PHARMACOGNOSTIC SPECIFICATION AND RUTIN CONTENT OF *SOPHORA JAPONICA* FLOWERING BUD

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ข้อกำหนดทางเภสัชเวทและปริมาณวิเคราะห์รูตินในดอกฮ่วยฮวย

นางสาวระพีพร ชนะภักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Field of Study	Public Health Sciences
Thesis Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.
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ระพีพร ชนะภักดิ์ : ข้อกำหนดทางเภสัชเวทและปริมาณวิเคราะห์รูตินในดอกฮ่วยฮวย (PHARMACOGNOSTIC SPECIFICATION AND RUTIN CONTENT OF *SOPHORA JAPONICA* FLOWERING BUD) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. นิจศิริ เรืองรังษี, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ชนิดา พลานุเวช, 64 หน้า.

ฮ่วยฮวยเป็นพืชที่อยู่ในตระกูลถั่ว ในทางการแพทย์แผนจีน ดอกตูมของฮ่วยฮวยนำมาใช้ในการห้ามเลือดและริดสีดวงทวาร อย่างไรก็ตาม ข้อกำหนดทางเภสัชเวทและการวิเคราะห์ปริมาณรูตินในดอกฮ่วยฮวยไม่เคยจัดทำในประเทศไทยมาก่อน งานวิจัยนี้จึงได้ศึกษาข้อกำหนดทางเภสัชเวทและวิเคราะห์ปริมาณสารรูตินในดอกฮ่วยฮ่วย โดยคัดเลือกจาก 12 แหล่งทั่วประเทศ การตรวจสอบลักษณะทางมหทรรศน์และจุลทรรศน์ของดอกฮ่วยฮวย ในการศึกษาเอกลักษณ์ทางเคมี – ฟิสิกส์ของดอกฮ่วยฮวย พบว่าปริมาณน้ำ น้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม และปริมาณเถ้าที่ไม่ละลายในกรด ไม่ควรเกินร้อยละ 7.2, 7.0, 7.4 และ 1.2 โดยน้ำหนักตามลำดับ ปริมาณสกัดด้วยเอทานอลและปริมาณสกัดด้วยน้ำ ไม่ควรน้อยกว่าร้อยละ 10.6 และ 25.7 โดยน้ำหนักตามลำดับ การวิเคราะห์ปริมาณสารรูตินด้วยเทคนิคทินเลเยอร์โครมาโทกราฟี-เด็นซิโทมีทรีโดยใช้แผ่นทินเลเยอร์โครมาโทกราฟีซิลิกาเจลเป็นวัฏภาคคงที่ เอทิลอะซิเตท เอทานอล น้ำ กรดอะซีติกและกรดฟอร์มิก (5:1:3:1:1) เป็นวัฏภาคเคลื่อนที่ ตรวจวัดภายใต้แสงอัลตราไวโอเลต 363 นาโนเมตร และวิธีวิเคราะห์ทินเลเยอร์โครมาโทกราฟีโดยภาพถ่ายวิเคราะห์โดยใช้โปรแกรมอิมเมจเจ การทดสอบความเที่ยงตรงของวิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทมีทรีและวิธีวิเคราะห์ทินเลเยอร์โครมาโทกราฟีจากภาพพบว่ามีช่วงวิเคราะห์แบบโพลีโนเมียล 0.3–0.9 ไมโครกรัมต่อจุด ค่าสัมประสิทธิ์การตัดสินใจเท่ากับ 0.9992 ค่าเฉลี่ยการคืนกลับร้อยละ 94.1–108.9 และ 95.2-109.6 ตามลำดับ ค่าความสามารถในการวัดซ้ำ มีค่าระหว่างร้อยละ 2.0-4.2 และ 1.1-6.2 ค่าความแม่นยำ มีค่าระหว่างร้อยละ 3.6–15.6 และ 4.3–8.6 ตามลำดับ ขีดจำกัดของการตรวจพบและขีดจำกัดของการหาปริมาณ มีค่า 0.02, 0.024 และ 0.06, 0.07 ไมโครกรัมต่อจุด ตามลำดับ ค่าความคงทนมีค่าสัมประสิทธิ์ของการกระจายร้อยละ 8.2 และ 1.3 ตามลำดับ การวิเคราะห์ปริมาณรูตินในดอกฮ่วยฮวยโดยใช้วิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทมีทรีและวิธีวิเคราะห์ทินเลเยอร์โครมาโทกราฟีจากภาพพบว่า มีค่าเฉลี่ยร้อยละ 16.09 ± 4.00 และ 14.73 ± 3.58 ตามลำดับ จากการศึกษาครั้งนี้สามารถจัดทำเป็นข้อกำหนดทางมาตรฐานของดอกฮ่วยฮวยในประเทศไทย เพื่อใช้เป็นประโยชน์ในด้านการใช้สมุนไพรต่อไป

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: RUTIN / SOPHORA JAPONICA / TLC-DENSITOMETRY / TLC-IMAGE ANALYSIS / PHARMACOGNOSTIC SPECIFICATION

RAPEEPORN CHANAPUK: PHARMACOGNOSTIC SPECIFICATION AND RUTIN CONTENT OF *SOPHORA JAPONICA* FLOWERING BUD. ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., CO-ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., 64 pp.

Sophora japonica L. (Fabaceae) has been used in traditional Chinese medicine for treatment of hemostatic and hemorrhoids. This study aimed to extablish the pharmacognostic specification and rutin content of *S. japonica* flowering bud in Thailand. Macroscopic and microscopic evaluation of *S. japonica* flowering bud were demonstrated. Physico-chemical parameters including water content, loss on drying, total ash and acid-insoluble ash should be less than 7.2, 7.0, 7.4 and 1.2 % of dry weight respectively. The ethanol-extractive and water-extractive should be more than 10.6, 25.7 % by dry weight respectively. Rutin in *S. japonica* flowering bud was extracted in 80% ethanol by Soxhlet apparatus. For quantitative analysis of rutin, TLC densitometry using TLC silica gel plates as stationary phase, ethyl acetate:ethanol:water:acetic acid:formic acid (5:1:3:1:1) as mobile phase. TLC image photographed was analyzed by TLC ImageJ software. The method validity of TLC–densitometry and TLC image analysis were shown that the calibration range were polynomial with 0.3–0.9 µg/spot (R^2 = 0.9992). The accuracy was 94.1–108.9% and 95.2–109.6 %recovery. The repeatability was 2.0–4.2 and 1.1–6.2%RSD. The intermediate precision was 3.6–15.6 and 4.3-8.6%RSD. LOD and LOQ were 0.02, 0.024 and 0.06, 0.07 µg/spot. The robustness was 8.2 and 1.3%RSD, respectively. The rutin content of *S. japonica* flowering bud was determined using TLC-densitometry and TLC image analysis were 16.09±4.00 and 14.73±3.58 % by dry weight, respectively. This study could be used for standardization of *S. japonica* flowering bud in Thailand.

Field of Study: Public Health Sciences Student's Signature .....
Academic Year: 2015 Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABRREVIATIONS

%	Percent
℃	Degree Celsius
µg	Microgram
µm	Micrometer
cm	Centimeter
g	Gram
kg	Kilogram
kHz	Kilohertz
L	Liter
LOD	Limit of detection
LOQ	Limit of quantification
mg	Milligram
ml	Milliliter
mm	Millimeter
nm	Nanometer
RSD	Relative standard deviation
SD	Standard deviation
TLC	Thin layer chromatography
UV	Ultraviolet

CHAPTER I
INTRODUCTION

Background and Rationale

Plants have been used for treatment of diseases for thousands of years before recorded history. The Egyptian and Chinese have written to explain about medicinal plants in papyrus and bamboo strips. Indigenous cultures, such as Africa and American used plants to healing on a ceremony. Most of the world's population still uses herbal medicines for primary health care and provide basic health service. In addition, people live far off areas where it is available health service, and people also live in poor areas where it suggests the inexpensive remedy. However, in modern medicine areas is available, an interest on herbal medicines continues to get more popular every year.

Herbal medicines are not always safe just because it come from natural. Some people have adverse reactions which caused by the use of herbal medicines; moreover, they may have side effects when obtain chemicals for a long time such as carcinogenic and hepatotoxic effects. It would be benefit for human health when suitably used herb as medicine. Therefore, quality control and standardization of herbal medicines are very important for safe using of herbal medicines [1].

Fabaceae (Leguminosae or pea family) is the third largest family of angiosperms. The Fabceae distributes throughout temperate and tropical areas of the world [2]. Since, they have been used in several ways both human and animal such as food, medicine, chemical and fertilizer in agricultural industry.

Sophora japonica L. belongs to family Fabaceae. It is known in common name as Japanese pagoda tree or Chinese scholar tree. This plant is a native plant in China and widely found either Japan or Korea. It is a deciduous trees, small to medium sized tree, flowering period is from July to August, and fruiting period is August to October [3]. In traditional Chinese medicine, the flowering buds of *S. japonica* have been used for treatment of hemostatic, hemorrhoids and

hematemesis [4, 5]. The fruit is used for stopping bleeding and lowering blood pressure, root bark and leaf are used for pain killer. The previous studies of pharmacological properties of this plant investigated antioxidant, antiplatelet, anti-inflammatory and *in vivo* antiobesity [5-8], and they found that *S. japonica* tended to be medicinal plant. The main chemical compound of *S. japonica* is rutin. Rutin is a flavone glycoside which is known as vitamin P. It was reported in antioxidant and anti-inflammation agent [9]. In Thailand, *S. japonica* is popular used for treatment of hemorrhoids. Eventually, the standardization of crude drug quality and determination of rutin content of *S. japonica* flowering buds have never been established in Thailand.

This study aimed to investigate the quality of *S. japonica* flowering buds in Thailand and established the pharmacognostic specification of this crude drug with reference to the rutin content determined by quantitative TLC.

Research problems

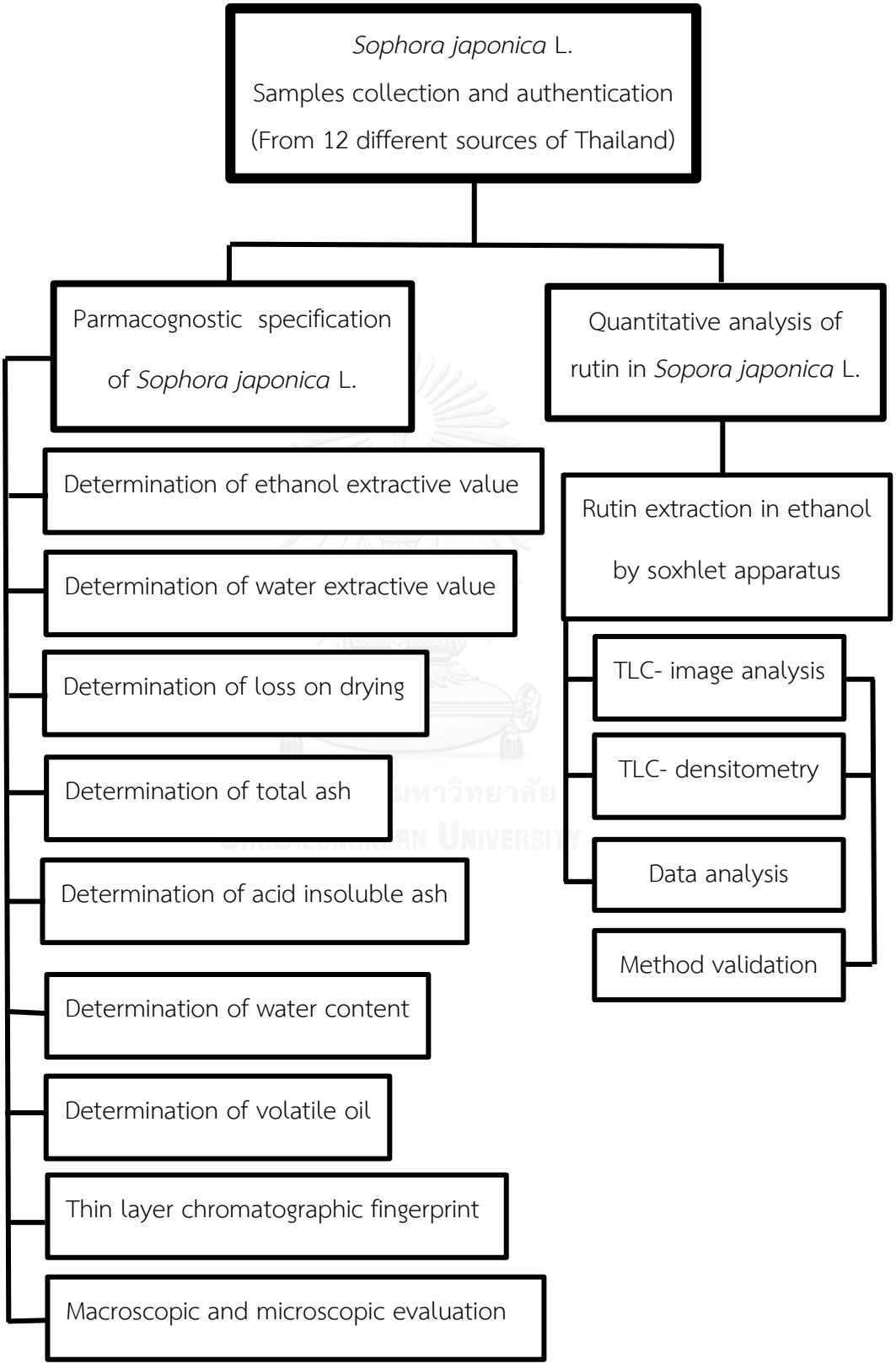
The quality parameters as well as rutin content of *S. japonica* crude drug in Thailand have never been established.

