PHARMACOGNOSTIC SPECIFICATIONS AND CHLOROGENIC ACID CONTENT OF *MORUS ALBA* LINN. LEAVES IN THAILAND

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ข้อกำหนดทางเภสัชเวทและปริมาณกรดคลอโรจีนิกของใบหม่อนในประเทศไทย

น.ส.พิมพ์กุล ไอยรากาญจนกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PHARMACOGNOSTIC SPECIFICATIONS AND CHLOROGENIC ACID CONTENT OF *MORUS ALBA* LINN. LEAVES IN THAILAND
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Field of Study	Public Health Sciences
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พิมพ์กุล ไอยรากาญจนกุล : ข้อกำหนดทางเภสัชเวทและปริมาณกรดคลอโรจีนิกของใบหม่อนในประเทศไทย . (PHARMACOGNOSTIC SPECIFICATIONS AND CHLOROGENIC ACID CONTENT OF *MORUS ALBA* LINN. LEAVES IN THAILAND) อ.ที่ปรึกษาหลัก : ดร.อนุชิต ภานุมาสวิวัฒน์

ต้นหม่อน (*Morus alba* Linn.) ซึ่งอยู่ในวงศ์ Moraceae เป็นสมุนไพรที่รู้จักกันอย่างแพร่หลาย ใบหม่อนเป็นสมุนไพรที่ใช้ในตำรับยาแพทย์แผนจีนและแพทย์แผนไทยเพื่อการรักษาไข้หวัดที่เกิดจากลมร้อน เจ็บคอ ไอ ตาอักเสบ เป็นต้น การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาข้อกำหนดทางเภสัชเวทและวิเคราะห์ปริมาณกรดคลอโรจีนิกในใบหม่อน โดยทำการเก็บรวบรวมตัวอย่างใบหม่อนจาก 15 แหล่งที่แตกต่างกันในประเทศไทย และได้ศึกษาลักษณะทางมหทรรศน์และจุลทรรศน์ของใบพืชสด สำหรับรายละเอียดทางพฤกษศาสตร์นำเสนอในรูปแบบลายเส้น ในส่วนของการศึกษาลักษณะทางจุลทรรศน์ได้แสดงรายละเอียดของค่าคงที่ของแผ่นใบและภาคตัดขวางของเส้นกลางใบ ใบหม่อนถูกทำให้แห้งเพื่อนำมาวิเคราะห์ข้อกำหนดทางเภสัชเวทซึ่งประกอบด้วยปริมาณความชื้น (7.97 ± 0.35%), ปริมาณสารสกัดด้วยน้ำ (16.14 ± 0.50%), ปริมาณสารสกัดด้วยเอทานอล (8.61 ± 0.39%), ปริมาณความชื้นที่หายไปเมื่ออบแห้ง (4.55 ± 0.21%), ปริมาณเถ้ารวม (14.38 ± 0.25%) และปริมาณเถ้าที่ไม่ละลายในกรด (6.21 ± 0.37%) การตรวจสอบทางพฤกษเคมีเบื้องต้นของใบหม่อนพบว่ามีสารประกอบฟีนอลิก ฟลาโวนอยด์ สเตียรอยด์ และไตรเทอร์ปีน ใบหม่อนแห้งถูกนำมาสกัดด้วยเอทานอล 95% โดยวิธี Soxhlet apparatus ซึ่งได้วิเคราะห์ปริมาณกรดคลอโรจีนิกด้วยวิธี RP-HPLC โดยใช้คอลัมน์ C_{18} เป็นวัฏภาคคงที่และใช้เมทานอล : 0.2% กรดฟอสโฟริก (45% : 55%) เป็นวัฏภาคเคลื่อนที่ พบว่าปริมาณกรดคลอโรจีนิกในใบหม่อนมีค่าเท่ากับ 0.4159 ± 0.20 กรัม ต่อ 100 กรัมของน้ำหนักพืชแห้ง

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อ นิสิต .....

ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Phimkun Aiyarakanchanakun : PHARMACOGNOSTIC SPECIFICATIONS AND CHLOROGENIC ACID CONTENT OF *MORUS ALBA* LINN. LEAVES IN THAILAND. Advisor: ANUCHIT PHANUMARTWIWATH, Ph.D.

Morus alba Linn., belonging to Moraceae family, is commonly used as an herb. *M. alba* leaves are widely used in traditional Chinese medicine and Thai traditional medicine. It used to treat colds caused by wind-heat, sore throat, cough, eye inflammation, etc. This study aimed to investigate the pharmacognostic specifications and analyze the content of chlorogenic acid in *M. alba* leaves. *M. alba* leaves were collected from 15 different sources throughout Thailand. The fresh mature leaves were used for macroscopic and microscopic evaluations. The macroscopic details were illustrated as drawings. The microscopic evaluation showed the details of the leaf constant number and the cross-section of the midrib. *M. alba* leaves were cleaned and dried for analysis of pharmacologic specification. The parameters included water content (7.97 ± 0.35%), water-extractive value (16.14 ± 0.50%), ethanol-extractive value (8.61 ± 0.39%), loss on drying (4.55 ± 0.21%), total ash (14.38 ± 0.25%) and acid-insoluble ash (6.21 ± 0.37%). The preliminary phytochemical examination of *M. alba* leaves showed the detection of phenolic compounds, flavonoids, steroids and triterpenes. The dried *M. alba* leaves were extracted with 95% ethanol by using Soxhlet apparatus. The quantitative analysis of chlorogenic acid content was analyzed by using RP-HPLC. A C_{18} column was used as a stationary phase and methanol: 0.2% phosphoric acid (45%: 55%) was used as a mobile phase. The content of chlorogenic acid of *M. alba* leaves was found to be 0.4159 ± 0.20 g/100g by dry weight.

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

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LIST OF ABBREVIATIONS

%	=	Percent
µg/ml	=	Microgram / Milliliter
µL	=	Microliter
µm	=	Micrometer
CCL_4	=	Carbon tetrachloride
CGA	=	Chlorogenic acid
cm	=	Centimeter
DOPA	=	3,4-dihydroxyphenylalanine
DPPH	=	2,2-diphenyl-1-picryl-hydrazyl-hydrate
etc.	=	et cetara
$FeCl_3$	=	Iron (III) chloride
g	=	Gram
g/kg	=	Gram / kilogram
g/L	=	Gram / Liter
H_2SO_4	=	Sulfuric acid
HBsAg	=	Hepatitis B surface antigen
HCl	=	Hydrochloric acid
HPLC	=	High performance liquid chromatography
hr	=	Hour
IC_{50}	=	Half maximal inhibitory concentration

ICH	=	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
l	=	Liter
LOD	=	Limit of detection
LOQ	=	Limit of quantitation
LPS	=	Lipopolysaccharide
M. alba	=	*Morus alba* Linn
mg	=	Milligram
mg/kg	=	Milligram / kilogram
MIC	=	Minimum Inhibitory Concentration
min	=	Minute
ml	=	Milliliter
mm	=	Millimetre
mm^2	=	Square millimeter
NaOH	=	Sodium hydroxide
^oC	=	Degree Celsius
PTFE	=	Polytetrafluoroethylene
R^2	=	R-squared
Rf	=	Retention factor
RP-HPLC	=	Reverse phase high performance liquid chromatography

RSD	=	Relative Standard Deviation
SD	=	Standard deviation
TLC	=	Thin Layer Chromatography
UV	=	Ultraviolet
WHO	=	World Health Organization

CHAPTER I
INTRODUCTION

Background and significance of the study

The definition of traditional medicine is “the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in prevention, diagnosis, improvement or treatment of physical and mental illnesses.” (World Health Organization, 2005)

"Herb" or "Herbal medicine" has been found to be useful in treatment and management of various health problems for a long time. Medicinal plants are main materials of traditional medicine which have been used for thousands of years. Medicinal plants also have a significant contribution to human health and have properties in health promotion, preventing illness and healing recovery. Nowadays, traditional medicine has been accepted and widely used in many countries such as China, Thailand, Korea, India, etc. Therefore, herbs are getting more and more attention, and there are also a lot of research studies on herbal medicine.

Although herbal medicine has been used widely; however, it doesn't mean that the herbs are safe and have no side effects. Moreover, the effectiveness of herbal medicine depends on the quality of the herbs. Therefore, it is important to require the quality control of the ingredients using pharmacognostic specification techniques. The pharmacognostic specification is to identify the purity of herbal material to ensures the correct substance in the amount for desired therapeutic effect (safety, quality and efficacy) and the determination of quality and purity by using various parameters like morphological, microscopic, physical, chemical, etc. (World Health Organization, 2011)

Morus alba Linn., a deciduous tree in the family Moraceae, is commonly known as a white mulberry, or in Thai name as "Mon", which is widely distributed in temperate to subtropical regions of the Northern hemisphere to the tropics of the Southern hemisphere. The white mulberry trees can grow in a wide range of climatic and topographic. The white mulberry is usually cultivated to feed the silkworms or cut for food for livestock. Nowadays, *M. alba* leaves are used in a variety of dishes. In

addition, *M. alba* leaves have medicinal properties, and it can also improve the taste of food. It is also popular to make an oriental tea. According to herbal medicine, every part of this plant can be used such as fruits, leaves, stems, etc. In both traditional Chinese medicine and traditional Thai medicine, *M. alba* leaves are used as an ingredient in drug recipes.

M. alba leaves used in traditional Chinese medicine have their properties in removing wind-heat, clearing the lungs and moisturizing, balancing the liver and improving the eyesight, cooling blood and stopping bleeding. Leaves of *M. alba* are used to treat fever caused by wind heat, cough caused by heat lungs, dizziness, hemoptysis and hematemesis (Bagachi, Semwal, & Bharadwaj, 2013). There are many remedies that contain *M. alba* leaves for example “Sang zhu yin” (桑菊饮) from the detailed analysis of warm febrile diseases《温病条辨》, “Du sheng san” (独圣散) from the general medical collection of Royal Benevolence《圣济总录》, “Sang ma wan” (桑麻丸) from medical treasure《医级宝鉴》etc. In Thai traditional medicine, *M. alba* leaves are often used as a secondary drug in cough syrup and expectorate and heat-releasing drugs, not as a primary drug.

M. alba leaves contain many bioactive compounds, especially chlorogenic acid. Chlorogenic acid is a phenolic compound forming an ester bond by condensation of a carboxyl group of the caffeic acid with a hydroxyl group of quinic acid. This compound shows several biological activities such as antioxidant, cardioprotective, antibacterial, hepatoprotective, anti-inflammatory, antipyretic, anti-obesity, neuroprotective, antiviral, anti-microbial, antihypertension activities as well as central nervous system (CNS) stimulator (Muhammad Naveed, et al., 2018).

In this research, we aim to study the pharmacognostic specification of *M. alba* leaves found in Thailand and evaluate their chlorogenic acid contents by using reversed-phase high-performance liquid chromatography (RP-HPLC) analysis Additionally, *M. alba* leaves are widely used as herbal medicine in many countries; however, there are no studies on the pharmacognostic specifications of *M. alba* leaves in Thailand.

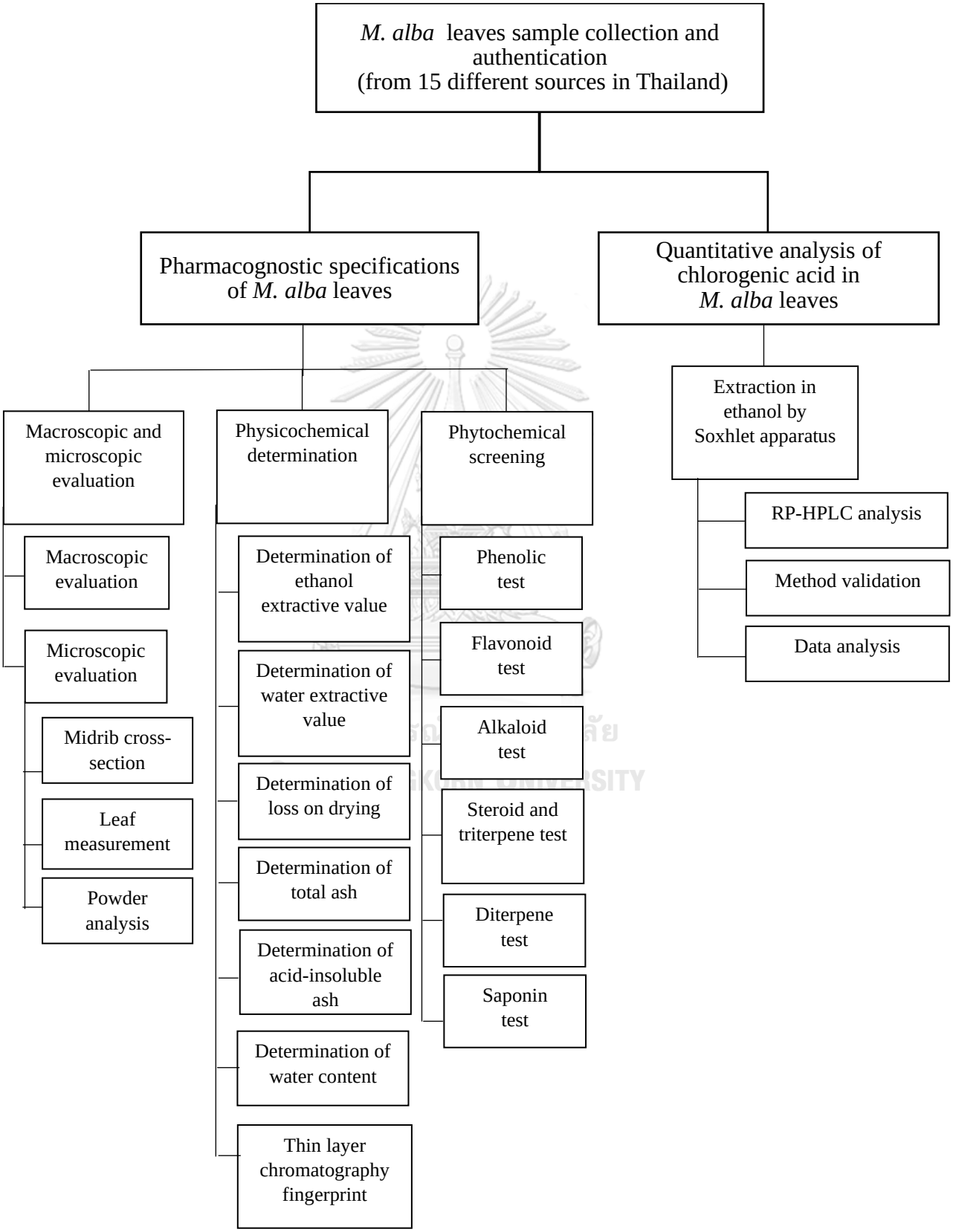
Research gap

M. alba leaves have been used in traditional medicine and their biological activities have also been studied, but the pharmacognostic specification parameters, phytochemical screening and chlorogenic acid contents of *M. alba* leaves in Thailand have never been determined.

Objective

1. To determine the pharmacognostic parameters of *M. alba* leaves in Thailand.
2. To investigate the chlorogenic acid contents in *M. alba* leaves by RP-HPLC analysis.
3. To investigate the phytochemical compounds of *M. alba* leaves by the preliminary phytochemical screening test.
4. To validate the method for quantitative analysis of chlorogenic acid using RP-HPLC.

Conceptual framework



CHAPTER II
LITERTURE REVIEWS

Introduction of *M. alba*

The genus *Morus*, belonging to the Moraceae family, is a deciduous tree which is commonly known as "Mulberry". The plant is growing in a wild and under cultivation in many temperate world regions. This genus originally grows on the low slopes of Himalayas bordering between China and India, and is widely distributed in Asia, Europe, North and South America and Africa. Normally, this genus has more than 200 species and is identified in taxonomy.

Taxonomy of *M. alba* Linn.

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Urticales
Family	Moraceae
Genus	*Morus*
Species	*Morus alba* Linn.

M. alba Linn. is the dominant species of *Morus* which is commonly known as a white mulberry. *M. alba* has different names in different countries which are summarized in the Table 1. It is economically important plants used as food for silkworms and cut for food for livestock (cattle, goats, etc.). In addition, leaves are also popular for making tea as well. Both fresh and dried fruits are edible, and they can be fermented to make a wine. The white mulberry tree is a native plant which are widely found in Pakistan, India and Nepal, Northern China, Indochina and Japan. It is

used as a food source for silkworms which are widely cultivated in many continentals such as Europe and Asia. In Thailand, most of them can be cultivated in North – eastern, which are summarized in the Table 2.

Synonyms (WCSP, 2012)

Morus multicaulis Perr., *Morus atropurpurea* Roxb., *Morus latifolia* Poir., *Morus chinensis* Lodd.

