EVALUATION OF ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY AND QUALITY DETERMINATION OF MADHUCA LONGIFOLIA (J. KOENIG EX L.) MACBR. AND MORUS ALBA L. PLANT MATERIALS

By
Mrs. Aye THIDA

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL SCIENCES
Graduate School, Silpakorn University
Academic Year 2022
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การประเมินฤทธิ์การยับยั้งอัลฟ่ากลูโคซิเดสและการควบคุมคุณภาพวัตถุดิบ *Madhuca longifolia* (J. Koenig ex L.) Macbr และ *Morus alba* L.

โดย
Mrs.Aye Thida

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปรัชญาดุษฎีบัณฑิต
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MRS. AYE THIDA : EVALUATION OF ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY AND QUALITY DETERMINATION OF MADHUCA LONGIFOLIA (J. KOENIG EX L.) MACBR. AND MORUS ALBA L. PLANT MATERIALS THESIS ADVISOR : MALAI SATIRAPHAN

The alpha-glucosidase inhibitory activity and the quality determination of *Madhuca longifolia* (J.Koenig ex L.) Macbr. and *Morus alba* L. plant materials were evaluated. Boiling water extract of leaves from both plants exhibited potent inhibitory effect on alpha-glucosidase with the IC_{50} values of 15.23±1.32 and 195.73±34.84 µg/mL for *M. longifolia* (L1) and *M. alba* cultivar Buriram 60 from Nakorn Ratchasima, respectively, compared with acarbose at 763.92±22.27 µg/mL. All samples were determined for loss on drying, heavy metal contamination, total phenolic content, microbial contamination, and the fingerprints of Fourier Transform Infrared (FT-IR) spectra, Gas Chromatography-Mass Spectroscopy (GC-MS) chromatograms and Liquid Chromatography-Diode Array Detector (LC-DAD) chromatograms. All tested samples complied with Thai Herbal Pharmacopoeia (THP) 2019, The Association of Southeast Asian Nations (ASEAN), The World Health Organization (WHO), The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), The United States Pharmacopeia (USP) 40 and Thai Pharmacopoeia (TP) 2005 guidelines, in loss on drying, heavy metal and microbial contamination. However, leaf samples of *M. longifolia* and *M. alba* cultivar Buriram 60 from Buriram province were contaminated with non-pathogenic *Clostridium* species. GC-MS chromatograms of *M. longifolia* leaf extract showed high amount of phytol, linolenyl alcohol, neophytadiene and palmitic acid, while that of *M. alba* showed high amount of phytol and fatty acids such as palmitic acid, oleic acid and stearic acid. The High-Performance Liquid Chromatographic (HPLC) method was developed to quantify gallic acid and quercetin in *M. longifolia*, and chlorogenic acid, caffeic acid, rutin and quercetin in *M. alba* leaf extracts. The FT-IR and chromatographic fingerprints could be used for the identification and authentication of *M. longifolia* leaf and flower, and *M alba* leaf. The developed HPLC method will be useful for the quantification of active constituents and quality assessment of *M. longifolia* and *M. alba* leaf in the future. Moreover, leaf of *M. longifolia* and *M. alba* will be potential sources for hypoglycemic compounds.

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LIST OF ABBREVIATIONS

Amu	Atomic mass unit
ATR FT-IR	Fourier transform Infra-red spectroscopy
BR-BR	Cultivar Buriram 60 from Buriram Province
BR-KB	Cultivar Buriram 60 from Kanjanaburi Province
BR-NR	Cultivar Buriram 60 from Nakhon Rajasima Province
˚C	Degree centigrate
cps	Count per second
EtOH	Ethanol
Fig	Figure
g	Gram (s)
GC MS	Gas Chromatography Mass Spectrophotometry
h	Hours
H_2O	water
HPLC	High Performance Liquid Chromatography
IR	Infrared
ICP-MS	Inductively Coupled Plasma Mass spectrophotometry
kg	Kilogram (s)
KP-BR	Cultivar Khun Pai from Buriram Province
KP-KB	Cultivar Khun Pai from Kanjanaburi Province
KP-NR	Cultivar Khun Pai from Nakhon Rajasima Province
mg	Milligram (s)
m/z	Mass to charge ratio
min	Minute (s)
mL	Milliliter
ng	Nanogram (s)
nm	Nanometer

LIST OF ABBREVIATIONS (Conti.)

PNPG	p-nitro phenyl-α-D-glucopyranoside
PCA	Principal Component Analysis
ppm	Part per million
R_t	Retention time
SD	Standard deviation
THP	Thai Herbal Pharmacopoeia
TP	Thai Pharmacopoeia
TPC	Total Phenolic Content
µg	Microgram (s)
µL	Microliter
UV-VIS	Ultraviolent Visible Spectroscopy
$\tilde{v}$, cm^{-1}	Wavenumber per centimeter
Vmax	Wavenumber at maximum absorption
λmax	Wavelength at maximum absorptivity
w/w	Weight by weight
%	Percentage

มหาวิทยาลัยศิลปากร

CHAPTER 1

INTRODUCTION

Among several diseases, diabetes mellitus is a life-threatening disease. In 2014, World Health Organization (WHO) announced that more than 422 million people worldwide lived with diabetes, compared to 108 million in 1980 and in 2016 diabetes was the 7th leading cause of death. The global prevalence of diabetes has nearly doubled since 1980, rising from 4.7% to 8.5% in adult population and risen faster in low- and middle-income countries. Diabetes caused 1.5 million deaths in 2012, and higher-than-optimal blood glucose caused an additional 2.2 million deaths, by increasing the risks of cardiovascular and other diseases. Forty-three percent of these 3.7 million deaths occurred before the age of 70 years (1, 2).

Natural products from natural resources have been used for the treatment or cure of ailments since long time ago. Medicinal plants are main sources of biologically active compounds (3). For the treatment of diabetes, several plants have been used based on their traditional knowledge and scientific reports, and WHO also legitimated the use of medicinal plants (4). Polyphenols, flavonoids, tannins, chalcones, and carotenoids are mostly together responsible for biological activity of natural products (5-7). Plants with phenolic phytochemicals have high antioxidant activity and are helpful for inhibiting and fighting human chronic diseases linked to oxidative stress (8, 9). Some plants comprised high amounts of α-amylase and α-glucosidase inhibitors which exhibited activities against hyperglycemia and related complications of hypertension (10-12).

Nowadays agents possessing α-glucosidase inhibitory activity have been claimed for the control of hyperglycemia (13). Among several plants, *Madhuca longifolia* (J.Koenig ex L.) Macbr.(14-16) as well as *Morus alba* L. were potential sources of compounds for blood sugar lowering activity (17-21).

Madhuca longifolia (J.Koenig ex L.) Macbr. or meze (a common name in Myanmar) belongs to the Sapotaceae family. In Myanmar, it is common to soak dried flower in boiling water for drinking as tea. Although, some parts of this plant elsewhere such as bark and seed have been reported for antidiabetic activity, there

were no study on the biological activity, especially α-glucosidase inhibitory activity of leaf and flower of Myanmar meze (22-24).

Morus alba L. or mulberry is included in the Moraceae Family. Several parts of mulberry tree possess various biological activities such as root, bark and leaf possess anti-hyperglycemic activity (25-27). However, *M. alba* leaf from different sources may show diverse anti-hyperglycemic activity, resulting from different amounts of active chemical constituents. The study of α-glucosidase inhibitory activity of *M. alba* leaf will benefit its consumption as herbal tea in Thailand.

Currently, herbal medicines are extensively used to treat and prevent many diseases. Consequently, standardization and quality assessment of herbal medicines are very important. Standardization requires the evaluation of both quality and purity of crude drug by means of physical, chemical, biological, morphological, macroscopical and microscopical observation. Physical parameters include moisture content, ash value, extractive value, viscosity, density, bitterness value, solubility, swelling index, foaming index, specific gravity; macroscopic parameters involve color, odor, taste, texture and fracture; microscopic parameters include scanning electron microscope (SEM) studies, powder studies; biological parameters include microbial contamination, pharmacological evaluation, toxicological studies; and chemical parameters include chromatographic techniques, heavy metal content, pesticide residue and mycotoxins (28). In Thai Herbal Pharmacopoeia, determination of moisture content (as loss on drying) is one of the criteria for the quality evaluation of mulberry leaf (29).

Toxic heavy metals in herbal materials are one of potentially hazardous contaminants and their amounts depend on sources and cultivar (30, 31). Therefore, WHO prescribed limits for toxic metals in herbal medicines and products (32).

Chemical fingerprinting is an important tool in the quality control of herbal medicines. Chromatographic fingerprint of an herbal medicine consists of pharmacologically active or chemically characteristic components. Fingerprint pattern is very useful for the identification and the authentication of herbal medicines (33). Although there were some reports on the chemical fingerprints of *M. longifolia* and *M. alba* leaves (34-40), the fingerprints from ATR-FT-IR and GC-MS analyses of leaves and flowers of Myanmar meze as well as leaves of Thai mulberry will be

performed. These fingerprints may be useful as references for the commercial products.

This study focuses on the quality determination of the extracts of *M. longifolia* leaf and flower from Myanmar, and *M. alba* leaf from both Thailand and Myanmar. The evaluation of α-glucosidase inhibitory activity will be determined as biological parameter, the moisture content as physical parameter, the preliminary phytochemical screening, the amount of heavy metals, total phenolic content and active constituents, and the chemical fingerprints from FT-IR, GC-MS and LC-MS as chemical parameters. The alpha-glucosidase inhibitory activity and the quality determination of *M. alba* tea products marketed in Thailand will also be evaluated.

OBJECTIVES

1. To evaluate the α-glucosidase inhibitory activity on leaf and flower extracts of *Madhuca longifolia* (J.Koenig ex L.) Macbr. as well as leaf extracts and extract of commercial tea products of *Morus alba* L.

2. To determine the quality of *Madhuca longifolia* (J.Koenig ex L.) Macbr. leaf and flower materials and leaf and commercial tea products of *Morus alba* L. as well as to establish a standardization method for the quality control of *Madhuca longifolia* (J.Koenig ex L.) Macbr. plant materials

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CHAPTER 2
LITERATURE REVIEW

2.1 Diabetes

Diabetes mellitus is a complex, chronic, progressive and life-threatening disease in worldwide population. It was one of the four priority noncommunicable diseases (NCDs) targeted by world leaders. WHO announced that diabetes mellitus may become the 7th biggest killer in the world by 2030. In 1985, 30 million people suffered from diabetes, and in 2010 that increased to 285 million. For WHO South-East Asia Region, the number of adults with diabetes has increased from 17 million in 1980 to 96 million in 2014. Almost 9% of the adult population of the WHO South-East Asia Region had diabetes and was the second highest prevalence after the Eastern Mediterranean Region. In 2012, nearly 1 million adults in the South-East Asia Region died of the consequences of high blood glucose due to diabetes (e.g., diabetic coma), as well as deaths from diabetes-attributable renal failure, tuberculosis and cardiovascular disease. In South-East Asia Region about 1 in 10 premature deaths were from the consequences of high blood glucose, while that number was 1 in 20 for the European Region. The International Diabetes Federation estimated that number of affected patients in 2019 would stand at 463 million, and in 2045, around 700 million people will suffer from diabetes. Self-management education and support for diabetes are important to prevent acute complications and reduce the risk of long-term complications (2, 13, 41-43). In Myanmar, the prevalence of diabetes mellitus is increasing. In Yangon, the capital of Myanmar, overall prevalence of adult-onset diabetes in 2003-2004 was 11.8% (urban prevalence is 13.9% and rural prevalence was 7.3%) (44). The national prevalence of diabetes was 10.5% for the adult aged between 24 and 65 years, and estimated number of people with diabetes was at least 2.5 million in 2013–2014 (45). Major NCD risk factors in Myanmar are smoking, smokeless tobacco use, exposure to second-hand smoke, current alcohol drinking (esp. in younger males), low consumption of fruit and vegetables, overweight and obesity (esp. in the women), hypertension, diabetes and abnormal lipid levels (46).

Type 1 diabetes (insulin-dependent, juvenile or childhood-onset diabetes) is considered by deficient insulin production in the body. People with type 1 diabetes

require daily administration of insulin to regulate the amount of glucose in their blood. If they do not have access to insulin, they cannot survive. The cause of type 1 diabetes is not known and not preventable. Symptoms comprise constant hunger, weight loss, excessive urination and thirst, fatigue and vision changes (2).

Type 2 diabetes (non-insulin-dependent or adult-onset diabetes) is due to the body's ineffective use of insulin. The vast majority of people around the world are reported with type 2 diabetes. Symptoms may be similar to those of type 1 diabetes and the disease may go undiagnosed for several years, until complications have already arisen. Type 2 diabetes occurred only in adults but it has begun to occur in children. Overweight and obesity are the strongest risk factors for type 2 diabetes. Populations in South-East Asia develop diabetes at a lower level of body mass index (BMI) than populations of European origin (2).

Impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG) are intermediate conditions in the transition between normal blood glucose levels and diabetes (especially type 2). People with those are at increased risk of heart attacks and strokes. Gestational diabetes (GDM) is a temporary condition that occurs in pregnancy and carries long-term risk of type 2 diabetes (42). For complication of diabetes, it can damage heart, blood vessels, eyes, kidneys and nerves, conducting to disability and premature death. A significant greater chance of incurring catastrophic medical expenditure was occurred in people with diabetes than individuals without diabetes and more noticeable in lower-income countries (2).

Currently, diabetes is usually controlled using synthetic oral hypoglycemic agents, proper diet and regular physical exercise. Although the oral hypoglycemic agents are excellent in controlling blood sugar level, they cannot take care of all complications associated with diabetes. On the other hand, herbal remedies with multiple phytoconstituents not only control the blood sugar but also ameliorate the diabetic complications. In the treatment of diabetes, WHO also allows the use of medicinal plants and encourages the documentation of ethnomedical data of medicinal plants based on their traditional knowledge (3-6) . Therefore, study of medicinal plants that possess antidiabetic activity will be useful for the control and treatment of diabetes.

In type 2 diabetes mellitus, an effective control of hyperglycemia includes the retarding, regulation and/or inhibition of carbohydrate hydrolyzing enzymes. From which, alpha-glucosidase is located in the brush border of the enterocytes of the jejunum and is the most important enzyme in carbohydrates digestion. It can selectively hydrolyze terminal (1→4)-linked alpha-glucose residues (starch or disaccharides) to release a single alpha-glucose molecule which is absorbed in the upper jejunum, resulting in hyperglycemia. Inhibitors of alpha-glucosidase can retard the decomposition and absorption of dietary carbohydrates by restricting the breakdown of linear or branched oligosaccharide units like a-limit dextrin, maltose and malt triose to produce glucose, thereby preventing glucose absorption into blood stream and suppressing the post-prandial hyperglycemia. Most alpha-glucosidase inhibitors can attach to the carbohydrate binding site of alpha-glucosidase due to their similarity with disaccharides or oligosaccharides in molecular structure and the complexes have a stronger affinity than the carbohydrate–glucosidase complexes. Some flavonoid-based compounds inhibit alpha-glucosidase by non-competitive, uncompetitive or mixed types (47, 48).

Inhibition of enzymes involved in the digestion of carbohydrates can significantly decrease the postprandial increase of glucose level in the blood after a mixed carbohydrate diet, which has been shown to be essential in preventing the progress of impaired glucose tolerance towards type-2 diabetes. Currently commercialized anti-glucosidase drugs are acarbose, miglitol and voglibose. However, these drugs associated with mild, short-lived and dose-dependent gastrointestinal side effects including diarrhea, abdominal pain and flatulence. Therefore, research is continuing to find novel inhibitors with improved efficacy and minimal side effects.

Natural products of plant origin have been a valuable source of therapeutic agents through lesser toxicity and side effects. Compounds from plants as potential candidates for alpha-glucosidase inhibitors are secondary metabolites such as alkaloids, phenolic acids, flavonoids, terpenoids, anthocyanins, and their glycosides (47, 49). Polyphenols, especially flavonoids, phenolic acids and tannins, have important property for inhibiting alpha-glucosidase. A flavonoid with two catechol

groups in A- and B-rings, together with a 3-OH group at C-ring (as quercetin in Table 3), gave the most active alpha-glucosidase inhibition than acarbose (48, 50).

Normally, the potency of alpha-glucosidase inhibitory activity was determined through a colorimetric method. PNPG (4-Nitrophenyl-β-D- glucopyranoside), a chromogenic β-D-glucosidase substrate, produced a yellow solution upon cleavage from the reaction with alpha-glucosidase. The yellow product of *p*-nitrophenol has been detected by visible light at 405 nm. To determine the activity, % inhibition was calculated from the reaction of PNPG, enzyme, and enzyme inhibitor compare to that of PNPG and enzyme. Then, IC_{50} was calculated from the calibration curve of % inhibition VS five concentration of sample. Acarbose was used a positive control of alpha-glucosidase inhibitor (51, 52).

2.2 *Madhuca longifolia*

The Sapotaceae is a family of flowering plants with 60 genera and about 1,343 species. It contains trees or shrubs with distribution worldwide, and the highest species diversity is discovered in the tropical and subtropical regions of Asia and South America. Several species produce edible fruits, with or without economic uses. Species noted for their edible fruits include *Manilkara* (Sapodilla, sapota), *Chrysophyllum cainito*, *Pouteria*, and *Planchonia careya* (53).

Madhuca longifolia (J.Koenig ex L.) Macbr. belongs to the Sapotaceae family. This plant is distributed in northern, central and southern part of India, Nepal, Sri Lanka and Myanmar. Its common names in Myanmar are meze or myintzu-thaka-natpan. In Myanmar it mostly distributes in central region such as Mandalay and Magway division and also distributes in lower region, i.e., Yangon division (22-24).

M. longifolia is a medium to large deciduous tree growing widely under dry tropical and sub-tropical climatic conditions and provides plenteous amount of shade (23). It is a drought-resistant, needs a huge amount of water and cannot grow well under shade. It can grow up in shallow, boulders, clay, and calcareous soil but grows best in sandy soil (54). It is found up to an altitude of 1200 m with rainfall from 550-1500 mm and a mean annual maximum temperature of 28-50°C (55). It can grow up to a height of 17-18m (56). Taxonomy of this plant includes, Family: Sapotaceae; Genus: *Madhuca*; Species: *M. longifolia*; Binomial name: *Madhuca longifolia*

(J.Konig) J.F.Macbr.; Common name: Myintzu-thaka- natpan, meze (Myanmar), honey tree, butter tree (English), mahua (India), miilluppai (SriLanka); Synonym(s): *Madhuca latifolia* Macb., *Bassia latifolia* Roxb., *Madhuca indica* J.F. Gmel. In Sanskrit the word Madhuca means honey. Leaves are leathery and are present in the form of bunches at the endings of branches, shortly acuminate, elliptic, shortly acuminate band base cuneate (56, 57). They are pointed tips and have a thick texture, a hairy surface on the beneath, and strong nerves (55, 58). Flowers are frequent, white in color, fascicled and are present at the branch endings, drooping on pedicels. They have a sweet flavour and are fleshy in texture (55-57). Fruits are green and fleshy which contain 3-4 brown-colored ellipsoidal seeds with shiny coat. The tree possesses oil-bearing seeds and flowers. Flowering of the plant occurs from March to April and collection of seeds can be performed in May, June and July (54) . Flowers contain 50.6% of reducing sugars, 3.43% of cane sugar, 6.37% protein, 8% Calcium, and 2% Phosphorous. Seed oil has Refractive index of about 1.452-1.462, Saponification value of 187-197, Iodine value 55-70, Unsaponifiable matter (%) 1-35, Palmitic Acid C 16:0 (%) 24.56, Stearic Acid C 18:0 (%) 22.77, Oleic Acid C 18:0 (%)37.08, Linolic Acid C 18:2 (%) 14.3. Many therapeutic formulations of *M. longifolia* Linn were suggested by the Ayurvedic Pharmacopoeia of India. Liquid dosage form was used for curing of bleeding, emaciation, tiredness, diseases of skin, constipation, piles, burning sensation, burning micturition, gynecological disorders (heavy and irregular periods metrorrhagia, menorrhagia), amoebiasis, bacterial dysentery, amoebic dysentery, bloody diarrhea. Powder form was used for curing of diabetes, urinary disorders, enhanced milk production and provided strength to lactating mothers (23, 24, 54). *M. longifolia* contains various secondary metabolites and possesses many biological activities. Bioactive compounds, activities and uses of *M. longifolia* were shown in Table 1. Some parts such as bark and seed have been tested for antidiabetic activity, while leaf and flower of Myanmar meze have not been reported (22-24). However, it was common to soak dried flowers for drinking in Myanmar.

Table 1 Bioactive compounds, activities and uses of *M. longifolia*

Part used	Uses	Chemical group	Compounds	Biological activities	Ref:
Leaf	Expectorant, Chronic Bronchitis, Gastropathy, Dermatopathy, Rheumatism, Haemorrhoids	terpenoids	β-carotene, xanthophylls, erthrodiol,		(23)
		long chain fatty acid	palmitic acid		
		polyphenolic flavonoid	myricetin and its 3- O-arabinoside and 3-O-L-rhamnoside, quercetin and its 3-galactoside		
		phytosterol	β-sitosterol and its 3-O-β-D-glucoside, stigmasterol, sitosterol		
			3β-caproxy and 3β-palmitoxy- olean-12-en-28-ol, n-hexacosanol, 3β-caproxyolcan- 12-en-28-ol, n-octacosanol,		

				Antihyperglycemic Activity	(15)
				Wound healing activity	(59)
				Nephro and heptoprotective activity,	(60)
				Antioxidant activity	(60)
				Cytotoxic activity	(61)
				Antimicrobial activity	(62)
Flower	Analgesic, Diuretic, Pharyngitis,	vitamin	vitamins A and C carotene,		(24)

	Bronchitis, Lactation, Acute and chronic tonsillitis, Tonic, Cooling agent, Astringent, Demulcent, for the treatment of helminths	essential amino acid	thiamine, riboflavin, niacin, folic acid, biotin, inositol	Analgesic Hepatoprotective activity Skin diseases Cure cough	(63) (64) (16) (60)
Bark	Rheumatism, Chronic bronchitis, Diabetes mellitus, Snake- bite poisoning, Bleeding and spongy Gums, Fractures,		sesquiterpene alcohol, α-terpeneol, 3β-monocaprylic ester of eythrodiol, 3β-capryloxy oleanolic acid, α- and β- amyrin acetates,		(23)

	Itch, Swelling	terpenoids	ethylcinnamate	Antidiabetic activity Antihyperglycemic and antioxidant Wound healing activity Antibacterial activity Antioxidant activity Rheumatism, bleeding and spongy gums	(65) (16) (59) (16) (66) (67)
Stem bark				Antimicrobial activity	(62)
Seeds	Rheumatism, Headache, Laxative, Piles. Skin disease, Emulcent property	polyphenolic flavonol long chain fatty acid	myricetin, quercetin arachidic acid, linolelic acid, oleic acid, myrisic acid, palmitic acid, stearic acid		(23)

		amino acids saponin	α-alanine, aspartic acid, cystine, glycine, isoleucine and leucine, lysine, methionine, proline, serine, threonine mi-saponin A, B	Anti-inflammatory Alleviate pain	(68) (69)
Seeds cake				Anti-inflammatory, antiulcer, hypoglycaemic activity	(69)
Nut shell		polyphenolic, flavonol phytosterol	*n*-hexacosanol, quercetin and dihyroquercetin, β-sitosterol, and its 3β Dglucoside		(23)

Fruits	Acute and chronic tonsillitis, Pharyngitis, Astringent, As lotion in chronic ulcer	terpenoids flavonoid phytosterol saturated fatty alcohol vitamin	α- and β- amyrin acetates quercetin, dihyro quercetin, β-sitosterol and its 3β-Dglucoside *n*-hexacosanol carotene, ascorbic acid	Asthma and Phithisis	(60)
Aerial parts				Anti- inflammatory, analgesic and antipyretic activity	(70)

2.3 *Morus alba*

The Moraceae is a family of flowering plants with 40 genera and about 1217 species. *Morus alba* L. belongs to the Moraceae family. The tree is distributed worldwide and cultivated in tropical, subtropical and temperate regions of Asia and Africa such as Japan, China, Korea, Thailand, India, Indonesia, Vietnam, Africa and Brazil. It is also distributed in South and North America, Himalayas, Europe and Myanmar (26, 71, 72). *Morus alba* (white mulberry) and *Morus nigra* (blackberry) are the most common species, which *Morus alba* L. (white mulberry) is the dominant species (27, 72).

The tree is a small deciduous tree with a height of about 30 m and a width of about 1.8 m a width (27). Leaves are simple, alternate, variable in size and shape, usually ovate to broadly ovate, 5 to 20 cm long, 2.5 to 14 cm wide, apex acute, acuminate or obtuse, base truncate, round or codate, margin serrate, serrate-crenate or irregularly lobed, basal nerve 3, blade abaxially glabrous or sparingly pubescent along major veins or tufted in axils of principle lateral veins and midrib, adaxially glabrous or sparsely pubescent; petiole up to 5.5 cm long; stipule ovate to lanceolate, 5 to 9 mm, caducous. Inflorescence catkin, axillary, sordid white or greenish white, monoecious or dioecious; male catkin lax, 1 to 4 cm long; female catkin ovoid, 0.5 to 2 cm long; peduncle as long as the catkin. Male flower sessile; sepals 4, imbricate, glabrous, marginally hairy; ovary 1-celled, glabrous, style, 0.5 to 1 mm long, 2-partite, stigma papillose. Fruit multiple, syncarpous, ovoid, ellipsoid or cylindric, 1 to 2.5 cm long, 0.5 to 1 cm wide, fleshy, becoming reddish, purplish, blackish or whitish when mature (29).

Taxonomy of this plant includes, Family: Moraceae; Genus: *Morus L*; Species: *Morus alba* L.; Binomial name: *Morus alba* L.; Common name: Po-Sa (Myanmar) (73), mulberry or mon (Thailand). Synonyms; Moral Blanco Leaf, Russian Mulberry Leaf, Silkworm Mulberry Leaf, White Mulberry Leaf, White-fruited Mulberry Leaf (29). For silkworms and animals, *M. alba* leaf is used as fodder. In Asian countries, especially in Japan, Korea and Thailand mulberry leaf is used as an infusion. In Thailand, mulberry tea is a commercial product available in the market.

Mulberry fruits are used as vegetables in different parts of the world and in European countries, it is grown for fruit production (27). *M. alba* leaf contains a high amount of important minerals such as calcium (Ca), potassium (K), magnesium (Mg), zinc (Zn) and also contains iron (Fe), sodium (Na) and organic acids. *M. alba* fruits contain higher amount of protein than other mulberry species (71).

Different parts of mulberry possess various bioactive compounds and various biological activities including anti-hyperglycemic activity. Root, bark and leaves of mulberry possess anti-hyperglycemic activity (27, 74, 75). Bioactive compounds, activities and uses of *M. alba* were shown in Table 2.

Table 2 Bioactive compounds, activities and uses of *M. alba*

Parts	Uses	Chemical group	Compounds	Biological activities	Ref:
Leaf	Expectorant, Fever, Sore and inflamed eyes, Sore throats, Headaches, Dizziness, Vertigo, Antibacterial, Diaphoretic, Hypoglycaemic	vitamin	ascorbic acid, carotene, folic acid, folinic acid, vitamin B1, D		(26, 74)
		volatile constituents	n-butanol, betagamma-hexenol, methyl-ethyl acetaldehyde, n-butylaldehyde, isobutylaldehyde, valeraldehyde, hexaldehyde, alpha-beta-hexenal, methyl-ethyl ketone, methyl-hexyl ketone, butylamine, acetic acid, propionic acid, isobutyric acid		
		mineral	calcium malate		
		natural acids	succinic acid, tartaric acids		

		chlorophyll	xanthophyll		
		tannin	tannins		
		flavonoid	isoquercitrin (quercetin 3-glucoside)		
			astragalin		(76)
			atalantoflavone, cyclomorusin, cyclomulberrin, 3′, 8-Diprenyl-4′, 5, 7-trihydroxyflavone, 8-geranylapigenin, kaempferol, kuwanons A−C, E, G, H, J, S, T		(77)
			dihydrokaempferol 7-*O*-*β*-D-glucopyranoside, kaempferol 3-*O*-*β*-D-rutinoside, kaempferol 3-*O*-*β*-D-glucopyranoside, quercetin, quercetin 3, 7-di-*O*-*β*-		(78)

			D-glucopyranoside, quercetin 3-*O*-(6″-*O*-acetyl)-*β*-D-, quercetin 7-*O*-*β*-D-glucopyranoside, 5, 7, 3′-Trihydroxy-flavanone-4′-*O*-*β*-D-glucopyranoside, 5, 7, 4′-Trihydroxy-flavanone-3′-*O*-*β*-D-glucopyranoside		
			quercetin-3, 7-di-*O*-*β*-D-glucopyranoside, quercetin 3-(6-malonylglucoside), rutin		(79, 80)
		polyhydroxylated piperidine alkaloid	1-deoxynojirimycin (DNJ)		(26, 71, 81)
		chalcone	morachalcones B, C		(26, 75)
		glucoside	benzyl d-glucopyranoside, roseoside, scopolin, skimmin,		(81)

			7-methoxyl-8-hydroxyethyl-4′-hydroxylflavane-2′-*O*-*β*-D-glucopyranoside		(19)
		cyclic polyol	5-*O*-caffeoylquinic acid		(82, 83)
		Phenolic compounds	*p*-coumaric acid, *m*-coumaric acid		
				Anti-diabetics	(26, 71, 72, 84-92)
				Antioxidant	(26, 71, 72, 85, 91, 93-100)
				Cytotoxic	(22, 26, 71, 72, 101-109)
				Antimicrobial	(26, 71, 72, 110-116)
				Skin toning activity	(26, 117)
				Anti-atherosclerotic effects	(26, 80, 118, 119)
				Neuroprotective	(26, 71, 72, 120-129)
				Hepatoprotective	(26, 72, 97, 130-134)
				Anxiolytic and antidepressant activities	(26, 135)
				Immune enhancing	(72, 136, 137)

				Anti-obesity	(71, 72, 97, 138-140)
Fruit	Cleansing, Tonic, As gargle, Mouthwash, Prevent premature graying of hair, Dizziness, Ringing in the ears, Blurred vision, Insomnia				(26, 74)
		glucoside	4-O-α-D-galactopyranosyl-calystegine B2, 3β,6β dihydroxynortropane		(26, 141)
		Phenolic and flavonoids compounds	cinnamic acid derivatives, flavonols, anthocyanins, benzoic acid derivatives, rutin, quercetin, chlorogenic acid, kaempferol, apigenin,		(71, 78, 142-144)

			caffeic acid, gallic acid flavonols, flavonoids, anthocyanins, hydroxynamic acids, benzoic acids,		
		cyclic polyol	caffeoylquinic acids		(71, 145)
		volatile compounds	aldehydes, esters, ketones, benzene terpenes, oxygenated terpenes		(71, 146)
		major fatty acids	linoleic acid, palmitic acid, oleic acid, stearic acids, myristic acid		(71, 147)
		vitamin	β-carotene, ascorbic acid		(71, 143, 144)
				Anti-diabetics	(71, 72, 78, 148-150)
				Cytotoxic	(71, 72, 151-156)
				Antioxidant	(71, 72, 78, 148, 153, 157-160)
				Antibacterial	(71, 72, 161-165)

				Hepatoprotective activity	(72, 166, 167)
				Neuroprotective	(71, 72, 168-173)
				Anti-obesity	(71, 72, 174-176)
				Immune enhancing	(72, 177-180)
Stem	Antirheumatic, Antispasmodic, Diuretic, Hypotensive				(26, 74)
				Antibacterial	(72, 181)
Bark	Vermifuge, Purgative				(26, 74)
		polyhydroxylated piperidine alkaloid	1-deoxynojirimycin (DNJ)		(26, 84)
				Antidiabetic	(26, 84)
				Antimicrobial	(26, 182)
Root	Anthelmintic, Astringent				(26, 74)
				Anticancer	(26, 72, 183-187)
				Immune enhancing	(72, 188)
				Antidiabetic	(18, 72, 93, 189)
				Antioxidant	(72, 190)

				Hepatoprotection	(72, 191)
				Neuroprotection	(72, 192)
Root bark	Toothache Laxative Antiasthmatic Antitussive Sedative				(26, 74)
		polyhydroxylated alkaloids	(2R,3R,4R)-2-hydroxymethyl-3,4-dihydroxy-pyrrolidine-N-propionamide		(26, 141)
		prenylated flavonoid	moralbanone, kuwanon S, mulberroside C, cyclomorusin, eudraflavone B hydroperoxide, oxydihydromorusin, leachianone G, alpha-acetyl-amyrin		(26, 193)
		glyco-protein	moran 20K		(26, 93)
		benzofurans	mulberrofuran G, albanol B		(26, 194)
				Anti-diabetics	(18, 26)
				Anticancer	(26, 188)

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				Skin toning	(26, 117)
Seed		flavonoids and phenolic compounds	rutin, 4-prenylmoracin, quercitrin, (+)-dihydroquercetin, quercetin, isoquercitrin, chlorogenic acid, moracin, procatechuic acid, dihydrokaempferol, trans-resveratrol, caffeic acid, 3,4-dihydroxybenzoic acid, cyaniding-3-rutinoside		(71, 195)
		Carbohydrate, Fatty acids, Protein			(71, 196-198)
				Antiviral	(71, 195)

2.4 gallic acid, chlorogenic acid, caffeic acid, rutin and quercetin

Gallic acid and quercetin with its derivatives were bioactive compounds discovered in *M. longifolia* plant (34, 199). Gallic acid (3,4,5-trihydroxybenzoic acid) (Table 3) is one of non-flavonoid polyphenolic compounds mostly distributed in herbs, fruits and vegetables. It possesses many biological effects such as antioxidant, anti-inflammatory, antibacterial, antiviral, cardioprotective, anti-diabetic effects and anti-metastasis (3, 200). Gallic acid extracted from plants and its derivative methyl gallate and propyl gallate showed strong alpha-glucosidase inhibitory activity (201).

Chlorogenic acid ((1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl) prop-2-enoyl] oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid) (Table 3) is a polyphenol and ester of caffeic and quinic acid and known as 5-O-caffeoylquinic acid. It was found in coffee, black tea, apples, carrots, grapes, kiwi fruit, pears, plums, potatoes. It possesses antioxidant, anticarcinogenic, anti-inflammatory, neuroprotective, antihyperlipidemic, hypoglycemic effect and benefits on type 2 diabetes, obesity, Alzheimer's disease and stroke. Moreover, a chlorogenic acid-rich fraction of *Smilax aristolochiifolia* root extract inhibited alpha-glucosidase activity in a noncompetitive mode. (202-204).

Caffeic acid ((E)-3-(3,4-dihydroxyphenyl) prop-2-enoic acid) (Table 3) is a polyphenol, found in coffee beans, olives, blueberries, apples, potatoes, carrots, propolis and cider. It acts as a carcinogenic inhibitor and possesses antioxidant and antibacterial activity in vitro, and can prevent atherosclerosis, and other cardiovascular diseases. Caffeic acid can inhibit alpha-glucosidase enzyme and is a safe and potent agent against diabetes in mice (205-207). Caffeic acid reveals a significant potential as an antidiabetic agent in mice by suppressing a progression of type 2 diabetic states suggested by an attenuation of hepatic glucose output and enhancement of adipocyte glucose uptake, insulin secretion, and antioxidant capacity (208).

Rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxymethyl] oxan-2-yl] oxychromen-4-one) (Table 3) is a glycoside of quercetin and it belongs to flavanol. It is found in black tea, mango, onion, broccoli, apples, cherries and berries.

It possesses anti-diabetic potentials. Some studies proposed that rutin exerted its hypoglycemic effect through multiple actions including increasing the proliferation of pancreatic β-cells, enhancing insulin sensitivity and stimulating insulin secretion. It has shown to strongly inhibit α-glucosidase. In previous study gallic acid, quercetin and rutin showed better inhibition in alpha-glucosidase inhibitory activity than standard acarbose (201).

Quercetin (3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one) (Table 3), a key individual from the flavonoid family, is found in tomato, onion, apple, berries, many nuts, seeds, barks, flowers, tea, lettuce, brassica vegetables and leaves. Greater part of quercetin exists as glycosides with sugar moiety (3). Quercetin possesses numerous biological functions on human health such as anti-diabetes, anticancer, anti-inflammatory, cardiovascular protection, cataract prevention, antiulcer, anti-allergic. antiviral, analgesic, anti-obesity, antidepressant as well as preventing asthma and hypertension. Moreover, it is useful for prevention and nutritional controlling of diabetic complication(209, 210). Quercetin was shown to strongly inhibit alpha-glucosidase (201).

Table 3 Structure of gallic acid, chlorogenic acid, caffeic acid, quercetin and rutin

Compound	Structure
Gallic acid	O, OH, HO, OH, OH
Chlorogenic acid	OH, COOH, HO, O, OH, OH, OH
Caffeic acid	O, HO, OH, HO
Rutin	OH, HO, O, OH, O, OH, O, OH, O, O, O, OH, HO, OH, OH, OH

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Compound	Structure
Quercetin	

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CHAPTER 3

STUDY OF MADHUCA LONGIFOLIA (J. KOENIG EX L.) MACBR.

3.1 Materials and Methods

3.1.1 Chemicals

Name	Source
Acarbose	Sigma Aldrich
Acetic acid	Merck, Germany
Alpha-glucosidase enzyme	Sigma Aldrich
Ammonia	Merck, Germany
Caffeic acid	Sigma Aldrich
Chloroform	Merck, Germany
Chlorogenic acid	Sisco Research Laborites, India
Columbia CAN Agar	BBL ™, USA
Copper sulphate	Sigma Aldrich
Ethanol, absolute	Merck, Germany
Ethanol (70%)	Merck, Germany
Ferric chloride	Sigma Aldrich
Folin Ciocalteu's reagent	Sisco Research Laborites, India
Gallic acid	Sisco Research Laborites, India
Iodine	Sigma Aldrich
Mac Conkey Agar	Difco ™, USA
Mac Conkey Broth	Difco ™, USA
Mannitol Salt Agar	BBL ™, USA
Methanol	HPLC grade, Merck, Germany
Monobasic potassium phosphate	Sigma Aldrich
Multi-element Calibration Standard 2A, (Agilent)	Agilent, USA
Nitric acid (65%)	Merck, Germany
Paraffin	PC Drug company, Thailand
p-nitro phenyl-α-D-glucopyranoside (PNPG)	Sigma Aldrich

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Name	Source
Potassium dihydrogen phosphate	Sigma Aldrich
Potassium hydroxide (pellets)	Sigma Aldrich
Quercetin	Sigma Aldrich
Rappaport Vassiliadis Broth	Difco ™, USA
Reinforced Clostridial Medium	Difco ™, USA
Rutin	ACROS, organics
Sodium carbonate	Sigma Aldrich
Sodium hydroxide (pellets)	Sigma Aldrich
Soybean-Casein Digest Medium	Bacto ™, USA
Sulphuric acid (concentrated)	Merck, Germany
Xylose-Lysine-Deoxycholate (XLD) Agar Medium	Difco ™, USA
Xylose-Lysine-Deoxycholate (XLD) Agar	Difco ™, USA
Whatman filter paper	

3.1.2 Instruments

Name	Source
Analytical balance	Sartorius, Germany
Autoclave, HIRAYAMA, HICLAVE, HVA 85	Hirayama, Japan
Centrifuge, Microfuge 16 centrifuge, Backman coulter	Backman coulter, USA
Column (HP-5MS, 0.25 mm x 30m x 0.25µm) (Agilent)	Agilent, USA
Fourier Transformed Infrared spectrophotometer (FT-IR 4700) (iD7 ATR) (Nicolet iS5)	Thermo Scientific Nicolet, USA

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Name	Source
Gas Chromatography-Mass Spectroscopy (GC-MS) (Agilent 6890N) 5973 mass selective detector	Agilent, USA
High Performance Liquid Chromatography (HPLC) (Agilent 1260)	Agilent, USA
Hot air oven, Heraeus	Heraeus, France
Incubator, Heraeus	Heraeus, France
Inductively Coupled plasma Mass Spectroscopy (ICP-MS) (Agilent 7500ce)	Agilent, USA
Liquid Chromatography Mass Spectroscopy LC-MS (Agilent 1100 series, G2445D)	Agilent, USA
Microplate reader (TECAN Infinite F 50)	Switzerland
Microwave digestion Instrument, Multi-wave 3000, ETHOS One MILESTONE	Austria
PH meter, Mettler toledo	Mettler Toledo, Germany
Rotary evaporator BUCHI Heating Bath B 100 BUCHI Rotavapor R-100	BUCHI, Switzerland
Sonicator, Elma, Model 890/H	Elma, Germany
Ultrasonic bath	CREST ultrasonics, USA
Ultraviolent Visible spectrophotometer (UV-Vis) (Agilent, Cary 60)	Agilent, USA
Vortex mixer	Vision Scientific, Korea

3.1.3 Plant Material

M. longifolia leaves were collected from Mandalay region and flowers were from Mandalay and Bagan region in Myanmar (Middle of Myanmar) in May 2016

and identified by an authorized botanist at the Department of Botany, University of Mandalay, Myanmar. All of samples were dried at 50˚C in oven and ground into powder. Voucher specimen were stored at the Department of Pharmaceutical Chemistry, Silpakorn University, Thailand. L1, L2 were leaf sample from Amarapura township and L3 was leaf sample from Chanayetharzan township in Mandalay Division. F M1 and F M2 were flower samples from Amarapura township and F B was flower sample from Bagan township in Mandalay Division.

3.1.4 Extraction (Maceration with water, boiling water and absolute ethanol)

Each of 2 g of *M. longifolia* leaf and flower powders was macerated with 20 mL of different solvents that were water, boiling water and absolute ethanol, in 3 conical flasks for 1 hour and shaken frequently every 10 minutes. In extracting with boiling water, boiling water was added to the sample containing flask and the flask was put into the water bath at 30-40˚C to keep the temperature of the extract. After 3 times maceration, the filtrate of water and boiling water extracts were freeze-dried, while ethanolic extract was dried under vacuum by a rotary evaporator. The % yield of extracts were calculated as % yield = (g of dried extract/ g of dried powder) $\times$ 100.

3.1.5 Evaluation of alpha-glucosidase inhibitory activity of leaf and flower extracts

3.1.5.1 Preparation of 50 mM phosphate buffer

Monobasic potassium phosphate (KH_2PO_4) 3.4 g and 0.448 g of sodium hydroxide (NaOH) were dissolved in ultra-pure water and made up to 500 mL. Adjust to pH 6.8 with 0.2 M NaOH.

3.1.5.2 Preparation of alpha-glucosidase 0.1 U/ mL solution

Twenty µL of 50-unit alpha-glucosidase enzyme solution was diluted with phosphate buffer to 10 mL.

3.1.5.3 Preparation of 1 mM p-nitrophenyl-α -D-glucopyranoside (PNPG) solution

Dissolve 0.301 g of *p*-nitrophenyl-α-D-glucopyranoside (PNPG) in ultra-pure water and make up to 100 mL.

