STANDARDIZATION OF *LONICERA JAPONICA* FLOWERING BUD AND CONTENTS OF CHLOROGENIC, ROSMARINIC AND CAFFEIC ACIDS IN SELECTED THAI MEDICINAL PLANTS

Mr. Chayanon Chaowuttikul

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มาตรฐานของดอกสายน้ำผึ้ง และปริมาณวิเคราะห์ของกรดคลอโรจีนิก โรสมารินิก และคาเฟอิก ในสมุนไพรไทยบางชนิด

นายชยานนท์ เชาวน์วุฒิกุล

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By	Mr. Chayanon Chaowuttikul
Field of Study	Public Health Sciences
Thesis Advisor	Assistant Professor Chanida Palanuvej, Ph.D.
Thesis Co-Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.

Accepted by the College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the College of Public Health Sciences
(Professor Sathirakorn Pongpanich, Ph.D.)

THESIS COMMITTEE

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(Assistant Professor Naowarat Kanchanakhan, Ph.D.)

.....Thesis Advisor
(Assistant Professor Chanida Palanuvej, Ph.D.)

.....Thesis Co-Advisor
(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

.....Examiner
(Assistant Professor Kanchana Rungsihirunrat, Ph.D.)

.....Examiner
(Tepanata Pumpaibool, Ph.D.)

.....External Examiner
(Assistant Professor Piyanuch Thongphasuk, Ph.D.)

ชยานนท์ เชาวน์วุฒิกุล : มาตรฐานของดอกสายน้ำผึ้ง และปริมาณวิเคราะห์ของกรดคลอโรจีนิก โรสมารินิก และคาเฟอิกในสมุนไพรไทยบางชนิด (STANDARDIZATION OF *LONICERA JAPONICA* FLOWERING BUD AND CONTENTS OF CHLOROGENIC, ROSMARINIC AND CAFFEIC ACIDS IN SELECTED THAI MEDICINAL PLANTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นิจศิริ เรืองรังษี, 244 หน้า.

การหาปริมาณกรดคลอโรจีนิก กรดโรสมารินิก และกรดคาเฟอิก ในพืชสมุนไพรไทย 111 ตัวอย่าง ด้วยเครื่องมือไฮเพอร์ฟอแมนซ์ลิควิดโครมาโตกราฟี พบว่ามีตัวอย่างสมุนไพรที่พบทั้ง 3 สาร คิดเป็นร้อยละ 39.64 พบ 2 สาร คิดเป็นร้อยละ 40.54 พบเพียง 1 สาร คิดเป็นร้อยละ 14.41 และตรวจไม่พบทั้ง 3 สาร คิดเป็นร้อยละ 5.41 ปริมาณกรดคลอโรจีนิกพบมากที่สุดในดอกสายน้ำผึ้ง กรดโรสมารินิกพบมากที่สุดในใบเลมอนบาล์ม และกรดคาเฟอิกพบมากที่สุดในเมล็ดกาแฟโรบัสตา การศึกษาเพื่อจัดทำข้อกำหนดทางเภสัชเวทและปริมาณวิเคราะห์ของกรดคลอโรจีนิกในดอกสายน้ำผึ้งจากร้านขายสมุนไพร 15 แหล่งทั่วประเทศไทย ได้แสดงลักษณะทางมหทรรศน์และจุลทรรศน์ของดอกสายน้ำผึ้ง และศึกษาเอกลักษณ์ทางกายภาพและเคมีของดอกสายน้ำผึ้ง พบว่ามีน้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด ปริมาณความชื้น ปริมาณสิ่งสกัดด้วยน้ำ และปริมาณสิ่งสกัดด้วยเอทานอล ร้อยละ 10.11, 6.59, 1.14, 10.82, 16.46 และ 28.88 โดยน้ำหนักตามลำดับ สำหรับปริมาณวิเคราะห์กรดคลอโรจีนิกในดอกสายน้ำผึ้งโดยวิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโตมีทรีเปรียบเทียบกับวิธีทินเลเยอร์โครมาโทกราฟีโดยวิเคราะห์ภาพถ่ายโดยใช้โปรแกรมอิมเมจเจ พบปริมาณกรดคลอโรจีนิก ร้อยละ 2.24 และ 2.09 โดยน้ำหนัก ซึ่งพบว่าปริมาณที่วิเคราะห์โดยทั้งสองวิธีไม่แตกต่างกัน (P = 0.13) โดยใช้สถิติ paired t-test การตรวจสอบความใช้ได้ของวิธีวิเคราะห์เชิงปริมาณทั้งสามวิธี ประเมินโดยการใช้แนวทางของไอซีเอช (ICH guideline) พบว่าวิธีไฮเพอร์ฟอแมนซ์ลิควิดโครมาโตกราฟี วิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโตมีทรี และวิธีทินเลเยอร์โครมาโทกราฟีโดยวิเคราะห์ภาพถ่าย มีความเหมาะสม เชื่อถือได้ และมีประสิทธิผลในการวิเคราะห์หาปริมาณ การทดสอบฤทธิ์ทางชีวภาพของดอกสายน้ำผึ้งเปรียบเทียบกับกรดคลอโรจีนิก กรดโรสมารินิก และกรดคาเฟอิก โดยการทดสอบความเป็นพิษต่อไรทะเล การทดสอบความเป็นพิษต่อเซลล์มะเร็งโดยวิธีเอ็มทีที การประเมินความเป็นพิษต่อดีเอ็นเอโดยวิธีโคเมท การทดสอบฤทธิ์ต้านจุลชีวิน การศึกษาฤทธิ์ต้านออกซิเดชัน และการศึกษาฤทธิ์ต้านเบาหวานโดยวัดการยับยั้งเอ็นไซม์แอลฟากลูโคซิเดสจากยีสต์แซคคาโรไมซีส ซีรีวิซิอี ผลการศึกษาพบว่าสารสกัดเอทานอลของดอกสายน้ำผึ้งไม่พบความเป็นพิษต่อไรทะเล ต่อเซลล์มะเร็ง และเซลล์ปกติ กรดคลอโรจีนิก กรดโรสมารินิก และกรดคาเฟอิกพบความเป็นพิษต่อไรทะเล และแสดงความเป็นพิษต่อเซลล์มะเร็งและเซลล์ปกติมากกว่าสารสกัด แต่ยังคงถือว่าไม่เป็นพิษเมื่อเทียบกับเกณฑ์มาตรฐาน สารสกัดและสารทดสอบทั้ง 3 สาร พบว่าสร้างความเสียหายต่อดีเอ็นเอจากเซลล์เม็ดเลือดขาวของมนุษย์ แต่ไม่พบฤทธิ์ในการต้านจุลชีวินทั้งหมดที่ศึกษา สารสกัดและสารทดสอบทั้ง 3 สาร พบว่ามีฤทธิ์ในการต้านออกซิเดชันด้วยวิธีการต้านอนุมูลอิสระดีพีพีเอช มีฤทธิ์ในการต้านไนตริกออกไซด์ และมีความสามารถในการรีดิวซ์ อย่างไรก็ตามมีเพียงสารทดสอบทั้ง 3 สารเท่านั้น ที่มีฤทธิ์ต้านอนุมูลอิสระโดยวิธีการฟอกสีเบตา-แคโรทีน นอกจากนี้สารสกัดและสารทดสอบทั้ง 3 สาร พบว่ามีฤทธิ์ในการยับยั้งเอ็นไซม์แอลฟากลูโคซิเดสจากยีสต์

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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CHAYANON CHAOWUTTIKUL: STANDARDIZATION OF *LONICERA JAPONICA* FLOWERING BUD AND CONTENTS OF CHLOROGENIC, ROSMARINIC AND CAFFEIC ACIDS IN SELECTED THAI MEDICINAL PLANTS. ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 244 pp.

Quantification of chlorogenic, rosmarinic and caffeic acids in 111 selected Thai medicinal plants using high performance liquid chromatography demonstrated that among 111 samples, 39.64% contained all of 3 compounds, 40.54% contained 2 compounds, 14.41% contained only 1 compound and 5.41% could not detect these 3 compounds. *Lonicera japonica* flowering buds were found to be the richest source for chlorogenic acid content, *Melissa officinalis* leaves showed the most rosmarinic acid content and the most caffeic acid content was found in *Coffea canephora* seeds. Pharmacognostic specification and chlorogenic acid content of *L. japonica* flowering bud from 15 various herbal drugstores throughout Thailand were established. Macroscopic and microscopic evaluation of flowering bud were demonstrated. Physico-chemical parameters including loss on drying, total ash, acid insoluble ash, water content, ethanol and water soluble extractive values were found to be 10.11, 6.59, 1.14, 10.82, 16.46 and 28.88 % by dry weight respectively. For quantitative analysis, chlorogenic acid content in flowering bud by TLC-densitometry compared to TLC-image analysis by imageJ software were found to be 2.24 and 2.09 g/100 g respectively which were not significantly different (P = 0.13). The validation parameters of all quantitative analysis were investigated according to ICH guideline. HPLC as well as TLC-densitometry and TLC-image analysis were demonstrated as suitable, reliable and efficient methods for the quantitative analyses. *In vitro* biological activities of *L. japonica* flowering bud compared to chlorogenic, rosmarinic and caffeic acids were evaluated by brine shrimp lethality assay, MTT cell viability assay, comet assay, antimicrobial activities, antioxidant activities and yeast alpha-glucosidase inhibition assay. The results demonstrated that flowering bud ethanolic extract showed non-toxicity on brine shrimp nauplii and 6 tested cell lines. Chlorogenic, rosmarinic and caffeic acids demonstrated toxicity against brine shrimp nauplii. They showed more cytotoxic potentials against tested cell lines than the extract but were still accepted as no cytotoxicity. The extract and 3 compounds showed human lymphocyte DNA damage by comet assay. They were no inhibitory activities against tested microorganisms. The extract and the compounds demonstrated the abilities of DPPH and nitric oxide scavenger and reducing power. However, only the compounds exhibited beta-carotene bleaching activity. Moreover, they inhibited enzyme activity in yeast alpha-glucosidase inhibition study.

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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	=	Percent
µg/ml	=	Microgram per millilitre
µl	=	Microliter
µM	=	Micromolar
µm	=	Micrometre
ATCC	=	American Type Culture Collection
BHT	=	Butylated hydroxytoluene
CCD	=	Charge-couple device
CECT	=	Colección Española de Cultivos Tipo (Spanish Type Culture Collection)
CFU	=	Colony forming unit
cm	=	Centimetre
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
EDTA	=	Ethylenediaminetetraacetic acid
$FeSO_4$	=	Iron (II) sulfate
FRAP	=	Ferric reducing antioxidant power
g	=	Gram
g/g	=	Gram per gram
g/mol	=	Gram per mole
HPLC	=	High performance liquid chromatography

HPLC-DAD	=	High performance liquid chromatography-diode array detector
HPTLC	=	High performance thin layer chromatography
hr	=	Hour
ICH	=	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IUPAC	=	International Union of Pure and Applied Chemistry
l	=	Litre
LOD	=	Limit of detection
LOQ	=	Limit of quantification
m	=	Metre
mAU	=	Milli-absorbance unit
mg	=	Milligram
mg/kg	=	Milligram per kilogram
mg/ml	=	Milligram per millilitre
min	=	Minute
ml	=	Millilitre
ml/min	=	Millilitre per minute
mM	=	Millimolar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	=	Nanometre
°C	=	Degree Celsius
PBS	=	Phosphate buffer saline

pH	=	Potential of hydrogen ion
PNPG	=	p-nitrophenyl-α-D-glucopyranoside
PTFE	=	Polytetrafluoroethylene
R^2	=	Coefficient of determination
Rf	=	Retention factor
rpm	=	Revolutions per minute
RSD	=	Relative standard deviation
SD	=	Standard deviation
spp.	=	Species
TLC	=	Thin layer chromatography
TPTZ	=	2,4,6-Tripyndyl-s-triazine
UV	=	Ultraviolet
v/cm	=	Volt per centimetre
v/v	=	Volume in a volume
WHO	=	World Health Organization

CHAPTER I
INTRODUCTION

Background and significance of the study

The herbal medicines have been using for immemorial time to treat and prevent of various health diseases [1]. They will be benefit for human health when used them as appropriate. The herbal medicines are natural products, thus they are not only always safe because of adverse reactions and long-term side effects from chemicals in herbs, but also the quality of them still has not been reported enough researches. For that reason, standardization and quality control of herbal medicines are necessary assessment [2].

Lonicera japonica Thunb. (Caprifoliaceae), commonly known in English as "Japanese Honeysuckle" and called "Sai Nam Phueng" in Thai, is native to the Eastern Asia and become naturalized throughout the world [3]. *L. japonica* has young stems with pubescence; ovate leaves with 3–8 cm long and 1–3.5 cm wide; flowers in axillary cymes; white corolla, turning yellowish or tinged pink, 2-lipped. Flowering bud is yellowish-green color, clavate shape, 2-3 cm in length with velvet surface. The pharmacological studies of *L. japonica* flowering bud have shown a wide biological activity, such as antibacterial, antiendotoxin, antiviral, anti-inflammatory and other activities [3]. In traditional Thai medicine, this plant is used for antipyretic effect [4]. In traditional Chinese medicine clinical practice, *L. japonica* flowering bud is usually used to treat various infectious diseases, anti-inflammatory and exopathogenic wind-heat [5]. The chemical constituents have been widely researched. The main compositions such as essential oils, organic acids, flavones, saponins, iridoids and inorganic elements were isolated and identified [3]. In Chinese Pharmacopoeia, the indicator compound of *L. japonica* is chlorogenic acid, which has been used as characteristic for the quality of this plant [6].

Phenolic acids are secondary metabolites of plants and commonly involved in defence against ultraviolet radiation or aggression by pathogens and also found in

edible plants. They have two main groups of phenolic acids: derivatives of benzoic acid and derivatives of cinnamic [7].

Caffeic acid, one of hydroxycinnamic acid as a secondary metabolite, is more widely distributed in many plant species. It is present in several food sources, such as berries, coffee drinks and dietary supplements. Caffeic acid has many biological activities such as antioxidant activity, antibacterial activity, prevention of atherosclerosis and other cardiovascular diseases [8].

Chlorogenic acid is an ester of caffeic acid and quinic acid. It is a kind of polyphenol derivative which widely distribute in plants, fruits and vegetables [9]. Chlorogenic acid has been shown its biological and physiological activities such as antioxidant, neuroprotective effects, protective effect against cardiovascular disease, alpha-glucosidase inhibitors, and so on [10].

In addition, rosmarinic acid, an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid, is commonly found in species of the Boraginaceae, Lamiaceae, and in some fern and hornwort. The pharmacological activities of rosmarinic acid possessed as antiviral, antibacterial, anti-inflammatory and antioxidant [11].

Chromatography is a technique to separate compounds in a mixture depend on the various times taken for each component to pass through a stationary phase. Thin layer chromatography (TLC) is one of liquid chromatography type which the stationary phase, usually silica gel, was layered on supporters such as glass, aluminum, or plastic. TLC is a simple method for separation, qualitative identification and semi-quantitative analysis of constituents in herbal medicines. High performance liquid chromatography (HPLC) is a popular technique for qualitative and quantitative analysis of chemical substances in medicinal plant. The HPLC analysis is a selective and sensitive analytical technique to quantify substances in the natural products. Quantitative analysis is a method to detect the interested compound in the plant extracts and can be characteristic of each plant.

The biological experimental assessments have been used as standard safety studies together with the efficacy tests. The herbal medicines, including natural products are mostly comprised of complex compounds, thus it is important to investigate the biological studies to get scientific information before clinical trials.

Oxidation is the loss of an electron from one atom to another atom and present in metabolism, but when the electron flow becomes unpaired single electrons then producing free radicals [12]. Free radicals cause many diseases including cancer, cardiovascular disease, ulcerative colitis, aging and so on [13]. Cancer is a group of diseases involving uncontrolled cell growth with the possibility to attack or spread to other organs. There are a lot of biochemical and physiological carcinogens such as tobacco smoke, infections by virus, bacteria and parasites, ultraviolet, contamination of food by mycotoxins and so forth [14]. Pathogenic microorganism is pathogen such as virus, bacteria and fungi that can cause infection diseases in humans. Diabetes mellitus is a chronic metabolic disease associated with a lack of insulin or insulin resistance causing high blood glucose levels [15]. Nowadays, there are many chemical drugs can be treated diseases, however side effects and drug resistant occurred frequently. Therefore, the medicinal plants have been increasing interest for alternative treatment due to their less side effects and risk to the body.

From the above, this research is interesting to reveal chlorogenic acid, rosmarinic acid and caffeic acid in various medicinal and edible herbs in Thailand and validated HPLC. The pharmacognostic specifications of *L. japonica* flowering bud and chlorogenic acid content were investigated by using TLC-densitometry compared with TLC-image analysis (ImageJ software). Densitometry is performed by measuring the intensity of the absorbance or fluorescence signal between the sample spots and background on the TLC plate using specific or non-specific wavelength. Another method to quantify the chemical constituents is image analysis, processing with software to measure the intensity of pixels in digital imaging of TLC chromatogram [16]. Moreover, *L. japonica* flowering bud ethanolic extract, standard chlorogenic acid, standard rosmarinic acid and standard caffeic acid were examined in the biological

activities including antimicrobial, antioxidant, alpha-glucosidase inhibition activity, as well as brine shrimp lethality assay, MTT assay and comet assay.

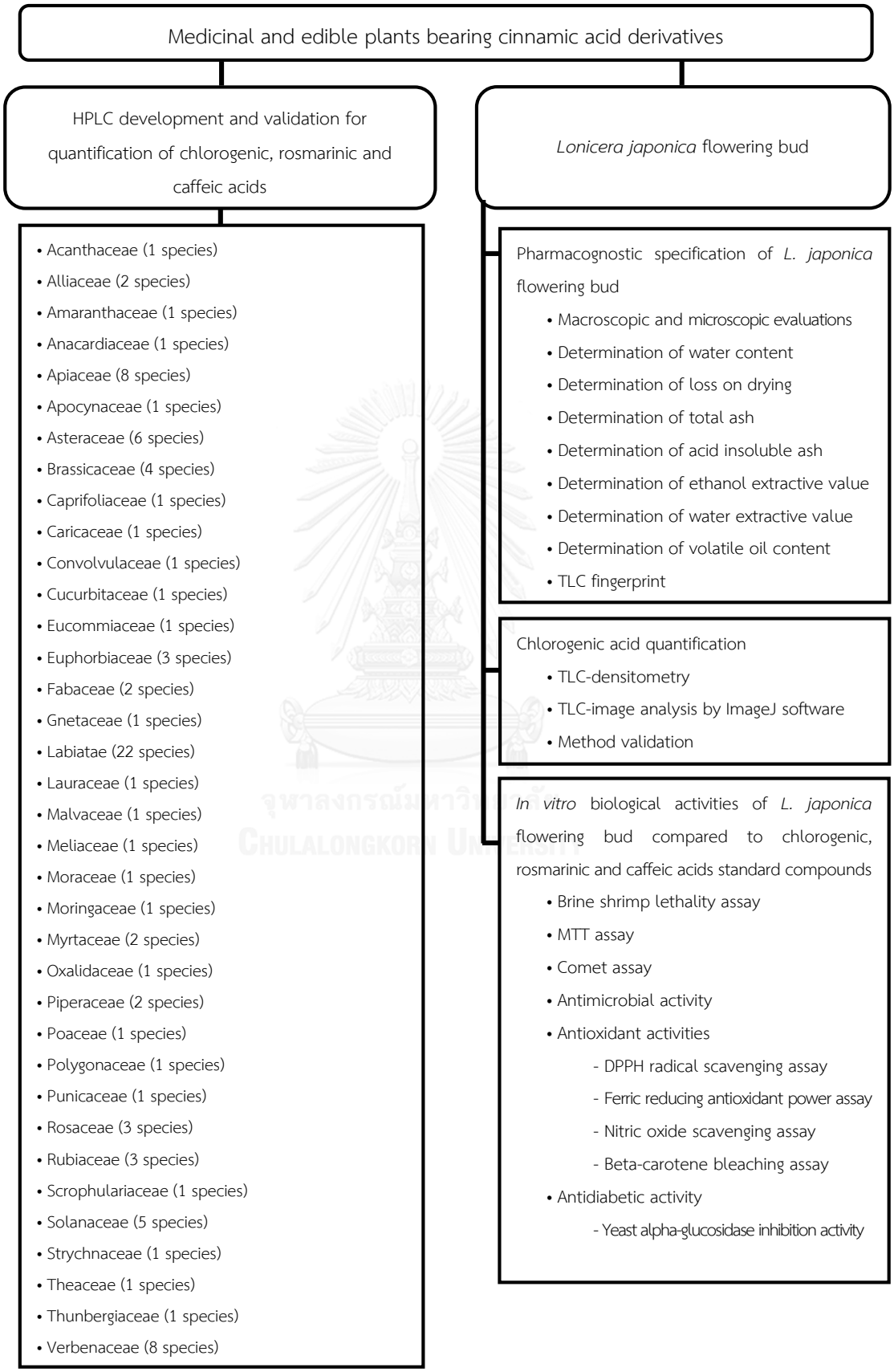
Objectives

1. To quantify chlorogenic acid, rosmarinic acid and caffeic acid contents in various medicinal and edible herbs using high performance liquid chromatography.
2. To establish the pharmacognostic specifications of *L. japonica* flowering bud.
3. To determine chlorogenic acid content in *L. japonica* flowering bud by TLC-densitometry compared to TLC-image analysis using ImageJ free software.
4. To examine *in vitro* biological activities of *L. japonica* flowering bud ethanolic extract compared to standard chlorogenic, rosmarinic and caffeic acids for their antimicrobial, antioxidant, alpha-glucosidase inhibition and cytotoxic activities.

Benefits of the study

1. This research provides the approximately quantification of chlorogenic, rosmarinic and caffeic acids in selected plants using high performance liquid chromatography.
2. This research provides the pharmacognostic specifications of *L. japonica* flowering bud.
3. This research provides the methodology to determine the chlorogenic acid content in *L. japonica* flowering bud.
4. This research provides the scientific evidences *in vitro* biological activities of *L. japonica* flowering bud ethanolic extract compared to standard chlorogenic, rosmarinic and caffeic acids.

Conceptual framework



CHAPTER II

LITERATURE REVIEWS

Part I: Quantification of chlorogenic acid, rosmarinic acid and caffeic acid contents in selected plants using high performance liquid chromatography

Polyphenol

Phenolic compounds or polyphenols are one of the most abundant and extensively distributed groups of substances in the plant kingdom which appear in all plant organ; however, the polyphenolic profile of plants differs between varieties of the same species. For decades, polyphenols have interested many researches for their antioxidant, antioxidative stress activities and great abundance in food. Polyphenols, the secondary metabolites of plant, are the active compounds in many medicinal plants.

The varieties of natural polyphenols range from simple molecules (such as phenolic acids) to highly polymerized compounds (so as tannins). Polyphenols occur primarily in conjugated form, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist. In addition, carboxylic acid, organic acids, amines, lipids, and linkages with other phenols are also common conjugated compounds [7, 17].

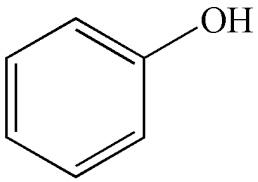
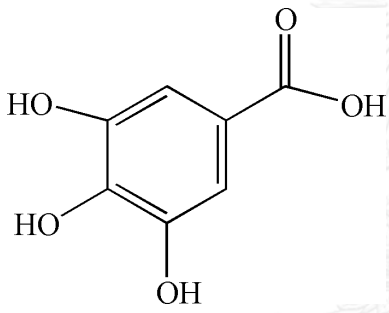


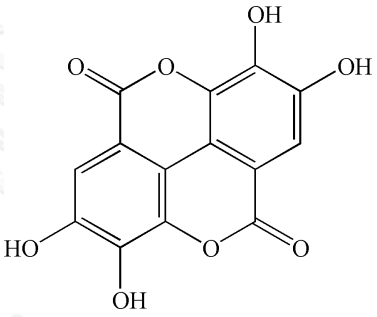
Figure 1 Phenol structure

Phenolic acids can be divided into two classes: benzoic acid derivatives, containing seven carbon atoms (C_6-C_1), and cinnamic derivatives, containing nine carbon atoms (C_6-C_3). They consist of benzene as a basis bond to a carboxylic group (benzoic acids) or to propenoic acid (cinnamic acids). Both structures can be found with different hydroxylation levels [18, 19].

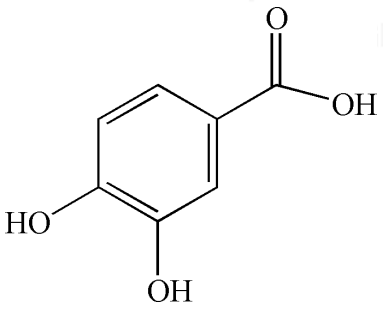
The hydroxybenzoic acid content in edible plants is commonly low, except in some red fruits, black radish, onion and potatoes skin. The main hydroxybenzoic acid are gallic acid, ellagic acid, protocatecuic acid and 4-hydroxybenzoic acid (Figure 2) [7, 18, 20].



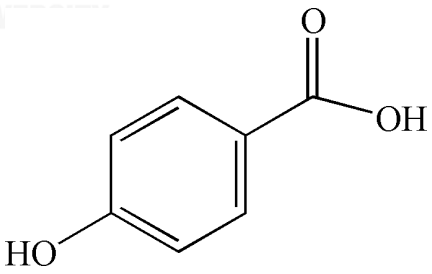
Gallic acid



Ellagic acid



Protocatecuic acid



4-hydroxybenzoic acid

Figure 2 The structures of hydroxybenzoic acid derivatives; gallic acid, ellagic acid, protocatecuic acid and 4-hydroxybenzoic acid

Hydroxycinnamic acid is usually found in plants compared to hydroxybenzoic acid. The hydroxycinnamic acid derivatives consist of a large group of simple phenolic acid, and bountiful in fruits, seed of fruits, vegetables and cereals. In addition, they have been arranged into structural and functional constituents of plant cell walls and also as bioactive ingredients of diets. The derivatives of hydroxycinnamic acids are synthesized through shikimate pathway in which phenylalanine and tyrosine are used as starting precursor molecules. The main hydroxycinnamic acid derivatives are ferulic acid, caffeic acid, *p*-coumaric acid, chlorogenic acid, sinapic acid and rosmarinic acid (Figure 3) [7, 18, 20].

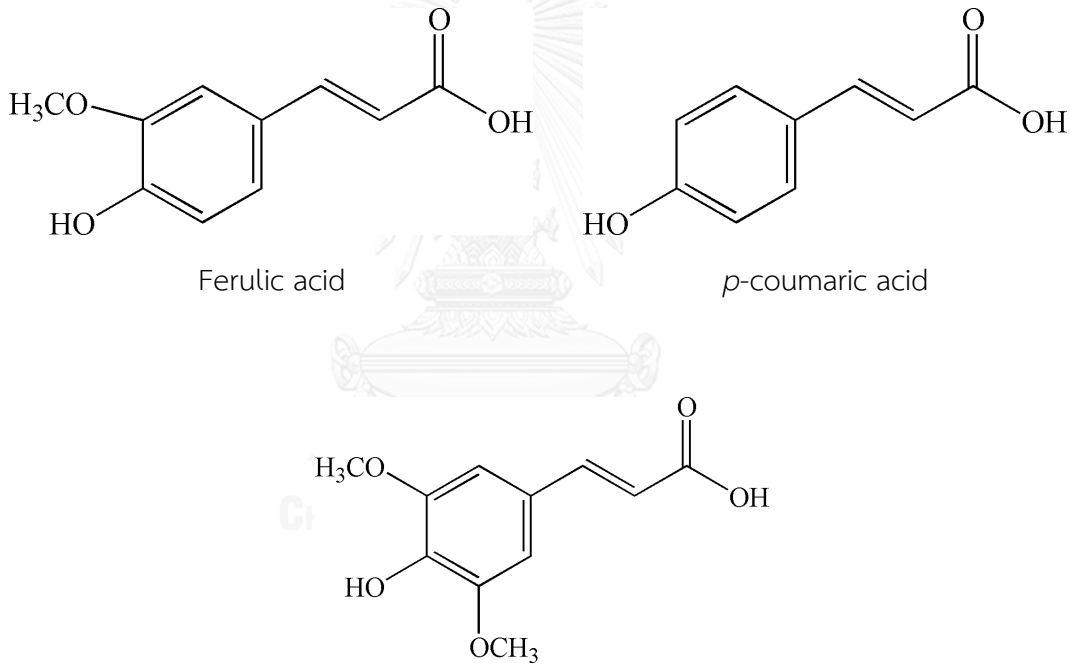


Figure 3 The structures of hydroxycinnamic acid derivatives; ferulic acid, *p*-coumaric acid and sinapic acid

Caffeic acid

Chemical name	Caffeic acid
Molecular formula	$C_9H_8O_4$
IUPAC name	(E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid
Molecular weight	180.159 g/mol

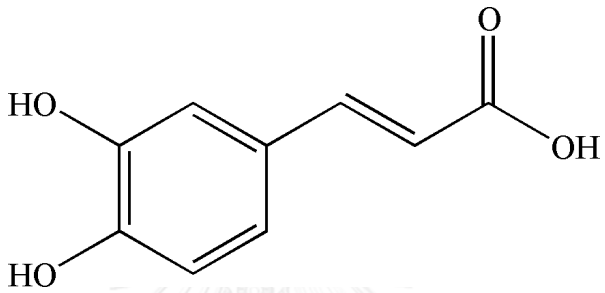


Figure 4 Structure of caffeic acid

Chemistry and occurrence

Caffeic acid (Figure 4), a secondary metabolite of shikimate pathway, is one of hydroxycinnamic acid derivatives widely distributed in many plant species. It is present in several food sources, such as berries, coffee drinks, vegetables and dietary supplements [8].

Biological activities

Gülçin evaluated the antioxidant properties of caffeic acid using various *in vitro* antioxidant methods. The DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical scavenging showed 93.9% inhibition of caffeic acid compared to those of BHT and BHA (99.7 and 86.2%, respectively), at the concentration of 20 µg/ml. For the ferrous ions chelating capacity, caffeic acid was found to be 53.2% chelation of ferrous ion at 10 µg/mL concentration compared to BHT and BHA (64.3% and 72.1%, respectively).

Furthermore, caffeic acid is proven as an effective superoxide anion radical scavenging, ABTS scavenging and total reducing power [21].

Almajano *et al.* studied antimicrobial activity of caffeic acid in vary pH range 5-7. The result showed pH dependent on MIC values, at pH 5 the MIC value (%) of *Pseudomonas aeruginosa* (CECT 108), *Bacillus cereus* (CECT 5144), *Micrococcus luteus* (CECT 5863), *Escherichia coli* (CECT 99), *Staphylococcus aureus* (CECT 239), *Listeria monocytogenes* (CECT 911) and *Candida albicans* (CECT 1002) as 0.25, 0.20, 0.20, 0.25, 0.25, 0.32 and 0.25% respectively. The sensitivity of caffeic acid with respect to the microorganisms was "*B. cereus, M. luteus* > *P. aeruginosa, C. albicans* > *E. coli, S. aureus,* and *L. monocytogenes*". The last three bacteria at pH 6.2 and 7 were not inhibited by caffeic acid at its highest concentration (0.4%) [22].

Stanifort *et al.* stated that the caffeic acid significantly reduced the mRNA expression of Interleucin-10 UVB-induced in murine and also inhibited the activation of p38-MAPK [23]. Moreover, in the study of Yang *et al.*, the caffeic acid lowered the migratory capacity of malignant keratinocytes [24].

Pang *et al*. studied the protective mechanism of caffeic acid in acetaminophen-induced liver injury. The results demonstrated that 400 mg/kg of acetaminophen induced the raise of serum alanine/aspartate aminotransferases, while caffeic acid at 30 mg/kg reduced the acetaminophen-induced increased alanine/aspartate aminotransferases. Moreover, caffeic acid at 10 and 30 mg/kg reversed acetaminophen-induced decreased the quantity of liver glutathione. Additionally, mice treated with 400 mg/kg of acetaminophen exhibited severe liver damage, indicated by intrahepatic hemorrhage, lymphocytes infiltration and the destruction of liver structure. After treatment with caffeic acid at 10 and 30 mg/kg, the damage cells were all ameliorated. Moreover, caffeic acid was found to reversed the decreased cell viability induced by acetaminophen in human normal liver L-02 cells and HepG2 cells [25].

Choi *et al*. reported that caffeic acid at 50 μg/ml showed a maximally protective effect against cisplatin-induced HEI-OC1 (mouse auditory cell line) cell

damage by the MTT assay. Furthermore, caffeic acid decreased cell death by apoptosis and necrosis [26].

Bouzaienea *et al*. studied the effect of caffeic acid on superoxide anion production, adhesion and migration of human lung (A549) and colon adenocarcinoma (HT29-D4) cancer cell lines. Caffeic acid at 200 μM significantly decreased superoxide production by 92% of A549 and 77% of HT29-D4 cell lines respectively. Migration assay examined with A549 cell line, showed that 200 μM of caffeic acid reduced significantly cell migration by 7.7% of the covered surface [27]. Moreover, Ye *et al*. reported that caffeic acid isolated from *Ocimum gratissimum* had anti-proliferative effects on cervical cancer cell lines (HeLa) [28].

Chlorogenic acid

Chemical name	Chlorogenic acid
Molecular formula	$C_{16}H_{18}O_9$
IUPAC name	(1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid
Molecular weight	354.311 g/mol

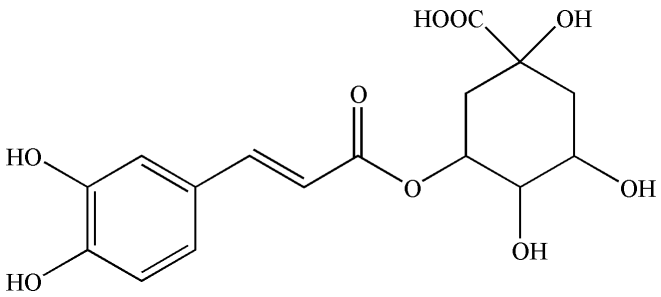


Figure 5 Structure of chlorogenic acid

Chemistry and occurrence

Chlorogenic acid (Figure 5) is an ester form of caffeic acid and quinic acid. It is one of polyphenol derivatives widely found in plants, fruits and vegetables. Especially, in coffee and some traditional Chinese medicines, such as *Lonicera japonica* bud and flower, and *Eucommia ulmodies* leaves [10, 19]. About 71 different species of chlorogenic acid have now been isolated and identified from different sources. According to the International Union of Pure and Applied Chemistry (IUPAC), chlorogenic acid is designated to be 5-caffeoylquinic acid (5-CQA) [29] due to its commercially available and extensive studies for the antioxidant activity.

Biological activities

Ohno *et al*. studied nitric oxide suppression and indicated that chlorogenic acid dose-dependently decreased the level of nitric oxide production, as IC_{50} of 652 ± 114 μM, which proved not to be cytotoxic to the hepatocytes [30].

Kweon *et al*. assessed IC_{50} of chlorogenic acid in the DPPH scavenging assay as 12.3 ± 0.12 μM compared to ascorbic acid as 49.5 ± 0.35 μM. The superoxide anion radical scavenging activity exhibited IC_{50} of chlorogenic acid as 6.9 ± 0.12 μM compared to ascorbic acid as 56.0 ± 1.01 μM [31].

Li *et al*. evaluated the antioxidant effect of chlorogenic acid against methylmercury (MeHg) in PC12 cells, and displayed the dose-dependent manner of chlorogenic acid which could protect PC12 cells against MeHg-induced damage. Chlorogenic acid not only suppressed the generation of reactive oxygen species, the decrease of activity in glutathione peroxidase (GPx), and the decrease of glutathione, but also attenuated caspase-3 activation in PC12 cells by MeHg. The result concluded that chlorogenic acid might exert neuroprotective effects through its antioxidant actions [32].

Oboh et al. studied *in vitro* α-glucosidase activities of chlorogenic acid, and revealed IC_{50} as 9.24 μg/ml compared to caffeic acid which showed the higher inhibitory effect as 4.98 μg/ml [33].

Mikami *et al*. examined the protective effects of chlorogenic acid on glutamate-induced neuronal cell death using primary cultures of mouse cerebral cortex. The results demonstrated that the treatment with chlorogenic acid was able to inhibit glutamate-induced neuronal cell death, as well as prevented the increased concentrations of Ca^{2+} in intracellular caused by the addition of glutamate to cultured neurons [34].

Hong *et al*. evaluated the effects of chlorogenic acid on diabetic mice. The anti-diabetes efficacy of chlorogenic acid was treated with 16-week-old mice, the result revealed that mice in the chlorogenic acid treatment groups demonstrated decreased blood glucose levels comparing with diabetes mellitus group. The hearing threshold and latency tests were performed using auditory brainstem responses to detect any improvement mediated by chlorogenic acid in peripheral auditory function damaged by diabetes mellitus. The hearing thresholds or latencies in response to clicks, 4-kHz TBs and 8-kHz TBs in the chlorogenic acid treatment groups decreased significantly compared to the diabetes mellitus group. Thus, the researchers indicated that chlorogenic acid might improve damaged peripheral auditory function in the diabetes mellitus mouse model [35].

Ji *et al*. observed the protective effect of chlorogenic acid against *in vivo* acetaminophen-induced liver injury in mice by histological observation. The results implied that mice treated with 300 mg/kg of acetaminophen induced the elevation of serum alanine transaminase, aspartate transaminase and also showed severe liver damage, indicated by intrahepatic hemorrhage, lymphocytes infiltration and the destruction of liver structure. Conversely, chlorogenic acid could reverse such liver injury in the dose-dependent manner, especially chlorogenic acid at the concentration of 40 mg/kg [36].

Cinkilic *et al*. studied the radioprotective effect of chlorogenic acid in human blood lymphocytes using the alkaline comet assay. The results proved that chlorogenic acid decreased the DNA damage induced by X-ray irradiation and provided a significant radioprotective effect in which the magnitude of protection for genetic damage index ranged from 4.49 - 48.15% [37].

Rosmarinic acid

Chemical name	Rosmarinic acid
Molecular formula	$C_{18}H_{16}O_8$
IUPAC name	(2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid
Molecular weight	360.318 g/mol

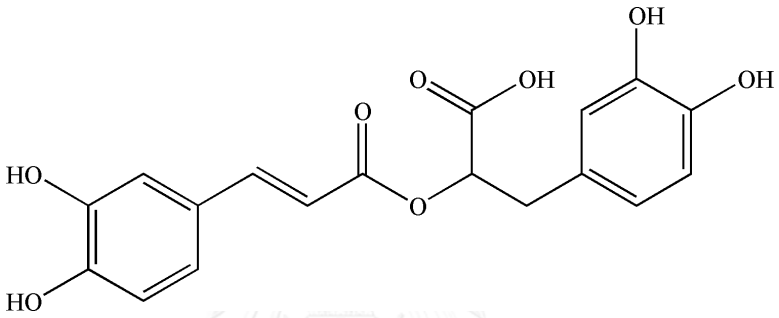


Figure 6 Structure of rosmarinic acid

Chemistry and occurrence

Rosmarinic acid (Figure 6) is an ester form of caffeic acid and 3,4-dihydroxyphenyllactic acid. The derivatives of rosmarinic acid from natural products comprising of rosmarinic acid conjugated with other aromatic moieties have been identified from higher plants such as isorinic acid and lithospermic acid. Rosmarinic acid is commonly found in Boraginaceae, subfamily Nepetoideae of the Lamiaceae. Moreover, it is also found in some ferns of the family Blechnaceae, in lower plants such as hornworts and in monocotyledonous plants as the sea grass family Zosteraceae [11, 38].

Biological activities

Pérez-Fons et al. reported the antioxidant activity of rosmarinic acid which exhibited in a trolox equivalent antioxidant capacity (TEAC) assay as 3.655 ± 0.073 mmol of Trolox/g of compound compared to carnosic acid, carnosol, rosmadial and genkwanin as 3.565 ± 0.050, 3.566 ± 0.21, 1.963 ± 0.083 and 1.045 ± 0.064 mmol of Trolox, respectively [39].

Vostálová *et al*. confirmed that rosmarinic acid also significantly eliminated ROS production and diminished IL-6 release, moreover, it could suppress UVB-induced alterations to human keratinocytes HaCaT [40].

Kim *et al.* indicated that rosmarinic acid was able to inhibit the retinal endothelial cells proliferation in a dose-dependent manner, and restrained the *in vitro* angiogenesis of tube formation. The rosmarinic acid also showed an anti-angiogenic activity against retinal neovascularization in a mouse model of retinopathy and no retinal toxicity [41].

Rahbardar *et al*. investigated the potential anti-inflammatory effects of rosmarinic acid using sciatic nerve chronic constriction injury (CCI)-induced neuropathic pain in a rat model. They demonstrated a significant increase of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE-2), nitric oxide (NO), interlukin-1ß (IL-1ß) and matrix metalloproteinases (MMP2) in the spinal cord of CCI rats on day 7 and day 14 after injury. The results of rosmarinic acid (40 mg/kg, intraperitoneal administration) reduced amount of inflammatory and oxidative markers on day 7 and day 14 [42].

Coelho *et al*. studied the effect of rosmarinic acid on seizures induced by pentylenotetrazole (PTZ) using the kindling model in male CF-1 mice. Mice were treated with rosmarinic acid (1, 2 or 4 mg/kg; i.p.) once every three days during 16 days, 30 min before PTZ administration (50 mg/kg; s.c.). The results demonstrated that 2 mg/kg of rosmarinic acid increased latency and decreased percentage of seizures, only on the 4th day of observation but the other tested doses did not show any effect. In addition, the alkaline comet assay using brain cortex revealed that rosmarinic acid at 4 mg/kg could reduce both DNA damage and damage index (DI) [43].

High performance liquid chromatography (HPLC)

Chromatography is a physical separation technique in a mixture that the components to be separated are distributed between two phases. Each compound travels through a stationary phase carried by a mobile phase for different time taken to move from the start position to the detected position. [44].

HPLC is a column chromatographic technique which consists of greatly small particles of stationary phase coated in solid supporter, generally placed inside a stainless steel column, and a liquid mobile phase. The separations of analysis or component are demonstrated by peak in the chromatogram. The detection of analysis can be performed using a variety of detectors (Table 1). HPLC has been extensively used for analysis in food, nutrition, pharmaceuticals industries, agriculture, and environment. Its applications include separation, purification, identification, and quantification of various components. Currently, it is the most commonly equipment used for both qualitative and quantitative analyses in herbal extracts by automatic operation and efficient separation [45, 46].

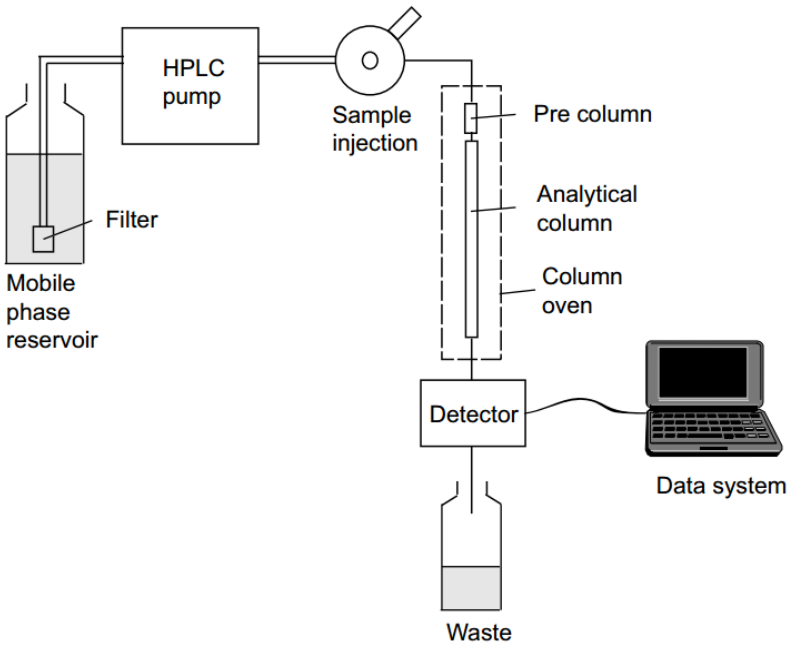


Figure 7 The main structure of HPLC system [47]

Table 1 Common HPLC detectors [44, 48]

Detector	Description
Ultraviolet	This detector displays the absorption of UV or visible light in the HPLC eluent which has this property. Different compounds do not absorb at the same wavelength, thus this is a selective detector.
Diode array detector (DAD)	This detector is also known as a photodiode array detector (PDA). It provides UV spectra of eluting peaks while simultaneously functioning as a full UV range.
Fluorescence	This detector displays the emitted fluorescent light of the fluorescent compound at a suitable wavelength. It is sensitive and selective but is appropriate to compounds with strong innate fluorescence.
Electrochemical detector (ECD)	This detector responds to compounds that can be oxidisable or reducible, in which electron flow generated by a reaction takes place at the surface of the electrodes.
Conductivity detector	This detector measures the electrical conductivity of the mobile phase and can be detected to ppm-ppb levels analysis of ions, organic acids, and surfactants.
Refractive index (RI)	This detector measures changes of refraction index when the analyte passes through the sample cell in the detector, the reference detector being filled with the mobile phase.
Evaporative light scattering (ELS)	This detector is based on the scattering of a beam of light by particles of analyte remaining after evaporation of the mobile phase.

Part II: Pharmacognostic specification of *Lonicera japonica* flowering bud and chlorogenic acid content by TLC-densitometry and TLC-image analysis

Caprifoliaceae

Caprifoliaceae, honeysuckle family, consist of approximately five genera and 207 species. This family is distributed throughout the world, mostly in temperate regions of East Asia and Eastern North America [49]. For economic importance, plants are grown as ornamental shrubs or vines in *Lonicera* (Honeysuckle) species [50].

"*Shrubs or woody climbers, rarely small trees or herbs. Leaves opposite, rarely whorled, simple or pinnatifid, conduplicate or involute in vernation; interpetiolar stipules absent or rarely well developed. Inflorescence thyrsoid, axillary or terminal, compact or lax, cymes 1-, 2-, or 3-flowered; paired flowers sometimes with ovaries ± fused. Cymes with a pair of bracts and 2 pairs of bracteoles, located at base of ovaries, ± fused, occasionally accrescent in fruit, rarely absent. Flowers bisexual, actinomorphic or zygomorphic. Calyx 4- or 5-lobed. Corolla epigynous, gamopetalous; lobes 4 or 5, spreading, sometimes bilabiate, aestivation imbricate. Stamens (4 or)5, didynamous, alternating with corolla lobes, sometimes exserted; anthers free, 2-celled, opening by longitudinal slits, introrse. Ovary inferior, carpels 2–8, fused; placenta axile; ovules 1 to many per locule, some of which can be abortive, pendulous; style solitary; stigmas capitate or lobed. Fruit a berry, a drupe with 2–5 pyrenes, or a leathery achene. Seeds 1 to many; embryo small, straight; endosperm copious* [49]."

Lonicera

Lonicera genus is one of five genera that belongs to Caprifoliaceae family. It includes around 180 species, typically found in North Africa, Asia, Europe and North America.

"*Shrubs erect or dwarf, rarely small trees, sometimes climbers, deciduous or evergreen. Branches hollow or solid with white or brown pith; winter buds with 1 to several pairs of scales, rounded or acutely 4-angular, inner scales sometimes*

accrescent and reflexed. Accessory buds sometimes present, occasionally terminal buds reduced and substituted by 2 lateral buds. Leaves opposite, rarely whorled, margin entire, rarely dentate or divided; leaves usually estipulate, occasionally with interpetiolar stipules or a swollen interpetiolar line; sometimes 1 or 2 pairs of leaves below inflorescence connate and forming involucral bracts. Inflorescence thyrsoid, terminal or axillary, cymes opposite and usually reduced to paired flowers, rarely 1-, sometimes 3-flowered. Inflorescence occasionally pedunculate; cymes sessile, sometimes forming a capitulum, or cymes pedunculate with a pair of bracts and 2 pairs of bracteoles; bracts usually small, sometimes leaflike; bracteoles usually free, sometimes ± fused and cupular occasionally enclosing ovaries, sometimes absent. Paired flowers with free or partially to completely fused ovaries. Calyx 5-lobed, rarely 4-lobed, sometimes truncate, base occasionally with a collarlike emergence. Corolla white, yellow, reddish, or purple-red, often changing color after anthesis, campanulate, funnelform, regularly or subregularly 5(or 4)-lobed, or bilabiate and upper lip 4-lobed; tube long or short, often shallowly to deeply gibbous on ventral side toward base, rarely spurred. Nectary of compact sessile glandular hairs on ventral side toward base of corolla tube, occasionally in 5 regular lines, rarely swollen at base of style. Stamens 5; anthers dorsifixed. Ovary 2 or 3(–5)-locular; style slender, hairy or glabrous; stigmas capitate. Fruit a berry, red, blue-black, black, green, or white sometimes pruinose, bracteoles occasionally accrescent in fruit and enclosing paired berries. Seeds 1 to numerous, smooth, pitted or granular, with rounded embryo [49]."

Lonicera japonica

Lonicera japonica, commonly known in English as "Japanese Honeysuckle" or "Jin Yin Hua or Ren Dong" in Chinese and called "Sai Nam Phueng" in Thai, is native to the eastern Asia and becomes naturalized in Argentina, Brazil, Mexico, Australia, New Zealand and United States [3, 4].

Scientific classification [51]

Kingdom: Plantae - Plants

Subkingdom: Tracheobionta - Vascular plants

Superdivision: Spermatophyta - Seed plants

Division: Magnoliophyta - Flowering plants

Class: Magnoliopsida - Dicotyledons

Subclass: Asteridae

Order: Dipsacales

Family: Caprifoliaceae - Honeysuckle family

Genus: *Lonicera* L. - honeysuckle

Species: *Lonicera japonica* Thunb. - Japanese honeysuckle

"*Sprawling and twining lianas, semievergreen. Branches becoming hollow. Branches, petioles, and peduncles with dense, yellow-brown spreading stiff pubescent, interspersed with long glandular hairs. Petiole 3–8 mm; leaves blade ovate, elliptic, oblong or broadly lanceolate, 3–8 × 1–4 cm, abaxially sparsely to densely hairy, adaxially hairy along veins, base rounded to subcordate, margin ciliate, occasionally sinuate, apex acute to acuminate. Flowers fragrant, paired and axillary toward apices of branchlets; peduncle 2–40 mm, shorter toward apex of branchlets; bracts leaflike, ovate to elliptic, 1–3 cm; bracteoles ca. 1 mm, pubescent, apex rounded or truncate and ciliate. Neighboring 2 ovaries free; ovary ca. 2 mm, glabrous. Calyx lobes triangular, ca. 1 mm, densely hairy abaxially and along margin, apex acute. Corolla bilabiate, white, becoming yellow, or purple outside and white inside, 3–5 cm, spreading hairy with interspersed long glandular hairs outside; tube 1.5–3 cm, not gibbous at base; upper lip irregularly 4-lobed, lobes 2–8 mm; lower lip recurved. Stamens and style glabrous, subequaling to exceeding corolla. Berries black when*

mature, glossy, globose, 6–7 mm in diam.; seeds brown, ovoid or ellipsoid, ca. 3 mm, shallowly pitted [49, 52]*."*

Lonicera japonica has two varieties; *L. japonica* var. *japonica* presents corolla white, later yellow-white, whereas *L. japonica* var. *chinensis* (Watson) Baker expresses corolla purple outside, white inside [49]. The flowering period of *L. japonica* is from May to September and duration of flowering is generally 5 - 8 days. The flowering stage can be separated into six stages:

Stage 1: The juvenile bud stage

Stage 2: The green stage

Stage 3: The white stage

Stage 4: The complete white stage

Stage 5: The silver flowering stage

Stage 6: The gold flowering stage

L. japonica often grows in warm subtropical areas, hillside scrub, rocks pile and roadside, and from sea level to 1,200 - 1,500 m elevation. Apart from natural wild growth, Japanese honeysuckle is cultured as an ornamental plant which conserves water and soil in the world due to its gorgeous flowers and strong roots [53, 54].

Figure 8 Flowering plant of *Lonicera japonica*

Chemical constituents

More than 140 chemical compounds have been isolated and identified from *L. japonica*. Organic acids, essential oil and flavonoids are top three important groups of bioactive compounds found in this plant [3].

Organic acids

Organic acids are one of the most important and effective components of *L. japonica*. The main compositions contain chlorogenic acid, isochlorogenic acid, caffeic acid, hexadecanoic acid and etc. [55] As a major compound of the flowers, chlorogenic acid has received much attention for its part of human diet and supplements with potential biological effects [10], and used as a standard compound in plant for quality control. In Chinese Pharmacopoeia, the content of chlorogenic acid in flowering bud should be not less than 1.5% [6].

Essential oil

Essential oil, one of the main compositions of *L. japonica*, is interesting in both wide activity and utilization. A total of eighty-nine volatile oil compounds were identified, and the main compound in flowers and leaves fractions was found to be linalool [56]. Moreover, the study of Ikeda *et al*. reported that linalool was the important components which characterize the volatile of honeysuckle flowers [57]. Due to the differences in geography, harvesting time, parts of used and processing methods, the contents and components of essential oil are different. Previous research indicated that the silver flowering stage is the most preferable harvest times for volatile oil [53]; the best medicinal part is flower; low temperature and no-lighting are in favor of the essential oil in the dry and extraction processes [3].

Flavonoids

The widespread biological activities of flavonoids have been significantly applied to treat many diseases, including cancer, cardiovascular disease, oxidative stress and neurodegenerative disorders [58]. Luteolin 7-*O*-glucoside, along with chlorogenic acid, has been noted in the Chinese Pharmacopoeia to control the quality of crude drug by HPLC method, the content of luteolin 7-*O*-glucoside in flowering bud should be not less than 0.1% [6]. Moreover, other flavonoids of *L. japonica* were identified as quercetin, hyperoside, lonicerin, loniceraflavone, luteolin and etc. Until now, about 30 flavones have been isolated and identified from this plant [3].

Ethnopharmacology

L. japonica has been cultured and used as a traditional medicine in many countries, especially in Eastern Asia. In China, Japanese honeysuckle has been used to treat dysentery, swellings, clear away the heat-evil and prolong life that has noted in 'Ben Cao Gang Mu', the well-known classical Chinese materia medica book [3, 59]. Additionally, some prescription in Chinese Pharmacopoeia used this plant as the main composition to heal various ailments such as curing headache, fever, cough, pruritus, upper respiratory tract infection, acute tonsillitis and etc. [3] In Thailand, this plant is widely used for antipyretic, diuretic and antidiarrheal effects [4, 60].

Pharmacological activities of *L. japonica* crude extract

Antioxidant activity

Cai *et al*. (2004) studied antioxidant activity and phenolic compounds in 112 species of traditional Chinese medicinal plants, the result of total phenolic content and Trolox equivalent antioxidant capacity (TEAC) values for *Lonicera japonica* flora bud methanolic extract were 3.63 gallic acid equivalent/100 g dry weight and 589.1 μmol Trolox equivalent/100 g dry weight, respectively [61].

Chio *et al*. (2007) reported the antioxidant effect of *L. japonica* flowers in ethyl acetate fraction ,via 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, total reactive oxygen species (ROS), hydroxyl radical (OH), and peroxynitrite ($ONOO^-$) assays as IC_{50} values of 4.37, 27.58 ± 0.71, 12.13 ± 0.79 and 0.47 ± 0.05 μg/mL, respectively [62].

Tsai *et al*. (2008) reported the Trolox equivalent antioxidant capacity (TEAC) of methanolic extract from honeysuckle to be 0.595 ± 0.011 mmole TE/ g extract and oxygen radical absorbing capacity (ORAC) was 1.66 ± 0.01 mmole TE/ g extract [63].

Seo *et al*. (2012) evaluated the antioxidant capacities of the leaf, flower and stem of *L. japonica* methanolic extract. The result of DPPH assay was EC_{50} as 79.3 ± 2.0, 90.7 ± 1.8 and 89.3 ± 2.4 mg/L, respectively compared to BHT as 180.1 ± 9.0 mg/L. For $ABTS^+$ assay, the result showed EC_{50} as 33.0 ± 0.7, 40.1 ± 1.0 and 43.1 ± 1.3 mg/L, respectively, while BHT as 156.1 ± 4.3 mg/L [64].

Anti-inflammatory activity

Lee *et al*. (1998) examined *n*-butanol fraction of *L. japonica* to anti-inflammatory activity. It demonstrated significant anti-inflammatory effects at oral doses of 400 mg/kg against arachidonic acid (AA) ear edema, croton-oil ear edema, carrageenan (CGN)-paw edema, rat cotton pellet granulomatic and rat adjuvant-induced arthritis (AIA) inflammation models, the inhibitions were 27%, 23%, 26%, 18% and 42%, respectively. On the contrary, the inhibition rate of aspirin (100 mg/kg), as positive drug, were 27%, 13%, 13%, 0% and 58% [65].

Tae *et al*. (2003) investigated the anti-inflammatory effects in proteinase-activated receptor 2 (PAR2)-mediated mouse paw edema of *L. japonica* water extract at dose as 10, 50, 100 and 200 mg/kg, orally administered. At doses of 50, 100 and 200 mg/kg, the water extract presented significant inhibition of both change in paw thickness and vascular permeability. The inhibition rate of paw thickness was 41.8%, 69.1%, 70.9%, and vascular permeability was 40.2%, 69.7%, 68.8%. Moreover, the water extract at 100 mg/kg also significantly inhibited PAR2 agonists-induced

myeloperoxidase (MPO) activity and tumor necrosis factor (TNF)-α expression in paw tissue [66].

Antibacterial and antiviral activities

Rahman *et al*. (2009) evaluated the antibacterial activity of ethanolic extract from *L. japonica* leaves and essential oil from flowers. The result exhibited the effect against *Bacillus cereus* (SCK 11), *Bacillus subtilis* (ATCC 6633), *Enterobacter aerogenes* (KCTC 2190), *Escherichia coli* (ATCC 8739), *Listeria monocytogenes* (ATCC 19116), *Staphylococcus aureus* (ATCC 6538 and KCTC 1916), *Salmonella enteritidis* (KCTC 12021) and *Salmonella typhimurium* (KCTC 2515) but no effect on *Escherichia coli* (O157:H7 ATCC 43888) and *Pseudomonas aeruginosa* (KCTC 2004). This study suggested that the extract and essential oil from *L. japonica* might be a potential source of preservatives for use in the foodstuff or pharmaceutical industries [67]. Furthermore, Shane *et al*. (2007) evidently confirmed that the floral bud from *L. japonica* was also against *Bacillus cereus* and *Staphylococcus aureus* [68].

