# PHARMACOGNOSTIC SPECIFICATION AND ROTENONE CONTENT IN *DERRIS ELLIPTICA* STEMS, MACROSCOPIC, MICROSCOPIC AND MOLECULAR IDENTIFICATION OF SELECTED *DERRIS* SPECIES IN THAILAND

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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# ข้อกำหนดทางเภสัชเวทและปริมาณโรตีโนนของเถาหางไหลแดง การประเมินลักษณะทางมหทรรศน์ จุลทรรศน์ และอณูโมเลกุลของพืชบางชนิดในสกุลเดอร์ริสในประเทศไทย

นายกฤตภาส ไพเราะ



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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กฤตภาส ไพเราะ : ข้อกำหนดทางเภสัชเวทและปริมาณโรตีโนนของเถาหางไหลแดง การประเมินลักษณะทางมหทรรศน์ จุลทรรศน์ และอณูโมเลกุลของพืชบางชนิดในสกุลเดอร์ริสในประเทศไทย . ( PHARMACOGNOSTIC SPECIFICATION AND ROTENONE CONTENT IN *DERRIS ELLIPTICA* STEMS, MACROSCOPIC, MICROSCOPIC AND MOLECULAR IDENTIFICATION OF SELECTED *DERRIS* SPECIES IN THAILAND) อ.ที่ปรึกษาหลัก : รศ. ดร.นิจศิริ เรืองรังษี, อ.ที่ปรึกษาร่วม : รศ. ดร.ชนิดา พลานุเวช,รศ. ดร. กาญจนา รังษีหิรัญรัตน์

พืชสกุลเดอริสจัดอยู่ในวงศ์ Fabaceae กระจายอยู่ในโซนเส้นศูนย์สูตรทั้งในแอฟริกาและเอเชีย ในประเทศไทยพบได้ 16 ชนิด กระจายทั่วภูมิภาค หางไหลแดง คือพืชชนิดหนึ่งในสกุลเดอริส เป็นที่รู้จักกันในหมู่ชาวประมงและชาวสวน มีสรรพคุณในการเบื่อปลาและใช้ในการกำจัดแมลงศัตรูพืช โดยเฉพาะส่วนเถาและรากมีสารสำคัญ ที่มีชื่อว่า โรตีโนน เป็นสารกลุ่ม ไอโซฟลาโวน การศึกษาครั้งนี้เพื่อจัดทำข้อกำหนดทางเภสัชเวท ของเถาหางไหลแดง และพัฒนาวิธีวิเคราะห์ปริมาณสารโรตีโนนในเถาหางไหลแดง โดยวิธีโครมาโทกราฟีชนิดแผ่นบางร่วมกับเด็นซิโทเมทรีและการวิเคราะห์เชิงภาพ ข้อกำหนดทางเคมี-ฟิสิกส์ ของเถาหางไหลแดงพบว่า มีปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด น้ำหนักที่หายไปเมื่อทำให้แห้งและปริมาณน้ำไม่เกินร้อยละ 7, 1, 5 และ 8 โดยน้ำหนักตามลำดับ ปริมาณสิ่งสกัดด้วยเอทานอลและปริมาณสิ่งสกัดด้วยน้ำไม่น้อยกว่าร้อยละ 4 และ 11 โดยน้ำหนัก ตามลำดับ ลักษณะทางจุลทรรศน์ (ภาคตัดขวางและผงยา) ของเถาหางไหลแดง แสดงในรูปแบบภาพวาดลายเส้น พบปริมาณสารโรตีโนนโดยวิธีวิเคราะห์ทั้งสองวิธีเท่ากับ 0.2870 ± 0.1242 และ 0.2844 ±0.1209 กรัม ในสมุนไพรแห้ง 100 กรัม ซึ่งไม่ต่างกันอย่างมีนัยสำคัญทางสถิติ ($P > 0.05$) สำหรับการทดสอบฤทธิ์ทางชีวภาพในหลอดทดลองพบว่า สิ่งสกัดเอทานอลของเถาหางไหลแดงและโรตีโนนมีฤทธิ์ในการต้านออกซิเดชัน ยับยั้งเอ็นไซม์แอลฟากูลโคซิเดส มีความเป็นพิษต่อไรทะเล ผลการทดสอบความเป็นพิษต่อเซลล์มะเร็ง (MAD-231, HepG2, HT-29, CaCo-2) โดยวิธีเอ็มทีที พบความเป็นพิษต่อเซลล์มะเร็งที่ศึกษา ($LC_{50} > 20$ ไมโครกรัม/มิลลิลิตร) การศึกษาลักษณะทางจุลทรรศน์ของใบพืชในสกุลเดอริส 8 ชนิด (ย่านสาวคำ หางไหลขาว หางไหลแดง ชะเอมเหนือ ถอบแถบน้ำ เถาวัลย์เปรียง หยีน้ำ *D. solorioides*) พบว่าค่าคงที่ทางจุลทรรศน์ของใบ ได้แก่ จำนวนปากใบ ค่าดัชนีปากใบ ค่าอัตราส่วนเซลล์รั้ว ค่าพื้นที่เซลล์ผิว จำนวนขน สามารถใช้เป็นกุญแจในการแยกชนิดของพืชทั้ง 8 ชนิด การประเมินทางอณูโมเลกุลด้วยลายพิมพ์ดีเอ็นเอชนิด ไอเอสเอสอาร์โดยใช้ไพรเมอร์ ไอเอสเอสอาร์ 11,12,14,17 และ 35 ให้แถบ DNA ที่มีความแตกต่างและคมชัดทั้งหมด 256 แถบ ให้ค่าดัชนีความคล้ายคลึงทางพันธุกรรมของพืชในสกุลเดอริสอยู่ระหว่าง 0.11 ถึง 0.43 ผลการศึกษาครั้งนี้สามารถนำไปประยุกต์ใช้ในการพิสูจน์เอกลักษณ์ของเถาหางไหลแดงที่พบในประเทศไทย และสร้างข้อกำหนดทางเภสัชเวท รวมทั้งปริมาณสารโรตีโนนในเถาหางไหลแดงและฤทธิ์ทางชีวภาพในหลอดทดลองของเถาหางไหลแดง



สาขาวิชา วิทยาศาสตร์สาธารณสุข
ปีการศึกษา 2563

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ..... ที่ปรึกษาหลัก.
ลายมือชื่อ อ..... ที่ปรึกษาร่วม.
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The genus *Derris* belonging to Fabaceae family are distributed along the equatorial zone in Africa and Asia. In Thailand, 16 species of *Derris* species can be found throughout the country. *Derris elliptica* (Hanglai-Dang), is well-known among fishermen and gardeners as fish poison and insecticide, especially the stem and root which contain isoflavonoid compound named rotenone. This study established the pharmacognostic specification of *Derris elliptica* dried stem crude drug and developed the quantitative analysis of rotenone content in *D. elliptica* stem using TLC-densitometry and image analysis. The physico-chemical parameters of *D. elliptica* stem included total ash, acid-insoluble ash, loss on drying and water contents that should not be more than 7, 1, 5 and 8 g/ 100g crude drug, respectively. The ethanol and water extractive values should not be less than 4 and 11 g/ 100 g crude drug, respectively. Microscopic characteristics (transverse section and powder) of *D.elliptica* stem were illustrated by hand drawing. Rotenone contents obtained from both quantitative TLC were 0.2870 ± 0.1242 and 0.2844 ±0.1209 g/100 g crude drug, respectively which was not statistically significantly different ($P > 0.05$). *In vitro* biological activities of the stem ethanolic extract and rotenone revealed the antioxidant, α-glucosidase inhibitory properties and brine shrimp toxicity. MTT assay revealed that the ethanolic extract and rotenone were marginal cytotoxic ($LC_{50}$ > 20 µg/ml) against the cancer cells (MAD-231, HepG2, HT-29, CaCo-2). Microscopic leaf characteristics including leaf constant numbers of 8 *Derris* ( *D. amonea*, D. *malaccensis*, *D. elliptica*, *D. reticulate*, *D. trifoliate*, *D. indica*, *D. solorioides* ) were shown that stomatal number, stomata index, palisade ratio, epidermal cell area, trichome number could be used as the identification key for selected 8 species. DNA fingerprinting using ISSR 11, 12, 14, 17 and 35 as primers produced a total of distinct and reproducible 256 bands. The genetic relationship among selected *Derris* species presented the similarity index between 0.11 to 0.43. The information from this research can be applied for identification of *D. elliptica* stems in Thailand as well as providing the information of pharmacogenetic specification, including rotenone content in and *in vitro* biological properties of *D. elliptica* stem.



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| | | Co-advisor's Signature ..... |
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Krittapat Phairoh

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## LIST OF ABBREVIATION

| | |
|---|---|
| AFLP | Amplified fragment length polymorphism |
| $AlCl_3$ | Aluminum chloride |
| BHT | 3, 5-Di-tert-4-butylhydroxytoluene |
| bp | Base pair |
| CTAB | Cetyl trimethyl ammonium bromide |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphates(dATP, dTTP,dGTP, dCT) |
| DPPH | 2, 2- diphenyl-1 picryl hydrazyl |
| EDTA | Ethylenediaminetetraacetic acid |
| $Fe^{2+}$ | Ferrous ion |
| $Fe^{3+}$ | Ferric ion |
| $FeSO_4$ | Ferrous sulfate |
| FRAP | Ferric reducing antioxidant power |
| GAE | Gallic acid equivalent |
| HCl | Hydrochloric acid |
| $IC_{50}$ | Half maximal inhibitory concentration |
| ISSR | Inter-simple sequence repeat |
| LOD | Limit of detection |
| LOQ | Limit of quantitation |

| | |
|---|---|
| M | Molar |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| ml | Milliliter |
| mm | Millimeter |
| mM | Millimolar |
| $mm^2$ | Square millimeter |
| ng | Nano gram |
| nm | Nanometer |
| $OH^-$ | Hydroxyl group |
| PCR | Polymerase chain reaction |
| pH | Potential of hydrogen ion |
| QE | Quercetin equivalent |
| RAPD | Random amplified polymorphic DNA |
| Rf | Retention factor |
| rpm | Round per minute |
| RSD | Relative standard deviation |
| SD | Standard deviation |
| SSR | Simple sequence repeat |
| ISSR | Inter simple sequence repeat |
| *Taq* | *Thermus aquaticus* |

| | |
|---|---|
| TBE buffer | Tris-boric and EDTA buffer |
| TE buffer | Tris-EDTA buffer |
| TLC | Thin-layer chromatography |
| TPTZ | 2, 4, 6-tripyridyl-S-triazine |
| Tris | Tris (hydroxymethyl) aminomethane |
| Tris-HCl | Tris-hydrochloride buffer |
| UPGMA | Unweighted pair group method with arithmetic mean |
| UV | Ultraviolet |
| v/v | Volume by volume |
| w/w | Weight by weight |
| w/v | Weight by volume |
| WHO | World Health Organization |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometer |
| µM | Micromolar |

# CHAPTER I

# INTRODUCTION

## Background and significance of the study

In present, natural and herbal medications gain more interests and get being used worldwide, promoting the economic values of health services and herbal products. In 2017, the Thai government releases the policy "Thailand 4.0: Herbal Products Roadmap" to transform Thailand's economic structure into "Value-Based Economy," for developing herbal plants and increasing the value of processed herbal products (Division of Research Administration and Educational Quality Assurance, 2017). In the meantime, Thai food and drug administration (Thai FDA) has announced the National List of Essential Medicinal plant (National Drug Information, 2018). However, the specification of all the medicinal plants cannot be included in the Thai herbal pharmacopoeia (Department of medical science, 2016) Therefore, it is crucial to conduct the quality control of those plants in order to guarantee not only their safety but also their efficacy.

The genus *Derris* Lour. is a member of the papilionoid Leguminosae with a small to medium size. There are approximately 50 species and 16 of them are distributed in Thailand (Adema, 2000). The roots, leaves and stems of *Derris* species and its allies contain isoflavonoid rotenone which can be used as an insecticide and fish poison. Some species, for instance, *D. malaccensis*, *D. elliptica* and *D. reticulate* are locally used to treat diseases in Thailand and other Southeast Asian countries. Rotenone is an active compound found in Leguminosae family, especially in the genus *Derris*. It is commonly used as an indicator to distinguish between species. Rotenone is used as an insecticide along with carbaryl, pyrethrins and lindane to prevent mites, ticks, lice, spiders, beetles and aphids on vegetables, fruits and flowers. Besides, the emulsion of rotenone can eliminate undesirable fish in water management system.

According to the United States Environmental Protection Agency survey in 1990, rotenone was one of the most widely used pesticides in household because it is very safe when properly used (U.S. Environmental Protection Agency, 1990). However, it turns toxic to living organisms if higher dose is applied. Therefore, rotenone is classified as a moderately hazardous Class II pesticide by WHO (World Health Organization & International Programme on Chemical Safety, 1992). It is somehow found to be involved in suicidal attempts as it can cause acute congestive heart failure. Rotenone has been popular during past decades due to its involvement with the Parkinson's disease (Pan-Montojo et al., 2010; Xiong et al., 2012). Discovered by researchers, rotenone is extensively used to produce mitochondrial dysfunction and reproduce Parkinson's disease in animal models (R. E. Drolet, J. R. Cannon, L. Montero, & J. T. Greenamyre, 2009; Xiong et al., 2012).

The standardization decisively assures the quality and therapeutic efficacy. The processes for authentication, quality control and method validation is preferred to standardize the herbal materials. The techniques for identification usually include macroscopic, microscopic characters and chemical fingerprinting, whereas physicochemical property and the content of active constituents are used to ensure the quality (Mukherjee, 2007; Singh, Sharma, Agrawal, & Marshall, 2010). Another method for authentication in molecular level is polymerase chain reaction (PCR)-based molecular markers, which provides genetic relationship of the species and evaluates the genetic evolution. Interestingly, the molecular marker is more advantageous than macroscopic, microscopic determination and chemical fingerprinting because it is not likely affected by the age of the plant, environmental and physicological conditions (Mishra et al., 2016; Pawar, Handy, Cheng, Shyong, & Grundel, 2017). Among PCR based DNA marker systems, the inter simple sequence repeat amplification (ISSR) is a simple,

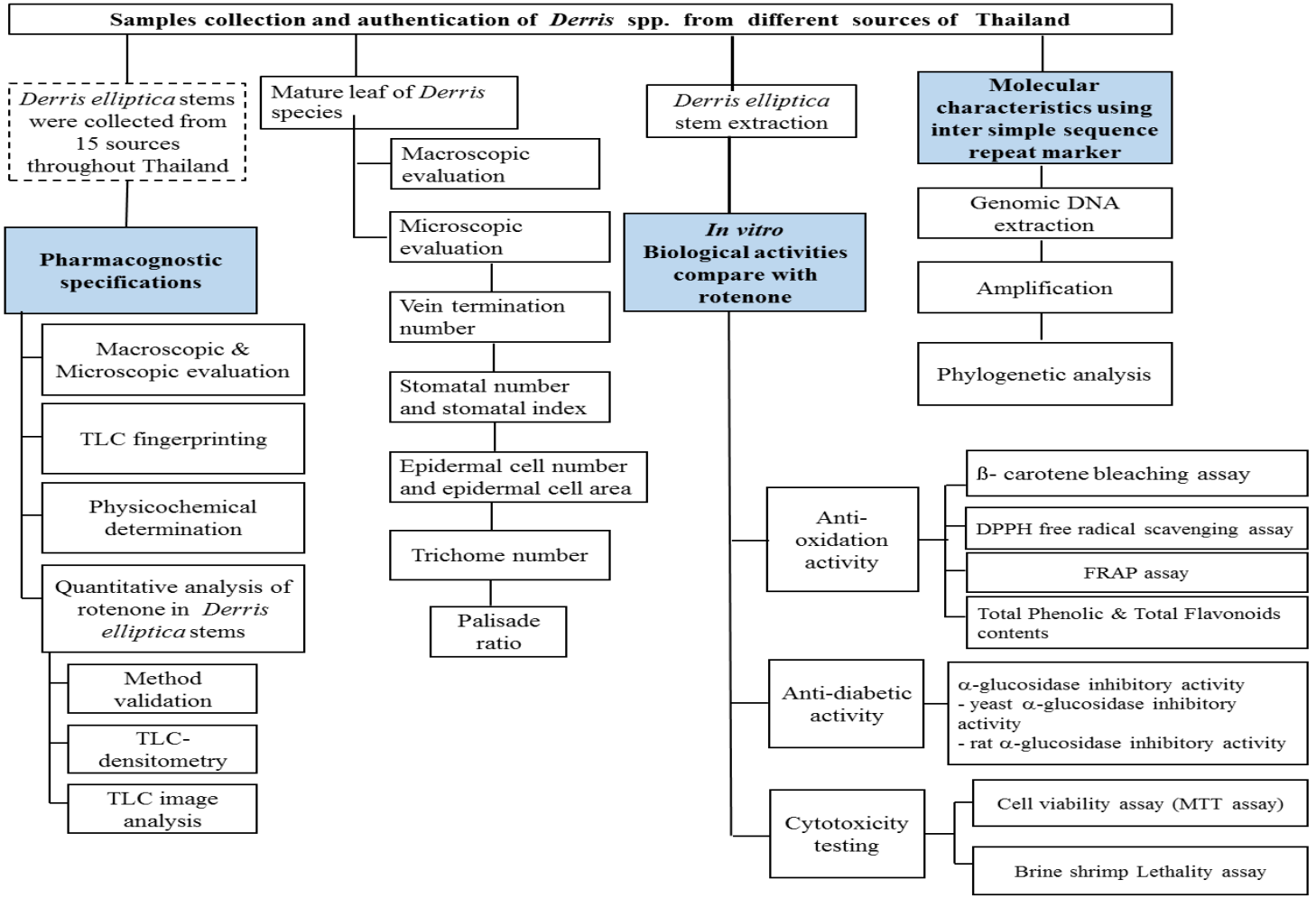
rapid and reliable technique used to assess polymorphism and genetic mapping of various species.

Therefore, this study aims to distinguish *Derris* species existing in Thailand using various methods including macroscopic, microscopic examination and ISSR technique. Furthermore, this study develops and validates TLC densitometry and TLC image analysis of rotenone content in *D. elliptica* stems. The results of this study will provide useful information on the correct identification and the rotenone content of this plant species.

**Objectives of the study**

1. To develop the pharmacognostic specification of *Derris elliptica* stems
2. To evaluate the rotenone content in *Derris elliptica* stems by TLC-image analysis using ImageJ software and TLC-densitometry method
3. To investigate *in vitro* biological activities of ethanolic extract of *Derris elliptica* stems and rotenone compound
4. To provide macroscopic, microscopic characteristics of leaf including the constant numbers among selected *Derris* species in Thailand
5. To investigate the genetic relationship of selected *Derris* species using inter simple sequence repeat marker (ISSR)

## Conceptual framework



# CHAPTER II

# LITERATURE REVIEWS

## Description of plant in genus *Derris*

*Scientific Classification* of plant in genus *Derris*

Kingdom: Plantae - Plants

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Subfamily: Faboideae

Genus: *Derris*

**The genus *Derris*** (Y. Sirichamorn, F. A. C. B. Adema, & P. C. Welzen, 2012)

The publication from the American Society of Plant Taxonomists reported various *Derris* species in Thailand as follows: Lianas. Twigs usually lenticellate. Leaves imparipinnate; stipules present, persistent or caducous; stipellae absent or present, persistent. Leaflets generally 3–9, rarely up to 15 per leaf, opposite, entire, variable in shape and size. Inflorescences axillary pseudoracemes, often combined with terminal or axillary pseudopanicles, rarely true panicles, sometimes an intermediate form present: a panicle in the lower part and a pseudoraceme in the upper part, or, a pseudoraceme in the lower part and flowers solitary in the upper part. Flowers usually 2–5 or occasionally up to 7, scattered throughout or on the top of the knob-like, club-shaped to long and slender brachyblasts. Bracts subtending brachyblasts and flowers persistent or caducous, shorter than the corresponding flower buds; bracteoles present, usually at the apex of the pedicel or at the base of the calyx. Calyx cup-shaped, often maroonish or reddish, bilabiate, upper lip 2-

lobed, often indistinctly so lower lip 3-lobed. Corolla generally whitish or pinkish; standard reflexed at base, without or with distinct basal callosities; wings approximately as long as keel petals, in some species curved backward towards the calyx, wings and keel petals adherent by lateral pockets or hooked together by auricles or twisted claws (or any combination). Stamens 10, diadelphous, with basal fenestrae; anthers all equal, fertile, glabrous or hairy. Disc generally inconspicuous or annular. Ovary with 2–5 (rarely up to 7) ovules. Pods indehiscent, thin and stiff, with a wing along both sutures or along the upper suture only, rarely wingless. Seed chambers absent but sometimes seeds surrounded by a thickening of the pod's vascular bundles. Seeds bean-shaped, 1 or 2(–3) to rarely more per pod; hilum usually central.

**Distribution**

This genus comprises about 50 species mainly in Southeast Asia, of which 16 can be found in Thailand (Adema, 2000).

**Habitat and ecology**

Species of *Derris* are sunlight and water-preferring plants, usually found in fully exposed or semi-shaded areas along the streams or rivers. *D. microphylla* and *D. robusta* are deciduous trees and found in mixed deciduous forests or at the edge of dry evergreen forests. *D. scandens and D. amoena* are also found in disturbed or degraded areas, *D. trifoliata* is typical for the mangrove, and, *D. tonkinensis*, only occurs on limestone.(Y. Sirichamorn et al., 2012).

***Derris amoena* Benth.**

**General description:**

"Lianas. Bark maroonish, lenticellate, glabrous. Twigs 3–5 mm diam., slightly strigose or glabrescent, lenticellate. Stipules triangular, ca. 1.5 by 0.7–1 mm, outside thinly sericeous, inside glabrous. Leaves with 5–9 leaflets, reddish when young; petiole 3.5–6 cm long, slightly grooved and striate, glabrous or with few hairs; rachis 3.5–11 cm long, grooved and striate, glabrescent; pulvinus 5–9 mm long, thinly strigose. Stipellae absent. Leaflets subcoriaceous; terminal one elliptic to obovate, 6–9 by 3–4.5 cm, length/width ratio ca. 2, base cuneate to rounded, apex acuminate, acumen 2–5 mm long, rounded, upper surface glabrous or slightly strigose, especially at the basal part of the midrib, lower surface glabrous or slightly strigose, usually glaucous, midrib flat or slightly raised above, veins flat on both sides, 7–13 per side, 2–10 mm apart, parallel, seemingly ending and anastomosing in the margin, venation reticulate; lateral ones mostly like terminal one, elliptic to obovate, 4.5–8.5 by 2–4 cm, length/width ratio 2.1–2.3; pulvinus 5.5–7 mm long, glabrous. Inflorescences axillary pseudoracemes or pseudopanicles, 7–18 cm long, peduncle 0.5–4.5 cm long, striate, glabrous or sometimes slightly sericeous. Bracts subtending brachyblasts ovate to triangular, ca. 0.8 by 0.6–0.7 mm, outside sericeous with ciliate margin, inside glabrous; bracts subtending flowers ovate, 0.4–0.5 by 0.2–0.5mm, outside sericeous, inside glabrous. Brachyblasts knob-like, 0.5–0.7 mm long, with 2 or 3 flowers, sericeous. Pedicels 3–7 mm long, sericeous. Bracteoles at the top or the upper part of the pedicels, ovate, 0.3–0.4 by 0.2–0.3 mm, outside slightly pubescent, inside glabrous. Calyx cup-shaped, 2–3 mm high, maroonish, outside thinly sericeous, margin ciliate, inside glabrous, tube 2.5–2.8 mm high, upper lip with 2 short lobes, shallowly notched, 0.3–0.4 by 3–4mm; lateral and lower lobes shortly triangular, 0.5–0.6 by 1.5–2 mm, apex obtuse or rounded. Corolla purplish. Standard: claw 2.7–3 mm long; blade broadly elliptic to orbicular, 6–6.5 by 8–8.5 mm, apex emarginate, basal callosities

absent, outside slightly ciliate at apex, inside glabrous. Wings: claw 1.7–3 mm long; blade elliptic, 6–7 by 1.7–2.3 mm, apex rounded, outside slightly hairy and ciliate at the apex, inside glabrous, auricle ca. 0.5 mm long. Keel petals: claw ca. 2 mm long; blade boat-shaped, 6–6.5 by ca. 3 mm, apex rounded, outside thinly sericeous near the apical part and ciliate at apex, inside glabrous, upper auricle indistinct, 0.1–0.2 mm long, lateral pocket 1.2–1.7 mm long. Stamens diadelphous, with basal fenestrae, 7–8 mm long, free part 2–3 mm long, glabrous; anthers ca. 0.5 by 0.4 mm, glabrous. Disc indistinct. Ovary 4.5–5 mm long, thinly sericeous; ovules 2 or 3; style 3.5–4 mm long, slightly hairy at base or sometimes glabrous. Pods broadly straplike, flat, 4–9.5 by 1.5–2.6 cm, glabrous, with wing along both sutures, upper wing 3–4 mm wide, lower wing ca. 2 mm wide.

**Distribution:**

Thailand (Peninsular); Malesia: Malay Peninsula, Sumatra, and Borneo

**Habitat:**

Edge of evergreen forests, along roadsides or rivers, in disturbed areas, in para-rubber (Hevea) plantations or open margins of swamp forest, up to 25 m (Y. Sirichamorn et al., 2012).

***Derris elliptica* (Wall.) Benth.**

**General description:**

"Lianas, up to 5 m high. Bark dull greenish to brown. Twigs 4–15 mm diam., hirsute, glabrescent in older parts, lenticellate. Stipules triangular or ovate, 3–3.5 by 1.7–3 mm, outside sericeous, inside glabrous. Leaves with 7–15 leaflets; petiole 4–14.5 cm long, striate, (thinly) hirsute; rachis 6–20.5 cm long, striate, (thinly) hirsute; pulvinus 5–11 mm long, (thinly) hirsute. Stipellae rarely present, acicular, ca. 1.5 mm long. Leaflets chartaceous to subcoriaceous, terminal one obovate to narrowly obovate, 7–16 by 2–5 cm, length/width ratio 3.2–4.3, base cuneate, apex acuminate, acumen 3–20 mm long, rounded, upper surface glabrous to thinly sericeous, lower surface more or less sericeous, older leaflets often less hairy, midrib flat above, rarely slightly sunken, raised below, nerves flat above, raised below, 7–14 per side, 3–16 mm apart, curving towards the apex and almost reaching the margin, sometimes anastomosing near the margin, venation reticulate; lateral leaflets mostly like terminal one, narrowly elliptic to obovate, 4–16 by 2–5.5 cm, length/width ratio 2–4; pulvinus 3–7 mm long, (thinly) sericeous. Inflorescences axillary pseudoracemes/ pseudopanicles, rarely terminal, up to 30 cm long; peduncle 0.5–5.5 cm long, sericeous; lateral branches 5–20 cm long. Bracts subtending brachyblasts ovate to triangular, 1.5–2.5 by 1–1.5 mm, outside sericeous, inside glabrous; bracts subtending flowers ovate, elliptic, triangular or cordate, 1.5–2.5 by 1–2 mm, outside sericeous, inside glabrous. Brachyblasts long cylindrical, 10–25 mm long, with 2 or 3 flowers at the apex. Pedicels 5–11 mm long, sericeous. Bracteoles at the top of the pedicels, (narrowly) ovate or triangular, 1–2.7 by 1–2 mm, outside sericeous, inside glabrous. Calyx 5.5–7 mm high, brown to dark maroonish, outside sericeous, inside sericeous at apex, tube 4–6 mm high; upper lip hardly visible or sometimes with 2 short semicircular lobes; lateral lobes (broadly) triangular, 1–1.5 by 3–5 mm; lower lobe triangular, 1.5–2.5 by 3–5mm. Corolla pale to deep pink, standard with green blotch at base. Standard: claw 3–4 mm long; blade

orbicular, 12–17 by 10–17 mm, apex emarginate, basal callosities large, outside sericeous, except for the margin and claw, inside sericeous at apex. Wings: claw 4.5–5 mm long; blade obliquely elliptic, 10–13 by 4.5–5 mm, apex rounded, upper auricle 0.5–1 mm long, lower auricle absent, lateral pocket 2–5 mm long, sometimes hardly visible, outside with some hairs at the ventral side part of the apex, inside sericeous at apex. Keel petals: claw 4.8–5 mm long; blade boat-shaped, 10–11 by 5–6 mm, apex rounded, auricle indistinct, lateral pocket large, 4–5 mm long, outside with some hairs at apex, inside sericeous at apex, rarely with some hairs at the apex. Stamens diadelphous, with basal fenestrae, 14–23 mm long, free part 3–7 mm long, glabrous, rarely with some hairs; anthers 0.3–0.6 by 0.2–0.4 mm, with some hairs outside. Disc indistinct, annular, usually with some hairs. Ovary 6–10 mm long, outside sericeous, inside with a row of hairs along the lower suture; stipe 1–1.7 mm long, sericeous; ovules 3–5; style 8–14 mm long, sericeous in lower part. Pods elliptic or strap-like, flat, 4–11 by 1.5–2 cm, thinly sericeous, sometimes also glaucous, with wing along the upper suture only or rarely winged on both sutures, upper wing 1.5–2 mm wide, lower wing usually undeveloped, up to 1.5 mm wide; seed chamber absent. Seeds seen immature in well-developed pods, bean-shaped, flat, ca. 13 by 8–8.5 by ca. 3 mm; hilum central, 2.5–3 mm long.



**Distribution:**

India, Myanmar, Laos, Vietnam, Cambodia, throughout Thailand, Malesia: Malay Peninsula, Philippines (Palawan); Caroline Islands, and probably introduced in Hawaii.

**Habitat:**

Primary and secondary forest, often along riverbanks, up to 1,450 m.(Sirichamorn, Adema, Gravendeel, & van Welzen, 2012; Y. Sirichamorn et al., 2012)

*Derris malaccensis* **Prain**

**General description:**

"Lianas. Twigs 3–6 mm diam., smooth or slightly lenticellate, thinly hirsute in young parts, older parts glabrescent. Stipules ovate, 1.5–2 by 2–2.5 mm, outside thinly sericeous, margin ciliate, inside glabrous. Leaves with 5–9 leaflets, rarely up to 15; petiole 5–7.5 cm long, striate; rachis 4–8 cm long, striate, glabrous to thinly hirsute at the upper side; pulvinus 4–8 mm long, hirsute, especially at the base. Stipellae absent. Leaflets chartaceous, terminal one obovate, 5–9 by 3.4–5 cm, length/width ratio 1.5–1.8, base cuneate, apex shortly acuminate or obtuse, acumen 3–4 mm long, rounded, upper surface glabrous, lower surface with widely scattered appressed hairs, especially along the midrib and veins, midrib flat to slightly sunken above, raised below, nerves flat or slightly raised above, raised below, 8–11 per side, 6–10 mm apart, curving towards the apex and almost reaching the leaf margin, anastomosing near or close to the margin, venation reticulate; lateral leaflets mostly like terminal one, elliptic or obovate, 6–7.5 by 3.3–4 cm, length/width ratio 1.8–1.9; pulvinus ca. 4.5 mm long, thinly sericeous. Inflorescences axillary or rarely terminal pseudoracemes rarely combined to pseudopanicles, 7–16 cm long; peduncle 1–4.5 cm long, glabrous to thinly strigose; lateral branches up to 7 cm long. Bracts subtending brachyblasts ovate to triangular, 1.7–1.8 by 1.5–1.6 mm, outside sericeous, margin ciliate, inside glabrous; bracts subtending flowers broadly elliptic to ovate, 1–1.1 by 0.7–0.8 mm, outside glabrous or thinly sericeous, margin ciliate, inside glabrous. Brachyblasts slender, cylindrical, 5–9 mm long, with 2 or 3 flowers at the apex. Pedicels 3–9.5 mm long, glabrous or thinly sericeous. Bracteoles at the top of the pedicels, elliptic to ovate or orbicular, ca. 1 by 0.7–0.8 mm, outside glabrous or thinly sericeous, margin ciliate, inside glabrous or thinly sericeous at the apex. Calyx 3–4.5 mm high, reddish, outside glabrous to thinly sericeous, margin ciliate, inside glabrous, tube 2.5–4 mm high; upper lip indistinct or sometimes with 2 short semicircular lobes; lateral lobes (broadly) triangular, 0.3–0.5

by 2–2.5 mm; lower lobe triangular, 0.4–0.5 by 2–3 mm. Corolla white or pink. Standard: claw 2–2.5 mm long; blade broadly ovate to orbicular, 11.5–12 by 10–13 mm, apex rounded, glabrous, basal callosities present. Wings: claw 2.5–4 mm long; blade elliptic, 10–11 by 3.8–4 mm, apex rounded, both sides glabrous or thinly ciliate at apex, upper auricle 0.5–1 mm long, lower auricle 0.2–0.4 mm long, lateral pocket 2.5–3.5 mm long, Keel petals: claw 4–4.5 mm long; blade boat-shaped, 9.6–10 by 3.5–3.7 mm, apex rounded, both sides glabrous or with some hairs at the apex, auricle up to 0.7 mm long or sometimes indistinct, lateral pocket 3–4 mm long. Stamens diadelphous with basal fenestrae, 9–11 mm long, free part 3–4 mm long, glabrous; anthers 0.6–0.9 by 0.3–0.5 mm, glabrous. Disc annular or with (up to) 10 lobes, 0.2–0.5 mm high, with some hairs. Ovary 3.5–7 mm long, sericeous; stipe usually indistinct but sometimes up to 1 mm long; ovules 3 or 4; style 6–7 mm long, sericeous in lower part. Pods elliptic or ovate, 40–50 by 20–23 mm, glabrous, with wing along both sutures, upper wing 3–4.5 mm wide, lower wing 3–4.5 mm wide, sometimes indistinct; seed chamber absent.

**Distribution:**

India, Nepal, Bangladesh, Myanmar, China, Laos, Vietnam and northern Thailand.



**Habitat:**

Primary and secondary dry evergreen forests, often along rivers(Y. Sirichamorn et al., 2012).

***Derris indica* (Lam.) Bennet**

**General description:**

"Pongamia pinnata is a medium-sized evergreen or briefly deciduous, glabrous shrub or tree 15-25 m high, with straight or crooked trunk 50-80 cm or more in diameter and broad crown of spreading or drooping branches. Branchlets hairless with pale stipule scars. Leaves alternate, imparipinnate with long slender leafstalk, hairless, pinkish-red when young, glossy dark green above and dull green with prominent veins beneath when mature. Leaflets 5-9, paired except at end, short-stalked, ovate elliptical or oblong, 5-25 x 2.5-15 cm, obtuseacuminate at apex, rounded to cuneate at base, not toothed at the edges, slightly thickened. Stipules are caducous. Inflorescence raceme-like, axillary, 6-27 cm long, bearing pairs of strongly fragrant flowers; calyx campanulate, 4-5 mm long, truncate, finely pubescent. Flower clusters at base of and shorter than leaves, to 15 cm long, slender, drooping. Flowers 2-4 together, short-stalked, pea-shaped, 15-18 mm long. Calyx campanulate, 4-5 mm long, truncate, finely pubescent; corolla white to pink, purple inside, brownish veined outside, 5- toothed, standard rounded obovate 1-2 cm long, with basal auricles, often with green central blotch and thin silky hairs on back; wings oblong, oblique, slightly adherent to obtuse keel. Pods borne in quantities, smooth, oblique oblong to ellipsoid, 3-8 x 2-3.5 x 1-1.5 cm, flattened but slightly swollen, slightly curved with short, curved point (beaked), brown, thick-walled, thick leathery to subwoody, hard, indehiscent, 1-2 seeded, short stalked. Seeds compressed ovoid or elliptical, bean-like, 1.5-2.5 x 1.2-2 x 0.8 cm, with a brittle coat long, flattened, dark brown, oily.

**Distribution:**

In tropical Asia, Australia, the Polynesia and Philippine Islands

**Habitat:**

The tree is used for afforestation, especially in watersheds, in drier parts of the country and also in social forestry plantations (Y. Sirichamorn et al., 2012).

***Derris reticulata* Craib**

**General description:**

"Lianas, basal diameter 3–10 cm. Bark thin, smooth, light brown. Twigs 3–5 mm diam., glabrous, lenticellate. Leaves usually with 5(–7) leaflets Stipules caducous, triangular, 1.2–1.5 x 1.4–1.5 mm, outside sericeous, inside glabrous. Petiole 2–6 cm long, grooved above, glabrous or with some scattered hairs, rachis 2.5–5 cm long, pulvinus 4–6 mm long. Stipellae absent or reduced to hairy knob-like structures. Leaflets: subcoriaceous, terminal one (narrowly) elliptic to (narrowly) obovate, 6.5–13 x 2.5–4 cm, length/width ratio 2.6–3.3, base cuneate to obtuse, apex acuminate, acumen ca. 9–15 mm long, rounded, upper surface glabrous, lower surface glabrous except along the midrib with some scattered hairs, midrib flat or slightly raised above, distinctly raised below, veins flat above, raised below, 6–11 per side, 8–13 mm apart, not reaching the margin but curving towards the apex, sometimes anastomosing near the margin, venation reticulate; lateral ones (narrowly) elliptic to (narrowly) ovate, 5.5–12 x 1.5–4 cm, length/width ratio 2.2–3.6, base broadly cuneate to obtuse, apex acuminate, acumen 5–7 mm long, rounded, above glabrous, below glabrous except along the midrib with some scattered hairs; pulvinus 3–4 mm long. Inflorescences axillary or (rarely) terminal, pseudoracemes/ pseudopanicles, 2.5–15 cm long, striate, sericeous; peduncle up to 1 cm long, striate, sericeous; lateral branches up to 7 cm long. Bracts subtending brachyblasts ovate to narrowly triangular, 1–2 x 0.6–0.8 mm, outside pubescent, inside glabrous. Brachyblasts knob-like to elongate and cylindrical, ca. 9 mm long, (thinly) sericeous, with 1–4 flowers throughout, if 1, 2 or 3-flowered, then flowers sometimes seemingly at apex of the brachyblast, near the top of the inflorescence brachyblast sometimes disappearing and then flowers solitary (see *D. tonkinensis*). Bracts subtending flowers ovate or triangular, ca. 1 x 0.4–0.6 mm, outside pubescent, inside glabrous. Pedicels 7–10 mm long, (thinly) sericeous. Bracteoles in the middle or upper part of the pedicels, ovate or triangular, 0.6-0.8 x 0.4 mm, outside pubescent, inside glabrous. Calyx reddish or greenish-red, cup-shaped, 3–4 mm high, outside (thinly) sericeous or

glabrescent, margin ciliate, inside glabrous, tube 2–3 mm high, upper lip with 2 short triangular lobes, 0.3–0.5 by 2–2.5 mm; lateral lobes triangular, 1–1.5 by 1.5–2 mm; lower lobe triangular, 1–1.3 by ca. 2 mm. Corolla white. Standard: claw 3–3.5 mm long; blade broadly obovate to orbicular, 8–10 by 7–8 mm, apex emarginate, basal callosities absent, outside thinly pubescent at apex, sometimes glabrescent, inside glabrous or with some hairs in the middle. Wings curved backward to the calyx claw 1.5–2 mm long; blade elliptic, 7–9 by 1–2 mm, apex rounded, outside glabrous or thinly ciliate at apex, inside glabrous, upper auricle up to 1.5 mm long, lower auricle indistinct, 0.1–0.5 mm long. Keel petals: claw 2–2.5 mm long; blade boat-shaped, 7–8 by 2.7–3 mm, apex rounded, outside glabrous or thinly ciliate at apex, inside glabrous, upper auricle ca. 1 mm long, lateral pocket 1–2 mm long. Stamens diadelphous, with basal fenestrae, 9–11 mm long, free part 2–3 mm long, glabrous; anthers ca. 0.2 by 0.25 mm, glabrous. Disc indistinct or annular, up to 0.1 mm high. Ovary up to 7 mm long, stipe indistinct, sericeous; ovules 2 or 3; style 2.5–3 mm long, thinly hairy at base. Pods strap-like or sometimes ellipsoid, 5–9.5 x 1.5–2.5 cm, thin, with a wing along both sutures, upper wing 4–7 mm wide, lower wing 2–3 mm wide, with some scattered short hairs, especially on both wings, or sometimes glabrescent, seed chamber indistinct, but usually slightly thickened and with reticulate veins around the seed. Seeds 1–2 per pod, discoid or bean-shaped, 11–13 x 6–6.5 x 1.5–2.5 mm; hilum central, 0.5–1 mm long.



**Distribution:**

Endemic to Thailand

**Habitat:**

Semi-shaded area of dry evergreen forests, edge of evergreen mixed (dipterocarp) forest, in bamboo forest, or along streams, sometimes also found in limestone area, 50–450 m. (Y. Sirichamorn et al., 2012).

***Derris scandens* (Roxb.) Benth.**

**General description:**

"Lianas, basal diameter 2–5 cm. Twigs 3–5 mm diam., thinly strigose to glabrous, lenticels throughout. Leaves with 9–13 leaflets. Stipules triangular, 1–1.5 +0.9–1.3 mm, outside sericeous, inside glabrous. Petiole 2–5 cm long, grooved above, thinly hirsute or sometimes glabrescent; rachis 5.5–10 cm long, grooved above, (thinly) hirsute to glabrous; pulvinus 3–7 mm long. Stipellae present or sometimes inconspicuous, acicular, up to 1.5 mm long. Leaflets subcoriaceous; terminal one elliptic to obovate, 4.5–8 +1.5–3.2 cm, length/width ratio 2.5–3, base cuneate to broadly cuneate or rounded, apex obtuse, usually emarginate or shortly obtusely acuminate, acumen 2–6 mm long, emarginate to rarely rounded, upper surface shiny, glabrous to slightly strigose along midrib, lower surface thinly strigose, midrib and veins slightly raised or flat above, raised below, veins 7–12 per side, 3–5 mm apart, curving towards the apex and sometimes reaching the margin, sometimes anastomosing near the margin, venation reticulate; lateral ones mostly like terminal, elliptic to ovate, rarely obovate, 3–6.8 +1.2–3.5 cm, length/width ratio 1.9– 2.5; pulvinus 1.5–5 mm long. Pseudoracemes axillary, rarely several together as pseudopanicles, 5–28 cm long, peduncle 1.5–5 cm long, thinly strigose. Bracts subtending brachyblasts ovate-triangular, 0.7–1 +0.5– 0.7 mm, outside sericeous, inside glabrous. Brachyblasts knob-like, 2–6 mm long, 5–10-flowered, sericeous. Bracts subtending flowers elliptic to ovate, 0.6– 0.7 +0.3–0.5 mm, outside sericeous, inside glabrous. Pedicels 5.5–9 mm long, sericeous. Bracteoles at the base of the calyx, elliptic or ovate, 0.5–0.8 +0.3–0.7 mm, outside pubescent, inside glabrous. Calyx reddish or greenish-red, cupshaped, 2.5–2.7 mm high, outside sericeous, inside glabrous, tube ca. 2 mm high; upper lip and lateral lobes hardly visible; lower lobe triangular, ca. 0.7 +1.5–2 mm. Corolla white or pale pink. Standard: claw 2–3 mm long; blade orbicular or obovate, 5–8 +6–8 mm, apex emarginate, without auricle or basal callosities, outside glabrous or sometimes

slightly sericeous at apex, ciliate in lower part to up to halfway, inside glabrous. Wings: claw 2–2.5 mm long; blade narrowly obovate, 4.5–6.5 +1–1.5 mm, apex rounded, outside slightly sericeous at the apex and distinctly strigose on both auricles, inside glabrous, upper auricle ca. 0.5 mm long, lower auricle hardly visible, lateral pocket inconspicuous. Keel petals: claw 2.3–2.5 mm long; blade boat-shaped, 6–6.5 +2.3– 2.5 mm, apex rounded, outside hairy at the apex, sometimes also along the major veins ventrally, inside glabrous, auricle and lateral pocket hardly visible. Stamens diadelphous, with basal fenestrae, white, 9–12 mm long, free part of filaments 1.5–3.5 mm long, glabrous; anthers 0.3–0.5 +0.3–0.4 mm, glabrous. Disc short-tubular, more or less distinctly (5-)10- lobed, up to 0.6 mm high. Ovary hairy, 4–5 mm long, stipe hairy, ca. 1 mm long; ovules 8–10; style 4–5.5 mm long, glabrous but slightly hairy at base. Pods strap-like, 4.5–9 + 0.7–1.5 cm, flat, bulging around the seeds, with a wing along the upper suture only, wing 1–2 mm wide, thinly sericeous; seed chamber hard, usually visible from outside. Seeds beanshaped, flat, ca. 7 +5 +1 mm; hilum central, ca. 1 mm long.

**Distribution**



*Derris scandens* is widespread species ranging from Reunion island to India, Sri Lanka, Bangladesh, Myanmar, thoughout Indochina, Thailand, Malay Peninsular, Sumatra, Borneo, Java, Philippines, Celebes, Lesser Sunda Islands, Papua New Guinea (Morobe Prov.), and Australia.

**Habitat and ecology**

This common species grows in various types of vegetation: open and disturbed or abandoned cultivated areas, shrubbery, agricultural areas, edges of rice fields, grasslands, or beach or mangrove forest, up to 750 m. (Y. Sirichamorn et al., 2012).

***Derris solorioides* Sirich. & Adema**

**General description:**

"Lianas. Twigs up to 10 mm diam, glabrous, lenticellate. Stipules caducous, triangular, 1–1.5 by c. 1.5 mm, outside glabrous or thinly sericeous, inside glabrous. Leaves with 5–9 leaflets; young leaves light green; petiole 3–9.5 cm long, striate, glabrous; rachis 4.5–11 cm long, striate, glabrous; pulvinus 5–10 mm long, glabrous. Stipellae absent. Leaflets chartaceous to subcoriaceous, broader leaflets usually found in plants growing in shadier areas; terminal one (narrowly) elliptic or (narrowly) obovate, 6–12.5 by 2.7–5.5 cm, length/width ratio 1.6–2.2, base cuneate, obtuse to rounded, apex obtuse or slightly emarginate, upper and lower surface glabrous, midrib slightly raised in a furrow above, distinctly raised below, veins raised on both sides, 7–15 per side, 5–20 mm apart, not reaching the margin but curving upwards, sometimes anastomosing near the margin, venation reticulate; lateral leaflets (narrowly) elliptic to (narrowly) ovate or (narrowly) obovate, 4.5–11 by 2.2–5.5 cm, length/width ratio 1.8–2.2; pulvinus of petiolules 4–6 mm long. Inflorescences axillary panicles or sometimes terminal, 11–21cm long, peduncle 5–20 mm long, glabrous or thinly strigose, striate; lateral branches 1–10 cm long. Bracts subtending lateral branches ovate-triangular, 0.8–1.2 by 0.8–1.3 mm, outside glabrous to thinly sericeous, inside glabrous but with some hairs at base, margin ciliate, sometimes leaf-like. Brachyblasts absent. Bracts subtending flowers elliptic to ovate, 0.8–1 by 0.3–0.4 mm, both sides glabrous or with few hairs, margin ciliate. Pedicels slender, 7–10 mm long, glabrous. Bracteoles at the upper part of pedicels, narrowly elliptic to ovate, 0.6–0.7 by 0.2–0.3 mm, both sides glabrous, margin ciliate. Calyx greenish, cup-shaped, 2.5–3 mm high, outside glabrous, with hairs near the lobes, inside glabrous, margin ciliate, tube 2–2.5 mm high, upper lip indistinct or with two short lobes, 0.2–0.3 by 0.7–1 mm; lateral lobes triangular or semi-circular, 0.5–0.7 by 1.2–1.7 mm; lower lobe triangular or semi-circular, c. 0.7 by 1.5–1.6 mm. Corolla white or slightly with pale green hue. Standard: claw 3.3–3.5 mm

long; blade broadly obovate to orbicular, 5.5–7 by 5–5.7 mm, basal callosities absent, apex emarginate, both sides glabrous. Wings: claw 3–3.5 mm long; blade elliptic, 6–7 by 1.7–2.3 mm, apex rounded, both sides glabrous, upper auricle c. 1 mm long, lower auricle indistinct, lateral pocket 0.7–1 mm long, sometimes indistinct. Keel petals: claw 2–2.5 mm long; blade boat-shaped, 5.5–6 by 2–2.5 mm, apex rounded, outside glabrous except for the apical part with few hairs, inside glabrous, upper auricle indistinct, lateral pocket 1–2 mm long. Stamens diadelphous, with basal fenestrae, 8–9.5 mm long, free part 2.5–3 mm long, glabrous; anthers 0.4–0.5 by 0.3–0.45 mm, glabrous. Disc annular or indistinct. Ovary 4–5 mm long, stipe indistinct, slightly hairy; ovules c. 8; style 4–4.5 mm long, slightly hairy. Pods elliptic to strap-like, 4.5–8 by 1.8–2.2 cm, glabrous, with a wing along the upper suture, 2–4 mm wide, seed chamber absent but darker area around seed present in pericarp of dry pods, without thickening of the pericarp. Seeds 1–2 per fruit or sometimes immature, discoid, flat, 13–14 by 10–11 by 1–2 mm; hilum central, 1–1.4 mm long.

**Distribution:**

Endemic to Thailand



**Habitat:**

Deciduous, partly open place, seasonal fire-damaged forest, rugged limestone terrain,100–375 m.(Sirichamorn, Adema, & Van Welzen, 2012, 2014).

***Derris trifoliata* Lour.**

**General description:**

"Lianas, sometimes more or less creeping, up to 8 m long, up to 5 cm diam. Twigs hollow, 4–7 mm diam., glabrous, lenticellate. Leaves with 3–7 leaflets, rarely unifoliolate. Stipules triangular, 1.5–2 x ca. 2 mm, glabrous, caducous. Petiole 1.5–10 cm long, grooved above, striate, glabrous; rachis 1.5–6 cm long, grooved above, striate, glabrous; pulvinus 3–10 mm long, glabrous. Stipellae sometimes present, acicular, 0.5–1(–1.5) mm long, glabrous. Leaflets subcoriaceous to coriaceous; terminal one ovate to elliptic, 3.5–16 x 1.5–8.5 cm, length/ width ratio 1.8–3, base slightly cordate to (sub)peltate, rarely rounded, apex emarginate, rounded or acuminate, acumen 3–25 mm long, upper and lower surface glabrous, midrib and nerves flat or slightly raised, veins 5–11 per side, 3–20mm apart, not reaching the margin but curving towards the apex, anastomosing before the margin, venation reticulate; lateral ones mostly like the terminal one, narrowly elliptic to ovate, 2.5–12 x 1–5.5 cm, length/width ratio 1.7–3.6; pulvinus 4–7 mm long. Inflorescences axillary or ramiflorous (then often on creeping branches), pseudoracemes rarely pseudo-panicles, 1–18 cm long, peduncle 0.2–4.5 cm long, thinly strigose. Bracts subtending brachyblasts triangular, 0.6 –1.4 x 0.5–1 mm, outside glabrous or thinly sericeous, margin ciliate, inside glabrous. Brachyblasts knob-like, up to 5 mm long, with 2 or 3(–7) flowers. Bracts subtending flowers elliptic to triangular, orbicular or cordate, 0.5–0.8 x 0.3–0.6 mm, both sides glabrous, margin ciliate. Pedicels 2–6 mm long, glabrous or with few hairs. Bracteoles at the apex or the upper part of the pedicels, elliptic, triangular, orbicular or cordate, 0.5–0.7 x 0.4–0.6 mm, both sides glabrous, margin ciliate. Calyx cup-shaped, 2.5–4 mm long, outside glabrous, teeth ciliate, inside sericeous in upper part, tube 2–3 mm long; upper lip usually shallowly notched, 0.25–0.5 x 2.5–4 mm; lateral lobes triangular or semi-circular, ca. 0.5 x 1–2 mm; lower lobe triangular, ca. 0.5 x 1.5–2 mm. Corolla white to pale pink. Standard with a green spot at the base; claw 2–3 mm long; blade orbicular,

7–9.5 x 7.5–10 mm, apex slightly emarginate, basal callosities absent, both sides glabrous. Wings: claw 2.5–4 mm long; blade elliptic, 6–9 x 2–3 mm, apex rounded, both sides glabrous, lateral pocket indistinct, upper auricle 0.5–1 mm long. Keel petals: claw 2.5–3.5 mm long; blade boat-shaped, 6–8 x 3–4 mm, apex rounded, both sides glabrous, lateral pocket ca. 2 mm long, upper auricle indistinct or sometimes up to 1 mm long. Stamens diadelphous, with basal fenestrae, 8–12 mm long, free part 1.5–4 mm long, glabrous; anthers 0.6–0.8 x 0.3–0.5 mm, glabrous. Disc absent. Ovary 3–5 mm long, stipe indistinct, sericeous; ovules 3–8; style 6–9 mm long, glabrous or sometimes sericeous for up to 2/3. Pods elliptic to more or less discoid or obovoid, 30–55 x 20–37 mm, with few hairs especially basally and on the beak, upper wing only, 1–5 mm wide; seed chamber absent. Seeds 1–3 per fruit, flat, bean-shaped, 13–23 x 12–21 x 2–5 mm; hilum central, ca. 1 mm long.