3.1.5.4 Preparation of 0.1 M sodium carbonate (Na_2CO_3) solution

Dissolve 1.06 g of sodium carbonate in ultra-pure water and make up to 100 mL.

3.1.5.5 Preparation of stock solution of acarbose

Ten mg/mL of acarbose stock solution was made up with phosphate buffer.

3.1.5.6 Preparation of stock solution of plant extract

Ten mg/mL of stock solution of plant extract were prepared with phosphate buffer.

3.1.5.7 Alpha-glucosidase inhibition assay

The extracts of *M. longifolia* leaf and flower were tested for alpha-glucosidase inhibitory activity using acarbose as a standard and PNPG as a substrate. Fifty µL of phosphate buffer (50mM, pH= 6.8), 20µL of varying concentrations (5-2000 µg/mL) of plant extract and 20 µL of alpha glucosidase (0.1U/mL) were added into 96 well plate and pre- incubated at 37˚C for 15 minutes. After that 20µL of PNPG (1mM) was added to the mixture as a substrate and incubated again at 37˚C for 30 minutes. Then, 50 µL of sodium carbonate solution (0.1M) was added to stop the reaction and yellow color produced was measured at 405 nm with a microplate reader (TECAN Infinite F 50). Blank and each experiment were performed in triplicates and acarbose at various concentrations (750-2500 µg/mL) was used as a standard. The percent inhibition of alpha glucosidase activity was calculated using the following equation and IC_{50} values were calculated (19).

$$\text{Inhibition } \% = [1 - (Aa - Ab)/ (Ac - Ad)] \times 100$$

Aa is the absorbance at 405 nm with test sample and enzyme

Ab is the absorbance at 405 nm with test samples but without enzyme

Ac is the absorbance at 405 nm with enzyme but without test sample

Ad is the absorbance at 405 nm without test samples and enzyme

3.1.6. Preliminary phytochemical screening

The extracts were diluted with distilled water and screened for the presence of the secondary metabolites such as alkaloids, flavonoids, terpenoids, steroids, phenol and tannins, cardiac glycosides, saponins, carbohydrates, proteins and anthraquinones by using the methods prescribed in reference (35, 211-213).

3.1.6.1 Preparation of diluted extract

About 20 mg of extract was mixed with 20 mL of distilled water.

3.1.6.2 Test for Alkaloids

To 6 mL of extract, 6 mL of 1% hydrochloric acid was added and heated gently. The solution was divided into three portions to be treated with three reagents: Dragendorff's, Mayer's and Wagner's reagents. Turbidity of the resulting precipitates in each test indicated the presence of alkaloids (35, 211, 212).

3.1.6.3 Test for Flavonoids

To 2 mL of extract, 2 mL of 10% NaOH was added, formation of pale-yellow precipitate which turned colorless on the addition of 2 mL of 1% hydrochloric acid representing a positive result (213).

3.1.6.4 Test for Terpenoids

Two mL of extract was mixed with 2 mL of chloroform, and 1 mL of concentrated H_2SO_4 was added carefully by the side of test tube. Clear upper layer and lower layer with a reddish-brown interphase represented the presence of terpenoids (213).

3.1.6.5 Test for Steroids

Two mL of extract was mixed with 2 mL of chloroform. Then 2 mL of each of acetic acid and concentrated H_2SO_4 were poured into the mixture. Formation of a greenish coloration represented a positive result (35).

3.1.6.6 Test for Phenol and Tannins

Two mL of extract was mixed with 1-2 drops of 2% solution of ferric chloride. A black or blue- green coloration represented a positive result with phenolic compounds (35).

3.1.6.7 Test for Cardiac Glycosides

Keller-kilani test

Two mL of glacial acetic acid and 1-2 drops of 2% solution of ferric chloride were added to the 2 mL of extract. The mixture was poured carefully to another test tube containing 2 mL of concentrated H_2SO_4. Formation of a brown ring at the interphase represented the presence of cardiac glycosides(35).

3.1.6.8 Test for Saponins

Three mL of distilled water was mixed with 2 mL of the extract in a test tube and shaken vigorously. The formation of stable foam represented a positive result (35).

3.1.6.9 Test for Carbohydrates

Iodine test

A few drops of iodine solution were added to 2 mL of the extract. A purple or dark blue color formation represented the presence of the carbohydrate (35).

3.1.6.10 Test for Proteins

Biuret's Test

Five drops of 2 % copper sulphate solution were added to 2 mL of the extract. One mL of ethanol solution and excess of potassium hydroxide pellets were added. Formation of a pink color represented a positive result (211, 212).

3.1.6.11 Test for Anthraquinones

Two mL of extract was boiled for 3 minutes. Two mL of chloroform and 5 drops of 10% ammonia were added. A rose-pink coloration represented the presence of anthraquinone (211).

3.1.7 Quality determination of *M. longifolia*

3.1.7.1 Determination of loss on drying

One gram of sample powder was dried at 105˚C in an oven until constant weight obtained. The procedure was described in Thai Herbal Pharmacopoeia (29). Percent loss on drying was calculated as followed:

% Loss on Drying = (weight before dry- weight after dry)/weight before dry $\times 100$

3.1.7.2 Determination of heavy metal content by ICP-MS

The dried samples of about 0.3 g were microwave digested with 65% nitric acid using Multi-wave 3000, ETHOS One. After digestion, clear liquid samples were injected into an ICP-MS for simultaneous determination of 5 elements (Arsenic, As, Cadmium, Cd, Lead, Pb, Nickel, Ni, and Barium, Ba) using inductively coupled plasma mass spectrometer (ICP-MS), Agilent 7500ce.

3.1.7.2.1. Microwave digestion

All glassware, plastic beakers and bottles, digestion vessels and sample tubes were soaked in 10% nitric acid overnight, rinsed with distilled water and dried before use. Herbal tea containing sugar was used as the reference material.

Zero-point three g of dried samples or reference was put into a vessel and 5 mL of concentrated ultrapure nitric acid (65%) was added, but for vessel-containing reference, 10 mL of acid was used. For a blank, 2 mL of ultrapure water and 5 mL of concentrated ultrapure nitric acid (65%) were used and microwave digested for 30 min. The digestion procedure was as follow: (1) 1400 W at 180 ºC for 15 min, (2) 1400 W at 180 ºC for 15 min (3) 0 W for 30 min for cooling. After cooling, the digested solutions were transferred quantitatively into the volumetric flasks (25 mL) and made up to volume with ultrapure water. The solutions were filtered with Whatman filter paper to plastic bottles and kept in a refrigerator. All samples were prepared in triplicate.

3.1.7.2.2 Preparation of 5% nitric acid solution

Concentrated nitric acid (65%, 6.7 mL) was diluted to 100 mL with ultrapure water.

3.1.7.2.3 Preparation of standard working solutions

One hundred μL of solution of Multi-element Calibration Standard 2A (Agilent) was taken and diluted to 10 mL with 5% nitric acid solution. From which 0.1, 2.5, 5, 7.5, 10, 50, 200 and 500 ng/mL of standard solutions were also prepared.

3.1.7.2.4 ICP-MS injection

After digestion, clear liquid samples were injected into an ICP-MS for simultaneous determination of 5 elements. Conditions for ICP-MS injection were shown in Table 4.

Table 4 Working conditions for ICP-MS detection

ICP-MS parameter	Value
RF power	1500 W
RF matching	1.72 V
S/C temperature	2 ˚C
Sample Depth	9 mm
Carrier gas flow rate	0.85 L/min (99.995 %) Argon
Make up gas flow rate	0.3 L/min
Nebulizer Pump flow rate	0.10 rps

3.1.7.3 Determination of total phenolic content

Total phenolic contents of both flower and leaf extract of *M. longifolia* were assayed by using Folin-Ciocalteu method slightly modified from (214). Gallic acid was used as a standard. The calibration curve was plotted using gallic acid. In brief, 10 mg of gallic acid was dissolved in 10 mL of distilled water. Dilution was made to obtain the concentration range of 1.25 - 120 µg/mL. Two hundred and fifty µL (1mg/mL) of solution was mixed with 125 µL of 50% Folin-Ciocalteu reagent in 1.5 mL micro centrifuge tube. After 5 minutes, 625 µL of 20% sodium carbonate was added and mixed by using a vortex mixer, then, incubated for 2 hours in a dark place at room temperature. A control was prepared without including gallic acid. When oxidation of phenolic compounds happened, phosphomolybdic and phosphotungstic acid in the Folin- Ciocalteu reagent were reduced to blue color molybdenum and tungsten oxides (214). The absorbance was measured in micro cuvette at 750 nm for leaf and 700 nm for flower using UV-Visible spectrophotometer (Agilent, Cary 60). Total phenolic content was calculated and expressed as mg GAE/g extract.

3.1.7.4 Determination of Microbial contamination

Microbial limit tests for *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* species and *Clostridia* species in *M. longifolia* leaf and flower powder were done according to USP 40 (215)(USP 40) and Thai Pharmacopoeia 2005 (216). Limit of microbial contamination in sample were prescribed in Table 5.

3.1.7.4.1 Test for *Escherichia coli*

One gram of substance to be examined was added to 100 mL of Fluid Soybean- Casein Digest Medium, mixed and incubated at 35°-37 °C for 18-48 hours. The container was shaken, then 1 mL was transferred to 100 mL of MacConkey Broth and incubated at 43°-45 °C for 18-24 hours. Subculture was done on plates of

MacConkey Agar Medium and incubated at 35°- 37°C for 18-72 hours. When examined, none of colonies confirmed that the sample met the requirements of the test for absence of *Escherichia coli*.

3.1.7.4.2 Test for *Staphylococcus aureus*

One gram of substance to be examined was added to 100 mL of Fluid Soybean- Casein Digest Medium, mixed and incubated at 35° - 37 °C for 18-48 hours. Subculture was done on plates of Mannitol-Salt Agar Medium and incubated at 35° - 37°C for 18-72 hours. When examined, none of colonies confirmed that the sample met the requirements of the test for absence of *Staphylococcus aureus*.

3.1.7.4.3 Test for *Salmonella* species

Ten grams of substance to be examined was added to 100 mL of Fluid Soybean- Casein Digest Medium, mixed and incubated at 35°- 37 °C for 18-24 hours. One mL of the enrichment culture was transferred to 10 mL of Rappaport Vassiliadis Broth, mixed and incubated at 35° - 37 °C for 18 - 24 hours. Subculture was done on plates of Xylose-Lysine-Deoxycholate Agar Medium and incubated at 35° - 37°C for 18-72 hours. When examined, none of colonies confirmed that the sample met the requirements of the test for absence of the genus *Salmonella*.

3.1.7.4.4 Test for *Clostridia* species

Ten grams of substance to be examined was added into 2 suitable containers, each containing 100 mL of Reinforced Medium for *Clostridia*. To distinguish between sporing and non-sporing organisms, one container was immediately sealed with a layer of sterile liquid paraffin or (mineral oil). Other container was heated at 80°C for 10 minutes, cooled rapidly and then similarly sealed. Both containers were incubated at 35°- 37 °C and examined every 24-hour period up to 2 - 3 days.

After incubation, subcultures were made from each container on plates of Columbia agar to which gentamicin had been added and incubated under anaerobic conditions at 35- 37 °C for 48 hours. When examined, none of colonies confirmed that the sample met the requirements of the test for absence of pathogenic *Clostridia*.

Table 5 Limit of microbial contamination in sample

Microbial	Limit
E. coli	No colony of *E. coli* in 1 g sample
S. aureus	No colony of *S. aureus* in 1 g sample
Salmonella species	No colony of *Salmonella* species in 10 g sample
Clostridia species	No colony of spore and non-spore *Clostridia* species in 10 g each test.

3.1.7.5 Infrared Spectroscopy (IR) profiling

3.1.7.5.1 ATR-FT- IR Analysis

FT-IR analysis of *M. longifolia* leaf and flower powder was done by Nicolet iS5 FT-IR Infra-Red spectrometer with the iD7 ATR accessory. The spectra were recorded across the entire mid-infrared region (500–4000 cm-1). The sample powder was pressed with a conical tip onto the diamond crystal and a pressure was applied. The tip and the diamond crystal were thoroughly washed with ethanol before each measurement to avoid cross contamination (217).

3.1.7.6 Gas Chromatography- Mass Spectroscopy (GC-MS) profiling

3.1.7.6.1 Preparation of Extract for GC-MS Analysis

Absolute ethanolic extracts of *M. longifolia* leaf and flower from 3.1.4 was dissolved in methanol to get 1 mg/mL solution. The solution was then filtered with 0.45 µm pore diameter syringe filter and added into small vials for GC-MS analysis.

3.1.7.6.2 GC-MS condition

The chemical constituents were analysed using Agilent GC-MS, model 6890 N equipped with 5973 mass selective detector. Chromatographic condition was modified from the reference (218). Two µL sample was injected into an HP-5MS column (0.25 mm i.d. × 30 m × film thickness 0.25 µm). The initial temperature was set at 60 °C, whereas the injector temperature was set at 250 °C, and throughout the process temperature was set to increase at a speed of 3°C/min. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The total run time was 63 minutes and mass scan range were 50-500 amu.

3.1.7.6.3 Identification of Chemical constituents

Identification of compounds was done based on comparison of mass spectra with spectra in the National Institute of Standard and Technologies (NIST 05.LIB) and wiley7n libraries. Percentage peak area (% peak area) higher than 0.04% was also determined for each constituent.

3.1.7.7 High Performance Liquid Chromatography (HPLC) profiling

3.1.7.7.1 Instrumentation and chromatographic condition

All analyses were operated on an Agilent 1260 HPLC system (Agilent, USA) equipped with a photodiode array detector. The analytical method prescribed in reference was modified (83). The analytical column used was C18 column (Synergi 4µ Polar-RP 80 °A, 150 x 2 mm, Phenomenex®). The ratio of mobile phase, flow rate, column temperature and wavelength were explored. The mobile phase contained the mixture of solvent A (0.1% formic acid in water) and solvent B (methanol). Solvents used were filtered through a 0.22 µm filter and degassed. The sample injection volume was 2 µL.

3.1.7.7.2 Preparation of standard solutions

Standard gallic acid and quercetin were weighed and dissolved in 80% ethanol to obtain a stock solution of 1 mg/mL. Working solutions were serially diluted from stock solution to 6 concentration of standard solutions with the range of 3.125 to 100 µg/mL for gallic acid and 0.78 to 50 µg/mL for quercetin.

3.1.7.7.3 Method validation

The developed HPLC method for the quantification of gallic acid and quercetin in aqueous extract of *M. longifolia* was validated for specificity, system suitability, linearity, sensitivity, precision and accuracy, according to ICH and AOAC guidelines (219, 220).

3.1.7.7.3.1 Specificity and system suitability

Specificity was assessed using standard and DAD detector. Peak identification was carried out based on retention time and UV spectrum of those of standards. To evaluate system suitability of the method, resolution together with repeatability of retention time and peak area of six replicate injections of 50 µg/mL standard gallic acid and 6.25 µg/mL of quercetin standard solutions were used. The %RSD values were calculated in each case.

3.1.7.7.3.2 Linearity and range

Linearity was established across 6 different concentrations (3.125 to 100 µg/mL) of gallic acid and 7 different concentrations (0.78 to 50 µg/mL) of quercetin. The regression equation and correlation coefficient were reported.

3.1.7.7.3.3 Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curves of gallic acid and quercetin. The LOD was calculated according to the expression 3.3*SD/S. While SD is the standard deviation of the response and S is the slope of the calibration curve. The LOQ was calculated using the expression 10*SD/S (219).

3.1.7.7.3.4 Accuracy and precision

The accuracy was assessed by means of recovery assays using standard addition method to the diluted sample solution. Prior to fortification, the diluted sample solutions were determined for background levels of gallic acid and quercetin. Standards were spiked at three different levels, 40, 50 and 60 µg/mL for gallic and 5, 6.25 and 7.5 µg/mL for quercetin. Each solution was injected in triplicate within 2 sequential days. Unspiked diluted samples were analysed to calculate actual recovery. Accuracy was revealed as the recovery percentage across the range of the assay. Intra- and inter-day precisions were shown as the percentage of relative standard deviation (%RSD) within 1 day and 2 sequential days, respectively.

3.1.7.7.4 Content of gallic acid and quercetin in the aqueous extract of *M. longifolia*

Dried aqueous extract (10 mg) was dissolved in 80% ethanol to get the final concentration of 10 mg/mL as sample solution. The developed HPLC method in the present study was used to quantify the amounts of gallic acid and quercetin in the aqueous extracts of *M. longifolia* leaf. The amount was expressed as mg/100 g dried extract.

3.1.7.8 LC-MS profiling

The extract used in HPLC analysis was also analysed by LC-MS in which a group of high response peak around the retention time of 24-28 min from LC-DAD

was identified using an Agilent 1100 series LC coupled to mass spectrometer (Agilent, G2445D).

3.1.8 Statistical analysis

All values of experimental measurements will be expressed as mean ± SD. One-way analysis of variance (ANOVA) and two-factor with replication was used by excel for the measurement of the statistical significance. The statistical significance level $p<0.05$ was used.

3.1.9 Principal component analysis (PCA)

To detect clustering of samples, principal component analysis (PCA) was used. Unsupervised pattern recognition method was called principal component analysis (PCA). To present different manner for interpretation and to reduce data, PCA is very useful (221). Data obtained from GC-MS, ICP-MS and HPLC were analyzed by PCA to differentiate about sources of leaves and flowers using WEKA version 3.8.5 open-source software, windows version 10.0 (available from: https://www.cs.waikato.ac.nz/ml/weka/).

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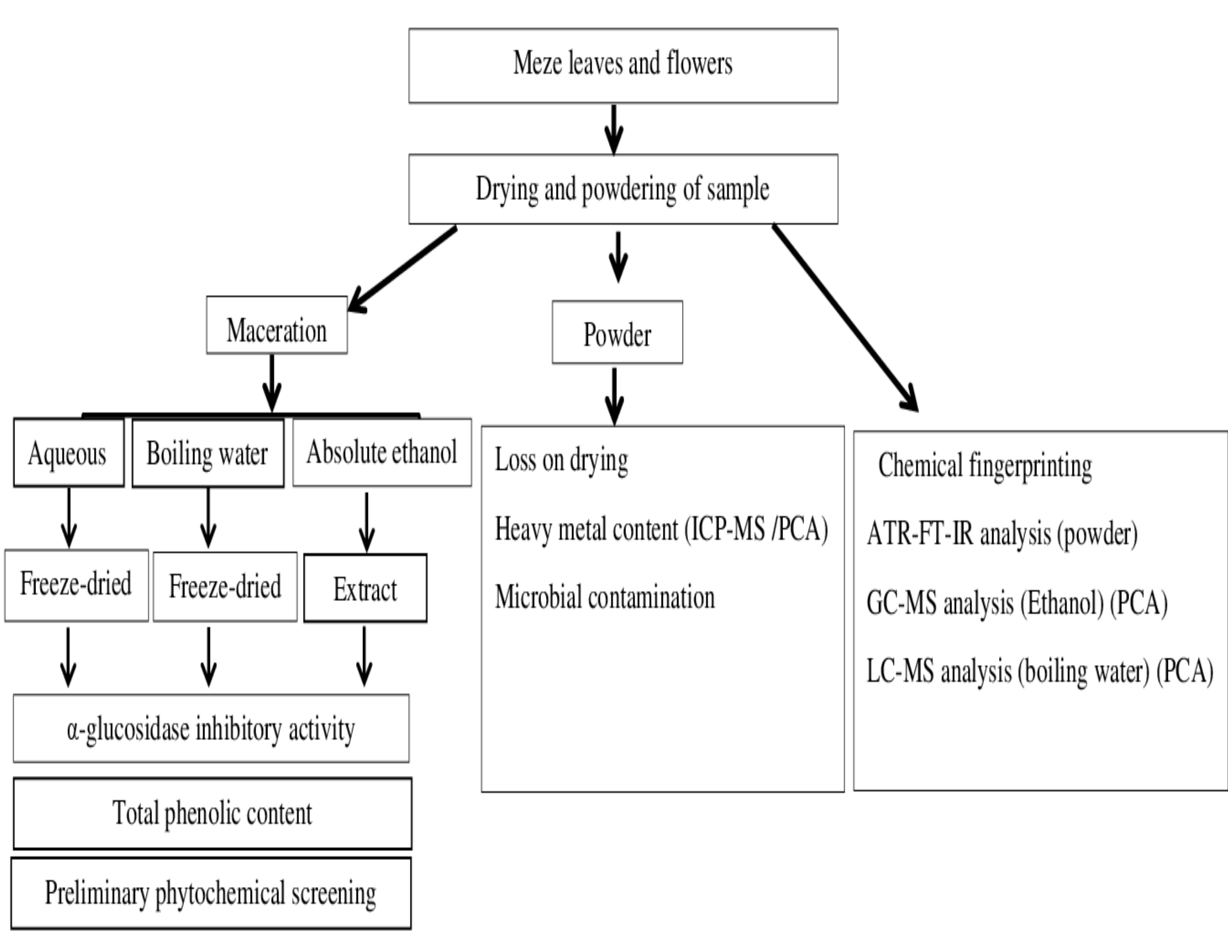
Flow chart



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3.2 Results and discussions

3.2.1 Extraction (% yield of extracts)

The % yield of water, boiling water and absolute ethanolic extracts of *M. longifolia* leaf and flower were shown in Table 6 and Figure 1. The % yield of all extracts were in the range of 2.55 -55.24 %. Flower extracts possessed more % yield value than leaf extracts. The boiling water extract of flowers from Amarapura area, sample FM2, possessed the highest % yield.

The result from two-factor with replication of ANOVA at 95% confidence interval, from 3 extraction solvents of *M. longifolia* leaves and flowers, showed that % yield of the sample from different sources presented significant differences (Appendix). Considering boiling water extracts of leaves from different source, the highest % yield was from Amarapura area (L2) followed by Chanayetharzan area (L3) and Amarapura area (L1), respectively.

Table 6 % Yield of different extracts of *M. longifolia* leaf and flower

Sample	% yield		
	aqueous extract (mean ± SD)	boiling water extract (mean ± SD)	absolute ethanolic extract (mean ± SD)
L1	8.45 ± 0.34	10.13± 0.60	2.55 ± 0.08
L2	9.88 ± 0.8	13.41± 0.66	3.52 ± 0.63
L3	8.47 ± 0.18	13.21± 0.5	4.05 ± 0.19
FM1	47.48 ± 0.19	33.63 ± 1.87	24.28 ± 2.82
FM2	46.13 ± 9.03	55.24 ± 2.61	35.08 ± 0.67
FB	49.15 ± 2.48	33.08 ± 2.54	24.52 ± 1.64

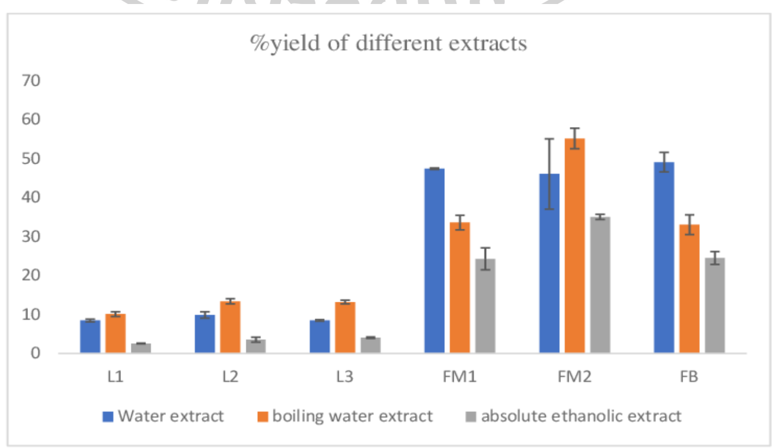

Figure 1 % yield of different extracts of *M. longifolia* leaf and flower

3.2.2 Evaluation of alpha-glucosidase inhibitory activity of leaf and flower extracts of *M. longifolia*

The alpha-glucosidase inhibitory activities of different extracts of leaf and flower of *M. longifolia* were shown as IC_{50} value (µg/mL) in Table 7 and Figures 2-3. From the mean of IC_{50}, leaf extracts were much more potent in inhibiting alpha-glucosidase than flower extracts. The ranges of IC_{50} for leaf were 40.35-59.84, 15.23-28.41 and 138.26-147.54 for aqueous, boiling water, and ethanol extracts, respectively. Those for flower were 197.05-1561.80 for boiling water extract. Focus on comparing leaf extracts to acarbose, IC_{50} values of all of leaf extracts were lower than the value of standard acarbose. As a result, all of leaf extracts possessed higher activity than acarbose. Moreover, boiling water with higher temperature of extraction resulted in more extracted bioactive compounds. The highest activity was found in leaf sample from Amarapura area, L1, followed by L2 and L3 and this sequence corresponded to the quercetin content. Quantification of quercetin showed 20.56, 9.86, and 7.31 mg/100g in L1, L2, and L3 respectively. It has been reported that quercetin could inhibit alpha-glucosidase enzyme (Kim J H et al.,2011, Shia G J et al., 2019). In the present study, boiling water extracts of *M. longifolia* leaf significantly showed higher alpha-glucosidase inhibitory activity than previous study (Sangeetha, R et al., 2018).

In extracts of flower samples, only boiling water extract possessed activity. Water and ethanol extracts of flowers did not show activity. The boiling water extract of flower from Amarapura area, sample FM2, possessed lower activity than acarbose. Other samples possessed higher activity than acarbose. Boiling water extract of flowers from Bagan, FB possessed the highest activity followed by FM1 and FM2. In this study, leaf extracts possessed higher activity than flower extracts.

The result from ANOVA: single factor in EXCEL showed that among leaf extract, different solvents could significantly extract different amounts of the bioactive compounds (Appendix). The mean values of IC_{50} showed that the strength was in the order of boiling water extract > aqueous extract > ethanolic extract. In boiling water extract, leaves and flowers from different sources also gave significantly different potency of biological effects at 95% confident interval (L1>L2>L3 and FB>FM1>FM2).

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Table 7 IC_{50} value of different extracts of *M. longifolia* leaf and flower

Sample	Aqueous (mean ± SD) (µg/mL)	Boiling water (mean ± SD) (µg/ mL)	Ethanol (mean ± SD) (µg/mL)
L1	40.35±2.57	15.23±1.32	143.54±6.84
L2	59.84±1.36	24.07±1.79	138.26±10.90
L3	44.89±1.83	28.41±1.65	147.54±3.80
FM1		197.06±2.92	-
FM2		1561.80±378.74	-
FB		156.18±22.71	-
Acarbose		763.92±22.27	

(-) = not show activity

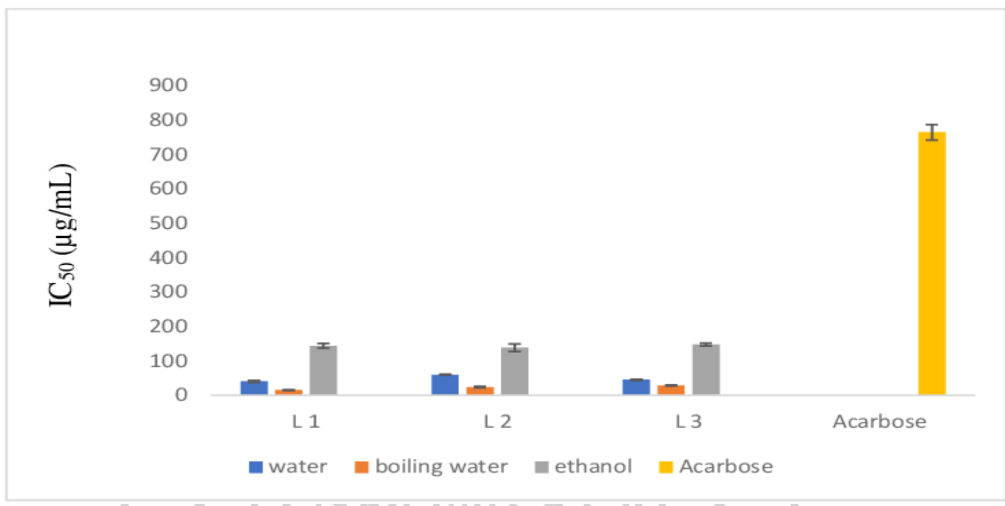

Figure 2 IC_{50} value of different extracts of *M. longifolia* leaf

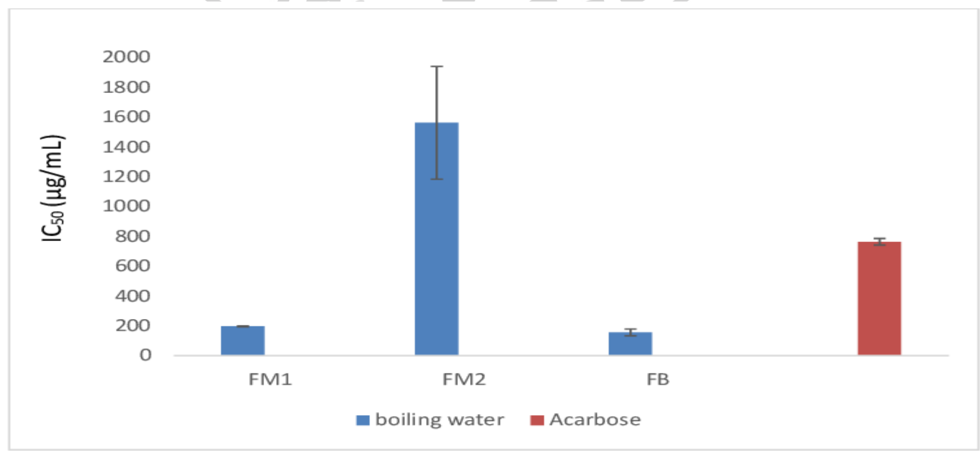

Figure 3 IC_{50} value of boiling water extract of *M. longifolia* flower

3.2.3 Preliminary phytochemical screening

Phytochemical screening of aqueous, boiling water and absolute ethanolic extracts of *M. longifolia* leaves and flowers from Mandalay and Bagan townships indicated the presence of secondary metabolites such as flavonoids, terpenoids, saponins, phenols and tannins, anthraquinone, carbohydrates, proteins and cardiac glycosides. The results were shown in Table 8. Alkaloids were not detected in all extracts. Anthraquinones did not showed in absolute ethanolic extract of flowers. Carbohydrates were not present in absolute ethanolic extracts of leaves and flowers. Proteins was not detected in water, boiling water and absolute ethanolic extracts of leaves.

In other study (222), *M. longifolia* leaf extracts showed the presence of alkaloids, carbohydrates, phenolics, flavonoids, proteins, amino acids, tannins, phytosterols and saponins. In another study (223), methanolic and aqueous extracts of leaf also possessed alkaloids, carbohydrates, proteins, saponins, tannins and triterpenoids.

Table 8 Preliminary phytochemical screening of different extracts of *M. longifolia* leaf and flower

Phytochemical	aqueous extract						boiling water extract						absolute ethanolic extract					
test	L1	L2	L3	FM1	FM2	FB	L1	L2	L3	FM1	FM2	FB	L1	L2	L3	FM1	FM2	FB
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	++	++	++	+	+	+	++	++	++	+	+	+	++	++	++	+	+	+
Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saponin	++	++	++	+ +	+ +	++	++	++	++	+ +	+ +	++	++	++	++	+ +	+ +	++
Phenols and Tannins	++	++	++	+ +	+ +	++	++	++	++	+ +	+ +	++	++	++	++	+ +	+ +	++
Anthraquinones	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Carbon hydrates	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Proteins	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+
Cardiac glycosides	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+

(-) = negative, (+) = weakly positive, (++) = positive

3.2.4 Quality determination of *M. longifolia*

3.2.4.1 Determination of loss on drying (LOD)

The % loss on drying of *M. longifolia* leaf and flower powder were shown in Table 9 and Figure 4. The values of % loss on drying of leaves were lower than the flowers. The values of all samples were not more than 10 % after drying at 105˚C to constant weight.

Table 9 % Loss on drying of *M. longifolia* leaf and flower powder

Sample	%LOD
L1	8.43
L2	7.24
L3	6.89
FM1	9.01
FM2	9.94
FB	8.61

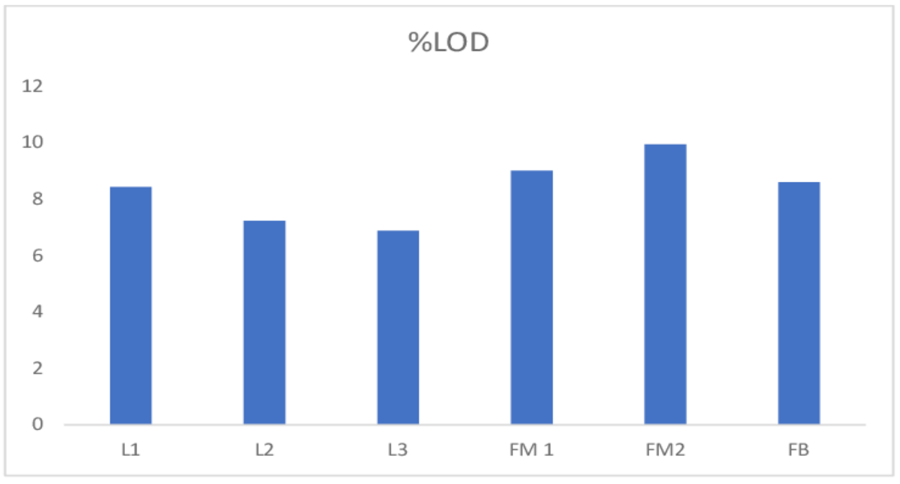

Figure 4 % Loss on drying of *M. longifolia* leaves and flowers powder

3.2.4.2 Determination of heavy metal content by ICP-MS

3.2.4.2.1 Standard curves

Heavy metals contents in *M. longifolia* leaf and flower powder were determined by ICP-MS. Calibration curve equations, linear range and LOD, LOQ of As, Cd, Pb, Ni and Ba were described in Table 10 and Figures 5-9, respectively.

Table 10 Calibration equations, LOD and LOQ for 5 elements

Elements	Calibration equations	Ranges(ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)
As	Y=763.1x+1770	0-500	0.9995	0.015	0.050
Cd	Y=5823.7x-13867	0-500	0.9994	0.004	0.013
Pb	Y=39788x-366213	0-500	0.998	0.034	0.113
Ni	Y=2515.3x+3202.5	0-500	0.9997	0.023	0.077
Ba	Y=9421.2x-62976	0-500	0.9985	0.042	0.140

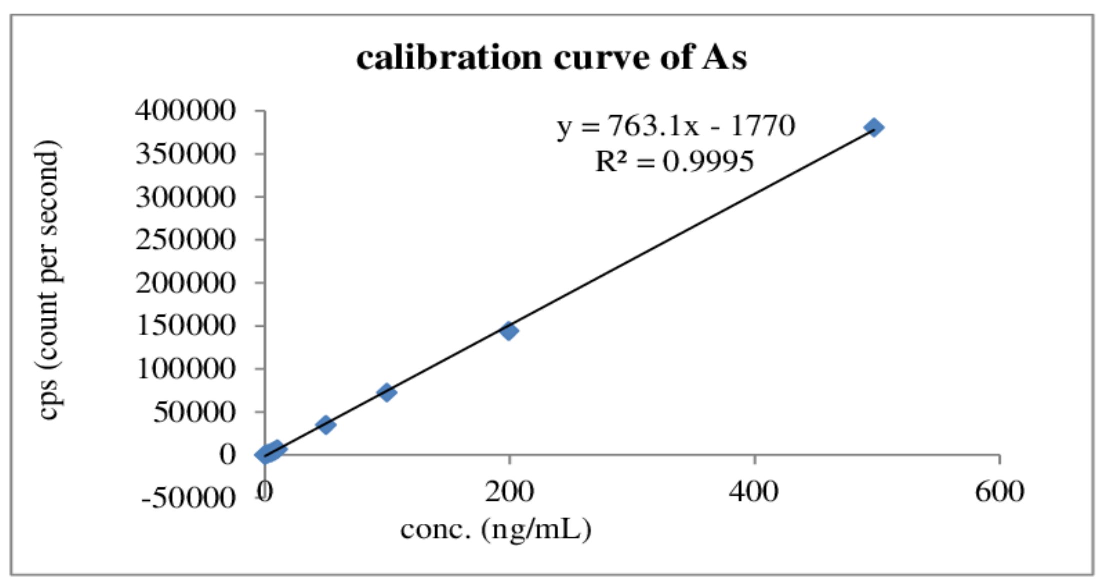

Figure 5 Calibration curve of As

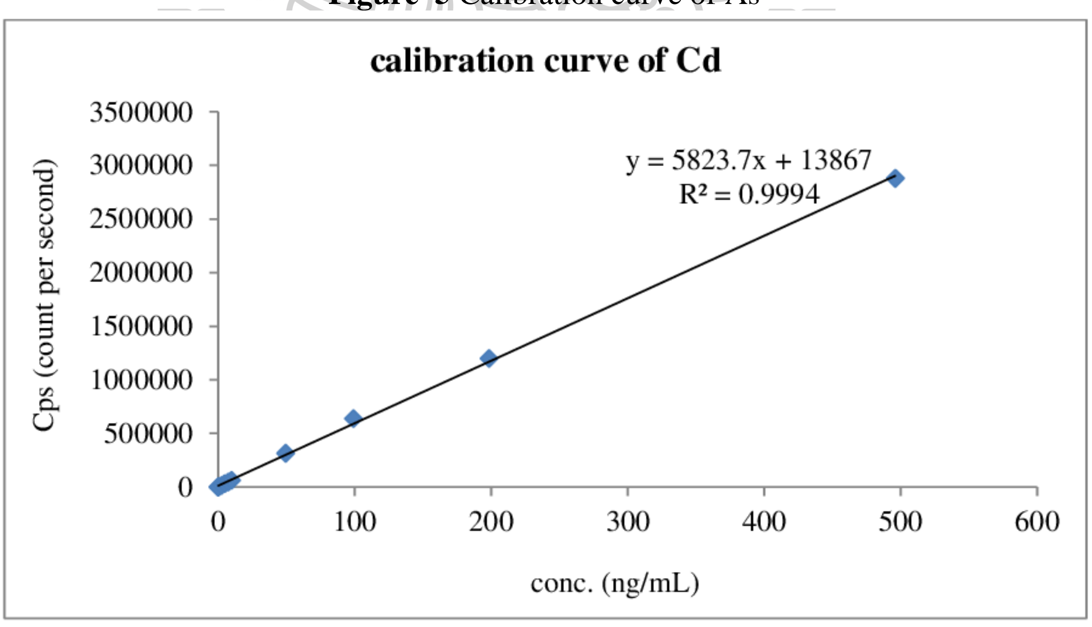

Figure 6 Calibration curve of Cd

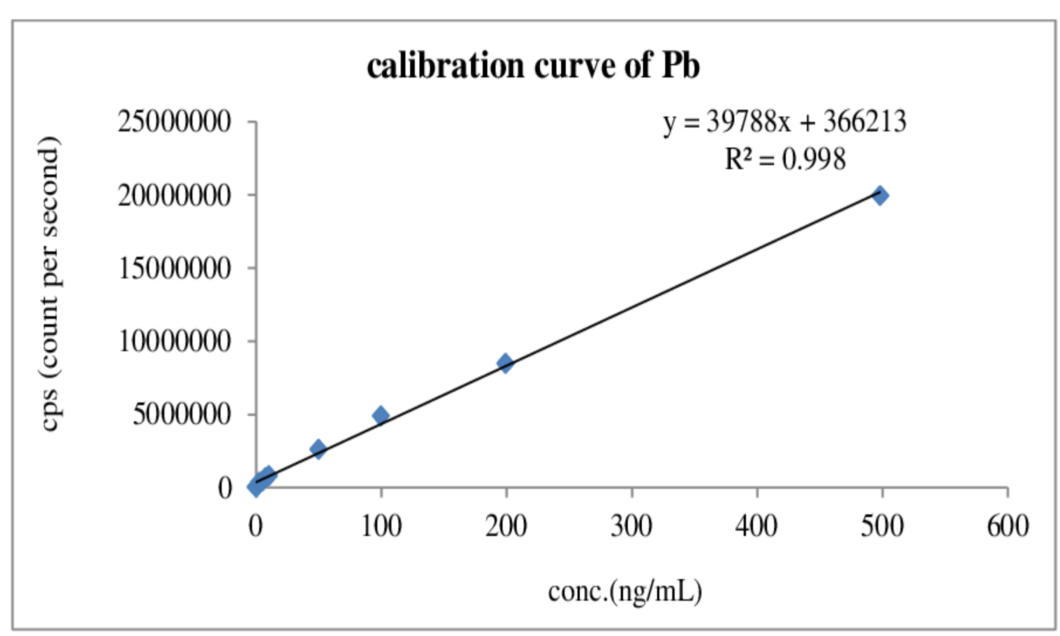

Figure 7 Calibration curve of Pb

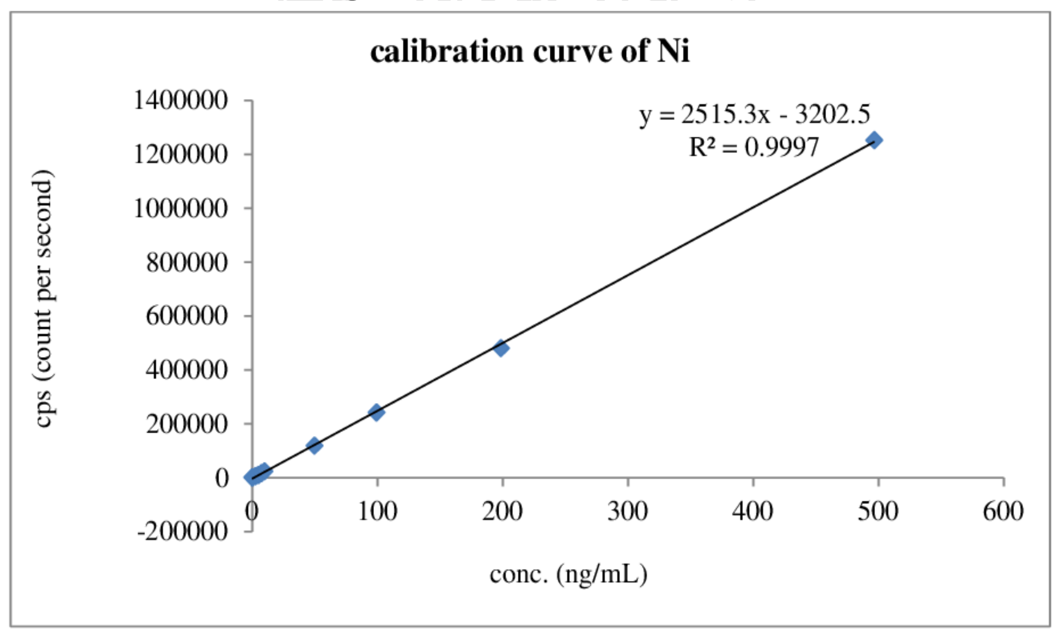

Figure 8 Calibration curve of Ni

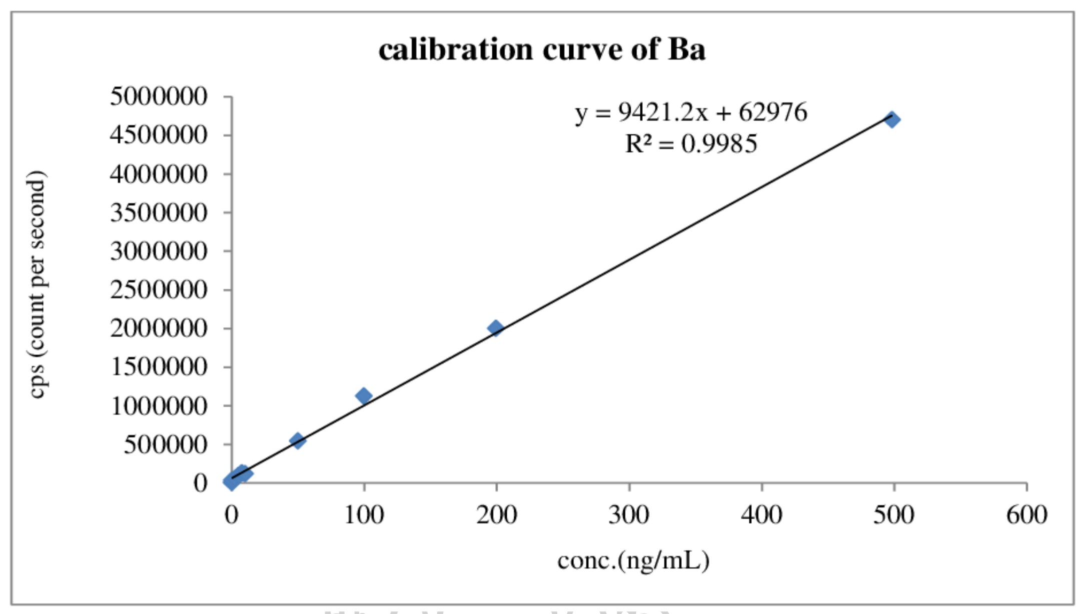

Figure 9 Calibration curve of Ba

3.2.4.2.2 Heavy metal contamination in *M. longifolia* leaves and flowers

Heavy metal content was expressed as mg/kg (mean ± SD). Arsenic, cadmium and lead are classified as class 1 human toxicants by The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). Nickel was considered as class 2A route-dependent human toxicant with high probability occurrence, while barium was class 3, low toxicity element by oral route.