Ma *et al.* (2002) demonstrated the antiviral activities against respiratory syncytial virus (RSV) by means of the cytopathologic effect (CPE) assay in 44 medicinal plants which applied to treat respiratory tract infectious diseases in China. The aqueous extracts of *L. japonica* flower bud exhibited potent antiviral activities against RSV (IC_{50} was 50.0 μg/ml) [69]. Wang *et al.* (2006) isolated flavonoids from *L. japonica* floral buds and proved the anti-virus (H9N2) activity [70]. Additionally, *L. japonica* has also been used to prevent and treat some viral diseases in human and veterinary for example SARS coronavirus, H1N1 (Swine) flu virus, and being called the 'bouvardin' [71].

Hepatoprotective effect

Sun *et al.* (2010) induced an acute stage of hepatic injury in Wistar rats by injecting a high dose (35 mg/kg) of dimethylnitrosamine (DMN) for 7 days, *L. japonica* ethanolic extract showed meaningfully hepatoprotective effect by histopathological analysis [72].

Toxicity activity

Thanabhorn *et al*. (2006) performed the acute and subacute toxicity of *L. japonica* leaves ethanol extract in male and female Sprague-Dawley rats. The ethanol extract at a dose of 5,000 mg/kg by orally did not present mortality or significant changes in the general behavior and gross examination of the internal organs of rats showed no detectable abnormalities. In the subacute toxicity study, the rats received repeated doses of ethanolic extract at 1,000 mg/kg/day for 14 consecutive days. The satellite group was treated with the same dose of ethanolic extract at the same period, and kept for further 14 days after treatment. There were no signs of toxicity and mortality in the treated group as compared to the control group of both sexes. Therefore, the results exposed that the ethanolic extract of leaves was fairly nontoxic [4].

Standardization parameters [73, 74]

"Quality control methods for herbal material guideline" has been published by World Health Organization to describe various analytical assessments information for the standardization parameters of medicinal plant materials. The following methods encourage to examine the quality of herbal material by using modern control techniques.

Macroscopic and microscopic examination

Medicinal plant materials are categorized according to sensory, macroscopic and microscopic characteristics. An examination to determine these characteristics is the first step towards establishing the identity, quality and purity of materials, and should be carried out before any further tests are performed. Visual inspection provides the simplest and quickest for investigation. Macroscopic identity of herbal materials is based on shape, size, color, surface characteristics, texture, fracture characteristics, odour and appearance of the cut surface. Microscopic evaluation is essential for the identification of powdered materials; the specimen may have to be treated with chemical reagents.

Determination of water content and loss on drying

An excess of water in herbal materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. Limits for water content should therefore be set for every given herbal material. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water.

The azeotropic method (toluene distillation method) is performed for the measurement of water present in the material. Toluene must be saturated with water before use for an accurate result.

The test for loss on drying determines both water and volatile matter in the material. It can be carried out by heating at 100 - 105 °C until constant weight.

Determination of ash

The ash residue after ignition of herbal materials is an inorganic material that varies within fairly wide limits, so it is important parameter for evaluation of crude drug. The ash value is determined by different methods to measure the total ash and acid insoluble ash.

The total ash method is designed to measure the total amount of material remaining after complete ignition. The total ash usually consists of carbonate, phosphates, silicates and silica as “physiological ash”, which is derived from the plant tissue itself, and “non-physiological ash”, which is the residue of the extraneous matter on the plant surface.

Acid insoluble ash is to measure the residual after boiling the total ash in diluted hydrochloric acid, and igniting the remaining insoluble matter. This ash measurement presents some inorganic elements such as silica.

Determination of extractable matter

This method determines the amount active constituent in plant material when extracted with solvent. The extraction of any crude drug with a specific solvent gives yield that contains different phyto-constituents, regarding the specific solvent used as well as the plant nature. Ethanol and water are the primary solvents for plant material extraction.

Determination of volatile oil

The characteristics of volatile oils are identified by their odour, oil-like appearance and ability to volatilize at room temperature. They are various compound such as monoterpenes, sesquiterpenes and their oxygenated derivatives. Aromatic compounds predominate in certain volatile oils. The determination of volatile oil is determined by water distillation. The dissolved volatile oils will then float on top of the aqueous phase in a graduated tube.

Thin layer chromatography

In the organic chemistry laboratory, thin layer chromatography (TLC) is one of the most extensive analytical methods for a long time. TLC is a rapid screening method used to identify and separate compounds in herbal extracts. TLC is more advantages than other chromatographic techniques, due to its low cost of instrumentation, short time analysis, and simplicity to use [75-77].

TLC is an adsorption chromatography in which substances are separated based on the interaction between stationary phase and mobile phase. The stationary phase is a layer of adsorbent coated on the plate, whereas the mobile phase is a selected solvent [78]. Silica gel is the most commonly used for adsorbents of TLC plates; moreover, the other adsorbents used as stationary phase include alumina, octadecasilica, cellulose, dextran gels, polyamide, or other ion exchange polymeric resin [46]. The support materials for the stationary phases are glass, plastic or aluminum plate. Among them, aluminum is most commonly used. The substances will be spotted onto the plate to be dissolve with solvent for separation. The selected solvent is allowed to flow up the plate by capillary action called “development step”. After the development, the solvent is removed from the plate by evaporation or heating, and detection is performed under the ultraviolet light of 254 and 365 nm wavelengths [79, 80].

An important qualitative parameter, which characterizes the position of a spot on TLC plate, is the retention factor (Rf) value. It is define as:

$$Rf = \frac{\text{Distance of the compound from original spot travelled to the developed spot}}{\text{Distance of the solvent from original line travelled to the developed line}}$$

TLC is frequently used for both qualitative and quantitative purposes. For qualitative analysis, it can be determined by the number of compounds in a mixture and identified substances. On the other hand, TLC is used for content determination of required testing substances in quantitative evaluation [80].

Quantitative analysis can be performed with data from scanning densitometry and image analysis method. Scanning densitometer contains a fixed wavelength to measure the difference in absorbance or fluorescence signal between a separated zone and the empty plate background. The peak area data of the unknowns are compared with data from calibration standards chromatographed on the same plate [16, 81].

TLC-densitometry

In situ densitometry is a simple way of quantifying the desired sample components or amount directly applied on the plate. The resolution of compounds to be separated on the plate is followed by measuring the optical density of the separated spots directly on the plate. The sample amounts are determined by comparing them to a standard curve from reference materials chromatographed simultaneously under the same condition [74].

A typical densitometer, which could also be used for scanning chromatogram, has the following operating characteristic: [82]

- Reflectance or transmission modes
- Absorbance or fluorescence measurements
- Accommodates plates up to 20 x 20 cm
- Wavelength range: 190-800 nm
- Multiwavelength scanning, up to 31 channels
- Computer controlled and data processed
- Full spectra available for qualitative analysis

Scanning densitometers are slit-scanning, single-beam, single-wavelength instruments and evaluation with software after scanning. The feature of the scanner principal design represented by TLC Scanner 3 is demonstrated in Figure 9.

The instruments comprise of an electronic part, a compartment for plate positioning, and the optical system. The three light sources including mercury vapor lamp, deuterium lamp and tungsten halogen lamp are positioned in the light path by motor drive. The deuterium and tungsten halogen lamps are continuum lamps; they emit light over a wide wavelength range. Different sources must be used to cover the entire UV-vis range. The tungsten-halogen lamp is used as the source for scanning colored zones in the 400 - 800 nm range (visible absorption). The deuterium lamp is directly used for scanning UV-absorbing zones, or quenched zones on phosphor-containing layers in the 190 - 450 nm range. The high-intensity mercury or xenon sources is for fluorescence excitation.

Monochromators or filters is a grating in modern instruments, in which some old instruments use a quartz prism to apply for wavelength selection and a photomutiplier tube or photodiode detector for signal measurement. Measurements are commonly conducted under the reflectance mode and occasionally the transmission mode by mounting the plate on a movable stage controlled by stepping motors that is mechanically operated in the x- and y-directions. The plate is scanned with a fixed beam of monochromatic light in the form of an adjustable rectangular slit, the height of which is matched to the width of the largest spot or band [81, 83, 84].

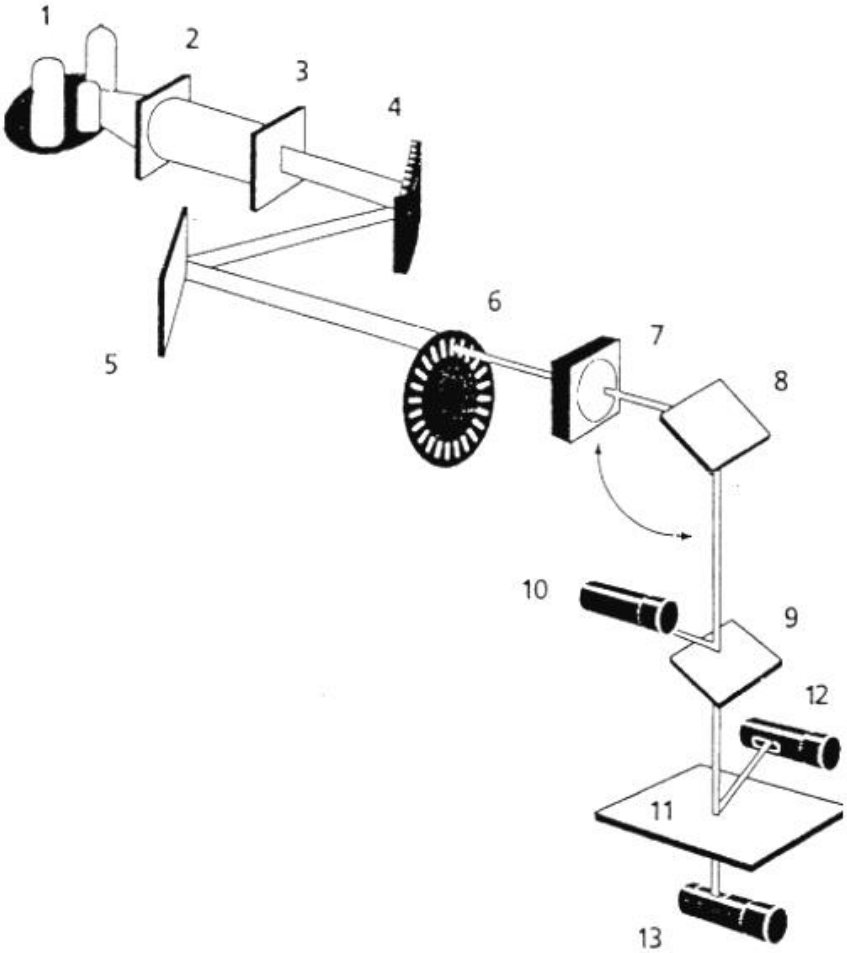


Figure 9 Light path diagram of TLC Scanner 3 [81]

1) Lamp selector; 2) entrance lens system; 3) entrance slit; 4) grating monochromator; 5) mirror; 6) 20 fixed-slit aperture disk; 7) lens system positioned in accordance with the slit size selected (choices are 0.5 - 12 mm length and 0.025 - 1.2 mm width); 8) mirror; 9) beam splitter; 10) reference photomultiplier; 11) plate to be scanned; 12) measuring photomultiplier; 13) photodiode (trans-mission).

TLC-image analysis

In previous study, the charge-couple device (CCD) camera is also used in quantitative method. CCD is two-dimensional detectors containing sensors capable for imaging an area in seconds or real time. The output from each sensor pixel on the CCD is a voltage, which is proportional to the intensity of light falling on the sensor and the exposure time. These series of voltages are digitized and transferred to a computer for storage and data processing. Coupling CCD detection with TLC, the entire chromatographic plate can be imaged in a single exposure yielding rapid quantification in shorter analysis time, compared to that of slit scanning densitometers. CCD detectors have demonstrated extremely low dark current and read noise characteristics, high sensitivity and excellent linearity. These features have made the CCD an excellent detector for many imaging applications in chemical analysis, such as fluorescence detection. The advantages of image analysis are fast data acquiring and simple instrument design [16, 81, 85].

ImageJ is a popular software from several image analysis software that requires images from CCD camera for analysis. ImageJ is an open source developed in Java programs, that users can manually develop program and fix the program. It is used in many fields such as medical researches and biological microscopy. It can be demonstrated in both Windows and Macintosh, available free download from website of the US National Institute of Mental Health. (http://rsbweb.nih.gov/ij/index.html) [85, 86].

Method validation [87, 88]

Method validation is the procedure to confirm the reliability of the method and demonstrate the useful analytical data in normal use. The purpose of these methods is to ensure that an analytical methodology is accurate, specific, reproducible and robust over the specified range of analysis.

According to ICH guideline for the validation of analytical procedures, the recommended validation parameters recommended are linearity, range, specificity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness.

Linearity and range

The linearity is the ability of an analytical process within a given range to obtain experiment results which are directly proportional to the analyte concentration in the sample. ICH guideline recommends a minimum of 5 concentrations for the establishment of linearity.

The range is the interval between the upper and lower concentrations of analyte in the sample which has been demonstrated that the analytical process has a suitable level of precision, accuracy and linearity.

Specificity

The specificity is the ability to determine the presence of components in the analyte. For chromatographic method, the peak identity of the interested compounds should be clearly separated from other components in the sample. Also, the UV absorbance spectra of selected compound and standard peaks should be matched. The peak purity is evaluated to check the impurity of UV absorbance spectra performed by up-slope, apex and down-slope of the selected compound.

Accuracy

The accuracy is the closeness of the test value obtained by the analytical method to the true value. ICH guideline recommends that the accuracy should be evaluated by a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range, such as 3 concentrations with 3 replicates each of the total analytical method. The accuracy result should be reported

as percent recovery that performed by spiked sample with known concentration of analyte.

Precision

The precision is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions and expressed as the percent relative standard deviation (RSD). The precision should be performed at 3 levels according to ICH guideline including repeatability, intermediate precision and reproducibility.

Limit of detection

The limit of detection (LOD) is described as the lowest concentration of an analyte in a sample that can be detected but not quantitated. There are several approaches for determining the LOD;

Base on visual evaluation, the LOD is evaluated by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Base on signal-to-noise, the LOD is evaluated by comparing between the sample signals with blank samples. The acceptable ratio of LOD is between 3 or 2:1.

Based on the standard deviation of the response (σ) and the slope of the calibration curve (*S*), the LOD may be expressed as: LOD = 3.3 (σ)/*S*. The standard deviation of the response can be evaluated based on the standard deviation (SD) of the blank, on the residual SD of the regression line, or the SD of *y*-intercepts of regression line.

Limit of quantitation

The limit of quantitation (LOQ) is described as the lowest concentration of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. There are several approaches for determining the LOQ;

Base on visual evaluation, the LOQ is evaluated by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Base on signal-to-noise, the LOQ is evaluated by comparing between the sample signals with blank samples. The acceptable ratio of LOQ is 10:1.

Based on the standard deviation of the response (σ) and the slope of the calibration curve (*S*), the LOQ may be expressed as: LOQ = 10 (σ)/*S*. The standard deviation of the response can be evaluated based on the standard deviation (SD) of the blank, on the residual SD of the regression line, or the SD of *y*-intercepts of regression line.

Robustness

The robustness is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It is performed by varying method parameters, in HPLC [89] such as pH in mobile phase, mobile phase ratio, different column, temperature, flow rate and etc. In HPTLC method [80, 90] is varied in small change of solvent composition, humidity, chamber size, the temperature of plate activation, the distance of development, the wavelength and etc.

Part III: *In vitro* biological activity evaluations

Cytotoxic activity (Brine shrimp lethality assay)

Brine shrimp (*Artemia salina*) is a species of aquatic crustaceans. The brine shrimp anatomy can be divided into 3 parts: head, a middle (thorax) and a tail (abdomen). Brine shrimps generally move on their backs, upside down with their leafy-legs uppermost [91]. They have been used as a "benchtop bioassay" for the investigation of bioactive natural products, and they are a good selection for elementary toxicity investigations of consumer products. The brine shrimp lethality assay might be used as a simple method to guide screening and fractionation of physiologically active plant extracts, where one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive [92, 93].

Cytotoxic activity (MTT cell viability assay)

The tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a rapid colorimetric assay, developed by Mosmann (1983), that measures only living cells for measurement of cytotoxicity and cell proliferation [94].

The principle of MTT assay is detected an increase or decrease in the number of viable cells that related to mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT into a purple colored formazan crystals (Figure 10). The formazan must be solubilized prior to recording absorbance readings because of its insoluble precipitate property, various solubilized agents such as acidified isopropanol, DMSO, dimethylformamide, SDS, and combinations of detergent and organic solvent. Therefore, any decrease or increase of viable cell number can be detected by measuring formazan concentration at 570 nm using a plate reader [95]. The results will be showed as 50% growth inhibition as compared to the growth of the untreated control (50% inhibitory concentration, IC_{50}). According to the US National Cancer Institute Plant Screening Program, a pure compound is generally considered to have in vitro cytotoxic activity with IC_{50} less than 4 µg/ml, while this value was considered at less than 20 µg/ml for a crude extract [96, 97].

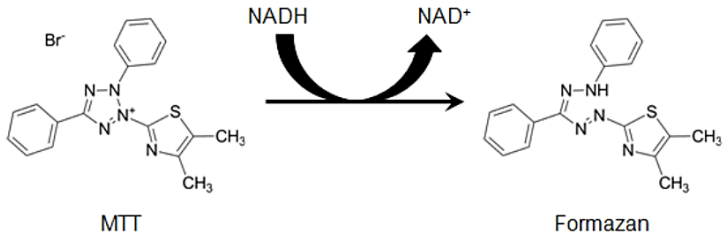


Figure 10 MTT structure and formazan product

DNA damage (Comet assay)

The comet assay or the single cell gel electrophoresis assay (SCG or SCGE assay) is a rapid and quantitative technique that has become one of the standard methods in measuring the DNA damage of eukaryotic cells. It is based on quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis. This assay acquires simplicity, sensitivity, versatility, speed and economy for detecting DNA strand break. The cell suspension can be isolated from whole blood, cell from tissue biopsies, buccal cells, plant cells, sperm cells and culture cells can be used [98-100]. Peripheral blood mononuclear cells can be isolated from whole blood sample, it is a convenient source of cells and the majority of human biomonitoring researches. Lymphocytes are normal diploid cells and suitable for study because they circulate through the whole body and can have a relatively long life-span [101].

Antimicrobial activity

The main objective of antimicrobial susceptibility testing is to suggest the researcher in the choice of agents for therapy. Agents are commonly used empirically and routine testing serves the latest information on suitable agents for empirical use. In addition to laboratory work, antimicrobial susceptibility tests are used to evaluate the *in vitro* activity of new agents [102]. *In vitro* antimicrobial susceptibility tests are depended on two roles, diffusion and dilution. The simple and widespread methods are agar diffusion and broth dilution methods [103].

Agar diffusion method

The agar diffusion test are evaluated by inoculating a nutrient agar medium in a standard method and applies test compound to the agar surface in some type of reservoir. The test compound is diffused surrounding medium, after incubation of appropriate time, there should be an inhibition zone of organism growth around the reservoir. The dimension of zone may be measured to estimate the degree of organism susceptibility [104]. Types of agar diffusion test are categorized by the techniques that apply the test compound solution to a seeded agar medium.

Agar disk diffusion protocol includes the filter paper disk containing the test compound at each concentration placed directly on agar surface. The plates are incubated under suitable conditions. Usually, test compound solution diffuses into the agar and inhibits germination and growth of the test microorganism. The diameters of inhibition growth zones are then measured.

The procedure of agar well diffusion method is similar to agar disk diffusion method, a hole with a diameter of 6 - 8 mm is punched aseptically with a sterile cork borer and pipetted the test compound solution into the well. After incubation, the agent diffuses in the agar medium and inhibits the growth of the microbial tested [103].

Broth microdilution method

The method is adaptation of the broth dilution method by using small volume in 96-well microplate. Next, well is inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale. After incubation under suitable conditions, the well microplate is examined for microbial growth as indicated by turbidity that detected by the unaided eye. The lowest concentration of test compound that completely inhibits growth of the organism demonstrates the minimal inhibitory concentration (MIC) [103].

Antioxidant activities

Oxidants (Free radicals)

Free radicals are the products of normal cellular metabolism. The free radicals are one or more unpaired electron atoms or molecules that make them unstable, short lived and highly reactive. The result from their highly reactivity, they can steal electrons from other substances for stability. Accordingly the attacked molecule loses its electron and becomes a free radical itself, source of a chain reaction which effectively damages the living cell. Free radicals are found in biological system, which are often associated with oxygen and other substances. They are often referred to reactive oxygen species (ROS) and reactive nitrogen species (RNS).

The oxidative stress, has been induced by free radicals, causes serious cell damage involved in several human diseases such as neurodegenerative disorders (Parkinson's disease, Alzheimer's disease), cardiovascular diseases (atherosclerosis and hypertension), arthritis, immunological incompetence, various cancers (colorectal, prostate, breast, lung, bladder cancers) and etc. [105, 106]

Antioxidants

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Important characteristics of antioxidant agents are the ability to donate electron to oxidant or reactive oxygen substances and inhibit oxidative stress reaction.

Antioxidants can be found in foods, fruits, vegetables or supplementations. Examples of antioxidants include beta-carotene, lutein, lycopene, vitamin A, vitamin C, vitamin E and other substances. Furthermore antioxidant can be found from natural sources or secondary metabolites such as phenolic compounds and flavonoids [107]. A variety of *in vitro* and *in vivo* methods are currently used for determination of antioxidant and free radical scavenging capacity of plant extracts. *In vitro* screening is the primary selective tool for finding potential antioxidants and free radical scavengers. There are many different methods to evaluate the *in vitro*

antioxidant activity of the medicinal plants which involve different mechanisms of antioxidation, based on chemically scavenging of ROS or RNS.

DPPH$^{\bullet}$ radical scavenging assay

The DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) radical is a popular stable free radical used for assessing radical scavenging or antioxidant activity. The DPPH$^{\bullet}$ assay is based on the ability of antioxidants to reduce the stable free radical DPPH$^{\bullet}$. The delocalization of the spare electron with DPPH$^{\bullet}$ molecule causes the stability of this free radical and gives characteristics of deep violet color with a maximum absorbance of 520 nm, resulting in a color transformation from violet to yellow [108].

When a solution of DPPH$^{\bullet}$ is mixed with a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color. Representing the DPPH$^{\bullet}$ radical by Z$^{\bullet}$ and the donor molecule by AH, the primary reaction is

$$Z^{\bullet} + AH = ZH + A^{\bullet}$$

Where, ZH is the reduced form and A$^{\bullet}$ is free radical produced in this first step [109]

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay is colorimetric method to measures the ability of antioxidant to reduce ferric ion based on the complex reduction between ferric ion and TPTZ to the deeply blue-colored ferrous complex, at low pH. The reduction of ferric to ferrus ions cause a change in color that can be detected by spectrophotometer at 593 nm. The results express as mM of ferrus equivalents or relative to an antioxidant standard. However, FRAP assay cannot detect species that act by radical quenching (H transfer), particularly SH group of antioxidant such as protein and glutathione [13, 110, 111].

Nitric oxide scavenging activity

The nitric oxide scavenging activity measures the ability of sample to scavenge nitric oxide. The nitric oxide ($NO^{\bullet}$) has been produced by sodium nitroprusside solution at physiological pH 7.2. $NO^{\bullet}$ reacts with oxygen under aerobic condition to produce stable product such as nitrate and nitrite. The quantity of scavenging activity can be evaluated using Griess reagent, the result showed pink color solution and measured at 546 nm [13].

Beta-carotene bleaching assay

Beta-carotene bleaching assay measures the ability of an antioxidation to inhibit lipid peroxidation. This method measures of the discoloration of the action between beta-carotene and linoleic which lack of antioxidant substance. The free linoleic acid radical, is an unsaturated fatty acid, formed upon the abstraction of a hydrogen atom from one of its methylene groups attacked the beta-carotene molecules, which lost the double bonds and therefore, its characteristic orange color. Bleaching is based on the loss of the yellow color of beta-carotene due to its reaction with radicals and measured by the difference between interval times at 470 nm [13, 112].

Antidiabetic activities

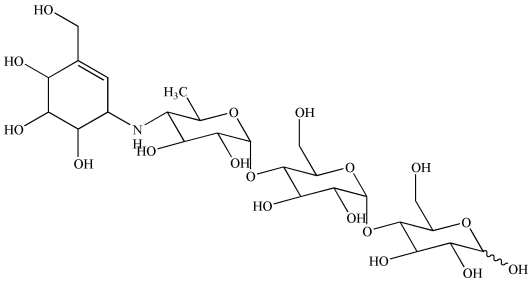
Diabetes mellitus is a group of chronic metabolic diseases in a person which has chronic hyperglycemia (high blood sugar level), resulting from defects of insulin secretion, insulin action, or both. This disease is induced long-term damage and dysfunction in many organs such as eyes, kidneys, heart and blood vessel. Also, associated with symptoms including polyuria, polydipsia, weight loss and blurred vision. There are 2 forms of diabetes mellitus [113, 114]:

Type 1 diabetes or in term as insulin-dependent diabetes or juvenile-onset diabetes, is due to autoimmune-mediated destruction of ß-cell islets of pancreas, resulting in lack of insulin. People with type 1 must treated with insulin injection.

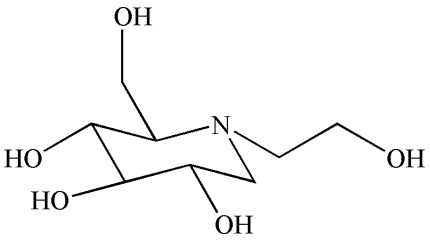
Type 2 diabetes or in term as non-insulin-dependent diabetes or adult-onset diabetes, is results from insulin resistance and/or abnormal insulin secretion. People with type 2 are not depend on insulin but may take it for control blood glucose level.

Due to the increased of type 2 diabetes patients in worldwide, this creates affected to demand for the research of developing anti-diabetic drugs.

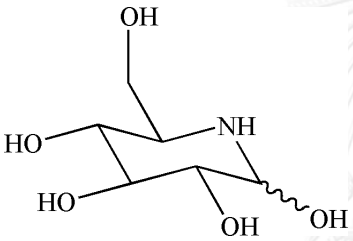
Alpha-glucosidase is a carbohydrate-hydrolyzing enzyme with high abundance in microorganisms, plants, and animals [115]. It is important enzyme to absorb carbohydrates in the brush border of small i0ntestine, and has been recognized as a therapeutic target for modulation of postprandial hyperglycaemia. Numerous alpha-glucosidase inhibitors, for example Acarbose, Miglitol, Nojirimycin and Voglibose (Figure 11) can inhibit alpha-glucosidase accordingly delaying the absorption of sugars from the gut and have been used for treatment of diabetes mellitus [116, 117].



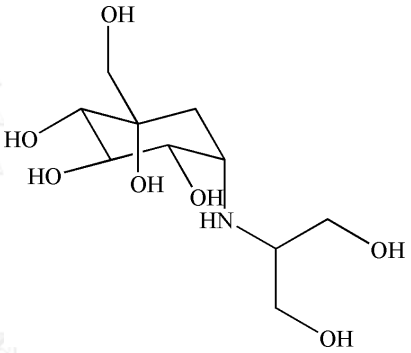
Acarbose



Miglitol



Nojirimycin



Voglibose

Figure 11 Alpha-glucosidase inhibitors

CHAPTER III

MATERIALS AND METHODOLOGY

Chemicals and reagents

2,2-Diphenyl-1-picylhydrazyl (DPPH)	Sigma-Aldrich, St. Louis, Missouri, USA
2,4,6-Tripyridyl-*s*-triazine (TPTZ)	Sigma-Aldrich, St. Louis, Missouri, USA
2,6-Di-*tert*-butyl-4-methylphenol (BHT) (CAS No. 128-37-0)	Sigma-Aldrich, St. Louis, Missouri, USA
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)	Life Technologies, California, USA
Acarbose	Sigma-Aldrich, St. Louis, Missouri, USA
Acetic acid glacial	BDH Chemicals, Poole, UK
Agarose	Research organics, Ohio, USA
Agarose, low gelling temperature	Sigma-Aldrich, St. Louis, Missouri, USA
Alpha-glucosidase from *Saccharomyces cerevisiae*	Sigma-Aldrich, St. Louis, Missouri, USA
Amikacin sulfate	T.P. Drug Laboratories (1969), Bangkok, Thailand
Ampicillin sodium	T.P. Drug Laboratories (1969), Bangkok, Thailand

Chemicals and reagents (Cont.)

Beta-carotene	Sigma-Aldrich, St. Louis, Missouri, USA
Caffeic acid (CAS No. 331-39-5, purity ≥98%)	Sigma-Aldrich, St. Louis, Missouri, USA
Chloroform, AR grade	RCI Labscan Limited, Bangkok, Thailand
Chlorogenic acid (CAS No. 327-97-9, purity ≥95%)	Sigma-Aldrich, St. Louis, Missouri, USA
Dimethysulfoxide (DMSO)	Merck, Darmstadt, Germany
di-Sodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich, St. Louis, Missouri, USA
Doxorubicin hydrochloride	Sigma-Aldrich, St. Louis, Missouri, USA
Ethanol, AR grade	RCI Labscan Limited, Bangkok, Thailand
Ethidium bromide (10mg/ml solution)	Bio Basic Canada, Ontario, Canada
Ethyl acetate, AR grade	RCI Labscan Limited, Bangkok, Thailand
Ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2)	Ajax Finechem, Auckland, New Zealand
Ferrozine	Sigma-Aldrich, St. Louis, Missouri, USA
Formic acid 98-100%, AR grade	Merck, Darmstadt, Germany

Chemicals and reagents (Cont.)

Histopaque-1077	Sigma-Aldrich, St. Louis, Missouri, USA
Hydrochloric acid 37%, AR grade	RCI Labscan Limited, Bangkok, Thailand
Hydrogen Peroxide 30%, AR grade	QRëC, New Zealand
Iron (II) chloride tetrahydrate ($FeCl_2$•$4H_2O$)	Sigma-Aldrich, St. Louis, Missouri, USA
Iron (II) sulfate heptahydrate ($FeCl_2$•$7H_2O$)	Sigma-Aldrich, St. Louis, Missouri, USA
Iron (III) chloride hexahydrate ($FeCl_3$•$6H_2O$)	Ajax Finechem, Auckland, New Zealand
Linoleic acid	Sigma-Aldrich, St. Louis, Missouri, USA
Methanol, AR grade	RCI Labscan Limited, Bangkok, Thailand
Methanol, HPLC grade	RCI Labscan Limited, Bangkok, Thailand
Mueller Hinton agar and broth	HiMedia Laboratories, Mumbai, India
N-(1-Naphthyl)ethylenediamine dihydrochloride	Sigma-Aldrich, St. Louis, Missouri, USA
Ortho-phosphoric acid 85%, AR grade	RCI Labscan Limited, Bangkok, Thailand
Petroleum ether 40-60, AR grade	RCI Labscan Limited, Bangkok, Thailand

Chemicals and reagents (Cont.)

p-Nitrophenyl α-D-glucopyranoside	Sigma-Aldrich, St. Louis, Missouri, USA
Potassium chloride (KCl)	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Quercetin hydrate (CAS No. 849061-97-8, purity ≥95%)	Sigma-Aldrich, St. Louis, Missouri, USA
Rosmarinic acid (CAS No. 20283-92-5, purity 96%)	Sigma-Aldrich, St. Louis, Missouri, USA
RPMI-1640 Medium	Life Technologies, California, USA
Sabouraud Dextrose agar and broth	HiMedia Laboratories, Mumbai, India
Sodium acetate ($C_2H_3NaO_2$)	Sigma-Aldrich, St. Louis, Missouri, USA
Sodium bicarbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Sodium carbonate (Na_2CO_3)	QRëC, New Zealand
Sodium chloride (NaCl)	Ajax Finechem, Auckland, New Zealand
Sodium nitroprusside dihydrate	Sigma-Aldrich, St. Louis, Missouri, USA
Sulfanilamide	Sigma-Aldrich, St. Louis, Missouri, USA
Toluene	RCI Labscan Limited, Bangkok, Thailand

Chemicals and reagents (Cont.)

Tris(hydroxymethyl)-methylamine	Fisher Scientific UK, Leicestershire, UK
Triton X-100	Sigma-Aldrich, St. Louis, Missouri, USA
Tween 20	Merck, Darmstadt, Germany
Ultra-pure water	NW20VF, Heal Force, China

Materials

Cover glasses (24 x 50 mm), Menzel Gläser	Thermo Scientific, Brunswick, Germany
Cuvettes, visible range	Bibby Scientific, Staffordshire, UK
Filter papers No. 4	Whatman, UK
Filter papers No. 40 Ashless	Whatman, UK
Hemocytometer	Digital Bio, Seoul, Korea
Inertsil® ODS-3 HPLC column (5 µm x 4.6 mm x 250 mm)	GL Sciences, Tokyo, Japan
Microscope slides (25.4 x 76.2 mm)	Sail Brand, China
Microtiter plates with 96 wells	BRANDplates, Wertheim, Germany
Nylon membrane filters (46 mm x 0.45 µm)	National Scientific, Tennessee, USA
PTFE membrane syringe filters (13 mm x 0.45 µm)	ANPEL Laboratory Technology (Shanghai), Shanghai, China

Materials (Cont.)

ReproSil®-Pur ODS-3 HPLC guard column (5 µm x 4.0 mm x 10 mm)	Dr. Maisch GmbH, Ammerbuch, Germany
Syringe	Nipro, Phra Nakhon Si Ayutthaya, Thailand
TLC aluminium sheet, silica gel 60 GF_{254}	Merck, Darmstadt, Germany

Instruments and equipments

Ashing Furnaces (AAF 11/18)	Carbolite, Hope Valley, UK
Autoclave (Model: HVE-50)	Hirayama, Tokyo, Japan
CAMAG TLC Chamber	CAMAG, Muttenz, Switzerland
CAMAG TLC Plate Heater III	CAMAG, Muttenz, Switzerland
CAMAG TLC Scanner 4	CAMAG, Muttenz, Switzerland
Centifuge (Model: SIGMA 1-14)	Sartorius, Göttingen, Germany
Digital balance (Model: SI-234)	Denver Instrument, New York, USA
Digital camera (Canon PowerShot A650 IS)	Canon Marketing (Thailand), Bangkok, Thailand
Digital Orbital Shaker (Model: SHO-2D)	Daihan Scientific, Gangwon-do, Korea
High performance liquid chromatography (LC-20A) equipped with photo diode array detector (SPD-M20A)	Shimadzu, Kyoto, Japan
Hot air oven	WTB binder, Tuttlingen, Germany
ImageJ software (Version: 1.50)	National Institutes of Health, USA

Instruments and equipments (Cont.)

Laminar hood (Model: Class II BSC)	ESCO, Singapore
Microplate reader, built-in cuvette UV/Vis spectrophotometer (Model: Anthos Zenyth 200rt)	Biochrom, Cambridge, UK
Microscope (Axio Imager.A2)	Carl Zeiss, Jena, Germany
Rotary evaporator (Model: R-210)	Buchi, Flawil, Switzerland
Ultra-pure water purification (Model: NW20VF)	Heal Force, China
Ultrasonic bath	Analytical Lab Science, Bangkok, Thailand
Ultraviolet viewing cabinet (Model: CC-80)	Spectronics Corporation, New York, USA
Water bath (Model: SC/48 R)	Brinkmann, USA
winCATS software (Version: 1.4.6.2002)	CAMAG, Muttenz, Switzerland

Materials and methods

Part I: Quantification of chlorogenic acid, rosmarinic acid and caffeic acid contents in selected plants using high performance liquid chromatography

Sample collection

Various 111 plant samples (Table 2) were purchased from local markets and convenience store markets in Thailand during 2015 and dried at 45 °C in hot air oven. All plant materials were authenticated by Associate Professor Nijsiri Ruangrungsi, Ph.D., and voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. After removal of any foreign matters, crude drugs were grounded into coarse powders before use.

Table 2 Selected plant samples in the study with Thai name and parts used

No.	Scientific plant name	Plant parts used	Thai plant name
	Family: Acanthaceae		
1	*Andrographis paniculata* (Burm.f.) Wall. ex Nees	Leaves	ใบฟ้าทะลายโจร
	Family: Alliaceae		
2	*Allium sativum* L.	Bulbs	กระเทียม
3	*Allium cepa* L.	Bulbs	หอมหัวใหญ่
	Family: Amaranthaceae		
4	*Spinacia oleracea* L.	Leaves	ใบป่วยเล้ง
	Family: Anacardiaceae		
5	*Mangifera indica* L. cv. Okrong	Leaves	ใบมะม่วงอกร่อง
	Family: Apiaceae		
6	*Anethum graveolens* L.	Aerial part	Dill
7	*Apium graveolens* L.	Aerial part	คื่นฉ่ายฝรั่ง
8	*Apium graveolens* L. var. *secalinum*	Aerial part	คื่นฉ่ายจีน
9	*Centella asiatica* (L.) Urb.	Aerial part	บัวบก
10	*Coriandrum sativum* L.	Seeds	เมล็ดผักชี
	Family: Apiaceae		
11	*Daucus carota* L. subsp. *sativus* (Hoffm.) Arcang.	Roots	แครอท
12	*Eryngium foetidum* L.	Leaves	ใบผักชีฝรั่ง
13	*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill	Aerial part	Parsley

Table 2 Selected plant samples in the study with Thai name and parts used (Cont.)

No.	Scientific plant name	Plant parts used	Thai plant name
	Family: Apocynaceae		
14	*Telosma cordata* (Burm. f.) Merr.	Flowers	ดอกขจร
	Family: Asteraceae		
15	*Artemisia dracunculus* L.	Aerial part	Tarragon
16	*Artemisia pallens* Wall. ex DC.	Aerial part	โกฐจุฬาลัมพา
17	*Chromolaena odorata* (L.) R. M. King & H. Rob.	Leaves	ใบสาบเสือ
18	*Gnaphalium polycaulon* Pers.	Aerial part	หญ้านางนวล
19	*Helianthus annuus* L.	Pericarps	เปลือกเมล็ดทานตะวัน
20	*Helianthus annuus* L.	Seeds	เนื้อในเมล็ดทานตะวัน
21	*Helianthus annuus* L.	Sprouts	ต้นอ่อนทานตะวัน
22	*Lactuca sativa* L.	Leaves	ผักกาดหอม
	Family: Brassicaceae		
23	*Brassica juncea* (L.) Czern.	Leaves	ผักกาดหิ่น
	Family: Brassicaceae		
24	*Brassica oleracea* L. Group *Capitata*	Aerial part	กะหล่ำปลี
25	*Brassica rapa* L. Group *Pekinensis*	Aerial part	ผักกาดขาว
26	*Raphanus sativus* L.	Roots	หัวผักกาดขาว
	Family: Caprifoliaceae		
27	*Lonicera japonica* Thunb.	Flowering bud	ดอกสายน้ำผึ้ง

Table 2 Selected plant samples in the study with Thai name and parts used (Cont.)

No.	Scientific plant name	Plant parts used	Thai plant name
	Family: Caricaceae		
28	*Carica papaya* L.	Leaves	ใบมะละกอฮอลแลนด์
	Family: Convolvulaceae		
29	*Ipomoea aquatica* Forssk.	Aerial part	ผักบุ้งไทย
	Family: Cucurbitaceae		
30	*Momordica charantia* L. (Thai varieties)	Fruits	ผลมะระขี้นก
31	*Momordica charantia* L. (Chinese varieties)	Fruits	ผลมะระจีน
	Family: Eucommiaceae		
32	*Eucommia ulmoides* Oliv.	Stem barks	โต่วต๋ง
	Family: Euphorbiaceae		
33	*Euphorbia hirta* L.	Aerial part	ต้นน้ำนมราชสีห์
34	*Phyllanthus emblica* L.	Fruits	ผลมะขามป้อม
35	*Ricinus communis* L.	Leaves	ใบละหุ่ง
	Family: Fabaceae		
36	*Pisum sativum* L.	Fruits	ถั่วลันเตา
37	*Pisum sativum* L. var. *macrocarpon*	Fruits	ถั่วลันเตาหวาน
38	*Sesbania grandiflora* (L.) Poir.	Flowers	ดอกแค
39	*Sesbania grandiflora* (L.) Poir.	Stem barks	เปลือกต้นแค

Table 2 Selected plant samples in the study with Thai name and parts used (Cont.)

No.	Scientific plant name	Plant parts used	Thai plant name
	Family: Gnetaceae		
40	*Gnetum gnemon* L. var. *tenerum* Markgr.	Leaves	ผักกะเหรี่ยง
	Family: Labiatae		
41	*Hyptis suaveolens* (L.) Poit.	Aerial part	ต้นแมงลักคา
42	*Leonotis nepetifolia* (L.) R. Br.	Leaves	ใบฉัตรพระอินทร์
43	*Leonurus sibiricus* L.	Aerial part	ต้นกัญชาเทศ
44	*Melissa officinalis* L.	Leaves	ใบ Lemon Balm
45	*Mentha arvensis* L. var. *piperascens* Malinv.	Leaves	Japanese mint
46	*Mentha cordifolia* Opiz ex Fresen	Leaves	ใบสะระแหน่
47	*Ocimum africanum* Lour.	Leaves	ใบแมงลัก
48	*Ocimum basilicum* L.	Leaves	ใบโหระพา
49	*Ocimum gratissimum* L. var. *macrophyllum* Briq.	Leaves	ใบกะเพราควาย
50	*Ocimum tenuiflorum* L.	Leaves	ใบกะเพรา
51	*Origanum majorana* L.	Leaves	ใบ Marjoram
52	*Origanum vulgare* L.	Leaves	ใบ Oregano
53	*Orthosiphon aristatus* (Blume) Miq.	Leaves	ใบหญ้าหนวดแมว
54	*Perilla frutescens* (L.) Britton	Leaves	ใบงาขี้ม่อน
55	*Plectranthus amboinicus* (Lour.) Spreng.	Leaves	ใบเนียมหูเสือ
56	*Plectranthus rotundifolius* (Poir.) Spreng.	Leaves	ใบมันขี้หนู
57	*Plectranthus rotundifolius* (Poir.) Spreng.	Tubers	หัวมันขี้หนู
58	*Plectranthus scutellarioides* (L.) R. Br.	Leaves	ใบฤาษีผสมแล้ว
59	*Rosmarinus officinalis* L.	Aerial part	Rosemary
60	*Salvia hispanica* L.	Seeds	เมล็ด Chia

Table 2 Selected plant samples in the study with Thai name and parts used (Cont.)

No.	Scientific plant name	Plant parts used	Thai plant name
	Family: Labiatae		
61	*Salvia officinalis* L.	Aerial part	Sage
62	*Thymus citriodorus* (Pers.) Schreb.	Aerial part	Lemon Thyme
63	*Thymus vulgaris* L.	Aerial part	Thyme
	Family: Lauraceae		
64	*Persea americana* Mill.	Flesh	เนื้อผลอะโวคาโด
65	*Persea americana* Mill.	Peels	เปลือกผลอะโวคาโด
66	*Persea americana* Mill.	Seeds	เมล็ดผลอะโวคาโด
	Family: Malvaceae		
67	*Hibiscus sabdariffa* L.	Leaves	ใบกระเจี๊ยบแดง
	Family: Meliaceae		
68	*Azadirachta indica* A. Juss.	Leaves	สะเดา
	Family: Moraceae		
69	*Morus alba* L.	Leaves	ใบหม่อน
	Family: Moringaceae		
70	*Moringa oleifera* Lam.	Leaves	ใบมะรุม
71	*Moringa oleifera* Lam.	Seeds	เมล็ดมะรุม

Table 2 Selected plant samples in the study with Thai name and parts used (Cont.)

No.	Scientific plant name	Plant parts used	Thai plant name
	Family: Myrtaceae		
72	*Psidium guajava* L.	Fruits	ผลฝรั่ง
73	*Syzygium antisepticum* (Blume) Merr. & L. M. Perry	Leaves	ใบเสม็ดชุน / ผักเม็ก
	Family: Oxalidaceae		
74	*Averrhoa carambola* L.	Fruits	ผลมะเฟือง
	Family: Piperaceae		
75	*Piper betle* L.	Leaves	ใบพลู
76	*Piper nigrum* L. (Black pepper)	Fruits	พริกไทยดำ
77	*Piper nigrum* L. (White pepper)	Seeds	พริกไทยล่อน
	Family: Poaceae		
78	*Cymbopogon citratus* (DC.) Stapf	Rhizomes	เหง้าตะไคร้
	Family: Polygonaceae		
79	*Persicaria odorata* (Lour.) Soják	Leaves	ผักแพว / ผักไผ่
	Family: Punicaceae		
80	*Punica granatum* L. var. *granatum*	Leaves	ใบทับทิม
81	*Punica granatum* L. var. *granatum*	Peels	เปลือกผลทับทิม
	Family: Rosaceae		
82	*Fragaria vesca* L.	Fruits	ผลสตรอว์เบอร์รี

Table 2 Selected plant samples in the study with Thai name and parts used (Cont.)

No.	Scientific plant name	Plant parts used	Thai plant name
	Family: Rosaceae		
83	*Malus domestica* Borkh.	Fruits	ผลแอปเปิล
84	*Pyrus communis* L.	Fruits	ผลสาลี่
	Family: Rubiaceae		
85	*Coffea arabica* L.	Seeds	เมล็ดกาแฟอาราบิกา
86	*Coffea canephora* Pierre ex A. Froehner	Seeds	เมล็ดกาแฟโรบัสตา
87	*Morinda citrifolia* L.	Fruits	ผลยอ
88	*Morinda citrifolia* L.	Leaves	ใบยอ
	Family: Scrophulariaceae		
89	*Limnophila aromatica* (Lam.) Merr.	Aerial part	ผักแขยง
	Family: Solanaceae		
90	*Capsicum annuum* L. (Green bell pepper)	Fruits	พริกหวานเขียว
91	*Capsicum annuum* L. (Orange bell pepper)	Fruits	พริกหวานส้ม
92	*Capsicum annuum* L. (Red bell pepper)	Fruits	พริกหวานแดง
93	*Capsicum annuum* L. (Yellow bell pepper)	Fruits	พริกหวานเหลือง
94	*Nicotiana tabacum* L.	Leaves	ใบยาสูบ
	Family: Solanaceae		
95	*Physalis angulata* L.	Aerial part	ต้นโทงเทง
96	*Physalis peruviana* L.	Fruits	ผลเคพกูสเบอร์รี

Table 2 Selected plant samples in the study with Thai name and parts used (Cont.)

No.	Scientific plant name	Plant parts used	Thai plant name
	Family: Solanaceae		
97	*Physalis peruviana* L.	Calyx	เปลือกผลเคพกูสเบอร์รี
98	*Solanum lycopersicum* L. var. *cerasiforme*	Fruits	ผลมะเขือเทศเชอร์รี่
99	*Solanum lycopersicum* L. var. *lycopersicum*	Fruits	ผลมะเขือเทศสีดา
	Family: Strychnaceae		
100	*Strychnos nux-vomica* L.	Seeds	เมล็ดแสลงใจ
	Family: Theaceae		
101	*Camellia sinensis* (L.) Kuntze var. *assamica* (Mast.) Kitam.	Leaves	ใบชา
	Family: Thunbergiaceae		
102	*Thunbergia laurifolia* Lindl.	Leaves	ใบรางจืด
	Family: Verbenaceae		
103	*Clerodendrum calamitosum* L.	Leaves	ใบราตรีสวรรค์
104	*Clerodendrum indicum* (L.) Kuntze	Leaves	ใบไม้เท้ายายม่อม
105	*Clerodendrum quadriloculare* (Blanco) Merr.	Leaves	ใบสาวสันทราย
106	*Clerodendrum serratum* (L.) Moon	Leaves	ใบอัคคีทวาร
107	*Clerodendrum thomsoniae* Balf. f.	Leaves	ใบพวงเงินดอกแดง
108	*Vitex agnus-castus* L.	Leaves	ใบคนทีดอกม่วง
109	*Vitex negundo* L.	Leaves	ใบคนทีเขมา
110	*Vitex trifolia* L. subsp. *litoralis* Steenis	Leaves	ใบคนทีสอทะเล
111	*Vitex trifolia* L. subsp. *trifolia*	Leaves	ใบคนทีสอ

Sample extraction

Ten grams of each selected plant sample were exhaustively extracted with petroleum ether and followed by 95% ethanol using a Soxhlet apparatus. The ethanolic extract was filtered through filter-paper Whatman No. 4 and evaporated to dryness under reduced pressure by rotary evaporator. The extract yields were weighed, recorded and stored at -20 ℃ to avoid the possibility of degradation of active compounds.

Chromatographic conditions

Shimadzu HPLC LC-20A system (Shimadzu, Japan) consists of a system controller (CMB-20A), two solvent delivery units (LC-20A), an on-line degassing unit (DGU-20A3), an auto-sample (SIL-20A), a column oven (CTO-20A) and a photo-diode array detector (SPD-M20A). System control and data analysis were processed with Shimadzu LC Solution software. The chromatographic separation was performed with an Inertsil® ODS-3 5 µm C_{18} column (4.6 x 250 mm) and coupled with ReproSil®-Pur ODS-3 C_{18} guard column (4.0 X 10 mm). The samples were analysed using 0.2% phosphoric acid in water (solvent A) and methanol (solvent B) as mobile phase. The isocratic program was set at 45% B for 20 minutes at a flow rate of 1.2 ml/min. The mobile phase was filtered through 0.45 µm nylon membrane filters and degassed using an ultrasonic bath before analysis. The column temperature was maintained at 30 ℃ and injection volume of standards and sample solutions was 5 µl. The wavelength was set at 325 nm for monitoring chromatographic profile. All measurement was done in triplicate.

Preparation of chlorogenic, rosmarinic and caffeic acid standard solutions

One milligram of each standards was dissolved in 1 ml of methanol. The solution was filtered through a 0.45 µm PTFE membrane syringe filter.

Preparation of sample solutions

Fifty milligrams of each extracts were dissolved in 1 ml of methanol and diluted to appropriate concentrations for further HPLC analysis. The solution was filtered through a 0.45 µm PTFE membrane syringe filter.

Method validation

According to the ICH guideline [88]: calibration range, specificity, accuracy, repeatability, intermediate precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness were validated for analytical method.

Calibration range

The calibration range was performed by plotting peak areas that obtained from HPLC analysis *versus* concentrations of standard. The stock solutions of chlorogenic acid, rosmarinic acid and caffeic acid were dissolved in methanol and diluted together to give concentration of 16.67, 33.33, 50.00, 66.67 and 83.33 µg/ml for evaluation of the calibration range. The calibration range of these standards was fitted by linear regression. The regression equation was calculated in the form of $y = ax + b$, where y is peak area and x is concentration.

Specificity

The specificity was evaluated by peak purity test. The peak purity index of the analyte was processed with Shimadzu LC Solution software. It was determined by comparing all the spectra within the chromatographic peak to the reference spectrum at the peak apex.

Accuracy

The accuracy of each sample was tested by recovery method. Three different levels of standard solutions (low, medium, high) was spiked in to the extract. The spiked and un-spiked samples were evaluated under the same condition in triplicate. The accuracy was calculated as percent recovery by using following formula:

$$\% \text{ recovery} = \left(\frac{C_1}{C_2 + C_3} \right) \times 100$$

Where: C_1 = the amount of compound found in spiked sample

C_2 = the amount of compound found in un-spiked sample

C_3 = the amount of standard added to sample

Precision

The precision was determined by repeatability (intra-day) and intermediate precision (inter-day) studies. The method was performed by analysing three level concentrations of sample solution in triplicate on the same day for repeatability and in the five different days for intermediate precision. The precision was calculated in term of percent relative standard deviation (% RSD) of compound content by following formula:

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

Where: SD = the standard deviation of each measurement

Limit of detection (LOD)

Limit of detection (LOD) which is the lowest concentration that can be detected but not accurately quantitated was determined from the calibration range using following formula:

$$LOD = \frac{3.3 \times \sigma}{S}$$

Where: σ = the residual standard deviation of regression line

S = the slope of regression line

Limit of quantitation (LOQ)

Limit of quantitation (LOQ) which is the lowest concentration that can be accurately quantitated was determined from the calibration range using following formula:

$$LOD = \frac{10 \times \sigma}{S}$$

Where: σ = the residual standard deviation of regression line

S = the slope of regression line

Robustness

The robustness was determined for variations in flow rates (1.195, 1.200 and 1.205 ml/min), variations in column temperature (29, 30 and 31 °C) and variations in wavelength (322, 325 and 328 nm). The robustness was calculated in term of percent relative standard deviation of peak area.

Data analysis

The data were evaluated by comparing the area under peak with the calibration curve. The area under peak was analysed using Shimadzu LC Solution software for determination of chlorogenic acid, rosmarinic acid and caffeic acid contents.

Part II: Pharmacognostic specification of *Lonicera japonica* flowering bud and chlorogenic acid content by TLC-densitometry and TLC-image analysis

Sample collection

Fifteen samples of dried *Lonicera japonica* flowering bud were purchased from 15 various herbal drugstores throughout Thailand. All plant materials were authenticated by Associate Professor Nijsiri Ruangrungsi, Ph.D., and voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. After removal of any foreign matter, crude drugs were grounded into coarse powders before use.

Sample extraction

Five grams of dried powered *L. japonica* flowering bud were exhaustively extracted with 95% ethanol using a Soxhlet apparatus. The ethanolic extract was filtered through filter-paper Whatman No. 4 and evaporated to dryness under reduced pressure by rotary evaporator. The extract yields were weighed, recorded and stored at -20 °C to avoid the possibility of degradation of active compound. Ten milligrams of the extract were dissolved in 1 ml of 95% ethanol for further analysis of chlorogenic acid by TLC-densitometry and TLC-image analysis.

Standardization parameters of *L. japonica* flowering bud

The standardization parameters were examined according to "Quality control methods for medicinal plant materials" established by World Health Organization (WHO) [73]. The physico-chemical parameters including water content, loss on drying, total ash, acid-insoluble ash, ethanol and water extractive values, and volatile oil content were determined.

Macroscopic examinations

Dried *L. japonica* flowering bud was observed by visual examination of surface characteristics, texture, size, color, and other inspection. The whole plant was illustrated by hand drawing in proportional scale related to the original size.

Microscopic examination

The microscopic evaluation of dried *L. japonica* flowering bud was examined. Transverse section of the corolla, and powdered of the crude drug was mounted onto a glass slide in water to observe under microscope with 10X, 20X, 40X objective lens magnifications and 10X eyepiece lens. Photographs were taken by digital camera and illustrated by hand drawing in proportional scale related to the original size.

Determination of water content (azeotropic method)

The accurate 50 g of dried powered *L. japonica* flowering bud were transferred to round bottom flask, 200 ml of water-saturated toluene were added and boiled until the water is completely distilled. The water and toluene layer were separated then the volume of water was recorded and calculated in the percentage.

Determination of loss on drying

The accurate 3 g of dried powered *L. japonica* flowering bud were transferred to a pre-weighed crucible and then dried at 105 °C in a hot air oven until constant weight. The crucible was allowed to cool at room temperature, weighed and calculated the loss of weight in percentage.

Determination of total ash

The accurate 3 g of dried powered *L. japonica* flowering bud were transferred to a pre-weighed crucible and then incinerated at 500 °C by gradually increasing temperature in ashing furnaces until white ash is obtained. The crucible was cooled in a desiccator. The content of ash was weighed without delay and calculated in percentage.

Determination of acid insoluble ash

The aforementioned crucible that containing the total ash was added with 25.0 ml of hydrochloric acid (70 g/l), covered with a watch-glass and boiled gently for 5 minutes, the insoluble matter was filtered through an ashless filter-paper Whatman No. 40, the filter-paper was transferred into the original crucible, dried on a hot plate and incinerated at 500 °C until ash remaining. The residue was cooled in a desiccator. The content of ash was weighed and calculated in percentage.

Determination of ethanol soluble extractive value

The accurate 5 g of dried powered *L. japonica* flowering bud were macerated with 70 ml of 95% ethanol in closed conical flask on orbital shaker for 6 hours under shaking, and standing for 18 hours. After that, the extract was filtered rapidly through filter-paper Whatman No. 4, the marc was washed and the filtrate was adjusted to 100 ml with 95% ethanol. Twenty millilitres of the filtrate were transferred to pre-weighed

small beaker and evaporated to dryness on a water-bath. Finally, the extract was dried at 105 ℃ for 6 hours, cooled in a desiccator, weighed and calculated in a percentage.

Determination of water soluble extractive value

The accurate 5 g of dried powered *L. japonica* flowering bud were macerated with 70 ml of water in closed conical flask on orbital shaker for 6 hours under shaking, and standing for 18 hours. After that, the extract was filtered rapidly through filter-paper Whatman No. 4, the marc was washed and the filtrate was adjusted to 100 ml with water. Twenty millilitres of the filtrate were transferred to pre-weighed small beaker and evaporated to dryness on a water-bath. Finally, the extract was dried at 105 ℃ for 6 hours, cooled in a desiccator, weighed and calculated in a percentage.

Determination of volatile oil content

The accurate 100 g of dried powered *L. japonica* flowering bud were transferred to round bottom flask, 600 ml of water were added and boiled using Clevenger apparatus until the volatile oil is completely distilled. The volatile oil and water were separated then the volume of volatile oil was recorded and calculated in the percentage.

Thin layer chromatographic fingerprint

Five milligrams of the crude extract were dissolved in 1 ml of ethanol. Three microliters of the crude extract solution were applied onto the silica gel GF_{254} TLC plate. The TLC plate was developed in a saturated TLC chamber with ethyl acetate : methanol : water : formic acid (50:4:4:2.5) as mobile phase. After development, the plate was removed and allowed it to dry at room temperature and observed for the spots on the plate under UV light with 254 nm and 365 nm. Then, the plate was sprayed with ferric chloride reagent and heated at 105 ℃ for 10 min in a hot air oven.

