STANDARDIZATION OF LEAF AND STEM OF *THUNBERGIA LAURIFOLIA* AND QUANTITATIVE ANALYSIS OF ROSMARINIC ACID IN SELECTED THAI MEDICINAL PLANTS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Public Health Sciences
Common Course
College of Public Health Sciences
Chulalongkorn University
Academic Year 2018

มาตรฐานของใบและเถาของรางจืด และปริมาณวิเคราะห์ของกรดโรสมารินิกในสมุนไพรไทยบางชนิด

น.ส.ปริชาติ หงษ์สิงห์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์สาธารณสุข ไม่สังกัดภาควิชา/เทียบเท่า
วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	STANDARDIZATION OF LEAF AND STEM OF *THUNBERGIA LAURIFOLIA* AND QUANTITATIVE ANALYSIS OF ROSMARINIC ACID IN SELECTED THAI MEDICINAL PLANTS
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ปริชาติ หงษ์สิงห์ : มาตรฐานของใบและเถาของรางจืด และปริมาณวิเคราะห์ของกรดโรสมารินิกในสมุนไพรไทยบางชนิด. (STANDARDIZATION OF LEAF AND STEM OF *THUNBERGIA LAURIFOLIA* AND QUANTITATIVE ANALYSIS OF ROSMARINIC ACID IN SELECTED THAI MEDICINAL PLANTS) อ.ที่ปรึกษาหลัก : รศ. ดร.ชนิดา พลานุเวช, อ.ที่ปรึกษาร่วม : รศ. ดร.นิจศิริ เรืองรังษี

รางจืดมีชื่อทางวิทยาศาสตร์ว่า *Thunbergia laurifolia* Lindl. ซึ่งจัดอยู่ในวงศ์ Thunbergiaceae ใบและเถาของรางจืดสามารถนำมาใช้ในการรักษาไข้ แก้อักเสบ รวมไปถึงแก้พิษ *Perilla frutescens* (L.) Britton หรืองาขี้ม้อน จัดอยู่ในวงศ์ Labiatae ใบงาขี้ม้อนถูกนำมาใช้เพื่อรักษาอาการแพ้ ไข้ และอักเสบ กรดโรสมารินิกเป็นสารออกฤทธิ์ทางชีวภาพที่พบได้ในพืชทั้งสองชนิดซึ่งถูกนำมาใช้เป็นตัวบ่งชี้มาตรฐานในการศึกษาครั้งนี้ การหาปริมาณกรดโรสมารินิกในตัวอย่างทั้งสามโดยใช้วิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรีและวิธีทินเลเยอร์โครมาโทกราฟีโดยวิเคราะห์ภาพถ่ายไม่แตกต่างกันอย่างมีนัยสำคัญ ($P > 0.05$) ข้อกำหนดทางเภสัชเวทของใบและเถารางจืดได้ถูกจัดทำขึ้นซึ่งประกอบด้วยการประเมินลักษณะทางจุลทรรณ์และมหทรรศน์ ลักษณะทางเคมี-ฟิสิกส์ และเอกลักษณ์ทางเคมี เพื่อให้มีการควบคุมคุณภาพของสมุนไพรตามคำแนะนำขององค์การอนามัยโลก การทดสอบฤทธิ์ทางชีวภาพในหลอดทดลองแสดงให้เห็นว่าสารสกัดเอทานอลของใบและเถาของรางจืดรวมไปถึงกรดโรสมารินิก มีฤทธิ์ในการต้านอนุมูลอิสระและเบาหวาน นอกจากนี้สารสกัดเอทานอลทั้งสองของรางจืดรวมถึงกรดโรสมารินิกไม่พบความเป็นพิษต่อเซลล์มะเร็ง และเซลล์ปกติ เมื่อเทียบกับเกณฑ์มาตรฐาน อย่างไรก็ตามการทดสอบด้วยวิธีโคเมทแสดงให้เห็นว่าสารทดสอบทั้งสามสร้างความเสียหายต่อดีเอ็นเอในเซลล์เม็ดเลือดขาวของมนุษย์เมื่อเพิ่มความเข้มข้น

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5779056353 : MAJOR PUBLIC HEALTH SCIENCES

KEYWORD: THUNBERGIA LAURIFOLIA, PERILLA FRUTESCENS, ROSMARINIC ACID, PHARMACOGNOSTIC SPECIFICATION, IN VITRO BIOLOGICAL ACTIVITY

Parichart Hongsing : STANDARDIZATION OF LEAF AND STEM OF *THUNBERGIA LAURIFOLIA* AND QUANTITATIVE ANALYSIS OF ROSMARINIC ACID IN SELECTED THAI MEDICINAL PLANTS. Advisor: Assoc. Prof. Chanida Palanuvej, Ph.D. Co-advisor: Assoc. Prof. Nijsiri Ruangrungsi, Ph.D.

Rang-Jued or *Thunbergia laurifolia* Lindl. is a species in family Thunbergiaceae. In Thailand, leaf and stem of *T. laurifolia* are used to treat fever, inflammation, as well as antidotes. *Perilla frutescens* (L.) Britton or Nga-Kee-Mon belongs to Labiatae family. The leaf of *P. frutescens* is used to treat allergy, fever and inflammation. Rosmarinic acid is the active compound in both plants which was used as a standard marker in this study. Quantification of rosmarinic acid in the three samples using TLC-densitometry and TLC-image analysis were not statistically significantly different ($P > 0.05$). The pharmacognostic specification of *T. laurifolia* leaf and stem including macroscopic evaluation, microscopic evaluation, physico-chemical parameters and chemical fingerprints were established in order to provide the quality control for medicinal plant as per World Health Organization recommended. *In vitro* biological activities indicated that the ethanolic extracts of *T. laurifolia* leaf and stem as well as rosmarinic acid had antioxidant and anti-diabetic potentials. Furthermore, both *T. laurifolia* ethanolic extracts and rosmarinic acid showed non-toxicity to cancer and normal cell lines according to standard criteria. However, the comet assay showed that the three test samples exhibited DNA damage in human lymphocytes which were proportional to the concentration.

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

Academic Year: 2018 Advisor's Signature .....

Co-advisor's Signature .....

ACKNOWLEDGEMENTS

The author wishes to express her deepest gratitude and appreciation to her beloved thesis advisor, Associate Professor Dr. Chanida Palanuvej and her thesis co-advisor, Associate Professor Dr. Nijsiri Ruangrungsi, for their valuable suggestion, guidance, encouragement and support with kindness throughout this study.

Gratitude is grateful to the thesis committee members, Assistant Professor Dr. Naowarat Kanchanakhan, Assistant Professor Dr. Piyanuch Thongphasuk, Dr. Tepanata Pumpaibool and Dr. Onuma Zongram for their valuable suggestions and advices to improve this thesis.

The author is thankful to “The 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship” and “Overseas Academic Presentation Scholarship for Graduate Students” for scholarship support. Appreciation is also extended to Ms. Napaporn Siripoon, Mr. Somchai Issaravanich, Dr. Woratouch Thitikornpong and the other staff members whose names have not been mentioned here in Chulalongkorn University for providing research facilities and their kind assistances throughout this study.

Last but not the least, the author is indebted to Mrs. Nuanjion Hongsing and Mr Pramai Hongsing, her parents for their supporting, understanding and encouraging to dedicate on her study. The author would like to express her deep gratitude and dedicate this degree to them.

Parichart Hongsing

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

%RSD	the percentage of relative standard deviation
µg	microgram
µM	micromolar
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AOAC	association of analytical communities
$A\beta_{1-42}$	1-42 peptides of amyloid beta-protein
$A\beta_{25-35}$	25-35 peptides of amyloid beta-protein
BHT	3, 5-Di-*tert*-4-butylhydroxytoluene
CAT	catalase activity
CE	*catechin equivalent*
CFU	colony-forming unit
cm	centimeter
DCF	2',7'-dichlorodihydrofluoescein
$DCFH_2$-DA	2',7'-Dichlorodihydrofluoresceindiacetate
	or 2',7'-dichlorofluorescin diacetate
DCFH-DA	2', 7'-dicchlorodihydrofluorescein diacetate

DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
FIC	ferrous ion chelating
FRAP	ferric reducing antioxidant power
GAE	*gallic acid* equivalent
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSH-Px	glutathione peroxidase
HMC-1	human leukemic mast cell line
HPLC-DAD	high-performance *liquid chromatography* with diode-array detection
IC_{50}	half maximal inhibition concentration
ICH	International Conference on Harmonisation
l	liter

LOD	limit of detection
LOQ	limitation of quantitation
m	meter
MDA	malondialdehyde
mg	milligram
ml	milliliter
mm	millimeter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIH	the National Institutes of Health
nm	nanometer
^{o}C	Celsius degree
PBS	*phosphate-buffered* saline
PC12	rat adrenal phenochromocytoma cells
R^2	coefficient of determination
R_f	retention factor
RNS	reactive nitrogen species

ROS	reactive oxygen species
RSD	relative standard deviation
SCGE	single cell gel electrophoresis
SD	standard deviation
TE	*trolox* equivalent
TEAC	total antioxidant capacity
TLC	thin-layer chromatography
TNF-α	tumor necrosis factor-*alpha*
TPC	total phenolic content
TPTZ	2, 4, 6-tripyridyl-s- triazine
Tris	*tris*(hydroxymethyl)aminomethane
UV	ultraviolet
VIS	visible light
WHO	World Health Organization

CHAPTER I
INTRODUCTION

Background and rationale

Plants and natural products are majority sources of both food and medicine for human basic needs. In fact, up to fifty percent of the world's most popular pharmaceutical drugs are based on chemical first came from natural sources (Patwardhan, 2007). The use of herbal medicine has also been globally recommended and promoted in national primary health care programs in many countries, especially in Asia due to the ease of use, accessibility and low cost (ASEAN Task Force on Traditional Medicine, 2014). Moreover, the use of herbal medicine has expanded remarkably as many herbal products continues to grow and introduce to the global market with the influence of the natural therapy health trend (Sharma, 2015). Although various herbal medicines have been used in traditional medicine for a long time, many of them still remain untested and the use also not monitored scientifically. Furthermore, some adverse events were detected due to the misidentification or mislabeling of the medicinal plants. According to World Health Organization, these aforementioned issues have become essential to provide the protection for people consuming the medicinal plants worldwide. Therefore, the assessment of quality control, safety and efficacy of medicinal plants must be provided (World Health Organization, 2011). In addition, the secondary metabolites

are abundantly in medicinal plants and are suggested to be used as the chemical marker, a tool for assuring the quality control (S. Li et al., 2008).

Thunbergia laurifolia Lindl., belonging to Thunbergiaceae family, is known in common name as Laurel clock vine or blue trumpet vine (English) and Rang Jued (Thai) (Scotland & Vollesen, 2000). This climber plant indigenous to Southeast Asia, but today it can be found in many parts of the world (Kosia, Jiraungkoorskul, & Jiraungkoorskul, 2015). In traditional Thai medicine, the plant is prescribed as antipyretic, antidote, anti-inflammatory and relief of heart-burn and quench (Phrommani & Uthitchalanon, 1973; Wuttithammawet, 2005). The use of juice obtained from crushed fresh leaves has been reported in Malaysia to treat menorrhagia, deafness and healing the wound from cuts and boils (Chan & Lim, 2006). Numerous studies have been reported on the chemical constituents from *Thunbergia laurifolia,* including steroids, phenolics and glycosides leading to many investigations of its biological activities. Unsurprisingly, several biological studies related to therapeutic effects and chemical constituents have been recently reported for *Thunbergia laurifolia*, including antioxidant (Sinsawat, Koomklang, & Sinsawat, 2013), antimicrobial (Ruksounjik & Khunkitti, 2016), antiproliferative (Jetawattana, Boonsirichai, Charoen, & Martin, 2015), antimutagenicity (Saenphet, Kantaoop, Saenphet, & Aritajat, 2005), hepatoprotective (Pramyothin, Chirdchupunsare, Rungsipipat, & Chaichantipyuth, 2005), detoxifying effect (Chattaviriya, Morkmek, Lertprasertsuke, & Ruangyuttikarn, 2010), anti-diabetic

(Aritajat, Wutteerapol, & Saenphet, 2004), anti-inflammatory and antinoceptive activates (Boonyarikpunchai, Sukrong, & Towiwat, 2014). However, the aforementioned studies are only emphasize on the investigation of *Thunbergia laurifolia* leaf. Hence, the biological study of *Thunbergia laurifolia* stem is also needed to be provided for the quality control.

Perilla frutescens (L.) Britton, belonging to Lamiaceae family, is known in common name as Perilla or beefsteak plant (English) and Nga-Kee-Mon (Thai) (Britton, 1894). The plant is native to South Asia and South East Asia, and is widely cultivated at the northern part of Thailand (S. K. Singh et al., 2017). The evidence record in Song Dynasty book revealed the utility of *Perilla frutescens* as the traditional medicine for a thousand of years. Perilla leaf has been used for many medicinal purposes, for instant, common cold, allergy and inflammation (Bachheti, Archana, & Tofik, 2014; Ravindran, 2017). In Thailand, the leaf and shoot of *Perilla frutescens* are prescribed to treat common cold, cough and dyspepsia (Chuakul, 1996). Previous phytochemical studies reported various chemical constituents in the plant, for example, phenolics, flavonoids and triterpenes. Many study of *Perilla frutescens* leaf revealed biological activities similar to that of *Thunbergia laurifolia*, including antioxidant (Zhu et al., 2014), antimicrobial (D. Kim, Kim, & Choi, 2011), anti-diabetic (D. H. Kim, Kim, Yu, Jeong, & Kim, 2018), antidepressant (Ji et al., 2014), hepatoprotective (S. Y. Yang, Hong, Lee, Kim, & Lee, 2013), anti-allergic and anti-inflammatory activities (Oh, Park, Ahn, Park, & Kim, 2011).

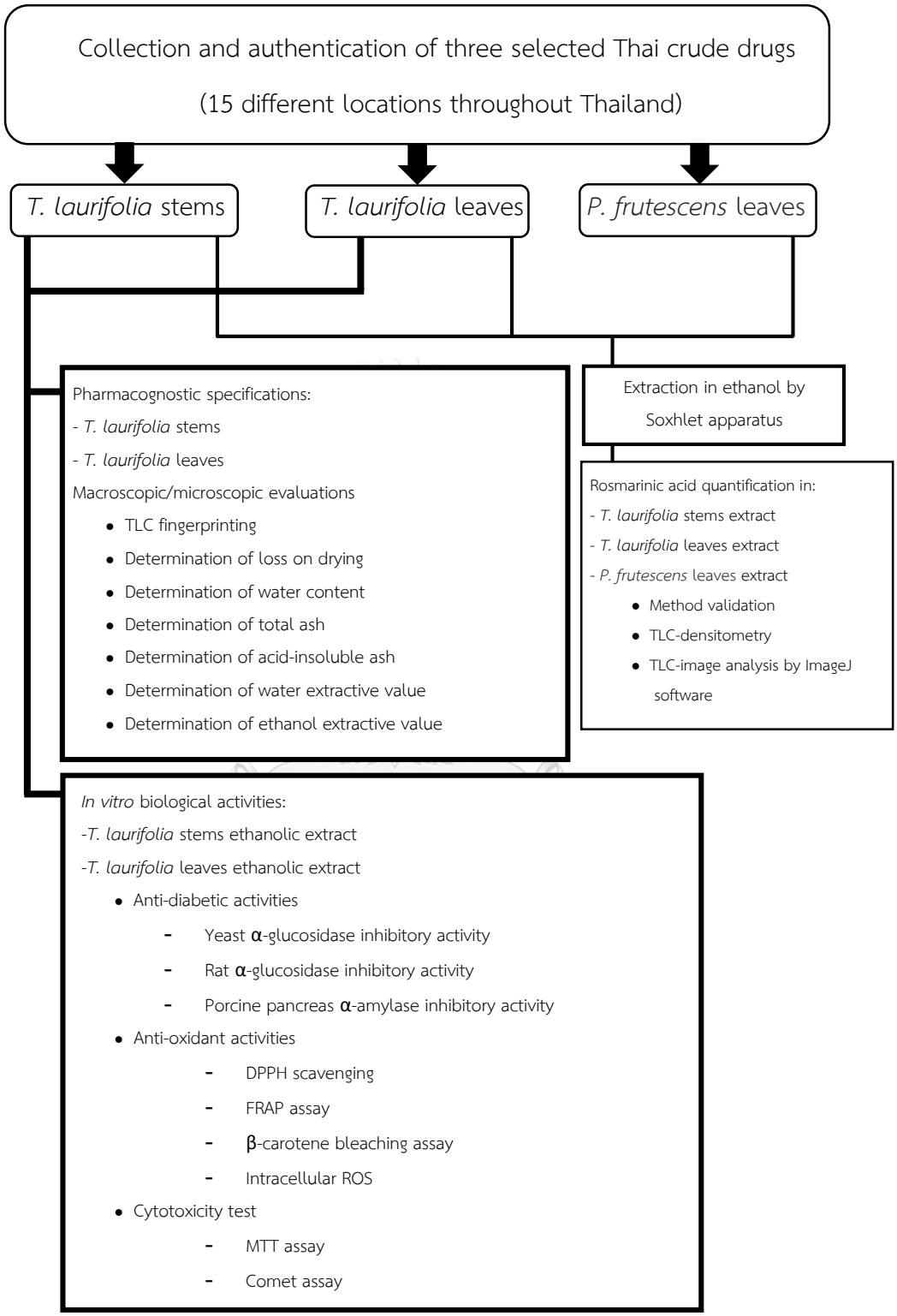
Rosmarinic acid is a phenol ester derived from the combination of caffeic *acid* and 3, 4-dihydroxyphenyllactic *acid*. This natural compound exhibited numerous remarkable pharmacological as well as biological activities which can be found abundantly in *Perilla frutescens* and *Thunbergia laurifolia* (Suwanchaikasem, Chaichantipyuth, & Sukrong, 2014; Zhu et al., 2014). Concerning in its therapeutic effects, many reviews of this compound have been reported in the last decade. (Amoah, Sandjo, Kratz, & Biavatti, 2016; Khojasteh, Mirjalili, Hidalgo, Corchete, & Palazon, 2014; Petersen, 2013).

Regarding to the promising potential of *Thunbergia laurifolia* in Thai traditional medicine, this research tempts to provide the assessment of quality control, safety and efficacy of *Thunbergia laurifolia* leaf and stem. Rosmarinic acid, a marker compound was quantified in *Thunbergia laurifolia* leaf and stem as well as *Perilla frutescens* leaf. Although various analytical methods to quantify rosmarinic acid content in medicinal plants have been reported, there is no report on rosmarinic acid quantification in *Thunbergia laurifolia* stem. Therefore, a quantification of rosmarinic acid content in *Thunbergia laurifolia* stem is in the interest to strengthen the quality of herbal medicine. Additionally, the development of analytical method for rosmarinic acid quantification in three selected herbal materials was investigated using TLC-densitometry and TLC-image analysis due to their simplicity and inexpensiveness.

Objectives of the study

1. To establish the pharmacognostic specification of *Thunbergia laurifolia* leaves and stems in Thailand.
2. To provide the scientific evidence in biological activities of *Thunbergia laurifolia* leaves and stems in Thailand with reference to rosmarinic acid.
3. To evaluate rosmarinic acid contents in *Perilla frutescens* (leaves) and *Thunbergia laurifolia* (leaves and stems) using TLC-densitometry compared to TLC-image analysis.

Conceptual framework



CHAPTER II
LITERATURE REVIEWS

Thunbergia laurifolia Lindl.

Taxonomic hierarchy

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta – Vascular plants

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliosida – Dicotyledons

Subclass: Asteridae

Order: Scrophulariales

Family: Thunbergiaceae

Genus: *Thunbergia*

Species: *Thunbergia laurifolia* Lindl.

Plant description

During the period of *eighteenth to nineteenth* century, Thunbergiaceae is a family of *Thunbergia laurifolia* as shown in Figure 1, which was separated from Acanthaceae due to the absence of cystoliths (Scotland & Vollesen, 2000). The global plant database provides the information of this plant that " Vigorous woody twiner to 25 m or more, forming large tangles and often completely covering large

trees; young branches glabrous or puberulous at nodes. Leaves glossy; petiole 1.5–5 cm long, glabrous; lamina lanceolate to triangular ovate, largest 13–20.5≈4–10.5 cm, apex acuminate, base truncate to cordate, without or with rounded to hastate lobes, margin subentire or with a few large teeth, glabrous, with whitish pustules along major veins above. Flowers in pendulous racemoid cymes to 30 cm long; peduncle to 11 cm long, glabrous, with a pair of leafy sessile bracts to 8≈4.5 cm at base of cyme; pedicels 2–4.5 cm long, glabrous; bracteoles oblong to obovate, 2.5–4≈1–2 cm, subacute to rounded, apiculate, truncate at base, glabrous. Calyx an entire or slightly undulate puberulous rim. Corolla pale mauve to mauve or purple; cylindric tube ± 1 cm long; throat broadly campanulate, 3–4 cm long, 2–3 cm in diameter apically; lobes 3–4≈3–4 cm. Filaments 9–15 and 11–17 mm long, glabrous; anthers narrowly oblong, 7–9 mm long, indistinctly apiculate, bearded at base and almost to apex along one side with long hairs with small lateral spinules; all thecae spurred, spurs 4–5 mm long, flattened. Capsule subglobose, 13–15 mm in diameter, glabrous, beak 25–30 mm long, parallel-sided. Seed 8–12 mm in diameter (Vollesen, 2008).”

Distribution

Thunbergia laurifolia is the plant that native to India, Thailand and Malaysia (Sultana, Chatterjee, Roy, & Chandra, 2015). Additionally, it can be found in South Asia, South East Asia, Eastern Africa and North America (Global Plants, 2018). Currently, this plant can be found in worldwide (Kosia et al., 2015).

Vernacular names

- Laurel clock vine or Blue trumpet vine (English)
- Rang Jued (Thai)
- Kar Tuau (Malay)
- Neel Lata (Hindi)
- Neel lota (Bengali)
- Liane mauve (French).

Synonyms

- *Thunbergia grandiflora* var. *laurifolia* (Lindl.) Benoist
- *Thunbergia harrisii* Hook.f.

Figure 1 Leaves and flowers of *Thunbergia laurifolia* Lindl.

Traditional uses

The principle in Thai traditional pharmacy classifies the taste of drug correlating to their healing properties. From the taste, *Thunbergia laurifolia*, or Rang Jued in Thai is classified as "cool" taste in which the whole plant is prescribed as antipyretic, antidote, anti-inflammatory and relief of heart-burn and quench (Phrommani & Uthitchalanon, 1973; Wuttithammawet, 2005). Fresh or dried leaves are famous for its therapeutic effect as the antidote for poisoning from chemical, food and animal. The dried bark and roots are also used as antidote, antipyretic and anti-inflammatory (Kanchanapoom, Kasai, & Yamasaki, 2002; Thongsaard & Marsden, 2002). According to locally ethno-medicinal uses in Malaysia, the juice of fresh leaves are taken orally for menorrhagia or putting into the ear for deafness. The crushed leaves can be used as a poultice topical application for cuts and boils (Chan & Lim, 2006).

Chemical constituents of *Thunbergia laurifolia*

Plants have been recognized as the important sources of natural medicine due to the chemical constituents containing in the plants. These chemical constituents represent the therapeutic effects and properties of the plant. Previous studies have been reported on the chemical constituents from various parts of *Thunbergia laurifolia* including steroids, phenolics and glycosides (Table 1).

Table 1 Chemical constituents reported of *Thunbergia laurifolia*

Part of plant	Chemical constituent	Reference
Leaf	▪ carotenoid ▪ glycine ▪ methionine ▪ serine ▪ steroids	(Jitpewngam, 1979)
Leaf	▪ 7-stigmasterol ▪ alpha-spinasterol ▪ arabinose ▪ beta-sitosterol ▪ galactose ▪ stigmasterol ▪ glucose ▪ glycine ▪ histidine ▪ methionine ▪ rhamnose ▪ serine ▪ xylose	(Tansuwan, 1983)
Leaf	▪ apigenin ▪ casmosiin ▪ clorogenic acid ▪ delphinidin-3-5-di-O-bata-D-glucoside	(Thongsaard & Marsden, 2002)
Leaf	▪ apigenin ▪ caffeic acid ▪ chlorophyll *a* ▪ chlorophyll *b* ▪ lutein ▪ pheophorbide *a*	(Oonsivilai, Cheng, Bomser, Ferruzzi, & Ningsanond, 2007)

Part of plant	Chemical constituent	Reference
Leaf	■ rosmarinic acid	(Suwanchaikasem et al., 2014)
Aerial part	■ (*E*)-2-hexenyl β-glucopyranoside ■ 3'-*O*-β-glucopyranosyl-stilbericoside ■ 6,8-di-*C*-glucopyranosylapigenin ■ 8-*epi*-grandifloric acid ■ benzyl β-(2' -*O*-β-glucopyranosyl)	(Kanchanapoom et al., 2002)
Aerial part	■ glucopyranoside ■ benzyl β-glucopyranoside ■ grandifloric acid ■ 6-*C*-glucopyranosylapigenin ■ hexanol β-glucopyranoside	(Kanchanapoom et al., 2002)
Flower	■ apigenin ■ apigenin-7-*O*-β-D-glucopyranoside ■ chlorogenic acid ■ delphinidin-3,5-di-*O*-β-D-glucopyranoside	(Pumima & Gupta, 1978)

Pharmacological and biological activities of *Thunbergia laurifolia*

Antioxidant activities

Numerous studies of *Thunbergia laurifolia* have been reported for its antioxidant potential through many *in vitro* and *in vivo* studies. Total phenolic content (TPC) is one of the most popular test to quantitate the antioxidant compound in herbal medicines. Methanolic extract of *Thunbergia laurifolia* fresh leaves showed TPC values of 477 GAE/100 g whereas different drying processes of the leaves using oven, sunlight and microwave reported TPC values of 102, 95 and

624 mg GAE/100 g. The dried leaves of *Thunbergia laurifolia* resulting from microwave drying process were extracted with hot water as normal tea brewing showed approximately 50% of the chelating ability at concentration of 1 mg/ml from ferrous ion chelating (FIC) assay. The TPC assay reported the value of 5170 mg GAE/100g while 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) activities had IC_{50} values of 0.07 mg GAE/ml and 39.5 mg GAE/g (Chan & Lim, 2006). Similar study of *Thunbergia laurifolia* tea from five different brands extracted in hot water reported the DPPH antioxidant activity (EC_{50}), ranging from 0.05- 0.56 mg/ml (Laovitthayanggoon, Charoenkul, Supavilai, & Aramphongphan, 2007).

The study of young, developing and mature leaves of *Thunbergia laurifolia* showed TPC values of 407, 513 and 298 mg GAE/100 g. The differences of collecting times (ranging from 532 to 795 mg GAE/100 g) and locations (ranging from 543 to 892 mg GAE/100 g) were done on TPC assay revealing the variation of phenolic contents in the leaves of *Thunbergia laurifolia* (Chan, Eng, Tan, & Wong, 2011).

Water, ethanol and acetone extracts from the leaves of *Thunbergia laurifolia* were subjected to TPC determination and yielded 24.33, 5.65 and 1.42 µg GAE/ml respectively. The three extracts of *Thunbergia laurifolia* leaves also showed the DPPH antioxidant assay at the EC_{50} values of 0.13, 0.26, 0.61 mg GAE/mL whereas FRAP assay showed the EC_{50} values of 0.93, 0.18, 0.04 mmol/g respectively (Oonsivilai, 2006; Oonsivilai, Ferruzzi, & Ningsanond, 2008).

Another TPC assay of hot water, ethanol and acetone extracts from *Thunbergia laurifolia* leaves reported the values of 2,634.87, 305.24 and 81.58 mg GAE /100 g respectively. Moreover, water extract of the leaves were done in three antioxidant activities including 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), DPPH and FRAP assays. It was found that the water extract showed the highest antioxidant activities at the IC_{50} of 3.920 mg/ml, 1.598 mg/ml and 0.254 mmol Fe^{2+}/g respectively (Oonsivilai, Oonmetta-area, & Singthong, 2011). Another study of ethanolic and water extracts of *Thunbergia laurifolia* leaves were done in DPPH assay (EC_{50} = 199.97 and 86.04 µg/ml), FRAP assay (155.05 and 148.41 µM TE/g dry weight) and TPC determination (26.54 and 35.84 mg CE/g dry weight) (Suwanchaikasem, 2011).

Water and ethanolic extracts of *Thunbergia laurifolia* leaves were subjected to evaluate antioxidant properties using ABTS assay (30.83 and 33.87 mg TE/g extracts) and DPPH assay (6.71 and 9.64 mg GAE/g extracts). Phenolic content in water extract (22.18 mg GAE/g extract) was lower than the ethanolic extract (33.13 mg GAE/g extract) (Pukumpuang, Thongwai, & Tragoolpua, 2012).

Fresh and dried leaves of *Thunbergia laurifolia* were used to evaluate the phenolic compound, flavonoid and antioxidant activity. It was found that ethanolic extracts yielded the highest amount of phenolic compound. The ethanolic fresh leaves extract (57.35 mg/ 100 g crude drug) had higher phenolic content than that of the ethanolic dried leaves extract (45.65 mg/ 100 g crude drug). The suitable

temperature and time for ethanolic extraction of the fresh leaves and dried leaves to quantitate phenolic compound was 90 °C and 4 hours. For flavonoids content, the leaves were extracted in ethanol at 90 °C for 6 hours yielded the highest amount of flavonoids in both fresh leaves (8.19 mg/ 100 g crude drug) and dried leaves (4.18 mg/ 100 g crude drug). For DPPH activity, ethanolic extracts at 90 °C for 4 hours of fresh leaves (94.60%) showed higher DPPH radical scavenging activity than that of dried leaves (92.94%) (Sinsawat et al., 2013). Similar test on DPPH activity of isolated rosmarinic acid compound from *Thunbergia laurifolia* leaves (EC_{50} = 2.71 µg/ml) had higher antioxidant activity comparing to trolox (EC_{50} = 3.51 µg/ml) but lower than that of quercetin (EC_{50} = 0.62 µg/ml) (Suwanchaikasem et al., 2014).

Water extract of *Thunbergia laurifolia* leaves at the concentrations of 100 and 200 mg/kg body weight, loading dose at 1g/L in drinking water for 8 weeks was treated to the mice with the condition as lead-induced oxidative stress level in the brain. The results showed significant elevation of glutathione peroxidase (GSH-Px) activity which is the antioxidant defense enzyme in the body. In contrast, the level of malondialdehyde (MDA) was reduced revealing the ability to decrease the lipid peroxidation in the brain and plasma of the lead-induced mice. Moreover, this study also reported the high level of TPC value (684.30 mg of GAE/ml) and total antioxidant capacity value (31.20 TEAC, mmol/ml) from the water extract of the leaves (Tangpong & Satarug, 2010).

Ethanolic extract of *Thunbergia laurifolia* leaves mixing with fish food was used to treat the lead nitrate-induced *Oreochromis niloticus* fish showing the reduction of a lipid peroxidation biomarker, MDA. On the other hand, the increasing of glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx) and catalase activity (CAT) revealed the ability of the leaves extract in the fish food to increase the intrinsic antioxidant against lipid peroxidation causing from lead toxicity (Palipoch et al., 2011).

Antimicrobial activities

The ethanolic extract of *Thunbergia laurifolia* leaves were analyzed for antimicrobial activity. The results showed that the leaf extracts were active against all pathogens giving the inhibition zone of 17.0, 12.8, 13.8 and 11.8 mm against *Staphylococcus aureus* DMST 2658, *Staphylococcus epidermidis* DMST 12853, *Bacillus subtilis* DMST 15896 and *Pityrosporum ovale* ATCC 64061, respectively (Ruksounjik & Khunkitti, 2016).

The endophytic actinomycetes (TL-19) isolated from *Thunbergia laurifolia* leaves exhibited antimicrobial activity. The results showed the inhibition zone from isolated TL-19 against *Candida albicans* ATCC90028 (2.03 mm), *Staphylococcus aureus* ATCC25932 (2.39 mm), *Pseudomonas aeruginosa* ATCC27853 (1.39 mm), *Alternaria porri* (0.40 mm), *Colletotrichum gloeosporioides* (1.47 mm), *Colletotrichum musae* (2.10 mm), *Curvularia* sp. (0.37 mm), *Drechslera* sp. (0.93 mm), *Exserohilum*

sp. (1.37 mm), *Fusarium oxysporum* (0.57) and *Verticillium sp.*(0.93 mm) (Chanaphant, 2010).

Antiproliferative activities

The aqueous extract of *Thunbergia laurifolia* fresh and dried leaves were used to evaluate the mutagenicity in male rats that induced by oral administration of the extract from *Pueraria mirifica* Airy Shaw & Suvatabundhu (600-800 mg/kg) resulting in polychromatic erythrocytes damage in rats. It was found that the combination between *Pueraria mirifica* root extract and *Thunbergia laurifolia* dried leaves extract in the proportion of 7:3 exhibited the highest effect of micronuclei reduction percentage at 99.08% (Saenphet et al., 2005).

The whole aerial parts of *Thunbergia laurifolia* was extracted in hot water and subjected to investigate for the antiproliferative activity in MCF-7 cells (human breast adenocarcinoma). The concentration dependent manner when cells were treated with the aqueous extract for a day was reported with the IC_{50} value of 843 µg/ml (Jetawattana et al., 2015).

Hepatoprotective activities

The investigation of ethanol induced liver injury of mice before receiving *Thunbergia laurifolia* leave aqueous extract at 200 mg/kg body weight showed the ability to reduce the activities of glutamic oxaloacetic transaminase at 1.3 fold, glutamic pyruvic transaminase at 1.9 fold and liver triglyceride at 1.4 fold comparing with mice treated with ethanol alone. Additionally, the leaf extract also revealed the

hepatoprotective effect by decreasing the hepatic lipid peroxidation (11.10%) and blood ethanol concentration (18.95%). Moreover, the elevation of hepatic alcohol dehydrogenase at 142.15% and aldehyde dehydrogenase at 187.09% also indicated the hepatoprotective effect (Chahawirat, 2000).

The *in vitro* study of rat hepatocyte as primary cell culture was used to evaluate the hepatoprotective effect from *Thunbergia laurifolia* leaf aqueous extract. The cells were treated with ethanol as hepatotoxic. It was found that the aqueous extract at the concentration of 2.5 and 5.0 mg/ml could promote the liver cell recovery and reduction of alanine aminotransferase and aspartate aminotransferase resulting from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The *in vivo* study of ethanol-treated rats also showed the ability of the leaf aqueous extract at concentration of 25 mg/kg body weight to normalize the levels of hepatic triglyceride, alanine aminotransferase and asparate aminotransferase in the rat's liver (Pramyothin et al., 2005).

Detoxifying effects

Hot water was used to extract *Thunbergia laurifolia* leaf for the *in vivo* study of dopaminergic neurotransmission with amphetamine in rat model. The results showed that the leaf extract (0.1g/ml) increased the release of K^+-stimulated dopamine in the same manner of amphetamine in the rat brain (striatum) by HPLC with electrochemical detection. It is possible that *Thunbergia laurifolia* leaves may

exerted its therapeutic properties for withdrawal and addiction (Thongsaard & Marsden, 2002).

The follow-up of previous study was conducted to investigate whether the methanolic extract of *Thunbergia laurifolia* leaves could alter neuronal activity in specific brain regions in rat by determining from the functional nuclear magnetic resonance imaging. The study reported that the methanolic extract increased signal intensity in many brain areas of the rats which related to the previous reports of amphetamine or cocaine administration. However, the study of addiction after consuming the plant, *Thunbergia laurifolia* leaves remains to be confirmed (Thongsaard, Marsden, Morris, Prior, & Shah, 2005).

The co-treatment of the rat receiving lead in drinking water together with *Thunbergia laurifolia* leaves extract at 100 or 200 mg/kg body weigh once a day could reduce the catalytic activity of caspase-3 resulting in prevention of neuronal cell death and memory loss in the lead-induced rats (Tangpong & Satarug, 2010). The lead toxicity was also done on Nile Tilapia fish (*Oreochromis niloticus*) receiving lead with food supplement containing *Thunbergia laurifolia* leaves extract. The food supplement containing 0.2 mg of the extract / g of fish food exhibited the reduction of lead concentration in muscle and liver of the fish. The improvement of blood chemistry, growth performance, histology and hematology were detected in the fish (Palipoch et al., 2011).

The effect of aqueous leaf extract of *Thunbergia laurifolia* for detoxifying effect on cadmium-induced rats has been reported. Before injection of cadmium chloride solution for 20 days (1.0 mg/kg body weigh), the rats were fed with drinking water containing the leaf extract at concentration of 0.1 mg/ml. It was found that the body weight of the rats receiving the extract were higher than that of rats given with cadmium alone. The study reported that the urine and blood of the rats receiving the leaf extract could not reduce the level of the cadmium. However, the histological study indicated no structural change or damage of kidney in the rats receiving the leaf extract comparing with the rat given only cadmium alone (Chattaviriya et al., 2010).

Antinociceptive and anti-inflammatory effects

Hexane and alcohol extracts of *Thunbergia laurifolia* leaves possessed the ability to reduce the swelling of mice paw by carrageenan-induced paw edema (Charumanee et al., 1998). The ethanolic extract of *Thunbergia laurifolia* leaves were used for rosmarinic acid isolation. The mice receiving isolated rosmarinic acid at dose 100 mg/ kg body weigh significantly increased the hot-plate latency comparing to the control and significantly decreased the analgesic response. In acetic acid-induced writhing test, at dose 100 mg/ kg body weight of isolated rosmarinic acid exhibited 85% inhibition of writhing. At the same dose of isolated rosmarinic acid (100 mg/ kg body weigh) significantly reduced licking time of early and late phases in formalin induced nociception test. The study indicated that the mice treated with isolated

rosmarinic acid solution possessed antinociceptive effect. Cotton pellet-induced granuloma formation and carrageenan-induced paw edema were used to determine anti-inflammatory effects. Isolated rosmarinic acid at100 mg/ kg body weight exhibited the similar inhibitory action of that of the standard anti-inflammatory agent (indomethacin). Moreover, isolated rosmarinic acid (100 mg/ kg body weigh) also showed the significantly suppression of carrageenan-induced paw edema after carrageenan injection. Therefore, rosmarinic acid isolated from *Thunbergia laurifolia* leaves possessed the anti-inflammatory effect (Boonyarikpunchai et al., 2014).

Anti-diabetic effects

The aqueous extract of *Thunbergia laurifolia* leaves, loading dose at 60 mg/ml/day for 15 days showed the ability to decrease blood glucose level in alloxan induced diabetic rats. The histological study revealed the recovery of some β-cell structure in the pancreas of diabetic rats (Aritajat et al., 2004). Similar study of diabetic animal model was done in diabetic cats. The result showed that the aqueous extract of *Thunbergia laurifolia* leaves, loading dose at 500 mg/kg/day for 28 days could reduce the blood glucose level in diabetic cats (Pitoolpong, Kanthawat, Thaiprodist, & Singh, 2014).

The culinary of Thai food for diabetes patients was investigated for diabetes risk reduction. Roasted *Thunbergia laurifolia* leaves (10 min roasting time) as a healthy vegetable were added to chili paste and then the paste was extracted with 50% methanol in water and evaporated to dryness. The extract was dissolved in

dimethyl sulfoxide (*DMSO)* (10 ml/g dry weight) showing 99.05% of porcine pancreatic **α**-amylase inhibitory (Jaiboon, Boonyanuphap, Suwansri, Ratanatraiwong, & Hansawasdi, 2011).

***Perilla frutescens* (L.) Britton**

Taxonomic hierarchy

Kingdom: Plantae – Plants

Subkingdom: Viridiplantae

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliosida – Dicotyledons

Subclass: Asteridae

Order: Lamiales

Family: Labiatae

Genus: *Perilla*

Species: *Perilla frutescens* (L.) Britton

Plant description

The flora of China provides the information of this plant that " Herbs erect. Stems 0.3-2 m tall, green or purple, finely pilose or densely villous. Petiole 3-5 cm; leaf blade broadly ovate to circular, 4.5-13 × 2.8-10 cm, green, purplish, or purple-black, pilose or adaxially pilose, abaxially appressed villous, base rounded to broadly cuneate, margin narrowly to coarsely serrate, apex short acuminate or mucronate. Verticillasters 1.5-15 cm, densely villous; bracts ca. 4 × 4 mm, short acuminate, red-brown glandular. Pedicel ca. 1.5 mm, densely villous. Calyx ca. 3 mm, erect, base villous, yellow glandular, lower lip longer than upper lip; fruiting calyx 4-11 mm, base

villous or pilose, glandular. Corolla 3-4 mm, slightly puberulent, tube 2-2.5 mm. Nutlets gray-brown or tawny, 1-1.5 mm in diameter (Britton, 1894).”

Distribution

Perilla frutescens is native to India and China. The plant can be found in South Asia and South East Asia. Additionally, this plant are cultivated mainly in Korea and Japan because of the highly consumption of the leaves and seeds. (S. K. Singh et al., 2017). In Thailand, the plant is widely cultivated at the northern part as food and medicinal herb as shown in Figure 2.

Vernacular names

- Perilla (English)
- Zisu (Chinese)
- Egoma or Shiso (Japanese)
- Kaennip (Korean)
- Nga-Kee-Mon (Thai)

Synonyms

- Melissa maxima Ard.
- Perilla avium Dunn
- Mentha perilloides Lam.
- Perilla ocymoides L.
- Ocimum frutescens L.

Figure 2 Cultivation and leaves of *Perilla frutescens* (L.) Britton in Thailand

Traditional uses

Perilla frutescens has a long history of being used as traditional medicine in China and many countries in Asia. The record of Song Dynasty book "Taiping Huimin Hejiju Fang" revealed the use of *Perilla frutescens* in many traditional medicine. Each part of the plant is prescribed for different condition of sickness. The leaf of *Perilla frutescens* is prescribed for common cold, fever, cough, digestive difficulty, lung complication, gastro-enteritis, allergy, balancing incorrect energy and counteract poisoning or allergic with fish and crab consumption. The stem of *Perilla frutescens* is used in the remedy for morning sickness. The medicinal remedies containing *Perilla frutescens* seed are prescribed for asthma and common cold. In Japanese traditional medicine, *Perilla frutescens* is prescribed for allergy and inflammation (Bachheti et al., 2014; Ravindran, 2017). In northern Thailand, the seed of *Perilla frutescens* is prescribed as muscle tonic. The seed oil mixing with root oil of *Zingiber montanum* is applied for massage to relieve muscle pain. Leaf and shoot of *Perilla frutescens* are used to treat common cold, cough and dyspepsia (Chuakul, 1996).

Chemical constituents of *Perilla frutescens*

Previous studies have been reported on the chemical constituents from various parts of *Perilla frutescens* including phenolics, flavonoids, triterpenes, polycosanols, tocopherols, phytosterols, fatty acids and volatile oil as summarized in Table 2.