Objectives of the study

1. To develop the pharmacognostic specification of *S. japonica* dried flowering buds in Thailand.

2. To investigate the rutin content of *S. japonica* flowering buds by TLC densitometry compared to TLC image analysis using ImageJ software.

Conceptual framework



CHAPTER II

INTRODUCTION

Taxonomy

Kingdom Plantae

Division Tracheophyta

Class Magnoliopsida

Order Fabales

Family Faboideae

Genus *Sophora*

Species *S. japonica*

Botanical characteristics

Sophora japonica L. is a deciduous trees, small to medium sized tree up to height 15-30 m. Bark gray-brown, longitudinally striate; branches of current year green, glabrous. Leaves 15-25 cm long; stipules ovate to linear, caducous; petiole inflated at base, bud hidden; leaflets 9-15; stipels subulate; blades ovate-lanceolate or ovate-oblong, 2.5-6 × 1.5-3 cm, papery, glaucous and sparsely to densely pubescent abaxially, usually becoming gla-brate, base broadly cuneate or rounded, apex acuminate, mucro-nate. Panicles terminal, to 30 cm long; bracteole subulate. Calyx shortly campanulate, 3.5-4.5 mm long; teeth 5, obtuse, pubescent. Corolla white or creamy yellow, rarely purple-red; standard broadly ovate, claw short, base cordate, apex retuse; wings ovate-oblong, ca. 10 × 4 mm long; keel similar to wings, but broader. Stamens 10, unequal, free, persistent. Ovary glabrescent. Leg-umes

green, moniliform, 2.5-5 × ca. 1 cm long, obviously con-stricted between seeds, indehiscent, fleshy. Seeds 1-6, yellow-green, black-brown when dry, ovoid [3].

S. japonica is well adapted to dry weather condition. It is native in temperate and troperate regions, cultivation in the tropics is only possible in drier or high altitude. It is native in China and Korea.

Common Names : Pagoda Tree, Japanese Pagoda Tree, Chinese Scholar Tree, Umbrella Tree

Synonyms *Styphnolobium japonicum* L. Schott

Sophora korolkowii Dieck

Sophora sinensis Forrest

Medicinal uses

In China, the flowers and buds of *S. japonica* are used for treatment of hemafecia, hemorrhoids blood, bloody flux, uterine bleeding, hematemesis, liver heat, red eyes, headache and dizziness [10]. In Korea, the buds and fruits of *S. japonica* are used to cure haemostatic agents [5].

Chemical constituents

In 2001, the pericarps of *S. japonica* were isolated with 95 % ethanol using an ultrasonic apparatus. The extract was found four new isoflavone triglycosides, genistein 7-*O*-*β*-D-glucopyranoside-4′-*O*-[(***α***-L-rhamnopyranosyl)-(1→2)-*β*-D-glucopyranoside], genistein 7-*O*-ß-D-glucopyranoside-4′-*O*-[(ß-D-glucopyranosyl)-(1→2)-ß-D-glucopyranoside], genistein 7-*O*-***α***-L-rhamnopyranoside-4′-*O*-[(***α***-L-rhamnopyranosyl)-(1→2)-*β*-D-glucopyranoside], and genistein 7-*O*-***α***-L-rhamnopyranoside-4′-*O*-[(*β*-D-glucopyranosyl)-(1→2)-*β*-D-glucopyranoside] and nine known compounds, genistein 7-*O*-*β*-D-glucopyranoside-4′-*O*-*β*-D-glucopyranoside,

sophorabioside, prunetin 4′-*O*-*ß*-D-glucopyranoside, sophororicoside, genistin, rutin, kaempferol 3-*O*-*ß*-rutinoside, quercetin 3-*O*-*ß*-D-glucopyranoside, and kaempferol 3-*O*-*ß*-D-glucopyranoside [11].

In the other study, the pericarps of *S. japonica* isolated by various chromatographic techniques were reported two kaempferol triglycosides, kaempferol 3-*O*-ß-D-sophoroside-7-*O*-**α**-L-rhamnoside and kaempferol 3-*O*-(2″-*O*-*ß*-D-glucosyl)-*ß*-D-rutinoside [12].

In 2002, Tang reported a new coumaronochromone, sophorophenolone and thirteen known compounds isolated from *S. japonica* pericarps [13].

In 2003, the powder of *S. japonica* seeds were extracted with 60 and 90 % ethanol. The study reported a flavonol tetraglyciside (kaempferol 3- *O*-**α**-L – rhamnopyranosyl(1→6)-*ß*-D-glucopyranosyl(1→2)-*ß*-D-glucopyranoside-7- *O*- **α** -L - Rhamnopyranoside) and nine known compounds [14]. .

In 2007, the extracts from pericarps of *S. japonica* determined by HPLC were demonstrated that six flavone compounds, genistein-7,4′-di-*O*-*ß*-D-glucoside, genistein-7-*O*-*ß*-D-glucopyranoside-4′-*O*-[(-L-rhamnopyransoyl)-(1-2)-*ß*-D-glucopyranoside], kaempferol-3-*O*-ß-D-sophoroside, quercetin-3-*O*-*ß*-L-ramnopyranosyl-(1 - 6)-*ß*-D-glucopyranoside, genistein-4′-*ß*-L-rhamnopyransoyl-(1 - 2)-**α**-D-glucopyranoside, and kaempferol-3-*O*-***ß***-L-ramnopyranosyl-(1 - 6)-*ß*-D-glucopyranoside [15].

In 2008, the isolate from small branches of *S. japonica* was reported two new isoflavone triglycisides, genistein 4'-*O*-(6''-*O*-**α**-L-rhamnopyranosyl)-*ß*-sophoroside, and genistein 4'-O-(6'''-*O*-**α**-L-rhamnopyranosyl)-*ß*-sophoroside , with five known compounds, sophorabioside, genistin, rutin, quercetin 3-*O*-*ß*-D-glucopyranoside, and kaempferol 3-*O*-*ß*-D-glucopyranoside [16].

In the other study, Tang reported that the extract from leaves of *S. japonica* was found two new isoflavone tetraglycosides included genistein 7-*O*-*β*-D-glucopyranoside-4′-*O*-(6‴-*O*-***α***-L-rhamnopyranosyl)-ß-sophoroside and genistein 7-*O*-***α***-L-rhamnopyranoside-4′-*O*-(6‴-*O*-***α***-L-rhamnopyranosyl)-ß-sophoroside and six known compounds, genistein 7-*O*-*β*-D-glucopyranoside-4′-*O*-*β*-D-glucopyranoside, sophorabioside, genistin, rutin, quercetin 3-*O*-*β*-D-glucopyranoside, and kaemgferol 3-*O*-*β*-D-glucopyranoside [17].

In 2010, the methanolic extract of *S. japonica* stem bark was found a new isoflavone glycoside, 6-methoxy-7-hydroxy-4′-*O*-ß-D-glucosyl isoflavone, glycitein-4′-*O*-ß-D-glucoside, and nine known flavonoids [18].

In 2013, the leaves of *S. japonica* were extracted with 90 % ethanol separated by colomn chromatography and were determined by spectroscopic methods. The extracts were found two new flavanones included japonicasins A, B [4].

In 2014, Abdallah reported seven compounds, genistin ,sophoricoside , sophorabioside , sophoraflavonoloside , genistein 7,4′-di-*O*-*β* -D-glucopyransoide , kaempferol 3- *O*-***α***–L-rhamnopyranosyl(1→6)*β* -D-glucopyranosyl(1→2)*β*-D-glucopyranoside and rutin in the methanolic extract from *S. japonica* seeds [19].

Pharmacological activities of *Sophora japonica* L.

Tyrosinase inhibitory activity

Lai *et al*. (2014) studies tyrosinase inhibitory activity in flavonoid-rich extract from *S. japonica* flowers using mushroom tyrosinases activity. The results showed that the extract has able tyrosinase inhibitory activity than ascorbic acid (0.1 % extract vs 1 % ascorbic acid). The IC_{50} was calculated to be 3.12 mg/ml [20].

Antiobesity

In 2009, Park reported the effect of antiobesity in high-fat diet-induced obese mice. The mice were randomized into 3 groups, fed a high-fat diet, high-fat diet with 1 % or 5 % *S. japonica* powder for 4 weeks. The results showed that the high-fat diet with crude drug powder significantly decreased body weight and reduced fat mass in induced obese mice [8].

Antioxidant

The ethanolic extract of *S. japonica* flowering buds was studies for the antioxidant property by DPPH, ABTS+ and method of oxygen consumption in food model (lard and sunflower). The results were found a high radical scavenging activity and slowing down lipid oxidation ability in food model [7].

Anti-inflammatory activity

The anti-inflammatory activity of extract from *S. japonica* flowers was examined by nitric oxide production and cell viability on RAW 264.7 macrophages. The result has showed significant inhibition of nitric oxide production and TNF- production with low IC_{50} values [6].

Antihyperglycemic activity

The ethanolic extract from *S. japonica* flowers was tested on lowering blood glucose levels and thiobarbituric acid reactive substances (TBARS) in streptozocin (STZ) – induced diabetic rats. The extract was showed reduced blood TBARS levels *versus* diabetic controls [21].

Anti-platelet activity

The methanolic extract from stems, fruits and leaves of *S. japonica* were used for study of anti-platelet activity. The rat platelet aggregation induced by arachidonic acid, ADP, collagen and a thromboxane A_2 mimetic agent compared with acetyl saticylic acid (ASA). The result has showed that the extract could inhibit better than ASA on induced aggregation [5].

Estrogenic activity

The methanolic extract of *S. japonica* treated with naringinase enzyme was found 12 compounds in this plant and the effect of estrogenic activity by yeast two-hybrid assay (ER*β*, ER***α***). Sophoricoside, kaempferol and genistein, the compounds of *S. japonica*, showed estrogenic activity at different concentration in both yeast. Sophoricoside, rutin, kaempferol and genistein demonstrated weak anti-estrogenic activity in yeast ER***α*** at concentration of 10^{-4} M and none of these compounds showed anti-estrogenic activity in yeast ERß [22].

In the other study on the estrogenic activities of methanolic extract from *S. japonica* seeds in the same method, the results have showed a significant estrogenic activity of the extract only after naringinase treatment [23].

Antinociceptive activity

Zhang *et al.* (2013) studied the antinociceptive activity of *S. japonica* flowering buds extract compared to quercetin tested in rat induced with bee venem lyophilized melittin. The result showed that methanolic extract (5-500 mg/kg) and quercetin (5-50 mg/kg) were established the inhibition of both neurogenic pain or acute pain and inflammatory pain or tonic pain of bee venom-induced pain [24].

Anti-adipogenic action

Jang *et al.* (2011) determined the anti-adipogenic action of *S. japonica* in C3H10T1/2 mesenchymal stem cells and 3T3-L1 preadipocyte cells. The ethyl acetate extracts from mature fruits of *S. japonica* inhibited adipocyte differentiation in the preadipocyte cells [25].

Aldose reductase inhibitory activity

Park *et al.* (2010) reported the inhibitory effects of methanolic extract from *S. japonica* on aldose reductase activity were determined in rat lens. The compounds in this extract were established inhibitory effects [18].

Antiosteoporotic activity

The isolated compounds from methanolic extract of *S. japonica* seeds for osteoprotective effect were tested in ovarectomized rats. After the treatment with compound (15 mg/kg and 30 mg/kg), the results showed the effect significant improved bone hardness (89.3 % and 97%, respectively) [19].

Rutin

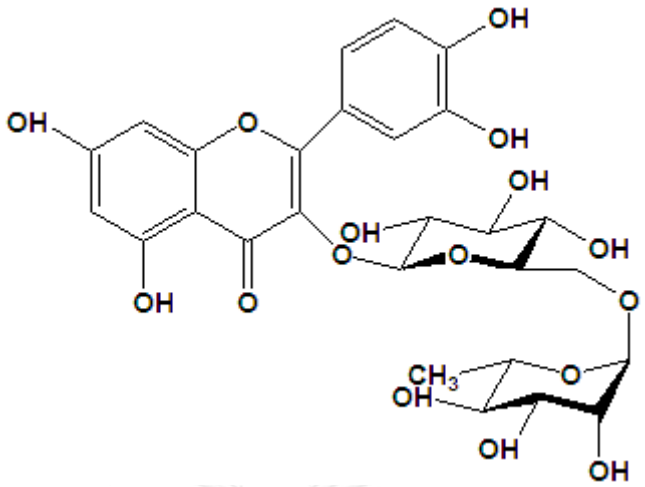


Figure 1 Structure of rutin

IUPAC : 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-({[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy}methyl)oxan-2-yl]oxy}-4H-chromen-4-one

Molecular formula : $C_{27}H_{30}O_{16}$

Molecular weight : 610.52 g·mol^{-1}

Description :

Rutin is a kind of flavone glycoside. It is also known as vitamin P. The chemical structure of rutin consists of quercetin and rutinose which has several acidic hydroxyl groups on aromatic rings. Rutin is generally found in plants including buckwheat (*Fagopyrum esculentum* Moench), *Ruta graveolens* L., *Maranta leuconeura* E. Morren, *Orchidantha maxillarioides* (Ridl.) Schum, *Strelitzia reginae* Banks ex Aiton, *Canna indica* L., *Canna edulis* Ker Gawl., *Labisia pumila* (Blume) Mez. and *Sophora japonica* L. [9].