**Distribution:**

The most widespread species of the genus *Derris*, occurring along southern and eastern coast of Africa, Madagascar, Reunion, Seychelles, India and Sri Lanka to China and the Ryukyus, southward to Southeast Asia, Thailand and throughout Malesia up toward Australia and the west Pacific.



**Habitat:**

Primary and secondary forests along coasts, on beaches, in mangrove, along dikes, river banks, and brackish swamps, up to 400 m.(Y. Sirichamorn et al., 2012).

**Pharmacological activities of *Derris Spp.***

*Derris* is locally known as a bio-insecticidal plant. *Derris* plants are evergreen, woody climber that are distributed in the wild, subtropical regions of Asia. They had been grown and used as natural pesticides for more than 100 years. Their roots, in powdered form, were once used for fishing. These *Derris* plants were also used as ectoparasiticides. There have been many reports on the *Derris* extract because of its abundance of flavonoids. Its root possesses main active compound, rotenone, which is reported to be a strong pesticide (Margarita Stoytcheva, 2011). To obtain higher yield of rotenone, the plant requires a calm region with less acidic soil, at least 75% moisture content in soil and 25-30 ºC surrounding temperature (S. I. Zubairi, M. R. Sarmidi, & R.A. Aziz, 2014b). In Malaysia, *D. elliptica* plants are grown plentifully in palm oil and rubber plantations. *D. elliptica* contains a strong knockdown ability to the pest, particularly for Homoptera, such as Diamondback moth and *Plutella xylostella* Linn. (Suraphon Visetson & Manthana Milne, 2001). *D. elliptica* (Tuba Kapur) and *D. malaccensis* (Tuba Gading) are generally located in the Peninsular of Malaysia. *D. elliptica* is easily found in laterite or clay soil, whereas *D. malaccensis* is found in peat soil. These two species are significantly different regarding the amount of fine and coarse roots collected in the pre-processing treatment. *D. malaccensis* provides greater amount of fine and coarse roots, as well as higher yield of rotenone, compared to *D. elliptica* (S. I. Zubairi, M. R. Sarmidi, & R.A. Aziz, 2014a).



*Derris* plants received much attention from phytochemical viewpoint because of their plentiful production of flavonoids. There are many reports about the insecticidal of *Derris* extracts (Table1).

**Table 1** Insecticidal activities of extracts from *derris* plants

| Source | Extract | Biological activity | References |
|---|---|---|---|
| *D. elliptica* | Ethanolic extract with Soxhlet extraction | Insecticidal activity ($3^{rd}$ instar larvae of *Plutella xylostella*, $LD_{50}$= 24.25 ppm) | (Visetson & Milne, 2001) |
| *D. elliptica* | Ethanolic extract | Insecticidal activity ($3^{rd}$- early $4^{th}$ instar larvae of *Aedes aegypti,* $LD_{50}$= 20.49 mg/l) | (Komalamisra, Trongtokit, Rongsriyam, & Apiwathnasorn, 2005) |
| *D. elliptica* | Water extract | Larvicidal activity (mosquito larvae, $LD_{50}$= 5.6 g/l) | (Sangmaneedet, Kanistanon, Papirom, & Tessiri, 2005) |
| *D. elliptica* | Petroleum ether extract | Insecticidal activity ($3^{rd}$- early $4^{th}$ instar larvae of *Aedes aegypti,* $LC_{50}$= 11.17 mg/l)<br>Insecticidal activity ($3^{rd}$- early $4^{th}$ instar larvae of *Culex quinquefasciatus* $LC_{50}$= 4.61 mg/l)<br>Insecticidal activity ($3^{rd}$- early $4^{th}$ instar larvae of *Anopheles* | (Komalamisra et al., 2005) |

| Source | Extract | Biological activity | References |
|---|---|---|---|
| | | *dirus,* $LC_{50}$= 8.07 mg/l)<br>Insecticidal activity (3rd- early 4th instar larvae of *Mansonia uniformis,* $LC_{50}$= 18.84 mg/l) | |
| *D. elliptica* | Methanolic ether extract | Insecticidal activity (3rd- early 4th instar larvae of *Aedes aegypti,* $LC_{50}$= 13.17 mg/l)<br>Insecticidal activity (3rd- early 4th instar larvae of *Culex quinquefasciatus* $LC_{50}$= 18.53 mg/l)<br>Insecticidal activity (3rd- early 4th instar larvae of *Anopheles dirus,* $LC_{50}$= 16.17 mg/l)<br>Insecticidal activity (3rd- early 4th instar larvae of *Mansonia uniformis,* $LC_{50}$= 45.16 mg/l) | (Komalamisra et al., 2005) |

*D. elliptica* fresh and dried powders were demonstrated for the potential in the treatment of cutaneous myiasis in pigs. The findings revealed that both forms of powder had high effectiveness in killing fly larvae. The dried powder showed greater cumulative death percentages of larvae (38%, 70%, 80% and 88% after 3, 6, 9 and 12 hours of exposure, respectively), whereas the fresh powder displayed lower percentages of 22%, 48%, 64% and 86% at the same exposure intervals. The dried powder had a potential to treat cutaneous myiasis in pig *in vivo*. After examined with the dried powder, all tested pigs were fed with normal food and stayed in normal condition without any side effect. Within 24 hours after the exposure of larvae and dried powder, the larvae were killed and the inflammation resulted from larval migration was recovered by seven days (Sangmaneedet et al., 2005).

Apart from insecticidal activities, some *Derris* plant extracts showed antibacterial potential. The ethyl acetate portion of *D. malaccensis* methanolic extract displayed potent inhibitory effect against *Helicobacter pylori*, with the $IC_{50}$ value less than 1 µg/ml (Takashima, Chiba, Yoneda, & Ohsaki, 2002).

Leaves and stems of *D. elliptica*; leaves, roots, barks and heart woods of *D. indica*; and leaves and roots of *D. trifoliata* were studied for antibacterial effect against twenty-five pathogens. All samples were serially extracted with petrol (60-80 °C), dichloromethane, ethyl acetate, butanol and methanol. *D. indica* methanolic fractions of leaves and heart wood showed strong inhibition, as well as the petrol, butanolic and methanolic fractions of *D. indica* root bark. The petrol and ethyl acetate fractions of *D. trifoliate* leaves and roots were also exhibited significant inhibitory. *D. elliptica* (Lotin or Hang lai daeng) and *D. malaccensis* (Hang lai khao) are commonly used to kill insects in China and South East Asia. Both of them have toxic compounds such as rotenone and its derivatives (sumatrol, deguelin, and toxicarol) (Fugami & Nagajima, 1971).

For dusting objectives, the commercially powdered *Derris* roots are grounded and diluted with a carrier, such as talc or clay, to 1% concentration. To get a homogeneous form, the coated dust is made by mixing the root liquid extract with the carrier, and allowing it to dry. For spraying objectives, although the root powder can be dissolved with water, organic solvents (i.e. ethylene dichloride, trichloroethylene, or chlorobenzene) can dissolve the powdered sample better. The rotenone-containing extract in oil (or paraffin oil) and emulsifier with an addition of perfume are a good combination for household- and cattle-used sprays. Rotenone, presented in sprays and aerosol with pyrethrins, helps stimulate the knockdown (Tyler, Brady, & Robbers, 1976).

The *Derris* raw root, when solely diluted with water or extracted with alcohol following with soap water dilution, can be used as a pesticide. However, the insecticidal potency of *Derris* active compound may be lost if contacted with water or basic solution for too long. The half-life of rotenone in soil and water conditions is around 1-3 days; otherwise, it is quickly broken down. Fortunately, rotenone neither leaches out of soil nor causes water pollutant. Besides, rotenone is sensitive to light and air, allowing its biological activity to last only for a short time. Rotenone, as well as other toxic compounds, is gradually decomposed when faced with sunlight – 5-6 days for spring sunlight and 2-3 days for summer sunlight. Rotenone is also heat- or high-temperature-sensitive (Gupta, 2018; Matsumura, 1985; Ray, 1991)

Photo-degradation of rotenone in various solvents and in solid form was observed. The main photoproducts produced after exposed to sunlight were evaluated. A few photochemical reactions were occurred due to the irradiation of rotenone towards oxygen, generating the oxidized derivatives (Cheng, Yamamoto, & Casida, 1972). Chemical constituents of some *Derris* species were show in Table2.

**Table 2** chemical constituents from some *Derris* species

| Species | Chemical constituent | Reference |
|---|---|---|
| *Derris amoena* Benth. (Seed) | 3,4-Dihydroxy-2,5-bis(hydroxymethyl)pyrrolidine | (S. V. Evans, Fellows, Shing, & Fleet, 1985) |
| *Derris elliptica* (Wall.) Benth. (Root) | Benzofuranoids<br>Tubaci acid; *(R)-form*<br>Benzopyranoids<br>ß-Tubaci acid<br>Flavonoids<br>Deguelin<br>Elliptinol<br>Rotenone<br>Tephrosin<br>Deguelin<br>Toxicarol<br>*dl*-toxicarol<br>*l*-elliptone<br>Maackiain (+)<br>Maackiain (-)<br>Oxygen heterocycle<br>Tubaic acid<br>Tubaic acid, β | (Ahmed, Nelson, & Gibson, 1989; Crombie & Thomas, 1965; Gaudin & Vacherat, 1938; Jones & Graham, 1938; Obara & Matubara, 1981; R.P. Rastogi et al., 1990; Toxopeus, 1952; Welter, Jadot, Dardenne, Marlier, & Casimir, 1976) |
| *Derris elliptica* (Wall.) Benth. (Leaf) | Amino acids and peptides<br>4,5-Dihydroxy-2-piperidinecarboxylic acid;(*2S,4R,5S*)-form | (Chen & Tsai, 1955; S. V. Evans et al., 1985; Marlier, |

| Species | Chemical constituent | Reference |
|---|---|---|
| | $\gamma$-Hydroxyhomoarginine<br>Pipecolic acid tubaic<br>β-tubaic acids<br>Piperidine, 2-(S)-carboxy-4-(R)-5-(S)-dihydroxy<br>Piperidine, 2-(S)-carboxy-4-(S)-5-(S)-dihydroxy<br>Alkaloid<br>Pyrrolidine, 3-4-dihydroxy-2-5-dihydroxy-methyl<br>Flavonoid<br>Rotenone | Dardenne, & Casimir, 1976; R. P. Rastogi & Mehrotra, 1993; Welter et al., 1976) |
| *Derris elliptica* (Wall.) Benth. (Seed) | Alkaloids<br>3,4-Dihydroxy-2,5-bis(hydroxymethyl)pyrrolidine;(2R,3R,4R,5R) -form<br>3,4-Dihydroxy-2,5-bis(hydroxymethyl)pyrrolidine<br>3,4-Dihydroxy-2,5-bis(hydroxymethyl)pyrrolidine;(2S,4R,5S)-form<br>3,4-Dihydroxy-2,5-bis(hydroxymethyl)pyrrolidine;(2S,4S,5S)-form | (Welter et al., 1976) |
| *Derris elliptica* (Wall.) Benth. (Stem) | Flavonoid<br>Rotenone | (Chen & Tsai, 1955) |
| *Derris malaccensis* Prain (Seed) | Amino acids and peptides<br>$\gamma$-Hydroxyhomoarginine | (S. V. Evans et al., 1985) |

| Species | Chemical constituent | Reference |
|---|---|---|
| | Alkaloids<br>3,4-Dihydroxy-2,5-bis(hydroxymethyl)pyrrolidine | |
| *Derris malaccensis* Prain (Root) | Flavonoid<br>Rotenone | (Toxopeus, 1952) |
| *Derris indica* (Lam.) Bennet (Stem) | Flavone<br>Fisetin tetramethyl ether<br>Luteolin<br>Methylenedioxy<br>Demethoxykangin<br>Kanugin<br>Furanoflavone<br>Karanjin<br>Pachycarin D<br>Millettocalyxin C<br>Pongapin 33<br>2´-methpxy-4´,5´-methylenedioxyfurano[7,8:4",5"]-flavone 11<br>3´-methoxypongapin<br>Pongamone A<br>Pongamone E<br>Pongamone D<br>Furanoflavanone<br>Chromenoflavaone<br>Isopongaflavone/candidin<br>Pongachromene<br>Isolonchocarpin<br>Pongachin | (Carcache-Blanco et al., 2003; Kitagawa et al., 1992; Koysomboon, van Altena, Kato, & Chantrapromma, 2006; L. Li et al., 2006; Minakawa et al., 2010; Pathak, Saini, & Khanna, 1983; Saha, Mallik, & Mallik, 1991; Talapatra, Mallik, & Talapatra, 1982; Tanaka, Iinuma, Yuki, Fujii, & |

| Species | Chemical constituent | Reference |
|---|---|---|
| | Pterocarpan<br>Maackiain<br>Medicarpin<br>Chromenochalcone<br>Obovatachalcone<br>Glabrachromene<br>Praecansone B<br>Pnogapinone A<br>7-methoxypraecansone B<br>Enolchalcone<br>Tunicatachalcone | Mizuno, 1992; H. Yin, Zhang, & Wu, 2004; Hao Yin, Zhang, Wu, & Nan, 2006) |
| *Derris indica* (Lam.) Bennet (Root) | Flavone<br>3,7-dimethoxyflavone<br>7-O-methyl chrysin<br>7,4´-dimethoxy-5-hydroxyflavone<br>Methylenedioxy flavone<br>3,4-methylenedioxy-10-methoxy-7-oxo[2]benzopyrano[4,3-b]benzopyran<br>Furanoflavone<br>Lanceolatin B<br>3,3´,4´-trihydroxy-4H-furo[2,3-h] chromen-4-one<br>Ponganone XI<br>Ovalifolin<br>Chromenoflavaone<br>Ponganone I<br>Ponganone II<br>Ponganone III | (Koysomboon et al., 2006; Minakawa et al., 2010; Ranga Rao et al., 2009; Saha et al., 1991; Tanaka et al., 1992) |

| Species | Chemical constituent | Reference |
|---|---|---|
| | Ponganone IV<br>Ponganone V<br>Ponganone VI<br>Ponganone VIII<br>Flavanone<br>Furanochalcone<br>Milletenone<br>Ponganone VII<br>Ponganone IX<br>Dihydromilletenone methyl ether<br>Pongamol<br>Ovalitenin B | |
| *Derris indica* (Lam.) Bennet (Leaf) | Flavonol<br>Kaempferol<br>Quercetin<br>Flavonoid glycoside<br>Vitexin<br>Isoquercitrin<br>Kaempferol 3-O-β-D-glucopyranoside<br>Kaempferol 3-O-β-D-rutinoside<br>Rutin<br>Vicenin-2 4′-O-methyl-genistein7-O-β-D-rutinoside<br>Rotenoid<br>Chalcone<br>Pongagallone A<br>Pongagallone B | (Gandhidasan, Neelakantan, Raman, & Devaraj, 1986; Marzouk, Ibrahim, El-Gindi, & Abou Bakr, 2008) |

| Species | Chemical constituent | Reference |
|---|---|---|
| *Derris indica* (Lam.) Bennet (Fruit) | Furanoflavone<br>3´-methoxyfuro[8,7:4",5"] flavone<br>Pongol<br>Pongapinnol A<br>Pongapinnol B<br>Pongapinnol C<br>Pongapinnol D<br>Flavonoid glycoside<br>Pongamoside A<br>Pongamoside B<br>Pongamoside C<br>Pongamoside D<br>Coumestan<br>Pongacoumestan<br>Furanoflavanone | (Ahmad, Yadav, & Maurya, 2004) |
| *Derris indica* (Lam.) Bennet (Flower) | Furanoflavone<br>Pongaglabol methyl ether 32<br>Pongaglabol 21<br>Isopongaglabol<br>Kanjone<br>Pinnatin<br>Glabone<br>6-methoxyisopongaglabol<br>6-methoxyisopongaglabol methylether<br>Chromenoflavaone<br>Ovalichromene B | (Talapatra, Mallik, & Talapatra, 1980; Talapatra et al., 1982) |
| *Derris indica* (Lam.) Bennet (Seed) | Furanoflavone<br>Pongaglabrone | (Koysomboon et al., 2006; |

| Species | Chemical constituent | Reference |
|---|---|---|
| | Pongapin 26 27 33 34<br>Chromenoflavone<br>Pongaflavone 11 37<br>Isopongachromene<br>Chromenochalcone<br>Obovatachalcone<br>Glabrachalcone<br>Glabrachalcone II | Minakawa et al., 2010; Pathak et al., 1983; Rashid, Abbasi, Tahir, Yusof, & Yamin, 2008; Saha et al., 1991; Tanaka et al., 1992) |
| *Derris reticulata* Craib<br>(Stem) | Flavanones<br>Lupinifolin<br>Epoxylupinifolin<br>Dereticulatin<br>Hydroxyl epoxylupinifolin<br>Flavonoids<br>Dihydroxy-hydroxymethyl-dimethylpyrano<br>Flavanone<br>Dihydroxylupinifolin<br>Lupiwighteone | (Prawat, Mahidol, & Ruchirawat, 2000; Songsak, 1995) |
| *Derris scandens* (Roxb.)<br>Benth.<br>(Root) | Benzopyranoids<br>Lonchocarpenin<br>Lonchocarpic acid<br>Robustic acid<br>Scandenin<br>Flavonoids<br>Chandalone<br>Lonchocarpenin | (Clark, 1943; Falshaw et al., 1969; Hussain et al., 2015; Johnson, Pelter, & Stainton, 1966; |

| Species | Chemical constituent | Reference |
|---|---|---|
| | Lonchocarpic acid<br>Osajin<br>Robustic acid<br>Scandenin<br>Scandenone<br>Scandinone<br>Flavone<br>Laxifolin<br>Isoflavone<br>Chandalone<br>Lupalbigenin<br>Isoflavone glycoside<br>Sphaerobioside<br>Triterpene<br>Betulinic acid<br>Lupeol | Laupattarakasem, Houghton, & Hoult, 2004; M. N. Rao, Krupadanam, & Srimannarayana, 1994; Sengupta, Das, & Saha, 1971; Sreelatha et al., 2010) |
| *Derris scandens* (Roxb.) Benth.<br>(Stem) | Terpenoids<br>Lupeol<br>Taraxerol<br>Steroids<br>ß-Sitosterol<br>Scandenin B<br>Isoflavone<br>Derrisisoflavone A<br>Derrisisoflavone B<br>Derrisisoflavone C<br>Derrisisoflavone D<br>Derrisisoflavone E | (Hussain et al., 2015; Laupattarakasem et al., 2004; Mahabusarakam, Deachathai, Phongpaichit, Jansakul, & Taylor, 2004; Pelter & Stainton, |

| Species | Chemical constituent | Reference |
|---|---|---|
| | Derrisisoflavone F<br>Erysenegalensein E<br>Eturunagarone<br>Genistein<br>Isochandalone<br>Isorobustone<br>Lupalbigenin<br>Lupinisoflavone G<br>Lupinisol A<br>Lupiwighteone<br>3′-Methylorobol<br>Santal<br>Scandenal<br>Warangalone<br>Scanderone<br>Scandinone<br>Ulexone A<br>5,7,4′-Trihydroxy-6,8-diprenylisoflavone<br>Isoflavone glycoside<br>Derriscandenoside A<br>Derriscandenoside B<br>Derriscandenoside C<br>Derriscandenoside D<br>Derriscandenoside E<br>Derriscanoside A<br>Derriscanoside B<br>Pterocarpan<br>Flemichapparin B | 1966; M. N. Rao et al., 1994; S. A. Rao et al., 2007; Rukachaisirikul, Sukpondma, Jansakul, & Taylor, 2002; Sekine, Inagaki, Ikegami, Fujii, & Ruangrungsi, 1999; Sengupta et al., 1971) |

| Species | Chemical constituent | Reference |
|---|---|---|
| | Flemichapparin C<br>Maackiain<br>Steroid<br>β-Sitosterol<br>β-Sitosterol glucopyranoside<br>β-Amyran-3-one<br>β-Amyrin | |
| *Derris scandens* (Roxb.) Benth.<br>(Whole plant) | Scandenin<br>Scandenin A<br>Isoflavone<br>Isoscandinone<br>4′-O-Methylosajin<br>4′-O-Methylscandinone<br>Osajin | (S. A. Rao et al., 2007) |
| *Derris scandens* (Roxb.) Benth.<br>(Seed) | Aminoacids and peptides<br>4-Hydroxyarginine<br>γ-Hydroxyhomoarginine<br>Alkaloids<br>2-Aminoimidazole | (Fellows, Polhill, & Arthur Bell, 1978) |
| *Derris trifoliata* Lour.<br>(Root) | Steroids<br>Campesterol<br>Cholesterol<br>beta-Sitosterol<br>Stigmast-7-en-3-ol<br>Stigmasterol | (Bose, Kirtaniya, & Adityachaudhury, 1976; Nair & Gunasegaran, 1982) |
| *Derris trifoliata* Lour.<br>(Leaf) | Flavonoids | (Swapan & Robert, 1985) |

| Species | Chemical constituent | Reference |
|---|---|---|
| | Dehydrorotenone; (R)-form<br>Quercetin<br>Rhamnetin<br>Terpenoids<br>alpha-Amyrin<br>beta-Amyrin<br>Lupeol | |
| *Derris trifoliata* Lour.<br>(Seed) | Amino acids and peptides<br>Homoarginine; (S)-form<br>4-Hydroxyarginine<br>Alkaloids<br>3,4-Dihydroxy-2,5-bis(hydroxymethyl)pyrrolidine | (S. V. Evans et al., 1985) |

**Table 3** Pharmacological activities of some *Derris* species

| Species | Pharmacological activity | Reference |
|---|---|---|
| *Derris amoena* Benth. | Cytotoxic activity | (Chiayvareesajja, Rittibhonbhun, Hongpromyart, & Wiriyachitra, 1997) |
| *Derris elliptica* (Wall.) Benth. | Antimicrobial<br>Insecticidal activity | (Hu et al., 2005; Khan, Omoloso, & Barewai, 2006) |
| *Derris malaccensis* Prain | Antioxidant<br>Antimicrobial<br>Anti-Vius<br>Cytotoxic activity | (Chokchaichamnankit et al., 2011; Harper, 1940; Wangteeraprasert & Likhitwitayawuid, 2009) |

| Species | Pharmacological activity | Reference |
|---|---|---|
| *Derris indica* (Lam.) Bennet | Antifungal<br>Antimicrobial<br>Anti-inflammatory<br>Antioxidant<br>Anti hyperglycemic<br>Antidiabetic | (Al Muqarrabun, Ahmat, Ruzaina, Ismail, & Sahidin, 2013; Mondal, Haque, Haque, & Khan, 2012) |
| *Derris reticulata* Craib | Antioxidant<br>Anti-inflammatory<br>Anti-Diabetic | (Kumkrai, Kamonwannasit, & Chudapongse, 2014; Kumkrai, Weeranantanapan, & Chudapongse, 2015; Vongnam, Wittayalertpanya, Raungrungsi, & Limpanasithikul, 2013) |
| *Derris scandens* (Roxb.) Benth. | Anti-inflammatory<br>Anti-migration<br>Anti-microbial<br>Antioxidant<br>Anti-dermatophyte activity Anti-fungal activity<br>Cytotoxicity against<br>Hypertensive activity | (Hussain et al., 2015; Jansakul, Srichanbarn, & Saelee, 1997; Laupattarakasem et al., 2004; Mahabusarakam et al., 2004; Metwally, Omar, Harraz, & El Sohafy, 2010; S. A. Rao et al., 2007; Sekine et al., 1999; Tedasen, Sukrong, Sritularak, Srisawat, & Graidist, 2016) |
| *Derris trifoliata* Lour. | Antimicrobial activity | (Khan et al., 2006) |

**Rotenone**

Rotenone or (Figure 1) is a member of a class of compounds of related molecular structure referred to as isoflavones. The chemical description is shown in Table 4.

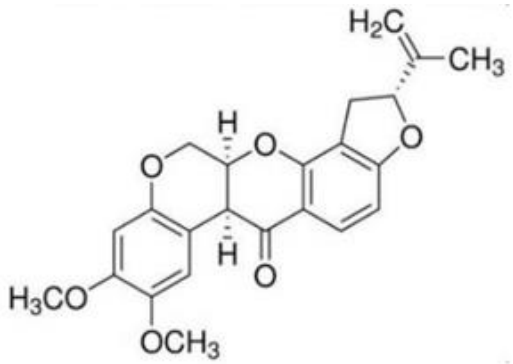



**Figure 1** Chemical structure of rotenone

**Table 4** Chemical description of rotenone

| | |
|---|---|
| **Chemical name** | Rotenone |
| **Molecular formula** | $C_{23}H_{22}O_6$ |
| **IUPAC name** | (2R,6aS,12aS)-2-Isopropenyl-8,9-dimethoxy-1,2,12,12a-tetrahydrochromeno[3,4-b]furo[2,3-h]chromen-6(6aH)-one |
| **Molecular weight** | 394.4 g/mol |
| **CAS number** | 83-79-4 |
| **Synonym** | Nicouline, Dactinol, Tubatoxin |
| **Appearance** | Colorless |

**Pharmacological activities of rotenone**

Rotenone is considered as one of the oldest natural insecticide. It has been used for several decades till today. It is a main active compound of *Derris*, *Lonchocarpus* and *Tephrosia* species. The dried products of *D. elliptica* and *D. malaccensis* (also called *derris* or trub) are economic products of Malaya and the East Indies; *L. utilis* and *L. urucu* (timbo or cube) are from South America; and *Tephrosia* species are from East Africa.

Rotenone, when extracted with solvent (e.g., chloroform), is usually in the roots, resins or crystals. Amount of rotenoids in the commercial extracts are varied depending on the areas of plants grown (Mutsumura, 1975; O'Brien, 1967).

The colorless, crystalline rotenone ($C_{23}H_{22}O_6$) is an isoflavonoid with the molar mass of 394.42 and the melting point of 165 °C. It is less soluble in water (15 mg/l at 100°C), but highly soluble in organic solvents and oils (Ray, 1991). It is easily oxidized by light, becoming yellow, orange to deep red.

Rotenone is a non-systemic insecticide to remove aphids, maggots, bagworms, codling moths, Japanese beetles, leaf hoppers, flea beetles and vegetable weevils for fruit and vegetable (Ray, 1991; Tyler et al., 1976; Ujváry, 2001). In household, it is used to control insects in the garden and on pets, as well as eliminate fish for water management. As a veterinary medicine, the powdered form rotenone is used to control parasitic mites on poultry, and lice ticks on dogs, cats and horses (Gupta, 2018).

Rotenone potentially decreased the survival of hatching affected female *Tetranychus urticae* by 85.14% mortality. The rate of ovipostion also declined after treating with rotenone. The hatching of *Phytoseiulus persimilis* eggs and the survival of affected female were reduced by 100% mortality (Duso et al., 2008).

Using the larval development assays, rotenone displayed anthelmintic activity to larvae of *Haemonchus contortus* ($LC_{50}$ = 0.54 µg/ml) and *Trichostrongylus colubriformis* ($LC_{50}$ = 0.64 µg/ml). The complete movement cessation of adult *H. contortus* after 72 hours was due to rotenone at 20 µg/ml. The toxicity of rotenone on the larvae of both plants was raised in the presence of piperonyl butoxide. This significant synergism revealed that theses nematode species could apply a cytochrome P450 enzyme system to detoxify rotenone and implied that a role might exist for cytochrome P450 inhibitors to act as synergists for other anthelmintics that were susceptible to oxidative metabolism within the nematode (Kotze, Dobson, & Chandler, 2006).

The acute toxicity of rotenone towards insects, fish and animals are involved with the NADH inhibition: ubiquinaone oxidoreductase (in the electron transport chain) as the primary target, terminating the transport of electrons to $O_2$ and hence inhibiting the formation of adenosine triphosphate (ATP), which is fatal for the living things. Poisoning insects include four steps: (1) inactive, movement instability, and refusal to eat; (2) knockdown; (3) paralysis and (4) death (Ujváry, 2001).

Rotenone is more active than other main constituents in *Derris* resins. For example, deguelin and toxicarol are different from rotenone because of the presence of pyran E-ring instead of dihydrofuran bearing an unsaturated side chain.



Rotenone and rotenoids have been reported for their anticancer activity in rats and mice. Dietary rotenone can reduce the background incidence of live tumors in mice and mammary tumors in rats. Rotenone can prevent cell proliferation triggered by peroxisome proliferator in mouse. Deguelin and its derivatives can restrain phorbol ester-induced ornithine decarboxylase (ODC) activity. The research found that NADH inhibitory: ubiquinone oxidoreductase activity can reduce the level of induced ODC activity, resulting in antiproliferative and anticancer effects (Fang & Casida, 1998).

Rotenoids, rotenone, rotenolone, dehydrorotenone, deguelin, tephrosin, toxicarol, dehydrodeguelin and elliptone, isolated from *D. malaccensis,* exhibited antibacterial action against *H. pylori* with the MIC values ranging from 0.3 to 9.8 mg/ml (Takashima et al., 2002).

Decades ago, rotenone was introduced to be used instead of lead- or arsenic-mediated pesticides because of its non-toxicity. Nevertheless, rotenone has been recorded as a moderately hazardous Class II pesticide by WHO. Several researches reported that acute oral toxicity of rotenone was moderate for mammals, but there was variation between species as shown in Table 5 (O'Brien, 1967). Orally, the $LD_{50}$ values of rotenone were varied from 132-1500 mg/kg in rats, due to the use of various plant extracts. Rotenone was reported to be more toxic to female rats than male rats. Rotenone, treated in mice, with $LD_{50}$ value of 350 mg/kg, could cause strong irritation to the skin rabbits (World Health Organization & International Programme on Chemical Safety, 1992).

**Table 5** Toxicity of rotenone

| **Organism** | **Route** | **$LD_{50}$ (mg/kg)** |
|---|---|---|
| Rat | Oral | 132 |
| Guinea pig | Oral | 200 |
| | Intraperitoneal | 15 |
| Chicken | Oral | 996 |
| Honeybees | Oral | 3 |
| Milkweed bug | Topical | 25 |
| American cockroach | Topical | 2000 |
| | Oral | 1000 |
| | Injected | 5 |
| Japanese beetle | Topical | 25 |
| | Injected | 40 |

Rotenone is thought to be fairly toxic to humans with the oral $LD_{50}$ ranging from 300-500 mg/kg. It causes irritation and sudden vomit, but hardly causes death. Rotenone is more toxic when inhaled than consumed. The degree of toxicity depends on the average particle size of powdered rotenone. The smaller-sized particles easily get into deeper area in human lung. On the other hand, the rate of absorption of rotenone in stomach and intestine is relatively slow, despite the acceleration by fats and oils.

Rotenone undergoes metabolism in the liver by nicotinamide adenine dinucleotide phosphate (NADP)-linked hepatic microsomal enzyme. The products obtained from the metabolism of rotenone are rotenoids (e.g., rotenolone I and II, hydroxyl and dihydroxyrotenones). The studies from animal suggest that other possible metabolites are carbon dioxide and a more water-soluble substance that can be excreted in the urine. According to the studies on rats and mice, around 20% of the applied oral concentration is excreted through urine within 24 h. The remaining rotenone that cannot be absorbed in the GI tract is resulted in the form of feces (Gupta, 2018).



Rotenone is a general use pesticide (GUP) for cranberries, but a limited use for fish control. It is either EPA toxicity Class I or III (highly toxic or slightly toxic), depending on the formulation. Rotenone is labelled “DANGERED” in emulsified-concentrated formulation because of its strong toxicity. For other forms of rotenone, it is labelled “CAUTION” (Extension Toxicology Network, 1996).

**Physico-chemical indication for quality control for herbal material**

Herbal material quality control methods are described in WHO guideline (World Health Organization, 2011).

**Extractable matters**

Extractable matters represent the active compounds obtained from the extraction of plant materials. The solvents commonly used are ethanol (for less polar compounds) and water (for more polar compounds).

**Water content**

The water content of medicinal plant materials can be evaluated by azeotropic distillation. The plant materials are distilled with water-immiscible solvent, such as toluene or xylene. Such solvent must be saturated with water before use to prevent error because organic solvent can absorb water gradually.

**Loss on drying**

Loss on drying is a gravimetric method for water determination. The plant materials are heated at 100-105 ℃ and cooled down in desiccator. However, any volatiles matters are also losing.

**Total ash and acid insoluble ash**

Ash value reveals the non-volatile inorganic matters or minerals in plant materials. After complete incineration (500 °C) of plant material, the amount of total ash is measured. The ash, which is not be solubilized in HCl (70 g/l) are acid-insoluble ash. The example of this type of minerals is silica usually found as silicic acid in plant cell wall.

**TLC image analysis and TLC-densitometry**

Thin-layer chromatography (TLC)

TLC is particularly valuable for both quantitative and qualitative analyses. This technique is extensively used as a basic screening tool to analyze many different substances, because it is quite simple, efficient, and economical method for identification and separation of chemical compounds. TLC technique is commonly applied in pharmaceutical industry for purification of herbal medicines. The process

includes three fundamental steps: sample application, chromatogram development and visualization.

TLC comprises of two phases: stationary and mobile phases. Silica gel and alumina are two most widely used types of stationary phase, due to their adsorption property. Good mobile phase normally has low boiling point, low viscosity, less toxicity, non-reactive with the analytical sample and inexpensive. The sample on TLC plate can be detected under UV-visible light (254 and 365 nm). TLC staining reagents can be used to improve the detectability of the sample by changing non-UV absorbing substances into detectable derivatives.

**TLC- densitometry**

TLC-densitometry is a technique used to identify organic compounds by which the chemical substances are separated by TLC and further measured the absorbance by densitometry (Stroka, Spangenberg, & Anklam, 2002). TLC-densitometer works in combination with digital software to quantitate the amount of interested compound in plant materials in reference to the standard compound. TLC-densitometer provides three light sources: deuterium lamp (190-450 nm) for UV region, halogen-tungsten lamp (350-900 nm) for visible region, and high pressure mercury lamp (254-578 nm) for fluorescence. Therefore, the densitometer can detect any compounds according to their physical properties in absorbance or fluorescence ranging from 190-900 nm (CAMAG, 2020).



**TLC-image analysis**

TLC-image analysis works in combination with a free software ImageJ developed by the National Institutes of Health, USA. The program manually measures the pixel intensity of digital image of developed TLC plate, and transforms to chromatographic peak (The National Institutes of Health, 2018).

**Biological activities evaluation**

**Anti-oxidant activities**

DPPH radical scavenging assay

2, 2 Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay examines the potency to scavenge DPPH free radical of antioxidants. DPPH is a stable free radical with a purple color that has a maximum wavelength of 517 nm. When received electron form the antioxidant compound, DPPH changes to a non-radical form (1,1-diphenyl-2-picryhydrazine; DPPHH), turning its purple color to yellow color (Figure 2) (Brand, Cuvelier, & Berset, 1995; Prior, Wu, & Schaich, 2005).

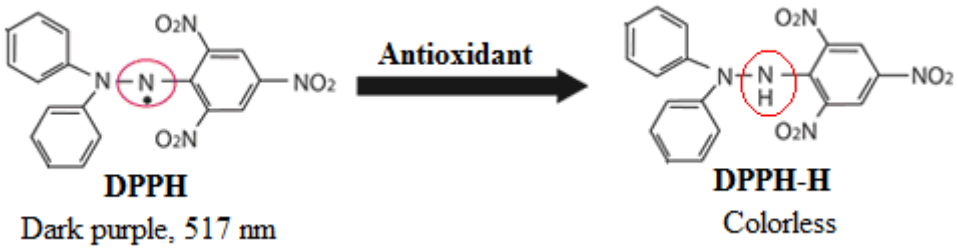



**Figure 2** DPPH and antioxidant reaction

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) assay evaluates the capability of antioxidants to reduce ferric ion ($Fe^{3+}$) to ferrous ion ($Fe^{2+}$) in the complex compound. Ferric tripyridyltriazine ($Fe^{3+}$-TPTZ) is used as the tested compound to undergo redox reaction, and then reduced by antioxidant to ferrous complex ($Fe^{2+}$-TPTZ) with purple-bluish color at the maximum absorbance of 593 nm (Benzie & Strain, 1996).

*ß*-carotene bleaching assay

*ß*-Carotene bleaching assay determines the ability of antioxidant compound to inhibit lipid peroxidation. The color bleaching of *ß*-carotene occurs when exposed to oxidizing species or free radicals. The rate of bleaching is reduced in the presence of antioxidant, due to the competition between *ß*-carotene and antioxidant with the subjected radicals (Ueno, Chiba, Matsumoto, Nakagawa, & Miyanaka, 2014).

Total phenolic and flavonoid content assays

To determine total phenolic and flavonoid contents of plant materials, colorimetric assay is world-widely performed in the UV-VIS spectrophotometric technique. The determination of total phenolic content uses Folin-Ciocalteu's reagent (phosphotungstic-phosphomolybdic reagent) to form a reduced blue complex of phosphotungstic-phosphomolybdenum that can be measured by visible-light spectrophotometry (Schofield, Mbugua, & Pell, 2001). For total flavonoid content, the aluminium chloride colorimetric assay is commonly used. The CO and OH groups in flavonoid reacts with aluminium metal ion ($Al^{3+}$) and form metal complex that can be detected and quantitated by visible-light spectrophotometry (Chang, Yang, Wen, & Chern, 2002; Mabry, Markham, & Thomas, 1970).

*In vitro* $\alpha$ -glucosidase inhibitory activity

People with high blood sugar is considered to be diabetes, which can be divided into two types. Type 1 diabetes (insulin-dependent/juvenile-onset diabetes) is the condition of producing less or no insulin. It is an autoimmune disorder where the immune system incorrectly attacked the pancreatic cells that are responsible for insulin production. Type 1 diabetes usually occurs in children. Type 2 diabetes (non-insulin dependent/adult onset diabetes) occurs mostly in adult, but can occur in children, particularly those with likely-to-be overweight. People with type 2 diabetes normally generate insulin but the body ineffectively responds to it (World Health Organization, 2016).

$\alpha$-Glucosidase and $\alpha$-amylase are considerable enzymes for the evaluation of anti-diabetic potential in plant material (Tadera, Minami, Takamatsu, & Matsuoka, 2006). These enzymes involve in the hydrolysis of polysaccharides to monosaccharides. Firstly, $\alpha$ -amylase in saliva and pancreatic juice hydrolyzes polysaccharides at $\alpha$-1,4-glycosidic bonds to dextrins and disaccharides. Secondly, $\alpha$-

glucosidase in brush border of small intestine breaks down dextrins and disaccharides to monosaccharides, resulting in an increase of blood sugar level. $\alpha$-Glucosidase and $\alpha$-amylase inhibitors lower the rate of carbohydrate hydrolysis in the small intestine, reducing the blood glucose level related to type 2 diabetes (Kazeem, Adamson, & Ogunwande, 2013; Kwon, Apostolidis, & Shetty, 2007).

**Toxicity activities**

MTT tetrazolium reduction assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is used to assess cell proliferation or cytotoxicity effect of tested compound or the extract. MTT is reduced by mitochondrial dehydrogenases in viable cells, changing the yellow MTT to purple MTT formazan crystal that can be detected at 570 nm. Based on the American NCI plant screening program, the crude extract that shows $IC_{50} \leq 20$ µg/ml has cytotoxic activity, while the pure substance that shows the $IC_{50} \leq 4$ µg/ml has cytotoxic activity (Geran, Greenberg, MacDonald, Schumacher, & Abbott, 1972).

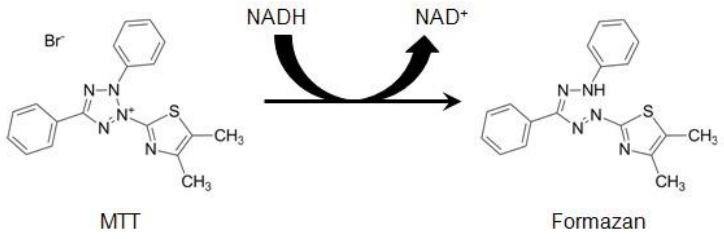



**Figure 3** MTT assay reaction

For the analysis of MTT assay, the result of tested sample is compared to that of the control cell. If the absorbance of tested sample is less than that of the control, it means that the sample is toxic to the cell. On the hand, for the interpretation of cell proliferation effect, the higher absorbance of tested sample indicates greater potential comparing with the control.

Brine shrimp lethality assay

*Artemia salina* or brine shrimp is a sensitive indicator, normally used for primary screening of toxicity test on plant materials. The brine shrimp lethality assay is to examine the ability of the plant materials to kill brine shrimp nauplii. The brine shrimp lethality is expressed as $LC_{50}$ value, the lethal concentration of the tested sample required to kill 50% of the brine shrimp population. The $LC_{50}$ value can be classified into three levels: $LC_{50} >$ 1000 µg/ml is non-toxic; $500 \leq LC_{50} \leq 1000$ µg/ml is weak-toxic; and $LC_{50} < 500$ µg/ml is toxic (Bastos et al., 2009; Vehovszky, Szabó, Acs, Gyori, & Farkas, 2010).

**Characterization of herbal materials**

Morphological characteristics

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

Macroscopic evaluation

Macroscopic evaluation generally plays the first role on the classification of the plants. Macroscopic analysis can be demonstrated by naked eyes or magnifying lens. The gross morphological and organoleptic characteristics can be used to differentiate the plants. Natural variations are commonly due to the environment factors.

Microscopic evaluation

Microscopic analysis is the study about cellular structures and histological characteristics of the plant using microscope.

Transverses section of midrib

In microscopic determination, transverse section of midrib and main veins shows vascular tissues and specifically morphological of cells, such as palisade cells, stomata cells, epidermal cells, etc. Cell type, size or distribution in the midrib transverse section are

characteristics that can be used for plant identification. Also, the transverse section can be taken into consideration for plant contamination and adulteration (Ferguson, 1956).

*Clearing reagents for microscopic examination*

The presence of various content within the cell such as starch gain, plastid and oil etc., may result to non-translucent section and obscure certain characteristics. There are some reagents that can dissolve of these contents and have been used to make an infiltrating effect. Some of most frequently used reagents are sodium hypochlorite and chloral hydrate as described below (Mukherjee, 2007).

*Sodium hypochlorite solution*

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

*Chloral hydrate solution*

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



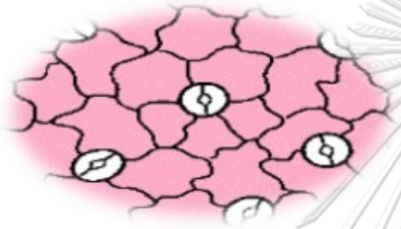
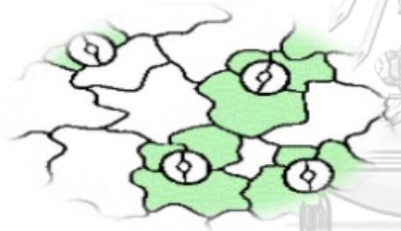
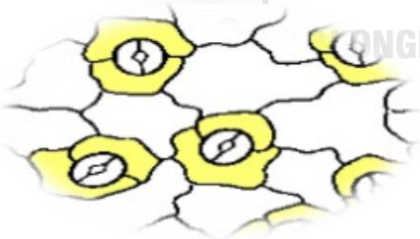
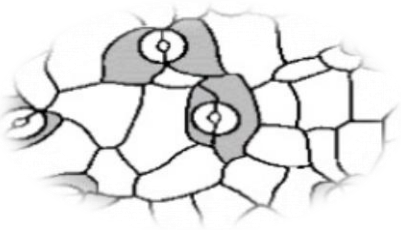
**Leaf measurement**

Leaf measurement is one of the parameters used to distinguish between some closely related species not easily characterized by general microscopy. Microscopic leaf constant values can be measured as stomatal number, stomatal index, vein-islet number, veinlet termination number, epidermal cell number, epidermal cell area and palisade ratio (Kokate, Purohit, & Gokhale, 2004).

Stomata classification

Shape of the epidermis and stomata are the first investigation in the microscopic examination of leaf. In the mature leaves, four significantly different types of stomata are distinguished by their form and the arrangement of the surrounding cells, especially the subsidiary cells. Four types of stomata are demonstrated in Table 6.

**Table 6** Stomata classification

| Surface view | Type of stomata | The arrangement of the surrounding cells |
|---|---|---|
|  | Anomocytic (irregular-celled) | The stoma is bordered by a varying number of cell, normally not different from the epidermis. |
|  | Anisocytic (unequal-celled) | The stoma is typically border by three or four subsidiary cells, one of which is clearly smaller than the other. |
|  | Diacytic (cross-celled) | The stoma is complemented by two subsidiary cells, the common wall of which is at right angles to the stoma. |
|  | Paracytic (parallel-celled) | The stoma has two subsidiary cells, that the long axes are parallel to the axis of the stoma. |

Stomatal number and stomatal index

Stoma is naturally found in both lower and upper epidermis of the leaf and sometimes in other organs such as stem, consisting of the pore and two guard cells. The guard cell is a specialized cell in the epidermis of leaves parenchyma cell which is responsible for controlling the size of the opening for gas exchange. The stomatal numbers are varied according to the environment and geographic sources, such as humidity, light intensity, temperature and concentration of carbon dioxide gas (Grant, 2004; Martin & Glover, 2007). In leaves, the stomatal patterning distribution is highly variable among species (Casson & Gray, 2008). The stomatal number means the average number of stomata in 1 $mm^2$ area, while the stomatal index is a ratio of the stomatal number to the sum of the epidermal cell number and that stomatal number.

Vein-islet number

Vein-islet is the small areas of green tissue outlined by the veinlets. It is counted as an average number of vein-islet numbers per mm2 of leaf surface (Kumar et al., 2012).

Palisade ratio

Palisade cell is irregularly a shaped plant cell of the mesophyll of leaves, mostly found in upper epidermal layer as well as in the plant stems. Palisade cell contains most of the chloroplasts which are subjected to the photosynthesis (Eames & MacDansls, 1974). The ratio of palisade can be used to identify the leaf regardless of geographical variation.

Trichome number

Trichome is considered as a hair of the plant that can be seen in leaves, stems and fruits. It is an identifying tool used to distinguish between plant species. Trichome is classified into two groups: glandular and non-glandular trichomes (Serna & Martin, 2006; Werker, 2000). Trichome plays a role in reduction of heat load, improvement of

water absorption, and defenses from insects. Trichome number is calculated as an average number of trichomes per $mm^2$

Epidermal cell area

Mean leaf epidermal cell surface area can be useful to identify plant species. Despite epidermal cell area are not fixed values, they are relatively constant within a narrow range for leaf species (Foroughbakhch, Ferry Sr, Hernández-Piñero, Alvarado-Vázquez, & Rocha-Estrada, 2008).

**Molecular evaluation**

The description, classification of genetic diversity is a main problem of biology (Clegg, 1993). Nowadays, many molecular markers are used to analyze the genetic diversity. The molecular techniques have been broadly used to authenticate the herbal medicine and to evaluate the relationship among or different cultivars and species. Mukherjee reported that the molecular methods are beneficial and accurate for examination in diversity of plants genetic (Mukherjee, 2007). Sukrong supported that the age, environment, physicological condition, temperature, harvesting, and processing are less affect when experiment with DNA-based markers or molecular technique (Sukrong, 2005). So, many researchers adore using the molecular evaluation for their research in worldwide.



Plant's DNA is spread in many areas of the cell such as nucleus, mitochondria and chloroplast. Mostly, DNA is loaded in nucleus. However, to classify and differentiate the phylogeny of plant, botanists generally use chloroplast DNA.

Numerous studies of *Derris* species were reported about genetic evaluation using molecular makers. The study of *Derris trifoliate* using ISSR marker to investigate the genetic diversity along the coasts located at the South of China Sea revealed that the fruit dispersal and habitat fragmentation may influence in the genetic pattern of

*Derris trifoliate*. Therefore, the management of conservation of the species was recommended in this research (Wu, Geng, & Shu, 2012).

The assessment of genetic diversity among 210 samples of *Derris elliptica* in China was determined using microsatellite amplification method. It was found that there were two major groups of genotypes (semi-wild and cultivated populations) resulted from UPGMA dendrogram which was correlated to the result from Principal Coordinate Analysis (PCA). According to the result of the study, future germplasm collection and breeding programs should be considered due to the improvement of economically important traits (H. Li & Geng, 2015).

Three chloroplast genes including trnK-matK , trnL-F IGS, psbA-trnH IGS as well as nuclear ribosomal ITS /5.8S using Parsimony and Bayesian techniques were used to define and differentiate 50 species of palaeotropic *Derris*-like taxa. The results showed that *Paraderris* exhibited the closely phylogenetic relationship to *Derris*, whereas *Brachypterum* and *Synapomorphic* were clearly separated from *Derris.* Therefore, *Paraderris* was subjected as a synonym with *Derris* sensu stricto (s.s.) (Yotsawate Sirichamorn, Frits A. C. B. Adema, et al., 2012).



The development of microsatellite markers from an AC-enriched genomic library using the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) method was successfully developed to improve the germplasm collection and breeding program for *Derris elliptica*. It was found that 37 microsatellite markers amplified totally 159 alleles, showing a range of 2-8 (4.3 per locus) as well as the polymorphic information content value across all loci was around 0.53 with a range of 0.05-0.75. The researchers also suggested that these potential markers can be used for genetic diversity studies, such as the distinction between the wild relatives from each

other and from the cultivated genotypes for the economic purpose. (H. Li & Geng, 2013).

*D. scandens* Benth. collected from 19 provinces and 1 callus culture in Thailand were investigated for genetic difference study using DNA fingerprint analysis, TLC fingerprint and morphological study. Twenty samples showed similarity pattern of TLC fingerprint which was correlated to the results obtained from morphological study. In contrast, the use of DNA fingerprint analysis by Winboot program had shown three different groups of collected samples (Mongkolchaipak, Marsud, & Suchantaboot, 2011).

Five *Derris* species (*D. scandens, D. elliptica, D. malaccensis, D. trifoliate* and *D. reticulata*) were subjected for identification and construction of genetic relationship using ramdom amplified polymorphic DNA (RAPD) method showing 9 primers which exhibited polymorphism. The unweighted pair-group analysis using arithmetic averages (UPGMA) was used to generate the dendrogram, indicating 2 clusters among five *Derris* species; cluster 1 contained four species, whereas *D. trifoliate* was separated into another group (Sukrong, 2005).



**Plant genomic DNA extraction**

DNA or deoxyribonucleic acid is a molecule which encrypts the genetic data of the living organisms and also viruses. Plant genomes are all the genetic essential in plant cell compose of nuclear genome and organelle genome (mitochondrial genome and chloroplast genome). DNA can be used for molecular analysis consist of PCR, cloning, electrophoresis, and DNA or protein sequencing.

Most plant DNA extraction methods are normally use young leaves as starting material. The fresh tissue has to keep for a short time, not frozen yet. However, if the researcher would like to store for long period, the DNA might freezing directly in -80

degree Celsius. Firstly, plant samples should be checked carefully because some might infect by bacterial or fungal parasites and these may present an altered DNA profile at the result. Using liquid nitrogen as a shock-freezing and store at -80 $^{o}$C, freeze-drying, chemical safety, or drying in silica gel is normally early step for researcher.

There are many reports presented about the five steps to isolate DNA from plant cell. At first, disruption or break the cell to release the DNA, then separate DNA from proteins and other cellular debris. Third is precipitation of DNA. Next step, the DNA cleaning and the last is to confirm the quality of DNA. In common, all steps implicate disruption then remove proteins and contaminants and restoration of the DNA in the final step.

DNA isolation Kit

The DNA commercial Kit is a favorable, but it quite expansive alternative. It is considered to be a widely isolation method and gets higher percent yields and high quality of DNA. It also relative simple, save time, and do not contain harmful chemical such as phenol, chloroform, involves minimal handling. Many laboratories use the commercial Kit for isolate and purify the genomic and plasmid DNA from variety of plant samples consists of tissues, cells, blood, or serum.



CTAB method

Cetrimonium bromide or CTAB is a cationic detergent that solubilizes membranes and forms a complex with DNA. CTAB also stand for the most popular procedure of plant DNA isolation. In 1990, Doyle and Doyle have been used CTAB to extract DNA with proteinase K and **β**-mercaptoethanol which is a reducing agent. This method is relative simple, save time, low cost and gets a great qualitative DNA (Doyle, 1987).

There are other methods to extract plant's DNA such as Sodium dodecyl sulfate (SDS). It helps cell membrane and nuclear envelop to break down and expose

the chromosome that contain DNA. To remove the membrane barriers, SDS helps to release DNA from histones and other DNA binding proteins by denaturing them. However, it's quite complicated and time consuming.

Polyvinylpyrrolidone or PVP is another one to remove polyphenols along with a high molar concentration of NaCl to obstruct the co-precipitation of DNA and polysaccharides.