Table 1. Vernacular name

Country	Vernacular name	Reference
Thailand	Mon	(Nijsiri Ruangrungsi, 2004)
China	Sang shu	(Chan, Phui-Yan, & Siu-Kuin, 2016)
English	White mulberry	(Bagachi et al., 2013)
Hindi	Chinni, Tut, tutri	
Bengail	Tut	
Marath	Tut, Ambat	
Gujarati	Shetur	
Telegu	Reshme chettu	
Tamil	Kambli chedi	
Kannada	Bili uppunerale	
Punjabi	Tut, Tutri	
Oriya	Tuto, Tuticoli	

Plant description

M. alba is defined as “the plant is usually a monoecious shrub or a medium sized tree with a cylindrical stem and rough, brown, vertically fissured bark. Leaves are variable in size and shape, usually 5 to 7.5 cm long, often deeply lobed, margins serrate or crenate-serrate, apex acute or shortly acuminate, base cordate or truncate; 3

basal nerves, lateral nerves forked near the margins. Flowers are inconspicuous and greenish: male spikes (catkins) are broad, cylindrical or ovoid, female spikes are ovoid and stalked. Fruit (syncarp) consists of many drupes enclosed in a fleshy perianth, ovoid or subglobose, up to 5 cm long, white to pinkish white, purple or black when ripe" (Bagachi et al., 2013)

Table 2. Distribution in Thailand

Northern	Nan, Chiang Mai, Chiang Rai, Phayao, Phitsanulok, Mae Hong Son
North – eastern	Khon Kaen, Maha Sarakham, Udon Thani, Roi Et Buri Ram, Yasothon
Eastern	Sa Kaeo, Prachin Buri, Chon Buri
Central	Bangkok, Ayutthaya, Nakhon Pathom, Suphan Buri, Kanchanaburi, Phetchabun
Western	Ratchaburi, Phetchaburi, Rayong
Southern	Chumphon, Surat Thani, Songkhla

Traditional uses of *M. alba*

The stem has been widely used in rheumatoid and joint pain. It is used to treat in cramp, diuretic, and hypotensive. The bark is used as deworming. (Bagachi et al., 2013; Boonworapat, 2011)

The root has been widely used in treatment of diuretic, febrifuge and diabetic. It is used to treat cough, asthma, bronchitis, fever, edema, wound and whiten the skin. The bark is used for treatment of hemoptysis and asthma. (Bagachi et al., 2013; Mahboubi, 2019)

The fruit has been widely used in preventing premature graying of the hair and treating dizziness, ringing in the ears, blurred vision, constipation and insomnia. (Bagachi et al., 2013; Mahboubi, 2019)

The leaves can be used in making healthy food whether it is made into a soup or fry. The mulberry leaves help improve a flavor of the food. The leaves have been widely used in expectorant, diabetic, febrifuge and dyslipidemia. The leaves are used to treat cough, asthma, bronchitis, fever, edema, sore throats, headaches, dizziness and vertigo, sedative, inflamed and sore eyes, beriberi and constipation. (Ann, Eo, & Lim, 2015; Bagachi et al., 2013)

Traditional Chinese medicine (TCM) and Thai traditional medicine are quite the same, use the leaves as an ingredient in the recipe. The leaves are bitter, sweet and cool active on the lungs and liver, and remove the symptoms of heat with origin of lungs and management of liver or kidney deficiency, auditory sharpness and eyes brightness. It is used to treat cough, sore throat, fever, and bronchitis. Additionally, *M. alba* has other medicinal properties, as shown in Table 3.

Table 3. Traditional uses of *M. alba*

Traditional uses	Plant parts used	References
Treatment of respiratory disease and asthma	Roots, Root barks	(Bagachi et al., 2013)
	Leaves	(Bagachi et al., 2013)
	Fruits	(Bagachi et al., 2013)
Treatment of fever	Leaves	(Mahboubi, 2019)
Treatment of obesity	Leaves	(Ann et al., 2015)
	Fruits	(Mahboubi, 2019)
Treatment of diabetic	Leaves	(Bagachi et al., 2013)
	Fruits	(Bagachi et al., 2013)
Treatment of edema	Root, Root bark	(Mahboubi, 2019)
	Leaves	(Bagachi et al., 2013)
Treatment of constipation	Leaves	(Mahboubi, 2019)
	Fruits	(Chan et al., 2016; Mahboubi, 2019)
Treatment of hyperlipidemia	Leaves	(Chan et al., 2016)
Treatment of rheumatoid	Stems	(Bagachi et al., 2013;

		Boonworapat, 2011)
Treatment of skin diseases (Skin toning)	Root barks	(Zafar et al., 2013)
	Leaves	(Bagachi et al., 2013)

Bioactivity of *M. alba*

Nowadays, there are various studies about bioactive ingredients derived from of *M. alba* leaves. The mulberry leaves are popular for brewing tea and cooking in many countries. It is also used as a therapeutic drug in both Chinese and Thai traditional medicines. There are many studies of biological activities of *M. alba* leaves such as antioxidant activity, antimicrobial activity, antidiabetic activity and anti-obesity activity, etc. In this report, some biological activities studies are summarized according to the topics as shown below.

Antioxidant activity

In 2005, Wattanapitayakul, et al., reported that both ethanol extract and aqueous extract of *M. alba* leaves showed antioxidant properties, but the aqueous extract had the highest antioxidant activity through Ferric reducing antioxidant power (FRAP) assay. Furthermore, the ethanol extract of *M. alba* leaves showed inhibitory effects against $FeSO_4/H_2O_2$-induced lipid peroxidation in microsomes of rat, and it was found that mulberroside A showed radical scavenging activity on DPPH antioxidant. (Zafar et al., 2013)

Antimicrobial activity

Kuwanon C, mulberrofuran G and albanol B isolated from the ethanolic extract of *M. alba* leaves showed strong antibacterial activity with minimum inhibitory concentrates (MIC's) ranging from 5 to 30 mg/mL. (Sohn, Son, Kwon, Kwon, & Kang, 2004) In 2015, Alisson Macário de Oliveira, et al., studied antimicrobial activity of the ethanolic extract of *M. alba* leaves. The study was conducted by using mice treated with the extract (300 and 2000 mg/kg) for 14 days,

and the result showed that the extract of *M. alba* leaves had antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus, Candida krusei, Candida albicans*, *Aspergillus flavus* and *Candida tropicalis*. Hence, the extract contained antimicrobial agents and was not harmful to mice when ingested. In 2003, Park KM, et al., reported that the methanol extract of *M. alba* root bark exhibited antibacterial activity against oral pathogens, with the minimum inhibitory concentration (MIC) of 8 μg/mL.

Antidiabetic activity

In 2009, Bahman Nickavara and Golboo Mosazadeha reported the ethanol extract of *M. alba* leaves inhibited α-amylase which was an important enzyme for controlling blood sugar levels in type II diabetes patients. The study reported the possible effects of *in vitro* starch breakdown by α-amylase, and the results showed that the extract inhibited α-amylase activities in a concentration-dependent manner [IC_{50} = 17.60 (17.39-17.80) mg/mL]. The aqueous extract of *M. alba* leaves exhibited potential hypoglycemic effects, and it was studied by using Goto-Kakizaki (GK) and Wistar rats. (J. M. Park et al., 2009) In 2012, Hunyadi A, et al., reported that after rat taking the aqueous ethanol extract of *M. alba* leaves, it was found that a decrease of blood glucose levels of type II diabetic in rats was observed after 11 days. Also, the *M. alba* leaf extract had therapeutic effects in diabetes-induced rats and could restore the diminished number of β-cells. (Mohammadi & Naik, 2008)

Another study reported that the ethanolic extract of fruit had a significant decrease of blood glucose and serum protein and an increase of antioxidant enzymatic activities in STZ-induced diabetic mice. (Y. Wang, Xiang, Wang, Tang, & He, 2013)

Anti-obesity activity

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health (WHO). In 2009, Oh KS et al., reported the effects of the ethanol extract of *M. alba* leaves on melanin-concentrating hormone receptor and

anti-obesity activities in diet-induced obese mice. The results showed that the extract exhibited a potent inhibitory activity, with IC_{50} value of 2.3 μg/mL.

Neuroprotective activity

The neurodegeneration is mostly caused by free radical production. Neurological disorders such as Alzheimer's and Parkinson's are caused by depletion of gamma-aminobutyric acid (GABA) in the brain (Zafar et al., 2013). The previous study reported about *M. alba* leaves extract provided a viable treatment for Alzheimer's disease through the inhibition of amyloid beta-peptide (1e42) fibril formation and attenuation of amyloid β peptide (1e42)-induced neurotoxicity (Niidome et al., 2007).

Anti-dopaminergic effect activity

In 2007, Niidome et al., reported that the methanol extract of *M. alba* leaves was evaluated on haloperidol and metoclopramide induced catalepsy, foot shock-induced aggression, amphetamine-induced stereotyped behavior and phenobarbitone induced sleeping in mice. The result showed that the *M. alba* extract possessed the antidopaminergic activity.

Hepatoprotective activity

In 2010, Hogade, et al., reported that ethanol extract and aqueous extract of *M. alba* leaves was against hepatotoxicity induced by carbon tetrachloride (CCl_4) by using standard drug Liv-52. for phytochemical tests. The result showed that both alcoholic and aqueous extracts had significant protective effect against the toxicity induced by CCl_4. Protective mechanisms of the aqueous extract of *M. alba* L. fruit in Wistar rats with CCl_4 induced hepatic injury were studied. The results showed that the extract exhibited protective and curative effects against liver damage and inhibition of pro-inflammatory gene expression (Hsu et al., 2012).

Skin toning activity

The leaf and root bark extracts of *M. alba* exhibited relatively high inhibition on the 3,4-dihydroxyphenylalanine (DOPA) oxidase and anti-tyrosinase activities. In 2002, Lee SH, et al., reported that the methanol extract of *M. alba* leaves containing mulberroside F exhibited anti-tyrosinase activity which was 4.5-fold stronger than kojic acid and also had an inhibitory effect on melanin formation in melan-a cells.

Cytotoxic activity

The aqueous and methanol extracts obtained from *M. alba* leaves contained two bioactive flavonoids which were quercetin-3-O-β-D-glucopyranoside and quercetin-3-7-di-O-β-D-glucopyranoside. The compounds inhibited the growth of human leukemia HL-60 cells. A flavanone (7, 2′, 4′, 6′-tetrahydoroxy-6-geranylflavanone) found in ethyl acetate root extract of *M. alba* exhibited cytotoxic activity against rat hepatoma dRLh84 cells with an IC_{50} value of 53 µg/mL (Kim SY, et al., 2000). Furthermore, the study reported that 11 flavonoids were isolated from the methanol extract of *M. alba* leaves. They showed cytotoxic activity against human cancer HeLa, MCF-7, and Hep-3B cells (Dat, Binh, Van Minh, Huong, & Lee, 2010). In 2010, Yang Y, et al., reported that two new chalcones, morachalcones B and C, were isolated from the ethanol extract *M. alba* leaves. The chalcones had moderate cytotoxic activity against human cancer HCT-8 and BGC-823 cells.

Chemical compounds of *M. alba*

The previous studies reported about the isolation of chemical constituents in *M. alba* from various parts of the plant including phenolics, flavonoids, anthocyanins, terpenoids, stilbenoids, coumarins and alkaloids.

Compounds isolated from stems and twigs of *M. alba*

The previous studies reported that stem and twig of *M. alba* contained flavonoids as 6-geranylapigenin, 6-geranylnorartocarpetin and quercetin. (W.-Y. Jin et al., 2002). In 2014, Riviere C, et al., reported the isolation of stilbenoids as mulberrosides A**,** dihydromorin-7-O-β-glucoside, resveratrol, moracins M, steppogenin, oxyresveratrol and dihydromorin. It also reported about isolation of coumarins from stem of *M. alba* gave isoscopoletin 6-(6-O-β-apiofuranosyl-β-glucopyranoside).

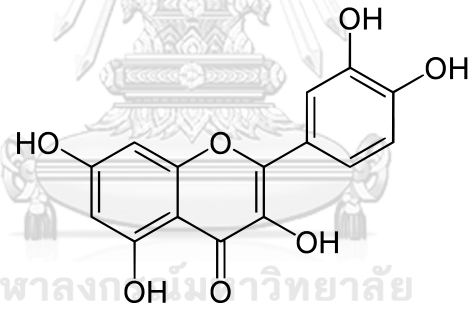


Figure 1. The structure of quercetin**.**

(Y.-M. Zhang, Zhang, & Wang, 2020)

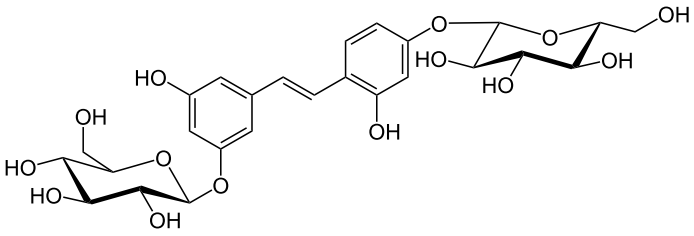


Figure 2. The structure of mulberrosides A

(Komaikul, Kitisripanya, Tanaka, Sritularak, & Putalun, 2014)

Compounds isolated from roots and root barks of *M. alba*

The root and root bark of *M. alba* contained flavonoids as cyclomorusin, morusin, oxydihydromorusin, sanggenons F, J, K and 7, 2′, 4′, 6′-tetrahydroxy-6-geranylflavanone. (Nomura, Hano, & Fukai, 2009; Z.-G. Yang, Matsuzaki, Takamatsu, & Kitanaka, 2011) In 2011, Yang ZG, et al., reported the isolation of terpenoids in root bark of *M. alba* as betulinic acid, ursolic acid and uvaol. There are studies about coumarin derivatives including 5, 7-dihydroxycoumarin 7-O-β-d-apiofuranosyl-(1→6)-O-β-D-glucopyranoside, 5, 7-dihydroxycoumarin 7-O-β-D-glucopyranoside. In 2010, Yang Y, et al., studied about isolation of stilbenoids from root bark of *M. alba* gave alabafuran A, artoindonesianin O, 3′, 5′-dihydroxy-6-methoxy-7-prenyl-2-arylbenzofuran, mulberrofurans L, Y and oxyresveratrol 2-O-β-D-glucopyranoside. (Z.-G. Yang et al., 2011) The previous studies reported about alkaloids as calystegins B2, C1, 1-deoxynojirimycin, 1, 4-dideoxy-1, 4-imino-pD-arabinitol, 1, 4-dideoxy-1, 4-imino-pD-ribitol, fagomine, 3-epi-fagomine, 2-O-β-D-glucopyranosyl-1-deoxynojirimycin, 3-O-β-D-glucopyranosyl-1-deoxynojirimycin, 4-O-β-D-glucopyranosyl-1-deoxynojirimycin and N-methyl-1-deoxynojirimycin.

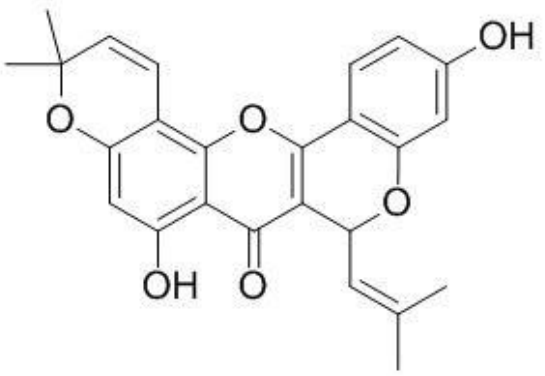


Figure 3. The structure of cyclomorusin

(Singab, El-Beshbishy, Yonekawa, Nomura, & Fukai, 2005)

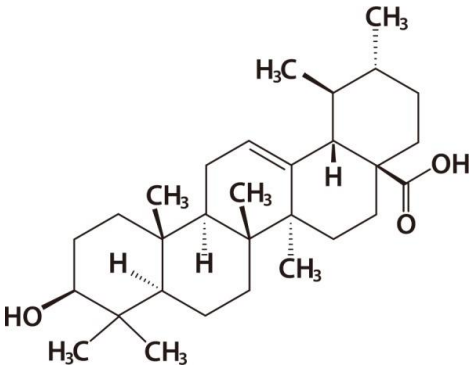


Figure 4. The structure of ursolic acid.