Pharmacological activities of rutin

Antioxidation

The antioxidant activity of rutin were analysed using various assays including total antioxidant activity and reducing power, hydroxyl radical scavenging assay, superoxide radical scavenging assay, DPPH radical scavenging assay and lipid peroxidation assay. The rutin showed less potent of various antioxidant activities and demonstrated strong DPPH radical scavenging activity [26].

Anti-inflammation

The anti-inflammatory activities were determined in acute and chronic inflammation rats. The treatment group were given rutin (80 mg/kg) 1 hour after induced inflammation in acute phase and once every day until day 21 in chronic phase. The results showed rutin inhibited in both acute and chronic phases of inflammation model [27].

Hepatoprotective activity

Janbaz *et al.* (2002) studied serum levels of liver transaminase enzymes activities on paracetamol- and CCl_4–induced hepatotoxicity using rodent model. The results showed that rutin could prevent liver from damage on paracetamol- and CCl_4- induced hepatotoxicity. The lethality test also showed that rutin could reduce the death rate to 40 % on paracetamol-induced hepatotoxicity [28].

Parameters standardization

Macroscopic and microscopic examination

Macroscopic identity of plants materials is based on shape, size, color, surface characteristics, texture, fracture characteristics and appearance of the cut surface. In addition, microscopic inspection of plants materials is a necessary method for identification of powdered materials. The specimen may have to be treated with chemical reagents and used in association with other analytical methods.

Determination of water content

Azeotropic method is use for measurement of the water content in crude drug. The crude drug is distilled with a solvent such as toluene or xylene. Before determination content of water, the solvent provided is anhydrous therefore the solvent should be saturated with water for accurate results.

Determination of extractable matter

Determination of extractable matter was used for determination of constituent from plant materials. The plant materials were extracted with water and ethanol. Water was used for the polar substances while ethanol was used for the slightly non-polar substances.

Determination of loss on drying

Loss on drying is a method which used for determination of water and volatile matter in suspect plant material. Drying method can be performed in several ways. The easiest way to determined water and volatile matter in plant materials was heating method. The heating method can be done by heating in oven at the temperature around 100-105 °C, keeping in desiccator with phosphorus pentoxide under atmospheric or reducing of pressure at room temperature for a specified period of time.

Determination of ash

Total ash is an important method for determination of organic and inorganic substances from plant material. The ash remaining following ignition of plant materials is determined by two different methods which measure total ash and acid-insoluble ash.

The total ash method determined the total of remaining after combustion. This includes ash which is derived from the plant tissue and ash which is the residue of the extraneous matter adhering to the plant surface.

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and combustion the remaining insoluble matter. This measures the amount silica present.

Determination of volatile oils

Volatile oils are characterized by smell and ability to volatilize at room temperature. The chemical of volatile oils are usually composed of mixtures of monoterpenes, sesquiterpenes and their oxygenated derivatives. The volatile oils can be described the physical properties of mixtures for extracts. In order to determinate of volatile oils, the crude drug is distilled in water using Clevenger apparatus [29].

Thin layer chromatography

Thin layer chromatography (TLC) is a form of liquid chromatographic method used to separate the extract using a thin stationary phase. It can be used to check purity of compounds, to identify the compounds in the extract and to achieve a quantitative analysis of the compounds [30]. TLC is a popular chromatographic technique because it is simple and inexpensive. It used for qualitative and quantitative results base on visual. TLC system composed of stationary phase and mobile phase. The stationary phase in TLC is an adsorbent and spongy particles coated with silica gel, aluminia or magnesium silicate. Mobile phase is the mixture of

solvents for separation components in extract. The suitable of stationary and mobile phase will be separated the extract effectively. The result of TLC can be detected when separated compound absorb UV light or illuminate fluorescence. Moreover, the developed TLC plates will be sprayed or dipped into the chemical reagent for ensuring a homogenous and reproducible [31].

Retention factor

The retention factor (Rf) is calculated value for the distance of the spots from compound appear from origin in TLC plates and the distance moved of the solvent from origin. The Rf value can be used for identify the compounds under the same conditions. The Rf values can be calculated using the formula below [30].

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

Quantitative analysis of rutin in *S. japonica*

The quantitative analysis can be executed from scanning by densitometry and scanning by Image analysis. Densitometry measurements transform the substance diffusion on a TLC plate into digital computer data. The systems that allow quantitative measurements have been available for fluorescence or ultraviolet absorption measurements, while the reflexion analysis is the most common application [32]. ImageJ is a public domain Java image processing and analysis program. It is equipment for quantitate the compounds and calculate pixel area in digital image by user. It supports standard image processing functions such as contrast manipulation, sharpening, smoothing, edge detection and median filtering [33].

CHAPTER III

MATERIALS AND METHODS

Chemicals and reagents

Acetic acid	BDH Limited Poole England
Acetone	E. Merck Darmstadt, Germany
Chloroform	E. Merck Darmstadt, Germany
Ethanol	RCI Labscan Limited, Bangkok, Thailand
Ethyl acetate	RCI Labscan Limited, Bangkok, Thailand
Formic acid	Ajax Finechem Pty LTD.
Hydrochloric acid	RCI Labscan Limited, Bangkok, Thailand
Rutin	ChromaDex, CA 92618
Sulfuric acid	BDH Limited Poole England
Toluene	RCI Labscan Limited, Bangkok, Thailand
Materials	
Filter paper No.4	Whatman™ Paper, UK
Filter paper No.40 ashless	WhatmanTM Paper, UK
TLC silica gel 60 GF_{254}	Merck, LTD, USA

Instrument and equipment

Aqua-shaker	Adolf Kuhner AG, Switzerland
CAMAG TLC Chamber	CAMAG, Switzerland
CAMAG TLC Scanner 3	CAMAG, Switzerland
Digital camera	Canon Marketing (Thailand) Co., LTD. Bangkok
Hot air oven	WTC Binder, Germany

Image J software	National Instutes of Health, USA
Incinerator	Carbilite, UK
Microscope	Zeiss Axioskop, Germany
Rotary vacuum evaporator	Buchi, Switzerland
Ultraviolet viewing cabinet	Spectronics corp., USA
WinCATS software	CAMAG, Switzerland

Plants materials

The flowering buds of *Sophora japonica* were collected from 12 different sources throughout Thailand and were authenticated by Associate Professor Nijsiri Ruangrungsi. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. After removal of any foreign matters, the flowering buds were crushed into powder.

Pharmacognostic specification of *Sophora japonica*

Macroscopic examination

Macroscopic examination of *S. japonica* flowering bud was carried out for the shape, size, color, surface characteristics, texture and other characters.

Microscopic examination

Microscopic appearances of the *S. japonica* flowering bud were examined in transverse section and in powder forms. The tissue section and powder were mounted with water in the glass slide for observation and were examined under the microscope with the objective lens magnification of 4x, 10x and 40x and the eyepiece lens magnification of 10x.

Determination of water content (Azeotropic method)

Fifty grams of *S. japonica* powder were added with 200 ml of water-saturated toluene and boiled by Azeotropic distillation. The volume of water was measured and calculated in percentage.

Determination of loss on drying

Three grams of *S. japonica* powder were weighed in previously weighed crucible and dried at 105°C for 6 hour. After that, the crucible was left to cool at room temperature. The loss of weight was calculated in a percentage of dried material.

Determination of total ash

Three grams of *S. japonica* powder were added into a pre-weighed crucible and incinerated at 500°C for 5 hours until white which indicating the absence of carbon. The crucible was left to cool in desiccator and weighed without delay. The content of total ash was calculated in a percentage of dried material.

Determination of acid-insoluble ash

Twenty milliliters of hydrochloric acid (70 g/L) were added into the crucible which contained the total ash then covered with the watch-glass and boiled for 5 minutes. The insoluble matters were collected on an ashless filter-paper, transferred to the original crucible, dried on a hotplate and incinerated to ash again. The crucible was left to cool in desiccator and weighed without delay. The content of acid-insoluble ash was calculated in a percentage of dried material.

Determination of alcohol soluble extractive value

Five grams of *S. japonica* powder were macerated with 70 ml of 95 % ethanol in conical flask under shaking for 6 hours and standing for 18 hours. The marc was washed and the filtrate was adjusted to 100 ml with ethanol. After that, twenty milliliters of the filtrate were transferred into a pre-weighed beaker and evaporated to dryness. The extract was dried at 105 °C for 6 hours, cooled in desiccator for 30 minutes and weighed without delay. The content of extractable matter was calculated in a percentage of dried material.

Determination of water soluble extractive value

Five grams of *S. japonica* powder were macerated with 70 ml of distilled water in conical flask under shaking for 6 hours and standing for 18 hours. The marc was washed and the filtrate was adjusted to 100 ml with distilled water. After that, twenty milliliters of the filtrate were transferred into a pre-weighed beaker and evaporate to dryness. The extract was dried at 105 ℃ for 6 hours, cooled in desiccator for 30 minutes and weighed without delay. The content of extractable matter was calculated in a percentage of dried material.

Determination of volatile oil

One hundred grams of *S. japonica* powder were added with 600 ml of water and boiled by Clevenger apparatus. The volume of volatile oil was measured and calculated in percentage.

Thin layer chromatographic fingerprint

One gram of *S. japonica* powder was macerated with 20 ml of 95% ethanol in conical flask for 6 hours under shaking and 18 hours under standing, filtered, evaporated to dryness and dissolved with 1 ml of 95 % ethanol. The extract was spotted on TLC silica gel 60 GF_{254} plate and developed in toluene : chloroform : acetone : formic acid (8:6:7:2). After that, the plate was examined under ultraviolet light (254, 365 nm) and dipped in 10 % sulfuric acid in ethanol then heated at 100℃ for 15 minutes.

Quantitative analysis of rutin in *Sophora japonica* flowering bud

Preparation of standard solutions

One milligram of standard rutin was dissolved in 1 ml of 95% ethanol. The stock solution was diluted to obtain the series of standard solution range from 0.1 to 0.3 mg/ml. These solutions were stored in refrigerator at -20℃.

Preparation of 80 % ethanol extracts of *Sophora japonica*

Three grams of *S. japonica* powders were extracted with 80 % ethanol by Soxhlet apparatus, filtered and evaporated to dryness. The extract was dissolved with ethanol to get the concentration of 0.5 mg/ml. This extract was further used for TLC densitometry and TLC image analysis.

TLC image analysis

Three microliters of *S. japonica* extract and standard rutin solution in ethanol were applied on the silica gel 60 GF_{254} TLC plate, developed in ethyl acetate : ethanol : water : acetic acid : formic acid (5:1:3:1:1). After development, the plate was observed under short wave (254 nm) ultraviolet light and photographed using digital camera.

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

TLC-densitometry

The rutin spots on the developed TLC plates were quantitatively analyzed by scanning with TLC densitometer. The calibration curve of rutin was constructed by plotting peak areas and concentrations of rutin in µg/spot.

Method validation

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

Calibration range

The calibration range was determined by plotting peak areas and concentrations of standard rutin applied.

Accuracy

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

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

A = actual calculated amount in recovery sample

B = amount of un-spiked sample

C = amount of standard rutin added to the sample

Precision

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

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

SD = the standard deviation of each measurement

Specificity

The developed TLC plate was scanned for absorption spectra under the wavelength of 200-700 nm by TLC scanner. The specificity represented by peak identity and peak purity was evaluated by the identity of the absorption spectra among the standard rutin and each sample as well as the identity of the absorption spectra at up-slope, apex and down-slope of the peak.

Limit of detection

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

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

σ = the residual standard deviation of regression line

S = the slope of regression line

Limit of quantitation

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

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

σ = the residual standard deviation of regression line.

S = the slope of regression line

Robustness

The robustness was examined by changing the ratio of mobile phase. The selected mobile phase ratio of ethyl acetate : ethanol : water : acetic acid : formic acid at the ratio of 5.1 : 1.1 : 3.1 : 1 : 1 , 4.9 : 0.9 : 2.9 : 1 : 1 , 4.8 : 0.8 : 2.8 : 1 : 1 were examined and calculated for % RSD of peak area.

Data analysis

The parameters due to standardization were expressed as grand mean ± pooled standard deviation. The rutin contents between TLC image analysis and TLC-densitometry were compared by paired *t*-test statistical analysis.

CHAPTER IV

RESULTS

Macroscopic evaluation

The dried flowering bud of *S. japonica* was yellow and brown color, 1 cm in length and 0.2 cm in width (Figure 2). The drawing of whole plant of *S. japonica* was shown in Figure 3.

Figure 2 Flowering bud dried of *Sophora japonica* L.