Table 2 Chemical constituents reported of *Perilla frutescens*

Part of plant	Chemical constituent	Reference
Leaf	■ 1-octen-3-ol ■ 3-hexen-1-ol ■ 3-octanol ■ beta-pinene ■ limonene ■ *p*-cymene ■ *p*-cymenene ■ alpha-ionone	(Nabeta, Ohnishi, Hirose, & Sugisawa, 1983)
Leaf	■ benzaldehyde ■ beta-elemene ■ caryophyllene ■ copaene ■ eugenol ■ isoegomaketone ■ linalool ■ nyristicin ■ perillaketone	(Nabeta et al., 1983)
Unknown	■ oxalic acid	(Ogawa, Takahashi, & Kitagawa, 1984)
Leaf	■ dillapiole ■ elemicin ■ elsholtziaketone ■ isoegomaketone ■ limonene ■ myristicin ■ naginataketone ■ perillaketone ■ perillaldehyde	(Koezuka, Honda, & Tabata, 1986)

Part of plant	Chemical constituent	Reference
Leaf	■ limonene ■ perillyl alcohol ■ *trans*-carveol ■ *trans*-isopiperitenol	(Karp, Mihaliak, Harris, & Croteau, 1990)
Leaf	■ perilloside A	(Fujita & Nakayama, 1992)
Leaf	■ perilloside B ■ perilloside C ■ perilloside D	(Fujita & Nakayama, 1993)
Leaf	■ perilloside E	(Fujita, Funayoshi, & Nakayama, 1994)
Leaf	■ 3,4-dihydroxybenzaldehyde ■ 6,7-dihydroxycoumarin ■ caffeic acid ■ methyl 3,4-dihydroxy-benzoate ■ methyl caffeate, 3',4',5,7-tetrahydroxy-flavone ■ rosmarinic acid ■ *trans-p*-menth-8-en-7-yl caffeate ■ vinyl caffeate	(Tada, Matsumoto, Yamaguchi, & Chiba, 1996)
Unknown	■ alpha-pinene ■ citral ■ elsholtziaketone ■ isoamyl-3-furylketone ■ limonene ■ naginataketone ■ perillaketone ■ perillaldehyde ■ perillene	(Fujita & Nakayama, 1997)

Part of plant	Chemical constituent	Reference
Aerial part	■ 1-octen-3-ol ■ camphene ■ elsholtziaketone ■ perillaldehyde ■ perillyl alcohol ■ pinene	(Fujita & Nakayama, 1997)
Aerial part	■ allofarnesene ■ alpha-farnesene ■ beta-caryophyllene ■ *cis*-shisool ■ dillapiole ■ elemicin ■ isoegomaketone ■ limonene ■ linalool ■ myristicin ■ naginataketone ■ perillaketone ■ perillaldehyde ■ perillyl alcohol ■ rosefuran ■ *trans*-shisool	
Fruit	■ beta-caryophyllene ■ carvone ■ elemicin ■ perillaldehyde ■ perillyl alcohol ■ phenethyl alcohol	

Part of plant	Chemical constituent	Reference
Leaf	■ 9-*cis*-beta-carotene ■ alpha-tocopherol ■ ascorbic acid ■ gamma-tocopherol ■ lutein ■ *trans*-beta-carotene	(L. Li et al., 2009)

Pharmacological and biological activities of *Perilla frutescens*

Antioxidant activities

Many phytochemical studies indicated that *Perilla frutescens* contained various natural antioxidant compounds, for instant, phenolics and flavonoids. Therefore, numerous antioxidant activities have been investigated in this plant.

Perilla frutescens leaves were extracted by acidified methanol including methanol: glacial acetatic acid: water (50:3.7:46.3) to obtain hydrophilic components. The lipophilic components were extracted by methanol followed by tetrahydrofuran. The combination of hydrophilic and lipophilic components were also extracted by acidified methanol and tetrahydrofuran. Two main antioxidant activities in this study were lipophilic and hydrophilic assays. For lipophilic assay, 2,2′-azobis (4-methoxiy-2,4-dimethylvaleronitrile) was used as a lipid soluble radical initiator along with 4,4-difluoro-5-(4-phenyl1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid as a lipophilic fluorescence probe. The results indicated that lipophilic components extracted from *Perilla frutescens* leaves had 84.02 ± 2.91 % inhibition of lipophilic

oxidation. For hydrophilic assay 2,2′- azobis-(2-amidinopropane) dihydrochloride was used as the radical generator and 2′,7′-dichlorodihydrofluorescein as the probe. It was found that hydrophilic components extracted from *Perilla frutescens* leaves had 78.93±4.3691 % inhibition of the hydrophilic oxidation. Finally, the combination of both components yielded 90.63±2.32 % inhibition of the hydrophilic oxidation and 82.18±2.03 % inhibition of the lipophilic oxidation. Therefore, the highest antioxidant potential was in the combination of both components extracted from *Perilla frutescens* leaves (L. Li et al., 2009).

Five grams of *Perilla frutescens* leaves were found to contain 5 mg lutein. Twelve healthy volunteers ingested powder of *Perilla frutescens* leaves 5g/day for 10 days, then the blood was collected for antioxidant activities. It was found that the MDA, a marker of lipid peroxidation in the plasma blood of the volunteers was decreased, thus the plant can help to reduce the lipid peroxidation in the body (Schirrmacher, Skurk, Hauner, & Grabmann, 2010).

Dried leaves of *Perilla frutescens* were extracted with water, ethanol and methanol. Then the obtained extracts were dissolved in water and partitioned with hexane, chloroform, ethyl acetate and butanol. It was found that ethyl acetate fraction from all extracts exhibited the highest values of total phenolic and flavonoid contents. Rosmarinic acid content in all extracts was investigated using HPLC method. The results showed that ethyl acetate fraction from methanol contained the highest content of rosmarinic acid (155.50 ± 5.20 mg/g fraction). Moreover, ethyl

acetate fractions from water extract at 0.5 mg fraction/ml yielded the highest values in DPPH free radical scavenging activity (90.74%), while ethyl acetate fractions from methanol extract at 0.5 mg fraction/ml had the strongest reducing power of 1.656 as determined by the potassium ferricyanide reduction method. Chloroform fraction from ethanol extract exhibited the highest antioxidant activity (36.78%) on beta-carotene bleaching assay using linoleic acid hydroperoxides to attack the beta-carotene molecule (Hong, Park, & Kim, 2010).

Aerial part of *Perilla frutescens* cv. Chookyoupjaso mutant was subjected to determine its phytochemical compounds using HPLC analysis. No new compound was investigated in this study; however, most of the phytochemical compounds in this plant were the same as in *Perilla frutescens*. Thus, this plant may have antioxidant effect. Aqueous extract of this plant were investigated for cytoprotective effect and reactive oxygen species (ROS) scavenging activity in human hepatoma HepG2 cells using 5-(and-6)-carboxy-2'7'-dichlorodihydrofluorescein diacetate. The production of ROS in the cells was induced by 3-morpholinosydnonimine (SIN-1). At the concentration of 100 µg/ml of the extract showed 50% reduction of ROS production in the cells. It was found that the extract significantly decreased the intracellular ROS levels in a concentration-dependent manner. Moreover, the extract also showed cytoprotective effect by increasing cell viability. Carbon tetrachloride (CCl_4) was used to induce oxidative damage in the liver of BALB/c mice. After the CCl_4-induced mice received the aqueous extract, lipid peroxidation and serum

alkaline phosphatase activity were decreased as evidenced by recovering the antioxidants enzyme activities. Therefore, the aqueous extract also had protective effect against oxidative damage in liver (Cho et al., 2011).

Methanolic extract of *Perilla frutescens* leaves was subjected to the 12-*O*-tetradecanoylphorbol-13-acetate induced-superoxide generation assay using human promyelocytic leukemia cell line (HL-60). The results were found to be dose-dependent manner of the test samples including the extract (IC_{50} = 21 μM), rosmarinic acid (IC_{50} = 29 μM) and caffeic acid (IC_{50} = 30 μM) (Takahashi et al., 2011).

Aqueous extract of *Perilla frutescens* leaves showed antioxidant activity by DPPH antioxidant assay at the IC_{50} values of 29 mg/ml while the total polyphenol content assay reported the value of 1.7 mg/ml. The inhibition effects of *Perilla frutescens* leaves extract on low-density lipoprotein oxidation and antioxidant enzyme expression using human umbilical vein endothelial cells were reported. The LDL oxidation assay reported significantly prolonged oxidation lag time of the extract at 51min comparing to the control (24 min). Additionally, the cells treated with the extract also showed the increasing of the antioxidant enzymes including catalase and Cu-Zn-superoxide dismutase (Saita et al., 2012).

The nuclear factor erythroid 2-related factor 2-antioxidant response element (Nrf2-ARE) pathway works on controlling the antioxidant enzymes against oxidative stress in the body. Rat adrenal phenochromocytoma (PC12) cells were treated with ethanol and ether extracts of *Perilla frutescens* leaves showed the elevation of

luciferase activity in a concentration-dependent manner. The isolated 2′,3′-dihydroxyl-4′,6′-dimethoxychalcone from the *Perilla frutescens* leaves (30μM) assisted in the enhancement of antioxidant enzyme induction through cellular defense system *via* this pathway. Additionally, protective action against 6-hydroxydopamine induced cytotoxicity was also found in the isolated compound (Izumi et al., 2012).

Supramolecular formation technique and solvent extraction was used to isolate rosmarinic acid from *Perilla frutescens* leaves. The rosmarinic acid extract showed high total phenolic content of 433.9 μg/mg of GAE and effective DPPH assay (IC_{50} = 5.5 μg/mL) (Zhu et al., 2014).

Antioxidant compound, rosmarinic acid in *Perilla frutescens* leaves was quantified by ultra-high performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry. It was found that rosmarinic acid content was ranging from 6.38 – 903.53 μg/g. The methanolic extract of *Perilla frutescens* leaves also provides the antioxidant effects on DPPH (ranging from 63-86 %inhibition) and ABTS (ranging from 73-90 %inhibition) assays (Y. H. Lee et al., 2017).

The study on antioxidant capacities and the amount of phenolic compound during the growth cycle of *Perilla frutescens* were reported. Two samples including methanolic extract and fresh sample (freeze-dried and ground the whole plant) were subjected to determine total phenolic contents. It was found that methanolic extract (123.2 ± 12.7 mg CE/g extract) and fresh sample (4.02 ± 0.42 mg CE/g fresh matter) at

full flowering stage of *Perilla frutescens* exhibited the highest total phenolic contents. Then, the separation of individual phenolic compounds in methanolic extract and fresh sample was done by using HPLC analysis. Rosmarinic acid was found to be the main compound in both samples. The highest content of rosmarinic acid in methanolic extract was at early flowering stage (66.17 ± 4.90 mg/g extract), while that of fresh sample was at full flowering stage (1.815 ± 0.170 mg/g fresh matter). Moreover, antioxidant capacities by TEAC, FRAP and DPPH radical scavenging activity were found to be the highest values during the early and full flowering stages in both samples (Gai, Peiretti, Karama, & Amarowicz, 2017).

Three main antioxidant compounds were found in *Perilla frutescens* var. *acuta* leaf by HPLC analysis: rosmarinic acid, luteolin-7-*O*-glucuronide, and apigenin-7-*O*-glucuronide. The plant was subjected to intracellular ROS scavenging activity using 2', 7'-dichlorodihydrofluorescein diacetate *(*DCFH-DA*)* on inhibitory effect of oxidative stress induced by hydrogen peroxide (H_2O_2) in mouse myoblast C2C12 cells. The results indicated that the aqueous extract of *Perilla frutescens* var. *acuta* leaf had no toxicity on the cells resulting from MTT assay. Moreover, the aqueous extract showed the potential to inhibit ROS production in H_2O_2-induced oxidative stress muscle cells in a dose-dependent manner. Additionally, this research reported the ability of the leaf aqueous extract to improve visual fatigue in animal model as they mentioned about the food containing antioxidant compound might have accommodative ability and ciliary muscle relaxant effects (J. Kim et al., 2017).

Comet assay is one of genotoxicity assessment which related to antioxidant property of the food containing antioxidant compound by preventing DNA from oxidative injury and damage. The leaf aqueous extracts of two varieties of *Perilla frutescens* (purple and bicolor) were used to determine the antioxidant potential on protecting DNA from oxidative damage. The results from MTT assay indicated that the leaf aqueous extracts had no toxicity to the isolated human peripheral blood lymphocytes. In comet assay, H_2O_2 was used to induce DNA damage in lymphocytes. Both extracts showed the ability to protect DNA damage in a dose dependent manner. The results were expressed in two different ways; DNA damage by tail DNA% and tail moment. At the concentration of 100 µg/ml, the purple leaf aqueous extract and bicolored leaf extract had 89.14 and 88.88 % tail moment, whereas they exhibited 85.37 and 70.60 % inhibition of tail DNA% (Chao et al., 2013).

Anti-allergic and anti-inflammatory activities

Water decoction of *Perilla frutescens* leaves was prepared for the investigation of type I allergic effect using passive cutaneous anaphylaxis reaction in the mice ears. Then the mice were fed with various dose of the leaves decoction for 30 min. Leaf decoction at the dose of 500 mg/ kg body weigh exhibited the highest suppression of this reaction showing 43% of the inhibition. Rosmarinic acid, one of the constituent in Perilla decoction also exhibited the reaction suppression of PCA reaction (41% inhibition) at the dose of 500 mg/ kg body weight (Toshiaki Makino et al., 2001).

Clinical study of *Perilla frutescens* leaves on the inhibition of mild seasonal allergic rhinoconjuctivitis was investigated on human. The leaves were extracted in 1.0% (w/v) citric acid aqueous and then the rosmarinic acid determination was investigated in the extract. The extract containing rosmarinic acid at the concentrations of 50 and 200 mg in the extract were prepared as the supplement. The patients consumed 50 and 200 mg of rosmarinic acid/ day had no adverse events and the treatment also reduced the number of eosinophils and neutrophils which detected in nasal lavage fluid of the patients. Moreover, the patients also reported that their symptoms were relieved (55.6% and 70% of the patients consumed 50 and 200 mg of rosmarinic acid/ day) after consuming *Perilla frutescens* leaves supplement (Hirohisa et al., 2004).

The leaves of *Perilla frutescens* were extracted with 30% ethanol and its constituent, rosmarinic acid were subjected to investigate the anti-allergic effects in allergic rhinitis and rhinoconjunctivitis both *in vivo* and *in vitro* model. It was found that the mice received both test samples before allergic induction by ovalbumin could reduce the number of rubbing nasal, ear and eye. Histamine level in the serum of the allergic mice receiving the test samples also reduced. The level of Immunoglobulin E was reduced as well as protein levels, mRNA expressions of interleukin; IL-1β, IL-6 and tumor necrosis factor-α obtained from nasal mucosa, spleen and serum of the allergic mice receiving both test samples. Caspase-1 activity and cyclooxygenase-2 protein expression were reduced as well as the mast cells and

eosinophil infiltration in the same nasal mucosa tissue of the allergic mice. The human leukemic mast cell line treated with the test samples exhibited the reduction on nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, v-rel avian reticuloendotheliosis viral oncogene homolog A (NF-kB/Rel A) and caspase-1 activation. Therefore, *Perilla frutescens* leaves and its constituent, rosmarinic acid could improve the allergic inflammatory reactions (Oh et al., 2011).

Antimicrobial activities

Perilla frutescens leaves were extracted in 50% of ethanol at 80 ℃ for 26 h exhibited the highest antibacterial activity against *Pseudomonas aeruginosa* using evolutionary operation-factorial design technique. The growth of bacterial population was reduced from 6.660 log CFU/ml to 4.060 log CFU/ml. Moreover, the damage of *Pseudomonas aeruginosa* morphology was observed after treated with ethanolic extract of the leaves (Choi, Lee, Lim, & Kim, 2010).

Similar antibacterial activity of *Perilla frutescens* leaves against *Staphylococcus aureus* ATCC6538 using the same technique exhibited the highest antibacterial activity when the leaves were extracted with 45% of ethanol at 75℃ for 24 h. The growth of bacterial population was reduced from 7.535 log CFU/ml to 4.865 log CFU/ml and the damage in bacterial morphology was observed (D. Kim et al., 2011).

The study of aromatic plant for cooking, *Perilla frutescens* leaves on antimicrobial activity was investigated. It was found that methanolic extract of *Perilla*

frutescens leaves revealed the antimicrobial property against *Salmonella enteritidis* (>6.0 log CFU/ml), *Vibrio parahaemolyticus* (<1.3 log CFU/ml), *Staphylococcus aureus* (1.9 log CFU/ml), *Escherichia coli* O157:H7 (>6.0 log CFU/ml) *and Listeria monocytogenes* (2.8 log CFU/ml) (Hara, Kobayashi, Sugita, & Kondo, 2004).

Anti-diabetic effects

Aqueous and methanolic extracts of *Perilla frutescens* leaves were subjected for anti-diabetic determination using rat intestinal α-glucosidase assay. It was found that both extracts possessed anti-diabetic potential. The IC_{50} values were expressed as mmol catechin equivalents in aqueous (IC_{50} = 0.49) and methanolic (IC_{50} = 0.34) extracts (Mai, Thu, Tien, & Chuyen, 2007).

The isolated compounds from *Perilla frutescens* seed were investigated for their inhibitory activities against yeast α–glucosidase and human aldose reductase. These two enzymes plays the important role in diabetes mellitus as α–glucosidase will breakdown oligosaccharides into glucose, thus increasing the blood glucose level. Aldose reductase will catalyze the reduction of glucose to sorbital leading to the onset of various diabetic complications that related to many organs including eye and kidney. Five compounds were isolated from the plant including caffeic acid-3-O-glucoside, rosmarinic acid-3-O-glucoside, rosmarinic acid, luteolin and apigenin. The ethanolic solutions of the isolated compounds were subjected to yeast α–glucosidase assay and luteolin exhibited the IC_{50} values of 45.5 μM. At the high level (>100 μM) of other compounds did not show the inhibitory effects. Quercetin, a positive control showed IC_{50} value of 26.7 μM. Only

luteolin was subjected to aldose reductase inhibitory activity and showed the IC_{50} value of 0.6 μM. additionally, the ethanolic solution of luteolin was found to be noncompetitive inhibitor for α–glucosidase (Ha et al., 2012).

Perilla frutescens sprout was extract with 40% ethanol for 5 h at 70 °C for the investigation of hypoglycemic effect using animal model. Induced-diabetic mice were treated with the extract at the concentrations of 300 and 1,000 mg/kg body weight for 4 weeks. It was found that the *Perilla frutescens* sprout contained rosmarinic acid as the major constituent (15.24 mg/g) quantitating by HPLC-DAD alnalysis. The results indicated the reduction of serum insulin, fasting blood glucose, total cholesterol and triglyceride levels in induced-diabetic mice consumed the extract. The study also revealed the improvement of insulin sensitivity and glucose intolerance and the reduction of gluconeogenic protein expression in the liver. The histological study indicated that there was no liver damage after the induced-mice consumed the *Perilla frutescens* sprout extract (D. H. Kim et al., 2018).

Perilla frutescens leaves extract and its constituent, rosmarinic acid were subjected to the investigation of anti-diabetic property using α-glucosidase assay. It was found that the rosmarinic acid extracted from (IC_{50} = 0.23 mg/ml) the leaves exhibited the highest α-glucosidase inhibition comparing to *Perilla frutescens* leaves extract (IC_{50} = 0.42 mg/ml) and standard rosmarinic acid (IC_{50} = 0.95 mg/ml) (Zhu et al., 2014).

Antidepressant activities

Animal model using mice were used to determine the antidepressant effect of *Perilla frutescens* leaves oil. Chronic unpredictable mild stress technique including 9 steps for 24 h was used to induce mild stress of mice. Three behavior evaluations were investigated. It was found that the stress-induced mice consumed *Perilla frutescens* leaves oil exhibited the reduction of open-field test, forced swimming test and tail suspension test. The study of mice hippocampus indicated the reduction of monoamine neurotransmitters and their metabolites contents including 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. The blood plasma of stress-induced mice indicated the lowering of serum interleukin; IL-1, IL-6, and tumor necrosis factor-*alpha* (TNF-α) levels (Ji et al., 2014).

Hepatoprotective activities

Cytotoxicity test of aqueous extract, caffeic acid and rosmarinic acid using human hepatoma (HepG2 cells) exhibited the increasing of cell viabilities. The hepatotoxicity indicators including serum enzymes aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase were reduced after giving *Perilla frutescens* leaves extract (1000 mg/kg body weight), caffeic acid (1.32 mg/kg body weight) and rosmarinic acid (26.84 mg/kg body weight) for the rats. Rat liver tissues indicated the increasing of antioxidant enzymes including catalase, glutathione peroxidase and superoxide dismutase leading to the reduction of lipid peroxidation in rat livers. (S. Y. Yang et al., 2013).

Rosmarinic acid

General information

Chemical name: Rosmarinic acid

IUPAC name: (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid

Molecular formula: $C_{18}H_{16}O_8$

Molecular weight: 360.318 g/mol

Melting point: 171 – 175 °C

Solubility: Soluble in most organic solvents

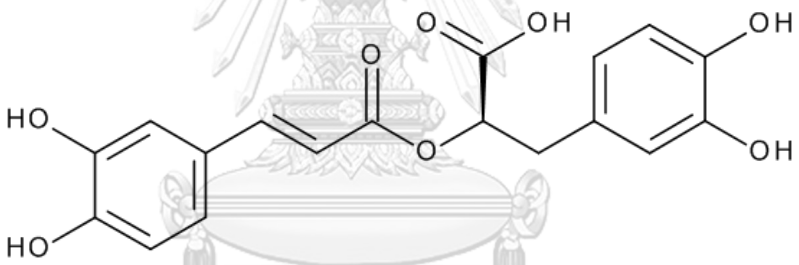


Figure 3 Molecular structure of rosmarinic acid

Rosmarinic acid is a phenolic ester derived from caffeic acid and lactic acid (3,4-dihydrophenyl) which has been discovered in 1958 by Scapati and Oriente (Scarpati & Oriente, 1958). It was first isolated and purified from the plant, Rosemary (*Rosmarinus officinalis*) in the family Lamiaceae. The occurrence of rosmarinic acid can be found in several plants, for instant, *Thunbergia laurifolia* (Thunbergiaceae) and *Perilla frutescens* (Lamiaceae) (Suwanchaikasem et al., 2014; Zhu et al., 2014).

Pharmacological and biological activities of rosmarinic acid

Antioxidant, anti-inflammatory and cytoprotective activities

Rosmarinic acid was investigated in apoptosis of various different cell lines: anaplastic thyroid cancers (ARO), *papillary* thyroid cancer (NPA) and erythroleukemia (K562). The cells were treated with sorbitol to induce hyperosmotic stress resulting in apoptosis. It was found that rosmarinic acid at the concentration of 25 μM inhibited sorbitol-induced apoptosis in all studied cell lines. Additionally, rosmarinic acid exhibited the suppression of ROS production as well as caspase-9 activation (Salimei et al., 2007).

Rosmarinic acid was subjected for the oxidative damage and anti-inflammatory activities of lipopolysaccharide-induced in primary cultured human gingival fibroblasts. The induction of inflammatory increased ROS level in the cells. The treatment of 1 μg/ml of rosmarinic acid significantly decreased ROS level in the cells. Moreover, GSH depletion in one of the indicator to determine oxidative damage in cells. At the same concentration, rosmarinic acid did not disturb the level of GSH. Lipid peroxidation was established relating to the increment of ROS level, however, the level of lipid peroxidation was decreased after the treatment of rosmarinic acid. For anti-inflammatory activity, rosmarinic acid was active for the reduction of TNF-α, a pro-inflammatory mediator. The compound also suppressed the lipopolysaccharide-induced nitric oxide synthase expression, a cellular signaling

for tissue damage, inflammation and cytotoxicity (Zdařilová, Svobodová, Šimánek, & Ulrichová, 2009).

Lipid peroxidation assay using 2,2′- azobis-(2-amidinopropane) dihydrochloride as a free radical generator treated with liposomes was used to investigate antioxidant efficiency of rosmarinic acid. In the result, rosmarinic acid had high efficiency against lipid peroxidation with the IC_{50} values of 1.51 μM. Moreover, the study also evidenced that rosmarinic acid exhibited its antioxidant potential due to its ability to insert inside membranes with a very small amount, approximately 1 mol% of rosmarinic acid (Fadel, El Kirat, & Morandat, 2011).

Rosmarinic acid was used to investigate cell viability, hepatotoxicity, endogenous antioxidant enzymes and lipid peroxidase assay using rat liver and human hepatocyte (HepG2). It was found that rosmarinic acid was active in the reduction of lipid peroxidation and hepatotoxicity serum enzymes (alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase). Additionally, the compound also active in the increment of cell viability and antioxidant enzymes (catalase, glutathione peroxidase and superoxide dismutase). Thus, this study indicated that rosmarinic acid is the potent antioxidant and hepatoprotective agents (S. Y. Yang et al., 2013).

Beta-carotene bleaching, DPPH free radical scavenging, ferric reducing power and chelating effect on ferrous ions was used to evaluate antioxidant potential of rosmarinic acid. The results indicated that rosmarinic acid possessed antioxidant

potential in all four tests. At 2 mg/ml of rosmarinic acid in water, the compound had 98.92% antioxidant activity in beta-carotene bleaching assay, whilst the compound exhibited lower antioxidant potential at 65.05 % in chelating effect on ferrous ion. Scavenging ability on DPPH free radical and ferric reducing power of rosmarinic acid showed its antioxidant potential in the concentration-dependent manners. Ultraviolet and hydrogen peroxide were used to elicit DNA damage for the protective effect of rosmarinic acid on pBR322 plasmid DNA. It was found that the compound showed DNA protective effect even at 0.002 mg/ml, a low concentration (Sevgi, Tepe, & Sarikurkcu, 2015).

The ability of rosmarinic acid to scavenge the free DPPH radical showed a concentration-dependent manner with EC_{50} value of 0.23 mM which was similar to the control, quercetin with EC_{50} value of 0.21 mM. The cellular protection assay using tert-Butyl hydroperoxide-induced oxidative stress in human liver cells (HepG2) was determined in short (5 hours) and long (20 hours) term exposure assays. It was found that rosmarinic acid was non-toxic to the cells and exhibited the antioxidant potential with EC_{50} values of 0.69 and 0.79 mM in short and long term cytoprotection assays respectively (Adomako-Bonsu, Chan, Pratten, & Fry, 2017).

Anti-diabetic effects

Rosmarinic acid (97% purity) was subjected to amylase inhibitory activity. The porcine pancreatic amylase using starch as a substrate was used in this study. Colorimetric measurement showed that 97% rosmarinic acid (0.07-0.42 mM) had the

strongest inhibitory activity comparing to other test compounds with the concentration dependent manner (McCue & Shetty, 2004).

Alloxan-induced diabetic kidney rats were subjected for diabetic nephropathy inhibition. Malondialdehyde, a marker for lipid peroxidation was also evaluated in this study. It was found that diabetic rats treated with 100 or 200 mg/kg/d rosmarinic acid could inhibit the elevation of MDA level and maintain the normal level of MDA when compared to untreated and control groups respectively. The elevation of biochemical substances as kidney function markers including serum creatinine and serum urea were inhibited and maintained at the normal level after received rosmarinic acid treatment. The histological study showed the ability of rosmarinic acid to inhibit glomerular hypertrophy, glomerular numbers reduction and glomerulosclerosis in diabetic rats. Thus, rosmarinic acid is one of anti-diabetic and nephroprotective agents (Tavafi, Ahmadvand, Khalatbari, & Tamjidipoor, 2011).

The inhibitory effects of rosmarinic acid at the concentrations of 0.01 - 0.4 mM on yeast **α**-glucosidase and mushroom tyrosinase were determined using colorimetric measurement. This study revealed the potential effects of rosmarinic acid as a noncompetitive tyrosinase inhibitor and competitive **α**-glucosidase inhibitor (Lin et al., 2011). Similar study on **α**-glucosidase inhibitory activity of the isolated rosmarinic acid from *Perilla frutescens* leaves showed IC_{50} value of 0.23 mg/ml (Zhu et al., 2014).

Animal model was used to investigate the anti-diabetic activity of rosmarinic acid using oral glucose tolerance test, postprandial glucose test, insulin tolerance test and homeostatic model assessment. Type 1 diabetic mice were induced by streptozocin and type 2 diabetic mice were induced by high-fat diet. It was found that rosmarinic acid decreased plasma glucose level in the blood as well as the calculated relative area under the glucose concentration curve of type 1 diabetic mice resulting in a dose-respond manner. In addition, a dose-respond manner of rosmarinic acid also found in the improvement of glucose utilization and insulin sensitivity in type 2 diabetic mice. Rosmarinic acid also ameliorated the gluconeogenesis in both types of diabetic mice by the reduction of phosphoenolpyruvate carboxykinase expression in the livers. On the other hand, the increment of glucose transporter expression in rat skeletal muscle was also found in both types of diabetic mice (Runtuwene et al., 2016).

Anti-allergic activities

The study of histamine inhibitory activity was done on rat mastocytes. Compound 48/80, as described in the study that “it is a polymer prepared by heating 4-methoxy-N-methylphenylamine with formaldehyde in acid solution and a very potent histamine liberator” was used to induce mast cell degranulation resulting in the release of histamine. It was found that rosmarinic acid at IC_{50} value of 18 μM showed the potent ability to inhibit histamine (Simpol, Otsuka, Ohtani, Kasai, & Yamasaki, 1994).

Ovalbumin-induced allergic mice treated with rosmarinic acid exhibited the reduction in the number of nose, ear and eye rubs as well as the histamine level in mice serum. The level of immunoglobulin E was also reduced and likewise mRNA expressions of interleukin; IL-1β, IL-6 and TNF-α obtained from nasal mucosa, spleen and serum of the allergic mice. Furthermore, cyclooxygenase-2 protein expression, caspase-1 activity, mast cells and eosinophil infiltration were decreased in the mice nasal mucosa tissue. The reduction of NF-kB/Rel A and caspase-1 activation was found in human leukemic mast cell line (HMC-1) (Oh et al., 2011).

Neuroprotective effects

Various studies on rosmarinic acid against Alzheimer's disease have been reported both *in vivo* and *in vitro* studies. The key pathological marker proteins (amyloids peptides) causing the induction of neuronal cell death were used as the target for the investigation. The cellular model using pheochromocytoma of the rat adrenal medulla (PC12) was treated with the 1-42 peptides of amyloid beta-protein ($A\beta_{1-42}$) to induce cell death. Rosmarinic acid at the concentration of 10 μM exhibited the reduction of $A\beta_{1-42}$ aggregation, lipid peroxidation, and ROS formation (Airoldi et al., 2013). The mice were injected with the 25-35 peptides of amyloid beta-protein ($A\beta_{25-35}$) to the cerebrospinal fluid in cerebral ventricles causing the amyloids peptide aggregation in the brain. The mice consumed rosmarinic acid every day at the concentration of 0.25 mg/kg body weight for 2 weeks showed the enhancement of learning and memory ability resulting from three different tests including T-maze,

object recognition and Morris water maze tests. Moreover, rosmarinic acid also reduced nitric oxide and malondialdehyde levels in the brain, liver and kidney of the mice (A. Y. Lee, Hwang, Lee, Lee, & Cho, 2016). The study on cholinesterase inhibitory activity was established to determine the ability of rosmarinic acid against Alzheimer's disease. Rosmarinic acid at the concentration of 10 µg/ml showed the inhibition of the key enzymes, acetylcholinesterase (29%) and butyrylcholinesterase (80%, IC50 = 6.59 µg/ml) resulting from the *in vitro* study (Senol et al., 2017).

Quality control methods for medicinal plant material

World Health Organization (2011) recommended the testing and analytical methods for quality assessment of the plants as follows:

Macroscopic and microscopic examinations

The first process that needed for the identity and purity of medicinal plant materials is macroscopic examination. This examination is emphasized on the morphological components of the medicinal plant materials. Organoleptic evaluation is the simplest method for identity and purity of medicinal plant materials based on many physical characteristics including surface of the crude drug, appearance of the cut surface, texture, shape, size and color. The fine structure in the form of sectioned or grounded plant materials can be determined by using microscope to investigate its anatomical and histological characteristics. Additionally, microscopic leaf constant parameters such as stomatal number, stomatal index, palisade ratio, epidermal cell

number and epidermal cell area can be used to confirm and ensure the identification of the plant materials.

Determination of loss on drying and water contents

The content of loss on drying and water of dried plant material in the natural state indicate the quality of crude drug. An excess water promotes the growth of some microorganisms, especially bacterium and fungus which deteriorate crude drug. Azeotopic distillation with water-immiscible solvent such as toluene is a suitable technique for determination of water content. It is important that the solvent is saturated with water before use because the anhydrous solvent will absorb the water leading to the inaccurate results.

Determination of total ash and acid insoluble ash

The inorganic substances in herbal material are represented by the ash content. The total ash means the total non-volatile inorganic matters which remains after incineration of the crude drug with high temperature. Acid-insoluble ash is the residue obtained after boiling the total ash with hydrochloric acid (70 g/L) and incineration of the remaining insoluble matters. This measures the amount of some inorganic matters which are not solubilized in hydrochloric solution. Adulterants in herbal materials; both physiological (the foreign plant materials) and non-physiological (adherent extraneous foreign matters) materials can affect the contents of total and acid insoluble ashes.

Determination of extractable matter

Extractable matters represent the particular phyto-constituents in the certain amount of plant materials extracted in selected solvent. Single solvent extraction, such as water and ethanol is commonly used for this method.

Thin-layer chromatography

The discovery of this technique was revealed by Kirchner and his team in the 1950s. In that time, it was called “Chromatostrips” because of the procedure was similar with paper chromatography but differ in the use of stationary phase. According to Kirchner work, it was the first time that the silica gel layer contained fluorescence indicator was attached on the glass plate with the aid of the binder. Later in the late 1950s, the term “Thin-layer chromatography or TLC” was introduced by Egon Stahl. Stahl offered the breakthrough of convenience manufactured thin-layer chromatography with more efficiency and accuracy. This still holds to the present for the qualitative and quantitative analysis of chemical substances (Sherma & Fried, 2003).

The basic of thin-layer chromatography to separate chemical substances for qualitative and quantitative analysis is described out as follows. The sample is placed onto one end of the TLC-plate forming the initial zone after dryness. The end of TLC-plate that closes to the initial zone is placed into the mobile phase, usually a single solvent or the combination of various solvents, inside a closed tank or chamber. The correct or suitable stationary phase and mobile phase will lead the

migration of the sample at different rates yielding the separation of the sample as seen in the chromatogram. The stationary phase (TLC-plate) is removed out of the chamber for dryness when the mobile phase has moved to the suitable distance. Then the obtained chromatogram is visualized under ultraviolet light (UV-light) or daylight with or without the assistance of specific spraying or dipping reagents.

The migrating behavior of the compounds provides the retardation factor (R_f) value which can be used for identification and authentication of the interested compounds. The R_f value can be calculated using the migration distance of the substance divided by the migration distance of the solvent front (Mukherjee, 2007; Sherma & Fried, 2003).

$$Rf = \frac{\text{migration distance of the substance}}{\text{migration distance of the solvent front}}$$

TLC-densitometric analysis

Densitometric analysis provides the detection and identification of the developed chromatogram which can be seen as separation tracts obtained from planar chromatographic method. Separated compounds on TLC-plate are measured in the reflectance from absorbance, fluorescence or a combination of absorbance-fluorescence modes. The compounds that absorb light in the ultraviolet (UV) or visible range can be measured with the maximum absorption wavelength. The light sources of absorption measurement are deuterium lamps (UV region, 190-450 nm)

and halogen or tungsten lamps (visible region, 350-900 nm). In addition, high pressure mercury lamp (UV/visible regions, 254-578 nm) is the light source for the compounds that emit the fluorescent light after irradiation in the specific wavelength, usually a long wavelength. The emitted light goes through the monochromator (filter) for wavelength selection. Finally, the light beam will go to photomultiplier or photodiode detectors for signal measurement. All measurement steps are automated by winCAT software (Sherma & Fried, 2003).

TLC-image software analysis

The National Institutes of Health (NIH), Department of Health and Human Services in America has established a free image processing software, ImgeJ software. It is an open-source platform written in Java which can be used in many operating systems. The software has been developed from its predecessor "NIH Image" as the tool for scientific image processing software (Schindelin, Rueden, Hiner, & Eliceiri, 2015). For TLC-quantitative analysis, the separated compounds on the TLC-plate is captured by digital camera with the high resolution. The digital image then transfers to the program, ImageJ. This free software provides the algorithms that calculate the intensity of image pixels assisting by the automated or defined selection of the interested regions within the image. The selected region is transformed to densitogram showing peak area. The area under the peak is proportional to the concentration of the sample which can be compared with the authentic standard curve of interested compound (The National Institutes of Health, 2018).

Method validation

Association of analytical communities (AOAC) gives three definitions of method validation, “First, validation is the process of demonstrating or confirming the performance characteristics of a method of analysis. Second, the method of analysis is the detailed set of directions, from the preparation of the test sample to the reporting of the results that must be followed exactly for the results to be accepted for the stated purpose. Third, the performance characteristics of a method of analysis are the functional qualities and the statistical measures of the degree of reliability exhibited by the method under specified operating conditions (Association of analytical communities, 2002).” According to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline, the validation of developed analytical procedure is required for the quality. Typical validation parameters are recommended including linearity, range, detection limit, quantitation limit, accuracy, precision, specificity and robustness (Validation of analytical procedures: text and methodology Q2(R1), 2005).

Linearity and range

Linearity and range are the fundamental of the analytical procedure. The degree of linearity is obtained from the calibration curve plotted between the measure value of analyte and the concentration of the substance. A minimum of five concentrations for the analytical procedure providing correlation coefficient, slope of

the regression line, y-intercept and residual sum of square are recommended for the linearity evaluation.

Range is typically obtained from linearity test as an interval of lower to upper concentrations of the analyte measure value in the substance. This parameter supports the degree of accuracy, precision and linearity in the sample containing the analyte for analytical procedure.

Limitation of detection

Limitation of detection (LOD) is the detectability of lowest amount of the analyte in substance from an individual analytical procedure. However, it is unnecessary to quantitate as the exact amount of the analyte in sample. There are three determinations suggested for limitation of detection based on visual evaluation, signal-to-noise (ratio between 3 or 2:1 is acceptable) and the calculation between standard deviation of the response (σ) and the slope (S) as the following equation:

$$\text{Limitation of detection} = 3.3\ \sigma / S$$

Limitation of quantitation

Limitation of quantitation (LOQ) is the ability to quantitate of lowest amount of the analyte in substance with proper accuracy and precision from an individual analytical procedure. There are three approaches similar to that of limitation of detection but differ in the process based on visual evaluation, signal-to-noise (ratio

10:1 is acceptable) and the calculation between standard deviation of the response (σ) and the slope (S) as the following equation:

$$\text{Limitation of detection} = 10\sigma / S$$

Accuracy

The accuracy is sometimes termed as trueness because it represents the closeness of the obtained values from the test to the reference or true value by spiking the known amount of analyte into the substance. According to ICH guideline, 3 concentrations covering the specific range with 3 replicates of each analytical method should be evaluated (the minimum requirement is 9 determinations) and expressed in percent recovery.

Precision

Precision of an analytical procedure is obtained from many sampling of the same sample, usually homogenous sample under the prescribed methodology. ICH guideline recommended 3 considerations including repeatability, reproducibility and intermediate precision and the results are given as the percentage of relative standard deviation (%RSD).

Specificity

Specificity provides the ability of a particular method to specifically determine the analyte in the substance. In chromatographic method, the analyte peak should be separated from the interference to obtain a good identification.

Purification is performed by comparison of the standard peak and UV-absorbance spectra of the analyte in substance. This can determine from up-slope, apex and down-slope of one component.

Robustness

The determination of robustness can be investigated in many ways depending on the study procedure. It measures the capacity of the method to give the similar results when slightly changes occurred. In the case of TLC-method, it can be investigated by varying the analytical condition, for instant, the proportion of mobile phase.

Biological activity

Antidiabetic activity

Diabetes mellitus is one of the serious medical problem due to the raising number of people with diabetes worldwide (World Health Organization, 2016). The condition of blood glucose level higher than the normal (hyperglycemia) is caused from the ineffectiveness of insulin utilization in the body. Diabetes mellitus is classified into 2 types.

Type 1 diabetes (insulin-dependent/ juvenile/ childhood-onset diabetes) has been described as the insufficient insulin in the body due to the malfunction of insulin production from the pancreases. Type 1 diabetes people require daily insulin administration to manage the blood glucose level in their bodies.

Type 2 diabetes (non-insulin dependent/ adult-onset diabetes) has been described as the condition when the pancreas produce inadequate insulin or the body cannot properly use the insulin for blood glucose regulation. Presently, type 2 diabetes is increased not only in adults but also in adolescent due to 2 risk factors, overweight and obesity.

Alpha-amylase and alpha-glucosidase, the key enzymes that have been used to evaluate the antidiabetic agents from various medicinal plants (Tadera, Minami, Takamatsu, & Matsuoka, 2006). Alpha-glucosidase is the complex enzyme which can be found mostly in the brush border of the enterocytes in small intestine, whereas alpha-amylase can be found abundantly in pancreatic juice and saliva (Krentz & Sinclair, 2012). These two enzymes are working on the hydroxylation of carbohydrate. Alpha-amylase breakdowns polysaccharides, such as starch into smaller forms, such as dextrins, disaccharides and glucose. Alpha-glucosidase will hydrolyze some dextrins and disaccharides into monosaccharides, then the body can absorb *via* the brush border of the small intestinal cells leading to the elevation of blood glucose level. Inhibition of these two key enzymes can significantly reduce blood glucose level. Currently, antidiabetic drugs acting as the competitive inhibitor of alpha-glucosidase for type 2 diabetes include acarbose, miglitol, nojirimycin and 1-deoxynorjirimycin (Rosa & Dias, 2014).

Antioxidant activity

Any molecules that contain the unpaired electron in the structure is called free radicals. Free radicals are highly reactive and unstable, so they behave as either oxidant or reductant. The free radicals consisting of oxygen is known as reactive oxygen species (ROS), for example, superoxide anion, peroxide, hydrogen peroxide and hydroxyl radical. On the other hand, the free radicals consisting of nitrogen is known as reactive nitrogen species (RNS), for example, peroxy nitrite and nitric oxide radical. Free radical are formed in our body every day through the normal metabolic processes or even the inflammation in our body. The free radical derived from external sources, for example, environment pollutants, UV-light, cigarette smoking and radiation (Lobo, Patil, Phatak, & Chandra, 2010). The excess amount of ROS can cause cell damage because it highly react to other molecules such as lipids, nucleic acid and protein (Serafini, 2006). High accumulation of ROS content affects in many health issues. Therefore, the imbalance between ROS content and endogenous protein antioxidants cause some health problems, for instant, Alzheimer's disease (Hureau & Faller, 2009), inflammation injuries, atherosclerosis (Valko et al., 2007), cardiovascular diseases, cancer (Gerber et al., 2002), and aging (Angelopoulou, Lavranos, & Manolakou, 2009).

DPPH radical scavenging assay

DPPH is a common abbreviation for the dark purple organic chemical compound 2,2-diphenyl-1-picrylhydrazyl with the maximum wavelength at 517 nm. It

is a stable free radical making ease of use for antioxidant investigation. The antioxidant substance may transfers an electron or donating a hydrogen which causes the discoloration from purple to yellow. The change of color can detect by spectrophotometric measurement (Figure 4) (Brand, Cuvelier, & Berset, 1995).