(Seo et al., 2018)

Compounds isolated from fruits of *M. alba*

In 2010, Memon A. A, et al. and Dat NT, et al., reported about isolation of phenolic acids from fruit of *M. alba* gave 3-O-Caffeoylquinic acid, 5-O-Caffeoylquinic acid, *m*-coumaric acid, *p*-coumaric acid, ellagic acid, ferulic acid, gallic acid, gentisic acid, syringic acid, vanillic acid and many more compound. In 2015, Natic MM, et al., studied the isolation of flavonoids as follows: epigallocatechin, epigallocatechin gallate, gallocatechin, gallocatechin gallate, isorhamnetin glucuronide, isorhamnetin hexoside, isorhamnetin hexosylhexoside, kaempferol glucuronide, morin, naringin, quercetin and rutin. Also, they reported that the isolation of anthocyanins found in *M. alba* fruit gave 2α, 3β-dihydroxynortropane, 2β, 3β-dihydroxynortropane, 3β, 6exo-dihydroxynortropane, morroles B, 2α, 3β, 6exo-trihydroxynortropane, 4-[formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl] butanoate, 4-[formyl-5-(methoxymethyl)-1H-pyrrol-1-yl] butanoic acid and etc. (Kim, Chang, Hwang, Kim, & Lee, 2014; Kusano et al., 2002)

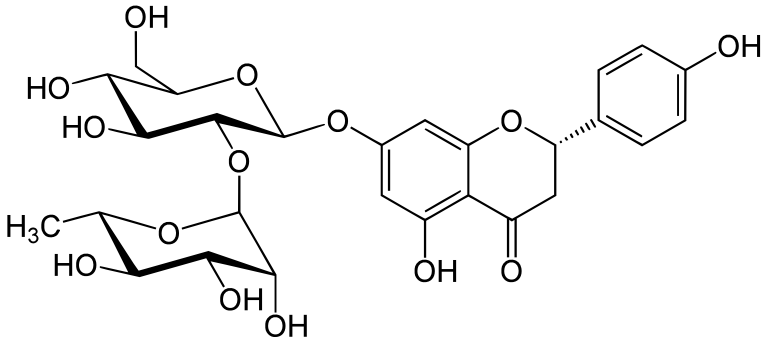


Figure 5. The structure of naringin.

(Izawa, Amino, Kohmura, Ueda, & Kuroda, 2010)

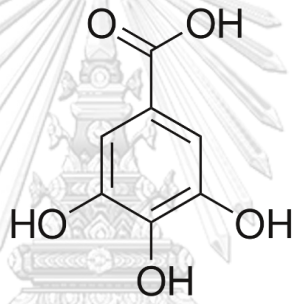


Figure 6. The structure of gallic acid

(Govea-Salas et al., 2018)

Compounds isolated from leaves of *M. alba*

In 2010, Memon A. A, et al., studied about the isolation of phenolic acids from leaves of *M. alba* by using HPLC method. The phenolic acids were identified as gallic acid, chlorogenic acid, protocatechuic, p-hydroxybenzoic acid, vanillic acid, syringic acid and p-coumaric acid. In 2010, Dat NT, et al., reported that the methanol extract of *M. alba* leaves contained flavonoid constituents as cyclomorusin, cyclomulberrin, 8-geranylapigenin, 3′, 8-diprenyl-4′, 5,7-trihydroxyflavone, kaempferol, morusin, sanggenon J and K, and atalantoflavone. In previous studies, stilbenoids isolated from *M. alba* leaves were chalcomoracin, moracins and mulberrosides A, B, F. (Z.-G. Yang et al., 2011; Z. Yang, Wang, Wang, & Zhang,

2012) Also, coumarin derivatives were isolated and identified as morachalcones B and C. (Y. Yang et al., 2010)

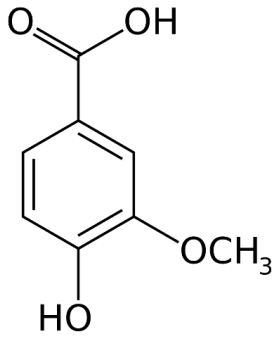


Figure 7. The structure of vanillic acid

(Almeida, Cavalcante, & Vicentini, 2016)

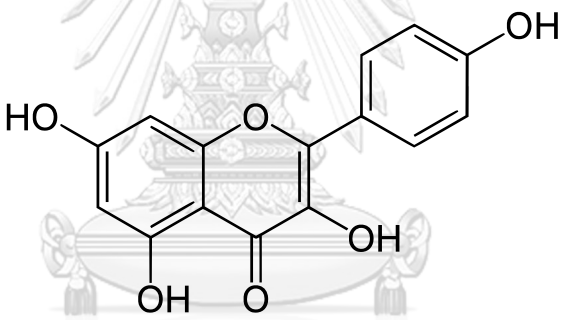


Figure 8. The structure of kaempferol

(Butt, Nazir, Sultan, & Schroën, 2008)

Introduction of Chlorogenic acid

Phenolic compounds are a large class of plant secondary metabolites mostly found in plant tissues, including fruits and vegetables (Cheynier, 2012). Phenolic compounds contain a phenol group which is a derivative of the benzene ring. There is at least one hydroxyl group (-OH group) substituted on the aromatic ring the simple phenolic compound is a phenol consisting of one benzene ring and one hydroxyl group.

Chlorogenic acid is one of the most available acids among polyphenol compounds which is an ester of caffeic acid and quinic acid, functioning as an intermediate in lignin biosynthesis. Despite the "chemical name begins with “chloro”, the chlorogenic acid contains no chlorine atom. Chloro originated from the Greek word means a green color. The chemical information of chlorogenic acid is shown in Table 4. The common form of chlorogenic acid is known as 5-O-caffeoylquinic acid (5-CQA), and chlorogenic acid has been found in human bladder tissue and is detected in multiple biofluids such as urine and blood. Inside the cell, chlorogenic acid is primarily in the cytoplasm. In the outside body, chlorogenic acid can be found in many foods such as chia seed, mulberry, coffee bean, tea etc. This compound is known as an antioxidant and also slows the release of glucose into the bloodstream after a meal as well.

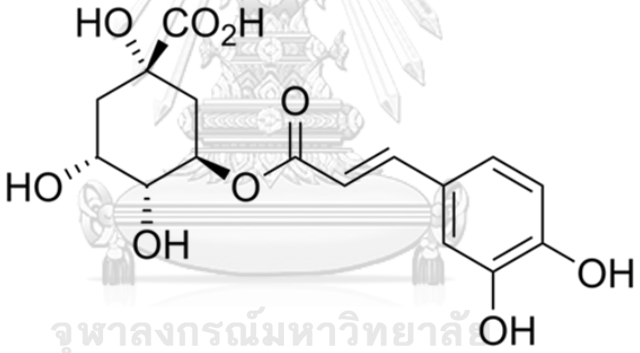


Figure 9. The structure of chlorogenic acid
(Plazas et al., 2013)

Table 4. Chemical information of chlorogenic acid

Chemical name	**Chlorogenic acid**
IUPAC Name:	(1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl) prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid
Formula:	$C_{16}H_{18}O_9$
Synonyms:	3-Caffeoylquinate, 3-Caffeoylquinic acid, 3-CQA, 3-O-Caffeoylquinic acid, Chlorogenate

Molecular weight:	354.31 g/mol
Melting point:	205-209°C
Solubility:	40 mg/mL at 25 °C (water)

Bioactivity of Chlorogenic acid

The chlorogenic acid is studied extensively because it is found in many plants and shows various health promotion and treatment properties. The chlorogenic acid is an important and biologically active dietary phenolic compound by playing several important and therapeutic roles such as anti-Hepatitis B Virus activity, antioxidant activity, antibacterial activity, anti-inflammatory, neuroprotective activity, antimicrobial etc.

Anti-Hepatitis B Virus activity

The chlorogenic acid is a polyphenol which is found in many plants and has a diverse antiviral activity. In 2009, Gui-Feng Wang, et al., reported that chlorogenic acid reduced HBV level of the DHBV-infected duckling model. In addition, the study showed anti-HBV activity of coffee beans extract containing a high content of chlorogenic acid. It showed an inhibitory effect on HBV replication. Furthermore, the study showed that the extracts inhibited HBV-DNA replication and suppressed Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) secretion *in vitro*, with IC_{50} values of 82.4 ± 23.2 μg/mL on HBV-DNA, 62.5 ± 6.5 μg/mL on HBsAg and 133.6 ± 29.2 μg/mL on HBeAg (Zuo, Tang, & Xu, 2015).

Antioxidant activity

The chlorogenic acid is a good natural antioxidant agent. In 2008, Zhinan Xiang and Zheng Xiang Ning, reported that chlorogenic acid had antioxidant potential by using DPPH assay. A study of microencapsulation techniques reported that chlorogenic acid had a success of microencapsulation. This resulted in formation of stable microparticles with good release properties, regulators and antioxidant effects,

indicating that increasing uses in the food and pharmaceutical industries. (Gonçalves et al., 2017)

Antibacterial activity

The previous study reported that chlorogenic acid showed antimicrobial activity against *P. aeruginosa* P1 which was a food-borne pathogen. The chlorogenic acid exhibited a decrease in the lipopolysaccharide (LPS) content of *P. aeruginosa* P1 and decreased the expression of major genes in LPS biosynthesis, however, chlorogenic acid might be inhibited by intracellular metabolism of *P. aeruginosa* P1 cells. (Su et al., 2019) In 2017, Zhonghao Zhang and Taowen Pan., reported biological evaluation of the chlorogenic acid in *Verbena officinalis* L. extract by using the Cylinder-plate method and MIC assay. The result showed that *V. officinalis* L. extract had chlorogenic acid content (30%) and showed strong antibacterial activity against Gram-negative bacteria.

Anti-inflammatory

The researchers investigated the effects of chlorogenic acid on experimental pancreatitis in mice. The chlorogenic acid decreased the histological severity of pancreatitis and pancreatitis-associated lung injury. It also inhibited the level of pancreatic enzyme activity and decreased levels of serum and pancreatic macrophage migration inhibitory factors in mice with pancreatitis caused by L-arginine (Ohkawara, Takeda, & Nishihira, 2017).

Neuroprotective activity

The chlorogenic acid inhibited the acetylcholinesterase activity in the hippocampus and frontal cortex, also decreased malondialdehyde levels in the hippocampus and frontal cortex. The *in vitro* study showed that chlorogenic acid inhibited acetylcholinesterase activity (IC_{50} = 98.17 μg/mL) and free radical

scavenging activity (IC_{50} = 3.09 μg/mL) where it depended on the quantity (Kwon et al., 2010) In 2014, Faisal Kabir, et al., reported that the antimicrobial activity of chlorogenic acid by using Gram-negative bacterium *Escherichia coli* IFO 3301. Bacteriostatic effects were assessed by spectrophotometer, and bactericidal effects based on viable cell distribution on MacConkey agar plates. The result showed that chlorogenic acid had not only bacteriostatic effects but also bactericidal effects.

Antidiabetic activity

In 2013, Ong, K.W. et al., reported that mice were treated with vehicle, 250 mg/kg oral gavage metformin or 250 mg/kg chlorogenic acid intraperitoneally daily for 2 weeks. The result showed that chlorogenic acid inhibited hepatic glucose-6-phosphatase expression and activity, and it decreased hepatic steatosis, improved lipid profiles and skeletal muscle glucose uptake, which improved fasting glucose levels, glucose tolerance, insulin sensitivity and dyslipidemia in the diabetic (db/db) mice.

Anti-obesity activity

The chlorogenic acid had a significantly decreased percentage of body fat, fasting plasma glucose, and HbA1c compared to the db/db-control group. This study was using female C57BL/BKS late diabetic mice, The mice were divided into four groups including db/db control, db/db- CGA groups and non-diabetic mice (db/m-control group, db/m- CGA group) The mice in CGA groups were given CGA (80 mg/kg BW) for 12 weeks. (S. Jin, Chang, C., Zhang, L., Liu, Y., Huang, X., & Chen, Z., 2015). In 2010, Ae-Sim Cho, et al., had a study of anti-obesity activity by using male induced-obese ICR mice, the mice were fed by a normal diet, a high-fat diet, a high-fat diet with chlorogenic acid (0.2 g/kg diet) for 8 weeks. The result showed that chlorogenic acid significantly reduced body weight, visceral fat mass, plasma leptin and insulin levels, triglycerides in liver and heart, and cholesterol in adipose tissue and heart compared to the high-fat control group.

Pharmacognostic specification

Pharmacognostic specification is a process to ensure the correct substance in amount for desired therapeutic effect (safety, quality and efficacy), as well as to confirm the identity and determination of quality and purity and detection of adulterant by various parameters like morphological, microscopic, physical, chemical, etc. In this study testing and analytical method will follow World Health Organization (WHO) guideline. (World Health Organization, 2011)

Macroscopic and microscopic examinations

Macroscopic examination

The first process used for identifying medicinal plant materials is macroscopic examination. The macroscopic examination is the morphological description of medicinal plant materials is based on size, shape, color, surface characteristics, fracture characteristics, texture, odor, and taste, but a test should be applied only if specifically required for a given herbal material. The method can be observed by using visual observation (World Health Organization, 2011).

Microscopic examination

The microscopic examination is used for the identification of plants on the cellular structure and their content of plant materials by using a microscope. It reveals plant histological characteristics. It is used as a primary screening test for the identification of plant material. It is used to determine the structure of herbal by their histological characters.

1. **Midrib cross-section**

Midrib cross-section is the study of plant's organs (xylem, phloem, epidermis, palisade cell, etc.) in the different parts of the plant by doing either transverse or longitudinal cross-section.

2. Leaf measurement

Leaf measurement is a method which can be used to distinguish plants in species level (Evans, 2002). The measurement has many parameters such as stomatal number / stomatal index, trichome number / trichome index, palisade ratio, epidermal cell number / epidermal cell area, vein islet etc.

2.1. Stomata

The stomata are apertures in the epidermis, each bounded by two guard cells, that facilitate gas exchange. In Greek, stoma means “mouth”. They occur in vascular plants, the stoma is the greenest aerial parts of plants, usually in the leaves part, and they also found in the stem but less than leaves. WHO guideline has divided the stomata into four types, as shown in Figure 10.

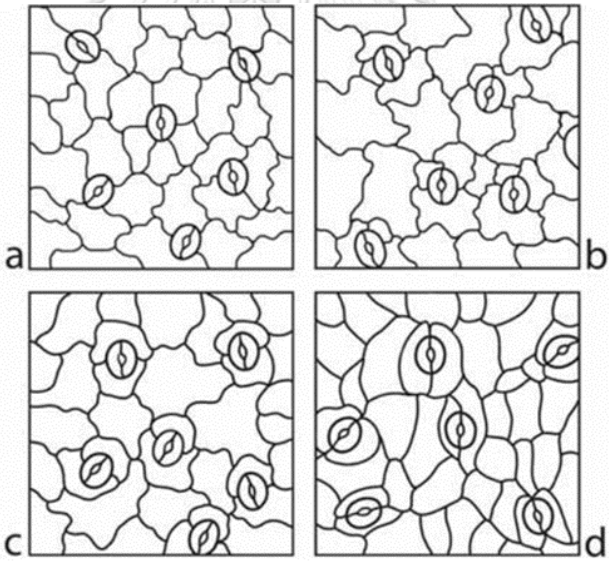


Figure 10. Type of leaf stoma

a). The anomocytic or ranunculaceous (irregular-celled) type,

b). The anisocytic or cruciferous (unequal-celled) type,

c). The diacytic or caryophyllaceous (cross-celled) type and

d). The paracytic or rubiaceous (parallel-celled) type

(World Health Organization, 2011)

The stomatal number is specific for identification and characterization of leaves. The stomata cell is counted in the area of one square millimeter (mm^2). The leaves of the same plant maybe have a difference of number because of different age of leaves and different growing environments.

The stomatal index is a percentage of the stomatal number to the total number of epidermal cells (each stoma is counted as one cell). The percentage proportion of stomatal index is calculated using the following equation.

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

Where, S = the number of stomata per one square millimeter of lower epidermis.

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

2.2. Trichome

Trichomes are specialized structures of epidermal and are found as extrusions or appendages on plant surfaces. Based on their shape and function, glandular and non-glandular trichomes is recognized, and the absence of a glandular head in the non-glandular trichomes is the main morphological difference between them (Werker, 2000).

Trichome number is the number of trichome per unit area of leaf, it can be counted in the area of one square millimeter (mm^2).

The percentage proportion of trichome index is calculated using the following equation.

$$\text{Trichome} = \frac{T}{E+T} \times 100$$

Where, T = the number of trichome or cicatrix per one square millimeter of upper epidermis.

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

2.3. Epidermal cell

Epidermal cell or epidermis includes several types of cells that make up the epidermis of plants, the word from Greek meaning as “over-skin”, is a single layer of cells that covers the leaves, flowers, roots and stems of plants. It forms a boundary between the plant and the external environment.

2.4. Palisade cell

Palisade cells derived from the outer epidermis of the outer integument, are a type of parenchyma cells that contain most of the chloroplasts in leaves. Their function is to enable photosynthesis to be carried out efficiently, and they have several adaptations.

3. Powder analysis

Powder analysis plays a significant role in the identification of a crude drug. These characters may help in the identification of the right variety and search for adulterants. Powder microscopy is one of the simplest and cheapest methods to start investigating the crude drug. It is useful for further pharmacological and therapeutic evaluation along with the standardization of the plant material.

Physicochemical parameter

Foreign matter

Medicinal plant materials should be totally free from insects or molds, including mineral origins such as stones, sand, soil, dust, etc. Also, harmful and poisonous foreign matters, chemical residues, and animal objects are such as insects or invisible microbial contaminants.

Water contents

Determination of the water content is important for plant material specification because the excess of water in herbal materials can encourage the microbial growth,

the presence of fungi or insects, and deterioration following hydrolysis. The azeotropic distillation method is specifically used for the measurement of water content in the plant material. The sample is distilled together with a water-immiscible solvent such as toluene or xylene, this solvent should be saturated with water before use of the accurate result (World Health Organization, 2011).