Quantitative analysis of chlorogenic acid in *Lonicera japonica* flowering bud

Preparation of chlorogenic acid standard solutions

Standard chlorogenic acid was dissolved in 95% ethanol and diluted to obtain the concentration of 0.6, 1.2, 1.8, 2.4, 3.0 mg/ml. These standard solutions were stored in refrigerator at 4 ℃.

TLC-image analysis by ImageJ software

Three microliters of *L. japonica* flowering bud ethanolic extract and standard chlorogenic acid solutions were applied on the silica gel 60 GF_{254} 20 × 10 cm TLC plate, and developed in the saturated TLC chamber using a mixture of ethyl acetate : formic acid : acetic acid : water (10:1.1:1.1:2.6) as mobile phase solvent. After development, the TLC plate was observed under short wave (254 nm) ultraviolet light in UV viewing cabinet and photographed using digital camera.

Quantitative analysis of the chlorogenic acid spot on TLC plate was analysed by ImageJ software. The calibration curve of chlorogenic acid was performed by plotting peak areas *versus* concentrations of chlorogenic acid in µg/spot.

TLC-densitometry

The developed TLC plate was scanned for the chlorogenic acid spot by CAMAG TLC Scanner 4 at the wavelength of 325 nm (maximum absorbance) and expressed as chromatographic peak by winCATS software. The calibration curve of chlorogenic acid was performed by plotting peak areas *versus* concentrations of chlorogenic acid in µg/spot.

Method validation

According to the ICH guideline [88]: calibration range, specificity, accuracy, repeatability, intermediate precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness of chlorogenic acid quantitative analysis in *L. japonica* flowering bud were validated.

Calibration range

The calibration range was performed by plotting peak areas *versus* concentrations of chlorogenic acid per spot applied.

Specificity

The developed TLC plate was scanned under the wavelength of 200 - 700 nm for absorption spectra by CAMAG TLC Scanner 4. The specificity was performed by comparing UV absorbance spectra of the peak apex among all samples and standard chlorogenic acid for peak identity, and the comparison of UV absorbance spectra recorded at up-slope, apex and down-slope of the peak for peak purity.

Accuracy

The accuracy of quantitative TLC analysis was tested by recovery method. Three different levels of standard chlorogenic acid solutions (low, medium, high) were spiked into the extract. The spiked and un-spiked samples were evaluated under the same condition in triplicate. The accuracy was calculated as percent recovery by using following formula.

$$\% \text{ recovery} = \left(\frac{C_1}{C_2 + C_3} \right) \times 100$$

Where: C_1 = the amount of chlorogenic acid found in spiked sample

C_2 = the amount of chlorogenic acid found in un-spiked sample

C_3 = the amount of standard chlorogenic acid added to sample

Precision

The precision of quantitative TLC analysis was determined by repeatability (intra-day) and intermediate precision (inter-day) studies. The method was performed by analysing three level concentrations of sample solution in triplicate on the same day for repeatability and in the three different days for intermediate precision. The precision was calculated in term of percent relative standard deviation (% RSD) of chlorogenic acid content by following formula.

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

Where: SD = the standard deviation of each measurement

Limit of detection (LOD)

LOD which is the lowest concentration that can be detected but not accurately quantitated was determined from the calibration range using following formula.

$$LOD = \frac{3.3 \times \sigma}{S}$$

Where: σ = the residual standard deviation of regression line

S = the slope of regression line

Limit of quantitation (LOQ)

LOQ which is the lowest concentration that can be accurately quantitated was determined from the calibration range using following formula.

$$LOD = \frac{10 \times \sigma}{S}$$

Where: σ = the residual standard deviation of regression line

S = the slope of regression line

Robustness

Mobile phase composition was selected for robustness parameter in this study. A little variation in a mixture ratio of ethyl acetate : formic acid : acetic acid : water was performed as 99.8 : 10.8 : 11.2 : 26.2, 100.0 : 11.0 : 11.0 : 26.0 and 100.2 : 11.2 : 10.8 : 25.8. The robustness was calculated in term of percent relative standard deviation (% RSD) of peak area.

Data analysis

The standardization parameters were expressed as grand mean ± pooled standard deviation.

The chlorogenic acid contents between TLC-densitometry and TLC-image analysis were compared by paired *t*-test statistical analysis.

Part III: *In vitro* biological activity evaluations

L. japonica flowering bud ethanolic extract, standard chlorogenic acid, standard rosmarinic acid, standard caffeic acid were performed for *in vitro* biological activities.

Cytotoxic activity (Brine shrimp lethality assay)

Brine shrimp lethality assay was carried out according to the procedure described by Meyer *et al*. [118]. The artificial sea water was prepared and oxygenated for 24 hours before use. Brine shrimp (*Artemia salina*) eggs were sprinkled into the larger compartment of the hatching box which was darkened, while the smaller compartment was illuminated and plastic divider with several 2 mm holes between both compartments. After 24 hours of incubation, hatched nauplii were swam to the light side of the hatching box, 10 nauplii were transferred by Pasteur pipette to vial containing artificial sea water and then adjusted the find volume to 5 ml. One hundred microliters of *L. japonica* flowering bud ethanolic extract at concentration of 1,000 - 10,000 µg/ml and three standards at concentration of 100 - 1,000 µg/ml dissolved in ethanol were pipetted on small filter papers and air dried. Then, dried filter papers were put into vials containing the nauplii and placed the vials under illumination. Each concentration was done in five replicates. The percent death of nauplii was evaluated at 6, 12, 18 and 24 hours and calculated for the LC_{50}.

Cytotoxic activity (MTT cell viability assay)

Cell survival assay was operated at the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. Five human cancer cell lines including BT-474 (breast ductal carcinoma), ChaGo-K-1 (bronchogenic carcinoma), Hep G2 (hepatocellular carcinoma), KATO III (gastric carcinoma), SW620 (colorectal adenocarcinoma), and 1 human normal cell line as WI-38 (lung fibroblast) were purchased from American Type Culture Collection.

Preparation of *L. japonica* flowering bud ethanolic extract, standard compounds and doxorubicin solutions

The ethanolic extract and three standard compounds were dissolved in DMSO to the concentrations of 0.01, 0.1, 1, 10 and 100 µg/ml.

Doxorubicin was dissolved in normal saline to the concentrations of 0.001, 0.01, 0.1, 1 and 10 µg/ml.

Preparation of MTT solution

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was dissolved in normal saline to obtain the concentrations at 5 mg/ml, and kept in the dark condition at 4 °C.

Preparation of cell lines

The cell lines were cultured in complete medium (RPMI-1640 medium containing 5% (v/v) fetal calf serum) in tissue culture flask at 37 °C with 5% (v/v) CO_2 for 3 days. Two hundreds microliters of cells were seeds in a 96-well culture plates at a density of 1 x 10^4 cells/well and cultured in a 5% (v/v) CO_2 incubator at 37 °C, 100% relative humidity for 24 hours.

MTT method

Cell viability using MTT assay was determined by the modified method as described by Mosmann [119]. Each cell line, 5 x 10^3 cells in 198 µl of complete media was transferred to each well of flat 96-well plate and incubated at 37 °C with 5% (v/v) CO_2 for 24 hours. After overnight, 2 µl of test samples in DMSO at different concentrations were added and incubated as above. In addition, doxorubicin in 2 µl of DMSO was used as positive control and pure DMSO as negative control. After 72 hours, 10 µl MTT solution were added into each well and incubated for 4 hours. The supernatant of medium was removed, 150 µl of DMSO and 25 µl of 0.1 M glycine were added and gently mixed to lyse the cells along with dissolved the formazan crystals prior to the absorbance measuring using a microplate reader at 540 nm. Four replicates of each sample were performed. The number of cell survival as a relative percentage of the sample absorbance and the negative control absorbance (DMSO set to 100% viability) was calculated using following formula.

$$\% \text{ cell survival} = \frac{\text{mean of sample absorbance}}{\text{mean of negative control absorbance}} \times 100$$

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

DNA damage (Comet assay)

The comet assay was performed by the modified method of Singh *et al*. [120]

Lymphocytes isolation

Fresh blood specimen from healthy donor was aseptically collected in sterile tube containing heparin. Six millilitres of diluted fresh blood was layered over 3 ml of Ficoll-Histopaque 1077 in a conical centrifuge tube, then centrifuge at 1,800 rpm, 4 °C

for 30 min. The lymphocyte cells were washed three times in phosphate buffer saline (pH 7.4) and added with 10 ml of incomplete RPMI-1640 medium to discharge the buffer. Each step was centrifuge at 1,600 rpm, 4 ℃ for 10 min. Then, complete RPMI-1640 medium was added to obtain the lymphocyte suspension about 4 x 10^5 cells/ml using hemocytometer. Four hundred microliters portions were aliquoted into microcentrifuge tube and kept at -80 ℃.

Comet assay

Each lymphocyte suspension was washed three times in PBS (pH 7.4) and added incomplete RPMI-1640 medium to obtain 4 ml as suspension. *L. japonica* flowering bud ethanolic extract and three standard compounds at three concentrations of 25, 50 and 100 µg/ml were dissolved in 2% DMSO. Hydrogen peroxide was used as a positive control whereas PBS (pH 7.4) and 2% DMSO as negative control. One hundred microliters of lymphocyte suspension was added into microcentrifuge tube that containing 100 µl of sample and incubated at 37 ℃ for 1 hour. Then the treat samples were centrifuged at 3,000 rpm, 4 ℃ for 5 min to discard the supernatant.

The slides and coverslips were cleaned with ethanol and air-dried before used. The slide was pre-coated with 1% normal agarose which melt in water as the first layer and kept in low humidity before use to ensure the agarose adhesion. The treated samples were mixed with 1% low melting agarose which melt with PBS (pH 7.4) as ratio 1:1 at 37 ℃ and spread onto the pre-coated slide, placed the coverslip over the second layer and kept on ice until agarose gel solidified. After agarose gel has harden, the coverslip was slided off and spread with 0.5% low melting agarose which melt with PBS (pH 7.4) as the third layer, cover with coverslip and kept in a cool temperature until agarose forming harden. Then the freshly lysis solution was prepared by mixed 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10) with 10% DMSO and 1% Triton X-100 being added just before use. The coverslip was slided off and the slide was immersed into a cool freshly lysis solution at 4 ℃ for 1 hour. After lysis process, the slides was

placed in horizontal gel electrophoresis chamber containing the electrophoresis solution (1 mM EDTA-Na_2 and 300 mM NaOH, pH>13). The electrophoresis was conducted at 0.7 v/cm for 25 min under dark condition. After electrophoresis process, the slide was placed horizontally and washed three times with the neutralization buffer containing 0.4M Tris buffer (pH 7.5) for 5 min.

Each slide was stained with 20 μg/ml ethidium bromide for 5 min, washed with water and covered with coverslip, kept in a cool temperature. The migrated DNA (comet) was observed under fluorescent microscope with the magnification of 400X. The degrees of damage was categorized into five classes of visual scoring (Table 3) based on the size and intensity of the comet tail. One hundred comets were scored from each slide to assign a value between 0 - 400 arbitrary units [37, 121].

Table 3 Classification of tail length DNA damage

Class	Description
0	no damage
1	little damage with a tail length that is shorter than the diameter of the nucleus
2	medium damage with a tail length one to two times the diameter of the nucleus
3	significant damage with a tail length between two and-a-half and three times the diameter of the nucleus
4	significant damage with a tail longer than three times the diameter of the nucleus

Antimicrobial activity

Microorganisms

The microorganisms include three non-spore forming gram-positive bacteria, two spore forming gram-positive bacteria, six non-spore forming gram-negative bacteria and two fungi strains were described in Table 4.

Table 4 Tested microorganisms

Tested microorganism	
Gram positive bacteria	*Kocuria rhizophila* ATCC 9341[3]
(Non-spore forming bacteria)	*Staphylococcus aureus* ATCC 6538P[1]
	Staphylococcus epidermidis (Isolates)[2]
Gram positive bacteria	*Bacillus cereus* ATCC 1177[3]
(Spore forming bacteria)	*Bacillus subtilis* ATCC 6633[1]
Gram negative bacteria	*Enterobacter aerogenes* ATCC 13048[3]
(Non-spore forming bacteria)	*Escherichia coli* ATCC 25922[1]
	Pseudomonas aeruginosa ATCC 9027[1]
	Salmonella typhi (Isolates)[2]
	Salmonella typhimurium ATCC 13311[2]
	Shigella spp. (Isolates)[2]
Non-mycelium fungi	*Candida albicans* ATCC 10230[1]
	Saccharomyces cerevisiae ATCC 9763[1]

Sources:

[1] Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University

[2] Department of Microbiology, Faculty of Science, Chulalongkorn University

[3] Department of Microbiology, Faculty of Science and Technology, Suan Sunandha Rajabhat University

Preparation of inoculum suspensions

The bacteria and fungi strains were cultivated in Mueller Hinton agar and Sabouraud Dextrose agar respectively then incubated at 37 °C on agar media for 18 - 24 hours (for bacteria) or 24 - 48 hours (for fungi). Four or five well isolated colonies were suspended in 0.85% normal saline. The turbidity of bacteria and fungi suspensions was adjusted to obtain 0.5 McFarland turbidity standard (optical density 0.08 - 0.10 at 625 nm, light path 1 cm) which equivalent to 1×10^8 CFU/ml.

Determination of zone inhibition (Agar well diffusion method)

Slightly modified agar well diffusion method using a two-layer agar technique was performed for antibacterial testing. One hundred microliters of the microbial suspension (1×10^8 CFU/ml) were mixed with 3 ml of sterile seed agar and poured on the sterile base agar. The plates were allowed to dry at room temperature. Agar wells were bored in seeded agar plates by a sterile 6 mm-diameter cork borer.

Twenty microliters of 200 mg/ml in DMSO of plant extract, positive and negative controls were transferred into each well. Ampicillin and amikacin (1 mg/ml) were used as positive control and DMSO as negative control. The plates were incubated at 37 °C for 18 - 24 hours (for bacteria) and 24 - 48 hours (for fungi). Then, the zone inhibition was measured in millimetre. Each sample was tested in triplicate [122, 123].

Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The broth microdilution method was performed in a sterile 96 well microplate with some modification [124, 125]. A microbial suspension in broth was prepared by adding 10 µl of 0.85% normal saline microbial suspensions to 1 ml of Mueller Hinton broth (for bacteria) or Sabouraud dextrose broth (for fungi).

The samples with zone inhibition were serially diluted two-fold in DMSO. Fifty microliters of the microbial suspension (1×10^8 CFU/ml) in broth were added to each

well containing 50 µl of samples, positive and negative controls, and incubated at 37 ℃ for 18 - 24 hours (for bacteria) and 24 - 48 hours (for fungi).

The lowest concentration of samples which expresses growth inhibition detected by the lack of visual turbidity compared to the negative control was defined as the MIC. The broth from the wells without turbidity was streaked onto the agar plates and incubated at 37 ℃ for 18 - 24 hours (for bacteria) and 24 - 48 hours (for fungi). the least concentration with no microbial growth on the plate was considered as MBC or MFC [125].

Antioxidant activities

2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay

The ability to scavenge DPPH free radical was assessed as described by Brand-William *et al*. [126] with minor modification. One hundred microliters of various concentrations of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic and caffeic acids or positive controls (quercetin and BHT) dissolved in methanol were added to 100 µl of 120 µM DPPH methanolic solution in 96 well-microplate. The plate was incubated for 30 minutes in the dark at room temperature. The absorbance at 517 nm was measured. Each sample was done in triplicate. Percent scavenging activity was calculated:

$$\% \text{ Inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

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

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was assessed by the method of Benzie and Strain [127] with minor modification. Twenty-five microliters of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic and caffeic acids (1 mg/ml) were mixed with 175 µl of FRAP reagent in 96 well-microplate and incubated for 30 minutes in the dark at room temperature. The absorbance at 593 nm was measured using microplate reader. Quercetin and BHT were used as a positive control. The FRAP value of samples was calculated using the linear relationship from the calibration curve of $FeSO_4$ methanolic solutions in the range of 0.5 - 1.5 mM. Each sample was done in triplicate. The samples and positive controls were expressed as mM of ferrous iron.

Nitric oxide scavenging assay

Nitric oxide scavenging assay was performed according to the method of Rai *et al*. [128] with minor modification. The mixture containing 50 µl of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic and caffeic acids or positive control (quercetin) at concentration 800 µg/ml dissolved in DMSO and 50 µl of sodium nitroprusside (10 mM) in phosphate buffer saline was incubated at 25 ℃ for 150 min. Then the mixture was reacted with 50 µl of Griess reagent (0.33% sulphanilamide in 20% glacial acetic acid) and incubated for 10 min. After incubation, 50 µl of 0.1% naphthyl ethylenediamine dihydrochloride were added and allowed to stand for 30 min. The absorbance at 540 nm was measured. Each sample was done in triplicate. Percent scavenging activity was calculated:

$$\% \text{ Inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

Beta-carotene bleaching assay

Beta-carotene bleaching assay was performed to measure the ability of the extract to inhibit lipid peroxidation according to the method of Jayaprakasha *et al*. [129] with minor modification. One millilitre of beta-carotene solution (2 mg/ml in chloroform) was mixed with 40 µl of linoleic acid (10 mg/ml) and 400 µl of Tween 20. The chloroform was removed at 40 ℃ under vacuum. The mixture was diluted with 50 ml of ultra-pure water and shaken to form an emulsion. Two hundred microliters of the emulsion were transferred in to the 96 well-microplate which contained 10 µl of the various concentrations of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic and caffeic acids or positive controls (quercetin and BHT) and heated at 50 ℃. The absorbance at 470 nm was recorded at 30 minutes intervals for 120 minutes. Each sample was done in triplicate. The antioxidant activity was calculated:

$$\% \text{ Antioxidant activity} = 1 - \left(\frac{A_0 - A_{120}}{C_0 - C_{120}}\right) \text{ X } 100$$

Where A_0, A_{120}: the absorbance values measured at zero time and end time of incubation for sample

C_0, C_{120}: the absorbance values measured at zero time and end time of incubation for control

Antidiabetic activity

Yeast alpha-glucosidase inhibition assay

The enzyme inhibition activity against *Saccharomyces cerevisiae* alpha-glucosidase was determined according to Wan *et al*. [130] with slight modifications using 1 mM of *p-Nitrophenyl*-α-D-glucopyranoside (PNPG) as a substrate. Ten microliters of tested inhibitors (*L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic and caffeic acids or acarbose) in DMSO were mixed with 20 µl of 0.5 U/ml alpha-glucosidase and 120 µl of 0.01 M sodium phosphate buffer (pH 6.9) in 96 well-microplate and preincubated at 37 °C for 10 minutes. Next, 20 µl of PNPG were added and then incubated at 37 °C for 10 minutes. After incubation, 80 µl of 0.2 µM sodium carbonate (Na_2CO_3) were added to terminate the reaction and the absorbance was measured at 405 nm using microplate reader. All tested inhibitors were analysed in triplicate. The percent inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

CHAPTER IV

RESULTS

Part I: Quantification of chlorogenic acid, rosmarinic acid and caffeic acid in selected plants using high performance liquid chromatography

Various 111 plant samples were exhaustively extracted with petroleum ether and followed by 95% ethanol using Soxhlet apparatus. The percent yields of crude extracts were shown in Table 5.

Quantitative analysis of chlorogenic, rosmarinic and caffeic acids in selected plants were performed by HPLC analysis. Chlorogenic, rosmarinic and caffeic acids in extracts were identified by comparing the retention time and UV spectrum of each peak with reference of standard compounds (Figure 12). The contents of chlorogenic, rosmarinic and caffeic acids in 111 plant sample ethanolic extracts were shown in Table 5. Among 111 plant samples, highest content of chlorogenic, rosmarinic and caffeic acids were found in *Lonicera japonica* flowering bud, *Melissa officinalis* leaves and *Coffea canephora* seed at the concentration of 9.8959 ± 0.0036, 19.9077 ± 0.1705 and 1.2332 ± 0.0033 g/100 g of dried plant respectively.

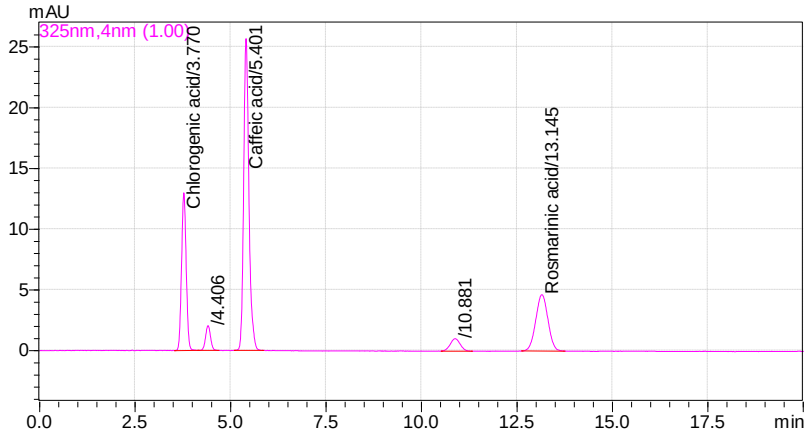


Figure 12 HPLC chromatograms of standard chlorogenic, rosmarinic and caffeic acids at 325 nm by HPLC-DAD

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Acanthaceae					
1	*Andrographis paniculata* (Burm.f.) Wall. ex Nees	Leaves	25.5946	0.6250 ± 0.0010	0.3379 ± 0.0007	0.0451 ± 0.0004
	Family: Alliaceae					
2	*Allium sativum* L.	Bulbs	10.3932	-	-	0.0051 ± 0.0000
3	*Allium cepa* L.	Bulbs	66.4674	0.0341 ± 0.0005	-	-
	Family: Amaranthaceae					
4	*Spinacia oleracea* L.	Leaves	21.1034	-	0.0386 ± 0.0004	0.0373 ± 0.0002
	Family: Anacardiaceae					
5	*Mangifera indica* L. cv. Okrong	Leaves	28.6979	0.3543 ± 0.0025	-	1.0075 ± 0.0078
	Family: Apiaceae					
6	*Anethum graveolens* L.	Aerial part	28.7136	7.3607 ± 0.0375	-	0.1844 ± 0.0017
7	*Apium graveolens* L.	Aerial part	36.7388	0.9130 ± 0.0033	2.8799 ± 0.0177	0.0879 ± 0.0012

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Apiaceae					
8	*Apium graveolens* L. var. *secalinum*	Aerial part	36.0661	2.5738 ± 0.0107	2.5563 ± 0.0135	0.1119 ± 0.0003
9	*Centella asiatica* (L.) Urb.	Aerial part	31.9171	0.8483 ± 0.0030	0.9096 ± 0.0031	0.0864 ± 0.0015
10	*Coriandrum sativum* L.	Seeds	4.7698	0.0277 ± 0.0000	0.0609 ± 0.0008	0.0236 ± 0.0001
11	*Daucus carota* L. subsp. *sativus* (Hoffm.) Arcang.	Roots	52.0628	0.3264 ± 0.0026	-	0.0853 ± 0.0004
12	*Eryngium foetidum* L.	Leaves	30.4513	4.9789 ± 0.0055	4.3024 ± 0.1001	0.1601 ± 0.0067
13	*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill	Aerial part	31.8261	0.0584 ± 0.0040	9.1534 ± 0.6017	0.0480 ± 0.0011
	Family: Apocynaceae					
14	*Telosma cordata* (Burm. f.) Merr.	Flowers	34.5841	0.3498 ± 0.0006	-	0.1841 ± 0.0016
	Family: Asteraceae					
15	*Artemisia dracunculus* L.	Aerial part	28.8849	5.2533 ± 0.0467	5.3589 ± 0.0369	0.0623 ± 0.0083
16	*Artemisia pallens* Wall. ex DC.	Aerial part	8.1407	0.0421 ± 0.0001	-	0.0112 ± 0.0001
17	*Chromolaena odorata* (L.) R. M. King & H. Rob.	Leaves	28.2391	4.1366 ± 0.0345	0.2790 ± 0.0089	0.6214 ± 0.0179
18	*Gnaphalium polycaulon* Pers.	Aerial part	23.4087	0.6993 ± 0.0091	1.2066 ± 0.0117	0.2355 ± 0.0090

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Asteraceae					
19	*Helianthus annuus* L.	Pericarps	2.8800	0.0381 ± 0.0000	0.0040 ± 0.0000	0.0033 ± 0.0000
20	*Helianthus annuus* L.	Seeds	11.5229	2.1444 ± 0.0058	0.0079 ± 0.0001	0.1103 ± 0.0004
21	*Helianthus annuus* L.	Sprouts	30.7260	2.1988 ± 0.0054	3.1169 ± 0.0291	0.0934 ± 0.0021
22	*Lactuca sativa* L.	Leaves	24.0878	1.3964 ± 0.0357	0.3430 ± 0.0013	0.2575 ± 0.0019
	Family: Brassicaceae					
23	*Brassica juncea* (L.) Czern.	Leaves	28.4137	0.3513 ± 0.0019	-	0.0834 ± 0.0012
24	*Brassica oleracea* L. Group *Capitata*	Aerial part	38.5096	0.1724 ± 0.0016	-	-
25	*Brassica rapa* L. Group *Pekinensis*	Aerial part	41.5730	0.1535 ± 0.0008	-	-
26	*Raphanus sativus* L.	Roots	52.5659	0.0722 ± 0.0016	-	0.0327 ± 0.0013
	Family: Caprifoliaceae					
27	*Lonicera japonica* Thunb.	Flowering bud	32.7340	9.8959 ± 0.0036	2.5433 ± 0.0066	0.1945 ± 0.0015

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Caricaceae					
28	*Carica papaya* L.	Leaves	17.7591	0.0492 ± 0.0000	0.0227 ± 0.0006	0.5083 ± 0.0004
	Family: Convolvulaceae					
29	*Ipomoea aquatica* Forssk.	Aerial part	30.5026	2.5411 ± 0.0135	8.3026 ± 0.0419	0.1127 ± 0.0017
	Family: Cucurbitaceae					
30	*Momordica charantia* L. (Thai varieties)	Fruits	28.3591	0.0420 ± 0.0001	-	<LOQ
31	*Momordica charantia* L. (Chinese varieties)	Fruits	35.7284	0.0853 ± 0.0005	-	<LOQ
	Family: Eucommiaceae					
32	*Eucommia ulmoides* Oliv.	Stem barks	10.4412	0.0392 ± 0.0001	-	0.0187 ± 0.0002
	Family: Euphorbiaceae					
33	*Euphorbia hirta* L.	Aerial part	11.1504	0.2336 ± 0.0001	-	0.0205 ± 0.0004

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Euphorbiaceae					
34	*Phyllanthus emblica* L.	Fruits	28.6710	0.2996 ± 0.0014	0.0774 ± 0.0003	0.0288 ± 0.0004
35	*Ricinus communis* L.	Leaves	22.5992	0.0392 ± 0.0003	-	0.3521 ± 0.0037
	Family: Fabaceae					
36	*Pisum sativum* L.	Fruits	45.8380	-	-	-
37	*Pisum sativum* L. var. *macrocarpon*	Fruits	43.7131	-	-	-
38	*Sesbania grandiflora* (L.) Poir.	Flowers	42.0929	0.0158 ± 0.0003	0.2040 ± 0.0066	<LOQ
39	*Sesbania grandiflora* (L.) Poir.	Stem barks	3.9595	0.0093 ± 0.0001	-	0.0062 ± 0.0000
	Family: Gnetaceae					
40	*Gnetum gnemon* L. var. *tenerum* Markgr.	Leaves	22.4241	0.0919 ± 0.0013	-	-
	Family: Labiatae					
41	*Hyptis suaveolens* (L.) Poit.	Aerial part	11.1941	0.0780 ± 0.0008	3.9612 ± 0.0057	0.1106 ± 0.0001
42	*Leonotis nepetifolia* (L.) R. Br.	Leaves	22.2871	0.1709 ± 0.0007	-	0.4179 ± 0.0103

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Labiatae					
43	*Leonurus sibiricus* L.	Aerial part	14.0978	0.0453 ± 0.0030	-	0.0121 ± 0.0001
44	*Melissa officinalis* L.	Leaves	20.3709	0.0478 ± 0.0039	19.9077 ± 0.1705	0.1735 ± 0.0058
45	*Mentha arvensis* L. var. *piperascens* Malinv.	Leaves	19.9680	1.0377 ± 0.0023	6.8089 ± 0.0860	0.1083 ± 0.0009
46	*Mentha cordifolia* Opiz ex Fresen	Leaves	21.4610	0.1167 ± 0.0002	7.5374 ± 0.0103	0.1000 ± 0.0013
47	*Ocimum africanum* Lour.	Leaves	15.4660	0.0333 ± 0.0003	1.6911 ± 0.0018	0.0920 ± 0.0002
48	*Ocimum basilicum* L.	Leaves	15.7563	0.2426 ± 0.0013	0.5965 ± 0.0025	0.3109 ± 0.0005
49	*Ocimum gratissimum* L. var. *macrophyllum* Briq.	Leaves	19.3723	0.1886 ± 0.0021	1.7564 ± 0.0008	0.2404 ± 0.0033
50	*Ocimum tenuiflorum* L.	Leaves	17.3166	0.2154 ± 0.0005	2.2919 ± 0.0030	0.1121 ± 0.0018
51	*Origanum majorana* L.	Leaves	24.1194	-	7.9537 ± 0.0284	0.0649 ± 0.0028
52	*Origanum vulgare* L.	Leaves	31.5926	0.1233 ± 0.0045	9.9020 ± 0.0905	0.4103 ± 0.0093
53	*Orthosiphon aristatus* (Blume) Miq.	Leaves	11.3322	0.0721 ± 0.0017	2.1012 ± 0.0028	0.1154 ± 0.0005
54	*Perilla frutescens* (L.) Britton	Leaves	21.6211	0.0418 ± 0.0005	13.1851 ± 0.0212	0.1887 ± 0.0011
55	*Plectranthus amboinicus* (Lour.) Spreng.	Leaves	12.3686	0.0485 ± 0.0011	0.2786 ± 0.0032	0.1208 ± 0.0005
56	*Plectranthus rotundifolius* (Poir.) Spreng.	Leaves	22.8489	-	0.3344 ± 0.0033	0.0860 ± 0.0012
57	*Plectranthus rotundifolius* (Poir.) Spreng.	Tubers	6.6283	-	0.6688 ± 0.0048	0.2695 ± 0.0042

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Labiatae					
58	*Plectranthus scutellarioides* (L.) R. Br.	Leaves	26.2736	-	2.5936 ±0.0162	0.2168 ± 0.0006
59	*Rosmarinus officinalis* L.	Aerial part	17.9891	0.1236 ± 0.0051	2.6113 ± 0.0228	0.1455 ± 0.0097
60	*Salvia hispanica* L.	Seeds	5.4273	0.0288 ± 0.0001	0.5758 ± 0.0245	0.0072 ± 0.0003
61	*Salvia officinalis* L.	Aerial part	20.7187	-	6.8293 ± 0.0704	0.1660 ± 0.0093
62	*Thymus citriodorus* (Pers.) Schreb.	Aerial part	19.9628	0.1654 ± 0.0009	10.1759 ± 0.4173	0.1284 ± 0.0100
63	*Thymus vulgaris* L.	Aerial part	21.4988	0.0928 ± 0.0045	4.3487 ± 0.0249	0.1552 ± 0.0021
	Family: Lauraceae					
64	*Persea americana* Mill.	Flesh	16.1418	0.0292 ± 0.0002	-	-
65	*Persea americana* Mill.	Peels	15.2587	0.5391 ± 0.0014	-	0.0081 ± 0.0002
66	*Persea americana* Mill.	Seeds	22.1428	1.3808 ± 0.0053	-	-
	Family: Malvaceae					
67	*Hibiscus sabdariffa* L.	Leaves	31.8288	1.1171 ± 0.0017	-	0.3514 ± 0.0015

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Meliaceae					
68	*Azadirachta indica* A. Juss.	Leaves	16.6648	-	-	-
	Family: Moraceae					
69	*Morus alba* L.	Leaves	20.0448	3.0277 ± 0.0051	0.0499 ± 0.0010	0.0249 ± 0.0005
	Family: Moringaceae					
70	*Moringa oleifera* Lam.	Leaves	16.5091	0.5254 ± 0.0016	-	0.0304 ± 0.0001
71	*Moringa oleifera* Lam.	Seeds	8.4142	-	-	0.0042 ± 0.0000
	Family: Myrtaceae					
72	*Psidium guajava* L.	Fruits	50.0348	-	-	0.0217 ± 0.0000
73	*Syzygium antisepticum* (Blume) Merr. & L. M. Perry	Leaves	23.8213	-	-	-
	Family: Oxalidaceae					
74	*Averrhoa carambola* L.	Fruits	63.4117	0.0445 ± 0.0013	-	0.0088 ± 0.0001

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Piperaceae					
75	*Piper betle* L.	Leaves	21.4311	0.1364 ± 0.0003	-	0.0406 ± 0.0002
76	*Piper nigrum* L. (Black pepper)	Fruits	7.9849	-	-	-
77	*Piper nigrum* L. (White pepper)	Seeds	5.6192	-	-	-
	Family: Poaceae					
78	*Cymbopogon citratus* (DC.) Stapf	Rhizomes	26.8861	0.1316 ± 0.0018	-	0.0733 ± 0.0026
	Family: Polygonaceae					
79	*Persicaria odorata* (Lour.) Soják	Leaves	17.3908	0.2890 ± 0.0171	-	0.0913 ± 0.0001
	Family: Punicaceae					
80	*Punica granatum* L. var. *granatum*	Leaves	43.5253	0.1938 ± 0.0091	-	0.3049 ± 0.0061
81	*Punica granatum* L. var. *granatum*	Peels	40.1724	-	0.0493 ± 0.0004	0.3632 ± 0.0032

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Rosaceae					
82	*Fragaria vesca* L.	Fruits	70.5059	0.0463 ± 0.0006	-	-
83	*Malus domestica* Borkh.	Fruits	85.3321	0.2772 ± 0.0006	-	-
84	*Pyrus communis* L.	Fruits	74.5221	0.4069 ± 0.0007	-	-
	Family: Rubiaceae					
85	*Coffea arabica* L.	Seeds	8.2275	5.9665 ± 0.0065	0.8446 ± 0.0021	0.3802 ± 0.0008
86	*Coffea canephora* Pierre ex A. Froehner	Seeds	12.2621	7.8425 ± 0.0371	1.6629 ± 0.0074	1.2332 ± 0.0033
87	*Morinda citrifolia* L.	Fruits	23.6855	0.0456 ± 0.0006	-	0.0098 ± 0.0001
88	*Morinda citrifolia* L.	Leaves	28.4407	0.0808 ± 0.0003	-	-
	Family: Scrophulariaceae					
89	*Limnophila aromatica* (Lam.) Merr.	Aerial part	16.2367	0.6485 ± 0.0012	-	0.0581 ± 0.0013
	Family: Solanaceae					
90	*Capsicum annuum* L. (Green bell pepper)	Fruits	51.8632	0.1447 ± 0.0001	-	0.0248 ± 0.0003

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Solanaceae					
91	*Capsicum annuum* L. (Orange bell pepper)	Fruits	55.3660	0.1689 ± 0.0008	-	0.0255 ± 0.0011
92	*Capsicum annuum* L. (Red bell pepper)	Fruits	58.1961	0.1638 ± 0.0016	-	0.0849 ± 0.0022
93	*Capsicum annuum* L. (Yellow bell pepper)	Fruits	58.2367	0.1747 ± 0.0034	-	0.0264 ± 0.0010
94	*Nicotiana tabacum* L.	Leaves	30.8379	3.3169 ± 0.0014	-	0.0571 ± 0.0003
95	*Physalis angulata* L.	Aerial part	12.7328	0.1959 ± 0.0017	-	0.0119 ± 0.0001
96	*Physalis peruviana* L.	Fruits	61.3757	0.0967 ± 0.0010	-	<LOQ
97	*Physalis peruviana* L.	Calyx	13.8569	0.4744 ± 0.0005	-	0.0858 ± 0.0007
98	*Solanum lycopersicum* L. var. *cerasiforme*	Fruits	45.3739	0.2252 ± 0.0097	-	0.0366 ± 0.0001
99	*Solanum lycopersicum* L. var. *lycopersicum*	Fruits	60.3556	0.2805 ± 0.0090	-	0.0346 ± 0.0002
	Family: Strychnaceae					
100	*Strychnos nux-vomica* L.	Seeds	3.2277	0.3891 ± 0.0001	-	0.0044 ± 0.0001
	Family: Theaceae					
101	*Camellia sinensis* (L.) Kuntze var. *assamica* (Mast.) Kitam.	Leaves	46.3630	0.7192 ± 0.0031	-	0.0511 ± 0.0008

* - = cannot be detected

Table 5 The contents of chlorogenic, rosmarinic and caffeic acids in plant sample (Cont.)

No.	Scientific plant name	Plant parts used	% yield (g/100g)	Content (g/100g of dried plant)		
				Chlorogenic acid	Rosmarinic acid	Caffeic acid
	Family: Thunbergiaceae					
102	*Thunbergia laurifolia* Lindl.	Leaves	14.2920	0.0815 ± 0.0044	11.4870 ± 0.0189	0.2180 ± 0.0005
	Family: Verbenaceae					
103	*Clerodendrum calamitosum* L.	Leaves	20.4012	0.0225 ± 0.0005	-	0.0103 ± 0.0002
104	*Clerodendrum indicum* (L.) Kuntze	Leaves	24.3995	-	-	0.0114 ± 0.0010
105	*Clerodendrum quadriloculare* (Blanco) Merr.	Leaves	21.4071	0.0657 ± 0.0006	-	-
106	*Clerodendrum serratum* (L.) Moon	Leaves	33.6863	1.8037 ± 0.0055	-	-
107	*Clerodendrum thomsoniae* Balf. f.	Leaves	19.3895	0.0179 ± 0.0019	0.9034 ± 0.0043	0.0770 ± 0.0018
108	*Vitex agnus-castus* L.	Leaves	32.9460	5.5568 ± 0.0678	3.0834 ± 0.0369	0.0844 ± 0.0006
109	*Vitex negundo* L.	Leaves	28.4407	1.2378 ± 0.0020	1.1353 ± 0.0053	0.0540 ± 0.0043
110	*Vitex trifolia* L. subsp. *litoralis* Steenis	Leaves	28.5451	0.3792 ± 0.0025	1.4967 ± 0.0076	0.1670 ± 0.0032
111	*Vitex trifolia* L. subsp. *trifolia*	Leaves	31.4025	2.1799 ± 0.0555	2.7448 ± 0.0978	0.1765 ± 0.0055

* - = cannot be detected

Method validation

Lonicera japonica flowering bud ethanolic extract was used as substitute of all 111 plant samples to evaluate the validity of an analytical method.

Calibration curve

Standard chlorogenic, rosmarinic and caffeic acids at 5 concentrations were investigated for linearity by HPLC method. The calibration curves of standard compounds were liner in the range of 16.67 - 83.33 µg/ml. The regression equation of chlorogenic, rosmarinic and caffeic acids were $y = 2874.5x + 813.03$, $y = 2833.8x - 1858.3$ and $y = 5202.2x + 673.32$ respectively (Figure 13 - 15). The coefficient of determination (R^2) of standard compounds were more than 0.999.

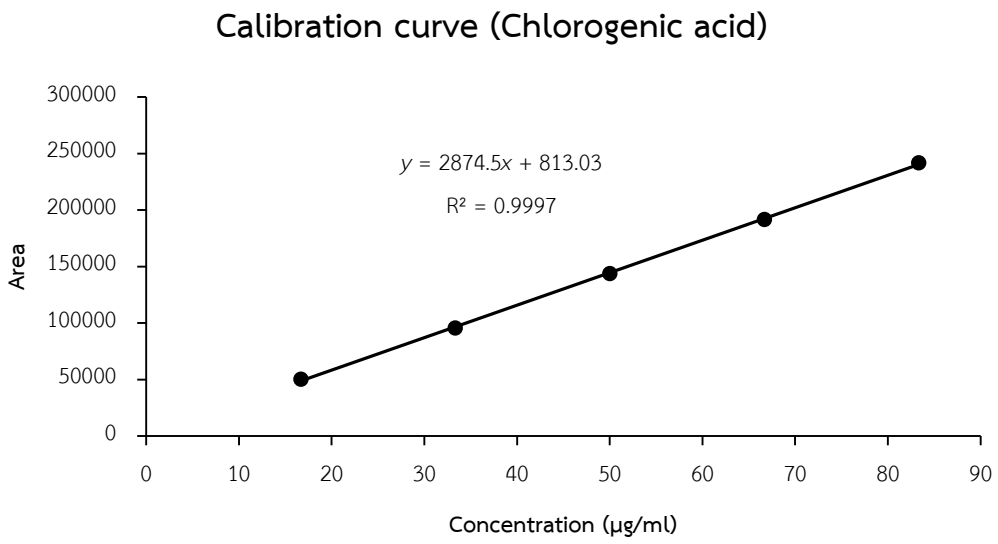


Figure 13 The calibration curve of chlorogenic acid

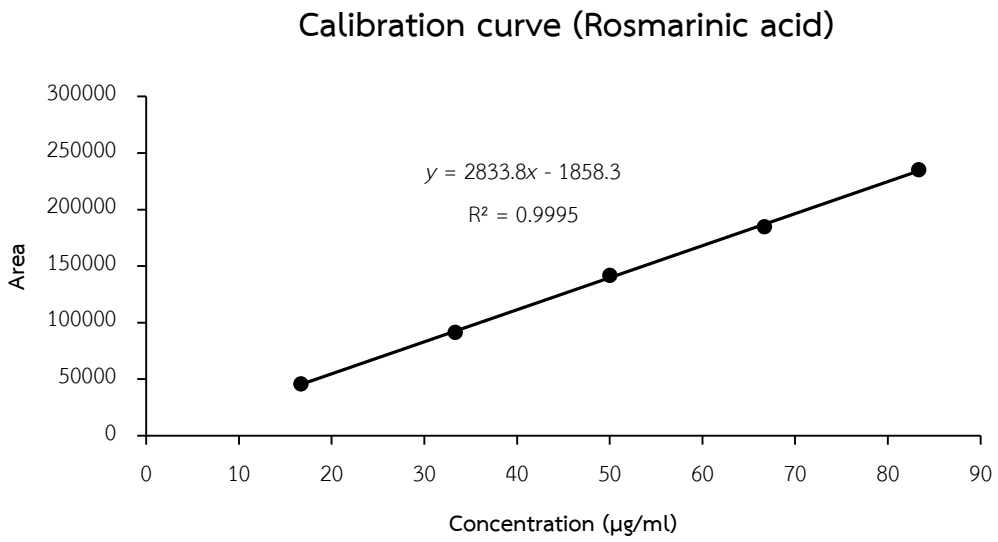


Figure 14 The calibration curve of rosmarinic acid

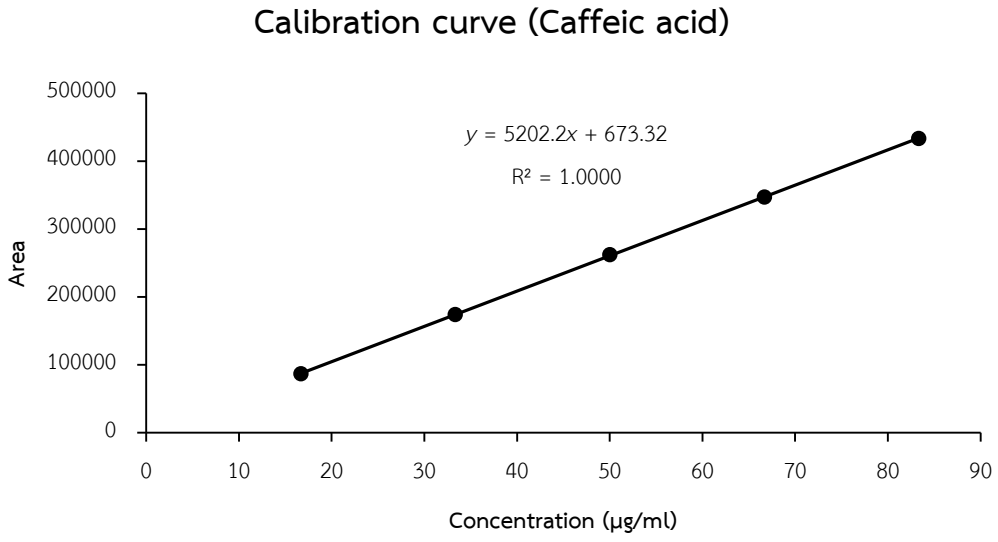


Figure 15 The calibration curve of caffeic acid

Specificity

The specificity which was evaluated by peak purity test confirmed that analyte chromatographic peak is not attributable with another compound. The peak purity index of three compounds were shown above 0.999 (Figure 16 - 18).

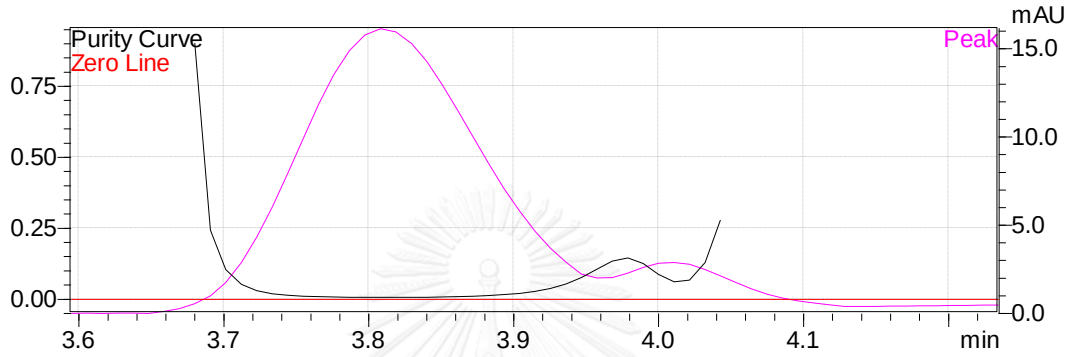


Figure 16 The peak purity of chlorogenic acid in *L. japonica* flowering bud ethanolic extract (Peak purity index: 1.000000)

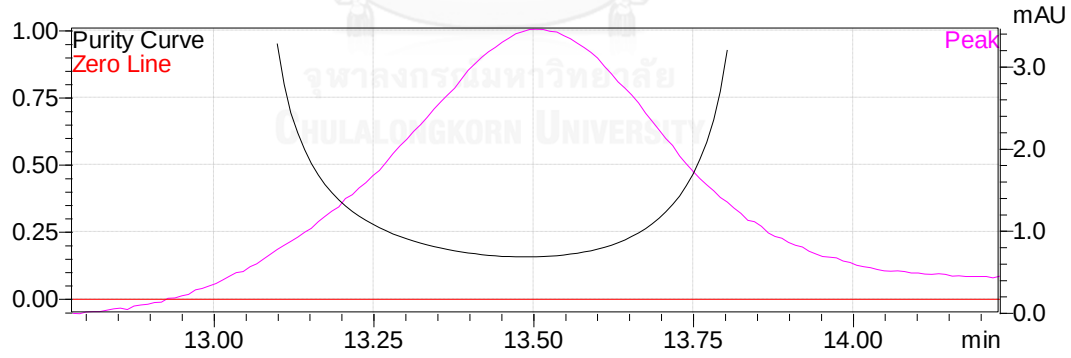


Figure 17 The peak purity of rosmarinic acid in *L. japonica* flowering bud ethanolic extract (Peak purity index: 0.999952)

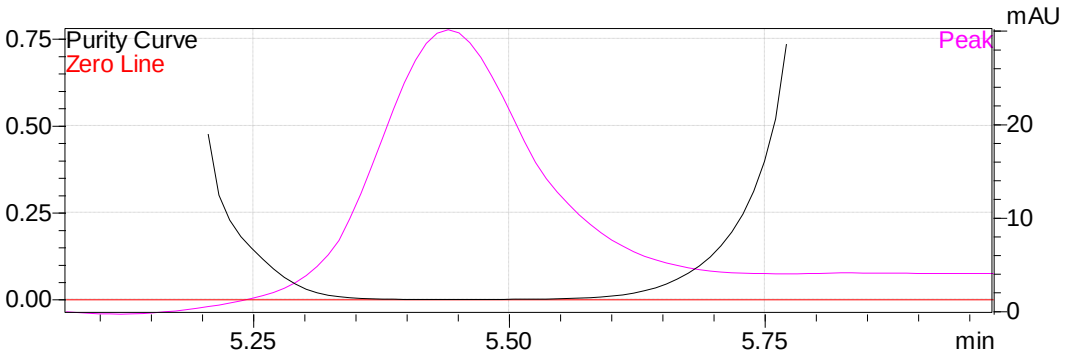


Figure 18 The peak purity of caffeic acid in *L. japonica* flowering bud ethanolic extract (Peak purity index: 0.999999)

Accuracy

The accuracy was evaluated by recovery method. Three concentrations of standard compounds were spiked into the sample. The accuracy of chlorogenic, rosmarinic and caffeic acids quantitative analysis in *L. japonica* flowering bud ethanolic extract ranged from 103.978 - 108.63, 97.23 - 99.09 and 99.41 - 100.85 %recoveries respectively as shown in Table 6.

Precision

The repeatability and intermediate precision were performed on sample with three different concentrations of standard compounds at same and five different days of experiments, respectively. The values were shown as %RSD which meant the error of the method. The repeatability and intermediate precision were shown in Table 6.

Table 6 Accuracy and precision of chlorogenic, rosmarinic and caffeic acids in *L. japonica* flowering bud

Compounds	Spike concentration (µg/ml)	% recovery (n = 3)	%RSD	
			Repeatability precision (n = 3)	Intermediate precision (n = 5)
Chlorogenic acid	10	108.632	0.130	0.989
	25	103.976	0.077	0.699
	50	107.396	0.054	1.770
Rosmarinic acid	10	97.230	0.259	1.522
	25	99.089	0.234	1.039
	50	98.328	0.135	1.415
Caffeic acid	10	100.447	0.169	6.468
	25	99.407	0.046	5.795
	50	100.851	0.074	3.119

Limit of detection and limit of quantitation

LOD and LOQ analysis were calculated by the residual standard deviation of a regression line and the slope of calibration curve. The LOD of chlorogenic, rosmarinic and caffeic acids that taken as the lowest concentration of analyte in a sample which could be detected were 1.64, 2.22 and 0.65 µg/ml respectively. The LOQ of chlorogenic, rosmarinic and caffeic acids that taken as the lowest concentration of analyte in a sample which could be accurately quantitated were 4.97, 6.72 and 1.97 µg/ml respectively.

Robustness

The robustness of sample and standard compounds was determined during the analysis of HPLC method when the flow rate of mobile phase was varied from 1.195 - 1.205 ml/min, the column temperature was varied from 29 - 31 °C and the wavelength was varied from 322 - 328 nm. The results were demonstrated that no differences (%RSD <4) in the area of the curve and retention time as shown in Table 7 - 8.

Table 7 Robustness of chlorogenic, rosmarinic and caffeic acids quantitation in *L. japonica* flowering bud

Compounds	% RSD of sample					
	Flow rate		Temperature		Wavelength	
	Rt	Area	Rt	Area	Rt	Area
Chlorogenic acid	0.31	0.50	0.79	0.78	0.06	1.14
Rosmarinic acid	0.19	0.66	2.63	0.89	0.02	1.33
Caffeic acid	0.27	0.95	1.11	3.14	0.07	2.08

Rt = Retention time

Table 8 Robustness of chlorogenic, rosmarinic and caffeic acids quantitation

Compounds	% RSD of standard compounds					
	Flow rate		Temperature		Wavelength	
	Rt	Area	Rt	Area	Rt	Area
Chlorogenic acid	0.24	0.33	0.78	0.14	0.14	1.13
Rosmarinic acid	0.11	1.38	2.57	1.10	0.03	2.41
Caffeic acid	0.23	0.29	1.20	0.25	0.09	0.87

Rt = Retention time

Part II: Pharmacognostic specification of *Lonicera japonica* flowering bud and chlorogenic acid content by TLC-densitometry and TLC-image analysis

Scientific name	*Lonicera japonica* Thunb.
Common name	Sai Nam Phueng
English name	Japanese honeysuckle
Family	Caprifoliaceae
Part used	Flowering bud

Macroscopic and microscopic examinations

Dried *L. japonica* flowering buds were yellowish-green color, clavate shape, 2 - 3 cm in length with velvet surface as shown in Figure 19. Anatomical characteristics of corolla part was illustrated in Figure 20. Both glandular and non-glandular trichomes were found. Histological characteristics of powered crude drug including corolla fragment, glandular and non-glandular trichomes, pollen grain, petal parenchyma and calcium oxalate prism as shown in Figure 21.

Figure 19 Dried *L. japonica* flowering bud crude drug

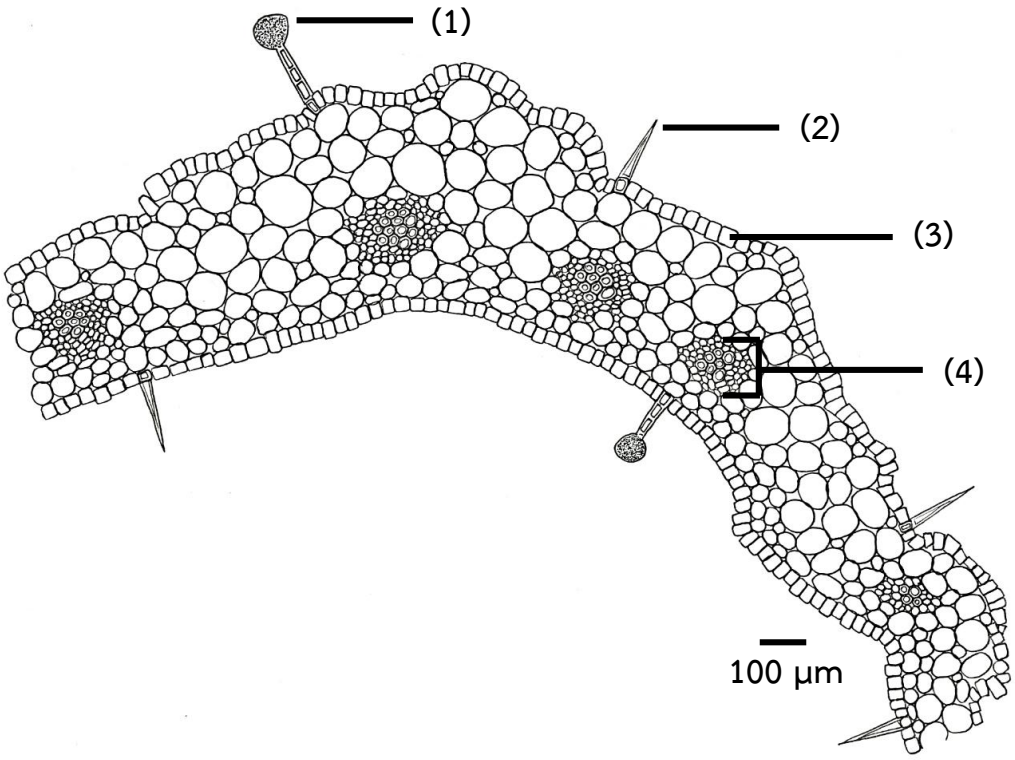


Figure 20 Anatomical characteristics of *L. japonica* flowering bud (corolla part cross section)

(1) Glandular trichome

(2) Non-glandular trichome

(3) Epidermis

(4) Vascular bundle

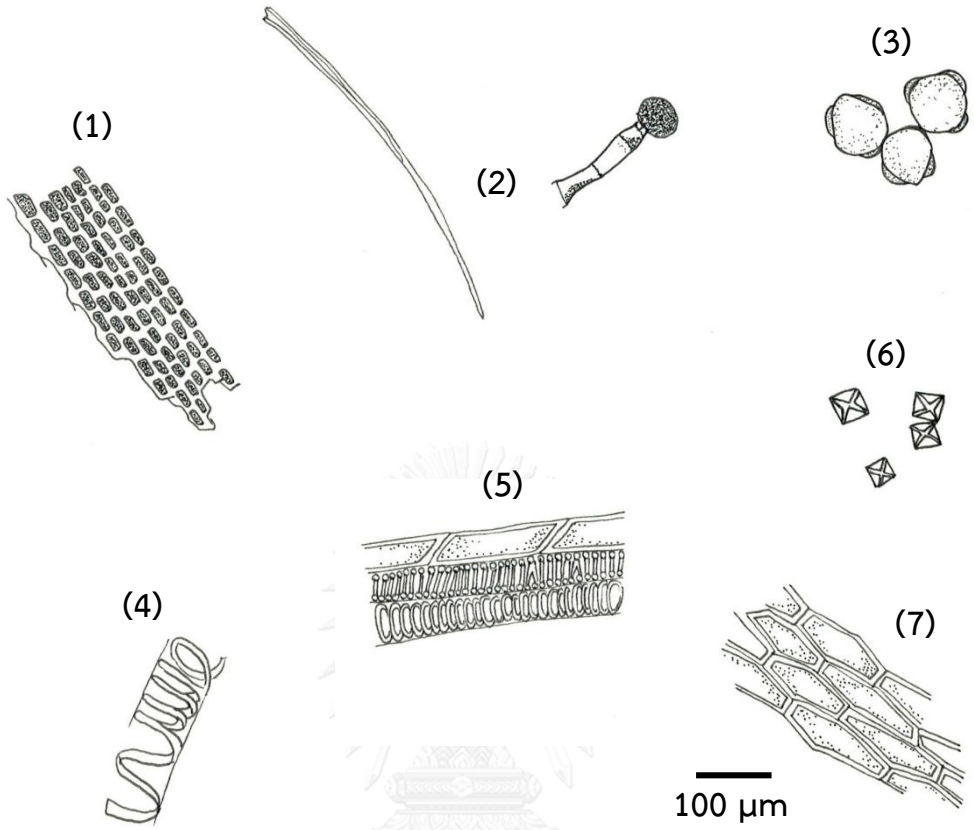


Figure 21 Histological characteristics of *L. japonica* flowering bud powders

(1) Corolla fragment

(2) Glandular and non-glandular trichome

(3) Pollen grain

(4) Spiral vessel

(5) Parenchyma and vascular bundle

(6) Prism of calcium oxalate

(7) Petal parenchyma

Physico-chemical evaluation

The pharmacognostic constant numbers due to the standardization parameters of *L. japonica* flowering bud were shown in Table 9. The results showed the contents of loss on drying, total ash, acid insoluble ash and water content should be not more than 10.11, 6.59, 1.14 and 10.82 % by dry weight respectively whereas ethanol and water soluble extractive values should be not less than 16.46 and 28.88 % by dry weight respectively.