The concentration of heavy metals in *M. longifolia* leaves and flowers from Myanmar were described in Table 11 and Figures 10-11. As, Cd, Pb, Ni and Ba contents in leaf samples were in the range of 0.0346-0.2519, 0.0309-0.0661, 0.4011-0.1819, 0.8390 -1.1435 and 22.3815-62.3907 mg/kg, respectively. Sample L1 from Amarapura area, showed the highest content of Cd and Ni but the lowest of As, Pb and Ba content. L3, another sample from Chanayetharzan area, showed the highest contamination of As, Pb and Ba. L2, sample from Amarapura area, showed the lowest content of Cd and Ni.

As, Cd, Pb, Ni and Ba content in flower samples were in the range of 0.0167-0.1812, 0.0083-0.0266 and 0.3831-1.3096, 0.8259 -1.2582 and 1.3079-8.8105 mg/kg, respectively. F.B, sample from Bagan, showed the highest content of As and Ba but

the lowest content of Cd and Pb. F.M1, sample from Mandalay, showed the highest content of Cd but the lowest content of As and Ni. F.M2, another sample from Mandalay, sample showed the highest content of Pb and Ni but the lowest content of Ba.

Among *M. longifolia* leaf and flower samples, As, Cd and Ba content in leaves were higher than in flowers, but Pb and Ni content in flowers were higher than in leaves.

The content of As, Cd and Pb in all samples was within the maximum limit prescribed in WHO and ASEAN guidelines for herbal medicines (32, 224). The content of Ni and Ba in all samples was also lower than the guideline limit of ICH (219). All samples in this research from different sources and different parts contained variable amount of heavy metals. These differences may be due to variability of exposure to environmental pollution, genetics, geographical region and composition of soil. However, heavy metal content was within acceptable and safe limit prescribed in ASEAN guidelines, WHO recommendation and ICH guideline.

From ANOVA, two-factor with replication table, most of metal levels in *M. longifolia* leaves and flowers were affected by cultivation source at 95% confidence interval (Appendix).

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Table 11 Heavy metal content in *M. longifolia* leaf and flower powder

sample	Metal Content)mg/kg ± SD(				
	As	**Cd**	**Pb**	**Ni**	**Ba**
L 1	0.0346 ± 0.0005	0.0661 ± 0.0026	0.4011 ± 0.0097	1.1435 ± 0.0958	22.3815 ± 0.8523
L 2	0.1149 ± 0.0016	0.0309 ± 0.0292	0.5847 ± 0.2090	0.8390 ± 0.0375	49.4727 ± 0.5569
L 3	0.2519 ± 0.0093	0.0546 ± 0.0113	1.1819 ± 0.0249	0.9522 ± 0.1561	62.3907 ± 3.7029
FM1	0.0167 ± 0.0021	0.0266 ± 0.0059	0.6125 ± 0.1030	0.8259 ± 0.0677	3.3618 ± 0.0909
FM2	0.0426 ± 0.0398	0.0195 ± 0.0169	1.3096 ± 1.1436	1.2582 ± 1.0924	1.3079 ± 1.1379
FB	0.1812 ± 0.0712	0.0083 ± 0.0018	0.3831 ± 0.0445	0.9956 ± 0.0117	8.8105 ± 0.4754
Limitation					
WHO		0.3	10		
ASEAN	5	0.3			
ICH				20	140

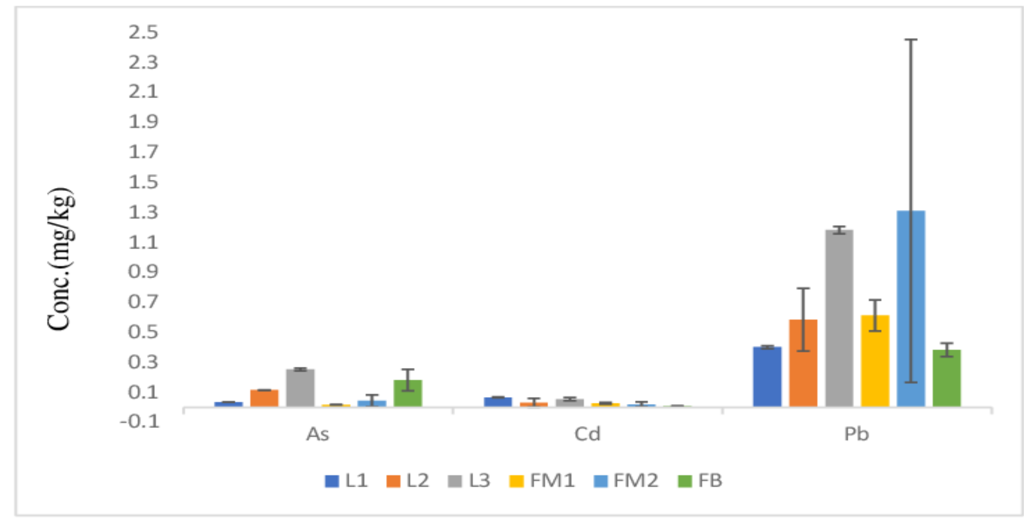


Figure 10 Heavy metal (As, Cd and Pb) content in *M. longifolia* leaves and flower powder

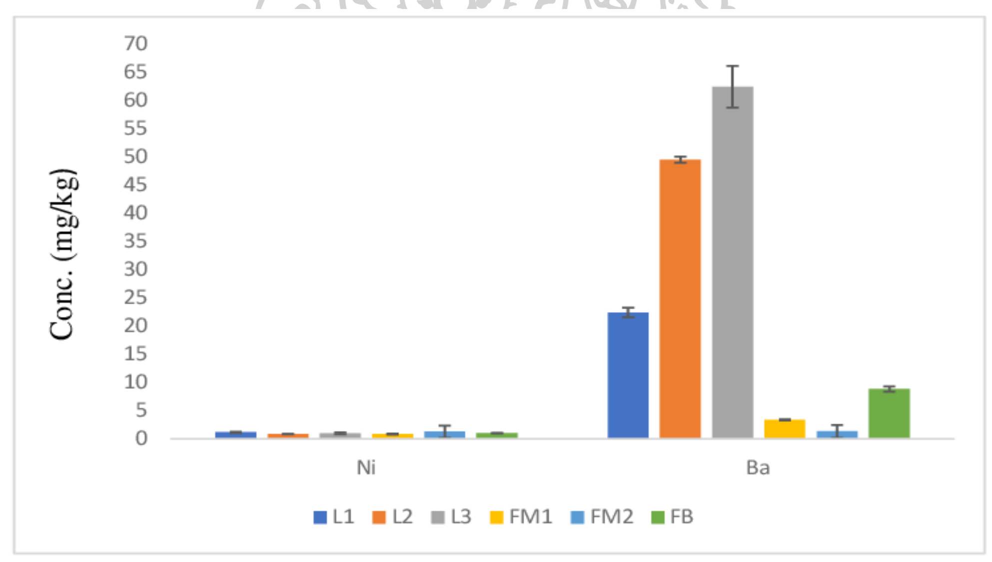


Figure 11 Heavy metal (Ni and Ba) content in *M. longifolia* leaves and flower powder

3.2.4.3 Determination of total phenolic content

Total phenolic content (TPC) of leaf and flower extracts of *M. longifolia* were expressed as mg GAE /g extract and shown in Table 12 and Figure 12 A and B. In leaf extracts, TPCs contents were in the range of 41.67-104.95, 86.89-179.43, 81.37-30.45 mg GAE /g extract for aqueous, boiling water and ethanolic extracts, respectively. Among leaf samples, boiling water extract of sample from Chanayetharzan area, L3 possessed the highest phenolic content.

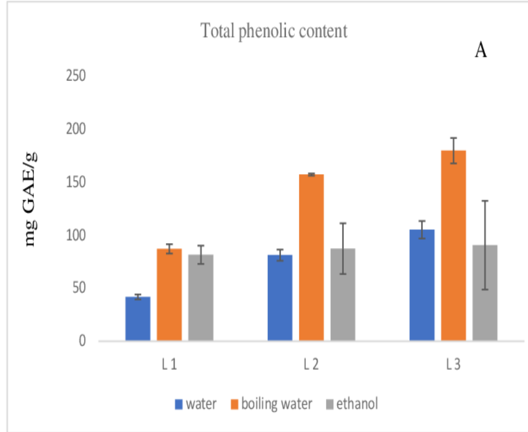
In flower samples, TPCs were in the range of 6.15-7.89, 8.46-9.86, 5.53-9.79 mg GAE /g extract, respectively. In all flower samples, boiling water extracts of sample from Amarapura area, FM2 showed the highest phenolic content.

When comparing with flower extracts, leaf extracts possessed higher TPCs. In all leaf and flower samples, boiling water extracts of leaf sample from Chanayetharzan area, L3, possessed the highest total phenolic content.

The result from ANOVA two-factor replication, TPCs content were affected by different solvents and sources and statistically significantly different at 95% confidence interval (Appendix). TPC contents in leaves were much higher than in flowers. Boiling extract of leaves present higher content than aqueous and ethanolic extracts.

Table 12 Total phenolic content (mg GAE /g) in different extracts of *M. longifolia* leaf and flower

Sample	Aqueous (mean ± SD)	Boiling water (mean ± SD)	Ethanol (mean ± SD)
Leaf			
L1	41.67±2.30	86.89± 4.37	81.37±8.47
L2	80.97±5.22	156.91±0.99	87.13±23.94
L3	104.95±8.18	179.43±11.9	90.45±41.74
Flower			
FM1	6.15±0.13	8.46±0.60	5.53±0.83
FM2	7.89±0.31	9.86±0.54	5.99±0.81
FB	6.84±0.22	9.22±0.04	9.79±1.25^{b}



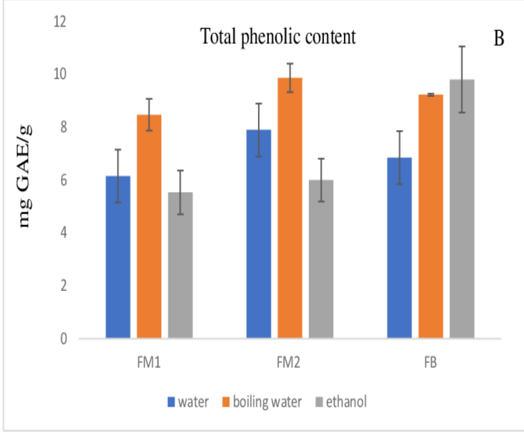


Figure 12 Total phenolic content in different extracts of *M. longifolia*. (A) leaf; (B) flower

3.2.4.4 Determination of microbial contamination

Microbial contamination of *M. longifolia* leaf and flower powder were determined according to USP 40 and Thai Pharmacopoeia 2005 and the results were shown in Table 13. Leaf was free from the contamination of *E. coli*, *S. aureus* and *Salmonella* species. However, they were contaminated with non-pathogenic *Clostridium* species, other than *C. tetani*, *C. botulinum* and *C. perfringers*, which are pathogenic. Flower powder was free from the contamination of all tested microbial. Result of all samples were within the limit prescribed in USP 40 and Thai Pharmacopoeia 2005, except for *Clostridium* spp. in leave samples. However, these contaminated species were not pathogenic species.

Table 13 Microbial contamination in *M. longifolia* leaf and flower powder

Sample	E. coli	S. aureus	Salmonella	Clostridium spp.
Leaf				
L1	-	-	-	+
L2	-	-	-	+
L3	-	-	-	+
Flower				
FM1	-	-	-	-
FM2	-	-	-	-
FB	-	-	-	-

(+) = present, (-) = absent

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3.2.4.5 Fourier Transformed-Infrared Spectroscopy (IR) profiling

In three samples of *M. longifolia* leaves, common peaks were found at wave number 1031.1, 1204.0, 1316.6, 1441.5, 1537.9, 1608.3, 2848.8, 2916.6 and 3277.0 (Table 14 and Figure 13). Most bands from sample L1 and L3 were in common. Peak at wave number 3277 was missing from L2 sample but other peaks were similar with L1 and L3.

In three samples of *M. longifolia* flowers, common peaks were found at wave number 776.2, 817.2, 865.6, 919.8, 1027.9, 1254.6, 1409.9, 1636.2, 2929.6 and 3278.2 cm^{-1} (Table 15 and Figure 14). ATR-FT-IR spectra of three flowers from Mandalay and Bagan were similar.

Overall, similar IR spectra were obtained and the same bands were present. Thus, FTIR spectra could be used to identify the characteristics of leaf and flower samples.

Table 14 Wave number and assignment of *M. longifolia* leaf powder

No	Sample	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number
1	L1	1031.1	1204.0	1316.6	1441.4	1537.9	1608.3	2848.8	2916.6	3277.0
2	L2	1033.2	1204.2	1318.2	1456.0	1516.0	1621.4	2847.4	2914.4	-
3	L3	1031.2	1204.2	1317.6	1442.6	1537.9	1605.9	2848.3	2916.0	3265.8
	Assignment	C-O stretching	C-O stretching	C=C stretching	C-H banding	C=C stretching	C=C stretching	C-H stretching	C-H stretching	O-H stretching

Table 15 Wave number and assignment of *M. longifolia* flower powder

No	Sample	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number	Wave-number
1	FM1	776.2	817.2	865.6	919.8	1027.9	1254.6	1409.9	1636.2	2929.6	3278.2
2	FM2	776.2	817.9	866.2	907.9	1034.4	1237.4	1409.9	1635.5	2930.4	3284.6
3	F B	776.2	818.1	866.3	907.9	1035.0	1236.8	1422.6	1633.3	2931.0	3286.9
	Assignment	C-H banding	C-H banding	C-H banding	C-H banding	C-O stretching	C-O stretching	C-H banding	C=C stretching	C-H stretching	O-H stretching

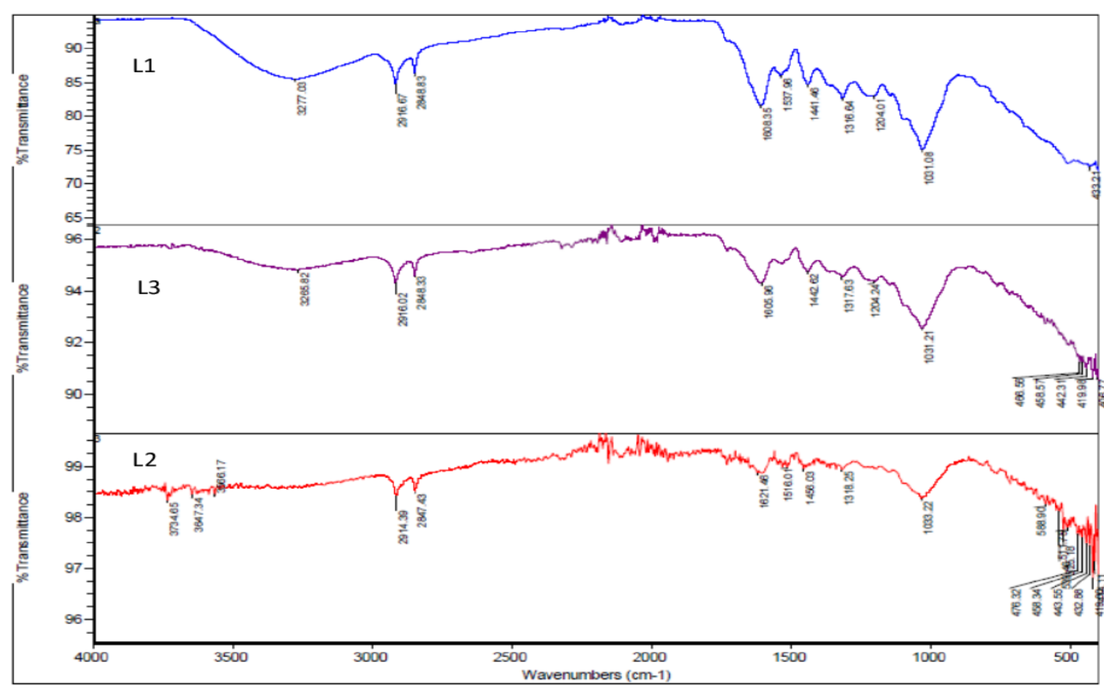

Figure 13 FT-IR spectra of *M. longifolia* leaves

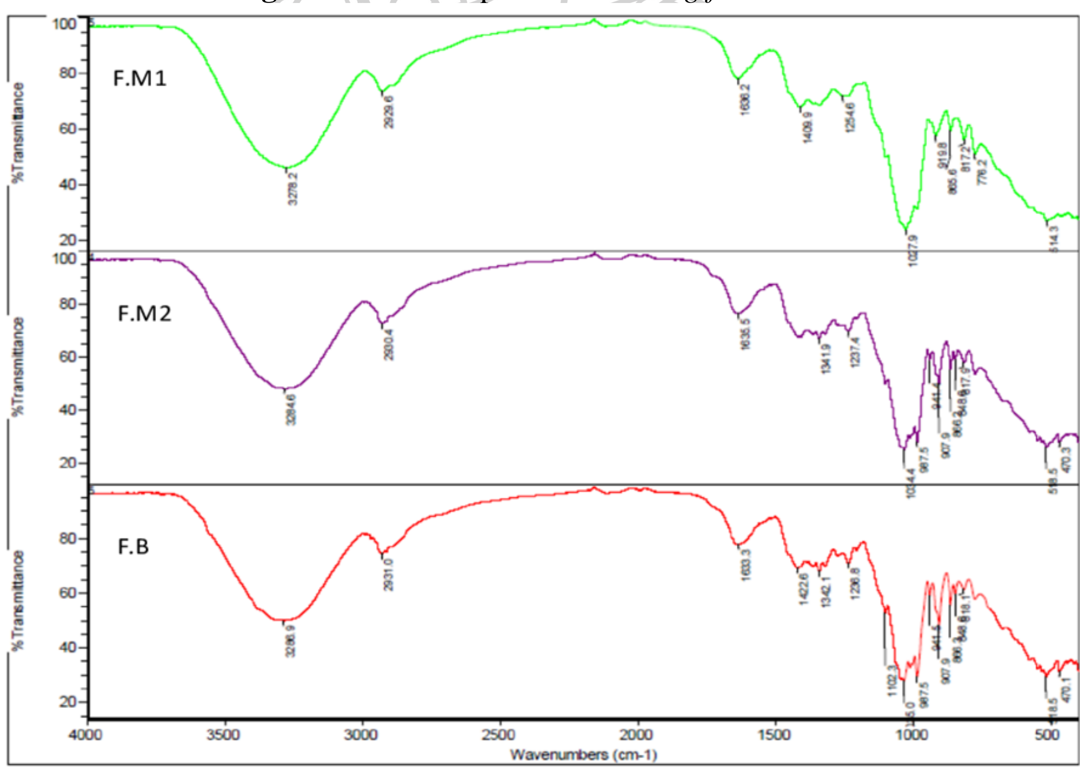

Figure 14 FT-IR spectra of *M. longifolia* flowers

3.2.4.6 Gas Chromatography- Mass Spectroscopy (GC-MS) profiling

3.2.4.6.1 GC-MS fingerprint of *M. longifolia* leaf extracts

The GC-MS chromatograms of the ethanolic extract of *M. longifolia* from three different sources from Mandalay Division, but different area, L1 from Amarapura area, L2 from different place in Amarapura area, L3 from Chanayetharzan area were analysed.

A total of 32 compounds containing terpenes, saturated fatty acid, unsaturated fatty acid and benzofuran were found. Table 16 showed compounds with % peak area that was > 0.04% in the chromatogram of L1, L2 and L3 leaf extract. Four major compounds in the chromatogram of leaf extracts were linolenyl alcohol, phytol, palmitic acid and neophytadiene (the retention time at 50.41, 49.47, 21.65 and 40.74 min, respectively. The percentage of peak area of each compound was different in each extract. The major compounds from L1extracts were found in the order from high to low; linolenyl alcohol, palmitic acid, phytol and neophytadiene. In L2 and L3 extracts, phytol was found as the most common compounds, following by linolenyl alcohol, neophytadiene and palmitic acid. The chromatograms of each extract were very similar. However, L1 extracts may contain all compounds in lower concentration. The GC-MS fingerprints of ethanolic extracts of M. longifolia leaf were shown in Figure 15.

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Table 16 Retention time and % peak area of compounds found in ethanolic leaf extracts of *M. longifolia* from Mandalay Division in Myanmar

Peak	Retention time (min)	% Peak area			Compound name
		L1	L2	L3	
1	24.46	-	-	0.601.12	Unknown
2	29.19	-	6.28 - 8.69	6.28-11.11	Unknown
3	31.89	-	0.971.45	-	diethyl phthalate
4	37.68	-	-	0.41-0.67	Unknown
5	38.21	-	-	0.45-0.54	myristic acid
6	38.55	-	-	1.54-2.58	(-)-loliolide
7	40.74	1.45-2.36	5.78-6.01	6.81-8.05	neophytadiene
8	40.94	-	-	0.95-1.83	Unknown
9	41.58	-	1.53-1.98	1.80-2.39	Unknown
10	42.18	-	1.91-1.99	2.12-2.55	Unknown
11	43.77	-	-	0.47-0.53	methyl palmitate
12	44.70	-	0.70-1.69	0.35-0.88	dibutyl phthalate
13	44.98	21.65-24.40	5.17-9.90	6.11-8.93	palmitic acid
14	49.47	6.10-7.64	12.46-16.95	11.37-13.16	phytol
15	50.04	-	0.91-1.13	0.45-0.66	methyl stearate
16	50.19	-	-	1.57-1.79	linoleic acid
17	50.41	45.56-46.72	10.20-14.90	10.86-11.83	linolenyl alcohol

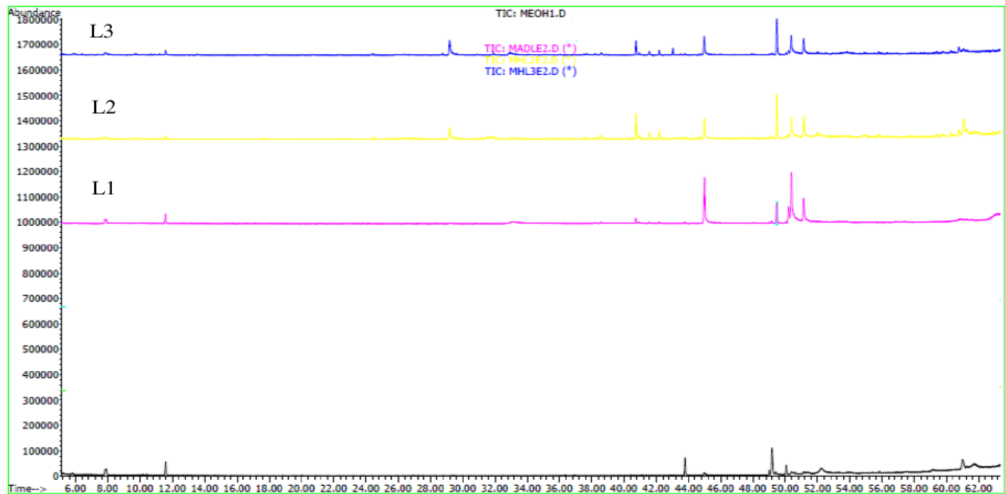

Figure 15 GC-MS fingerprint of ethanolic extracts of *M. longifolia* leaf

3.2.4.6.2 GC-MS fingerprint of *M. longifolia* flower extracts

GC-MS chromatograms of the ethanolic extract of *M. longifolia* flower from two different sources from Mandalay- (FM1 and FM2) and Bagan, FB were analysed. Table 17 showed the compounds found in each extract. All 3 extracts were similar and they were also rich in linolenyl alcohol, palmitic acid and stearic acid. The GC-MS fingerprints of the ethanolic extracts of *M. longifolia* flowers were shown in figure16.

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Table 17 Retention time and % peak area of compounds found in ethanolic flower extracts of *M. longifolia* from Mandalay and Bagan Divisions in Myanmar

Peak	Retention time (min)	% Peak area			Compound name
		FM1	FM2	FB	
1	5.92	-	3.11-3.66	-	Unknown
2	10.88	3.25-3.85	2.04-2.43	-	Unknown
3	13.14	-	0.43-0.67	-	Unknown
4	17.21	-	1.28-2.90	-	5-Hydroxymethylfurfural
5	24.33	1.81-3.25	7.08-7.97	-	Unknown
6	32.91		0.09-0.67		Unknown
7	33.51	-	0.10-1.07		Unknown
8	44.95	0.85-7.28	1.87-3.16	1.77-30.85	palmitic acid
9	50.36	0.85-8.28	0.65-2.49	0.85-8.28	linolenyl alcohol
10	51.11	0.53-1.70	0.69-2.95	1.95-20.20	stearic acid

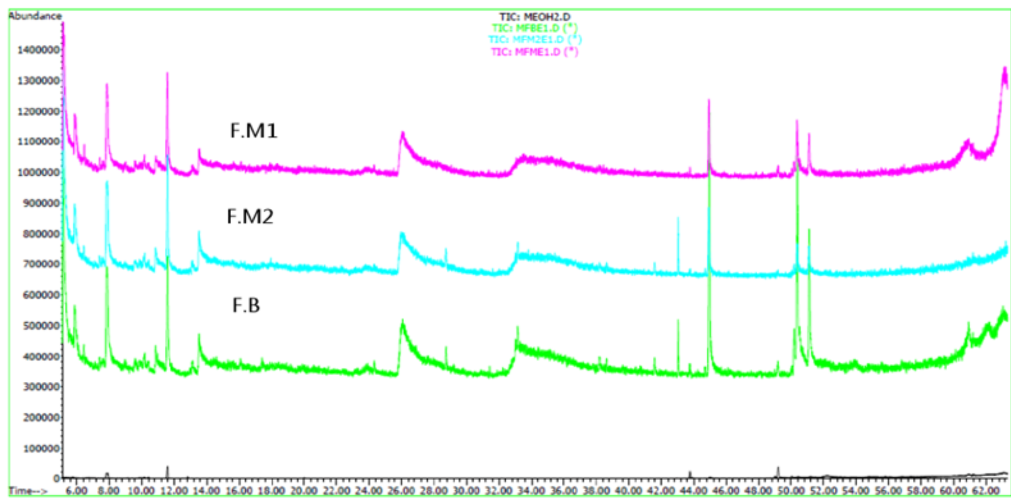


Figure 16 GC-MS fingerprint of ethanolic extracts of *M. longifolia* flower

3.2.4.7 High Performance Liquid Chromatography (HPLC) profiling

3.2.4.7.1 Method development

The optimal condition for simultaneous determination of gallic acid and quercetin was developed. Methanol, a mobile phase (B), gave good separation and reduced the risk of toxic organic solvent. Adding formic acid in the mobile phase (A, 0.1% formic acid in water) could improve the resolution and eliminate peak tailing (225). This method used only 0.3 mL/min flow rate that could decrease the amount of solvent used. Gradient elution was set from 0% (B) to 100% (B) in 50 min and maintained at 100%(B) for 5 min. Then, the mobile phase was brought back to 0% (B) and held for 10 min for column equilibration. The column temperature was kept at 30ºC and the detection wavelength was designed at 270 nm for gallic acid and 360 nm for quercetin based on UV spectrum of each compound. The retention time of gallic acid and quercetin were 4 min and 30.6 min, respectively. Chromatogram of the standards and sample extract were shown in Figure 17(A) and (B).

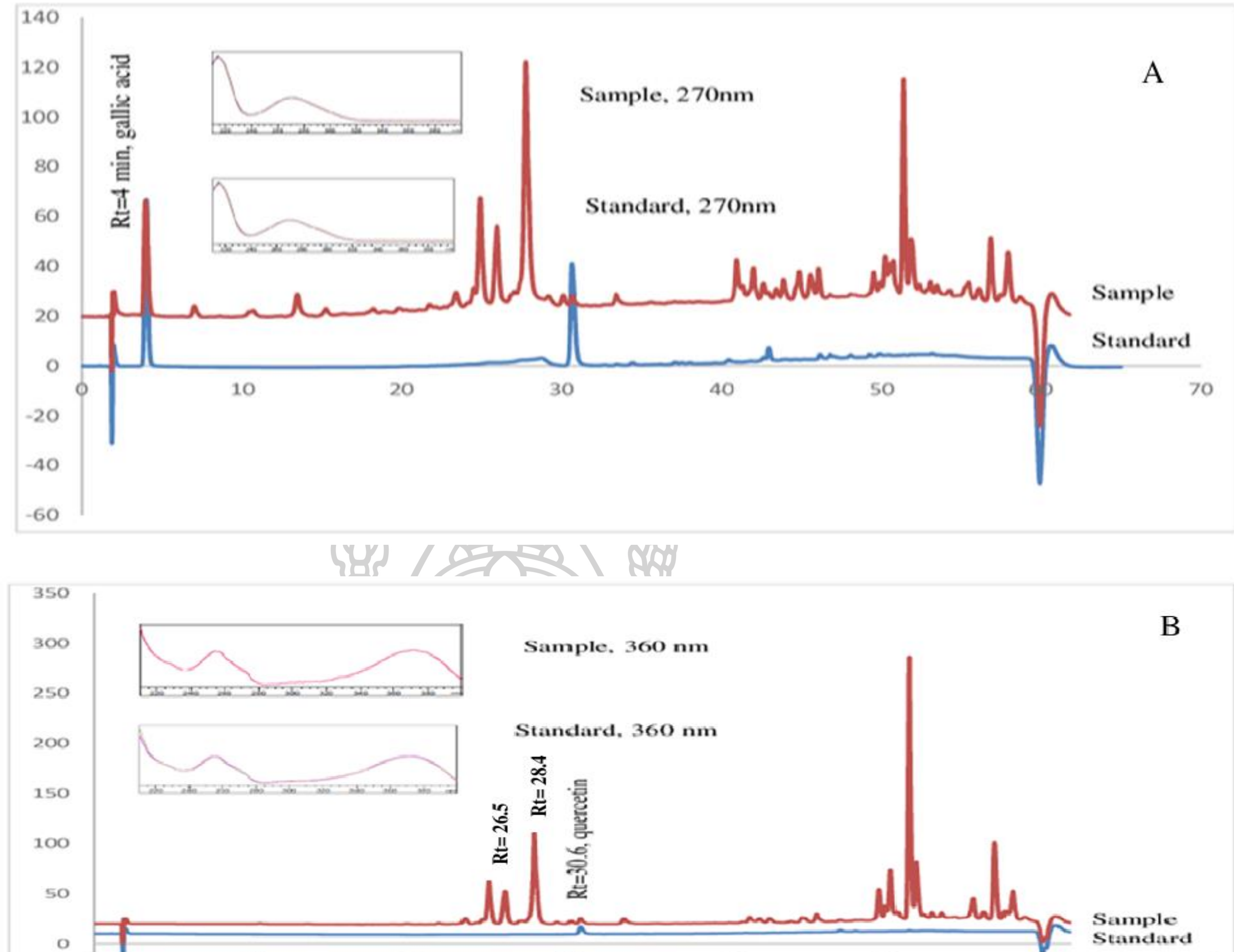


Figure 17 Chromatograms of standard solution and sample extract. (A) standard gallic acid and sample extract at 270 nm; (B) standard quercetin and sample extract at 360 nm

3.2.4.7.2 Method Validation

Table 18 System suitability of gallic acid and quercetin

Compound	Average retention time (min)	Resolution (Rs)	Repeatability of Retention Time (%RSD)	Repeatability of peak area (%RSD)	Conclusion
Accepted criteria		R≥1.5	RSD%<1.0	RSD%<6	Satisfaction
Gallic acid	4.005	3.06	0.04	0.20	Satisfaction
Quercetin	30.667	40.39	0.04	0.69	Satisfaction

3.2.4.7.3 Specificity and System suitability

Specificity was assessed by retention time of the examined compounds on the chromatogram and UV spectrum of the related peaks. UV spectrum of peaks at retention time of 4.0 and 30.7 min from the sample chromatogram corresponded to UV spectrum of gallic acid and quercetin from the standard chromatogram, respectively. The purity of peak values higher than 950 indicated that no interference of other chemicals. This exhibited high specificity of the system for the determination of both compounds. Results from system suitability testing were shown in Table 18. All results were within acceptable range prescribed in (220, 226, 227).

3.2.4.7.4 Linearity and range

The developed method demonstrated linearity within the concentration range of 3.125 to 100 µg/mL for gallic acid and 0.78 to 50 µg/mL for quercetin and the goodness of fit (r^2) was found to be 1 for gallic acid and 0.9999 for quercetin and were shown in Table 19. These results indicated a linear relationship between the concentration of analyte and area under the peak.

3.2.4.7.5 Sensitivity

The LOD value was 0.24 and 0.21 (µg/mL) and LOQ value was 0.73 and 0.63 (µg/mL) for gallic acid and quercetin, respectively and were shown in Table 19.

Table 19 Calibration equations, LOD and LOQ of gallic acid and quercetin

Analyte	Calibration equations	r^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Gallic acid	y=20.019x-3.232	1	3.125-100	0.24	0.73
Quercetin	y=22.141x-6.108	0.9999	0.78-50	0.21	0.63

3.2.4.7.6 Accuracy and Precision

The overall recovery percentages of 103.86 to 104.98% for gallic acid and 100.10 to 102.97% for quercetin were within the acceptance range of 80-110% (220). Intra and inter-day precision results were shown in Table 20. The value of relative standard deviation of intra and inter-day precision were in the range of 0.18 to 1.55 and lower than 2%. According to the result, the developed method was found to be good precise and accurate.

Table 20 Accuracy and intra-day and inter-day precision

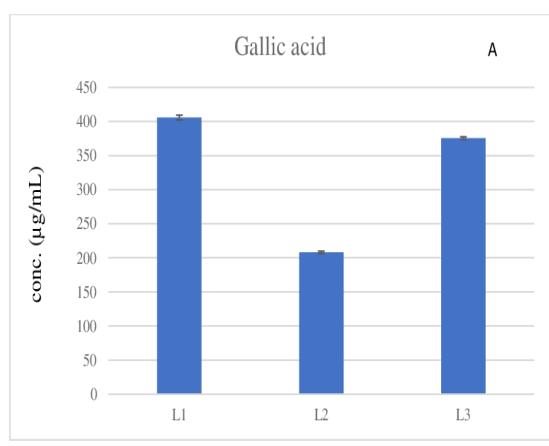
Analyte	Amount added (µg/mL)	Average amount recovery (µg/mL) (Mean±SD)	%Recovery (Mean ± SD)	Intra-day precision (%RSD)	Inter-day precision (%RSD)
	40	41.84±0.16	104.59±0.41	0.39	0.37
Gallic acid	50	51.93±0.27	103.86±0.55	0.53	0.67
	60	62.99±0.12	104.98±0.19	0.18	0.48
	5	5.00±0.07	100.10±1.31	1.32	1.76
Quercetin	6.25	6.43±0.03	102.97±0.41	0.40	1.28
	7.5	7.71±0.11	102.80±1.59	1.55	1.18

3.2.4.8 Content of gallic acid and quercetin in the aqueous extract

The amounts of active compounds in the extracts were expressed as mg/100g of extract weight and shown in Table 21 and Figure 18. The range of concentration of active compounds in boiling water extracts were 207.95 to 405.79 mg/100 g for gallic acid and 7.31 to 20.56 mg/100 g for quercetin. Although in *M. longifolia*, quercetin was not found in high amount, a group of high response peak around the retention time of 24-28 min showed the pattern of UV spectrum similar to that of quercetin. The results showed that different sources gave different quality of leaf extracts. The amounts of both compounds from 3 different areas showed significant difference at 95% confidence interval among one another in ANOVA two-factor replication result (Appendix). Leaf from the Amarapura area, L1, possessed the highest quantity of both bioactive compounds.

Table 21 Content of gallic acid and quercetin in the aqueous extracts of *M. longifolia* leaf

Sample	Aqueous extract (mg/100 g) (mean ± SD)	
.	Gallic acid	Quercetin
L1	405.79±3.50	20.56±0.41
L2	207.95±1.67	9.86± 0.06
L3	375.78±1.68	7.31±0.07



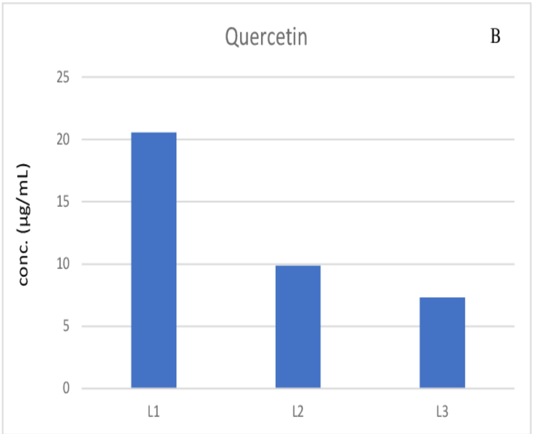


Figure 18 Content of active compounds in aqueous extracts of *M. longifolia* leaf. (A) Gallic acid; (B) Quercetin

3.2.4.9 LC-MS profiling

Table 22 showed the identified compounds in aqueous extract of *M. longifolia* leaf by LC-MS. High peak at retention time 25.5 min showed *m/z* values at 271, 316, 463 amu which expected to be dihydroisorhamnetin-3-*o*-L-rhamnoside. Peak at retention time 26.5 min showed m/z value at 179, 301, 463 amu and could be further identified as quercetin hexoside. Peak at retention time 28.4 min showed m/z value at 447 amu and expected to be quercetin rhamnoside. Quercetin was eluted at 31.3 min and showed the 179, 301amu. The m/z values in the present study complied with other studies (228-231). Previous researches reported flavonoids (quercetin) in *M. longifolia* leaf extracts (26, 54, 57, 232). Alpha-glucosidase inhibitory activities might be due to quercetin and quercetin related compounds in the extract found in LC-MS study (209, 233).

Table 22 m/z of compounds with similar UV spectrum

No	Retention time(min)	*m/z* (amu)	Expected compound
1	25.5	463, 316, 271	dihydroisorhamnetin-3-*o*-L-rhamnoside
2	26.5	463, 301, 179	Quercetin hexoside
3	28.4	447, 301, 255	Quercetin rhamnoside
4	31.3	301, 179	Quercetin

3.2.5 Principal Component Analysis (PCA)

3.2.5.1 PCA study on *M. longifolia* leaf

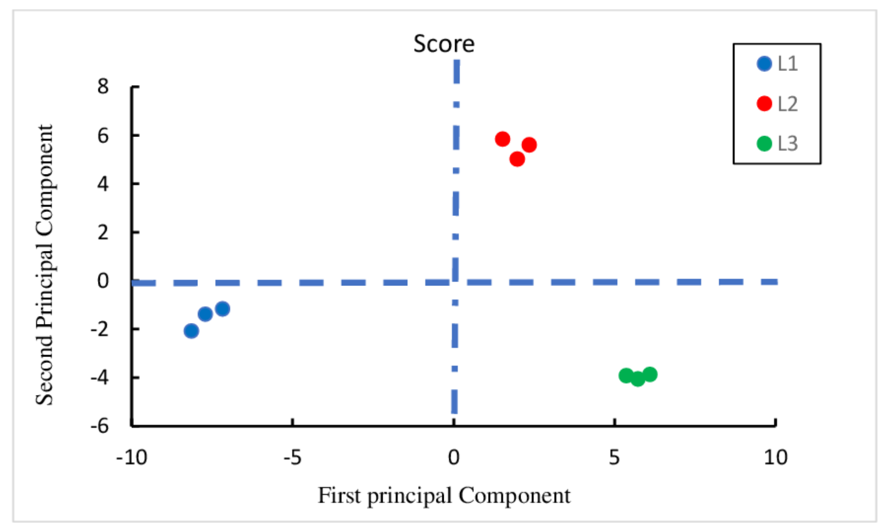


Figure 19 Score plot of *M. longifolia* leaf from different sources

Generally, PCA interpretation was displayed as a two-dimensional graph where the principal axis represented the directions of the first two main principal components (First principal component, PC1 vs Second principal component, PC2): 1) scores plot and 2) loadings plot. PC1 in the scores plot of Figure 19 presented differentiation among 3 groups. Dominant compounds in leaf sample from Amarapura area (L2) and leaf sample from Chanayetharzan area (L3) were strongly positively correlated with PC1 and dominant compounds in leaf from Amarapura area (L1) were negatively correlated with PC1. This score plot could differentiate leaf sample from 3 different sources.