Polymerase chain reaction

Polymerase chain reaction (PCR) or *in vitro* enzymatic gene amplification is the technique that increases DNA fragments. It is quick and easy method to characterize, analyzes, and generates unlimited copies of any DNA or RNA pieces. The components of the PCR reaction are consist of DNA template, thermostable DNA polymerase, deoxyribonucleotide triphosphates (dNTPs), oligonucleotide primers, suitable buffers, and magnesium or manganese ions ($Mg^{2+}$) (Williams, Kubelik, Livak, Rafalski, & Tingey, 1990).

To obtain the copying method start, DNA template and two primers were required. The primers are short chain of four different chemical constituents to build the strand of genetic materials. These are consisting of the 3' ends of each of the sense and anti-sense strand of DNA target.

The efficacy of PCR can stimulate from three factors. They are specificity, efficiency or yield and fidelity. There are three steps for PCR. First step is denaturation. In this step, the target genetic material must be denatured. The strands of its helix should be split by heating around 90-96 °C. Next, it called annealing step, the primers bind to their target sequences on the template DNA. This step, the temperature was decrease to about 35 to 65 °C (depend on primer sequence and technique). The last step is elongation step, DNA was synthesis by polymerase. The temperature is chosen

at which the activity of the thermostable polymerase is optimal (usually 65 to 72 °C). To gain more DNA, repeating the three steps cycle 25 to 50 times result in the exponential amplification.

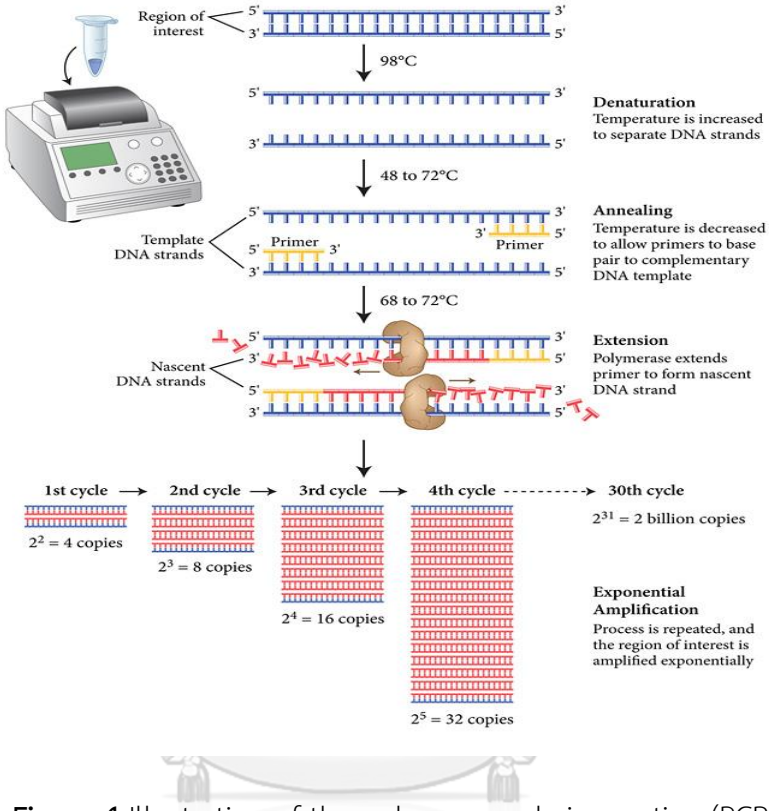



**Figure 4** Illustration of the polymerase chain reaction (PCR)



However, there are some problems that can present with PCR technique such as the plant sample contamination. It might be contaminate with the external genetic material which can make many copies of unrelated DNA. For the reason, it may present an error result. But there are also have many applications of PCR such as cloning, genetic engineering, etc.

**DNA markers and DNA fingerprinting**

DNA marker is broadly applied to specify the characteristics of all living things. It is one type of DNA which is positioned in nucleus, chloroplast or mitochondria. DNA marker is served as an indicator because of the variation of nucleotides in DNA molecules or DNA polymorphism of nucleotide sequencing.

DNA fingerprinting (also known as DNA typing or DNA profiling) is a technique used to determine nucleotide sequences of certain areas of DNA which are unique to each individual. DNA fingerprinting can be used to test plant polymorphism by cutting the genomic DNA with restriction enzymes and undergoing gel electrophoresis. The separated DNA is then transferred to a membrane filter for hybridization using probe, resulting in particular fingerprinting for individual characteristic. Regarding plant biology, the DNA fingerprinting is mainly performed for identification of genetic diversity and variation. The most commonly used techniques are restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and etc.

**Inter simple sequence repeat amplification (ISSR)**

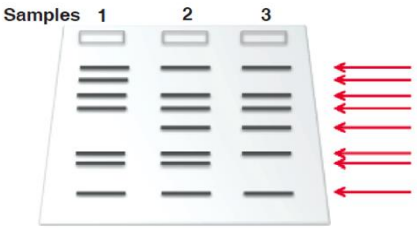
ISSR is a PCR-based DNA technique is a rapid and simple technique to study genetic relationship of plants in species and genus level. ISSRs are DNA fragments with microsatellite segments at the front and back ends. ISSR primer, containing the repeated sequences complementary to the microsatellite segments, is subjected to PCR to amplify the DNA fragments. Compared to other PCR-based molecular techniques, ISSR gives more reproducible products than RAPD and requires lower cost than AFLP (Ng & Tan, 2015; Sachindra, Airanthi, Hosokawa, & Miyashita, 2010).

Normal PCR reaction usually contains double primers (forward and reverse primers), but ISSR primer contains a single primer because it already acts as both forward and reverse primers. ISSR primer is classified into three types: Unanchored (consisting of a repeated motif); 5'-anchored (consisting of a repeated motif with one

or several non-motif nucleotides at the 5'-end); and 3'-anchored (consisting of a repeated motif with one or several non-motif nucleotides at the 3'-end). The annealing of temperature of ISSR-PCR is usually between 45-60 ℃, depending on the melting temperature of the primer (Pradeep Reddy, Sarla, & Siddiq, 2002).

Gel electrophoresis of the ISSR amplified products is commonly conducted using 1.0-2.0% w/v agarose gel. Bornet and Branchard reported that 2.0% w/v agarose gel was the most suitable concentration for visualizing ISSR bands (Bornet & Branchard, 2001). Besides agarose gel, polyacrylamide gel can be used (Ng & Tan, 2015; Pradeep Reddy et al., 2002). For scoring ISSR amplified bands, it is quite subjective because the results may vary from person to person (Meudt & Clarke, 2007; Pompanon, Bonin, Bellemain, & Taberlet, 2005). However, there are a few tips of scoring ISSR bands that can be individually adaptable. First, the range of band-scoring size should be set as a standard before scoring (usually 100-2000 base pairs). Next, only the clear, distinctive bands with strong intensities are counted. Smeared bands and bands with weak intensities may be due to unspecific binding of primers, causing unintended amplification (Ng & Tan, 2015).

A "band" scored in ISSR can be called "locus." Each ISSR amplified band is separately considered as single locus, reflecting one data point in the analysis. The total number of different ISSR band sizes in a study is called "the total number of bands." The number of ISSR bands that show variation (the bands are present for some samples and absent for the others) is called "the number of polymorphic bands." The percentage of the polymorphic bands is expressed as percent polymorphism. Example of ISSR band scoring is shown in Figure 5.




| Locus | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| L1 | 1 | 1 | 1 |
| L2 | 1 | 0 | 0 |
| L3 | 1 | 1 | 1 |
| L4 | 1 | 1 | 1 |
| L5 | 0 | 1 | 1 |
| L6 | 1 | 1 | 1 |
| L7 | 1 | 1 | 0 |
| L8 | 1 | 1 | 1 |

$$\% \text{ Polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Total number of bands = 8
Number of polymorphic bands = 3

$$\% \text{ Polymorphism} = \frac{3}{8} \times 100 = 37.5\%$$

**Figure 5** ISSR band scoring

# CHAPTER III

# MATERIALS AND METHODS

Chemicals and reagents

| | |
|---|---|
| 2, 2-Diphenyl-1-picylhydrazyl | Sigma-Aldrich, USA |
| 2, 4, 6-Tripyridyl-s-triazine | Sigma-Aldrich, USA |
| 3, 5-Di-tert-4-butylhydroxytoluene | Sigma-Aldrich, USA |
| Acetic acid | BDH Chemicals, UK |
| Acetone | Merck, Germany |
| Absolute ethanol | Merck, Daemstadt, Germany |
| Aluminium chloride | Ajax Finechem Pty. Ltd., NewZealand |
| Agarose | P2 Innovation Ltd., UK |
| Boric acid | Ajax Finechem Pty. Ltd., New Zealand |
| Carotene | Fulka, USA |
| Chloral hydrate | Ajax Finechem Pty. Ltd., New Zealand |
| Chloroform | RCI Labscan Limited, Thailand |
| DNA marker | Thermo Fisher Scientific Inc., USA |
| DNeasy plant mini kit | QIAGEN, USA |
| DNTPs | Thermo Fisher Scientific Inc., USA |
| EDTA | Ajax Finechem Pty. Ltd., New Zealand |
| Ethanol | RCI Labscan Limited, Thailand |
| Ethidium bromide | Bio Basic Canada, Canada |
| Ethyl acetate | RCI Labscan Limited, Thailand |
| Formic acid | Merck, Daemstadt, Germany |
| Ferrozine | Sigma-Aldrich, USA |
| Folin-Ciocalteu's reagent | Merck, Germany |
| Gallic acid | Sigma-Aldrich, USA |

| | |
|---|---|
| GeneRuler 1 Kb DNA ladder | Thermo Fisher Scientific Inc., USA |
| Haiter solution (6% sodium hypochlorite) | Kao Corp., Japan |
| Hydrochloric acid | RCI Labscan Limited, Thailand |
| Isoamyl alcohol | Sigma-Aldrich Company Co., St.Louis,MO,USA |
| ISSR primer | Eurofins MWG Operon Inc., USA |
| Liquid nitrogen | |
| Loading dye | Thermo Fisher Scientific Inc., USA |
| Magnesium chloride | Thermo Fisher Scientific Inc., USA |
| Mercaptoethanol | Sigma-Aldrich Company Co., St. Loi,MO,USA |
| Methanol | RCI Labscan Limited, Thailand |
| Rotenone | Sigma-Aldrich Company Co., St. Louis, MO,USA |
| Sodium acetate | BDH Laboratory supplies, Poole, England |
| Sodium chloride | BDH Laboratory supplies, Poole, England |
| Taq DNA polymerase | Thermo Fisher Scientific Inc., USA |
| Toluene | RCI Labscan Limited, Thailand |
| Tris (hydroxymethyl)-aminomethane | Fluka, Biochemika, Germany |
| 1 kb DNA ladder | Promega, USA |
| 100 kb DNA ladder | Promega, USA |

Materials, instruments and equipment

| | |
|---|---|
| TLC Chamber | CAMAG Switzerland |
| TLC Scanner4 | CAMAG Switzerland |
| TLC Visualizer | CAMAG Switzerland |
| Centrifuge machine | Labnet International, Inc., USA |
| Combi-Spin | BioSan, USA |
| Digital camera | (Canon PowerShot A650 IS) Canon Inc., Japan |
| Filter paper No.4 | Whatman™ paper, UK |

| | |
|---|---|
| Filter paper No.40 ashless | Whatman™ paper, UK |
| Gel electrophoresis apparatus and power supply | Biometra |
| Glass slide and coverglass | HAD, China |
| Hot air oven | WTB binder, Tuttlingen, Germany |
| ImageJ software | National Instiutes of Health, USA |
| Incinerator | Carbolite, UK |
| InGenius 3 with GeneSis software | Syngene, UK |
| Microscope (Axio imager A2) | Zeiss inc., Germany |
| NanoDrop Spectrophotometer ND-1000 | NanoDrop Technologies, Inc., Wilmington, DE, USA |
| PCR Thermal Cycler | GenePlus, Thailand |
| Photomicroscope | Zeiss Imager A.2 Axio, Germany |
| Proflex PCR system thermocycler | Thermo Fisher Scientific Inc., USA |
| Rotary vacuum evaporator | Buchi, Switzerland |
| TLC aluminium sheet Silica gel 60 F254 | Merk, Germany |
| Ultraviolet viewing cabinet | Spectronics Corporation, USA |
| Ultrapure water system | NW20VF, Heal Force, China |
| UV visualize gel documentation machine | Auto Chemi System |
| winCATS software (Version: 1.4.6.2002) | CAMAG, Switzerland |

**Part I : Pharmacognostic evaluation of *Derris elliptica* stems**

Plant materials

*Derris elliptica* stems were collected from 15 locations throughout Thailand. The plants were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University.

Macroscopic evaluation

The appearance of physical characteristics including size, shape, texture and color of the crude drugs were examined by visual inspection.

Microscopic evaluation

*Anatomical character investigation*

Transverse section was examined for anatomical characteristics. The cells and tissues were photographed under microscope with the magnification of 10X to 40X and illustrated by hand drawing.

*Histological character investigation*

Dried stems of *Derris elliptica* were ground through the number-60 sieve mesh. The powdered sample were placed on the glass slide, mounted with a few drops of water, and covered with the cover slip. The histological characteristics of the powdered sample were observed under 40X-magnification microscope, photographed and illustrated by hand drawing.



Physicochemical parameters

According to the World Health Organization (WHO) and Thai Herbal Pharmacopoeia, the standardization parameters were assessed in *D. elliptica* stem crude drug from 15 locations. Each location was done in triplicate.

Determination of loss on drying

Three grams of dried stem powder were placed in a pre-weighed crucible. The samples were dried at 105 ℃ for 6 hours until constant weight and allowed to cool to room temperature. The samples were weighed and calculated for loss on drying value in percentage.

Determination of total ash

Three grams of the plant samples were weighed in a pre-weighed crucible and incinerated at 500 ℃ for 5 hours. The samples were allowed to cool down in a desiccator and the constant weight were recorded. The total ash value was evaluated in percentage.

Determination of acid-insoluble ash

The crucible containing the abovementioned total ash, twenty-five milliliters of hydrochloric acid (70 g/L) were added and boiled for 5 minutes. The samples were filtered with number-40 ashless filter papers. The insoluble matters on the filter paper were dried, incinerated to ash and cooled in the desiccator. The acid-insoluble ash was weighed and calculated in percentage.



Determination of extractive value

In this study, water and 95% ethanol were used as the solvents for the determination of extractive value. Five grams of the plant materials were macerated in the solvent (70 ml water or 95% ethanol) with shaking for 6 hours and standing for 18 hours. After filtration through filter-paper Whatman No. 4, the marc was washed with the solvent used and the filtrate was finally adjusted to 100 ml. Twenty milliliters of the filtrate were transferred to pre-weighed small beaker and evaporated to dryness on water bath then further heated in an oven at 105 ℃ for 6 hours and cooled to room temperature in a desiccator for 30 minutes. The extractive value were weighed and calculated in percentage of crude drug.

Determination of water content

Fifty grams of the plant sample in 200 ml of water-saturated toluene were boiled via azeotropic distillation. The water content was recorded and expressed in percentage.

**Thin layer chromatography fingerprint**

Twenty milliliter of the ethanolic filtrate from aforementioned ethanolic extractive value determination were transferred to another beaker. Evaporated to dryness on a water bath and dissolved in 1 ml of methanol. Three microliters of the extracted solution were performed onto TLC silica gel 60 GF254 plate and developed in the suitable solvent system (toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2)). The TLC plate was dried and observed under UV light, both short and long wavelengths (254 and 365 nm), and then sprayed with 10% sulfuric acid in methanol.

The retention factor (Rf) of the spots on TLC plate were determined using the following equation:

$$R_f = \frac{\text{Distance of the analytical sample travelled}}{\text{Distance of the solvent travelled}}$$



**Part II : Quantitative analysis of rotenone in *Derris elliptica* stems**

Preparation of ethanolic extracts of *Derris elliptica* stems

Five grams of *D. elliptica* stems dried powder were exhaustively extracted with 95% ethanol using Soxhlet apparatus. The ethanolic extracts were filtered through Whatman number-4 filter paper and evaporated to dryness. The percent yield of the extract was calculated. The extracts were redissolved in methanol to obtain various concentrations and kept at 4°C for rotenone quantification. The remaining extracted samples were stored at -20°C.

Preparation of standard solution of rotenone

The stock solution of standard rotenone was prepared by dissolving 1 mg of standard rotenone in 1 ml methanol. The stock solution was then diluted to the series of standard solution ranging from 0.2 to 1 mg/ml, and stored at 4°C.

TLC-densitometry

Three microliters of the ethanolic extract of *Derris elliptica* stems and standard solution of rotenone were spotted onto the TLC silica gel 60 $GF_{254}$ plate using Linomat 5 applicator. The TLC plate was developed in a solvent system containing toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2). After the development, the plate was left to dryness and scanned with CAMAG TLC Scanner 4 (CAMAG, Switzerland) at 301 nm. The rotenone content was calculated from the peak area of each chromatographic band against the standard calibration curve.

TLC image analysis by ImageJ software

The TLC plates under the wavelengths 254 nm were photographed using TLC visualizer and transform the band to chromatographic peak by ImageJ software. The content of rotenone was evaluated by comparing peak area to the standard calibration curve obtained from the same TLC plate.



**Method validation**

The method were validated in terms of calibration range, specificity, accuracy, precision, LOD, LOQ and robustness, according to the ICH guideline (ICH, 2005).

Calibration range

The peak areas and concentrations of the standard rotenone were plotted to obtain the calibration range. The coefficient of determination ($r^2$) was evaluated using Excel software.

Specificity

The specificity was assessed in terms of peak identity and peak purity. Peak identity was evaluated by comparing the absorption spectra of rotenone among the sample and the standard rotenone, while peak purity was determined from the comparison of the absorption spectra at up-slope, apex and down-slope of the peak.

Accuracy

The recovery study was performed by spiking the standard solution of rotenone into the ethanolic extract to obtain three different levels (low, medium and high) along the calibration range. All spiked and un-spiked samples was examined in triplicate. The accuracy was expressed as percent recovery.

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

Where, A = amount of spiked sample

B = amount of un-spiked sample

C = amount of standard spiked into the recovery sample

Precision



The repeatability (intra-day) precision and intermediate (inter-day) precision were evaluated by analyzing the sample solution of three concentrations on the same day and three different days, respectively. All tested samples were performed in triplicate and expressed as % relative standard deviation (% RSD).

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

Limit of detection and limit of quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were examined regarding the calibration curve.

$$LOD = \frac{3.3\ x\ SD_{residual}}{S}$$

$$LOQ = \frac{10\ x\ SD_{residual}}{S}$$

Where, $SD_{residual}$ = the residual standard deviation of regression line

S = the slope of regression line

Robustness

The robustness was conducted by altering a ratio of the solvent system used to develop the TLC plate. The percent RSD (%RSD) of the peak area was calculated as the result.

Data analysis

Grand mean and pooled standard deviation was used for the analysis of physicochemical parameters. The rotenone contents obtained from TLC-densitometry and TLC image analysis were statistically compared by paired student t-test ($P \leq 0.05$).



**Part III Biological activity assessment of ethanolic extract of *Derris elliptica* stems**

**Antioxidant activities**

DPPH radical scavenging assay

The radical scavenging property was evaluated based on the study written by Brand-William *et al*. (Brand-Williams, Cuvelier, & Berset, 1995). To the 96-well microplate, 100 µl of the ethanolic extract at different concentrations, standard rotenone and positive controls (i.e., BHT and quercetin) were mixed with 100 µl of

DPPH solution (120 µM in methanol). The samples were incubated in the dark at room temperature for one hour. The absorbance was measured at 517 nm. All tested samples were performed in triplicate. The antioxidation regarding radical scavenging activity was expressed as $IC_{50}$ value.

$$\%\ Inhibition = \frac{[Absorbance\ control - Absorbance\ sample]}{Absorbance\ control} \times 100$$

Ferric reducing antioxidant power (FRAP) assay

The reducing power was assessed as described by Benzie and Strain (Benzie & Strain, 1996). Firstly, FRAP working reagent was freshly prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM of TPTZ in 40 mM HCl and 20 mM of $FeCl_3.H_2O$ in ratio of 10:1:1. Secondly, to the 96-well microplate, 25 µl of the ethanolic extract (0.5 mg/ml in methanol) and positive controls (i.e., BHT and quercetin) were mixed with 175 µl of FRAP working reagent. The mixture was then incubated at room temperature for 30 minutes. The absorbance was measured at 593 nm. All samples were performed in triplicate. The antioxidant activity regarding the reducing power was calculated as the amount of mM of ferrous iron (Fe (II)) per mg extract obtained from ferrous sulfate calibration curve.



ß-carotene bleaching assay

ß-Carotene bleaching test was demonstrated in accordance with Jayaprakasha and team (Jayaprakasha, Jena, Negi, & Sakariah, 2002). To the 96-well microplate, 1 µl of ß-carotene solution (2 mg/ml in chloroform) was mixed with 20 µl of linoleic acid and 200 µl of Tween 20. The mixture was taken under vacuum at 40 °C to remove chloroform, following with the addition of 50 ml water to generate the emulsion. The emulsion (200 µl) was transferred to 96-well microplate that contains various concentrations of extract, standard rotenone and positive controls (i.e., BHT and quercetin). The mixture reaction was heated at 50 °C before measuring the absorbance

at 470 nm at 30 minutes intervals for 120 minutes. All samples were performed in triplicate. The antioxidant activity was calculated in percentage.

$$\%\ Antioxidant\ activity = \left(\frac{(C_0 - C_{120}) - (A_0 - A_{120})}{C_0 - C_{120}}\right) \times 100$$

Where $A_0$ = the absorbance values measured at zero time of sample

$A_{120}$ = the absorbance values measured at end time of sample

$C_0$ = the absorbance values measured at zero time of control

$C_{120}$ = the absorbance values measured at end time of control

Total phenolic content

To quantitate the content of phenolic compounds, 25 µl of the ethanolic extract (0.5 mg/ml) were undergone reaction with 125 µl of 10% Folin-Ciocalteu's phenol reagent in a 96-well microplate. One hundred microliter of 7.5% $Na_2CO_3$ solution were added to the mixture reaction and then incubated at room temperature for an hour. The absorbance was measured at the wavelength of 756 nm. All the tested samples were performed in triplicate. The total phenolic content was evaluated with respect to the calibration curve of gallic acid, and displayed in terms of gallic acid equivalents per mg extract.



Total Flavonoids

The determination of total flavonoid content by aluminium chloride colorimetric method was conducted according to Chang and his team with minor modifications (Chang et al., 2002). Fifty microliters of 0.5 mg/ml extract were placed into 96-well plate and mixed with 10 µl of 10% aluminium chloride solution. Then, 150 µl of water and 10 µl of 1 M of sodium acetate solution were added into the mixture solution. The mixture solution was incubated at room temperature, under darkness for 40 min, and then measured at 510 nm. All tested samples were performed in triplicates. The results were expressed as quercetin equivalents per mg extract.

Antidiabetic activities

Inhibition of yeast alpha-glucosidase activity

The property to inhibit α-glucosidase from *Saccharomyces cerevisiae* was evaluated as describe by previous study with some modifications (Wan, Min, Wang, Yue, & Chen, 2013). To the 96-well microplate, 10 µl of the extract at various concentrations, standard of rotenone and positive control (i.e., acarbose) in DMSO were added with 120 µl of 0.1 M sodium phosphate buffer (pH 6.9) and 20 µl of 0.5 U/ml α-glucosidase. After incubating at 37 °C for 15 minutes, 20 µl of *p*-nitrophenyl-α-D-glucopyranoside was added to the mixture and incubated at 37 °C for another 30 minutes. After adding 80 µl of 0.2 µM $Na_2CO_3$ to the mixture, the absorbance was measured at 405 nm. Each sample was performed in triplicate. The inhibitory activity was expressed as percent inhibition.

$$\%\ Inhibition = \frac{[Absorbance\ control - Absorbance\ sample]}{Absorbance\ control} \times 100$$

Rat intestinal α-glucosidase inhibitory activity

The test in this part was slightly modified from the two previous studies (Ganogpichayagrai, Palanuvej, & Ruangrungsi, 2017; Gayle, 2012). Various concentrations of extracts, rotenone and positive control (acarbose) were prepared in DMSO. The rat intestinal acetone powder was suspended in 0.1 M sodium phosphate buffer (pH 6.9) at the concentration of 30 mg/ml. The suspension was sonicated on ice for 20 min and then centrifuged at 3000 rpm, 4 °C for 30 min. The supernatant was collected for the test. The 96-well plate containing 50 µl of the sample solutions, 100 µl of 1mM *p-nitrophenyl α-D-glucopyranoside* and 50 µl of supernatant cantaining α-glucosidase enzyme was incubated for 30 min at 37 °C, then measured the absorbance at 405 nm. The test was done in triplicate and the inhibition percentage was calculated following this formula:

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample}) \times 100}{\text{Absorbance of the control}}$$

Cytotoxic activity on cancer cell

Human colorectal adenocarcinoma (HT-29), human hepatocellular carcinoma (HepG2), human breast cancer (MDA-MB-231) and human intestinal epithelial (Caco-2) were purchased from the American Type Culture Collection (ATCC) and were cultured in complete medium containing 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin–streptomycin and basal media (DMEM).

Two hundred microliters of cultured cells were seeded in 96-well plate (density of complete medium/well = $1x10^5$ cells/ml) and incubated at 37 °C in a humidified condition with 5% v/v carbon dioxide for 24 h. Then, the cells were mixed with different concentrations of the *D. elliptica* extract or standard rotenone for another 24 h. The MTT solution (0.4 mg/ml) was substituted for the medium and incubated for 4 h. The MTT solution was removed and replaced by DMSO. The optical density was detected at 570 nm. The test was performed in triplicate. The data obtained was expressed as $IC_{50}$ in reference to the control.

Brine shrimp lethality assay



As described by Meyer et al., the test on brine shrimp lethality was implemented (Meyer et al., 1982). The hatching box containing 24-hour aerated artificial sea water (36.66 %w/v) was divided into two parts using a divider with 2-mm holes. One section was darkened whereas another one was illuminated. *A saline* eggs were sprinkled into the dark part and incubated at ambient temperature for 48 hours. Then, ten brine shrimps were collected from the light side and transferred to the sample vials containing 5-ml artificial sea water. The filter papers containing various concentrations of extract in methanol were dried then placed into each vial. Five replications were performed on each sample concentration. The percent death of nauplii was evaluated at 6, 12, 18 and 24 hours, and the $LD_{50}$ was calculated.

**Part IV : Plant morphology and microscopic characteristics including leaf constant number of selected *Derris* species**

Plant collection

Fresh mature leaves of selected *Derris* species (*D. elliptica D. malaccensis D. scandens D. trifoliate D. indica D. reticulate D. amoena D. solorioides*) were collected from 3 different locations in Thailand. All samples were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Macroscopic evaluation

The botanical morphologies of selected *Derris* species were observed for their visual characters such as shape, size, and illustrated by hand drawing.

Microscopic leaf anatomical evaluation

Fresh leaves of selected *Derris* species were transversely cut by razor blade to observe the anatomical structures of plant midribs. The leaf cross section was placed on a glass slide, mounted with a few drops of water and covered with cover slip. Each sample was evaluated under microscope. The characteristics of the plant midribs were manually illustrated by hand drawing with the proportion size correlated to the plant.



Microscopic leaf constant numbers

The leaves of selected *Derris* species were cleaned. The central parts of the lamina, midway between the midrib and the margin were cut into small size, approximately 10 x 10 mm2. To remove chlorophyll, each sample was soaked in Haiter bleaching solution (6% w/w sodium hypochlorite) diluted with water (1:1). The leaf sample was washed with water and soaked in chloral hydrate solution (chloral hydrate : water (4:1)) under low heat. The specimen was placed on the glass slide, mounted with water and covered with the cover slip. The suitable magnification of microscope was chosen with respect to the selected cells. The interested cells in a unit area were

traced, photographed, and counted for 30 fields per each location of species collected (three different locations per species) as follows :

Vein-islet number

The small areas of tissue surrounded by the veinlets were counted. The vein-islet numbers were determined as the number of vein-islets per 1 square millimeter of each field.

Stomatal number

The stomatal numbers were counted and determined as the number of stomata in 1 square millimeter area,

Trichome number

The trichomes and the cicatrices were counted and determined per 1 $mm^2$ of each field.

Epidermal cell number

The epidermal cells in the same unit area of stomata and trichomes were counted. The epidermal cells numbers were expressed per 1 square millimeter of area studied.



Stomatal index

The stomatal index were calculated as a ratio of the stomatal number to the ordinary epidermal cell number in the same unit area.

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

S = number of stomata per unit area

E = number of epidermal cells including trichomes and cicatrices in the same unit area

Palisade ratio

The palisade cells beneath four contiguous epidermal cells were counted and divided by four.

Epidermal cell area

The numbers of epidermal cell in one square millimeter were used to estimate the epidermal cell area.

$$\text{Epidermal cell area} = \frac{1}{\text{Number of ordinary epidermal cell}}$$

**Part V : Molecular identification of selected *Derris* species**

Plant collection

The leaves of selected *Derris* species were collected from three different sources throughout Thailand. All samples were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi

Preparation of CTAB buffer

Genomic DNA was extracted from the fresh young leaves using a modified CTAB method (Doyle, 1987). The preparation of CTAB buffer is shown in Table 7.

**Table 7** Preparation of CTAB buffers

| Stock reagent | Final concentration | Final amount |
|---|---|---|
| CTAB | 2% (w/v) | 2 g |
| 1 M Tris-HCl pH 8 | 100 mM | 10 ml |
| 0.5 M EDTA | 20 mM | 4 ml |
| 5 M NaCl | 1.4 M | 28 ml |
| PVP | 1% | 1 g |
| Adjust to 100 ml with water | | |

Procedure

Firstly, the young and fresh leaf materials was grinded to powder using liquid nitrogen. Secondly, the powdered samples were transferred into 1.5 microcentrifuge tube, and was added with 500 µl of CTAB buffer (4 µl of 2-mercaptoethanol was added to each 1 ml of CTAB buffer before used). The mixture was incubated in a shaking water bath at 65 °C for an hour, and then centrifuged at 10,000 round per minute (rpm) for 10 minutes. After that, the supernatant was transferred into a clean microcentrifuge tube, added with chloroform and centrifuged at 10,000 rpm for 10 minutes. The upper layer is then transferred into a new microcentrifuge tube, added with 500 µl of chloroform: isoamyl alcohol (24: 1), and centrifuged at 10,000 rpm for 10 minutes. Next, the upper layer is transferred into a new microcentrifuge tube, added with 1: 10

volume of 3M sodium acetate pH 5.0, inverted the tube gently and kept it at -20 ℃ for an hour. Then, the absolute ethanol was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was discarded. The DNA pellet was washed with 1 ml of cold 70% ethanol, inverted gently and centrifuged at 10,000 rpm for 10 minutes. All the supernatant were removed and the DNA pellet were dried at room temperature. Finally, the dried DNA pellet was re-dissolved in 100 µl TE buffer (10 mM Tris pH 8, 0.1 mM EDTA ph 8), stored at 4 ℃ refrigerator for days to weeks and kept at -20 ℃ for further use.

DNA extraction by DNeasy® plant mini kit

*Derris* genomic DNA was extracted from the fresh young leaves using DNeasy® plant mini kit. Sample was disrupted using mortar and pestle. The powdered samples were transferred into 1.5 microcentrifuge tube. Four microliter of RNase A was added in 400 µl Buffer API then was vortexed and was incubated for 10 minutes at 65ºC. The tube was inverted 2-3 times during incubation then was added 130 µl in buffer P3. It was mixed and incubated for 5 minutes on ice. The lysate was centrifuged for 5 minutes at 20,000 x g then was pipeted into QIAshredder spin column placed in a 2 ml collection tube and was centrifuged for 2 minutes at 20,000 x g. The flow-through was transfered into a new tube without disturbing the pellet if present. 1.5 volumes of buffer AW1 was added, and was mixed by pipetting. 650 µl of the mixture was transfered into a DNeasy mini spin column that was placed in 2 ml collection tube, was centrifuged for 1 minute at ≥6000 x g (≥8000 rpm), was discarded the flow-through. This step was repeated with the remaining sample. The spin column was placed into a new 2 ml collection tube, 500 µl buffer AW2 was added, and was centrifuged for 1 minute at ≥6000 x g, then the flow-through was discarded. Another 500 µl buffer AW2 was added, was centrifuge for 2 minutes at 20,000 x g. The spin column was transfered to a new 1.5 ml or 2 ml microcentrifuge tube. 100 µl of Buffer AE was add for elution,

was incubate for 5 minutes at room temperature (15-25°C(, then was centrifuged for 1 minute at ≥6000 x g. stored at 4 °C refrigerator for days to weeks and kept at -20 °C for further use.

Preparation of 1.5% agarose gel

The agarose powder (1.5 g) was weight in 100 ml of 1X TBE buffer and then dissolved using microwave. After that, the gel solution was allowed to stand at room temperature until warm, and then poured into the plastic tray. After the gel becomes solid, the comb was taken off. The tray was put into the gel electrophoresis apparatus and filled with 1X TBE buffer.

DNA qualification

The concentration and purity of DNA were measured using NanoDrop One spectrophotometer to determine the absorbance ratio at λ260/280 nm (Good-quality DNA were λ260/280 ratio of 1.7–2.0)

ISSR amplification

Primer were used to screen among 15 ISSR primers selected to produce amplified genomic DNA. ISSR-PCR amplification was conducted in 20 µl reaction volume, containing 2 µl( 50ng of DNA) of extracted genomic DNA, 0.4 µl of 0.2 µM primer, 2 µl of 2.5 mM $MgCl_2$, 0.4 µl of 2 µM dNTPs, 0.2 µl of 1 U *Taq* DNA polymerase. The PCR reaction to were performed using a Proflex PCR system thermocycler. Firstly, the initial denaturation step for 5 minutes at 95°C. Next denaturation at 95 °C for 45 seconds, follow the different annealing temperature of each primer for 45 seconds; and extension at 72 °C for 60 seconds and the cycle was repeated 45 cycles, final extension was performed at 72°C for 10 minutes and a hold temperature of 4 °C at the end.

Detection of PCR product

For gel electrophoresis, 8 µl of the ISSR-PCR product solution was mixed with 2 µl 6X loading dye, and then loaded into a 1.5% agarose gel in an electrophoresis chamber filled with 1X TBE buffer. The gel with DNA fragment was developed at 100 Volt for 45 minute. After the electrophoresis, the gel was stained with ethidium bromide, destained with sterile deionized water, and observed under UV light. The band intensity of the PCR product was compared with 1 kb DNA ladder marker to obtain the size of the PCR product.

Data analysis

DNA fragment size was evaluated after photographed an agarose gel. The reproducible amplified bands were used to analyze the result by CLIDS 1D Pro software. The similarity index was calculate using Jaccard's UGMA and pairwise distance matrix was originated a dendrogram using UPGMA ground on the character deviations.

# CHAPTER IV

# RESULTS

## Part I : Pharmacognostic evaluation of *Derris elliptica* stems

### Macroscopic characteristics

*Derris elliptica* (Wall.) Benth. was illustrated showing botanical appearance as mentioned in flora of China that "a climbers, lianas, 7-10 m, robust. Young branchlets, leaf rachises, petioles, and petiolules densely brown pubescent. Branches glabrous when old, scattered with brown lenticels. Leaves 9-13-foliolate; rachis 20-35 cm, including petiole 4-8 cm; leaflet blades oblong, obovate-oblong, or oblanceolate, 6-15 × 2-4 cm, thickly papery, abaxially greenish white and finely brown sericeous, adaxially glabrous or only pubescent along veins, base cuneate to broadly cuneate, apex shortly obtusely acuminate. Pseudoracemes axillary, 15-25 cm, rachis densely pubescent; rachis nodes with 3 or 4 flowers fascicled on short branchlets; peduncle 8-12 cm or much longer, densely pubescent. Pedicel 6-8 mm, densely pubescent. Flowers ca. 2 cm. Calyx shallowly cup-shaped, ca. 4 × 6-7 mm, densely sericeous. Corolla pink to whitish, 1.5-1.8 cm; standard suborbicular, 1.2-1.5 cm wide, outside brown pubescent, apex emarginate. Ovary densely pubescent. Legume oblong, 3.5-8 × 1.7-2 cm, compressed, pubescent when young, glabrescent; abaxial suture with a ca. 0.5 mm wide wing, adaxial suture with a ca. 2 mm wide wing. Seeds 1-4 per legume (Flora of China, 2001)" (Figure 6).

*D. elliptica* dried stem crude drug was reddish-light yellow covered with reddish-brown bark (Figure 7).

Microscopic characteristics

The histological characteristics of *D. elliptica* stem powder included sclereids vessel, cork cell, prism crystal, part of group fiber, wood fiber, starch granules, bordered pitted, fiber, sclereids and spiral vessel (Figure 8). The transverse section of *D. elliptica* dried stem was performed to reveal the anatomical characteristics including cork cambium, vascular cambium, vascular bundle and pith (Figure 9).

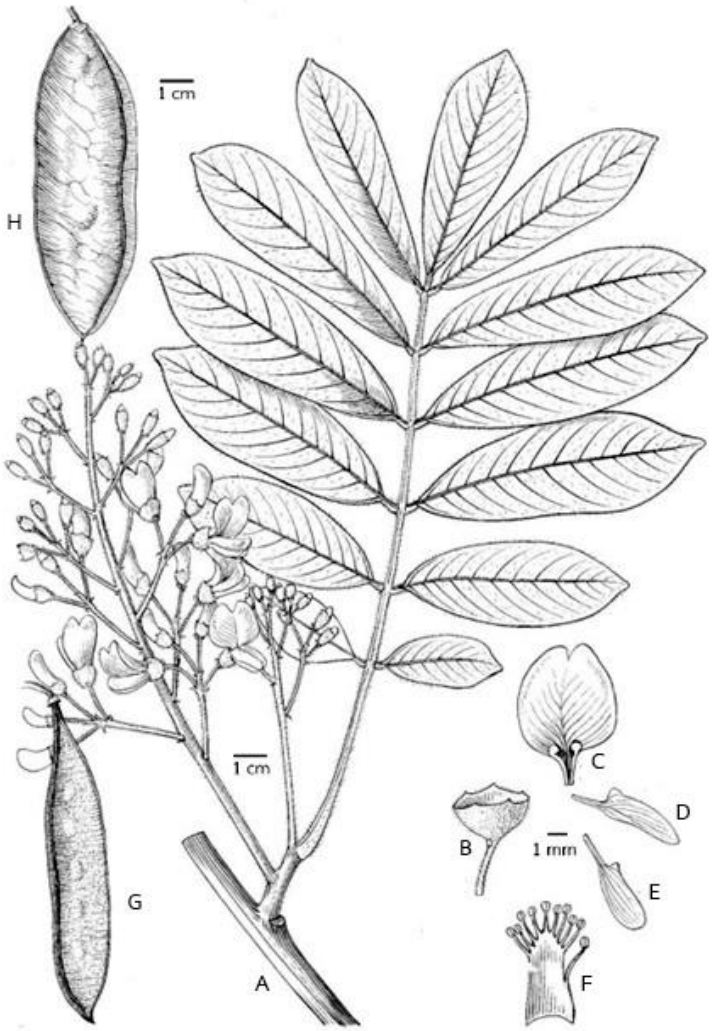



**Figure 6** The branches of *Derris elliptica*

A. flowering branch, B. calyx, C. standard, D. wing petal, E. keel petal, F. stamens, G. narrowly strap-like pod with two indistinct wings (occasional), H. broadly elliptic pod with one wing (commonly found)


**Figure 7** *Derris elliptica* dried stem crude drugs

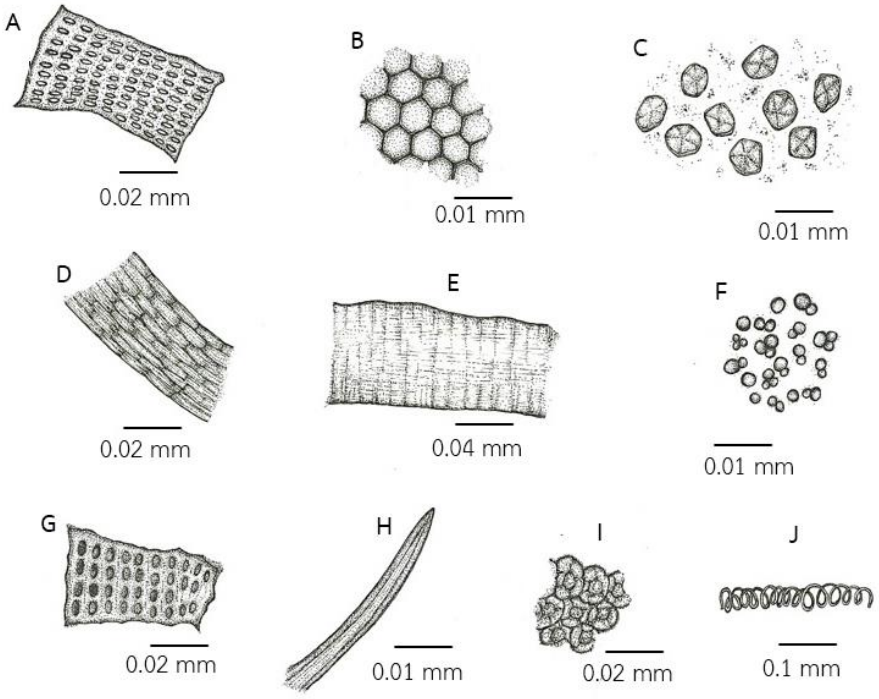



**Figure 8** Histological characteristics of *Derris elliptica* stem powder

A. sclereids vessel, B. cork cell, C. prism crystal, D. part of group fiber, E. wood fiber, F. starch granules, G. bordered pitted, H. fiber, I. Sclereids, J. spiral vessel

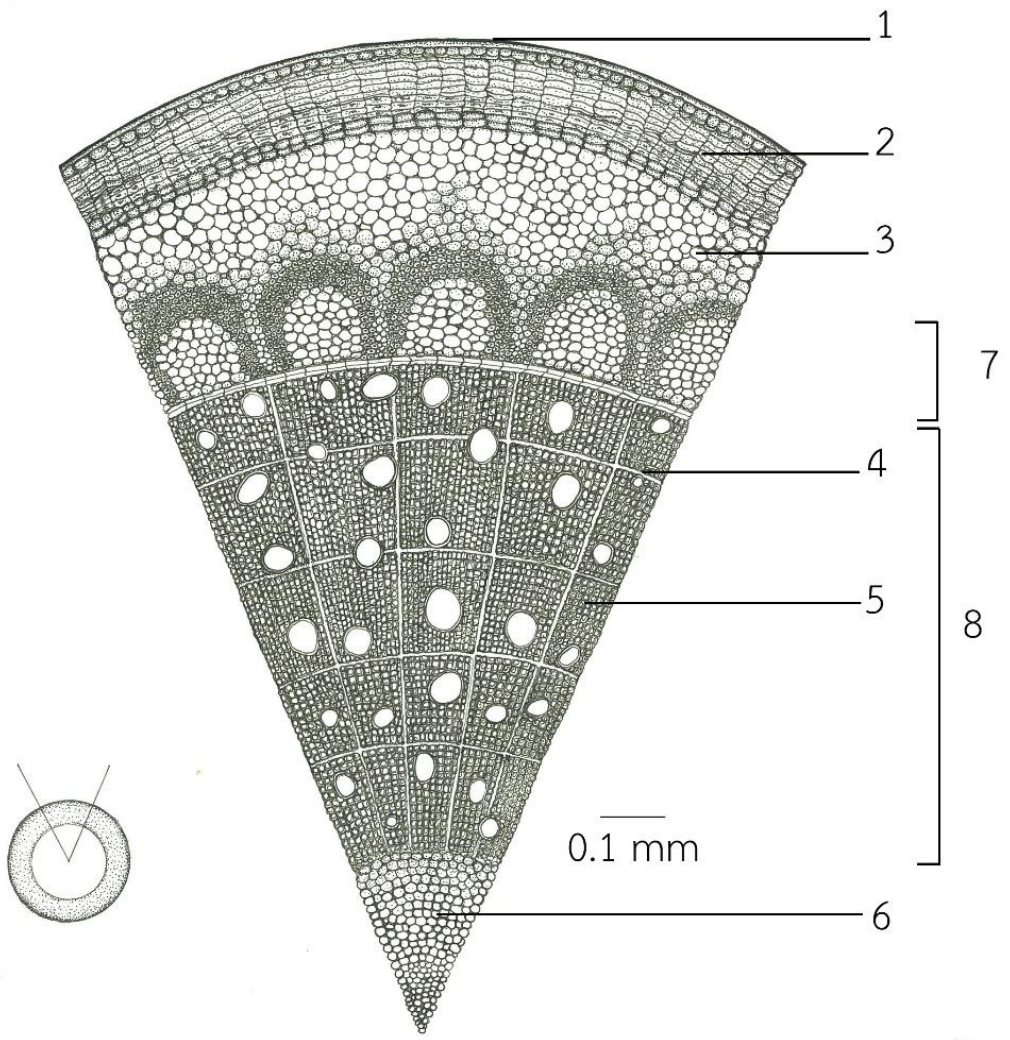





**Figure 9** The transverse section of *Derris elliptica* stem

1.epidermis, 2.cork cambium, 3. parenchyma, 4. xylem vessel, 5. xylem ray 6. pith, 7. vascular cambium, 8. vascular bundle

**Physico-chemical parameters of *Derris elliptica* stem**

The physico-chemical parameters of *D. elliptica* stem were shown in Table 8. The results indicated the specification for the quality control of *D. elliptica* stem crude drug that acid-insoluble ash, total ash, loss on drying and water contents should not be more than 1, 7, 5 and 8% by dry weight, respectively. In addition, ethanol and water soluble extractive matters should not be less than 4 and 11% by dry weight, respectively.

**Table 8** Physico-chemical parameters of *Derris elliptica* stem

| Parameter | Mean ± SD Content (% by weight)* | Min-Max |
|---|---|---|
| Acid-insoluble ash | 1.22±0.20 | 0.92-1.67 |
| Total ash | 7.35±0.63 | 6.06-8.44 |
| Ethanol-soluble extractive | 4.07±1.23 | 1.34-6.06 |
| Water-soluble extractive | 11.31±1.68 | 8.46-13.73 |
| Loss on drying | 5.77±0.92 | 4.15-7.50 |
| Water content | 8.81±1.30 | 7.22-11.89 |

*The parameters were shown as grand mean ± pooled SD. The samples from 15 locations were tested in triplicate per location.



**Thin layer chromatographic fingerprint of *Derris elliptica* stem**

Three microliters of *D. elliptica* stem ethanolic extract (1 mg/ml) was applied onto TLC silica gel 60 $GF_{254,}$ plate, developed in the solvent system (toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2)). and observed under UV light, both short and long wavelengths (254 and 365 nm), and then sprayed with 10% sulfuric acid in methanol as shown in Figure 10.

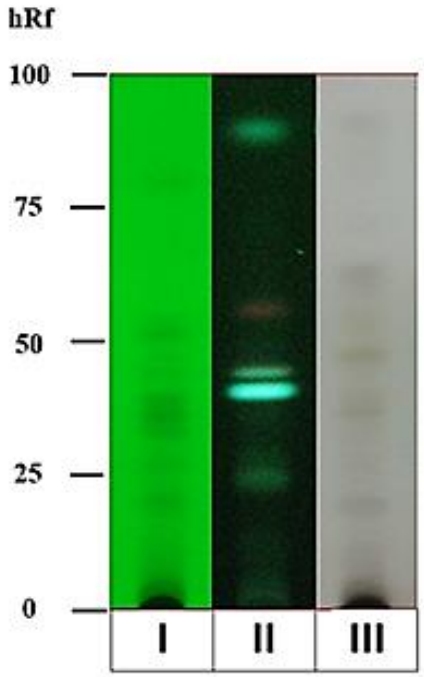



**Figure 10** TLC fingerprint of the ethanolic extract of *Derris elliptica* stem

**Solvent system**

Toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2)

**Detection**

I = detection under UV light 254 nm

II = detection under UV light 365 nm

III = detection with 10% sulfuric acid staining reagent and heat

**Part II Quantitative analysis of rotenone by TLC-densitometry and TLC-image analysis**

The dried stems powder of *D. elliptica* from 15 different sources throughout Thailand were exhaustively extracted with 95% ethanol by soxhlet apparatus. The average yield of *D. elliptica* stem extract was 13.93 ± 2.81 % by weight (Table 9).

**Table 9** The yield of *Derris elliptica* stems ethanolic extracts from 15 different sources throughout Thailand

| Source | weight of sample (g) | weight of ethanolic extract (g) | yield (g/100g) |
|---|---|---|---|
| 1.Bangkok | 5.0360 | 0.6045 | 12.0036 |
| 2.Angthong | 5.0049 | 0.6419 | 12.8254 |
| 3.Phuket | 5.0267 | 0.5964 | 11.8646 |
| 4.Chanthaburi | 5.0057 | 0.4841 | 9.6710 |
| 5.Khon Kaen | 5.0266 | 0.5281 | 10.5061 |
| 6.Trang | 5.0287 | 0.6308 | 12.5440 |
| 7. Sakon Nakhon | 5.0335 | 0.7975 | 15.8438 |
| 8. Chiang Rai | 5.0050 | 0.5207 | 10.4036 |
| 9. Nakhon Sawan | 5.0466 | 0.7816 | 15.4877 |
| 10. Uthai Thani 1 | 5.0852 | 0.8576 | 16.8646 |
| 11. Uthai Thani 2 | 5.0079 | 0.9766 | 19.5012 |
| 12. Chiang Mai | 5.0341 | 0.7168 | 14.2389 |
| 13. Lampang | 5.0462 | 0.7379 | 14.6229 |
| 14. Prachinburi | 5.0802 | 0.8248 | 16.2356 |
| 15. Lamphun | 5.0207 | 0.8230 | 16.3921 |
| | | **Mean ± SD** | 13.9337 ± 2.8171 |
| | | **Min – Max** | 9.6710 - 19.5012 |

**Rotenone content in *Derris elliptica* stem by TLC densitometry**

Standard rotenone standards and sample extracts were developed with the solvent system of toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2, v/v). CAMAG TLC scanner with winCAT software was used to detect rotenone on the silica gel 60 $GF_{254}$ TLC plate under 301 nm (λmax) providing the 3D TLC-densitogram of rotenone (Figure 11) and its peak areas. The amounts of rotenone in each extract and each crude drug were exhibited. The average content of rotenone in *D. elliptica* stem crude drug was 0.2870 ± 0.1242 g/100g (Table 10).

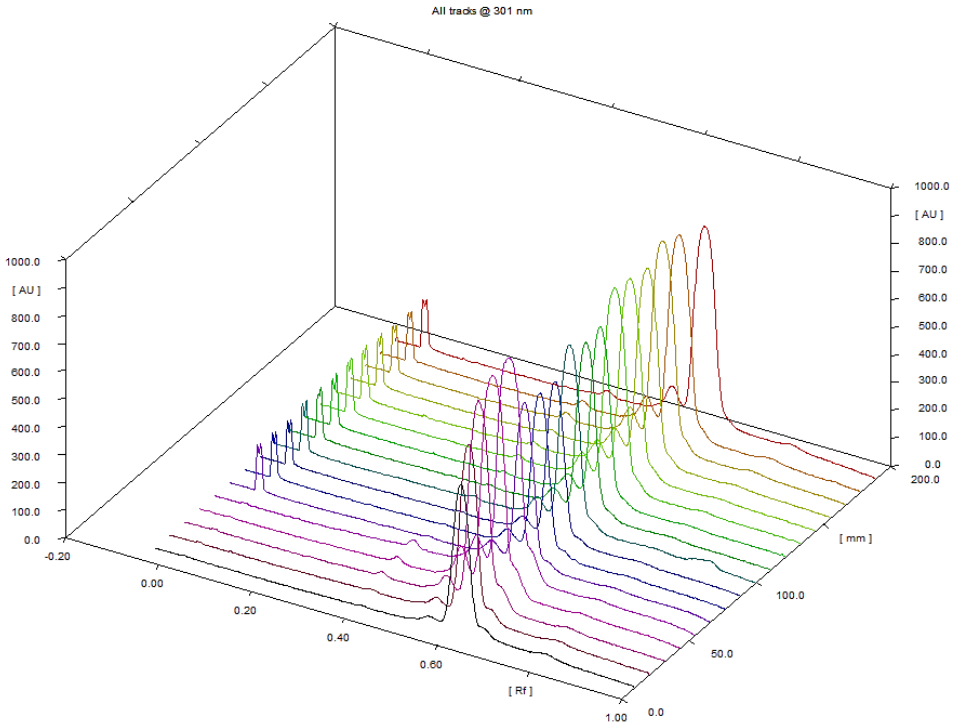



**Figure 11** TLC-densitogram of rotenone standards and *Derris elliptica* stem ethanolic extracts under UV 301 nm

**Table 10** The amounts of rotenone in *Derris elliptica* stems from 15 different sources throughout Thailand by TLC-densitometry

| Source | Rotenone in the ethanolic extract (g/g) | Yield of the ethanolic extract (g/ 100g of dried crude drug) | Rotenone in *D. elliptica* stem (g/ 100g of dried crude drug) |
|---|---|---|---|
| 1.Bangkok | 0.0083 | 12.0036 | 0.0992 |
| 2.Angthong | 0.0139 | 12.8254 | 0.1784 |
| 3.Phuket | 0.0160 | 11.8646 | 0.1903 |
| 4.Chanthaburi | 0.0194 | 9.6710 | 0.1875 |
| 5.Khon Kaen | 0.0136 | 10.5061 | 0.1428 |
| 6.Trang | 0.0274 | 12.5440 | 0.3440 |
| 7. Sakon Nakhon | 0.0136 | 15.8438 | 0.2157 |
| 8. Chiang Rai | 0.0169 | 10.4036 | 0.1756 |
| 9. Nakhon Sawan | 0.0208 | 15.4877 | 0.3224 |
| 10. Uthai Thani 1 | 0.0272 | 16.8646 | 0.4582 |
| 11. Uthai Thani 2 | 0.0242 | 19.5012 | 0.4710 |
| 12. Chiang Mai | 0.0261 | 14.2389 | 0.3710 |
| 13. Lampang | 0.0302 | 14.6229 | 0.4410 |
| 14. Prachinburi | 0.0246 | 16.2356 | 0.3994 |
| 15. Lamphun | 0.0188 | 16.3921 | 0.3084 |
| | | **Mean ± SD** | 0.2870 ± 0.1242 |
| | | **Min - Max** | 0.0992 – 0.4710 |

**Method validation (TLC-densitometry)**

The tested parameters for method validation including calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were performed according to ICH guideline.