Table 9 Physico-chemical characteristics of *L. japonica* flowering bud

Parameter	Content (% by weight)*
Loss on drying content	10.11 ± 0.06
Total ash content	6.59 ± 0.05
Acid-insoluble ash content	1.14 ± 0.06
Ethanol extractive value	16.46 ± 0.25
Water extractive value	28.88 ± 0.59
Water content	10.82 ± 0.49
Volatile oils content	0

*The parameters were shown as grand mean ± pooled standard deviation. Samples were from 15 different sources throughout Thailand. Each sample was analysed in triplicate.

Thin layer chromatographic fingerprint

TLC fingerprint of *L. japonica* flowering bud ethanolic extract was observed under UV light at 254 and 365 nm. The ferric chloride reagent was used to detect the present of phenol compounds as shown in Figure 22.

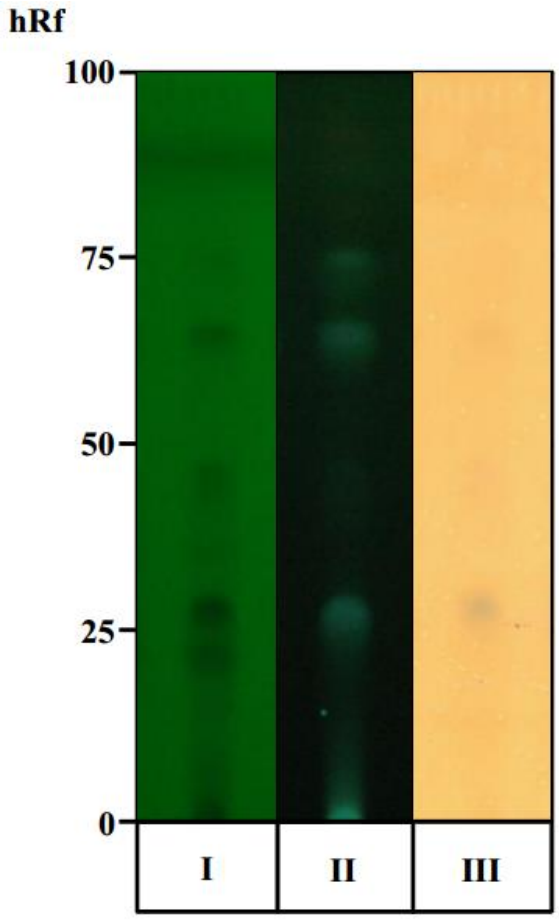


Figure 22 TLC fingerprint of *L. japonica* flowering bud ethanolic extract

Stationary phase Silica gel 60 GF_{254} TLC plate

Mobile phase Ethyl acetate : methanol : water : formic acid (50 : 4 : 4 : 2.5)

Detection I = detection under UV 254 nm

II = detection under UV 365 nm

III = detection with ferric chloride reagent

Quantitative analysis of chlorogenic acid in *Lonicera japonica* flowering bud

Ethanolic extract of *L. japonica* flowering bud

The dried powders of *L. japonica* flowering bud from 15 different sources were exhaustively extracted with 95% ethanol by Soxhlet apparatus. The percent yields of crude extracts were shown in Table 10. The average percent yield of *L. japonica* flowering bud ethanolic extract was 39.44 ± 5.83 g/100 g by dry weight.

Table 10 Yield of *L. japonica* flowering bud ethanolic extract from 15 different sources in Thailand

Source	Dried crude drug (g)	Ethanolic extract (g)	% yield (g/100g)
1	5.0082	2.1774	43.48
2	5.0053	1.8132	36.23
3	5.0041	1.9876	39.72
4	5.0015	2.1869	43.72
5	5.0033	2.0512	41.00
6	5.0088	2.1763	43.45
7	5.0023	2.2115	44.21
8	5.0052	2.0000	39.96
9	5.0012	2.0619	41.23
10	5.0084	1.8370	36.68
11	5.0016	1.9216	38.42
12	5.0051	2.0102	40.16
13	5.0028	1.0183	20.35
14	5.0029	2.0081	40.14
15	5.0028	2.1437	42.85
		Average	**39.44 ± 5.83**

TLC-image analysis by ImageJ software

Method validation

Calibration curve

The calibration curve of standard chlorogenic acid ranged from 0.6 - 3.0 µg/spot as shown in Figure 23. The polynomial equation was $y = -1787x^2 + 14904x - 3015.4$ and the coefficient of determination (R^2) of the curve was 0.9999.

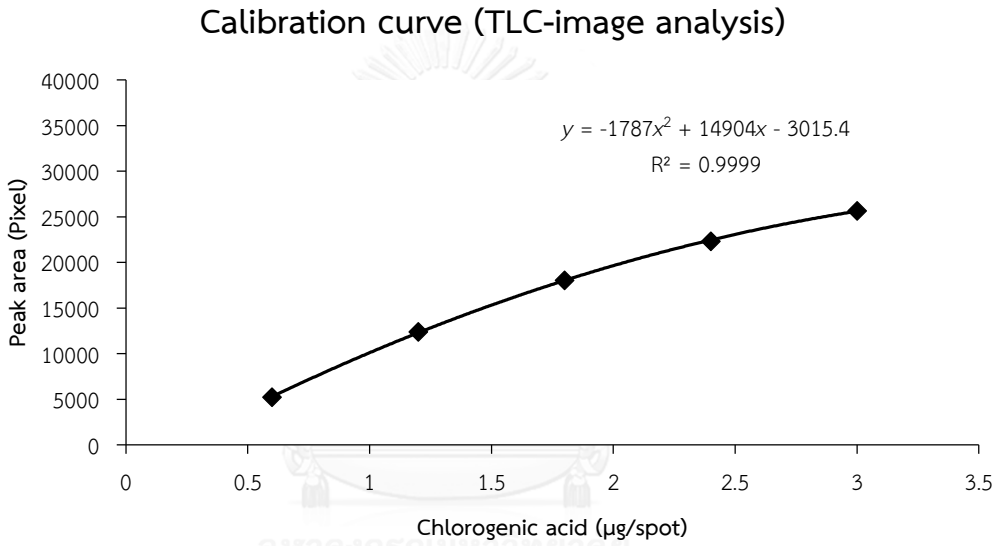


Figure 23 The calibration curve of standard chlorogenic acid by TLC-image analysis

Accuracy

The recovery assay was used to validate the accuracy of chlorogenic acid quantitation by TLC-image analysis method. Three concentrations of standard chlorogenic acid were spiked into the sample. The accuracy of chlorogenic acid quantitative analysis in *L. japonica* flowering bud ethanolic extract were between 84.08 - 105.29 %recoveries as shown in Table 11.

Table 11 Accuracy of chlorogenic acid quantitation in *L. japonica* flowering bud by TLC-image analysis (n = 3)

Chlorogenic acid added (µg/spot)	Chlorogenic acid found (µg/spot)	% Recovery
0.0	0.719	-
0.6	1.108	84.08
1.2	1.957	102.02
1.8	2.654	105.29
	Average	**97.13 ± 11.42**

Precision

The repeatability and intermediate precision were performed on sample with four different concentrations of standard chlorogenic acid at same and three different days of experiments, respectively. The values were shown as %RSD which meant the error of the method. The repeatability and intermediate precision were between 5.86 - 14.65 %RSD and 5.87 - 13.95 %RSD respectively (Table 12).

Table 12 Repeatability and intermediate precision of chlorogenic acid quantitation in *L. japonica* flowering bud by TLC-image analysis (n = 3)

Repeatability		Intermediate precision	
Chlorogenic acid (µg/spot)	%RSD	Chlorogenic acid (µg/spot)	%RSD
0.65 ± 0.04	5.86	0.79 ± 0.05	5.87
1.28 ± 0.19	14.65	1.22 ± 0.17	13.95
1.97 ± 0.20	9.89	1.90 ± 0.15	7.93
2.30 ± 0.19	8.22	2.45 ± 0.33	13.33
Average	**9.66 ± 3.72**	**Average**	**10.27 ± 3.99**

Limit of detection and limit of quantitation

LOD and LOQ of TLC-image analysis were calculated by the residual standard deviation of a regression line and the slope of calibration curve. The LOD and LOQ of chlorogenic acid for TLC-image analysis were 0.03 and 0.09 µg/spot, respectively.

Robustness

The robustness of chlorogenic acid quantitation in *L. japonica* flowering bud by TLC-image analysis was determined in three mobile phase ratios. The result of robustness was 9.17 %RSD of peak area (Table 13).

Table 13 Robustness of chlorogenic acid quantitation in *L. japonica* flowering bud and standard compound by TLC-image analysis

Mobile phase ratio (v/v)	Peak area	
Ethyl acetate : formic acid : acetic acid : water	Sample	Standard chlorogenic acid
49.9 : 5.4 : 5.6 : 13.1	9128.32	43095.95
50.0 : 5.5 : 5.5 : 13.0	7694.89	39270.50
50.1 : 5.6 : 5.4 : 12.9	8982.50	48530.94
Average	**8601.90 ± 788.87**	**43632.46 ± 4653.47**
%RSD	**9.17**	**10.67**

The chlorogenic acid content in *L. japonica* flowering bud by TLC-image analysis

The amount of chlorogenic acid in *L. japonica* flowering bud ethanolic extract were done in triplicate using TLC-image by ImageJ software. The content values were calculated and shown as grams of chlorogenic acid per 100 grams of dried crude drug (Table 14). The average of chlorogenic acid content was 2.09 ± 0.44 g/100 g.

Table 14 The chlorogenic acid content in *L. japonica* flowering bud by TLC-image analysis

Source	Chlorogenic acid in the ethanolic extract (g/g of dried crude drug)	Yield of the ethanolic extract (g/100g of dried crude drug)	Chlorogenic acid in *L. japonica* flowering bud (g/100g of dried crude drug)
1	0.065	43.477	2.828
2	0.052	36.226	1.886
3	0.060	39.719	2.364
4	0.062	43.725	2.716
5	0.056	40.997	2.283
6	0.057	43.450	2.464
7	0.051	44.210	2.240
8	0.045	39.958	1.800
9	0.050	41.228	2.052
10	0.044	36.678	1.606
11	0.047	38.420	1.798
12	0.050	40.163	1.990
13	0.056	20.355	1.131
14	0.057	40.139	2.271
15	0.044	42.850	1.889
		Average	**2.09 ± 0.44**

TLC-densitometry

Method validation

Calibration curve

The calibration curve of standard chlorogenic acid ranged from 0.6 - 3.0 µg/spot was shown in Figure 24. The polynomial equation was $y = -2681.5x^2 + 17419x + 3699.2$ and the coefficient of determination (R^2) of the curve was 0.9990.

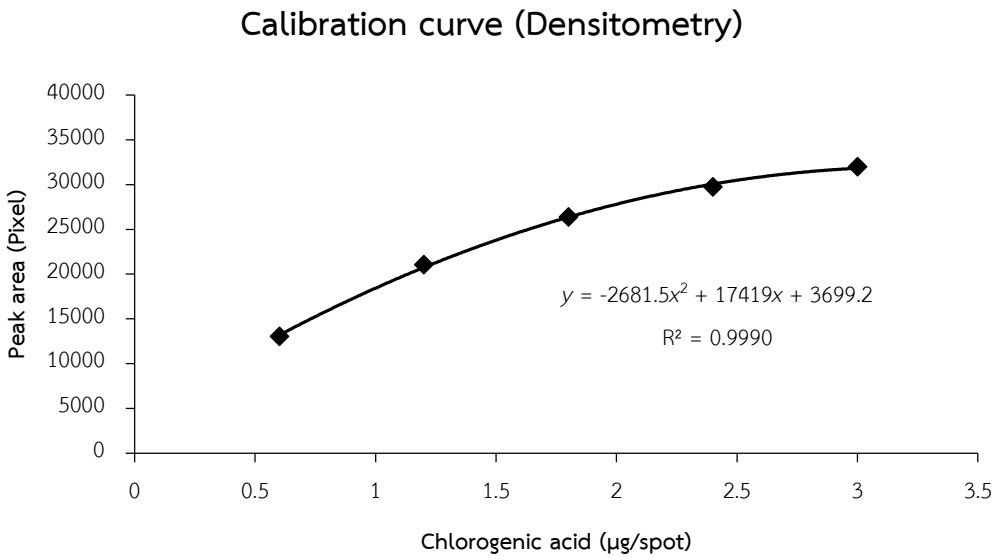


Figure 24 The calibration curve of standard chlorogenic acid by TLC-densitometry

Specificity

The specificity was established by comparing light absorbance spectra of all samples and standard chlorogenic acid for peak identity as shown in Figure 25, and the peak purity was performed by comparison of spectra at up-slope, apex and down-slope of the peak as demonstrated in Figure 26.

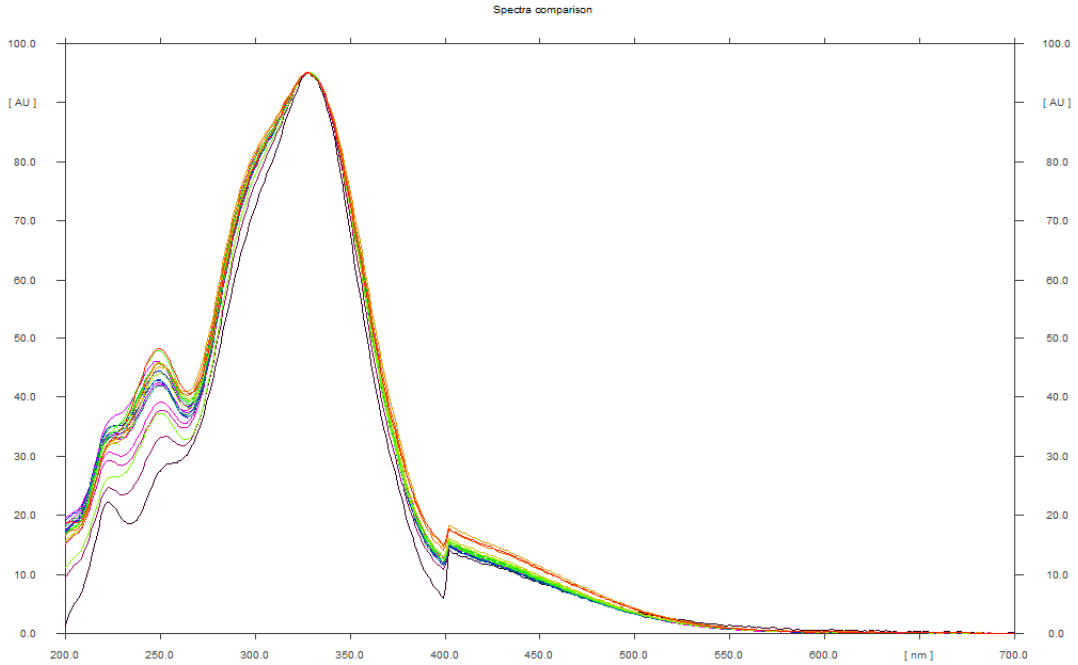


Figure 25 The ultraviolet absorbance spectra of chlorogenic acid in samples and standard chlorogenic acid bands

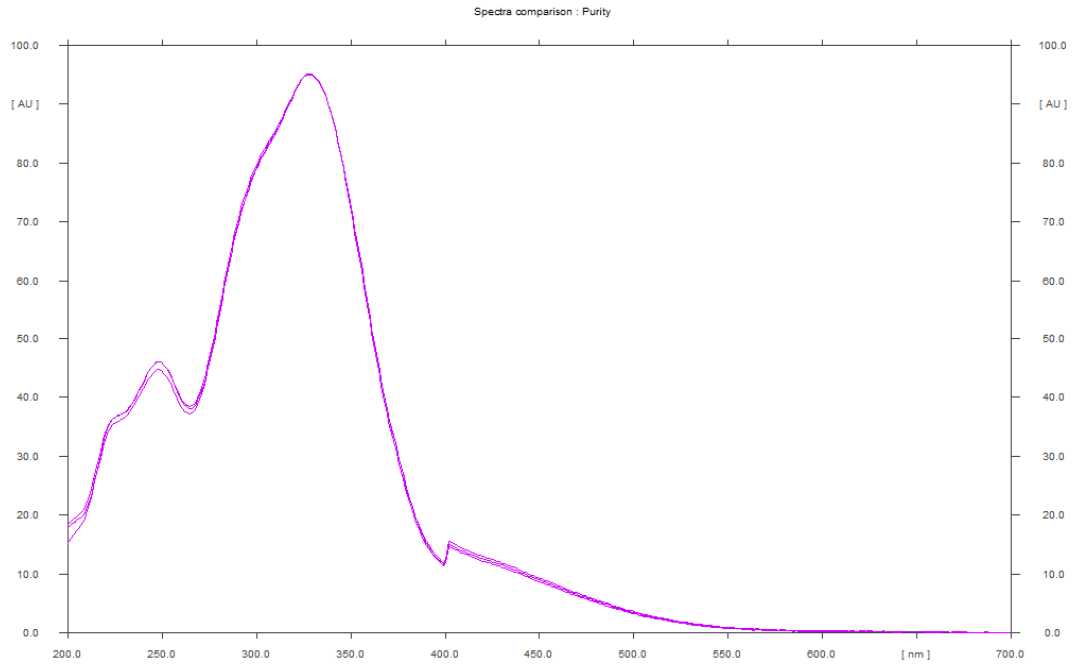


Figure 26 Peak purity determination using up-slope, apex and down-slope of the peak

Accuracy

The recovery assay was used to validate the accuracy of chlorogenic acid quantitation by TLC-densitometry. Three concentrations of standard chlorogenic acid were spiked into the sample. The accuracy of chlorogenic acid quantitative analysis in *L. japonica* flowering bud ethanolic extract were between 89.99 - 98.16 %recoveries as shown in Table 15.

Table 15 Accuracy of chlorogenic acid quantitation in *L. japonica* flowering bud by TLC-densitometry (n = 3)

Chlorogenic acid added (µg/spot)	**Chlorogenic acid found (µg/spot)**	**% Recovery**
0.0	0.851	-
0.6	1.306	89.99
1.2	1.931	94.15
1.8	2.602	98.16
	Average	**94.10 ± 4.09**

Precision

The repeatability and intermediate precision were performed on sample with four different concentrations of standard chlorogenic acid at same and three different days of experiments, respectively. The values were shown as %RSD which meant the error of the method. The repeatability and intermediate precision were between 0.73 - 1.46 %RSD and 2.87 - 4.13 %RSD respectively (Table 16).

Table 16 Repeatability and intermediate precision of chlorogenic acid quantitation in *L. japonica* flowering bud by TLC-densitometry (n = 3)

Repeatability		**Intermediate precision**	
Chlorogenic acid (µg/spot)	**%RSD**	**Chlorogenic acid (µg/spot)**	**%RSD**
0.88 ± 0.01	1.20	0.89 ± 0.03	2.87
1.32 ± 0.02	1.46	1.30 ± 0.05	4.01
1.88 ± 0.01	0.73	1.94 ± 0.08	3.92
2.40 ± 0.03	1.28	2.36 ± 0.10	4.13
Average	**1.17 ± 0.31**	**Average**	**3.73 ± 0.58**

Limit of detection and limit of quantitation

LOD and LOQ of TLC-image analysis were calculated by the residual standard deviation of a regression line and the slope of calibration curve. The LOD and LOQ of chlorogenic acid for TLC-densitometry were 0.07 and 0.21 µg/spot, respectively.

Robustness

The robustness of chlorogenic acid quantitation in *L. japonica* flowering bud by TLC-densitometry was determined in three mobile phase ratios. The result of robustness was 8.59 %RSD of peak area (Table 17).

Table 17 Robustness of chlorogenic acid quantitation in *L. japonica* flowering bud and standard compound by TLC-densitometry

Mobile phase ratio (v/v)	**Peak area**	
Ethyl acetate : formic acid : acetic acid : water	**Sample**	**Standard chlorogenic acid**
49.9 : 5.4 : 5.6 : 13.1	15631.65	34295.20
50.0 : 5.5 : 5.5 : 13.0	18244.94	33611.69
50.1 : 5.6 : 5.4 : 12.9	15948.99	32684.48
Average	**16608.53 ± 1426.03**	**33530.46 ± 808.428**
%RSD	**8.59**	**2.41**

The chlorogenic acid content in *L. japonica* flowering bud by TLC-densitometry

The amount of chlorogenic acid in *L. japonica* flowering bud ethanolic extract were done in triplicate using TLC-densitometry. The content values were calculated and shown as grams of chlorogenic acid per 100 grams of dried crude drug (Table 18). The average of chlorogenic acid content was 2.24 ± 0.50 g/100 g.

Table 18 The chlorogenic acid content in *L. japonica* flowering bud by TLC-densitometry

Source	Chlorogenic acid in the ethanolic extract (g/g of dried crude drug)	Yield of the ethanolic extract (g/100g of dried crude drug)	Chlorogenic acid in *L. japonica* flowering bud (g/100g of dried crude drug)
1	0.056	43.477	2.444
2	0.057	36.226	2.064
3	0.074	39.719	2.926
4	0.066	43.725	2.885
5	0.056	40.997	2.310
6	0.062	43.450	2.690
7	0.060	44.210	2.634
8	0.060	39.958	2.384
9	0.058	41.228	2.374
10	0.057	36.678	2.102
11	0.047	38.420	1.824
12	0.048	40.163	1.912
13	0.049	20.355	0.994
14	0.044	40.139	1.762
15	0.052	42.850	2.247
		Average	**2.24 ± 0.50**

The comparison of chlorogenic acid content between TLC-densitometry and TLC-image analysis

The comparison of chlorogenic acid content between TLC-densitometry and TLC-image analysis (Table 19) were statistically tested using paired *t*-test. It was found that the chlorogenic acid content by two methods were not significantly different (t = 1.62, *P* = 0.13).

Table 19 The comparison of chlorogenic acid contents between TLC-densitometry and TLC-image analysis

Source	Chlorogenic acid in the ethanolic extract (g/g)	
	TLC-densitometry	TLC-image analysis
1	0.056	0.065
2	0.057	0.052
3	0.074	0.060
4	0.066	0.062
5	0.056	0.056
6	0.062	0.057
7	0.060	0.051
8	0.060	0.045
9	0.058	0.050
10	0.057	0.044
11	0.047	0.047
12	0.048	0.050
13	0.049	0.055
14	0.044	0.057
15	0.052	0.044

Part III: *In vitro* biological activity evaluations

Cytotoxic activity (Brine shrimp lethality assay)

The results from brine shrimp lethality assay were expressed as the concentrations of tested samples necessary to cause 50% of lethality (LC_{50}) that shown in Table 20. Toxic strength was classified as toxic if LC_{50} value <1000 µg/ml and non toxic if LC_{50} ≥1000 µg/ml [118]. The result demonstrated that *L. japonica* flowering bud ethanolic extract was not toxic to brine shrimp due to LC_{50} value of 2286.20 µg/ml. On the contrary, standard chlorogenic, rosmarinic and caffeic acids demonstrated potential to be toxic in brine shrimp with LC_{50} value of 266.05, 289.66 and 231.82 µg/ml respectively.

Table 20 Cytotoxic activity as LC_{50} of brine shrimp lethality

Tested samples	LC_{50} (µg/ml)
Extract	2286.20
Chlorogenic acid	266.05
Rosmarinic acid	289.66
Caffeic acid	231.82

Cytotoxic activity (MTT cell viability assay)

The cytotoxic activities against 5 cancer cell lines and 1 normal cell line of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic and caffeic acids were evaluated by MTT assay. The results were shown in Table 21. Doxorubicin was used as a positive control. U.S. National Cancer Institute (NCI) establishes the criteria of cytotoxicity that plant extract and pure compound, with IC_{50} value <20 µg/ml and <4 µg/ml respectively, are considered to have cytotoxic activity [96, 97]. All tested samples demonstrated no significant activity against six cell lines

with IC_{50} more than standard criteria. However, caffeic acid showed more cytotoxic potential against breast ductal carcinoma (BT-474) with IC_{50} = 7.02 µg/ml.

Table 21 Cytotoxic activity as IC_{50} of MTT cell viability

Tested samples	IC_{50} (µg/ml)					
	BT-474	ChaGo-K-1	Hep G2	KATO III	SW620	WI-38
Extract	>100	>100	>100	>100	>100	>100
Chlorogenic acid	>100	78.72	>100	>100	69.59	>100
Rosmarinic acid	88.54	82.98	53.33	62.43	49.71	>100
Caffeic acid	7.02	84.46	66.11	80.70	48.82	75.41
Doxorubicin	0.80	0.65	0.12	0.71	2.57	0.22

DNA damage (Comet assay)

The comet scores were obtained from 100 comets per slide in each concentration multiplying with the number of class cell to score between 0 - 400 arbitrary unit. The total scores of DNA damage were showed in Figure 27. Hydrogen peroxide was used as positive control. Phosphate buffer saline (PBS) pH 7.4 and 2% DMSO were used as negative control. The tested samples showed a dose-dependent relationship between the degree of DNA damage and concentration of the sample. Chlorogenic acid at 100 µg/ml showed the highest DNA damage. Flowering bud ethanolic extract showed a potentiating effect on DNA damage more than rosmarinic and caffeic acids.

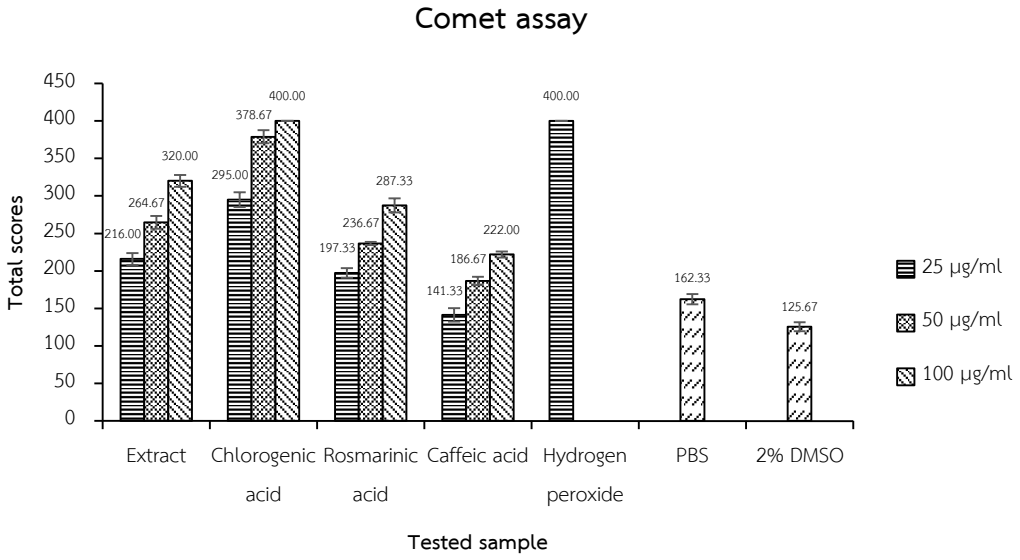


Figure 27 Total scores of DNA damage in human lymphocyte cells

Antimicrobial activity

The results of tested samples against 13 microorganisms were evaluated by agar well diffusion method (Table 22) and broth microdilution method (Table 23). It was found that *L. japonica* flowering bud ethanolic extract presented no inhibitory activity against any tested microorganisms. Moreover, all tested samples exhibited no inhibitory activity against *Candida albicans* and *Saccharomyces cerevisiae*. For agar well diffusion method, chlorogenic acid demonstrated inhibition zone against tested microorganisms ranging from 6.67 - 10.00 mm, the widest of inhibition zone was found against *Enterobacter aerogenes* of 10.00 mm. Rosmarinic acid demonstrated inhibition zone against tested microorganisms ranging from 6.67 - 16.33 mm, the widest of inhibition zone was found against *Kocuria rhizophila* of 16.33 mm. Caffeic acid demonstrated inhibition zone against tested microorganisms ranging from 8.67 - 12.33 mm, the widest of inhibition zone was found against *Shigella* spp. of 12.33 mm. However, MIC value demonstrated that all tested samples had no potential against tested microorganisms (>4,000 µg/ml).

Table 22 Antimicrobial activity of *L. japonica* flowering bud ethanolic extract, chlorogenic acid, rosmarinic acid, caffeic acid, ampicillin and amikacin using agar well diffusion method

Microorganisms	Inhibition zone (mm)*					
	Extract	Chlorogenic acid	Rosmarinic acid	Caffeic acid	Ampicillin	Amikacin
Bacillus cereus	NA	NA	NA	NA	22.33 ± 0.58	22.00 ± 1.00
Bacillus subtilis	NA	10.00 ± 0.00	9.67 ± 0.58	10.33 ± 0.58	13.33 ± 0.58	21.00 ± 0.00
Enterobacter aerogenes	NA	7.33 ± 1.15	NA	9.67 ± 0.58	9.00 ± 0.00	16.33 ± 0.58
Escherichia coli	NA	6.67 ± 1.15	6.67 ± 1.15	9.33 ± 0.58	17.67 ± 0.58	18.33 ± 0.58
Kocuria rhizophila	NA	15.00 ± 0.00	16.33 ± 0.58	11.33 ± 0.58	40.67 ± 0.58	24.67 ± 0.58
Pseudomonas aeruginosa	NA	8.67 ± 0.58	8.67 ± 0.58	8.67 ± 0.58	NA	20.00 ± 0.00
Salmonella typhi	NA	8.00 ± 0.00	7.67 ± 1.53	9.67 ± 0.58	26.33 ± 0.58	18.33 ± 0.58
Salmonella typhimurium	NA	8.67 ± 0.58	7.67 ± 1.53	9.67 ± 0.58	30.33 ± 0.58	20.00 ± 0.00
Shigella spp.	NA	NA	9.33 ± 0.58	12.33 ± 0.58	23.33 ± 0.58	22.23 ± 0.58
Staphylococcus aureus	NA	9.67 ± 0.58	8.33 ± 0.58	9.33 ± 0.88	40.33 ± 0.58	12.33 ± 0.58
Staphylococcus epidermidis	NA	9.33 ± 0.58	8.33 ± 0.58	11.67 ± 0.58	25.67 ± 0.58	23.00 ± 0.00
Candida albicans	NA	NA	NA	NA	NA	NA
Saccharomyces cerevisiae	NA	NA	NA	NA	NA	NA

*mean ± SD, NA = no activity, 6 millimeter of well. The tests were done in triplicate.

Table 23 Antimicrobial activity of *L. japonica* flowering bud ethanolic extract, chlorogenic acid, rosmarinic acid, caffeic acid, ampicillin and amikacin using broth microdilution method

Microorganisms	Extract		Chlorogenic acid		Rosmarinic acid		Caffeic acid		Ampicillin		Amikacin	
	MIC (µg/ml)	MBC/ MFC (µg/ml)	MIC (µg/ml)	MBC/ MFC (µg/ml)	MIC µg/ml)	MBC/ MFC (µg/ml)	MIC (µg/ml)	MBC/ MFC (µg/ml)	MIC (µg/ml)	MBC/ MFC (µg/ml)	MIC (µg/ml)	MBC/ MFC (µg/ml)
Bacillus cereus	NA	NA	NA	NA	NA	NA	NA	NA	0.39	0.39	1.56	1.56
Bacillus subtilis	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	>200	>200	12.5	12.5
Enterobacter aerogenes	NA	NA	>4000	>4000	NA	NA	4000	>4000	>200	>200	25	25
Escherichia coli	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	6.25	25	25	200
Kocuria rhizophila	NA	NA	>4000	>4000	4000	>4000	4000	>4000	0.39	0.39	6.25	6.25
Pseudomonas aeruginosa	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	NA	NA	50	100
Salmonella typhi	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	1.562	50	200	>200
Salmonella typhimurium	NA	NA	>4000	>4000	>4000	>4000	>4000	>4000	0.781	6.25	50	100
Shigella spp.	NA	NA	NA	NA	>4000	>4000	4000	4000	6.25	12.5	50	50
Staphylococcus aureus	NA	NA	>4000	>4000	>4000	>4000	4000	>4000	0.39	25	6.25	100
Staphylococcus epidermidis	NA	NA	>4000	>4000	4000	4000	4000	>4000	12.5	12.5	3.125	12.5
Candida albicans	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Saccharomyces cerevisiae	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*NA = no activity. The tests were done in triplicate.

Antioxidant activities

2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay

The results of DPPH radical scavenging assay of tested samples were demonstrated in Table 24. Caffeic acid showed highest potential radical scavenging activity with IC_{50} of 4.27 µg/ml followed by quercetin which used as positive control (IC_{50} = 4.84 µg/ml). *Lonicera japonica* flowering bud ethanolic extract showed the weakest potential radical scavenging activity in this study.

Table 24 DPPH radical scavenging activity (IC_{50}) of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls

Tested samples	IC_{50} (µg/ml)
L. japonica flowering bud ethanolic extract	54.78
Chlorogenic acid	7.83
Rosmarinic acid	5.99
Caffeic acid	4.27
Quercetin	4.84
Butylated hydroxytoluene (BHT)	24.82

Ferric reducing antioxidant power (FRAP) assay

The results of ferric reducing antioxidant power assay of tested samples were demonstrated in Table 25. Rosmarinic acid showed highest reducing power ability with FRAP value of 1.57 ± 0.04 mM $FeSO_4$/mg sample, followed by BHT and quercetin which used as positive controls with FRAP value of 1.51 ± 0.01 and 1.48 ± 0.06 mM $FeSO_4$/mg sample respectively. Chlorogenic acid showed the lowest reducing power ability in this study.

Table 25 FRAP value of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls

Tested samples	**FRAP value (mM $FeSO_4$/mg sample)**
L. japonica flowering bud ethanolic extract	1.24 ± 0.02
Chlorogenic acid	1.17 ± 0.02
Rosmarinic acid	1.57 ± 0.04
Caffeic acid	1.32 ± 0.13
Quercetin	1.48 ± 0.06
Butylated hydroxytoluene (BHT)	1.51 ± 0.01

Nitric oxide scavenging assay

The capability of nitric oxide scavenger of tested samples were demonstrated in Table 26. Chlorogenic acid showed highest inhibitory potential of 75.97%, followed by quercetin which used as positive controls of 72.27%. *Lonicera japonica* flowering bud ethanolic extract showed lowest inhibitory potential of nitric oxide scavenger in this study of 49.86%.

Table 26 Nitric oxide inhibition of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive control

Tested samples	Nitric oxide inhibition (%)*
L. japonica flowering bud ethanolic extract	49.86 ± 0.48
Chlorogenic acid	75.97 ± 1.82
Rosmarinic acid	70.35 ± 2.46
Caffeic acid	64.73 ± 1.53
Quercetin	72.27 ± 1.88

*Concentration of all tested samples at 800 µg/ml

Beta-carotene bleaching assay

Beta-carotene bleaching assay of tested samples demonstrated antioxidant ability with dose-response relationship (Figure 28) and the results were shown in Table 27. Butylated hydroxytoluene (BHT) and quercetin which used as positive controls showed highest peroxidation inhibition of 91.81% and 78.10% respectively, followed by rosmarinic acid with peroxidation inhibition of 53.68%. *Lonicera japonica* flowering bud ethanolic extract showed the lowest peroxidation inhibition in this study.

Table 27 Beta-carotene bleaching of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls

Tested samples	Beta-carotene bleaching (%)*
L. japonica flowering bud ethanolic extract	2.71 ± 1.03
Chlorogenic acid	40.20 ± 7.56
Rosmarinic acid	53.68 ± 0.79
Caffeic acid	49.28 ± 1.24
Quercetin	78.12 ± 0.57
Butylated hydroxytoluene (BHT)	91.81 ± 0.24

*Concentration of all tested samples at 1 mg/ml

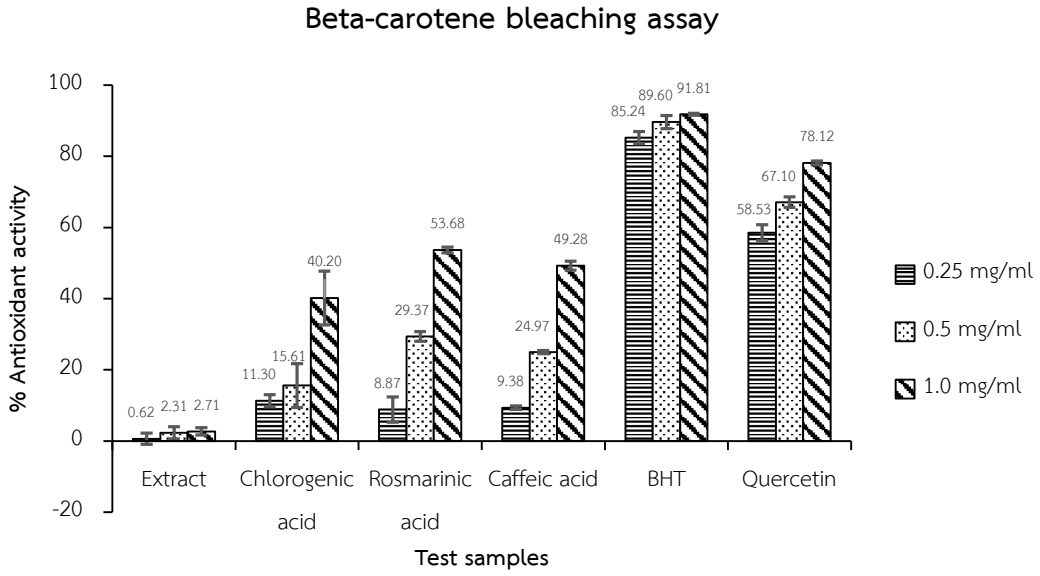


Figure 28 The antioxidant activity of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls by beta-carotene bleaching assay

Antidiabetic activity

Yeast alpha-glucosidase inhibition assay

The results of yeast alpha-glucosidase inhibition assay of tested samples were demonstrated in Table 28. Rosmarinic acid showed highest potential effect on alpha-glucosidase inhibition with IC_{50} of 8.24 mg/ml compared to acarbose which used as positive controls (IC_{50} = 10.16 mg/ml). Chlorogenic acid showed lowest potential effect on alpha-glucosidase inhibition in this study.

Table 28 Yeast alpha-glucosidase inhibition of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive control

Tested samples	**IC_{50} (mg/ml)**
L. japonica flowering bud ethanolic extract	20.29
Chlorogenic acid	24.95
Rosmarinic acid	8.24
Caffeic acid	12.59
Acarbose	10.16

CHAPTER V
DISCUSSION AND CONCLUSION

High performance liquid chromatography (HPLC) is a primary method for separation and analysis of chemical compounds in many fields, for example agriculture, cosmetics, pharmaceutical industries, environments and food. Presently, it is commonly used for qualitative and quantitative analyses of chemicals in herbal extracts. The identification of compounds depends on retention time and light spectral characteristics of each chromatographic peak [45]. This study was performed with reverse phase (C_{18}) column and detected by photo diode array detector (PDA). The PDA establishes a large amount of spectral information with optimal sensitivity and wavelength resolution. Moreover, this detector collects data with a maximum wavelength bandwidth of 190 - 800 nm [131]. Chlorogenic, rosmarinic and caffeic acids are phenolic compounds containing conjugated double bonds which have strong UV absorption; thus, PDA is a suitable detector for analysis. The reverse phase HPLC column is wildly used to separate phenolic compounds in fruits, plant extracts and derivatives. Octadecilsilane (ODS, or simply C_{18}) column is preferred for polar compounds analysis because there is no danger that those highly polar substances may be irreversibly retained in the column and gradually changing the separation characteristics of the column [132, 133]. The chromatographic condition optimization including mobile phase, gradient elution procedure, flow rate, column temperature and wavelength detection were performed to provide good separation of constituents. Numerous mobile phases and gradient program were trialled using various proportions of different aqueous phases and organic modifiers. Formic acid, phosphoric acid and acetic acid were usually employed to the aqueous phase to enhance the resolution, restrain the ionization and reduced the peak tailing of compounds [134]. The most suitable mobile phase that showed good resolution and symmetric peak shape were obtained using two parts as Solvent A (0.2% phosphoric acid in water) and Solvent B (methanol) with an isocratic program. Increasing column temperature by 1 °C affected to decrease the retention time about 1 - 2%, moreover the increasing temperature

also improved resolution and increased the production rate [135, 136]. Thus, the column temperature was hold at 30 °C for the duration of analysis to improve the retention time precision. Hydroxycinnamic acids have the maximum wavelength during 270 - 360 nm [137]. The UV spectra of standard chlorogenic, rosmarinic and caffeic acids were compared at varying wavelengths, and based on the data from the literatures. The optimal detection wavelength in this study was to be 325 nm [138, 139]. In the present HPLC analysis, the samples were selected by interestingly or widely edible vegetables, fruits, and herbal plants in Thailand. The standard markers to quantify in this study are chlorogenic, rosmarinic and caffeic acid which are hydroxycinnamic acid derivatives. Hydroxycinnamic acid derivatives, a subgroup of phenylpropanoids, are synthesized by shikimate pathway where the starter precursor molecules are phenylalanine and tyrosine. The results of HPLC analysis demonstrated that the distribution of these 3 phenolic compounds varied in many samples. Among 111 samples, 39.64% contained all of 3 compounds, 40.54% contained 2 compounds, 14.41% contained only 1 compound and 5.41% could not detect these 3 compounds. Rosmarinic acid was mostly found in Labiatae family, chlorogenic and caffeic acid were found in many families. *Lonicera japonica* flowering buds were found to be the richest source for chlorogenic acid content as 9.90 g/100 g of dried crude drug and *Melissa officinalis* leaves showed the most rosmarinic acid content as 19.91 g/100 g of dried crude drug. Moreover, the most caffeic acid content was found in *Coffea canephora* seeds as 1.23 g/100 g of dried crude drug. The analytical method validation is the process that confirms precise, accurate and reliable quantitative data. According to the ICH guideline: calibration range, specificity, accuracy, repeatability, intermediate precision, limit of detection, limit of quantitation and robustness should be validated for analytical analysis. The specificity was evaluated by peak identity and peak purity. The test is performed to demonstrate that the chromatographic peak does not contain multiple compounds. This test is based on absorbance spectrum which detected by diode array detectors. If all the individual spectra recorded during elution of a peak are identical even detected at any periods of a peak, the peak is considered pure [140]. An identical peak will result a peak purity index of 100% or peak purity index of 1.0, indicating that all spectra are similar [141]. The results showed peak purity index of

chlorogenic, rosmarinic and caffeic acids were more than 0.999, it could represent that no impurity detected in these peaks. The calibration curves of standard compounds were performed by 5 concentrations in the range of 16.67 - 83.33 µg/ml. The linearity showed good correlation ($R^2 \geq 0.999$). An analytical technique is acceptable which the correlation of method (R^2) value achieved is 0.99 or better. Furthermore, the greatest results is obtained when the concentration of the sample is within the concentration range performed [142]. The acceptable range of recovery is during 80 - 120% of the test concentration [88] and the criteria of repeatability and intermediate precision was not more than 15 %RSD [143]. Thus, the results indicated that this HPLC analysis was accurate and precise for quantification of 3 compounds in plant samples. The robustness should be evaluated during HPLC analysis and depends on the type of parameters under testing. It should demonstrate the reliability of an analysis with respect to deliberate variations in method parameters [88]. This study showed that there were no differences (%RSD <4) in the retention time and the peak area of 3 phenolic compounds in the robustness validation. The HPLC analysis in this study demonstrated the contents of 3 phenolic compounds in selected plants that could indicate the active constituents to pick up the interesting plants for further development of the herbal medicinal drug.

Currently, not only trend in natural products has become increasing but also the traditional medicine using crude drugs or herbal remedies to treat and alleviate the diseases, leading to the large supply of herbal medicinal consumption. Thus, the evaluations of their quality, efficacy and safety are important. World Health Organization has established assessment for the quality control of medicinal plants including the classification, plant identification, determination of active compound, and identification of contamination. Pharmacognosy is the study of the medicines obtained from natural source, especially from plants. The pharmacognosy researches have been evaluated in identity, purity and quality of the plant material. The main experiments in pharmacognostic study are macroscopic and microscopic characteristics, physico-chemical parameters, TLC fingerprint and active chemical compound [144]. Macroscopic and microscopic evaluations are the first step that simply and rapid

methods to establish the identification of plant materials [73]. This study demonstrated the macroscopic and microscopic characteristics of *L. japonica* flowering bud. Corolla part cross section of flowering bud presented the anatomical structure of glandular and non-glandular trichomes, epidermis and vascular bundle. The previous research observed transverse section buds under fluorescence microscope, also presented non-glandular trichomes and vascular bundles [145]. The main characteristics of powdered flower are pollen grains, inner wall cells of anther sac, non-glandular hairs and glandular hairs. Additionally, crystals of calcium oxalate, secretory tissue, pigment cells, and others could be identified [146]. The histological characteristics in this study presented corolla fragment, glandular and non-glandular trichomes, pollen grains, spiral vessel, parenchyma and vascular bundle, prism of calcium oxalate and petal parenchyma. The previous research also showed glandular and non-glandular hairs, pollen grains and cluster of calcium oxalate [145]. The physico-chemical parameters are an essential for qualification of crude drug. Extractive matters in specific solvents represent chemical compounds in crude drug. The ethanol and water soluble extractive values of *L. japonica* flowering bud should not be less than 16.5 and 28.9% by weight respectively. The results showed water soluble extractive yield higher than ethanol extractive yield, it indicated that more polar components were existed in this flowering bud. The loss on drying, total ash, acid-insoluble ash and water content should not be more than 10.1, 6.6, 1.1, and 10.8% by dry weight respectively. These parameter values were lower than previous study which total ash, acid-insoluble ash and water content were found to be 10.6, 1.2 and 17.5% by dry weight respectively [147]. Moreover, Chinese Pharmacopoeia established the standard criteria of total ash, acid-insoluble ash and water content in *L. japonica* flowering bud were not more than 10.0, 3.0 and 12.0% by dry weight respectively [148]. Total ash and acid-insoluble ash contents are composed of the non-volatile inorganic matters or minerals remaining after incineration of crude drugs. The ash study is helpful to determine the quality, purity, and to control adulteration or contamination of powdered crude drug. The total ash presents mineral compounds, for example carbonates, phosphates, oxides or silicates in plant tissues while acid-insoluble ash indicates some mineral compounds that cannot soluble in hydrochloric acid to form metal salts such as silicates [74, 149].

Loss on drying value is to determine any volatilized matter in the crude drug by oven-drying method. Water content determined by azeotropic distillation demonstrated only water that containing in the crude drug. The water content should be kept at a minimum in order to prevent chemical degradation as well as microbial growth during storage [74]. The volatile oil was undetected in this dried crude drug and the previous study showed the lowest volatile oil content at flowering bud stage [53]. Fingerprint analysis is effective tool for quality control of crude drug due to its simplicity and reliability. Thin layer chromatography is a method used to obtain a fingerprint profile to identify and authenticate compounds in herbal medicines [150]. The mobile phase consisted of ethyl acetate : methanol : water : formic acid (50 : 4 : 4 : 2.5) and silica gel GF_{254} TLC plate as stationary phase were suitable to separate chemical compounds and obtain TLC chromatogram capable to be chemical fingerprint in standardization of this crude drug.

For quantitative analysis, the percentage yield of exhausted ethanolic extract was 39.44 ± 5.83 g/100 g crude drug in average. The outstanding advantages of Soxhlet extraction include simple method, low cost of the basic equipment, and continuous process. In conversely, the disadvantage include long time required for extraction, no suitability for thermolabile compounds as long period boiling may lead to chemical degradation, and large amount of solvent extraction [151]. Chlorogenic acid content in *L. japonica* flowering bud was performed by TLC-densitometry compared to TLC-image analysis by imageJ software. TLC-densitometry is a reliable method that measures the difference in absorbance or fluorescence signal between a compound band and surrounding plate background. Whereas, TLC-image analysis uses a CCD camera to capture the image of TLC chromatogram and interprets the intensity of color of compound band and contrast background to chromatographic peak by ImageJ software. This study demonstrated that chlorogenic contents in flowering bud by TLC-densitometry compared to TLC-image analysis were found to be 2.24 ± 0.50 and 2.09 ± 0.44 g/100 g respectively. The previous study reported that chlorogenic acid content in crude drug was 2.62 - 3.66% by dry weight [152]. In addition, according to Chinese Pharmacopoeia, the content of chlorogenic acid in flowering bud should not

be less than 1.5% [148]. The variations of chemical content may depend on environmental conditions such as geography, temperature, type of soil and etc. [153]. The validation of these quantitative methods were performed according to ICH guideline. The calibration range of chlorogenic acid was polynomial ranged from 0.6 - 3.0 µg/spot. Although the calibration curves of chlorogenic acid by both methods were polynomial, the coefficient of determination were shown to be more than 0.999. The specificity was shown by comparing UV spectrum of the peak apex among all samples and standard chlorogenic acid for peak identity, and the comparison of UV spectrum recorded at up-slope, apex and down-slope of the peak for peak purity. The spectra showed the maximum absorbance of chlorogenic acid at the wavelength of 325 nm which in accordance with the previous study that densitometric analysis of chlorogenic acid could be detected at 330 nm [152]. The recovery was determined to evaluate the accuracy of the method by spiking known three concentrations of standard chlorogenic acid in a sample extract. The percent recovery values of TLC-densitometry and TLC-image analysis were 94.10 ± 4.09 and 97.13 ± 11.42 respectively. The results were accepted in range of 80 - 120% [88], thus these methods were accurate. The repeatability and the intermediate precision were determined on the same day and in three different days. The repeatability and the intermediate precision of TLC-densitometry and TLC-image analysis were 1.17, 3.73 and 9.66, 10.27 %RSD respectively, that were not more than 15 %RSD of standard criteria [143]. The LOD and LOQ of TLC-densitometry and TLC-image analysis were calculated by the residual standard deviation of a regression line and were found to be 0.07, 0.21 and 0.03, 0.09 µg/spot respectively. The robustness of TLC-densitometry and TLC-image analysis performed by varying the mobile phase ratio showed the values of 8.59 and 9.17 %RSD of sample peak area, and 2.41 and 10.67 %RSD of standard chlorogenic acid peak area. The validation results of TLC-densitometry were close to previous study which reported that the accuracy, repeatability, intermediate precision, LOD and LOQ were 99.66-101.59 %recovery, 1.01-1.32 %RSD, 3.21 %RSD, 0.12 µg/spot and 0.20 µg/spot respectively [152]. Thus, these methods were suitable, reliable and efficient to evaluate the quantitative analysis of chlorogenic acid content in *L. japonica* flowering bud.

Brine shrimp lethality assay has been used as an indicator for preliminary cytotoxicity. The advantages of this assay are simple, rapid and inexpensive. Tested sample was classified as toxic if LC_{50} value <1000 µg/ml and non toxic if LC_{50} ≥1000 µg/ml [93]. It was found that caffeic acid demonstrated higher toxicity against brine shrimp nauplii than chlorogenic and rosmarinic acids (LC_{50} = 231.82, 266.05 and 289.66 µg/ml respectively), while the flowering bud ethanolic extract exhibited non toxicity. The previous study also reported the toxicity of chlorogenic acid against brine shrimp nauplii with LC_{50} of 300 µg/ml [154].

MTT cell viability assay is the traditional method for anti-cancer drug recovery and also used to investigate the proliferation and cytotoxicity of medicinal agents based on the mitochondrial dehydrogenase enzymes activity in cells [155]. U.S. National Cancer Institute (NCI) establishes the criteria of cytotoxicity that plant extract and pure compound, with IC_{50} value <20 µg/ml and <4 µg/ml respectively, are considered to have cytotoxic activity [96, 97]. All tested samples demonstrated cell viable inhibition potential. However, as cytotoxic standard criteria, they were classified as no cytotoxicity. Park *et al*. reported that the extract of *L. japonica* did not significantly changed WI-38 lung-derived cell line viability [156]. Moreover, caffeic acid also showed no cytotoxic activity to HepG2 cells (IC_{50} = 781.8 µg/ml) [157].

Comet assay is a rapid standard method to observe DNA damage in eukaryotic cells based on quantification of denatured DNA fragments migrating out of the cell nucleus during electrophoresis [99]. Flowering bud ethanolic extract and 3 standard compounds showed DNA damage potential with a dose-dependent relationship between the degree of DNA damage and concentration of the sample. Estefanía *et al*. reported high level of DNA damage of K562 leukemia cells after exposure to chlorogenic acid (177 - 1,771 µg/ml) for 24 hours [158]. Devipriya *et al*. found that caffeic acid (10 µg/ml) demonstrated low DNA damage on human lymphocytes and no significant increase of comet formation compared to 0.2% DMSO [159]. Those results were related to present study that chlorogenic acid was high DNA damage potential and the lowest concentration of caffeic acid demonstrated less DNA damage closely to negative controls.

The antimicrobial agents derived from medicinal plants become increasingly interested. The ideal performances of antimicrobial activity assay should be inexpensive, simple, rapid, reproducible and maximize sample throughput in order to screen with a various number of plant extracts [160]. Agar well diffusion assay showed antibacterial potential of chlorogenic, rosmarinic and caffeic acids. However, MIC were found to be >4,000 µg/ml representing low inhibitory potential against tested microorganisms. Zaixiang *et al*. reported MIC of chlorogenic acid by agar dilution method against *Bacillus subtilis* (ATCC 9372), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922) and *Salmonella Typhimurium* (ATCC 50013) as 40, 40, 80 and 40 µg/ml respectively, that seemed to be active potential to antimicrobial activity [161]. Fu *et al*. demonstrated MIC and MBC of *L. japonica* flowering bud 50% ethanolic extract against *E. coli* (ATCC 25922) of 500 and >1000 mg/ml respectively [162]. Moreover, Abedini *et al*. showed MIC and MBC of rosmarinic acid that isolated from *Hyptis atrorubens* stem against *Staphylococcus epidermidis* 5001 of 0.3 and 0.3 mg/ml respectively and against *Pseudomonas aeruginosa* (ATCC 27583) of 2.5 and >2.5 mg/ml respectively [163].

An antioxidant is substances that can inhibit or delay oxidative damage to a target molecule. Its ability to trap free radicals is the main character of the antioxidant [164]. Antioxidative activity assays revealed DPPH and nitric oxide scavenging potentials as well as promising reducing power of flowering bud ethanolic extract and tested polyphenolic compounds. However, beta-carotene bleaching inhibitory activity was possessed only from the polyphenols. Phenolic derivatives are very important secondary metabolites in plant because of their scavenging ability due to their hydroxyl groups [165].

DPPH is stable free-radical molecules with violet color due to the delocalization of its electrons, the antioxidant compounds can donate a hydrogen atom to DPPH to form DPPH:H with yellow color. The antioxidant potential by DPPH radical scavenging test demonstrated that caffeic acid was most potent followed by quercetin, rosmarinic acid, chlorogenic acid, BHT and flowering bud ethanolic extract. This result related to the previous study which demonstrated that IC_{50} of chlorogenic

acid and quercetin were found to be 10.59 and 3.82 μg/ml respectively [166]. Caroline *et al*. reported that IC_{50} of caffeic acid was found to be 2.39 μg/ml which related to this study [157]. Moreover, dried flowering bud extracted with 75% ethanol showed DPPH scavenging activity with IC_{50} of 56.8 ± 0.5 μg/ml that also related to this study [167].

FRAP assay is the assay to measure the power of antioxidant on reducing ferric iron and 2, 4, 6-tripyridyl-s-triazine (TPTZ) to ferrous tripyridyltriazine (Fe(II)-TPTZ). Reducing power activity of flowering bud ethanolic extract and 3 standard compounds were revealed. Sha *et al*. demonstrated FRAP value of flowering bud aqueous extract as 345.26 ± 3.18 μmol $FeSO_4$/g dry weight which was less than in this study [168], thus the ethanolic extract of flowering bud demonstrated more reducing power potential than aqueous extract. Piazzon *et al*. studied the antioxidant's reducing capacity and the results related to this study that caffeic acid showed more reducing power potential than chlorogenic acid [169].

The nitric oxide plays an important role in various inflammatory processes that toxicity of nitric oxide increases greatly when it reacts with superoxide radical. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The antioxidant substance inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide [170]. Chlorogenic acid inhibited nitrite radical more than quercetin and rosmarinic acid while caffeic acids inhibited the nitrite radical less than quercetin. Flowering bud ethanolic extract showed 49.86% inhibitory potential. Chen *et al*. studied the methanolic extract of bud and flower and demonstrated their inhibitory effects on nitric oxide induced by LPS in mouse macrophages RAW 264.7 (IC_{50} = 125.45 μg/ml) [171]. Moreover, chlorogenic acid which isolated from flowers and buds of *L. japonica* could suppressed the nitric oxide induction in IL-1β-stimulated hepatocytes (IC_{50} = 231 μg/ml) [30].

Beta-carotene bleaching assay measures the ability of an antioxidant to inhibit lipid peroxidation which produced by linoleic acid. Chlorogenic, rosmarinic and caffeic acids had lower antioxidant potential than positive controls. Rosmarinic acid showed highest antioxidant activity whereas flowering bud ethanolic extract showed slightly

bleaching inhibitory activity. By this method, the polar compounds were considered as weak antioxidants because the polar compounds remained in the aqueous phase of the emulsion and were thus less effective in protecting the linoleic acid in lipid phase [172].

Alpha-glucosidase is the enzyme that digests maltose and dextrins into glucose at luminal surface of the small intestine and promoted the leading of blood glucose level. Acarbose, voglibose, and miglitol are the alpha-glucosidase inhibitors that used in clinical treatment as oral antihyperglycemic drugs [173]. In this *in vitro* yeast alpha-glucosidase inhibition study, rosmarinic acid inhibited enzyme activity more than acarbose while caffeic acids and flowering bud ethanolic extract inhibited enzyme activity less than acarbose. Chlorogenic acid showed the lowest potential to inhibit this enzyme activity. This result related to the previous study which demonstrated that IC_{50} of acarbose was found to be 11.93 mg/ml [174]. Oboh *et al*. studied yeast alpha-glucosidase inhibitory activity and the results related to this study that caffeic acid showed more effective activity to inhibit enzyme than chlorogenic acid (IC_{50} = 4.98 and 9.24 µg/mL) [175]. Kubínová *et. al*. showed that rosmarinic acid isolated from *Plectranthus madagascariensis* methanolic extract exhibited inhibitory activity against alpha-glucosidase (IC_{50} = 92 mg/ml) [176].

