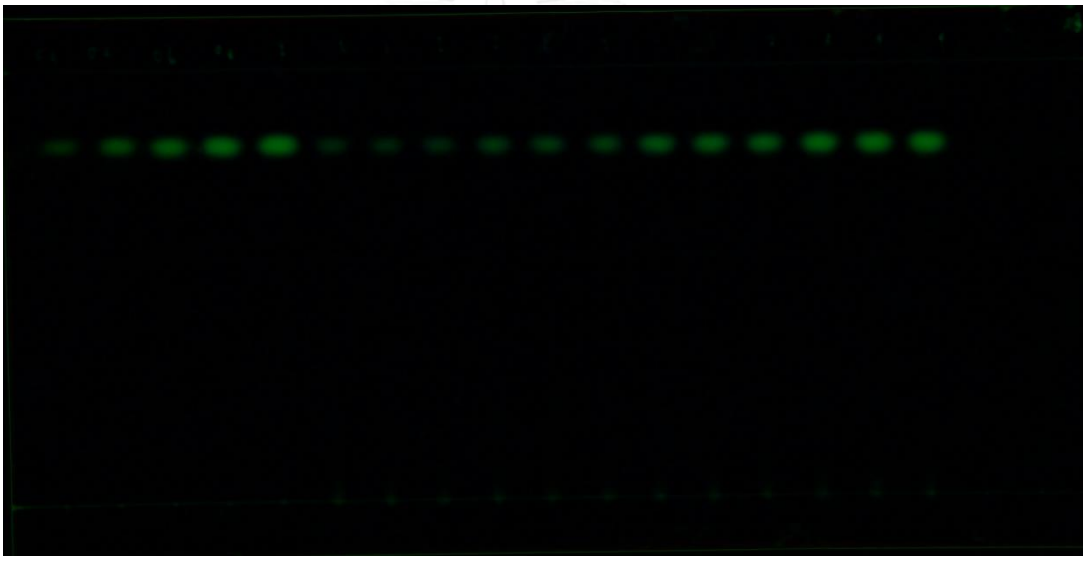
spiked standard 0.9 µg/spot NO. 12-14,

spiked standard 1.5 µg/spot NO. 15-17)

visual under UV 254 nm (A), with subtract background by ImageJ software (B)



(A)



(B)

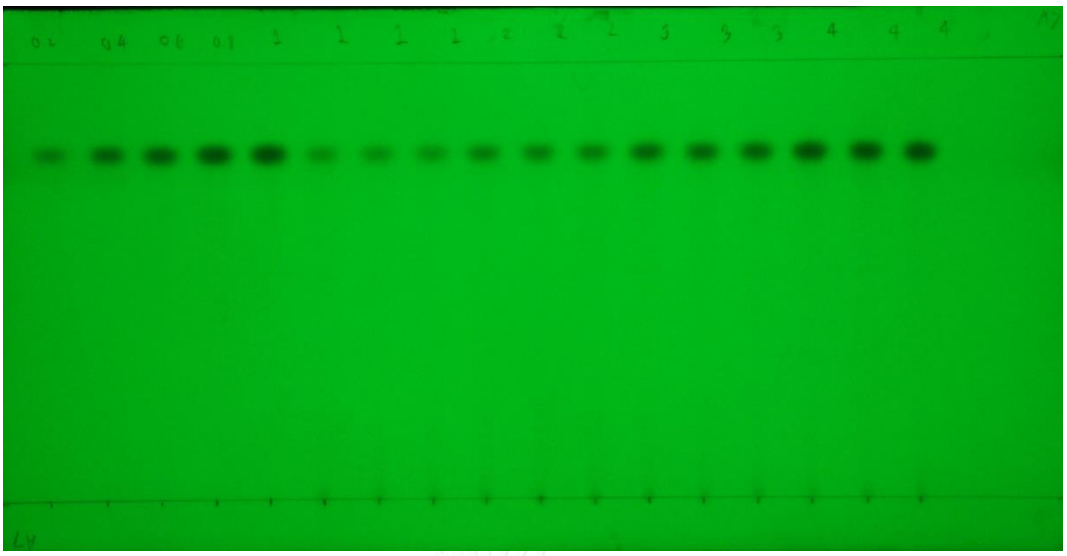
Figure 35 The TLC Plate F Precision

(standard lupinifolin NO.1-5, sample NO.6-8, spiked standard 0.3 µg/spot NO. 9-11,