Calibration range

The calibration curve of standard rotenone was shown as polynomial regression in the range of 0.90 – 6.00 µg/spot with the regression equation of $y = -438.02x^2 + 5930.3x + 11961$. The coefficient of determination ($R^2$) of rotenone was 0.9991 Figure 12

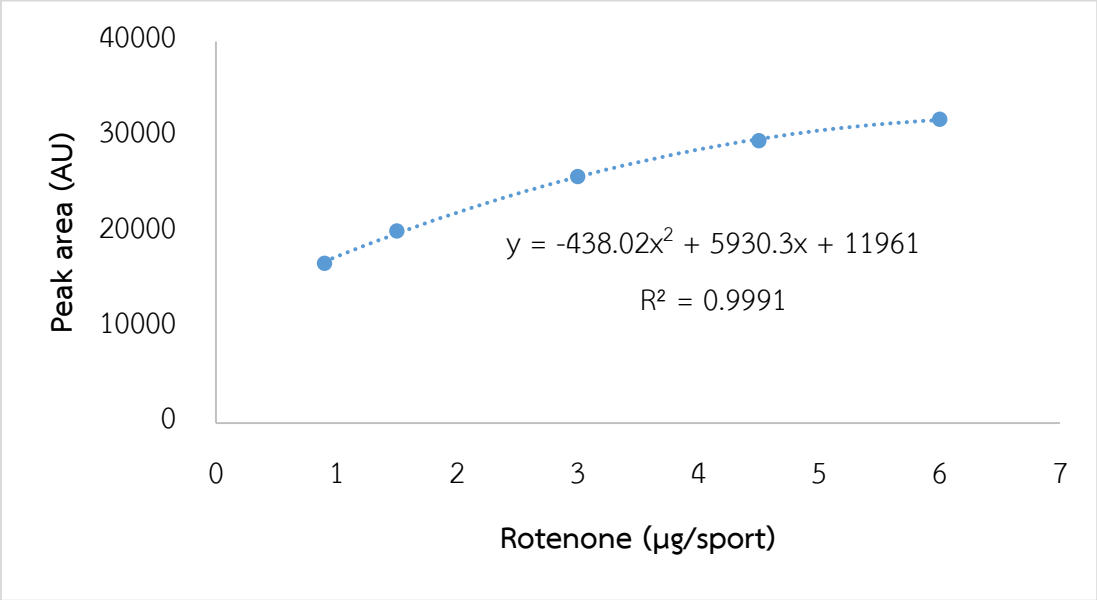



**Figure 12** The calibration curve of standard rotenone by TLC-densitometry

Detection limit and quantitation limit

The detection limit (LOD) and quantitation limit (LOQ) were obtained from the calculation based on the slope of the calibration curve and the standard deviation of regression line. The lowest concentration of analyte in a sample that could be detected was found to be 0.13 µg/spot, whereas the lowest concentration of analyte in a sample that could be quantitatively defined was found to be 0.39 µg/spot.

Accuracy

The accuracy of rotenone quantification in the ethanolic extracts of *D. elliptica* stem was validated using the recovery analysis. Standard rotenone was spiked into the extract, providing low, medium and high concentrations of rotenone. The recovery values were 96.23 – 105.08% as shown in Table 11.

**Table 11** Accuracy of quantification of rotenone in *Derris elliptica* stem by TLC-densitometry (n=3)

| **Rotenone added (µg/spot)** | **Rotenone found (µg/spot)** | **% Recovery** |
|---|---|---|
| 0.0 | 1.81±0.05 | - |
| 1.2 | 3.16±0.06 | 104.89±3.77 |
| 2.8 | 4.85±0.07 | 105.09±2.69 |
| 4.8 | 6.36±0.14 | 96.23±2.74 |
| **Average** | | 99.62±2.35 |

Precision

The precision was interpreted as %RSD at four concentrations of rotenone in the extracts and determined by repeatability and intermediate precision on the same day and three different days, respectively. The repeatability and intermediate precision were found to be 1.67 and 3.32 %RSD, respectively Table 12

**Table 12** Repeatability and intermediate precision of rotenone in *Derris elliptica* stem by TLC-densitometry (n=3)

| Repeatability | | Intermediate precision | |
|---|---|---|---|
| Amount (µg/spot) | %RSD | Amount (µg/spot) | %RSD |
| 1.70 ± 0.01 | 0.86 | 1.78 ± 0.09 | 4.90 |
| 2.98 ± 0.08 | 2.69 | 3.05 ± 0.09 | 2.88 |
| 4.51 ± 0.06 | 1.26 | 4.63 ± 0.17 | 3.58 |
| 6.18 ± 0.11 | 1.85 | 6.15 ± 0.12 | 1.91 |
| **Average** | 1.67±0.80 | | 3.32 ± 1.25 |

Specificity

*Peak identity and peak purity*

The similarity of light absorption spectra of the peaks at apex among standard rotenone and all samples and the similarity of light absorption spectra of the sample peak at up-slope, apex and down-slope represented peak identity (Figure 13) and peak purity (Figure 14), respectively. The maximum absorbance of rotenone was at the wavelength of 301 nm.

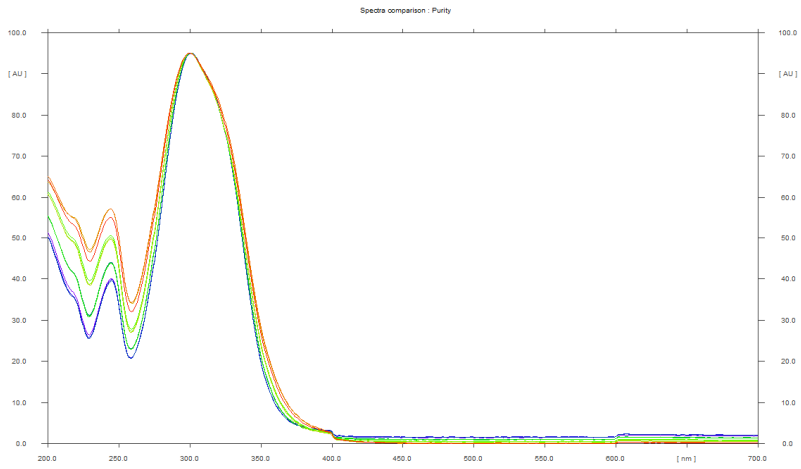



**Figure 13** The absorbance spectra of rotenone in *Derris elliptica* stem extracts from 15 different sources and standard rotenone presenting peak identity

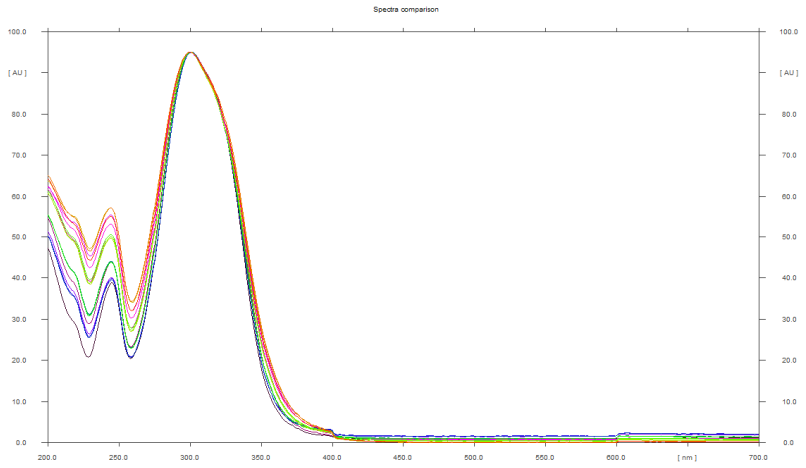



**Figure 14** Peak purity measurement using up-slope, apex and down-slope of the peak

Robustness

The robustness of rotenone quantification in *Derris elliptica* stem ethanolic extracts by TLC-densitometric analysis was investigated by varying the ratio of toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2, v/v) as shown in Table 13. The robustness result was found to be 0.75 %RSD of peak area.

**Table 13** Robustness of rotenone in *Derris elliptica* stem by TLC-densitometry

| Mobile phase ratio | | | | |
|---|---|---|---|---|
| **Toluene** | **Chloroform** | **Acetone** | **Formic acid** | **Peak area** |
| 5.0 | 4.0 | 1.0 | 0.2 | 28572.44 |
| 5.2 | 3.8 | 0.8 | 0.4 | 28211.09 |
| 5.1 | 3.9 | 1.1 | 0.1 | 28164.05 |
| 4.9 | 4.1 | 0.7 | 0.3 | 28555.66 |
| **Mean ± SD** | | | | 28310.27± 213.82 |
| **%RSD** | | | | 0.75 |

**Rotenone contents in *Derris elliptica* stem by TLC-image analysis**

The same developed TLC plate used in TLC-densitometry was photographed under UV 254 nm by TLC visualizer. The obtained image was analyzed for rotenone peak area by ImageJ software. The average amount of rotenone was found to be 0.2843 ± 0.1209 g/ 100g of the crude drug (Table 14).

**Table 14** The amounts of rotenone in *Derris elliptica* stems from 15 different sources throughout Thailand by TLC-image analysis

| Source | Rotenone in the ethanolic extract (g/g) | Yield of the ethanolic extract (g/ 100g of dried crude drug) | Rotenone in *D. elliptica* stem (g/ 100g of dried crude drug) |
|---|---|---|---|
| 1.Bangkok | 0.0086 | 12.0036 | 0.1035 |
| 2.Angthong | 0.0138 | 12.8254 | 0.1773 |
| 3.Phuket | 0.0164 | 11.8646 | 0.1945 |
| 4.Chanthaburi | 0.0187 | 9.6710 | 0.1812 |
| 5.Khon Kaen | 0.0138 | 10.5061 | 0.1448 |
| 6.Trang | 0.0264 | 12.5440 | 0.3308 |
| 7. Sakon Nakhon | 0.0134 | 15.8438 | 0.2127 |
| 8. Chiang Rai | 0.0174 | 10.4036 | 0.1814 |
| 9. Nakhon Sawan | 0.0210 | 15.4877 | 0.3245 |
| 10. Uthai Thani 1 | 0.0267 | 16.8646 | 0.4506 |
| 11. Uthai Thani 2 | 0.0245 | 19.5012 | 0.4777 |
| 12. Chiang Mai | 0.0259 | 14.2389 | 0.3695 |
| 13. Lampang | 0.0293 | 14.6229 | 0.4279 |
| 14. Prachinburi | 0.0235 | 16.2356 | 0.3808 |
| 15. Lamphun | 0.0188 | 16.3921 | 0.3080 |
| | | **Mean ± SD** | 0.2844 ± 0.1209 |
| | | **Min – Max** | 0.1035 – 0.4777 |

Method validation (TLC-image analysis)

Calibration range

The calibration curve of standard rotenone was shown as polynomial regression in the range of 0.90 – 6.00 µg/spot with the regression equation of $y = 14.215x^2 + 10891x - 5906.3$. The coefficient of determination ($R^2$) of rotenone was 0.9992 (Figure 15).

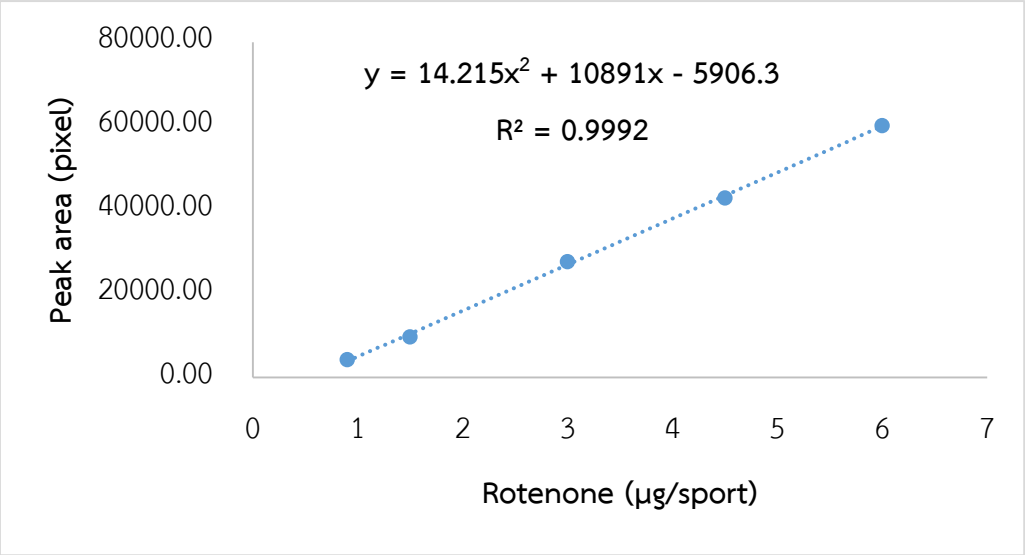



**Figure 15** The calibration curve of standard rotenone by TLC-image analysis



Detection limit and quantitation limit

The lowest concentration for analytic in a sample that could be detected (LOD) was found to be 0.27 µg/spot, whereas the lowest concentration for analyte in a sample that could be quantitatively defined (LOQ) was found to be 0.83 µg/spot.

Accuracy

The recovery values were 96.57-105.71 % as demonstrated in Table 15.

**Table 15** Accuracy of quantification of rotenone in *Derris elliptica* stem by TLC-image analysis (n=3)

| Rotenone added (µg/spot) | Rotenone found (µg/spot) | % Recovery |
|---|---|---|
| 0.0 | 1.82±0.05 | - |
| 1.2 | 3.18±0.05 | 105.20±3.88 |
| 2.8 | 4.89±0.06 | 105.71±2.54 |
| 4.8 | 6.40±0.15 | 96.57±3.00 |
| **Average** | | 102.49±5.30 |

Precision

The repeatability and intermediate precision values were found to be 2.12 and 3.43 %RSD, respectively (Table 16).

**Table 16** Repeatability and intermediate precision of rotenone in *Derris elliptica* stem by TLC-image analysis (n=3)

| Repeatability | | Intermediate precision | |
|---|---|---|---|
| **Amount (µg/spot)** | **%RSD** | **Amount (µg/spot)** | **%RSD** |
| 1.82 ± 0.06 | 3.05 | 1.78 ± 0.08 | 4.30 |
| 3.18 ± 0.06 | 1.84 | 3.02 ± 0.12 | 3.84 |
| 4.89 ± 0.06 | 1.24 | 4.70 ± 0.12 | 2.56 |
| 6.40 ± 0.15 | 2.35 | 6.33 ± 0.19 | 3.03 |
| **Average** | 2.12±0.77 | | 3.43±0.78 |

Robustness

The robustness of rotenone quantification in *D. elliptica* stem ethanolic extracts by TLC-image analysis was investigated by varying the ratio of the toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2, v/v) as shown in Table 17 The robustness result was found to be 0.20 %RSD of peak area.

**Table 17** Robustness of rotenone in *Derris elliptica* stem by TLC-image analysis

| Mobile phase ratio | | | | Peak area |
|---|---|---|---|---|
| **Toluene** | **Chloroform** | **Acetone** | **Formic acid** | |
| 5.0 | 4.0 | 1.0 | 0.2 | 25515.35 |
| 5.2 | 3.8 | 0.8 | 0.4 | 25097.22 |
| 5.1 | 3.9 | 1.1 | 0.1 | 25078.09 |
| 4.9 | 4.1 | 0.7 | 0.3 | 25174.10 |
| | | **Mean ± SD** | | 25116.47 ± 50.82 |
| | | **%RSD** | | 0.20 |

Method comparison

Rotenone contents determined by TLC-densitometry and TLC-image analysis were demonstrated in Table 18. The obtained contents were statistically analyzed by paired t-test. The result showed that rotenone contents obtained from both methods were not significantly different ($P > 0.05$).

**Table 18** rotenone contents in *Derris elliptica* stem by TLC-densitometry and TLC-image analysis

| Source | Rotenone content (g/ 100 g of dried crude drug) | |
|---|---|---|
| | TLC-densitometry | TLC-image analysis |
| 1.Bangkok | 0.0992 | 0.1035 |
| 2.Angthong | 0.1784 | 0.1773 |
| 3.Phuket | 0.1903 | 0.1945 |
| 4.Chanthaburi | 0.1875 | 0.1812 |
| 5.Khon Kaen | 0.1428 | 0.1448 |
| 6.Trang | 0.3440 | 0.3308 |
| 7. Sakon Nakhon | 0.2157 | 0.2127 |
| 8. Chiang Rai | 0.1756 | 0.1814 |
| 9. Nakhon Sawan | 0.3224 | 0.3245 |
| 10. Uthai Thani 1 | 0.4582 | 0.4506 |
| 11. Uthai Thani 2 | 0.4710 | 0.4777 |
| 12. Chiang Mai | 0.3710 | 0.3695 |
| 13. Lampang | 0.4410 | 0.4279 |
| 14. Prachinburi | 0.3994 | 0.3808 |
| 15. Lamphun | 0.3084 | 0.3080 |
| **Mean ± SD** | 0.2870 ± 0.1242 | 0.2844 ± 0.1209 |
| **Min – Max** | 0.0992 – 0.4710 | 0.1035 – 0.4777 |

**Part III : *In vitro* biological activities**

**Antioxidant activities**

DPPH radical scavenging activity

The DPPH radical scavenging abilities of *D. elliptica* stem ethanolic extract and standard rotenone with reference to the positive controls, quercetin and BHT, were demonstrated in Table 19. *D. elliptica* stem extract exhibited the highest antioxidant activity ($IC_{50}$ = 0.02 µg/ml), followed by rotenone ($IC_{50}$ = 0.62 µg/ml), quercetin ($IC_{50}$ = 3.69 µg/ml) and BHT ($IC_{50}$ = 28.59 µg/ml). The results of all tested samples demonstrated a dose-response relationship (Figure 16).

**Table 19** DPPH radical scavenging activity ($IC_{50}$) of *Derris elliptica* ethanolic extracts, rotenone and positive controls

| Tested sample | $IC_{50}$ (µg/ml) |
|---|---|
| *Derris elliptica* stem ethanolic extract | 0.02 |
| Rotenone | 0.62 |
| Quercetin | 3.69 |
| BHT | 28.59 |

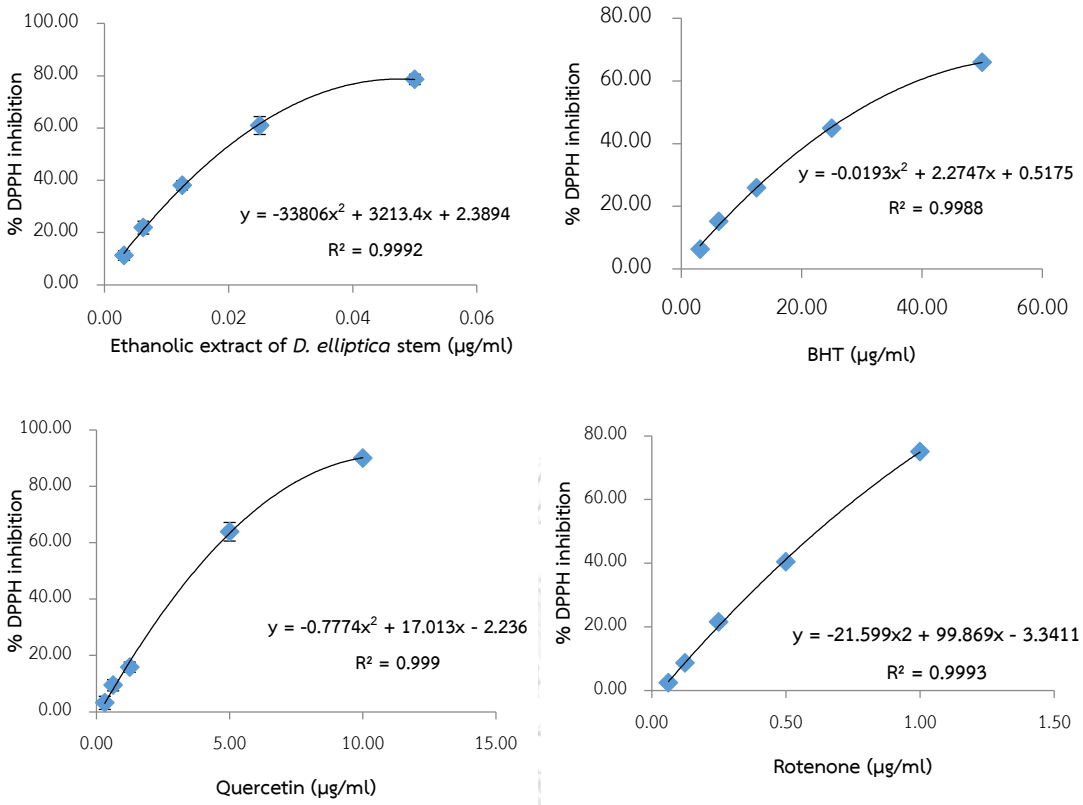



**Figure 16** DPPH scavenging activity of tested samples

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was used to evaluate antioxidant activity on ferric ion reduction of the ethanolic extracts of *Derris elliptica* stem and the standard rotenone. The amounts of ferrous ion occurred were compared from the calibration curve of ferrous sulfate (Figure 17). Rotenone had the lowest antioxidant effect with FRAP value (0.02 mM Fe(II)/mg) when compared to the positive controls, quercetin and BHT (2.56 and 1.02 mM Fe(II)/mg, respectively). *D. elliptica* extracts was likely to express the reducing power ability as same as BHT with FRAP values of 1.01 and 1.02 mM Fe(II)/mg, respectively (Table 20).

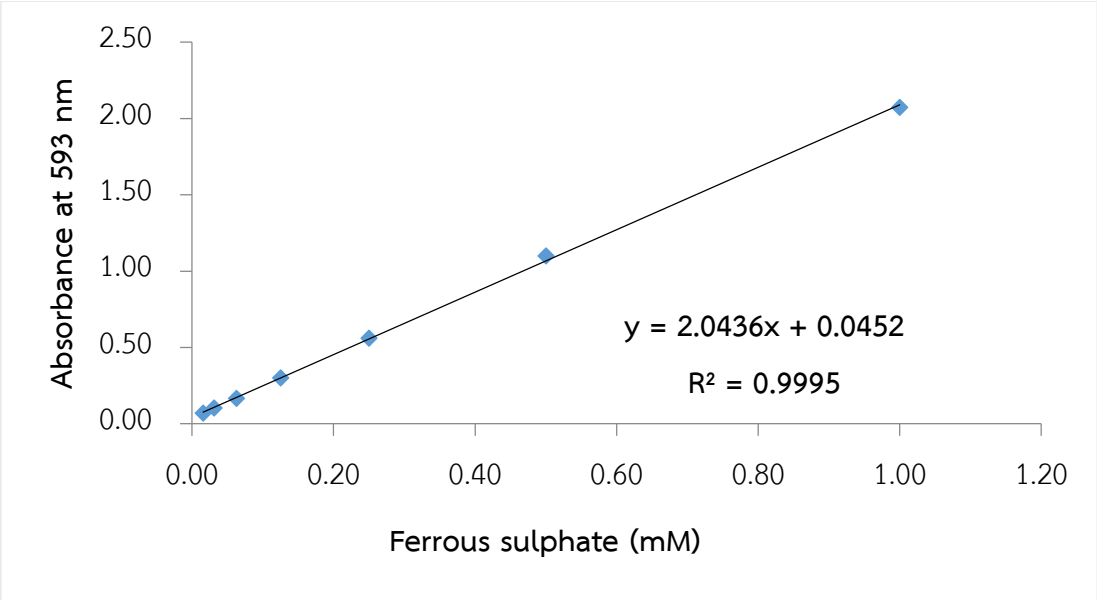



**Figure 17** Standard curve for determination of antioxidant capacity by ferric ion reducing antioxidant power

**Table 20** Ferric reducing antioxidant power of *Derris elliptica* ethanolic extracts, rotenone and positive controls

| Tested sample | FRAP value (mM Fe(II)/mg) |
|---|---|
| *Derris elliptica* stem | 1.01 |
| Rotenone | 0.02 |
| Quercetin | 2.56 |
| BHT | 1.02 |

Beta-carotene bleaching assay

The peroxidation inhibitions in the beta-carotene bleaching assay were demonstrated in a dose-response relationship (Table 21; Figure 18). *D. elliptica* stem ethanolic extract at 1 mg/ml showed 71.13% antioxidant activity compared to 10.04% of rotenone at the same concentration. BHT and quercetin which were used as positive controls showed peroxidation inhibition of 93.90% and 92.02%, respectively.

**Table 21** Beta-carotene bleaching inhibitory activity of *Derris elliptica* ethanolic extracts rotenone and positive control at the concentration of 1 mg/ml

| Tested sample | % Beta-carotene bleaching inhibition |
|---|---|
| *Derris elliptica* stem ethanolic extract | 71.13 |
| Rotenone | 10.04 |
| Quercetin | 92.02 |
| BHT | 93.90 |

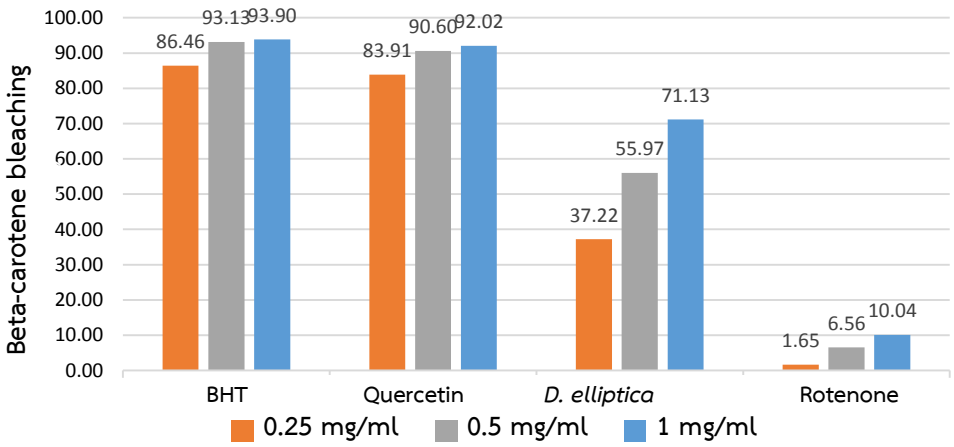



**Figure 18** The antioxidant activity of various concentrations of tested sample compared to BHT and quercetin by beta-carotene bleaching assay

Total phenolic content

The ethanolic extract of *D. elliptica* stem was assessed for the total phenolic content using Folin-Ciocalteu's phenol reagent. Gallic acid was used to generate a calibration curve (Figure 19). The *D. elliptica* ethanolic extract revealed the total phenolic content of 265.48 ± 9.042 mg GAE/g extract.

**Table 22** Total phenolic content of the ethanolic extract of *Derris elliptica* stem, which calculated using equation from standard curve of gallic acid

| Sample (0.5 mg/ml) | Absorbance at 756 nm | | | | Total phenolic content (mg GAE/g extract) |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | mean ± SD | |
| *D. elliptica* stem | 0.216 | 0.208 | 0.223 | 0.216 ± 0.008 | 265.48 ± 9.042 |

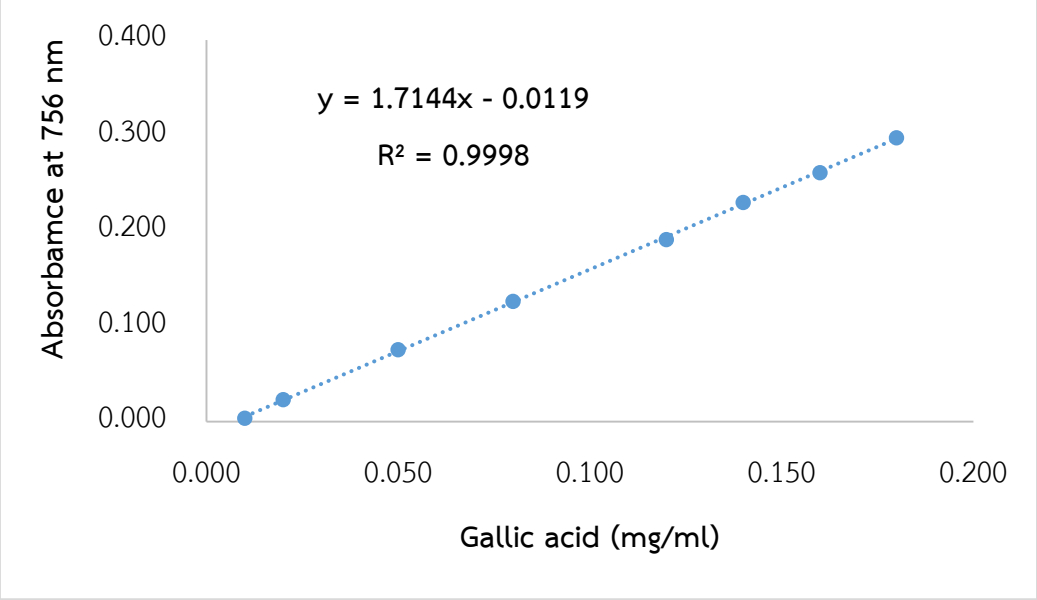



**Figure 19** Gallic acid calibration curve for total phenolic quantification

Total flavonoid content

The ethanolic extract of *D. elliptica* was evaluated for the flavonoid content using the aluminum chloride colorimetric method. The result indicated that the extract had the total flavonoid content of 9.48 ± 0.68 mg quercetin equivalent/g extract.

**Table 23** Total flavonoid content of the ethanolic extract of *Derris elliptica*, which calculated using equation from standard curve of quercetin

| Sample (0.5 mg/ml) | Absorbance at 510 nm | | | | Total flavonoid content (mg QE/g extract) |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | mean ± SD | |
| Ethanolic extract *D. elliptica* stem | 0.029 | 0.031 | 0.033 | 0.031 ± 0.008 | 9.48 ± 0.68 |

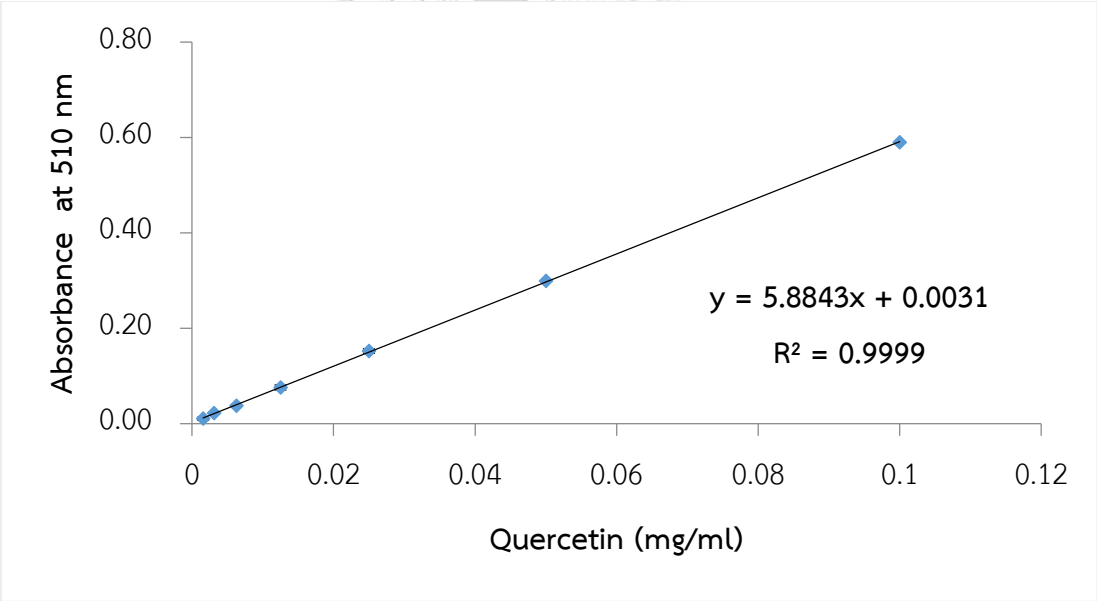



**Figure 20** Quercetin acid calibration curve for total flavonoid quantification

Antidiabetic activities

The results of antidiabetic activities of *D. elliptica* stem ethanolic extracts, standard rotenone and positive control were obtained from 2 types of α-glucosidase assays as shown in Table 24. All tested samples displayed a concentration-response relationship (Figure 21,22).

Rotenone and *D. elliptica* stem extract exhibited greater potential effect on yeast α-glucosidase inhibition than a positive control, acarbose with $IC_{50}$ values of 0.86, 0.06 and 7.69 mg/ml, respectively.

Rat intestinal α-glucosidase inhibitory assay indicated that rotenone ($IC_{50}$ value > 0.6 mg/ml) and *D. elliptica* stem extract ($IC_{50}$ value = 0.59 mg/ml) had antidiabetic effects, but still lesser than acarbose ($IC_{50}$ value of 0.39 mg/ml).

**Table 24** In vitro antidiabetic activities of *Derris elliptica* ethanolic extracts, rotenone acid and positive control

| Tested sample | $IC_{50}$ (mg/ml) | |
|---|---|---|
| | **Rat intestinal α-glucosidase inhibition** | **Yeast α-glucosidase inhibition** |
| *D. elliptica* stem ethanolic | 0.59 | 0.07 |
| extract | > 0.6* | 0.87 |
| Rotenone | 0.39 | 7.70 |
| Acarbose | | |

***** Rotenone > 0.6 (mg/ml) could not be determined because of turbidity

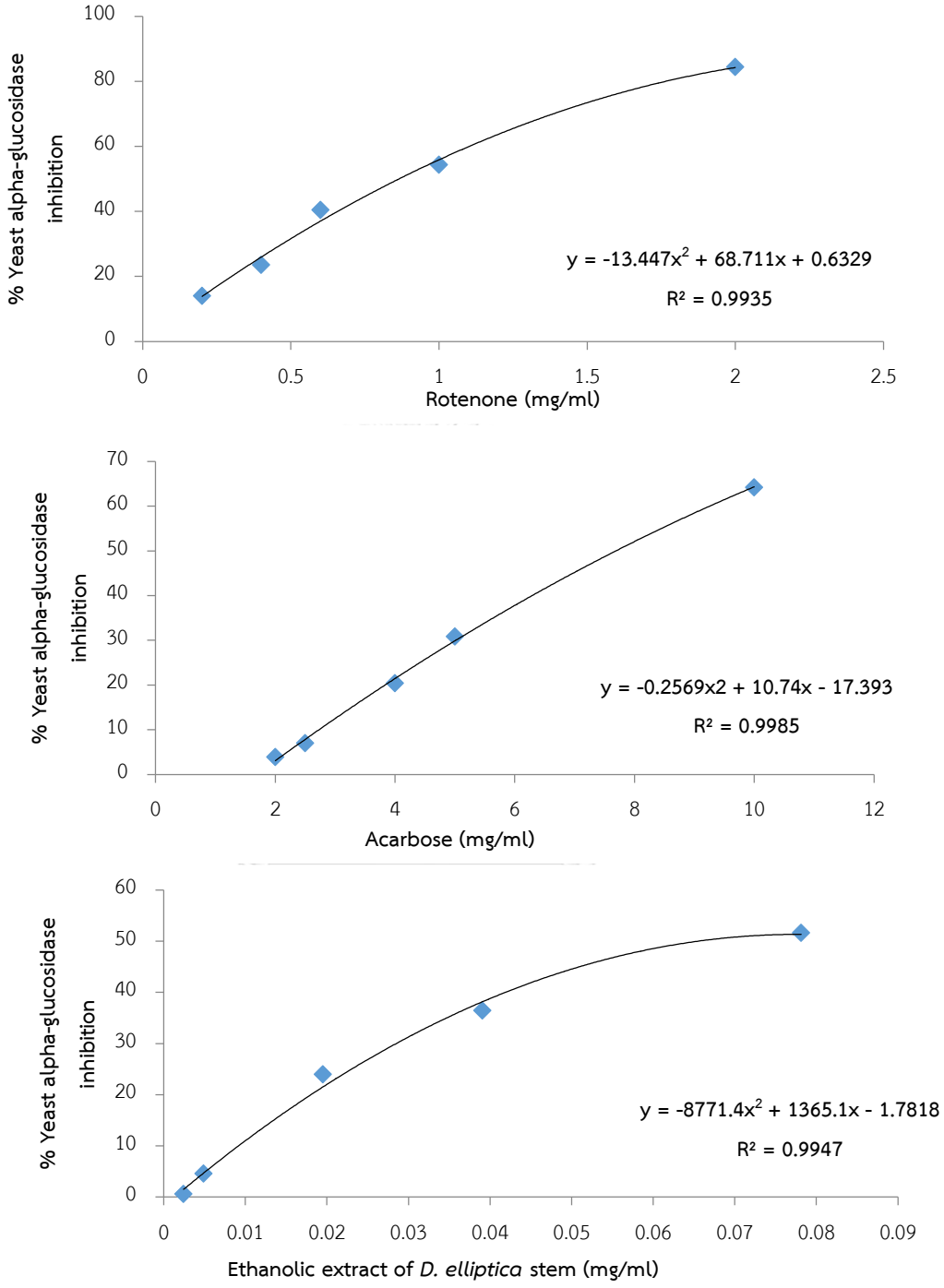



**Figure 21** Yeast α-glucosidase inhibition of test samples

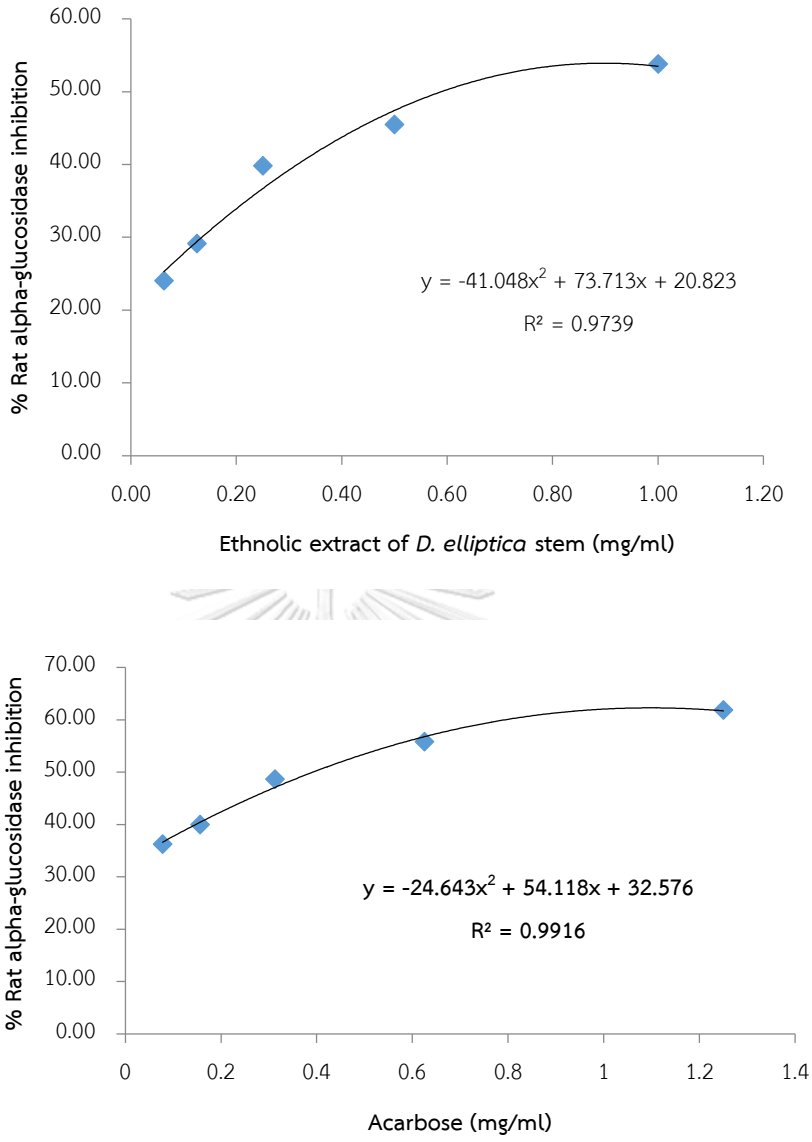



**Figure 22** Rat intestinal α-glucosidase inhibition of test samples

**Cytotoxic activities**

Brine shrimp lethality activity

The results of brine shrimp lethality activity of *D. elliptica* stem ethanolic extract and rotenone were shown in Figure 23. According to $LC_{50}$ values: $LC_{50}$ values > 1000 µg/ml (non-toxic), ≥ 500 ≤ 1000 µg/ml (weak toxic) and < 500 µg/ml (toxic). *D. elliptica* stem extract revealed the toxicity against brine shrimp nauplii with $LC_{50}$ of 56.02 µg/ml (toxic), and the standard rotenone displayed $LC_{50}$ of 0.16 µg/ml (toxic).(Table 25)

**Table 25** Brine shrimp lethality ($LC_{50}$) of the ethanolic extract of *Derris elliptica* stem

| **Sample** | **Brine shrimp lethality (24 hrs) $LC_{50}$ (µg/ml)** |
|---|---|
| Ethanolic extract of *D. elliptica* stem | 56.02 |
| Rotenone | 0.16 |

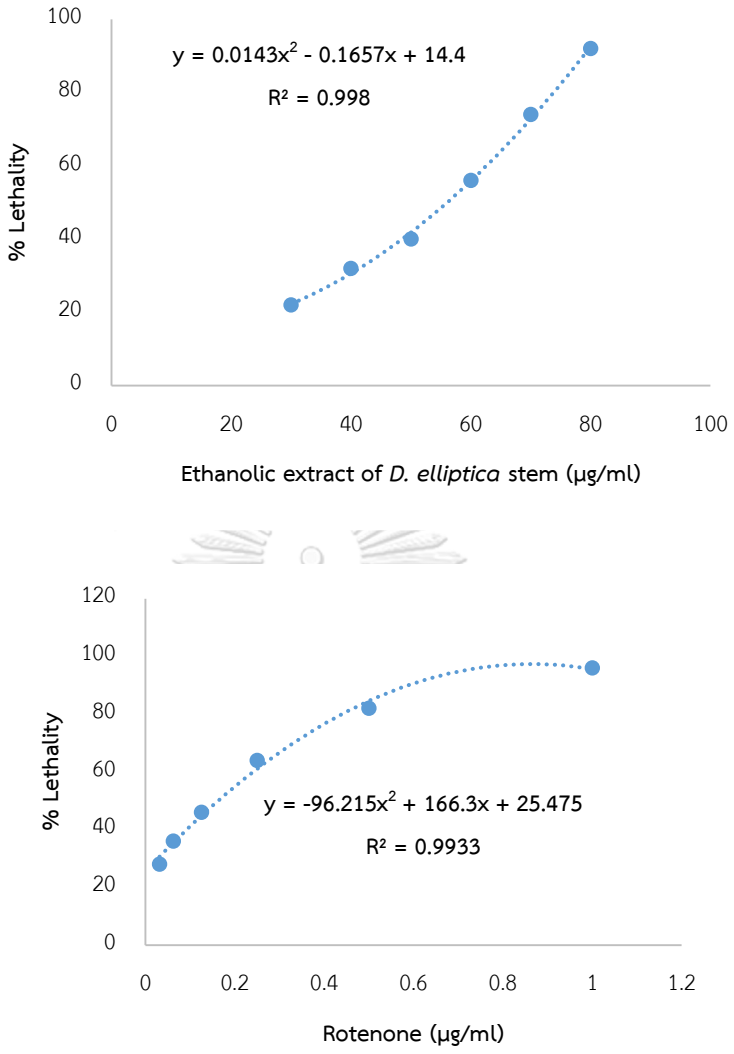



**Figure 23** Cytotoxic activity of tested samples due to brine shrimp lethality testing

Cytotoxic activity against cancer cell lines

The cytotoxicity on human cancer cell lines of the standard rotenone and the ethanolic extract of *D. elliptica* was demonstrated with concentration-dependent relationship (Appendix D, Table 41-42). The *D. elliptica* ethanolic extract showed $IC_{50}$ of 80.40 µg/ml against HT-29. Rotenone showed $IC_{50}$ of 73.59 µg/ml for MDA-231, 50.29 µg/ml for HepG2, and 67.96 µg/ml for HT-29 (Table26). The $IC_{50}$ values against other cell lines of the extract and rotenone were more than 100 µg/ml (Table 26). As a result, the *D. elliptica* ethanolic extract and rotenone presented marginal cytotoxic activity

against four cell lines with the $IC_{50}$ value more than that of the standard criteria (20 µg/ml).

**Table 26** Cytotoxic activities of *Derris elliptica* stem ethanolic extract, and rotenone against cancer cell lines

| Sample | $IC_{50}$ (µg/ml)* | | | |
|---|---|---|---|---|
| | MDA-231 | HepG2 | HT-29 | Caco-2 |
| Ethanolic extract of *D. elliptica* stem | >100 | >100 | 80.40 ± 1.74 | >100 |
| Rotenone | 73.59± 1.86 | 50.29 ± 3.09 | 67.96 ± 1.51 | >100 |

* Mean ± standard deviation (n = 3)

**Part IV : Plant morphology and microscopic characteristics including leaf constant numbers of selected *Derris* species**

**Plant morphology of selected *Derris* species**

Eight *Derris* species including *Derris amoena* Benth., *Derris elliptica* (Wall.) Benth., *Derris malaccensis* Prain, *Derris indica* (Lam.) Bennet, *Derris reticulata* Craib, *Derris scandens* (Roxb.) Benth., *Derris solorioides* Sirich.& Adema, *Derris trifoliata* (Lour.) Tabu. were observed, photographed, and illustrated by hand drawing in the proportion size related to an original scale (Figur 24-31).