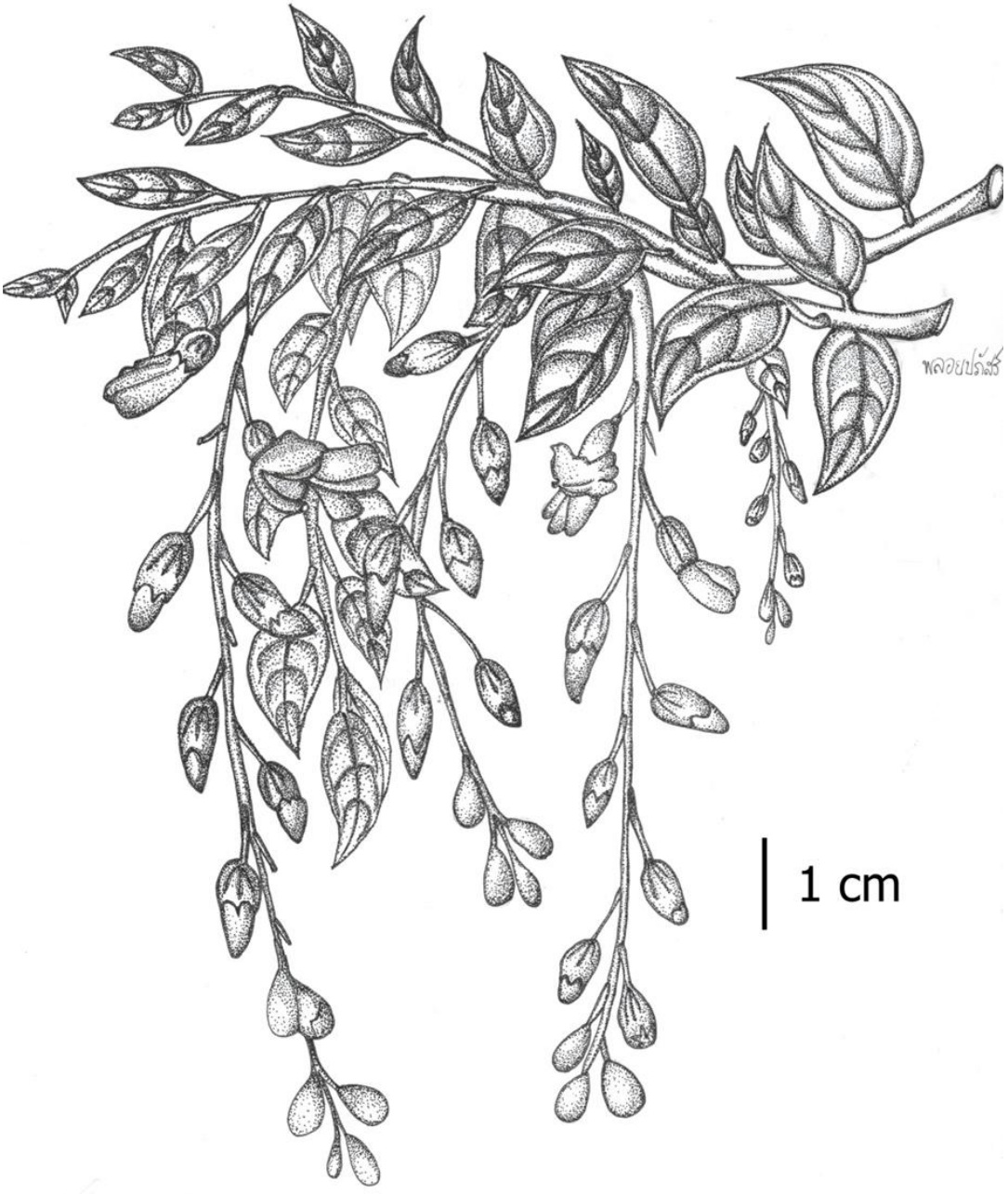


Figure 3 Flowering bud of *Sophora japonica* L.

Microscopic evaluation

The anatomical characterization of *S. japonica* flowering bud showed multicellular trichome, parenchyma, phloem, cavity and spiral vessel (Figure 4). The histological evaluation of *S. japonica* flowering bud powder demonstrated parenchyma, multicellular trichome, spiral vessel and pollen grain (Figure 5).

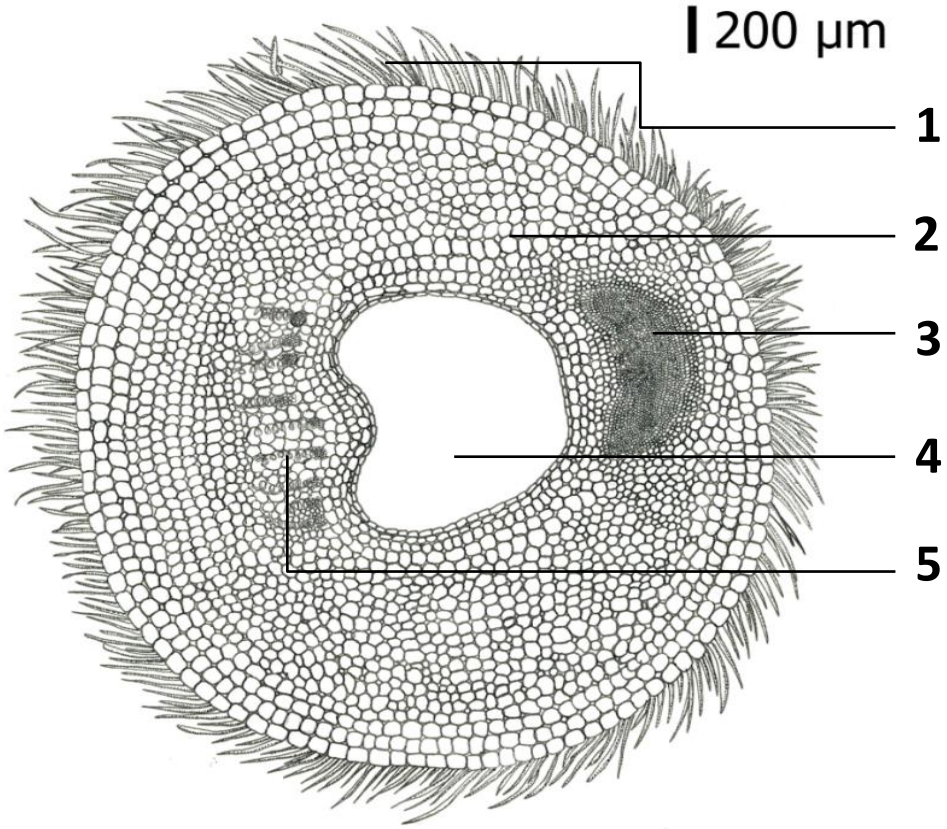


Figure 4 Transverse section of *S. japonica* flowering bud

1. multicellular trichome
2. parenchyma
3. phloem
4. cavity
5. spiral vessel

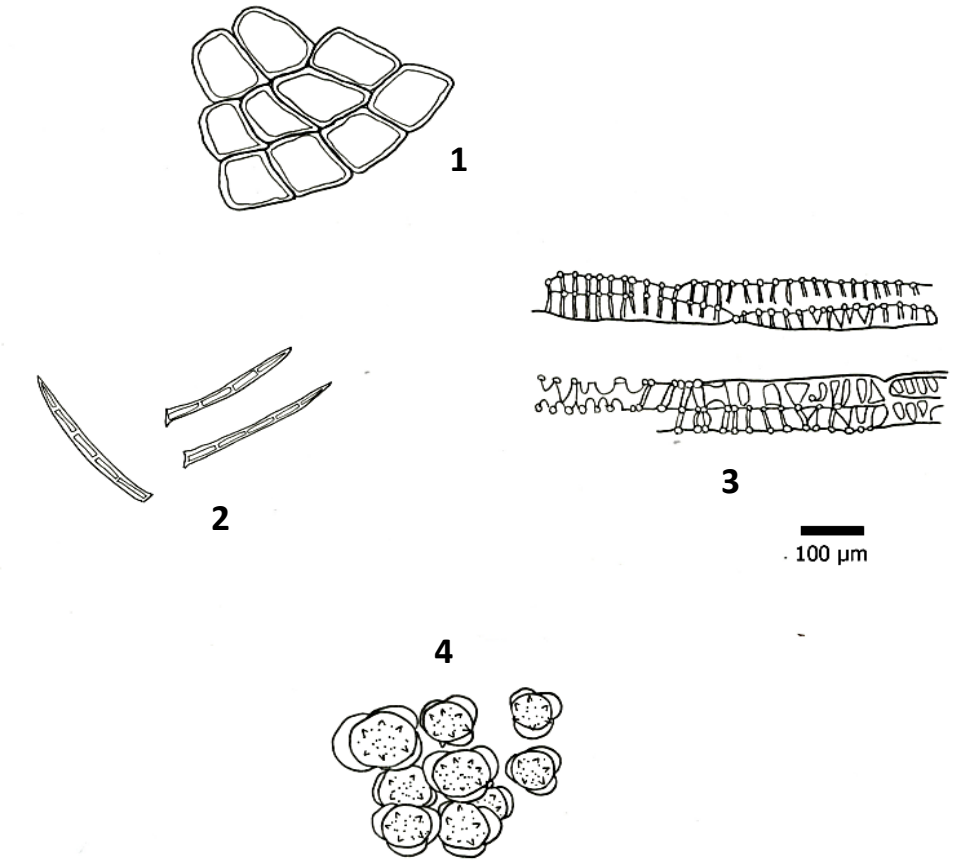


Figure 5 The histological characters of *S. japonica* flowering bud powder

1. Parenchyma
2. Multicellular trichome
3. Spiral vessel
4. Pollen grain

Physico-chemical parameters of dried *S. japonica* flowering bud

The results of physico-chemical parameters of *S. japonica* flowering bud were shown in Table 1. The water content, loss on drying, total ash and acid insoluble ash should be not more than 7.2, 7.0, 7.4 and 1.2 % of dry weight respectively. The ethanol soluble extractive value and water soluble extractive value should be not less than 10.6, 25.7 % by dry weight respectively.

Table 1 Physico-chemical contants of *S. japonica* flowering bud (% by weight)

Parameter	Mean	SD
Water content	7.19	1.17
Loss on drying	7.05	0.9
Total ash	7.37	0.49
Acid-insoluble ash	1.19	0.47
Ethanol soluble extractive value	10.56	1.91
Water soluble extractive value	25.74	1.59
Volatile oil	0	0

*The samples were from 12 different sources throughout Thailand. Each sample was performed in triplicate.

Thin layer chromatographic fingerprint

The ethanolic extract of *S. japonica* was spotted on TLC silica gel 60 GF_{254} plate developed in toluene : chloroform : acetone : formic acid (8 : 6 : 7 : 2) and observed under ultraviolet light (254, 365 nm) and dipped with 10 % sulfuric acid in ethanol then heated at 100 ℃ for 15 minutes.

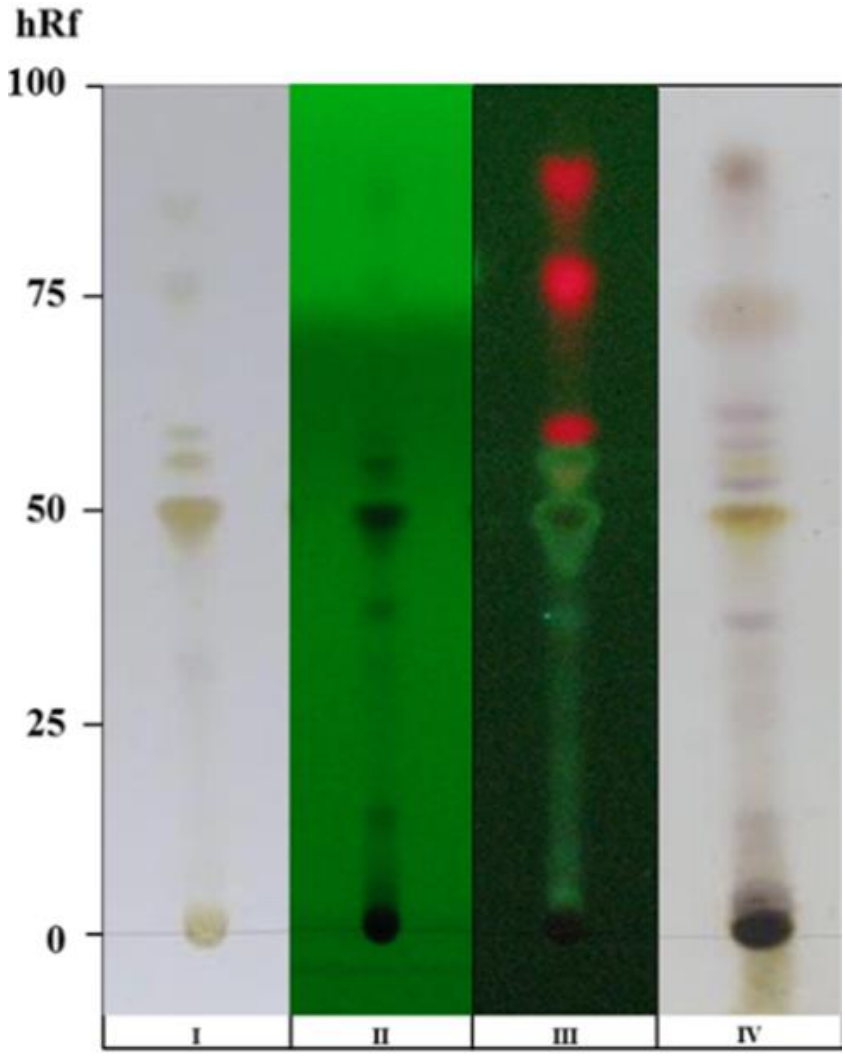


Figure 6 TLC fingerprint of the ethanolic extract of *S. japonica* flowering bud

I detection under daylight
II detection under UV 254 nm
III detection under UV 365 nm
IV detection with 10 % sulfuric acid in ethanol

Ethanolic extraction of *S. japonica* flowering bud

The percent yield of 80 % ethanolic extract of *S. japonica* flowering bud by Soxhlet extraction was 46.65 ± 9.40 % by weight in average (Table 2).