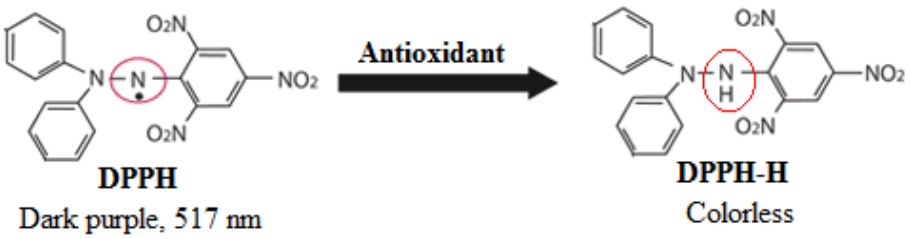


Figure 4 DPPH and antioxidant reaction

Ferric reducing antioxidant power assay

FRAP assay is a common abbreviation for this method. The principle is based on the ability of antioxidant agent to reduce ferric ion into ferrous ion. The ferric tripyridyltriazine is used as the test compound. Antioxidant compound reduces the iron atom of ferric ion (Fe^{3+}-TPTZ) yielding the dark blue complex compound of ferrous ion (Fe^{2+}-TPTZ) with the maximum wavelength at 593 nm (Figure 5) (Benzie & Strain, 1996).

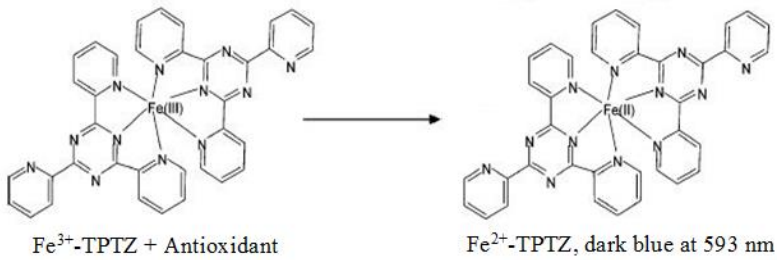


Figure 5 Mechanism reaction of FRAP assay

β-carotene bleaching assay

β-carotene is a reddish-orange organic compound with the ability to protect cell membrane by quenching singlet oxygen and scavenging free radicals. However, the compound can be oxidized during the autoxidation process of linoleic acid, thus the plant extract containing antioxidant compound can retard the β-carotene decay. In addition, blenching is based on the discoloration from reddish-orange to yellow due to its reaction with radicals (Jayaprakasha, Jena, Negi, & Sakariah, 2002).

Intracellular ROS measurement

Reactive oxygen species is one of the majority cause of intracellular oxidation. ROS can cause damage of cell membrane by reacting with unsaturated fatty acid in phospholipids. Additionally, an excess of ROS also cause oxidative stress leading to cell damage. Therefore, the study of antioxidant activity in cell has gained more attention for the investigation of antioxidant compound derived from medicinal plants as it can stimulate cellular biochemical processes. As aforementioned, it represents the closer relationship to the livings comparing to the *in vitro* chemical antioxidant assays. $DCFH_2$-DA is an abbreviation for 2',7'-Dichlorodihydrofluores-ceindiacetate or 2',7'-dichlorofluorescin diacetate. This compound is able to diffuse through the cell membrane where it is enzymatically hydrolyzed by non-specific intracellular esterase by cleaving off the lipophilic groups and becoming a charged compound trapped inside the cells. Non-fluorescent $DCFH_2$ is oxidized by intracellular ROS which covert to a highly fluorescent, 2',7'-dichlorodihydrofluoescein

(DCF) with the excitation at 485 nm and the emission at 535 nm (Shirai, Yamanishi, Moon, Murota, & Terao, 2002).

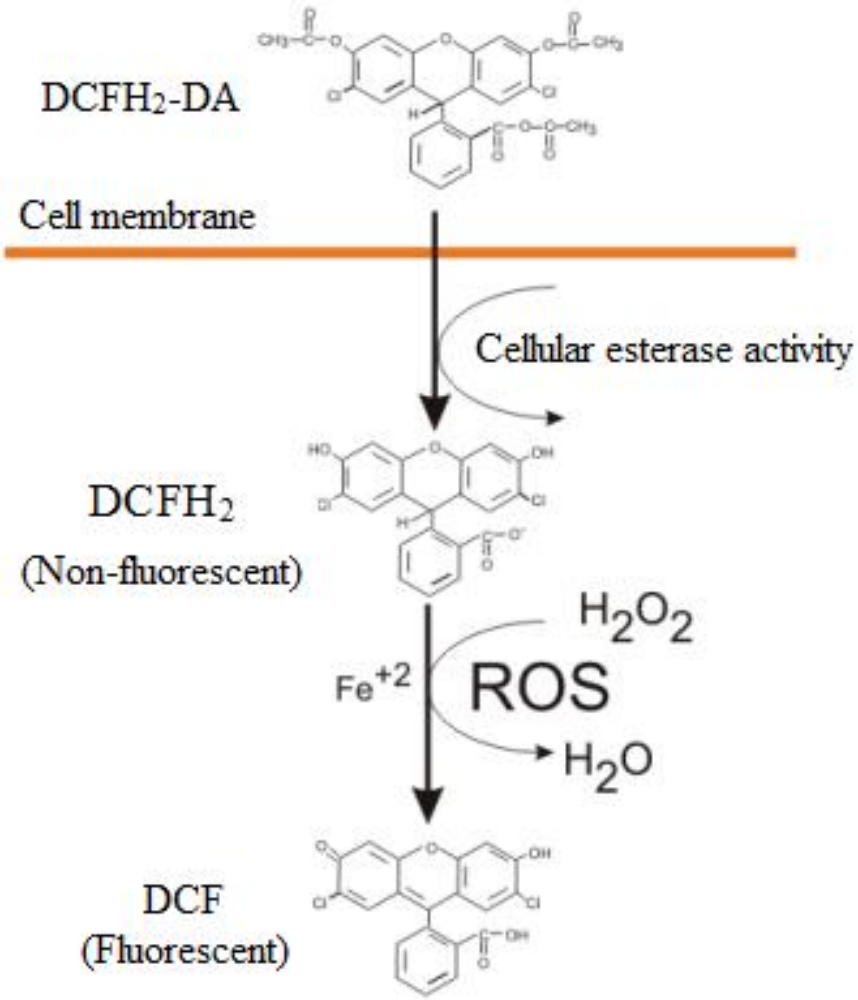


Figure 6 Formation of fluorescent compound DCF by ROS

Cytotoxicity

Comet assay

The comet assay is a single cell gel electrophoresis assay (SCGE) for evaluating DNA damage in cells. The migration of DNA strands from nuclei which were exposed to an electric field under neutral conditions made the cells appeared as the comet was demonstrated in 1984 by Ostling and Johanson (Östling & Johanson, 1984). Later in 1988, alkaline conditions with some modification of the previous study was established by Singh and his co-workers providing more sensitivity, specificity and reproducibility of the method (N. P. Singh, McCoy, Tice, & Schneider, 1988). Determination of denatured DNA fragments begins when the cells are treated with non-ionic detergent and lysis solution to remove cell membrane, nucleoplasm, cytoplasm and nucleosomes. The leftover nucleoid is treated with alkaline solution making the unwinding of DNA supercoils. The exposure of alkali labile sites, apurinic and apyrimidinic sites makes the break of DNA. The DNA fragments will migrate to the anode during the electrophoresis producing the tail next to the cell. This makes the cells looks like comet. Various cells were subjected for the comet assay, for example, sperm cells and culture cells. Peripheral blood lymphocytes are one of the convenient sources of cells because the cells can be isolated from the blood sample. Lymphocyte, a normal diploid cell in human circulates mostly in the blood making it suitable for human biomonitoring research (Nandhakumar et al., 2011).

MTT assay for cell viability determination

MTT is an abbreviation for the yellow chemical compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The compound is commonly used in cytotoxicity and proliferation of cells. MTT assay is based on the reduction of MTT compound by the enzyme, mitochondrial dehydrogenases in the cells causing the change of color from yellow (tetrazole) to purple (formazan crystals). Dimethyl sulfoxide is used to solubilize the formazan crystals inside the cells. The purple solution containing formazan crystals can be measured with the wavelength at 570 nm. The MTT assay can be used to indicate the degree of toxicity of the sample to the cells by comparing the cells treated with sample to the control cells. Higher absorbance value of the cell treated with sample indicates a good cell proliferation effect (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987).

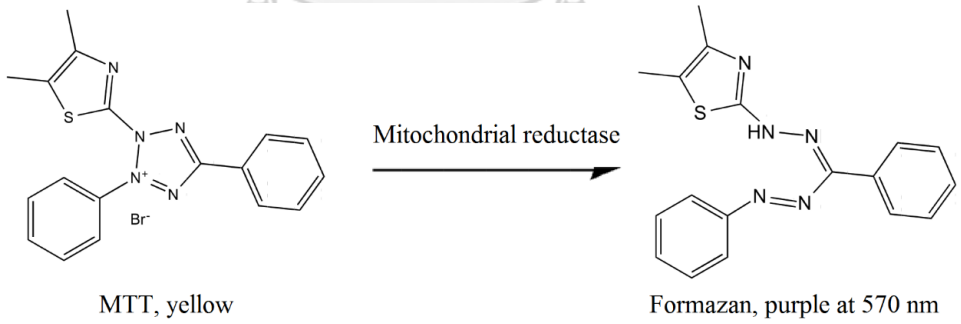


Figure 7 The chemical reaction of MTT assay

CHAPTER III

MATERIALS AND METHODS

Chemicals and reagents

2, 2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma, USA
2, 4, 6-tripyridyl-s- triazine (TPTZ)	Sigma, USA
2', 7'-Dicchlorodihydrofluorescein diacetate (DCFH-DA)	Sigma, USA
2-chloro-4-nitrophenol-a-D-maltotrioside (CNPG3)	Sigma, USA
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma, USA
3, 5-Di-*tert*-4-butylhydroxytoluene (BHT)	Sigma, USA
Acarbose	Sigma, USA
Acetone	Merk, Germany
Alpha-amylase from porcine pancreases	Sigma, USA
Alpha-glucosidase from rat intestine	Sigma, USA
Alpha-glucosidase from *Saccharomyces cerevisiae*	Sigma, USA
Beta-carotene	Fulka, USA
Chloroform, AR grade	RCI Labscan, Thailand
Dimethyl sulfoxide (DMSO)	Merk, Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma, USA
Ethanol, AR grade	RCI Labscan, Thailand
Ethidium bromide (10mg/ml solution)	Bio Basic, Canada

Chemicals and reagents (Cont.)

Ethyl acetate, AR grade	RCI Labscan, Thailand
Ethylenediaminetetraacetic acid (EDTA)	Univar, Australia
Fetal bovine serum (FBS)	Hyclone, UK
Folin-Ciocalteu reagent	Merk, Germany
Formic acid	Fisher Scientific, UK
Histopaque-1077	Sigma, USA
Hydrochloric acid 37%, AR grade	RCI Labscan, Thailand
Hydrogen peroxide	Fisher Scientific, USA
Intestinal acetone powders from rat	Sigma, USA
Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$)	Sigma, USA
Iron (III) chloride ($FeCl_3 \cdot 6H_2O$)	Ajax Finechem, New Zealand
Linoleic acid	Sigma, USA
Medium (DMEM)	Gibco, New Zealand
Medium (RPMI-1640)	Life Technological, USA
Methanol, AR grade	RCI Labscan, Thailand
Penicillin streptomycin	Gibco, New Zealand
p-nitrophenyl ***α****- D –glucopyranoside (PNPG)*	Sigma, USA
Potassium chloride (KCl)	Merk, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merk, Germany
Quercetin hydrate	Sigma, USA

Chemicals and reagents (Cont.)

Rosmarinic acid	Sigma, USA
Sodium bicarbonate ($NaHCO_3$)	Sigma, USA
Sodium carbonate (Na_2CO_3)	QREC, New Zealand
Sodium chloride (NaCl)	Ajax Finechem, New Zealand
Toluene	RCI Labscan, Thailand
Tris(hydroxymethyl)-methylamine	Fisher Scientific, UK
Trition X-100	Sigma, USA
Tween 20	Merk, Germany

Materials

Cover glasses (24 x 50 mm), Menzel Gläser	Thermo Scientific, Germany
Filter paper No.4	Whatman™ paper, UK
Filter paper No.40 ashless	Whatman™ paper, UK
Hemocytometer	Digital Bio, Korea
Microscope slides (25.4 x 76.2 mm)	Sail Brand, China
Microtiter plate with 96 wells	BRAND plates, Wertheim®, Germany
TLC aluminium sheet, silica gel 60 GF_{254}	Merck, Germany

Instruments and equipments

5% CO_2 incubator	Thermo Fisher Scientific, USA
Ashing furnaces	Carbolite, Scientific Promotion, Thailand
Autoclave	HPV-50, Hirayama, USA

Instruments and equipments (Cont.)

AxioVision40 software (V 4.6.3.0)	Zeiss Inc., Germany
CAMAG TLC Linomat 5	CAMAG, Switzerland
CAMAG TLC Plate Heater III	CAMAG, Switzerland
CAMAG TLC Scanner 4	CAMAG, Switzerland
Centrifuge	Hettich Lab Technology, Germany
Digital balance (Model: SI-234)	Denver Instrument, USA
Digital camera (Canon PowerShot A640)	Canon Inc., Japan
Digital orbit shaker (Model: SHO-2D)	Daihan Scientific, Korea
Hot air oven	WTB binder, Scientific Promotion, Thailand
Image-J software	National Institutes of Health, USA
Laminar hood (Model: Class II BSC)	ESCO, Singapore
Microplate reader (Axio imager A2)	Zeiss Inc., Germany
PowerPac™ Basic Power Supply	Bio-Rad, USA
Refrigerated centrifuge	Sigma, Germany
Rotary evaporation	Buchi, Switzerland
Ultra-pure water purification NW20VF	Heal Force, China
Ultrasonic bath	Analytical Lab Science Co., LTD, Thailand
UV-Viewing cabinet	Spectronics Crop., USA
winCATS software	CAMAG, Switzerland

Sample collection

Fifteen samples of each selected Thai crude drug including *Thunbergia laurifolia* leaves, *Thunbergia laurifolia* stems and *Perilla frutescens* leaves were collected from 15 different locations throughout Thailand. All of the obtained crude drugs were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. Any foreign matters in each authentic sample were removed before drying at 50 °C in the hot air oven. Finally, the dried samples were ground into powder for testing.

Plant extraction

The powder of each selected plant material was exhaustively extracted with ethanol by Soxhlet apparatus, then the extract was filtered through Whatman No.4 filter paper and evaporated *in vacuo.* The percent yield of each extract was calculated and recorded. The extract was kept in clean and air-tight container at -20°C.

Pharmacognostic specification of *Thunbergia laurifolia* leaves and stems

The standardization parameters were performed for quality of the crude drugs

Macroscopic examination

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

Microscopic examination

Cells and tissues of each plant material has a characteristic microscopic appearance, thus the following methods were used to identify the plant materials under the microscope. The results of all methods were taken with digital camera and illustrated by hand drawing.

1) Histological examination

Cutting the midrib of a mature leaf and dried stems by freehand transverse section technique were performed for examining structures or characteristics of plant cells and tissues.

2) Powdered drug examination

Each dried crude drug sample was ground to fine powder and mounted with water on the glass-slide for examining structures or characteristics of plant cells and tissues.

3) Microscopic leaf constant values determination

The mature leaves were collected from three different locations. The leaves were cut into suitable length and then clarified by boiling with gentle heat in chloral hydrate (4g/ml in water) until obtain the transparent leaves. The transparent leaves were rinsed twice with water and kept in glycerin for further investigations.

3.1) Determination of the stomatal number

Thirty fields of the viewing areas under microscope were subjected for counting the number of stomata in leaves. Additionally, the half-view stoma cell in

the viewing area was also counted as a half cell. The stomatal number was calculated using the following formula:

$$\text{Stomatal number} = \frac{\text{Number of stomata}}{\text{Area of epidermal cells (mm}^2\text{)}}$$

3.2) Determination of the stomatal index

The proportion of the number of stomata and epidermal cells in the same microscopic field were used to determine stomatal index by the following formula:

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

Both stomata and epidermal cells must be in the same unit area for the calculation, where 'S' is number of stomata and 'E' is number of epidermal cells.

3.3) Determination of palisade ratio

The number of palisade cells underneath one epidermal cell was counted and divided by four for the determination.

3.4) Determination of the upper epidermal cell area

The epidermal cell and also the half-view epidermal cell in each viewing area of upper epidermis (thirty fields) were counted and calculated using the following formula:

$$\text{Epidermal cell area} = \frac{\text{Number of epidermal cell}}{\text{Area of view (mm}^2\text{)}}$$

Thin layer chromatography fingerprint

The ethanolic extract of each plant material was dissolved with 1 ml of 95% ethanol. Three microliters of the mixture were applied onto the TLC-silica gel 60 GF_{254} plate and developed in the suitable solvent system. The plate was removed from the solvent chamber and allowed to dry before detection with visible day light, ultraviolet light 245 nm, ultraviolet light 365 nm and anisaldehyde staining reagent.

Physico-chemical determination

Determination of loss on drying

Three grams of crude drug powder were placed in the pre-weighed porcelain crucible and dried at 105 °C to constant weight. The outcome was calculated in percentage.

Determination of water content

Fifty grams of crude drug powder were transferred into the flask containing 200 ml of water-saturated toluene. The flask was connected with the apparatus for azeotopic distillation. The amount of water was measured and calculated in percentage.

Determination of total ash

Three grams of crude drug powder in the pre-weighed porcelain crucible were incinerated at 500 °C until the sample turns into white matter and obtains the constant weight. The porcelain crucible was transferred to cool down in a desiccator and then weighed.

Determination of acid-insoluble ash

The porcelain crucible containing ash was boiled gently with 25 ml of hydrochloric acid (70 g/L) for 5 min. The ashless filter paper was used to separate the insoluble matter from the mixture. The insoluble matter on the ashless filter paper was incinerated at 500 °C. The porcelain crucible was transferred to cool down in a desiccator and weighed.

Determination of water extractive value

Seventy milliliters of water were used to macerate 5 g of crude drug powder under shaking for 6 h and standing for 18 h in the room temperature. The extract solution was filtered through Whatman No.4. The marc was washed with the solvent and adjusted the final volume to 100 ml. Twenty five milliliters of the filtrate was transferred to a pre-weighed beaker, evaporated on the water-bath, and then dried in a hot air oven at 105℃ to constant weight.

Determination of ethanolic extractive value

Seventy milliliters of ethanol were used to macerate 5 g of crude drug powder under shaking for 6 h and standing for 18 h in the room temperature. The extract solution was filtered through Whatman No.4. The marc was washed with the solvent and adjusted the final volume to 100 ml. Twenty five milliliters of the filtrate were transferred to a pre-weighed beaker, evaporated on the water-bath, and then dried in a hot air oven at 105℃ to constant weight.

Quantification of rosmarinic acid in *Perilla frutescens* leaves, *Thunbergia laurifolia* leaves and stems

The selected Thai medicinal plant materials including *Thunbergia laurifolia* leaves, *Thunbergia laurifolia* stems and *Perilla frutescens* leaves contain the same compound, rosmarinic acid, which is the interested constituent in this study as the powerful natural antioxidant.

Preparation of rosmarinic acid standard solution

Rosmarinic acid stock solution was prepared by dissolving in 95% ethanol. The series of rosmarinic acid solution were obtained by diluting the stock solution to give concentrations of 0.25, 0.4, 0.8, 1 and 2 mg/ml.

Preparation of ethanolic extract

Five grams of each crude drug powder were exhaustively extracted in 95% ethanol by using Soxhlet apparatus. Each ethanolic extract was filtered through Whatman filter paper No.4 and allowed to evaporate at 40 ^{o}C. The ethanolic extract was calculated in percent yield and recorded.

TLC-densitometric method

Four microliters of rosmarinic acid standard and the crude drug ethanolic extracts were applied in triplicate as bands onto the silica gel60 GF_{254} TLC plate by using CAMAG Linomat 5 with nitrogen compressed air. Two sets of mobile phase were applied in this study. First, the spotted plate was developed using toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v). After the mobile phase migrated to

the eluent front, the plate was removed and allowed to dry at room temperature. Then the plate was developed again with the same process but using the second mobile phase including toluene-ethyl acetate-formic acid (5:4:1, v/v). Rosmarinic acid content was carried out by CAMAG TLC Scanner 4 with the absorbance scanning at the maximum wavelength of 330 nm. All chromatographic developments providing the peak area were subjected to quantify the rosmarinic acid content using the winCATS version 1.4.9 software for the integration.

TLC-image analysis method

The image of rosmarinic acid bands on the TLC plates was captured with Canon PowerShot A560 IS digital camera in a UV-Fluorescence analysis cabinet with 365 nm UV lamp. The digital images of TLC plates were transferred to the computer and saved in TIFF (.tif) format. ImageJ, a Java-based image processing program was used to analyze the color intensity of rosmarinic acid bands interpreting as chromatographic peaks which can be measured the peak area. This process was done on the spotted bands in the same TLC plate using with TLC-densitometric method.

Method validation

The validation of both analytical procedures for rosmarinic acid in the selected plant materials using TLC-densitometer and ImageJ software was performed by following the International Conference on Harmonisation guideline (Validation of analytical procedures: text and methodology Q2(R1), 2005).

Linearity and range

The peak area and different rosmarinic acid standard concentrations (0.25, 0.4, 0.8, 1 and 2 mg/ml) were used to construct the calibration curve.

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

Both methods are based on the residual standard deviation of a regression line and the slope from the calibration curve. The calculation for LOD and LOQ was obtained from these following formula:

$$\text{Limit of detection (LOD)} = \frac{3.3 \times \sigma}{S}$$

$$\text{Limit of quantitation (LOQ)} = \frac{10 \times \sigma}{S}$$

where σ = standard deviation of the regression line

S = the slope from the calibration curve

Accuracy

The sample was spiked with the known amount of rosmarinic acid standard at low, medium and high concentrations which are in the calibration range. The accuracy was assessed and calculated as the mean percentage recovery using the following formula:

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

where A = the amount of spiked sample

B = the amount of un-spiked sample

C = the amount of standard spiked into the recovery sample

Precision

Nine determinations (3 concentration/ 3 replicate each) covering the specified range were performed for the intra-day precision (in the same day) and inter-day precision (3 different days). The repeatability and intermediate precision were expressed as %RSD by the following formula:

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

where SD = the standard deviation of each measurement

Specificity

Absorbance spectrum of the peak apex among all samples were subjected to compare with the standard rosmarinic acid for peak identity under the range of 200 – 700 nm. The absorbance spectrums which are recorded at up-slope, apex, and down-slope of the peak were compared for peak purity.

Robustness

The ratio of solvents in the second system was varied for the evaluation of the robustness and expressed as %RSD.

In vitro biological activities of *Thunbergia laurifolia* leaf and stem

Anti-diabetic activities

Yeast α-glucosidase inhibitory activity

The test in this part was slightly modified from the previous study of Wan and others in 2013 (Wan, Min, Wang, Yue, & Chen, 2013). Various concentrations of extracts, rosmarinic acid and positive control (acarbose) were prepared in DMSO, then 10 µl of the sample solutions were mixed with 120 µl of 0.1 M sodium phosphate buffer (pH 6.9) and 20 µl of 0.5 Unit/ml yeast α-glucosidase. The 96-well plate containing the mixture was incubated for 15 minutes at 37 ^{o}C. Then, 20 µl of *p-nitrophenyl α-D*-glucopyranoside at the concentration of 1 mM were added and incubated for 30 minutes at 37 ^{o}C. Finally, 80 µl of sodium carbonate were added to terminate the reaction and measured the absorbance at 405 nm. The test was done in triplicate and the inhibition percentage was calculated following this formula:

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

Rat intestinal α-glucosidase inhibitory activity

The test in this part was slightly modified from the previous studies of Ganogpichayagrai and others in 2017 and Shipp and others in 2012 (Ganogpichayagrai, Palanuvej, & Ruangrungsi, 2017; Gayle, 2012). Various concentrations of extracts, rosmarinic acid 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 ^{o}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 α-glucosidase enzyme was incubated for 30 min at 37 ^{o}C, then measured the absorbance at 405 nm. The test was done in triplicate and the inhibition percentage was calculated following this formula:

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

Pancreatic α-amylase inhibitory activity

The test in this part was slightly modified from the previous studies of Gella and others in 1996 and Kumar and others in 2011 (Gella, Gubern, Vidal, & Canalias, 1997; Kumar et al., 2011). Various concentrations of extracts, rosmarinic acid and positive control (acarbose) were prepared in DMSO. The control reaction was carried out without the extracts or acarbose by using only 0.1 M sodium phosphate buffer (pH 6.9). The 96-well plate containing 30 µl of the test solutions was mixed with 30 µl of 25 U/ml of α-amylase in 0.1 M sodium phosphate buffer (pH 6.9). The mixture was incubated for 10 minutes at 37 ^{o}C. After that, 30 µl of 1mM 2-chloro-4-nitrophenyl-α-D-maltotrioside were added and incubated for 20 minutes at 37 ^{o}C.

The 96-well plate was measured the absorbance at 405 nm. The test was done in triplicate and the inhibition percentage was calculated following this formula:

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

Antioxidant activities

DPPH radical scavenging assay

The test in this part was slightly modified from the previous study of Brand-William and others in 1995 (Brand et al., 1995). Fifty microliters of various concentrations of extracts, rosmarinic acid and controls (ascorbic and BHT) in ethanol were added to 150 microliters of 120 μM DPPH ethanolic solution. Then the 96-well plate containing the mixture was incubated in the dark at room temperature for 30 minutes and subjected to measure the absorbance at 517 nm. The test was done in triplicate. The half maximal inhibitory concentration (IC_{50}) was evaluated from the curve fitting between the scavenging percentages of the test samples and their concentrations following this formula:

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

Ferric reducing antioxidant power (FRAP) assay

The test in this part was slightly modified from the previous study of Benzie and Strain in 1996 (Benzie & Strain, 1996). The FRAP reagent solution was prepared in the ratio of 10:1:1 including 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-trlazine (TPTZ) solution and 20 mM ferric chloride respectively. Twenty-five microliters of the extract, rosmarinic acid and controls (ascorbic and BHT) in ethanol were added to 175 microliters of FRAP reagent solution. Then the 96-well plate containing the mixture was incubated at room temperature for 30 min and subjected to measure the absorbance at 593 nm. The test was done in triplicate. Ferrous sulfate calibration curve was established to evaluate the reducing antioxidant power expressing in mM of ferrous iron per milligram of the samples.

β-carotene bleaching assay

The test in this part was slightly modified from the previous studies of Andrade and others in 2013 and minor modification of Jayaprakasha and others in 2002 (Andrade et al., 2013; Jayaprakasha et al., 2002). The β-carotene solution was prepared by mixing 1 ml of β-carotene (1mg/ 5ml in chloroform), 20 µl of linoleic acid and 200 µl of tween 20. The chloroform was removed at 40 ^{o}C using a rotary evaporator. Then the residue was dissolved in 50 ml of ultra-pure water. Ten microliters of various concentrations of extracts, rosmarinic acid and positive control (BHT) were mixed with 200 µl of the β-carotene solution and then immediately measured the absorbance at 470 nm. Subsequently, the 96-well plate was incubated

at 50 °C for 60 minutes and measured the absorbance at 470 nm again. The test was done in triplicate and the half maximal inhibitory concentration (IC_{50}) was evaluated from the curve fitting between the antioxidant activity percentages of the test samples and their concentrations following this formula:

$$\% \text{ Antioxidant activity} = [1- \{(A_0 - A_{60})/ (C_0 - C_{60})\}] \times 100$$

where A_0 = the absorbance at time zero

A_{60} = the absorbance at 60 min

C_0 = the absorbance at time zero of control

C_{60} = the absorbance at 60 min of control

Intracellular ROS measurement

The purchase of human umbilical vein endothelium (EA.hy926) was obtained from American Type Culture Collection (ATCC). The endothelial cells were grown in Dulbecco's Modified Eagle's medium supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin to achieve the density of $1x10^5$ cells/ml in 5% CO_2 incubator at 37 °C. The old medium was changed and the subcultivation process was performed by using 0.25% trypsin in PBS buffer (pH 7.4) in every 4 days. Dimethyl sulfoxide (DMS0) at 0.5% final concentration (v/v) was used as the vehicle control. The test in this part was slightly modified from the previous study of Carmichael and others in 1987 (Carmichael et al., 1987). First, the cell at the density of $1x10^5$ cells/ml was seeded into 96-well plate and incubated at 37 °C for 24 hours. After that, various concentrations of extracts, rosmarinic acid and hydrogen peroxide were treated to

the cell incubating at 37 °C for 24 hours. The medium was removed and 0.4 mg/ml of MTT solution was treated into the cells and incubated for 4 hours. After removing MTT solution, 100% DMS0 was added and measured the absorbance at 570 nm. The test was done in triplicate and the half inhibition concentration (IC_{50}) was evaluated from the curve fitting between the percentage of cell survival and concentrations following this formula:

$$\% \text{ Cell survival} = (A_{treat} / A_{control}) \times 100$$

where A_{treat} = the absorbance of treated cell

$A_{control}$ = the absorbance of control

The obtained IC_{50} value of hydrogen peroxide was used to induce intracellular ROS in the cells.

DCFH-DA assay was slightly modified from the previous study of Shirai and others in 2002 (Shirai et al., 2002). First, the cell at the density of $1x10^5$ cells/ml was seeded into 96-well plate and incubated at 37 °C for 24 hours. After that, various concentrations of extracts and rosmarinic acid were treated to the cell incubating at 37 °C for 24 hours. The medium was removed and immediately washed the cell twice with PBS buffer (pH 7.4). DCFH-DA solution in PBS buffer at the concentration of 5 μM was added into the cell for 30 minutes. DCFH-DA solution was removed and immediately washed the cell twice with PBS buffer, then incubated the cell with 0.05 mg/ml of hydrogen peroxide for 30 min. The solution in the 96-well plate was

measured with excitation at 485 nm and emission at 535 nm. The percentage of intracellular ROS was calculated following this formula:

$$\% \text{ Intracellular ROS} = (A_{test} / A_{control}) \times 100$$

where A_{treat} = the absorbance of cell with interested substances/ 0.05 mg/ml of hydrogen peroxide pre-incubation for 30 minutes

$A_{control}$ = the absorbance of untreated cell (control)

Cytotoxicity

Comet assay

The test in this part was slightly modified from the previous study of Singh and others in 1988 (N. P. Singh et al., 1988). There were two main parts includes isolation of lymphocyte and comet assay procedure.

1.1) Isolation of lymphocytes

The isolated lymphocytes were obtained from fresh blood specimen of a healthy donor. Briefly, 6 µl of diluted fresh blood were mixed with 3 ml of Ficoll-Histopaque 1077, then the mixture was centrifuged at 1,800 rpm, 4 °C for 30 min. The lymphocytes were washed with PBS buffer (pH 7.4), then centrifuged at 1,600 rpm, 4 °C for 10 min, this step was repeated three times. After that, 5 ml of RPMI 1640 (incomplete medium) were added and the mixture was centrifuged at 1,600 rpm, 4 °C for 10 min to discharge the buffer. Finally, RPMI 1640 (complete medium)

was added to obtain the lymphocyte suspension at $4x10^5$ cells/ml. The cell suspension was aliquoted into 400 µl in each microcentrifuge tube and kept at -80 °C.

1.2) Comet assay procedure

The test in this part was slightly modified from the previous study of Peggy Olive and Judit Banáthin in 2006 (Olive & Banáth, 2006). Four microliters of lymphocyte suspension ($4x10^5$ cells/ml) were washed three times with PBS buffer (pH 7.4). Incomplete RPMI 1640 medium was added to obtain 4 ml of the suspension. For the test sample, the extracts and rosmarinic acid in 2% DMSO at the concentrations of 25, 50, 100 µg/ml were prepared. Additionally, hydrogen peroxide was used as a positive control and PBS buffer (pH 7.4) was used as negative control. Therefore, 100 µl of test sample were mixed with 100 µl of the cell suspension incubating at 37 °C for 1 hours. Then, the mixture was centrifuged at 3,000 rpm, 4 °C for 5 min. The slide was coated with 1 % normal agarose in water as the first layer and let it dry on the flat surface. After the agarose gel was firmly attached to the slide, the mixture of 1 % low melt agarose in PBS buffer (pH 7.4) and treated samples in the ratio of 1:1 at 37 °C were spread onto the gel. This second layer was covered with the coverslip, rested on the ice tray to harden the gel. Then, the coverslip was gently sided off and 0.5 % low melt agarose in PBS buffer (pH 7.4) was spread onto the second layer, rested on the ice tray to harden the gel. The slides without coverslips were immersed into a cold lysis solution for an hour. The lysis

solution was prepared from 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO and 1% Triton X-100. Next, the slides were transferred to gel electrophoresis box containing the alkaline unwinding solution (pH >13) at 0.7 v/cm for 25 minutes. The alkaline unwinding solution or electrophoresis buffer (pH >13) was prepared from 10 M NaOH and 200 mM EDTA. After the electrophoresis running, the slides were rinsed with the neutralization buffer containing 0.4 M Tris buffer (pH 7.5) for three times. The slides were stained with 20 µg/ml ethidium bromide for 5 min, then rinsed with water and covered with the coverslip. Finally, the migration of DNA (comet) was detected under the fluorescent microscope with the magnification of 400X. The captured images of the 100 comets per a slide were used for visual scoring. The degree of damage between 0 – 4 was reported as the value between 0 – 400 in arbitary units (AU) (Collins, 2002).

Cytotoxic activity using MTT assay

The purchase of various cell lines including human lung fibroblast (WI-38), human breast ductal carcinoma (BT-474), human bronchogenic carcinoma (ChaGo-K-1), human hepatocellular carcinoma (Hep G2), human gastric carcinoma (KATO III) and human colorectal adenocarcinoma (SW620) was obtained from American Type Culture Collection (ATCC). The normal cell line was WI-38 cells whereas other 5 cell lines were cancer cells. All cell lines were grown in RPMI-1640 medium, supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin to achieve the density of $1x10^5$ cells/ml in 5% CO_2 incubator at 37 ^{o}C. Leaf and stem ethanolic

extracts of *T. laurifolia*, rosmarinic acid and doxorubicin (positive control) was subjected for cytotoxic determination using MTT assay as described in the previous part.

Data analysis

The standardization parameters were carried out as grand mean ± pooled SD. Rosmarinic acid contents determined by TLC densitometry and TLC image analysis were compared using paired *t*-test ($P<0.05$). The biological activities expressed as IC_{50} were calculated from dose-response curve and equation fit by non-linear regression function of Microsoft Excel.

CHAPTER IV

RESULTS

Pharmacognostic specification of *Thunbergia laurifolia* Lindl.

Common Name	RANG-JUED
Other Names	Kamlang Chang Phueak, Khop Cha Nang, Khruea Khao Khiao
English Name	Purple Allamanda, Laurel-leaved Thunbergia, Laurel Clock Vine
Scientific Name	*Thunbergia laurifolia* Lindl.
Synonym	*Thunbergia grandiflora* Roxb.
Family	THUNBERGIACEAE
Distribution	Tropical regions worldwide
Used Part	Leaf and stem
Ethnomedical Uses	Relief of fever; for detoxifying and antidotal purposes

Macroscopic evaluation

The plant was illustrated showing botanical appearance as mentioned in flora of China that “vines 10 m or longer, petiole 1-7 cm, grooved, pubescent; leaf blade ovate to triangular-ovate, 5-10 × 4-8 cm, papery, both surfaces pubescent, palmately 3-7-veined, base subcordate to truncate, margin undulate, irregularly angular on basal half, or rarely entire, apex acuminate to acute. Flowers solitary, paired in leaf axils, or arranged in terminal racemes with 2-4 flowers per node; peduncle 4-7 cm, sulcate, pubescent; rachis pubescent with large cyathiform glands; apical

inflorescence bracts subulate to linear-subulate, 2-6 × 1-1.5 mm, pubescent; bracteoles oblong to ovate, 2.5-4 × 1.5-2.2 cm, both surfaces pubescent, 5-7-veined, base truncate, margin entire or ciliate, apex acute with a short mucro. Calyx ca. 2 mm, annular, unlobed, densely pubescent. Corolla bluish with a yellowish throat, 4-6 cm, outside glabrous; tube basally cylindric and ca. 3 mm wide for ca. 7 mm then gradually widened to ca. 5 cm at throat; limb subactinomorphic; lobes ovate, ca. 3 × 2.5 cm. Staminal filaments 7-9 mm; anther thecae pubescent, basally appendaged. Style glabrous; stigma with 2 subequal lobes. Capsule 1.2-1.5 cm, pubescent, basal part 1.3-1.8 cm in diam., beak ca. 2.5 cm (Hu, Deng, & Thomas, 2011) ” (Figure 8).

The dried leaf crude drug was green and grayish-brown while the dried stem crude drug was light yellow with gray and reddish-brown bark (Figure 9).

Microscopic evaluation

The transverse section of *T. laurifolia* dried stem was performed to reveal the anatomical characteristics including cork, parenchyma of cortex, endodermis, vessel and pith (Figure 10). The histological characteristics of *T. laurifolia* stem powder included raphide crystals, fragment of vessel, fragment of bordered pitted vessel, fragment of fiber, cork in sectional view, lignified parenchyma (Figure 11). The anatomical characteristics of *T. laurifolia* leaf midrib was shown in transverse section including upper epidermis, spongy mesophyll, palisade mesophyll, xylem vessel, parenchyma cell, collenchyma cell, phloem and lower epidermis (Figure 12). Leaf powder was investigated for the histological characteristics showing calcium oxalate

prisms, raphide crystals, fiber, stomata, scale leaf, spiral vessels, parenchyma cells, and fragment of epidermis (Figure 13).

Figure 8 Whole plant of *Thunbergia laurifolia* Lindl.

Figure 9 *Thunbergia laurifolia* Lindl. dried leaf and stem crude drugs

Microscopic

Anatomical character

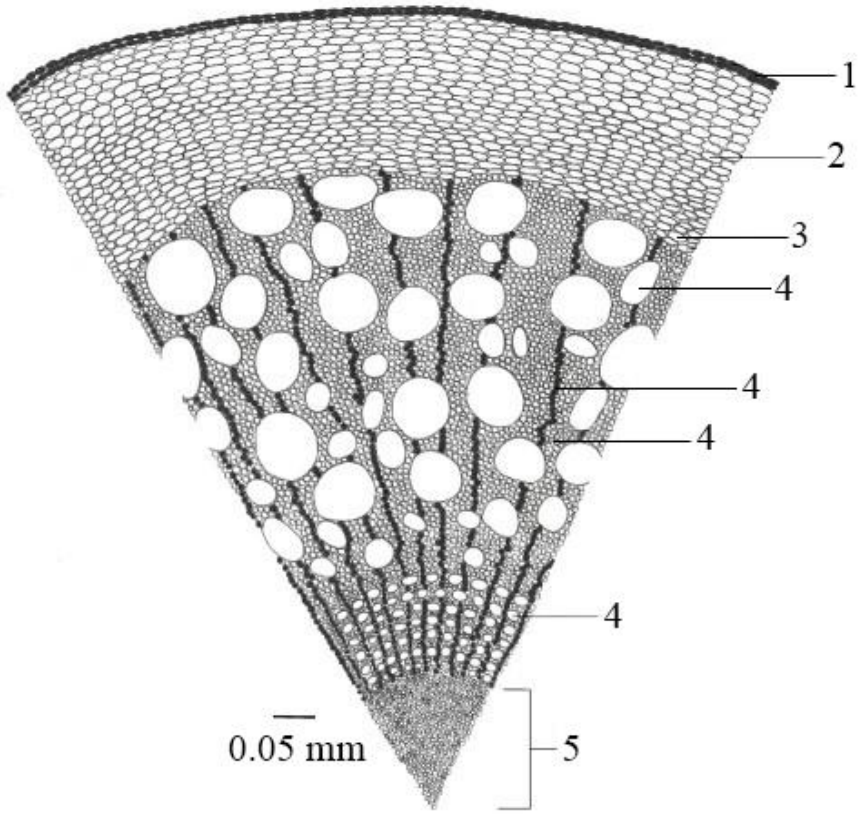


Figure 10 Transverse section of *Thunbergia laurifolia* stem

1. Cork
2. Parenchyma of cortex
3. Endodermis
4. Vessel
5. Pith

Microscopic

Histological character

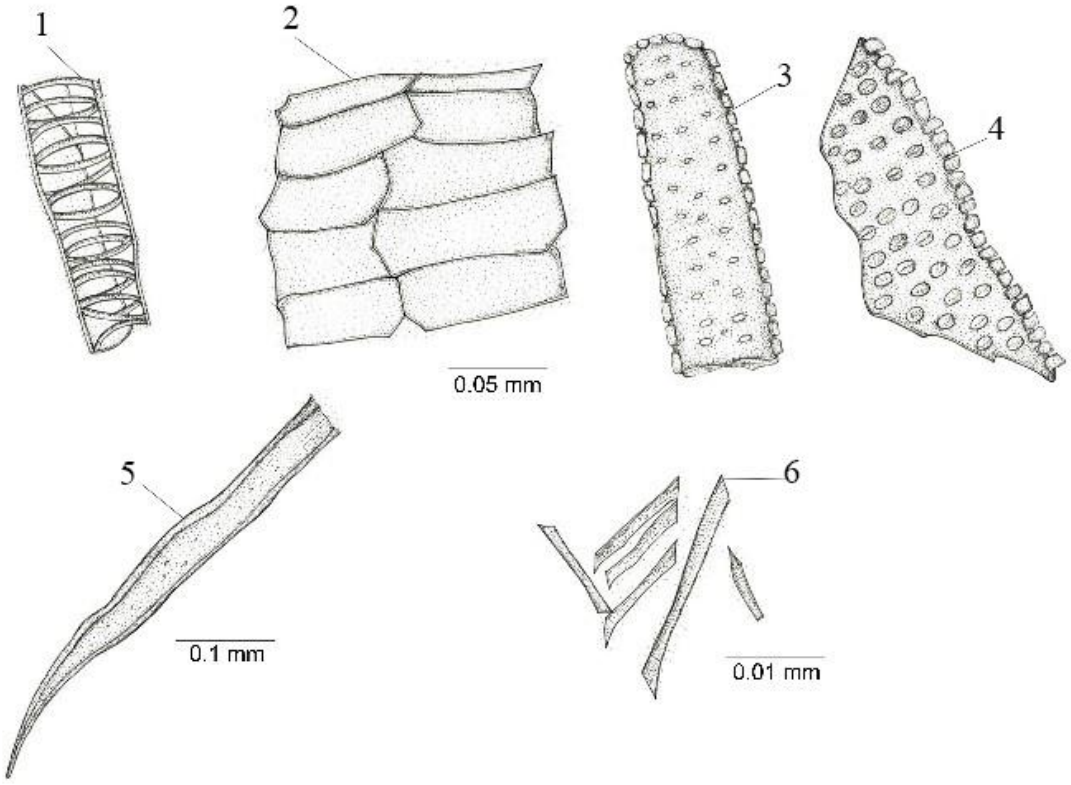


Figure 11 Powder of *Thunbergia laurifolia* stem

1. Fragment of vessel

2. Cork in sectional view

3. Lignified parenchyma

4. Fragment of bordered pitted vessel

5. Fragment of fiber

6. Raphide crystals

Microscopic

Anatomical character

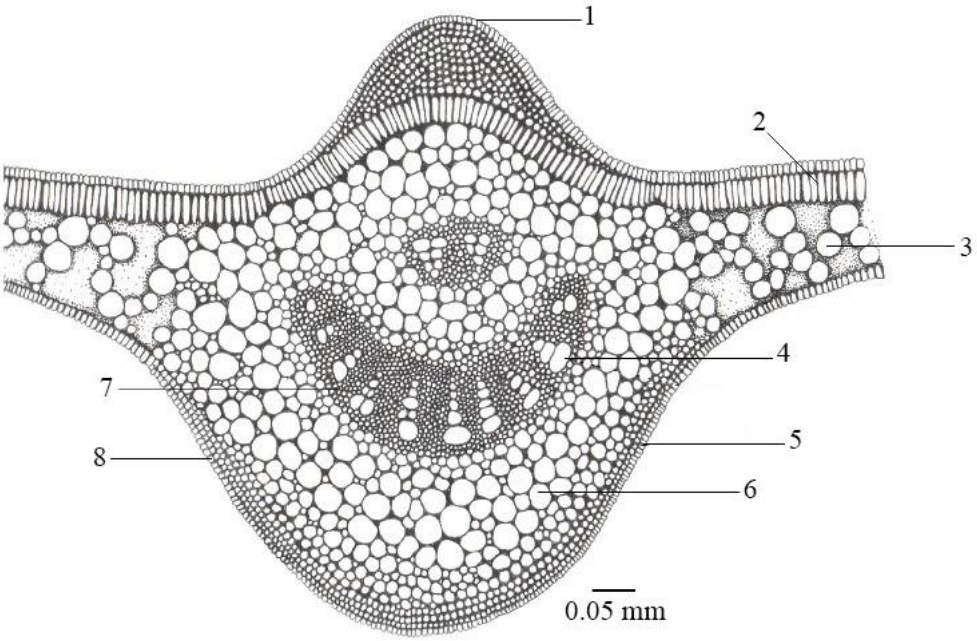


Figure 12 Transverse section of the midrib of *Thunbergia laurifolia* leaf

1. Upper epidermis
2. Palisade mesophyll
3. Spongy mesophyll
4. Xylem vessel
5. Collenchyma cell
6. Parenchyma cell
7. Phloem
8. Lower epidermis

Microscopic

Histological character

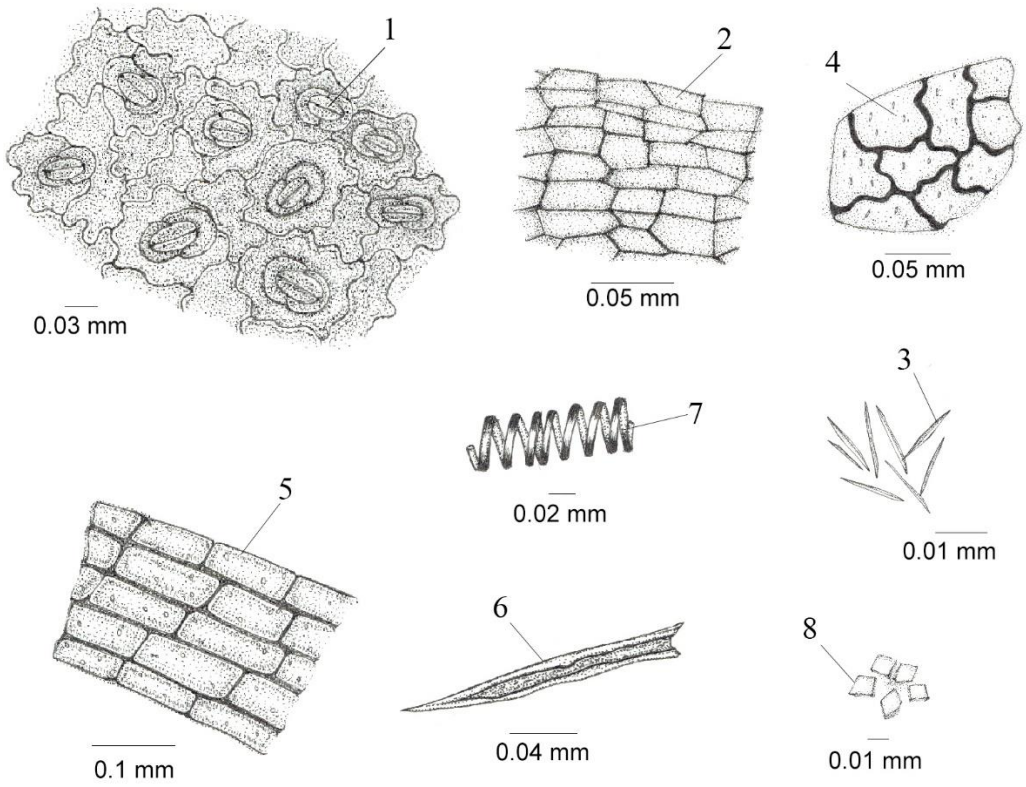


Figure 13 Powder of *Thunbergia laurifolia* leaf

1. Stomata
2. Fragment of epidermis
3. Raphide crystals
4. Scale leaf
5. Parenchyma cells
6. Fiber
7. Spiral vessels
8. Calcium oxalate prisms

Thin layer chromatographic fingerprint of *Thunbergia laurifolia* stem

One gram of *T. laurifolia* stem powder crude drug was macerated with 10 ml of 95% ethanol for 6 hours, filtered and evaporated to dryness. The residue was dissolved in 1 ml of 95% ethanol and then applied 5 μl to the thin-layer chromatographic plate, using silica gel 60 GF_{254} as the coating substance (Figure 14).