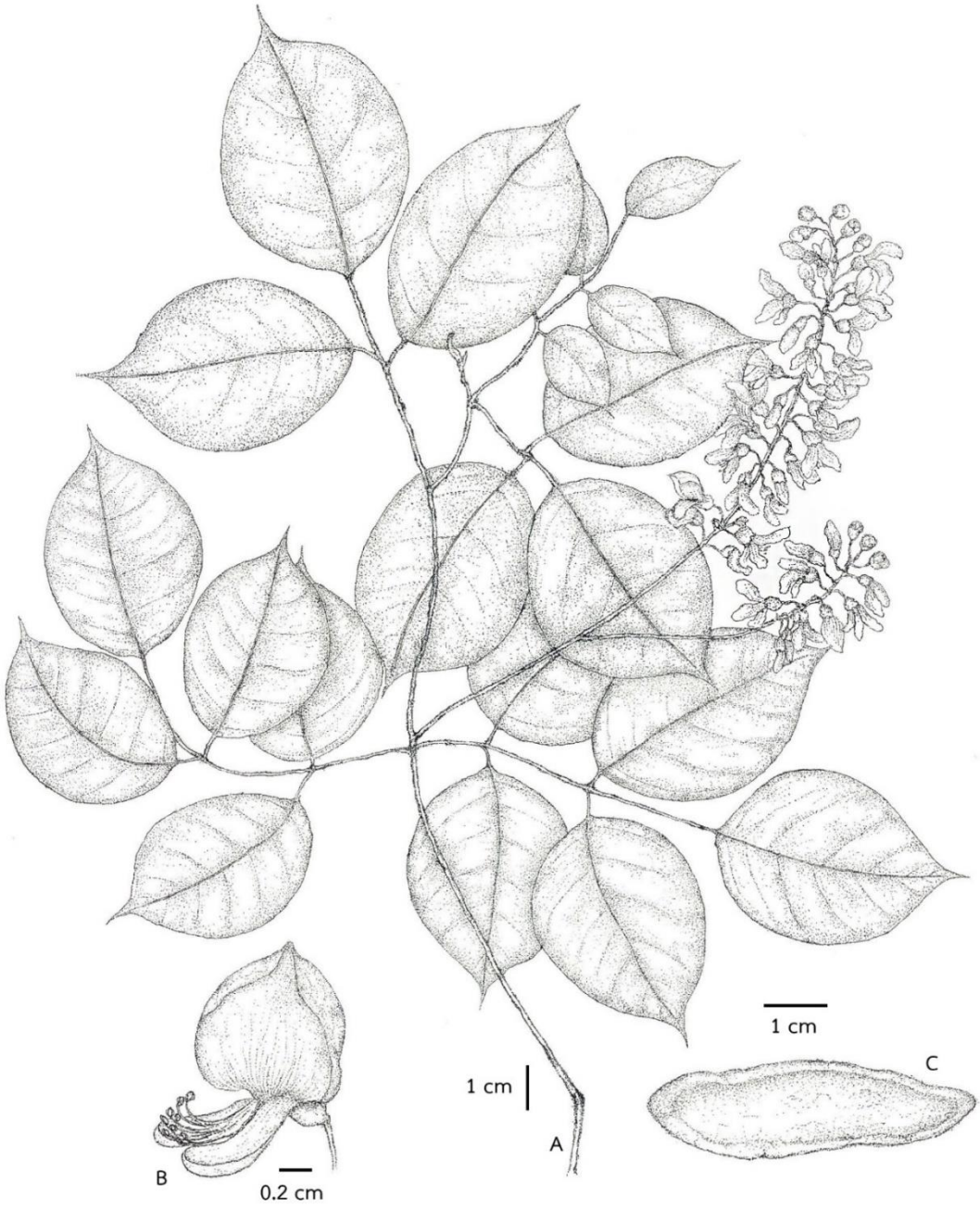



**Figure 24** The branches of *Derris amoena*

A. flowering branch, B. flower, C. Pod

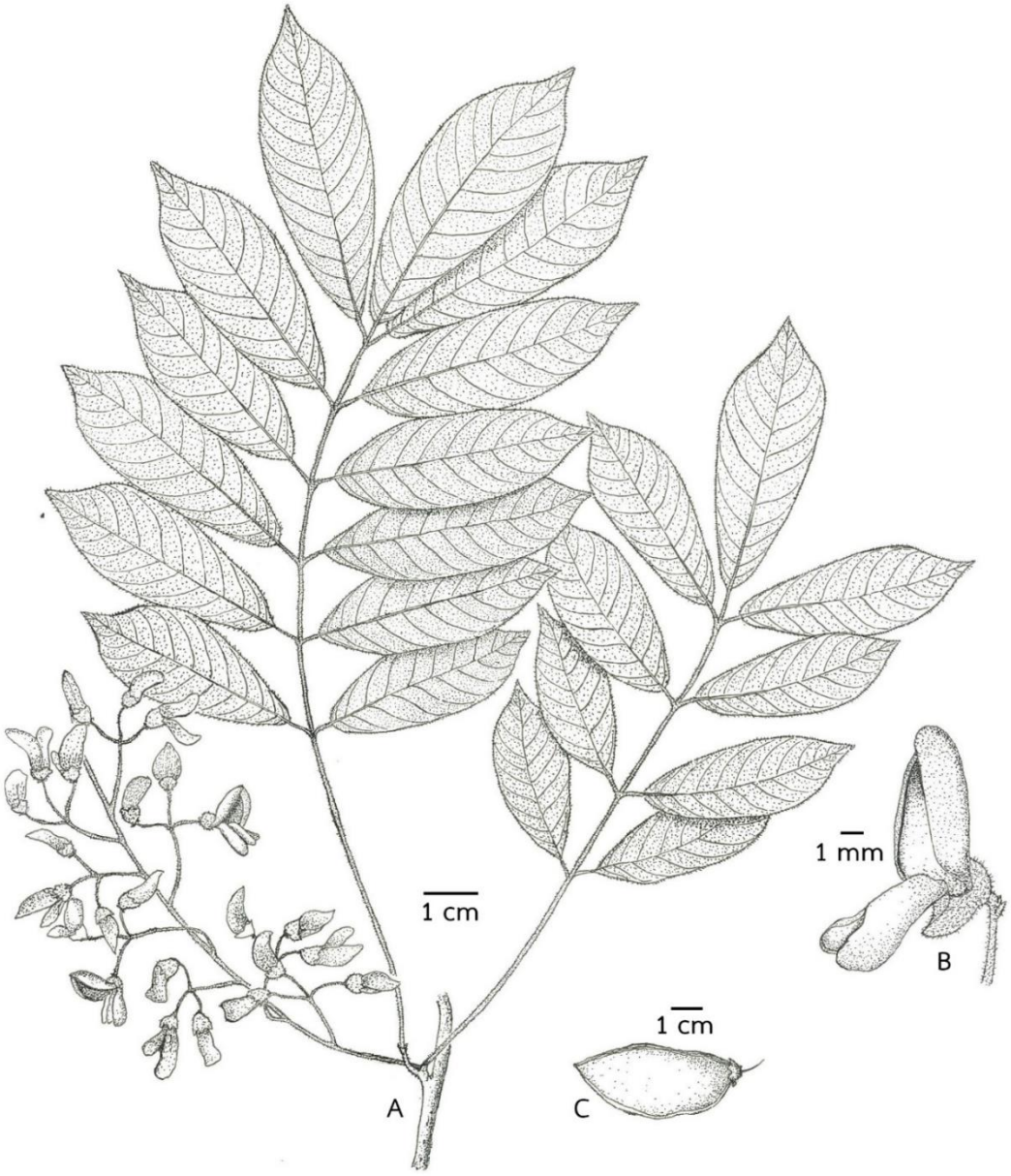



**Figure 25** The branches of *Derris elliptica*

A. flowering branch, B. flower, C. Pod


**Figure 26** The branches of *Derris malaccensis*

A. flowering branch, B. flower

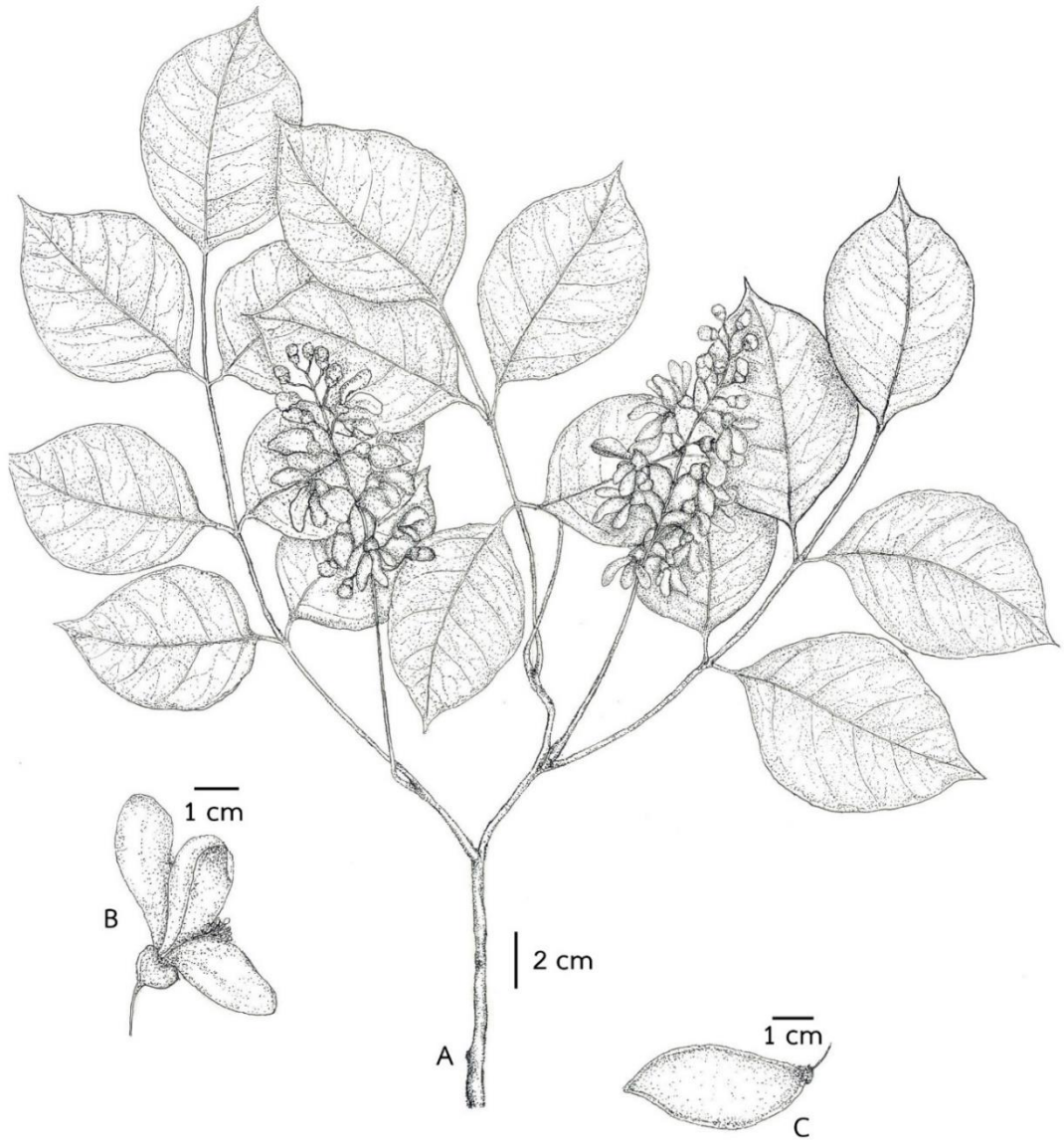



**Figure 27** The branches of *Derris indica*

A. flowering branch, B. flower, C. Pod


**Figure 28** The branches of *Derris reticulata*

A. flowering branch, B. flower, C. Pod

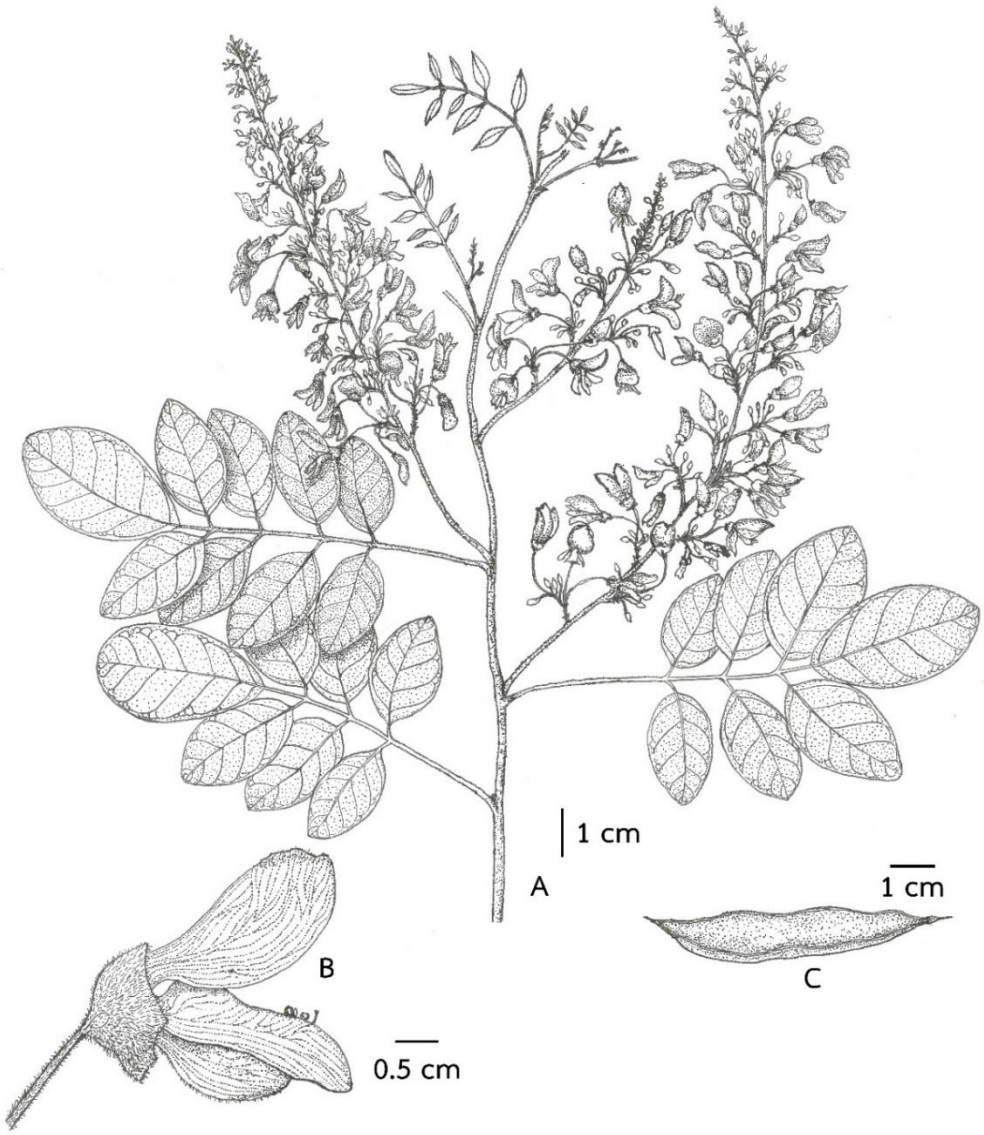



**Figure 29** The branches of *Derris scandens*

A. flowering branch, B. flower, C. Pod

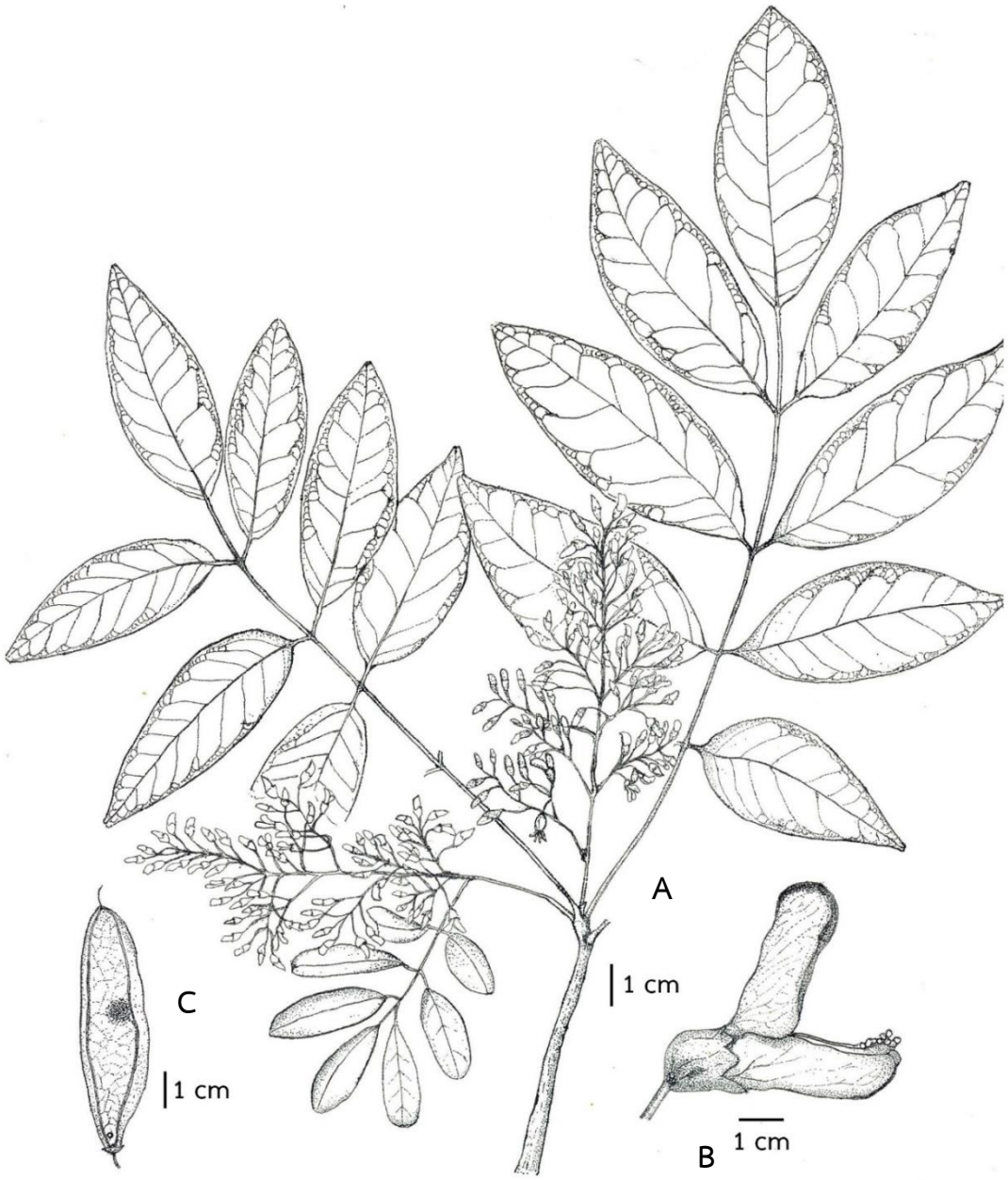



**Figure 30** The branches of *Derris solorioides*

A. flowering branch, B. flower, C. Pod

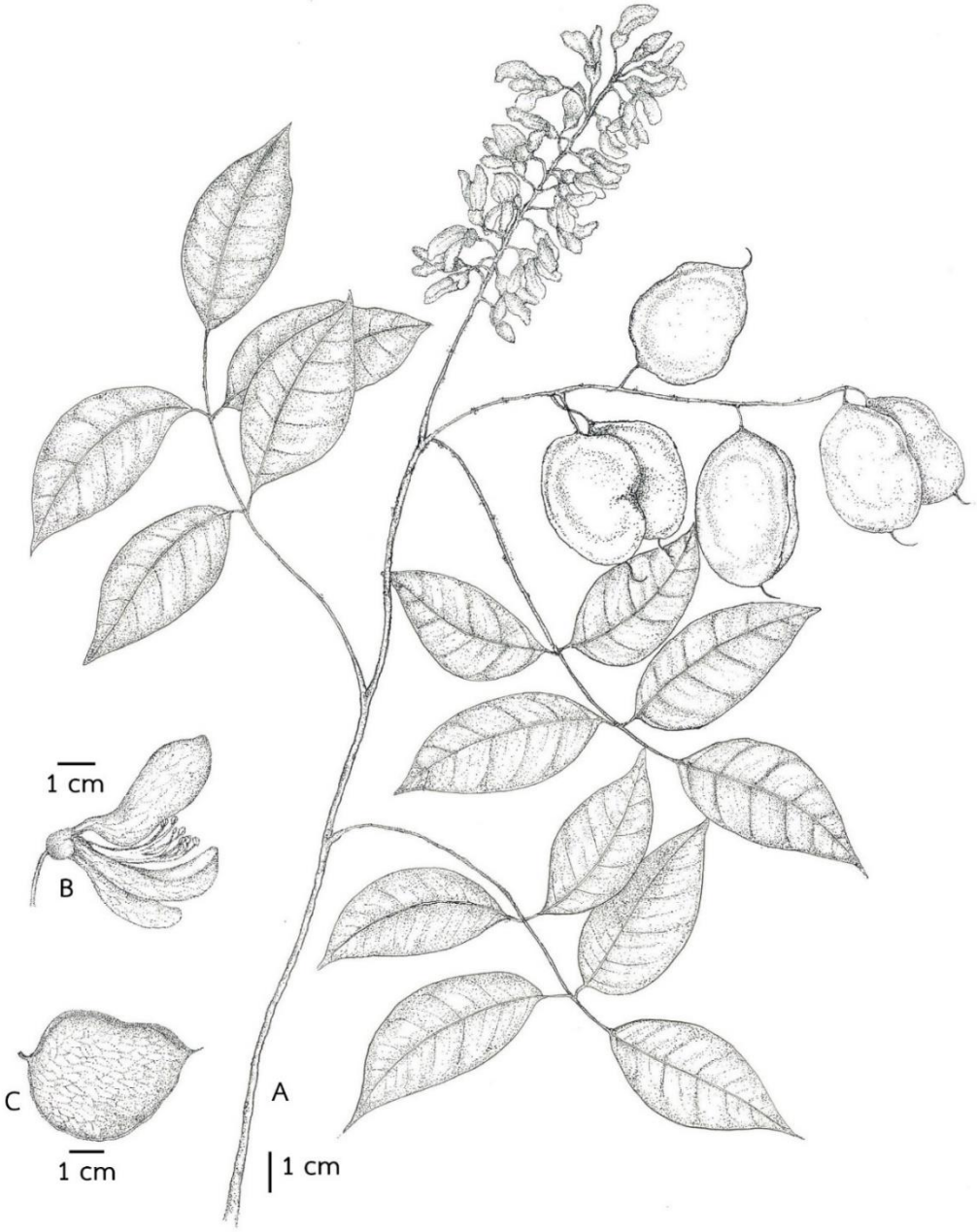
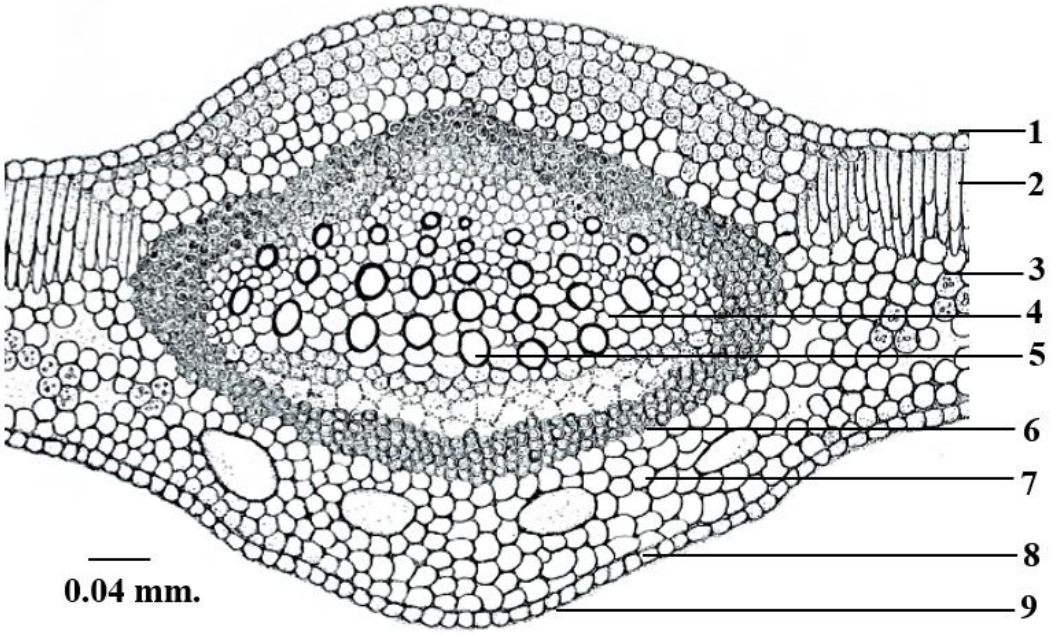



**Figure 31** The branches of *Derris trifoliata*

A. flowering branch, B. flower, C. Pod

Microscopic leaf anatomical evaluation

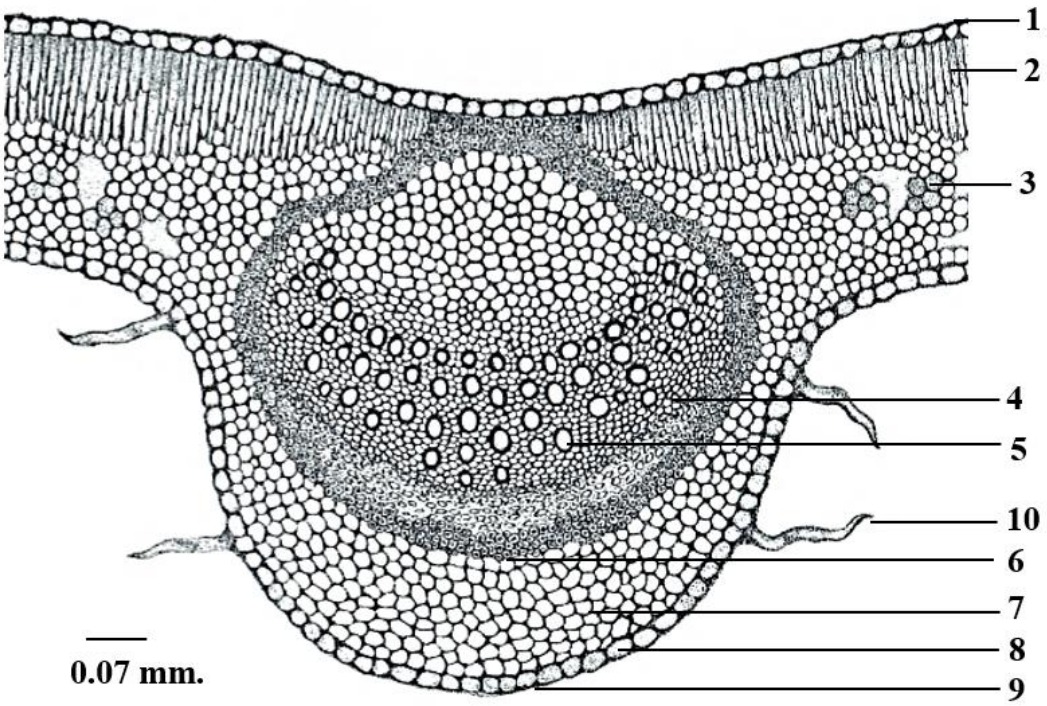
The midrib cross section of the leaf of eight *Derris* species were investigated and illustrated (Figure 32-39) The structures, consisted of upper epidermis, palisade cell, spongy cell, sclerenchyma, xylem tissue, phloem tissue, parenchyma, collenchyma and lower epidermis, were presented from all species. Trichomes were obviously found only in two species, at the midrib of the leaf (*D. elliptica* and *D. scandens*).




**(a) *Derris amoena***

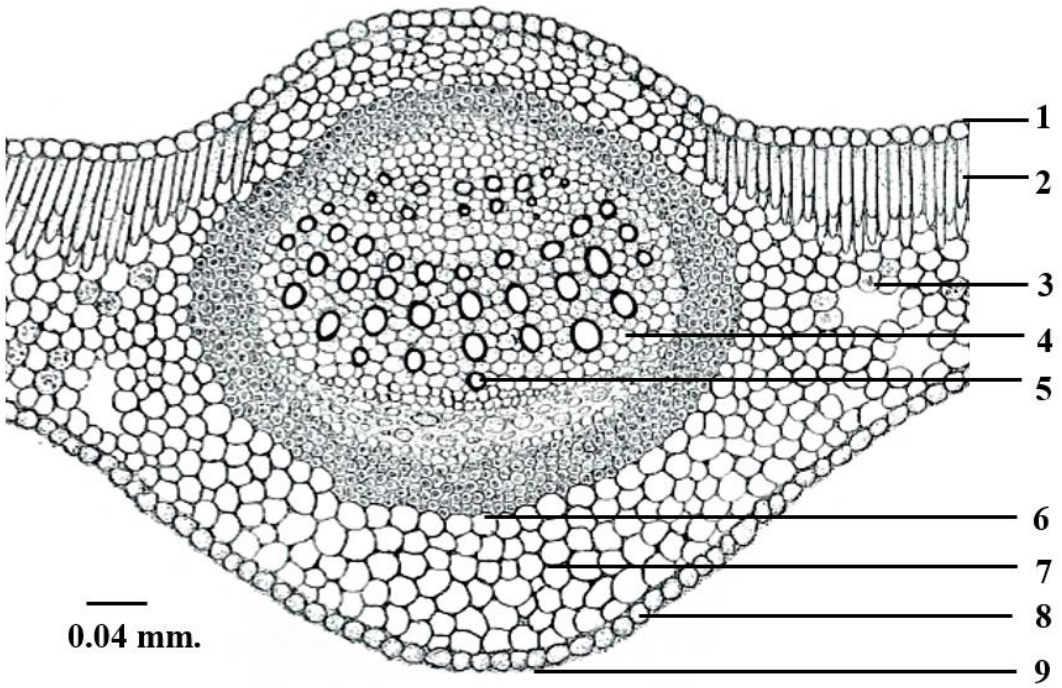


**Figure 32** Anatomical characteristics of *Derris amonena* leaf midrib (cross-section) 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma,9. Lower epidermis



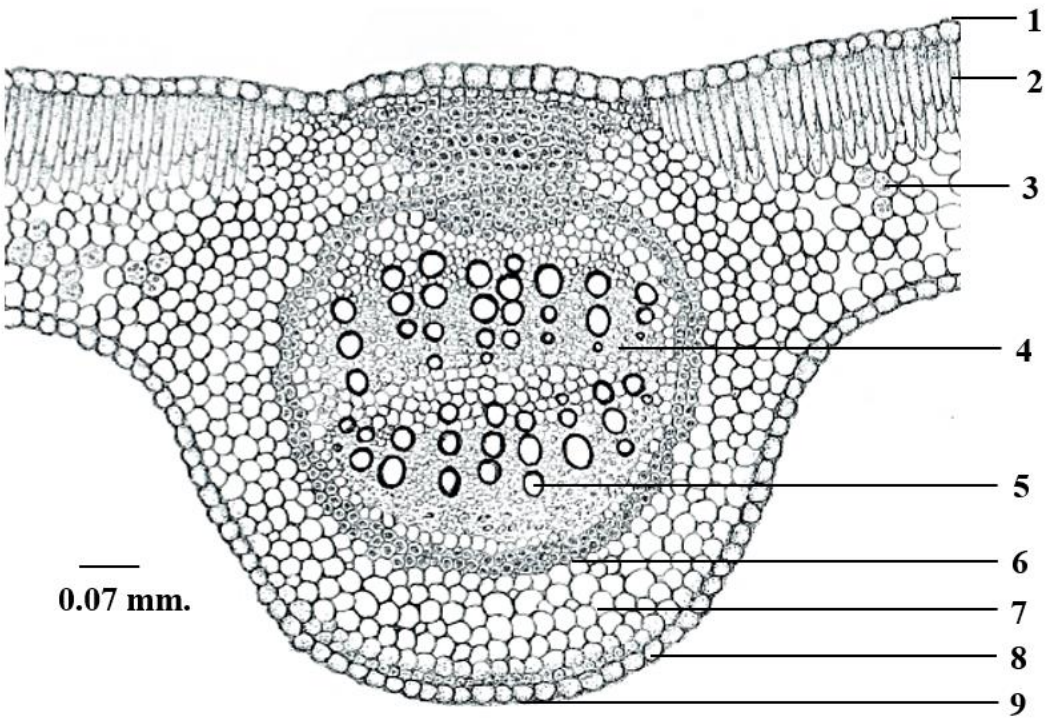

**(b) *Derris elliptica***

**Figure 33** Anatomical characteristics of *Derris elliptica* leaf midrib (cross-section) 1.Upper epidermis, 2.Palisade cell, 3.Spongy cell, 4.Sclerenchyma, 5.Xylem tissue, 6.Phloem tissue, 7.Parenchyma, 8.Collenchyma, 9.Lower epidermis, .10 Unicellular non-glandular trichome



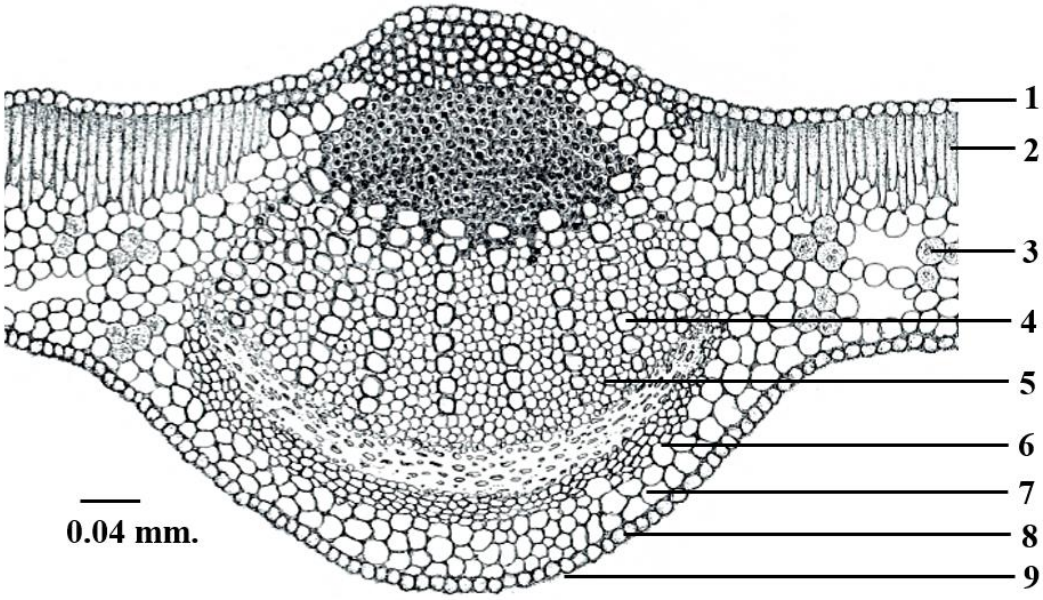

**(c) *Derris malaccensis***

**Figure 34** Anatomical characteristics of *Derris malaccensis* leaf midrib (cross-section) 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis



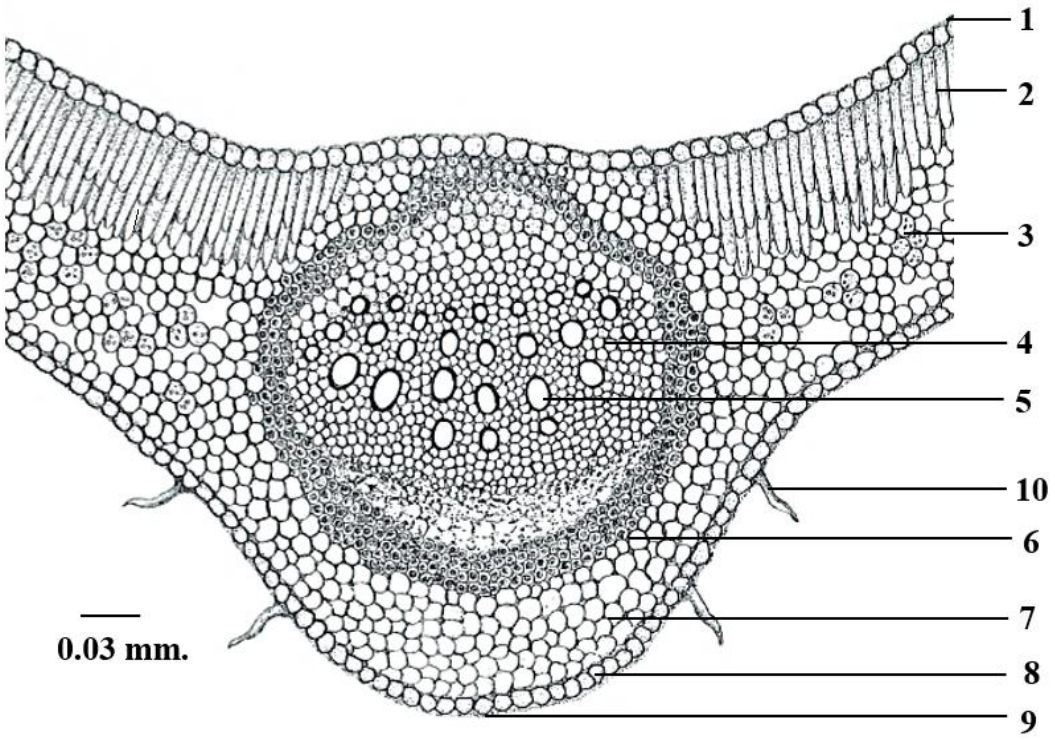

(d) *Derris indica*

**Figure 35** Anatomical characteristics of *Derris indica* leaf midrib (cross-section)
1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis



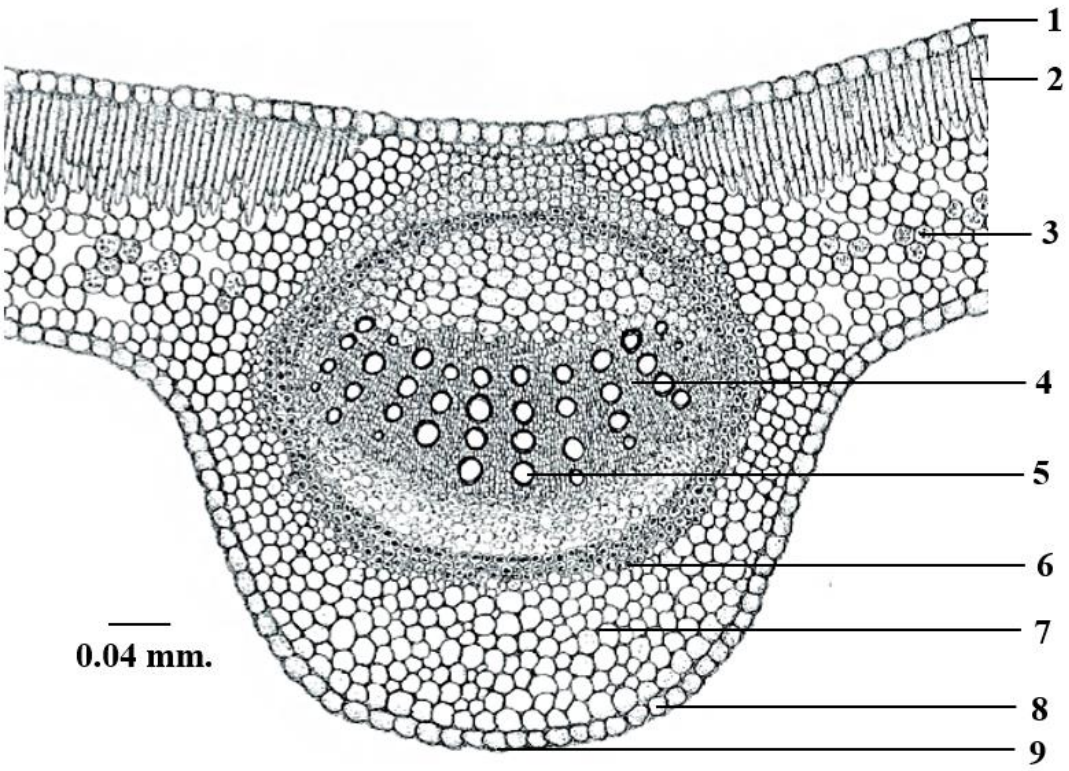

**(e) *Derris reticulata***

**Figure 36** Anatomical characteristics of *Derris reticulata* leaf midrib (cross-section) 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis



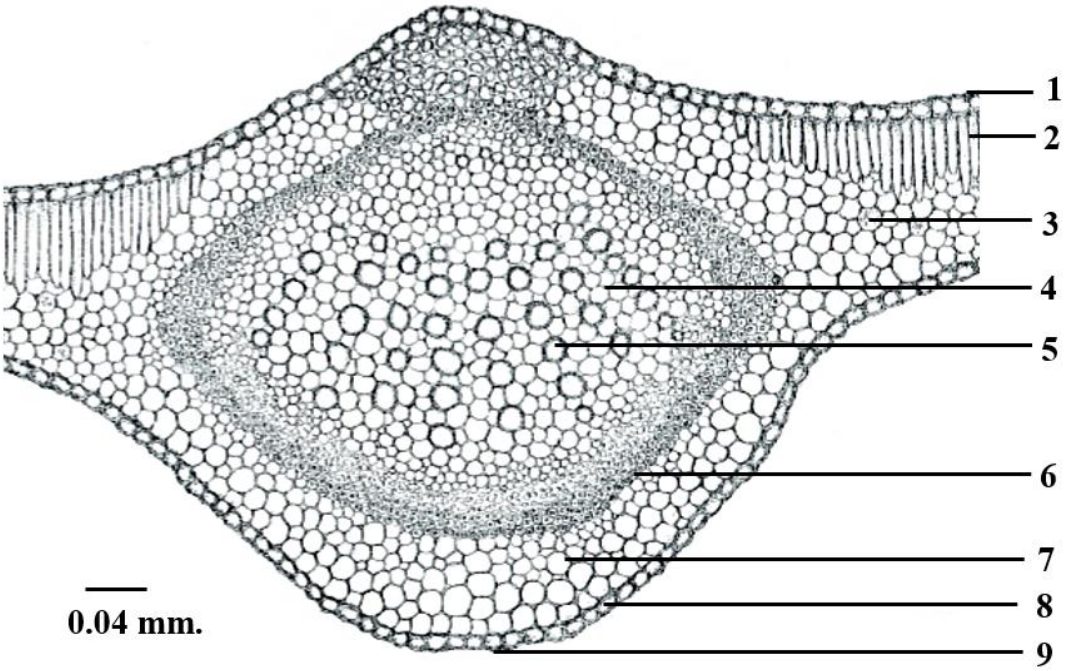

**(f) *Derris scandens***

**Figure 37** Anatomical characteristics of *Derris scandens* leaf midrib (cross-section)
1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue,
6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis,
10. Unicellular non-glandular trichome




(g) ***Derris solorioides***

**Figure 38** Anatomical characteristics of *Derris solorioides* leaf midrib (cross-section) 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis



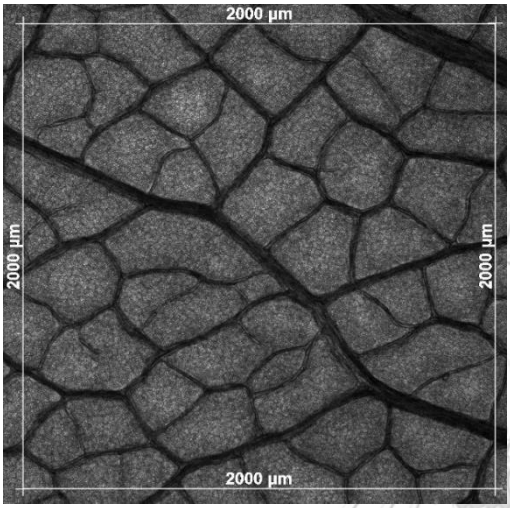

**(h) *Derris trifoliata***

**Figure 39** Anatomical characteristics of *Derris trifoliate* leaf midrib (cross-section)
1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Sclerenchyma, 5. Xylem tissue, 6. Phloem tissue, 7. Parenchyma, 8. Collenchyma, 9. Lower epidermis

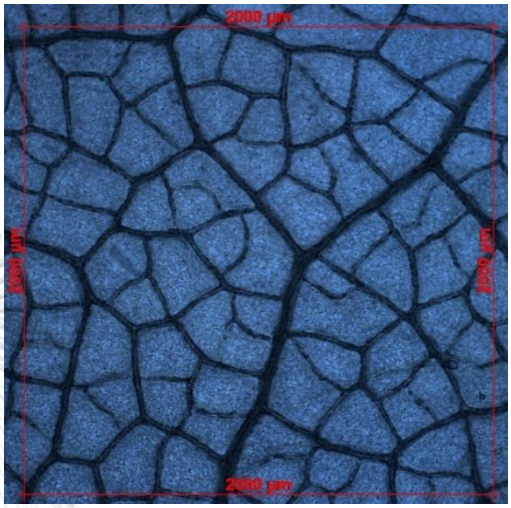
**Microscopic leaf constant numbers**

Vein-islet number

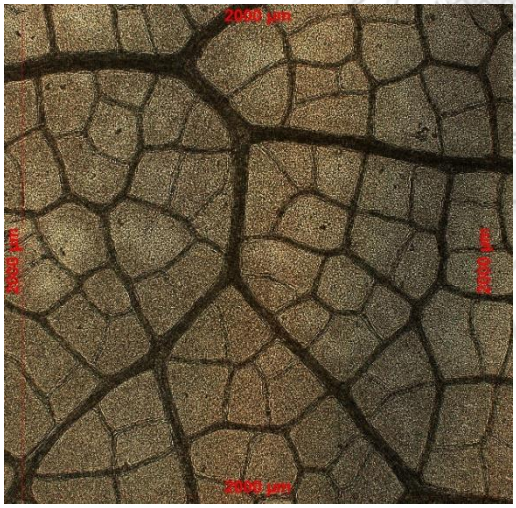
Vein islet number of eight species were shown in Table 28, and the vein islet characteristics were shown in Figure 40 (A-H).



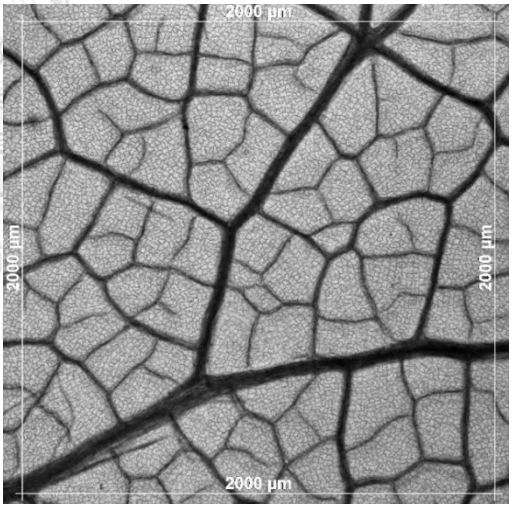

(A) *Derris amoena*



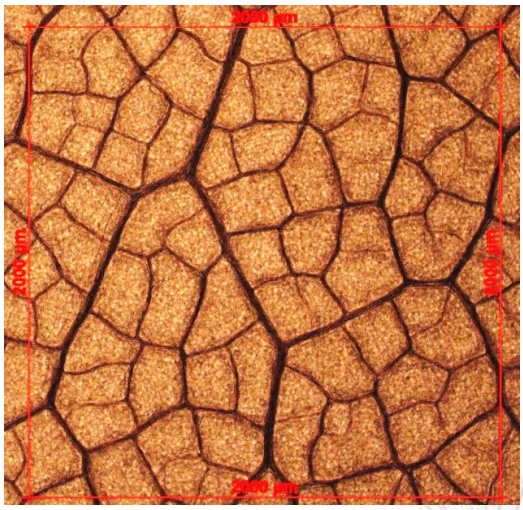

(B) *Derris elliptica*



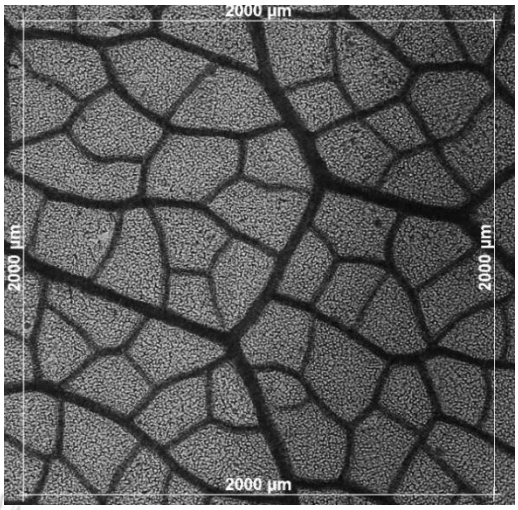

(C) *Derris indica*



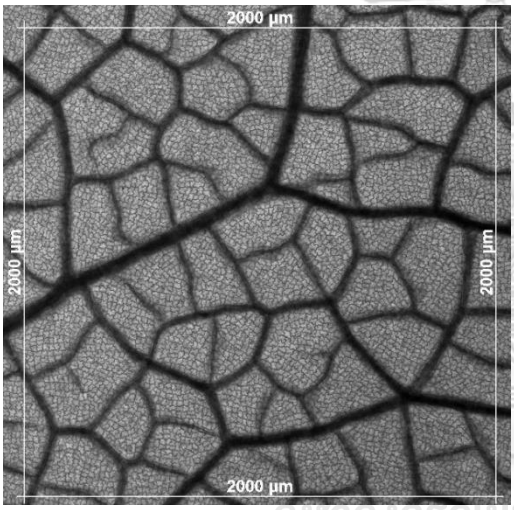

(D) *Derris malaccensis*



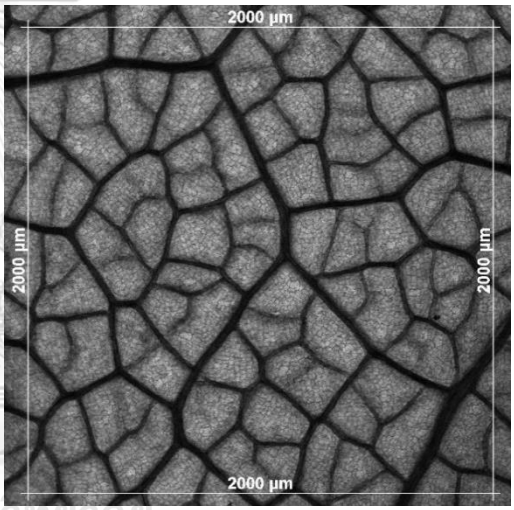
(E) *Derris reticulata*




(F) *Derris scandens*




(G) *Derris solorioides*




(H) *Derris trifoliate*

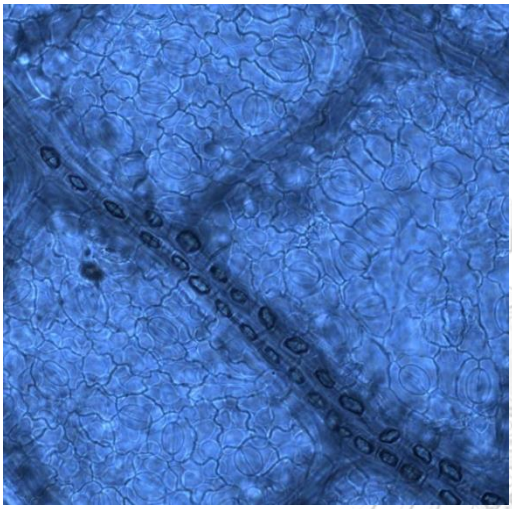


**Figure 40** Photographs of vein islet cells

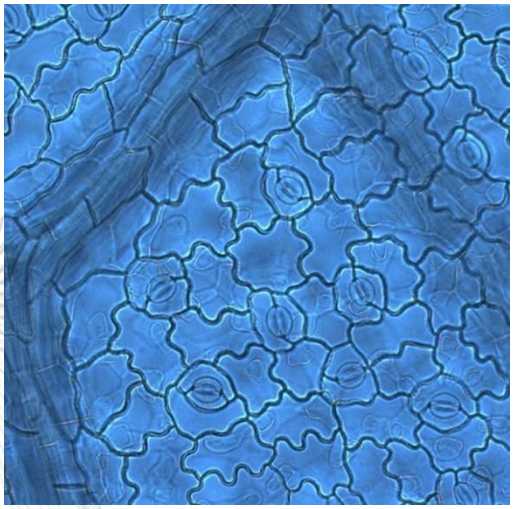
*(A)* D. amoena, *(B)* D. elliptica, *(C)* D. indica, *(D)* D. malaccensis, *(E)* D. reticulata, *(F)* D. scandens, *(G)* D. solorioides, *(H)* D. trifoliata

Stomatal cells

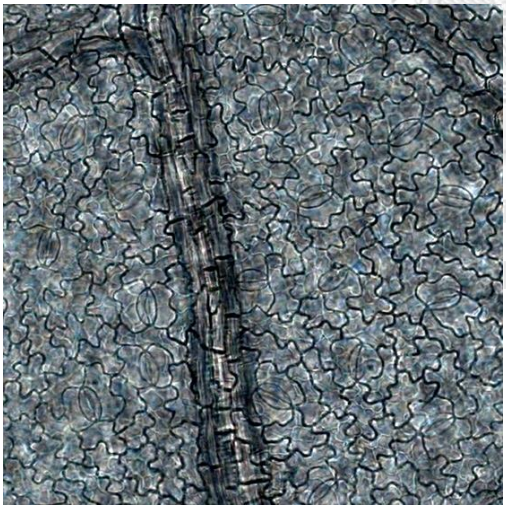
The stomatal cells of eight *Derris* species were distributed on the lower epidermis, except *D. trifoliate* was found on both sides of leaf. *Derris* species in this study showed paracytic type of stomata as shown in Figure 41.



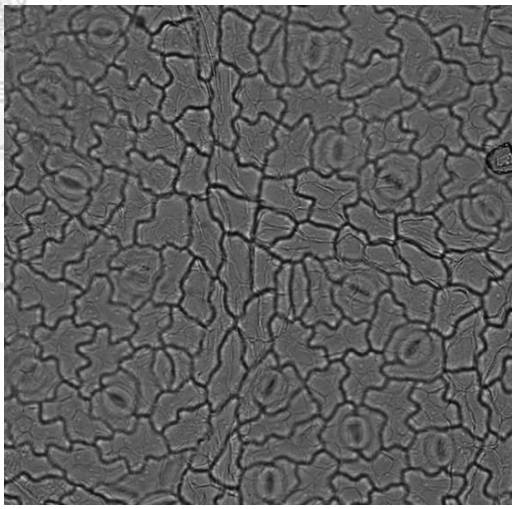
(A) *Derris amoena*



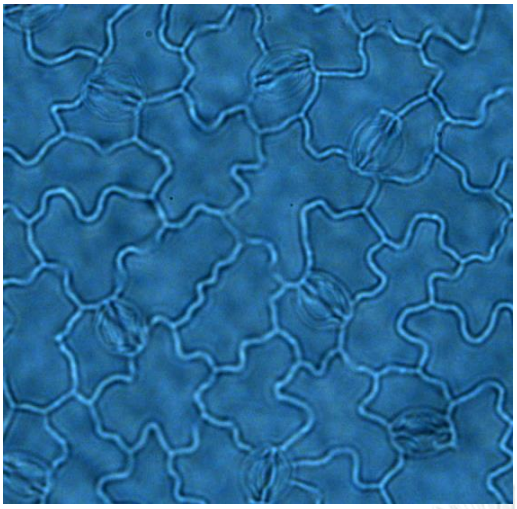
(B) *Derris elliptica*



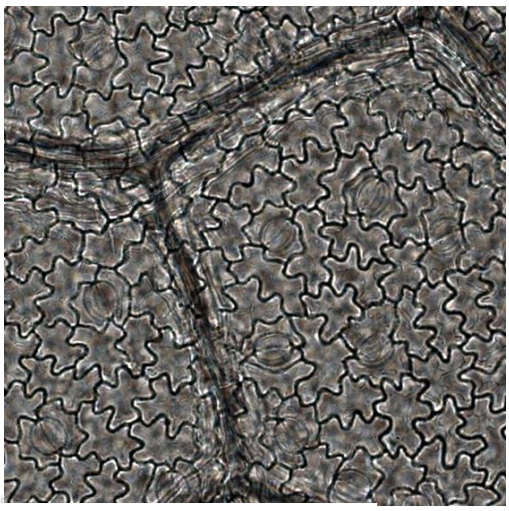
(C) *Derris indica*



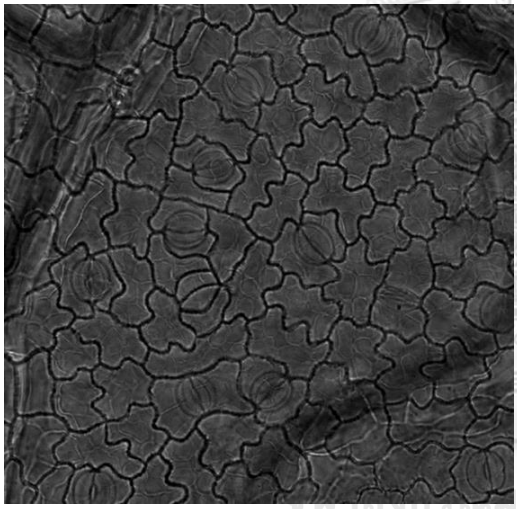
(D) *Derris malaccensis*



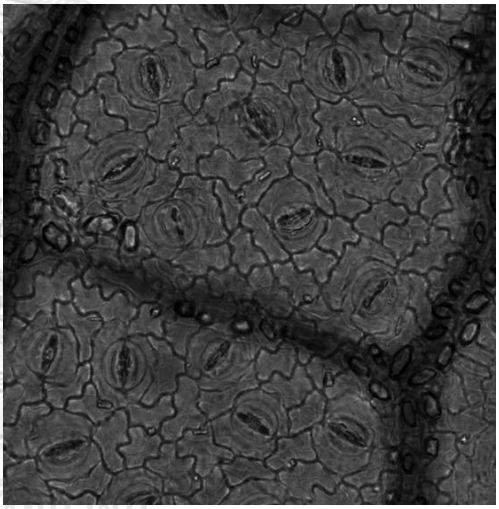
(E) *Derris reticulata*



(F) *Derris scandens*



(G) *Derris solorioides*



(H) *Derris trifoliata*

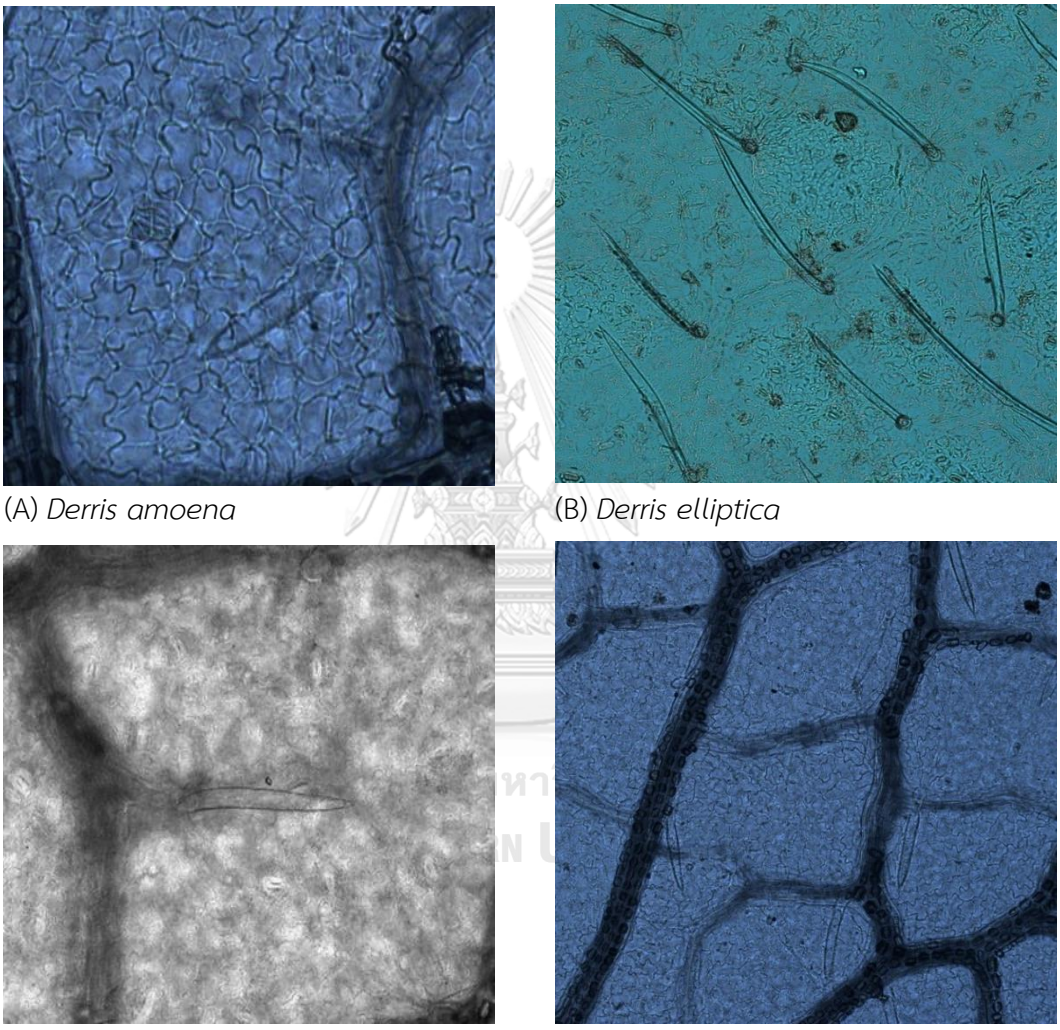


**Figure 41** Photographs of stomatal cells

(A) *D. amoena*, (B) *D. elliptica*, (C) *D. indica*, (D) *D. malaccensis*, (E) *D. reticulata*, (F) *D. scandens*, (G) *D. solorioides*, (H) *D. trifoliata*

Trichome

The trichomes found on hypostomatic leaf of *D. amoena, D. elliptica, D. malaccensis* and *D. scandens* were unicellular non-glandular type. The photograph of trichomes in four *Derris* species were present in Figure 42 (A-D).The trichome numbers were shown in the Table 27., while *D. amoena* and *D. malaccensis* trichomes were trace and could not be quantified.