Loading bar plot in Figure 20 illustrated that 15 compounds and 2 of heavy metal content were dominant in leaf from Amarapura area (L1) with loading (PC1<-0.05, PC2<0.00). In loading plot, 3 compounds were dominant in GC-MS result at retention time 44.98 (r18, palmitic acid), 50.41(r22, linolenyl alcohol) 51.14 (r23, stearic acid), 12 compounds including quercetin (t26) and other 11 unknown compounds (t2, t8, t9, t11, t13, t14, t28, t30, t31, t32, t34) were dominant in HPLC result and content of heavy metal Nickel (Ni) and Cadmium (Cd) were dominant. In leaf sample from Amarapura area (L2), 12 compounds were dominant with loading

(PC1>0.00, PC2>0.08). In loading plot, 8 compounds were dominant in GC-MS result at retention time 28.75 (r5, unknown), 31.89 (r7, Diethyl phthalate), 43.04(r15, unknown), 44.7(r17, Dibutyl phthalate), 49.47 (r19, phytol), 50.04 (r20, Methyl stearate), 59.76(r25, unknown), 61.22(r29, unknown) and 4 compounds (t12, t16, t19, t24) were dominant in HPLC result. In leaf sample from Chanayetharzan area (L3), 12 compounds and 2 heavy metals were dominant with loading (PC1>0.07, PC2<-0.02). In loading plot, 8 compounds were dominant in GC-MS result at retention time 24.46 (r3, unknown), 26.4 (r4, unknown), 37.68(r 8, unknown), 38.55(r10, (-)-Loliolide), 40.94(r12, unknown), 60.23(r26, unknown), 61.75(r30, unknown), 62.86(r31, unknown), 4 compounds (t22, t23, t25, t27) were dominant in HPLC result and content of heavy metal Arsenic (As), Lead (Pb) and Barium (Ba) were dominant. The result was consistent with the score plot.

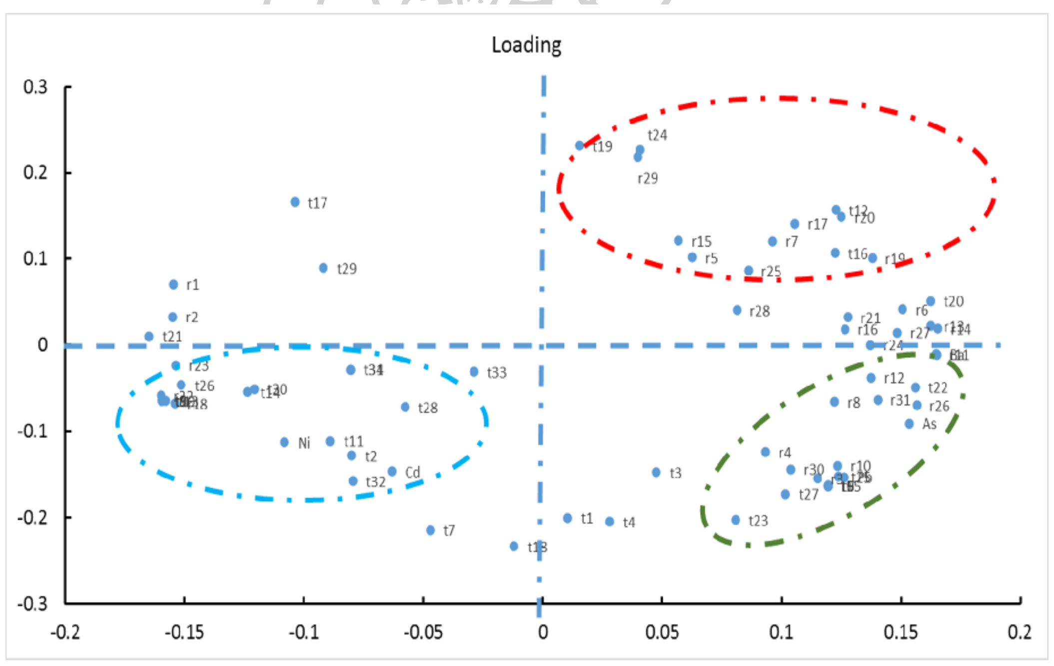


Figure 20 Loading plot of *M. longifolia* leaf from different sources

3.2.5.2 PCA study on *M. longifolia* flower

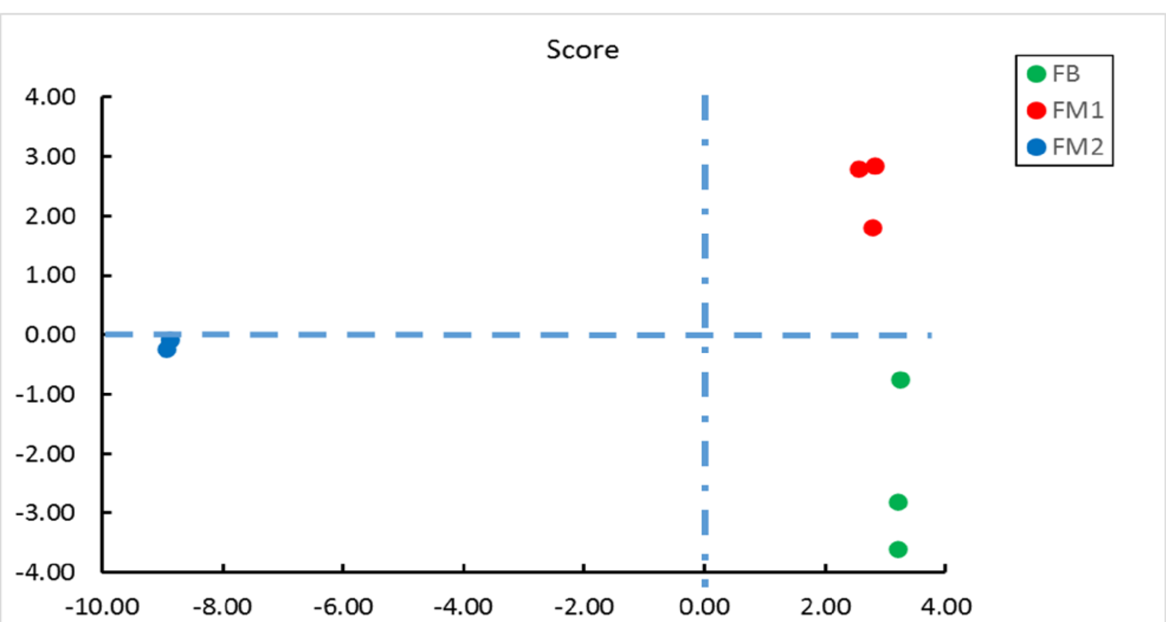


Figure 21 Score plot of *M. longifolia* flower from different sources

PC1 in the scores plot of Figure 21 presented differentiation among 3 groups. Dominant compounds in flower sample from Amarapura area (FM1) and flower sample from Bagan (FB) were strongly positively correlated with PC1 and dominant compounds in flower sample from Amarapura area (FM2) were strongly negatively correlated with PC1. This score plot could differentiate flower sample from different source.

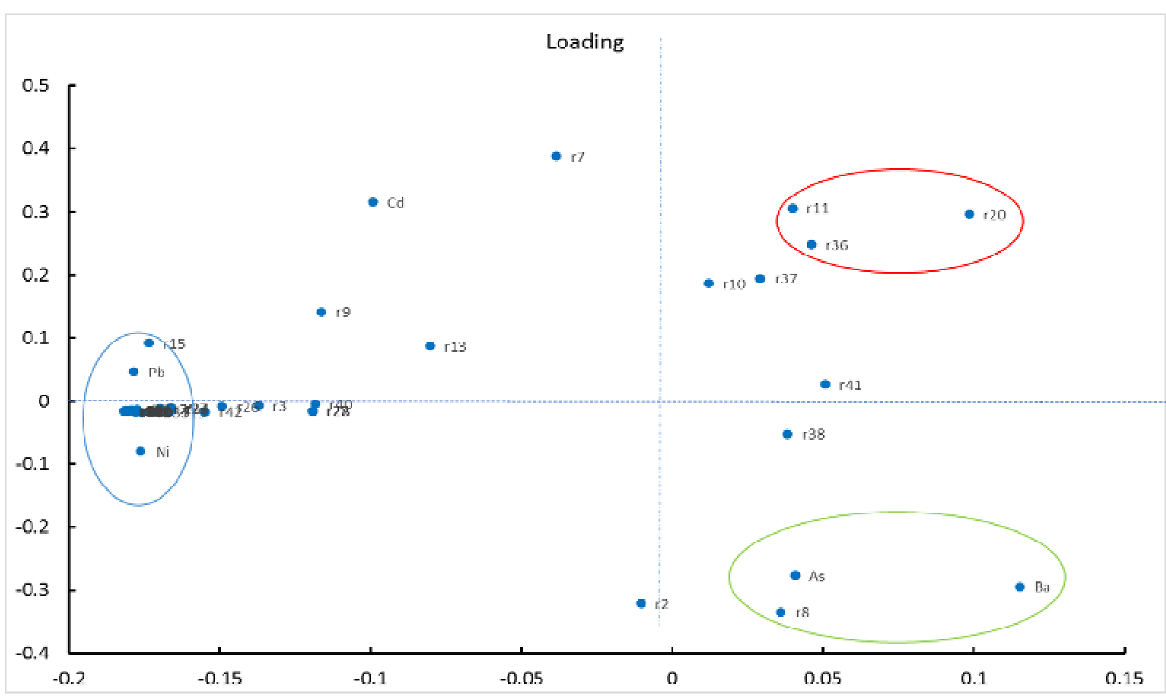


Figure 22 Loading plot of *M. longifolia* flower from different sources

Loading bar plot in Figure 22 illustrate that 3 compounds were dominant in flower sample from Amarapura area (FM1) with loading (PC1>0.02, PC2<0.20). In loading plot, 3 compounds were dominant in GC-MS result at retention time 14.11 (r11, unknown), 27.21(r20, unknown) and 33.32 (r36, unknown). In flower sample from Amarapura area (FM2), 17 compounds and 2 heavy metals were dominant with loading (PC1<-0.17, PC2<0.1). In loading plot, 17 compounds were dominant in GC-MS result at retention time 5.92 (r1, unknown), 7.86 (r2, decane), 17.41 (r 14, unknown), 24.33 (r15, unknown), 26.43 (r16, unknown), 26.5 (r17, unknown), 26.89 (r18, unknown), 27.79 (r25, unknown), 28.14 (r29, unknown), 30.09 (r30, unknown), 30.60 (r31, unknown), 31.46 (r32, unknown), 31.90 (r33, unknown), 32.02 (r34, unknown), 33.18 (r35, unknown), 33.87 (r39, unknown), 38.22 (r43, unknown) and content of heavy metal Lead (Pb) and Nickel (Ni) were dominant. In flower sample from Bagan area (FB), 1 compound and 2 heavy metals were dominant with loading (PC1>0.02, PC2>-0.2). In loading plot, 1 compound were dominant in GC-MS result at retention time 11.58 (r8, undecane) and heavy metal Arsenic (As) and Barium (Ba) were dominant. The result was consistent with the score plot.

3.3 Conclusion

Boiling water extracts of *M. longifolia* leaf from Mandalay (L1, L2, L3) possesed higher alpha-glucosidase inhibitory activity than flower extracts. This might be due to the presence of high total phenolic content i.e., phenolic compounds in the form of flavonoids, phenols, terpenoids, tannins, quercetin and various forms of quercetin related compounds in the extracts. Water and ethanolic extracts of leaf also possessed higher activity when compared to acarbose. Consequently, *M. longifolia* leaf and flower would be potential sources of compounds for the control of *Diabetes mellitus.*

In the Quality determination of *M. longifolia*, % loss on drying of leaf and flower powder were not more than 10%. Moreover, heavy metals such as As, Cd, Pb, Ni and Ba contents in all samples were within acceptable and safe limit prescribed in ASEAN guidelines, WHO recommendation and ICH guideline Q3D. Furthermore, leaf and flower powder were free from the contamination of pathogenic microorganism such as *E. coli*, *Salmonella* spp., *S. aureus*, *Clostridium* spp., including *C. tetani*, *C. botulinum* and *C. perfringers*. Therefore, *M. longifolia* leaf and flower powder can be used safely as herbal materials or as raw materials for herbal medicines.

ATR-FT-IR spectroscopy enabled short analysis time, simple, not complicated sample preparation and non-destructive method. Moreover, this technique could be valuable for spectral acquirement of *M. longifolia* leaves and flowers. This ATR-FT-IR fingerprint pattern could be useful for the identification and authentication of *M. longifolia* leaf and flower powders. Therefore, ATR-FT-IR technique can be beneficial in the quality control of herbal materials.

GC-MS analysis of ethanolic extracts of *M. longifolia* leaf showed a total of 32 compounds containing terpenes, saturated fatty acid, unsaturated fatty acids and benzofuran. Four compounds, including neophytadiene, palmitic acid, phytol and linolenyl alcohol were found in common in samples from three different sources from Mandalay area (L1 from Amarapura area, L2 from different place in Amarapura area, L3 from Chanayetharzan area). Ethanolic extracts of flower exhibited a total of 18 compounds comprising furan and saturated fatty acids. Three compounds such as palmitic acid, 9,12,15-octadecatrien-1-ol, (Z, Z, Z) and stearic acid were found in

common in three samples from Mandalay and Bagan area (FM1 from Amarapura area, FM2 from different place in Amarapura area, FB from Bagan area). GC-MS fingerprint can be used for the identification and authentication of *M. longifolia* leaves and flowers from different sources.

The obtained **HPLC** method for the simultaneously determination of gallic acid and quercetin in aqueous leaf extract of *M. longifolia* was simple, precise, accurate, reproducible, and highly sensitive. In this study, DAD detector was suitable for the determination of compounds with different UV maxima. The chromatogram could be used as a chemical fingerprint of this aqueous extract. This newly developed HPLC method will be helpful for the quantification of active constituents and quality assessment of *M. longifolia* leaf in the future. LC-MS study demonstrated quercetin and various forms of quercetin related compounds in the extract.

Heavy metal contamination might be due to variability of exposure to environmental pollution, geographical region and composition of soil. The attention should be paid during both collection and equipment used in analysis. Moreover, the GC-MS fingerprints combining with chemometric analysis were preferred approaches for the authentication and characterization of *M. longifolia* leaves and flowers from various sources. Additionally, with further studies of the chemical constituents from other chromatographic and spectroscopic analyses, a complete metabolomic fingerprint for *M. longifolia* leaves and flowers from Myanmar will be established. PCA study on ICP-MS, GC-MS and HPLC data could differentiate different sources of leaves and flowers from Mandalay and Bagan townships.

CHAPTER IV
STUDY OF *MORUS ALBA* L.

4.1 Materials and Methods

4.1.1 Chemicals

Name	Source
Acarbose	Sigma Aldrich
Acetic acid	Merck, Germany
Alpha-glucosidase enzyme	Sigma Aldrich
Ammonia	Merck, Germany
Caffeic acid	Sigma Aldrich
Chloroform	Merck, Germany
Chlorogenic acid	Sisco Research Laborites, India
Columbia CAN Agar	BBL ™, USA
Copper sulphate	Sigma Aldrich
Ethanol, absolute	Merck, Germany
Ethanol (70%)	Merck, Germany
Ferric chloride	Sigma Aldrich
Folin Ciocalteu's reagent	Sisco Research Laborites, India
Gallic acid	Sisco Research Laborites, India
Iodine	Sigma Aldrich
Mac Conkey Agar	Difco ™, USA
Mac Conkey Broth	Difco ™, USA
Mannitol Salt Agar	BBL ™, USA
Methanol	HPLC grade, Merck, Germany
Monobasic potassium phosphate	Sigma Aldrich
Multi-element Calibration Standard 2A, (Agilent)	Agilent, USA
Nitric acid (65%)	Merck, Germany
Paraffin	PC Drug company, Thailand

Name	Source
p-nitro phenyl-α-D-glucopyranoside (PNPG)	Sigma Aldrich
Potassium dihydrogen phosphate	Sigma Aldrich
Potassium hydroxide (pellets)	Sigma Aldrich
Quercetin	Sigma Aldrich
Rappaport Vassiliadis Broth	Difco ™, USA
Reinforced Clostridial Medium	Difco ™, USA
Rutin	ACROS, organics
Sodium carbonate	Sigma Aldrich
Sodium hydroxide (pellets)	Sigma Aldrich
Soybean-Casein Digest Medium	Bacto ™, USA
Sulphuric acid (concentrated)	Merck, Germany
Xylose-Lysine-Deoxycholate (XLD) Agar Medium	Difco ™, USA
Xylose-Lysine-Deoxycholate (XLD) Agar	Difco ™, USA

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4.1.2 Instruments

Name	Source
Analytical balance	Sartorius, Germany
Autoclave, HIRAYAMA, HICLAVE, HVA 85	HIRAYAMA, Japan
Centrifuge, Microfuge 16 centrifuge, Backman coulter	Backman coulter, USA
Column (HP-5MS, 0.25 mm x 30m x 0.25µm) (Agilent)	Agilent, USA
Fourier Transformed Infrared spectrophotometer (FT-IR 4700) (iD7 ATR) (Nicolet iS5)	Thermo Scientific Nicolet, USA
Gas Chromatography-Mass Spectroscopy (GC-MS) (Agilent 6890N) 5973 mass selective detector	Agilent, USA
High Performance Liquid Chromatography (HPLC) (Agilent 1260)	Agilent, USA
Hot air oven, Heraeus	Heraeus, France
Incubator, Heraeus	Heraeus, France
Inductively Coupled plasma Mass Spectroscopy (ICP-MS) (Agilent 7500ce)	Agilent, USA
Liquid Chromatography Mass Spectroscopy (LC-MS) (Agilent 1100series, G2445D)	Agilent, USA
Microplate reader (TECAN Infinite F 50)	Switzerland
Microwave digestion Instrument, Multi-wave 3000, ETHOS One MILESTONE	Austria
PH meter, Mettler toledo	Mettler Toledo, Germany

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Name	Source
Rotary evaporator	BUCHI, Switzerland
BUCHI Heating Bath B 100	
BUCHI Rotavapor R-100	
Sonnicator, Elma, Model 890/H	Elma, Germany
Ultrasonic bath	CREST ultrasonics, USA
Ultraviolent Visible spectrophotometer (UV-Vis) (Agilent)	Agilent, USA
Vortex mixer	Vision Scientific, Korea

4.1.3 Plant Material

4.1.3.1 Plant samples

There were 2 groups of *Morus alba* L. (mulberry) leaves in this study; sample leaves and reference leaves. There were 5 sample leaves, collecting from Taunggyi region in Myanmar (M) (Eastern of Myanmar) and from Bangkok (T1), Nonthaburi (T2) Nakhon Pathom (T3) province in Thailand. The upper top leaf from Bangkok (UL-T1) were also specifically collected. The second group were reference leaves which consisted of 2 cultivars; Buriram 60 (BR) and Khun Pai (KP). Both cultivars were collected from Queen Sirikit Department of Sericulture in Nakhon Ratchasima (NR), Kanjanaburi (KB) and Buriram (BR) province in Thailand. All of samples were dried at 50˚C in oven and ground into powder.

4.1.3.2 Tea products

Fourteen mulberry tea products were purchased from various markets in Thailand.

4.1.4 Extraction (Maceration with water, boiling water and absolute ethanol)

Both reference leaves and sample leaves of *M. alba* were extracted with water, boiling water and absolute ethanol according to methods used for *M. longifolia* leaves and flowers. *M. alba* tea powder was extracted with boiling water. Water and boiling water extracts were freeze dried for further studies.

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4.1.5 Evaluation of alpha-glucosidase inhibitory activity

All extracts from 4.1.4 were tested for alpha-glucosidase inhibitory activity according to methods used for *M. longifolia* leaf and flower extracts.

4.1.6 Preliminary phytochemical screening

Boiling water, water and absolute ethanolic extracts of *M. alba* leaf from 4.1.3.1 were tested for preliminary phytochemical screening according to methods used for *M. longifolia* leaf and flower extract.

4.1.7 Quality determination of *M. alba* L.

4.1.7.1 Determination of loss on drying

M. alba leaves and tea powder from 4.1.3.1 and 4.1.3.2, respectively, were determined for loss on drying according to methods used for *M. longifolia* leaf and flower powder.

4.1.7.2 Determination of heavy metal content by ICP-MS

M. alba leaves and tea powder from 4.1.3.1 and 4.1.3.2 respectively, were determined for heavy metal content according to methods used for *M. longifolia* leaf and flower powder.

4.1.7.3 Determination of total phenolic contents

Boiling water, water and absolute ethanolic extracts of *M. alba* leaf from 4.1.3 and boiling water extract of *M. alba* tea from 4.1.4 were determined for total phenolic contents according to methods used for *M. longifolia* leaf and flower extracts.

4.1.7.4 Determination of microbial contamination

M. alba leaves and tea powder were determined microbial contamination according to methods used for *M. longifolia* leaf and flower powder. Note. The amount of some tea products was not enough for the test, so 6 tea products were tested.

4.1.7.5 Fourier Transformed-Infrared spectroscopy (FT-IR) analysis

M. alba leaves and tea powder from 4.1.3.1 and 4.1.3.2, respectively, were examined for FT-IR analysis according to methods used for *M. longifolia* leaf and flower powder.

4.1.7.6 Gas Chromatography- Mass Spectroscopy (GC-MS) profiling

Absolute ethanolic extract of *M. alba* leaf and tea were carried out in GC-MS profiling according to methods used for *M. longifolia* leaf and flower extracts.

4.1.7.7 High Performance Liquid Chromatography (HPLC) profiling

M. alba leaves and tea powder from 4.1.3.1 and 4.1.3.2, respectively, were extracted and determined for the content of chlorogenic acid, caffeic acid, rutin, and quercetin.

4.1.7.7.1 Preparation of the Extract

Each of 0.5 g of sample was sonicated with 8 mL of boiling water for 1 hour. Then, the extract was made up to 10 mL volume with boiling water, centrifuged at 4000 rpm about 30 min, and filtered under vacuum.

4.1.7.7.2 Instrumentation and chromatographic condition

All analyses were operated on an Agilent 1260 HPLC system (Agilent, USA) equipped with a photodiode array detector. The analytical method prescribed in reference (83) was modified. The analytical column used was Agilent (Poroshell 120) extended with C18 (150 mm x 4.6 mm x 2.7µm). The ratio of mobile phase, flow rate and column temperature were explored. The mobile phase contained the mixture of solvent A (0.1% formic acid in water) and solvent B (methanol). Solvents used were filtered through a 0.22 µm filter and degassed. The sample injection volume was 2 µL. Chlorogenic acid and caffeic acid were detected at the wavelength of 320 nm while rutin and quercetin were detected at the wavelength of 360 nm.

4.1.7.7.3 Preparation of standard solutions

Standard chlorogenic acid, caffeic acid, rutin and quercetin were weighed and dissolved in 80% ethanol to obtain a stock solution of 1 mg/mL. Working solutions were serially diluted from stock solution to 5 concentrations of standard solutions in the range of 100-525 µg/mL for chlorogenic acid, 1.2-15 µg/mL for caffeic acid, 15-100 µg/mL for rutin and 5-20 µg/mL for quercetin.

4.1.7.7.4 Method validation

The developed HPLC method for the quantification of chlorogenic acid, caffeic acid, rutin and quercetin in aqueous extract of *M. alba* was validated for specificity, system suitability, linearity, sensitivity, precision and accuracy, according to ICH and AOAC guidelines (219, 220).

4.1.7.7.4.1 Specificity and system suitability

Specificity was assessed using standard compounds and DAD detector. Peak identification was carried out based on retention time and UV spectrum of those of standards. To evaluate system suitability of the method, repeatability of retention time and peak area of five replicate injections of 262.5 µg/mL of chlorogenic acid, 5 µg/mL of caffeic acid, 25 µg/mL of rutin and 10 µg/mL of quercetin standard solutions were used. The %RSD values were calculated in each case.

4.1.7.7.4.2 Linearity and range

Linearity was established across 5 different concentrations: 100-525 µg/mL for chlorogenic acid, 1.2 -15 µg/mL for caffeic acid, 15-100 µg/mL for rutin and 5-20 µg/mL for quercetin. The regression equations and correlation coefficients were reported.

4.1.7.7.4.3 Sensitivity

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curves of chlorogenic acid, caffeic acid, rutin and quercetin. The LOD was calculated according to the expression 3.3*SD/S. While SD is the standard deviation of the response and S is the slope of the calibration curve. The LOQ was calculated using the expression 10*SD/S (219).

4.1.7.7.4.4 Accuracy and precision

The accuracy was assessed by means of recovery assays using standard addition method to the diluted sample solution. Prior to fortification, the diluted sample solutions were determined for background levels of chlorogenic acid, caffeic acid, rutin and quercetin. Standards were spiked at three different levels, 160, 200 and 240 µg/mL for chlorogenic acid, 3, 3.75 and 4.5 µg/mL for caffeic acid, 32, 40 and 52.80 µg/mL for rutin and 8, 10 and 12 µg/mL for quercetin. Each solution was injected in triplicate within 2 sequential days. Unspiked diluted samples were analyzed to calculate actual recovery. Accuracy was revealed as the recovery percentage across the range of the assay. Intra- and inter-day precisions were shown as the percentage of relative standard deviation (%RSD) within 1 day and 2 sequential days, respectively.

4.1.7.7.5 Content of chlorogenic acid, caffeic acid, rutin and quercetin in the aqueous extracts

About 1 ml of the extract was taken and filter with 0.22 µm syringe filter before HPLC injection. The developed HPLC method in the present study was used to quantify the amounts of chlorogenic acid, caffeic acid, rutin and quercetin in the aqueous extracts of *M. alba* leaf. The amount was expressed as mg/100 g dried extract.

4.1.7.8 LC-MS Analysis

The extract used in HPLC analysis was also analyzed by LC-MS in which high peak area around 20 to 40 min from LC-DAD was identify using an Agilent 1100 series LC coupled to mass spectrometer (Agilent, G2445D).

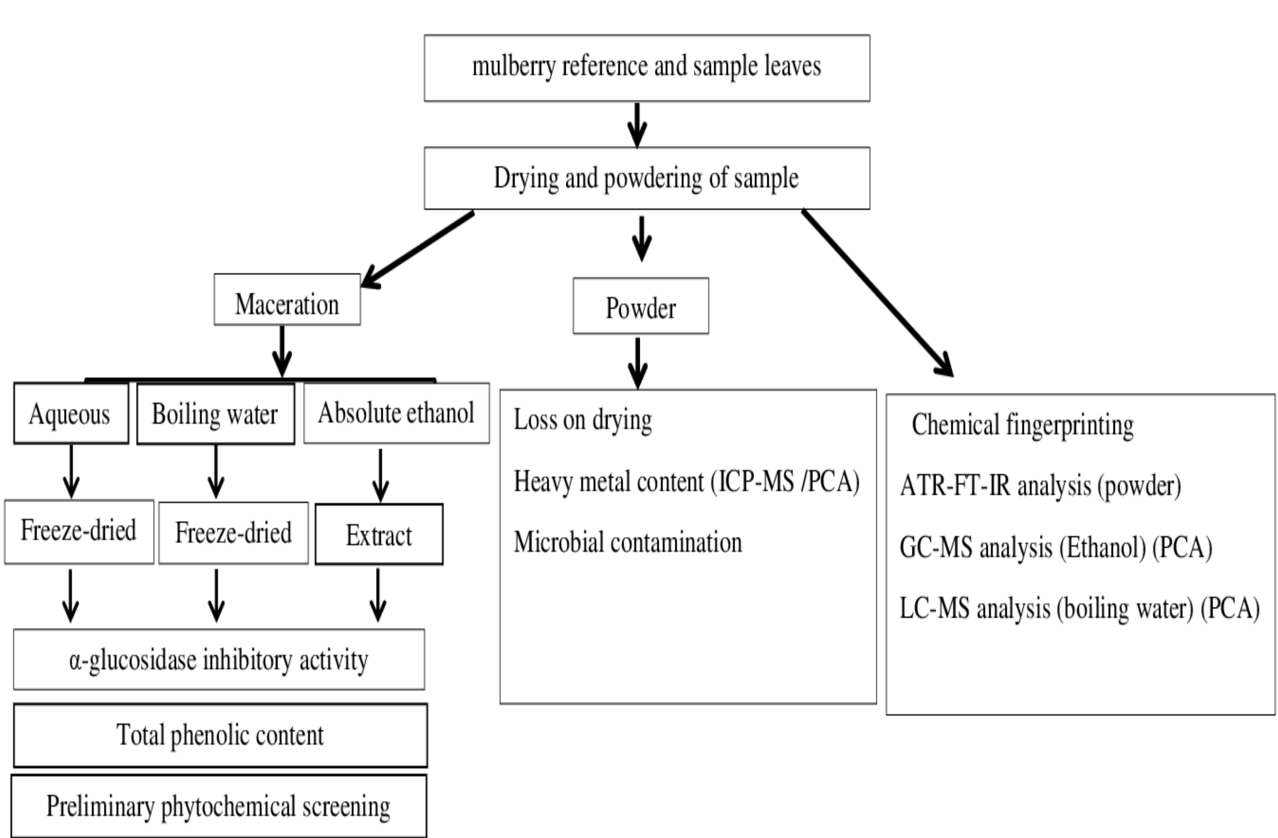
4.1.8 Statistical Analysis

All values of experimental measurements will be expressed as mean ± SD. One-way analysis of variance (ANOVA) and two-factor with replication was used by excel for the measurement of the statistical significance. The statistical significance level $p<0.05$ was used.

4.1.9 Principal component analysis (PCA)

To detect clustering of samples, principal component analysis (PCA) was used. To present different manner for interpretation and to reduce data, PCA is very useful (221). Data obtained from GC-MS, ICP-MS and HPLC were analyzed by PCA to differentiate about the cultivar and sources of reference leaves using WEKA version 3.8.5 open-source software, Windows version 10.0 (available from: https://www.cs.waikato.ac.nz/ml/weka/). Pearson correlation (with Minitab Trial version) was used to evaluate linear relation between each pair of metal contents.

Flow chart



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Flow chart

mulberry tea products

Maceration

Boiling water

Freeze-dried

α-glucosidase inhibitory activity

Total phenolic content

Powder

Loss on drying

Heavy metal content (ICP-MS /PCA)

Microbial contamination

Chemical fingerprinting

GC-MS analysis (Ethanol) (PCA)

LC-MS analysis (boiling water) (PCA)

4.2 Result and Discussion

4.2.1 Extraction (% yield of different extracts)

The % yield of aqueous, boiling water and absolute ethanolic *M. alba* L. leaf and tea extracts were shown in Table 23 and Figures 23-25. Among reference leaves, % yield value of different extracts were in the range of 13.14-24.56 for aqueous extracts, 15.10-23.79 for boiling water extracts, and 2.98-5.14 for ethanol extracts. Aqueous extract of BR-BR showed the highest % yield. Among sample leaves, % yield values were in the range of 14.72-19.16 for aqueous extracts, 19.36-25.60 for boiling water extracts, and 4.07-8.77 for ethanol extracts. Boiling water extract of sample M from Myanmar, showed the highest % yield (25.60). In boiling water extract of tea products, % yield values were in the range of 9.02 - 23.46. Extract of Tea 1 possessed the highest % yield (23.46) while that of Tea 13 was the lowest.

Boiling water extracts gave the highest % yield in reference leaves and sample leaves except BR-BR and KP-NR, while ethanolic extracts gave the lowest % yield value. Aqueous and boiling water extracts of reference leaves from Buriram (BR-BR and KP-BR) gave higher % yield than other provinces. The result from two-factor with replication of ANOVA at 95% confidence interval (Appendix), from 3 extraction solvents of reference leaves, % yield of the sample from different provinces presented significant difference, regardless of the cultivars. Considering boiling water extracts of reference leaves from different source, the highest % yield was from Buriram following by Nakorn Ratchasima and Kanjanaburi province, respectively. This implied that leaves from different source have different matrix that could be extracted when maceration in hot water.

Table 23 % yield of *M. alba* leaf and tea products extracts

Sample	Aqueous extract (mean ± SD)	Boiling water extract (mean ± SD)	Absolute ethanolic extract (mean ± SD)
Reference leaf			
BR-KB	13.14±1.14	15.10±1.05	4.31±0.05
BR-NR	19.89±2.25	19.83±1.01	5.14±0.27
BR-BR	24.56±6.61	21.67±2.38	3.61±0.07
KP-KB	13.33±2.09	17.40±0.45	3.88±0.20
KP-NR	17.07±1.45	16.92±2.13	4.21±0.10
KP-BR	18.98±0.66	23.79±1.58	2.98±0.11
Sample leaf			
M	15.52±0.26	25.60±1.43	8.77±0.48
T1	17.15±0.52	21.96±1.39	6.90±0.16
T2	14.72±0.36	21.18±0.90	6.55±0.05
T3	17.89±0.69	21.15±0.63	7.40±0.08
UL-T1	19.16±0.52	19.36±1.04	4.07±0.06
Tea products			
Tea 1		23.46±0.91	
Tea 2		15.80±0.48	
Tea 3		22.32±0.49	
Tea 4		16.16±0.22	
Tea 5		18.38±1.02	
Tea 6		21.84±0.67	
Tea 7		13.16±0.45	
Tea 8		16.79±1.77	
Tea 9		12.29±0.89	
Tea10		14.62±0.34	
Tea 11		22.03±1.91	
Tea 12		18.88±1.25	
Tea 13		9.02±1.23	
Tea 14		12.16±0.06	

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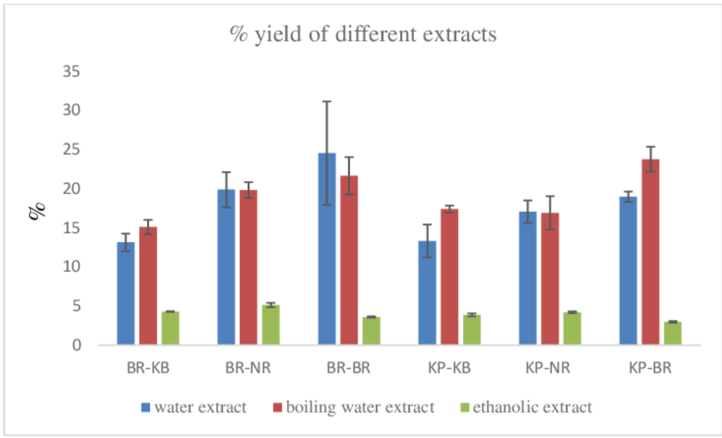

Figure 23 % yield of different reference leaf extracts of *M. alba*

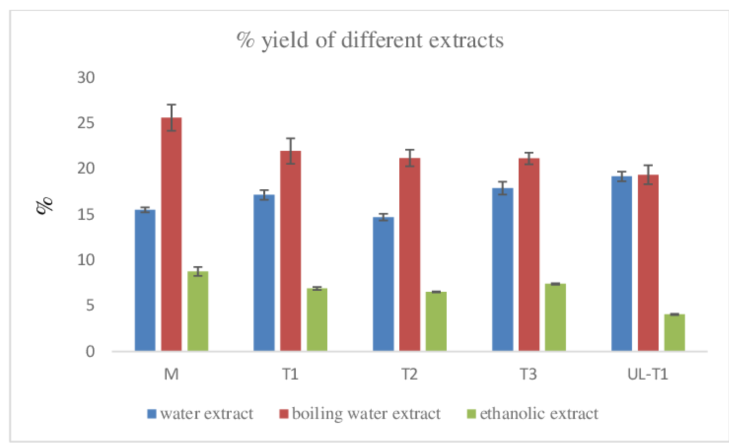

Figure 24 % yield of different sample leaf extracts of *M. alba*

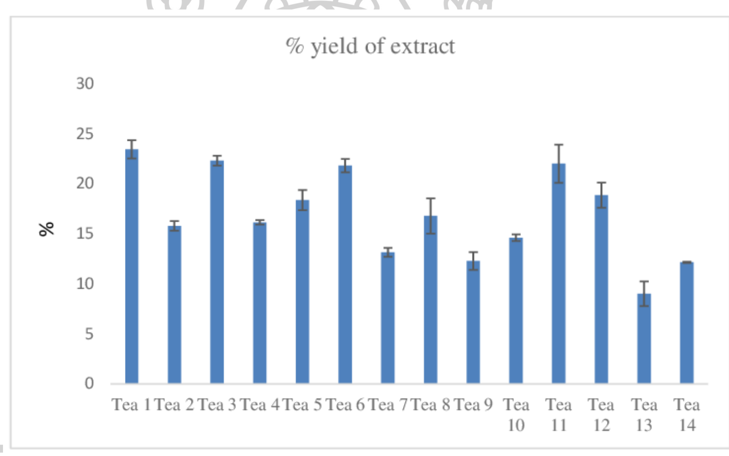

Figure 25 % yield of boiling water extract of *M. alba* tea products

4.2.2 Evaluation of alpha-glucosidase inhibitory activity of *M. alba* leaf and tea product extracts

The alpha-glucosidase inhibitory activities of *M. alba* leaf and tea product extracts were expressed as IC_{50} value and were shown in Table 24 and Figures 26-28. Among reference leaves, the range of IC_{50} values were 269.86 µg/mL -no activity for aqueous extracts, 195.75-928.31 µg/mL for boiling water extracts, and 132.85 µg/mL -no activity for ethanolic extracts compared to standard acarbose at 763.92 µg/mL. Boiling water and ethanolic extracts of BR-BR, aqueous extract of BR-KB and boiling water extract of KP-NR possessed higher IC_{50} value when compared to IC_{50} value of standard acarbose. Therefore, these extracts possessed lower alpha-glucosidase inhibitory activity than acarbose while all other extracts of leaves

possessed lower IC_{50} value and higher activity than acarbose. BR-NR extracts in every solvent showed higher activity than other samples with the same extraction solvent. The highest activity was found in ethanolic extract of BR-NR while the lowest activity was in ethanolic extract of BR-BR.

Among sample leaves, the range of IC_{50} values were 218.29-921.08 µg/mL for aqueous extracts, 243.46-439.81 µg/mL for boiling water extracts, and 152.73-771.68 µg/mL for ethanolic extracts. Only ethanolic extracts of T1 and aqueous extract of UL-T1 possessed higher IC_{50} value and lower activity than acarbose while other extracts of sample leaves show higher activity than acarbose. The highest activity was found in ethanolic extract of *M. alba*. In previous study, IC_{50} value of 60% alcohol extract was 0.4113µg/mL, and 100% alcohol extract was 0.6315 µg/mL, which possessed higher activity than the present study (234). In another study, 65% ethanolic extract of mulberry leaf showed the IC_{50} of 309.82 µg/mL (235).

In tea products, IC_{50} values were in the range of 148.77-3219.07 µg/mL. All tea products, except tea 7 and 14, possessed lower IC_{50} value and higher activity than acarbose. The highest activity was found in tea 12. There has been no other study of alpha-glucosidase inhibitory activity on mulberry teas. Manufactures should consider alpha-glucosidase inhibitory activity of raw materials to make a good benefit to the customers.

From ANOVA, two-factor with replication table, both cultivars and source of reference leaves affected the alpha-glucosidase inhibitory activity at 95% confidence interval as in Table 35 (Appendix).

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Table 24 IC_{50} values of reference leaf, sample leaf and tea product extracts of *M. alba*

Sample	IC_{50} (µg/mL)		
	Aqueous extract (mean ± SD)	Boiling water extract (mean ± SD)	Absolute ethanolic extract (mean ± SD)
Reference leaf			
BR-KB	921.08±312.51	363.15±32.13	249.24±36.86
BR-NR	269.86±11.87	195.73±34.84	132.85±38.85
BR-BR	362.24±35.58	928.31±84.5	776.94±120.53
KP-KB	483.36±24.05	386.24±22.9	-
KP-NR	-	886.42±38.34	171.52±19.07
KP-BR	290.06±24.44	369.08±20.99	385.10±7.92
Sample leaf			
M	500.77±91.89	439.81±42.72	152.73±31.23
T1	218.29±25.48	322.65±32.70	771.68±93.82
T2	582.57±95.72	243.46±13.33	659.47±71.18
T3	251.48±7.67	269.37±23.82	174.84±4.22
UL-T1	921.08±312.51	299.19±14.25	374.41±27.23
Tea products			
Tea 1		344.27±9.83	
Tea 2		239.90±20.93	
Tea 3		397.01±33.66	
Tea 4		537.42±98.91	
Tea 5		702.31±94.31	
Tea 6		452.31±90.70	
Tea 7		1256.97±273.42	
Tea 8		169.03±24.89	
Tea 9		689.81±88.66	
Tea 10		704.00±317.45	
Tea 11		915.48±93.97	
Tea 12		148.77±28.10	
Tea 13		543.91±154.13	
Tea 14		3219.07±62.65	
Acarbose		763.92±22.27	

(-) = not show activity

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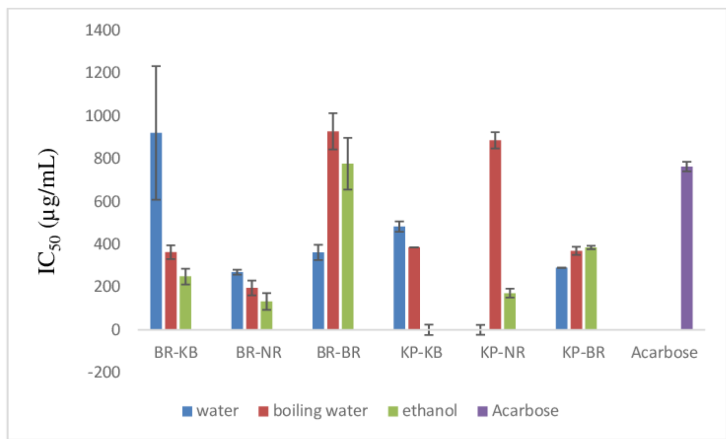

Figure 26 IC_{50} value of different reference leaf extract of *M. alba*

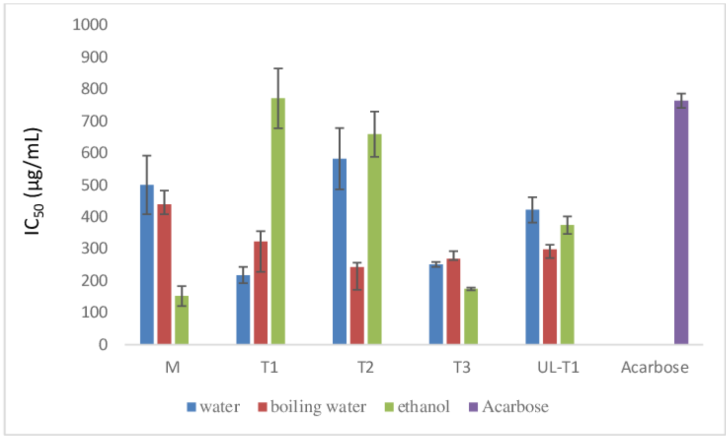

Figure 27 IC_{50} value of different sample leaf extracts of *M. alba*

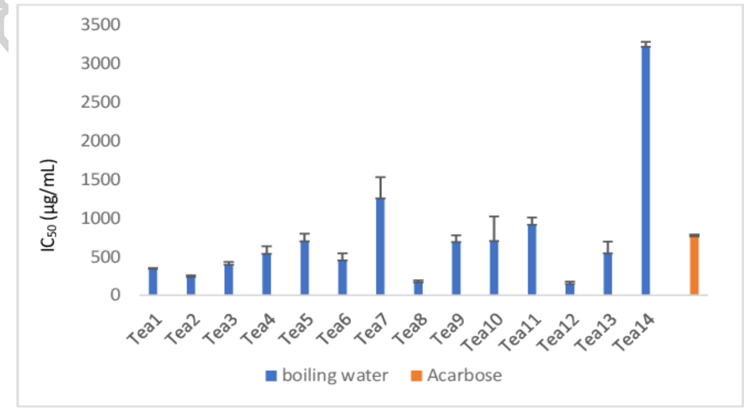

Figure 28 IC_{50} value of boiling water extract of *M. alba* tea products

4.2.3 Preliminary phytochemical screening

The extracts of reference leaves and sample leaves were screened for phytochemistry. Phytochemical screening of boiling water, aqueous and absolute

ethanolic extracts of *M. alba* reference leaves indicated the presence of some secondary metabolites such as flavonoids, terpenoids, steroids, saponin, phenols and tannins and cardiac glycosides. However, proteins were only present in boiling water extract of KP-KB and aqueous extract of BR-BR and KP-NR. The results were shown in Table 25. Alkaloids, anthraquinones and carbohydrates were not detected in all extracts.