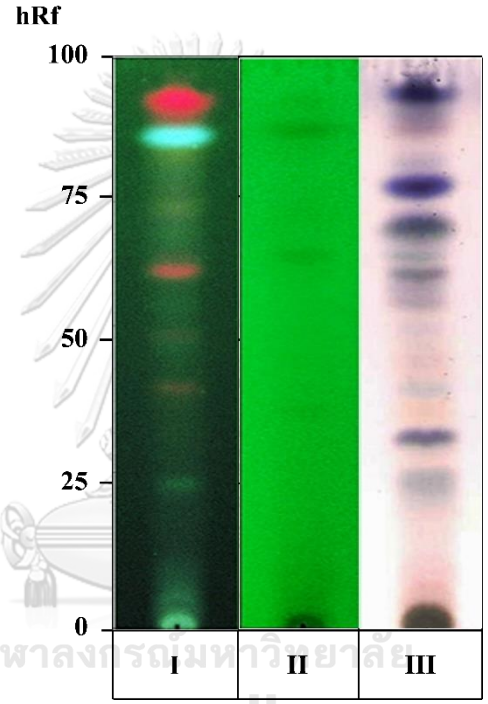


Figure 14 TLC fingerprint of ethanolic extract of *Thunbergia laurifolia* stem

Solvent system

Hexane : Dichloromethane : Methanol 12 : 80 : 12

Detection

I = detection under UV light 365 nm

II = detection under UV light 254 nm

III = detection with anisaldehyde staining reagent

Physico-chemical parameters of dried *Thunbergia laurifolia* stem

The contents of physico-chemical parameters of *T. laurifolia* stem were shown in Table 3. The results indicated the specification for the quality control of *T. laurifolia* stem crude drug that acid-insoluble ash, total ash, loss on drying and water content should not be more than 3.33, 8.44, 7.31 and 10.61% of dry weight, respectively. On the other hand, ethanol and water soluble extractive matters should not be less than 3.04 and 10.95% of dry weight, respectively. The volatile oil content in the stem was likely to be too small to give a representative yield, therefore the volatile oil content is not shown in this study.

Table 3 Physico-chemical content of *T. laurifolia* stem (% by weight)

Specification	Content (% by dry weight)*
Acid-insoluble ash	3.33 ± 0.39
Total ash	8.44 ± 0.55
Ethanol-soluble extractive	3.04 ± 0.19
Water-soluble extractive	10.95 ± 0.19
Loss on drying	7.31 ± 0.16
Water content	10.61 ± 0.43

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

Thin layer chromatographic fingerprint *Thunbergia laurifolia* leaf

One gram of *T. laurifolia* leaf powder crude drug was macerated with 10 ml of 95% ethanol for 6 hours, filtered and evaporated to dryness. The residue was dissolved in 1 ml of 95% ethanol and then applied 5 μl to the thin-layer chromatographic plate, using silica gel 60 GF_{254} as the coating substance (Figure 15).

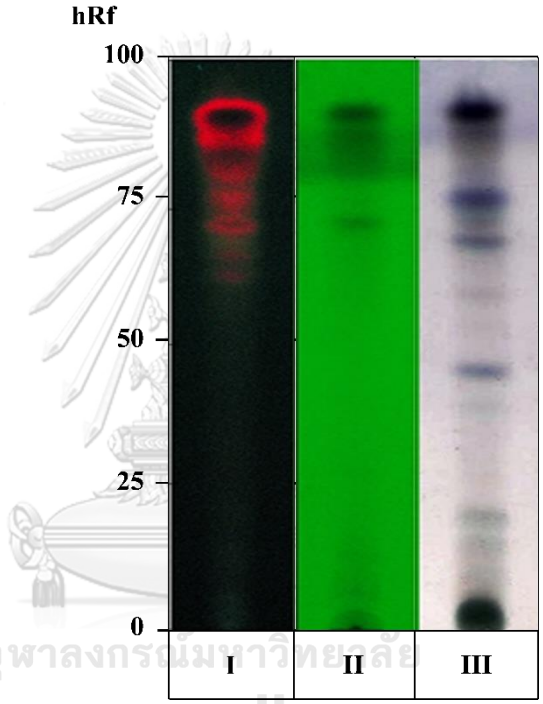


Figure 15 TLC fingerprint of ethanolic extract of *Thunbergia laurifolia* leaf

Solvent system

Hexane : Dichloromethane : Methanol 12 : 80 : 12

Detection

I = detection under UV light 365 nm

II = detection under UV light 254 nm

III = detection with anisaldehyde staining reagent

Physico-chemical parameters of dried *Thunbergia laurifolia* leaf

The contents of physico-chemical parameters of *T. laurifolia* leaf were shown in Table 4. The results indicated the specification for the quality control of *T. laurifolia* leaf crude drug that acid-insoluble ash, total ash, loss on drying and water content should not be more than 12.29, 19.71, 8.42 and 12.33% of dry weight, respectively. On the other hand, ethanol and water soluble extractive matters should not be less than 3.51 and 10.46% of dry weight, respectively. The volatile oil content in the leaf was likely to be too small to give a representative yield, therefore the volatile oil content is not shown in this study.

Table 4 Physico-chemical content of *T. laurifolia* leaf (% by weight)

Specification	Content (% by dry weight)*
Acid-insoluble ash	12.29 ± 0.43
Total ash	19.71 ± 0.20
Ethanol-soluble extractive	3.51 ± 0.16
Water-soluble extractive	10.46 ± 0.08
Loss on drying	8.42 ± 0.21
Water content	12.33 ± 0.76

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

Microscopic leaf measurement

The palisade, stomata and epidermal cells were quantitatively analyzed from 90 fields of lamina obtained from fresh mature leaves of *T. laurifolia* in three different places. Microscopic leaf constant numbers were shown in (Table 5). The images were shown in Figure 16-18. It was demonstrated that the leaf had anisocytic stomata type.

Table 5 In macroscopic leaf constant numbers of *Thunbergia laurifolia*

T. laurifolia	Lower epidermal cell number	Stomatal number	Stomatal index	Palisade ratio	Upper epidermal cell area(μm^2)
Min	328	120	22.54	6.25	183.82
Max	544	228	32.52	14.25	304.88
Mean	424.72	161.29	27.20	9.59	236.41
SD	44.13	11.72	2.13	1.31	23.85

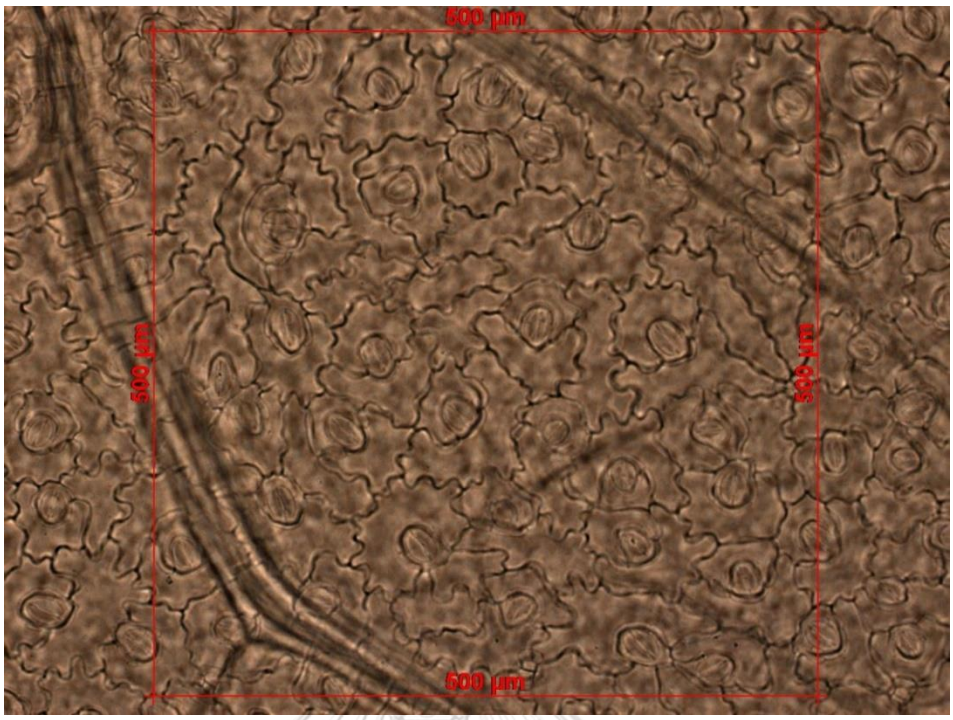


Figure 16 Stomata of *Thunbergia laurifolia* leaf (Anicocytic type)

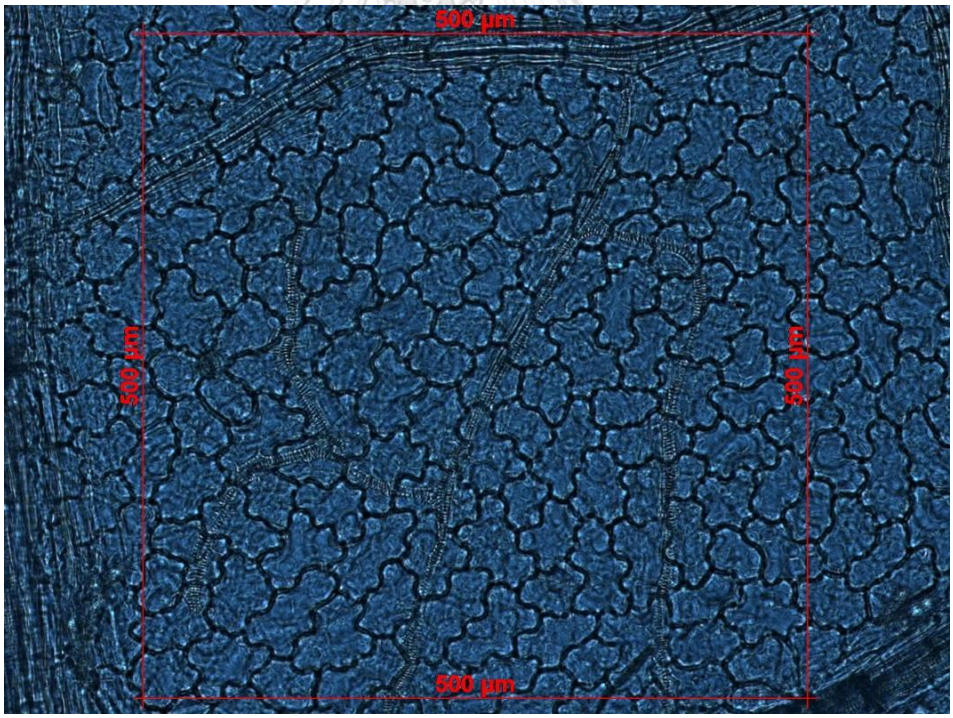


Figure 17 Epidermal cell in the upper side of *Thunbergia laurifolia* leaf

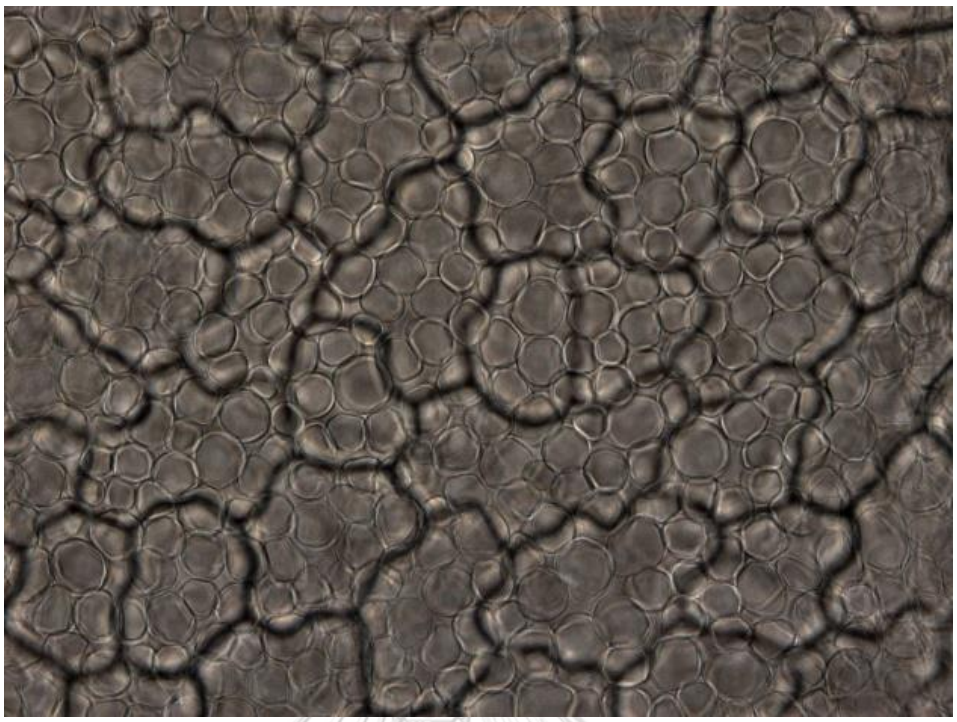


Figure 18 Palisade cells in the upper epidermis of *Thunbergia laurifolia* leaf

Quantitative analysis of rosmarinic acid in selected plant materials by TLC-densitometry and TLC-image analysis

***Thunbergia laurifolia* Lindl.**

Ethanolic extract of *Thunbergia laurifolia* leaf

The dried leaf powders of *T. laurifolia* obtained from 15 different sources throughout Thailand were exhaustively extracted with 95% ethanol by Soxhlet apparatus. The yield of *T. laurifolia* leaf ethanolic extract was 11.69 ± 4.16 % by weight. (Table 6)

Table 6 The yield of *Thunbergia laurifolia* leaf ethanolic extracts from 15 different sources throughout Thailand

Source	Weight of sample (g)	Weight of extractive matter (g)	yield (g/100g)
Bangkok	5.00	0.76	15.15
Chachoengsao	5.00	0.78	15.66
Chiang Mai	5.00	0.27	5.36
Chiangrai	5.00	0.51	10.12
Kanchanaburi	5.00	0.46	9.18
Khon Kaen	5.00	0.57	11.46
Lampang	5.00	0.43	8.66
Nakhon Pathom	5.00	0.47	9.37
Nakhon Ratchasima	5.00	0.52	10.36
Nong khai	5.00	0.67	13.40
Phitsanoulok	5.00	0.68	13.65
Prachuap Khiri Khan	5.00	0.83	16.66
Songkhla	5.00	0.37	7.30
Suphan Buri	5.00	0.40	8.09
Surin	5.00	1.05	20.91
		Average	11.69 ± 4.16
		Min	5.36
		Max	20.91

Quantitative analysis of rosmarinic acid contents in *Thunbergia laurifolia* leaf by TLC-densitometry

Rosmarinic acid standard and the leaf ethanolic extracts were developed in two solvent systems. Firstly, toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v) was used to decrease the spot tailing of the test samples. The second solvent system for rosmarinic acid separation from the extract was toluene-ethyl acetate-formic acid (5:4:1, v/v). CAMAG TLC scanner under 330 nm was used to detect rosmarinic acid on the developed silica gel60 GF_{254} TLC plate, providing the 3D TLC-densitogram of rosmarinic acid in the test samples (Figure 19). Additionally, the peak areas were computed by winCAT software exhibiting the average of rosmarinic acid content in the extracts as 0.32 ± 0.23 g/ 100g of the crude drug (Table 7).

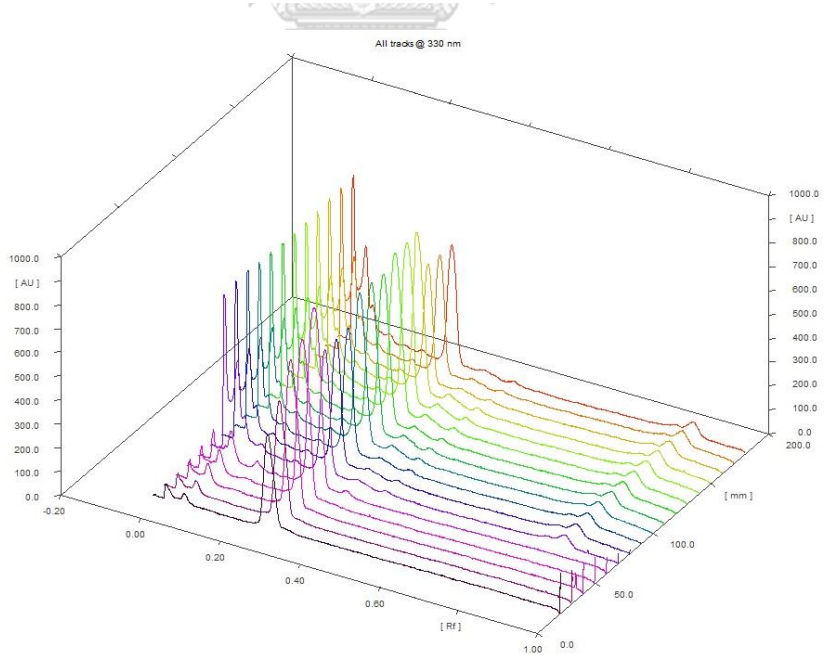


Figure 19 TLC-densitogram of rosmarinic acid standards and *Thunbergia laurifolia* leaf ethanolic extracts under UV 330 nm.

Table 7 The amount of rosmarinic acid in *Thunbergia laurifolia* leaf ethanolic extracts from 15 different sources throughout Thailand by TLC-densitometry

Source	Rosmarinic acid in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/ 100g of dried crude drug)	Rosmarinic acid in *T. laurifolia* leaf (g/ 100g of dried crude drug)
Bangkok	0.061	15.15	0.93
Chachoengsao	0.016	15.66	0.25
Chiang Mai	0.031	5.36	0.16
Chiangrai	0.037	10.12	0.37
Kanchanaburi	0.009	9.18	0.08
Khon Kaen	0.039	11.46	0.44
Lampang	0.010	8.66	0.08
Nakhon Pathom	0.031	9.37	0.29
Nakhon Ratchasima	0.019	10.36	0.19
Nong khai	0.022	13.40	0.30
Phitsanoulok	0.022	13.65	0.30
Prachuap Khiri Khan	0.013	16.66	0.22
Songkhla	0.021	7.30	0.15
Suphan Buri	0.034	8.09	0.28
Surin	0.032	20.91	0.66
Average			0.32 ± 0.23

Method validation (TLC-densitometry)

All test parameters for method validation including calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were performed by following ICH guideline.

Calibration range

The calibration curve of rosmarinic acid standard was shown as polynomial regression in the range of 1.00 – 8.00 µg/spot with the regression equation of $y = -701.95x^2 + 12286x + 12231$. The coefficient of determination (R^2) of rosmarinic acid was 0.9979 (Figure 20).

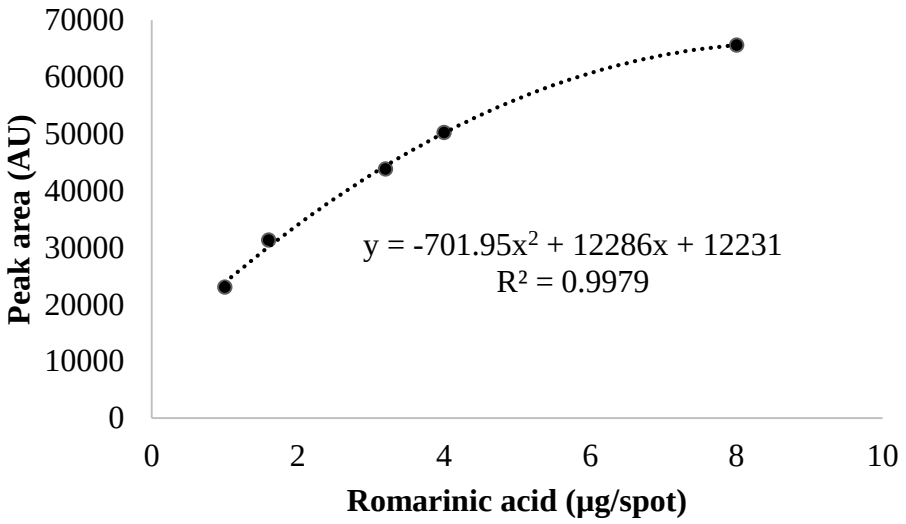


Figure 20 The calibration curve of rosmarinic acid standard by TLC-densitometry

Detection limit and quantitation limit

The detection limit (LOD) and quantitation limit (LOQ) determinations were obtained from the calculation based on the slope of the calibration curve and the standard deviation of regression line. The lowest concentration for analyte in a

sample that could be detected was found to be 0.14 µg/spot, whereas the lowest concentration for analyte in a sample that could be quantitatively defined was found to be 0.42 µg/spot.

Accuracy

The accuracy of rosmarinic acid quantification in the ethanolic extracts of *T. laurifolia* leaf was validated by using the recovery analysis. Rosmarinic acid standard was spiked into the extract, providing low, medium and high concentrations of the standard. The recovery values were 89.31 – 96.43% as shown in Table 8.

Table 8 Accuracy of quantification of rosmarinic acid in *Thunbergia laurifolia* leaf by TLC-densitometry (n=3)

Rosmarinic acid added (µg/spot)	Rosmarinic acid found (µg/spot)	% Recovery
0.00	1.27 ± 0.02	-
0.40	1.54 ± 0.02	92.14 ± 2.31
2.80	3.64 ± 0.07	89.31 ± 2.04
4.80	5.85 ± 0.23	96.41 ± 3.86
Average		92.62 ± 3.58

Precision

The precision was interpreted as %RSD at four concentrations of rosmarinic acid 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 2.22 and 3.26 %RSD, respectively (Table 9).

Table 9 Repeatability and intermediate precision of rosmarinic acid in *Thunbergia laurifolia* leaf by TLC-densitometry (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	%RSD	Amount (µg/spot)	%RSD
1.27 ± 0.02	1.50	1.25 ± 0.05	4.04
1.54 ± 0.02	1.54	1.48 ± 0.07	4.88
3.64 ± 0.07	1.94	3.65 ± 0.10	2.82
5.85 ± 0.23	3.89	5.78 ± 0.08	1.29
Average	2.22 ± 1.14		3.26 ± 1.56

Specificity

Peak identity and peak purity

The comparison of light absorption spectrum of the peak at apex among rosmarinic acid standard and all samples and the comparison of light absorption spectrum of the sample peak at up-slope, apex and down-slope were determined for peak identity (Figure 21) and peak purity (Figure 22), respectively. The maximum absorbance of rosmarinic acid was at the wavelength of 330 nm.

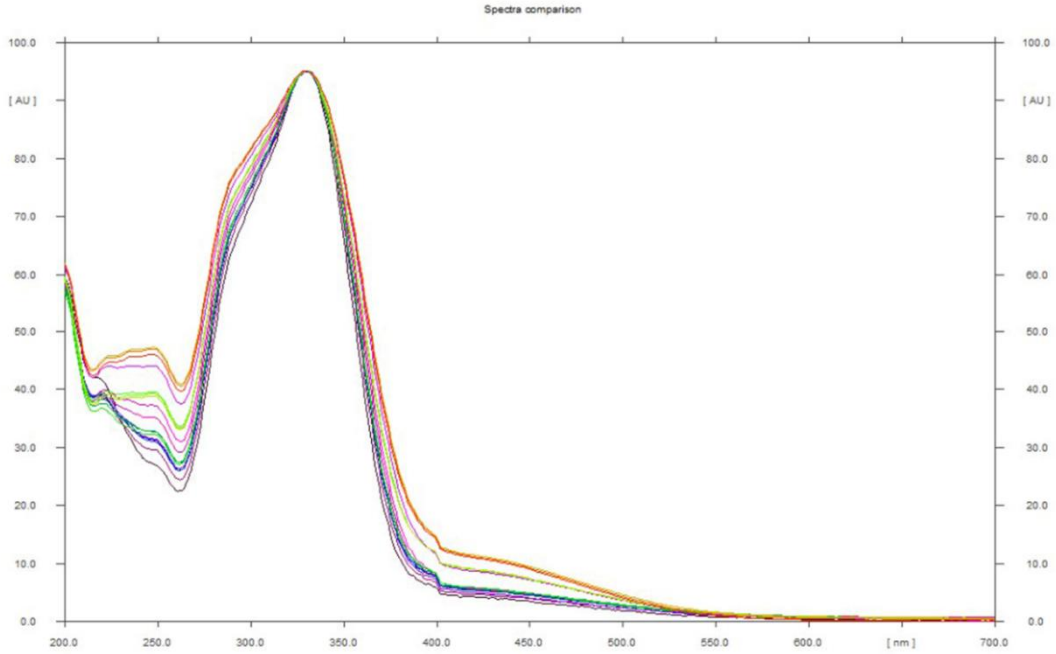


Figure 21 The absorbance spectra of rosmarinic acid in *Thunbergia laurifolia* leaf extracts from 15 different sources and rosmarinic acid standard presenting peak identity

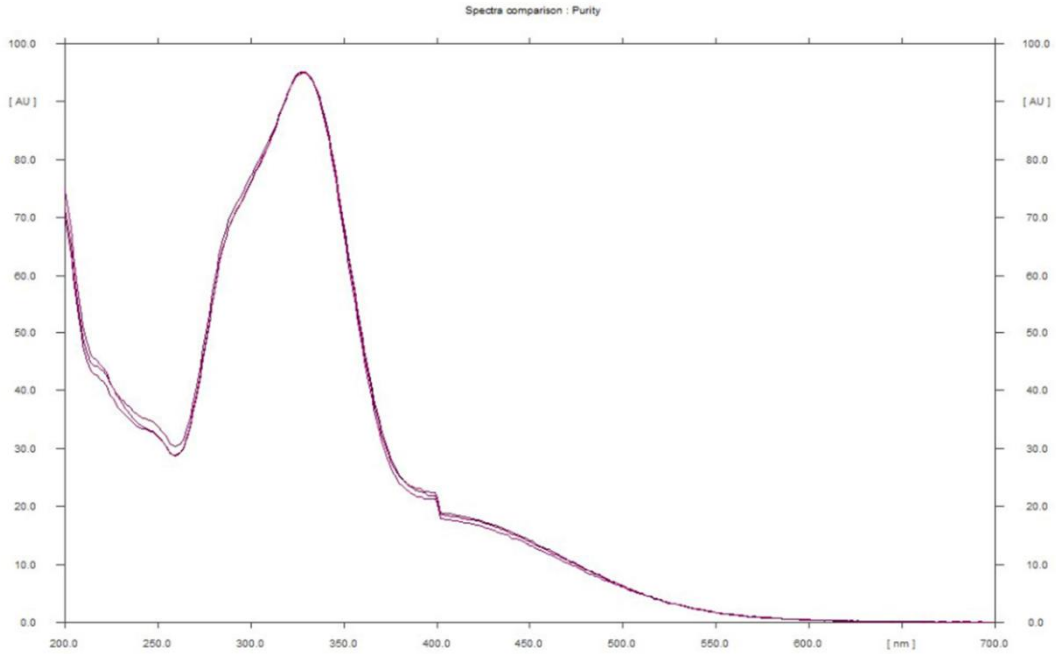


Figure 22 Peak purity measurement using up-slope, apex and down-slope of the peak

Robustness

The robustness of rosmarinic acid quantification in *Thunbergia laurifolia* leaf ethanolic extracts by TLC-densitometric analysis was investigated by varying the ratio of the second solvent system as shown in Table 10. The robustness result was found to be 3.35 %RSD of peak area.

Table 10 Robustness of rosmarinic acid in *T. laurifolia* leaf by TLC-densitometry

Mobile phase composition **Toluene: Ethyl acetate: Formic acid**	**Peak area**
5.1: 3.9: 0.9	32802.35
4.9: 4.1: 1.1	32044.66
4.8: 4.2: 0.8	34221.40
Mean ± SD	33022.80 ± 1104.99
% RSD	3.35

Quantitative analysis of rosmarinic acid contents in *Thunbergia laurifolia* leaf by TLC-image analysis

The same developed TLC plate used in TLC-densitometry was photographed under UV 365 nm by a digital camera. Then the obtained digital photograph was analyzed for rosmarinic acid peak area by ImageJ software. The average amount of rosmarinic acid was found to be 0.32 ± 0.23 g/ 100g of the crude drug (Table 11).

Table 11 The amount of rosmarinic acid in *Thunbergia laurifolia* leaf ethanolic extracts from 15 different sources throughout Thailand by TLC-image analysis

Source	Rosmarinic acid in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/ 100g of dried crude drug)	Rosmarinic acid in *T. laurifolia* leaf (g/ 100g of dried crude drug)
Bangkok	0.061	15.15	0.98
Chachoengsao	0.016	15.66	0.29
Chiang Mai	0.031	5.36	0.20
Chiangrai	0.037	10.12	0.43
Kanchanaburi	0.009	9.18	0.09
Khon Kaen	0.039	11.46	0.44
Lampang	0.010	8.66	0.09
Nakhon Pathom	0.031	9.37	0.29
Nakhon Ratchasima	0.019	10.36	0.20
Nong khai	0.022	13.40	0.31
Phitsanoulok	0.022	13.65	0.41
Prachuap Khiri Khan	0.013	16.66	0.20
Songkhla	0.021	7.30	0.14
Suphan Buri	0.034	8.09	0.27
Surin	0.032	20.91	0.59
Average			0.33 ± 0.23

Method validation (TLC-image analysis)

Similar to method validation in TLC-densitometry, all test parameters for method validation including calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were performed by following ICH guideline.

Calibration range

The calibration curve of rosmarinic acid standard was shown as polynomial regression in the range of 1.00 – 8.00 µg/spot with the regression equation of $y = -232.48x^2 + 4671.9x - 1739$. The coefficient of determination (R^2) of rosmarinic acid was 0.9987 (Figure 23).

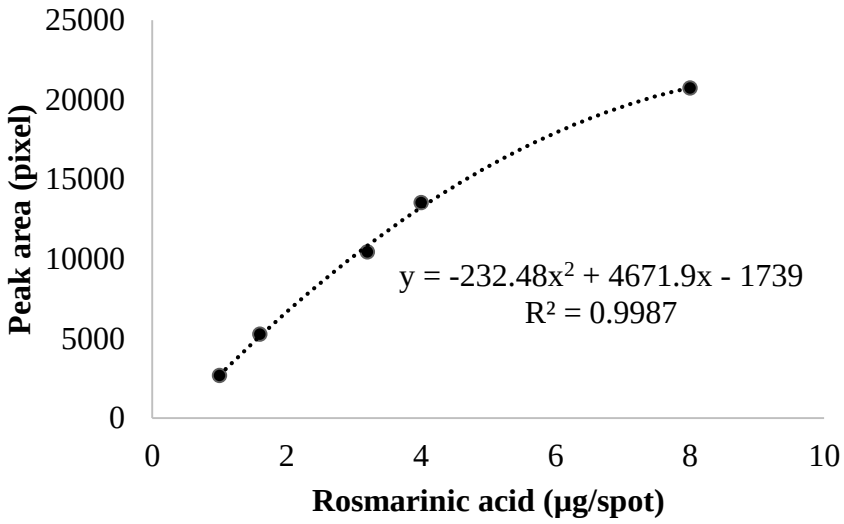


Figure 23 The calibration curve of rosmarinic acid standard by TLC-image analysis

Detection limit and quantitation limit

The lowest concentration for analyte in a sample (LOD) that could be detected was found to be 0.20 µg/spot, whereas the lowest concentration for

analyte in a sample (LOQ) that could be quantitatively defined was found to be 0.61 µg/spot.

Accuracy

The recovery values were 89.47 - 98.06 % as demonstrated in Table 12.

Table 12 Accuracy of quantification of rosmarinic acid in *Thunbergia laurifolia* leaf by TLC-image analysis (n=3)

Rosmarinic acid added (µg/spot)	Rosmarinic acid found (µg/spot)	% Recovery
0.00	1.26 ± 0.01	-
0.40	1.54 ± 0.02	92.45 ± 0.86
2.80	3.64 ± 0.06	89.47 ± 1.76
4.80	5.95 ± 0.10	98.06 ± 1.81
Average		93.33 ± 4.36

Precision

The repeatability and intermediate precision values were found to be 1.35 and 2.68 %RSD, respectively (Table 13).

Table 13 Repeatability and intermediate precision of rosmarinic acid in *Thunbergia laurifolia* leaf by TLC-image analysis (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	%RSD	Amount (µg/spot)	%RSD
1.26 ± 0.01	0.93	1.25 ± 0.04	2.91
1.54 ± 0.02	1.04	1.52 ± 0.04	2.66
3.64 ± 0.06	1.68	3.80 ± 0.11	3.00
5.95 ± 0.10	1.76	5.80 ± 0.12	2.14
Average	1.35 ± 0.43		2.68 ± 0.38

Robustness

The robustness of rosmarinic acid quantification in *Thunbergia laurifolia* leaf ethanolic extracts by TLC-image analysis was investigated by varying the ratio of the second solvent system as shown in Table 14. The robustness result was found to be 0.20 %RSD of peak area.

Table 14 Robustness of rosmarinic acid in *T. laurifolia* leaf by TLC-image analysis

Mobile phase composition **Toluene: Ethyl acetate: Formic acid**	**Peak area**
5.1: 3.9: 0.9	25097.22
4.9: 4.1: 1.1	25078.09
4.8: 4.2: 0.8	25174.10
Mean ± SD	25116.47 ± 50.82
% RSD	0.20

Method comparison

Rosmarinic acid content determined by TLC-densitometry and TLC-image analysis were demonstrated in Table 15. The obtained contents were statistically analyzed by paired t-test. The result showed that rosmarinic acid contents obtained from both methods were not significantly different ($P > 0.05$).

Table 15 Rosmarinic acid contents in Thunbergia laurifolia leaf by TLC-densitometry and TLC-image analysis

Source	Rosmarinic acid contents (g/ 100g of dried crude drug)	
	TLC-densitometry	TLC-image analysis
Bangkok	0.93	0.98
Chachoengsao	0.25	0.29
Chiang Mai	0.16	0.20
Chiangrai	0.37	0.43
Kanchanaburi	0.08	0.09
Khon Kaen	0.44	0.44
Lampang	0.08	0.09
Nakhon Pathom	0.29	0.29
Nakhon Ratchasima	0.19	0.20
Nong khai	0.30	0.31
Phitsanoulok	0.30	0.41
Prachuap Khiri Khan	0.22	0.20
Songkhla	0.15	0.14
Suphan Buri	0.28	0.27
Surin	0.66	0.59
Average	0.32 ± 0.23	0.33 ± 0.23

***Thunbergia laurifolia* stem**

Ethanolic extract of *Thunbergia laurifolia* stem

The dried stem powders of *T. laurifolia* obtained from 15 different sources throughout Thailand were exhaustively extracted with 95% ethanol by Soxhlet apparatus. The yield of *T. laurifolia* stem ethanolic extract was 11.08 ± 3.33 % by weight as demonstrated in Table 16.

Table 16 The yield of *Thunbergia laurifolia* stem ethanolic extracts from 15 different sources throughout Thailand

Source	Weight of sample (g)	Weight of extractive matter (g)	yield (g/100g)
Bangkok	5.00	0.45	8.99
Chachoengsao	5.00	0.36	7.24
Chiang Mai	5.00	0.84	16.74
Chiangrai	5.00	0.48	9.62
Kanchanaburi	5.00	0.36	7.11
Khon Kaen	5.00	0.46	9.12
Lampang	5.00	0.47	9.40
Nakhon Pathom	5.00	0.54	10.78
Nakhon Ratchasima	5.00	0.67	13.44
Nong khai	5.00	0.78	15.55
Phitsanoulok	5.00	0.41	8.13
Prachuap Khiri Khan	5.00	0.83	16.55
Songkhla	5.00	0.45	8.92
Suphan Buri	5.00	0.51	10.27
Surin	5.00	0.71	14.29
		Average	11.08 ± 3.33
		Min	7.11
		Max	16.74

Quantitative analysis of rosmarinic acid contents in *Thunbergia laurifolia* stem by TLC-densitometry

Similar to the quantitative analysis in *Thunbergia laurifolia* leaf, rosmarinic acid standard and the stem ethanolic extracts were developed in two solvent systems. Firstly, toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v) and the second solvent system for rosmarinic acid separation from the extract was toluene-ethyl acetate-formic acid (5:4:1, v/v). CAMAG TLC scanner under 330 nm was used to detect rosmarinic acid on the developed silica gel60 GF_{254} TLC plate, providing the 3D TLC-densitogram of rosmarinic acid in the test samples (Figure 24). Additionally, the peak areas were computed by winCAT software exhibiting the average of rosmarinic acid content in the stem extracts as 0.12 ± 0.08 g/ 100g of the crude drug (Table 17).

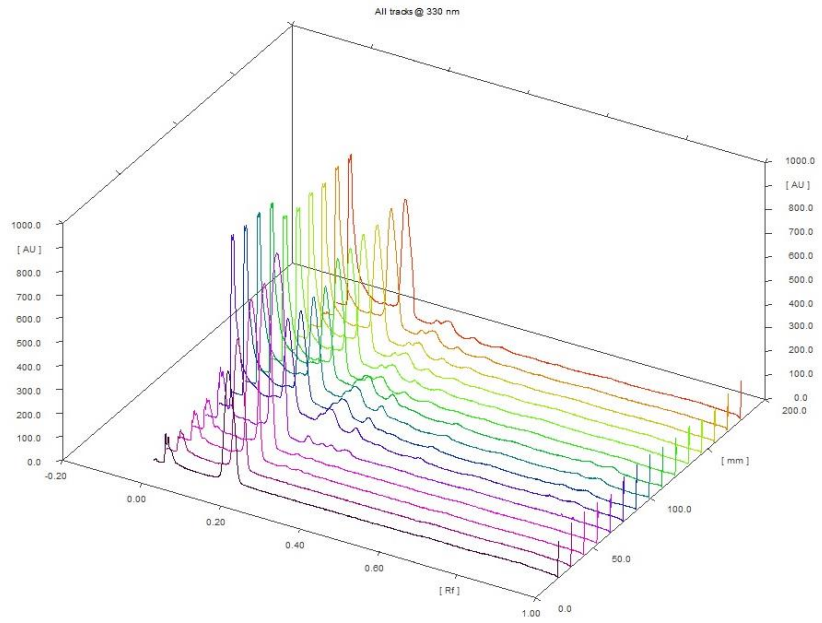


Figure 24 TLC-densitogram of rosmarinic acid standards and *Thunbergia laurifolia* stem ethanolic extracts under UV 330 nm.