(A) *Derris amoena*

(B) *Derris elliptica*

(C) *Derris malaccensis*

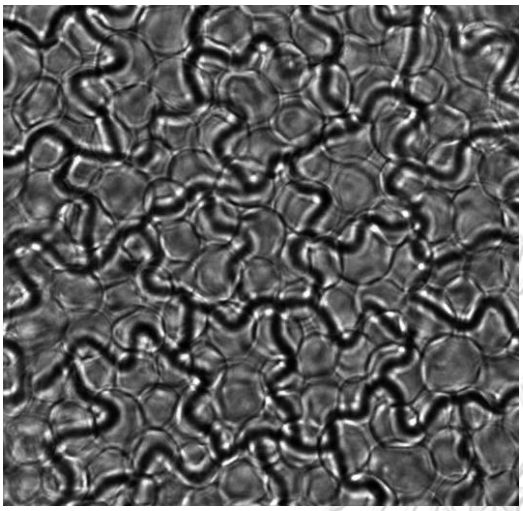
(D) *Derris scandens*

**Figure 42** Photographs of thrichome

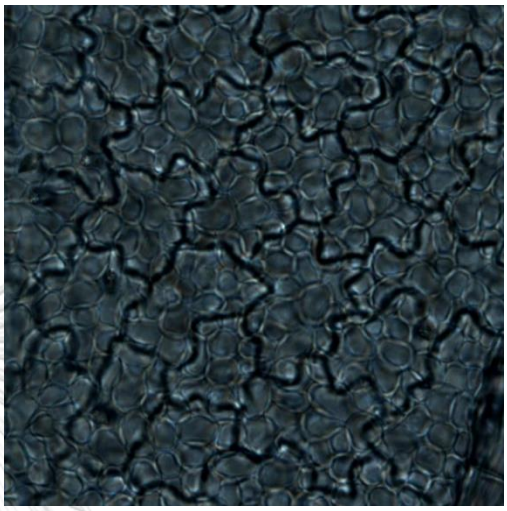
(A) *D. amoena*, (B) *D. elliptica*, (C) *D. malaccensis*, (D) *D. scandens*

Palisade cells

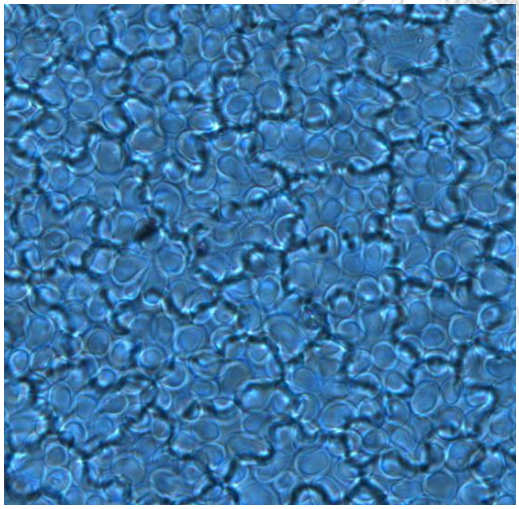
The palisade ratios were shown in Table28. The photographs of palisade cells under upper epidermal cells of selected *Derris* species were presented in Figure 43 (A-H).



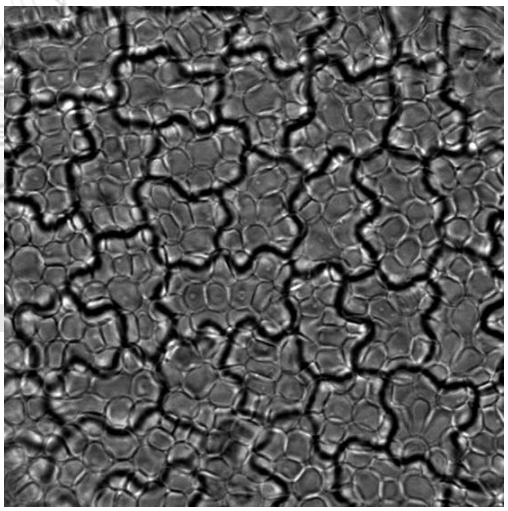
(A) *Derris amoena*



(B) *Derris elliptica*



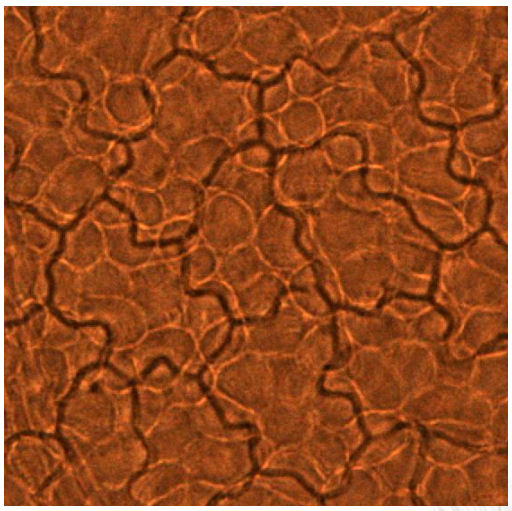
(C) *Derris indica*



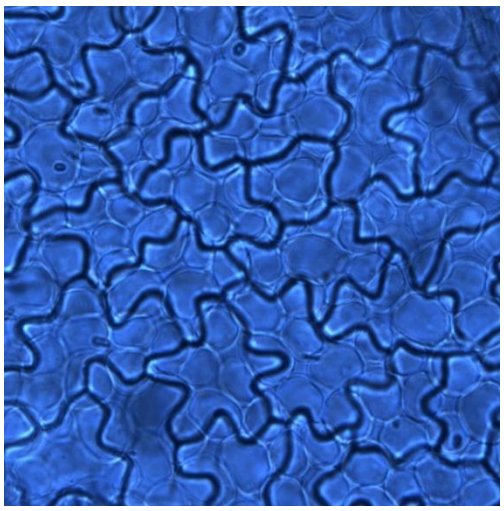
(D) *Derris malaccensis*

Epidermal cell area

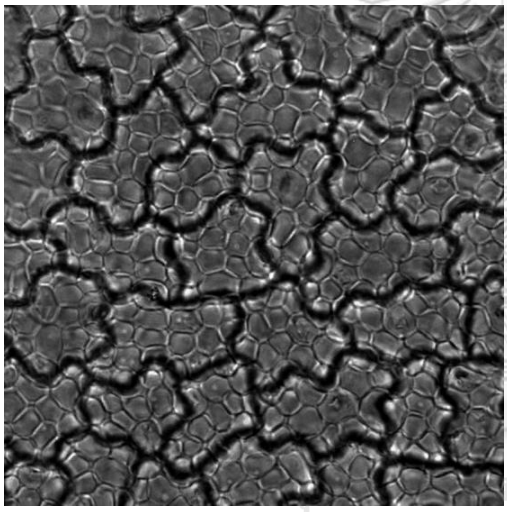
The lower epidermal cell areas estimated from the epidermal cell number, the stomatal number and the trichome number in the same unit area of 1 $mm^2$ were shown in table 28



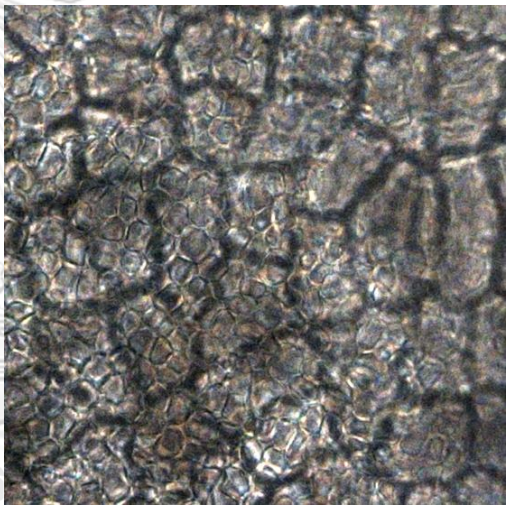
(E) *Derris reticulata*



(F) *Derris scandens*



(G) *Derris solorioides*



(H) *Derris trifoliata*



**Figure 43** Photographs of palisade cells

(A) *D. amoena*, (B) *D. elliptica*, (C) *D. indica*, (D) *D. malaccensis*, (E) *D. reticulata*, (F) *D. scandens*, (G) *D. solorioides*, (H) *D. trifoliata*

**Table 27** Microscopic leaf constant numbers of eight *Derris* species based on stomatal number, stomatal index and trichome number

| *Derris* species | Lower stomatal number | Lower stomatal index | Upper stomatal number | Upper stomatal index | Trichome number |
|---|---|---|---|---|---|
| *Derris amoena* | 186.71 ± 20.16 (116 - 324 ) | 13.46 ± 1.33 (10.50 – 18.66) | - | - | trace* |
| *D. elliptica* | 218.31 ± 21.48 (104 - 334) | 11.23 ± 1.17 (6.79 - 16.01) | - | - | 16.53 ± 2.75 (6.25 - 26) |
| *D. malaccensis* | 176 ± 13.43 (128 - 260) | 10.27 ± 0.98 (8.09 - 17.08) | - | - | trace* |
| *D. indica* | 195.11 ± 21.69 (128-264) | 8.93 ± 0.91 (5.45 - 11.83) | - | - | - |
| *D. reticulata* | 316.53 ± 23.03 (264 - 356) | 20.21 ± 1.53 (17.07 - 22.28) | - | - | - |
| *D. scandens* | 223.96 ± 20.38 (132 - 336) | 14.35 ± 1.25 (8.66 - 18.77 ) | - | - | 14.15 ± 1.92 (8 - 20) |
| *D. solorioides* | 155.87 ± 11.67 (116 - 192) | 9.57 ± 0.70 (7.24 - 12.38) | - | - | - |
| *D. trifoliate* | 194.71 ± 17.53 (152 - 272) | 9.59 ± 0.86 (8.11 - 11.86) | 35.46 ± 7.76 (34-52) | 3.55 ± 0.98 (3.02-5.25) | - |



* Trace of trichome were found at the lamina of the leaf.

**Table 28** Microscopic leaf constant numbers of eight *Derris* species based on lower epidermal cell area, palisade ratio and vein islet number

| ***Derris* species** | **Lower epidermal cell area ($\mu m^2$)** | **Palisade ratio** | **Vein islet number** |
|---|---|---|---|
| *Derris amoena* | 886.65 ± 45.34 | 7.65 ± 0.87 | 8.88 ± 1.15 |
| | (586.85 – 1136.36) | (4.75 -10.5) | (5 – 12.75) |
| *D. elliptica* | 612.78 ± 27.78 | 10.53 ± 0.84 | 18.41 ± 0.87 |
| | (499 - 817) | (6.25 - 17) | (17 - 21.75) |
| *D. malaccensis* | 656.8 ± 47.55 | 7.96 ± 0.83 | 14.03 ± 1.30 |
| | (537.05 - 825.08) | (6 - 10.75) | (9 - 16.75) |
| *D. indica* | 606.59 ± 30.32 | 7.70 ± 0.89 | 15.36 ± 0.99 |
| | (428.08 - 984.25) | (5.5 - 10) | (10.5 - 19.375) |
| *D. reticulata* | 1172.99 ± 56.25 | 9.01 ± 1.96 | 15.05 ± 0.90 |
| | (1033.06 -1308.90) | (6.25 - 13.75) | (13.5-17.5) |
| *D. scandens* | 756.56 ± 32.93 | 5.53 ± 0.63 | 15.37 ± 1.62 |
| | (683.06 - 836.12) | (2.75 - 7.75 ) | (10- 18.50) |
| *D. solorioides* | 688.95 ± 32.67 | 10.66 ± 1.12 | 12.54 ± 1.07 |
| | (551.88 - 961.54) | (7.5 - 13.75) | (9.5 - 14.75) |
| *D. trifoliata* | 555.44 ± 25 | 10.83 ± 1.66 | 17.97 ± 1.10 |
| | (422.30 - 737.46) | (7.5 - 14.75) | (14.5 - 21) |

**Part V : Molecular identification**

DNA isolation

The genomic DNA obtained from young fresh leaves of each *Derris* species was extracted using modified CTAB method (Doyle & Doyle, 1990). The concentration and purity of DNA were measured using NanoDrop One spectrophotometer (Table 29).

**Table 29** The concentration and purity of the genomic DNA detected by NanoDrop One spectrophotometer

| Sample | DNA concentration (ng/µl) | A260/A280 |
|---|---|---|
| *Derris amoena* | 460.90 | 1.75 |
| *D. elliptica* | 122.96 | 1.94 |
| *D. malaccensis* | 156.35 | 1.99 |
| *D. indica* | 47.00 | 2.16 |
| *D. reticulata* | 227.51 | 1.78 |
| *D. scandens* | 302.50 | 2.05 |
| *D. solorioides* | 98.8 | 1.61 |
| *D. trifoliata* | 52.30 | 2.15 |
| *Ceasalpinia sappan* | 73.06 | 2.08 |
| *Clerodendrum paniculatum* | 251.11 | 2.13 |

ISSR analysis

In the present study, five out of fifteen ISSR primers were selected to produce amplified genomic DNA. The total of 256 reproducible and polymorphic bands were obtained with the average of 51.20 bands per primer with 100% polymorphism. The DNA fragment size ranged from 246-2043 base pairs. The sequences of five ISSR primers together with the number of ISSR amplified products of selected eight *Derris* plant species were displayed in Table 30. The primer ISSR 12 demonstrated the highest polymorphic bands (68 bands), whereas the primer ISSR 35 showed the lowest number (41 bands). No band was appeared in the amplification with negative control. The example of ISSR fingerprints of selected *Derris* species and outgroup plants obtained from primer ISSR 17 were shown in Figure 45

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

| Primer | Primer sequence* | Annealing Tm (°C) | Fragment size range (bps) | Total band | Polymorphic band | Polymorphism (%) |
|---|---|---|---|---|---|---|
| ISSR11 | AGAGAGAGAGAGAGAGYT | 45 | 330.90-1895.33 | 49 | 49 | 100 |
| ISSR12 | AGAGAGAGAGAGAGAGYC | 45 | 246.15-1759.85 | 68 | 68 | 100 |
| ISSR14 | GAGAGAGAGAGAGAGAYT | 45 | 294.32-1921.83 | 43 | 43 | 100 |
| ISSR17 | CACACACACACACACARG | 45 | 303.54-1695.82 | 55 | 55 | 100 |
| ISSR35 | CTCTCTCTTCTCTA | 42 | 444.06-2043.09 | 41 | 41 | 100 |
| Total | | | 246.15-2043.09 | 256 | 256 | 100 |

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

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

The Jaccard's similarity matrix was used to analyze the genetic similarity coefficients among selected *Derris* species in Thailand. The highest similarity index (0.43) was presented between *D. elliptica* and *D. trifoliata* at 0.43 while the lowest similarity index (0.11) was found between *D. trifoliata* and *D. amoena.* Outgroup plants (*Clerodendrum paniculatum* and *Ceasalpinia sappan*) were separated from the *Derris*

species. The similarity index of selected eight *Derris* species and outgroup were shown in Table 31.

**Table 31** Similarity index of selected *Derris* species and outgroup plants

| ***Derris* species** | | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Derris elliptica* | **1** | 1.00 | | | | | | | | | |
| *D. malaccensis* | **2** | 0.28 | 1.00 | | | | | | | | |
| *D. scandens* | **3** | 0.27 | 0.31 | 1.00 | | | | | | | |
| *D. trifoliata* | **4** | 0.43 | 0.31 | 0.22 | 1.00 | | | | | | |
| *D. indica* | **5** | 0.23 | 0.26 | 0.23 | 0.31 | 1.00 | | | | | |
| *D. reticulata* | **6** | 0.28 | 0.25 | 0.21 | 0.31 | 0.21 | 1.00 | | | | |
| *D. amoena* | **7** | 0.20 | 0.28 | 0.27 | 0.11 | 0.20 | 0.24 | 1.00 | | | |
| *D. solorioides* | **8** | 0.22 | 0.33 | 0.36 | 0.27 | 0.30 | 0.23 | 0.30 | 1.00 | | |
| *Ceasalpinia sappan* | **9** | 0.22 | 0.19 | 0.25 | 0.26 | 0.18 | 0.22 | 0.17 | 0.17 | 1.00 | |
| *Clerodendrum paniculatum* | **10** | 0.19 | 0.20 | 0.23 | 0.16 | 0.14 | 0.27 | 0.18 | 0.11 | 0.20 | 1.00 |

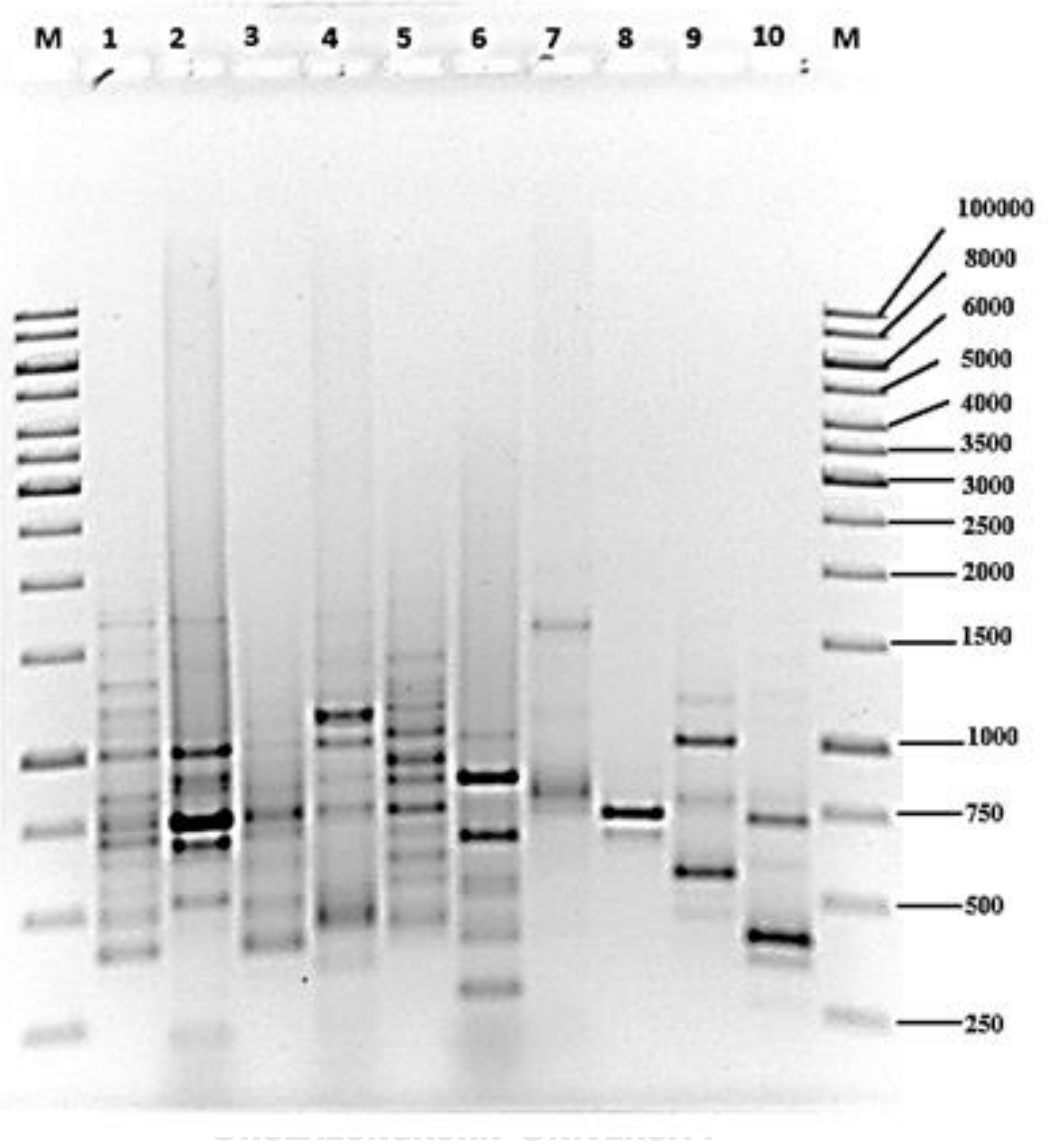



**Figure 44** Fingerprint of ISSR-17

[M = 1kb molecular weight marker, lane1 = *D. elliptica*, lane2 = *D. malaccensis*, lane 3 = *D. scandens*, lane 4 = *D. trifoliata*, lane 5 = *D. indica*, lane 6 = *D. reticulata*, lane 7 = *D. amoena*, lane 8 = *D .solorioides*, lane 9 = *Ceasalpinia sappan*, lane 10 = *Clerodendrum paniculatum*]

Based on the phylogenetic dendrogram, *Derris* species were divided into two major groups (Figure 46). Cluster I included *D. amoena, D. malaccensis, D. solorioides* and *D. scandens;* whereas, Cluster II consisted of *D. indica, D. reticulata, D. trifoliata* and *D. elliptica.* The outgroup plants (*Clerodendrum paniculatum* and *Ceasalpinia sappan*) were clearly separated from the *Derris* species. The similarity among eight *Derris* species were measured by Jaccard's similarity matrix (Table 31). *D. elliptica* and *D. trifoliata* presented the highest similarity index of 43, which were grouped in Cluster II. On the contrary, the lowest similarity index of 11 was found between *D. trifoliata* (Cluster II) and *D. amoena* (Cluster I).


**Figure 45** Phylogenetic dendrogram of eight *Derris* species and outgroup clustered by UPGMA

# CHAPTER V
# DISCUSSION AND CONCLUSION

The utility of plants as the natural therapeutic drug has been passed down from generation to generation in Thai traditional medicine. Presently, the use of herbal medicine has been applied in the primary health care system in Thailand. Therefore, the proper investigations which are necessary for quality, efficacy, and safety controls of the plants are recommended to provide sufficient scientific evidence for the public and health professionals. This research followed a guideline "Quality control method for medicinal plants material" which includes many tests procedures to evaluate plant materials to use in drug quality control laboratory (World Health Organization, 2011).

*Derris* is a genus of Papilionoid Leguminosae which can be found abundantly in Southeast Asia countries. Previous report indicated that there are 16 species found in Thailand among approximately 50 species worldwide. (Adema, 2000) The current research has established pharmacognostic specifications of *Derris elliptica* (Wall.) Benth. stem. Macroscopic and microscopic examinations are preliminary techniques for plant material identification. The macroscopic and microscopic characteristics of *D. elliptica* were illustrated by hand drawing. The illustrated characteristics were considered to be the simplest and the most inexpensive tools to identify this crude drug. The histological characteristics of *D. elliptica* stem powder included fragments of spiral vessels, sclereids, groups of fibers, bordered pitted, starch granules, prisms. The transverse section of *D. elliptica* dried stem revealed the anatomical characteristics including epidermis, cork cambium, parenchyma, vascular cambium, xylem vessel, phloem vascular bundle and pith. The dried stem of crude drug was light yellow with dark grayish-brown bark. TLC chromatographic pattern can be used together as the potential tools for authentication of *D. elliptica* stem crude drug sold in the market.

Moreover, the physicochemical evaluation of *D. elliptica* stem crude drug is very useful for the detection of adulterant or improper handling. The specified contents of loss on drying and water (moisture) are important because the high amount of water in the plant material can be a factor to cause the growth of bacteria and fungi, resulting in microorganism contamination and decomposing of the plant material. Moreover, total ash and acid insoluble ash values represent the inorganic matters (minerals) in plant material. One of the minerals remaining in acid insoluble ash is silicon that is essential in plant cell wall by a form of silicic acid. Adulteration and contamination from soil, sand, etc. result in higher ash values, therefore these parameters can indicate the purity and quality of crude drug. The results obtained from this study indicated that the loss on drying, total ash, acid-insoluble ash and water content should not be more than 5, 7, 1, 8% of dry weight, respectively. Moreover the extractive values obtained from various solution such as ethanol and water are also the indicator for controlling the phytochemical constituents of the crude drugs. For *D. elliptica* stems crude drug, the ethanol and water soluble extractive values should not be less than 4 and 11% of dry weight, respectively. The results indicated that *D. elliptica* stems contained great amounts of polar active compounds. Apart from that, the phytochemical fingerprinting is considered to be a reliable tool in primary identifying the herbal materials. TLC fingerprinting displayed the characteristics of active compounds found in *D. elliptica* stems, which could be used as a reference for further plant identification.



Rotenone is one of the main compounds in *D. elliptica* stem which can be used as the chemical marker to identify the plant and to evaluate the amount of active compound. Validated TLC-densitometry and TLC-image analysis for quantitative determination of rotenone content in *D. elliptica* stem were successfully developed in this study. It was found that rotenone contents in the ethanolic extracts of *D. elliptica* stem using Soxhlet extraction technique were not significantly different

(*P*>0.05) from both quantitative methods; TLC-densitometry (0.2870 ± 0.1242 g/ 100 g of dried crude drug) and TLC-image analysis (0.2844 ± 0.1209 g/ 100 g of dried crude drug). Thus, the new developed TLC-image analysis in this study could be used as the alternative technique to quantify rotenone content in *D. elliptica* stem due to its ease of use and economy of cost comparing to HPLC technique. The optimized TLC-densitometry and TLC-image analysis were validated for the determination of rotenone content in *D. elliptica* stem extracts. The specificity was established by comparing UV spectra of the peaks at the peak apex among standard rotenone and all samples as well as comparing the sample peak at three positions of the peak (apex, up slope, and down-slope). The polynomial calibration curves ranged from 0.9 to 6.0 μg/spot. The values of accuracy represented by % recovery of both methods were within acceptable limits (96.23–105.71% recovery) i.e. the percent recovery has to be in between 80-120% (*Validation of analytical procedures: text and methodology Q2(R1)*, 2005). The repeatability precision and the intermediate precision of both methods were less than 5% RSD. The limit of detection and limit of quantitation were calculated by the residual standard deviation of a regression line and displayed as 0.27 and 0.81 μg/spot for TLC-densitometry, and 0.16 and 0.47 μg/spot for TCL-image analysis. These values revealed satisfactory sensitivity of both methods. The robustness expressed the values of 0.97% RSD for TLC-densitometry and 0.51% RSD for TLC image analysis. This implied that the changes in the mobile phase ratio did not affect both methods.



Antioxidant properties were investigated, It was fond that *D. elliptica* stem exhibited the highest antioxidant potential ($IC_{50}$ = 0.02 μg/ml) in DPPH radical scavenging activity comparing to the values from those of positive controls. In this study, the ethanolic extract of *D. elliptica* stem and BHT showed similar reducing power ability with FRAP value of 1.01 and 1.02 mM Fe(II)/mg respectively. The result indicated that rotenone showed lowest reducing potential (0.02 mM Fe(II)/mg). The

FRAP assay of another *Derris* species, *Derris trifoliate* stem was extracted with ethanol by maceration and the result revealed that the ethanolic extract exhibited 464 μM Fe(II)/ 100g extract (Sharief, Srinivasulu, Veni, & Rao, 2014). Similar study of FRAP assay on *Derris brevipes* aerial part (leaf and stem) extracted with methanol revealed slightly antioxidant effect at the concentration of 1.79 mM Fe(II)/ 1ml extract which indicated slightly antioxidant activity same as in the DPPH activity of the methanolic extract ($IC_{50}$ at 167.5 μg/ml) (Telekone & Khan, 2014). In Philippine, ethanolic extract of *Derris elliptica* leaves at the concentration of 500 ppm exhibited DPPH radical scavenging activity with $IC_{50}$ of 50.79 ppm.(Uy & Villazorda, 2015). *Derris robusta* leaves were macerated in methanol to evaluate antioxidant activities. It was found that the methanolic extract exhibited $IC_{50}$ of 91.45 μg/ml, 43.17 GAE/g, and 192.58 QE/g for DPPH radical scavenging activity, total phenolic content and total flavonoid content, respectively (Paul, Sumon, & Hoque, 2019). However, the antioxidant activities in this current study indicated that rotenone is not a good antioxidant compound, which was related to previous report that rotenone-induced oxidant injury in liver tissue (Terzi et al., 2004). Additionally, rotenone has been used as the agent to induce Parkinson's disease in animal model (Robert E Drolet, Jason R Cannon, Laura Montero, & J Timothy Greenamyre, 2009; Xiong et al., 2012). In β-carotene bleaching test, the results indicated that the extract showed higher ability to inhibit the bleaching of beta-carotene than rotenone. The antioxidant activities of the extract, standard rotenone and positive controls demonstrated the dose response relationship. The results of beta-carotene bleaching inhibition of *D. elliptica* ethanolic extract, rotenone, quercetin and BHT at a concentration at 1 mg/ml were 71.13, 10.04, 92.02, 93.90 % respectively in this study.



Alpha-glucosidase activities were investigated for antidiabetic property of *D. elliptica* stem and rotenone. Ethanolic extract of *D. elliptica* stem and rotenone

exhibited higher effects than a positive control, acarbose on yeast alpha-glucosidase activity; $IC_{50}$ = 0.07, 0.87 and 7.70 mg/ml, respectively. However, the results from rat intestinal alpha-glucosidase activity were not similar to those results in yeast alpha-glucosidase activity. The different results from both activities might get affected by a difference of the primary structure of the enzyme obtained from baker's yeast and mammalian small intestine. Rotenone inhibition on rat intestinal alpha-glucosidase assay could not be determined because of turbidity at the rotenone concentration more than 0.6 mg/ml. *In vivo* study showed that n-octacosanol isolated from *D. elliptica* exhibited hypoglycemic potential on hydrocortisone sodium succinate-induced diabetic mice. Blood glucose concentration showed dose-response relationship i.e. 120 ± 22 and 141 ± 24 mg/dL for 10 and 5 mg/kg n-octacosanol administration, respectively. Blood glucose concentration of normal mice, diabetic mice and gliclazide (positive control) administered mice were shown as 123 ± 9, 246 ± 31 and 117 ± 12 mg/dL, respectively (Ralph P. Morco, Gigimonette Ramos, & Miguel, 2012).

Cytotoxicity activity on 4 different cancer cells treated with *D. elliptica* ethanolic extract and rotenone exhibited marginal toxicity on all tested cancer cells ($IC_{50}$ > 20 µg/ml). Rotenone showed more toxicity than the extract especially against HepG2 ($IC_{50}$ = 50.29 µg/ml) which is related to the result from previous report of HepG2 cells treated with rotenone ($LC_{50}$ = 70.99 µg/ml) (Upegui et al., 2014). Another study of *D. elliptica* leaf was investigated for anticancer activity. It was found that the ethanolic extract of the leaf exhibited a potential cytotoxicity against HepG2 (IC50 = 118.12 µg/ml) investigated by MTT assay. Moreover, the study indicated that the leaf extract processes anti-proliferative effect due to its ability to induce apoptosis as well as DNA fragmentation in the tumor cell lines, but not in normal cells (peripheral blood mononuclear cells, PBMCs). (Sittisart, Piakaew, Chuea-Nongthon, & Dunkhunthod,

2019). Another toxicity test using brine shrimp lethality assay revealed that both *D. elliptica* stem ethanolic extract and rotenone were toxic, especially rotenone ($LC_{50}$ = 0.16 µg/ml) which was evidenced by previous research that reported the toxicity of rotenone to reduce hatching success rate of brine shrimp by blocking the development (Covi, Hutchison, Neumeyer, & Gunderson, 2016). Moreover, the study of different processing parameters used for toxicity study on brine shrimp also indicated that rotenone produced $LC_{50}$ less than 1 ppm (Saiful Irwan Zubairi, Mohamad Roji Sarmidi, & Ramlan Abdul Aziz, 2014). In another species of *Derris*, *D. robusta* leaf methnolic extract also exhibited cytotoxicity against the brine shrimp nauplii (Paul et al., 2019).

Microscopic leaf constant numbers are important quantitative parameters that can be used to identify and distinguish between some closely related species not easily characterized by qualitative microscopic evaluation (W. C. Evans, 2009).The characteristics of microscopic leaf constant numbers among eight *Derris* species in Thailand were revealed. The variation within species may be due to age of leaf, environmental condition and geographical source (Gokhale, 2008; Woodward, Lake, & Quick, 2002) The results in this study were obtained from the mature leaves collected from 3 different locations. Regarding stomata, only *D. trifoliate* was found as amphistomatic leaf, whereas the other species were hypostomatic. The paracytic stomata found in the study was in agreement with the previous report (Das & Ghose, 1993). The lower stomatal index of *D. trifoliata* reported by Das & Ghose was found to be 10.74, which was consistent with that of *D. trifoliata* obtained from the study (9.59 ± 0.86). Among eight *Derris* species, *D. reticulata* had the highest value of stomatal index (20.21 ± 1.53), while *D. indica* showed the lowest value (8.93 ± 0.91).



Epidermal cell area of leaf have been reported considered to be relatively constant within a narrow range for each species which could be used for species identification, (Foroughbakhch et al., 2008). The lower epidermal cell area among eight

*Derris* species were demonstrated, *D. reticulata* possessed the greatest epidermal cell area (1,172.99 ± 56.25 $\mu m^2$).

Type and the density of trichomes were capable to be a tool for identification of plant samples (Intakhiao, 2019), although the trichome number may be varied caused by seasonal and environmental conditions (Pérez-Estrada, Cano-Santana, & Oyama, 2000). In this study, the trichomes that were found on lower epidermis of laminae of *D. amoena, D. elliptica, D. malaccensis* and *D. scandens* were unicellular non-glandular type. *D. scandens* trichome type was in agreement with the previous study that reported as unicellular trichome (Leelavathi & Ramayya, 1983). The trichome number of *D. elliptica and D. scandens* were 16.53 ± 2.75 and 14.15 ± 1.92, respectively, while *D. amoena* and *D. malaccensis* trichomes were too less to be quantified. Based on the presence of trichomes, it could be used to separate eight *Derris* species into 2 groups: trichome-containing group (group 1) and non-trichome-containing group (group 2). Group 1 consisted of four species which were *D. amoena, D. elliptica, D. malaccensis* and *D. scandens*; whereas, group 2 included *D. indica, D. reticulata, D. solorioides* and *D. trifoliata.*



The other important leaf constant parameters used as a diagnostic value for differentiating of plant species are palisade ratio and vein islet number. The vein islet is used to point towards the small areas of the photosynthetic tissues encircled by the ultimate division of the vascular stands. The palisade ratio does not alter based on geographical variations and can be determined on fine powder of crude drug (Mukherjee, 2007).

Furthermore, molecular identification of selected eight *Derris* species was also achieved for evaluation of the genetic relationship in this genus. DNA markers provide an accurate and efficient identification of medicinal plants without an effect of

environmental factors (Joshi, Chavan, Warude, & Patwardhan, 2004). ISSR markers is one of popular technique that used to identify plant species, genetic diversity, molecular ecology etc. This technique provides a reliable and high informative system for DNA fingerprinting. ISSR primer is universal primer, easy to assess, and not require the previous sequence information. It is not complicated and suitable for beginner to study the relationship in plants (Wang, 2002).

Because ISSR marker is easy to perform, costs low price and requires no prior genetic information, it was chosen in the present study. The annealing temperature around 45 ºC was in practice compatible with the previous papers (Pradeep Reddy et al., 2002). According to Sukrong *et al*., the annealing temperature of RAPD technique was 36 ºC, due to smaller amount of nucleotide bases in each RAPD primer. *D. scandens, D. elliptica, D. malaccensis* and *D. reticulata* were in the same cluster, while *D. trifoliata* was individually separated (Sukrong, 2005). This was nearly in agreement with the present study where *D. elliptica* and *D. reticulata* were both in Cluster II, but *D. scandens* and *D. malaccensis* were in Cluster I. In ISSR, the genomic DNA fragments are randomly amplified using microsatellite-sequences-containing primers, generating higher reproducibility of the ISSR-PCR products, compared to RAPD. However, the variation could be occurred by the mutation at PCR priming sites and Indels (insertion/deletion) within amplified DNA sections (Ng & Tan, 2015).



Reported by Sirichamorn *et al.*, the phylogenic results based on the parsimony and Bayesian analyses of combined nuclear ribosomal ITS/5.8S and three chloroplast genes (*trnK-matK*, *trnL-F* IGS and *psbA-trnH* IGS) revealed that *D. trifoliata* and *D. reticulata* were relatively in the same cluster while *D. amoena* was separately in other group (Y. Sirichamorn, F. Adema, & P. C. v. Welzen, 2012). This was consistently agreed with the present study where *D. amoena* was in Cluster I; on the other hand, *D. trifoliata* and *D. reticulata* were in Cluster II. Another study from Sirichamorn *et al.* in

2014 suggested that the combined molecular and morphological (i.e. inflorescence type, number of ovules, pods' wings, floral disk and seed chamber) dataset expressed the similar phylogenic structure to those found in the complete molecular analysis discovered in 2012 (Sirichamorn, Adema, Roos, & van Welzen, 2014).

**Conclusion**

TLC-densitometry and TLC-image analysis were developed and validated for quantification of rotenone in *D. elliptica* stem. This study provided pharmacognostic specification of *D. elliptica* stem crude drug as well as its rotenone contents in Thailand. Various biological activities including antioxidant, antidiabetic and cytotoxicity activities of the ethanolic extract of *D. elliptica* stem crude drug were demonstrated. The research also provided botanical morphology and microscopic leaf characteristics of the midrib as well as microscopic leaf constant numbers of 8 *Derris* species in Thailand that could be used as the tool for identification of these *Derris* species. Furthermore, ISSR fingerprints and phylogenetic dendrogram of 8 *Derris* species were demonstrated.

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# APPENDICES

# APPENDIX A

Quality parameter of *Derris elliptica* stems

Table 32 Physicochemical parameters of *D. elliptica* stems (g/100g)

| Source | Order | Loss on drying | Total ash | Acid-insoluble ash | Water extractable value | Ethanol extractable value | water content |
|---|---|---|---|---|---|---|---|
| Bangkok | 1 | 6.89 | 7.336 | 1.16 | 12.71 | 5.40 | 8.33 |
| | 2 | 7.16 | 7.296 | 0.92 | 13.46 | 5.89 | 8.00 |
| | 3 | 7.10 | 7.346 | 1.20 | 13.53 | 6.29 | 8.00 |
| Angthong | 4 | 7.11 | 7.902 | 1.36 | 12.58 | 4.13 | 9.00 |
| | 5 | 7.51 | 8.111 | 1.160 | 13.32 | 4.27 | 9.33 |
| | 6 | 7.10 | 8.112 | 1.580 | 12.61 | 4.38 | 10.00 |
| Phuket | 7 | 5.8 | 7.219 | 1.32 | 13.960 | 5.28 | 8.33 |
| | 8 | 6.28 | 7.106 | 1.29 | 13.73 | 4.98 | 8.33 |
| | 9 | 6.07 | 7.108 | 1.61 | 13.66 | 5.39 | 9.67 |
| Chanthaburi | 10 | 5.43 | 6.160 | 1.23 | 10.89 | 4.86 | 9.00 |
| | 11 | 5.94 | 7.535 | 1.64 | 11.59 | 4.27 | 8.33 |
| | 12 | 6.38 | 7.027 | 1.67 | 11.20 | 4.02 | 9.00 |
| Khon Kaen | 13 | 4.47 | 6.058 | 1.490 | 9.93 | 4.18 | 7.67 |
| | 14 | 4.79 | 6.400 | 1.56 | 9.39 | 4.05 | 7.33 |
| | 15 | 4.74 | 6.194 | 1.02 | 9.61 | 4.43 | 8.00 |
| Trang | 16 | 7.20 | 7.172 | 1.39 | 13.45 | 2.99 | 9.33 |
| | 17 | 7.07 | 7.435 | 1.22 | 12.10 | 2.72 | 8.67 |
| | 18 | 7.26 | 6.848 | 1.15 | 11.62 | 2.64 | 9.33 |
| Sakon Nakhon | 19 | 5.20 | 8.306 | 1.21 | 8.91 | 1.34 | 7.22 |
| | 20 | 5.57 | 7.914 | 1.28 | 7.85 | 1.32 | 7.67 |
| | 21 | 5.44 | 8.442 | 1.57 | 8.61 | 1.48 | 7.00 |
| Chiang Rai | 22 | 6.45 | 7.499 | 1.21 | 12.60 | 6.23 | 8.33 |
| | 23 | 6.28 | 7.207 | 1.67 | 12.65 | 6.06 | 8.67 |
| | 24 | 6.22 | 7.123 | 1.05 | 12.74 | 6.14 | 8.33 |
| Nakhon Sawan | 25 | 4.83 | 7.172 | 1.19 | 13.34 | 4.74 | 8.33 |
| | 26 | 5.23 | 7.078 | 1.02 | 13.31 | 4.61 | 7.67 |
| | 27 | 5.34 | 7.426 | 1.29 | 12.68 | 4.77 | 8.00 |
| Uthai Thani 1 | 28 | 4.54 | 6.950 | 1.20 | 10.60 | 3.35 | 7.00 |
| | 29 | 5.36 | 7.470 | 1.14 | 10.81 | 3.19 | 7.33 |
| | 30 | 5.55 | 7.301 | 1.25 | 10.07 | 3.73 | 7.33 |

| Source | Order | Loss on drying | Total ash | Acid-insoluble ash | Water extractable value | Ethanol extractable value | water content |
|---|---|---|---|---|---|---|---|
| Uthai Thani 2 | 31 | 6.31 | 8.295 | 1.00 | 10.10 | 4.36 | 11.67 |
| | 32 | 6.08 | 7.936 | 1.35 | 10.51 | 4.20 | 10.67 |
| | 33 | 5.75 | 8.005 | 1.00 | 12.13 | 4.38 | 11.89 |
| Chiang Mai | 34 | 6.06 | 6.145 | 1.04 | 10.81 | 2.53 | 12.00 |
| | 35 | 6.07 | 6.065 | 0.97 | 10.91 | 2.51 | 11.67 |
| | 36 | 5.92 | 6.409 | 1.01 | 11.13 | 2.54 | 12.00 |
| Lampang | 37 | 4.15 | 7.174 | 1.41 | 9.39 | 3.71 | 9.67 |
| | 38 | 4.71 | 7.481 | 0.92 | 9.14 | 4.19 | 9.33 |
| | 39 | 4.48 | 7.740 | 1.12 | 10.44 | 3.78 | 9.67 |
| Prachinburi | 40 | 4.57 | 7.837 | 1.05 | 11.53 | 4.34 | 7.33 |
| | 41 | 4.93 | 7.836 | 1.26 | 11.32 | 4.47 | 8.00 |
| | 42 | 5.58 | 8.211 | 1.12 | 11.61 | 4.76 | 8.33 |
| Lamphun | 43 | 4.93 | 7.871 | 1.04 | 8.95 | 3.42 | 9.33 |
| | 44 | 5.18 | 7.710 | 0.98 | 9.04 | 3.36 | 8.67 |
| | 45 | 4.45 | 7.564 | 1.03 | 8.46 | 3.72 | 9.00 |
| **Min** | | **4.15** | **6.06** | **0.92** | **8.46** | **1.34** | **7.22** |
| **Max** | | **7.50** | **8.44** | **1.67** | **13.73** | **6.06** | **11.89** |
| **Grand mean** | | **5.77** | **7.35** | **1.22** | **11.31** | **4.07** | **8.81** |
| **Pooled S.D.** | | **0.92** | **0.63** | **0.20** | **1.68** | **1.23** | **1.30** |

**APPENDIX B**

Data of TLC quantitative analysis of rotenone

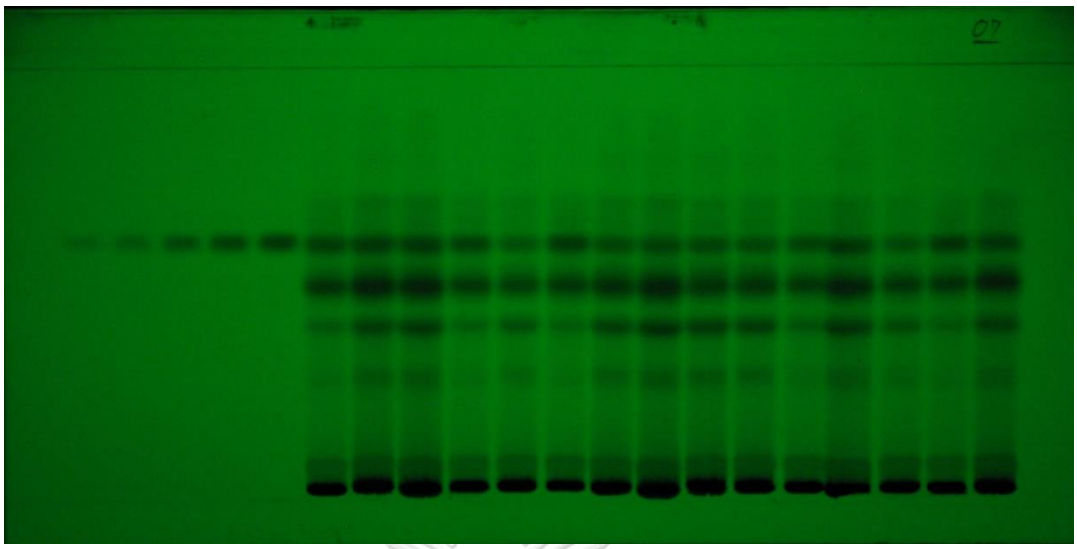

**Figure 46** The TLC plate under UV 254 nm

standard rotenone 1st- 5th spots and *D. elliptica* stem ethanolic extract from 15 different locations

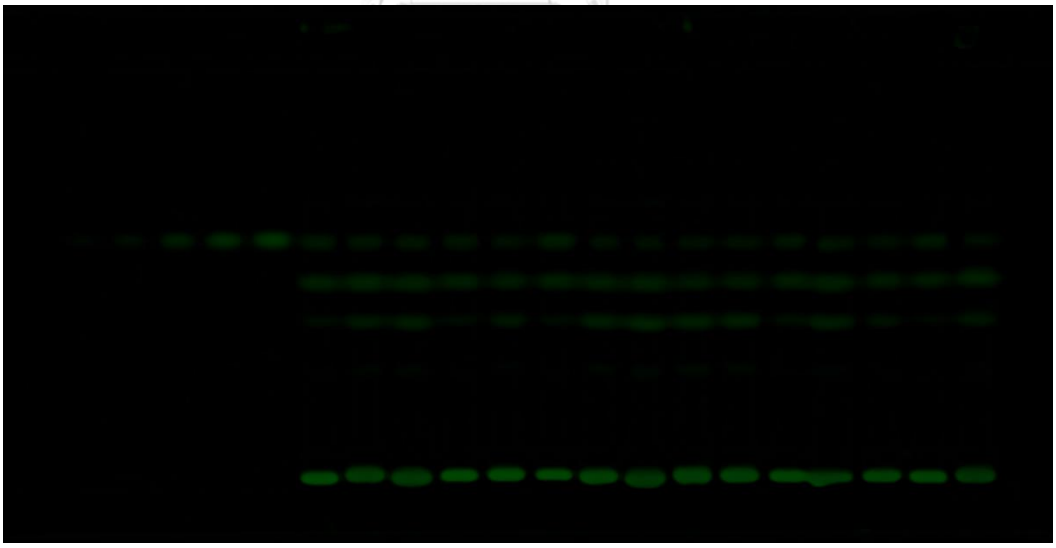

**Figure 47** The TLC plate under UV 365 nm

standard rotenone 1st- 5th spots and *D. elliptica* stem ethanolic extract from 15 different locations

**APPENDIX C**

Data of antioxidant activities

DPPH radical scavenging activity

**Table 33** DPPH radical scavenging activity of ethonolic extract of *D. elliptica* stems

| Conc. (µg/ml) | $OD_{517}$ (reaction mixture) | | | DPPH inhibition (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| | Exp1 | Exp2 | Exp3 | Exp1 | Exp2 | Exp3 | Mean | SD |
| 0.00000 | 0.196 | 0.190 | 0.195 | | | | | |
| 0.00313 | 0.169 | 0.176 | 0.171 | 0.004 | 12.737 | 9.122 | 11.704 | 11.19 |
| 0.00625 | 0.146 | 0.153 | 0.155 | 0.005 | 24.613 | 20.998 | 19.966 | 21.86 |
| 0.01250 | 0.116 | 0.123 | 0.121 | 0.004 | 40.103 | 36.489 | 37.522 | 38.04 |
| 0.02500 | 0.068 | 0.080 | 0.079 | 0.007 | 64.888 | 58.692 | 59.208 | 60.93 |
| 0.05000 | 0.037 | 0.043 | 0.044 | 0.004 | 80.895 | 77.797 | 77.281 | 78.66 |