Phytochemical screening of boiling water, aqueous and absolute ethanolic leaf extracts in sample leaf group also indicated the presence of flavonoids, terpenoids, steroids, saponins, phenols and tannins but not cardiac glycosides. The results were shown in Table 26. Alkaloids, anthraquinones, carbohydrates and proteins also were not detected in all extracts. Therefore, the present study complied with previous studies (235, 236).

Table 25 Phytochemical screening of different reference leaf extracts of *M. alba*

Phytochemical test	aqueous extract						boiling water extract						absolute ethanolic extract					
	BR-KB	BR-NR	BR-BR	KP-KB	KP-NR	KP-BR	BR-KB	BR-NR	BR-BR	KP-KB	KP-NR	KP-BR	BR-KB	BR-NR	BR-BR	KP-KB	KP-NR	KP-BR
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Steroids	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+
Saponin	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Phenols and Tannins	++	++	++	++	++	++	++	++	++	++	++	++	-	-	-	-	-	++
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbon hydrates	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Proteins	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
Cardiac glycosides	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(-) = negative, (+) = weakly positive, (++) = positive

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Table 26 Phytochemical screening of different sample leaf extracts of *M. alba*

Phytochemical test	aqueous extract					boiling water extract					absolute ethanolic extract				
	M	T1	T2	T3	UL-T1	M	T1	T2	T3	UL-T1	M	T1	T2	T3	UL-T1
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saponin	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Phenols and Tannins	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbon hydrates	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Proteins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(-) = negative, (+) = weakly positive, (++) = positive

4.2.4 Quality determination of *Morus alba* L.

4.2.4.1 Determination of loss on drying

The % loss on drying of *M. alba* leaves and teas were shown in Table 27 and Figure 29. The LOD percentage of reference leaves, sample leaves and tea products were in the range of 8.93-9.94 %, 7.51-8.98 % and 7.21-10.11 %, respectively. Limit of % LOD value for mulberry or mon in Thai Herbal Pharmacopoeia is not more than 11% w/w after drying at 105˚C to constant weight. Therefore % LOD of all *M. alba* leaves and teas were within the limit prescribed in Thai Herbal Pharmacopoeia 2019.

Table 27 Percent LOD of leaf powder of *M. alba* and tea products

Sample	% LOD
Reference leaves	
BR-KB	8.93
BR-NR	9.51
BR-BR	9.36
KP-KB	9.66
KP-NR	9.94
KP-BR	9.91
Sample leaves	
M	8.98
T1	7.51
T2	7.62
T3	7.63
UL-T1	8.46
Tea products	
Tea 1	10.11
Tea2	8.80
Tea 3	9.03
Tea 4	9.74
Tea 5	9.20
Tea 6	9.84
Tea 7	8.52
Tea 8	7.84
Tea 9	8.53
Tea 10	7.21
Tea 11	7.27
Tea 12	9.43
Tea 13	9.21
Tea 14	9.18

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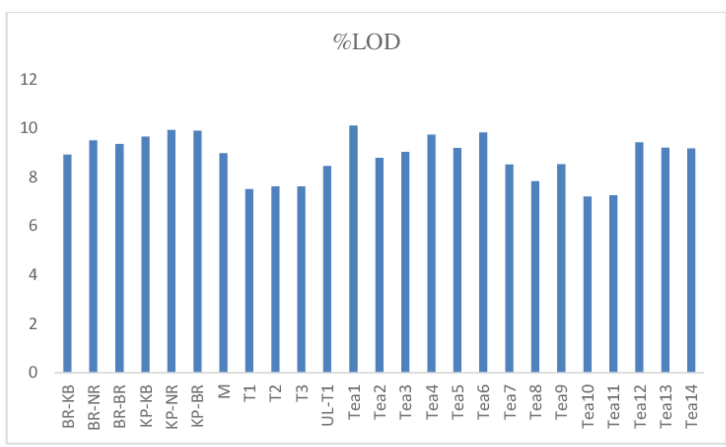

Figure 29 Percent LOD of reference leaves, sample leaves and tea products of *M. alba*

4.2.4.2 Determination of heavy metal contents by ICP-MS

4.2.4.2.1 Standard curves

The result was the same as the result in chapter 3.

4.2.4.2.2 Heavy metal content in *M. alba* leaves and tea samples

Arsenic, As, could distribute into the environment via geological (geogenic) e.g., geothermal/volcanic activities, anthropogenic (human activities) sources e.g., agricultural using pesticides, fungicides and fertilizers, industrial of paints/ chemicals, electroplating others like sewage and mining and biological source (biogenic) e.g., plants, animals and microorganism (237). Cadmium, Cd, could reach the food chain by various geogenic and anthropogenic activities (238). Contaminated sources of lead, Pb, were from mining, manufacturing, recycling activities, paints, lead pipe, stained glass, lead crystal glassware, and petroleum engine (239). Nickel, Ni, may be contaminated from environment (liquid and solid fuel, industrial waste), air and soil (240). Barium, Ba, may be contaminated from environment (mining) and food (Brazil nuts, seaweed, fish).

The concentration of heavy metals in reference leaves were shown in Table 28 and Figures 8-9. The content of As, Cd and Pb were in the range of 0.0253-0.0863, 0.0043-0.0089 and 0.1184-0.2463 mg/kg, respectively. The content of Ni and Ba were in the range of 0.3783-0.7724 and 10.5743-49.3895 mg/kg. BR-NR showed the highest content of As, Cd and Pb and the highest content of Ni was found in BR-KB. However, KP-NR showed the highest content of Ba. KP-KB possessed the lowest

content of As, Cd and Pb and the lowest content of Ni was found in KP-NR. The lowest content of Ba was found in BR-KB.

The concentration of heavy metals in sample leaves from different sources were described in Table 28 and Figures 8 -9. The content of As, Cd and Pb were in the range of 0.0552-0.1678, 0.0095-0.0136 and 0.3307-0.4694 mg/kg, respectively. The content of Ni and Ba were in the range of 1.0319-2.1349 and 11.4302-47.1561 mg/kg, respectively.

The concentration of heavy metals in 14 *M. alba* L. tea products were shown in Table 28 and Figures 30-31. The content of As, Cd and Pb were in the range of 0.0166-0.3996, 0.0044-0.0183 and 0.0103-1.0069 mg/kg respectively. The content of Ni and Ba were in the range of 0.5656-3.0988 and 10.98682-133.4450 mg/kg.

Reference leaves possessed lower content of heavy metals than sample leaves except for Ba while tea products possessed the highest content among all sample investigated. Lower As and Pb contents of reference leaves represented the closed control of pesticide and fertilizer utilization in the agricultural environment as well as the control of distance from roadside. High levels of Cd, Ni and Ba demonstrated that contamination from high technology equipment in industrial production should be aware.

The content of As, Cd and Pb in all samples was within the maximum limit prescribed in WHO and ASEAN guidelines for herbal medicines. The content of Ni and Ba in all samples was also lower than the guideline limit of ICH. All samples in this research from different sources and different parts contained variable amount of heavy metals. These differences may be due to variability of exposure to environmental pollution, genetics, geographical region composition of soil. However, heavy metal content was within acceptable and safe limit prescribed in ASEAN guidelines, WHO recommendation and ICH guideline. However, the attention should be paid during both cultivation process and tea infusion production.

From ANOVA, two-factor with replication table, most of metal levels in mulberry leaves were affected by both cultivar and cultivation source except Pb and Cd, at 95% confidence interval (Appendix). Cd content were affected only by cultivation area whereas no difference in Pb content was made by cultivar and source. Mulberry leaves cultivar Buriram 60 seemed to have higher metal levels than cultivar

Khun Pai, especially that from NR that contained the highest amount of As and Cd while the reference leaves BR-KB contained the highest level of Ni. Only reference leaves cultivar Khun Pai, KP-NR, contained the highest in Ba content. Leaves, KP-KB, contained the least content of As and Cd and KP-NR contained the least content in Ni.

Table 28 Heavy metal content in powder of reference leaves, sample leaves and tea products of *M. alba*

sample	Metal Content)mg/kg ± SD(				
	As	**Cd**	**Pb**	**Ni**	**Ba**
Reference leaves					
BR- KB	0.0285 ± 0.0010	0.0081 ± 0.0027	0.2404 ± 0.0423	0.7724 ± 0.0355	10.5743 ± 0.5751
BR-NR	0.0863± 0.0030	0.0089 ± 0.0009	0.2463 ± 0.1278	0.5797 ± 0.0408	33.2780 ± 1.6611
BR-BR	0.0285 ± 0.0011	0.0050 ± 0.0003	0.1256 ± 0.0692	0.6776 ± 0.2621	49.3895 ± 1.2510
KP- KB	0.0253 ± 0.0037	0.0043 ± 0.0013	0.1184 ± 0.0467	0.6676 ± 0.1151	25.5653 ± 18.9073
KP-NR	0.0474 ± 0.0008	0.0046 ± 0.0001	0.1595 ± 0.0076	0.3783 ± 0.0196	52.1426 ± 1.5176
KP-BR	0.0303 ± 0.0013	0.0057 ± 0.0009	0.2346 ± 0.0883	0.4989 ± 0.0383	46.6449 ± 1.0847
Sample leaves					
M	0.1678 ± 0.0059	0.0124 ± 0.0036	0.3470 ± 0.0754	1.1405 ± 0.0610	11.4302 ± 0.3569
T1	0.0608 ± 0.0007	0.0104 ± 0.0023	0.4204 ± 0.2182	2.1349 ± 0.0141	41.4144 ± 0.0819
T2	0.1018 ± 0.0010	0.0107 ± 0.0025	0.3307 ± 0.0290	1.4194 ± 0.0554	47.1561 ± 0.8913
T3	0.1420 ± 0.0095	0.0095 ± 0.0015	0.4694 ± 0.0547	1.2854 ± 0.1142	18.1827 ± 1.9070
UL-T1	0.0552 ± 0.0013	0.0136 ± 0.0046	0.4600 ± 0.1234	1.0319 ± 0.1055	29.0419 ± 1.4721
Limitation					
WHO		0.3	10		
ASEAN	5	0.3			
ICH				20	140

Heavy metal content in powder of reference leaves, sample leaves and tea products of *M. alba*

sample	Metal Content)mg/kg ± SD(				
	As	**Cd**	**Pb**	**Ni**	**Ba**
Tea products					
Tea1	0.0525 ± 0.0034	0.0095 ± 0.0008	0.3668 ± 0.0237	0.7647 ± 0.0460	86.4393 ± 8.1882
Tea 2	0.1437 ± 0.0062	0.0057 ± 0.0012	0.3104 ± 0.0832	0.5656 ± 0.0637	45.9991 ± 0.7302
Tea 3	0.0898 ± 0.0046	0.0084 ± 0.0005	0.0642 ± 0.0242	1.0364 ± 0.0885	66.3910 ± 2.8278
Tea 4	0.0166 ± 0.0006	0.0075 ± 0.0017	0.0225 ± 0.0093	2.6832 ± 2.2391	39.9134 ± 0.3526
Tea 5	0.1158 ± 0.0102	0.0044 ± 0.0017	0.0224 ± 0.0109	1.0091 ± 0.9380	103.9880 ± 4.2651
Tea 6	0.3996 ± 0.0555	0.0060 ± 0.0010	0.0103 ± 0.0059	1.1525 ± 1.1494	133.4450 ± 20.6112
Tea 7	0.0602 ± 0.0052	0.0052 ± 0.0033	0.0428 ± 0.0742	1.4186 ± 0.1044	53.9487 ± 2.2281
Tea 8	0.0902 ± 0.0063	0.0160 ± 0.0045	0.2330 ± 0.1461	2.6166 ± 0.1751	83.4776 ± 0.6701
Tea 9	0.2372 ± 0.0244	0.0073 ± 0.0014	0.3986 ± 0.3743	1.9045 ± 0.0181	69.1649 ± 0.8640
Tea10	0.1911 ± 0.0103	0.0139 ± 0.0033	0.1575 ± 0.0899	1.6515 ± 0.0591	46.0273 ± 1.5347
Tea 11	0.0913 ± 0.0067	0.0183 ± 0.0150	0.0513 ± 0.0889	3.0988 ± 0.4319	90.8006 ± 2.9050
Tea 12	0.0679 ± 0.0237	0.0065 ± 0.0031	0.0381 ± 0.0660	1.7527 ± 0.2946	59.8613 ± 44.8930
Tea 13	0.0539 ± 0.0183	0.0057 ± 0.0028	0.0848 ± 0.0810	1.8135 ± 0.4329	10.9868 ± 5.9978
Tea 14	0.0699 ± 0.0039	0.0137 ± 0.0145	1.0069 ± 1.4371	2.0575 ± 0.1135	13.1700 ± 8.6852
Limitation					
WHO		0.3	10		
ASEAN	5	0.3			
ICH				20	140

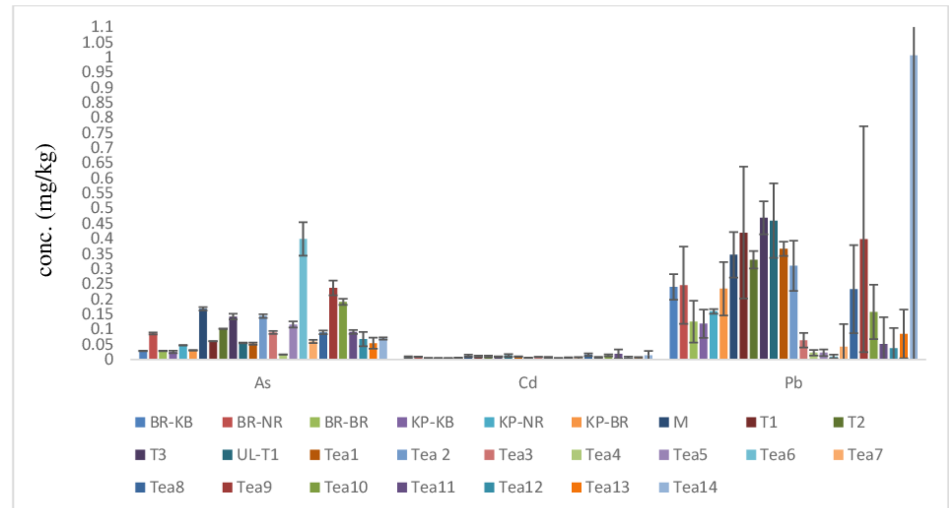

Figure 30 Heavy metal (As, Cd, Pb) content in powder of reference leaves, sample leaves and tea products of *M. alba*

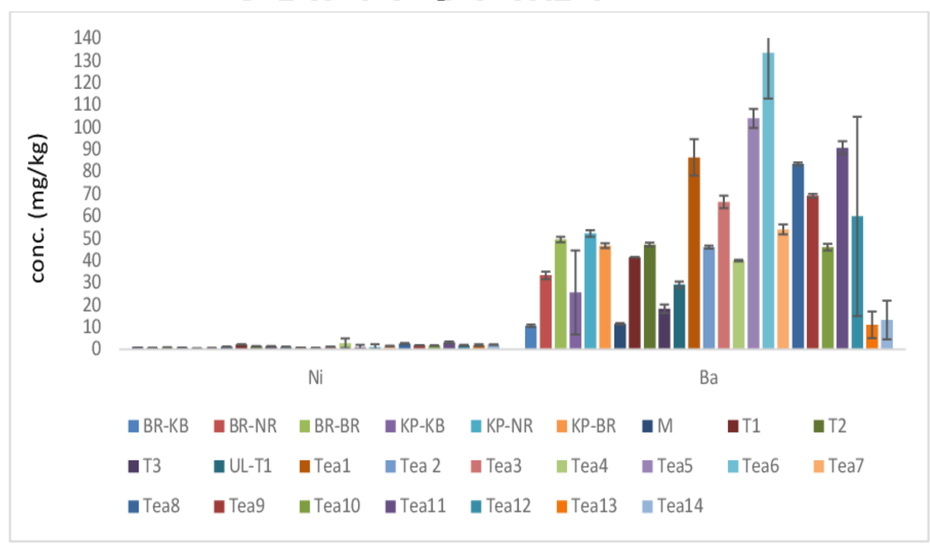

Figure 31 Heavy metal (Ni, Ba) content in in powder of reference leaves, sample leaves and tea products of *M. alba*

4.2.4.3 Determination of total phenolic content

Total phenolic content (TPC) of leaf and tea extracts of *M. alba* was expressed as mg GAE/ g extract and shown in Table 29 and Figures 32-34. TPCs of references leaves were in the range of 12.95-34.03, 14.16-37.99 and 7.26-27.22 mg GAE/g extract for aqueous, boiling water and ethanol extract, respectively. Boiling water extract of KP-NR possessed the highest TPC.

Among extracts of sample leaves, TPCs were in the range of 19.87-31.95, 28.36-47.36, 10.55-28.67 mg GAE/g extract for aqueous, boiling water and ethanolic extract, respectively. Boiling water extract of top leaf UL-T1 possessed the highest TPC. In three types of extracts of all samples, boiling water extracts possessed much higher content than aqueous extracts and ethanol extracts.

TPCs of reference and sample leaves from the present study were found higher than those in previous studies (99, 241, 242).

In boiling water extracts of *M. alba* tea products, TPCs were in the range of 16.83-49.54 mg GAE/g extract. Tea 3 possessed the highest TPC among all test samples. TPCs of all tea products in this study were higher than previous study (243).

Considering reference leaves, the result from ANOVA showed that different extraction solvents gave significant difference in TPCs (Appendix). From mean values, extraction with ethanol gave the lowest TPC among 3 solvents. TPC of aqueous extracts gave the mean values closed to those from boiling water extracts. Cultivars and province sources of leaves caused significant difference at 95% confidence interval within all solvent extractions. Considering mean values, cultivar BR gave lower TPC than cultivar KP in all solvents and leaves from KB gave the lowest TPC in all solvents.

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Table 29 Total phenolic content in extracts of reference leaf, sample leaf and tea product of *M. alba*

Sample	Aqueous (mean ± SD)	Boiling water (mean ± SD)	Ethanol (mean ± SD)
Reference leaves			
BR-KB	12.95±0.54	14.16±0.76	9.28±1.67
BR-NR	25.39±2.86	29.73±0.66	18.64±5.40
BR-BR	22.73±0.51	21.51±0.50	7.26±1.85
KP-KB	22.29±0.88	20.44±1.37	8.40±0.74
KP-NR	34.03±3.77	37.99±1.84	27.22±5.45
KP-BR	33.12±2.78	37.24±1.15	15.84±1.09
Sample leaves			
M	30.85±1.40	42.77±1.88	28.67±0.43
T1	25.95±3.24	31.74±0.72	10.55±3.39
T2	19.87±5.60	28.36±0.70	12.96±2.57
T3	28.19±5.09	31.68±0.98	13.88±1.51
UL-T1	31.95±3.36	47.36±2.01	24.63±5.01
Tea products			
Tea 1		45.95 ± 0.62	
Tea 2		44.22 ± 0.61	
Tea 3		49.54 ± 0.65	
Tea 4		39.72 ± 0.14	
Tea 5		36.43 ± 0.92	
Tea 6		29.95 ± 0.33	
Tea 7		31.16±1.04	
Tea 8		35.59±0.88	
Tea 9		32.55 ± 2.47	
Tea 10		33.86 ± 1.70	
Tea 11		42.30 ± 2.89	
Tea 12		30.19±0.86	
Tea 13		25.15 ± 2.28	
Tea 14		16.83±0.27	

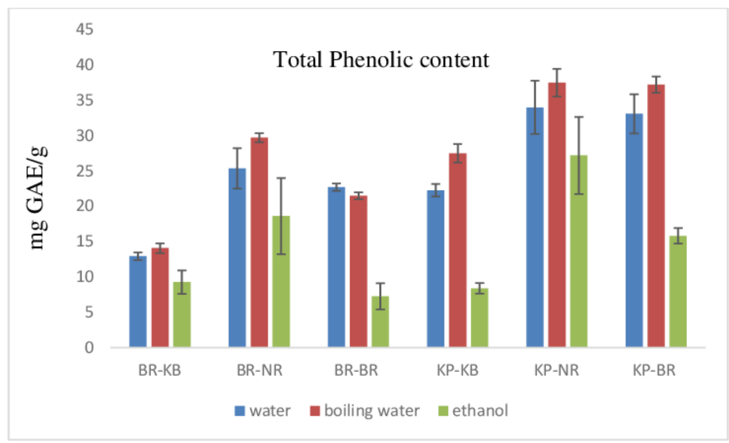

Figure 32 Total phenolic content in different reference leaf extracts of *M. alba*

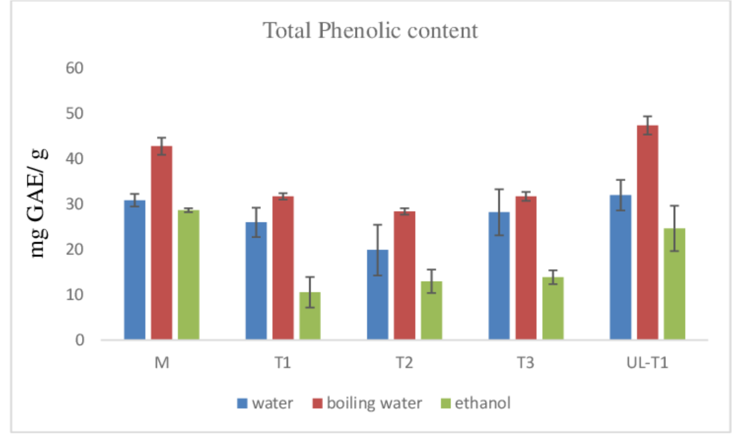

Figure 33 Total phenolic content in different sample leaf extracts of *M. alba*

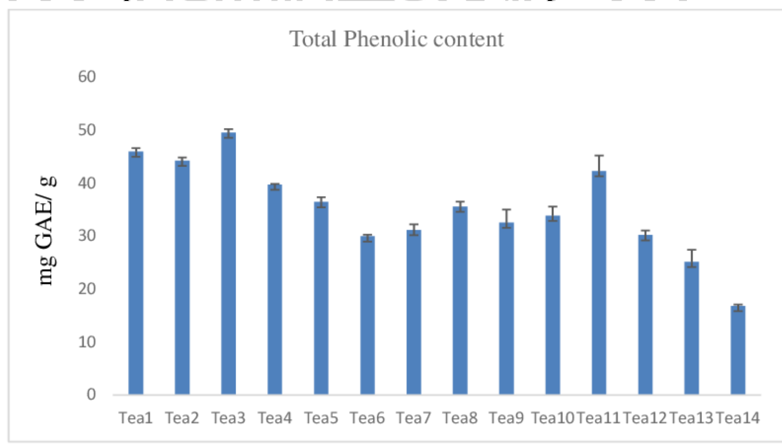

Figure 34 Total phenolic content in boiling water extracts of *M. alba* tea products

4.2.4.4 Determination of microbial contamination

Results for microbial contamination in powder of reference leaves, sample leaves and tea products were shown in Table 30. All reference leaves, except BR-BR

were free from the contamination of *E. coli*, *S. aureus*, *Salmonella* species and *Clostridium* species. The reference leaves BR-BR showed the contamination of *Clostridium* species. However, this contaminated species was not pathogenic. Pathogenic *Clostridium* spp. were *C. tetani*, *C. botulinum* and *C. perfringers*.

Table 30 Microbial contamination in reference leaves, sample leaves and tea products of *M. alba*

Sample	*E. coli*	*S. aureus*	*Salmonella*	*Clostridium. spp*
Reference leaves				
BR-BR	-	-	-	+
BR-KB	-	-	-	-
BR-NR	-	-	-	-
KP-BR	-	-	-	-
KP-KB	-	-	-	-
KP-NR	-	-	-	-
Sample leaves				
M	-	-	-	-
T1	-	-	-	-
T2	-	-	-	+
T3	-	-	-	-
UL-T1	-	-	-	-
Tea products				
Tea 1	-	-	-	+
Tea 7	-	-	-	-
Tea 8	-	-	-	-
Tea 10	-	-	-	+
Tea 12	-	-	-	-
Tea 14	-	-	-	-

(+) = present, (-) = absent

In samples leaves and tea products, T2, Tea 1 and Tea 10 showed the contamination of nonpathogenic *Clostridium* species.

Limit of microbial contamination prescribed in Thai Pharmacopoeia 2005 and USP 40 is described as follow. For *E. coli* and *S. aureus* there is none of the colonies in 1g of test sample. There should be no colonies of *Salmonella* in 10 g of test sample as well as for spore and non-spore pathogenic *Clostridium* spp., there should be no colonies in 10 g of each test.

4.2.4.5 Fourier Transformed-Infrared Spectroscopy (IR) profiling

In reference leaves of *M. alba*, common peaks were found at wave number 1046.77, 1416.64, 1598.09, 1741.81, 2851, 2919.85, 3270.2 in Table 9 and Figure 35. Assignment of most bands were done and shown in Table 31. It was found that peaks of sample from different sources were similar.

In *M. alba* sample leaves from Myanmar and Thailand, common peaks were found at wave number 1046.77, 1416.64, 1598.09, 1741.81, 2851, 2919.85, 3270.2 cm^{-1} in Table 31 and Figure 36. Assignment of most bands were done and shown in Table 52. It was found that peaks of sample from different sources were similar. The present study complied with the previous study (244). Overall, in all samples, similar spectral shapes were obtained, and the same bands were present in all spectra with slight differences in mutual intensities.

Table 31 Wave number ($\tilde{v}$) of mulberry leave

Sample	($\tilde{v}$, cm^{-1})	($\tilde{v}$, cm^{-1})	($\tilde{v}$, cm^{-1})	($\tilde{v}$, cm^{-1})	($\tilde{v}$, cm^{-1})	($\tilde{v}$, cm^{-1})	($\tilde{v}$, cm^{-1})
Reference leaves							
BR-KB	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
BR-NR	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
BR-BR	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
KP-KB	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
KP-NR	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
KP-BR	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
Sample leaves							
M	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
T1	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
T2	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
T3	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
UL-T1	1046.77	1416.64	1598.09	1741.81	2851	2919.85	3270.2
Assignment	C-O stretching	C-H banding	C=C stretching	C=O stretching	C-H stretching	C-H stretching	O-H stretching

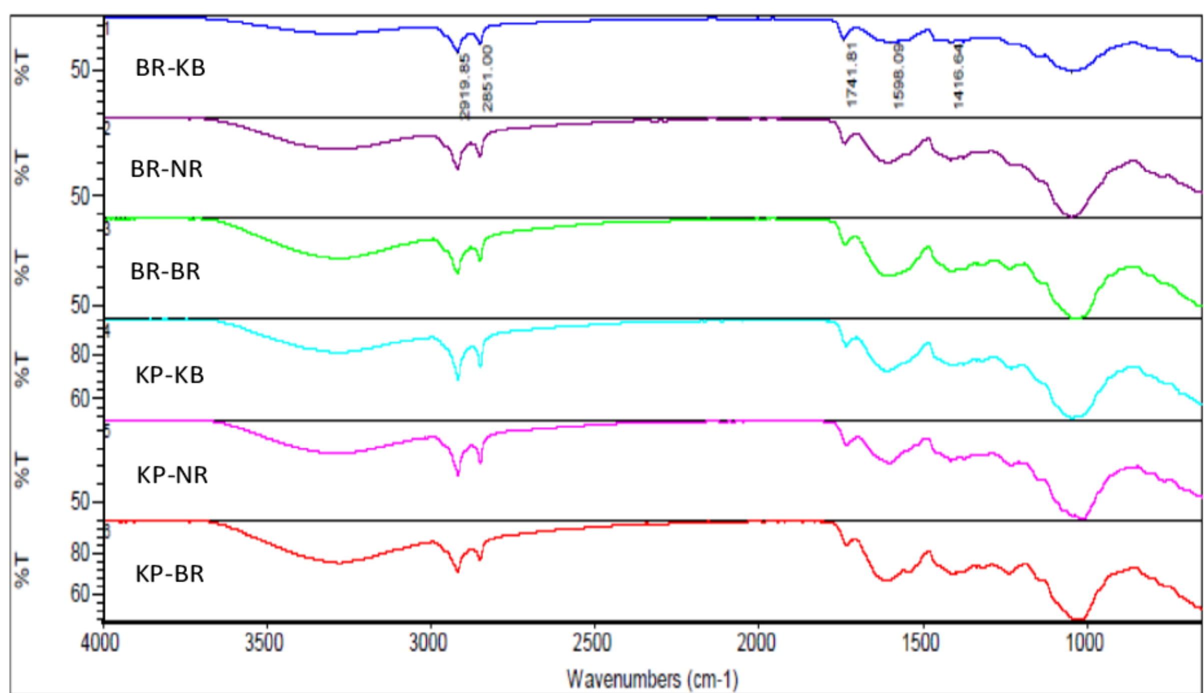

Figure 35 FT-IR spectrum of reference leaves of *M. alba* cultivar

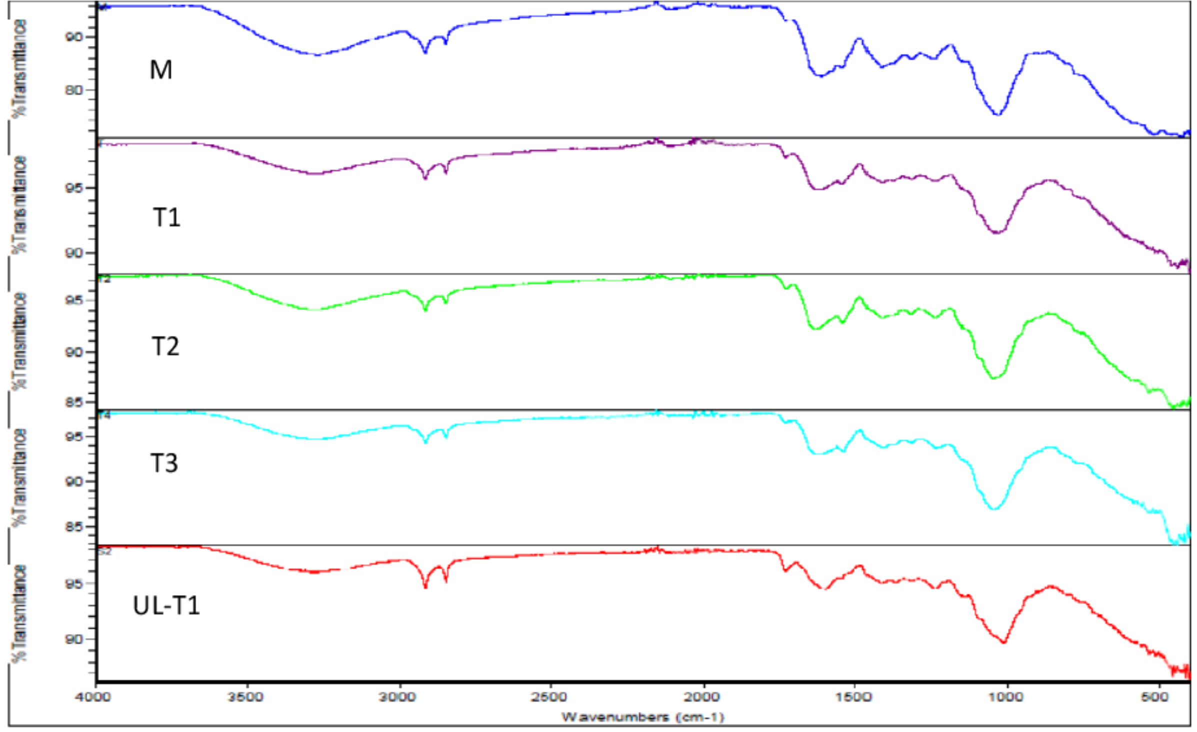

Figure 36 FT-IR spectrum of sample leaves of *M. alba*

4.2.4.6 Gas Chromatography- Mass Spectroscopy (GC-MS) profiling

4.2.4.6.1 GC-MS fingerprint of *M. alba* reference and sample leaf extracts

GC-MS chromatograms of the ethanolic extracts of *M. alba* leaf and teas were analyzed. A total of 83 compounds containing terpenes, saturated fatty acids, unsaturated fatty acids and benzofurans were found from reference leaf. Table 32 with % area cut off at 0.04 showed that the ethanolic extracts of cultivar Buriram 60 from KB, NR and BR contained 19, 16, and 26 compounds, respectively. Phytol was the most abundant compound in BR-KB and BR-BR, following by palmitic acid and oleic acid. While oleic acid was the most common substances in BR-NR, following by phytol, and palmitic acid. The compounds with more than 0.04% peak area of the ethanolic extracts of *M. alba* cultivar Khun Pai were shown in Table 33. There were 23, 18, 21 compounds found in the leaves from KB, NR and BR, respectively. Phytol, oleic acid and palmitic acid were the most abundant compounds in the leaves from NR and BR but in different order while the leaves from KB were rich in phytol, methyl linolenate and stearic acid. It was remarkable that for both cultivars, % peak area of phytol was much less and % peak area of palmitic acid, oleic acid and linoleic acid were much higher in leaves from NR than in leaves from KB and BR. The GC-MS chemical fingerprints of ethanolic leaf extracts from 2 cultivar of *M. alba* were shown in Figure 37.

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Table 32 Retention time and % peak area of compounds found in ethanolic extracts of reference leaves of *M. alba* cultivar Buriram 60 (BR) from KB, NR and BR provinces

No	Retention Time (min)	% peak area			Compound name
		KB	NR	BR	
1	5.79	0.09 -0.23	0.06-0.21	0.04- 0.05	N, N-dimethylethanethioamide
2	29.66	0.18-0.50	0.16-0.31	0.22- 0.30	4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one
3	38.26	-	-	0.11- 0.36	myristic acid
4	38.61	0.21-0.46	0.24-1.07	0.45- 0.54	(-)-loliolide
5	40.18	0.17-0.59	-	0.21- 0.41	Myristaldehyde
6	40.38	-	-	0.12- 0.64	propan-2-yl-tetradecanoate
7	40.75	0.34-0.66	0.27-0.50	0.64- 0.98	Neophytadiene
8	40.96	0.66-1.25	0.61-0.84	0.39- 1.56	Phytone
9	42.18	-	-	0.27- 0.44	Unknown
10	43.04	-	-	0.07- 0.34	7,9-di-tert-butyl-1-oxaspiro [4.5] deca-6,9-diene-2,8-dione
11	43.76	0.56-6.51	0.61-0.84	1.70- 3.50	methyl palmitate
12	44.99	2.21-5.02	13.91-17.50	5.08- 11.28	palmitic acid
13	45.48	-	-	0.52- 0.58	Unknown
14	45.97	-	0.44- 0.55	0.36- 0.53	ethyl palmitate
15	48.99	0.29-3.34	0.39- 0.55	0.82- 2.28	methyl linoleate
16	49.19	2.48-18.33	2.01- 3.39	4.57- 12.72	methyl linolenate
17	49.52	33.14-41.11	10.25- 16.29	26.84-32.30	Phytol
18	50.05	0.28-1.75	0.06- 0.20	0.78- 0.94	methyl stearate
19	50.18	0.40-1.16	2.31- 2.91	0.44-0.61	linoleic acid
20	50.39	3.36-8.67	22.35- 32.25	5.93- 8.96	oleic acid
21	51.20	1.59-2.22	5.91- 6.57	4.70-6.61	stearic acid
22	52.57	0.33-0.59	-	0.88-1.04	Unknown
23	55.59	-	-	0.26-0.36	trans-ferulic acid
24	56.97	0.28-0.45	-	0.11-0.17	farnesyl acetone
25	60.74	2.22-4.54	0.74- 1.23	1.58-2.45	Monopalmitin
26	60.94	0.15-2.22	-	1.08-1.35	(6E,10E,14E,18E)-2,6,10,19,23-pentamethyltetracosa-2,6,10,14,18,22-hexaene

Table 33 Retention time and % peak area of compounds found in ethanolic extracts of reference leaves of *M. alba* cultivar Khun Pai (KP) from KB, NR and BR provinces

No	Retention Time (min)	% peak area KB	% peak area NR	% peak area BR	Compound name
1	5.79	0.15-0.19	0.09-0.18	0.07-0.18	N, N-dimethylethanethioamide
2	29.66	0.20-0.54	0.13-0.29	0.27-0.45	4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one
3	38.61	0.37-0.95	0.27-0.56	0.60-1.01	(-)-loliolide
4	40.18	0.53-0.82	-	-	Myristaldehyde
5	40.75	0.45-0.93	0.50-1.04	0.75-1.31	Neophytadiene
6	40.96	0.92-1.78	0.32-0.63	0.62-1.08	6,10,14-trimethylpentadecan-2-one
7	42.18	0.14- 0.42	0.20-0.30	0.22-0.52	Unknown
8	43.76	2.29-3.88	0.65-1.62	0.40-2.76	methyl palmitate
9	44.99	4.46-5.96	11.07-17.57	5.02-9.28	palmitic acid
10	45.48	0.16-0.41	-	-	Unknown
11	45.81	0.14-0.21	-	-	Farnesol
12	45.97	0.27-0.38	0.34-1.08	0.43-0.52	ethyl palmitate
13	48.99	1.54-3.33	0.32-0.59	0.28-2.30	methyl linoleate
14	49.19	5.69-11.47	1.24-4.16	1.29-9.51	methyl linolenate
15	49.52	23.28-34.40	6.54-10.54	26.16-31.62	Phytol
16	50.05	-	-	0.14-0.83	methyl stearate
17	50.18	0.69-1.16	1.59-3.68	0.45-3.63	linoleic acid
18	50.39	0.74-1.22	15.39-25.65	8.05-20.69	oleic acid
19	51.20	7.59-9.10	6.13-7.70	4.46-5.38	stearic acid
20	52.57	0.74-1.01	-	0.73-0.97	Unknown
21	55.59	0.13-0.26	-	0.04-0.15	trans-ferulic acid
22	56.97	0.20-0.37	0.15-0.23	0.13-0.29	farnesyl acetone
23	60.74	2.82-2.98	0.36-2.68	2.34-2.82	Monopalmitin
24	60.94	0.64-2.31	1.26-4.72	0.60-1.15	(6E,10E,14E,18E)-2,6,10,19,23-pentamethyltetracosa-2,6,10,14,18,22-hexaene

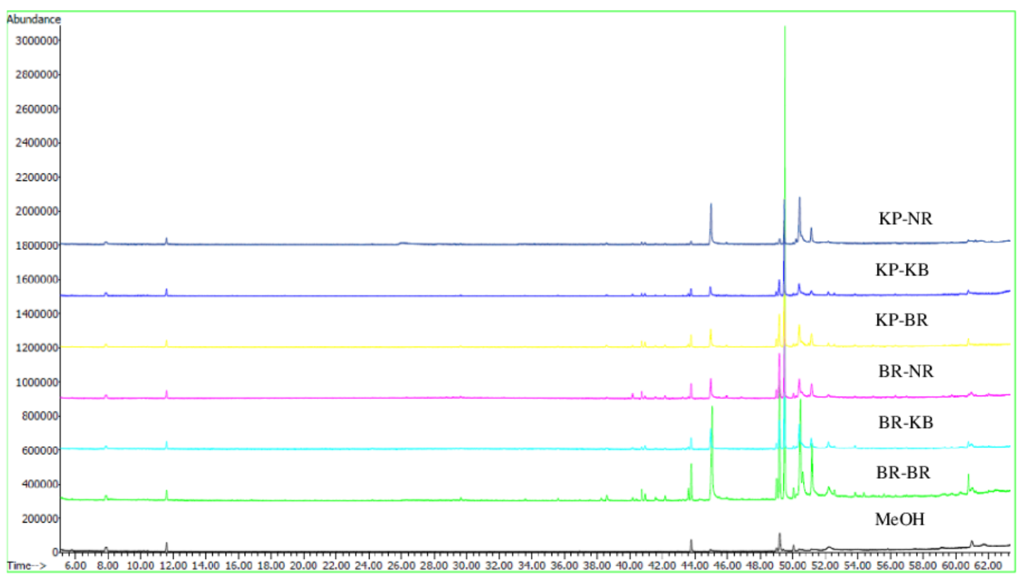


Figure 37 GC-MS fingerprints of ethanolic extracts of reference leaves of *M. alba*

A total of 73 compounds containing terpenes, saturated fatty acids, unsaturated fatty acids and benzofurans were found in sample leaves of *M. alba*. The extracts of M, T1, T2, T3 and ULT1 were found 23, 25, 24, 23, and 26 compounds with %area >0.04%, respectively as shown in Table 34. Twenty compounds such as oleic acid (or (Z)-octadec-9-enoic acid) (% peak area of 13.43-30.95%), phytol or 3, 7, 11, 15-tetramethylhexadec-2-en-1-ol) (10.32- 25.28%), linoleic acid or (9Z,12Z)-octadeca-9,12-dienoic acid (1.25-22.25%), palmitic acid (or hexadecenoic acid) (6.97-15.45%), stearic acid or octadecanoic acid (2.73-9.76%) and methyl linolenate (or methyl (9Z,12Z,15Z)-octadeca-9,12,15-trienoate) (1.03-4.16%), with respect to % peak area were found in common in samples from both Myanmar and Thailand. In leaf extracts of *M. alba* from both countries, % peak area of linoleic acid and stearic acid were much higher in leaves from Myanmar and % peak area of oleic acid and phytol were much higher in leaves from Thailand. The GC-MS chemical fingerprints of ethanolic leaf extracts of *M. alba* from Myanmar and Thailand were shown in Figure 38. The fingerprint patterns of sample leaves were similar to those of reference leaves. Our study complied with the previous studies (36, 40).