Table 2 The percent yield of 80 % ethanolic extract of *S. japonica* flowering bud from 12 different sources throughout Thailand

Source	Weight of sample (g)	Weight of extractive matter (g)	% yield
1	3.00	1.31	43.67
2	3.00	1.42	47.21
3	3.00	1.22	40.47
4	3.00	1.44	48.04
5	3.00	1.39	46.21
6	3.00	1.21	40.30
7	3.00	1.57	52.04
8	3.00	1.47	49.05
9	3.00	0.86	28.67
10	3.00	2.09	69.50
11	3.00	1.41	47.02
12	3.00	1.43	47.66
		Average	**46.65 ± 9.40**

Specificity

Peak identity

The identity in absorbance spectra determined at the peak apex among rutin standards and samples was shown in Figure 7. The maximum absorbance of rutin was at the wavelength 363 nm.

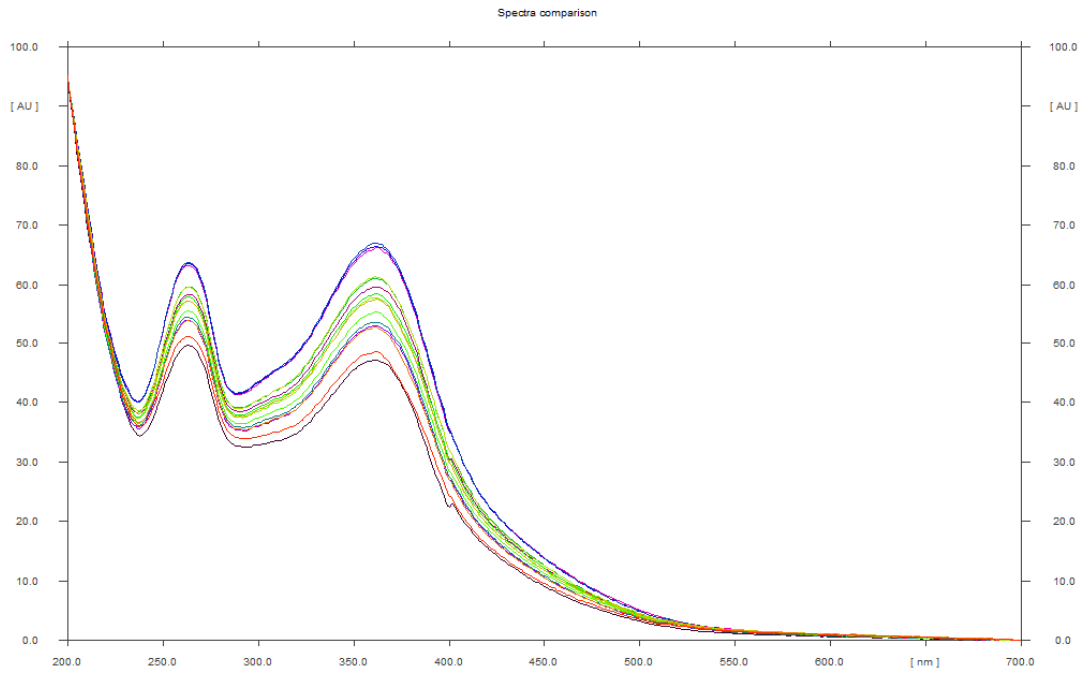


Figure 7 The absorbance spectra of rutin in *S. japonica* flowering bud extracts from 12 different sources and standard rutin representing peak identity

Peak purity

Peak purity of rutin was represented in Figure 8. The absorbance spectra from up-slope, apex and down-slope of the peak were identical.

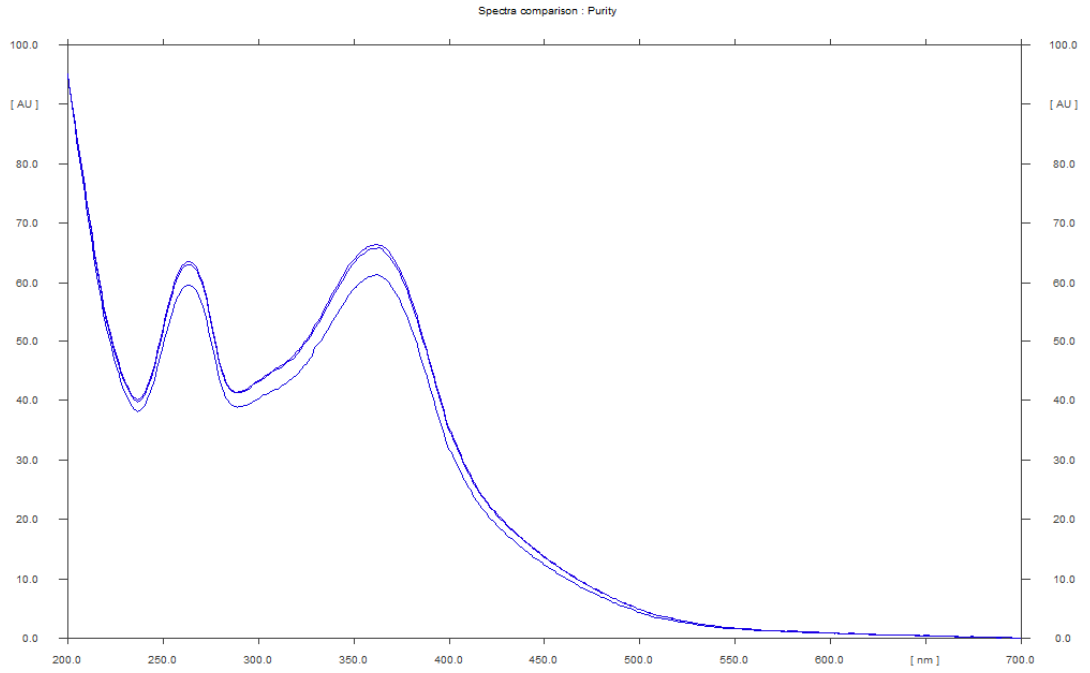


Figure 8 Peak purity measurement using up-slope, apex and down-slope of the peak.

Method validation (TLC image analysis)

Calibration range

The calibration curve of rutin obtained by the peak area of standard (0.3 – 0.9 mg/spot) was polynomial with the regression equation of y = -9344.1x2 + 50711x - 6884.5. The coefficient of determination (R^2) of rutin was 0.9992.

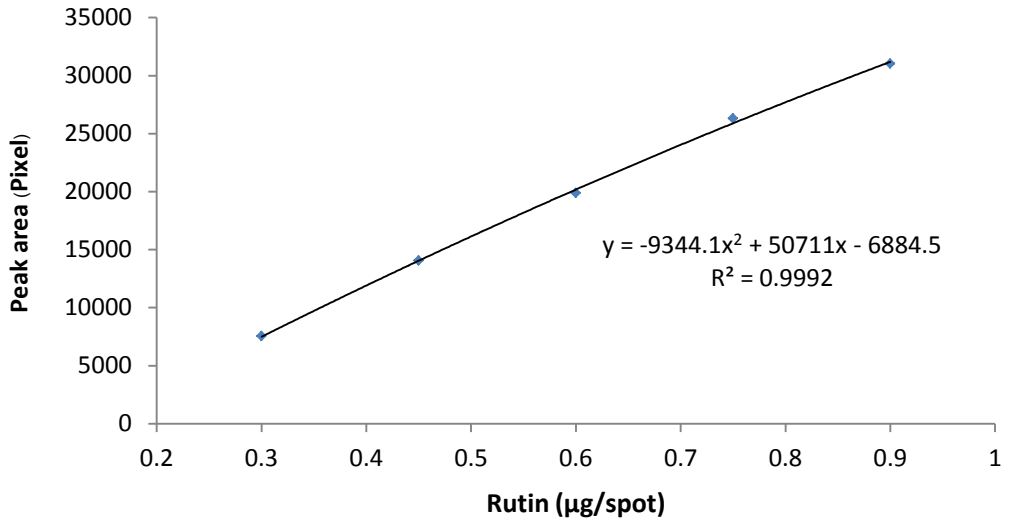


Figure 9 The calibration curve of rutin in *S. japonica* flowering bud by TLC image analysis

Accuracy

The accuracy was tested by recovery method. Standard rutin was spiked into the extract to have three different levels of rutin (low, medium, high). The percent recoveries were shown in Table 3.

Table 3 Accuracy of quantitation of rutin in *S. japonica* flowering bud by TLC image analysis

Rutin added (µg/spot)	**Rutin found (µg/spot)**	**% Recovery**
0.00	0.31 ± 0.01	-
0.06	0.37 ± 0.02	99.06 ± 5.17
0.24	0.52 ± 0.01	93.22 ± 2.69
0.48	0.74 ± 0.04	93.41 ± 5.00
	Average	**95.24 ± 3.32**

Precision

The precision was examined by repeatability (intra-day) and intermediate precision (inter-day). The method was performed by analyzing sample solution of three concentrations in three replicates on the same day and three different days respectively. The repeatability and intermediate precision were showed in Table 4.

Table 4 Repeatability and intermediate precision of quantitation of rutin in *S. japonica* flowering by TLC image analysis

Repeatability		**Intermediate precision**	
Amount (µg/spot)	**%RSD**	**Amount (µg/spot)**	**%RSD**
0.37 ± 0.02	6.17	0.39 ± 0.03	7.52
0.52 ± 0.01	2.41	0.59 ± 0.05	8.58
0.74 ± 0.04	5.00	0.76 ± 0.03	4.31
Average	**3.67 ± 2.32**		**6.63 ± 1.85**

Detection limit and quantitation limit

The detection limit and quantitation limit determination were based on the standard deviation of regression line and the slope of the calibration curve. The detection limit and quantitation limit were 0.024 and 0.074 µg/spot.

Robustness

The robustness was examined by changing the mobile phase ratio. The selected mobile phase ratio was shown in Table 5. The robustness was 1.29 % RSD.

Table 5 Robustness of rutin in *S. japonica* flowering bud by Image analysis

Mobile phase composition **Ethyl Acetate : Ethanol : H_2O : Acetic acid : Formic acid**	**Peak area**
5.1 : 1.1 : 3.1 : 1 : 1	26691.67
4.9 : 0.9 : 2.9 : 1 : 1	26031.09
4.8 : 0.8 : 2.8 : 1 : 1	26230.74
Mean ± SD	**26317.83 ± 338.79**

The content of rutin in *S. japonica* flowering bud by TLC image analysis

The ethanolic extracts of *S. japonica* flowering bud were determined for the rutin content in triplicate by TLC image analysis and calculated as grams per 100 grams of the crude drug (Table 6).

Table 6 The amount of rutin in *S. japonica* flowering bud by TLC image analysis (% by weight)

Source	Rutin in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100 g of dried crude drug)	Rutin in *S. japonica* flowering bud (g/100 g of dried crude drug)
1	0.28	43.67	12.12
2	0.28	47.21	13.07
3	0.29	40.47	11.78
4	0.37	48.04	17.91
5	0.33	46.21	15.15
6	0.36	40.30	14.38
7	0.31	52.04	15.87
8	0.34	49.05	16.83
9	0.34	28.67	9.83
10	0.33	69.50	23.14
11	0.33	47.02	15.50
12	0.24	47.66	11.26
	Average		**14.74 ± 3.59**

Method validation (TLC densitometry)

Calibration range

The calibration curve of rutin was obtained by the peak area of standard (0.3 – 0.9 mg/spot) was polynomial with the regression equation of y = -6307.9x2 + 18665x + 1443.5. The coefficient of determination (R^2) of rutin was 0.9992.

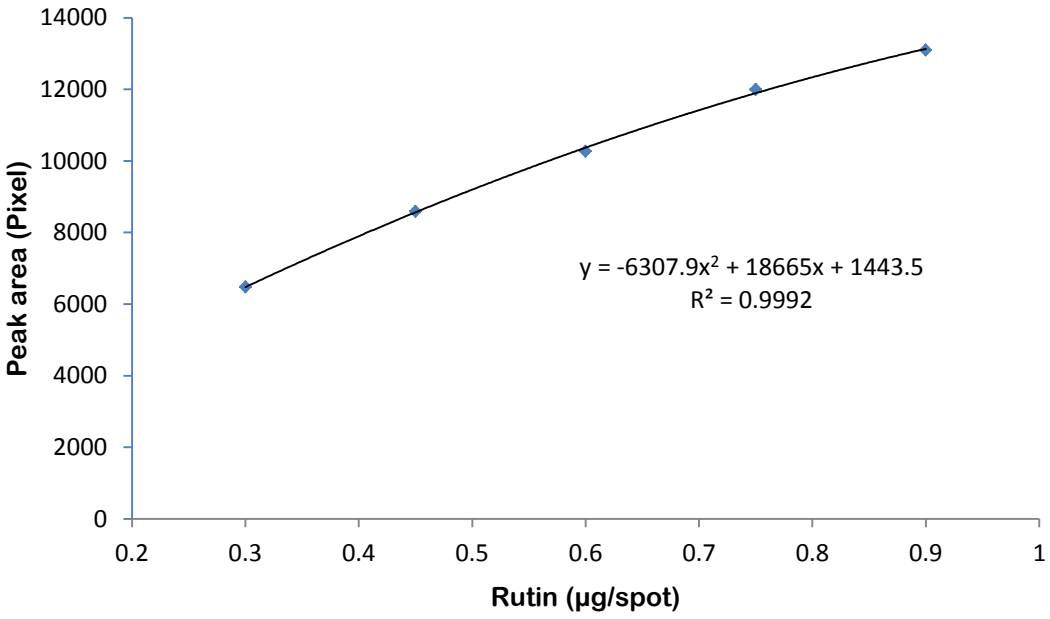


Figure 10 The calibration curve of rutin in *S. japonica* flowering bud by TLC densitometry

Accuracy

The accuracy was tested by recovery method. Standard rutin was spiked into the extract to have three different levels of rutin (low, medium, high). The percent recoveries were shown in Table 7.