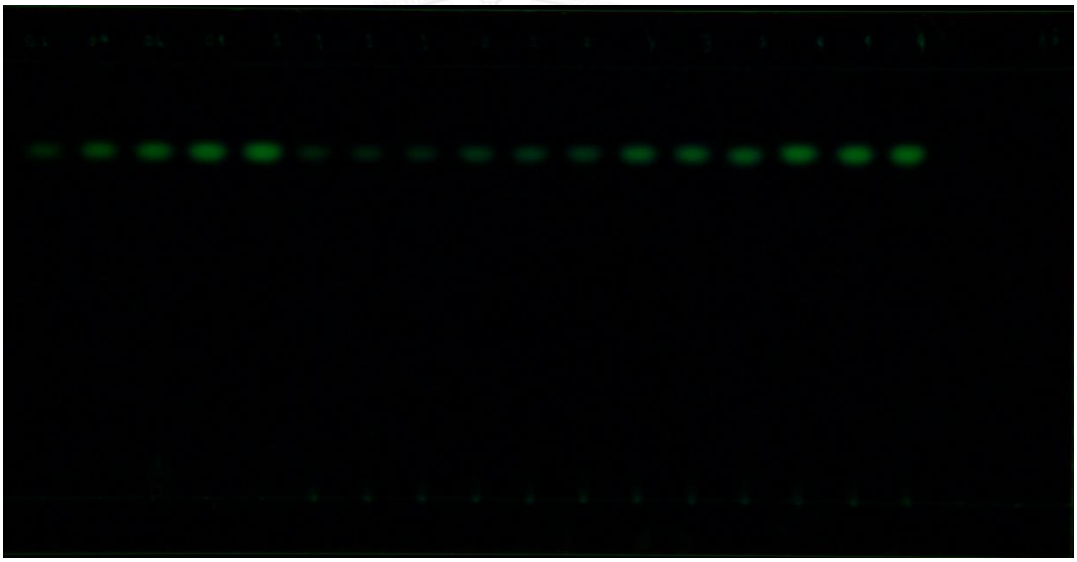
spiked standard 0.9 µg/spot NO. 12-14,

spiked standard 1.5 µg/spot NO. 15-17)

visual under UV 254 nm (A), with subtract background by ImageJ software (B)



(A)



(B)

Figure 36 The TLC Plate G Precision

(standard lupinifolin NO.1-5, sample NO.6-8, spiked standard 0.3 µg/spot NO. 9-11,

spiked standard 0.9 µg/spot NO. 12-14,

spiked standard 1.5 µg/spot NO. 15-17)

visual under UV 254 nm (A), with subtract background by ImageJ software (B)

APPENDIX B

Data of cytotoxicity determination of *Derris reticulata* stem wood

Table 26 Cytotoxicity effect of *D. reticulata* stem wood ethanolic extract on 5 human cancer cell lines and 1 human normal cell line

Ethanolic extract (µg/ml)	BT474		CHAGO-K1		Hep-G2	
	Absorbance (Mean)	PS	Absorbance (Mean)	PS	Absorbance (Mean)	PS
100	0.234	33	0.327	59	0.229	30
10	0.621	87	0.510	92	0.629	83
1	0.672	94	0.460	83	0.712	94
0.1	0.610	85	0.477	86	0.669	88
0.01	0.707	98	0.535	97	0.624	82
0.001	0.661	92	0.553	100	0.721	95
0.0001	0.638	89	0.599	108	0.761	100
DMSO	0.718	100	0.553	100	0.758	100

Ethanolic extract (µg/ml)	KATO-3		SW620		Wi-38	
	Absorbance (Mean)	PS	Absorbance (Mean)	PS	Absorbance (Mean)	PS
100	0.620	67	0.395	31	0.281	45
10	0.787	85	1.273	100	0.586	93
1	0.826	90	1.270	100	0.617	98
0.1	0.857	93	1.275	100	0.567	90
0.01	0.867	94	1.250	98	0.622	98
0.001	0.784	85	1.339	105	0.587	93
0.0001	0.768	83	1.304	102	0.575	91
DMSO	0.922	100	1.275	100	0.632	100

*PS (Percentage of cell survival)

Table 27 Cytotoxicity effect of doxorubicin on 5 human cancer cell lines and 1 human normal cell line