Table 17 The amount of rosmarinic acid in *Thunbergia laurifolia* stem ethanolic extracts from 15 different sources throughout Thailand by TLC-densitometry

Source	Rosmarinic acid in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/ 100g of dried crude drug)	Rosmarinic acid in *T. laurifolia* stem (g/ 100g of dried crude drug)
Bangkok	0.017	8.99	0.15
Chachoengsao	0.011	7.24	0.08
Chiang Mai	0.018	16.74	0.31
Chiangrai	0.023	9.62	0.22
Kanchanaburi	0.015	7.11	0.10
Khon Kaen	0.003	9.12	0.03
Lampang	0.005	9.40	0.04
Nakhon Pathom	0.008	10.78	0.09
Nakhon Ratchasima	0.007	13.44	0.09
Nong khai	0.007	15.55	0.11
Phitsanoulok	0.008	8.13	0.06
Prachuap Khiri Khan	0.015	16.55	0.24
Songkhla	0.008	8.92	0.07
Suphan Buri	0.008	10.27	0.09
Surin	0.008	14.29	0.11
	Average		0.12 ± 0.08

Method validation (TLC-densitometry)

All test parameters for method validation including calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were performed by following ICH guideline.

Calibration range

Similar to the method validation for quantitative analysis in *Thunbergia laurifolia* leaf**,** the calibration curve of rosmarinic acid standard was shown as polynomial regression in the range of 1.00 – 8.00 µg/spot with the regression equation of $y = -577.95x^2 + 8847x + 13644$. The coefficient of determination (R^2) of rosmarinic acid was 0.996 (Figure 25).

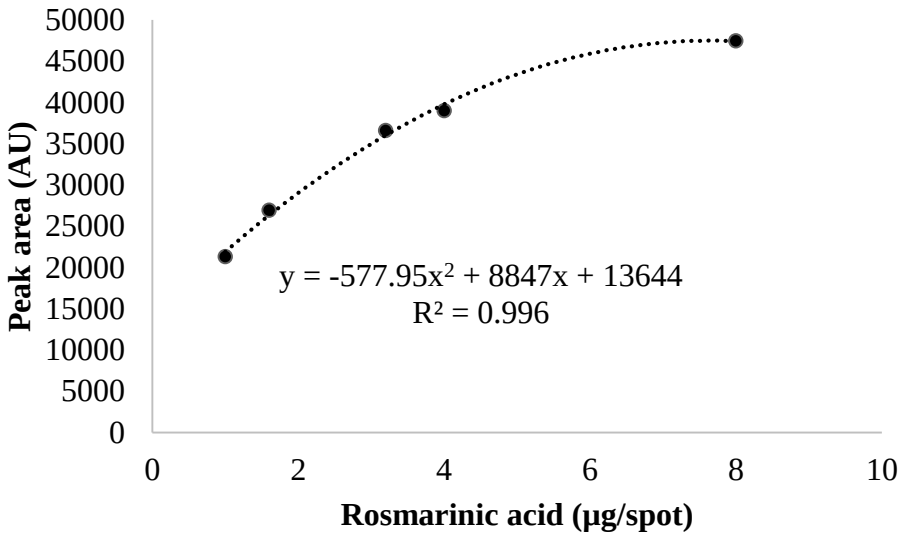


Figure 25 The calibration curve of rosmarinic acid standard by TLC-densitometry

Detection limit and quantitation limit

The lowest concentration of rosmarinic acid to analyte in a sample that could be detected was found to be 0.24 µg/spot, whereas the lowest concentration that could be quantitatively defined was found to be 0.73 µg/spot.

Accuracy

The accuracy of rosmarinic acid quantification in the ethanolic extracts of *T. laurifolia* stem was validated by using the recovery analysis. Rosmarinic acid standard was spiked into the stem extract, providing low, medium and high concentrations of the standard. The recovery values were 98.12 – 102.61% as shown in Table 18.

Table 18 Accuracy of quantification of rosmarinic acid in *Thunbergia laurifolia* stem by TLC-densitometry (n=3)

Rosmarinic acid added (µg/spot)	Rosmarinic acid found (µg/spot)	% Recovery
0.00	1.23 ± 0.10	-
0.40	1.69 ± 0.04	102.61 ± 5.25
2.40	3.58 ± 0.05	98.12 ± 2.12
4.80	6.06 ± 0.08	100.26 ± 0.95
Average		100.33 ± 2.24

Precision

The precision was interpreted as %RSD at four concentrations of rosmarinic acid in the stem 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 3.20 and 5.56 %RSD, respectively (Table 19).

Table 19 Repeatability and intermediate precision of rosmarinic acid in *Thunbergia laurifolia* stem by TLC-densitometry (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	%RSD	Amount (µg/spot)	%RSD
1.25 ± 0.01	7.99	1.24 ± 0.11	9.12
1.69 ± 0.04	2.20	1.60 ± 0.08	5.07
3.58 ± 0.05	1.34	3.43 ± 0.14	4.01
6.06 ± 0.08	1.25	6.47 ± 0.26	4.04
Average	3.20 ± 3.23		5.56 ± 2.42

Specificity

Peak identity and peak purity

The comparison of light absorption spectrum of the peak at apex among rosmarinic acid standard and stem ethanolic samples and the comparison of light absorption spectrum of the sample peak at up-slope, apex and down-slope were determined for peak identity (Figure 26) and peak purity (Figure 27), respectively. The maximum absorbance of rosmarinic acid was at the wavelength of 330 nm.

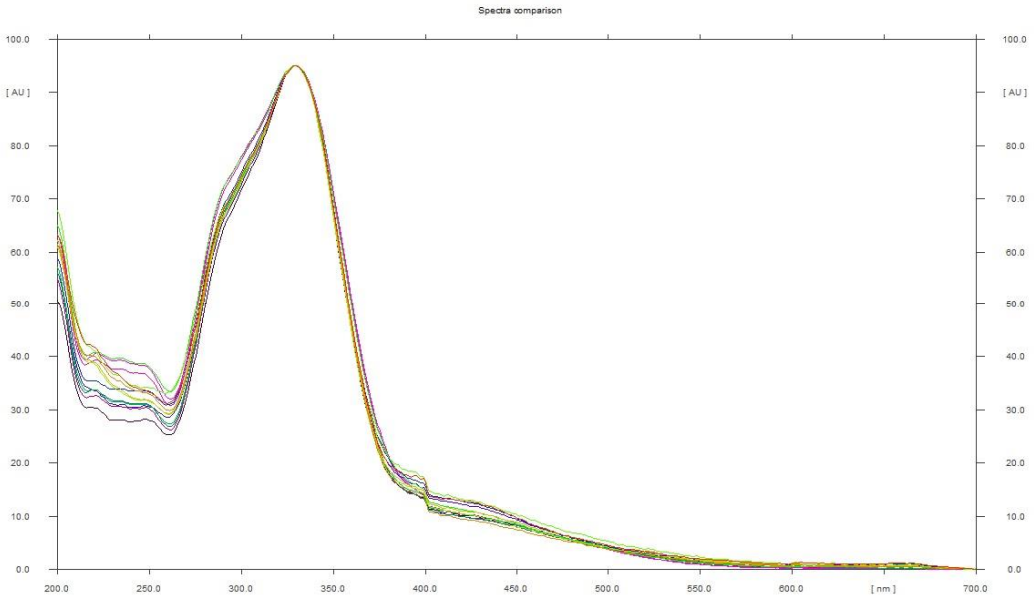


Figure 26 The absorbance spectra of rosmarinic acid in *Thunbergia laurifolia* stem extracts from 15 different sources and rosmarinic acid standard presenting peak identity

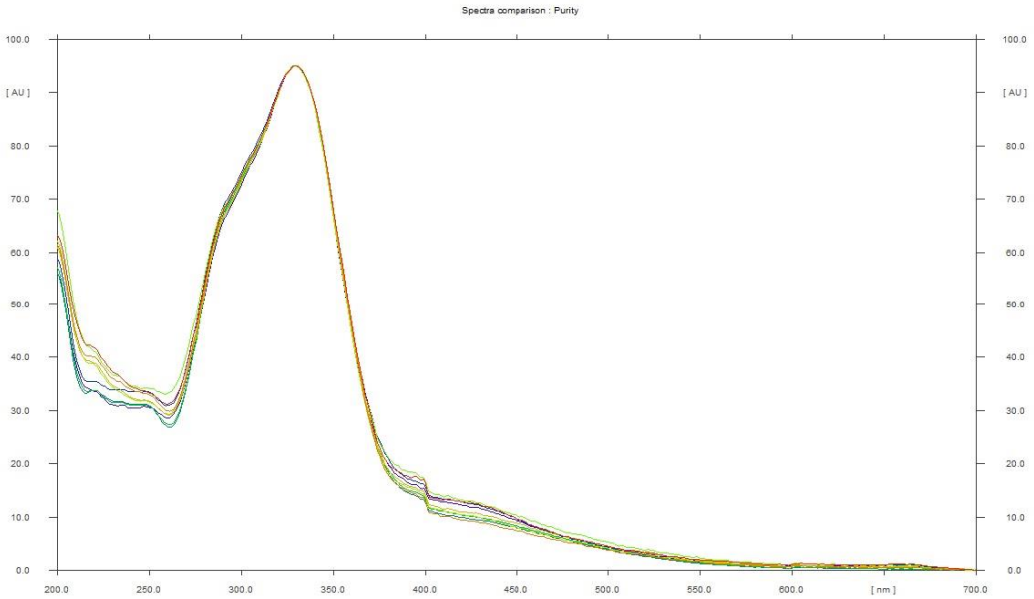


Figure 27 Peak purity measurement using up-slope, apex and down-slope of the peak

Robustness

The robustness of rosmarinic acid quantification in *Thunbergia laurifolia* stem ethanolic extracts by TLC-densitometric analysis was investigated by varying the ratio of the second solvent system as shown in Table 20. The robustness result was found to be 1.78 %RSD of peak area.

Table 20 Robustness of rosmarinic acid in *Thunbergia laurifolia* stem by TLC-densitometry

Mobile phase composition **Toluene: Ethyl acetate: Formic acid**	**Peak area**
5.1: 3.9: 0.9	28155.87
4.9: 4.1: 1.1	28375.64
4.8: 4.2: 0.8	29124.81
Mean ± SD	28552.11 ± 508.00
% RSD	1.78

Quantitative analysis of rosmarinic acid contents in *Thunbergia laurifolia* stem by TLC-image analysis

The digital photograph under UV 365 nm obtained from developed TLC plate used in TLC-densitometry was analyzed for rosmarinic acid peak area by ImageJ software. The average amount of rosmarinic acid was found to be 0.13 ± 0.09 g/ 100g of the crude drug (Table 21).

Table 21 The amount of rosmarinic acid in *Thunbergia laurifolia* stem ethanolic extracts from 15 different sources throughout Thailand by TLC-image analysis

Source	Rosmarinic acid in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/ 100g of dried crude drug)	Rosmarinic acid in *T. laurifolia* stem (g/ 100g of dried crude drug)
Bangkok	0.015	8.99	0.14
Chachoengsao	0.011	7.24	0.08
Chiang Mai	0.023	16.74	0.38
Chiangrai	0.021	9.62	0.21
Kanchanaburi	0.015	7.11	0.11
Khon Kaen	0.003	9.12	0.03
Lampang	0.005	9.40	0.05
Nakhon Pathom	0.008	10.78	0.09
Nakhon Ratchasima	0.006	13.44	0.08
Nong khai	0.008	15.55	0.13
Phitsanoulok	0.008	8.13	0.07
Prachuap Khiri Khan	0.017	16.55	0.28
Songkhla	0.009	8.92	0.08
Suphan Buri	0.009	10.27	0.09
Surin	0.007	14.29	0.11
Average			0.13 ± 0.09

Method validation (TLC-image analysis)

Similar to method validation in TLC-densitometry, all test parameters for method validation including calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were performed by following ICH guideline.

Calibration range

The calibration curve of rosmarinic acid standard was shown as polynomial regression in the range of 1.00 – 8.00 µg/spot with the regression equation of $y = -427.6x^2 + 11325x - 5009.7$. The coefficient of determination (R^2) of rosmarinic acid was 0.9998 (Figure 28).

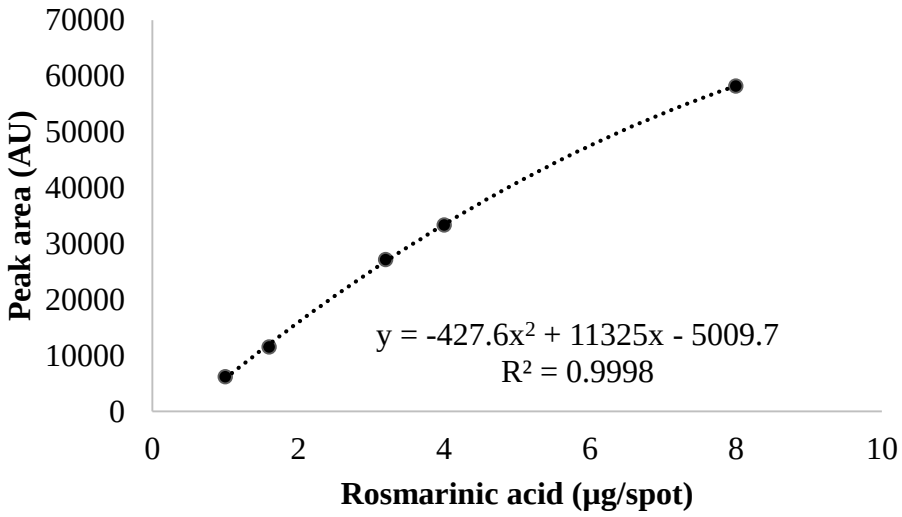


Figure 28 The calibration curve of rosmarinic acid standard by TLC-image analysis

Detection limit and quantitation limit

The lowest concentration for analyte in a sample that could be detected was found to be 0.20 µg/spot, whereas the lowest concentration for analyte in a sample that could be quantitatively defined was found to be 0.61 µg/spot.

Accuracy

The recovery values were 97.45 - 99.27 % as demonstrated in Table 22.

Table 22 Accuracy of quantification of rosmarinic acid in *Thunbergia laurifolia* stem by TLC-image analysis (n=3)

Rosmarinic acid added (µg/spot)	Rosmarinic acid found (µg/spot)	% Recovery
0.00	1.29 ± 0.10	-
0.40	1.67 ± 0.06	98.73 ± 4.33
2.40	3.60 ± 0.05	97.45 ± 1.21
4.80	6.05 ± 0.08	99.27 ± 0.82
Average		98.48 ± 0.93

Precision

The repeatability and intermediate precision values were found to be 3.39 and 4.51 %RSD, respectively (Table 23).

Table 23 Repeatability and intermediate precision of rosmarinic acid in *Thunbergia laurifolia* stem by TLC-image analysis (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	%RSD	Amount (µg/spot)	%RSD
1.29 ± 0.10	7.37	1.24 ± 0.11	8.66
1.67 ± 0.06	3.45	1.57 ± 0.06	3.79
3.60 ± 0.05	1.47	3.45 ± 0.14	4.05
6.05 ± 0.08	1.27	6.24 ± 0.10	1.53
Average	3.39 ± 2.83		4.51 ± 2.99

Robustness

The robustness of rosmarinic acid quantification in *Thunbergia laurifolia* stem ethanolic extracts by TLC-image analysis was investigated by varying the ratio of the second solvent system as shown in Table 24. The robustness result was found to be 0.34 %RSD of peak area.

Table 24 Robustness of rosmarinic acid in *Thunbergia laurifolia* stem by TLC-image analysis

Mobile phase composition **Toluene: Ethyl acetate: Formic acid**	**Peak area**
5.1: 3.9: 0.9	29323.96
4.9: 4.1: 1.1	29494.16
4.8: 4.2: 0.8	29323.04
Mean ± SD	29380.39 ± 98.53
% RSD	0.34

Method comparison

Rosmarinic acid content in *Thunbergia laurifolia* stem determined by TLC-densitometry and TLC-image analysis were demonstrated in Table 25. The obtained contents were statistically analyzed by paired t-test. The result showed that rosmarinic acid contents obtained from both methods were not significantly different ($P > 0.05$).

Table 25 Rosmarinic acid contents in *Thunbergia laurifolia* stem by TLC-densitometry and TLC-image analysis

Source	Rosmarinic acid contents (g/ 100g of dried crude drug)	
	TLC-densitometry	TLC-image analysis
Bangkok	0.15	0.14
Chachoengsao	0.08	0.08
Chiang Mai	0.31	0.38
Chiangrai	0.22	0.21
Kanchanaburi	0.10	0.11
Khon Kaen	0.03	0.03
Lampang	0.04	0.05
Nakhon Pathom	0.09	0.09
Nakhon Ratchasima	0.09	0.08
Nong khai	0.11	0.13
Phitsanoulok	0.06	0.07
Prachuap Khiri Khan	0.24	0.28
Songkhla	0.07	0.08
Suphan Buri	0.09	0.09
Surin	0.11	0.11
Average	0.12 ± 0.08	0.13 ± 0.09

***Perilla frutescens* (L.) Britton**

Ethanolic extract of *Perilla frutescens* leaf

The dried leaf powders of *P. frutescens* obtained from 15 different sources throughout Thailand were exhaustively extracted with 95% ethanol by Soxhlet apparatus. The yield of *P. frutescens* leaf ethanolic extract was 22.45 ± 4.48 % by weight in as demonstrated in Table 26.

Table 26 The yield of *Perilla frutescens* leaf ethanolic extracts from 15 different sources throughout Thailand

Source	Weight of sample (g)	Weight of extractive matter (g)	yield (g/100g)
Chiang Mai, Mueang	5.00	1.17	23.37
Chiang Rai, Mae Sai	5.00	1.20	23.89
Chiang Rai, Mueang 1	5.00	1.26	25.21
Chiang Rai, Mueang 2	5.00	1.28	25.49
Chiang Rai, Thoeng	5.00	1.30	25.96
Chiang Rai, Wiang Kaen	5.00	1.20	24.00
Lampang, Mueang 1	5.00	0.83	16.65
Lampang, Mueang 2	5.00	0.95	18.97
Lampang, Thoen	5.00	0.86	17.23
Lamphun, Mueang 1	5.00	1.04	20.78
Lamphun, Mueang 2	5.00	1.23	24.54
Lamphun, Pa Sang	5.00	1.37	27.37
Lamphun, Thung Hua Chang	5.00	1.53	30.50
Sisaket, Mueang 1	5.00	0.89	17.79
Sisaket, Mueang 2	5.00	0.75	15.02
		Average	22.45 ± 4.48
		Min	15.02
		Max	30.50

Quantitative analysis of rosmarinic acid contents in *Perilla frutescens* leaf by TLC-densitometry

Rosmarinic acid standard and *P. frutescens* leaf ethanolic extracts were developed in two solvent systems. Similar to that of *Thunbergia laurifolia*, toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v) was used as the first solvent system to decrease the spot tailing of the test samples. Then toluene-ethyl acetate-formic acid (5:4:1, v/v), the second solvent system was used for rosmarinic acid separation from the extract. CAMAG TLC scanner under 330 nm was used to detect rosmarinic acid on the developed silica gel60 GF_{254} TLC plate, providing the 3D TLC-densitogram of rosmarinic acid in the samples (Figure 29). Additionally, the peak areas were computed by winCAT software exhibiting the average of rosmarinic acid content in the extracts as 2.50 ± 1.63 g/ 100g of the crude drug (Table 27).

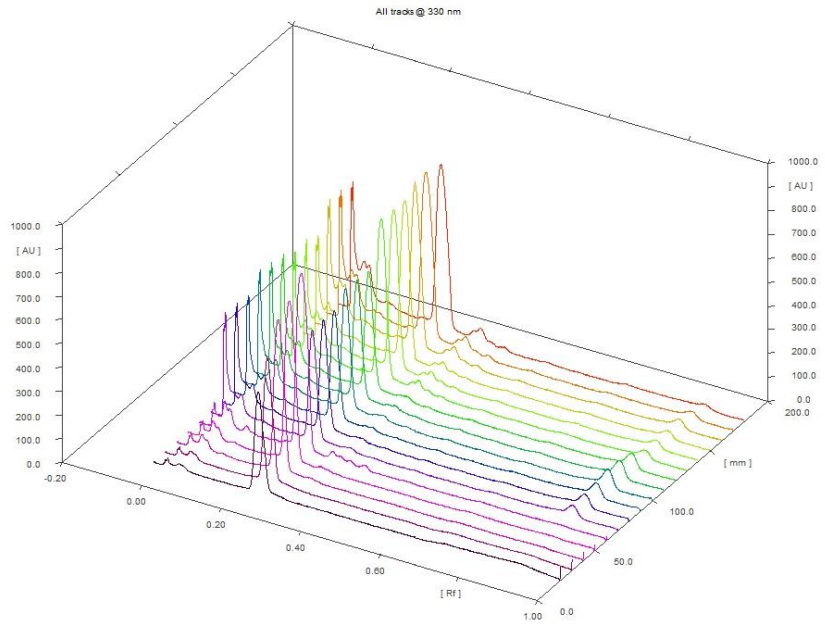


Figure 29 TLC-densitogram of rosmarinic acid standards and *Perilla frutescens* leaf ethanolic extracts under UV 330 nm.

Table 27 The amount of rosmarinic acid in *Perilla frutescens* leaf ethanolic extracts from 15 different sources throughout Thailand by TLC-densitometry

Source	Rosmarinic acid in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/ 100g of dried crude drug)	Rosmarinic acid in *P. frutescens* leaf (g/ 100g of dried crude drug)
Chiang Mai, Mueang	0.083	23.37	1.93
Chiang Rai, Mae Sai	0.139	23.89	3.32
Chiang Rai, Mueang 1	0.160	25.21	4.04
Chiang Rai, Mueang 2	0.194	25.49	4.94
Chiang Rai, Thoeng	0.136	25.96	3.53
Chiang Rai, Wiang Kaen	0.137	24.00	3.29
Lampang, Mueang 1	0.011	16.65	0.19
Lampang, Mueang 2	0.014	18.97	0.27
Lampang, Thoen	0.017	17.23	0.30
Lamphun, Mueang 1	0.136	20.78	2.82
Lamphun, Mueang 2	0.121	24.54	2.96
Lamphun, Pa Sang	0.130	27.37	3.57
Lamphun, Thung Hua Chang	0.151	30.50	4.60
Sisaket, Mueang 1	0.062	17.79	1.09
Sisaket, Mueang 2	0.047	15.02	0.71
Average			2.50 ± 1.63

Method validation (TLC-densitometry)

All test parameters for method validation including calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were performed by following ICH guideline.

Calibration range

The calibration curve of rosmarinic acid standard was shown as polynomial regression in the range of 1.00 – 8.00 µg/spot with the regression equation of $y = -545.65x^2 + 9073.1x + 11191$. The coefficient of determination (R^2) of rosmarinic acid was 0.9979 (Figure 30).

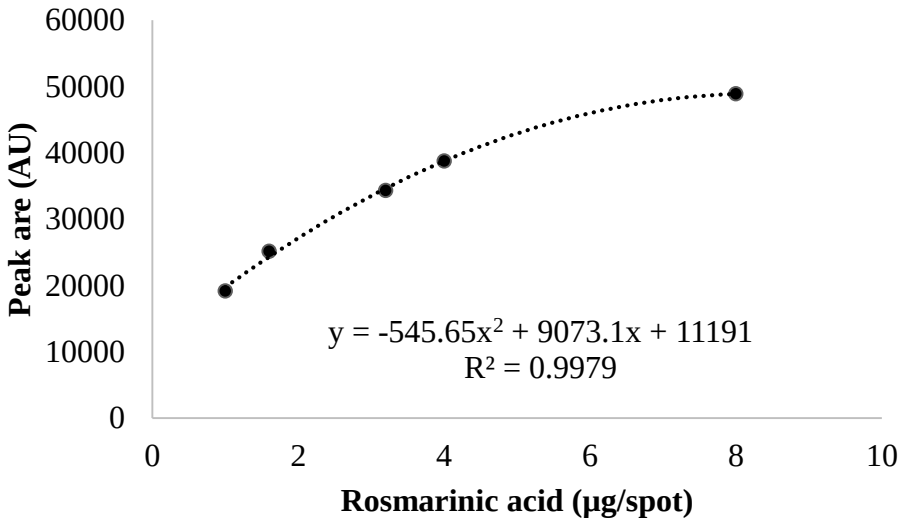


Figure 30 The calibration curve of rosmarinic acid standard by TLC-densitometry

Detection limit and quantitation limit

The lowest concentration for analyte in a sample that could be detected was found to be 0.18 µg/spot, whereas the lowest concentration for analyte in a sample that could be quantitatively defined was found to be 0.55 µg/spot.

Accuracy

The accuracy of rosmarinic acid quantification in the ethanolic extracts of *P. frutescens* leaf was validated by using the recovery analysis. Rosmarinic acid standard was spiked into the extract, providing low, medium and high concentrations of the standard. The recovery values were 98.75 – 101.73% as shown in Table 28.

Table 28 Accuracy of quantification of rosmarinic acid in *P. frutescens* leaf by TLC-densitometry (n=3)

Rosmarinic acid added (µg/spot)	Rosmarinic acid found (µg/spot)	% Recovery
0.00	1.07 ± 0.01	-
0.40	1.45 ± 0.03	98.75 ± 1.37
2.80	3.55 ± 0.02	99.38 ± 0.54
4.80	5.97 ± 0.10	101.73 ± 1.93
Average		99.95 ± 1.57

Precision

The precision was interpreted as %RSD at four concentrations of rosmarinic acid in *P. frutescens* leaf 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 1.38 and 4.32 %RSD, respectively (Table 29).

Table 29 Repeatability and intermediate precision of rosmarinic acid in *Perilla frutescens* leaf by TLC-densitometry (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	%RSD	Amount (µg/spot)	%RSD
1.07 ± 0.01	1.27	1.16 ± 0.07	6.14
1.45 ± 0.03	2.03	1.49 ± 0.01	0.45
3.55 ± 0.02	0.47	3.41 ± 0.13	3.67
5.97 ± 0.10	1.75	5.30 ± 0.37	7.02
Average	1.38 ± 0.68		4.32 ± 2.95

Specificity

Peak identity and peak purity

The comparison of light absorption spectrum of the peak at apex among rosmarinic acid standard and all samples and the comparison of light absorption spectrum of the sample peak at up-slope, apex and down-slope were determined for peak identity (Figure 31) and peak purity (Figure 32), respectively. The maximum absorbance of rosmarinic acid was at the wavelength of 330 nm.

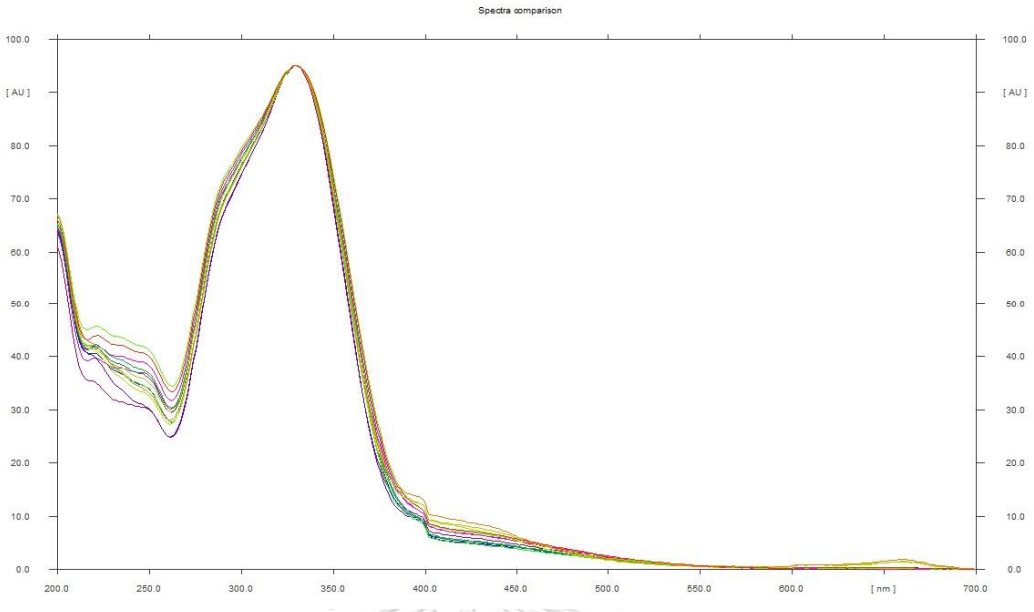


Figure 31 The absorbance spectra of rosmarinic acid in *Perilla frutescens* leaf extracts from 15 different sources and rosmarinic acid standard presenting peak identity

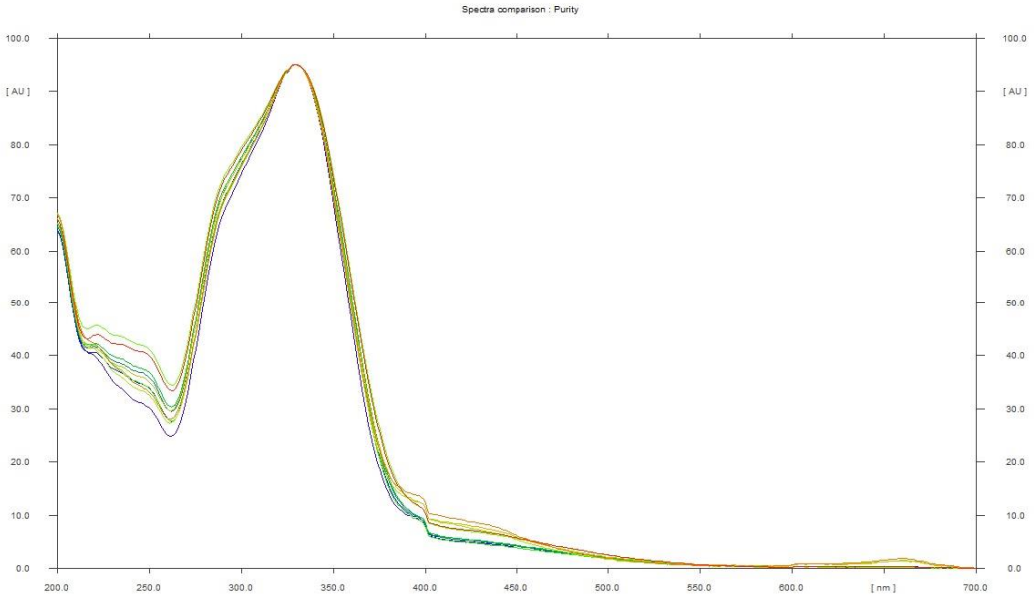


Figure 32 Peak purity measurement using up-slope, apex and down-slope of the peak

Robustness

The robustness of rosmarinic acid quantification in *Perilla frutescens* leaf ethanolic extracts by TLC-densitometric analysis was investigated by varying the ratio of the second solvent system as shown in Table 30. The robustness result was found to be 6.93 %RSD of peak area.

Table 30 Robustness of rosmarinic acid in *Perilla frutescens* leaf by TLC-densitometry

Mobile phase composition **Toluene: Ethyl acetate: Formic acid**	**Peak area**
5.1: 3.9: 0.9	29402.11
4.9: 4.1: 1.1	28556.44
4.8: 4.2: 0.8	25714.96
Mean ± SD	27891.17 ± 1931.50
% RSD	6.93

Quantitative analysis of rosmarinic acid contents in *Perilla frutescens* leaf by TLC-image analysis

The same developed TLC plate used in TLC-densitometry was photographed under UV 365 nm by a digital camera. Then the obtained digital photograph was analyzed for rosmarinic acid peak area by ImageJ software. The average amount of rosmarinic acid was found to be 2.49 ± 1.61 g/100g of the crude drug (Table 31).

Table 31 The amount of rosmarinic acid in *Perilla frutescens* leaf ethanolic extracts from 15 different sources throughout Thailand by TLC-image analysis

Source	Rosmarinic acid in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/ 100g of dried crude drug)	Rosmarinic acid in *P. frutescens* leaf (g/ 100g of dried crude drug)
Chiang Mai, Mueang	0.086	23.37	2.02
Chiang Rai, Mae Sai	0.138	23.89	3.30
Chiang Rai, Mueang 1	0.164	25.21	4.13
Chiang Rai, Mueang 2	0.187	25.49	4.78
Chiang Rai, Thoeng	0.138	25.96	3.58
Chiang Rai, Wiang Kaen	0.132	24.00	3.16
Lampang, Mueang 1	0.011	16.65	0.19
Lampang, Mueang 2	0.015	18.97	0.28
Lampang, Thoen	0.017	17.23	0.30
Lamphun, Mueang 1	0.134	20.78	2.78
Lamphun, Mueang 2	0.122	24.54	3.01
Lamphun, Pa Sang	0.130	27.37	3.55
Lamphun, Thung Hua Chang	0.146	30.50	4.46
Sisaket, Mueang 1	0.059	17.79	1.04
Sisaket, Mueang 2	0.047	15.02	0.71
Average			2.49 ± 1.61

Method validation (TLC-image analysis)

Similar to method validation in TLC-densitometry, all test parameters for method validation including calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were performed by following ICH guideline.

Calibration range

The calibration curve of rosmarinic acid standard was shown as polynomial regression in the range of 1.00 – 8.00 µg/spot with the regression equation of $y = -693.45x^2 + 10535x - 1677.4$. The coefficient of determination (R^2) of rosmarinic acid was 0.9963 (Figure 33).

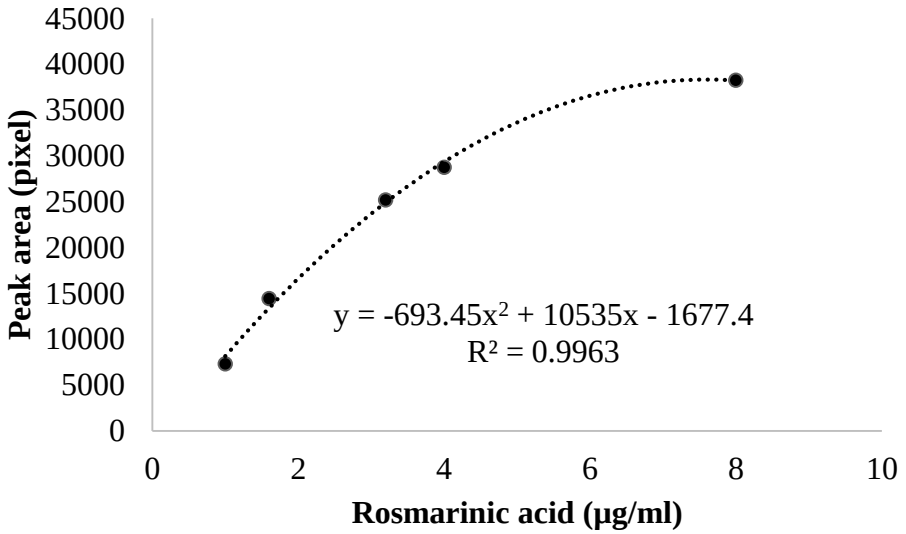


Figure 33 The calibration curve of rosmarinic acid standard by TLC-image analysis

Detection limit and quantitation limit

The lowest concentration for analyte in a sample (LOD) that could be detected was found to be 0.20 µg/spot, whereas the lowest concentration for

analyte in a sample (LOQ) that could be quantitatively defined was found to be 0.60 µg/spot.

Accuracy

The recovery values were 92.08 – 102.25 % as demonstrated in Table 32.

Table 32 Accuracy of quantification of rosmarinic acid in *Perilla frutescens* leaf by TLC-image analysis (n=3)

Rosmarinic acid added (µg/spot)	Rosmarinic acid found (µg/spot)	% Recovery
0.00	1.07 ± 0.01	-
0.40	1.44 ± 0.00	97.81 ± 0.08
2.80	3.57 ± 0.01	92.08 ± 0.11
4.80	6.01 ± 0.09	102.25 ± 1.38
Average		97.38 ± 5.10

Precision

The repeatability and intermediate precision values were found to be 0.70 and 5.15 %RSD, respectively (Table 33).

Table 33 Repeatability and intermediate precision of rosmarinic acid in *Perilla frutescens* leaf by TLC-image analysis (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	%RSD	Amount (µg/spot)	%RSD
1.07 ± 0.01	0.82	1.17 ± 0.08	6.64
1.44 ± 0.00	0.28	1.47 ± 0.03	2.01
3.57 ± 0.01	0.21	3.44 ± 0.12	3.48
6.01 ± 0.09	1.50	5.30 ± 0.45	8.49
Average	0.70 ± 0.60		5.15 ± 2.95

Robustness

The robustness of rosmarinic acid quantification in *Perilla frutescens* leaf ethanolic extracts by TLC-image analysis was investigated by varying the ratio of the second solvent system as shown in Table 34. The robustness result was found to be 6.61 %RSD of peak area.

Table 34 Robustness of rosmarinic acid in *Perilla frutescens* leaf by TLC-image analysis

Mobile phase composition **Toluene: Ethyl acetate: Formic acid**	**Peak area**
5.1: 3.9: 0.9	18221.64
4.9: 4.1: 1.1	18688.53
4.8: 4.2: 0.8	16457.47
Mean ± SD	1176.71 ± 1176.71
% RSD	6.61

Method comparison

Rosmarinic acid content determined by TLC-densitometry and TLC-image analysis were demonstrated in Table 35. The obtained contents were statistically analyzed by paired t-test. The result showed that rosmarinic acid contents obtained from both methods were not significantly different ($P > 0.05$).

Table 35 Rosmarinic acid contents in *Perilla frutescens* leaf by TLC-densitometry and TLC-image analysis

Source	Rosmarinic acid contents (g/ 100g of dried crude drug)	
	TLC-densitometry	TLC-image analysis
Chiang Mai, Mueang	1.93	2.02
Chiang Rai, Mae Sai	3.32	3.30
Chiang Rai, Mueang 1	4.04	4.13
Chiang Rai, Mueang 2	4.94	4.78
Chiang Rai, Thoeng	3.53	3.58
Chiang Rai, Wiang Kaen	3.29	3.16
Lampang, Mueang 1	0.19	0.19
Lampang, Mueang 2	0.27	0.28
Lampang, Thoen	0.30	0.30
Lamphun, Mueang 1	2.82	2.78
Lamphun, Mueang 2	2.96	3.01
Lamphun, Pa Sang	3.57	3.55
Lamphun, Thung Hua Chang	4.60	4.46
Sisaket, Mueang 1	1.09	1.04
Sisaket, Mueang 2	0.71	0.71
Average	2.50 ± 1.63	2.49 ± 1.61

In vitro biological activities

Antioxidant activities

DPPH radical scavenging activity

The antioxidant ability of the ethanolic extracts of *Thunbergia laurifolia* leaf and stem as well as rosmarinic acid was demonstrated in Table 36. Rosmarinic acid exhibited the highest antioxidant activity more than ascorbic acid, a positive control, followed by the leaf and stem extracts. The result of all samples exhibited a dose-response relationship (Figure 34).

Table 36 DPPH radical scavenging activity (IC_{50}) of *Thunbergia laurifolia* ethanolic extracts, rosmarinic acid and positive control

Test samples	IC_{50} (µg/ml)
Thunbergia laurifolia leaf extract	151.47
Thunbergia laurifolia stem extract	195.12
Rosmarinic acid	52.11
Ascorbic acid	118.29

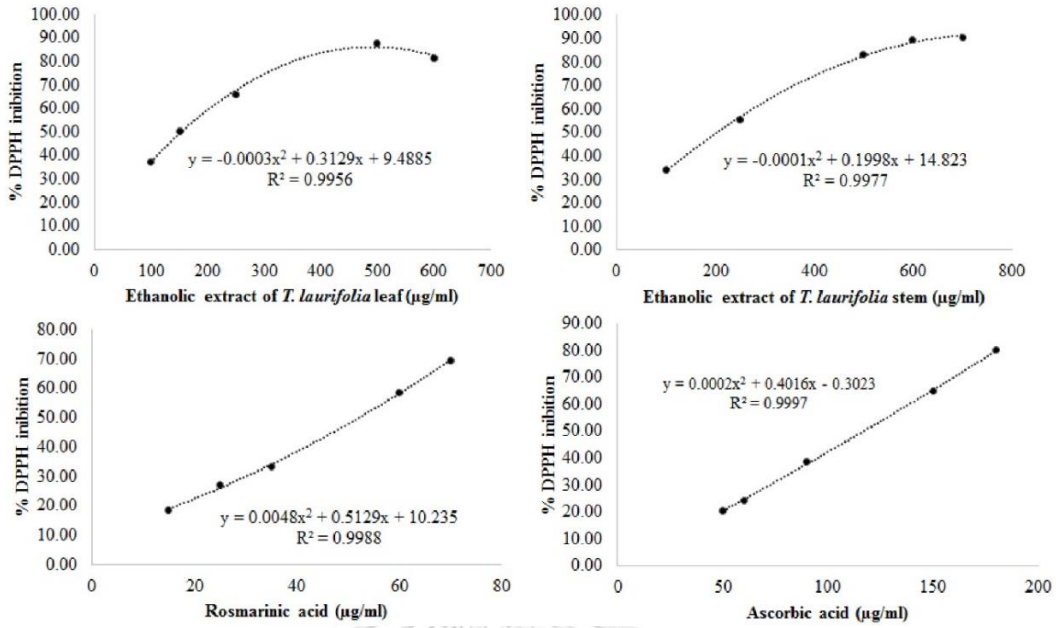


Figure 34 DPPH scavenging activity of test samples

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was used to determine antioxidant activity in the ethanolic extracts of *Thunbergia laurifolia* leaf and stem as well as rosmarinic acid (Table 37). The results were expressed as the amount of ferrous sulfate ion concentration (Figure 35). Rosmarinic acid seemed to be a good antioxidant compound due to its similar value as those of the positive controls, ascorbic acid and BHT showing FRAP values of 0.46, 0.42 and 0.55 mM Fe(II)/mg extract, respectively. Both leaf and stem ethanolic extracts exhibited reducing power ability with FRAP values of 0.26 and 0.18 mM Fe(II)/mg extract, respectively.