**Table 34** DPPH radical scavenging activity of rotenone

| Conc. (µg/ml) | $OD_{517}$ (reaction mixture) | | | DPPH inhibition (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| | Exp1 | Exp2 | Exp3 | Exp1 | Exp2 | Exp3 | Mean | SD |
| 0 | 0.196 | 0.190 | 0.195 | | | | | |
| 0.0625 | 0.186 | 0.192 | 0.189 | 3.959 | 0.861 | 2.410 | 2.41 | 1.549 |
| 0.1250 | 0.176 | 0.181 | 0.174 | 9.122 | 6.540 | 10.155 | 8.61 | 1.862 |
| 0.2500 | 0.150 | 0.154 | 0.152 | 22.547 | 20.482 | 21.515 | 21.51 | 1.033 |
| 0.5000 | 0.112 | 0.122 | 0.112 | 42.169 | 37.005 | 42.169 | 40.45 | 2.981 |
| 1.0000 | 0.047 | 0.053 | 0.045 | 75.731 | 72.633 | 76.764 | 75.04 | 2.150 |

**Table 35** DPPH radical scavenging activity of positive control (BHT)

| Conc. (µg/ml) | $OD_{517}$ (reaction mixture) | | | DPPH inhibition (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| | Exp1 | Exp2 | Exp3 | Exp1 | Exp2 | Exp3 | Mean | SD |
| 0 | 0.161 | 0.161 | 0.163 | | | | | |
| 3.13 | 0.151 | 0.151 | 0.152 | 6.598 | 6.598 | 5.979 | 6.39 | 0.357 |
| 6.25 | 0.133 | 0.139 | 0.139 | 17.732 | 14.021 | 14.021 | 15.26 | 2.143 |
| 12.50 | 0.119 | 0.119 | 0.121 | 26.392 | 26.392 | 25.155 | 25.98 | 0.714 |
| 25.00 | 0.085 | 0.089 | 0.093 | 47.423 | 44.948 | 42.474 | 44.95 | 2.474 |
| 50.00 | 0.051 | 0.056 | 0.058 | 68.454 | 65.361 | 64.124 | 65.98 | 2.230 |

**Table 36** DPPH radical scavenging activity of positive control (Quercetin)

| Conc. (µg/ml) | $OD_{517}$ (reaction mixture) | | | DPPH inhibition (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| | Exp1 | Exp2 | Exp3 | Exp1 | Exp2 | Exp3 | Mean | SD |
| 0.00 | 0.198 | 0.196 | 0.199 | | | | | |
| 0.31 | 0.194 | 0.186 | 0.194 | 0.005 | 1.855 | 5.902 | 1.855 | 3.20 |
| 0.625 | 0.179 | 0.175 | 0.183 | 0.004 | 9.444 | 11.467 | 7.420 | 9.44 |
| 1.250 | 0.166 | 0.163 | 0.170 | 0.004 | 16.020 | 17.538 | 13.997 | 15.85 |
| 2.500 | 0.132 | 0.119 | 0.135 | 0.009 | 33.221 | 39.798 | 31.703 | 34.91 |
| 5.000 | 0.071 | 0.065 | 0.078 | 0.007 | 64.081 | 67.116 | 60.540 | 63.91 |
| 10.000 | 0.019 | 0.019 | 0.021 | 0.001 | 90.388 | 90.388 | 89.376 | 90.05 |

**Ferric ion reducing antioxidant power (FRAP)**

**Table 37** FRAP value of *D. elliptica* stems ethanolic extract , rotenone, quercetin and BHT

| Tested samples (0.5mg/ml) | Absorbance at 593 nm | | | | Ferrous sulphate equivalent (mM) | FRAP value (mM Fe(II)/mg) |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Mean ± SD | | |
| *D. elliptica* extract | 1.093 | 1.165 | 0.968 | 1.075 ± 0.1 | 0.5041 | 1.0082 |
| Rotenone | 0.073 | 0.07 | 0.071 | 0.071±0.002 | 0.0128 | 0.0256 |
| Quercetin | 2.65 | 2.668 | 2.67 | 2.663±0.011 | 1.2808 | 2.5616 |
| BHT | 1.132 | 1.04 | 1.103 | 1.092±0.047 | 0.5121 | 1.0241 |

**Beta-carotene bleaching inhibition**

**Table 38** The absorbance of beta-carotene bleaching of tested samples at 0.25 mg/ml

| Tested sample | Time | $OD_{470}$ (reaction mixture) | | | | |
|---|---|---|---|---|---|---|
| | | Exp1 | Exp2 | Exp3 | Average | SD |
| BHT | 0 | 2.645 | 2.639 | 2.663 | 2.649 | 0.012 |
| | 30 | 2.554 | 2.612 | 2.606 | 2.591 | 0.032 |
| | 60 | 2.475 | 2.584 | 2.548 | 2.536 | 0.056 |
| | 90 | 2.379 | 2.465 | 2.464 | 2.436 | 0.049 |
| | 120 | 2.340 | 2.433 | 2.417 | 2.397 | 0.050 |
| Quercetin | 0 | 2.66 | 2.715 | 2.706 | 2.694 | 0.030 |
| | 30 | 2.566 | 2.574 | 2.6 | 2.580 | 0.018 |
| | 60 | 2.507 | 2.588 | 2.587 | 2.561 | 0.046 |
| | 90 | 2.43 | 2.491 | 2.505 | 2.475 | 0.040 |
| | 120 | 2.398 | 2.473 | 2.484 | 2.452 | 0.047 |
| *D. elliptica* ethanolic extract | 0 | 2.676 | 2.64 | 2.685 | 2.667 | 0.024 |
| | 30 | 2.346 | 2.335 | 2.362 | 2.348 | 0.014 |
| | 60 | 1.988 | 1.966 | 2.008 | 1.987 | 0.021 |
| | 90 | 1.675 | 1.637 | 1.724 | 1.679 | 0.044 |
| | 120 | 1.431 | 1.384 | 1.497 | 1.437 | 0.057 |
| Rotenone | 0 | 2.622 | 2.689 | 2.679 | 2.663 | 0.036 |
| | 30 | 1.832 | 2.042 | 2.033 | 1.969 | 0.119 |
| | 60 | 1.056 | 1.399 | 1.368 | 1.274 | 0.190 |
| | 90 | 0.638 | 0.96 | 0.926 | 0.841 | 0.177 |
| | 120 | 0.417 | 0.683 | 0.652 | 0.584 | 0.145 |
| Negative control | 0 | 2.675 | 2.674 | 2.680 | 2.676 | 0.003 |
| | 30 | 1.901 | 1.934 | 1.956 | 1.930 | 0.028 |
| | 60 | 1.211 | 1.271 | 1.281 | 1.254 | 0.038 |
| | 90 | 0.790 | 0.851 | 0.811 | 0.817 | 0.031 |
| | 120 | 0.343 | 0.537 | 0.587 | 0.556 | 0.027 |

**Table 39** The absorbance of beta-carotene bleaching of tested samples at 0.5 mg/ml (Cont.)

| Tested samples | Time | $OD_{470}$ (reaction mixture) | | | | |
|---|---|---|---|---|---|---|
| | | Exp1 | Exp2 | Exp3 | Average | SD |
| BHT | 0 | 2.624 | 2.668 | 2.642 | 2.645 | 0.022 |
| | 30 | 2.601 | 2.626 | 2.662 | 2.630 | 0.031 |
| | 60 | 2.544 | 2.597 | 2.611 | 2.584 | 0.035 |
| | 90 | 2.476 | 2.532 | 2.55 | 2.519 | 0.039 |
| | 120 | 2.47 | 2.513 | 2.516 | 2.500 | 0.026 |
| Quercetin | 0 | 2.629 | 2.647 | 2.64 | 2.639 | 0.009 |
| | 30 | 2.573 | 2.638 | 2.66 | 2.624 | 0.045 |
| | 60 | 2.564 | 2.631 | 2.606 | 2.600 | 0.034 |
| | 90 | 2.481 | 2.546 | 2.53 | 2.519 | 0.034 |
| | 120 | 2.438 | 2.512 | 2.499 | 2.483 | 0.040 |
| *D. elliptica* ethanolic extract | 0 | 2.657 | 2.657 | 2.64 | 2.651 | 0.010 |
| | 30 | 2.47 | 2.434 | 2.437 | 2.447 | 0.020 |
| | 60 | 2.221 | 2.186 | 2.185 | 2.197 | 0.021 |
| | 90 | 2.01 | 1.975 | 1.969 | 1.985 | 0.022 |
| | 120 | 1.835 | 1.792 | 1.787 | 1.805 | 0.026 |
| Rotenone | 0 | 2.636 | 2.659 | 2.544 | 2.613 | 0.061 |
| | 30 | 2.01 | 1.948 | 1.866 | 1.941 | 0.072 |
| | 60 | 1.38 | 1.288 | 1.242 | 1.303 | 0.070 |
| | 90 | 0.967 | 0.871 | 0.845 | 0.894 | 0.064 |
| | 120 | 0.712 | 0.621 | 0.601 | 0.645 | 0.059 |
| Negative control | 0 | 2.675 | 2.674 | 2.680 | 2.676 | 0.003 |
| | 30 | 1.901 | 1.934 | 1.956 | 1.930 | 0.028 |
| | 60 | 1.211 | 1.271 | 1.281 | 1.254 | 0.038 |
| | 90 | 0.790 | 0.851 | 0.811 | 0.817 | 0.031 |
| | 120 | 0.343 | 0.537 | 0.587 | 0.556 | 0.027 |

**Table 40** The absorbance of beta-carotene bleaching of tested samples at 1 mg/ml (Cont.)

| Tested samples | Time | $OD_{470}$ (reaction mixture) | | | | |
|---|---|---|---|---|---|---|
| | | Exp1 | Exp2 | Exp3 | Average | SD |
| BHT | 0 | 2.633 | 2.648 | 2.668 | 2.650 | 0.018 |
| | 30 | 2.609 | 2.62 | 2.663 | 2.631 | 0.029 |
| | 60 | 2.633 | 2.619 | 2.669 | 2.640 | 0.026 |
| | 90 | 2.536 | 2.585 | 2.599 | 2.573 | 0.033 |
| | 120 | 2.534 | 2.538 | 2.596 | 2.556 | 0.035 |
| Quercetin | 0 | 2.675 | 2.722 | 2.68 | 2.692 | 0.026 |
| | 30 | 2.596 | 2.64 | 2.652 | 2.629 | 0.029 |
| | 60 | 2.578 | 2.611 | 2.636 | 2.608 | 0.029 |
| | 90 | 2.539 | 2.569 | 2.574 | 2.561 | 0.019 |
| | 120 | 2.542 | 2.555 | 2.576 | 2.558 | 0.017 |
| *D. elliptica* ethanolic extract | 0 | 2.628 | 2.643 | 2.604 | 2.625 | 0.020 |
| | 30 | 2.538 | 2.522 | 2.456 | 2.505 | 0.043 |
| | 60 | 2.372 | 2.356 | 2.307 | 2.345 | 0.034 |
| | 90 | 2.213 | 2.2 | 2.177 | 2.197 | 0.018 |
| | 120 | 2.104 | 2.08 | 2.055 | 2.080 | 0.025 |
| Rotenone | 0 | 2.659 | 2.671 | 2.674 | 2.668 | 0.008 |
| | 30 | 2.029 | 1.89 | 2.008 | 1.976 | 0.075 |
| | 60 | 1.458 | 1.277 | 1.411 | 1.382 | 0.094 |
| | 90 | 1.091 | 0.908 | 1.017 | 1.005 | 0.092 |
| | 120 | 0.844 | 0.676 | 0.761 | 0.760 | 0.084 |
| Negative control | 0 | 2.675 | 2.674 | 2.680 | 2.676 | 0.003 |
| | 30 | 1.901 | 1.934 | 1.956 | 1.930 | 0.028 |
| | 60 | 1.211 | 1.271 | 1.281 | 1.254 | 0.038 |
| | 90 | 0.790 | 0.851 | 0.811 | 0.817 | 0.031 |
| | 120 | 0.343 | 0.537 | 0.587 | 0.556 | 0.027 |

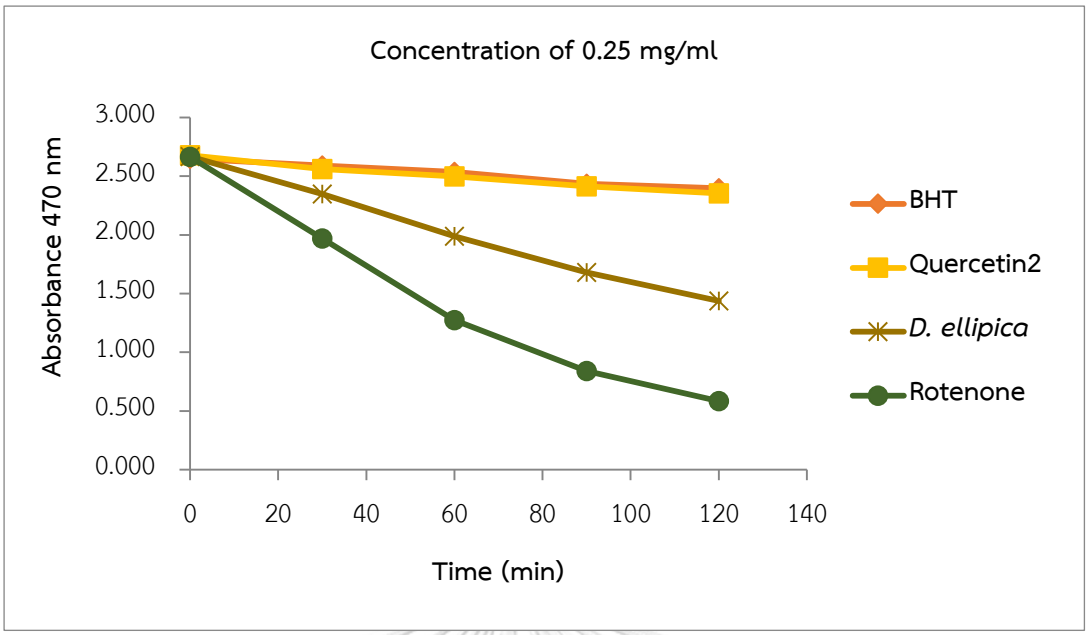


**Figure 48** The absorbance of beta-carotene bleaching of tested samples at 0.25 mg/ml

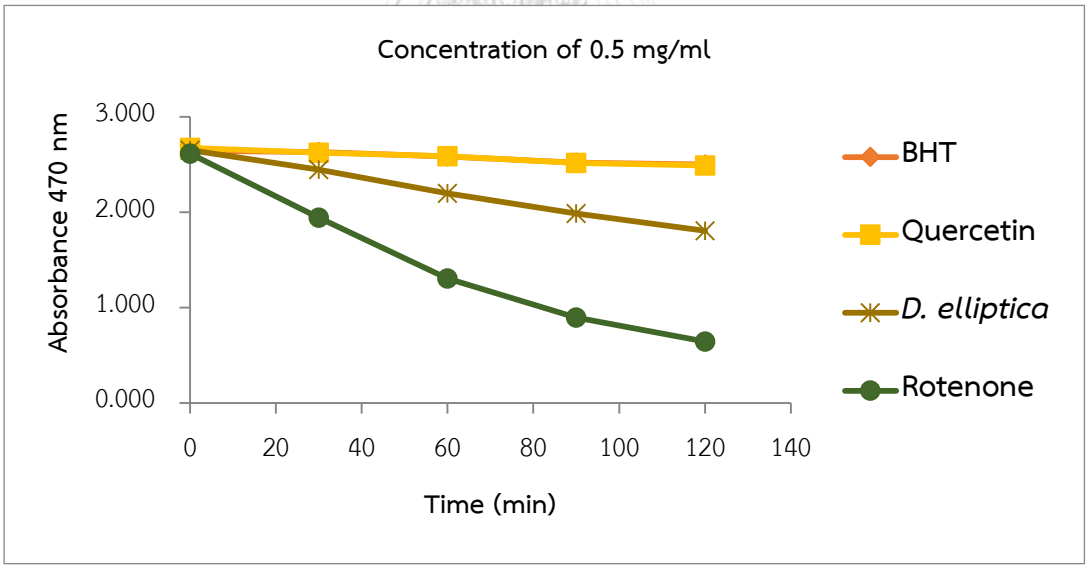


**Figure 49** The absorbance of beta-carotene bleaching of tested samples at 0.5 mg/ml

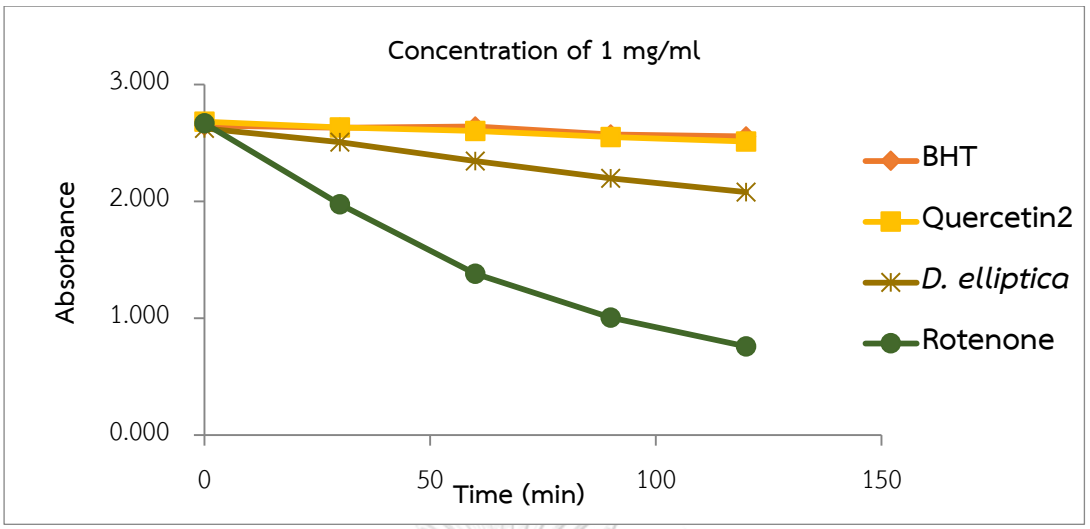


**Figure 50** The absorbance of beta-carotene bleaching of tested samples at 1 mg/ml

# APPENDIX D

Data of brine shrimp lethality and MTT cell viability assays

**Table 41** Number of survivor nauplii at each time among various concentration of *D. elliptica* ethnalic extract

| Conc. (µg/ml) | Time (hr.) | | | | | | | | | | | | | | | | | | | | | | | | % Lethality |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6 | | | | | | 12 | | | | | | 18 | | | | | | 24 | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | mean | 1 | 2 | 3 | 4 | 5 | mea | 1 | 2 | 3 | 4 | 5 | mean | 1 | 2 | 3 | 4 | 5 | mean | SD | |
| 80 | 3 | 3 | 3 | 2 | 4 | 3 | 2 | 3 | 2 | 3 | 2 | 2.4 | 1 | 2 | 1 | 1 | 2 | 1.4 | 1 | 2 | 0 | 0 | 1 | 0.8 | 0.84 | 92 |
| 70 | 6 | 4 | 6 | 5 | 7 | 5.6 | 5 | 4 | 4 | 4 | 6 | 4.6 | 4 | 4 | 4 | 3 | 5 | 4 | 2 | 3 | 2 | 2 | 4 | 2.6 | 0.89 | 74 |
| 60 | 7 | 8 | 7 | 7 | 7 | 5.6 | 6 | 7 | 6 | 6 | 5 | 6 | 6 | 5 | 5 | 5 | 4 | 5 | 4 | 5 | 4 | 5 | 4 | 4.4 | 0.55 | 56 |
| 50 | 10 | 10 | 10 | 10 | 9 | 9.8 | 8 | 9 | 9 | 9 | 8 | 8.6 | 8 | 8 | 7 | 9 | 7 | 7.8 | 7 | 6 | 6 | 6 | 5 | 6 | 0.71 | 40 |
| 40 | 10 | 10 | 10 | 10 | 10 | 10 | 9 | 10 | 9 | 9 | 9 | 9.2 | 8 | 8 | 8 | 9 | 8 | 8.2 | 6 | 7 | 7 | 7 | 7 | 6.8 | 0.45 | 32 |
| 30 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 9 | 10 | 9 | 10 | 9.6 | 9 | 8 | 9 | 9 | 8 | 8.6 | 8 | 8 | 8 | 8 | 7 | 7.8 | 0.45 | 22 |
| Negative control | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 0 | 100 |

**Table 42** Number of survivor nauplii at each time among various concentration of rotenone

| Conc. (µg/ml) | Time (hr.) | | | | | | | | | | | | | | | | | | | | | | | | % Lethality |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6 | | | | | | 12 | | | | | | 18 | | | | | | 24 | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | mean | 1 | 2 | 3 | 4 | 5 | mean | 1 | 2 | 3 | 4 | 5 | mean | 1 | 2 | 3 | 4 | 5 | mean | SD | |
| 1 | 4 | 3 | 2 | 3 | 4 | 3.2 | 4 | 3 | 2 | 2 | 2 | 2.6 | 2 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0.4 | 0.55 | 96 |
| 0.5 | 4 | 5 | 5 | 5 | 6 | 5 | 2 | 4 | 5 | 4 | 5 | 4 | 2 | 3 | 3 | 2 | 3 | 2.6 | 1 | 2 | 2 | 2 | 2 | 1.8 | 0.45 | 82 |
| 0.25 | 6 | 7 | 6 | 6 | 5 | 6 | 6 | 6 | 5 | 6 | 4 | 5.4 | 5 | 5 | 4 | 6 | 3 | 4.6 | 4 | 4 | 3 | 5 | 2 | 3.6 | 1.14 | 64 |
| 0.125 | 8 | 9 | 9 | 8 | 8 | 8.4 | 8 | 8 | 7 | 6 | 6 | 7 | 7 | 7 | 6 | 5 | 5 | 6 | 6 | 6 | 5 | 6 | 4 | 5.4 | 0.89 | 46 |
| 0.0625 | 9 | 10 | 9 | 9 | 8 | 9 | 9 | 9 | 9 | 8 | 8 | 8.6 | 8 | 8 | 7 | 8 | 7 | 7.6 | 7 | 6 | 6 | 7 | 6 | 6.4 | 0.55 | 36 |
| 0.03125 | 10 | 10 | 10 | 10 | 9 | 9.8 | 10 | 9 | 10 | 10 | 9 | 9.6 | 10 | 8 | 9 | 9 | 8 | 8.8 | 8 | 7 | 7 | 7 | 7 | 72 | 0.45 | 22 |
| Negative control | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 0 | 100 |

**Table 43** Cytotoxicity effect of *Derris elliptica* stems ethanolic extract by MTT cell viability

| | Conc. | CaCo-2 (OD540) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (ug/ml) | 1 | %viability | 2 | %viability | 3 | %viability | 4 | %viability |
| Ethanolic extract | 100 | 0.580 | 74 | 0.589 | 73 | 0.592 | 71 | 0.601 | 76 |
| | 50 | 0.659 | 85 | 0.653 | 81 | 0.675 | 81 | 0.647 | 82 |
| | 10 | 0.703 | 90 | 0.711 | 89 | 0.725 | 87 | 0.712 | 90 |
| | 5 | 0.722 | 93 | 0.734 | 91 | 0.788 | 95 | 0.769 | 97 |
| | 1 | 0.746 | 96 | 0.768 | 96 | 0.801 | 96 | 0.793 | 100 |
| DMSO | | 0.779 | 96 | 0.803 | 99 | 0.832 | 101 | 0.791 | 99 |
| Control | | 0.811 | | 0.808 | | 0.826 | | 0.802 | |

| | Conc. | HT-29 (OD54) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (ug/ml) | 1 | %viability | 2 | %viability | 3 | %viability | 4 | %viability |
| Ethanolic extract | 100 | 0.565 | 45 | 0.544 | 45 | 0.531 | 44 | 0.539 | 45 |
| | 50 | 0.712 | 57 | 0.707 | 59 | 0.701 | 58 | 0.714 | 59 |
| | 10 | 0.813 | 65 | 0.847 | 71 | 0.855 | 70 | 0.841 | 70 |
| | 5 | 0.932 | 75 | 0.956 | 80 | 0.925 | 76 | 0.946 | 78 |
| | 1 | 1.034 | 83 | 1.011 | 84 | 1.022 | 84 | 1.003 | 83 |
| DMSO | | 1.246 | 98 | 1.198 | 96 | 1.213 | 95 | 1.207 | 93 |
| Control | | 1.265 | | 1.254 | | 1.274 | | 1.296 | |

| | Conc. (ug/ml) | MDA-231 (OD540) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | %viability | 2 | %viability | 3 | %viability | 4 | %viability |
| Ethanolic extract | 100 | 0.634 | 66 | 0.622 | 67 | 0.618 | 66 | 0.601 | 64 |
| | 50 | 0.717 | 74 | 0.693 | 75 | 0.666 | 71 | 0.679 | 72 |
| | 10 | 0.778 | 81 | 0.756 | 82 | 0.742 | 80 | 0.751 | 80 |
| | 5 | 0.878 | 91 | 0.856 | 93 | 0.833 | 89 | 0.845 | 90 |
| | 1 | 0.921 | 96 | 0.910 | 99 | 0.911 | 98 | 0.909 | 96 |
| DMSO | | 0.963 | 97 | 0.922 | 99 | 0.932 | 95 | 0.944 | 99 |
| Control | | 0.989 | | 0.936 | | 0.977 | | 0.952 | |

| | Conc. (ug/ml) | HepG2 (OD540) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | %viability | 2 | %viability | 3 | %viability | 4 | %viability |
| Ethanolic extract | 100 | 0.611 | 66 | 0.644 | 68 | 0.606 | 64 | 0.591 | 62 |
| | 50 | 0.687 | 75 | 0.672 | 70 | 0.689 | 73 | 0.691 | 72 |
| | 10 | 0.789 | 86 | 0.792 | 83 | 0.771 | 82 | 0.777 | 81 |
| | 5 | 0.849 | 92 | 0.866 | 91 | 0.859 | 91 | 0.842 | 88 |
| | 1 | 0.871 | 95 | 0.899 | 94 | 0.891 | 95 | 0.883 | 92 |
| DMSO | | 0.921 | 93 | 0.954 | 102 | 0.942 | 99 | 0.956 | 98 |
| Control | | 0.986 | | 0.939 | | 0.948 | | 0.977 | |

**Table 44** Cytotoxicity effect of rotenone by MTT cell viability

| | Conc. | Caca-2 (OD540) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (ug/ml) | 1 | %viability | 2 | %viability | 3 | %viability | 4 | %viability |
| Rotenone | **100** | 0.487 | **63** | 0.512 | **64** | 0.479 | **58** | 0.496 | **63** |
| | **50** | 0.564 | **72** | 0.579 | **72** | 0.568 | **68** | 0.551 | **70** |
| | **10** | 0.624 | **80** | 0.647 | **81** | 0.652 | **78** | 0.621 | **79** |
| | **5** | 0.705 | **91** | 0.722 | **90** | 0.711 | **85** | 0.678 | **86** |
| | **1** | 0.742 | **95** | 0.774 | **96** | 0.796 | **96** | 0.721 | **91** |
| **DMSO** | | 0.779 | **96** | 0.803 | **99** | 0.832 | **101** | 0.791 | **99** |
| **Control** | | 0.811 | | 0.808 | | 0.826 | | 0.802 | |

| | Conc. | HT-29 (OD540) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (ug/ml) | 1 | %viability | 2 | %viability | 3 | %viability | 4 | %viability |
| Rotenone | **100** | 0.437 | **35** | 0.418 | **35** | 0.439 | **36** | 0.455 | **38** |
| | **50** | 0.655 | **53** | 0.634 | **53** | 0.619 | **51** | 0.642 | **53** |
| | **10** | 0.789 | **63** | 0.776 | **65** | 0.768 | **63** | 0.767 | **64** |
| | **5** | 0.914 | **73** | 0.925 | **77** | 0.902 | **74** | 0.929 | **77** |
| | **1** | 1.011 | **81** | 1.022 | **85** | 1.009 | **83** | 1.023 | **85** |
| **DMSO** | | 1.246 | **98** | 1.198 | **96** | 1.213 | **95** | 1.207 | **93** |
| **Control** | | 1.265 | | 1.254 | | 1.274 | | 1.296 | |

| | Conc. | MDA-231 (OD540) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (ug/ml) | 1 | %viability | 2 | %viability | 3 | %viability | 4 | %viability |
| Rotenone | **100** | 0.403 | **42** | 0.379 | **41** | 0.386 | **41** | 0.404 | **43** |
| | **50** | 0.518 | **54** | 0.509 | **55** | 0.541 | **58** | 0.532 | **56** |
| | **10** | 0.625 | **65** | 0.618 | **67** | 0.649 | **70** | 0.627 | **66** |
| | **5** | 0.758 | **79** | 0.747 | **81** | 0.751 | **81** | 0.749 | **79** |
| | **1** | 0.838 | **87** | 0.836 | **91** | 0.821 | **88** | 0.849 | **90** |
| **DMSO** | | 0.963 | **97** | 0.922 | **99** | 0.932 | **95** | 0.944 | **99** |
| **Control** | | 0.989 | | 0.936 | | 0.977 | | 0.952 | |

| | Conc. | HepG2 (OD540) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (ug/ml) | 1 | %viability | 2 | %viability | 3 | %viability | 4 | %viability |
| Rotenone | **100** | 0.272 | **30** | 0.239 | **25** | 0.254 | **27** | 0.241 | **25** |
| | **50** | 0.414 | **45** | 0.408 | **43** | 0.422 | **45** | 0.434 | **45** |
| | **10** | 0.617 | **67** | 0.579 | **61** | 0.591 | **63** | 0.568 | **59** |
| | **5** | 0.703 | **76** | 0.711 | **75** | 0.726 | **77** | 0.718 | **75** |
| | **1** | 0.813 | **88** | 0.821 | **86** | 0.841 | **89** | 0.804 | **84** |
| **DMSO** | | 0.921 | **93** | 0.954 | **102** | 0.942 | **99** | 0.956 | **98** |
| **Control** | | 0.986 | | 0.939 | | 0.948 | | 0.977 | |

# APPENDIX E

Microscopic leaf constant numbers

**Table 45** Stomatal number, stomatal index, and trichome number of *D. amonea*

| Field | Stomatal number (/mm$^2$) | | | Stomatal index | | | Trichome number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 324 | 120 | 172 | 18.66 | 11.32 | 13.87 | - | - | - |
| 2 | 256 | 172 | 140 | 15.80 | 15.01 | 11.99 | - | - | - |
| 3 | 248 | 152 | 152 | 14.12 | 14.05 | 12.71 | - | - | - |
| 4 | 296 | 132 | 176 | 15.68 | 12.29 | 14.15 | - | - | - |
| 5 | 264 | 136 | 144 | 14.35 | 12.45 | 12.33 | - | - | - |
| 6 | 244 | 140 | 132 | 13.96 | 12.32 | 11.30 | 1 | - | - |
| 7 | 244 | 144 | 168 | 14.91 | 12.61 | 13.21 | - | - | - |
| 8 | 256 | 144 | 136 | 14.61 | 12.18 | 12.01 | - | - | - |
| 9 | 240 | 128 | 156 | 14.08 | 10.87 | 12.62 | - | - | - |
| 10 | 256 | 144 | 128 | 13.33 | 12.83 | 11.11 | - | - | - |
| 11 | 284 | 148 | 168 | 14.84 | 12.71 | 14.38 | - | - | - |
| 12 | 256 | 152 | 152 | 15.02 | 12.84 | 13.29 | - | - | - |
| 13 | 288 | 144 | 188 | 15.58 | 12.86 | 16.21 | - | - | - |
| 14 | 288 | 132 | 136 | 16.29 | 11.54 | 11.62 | - | - | - |
| 15 | 260 | 144 | 168 | 15.19 | 12.54 | 15.03 | - | - | - |
| 16 | 232 | 112 | 136 | 13.84 | 10.07 | 12.45 | - | 1 | - |
| 17 | 272 | 152 | 160 | 16.08 | 13.36 | 15.38 | - | - | - |
| 18 | 272 | 120 | 120 | 15.35 | 11.41 | 10.56 | - | - | - |
| 19 | 260 | 128 | 136 | 14.91 | 11.23 | 12.06 | - | - | - |
| 20 | 300 | 144 | 156 | 16.63 | 12.52 | 12.83 | - | - | - |
| 21 | 224 | 128 | 164 | 13.05 | 11.31 | 14.59 | - | - | - |
| 22 | 280 | 156 | 148 | 14.55 | 13.09 | 13.91 | - | - | 1 |
| 23 | 296 | 164 | 132 | 15.29 | 13.20 | 11.83 | - | - | - |
| 24 | 200 | 168 | 160 | 10.50 | 14.17 | 13.51 | - | - | - |
| 25 | 292 | 132 | 168 | 14.93 | 11.83 | 13.95 | - | - | - |
| 26 | 248 | 116 | 144 | 13.69 | 10.36 | 12.86 | - | - | - |
| 27 | 304 | 148 | 152 | 15.32 | 13.73 | 14.18 | 1 | - | - |
| 28 | 320 | 140 | 144 | 17.58 | 11.99 | 13.19 | - | - | - |
| 29 | 248 | 152 | 132 | 13.60 | 12.18 | 12.18 | - | - | - |
| 30 | 312 | 136 | 144 | 17.49 | 11.18 | 12.95 | - | - | - |

Source 1 = Trang 1, Source 2 = Trang 2, Source 3 = Nakhon Si Thammarat

**Table 46** Vein islet number, palisade ratio, and epidermal cell number of *D. amonea*

| Field | Vein islet number (/mm$^2$) | | | Palisade ratio | | | Lower epidermal cell number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 12.38 | 5.13 | 5.75 | 7 | 8 | 7.5 | 1412 | 940 | 1068 |
| 2 | 10.00 | 5.00 | 6.13 | 7.5 | 7 | 9.5 | 1364 | 974 | 1028 |
| 3 | 11.75 | 5.75 | 5.50 | 7.75 | 7.5 | 8.5 | 1508 | 930 | 1044 |
| 4 | 12.00 | 6.00 | 6.00 | 7.25 | 7.5 | 9.5 | 1592 | 942 | 1068 |
| 5 | 11.25 | 6.00 | 5.50 | 7.5 | 7 | 9 | 1576 | 956 | 1024 |
| 6 | 11.13 | 5.25 | 5.00 | 7.5 | 9.5 | 8.75 | 1504 | 996 | 1036 |
| 7 | 11.00 | 5.63 | 5.25 | 7.75 | 9 | 7.5 | 1392 | 998 | 1104 |
| 8 | 15.75 | 6.00 | 5.88 | 7.75 | 8 | 7 | 1496 | 1038 | 996 |
| 9 | 16.00 | 5.50 | 5.75 | 7.5 | 8.5 | 9 | 1464 | 1050 | 1080 |
| 10 | 16.75 | 6.00 | 5.75 | 8.75 | 8.5 | 7.5 | 1664 | 978 | 1024 |
| 11 | 15.50 | 5.25 | 5.75 | 7 | 8.25 | 9.75 | 1630 | 1016 | 1000 |
| 12 | 13.50 | 5.38 | 5.63 | 8 | 7.5 | 10.5 | 1448 | 1032 | 992 |
| 13 | 15.75 | 5.25 | 5.88 | 7.5 | 9 | 9.5 | 1560 | 976 | 972 |
| 14 | 16.50 | 5.50 | 5.63 | 7.25 | 8 | 7.5 | 1480 | 1012 | 1034 |
| 15 | 15.00 | 5.75 | 5.00 | 7.75 | 9 | 8 | 1452 | 1004 | 950 |
| 16 | 15.25 | 6.00 | 5.50 | 7.5 | 7.5 | 9 | 1444 | 1000 | 956 |
| 17 | 15.50 | 5.75 | 5.50 | 8.75 | 7.5 | 9.5 | 1420 | 986 | 880 |
| 18 | 15.63 | 5.38 | 5.88 | 8.5 | 8 | 9.5 | 1500 | 932 | 1016 |
| 19 | 19.75 | 5.75 | 5.50 | 8.75 | 9.5 | 9 | 1484 | 1012 | 992 |
| 20 | 17.50 | 5.63 | 6.00 | 8.25 | 8 | 7.5 | 1504 | 1006 | 1060 |
| 21 | 18.75 | 5.13 | 6.00 | 7.5 | 8.5 | 8 | 1492 | 1004 | 960 |
| 22 | 18.13 | 5.13 | 5.25 | 7.75 | 9.5 | 9.5 | 1644 | 1036 | 916 |
| 23 | 20.00 | 5.13 | 5.13 | 7.5 | 9.5 | 8.25 | 1640 | 1078 | 984 |
| 24 | 18.00 | 5.13 | 5.50 | 7 | 9 | 8.75 | 1704 | 1018 | 1024 |
| 25 | 17.25 | 5.13 | 5.25 | 7.5 | 7.5 | 9 | 1664 | 984 | 1036 |
| 26 | 17.38 | 5.75 | 6.00 | 8 | 9 | 7.5 | 1564 | 1004 | 976 |
| 27 | 19.50 | 5.50 | 5.38 | 7.25 | 7.5 | 7.5 | 1680 | 930 | 920 |
| 28 | 17.00 | 6.00 | 6.25 | 8.5 | 7.5 | 9.5 | 1500 | 1028 | 948 |
| 29 | 14.25 | 6.00 | 5.63 | 7.25 | 8.5 | 10 | 1576 | 1096 | 952 |
| 30 | 16.00 | 5.50 | 5.75 | 8.75 | 10.5 | 9 | 1472 | 1080 | 968 |

Source 1 = Trang 1, Source 2 = Trang 2, Source 3 = Nakhon Si Thammarat

**Table 47** Stomatal number, stomatal index, and trichome number of *D. elliptica*

| Field | Stomatal number **(/mm$^2$)** | | | Stomata index | | | Trichome number **(/mm$^2$)** | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 224 | 120 | 344 | 11.50 | 8.76 | 16.01 | 12 | 16 | 12 |
| 2 | 208 | 136 | 320 | 10.34 | 9.16 | 13.77 | 11 | 16 | 15 |
| 3 | 240 | 124 | 300 | 11.32 | 8.93 | 13.81 | 19 | 17 | 13 |
| 4 | 232 | 116 | 312 | 12.18 | 7.59 | 14.03 | 19 | 17 | 9 |
| 5 | 264 | 156 | 332 | 12.84 | 10.66 | 15.43 | 10 | 17 | 10 |
| 6 | 224 | 152 | 304 | 11.48 | 10.86 | 13.72 | 11 | 16 | 11 |
| 7 | 200 | 140 | 340 | 9.73 | 9.67 | 15.32 | 10 | 18 | 12 |
| 8 | 232 | 132 | 272 | 11.53 | 9.57 | 12.52 | 13 | 17 | 10 |
| 9 | 224 | 124 | 292 | 10.65 | 8.38 | 14.04 | 13 | 18 | 9 |
| 10 | 248 | 116 | 332 | 11.42 | 8.36 | 15.99 | 10 | 16 | 15 |
| 11 | 272 | 140 | 268 | 12.81 | 9.70 | 12.50 | 13 | 17 | 17 |
| 12 | 208 | 128 | 268 | 9.73 | 9.28 | 12.03 | 13 | 16 | 16 |
| 13 | 200 | 124 | 324 | 10.26 | 8.45 | 15.06 | 10 | 14 | 13 |
| 14 | 216 | 144 | 280 | 9.89 | 10.23 | 14.00 | 11 | 16 | 10 |
| 15 | 252 | 104 | 280 | 12.28 | 7.37 | 12.70 | 12 | 16 | 16 |
| 16 | 208 | 120 | 308 | 9.58 | 8.15 | 14.95 | 18 | 15 | 12 |
| 17 | 224 | 152 | 324 | 10.98 | 10.50 | 14.46 | 16 | 17 | 11 |
| 18 | 272 | 132 | 304 | 12.77 | 9.73 | 13.22 | 14 | 15 | 9 |
| 19 | 248 | 104 | 324 | 12.33 | 7.14 | 15.49 | 13 | 15 | 10 |
| 20 | 200 | 124 | 308 | 9.60 | 8.14 | 14.58 | 12 | 16 | 12 |
| 21 | 220 | 124 | 272 | 10.38 | 8.49 | 12.48 | 16 | 17 | 14 |
| 22 | 232 | 132 | 288 | 11.13 | 9.22 | 14.20 | 10 | 15 | 16 |
| 23 | 192 | 104 | 320 | 8.99 | 7.24 | 15.41 | 11 | 16 | 11 |
| 24 | 260 | 152 | 272 | 12.60 | 9.92 | 13.36 | 10 | 15 | 12 |
| 25 | 192 | 120 | 296 | 9.07 | 8.70 | 13.31 | 18 | 16 | 10 |
| 26 | 192 | 136 | 316 | 8.96 | 9.74 | 13.67 | 19 | 16 | 14 |
| 27 | 232 | 104 | 280 | 10.84 | 6.79 | 12.50 | 10 | 16 | 16 |
| 28 | 192 | 112 | 328 | 9.34 | 7.22 | 14.70 | 12 | 17 | 14 |
| 29 | 184 | 120 | 272 | 8.95 | 7.89 | 13.26 | 17 | 17 | 13 |
| 30 | 208 | 152 | 324 | 10.24 | 9.55 | 14.86 | 19 | 16 | 12 |

Source 1 = Bangkok, Source 2 = Nakhon Ratchasima, Source 3 = Nakhon Pathom

**Table 48** Vein islet number, palisade ratio, and epidermal cell number of *D. elliptica*

| Field | Vein islet number (/mm$^2$) | | | Palisade ratio | | | Lower epidermal cell number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 8.25 | 18.25 | 29 | 17 | 10 | 9 | 1724 | 1250 | 1804 |
| 2 | 8.25 | 17.375 | 30.25 | 15 | 8.75 | 6.25 | 1804 | 1348 | 2004 |
| 3 | 8.13 | 16.75 | 30 | 13.5 | 9.25 | 6.5 | 1880 | 1264 | 1872 |
| 4 | 8.50 | 17.75 | 31.25 | 16.5 | 8.75 | 7.25 | 1672 | 1412 | 1912 |
| 5 | 8.25 | 16.75 | 30.75 | 14.5 | 8.5 | 8.5 | 1792 | 1308 | 1820 |
| 6 | 8.25 | 17.25 | 29 | 15.5 | 8.5 | 8.25 | 1728 | 1248 | 1912 |
| 7 | 8.00 | 17.5 | 30.75 | 13.5 | 8.75 | 8.75 | 1856 | 1308 | 1880 |
| 8 | 7.00 | 18 | 28 | 15 | 9.5 | 7.25 | 1780 | 1248 | 1900 |
| 9 | 8.63 | 17.25 | 29.5 | 16 | 8.75 | 7.75 | 1880 | 1356 | 1788 |
| 10 | 8.75 | 17.5 | 31.5 | 14 | 8.5 | 9.75 | 1924 | 1272 | 1744 |
| 11 | 8.38 | 17.75 | 29.75 | 15 | 7.75 | 8 | 1852 | 1304 | 1876 |
| 12 | 8.25 | 16.5 | 32.25 | 15.5 | 8.75 | 8.25 | 1930 | 1252 | 1960 |
| 13 | 8.38 | 16.25 | 30.25 | 17 | 8 | 8 | 1750 | 1344 | 1828 |
| 14 | 8.00 | 17.5 | 31.5 | 13 | 9 | 8 | 1968 | 1264 | 1720 |
| 15 | 8.00 | 15.75 | 30 | 13.5 | 9.75 | 7 | 1800 | 1308 | 1924 |
| 16 | 8.50 | 15.5 | 29 | 14.5 | 9.5 | 8.75 | 1964 | 1352 | 1752 |
| 17 | 8.00 | 17.25 | 31 | 16 | 9 | 8 | 1816 | 1296 | 1916 |
| 18 | 8.50 | 17.25 | 29.75 | 15.5 | 9.25 | 7 | 1858 | 1224 | 1996 |
| 19 | 8.50 | 18 | 28.75 | 15 | 9.25 | 7.5 | 1764 | 1352 | 1768 |
| 20 | 7.75 | 17 | 28.25 | 13.5 | 8.75 | 7.5 | 1884 | 1400 | 1804 |
| 21 | 8.00 | 16.5 | 29.25 | 14.5 | 9.75 | 7 | 1900 | 1336 | 1908 |
| 22 | 8.13 | 16.25 | 32.75 | 15 | 9.75 | 7.5 | 1852 | 1300 | 1740 |
| 23 | 7.50 | 16.25 | 32.5 | 15.5 | 9 | 7 | 1944 | 1332 | 1756 |
| 24 | 8.13 | 17.25 | 31.75 | 16 | 9.25 | 7.5 | 1804 | 1380 | 1764 |
| 25 | 7.75 | 17 | 30 | 14.5 | 9.75 | 8 | 1924 | 1260 | 1928 |
| 26 | 6.50 | 16.75 | 29 | 14 | 8 | 8.25 | 1952 | 1260 | 1996 |
| 27 | 7.50 | 17.75 | 30 | 17 | 8 | 7.5 | 1908 | 1428 | 1960 |
| 28 | 8.00 | 15.75 | 30.25 | 15.5 | 8.25 | 6.5 | 1864 | 1440 | 1904 |
| 29 | 8.25 | 18.5 | 30.5 | 15 | 8 | 6.5 | 1872 | 1400 | 1780 |
| 30 | 8.50 | 14.25 | 28.75 | 14 | 9.25 | 7.5 | 1824 | 1440 | 1856 |

**Source 1 = Bangkok, Source 2 = Nakhon Ratchasima, Source 3 = Nakhon Pathom**

**Table 49** Stomatal number, stomatal index, and trichome number of *D. indica*

| Field | Stomatal number (/mm$^2$) | | | Stomata index | | | Trichome number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 160 | 232 | 192 | 6.91 | 10.49 | 9.30 | - | - | - |
| 2 | 152 | 224 | 212 | 6.40 | 10.71 | 9.83 | - | - | - |
| 3 | 160 | 192 | 212 | 6.57 | 8.71 | 10.47 | - | - | - |
| 4 | 168 | 224 | 176 | 7.00 | 10.87 | 9.19 | - | - | - |
| 5 | 144 | 200 | 228 | 5.92 | 9.58 | 10.17 | - | - | - |
| 6 | 152 | 208 | 208 | 6.67 | 9.96 | 9.40 | - | - | - |
| 7 | 128 | 192 | 208 | 5.45 | 9.07 | 9.49 | - | - | - |
| 8 | 184 | 184 | 176 | 7.41 | 8.80 | 8.15 | - | - | - |
| 9 | 192 | 200 | 232 | 7.62 | 9.19 | 10.90 | - | - | - |
| 10 | 176 | 224 | 200 | 7.30 | 10.53 | 9.21 | - | - | - |
| 11 | 200 | 184 | 208 | 8.26 | 8.66 | 9.83 | - | - | - |
| 12 | 144 | 232 | 192 | 6.12 | 10.49 | 9.74 | - | - | - |
| 13 | 152 | 200 | 184 | 6.59 | 9.49 | 9.26 | - | - | - |
| 14 | 184 | 224 | 176 | 7.43 | 10.59 | 8.84 | - | - | - |
| 15 | 160 | 208 | 160 | 6.71 | 9.58 | 7.97 | - | - | - |
| 16 | 160 | 248 | 224 | 6.94 | 11.55 | 10.59 | - | - | - |
| 17 | 152 | 208 | 200 | 6.53 | 10.38 | 9.33 | - | - | - |
| 18 | 144 | 240 | 212 | 6.21 | 11.24 | 10.25 | - | - | - |
| 19 | 176 | 192 | 192 | 7.30 | 9.64 | 9.16 | - | - | - |
| 20 | 192 | 248 | 208 | 9.09 | 11.83 | 9.81 | - | - | - |
| 21 | 208 | 192 | 184 | 8.18 | 9.28 | 8.90 | - | - | - |
| 22 | 224 | 192 | 188 | 8.99 | 9.18 | 8.79 | - | - | - |
| 23 | 184 | 264 | 200 | 7.90 | 12.00 | 9.03 | - | - | - |
| 24 | 144 | 240 | 220 | 6.12 | 11.43 | 10.19 | - | - | - |
| 25 | 208 | 192 | 196 | 8.80 | 8.74 | 9.28 | - | - | - |
| 26 | 176 | 240 | 220 | 7.42 | 11.17 | 9.87 | - | - | - |
| 27 | 192 | 216 | 192 | 8.00 | 10.27 | 9.02 | - | - | - |
| 28 | 144 | 232 | 232 | 5.87 | 10.86 | 11.28 | - | - | - |
| 29 | 136 | 232 | 176 | 5.78 | 10.80 | 8.46 | - | - | - |
| 30 | 192 | 192 | 208 | 7.75 | 9.50 | 10.10 | - | - | - |

Source 1 = Chonburi 1, Source 2 = Chonburi 2, Source 3 = Samut Sakhon

**Table 50** Vein islet number, palisade ratio, and epidermal cell number of *D. indica*

| Field | Vein islet number (/mm$^2$) | | | Palisade ratio | | | Lower epidermal cell number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 17.00 | 11.50 | 16.25 | 7.75 | 6.50 | 6.00 | 2156 | 1980 | 1872 |
| 2 | 17.13 | 12.50 | 17.50 | 8.75 | 5.50 | 6.75 | 2224 | 1868 | 1944 |
| 3 | 16.25 | 12.50 | 16.00 | 6.25 | 6.50 | 6.75 | 2276 | 2012 | 1812 |
| 4 | 16.25 | 12.50 | 17.00 | 7.75 | 7.00 | 5.50 | 2232 | 1836 | 1740 |
| 5 | 16.25 | 12.75 | 16.00 | 9.50 | 6.00 | 6.75 | 2288 | 1888 | 2014 |
| 6 | 17.00 | 12.50 | 17.50 | 7.75 | 7.00 | 7.50 | 2128 | 1880 | 2004 |
| 7 | 17.25 | 13.00 | 17.75 | 8.50 | 8.00 | 8.00 | 2220 | 1924 | 1984 |
| 8 | 18.00 | 12.50 | 18.00 | 8.00 | 7.50 | 7.00 | 2300 | 1908 | 1984 |
| 9 | 19.25 | 14.25 | 19.38 | 8.50 | 8.00 | 6.00 | 2328 | 1976 | 1896 |
| 10 | 17.50 | 13.50 | 18.25 | 8.75 | 8.00 | 7.50 | 2236 | 1904 | 1972 |
| 11 | 18.25 | 12.25 | 18.75 | 8.50 | 6.50 | 7.00 | 2220 | 1940 | 1908 |
| 12 | 15.25 | 11.75 | 15.38 | 8.50 | 9.00 | 6.75 | 2208 | 1980 | 1780 |
| 13 | 15.75 | 11.25 | 16.00 | 10.00 | 6.50 | 7.50 | 2156 | 1908 | 1804 |
| 14 | 15.75 | 12.00 | 16.13 | 9.50 | 7.50 | 6.25 | 2292 | 1892 | 1816 |
| 15 | 18.75 | 12.75 | 18.00 | 8.50 | 6.50 | 7.50 | 2224 | 1964 | 1848 |
| 16 | 16.25 | 12.50 | 16.75 | 8.00 | 8.50 | 7.25 | 2144 | 1900 | 1892 |
| 17 | 15.75 | 11.75 | 15.50 | 8.50 | 6.50 | 8.50 | 2176 | 1796 | 1944 |
| 18 | 17.50 | 12.50 | 18.75 | 8.00 | 7.50 | 6.25 | 2176 | 1896 | 1856 |
| 19 | 15.25 | 12.25 | 15.50 | 10.00 | 7.50 | 8.25 | 2236 | 1800 | 1904 |
| 20 | 15.25 | 11.50 | 16.00 | 9.00 | 7.00 | 7.25 | 1920 | 1848 | 1912 |
| 21 | 16.00 | 12.25 | 16.50 | 8.00 | 8.50 | 7.50 | 2336 | 1876 | 1884 |
| 22 | 16.00 | 11.25 | 17.25 | 10.00 | 10.00 | 7.00 | 2268 | 1900 | 1952 |
| 23 | 15.25 | 10.50 | 18.75 | 8.50 | 7.00 | 6.50 | 2144 | 1936 | 2016 |
| 24 | 17.50 | 12.75 | 16.13 | 8.50 | 7.00 | 8.00 | 2208 | 1860 | 1940 |
| 25 | 16.00 | 12.50 | 17.38 | 11.00 | 7.50 | 9.00 | 2156 | 2004 | 1916 |
| 26 | 17.50 | 13.25 | 18.00 | 8.00 | 7.00 | 6.75 | 2196 | 1908 | 2008 |
| 27 | 17.00 | 12.25 | 15.75 | 9.00 | 7.50 | 6.25 | 2208 | 1888 | 1936 |
| 28 | 16.00 | 11.25 | 18.25 | 9.00 | 7.00 | 7.50 | 2308 | 1904 | 1824 |
| 29 | 16.50 | 12.50 | 18.25 | 10.00 | 8.00 | 6.75 | 2216 | 1916 | 1904 |
| 30 | 17.00 | 12.50 | 16.50 | 9.50 | 6.50 | 6.75 | 2284 | 1828 | 1852 |