Doxorubicin (µg/ml)	BT474		CHAGO-K1		Hep-G2	
	Absorbance (Mean)	PS	Absorbance (Mean)	PS	Absorbance (Mean)	PS
10	0.146	16	0.092	8	0.415	21
1	0.234	26	0.333	28	0.111	6
0.1	0.760	83	1.093	91	0.867	45
0.01	0.868	95	1.203	100	1.740	90
0.001	0.949	104	1.233	102	1.989	103
Control	0.912	100	1.204	100	1.939	100

Doxorubicin (µg/ml)	KATO-3		SW620		Wi-38	
	Absorbance (Mean)	PS	Absorbance (Mean)	PS	Absorbance (Mean)	PS
10	0.766	46	0.213	14	0.237	27
1	1.085	65	0.521	34	0.172	19
0.1	1.334	80	0.760	49	0.504	57
0.01	1.637	99	1.523	98	0.951	108
0.001	1.687	102	1.469	95	0.967	110
Control	1.660	100	1.551	100	0.882	100

*PS (Percentage of cell survival)

APPENDIX C

Data of antioxidant activities of *D. reticulata* stem wood

Table 28 DPPH scavenging activity of ethanolic extract of *Derris reticulata* stem wood

Ethanolic extracts of *D. reticulata* (μg/ml)	OD_{517} (reaction mixture)			DPPH scavenging (%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.00	0.271	0.264	0.281					
800	0.152	0.151	0.148	44.118	44.485	45.588	44.730	0.765
1000	0.124	0.123	0.123	54.412	54.779	54.779	54.657	0.212
1200	0.103	0.106	0.105	62.132	61.029	61.397	61.520	0.562
1400	0.085	0.085	0.087	68.750	68.750	68.015	68.505	0.425
1600	0.067	0.068	0.066	75.368	75.000	75.735	75.368	0.368

Table 29 DPPH scavenging activity of positive control (BHT)

BHT (μg/ml)	OD_{517} (reaction mixture)			DPPH scavenging (%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.00	0.271	0.264	0.281					
12.5	0.266	0.270	0.265	2.206	0.735	2.574	1.838	0.973
25	0.243	0.241	0.240	10.662	11.397	11.765	11.275	0.562
50	0.204	0.203	0.205	25.000	25.368	24.632	25.000	0.368
100	0.145	0.144	0.144	46.691	47.059	47.059	46.936	0.212
200	0.090	0.087	0.086	66.912	68.015	68.382	67.770	0.765

Table 30 DPPH scavenging activity of positive control (Quercetin)

Quercetin (µg/ml)	OD_{517} (reaction mixture)			DPPH scavenging (%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.00	0.295	0.299	0.295					
1.25	0.281	0.277	0.279	5.068	6.419	5.743	5.743	0.676
2.5	0.255	0.247	0.248	13.851	16.554	16.216	15.541	1.473
5	0.206	0.196	0.199	30.405	33.784	32.770	32.320	1.734
10	0.096	0.085	0.082	67.568	71.284	72.297	70.383	2.490
20	0.030	0.029	0.029	89.865	90.203	90.203	90.090	0.195

Table 31 The calculated FRAP value of *D. reticulata* ethanolic extract, quercetin and BHT, which calculated by using the equation from standard curve of ferrous sulphate

Sample (0.5 mg/ml)	Absorbance at 593 nm				Ferrous sulphate equivalent (mM)
	1	2	3	mean ± SD	
D. reticulata ethanolic extract	0.072	0.073	0.075	0.073 ± 0.002	0.066
Quercetin	0.396	0.408	0.403	0.402 ± 0.006	0.457
BHT	0.429	0.435	0.424	0.429 ± 0.006	0.489

Table 32 The calculated total phenolic content of *D. reticulata* ethanolic extract, which calculated by using the equation from standard curve of gallic acid

Sample (0.5 mg/ml)	Absorbance at 756 nm				Gallic acid equivalents (mg GAE/g extract)
	1	2	3	mean ± SD	
D. reticulata ethanolic extract (1)	0.036	0.033	0.043	0.037 ± 0.005	43.51
D. reticulata ethanolic extract (2)	0.039	0.042	0.033	0.038 ± 0.005	45.46
D. reticulata ethanolic extract (3)	0.042	0.036	0.035	0.038 ± 0.005	44.16
				mean ± SD	44.37 ± 0.99

VITA

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Proceedings

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