Table 37 Ferric reducing antioxidant power of *Thunbergia laurifolia* ethanolic extracts, rosmarinic acid and positive control

Test samples	mM Fe(II)/mg extract
Thunbergia laurifolia leaf extract	0.26
Thunbergia laurifolia stem extract	0.18
Rosmarinic acid	0.46
Ascorbic acid	0.42
BHT	0.55

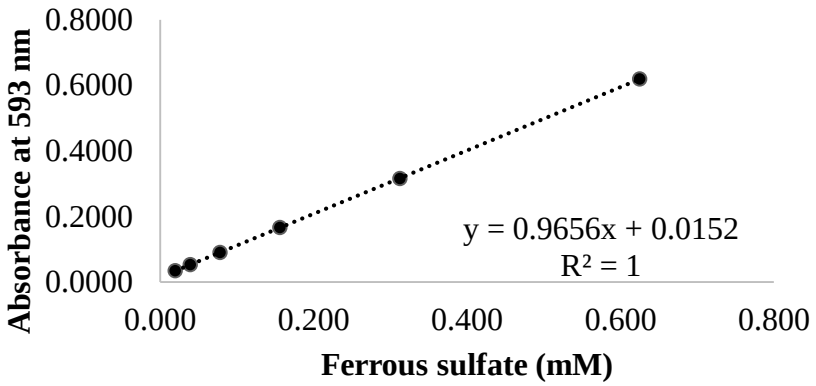


Figure 35 Standard curve of ferrous sulfate ion for ferric ion reducing antioxidant power

Beta-carotene bleaching assay

Inhibition of linoleic acid induced β-carotene bleaching was used to determine antioxidant activity of the ethanolic extracts of *Thunbergia laurifolia* leaf and stem as well as rosmarinic acid (Table 38). The highest inhibitory activity was found in rosmarinic acid whereas leaf and stem extracts had higher antioxidant

activity than the control, BHT with IC_{50} 0f 0.01, 0.19, 0.13 and 1.04 mg/ml, respectively (Figure 36).

Table 38 Beta-carotene bleaching inhibitory activity of *Thunbergia laurifolia* ethanolic extracts, rosmarinic acid and positive control

Test samples	IC_{50} (mg/ml)
Thunbergia laurifolia leaf extract	0.19
Thunbergia laurifolia stem extract	0.13
Rosmarinic acid	0.01
BHT	1.04

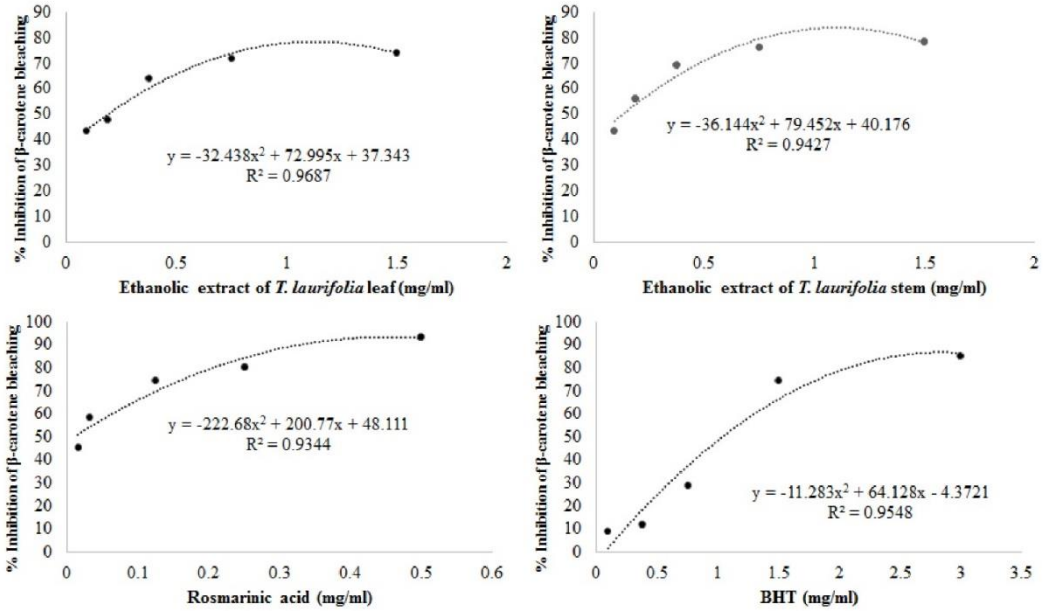


Figure 36 Inhibition of β-carotene bleaching of test samples

Effect of *T. laurifolia* leaf extract, *T. laurifolia* stem extract and rosmarinic acid on H_2O_2-induced oxidative stress in the EA.hy926 endothelial cells

Intracellular ROS measurement using DCFH-DA assay

The EA.hy926 endothelial cells were treated with various concentrations of *T. laurifolia* leaf and stem extracts, rosmarinic acid and H_2O_2 for 2 hr. It was found that the IC_{50} of cell viability assayed by MTT were 0.48, >1, 0.23 and 0.05 mg/ml, respectively (Figure 37). The IC_{50} value of H_2O_2 (0.05mg/ml) was then selected to induce intracellular ROS production which was investigated using the cell-permeable probe DCFH-DA. The results were shown as the percentage of ROS. At 1 mg/ml of the test sample, it was found that *T. laurifolia* leaf extract, rosmarinic acid and *T. laurifolia* stem extract showed marginal protection of H_2O_2 –induced oxidative stress as intracellular ROS reduction comparing to the control (100%) in 77.31 ± 0.66, 88.10 ± 6.10 and 91.30 ± 0.86% intracellular ROS, respectively (Figure 38).

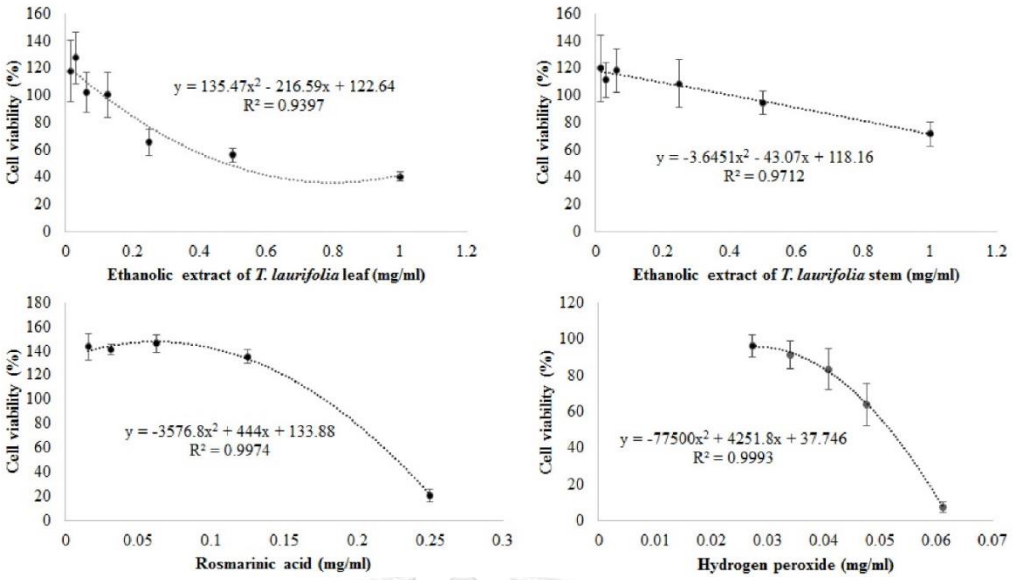


Figure 37 Effects of *Thunbergia laurifolia* ethanolic extracts, rosmarinic acid and hydrogen peroxide on cell viability of EA.hy926 cells determined by MTT assay

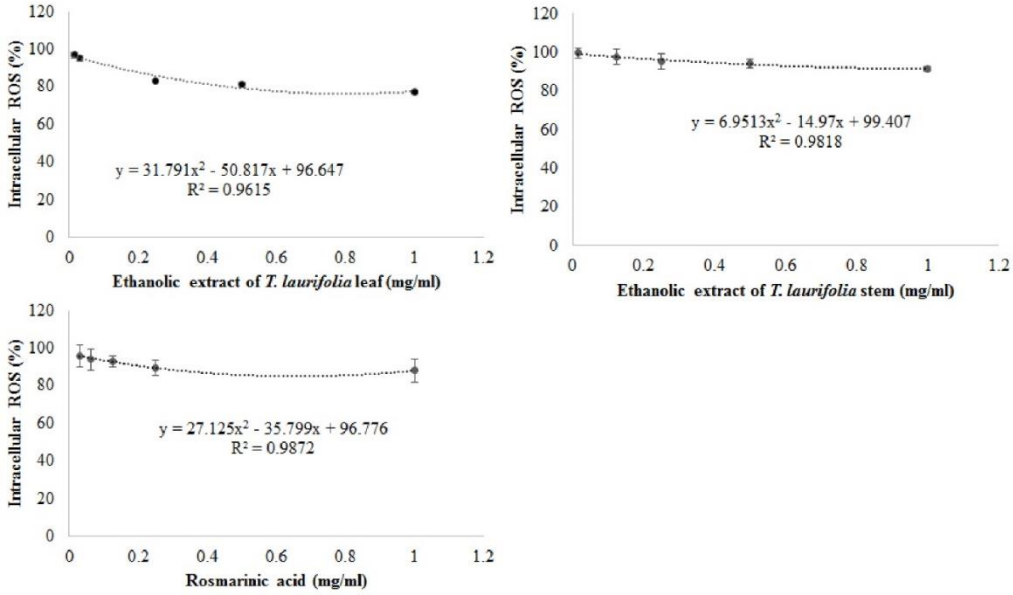


Figure 38 Effects of *Thunbergia laurifolia* ethanolic extracts and rosmarinic acid on the level of intracellular ROS production in EA.hy926 cells determined by DCFH-DA assay

Antidiabetic activities

The results of antidiabetic activities of *Thunbergia laurifolia* ethanolic extracts, rosmarinic acid and positive control were obtained from three different assays as shown in Table 39. Additionally, all test samples exhibited a dose-response relationship (Figure 39-41).

Yeast α-glucosidase inhibitory activity revealed that rosmarinic acid and *T. laurifolia* leaf extract exhibited greater potential effect than a positive control, acarbose on yeast α-glucosidase inhibition with IC_{50} of 0.31, 0.80 and 1.48 mg/ml, respectively. *T. laurifolia* stem extract exhibited the lowest potential effect on yeast α-glucosidase inhibition with IC_{50} of 5.89 mg/ml.

Rat intestinal α-glucosidase inhibitory activity revealed that rosmarinic acid exhibited greater potential effect than acarbose on rat α-glucosidase inhibition with IC_{50} of 1.68 and 1.97 mg/ml, respectively. *T. laurifolia* leaf extract showed IC_{50} of 10.13 mg/ml, whereas *T. laurifolia* stem extract still exhibited the lowest potential effect on rat α-glucosidase inhibition with IC_{50} of 77.47 mg/ml.

Porcine pancreas α-amylase inhibitory activity revealed that *T. laurifolia* leaf extract exhibited greater potential effect than rosmarinic acid and *T. laurifolia* stem extract on porcine pancreas α-amylase inhibition with IC_{50} of 4.38, 9.71 and >20 mg/ml, respectively. Acarbose showed the highest inhibition with IC_{50} of 0.01 mg/ml.

Table 39 In vitro antidiabetic activities of *Thunbergia laurifolia* ethanolic extracts, rosmarinic acid and positive control

Test samples	IC_{50} (mg/ml)		
	Yeast α-glucosidase inhibition	Rat intestinal α-glucosidase inhibition	Porcine pancreas α-amylase inhibition
T. laurifolia leaf extract	0.80	10.13	4.38
T. laurifolia stem extract	5.89	77.47	> 20
Rosmarinic acid	0.31	1.68	9.71
Acarbose	1.48	1.97	0.01

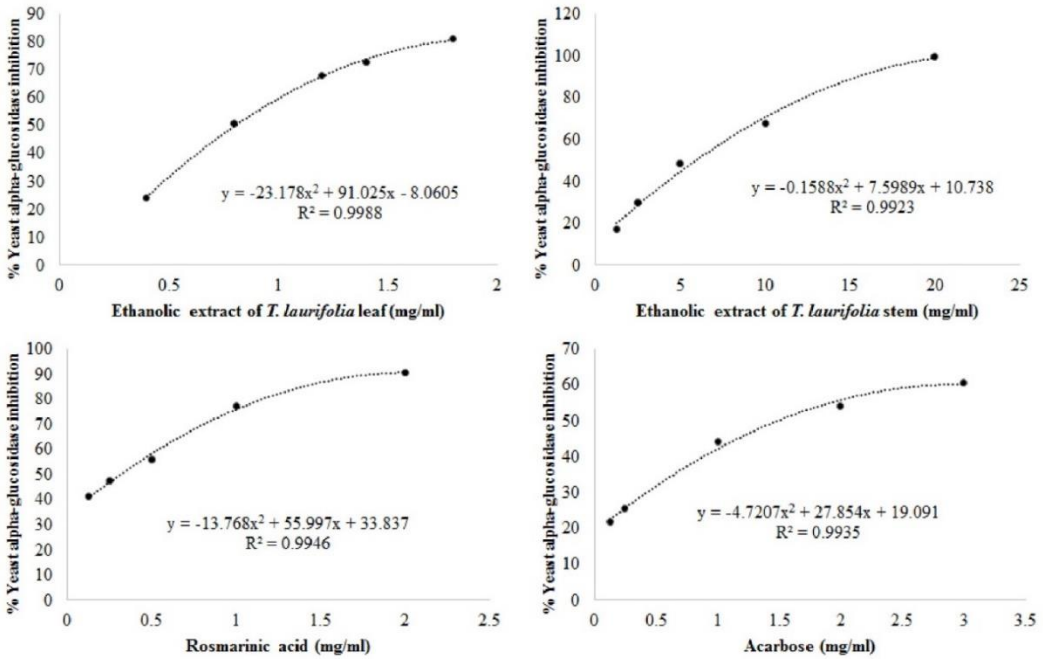


Figure 39 Yeast α-glucosidase inhibition of test samples

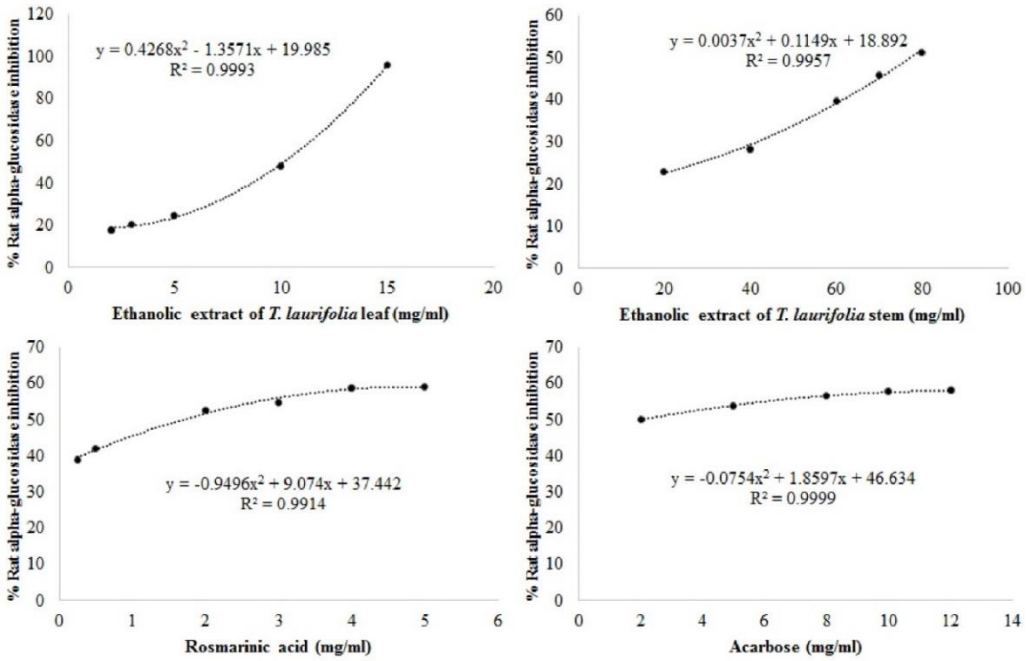


Figure 40 Rat intestinal α-glucosidase inhibition of test samples

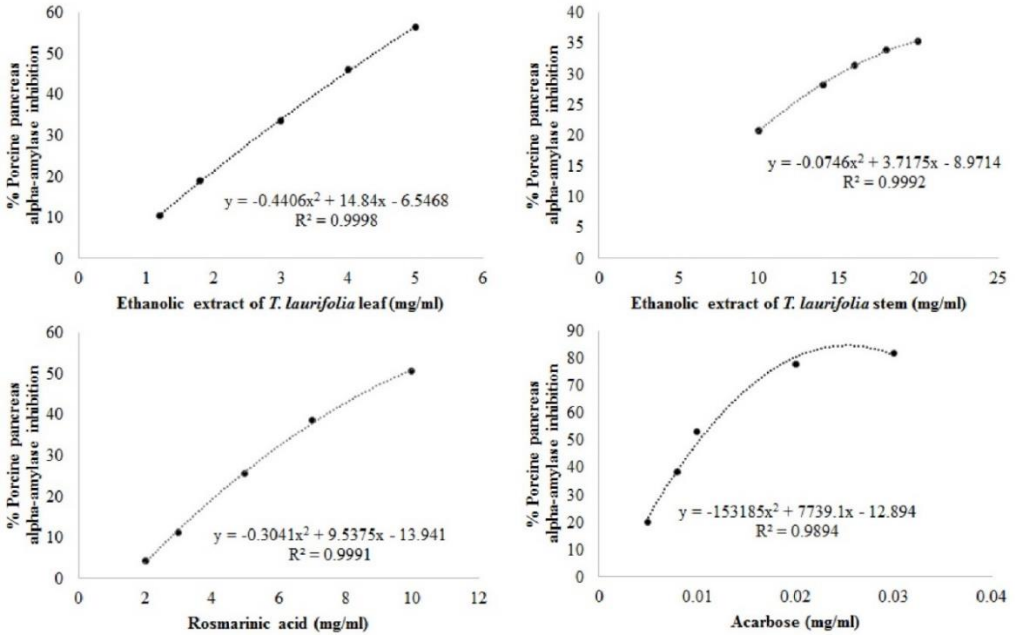


Figure 41 Porcine pancreas α-amylase inhibition of test samples

Cytotoxicity

Effect of *T. laurifolia* leaf extract, *T. laurifolia* stem extract and rosmarinic acid on lymphocyte DNA damage (Comet assay)

The assessment of DNA damage in lymphocytes treated with *T. laurifolia* ethanolic extracts, rosmarinic acid, hydrogen peroxide (positive control) and phosphate buffer saline (negative control) was expressed as the comet scores. All test samples exhibited a dose-dependent manner. Rosmarinic acid at 100 µg/ml showed the highest DNA damage followed by ethanolic stem extract and ethanolic leaf extract of *T. laurifolia* as shown in Figure 42.

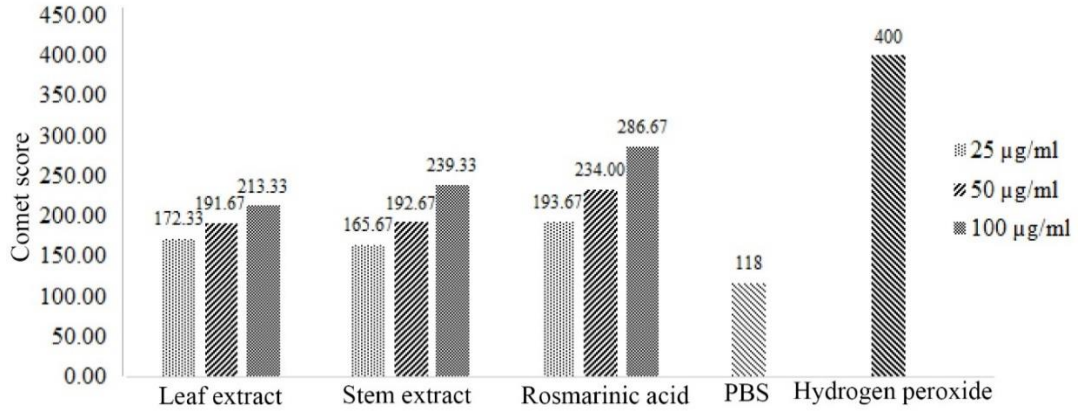


Figure 42 Total scores of DNA damage in human lymphocyte cells using comet assay

Cytotoxicity against cancer cell lines

The cytotoxicity of *T. laurifolia* ethanolic extracts and rosmarinic acid were evaluated in 5 cancer cell lines (BT-474, ChaGo-K-1, Hep G2, KATO III, and SW620) and 1 normal cell line (WI-38) using MTT cell viability assay. Doxorubicin, one of the most effective anti-cancer agent was used as a positive control in this study. The results were expressed as IC_{50} values as shown in Table 40. *T. laurifolia* leaf ethanolic extract showed the highest effect against all test cell lines.

Table 40 Cytotoxic activity (IC50) of *Thunbergia laurifolia* ethanolic extracts, rosmarinic acid and positive control against selected cell lines.

Test samples	IC_{50} (µg/ml)					
	WI-38	BT-474	ChoGo-K-1	Hep G2	KATO III	SW620
Leaf extract	45.30	38.54	29.92	31.28	36.46	63.71
Stem extract	>100	>100	>100	>100	>100	>100
Rosmarinic acid	>100	93.01	79.86	>100	>100	81.69
Doxorubicin	0.22	0.80	0.65	0.12	0.71	2.57

CHAPTER V
DISSCUSSION AND CONCLUSION

Pharmacognostic specification of *Thunbergia laurifolia* leaf and stem

Thunbergia laurifolia Lindl has been used as the traditional medicine in many Asian countries. This plant is one of the well-known medicinal plants in Thailand due to its therapeutic effects and can be locally cultivate in many parts of Thailand. Leaf and stem of the plant are commonly used to treat inflammation, poisoning and fever (Thongsaard & Marsden, 2002). Thus, the plant can be found in many drug forms for the ease of use including powder, capsule, tablet and tea. Currently, herbal plants are increasingly being sought out as medicinal products widely used for health care throughout many countries. It is therefore essential to ensure the quality of plant materials for the consumers (Sharma, 2015). Pharmacognostic specification indicates the important factors for quality assurance of herbal drugs leading to the development of standardization parameters including morphological examination, microscopical evaluation, physico-chemical parameters, leaf constant numbers, chemical fingerprint profile and active phytochemical compound.

The conventional methods which based on sensory evaluation for standardization of medicinal plant materials are macroscopic and microscopic examinations. These examinations are taken as the first step to determine identity, purity and characteristics of medicinal plant materials. Even though some characteristic appearances of the dried plant materials obtained from visual

inspection can be used for plant identification, however it is recommended to substantiate the findings by microscopic examination. In this study, anatomical and histological characteristics of *T. laurifolia* leaf and stem revealed that both samples contain raphide crystals, whereas cystoliths were not present (Figure 10-13). These findings are in agreement with the character of *Thunbergia* genus (Carlquist & Zona, 1988). Previous study of *T. laurifolia* leaf reported that the transverse section of the leaf presented the trichome at the upper epidermis which was also in agreement with this study. Moreover, the stomata type reported in the previous study was anomocytic stomata which was different from this study (anisocytic stomata). However, the stomatal index showing in Table 5 of this study (27.20) were found to be similar to that previous report (27.75) (Putiyanan, Chansakaow, Phrutivorapongkul, & Charoensup, 2008).

The physico-chemical parameters of *T. laurifolia* leaf and stem crude drugs were established (Table 3, 4). These parameters could be used as the criteria for quality control and quality assurance of *T. laurifolia* leaf and stem. The finding values of acid-insoluble ash, total ash, loss on drying and water content in both leaf and stem of *T. laurifolia* indicated the limit values to maintain good quality of the plant materials. Ash values are the criteria to judge the purity or identity of the plant materials based on the remaining inorganic substances, thus these values can be used to indicate contamination, adulteration or substitution of the plant materials. Another factor that can cause the deterioration in plant materials is water because

high amount of water in the plant materials encourages bacteria and fungi growths. Thus the limit of water content obtained from loss on drying and water content were investigated in this study. Determination of solvent extractive matter can be used to indicate the nature of chemical constituents present in the plant materials and this also assists in identification of the plants when used such a certain solvent and extraction method. Ethanol and water were used in this study because of their solvent strength. Water soluble extractive value was higher than that of ethanol in both leaf and stem of *T. laurifolia* showing that the plant materials contain high amount of polar compounds. In TLC fingerprint, leaf and stem ethanolic extracts were separated on TLC plate showing good separation of the spots when detected under ultraviolet light (254, 365 nm) and staining reagent. The obtained unique pattern of phytochemical on TLC plates were investigated to ensure the reliability of the plant materials which could be used as reference standard for further plant identification.

Quantitative analysis of rosmarinic acid in *Perilla frutescens* leaf, *Thunbergia laurifolia* leaf and stem by TLC-densitometry and TLC-image analysis

Phytochemical constituents occuring naturally in medicinal plant materials have been considered to be use as the chemical marker for identification of the plant materials. In accordance with the European Medicines Agency (EMEA) herbal quality guidelines, the quantification of a chemical maker is also recommended to strengthen the quality of the plant materials due to its therapeutic activity (European Medicines Agency, 2008). The American Herbal Pharmacopoeia (AHP) has also recommended the use of chemical marker, whether a single or several compounds in medicinal plant materials to assure the quality control (Upton, 2011). The chemical markers in the plant materials are selected from the interested chemical constituents in the plant materials with sufficient quantities and being specific for the certain plant materials. Moreover, the selected chemical makers should be detectable and quantifiable by relevant analytical method with available instruments (World Health Organization, 2017).

In this study, three medicinal plant materials including *Perilla frutescens* leaf, *Thunbergia laurifolia* leaf and stem were selected as the interested herbal medicine which commonly used and found in Thailand. Additionally, these plants also contain the same interesting compound named, rosmarinic acid, which has been proved to show numerous remarkable pharmaceutical and biological activities (Amoah et al., 2016). Therefore, rosmarinic acid was selected to be the chemical marker in

Thunbergia laurifolia leaf and stem as well as *Perilla frutescens* leaf, as the compound has been proved to be the main antioxidant agent and could be found abundantly in the plant materials (Suwanchaikasem et al., 2014; Zhu et al., 2014). The analytical method used in this study was thin layer chromatographic technique due to its ease of use, less time consuming and accessibility (Sherma & Fried, 2003). The use of two mobile phases was successful to separate rosmarinic acid from the ethanolic extracts on TLC plate. The first mobile phase was toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v) and the second mobile phase was toluene-ethyl acetate-formic acid (5:4:1, v/v). The first solvent system was developed to decrease the spot tailing of rosmarinic acid by migrating the plant pigment such as chlorophyll without any effect to rosmarinic acid. Then, the second solvent system migrated the interesting compound, rosmarinic acid showing good separation. Quantification of rosmarinic acid on TLC plate were determined using TLC-densitometer and ImageJ analysis. Densitometry is the qualitative and quantitative measurements of optical density evaluated from absorbed visible, UV light or emitted fluorescence upon excitation with UV light in the TLC plate as a light-sensitive materials (Stroka, Spangenberg, & Anklam, 2002). ImageJ analysis is a free software which can be used to quantitate the amount of chemical markers based on the pixel intensity obtained from the picture of developed TLC plate generating chromatographic peak (National Institutes of Health, 2018). Additionally, this two analytical methods are simple, inexpensive and less time consuming when compared to those complicated

instruments such as HPLC or GC analytical method. In this study, the data obtained from TLC-densitometer and TLC-ImageJ analysis were validated by following the ICH guideline, exhibiting calibration range, LOD, LOQ, accuracy, precision, specificity, and robustness.

Method validation of developed TLC-densitometric analysis and TLC-image analysis for rosmarinic acid quantification in *Perilla frutescens* leaf, *Thunbergia laurifolia* leaf and stem were found to be valid. The comparison of light absorption spectrum of the peak at apex among standard, rosmarinic acid and all samples exhibited the peak identity, whereas the comparison of the absorption spectrum of the sample peak at up-slope, apex and down-slope exhibited peak purity. The maximum absorbance of rosmarinic acid was at the wavelength of 330 nm which in accordance to previous studies that the maximum UV spectrum of rosmarinic acid could be detect at the wavelength of 330 nm (Gudzenko, 2013; Shekarchi, Hajimehdipoor, Saeidnia, Gohari, & Hamedani, 2012). Thus, the proposed methods in this study demonstrated the optimal wavelength which accurately quantified rosmarinic acid in the selected plant materials as active compound content. The recovery was investigated to determine the accuracy in the proposed methods by adding known three concentrations of standard compound in a sample. The recovery in this study was found to be within acceptable limits (80-115%) (AOAC international, 2002). The repeatability was done in the same day and the intermediate precision was done in three different days expressing as %RSD. It was found that the two

proposed methods exhibited < 10% RSD. The calibration curves were polynomial relationships with good correlation coefficients (R^2 > 0.99) in both proposed methods. The limit of detection (LOD) and limit of determination (LOQ) were based on the slope of the calibration curve and the standard deviation of regression line. In this study, the lowest concentration of rosmarinic acid in samples that could be determined with suitable precision and accuracy were found in both proposed methods. The robustness was done by varying the ratio of the second mobile phase and the results were found to be robust with no alteration of the resolution in both methods. Therefore, all of the validated data obtained from both proposed methods in *Perilla frutescens* leaf, *Thunbergia laurifolia* leaf and stem could fulfill the requirement of International Conference on Harmonisation guideline.

P. frutescens leaf exhibited the highest content of rosmarinic acid followed by *T. laurifolia* leaf and stem. The content of rosmarinic acid in *P. frutescens* leaf determined by TLC-densitometry and TLC-image analysis were 2.50 ± 1.63 (Min-max: 0.19-4.94) and 2.49 ± 1.61 (Min-max: 0.19-4.78) % w/w in the leaf crude drug, respectively. The quantitative analysis of rosmarinic acid content in *T. laurifolia* leaf determined by TLC-densitometry and TLC-image analysis were 0.32 ± 0.23 (Min-max: 0.08-0.93) and 0.33 ± 0.23 (Min-max: 0.09-0.98) % w/w in the leaf crude drug, respectively. The contents of rosmarinic acid were found to be 0.120 ± 0.08 (Min-max: 0.03-0.31) and 0.13 ± 0.09 (Min-max: 0.03-0.38) % w/w in the stem crude drug by TLC-densitometry and TLC-image analysis, respectively. Previous study on

rosmarinic acid quantification in *T. laurifolia* leaf was investigated using HPLC technique. The leaves were collected from six different places in Thailand, then the sample was macerated with 95% ethanol and evaporated to dryness. The obtained ethanolic extract of the leaf was subjected to HPLC analysis, showing rosmarinic acid content ranging from 0.16 to 5.30% by dry weight (Suwanchaikasem et al., 2014). Another study of rosmarinic acid content in *T. laurifolia* leaf was investigated using LC-MS technique. *T. laurifolia* leaf was purchased from a local farmer in Bangkok, Thailand. The leaves were extracted in hot water for an hour and filtered through filter paper. The obtained filtrate was freeze-dried. The crude extract was dissolved with water before taking to quantitatively measure the amount of rosmarinic acid by LC-MS. The aqueous extract of *T. laurifolia* leaf was subjected to LC-MS analysis, showing rosmarinic acid content 90.28±14.51 mg/kg of crude drug extract, approximately 0.00009 % by weight (Junsi, Siripongvutikorn, Yupanqui, & Usawakesmanee, 2017). The content might be varied due to many factors including the collected location, extraction technique, used solvent and quantitative analytic method (W. Yang et al., 2017). There is no report of rosmarinic acid quantification in *T. laurifolia* stem, thus this study is the first report on determination of rosmarinic acid content in *T. laurifolia* stem.

In late nineteenth-century, rosmarinic acid was isolated from *P. frutescens* leaf. The leaves were macerated in methanol and column chromatography was used to quantitatively separate the compound, yielding 0.12 mg/g (approximately, 0.012%

by weight) (Tada et al., 1996). Later in 1998, fresh leaves of *P. frutescens* were extracted with methanol, then rosmarinic acid quantitative analysis was performed using preparative HPLC technique exhibiting 0.1% of rosmarinic acid (Nakamura, Ohto, Murakami, & Ohigashi, 1998). Another study on rosmarinic acid isolation in *P. frutescens* leaf was done by using column chromatography. Rosmarinic acid content was detected by comparison of H-NMR and retention time of HPLC with authentic sample. The leaves were extracted by boiling in water for an hour, then the extract was subjected to the aforementioned methods yielding rosmarinic acid content 190 mg/ 5 g dried weight (approximately, 3.8%) (Toshiaki Makino et al., 2003). Later in 2005, the determination of rosmarinic acid content in *P. frutescens* leaf was investigated using capillary electrophoresis with electrochemical detection. The leaves was extracted with 80% ethanol in ultrasonic bath at room temperature for 2 hours. The amount of rosmarinic acid content was ranged from 0.11 - 0.18% by weigh (Peng, Ye, & Kong, 2005). Rosmarinic acid content was also evaluated in *P. frutescens* leaf as one of the main polyphenolic substances. The leaves were extracted with water–acetone–hydrochloric acid (20:80:1, v/v/v) and rosmarinic acid content was determined using HPLC technique. It was found that rosmarinic acid level was highest during April (14.3 ± 1.3 mg/g). Additionally, the leaves were obtained from five different places exhibiting rosmarinic acid content ranging from 4.69 – 14.4 mg/g (0.47 – 1.44 % by weight). Thus, the amount of rosmarinic acid acuminated in the leaves was varied depend on the season as well as the cultivated

area (Natsume, Muto, Fukuda, Tokunaga, & Osakabe, 2006). HPLC-DAD method was set up to investigate rosmarinic acid content in methanolic extract of *P. frutescens* leaf obtained from 13 different locations. It was found that the leaves yielded rosmarinic acid content ranging from 1033.00 – 3471.30 mg/kg by weight (approximately, 0.10 – 0.35%) (Liu, Wan, Zhao, & Chen, 2013). In accordance to previous reports on rosmarinic acid content in *P. frutescens* leaf which the amount of rosmarinic acid was ranged from 0.10 – 3.8% by weight, the results in this work showed the amount of rosmarinic acid from *P. frutescens* leaf ranged from 0.19 – 4.94% by weight resulting from TLC-densitometric analysis and 0.19 – 4.78% by weight resulting from TLC-image analysis. This work presents the first application of TLC-densitometric and TLC-image analyses for the determination of rosmarinic acid content in *P. frutescens* leaf.

The developed method showed its validity for rosmarinic acid quantification in three selected plant materials and could be used as an alternative quantitative method to evaluate rosmarinic acid content as the active compound in *P. frutescens* leaf, *T. laurifolia* stem and leaf. The realization of such analysis is more economical and simple in comparison to HPLC technique.

Antioxidant activities

Accumulation of free radicals or oxidants causes cellular oxidative stress which contributes towards various diseases. Therapeutic focus has therefore shifted towards the ability of medicinal plant material and its phytoconstituent, especially polyphenolic substances as the natural source of antioxidant agent (Kähkönen et al., 1999). The main action of phenolics on antioxidant activity is based on their redox properties, which allow them to have a metal chelation potential or act as hydrogen donators, singlet oxygen quenchers and reducing agents (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). There are three antioxidant activities were used to investigate the antioxidant property of *T. laurifolia* stem and leaf, including DPPH free radical scavenging activity, ferric ion reducing antioxidant power assay and β-carotene bleaching assay. In this study, *T. laurifolia* stem seemed to have lower antioxidant activity than the leaf and rosmarinic acid. This might be due to the lesser amount of rosmarinic acid in the stem than in the leaf of *T. laurifolia.*

DPPH radical scavenging activity

The purple compound of 2,2-diphenyl-1-picrylhydrazyl. (DPPH), a stable free radical was used to investigate the scavenging ability of antioxidant agent, whether through the donation of hydrogen or electron to quench the DPPH radical, resulting in delocalization of DPPH from purple to yellow (Brand et al., 1995).

The current study used exhaustively Soxhlet extraction for the ethanolic crude extracts and it was revealed that rosmarinic acid exhibited the highest antioxidant activity followed by ascorbic acid (positive control), *T. laurifolia* leaf and stem extracts with IC_{50} of 52.11, 118.29, 151.47 and 195.12 µg/ml, respectively. Chan and Lim (2006) studied dried and fresh leaves of *T. laurifolia* using microwave extraction representing as normal tea brewing with water. The results showed the microwave-dried and fresh leaves antioxidant activity with IC_{50} of 0.50 and 0.99 mg/ml, respectively. Suwanchaikasem (2011) reported that ethanolic and water extracts of *T. laurifolia* leaves exhibited IC_{50} of 199.97 and 86.04 µg/ml, respectively on DPPH assay. The use of different solvents for the extraction of *T. laurifolia* leaf revealed that water extract showed higher antioxidant activity more than ethanol and acetone extracts with IC_{50} of 3.92, 9.02 and 52.91 mg/ml, respectively (Oonsivilai et al., 2011). Sinsawat et al. (2013) indicated that the optimal temperature and time for extraction of *T. laurifolia* leaves in methanol were found to be at 90 °C for 4 hours. The fresh and dried leaves using that condition exhibited 94.60 and 92.94% of DPPH inhibition. Rosmarinic acid isolated that *T. laurifolia* leaves was reported the higher DPPH scavenging (2.71 ± 0.08 µg/ml) more than trolox (positive control) and ethanolic extract of *T. laurifolia* leaf (30.62 – 114.51 µg/ml) (Suwanchaikasem et al., 2014). In another study, rosmarinic acid standard also exhibited the highest antioxidant activity comparing to positive controls, trolox and BHT with IC_{50} of 4.6, 5.1 and 6.6 µg/ml, respectively (Zhu et al., 2014). Similarly, rosmarinic acid had higher

antioxidant activity compared to BHT and ascorbic acid with IC_{50} of 2.90, 18.80 and 3.80 µg/ml, respectively (Tepe, 2008). It can be summarized that rosmarinic acid possessed higher antioxidant activity comparing to *T. laurifolia* leaf extracts and positive control in some studies. The overall IC_{50} values of *T. laurifolia* leaf extracts may be varied due to the different solvent for extraction, plant collected location as well as method for plant preparation. This study revealed the DPPH scavenging activity of *T. laurifolia* stem with the IC_{50} of 195.12 µg/ml.

Ferric reducing antioxidant power

FRAP assay is used to determine antioxidant potential of plant materials through the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) resulting in the development of blue color which can be measured at 593 nm. The antioxidant capacity is determined as the amount of Fe^{2+} produced by the plant materials (Benzie & Strain, 1996).

In this current study, rosmarinic acid demonstrated higher reducing power potential than ascorbic acid (positive control), *T. laurifolia* leaf and stem with FRAP value of 0.47, 0.42, 0.26 and 0.18 mM Fe(II)/mg compound or extract, respectively. BHT, a positive control exhibited the highest FRAP value of 0.55 mM Fe(II)/mg. Different solvents could be used to extract *T. laurifolia* leaf including water, ethanol and acetone. It was shown that water extract exhibited the highest reducing power ability followed by ethanol and acetone. The positive controls, BHT, ascorbic acid

and trolox exhibited higher FRAP values of 1.42, 119.50 and 7.20 mmol Fe(II)/g, respectively (Oonsivilai, 2006). Likewise, water extract of *T. laurifolia* also showed the highest antioxidant activity followed by ethanol and acetone with FRAP values of 0.25, 0.04 and 0.01 mmol Fe(II)/g, respectively. However, BHT (2.37 mmol Fe(II)/g) still exhibited the highest FRAP value in the test (Oonsivilai et al., 2011). Another study reported slightly more reducing power of ethanolic extract of *T. laurifolia* leaf than water extract with FRAP values of 155.05 and 148.41 μM TE/g dry weight (Suwanchaikasem, Phadungcharoen, & Sukrong, 2013). Pure compound, rosmarinic acid dissolved in water showed reducing potential with FRAP value of 5.25 ± 0.18 mM Fe (II)/L, which was stronger than trolox and BHT with FRAP values of 1.00 and 1.11 mM Fe (II)/L, respectively (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005). In this study, rosmarinic acid demonstrated highest reductive ability compared to ascorbic acid, a positive control.

Beta-carotene bleaching inhibitory activity

Beta-carotene can be found abundantly in plants containing reddish-orange pigment and is known as antioxidant agent (Paiva & Russell, 1999). The use of this compound to investigate antioxidant activity is based on the ability of the test compound to inhibit or delay lipid peroxidation which causes the bleaching of β-carotene resulting in the reduction of color intensity due to the interruption of its double bond by oxidation (Mueller & Boehm, 2011). In this study, linoleic acid was used to initiate fatty acid oxidation, producing conjugated dienes and other volatile

products to attack and bleach the color of β-carotene. Thus, inhibition of linoleic oxidation can slow down the rate of β-carotene bleaching (Ismail, Mariod, Bagalkotkara, & Lingb, 2010).

The current study revealed that rosmarinic acid had the highest capacity to alleviate the oxidation in β-carotene bleaching. Both *T. laurifolia* stem and leaf extracts demonstrated good activity compared to BHT, a positive control with IC_{50} of 0.01, 0.13, 0.19, 1.04 mg/ml, respectively. Another study on the inhibition of linoleic acid oxidation revealed that the rosmarinic acid had the greatest percentage (100%) to inhibit β-carotene bleaching compared to BHT (96%) and ascorbic acid (95%) (Tepe, 2008). At 2 mg/ml concentration, the similar results were found by Sevgi et al. (2015) that rosmarinic acid exhibited the highest percentage (99%) to inhibit β-carotene bleaching compared to BHT (86%). Rosmarinic acid as well as *T. laurifolia* stem and leaf extracts were capable to suppress lipid peroxidation *in vitro.*

Intracellular ROS activity

Human umbilical vein endothelial cells (EA.hy926) were used for intracellular ROS assay. This immortalized cell line has been developed from human umbilical vein endothelial cells (HUVECs) fusing with lung adenocarcinoma cell line A549 (Edgell, McDonald, & Graham, 1983). This cell line has the better characterized permanent human vascular, especially the large vessel endothelium compared to other endothelial cell lines. EA.hy926 cell line is one of the most used for human

vascular investigation (Bouïs, Hospers, Meijer, Molema, & Mulder, 2001). Hydrogen peroxide was used to induce intercellular ROS as mentioned in the previous report that it could be used to encourage oxidative stress, leading to cellular dysfunction on HUVECs cell line (Chen, Gu, Shao, Luo, & Tan, 2010; Gong et al., 2010). In this study, EA.hy926 cells were treated with various concentrations of *T. laurifolia* extracts, rosmarinic acid and H_2O_2 to evaluate IC_{50} of cell lethality. The results showed does-respond relationship on EA.hy926 cells viability. However, the IC_{50} were > 200 µg/ml which were considered as no cytotoxic effect (Geran, Greenberg, MacDonald, Schumacher, & Abbott, 1972). The IC_{50} of H_2O_2 (0.05 mg/ml) was used to induce intracellular ROS in EA.hy926 cells. It was found that the intracellular ROS production seemed to be slightly decreased when the cells were treated with *T. laurifolia* leaf extract, rosmarinic acid and *T. laurifolia* stem extract. On the contrary, *T. laurifolia* leaf extract was mentioned for the decrease of ROS production in HepG2 cell line, detected by DCFH-DA assay (Rocejanasaroj, Tencomnao, & Sangkitikomol, 2014). In addition, pretreatment of HUVEC with rosmarinic acid (25-200 µM) could reduce intracellular ROS in a dose-dependent manner by 31-59% after 12 hours of incubation (Huang & Zheng, 2006). EA.hy926 cells might not be suitable cell lines for testing rosmarinic acid and the extracts containing rosmarinic acid according to tis cytotoxicity on this cell line.