Table 34 Retention time and % peak area of compounds found in ethanolic extracts of sample leaves *M. alba*

No	Retention time (min)	% peak area					Compound name
		M	T1	T2	T3	UL-T1	
1	10.17	0.36-0.44	0.21-0.27	0.15-0.28	0.18-0.22	0.12-0.27	Unknown
2	29.66	0.32-0.42	0.32-0.32	0.27-0.33	0.24-0.33	0.17-0.38	4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one
3	35.49	-	0.15-0.21	0.10-0.22	-	-	Unknown
4	38.26	0.15-0.32	0.12-0.34	0.22-0.24	0.23-0.45	0.10-0.26	myristic acid
5	38.61	0.57-0.85	0.48-0.88	0.52-0.84	0.78-1.10	0.48-0.76	(-)-loliolide
6	40.18	0.39-0.51	0.73-1.05	0.32-0.41	0.39-0.66	0.39-0.71	myristaldehyde
7	40.75	1.27-1.5	1.03-1.39	1.36-1.78	2.57-3.62	1.13-1.62	neophytadiene
8	40.96	0.41-0.57	0.39-0.47	0.38-0.44	0.60-0.97	0.29-0.50	6,10,14-trimethylpentadecan-2-one
9	41.56	0.59-0.64	0.52-0.55	0.5-0.53	0.79-0.96	0.53-0.60	Unknown
10	42.18	0.47-0.61	0.35-0.53	0.40-0.57	0.83-1.14	0.34-0.59	Unknown
11	43.76	0.55-0.95	0.68-1.08	0.67-0.99	0.81-1.14	0.59-0.78	methyl palmitate
12	44.99	11.94-14.41	8.96-15.45	9.67-11.19	6.97-11.26	8.99-10.45	palmitic acid
13	45.19	0.23-0.50	-	0.15-0.58	0.17-0.31	0.24-0.74	Unknown
14	45.81	-	0.08-0.28	-	-	0.11-0.15	farnesol
15	45.97	0.79-0.99	0.25-0.30	-	0.31-0.40	0.30-0.59	ethyl palmitate
16	48.99	0.28-0.44	0.26-0.52	0.35-0.57	0.28-0.34	0.46-0.53	methyl linoleate
17	49.19	1.03-1.96	1.63-2.82	2.36-4.17	1.80-2.65	1.66-2.45	methyl linolenate
18	49.52	10.33-15.61	17.86-25.28	17.71-19.15	17.55-20.10	10.81-16.82	phytol
19	50.05	0.24-0.58	0.21-0.57	0.2-0.43	0.31-0.57	0.22-2.20	methyl stearate

Retention time and % peak area of compounds found in ethanolic extracts of sample leaves *M. alba*

No	Retention time (min)	% peak area					Compound name
		M	T1	T2	T3	UL-T1	
20	50.18	1.99-2.25	2.10-2.87	3.03-3.65	1.26-1.82	3.80-4.19	linoleic acid
21	50.39	13.43-18.47	14.35-19.55	25.77-30.95	16.92-22.53	26.87-29.02	oleic acid
22	51.20	7.07-9.77	2.73-4.52	3.30-5.60	4.81-5.62	3.45-5.64	stearic acid
23	52.57	-	0.15-0.37	0.12-0.19	0.18-0.50	0.30-0.87	Unknown
24	55.59	-	-	0.18-0.24	-	0.13-0.35	trans-ferulic acid
25	56.97	0.61-0.66	0.55-0.59	0.26-1.62	0.71-3.49	0.23-0.93	farnesyl acetone
26	60.74	0.40-1.17	-	-	-	0.98-1.18	monopalmitin
27	60.94	0.18-4.86	2.19-12.46	1.88-8.71	3.50-6.99	3.40-4.88	(6E,10E,14E,18E)-2,6,10,19,23-pentamethyltetracosa-2,6,10,14,18,22-hexaene

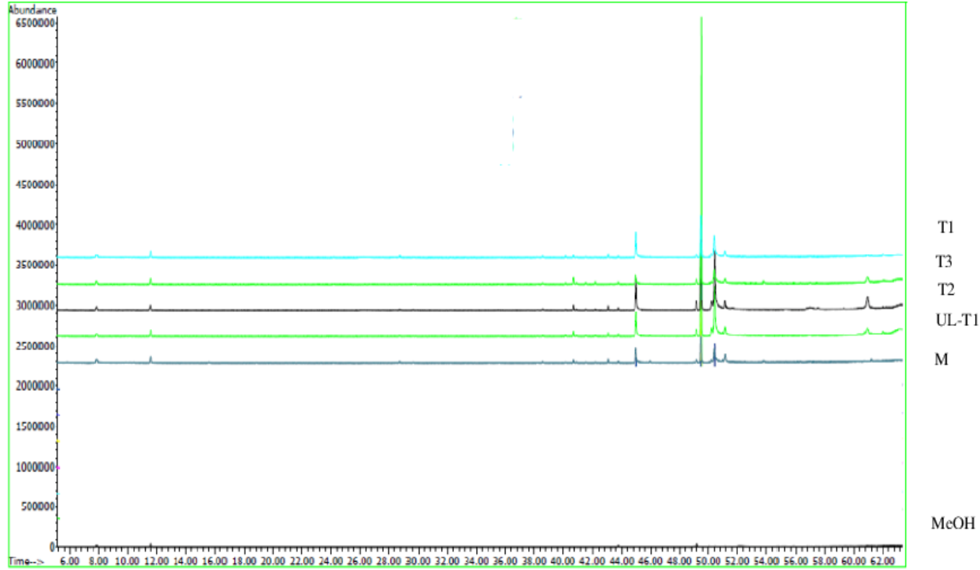


Figure 38 GC-MS fingerprint of ethanolic extracts of sample leaves of *M. alba*

4.2.4.6.2 GC-MS profiles of *M. alba* tea product extracts

The GC-MS chromatograms of the ethanolic leaf extracts of 14 *M. alba* commercial tea products were analyzed. All tea products were labelled *M. alba* leaves without other herbal plants except for tea 10. Tea products contained total of 18 compounds in which 6 compounds were common including phytol (5.13-38.94 %), oleic acid (1.90-24.23 %), methyl linolenate (0.44-12.54%), methyl palmitate (0.29-8.18 %), palmitic acid (1.60-11.25 %), stearic acid (0.49-6.58 %), which also were found in both cultivars of *M. alba* leaves and shown in Table 35. However, stearic acid was not found in tea 10 and 11. Tea 7 possessed the highest % peak area of methyl palmitate and stearic acid. Tea 3 possessed the highest % peak area of oleic acid and palmitic acid. The highest % peak area of phytol and methyl linolenate were found in Tea 13 and Tea 9 respectively. The GC-MS chemical fingerprints of the ethanolic extracts of tea products were shown in Figure 39.

Table 35 Retention time and % peak area of compounds found in ethanolic extracts of *M. alba* tea products

No	Retention time (min)	% peak area														Compound name
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1	26.43	-	-	-	-	-	-	6.15-12.51	3.34-10.28	0.81-6.56	0.45-2.16	-	-	-	-	dimethyl phthalate
2	29.66	-	-	0.19-0.45	-	-	-	-	0.60-1.25	-	-	-	0.88-1.63	-	-	4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one
3	31.91	-	-	-	-	-	-	26.95-34.89	13.86-28.80	3.39-23.52	1.57-8.09	3.29-8.10	1.53-3.58	1.51-2.25	1.27-1.53	diethyl phthalate
4	35.58	-	-	-	-	-	-	-	1.13-1.36	-	-	-	0.96-1.71	-	-	Unknown
5	40.18	-	0.50-0.90	0.20-1.19	-	-	-	0.41-0.60	0.95-1.48	0.78-1.20	-	0.47-2.38	0.82-1.54	-	-	Unknown
6	40.96	-	0.83-0.93	0.46-0.62	0.38-0.63	-	-	-	-	-	-	-	0.41-0.81	-	0.53-0.74	Unknown
7	41.59	-	-	0.14-0.35	0.31-0.40	-	-	0.34-0.48	0.41-1.27	-	-	-	-	-	0.66-1.45	Unknown
8	43.76	0.36-1.00	0.96-1.62	0.29-0.43	0.78-1.38	0.48-1.71	0.48-1.70	4.53-8.18	2.36-5.11	3.44-6.37	1.23-4.32	2.30-5.10	1.09-1.91	1.97-2.29	1.50-1.82	methyl palmitate
9	44.99	5.35-6.80	3.46-5.25	4.52-11.25	4.16-4.79	1.60-7.74	3.37-5.94	2.65-5.15	4.37-4.89	3.26-7.18	3.22-4.45	3.20-7.97	3.63-5.07	2.51-4.62	4.02-6.18	palmitic acid
10	48.99	-	0.55-0.80	-	-	0.21-0.65	-	0.98-2.47	0.61-1.22	1.77-2.81	1.08-2.42	1.01-2.56	0.44-0.96	1.13-1.20	0.95-1.39	methyl linoleate
11	49.19	0.44-1.93	1.72-2.28	0.88-1.04	1.65-2.45	0.90-2.71	1.12-3.45	6.42-11.87	2.76-6.11	6.95-12.54	2.74-8.35	4.08-9.18	1.62-2.67	6.30-7.04	3.98-5.19	methyl linolenate

Retention time and % peak area of compounds found in ethanolic extracts of *M. alba* tea products

No	Retention time (min)	% peak area														Compound name
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
12	49.5	11.78-19.95	29.65-33.71	15.47-20.47	12.57-20.78	14.27-33.00	19.50-24.26	5.13-8.61	8.83-10.15	8.28-17.06	23.82-33.56	5.66-12.53	12.23-18.98	27.44-38.94	14.65-20.69	phytol
13	50.05	-	0.44-0.75	-	0.17-0.39	0.16-0.61	-	1.83-2.66	0.97-1.94	1.19-1.94	0.66-1.11	0.95-1.74	0.63-1.15	0.64-0.73	0.49-0.58	methyl stearate
14	50.39	6.82-10.54	3.4-5.51	2.42-24.23	6.05-7.49	3.84-7.08	3.99-6.47	2.09-2.74	3.72-6.01	1.90-4.51	2.15-11.52	4.71-9.27	8.49-11.69	5.43-11.25	13.38-17.06	oleic acid
15	51.2	0.49-2.35	0.66-2.22	2.02-2.19	0.75-1.24	0.91-2.35	0.08-1.70	2.45-6.58	2.48-3.12	1.83-2.32	-	-	1.43-2.75	1.26-1.35	1.03-1.41	stearic acid
16	54.93	-	-	-	0.14-0.69	-	-	0.82-1.28	0.81-1.16	-	-	1.50-2.50	0.51-1.52	1.14-1.75	1.07-1.53	Unknown
17	55.82	-	-	-	-	-	-	0.29-0.62	0.42-1.61	-	-	-	-	-	-	Unknown
18	59.78	-	0.11-0.36	0.23-0.36	-	0.18-0.39	0.13-0.68	0.93-1.49	0.68-1.45	-	-	1.96-3.67	0.93-2.54	1.94-3.71	1.77-3.19	Unknown

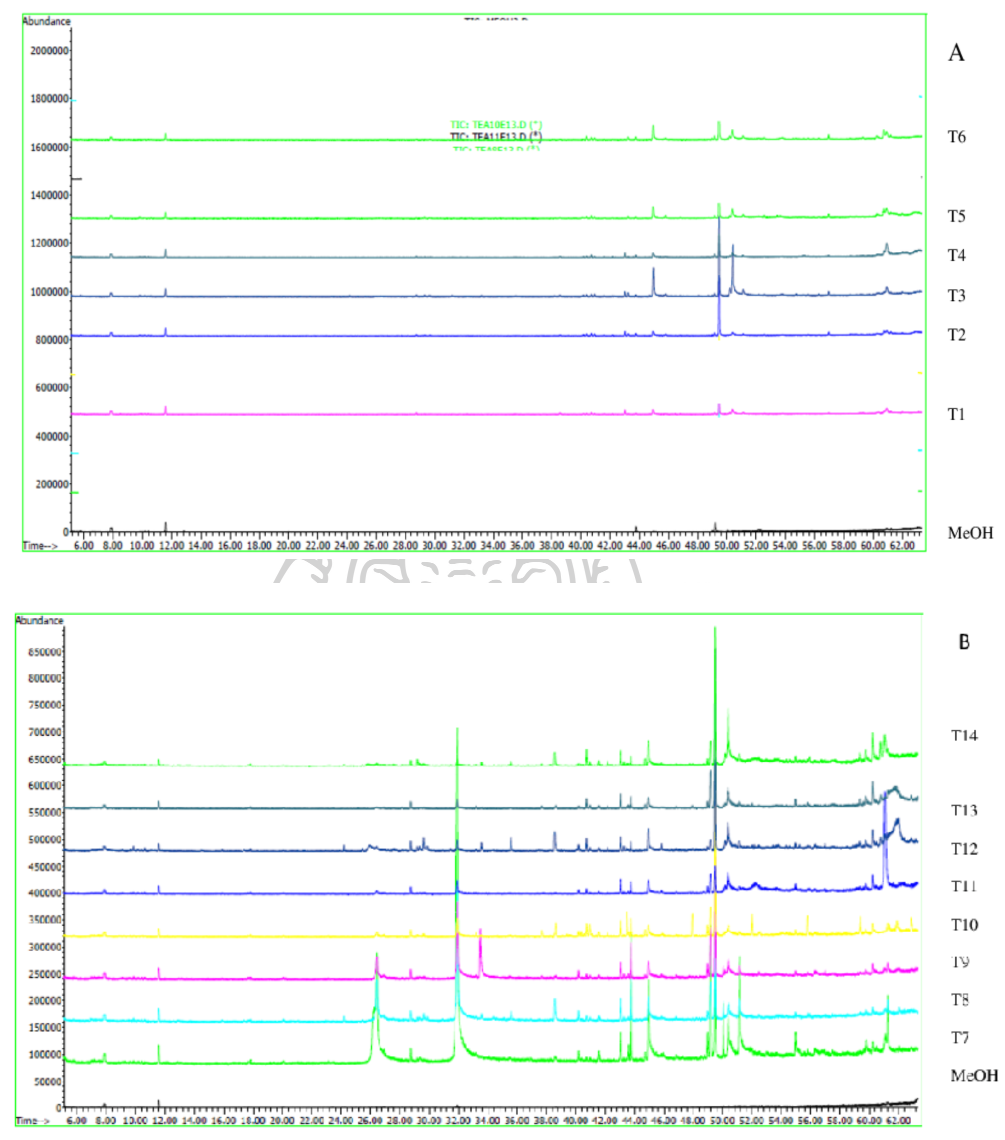


Figure 39 GC-MS fingerprint of ethanolic extracts of *M. alba* tea product, (A) T1-T6=Tea1-6, (B) T7-T14= Tea 7-14

4.2.4.7 High Performance Liquid Chromatography (HPLC) profiling

4.2.4.7.1 Method development

The analytical method described in reference (83) was modified to get good separation. The ratio of mobile phase, flow rate, column temperature and wavelength were explored. Although methanol (B) was used as a mobile phase in place of acetonitrile, the method could give better separation and reduce the more toxic solvent. Adding formic acid in the mobile phase (A, 0.1% formic acid in water) could

improve the resolution and eliminate peak tailing (225). Using flow rate just 0.3 mL/min also decreased the amount of solvent use. Gradient elution was set from 5% (B) to 95% (B) in 45 min and maintained at 95%(B) for 5 min. Then, the mobile phase was brought back to 5% (B) and held for 10 min for column equilibration. The column temperature was maintained at 35ºC and the detection wavelength was set at 320 nm for chlorogenic acid, caffeic acid and 360 nm for rutin and quercetin. Solvent used were filtered through a 0.22µm filter and degassed. The sample injection volume was 2 µL.

4.2.4.7.2 Method validation

Table 36 System suitability of chlorogenic acid, caffeic acid, rutin and quercetin

Compound	Average retention time (min)	Repeatability of Retention time %(RSD)	Repeatability of peak area %(RSD)	Conclusion
Accepted criteria		%RSD<1.0	%RSD<6	Satisfaction
Chlorogenic acid	25.63	0.02	0.07	Satisfaction
Caffeic acid	28.21	0.02	0.13	Satisfaction
Rutin	33.93	0.02	0.87	Satisfaction
Quercetin	39.11	0.02	1.25	Satisfaction

4.2.4.7.3 Specificity and System suitability

Specificity was assessed by retention time of the examined compounds on the chromatogram and UV spectrum of the related compounds. UV spectrum of peaks at retention time of 25.63, 28.21, 33.93 and 39.11min from the sample chromatogram corresponded to UV spectrum of chlorogenic acid, caffeic acid, rutin and quercetin from the standard chromatogram, respectively. Results from system suitability testing were shown in Table 36. All results were within acceptable range prescribed in (220, 226, 227) expect for %RSD of repeatability of peak area of quercetin.

4.2.4.7.4 Linearity and Range

The developed method exhibited linearity within the concentration range of 100-525 µg/mL for chlorogenic acid, 1.2-15.0 µg/mL for caffeic acid, 15-100 µg/mL for rutin and 5-20 for quercetin. R^2 was found to be 0.9975 for chlorogenic acid, 0.9997 for caffeic acid, 0.9975 for rutin and 0.9962 for quercetin and were shown in

Table 37. These results indicated a linear relationship between the concentration of analyte and area under the peak.

4.2.4.7.5 Sensitivity

The LOD value was 30.76, 0.26, 4.90, 1.45 μg/mL and LOQ value was 93.22, 0.77, 14.85 and 4.40 μg/mL for chlorogenic acid, caffeic acid, rutin and quercetin, respectively and were shown in Table 37.

Table 37 Calibration equation, LOD and LOQ of chlorogenic acid, caffeic acid, rutin and quercetin

Analyte	Calibration equation	r^2	Linear range	LOD (μg/mL)	LOQ (μg/mL)
Chlorogenic acid	Y=16.784x-161	0.9975	100-525	30.76	93.22
Caffeic acid	Y=31.621x-2.3527	0.9997	1.2-15.0	0.26	0.77
Rutin	Y=8.3548x-8.1538	0.9975	15-100	4.90	14.85
Quercetin	Y=16.97x-12.904	0.9962	5-20	1.45	4.40

4.2.4.7.6 Accuracy and Precision

The overall recovery percentage of 91.50-108.97 for chlorogenic acid, 83.51-98.92 for caffeic acid, 102.29-106.15 for rutin and 105.37-112.04 for quercetin were within the acceptable range of 80-115 % (145) and shown in Table 38. Intra and inter-day precision results were shown in Table 39. The value of relative standard deviation of intra and inter-day precision were in the range of 0.1-1.87 and lower than 2 % except for the value of quercetin. According to the results, the developed method was found to be good precise and accurate.

Table 38 Accuracy

Analyte	Amount added (μg/mL)	Average amount recovery (μg/mL)	% Recovery	Criteria (AOAC)
Chlorogenic acid	160-240	174.35-223.94	93.31-108.97 %	85-110
Caffeic acid	3.0-4.5	2.97-4.43	83.51-98.92 %	80-115
Rutin	32.0-52.8	33.36-56.05	102.29-106.15	85-110
Quercetin	8-12	8.96-12.64	105.37-112.04	80-115

Table 39 Intra-day and Inter-day precision

Analyte	Conc. (µg/mL)	Intra-day precision (% RSD)	Criteria (AOAC)	Inter-day precision (% RSD)	Criteria (AOAC)
Chlorogenic acid	131.25-394	0.16-0.46	3	0.21-0.96	4
Caffeic acid	2.5-7.5	0.10-0.30	6	0.24-0.79	8
Rutin	10.0-50	0.32-0.81	4-6	0.34-0.62	6
Quercetin	5-15	0.31-1.87	4-6	2.93-6.81	6-8

4.2.4.8 Content of chlorogenic acid, caffeic acid, rutin and quercetin in the aqueous extracts

Chlorogenic acid, caffeic acid, rutin and quercetin were reported in the previous studies as the major bioactive compounds in mulberry leaves. Chlorogenic acid was derivatives of caffeic acid whereas rutin was quercetin glycoside. Rutin was indicated in THP as a marker compound for identification. Chlorogenic acid and caffeic acid rutin and quercetin were found to present antioxidant and anti-diabetes activity (83, 145, 245). Quantification of these four compounds were interesting and could certify quality of raw material.

The amount of active in the extracts were expressed as mg/100g of extract weight. The concentration of active compounds in aqueous extracts of *M. alba* reference leaves were 212.06-838.78 mg/100g for chlorogenic acid, 0-14.64 mg/100g for caffeic acid, and 0-202.36 mg/100g for rutin, however quercetin was not found and results were shown in Table 40 and Figure 40 (A) and (B) and Figure 41. KP-NR leaves possessed the highest quantity of three bioactive compounds. The content of active compound was statistically significantly different at 95% confidence interval (Appendix).

Table 40 Content of chlorogenic acid, caffeic acid, rutin and quercetin in aqueous extracts of reference leaf, sample leaf and tea products of *M. alba*

Sample	Aqueous Extract (mg/ 100g) (mean ± SD)			
	Chlorogenic acid	Caffeic acid	Rutin	Quercetin
Reference leaves				
BR-BR	212.06 ± 14.64	BLQ	31.32± 0.51	BLQ
BR-KB	BLQ	BLQ	BLQ	BLQ
BR-NR	217.26 ± 22.07	BLQ	BLQ	BLQ
KP-BR	617.16 ± 37.55	11.71 ± 2.46	138.84 ± 31.18	BLQ
KP-KB	BLQ	BLQ	BLQ	BLQ
KP-NR	838.78 ± 30.48	14.64 ± 1.22	202.36 ± 14.30	BLQ
Sample leaves				
M	136.84 ± 0.49	1.92± 0.29	5.17 ± 0.49	BLQ
T1	137.07 ± 0.34	1.90± 0.25	4.42 ± 0.09	BLQ
T2	135.36 ± 0.20	0.19± 0.03	3.91 ± 0.004	BLQ
T3	170.42 ± 6.10	1.89± 0.04	6.95 ± 0.19	BLQ
UL-T1	150.27 ± 3.68	1.35± 0.07	4.34± 0.51	BLQ
Tea products				
Tea1	31.20 ± 1.87	14.70 ± 27.78	5.99 ± 0.24	BLQ
Tea 2	BLQ	BLQ	BLQ	BLQ
Tea 3	211.57 ± 19.30	BLQ	38.74 ± 0.50	BLQ
Tea 4	177.54 ± 14.72	3.96 ± 0.39	94.95 ± 14.65	BLQ
Tea 5	BLQ	BLQ	BLQ	BLQ
Tea 6	58.70 ± 2.92	2.07 ± 0.27	10.87 ± 2.09	BLQ
Tea 7	BLQ	15.40 ± 3.95	BLQ	BLQ
Tea 8	BLQ	61.44 ± 7.45	49.98 ± 2.81	BLQ
Tea 9	BLQ	BLQ	BLQ	BLQ
Tea 10	BLQ	32.36 ± 2.61	46.22 ± 5.70	BLQ
Tea 11	189.48 ± 0.71	BLQ	50.89 ± 6.37	BLQ
Tea 12	BLQ	BLQ	BLQ	BLQ
Tea 13	57.96 ± 1.87	1.52 ± 0.03	17.15 ± 6.06	BLQ
Tea14	BLQ	BLQ	BLQ	BLQ

BLQ = below limit of quantification

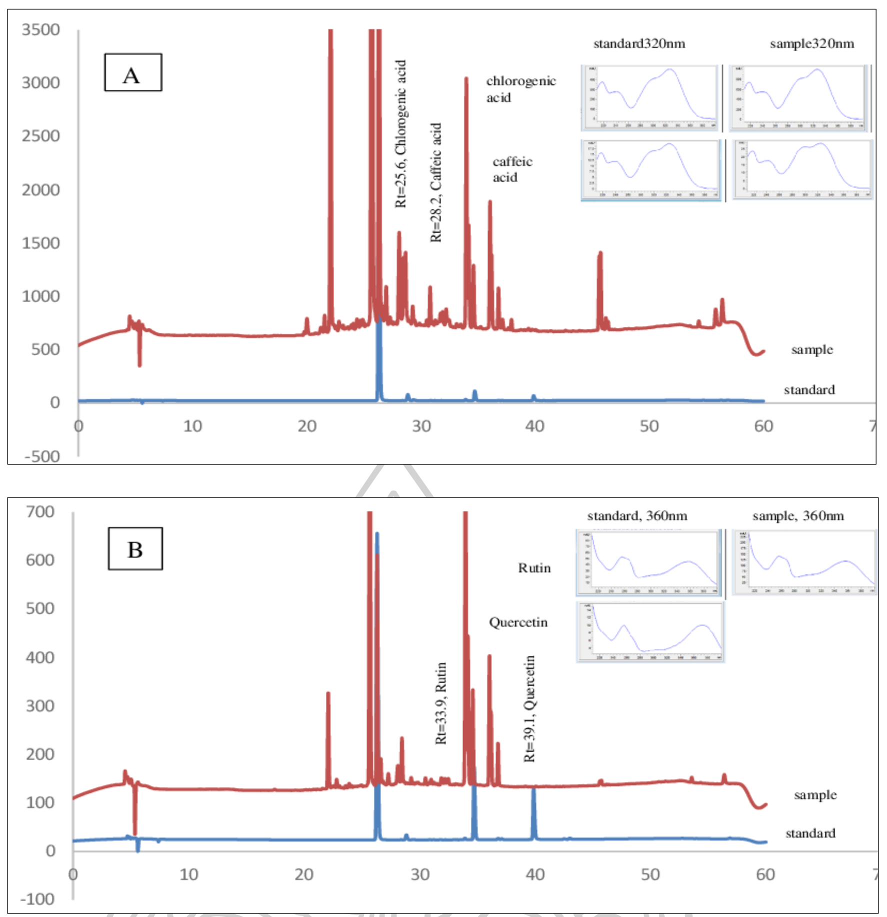


Figure 40 Chromatogram of standard solution and sample extract (KP-NR). (A) standard chlorogenic acid and caffeic acid, detected at 320 nm (B) rutin and quercetin, detected at 360 nm

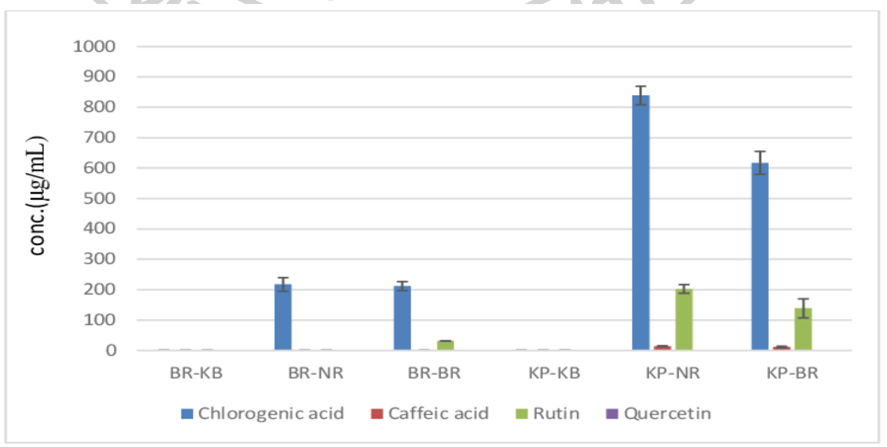


Figure 41 Content of active compounds in aqueous extracts of reference leaves of *M. alba*

The concentration range of active compounds in aqueous extracts of sample leaves of *M. alba* were 135.36-170.42 mg/100g for chlorogenic acid, 0.19-1.92 mg/100g for caffeic acid and 3.91-6.95 mg/100g for rutin. Quercetin was not found in all samples and this may be due to the solvent used and the results were shown in Table 40 and Figure 42. T3 possessed the highest quantity of chlorogenic acid and rutin. Sample M possessed the highest quantity of caffeic acid. The present study detected more content of chlorogenic acid and rutin than previous studies (83, 145, 245). Our study showed that chlorogenic acid was the most abundant compound in the leaves except leaves from Kanjanaburi (BR-KB, KP-KB).

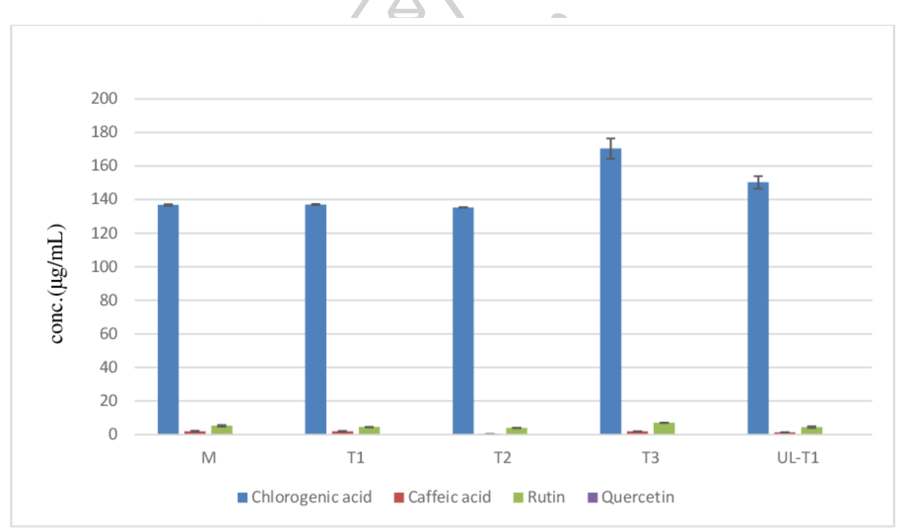


Figure 42 Content of active compound in aqueous extracts of sample leaves of *M. alba*

The concentration of active compounds in aqueous extract of *M. alba* tea products were 0-211.57 mg/100g for chlorogenic acid, 0-61.44 mg/100g for caffeic acid, 0-94.95 mg/ 100g for rutin, and quercetin was not found. Results were shown in Table 40 and Figure 43. Among 14 samples, Tea 3 possessed the highest quantity of chlorogenic acid, Tea 8 possessed the highest quantity of caffeic acid and Tea 4 possessed the highest quantity of rutin. Tea 2, 9, 12 and 14 did not showed the four active compounds. Results showed that different brands gave different quality of leaf extracts. Comparing among references leaves and tea products, reference leaves possessed the highest amount of chlorogenic acid and rutin while tea products possessed the highest amount of caffeic acid.

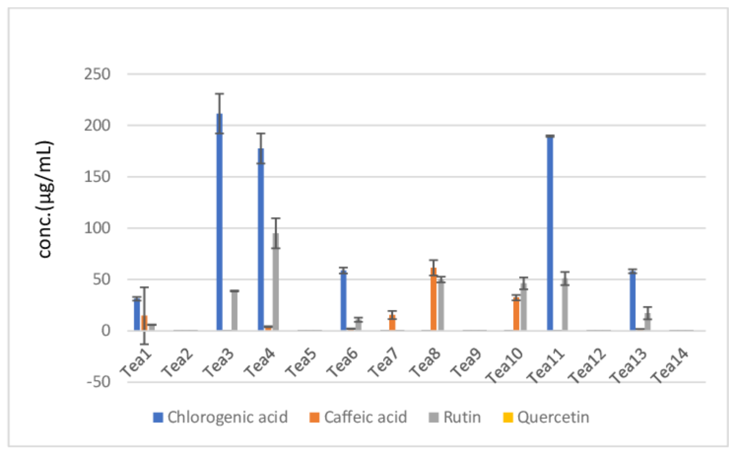


Figure 43 Content of active compounds in aqueous extracts of *M. alba* tea products

4.2.4.9 LC-MS profiling

Table 41 showed the identified compounds in aqueous extract of *Morus alba* leaf by LC-MS. Peak at retention time 25.68 min with m/z value at 191, 353 amu could be further identified as chlorogenic acid (5-caffeoylquinic acid). Peak at retention time 28.03 min with m/z value at 179 amu could be further identified as caffeic acid. Peak at retention time 33.97 min with m/z value at 609 could be further identified as rutin. Peak at retention time 39.11 min with m/z value at 301 and could be further identified as quercetin. Previous researches reported various derivatives of phenolic compounds (chlorogenic acid, caffeic acid) and flavonoids (rutin) in mulberry leaf extracts (39, 246-248).

The UV spectrum of the compounds at the retention time of 22.09 and 26.32 min from reference leaves were similar to that of chlorogenic acid whereas those of 30.99 and 34.18 min were similar to rutin. The injection of reference leaf extract to LC-MS showed the m/z as in Table 63. It supported the fact that there was different form of caffeic acid and quercetin in mulberry leaves.

Table 41 *m/z* of compounds with similar UV spectrum

No	Retention time(min)	m/z (amu)	Expected compound
1	22.09	353, 191, 135	neochlorogenic acid (3-caffeoylquinic acid)
2	25.68 (chlorogenic acid)	353, 191	chlorogenic acid (5-caffeoylquinic acid)
3	26.32	353, 173	cryptochlorogenic acid (4-caffeoylquinic acid)
4	28.03 (caffeic acid)	179	caffeic acid
5	30.99	755	quercetin-rhamnose-hexose-rhamnose
6	33.97 (rutin)	609	rutin
7	34.18	609	quercetin rutinoside isomer
8	39.11 (quercetin)	301	quercetin

4.2.5. Principle Component Analysis (PCA)

PCA was an unsupervised machine learning method that transformed a larger number of original variables which had correlations into a smaller number of uncorrelated variables called factors or principal components. Generally, PCA interpretation was displayed as a two-dimensional graph where the principal axis represented the directions of the first two main principal components (First principal component, PC1 vs Second principal component, PC2): 1) scores plot and 2) loadings plot.

4.2.5.1 ICP-MS

Table 42 showed eigenvalues (percentage of variance for each component), correlations of each variable with component 1 (PC1) and 2 (PC2).

Table 42 PCA: eigenvalues (percentage of variance for each component), correlations of each variable with component 1 (PC1) and 2 (PC2)

	eigenvalue	proportion	cumulative
PC1	1.6998	0.3400	0.3400
PC2	1.3458	0.2692	0.6092
PC3	1.1362	0.2272	0.8364
PC4	0.5966	0.1193	0.9557

Score plot showed that the present of five heavy metals in all reference leaves were in the same quadrant (negative to PC1 but positive to PC2) and different from many tea products and sample leaves. Loading plot showed that reference leaves were

in good control of environment and have less contamination of all five heavy metals. The sample leaves showed major contamination of Pb that might be from no environmental control such as proximity to roadside. Major contamination of Cd, Ni, As and Ba were found in some tea products.

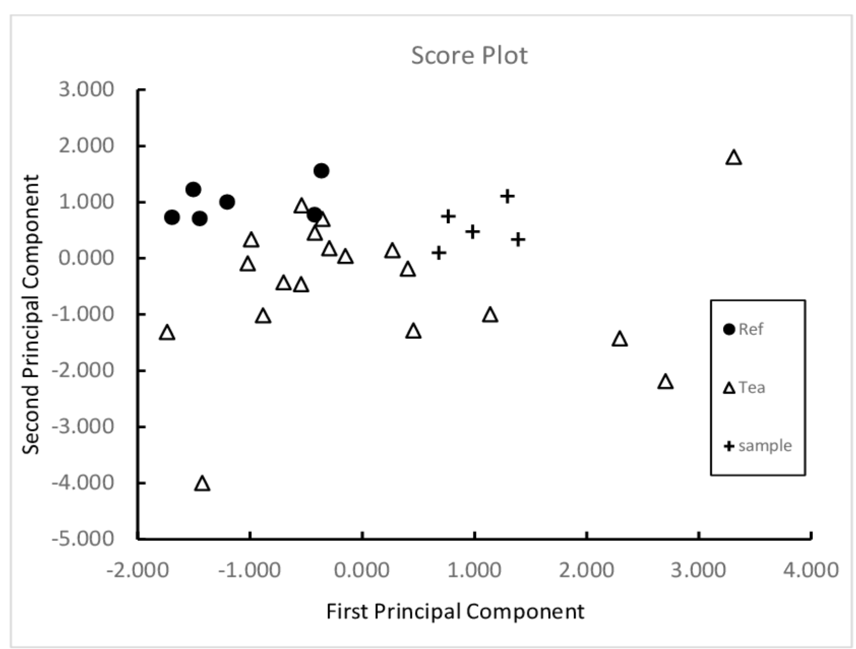


Figure 44 Scores plots obtained from heavy metal (As, Cd, Pb, Ni and Ba) contents of 3 sources of mulberry leaves

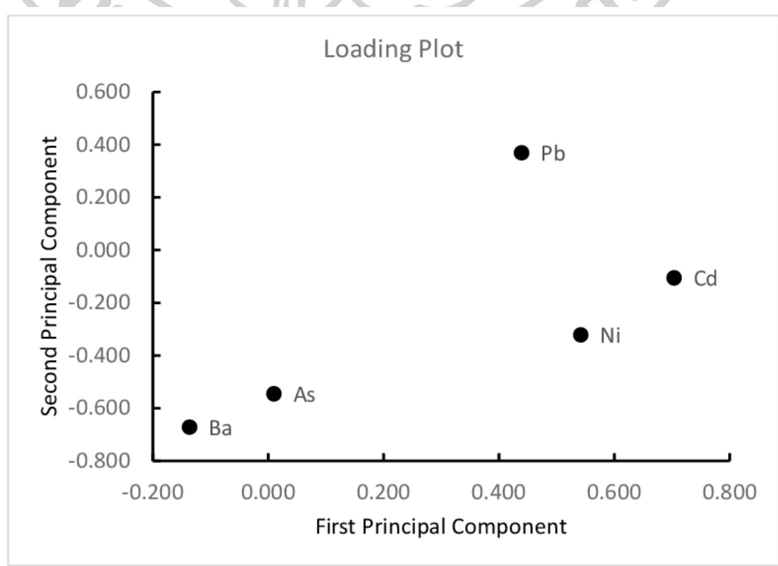


Figure 45 Loadings plots of 5 heavy metals (As, Cd, Pb, Ni and Ba) from 3 sources

Pearson's correlations coefficient at the significance level of 0.05 among five metals in references and tea products were shown in Table 43. Cadmium level revealed a medium positive relationship with Ni level. Both metals were found predominantly in products from tea products. A small relationship between As and Ba contents were also found whereas there was a small negative relationship between Ni and Pb levels.

Table 43 Correlation among As, Cd, Pb, Ni and Ba contents in references and all samples

	As	Cd	Pb	Ni	Cell content
Cd	0.030				Pearson correlation
	0.797				P-value
Pb	-0.009	0.197			
	0.936	0.091			
Ni	0.043	0.593	-0.257		
	0.714	0.000	0.026		
Ba	0.388	0.078	0.026	0.104	
	0.001	0.508	0.824	0.374	

4.2.5.2 GC-MS

The percentage peak area of 27 variables corresponding to all chemical constituents from GC-MS chromatograms which was analyzed by PCA. PC1 in the scores plot of Figure 46 presented differentiation among 2 groups. PC1 and PC2 collectively were able to explain the highest variance of 53% information and the first seven uncorrelated principal components explained 88% of the total variation.

Figure 46 showed that all leaf samples from NR province strongly negatively correlated with PC1, whereas samples from BR and KB provinces weakly positively correlated with PC1. The loading bar plot in Figure 47 illustrated that 14 marker peaks out of 27 peaks (with loadings <-0.15 or >0.15) at retention time 40.18 (c5, myristaldehyde), 43.76 (c11, methyl palmitate), 44.99 (c12, palmitic acid), 45.97 (c15, ethyl palmitate), 48.99 (c16, methyl linoleate), 49.19 (c17, methyl linolenate), 49.52 (c18, phytol), 50.05 (c19, methyl stearate), 50.18 (c20, linoleic acid), 50.39 (c21, oleic acid), 51.20 (c22, stearic acid), 52.57 (c23, unknown), 55.59 (c24, trans-

ferulic acid), and 60.74 (c26, monopalmitin) were able to discriminate among the leaves from KB, NR, and BR provinces. With negative PC1-loadings, 5 marker peaks at retention time 44.99 (c12), 45.97 (c15), 50.18 (c20), 50.39 (c21) and 51.20 (c22) corresponded to samples from NR. On the other hand, with positive PC1-loadings, 9 marker peaks at retention time 40.18 (c5), 43.76 (c11), 48.99 (c16), 49.19 (c17), 49.52 (c18), 50.05 (c19), 52.57 (c23), 55.59 (c24) and 60.74 (c26) corresponded to samples from KB and BR provinces.

From the chemometric analysis, for both cultivars, samples from NR province could be characterized by 5 chemical constituents, i.e., palmitic acid, ethyl palmitate, linoleic acid, oleic acid and stearic acid. However, the GC-MS fingerprints showed that the % peak area of palmitic acid, oleic acid and linoleic acid were much higher in leaves from NR than KB and BR provinces.

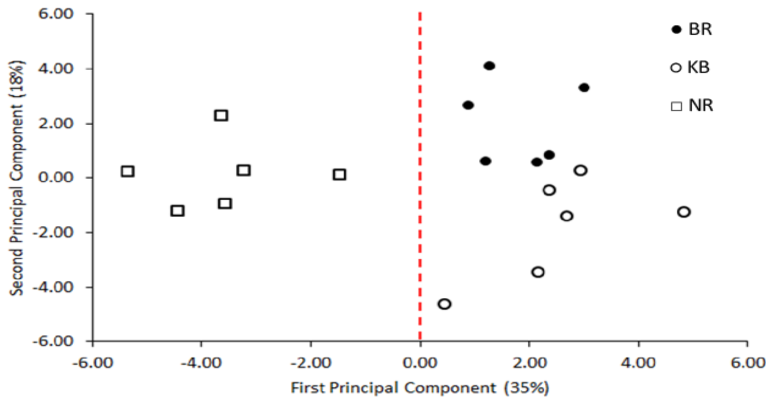


Figure 46 Scores plots obtained from the PCA of *M. alba* leaves

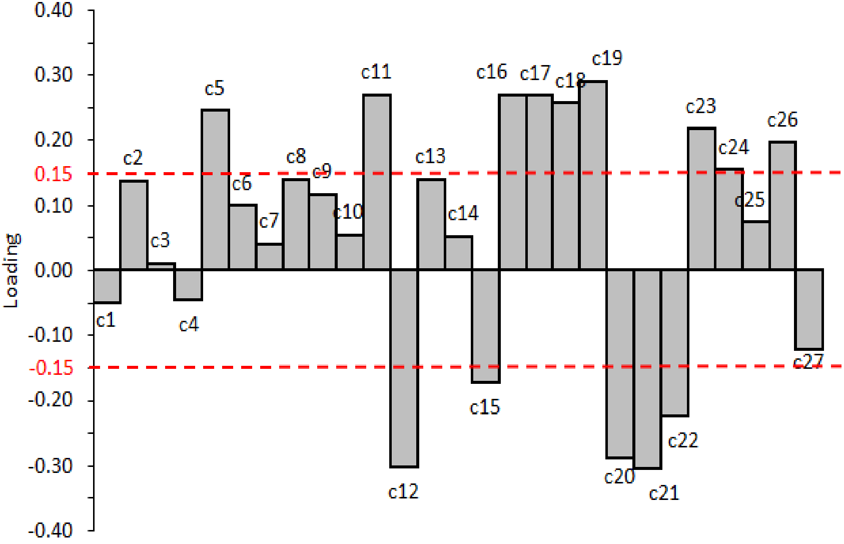


Figure 47 Loadings bar plots obtained from the PCA of *M. alba* leaves

4.2.5.3 HPLC

The result of PCA on HPLC result of six reference leaves were shown in Figures 48-49. Figure 26 showed the score plot of different sources of six reference leaves. In which leaf sample from Kanjanaburi (KB) province strongly positively correlated with PC1, whereas samples from Buriram (BR) and Nakhon Ratchasima (NR) provinces weakly positively to strongly negatively correlated with PC1.