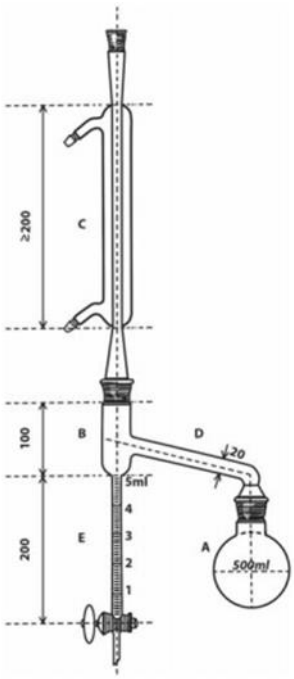


Figure 11. Apparatus for azeotropic distillation method.
(World Health Organization, 2011)

Loss on drying

Determination of loss on drying is the method used for determining the water content in the plant material. Drying can be carried out by heating, and the heating method uses the oven at 100-105 °C, after that keeping in the desiccator at room temperature (World Health Organization, 2011).

Extractable matter

Determination of the extractable matter is used to determine the amounts of active constituents in the plant material. The sample can be extracted with a specific solvent such as water and ethanol. The water is used for the polar substances, on the other hand, ethanol is used for less polar substances (World Health Organization, 2011).

Total ash

Determination of the total ash is useful for the purity and quality which signifies the inorganic substances in plant materials. This method determines the non-volatile inorganic matter after complete incineration at 500°C for 5 hours or until it becomes white (World Health Organization, 2011).

Acid-insoluble ash

Determination of acid-insoluble ash is the residue obtained after boiling total ash with hydrochloric acid (70 g/L). This method measures the amount of inorganic matters which are not solubilized in hydrochloric solution (World Health Organization, 2011).

Thin layer chromatography

Chromatography is the most widely used in a separation technique, and this technique uses for separating the components or solutes of a mixture between two phases: mobilized one is called mobile phases and the other is called the stationary phase (Tistaert, Dejaegher, & Vander Heyden, 2011).

Thin layer chromatography (TLC) is one type of chromatography technique which is a cheap, fast, and efficient way to separate a mixture into its components for analytical purposes. TLC comprises of a stationary phase and a mobile phase that compounds can be separated by their polarities. The sample compound in the mobile

phase moves over the surface of the stationary phase. Non-polar compounds are strongly held on the stationary phase and will move quickly through it, polar compounds can be slowed on their process through the stationary phase by their strong interactions with it.

The retention factor (R_f) is the ratio of distance traveled by a spot of sample origin in TLC plates to distance traveled by a solvent front from the origin. The R_f value can be used to identify compounds due to their uniqueness to each compound. If same R_f value in same solvent, it may be same compound (Bele & Khale, 2011).

The R_f value can be calculated using the formula below:

$$R_f = \frac{\text{Distance of compound from origin}}{\text{Distance of solvent front from origin}}$$

Phytochemical screening

The phytochemical screening is a technique use for detecting the secondary metabolites of herbal medicine by using chemical screening test such as ferric chloride test for phenolics, Shinoda test and alkaline test for flavonoids, Dragendorff's test, Wagner's test and Hager's test for alkaloids, etc. (Srivastava, Singh, Devi, & Chaturvedi, 2014)

Phenolics

Phenolics are the largest group of secondary metabolites which can be found in many plants. The ferric chloride test is used to determine a phenolic compound. The method detects a phenol group of the phenolic compound by color changing as blue, purple, green, or red brown depends on the nature of the phenol when adding a ferric chloride solution. (Pasto & Johnson, 1979)

Flavonoids

Flavonoids are polyphenolic compounds which are commonly found in various parts of plants. The Shinoda test and alkaline test are used to determine flavonoids. For the Shinoda test, this method detects a flavonoid by color changing as orange or deep red color when a small piece of magnesium ribbon and a few drops of concentrated HCl are added.(Scholz & Liebezeit, 2006). The alkaline test, when adding a few drops of 5% NaOH solution the ethanol extract will change to yellow color and then turns to colorless when adding a few drops of 2M HCl. That result shows this ethanol extract contain flavonoids. (R. Gul, Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N., 2017)

Alkaloids

Dragendorff's, Wagner's and Hager's tests are used for determining the presence of alkaloid in a sample. There are few steps for the extraction: dissolving a sample with chloroform, then adding 25% ammonia and mixing it, selecting a chloroform part and adding 2M HCl and mixing it, after that selecting HCl part. In the Dragendorff's test, adding a few drops of Dragendorff's reagent in the sample solution will show the reddish-brown precipitate. In the Wagner's test, adding a few drops of Wagner's reagent in the sample solution will show the reddish-brown precipitate. In the Hager's test, adding a few drops of picric acid in the sample solution will show the yellow precipitate. (Parbuntari, 2018; Scholz & Liebezeit, 2006)

Steroid and triterpenes

The Salkowski's test and the Libermann Burchard test are used to determine steroid and triterpenes compound. For the Salkowski's test, there are few steps: dissolving the sample with chloroform, then adding a few drops of concentrated sulfuric acid and shaking it. A red color in the lower layer indicates the presence of sterols, but a yellow color in the lower layer indicates the presence of triterpenoids. The Libermann Burchard test, there are few steps: dissolving the sample with

chloroform, then adding a few drops of concentrated sulfuric acid and shaking it. If a brown ring at the junction of two layers and the upper layer turns green, it indicates the presence of sterols. If a solution shows a dark red color, it indicates the presence of triterpenoids. (R. Gul, Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N., 2017)

Saponin

A foam test is used to determine a saponin. The water is added to the sample and the mixture is shaken for about 1 min. If there is a froth stable for 30 min, it indicates the presence of the saponin. (R. Gul, Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N., 2017)

Quantitative analysis

Soxhlet extraction

The Soxhlet extraction is widely used for many types of solid samples, especially biological samples. The equipment for the Soxhlet extraction apparatus consists of a distillation flask, sample holder (thimble), siphon and condenser. The steps of preparation of the Soxhlet extraction method are as follows: packing plant sample materials in a filter paper, loading into a thimble, and adding an extraction solvent in the solvent flask. The solvent is heated and subsequently condensed, and the freshly distilled solvent passes through the thimble containing the sample to be extracted. When the solvent in the thimble is overflow, the solvent will fall back into the solvent flask (Słomińska, 2012).

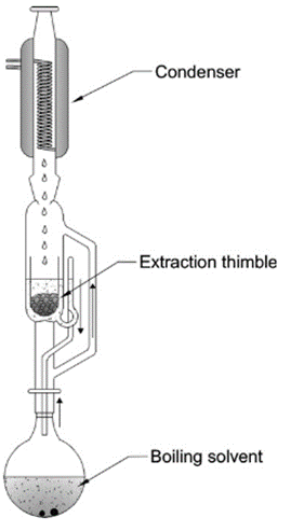


Figure 11. Soxhlet apparatus.
(Słomińska, 2012)

High Performance Liquid Chromatography (HPLC)

Chromatography is a technique for separation, purification and identification of a mixture for quantitative and qualitative analysis (Coskun, 2016). It is a technique for separating the components between two phases: the mobile phases and the stationary phase. Molecules in the mixture can be separated from each other while moving with the aid of a mobile phase.

High-performance liquid chromatography (HPLC) or high-pressure liquid chromatography is one type of liquid chromatographic method. It is widely used to separate a mixture of compounds in analytical chemistry and biochemistry to identify and quantify the individual components of the mixture. In the HPLC system, the stationary phase is a solid and the mobile phase is a liquid. The mobile phase can move through a pre-packed column (the stationary phase), the sample is loaded on the inject loop and pass through the column with a flow of the mobile phase. Before entering the separation column, the outlet of the column is connected with a detector which serves to detect substances. The detection of a compound can be done by using a variety of detectors (Hansen, Pedersen-Bjergaard, & Rasmussen, 2011).

Reverse Phase HPLC (RP-HPLC) is a commonly used mode of HPLC. Whereby the stationary phase is hydrophobic, the column packing materials commonly made from silica derivatized with reagents to form a hydrophobic surface on the silica particle. Typically, silica used in a column is made by chemical modification of silanol groups using chlorosilanes or other organic silane reagents such as octadecyl carbon chain (C_{18}), C_8-bonded silica (C_8), cyano-bonded silica etc. Generally, a C_{18} column is mostly used in the reversed phase HPLC. The mobile phase of RP-HPLC is a more polar aqueous solution, which consists of a mixture of water and one or more organic solvents that are soluble with water (Hansen et al., 2011).

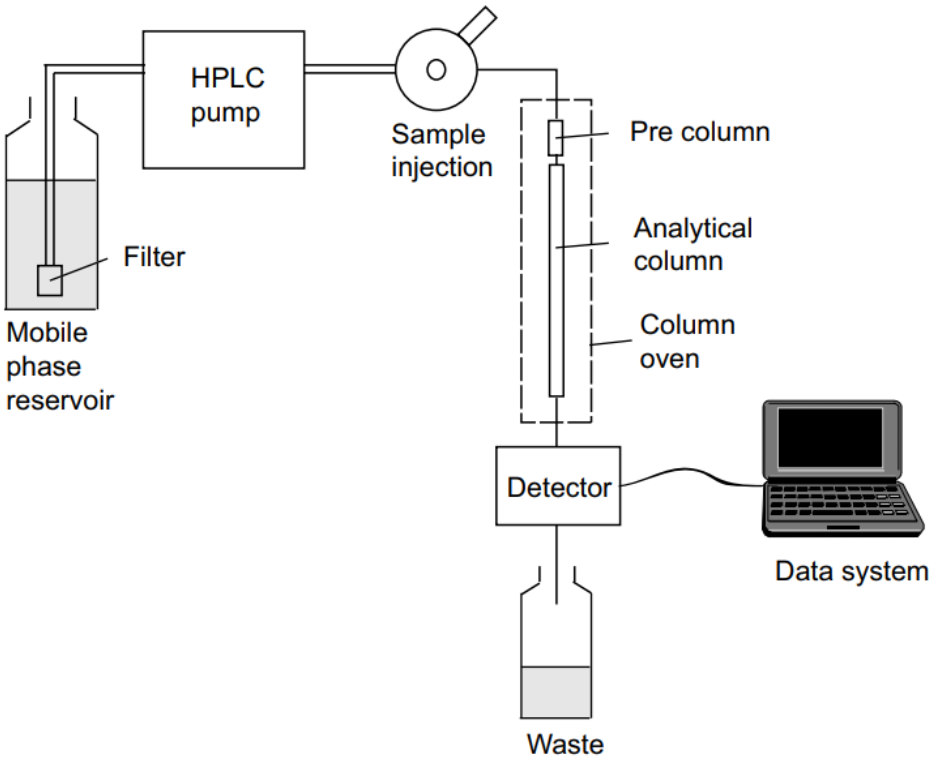


Figure 12. The main structure of HPLC system.
(Hansen et al., 2011)

CHAPTER III

MATERIALS AND METHODS

Chemicals and reagents

1. Ethyl acetate, AR grade	RCL Labscan Limited, Bangkok, Thailand
2. Methanol, AR grade	RCI Labscan Limited, Bangkok, Thailand
3. Formic acid 98-100%, AR grade	Merck, Darmstadt, Germany
4. Ethanol, AR grade	RCI Labscan Limited, Bangkok, Thailand
5. Toluene	RCI Labscan Limited, Bangkok,Thailand
6. Chlorogenic acid	Sigma-Aldrich, St. Louis, Missouri, USA (CAS No. 327-97-9, purity ≥95%)
7. Chloral hydrate	
8. Hydrochloric acid 37%, AR grade	RCI Labscan Limited, Bangkok,Thailand
9. Methanol, HPLC grade	RCI Labscan Limited, Bangkok,Thailand
10. Phosphoric acid	
11. Haiter®	

Materials

1. Filter paper No.4	Whatman™ Paper, UK
2. Filter paper No.40 ashless	Whatman™ Paper, UK
3. TLC silica gel 60 GF254	Merck, LTD, USA
4. Microscope slides (25.4 x 76.2 mm)	Sail Brand, China
5. Cover glasses (24 x 50 mm)	Thermo Scientific, Brunswick, Germany

6. Nylon membrane filters (46 mm x 0.45 µm) PTFE membrane syringe filter (13 mm x0.45 µm)	National Scientific, Tennessee, USA ANPEL Laboratory Techno (Shanghai), Shanghai, China.

Instrument and equipment

1. Aqua shaker	Adolf Kuhner AG, Switzerland
2. Ashing furnaces	Carbolite, Scientific Promotion, Bangkok, Thailand
3. CAMAG TLC plate Heater III	CAMAG, Switzerland
4. CAMAG TLC chamber	CAMAG, Switzerland
5. Digital balance (Model: SI-234)	Denver Instrument, New York, USA
6. CAMAG TLC visualizer 2	CAMAG, Switzerland
7. Digital orbital shaker (Model: SHO-2D)	Daihan Scientific, Korea
8. Hot air oven	WTC Binder, Germany
9. Incinerator	Carbilite, UK
10. Rotary vacuum evaporator	Buchi, Switzerland
11. Microscope	Zeiss Axioskop, Germany
12. Vortex mixer	Scientific Industries, USA
13. High performance liquid chromatography (LC-20A) equipped with photo diode array detector (SPD-M20A)	Shimadzu, Kyoto, Japan
14. Ultrasonic bate	Analytical Lab Science, Bangkok, Thailand.

Plant materials

M. alba leaves were collected from 15 different sources throughout Thailand. The sample was authenticated by Associate Professor Nijsiri Ruangrugsi as an expert in the fields of Pharmaceutical Botany and Pharmacognosy. The voucher specimens are deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. After removing any foreign matters, the sample was dried by hot air oven and ground into powder before testing.

Pharmacognostic specification of *M. alba*

Macroscopic and microscopic examinations

Macroscopic examination

Macroscopic examinations of *M. alba* were identified based on surface characteristics, shape, size, color, texture and other inspection. The leaves of *M. alba* in Thailand were picked by hand in a proportional scale to the original size.

Microscopic examination

Microscopic examination of the leaves of *M. alba* was examined in midrib cross section, leaf measurement and powder from. Photographs are taken by digital camera and drawn by hand in a proportional scale to the original size.

1. **Midrib cross-section**

Midrib cross-section was conducted by free hand cross-section. The sources of fresh midrib part of *M. alba* mature leaves were cut by using free hand cross-section by razor and put the tissue section with water in a glass slide for observation, and examine under the microscope with magnification of 5x, 10x, 20x and 40x objective lens.

2. Leaf measurement

The mature leaves of 3 different sources *M. alba* were cut into a square by avoiding the midrib, about 1x1 cm. Leaves were put in a mixture of haiter® (6% sodium hypochlorite) and water (1:1) and the leaf sample was left until the green color of chlorophyll disappears. After that the leaf sample was moved from haiter® solvent to a solution of chloral hydrate and water (4:1) for approximately 2-3 days, and the leaf sample was placed into a glass slide with a drop of water for observing and examining under the microscope with magnification of 5x, 10x, 20x and 40x objective lens. Every source was carried out with 30 fields, the total is 90 fields.

2.1 Epidermal cell

Epidermal cell number was counted and calculated per one square millimeter (mm^2) of the upper epidermis of the leaf. The cells on the four edges were counted only the top and the left sides.

2.2 Palisade cell

The palisade cell was counted under the four epidermal cells. The cells on the four edges were counted only the top and the left sides.

2.3 Stoma

The stomata cell was counted in the area of one square millimeter (mm^2) of the lower epidermis of the leaf. The cells on the four edges were counted only the top and the left sides. The percentage proportion of stomatal index was calculated using the following equation:

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

Where, S = the number of stomata per one square millimeter of lower epidermis.

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

2.4 Trichome

The trichome cell was counted in the area of one square millimeter (mm^2) of the upper epidermis of the leaf. The cells on the four edges were counted only the top and the left sides. The percentage proportion of trichome index was calculated using the following equation:

$$\text{Trichome} = \frac{T}{E+T} \times 100$$

Where, T = the number of trichome or cicatrix per one square millimeter of upper epidermis.

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

3. Powder study

The dried leaf powder of *M. alba* was mixed with a solution of chloral hydrate in a glass slide for observation and examination under the microscope with magnification of 5x, 10x, 20x and 40x objective lens.

Physicochemical determination

Determination of water content (Azeotropic method)

Fifty grams of *M. alba* dried leaf powders were transferred to a round bottom flask. A 300 mL of water saturated toluene was added to the flask containing the powders and the mixture was boiled by using azeotropic distillation method until the water was completely distilled. The volume of water was measured and calculated in a percentage.

Determination of loss on drying

Three grams of *M. alba* dried leaf powders were weighed in a previously weighed crucible and dried at 105 ºC for 6 hours until the weight was constant. After that, the crucible was left to cool at room temperature, weighed and calculated the loss of weight in a percentage.

Determination of total ash

The crucible which contains ash from loss on drying was incinerated at 500 ºC for 5 hours until it became white. The crucible was left to cool in a desiccator and then weighed without the delay. The content of total ash was calculated in a percentage.