Anti-diabetic activity

Dietary carbohydrate is the main source of blood sugar which can be hydrolyzed by pancreatic **α**-amylase and **α**-glucosidase at the small intestine. These two enzymes are therapeutic target to manage hyperglycemia. Therefore, suppression or inhibition of these key enzymes are considered to be the treatment for diabetes (Krentz & Bailey, 2005). Acarbose, a competitive inhibitor of **α**-glucosidase and pancreatic **α**-amylase was used as a positive control in this study due to its mechanism to interrupt substrate-binding site of the enzyme with the substrate. Additionally, it is one of the recommend medicine to treat type 2 diabetes (Calder & Geddes, 1989). It was found that rosmarinic acid exhibited the highest inhibitory activity on yeast and rat intestinal **α**-glucosidase showing IC_{50} of 0.31 and 1.68 mg/ml, respectively. *T. laurifolia* leaf extract exhibited higher inhibitory activity than the stem extract with IC_{50} of 0.80 and 5.89 mg/ml on yeast **α**-glucosidase, as well as the IC_{50} of 10.13 and 77.47 mg/ml, respectively on rat **α**-glucosidase. Furthermore, *T. laurifolia* leaf extract exhibited the higher inhibitory activity on **α**-amylase (4.38 mg/ml) more than that of rosmarinic acid (9.71 mg/ml) and *T. laurifolia* stem extract (>20 mg/ml). The study on diabetic food indicated that the chili paste mixing with roasted *T. laurifolia* leaf exhibited 99% of **α**-amylase inhibition (Jaiboon et al., 2011). Previous study by Zhu et al. (2014) on **α**-glucosidase inhibitory activity reported that the isolated rosmarinic acid showed IC_{50} value of 0.23 mg/ml. Sompong et al. (2 0 1 6) extracted *T. laurifolia* leaf and indicated that 1 mg/ml

exhibited 11.40 ± 2.58% inhibition of pancreatic α-amylase. In summary, *T. laurifolia* leaf and stem with reference to rosmarinic acid demonstrated *in vitro* inhibitory potential on starch digestive enzymes.

Cytotoxicity

MTT assay has been used as a conventional method to detect reductive metabolism in cells for viability, proliferation, cytotoxicity assays as well as screening for anti-cancer drug (Y. Li, Huang, Huang, Du, & Huang, 2012). *T. laurifolia* leaf extract demonstrated cytotoxic potential against all 5 cancer cell lines as well as 1 normal cell line used in this study. The National Cancer Institute (NCI) has set the criteria for cytotoxicity evaluation in plant extract and pure compound. The plant extract must have IC_{50} lower than 20 µg/ml, whereas the pure compound must have IC_{50} lower than 4 µg/ml (Geran et al., 1972). According to the criteria, all test samples in this study were not effective compound against cancer cell lines. In previous cytotoxic study, four cancer cell lines were treated with water, ethanolic and petroleum ether extracts of *T. laurifolia* leaf. It was found that all *T. laurifolia* leaf extracts showed IC_{50} >100 µg/ml against all the test cell lines, indicating low cytotoxicity (Oonsivilai et al., 2008). In another study, rosmarinic acid decreased cell viability of HepG2 cell lines as its concentration increased in time-dependent manner with IC50 of 33 ± 0.74 µg/ml after 48 hours of incubation (Ma et al., 2018). Another study of rosmarinic acid against the viability of HepG2 cell lines indicated the IC_{50} of 53.33 µg/ml after 5 hours

of incubation (Chaowuttikul, Palanuvej, & Ruangrungsi, 2018). This study found that *T. laurifolia* ethanolic leaf extract was more toxic against ChoGo-K-1 than other types of cell lines (IC_{50} 29.92 µg/ml). Further fractionation of this extract could be beneficial for the study on the anticancer potential of *T. laurifolia* leaf.

DNA damage effect

Comet assay was used to determine DNA damage at the level of single cell. Detection of damage cells was expressed in comet score in this study showing the does-dependent manner in all test samples. In this study, DNA damage effects were shown as a dose-dependent manner in all test samples. Previous study showed 7.00 ± 1.15, 9.33 ± 1.66 and 9.66 ± 1.33 of the comet scores obtained from *T. laurifolia* leaf extract at 25, 50, 100 µg/ml in human peripheral blood leukocytes by comet assay (Rana & Tangpong, 2017). This previous study indicated that *T. laurifolia* leaf extract exhibited dose-dependent manner relationship which was in agreement with this current study. However, the level of comet score might be varied due to many factors such as different location for plant collection, chemical used in the study, incubation time and other environment effects.

Conclusion

TLC-densitometry and TLC-image analysis were established for quantification of rosmarinic acid in *T. laurifolia* leaf and stem as well as *P. frutescens* leaf. The developed methods were found to be valid as per ICH guide line, confirming that the proposed analytical procedure employed for a quantification is suitable to determine rosmarinic acid content. This current study also provides pharmacognostic specification of *T. laurifolia* leaf and stem in Thailand for quality control and standardization of these medicinal plant materials. *T. laurifolia* leaf and stem were revealed their margined potentials on *in vitro* antioxidation, anti-diabetes and anticancer. Toxicity as in vitro DNA damage and cytotoxic potentials were demonstrated.

REFERENCES

Adomako-Bonsu, A. G., Chan, S. L., Pratten, M., & Fry, J. R. (2017). Antioxidant activity of rosmarinic acid and its principal metabolites in chemical and cellular systems: Importance of physico-chemical characteristics. *Toxicology in Vitro, 40*, 248-255.

Airoldi, C., Sironi, E., Dias, C., Marcelo, F., Martins, A., Raute, r. A. P., . . . Jimenez-Barbero, J. (2013). Natural Compounds against Alzheimer's Disease: Molecular Recognition of Aβ1–42 Peptide by Salvia sclareoides Extract and its Major Component, Rosmarinic Acid, as Investigated by NMR. *Chemistry – An Asian Journal, 8*(3), 596-602.

Amoah, S. K., Sandjo, L. P., Kratz, J. M., & Biavatti, M. W. (2016). Rosmarinic Acid – Pharmaceutical and Clinical Aspects. *Planta Medica, 82*(05), 388-406.

Andrade, M. A., das Graças Cardoso, M., de Andrade, J., Silva, L. F., Teixeira, M. L., Valério Resende, J. M., . . . Barroso, J. G. (2013). Chemical Composition and Antioxidant Activity of Essential Oils from *Cinnamodendron dinisii* Schwacke and Siparuna guianensis Aublet. *Antioxidants, 2*(4).

Angelopoulou, R., Lavranos, G., & Manolakou, P. (2009). ROS in the aging male: Model diseases with ROS-related pathophysiology. *Reproductive Toxicology, 28*(2), 167-171.

AOAC international. (2002). *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* Arlington:

Association of Official Analytical Chemists.

Aritajat, S., Wutteerapol, S., & Saenphet, K. (2004). Anti-diabetic effect of *Thunbergia laurifolia* Linn. aqueous extract. *The Southeast Asian Journal of Tropical Medicine and Public Health, 35*(2), 53-58.

ASEAN Task Force on Traditional Medicine. (2014). *Herbal Medicines Used in Primary Health Care in ASEAN* (Anchalee Chuthaputi, Pornthip Termwiset, & Benjama Boonterm Eds.). Thailand: Department of Thai Traditional and Alternative Medicine.

Association of analytical communities. (2002). *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*. Retrieved from https://www.aoac.org/aoac_prod_imis/AOAC_Docs/Standards Development/SLV_Guidelines_Dietary_Supplements.pdf

Bachheti, R. K., Archana, J., & Tofik, A. (2014). A Phytopharmacological Overview on Perilla frutescens. *International Journal of Pharmaceutical Sciences Review and Research, 26*(2), 55-61.

Benzie, I. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry, 239*(1), 70-76.

Boonyarikpunchai, W., Sukrong, S., & Towiwat, P. (2014). Antinociceptive and anti-inflammatory effects of rosmarinic acid isolated from *Thunbergia laurifolia*

Lindl. *Pharmacology Biochemistry and Behavior, 124*, 67-73.

Bouïs, D., Hospers, G. A. P., Meijer, C., Molema, G., & Mulder, N. H. (2001). Endothelium in vitro: A review of human vascular endothelial cell lines for blood vessel-related research. *Angiogenesis, 4*(2), 91-102. doi:10.1023/a:1012259529167

Brand, W. W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology, 28*(1), 25-30.

Britton, N. L. (1894). *Perilla frutescens* (Linnaeus) Britton Retrieved from http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=124553. Flora of China Retrieved 4th April 2018, from Torrey Botanical Club http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=124553

Calder, P. C., & Geddes, R. (1989). Acarbose is a competitive inhibitor of mammalian lysosomal acid α-d-glucosidases. *Carbohydrate Research, 191*(1), 71-78. doi:https://doi.org/10.1016/0008-6215(89)85047-5

Carlquist, S., & Zona, S. (1988). Wood anatomy of Acanthaceae: a survey. *Aliso, 12*(1), 201-227.

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., & Mitchell, J. B. (1987). Evaluation of a Tetrazolium-based Semiautomated Colorimetric Assay: Assessment of Chemosensitivity Testing. *Cancer Research, 47*(4), 936.

Chahawirat, A. (2000). *The protective effect of Thunbergia laurifolia extract on ethanol induced hepatotoxicity in mice.* (Master Degree), Mahidol University, Mahidol

University. (20000245)

Chan, E. W. C., Eng, S. Y., Tan, Y. P., & Wong, Z. C. (2011). Phytochemistry and Pharmacological Properties of *Thunbergia laurifolia*: A Review. *Pharmacognosy Journal, 3*(24), 2.

Chan, E. W. C., & Lim, Y. Y. (2006). Antioxidant activity of *Thunbergia laurifolia* tea. *Journal of Tropical Forest Science, 18*(2), 130-136.

Chanaphant, S. (2010). *ISOLATION AND SCREENING FOR ENDOPHYTIC ACTINOMYCETES WITH INHIBITORY EFFECTS ON TESTED MICROORGANISMS AND THEIR APPLICATIONS.* (Master of Science), Silpakorn University, Silpakorn University. (51313209)

Chao, P. Y., Huang, W. Y., Hu, S. P., Lo, H. F., Lin, K. H., Huang, M. Y., . . . Yang, C. M. (2013). Indigenous Purple Vegetable Extracts Protect against Hydrogen Peroxide-Induced DNA Damage in Human Lymphocytes. *Food and Nutrition Sciences, 4*, 62-70.

Chaowuttikul, C., Palanuvej, C., & Ruangrungsi, N. (2018). IN VITRO BIOLOGICAL ACTIVITIES OF Lonicera japonica FLOWERING BUD COMPARED TO CHLOROGENIC ACID, ROSMARINIC ACID AND CAFFEIC ACID. *Thai Bulletin of Pharmaceutical Sciences, 13*(1), 27-34.

Charumanee, S., Vejabhikul, S., Taesotikul, T., Netsingha, W., Sirisaad, P., & Leelapornpisit, P. (1998). *Development of topical anti-inflammatory*

preparations from Thunbergia laurifolia Lindl. Retrieved from Chiangmai University, Thaialnd:

Chattaviriya, P., Morkmek, N., Lertprasertsuke, N., & Ruangyuttikarn, W. (2010). Drinking *Thunbergia laurifolia* Lindl. Leaf Extract Helps Prevent Renal Toxicity Induced by Cadmium in Rats. *Thai Journal of Toxicology, 25*(2), 124-132.

Chen, J., Gu, Y., Shao, Z., Luo, J., & Tan, Z. (2010). Propofol protects against hydrogen peroxide-induced oxidative stress and cell dysfunction in human umbilical vein endothelial cells. *Molecular and Cellular Biochemistry, 339*(1), 43-54. doi:10.1007/s11010-009-0368-y

Cho, B. O., Park, H. Y., Ryu, H. W., Jin, C. H., Choi, D. S., Kim, D. S., . . . Jeong, I. Y. (2011). Protective Effect of *Perilla frutescens* cv. Chookyoupjaso Mutant Water Extract against Oxidative Injury *in vitro* and *in vivo*. *Food Science and Biotechnology, 20*(6), 1705-1711.

Choi, U. K., Lee, O. H., Lim, S. I., & Kim, Y. C. (2010). Optimization of Antibacterial Activity of *Perilla frutescens* var. *acuta* Leaf against Pseudomonas aeruginosa Using the Evolutionary Operation-Factorial Design Technique. *International Journal of Molecular Sciences, 11*(10), 3922-3932.

Chuakul, W. (1996). *Herbal medicine of local Lanna* (W. Chuakul Ed.). Bangkok, Thailand: Department of pharmaceutical botany, aculty of pharmacy, Mahidol University.

Collins, A. R. (2002). The Comet Assay. In V. V. Didenko (Ed.), *In Situ Detection of DNA Damage: Methods and Protocols* (pp. 163-177). Totowa, NJ: Humana Press.

Edgell, C. J., McDonald, C. C., & Graham, J. B. (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proceedings of the National Academy of Sciences of the United States of America, 80*(12), 3734-3737. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6407019

European Medicines Agency. (2008). Reflection paper on markers used for quantitative and qualitative analysis of herbal medicinal products and traditional herbal medicinal products. Retrieved from https://www.ema.europa.eu/documents/scientific-guideline/draft-reflection-paper-markers-used-quantitative-qualitative-analysis-herbal-medicinal-products_en.pdf

Fadel, O., El Kirat, K., & Morandat, S. (2011). The natural antioxidant rosmarinic acid spontaneously penetrates membranes to inhibit lipid peroxidation in situ. *Biochimica et Biophysica Acta (BBA) - Biomembranes, 1808*(12), 2973-2980. doi:https://doi.org/10.1016/j.bbamem.2011.08.011

Fujita, T., Funayoshi, A., & Nakayama, M. (1994). A Phenylpropanoid Glucoside from *Perilla Frutescens*. *Phytochemistry, 37*(2), 543-546.

Fujita, T., & Nakayama, M. (1992). Perilloside A, A Monoterpene Glucoside from *Perilla Frurescens*. *Phytochemistry, 31*(9), 3265-3267.

Fujita, T., & Nakayama, M. (1993). Monoterpene Glucosides and Other Constituents from

Perilla Frutescens. *Phytochemistry, 34*(6), 1545-1548.

Fujita, T., & Nakayama, M. (1997). *Chemical Studies on the Constituents of Perilla Frutescens*. Retrieved from Japan:

Gai, F., Peiretti, P. G., Karama, M., & Amarowicz, R. (2017). Changes in the Total Polyphenolic Content and Antioxidant Capacities of Perilla (*Perilla frutescens* L.) Plant Extracts during the Growth Cycle. *Journal of Food Quality, 2017*, 8.

Ganogpichayagrai, A., Palanuvej, C., & Ruangrungsi, N. (2017). Antidiabetic and anticancer activities of Mangifera indica cv. Okrong leaves. *Journal of Advanced Pharmaceutical Technology & Research, 8*(1), 19-24.

Gayle, M. S. (2012). *Purification of Alpha-Glucosidase Inhibitiors from Grape Extract.* (Master of Science), Wayne State University, The Graduate School of Wayne State University.

Gella, J., Gubern, G., Vidal, R., & Canalias, F. (1997). Determination of total and pancreatic e-amylase in human serum with 2-chloro-4-nitrophenyl-a-D-maltotrioside as substrate. *Clinica Chimica Acta, 259*, 147-160.

Geran, R., Greenberg, N., MacDonald, M., Schumacher, A., & Abbott, B. (1972). Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *CANCER CHEMOTHERAPY REPORTS, 3*(2), 1-103.

Gerber, M., Boutron-Ruault, M. C., Hercberg, S., Riboli, E., Scalbert, A., & Siess, M. H. (2002). Food and cancer: state of the art about the protective effect of fruits

and vegetables. *Bulletin du Cancer, 89*(3), 293-312.

Global Plants. (2018). Thunbergia laurifolia Retrieved from http://plants.jstor.org/search?filter=name&so=ps_group_by_genus_species+asc&Query=thunbergia+laurifolia. Retrieved 15 Febuary 2018, from Journal storage http://plants.jstor.org/search?filter=name&so=ps_group_by_genus_species+asc&Query=thunbergia+laurifolia

Gong, G., Qin, Y., Huang, W., Zhou, S., Yang, X., & Li, D. (2010). Rutin inhibits hydrogen peroxide-induced apoptosis through regulating reactive oxygen species mediated mitochondrial dysfunction pathway in human umbilical vein endothelial cells. *European Journal of Pharmacology, 628*(1), 27-35. doi:https://doi.org/10.1016/j.ejphar.2009.11.028

Gudzenko, A. (2013). Development and validation of a method for the determination of rosmarinic acid in Mentha piperita L. using solid-phase extraction and RP-HPLC with photodiode array detection *Journal of Chemical and Pharmaceutical Research, 5*(9), 40-45.

Ha, T. J., Lee, J. H., Lee, M. H., Lee, B. W., Kwon, H. S., Park, C. H., . . . Jang, D. S. (2012). Isolation and identification of phenolic compounds from the seeds of *Perilla frutescens* (L.) and their inhibitory activities against α-glucosidase and aldose reductase. *Food Chemistry, 135*(3), 1397-1403.

Hara, K. Y., Kobayashi, A., Sugita, K. Y., & Kondo, K. (2004). Antibacterial Activity of Plants

Used in Cooking for Aroma and Taste. *Journal of Food Protection, 67*(12), 2820-2824.

Hirohisa, T., Naomi, O., Chiaki, S., Rie, Y., Ken-ichiro, I., Akiko, Y., . . . Toshikazu, Y. (2004). Extract of *Perilla frutescens* Enriched for Rosmarinic Acid, a Polyphenolic Phytochemical, Inhibits Seasonal Allergic Rhinoconjunctivitis in Humans. *Experimental Biology and Medicine, 229*(3), 247-254.

Hong, E., Park, K. H., & Kim, G. H. (2010). PHENOLIC-ENRICHED FRACTIONS FROM PERILLA FRUTESCENS VAR. ACUTA: DETERMINATING ROSMARINIC ACID AND ANTIOXIDANT ACTIVITY. *Journal of Food Biochemistry, 35*, 1637-1645.

Hu, J., Deng, Y., & Thomas, F. D. (2011). Thunbergia laurifolia Lindl desceiption. Retrieved from http://www.efloras.org/florataxon.aspx?flora_id=610&taxon_id=132906

Huang, S.-s., & Zheng, R.-l. (2006). Rosmarinic acid inhibits angiogenesis and its mechanism of action in vitro. *Cancer Letters, 239*(2), 271-280. doi:https://doi.org/10.1016/j.canlet.2005.08.025

Hureau, C., & Faller, P. (2009). Aβ-mediated ROS production by Cu ions: Structural insights, mechanisms and relevance to Alzheimer's disease. *Biochimie, 91*(10), 1212-1217.

Ismail, M., Mariod, A., Bagalkotkara, G., & Lingb, H. S. (2010). Fatty acid composition and antioxidant activity of oils from two cultivars of Cantaloupe extracted by

supercritical fluid extraction. *Grasas y Aceites, 61*(1), 37-44.

Izumi, Y., Matsumura, A., Wakita, S., Akagi, K., Fukuda, H., Kume, T., . . . Akaike, A. (2012). Isolation, identification, and biological evaluation of Nrf2-ARE activator from the leaves of green perilla (*Perilla frutescens* var. *crispa* f. *viridis*). *Free Radical Biology and Medicine, 53*(4), 669-679.

Jaiboon, V., Boonyanuphap, J., Suwansri, S., Ratanatraiwong, P., & Hansawasdi, C. (2011). Alpha amylase inhibition and roasting time of local vegetables and herbs prepared for diabetes risk reducing chili paste *Asian Journal of Food and Agro-Industry, 4*(02), 103-113.

Jayaprakasha, G. K., Jena, B. S., Negi, P. S., & Sakariah, K. K. (2002). Evaluation of antioxidant activities and antimutagenicity of turmeric oil: a byproduct from curcumin production. *Zeitschrift für Naturforschung C, 57*(9-10), 828-835.

Jetawattana, S., Boonsirichai, K., Charoen, S., & Martin, S. (2015). Radical Intermediate Generation and Cell Cycle Arrest by an Aqueous Extract of *Thunbergia Laurifolia* Linn. in Human Breast Cancer Cells. *Asian Pacific Journal of Cancer Prevention, 16*, 4357-4361.

Ji, W. W., Li, R. P., Li, M., Wang, S. Y., Zhang, X., Niu, X. X., . . . Ma, S. P. (2014). Antidepressant-like effect of essential oil of Perilla frutescens in a chronic, unpredictable, mild stress-induced depression model mice. *Chinese Journal of Natural Medicines, 12*(10), 753-759.

Jitpewngam, W. (1979). *The studies of compounds in Thunbergia laurifolia Lindl.* (Master degree), Chiang Mai University, Chiang Mai University. (859)

Junsi, M., Siripongvutikorn, S., Yupanqui, C. T., & Usawakesmanee, W. (2017). Phenolic and flavonoid compounds in aqueous extracts of Thunbergia laurifolia leaves and their effect on the toxicity of the carbamate insecticide methomyl to murine macrophage cells. *Functional Foods in Health and Disease, 7*(7), 529-544.

Kähkönen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J.-P., Pihlaja, K., Kujala, T. S., & Heinonen, M. (1999). Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. *Journal of Agricultural and Food Chemistry, 47*(10), 3954-3962. doi:10.1021/jf990146l

Kanchanapoom, T., Kasai, R., & Yamasaki, K. (2002). Iridoid glucosides from *Thunbergia laurifolia*. *Phytochemistry, 60*(8), 769-771.

Karp, F., Mihaliak, C. A., Harris, J. L., & Croteau, R. (1990). Monoterpene biosynthesis: specificity of the hydroxylations of (-)-limonene by enzyme preparations from peppermint (Mentha piperita), spearmint (Mentha spicata), and perilla (Perilla frutescens) leaves. *Archives of Biochemistry and Biophysics, 276*(1), 219-226.

Khojasteh, A., Mirjalili, M. H., Hidalgo, D., Corchete, P., & Palazon, J. (2014). New trends in biotechnological production of rosmarinic acid. *Biotechnology Letters, 36*(12), 2393-2406.

Kim, D., Kim, Y., & Choi, U. (2011). Optimization of Antibacterial Activity of *Perilla frutescens* var. *acuta* Leaf against Staphylococcus aureus Using Evolutionary Operation Factorial Design Technique. *International Journal of Molecular Sciences, 12*(4), 2395-2407.

Kim, D. H., Kim, S. J., Yu, K. Y., Jeong, S. I., & Kim, S. Y. (2018). Anti-hyperglycemic effects and signaling mechanism of *Perilla frutescens* sprout extract. *Nutrition Research and Practice, 12*(1), 20-28.

Kim, J., Choi, H., Kim, M. R., Oh, D. R., Kim, Y., Kang, H., . . . Choi, C. Y. (2017). Amelioration of Visual Display Terminal-induced Ocular Fatigue by Aqueous Extracts of *Perilla frutescens* var. acuta. *Journal of Food and Nutrition Research, 5*(8), 553-561.

Koezuka, Y., Honda, G., & Tabata, M. (1986). Genetic Control of the Chemical Composition of Volatile Oils in *Perilla Frutescens*. *Phytochemistry, 25*(4), 859-863.

Kosia, P., Jiraungkoorskul, K., & Jiraungkoorskul, W. (2015). Review of antidiabetic activity of “Rang Jeud” *Thunbergia laurifolia*. *Journal of Applied Pharmaceutical Science, 5*(2), 99-103.

Krentz, A. J., & Bailey, C. J. (2005). Oral Antidiabetic Agents. *Drugs, 65*(3), 385-411. doi:10.2165/00003495-200565030-00005

Krentz, A. J., & Sinclair, A. J. (2012). Chapter 36 - The Evolution of Glucose-Lowering

Drugs for Type 2 Diabetes A2 - Bagchi, Debasis. In N. Sreejayan (Ed.), *Nutritional and Therapeutic Interventions for Diabetes and Metabolic Syndrome* (pp. 459-474). San Diego: Academic Press.

Kumar, A., Lakshman, K., Jayaveera, K. N., Sheshadri-Shekar, D., Narayan-Swamy, V. B., Khan, S., & Velumurga, C. (2011). In Vitro α-Amylase Inhibition and Antioxidant Activities of Methanolic Extract of *Amaranthus Caudatus* Linn. *Oman Medical Journal, 26*(3), 166-170.

Laovitthayanggoon, S., Charoenkul, O., Supavilai, P., & Aramphongphan, A. (2007). Safety Evaluation of Ran-jued (*Thunbergia laurifolia*) Tea. *Thai Journal of Pharmacology, 29*(1), 81-83.

Lee, A. Y., Hwang, B. R., Lee, M. H., Lee, S., & Cho, E. J. (2016). *Perilla frutescens* var. japonica and rosmarinic acid improve amyloid-β25-35 induced impairment of cognition and memory function. *Nutrition Research and Practice, 10*(3), 274-281.

Lee, Y. H., Kim, B., Kim, S., Kim, M. S., Kim, H., Hwang, S. R., . . . Lee, J. H. (2017). Characterization of metabolite profiles from the leaves of green perilla (*Perilla frutescens*) by ultra high performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry and screening for their antioxidant properties. *Journal of Food and Drug Analysis, 25*(4), 776-788.

Li, L., Chen, C. Y. O., Chun, H. K., Cho, S. M., Park, K. M., Lee-Kim, Y. C., . . . Yeum, K. J.

(2009). A fluorometric assay to determine antioxidant activity of both hydrophilic and lipophilic components in plant foods. *The Journal of Nutritional Biochemistry, 20*(3), 219-226.

Li, S., Han, Q., Qiao, C., Song, J., Lung, C. C., & Xu, H. (2008). Chemical markers for the quality control of herbal medicines: an overview. *Chinese Medicine, 3*, 7-7.

Li, Y., Huang, W., Huang, S., Du, J., & Huang, C. (2012). Screening of anti-cancer agent using zebrafish: Comparison with the MTT assay. *Biochemical and Biophysical Research Communications, 422*(1), 85-90.

Lin, L., Dong, Y., Zhao, H., Wen, L., Yang, B., & Zhao, M. (2011). Comparative evaluation of rosmarinic acid, methyl rosmarinate and pedalitin isolated from Rabdosia serra (MAXIM.) HARA as inhibitors of tyrosinase and **α**-glucosidase. *Food Chemistry, 129*(3), 884-889.

Liu, J., Wan, Y., Zhao, Z., & Chen, H. (2013). Determination of the content of rosmarinic acid by HPLC and analytical comparison of volatile constituents by GC-MS in different parts of Perilla frutescens (L.) Britt. *Chemistry Central Journal, 7*(1), 61. doi:10.1186/1752-153X-7-61

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews, 4*(8), 118-126.

Ma, Z.-J., Yan, H., Wang, Y.-J., Yang, Y., Li, X.-B., Shi, A.-C., . . . Wang, X.-X. (2018).

Proteomics analysis demonstrating rosmarinic acid suppresses cell growth by blocking the glycolytic pathway in human HepG2 cells. *Biomedicine & Pharmacotherapy, 105*, 334-349.

Mai, T. T., Thu, N. N., Tien, P. G., & Chuyen, N. V. (2007). Alpha-Glucosidase Inhibitory and Antioxidant Activities of Vietnamese Edible Plants and Their Relationships with Polyphenol Contents. *Journal of Nutritional Science and Vitaminology, 53*(3), 267-276.

Makino, T., Furuta, Y., Fujii, H., Nakagawa, T., Wakushima, H., Saito, K., & Kano, Y. (2001). Effect of Oral Treatment of *Perilla frutescens* and Its Constituents on Type-I Allergy in Mice. *Biological and Pharmaceutical Bulletin, 24*(10), 1206-1209.

Makino, T., Furuta, Y., Wakushima, H., Fujii, H., Saito, K.-i., & Kano, Y. (2003). Anti-allergic Effect of Perilla frutescens and Its Active Constituents. *Phytotheraphy Research, 17*.

McCue, P. P., & Shetty, K. (2004). Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase in vitro. *Asia Pacific Journal of Clinical Nutrition, 13*(1), 101-106.

Mueller, L., & Boehm, V. (2011). Antioxidant Activity of β-Carotene Compounds in Different in Vitro Assays. *Molecules, 16*(2). doi:10.3390/molecules16021055

Mukherjee, P. K. (2007). *Quality Control Herbal Drugs: An Approach to Evaluation of Botanicals* (first edition ed.). New Delhi, India: Business Horizons.

Nabeta, K., Ohnishi, Y., Hirose, T., & Sugisawa, H. (1983). Monoterpene Biosynthesis by Callus Tissues and Suspension Cells from Perilla Species. *Phytochemistry, 22*(2), 423-425.

Nakamura, Y., Ohto, Y., Murakami, A., & Ohigashi, H. (1998). Superoxide Scavenging Activity of Rosmarinic Acid from Perilla frutescens Britton Var. acuta f. viridis. *Journal of Agricultural and Food Chemistry, 46*, 4545–4550.

Nandhakumar, S., Parasuraman, S., Shanmugam, M., Rao, R. K., Chand, P., & Bhat, B. V. (2011). Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). *Journal of Pharmacology and Pharmacotherapeutics, 2*(2), 107-111.

National Institutes of Health. (2018). Basic concepts. Retrieved from https://imagej.nih.gov/ij/docs/concepts.html

Natsume, M., Muto, Y., Fukuda, K., Tokunaga, T., & Osakabe, N. (2006). Determination of rosmarinic acid and luteolin in Perilla frutescens Britton (Labiatae). *Journal of the Science of Food and Agriculture, 86*, 897-901.

Ogawa, Y., Takahashi, S., & Kitagawa, R. (1984). Oxalate Concent in Common Japanes Foods. *Acta Urologica Japonica, 30*(3), 305-310.

Oh, H. A., Park, C. H., Ahn, H. J., Park, Y. S., & Kim, H. M. (2011). Effect of *Perilla frutescens* var. *acuta* Kudo and rosmarinic acid on allergic inflammatory reactions. *Experimental Biology and Medicine, 236*(1), 99-106.

Olive, P. L., & Banáth, J. P. (2006). The comet assay: a method to measure DNA damage

in individual cells. *Nature Protocols, 1*, 23.

Oonsivilai, R. (2006). *Functional and Nutraceutical Properties of Rang Chuet (Thunbergia laurifolia Lindl.) Extracts.* (Degree of Docotr of Philosophy in Food Technology), Suranaree University of Technology, Suranaree University of Technology.

Oonsivilai, R., Cheng, C., Bomser, J., Ferruzzi, M. G., & Ningsanond, S. (2007). Phytochemical profiling and phase II enzyme-inducing properties of *Thunbergia laurifolia* Lindl. (RC) extracts. *Journal of Ethnopharmacology, 114*(3), 300-306.

Oonsivilai, R., Ferruzzi, M. G., & Ningsanond, S. (2008). Antioxidant activity and cytotoxicity of Rang Chuet (*Thunbergia laurifolia* Lindl.) extracts. *Asian Journal of Food and Agro-Industry, 1*(02), 116-128.

Oonsivilai, R., Oonmetta-area, J., & Singthong, J. (2011). *Bioactivity and Functional Properties of Yanang, Krueo Manoy and Rang Chuet extracts*. Retrieved from Suranaree University of Technology:

Östling, O., & Johanson, K. J. (1984). Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochemical and Biophysical Research Communications, 123*, 291–298.

Paiva, S., & Russell, R. (1999). Beta-carotene and other carotenoids as antioxidants. *Journal of the American College of Nutrition, 18*(5), 426-433.

Palipoch, S., Jiraungkoorskul, W., Tansatit, T., Preyavichyapugdee, N., Jaikua, W., & Kosai,

P. (2011). Effect of *Thunbergia laurifolia* (Linn) Leaf Extract Dietary Supplement Against Lead Toxicity in Nile Tilapia (*Oreochromis niloticus*). *World Journal of Fish and Marine Sciences, 3*(1), 01-09.

Patwardhan, B. (2007). *Drug discovery and development: traditional medicine and ethnopharmacology*. New Delhi, India: New India Publishing Agency.

Peng, Y., Ye, J., & Kong, J. (2005). Determination of Phenolic Compounds in Perilla frutescens L. by Capillary Electrophoresis with Electrochemical Detection. *Journal of Agricultural and Food Chemistry, 53*, 8141–8147.

Petersen, M. (2013). Rosmarinic acid: new aspects. *Phytochemistry Reviews, 12*(1), 207-227.

Phrommani, P., & Uthitchalanon, P. (1973). *Textbook of Thai Traditional Pharmacy*. Bangkok: Collage of Traditional Medicine Wat Mahathat.

Pitoolpong, N., Kanthawat, S., Thaiprodist, S., & Singh, R. (2014). Effect of *Thunbergia laurifolia* Linn. extract in hyperglycemic cats. *The Journal of Thai Veterinary Practitioners, 26*(1-2), 79-85.

Pramyothin, P., Chirdchupunsare, H., Rungsipipat, A., & Chaichantipyuth, C. (2005). Hepatoprotective activity of *Thunbergia laurifolia* Linn extract in rats treated with ethanol: In vitro and in vivo studies. *Journal of Ethnopharmacology, 102*(3), 408-411.

Pukumpuang, W., Thongwai, N., & Tragoolpua, Y. (2012). Total Phenolic Contents,

Antibacterial and Antioxidant Activities of Some Thai Medicinal Plant Extracts. *Journal of Medicinal Plants Research, 6*(35), 4953-4960.

Pumima, M., & Gupta, P. C. (1978). Colouring matters from the flowers of *Thunbergia laurifolia*. *Journal of Indian Chemistry Sciety, 55*, 622-623.

Putiyanan, S., Chansakaow, S., Phrutivorapongkul, A., & Charoensup, W. (2008). Standard Pharmacognostic Characteristic of Some Thai Herbal Medicine. *Chiang Mai University Journal of Natural Sciences, 7*(2), 239-255.

Rana, M. N., & Tangpong, J. (2017). In Vitro free radical scavenging and anti-genotoxic activities of Thunbergia laurifolia aqueous leaf extract. *Journal of Health Research, 31*(2), 127-133.

Ravindran, P. N. (2017). *The Encyclopedia of Herbs and Spices* (Vol. 2). London, United Kingdom: Centre for Agriculture and Bioscience International.

Rice-Evans, C., Miller, N., Bolwell, P., Bramley, P., & Pridham, J. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research, 22*(4), 375-383.

Rocejanasaroj, A., Tencomnao, T., & Sangkitikomol, W. (2014). Thunbergia laurifolia extract minimizes the adverse effects of toxicants by regulating P-glycoprotein activity, CYP450, and lipid metabolism gene expression in HepG2 cells. *Genetics and Molecular Research Journal, 13*(1), 205-219.

Rosa, M. M., & Dias, T. (2014). Chapter 54 - Commonly used endocrine drugs. In J. Biller

& J. M. Ferro (Eds.), *Handbook of Clinical Neurology* (Vol. 120, pp. 809-824): Elsevier.

Ruksounjik, O., & Khunkitti, W. (2016). The comparative study in bioactivities of Rang jeud, Butterfly pea and red grape peel. *Isan Journal of Pharmaceutical Sciences, 12*(1), 61-69.

Runtuwene, J., Cheng, K. C., Asakawa, A., Amitani, H., Amitani, M., Morinaga, A., . . . Inui, A. (2016). Rosmarinic acid ameliorates hyperglycemia and insulin sensitivity in diabetic rats, potentially by modulating the expression of PEPCK and GLUT4. *Drug Design, Development and Therapy, 10*, 2193-2202.

Saenphet, K., Kantaoop, P., Saenphet, S., & Aritajat, S. (2005). Mutagenicity of Pueraria mirifica Airy Shaw & Suvatabandhu and antimutagenicity of *Thunbergia laurifolia* Linn. *The Southeast Asian Journal of Tropical Medicine and Public Health, 36*(4), 238-241.

Saita, E., Kishimoto, Y., Tani, M., Iizuka, M., Toyozaki, M., Sugihara, N., & Kondo, K. (2012). Antioxidant activities of *Perilla frutescens* against low-density lipoprotein oxidation in vitro and in human subjects. *Journal of Oleo Science, 61*(3), 113-120.

Salimei, P. S., Marfè, G., Renzo, D. L., Stefano, D. C., Giganti, M. G., Filomeni, G., & Ciriolo, M. R. (2007). The interference of rosmarinic acid in the DNA fragmentation induced by osmotic shock. *Frontiers in Bioscience, 12*(4), 1308-1317.

Scarpati, M. L., & Oriente, G. (1958). Isolamento e costituzione dell'acido rosmarinico (dal rosmarinus off.). *La Ricerca Scientifica, 28*, 2329–2333.

Schindelin, J., Rueden, C. T., Hiner, M. C., & Eliceiri, K. W. (2015). The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular Reproduction and Development, 82*(7-8), 518-529.

Schirrmacher, G., Skurk, T., Hauner, H., & Grabmann, J. (2010). Effect of *Spinacia oleraceae* L. and *Perilla frutescens* L. on Antioxidants and Lipid Peroxidation in an Intervention Study in Healthy Individuals. *Plant Foods for Human Nutrition, 65*(1), 71-76.

Scotland, R. W., & Vollesen, K. B. (2000). Classification of Acanthaceae. *Kew Bulletin, 55*(3), 513-589.

Senol, F. S., Ślusarczyk, S., Matkowski, A., Pérez-Garrido, A., Girón-Rodríguez, F., Cerón-Carrasco, J. P., . . . Orhan, I. E. (2017). Selective *in vitro* and *in silico* butyrylcholinesterase inhibitory activity of diterpenes and rosmarinic acid isolated from *Perovskia atriplicifolia* Benth. and *Salvia glutinosa* L. *Phytochemistry, 133*, 33-44.

Serafini, M. (2006). The role of antioxidants in disease prevention. *Medicine, 34*(12), 533-535.

Sevgi, K., Tepe, B., & Sarikurkcu, C. (2015). Antioxidant and DNA damage protection potentials of selected phenolic acids. *Food and Chemical Toxicology, 77*, 12-21.

doi:https://doi.org/10.1016/j.fct.2014.12.006

Sharma, S. (2015). Current status of herbal product: Regulatory overview. *Journal of Pharmacy & Bioallied Sciences, 7*(4), 293-296.

Shekarchi, M., Hajimehdipoor, H., Saeidnia, S., Gohari, A. R., & Hamedani, M. P. (2012). Comparative study of rosmarinic acid content in some plants of Labiatae family. *Pharmacognosy magazine, 8*(29), 37-41. doi:10.4103/0973-1296.93316

Sherma, J., & Fried, B. (2003). *Handbook of Thain-Layer Chromatography* (Vol. 89). USA: Marcel Dekker, Inc. .

Shirai, M., Yamanishi, R., Moon, J. H., Murota, K., & Terao, J. (2002). Effect of Quercetin and Its Conjugated Metabolite on the Hydrogen Peroxide-induced Intracellular Production of Reactive Oxygen Species in Mouse Fibroblasts. *Bioscience, Biotechnology, and Biochemistry, 66*(5), 1015-1021.

Simpol, L. R., Otsuka, H., Ohtani, K., Kasai, R., & Yamasaki, K. (1994). Nitrile glucosides and rosmarinic acid, the histamine inhibitor from *Ehretia philippinensis*. *Phytochemistry, 36*(1), 91-95.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research, 175*(1), 184-191.

Singh, S. K., Koleb, P. C., Misra, A. K., Roy, S., Arya, L., Verma, M., . . . Singh, R. (2017). Characterization of *Perilla frutescens* (Linn.) Britt based on morphological,

biochemical and STMS markers. *Industrial Crops and Products, 109*, 773-785.

Sinsawat, W., Koomklang, N., & Sinsawat, P. (2013). *Comparison of Phenolic Compounds, Flavonoids and Antioxidant Capacity from Thunbergia Laurifolia Linn. Fresh and Dry Leaves*. Retrieved from Rajamangala University of Technology Suvarnabhumi:

Sompong, W., Muangngam, N., Kongpatpharnich, A., Manacharoenlarp, C., Amorworasin, C., Suantawee, T., . . . Adisakwattana, S. (2016). The inhibitory activity of herbal medicines on the keys enzymes and steps related to carbohydrate and lipid digestion. *BMC complementary and alternative medicine, 16*(1), 439-439. doi:10.1186/s12906-016-1424-2

Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I., & Bahorun, T. (2005). Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 579*(1), 200-213.

Stroka, J., Spangenberg, B., & Anklam, E. (2002). NEW APPROACHES IN TLC-DENSITOMETRY. *Journal of Liquid Chromatography & Related Technologies, 25*(10-11), 1497-1513. doi:10.1081/JLC-120005700

Sultana, K. W., Chatterjee, S., Roy, A., & Chandra, I. (2015). An Overview on Ethnopharmacological and Phytochemical properties of *Thunbergia sp. Medical & Aromatic Plants, 4*(5), 4.

Suwanchaikasem, P. (2011). *DNA Fingerprints, Chromatographic Patterns and Bioactivities of "Rang Chuet".* (Degree of Master of Sciences in Pharmacy Program in Pharmacognosy), Chulalongkorn University, Faculty of Pharmaceutical Sciences. (5376581533)

Suwanchaikasem, P., Chaichantipyuth, C., & Sukrong, S. (2014). Antioxidant-guided Isolation of Rosmarinic Acid, a Major Constituent from *Thunbergia laurifolia*, and Its Use as a Bioactive Marker for Standardization. *Chiang Mai Journal of Science, 41*(1), 117-127.

Suwanchaikasem, P., Phadungcharoen, T., & Sukrong, S. (2013). Authentication of the Thai medicinal plants sharing the same common name 'Rang Chuet': Thunbergia laurifolia, Crotalaria spectabilis, and Curcuma aff. amada by combined techniques of TLC, PCR-RFLP fingerprints, and antioxidant activities. *Journal of The Science Society of Thailand, 39*, 124-133.

Tada, M., Matsumoto, R., Yamaguchi, H., & Chiba, K. (1996). Novel Antioxidants Isolated from *Perilla frutescens* Britton var. crispa (Thunb.). *Bioscience, Biotechnology, and Biochemistry, 60*(7), 1093-1095.

Tadera, K., Minami, Y., Takamatsu, K., & Matsuoka, T. (2006). Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *Journal of Nutritional Science and Vitaminology, 52*(2), 149-153.

Takahashi, M., Sugiyama, Y., Kawabata, K., Takahashi, Y., Irie, K., Murakami, A., . . .

Ohigashi, H. (2011). 1,2-Di-O-α-linolenoyl-3-O-β-galactosyl-sn-glycerol as a superoxide generation inhibitor from *Perilla frutescens* var. *crispa*. *Bioscience, Biotechnology, and Biochemistry, 75*(11), 2240-2242.

Tangpong, J., & Satarug, S. (2010). Alleviation of lead poisoning in the brain with aqueous leaf extract of the *Thunbergia laurifolia* (Linn.). *Toxicology Letters, 198*(1), 83-88.

Tansuwan, K. (1983). *Studies on Chemical Constituents of Thunbergia Laurifolia Linn. Leaves.* (Master degree), Chulalongkorn University, Chulalongkorn University. (456)

Tavafi, M., Ahmadvand, H., Khalatbari, A., & Tamjidipoor, A. (2011). Rosmarinic Acid Ameliorates Diabetic Nephropathy in Uninephrectomized Diabetic Rats. *Iranian Journal of Basic Medical Sciences, 14*(3), 275-283.

Tepe, B. (2008). Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of Salvia virgata (Jacq), Salvia staminea (Montbret & Aucher ex Bentham) and Salvia verbenaca (L.) from Turkey. *Bioresource Technology, 99*(6), 1584-1588. doi:https://doi.org/10.1016/j.biortech.2007.04.008

The National Institutes of Health (Producer). (2018, 14 April 2018). ImageJ: Image Processing and Analysis in Java. Retrieved from https://imagej.nih.gov/ij/index.html

Thongsaard, W., Marsden, C., Morris, P., Prior, M., & Shah, Y. (2005). Effect of *Thunbergia*

laurifolia, a Thai natural product used to treat drug addiction, on cerebral activity detected by functional magnetic resonance imaging in the rat. *Psychopharmacology, 180*, 752-760.