Source 1 = Chonburi 1, Source 2 = Chonburi 2, Source 3 = Samut Sakhon

**Table 51** Stomatal number, stomatal index, and trichome number of *D. malaccensis*

| Field | Stomatal number (/mm$^2$) | | | Stomatal index | | | Trichome number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 208 | 136 | 156 | 10.18 | 8.83 | 9.79 | - | - | - |
| 2 | 212 | 148 | 156 | 10.73 | 9.30 | 9.92 | - | - | - |
| 3 | 228 | 156 | 184 | 11.33 | 9.92 | 11.12 | 1 | - | - |
| 4 | 188 | 160 | 172 | 9.91 | 9.76 | 10.58 | - | - | - |
| 5 | 208 | 148 | 168 | 14.44 | 9.46 | 9.31 | - | - | - |
| 6 | 208 | 180 | 164 | 14.65 | 10.69 | 8.09 | - | - | - |
| 7 | 260 | 140 | 144 | 17.08 | 8.75 | 8.40 | - | - | - |
| 8 | 228 | 136 | 188 | 11.40 | 8.27 | 10.65 | - | 2 | - |
| 9 | 200 | 144 | 172 | 11.26 | 8.65 | 9.32 | - | - | - |
| 10 | 216 | 152 | 176 | 11.21 | 9.69 | 10.54 | - | - | - |
| 11 | 192 | 144 | 160 | 10.00 | 9.30 | 9.36 | - | - | - |
| 12 | 196 | 160 | 172 | 9.88 | 9.43 | 9.71 | - | - | - |
| 13 | 228 | 144 | 188 | 11.83 | 8.70 | 11.24 | - | - | - |
| 14 | 200 | 148 | 160 | 10.02 | 8.94 | 10.00 | - | - | - |
| 15 | 216 | 168 | 164 | 10.53 | 9.68 | 9.23 | - | - | - |
| 16 | 232 | 144 | 172 | 11.42 | 9.14 | 9.93 | - | - | - |
| 17 | 220 | 164 | 156 | 11.41 | 10.41 | 10.08 | - | - | - |
| 18 | 216 | 152 | 164 | 12.16 | 9.18 | 9.86 | - | - | - |
| 19 | 200 | 152 | 168 | 11.19 | 9.36 | 10.57 | - | - | 1 |
| 20 | 212 | 160 | 164 | 11.86 | 10.05 | 10.46 | - | - | - |
| 21 | 220 | 144 | 180 | 11.53 | 8.74 | 10.40 | - | - | - |
| 22 | 180 | 160 | 168 | 9.43 | 9.93 | 10.46 | - | - | - |
| 23 | 212 | 136 | 160 | 11.06 | 8.90 | 9.83 | - | - | - |
| 24 | 216 | 172 | 148 | 12.27 | 10.31 | 9.32 | - | - | - |
| 25 | 220 | 128 | 156 | 11.73 | 8.67 | 9.61 | - | - | - |
| 26 | 212 | 140 | 152 | 10.77 | 9.38 | 9.60 | - | - | - |
| 27 | 204 | 144 | 160 | 10.83 | 9.00 | 9.71 | - | - | - |
| 28 | 228 | 144 | 184 | 11.78 | 9.28 | 11.59 | - | - | - |
| 29 | 204 | 136 | 148 | 11.04 | 8.88 | 9.66 | - | - | - |
| 30 | 208 | 176 | 156 | 11.63 | 10.89 | 9.79 | - | - | - |

Source 1 = Trang, Source 2 = Nakhon Ratchasima, Source 3 = Nonthaburi,

**Table 52** Vein islet number, palisade ratio, and epidermal cell number of *D. malaccensis*

| Field | Vein islet number (/mm$^2$) | | | Palisade ratio | | | Lower epidermal cell number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 17.50 | 14.00 | 14.25 | 7.75 | 6.75 | 9.00 | 1836 | 1404 | 1438 |
| 2 | 16.75 | 13.75 | 15.00 | 9.25 | 7.00 | 8.50 | 1764 | 1444 | 1416 |
| 3 | 17.00 | 13.00 | 11.50 | 8.25 | 7.00 | 6.50 | 1784 | 1416 | 1470 |
| 4 | 17.50 | 11.50 | 9.50 | 9.00 | 6.50 | 7.00 | 1710 | 1480 | 1454 |
| 5 | 16.25 | 11.50 | 10.25 | 8.00 | 6.00 | 6.50 | 1232 | 1416 | 1636 |
| 6 | 16.25 | 13.13 | 14.25 | 8.50 | 8.25 | 9.00 | 1212 | 1504 | 1862 |
| 7 | 15.75 | 11.75 | 12.25 | 8.75 | 7.75 | 7.50 | 1262 | 1460 | 1570 |
| 8 | 16.50 | 13.13 | 9.25 | 7.25 | 7.00 | 8.00 | 1772 | 1508 | 1578 |
| 9 | 15.25 | 13.00 | 11.00 | 7.75 | 7.00 | 7.00 | 1576 | 1520 | 1674 |
| 10 | 14.50 | 12.38 | 9.63 | 8.75 | 7.00 | 9.00 | 1710 | 1416 | 1494 |
| 11 | 15.75 | 14.00 | 12.25 | 9.50 | 6.25 | 8.50 | 1728 | 1404 | 1550 |
| 12 | 15.50 | 14.25 | 13.75 | 8.75 | 6.75 | 7.00 | 1788 | 1536 | 1600 |
| 13 | 15.75 | 15.50 | 12.75 | 8.00 | 7.25 | 8.00 | 1700 | 1512 | 1484 |
| 14 | 16.88 | 14.25 | 13.25 | 9.00 | 7.50 | 7.50 | 1796 | 1508 | 1440 |
| 15 | 15.75 | 15.00 | 12.38 | 9.50 | 7.50 | 6.50 | 1836 | 1568 | 1612 |
| 16 | 14.00 | 15.25 | 12.00 | 9.25 | 6.50 | 8.50 | 1800 | 1432 | 1560 |
| 17 | 14.00 | 14.88 | 11.50 | 9.75 | 7.75 | 9.00 | 1708 | 1412 | 1392 |
| 18 | 16.75 | 14.00 | 12.25 | 7.50 | 7.25 | 7.00 | 1560 | 1504 | 1500 |
| 19 | 17.00 | 12.75 | 13.00 | 8.50 | 8.00 | 8.00 | 1588 | 1472 | 1422 |
| 20 | 16.88 | 11.75 | 11.88 | 10.75 | 7.75 | 7.50 | 1576 | 1432 | 1404 |
| 21 | 16.13 | 12.50 | 14.25 | 9.00 | 6.25 | 8.00 | 1688 | 1504 | 1550 |
| 22 | 16.50 | 12.50 | 13.75 | 9.50 | 7.00 | 7.00 | 1728 | 1452 | 1438 |
| 23 | 15.75 | 13.75 | 14.00 | 9.00 | 6.75 | 6.00 | 1704 | 1392 | 1468 |
| 24 | 15.75 | 12.75 | 15.00 | 10.00 | 6.50 | 9.00 | 1544 | 1496 | 1440 |
| 25 | 15.75 | 11.00 | 14.00 | 8.00 | 8.00 | 8.00 | 1656 | 1348 | 1468 |
| 26 | 17.75 | 11.50 | 13.00 | 9.75 | 6.50 | 7.50 | 1756 | 1352 | 1432 |
| 27 | 15.25 | 13.75 | 14.00 | 8.75 | 6.00 | 9.00 | 1680 | 1456 | 1488 |
| 28 | 14.50 | 13.00 | 15.00 | 8.50 | 8.00 | 7.50 | 1708 | 1408 | 1404 |
| 29 | 17.50 | 12.25 | 14.00 | 9.75 | 6.50 | 9.50 | 1644 | 1396 | 1384 |
| 30 | 16.38 | 15.00 | 14.25 | 8.75 | 6.75 | 9.00 | 1580 | 1440 | 1438 |

Source 1 = Trang, Source 2 = Nakhon Ratchasima, Source 3 = Nonthaburi,

**Table 53** Stomatal number, stomatal index, and trichome number of *D. scandens*

| Field | Stomatal number (/mm$^2$) | | | Stomata index | | | Trichome number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 132 | 240 | 248 | 9.12 | 15.11 | 16.06 | 15.25 | 12.00 | 19.00 |
| 2 | 160 | 256 | 248 | 10.72 | 16.04 | 15.94 | 15.25 | 10.00 | 12.00 |
| 3 | 160 | 240 | 264 | 10.15 | 14.83 | 17.28 | 16.00 | 10.00 | 17.00 |
| 4 | 136 | 232 | 272 | 10.21 | 15.51 | 16.67 | 16.25 | 13.00 | 12.00 |
| 5 | 168 | 248 | 280 | 11.14 | 15.78 | 16.95 | 17.25 | 14.00 | 15.00 |
| 6 | 140 | 240 | 288 | 10.06 | 15.15 | 17.52 | 15.75 | 10.00 | 15.00 |
| 7 | 160 | 260 | 320 | 11.17 | 17.29 | 20.10 | 16.75 | 13.00 | 16.00 |
| 8 | 144 | 248 | 248 | 10.71 | 15.74 | 15.54 | 15.50 | 10.00 | 14.00 |
| 9 | 152 | 244 | 344 | 10.38 | 15.60 | 19.86 | 15.50 | 8.00 | 14.00 |
| 10 | 160 | 232 | 248 | 10.13 | 14.76 | 16.45 | 16.25 | 10.00 | 12.00 |
| 11 | 152 | 236 | 264 | 10.30 | 14.68 | 16.92 | 16.00 | 9.00 | 12.00 |
| 12 | 144 | 220 | 272 | 9.63 | 15.49 | 18.18 | 15.50 | 12.00 | 14.00 |
| 13 | 152 | 240 | 320 | 9.79 | 15.63 | 20.05 | 15.75 | 10.00 | 12.00 |
| 14 | 136 | 256 | 344 | 9.74 | 16.08 | 22.05 | 15.75 | 16.00 | 16.00 |
| 15 | 160 | 208 | 272 | 10.31 | 13.44 | 16.71 | 17.50 | 14.00 | 14.00 |
| 16 | 156 | 232 | 280 | 10.32 | 14.46 | 17.72 | 16.25 | 10.00 | 12.00 |
| 17 | 132 | 240 | 272 | 9.12 | 14.67 | 16.79 | 17.50 | 8.00 | 12.00 |
| 18 | 164 | 208 | 296 | 11.52 | 13.33 | 19.27 | 16.75 | 10.00 | 14.00 |
| 19 | 140 | 216 | 264 | 9.75 | 13.85 | 15.71 | 15.25 | 13.00 | 18.00 |
| 20 | 132 | 232 | 264 | 8.66 | 14.65 | 16.10 | 16.75 | 15.00 | 20.00 |
| 21 | 144 | 252 | 248 | 10.20 | 16.80 | 15.74 | 17.00 | 10.00 | 20.00 |
| 22 | 160 | 240 | 344 | 10.42 | 15.46 | 20.77 | 15.75 | 8.00 | 16.00 |
| 23 | 160 | 260 | 320 | 11.43 | 17.38 | 18.69 | 17.50 | 12.00 | 11.00 |
| 24 | 148 | 208 | 304 | 10.39 | 13.30 | 17.72 | 15.25 | 16.00 | 14.00 |
| 25 | 156 | 260 | 296 | 10.68 | 15.93 | 18.18 | 16.75 | 12.00 | 12.00 |
| 26 | 144 | 220 | 272 | 10.14 | 14.59 | 17.09 | 17.00 | 16.00 | 16.00 |
| 27 | 160 | 248 | 336 | 11.20 | 16.06 | 20.10 | 16.25 | 14.00 | 12.00 |
| 28 | 168 | 272 | 272 | 11.38 | 16.63 | 15.67 | 16.50 | 10.00 | 16.00 |
| 29 | 132 | 208 | 256 | 8.66 | 12.81 | 15.13 | 17.50 | 10.00 | 18.00 |
| 30 | 156 | 200 | 296 | 10.57 | 12.99 | 18.23 | 15.00 | 12.00 | 14.00 |

Source 1 = Bangkok, Source 2 = Nakhon Ratchasima, Source 3 Nakhon Pathom

**Table 54** Vein islet number, palisade ratio, and epidermal cell number of *D. scandens*

| Field | Vein islet number (/mm$^2$) | | | Palisade ratio | | | Lower epidermal cell number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 15.25 | 12.38 | 16.00 | 5.25 | 5.50 | 3.50 | 1316 | 1348 | 1296 |
| 2 | 15.25 | 12.25 | 16.00 | 5.75 | 6.00 | 4.75 | 1332 | 1340 | 1308 |
| 3 | 16.00 | 10.00 | 17.00 | 6.50 | 6.25 | 4.75 | 1416 | 1378 | 1264 |
| 4 | 16.25 | 12.50 | 16.50 | 6.50 | 5.25 | 5.50 | 1196 | 1264 | 1360 |
| 5 | 17.25 | 13.00 | 17.00 | 6.75 | 6.25 | 4.50 | 1340 | 1324 | 1372 |
| 6 | 15.75 | 13.13 | 17.50 | 6.50 | 5.75 | 4.50 | 1252 | 1344 | 1356 |
| 7 | 16.75 | 12.50 | 18.00 | 5.00 | 5.75 | 4.00 | 1272 | 1244 | 1272 |
| 8 | 15.50 | 12.38 | 15.50 | 6.25 | 5.50 | 4.75 | 1200 | 1328 | 1348 |
| 9 | 15.50 | 12.00 | 18.00 | 6.25 | 5.00 | 4.00 | 1312 | 1320 | 1388 |
| 10 | 16.25 | 13.00 | 16.50 | 5.50 | 5.25 | 5.00 | 1420 | 1340 | 1260 |
| 11 | 16.00 | 18.25 | 17.00 | 5.75 | 5.25 | 5.50 | 1324 | 1372 | 1296 |
| 12 | 15.50 | 17.75 | 18.00 | 7.00 | 5.75 | 4.75 | 1352 | 1200 | 1224 |
| 13 | 15.75 | 17.00 | 18.00 | 6.75 | 5.00 | 4.50 | 1400 | 1296 | 1276 |
| 14 | 15.75 | 18.25 | 18.00 | 6.00 | 5.00 | 2.75 | 1260 | 1336 | 1216 |
| 15 | 17.50 | 17.00 | 16.50 | 6.50 | 5.75 | 5.25 | 1392 | 1340 | 1356 |
| 16 | 16.25 | 17.00 | 18.00 | 7.00 | 7.00 | 5.25 | 1356 | 1372 | 1300 |
| 17 | 17.50 | 15.75 | 17.00 | 6.50 | 5.13 | 5.00 | 1316 | 1396 | 1348 |
| 18 | 16.75 | 17.00 | 18.00 | 6.75 | 5.75 | 5.25 | 1260 | 1352 | 1240 |
| 19 | 15.25 | 18.50 | 16.00 | 7.00 | 5.75 | 5.50 | 1296 | 1344 | 1416 |
| 20 | 16.75 | 11.75 | 16.25 | 6.00 | 5.50 | 4.00 | 1392 | 1352 | 1376 |
| 21 | 17.00 | 13.25 | 15.75 | 6.00 | 5.63 | 4.00 | 1268 | 1248 | 1328 |
| 22 | 15.75 | 13.25 | 17.50 | 6.25 | 5.75 | 5.25 | 1376 | 1312 | 1312 |
| 23 | 17.50 | 15.75 | 18.50 | 6.75 | 4.75 | 5.50 | 1240 | 1236 | 1392 |
| 24 | 15.25 | 12.75 | 17.50 | 6.75 | 4.75 | 4.50 | 1276 | 1356 | 1412 |
| 25 | 16.75 | 14.00 | 18.00 | 6.75 | 4.75 | 3.75 | 1304 | 1372 | 1332 |
| 26 | 17.00 | 12.50 | 17.00 | 6.25 | 5.75 | 5.25 | 1276 | 1288 | 1320 |
| 27 | 16.25 | 13.50 | 20.00 | 7.50 | 6.00 | 5.75 | 1268 | 1296 | 1336 |
| 28 | 16.50 | 13.75 | 15.50 | 6.00 | 7.25 | 5.25 | 1308 | 1364 | 1464 |
| 29 | 17.50 | 13.25 | 15.50 | 6.25 | 4.75 | 5.00 | 1392 | 1416 | 1436 |
| 30 | 15.00 | 13.75 | 18.50 | 5.00 | 5.50 | 4.50 | 1320 | 1340 | 1328 |

Source 1 = Bangkok, Source 2 = Nakhon Ratchasima, Source 3 Nakhon Pathom

**Table 55** Stomatal number, stomatal index, and trichome number of *D. solorioides*

| Field | Stomatal number (/mm$^2$) | | | Stomata index | | | Trichome number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 144 | 152 | 152 | 11.43 | 7.74 | 8.07 | - | - | - |
| 2 | 132 | 168 | 160 | 11.22 | 8.71 | 8.26 | - | - | - |
| 3 | 124 | 140 | 176 | 10.06 | 7.25 | 9.44 | - | - | - |
| 4 | 144 | 148 | 168 | 11.11 | 7.76 | 8.79 | - | - | - |
| 5 | 168 | 148 | 152 | 12.39 | 7.71 | 8.26 | - | - | - |
| 6 | 116 | 152 | 144 | 9.51 | 8.15 | 7.69 | - | - | - |
| 7 | 152 | 172 | 160 | 11.55 | 9.00 | 8.20 | - | - | - |
| 8 | 120 | 168 | 168 | 9.62 | 9.01 | 8.59 | - | - | - |
| 9 | 144 | 152 | 176 | 11.54 | 8.72 | 9.40 | - | - | - |
| 10 | 144 | 168 | 160 | 10.88 | 8.82 | 8.66 | - | - | - |
| 11 | 156 | 160 | 152 | 11.71 | 8.57 | 7.85 | - | - | - |
| 12 | 144 | 144 | 176 | 12.16 | 8.00 | 9.00 | - | - | - |
| 13 | 136 | 168 | 180 | 11.49 | 9.07 | 9.62 | - | - | - |
| 14 | 148 | 148 | 156 | 11.90 | 8.10 | 8.23 | - | - | - |
| 15 | 140 | 156 | 160 | 11.48 | 8.65 | 8.23 | - | - | - |
| 16 | 144 | 160 | 168 | 11.80 | 8.83 | 8.84 | - | - | - |
| 17 | 148 | 176 | 152 | 11.42 | 10.11 | 8.52 | - | - | - |
| 18 | 160 | 156 | 176 | 12.16 | 10.10 | 8.89 | - | - | - |
| 19 | 136 | 148 | 176 | 11.18 | 8.64 | 9.32 | - | - | - |
| 20 | 136 | 168 | 184 | 11.53 | 9.79 | 9.35 | - | - | - |
| 21 | 136 | 160 | 192 | 11.30 | 9.30 | 9.86 | - | - | - |
| 22 | 144 | 160 | 160 | 11.50 | 9.11 | 8.15 | - | - | - |
| 23 | 144 | 168 | 148 | 10.11 | 9.44 | 7.68 | - | - | - |
| 24 | 136 | 184 | 168 | 10.76 | 10.09 | 8.57 | - | - | - |
| 25 | 140 | 168 | 160 | 10.80 | 9.21 | 8.71 | - | - | - |
| 26 | 152 | 164 | 176 | 11.41 | 9.34 | 9.24 | - | - | - |
| 27 | 152 | 172 | 152 | 10.86 | 10.00 | 8.03 | - | - | - |
| 28 | 132 | 160 | 148 | 9.97 | 9.17 | 7.72 | - | - | - |
| 29 | 172 | 168 | 176 | 12.11 | 9.35 | 9.19 | - | - | - |
| 30 | 144 | 148 | 160 | 10.94 | 8.53 | 8.33 | - | - | - |

Source 1 = Bangkok 1, Source 2 = Bangkok 2, Source 3 Nakhon Pathom

**Table 56** Vein islet number, palisade ratio, and epidermal cell number of *D. solorioides*

| Field | Vein islet number (/mm$^2$) | | | Palisade ratio | | | Lower epidermal cell number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 13.50 | 13.00 | 11.00 | 10.25 | 9.50 | 10.50 | 1116 | 1812 | 1732 |
| 2 | 13.50 | 12.25 | 11.75 | 12.00 | 10.25 | 7.50 | 1044 | 1760 | 1776 |
| 3 | 13.25 | 12.50 | 11.25 | 12.50 | 11.25 | 9.50 | 1108 | 1792 | 1688 |
| 4 | 14.00 | 11.75 | 11.00 | 10.75 | 9.00 | 7.50 | 1152 | 1760 | 1744 |
| 5 | 12.75 | 13.00 | 11.63 | 12.75 | 12.50 | 8.50 | 1188 | 1772 | 1688 |
| 6 | 13.00 | 14.50 | 10.00 | 11.50 | 13.00 | 9.00 | 1104 | 1712 | 1728 |
| 7 | 13.00 | 13.75 | 11.75 | 11.25 | 11.00 | 9.50 | 1164 | 1740 | 1792 |
| 8 | 13.25 | 12.25 | 10.25 | 13.75 | 11.50 | 7.50 | 1128 | 1696 | 1788 |
| 9 | 13.63 | 13.25 | 11.00 | 14.50 | 10.00 | 9.50 | 1104 | 1592 | 1696 |
| 10 | 13.25 | 12.00 | 11.75 | 12.75 | 10.50 | 8.50 | 1180 | 1736 | 1688 |
| 11 | 14.50 | 12.50 | 11.75 | 13.50 | 10.00 | 8.75 | 1176 | 1708 | 1784 |
| 12 | 14.00 | 13.25 | 12.00 | 12.50 | 12.00 | 9.00 | 1040 | 1656 | 1780 |
| 13 | 14.25 | 13.50 | 11.25 | 12.25 | 11.50 | 9.25 | 1048 | 1684 | 1692 |
| 14 | 12.00 | 13.25 | 10.25 | 11.00 | 10.50 | 9.50 | 1096 | 1680 | 1740 |
| 15 | 12.50 | 13.50 | 10.25 | 11.00 | 10.50 | 9.50 | 1080 | 1648 | 1784 |
| 16 | 12.50 | 13.25 | 12.25 | 13.75 | 10.00 | 10.00 | 1076 | 1652 | 1732 |
| 17 | 12.50 | 11.25 | 11.25 | 13.50 | 11.25 | 8.50 | 1148 | 1564 | 1632 |
| 18 | 12.50 | 14.25 | 11.50 | 10.00 | 11.50 | 10.50 | 1156 | 1388 | 1804 |
| 19 | 12.50 | 12.50 | 11.38 | 12.75 | 10.00 | 10.00 | 1080 | 1564 | 1712 |
| 20 | 12.25 | 12.75 | 9.50 | 13.25 | 11.00 | 8.00 | 1044 | 1548 | 1784 |
| 21 | 11.75 | 15.50 | 11.25 | 11.00 | 9.50 | 7.50 | 1068 | 1560 | 1756 |
| 22 | 12.75 | 14.00 | 14.25 | 9.75 | 10.00 | 10.00 | 1108 | 1596 | 1804 |
| 23 | 11.75 | 12.50 | 14.50 | 13.25 | 11.00 | 8.00 | 1280 | 1612 | 1780 |
| 24 | 12.38 | 13.25 | 14.50 | 12.25 | 10.50 | 8.50 | 1128 | 1640 | 1792 |
| 25 | 10.75 | 12.00 | 12.75 | 11.50 | 13.25 | 10.00 | 1156 | 1656 | 1676 |
| 26 | 11.25 | 13.75 | 11.50 | 9.25 | 12.25 | 8.50 | 1180 | 1592 | 1728 |
| 27 | 12.75 | 15.50 | 11.50 | 11.75 | 10.00 | 10.50 | 1248 | 1548 | 1740 |
| 28 | 12.00 | 15.00 | 11.38 | 11.25 | 10.50 | 11.00 | 1192 | 1584 | 1768 |
| 29 | 11.88 | 14.75 | 12.00 | 11.50 | 10.50 | 9.50 | 1248 | 1628 | 1740 |
| 30 | 11.75 | 12.50 | 13.50 | 12.25 | 12.50 | 9.00 | 1172 | 1588 | 1760 |

Source 1 = Bangkok 1, Source 2 = Bangkok 2, Source 3 Nakhon Pathom

**Table 57** Upper Stomatal number, Upper stomatal index, and Upper Epidermal cell number of *D. trifoliata*

| Field | Upper stomatal number (/mm$^2$) | | | Upper stomata index | | | Upper epidermal cell number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 160 | 164 | 220 | 8.62 | 8.87 | 8.69 | 1696 | 1684 | 2312 |
| 2 | 168 | 180 | 200 | 8.94 | 9.16 | 8.17 | 1712 | 1784 | 2248 |
| 3 | 184 | 196 | 232 | 10.22 | 10.10 | 9.15 | 1616 | 1744 | 2304 |
| 4 | 160 | 176 | 240 | 8.73 | 10.48 | 9.65 | 1672 | 1504 | 2248 |
| 5 | 152 | 160 | 232 | 8.33 | 8.70 | 10.18 | 1672 | 1680 | 2048 |
| 6 | 164 | 184 | 220 | 9.36 | 11.47 | 9.29 | 1588 | 1420 | 2148 |
| 7 | 160 | 184 | 224 | 8.46 | 10.95 | 8.83 | 1732 | 1496 | 2312 |
| 8 | 196 | 188 | 208 | 10.89 | 10.13 | 8.44 | 1604 | 1668 | 2256 |
| 9 | 200 | 176 | 216 | 10.53 | 9.82 | 8.45 | 1700 | 1616 | 2340 |
| 10 | 160 | 204 | 256 | 9.17 | 11.86 | 10.19 | 1584 | 1516 | 2256 |
| 11 | 168 | 180 | 232 | 8.97 | 9.70 | 9.28 | 1704 | 1676 | 2268 |
| 12 | 152 | 168 | 192 | 8.30 | 9.46 | 8.45 | 1680 | 1608 | 2080 |
| 13 | 188 | 196 | 220 | 10.11 | 10.99 | 8.66 | 1672 | 1588 | 2320 |
| 14 | 200 | 180 | 248 | 10.66 | 10.02 | 10.30 | 1676 | 1616 | 2160 |
| 15 | 176 | 172 | 208 | 9.32 | 9.15 | 8.44 | 1712 | 1708 | 2256 |
| 16 | 192 | 184 | 272 | 10.53 | 10.48 | 11.20 | 1632 | 1572 | 2156 |
| 17 | 160 | 196 | 216 | 8.71 | 10.21 | 8.52 | 1676 | 1724 | 2320 |
| 18 | 164 | 168 | 264 | 9.28 | 9.23 | 10.03 | 1604 | 1652 | 2368 |
| 19 | 156 | 196 | 232 | 8.59 | 10.68 | 9.11 | 1660 | 1640 | 2316 |
| 20 | 192 | 188 | 208 | 10.21 | 10.54 | 9.22 | 1688 | 1596 | 2048 |
| 21 | 168 | 192 | 240 | 8.92 | 10.53 | 10.08 | 1716 | 1632 | 2140 |
| 22 | 204 | 156 | 216 | 10.90 | 8.84 | 8.49 | 1668 | 1608 | 2328 |
| 23 | 184 | 160 | 220 | 9.75 | 8.46 | 9.63 | 1704 | 1732 | 2064 |
| 24 | 152 | 160 | 260 | 8.12 | 10.55 | 10.76 | 1720 | 1356 | 2156 |
| 25 | 184 | 180 | 296 | 10.18 | 10.02 | 11.65 | 1624 | 1616 | 2244 |
| 26 | 196 | 184 | 224 | 10.84 | 10.20 | 8.78 | 1612 | 1620 | 2328 |
| 27 | 164 | 176 | 232 | 8.82 | 9.30 | 9.29 | 1696 | 1716 | 2264 |
| 28 | 160 | 192 | 256 | 9.09 | 10.06 | 9.89 | 1600 | 1716 | 2332 |
| 29 | 180 | 184 | 240 | 9.76 | 9.58 | 9.93 | 1664 | 1736 | 2176 |
| 30 | 156 | 160 | 216 | 8.48 | 9.39 | 8.67 | 1684 | 1544 | 2276 |

Source 1 = Nakhon Pathom, Source 2 = Ang Thong, Source 3 Samut Sakhon

**Table 58** Lower Stomatal number, Lower stomatal index, and Lower Epidermal cell number of *D. trifoliata*

| Field | Lower stomatal number (/mm$^2$) | | | lower stomata index | | | Lower epidermal cell number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 34 | 36 | 40 | 3.03 | 3.17 | 4.31 | 1088 | 1100 | 888 |
| 2 | 34 | 42 | 40 | 3.05 | 3.13 | 4.69 | 1080 | 1300 | 812 |
| 3 | 34 | 40 | 44 | 3.04 | 3.03 | 4.21 | 1086 | 1278 | 1000 |
| 4 | 34 | 40 | 44 | 3.05 | 3.06 | 4.87 | 1080 | 1266 | 860 |
| 5 | 34 | 36 | 42 | 3.03 | 3.05 | 4.63 | 1088 | 1144 | 866 |
| 6 | 38 | 36 | 44 | 3.02 | 3.17 | 4.21 | 1222 | 1100 | 1000 |
| 7 | 40 | 34 | 42 | 3.03 | 3.03 | 4.08 | 1278 | 1088 | 988 |
| 8 | 34 | 40 | 44 | 3.05 | 3.23 | 4.66 | 1080 | 1200 | 900 |
| 9 | 34 | 40 | 44 | 3.09 | 3.03 | 4.72 | 1066 | 1280 | 888 |
| 10 | 34 | 40 | 44 | 3.09 | 3.03 | 4.72 | 1066 | 1278 | 888 |
| 11 | 34 | 34 | 46 | 3.09 | 3.03 | 4.40 | 1066 | 1088 | 1000 |
| 12 | 34 | 36 | 44 | 3.03 | 3.11 | 4.69 | 1088 | 1122 | 894 |
| 13 | 34 | 38 | 48 | 3.04 | 3.07 | 4.58 | 1086 | 1200 | 1000 |
| 14 | 34 | 40 | 46 | 3.03 | 3.06 | 4.40 | 1088 | 1266 | 1000 |
| 15 | 34 | 40 | 48 | 3.02 | 3.09 | 4.90 | 1090 | 1254 | 932 |
| 16 | 34 | 42 | 48 | 3.02 | 3.03 | 4.90 | 1092 | 1346 | 932 |
| 17 | 42 | 34 | 48 | 3.03 | 3.03 | 4.63 | 1342 | 1088 | 988 |
| 18 | 42 | 34 | 48 | 3.06 | 3.09 | 5.13 | 1330 | 1066 | 888 |
| 19 | 46 | 34 | 42 | 3.09 | 3.16 | 4.08 | 1444 | 1042 | 988 |
| 20 | 44 | 34 | 48 | 3.05 | 3.03 | 4.96 | 1400 | 1088 | 920 |
| 21 | 46 | 36 | 52 | 3.07 | 3.02 | 5.24 | 1454 | 1156 | 940 |
| 22 | 46 | 34 | 44 | 3.06 | 3.09 | 4.21 | 1456 | 1066 | 1000 |
| 23 | 34 | 42 | 44 | 3.06 | 3.02 | 4.44 | 1078 | 1350 | 948 |
| 24 | 34 | 34 | 46 | 3.05 | 3.03 | 4.40 | 1082 | 1088 | 1000 |
| 25 | 36 | 36 | 44 | 3.06 | 3.11 | 4.22 | 1142 | 1122 | 998 |
| 26 | 36 | 34 | 46 | 3.05 | 3.03 | 4.41 | 1144 | 1088 | 998 |
| 27 | 44 | 34 | 44 | 3.05 | 3.03 | 4.26 | 1400 | 1088 | 988 |
| 28 | 42 | 34 | 48 | 3.04 | 3.09 | 4.58 | 1340 | 1066 | 1000 |
| 29 | 36 | 36 | 50 | 3.04 | 3.05 | 4.35 | 1150 | 1144 | 1100 |
| 30 | 38 | 34 | 48 | 3.02 | 3.05 | 4.58 | 1222 | 1080 | 1000 |

Source 1 = Nakhon Pathom, Source 2 = Ang Thong, Source 3 Samut Sakhon

**Table 59** Vein islet number, palisade ratio, and trichome number of *D. trifoliata*

| Field | Vein islet number (/mm$^2$) | | | Palisade ratio | | | Trichome number (/mm$^2$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 18.50 | 6.50 | 16.00 | 13.75 | 10.00 | 6.75 | - | - | - |
| 2 | 21.00 | 8.50 | 16.25 | 14.50 | 9.00 | 9.00 | - | - | - |
| 3 | 19.25 | 10.00 | 14.50 | 15.00 | 8.00 | 8.50 | - | - | - |
| 4 | 17.88 | 8.00 | 15.63 | 10.50 | 12.00 | 8.50 | - | - | - |
| 5 | 18.00 | 10.00 | 14.75 | 13.00 | 12.50 | 8.50 | - | - | - |
| 6 | 19.00 | 9.50 | 17.75 | 9.50 | 11.00 | 9.50 | - | - | - |
| 7 | 18.50 | 8.50 | 15.25 | 12.50 | 9.00 | 6.75 | - | - | - |
| 8 | 17.25 | 10.50 | 14.88 | 10.50 | 8.75 | 6.50 | - | - | - |
| 9 | 19.00 | 7.00 | 15.63 | 10.00 | 10.00 | 9.50 | - | - | - |
| 10 | 17.63 | 9.00 | 15.25 | 15.50 | 11.25 | 8.25 | - | - | - |
| 11 | 16.88 | 5.00 | 14.75 | 13.50 | 9.50 | 9.50 | - | - | - |
| 12 | 17.50 | 8.50 | 16.00 | 12.50 | 7.50 | 8.50 | - | - | - |
| 13 | 16.75 | 8.00 | 16.38 | 13.00 | 9.75 | 8.50 | - | - | - |
| 14 | 16.25 | 8.00 | 17.75 | 13.50 | 10.50 | 11.50 | - | - | - |
| 15 | 16.50 | 11.00 | 14.88 | 12.00 | 13.00 | 9.00 | - | - | - |
| 16 | 18.25 | 10.00 | 16.25 | 10.50 | 12.50 | 9.50 | - | - | - |
| 17 | 19.75 | 8.00 | 15.50 | 9.50 | 12.00 | 8.00 | - | - | - |
| 18 | 19.63 | 8.00 | 15.50 | 14.00 | 13.50 | 9.00 | - | - | - |
| 19 | 21.25 | 9.50 | 15.00 | 16.00 | 8.25 | 8.00 | - | - | - |
| 20 | 18.13 | 10.00 | 17.75 | 13.50 | 11.50 | 10.00 | - | - | - |
| 21 | 17.75 | 9.00 | 15.50 | 11.00 | 14.00 | 9.50 | - | - | - |
| 22 | 19.25 | 9.50 | 15.50 | 12.25 | 11.50 | 7.00 | - | - | - |
| 23 | 20.25 | 8.50 | 18.50 | 14.50 | 12.50 | 10.50 | - | - | - |
| 24 | 19.75 | 8.50 | 16.00 | 15.50 | 12.00 | 7.50 | - | - | - |
| 25 | 20.25 | 9.00 | 17.25 | 15.00 | 13.00 | 8.00 | - | - | - |
| 26 | 17.75 | 9.00 | 16.50 | 9.00 | 11.75 | 8.75 | - | - | - |
| 27 | 19.00 | 10.00 | 14.88 | 11.00 | 13.75 | 10.00 | - | - | - |
| 28 | 21.50 | 9.00 | 15.63 | 12.00 | 9.75 | 8.00 | - | - | - |
| 29 | 19.50 | 10.00 | 15.75 | 14.75 | 12.00 | 11.00 | - | - | - |
| 30 | 20.75 | 9.00 | 15.50 | 13.00 | 11.75 | 9.00 | - | - | - |

Source 1 = Nakhon Pathom, Source 2 = Ang Thong, Source 3 Samut Sakhon

**APPENDIX F**

ISSR fingerprint of selected eight *Derris* species

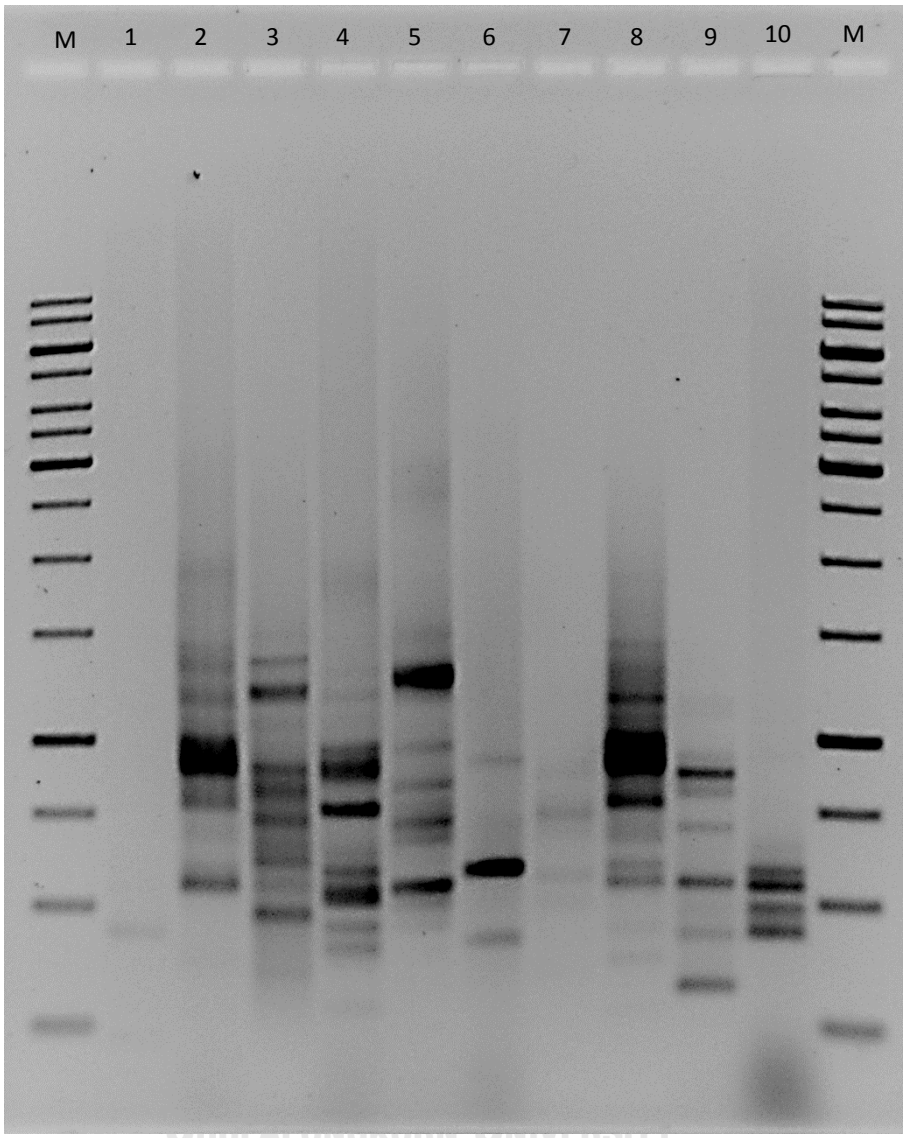


**Figure 51** Fingerprint of ISSR-11

[M = 1kb molecular weight marker, lane1 = *D. elliptica*, lane2 = *D. malaccensis*, lane 3 = *D. scandens*, lane 4 = *D. trifoliata*, lane 5 = *D. indica*, lane 6 = *D. reticulata*, lane 7 = *D. amoena*, lane 8 = *D .solorioides*, lane 9 = *Ceasalpinia sappan*, lane 10 = *Clerodendrum paniculatum*]

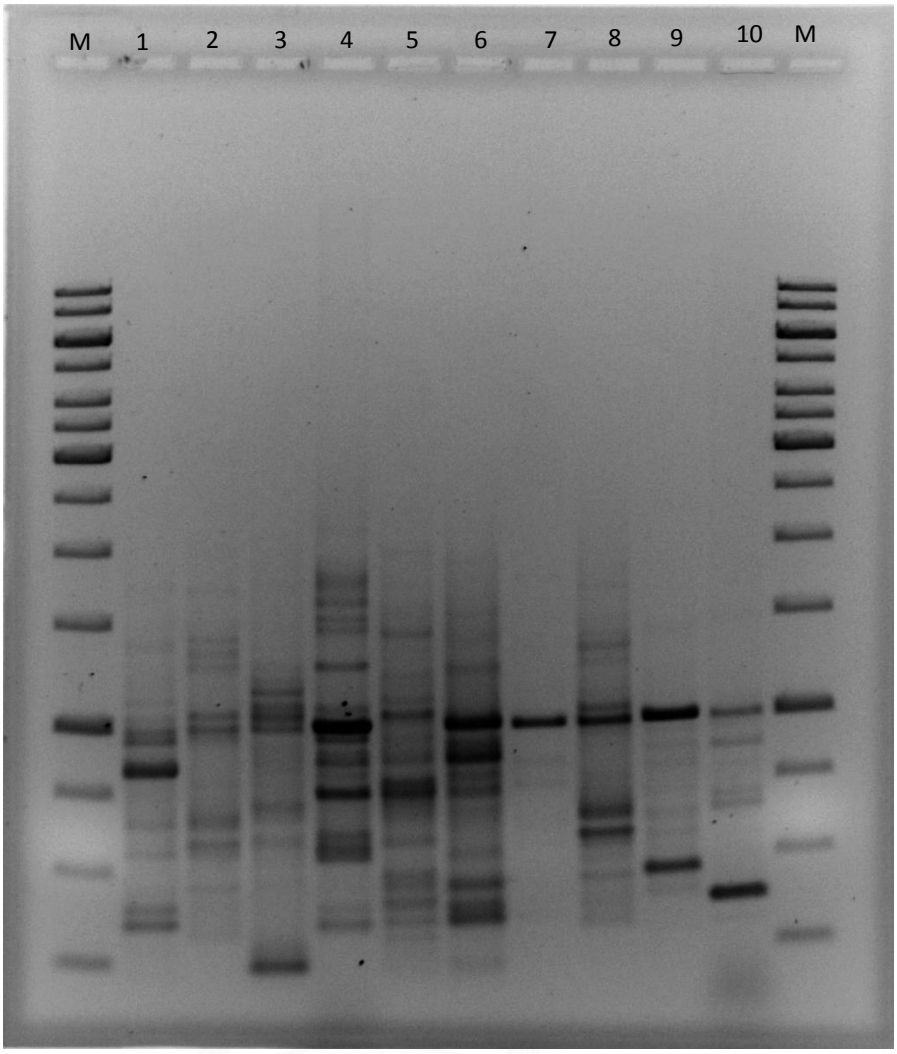


**Figure 52** Fingerprint of ISSR-12

[M = 1kb molecular weight marker, lane1 = *D. elliptica*, lane2 = *D. malaccensis*, lane 3 = *D. scandens*, lane 4 = *D. trifoliata*, lane 5 = *D. indica*, lane 6 = *D. reticulata*, lane 7 = *D. amoena*, lane 8 = *D .solorioides*, lane 9 = *Ceasalpinia sappan*, lane 10 = *Clerodendrum paniculatum*]

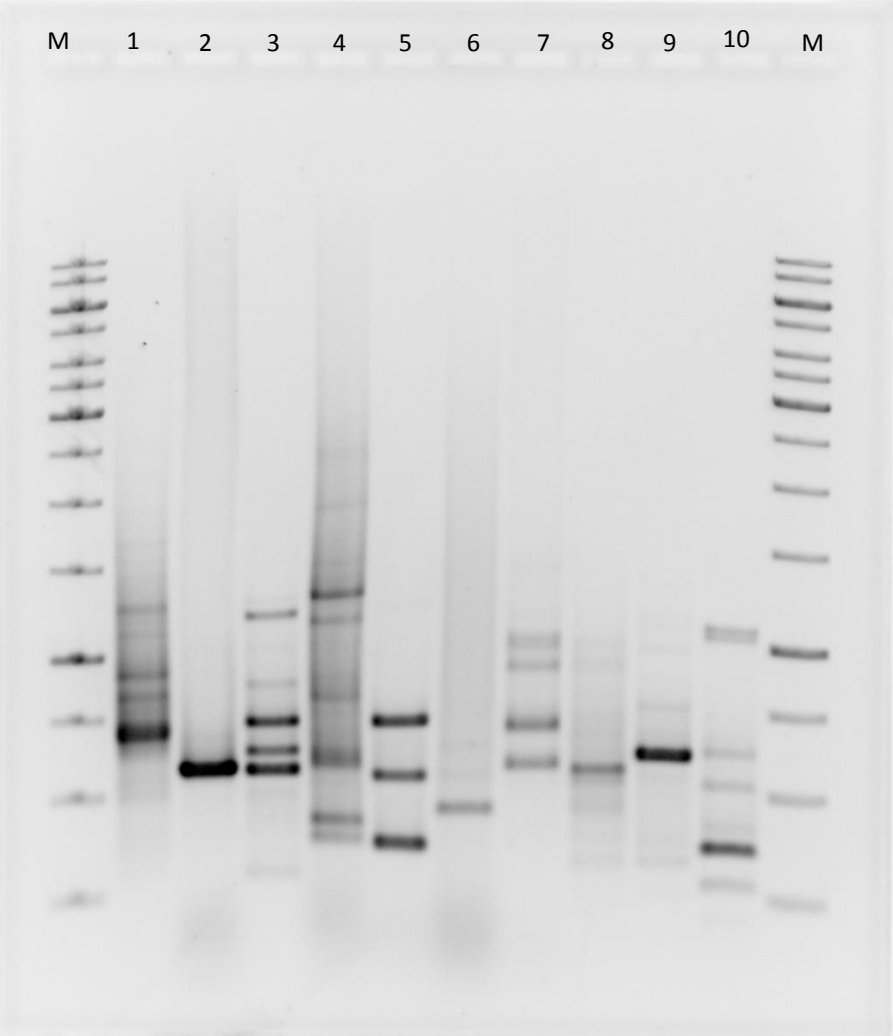




**Figure 53** Fingerprint of ISSR-14

[M = 1kb molecular weight marker, lane1 = *D. elliptica*, lane2 = *D. malaccensis*, lane 3 = *D. scandens*, lane 4 = *D. trifoliata*, lane 5 = *D. indica*, lane 6 = *D. reticulata*, lane 7 = *D. amoena*, lane 8 = *D .solorioides*, lane 9 = *Ceasalpinia sappan*, lane 10 = *Clerodendrum paniculatum*]

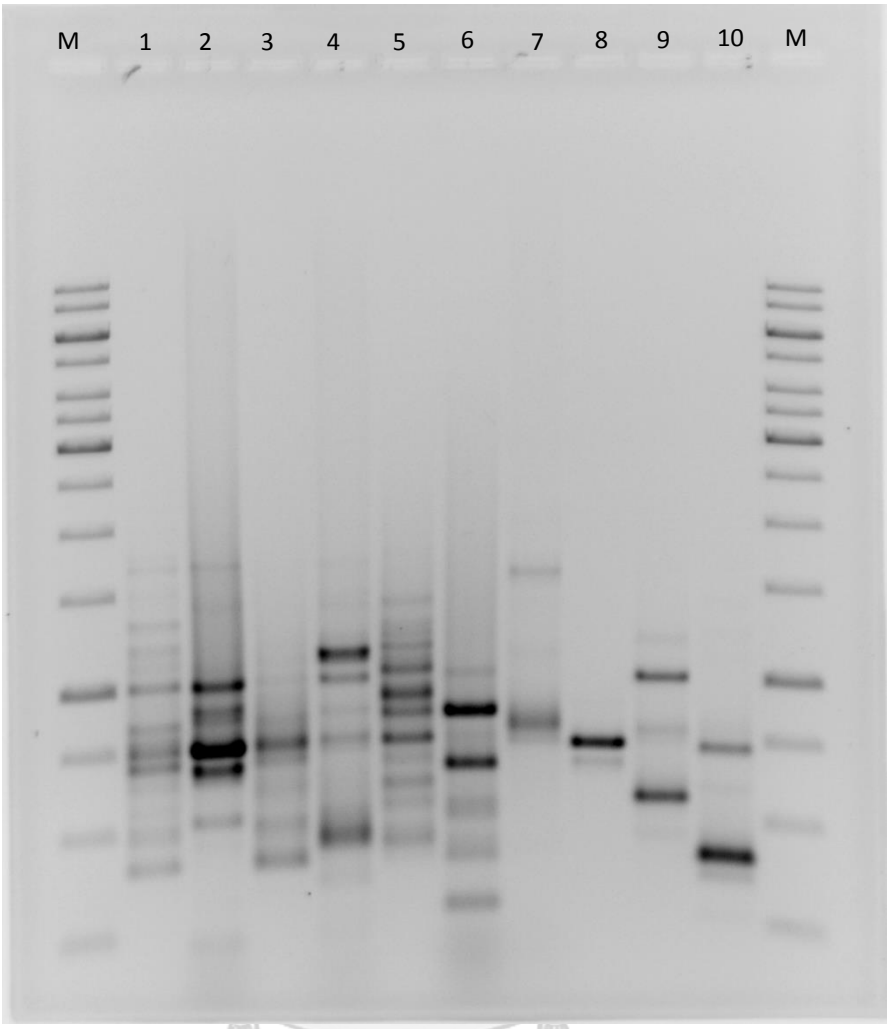




**Figure 54** Fingerprint of ISSR-17

[M = 1kb molecular weight marker, lane1 = *D. elliptica*, lane2 = *D. malaccensis*, lane 3 = *D. scandens*, lane 4 = *D. trifoliata*, lane 5 = *D. indica*, lane 6 = *D. reticulata*, lane 7 = *D. amoena*, lane 8 = *D .solorioides*, lane 9 = *Ceasalpinia sappan*, lane 10 = *Clerodendrum paniculatum*]

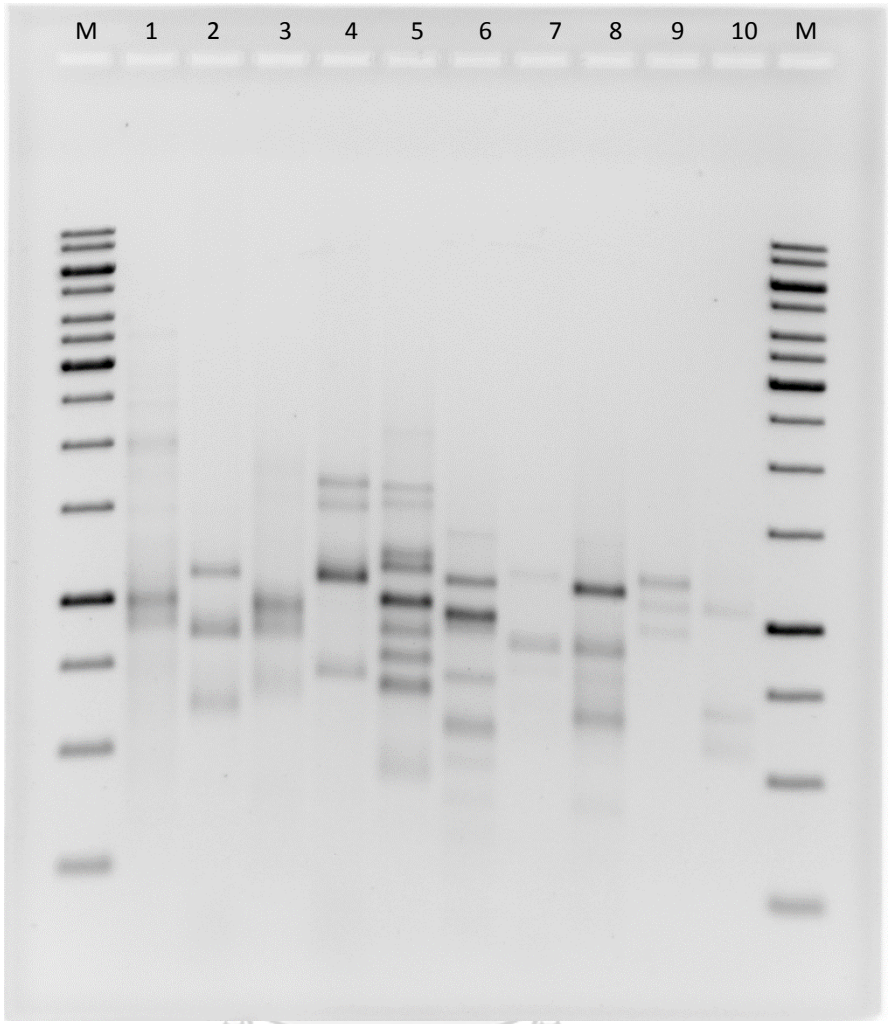


**Figure 55** Fingerprint of ISSR-35

[M = 1kb molecular weight marker, lane1 = *D. elliptica*, lane2 = *D. malaccensis*, lane 3 = *D. scandens*, lane 4 = *D. trifoliata*, lane 5 = *D. indica*, lane 6 = *D. reticulata*, lane 7 = *D. amoena*, lane 8 = *D .solorioides*, lane 9 = *Ceasalpinia sappan*, lane 10 = *Clerodendrum paniculatum*]

# VITA

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