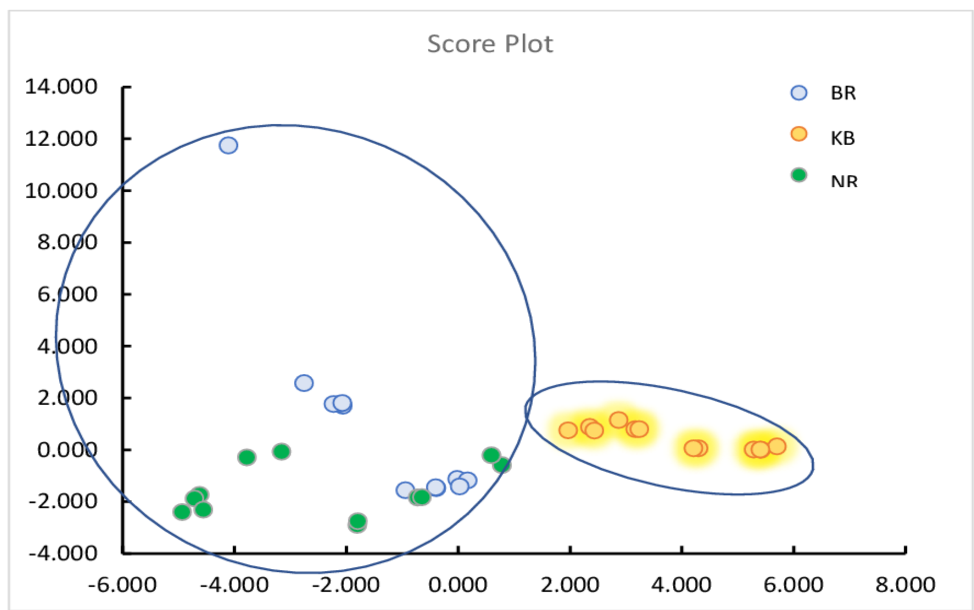


Figure 48 Score plot of different sources of reference mulberry leaves

Table 44 showed that % area of compound c6 from HPLC-DAD (360 nm) were due to chlorogenic acid group (derivative of caffeic acid) at retention time 22.09 and 26.32 min and rutin group (derivative of quercetin) at retention time 30.99 and 34.18-min. *M. alba* can show antioxidant and antidiabetic activities because various forms of caffeic acid and quercetin. Therefore, in the present study, alpha-glucosidase inhibitory activity of extract of *M. alba* may be due to various forms of caffeic acid and quercetin in the extract.

Table 44 % area of compounds C6, chlorogenic acid group and rutin group from HPLC-DAD (360 nm)

	AVE % area		
	C6	Chlorogenic acid	Rutin
BR-BR	22.36	35.26	12.74
BR-KB	43.24	0.65	-
BR- NR	23.73	33.42	10.71
KP-BR	5.57	50.89	25.06
KP-KB	41.77	6.99	10.39
KP-NR	6.69	42.36	28.88

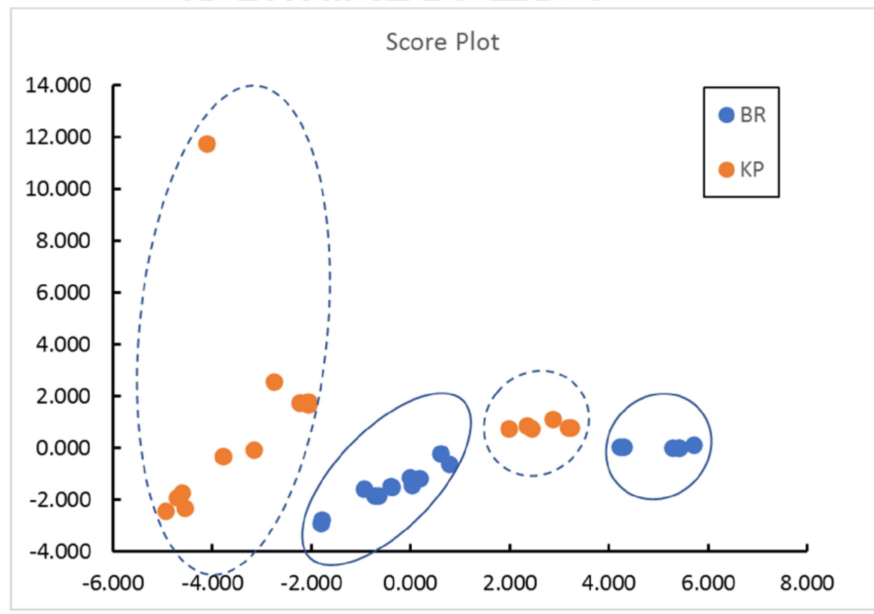


Figure 49 Score plot of different cultivar of reference mulberry leaves

Figure 49 illustrated score plot of different cultivar of reference mulberry leaves whereas it could differentiate the cultivar (BR) and (KP) of reference mulberry leaves. Therefore, PCA study on HPLC results could differentiate between different sources from (BR, KB, NR) and could not differentiate between different cultivar Buriram (BR) and Khun Pai (KP).

4.3 Conclusion

In this study, mulberry reference leaves, leaves from various source, and mulberry tea infusion products were evaluated. Our study focused on biological effects, anti-glucosidase activity, of tea infusion drinking and raw material quality control, including the classification of the source of raw material.

Anti-glucosidase activity of mulberry leaves as well as commercial tea infusion extracted with boiling water was compared to acarbose. The result showed that all boiling water extracts in this study showed this activity. However, the activity of 2 reference leaves, KP-NR and KP-BR, as well as 3 infusion tea products, Tea7, Tea11 and Tea14, possessed lower activity than acarbose. The extracts of leaves BR-NR, Tea8 and Tea12, were interesting for their high anti-glucosidase activity with IC_{50} less than 200 μg/mL. The extract of upper leaves of T1 (UL-T1) showed a little better activity than extracts of normal leaves T1. The anti-glucosidase effect was expected to come from various forms of caffeic acid (chlorogenic acid group) and quercetin (rutin group) in the extracts.

In **preliminary phytochemical screening**, the extract showed the present of flavonoids, phenols, terpenoids, tannins, steroids and saponin.

For the **quality control** purpose, all sample were determined for loss on drying, heavy metal contamination, TPC, microbial contamination, and the fingerprints of FTIR spectrum, GC-MS chromatograms, LC-DAD chromatograms. All tested samples complied with the criteria of THP, ASEAN guidelines, WHO guidelines, and ICH guidelines in loss on drying, heavy metal and microbial contamination. FTIR spectrum of sample leaves and infusion tea products were similar to reference leaves. GC-MS chromatograms showed high amount of phytol and fatty acid such as palmitic acid, oleic acid and stearic acid. Different sources caused variation in the amount of these compounds. However, the fingerprints were similar in all samples. LC-MS chromatograms showed more variation in the amounts of major compounds. Moreover, the major compounds were found in various derivatives of caffeic acid and quercetin. These three types of fingerprints could be used for identification of mulberry leaves.

PCA analysis was performed with 2 main principal components, PC1 and PC2, through data of heavy metal contents and chromatogram of GC-MS and HPLC-

DAD. Heavy metal contents of reference leaves showed similar of PC1 and PC2 that presented a good control in the environment of cultivation and different from sample leaves and tea products. This might be from the different in cultivation control and manufacturing process. PCA of GC-MS and HPLC-DAD implied obvious difference in the original province of reference leaves but cultivars could not be discriminated.

CHAPTER V
OVERALL CONCLUSION

The extracts of *M. longifolia* leaf and flower from Myanmar, and *M. alba* leaf from both Thailand and Myanmar were studied for their alpha-glucosidase inhibitory activity, preliminary phytochemical screening, and quality determination. In addition, alpha-glucosidase inhibitory activity and quality determination of *M. alba* tea products marketed in Thailand were evaluated.

Boiling water extract of *M. longifolia* leaf (L1) exhibited the highest alpha-glucosidase inhibitory activity with the IC_{50} of 15.23±1.32 µg/mL, higher than that of a standard acarbose (IC_{50} = 763.92±22.27 µg/mL), and *M. alba* leaves (IC_{50} = 195.73±34.84 - 928.31±84.5 µg/mL) and tea products (169.03±24.89 - 3219.07±62.65 µg/mL). From the IC50, extract from *M. longifolia* leaf were 10 times more potent than that of *M. alba* in the inhibition of alpha-glucosidase activity *in vitro*.

For the quality determination, the moisture content (%LOD) of all samples from both plants were within the limit of Thai Herbal Pharmacopoeia (less than 11%). The amounts of arsenic, cadmium and lead were within the limit of WHO and ASEAN Guideline for Herbal Medicines, and the amount of nickel and barium were within the limit of ICH Guidelines for Quality Control of Herbal Drugs (Q3D). Flower samples of *M. longifolia* were free from microbial contamination according to USP 40 and Thai Pharmacopoeia 2005 criteria, while leaf samples were contaminated with non-pathogenic *Clostridium* species. Almost all leaf samples of *M. alba* were free from microbial contamination, except cultivar Buriram 60 from Buriram province and leaf from Nonthaburi province (T2) that non-pathogenic *Clostridium* species were found. The ATR-FT-IR and GC-MS fingerprints were established for the identification and authentication of *M. longifolia* leaf and flower, and *M alba* leaf. The HPLC method was developed and validated for the determination of chlorogenic acid, caffeic acid, rutin, and quercetin in both plant leaf extracts. This developed method will be useful for the quantification of active constituents and quality assessment of *M. longifolia* leaf in the future.

From these results, leaf and flower of *M. longifolia* and *M. alba* will be potential sources for hypoglycemic compounds, and regularly consumption of these two plants as herbal tea may result in the reduction of blood sugar. Although the plant materials in this study contained microbial organisms and heavy metals within the ranges limited for herbal medicines, pesticide contamination and toxicity assessment should be evaluated. Moreover, harvest time and plant sources should be considered to assure the content of active constituents and the quality of the plant materials.

Standardization methods for *M. longifolia* raw material such as evaluation of alpha-glucosidase inhibitory activity and microbial contamination as biological parameter; determination of loss on drying as physical parameter; together with determination of total phenolic content, determination of heavy metal contamination, and chemical fingerprints from ATR-FT-IR, GC-MS and HPLC spectra as chemical parameter were performed and could be established as the quality control method for this plant.

The official monograph of *M. longifolia* raw material has never been presented in any pharmacopoeia. With the results from this project, the quality standard of this plant has been established with guidelines from Thai herbal pharmacopoeia. This standard can ensure consistency in the quality of use of this plant in the future.

Madhuca longifolia

Meze leaf

Synonyms Myintzu- thaka- natpan Leaf, honey tree Leaf, butter tree Leaf, mahua Leaf, miilluppai Leaf

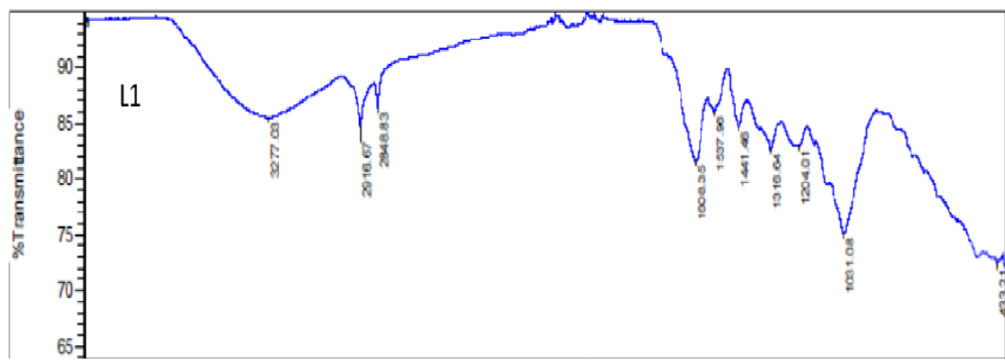
Meze leaf is a dried leaf of *Madhuca longifolia* (J. Koenig ex L.) Macbr. Family (Sapotaceae).

Constituents Meze leaf contains flavonoid (e.g., quercetin), polyphenolic (e.g., gallic acid), long chain fatty acid (e.g. palmitic acid), terpenoids (e.g. β-carotene), etc. (23).

Description of the plant Medium to large deciduous tree growing widely under dry tropical and sub-tropical climatic conditions and provides plenteous amount of shade. It is a drought-resistant, needs a huge amount of water and cannot grow well under shade. It can grow up in shallow, boulders, clay, and calcareous soil but grows best in sandy soil. It is found up to an altitude of 1200 m with rainfall from 550-1500 mm and a mean annual maximum temperature of 28-50°C. It can grow up to a height of 17-18m. Leaves are leathery and are present in the form of bunches at the endings of branches, shortly acuminate, elliptic, shortly acuminate band base cuneate. They are pointed tips and have a thick texture, a hairy surface on the beneath, and strong nerves. Flowers are frequent, white in color, fascicled and are present at the branch endings, drooping on pedicels. They have a sweet flavour and are fleshy in texture. Fruits are green and fleshy which contain 3-4 brown-colored ellipsoidal seeds with shiny coat. The tree possesses oil-bearing seeds and flowers. Flowering of the plant occurs from March to April and collection of seeds can be performed in May, June and July.

Packaging and storage Meze leaf shall be kept in well-closed containers, protected from light, and stored in a dry place (29).

Identification ATR-FT-IR, **(Fig. 50),** wave number (cm^{-1})**,** 1031.1(C-O stretching), 1204.0 (C-O stretching), 1316.6 (C=C stretching), 1441.5 (C-H banding), 1537.9 (C=C stretching), 1608.3 (C=C stretching), 2848.8 (C-H stretching), 2916.6 (C-H stretching), and 3277.0 (O-H stretching).



Wavenumbers (cm^{-1})

Figure 50 FT-IR spectra of *M. longifolia* leaf

Microbial contamination No colony of *E. coli* in 1 g sample, no colony of S. *aureus* in 1 g sample, no colony of *Salmonella* species in 10 g sample, no colony of spore and non-spore *Clostridia* species in 10 g sample (216).

Loss on Drying Not more than 10.0 percent w/w after drying at 105° to constant weight (29).

Total Phenolic content 86.89-179.43 mg GAE /g extract for boiling water extract.

Heavy metal content Not more than 5 mg/kg of As (224), 0.3 mg of Cd (32, 224), 10 mg/kg of Pb (32), 20 mg/kg of Ni (219) and 140 mg/kg of Ba (219).

Meze flower

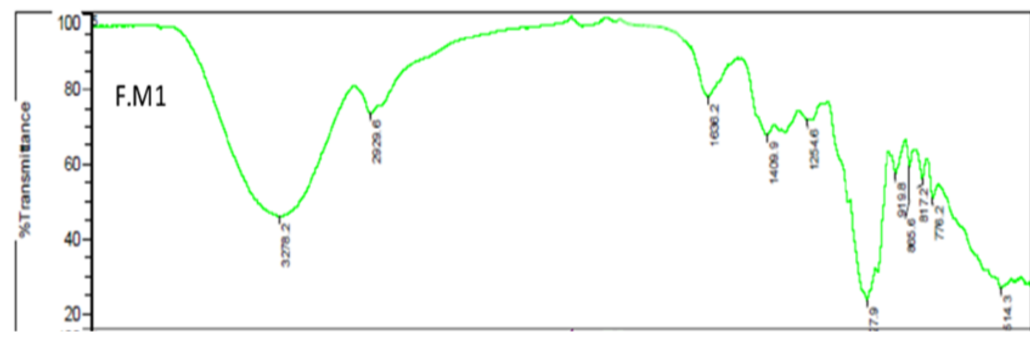
Synonyms Myintzu- thaka- natpan Flower, honey tree Flower, butter tree Flower, mahua Flower, miilluppai Flower

Meze flower is a dried flower of *Madhuca longifolia* (J. Koenig ex L.) Macbr. Family (Sapotaceae).

Constituents Vitamin (e.g., vitamin A, vitamin C), essential amino acid (thiamine, niacin), etc. (24).

Packaging and storage Meze flower shall be kept in well-closed containers, protected from light, and stored in a dry place (29).

Identification ATR-FT-IR**, (Fig. 51**), wave number (cm^{-1}) 776.2 (C-H banding), 817.2 (C-H banding), 865.6 (C-H banding), 919.8 (C-H banding), 1027.9 (C-O stretching), 1254.6 (C-O stretching), 1409.9 (C-H banding), 1636.2 (C=C stretching), 2929.6 (C-H stretching) and 3278.2 (O-H stretching).



Wavenumbers (cm^{-1})

Figure 51 FT-IR spectra of *M. longifolia* flower

Microbial contamination No colony of *E. coli* in 1 g sample, no colony of S. *aureus* in 1 g sample, no colony of *Salmonella* species in 10 g sample, no colony of spore and non-spore *Clostridia* species in 10 g sample (216).

Loss on Drying Not more than 10.0 percent w/w after drying at 105° to constant weight (29).

Total Phenolic content 8.46-9.86 mg GAE /g extract for boiling water extract.

Heavy metal content Not more than 5 mg/kg of As (224), 0.3 mg of Cd (32, 224), 10 mg/kg of Pb (32), 20 mg/kg of Ni (219) and 140 mg/kg of Ba (219).

REFERENCES

1. World Health Organization, World Diabetes Day 2018; Family and Diabetes [Internet]. 2018. Available from: https://www.int.
2. Roglic G, Organization WH. Global Report on Diabetes: World Health Organization; 2016.
3. Raut N, Dhore P, Saoji S, Kokare D. Selected bioactive natural products for diabetes mellitus. Studies in Natural Products Chemistry. 2016;48:287-322.
4. Gupta RK, Kesari AN, Watal G, Murthy P, Chandra R, Maithal K, et al. Hypoglycaemic and antidiabetic effect of aqueous extract of leaves of Annona squamosa (L.) in experimental animal. Current Science. 2005:1244-54.
5. Raut NA, Gaikwad NJ. Anti-diabetic potential of fractions of hydro-ethanol extract of Crataeva nurvala Buch. Ham Int J Pharm Phytopharmacol. 2014;3:281-3.
6. Samy RP, Ignacimuthu S. Antibacterial activity of some folklore medicinal plants used by tribals in Western Ghats of India. Journal of Ethnopharmacology. 2000;69(1):63-71.
7. Raut NA, Gaikwad NJ. Antidiabetic potential of fractions of hydro-ethanol extract of Cyperus rotundus L.(Cyperaceae). Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2012;3(4):1014-19.
8. Raut NA, Gaikwad NJ. Antidiabetic activity of hydro-ethanolic extract of Cyperus rotundus in alloxan induced diabetes in rats. Fitoterapia. 2006;77(7-8):585-8.
9. Kwon Y-II, Vattem DA, Shetty K. Evaluation of clonal herbs of Lamiaceae species for management of diabetes and hypertension. Asia pacific journal of clinical nutrition. 2006;15(1):107.
10. Shetty K, Wahlqvist M. A model for the role of the proline-linked pentose-phosphate pathway in phenolic phytochemical bio-synthesis and mechanism of action for human health and environmental applications. Asia Pacific Journal of Clinical Nutrition. 2004;13(1).
11. Paliyath G, Murr DP, Handa AK, Lurie S. Postharvest Biology and Technology of Fruits, Vegetables, and Flowers: Wiley; 2009.
12. Qian M, Rimando AM. Flavor and health benefits of small fruits: American Chemical Society; 2010.
13. Miao J, Li X, Zhao C, Gao X, Wang Y, Gao W. Active compounds, antioxidant activity and α-glucosidase inhibitory activity of different varieties of Chaenomeles fruits. Food Chemistry. 2018;248:330-9.
14. Ganapaty S, Nandeesh R, Veerapur V, Thippeswamy B, Shivasharan D. In vivo and In vitro Anti-diabetic effects of Madhuca indica Roxb. Alloxan-Induc Diabet Rats IJAPBC. 2013;2:282-90.
15. Ghosh R, Dhande I, Kakade V, Vohra R, Kadam V. Mehra. Antihyperglycemic activity of Madhuca longifolia in alloxan-induced diabetic rats. The Internet Journal of Pharmacology. 2009;6(2):1-12.
16. Prashanth S, Kumar AA, Madhu B, Kumar YP, Prashanth S. Antihyperglycemic and antioxidant activity of ethanolic extract of Madhuca longifolia bark. International Journal of Pharmaceutical Sciences Review and Research. 2010;5(3):89-94.
17. Nakamura S. Hypoglycemic effect of Morus alba leaf extract on postprandial blood glucose and insulin levels in patients with type 2 diabetes treated with sulfonylurea hypoglycemic agents. J Diabetes Metab. 2011;9.
18. Singab ANB, El-Beshbishy HA, Yonekawa M, Nomura T, Fukai T. Hypoglycemic effect of Egyptian Morus alba root bark extract: effect on diabetes and lipid peroxidation of streptozotocin-induced diabetic rats. Journal of ethnopharmacology. 2005;100(3):333-8.

19. Yang Z, Wang Y, Wang Y, Zhang Y. Bioassay-guided screening and isolation of α-glucosidase and tyrosinase inhibitors from leaves of Morus alba. Food chemistry. 2012;131(2):617-25.
20. Zhang M, Chen M, Zhang H-Q, Sun S, Xia B, Wu F-H. In vivo hypoglycemic effects of phenolics from the root bark of Morus alba. Fitoterapia. 2009;80(8):475-7.
21. Zhang Y, Ren C, Lu G, Cui W, Mu Z, Gao H, et al. Purification, characterization and anti-diabetic activity of a polysaccharide from mulberry leaf. Regulatory Toxicology and Pharmacology. 2014;70(3):687-95.
22. Akshatha KN, Murthy SM, Lakshmidevi N. Ethnomedical uses of Madhuca longifolia - a review. International Journal of Life science & Pharma Research. 2013;3(1):44-53.
23. Sunita M, Sarojini P. Madhuca lonigfolia (Sapotaceae): A review of its traditional uses and nutritional properties. International Journal of Humanities and Social Science Invention. 2013;2(5):30-6.
24. Verma N, Jha K, Kumar U, Deepak K, Singh NK, Singh AK, et al. Biological properties, phytochemistry and traditional uses of Mahua (Madhuca longifolia): A review. Int J Adv Res Innov. 2014;2(3):630-8.
25. Chan EW-C, Phui-Yan L, Siu-Kuin W. Phytochemistry, pharmacology, and clinical trials of Morus alba. Chinese journal of natural medicines. 2016;14(1):17-30.
26. eep S, Anindya B, Alok S, Abhishek B. Traditional uses, phytochemistry and pharmacology of Morus alba Linn.: A review. Journal of Medicinal Plants Research. 2013;7(9):461-9.
27. Zafar MS, Muhammad F, Javed I, Akhtar M, Khaliq T, Aslam B, et al. White mulberry (Morus alba): A brief phytochemical and pharmacological evaluations account. International journal of agriculture and biology. 2013;15(3).
28. Kulkarni KM, Patil LS, Khanvilkar VV, Kadam VJ. Fingerprinting techniques in herbal standardization. Indo American Journal of Pharmaceutical Research. 2014;4(2):1049-62.
29. Sāthāranasuk TK, Kānphæt TKW, Pharmacopoeia SotEotTH, Sciences MoPHDoM. Thai Herbal Pharmacopoeia 2018: Department of Medical Sciences, Ministry of Public Health; 2018.
30. Filipiak-Szok A, Kurzawa M, Szłyk E. Determination of toxic metals by ICP-MS in Asiatic and European medicinal plants and dietary supplements. Journal of Trace Elements in Medicine and Biology. 2015;30:54-8.
31. Kalkisim O, Ozdes D, Baltaci C, Duran C. Assessment of heavy metal contents of mulberry samples (fruit, leaf, soil) grown in Gumushane Province. Erwerbs-Obstbau. 2019;61(1):85-96.
32. Organization WH. WHO Guidelines for Assessing Quality of Herbal Medicines with Reference to Contaminants and Residues: World Health Organization; 2007.
33. Liang Y-Z, Xie P, Chan K. Quality control of herbal medicines. Journal of chromatography B. 2004;812(1-2):53-70.
34. Annalakshmi R, Mahalakshmi S, Charles A, Sahayam CS. GC–MS and HPTLC analysis of leaf extract of Madhuca longifolia (Koenig) Linn. Drug Invention Today. 2013;5(2):76-80.
35. Thirumalaisamy R, Vaijayanthimala M, Govindaraju S, Subramanian A. Phytochemical screening and GC-MS Analysis of Madhuca longifolia (L) Macbr. Int J Adv Sci Eng. 2015;2:87-92.
36. Emniyet AA, Emre A, Ozcelik B, Avci GA, Kose DA. Antioxidant and antimicrobial activities with GC/MS analysis of the Morus alba L. leaves. Hittite Journal of Science and Engineering. 2014;1(1):37-41.
37. Sun F, Shen L, Ma Z. Screening for ligands of human aromatase from mulberry (Mori alba L.) leaf by using high-performance liquid chromatography/tandem mass spectrometry. Food chemistry. 2011;126(3):1337-43.
38. Radulović NS, Miljković VM, Mladenović MZ, Nikolić GS. Essential oils of Morus alba

and M. nigra leaves: Effect of drying on the chemical composition. Natural Product Communications. 2017;12(1):1934578X1701200133.
39. Sánchez-Salcedo EM, Tassotti M, Del Rio D, Hernández F, Martínez JJ, Mena P. (Poly) phenolic fingerprint and chemometric analysis of white (Morus alba L.) and black (Morus nigra L.) mulberry leaves by using a non-targeted UHPLC–MS approach. Food chemistry. 2016;212:250-5.
40. Yashvanth S, Rani SS, Madhavendra S. Morus alba L., a new perspective: scanning electron microscopic, micro chemical, GC-MS and UPLC-MS characterisation. International Journal of Research in Pharmacy and Chemistry. 2015;5(1):106-15.
41. Abdi A, Jalilian M, Sarbarzeh PA, Vlaisavljevic Z. Diabetes and COVID-19: A systematic review on the current evidences. Diabetes research and clinical practice. 2020;166:108347.
42. Association AD. Introduction: Standards of medical care in diabetes—2022. Am Diabetes Assoc; 2022. p. S1-S2.
43. Roglic G, Varghese C, Thamarangsi T. Diabetes in South-East Asia: burden, gaps, challenges and ways forward. WHO South-East Asia Journal of Public Health. 2016;5(1):1-4.
44. Aye TT, Aung MW, Oo ES. Diabetes mellitus in Myanmar: Socio-cultural challenges and strength. Journal of Social Health and Diabetes. 2014;2(01):009-13.
45. Latt TS, Aye TT, Sandar E, Win YY, Wint KT, Khin ET. Traditional medicine and diabetes care in Myanmar. Journal of Social Health and Diabetes. 2019;7(01):016-21.
46. Organization WH. Noncommunicable disease risk factory survey, Myanmar 2009. WHO Regional Office for South-East Asia; 2012.
47. Assefa ST, Yang E-Y, Chae S-Y, Song M, Lee J, Cho M-C, et al. Alpha Glucosidase Inhibitory Activities of Plants with Focus on Common Vegetables. Plants. 2020;9(1):2.
48. Proença C, Freitas M, Ribeiro D, Oliveira EF, Sousa JL, Tome SM, et al. α-Glucosidase inhibition by flavonoids: an in vitro and in silico structure–activity relationship study. Journal of enzyme inhibition and medicinal chemistry. 2017;32(1):1216-28.
49. Dirir AM, Daou M, Yousef AF, Yousef LF. A review of alpha-glucosidase inhibitors from plants as potential candidates for the treatment of type-2 diabetes. Phytochemistry Reviews. 2021.
50. Lin D, Xiao M, Zhao J, Li Z, Xing B, Li X, et al. An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. Molecules. 2016;21(10):1374.
51. Sakulkeo O, Wattanapiromsakul C, Pitakbut T, Dej-Adisai S. Alpha-Glucosidase Inhibition and Molecular Docking of Isolated Compounds from Traditional Thai Medicinal Plant, Neuropeltis racemosa Wall. Molecules. 2022;27(3):639.
52. Elya B, Basah K, Mun'im A, Yuliastuti W, Bangun A, Septiana EK. Screening of α-glucosidase inhibitory activity from some plants of Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae. Journal of Biomedicine and Biotechnology. 2012;2012.
53. Baky MH, Kamal AM, Elgindi MR, Haggag EG. A review on phenolic compounds from family Sapotaceae. Journal of Pharmacognosy and Phytochemistry. 2016;5(2):280.
54. Kendre N, Wakte P. A review on Phytochemicals and biological attributes of Madhuca longifolia. Asian Journal of Pharmacy and Pharmacology. 2021;7(2):74-84.
55. Sinha J, Singh V, Singh J, Rai A. Phytochemistry, ethnomedical uses and future prospects of Mahua (Madhuca longifolia) as a food: a review. J Nutr Food Sci. 2017;7(573):10.4172.
56. Bhaumik A, Kumar M, Khan KA, Srinivas C. The bioactive compounds obtained from the fruit-seeds of Madhuca longifolia (L) act as potential anticancer agents. Sch J App Med Sci. 2014;2(4A):1235-8.
57. Singh SK SR, Akki KV. . A review on Madguca longigolia. Asean Journal of

Pharmaceutical Education and Research. 2014;3(2).
58. Priyanka Y, Anurabha M, Nayak S. Microscopic studies of Madhuca longifolia. Journal of Natural Product and Plant Resources. 2011;1(4):66-72.
59. Sharma S, Sharma MC, Kohli D. Wound healing activity and formulation of ether-benzene-95% ethanol extract of herbal drug Madhuca longifolia leaves in albino rats. Journal of optoelectronics and Biomedical materials. 2010;1(1):13-5.
60. Palani S, Raja S, Karthi S, Archana S, Kumar BS. In vivo analysis of nephro & hepato protective effects and antioxidant activity of Madhuca longifolia against acetaminophen-induced toxicity & oxidative stress. Journal of Pharmacy research. 2010;3(1):9-16.
61. Saluja M, Sangameswaran B, Hura I, Sharma A, Gupta S, Chaturvedi M. In Vitro cytotoxic activity of leaves of Madhuca longifolia against Ehrlich Ascites Carcinoma (EAC) cell lines. Int J Drug Discov Herbal Res. 2011;1:55-7.
62. Khond M, Bhosale J, Arif T, Mandal T, Padhi M, Dabur R. Screening of some selected medicinal plants extracts for in-vitro antimicrobial activity. Middle-East J Sci Res. 2009;4(4):271-8.
63. Chandra D. Analgesic effect of aqueous and alcoholic extracts of Madhuka Longifolia (Koeing). Indian Journal of Pharmacology. 2001;33(2):108-11.
64. Umadevi M, Maheswari C, Jothi R, Paleti SK, Reddy YS, Narayanan RV. Hepatoprotective Activity of Flowers of Madhuca longifolia (Koen.) Macbr. against paracetamol-induced hepatotoxicity. Research Journal of Pharmacy and Technology. 2011;4(2):259-62.
65. Kumar KP, Vidyasagar G, Ramakrishna D, Reddy IM, Gupta V, Raidu C. Screening of Madhuca indica for antidiabetic activity in streptozotocin and streptozotocin–nicotinamide induced diabetic rats. Int J PharmTech Res. 2011;3(2):1073-7.
66. Odili V, Akpe A, Arigbe-Osula M, Igbinaduwa P. Antioxidant and hepatoprotective activity of Madhuca longifolia (koenig) bark against CCl4-induced hepatic injury in rats: in vitro and in vivo studies. Research Journal of Pharmaceutical Biological and Chemical Sciences. 2010;1(1):1-10.
67. Tambekar D, Khante B. Antibacterial properties of traditionally used medicinal plants for enteric infections by adivasi's (Bhumka) in Melghat forest (Amravati district). Int J Pharm Sci Res. 2010;1:120-8.
68. Gaikwad RD, Ahmed ML, Khalid MS, Swamy P. Anti-inflammatory activity of Madhuca longifolia seed saponin mixture. Pharmaceutical biology. 2009;47(7):592-7.
69. RD G. Anti-inflammatory, anti-ulcer and hypoglycaemic activities of ethanolic and crude alkaloid extracts of Madhuca indica (Koenig) Gmelin seed cake. Advances in Traditional Medicine. 2007;7(2):141-9.
70. Shekhawat N, Vijayvergia R. Investigation of anti-inflammatory, analgesic and antipyretic properties of Madhuca indica GMEL. European journal of inflammation. 2010;8(3):165-71.
71. Chen C, Mohamad Razali UH, Saikim FH, Mahyudin A, Mohd Noor NQI. Morus alba L. plant: Bioactive compounds and potential as a functional food ingredient. Foods. 2021;10(3):689.
72. Chang B-Y, Koo B-S, Kim S-Y. Pharmacological activities for Morus alba L., focusing on the immunostimulatory property from the fruit aqueous extract. Foods. 2021;10(8):1966.
73. Oo PP, Min TS, San NK. Screening of Phytochemical Constituents, Elemental Composition and Evaluation of Antioxidant and Antibacterial Activity of Leaf Extract of Morus alba L.(White Mulberry). 3rd Myanmar Korea Conference Research Journal. 2020;3(2):679.
74. Sastri B. The Wealth of India, A dictionary of raw material and industrial products. Publ Inf Dir. 1950;5:285-93.

75. Kimura T, Nakagawa K, Kubota H, Kojima Y, Goto Y, Yamagishi K, et al. Food-grade mulberry powder enriched with 1-deoxynojirimycin suppresses the elevation of postprandial blood glucose in humans. Journal of agricultural and food chemistry. 2007;55(14):5869-74.
76. Yang Y, Zhang T, Xiao L, Yang L, Chen R. Two new chalcones from leaves of Morus alba L. Fitoterapia. 2010;81(6):614-6.
77. Dat NT, Binh PTX, Van Minh C, Huong HT, Lee JJ. Cytotoxic prenylated flavonoids from Morus alba. Fitoterapia. 2010;81(8):1224-7.
78. Wang Y, Xiang L, Wang C, Tang C, He X. Antidiabetic and antioxidant effects and phytochemicals of mulberry fruit (Morus alba L.) polyphenol enhanced extract. PloS one. 2013;8(7):e71144.
79. Kim SY, Gao JJ, Kang HK. Two flavonoids from the leaves of Mours alba induce differentiation of the human promyelocytic leukemia (HL-60) cell line. Biological and Pharmaceutical Bulletin. 2000;23(4):451-5.
80. Katsube T, Imawaka N, Kawano Y, Yamazaki Y, Shiwaku K, Yamane Y. Antioxidant flavonol glycosides in mulberry (Morus alba L.) leaves isolated based on LDL antioxidant activity. Food chemistry. 2006;97(1):25-31.
81. Doi K, Kojima T, Makino M, KIMURA Y, FUJIMOTO Y. Studies on the constituents of the leaves of Morus alba L. Chemical and pharmaceutical bulletin. 2001;49(2):151-3.
82. Hisayoshi K, Masashi Y, Koichi S. A novel cytotoxic prenylated flavonoid from the root of Morus alba. Journal of Insect Biotechnology and Sericology. 2004;73(3):113-6.
83. Memon AA, Memon N, Luthria DL, Bhanger MI, Pitafi AA. 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. 2010;60(1).
84. Oku T, Yamada M, Nakamura M, Sadamori N, Nakamura S. Inhibitory effects of extractives from leaves of Morus alba on human and rat small intestinal disaccharidase activity. British journal of nutrition. 2006;95(5):933-8.
85. Leyva-Jiménez FJ, Ruiz-Malagón AJ, Molina-Tijeras JA, Diez-Echave P, Vezza T, Hidalgo-García L, et al. Comparative study of the antioxidant and anti-inflammatory effects of leaf extracts from four different Morus alba genotypes in high fat diet-induced obesity in mice. Antioxidants. 2020;9(8):733.
86. Hunyadi A, Veres K, Danko B, Kele Z, Weber E, Hetenyi A, et al. In vitro anti-diabetic activity and chemical characterization of an apolar fraction of Morus alba leaf water extract. Phytotherapy Research. 2013;27(6):847-51.
87. Park JM, Bong HY, Jeong HI, Kim YK, Kim JY, Kwon O. Postprandial hypoglycemic effect of mulberry leaf in Goto-Kakizaki rats and counterpart control Wistar rats. Nutrition Research and Practice. 2009;3(4):272-8.
88. Hunyadi A, Martins A, Hsieh T-J, Seres A, Zupkó I. 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. 2012;7(11):e50619.
89. Król E, Jeszka-Skowron M, Krejpcio Z, Flaczyk E, Wójciak RW. The effects of supplementary mulberry leaf (Morus alba) extracts on the trace element status (Fe, Zn and Cu) in relation to diabetes management and antioxidant indices in diabetic rats. Biological trace element research. 2016;174(1):158-65.
90. Hunyadi A, Liktor-Busa E, Márki Á, Martins A, Jedlinszki N, Hsieh T, et al. Metabolic effects of mulberry leaves: exploring potential benefits in type 2 diabetes and hyperuricemia. Evidence-Based Complementary and Alternative Medicine. 2013;2013.
91. Jha S, Gupta SK, Bhattacharyya P, Ghosh A, Mandal P. In vitro antioxidant and antidiabetic activity of oligopeptides derived from different mulberry (Morus alba L.) cultivars. Pharmacognosy Research. 2018;10(4).

92. Eruygur N, Dural E. Determination of 1-deoxynojirimycin by a developed and validated HPLC-FLD method and assessment of in-vitro antioxidant, α-amylase and α-glucosidase inhibitory activity in mulberry varieties from Turkey. Phytomedicine. 2019;53:234-42.
93. Kim E-S, Park S-J, Lee E-J, Kim B-K, Huh H, Lee B-J. Purification and characterization of Moran 20K fromMorus alba. Archives of Pharmacal Research. 1999;22(1):9-12.
94. Wattanapitayakul SK, Chularojmontri L, Herunsalee A, Charuchongkolwongse S, Niumsakul S, Bauer JA. Screening of antioxidants from medicinal plants for cardioprotective effect against doxorubicin toxicity. Basic & clinical pharmacology & toxicology. 2005;96(1):80-7.
95. Kwon DH, Cheon JM, Choi E-O, Jeong JW, Lee KW, Kim KY, et al. The immunomodulatory activity of mori folium, the leaf of Morus alba L., in raw 264.7 macrophages in vitro. Journal of cancer prevention. 2016;21(3):144.
96. Liao B-Y, Zhu D-Y, Thakur K, Li L, Zhang J-G, Wei Z-J. Thermal and antioxidant properties of polysaccharides sequentially extracted from mulberry leaves (Morus alba L.). Molecules. 2017;22(12):2271.
97. Ann J-Y, Eo H, Lim Y. Mulberry leaves (Morus alba L.) ameliorate obesity-induced hepatic lipogenesis, fibrosis, and oxidative stress in high-fat diet-fed mice. Genes & Nutrition. 2015;10(6):46.
98. Cui H, Lu T, Wang M, Zou X, Zhang Y, Yang X, et al. Flavonoids from Morus alba L. leaves: Optimization of extraction by response surface methodology and comprehensive evaluation of their antioxidant, antimicrobial, and inhibition of α-amylase activities through analytical hierarchy process. Molecules. 2019;24(13):2398.
99. Yu Y, Li H, Zhang B, Wang J, Shi X, Huang J, et al. Nutritional and functional components of mulberry leaves from different varieties: Evaluation of their potential as food materials. International Journal of Food Properties. 2018;21(1):1495-507.
100. Tu J, Shi D, Wen L, Jiang Y, Zhao Y, Yang J, et al. Identification of moracin N in mulberry leaf and evaluation of antioxidant activity. Food and Chemical Toxicology. 2019;132:110730.
101. Choi E-M, Hwang J-K. Effects of Morus alba leaf extract on the production of nitric oxide, prostaglandin E2 and cytokines in RAW264. 7 macrophages. Fitoterapia. 2005;76(7-8):608-13.
102. Dabili S, Fallah S, Aein M, Vatannejad A, Panahi G, Fadaei R, et al. Survey of the effect of doxorubicin and flavonoid extract of white Morus alba leaf on apoptosis induction in a-172 GBM cell line. Archives of Physiology and Biochemistry. 2019;125(2):136-41.
103. Chan EWC, Wong SK, Tangah J, Inoue T, Chan HT. Phenolic constituents and anticancer properties of Morus alba (white mulberry) leaves. Journal of Integrative Medicine. 2020;18(3):189-95.
104. Sithara T, Arun K, Syama H, Reshmitha T, Nisha P. Morin inhibits proliferation of SW480 colorectal cancer cells by inducing apoptosis mediated by reactive oxygen species formation and uncoupling of warburg effect. Frontiers in Pharmacology. 2017;8:640.
105. Soltanian S, Sheikhbahaei M, Mohamadi N. Cytotoxicity evaluation of methanol extracts of some medicinal plants on P19 embryonal carcinoma cells. Journal of Applied Pharmaceutical Science. 2017;7(7):142-9.
106. Fathy SA, Singab ANB, Agwa SA, El Hamid D, Zahra FA, El Moneim S. The antiproliferative effect of mulberry (Morus alba L.) plant on hepatocarcinoma cell line HepG2. Egyptian Journal of Medical Human Genetics. 2013;14(4):375-82.
107. Zhang Q, Zhang F, Thakur K, Wang J, Wang H, Hu F, et al. Molecular mechanism of anti-cancerous potential of Morin extracted from mulberry in Hela cells. Food and Chemical Toxicology. 2018;112:466-75.
108. Fallah S, Karimi A, Panahi G, Nejad SG, Fadaei R, Seifi M. Human colon cancer HT-29 cell death responses to doxorubicin and Morus Alba leaves flavonoid extract. Cellular and

Molecular Biology. 2016;62(3):72-7.
109. Ramis ES, Hammoud GM, ElSawy KM. In vitro and in vivo studies on mulberry extracts: evaluation of chemical and anticancer activities and attenuation of lead toxicity. cerebellum. 2018;4:5.
110. Sohn H-Y, Son K, Kwon C-S, Kwon G-S, Kang S. 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. 2004;11(7-8):666-72.
111. De Oliveira AM, Mesquita MdS, da Silva GC, de Oliveira Lima E, de Medeiros PL, Paiva PMG, et al. Evaluation of toxicity and antimicrobial activity of an ethanolic extract from leaves of Morus alba L.(Moraceae). Evidence-Based Complementary and Alternative Medicine. 2015;2015.
112. Sheikhlar A, Alimon AR, Daud H, Saad CR, Webster CD, Meng GY, et al. White mulberry (Morus alba) foliage methanolic extract can alleviate Aeromonas hydrophila infection in African Catfish (Clarias gariepinus). The Scientific World Journal. 2014;2014.
113. Islam B, Khan SN, Haque I, Alam M, Mushfiq M, Khan AU. Novel anti-adherence activity of mulberry leaves: inhibition of Streptococcus mutans biofilm by 1-deoxynojirimycin isolated from Morus alba. Journal of antimicrobial chemotherapy. 2008;62(4):751-7.
114. Suwansri S, Khaoprasert S, Ratanatriwong P, Promboon A. Natural preservative from Thai mulberry: The antioxidant and antibacterial properties of leaf extracts from different cultivars. Acta horticulturae. 2008.
115. Thabti I, Elfalleh W, Tlili N, Ziadi M, Campos MG, Ferchichi A. Phenols, flavonoids, and antioxidant and antibacterial activity of leaves and stem bark of Morus species. International journal of food properties. 2014;17(4):842-54.
116. KOSTIĆ DSDDA, MITIĆ GSSSS, ĐORĐEVIĆ MNMAS. Phenolic composition, antioxidant activity, mineral content and antimicrobial activity of fresh fruit extracts of Morus alba L. Journal of Food and Nutrition Research (ISSN 1336-8672). 2014;53(1):22-30.
117. Lee SH, Choi SY, Kim H, Hwang JS, Lee BG, Gao JJ, et al. Mulberroside F isolated from the leaves of Morus alba inhibits melanin biosynthesis. Biological and Pharmaceutical Bulletin. 2002;25(8):1045-8.
118. Enkhmaa B, Shiwaku K, Katsube T, Kitajima K, Anuurad E, Yamasaki M, et al. Mulberry (Morus alba L.) leaves and their major flavonol quercetin 3-(6-malonylglucoside) attenuate atherosclerotic lesion development in LDL receptor-deficient mice. The Journal of nutrition. 2005;135(4):729-34.
119. Doi K, Kojima T, FUJIMOTO Y. Mulberry leaf extract inhibits the oxidative modification of rabbit and human low density lipoprotein. Biological and Pharmaceutical Bulletin. 2000;23(9):1066-71.
120. Niidome T, Takahashi K, Goto Y, Goh S, Tanaka N, Kamei K, et al. Mulberry leaf extract prevents amyloid beta-peptide fibril formation and neurotoxicity. Neuroreport. 2007;18(8):813-6.
121. Chen X-y, Zhang T, Wang X, Hamann MT, Kang J, Yu D-q, et al. A Chemical Investigation of the Leaves of Morus alba L. Molecules. 2018;23(5):1018.
122. Yadav AV, Nade VS. Anti-dopaminergic effect of the methanolic extract of Morus alba L. leaves. Indian journal of pharmacology. 2008;40(5):221.
123. Kang TH, Oh HR, Jung SM, Ryu JH, Park MW, Park YK, et al. Enhancement of neuroprotection of mulberry leaves (Morus alba L.) prepared by the anaerobic treatment against ischemic damage. Biological and Pharmaceutical Bulletin. 2006;29(2):270-4.
124. Rebai O, Belkhir M, Boujelben A, Fattouch S, Amri M. Morus alba leaf extract mediates neuroprotection against glyphosate-induced toxicity and biochemical alterations in the brain.