Determination of acid insoluble ash

Twenty-five milliliters of hydrochloric acid (70 g/L) were added to the crucible, which contained ash from the total ash than covered with the watch-glass and boiled for 5 minutes. The insoluble matters were collected on a filter-paper No. 40 ashless, after that they were transferred to the original crucible and dried on a hotplate and incinerated at 500 ºC for 5 hours. The crucible was left to cool in a desiccator and weighed without the delay. The content of acid-insoluble ash was calculated in a percentage.

Determination of ethanol soluble extractive value

Five grams of *M. alba* dried leaf powders were macerated with 70 mL of 95% ethanol in conical flask and used aluminum foil to cover the lid tightly. After that, the conical flask was placed in a shaker for 6 hours and stood for 18 hours. The marc was filtered and washed by using filter paper No. 4. The filtrate was adjusted to 100 mL with ethanol. After that, 20 mL of the filtrate was transferred to a pre-weighed beaker and evaporated to dryness. The extract was dried at 105 ºC for 6 hours, cooled in a

desiccator for 30 minutes and weighed without the delay. The content of extractable matter was calculated in a percentage.

Determination of water-soluble extractive value

Five grams of *M. alba* dried leaf powders were macerated with 70 mL of water in a conical flask and used aluminum foil to cover the lid tightly. After that, the conical flask was place in a shaker for 6 hours and stood for 18 hours. The marc was filtered and washed by using filter paper No. 4. The filtrate was adjusted to 100 mL with water. After that, 20 mL of the filtrate was transferred to a pre-weighed beaker and evaporated to dryness. The extract was dried at 105 ^{o}C for 6 hours, cooled in a desiccator for 30 minutes and weighed without the delay. The content of extractable matter was calculated in a percentage.

Thin layer chromatographic fingerprint

According to the ethanol extractive value determination, the 20 mL of extract was evaporated to dryness and then dissolved by adding 1 mL of 95% ethanol. The extract solution (3 µL) was spotted on a TLC Silica gel 60 GF_{254} plate and developed in a saturated TLC chamber with ethyl acetate: methanol: water: formic acid (16.8:1.2:1.2:0.4) as a mobile phase. After development, the plate was removed and dried at room temperature and examined under ultraviolet light with 254 and 366 nm. After that, the plate was sprayed with sulfuric acid reagent and heated at 105 °C for 10 min in CAMAC TLC plate heater III.

Phytochemical screening

Preparation of samples

Five grams of *M. alba* dried leaf powders were exhaustively extracted with 300 mL of 95% ethanol in a Soxhlet apparatus. The ethanolic extract was filtered and evaporated under reduced pressure in a rotary evaporator until dryness. The extracted sample was stored at 4 °C for phytochemical screening test.

Table 5. Preparation of chemical test

No.	Compound screening	chemical test	chemical	Concentrated
1	Phenols	Ferric chloride test	Ferric chloride ($FeCl_3$)	5%
2	Flavonoids	Shinoda test	Magnesium (Mg) chip	
			Hydrochloric acid (HCl)	Concentrate
		Alkaline test	Sodium hydroxide (NaOH)	5%
			Hydrochloric acid (HCl)	2M
3	Alkaloids	Dragendorff's test	Bismuth subnitrate	
			Potassium iodide	
			Glacial acetic acid	
			Water	
		Wagner's test	Potassium iodide	
			Iodine	
			Water	
		Hager's test	Picric acid	
4	Steroid and Triterpenes	Salkowski's test	Chloroform	
			Sulfuric acid (H_2SO_4)	
		Liebermann Burchard test	Acetic anhydride	
			Sulfuric acid (H_2SO_4)	
5	Saponin	Foam test	Water	

Phenols

The ferric chloride test was used for determining the presence of phenols in a sample. The 1 mL of the ethanol extract sample was loaded in a test tube, after that the sample was added with a few drops of 5% $FeCl_3$ solution (Pasto & Johnson, 1979).

Flavonoid

Shinoda test and alkaline test were used for determining the presence of flavonoid in a sample. For the Shinoda test, 1 mL of the ethanol extract sample was loaded in a test tube and then put Mg chip, after that was added a few drops of concentrated HCl. For the alkaline test, 1 mL of the ethanol extract sample was loaded in a test tube, after that was added a few drops of 5% NaOH and then added a few drops of 2M HCl (R. Gul, Jan, Faridullah, Sherani, & Jahan, 2017).

Alkaloid

Dragendorff's, Wagner's and Hager's tests were used for determining the presence of alkaloid in a sample. The 1 mL of the chloroform extract sample was loaded in a test tube and added 1 mL of 25% ammonia, after that was used a vortex mixer to mix a solution, and then collected a chloroform part which was transferred into another a test tube. The 1 mL of 2M HCl was added in the chloroform part, then used a vortex mixer to mix a solution and collected a HCl part and separated into 3 parts which were loaded in another test tube. Then, a few drops of Dragendorff's solution, Wagner's solution and Hager's solution were added in each test tube. (Parbuntari, 2018)

Steroid and triterpenes

Salkowski and Liebermann Burchard tests were used for determining the presence of steroid and triterpenes in a sample. For the Salkowski test, the 1 mL of the chloroform extract sample was loaded in a test tube and then added a few drops of concentrated H_2SO_4. For the Liebermann Burchard test, the 1 mL of the chloroform extract sample was loaded in a test tube and then added a few drops of acetic anhydride and concentrated H_2SO_4 (R. Gul, Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N., 2017).

Saponin

Foam test was used for determining the presence of saponin in a sample. One milligram of dried *M. alba* leaf powers was put in a test tube and then added 1-2 mL in the test tube which was further shaken. (R. Gul, Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N., 2017)

Quantitative analysis of chlorogenic acid in *M. alba* leaves

Preparation of chlorogenic acid solution

Ten milligrams of standard chlorogenic acid were dissolved in 1 mL of methanol. The stock solutions were diluted to obtain the series of standard solutions with concentrations of 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL, respectively. These solutions are stored in a -20 ℃ freezer.

Preparation of ethanol extracts of *M. alba* leaves

The accurate 5 g of 15 different sources *M. alba* dried leaf powders were exhaustively extracted with 300 mL of 95% ethanol in a Soxhlet apparatus. The ethanolic extract was filtered by filter paper No. 4 and evaporated under reduced pressure in a rotary evaporator until dryness. The extracted sample was stored at -20 ℃.

Ten milligrams of each extract were dissolved in 1 mL of methanol and diluted to appropriate concentration for HPLC analysis after that the solution was filtered through a 0.45 µm PTFE membrane syringe filter.

High Performance Liquid Chromatography (HPLC)

HPLC analysis was processed by using Shimadzu LC solution software. Shimadsu HPLC LL-20A system (Shimaszu, Japan) consists of a controller (CMB-20A), an auto-sampler (SIL-20A), an online degassing unit (DGU-20A3), two solvents delivery units (LC-20A) and photo-diode array detector (SPD- M20A). The sample was analyzed by using 0.2% phosphoric acid in water (solvent A) and

methanol (solvent B) as a mobile phase. The methanol was filtered through 0.45 PTEF membrane filters, and 0.2% phosphoric acid solution was filtered through 0.45 nylon membrane filters. Both were degassed by using the ultrasonic bath for 15 minutes before analysis. The chromatographic separation was performed with a C_{18} column (4.6 x 250 mm) and coupled with a C_{18} guard column (4.6 x 10 mm), temperature will be maintained at 30 °C. Injection of standard and sample was 10 µl, the concentration of sample is varied between different sources. The program was set in isocratic mode at 45% methanol for 50 minutes at flow rate 0.5 mL/min. The wavelength was set at 330 nm for monitory chromatographic profile. The measurement was carried out in triplicate.

Method validation

According to the ICH guidelines, the method validation including calibration range, accuracy, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ) and robustness of chlorogenic acid quantitative analysis in *M. alba* leaves were validated (Guideline, 2005).

Calibration range

The calibration range was calculated by plotting peak areas and concentrations of standard chlorogenic acid.

Accuracy

The accuracy was tested by recovery method. Standard chlorogenic solution was spiked into the extract to have three different levels of chlorogenic acid (low, medium, high). The spiked and un-spiked samples were analyzed under the same conditions in triplicate. The accuracy was determined by using a following formula. The acceptable range of recovery was a range of 80-120 % of the test concentration.

$$\% \text{ Recovery} = \left(\frac{A}{B+C}\right) \times 100$$

Where: A = the actual calculated amount in recovery sample

B = the amount un-spiked into the sample

C = the amount standard added to the sample

Precision

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

$$\% \text{ RSD} = \left(\frac{\text{SD}}{\text{Mean}}\right) \times 100$$

Where: SD = the standard deviation of each measurement

Specificity

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

Limit of detection (LOD)

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

$$\text{LOD} = \frac{3.3 \times \text{SD}}{\text{S}}$$

Where: SD = the residual standard deviation of regression line

S = the slope of regression line

Limit of quantitation (LOQ)

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

$$\text{LOQ} = \frac{10 \times SD}{S}$$

Where: SD = the residual standard deviation of regression line

S = the slope of regression line

Robustness

The robustness was examined by variations in flow rates (0.495, 0.500 and 0.505 mL/min), The robustness was calculated in term of percent relative standard deviation of peak area.

Data analysis

The data was evaluated by comparing the peak area with the calibration curve. The area under peak was analyzed by using Shimadzu LC Solution software for determination of chlorogenic acid content.

Statistical analysis

The descriptive statistical analysis of pharmacognostic specification; quantitative analysis of leaf constant number part was reported by using mean ± SD. The physicochemical determination part was reported by using grand mean ± pooled SD. The quantification by RP-HPLC analysis was reported by using mean ± SD. Method validation was reported by using mean, SD and %RSD.

CHAPTER IV
RESULTS

Pharmacognostic specification of *Morus alba* Linn. leaves

Common Thai Name	MON
Another Name	Sang shu (Chinese)
English Name	White mulberry
Scientific Name	*Morus alba* Linn.
Family	MOEACEAE
Distribution	Tropical
Plant Past	Leaves
Ethnomedical Uses	Treatment of diabetic, respiratory disease, fever.

Macroscopic and Microscopic evaluation

Macroscopic evaluation

The plant morphology of *M. alba* was a shrub with 3-10 m height which had shallowly furrowed gray barks, finely hairy branches, and reddish-brown and finely hairy ovoid winter buds. Stipules which lanceolate had 2-3.5 cm long, it was covered with a short trichome. Petiole had 1.5-5.5 cm long and trichome was also found. The leaves were single leaves and arrange alternately, it had oval-shaped or wide oval and long pointed tip. The concave base was heart shaped. The edge of the leaf was smooth or serrated. The young leaves were unequal in both sides of the leaf, the leaves were about 8-14 cm wide and 12-16 cm long. The flowers were single-sex catkins and greenish, male catkins were broad, cylindrical or ovoid had 2-3.5 cm long, and female catkins were ovoid 1–2 cm long. Male and female flowers were usually on separate trees although they might occur on the same tree. The fruit was 1–1.5 cm long and was deep purple in the wild, but in many cultivated plants it varied from white to pink.

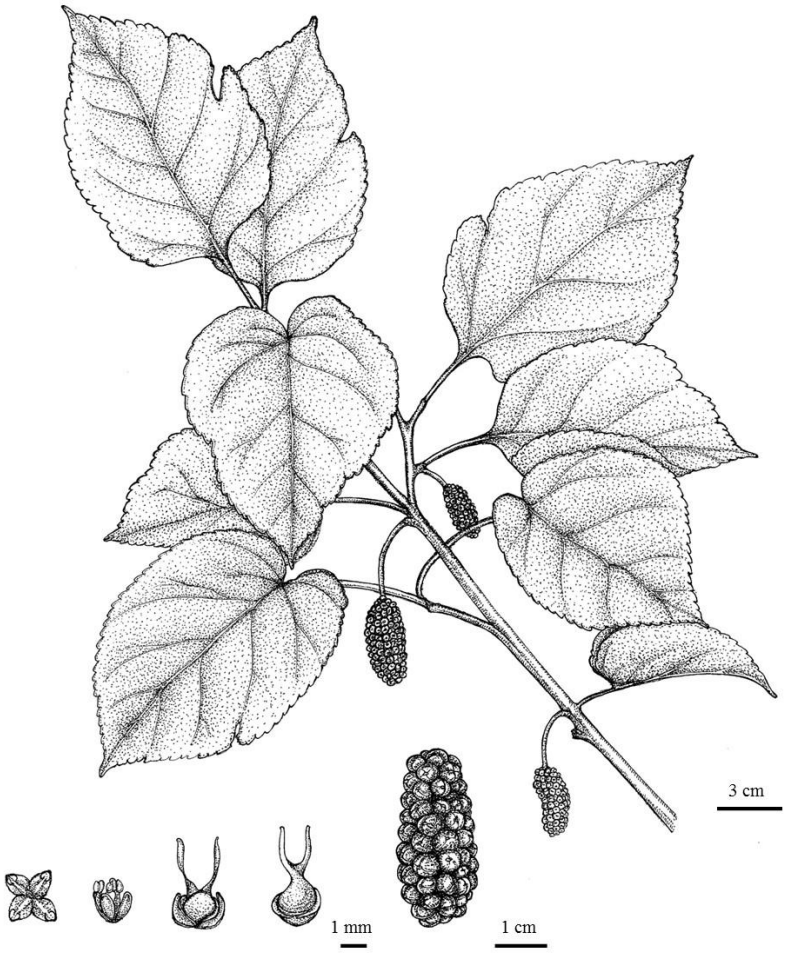


Figure 13. *M. alba* branch drawing picture.

Microscopic evaluation

Midrib cross-section

The mature leaves of *M. alba* were collected and cleaned, after that the midrib was cut thinly by hand. The transverse midrib cross-section of *M. alba* leaves including the epidermis layer which was the outline of the midrib cross-section slightly channeled on a rounded surface. It was made up of an epidermal cell and could be found as non-glandular trichome and unicellular trichome. The next layer found in chlorenchyma and parenchyma cells were multilayered without intercellular spaces. The middle part of the midrib cross-section had a vascular bundle including xylem and phloem. The midrib cross-section is shown in Figure 14.

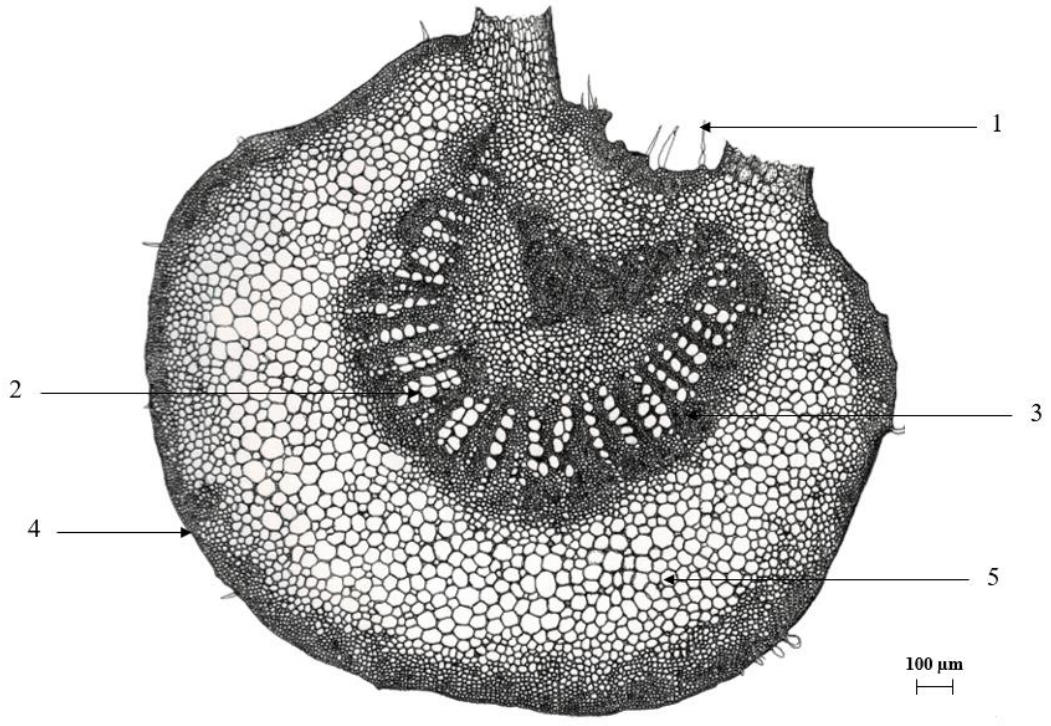


Figure 14. Midrib cross section drawing picture.

(1). Non-granular trichome, (2). Xylem, (3). Phloem, (4). Epidermis and (5). Chlorenchyma

Leaf measurement

Epidermal cell and palisade cell

The epidermal cell found in the upper epidermis cell had a thick-wall polygonal cell wall (Figure 15 A), and the lower epidermal cell had a thin-wall wavy cell wall (Figure 15 B). The palisade cell formed as the layer which was below the epidermal cell in the upper side of leaf. It was round shaped and clear. The structure of palisade cell is shown in Figure 16.