In conclusion, this present study revealed the content of chlorogenic, rosmarinic and caffeic acids in 111 selected Thai medicinal plants that performed by high performance liquid chromatography equipped with diode array detection. Moreover, pharmacognostic specification and chlorogenic acid content of *L. japonica* flowering bud in Thailand were established. The simple TLC combined with image analysis software could be used for quantification of chlorogenic acid in this crude drug. In addition, the *in vitro* biological activities of flowering bud ethanolic extract and its hydroxycinnamic acid constituents were demonstrated.

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APPENDICES

APPENDIX A

HPLC chromatogram of 111 plant sample ethanolic extracts

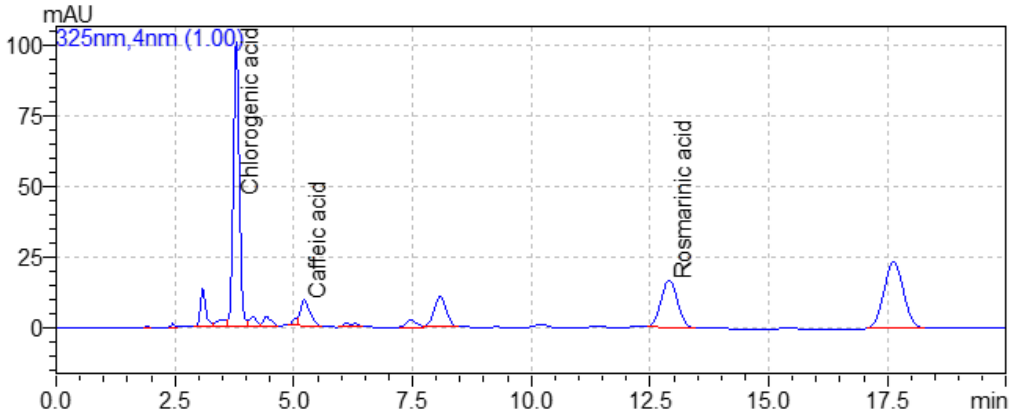

Figure 29 HPLC chromatogram of *Andrographis paniculata* leaf extract

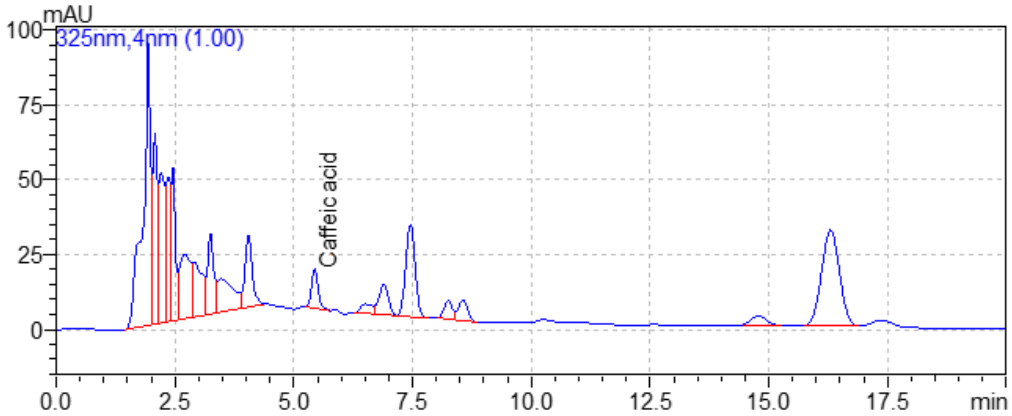

Figure 30 HPLC chromatogram of *Allium sativum* bulb extract

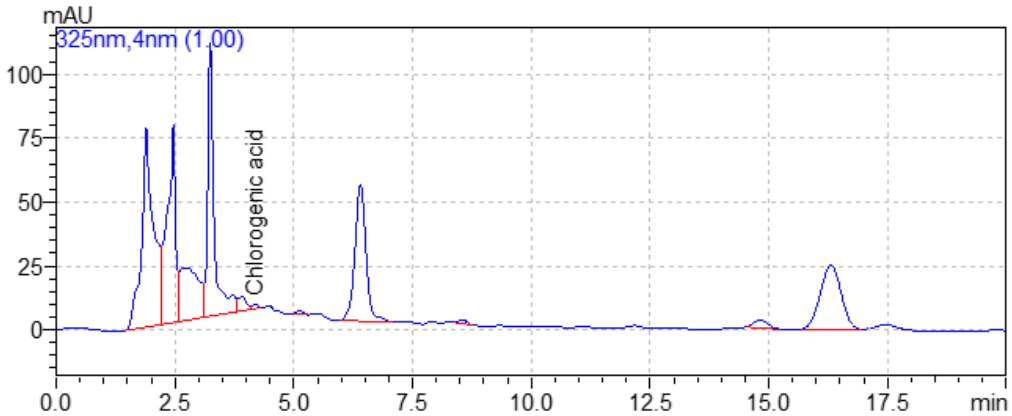

Figure 31 HPLC chromatogram of *Allium cepa* bulb extract

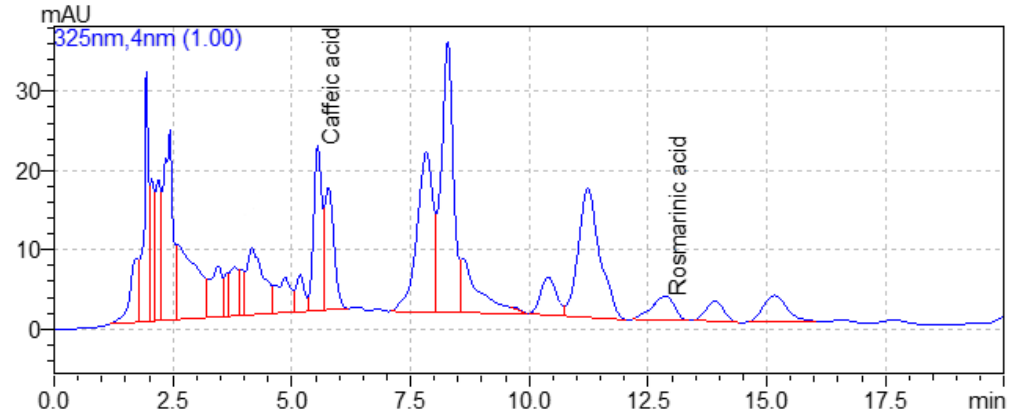

Figure 32 HPLC chromatogram of *Spinacia oleracea* leaf extract

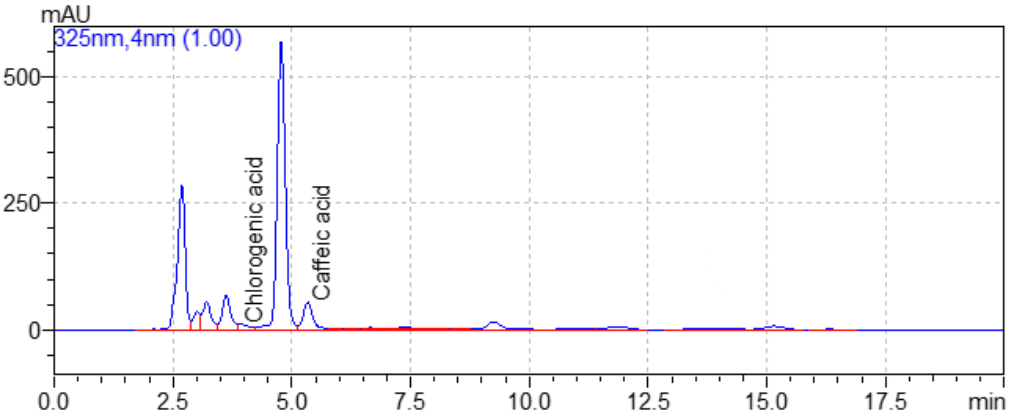

Figure 33 HPLC chromatogram of *Mangifera indica* leaf extract

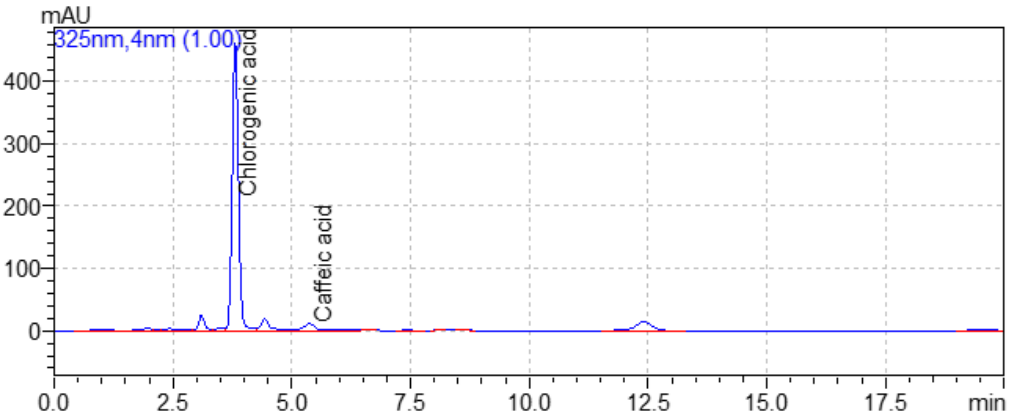

Figure 34 HPLC chromatogram of *Anethum graveolens* aerial part extract

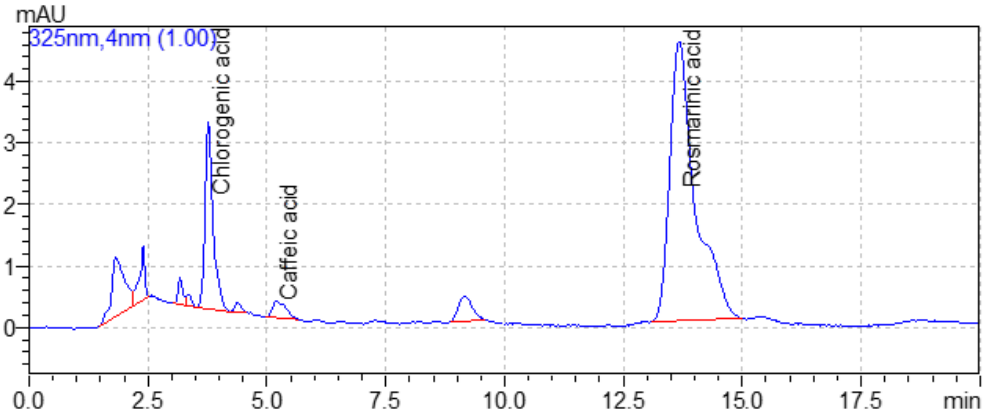

Figure 35 HPLC chromatogram of *Apium graveolens* aerial part extract

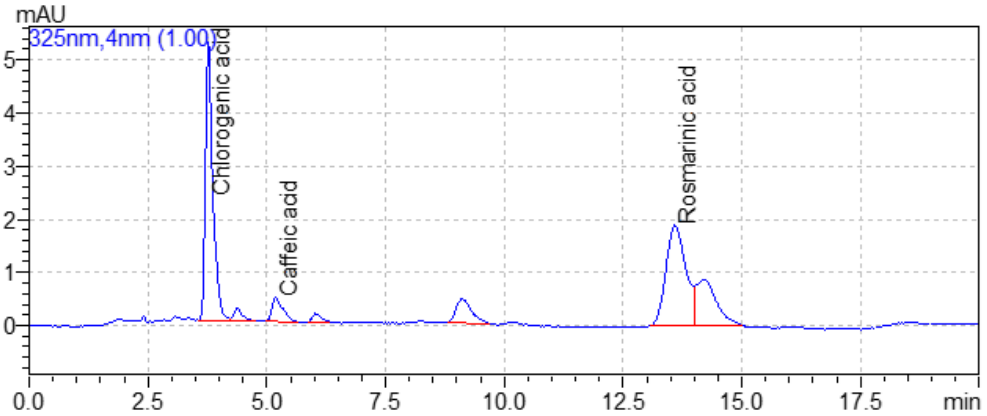

Figure 36 HPLC chromatogram of *Apium graveolens var. secalinum* aerial part extract

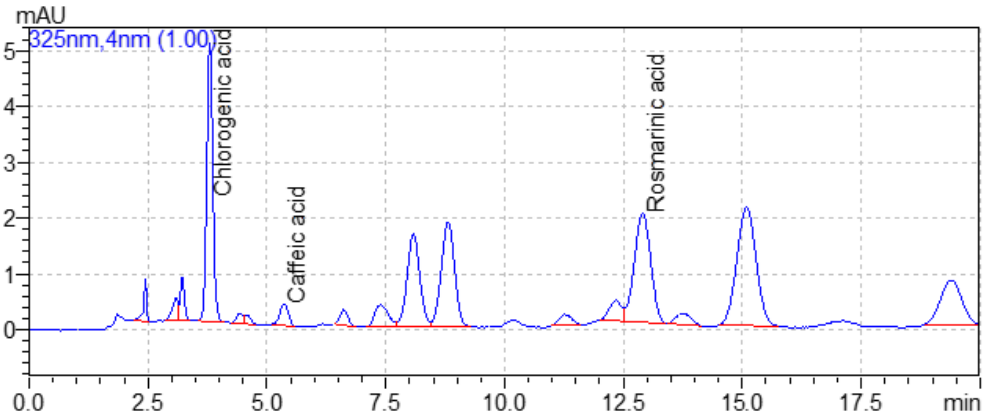

Figure 37 HPLC chromatogram of *Centella asiatica* aerial part extract

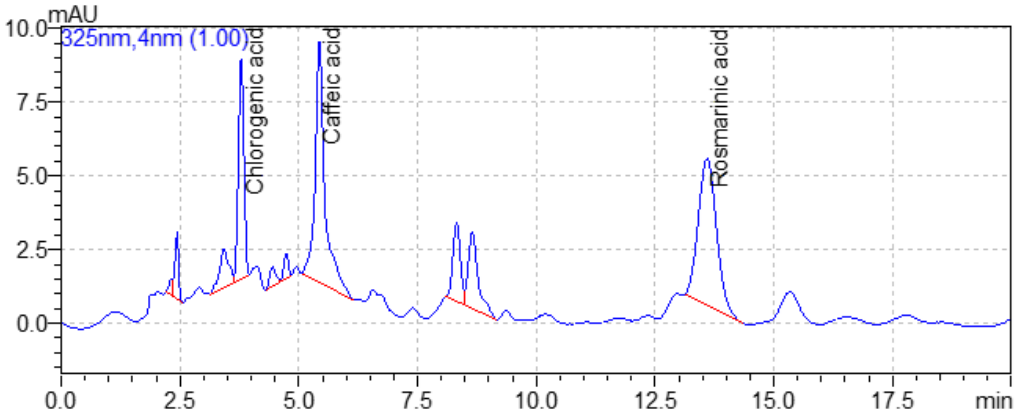

Figure 38 HPLC chromatogram of *Coriandrum sativum* seed extract

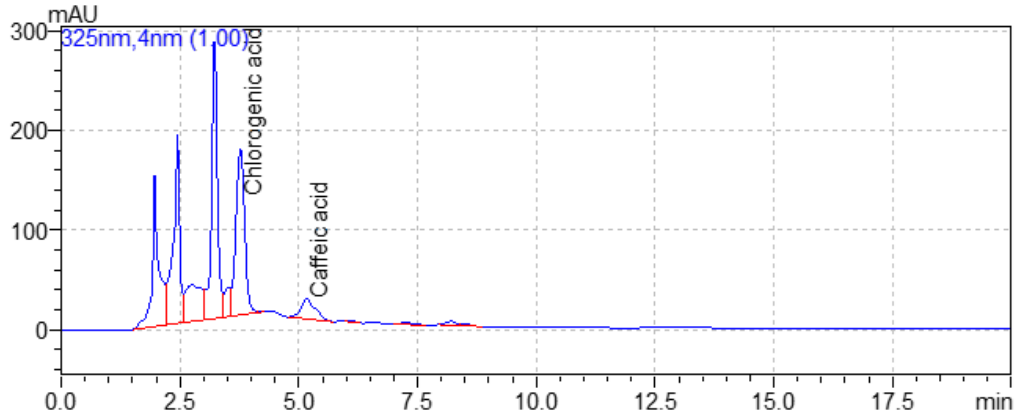

Figure 39 HPLC chromatogram of *Daucus carota* root extract

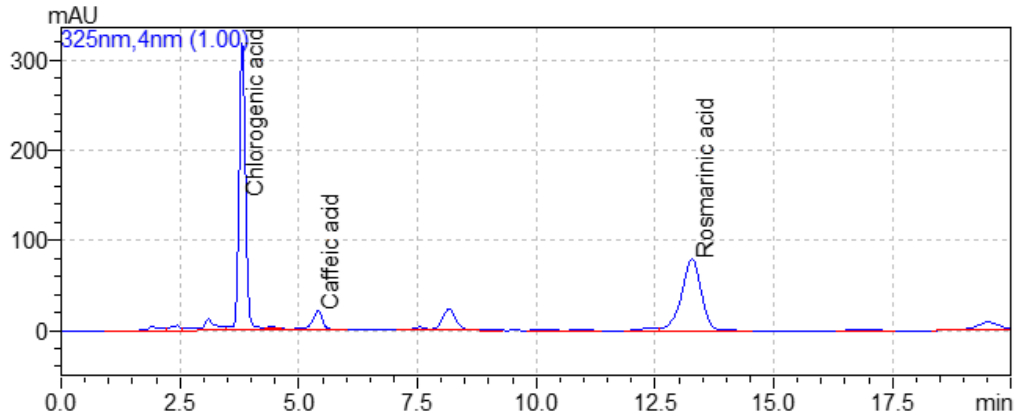

Figure 40 HPLC chromatogram of *Eryngium foetidum* leaf extract

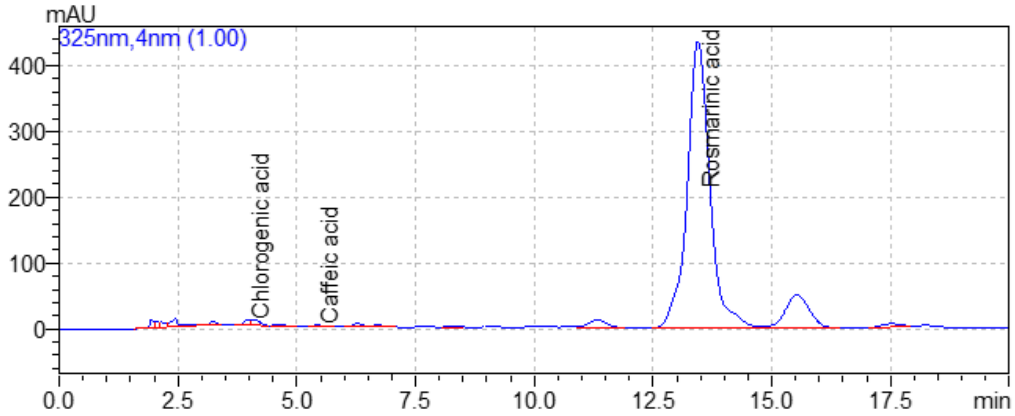

Figure 41 HPLC chromatogram of *Petroselinum crispum* aerial part extract

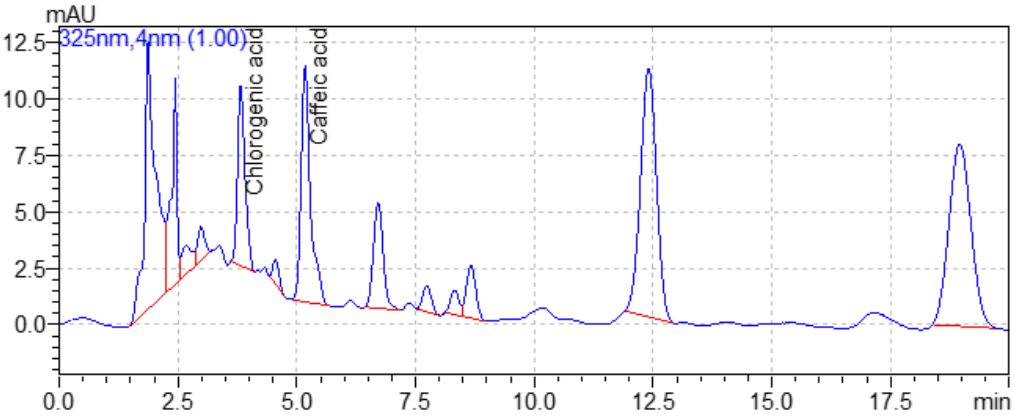

Figure 42 HPLC chromatogram of *Telosma cordata* flower extract

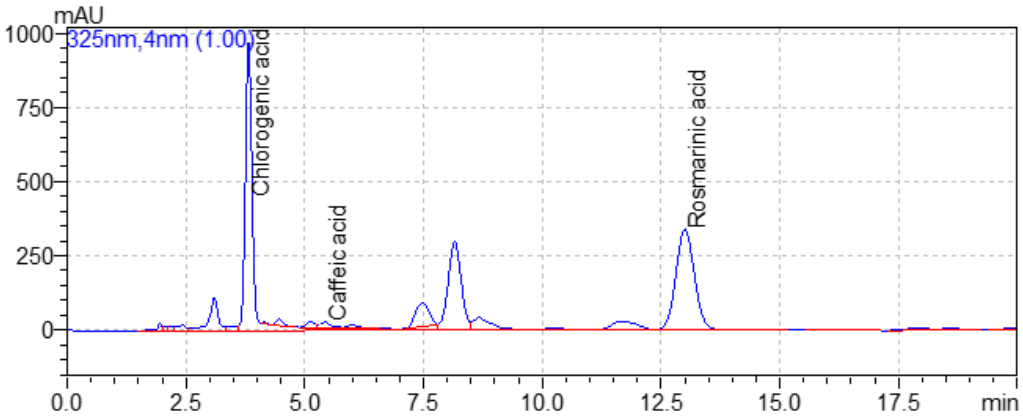

Figure 43 HPLC chromatogram of *Artemisia dracunculus* aerial part extract

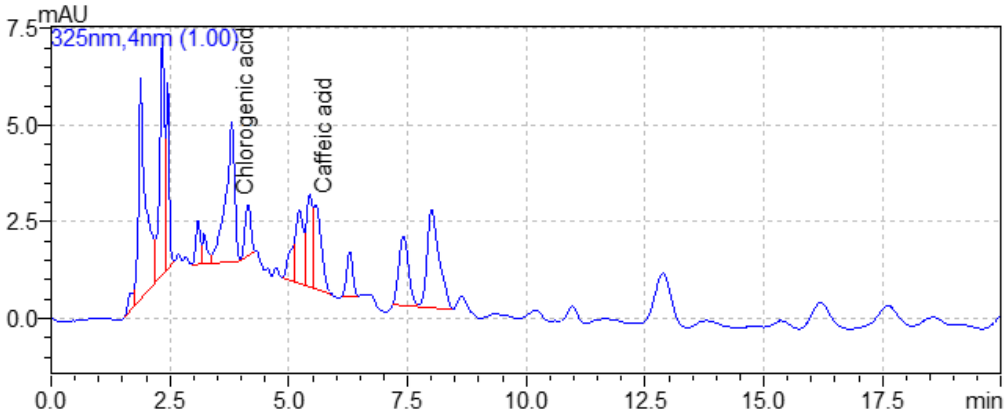

Figure 44 HPLC chromatogram of *Artemisia pallens* aerial part extract

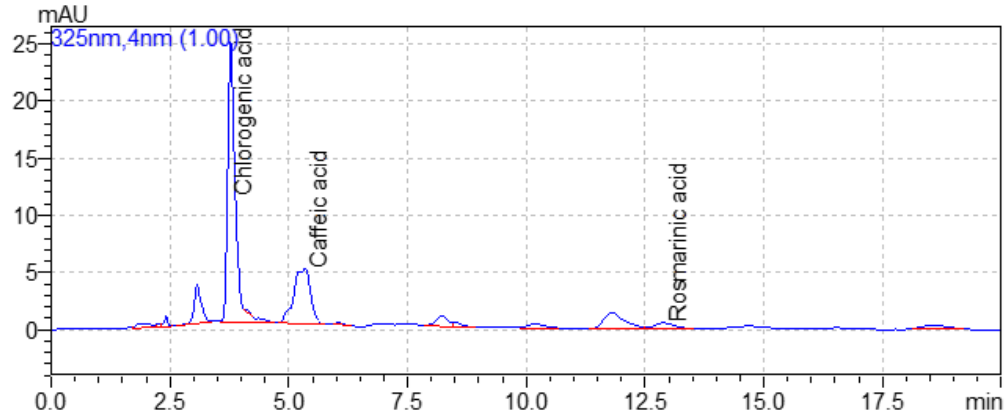

Figure 45 HPLC chromatogram of *Chromolaena odorata* leaf extract

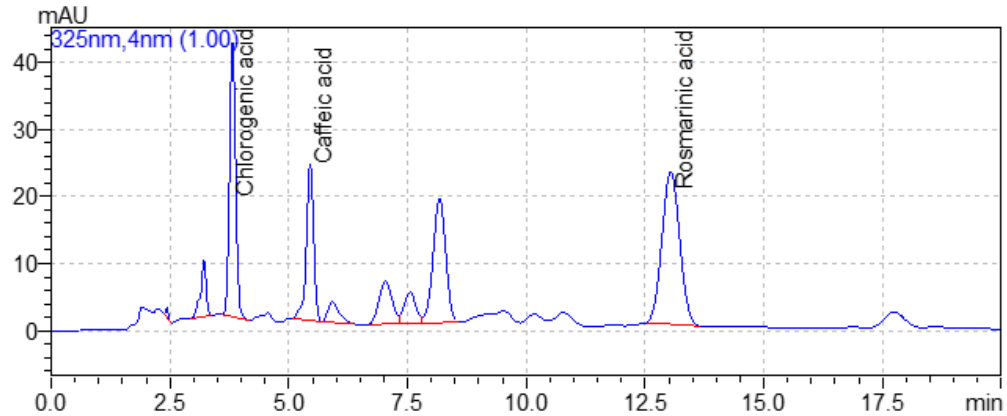

Figure 46 HPLC chromatogram of *Gnaphalium polycaulon* aerial part extract

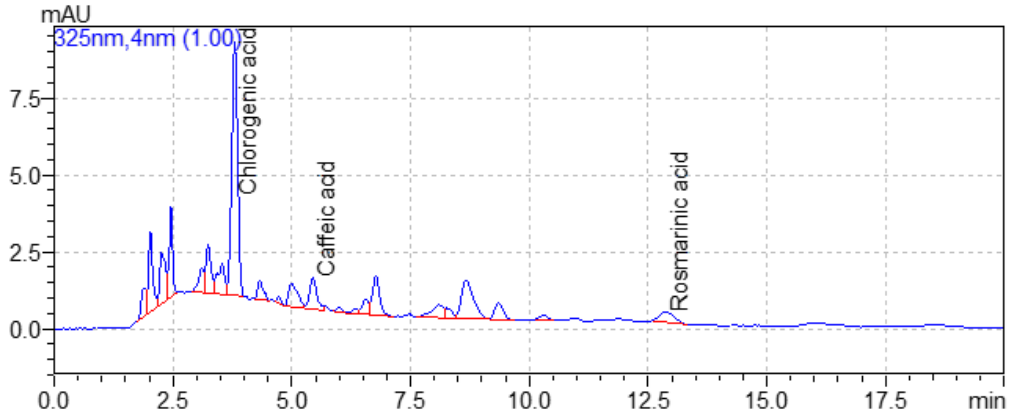

Figure 47 HPLC chromatogram of *Helianthus annuus* pericarp extract

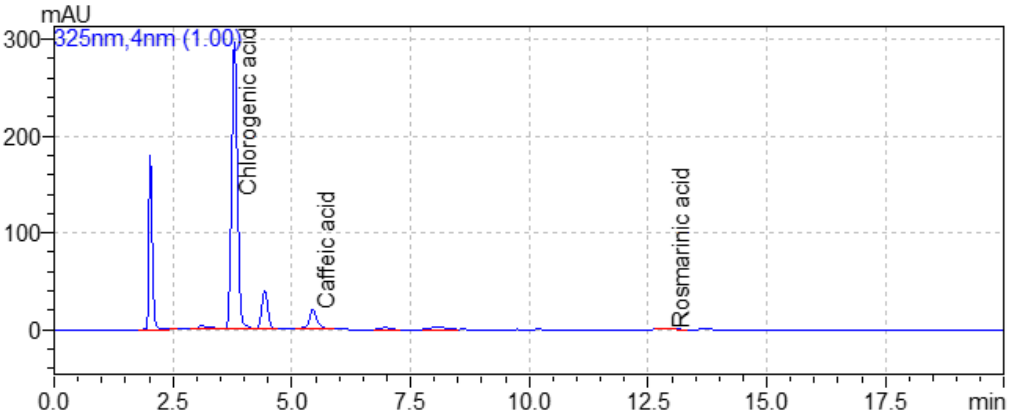

Figure 48 HPLC chromatogram of *Helianthus annuus* seed extract

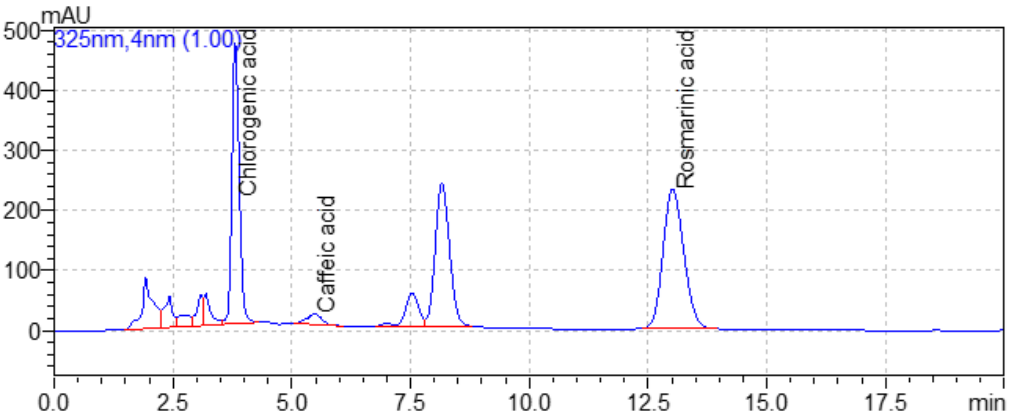

Figure 49 HPLC chromatogram of *Helianthus annuus* sprout extract

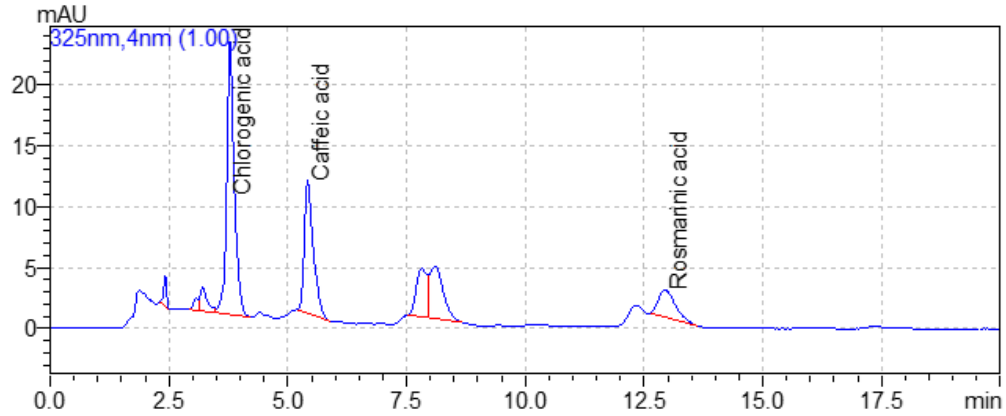

Figure 50 HPLC chromatogram of *Lactuca sativa* leaf extract

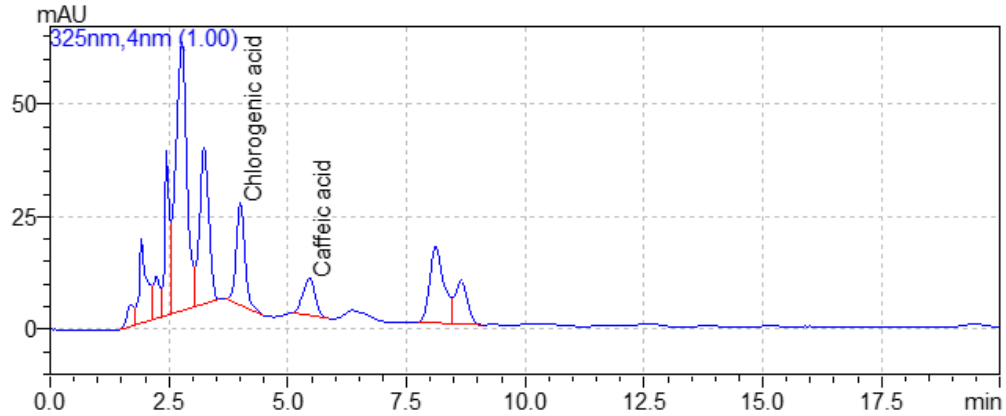

Figure 51 HPLC chromatogram of *Brassica juncea* leaf extract

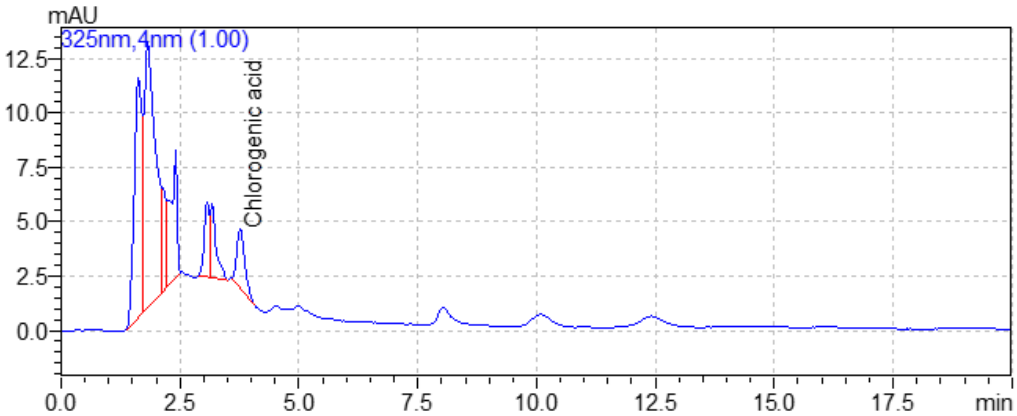

Figure 52 HPLC chromatogram of *Brassica oleracea* aerial part extract

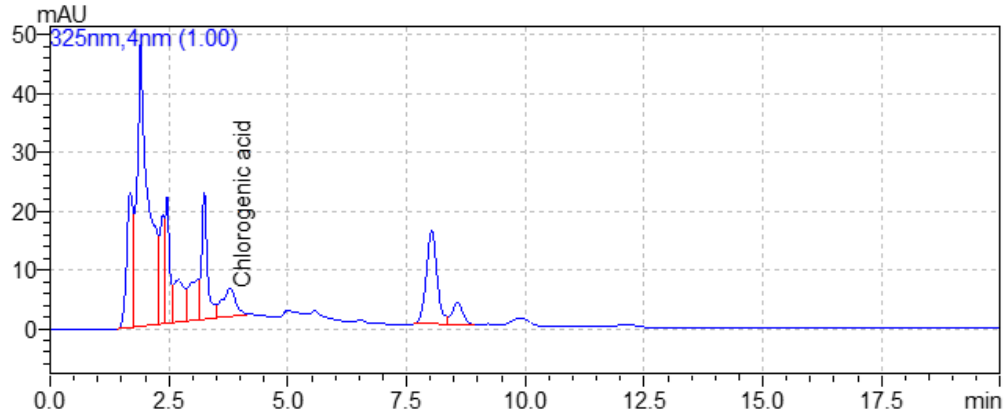

Figure 53 HPLC chromatogram of *Brassica rapa* aerial part extract

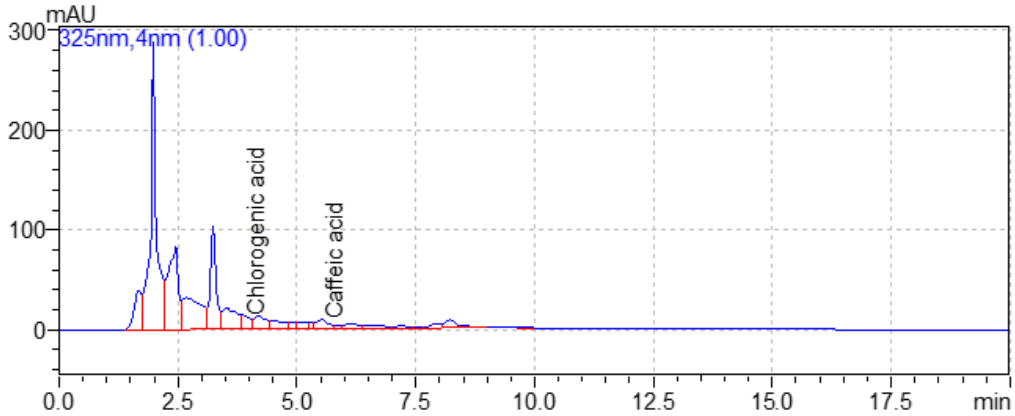

Figure 54 HPLC chromatogram of *Raphanus sativus* root extract

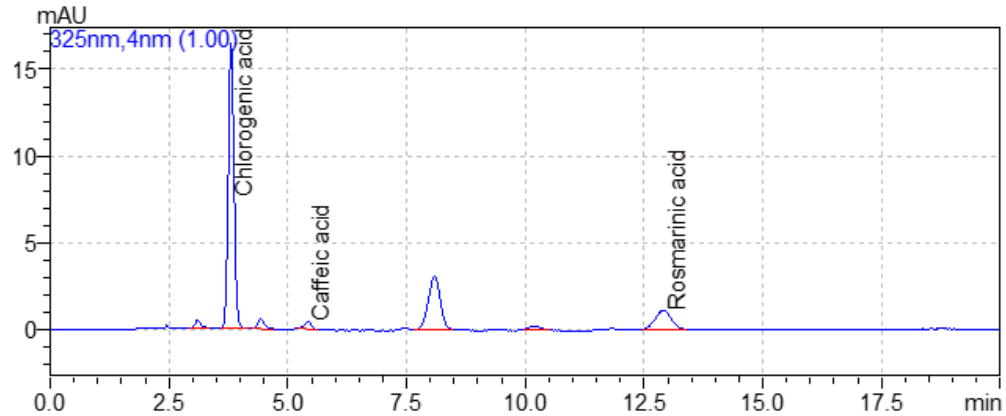

Figure 55 HPLC chromatogram of *Lonicera japonica* flowering bud extract

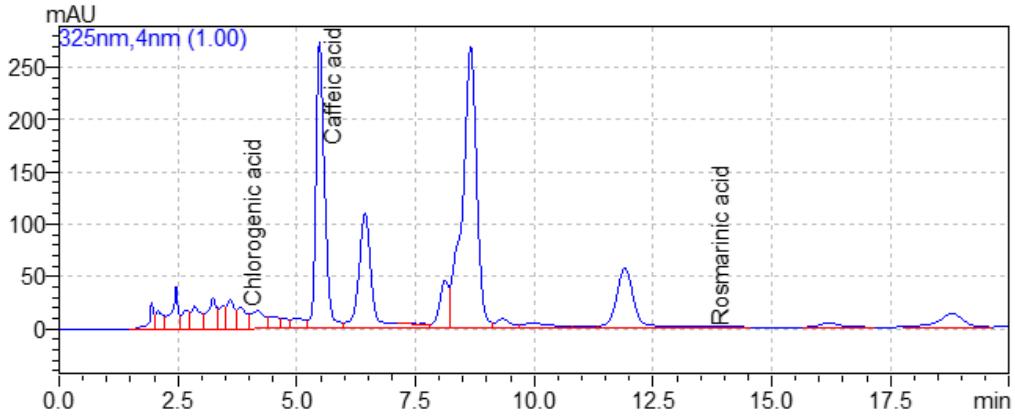

Figure 56 HPLC chromatogram of *Carica papaya* leaf extract

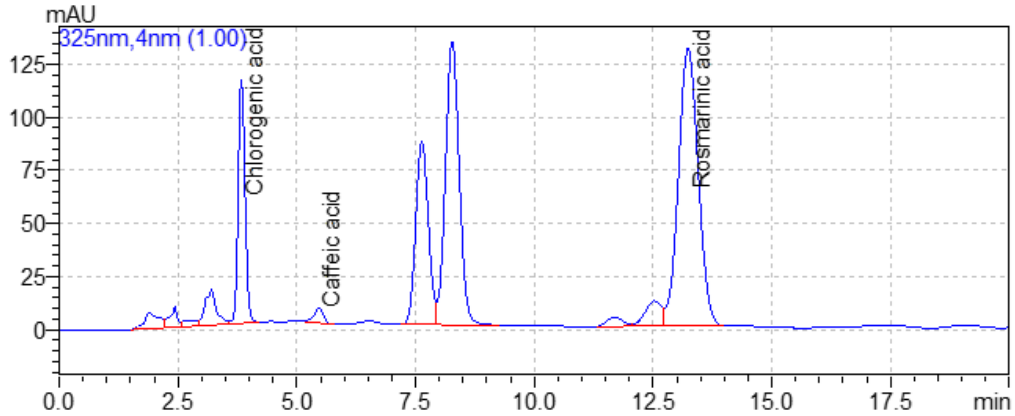

Figure 57 HPLC chromatogram of *Ipomoea aquatica* aerial part extract

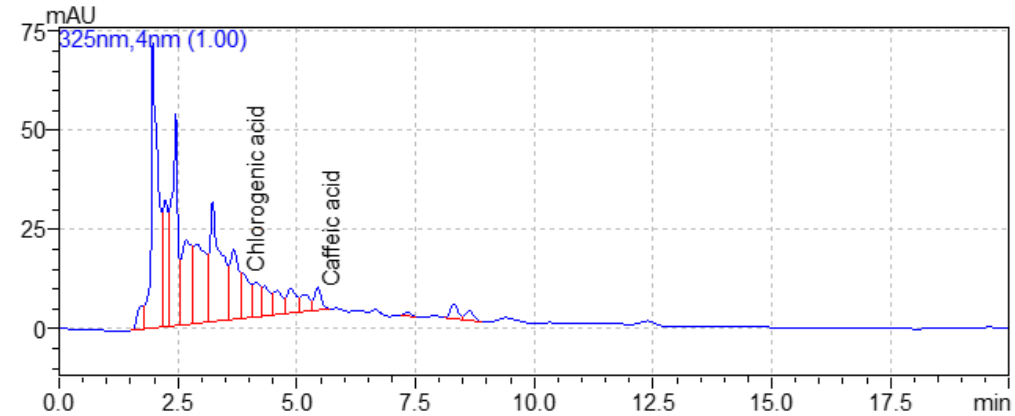

Figure 58 HPLC chromatogram of *Momordica charantia* (Thai varieties) fruit extract

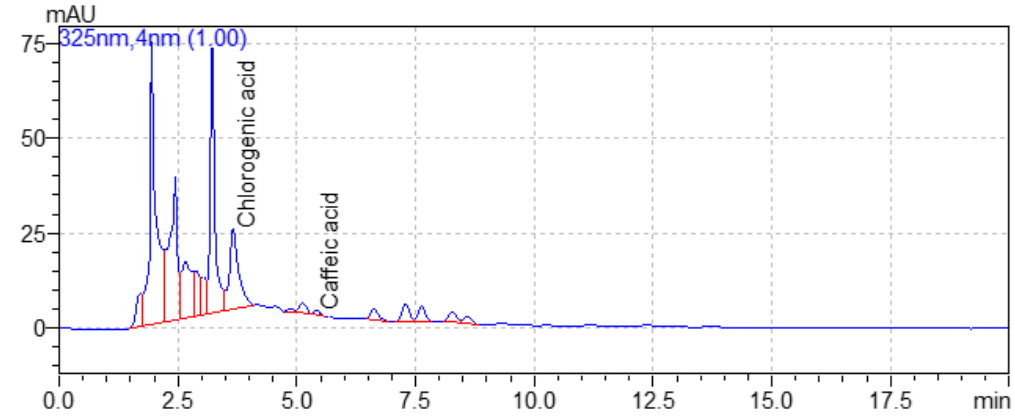

Figure 59 HPLC chromatogram of *Momordica charantia* (Chinese varieties) fruit extract