Table 7 Accuracy of quantitation of rutin in *S. japonica* flowering bud by TLC densitometry

Rutin added (µg/spot)	**Rutin found (µg/spot)**	**% Recovery**
0.00	0.32 ± 0.01	-
0.06	0.37 ± 0.01	98.48 ± 4.33
0.24	0.51 ± 0.02	91.99 ± 5.47
0.48	0.73 ± 0.03	91.75 ± 4.00
	Average	**94.07 ± 3.82**

Precision

The precision was examined by repeatability (intra-day) and intermediate precision (inter-day). The method was performed by analyzing sample solution of three concentrations in three replicates on the same day and three different days respectively. The repeatability and intermediate precision were showed in Table 8.

Table 8 Repeatability and intermediate precision of quantitation of rutin in *S. japonica* flowering by TLC densitometry

Repeatability		**Intermediate precision**	
Amount (µg/spot)	**%RSD**	**Amount (µg/spot)**	**%RSD**
0.37 ± 0.01	1.97	0.35 ± 0.02	4.69
0.51 ± 0.02	3.82	0.58 ± 0.09	15.62
0.73 ± 0.03	4.22	0.81 ± 0.04	5.17
Average	**3.57 ± 1.08**		**7.26 ± 5.61**

Detection limit and quantitation limit

The detection limit and quantitation limit determination were based on the standard deviation of regression line and the slope of the calibration curve. The detection limit and quantitation limit were 0.02 and 0.06 µg/spot.

Robustness

The robustness was examined by changing the mobile phase ratio. The selected mobile phase ratio was shown in Table 9. The robustness was 8.20 % RSD.

Table 9 Robustness of rutin in *S. japonica* flowering bud by TLC densitometry

Mobile phase composition **Ethyl Acetate : Ethanol : H_2O : Acetic acid : Formic acid**	**Peak area**
5.1 : 1.1 : 3.1 : 1 : 1	6746.01
4.9 : 0.9 : 2.9 : 1 : 1	7133.75
4.8 : 0.8 : 2.8 : 1 : 1	6058.26
Mean ± SD	**6646.01 ± 544.67**

The content of rutin in *S. japonica* flowering bud by TLC densitometry

The ethanolic extracts of *S. japonica* flowering bud were determined in triplicate by TLC densitometry and calculated as grams per 100 grams of the crude drug (Table 10).

Table 10 The amount of rutin in *S. japonica* flowering bud by TLC densitometry (% by weight)

Source	Rutin in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100 g of dried crude drug)	Rutin in *S. japonica* flowering bud (g/100 g of dried crude drug)
1	0.29	43.67	12.51
2	0.29	47.21	13.86
3	0.30	40.47	12.30
4	0.43	48.04	20.74
5	0.35	46.21	16.10
6	0.39	40.30	15.90
7	0.33	52.04	17.24
8	0.41	49.05	20.29
9	0.37	28.67	10.49
10	0.34	69.50	23.68
11	0.37	47.02	17.55
12	0.26	47.66	12.51
	Average		**16.10 ± 4.01**

Comparison of rutin contents between TLC image analysis and TLC densitometry

The rutin contents in *S. japonica* flowering bud ethanolic extracts performed by TLC image analysis were in accordance with the one determined by TLC densitometry (Table 11). However, the results by TLC image analysis was slightly less than the results by TLC densitometry ($P<0.05$).

Table 11 Comparison of rutin contents of *S. japonica* flowering bud between TLC image analysis and TLC densitometry

Source	Rutin in the ethanolic extract (g/g)	
	TLC image analysis	TLC densitometry
1	0.28	0.29
2	0.28	0.29
3	0.29	0.30
4	0.37	0.43
5	0.33	0.35
6	0.36	0.39
7	0.31	0.33
8	0.34	0.41
9	0.34	0.37
10	0.33	0.34
11	0.33	0.37
12	0.24	0.26
Average	**0.32 ± 0.04**	**0.34 ± 0.05**

CHAPTER V
DISCUSSION AND CONCLUSION

Herbal medicines are still commonly used for primary health care because it is easy and inexpensive. It would be good for human health when suitably used herb medicine. Accordingly, adulteration is very important for safety used on herbal medicines [1].

Pharmacognostic specification is a part of quality control of herbal medicines. In this study, standardization parameters of *Sophora japonica* flowering bud were established according to *World Health Organization* (WHO) guidelines [29]. Macroscopic and microscopic examinations are methods for identification of crude drugs. The macroscopic examination of *S. japonica* flowering bud was shown in Figure 3. Microscopic evaluation demonstrated the anatomical structure of multicellular trichome, parenchyma, phloem, cavity and spiral vessel as shown in Figure 4. Histological characteristics of *S. japonica* flowering bud powder were found parenchyma, multicellular trichome, spiral vessel and pollen grain (Figure 5).

The physico-chemical parameters of *S. japonica* dried flowering bud showed that the contents of water, loss on drying, total ash and acid insoluble ash should not be more than 7.2, 7.0, 7.4 and 1.2 % by weight respectively. The Japanese Pharmacopoeia (15^{th} edition, 2006) specified that the loss on drying and acid insoluble ash of *S. japonica* should not be more than 10 and 1.5 % by weight respectively. For Pharmacopoeia of the People's Republic of China (2005), the total ash of *S. japonica* should not be more than 9.0 % by weight [34]. The ethanol soluble extractive value and water soluble extractive value should not be less than 10.6, 25.7 % by weight respectively.

Thin layer chromatography is a technique for separation and identification of crude drugs. TLC fingerprint of *S. japonica* flowering bud was established for crude drug identification according to quantitative evaluated components.

For quantitative analysis, in this study the rutin content in *S. japonica* flowering bud performed by TLC-densitometry and TLC image analysis were found to be 16.09 ± 4.00 and 14.73 ± 3.58 % by weight of crude drugs, respectively. The Public Health Department of China stated of that the rutin content from dried flowers and flower buds of *S. japonica* should be not less than 8 and 20 % respectively [35]. The previous study by Liao *et al*. (2015) reported the rutin content 15.39 ± 9.41 % by weight in *S. japonica* flowering bud by ethanolic extraction using Soxhlet apparatus. The study also found that higher amount as 18.23 ± 13.38 % by weight was obtained by ultrasonic extraction and the frequency of 60-62 kHz [36]. Chen *et al*. (2000) reported the rutin content of 20.28 % by weight in the methanolic extract of *S. japonica* flower bud using capillary electrophoresis with electrochemical detection (CE-ED) [35]. Moreover, Chu *et al*. (2005) studies the ethanolic extract from the pericarp and seeds of *S. japonica* using the same method and demonstrated that amount of 0.219 and 0.016 % by weight respectively [37].

TLC-densitometry and TLC image analysis in this study were validated according to ICH guideline including calibration curve, accuracy, precision, detection limit (LOD), quantitation limit (LOQ), specificity, and robustness. The calibration curves were polynomial in TLC image analysis and TLC-densitometry with the range of 0.3 – 0.9 µg/spot (R^2 = 0.9992). The accuracy of TLC image analysis and TLC-densitometry were 95.2 – 109.6 % and 94.1 – 108.9 % recovery respectively (85 -110 % recovery are in the acceptable [38]). Repeatability of TLC-densitometry was in the acceptable (< 4 % RSD [38]). Nevertheless, intermediate precision of TLC-densitometry and repeatability and intermediate precision of TLC image analysis represented lower precision (3.6 – 15.6, 1.1 – 6.2 and 4.3 – 8.6 %RSD respectively). LOD and LOQ of TLC image analysis and TLC-densitometry indicated similar sensitivity. Specificity was confirmed by identical absorption spectra under the wavelength of 200-700 nm among the standard rutin and each sample and identical absorption spectra among up-slope, apex and down-slope of the peak. In this study, the maximum absorption of rutin was 363 nm as shown in Figure 7 which represented peak identity and peak purity of rutin. Robustness is a properly of the

method for indicating the reliability by deliberate variations of condition in method [39]. The robustness test in this study was designed by changing the mobile phase ratio and %RSD was found to be 8.2 and 1.3 %RSD respectively. Comparison of rutin content between TLC image analysis and TLC-densitometry showed that the results by TLC image analysis was slightly less than the results by TLC densitometry might be due to the peak that TLC densitometry scanned the sample rutin band at wavelength of 363 nm (maximum absorption) whereas TLC image analysis detected the rutin band under 254 nm in Ultraviolet viewing cabinet. However, TLC image analysis can be used as an alternative method for chemical quantitation in crude drug because it is simple and inexpensive.

In conclusion, the physico-chemical parameters are important for quality control. This pharmacognostic specification can be used for a part of quality control of *S. japonica* flowering bud. Furthermore, quantitative TLC represented its good reliability for analysis of rutin content in *S. japonica* flowering bud.

REFERENCES

1. WHO, *Guidelines for the appropriate use of herbal medicines*. Western Pacific series. 1998, Manila: WHO regional publications.
2. Wojciechowski M.F., Mahn J., and Jones B. *Fabaceae*. The Tree of Life Web Project 2006.
3. Bao and Bojian, *Sophora japonica Linnaeus*. Flora of China. Vol. 10. 2010.
4. Zhang L.B., Lv J.L., and Chen H.L., *Japonicasins A and B, two new isoprenylated flavanones from Sophora japonica.* Fitoterapia, 2013. **87**: p. 89-92.
5. Kim J.M. and Yun-Choi H.S., *Anti-platelet effects of flavonoids and flavonoid-glycosides from Sophora japonica.* Archives of Pharmacal Research, 2008. **31**(7): p. 886-890.
6. Zhang L., et al., *Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds.* Journal of Agricultural and Food Chemistry, 2011. **59**: p. 12361-12367.
7. Schalow S. and Mihaylova D., *Antioxidant and stabilization activity of a quercetin-containing flavonoid extract obtained from Bulgarian Sophora japonica L.* Brazilian Archives of Biology and Technology, 2013. **56**(3): p. 431-438.
8. Park K.W., Lee J.E., and Park K.M., *Diets containing Sophora japonica L. prevent weight gain in high-fat diet-induced obese mice* Nutrition Research, 2009. **29**: p. 819-824.
9. Chua L.S., *A review on plant-based rutin extraction methods and its pharmacological activities.* Journal of Ethnopharmacology, 2013. **150**: p. 805-817.
10. Zhang B., et al., *Antinociceptive activity of flower buds extract of Sophora japonica and its main active ingredient quercetin in bee venom-induced rat model.* Journal of Intercultural Ethnopharmacology, 2014. **3**(1): p. 9-14.
11. Tang Y., Lou F., Wang J., Zhuang S., *Four new isoflavone triglycosides from Sophora japonica.* J.Nat.Prod., 2001. **64**: p. 1107-1110.
12. Tang Y.P., Lou F.C., Wang J.H., *Two kaempferol triglycosides from pericarps of Sophora japonica L.* Zhongguo Zhong Yao Za Zhi, 2001. **26**(12): p. 839-841.

13. Tang Y.P., Hu J., Wang J.H., Lou F.C., *A new coumaronochromone from Sophora japonica.* J Asian Nat Prod Res, 2002. **4**(1): p. 1-5.
14. Wang J.H., et al., *A flavonol tetraglycoside fromvSophora japonica seeds.* Phytochemistry, 2003. **63**: p. 463-465.
15. Sun A., Sun Q., and Liu R., *Preparative isolation and purification of flavone compounds from Sopora japonica L. by high-speed counter-current chromatography combined with macroporous resin column separation.* J Sep Sci 2007. **30**(7): p. 1013-1018.
16. Tang Y.P., Zhu H.X., and Duan J.A., *Two new isoflavone triglycoside from the small branches of Sophora japonica.* J Asian Nat Prod Res, 2008. **10**(1-2): p. 65-70.
17. Tang Y., et al., *Isoflavone tetraglycosides from Sophora japonica leaves.* J.Nat.Prod., 2008. **71**: p. 448-450.
18. Park H.Y., et al., *A new isoflavone glycoside from the stem bark of Sophora japonica.* Archives of Pharmacal Research, 2010. **33**(8): p. 1165-1168.
19. Abdallah H.M., et al., *Isolation of antiosteoporotic compounds from seeds of Sophora japonica.* PLOS ONE, 2014. **9**(6): p. 1-8.
20. Lai J.S., Lin C.C., and Chiang T.M., *Tyrosinase inhibitory activity and and Thermostability of the flavonoid complex from Sophora japonica L (Fabaceae).* Tropical Journal of Pharmaceutical Research, 2014. **13**(2): p. 243-247.
21. Jung C.H., et al., *Antihyperglycemic activity of herb extracts on streptozotocin-induced diabetic rats.* Bioscience biotechnology and biochemistry, 2006. **70**(10): p. 2556-2559.
22. Halawany A.M.E., Chung M.H., Abdallah H.M., Nishihara T., Hattori M., *Estrogenic activity of a naringinase-treated extract of Sophora japonica cultivated in Egypt* Pharmaceutical Biology, 2010. **48**(2): p. 2010.
23. Halawany A.M.E., et al., *Screening for estrogenic and antiestrogenic activities of plants growing in Egypt and Thailand.* Pharmacognosy Research, 2011. **3**(2): p. 107-113.