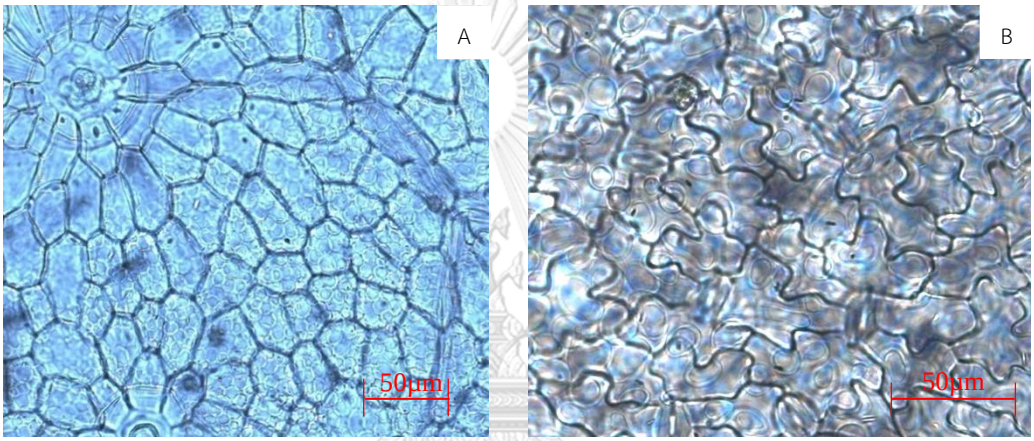


Figure 15. Epidermal cells of *M. alba* leaves.
(A) Upper side of *M. alba* leaves., (B) Lower side of *M. alba* leaves.

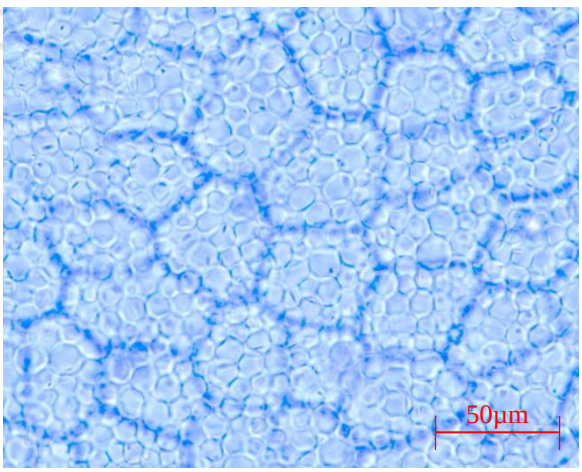


Figure 16. Palisade cells of *M. alba* leaves.

Stomatal cell

The stomatal cell of *M. alba* leaves was distributed only on the lower side of leaf surface. The type of stomatal was an anomocytic type, which was surrounded by various numbers of cells, and was generally not different from those of the epidermal cells. The characteristics of stomatal are shown in Figure 17.

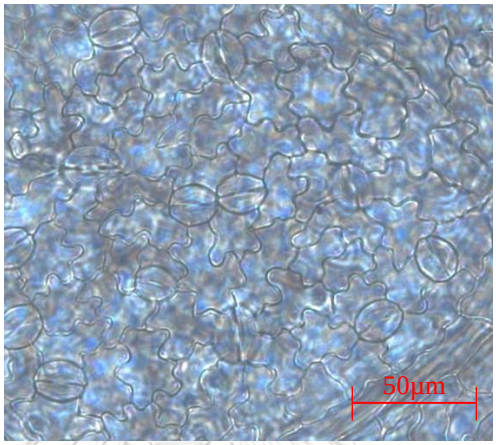


Figure 17. Stomatal cells of *M. alba* leaves.

Trichome

The non-glandular trichome and unicellular trichome were found in the upper side of leaf surface. Normally, *M. alba* leaves when soaked in sodium hypochlorite solution and chloral hydrate solution trichome were fallen off from the epidermis surface leaving cicatrices, which was a characteristic scar. The characteristics of cicatrices are shown in Figure 18.

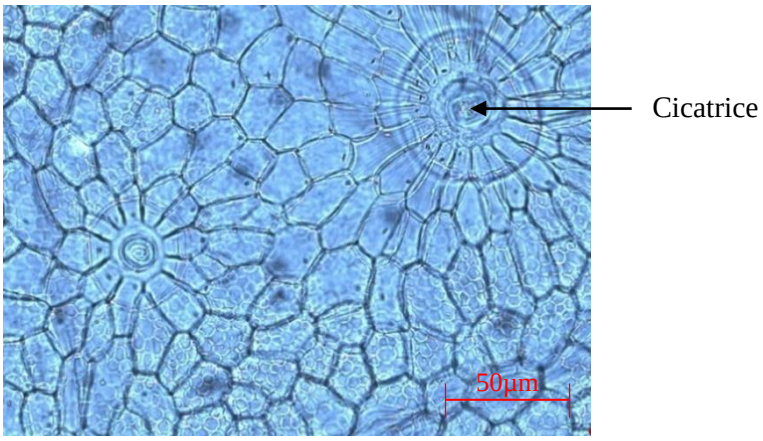


Figure 18. The cicatrices of *M. alba* leaves

Quantitative analysis of leaf constant number

The quantitative analysis of leaf constant number showed epidermal cell number, epidermal cell area, palisade ratio, stomatal number, stomatal index, trichome number and trichome index. The epidermal cell area and palisade ratio were determined with the upper epidermis. The stomatal number and stomatal index were determined with lower epidermis, the trichome number and trichome index were determined with the upper epidermis. The results are shown in Table. 6.

Table 6. Microscopic evaluation of leaf constant numbers of *M. alba* leaves throughout Thailand.

Leaf constant values	**Upper epidermis**		**Lower epidermis**	
	Min - max	**Mean ± SD**	**Min - max**	**Mean ± SD**
Epidermal cell number (per mm^2)	1008–1540	1216.53 ± 141.65	284–588	438.89 ± 81.88
Epidermal cell area (µm^2)	649.35–992.06	832.68 ± 93.16	1700.68–3521.13	2361.30 ± 456.31
Stomatal number (per mm^2)	–	–	140–280	193.56 ± 33.63
Stomatal index (per mm^2)	–	–	22.35–37.57	30.80 ± 3.82
Trichome number	12–28	15.87 ± 4.00	–	–
Trichome index	0.80–2.19	1.29 ± 0.30	–	–
Palisade ratio	13.25–17.75	15.10 ± 1.17	–	–

Powder analysis

The dried *M. alba* leaves were green to dark green color, the histological characteristics of *M. alba* leaves powder were presented i.e., epidermis cell, trichome, anomocytic stomatal and parenchyma cell. The histological characteristics of *M. alba* leaves powder are shown in Figure 19.

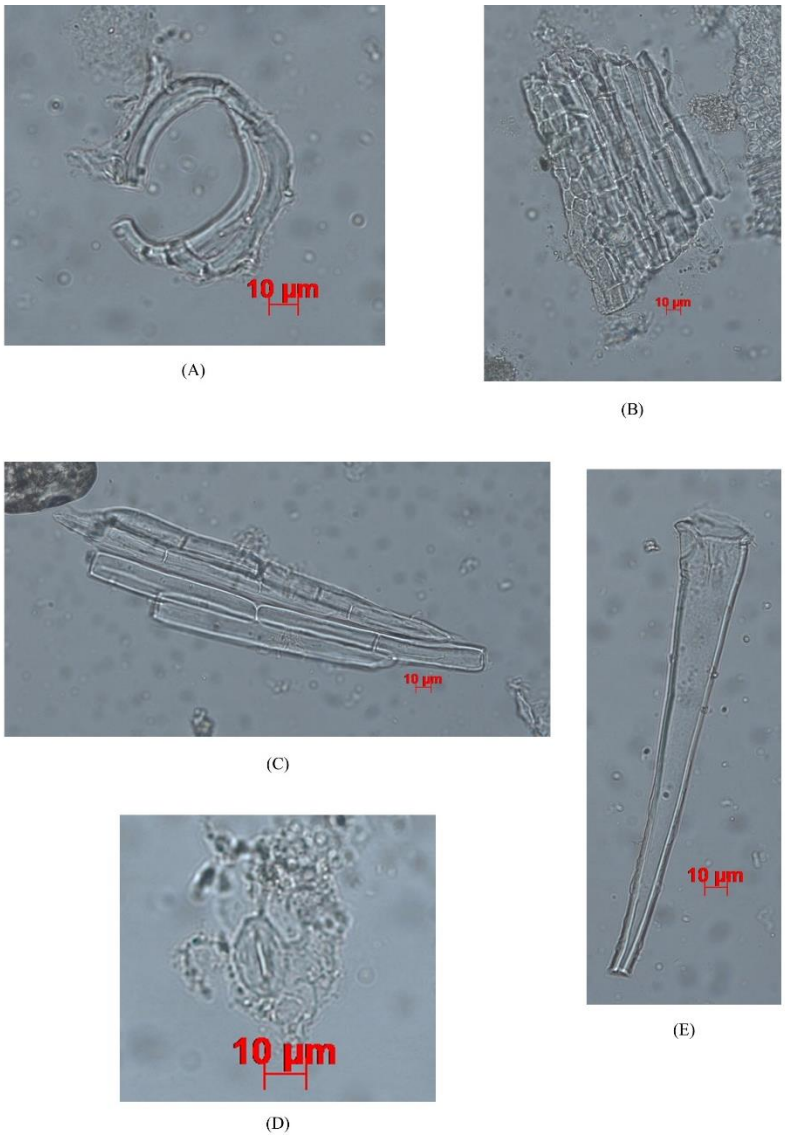


Figure 19. The histological characteristics of *M. alba* leaves.
(A) Non-glandular trichome, (B) Epidermal cell, (C) Longitudinal parenchyma cell, (D) Anomocytic type of stoma, (E) Non-glandular trichome

Physicochemical determination

The contents of physicochemical determination of *M. alba* leaves are shown in Table 7. The parameters were shown as grand mean ± pooled SD. The sample were collected from 15 different sources in Thailand. Each sample was tested in triplicate. The specification for quality control of *M. alba* leaves of water content, loss on drying, total ash and acid insoluble ash should not be higher than 7.79%, 4.55%, 14.38% and 6.20% of dry weight, respectively. The water-ethanol soluble extractive value should not be below than16.14% and 8.61% of dry weight.

Table 7. Physicochemical determination of *M. alba* leaves (% by weight)

Parameter	Content (% by weight) (Grand mean ± pooled SD)
Water content	7.97±0.35
Water-extractive value	16.14±0.50
Ethanol-extractive value	8.61±0.39
Loss on drying	4.55±0.21
Total ash	14.38±0.25
Acid insoluble ash	6.21±0.37

Thin layer chromatographic fingerprint

TLC fingerprint of ethanolic extract of *M. alba* leaves was observed under ultraviolet (UV) light with 254 and 366 nm and detected by sprayed with 10% sulfuric acid in ethanol for the detection of phenolic compounds. The result is shown in Figure 20. The TLC fingerprint under visible light showed about 6 bands, but their observation under UV radiation at 254 and 366 nm was showed about 8–9 bands. Additionally, the TLC fingerprint detected with 10% sulfuric acid in ethanol was showed about 10 bands.

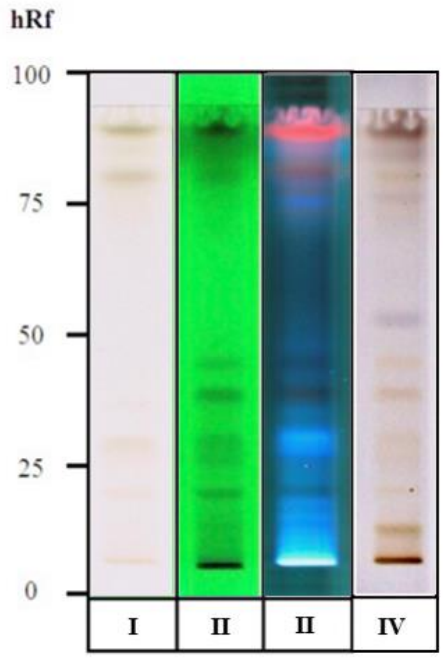


Figure 20. TLC fingerprint of ethanolic leaf extract of *M. alba* leaves.
Stationary phase: Silica gel 60 GF_{254} TLC plate.

Mobile phase: Ethyl acetate: methanol: water: formic acid (16.8:1.2:1.2:0.4, v/v/v/v)

Detection: I = under visible light
II = under UV 254 nm
III = under UV 366 nm
IV= sprayed with 10 % sulfuric acid in methanol.

Phytochemical screening

The phytochemical screening is a technique use for detecting the secondary metabolites of herbal medicine by using chemical screening tests. The results of phytochemical screening are shown in Table 8. *M. alba* leaves (Source from Maha Sarakham) showed the presence of phenols, flavonoids, steroids and triterpenes.

Table 8. Phytochemical screening tests of the crude ethanolic extract of *M. alba* leaves.

Phenols	**Ferric chloride test**	**+**
Flavonoids	Shinoda test	+
	Alkaline test	+
Steroids and triterpenes	Salkowski test	+
	Liebermann Burchard test	+
Alkaloid	Dragendorff's test	-
	Wagner's test	-
	Hager's test	-
saponin	Form test	-

+ = present, - = not present

Quantification by RP-HPLC analysis

Ethanolic extraction of *M. alba* leaves

The dried powders of *M alba*. leaves, which were collected from 15 different sources throughout Thailand, were extracted with 95 % ethanol by Soxhlet apparatus. The percentage yield of leaf ethanolic extract were found to be 26.54 ± 3.98%, the results are shown in Table 9.

Table 9. The percentage yield of the ethanolic extract of *M. alba* leaves from 15 different sources in Thailand.

Sources	Weight of sample (g)	Weight of extractive matter (g)	Percentage yield (%)
Suphan Buri	5.00	1.15	22.76
Roi Et	5.00	1.31	26.29
Ratchaburi	5.00	1.63	32.48
Chanthaburi	5.00	1.13	22.61
Phitsanulok	5.00	0.95	18.93
Rayong	5.00	1.16	23.08
Nakhon Ratchasima	5.00	1.41	28.21
Nakhon Pathom	5.00	1.56	31.07
Khon Kaen	5.00	1.54	30.59
Phetchabun	5.00	1.49	29.88
Maha Sarakham	5.00	1.20	23.88
Yasothon	5.00	1.18	23.23
Buri Ram	5.00	1.52	30.22
Prachin Buri	5.00	1.32	26.38
Nakhon Phanom	5.00	1.43	28.49
		Average	26.54 ± 3.98
		Min	18.93
		Max	32.48

Quantitative analysis of chlorogenic acid content in *Morus* alba leaves by RP-HPLC analysis

The chlorogenic acid contents of *M. alba* leaves were analyzed by using RP-HPLC. The results are shown in Table 10. The average of chlorogenic acid contents in 15 different sources were 0.4159 ± 0.20 g/100g by dry weight. The RP-HPLC chromatograms of chlorogenic acid (standard) and chlorogenic acid in *M. alba* leaf extract are shown in Figure 21.

Table 10. Chlorogenic acid content of *M. alba* leaves from 15 different sources throughout Thailand.