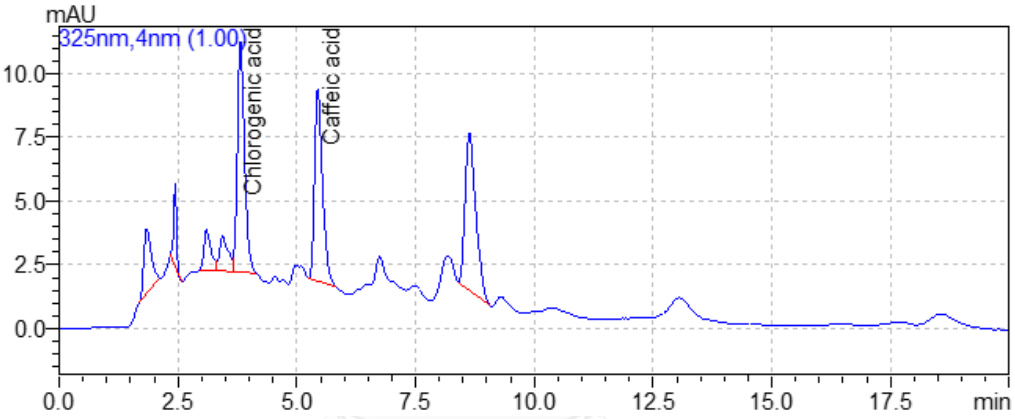

Figure 60 HPLC chromatogram of *Eucommia ulmoides* stem bark extract

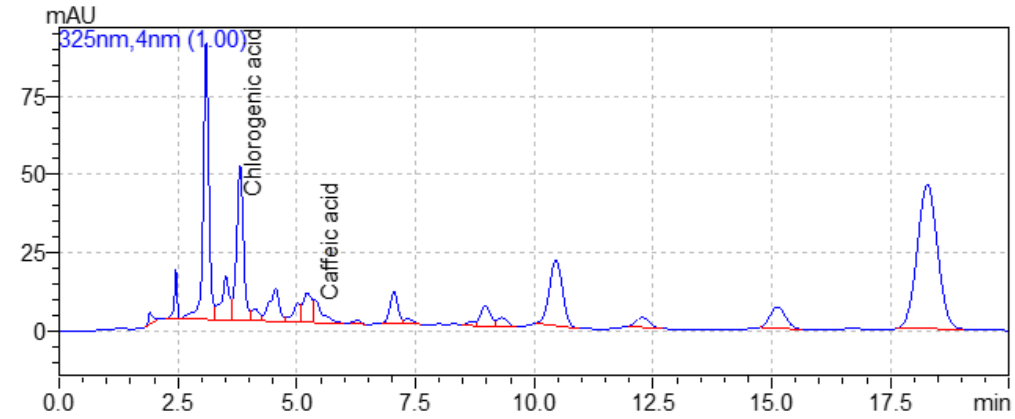

Figure 61 HPLC chromatogram of *Euphorbia hirta* aerial part extract

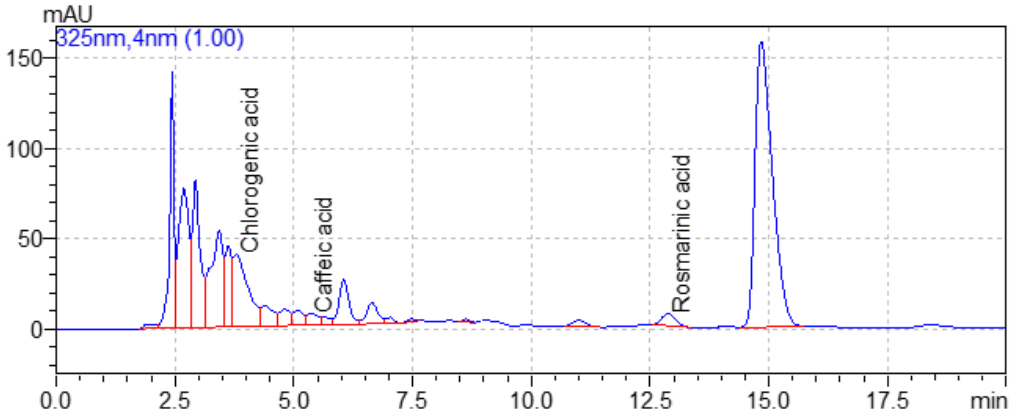

Figure 62 HPLC chromatogram of *Phyllanthus emblica* fruit extract

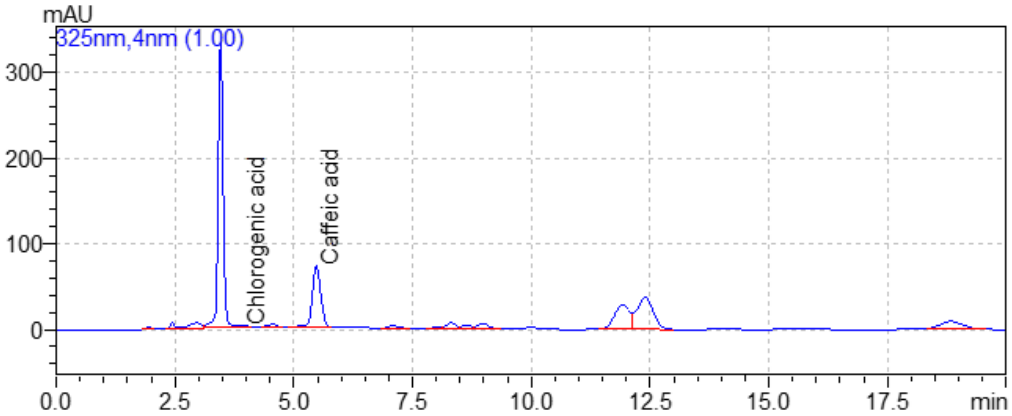

Figure 63 HPLC chromatogram of *Ricinus communis* leaf extract

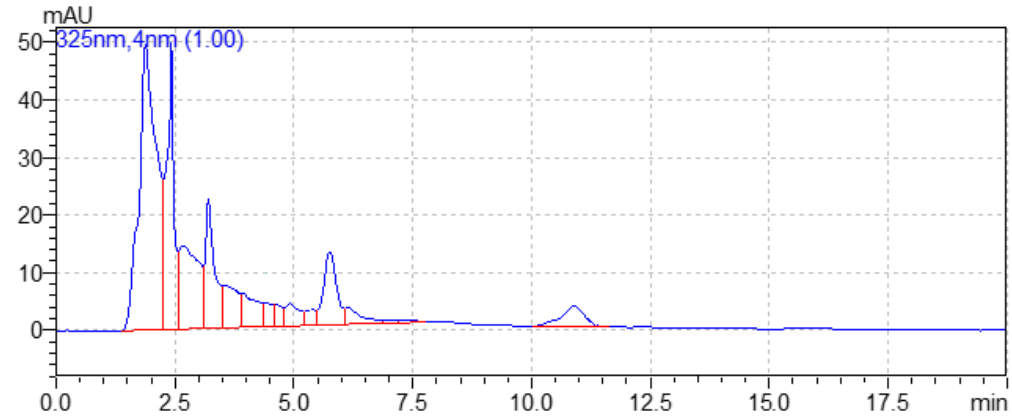

Figure 64 HPLC chromatogram of *Pisum sativum* fruit extract

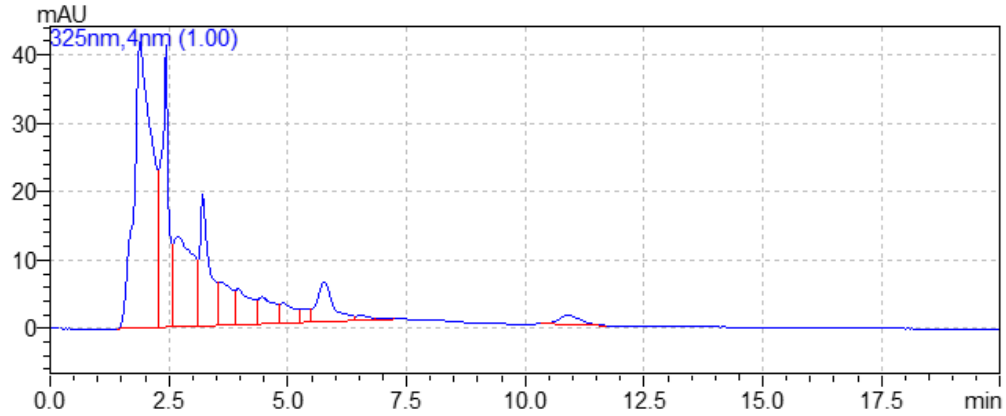

Figure 65 HPLC chromatogram of *Pisum sativum* var. *macrocarpon* fruit extract

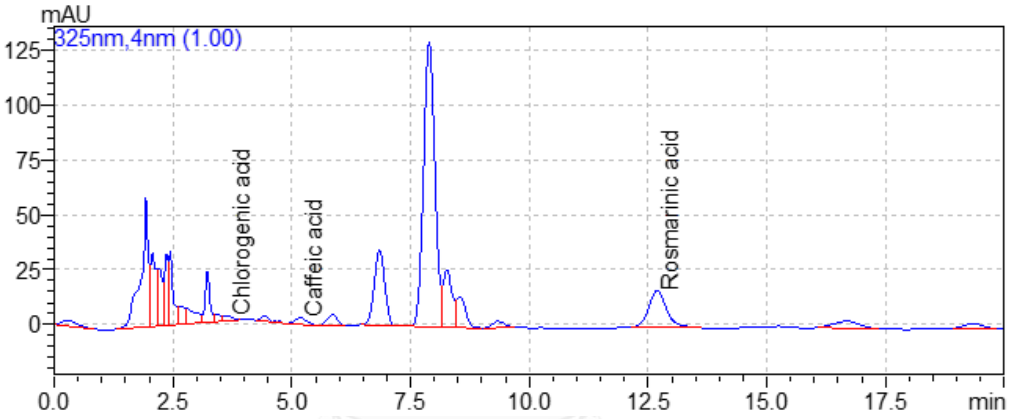

Figure 66 HPLC chromatogram of *Sesbania grandiflora* flower extract

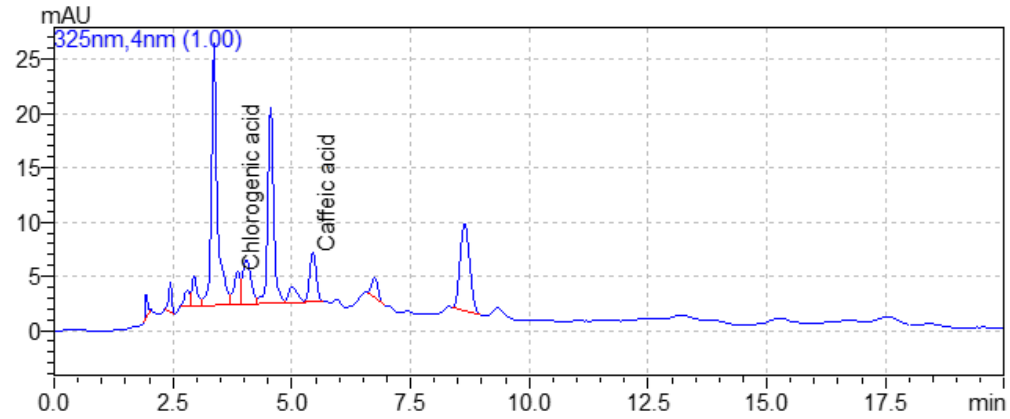

Figure 67 HPLC chromatogram of *Sesbania grandiflora* stem bark extract

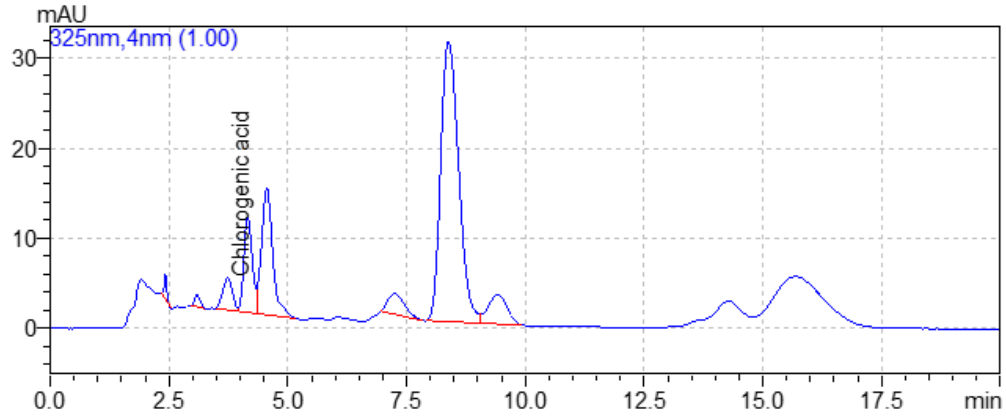

Figure 68 HPLC chromatogram of *Gnetum gnemon* leaf extract

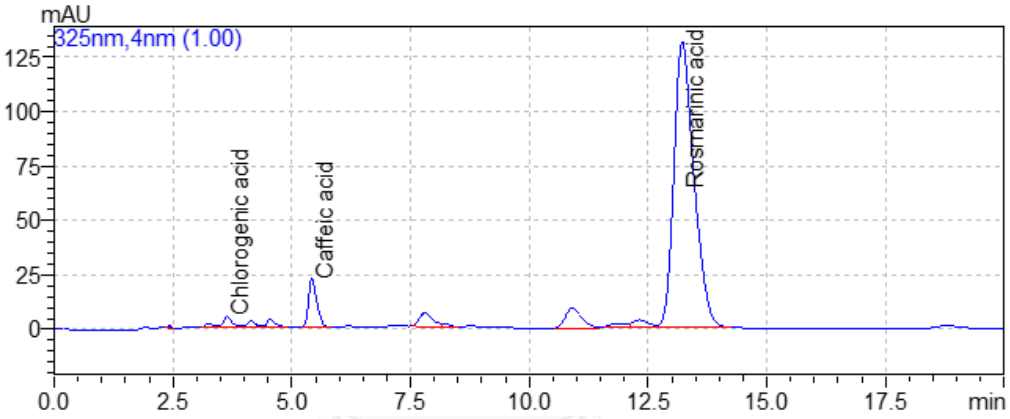

Figure 69 HPLC chromatogram of *Hyptis suaveolens* aerial part extract

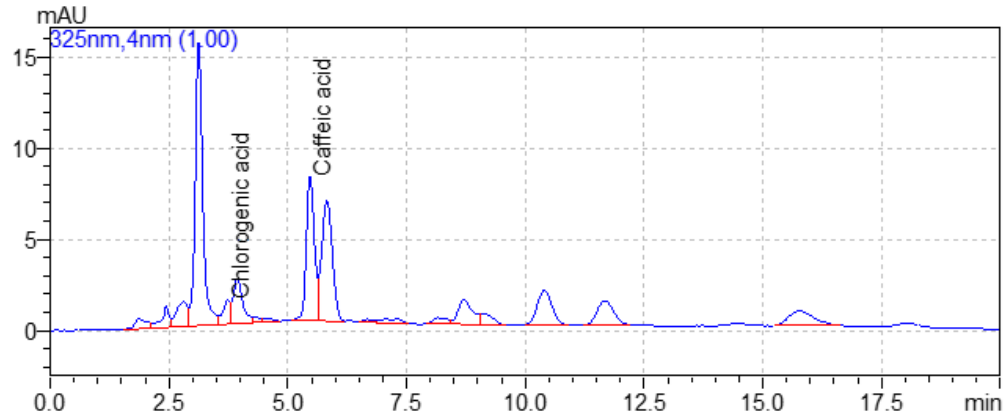

Figure 70 HPLC chromatogram of *Leonotis nepetifolia* leaf extract

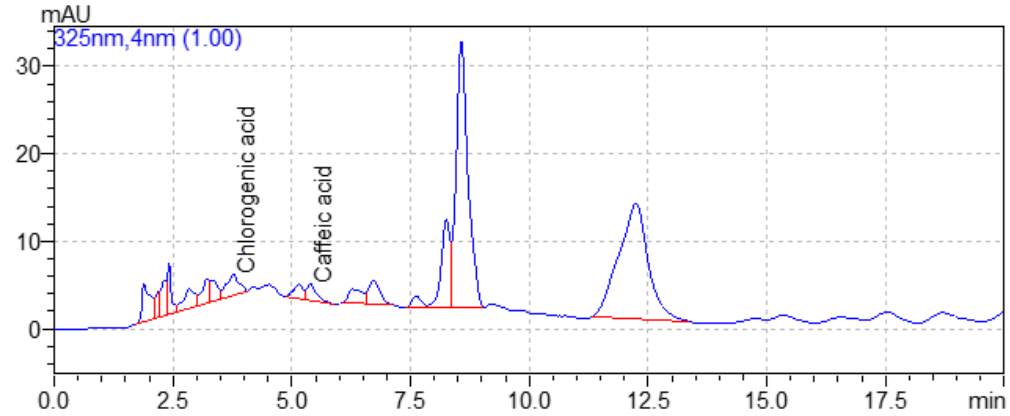

Figure 71 HPLC chromatogram of *Leonurus sibiricus* aerial part extract

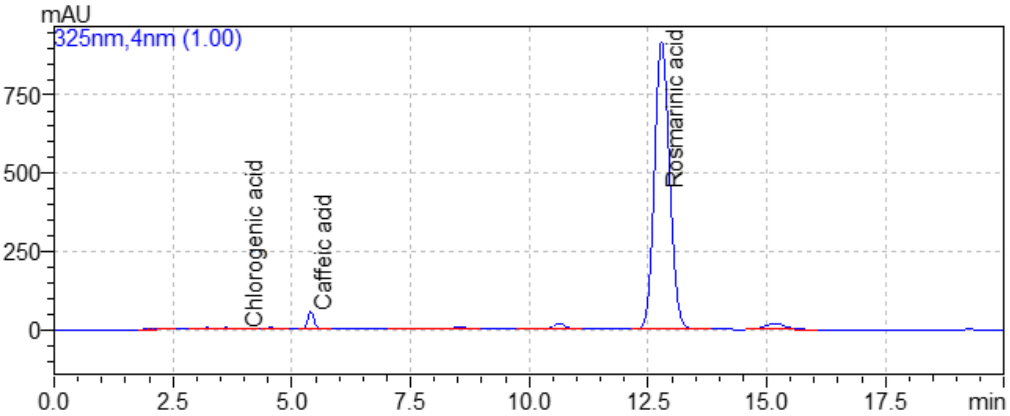

Figure 72 HPLC chromatogram of *Melissa officinalis* leaf extract

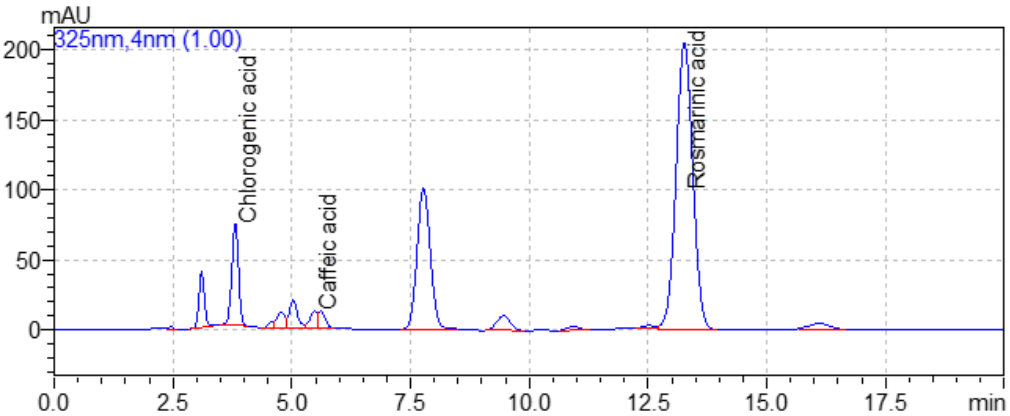

Figure 73 HPLC chromatogram of *Mentha arvensis* leaf extract

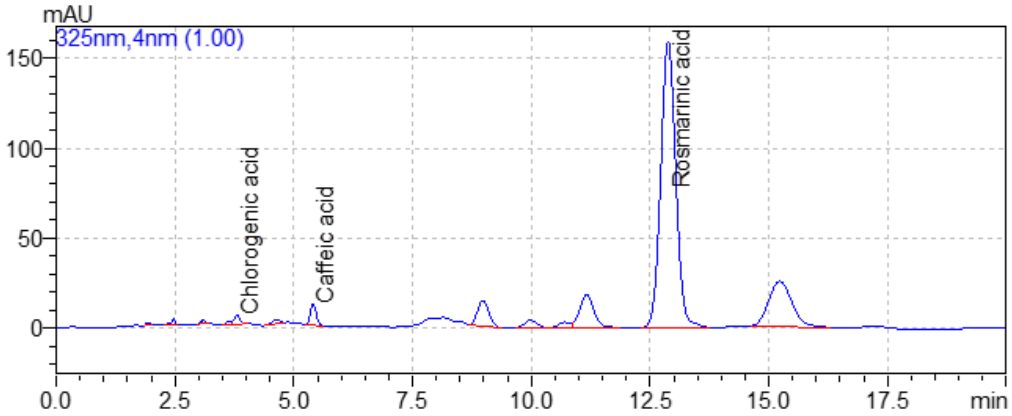

Figure 74 HPLC chromatogram of *Mentha cordifolia* leaf extract

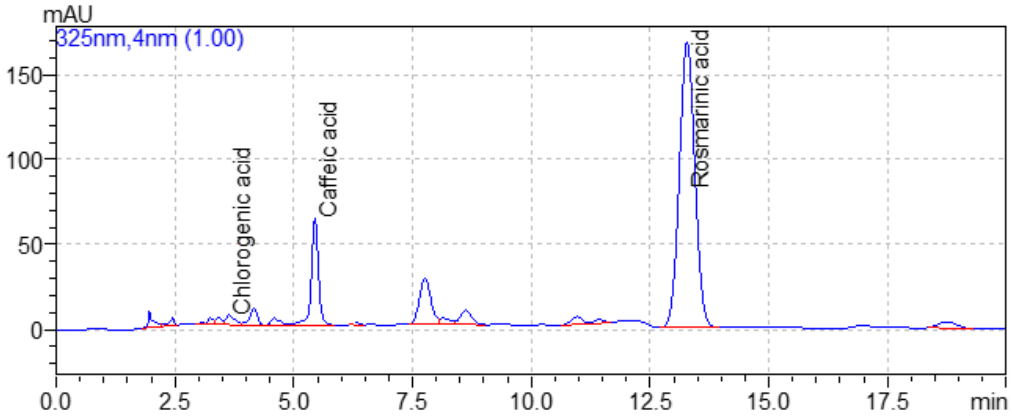

Figure 75 HPLC chromatogram of *Ocimum africanum* leaf extract

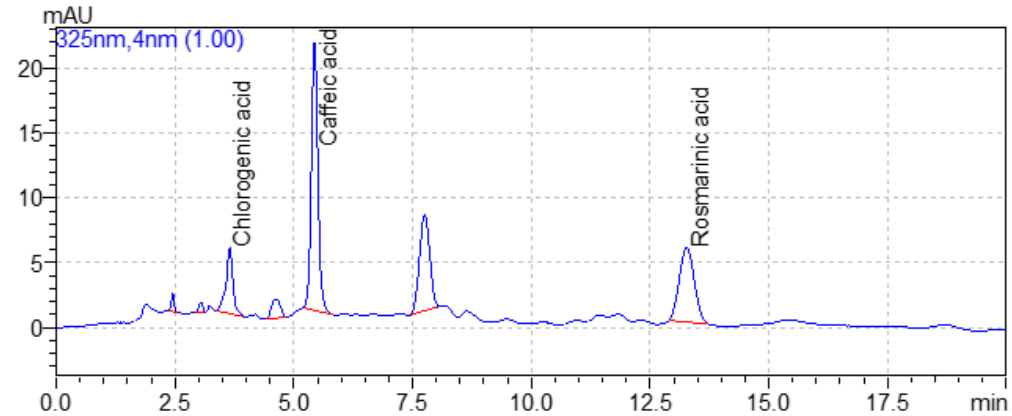

Figure 76 HPLC chromatogram of *Ocimum basilicum* leaf extract

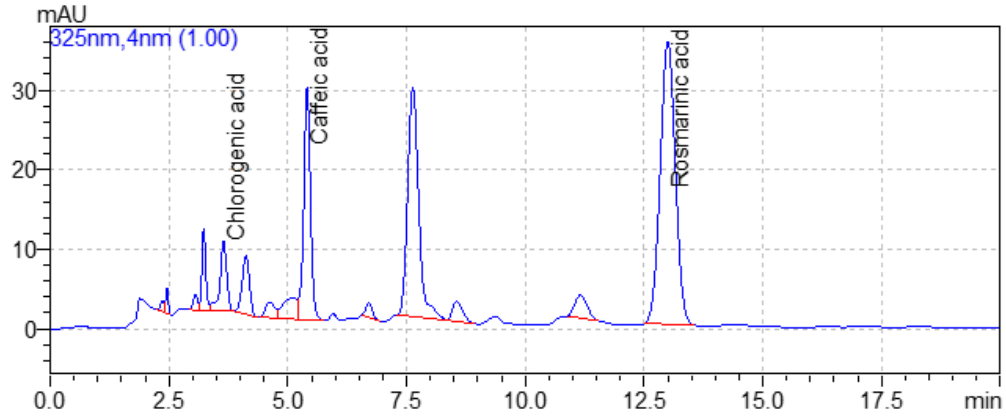

Figure 77 HPLC chromatogram of *Ocimum gratissimum* leaf extract

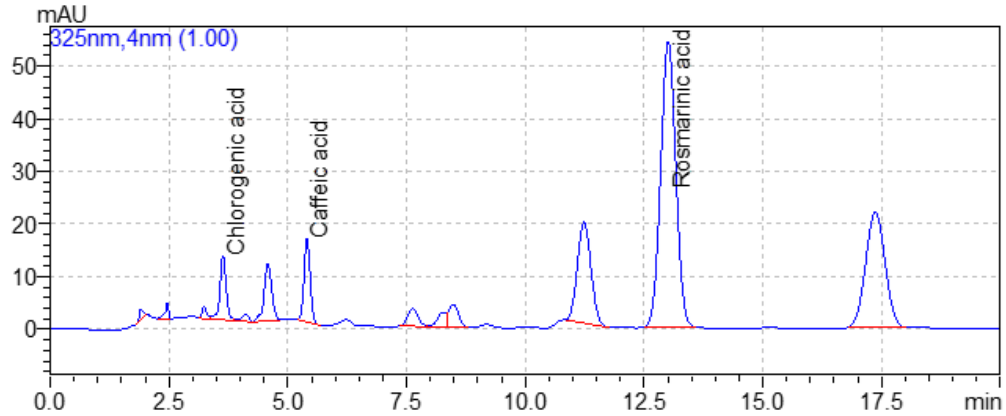

Figure 78 HPLC chromatogram of *Ocimum tenuiflorum* leaf extract

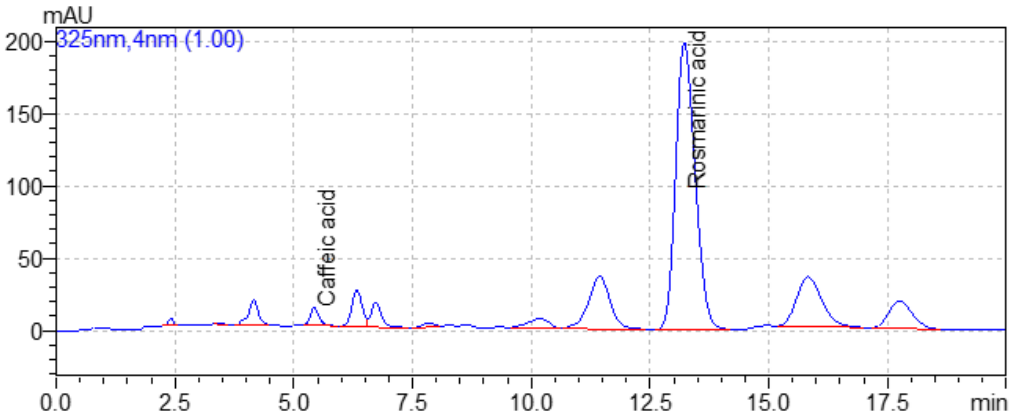

Figure 79 HPLC chromatogram of *Origanum majorana* leaf extract

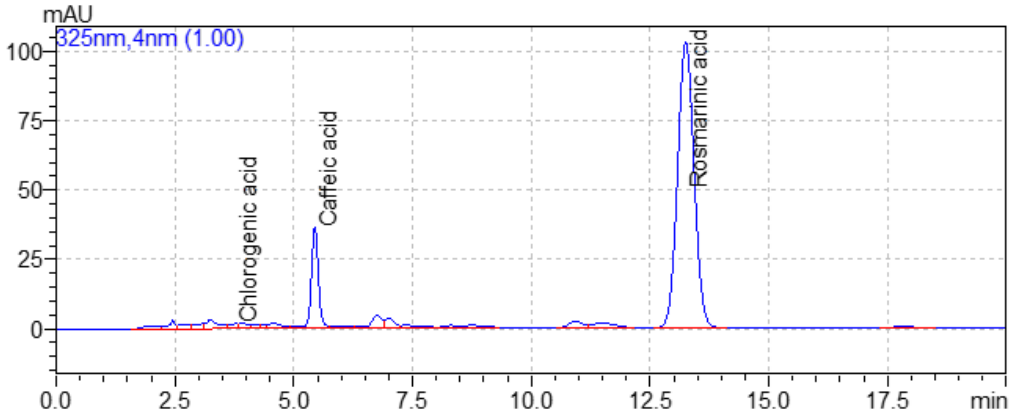

Figure 80 HPLC chromatogram of *Origanum vulgare* leaf extract

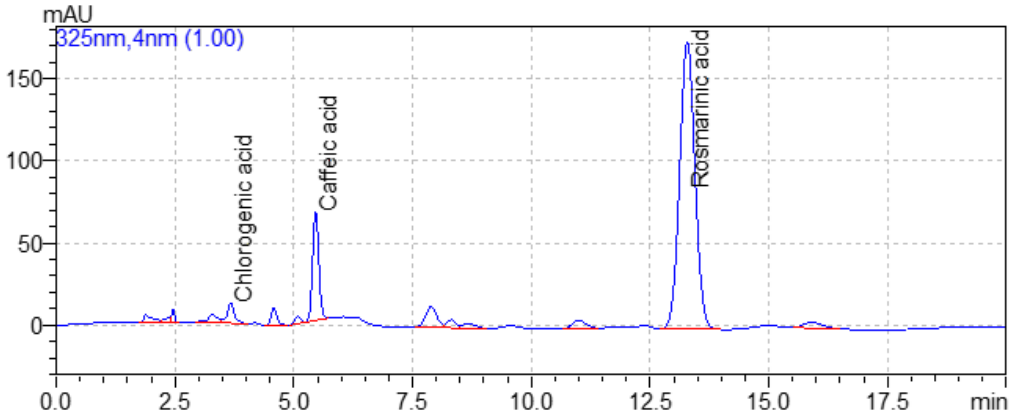

Figure 81 HPLC chromatogram of *Orthosiphon aristatus* leaf extract

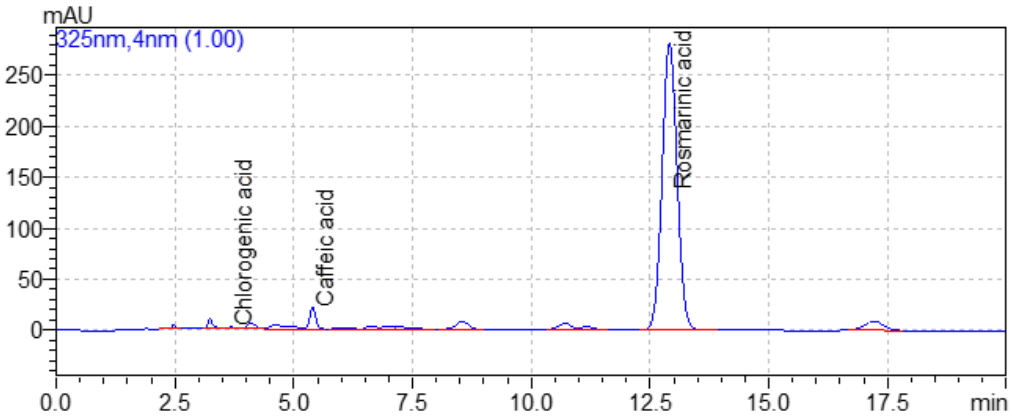

Figure 82 HPLC chromatogram of *Perilla frutescens* leaf extract

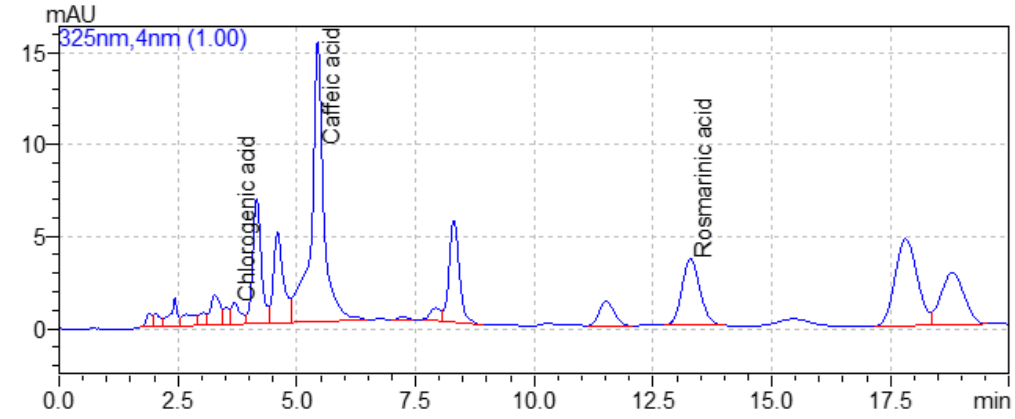

Figure 83 HPLC chromatogram of *Plectranthus amboinicus* leaf extract

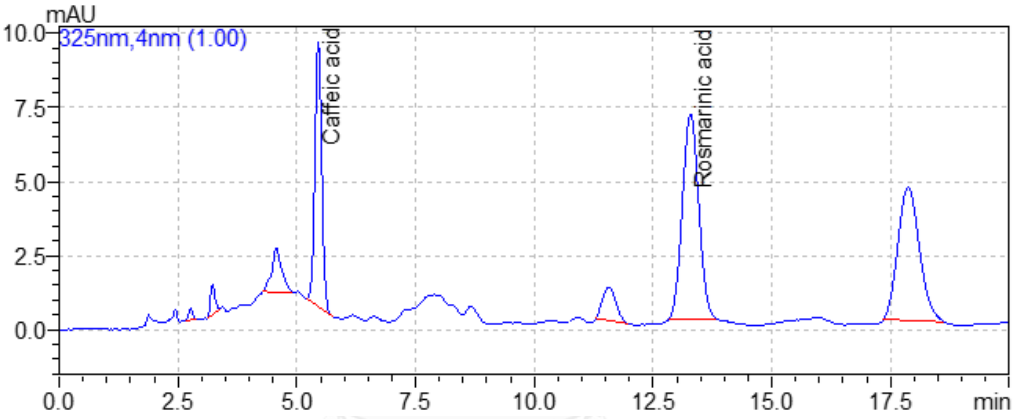

Figure 84 HPLC chromatogram of *Plectranthus rotundifolius* leaf extract

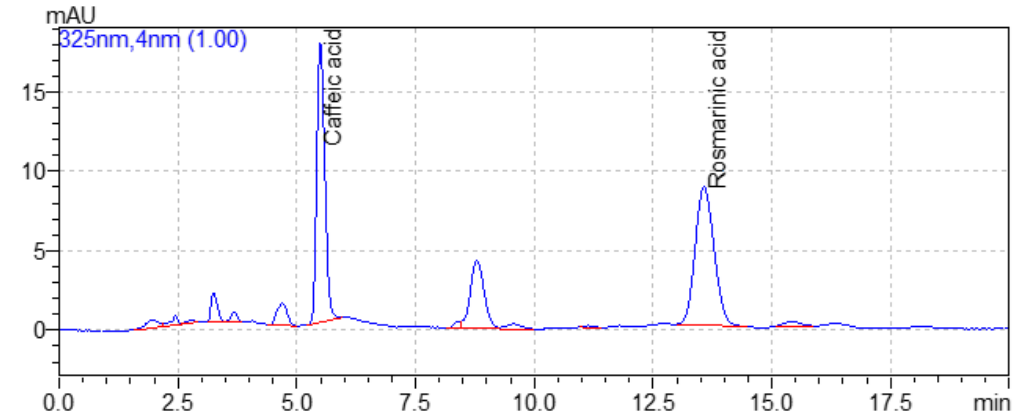

Figure 85 HPLC chromatogram of *Plectranthus rotundifolius* tuber extract

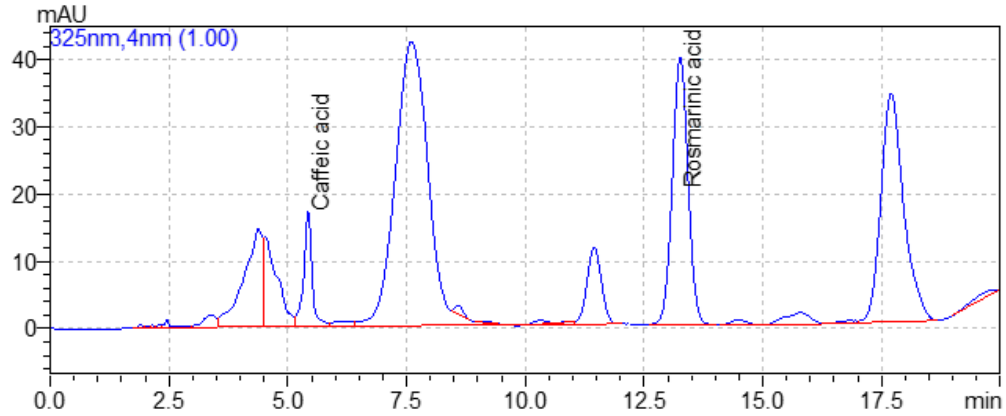

Figure 86 HPLC chromatogram of *Plectranthus scutellarioides* leaf extract

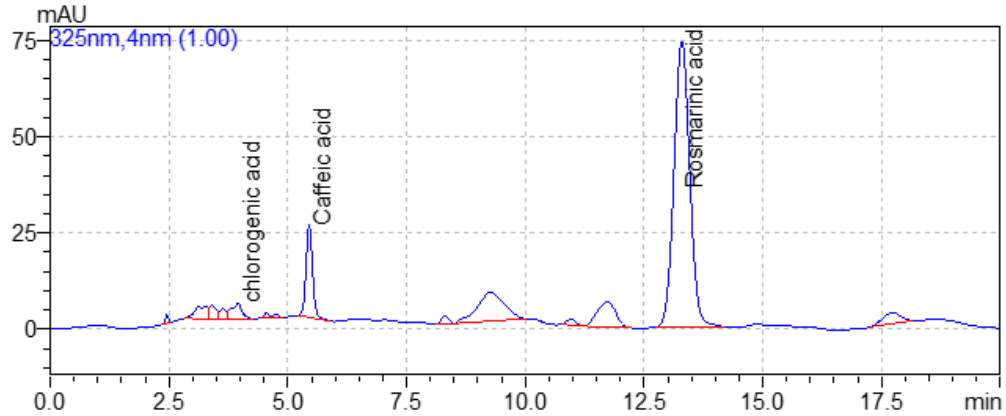

Figure 87 HPLC chromatogram of *Rosmarinus officinalis* aerial part extract

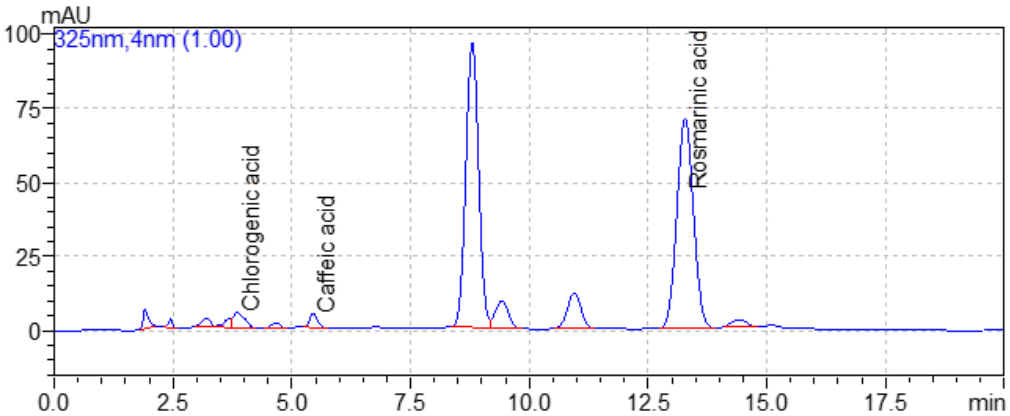

Figure 88 HPLC chromatogram of *Salvia hispanica* seed extract

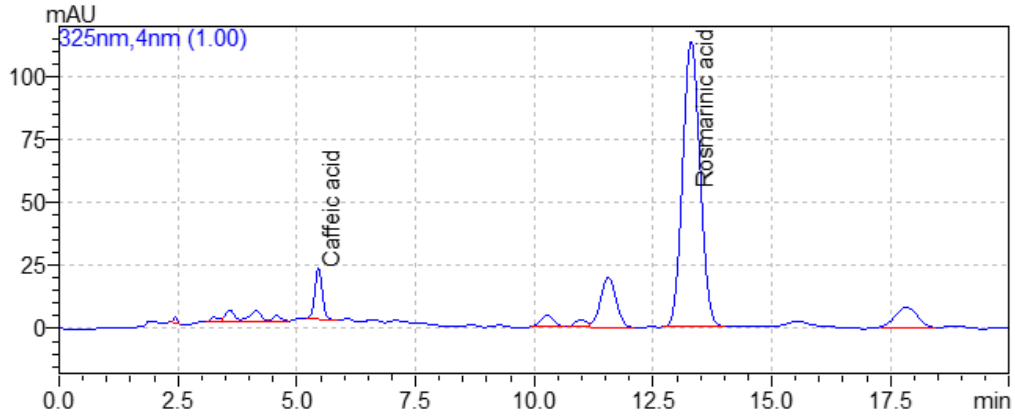

Figure 89 HPLC chromatogram of *Salvia officinalis* aerial part extract

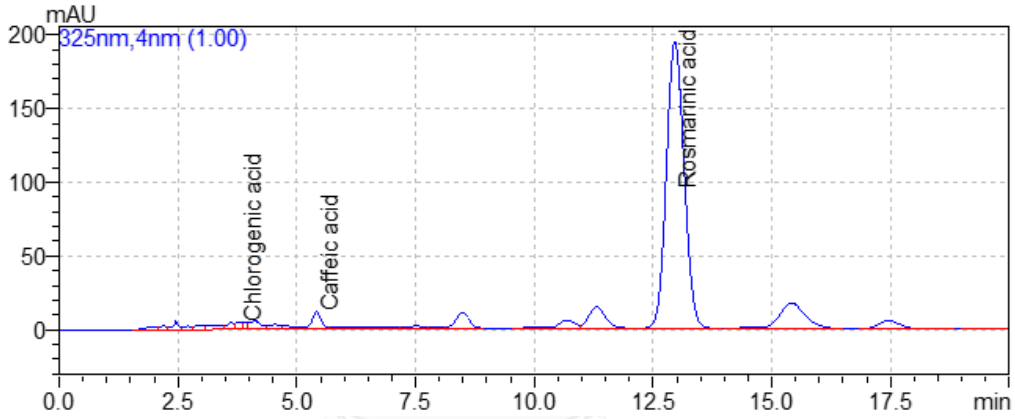

Figure 90 HPLC chromatogram of *Thymus citriodorus* aerial part extract

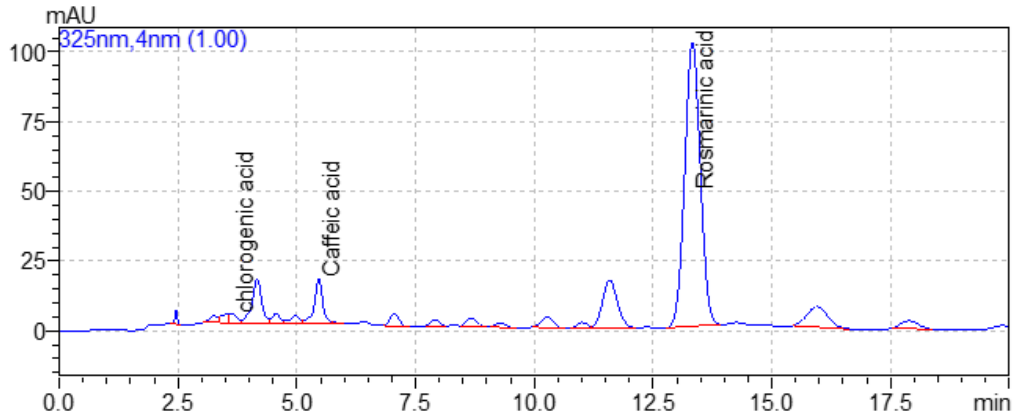

Figure 91 HPLC chromatogram of *Thymus vulgaris* aerial part extract

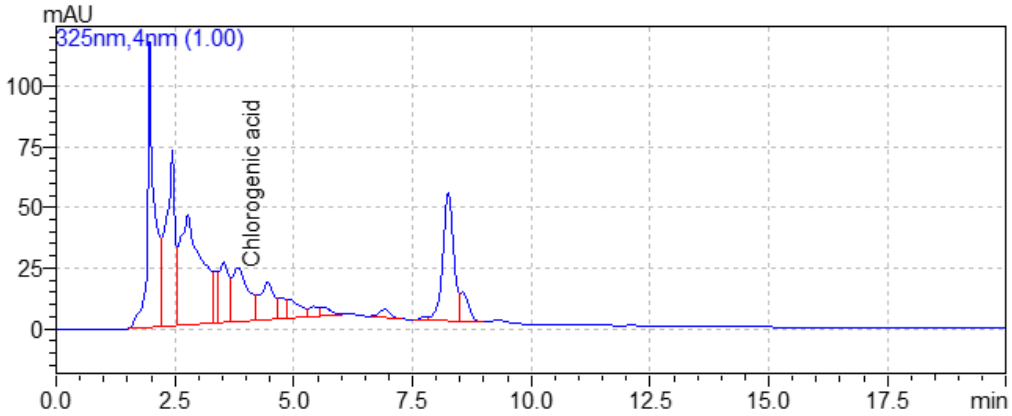

Figure 92 HPLC chromatogram of *Persea americana* flesh extract

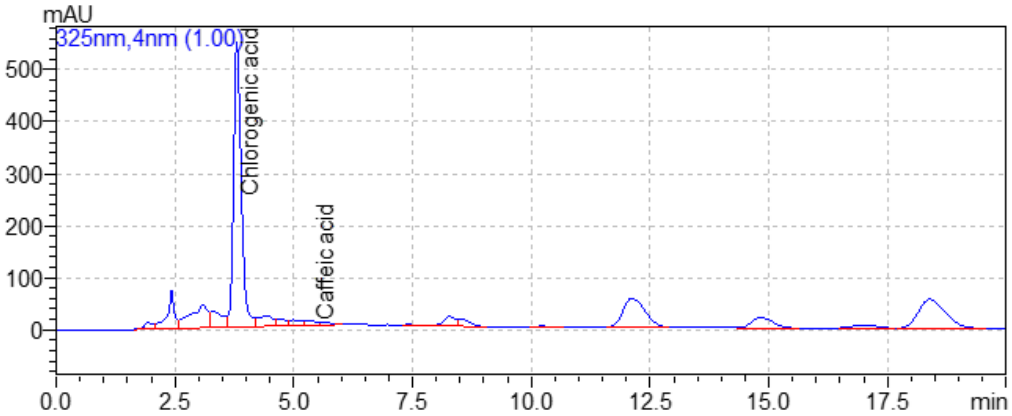

Figure 93 HPLC chromatogram of *Persea americana* peel extract

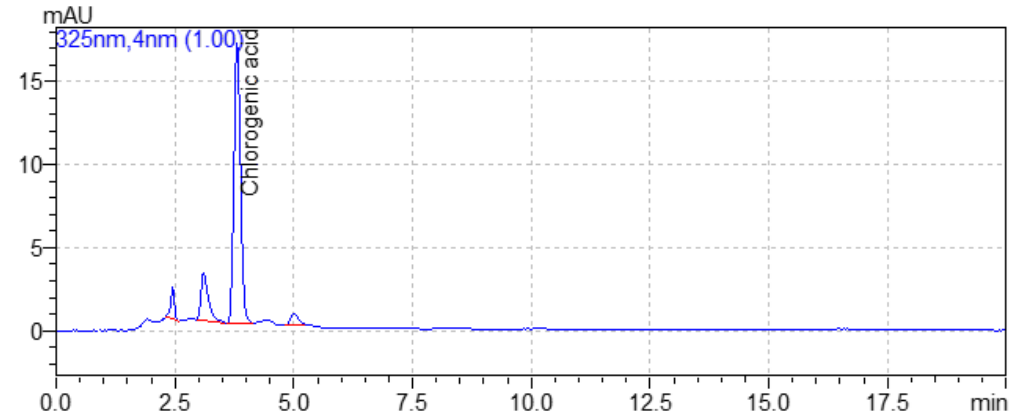

Figure 94 HPLC chromatogram of *Persea americana* seed extract

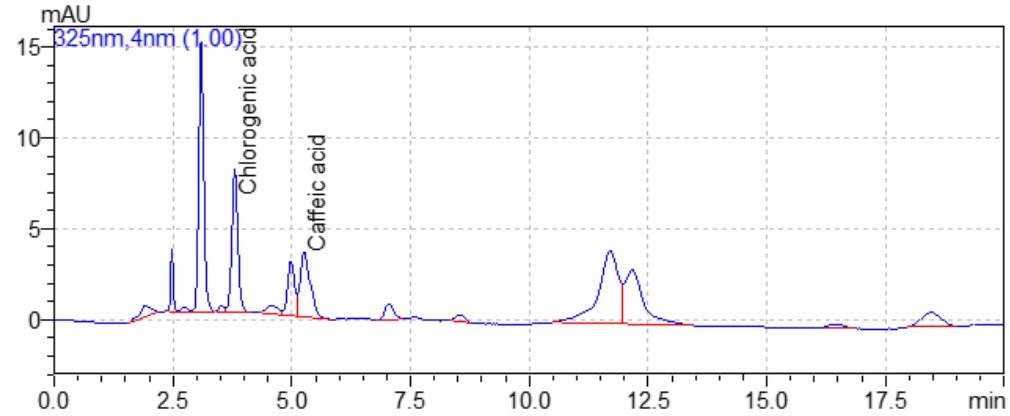

Figure 95 HPLC chromatogram of *Hibiscus sabdariffa* leaf extract

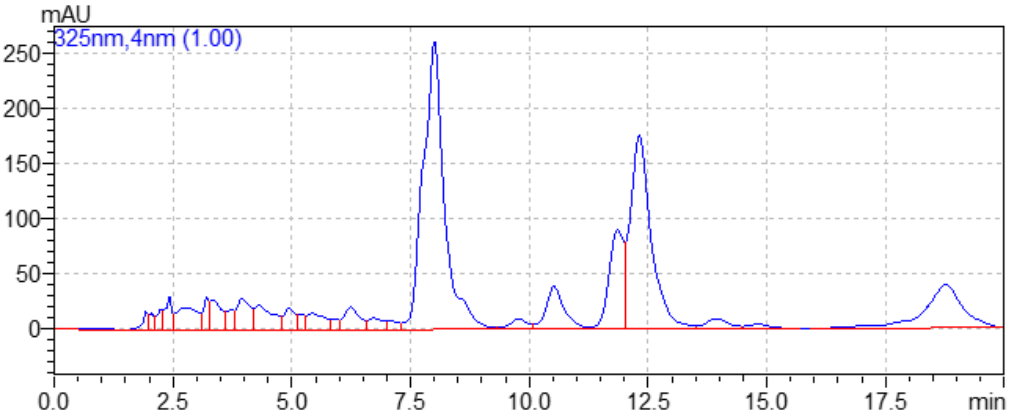

Figure 96 HPLC chromatogram of *Azadirachta indica* leaf extract

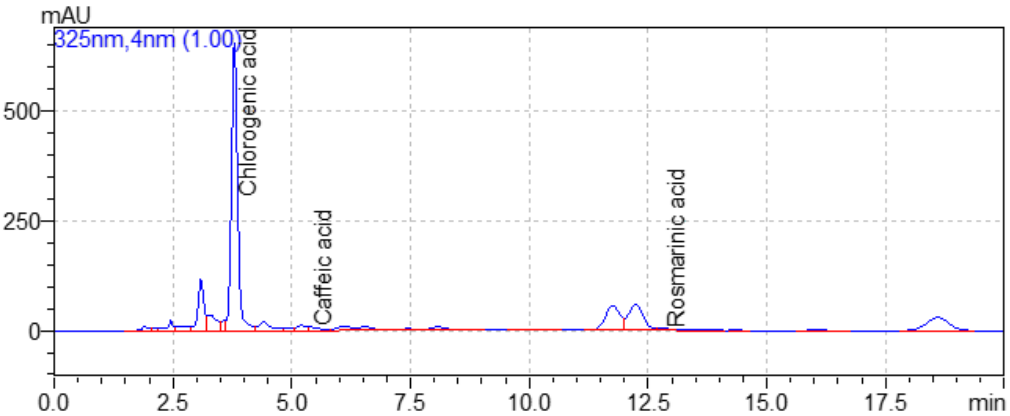

Figure 97 HPLC chromatogram of *Morus alba* leaf extract

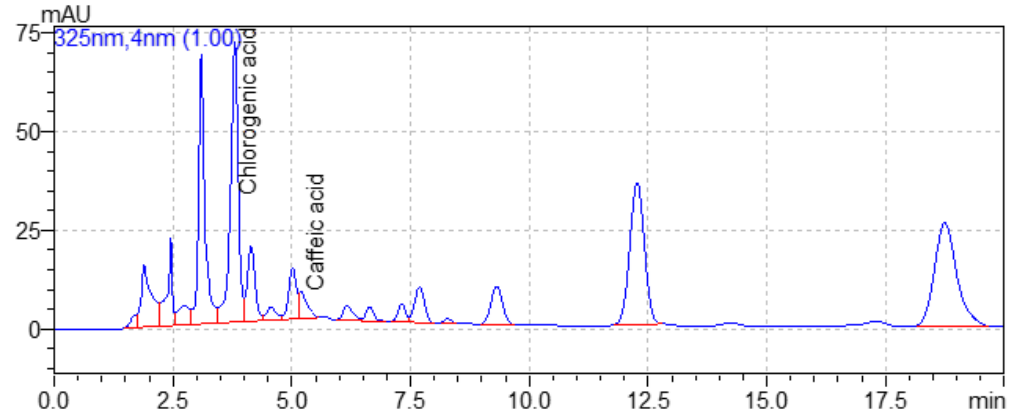

Figure 98 HPLC chromatogram of *Moringa oleifera* leaf extract

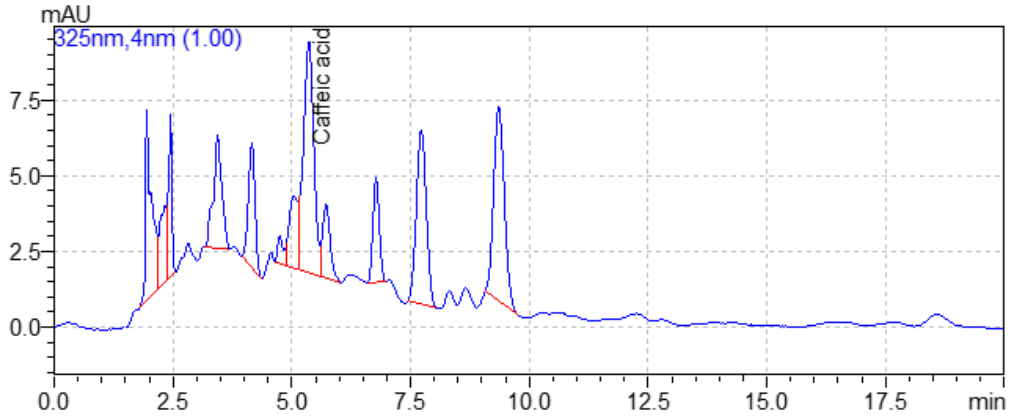

Figure 99 HPLC chromatogram of *Moringa oleifera* seed extract

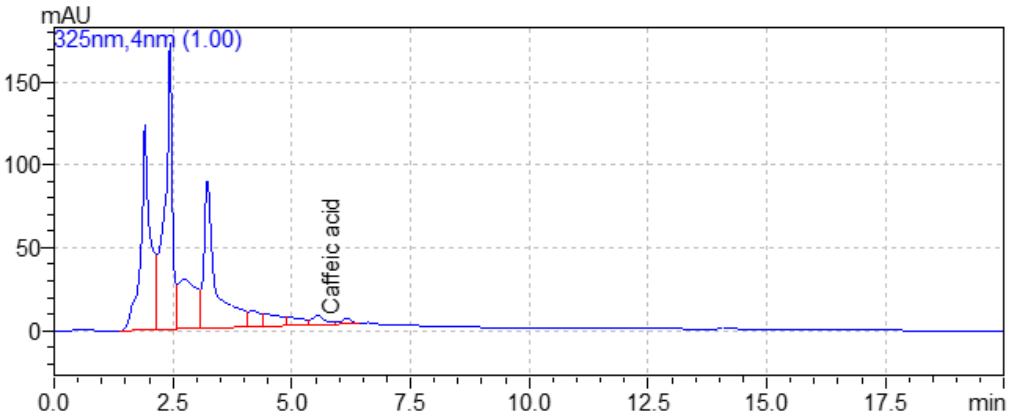

Figure 100 HPLC chromatogram of *Psidium guajava* fruit extract

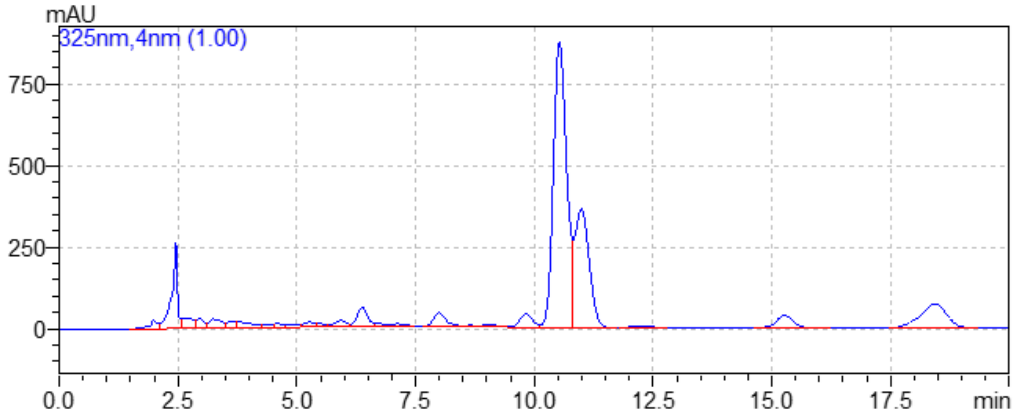

Figure 101 HPLC chromatogram of *Syzygium antisepticum* leaf extract

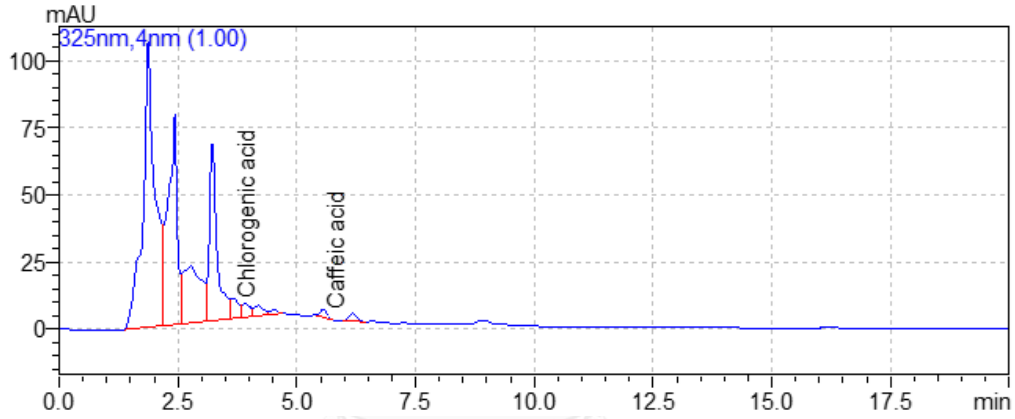

Figure 102 HPLC chromatogram of *Averrhoa carambola* fruit extract

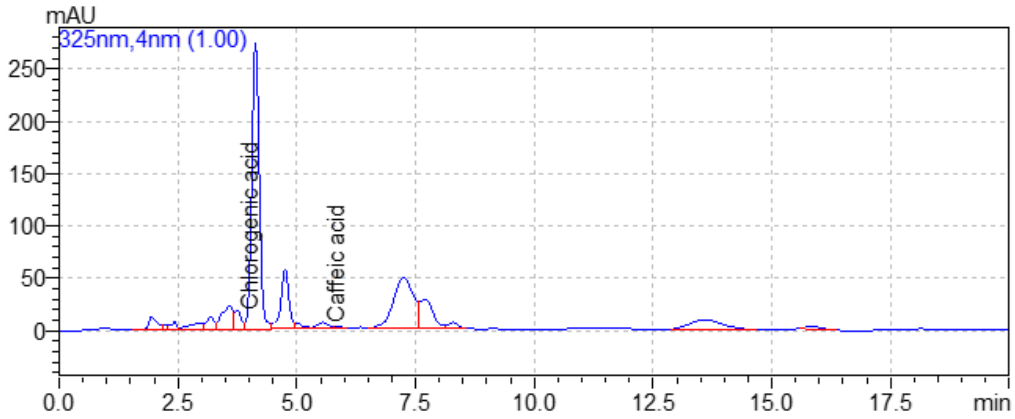

Figure 103 HPLC chromatogram of *Piper betle* leaf extract

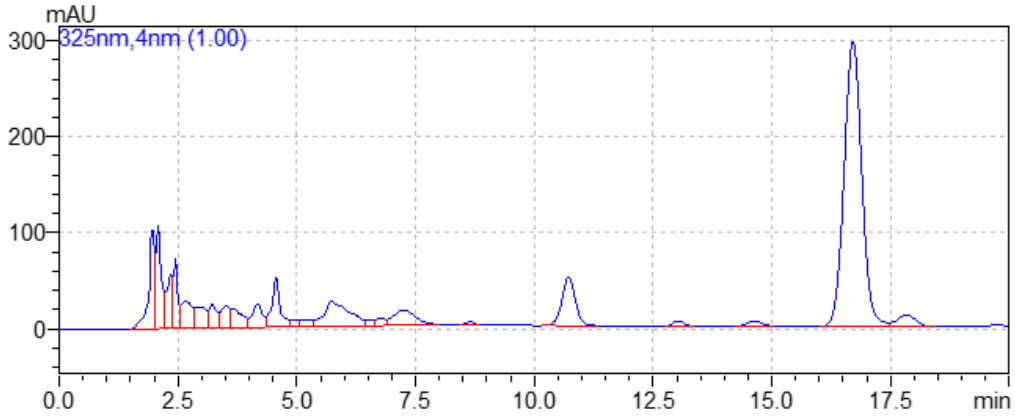

Figure 104 HPLC chromatogram of *Piper nigrum* (Black pepper) fruit extract

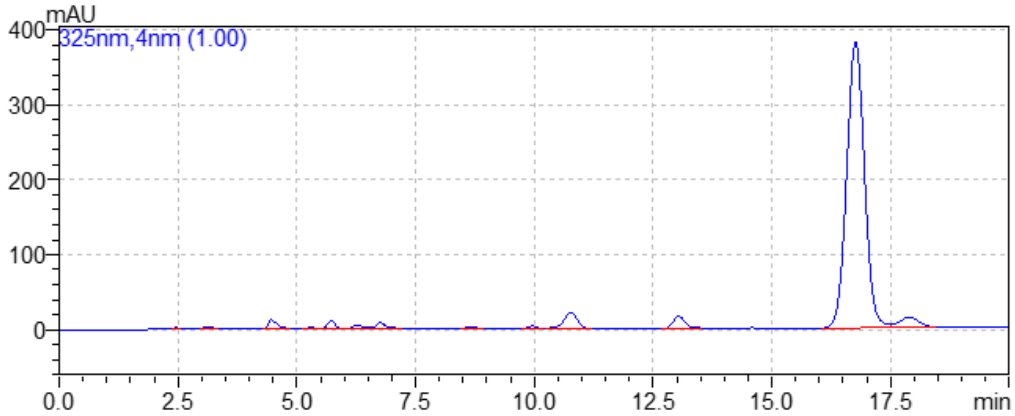

Figure 105 HPLC chromatogram of *Piper nigrum* (White pepper) seed extract

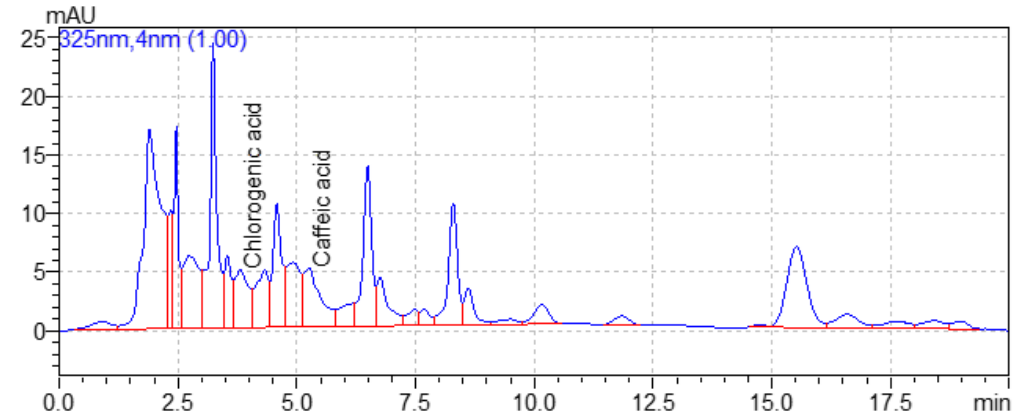

Figure 106 HPLC chromatogram of *Cymbopogon citratus* rhizome extract

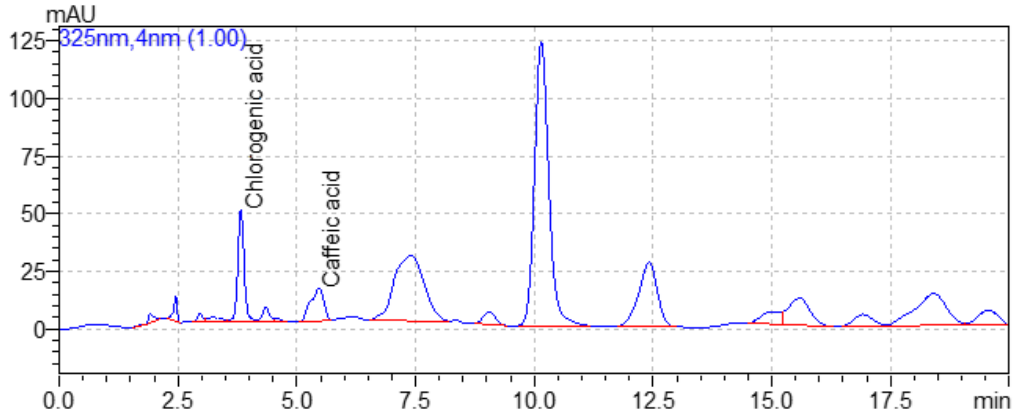

Figure 107 HPLC chromatogram of *Persicaria odorata* leaf extract

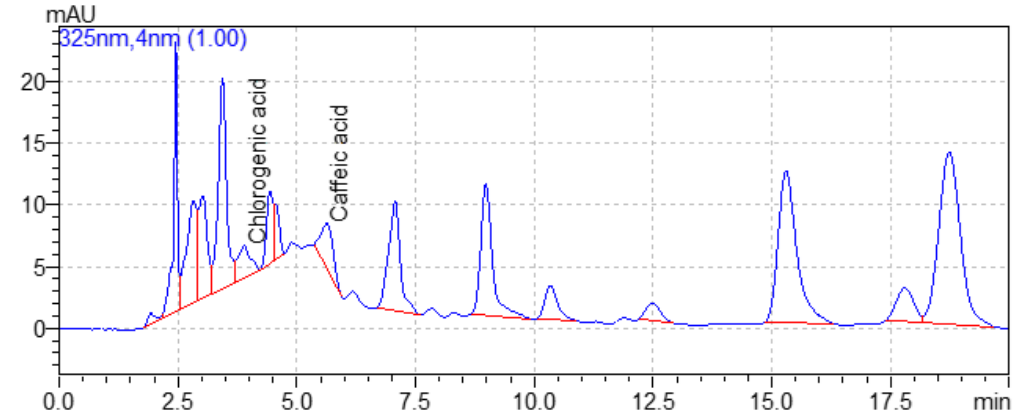

Figure 108 HPLC chromatogram of *Punica granatum* leaf extract

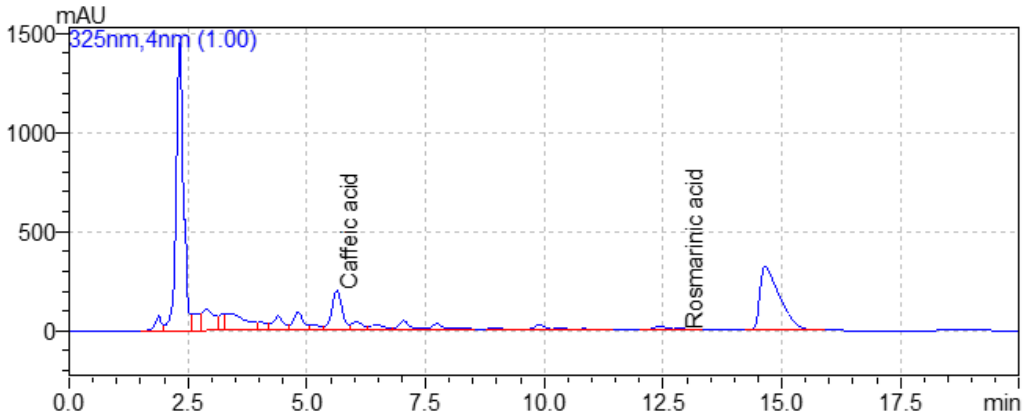

Figure 109 HPLC chromatogram of *Punica granatum* peel extract

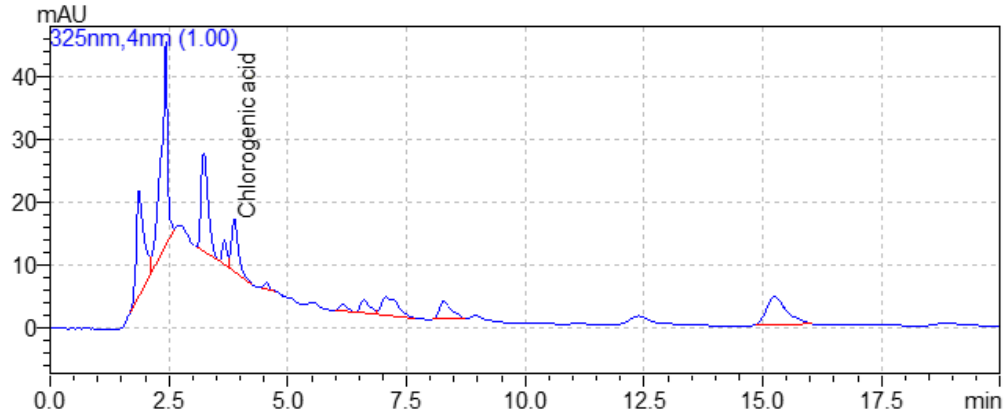

Figure 110 HPLC chromatogram of *Fragaria vesca* fruit extract

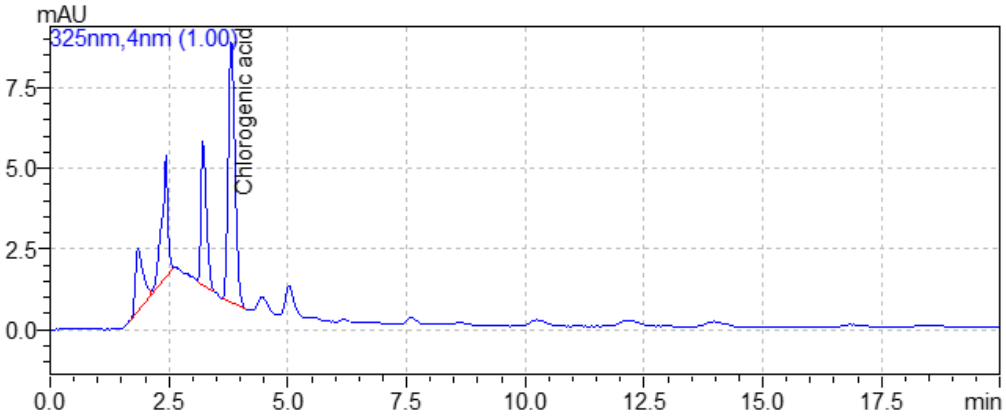

Figure 111 HPLC chromatogram of *Malus domestica* fruit extract

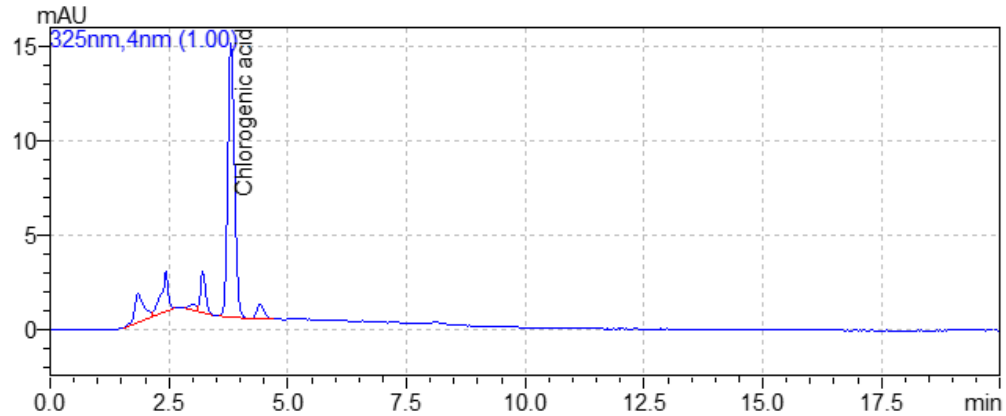

Figure 112 HPLC chromatogram of *Pyrus communis* fruit extract

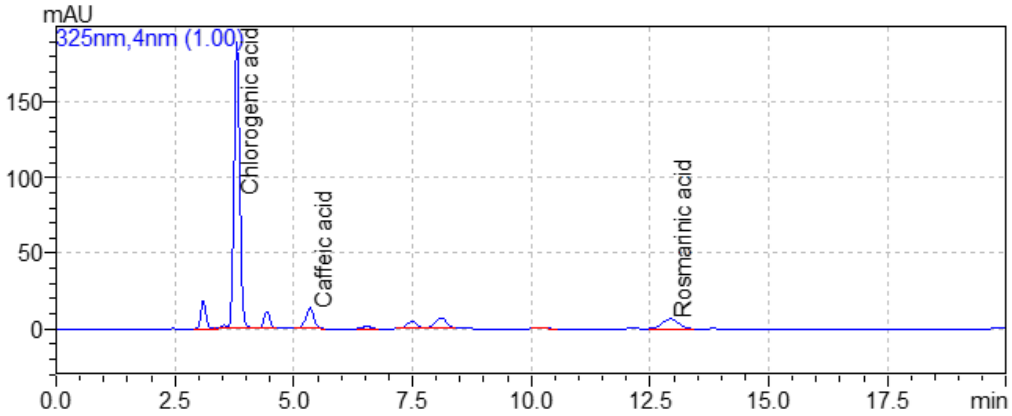

Figure 113 HPLC chromatogram of *Coffea arabica* seed extract

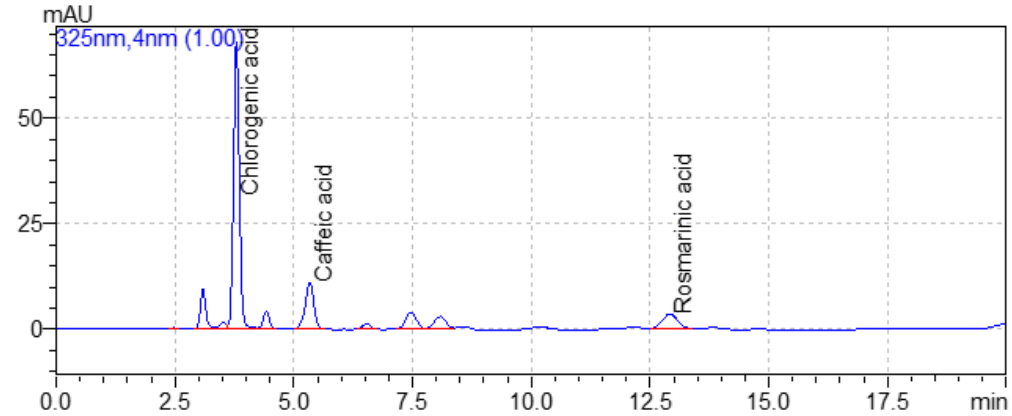

Figure 114 HPLC chromatogram of *Coffea canephora* seed extract

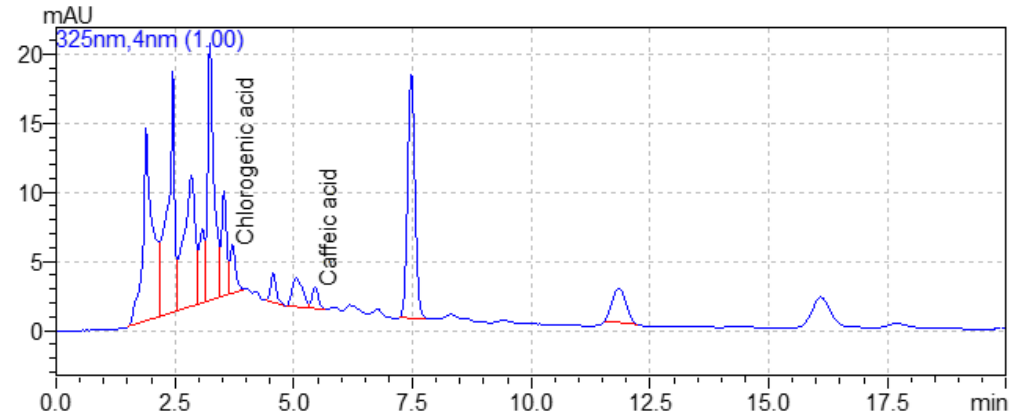

Figure 115 HPLC chromatogram of *Morinda citrifolia* fruit extract

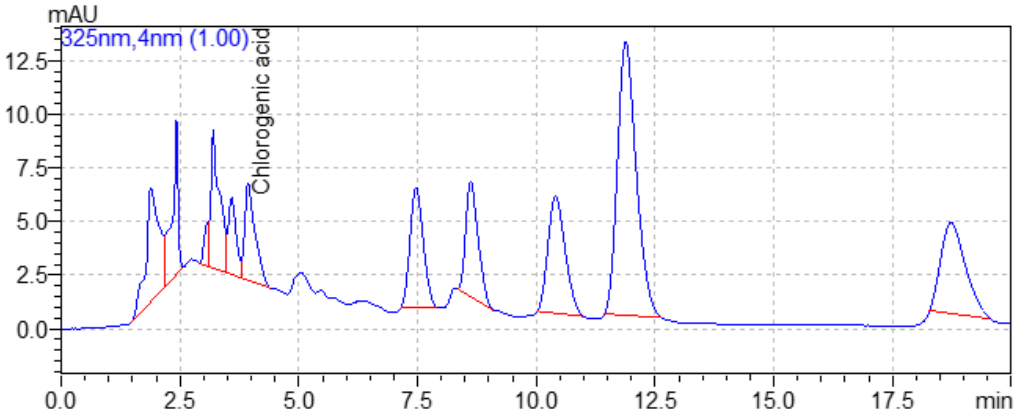

Figure 116 HPLC chromatogram of *Morinda citrifolia* leaf extract

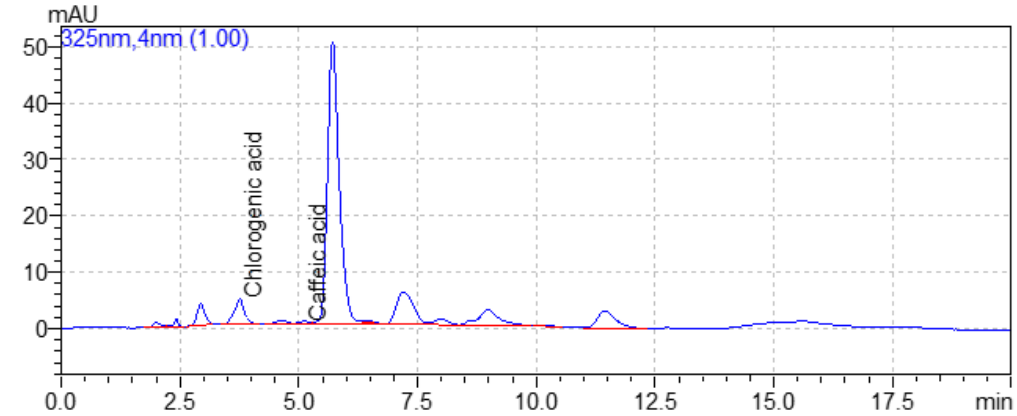

Figure 117 HPLC chromatogram of *Limnophila aromatica* aerial part extract

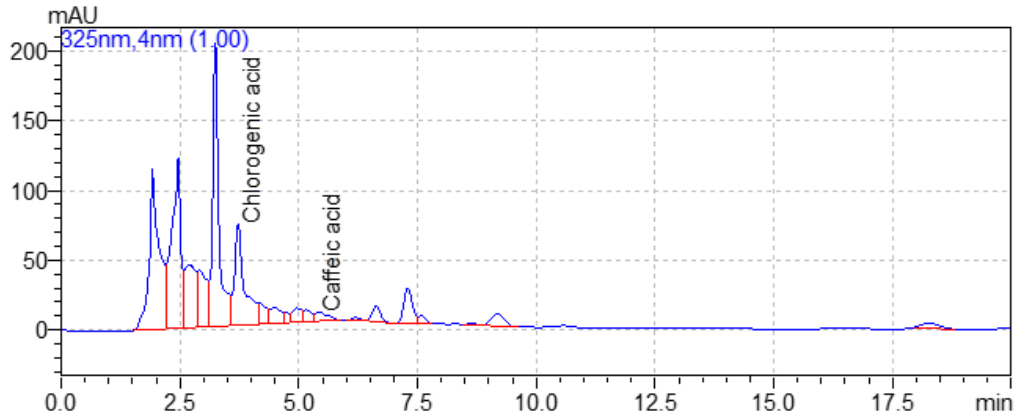

Figure 118 HPLC chromatogram of *Capsicum annuum* (Green bell pepper) fruit extract

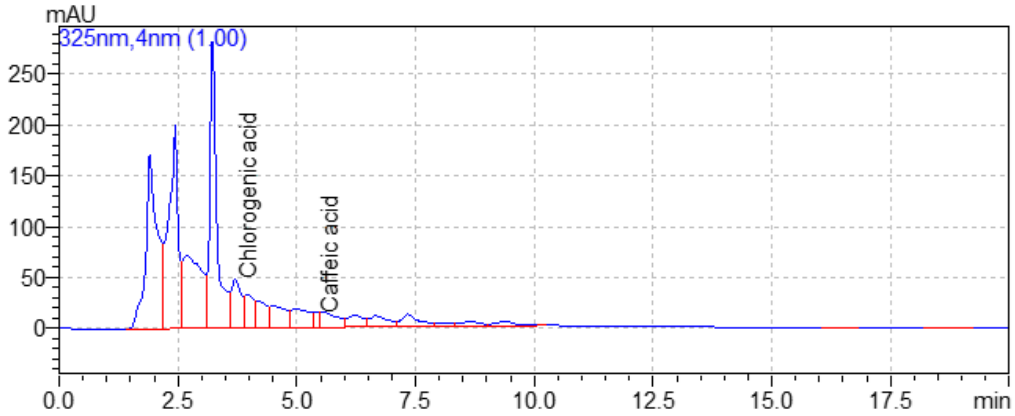

Figure 119 HPLC chromatogram of *Capsicum annuum* (Orange bell pepper) fruit extract

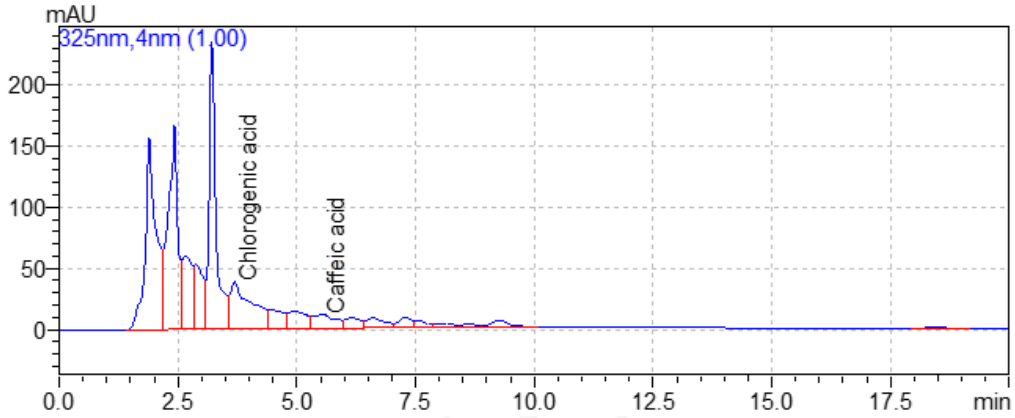

Figure 120 HPLC chromatogram of *Capsicum annuum* (Red bell pepper) fruit extract

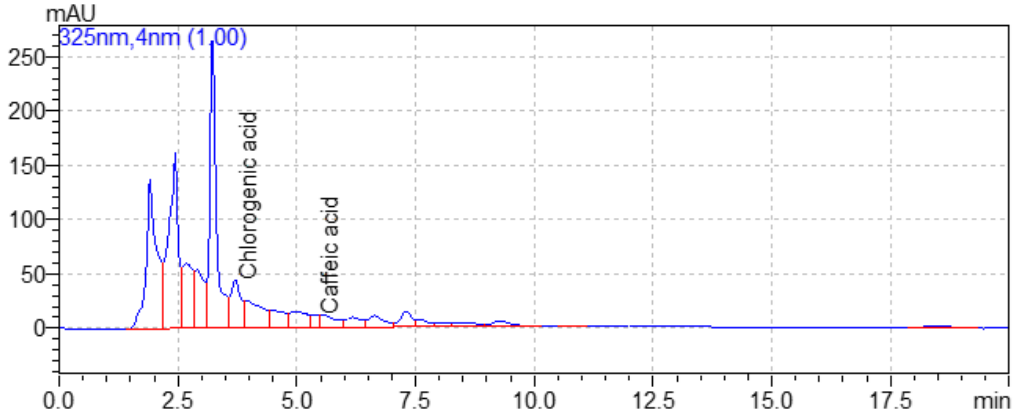

Figure 121 HPLC chromatogram of *Capsicum annuum* (Yellow bell pepper) fruit extract

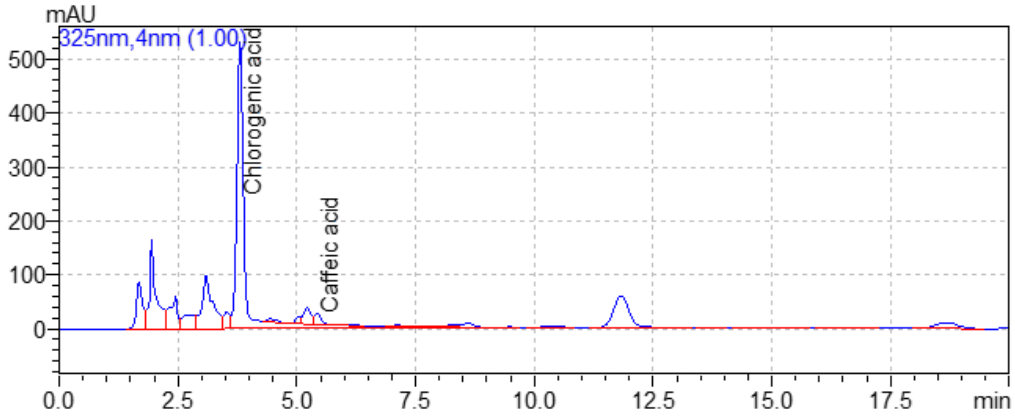

Figure 122 HPLC chromatogram of *Nicotiana tabacum* leaf extract

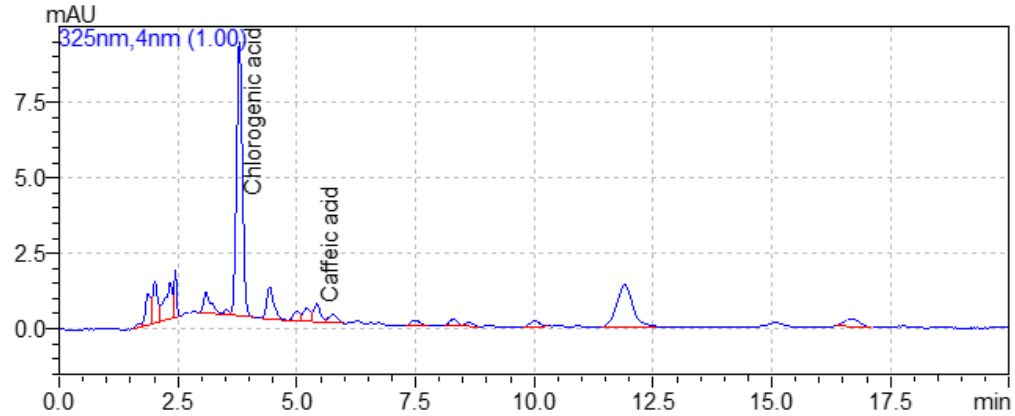

Figure 123 HPLC chromatogram of *Physalis angulata* aerial part extract

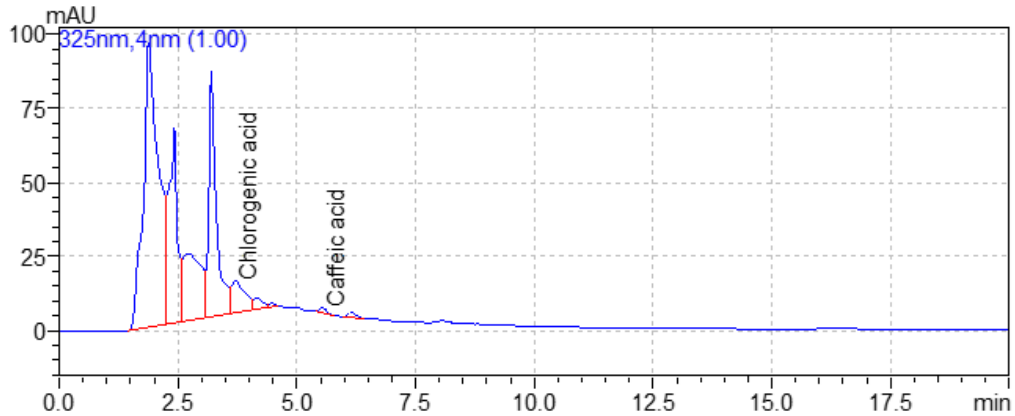

Figure 124 HPLC chromatogram of *Physalis peruviana* fruit extract

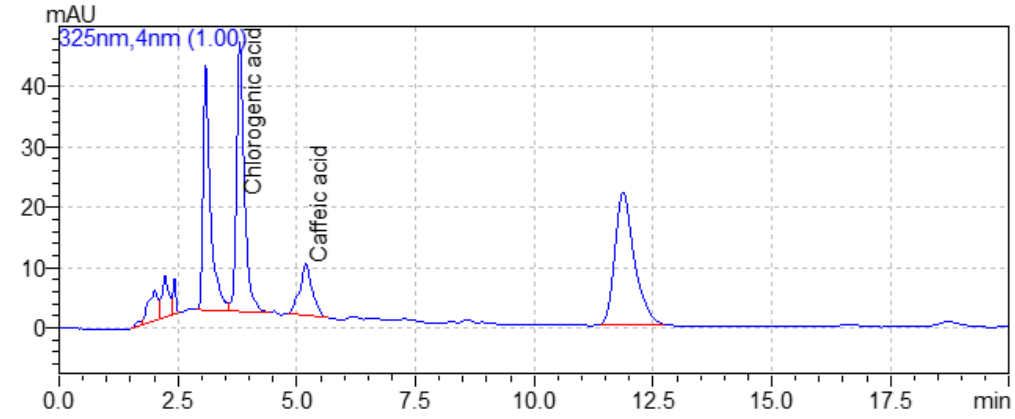

Figure 125 HPLC chromatogram of *Physalis peruviana* calyx extract

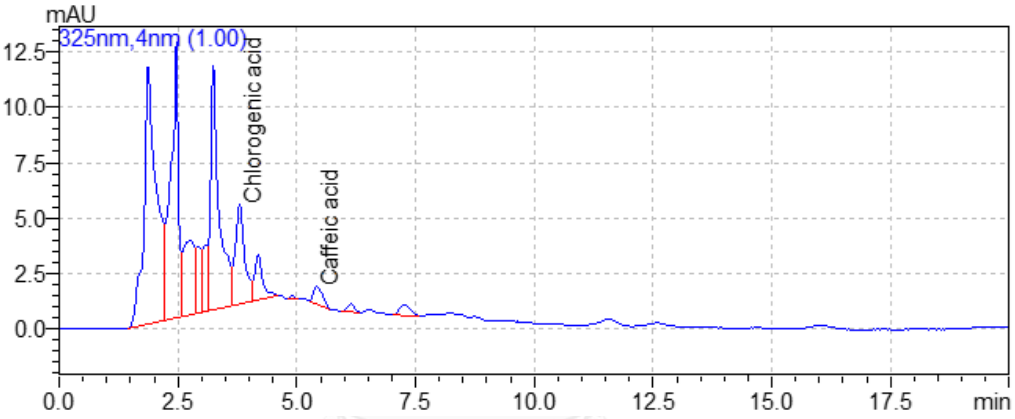

Figure 126 HPLC chromatogram of *Solanum lycopersicum* var. *cerasiforme* fruit extract

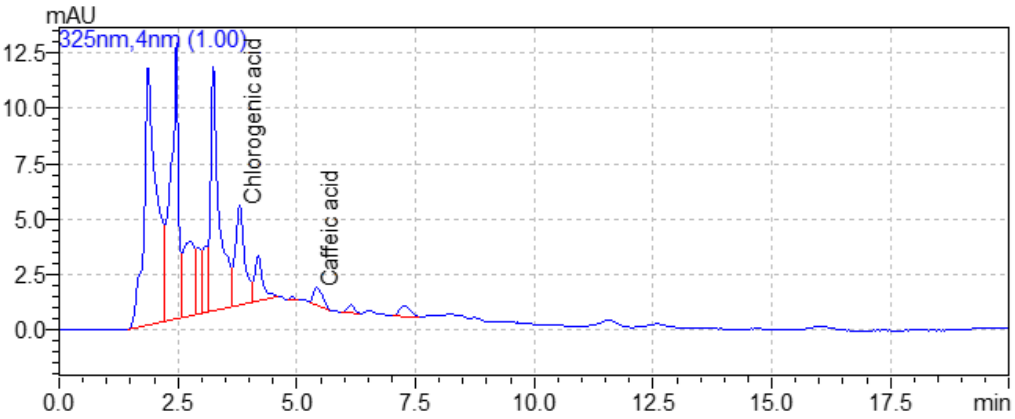

Figure 127 HPLC chromatogram of *Solanum lycopersicum* var. *lycopersicum* fruit extract

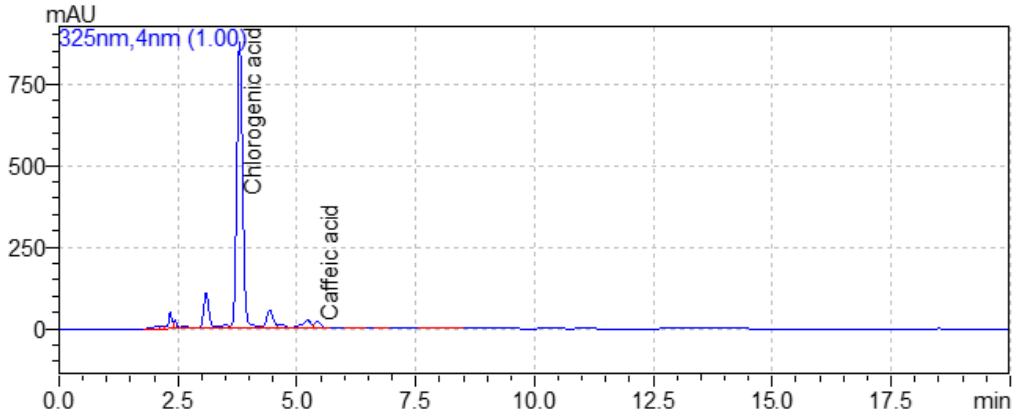

Figure 128 HPLC chromatogram of *Strychnos nux-vomica* seed extract

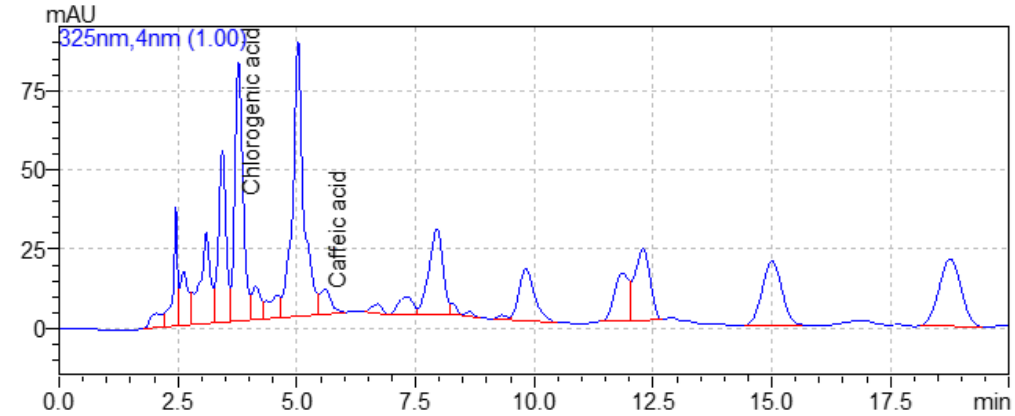

Figure 129 HPLC chromatogram of *Camellia sinensis* leaf extract

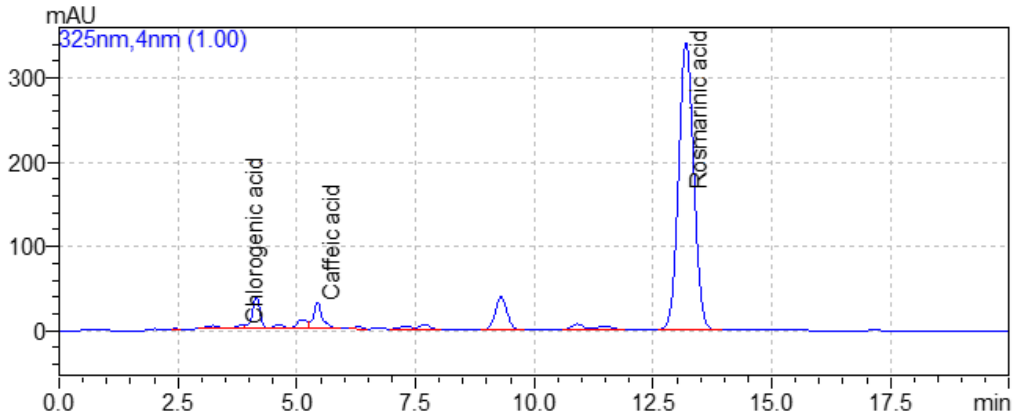

Figure 130 HPLC chromatogram of *Thunbergia laurifolia* leaf extract

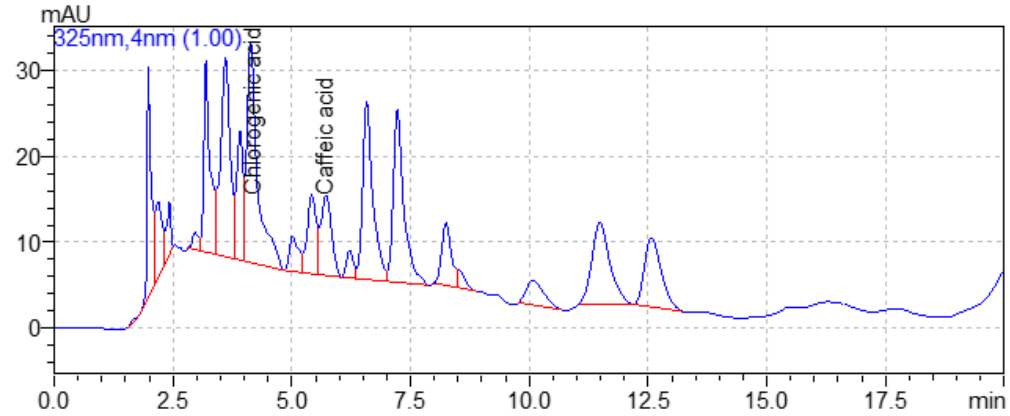

Figure 131 HPLC chromatogram of *Clerodendrum calamitosum* leaf extract

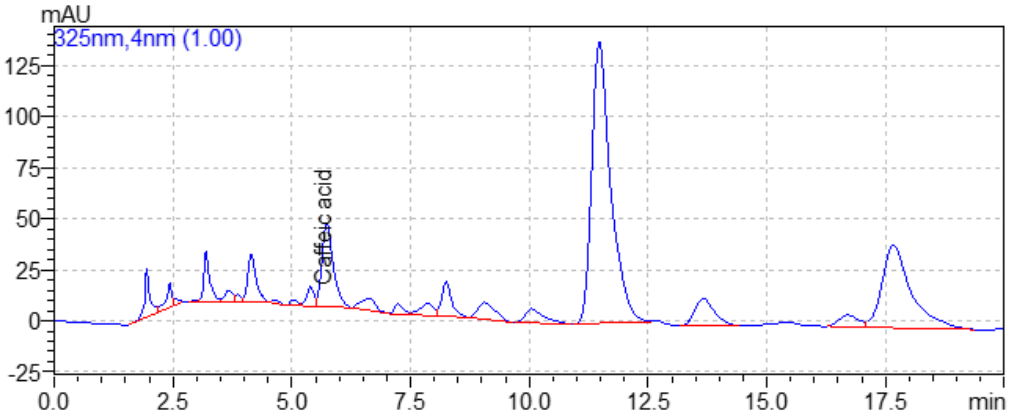

Figure 132 HPLC chromatogram of *Clerodendrum indicum* leaf extract

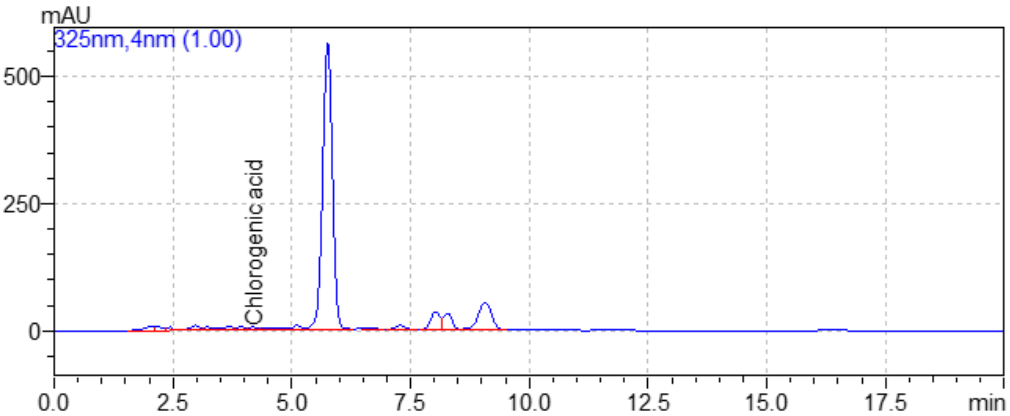

Figure 133 HPLC chromatogram of *Clerodendrum quadriloculare* leaf extract

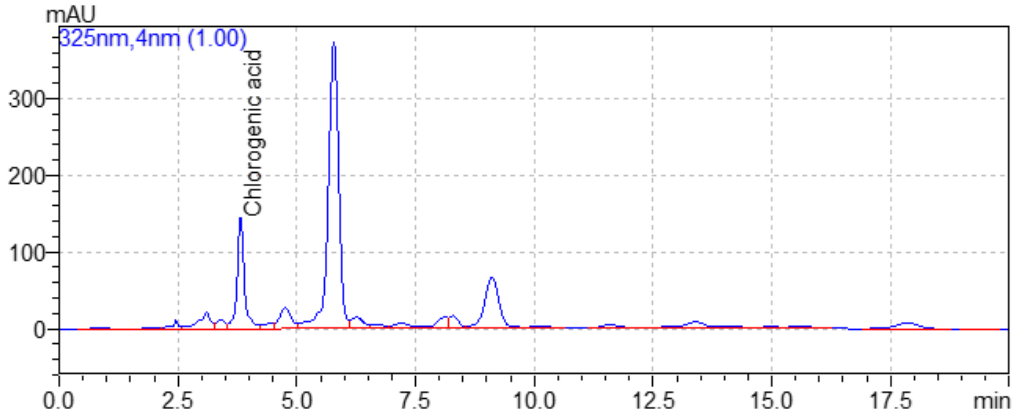

Figure 134 HPLC chromatogram of *Clerodendrum serratum* leaf extract

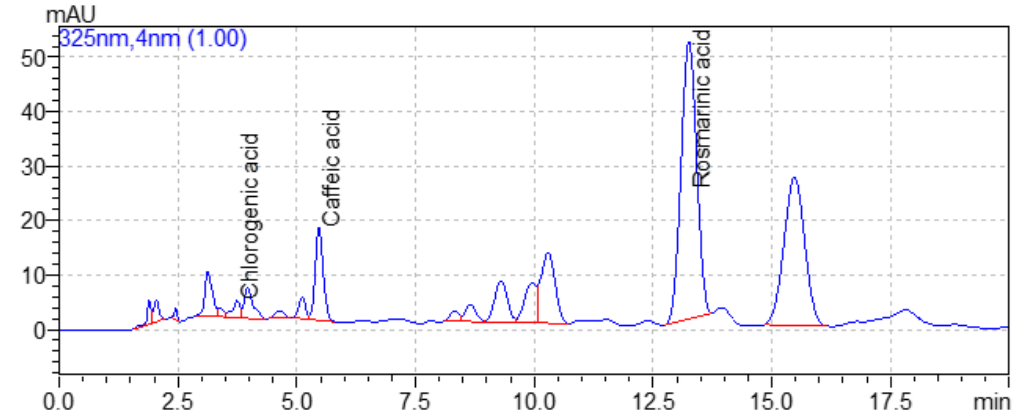

Figure 135 HPLC chromatogram of *Clerodendrum thomsoniae* leaf extract

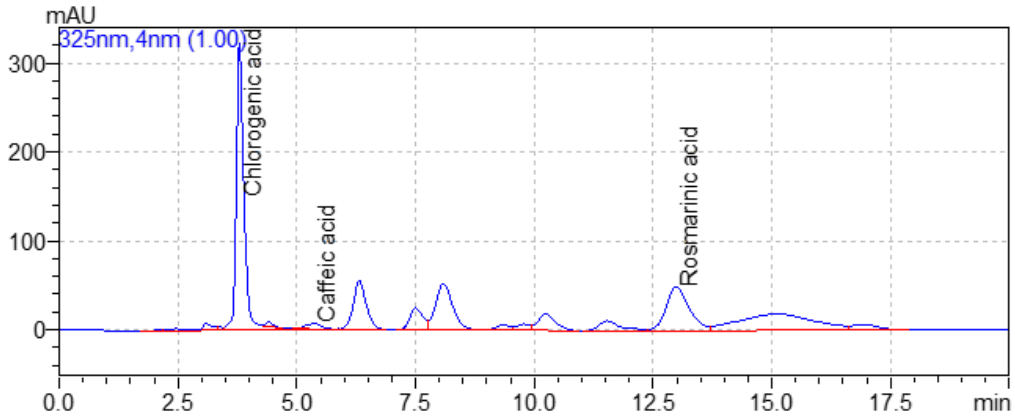

Figure 136 HPLC chromatogram of *Vitex agnus-castus* leaf extract

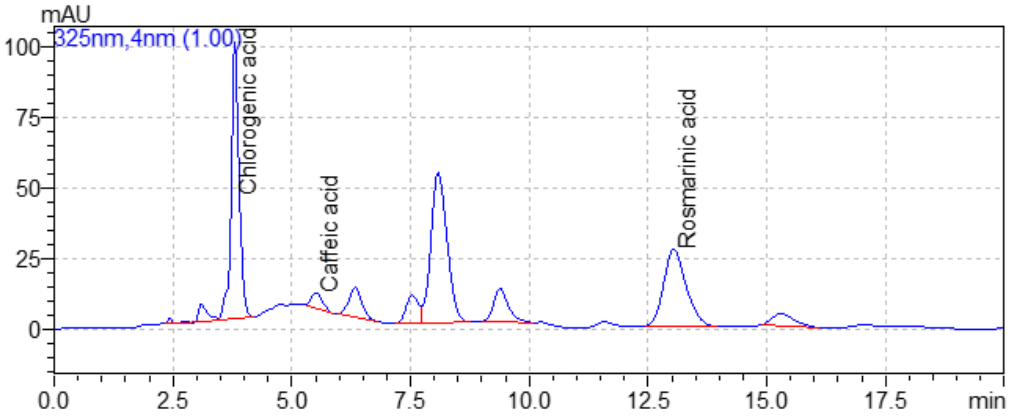

Figure 137 HPLC chromatogram of *Vitex negundo* leaf extract

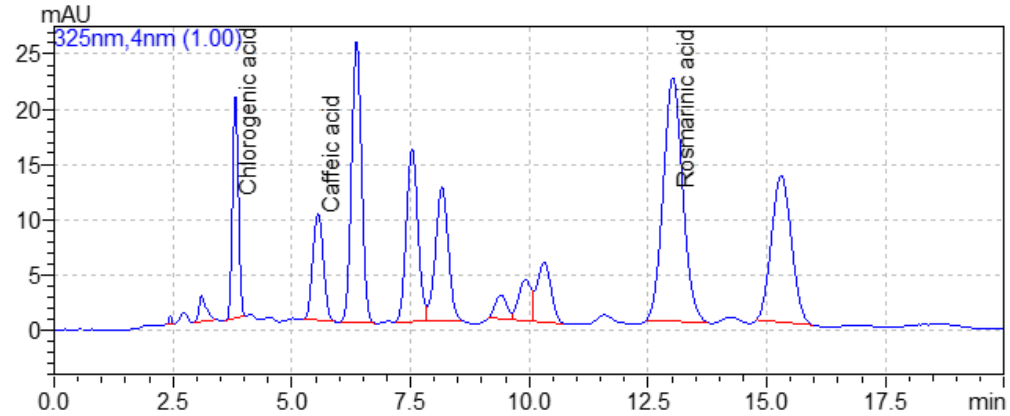

Figure 138 HPLC chromatogram of *Vitex trifolia* subsp. *litoralis* leaf extract

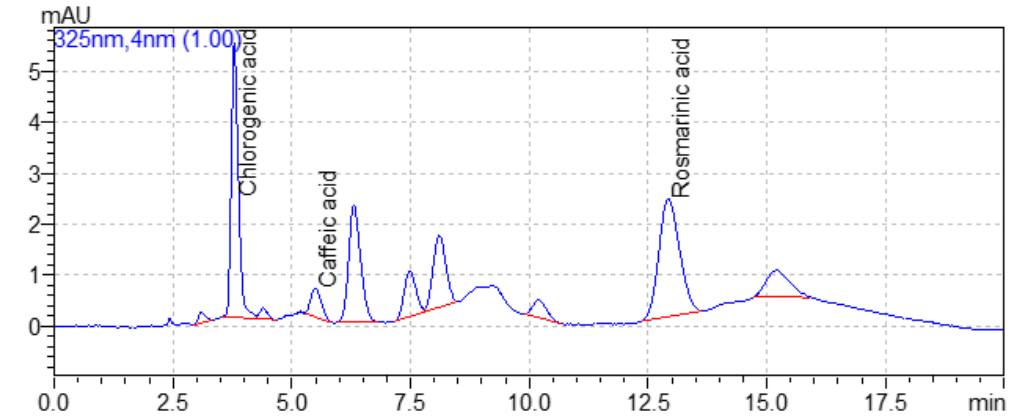

Figure 139 HPLC chromatogram of *Vitex trifolia* subsp. *trifolia* leaf extract

APPENDIX B

Physico-chemical parameters of *L. japonica* flowering bud

Table 29 The percent yield of *L. japonica* flowering bud crude drug

Sources	Weight of crude drug (g)	Weight of extractable matter (g)	%yeild
1	5.0082	2.1774	43.4767
2	5.0053	1.8132	36.2256
3	5.0041	1.9876	39.7194
4	5.0015	2.1869	43.7249
5	5.0033	2.0512	40.9969
6	5.0088	2.1763	43.4495
7	5.0023	2.2115	44.2097
8	5.0052	2.0000	39.9584
9	5.0012	2.0619	41.2281
10	5.0084	1.8370	36.6784
11	5.0016	1.9216	38.4197
12	5.0051	2.0102	40.1630
13	5.0028	1.0183	20.3546
14	5.0029	2.0081	40.1387
15	5.0028	2.1437	42.8500
		Mean	**39.4396**
		SD	**5.8269**

Table 30 Loss on drying content of *L. japonica* flowering bud crude drug