Thongsaard, W., & Marsden, C. A. (2002). A herbal medicine used in the treatment of addiction mimics the action of amphetamine on in vitro rat striatal dopamine release. *Neuroscience Letters, 329*(2), 129-132.

Upton, R. (2011). American Herbal Pharmacopoeia. Retrieved from http://www.her bal-ahp.org/

Validation of analytical procedures: text and methodology Q2(R1). (2005, April 25-27). Paper presented at the Proceedings of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*(1), 44-84.

Vollesen, K. (2008). Flora of Tropical East Africa: *Thunbergia laurifolia Lindl.* Retrieved from http://plants.jstor.org/compilation/thunbergia.laurifolia. Retrieved 8 Febuary 2018, from Royal Botanic Gardens, Kew http://plants.jstor.org/com pilation/thunbergia.laurifolia

Wan, L.-S., Min, Q.-X., Wang, Y.-L., Yue, Y.-D., & Chen, J.-C. (2013). Xanthone Glycoside

Constituents of Swertia kouitchensis with α-Glucosidase Inhibitory Activity. *Journal of Natural Products, 76*(7), 1248-1253.

World Health Organization. (2011). *Quality control methods for herbal materials*. Geneva, Switzerland: WHO press.

World Health Organization. (2016). *Global report on diabetes*. Retrieved from France: http://apps.who.int/iris/bitstream/handle/10665/204871/9789241565257_eng.pdf;jsessionid=2BCCB91CD6DAAEAC8DFA0BD9CB6C74A3?sequence=1

World Health Organization. (2017). WHO guidelines for selecting marker substances of herbal origin for quality control of herbal medicines. Retrieved from http://apps.who.int/medicinedocs/documents/s23240en/s23240en.pdf

Wuttithammawet, W. (2005). Synopsis of Thai traditional pharmacy and herbal properties (Vol. 2, pp. 183 - 184). Bangkok, Thailand: Silpa Siam Packaging & Printing.

Yang, S. Y., Hong, C. O., Lee, G. P., Kim, C. T., & Lee, K. W. (2013). The hepatoprotection of caffeic acid and rosmarinic acid, major compounds of *Perilla frutescens*, against t-BHP-induced oxidative liver damage. *Food and Chemical Toxicology, 55*, 92-99.

Yang, W., Zhang, Y., Wu, W., Huang, L., Guo, D., & Liu, C. (2017). Approaches to establish Q-markers for the quality standards of traditional Chinese medicines. *Acta Pharmaceutica Sinica B, 7*(4), 439-446.

Zdařilová, A., Svobodová, A., Šimánek, V., & Ulrichová, J. (2009). Prunella vulgaris extract and rosmarinic acid suppress lipopolysaccharide-induced alteration in human gingival fibroblasts. *Toxicology in Vitro, 23*(3), 386-392.

Zhu, F., Asada, T., Sato, A., Koi, Y., Nishiwaki, H., & Tamura, H. (2014). Rosmarinic Acid Extract for Antioxidant, Antiallergic, and α-Glucosidase Inhibitory Activities, Isolated by Supramolecular Technique and Solvent Extraction from Perilla Leaves. *Journal of Agricultural and Food Chemistry, 62*(4), 885-892.

APPENDICES

APPENDIX A

Data of quantitative analysis of rosmarinic acid content

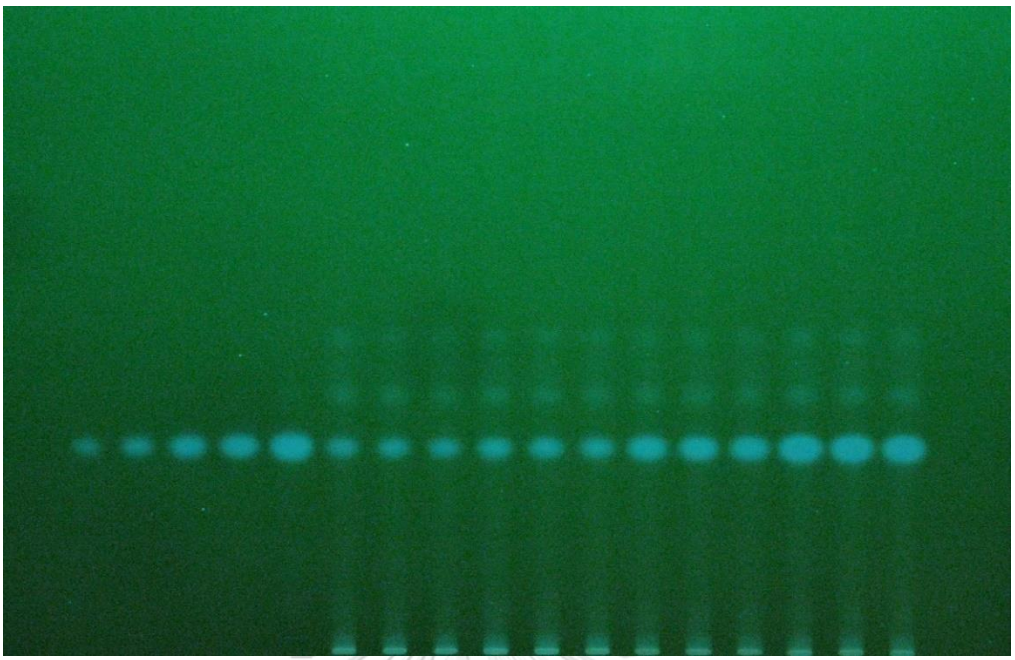


Figure 43 TLC photo-documentation of rosmarinic acid as the standard at 1st – 5th spots and *T. laurifolia* stem ethanolic extracts from 12 different locations at 365 nm

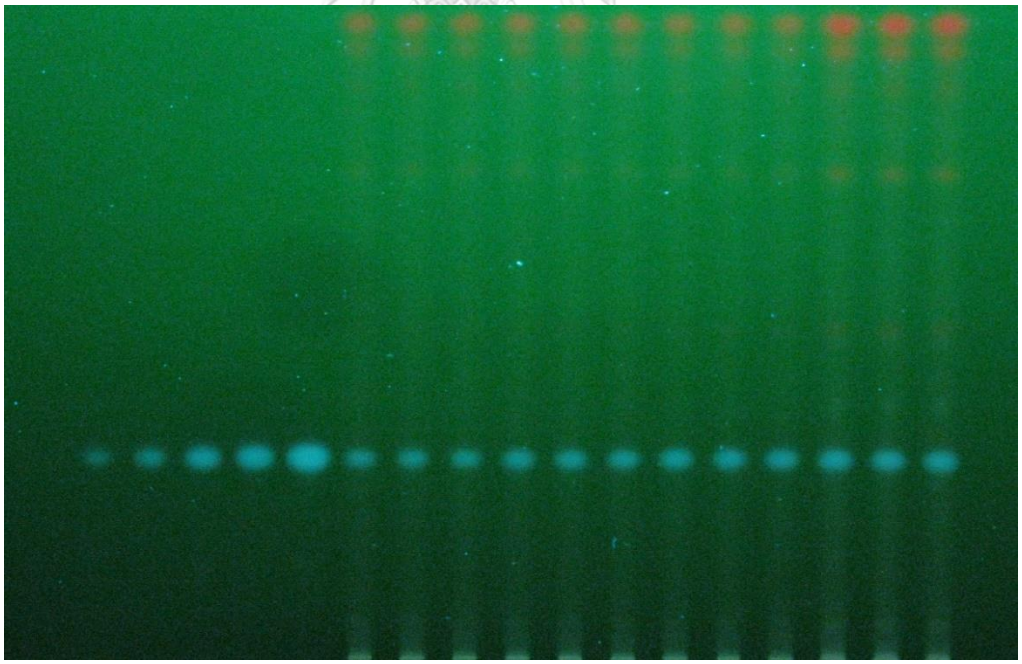


Figure 44 TLC photo-documentation of rosmarinic acid as the standard at 1st – 5th spots and *T. laurifolia* leaf ethanolic extracts from 12 different locations at 365 nm

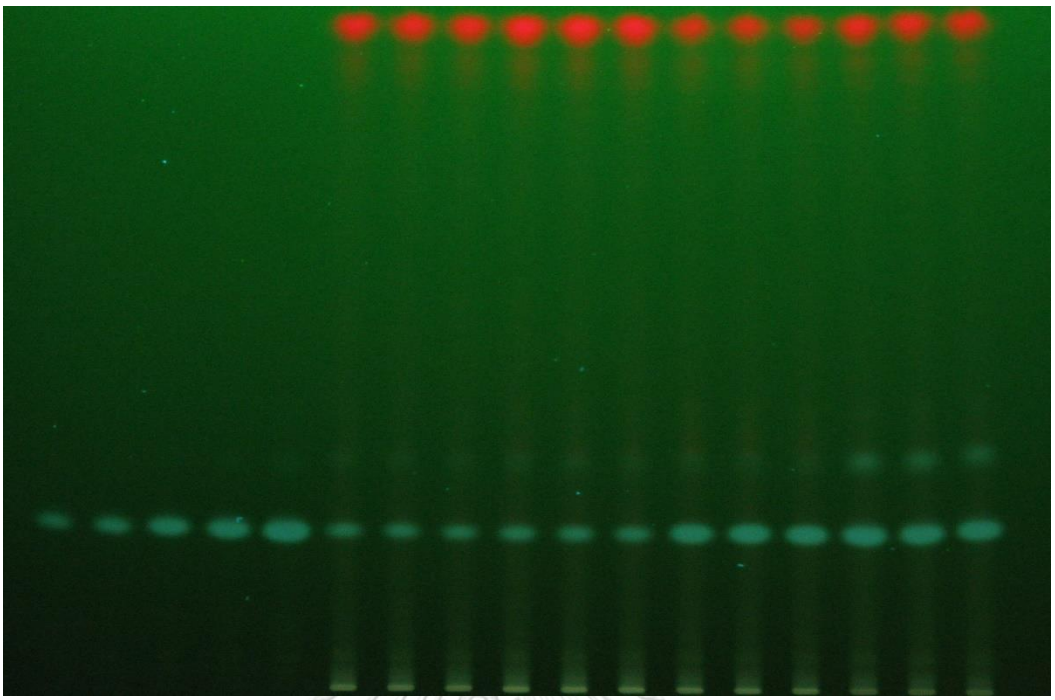


Figure 45 TLC photo-documentation of rosmarinic acid as the standard at 1st – 5th spots and *P. frutescens* leaf ethanolic extracts from 12 different locations at 365 nm

APPENDIX B

Data of antioxidant activities

DPPH radical scavenging activity

Table 41 DPPH radical scavenging activity of ethanolic extracts of *T. laurifolia* leaf

Conc. (µg/ml)	OD_{517} (reaction mixture)			DPPH inhibition (%)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.00	0.211	0.211	0.211					
100	0.135	0.131	0.13	36.02	37.91	38.39	37.44	1.25
150	0.111	0.103	0.1	47.39	51.18	52.61	50.39	2.69
250	0.07	0.048	0.08	66.82	77.25	62.09	68.72	7.76
500	0.033	0.023	0.021	84.29	89.05	90.00	87.78	3.06
600	0.037	0.037	0.043	82.38	82.38	79.52	81.43	1.65

Table 42 DPPH radical scavenging activity of ethanolic extracts of *T. laurifolia* stem

Conc. (µg/ml)	OD_{517} (reaction mixture)			DPPH inhibition (%)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.00	0.211	0.211	0.211					
100	0.14	0.14	0.136	33.65	33.65	35.55	34.28	1.09
250	0.103	0.091	0.089	51.18	56.67	57.62	55.16	3.47
500	0.042	0.036	0.029	80.09	82.86	86.19	83.05	3.05
600	0.023	0.023	0.021	89.10	89.05	90.00	89.38	0.54
700	0.02	0.02	0.02	90.52	90.48	90.48	90.49	0.03

Table 43 DPPH radical scavenging activity of rosmarinic acid

Conc. (µg/ml)	OD_{517} (reaction mixture)			DPPH inhibition (%)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.00	0.188	0.189	0.189					
15	0.15	0.16	0.15	18.90	17.89	18.95	18.58	0.60
25	0.14	0.14	0.14	27.37	27.89	26.32	27.19	0.80
35	0.13	0.13	0.13	32.63	34.21	32.63	33.16	0.91
60	0.08	0.08	0.08	56.84	58.95	59.47	58.42	1.39
70	0.06	0.06	0.06	67.37	70.00	71.05	69.47	1.90

Table 44 DPPH radical scavenging activity of ascorbic acid

Conc. (µg/ml)	OD_{517} (reaction mixture)			DPPH inhibition (%)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.00	0.188	0.189	0.189					
50	0.15	0.15	0.15	20.53	21.58	19.47	20.53	1.05
60	0.14	0.14	0.15	24.21	25.26	23.16	24.21	1.05
90	0.12	0.12	0.11	36.32	38.42	40.53	38.42	2.11
150	0.07	0.06	0.07	62.63	66.32	65.79	64.91	1.99
180	0.05	0.03	0.03	75.79	82.63	82.11	80.18	3.81

Ferric reducing antioxidant power (FRAP) assay

Table 45 FRAP values of *T. laurifolia* leaf ethanolic extract, *T. laurifolia* stem ethanolic extract, rosmarinic acid, ascorbic acid and BHT

Test samples (0.5 mg/ml)	OD_{593}			Ferrous sulphate equivalent (mM)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
T. laurifolia leaf	0.240	0.269	0.259	0.247	0.275	0.265	0.262	0.01
T. laurifolia stem	0.233	0.133	0.137	0.240	0.144	0.147	0.177	0.06
Rosmarinic acid	0.438	0.490	0.457	0.438	0.488	0.456	0.461	0.03
Ascorbic acid	0.426	0.410	0.427	0.427	0.411	0.427	0.422	0.01
BHT	0.557	0.552	0.549	0.553	0.548	0.545	0.549	0.00

β-carotene bleaching assay

Table 46 The absorbance of beta-carotene bleaching of *T. laurifolia* leaf ethanolic extract

Conc. (mg/ml)	OD_{470} (reaction mixture)					% inhibition (C_0-C_{60} = 0.35)
	A_0-A_{60}			Mean	SD	
	exp1	exp2	exp3			
0.09	0.21	0.18	0.19	0.19	0.01	43.68
0.19	0.18	0.20	0.15	0.18	0.02	47.90
0.38	0.13	0.13	0.10	0.12	0.02	64.12
0.75	0.10	0.10	0.08	0.10	0.01	71.79
1.50	0.09	0.09	0.08	0.09	0.01	74.15

Table 47 The absorbance of beta-carotene bleaching of *T. laurifolia* stem ethanolic extract

Conc. (mg/ml)	OD_{470} (reaction mixture)					% inhibition (C_0-C_{60} = 0.35)
	A_0-A_{60}			Mean	SD	
	exp1	exp2	exp3			
0.09	0.20	0.19	0.18	0.19	0.01	43.48
0.19	0.17	0.14	0.14	0.15	0.02	56.16
0.38	0.12	0.10	0.10	0.10	0.01	69.13
0.75	0.09	0.08	0.07	0.08	0.01	76.11
1.50	0.09	0.07	0.06	0.07	0.01	78.57

Table 48 The absorbance of beta-carotene bleaching of rosmarinic acid

Conc. (mg/ml)	OD_{470} (reaction mixture)					% inhibition (C_0-C_{60} = 0.42)
	A_0-A_{60}			Mean	SD	
	exp1	exp2	exp3			
0.02	0.245	0.255	0.19	0.23	0.03	45.30
0.03	0.198	0.163	0.163	0.17	0.02	58.46
0.13	0.101	0.149	0.07	0.11	0.04	74.63
0.25	0.106	0.118	0.023	0.08	0.05	80.42
0.50	0.033	0.028	0.021	0.03	0.01	93.50

Table 49 The absorbance of beta-carotene bleaching of BHT

Conc. (mg/ml)	OD_{470} (reaction mixture)					% inhibition (C_0-C_{60} = 0.35)
	A_0-A_{60}			Mean	SD	
	exp1	exp2	exp3			
0.09	0.32	0.33	0.30	0.31	0.01	9.23
0.38	0.30	0.29	0.32	0.30	0.02	12.21
0.75	0.27	0.24	0.23	0.25	0.02	29.04
1.50	0.10	0.08	0.09	0.09	0.01	74.33
3.00	0.01	0.09	0.06	0.05	0.04	85.10

APPENDIX C

Data of anti-diabetic activities

Yeast α-glucosidase inhibitory activity

Table 50 Yeast α-glucosidase inhibitory activity of *T. laurifolia leaf* extract

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 0.41)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.4	0.37	0.37	0.35	23.05	22.42	27.36	24.27	2.69
0.8	0.24	0.24	0.23	49.48	50.25	52.40	50.71	1.51
1.2	0.16	0.15	0.15	66.90	68.71	68.84	68.15	1.08
1.4	0.14	0.13	0.12	70.09	73.09	75.02	72.73	2.49
1.8	0.10	0.09	0.09	80.02	81.20	82.16	81.13	1.07

Table 51 Yeast α-glucosidase inhibitory activity of *T. laurifolia* stem extract

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 0.46)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
1.25	0.39	0.36	0.39	14.69	21.93	14.69	17.11	4.18
2.5	0.31	0.31	0.34	32.89	31.80	25.88	30.19	3.78
5	0.25	0.23	0.22	44.96	49.34	51.32	48.54	3.26
10	0.15	0.14	0.15	66.23	69.74	68.20	68.06	1.76
20	0.00	0.00	0.01	100.88	100.44	97.59	99.63	1.79

Table 52 Yeast α-glucosidase inhibitory activity of rosmarinic acid

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 0.46)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.125	0.23	0.24	0.17	49.78	47.37	63.82	53.65	8.88
0.25	0.19	0.18	0.16	59.43	60.96	64.04	61.48	2.34
0.5	0.13	0.14	0.13	72.37	70.18	71.27	71.27	1.10
1	0.08	0.08	0.09	83.55	82.02	79.82	81.80	1.87
2	0.04	0.03	0.04	91.23	94.30	91.23	92.25	1.77

Table 53 Yeast α-glucosidase inhibitory activity of acarbose

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 0.46)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.125	0.36	0.34	0.37	21.19	24.93	19.65	21.93	2.72
0.25	0.34	0.35	0.33	25.82	23.83	27.14	25.60	1.66
1	0.25	0.25	0.26	44.75	44.31	43.43	44.16	0.67
2	0.21	0.20	0.22	54.65	56.19	51.57	54.14	2.35
3	0.18	0.18	0.18	61.26	59.72	61.26	60.74	0.89

Rat intestinal α-glucosidase inhibitory activity

Table 54 Rat α-glucosidase inhibitory activity of *T. laurifolia* leaf extract

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 0.53)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
2	0.43	0.44	0.44	19.11	17.99	16.87	17.99	1.12
3	0.41	0.43	0.43	23.05	18.93	19.30	20.43	2.28
5	0.38	0.42	0.41	28.85	22.11	23.42	24.79	3.57
10	0.24	0.33	0.26	55.44	38.59	50.57	48.20	8.67
15	-0.02	0.04	0.04	103.00	92.32	92.51	95.94	6.11

Table 55 Rat α-glucosidase inhibitory activity of *T. laurifolia* stem extract

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 0.53)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
20	0.40	0.42	0.42	25.67	21.55	21.92	23.05	2.28
40	0.37	0.39	0.40	31.66	27.54	26.04	28.41	2.91
60	0.31	0.32	0.33	41.40	40.46	37.46	39.77	2.05
70	0.28	0.29	0.30	47.01	46.08	44.39	45.83	1.33
80	0.26	0.24	0.29	51.69	55.63	46.45	51.26	4.60

Table 56 Rat α-glucosidase inhibitory activity of rosmarinic acid

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 0.48)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.25	0.29	0.29	0.30	40.12	39.49	37.61	39.07	1.30
0.5	0.27	0.28	0.28	43.46	42.00	41.16	42.20	1.16
2	0.22	0.23	0.24	54.31	53.05	50.76	52.71	1.80
3	0.21	0.22	0.22	57.02	53.89	53.68	54.86	1.87
4	0.20	0.20	0.20	59.10	58.90	58.90	58.97	0.12

Table 57 Rat α-glucosidase inhibitory activity of acarbose

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 0.53)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
2	0.27	0.27	0.26	49.45	50.20	50.57	50.07	0.57
5	0.24	0.26	0.24	54.69	52.26	55.06	54.00	1.52
8	0.24	0.23	0.23	55.81	57.50	56.75	56.69	0.84
10	0.23	0.23	0.22	56.37	57.69	59.18	57.75	1.41
12	0.22	0.22	0.23	58.25	58.25	57.69	58.06	0.32

Porcine pancreas α-amylase inhibition

Table 58 Porcine pancreas α-amylase inhibitory activity of *T. laurifolia* leaf extract

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 1.07)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
1.2	0.97	0.96	0.94	9.18	10.21	12.26	10.55	1.57
1.8	0.88	0.86	0.87	17.95	20.19	18.69	18.94	1.14
3	0.73	0.70	0.70	31.75	34.45	34.83	33.68	1.68
4	0.61	0.57	0.55	42.94	46.76	48.44	46.05	2.82
5	0.48	0.45	0.46	54.87	57.67	57.11	56.55	1.48

Table 59 Porcine pancreas α-amylase inhibitory activity of *T. laurifolia* stem extract

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 1.06)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
10	0.83	0.814	0.87	21.55	23.06	17.77	20.79	2.73
14	0.757	0.752	0.767	28.45	28.92	27.50	28.29	0.72
16	0.713	0.725	0.739	32.61	31.47	30.15	31.41	1.23
18	0.71	0.675	0.709	32.89	36.20	32.99	34.03	1.88
20	0.687	0.681	0.682	35.07	35.63	35.54	35.41	0.30

Table 60 Porcine pancreas α-amylase inhibitory activity of rosmarinic acid

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 1.06)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
2	1.031	1.024	0.985	2.74	3.40	7.08	4.40	2.34
3	0.968	0.93	0.919	8.68	12.26	13.30	11.42	2.43
5	0.805	0.783	0.776	24.06	26.13	26.79	25.66	1.43
7	0.672	0.642	0.637	36.60	39.43	39.91	38.65	1.79
10	0.539	0.516	0.509	49.15	51.32	51.98	50.82	1.48

Table 61 Porcine pancreas α-amylase inhibitory activity of acarbose

Conc. (mg/ml)	OD_{405} (reaction mixture)			Yeast α-glucosidase inhibition (%) ($OD_{Control}$ = 1.16)				
	exp1	exp2	exp3	exp1	exp2	exp3	Mean	SD
0.005	0.93	0.92	0.94	19.82	21.28	19.39	20.16	0.99
0.008	0.72	0.71	0.72	38.45	38.70	38.45	38.53	0.15
0.01	0.54	0.54	0.55	53.81	53.64	52.96	53.47	0.45
0.02	0.25	0.25	0.26	78.37	78.37	77.42	78.05	0.55
0.03	0.21	0.21	0.21	82.32	82.14	82.06	82.17	0.13

APPENDIX D

Intracellular ROS measurement using DCFH-DA assay

MTT cell viability assay

Table 62 Cytotoxic effect of *T. laurifolia* leaf extract by MTT cell viability

Conc. (mg/ml)	EA.hy926 (OD_{570})					
	Percent survival					
	exp1	exp2	exp3	exp4	Mean	SD
0.016	145.91	118.65	114.06	91.33	117.49	22.40
0.031	142.20	145.14	112.87	109.64	127.46	18.80
0.063	113.43	110.29	103.32	81.13	102.04	14.57
0.125	121.88	103.23	95.17	81.79	100.52	16.76
0.250	79.32	63.02	56.42	63.07	65.46	9.75
0.500	56.91	61.79	55.92	49.81	56.11	4.92
1.000	36.26	42.48	38.38	43.83	40.24	3.52

Table 63 Cytotoxic effect of *T. laurifolia* stem extract by MTT cell viability

Conc. (mg/ml)	EA.hy926 (OD_{570})					
	Percent survival					
	exp1	exp2	exp3	exp4	Mean	SD
0.016	102.15	101.18	122.52	153.22	119.77	24.37
0.031	122.01	122.26	98.66	102.06	111.25	12.65
0.063	104.90	129.69	134.36	104.26	118.30	15.96
0.125	131.80	170.45	154.58	151.29	152.03	15.87
0.250	107.27	129.24	86.51	111.51	108.63	17.55
0.500	96.04	95.76	83.10	103.05	94.49	8.31
1.000	62.66	66.04	75.04	82.90	71.66	9.13

Table 64 Cytotoxic effect of rosmarinic acid by MTT cell viability

Conc. (mg/ml)	EA.hy926 (OD_{570})					
	Percent survival					
	exp1	exp2	exp3	exp4	Mean	SD
0.016	153.13	149.73	142.23	128.61	143.42	10.88
0.031	134.62	144.31	143.35	140.89	140.79	4.36
0.063	152.51	145.63	150.66	135.71	146.13	7.53
0.125	134.06	137.83	141.46	128.05	135.35	5.73
0.250	27.64	22.05	15.69	18.66	21.01	5.13
0.500	19.31	16.81	19.50	17.87	18.37	1.27
1.000	28.15	29.81	23.26	32.36	28.40	3.84

Table 65 Cytotoxic effect of H_2O_2 by MTT cell viability

Conc. (mg/ml)	EA.hy926 (OD_{570})					
	Percent survival					
	exp1	exp2	exp3	exp4	Mean	SD
0.8	88.23	95.95	102.59	99.17	96.48	6.14
1.0	83.93	99.21	96.23	86.25	91.41	7.45
1.2	78.58	96.47	87.93	71.00	83.50	11.08
1.4	67.29	72.49	69.41	46.86	64.01	11.63
1.8	11.68	5.85	6.60	6.06	7.55	2.77

Intracellular ROS measurement using DCFH-DA assay

Table 66 Intracellular ROS measurement of *T. laurifolia* leaf extract by DCFH-DA assay

Conc. (mg/ml)	EA.hy926 (excitation at 485 nm and emission at 535 nm.)				
	Intracellular ROS (%)				
	exp1	exp2	exp3	Mean	SD
0.015	98.66	95.83	96.45	96.98	1.49
0.031	94.35	94.06	96.70	95.03	1.45
0.250	82.61	83.60	83.72	83.31	0.61
0.500	81.51	79.34	82.43	81.09	1.58
1.000	77.31	76.64	77.97	77.31	0.66

Table 67 Intracellular ROS measurement of *T. laurifolia* stem extract by DCFH-DA assay

Conc. (mg/ml)	EA.hy926 (excitation at 485 nm and emission at 535 nm.)				
	Intracellular ROS (%)			Mean	SD
	exp1	exp2	exp3		
0.015	96.47	100.79	101.34	99.53	2.17
0.031	93.32	97.91	101.14	97.46	3.21
0.250	90.95	97.61	98.04	95.53	3.25
0.500	91.78	94.48	96.17	94.14	1.81
1.000	92.52	90.73	90.66	91.30	0.86

Table 68 Intracellular ROS measurement of rosmarinic acid by DCFH-DA assay

Conc. (mg/ml)	EA.hy926 (excitation at 485 nm and emission at 535 nm.)				
	Intracellular ROS (%)			Mean	SD
	exp1	exp2	exp3		
0.031	89.39	99.48	99.24	96.04	5.76
0.063	87.80	98.21	96.15	94.05	5.51
0.125	90.00	93.69	95.32	93.00	2.72
0.250	87.32	86.99	94.15	89.49	4.04
1.000	85.67	83.59	95.05	88.10	6.11

APPENDIX E

DNA damage (Comet assay)

Table 69 Total score of DNA damage in human lymphocyte cells

Concentration (µg/ml)	T. laurifolia leaf extract	T. laurifolia stem extract	Rosmarinic acid	PBS
25	170	173	189	100
	178	156	190	-
	169	168	202	-
Mean	172.33	165.67	193.67	-
SD	4.93	8.74	7.23	-
50	195	184	230	-
	195	207	239	-
	185	187	233	-
Mean	191.67	192.67	234.00	-
SD	5.77	12.50	4.58	-
100	188	225	295	-
	223	237	282	-
	229	256	283	-
Mean	213.33	239.33	286.67	-
SD	22.14	15.63	7.23	-
0	-	-	-	124
	-	-	-	105
	-	-	-	125
Mean	-	-	-	118.00
SD	-	-	-	11.27

APPENDIX F

Cytotoxicity against cancer cell lines

Table 70 Cytotoxic activities of *T. laurifolia* leaf extract by MTT cell viability

Concentration (µg/ml)	BT474 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.107	0.146	0.145	0.134	0.133	17
50	0.340	0.327	0.321	0.272	0.315	40
10	0.594	0.460	0.679	0.638	0.593	75
5	0.532	0.659	0.692	0.605	0.622	78
1	0.784	0.644	0.692	0.610	0.683	86
0.5	0.712	0.697	0.825	0.596	0.708	89
0.1	0.725	0.677	0.694	0.600	0.674	85
0.05	0.696	0.782	0.711	0.627	0.704	89
0.01	0.576	0.764	0.903	0.592	0.709	89
DMSO	0.881 0.620	0.742 0.849	0.746 0.780	0.891 0.848	0.795	100
Control	1.022 1.010	1.069 0.958	1.015 1.065	1.023 1.041	1.025	
Concentration (µg/ml)	Chago-K1 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.215	0.154	0.174	0.219	0.191	30
50	0.171	0.141	0.180	0.128	0.155	25
10	0.489	0.375	0.584	0.485	0.483	77
5	0.526	0.581	0.506	0.444	0.514	82
1	0.370	0.446	0.656	0.548	0.505	81
0.5	0.477	0.356	0.503	0.492	0.457	73
0.1	0.417	0.441	0.553	0.559	0.503	80
0.05	0.338	0.403	0.546	0.569	0.464	74
0.01	0.278	0.484	0.457	0.485	0.426	68
DMSO	0.525 0.634	0.603 0.695	0.637 0.802	0.578 0.536	0.627	100
Control	0.938 0.910	0.967 0.963	1.004 0.845	0.943 0.935	0.938	

Table 70 Cytotoxic activities of T. laurifolia leaf extract by MTT cell viability (Cont.)

Concentration (µg/ml)	Hep-G2(A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.132	0.134	0.275	0.131	0.168	17
50	0.339	0.154	0.254	0.173	0.230	23
10	0.809	0.712	0.665	0.981	0.792	79
5	1.147	0.746	0.842	0.728	0.866	87
1	1.065	1.009	0.980	0.854	0.977	98
0.5	0.762	0.948	0.707	0.666	0.771	77
0.1	1.012	0.815	0.849	0.741	0.854	86
0.05	1.166	0.876	0.964	0.740	0.937	94
0.01	0.910	0.903	0.965	0.981	0.940	94
DMSO	1.226 0.933	0.983 0.932	0.994 0.957	0.978 0.983	0.998	100
Control	1.717 1.776	1.404 1.649	1.462 1.558	1.665 1.650	1.609	
Concentration (µg/ml)	KATO -III (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.262	0.286	0.159	0.147	0.214	22
50	0.516	0.342	0.490	0.370	0.430	45
10	0.762	0.722	0.436	0.405	0.581	61
5	0.937	0.800	0.702	0.566	0.751	79
1	0.982	0.860	0.767	0.549	0.790	83
0.5	1.059	0.893	0.946	0.749	0.912	96
0.1	1.143	0.819	0.844	0.842	0.912	96
0.05	0.883	0.964	0.892	0.718	0.864	91
0.01	1.150	0.950	0.899	0.862	0.965	101
DMSO	1.459 0.814	0.955 0.859	0.819 1.062	0.692 0.966	0.953	100
Control	1.477 1.369	1.379 1.406	1.454 1.419	1.252 1.530	1.411	

Table 70 Cytotoxic activities of T. laurifolia leaf extract by MTT cell viability (Cont.)

Concentration (µg/ml)	SW620 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.256	0.131	0.188	0.279	0.214	19
50	0.866	0.862	0.534	0.628	0.723	63
10	1.216	1.147	1.058	1.019	1.110	97
5	1.322	1.141	1.044	1.027	1.134	99
1	1.444	1.177	1.064	1.066	1.188	103
0.5	1.311	1.159	1.075	1.031	1.144	99
0.1	1.353	1.143	1.040	1.049	1.146	100
0.05	1.454	1.156	1.071	1.044	1.181	103
0.01	1.291	1.156	1.112	1.078	1.159	101
DMSO	1.186 1.004	1.124 1.118	1.118 1.098	1.107 1.443	1.150	100
Control	1.490 1.387	1.382 1.379	1.307 1.418	1.365 1.526	1.407	
Concentration (µg/ml)	Wi-38 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.162	0.208	0.249	0.253	0.218	39
50	0.384	0.226	0.179	0.225	0.253	45
10	0.602	0.569	0.440	0.403	0.504	89
5	0.542	0.507	0.516	0.492	0.514	91
1	0.574	0.544	0.556	0.403	0.520	92
0.5	0.591	0.581	0.562	0.463	0.549	98
0.1	0.634	0.576	0.554	0.479	0.561	100
0.05	0.703	0.560	0.550	0.462	0.569	101
0.01	0.641	0.518	0.592	0.481	0.558	99
DMSO	0.601 0.516	0.608 0.562	0.600 0.487	0.522 0.606	0.563	100
Control	1.139 1.047	1.062 1.009	1.011 1.060	1.003 1.107	1.055	

Table 71 Cytotoxic activities of *T. laurifolia* stem extract by MTT cell viability

Concentration (µg/ml)	BT474 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.666	0.670	0.671	0.552	0.640	80
50	0.705	0.668	0.866	0.870	0.777	98
10	0.781	0.778	0.812	0.757	0.782	98
5	0.764	0.867	0.892	0.936	0.865	109
1	0.854	0.703	0.666	0.775	0.750	94
0.5	0.757	0.830	0.767	0.870	0.806	101
0.1	0.745	0.867	0.795	0.905	0.828	104
0.05	0.588	0.732	0.759	0.761	0.710	89
0.01	0.687	0.741	0.717	0.875	0.755	95
DMSO	0.881 0.620	0.742 0.849	0.746 0.780	0.891 0.848	0.795	100
Control	1.022 1.010	1.069 0.958	1.051 1.065	1.023 1.041	1.025	
Concentration (µg/ml)	Chago-K1 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.517	0.576	0.506	0.416	0.504	80
50	0.538	0.551	0.613	0.559	0.565	90
10	0.562	0.613	0.675	0.630	0.620	99
5	0.657	0.655	0.707	0.647	0.667	106
1	0.648	0.677	0.739	0.692	0.689	110
0.5	0.594	0.711	0.740	0.639	0.671	107
0.1	0.721	0.725	0.719	0.717	0.721	115
0.05	0.529	0.588	0.597	0.489	0.551	88
0.01	0.627	0.623	0.721	0.652	0.656	105
DMSO	0.525 0.634	0.603 0.695	0.637 0.802	0.587 0.536	0.627	100
Control	0.938 0.910	0.967 0.963	1.004 0.845	0.943 0.935	0.938	

Table 71 Cytotoxic activities of *T. laurifolia* stem extract by MTT cell viability (Cont.)

Concentration (µg/ml)	Hep-G2 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.745	0.799	0.922	0.798	0.816	82
50	0.763	0.689	0.935	1.010	0.849	85
10	0.907	0.946	0.918	1.009	0.945	95
5	0.889	0.974	1.139	1.057	1.015	102
1	0.887	0.932	1.108	0.902	0.957	96
0.5	0.830	0.809	1.008	0.938	0.896	90
0.1	0.996	0.949	1.094	1.008	1.012	101
0.05	1.002	0.986	1.219	0.945	1.038	104
0.01	0.807	0.862	1.040	1.028	0.934	94
DMSO	1.226 0.933	0.983 0.932	0.994 0.957	0.978 0.983	0.998	100
Control	1.717 1.776	1.404 1.649	1.462 1.558	1.655 1.650	1.609	
Concentration (µg/ml)	KATO -III (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.872	0.642	0.808	1.010	0.833	87
50	0.897	0.790	1.009	1.058	0.939	98
10	0.715	0.932	0.971	1.011	0.907	95
5	0.879	1.102	1.108	1.076	1.041	109
1	0.810	0.902	1.034	1.108	0.964	101
0.5	0.839	0.932	1.028	1.070	0.967	101
0.1	0.900	0.889	0.897	0.997	0.921	97
0.05	0.830	1.022	0.991	1.168	1.003	105
0.01	0.892	0.909	0.889	1.076	0.942	99
DMSO	1.459 0.814	0.955 0.859	0.819 1.062	0.692 0.966	0.953	100
Control	1.477 1.369	1.379 1.406	1.454 1.419	1.252 1.530	1.411	

Table 71 Cytotoxic activities of *T. laurifolia* stem extract by MTT cell viability (Cont.)

Concentration (µg/ml)	SW620 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.932	0.951	0.887	0.907	0.919	80
50	0.957	0.998	1.008	1.062	1.006	88
10	1.113	1.083	1.185	1.225	1.159	101
5	1.087	1.055	1.202	1.390	1.184	103
1	1.132	1.100	1.214	1.347	1.198	104
0.5	1.130	1.073	1.068	1.377	1.162	101
0.1	1.134	1.104	1.199	1.386	1.207	105
0.05	1.069	1.087	1.123	1.255	1.134	99
0.01	1.126	1.068	1.174	1.332	1.175	102
DMSO	1.186 1.004	1.124 1.118	1.118 1.098	1.107 1.443	1.150	100
Control	1.490 1.387	1.382 1.379	1.307 1.418	1.365 1.526	1.407	
Concentration (µg/ml)	Wi 38 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.466	0.423	0.537	0.531	0.489	87
50	0.450	0.518	0.458	0.473	0.475	84
10	0.530	0.527	0.606	0.612	0.569	101
5	0.488	0.522	0.563	0.635	0.552	98
1	0.444	0.505	0.548	0.576	0.518	92
0.5	0.554	0.564	0.577	0.620	0.579	103
0.1	0.507	0.658	0.604	0.601	0.593	105
0.05	0.484	0.484	0.465	0.569	0.501	89
0.01	0.486	0.583	0.634	0.633	0.584	104
DMSO	0.601 0.516	0.608 0.562	0.600 0.487	0.522 0.606	0.563	100
Control	1.139 1.047	1.062 1.009	1.011 1.060	1.003 1.107	1.055	

Table 72 Cytotoxic activities of rosmarinic acid extract by MTT cell viability

Concentration (µg/ml)	BT474 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.143	0.320	0.360	0.256	0.270	43
50	0.512	0.494	0.659	0.396	0.515	83
10	0.608	0.646	0.879	0.511	0.661	106
5	0.555	0.514	0.900	0.589	0.640	103
1	0.645	0.832	0.764	0.737	0.745	120
0.5	0.748	0.744	0.787	0.559	0.710	114
0.1	0.573	0.563	0.519	0.505	0.540	87
0.05	0.389	0.799	0.741	0.637	0.642	103
0.01	0.733	0.904	0.835	0.564	0.759	122
DMSO	0.568	0.562	0.726	0.630	0.622	100
Control	0.860	0.927	0.963	0.803	0.888	
Concentration (µg/ml)	Chago-KI (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.289	0.181	0.400	0.331	0.300	36
50	0.918	1.017	1.032	1.064	1.008	122
10	0.816	0.820	0.831	0.909	0.844	103
5	0.636	0.832	0.941	0.821	0.808	98
1	0.853	0.667	0.850	0.943	0.828	101
0.5	0.837	0.778	0.827	0.916	0.840	102
0.1	0.683	0.781	0.691	0.848	0.751	91
0.05	0.717	0.703	0.824	0.786	0.758	92
0.01	0.726	0.965	0.846	0.781	0.830	101
DMSO	0.726	0.815	0.823	0.927	0.823	100
Control	1.074	1.160	0.968	1.114	1.079	

Table 72 Cytotoxic activities of rosmarinic acid extract by MTT cell viability (Cont.)

Concentration (µg/ml)	Hep (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.970	0.638	0.812	0.762	0.769	74
50	1.311	1.183	1.133	1.408	1.259	116
10	0.900	1.119	0.955	1.150	1.031	95
5	1.209	1.240	1.039	1.004	1.123	104
1	0.958	1.090	1.111	1.188	1.087	101
0.5	1.066	1.086	1.067	1.103	1.081	100
0.1	0.823	0.836	1.104	0.899	0.916	85
0.05	1.142	1.139	1.089	1.033	1.101	102
0.01	1.011	1.006	1.008	0.924	0.987	91
DMSO	1.056	1.236	1.083	0.949	1.081	100
Control	1.530	1.484	1.544	1.392	1.488	
Concentration (µg/ml)	KATO-III (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	1.113	0.641	1.002	1.273	1.007	112
50	1.370	1.341	1.231	1.362	1.326	147
10	1.025	1.253	1.167	1.139	1.146	127
5	1.112	1.013	1.120	1.248	1.123	125
1	0.938	0.873	1.082	1.293	1.047	116
0.5	0.912	0.848	0.982	1.194	0.984	109
0.1	0.765	0.780	0.860	0.952	0.839	93
0.05	0.820	1.566	1.085	1.023	1.124	125
0.01	0.831	0.989	1.145	1.138	1.026	114
DMSO	0.885	0.864	0.806	1.051	0.902	100
Control	1.491	1.574	1.556	1.592	1.553	

Table 72 Cytotoxic activities of rosmarinic acid extract by MTT cell viability (Cont.)

Concentration (µg/ml)	SW620 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.259	0.332	0.436	0.170	0.299	24
50	1.303	1.153	1.228	1.306	1.248	100
10	1.358	1.257	1.167	1.324	1.277	102
5	1.376	1.218	1.174	1.165	1.233	99
1	1.378	1.137	1.152	1.110	1.194	95
0.5	1.313	1.132	1.143	1.091	1.170	93
0.1	1.281	1.100	1.132	1.119	1.158	92
0.05	1.271	1.144	1.152	1.135	1.176	94
0.01	1.379	1.204	1.134	1.082	1.200	96
DMSO	1.463	1.112	1.125	1.183	1.252	100
Control	1.497	1.408	1.420	1.488	1.453	
Concentration (µg/ml)	Wi - 38 (A_{540})					Percent survival
	Exp 1	Exp 2	Exp 3	Exp 4	Mean	
100	0.857	1.080	0.884	1.019	0.960	170
50	0.955	1.132	0.979	1.040	1.027	181
10	0.602	0.789	0.783	0.821	0.749	132
5	0.575	0.695	0.797	0.756	0.706	125
1	0.595	0.595	0.699	0.647	0.634	112
0.5	0.535	0.564	0.554	0.689	0.586	103
0.1	0.455	0.479	0.570	0.454	0.490	86
0.05	0.477	0.573	0.606	0.515	0.543	96
0.01	0.520	0.589	0.619	0.573	0.575	102
DMSO	0.514	0.610	0.571	0.569	0.566	100
Control	0.807	0.849	0.916	0.864	0.859	

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"Pharmacognostic Specification and Evaluation of Antidiabetic Activity Using Yeast Alpha Glucosidase in Thunbergia laurifolia Stems and Leaves" International Conference on Natural Science, Engineering, and Technology", January 19-21, 2019, Sapporo Convention Center, Japan.

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