Source	The percentage yield by Soxhlet extraction (g/100g)	Chlorogenic acid content (g/100 g dry weight)
Ratchaburi	32.48	0.8756
Yasothon	23.23	0.6095
Prachin Buri	26.34	0.5785
Nakhon Pathom	31.07	0.5601
Phetchabun	29.88	0.5032
Phitsanulok	18.93	0.4615
Maha Sarakham	23.88	0.4583
Buri Ram	30.22	0.4475
Suphan Buri	22.76	0.3561
Nakhon Ratchasima	28.21	0.3272
Khon Kaen	30.59	0.3122
Roi Et	26.29	0.2766
Rayong	23.08	0.2070
Chanthaburi	22.61	0.1946
Nakhon Phanom	28.49	0.0703
Average	26.54±3.98	0.4159±0.20

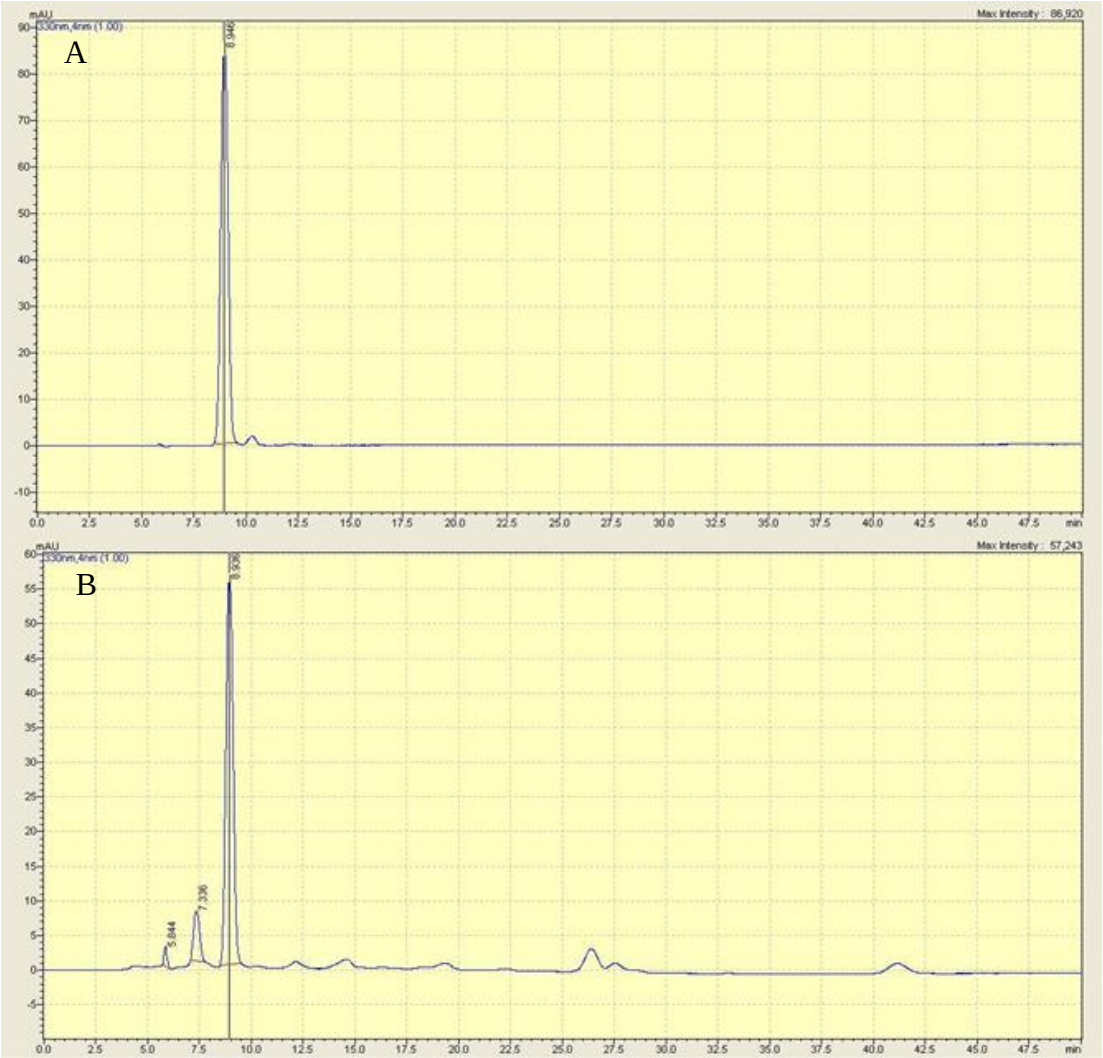


Figure 21. RP-HPLC quantitative analysis of chlorogenic acid content in the ethanolic leaf extract of *M. alba*:

(A) RP-HPLC profile of chlorogenic acid (standard); (B) RP-HPLC profile of the ethanolic leaf extract of *M. alba* leaves.

Method validation (HPLC)

The method validation parameters for the RP-HPLC quantitative analysis of chlorogenic acid contents were evaluated and are shown in Table 11.

Table 11. The method validation of RP-HPLC quantitative analysis of chlorogenic acid contents in *M. alba* leaves.

Parameter	Validation
Linearity	y = 55074x - 135166 (R^2 = 0.9981)
Accuracy (%recovery)	94.84
Limit of detection (mg/mL)	0.0028
Limit of quantitation (mg/mL)	0.0086
Repeatability (%RSD: 5, 20, 40 µg/mL)	1.10, 0.24, 0.14
Intermediate precision (%RSD: 5, 20, 40 µg/mL)	0.73, 1.06, 3.01
Robustness (%RSD)	0.17
Specificity (Peak purity index)	1.00

Calibration curve

Calibration curve was generated by a plot between peak area and six different concentration levels of standard chlorogenic acid. The regression equation of y = 55074x - 135166, the coefficient of determination (R^2) of chlorogenic acid was 0.9981 (Figure 21).

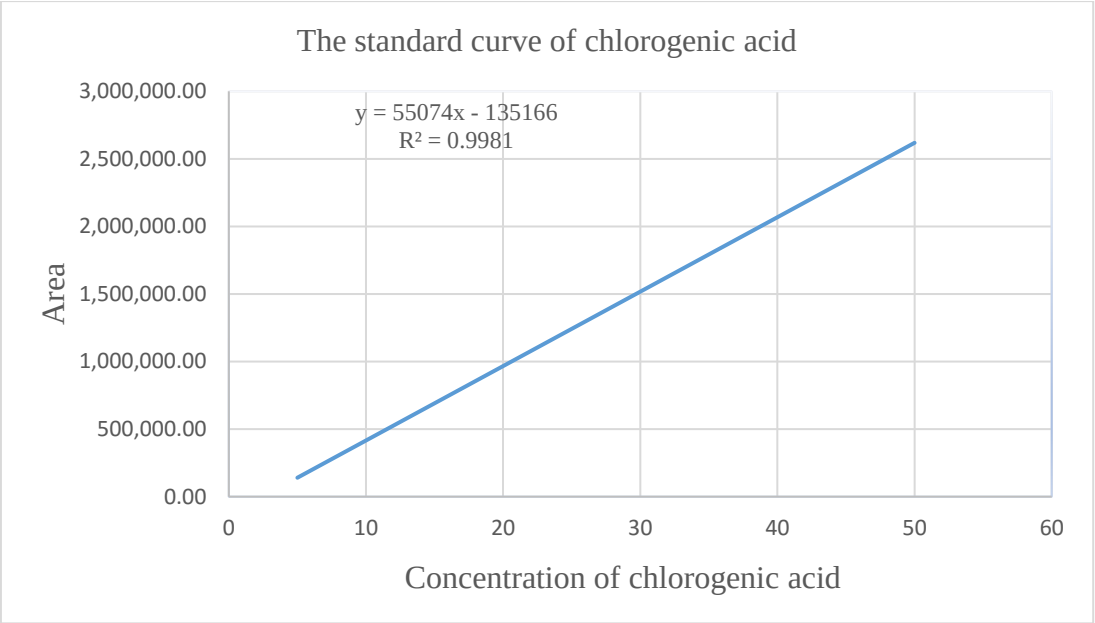


Figure 22. The calibration curve of standard chlorogenic acid.

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

The limit of detection (LOD) and limit of quantitation (LOQ) were obtained from calculation based on standard deviation of regression line and the slope of calibration curve. The LOD value which was the lowest concentration for analyte in a sample that could be detected was found to be 0.0028 mg/mL. The LOQ value which was the lowest concentration of analyte in a sample that could be quantitatively defined was found to be 0.0086 mg/mL.

Accuracy

The accuracy was analyzed by adding 3 different concentrations of standard chlorogenic acid (5, 20, 40 µg/mL) into the sample, all experiments were done in triplicate. The accuracy of HPLC for chlorogenic acid of *M. alba* leaves was calculated as a percentage recovery of chlorogenic acid (Table 12).

Table 12. Accuracy of HPLC for chlorogenic acid of *M. alba* leaves.

Chlorogenic acid (µg/mL)	Chlorogenic acid calculated (µg/mL)	Chlorogenic acid found (µg/mL)	% Recovery
-	20.4105	20.4105	100
5	22.9646	22.0769	96.36
20	30.4646	29.6172	97.39
40	40.4646	36.0257	85.62
		Average	94.84

Precision

The precision was examined by repeatability (intra-day) and intermediate precision (inter-day). The precision was evaluated with 3 different concentrations of chlorogenic acid and analyzed on the same day and 3 different days. The values were shown as % RSD. The results are shown in Table 13.

Table 13. Repeatability precision and intermediate precision of HPLC for chlorogenic acid of *M. alba* leaf

Concentration of chlorogenic acid (µg/mL)	Repeatability precision (% RSD)	Intermediate precision (% RSD)
5	1.10	0.73
20	0.24	1.06
40	0.14	3.01
Average	0.49	1.60

Specificity

The specificity was evaluated by analysis of peak purity test. The peak purity test confirmed that analyte chromatographic peak was not attributable with another compound. Moreover, the peak purity index of chlorogenic acid of *M. alba* leaf was found to be 1.00 (Figure 22.)

Figure 23. Peak purity of chlorogenic acid of *M. alba* leaf extract (Peak purity index: 1.00).

Robustness

The robustness of sample was evaluated during the analysis of HPLC method by changing the flow rate was varied from 0.495, 0.500 and 0.505 mL/min. The results are shown in Table 14.

Table 14. Robustness of HPLC for chlorogenic acid of *M. alba* leaf.

Flow rate (mL/min)	Retention time (min)	Peak area	Concentration of chlorogenic acid (μg/mL)
0.495	9.107	944,631.67	19.6063
0.500	8.980	988,919.67	20.4105
0.505	8.852	930,177.00	19.3438
Average	8.979	954,576.11	19.7869
SD	0.111	30,607.91	0.0332
%RSD	1.23	0.19	0.17

CHAPTER V
DISCUSSION AND CONCLUSION

The standardization and quality control of herbal material are important for quality of herbal medicine. The pharmacognostic specification was set by macroscopic and microscopic evaluations, physicochemical determination, and fingerprint profile (World Health Organization, 2011).

M. alba Linn. is one of medicinal plants used in many countries. In Thailand, *M. alba* leaves known as “Mon”, The leaves are bitter, sweet and cool active on the lungs and liver, and remove the symptoms of heat with origin of lungs and management of liver or kidney deficiency, auditory sharpness and eyes brightness. It is used to treat cough, sore throat, fever, and bronchitis (Bagachi et al., 2013).

Morphological characteristics is the first step that can help to identify and authenticate plant material (Evans, 2009). The genus *Morus* belonging to the family moraceae is comprised of about 37 genera and over 1100 species (Clement & Weiblen, 2009). *M. alba* is mainly found in temperate to subtropical regions of the Northern hemisphere to the tropics of the Southern hemisphere, it can grow in a wide range of climatic and topographic variables. The transverse midrib cross-section of *M. alba* leaves presented the epidermis layer which was the outline, and it slightly channeled on a rounded surface. Both chlorenchyma and parenchyma cells were found in the layer. The middle part had a vascular bundle including xylem and phloem. Non-glandular trichome and unicellular trichome formed on surface. The stoma of *M. alba* leaves was found to be an anomocytic type which had a same size and shape guard cell arrangement as the rest of the epidermis cells, and the non-glandular trichome was round, oval and short tip. The results were correlated with the previous study (KliMKo, 2016). The stomata are one of histological characteristic evaluation for identification of plant species. The epidermal cell has irregularly polygonal cell wall (KliMKo, 2016). Quantitative analysis of microscopic leaf constant number was used to identify between some closely species that cannot be differentiated by general microscopic (Evans, 2009). The stomatal cells were found on the lower leaf surface and the trichome were found on the upper leaf surface,

epidermal cells were found on both the upper and lower leaf surfaces. The palisade cell was found in the mesophyll under the epidermal cell on upper side of leaf.

The physicochemical parameters were evaluated. The quality parameters of *M. alba* leaves consisted of water content, loss on drying, total ash, acid insoluble ash, water extractive value and ethanol extractive value. Loss on drying and water content of leaf should not be more than 4.55 ± 0.21% and 7.97 ± 0.35% of dry weight, respectively. Loss on drying used to determine and control the moisture content of crude drugs by heating the sample, it not only removes water but also other volatile oil from crude drugs. Water content used to determine excessive amounts of water in crude drugs, whose water in crude drugs may cause microbial, bacteria, fungi or insect growth. The water extractive value and ethanol extractive value used to refer to the amount of an active compound in crude drugs when extracting with water and ethanol. This study showed that the water-extractive value was higher than the ethanol-extractive value which indicated that an enormous amount of highly water-soluble polar compounds in *M. alba* leaves. Both water extractive value and ethanol extractive value should not be less than 16.14 ± 0.50% and 8.61 ± 0.39% of dry weight, respectively. Total ash and acid insoluble ash should not be more than 14.38 ± 0.25% and 6.21 ± 0.37% of dry weight, respectively. Total ash was evaluated for inorganic substances in crude drugs after incineration with high temperatures such as Na, Ca, K, etc. Acid-insoluble ash was a measurement of amounts of inorganic matters which were not solubilized in a hydrochloric acid solution.

Thin layer chromatography is a technique used for separating the components or solutes of a mixture between two phases. The mobile phase consisted of ethyl acetate: methanol: water: formic acid (16.8:1.2:1.2:0.4, v/v/v/v) and silica gel 60 GF254 TLC plate as a stationary phase. The TLC fingerprint of ethanolic extract of *M. alba* leaves showed separation of compounds that spots were detected under UV at 254 and 366 nm and staining with sulfuric acid to visualize bands of their oxidized forms.

The phytochemical analysis of an ethanolic extract of *M. alba* leaves showed phenolics and flavonoids which were major bioactive compounds found in *M. alba* leaves. In addition, it showed the detection of steroids and triterpenes, but alkaloids

and saponins in the sample were not detected. A study being conducted in India reported that the ethanolic extract of *M. alba* leaves showed detection of flavonoids, steroids, triterpenes and alkaloids, but saponins was not detected (Ali, Rathaur, & Nishad, 2020).

The quantitative analysis of chlorogenic content in *M. alba* leaves was analyzed by RP-HPLC. The HPLC is a technique that is widely used for separating a mixture of natural compounds. RP-HPLC is commonly used in separation techniques in HPLC because of its wide usage range; more than 65% of all HPLC separations are performed in the reversed-phase mode (Boligon & Athayde, 2014). This study was performed with a reverse phase (C_{18}) column and detected by the photo diode array (PDA). The PDA establishes a large amount of spectral information with optimal sensitivity and wavelength resolution. This detector can collect data with various wavelengths between 190–800 nm. Chlorogenic acid is a phenolic compound containing conjugated double bonds which have strong UV absorption. So, the PDA is a suitable detector for analysis of chlorogenic acid. The reverse phase HPLC column is widely used to separate phenolic compounds in plant extracts. C_{18} column is preferred for polar compound analysis. The highly polar substances may be irreversibly retaining in the column and gradually changing the separation characteristics of the column (Dos Santos et al., 2017). The mobile phase of RP-HPLC is a more polar aqueous solution which consists of a mixture of water and one or more organic solvents that are miscible with water (Hansen et al., 2011). In the previous study, the condition of chromatography was using 0.2% phosphoric acid in water (solvent A), and methanol (solvent B) as a mobile phase. The isocratic program was set at 45% B for 20 minutes at a flow rate of 1.2 mL/min (Chaowuttikul, Palanuvej, & Ruangrungsi, 2020). In this study, the optimized condition was using 0.2% phosphoric acid in water (solvent A) and methanol (solvent B) as a mobile phase. The program was set in an isocratic mode at 45% methanol for 50 minutes at flow rate 0.5 mL/min. The temperature was maintained at 30º C. The main compound of *M. alba* leaves is chlorogenic acid, the highest content of chlorogenic acid was found in the ethanolic extract of *M. alba* leaves from Ratchaburi (0.8756 g/100 g dry weight). In addition, the lowest chlorogenic acid content of *M. alba* leaves was from Nakhon

Phanom (0.0703 g/100 g dry weight). The average of chlorogenic acid contents from 15 sources was 0.4159 g/100 g dry weight. For a previous study done in Japan, it showed that a content of chlorogenic acid in *M. alba* leaves was found to be 616–1014mg/100g by using the UPLC analysis (Sugiyama, Katsube, Koyama, & Itamura, 2017).

In conclusion, the pharmocognostic specification of *M. alba* leaves in Thailand is established. This study can further be used for quality control of this plants. For the quantitative analysis, RP-HPLC used for analyzing of chlorogenic acid of *M. alba* leaves is also developed.

REFERENCES

Ali, M., Rathaur, B., & Nishad, U. (2020). Phytochemical Screening, Physicochemical and Antioxidant Activity, TLC & Finger Print of HPTLC, Morus Alba Ethanol Extraction. *Int. J. Innov. Res. Sci. Eng. Technol., 5*(3).

Almeida, I., Cavalcante, F., & Vicentini, V. (2016). Different responses of vanillic acid, a phenolic compound, in htc cells: Cytotoxicity, antiproliferative activity, and protection from DNA-induced damage. *Genet. Mol. Res, 15*.

Ann, J.-Y., Eo, H., & Lim, Y. (2015). Mulberry leaves (Morus alba L.) ameliorate obesity-induced hepatic lipogenesis, fibrosis, and oxidative stress in high-fat diet-fed mice. *Genes & nutrition, 10*(6), 1-13.

Bagachi, A., Semwal, A., & Bharadwaj, A. (2013). Traditional uses, phytochemistry and pharmacology of Morus alba Linn.: a review. *Journal of Medicinal Plants Research, 7*(9), 461-469.

Bele, A. A., & Khale, A. (2011). An overview on thin layer chromatography. *International Journal of Pharmaceutical Sciences and Research, 2*(2), 256.

Boligon, A., & Athayde, M. (2014). Importance of HPLC in analysis of plants extracts. *Austin Chromatogr, 1*(3), 2.