Sources	Weight of crude drug (g)	Loss of weight (g)	Amount (% by weight)	Mean	SD
1	3.0009	0.2992	9.9703	9.9964	0.0407
	3.0013	0.2994	9.9757		
	3.0010	0.3014	10.0433		
2	3.0029	0.3734	12.4346	12.4993	0.0560
	3.0023	0.3762	12.5304		
	3.0041	0.3765	12.5329		
3	3.0024	0.3523	11.7339	11.7984	0.0928
	3.0030	0.3575	11.9048		
	3.0026	0.3530	11.7565		
4	3.0030	0.2860	9.5238	9.4378	0.0863
	3.0026	0.2834	9.4385		
	3.0039	0.2809	9.3512		
5	3.0038	0.2877	9.5779	9.6169	0.0340
	3.0031	0.2893	9.6334		
	3.0074	0.2899	9.6396		
6	3.0013	0.2823	9.4059	9.4190	0.0236
	3.0054	0.2839	9.4463		
	3.0027	0.2824	9.4049		
7	3.0035	0.3053	10.1648	10.1585	0.0322
	3.0028	0.3059	10.1872		
	3.0009	0.3038	10.1236		
8	3.0050	0.3052	10.1564	10.0496	0.0990
	3.0036	0.3013	10.0313		
	3.0037	0.2992	9.9610		
9	3.0016	0.2931	9.7648	9.7387	0.0231
	3.0018	0.2918	9.7208		
	3.0040	0.2923	9.7304		
10	3.0018	0.3283	10.9368	11.0313	0.0829
	3.0041	0.3332	11.0915		
	3.0057	0.3326	11.0656		

Table 30 Loss on drying content of *L. japonica* flowering bud crude drug (Cont.)

Sources	Weight of crude drug (g)	Loss of weight (g)	Amount (% by weight)	Mean	SD
11	3.0022	0.3064	10.2058	10.1890	0.0496
	3.0040	0.3044	10.1332		
	3.0016	0.3070	10.2279		
12	3.0049	0.3062	10.1900	10.1646	0.0274
	3.0035	0.3054	10.1681		
	3.0023	0.3043	10.1356		
13	3.0088	0.2607	8.6646	8.6656	0.0009
	3.0060	0.2605	8.6660		
	3.0025	0.2602	8.6661		
14	3.0014	0.2704	9.0091	9.0755	0.0591
	3.0028	0.2731	9.0948		
	3.0036	0.2740	9.1224		
15	3.0028	0.2928	9.7509	9.7464	0.0118
	3.0042	0.2924	9.7330		
	3.0025	0.2929	9.7552		
			Grand mean	**10.1058**	
			Pooled SD	**0.0564**	

Formulas:

$$\text{Grand mean} = \frac{\bar{x}_1 n_1 + \bar{x}_2 n_2 + \ldots + \bar{x}_k n_k}{n_1 + n_2 + \ldots + n_k}$$

$$\text{Pooled SD} = \sqrt{\frac{\left((n_1 - 1) \times SD_1^2\right) + \left((n_2 - 1) \times SD_2^2\right) + \ldots + \left((n_k - 1) \times SD_k^2\right)}{(n_1 + n_2 + \ldots + n_k) - k}}$$

Table 31 Total ash content of *L. japonica* flowering bud crude drug

Sources	Weight of crude drug (g)	Weight of ash (g)	Amount (% by weight)	Mean	SD
1	3.0009	0.2024	6.7446	6.7631	0.0217
	3.0013	0.2037	6.7871		
	3.0010	0.2028	6.7577		
2	3.0029	0.2198	7.3196	7.3313	0.0249
	3.0023	0.2196	7.3144		
	3.0041	0.2211	7.3599		
3	3.0024	0.1885	6.2783	6.2533	0.0225
	3.0030	0.1876	6.2471		
	3.0026	0.1872	6.2346		
4	3.0030	0.1908	6.3536	6.3489	0.0449
	3.0026	0.1919	6.3911		
	3.0039	0.1893	6.3018		
5	3.0038	0.1872	6.2321	6.2534	0.0527
	3.0031	0.1896	6.3135		
	3.0074	0.1869	6.2147		
6	3.0013	0.1934	6.4439	6.4344	0.0120
	3.0054	0.1935	6.4384		
	3.0027	0.1928	6.4209		
7	3.0035	0.1921	6.3959	6.4038	0.0078
	3.0028	0.1923	6.4040		
	3.0009	0.1924	6.4114		
8	3.0050	0.1950	6.4892	6.4057	0.0789
	3.0036	0.1902	6.3324		
	3.0037	0.1921	6.3954		
9	3.0016	0.1841	6.1334	6.1427	0.0237
	3.0018	0.1852	6.1696		
	3.0040	0.1840	6.1252		
10	3.0018	0.1965	6.5461	6.5382	0.0275
	3.0041	0.1971	6.5610		
	3.0057	0.1956	6.5076		

Table 31 Total ash content of *L. japonica* flowering bud crude drug (Cont.)

Sources	Weight of crude drug (g)	Weight of ash (g)	Amount (% by weight)	Mean	SD
11	3.0022	0.1904	6.3420	6.3745	0.0792
	3.0040	0.1942	6.4647		
	3.0016	0.1896	6.3166		
12	3.0049	0.1938	6.4495	6.3913	0.0544
	3.0035	0.1917	6.3826		
	3.0023	0.1904	6.3418		
13	3.0088	0.2497	8.2990	8.3784	0.0697
	3.0060	0.2527	8.4065		
	3.0025	0.2531	8.4296		
14	3.0014	0.1930	6.4303	6.3900	0.0415
	3.0028	0.1906	6.3474		
	3.0036	0.1920	6.3923		
15	3.0028	0.1948	6.4873	6.4798	0.0083
	3.0042	0.1944	6.4709		
	3.0025	0.1946	6.4813		
			Grand mean	**6.5926**	
			Pooled SD	**0.0447**	

Table 32 Acid insoluble ash content of *L. japonica* flowering bud crude drug

Sources	Weight of crude drug (g)	Weight of ash (g)	Amount (% by weight)	Mean	SD
1	3.0009	0.0364	1.2130	1.2296	0.0207
	3.0013	0.0376	1.2528		
	3.0010	0.0367	1.2229		
2	3.0029	0.0479	1.5951	1.5640	0.0723
	3.0023	0.0485	1.6154		
	3.0041	0.0445	1.4813		
3	3.0024	0.0420	1.3989	1.3310	0.0740
	3.0030	0.0376	1.2521		
	3.0026	0.0403	1.3422		
4	3.0030	0.0352	1.1722	1.2187	0.0590
	3.0026	0.0360	1.1990		
	3.0039	0.0386	1.2850		
5	3.0038	0.0304	1.0121	0.9075	0.0906
	3.0031	0.0256	0.8525		
	3.0074	0.0258	0.8579		
6	3.0013	0.0263	0.8763	0.8458	0.0553
	3.0054	0.0235	0.7819		
	3.0027	0.0264	0.8792		
7	3.0035	0.0230	0.7658	0.8516	0.0747
	3.0028	0.0271	0.9025		
	3.0009	0.0266	0.8864		
8	3.0050	0.0383	1.2745	1.3448	0.0686
	3.0036	0.0424	1.4116		
	3.0037	0.0405	1.3483		
9	3.0016	0.0290	0.9662	1.0580	0.0858
	3.0018	0.0341	1.1360		
	3.0040	0.0322	1.0719		
10	3.0018	0.0313	1.0427	1.0475	0.0487
	3.0041	0.0330	1.0985		
	3.0057	0.0301	1.0014		

Table 32 Acid insoluble ash content of *L. japonica* flowering bud crude drug (Cont.)

Sources	Weight of crude drug (g)	Weight of ash (g)	Amount (% by weight)	Mean	SD
11	3.0022	0.0367	1.2224	1.2422	0.0341
	3.0040	0.0385	1.2816		
	3.0016	0.0367	1.2227		
12	3.0049	0.0275	0.9152	0.9800	0.0772
	3.0035	0.0320	1.0654		
	3.0023	0.0288	0.9593		
13	3.0088	0.0509	1.6917	1.6568	0.0366
	3.0060	0.0499	1.6600		
	3.0025	0.0486	1.6187		
14	3.0014	0.0258	0.8596	0.8770	0.0249
	3.0028	0.0260	0.8659		
	3.0036	0.0272	0.9056		
15	3.0028	0.0244	0.8126	0.8680	0.0481
	3.0042	0.0268	0.8921		
	3.0025	0.0270	0.8993		
			Grand mean	**1.1348**	
			Pooled SD	**0.0618**	

Table 33 Ethanol soluble extractive value of *L. japonica* flowering bud crude drug

Sources	Weight of crude drug (g)	Weight of extractable matter (g)	Amount (% by weight)	Mean	SD
1	5.0017	1.003	20.0532	20.1261	0.0695
	5.0016	1.007	20.1336		
	5.0021	1.010	20.1915		
2	5.0046	0.858	17.1442	17.1950	0.0476
	5.0062	0.863	17.2386		
	5.0023	0.861	17.2021		
3	5.0023	0.962	19.2312	19.6722	0.5245
	5.0017	0.977	19.5334		
	5.0044	1.014	20.2522		
4	5.0039	0.973	19.4448	19.6435	0.1764
	5.0015	0.986	19.7041		
	5.0021	0.990	19.7817		
5	5.0049	0.947	18.9215	19.1311	0.3510
	5.0065	0.948	18.9354		
	5.0035	0.978	19.5363		
6	5.0079	0.951	18.9900	19.0350	0.1961
	5.0079	0.964	19.2496		
	5.0039	0.944	18.8653		
7	5.0015	0.951	19.0043	18.7485	0.2342
	5.0090	0.937	18.6963		
	5.0068	0.929	18.5448		
8	5.0052	0.953	19.0402	19.3099	0.2410
	5.0012	0.970	19.3853		
	5.0015	0.976	19.5041		
9	5.0068	1.034	20.6519	20.5076	0.2679
	5.0078	1.012	20.1985		
	5.0091	1.036	20.6724		
10	5.0021	0.792	15.8333	15.9779	0.1508
	5.0043	0.799	15.9663		
	5.0018	0.807	16.1342		

Table 33 Ethanol soluble extractive value of *L. japonica* flowering bud crude drug (Cont.)

Sources	Weight of crude drug (g)	Weight of extractable matter (g)	Amount (% by weight)	Mean	SD
11	5.0079	0.701	13.9879	13.5135	0.4211
	5.0041	0.669	13.3690		
	5.0062	0.660	13.1837		
12	5.0066	0.661	13.2026	13.0457	0.1369
	5.0023	0.650	12.9840		
	5.0075	0.649	12.9506		
13	5.0072	0.204	4.0641	4.0561	0.0250
	5.0025	0.202	4.0280		
	5.0048	0.204	4.0761		
14	5.0025	0.693	13.8431	13.7502	0.0890
	5.0016	0.684	13.6656		
	5.0029	0.688	13.7420		
15	5.0045	0.659	13.1681	13.1437	0.1087
	5.0083	0.663	13.2380		
	5.0020	0.652	13.0248		
			Grand mean	**16.4571**	
			Pooled SD	**0.2448**	

Table 34 Water soluble extractive value of *L. japonica* flowering bud crude drug

Sources	Weight of crude drug (g)	Weight of extractable matter (g)	Amount (% by weight)	Mean	SD
1	5.0034	1.647	32.9176	32.5347	0.5885
	5.0016	1.642	32.8295		
	5.0083	1.596	31.8571		
2	5.0040	1.636	32.6839	32.5553	0.1537
	5.0020	1.631	32.5970		
	5.0023	1.620	32.3851		
3	5.0035	1.243	24.8426	25.0324	0.3509
	5.0086	1.243	24.8173		
	5.0084	1.274	25.4373		
4	5.0097	1.640	32.8463	32.7030	0.3262
	5.0016	1.617	32.3297		
	5.0056	1.649	32.9331		
5	5.0036	1.673	33.4359	33.4089	0.7330
	5.0057	1.635	32.6628		
	5.0047	1.708	34.1279		
6	5.0082	1.439	28.7229	29.1268	0.9415
	5.0078	1.513	30.2029		
	5.0027	1.424	28.4546		
7	5.0023	1.483	29.6464	29.7389	0.7848
	5.0044	1.452	29.0045		
	5.0023	1.529	30.5659		
8	5.0039	1.566	31.2856	30.8174	0.5448
	5.0037	1.549	30.9471		
	5.0067	1.548	30.2195		
9	5.0064	1.160	23.1703	23.7679	0.5181
	5.0039	1.206	24.0912		
	5.0037	1.203	24.0422		
10	5.0012	1.274	25.4739	25.2296	0.2163
	5.0055	1.255	25.0624		
	5.0015	1.258	25.1525		

Table 34 Water soluble extractive value of *L. japonica* flowering bud crude drug (Cont.)

Sources	Weight of crude drug (g)	Weight of extractable matter (g)	Amount (% by weight)	Mean	SD
11	5.0015	1.439	28.7714	29.1629	0.8691
	5.0038	1.429	28.5583		
	5.0035	1.509	30.1589		
12	5.0040	1.503	30.0360	30.7283	0.6063
	5.0041	1.551	30.9846		
	5.0025	1.559	31.1644		
13	5.0075	1.117	22.3065	22.5072	0.1863
	5.0088	1.129	22.5403		
	5.0056	1.135	22.6746		
14	5.0041	1.283	25.6290	26.1356	0.6548
	5.0065	1.346	26.8751		
	5.0091	1.298	25.9029		
15	5.0063	1.509	30.1420	29.7410	0.6075
	5.0031	1.453	29.0420		
	5.0035	1.503	30.0390		
			Grand mean	**28.8793**	
			Pooled SD	**0.5892**	

Table 35 Water content of *L. japonica* flowering bud crude drug

Sources	Weight of crude drug (g)	Water content (g)	Amount (% by weight)	Mean	SD
1	50.03	4.70	9.3944	9.5309	0.6124
	50.00	5.10	10.2000		
	50.01	4.50	8.9982		
2	50.03	6.30	12.5924	12.4625	0.6103
	50.01	6.50	12.9974		
	50.01	5.90	11.7976		
3	50.01	5.40	10.7978	11.4636	0.5766
	50.02	5.90	11.7953		
	50.01	5.90	11.7976		
4	50.02	5.50	10.9956	10.8631	0.6118
	50.01	5.70	11.3977		
	50.02	5.10	10.1959		
5	50.00	4.90	9.8000	9.6000	0.2000
	50.00	4.80	9.6000		
	50.00	4.70	9.4000		
6	50.00	5.20	10.4000	10.6000	0.2000
	50.00	5.40	10.8000		
	50.00	5.30	10.6000		
7	50.01	5.10	10.1980	10.5319	0.3064
	50.00	5.40	10.8000		
	50.01	5.30	10.5979		
8	50.01	5.10	10.1980	10.5979	0.5307
	50.02	5.20	10.3958		
	50.00	5.60	11.2000		
9	50.00	4.90	9.8000	10.4660	0.6108
	50.01	5.30	10.5979		
	50.00	5.50	11.0000		
10	50.00	6.40	12.8000	12.9307	0.4143
	50.02	6.70	13.3946		
	50.01	6.30	12.5975		

Table 35 Water content of *L. japonica* flowering bud crude drug (Cont.)