Environmental Science and Pollution Research. 2017;24(10):9605-13.
125. Gupta G, Afzal M, David SR, Verma R, Candaswamy M, Anwar F. Anticonvulsant activity of Morus alba and its effect on brain gamma-aminobutyric acid level in rats. Pharmacognosy research. 2014;6(2):188.
126. Rayam S, Kudagi B, Buchineni M, Pathapati RM. Assessment of Morus alba (Mulberry) leaves extract for anti-convulsant property in rats. 2019.
127. Diniz TC, Silva JC, Lima-Saraiva SRGd, Ribeiro FPRdA, Pacheco AGM, de Freitas RM, et al. The role of flavonoids on oxidative stress in epilepsy. Oxidative medicine and cellular longevity. 2015;2015.
128. Tamtaji OR, Mohammadifar M, Behnam M, Taghizadeh M, Talaei SA. Investigation of the effect of alcoholic Morus alba leave extract on scopolamine-induced spatial memory impairment in rats. Scientific Journal of Kurdistan University of Medical Sciences. 2016;21(1):30-9.
129. Chen W, Liang T, Zuo W, Wu X, Shen Z, Wang F, et al. Neuroprotective effect of 1-Deoxynojirimycin on cognitive impairment, β-amyloid deposition, and neuroinflammation in the SAMP8 mice. Biomedicine & Pharmacotherapy. 2018;106:92-7.
130. Hogade M, Patil K, Wadkar G, Mathapati S, Dhumal P. Hepatoprotective activity of Morus alba (Linn.) leaves extract against carbon tetrachloride induced hepatotoxicity in rats. Afr J Pharm Pharmacol. 2010;4(10):731-4.
131. Kujawska M, Ewertowska M, Adamska T, Ignatowicz E, Flaczyk E, Przeor M, et al. Protective effect of Morus alba leaf extract on N-nitrosodiethylamine-induced hepatocarcinogenesis in rats. in vivo. 2016;30(6):807-12.
132. Liang H-W, Yang T-Y, Teng C-S, Lee Y-J, Yu M-H, Lee H-J, et al. Mulberry leaves extract ameliorates alcohol-induced liver damages through reduction of acetaldehyde toxicity and inhibition of apoptosis caused by oxidative stress signals. International Journal of Medical Sciences. 2021;18(1):53.
133. Lee MR, Kim JE, Park JW, Kang MJ, Choi HJ, Bae SJ, et al. Fermented mulberry (Morus alba) leaves suppress high fat diet-induced hepatic steatosis through amelioration of the inflammatory response and autophagy pathway. BMC complementary medicine and therapies. 2020;20(1):1-17.
134. Hu Y, Xu J, Chen Q, Liu M, Wang S, Yu H, et al. Regulation effects of total flavonoids in Morus alba L. on hepatic cholesterol disorders in orotic acid induced NAFLD rats. BMC complementary medicine and therapies. 2020;20(1):1-12.
135. Yadav A, Kawale L, Nade V. Effect of Morus alba L.(mulberry) leaves on anxiety in mice. Indian journal of pharmacology. 2008;40(1):32.
136. Chen X, Sheng Z, Qiu S, Yang H, Jia J, Wang J, et al. Purification, characterization and in vitro and in vivo immune enhancement of polysaccharides from mulberry leaves. PLoS One. 2019;14(1):e0208611.
137. Chen X, Yang H, Jia J, Chen Y, Wang J, Chen H, et al. Mulberry leaf polysaccharide supplementation contributes to enhancing the respiratory mucosal barrier immune response in Newcastle disease virus–vaccinated chicks. Poultry science. 2021;100(2):592-602.
138. Peng C-H, Lin H-T, Chung D-J, Huang C-N, Wang C-J. Mulberry Leaf Extracts prevent obesity-induced NAFLD with regulating adipocytokines, inflammation and oxidative stress. Journal of food and drug analysis. 2018;26(2):778-87.
139. Jambhale SM, Khyade VB. Utilization of Mulberry Methanolic Leaf Extractive (MMLE) for Treating Hypercholesterolemia in Rat, Rattus norvegicus (L). International Journal of Chemical Engineering and Processing. 2019;5(2):44-68.
140. Metwally FM, Rashad H, Mahmoud AA. Morus alba L. Diminishes visceral adiposity, insulin resistance, behavioral alterations via regulation of gene expression of leptin, resistin

and adiponectin in rats fed a high-cholesterol diet. Physiology & behavior. 2019;201:1-11.
141. Asano N, Yamashita T, Yasuda K, Ikeda K, Kizu H, Kameda Y, et al. Polyhydroxylated Alkaloids Isolated from Mulberry Trees (Morus alba L.) and Silkworms (Bombyx mori L.). Journal of Agricultural and Food Chemistry. 2001;49(9):4208-13.
142. Chu Q, Lin M, Tian X, Ye J. Study on capillary electrophoresis–amperometric detection profiles of different parts of Morus alba L. Journal of Chromatography A. 2006;1116(1-2):286-90.
143. Zhang H, Ma ZF, Luo X, Li X. Effects of mulberry fruit (Morus alba L.) consumption on health outcomes: A mini-review. Antioxidants. 2018;7(5):69.
144. Aramwit P, Bang N, Srichana T. The properties and stability of anthocyanins in mulberry fruits. Food Research International. 2010;43(4):1093-7.
145. Sánchez-Salcedo EM, Mena P, García-Viguera C, Hernández F, Martínez JJ. (Poly) phenolic compounds and antioxidant activity of white (Morus alba) and black (Morus nigra) mulberry leaves: Their potential for new products rich in phytochemicals. Journal of Functional Foods. 2015;18:1039-46.
146. Calín-Sánchez Á, Martínez-Nicolás JJ, Munera-Picazo S, Carbonell-Barrachina ÁA, Legua P, Hernández F. Bioactive compounds and sensory quality of black and white mulberries grown in Spain. Plant foods for human nutrition. 2013;68(4):370-7.
147. Xu X, Huang Y, Xu J, He X, Wang Y. Anti-neuroinflammatory and antioxidant phenols from mulberry fruit (Morus alba L.). Journal of Functional Foods. 2020;68:103914.
148. Chen C, Zhang B, Fu X, Liu RH. A novel polysaccharide isolated from mulberry fruits (Murus alba L.) and its selenide derivative: structural characterization and biological activities. Food & function. 2016;7(6):2886-97.
149. Sarikaphuti A, Nararatwanchai T, Hashiguchi T, Ito T, Thaworanunta S, Kikuchi K, et al. Preventive effects of Morus alba L. anthocyanins on diabetes in Zucker diabetic fatty rats. Experimental and Therapeutic Medicine. 2013;6(3):689-95.
150. Choi KH, Lee HA, Park MH, Han J-S. Mulberry (Morus alba L.) fruit extract containing anthocyanins improves glycemic control and insulin sensitivity via activation of AMP-activated protein kinase in diabetic C57BL/Ksj-db/db mice. Journal of medicinal food. 2016;19(8):737-45.
151. Lee D, Yu JS, Lee SR, Hwang GS, Kang KS, Park JG, et al. Beneficial effects of bioactive compounds in mulberry fruits against cisplatin-induced nephrotoxicity. International Journal of Molecular Sciences. 2018;19(4):1117.
152. Lee SR, Park JY, Yu JS, Lee SO, Ryu J-Y, Choi S-Z, et al. Odisolane, a novel oxolane derivative, and antiangiogenic constituents from the fruits of mulberry (Morus alba L.). Journal of agricultural and food chemistry. 2016;64(19):3804-9.
153. El-Baz FK, Hassan AZ, Abd-Alla HI, Aly HF, Mahmoud K. Phytochemical analysis, assessment of antiproliferative and free radical scavenging activity of Morus alba and Morus rubra fruits. Asian Journal of Pharmaceutical and Clinical Research. 2017:189-99.
154. Jyoti A, Anand K, Sunanda P. Synergistic action of phytochemicals augments their antioxidative efficacy: an in vitro comparative study. Asian J Pharm Clin Res. 2013;6(4):121-6.
155. Cho E, Chung EY, Jang H-Y, Hong O-Y, Chae HS, Jeong Y-J, et al. Anti-cancer effect of cyanidin-3-glucoside from mulberry via caspase-3 cleavage and DNA fragmentation in vitro and in vivo. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2017;17(11):1519-25.
156. Yu JS, Lee D, Lee SR, Lee JW, Choi C-I, Jang TS, et al. Chemical characterization of cytotoxic indole acetic acid derivative from mulberry fruit (Morus alba L.) against human cervical cancer. Bioorganic chemistry. 2018;76:28-36.
157. Zhang J, Chen C, Fu X. Fructus mori L. polysaccharide-iron chelates formed by self-embedding with iron (iii) as the core exhibit good antioxidant activity. Food & Function.

2019;10(6):3150-60.
158. Raman ST, Ganeshan AKPG, Chen C, Jin C, Li S-H, Chen H-J, et al. In vitro and in vivo antioxidant activity of flavonoid extracted from mulberry fruit (Morus alba L.). Pharmacognosy Magazine. 2016;12(46):128.
159. Li M, Li T, Hu X, Ren G, Zhang H, Wang Z, et al. Structural, rheological properties and antioxidant activities of polysaccharides from mulberry fruits (Murus alba L.) based on different extraction techniques with superfine grinding pretreatment. International Journal of Biological Macromolecules. 2021;183:1774-83.
160. Khan MS, Chen C, Fu X. The effect of geographic variation on chemical composition, antioxidant and hypoglycemic activities of Morus alba L. polysaccharides. Journal of Food Processing and Preservation. 2019;43(11):e14206.
161. Salimi F, Moradi M, Tajik H, Molaei R. Optimization and characterization of eco-friendly antimicrobial nanocellulose sheet prepared using carbon dots of white mulberry (Morus alba L.). Journal of the Science of Food and Agriculture. 2021;101(8):3439-47.
162. Yang J-Y, Lee H-S. Evaluation of antioxidant and antibacterial activities of morin isolated from mulberry fruits (Morus alba L.). Journal of the Korean Society for Applied Biological Chemistry. 2012;55(4):485-9.
163. Kumar RV, Srivastava D, Singh V, Kumar U, Vishvakarma VK, Singh P, et al. Characterization, biological evaluation and molecular docking of mulberry fruit pectin. Scientific Reports. 2020;10(1):1-16.
164. Kim BS, Kim H, Kang S-S. In vitro anti-bacterial and anti-inflammatory activities of lactic acid bacteria-biotransformed mulberry (Morus alba Linnaeus) fruit extract against Salmonella Typhimurium. Food Control. 2019;106:106758.
165. Jacob JR, Mansfield K, You J-E, Tennant BC, Kim Y-H. Natural iminosugar derivatives of 1-deoxynojirimycin inhibit glycosylation of hepatitis viral envelope proteins. Journal of Microbiology. 2007;45(5):431-40.
166. Hu D, Bao T, Lu Y, Su H, Ke H, Chen W. Polysaccharide from mulberry fruit (Morus alba L.) protects against palmitic-acid-induced hepatocyte lipotoxicity by activating the Nrf2/ARE signaling pathway. Journal of Agricultural and Food Chemistry. 2019;68(46):13016-24.
167. Pel P, Chae H-S, Nhoek P, Kim Y-M, Chin Y-W. Chemical constituents with proprotein convertase subtilisin/kexin type 9 mRNA expression inhibitory activity from dried immature Morus alba fruits. Journal of agricultural and food chemistry. 2017;65(26):5316-21.
168. Seo K-H, Lee D-Y, Jeong R-H, Lee D-S, Kim Y-E, Hong E-K, et al. Neuroprotective effect of prenylated arylbenzofuran and flavonoids from Morus alba fruits on glutamate-induced oxidative injury in HT22 hippocampal cells. Journal of Medicinal Food. 2015;18(4):403-8.
169. Liu D, Du D. Mulberry fruit extract alleviates cognitive impairment by promoting the clearance of amyloid-β and inhibiting neuroinflammation in Alzheimer’s disease mice. Neurochemical Research. 2020;45(9):2009-19.
170. Shin S, Yoo J, Li F, Baek S, Kim M. Mulberry fruit improves memory in scopolamine-treated mice: role of cholinergic function, antioxidant system, and TrkB/Akt signaling. Nutritional Neuroscience. 2019:1-11.
171. El-Baz FK, Aly HF, Abd-Alla HI, Ali SA. Neurorestorative mulberries potential of alzheimer’s disease in animal model. Asian J Pharm Clin Res. 2018;11(10):318-24.
172. Gu PS, Moon M, Choi JG, Oh MS. Mulberry fruit ameliorates Parkinson's-disease-related pathology by reducing α-synuclein and ubiquitin levels in a 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine/probenecid model. The Journal of Nutritional Biochemistry. 2017;39:15-21.
173. Bhuiyan MIH, Kim H-B, Kim SY, Cho K-O. The neuroprotective potential of cyanidin-3-glucoside fraction extracted from mulberry following oxygen-glucose deprivation. The Korean

Journal of Physiology & Pharmacology. 2011;15(6):353-61.
174. Kim SB, Chang BY, Hwang BY, Kim SY, Lee MK. Pyrrole alkaloids from the fruits of Morus alba. Bioorganic & medicinal chemistry letters. 2014;24(24):5656-9.
175. Yang X, Yang L, Zheng H. Hypolipidemic and antioxidant effects of mulberry (Morus alba L.) fruit in hyperlipidaemia rats. Food and Chemical Toxicology. 2010;48(8-9):2374-9.
176. El-Baz FK, Aly HF, Abd-Alla HI, Biomy D. Therapeutic impact of berries (Morus alba and Morus rubra) fruit extract in the regression of high-fat diet-induced cardiac dysfunction in rats. Asian J Pharm Clin Res. 2018;11:314-20.
177. Shin BR, Kim HS, Yun MJ, Lee HK, Kim YJ, Kim SY, et al. Promoting effect of polysaccharide isolated from Mori fructus on dendritic cell maturation. Food and chemical toxicology. 2013;51:411-8.
178. Chang BY, Kim SB, Lee MK, Park H, Kim SY. Improved chemotherapeutic activity by Morus alba fruits through immune response of toll-like receptor 4. International journal of molecular sciences. 2015;16(10):24139-58.
179. Wang D, Li H, Li B, Ma R, Zhang N, Zhang X, et al. Systematic fractionation and immunoenhancement of water-soluble polysaccharides isolated from fruit of Morus alba L. International journal of biological macromolecules. 2018;116:1056-63.
180. Kim SB, Chang BY, Jo YH, Lee SH, Han S-B, Hwang BY, et al. Macrophage activating activity of pyrrole alkaloids from Morus alba fruits. Journal of ethnopharmacology. 2013;145(1):393-6.
181. Chauhan S, Devi U, Kumar VR, Kumar V, Anwar F, Kaithwas G. Dual inhibition of arachidonic acid pathway by mulberry leaf extract. Inflammopharmacology. 2015;23(1):65-70.
182. Nomura T. Chemistry and anti-tumor promoting activity of Morus flavonoids. Prog Clin Bio Res. 1988;280:267-81.
183. Chung K-O, Kim B-Y, Lee M-H, Kim Y-R, Chung H-Y, Park J-H, et al. In-vitro and in-vivo anti-inflammatory effect of oxyresveratrol from Morus alba L. Journal of Pharmacy and Pharmacology. 2003;55(12):1695-700.
184. Min T-R, Park H-J, Park MN, Kim B, Park S-H. The Root Bark of Morus alba L. Suppressed the Migration of Human Non-Small-Cell Lung Cancer Cells through Inhibition of Epithelial–Mesenchymal Transition Mediated by STAT3 and Src. International journal of molecular sciences. 2019;20(9):2244.
185. Eo HJ, Park JH, Park GH, Lee MH, Lee JR, Koo JS, et al. Anti-inflammatory and anti-cancer activity of mulberry (Morus alba L.) root bark. BMC complementary and alternative medicine. 2014;14(1):1-9.
186. Kikuchi T, Nihei M, Nagai H, Fukushi H, Tabata K, Suzuki T, et al. Albanol A from the root bark of Morus alba L. induces apoptotic cell death in HL60 human leukemia cell line. Chemical and Pharmaceutical Bulletin. 2010;58(4):568-71.
187. Lim S-L, Park S-Y, Kang S, Park D, Kim S-H, Um J-Y, et al. Morusin induces cell death through inactivating STAT3 signaling in prostate cancer cells. American journal of cancer research. 2015;5(1):289.
188. Kim HM, Han SB, Lee KH, Lee CW, Kim CY, Lee EJ, et al. Immunomodulating activity of a polysaccharide isolated from Mori Cortex Radicis. Archives of pharmacal research. 2000;23(3):240-2.
189. Zhao Y, Kongstad KT, Jäger AK, Nielsen J, Staerk D. Quadruple high-resolution α-glucosidase/α-amylase/PTP1B/radical scavenging profiling combined with high-performance liquid chromatography–high-resolution mass spectrometry–solid-phase extraction–nuclear magnetic resonance spectroscopy for identification of antidiabetic constituents in crude root bark of Morus alba L. Journal of Chromatography A. 2018;1556:55-63.
190. Khan MA, Rahman AA, Islam S, Khandokhar P, Parvin S, Islam MB, et al. A comparative

study on the antioxidant activity of methanolic extracts from different parts of Morus alba L.(Moraceae). BMC Research Notes. 2013;6(1):1-9.
191. Tseng T-H, Lin W-L, Chang C-K, Lee K-C, Tung S-Y, Kuo H-C. Protective effects of Morus Root Extract (MRE) against lipopolysaccharide-activated RAW264. 7 cells and CCl4-induced mouse hepatic damage. Cellular physiology and biochemistry. 2018;51(3):1376-88.
192. Kuk EB, Jo AR, Oh SI, Sohn HS, Seong SH, Roy A, et al. Anti-Alzheimer's disease activity of compounds from the root bark of Morus alba L. Archives of pharmacal research. 2017;40(3):338-49.
193. Du J, He Z-D, Jiang R-W, Ye W-C, Xu H-X, But PP-H. Antiviral flavonoids from the root bark of Morus alba L. Phytochemistry. 2003;62(8):1235-8.
194. Chi YS, Jong HG, Son KH, Chang HW, Kang SS, Kim HP. Effects of naturally occurring prenylated flavonoids on enzymes metabolizing arachidonic acid: cyclooxygenases and lipoxygenases. Biochemical pharmacology. 2001;62(9):1185-91.
195. Oh M, Bae SY, Chung MS. Mulberry (Morus alba) seed extract and its polyphenol compounds for control of foodborne viral surrogates. Journal of the Korean Society for Applied Biological Chemistry. 2013;56(6):655-60.
196. ARGON ZÜ, İlhan N, Gökyer A, ÖZTÜRK SB, KOPARAL B. Phytochemical Evaluation of Morus alba Seeds and Cold Pressed Oil. Journal of the Turkish Chemical Society Section A: Chemistry. 2019;6(1):41-50.
197. Sbihi HM, Nehdi IA, Mokbli S, Romdhani-Younes M, Al-Resayes SI. Hexane and ethanol extracted seed oils and leaf essential compositions from two castor plant (Ricinus communis L.) varieties. Industrial Crops and Products. 2018;122:174-81.
198. Trigui I, Zarai Z, Chevance S, Cheikh-Rouhou S, Attia H, Ayadi M. Physicochemical properties, antioxidant activity and in vitro gastrointestinal digestion of purified proteins from black cumin seeds. International journal of biological macromolecules. 2019;126:454-65.
199. Yadav P, Singh D, Mallik A, Nayak S. Madhuca longifolia (Sapotaceae), a review of its traditional uses, phytochemistry and pharmacology. International Journal of Biomedical Research. 2012;3(7):291-305.
200. Kang J, Liu L, Liu M, Wu X, Li J. Antibacterial activity of gallic acid against Shigella flexneri and its effect on biofilm formation by repressing mdoH gene expression. Food Control. 2018;94:147-54.
201. Limanto A, Simamora A, Santoso AW, Timotius KH. Antioxidant, α-glucosidase inhibitory activity and molecular docking study of gallic acid, quercetin and rutin: A comparative study. Molecular and Cellular Biomedical Sciences. 2019;3(2):67-74.
202. Santana-Gálvez J, Cisneros-Zevallos L, Jacobo-Velázquez DA. Chlorogenic acid: Recent advances on its dual role as a food additive and a nutraceutical against metabolic syndrome. Molecules. 2017;22(3):358.
203. Tajik N, Tajik M, Mack I, Enck P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. European journal of nutrition. 2017;56(7):2215-44.
204. Pérez-Nájera VC, Gutiérrez-Uribe JA, Antunes-Ricardo M, Hidalgo-Figueroa S, Del-Toro-Sánchez CL, Salazar-Olivo LA, et al. Smilax aristolochiifolia root extract and its compounds chlorogenic acid and astilbin inhibit the activity of α-amylase and α-glucosidase enzymes. Evidence-Based Complementary and Alternative Medicine. 2018;2018.
205. Espíndola KMM, Ferreira RG, Narvaez LEM, Silva Rosario ACR, Da Silva AHM, Silva AGB, et al. Chemical and pharmacological aspects of caffeic acid and its activity in hepatocarcinoma. Frontiers in oncology. 2019:541.
206. Oršolić N, Sirovina D, Odeh D, Gajski G, Balta V, Šver L, et al. Efficacy of caffeic acid on diabetes and its complications in the mouse. Molecules. 2021;26(11):3262.

207. Magnani C, Isaac VLB, Correa MA, Salgado HRN. Caffeic acid: a review of its potential use in medications and cosmetics. Analytical Methods. 2014;6(10):3203-10.
208. Jung UJ, Lee M-K, Park YB, Jeon S-M, Choi M-S. Antihyperglycemic and antioxidant properties of caffeic acid in db/db mice. Journal of pharmacology and experimental therapeutics. 2006;318(2):476-83.
209. Shi G-J, Li Y, Cao Q-H, Wu H-X, Tang X-Y, Gao X-H, et al. In vitro and in vivo evidence that quercetin protects against diabetes and its complications: A systematic review of the literature. Biomedicine & Pharmacotherapy. 2019;109:1085-99.
210. Wang W, Sun C, Mao L, Ma P, Liu F, Yang J, et al. The biological activities, chemical stability, metabolism and delivery systems of quercetin: A review. Trends in Food Science & Technology. 2016;56:21-38.
211. Harborne JB. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis: Springer Netherlands; 2012.
212. Sofowora A. Screening plants for bioactive agents. Medicinal Plants and Traditional Medicinal in Africa 2nd Ed Spectrum Books Ltd, Sunshine House, Ibadan, Nigeria. 1993:134-56.
213. Evans W. Trease and Evans Pharmacognosy. 15th (ed.) Saunders Publishers, an imprint of Elsevier Science Ltd. India; 2002.
214. Juranović Cindrić I, Kunštić M, Zeiner M, Stingeder G, Rusak G. Sample preparation methods for the determination of the antioxidative capacity of apple juices. Croatica chemica acta. 2011;84(3):435-8.
215. Pharmacopeia U, editor The United States Pharmacopeia, USP 40/The National Formulary, NF 35, Microbial contamination of Nonsterile products: Test for specific organisms. Rockville, MD: US Pharmacopeial Convention; 2017.
216. The Drug Committee and the Food and Drug Administration of Thailand. Department of Medical Sciences MoPH, Bangkok, Thailand. Thai Pharmacopoeia Vol. I and II, Supplement 2005. Microbial limit test.2005. Appendix 11-26 p.
217. Peets P, Leito I, Pelt J, Vahur S. Identification and classification of textile fibres using ATR-FT-IR spectroscopy with chemometric methods. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2017;173:175-81.
218. Lv S, Wu Y, Zhou J, Lian M, Li C, Xu Y, et al. The study of fingerprint characteristics of Dayi Pu-erh tea using a fully automatic HS-SPME/GC–MS and combined chemometrics method. PLoS One. 2014;9(12):e116428.
219. Guideline IHT. Validation of analytical procedures: text and methodology. Q2 (R1). 2005;1(20):05.
220. AOAC Official methods of Analysis, Guidelines for Standard Method Performance Requirements2016.
221. Tokalıoğlu Ş. Determination of trace elements in commonly consumed medicinal herbs by ICP-MS and multivariate analysis. Food Chemistry. 2012;134(4):2504-8.
222. Annalakshmi R, Uma R, Chandran GS, Sahayam CS, Charles A. Evaluation of physico-chemical constants and phytochemical analysis of Madhuca longifolia. Int J Nat Prod Res. 2012;1(3):64-6.
223. Kamal A. Qualitative phytochemical analysis of Madhuca longifolia. Indian J Plant Sci. 2014;3(4):38-41.
224. Nations AoSE. Annex III Asean Guidelines on limit of Contaminants for Traditional Medicines and Health2014. 10 p.
225. Donno D, Cerutti AK, Prgomet I, Mellano MG, Beccaro GL. Foodomics for mulberry fruit (Morus spp.): Analytical fingerprint as antioxidants' and health properties' determination tool. Food Research International. 2015;69:179-88.
226. Hsu H-C, Chien C-S. Validation of analytical methods: a simple model for HPLC assay

methods. Journal of Food and Drug Analysis. 1994;2(3):161.
227. Seo J-H, Kim J-E, Shim J-H, Yoon G, Bang M-A, Bae C-S, et al. HPLC analysis, optimization of extraction conditions and biological evaluation of Corylopsis coreana Uyeki Flos. Molecules. 2016;21(1):94.
228. Chen Y, Yu H, Wu H, Pan Y, Wang K, Jin Y, et al. Characterization and quantification by LC-MS/MS of the chemical components of the heating products of the flavonoids extract in pollen typhae for transformation rule exploration. Molecules. 2015;20(10):18352-66.
229. Shen J, Jia Q, Huang X, Yao G, Ma W, Zhang H, et al. Development of a HPLC-MS/MS method to determine the 13 elements of semen cuscutae and application to a pharmacokinetic study in rats. Evidence-Based Complementary and Alternative Medicine. 2019;2019.
230. Macedo SKS, Almeida TdS, Ferraz CAA, Oliveira AP, Siqueira FJA, Arajo ECdC, et al. Identification of flavonol glycosides and in vitro photoprotective and antioxidant activities of Triplaris gardneriana Wedd. Journal of Medicinal Plants Research. 2015;9(7):207-15.
231. Fathoni A, Saepudin E, Cahyana AH, Rahayu D, Haib J, editors. Identification of nonvolatile compounds in clove (Syzygium aromaticum) from Manado. AIP Conference Proceedings; 2017: AIP Publishing LLC.
232. Khare P, Kishore K, Sharma DK. Medicinal uses, Phytochemistry and Pharmacological profile of Madhuca longifolia. Asian Journal of Pharmacy and Pharmacology. 2018;4(5):570-81.
233. Kim J-H, Kang M-J, Choi H-N, Jeong S-M, Lee Y-M, Kim J-I. Quercetin attenuates fasting and postprandial hyperglycemia in animal models of diabetes mellitus. Nutrition research and practice. 2011;5(2):107-11.
234. Habeeb MN, Naik PR, Moqbel FS. Inhibition of α-glucosidase and α-amylase by Morus alba Linn leaf extracts. J Pharm Res. 2012;5(1):285-9.
235. Danuri HM, Lestari WA, Sugiman U, Faridah DN. In vitro α-glucosidase inhibition and antioxidant activity of mulberry (Morus alba L.) leaf ethanolic extract. Jurnal Gizi dan Pangan. 2020;15(1):45-52.
236. Ayoola O, Baiyewu R, Ekunola J, Olajire B, Egunjobi J, Ayeni E, et al. Phytoconstituent screening and antimicrobial principles of leaf extracts of two variants of Morus alba (S30 and S54). Afr J Pharm Pharmacol. 2011;5:2161-5.
237. Mahimairaja S, Bolan N, Adriano D, Robinson B. Arsenic contamination and its risk management in complex environmental settings. Advances in Agronomy. 2005;86:1-82.
238. Sparks DL. Advances in Agronomy: Elsevier Science; 2013.
239. Lead poisoning [Internet]. 2021 [cited 25 April 2022]. Available from: https://www.who.int.
240. Genchi G, Carocci A, Lauria G, Sinicropi MS, Catalano A. Nickel: Human health and environmental toxicology. International journal of environmental research and public health. 2020;17(3):679.
241. Arabshahi-Delouee S, Urooj A. Antioxidant properties of various solvent extracts of mulberry (Morus indica L.) leaves. Food chemistry. 2007;102(4):1233-40.
242. Iqbal S, Younas U, Chan KW, Sarfraz RA, Uddin K. Proximate composition and antioxidant potential of leaves from three varieties of Mulberry (Morus sp.): a comparative study. International journal of molecular sciences. 2012;13(6):6651-64.
243. Nammatra R. Phytochemical contents and antioxidant activities of Thai herbal tea from leaves of Morus alba and Citrus hystrix. Journal of Science and Agricultural Technology. 2021;2(1):21-7.
244. Khaskheli MI, Chandio ZA, Khokhar FM, Jatoi WB, Khan S, Channa AM. Morus alba-A Potential Sorbent for the Removal of Arsenic: Equilibrium, Kinetic and Thermodynamic Study. Pakistan Journal of Analytical & Environmental Chemistry. 2020;21(1):54-63.
245. Thabti I, Elfalleh W, Hannachi H, Ferchichi A, Campos MDG. Identification and

quantification of phenolic acids and flavonol glycosides in Tunisian Morus species by HPLC-DAD and HPLC–MS. Journal of Functional Foods. 2012;4(1):367-74.
246. Zhang D-Y, Wan Y, Hao J-Y, Hu R-Z, Chen C, Yao X-H, et al. Evaluation of the alkaloid, polyphenols, and antioxidant contents of various mulberry cultivars from different planting areas in eastern China. Industrial Crops and Products. 2018;122:298-307.
247. Mena P, Sánchez-Salcedo EM, Tassotti M, Martínez JJ, Hernández F, Del Rio D. Phytochemical evaluation of eight white (Morus alba L.) and black (Morus nigra L.) mulberry clones grown in Spain based on UHPLC-ESI-MSn metabolomic profiles. Food research international. 2016;89:1116-22.
248. Dugo P, Donato P, Cacciola F, Paola Germanò M, Rapisarda A, Mondello L. Characterization of the polyphenolic fraction of Morus alba leaves extracts by HPLC coupled to a hybrid IT-TOF MS system. Journal of Separation Science. 2009;32(21):3627-34.

APPENDIX

Table 45 Anova: Two-Factor with Replication table of % yield of *M. longifolia* leaf extracts with different sources and different solvents

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Source	18.29169	2	9.145845	35.31492	5.88E-07	3.554557
Solvent	362.2749	2	181.1375	699.4275	8.62E-18	3.554557
Interaction	9.575737	4	2.393934	9.243716	0.000305	2.927744
Within	4.661633	18	0.25898			
Total	394.804	26				

Table 46 Anova: Two-Factor with Replication table of % yield of *M. longifolia* flower extracts with different sources and different solvents

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Source	234.3736	2	117.1868	9.107346	0.001851	3.554557
Solvent	2414.514	2	1207.257	93.82374	3.02E-10	3.554557
Interaction	231.3666	4	57.84164	4.495249	0.010789	2.927744
Within	231.6111	18	12.86728			
Total	3111.865	26				

Table 47 Anova: Two-Factor with Replication table of IC_{50} of *M. longifolia* leaf extracts with different sources and different solvents

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
source	335.1396	2	167.5698	7.559588	0.004137489	3.554557
solvent	72519.46	2	36259.73	1635.788	4.39738E-21	3.554557
Interaction	689.9447	4	172.4862	7.78138	0.000797836	2.927744
Within	398.9975	18	22.16653			
Total	73943.54	26				

Table 48 Anova: Single Factor table of IC_{50} of boiling water extracts of *M. longifolia* leaf with different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	270.7496482	2	135.3748	52.72841	0.000156	5.143253
Within Groups	15.40439	6	2.567398			
Total	286.1540382	8				

Table 49 Anova: Single factor table of IC_{50} of boiling water extracts of *M. longifolia* flower with different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3839977	2	1919989	40.00693	0.000339	5.143253
Within Groups	287948.4	6	47991.4			
Total	4127926	8				

Table 50 Anova: Two-Factor with Replication table of heavy metal content of *M. longifolia* leaf with different sources and different metal

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Source	516.9986	2	258.4993	261.4851	1.04E-19	3.31583
Metal	14120.79	4	3530.198	3570.974	1.06E-39	2.689628
Interaction	1985.76	8	248.2199	251.0871	2.57E-25	2.266163
Within	29.65744	30	0.988581			
Total	16653.21	44				

Table 51 Anova: Two-Factor with Replication table of heavy metal content of *M. longifolia* flower with different sources and different metal

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	14.58898	2	7.294491	26.98248	1.97E-07	3.31583
Columns	123.1556	4	30.78891	113.8888	1.1E-17	2.689628
Interaction	77.33692	8	9.667115	35.75887	2.82E-13	2.266163
Within	8.110252	30	0.270342			
Total	223.1918	44				

Table 52 Anova: Two-Factor with Replication table of TPCs of *M. longifolia* leaf extracts with different sources and different solvents

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Source	14307.86	2	7153.929	24.32402	7.64E-06	3.554557
Solvent	22083.16	2	11041.58	37.54239	3.78E-07	3.554557
Interaction	5918.676	4	1479.669	5.031011	0.006705	2.927744
Within	5293.974	18	294.1097			
Total	47603.67	26				

Table 53 Anova: Two-Factor with Replication table of TPCs of *M. longifolia* flower with different sources and different solvents

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	16.70263	2	8.351317	19.92986	2.73E-05	3.554557
Columns	27.77886	2	13.88943	33.1462	9.23E-07	3.554557
Interaction	23.67286	4	5.918215	14.12343	2.2E-05	2.927744
Within	7.542637	18	0.419035			
Total	75.69699	26				

Table 54 Anova**:** Two-Factor with Replication table of gallic acid and quercetin in aqueous extract of *M. longifolia* leaf

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	24227.17	2	12113.58	4033.338	4.11E-10	5.143253
Columns	301964.2	1	301964.2	100542	6.64E-14	5.987378
Interaction	21443.21	2	10721.61	3569.866	5.92E-10	5.143253
Within	18.02018	6	3.003364			
Total	347652.6	11				

Table 55 Anova: two-factor with replication table of % yield of boiling water extracts of *M. alba* leaves

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
cultivar	1.15	1.00	1.15	0.46	0.51	4.75
province	130.71	2.00	65.36	26.35	4.07E-05	3.89
Interaction	26.26	2.00	13.13	5.29	2.25E-02	3.89
Within	29.77	12.00	2.48			
Total	187.88	17				

Table 56 Anova: two-factor with replication table of % yield of aqueous extracts of *M. alba* leaves

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
cultivar	11.91	1.00	11.91	4.64	0.05	4.75
province	154.73	2.00	77.36	30.16	2.09E-05	3.89
Interaction	7.68	2.00	3.84	1.50	0.26	3.89
Within	30.78	12.00	2.56			
Total	205.0872	17				

Table 57 Anova: two-factor with replication table of % yield of ethanolic extracts of *M. alba* leaves

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
cultivar	2.03	1	2.03	82.89	9.78E-07	4.75
Province	5.62	2	2.81	114.46	1.53E-08	3.89
Interaction	0.22	2	0.11	4.50	0.03	3.89
Within	0.29	12	0.02			
Total	8.164628	17				

Table 58 Anova: two-factor with replication table of IC_{50} of *M. alba* leaf extracts with different cultivars and different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Cultivar	11940.9	1	11940.9	6.053423	0.030018114	4.747225
Province	228678.3	2	114339.2	57.96406	6.81226E-07	3.885294
Interaction	1173548	2	586773.8	297.464	5.97398E-11	3.885294
Within	23671.05	12	1972.587			
Total	1437838	17				

Table 59 Anova: two-factor with replication table of As content in *M. alba* leaves with different cultivars and different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
cultivar	0.000812	1	0.000812	173.05	1.73E-08	4.75
province	0.006	2	0.003	639.27	6.46E-13	3.89
Interaction	0.001475	2	0.000738	157.18	2.47E-09	3.89
Within	5.63E-05	12	4.69E-06			
Total	0.008344	17				

Table 60 Anova: two-factor with replication table of Cd content in *M. alba* leaves with different cultivars and different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
cultivar	2.76E-05	1	2.76E-05	15.00	0.0022	4.75
province	6.06E-06	2	3.03E-06	1.64	0.2339	3.89
Interaction	2.35E-05	2	1.18E-05	6.39	0.0129	3.89
Within	2.21E-05	12	1.84E-06			
Total	7.93E-05	17				

Table 61 Anova: two-factor with replication table of Pb content in *M. alba* leaves with different cultivars and different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
cultivar	0.003447	1	0.003447	2.39	0.1478	4.75
province	0.003162	2	0.001581	1.10	0.3650	3.89
Interaction	0.053877	2	0.026939	18.70	0.0002	3.89
Within	0.017283	12	0.00144			
Total	0.077769	17				

Table 62 Anova: two-factor with replication table of Ni content in *M. alba* leaves with different cultivars and different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
cultivar	0.117602	1	0.117602	14.15	0.0027	4.75
province	0.174758	2	0.087379	10.51	0.0023	3.89
Interaction	0.007653	2	0.003827	0.46	0.6417	3.89
Within	0.099734	12	0.008311			
Total	0.399748	17				

Table 63 Anova: two-factor with replication table of Ba content in *M. alba* leaves with different cultivars and different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
variety	202.2256	1	202.2256	148.36	4.1E-08	4.75
province	4386.261	2	2193.131	1,608.93	2.63E-15	3.89
Interaction	366.773	2	183.3865	134.54	6.06E-09	3.89
Within	16.35717	12	1.363098			
Total	4971.617	17				

Table 64 Anova: Single Factor table of TPCs of *M. alba* reference leaves with different extraction solvents

Groups	*Count*	*Sum*	*Average*	*Variance*
B	18	483.251	26.847278	83.777739
W	18	451.626	25.090333	57.572355
E	18	260.01981	14.445545	60.395383

Table 65 Anova: Single Factor table of TPCs of *M. alba* reference leaves with different sources

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1621.208	2	810.60401	12.053862	5.16514E-05	3.178799292
Within Groups	3429.6731	51	67.248492			
Total	5050.8811	53				

Table 66 Anova: Two-Factor with Replication table of TPCs of boiling water extract of *M. alba* reference leaves

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Cultivar	458.10618	1	458.10618	445.72139	7.4085E-11	4.7472253
Province	879.39742	2	439.69871	427.81156	7E-12	3.8852938
Interaction	74.384529	2	37.192265	36.186781	8.2765E-06	3.8852938
Within	12.333431	12	1.0277859			
Total	1424.2216	17				

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Table 67 Anova: Two-Factor with Replication table of TPCs of aqueous extract of *M. alba* reference leaves

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	402.54194	1	402.54194	76.563701	1.48501E-06	4.7472253
Columns	510.76823	2	255.38411	48.574201	1.76597E-06	3.8852938
Interaction	2.328571	2	1.1642855	0.2214478	0.804560586	3.8852938
Within	63.091298	12	5.2576082			
Total	978.73004	17				

Table 68 Anova: Two-Factor with Replication table of TPCs of ethanolic extract of *M. alba* reference leaves

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Cultivar	132.71569	1	132.71569	11.892836	0.00481742	4.7472253
Province	670.5018	2	335.2509	30.042295	2.1283E-05	3.8852938
Interaction	89.592452	2	44.796226	4.0142514	0.04625903	3.8852938
Within	133.91157	12	11.159297			
Total	1026.7215	17				

Table 69 Anova**:** Two-Factor with Replication table of chlorogenic acid, caffeic acid and rutin in aqueous extract of *M. alba* leaf

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	626520.6	5	125304.1	722.1783	4.45E-20	2.772853
Columns	654211.6	2	327105.8	1885.243	1.23E-21	3.554557
Interaction	617219.9	10	61721.99	355.7288	1.46E-18	2.411702
Within	3123.154	18	173.5086			
Total	1901075	35				

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