Boonworapat, W. (2011). *Thai Chinese Herbal Encyclopedia Book*.

Butt, M. S., Nazir, A., Sultan, M. T., & Schroën, K. (2008). Morus alba L. nature's functional tonic. *Trends in food science & technology, 19*(10), 505-512.

Chan, E. W.-C., Phui-Yan, L., & Siu-Kuin, W. (2016). Phytochemistry, pharmacology, and clinical trials of Morus alba. *Chinese journal of natural medicines, 14*(1), 17-30.

Chaowuttikul, C., Palanuvej, C., & Ruangrungsi, N. (2020). Quantification of chlorogenic acid, rosmarinic acid, and caffeic acid contents in selected Thai medicinal plants using RP-HPLC-DAD. *Brazilian Journal of Pharmaceutical Sciences, 56*.

Cheynier, V. (2012). Phenolic compounds: from plants to foods. *Phytochemistry reviews, 11*(2), 153-177.

Cho, A.-S., Jeon, S.-M., Kim, M.-J., Yeo, J., Seo, K.-I., Choi, M.-S., & Lee, M.-K. (2010). Chlorogenic acid exhibits anti-obesity property and improves lipid

metabolism in high-fat diet-induced-obese mice. *Food and chemical toxicology, 48*(3), 937-943.

Clement, W. L., & Weiblen, G. D. (2009). Morphological evolution in the mulberry family (Moraceae). *Systematic Botany, 34*(3), 530-552.

Coskun, O. (2016). Separation techniques: chromatography. *Northern clinics of Istanbul, 3*(2), 156.

Dat, N. T., Binh, P. T. X., Van Minh, C., Huong, H. T., & Lee, J. J. (2010). Cytotoxic prenylated flavonoids from Morus alba. *Fitoterapia, 81*(8), 1224-1227.

De Oliveira, A. M., Mesquita, M. d. S., da Silva, G. C., de Oliveira Lima, E., de Medeiros, P. L., Paiva, P. M. G., . . . Napoleão, T. H. (2015). Evaluation of toxicity and antimicrobial activity of an ethanolic extract from leaves of Morus alba L.(Moraceae). *Evidence-Based Complementary and Alternative Medicine, 2015*.

Dos Santos, W. N. L., da Silva Sauthier, M. C., dos Santos, A. M. P., de Andrade Santana, D., Azevedo, R. S. A., & da Cruz Caldas, J. (2017). Simultaneous determination of 13 phenolic bioactive compounds in guava (Psidium guajava L.) by HPLC-PAD with evaluation using PCA and Neural Network Analysis (NNA). *Microchemical Journal, 133*, 583-592.

Evans, W. C. (2009). *Trease and evans' pharmacognosy E-book*: Elsevier Health Sciences.

Gonçalves, B., Moeenfard, M., Rocha, F., Alves, A., Estevinho, B. N., & Santos, L. (2017). Microencapsulation of a natural antioxidant from coffee—chlorogenic acid (3-caffeoylquinic acid). *Food and Bioprocess Technology, 10*(8), 1521-1530.

Govea-Salas, M., Rivas-Estilla, A. M., Ascacio-Valdés, J., Zugasti-Cruz, A., Rodríguez-Herrera, R., Belmares-Cerda, R., & Morlett-Chávez, J. (2018). Gallic Acid as a Putative Antioxidant in Usage Against Liver Disease. In *The Liver* (pp. 317-322): Elsevier.

Guideline, I. H. T. (2005). Validation of analytical procedures: text and methodology. *Q2 (R1), 1*(20), 05.

Gul, R., Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N. (2017). Preliminary

phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extracts from Ephedra intermedia indigenous to Balochistan. *The Scientific World Journal, 2017*.

Gul, R., Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N. (2017). Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extracts from Ephedra intermedia indigenous to Balochistan. *The Scientific World Journal*.

Hansen, S. H., Pedersen-Bjergaard, S., & Rasmussen, K. (2011). *Introduction to pharmaceutical chemical analysis*: John Wiley & Sons.

Hogade, M., Patil, K., Wadkar, G., Mathapati, S., & Dhumal, P. (2010). Hepatoprotective activity of Morus alba (Linn.) leaves extract against carbon tetrachloride induced hepatotoxicity in rats. *African journal of pharmacy and pharmacology, 4*(10), 731-734.

Hsu, L.-S., Ho, H.-H., Lin, M.-C., Chyau, C.-C., Peng, J.-S., & Wang, C.-J. (2012). Mulberry water extracts (MWEs) ameliorated carbon tetrachloride-induced liver damages in rat. *Food and chemical toxicology, 50*(9), 3086-3093.

Hunyadi, A., Martins, A., Hsieh, T.-J., Seres, A., & Zupkó, I. (2012). Chlorogenic acid and rutin play a major role in the in vivo anti-diabetic activity of Morus alba leaf extract on type II diabetic rats. *PloS one, 7*(11), e50619.

Information, N. C. f. B. PubChem Compound Summary for CID 1794427, Chlorogenic acid. Retrieved from https://pubchem.ncbi.nlm.nih.gov/compound/Chlorogenic-acid.

Izawa, K., Amino, Y., Kohmura, M., Ueda, Y., & Kuroda, M. (2010). 4.16-human–environment interactions—Taste. *Comprehensive Natural Products II*, 631-671.

Jin, S., Chang, C., Zhang, L., Liu, Y., Huang, X., & Chen, Z. (2015). Chlorogenic acid improves late diabetes through adiponectin receptor signaling pathways in db/db mice. *PloS one, 10(4)*.

Jin, W.-Y., Na, M.-K., An, R.-B., Lee, H.-Y., Bae, K.-H., & Kang, S.-S. (2002). Antioxidant compounds from twig of Morus alba. *Natural Product Sciences, 8*(4), 129-132.

Kabir, F., Katayama, S., Tanji, N., & Nakamura, S. (2014). Antimicrobial effects of

chlorogenic acid and related compounds. *Journal of the Korean Society for Applied Biological Chemistry, 57*(3), 359-365.

Kim, S. B., Chang, B. Y., Hwang, B. Y., Kim, S. Y., & Lee, M. K. (2014). Pyrrole alkaloids from the fruits of Morus alba. *Bioorganic & medicinal chemistry letters, 24*(24), 5656-5659.

KliMKo, M. (2016). Micromorphology and anatomy of leaves of Morus alba L.(Moraceae) cultivars from Poland. *Steciana, 20*(2).

Komaikul, J., Kitisripanya, T., Tanaka, H., Sritularak, B., & Putalun, W. (2014). Development of an enzyme-linked immunosorbent assay for specific detection of mulberroside A in mulberry (Morus alba L.) using anti-mulberroside A polyclonal antibody. *Food Analytical Methods, 7*(1), 58-63.

Kusano, G., Orihara, S., Tsukamoto, D., Shibano, M., Coskun, M., Guvenc, A., & Erdurak, C. S. (2002). Five new nortropane alkaloids and six new amino acids from the fruit of Morus alba L INNÉ growing in Turkey. *Chemical and pharmaceutical bulletin, 50*(2), 185-192.

Kwon, S.-H., Lee, H.-K., Kim, J.-A., Hong, S.-I., Kim, H.-C., Jo, T.-H., . . . Lee, S.-Y. (2010). Neuroprotective effects of chlorogenic acid on scopolamine-induced amnesia via anti-acetylcholinesterase and anti-oxidative activities in mice. *European journal of pharmacology, 649*(1-3), 210-217.

Lee, S. H., Choi, S. Y., Kim, H., Hwang, J. S., Lee, B. G., Gao, J. J., & Kim, S. Y. (2002). Mulberroside F isolated from the leaves of Morus alba inhibits melanin biosynthesis. *Biological and Pharmaceutical Bulletin, 25*(8), 1045-1048.

Mahboubi, M. (2019). Morus alba (mulberry), a natural potent compound in management of obesity. *Pharmacological research, 146*, 104341.

Memon, A. A., Memon, N., Luthria, D. L., Bhanger, M. I., & Pitafi, A. A. (2010). Phenolic acids profiling and antioxidant potential of mulberry (Morus laevigata W., Morus nigra L., Morus alba L.) leaves and fruits grown in Pakistan. *Polish Journal of Food and Nutrition Sciences, 60*(1).

Mohammadi, J., & Naik, P. R. (2008). Evaluation of hypoglycemic effect of Morus alba in an animal model. *Indian journal of pharmacology, 40*(1), 15.

Natić, M. M., Dabić, D. Č., Papetti, A., Akšić, M. M. F., Ognjanov, V., Ljubojević, M.,

& Tešić, Ž. L. (2015). Analysis and characterisation of phytochemicals in mulberry (Morus alba L.) fruits grown in Vojvodina, North Serbia. *Food chemistry, 171*, 128-136.

Niidome, T., Takahashi, K., Goto, Y., Goh, S., Tanaka, N., Kamei, K., . . . Kihara, T. (2007). Mulberry leaf extract prevents amyloid beta-peptide fibril formation and neurotoxicity. *Neuroreport, 18*(8), 813-816.

Nijsiri Ruangrungsi, T. M. (2004). *Thai herbs 1*

NIKAVAR, B., & MOUSAZADEH, G. (2009). Influence of three Morus species extracts on α-amylase activity.

Nomura, T., Hano, Y., & Fukai, T. (2009). Chemistry and biosynthesis of isoprenylated flavonoids from Japanese mulberry tree. *Proceedings of the Japan Academy, Series B, 85*(9), 391-408.

Oh, K.-S., Ryu, S. Y., Lee, S., Seo, H. W., Oh, B. K., Kim, Y. S., & Lee, B. H. (2009). Melanin-concentrating hormone-1 receptor antagonism and anti-obesity effects of ethanolic extract from Morus alba leaves in diet-induced obese mice. *Journal of ethnopharmacology, 122*(2), 216-220.

Ohkawara, T., Takeda, H., & Nishihira, J. (2017). Protective effect of chlorogenic acid on the inflammatory damage of pancreas and lung in mice with l-arginine-induced pancreatitis. *Life sciences, 190*, 91-96.

Ong, K. W., Hsu, A., & Tan, B. K. H. (2013). Anti-diabetic and anti-lipidemic effects of chlorogenic acid are mediated by ampk activation. *Biochemical pharmacology, 85*(9), 1341-1351.

Parbuntari, H., Prestica, Y., Gunawan, R., Nurman, M. N., & Adella, F. (2018). Preliminary phytochemical screening (qualitative analysis) of cacao leaves (Theobroma cacao L.). *Eksakta: Berkala Ilmiah Bidang MIPA, 19(2)*, 40-45.

Park, J. M., Bong, H. Y., Jeong, H. I., Kim, Y. K., Kim, J. Y., & Kwon, O. (2009). Postprandial hypoglycemic effect of mulberry leaf in Goto-Kakizaki rats and counterpart control Wistar rats. *Nutrition Research and Practice, 3*(4), 272.

Park, K., You, J., Lee, H., Baek, N., & Hwang, J. (2003). Kuwanon G: an antibacterial agent from the root bark of Morus alba against oral pathogens. *Journal of ethnopharmacology, 84*(2-3), 181-185.

Pasto, D. J., & Johnson, C. R. (1979). *Laboratory text for organic chemistry*.

Plazas, M., Andujar, I., Vilanova, S., Hurtado, M., Gramazio, P., Herraiz, F. J., & Prohens, J. (2013). Breeding for chlorogenic acid content in eggplant: interest and prospects. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 41*(1), 26-35.

Rivière, C., Krisa, S., Péchamat, L., Nassra, M., Delaunay, J.-C., Marchal, A., . . . Mérillon, J.-M. (2014). Polyphenols from the stems of Morus alba and their inhibitory activity against nitric oxide production by lipopolysaccharide-activated microglia. *Fitoterapia, 97*, 253-260.

Scholz, B., & Liebezeit, G. (2006). Chemical screening for bioactive substances in culture media of microalgae and cyanobacteria from marine and brackish water habitats: first results. *Pharmaceutical biology, 44*(7), 544-549.

Seo, D. Y., Lee, S. R., Heo, J.-W., No, M.-H., Rhee, B. D., Ko, K. S., . . . Han, J. (2018). Ursolic acid in health and disease. *The Korean journal of physiology & pharmacology: official journal of the Korean Physiological Society and the Korean Society of Pharmacology, 22*(3), 235.

Singab, A. N. B., El-Beshbishy, H. A., Yonekawa, M., Nomura, T., & Fukai, T. (2005). Hypoglycemic effect of Egyptian Morus alba root bark extract: effect on diabetes and lipid peroxidation of streptozotocin-induced diabetic rats. *Journal of ethnopharmacology, 100*(3), 333-338.

Słomińska, M., Zygler, A., & Namieśnik, J. . (2012). Soxhlet Extraction and New Developments Such as Soxtec in: Comprehansive Sampling and Sample Preparation.

Sohn, H.-Y., Son, K., Kwon, C.-S., Kwon, G.-S., & Kang, S. (2004). Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: Morus alba L., Morus mongolica Schneider, Broussnetia papyrifera (L.) Vent, Sophora flavescens Ait and Echinosophora koreensis Nakai. *Phytomedicine, 11*(7-8), 666-672.

Srivastava, P., Singh, M., Devi, G., & Chaturvedi, R. (2014). Herbal medicine and biotechnology for the benefit of human health. In *Animal Biotechnology* (pp. 563-575): Elsevier.

Su, M., Liu, F., Luo, Z., Wu, H., Zhang, X., Wang, D., . . . Miao, Y. (2019). The antibacterial activity and mechanism of chlorogenic acid against foodborne pathogen pseudomonas aeruginosa. *Foodborne pathogens and disease, 16*(12), 823-830.

Sugiyama, M., Katsube, T., Koyama, A., & Itamura, H. (2017). Seasonal changes in functional component contents in mulberry (Morus alba L.) leaves. *The Horticulture Journal*, OKD-053.

Tistaert, C., Dejaegher, B., & Vander Heyden, Y. (2011). Chromatographic separation techniques and data handling methods for herbal fingerprints: a review. *Analytica chimica acta, 690*(2), 148-161.

Wang, G.-F., Shi, L.-P., Ren, Y.-D., Liu, Q.-F., Liu, H.-F., Zhang, R.-J., . . . Tang, W. (2009). Anti-hepatitis B virus activity of chlorogenic acid, quinic acid and caffeic acid in vivo and in vitro. *Antiviral research, 83*(2), 186-190.

Wang, Y., Xiang, L., Wang, C., Tang, C., & He, X. (2013). Antidiabetic and antioxidant effects and phytochemicals of mulberry fruit (Morus alba L.) polyphenol enhanced extract. *PloS one, 8*(7), e71144.

Wattanapitayakul, S. K., Chularojmontri, L., Herunsalee, A., Charuchongkolwongse, S., Niumsakul, S., & Bauer, J. A. (2005). Screening of antioxidants from medicinal plants for cardioprotective effect against doxorubicin toxicity. *Basic & clinical pharmacology & toxicology, 96*(1), 80-87.

Werker, E. (2000). *Trichome diversity and development.*

World Health Organization. (2005). *National policy on traditional medicine and regulation of herbal medicines: Report of a WHO global survey*: World Health Organization.

World Health Organization. (2011). *Quality control methods for herbal materials*: World Health Organization.

Xiang, Z., & Ning, Z. (2008). Scavenging and antioxidant properties of compound derived from chlorogenic acid in South-China honeysuckle. *LWT-Food Science and Technology, 41*(7), 1189-1203.

Yang, Y., Gong, T., Liu, C., & Chen, R.-Y. (2010). Four new 2-arylbenzofuran derivatives from leaves of Morus alba L. *Chemical and pharmaceutical bulletin,*

58(2), 257-260.

Yang, Z.-G., Matsuzaki, K., Takamatsu, S., & Kitanaka, S. (2011). Inhibitory effects of constituents from Morus alba var. multicaulis on differentiation of 3T3-L1 cells and nitric oxide production in RAW264. 7 cells. *Molecules, 16*(7), 6010-6022.

Yang, Z., Wang, Y., Wang, Y., & Zhang, Y. (2012). Bioassay-guided screening and isolation of α-glucosidase and tyrosinase inhibitors from leaves of Morus alba. *Food chemistry, 131*(2), 617-625.

Zafar, M. S., Muhammad, F., Javed, I., Akhtar, M., Khaliq, T., Aslam, B., . . . Zafar, H. (2013). White mulberry (Morus alba): A brief phytochemical and pharmacological evaluations account. *International journal of agriculture and biology, 15*(3).

Zhang, Y.-M., Zhang, Z.-Y., & Wang, R.-X. (2020). Protective mechanisms of quercetin against myocardial ischemia reperfusion injury. *Frontiers in Physiology, 11*, 956.

Zhang, Z., & Pan, T. (2017). HPLC determination of chlorogenic acid in Verbena officinalis L. extract and its in-vitro antibacterial activity. *Biomedical Research, 28*(9).

Zuo, J., Tang, W., & Xu, Y. (2015). Anti-hepatitis B virus activity of chlorogenic acid and its related compounds. In *Coffee in health and disease prevention* (pp. 607-613). Academic Press.

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