Sources	Weight of crude drug (g)	Water content (g)	Amount (% by weight)	Mean	SD
11	50.02	4.80	9.5962	10.1987	0.5313
	50.00	5.30	10.6000		
	50.00	5.20	10.4000		
12	50.01	5.20	10.3979	11.0645	0.5772
	50.01	5.70	11.3977		
	50.01	5.70	11.3977		
13	50.00	5.50	11.0000	10.6653	0.4171
	50.01	5.40	10.7978		
	50.01	5.10	10.1980		
14	50.01	5.10	10.1980	10.3327	0.2315
	50.00	5.10	10.2000		
	50.00	5.30	10.6000		
15	50.01	5.60	11.1978	10.9985	0.5279
	50.00	5.20	10.4000		
	50.01	5.70	11.3977		
			Grand mean	**10.8204**	
			Pooled SD	**0.4883**	

APPENDIX C

Cytotoxic activity (MTT cell viability)

Table 36 Cytotoxic activities of chlorogenic acid by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.422	0.562	0.638	0.474	0.524	101
10	0.561	0.665	0.673	0.560	0.615	119
1	0.487	0.622	0.637	0.558	0.576	111
0.1	0.468	0.630	0.599	0.535	0.558	108
0.01	0.554	0.489	0.527	0.533	0.526	101
DMSO	0.518	0.501	0.506	0.549	0.519	100
Control	0.647	0.550	0.717	0.661	0.644	

Concentration	ChaGo-K-1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.166	0.159	0.169	0.139	0.158	36
10	0.419	0.448	0.409	0.376	0.413	95
1	0.409	0.486	0.482	0.431	0.452	104
0.1	0.508	0.440	0.380	0.411	0.435	100
0.01	0.393	0.455	0.413	0.419	0.420	96
DMSO	0.448	0.442	0.420	0.434	0.436	100
Control	0.634	0.772	0.779	0.761	0.737	

Concentration	Hep G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.288	0.726	0.584	0.762	0.590	65
10	0.837	0.773	0.810	0.821	0.810	89
1	0.669	0.758	0.857	0.826	0.778	86
0.1	0.864	0.869	0.801	1.010	0.886	98
0.01	0.792	0.875	0.831	0.669	0.792	87
DMSO	0.823	0.925	0.978	0.900	0.907	100
Control	1.068	1.144	1.016	1.185	1.103	

Table 36 Cytotoxic activities of chlorogenic acid by MTT cell viability (Cont.)

Concentration	KATO III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.437	0.745	0.506	0.630	0.580	70
10	0.903	0.662	0.756	1.030	0.838	102
1	0.995	0.870	0.848	0.690	0.851	103
0.1	0.854	0.647	0.628	0.544	0.668	81
0.01	0.919	0.761	0.712	0.758	0.788	95
DMSO	0.814	0.808	0.841	0.837	0.825	100
Control	1.778	1.954	1.901	1.994	1.907	

Concentration	SW620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.554	0.551	0.411	0.909	0.606	33
10	1.691	1.314	1.682	1.626	1.578	85
1	1.622	1.629	1.447	1.491	1.547	83
0.1	1.842	1.706	1.542	1.608	1.675	90
0.01	1.643	1.599	1.723	1.907	1.718	93
DMSO	1.750	2.051	1.815	1.805	1.855	100
Control	2.193	2.616	2.494	2.817	2.530	

Concentration	WI-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.622	0.674	0.679	0.763	0.685	100
10	0.747	0.845	0.767	0.753	0.778	114
1	0.715	0.760	0.733	0.714	0.731	107
0.1	0.613	0.790	0.746	0.876	0.756	111
0.01	0.837	0.791	0.796	0.732	0.789	115
DMSO	0.702	0.757	0.674	0.701	0.684	100
	0.612	0.705	0.672	0.647		
Control	1.085	1.102	1.075	1.098	1.079	
	1.085	1.079	1.053	1.054		

Table 37 Cytotoxic activities of rosmarinic acid by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.332	0.167	0.211	0.156	0.217	42
10	0.501	0.517	0.578	0.671	0.567	109
1	0.610	0.572	0.581	0.625	0.597	115
0.1	0.442	0.375	0.524	0.584	0.481	93
0.01	0.505	0.400	0.562	0.482	0.487	94
DMSO	0.518	0.501	0.506	0.549	0.519	100
Control	0.647	0.550	0.717	0.661	0.644	

Concentration	ChaGo-K-1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.168	0.200	0.160	0.151	0.170	39
10	0.341	0.458	0.486	0.410	0.424	97
1	0.440	0.435	0.464	0.468	0.452	104
0.1	0.478	0.452	0.500	0.458	0.472	108
0.01	0.405	0.423	0.420	0.498	0.437	100
DMSO	0.448	0.442	0.420	0.434	0.436	100
Control	0.634	0.772	0.779	0.761	0.737	

Concentration	Hep G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.162	0.220	0.151	0.207	0.185	20
10	0.819	0.685	0.806	0.658	0.742	82
1	0.606	0.555	0.906	0.877	0.736	81
0.1	0.742	0.722	0.811	0.839	0.779	86
0.01	0.706	0.768	0.786	0.566	0.707	78
DMSO	0.823	0.925	0.978	0.900	0.907	100
Control	1.068	1.144	1.016	1.185	1.103	

Table 37 Cytotoxic activities of rosmarinic acid by MTT cell viability (Cont.)

Concentration	KATO III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.181	0.200	0.206	0.164	0.188	23
10	0.851	0.770	0.778	0.636	0.759	92
1	1.005	0.838	0.781	0.491	0.779	94
0.1	0.813	0.791	0.915	0.772	0.823	100
0.01	0.730	0.688	0.687	0.669	0.694	84
DMSO	0.814	0.808	0.841	0.837	0.825	100
Control	1.778	1.954	1.901	1.994	1.907	

Concentration	SW620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.243	0.216	0.183	0.167	0.202	11
10	1.790	1.271	1.110	1.503	1.419	76
1	1.770	1.623	1.931	1.747	1.768	95
0.1	1.678	1.396	1.670	1.778	1.631	88
0.01	1.716	1.574	1.549	1.625	1.616	87
DMSO	1.750	2.051	1.815	1.805	1.855	100
Control	2.193	2.616	2.494	2.817	2.530	

Concentration	WI-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.591	0.555	0.615	0.614	0.594	87
10	0.762	0.783	0.752	0.772	0.767	112
1	0.739	0.742	0.754	0.911	0.787	115
0.1	0.798	0.737	0.723	0.732	0.748	109
0.01	0.774	0.757	0.761	0.754	0.762	111
DMSO	0.702	0.757	0.674	0.701	0.684	100
	0.612	0.705	0.672	0.647		
Control	1.085	1.102	1.075	1.098	1.079	
	1.085	1.079	1.053	1.054		

Table 38 Cytotoxic activities of caffeic acid by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.302	0.369	0.211	0.324	0.302	58
10	0.118	0.106	0.124	0.123	0.118	23
1	0.547	0.574	0.554	0.473	0.537	104
0.1	0.562	0.568	0.528	0.481	0.535	103
0.01	0.618	0.552	0.526	0.549	0.561	108
DMSO	0.518	0.501	0.506	0.549	0.519	100
Control	0.647	0.550	0.717	0.661	0.644	

Concentration	ChaGo-K-1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.145	0.171	0.206	0.188	0.178	41
10	0.392	0.371	0.401	0.452	0.404	93
1	0.409	0.373	0.416	0.530	0.432	99
0.1	0.462	0.470	0.445	0.528	0.476	109
0.01	0.334	0.351	0.365	0.340	0.348	80
DMSO	0.448	0.442	0.420	0.434	0.436	100
Control	0.634	0.772	0.779	0.761	0.737	

Concentration	Hep G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.225	0.173	0.195	0.272	0.216	24
10	0.988	0.955	0.921	0.869	0.933	103
1	1.020	0.964	0.920	0.696	0.900	99
0.1	0.686	1.001	0.803	0.991	0.870	96
0.01	0.684	0.899	0.734	0.941	0.815	90
DMSO	0.823	0.925	0.978	0.900	0.907	100
Control	1.068	1.144	1.016	1.185	1.103	

Table 38 Cytotoxic activities of caffeic acid by MTT cell viability (Cont.)

Concentration	KATO III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.330	0.253	0.275	0.286	0.286	35
10	0.921	0.963	0.975	1.056	0.979	119
1	0.839	0.896	0.843	0.937	0.879	107
0.1	0.796	0.693	0.857	0.890	0.809	98
0.01	0.614	0.573	0.860	1.039	0.772	94
DMSO	0.814	0.808	0.841	0.837	0.825	100
Control	1.778	1.954	1.901	1.994	1.907	

Concentration	SW620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.181	0.177	0.190	0.238	0.197	11
10	1.183	1.882	1.624	1.735	1.606	87
1	1.334	1.559	1.840	1.729	1.616	87
0.1	1.330	1.465	1.685	1.643	1.531	83
0.01	1.383	1.465	1.662	1.709	1.555	84
DMSO	1.750	2.051	1.815	1.805	1.855	100
Control	2.193	2.616	2.494	2.817	2.530	

Concentration	WI-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.375	0.239	0.316	0.233	0.291	43
10	0.780	0.625	0.680	0.660	0.686	100
1	0.751	0.687	0.720	0.727	0.721	105
0.1	0.680	0.664	0.690	0.690	0.681	100
0.01	0.791	0.687	0.733	0.701	0.728	106
DMSO	0.702	0.757	0.674	0.701	0.684	100
	0.612	0.705	0.672	0.647		
Control	1.085	1.102	1.075	1.098	1.079	
	1.085	1.079	1.053	1.054		

Table 39 Cytotoxic activities of *L. japonica* flowering bud by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.612	0.603	0.599	0.568	0.596	105
10	0.428	0.593	0.553	0.411	0.496	88
1	0.465	0.743	0.640	0.518	0.592	105
0.1	0.458	0.596	0.466	0.457	0.494	87
0.01	0.687	0.681	0.508	0.574	0.613	108
DMSO	0.566	0.607	0.511	0.574	0.566	100
	0.547	0.600	0.529	0.594		
Control	0.585	0.755	0.785	0.702	0.694	
	0.559	0.728	0.763	0.674		

Concentration	ChaGo-K-1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.660	0.575	0.657	0.749	0.660	108
10	0.754	0.555	0.626	0.660	0.649	106
1	0.575	0.741	0.572	0.551	0.610	100
0.1	0.616	0.074	0.548	0.480	0.429	70
0.01	0.605	0.726	0.545	0.469	0.586	96
DMSO	0.625	0.865	0.539	0.489	0.612	100
	0.622	0.643	0.583	0.532		
Control	0.846	1.006	0.835	0.782	0.927	
	0.974	1.111	1.054	0.811		

Concentration	Hep G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.876	0.923	0.921	0.885	0.901	106
10	0.788	0.921	0.908	0.835	0.863	102
1	0.773	1.019	0.840	0.716	0.837	99
0.1	0.795	0.860	0.918	0.884	0.864	102
0.01	0.785	1.029	0.874	0.710	0.850	100
DMSO	0.839	0.985	0.931	0.970	0.847	100
	0.731	0.729	0.759	0.832		
Control	1.166	1.180	1.075	1.091	1.127	
	1.062	1.176	1.086	1.181		

Table 39 Cytotoxic activities of *L. japonica* flowering bud by MTT cell viability (Cont.)

Concentration	KATO III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.917	0.843	0.793	0.798	0.838	100
10	1.037	1.085	1.087	1.023	1.058	126
1	0.969	0.979	0.763	0.932	0.911	109
0.1	0.629	0.899	0.834	0.820	0.796	95
0.01	0.745	0.879	0.841	0.734	0.800	96
DMSO	0.749	0.794	0.692	0.824	0.836	100
	0.929	0.921	0.926	0.856		
Control	1.512	1.516	1.655	1.452	1.589	
	1.458	1.653	1.678	1.790		

Concentration	SW620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	1.134	0.977	0.950	1.212	1.068	107
10	1.152	1.032	1.084	1.183	1.113	111
1	1.162	1.150	1.107	0.865	1.071	107
0.1	0.868	0.945	0.923	0.889	0.906	91
0.01	0.911	0.995	0.914	0.894	0.929	93
DMSO	1.351	1.138	0.897	0.909	1.000	100
	1.011	0.956	0.858	0.881		
Control	1.018	1.168	1.144	1.107	1.072	
	1.080	1.044	1.060	0.956		

Concentration	WI-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.571	0.594	0.565	0.581	0.578	116
10	0.703	0.693	0.643	0.548	0.647	130
1	0.607	0.749	0.688	0.737	0.695	140
0.1	0.578	0.794	0.691	0.510	0.643	130
0.01	0.994	1.076	1.092	0.877	1.010	203
DMSO	0.543	0.506	0.429	0.520	0.496	100
	0.551	0.489	0.420	0.513		
Control	0.920	0.998	1.058	1.087	1.005	
	0.974	0.959	1.120	0.924		

Table 40 Cytotoxic activities of doxorubicin by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.106	0.103	0.092	0.085	0.097	15
1	0.126	0.127	0.124	0.108	0.121	19
0.1	0.563	0.535	0.537	0.385	0.505	78
0.01	0.687	0.664	0.587	0.590	0.632	98
0.001	0.782	0.805	0.674	0.692	0.738	115
Control	0.647	0.550	0.717	0.661	0.644	100

Concentration	ChaGo-K-1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.079	0.086	0.085	0.088	0.085	11
1	0.138	0.146	0.148	0.125	0.139	19
0.1	0.603	0.664	0.635	0.653	0.639	87
0.01	0.865	0.815	0.741	0.738	0.790	107
0.001	0.778	0.754	0.802	0.660	0.749	102
Control	0.634	0.772	0.779	0.761	0.737	100

Concentration	Hep G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.325	0.397	0.407	0.363	0.373	34
1	0.086	0.071	0.074	0.092	0.081	7
0.1	0.581	0.565	0.573	0.523	0.561	51
0.01	1.078	1.263	1.138	1.164	1.161	105
0.001	1.193	1.043	1.219	1.378	1.208	110
Control	1.068	1.144	1.016	1.185	1.103	100

Table 40 Cytotoxic activities of doxorubicin by MTT cell viability (Cont.)

Concentration	KATO III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.532	0.487	0.496	0.519	0.509	27
1	0.564	0.534	0.580	0.564	0.561	29
0.1	0.698	0.942	0.920	1.125	0.921	48
0.01	1.932	2.011	1.876	2.175	1.999	105
0.001	1.707	1.558	2.089	2.392	1.937	102
Control	1.778	1.954	1.901	1.994	1.907	100

Concentration	SW620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.088	0.096	0.095	0.087	0.092	4
1	0.214	0.213	0.221	0.189	0.209	8
0.1	0.551	0.646	0.483	0.445	0.531	21
0.01	1.867	1.861	2.033	1.769	1.883	74
0.001	2.649	2.981	2.695	2.350	2.669	105
Control	2.193	2.616	2.494	2.817	2.530	100

Concentration	WI-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.269	0.289	0.332	0.293	0.296	34
1	0.232	0.200	0.250	0.225	0.227	26
0.1	0.506	0.514	0.607	0.562	0.547	62
0.01	0.888	0.930	0.902	0.820	0.885	101
0.001	1.066	0.782	0.792	0.766	0.852	97
Control	0.856	0.858	0.842	0.965	0.879	100
	0.853	0.825	0.849	0.986		

APPENDIX D

DNA Damage (Comet assay)

Table 41 Total scores of DNA damage in human lymphocyte cells

Concentrations (µg/ml)	Extract	Chlorogenic acid	Rosmarinic acid	Caffeic acid	H_2O_2	PBS	2% DMSO
25	222	298	190	147	400	-	-
	207	284	202	146	400	-	-
	219	303	200	131	400	-	-
Mean	216.00	295.00	197.33	141.33	400.00	-	-
SD	7.94	9.85	6.43	8.96	0.00	-	-
50	256	386	239	185	-	-	-
	273	369	235	182	-	-	-
	265	381	236	193	-	-	-
Mean	264.67	378.67	236.67	186.67	-	-	-
SD	8.50	8.74	2.08	5.69	-	-	-
100	320	400	283	226	-	-	-
	328	400	298	222	-	-	-
	312	400	281	218	-	-	-
Mean	320.00	400.00	287.33	222.00	-	-	-
SD	8.00	0.00	9.29	4.00	-	-	-
0	-	-	-	-	-	160	119
	-	-	-	-	-	170	128
	-	-	-	-	-	157	130
Mean	-	-	-	-	-	162.33	125.67
SD	-	-	-	-	-	6.81	5.86

APPENDIX E

Antioxidant activities

Table 42 DPPH radical scavenging activity of chlorogenic acid

Concentration	OD_{517}			DPPH inhibition (%)				
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
1.25	0.159	0.173	0.177	20.896	13.930	11.940	15.589	4.702
2.5	0.141	0.158	0.159	29.851	21.393	20.896	24.046	5.033
5	0.122	0.127	0.129	39.303	36.816	35.821	37.313	1.794
10	0.074	0.086	0.090	63.184	57.214	55.224	58.541	4.143
20	0.019	0.022	0.022	90.547	89.055	89.055	89.552	0.862

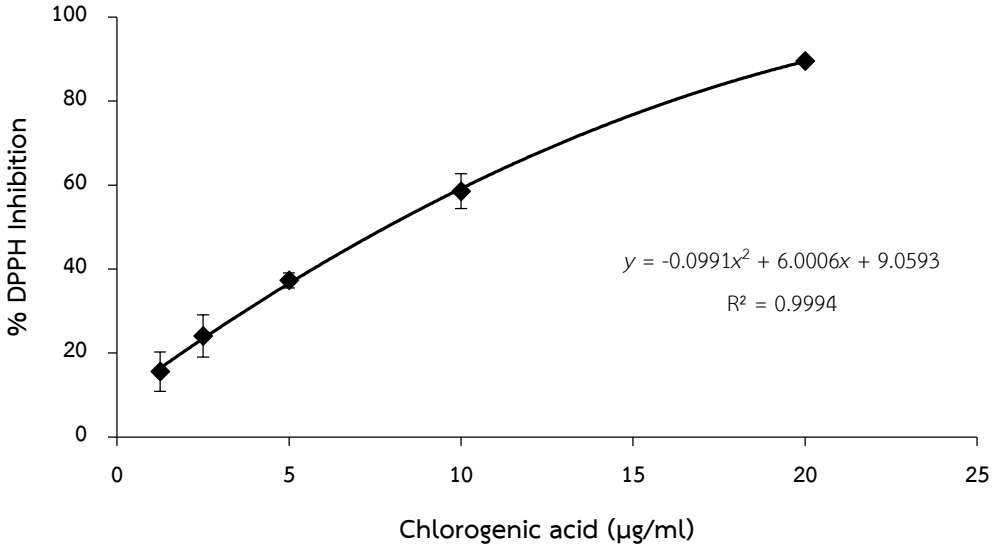

Figure 140 Percent DPPH inhibition of chlorogenic acid

Table 43 DPPH radical scavenging activity of rosmarinic acid

Concentration	OD_{517}			DPPH inhibition (%)				
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
0.625	0.185	0.186	0.189	7.960	7.463	5.970	7.131	1.036
1.25	0.171	0.172	0.175	14.925	14.428	12.935	14.096	1.036
2.5	0.163	0.165	0.164	18.905	17.910	18.408	18.408	0.498
5	0.112	0.111	0.113	44.279	44.776	43.781	44.279	0.498
10	0.040	0.039	0.042	80.100	80.597	79.104	79.934	0.760

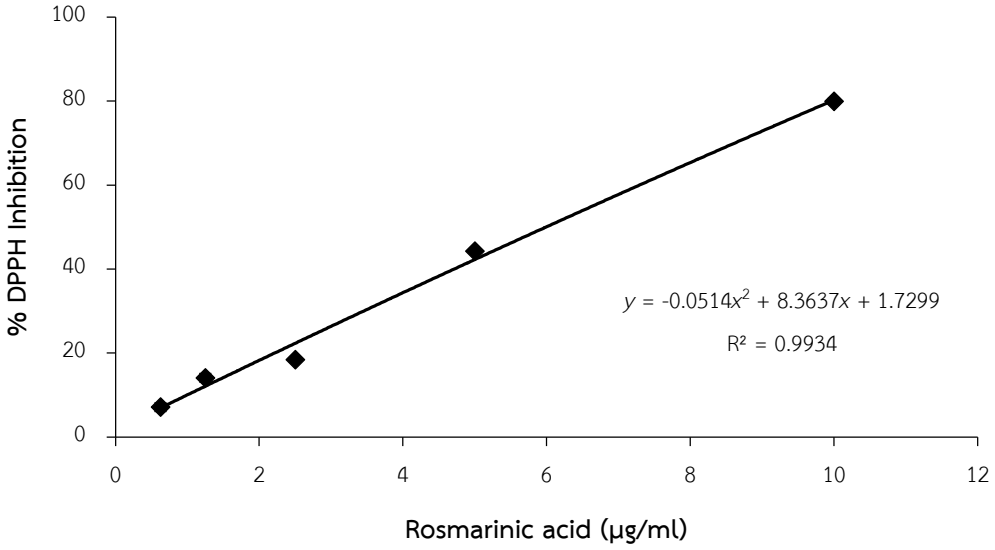

Figure 141 Percent DPPH inhibition of rosmarinic acid

Table 44 DPPH radical scavenging activity of caffeic acid

Concentration	OD_{517}			DPPH inhibition (%)				
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
0.625	0.189	0.189	0.188	5.970	5.970	6.468	6.136	0.287
1.25	0.171	0.177	0.175	14.925	11.940	12.935	13.267	1.520
2.5	0.153	0.152	0.152	23.881	24.378	24.378	24.212	0.287
5	0.080	0.078	0.079	60.199	61.194	60.697	60.697	0.498
10	0.019	0.019	0.019	90.547	90.547	90.547	90.547	0.000

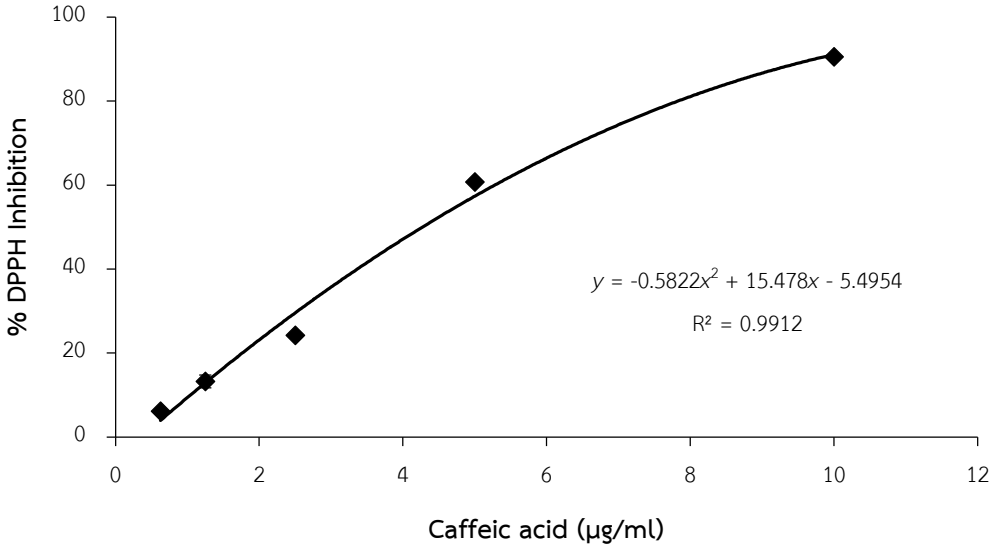

Figure 142 Percent DPPH inhibition of caffeic acid

Table 45 DPPH radical scavenging activity of *L. japonica* flowering bud ethanolic extract

Concentration	OD_{517}			DPPH inhibition (%)				
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
9.375	0.185	0.181	0.175	7.960	9.950	12.935	10.282	2.504
18.75	0.164	0.159	0.155	18.408	20.896	22.886	20.730	2.243
37.5	0.136	0.136	0.133	32.338	32.338	33.831	32.836	0.862
75	0.070	0.069	0.070	65.174	65.672	65.174	65.340	0.287
150	0.020	0.019	0.018	90.050	90.547	91.045	90.547	0.498

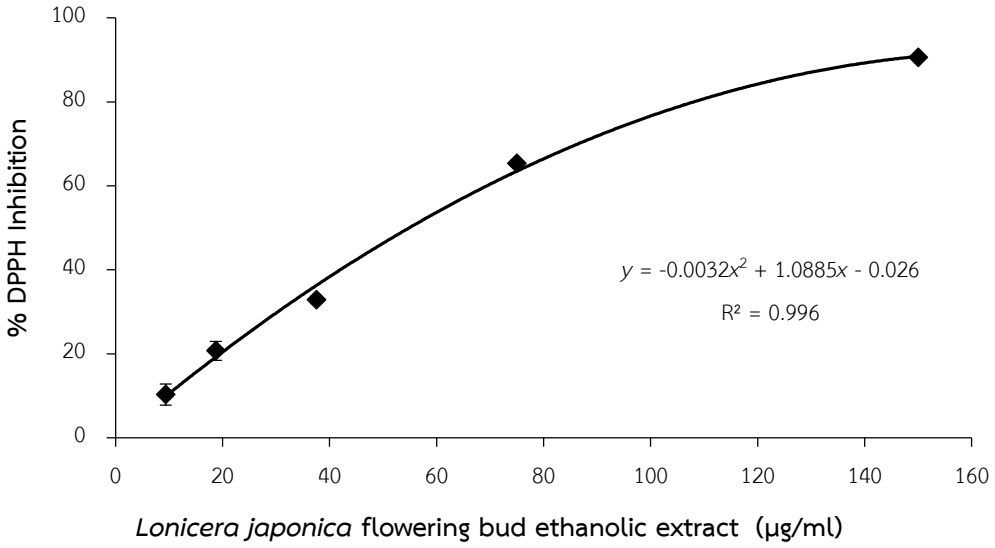

Figure 143 Percent DPPH inhibition of *L. japonica* flowering bud ethanolic extract

Table 46 DPPH radical scavenging activity of quercetin

Concentration	OD_{517}			DPPH inhibition (%)				
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
0.625	0.181	0.186	0.189	9.950	7.463	5.970	7.794	2.011
1.25	0.171	0.176	0.175	14.925	12.438	12.935	13.433	1.316
2.5	0.138	0.143	0.147	31.343	28.856	26.866	29.022	2.243
5	0.090	0.103	0.104	55.224	48.756	48.259	50.746	3.886
10	0.018	0.020	0.020	91.045	90.050	90.050	90.381	0.574

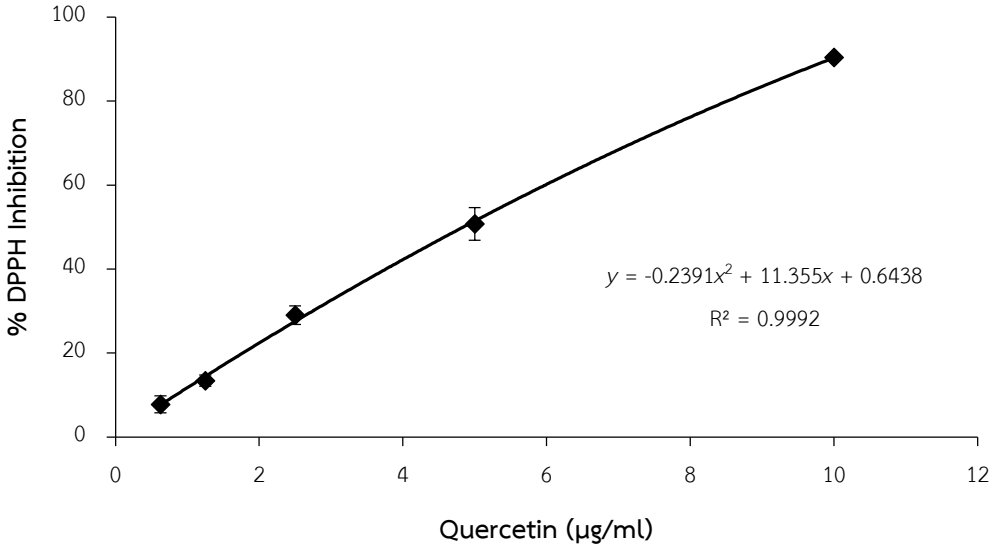

Figure 144 Percent DPPH inhibition of quercetin

Table 47 DPPH radical scavenging activity of butylated hydroxytoluene (BHT)

Concentration	OD_{517}			DPPH inhibition (%)				
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
3.125	0.177	0.183	0.171	11.940	8.955	14.925	11.940	2.985
6.25	0.171	0.164	0.169	14.925	18.408	15.920	16.418	1.794
12.5	0.137	0.141	0.141	31.841	29.851	29.851	30.514	1.149
25	0.100	0.099	0.101	50.249	50.746	49.751	50.249	0.498
50	0.053	0.051	0.056	73.632	74.627	72.139	73.466	1.252

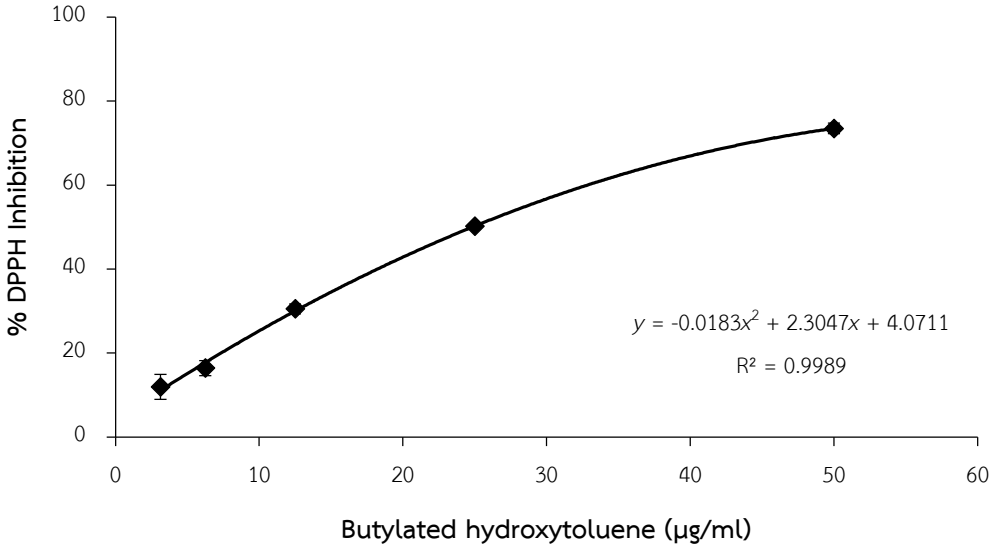

Figure 145 Percent DPPH inhibition of butylated hydroxytoluene (BHT)

Table 48 FRAP value of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls

Tested samples	OD_{593}			Ferrous sulphate equivalent (mM)				
(1 mg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
Extract	0.636	0.614	0.631	1.254	1.210	1.244	1.236	0.023
Chlorogenic acid	0.593	0.584	0.608	1.168	1.150	1.198	1.172	0.024
Rosmarinic acid	0.778	0.791	0.815	1.537	1.563	1.611	1.570	0.037
Caffeic acid	0.639	0.631	0.750	1.260	1.244	1.481	1.328	0.133
Quercetin	0.732	0.737	0.782	1.445	1.455	1.545	1.482	0.055
BHT	0.757	0.770	0.759	1.495	1.521	1.499	1.505	0.014

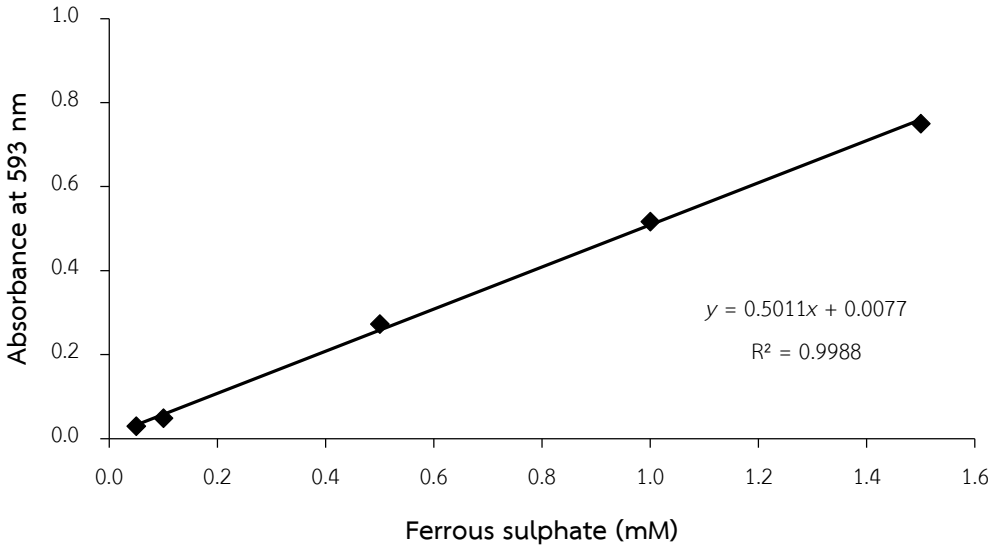

Figure 146 Calibration curve of $FeSO_4$ that used for calculated the FRAP value of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls

Table 49 Nitric oxide inhibition of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive control

Tested samples	OD_{450}			Nitric oxide inhibition (%)				
(800 µg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
Extract	0.184	0.181	0.184	49.589	50.411	49.589	49.863	0.475
Chlorogenic acid	0.071	0.061	0.066	74.150	77.791	75.971	75.971	1.820
Rosmarinic acid	0.050	0.043	0.044	67.532	72.078	71.429	70.346	2.458
Caffeic acid	0.087	0.082	0.080	63.031	65.156	66.006	64.731	1.532
Quercetin	0.087	0.076	0.083	70.575	74.295	71.928	72.266	1.883

Table 50 Beta-carotene bleaching of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls

Tested sample (0.25 mg/ml)	Time	OD_{470}			Beta-carotene bleaching (%)	
		Exp 1	Exp 2	Exp 3	Mean	SD
Extract	0	1.097	1.089	1.082		
	30	0.465	0.499	0.459		
	60	0.105	0.123	0.112		
	90	0.048	0.045	0.041		
	120	0.039	0.033	0.040	0.622	1.573
Chlorogenic acid	0	1.047	1.049	1.045		
	30	0.681	0.692	0.643		
	60	0.327	0.342	0.310		
	90	0.172	0.192	0.185		
	120	0.095	0.112	0.117	11.296	1.724
Rosmarinic acid	0	1.117	1.108	1.108		
	30	0.746	0.756	0.760		
	60	0.416	0.436	0.471		
	90	0.254	0.273	0.308		
	120	0.117	0.152	0.170	8.865	3.544
Caffeic acid	0	1.092	1.091	1.099		
	30	0.753	0.749	0.747		
	60	0.409	0.404	0.404		
	90	0.241	0.232	0.219		
	120	0.138	0.130	0.136	9.381	0.512
Quercetin	0	1.112	1.102	1.102		
	30	0.919	0.925	0.915		
	60	0.788	0.807	0.802		
	90	0.689	0.715	0.710		
	120	0.648	0.681	0.670	58.528	2.224
BHT	0	1.051	1.043	1.054		
	30	0.995	0.998	1.001		
	60	0.943	0.950	0.948		
	90	0.906	0.904	0.914		
	120	0.897	0.905	0.877	85.241	1.745
Control	0	1.089	1.085	1.097		
	30	0.262	0.266	0.259		
	60	0.051	0.052	0.049		
	90	0.042	0.042	0.041		
	120	0.035	0.031	0.029	-	-

Table 50 Beta-carotene bleaching of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls (Cont.)

Tested sample (0.5 mg/ml)	Time	OD_{470}			Beta-carotene bleaching (%)	
		Exp 1	Exp 2	Exp 3	Mean	SD
Extract	0	1.148	1.150	1.129		
	30	0.638	0.634	0.617		
	60	0.242	0.249	0.222		
	90	0.111	0.115	0.089		
	120	0.058	0.063	0.057	2.307	1.680
Chlorogenic acid	0	1.094	1.108	1.099		
	30	0.814	0.822	0.832		
	60	0.547	0.504	0.432		
	90	0.250	0.330	0.389		
	120	0.275	0.205	0.140	15.609	6.146
Rosmarinic acid	0	1.091	1.090	1.086		
	30	0.855	0.842	0.853		
	60	0.615	0.610	0.619		
	90	0.460	0.462	0.454		
	120	0.336	0.340	0.348	29.370	1.345
Caffeic acid	0	1.087	1.086	1.083		
	30	0.844	0.845	0.844		
	60	0.577	0.576	0.584		
	90	0.409	0.418	0.423		
	120	0.291	0.298	0.284	24.968	0.425
Quercetin	0	1.075	1.057	1.062		
	30	0.937	0.943	0.942		
	60	0.839	0.845	0.837		
	90	0.752	0.760	0.753		
	120	0.711	0.724	0.714	67.096	1.496
BHT	0	1.101	1.081	1.073		
	30	1.055	1.046	1.033		
	60	1.023	1.015	1.008		
	90	0.978	0.979	0.972		
	120	0.970	0.976	0.979	89.603	1.852
Control	0	1.089	1.085	1.097		
	30	0.262	0.266	0.259		
	60	0.051	0.052	0.049		
	90	0.042	0.042	0.041		
	120	0.035	0.031	0.029	-	-

Table 50 Beta-carotene bleaching of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls (Cont.)

Tested sample (1.0 mg/ml)	Time	OD_{470}			Beta-carotene bleaching (%)	
		Exp 1	Exp 2	Exp 3	Mean	SD
Extract	0	1.159	1.179	1.157		
	30	0.761	0.741	0.731		
	60	0.428	0.421	0.385		
	90	0.253	0.247	0.207		
	120	0.140	0.141	0.124	2.705	1.028
Chlorogenic acid	0	1.104	1.116	1.109		
	30	0.920	0.936	0.929		
	60	0.696	0.725	0.756		
	90	0.546	0.583	0.635		
	120	0.563	0.460	0.406	40.203	7.550
Rosmarinic acid	0	1.108	1.109	1.103		
	30	0.975	0.973	0.971		
	60	0.822	0.826	0.825		
	90	0.716	0.716	0.701		
	120	0.615	0.616	0.618	53.680	0.786
Caffeic acid	0	1.097	1.095	1.101		
	30	0.947	0.945	0.941		
	60	0.798	0.774	0.782		
	90	0.683	0.653	0.652		
	120	0.575	0.547	0.560	49.276	1.235
Quercetin	0	1.102	1.107	1.109		
	30	1.031	1.025	1.018		
	60	0.963	0.959	0.960		
	90	0.900	0.896	0.895		
	120	0.877	0.877	0.869	78.120	0.565
BHT	0	1.107	1.099	1.103		
	30	1.076	1.077	1.076		
	60	1.055	1.051	1.045		
	90	1.019	1.020	1.028		
	120	1.018	1.015	1.016	91.813	0.240
Control	0	1.089	1.085	1.097		
	30	0.262	0.266	0.259		
	60	0.051	0.052	0.049		
	90	0.042	0.042	0.041		
	120	0.035	0.031	0.029	-	-

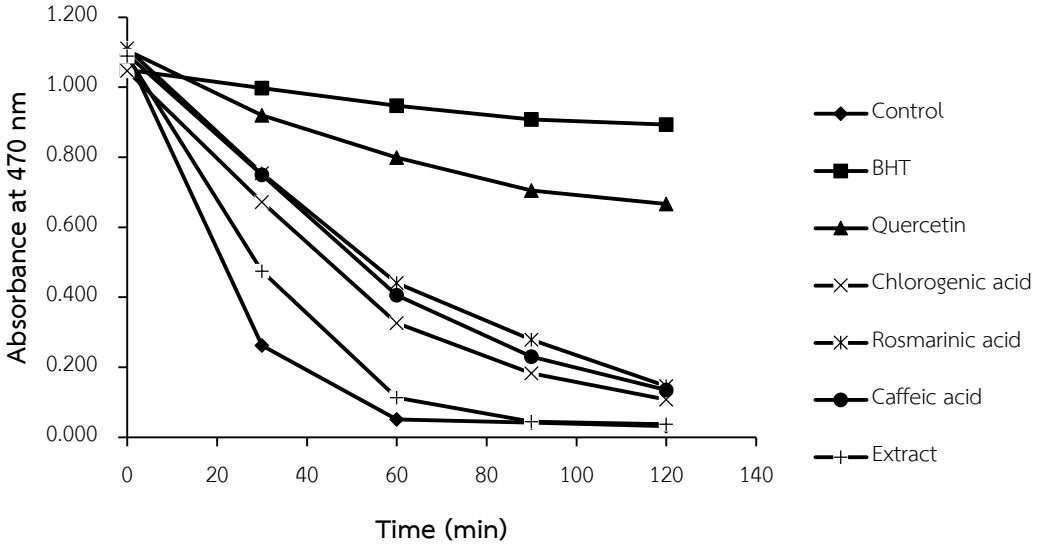

Figure 147 The absorbance of beta-carotene bleaching of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls at 0.25 mg/ml

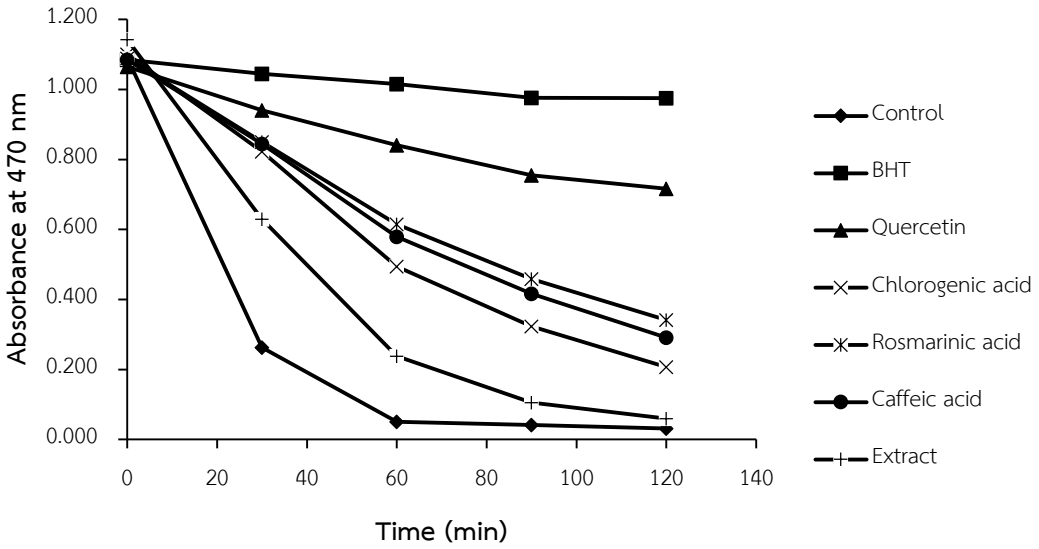

Figure 148 The absorbance of beta-carotene bleaching of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls at 0.5 mg/ml

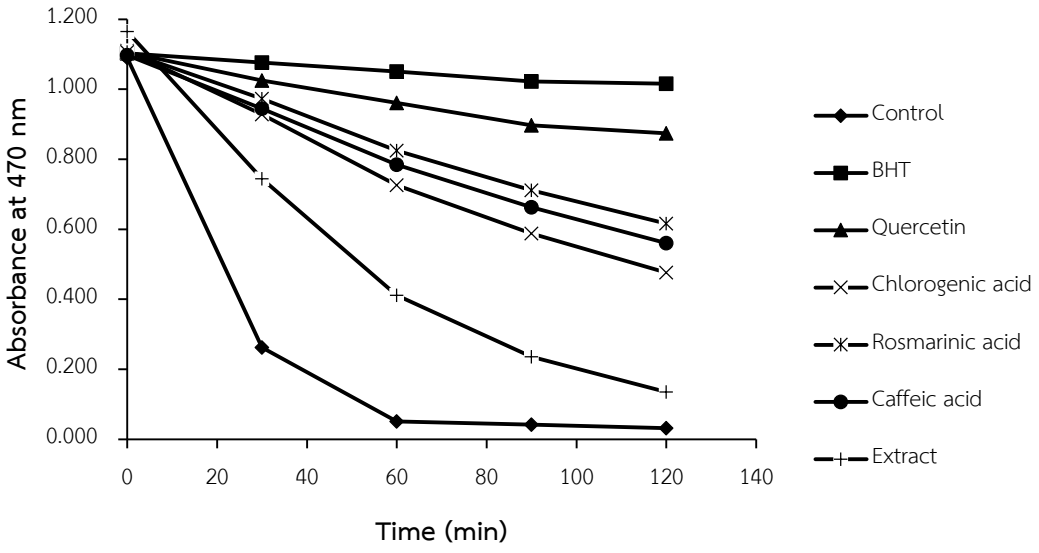

Figure 149 The absorbance of beta-carotene bleaching of *L. japonica* flowering bud ethanolic extract, standard chlorogenic, rosmarinic, caffeic acids and positive controls at 1.0 mg/ml

APPENDIX F

Antidiabetic activity

Table 51 Yeast alpha-glucosidase inhibition of *L. japonica* flowering bud ethanolic extract

Concentration	OD_{405}			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
DMSO	0.314	0.314	0.309					
5	0.305	0.287	0.279	2.866	8.599	9.709	7.058	3.672
10	0.248	0.258	0.241	21.019	17.834	22.006	20.287	2.180
15	0.202	0.206	0.191	35.669	34.395	38.188	36.084	1.930
20	0.150	0.167	0.159	52.229	46.815	48.544	49.196	2.765
25	0.125	0.104	0.121	60.191	66.879	60.841	62.637	3.688

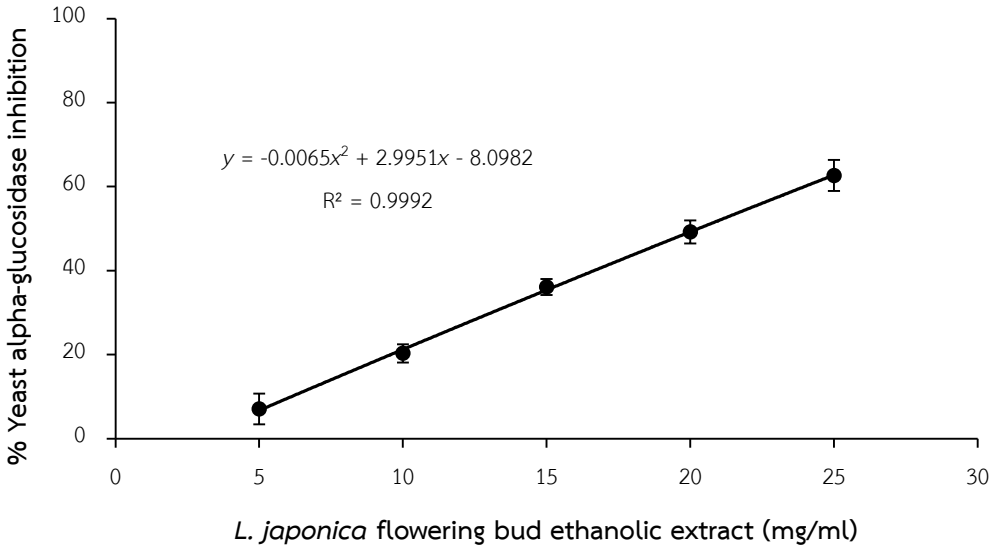

Figure 150 Percent yeast alpha-glucosidase inhibition of *L. japonica* flowering bud ethanolic extract

Table 52 Yeast alpha-glucosidase inhibition of chlorogenic acid

Concentration	OD_{405}			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
DMSO	0.356	0.361	0.368					
10	0.319	0.321	0.322	10.393	11.080	12.500	11.325	1.074
15	0.266	0.265	0.277	25.281	26.593	24.728	25.534	0.958
20	0.241	0.237	0.239	32.303	34.349	35.054	33.902	1.429
25	0.194	0.183	0.167	45.506	49.307	54.620	49.811	4.578
30	0.119	0.119	0.117	66.573	67.036	68.207	67.272	0.842

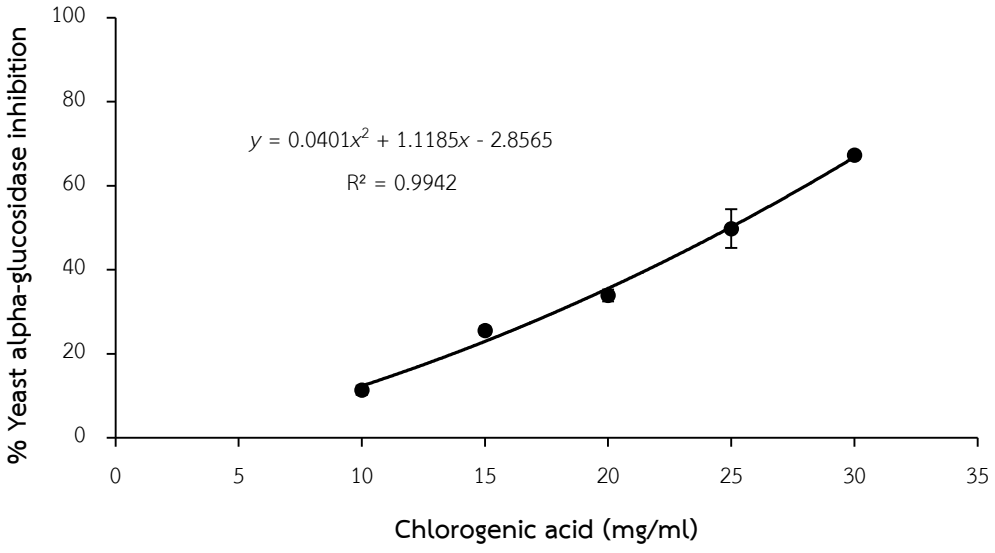

Figure 151 Percent yeast alpha-glucosidase inhibition of chlorogenic acid

Table 53 Yeast alpha-glucosidase inhibition of rosmarinic acid

Concentration	OD_{405}			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
DMSO	0.359	0.354	0.354					
5	0.214	0.215	0.229	40.390	39.266	35.311	38.322	2.668
10	0.166	0.170	0.172	53.760	51.977	51.412	52.383	1.226
15	0.071	0.101	0.106	80.223	71.469	70.056	73.916	5.507
20	0.054	0.046	0.053	84.958	87.006	85.028	85.664	1.162
25	0.035	0.046	0.031	90.251	87.006	91.243	89.500	2.216

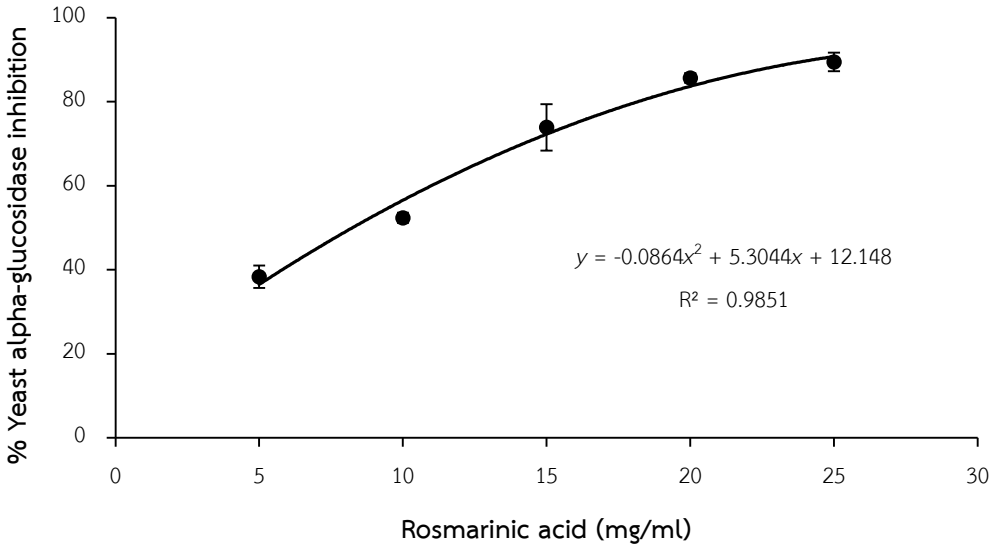

Figure 152 Percent yeast alpha-glucosidase inhibition of rosmarinic acid

Table 54 Yeast alpha-glucosidase inhibition of caffeic acid

Concentration	OD_{405}			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
DMSO	0.359	0.354	0.354					
5	0.315	0.317	0.310	12.256	10.452	12.429	11.713	1.095
10	0.251	0.250	0.248	30.084	29.379	29.944	29.802	0.373
15	0.138	0.129	0.132	61.560	63.559	62.712	62.610	1.004
20	0.020	0.074	0.038	94.429	79.096	89.266	87.597	7.801
25	0.017	0.018	0.004	95.265	94.915	98.870	96.350	2.189

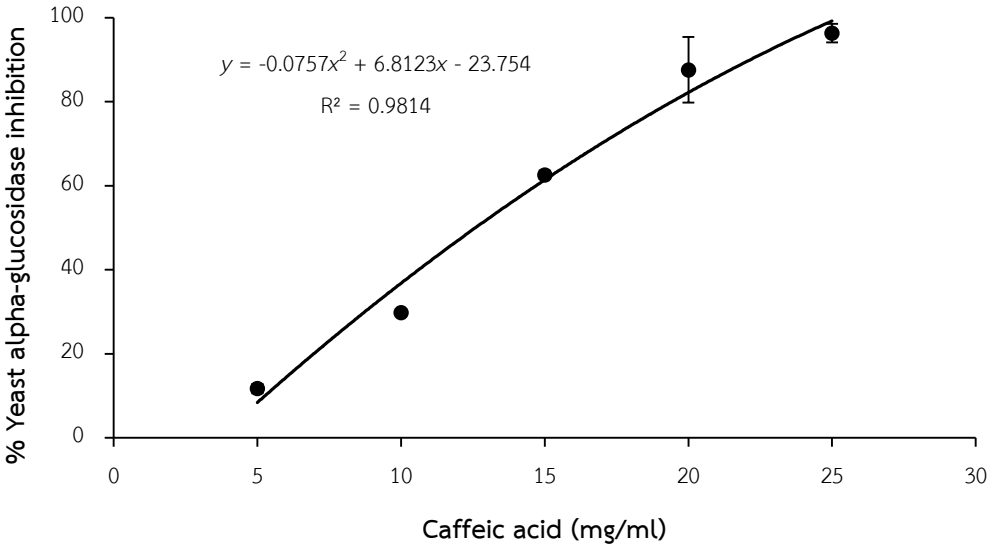

Figure 153 Percent yeast alpha-glucosidase inhibition of caffeic acid

Table 55 Yeast alpha-glucosidase inhibition of acarbose

Concentration	OD_{405}			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Mean	SD
DMSO	0.356	0.361	0.368					
2.5	0.304	0.316	0.303	14.607	12.465	17.663	14.912	2.612
5	0.233	0.222	0.219	34.551	38.504	40.489	37.848	3.023
10	0.200	0.184	0.179	43.820	49.030	51.359	48.070	3.860
15	0.156	0.138	0.149	56.180	61.773	59.511	59.154	2.814
20	0.110	0.116	0.118	69.101	67.867	67.935	68.301	0.694

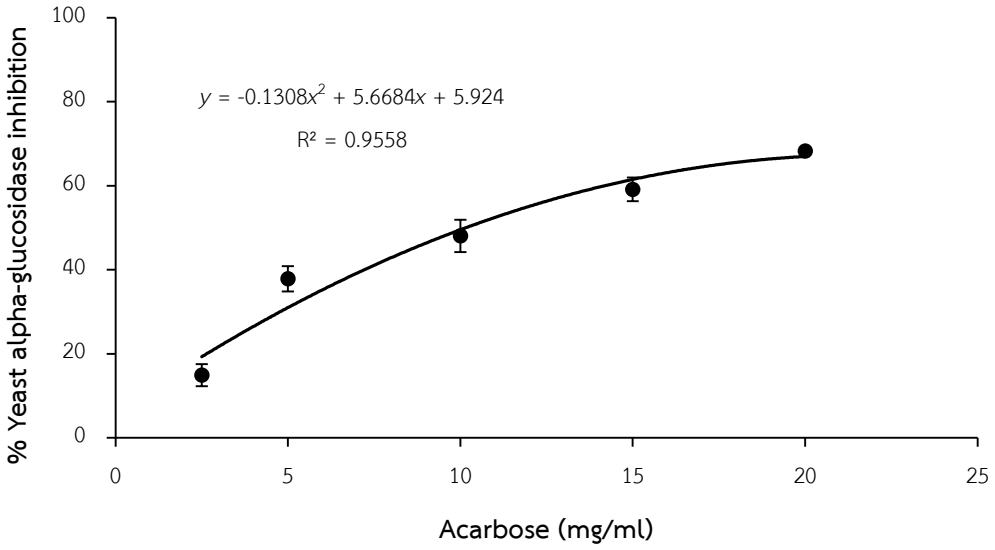

Figure 154 Percent yeast alpha-glucosidase inhibition of acarbose

VITA

Mr. Chayanon Chaowuttikul was born on July 16, 1988 in Trang, Thailand. He received his Bachelor's degree in Science (Thai Traditional Medicine) from Prince of Songkla University in 2011 and got a Master of Science in 2013 from College of Public Health Sciences, Chulalongkorn University, Thailand.

Publication

Chaowuttikul, C., C. Palanuvej, and N. Ruangrungsi, Pharmacognostic specification, chlorogenic acid content, and In vitro antioxidant activities of Lonicera japonica flowering bud. Pharmacognosy Research, 2017. 9(2): p. 128-132

Oral presentation

Chayanon Chaowuttikul, Chanida Palanuvej and Nijsiri Ruangrungsi. “Pharmacognostic specification of Lonicera japonica flowering bud and quantitative analysis of chlorogenic acid by thin layer chromatography-densitometry” Mae Fah Luang University International Conference on “Advance in Medical and Health Sciences” and Kaleidoscope of Traditional and Complementary Medicines International Conference on “Fostering Traditional and Complementary Medicine Through Research”, November 23-25, 2016, Mae Fah Luang University, Chiang Rai, Thailand.

Chayanon Chaowuttikul, Chanida Palanuvej and Nijsiri Ruangrungsi. “Chlorogenic acid content in Lonicera japonica flowering bud by quantitative thin-layer chromatography” IASTEM - 226th International Conference on Medical, Biological and Pharmaceutical Sciences (ICMBPS), August 6-7, 2017 at Kobe, Japan.