24. Zhang B., et al., *Antinociceptive activity of flower buds extract of Sophora japonica and its main active ingredient quercetin in bee venom-induced rat model.* Journal of Intercultural Ethnopharmacology, 2014. **3**(1): p. 9-14.
25. Jung S.R., et al., *Genistein mediates the anti-adipogenic actions of Sophora japonica L. extracts.* Journal of Medicinal Food, 2011. **14**(4): p. 360-368.
26. Yang J., Guo J., and Yuan J., *In vitro antioxidant properties of rutin.* LWT, 2008. **41**: p. 1060-1066.
27. Guardia T., et al., *Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin andhesperidin on adjuvant arthritis in rat.* Il Farmaco, 2001. **56**: p. 683-687.
28. Janbaz K.H., Saeed S.A., and Gilani A.H., *Protetive effect of rutin on paracetamol- and CCl_4-induced hepatotoxicity in rodents.* Fitoterapia, 2002. **73**: p. 557-563.
29. WHO, *Quality control methods for medicinal plant materials*. 1998, Geneva: World Heath Organization.
30. Touchstone J.C., *Practice of Thin Layer Chromatography*. 1992: A wiley-interscience publication.
31. Reich E. and Widmer V.M., *Thin-Layer Chromatography Overview*. 2013, Muttenz, Switzerland: CAMAG Laboratory.
32. Stroka J., Spengenberg B., and Anklam E., *New approaches in TLC-densitometry.* Journal of Liquid Chromatography & Related Technologies, 2002. **25**(10-11): p. 1497-1513.
33. Ferreira T. and Rasband W., *ImageJ User Guide*. Vol. IJ1.46r. 2012.
34. Satake M. and Goda Y., *Comparative Studies on Pharmacopoeial Definitions, Requirements and Information for Crude Drugs among FHH Member Countries in 2007*, in *The Sub-Committee I of the Western Pacific Regional Forum for the Harmonization of Herbal Medicines (FHH)*, K. N., Editor. 2011: Japan.

35. Chen G., Zhang H., and Ye J., *Determination of rutin and quercetin in plants by capillary eletrophoresis with eletrochemical detection.* Analytica Chimica Acta, 2000. **423**: p. 69-76.

36. Liao J, et al., *New method to enhance the extraction yield of rutin from Sophora japonica using a novel ultrasonic extraction system by determining optimum ultrasonic frequency.* Ultrasonics Sonochemistry, 2015. **27**: p. 110-116.

37. Chu Q., et al., *Simultaneous determination of phytoestrogens in different medicinal parts of Sophora japonica L. by capillary electrophoresis with electrochemical detection.* Biomedical chromatography, 2005. **19**: p. 149-154.

38. *AOAC Guidelines for single labratory validationof chemical methods for dietary supplement and botanicals*. 2002.

39. Guideline, I.H.T., *Validation of Anlytical Procedures: Text and Methodology, Q2 (R2)*. 2005, Geneva: The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

APPENDIX

Table 12 Determination of water content of *S. japonica* dried crude drug

Source		No.	Amount (% by weight)	Mean	SD
1	Bangkok 1	1	6.00	6.00	0.33
		2	6.33		
		3	5.67		
2	Bangkok 2	1	7.67	7.78	0.19
		2	7.67		
		3	8.00		
3	Bangkok 3	1	5.67	5.89	0.69
		2	6.67		
		3	5.33		
4	Chiangmai	1	8.67	8.56	0.19
		2	8.67		
		3	8.33		
5	Lumpang	1	6.33	6.44	0.51
		2	7.00		
		3	6.00		
6	Roi-Et	1	5.33	5.89	0.51
		2	6.33		
		3	6.00		
7	Khonkean	1	7.00	7.67	0.88
		2	8.67		
		3	7.33		
8	Chanthaburi	1	6.33	6.67	0.33
		2	7.00		
		3	6.67		
9	Suratthani	1	9.00	9.44	0.51
		2	10.00		
		3	9.33		
10	Phuket	1	6.67	7.00	0.33
		2	7.33		
		3	7.00		
11	Songkhla	1	6.33	7.00	0.67
		2	7.67		
		3	7.00		
12	Nakhonpathom	1	8.00	8.00	0.33
		2	8.33		
		3	7.67		
Grand mean				**7.19**	
Pooled SD				**0.50**	

Table 13 Determination of loss on drying of *S. japonica* dried crude drug

Source		No.	Amount (% by weight)	Mean	SD
1	Bangkok 1	1	6.49	6.41	0.10
		2	6.44		
		3	6.30		
2	Bangkok 2	1	6.24	6.44	0.18
		2	6.58		
		3	6.49		
3	Bangkok 3	1	6.45	6.41	0.21
		2	6.60		
		3	6.20		
4	Chiangmai	1	7.34	7.35	0.17
		2	7.17		
		3	7.52		
5	Lumpang	1	6.75	6.75	0.02
		2	6.78		
		3	6.74		
6	Roi-Et	1	6.78	6.80	0.04
		2	6.85		
		3	6.77		
7	Khonkean	1	5.98	5.88	0.10
		2	5.88		
		3	5.78		
8	Chanthaburi	1	8.00	7.99	0.05
		2	8.03		
		3	7.94		
9	Suratthani	1	6.58	6.52	0.08
		2	6.55		
		3	6.42		
10	Phuket	1	7.29	7.189	0.13
		2	7.23		
		3	7.05		
11	Songkhla	1	6.43	6.34	0.10
		2	6.23		
		3	6.37		
12	Nakhonpathom	1	9.11	9.28	0.15
		2	9.32		
		3	9.40		
Grand mean				**6.95**	
Pooled SD				**0.12**	

Table 14 Determination of total ash of *S. japonica* dried crude drug

Source		No.	Amount (% by weight)	Mean	SD
1	Bangkok 1	1	7.29	7.11	0.35
		2	6.71		
		3	7.35		
2	Bangkok 2	1	7.74	7.74	0.06
		2	7.67		
		3	7.80		
3	Bangkok 3	1	6.96	6.95	0.01
		2	6.94		
		3	6.95		
4	Chiangmai	1	7.85	7.71	0.25
		2	7.85		
		3	7.42		
5	Lumpang	1	6.94	6.92	0.05
		2	6.87		
		3	6.96		
6	Roi-Et	1	7.56	7.61	0.07
		2	7.58		
		3	7.70		
7	Khonkean	1	7.50	7.32	0.16
		2	7.19		
		3	7.28		
8	Chanthaburi	1	8.47	8.44	0.14
		2	8.57		
		3	8.29		
9	Suratthani	1	7.72	7.80	0.08
		2	7.89		
		3	7.80		
10	Phuket	1	7.09	7.03	0.05
		2	7.02		
		3	6.99		
11	Songkhla	1	6.89	6.91	0.03
		2	6.90		
		3	6.95		
12	Nakhonpathom	1	6.84	6.93	0.08
		2	7.00		
		3	6.96		
Grand mean				**7.37**	
Pooled SD				**0.15**	

Table 15 Determination of acid insoluble ash of *S. japonica* dried crude drug

Source		No.	Amount (% by weight)	Mean	SD
1	Bangkok 1	1	1.11	1.01	0.10
		2	0.91		
		3	1.03		
2	Bangkok 2	1	1.65	1.40	0.28
		2	1.45		
		3	1.10		
3	Bangkok 3	1	0.70	0.74	0.05
		2	0.71		
		3	0.80		
4	Chiangmai	1	2.11	1.98	0.28
		2	2.18		
		3	1.66		
5	Lumpang	1	0.78	0.74	0.06
		2	0.67		
		3	0.76		
6	Roi-Et	1	1.15	1.30	0.17
		2	1.28		
		3	1.48		
7	Khonkean	1	1.17	1.08	0.10
		2	0.98		
		3	1.09		
8	Chanthaburi	1	2.24	2.18	0.08
		2	2.09		
		3	2.22		
9	Suratthani	1	1.20	1.21	0.03
		2	1.20		
		3	1.24		
10	Phuket	1	0.91	0.85	0.07
		2	0.77		
		3	0.88		
11	Songkhla	1	0.85	0.80	0.05
		2	0.78		
		3	0.76		
12	Nakhonpathom	1	0.80	0.94	0.14
		2	1.08		
		3	0.96		
Grand mean				**1.19**	
Pooled SD				**0.14**	

Table 16 Determination of ethanol soluble extractive of *S. japonica* dried crude drug

Source		No.	Amount (% by weight)	Mean	SD
1	Bangkok 1	1	5.72	6.77	0.92
		2	7.43		
		3	7.17		
2	Bangkok 2	1	11.94	11.90	0.21
		2	12.09		
		3	11.67		
3	Bangkok 3	1	12.18	12.04	0.14
		2	12.06		
		3	11.89		
4	Chiangmai	1	12.76	12.70	0.17
		2	12.84		
		3	12.51		
5	Lumpang	1	13.17	12.91	0.25
		2	12.89		
		3	12.66		
6	Roi-Et	1	10.81	10.04	0.67
		2	9.74		
		3	9.58		
7	Khonkean	1	9.80	9.43	0.44
		2	9.55		
		3	8.94		
8	Chanthaburi	1	11.40	11.11	0.65
		2	11.55		
		3	10.36		
9	Suratthani	1	10.27	9.62	0.57
		2	9.35		
		3	9.23		
10	Phuket	1	8.65	8.80	0.16
		2	8.80		
		3	8.97		
11	Songkhla	1	9.26	8.94	0.30
		2	8.91		
		3	8.67		
12	Nakhonpathom	1	13.11	12.47	0.77
		2	12.69		
		3	11.62		
Grand mean				**10.56**	
Pooled SD				**0.51**	

Table 17 Determination of water soluble extractive of *S. japonica* dried crude drug

Source		No.	Amount (% by weight)	Mean	SD
1	Bangkok 1	1	24.93	24.24	0.69
		2	23.54		
		3	24.25		
2	Bangkok 2	1	24.50	24.34	2.50
		2	21.75		
		3	26.75		
3	Bangkok 3	1	27.41	27.21	0.28
		2	26.88		
		3	27.33		
4	Chiangmai	1	25.33	25.14	0.40
		2	25.40		
		3	24.68		
5	Lumpang	1	23.95	24.32	0.32
		2	24.52		
		3	24.48		
6	Roi-Et	1	26.88	26.23	0.97
		2	26.69		
		3	25.11		
7	Khonkean	1	25.24	24.95	0.33
		2	24.59		
		3	25.03		
8	Chanthaburi	1	25.33	25.14	0.40
		2	25.40		
		3	24.68		
9	Suratthani	1	25.00	25.35	0.37
		2	25.30		
		3	25.74		
10	Phuket	1	23.99	24.54	0.48
		2	24.85		
		3	24.77		
11	Songkhla	1	25.77	26.35	0.52
		2	26.79		
		3	26.49		
12	Nakhonpathom	1	27.75	28.40	0.68
		2	29.11		
		3	28.34		
Grand mean				**25.74**	
Pooled SD				**0.88**	

Formulas

Grand Mean

$$\bar{\bar{x}} = \left(\frac{n_1}{n}\right)\bar{x}_1 + \left(\frac{n_2}{n}\right)\bar{x}_2 + \left(\frac{n_3}{n}\right)\bar{x}_3 + \ldots + \left(\frac{n_k}{n}\right)\bar{x}_k$$

Residual standard deviation: σ

$$\sigma = \sqrt{\frac{\sum(Y - Y_{est})^2}{n-2}}$$

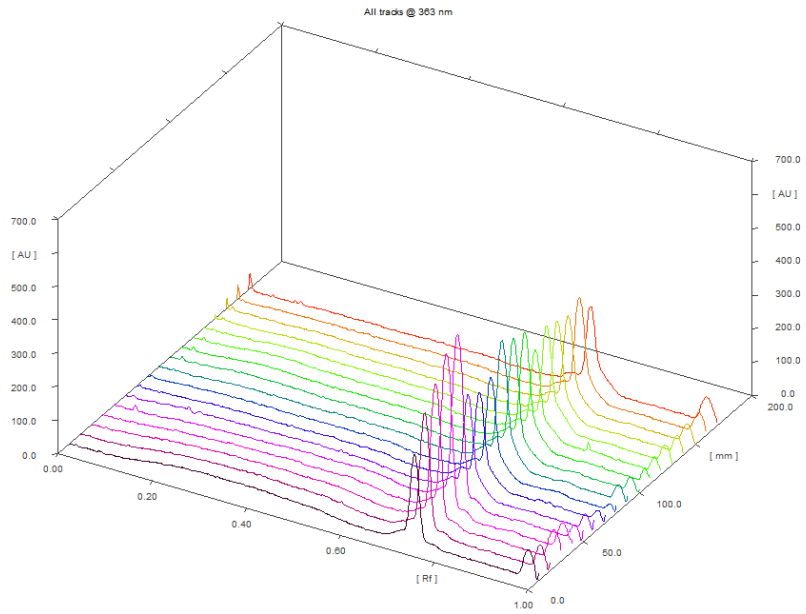


Figure 11 TLC – densitogram of rutin in *S. japonica* flowering bud extracts from 12 different sources and standard rutin for calibration range (1)

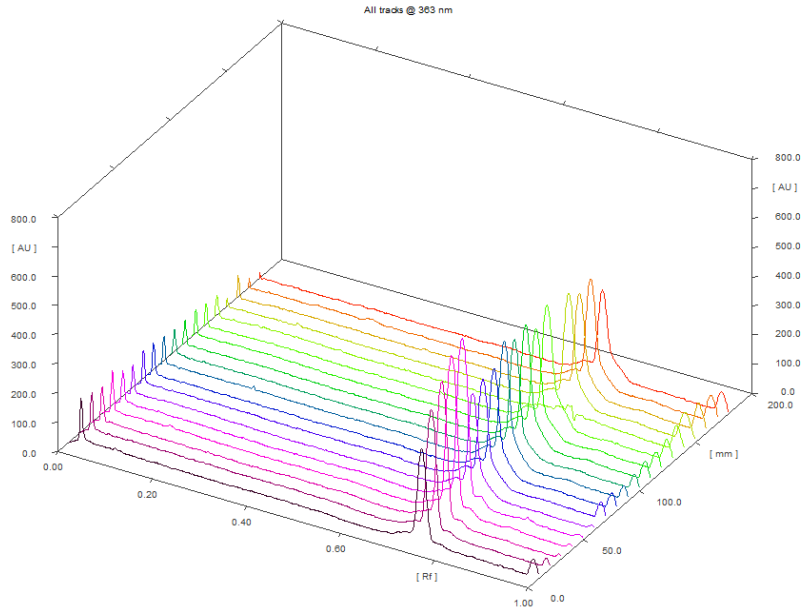


Figure 12 TLC – densitogram of rutin in *S. japonica* flowering bud extracts from 12 different sources and standard rutin for calibration range (2)

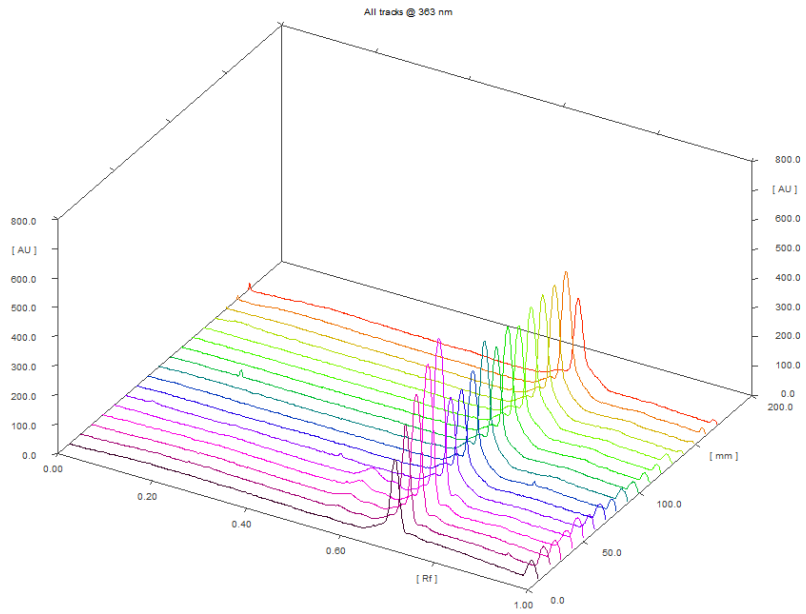


Figure 13 TLC – densitogram of rutin in *S. japonica* flowering bud extracts from 12 different sources and standard rutin for calibration range (3)

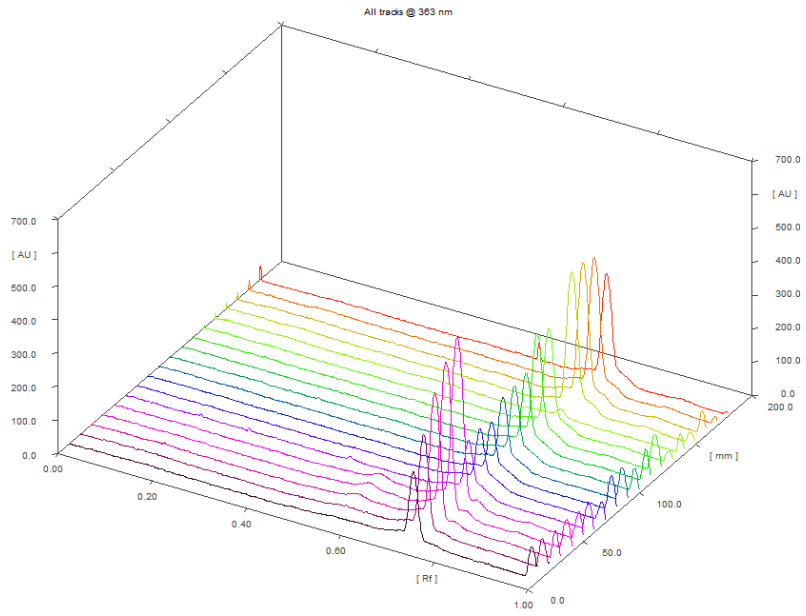


Figure 14 TLC – densitogram of standard rutin, *S. japonica* flowering bud extract unspiked and spiked to three different levels of rutin (low, medium, high) for accuracy (1)

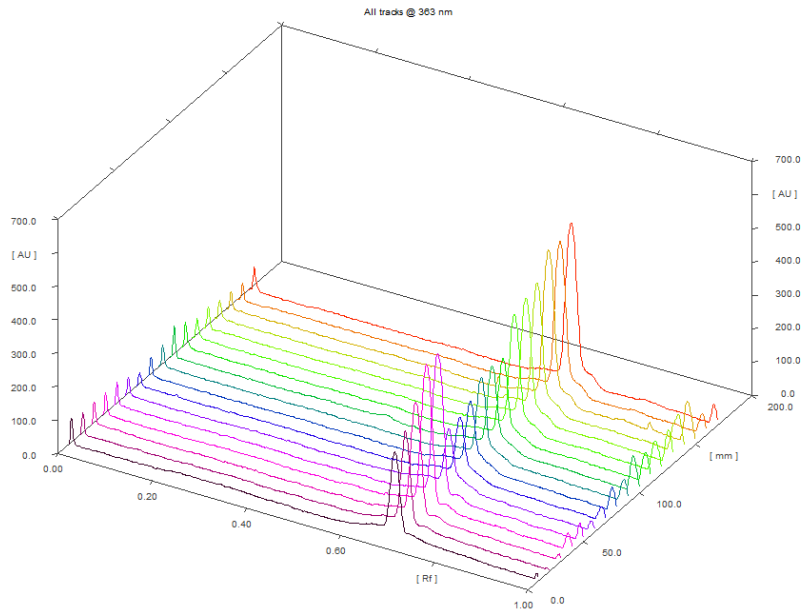


Figure 15 TLC – densitogram of standard rutin, *S. japonica* flowering bud extract unspiked and spiked to three different levels of rutin (low, medium, high) for accuracy (2)

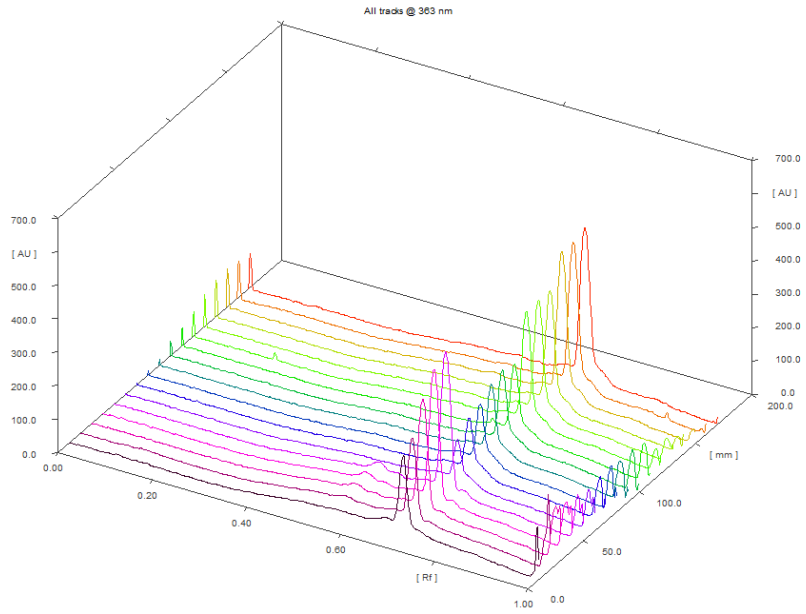


Figure 16 TLC – densitogram of standard rutin, *S. japonica* flowering bud extract unspiked and spiked to three different levels of rutin (low, medium, high) for accuracy (3)

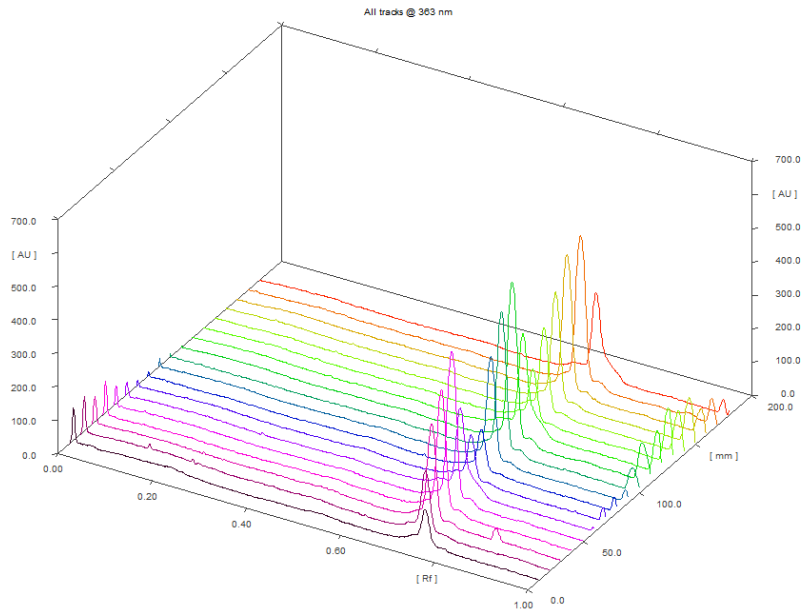


Figure 17 TLC – densitogram of standard rutin and *S. japonica* flowering bud extract for robustness (1)

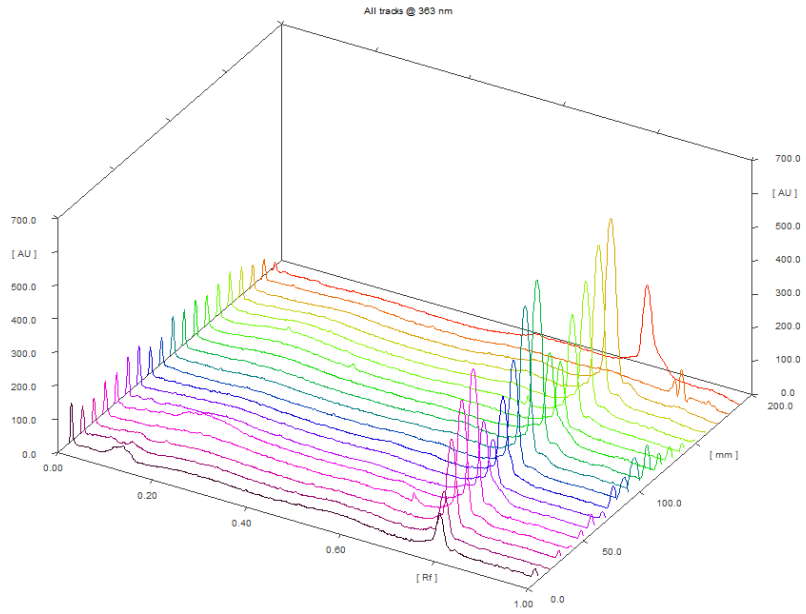


Figure 18 TLC – densitogram of standard rutin and *S. japonica* flowering bud extract for robustness (2)

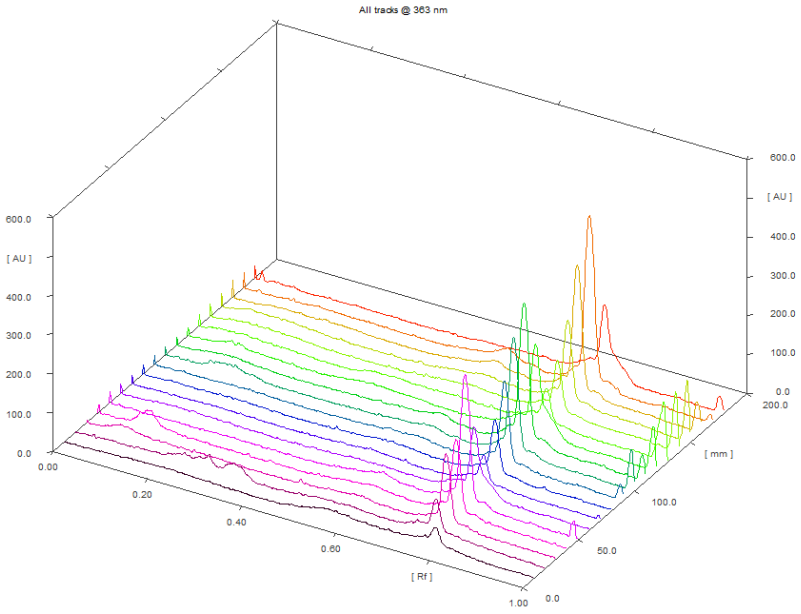


Figure 19 TLC – densitogram of standard rutin and *S. japonica* flowering bud extract for robustness (3)

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